Transcriptional cofactors in modulation of nuclear hormone receptor function

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Nuclear hormone receptors (NHRs) act as ligand-dependent transcription factors in cooperation with multiple cofactor complexes that repress or activate target gene transcription. This thesis is focused on the role of the transcriptional cofactor called Ski-interacting protein (SKIP/NCoA-62) in NHR and transforming growth factor-β (TGF-β) signalling.

During this thesis, SKIP was identified as a VDR- and RXR-interacting factor in a yeast two-hybrid screen. In mammalian cells, SKIP acted not only as a coactivator, but also as a repressor of RXR- and VDR-dependent gene transcription. These transcriptional effects appeared to be mediated through an AF-2 domain-dependent interaction with RXR and/or a RXR-associated-protein. SKIP possessed two functional repression domains: a N-CoR/SMRT interaction domain (aa1-200) and a N-CoR/SMRT independent domain (aa201-333) which repressed basal as well as autonomous activities of transcriptional activators such as SRC-1 and SRC-2. A third region in the C-terminus (334-536aa) also appeared to be required for cell-specific repression of RXR-dependent transactivation. Furthermore, cell-specific expression of N-CoR was correlated with SKIP repressor action, which was reversed by *all-trans*-retinoic-acid-induced differentiation of P19 cells. These results suggested that the novel actions of SKIP as both a positive and a negative modulator of NHR-dependent transcription, and possibly other signalling pathways, may be related to cell- and differentiation-specific interactions between SKIP and corepressors and coactivators.

As SKIP interacts with the Ski oncoprotein, a TGF-β repressor which associates with N-CoR, it was hypothesised that SKIP may modulate TGF-β signalling, directly or indirectly, via its interaction with Ski and/or the related Sno protein. In yeast and mammalian cells and *in vitro* SKIP interacted with Smad2 and Smad3 proteins (the intracellular transducers of TGF-β signalling). SKIP, in contrast to Ski/Sno, activated TGF-β-dependent transcription and counteracted Ski/Sno-mediated repression. In summary, SKIP appeared to act as a cell- and pathway-specific bifunctional (activator and repressor) transcriptional cofactor involved in modulation of both the NHR and TGF-β signalling pathways. The *in vivo* action of SKIP in these pathways and a potential role in cell proliferation and differentiation indicates that it has novel regulatory interactions of relevance to human development and disease.

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Transcriptional Cofactors in Modulation of Nuclear Hormone Receptor Function

Gary Martin Chee Mun Leong

A thesis submitted for the degree of

Doctor of Philosophy

Bone & Mineral Research Program,

Garvan Institute of Medical Research

and

Faculty of Medicine,

University of New South Wales

2001

DEDICATION

This thesis is dedicated to my family,

first and fore-most to Micky,

my ever-patient and supportive wife

and my three beautiful children:

Martin, Julia and Vivienne Mitzi Shelley.

Without them I could not have achieved anything.

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I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text.

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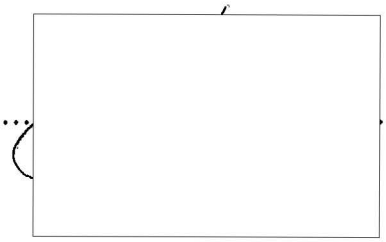
(signed).....  .....GARY M. LEONG

ABSTRACT

Nuclear hormone receptors (NHRs) act as ligand-dependent transcription factors in cooperation with multiple cofactor complexes that repress or activate target gene transcription. This thesis is focused on the role of the transcriptional cofactor Ski-interacting protein (SKIP/NCoA-62) in NHR and transforming growth factor-β (TGF-β) signalling.

In initial studies for this thesis, SKIP was identified as a VDR-interacting factor in a yeast two-hybrid screen and shown to interact with VDR and RXR in yeast. In mammalian cells, SKIP was found to act not only as a coactivator, but also as a repressor of RXR- and VDR-dependent gene transcription. This transcriptional effect appeared to be mediated through an AF-2 domain-dependent interaction with RXR and/or a RXR-associated-protein. SKIP possessed at least two functional domains: a N-CoR/SMRT interaction domain (aa1-200) and a N-CoR/SMRT-independent interaction domain (aa201-333) that repressed basal as well as autonomous activities of various transcriptional activators such as SRC-1 and SRC-2. A third region in the C-terminus (334-536aa) also appeared to be required for cell-specific repression of RXR-dependent transactivation. Furthermore, cell-specific expression of N-CoR was correlated with SKIP repressor action, which was reversed by *all-trans*-retinoic-acid-induced differentiation of P19 cells. These results thus suggest that the novel actions of SKIP as both a positive and a negative modulator of NHR-dependent transcription, and possibly other signalling pathways, may be related to cell- and differentiation-specific interactions between SKIP and corepressors and/or coactivators.

Furthermore, as SKIP interacts with the Ski oncoprotein, a TGF-β repressor which associates with N-CoR, it was hypothesised that SKIP may modulate TGF-β signalling. In yeast and mammalian cells and *in vitro* , SKIP interacted directly with Smad2 and Smad3 proteins (the intracellular transducers of TGF-β signalling). SKIP, in contrast to the transcriptional repressors Ski and the related Sno protein, activated TGF-β-dependent transcription and counteracted Ski/Sno-mediated repression. SKIP also synergistically activated the vitamin D receptor-responsive human osteocalcin promoter suggesting that SKIP may facilitate transcriptional crosstalk between the VDR and TGF-β signalling pathways.

In summary, SKIP appeared to act as a cell- and pathway-specific bifunctional (activator and repressor) transcriptional cofactor involved in modulation of both the NHR and TGF-β signalling pathways. The *in vivo* action of SKIP in these pathways and a potential role in cell proliferation and differentiation suggests that it has novel regulatory interactions of relevance to human development and disease.

PUBLICATIONS ARISING FROM THIS THESIS

Issa, L.L., **Leong G.M.,** Eisman J.A.

Molecular mechanism of vitamin D receptor action.

Inflammation Research 1998; 47:451-475.

Issa L.L., **Leong G.M.,** Barry J.B., Sutherland R.L., Eisman J.A.

The coactivators mGRIP1 and hRAC3 differentially interact with the VDR and diverge in their regulation of VDR-RXR mediated transcription. Endocrinology, 2001, 142:1606-1615.

Leong G.M., Subramaniam N., Figueroa J., Flanagan J.L., Hayman M.J., Eisman J.A., Kouzmenko A.P.

Ski-interacting protein (SKIP) interacts with Smad proteins to augment TGF-β–dependent transcription.

Journal of Biological Chemistry, 2001, 276:18243-18248.

Subramaniam N., **Leong G.M.,** Flanagan J.L., Cock T.A., Eisman J.A., Kouzmenko A.P.

Transcriptional synergism of 1,25-dihydroxyvitamin D3 and transforming growth factor-β signalling requires simultaneous binding of VDR and Smad3 proteins to their cognate DNA recognition elements.

Journal of Biological Chemistry, 2001, 276:15741-15746.

Abad V., Chrousos G.P., Reynolds J.C., Nieman L.K., Hill S.C., Weinstein R.S., **Leong G.M.**

Glucocorticoid excess during adolescence leads to a major persistent deficit in bone mass and increase in central body fat.

Journal of Bone & Mineral Research, 2001;16:1879-1885.

Chapter in Textbook

Gary M. Leong, Jacqueline R. Center, N. Kathryn Henderson, John A. Eisman. *Glucocorticoid-Induced Osteoporosis: Basic Pathological Mechanisms, Clinical Features And Management In The New Millenium.*

In "Osteoporosis" 2nd edition Eds. Marcus, R., Feldman D. & Kelsey, J. Academic Press. San Diego, CA. 2001 (Vol 2: 169-193).

Submitted to peer-reviewed journals

Leong G.M., Subramaniam N., Barry J.B., Issa L.L., Dahl R., Driggers P., Hayman M.J., Eisman J.A., Kouzmenko A.P., Gardiner E.M.

SKIP acts as a cell-line specific nuclear receptor coregulator that interacts with both corepressors and coactivators.

Issa L.L., **Leong G.M.**, Sutherland R.L., Eisman J.A.

Vitamin D analogue-specific recruitment of vitamin D receptor coactivators.

Barry J.B., **Leong G.M.**, Church W.B., Issa L.L., Eisman J.A., Gardiner E.M.

Vitamin D Receptor helix 10 interface required for interaction with SKIP, TFIIB and RXR.

Flanagan J.L., **Leong G.M.**, Subramaniam N., Sunn K.L., Cock, T., Crofts, L.A., Eisman J.A., Gardiner E.M. and Kouzmenko A.P.

AF-1 Activity and Differential Interaction with Ligand Binding Domain of N-Terminal A/B Domains of TwoHuman Vitamin D Receptor Isoforms.

ABSTRACTS ARISING FROM THIS THESIS

Oral presentations

Leong G.M., Eisman J.A., Hayman M.J. and Gardiner E.M. *Identification and characterisation of transcriptional cofactors important for vitamin D-dependent gene regulation.* Australian & New Zealand Bone Mineral Society Annual Meeting, Perth, 1998.

Leong G.M., Eisman J.A., Hayman M.J. and Gardiner E.M. *Identification and characterisation of the v-Ski interacting oncoprotein (hSkip) as a cofactor for vitamin D and retinoid X receptor dependent gene regulation.* Endocrine Society of Australia satellite symposium "Steroid Hormone Receptors and Cancer", Perth, 1998.

Leong G.M., Barry J.B., Issa L.L., Eisman J.A. , Hayman M.J. and Gardiner E.M. *The transcriptional co-activator hSkip acts in a cell-line specific manner to regulate nuclear hormone receptor gene function.* Australian & New Zealand Bone Mineral Society Annual Meeting, Cairns, 1999.

Leong G.M., Subramaniam N., Flanagan J.L., Eisman J.A., Gardiner E.M. and Kouzmenko A.P. *Intersection Of Nuclear Hormone Receptor And Transforming Growth Factor-β Signalling Through the Nuclear Cofactor Skip.* Australian & New Zealand Bone Mineral Society Annual Meeting, Hamilton Island, 2000.

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1998 for Plenary Poster and Young Investigator award at 2nd Joint Meeting of the American Society for Bone & Mineral Research and the International Bone & Mineral Society, San Francisco Dec 1-6, 1998.

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ABBREVIATIONS

$1,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
9-*cis*-RA	9-*cis*-retinoic acid
24-hydroxylase	25-hydroxyvitamin D 24-hydroxylase
3AT	3-aminotriazole
aa	amino acid
ACF	ATP-utilising-chromatin assembly factor
AD1	activation domain 1
AD2	activation domain 2
AF-1	activation function 1
AF-2	activation function -2
AIB-1	amplified in breast cancer 1
AML	acute myeloid leukaemia
APS	ammonium persulfate
AR	androgen receptor
ATRA	all-*trans*-retinoic acid
Bp	base pair(s)
BSA	bovine serum albumin
cAMP	3',5'-cyclic adenosine monophosphate
CARM1	coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
ChIP	chromatin immunoprecipitation (assay)
CHRAC	chromatin accessibility complex
CIA	coactivator independent of AF-2 function
CIP	calf intestinal alkaline phosphatase
DBD	DNA binding domain

DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
DR3	direct repeat element with 3 nucleotides spacing
DRIP	vitamin D receptor interacting protein
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EcR	ecydysone receptor
EGTA	ethylene glycol-bis-N,N,N',N'-tetraacetic acid
ER	estrogen receptor
ERAP 140/160	estrogen receptor-associated proteins 140/160 kDa
FBS	fetal bovine serum
GAL4AD	GAL4 activation domain
GAL4DBD	GAL4 DNA binding domain
GR	glucocorticoid receptor
GRE	glucocorticoid response elements(s)
GRIP1	glucocorticoid receptor interacting protein 1
GST	glutathione sepharose trasnferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLH	helic-loop-helix
HNF-4	Hepatocyte nuclear factor-4
HRE	hormone response element
IPTG	isopropyl-β-D-thio-galactopyranoside
LBD	Ligand binding domain
LBP	ligand binding pocket

LEXADBD	LEXA DNA binding domain
MAPK	mitogen activated protein kinase
MH1/2	mad homology domain 1/2
MOPS	3-[N-morpholino]propane sulfonic acid
MOZ	monocytic leukaemia zinc finger
MR	mineralocorticoid receptor
NCoA-62	Nuclear Receptor Coactivator-62 kDa
N-CoR	nuclear receptor corepressor
NHR	nuclear hormone receptor
NID	nuclear hormone receptor interaction domain
NLS	nuclear localisation signal
NR-box	nuclear receptor box
NSD1	NHR-binding SET domain containing protein-1
NURF	nucleosome remodelling
PAI-1	plasminogen activator inhibitor-1
PBP	PPAR-binding protein
PEG	polyethylene glycol
p/CAF	p300/CBP associated factor
p/CIP	p300/CBP interacting protein
PCR	polymerase chain reaction
PIC	preinitiation complex
PML	promyelocytic leukaemia
PMSF	phenylmethyl-sulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PSF	polypyrimidine tract-binding protein-associated splicing factor

RAC3	receptor associated coactivator 3
RAR	retinoic receptor
RARE	retinoic acid response elements
Rb	retinoblastoma protein
RID	receptor-interacting domain
RIP13	RXR-interacting protein 13
RIP140	receptor-interacting protein 140
RNA	ribonucleic acid
RSC	remodelling the structure of chromatin
RXR	retinoid X receptor
RXRE	retinoid X response elements
SAC	SET-associated Cys-rich domain
SAGA	*S*pt, *A*DA, *G*cn5 *A*cetyltransferase
SBE	Smad-binding element
SDS	sodium dodecyl sulfate
SID	Sin3 interaction domain
SKIP	Ski interacting protein
SMRT	silencing mediator for retinoic acid and thyroid hormone receptors
SRC-1-3	steroid receptor coactivator 1-3
$TAF_{II}s$	TBP-associated factors
T3	3,3',5-triiodo-$_L$-thyronine
TBP	TATA-binding protein
TEMED	N,N,N',N'-tetramethylethylenediamine
TFIIA	transcription factor IIA
TFIIB	transcription factor IIB
TFIID	transcription factor IID

TFIIF	transcription factor IIF
TGF-β	transforming growth factor-β
TIF2	transcription intermediary factor 2
TR	thyroid receptor
TRAC2	T3-associated cofactor
TRE	thyroid response elements
TRAP	thyroid receptor associated proteins
TRAM-1	TR activator molecule 1
TSA	trichostatin
UAS	upstream activating sequence
VDR	vitamin D receptor
VDRE	vitamin D response elements

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Chapter One

Introduction

1.1 OVERVIEW

The nuclear hormone receptor (NHR) superfamily mediate multiple functions in metazoan physiology by binding DNA hormone response elements in target promoters to modulate gene expression. In the absence of ligand, class 1 NHRs are bound to corepressors within large protein complexes that exhibit histone deacetylation (HDAC) activity. In response to ligand the NHR ligand-binding domain (LBD) undergoes a conformational change that facilitates interaction with a large range of coactivator complexes, including CBP/p300, SRCs, p/CAF and human mediator protein complexes. Some of these mediate interactions with the basal transcription machinery and others act as histone acetyltransferase (HAT) enzymes. Thus, the regulation of activated transcription controlled by NHRs is under the opposing influences of chromatin "opening" HAT or chromatin "repressing" HDAC activities that modulate the accessibility of regulatory transcription factors to the underlying DNA. In this model, a ligand-induced conformational change in the NHR LBD acts as a molecular switch that leads to corepressor release and coactivator binding to a ligand-exposed hydrophobic cleft within the NHR LBD. Coactivator binding, e.g. SRC-1, is followed by interaction with multiple HAT-containing and chromatin remodelling complexes (e.g. CBP/p300 and others) prior to recruitment of the RNA Pol II holoenzyme and formation of the pre-initiation complex. The mechanisms that control the specificity of action of these complexes *in vivo* are unclear, but it is hypothesised that cell-, receptor and promoter-specific factors may play a role in mediating the tissue- and developmental-specific properties ascribed to NHRs.

Resolution of the crystal structures of unliganded and liganded NHRs, the latter in association with coactivator peptides, have revealed structural determinants of coactivator binding. NHR coactivators bind NHRs through a conserved helical motif consisting of the pentapeptide leucine-containing sequence LXXLL. In a parallel manner corepressors bind NHRs through a similar slightly extended α-helical motif consisting of the sequence LXXI/HIXXXI/L found in various corepressors including N-CoR and SMRT. Ligand binding to NHRs leads to a

conformational change with formation of a hydrophobic cleft in the LBD and reorientation of specific helical regions which entraps the ligand and forms a "charge clamp" created by helix 3 at one end and the extreme C-terminal AF-2 activation helix 12. This "charge clamp" stabilises binding of the coactivator within the LBD and is predicted to preclude corepressor binding due to the unfavourable extended length of the corepressor helix.

This chapter reviews the structural and functional properties of NHRs, the potential roles of NHRs *in vivo* and the mechanisms of NHR transcriptional activation and repression, including their association with chromatin remodelling complexes. Emphasis is placed on the role of histone acetylation/deacetylation in NHR-mediated transcription and the potential role of different coactivators and corepressors and other novel cofactors in mediating the tissue and developmental-specific effects of the NHR gene superfamily.

1.2 THE NUCLEAR RECEPTOR SUPERFAMILY

1.2.1 Classification and Phylogeny

Nuclear hormone receptors (NHRs) are ligand-dependent transcription factors that play critical roles in human development, cell differentiation, cancer, metabolism, reproduction and inflammation (Glass and Rosenfeld, 2000; Issa *et al.*, 1998). The members of this superfamily are diverse with over 300 members defined in metazoans. Although these are generally known as NHRs the largest group are orphan receptors with no known ligands (Mangelsdorf and Evans, 1995; Whitfield *et al.*, 1999). Based on the evolution of the conserved DNA- and ligand-binding domains (LBD) the superfamily can be classified into 6 subfamilies and 26 groups of receptors (Figure 1.1) (Nuclear Receptor Subcommitee, 1999). This thesis focuses on members belonging to the first three NHR classes.

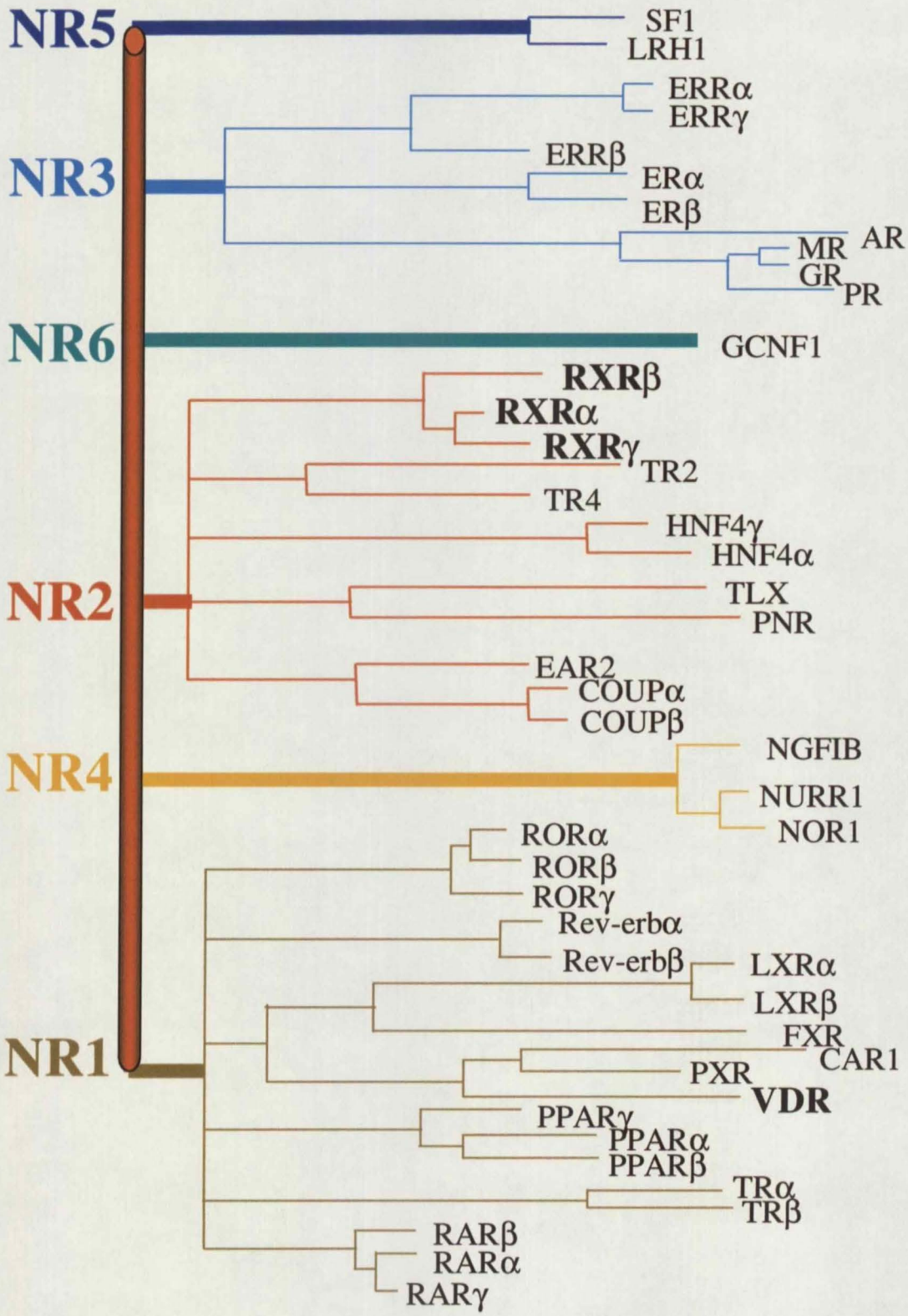


Fig. 1.1 Human Nuclear Hormone Receptor Phylogeny. Adapted with permission from Drs.V.Laudet and M.Robinson from Nuclear Receptors Nonmenclature Committee 1999.

The largest group, the class 1 receptors, in general heterodimerise with the class 2 receptor, the retinoid X receptor (RXR). Class 1 includes the steroid-thyroid-retinoic acid-vitamin D subfamily, consisting of the retinoic acid (RAR), thyroid hormone (TR), the vitamin D (VDR), the peroxismal-proliferator-activated receptors (PPAR) and another group of receptors which are activated by sterol ligands and are involved in hepatic function, including cholesterol and xenobiotic metabolism (Evans, 1988; Issa *et al.*, 1998; Wan *et al.*, 2000; Whitfield *et al.*, 1999). The classical steroid hormone receptors which bind as homodimers are grouped in class 3 and include the oestrogen (ERα and ERβ), androgen (AR), glucocorticoid (GR), progesterone (PR) and mineralocorticoid (MR) receptors. Most of the major human NHRs bind to ligands that consist mainly of compounds derived from acetyl CoA, including retinoids, sterols/steroids and vitamin D. The Thyroid hormone receptor has a ligand T3 (3,3',5-triiodo-$_L$-thyronine), which is derived from coupling of the amino acid tyrosine with iodination.

1.2.2 NHR Gene Knock-out Studies in Mice

NHR gene knockout studies in mice strongly support a role *in vivo* for these receptors in a wide variety of key developmental and homeostatic cell processes (Table 1.1) While the majority of these single gene knockout studies were associated with clear phenotypic effects post-natally, gene knockouts of RXR and RAR isoforms have revealed a surprising redundancy of receptor function with compensatory changes in isoform receptor expression and a lack of phenotype in knockout mice. Alternatively, some of the knockouts have been associated with embryonic lethality which precluded investigation of receptor function post-natally (Kastner *et al.*, 1995; Kastner *et al.*, 1997; Mendelsohn *et al.*, 1994; Ross *et al.*, 2000). To address this problem Cre-recombinase gene knockout technology has recently been used to investigate the tissue-specific roles of NHR function *in vivo* (Feil *et al.*, 1996; Wan *et al.*, 2000). As an example, adipocyte-specific

Gene knockout	Phenotype	References
VDR	Normal pre-natal growth and development; post-weaning growth retardation, reduced fertility, hypocalcaemia and abnormal bone formation consistent with rickets and alopecia	(Li *et al.*, 1997b; Yoshizawa *et al.*, 1997)
RXRα	Embryonic lethality E13.5 –16.5. Hypoplastic heart; resistance to limb RA-induced malformations	(Sucov *et al.*, 1994; Sucov *et al.*, 1995)
RXRβ	Peri-natal lethality 50%. Remainder phenotypically normal but sterile due to oligo-astheno-teratospermia with mutant Sertoli cells	(Kastner *et al.*, 1996)
RAR*	Normal, suggesting redundancy between receptor subtypes	(Kastner *et al.*, 1995)
RARα	High post-natal lethality and testis degeneration	(Lufkin *et al.*, 1993)
RARγ	Malformations of cervical vertebra and occipital skull, fusion of 1st and 2nd ribs	(Lohnes *et al.*, 1993)
RAR dk	α/γ, α/β2, β2/γ2: embryonic lethal with malformations similar to those observed with foetal Vitamin A deficiency	(Ross *et al.*, 2000)
RXRβ/ RXRγ (dk)	Double knockouts viable with no obvious congenital or postnatal abnormality except growth deficiency and male sterility due to loss of RXRβ. Compound mutant with one RXRα allele sufficient for normal RXR function *in vivo*	(Krezel *et al.*, 1996)
RXRα+/- RXRβ RXRγ		
RAR/RXR double mutants	Various developmental abnormalities as present in foetal vitamin A deficiency. RARα/RXRα complete absence of aortopulmonary septum. Studies suggest RXRα main isoform for RAR developmental functions; and little functional redundancy of RAR subtypes, though RXRβ can replace RXRα as heterodimerisation partner with RAR for many processes	(Kastner *et al.*, 1997; Mendelsohn *et al.*, 1994)
ERα	Infertility; Uterine hypoplasia with lack of responsiveness to oestradiol; ovarian dysfunction with cysts; in males abnormal sperm function. Less aggressive male sexual behaviour	(Das *et al.*,1997; Ogawa *et al.*, 1997)
ERβ	Females sub-fertile with impaired ovarian function; CNS maldevelopment e.g substantia nigra	(Wang *et al.*, 2001)
ERα/ERβ (dk)	Infertility; Male mice lack normal sexual and aggressive behaviour	(Couse and Korach, 1999; Ogawa *et al.*, 2000)
PRA	Females infertile with severe impairment in ovulation; normal mammary development	(Conneely and Lydon, 2000)

Table 1.1 Some of the published NHR gene knockout studies illustrating the diversity of developmental, hormonal and metabolic effects of NHRs *in vivo*. This list is in no way exhaustive but simply serves to illustrate the range of possible roles of NHRs *in vivo*. Many other NHR gene knockout studies not listed here have been performed, including with TRs, PPARs, LXRs, and some orphan receptors, such as COUP-TF and Nurr1. RAR*=RARα1,RARβ2,RARγ2; Dk = double knockout

RXRα knockout in 4 week transgenic mice reveals a central role for RXRα in adipogenesis probably as a heterodimer partner of PPARγ (Imai *et al.*, 2001). Thus future studies in this area are expected to provide further novel insights into developmental- and tissue-specific roles of different NHRs *in vivo* (Alfred, 2001).

1.3 NUCLEAR HORMONE RECEPTOR STRUCTURE AND TRANSCRIPTIONAL REGULATION

1.3.1 General Structure of Nuclear Hormone Receptors

Though structural domains within NHRs have been extensively defined *in vitro* by domain swapping and point mutation/deletion studies, seminal insights into NHR structure have arisen from x-ray crystallography studies (Renaud and Moras, 2000). In general NHRs appear to display a structural similarity and organisation with 5-6 functional modular domains designated A-F (Figure 1.2). These include a highly variable amino-terminal A/B domain ranging in length from 23 aa in VDR to 602 aa in MR. This domain in most other NHR contains an intrinsic activation function-1, or AF-1 ligand-independent transactivation domain. Regions C and D include the DNA-binding domain (DBD ~70aa) consisting of two zinc fingers (region C) and a linker hinge region D which is highly variable in length and contains nuclear localisation signals. The C-terminal region comprises the ligand-binding domain (LBD) or E region (~250aa) which extends from the DBD to the extreme C-terminus, and includes regions for dimerisation, ligand-binding and ligand-dependent transactivation through the activation-function-2 (AF-2) domain. The LBD also forms interaction surfaces for corepressors, coactivators and other factors that are involved in bridging communication with the basal transcription machinery (Glass and Rosenfeld, 2000). An extreme C-terminal F region function is poorly understood but in certain NHRs may be involved in masking corepressor binding as has been suggested for RXR (Zhang *et al.*, 1999b).

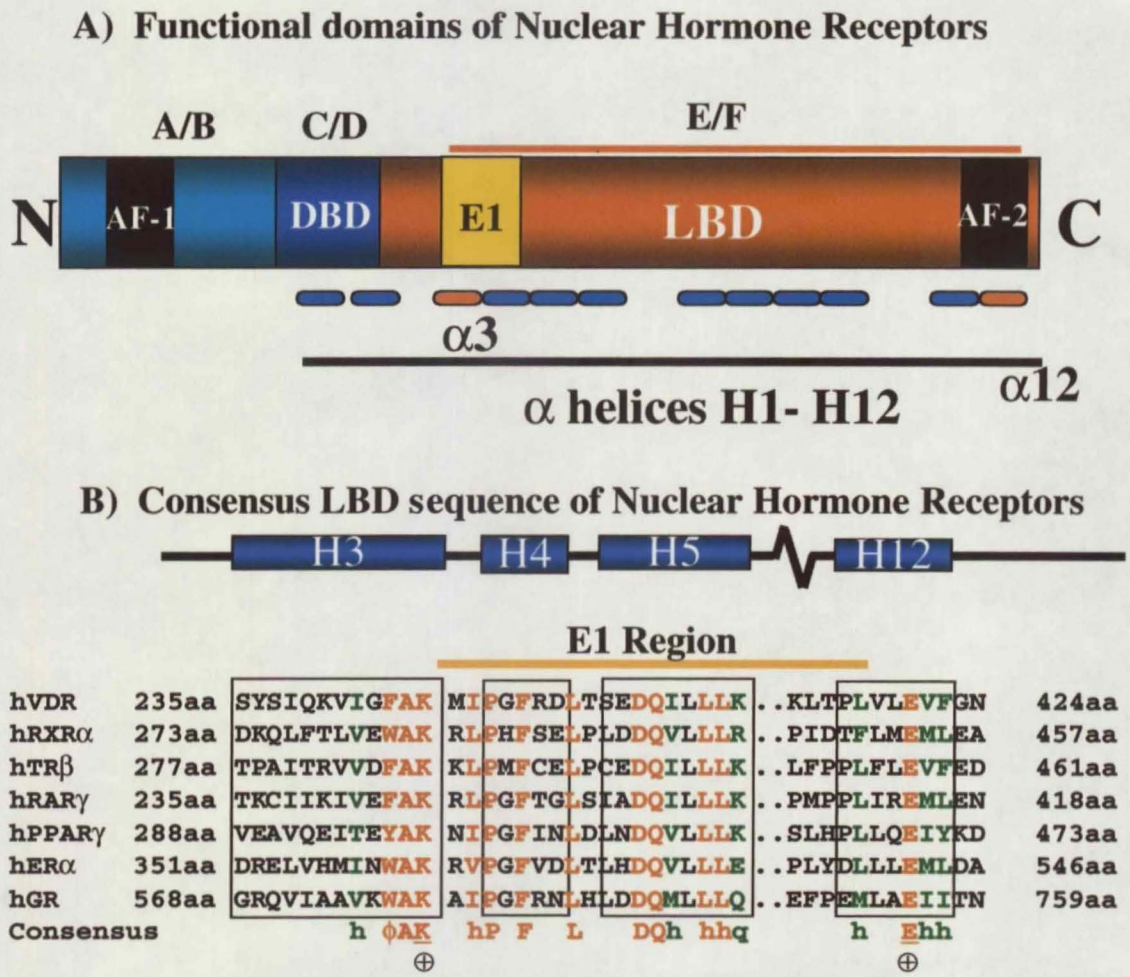


Fig. 1.2 General Structure of Nuclear Hormone Receptors

A) Schematic representation of functional domains within NHRs are shown from N- to C-terminus including A/B (light blue), DNA-binding domain (DBD) (C/D, dark blue) and ligand-binding domains (LBD) (E/F, red), the latter containing the highly conserved E1 region. Most NHRs harbour ligand-independent activation function-1 (AF-1) and ligand-dependent AF-2 domains. Overlapping corepressor and coactivator binding interface indicated by the red overline. Helices 1-12 represented underneath figure as as small ovals, with helices 3 and 12 shown in red. Not shown are potential phosphorylation and nuclear localisation sites.

B) Sequence alignment of LBD helices 3, 4, 5 and 12 (Wurz *et al.*, 1996 and Darimont *et al.*, 1998) which represent regions that interact with coactivators and corepressors. Residues representing the NHR signature consensus are shown in red and conserved hydrophobic (h) or strong polar (q) are in green. Absolutely conserved K residue in H3 and E residue in H12 form the "charge clamp" for stabilisation of coactivator binding are indicated by symbol ⊕. (ϕ) Bulky hydrophobic residue.

1.3.2 The DNA Binding Domain (DBD)

The most highly conserved regions of NHR are the DBD and the LBD. The NHR DBD consensus sequence is about 66aa in length organised about eight conserved cysteine residues which coordinate two Zn2+ ions, each one coordinated in a tetrahedral arrangement by four cysteines (Freedman *et al.*, 1988). The first zinc module (P box) determines half-site recognition and the second module is involved in dimerisation including half-site spacing recognition and orientation (D box) (Renaud and Moras, 2000; Umesono *et al.*, 1991). NHR bind to specific DNA sequences (hormone response elements, HRE) in target promoters. These consist of a consensus sequence of AGGTCA, or variations thereof, and bind HRE either as monomers, homodimers and/or heterodimers (Glass, 1994). There are 3 main HRE classes: 1) simple half-sites which are bound by monomers such as NGFI-B and Rev-Erb; 2) palindromic repeats with 3bp spacing such as the ER; and 3) direct repeats (DR) with variable spacing between half-sites (designated as +) which bind RXR heterodimers. These include HREs for RXR (DR+1), VDR (DR3+), RAR (DR+1, 2 or 5), TR (DR+4) and PPAR (DR1+) and others (Umesono *et al.*, 1991). Variations in the half-site consensus sequence AGGTCA and these variable spacer lengths increase the repertoire of response elements, and by overlapping with other DNA elements e.g. Smad-binding or AP-1 sites, facilitate NHR modulation of other cell signalling pathways i.e. transcriptional crosstalk (Kamei *et al.*, 1996; Subramaniam *et al.*, 2001).

1.3.3 The Ligand Binding Domain (LBD)

The LBD of NHRs is the second most highly conserved region and contains 11 to 13 α–helical regions and several small β-strands organised around a lipophilic ligand binding pocket (LBP) (Bourguet *et al.*, 1995; Williams and Sigler, 1998). Alignment of VDR, RXR, RAR and other LBD sequences from helices H3, H4, H5 and H12 reveals two common features of the LBD: a conserved hydrophobic core and a hypervariable region corresponding to the ligand-binding site (Wurtz *et al.*, 1996). The conserved core is composed of hydrophobic residues mostly clustered in a 34 aa peptide ranging from the middle of H3 to H5. This segment contains the

LBD signature motif consisting of F/WAKxxxxFxxLxxxDQxxLL that acts to stabilise the LBD (Darimont *et al.*, 1998; Renaud and Moras, 2000) (Figure 1.2). The high sequence variability of the ligand binding pocket constitutes the basis of ligand specificity (Wagner *et al.*, 2001). For example, 24 residues contribute to the ligand binding pocket in RARγ (Renaud *et al.*, 1995) and 22 in TRα (Wagner *et al.*, 1995), 16 of which share a common alignment position, but only 4 of which are identical (Renaud and Moras, 2000). Similarly, the recently described crystal structures of the VDR-LBD bound to $1,25(OH)_2D_3$ and synthetic vitamin D analogues suggest specific critical residues in the VDR make contact with the ligand in the LBP (Rochel *et al.*, 2000; Tocchini-Valentini *et al.*, 2001).

1.3.4 "Mouse-trap" Model of Ligand Binding and Role of Helix 12 in Transcriptional Regulation

Comparison of crystal structures of apo- versus holo-receptors, e.g. RXR with its ligand 9-*cis*-RA (Bourguet *et al.*, 1995; Egea *et al.*, 2000), provides a structural explanation for earlier *in vitro* observations which suggested that ligand induced a conformational change in the LBD (Keidel *et al.*, 1994; Leid, 1994; Leng *et al.*, 1993). In the RXR-apo-LBD crystal structure the LBD forms an anti-parallel α-helical sandwich fold consisting of a three-layer structure with helices H4, H5, H8, H9 and H11 sandwiched between H1-H3 on one side and H6, H7 and H10 on the other. The C-terminal activation helix H12 points away from the LBD core (Figure 1.3). The liganded RAR and TR LBD crystal structures confirmed this general structural organisation, but also suggested that ligand induced a unique positional change in the C-terminal H12. This observation led to the proposal of a "mouse-trap" model of ligand binding, in which ligand triggers a conformational change in the NHR-LBD that repositions mainly H12 to form a lid over the ligand-binding pocket, but to a lesser extent also involves movement of helices H3, H6 and H11, thereby creating a new surface where coactivators can bind (Renaud *et al.*, 1995) (Figure 1.3). This has since been substantiated by the crystal structure of the RXR-holo LBD (Egea *et al.*, 2000).

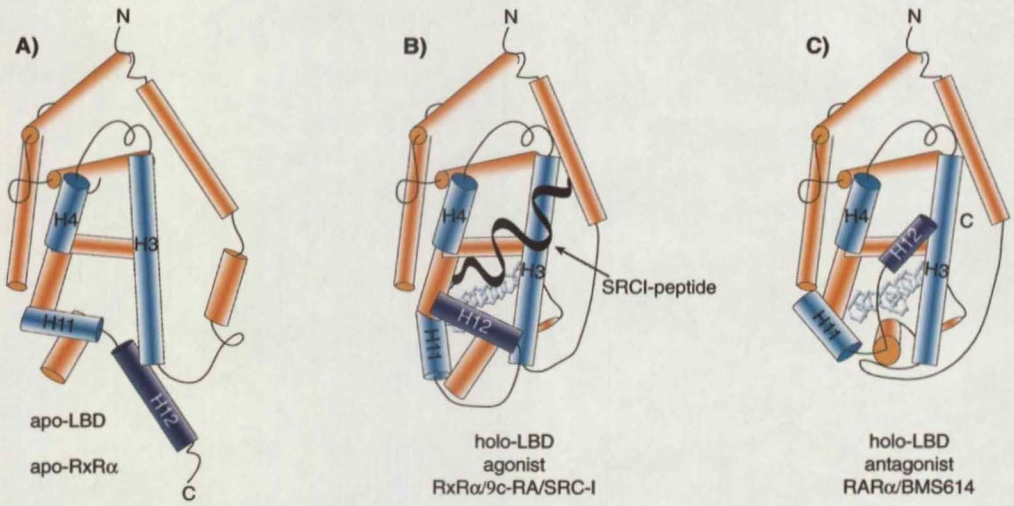


Fig.1.3 Conformational states of NHR LBDs.
Shown schematically are A) hRXRα apo-LBD, B) hRXRα holo-LBD bound to 9-*cis*-RA (central silver blue figure) (Egea *et al.*, 2000) and coactivator SRC-1 peptide (black ribbon) from PPAR model (Nolte *et al.*, 1998) and C) hRARαbound to antagonist BMS614 (Bourguet *et al.*, 2000). Note different positions of Helix H12 (dark blue cylinder) and other key helices (H3, H4 and H11 light blue cylinders) involved in coactivator and corepressor binding. In B), holo-LBD the helix H12 undergoes an agonist-induced conformational change in the so-called "mouse-trap" model. C), In the antagonist-bound LBD the helix H12 occupies the precise position of the SRC-1 peptide preventing coactivator binding i.e the molecular basis of NHR antagonism. In antagonist-bound ERα with raloxifene or tamoxifen (Shiau *et al.*, 1998) the position of H12 is analogous to that shown in C) with antagonist-bound hRARα. Figures adapted from Egea *et al.*, 2000.

The NHR-LBD may, however, have a more dynamic character than the RXR-apo- and holo-LBD structures suggest. This conclusion is supported by the crystal structure of the PPARγ-apo-LBD homodimer, in which the H12 in one monomer assumes a conformation as in RXR-apo-LBD, while H12 in the other monomer is positioned as in the RAR-holo-LBD (Nolte *et al.*, 1998). This latter "active" conformation is also observed in the monomer crystal structures of apo-LBDs of PPARγ and PPARβ (Uppenberg *et al.*, 1998; Xu *et al.*, 1999). This contrasts with the position assumed by H12 in the RXRα crystal structure after binding 9-*cis*-RA (Bourguet *et al.*, 1995; Egea *et al.*, 2000). Furthermore, resolution of the 9-*cis*-RA-bound PPARγ/RXRα heterodimer suggests that the PPAR H12 helix interacts with H7 and H10 of RXRα to stabilise the PPAR H12 helix in an active holo-position even in the absence of a PPAR agonist. This may also explain the "permissive" nature of some, but not all heterodimers, e.g. PPAR-RXR, which is activated by 9-*cis*-RA or a PPAR agonist, whilst non-permissive heterodimers, e.g. RAR/RXR, only respond to a RAR agonist, but not to 9-*cis*-RA (Renaud and Moras, 2000).

The AF-2 helix of RXR *in vitro* may also interfere with coactivator binding to its heterodimer partner e.g. VDR/RXR and RAR/RXR (Leo *et al.*, 2000; Leo *et al.*, 2001). This is consistent with studies suggesting deletion of the RXR AF-2 domain enhances coactivator binding to RAR/RXR (Westin *et al.*, 1998). The crystal structure of the unliganded RXR-LBD indicates that it exists as a tetramer consisting of two symmetric dimers. In each momomer the AF-2 helix masks the coactivator binding site so the tetramer could act to autorepress transcription. It has been proposed that addition of ligand dissociates the tetramer to form two activated homodimers (Gampe *et al.*, 2000). Resolution of the crystal structures of more agonist-bound NHR dimers, including VDR/RXR, with the receptor bound to coactivators or corepressors will provide greater insight into the role and relationship of the various functional domains, including the AF-1 and AF-2 H12 helix, in modulating NHR action.

1.4 GENERAL TRANSCRIPTION FACTORS AND ROLE IN POLYMERASE-II-DEPENDENT TRANSCRIPTION

1.4.1 The Preinitiation Complex (PIC)

RNA polymerase II-dependent transcription requires a large number of associated factors for pre-intiation complex (PIC) assembly *in vivo* (Cramer *et al.*, 2001; Gnatt *et al.*, 2001; Lemon and Tjian, 2000). The stepwise model of PIC assembly describes the ordered cooperation of multiple factors initially involving transcription factor IID (TFIID) in a multiprotein complex with TATA-binding protein (TBP) and the highly conserved TBP-associated factors (TAF_{II}s). Human TFIID comprises at least two distinct sets of factors: a core group containing $TAF_{II}250$, $TAF_{II}135$, $TAF_{II}100$ and $TAF_{II}28$; and another set of promoter-specific TAFs, including $TAF_{II}30$, $TAF_{II}20$ and $TAF_{II}18$ (Jacq *et al.*, 1994; Mengus *et al.*, 1995). TFIID binding is followed by TFIIA and TFIIB binding to the TATA element, which then associate with RNA Pol II and TFIIF, TFIIE and TFIIH to form the complete PIC (Hirose and Manley, 2000). However, this stepwise assembly model now appears less attractive in the light of the numerous other factors recently described to be involved in PIC assembly, especially within the context of a chromatin environment and the relatively rapid transcriptional responses observed *in vivo* (McNally *et al.*, 2000).

Thus a preassembly model with the RNA Pol II holoenzyme has been proposed as an alternative model to explain the requirements for regulated transcription (Lemon and Tjian, 2000). This model is supported by the purification of multiple proteins associated with the RNA Pol II complex. These include chromatin remodelling factors, such as SWI/SNF and CREB-binding protein (CBP), as well as mammalian coactivator complexes, such as the Mediator complex which share identical components to those within a TR/VDR-interacting complex called the TRAP/DRIP complex (Fondell *et al.*, 1996; Rachez and Freedman, 2001; Rachez *et al.*, 1999; Rachez *et al.*, 1998). As TFIID has not been detected in these complexes this suggests that recruitment of TFIID forms part of at least a two-step process with intial recruitment of chromatin remodelling factor complexes prior to recruitment of the RNA holoenzyme complex (Cosma *et al.*, 1999; Krebs *et al.*, 1999; Shang *et al.*, 2000).

Multiple NHRs have been shown to interact with different components of the basal transcription machinery, including TFIIB, TFIID and various TAFs and TBP itself (Baniahmad *et al.*, 1993; Blanco *et al.*, 1995; Fondell *et al.*, 1993; Ing *et al.*, 1992; Leong *et al.*, 1998b; MacDonald *et al.*, 1995; Mengus *et al.*, 1997). Thus, part of the mechanism of activated transcription by NHRs presumably involves direct communication with components of the PIC.

1.5. CHROMATIN, HISTONE MODIFICATIONS AND TRANSCRIPTION

1.5.1 The Nucleosome

Eukaryotic DNA is packaged with two copies of core histones H2A, H2B, H3 and H4 into nucleosomes, which form the basic repeating units of chromatin. Transcription of these tightly packaged genes is highly regulated by enzymatic events that alter the accessibility of the DNA to transcription factors (Kornberg and Lorch, 1999). Each nuclesosomal unit is formed by wrapping approximately 146 base pairs of DNA around a histone octamer particle containing one H3-H4 tetramer and two H2A-H2B dimers (Kornberg, 1974; Kornberg and Thomas, 1974). This model has been supported by resolution of the crystal structure of the histone octamer and of the nucleosome particle (Arents *et al.*, 1991; Luger *et al.*, 1997; Suto *et al.*, 2000). These studies revealed that the C-terminal histone-fold domains of core histones, but not the N-terminal histone tails, are essential for nucleosome assembly. The N-terminal domains however function as targets for a number of key enzymatic modifications, including acetylation, phosphorylation, methylation and ubiquitination, which may lead to changes in the accessibility of the underlying genome (Cheung *et al.*, 2000a; Luger *et al.*, 1997; Wolffe *et al.*, 2000). Part of this thesis addresses the potential roles of histone acetylation on NHR function.

1.5.2 Histone Acetylation and Transcription

Lysine residues within the N-terminal histone tails are sites of reversible acetylation. Numerous studies have observed the correlation between histone acetylation and transcriptionally active chromatin and conversely histone hypoacetylation and repressed chromatin (Alfrey *et al.*, 1964; Struhl, 1998). However, it was only as recently as 1996 that the first transcription-related histone acetyltransferase (HAT) enzyme was identified in tetrahymena. This HAT protein is related to yeast Gcn5 and its mammalian homologue p/CAF (p300/CBP-associated factor) (Blanco *et al.*, 1998; Brownell *et al.*, 1996; Yang *et al.*, 1996). Acetylation and deacetylation of these histone tails are executed by two opposing classes of factors: histone acetyltransferase enzymes or HATs, and histone deacetylases or HDACs. It appears that acetylated histones have a number of key effects on nucleosome function, including significantly reducing the affinity of the histone N-terminal tail for DNA. This leads to disruption of higher order chromatin structure and "opening" of chromatin which may facilitate transcription by allowing access of regulatory factors to the underlying DNA (Hong *et al.*, 1993).

1.5.3 Coactivators, HATS and Bromodomains

The demonstration that a number of key NHR coactivators, such as CBP/p300, p/CAF and the SRC (steroid receptor coactivator)/p160 family, contain HAT activity suggested a role for remodelling of chromatin as a mechanism for activated transcription by NHRs (Chen *et al.*, 1997b; Ogryzko *et al.*, 1996; Spencer *et al.*, 1997; Yang *et al.*, 1996). Recent evidence also suggests that acetylated lysines act as targets for a group of highly conserved HAT proteins which contain a 110aa sequence called the bromodomain (Winston and Allis, 1999). Bromodomains are present in a large number of conserved proteins from yeast to mammals, including Gcn5 and p/CAF (Dhalluin *et al.*, 1999), CBP/p300 and the general transcription factor TAFII250 (Jacobson *et al.*, 2000). These proteins appear to play a role in recruitment and stabilisation of other HAT containing remodelling complexes to increase local nucleosomal acetylation and may therefore be involved in cell- and promoter-specific gene transcription (Cheung *et al.*, 2000b; Winston and Allis, 1999).

1.5.4 Histone Acetyltransferases (HATs)

The carboxyl termini of two coactivators, SRC-1 and SRC-3, have been reported to possess HAT activity (Chen *et al.*, 1997a; Spencer *et al.*, 1997). This activity is weaker than and functionally different from the HAT activity of CBP/p300 and p/CAF. This may be related to differences in the carboxy termini of the SRC-1 and SRC-3 which do not appear to contain features corresponding to the HAT domains of p/CAF or Gcn5 (Clements *et al.*, 1999; Trievel *et al.*, 1999). Apart from the SRCs, p/CAF and CBP/p300, a large number of other proteins have now been demonstrated to possess HAT activity, including at least one sequence-specific DNA binding transcriptional activator, ATF-2 (Chen *et al.*, 2001; Kawasaki *et al.*, 2000). Chromatin immunoprecipitation experiments using antibodies specific for acetylated histone isoforms indicate that these HAT proteins co-immunoprecipitate in promoter regions associated with acetetylated histones i.e. remodelled "open" chromatin facilitating the binding of other transcription factors. HATs may also be involved in other cell functions, such as cell cycle progression e.g. via the actions of the general transcription factor and the bromodomain protein, TAFII250 (Dunphy *et al.*, 2000; O'Brien and Tjian, 1998), or in DNA repair and apoptosis e.g Tat-interactive protein-60 (TIP60) (Ikura *et al.*, 2000), as well as in DNA replication and RNA elongation, thus suggesting a wider role for histone acetylation than previously recognised (Chen *et al.*, 2001).

1.6. NUCLEAR RECEPTORS, COACTIVATORS AND HISTONE ACETYLATION

1.6.1 Nuclear Receptor Coactivators

Transcriptional "squelching" or interference observed between NHRs in transient receptor reporter transactivation assays first hinted at the presence of limiting factors required for transcription initiation in the cell (Meyer *et al.*, 1989). Numerous NHR coactivators have been cloned over the last 7 years since the initial identification of the ER-associated-proteins ERAP140 and ERAP160 (also known as RIP140 and RIP160/SRC-1, respectively) and the adenovirus E1A-associated p300 and related CBP proteins (Cavailles *et al.*, 1994; Cavailles *et al.*, 1995; Chakravarti *et*

al., 1996; Halachmi *et al.*, 1994; Hanstein *et al.*, 1996; Voegel *et al.*, 1996) (Table 1.2). These include the three major members of the p160 SRC family, the TRAP/DRIP complex, p/CAF and other proteins, many of which are present in multi-subunit complexes and act as bridging factors to the basal transcription machinery and/or possess chromatin remodelling activity.

1.6.2 Steroid Receptor Coactivator (SRC) p160 Family

The first NHR coactivators isolated, the ERAPs, were identified on the basis of ligand- and AF-2-dependent interaction with ERα. Subsequently, through expression cloning and yeast-two hybrid screening approaches, three related genes that encode the p160 factors were identified: SRC-1, SRC-2 and SRC-3 (see Table 1.2 for related acronyms). The SRCs share an overall 40% protein homology, with SRC-1 and SRC-2 more closely related to each other than to SRC-3. The protein homology between SRC-1 and SRC-2 is consistent with *in vitro* and *in vivo* experiments which show that SRC-2, but not SRC-3, can partially compensate for loss of SRC-1 function (Torchia *et al.*, 1997; Xu *et al.*, 2000a; Xu *et al.*, 1998). The SRCs have been reported to interact with a wide range of NHRs and other classes of factors (Table 1.2). In general they appear to interact with the AF-2 domain of NHRs (Hong *et al.*, 1997; Jeyakumar *et al.*, 1997) though SRCs also associate and potentiate the functions of the AF-1 domain of a subset of NHRs e.g ERα and AR (Bevan *et al.*, 1999; Ma *et al.*, 1999; Webb *et al.*, 1998). SRC-1 and/or SRC-2 also have been reported to modulate intramolecular interaction between the AF-1 and AF-2 domain of the PR, GR, ERα and VDR (Benecke *et al.*, 2000; Masuyama *et al.*, 1997; Onate *et al.*, 1998).

Table 1.2 Nuclear Receptor Coactivators "1994-2001"

Cofactor	Synonyms	Interacting NHRs	Interacting proteins	References
SRC-1	NCoA-1/p160 ERAP160/RIP160/ mSRC-1	PR, GR, ER, TR, RXR, VDR,HNF-4 PPARγ	CBP, AP-1, SRF, NF-κB, p53, c-jun/c-fos and others. HAT. AF2D, LD	(Cavailles *et al.*, 1994; Halachmi *et al.*, 1994; Onate *et al.*, 1995; Lee *et al.*, 1998; Na *et al.,1998;* Wang *et al;* 1998; Kodera *et al.*, 2000)
SRC-2	NcoA2/p160/hTIF2 p/CIP/mGRIP1/ mSRC-2	ER,GR,TR,RAR,VDR, PPARγ, RORα	CBP/p300 AF2D, LD	(Hong *et al.*, 1997; Hong *et al.*,1996 Voegel *et al.*, 1996) (Atkins *et al.*, 1999; Kodera *et al.*, 2000)
SRC-3	ACTR/p/CIP/ RAC3/TRAM1/ p160/mSRC-3/ xSRC3/AIB1	ERα>ERβ, PR,RAR,TR, VDR, PPARγ, RXR	CBP/p300/p/CAF/ SRC-1; HAT; overexpressed in breast cancer (AIB1) AF2D, LD	(Anzick *et al.*, 1997; Chen *et al.*, 1997b; Li *et al.*, 1997a; Torchia *et al.*, 1997) (Kodera *et al.*, 2000; Suen *et al.*, 1998; Takeshita *et al.*, 1997)
CBP/p300		RAR,RXR, ER,TR, PR, PPARγ	Multiple (see Fig 1.5). HAT; Bromodomain. AF2D and LD	(Chakravarti *et al.*, 1996; Kodera *et al.*, 2000; Ogryzko *et al.*, 1996) (Vo and Goodman, 2001) and others
TRAP220 or PBP/TRIP2	DRIP/ARC/SMCC Component of the Human Mediator Complex	TR,VDR,RAR,RXR, PPARα,PPARγ, RORα (PBP)	PML-RARα fusion protein No HAT activity LD2	(Fondell *et al.*, 1996; Rachez *et al.*, 1999; Rachez *et al.*, 1998; Yuan *et al.*, 1998);Atkins *et al.*, 1999; Kodera *et al.*, 2000; Shao *et al.*, 2000b).
DRIP150 DRIP205	Component of DRIP complex	GR AF-1 (DRIP150) GAR LBD (DRIP205)	DRIP 150 & 205 bridge GR AF-1 with AF2 domains	(Hittelman *et al.*, 1999)
p/CAF	Gcn5	RXR, RAR, ERα, GR, AR	E1A,CBP/p300;HAT; Bromodomain; AF2D, LD	(Blanco *et al.*, 1998; Yang *et al.*, 1996)

Footnote: Abbreviations: LD = LD; LI = ligand-independent; AF2D = AF-2 dependent; AF-1= AF1D= AF-1-dependent

Table 1.2 Nuclear Receptor Coactivators "1994-2001"

Cofactor	Synonyms	Interacting NHRs	Interacting proteins	References
SKIP	NcoA-62 (mSKIP)	VDR,RXR,RAR,ER,GR	Ski/Sno,Smad2 and 3, Notch AF-2 independent (VDR)	(Baudino *et al.*, 1998; Dahl *et al.*, 1998b; Leong *et al.*, 2001)
$TAF_{II}250$			General transcription factor; HAT; bromodomain	(Jacobson *et al.*, 2000) and others
TFIIB		VDR,ER, PR, RXR	SRC-1	(Baniahmad *et al.*, 1993; Ing *et al.*, 1992; MacDonald *et al.*, 1995) (Blanco *et al.*, 1995; Leong *et al.*, 1998b)
$TAF_{II}135$		RAR,VDR,TR	AF2D	(Mengus *et al.*, 1997)
$dTAF_{II}110$		TR, RXR		(Petty *et al.*, 1996; Schulman *et al.*, 1995)
$TAF_{II}30$		ERα	AF2D	(Jacq *et al.*, 1994)
$TAF_{II}55$		VDR, TRα	Ligand-independent	(Lavigne *et al.*, 1999)
BRG-1	Brahma/ SWI2/SNF2	GR/ER	ATP-dependent chromatin remodelling complex	(Fryer and Archer, 1998; Muchardt and Yaniv, 1993; Yoshinaga *et al.*, 1992).
PRMT1		ERα/TRβ1/AR Synergistic acts with CARM1 and SRC-2	SRC-1, SRC-2, SRC-3; Arginine-specific methyltransferase	(Koh *et al.*, 2001)
CARM1		ERα	SRC-1,SRC-2; related to PRMT1; can methylate histone H3 in vitro	(Chen *et al.*, 2000a; Chen *et al.*, 1999a)
p68/p70	Dead box protein	ERα AF-1	SRA/SRC-1	(Endoh *et al.*, 1999; Watanabe *et al.*, 2001)
Cyclin D1		ERα	SRC-1/SRC-3;ligand-independent	(Zwijsen *et al.*, 1998)

Footnote: Abbreviations: LD = LD; LI = ligand-independent; AF2D = AF-2 dependent; AF-1= AF1D= AF-1-dependent

Table 1.2 Nuclear Receptor Coactivators "1994-2001"

Cofactor	Synonyms	Interacting NHRs	Interacting proteins	References
PGC-1		PPARγ, ERα	SRC-1,CBP/p300; Tissue-restricted ligand-independent coactivator in brown fat	(Puigserver *et al.*, 1999; Tcherepanova *et al.*, 2000)
ARA70		AR	Enhances partial agonist activity of hormonal antagonists	(Miyamoto *et al.*, 1998; Yeh and Chang, 1996)
E6-AP	E3 ubiquitin ligase	AR,ER, PR,GR	RPF-1(hRSP5)	(Nawaz *et al.*, 1999)
L7/SPA		PR-A (RU486 bound)		(Jackson *et al.*, 1997)
SRA		PR-A	SRC-1;RNA coactivator; AF1D	(Lanz *et al.*, 1999)
TRIP1	SUG1	TR, VDR	26S Proteasome	(Lee *et al.*, 1995b; Masuyama and MacDonald, 1998)
BAG1L		VDR	Hsp70	(Guzey *et al.*, 2000)
WT1		VDR	Wilm's Tumor gene	(Maurer *et al.*, 2001)
p120		TRβ1, RXR	Coactivates through RXR on PPARγ/RXR dimer	(Monden *et al.*, 1999; Monden *et al.*, 1997)
EIA		TRβ1	CBP/p300;ligand-independent;bifunctional	(Wahlstrom *et al.*, 1999)
hNRC	ASC-2 RAP250/TRBP AIB3	TRα, TRβ, GR, RARα, RXRα, hERα and VDR, ERβ & PPAR (RAP250)	SRC-1, CBP, DRIP130 (TRBP); AF2D; AIB3 amplified in breast cancer	(Caira *et al.*, 2000; Ko *et al.*, 2000; Lee *et al.*, 1999; Mahajan and Samuels, 2000)
NRIF3	Isoforms β3-endonexin short and long (EnS & EnL)	TR & RXR, but not GR, PR, ERα nor VDR	AF2D, LD	(Li *et al.*, 1999a)
NRBF-1	Related to MRF-1	PPARα and others		(Masuda *et al.*, 1998)
PRIP	PPAR-interacting protein	PPARγ, PPARα , RARα, RXRα, ERα, TRβ1	Two LXXLL motifs	(Zhu *et al.*, 2000)

Footnote: Abbreviations: LD = LD; LI = ligand-independent; AF2D = AF-2 dependent; AF-1= AF1D= AF-1-dependent

1.6.3 Interaction Domains of SRCs

The SRCs contain a number of conserved functional domains (Figure 1.4). An amino-terminal basic helix-loop helix (bHLH)-Per-Arnt-Sim (PAS) domain, present in members of the PAS family of transcription factors (Hankinson, 1995). A central NHR-interacting domain (NID), which contains the 3 LXXLL motifs or NR boxes, mediates interaction with the AF-2 domain (Heery *et al.*, 1997; Voegel *et al.*, 1998). The SRCs contain two activation domains AD1 and AD2. AD1 appears to mediate AF-2 dependent interactions with most NHRs by functioning as a CBP/p300 interaction domain (CID) (Chen *et al.*, 1997a; Sheppard *et al.*, 2001; Voegel *et al.*, 1998). The CID has been mapped to the AD1 domain in SRC-1 and SRC-2 between aa 926-970 and 1095-1106aa, respectively (Ma *et al.*, 1999; Sheppard *et al.*, 2001). Different regions within the SRCs appear to interact differentially with NHRs. SRC-2/GRIP1 contains an auxilary domain designated NIDaux (aa1011-1121), which is required *in vitro* and in yeast for interaction with a subset of NHRs including the GR, AR and RARα, whereas the NID domain alone was sufficient for interaction with PR, RXRα, TRβ1 and VDR. Furthermore, the SRC-1 splice variant, SRC-1a, contains a fourth C-terminal NR box that is able to interact with the AR LBD. Another splice variant, SRC-1e, which lacks the fourth NR box is unable to interact with AR. In SRC-2/GRIP1 a separate domain between AD1 and AD2 designated NID_{AF-1} was necessary for interaction with the AR AF-1 domain (Ma *et al.*, 1999). Understanding the exact molecular mechanisms that govern NHR AF-1 domain interactions with the SRCs awaits resolution of the crystal structures of various NHR AF-1 domains bound with different coactivator peptides.

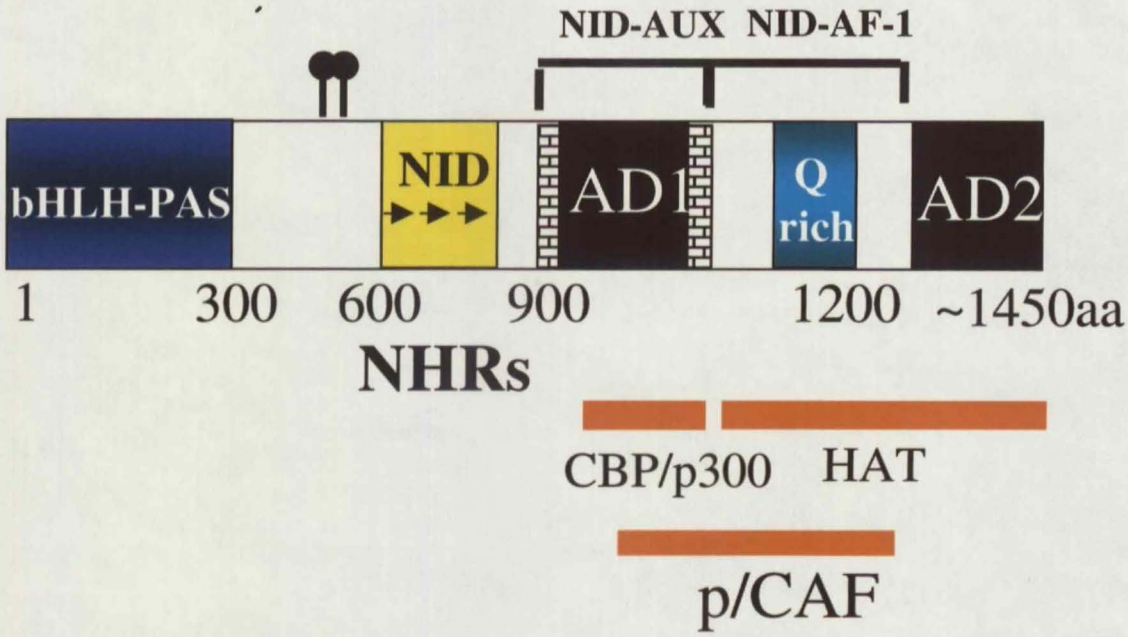


Fig. 1.4. General structure of p160 Steroid Receptor Co-activator (SRC) Family Members. This schematic representation is based on sequence alignment of SRC-1, SRC-2 and SRC-3 and their orthologues and on deletion analyses. Functional domains include a basic-helix-loop-helix motif (bHLH)-PAS (Per-Arnt-Sim) domain and a glutamine (Q)-rich domain. The NR box LXXLL motifs in the nuclear receptor interaction domain (NID) are indicated by 3 arrows. A fourth NR box (not shown here) which mediates AR interaction is found at the extreme C-terminus of a SRC-1a splice variant. An auxiliary domain NID-AUX (brick shading) mediates AR AF-2- dependent interaction while a region between the two transactivation domains AD1 and AD2 mediates AR AF-1 interaction (Ma *et al.*, 1999). CBP/p300 and p/CAF interaction and the C-terminal HAT domains are indicated by red bars. Two serine phosphorylation sites conserved in SRC-1 and SRC-2 (Ser^{569}) and all three SRCs (Ser^{517}) (Rowan *et al.*, 2000) are represented by two lolly-pops.

The AD2 domain interacts with other transcription factors such as the novel arginine methyltransferase proteins CARM1/PRMT1 which can methylate histone H3 *in-vitro* (Chen *et al.*, 1999a) and act synergistically with SRCs to augment ER- and AR-dependent transcription (Chen *et al.*, 2000a; Koh *et al.*, 2001). These *in vitro* studies suggest that SRCs can mediate various combinations of interactions with different domains of NHRs and other transcription factors (Zhou *et al.*, 1998). This may increase the diversity of responses mediated by different SRCs, and potentially may play a role in cell- and promoter-specific transcriptional regulation by NHR.

1.6.4 Functional Effects of Phosphorylation and Histone Acetylation on Coactivators.

Coactivator interaction with NHR may also be modulated by phosphorylation, as occurs within the AF-1 domain of ERβ to facilitate interaction with SRC-1 (Tremblay *et al.*, 1999). Furthermore, SRC-1, SRC-2 and SRC-3 themselves contain multiple phosphorylation sites, some of which lie within the domains that interact with CBP/p300 and p/CAF (Lopez *et al.*, 2001; Rowan *et al.*, 2000a; Rowan *et al.*, 2000b). MAPK-dependent phosphorylation of SRC-3 enhances recruitment and increased HAT activity of p300 (Font de Mora and Brown, 2000), and similarly it appears epidermal growth factor (EGF) regulates SRC-2/GRIP1 activity through a phosphorylation-dependent mechanism (Lopez *et al.*, 2001). The HAT activity of CBP is also enhanced by Cdk2-mediated phosphorylation (Ait-Si-Ali *et al.*, 1999).

SRC-3, a HAT protein, is itself acetylated by CBP/p300 disrupting its association with ER so inhibiting transcription (Chen *et al.*, 1997b; Chen *et al.*, 1999c). In these studies hormone-induced histone hyperacetylation was observed to be a transient event, peaking within a hour after hormone induction and downregulated thereafter. These and other observations suggest that NHR activation by ligand and subsequent association with coactivators is a dynamic process involving recycling of different cofactor complexes (Chen *et al.*, 1999c; Shang *et al.*, 2000). This hypothesis has been supported by a series of studies showing an ordered and interdependent recruitment of various chromatin remodelling and HAT

complexes. Using "purified" chromatin templates to examine RAR/RXR heterodimer-dependent transcription *in vitro*, efficient transcription followed a step-wise pattern, requiring the initial action of an ATP-dependent chromatin remodelling complex, followed by a CBP- and SRC-2-mediated acetylation event prior to recruitment of general transcription factors and RNA Pol II (Dilworth *et al.*, 2000). In a similar, but distinct series of events, transcription of the interferon-β (IFN-β) promoter required ordered recruitment of different chromatin remodelling complexes, including Gcn5, CBP-Pol II holoenzyme and SWI/SNF complexes (Agalioti *et al.*, 2000). These and other studies thus suggest there are gene-specific orders of recruitment of different HAT and chromatin remodelling complexes (Agalioti *et al.*, 2000; Reinke *et al.*, 2001).

1.6.5 *In vivo* roles of SRCs

The *in vivo* roles of two of the SRCs have now been analysed by gene knock-out experiments in mice. Mice with SRC-1 gene knock-out had a partial resistance to oestrogen, progesterone and thyroid hormones, with modest effects on growth and development, as well as effects on reproductive tissues, including the breast and uterus (Qi *et al.*, 1999; Weiss *et al.*, 1999; Xu *et al.*, 1998). The partial rather than total hormone resistance in these animals may be explained by compensatory increases in SRC-2 expression (Xu *et al.*, 1998). Interestingly, no apparent defects in PPARγ function have been documented in SRC-1 deficient mice (Qi *et al.*, 1999), whilst reduction of SRC-1 protein during sexual differentiation of the brain altered sex-specific brain morphology and behaviour (Auger *et al.*, 2000).

Knock-out of the SRC-3 gene in mice, the least homologous of the SRCs with the most tissue-restricted pattern of expression, shows a phenotype with moderate to severe growth retardation due to decreased IGF-1 secretion, delayed puberty, reduced female reproductive function and impaired mammary gland development (Xu *et al.*, 2000a).

1.6.6 Determinants of Coactivator Binding

The amino acid sequences of the NHR interaction domains of the SRCs and the coregulator RIP140 include leucine-rich pentapeptide motifs with the consensus sequence LXXLL, where X represents any other amino acid (Heery *et al.*, 2001; Heery *et al.*, 1997). These motifs, designated as NR boxes, have been found to be present in a number of other proteins known to interact with NHRs in a ligand-dependent manner (Heery *et al.*, 2001). Crystallisation of SRC LXXLL-containing peptides bound to liganded NHRs include the TRα and ERα holo-LBDs with peptides derived from NR box 1 and 2 of SRC-2/GRIP1 (Darimont *et al.*, 1998; Shiau *et al.*, 1998) and the PPARγ holo-LBD homodimer with an 88 residue fragment of SRC-1 (Nolte *et al.*, 1998). In these crystal structures, the LXXLL motif forms a short α helix (Darimont *et al.*, 1998; Nolte *et al.*, 1998; Shiau *et al.*, 1998). In the PPAR homodimer, the SRC-1 fragment containing the NR boxes 1 and 2 with each LXXLL motif binds one PPARγ LBD monomer (Nolte *et al.*, 1998). The coactivator helix docks to a hydrophobic cleft on the surface of the LBD, bounded on one side by the AF-2 H12 and on the other by the C-terminus of H3 (Feng *et al.*, 1998a). A highly conserved glutamate residue in the AF-2 domain makes contact with the amino-terminal end of the LXXLL peptide backbone while the C-terminal end of the LXXLL helix is held by a conserved lysine residue in helix 3. This forms a "charge clamp" around the coactivator NHR motif which acts to stabilise the NHR box in its binding site on the LBD surface. The crystal structure suggests the importance of the H3 lysine in transcriptional activation as observed *in vitro* (Feng *et al.*, 1998a; Henttu *et al.*, 1997; Jimenez-Lara and Aranda, 1999; Kraichely *et al.*, 1999; Mak *et al.*, 1999). Sequences amino- and carboxy-terminal to the LXXLL motif also appear to make additional contacts with the LBD and may play roles in determining the specificity of coactivator interactions with a particular NHR dimer or heterodimer (Chang *et al.*, 1999; Darimont *et al.*, 1998; Heery *et al.*, 2001; McInerney *et al.*, 1998; Shao *et al.*, 2000a).

1.6.7 CREB-binding–protein (CBP)/p300.

CREB-binding protein (CBP) and the related factor adenovirus E1A-associated protein p300 act as ligand-dependent AF-2 domain coactivators for a number of NHRs, including RAR, TR, RXR, and ERα and ERβ (Chakravarti *et al.*, 1996; Hanstein *et al.*, 1996; Kamei *et al.*, 1996; Kobayashi *et al.*, 2000). Interestingly, p300 interaction with the ERs occurs through the AF-1 domain which may facilitate ER AF-1 and AF-2 transcriptional synergism (Kobayashi *et al.*, 2000). NHRs interact through the extreme amino-terminus of CBP/p300. Competition for CBP/p300 occurs between AP-1 factors and NHRs (e.g. GR and RAR) which may lead to transrepression (Kamei *et al.*, 1996). The coactivation function of CBP/p300 is in a large part mediated by its HAT activity, which is considered to lead to local nucleosomal effects around target gene promoter regions to overcome chromatin-mediated repression (Bannister and Kouzarides, 1996; Kraus and Kadonaga, 1998; Kraus *et al.*, 1999; Ogryzko *et al.*, 1996). As CBP/p300 interacts with a large number of different classes of transcription factors, this suggests one function of CBP/p300 may be to act as transcriptional integrators in the cell (Vo and Goodman, 2001) (Figure 1.5). The long list of CBP/p300-interacting proteins includes general transcription factors such as TFIID, TFIIB and the RNA polymerase II holoenzyme, as well as other HAT proteins such as SRC-1, SRC-3 and p/CAF (Vo and Goodman, 2001). Thus, the ability of CBP to coordinate assembly of additional protein complexes appears to be an essential aspect of its function as a co-activator.

In addition to histones, CBP and p300 acetylate other substrates including sequence-specific transcription factors such as the tumour suppressor p53 and HNF-4 (Hepatocyte nuclear factor-4) modulating DNA binding of these factors (Gu and Roeder, 1997; Gu *et al.*, 1997; Soutoglou *et al.*, 2000). CBP/p300-mediated acetylation also can lead to negative effects on transcription, as in the case of T-cell

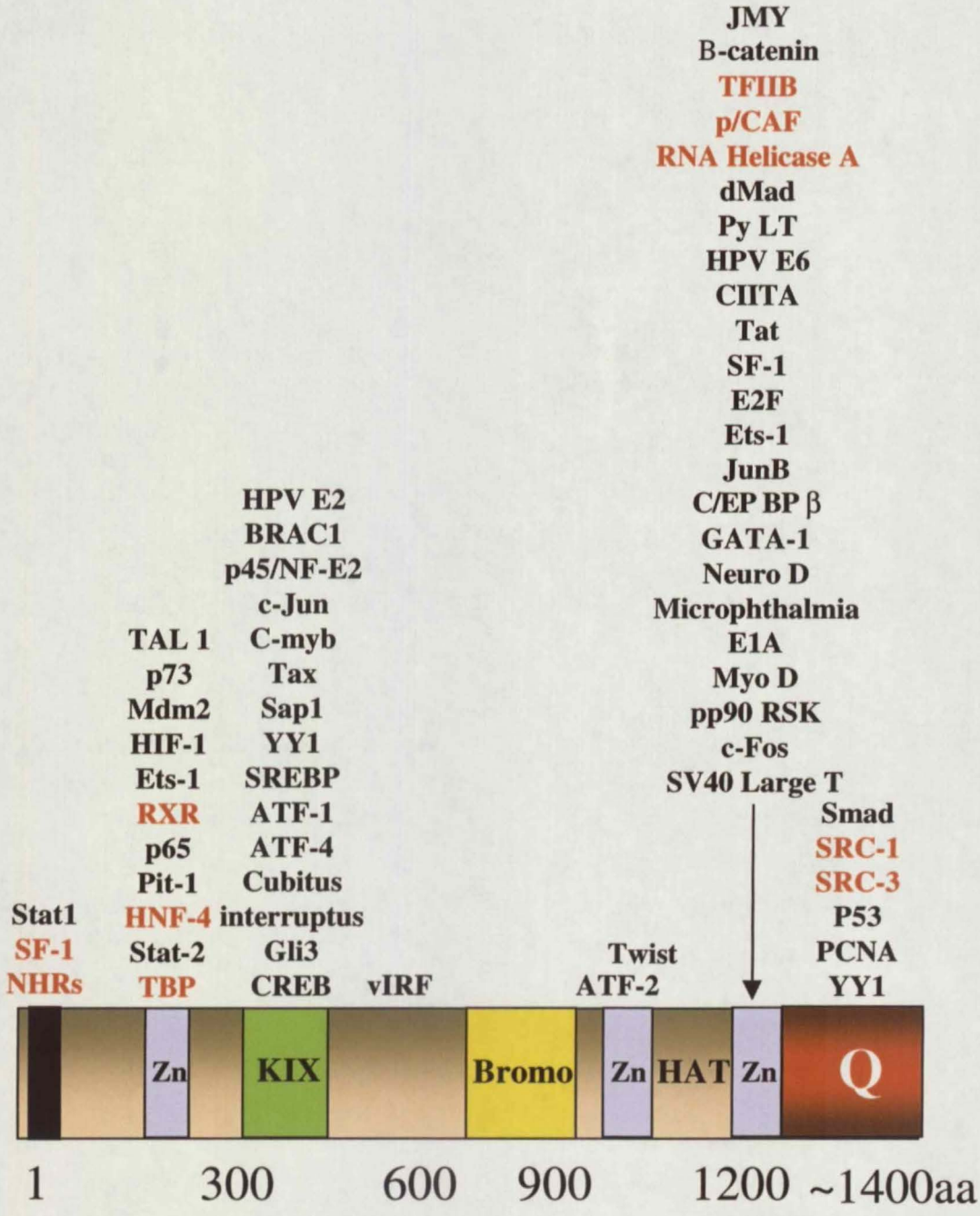


Fig.1.5 CBP/p300 structure and binding proteins.
The zinc fingers (Zn), CREB binding domain (KIX), bromodomain (Bromo), HAT domain and glutamine-rich domain (Q) are indicated. Interacting proteins are listed above their respective binding domains. NHRs, basal transcription factors and HAT proteins are indicated by red lettering.

factors (TCF) repression of Wingless signalling in *Drosophila* (Waltzer and Bienz, 1998). It also has been proposed that CBP HAT activity is modulated by other factors such as the adenovirus E1A protein, which inhibits acetylation of CBP/p300 and its associated transactivation function (Arany *et al.*, 1995; Chakravarti *et al.*, 1999; Eckner *et al.*, 1994; Hamamori *et al.*, 1999; Kurokawa *et al.*, 1998; Li *et al.*, 1999b). These latter findings suggest that E1A inhibits the function of CREB by preventing the assembly of CBP-co-activator complexes that contain RNA Poll II. In the case of RAR, interaction of CBP with SRC-1 appears to be critical for ligand dependent transcription (Kurokawa *et al.*, 1998). More recently, EIA has been shown to bind a HDAC-associated repressor protein called CtBP (carboxy-terminal binding protein) which has properties opposite to those of CBP/p300 (Boyd *et al.*, 1993; Zhang *et al.*, 2000b). CBP/p300 and p/CAF appear to be able to acetylate E1A at a site also important for CtBP binding and repression, suggesting that one mechanism for coactivator-mediated activation by CBP/p300 is through disruption of a CtBP-HDAC-repressor complex (Zhang *et al.*, 2000b). Furthermore, there is evidence that NHRs may modulate CBP/p300 HAT activity directly, as was recently shown for two naturally occuring mutants of HNF-1α (Hepatocyte nuclear factor–1α) associated with maturity-onset diabetes of the young (MODY). The mutant HNF-1α receptors bound more avidly to CBP and p/CAF than the wild-type receptor, but in contrast, were unable to stimulate the HAT activity of these proteins (Soutoglou, *et al.*, 2001).

Differences in p300 and CBP function have been suggested by an analysis of gene deletion experiments in mice which revealed distinct phenotypes (Goodman and Smolik, 2000; Kung *et al.*, 2000). Furthermore, in F9 cells abrogation of either CBP or p300 expression by cofactor-specific hammerhead ribosomes suggested that p300, but not CBP, was required for all-*trans*-retinoic acid-induced (RA)-differentiation of these cells. Additionally, transcriptional upregulation of the cell-cycle inhibitors p21 and p27 required either normal levels of p300 for the former or CBP for the latter, respectively. In contrast, both CBP and p300 was required for RA-induced apoptosis (Kawasaki *et al.*, 1998). In addition, loss of a single CBP allele in humans results in severe developmental defects as occurs in Rubinstein-

Taybi syndrome (Petrij *et al.*, 1995), suggesting that CBP/p300 levels may be limiting *in vivo* with deleterious effects of even relatively small decreases in expression levels. Furthermore, distinct roles for these two proteins have been demonstrated recently via the differential effects of growth factor-induced phosphorylation of these proteins. In these studies, CBP was recruited to POU homeodomain (Pit1) and AP-1 response element reporters after growth factor-induced serine phosphorylation. In contrast, p300 which lacks a phosphorylation site, bound only weakly to this transcription complex (Zanger, *et al.*, 2001,).

1.7 NHR COACTIVATOR COMPLEXES

1.7.1 The Mediator Family of Coactivator Complexes

Identification of other large protein co-activator complexes has increased the complexity of our understanding of NHR gene transcription. Apart from SRC p160 family and CBP/p300-associated protein complexes, these include the TR-associated proteins or TRAPs (Fondell *et al.*, 1996). An almost identical complex to the TRAP complex containing at least 9 proteins ranging from 70 to 250 kD was isolated as a complex of ligand-dependent VDR-interacting proteins (DRIPs) (Rachez *et al.*, 1999; Rachez *et al.*, 1996; Rachez *et al.*, 1998). Subsequently, other groups have isolated similar, if not identical complexes in yeast and mammals which include components of the ARC (activator recruited co-factor complex), CRSP/SMCC, NAT and PC-2 complexes (Hampsey and Reinberg, 1999; Malik *et al.*, 2000; Naar *et al.*, 1999; Ryu *et al.*, 1999; Sun *et al.*, 1998). This large composite co-activator complex that belongs to a family of related cofactors corresponding to the yeast Srb/Mediator complex (Gu *et al.*, 1999), and hence has also been termed the Mediator Complex (Ito and Roeder, 2001; Ito *et al.*, 1999; Ito *et al.*, 2000). A recent unifying nonmenclature has been proposed for the various Mediator complexes that designates TRAP/SMCC as Mediator (M)-T/S, DRIP as M-D, ARC as M-A, CRSP as M-C, NAT as M-N, PC-2 as M-P, human Mediator as M-S and the original yeast Mediator complex as Mediator (Rachez and Freedman, 2001). Whilst the Mediator-D and –A complexes have stimulating activity on chromatin templates *in vitro*, they do not contain intrinsic HAT activity. However, the yeast Mediator complex through one of its subunits, Nut1, does contain HAT activity (Lorch *et al.*,

2000). Among other proteins, these complexes contain another factor (Med 220/TRAP220/DRIP205/TRIP2/MPIP1) identical to a PPARγ-interacting protein (PBP) which mediates interaction with RNA Polymerase II, thus linking it directly to the basal transcription machinery (Li *et al.*, 1995; Myers and Kornberg, 2000; Rachez *et al.*, 1998; Yuan *et al.*, 1998; Zhu *et al.*, 1997). Med220/DRIP205/TRAP220 has also been shown to bind the VDR-LBD and other NHRs co-operatively with other cofactor complexes such as the SRCs (Rachez *et al.*, 2000; Treuter *et al.*, 1999; Yang *et al.*, 2000).

1.7.2 The p/CAF and SAGA complexes

p/CAF and Gcn5e are homologues of the yeast protein Gcn5, which is an essential component of a conserved family of multi subunit co-activator complexes referred to as the SAGA (*S*pt, *A*DA, *G*cn5 *A*cetyltransferase) complexes (Grant *et al.*, 1997). Purification of the SAGA and mammalian core p/CAF complexes has revealed the presence of a subset of TAFs including $TAF_{II}90$, $TAF_{II}68/61$, $TAF_{II}60$, $TAF_{II}25/23$ and $TAF_{II}20/17$ (Grant *et al.*, 1998; Ogryzko *et al.*, 1998). As p/CAF has also been demonstrated to interact with p300 and CBP (Yang *et al.*, 1996), and act as a coactivator for NHRs it suggests a potential role for the SAGA complex in NHR action (Blanco *et al.*, 1998; Chen *et al.*, 1997a; Korzus *et al.*, 1998).

1.7.3 ATP-dependent Chromatin Remodelling Complexes

NHRs also appear to require the action of a group of highly-conserved ATP-dependent chromatin remodelling complexes (Glass and Rosenfeld, 2000; Lemon and Tjian, 2000). Two related ATP-dependent remodelling complexes have been described in yeast as RSC (remodelling the structure of chromatin) (Cairns *et al.*, 1996; Pollard and Peterson, 1998) and the SWI/SNF complex (Owen-Hughes *et al.*, 1999). Homologues of SWI2/SNF2 are present in flies (Brahma) and mammals (BRG1, hBrm). In *Drosophila* a similar complex has been characterised called variously the NURF (nucleosome remodelling factor) (Tamkun *et al.*, 1992), ACF (ATP-utilising-chromatin assembly and the remodelling factor) (Ito *et al.*, 1997) and CHRAC (chromatin accessibility complex) (Varga-Weisz *et al.*, 1997). All these complexes contain ISWI, a member of the SWI2/SNF2 family, suggesting this protein may serve as the energy transducing component of the chromatin

remodelling machinery. Although yeast do not contain nuclear receptors, the activity of SWI/SNF complexes was required for GR activation in yeast (Yoshinaga *et al.*, 1992), whereas both hBrm and BRG1 have been shown to interact in a ligand-dependent manner with ER (Ichinose *et al.*, 1997). Furthermore studies of GR and PR activation of the MMTV promoter support a role for BRG1 and related ISWI complexes in chromatin remodelling and gene activation (Di Croce *et al.*, 1999; Fryer and Archer, 1998).

1.7.4 Dynamic Model of Cofactor-NHR Promoter Interaction

In a recent key study using chromatin immunoprecipitation assays (ChIP), endogenous ER cycled on and off its cognate promoter following ligand-activation in MCF7 breast cancer cells (Shang *et al.*, 2000). The ER bound to the promoter within 15 minutes of ligand treatment, but cycled on and off the promoter over a two hour period, as did associated cofactors such as SRC-3, PBP/TRAP220/DRIP205 and p300 with RNA Pol II, followed later by CBP and p/CAF. Furthermore, N-CoR and SMRT were recruited to the promoter following treatment with the anti-oestrogen tamoxifen. These studies are consistent with those observed *in vivo* for the GR on the MMTV promoter using fluorescence bleaching studies (McNally *et al.*, 2000), which also show a dynamic process with the receptor cycling rapidly on and off the promoter after ligand stimulation. Similar studies examining fluorescent-tagged SRC-1 and CBP to study ERα action also support a very rapid interaction dynamic (in the order of seconds) between the receptor and coactivator in response to agonists or antagonists in living cells (Stenoien *et al.*, 2001a; Stenoien *et al.*, 2001b). Studies of TRα recruitment of cofactor complexes in HeLa cells suggested that TRα interaction with SRCs, CBP and p/CAF, like ERα, occurs rapidly (~10min), whereas recruitment of TRAP220 complexes occurred much more slowly at about 3 hours post-T3 treatment (Sharma and Fondell, 2001). This contrasted with the study of Shang et al., 2000 (Shang *et al.*, 2000), in which ERα recruitment of αPBP, a component of the TRAP complex, occurred as rapidly as 30 minutes post-hormone treatment. Whether these differences in cofactor recruitment reflect receptor-, cell- or study technique differences remains to be determined. Nevertheless, it may be anticipated using

these types of studies that tissue- and NHR-specific cofactor-promoter interactions will be indentified. Temporal coordination of these interactions on and off the promoter in response to ligand and other stimuli may provide the subtlety and variation in mRNA synthesis required for precise tissue-, promoter and developmental stage-specific actions of the diverse NHR gene superfamily.

1.8 NUCLEAR RECEPTOR, COREPRESSORS AND HISTONE DEACETYLATION

1.8.1 N-CoR and SMRT Corepressors

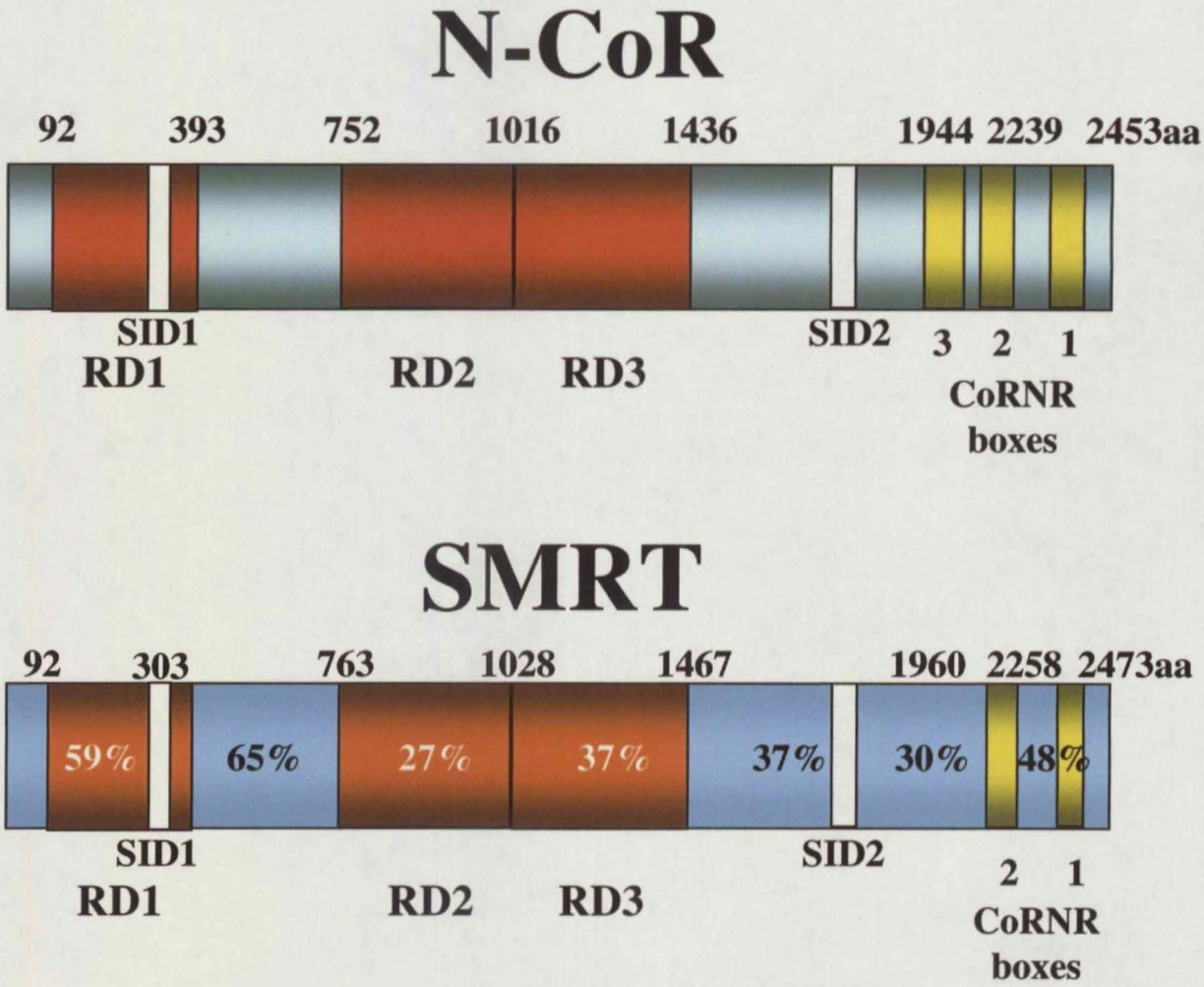
Two major nuclear corepressors have been cloned: N-CoR (Nuclear Corepressor) or RIP13 (Lee *et al.*, 1995a) and SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) or T3-associated cofactor (TRAC2) (Sande and Privalsky, 1996). N-CoR and SMRT interact, in the absence of ligand, with the TR, RAR, RXR and VDR and the orphan receptors Rev-erb and DAX1 (Chen and Evans, 1995; Crawford *et al.*, 1998; Horlein *et al.*, 1995; Tagami *et al.*, 1998; Zamir *et al.*, 1997a; Zamir *et al.*, 1996). Ligand binding causes decreased interaction of N-CoR to NHRs on most DNA sites *in vitro* and in intact cells (Heinzel *et al.*, 1997). N-CoR and SMRT also interact with many other classes of transcription factors including general transcription factors, such as various TAFs and TFIIB, as well as the TGF-β repressor and proto-oncogene c-Ski, helix-loop-helix (HLH) proteins Mad and partner Max, Notch and the interacting CBF1 protein and homeodomain proteins Pbx and Pit1, thus suggesting that corepressors play more general roles in the regulation of gene expression (Glass and Rosenfeld, 2000; Kao *et al.*, 1998; Muscat *et al.*, 1998; Nomura *et al.*, 1999; Wong and Privalsky, 1998; Zhou *et al.*, 1999; Zhou *et al.*, 2000b). These corepressors are also components of multi-protein repressor complexes, including mSin3 and various histone deacetylase enzymes (HDAC-1-8) that act together to compact chromatin and repress gene transcription (Alland *et al.*, 1997; Gray and Ekstrom, 2001; Jones *et al.*, 2001; Wen *et al.*, 2000).

1.8.2 Functional Domains and Effects of N-CoR and SMRT

N-CoR and SMRT are both approximately 270kD and contain a conserved NHR interaction domain in the C-terminus (Ordentlich *et al.*, 1999; Park *et al.*, 1999) (Figure 1. 6). In addition these proteins contain a highly related amino-terminal region with at least 3 independent repression domains capable of transferring active repression to a heterologous DNA binding domain (Chen and Evans, 1995; Horlein *et al.*, 1995). Interestingly thyroid hormone resistance syndromes are associated with mutations in the TR-β1-LBD that enhance ligand-independent interactions with N-CoR and SMRT (Yoh *et al.*, 1997). Remarkably, recruitment of N-CoR and SMRT appears to be essential for the antagonist activity of ER- and PR-specific antihormones, possibly by blocking the constitutive AF-1 trans-activation domain, or preventing coactivator binding (Jackson *et al.*, 1997; Lavinsky *et al.*, 1998; Shiau *et al.*, 1998; Smith *et al.*, 1997). N-CoR has also been proposed to serve as a co-activator in association with mSin3 and HDAC-2, but only in promoters harbouring negative T3 represser elements such as the TSH-β promoter (Sasaki *et al.*, 1999; Tagami *et al.*, 1997; Tagami *et al.*, 1999). SMRT repression may also be negatively modulated by the tyrosine kinase and the MAPK phosphorylation pathways, possibly leading to cellular redistribution of SMRT from the nuclear to the perinuclear or cytoplasmic compartment (Hong and Privalsky, 2000; Hong *et al.*, 1998; Lavinsky *et al.*, 1998).

1.8.3 Corepressor Nuclear Receptor Interactions

The two NHR interaction domains within N-CoR and SMRT each contain a conserved sequence consisting of the motif LXXI/HIXXXI/L referred to as a CoRNR box (Hu and Lazar, 1999; Nagy *et al.*, 1999; Perissi *et al.*, 1999) (Figure 1.7). This extended helix is similar to the NR box coactivator LXXLL motif and appears to be required for binding to unliganded NHRs (Zamir *et al.*, 1997b). Whereas N-CoR contains three CoRNR boxes, sequence comparison suggests SMRT only contains two such boxes (Webb *et al.*, 2000). Based on the stoichiometry of corepressor binding, it is likely that each of the CoRNR boxes interacts with a single NHR in a DNA-bound dimer (Cohen *et al.*, 1998; Zamir *et al.*, 1997b). The corepressor binding site on the LBD of interacting NHRs requires helices H3, H4



N-CoR	CoRNR 1	HSFADPASNLGLEDIIRKALMGSFDD	2276-2280aa
SMRT	CoRNR 1	AVQEHASTNMGLEAIIRKALMGKYDQ	
N-CoR	CoRNR 2	RTHRLITLADHICQIITQDFARNQVP	2073-2077aa
SMRT	CoRNR 2	GHQRVITLAQHISEVITQDYTRHH.P	
N-CoR	CoRNR 3	RGKTTITAANFIDVIITRQIASDKD	1949-1953aa
Consensus		I/LXXI/V I (Zhu *et al.*, 1999)	
		L XXI/H IXXXI/L (Perissi *et al.*,1999)	

Fig. 1.6. Schematic representation of corepressors N-CoR and SMRT indicating extent of amino acid homology between the two proteins and functional repression domains (RD1-3) in the N-terminal region. Sin3-interacting domains (SID1 and SID2) and nuclear receptor interaction domains (CoRNR boxes 1 and 2) are present in both N-CoR and SMRT with a third CoRNR box only within the C-terminal region of N-CoR (Webb *et al.*, 2000). Below, sequence alignment, with CoRNR boxes shown in **red** and surrounding conserved residues shown in **green**.

and H11 which overlap the coactivator interaction surface (Hu and Lazar, 1999; Nagy *et al.*, 1999; Perissi *et al.*, 1999; Renaud *et al.*, 2000). In contrast to coactivator binding to the LBD with the AF-2 activating helix H12 and H3 forming the "charge clamp", helix H12 inhibits corepressor binding to RXR and other NHRs (Schulman *et al.*, 1996; Zhang *et al.*, 1999a). The ligand activated "charge clamp" fits the shorter coactivator helix, blocking binding of the extended corepressor helix. This potentially represents the molecular mechanism for ligand-dependent displacement of the corepressor from the NHR LBD. Although no crystal structures have yet been resolved with a NHR-LBD bound to N-CoR or SMRT to confirm this model, modelling of the orphan receptors Rev-erbA and RVR, which lack the Helix 12, are consistent with this mechanism of corepressor-coactivator ligand-mediated switching (Renaud *et al.*, 2000). Thus a model has been proposed whereby ligand acts as a molecular switch to favourably alter the conformation of the LBD for coactivator binding at the expense of corepressor binding to the same overlapping interface (Hu and Lazar, 1999; Hu *et al.*, 2001; Nagy *et al.*, 1999; Perissi *et al.*, 1999).

1.8.4 Nuclear Receptor-Hormone Antagonist Crystal Structures

Crystal structures of antagonist-bound NHR-LBDs have been solved, including the ERα-LBD bound to raloxifene and tamoxifen, two different selective oestrogen receptor modulators (SERMs) (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998) (Figure 1.3). Raloxifene binds at the same site in ERα as the natural agonist 17β-oestradiol but induces a distinct H12 conformation. H12 is displaced by the long side chain of raloxifene so that it lies in a hydrophobic groove formed by H3, loop 3-4 and H4 with H12 partially burying the conserved lysine in H3 (Lys362) involved in formation of the coactivator "charge clamp". This presumably would interfere with coactivator NR box binding and provides a molecular mechanism for the antagonist activity of these ligands. Overall, therefore, it appears that the degree of agonist/antagonist effect correlates with the positioning of the H12 activation helix over the LBD (Gangloff *et al.*, 2001). In the ER-raloxifene and tamoxifen-bound structures, H12 contacts H3 and H5 through a hydrophobic surface (LXXML) similar to the LXXLL coactivator motif which orientates the H12 to mimic

coactivator interaction, but is incorrectly orientated for coactivator binding (Darimont *et al.*, 1998; Shiau *et al.*, 1998).

1.8.5 Differential interactions of N-CoR and SMRT with NHRs

Subtle but distinct differences in interaction occur between N-CoR and SMRT and different NHRs. TR and RevErb interacts more strongly with N-CoR than SMRT, and in gel shift assays N-CoR appears to bind TR homodimers in preference to TR-RXR heterodimers (Cohen *et al.*, 1998). RXR and PPAR showed no preference between N-CoR and SMRT, whereas RAR preferably interacted with SMRT (Cohen *et al.*, 1998). Furthermore, there appear to be differences in CoRNR box preference between NHRs. For example, TR interacts with both CoRNR boxes to mediate its repressive effects, whereas RXR, PPAR and RevErb primarily bind CoRNR2 box and RAR interacts strongly with CoRNR1, but minimally with CoRNR2 box (Cohen *et al.*, 1998; Hu and Lazar, 1999; Webb *et al.*, 2000).

1.8.6 N-CoR Knockout Mice Phenotype

A N-CoR gene knockout mouse, using a targeting construct to delete the 5′ Sin3 interaction domain (SID1), has demonstrated that N-CoR is indeed required for transcriptional repression *in vivo*. These mice develop defects in erythrocyte, T cell and neurological development and die in utero from severe anaemia (Jepsen *et al.*, 2000). In this same study, microinjection of IgG targeted against N-CoR and HDAC-3 in mouse fibroblasts transfected with a DR+1 RARE reporter paradoxically abolished ligand-dependent activation, suggesting that in some contexts N-CoR may act as a transactivator rather than repressor (Jepsen *et al.*, 2000). The mechanism for this effect remains unclear but indicate that N-CoR may serve bifunctional roles analogous to those of the Sin3 and Rpd3 proteins in yeast (Struhl *et al.*, 1998).

1.9 MAMMALIAN COREPRESSOR COMPLEXES

1.9.1 Sin3 and NuRD Repressor Complexes

As for coactivator complexes, a number of distinct but overlapping repressor complexes have been identified and purified. These include complexes with Sin3A, NuRD (nucleosome remodelling histone deacetylase), and N-CoR and SMRT, all of which may have distinct functional roles in mammalian cells. What role, if any, this redundancy and sharing plays is unclear. Nevertheless there is some evidence that corepressor protein complexes may be expressed in a cell-specific manner, and that this may be involved in cell- and differentiation specific repression events (Lavinsky *et al.*, 1998; Soderstrom *et al.*, 1997).

1.9.2 Sin3-HDAC Repressor Complexes

Critical insights into the mechanism of transcriptional repression by NHRs were provided by the discovery of mammalian homologues of yeast Sin3 (Ayer *et al.*, 1995) and the subsequent finding that these proteins interact with N-CoR and SMRT (Heinzel *et al.*, 1997). The yeast and mammalian Sin3 proteins are components of corepressor complexes that contain HDACs (RPD3 in yeast and HDAC-1/HDAC-2 in mammals). The list of mammalian proteins that repress via the Sin3 complex is now extensive (Knoepfler and Eisenman, 1999). For NHRs these include unliganded RAR and TR and antagonist-bound ERα and PR and VDR, which also all interact with SMRT and N-CoR (Lavinsky *et al.*, 1998; Shang *et al.*, 2000). Bound SMRT and N-CoR bring about transcriptional repression by recruiting the Sin3-HDAC complex. The Sin3-HDAC complex also mediates the effects of the cytokine transforming growth factor-β (TGF-β) signalling pathway. c-Ski, the viral homologue of the avian viral oncogene v-Ski, and the related Sno protein act as TGF-β repressors through an association with N-CoR and HDAC1 (Nomura *et al.*, 1999).

These studies provide evidence for a trancriptional model in which DNA binding factors recruit the Sin3-HDAC complex, while deacetylates histones leading to

transcriptional silencing. The mammalian Sin3 complex comprises at least 7 subunits, including the two deacetylases HDAC-1 and -2 plus Sin3 and two Sin-associated proteins, SAP30 and SAP18, and the Rb-associated polypeptides RbAp48 and RbAp46, which bind directly to core histones.

1.9.3 NuRD Repressor Complex

The NuRD complex is a Sin3-independent repressor complex but shares components with the Sin3 repressor complex, including HDAC-1, HDAC-2 and RbAp46 and RbAp48. It contains unique subunits including Mi-2, a protein related to the SWI/SNF chromatin remodelling family, MTA2 (metastasis-associated-protein 2) and MBD3 (methyl-CpG-binding domain-containing protein). Thus, the NURD complex restablishes a connection between gene repression and DNA methylation (Wolffe *et al.*, 1999; Zhang *et al.*, 1999c). The NuRD complex appears to function by repressing relatively large regions of chromatin in contrast to more localised gene-specific effects of Sin3 complexes (Knoepfler and Eisenman, 1999). More recently, the NuRD complex has been linked to NHR repression through its interaction with the novel corepressor Sharp (see below) (Shi *et al.*, 2001).

1.9.4 Other NHR Corepressor Proteins

As in the case of co-activators, a large number of other co-repressors have been cloned which may serve specific roles (Burke and Baniahmad, 2000). These include TRUP (TR uncoupling protein) (Burris *et al.*, 1995), SUN-CoR (Small Unique Nuclear receptor Corepressor) (Zamir *et al.*, 1997a), Alien (Dressel *et al.*, 1999; Polly *et al.*, 2000) and others (Burke and Baniahmad, 2000). TRUP inhibits transactivation by TR and RAR, but not RXR or ERα, possibly interfering with receptor DNA binding. SUN-CoR, a 16kD highly basic nuclear protein unrelated to N-CoR and SMRT, potentiates transcriptional repression by TR and RevErb *in vivo* and interacts with both N-CoR and SMRT (Zamir *et al.*, 1997a). Alien is a 34kDa protein that represses TR, EcR and VDR, but not RAR function. Alien is also involved with N-CoR in DAX-1 silencing of the orphan receptor steroidogenic factor–1 (SF-1) (Altincicek *et al.*, 2000; Crawford *et al.*, 1998). The *Drosophila*

homologue of N-CoR and SMRT, SMRTER, interacts with Sin3a and is involved in repression mediated by the heterodimer of ecdysone receptor (EcR) and *Drosophila* RXR homologue ultraspiracle (Tsai *et al.*, 1999). In addition an ER-specific repressor REA (Repressor of Estrogen Activity) and enhancer of anti-oestrogen activity has been recently cloned (Montano *et al.*, 1999). REA appears to compete with the coactivator SRC-1 for binding to liganded ERα via a NR box LKLLL motif (Delage-Mourroux *et al.*, 2000).

An interesting new group of corepressors which appear to also act as RNA binding proteins, PSF (polypyrimidine tract-binding protein-associated splicing factor) and the related NonO/p54nrb, interact with Sin3a independent of N-CoR and SMRT to mediate repression by unliganded TR and RXR (Mathur *et al.*, 2001). Interestingly, both genes are also disrupted by chromosomal translocations associated with renal cell carcinomas (Clark *et al.*, 1997). Another NHR corepressor, Sharp, (SMRT-HDAC-1 Associated Repressor Protein) interacts directly with SMRT, HDAC-1 and HDAC-2 and components of the NuRD repressor complex. It also contains RNA binding domains and interacts with the RNA coactivator SRA, with its expression upregulated by oestrogen treatment (Shi *et al.*, 2001). This increase in expression may act as a regulatory feedback loop analogous to that also observed with the orphan NHR and repressor SHP (Goodwin *et al.*, 2000; Lu *et al.*, 2000). Thus, there appear to be N-CoR/SMRT-dependent and -independent pathways by which NHRs can repress target gene transcription. How these different repression pathways are integrated *in vivo* to modulate the actions of NHRs remains an unanswered question in the field.

1.9.5 Purification and Identification of N-CoR and SMRT Core Complexes.

Through protein affinity purification and other techniques, a large number of distinct but overlapping N-CoR and SMRT protein complexes have been identified. Most studies suggest that N-CoR and SMRT exist in complexes of 1 to 2MDa in molecular mass (Underhill *et al.*, 2000). Until recently N-CoR and SMRT had not been co-isolated within a complex with the Sin3 repressor protein. However, two N-CoR/HDAC complexes, both > 1 MDa in size, have since been

purified from Xenopus oocytes, with one complex containing Sin3 (Jones *et al.*, 2001). In that study, the N-CoR1 complex contained Sin3A/HDAC-1 and RbAP48, whereas the N-CoR2 complex contained HDAC activity from a non-Sin3 associated HDAC, possibly a class II type HDAC. Other studies suggest that N-CoR and SMRT associate with other distinct HDAC family members, including HDAC-3, -4 and -5, the latter two HDACs binding *in vitro* to N-CoR through different domains than HDAC-3 (Wen *et al.*, 2000). N-CoR has also been isolated in association with components of the SWI/SNF remodelling complex, including BRG1-associated-factors (BAFs) and the SWI/SNF-related CBP activator protein (SRCAP) (Underhill *et al.*, 2000). Similar studies identifying components of SMRT complexes reveal that, like N-CoR, SMRT associates with HDAC-3 and HDAC-4, (Guenther *et al.*, 2000; Huang *et al.*, 2000; Li *et al.*, 2000a), but also associates with distinct HDACs not present in N-CoR complexes, including the type II Sin3a-interacting HDACs, HDAC-5 and -7.

1.9.6 Histone deacetylases (HDACS)

Histone deacetylation is mediated by three different classes of enzymes. Class 1 enzymes are all homologous to yeast Rpd3 and consist of HDACs-1, -2, -3 and -8. HDAC-1 and HDAC-2 are parts of both the Sin3 and NuRD multi-protein complexes. HDAC-3 appears to be functionally distinct as it is the only member of the Class 1 HDACs which is known to translocate between the nucleus and cytoplasm (Kohchbin, 2001). The Class 2 HDACs, comprising HDAC 4-7, were identified on the basis of homology to yeast HDA1 protein. SMRT and N-CoR have been found to be associated with both class 1 and 2 HDACs, including HDAC-3, -5 and -7 [Wen, 2000 #7668}. The function of class 2 HDACs, like HDAC3, appears to be regulated in part by nucleo-cytoplasmic shuttling. In specific cell lines HDAC-4 and -5 are retained in the cytoplasm, depending on the activity of the 14-3-3 anchoring protein; the latter being involved in subcellular localisation of a number of interacting proteins including NHRs and their associated cofactors, e.g. GR and RIP140 (Kohchbin, 2001; Zilliacus *et al.*, 2001). Consequently, the functioning of nuclear complexes containing HDAC-4, -5, or -7 depends on the intracellular locations of these HDACs (Dressel *et al.*, 2001; McKinsey *et al.*, 2000). A third class of recently cloned histone deacetylases are

related to the yeast repressors of transcription, the SIR family; their role in NHR-dependent transcription, if any, remains to be determined.

1.9.7 Bifunctional Cofactors Involved in both Activation and Repression of NHR-dependent Transcription.

In addition to transcriptional coactivators and corepressors, recent evidence suggests that there is a third loosely defined group of cofactors that possess bifunctional (i.e activator and repressor) properties. The bifunctional nature of these cofactors suggests that they may play roles in the tissue-, receptor- and promoter-specific actions of NHRs. This group includes RIP140, which was originally isolated as an ERα and AR coactivator (Cavailles *et al.*, 1995; Ikonen *et al.*, 1997; Kumar *et al.*, 1999). It was subsequently determined that RIP140 does not act as a coactivator for PPAR, RAR or TR, but represses GR and RAR signalling (Subramaniam *et al.*, 1999; Wei *et al.*, 2001). These effects may possibly occur through competition by RIP140 with SRC-1 for NHR binding and/or through RIP140 interaction with HDAC repressor complexes (Treuter *et al.*, 1998; Wei *et al.*, 2000).

The bifunctional protein mZac1, isolated as a SRC-2/GRIP1-interacting protein in a yeast two hybrid screen, interacted *in vitro* with SRC-2 and CBP/p300 and acted as a NHR coactivator. In the same cells, however, mZac1 repressed SRC-2-enhancement of ER-dependent reporter activity, suggesting possible competition between mZac1 and SRC-2 for ER (Huang and Stallcup, 2000). Furthermore, these effects were cell- and promoter-type specific. Similarly, Ying-yang-1 (YY1) and the general transcription factor TFIIB can behave as cell- and/or promoter-specific activators or repressors of NHR function (Blanco *et al.*, 1995; Ericsson *et al.*, 1999). Another bifunctional protein, NSD1 (NHR-binding SET domain containing protein-1), was identified by interaction with the RARα-LBD in a yeast two hybrid screen. NSD1 interacts with both unliganded and liganded NHRs through distinct ligand-independent repression and ligand-dependent activation domains. In addition, NSD1 has a number of functional domains implicated in chromatin-mediated transcriptional regulation, including a SET domain, PHD fingers and a SET-associated Cys-rich domain (SAC) domain (Huang *et al.*, 1998).

Another possible bifunctional protein, CIA (Coactivator independent of AF-2 function) was identified through its interaction with the orphan receptor Rev-erb family member RVR, which lacks an AF-2 domain (Suave *et al.*, 2001). CIA coactivation of ER-dependent transcription required a NHR binding element with an overlapping NR coactivator box and a CoRNR repressor motif. Specific domains within CIA contained either activation or repression function. Although not yet tested, these observations suggest that CIA, like NSD1, could potentially act as a both a coactivator and repressor.

Thus, there is a growing set of potential bifunctional coregulator proteins which may play specific roles in NHR-mediated effects on gene expression. The biological importance of these proteins remain to be determined. The possibility that different NHR-interacting coactivators, corepressors and bifunctional factors play intersecting roles in modulating the cell-, promoter- and receptor-dependent actions of the NHR superfamily will be an informative extension of current investigations.

1.10 ROLE AND SIGNIFICANCE OF COACTIVATORS/COREPRESSORS IN HUMAN DISEASE

There is substantial evidence that abnormal levels or function of NHR coactivators and corepressors and their associated HAT and/or HDAC protein complexes play roles in the development of human disease (Archer and Hodin, 1999; Cress and Seto, 2000; Graham *et al.*, 2000; Minucci and Pelicci, 1999). Th evidence includes effects on cellular proliferation and differentiation in relation to various human malignancies such as acute leukaemia and breast cancer. Additionally, hormone resistance states and developmental abnormalities related to NHRs and associated cofactors have also been defined in gene knockout studies in mice, but most await confirmation of equivalent gene defects in human genetic disease (Adachi *et al.*, 2000).

A number of tumour suppressor genes or proto-oncogenes, such as the retinoblastoma (Rb) and the TGF-β Smad-repressor protein c-Ski, also interact with HDACs, whereas the Rb-associated E2F activator protein associates with HATs (de Caestecker *et al.*, 2000; Harbour and Dean, 2000; Massague *et al.*, 2000; Nomura *et al.*, 1999; Vassilev *et al.*, 1998). Rb may also interact with other chromatin remodelling complexes like the SWI/SNF family (Zhang *et al.*, 2000a). These interactions thus appear to play roles in the underlying tumour-promoting effects following their dysregulation.

Disordered interactions with corepressors or coactivators underly the pathogenesis of various human leukaemias associated with chromosomal translocations. In acute promyelocytic leukaemia (PML), the RARα gene is fused to the PML gene or the related PLZF gene to yield the fusion protein PML/RAR or PLZF/RAR (Minucci and Pelicci, 1999). Both fusion proteins retain the ability to interact with a N-CoR/HDAC repressor complex. Dissociation of N-CoR from the PML/RAR fusion protein requires pharmacological doses of all-trans-retinoic acid (RA) *in vitro*, which is consistent with the similarly large doses required clinically to induce differentiation of PML/RAR expressing myeloid blasts and disease remission. In contrast, PLZF/RAR fusion proteins associate with N-CoR in a RA-resistant manner and require combined RA and histone deacetylase inhibitors, such as trichostatin, to switch the function of these fusion proteins from an inhibitor to an activator of RA signalling (Minucci and Pelicci, 1999). In acute myeloid leukaemia, the AML1 transcription factor is fused to the N-CoR/SMRT-interacting nuclear activator ETO (Minucci *et al.*, 2000; Zhang *et al.*, 2001b). The p300-interacting domain of AML1 is lost in the chromosomal translocation but replaced by the ETO protein, which retains a N-CoR interacting domain. Hence, the resultant fusion protein AMTL1/ETO is converted from an activator to a constitutive repressor, a change which is fundamental to its role in leukaemogenesis (Cress and Seto, 2000).

In an analogous manner to chromosomal translocations involving corepressors, various translocations which disrupt the CBP/p300 gene are associated with haematological malignancies. These include translocations that lead to fusion proteins of the MOZ (monocytic leukaemia zinc finger) or MLL (mixed lineage leukaemia) proteins with CBP/p300 (Goodman and Smolik, 2000). Though it is not totally clear, it appears these translocations lead to aberrant histone acetylation and hence increase the oncogenic potential of the rearranged gene products (Goodman and Smolik, 2000). Thus various key proteins involved in cellular proliferation and differentiation and cell-cycle progression require the action of histone modifying proteins. As such, it is highly likely that further insights from human disease will arise from dissection and study of these molecular pathways.

1.11 MODEL OF NHR-MEDIATED TRANSCRIPTION

NHRs mediate a vast range of effects on gene expression through the influence of a complex series of interactions that maintain the receptor and its target gene in either a repressed or an activated state. The roles of various protein complexes that interact with NHRs to mediate effects on chromatin structure are fundamental to this regulation (Figure 1.7). Unliganded NHRs interact with the corepressors N-CoR and SMRT, which form molecular platforms for interaction with large protein complexes that act as chromatin deacetylation machines. In response to ligand-stimulation, the NHR LBD undergoes a conformational change that leads to corepressor release and recruitment of coactivators, such as the SRCs, CBP/p300 or p/CAF, within large protein complexes that mediate localised nucleosomal acetylation. This is followed by a second round of nucleosomal acetylation through the action of chromatin remodelling complexes (e.g. SWI/SNF complexes) which promote larger regions of target promoter acetylation. Finally, transcription initiation proceeds with recruitment of the RNA holoenzyme Pol II complex directly or within an associated remodelling complex (e.g. with CBP or Mediator complex) (Hassan *et al.*, 2001). In keeping with recent evidence, there appears to be a dynamic recycling of both receptor and associated cofactor complexes until other histone modification events, or possibly other mechanisms, lead to termination of

transcription initiation (e.g., phosphorylation of C-terminal domain of RNA Pol II, or acetylation by CBP/p300 of SRC-3, or targeting of receptors and/or activators for ubiquitin-mediated destruction). Eventually these events return the target promoter region to a repressed deacetylated state ready for further rounds of transcription intiation (Chen *et al.*, 1999b; Cheung *et al.*, 2000a; Chi *et al.*, 2001; Lonard *et al.*, 2000; Shang *et al.*, 2000; Tansey, 2001). Some or many of these factors and complexes may act in a cell- and promoter-specific and temporally regulated manner to effect the final transcriptional outcome required for precise effects of NHR-mediated target gene expression.

Figure 1.7 Model for Nuclear Hormone Receptor-Dependent Transcription.

Shown schematically in this figure are a series of steps (A-H) that involve interactions between NHRs and multiple cofactor complexes.

A) In the centre of this transcriptional "circle" is the basal repressed state of transcription with an unliganded NHR heterodimer (red and yellow ovals e.g RXR/TR heterodimer) binding a hormone response element (HRE shown by red box) within a target gene promoter. The unliganded NHR heterodimer interacts with a N-CoR/SMRT/HDAC-Sin3 repressor complex and possibly other repressor complexes and/or factors e.g NuRD/HDAC complex, to maintain the chromatin environment in a histone deacetylated and/or methylated silent state.

B) Entry into the transcriptional "circle" by NHRs is initiated by a ligand-induced "switch" (represented as "lightning bolt") that causes a critical conformational change in the NHR-LBD.

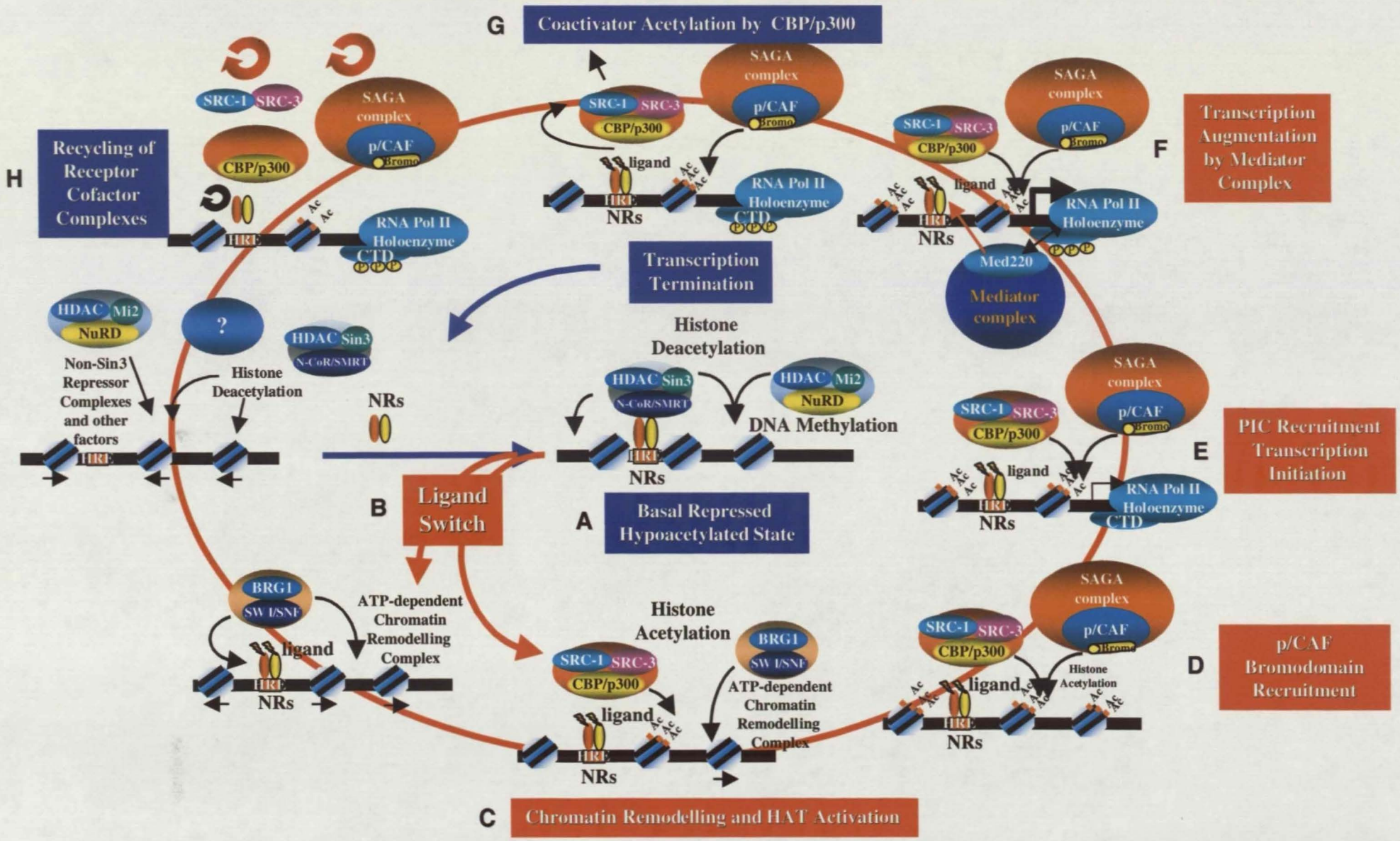
C) This leads to release of corepressor binding and recruitment of coactivator histone acetyltransferase (HAT) complexes that "open" chromatin. An alternative pathway may firstly involve recruitment of an ATP-dependent chromatin remodelling complex (e.g. SWI/SNF/BRG1) which leads to nucleosomal "shifting" (indicated by arrows). This is followed by coactivator HAT complex recruitment that, with the remodelling complex, synergistically prepares the target promoter region for later recruitment of RNA Pol II enzyme (Mizuguchi *et al.*, 2001). Gene–specific and/or other differences may possibly dictate the order of recruitment of these various complexes (Agalioti *et al.*, 2000; Dilworth *et al.*, 2000; Hassan *et al.*, 2001; Reinke *et al.*, 2001; Shang *et al.*, 2000).

D) Acetylated histones then act as targets for a second round of histone acetylation through recruitment of bromodomain HAT coactivator complexes e.g p/CAF within the SAGA complex.

E) This is followed by recruitment of the RNA Pol II holoenzyme complex at or near transcription initiation which is associated with carboxy-terminal domain (CTD) phosphorylation, **(F)** the latter having a possible role in transcription elongation as well as receptor-cofactor recycling (Shang *et al.*, 2000). The RNA Pol II holoenzyme complex may in turn recruit other coactivator complexes such as the Mediator complex, thereby maximising the transcriptional response.

G) Transcriptional termination may occur following CBP/p300-mediated acetylation of SRCs, which disrupts the NHR interaction and removes both receptor and coactivators and eventually RNA Pol II from the target promoter environment. This presumably would lead to decreased local histone acetylation and a less "open" chromatin environment. Other possible roles of specific components of the RNA Pol II enzyme complex such as targeting coactivators for ubiquitin-mediated destruction remain to be determined in the context of NHR-dependent transcription (Chi *et al.*, 2001).

H) Recycling of receptor and cofactor complexes may lead to further rounds of transcription initiation. Alternatively, if the effects of the ligand-stimulus wane, unliganded NHRs may recruit HDAC repressor complexes and perhaps other cell-specific factors to return the target promoter to a basal repressed hypoacetylated state.


G
Coactivator Acetylation by CBP/p300
SAGA complex
p/CAF
Bromo
SRC-1
SRC-3
CBP/p300
ligand
HRE
NRs
RNA Pol II Holoenzyme
CTD
F
Transcription Augmentation by Mediator Complex
Med220
Mediator complex
H
Recycling of Receptor Cofactor Complexes
Transcription Termination
HDAC
Mi2
NuRD
?
Sin3
N-CoR/SMRT
Non-Sin3 Repressor Complexes and other factors
Histone Deacetylation
NRs
B
Ligand Switch
A
Basal Repressed Hypoacetylated State
DNA Methylation
E
PIC Recruitment Transcription Initiation
BRG1
SW I/SNF
ATP-dependent Chromatin Remodelling Complex
Histone Acetylation
D
p/CAF Bromodomain Recruitment
C
Chromatin Remodelling and HAT Activation

1.12 RESEARCH AIMS

A fundamental question in understanding the molecular mechanism(s) of nuclear hormone receptor action is the precise milieu of regulatory factors required for tissue-specific gene regulation. The diversity of NHRs, their ligands and the large range of associated factors, many of which modify the chromatin environment, have revealed over the last 5 years a complexity previouly underestimated by those in the field. Through the diversity and complexity of the factors involved in these processes, a mammalian cell achieves the homeostatic nuclear environment essential for normal function and gene expression. Identification of novel and potentially key coregulators in this process may lead to an improved understanding of the transcriptional regulation mediated by the NHR gene superfamily and provide further insights into mechanism(s) of human development and disease.

Thus, the primary aim of this thesis was to investigate whether novel NHR cofactors may mediate tissue- or cell-line specific actions of NHRs. It was hypothesised that such factors may modulate NHR transcriptional effects in relation to cell growth and differentiation. A secondary aim was to determine the molecular mechanisms by which these cofactors mediate effects on NHR-dependent gene transcription. These studies led to identification of novel as well as previously characterised cofactors that interacted with NHRs (Chapter 3). The remainder of this thesis then focused on one of these factors, the Ski-interacting protein (SKIP), which behaved as a cell line- and receptor-specific bifunctional coregulator (coactivator and corepressor) of NHR gene transcription (Chapters 4 & 5). Subsequently, a third aim was to determine whether SKIP could mediate transcriptional crosstalk between the NHRs and other signalling pathways involved in cellular growth and differentiation. This led to studies of the role of SKIP in modulation of the TGF-β signalling pathway by examination of its interaction with Smad proteins and the Smad-repressors, c-Ski and Sno (Chapter 6).

In summary, a transcriptional coregulator, SKIP, was identified as a NHR-interacting factor and characterised to be a modulator of both NHR and TGF-β signaling, thus suggesting that it may mediate cross-talk between the NHRs and cell surface receptor stimulation of a cell.

Chapter Two

Materials and General Methods

2.1 MATERIALS

2.1.1 Chemicals/reagents and suppliers

All organic and inorganic chemicals were of Analytical Reagent grade or higher. Sodium dodecyl sulfate (SDS), sodium hydroxide, acetic acid (glacial), ethanol, chloroform, glycerol, hydrochloric acid, boric acid, polyvinylpyrrolidone, urea, formamide, formaldehyde solution (37%), sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium bicarbonate, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and ammonium persulfate (APS) were from BDH Laboratory Supplies, Poole, England. Sodium chloride, D-glucose, trisodium citrate, sodium acetate, potassium acetate, iso-amyl alcohol, calcium chloride, methanol, sodium azide, magnesium chloride, potassium chloride, magnesium sulfate, potassium hydroxide, glycine and sodium pyrophosphate were from Ajax Chemicals, Auburn, NSW, Australia. Tris base, ethidium bromide, 2-mercaptoethanol, bromophenol blue, xylene cyanol, Coomassie brilliant blue R250, bovine serum albumin (fraction V; BSA), salmon sperm DNA,), o-nitrophenyl β-D-galactoside (ONPG), dithiothreitol (DTT), phenylmethyl-sulfonyl fluoride (PMSF), aprotinin, leupeptin, Triton X-100, NP40, ethylenediamine tetraacetic acid (EDTA), ethylene glycol-bis-N,N,N',N'-tetraacetic acid (EGTA), sodium fluoride, 5-bromo-4-choloro-3-indolyl-β-galactopyranoside (X-GAL), lithium acetate, dimethyl sulfoxide (DMSO), 50% PEG 4000 (polyethylene glycol), 3-aminotriazole (3AT) and acid washed glass beads (425-600 microns) were from Sigma Aldrich Chemical Company, St Louis, MO, USA.

Cesium chloride was from Boehringer Mannheim, Mannheim, Germany and ampicillin was from CSL, Parkville, Victoria, Australia. DNA grade agarose and dextran sulfate were from Progen Industries, Brisbane, QLD, Australia. Isopropanol was from Mallinckrodt, Chesterfield, MO, USA. Guanidine thiocyanate and acrylamide (40% solution) 29:1 and 19:1 acryl:bis were from Amresco, Solon, Ohio, USA. Sarkosyl and N,N,N',N'- tetramethylethylenediamine (TEMED) were from International Biotechnologies Inc., Newhaven, CT, USA.

Ficoll (Type 400) was from ICN Biomedicals Australasia, Sydney, NSW, Australia and Sequagel™ concentrate acrylamide solution was from National Diagnostics, Atlanta, GA, USA. 3-[N-morpholino]propane sulfonic acid (MOPS) was from Research Organics, Cleveland, Ohio, USA and BCS scintillant was from Amersham International, Little Chalfont, Buckinghamshire, England. Ultrapure deoxynucleotide triphosphates were from Promega, Madison, WI, USA and poly-dIdC.poly-dIdC was from Pharmacia Biotech, Uppsala, Sweden. Phenol/chloroform and blue dextran dye were from Applied Biosystems, Foster City, CA, USA. HAP Bio-Gel was from Biorad, Hercules, CA, USA. $1,25(OH)_2D_3$ was from Tetrionics Inc., Madison, WI, USA and 9-*cis*- retinoic acid, all-*trans*-retinoic acid, dexamethasone and T3 (3,3',5-triiodo-$_L$-thyronine) were from Sigma-Aldrich.

2.1.2 Radiochemicals

[α^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml), [γ^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml), [α^{35}S]dATP (>1000 Ci/mmol, 10 µCi/ml) were from Amersham International. L-[^{35}S]-methionine (1175 Ci/mmol, 10 mCi/ml) was from NEN™ Life Science Products, Boston, MA, USA.

2.1.3 Autoradiography

Kodak Biomax™-MR and -ML X-ray film was used (Eastman Kodak, Rochester, NY, USA). Biomax™-MR film was used with Lightning Plus™ intensifying screens (NEN™).

2.1.4 Enzymes

All restriction endonucleases, DNA polymerase I (Klenow fragment), T4 DNA Ligase, calf intestinal alkaline phosphatase (CIP) and T4 polynucleotide kinase were from Promega. T7 DNA polymerase was from Perkin Elmer, Foster City, CA, USA. All enzyme buffers were supplied by the manufacturer.

2.1.5 Molecular Biology Kits

The Wizard™ Plus Miniprep System, Luciferase Assay System, Wizard™ PCR Preps DNA purification system and TNT® T7 Quick Coupled *in vitro* Translation System were from Promega. The QIAfilter Plasmid Maxi kit was from Qiagen Inc. Valencia, CA, USA. The Super-base sequencing kit was from Bresatec, Adelaide, Australia and the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit was from Perkin-Elmer. The Galacto-Light™ and Galactostar™ chemiluminescent assay systems were from TROPIX, Inc., Bedford, MA, USA. The ECL Plus™ Western blotting detection system was from Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England, and the Alkali Cation Yeast transformation kit was from BIO 101, Inc., La Jolla, CA, USA. Kits were used according to the manufacturer's instructions.

2.1.6 Synthetic oligonucleotides

Oligonucleotides were synthesised and reverse phase purified by Macromolecular Resources, Fort Collins, Colorado, USA, Beckman Instruments (Australia) Pty. Ltd., Sydney, Australia, or Sigma-Aldrich Pty. Ltd., Sydney, Australia.

2.1.7 Media

2.1.7.1 Media for bacterial culture

Bacterial cultures were grown in L-broth (1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 1% (w/v) sodium chloride; pH 7.5), sterilised by autoclaving. For solid medium (L-agar) 1.5% (w/v) agar was added to the L-broth. Bacto-agar, tryptone and yeast extracts were from Difco Laboratories, Detroit, MI, USA.

2.1.7.2 Media for mammalian cell culture

DMEM (5x liquid), αMEM (5x liquid), HEPES buffer (1 M) and penicillin/streptomycin (5000 IU/ml, 5000 μg/ml) were obtained from Trace Biosciences, Sydney, Australia. HAM-F12 (powdered stock) and EDTA solution (2% v/v) were from ICN, Sydney, Australia. Sodium bicarbonate solution (7.5% w/v), L-glutamine solution (200 mM) and fetal bovine serum (FBS) were from

Gibco BRL, Life Technologies, Gaithersberg, MD, USA. Gentamycin (40 mg/ml) was from Pharmacia and Upjohn, Perth, Australia. PBS tablets and trypsin (lyophilised) were from Amresco and CSL, respectively.

2.1.7.3 Media for yeast culture

Bacto-yeast extract, Bacto-peptone, Bacto-agar and yeast nitrogen base without amino acids with ammonium sulfate were from Difco Laboratories. Complete synthetic medium (CSM; powder) lacking in Histidine (His), Leucine (Leu), Tryptophan (Trp), CSM-HLT, CSM-LT or CSM-T were from BIO 101 Inc.

Untransformed yeast cells were grown in YEPD media (1% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-peptone, 2% (w/v) glucose, 40 mg/ml adenine sulfate), sterilised by autoclaving. YEPD agar contained 2% (w/v) Bacto-agar. Transformed yeast were grown in CSM (drop-out base; DOB) liquid medium (0.67% (w/v) yeast nitrogen base without amino acids with ammonium sulfate, 2% (w/v) glucose) with 0.064% (w/v) CSM-LT, sterilised by autoclaving. CSM agar contained 2% (w/v) Bacto-agar. Following sterilisation, all liquid media were supplemented with 0.3 mM Ade. Yeast transformed with a single plasmid were grown in CSM-LT medium supplemented with 0.4 mM Trp or a mixture of 1 mM Leu, 0.25 mM Ile and 0.25 mM Val. The following amino acids for 1x dropout media were obtained from Sigma: L-isoleucine (30mg/L), L-valine 150mg/L), L-adenine hemisulfate salt 20 mg/L, L-arginine (20mg/L), L-histidine HCL monohydrate (20mg/L), L-leucine (100mg/L), L-lysine-HCl 30mg/L), L-methionone (20mg/L), L-phenylalanine (50mg/L), L-threonine (200mg/L), L-tryptophan (20mg/L), L-tyrosine (30mg/L) and L-uracil (20mg/L).

2.1.8 *E. coli* strains

Escherichia coli strain XL1-Blue (Stratagene, La Jolla, CA, USA) (F'::Tn10 *pro*A^{+}B^{+}*lac*I^{q}D(*lac*Z)M15/*rec*A1 *end* A1 *gyr*96 (Na1^{r}) *thi* *hsd*R17 (r_k-m_k+) *sup*E44 *rel*A1 *lac*) was used in all transformations except those to express GST-fusion proteins (section 2.2.18.1).

Escherichia coli strain BL21-Gold (Stratagene) was used to express GST-fusion proteins. The genotype is: *E. coli* B F$^-$ *ompT hsdS*(r_B-m_B-) *dcm*$^+$ Tet^r *gal endA* Hte.

Escherichia coli strain HB101 was used for leucine complimentation (section 2.2.2.3). The genotype of this E. *coli leuB* mutation strain (F$^-$ *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*14 *gal*K2 *lac*Y1*pro*A2 *rps*L20(Sm^r) *xyl*5λ- *leu mtl*1).

2.1.9 Mammalian cell lines

NIH3T3 mouse fibroblast cells, P19 mouse embryonal carcinoma cells and COS-1 and CV-1 monkey kidney cells were obtained from the American Type Culture Collection, Manassas, VA, USA. ROS24/1 and ROS17/2.8 cells were a gift from G. Rodan, Merck, Sharp and Dohme, West Point, PA, USA. Mouse (FVBN) osteoblast cells from primary cultures were prepared and cultured as described (Thomas *et al.*, 2000).

2.1.10 Yeast strains

The strains used in yeast two- and three-hybrid assays were obtained from Clontech Laboratories Inc., Palo Alto, CA, USA. Yeast strain YRG2 used for cDNA library screening was obtained from Stratagene (Table 2.1).

2.1.11 Commercial plasmid vectors

pSG5 and pBluescript SK- were from Stratagene. pAS2-1, pBridge, pACTII, pLEXA, pM and pVP16 were from Clontech Laboratories. pCR3.1 was from Invitrogen, Carlsbad, CA, USA. Mammalian two-hybrid plasmids pACT and pBIND were from Promega.

Table 2.1 Yeast strains, genotype and selectable markers.

Strain	Genotype	Reporter	Transformation Markers
SFY526	*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp 1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3* :: G_AL1U_{AS}-GAL1TATA-lacZ	*lacZ*	*trp1, leu2*
CG1945	*MATa,* ura3-52, *his3-2*^{0}0, ade_2-1_{01}, lys2-$80_{1,t}$rp $_1$-*901, leu2-3,* 112, *gal4-542, gal80-538,cyhr2,LYS2::GAL1UASGAL1TATA-HIS3, URA3::GAL417mer(x3)-CyC1TATA-lacZ;*	*lacZ*	*trp1, leu2*
Y187	*MATα, ura3-52, his3-200, ade2-101, trp 1-901, leu2-3, 112, gal4Δ, met$^-$, gal80Δ, URA3* :: $GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ*	*lacZ*	*trp1, leu2*
YRG2	*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp 1-901, leu2-3,* 112, *gal4-542, gal80-538,LYS2*::UAS_{GAL1}-$TATA_{GAL1}$-*HIS3, URA3* :: UAS-$GAL4_{17mer(x3)}$-$CYC1_{TATA}$-*lacZ*	*lacZ*	*trp1, leu2*

2.2 METHODS

2.2.1 Yeast two-hybrid analysis

2.2.1.1 Principle of the yeast two-hybrid system

The yeast two-hybrid assay (section 3.3.1) uses components of the yeast GAL4 transcription system to assess interactions between proteins. In this assay, candidate interacting proteins are expressed as fusions with either the DNA binding or activation domain of the GAL4 transcriptional activator (GAL4DBD and GAL4AD, respectively). Interaction between the recombinant proteins

reconstitutes the GAL4DBD and GAL4AD, and promotes their binding to the DNA element. This element comprises the UAS (upstream activating sequence) consensus within the *GAL1* promoter linked to the integrated*lacZ* reporter gene (Fields and Song, 1989; Fields and Sternglanz, 1994). Binding thus induces *lacZ* expression, and β-galactosidase accumulation, the latter measured by a liquid reporter assay to quantitate the strength of the interaction between the two candidate proteins.

2.2.1.2 Transformation of yeast and selection of transformants

YRG-2, CG1945, SFY526 or Y187 (section 2.1.10) yeast colonies were picked from YEPD plates into 5 ml YEPD cultures and grown to saturation at 30°C. Fresh 100 ml YEPD cultures were inoculated and grown to OD_{600} = 0.4 - 0.6. Yeast were transformed using the Alkali Cation Yeast transformation kit, according to the manufacturer's instructions. Cells were pelleted by centrifugation at 2000 rpm for 5 min, rinsed in 10 ml TE pH 7.5 and centrifuged again. Cells were made chemically competent by incubation in 5 ml lithium/cesium acetate solution at 30°C for 30 min with gentle shaking. Cells were then centrifuged as above and resuspended in 1 ml TE pH 7.5.

100 µl competent cells were incubated with 1 ug plasmid DNA, 5 µl carrier DNA and 5 µl histamine solution in 10 µl H_2O for 15 min at room temperature. The cells were mixed into 0.8 ml polyethyleneglycol and 0.2 ml TE / cation mix and incubated for 10 min at 30°C. Cells were then incubated at 42°C for 10 min, followed by cooling to 30°C. Cells were centrifuged as above and resuspended in 200 µl CSM medium lacking the appropriate amino acid for selection, then spread onto appropriate agar plates and grown for 3 to 4 days at 30°C. Transformants were restreaked and grown for a further 3 to 4 days.

Transformants were inoculated to CSM cultures lacking the appropriate amino acid for selection, and grown to saturation at 30°C with vigorous shaking. Glycerol

(20%) stocks were prepared from these cultures, and used for inoculation of subsequent test cultures.

2.2.1.3 Mating of yeast for two- and three-hybrid assays

Haploid CG1945 or SFY526 transformants containing GAL4DBD-expressing plasmids were mated with Y187 haploid strains that harbour GAL4AD-expressing plasmids. A single colony of CG1945 or SFY526 was suspended in 1 ml YEPD medium, and six Y187 colonies were suspended in 3 ml YEPD. 20 µl of each suspension was combined in 160 µl YEPD in a 96-well plate and grown at 30°C overnight with shaking. The diploid cultures were spread to CSM-LT plates and grown for 2 to 3 days at 30°C, then several colonies were restreaked to select against contaminating haploid colonies.

2.2.1.4 Yeast two-hybrid protein interaction assay

Diploid mated strains were inoculated to 5 ml CSM-LT cultures and grown to saturation at 30°C with shaking. These cultures were inoculated to test cultures of 5 ml CSM-LT, at OD_{600} = 0.1. Test cultures were grown at 30°C with shaking for 4 - 5 h then incubated with 10^{-8} M $1,25(OH)_2D_3$ or ethanol vehicle control for ~ 16 h at 30°C. Cells were centrifuged at 3,500 rpm for 4 min at room temperature and the pellets lysed in 100 µl breaking buffer (100 mM Tris-HCl pH 8.0, 20% glycerol, 2 mM DTT, 5 mM PMSF) by vortexing for 30 sec followed by 30 sec break, 6 times, at 4°C, using one packed cell volume of glass beads. The cellular extract was fractionated by centrifugation at 14,000 rpm for 1 min at 4^0C and the protein concentration was determined using the Biorad assay (section 2.2.12.3). β-galactosidase activity of the extracts was measured using the Galacto-Light™ or Galactostar™ chemiluminescent assay (section 2.2.1.5).

2.2.1.5 Chemilumiscent β–galactosidase assay

10 - 20 µl lysate was incubated with 100 µl of Galacton™ substrate at room temperature for 1 h, protected from light. Luminescence was measured for 10 sec using an Autolumat LB 953 luminometer (Berthold, Bad Wildbad, Germany), after the injection of 100 µl Light Emission Accelerator. For yeast three-hybrid assays β-galactosidease was measured using the Galactostar™ chemiluminescent assay in

96 well-plates (Opti-plate™) using the Topcount™ microplate scintillation counter as per the manufacturer's instructions (Canberra Packard, Five Dock, Australia).

2.2.2 cDNA library screening protocols

2.2.2.1 Yeast two-hybrid library screening

Library screening was performed as per the manufacturer's instructions (Clontech) using a sequential lithium acetate transformation protocol with the yeast strain YRG2, which had previously been transformed with the VDR wildtype-pAS2-1 plasmid and expressed the VDR-GAL4DBD fusion protein (Figure 3.3). Three VDR-expressing yeast colonies were inoculated into 100 mL of YEPD media and incubated at 30°C for 16 hours with shaking at 230rpm. Approximately 75ml of this saturated culture was then inoculated into one litre of fresh YEPD and incubated at 30°C with shaking at 230rpm till mid-log phase (~OD_{595} 0.5). The cell pellet was resuspended in 500mL of distilled water, re-pelleted by centrifugation and resuspended in 8mL of 1x TE/lithium acetate.

Five hundred µg of DNA from a human adult liver cDNA library (Clontech) and 20 mg of calf testes carrier DNA (Clontech) was mixed with 8 mL of competent cells with 60 mL of sterile polyethylene glycol (PEG 4000)/lithium acetate, which were mixed by brief vortexing and incubated at 30°C for 30 min with shaking at 230 rpm. DMSO to 10% (7 mL) was then added and gently mixed and the cells heat shocked at 42°C in a water bath for 15 minutes before being chilled on ice and pelleted by centrifugation and resuspended in 10 mL 1x TE buffer. The transformation mix was then plated (200µL per 150mm plate) onto 50 CSM-His-Leu-Trp plates supplemented with 10mM 3-AT, half of which were supplemented with 30µL 10^{-5}M $1,25(OH)_2D_3$ tper 30mL media to a final concentration of 10^{-8}M. Plates were then incubated for between 4-7 days at 30°C to allow for colony growth.

2.2.2.2 β-galactosidase colony filter assays

Histidine positive clones were restreaked on CSM-HLT media in absence of presence of 10^{-8}M $1,25(OH)_2D_3$ to ensure a second round of selection. All persistent histidine positive clones were then streaked on Whatman 5 filter paper on top of CSM-HLT media in the absence or presence of 10^{-8}M $1,25(OH)_2D_3$ and grown at 30°C for 3-4 days. Filters were then permeabilised by snap freezing for ~10 secs in liquid nitrogen, thawed and dried at room temperature and placed on top of a clean filter presoaked in Z buffer/X-gal solution (Z buffer {Na_2HPO_4.7H20 16.1g/L; NaH_2PO_4.H20 5.5 g/L; KCl 0.75g/L and MgSO4.7H20 0.246 g/L pH 7.0} with 0.27 mL β-mercaptoethanol and 1.67 mL X-gal (20mg/mL) per 100mL of Z buffer) and incubated at 30°C for up to 8 hours to allow colonies to express visible levels of β-galactosidase.

2.2.2.3 Leucine complimentation to isolate interacting cDNA plasmid

Isolation of the leucine pACTII plasmid containing the "putative" interacting cDNA was performed by leucine complimentation with the HB101 E. *coli leuB* mutation strain (section 2.1.8). His+ and LacZ+ yeast colonies were inoculated into 5mL of CSM –Leu (Trp+) for 48 hours. Yeast DNA was isolated by vortexing cell pellet for 2 min with 0.3 grams of acid washed glass beads in 200uL yeast lysis solution (Triton X-100 2% v/v; SDS 1% V/V; NaCl 100mM;Tris-Cl (pH 8.0) 10 mM and EDTA 1mM) and 200uL of pehnol: choloroform: isoamyl alcohol (25:24:1) (Promega); following centrifugation to pellet cells the supernatant was removed and DNA precipitated (section 2.2.4.4). The DNA pellet was then washed in 70% ethanol and dried under vacuum and resuspended in 20-40uL of TE buffer.

HB101 E.*coli* cells were electoporated on ice as per the manufacturer's instructions using a Biorad Gene Pulser (Hercules, CA, USA). One uL of plasmid DNA were mixed in 50 uL of HB101 cells in a 0.2cm cuvette (Biorad) and electroporated at 2.0V and 200 ohms with 25uFd capacitance. One mL of LB without antibiotics was added to cells which were then incubated at 37°C for 60 mins shaking at 230 rpm. Cells were pelleted by centrifugation and washed twice in 1.5 ml of M9 minimal media (consisting of 200 mL of 5X M9 salts, 100mL of 10X dropout-Leu mixture,

2mL of 1M $MgSO_4$, 20 mL of 20% glycerol and 0.1mL of $CaCl_2$. 5X M9 medium per litre consists of 30g Na_2HPO_4, 15g KH_2PO_4; 5g NH_4Cl; 2.5g NaCl; and 15mg $CaCl_2$ (Sambrook *et al.*, 1989)). Cells were then plated on M9 medium containing 50ug/mL ampicillin, 40ug/mL proline and 1mM thiamine-HCl and 1X CSM-Leu dropout mixture. Leu+, Amp resistant transformants were isolated by growth in 3 mL LB ampicillin using the Wizard minipreps DNA kit (Promega) (section 2.2.4.1).

2.2.2.4 Screening Genbank/EMBL using ANGIS

Sequencing was performed by automatic flourescent ABI Perkin Elmer as per manufacturer's instructions (section 2.2.9.2) using a GAL4AD forward primer: 5'-ACGGACCAAACTGCGTATAACGCG-3' corresponding to 851-859aa of GAL4AD and approximately 100 bp upstream of cDNA cloning site in the pACTII vector. Sequences were assembled by Autoassembler (Perkin Elmer) and compared to available sequences in the NR Nucleic and Protein (National Center for Biotechnology Information (NCBI), Bethesda, MD, USA and Australian National Genomic Information Service (ANGIS), (Sydney, Australia) assembled databases using the BLASTN, BLASTP, BLASTX and FASTA DNA and protein search programs.

2.2.3 Mammalian Cell Culture

COS-1, CV-1 cells and NIH3T3, were maintained in DMEM with 10% FBS, 4 mM L-glutamine, 14 mM sodium bicarbonate, 20 mM HEPES and penicillin/streptomycin (100 IU/ml, 100 µg/ml). The cell lines ROS17/2.8 and ROS24/1 were maintained in HAM-F12 with 7.5% FBS, 1.52 mM L-glutamine, 0.8 mM calcium chloride, 14 mM sodium bicarbonate, 28 mM HEPES and 10 µg/ml gentamycin. P19 cells were maintained in αMEM with 5% FBS, 4 mM L-glutamine, 14 mM sodium bicarbonate, 20 mM HEPES and penicillin/streptomycin (100 IU/ml, 100 µg/ml). All media was adjusted to pH 7.2 - 7.4. All cells were maintained at 37°C/5% CO_2 and passaged by incubation with trypsin (0.025%)/EDTA (0.02%) in PBS at 37°C for 2-5 min, followed by dilution into fresh medium.

2.2.4 Preparation of nucleic acids

2.2.4.1 Small scale preparation of plasmid DNA

Small quanitities of plasmid DNA were prepared using the Wizard™ Plus Minipreps DNA Purification System, which uses alkali lysis followed by purification of plasmid DNA from the cleared lysate using a silica-based resin.

A single colony was inoculated into 2 ml of L-broth containing 100 µg/ml ampicillin, and incubated with shaking at 37°C overnight. Plasmid DNA was isolated according to the manufacturer's intructions, and eluted from the resin into 50 µl water or 1 x TE (20 mM Tris-HCl pH 8.0, 1 mM EDTA). Typically ~ 10 µg of DNA was recovered using this method.

2.2.4.2 Large scale preparation of plasmid DNA

Large amounts of plasmid DNA were prepared using the QIAfilter Plasmid Maxi Kit, which uses alkali lysis followed by purification of plasmid DNA from the cleared lysate using anion-exchange resin. The DNA is then concentrated and desalted by isopropanol precipitation.

A single colony was inoculated into 5 ml of of L-broth containing 100 µg/ml ampicillin, and incubated with shaking at 37°C overnight. The saturated overnight culture was diluted 1:100 into 200 ml of L-broth containing 100 µg/ml ampicillin and incubated with shaking at 37°C overnight. Plasmid DNA was isolated according to the manufacturer's instructions, and the final pellet resuspended in 200 - 400 µl TE buffer.

2.2.4.3 Extraction of RNA from cell lines

Total RNA was extracted from cultured mammalian cells by denaturation in guanidinum, followed by purification using a cesium chloride step gradient (Chirgin *et al.*, 1979).

A confluent 150 cm^2 flask of cells was washed with PBS and incubated with 3.5 ml GT buffer (4 M guanidine thiocyanate, 25 mM trisodium citrate pH 7.0, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol; pH 7.0) for 5 min at room temperature. The lysate from two flasks was pooled and sheared by drawing through a 21 gauge needle five times. The lysate was loaded onto a 2.5 ml cesium chloride cushion (5.7 M cesium chloride, 0.1 M EDTA pH 7.5, 0.05 M sodium acetate; pH 7.5) and centrifuged at 31,000 rpm for approximately 16 h, at 25°C, in a Beckman L-80 ultracentrifuge (SW41 rotor). The supernatant was carefully removed from the RNA pellet, which was washed with 70% ethanol, air dried and resuspended in 200 µl of TES buffer (10 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% SDS). The sample was extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and the upper aqueous phase containing the RNA removed to a new tube. The original sample was back-extracted with 200 µl of TES buffer, and the aqueous phases pooled. The RNA was precipitated (section 2.2.4.4), washed with 70% then 100% ethanol, resuspended in 200 µl water and quantitated (section 2.2.4.5).

2.2.4.4 Precipitation of DNA and RNA

Nucleic acids were purified and concentrated by precipitation with 0.3 M sodium acetate (pH 5.2) and 2 (DNA) or 2.5 (RNA) volumes of ethanol, at -70°C for 30 min to 1 h. The DNA or RNA was pelleted by centrifugation at 13,000 rpm for 15 min at 4°C in a benchtop microfuge, and the pellet air-dried and resuspended in water or TE.

2.2.4.5 Quantitation of nucleic acids

DNA, RNA and synthetic oligonucleotides were quantitated by measurement of absorbance at 260 nm wavelength using a Beckman DU 650 spectrophotometer

(Beckman Instruments, Fullerton, CA, USA). Concentration was estimated by assuming that A_{260} = 1 for 50, 40 and 20 µg/ml solutions of DNA, RNA and single-stranded oligonucleotide, respectively.

2.2.5 Electrophoresis of DNA

2.2.5.1 Agarose gel electrophoresis of DNA

DNA samples were prepared with 0.1 volume of 10 x loading buffer (0.4% (w/v) bromophenol blue, 0.4% xylene cyanol (w/v), 50% (v/v) glycerol). DNA fragments were separated by horizontal slab gel electrophoresis through 0.8 to 2% agarose (w/v), with 1 µg/ml ethidium bromide, in 1 x TBE buffer (90 mM Tris-borate, 2 mM EDTA; pH 8.0) at 80 V. Molecular weight markers SPP1/EcoRI (500 ng; Bresatec) were run alongside samples. Following electrophoresis the DNA fragments were visualised by UV transillumination and photographed with a Gel Doc 1000 gel documentation system (Biorad).

2.2.5.2 Purification of DNA from agarose

DNA fragments were purified either using low-melt agarose (Sigma-Aldrich) gels using the PCR DNA purification kit (Promega) as per the manufacturer's instructions, or by electrophoresis onto DEAE-cellulose membrane. Following separation of DNA fragments a slit was cut in the gel immediately ahead of the DNA fragment of interest, a piece of membrane (NA-45; Schleicher and Schuell, Dassel, Germany) was inserted and electrophoresis continued until all of the DNA in the band was collected onto the membrane. The membrane was trimmed and the DNA eluted by incubation for 1 h at 65°C in elution buffer (1.5 M NaCl in 1 x TE). The membrane was removed and the buffer centrifuged at 13,000 rpm for 5 min to remove residual agarose, the supernatant was removed to a new tube and the DNA precipitated and resuspended in water.

2.2.6 Radioactive labelling of DNA

2.2.6.1 Random prime labelling

The Prime-a-Gene® Labeling System was used to label double stranded DNA fragments from ~ 400 to 2000 bp, by the incorporation of [α32P] dCTP into a newly synthesised random primed DNA strand, according to the manufacturer's instructions.

Typically 50 ng of heat-denatured DNA template was incubated in 1 x labelling buffer (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 2 mM DTT, 200 mM HEPES pH 6.6, 5.2 A_{260} units/ml random hexadeoxyribonucleotides; Promega) with 50 μM each dNTP (G, A, T), 75 μCi [α32P] dCTP, 0.4 mg/ml BSA and 5 U DNA Polymerase I (Klenow fragment), in a total volume of 50 μl for 1 h at 37°C. Unincorporated radionucleotide was removed using a Nick column (section 2.2.6.3).

2.2.6.2 End labelling

Synthetic oligonucleotides were radiolabelled using T4 Polynucleotide Kinase to transfer the γ-phosphate of [γ^{32}P] ATP to the 5′ hydroxy terminus.

Typically, 200 ng of oligonucleotide (~21 mer) was incubated in 1 x kinase buffer (70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT; Promega), with 50 μCi [γ^{32}P] ATP and 10 U T4 Polynucleotide Kinase in a total volume of 20 μl for 1 h at 37°C. Unincorporated radionucleotide was removed using a Nick column (section 2.2.6.3).

2.2.6.3 Removal of unincorporated nucleotides

Unincorporated ^{32}P-labelled nucleotides were removed from labelled probe DNA using a Sephadex® G-50 Nick™ Column (Pharmacia Biotech). The sample volume was adjusted to 100 μl with TE and added to the column that had been pre-equilibrated with 3 ml TE. The sample was washed through the column with 300 μl TE and eluted with a further 500 μl TE.

2.2.7 Polymerase Chain Reaction (PCR)

PCR amplification was carried out in 1 x reaction buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA; Stratagene), with 2 mM $MgCl_2$, 200 µM each dNTP (G,A,T,C), 0.5 µM each primer, 1 U of Taq DNA polymerase (Perkin Elmer) and typically 1 ng of plasmid, in a final volume of 20 µl. Cycling conditions were typically as follows, with extension times of ~ 2 min/kb. For primers with sequence mismatches the annealing temperature was reduced to between 62°C and 48°C.

cycle 1: (1x)	94°C	2:00 min
cycle 2: (30x)	94°C	1:00 min
	62°C	0:30 sec
	72°C	2:00 min
cycle 3: (1x)	72°C	5:00 min

Cycling reactions were carried out on a Corbett FTS-4000 Capillary Thermal Sequencer (Corbett Research, Sydney, Australia).

2.2.8 Cloning of DNA

2.2.8.1 Preparation of vector and insert

Insert (from plasmid or PCR product) and vector DNA was prepared for cloning by digestion with the appropriate restriction enzyme(s) in 1 x restriction buffer supplied by the manufacturer, using enzyme concentrations of ~ 3-5 U/µg of DNA, at the temperature recommended by the manufacturer, for 2 h.

Vector DNA that had been prepared by digestion with a single restriction enzyme was purified and treated with calf intestinal alkaline phosphatase (CIP) to prevent self-ligation. The DNA was CIP treated in 1 x buffer (50 mM Tris-HCl pH 9.3, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine; Promega) with 1 U CIP/pmol 5′ termini (1 µg of 1 kb DNA = 3.03 pmol 5′ termini) at 37°C for 30 min. CIP was inactivated by incubation at 75°C for 15 min.

Both insert and vector DNA were gel purified (section 2.2.5.2) and quantified (section 2.2.4.5) before ligation.

2.2.8.2 Ligation of DNA

Vector and insert DNA were ligated using vector:insert ratios of ~ 1:1 to 1:4. Ligation reactions were carried out in 1 x ligation buffer (10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM ATP; Promega) and 3 U T4 DNA ligase, in a total of 10 µl, at 16°C for ~16 h. Ligated DNA was transformed into competent bacterial cells (section 2.2.8.4).

2.2.8.3 Preparation of competent cells

E. coli strain XL1-Blue (section 2.1.8) were made competent using rubidium chloride and calcium chloride, according to the method of (Hanahan et al., 1991).

2.2.8.4 Transformation of competent cells and selection of recombinants

Ligated plasmid DNA (10-100 ng) was mixed with 100 µl of competent XL1-Blue cells and incubated on ice for 30 min. The cells were 'heat shocked' at 42°C for 90 sec, cooled on ice for 2 min, and 1 ml of SOC medium (no antibiotic) was added and the cells incubated at 37°C for 1 h. For simple propogation of DNA plasmids, 100uL of the tranformation mix, or for ligation reactions the entire tranformation mix was pelleted by centrifugation and resuspended in 100uL of LB broth and mixtures plated onto L-agar plates containing 100 µg/ml ampicillin, and the plates were incubated at 37°C overnight. Transformants were screened for recombinant

clones by small scale plasmid preparation (section 2.2.4.1), restriction digests (section 2.2.8.1) and comparison of resulting restriction fragments with known controls, using agarose gel electrophoresis (section 2.2.5.1).

2.2.9 DNA sequencing

The sequence of all plasmid contructs was determined using either a manual or automated sequencing protocol.

2.2.9.1 Manual sequencing

Manual sequencing was performed using the dideoxy chain termination method of (Sanger et al., 1977), using T7 DNA Polymerase and the commercially available Super-base sequencing reagent kit, with the incorporation of [α^{35}S]dATP.

Double-stranded plasmid DNA template (~1 µg) was denatured with 0.17 M NaOH and 0.17 mM EDTA (pH 8.0) at 85°C for 5 min. The DNA was ethanol precipitated (section 2.2.4.4) and the pellet resuspended in 7 µl water. Primer (10 ng) was annealed to the template DNA in annealing buffer (40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 20 mM DTT; Bresatec) in a total volume of 10 µl, by heating to 70°C for 2 min and allowing to cool slowly to room temperature.

Labelling/extension and termination reactions were carried out according to the manufacturer's instructions. Half of each sequencing reaction was resolved on a 0.2 mm polyacrylamide sequencing gel (6% acryl:bis (19:1), 7.5 M urea, 1 x TBE), which was vacuum dried for 1 h at 80°C and exposed to X-ray film (Biomax™-MR).

2.2.9.2 Automated sequencing

Automated sequencing was performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit.

Double-stranded plasmid DNA template (~ 500 ng) was mixed with primer (3.2 pmol) and 8 µl of Terminator Ready Reaction Mix, in a total volume of 20 µl. This mixture was subjected to thermal cycling according to the manufacturer's

instructions, and the product was then precipitated (section 2.2.4.4) and the pellet resuspended in 3 µl of loading mix (14% formamide (v/v), 3.6 mM EDTA pH 8.0, 1 x blue dextran dye). The sample was denatured at 90°C for 2 min, and half of each sequencing reaction was resolved using the ABI PRISM™ 377 sequencing apparatus. Fluorescent labelling was tracked using the program Factura, and sequence data analysed using Autoassembler (Perkin Elmer).

2.2.10 Northern blot analysis

2.2.10.1 Electrophoresis of RNA

RNA samples (16 µg in 4 µl; section 2.2.4.3) were diluted in 16 µl of RNA sample buffer (50% formamide (v/v), 20% formaldehyde (v/v), 1 x MOPS buffer (see below), 1 x loading buffer (section 2.2.4.1), 1 µg/ml ethidium bromide) and incubated at 65°C for 10 min, followed by ice for 2 min. Samples were separated by horizontal slab gel electrophoresis through a 1% agarose (w/v) gel, containing 6.7% formaldehyde (v/v) and 1 µg/ml ethidium bromide, in 1 x MOPS buffer (40 mM MOPS, 1 mM EDTA pH 8.0, 5 mM sodium acetate, 9 mM NaOH; pH 7.0) at 15 V for ~ 16 h. Following electrophoresis the RNA was visualised by UV transillumination and photographed with a Gel Doc 1000 gel documentation system (Biorad Laboratories).

2.2.10.2 Electrotransfer of RNA

RNA was electrotransferred to Hybond™-N+ nylon membrane (Amersham Pharmacia Biotech) in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA; pH 8.0) at 800 mA for 2 h, using a Hoefer TE 50X electroblotter (Hoefer Scientific Instruments, San Francisco, CA, USA). The membrane was rinsed in 2 x SSC (0.3 M NaCl, 30 mM sodium citrate; pH 7.0), air-dried and UV fixed for 4.5 min.

2.2.10.3 Northern hybridisation

RNA bound to nylon membranes was prehybridised at 50°C in a buffer containing 10 x Denhardt's solution (0.2% (w/v) Ficoll (Type 400), 0.2% BSA (fraction V), 0.2% polyvinylpyrrolidone), 5 x SSC, 50 mM Tris-HCl pH 7.5, 1% (w/v) SDS, 0.2 x SSPE and 100 µg/ml denatured salmon sperm DNA, for at least 2 h. Radiolabelled probe DNA (section 2.2.6) was denatured at 95°C for 5 min and added to hybridisation buffer containing 1 x Denhardt's solution, 4 x SSC, 50 mM Tris-HCl (pH 7.5), 1% SDS, 0.2 x SSPE, 10% dextran sulfate, 50% formamide and 100 µg/ml denatured salmon sperm DNA. The prehybridisation buffer was replaced with the hybridisation buffer containing the probe, and incubation was continued at 50°C for ~ 16 h.

After hybridisation, membranes were rinsed in 2 x SCC at 63°C, followed by two low stringency washes in 2 x SSC / 0.1% SDS at 63°C for 30 min, and two high stringency washes in 0.2 x SSC / 0.1% SDS at 63°C for 15 min. Additional high stringency washes were performed at 65°C depending on background levels of radioactivity. Finally membranes were rinsed in 2 x SSC and exposed to X-ray film (Biomax™-MR), using Lightning Plus™ intensifying screens.

2.2.10.4 Densitometric analysis

Hybridised membranes were also exposed to phosphor screens which were scanned, and the signal quantified (Phosphorimager™ 445 SI, Molecular Dynamics, Kew East, Victoria, Australia). All samples were normalised to the signal from the 18S ribosomal RNA oligonucleotide probe.

2.2.10.5 Stripping Northern blots

Membranes were stripped of hybridised probe by immersion in boiling 0.1% SDS, and allowed to cool with gentle shaking for 1 h. Stripped membranes were rinsed in 2 x SSC.

2.2.11 *In vitro* translation of protein

Protein was translated *in vitro* using the TNT® T7 Quick Coupled Transcription/Translation System, which comprises a single master mix incorporating rabbit reticulocyte lysate with RNA polymerase, nucleotides, salts and RNase inhibitors.

Circular plasmid template DNA (1µg) was mixed with 40 µl TNT® T7 Quick Master mix and 20 µCi L-[^{35}S]-methionine, in a total volume of 50µl, and incubated at 30°C for 90 min. The products of translation were analysed by 10% SDS-PAGE (section 2.2.13), the gel fixed in Coomassie Destain (15% methanol, 10% acetic acid in H_20) for 10 min, vacuum dried at 80°C for 1 h and exposed to X-ray film (Biomax™-MR). For preparation of unlabelled proteins, the L-[^{35}S]-methionine was omitted from the reaction mix. In this case, a small-scale L-[^{35}S]-methionine-containing reaction was performed in parallel and analysed as described to ensure the integrity of the reagents.

2.2.12 Preparation of nuclear extracts

2.2.12.1 Preparation of nuclear lysates from mammalian cells

Nuclear lysates were prepared from COS-1 cells transfected (section 2.2.15.1) with 10 µg of a mammalian expression plasmid (e.g. SKIP-pCGN) / 1.1 x 10^6 cells / 13 cm dish for ~ 24 h. The protocol of (Andrews and Faller, 1991) was followed. Cells were scraped into 450 µl cold 1 x PBS, pooled and centrifuged at 13,000 rpm for 1 min at 4°C. The pellet was resuspended in 400 µl cold buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and allowed to swell on ice for 15 min. Cells were vortexed for 10 sec and centrifuged at 13,000 rpm for 1 min at 4°C. The pellet was resuspended in 100 µl cold buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and allowed to swell on ice for 30 min, followed by brief vortexing. The lysate was centrifuged at 13,000 rpm for 5 min at

4°C and the protein concentration of the supernatant determined (section 2.2.12.3) before storage at -80°C.

2.2.12.2 Preparation of whole cell lysates from yeast cells

4 ml cultures were inoculated from saturated cultures at OD_{600} = ~ 0.15 and grown to OD600 = 0.45 to 0.7 (~ 4 to 6 h). Cultures were centrifuged at 13,000 rpm for 3 min at RT and the supernatant removed. The pellet was resuspended in 50 µl 2 x Laemmli buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol), freeze-thawed at -80°C and boiled for 5 min, followed by centrifugation at 13,000 rpm for 5 sec. The protein content of the supernatant was estimated (section 2.2.12.3) before storage at -80°C.

2.2.12.3 Estimation of protein content

Protein concentrations of mammalian and yeast cell lysates were determined using the Biorad protein assay reagent (Biorad). A standard curve was established using BSA (0.5 - 25 µg). Standard and test samples were diluted (1:100 - 1:1000) in deionised H2O to a final volume of 800 µl, 200µl Biorad reagent was added and the mixture vortexed. The reactions were incubated at room temperature for 10 min and the absorbances at A595 nm were determined using a Beckman DU 650 spectrophotometer (Beckman Instruments).

2.2.13 Polyacrylamide gel electrophoresis (PAGE) of protein

Protein samples (~5-20 µg) were prepared with 1/3 volume of 3 x loading buffer (150 mM Tris-HCl pH 6.8, 6 mM EDTA, 3 % SDS, 3% 2-mercaptoethanol, 0.3 M DTT, 30 % glycerol). Samples were denatured at 95°C for 2 min and separated by PAGE. Separating gels contained 10% acrylamide (29:1 acryl:bis), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% APS and 0.05% TEMED. Stacking gels contained 5% acrylamide (29:1 acryl:bis), 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% APS and 0.2% TEMED. Gels were run in 1 x running buffer (25 mM Tris-HCl pH 8.3, 0.2 M glycine, 0.1% SDS; pH 8.3) at 100 - 120 V for 1 - 2 h. Rainbow™ coloured protein molecular weight markers (Amersham Pharmacia Biotech) were run alongside samples. Where appropriate, protein bands were visualised by

Coomassie blue staining (0.25% Coomassie brilliant blue R250, 45% methanol, 10% acetic acid). Gels were counterstained using destain solution (15% methanol, 10% acetic acid in H_2O).

2.2.14 Western blot analysis

2.2.14.1 Electrotransfer of protein

Protein samples subjected to PAGE (section 2.2.13) were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) using a Trans-blot electroblotting cell (Biorad), in 1 x transfer buffer (25 mM Tris, 200 mM glycine; pH 8.3) at 250 mA at 4°C for 2 h. Membranes were fixed by air drying.

2.2.14.2 Western hybridisation

Membranes containing immobilised proteins were blocked in 1 x TBST containing 5% (w/v) skim milk powder, 1% (w/v) BSA and 0.05% (v/v) sodium azide, at 4°C for ~ 16 hr. After three washes in 1 x TBST at room temperature for 10 min, the membranes were incubated with the primary antibody (0.4 - 1.0 µg/ml) in 1 x TBST with 0.05% (v/v) sodium azide at room temperature for ~ 2 h. Following three washes in 1 x TBST at room temperature for 10 min, the membranes were incubated with the secondary antibody (at dilutions of 1:20,000 - 1:50,000) in 1 x TBST with 5% (w/v) skim milk powder at room temperature for ~ 2 h. The membranes were washed three times in 1 x TBST at room temperature for 10 min, and the bound antibodies detected using the ECL Plus™ Western blotting detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The membranes were exposed to X-ray film (Biomax™-ML).

2.2.14.3 Stripping Western blots

Western blots were stripped by gentle shaking in a solution of 60 mM Tris-HCl pH 6.7, 2% SDS and 0.7% 2-mercaptoethanol, at 55°C for 30 min, followed by rinsing in 1 x TBST.

2.2.15 Transient transfection of mammalian cells

2.2.15.1 Transfection using FuGENE™ 6 transfection reagent

Mammalian cells were transfected using the FuGENE™ 6 non-liposomal transfection reagent (Boehringer Mannheim), according to the manufacturer's instructions. FuGENE/DNA transfection mixes were prepared by combining 2 µl FuGENE/µg total DNA in 100µl serum-free DMEM/3µl FuGENE, followed by incubation for 5 min at room temperature. The DNA was diluted from stocks and added to the FuGENE/serum-free medium mix, followed by incubation at room temperature for 15 min. The final transfection mix was added to the cell culture medium, mixed evenly and incubated at 37°C/5% CO_2.

2.2.15.2 Harvesting cells for assay of reporter gene activity

Following transfection with FuGENE™, the medium was removed from the wells and cells were lysed in 100 µl 1 x lysis buffer (Promega) at room temperature for ~ 30 min.

2.2.15.3 Luciferase Assay

This assay measures the light emitted upon oxidation of luciferin substrate by firefly luciferase (Luciferase Assay System). Cell lysate (20µl) in 1 x lysis buffer was mixed with 100 µl reconstituted Luciferase Assay Reagent and luminescence was measured for 10 sec with an Autolumat LB 953 luminometer (Berthold). Luciferase assays were carried out in triplicate and each transfection performed at least 3 times.

2.2.16 Far Western Assay

2.2.16.1 Far Western and immunoblot analysis

Far Western analysis and preparation of nuclear extracts were as previously described (Leong *et al.*, 1998b) (section 2.2.12.1). COS-1 nuclear extracts overexpressing Smad3 or HA-SKIP were run on 10% SDS-PAGE (section 2.2.13)

and electroblotted onto a polyvinylidne fluoride membrane (PVDF) (Millipore, Bedford, MA) (Figure 6.4C). Proteins were denatured with 6M guanidine hydrochloride and renatured by the stepwise dilution of guanidine hydrochloride. The Smad3 membrane was then blocked and hybridized overnight at 4°C with 20 ug of COS-1 nuclear extracts expressing HA-SKIP. The filter was rinsed three times in HYB (20mM Hepes-KOH, pH 7.4, 75mM KCL, 0.1mM EDTA, 2.5 mM Mg Cl2, 1% nonfat milk, 0.05% IPEGAL and 1mM DTT) and then probed with an anti-HA antibody (Boeringher-Mannheim) which detected HA-SKIP, followed by probing with a anti-mouse–HRP secondary antibody (Santa Cruz) prior to ECL chemiluminescent detection (Amersham) and autoradiography (section 2.2.14.2).

2.2.17 Electromobility Shift Assay (EMSA)

EMSA was performed with the PE2 32P radiolabelled probe (section 2.2.6, Figure 6.7) from the PAI-1 promoter (Hua *et al.*, 1999), as previously described (Subramaniam *et al.*, 2001), using *in vitro* translated cold Smad3 and Smad4 proteins (section 2.2.11) with COS-1 nuclear extracts overexpressing either SKIP, Sno or Ski (section 2.2.12.1).

2.2.18 GST binding assays

2.2.18.1 Expression and preparation of GST fusion proteins

Plasmids expressing GST fusion proteins were transformed to BL21-Gold (section 2.1.8) competent cells according to the manufacturer's protocol (Stratagene). The transformation mix was plated onto L-agar plates containing 100 µg/ml ampicillin, and the plates were incubated at 37°C overnight. A 20 ml culture of L-broth with 100 µg/ml ampicillin was inoculated with a single transformant and incubated at 37°C overnight with shaking. This saturated culture provided the inoculum for a 200 ml culture of L-broth with 100 µg/ml ampicillin, which was incubated at 37°C with shaking for 1 h. Protein expression was induced with 0.2 - 1.0mM IPTG and incubation was continued for 4 h. The cells were centrifuged at 6000 rpm for 15 min at 4°C and the pellet resuspended in 1 x PBS with Complete™ protease inhibitors (Boehringer Mannheim) and 2 mM DTT. Cells were sonicated for 10 sec

using a Branson Sonifier 250 (Branson Ultrasonics Corporation) followed by 1 min break, 7 times, at 4°C. Triton X-100 (to 1% (v/v) was added and the lysates were mixed for 30 min at 4°C, followed by centrifugation at 8000 rpm for 10 min at 4°C. 450µl Glutathione Sepharose® 4B beads (Pharmacia Biotech; washed and diluted to a 50% slurry in 1 x PBS) were added to the supernatant, followed by mixing for 30 min at 4°C. The mixture was centrifuged at 1500 rpm for 1 min at 4°C and the pelleted beads washed 3 times with 50 ml 1 x PBS + Complete™ protease inhibitors, with centrifugation between. The GST-fusion protein-coupled beads were resuspended in 5 ml 1 x PBS + Complete™ protease inhibitors and stored at 4°C. Protein expression was confirmed by 10% PAGE (section 2.2.13).

2.2.18.2 GST Binding reactions

GST-fusion protein-coupled Sepharose beads (10 µl/reaction) were preincubated in binding buffer (75 mM KCl, 40 mM Hepes pH 7.5, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/ml BSA, 0.05 % NP40 and Complete™ protease inhibitors) at 4°C overnight with mixing, centrifuged at 1500 rpm for 1 min at 4°C and resuspended again in binding buffer. Beads were then incubated with *in vitro* translated proteins (section 2.2.11) in the presence of appropriate ligand or vehicle in a total of 300 µl at 4°C for 2 h with mixing. Unbound proteins were removed by washing the beads 5 times in 1ml each of binding buffer, with centrifugation at 1500 rpm for 1 min at 4°C between. Bound proteins were eluted by heat denaturation at 95°C for 3 min in 3 x loading buffer (section 2.2.13) then resolved by 10% PAGE (section 2.2.13).

Chapter Three

Identification of Nuclear Hormone Receptor-Interacting Cofactors.

3.1 INTRODUCTION

Gene transcription modulation by nuclear hormone receptors (NHRs) involves a complex interplay of multiple factors which direct RNA polymerase II-dependent transcriptional initiation (Glass and Rosenfeld, 2000). Identification of numerous cofactors involved in modulation of NHR action has contributed to a greater understanding of the cell- and development-specific roles of NHRs and provided insights into their complex regulation *in vivo*. The first NHR coactivators and corepressors were identified using the yeast two-hybrid system, an *in vivo* genetic protein-protein-interaction assay (Fields and Song, 1989; Fields and Sternglanz, 1994; Le Douarin *et al.*, 1995a; MacDonald, 1998). These included the corepressors N-CoR and SMRT and the SRC p160 coactivator family (Chen and Evans, 1995; Hong *et al.*, 1996; Horlein *et al.*, 1995; Le Douarin *et al.*, 1995b; Lee *et al.*, 1995b; Onate *et al.*, 1995). Numerous other transcription factors involved in a diverse range of signalling pathways have also been cloned using the yeast two-hybrid system, some of which had not been identifiable through other cloning methods (Fields and Sternglanz, 1994). Therefore the system has played an instrumental role in the identification of key cofactors involved in NHR-dependent gene transcription.

Thus, to identify such cofactors, VDR was used to screen a cDNA library using the yeast two-hybrid system. The VDR is involved in the regulation of a wide variety of cellular processes, including the control of proliferation and differentiation of monocytes, macrophages, as well as breast cancer and leukaemic cells (Eisman, 1994; Evans, 1988; Freedman *et al.*, 1997; Liu *et al.*, 1996b). It is expressed ubiquitously in most tissues, yet appears to have cell-specific transcriptional effects. It was therefore hypothesised that transcriptional factors may play roles in determining the tissue-specific actions of VDR. At the time these studies were initiated, no VDR-specific coactivators had been identified. This screen lays the foundation for subsequent examination of interactions in this thesis of identified VDR-interacting cofactors with other NHRs and with other signalling pathways involving VDR.

3.1.1 Aims

The primary aim of the studies described in this chapter was to identify novel NHR-protein interactions. Thus, VDR was used as a "bait" protein to screen a human liver cDNA library using the yeast two-hybrid system. These studies demonstrated the power of this system in identifying novel protein-protein interactions, but also illustrated its limitations, including the identification of numerous "false" positives. In this chapter, the strategies used to identify "true" positives and eliminate "false" positives are outlined. Furthermore, the rationale is explored for the subsequent focus on a cofactor, Ski-interacting protein (SKIP), identified during the library screening.

3.2 METHODS

3.2.1 The yeast two-hybrid system

One of the major advantages in using yeast to study NHR-dependent gene regulation is that yeast do not express NHRs or the majority of associated coactivators and corepressors, although highly conserved basal transcription factors such as TBP and TAFs are present (Kokubo *et al.*, 1998; McEwan, 2001).

In the yeast two-hybrid system, two fusion proteins are expressed and their interaction *in vivo* is monitored using a combination of integrated reporter genes. These include the lacZ gene which allows colour screening for the presence of interacting proteins, and a second auxotrophic selectable marker which renders yeast harbouring interacting proteins able to grow in the absence of a specific amino acid, such as histidine.

The "bait" protein consists of the DNA-binding domain of a transactivator, such as the yeast GAL4 gene (aa1-147), fused to the protein, or part of the protein, for which interacting proteins are being sought. The second fusion protein, termed the "prey" or target, contains the activation domain (AD) of the transactivator e.g. GAL4 gene (aa768-881), fused to a single protein or to a range of proteins or protein fragments from an expression library. If the "bait" protein interacts with the "prey" protein when coexpressed in yeast, the GAL4DBD and GAL4AD

transcription unit are brought into close proximity and expression of the GAL4-responsive reporter genes is enhanced. Construction of a cDNA library in the GAL4AD vector thus allows screening for novel proteins that interact with a protein of interest, such as VDR-interacting factors (Figure 3.1).

It is well recognised that this system leads to identification of both false negatives and false positives secondary to various possible causes (Golemis, 2001). Thus, it is necessary to employ strategies to identify these false positive and negative clones and interpret putative interactions in the light of these results. Furthermore, as reporter activity in this system is dependent on nuclear interaction of the bait and its prey protein, this system may not necessarily identify protein-protein interactions that occur *in vivo*. Despite this apparent limitation, cytoplasmic protein-protein interactions or protein-protein interactions between cytoplasmic proteins that translocate into the nucleus have been identified in this system. Furthermore, variations in the original nuclear-based system to specifically allow detection of cytoplasmic protein-protein interactions have been developed (Fields and Sternglanz, 1994; Golemis, 2001). Other interactions that depend on post-translational modifications, which may not occur in yeast, such as glycosylation, may limit the ability to identify "true" interactions in this system (Fields and Sternglanz, 1994). Additionally, a protein-protein interaction that occurs in mammalian cells may not be apparent in yeast, if proteins not expressed in yeast are required for an effective interaction to occur. The reverse may also be the case, i.e. protein-protein interactions observable in yeast may not be detectable in mammalian cells because of factors that inhibit these interactions in mammalian cells. Thus, alternative systems have been developed which may in some circumstances overcome these problems, such as the mammalian two-hybrid and reverse yeast two-hybrid systems (Braselmann *et al.*, 1993; Golemis, 2001; Leanna and Hannink, 1996; Shih *et al.*, 1996; Vidal *et al.*, 1996). Despite all these limitations, however, the success of the yeast system in identifying novel protein-protein interactions has been verified in other model systems *in vitro* and *in vivo* (Fields, 1997; Golemis, 2001; Hazbun and Fields, 2001; Ito *et al.*, 2001). For the studies presented in this chapter, the GAL4-based yeast two-hybrid system was chosen as

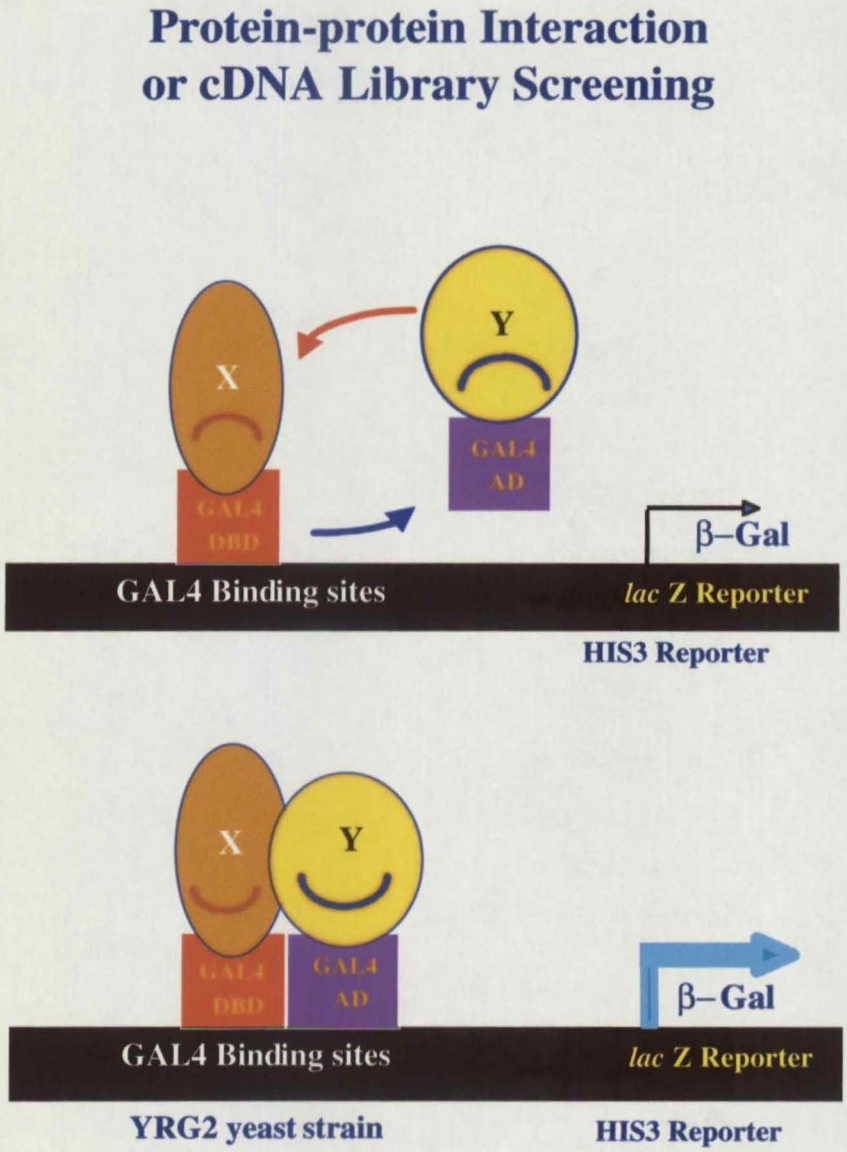


Fig. 3.1 The Yeast Two-hybrid system.

it was the best characterised system with proven success in identifying NHR-interacting cofactors.

3.2.2 Yeast strains and plasmids

The yeast strain YRG2 was chosen for library screening because it was purported to have a 10-fold higher transformation efficiency than other yeast strains, such as CG1945 of HF7c (section 2.1.10). This would allow a greater number of clones to be screened and hence increase the likelihood of identifying interacting clones within the library (section 2.2.2.1).

The high-copy episomal yeast two-hybrid plasmids, pAS2-1 and pACTII, were used for library screening and protein-protein interactions studies (section 2.1.11). VDR cDNAs were cloned into the pAS2-1 plasmid in frame with the GAL4DBD (section 3.3.4). The cDNAs of prey proteins and the expression library were cloned into the pACTII plasmid containing the Haemophilus Influenzae haemaglutinin protein (HA) epitope for immunoblot analysis. The pAS2-1 and pACTII plasmids contain two different auxotrophic markers, tryptophan and leucine, respectively, for selection in yeast strains such as YRG2, that contain inactivating mutations of these essential amino acid genes.

3.2.3 Human adult liver cDNA library

A cDNA library from human, adult liver was used in the two-hybrid library system (Horst and Reinhardt, 1997). In this commercially available library, liver cDNAs were cloned in frame with the GAL4AD in the vector pACTII.

3.2.4 Construction and characterisation of the VDR-GAL4DBD bait proteins

Two VDR "bait"-GAL4DBD pAS2-1 plasmids were constructed by PCR cloning for potential use in library screening, one including a full-length VDR (1-427aa) and the other a VDR C-terminal LBD construct (191-427aa) (Baker *et al.*, 1988). The forward VDR primer GL1:
5′-CCCGCCGGGATCCGTATGGAGGCAATGGCGGCCAGC-3 encoding the first 7 aa of human VDR cDNA (ATG intiating codon underlined) containing a 5′ B*amH*1 site and the reverse GL-2 primer:
5′-CCGGGCCCGGATCCTCAGGAGATCTCATTGCCAAACAC-3′ encoding the

last 7aa of VDR cDNA and a 3′ B*amH*1 site were used to amplify full-length VDR cDNA. The forward primer GL3 encoding aa192-198 of the hVDR cDNA: 5′- CCCGCCGGGATCCGTACCTCTTCAGACATGATGGAC-3′ with a 5′ B*amH*1 site with the reverse primer GL2 were used to amplify 192-427aa of the VDR cDNA. Both the full-length and 192-427aa VDR PCR products were digested with B*amH*1 restriction enzyme, purified and cloned (section 2. 2.8.1) into the B*amH*1 site of pAS2-1 and pM vectors (section 4.4.1) (Clontech). When overexpressed in yeast, neither the full-length nor 192-427aa VDR construct inhibited growth of the host YRG-2 yeast strain in comparison to the GAL4DBD control, indicating that VDR overexpression had no toxic effect on the host and should not adversely affect the transformation efficiency of the cDNA library (Figure 3.2).

Neither bait protein alone exhibited ligand-independent activation of the β-gal or histidine reporter genes, although there was a small increase in ligand-dependent activation by the wild-type VDR construct (Figure 3.3). Functional interactions of the two VDR constructs with a known VDR-interacting protein, TFIIB, were tested in the yeast two-hybrid system (Blanco *et al.*, 1995; MacDonald *et al.*, 1995). Wild-type VDR interacted as expected with TFIIB and had some ligand ($1,25(OH)_2D_3$ responsiveness (Figure 3.3). The C-terminal VDR bait, in contrast to the wild-type VDR construct, failed to respond to ligand or to interact with TFIIB. The ligand-responsiveness of the wild-type VDR-GAL4DBD construct alone was modest in comparison to its stronger ligand-dependent interaction with TFIIB. On the basis of these results, the full-length VDR rather than the C-terminal VDR bait was used for the subsequent library screening.

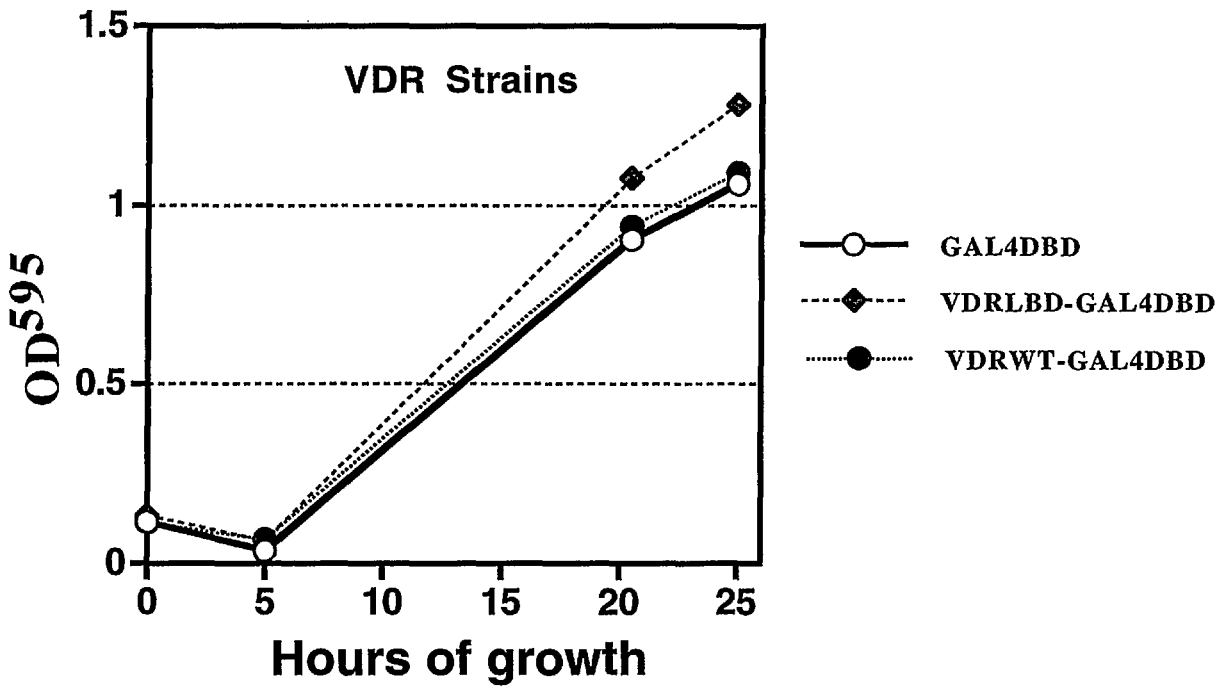


Fig. 3.2. Expression of VDR bait constructs does not adversely affect yeast growth.
Growth of YRG-2 yeast strains expressing VDR-LBD (191-427aa)-GAL4DBD or wild-type VDR-GAL4DBD with GAL4DBD control strain for 10-25 hours in CSM-Tryptophan medium.

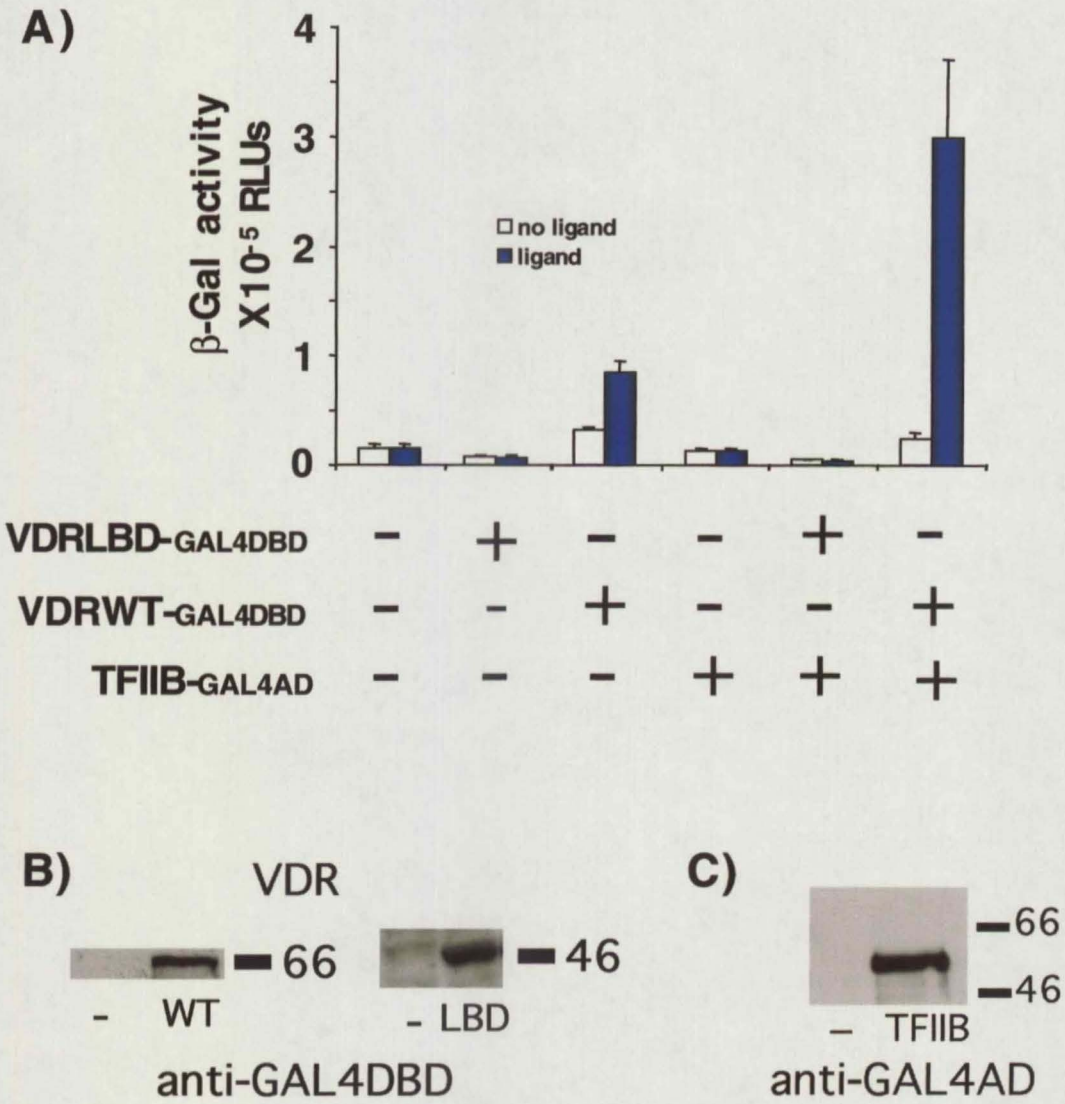


Fig. 3.3 VDR interaction with TFIIB.
A) Yeast two-hybrid analysis of VDR LBD (191-427aa) and wild-type (WT) bait constructs with TFIIB "prey". Values are mean ± SEM of at least 3 colonies from 2 independent experiments.
B) Immunoblot analysis of VDR and TFIIB yeast strains with empty vector indicated by -.

3.2.5 Screening of Liver cDNA library

Screening of the cDNA library was performed by sequential transformation of the YRG-2 yeast strain (section 2.2.2.1). Half the transformants were grown on solid medium without added ligand and the other half supplemented with 10^{-8} M $1,25(OH)_2D_3$. A total of 720 histidine positive clones (410 from the non-ligand and 310 from ligand screen) were isolated from 500,000 clones examined (Figure 3. 4). Of these 720 clones, only 328 tested positive for both histidine and β-galactosidase (β-gal) production [234 (71%) from the non-ligand and 94 (29%) from the ligand screen]. Based on the comparative strength of the β-gal colony filter assays to positive and negative control yeast strains, these colonies were divided into 131 strong (97 from non-ligand and 34 from ligand screen) and 197 weak (137 from non-ligand and 60 from ligand screen) β-gal positives.

Half the strong positive clones (i.e 66 of 131) were directly sequenced. Because of the rather large number of putative positive clones isolated, a functional assessment of the 197 weak β-gal positives colonies was undertaken (Figure 3.5). This assessment was performed by a yeast mating strategy (Figure 3.5) in which loss of the VDR-GAL4DBD tryptophan plasmid was induced by growth of yeast over 3 days in medium supplemented with tryptophan, but lacking leucine (Complete Synthetic Medium (CSM) minus leucine (CSM-Leucine) (section 2.2.2.3) (Figure 3.6). Loss of the tryptophan–selectable VDR-GAL4DBD plasmid was confirmed by replica plating on CSM-Leucine-Tryptophan and CSM-Leucine solid medium. Yeast that had lost the VDR bait tryptophan plasmid, but retained the prey leucine plasmid, grew on solid medium lacking leucine, but not on medium lacking both leucine and tryptophan. Over 95% of yeast were induced to lose the VDR bait plasmid using this method.

Each YRG2 strain retaining a prey cDNA leucine-producing plasmid was then mated in parallel cultures with Y187 strains expressing either the VDR bait or the empty GAL4DBD vector. The pairs of mated diploid strains were then plated on

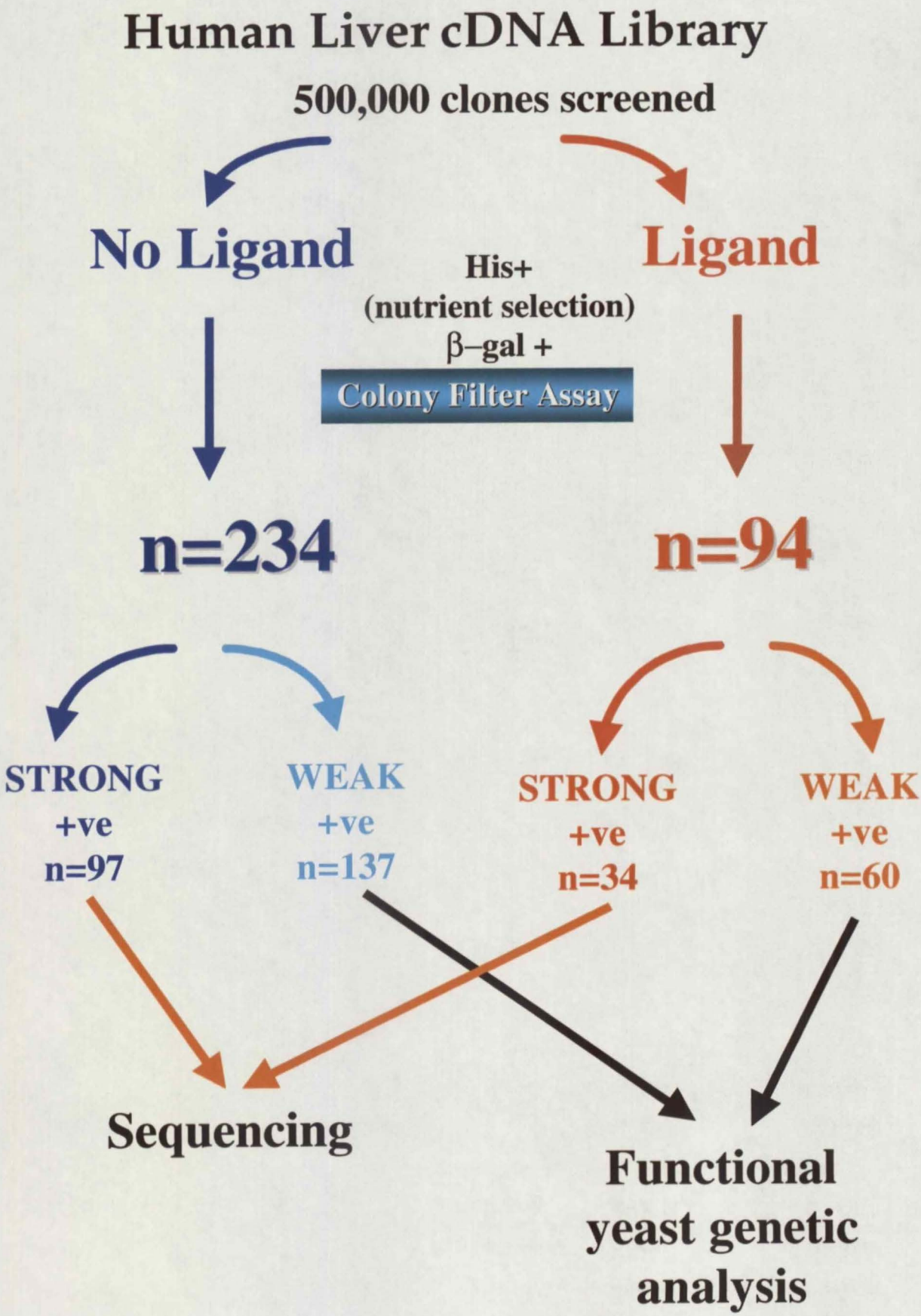


Fig. 3.4. Results of liver cDNA library screen.

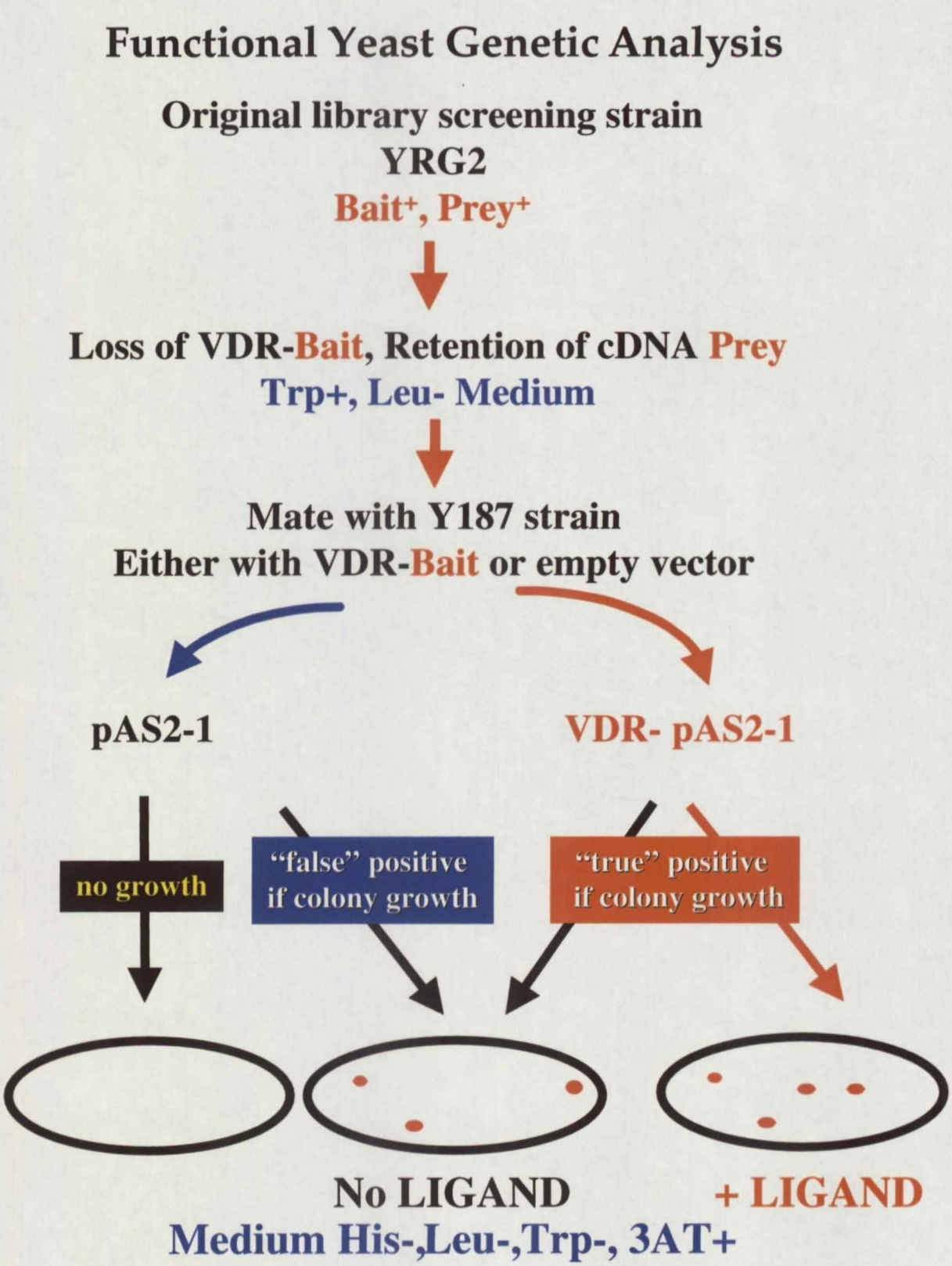


Fig. 3.5. Yeast genetic analysis to identify "true" and "false"positive two-hybrid clones. See Fig. 3.7 for outcome of analysis.

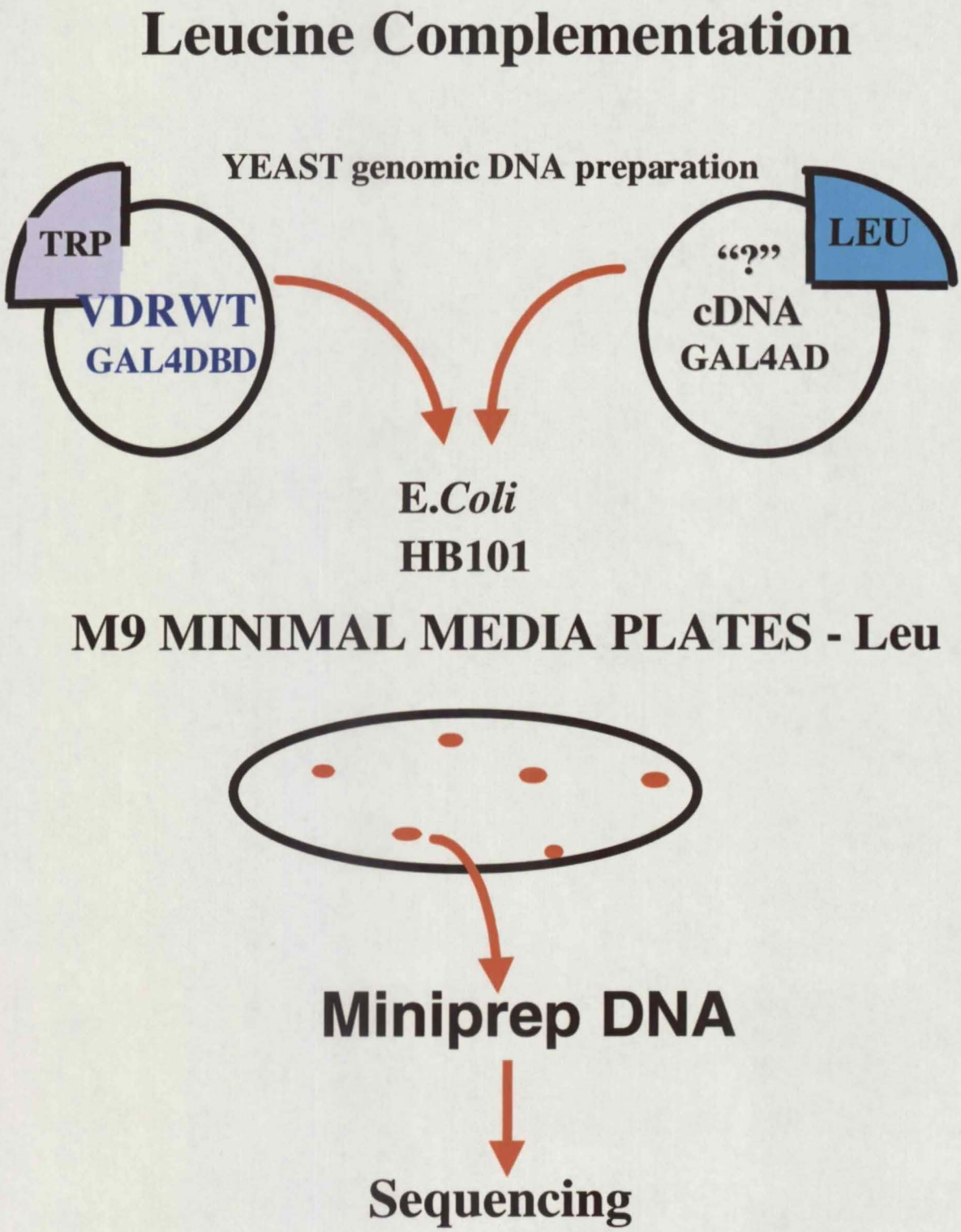


Fig. 3.6 Leucine complementation in HB101 Leucine negative E.*Coli*.
Whole yeast genomic DNA preparations were transformed by electroporation into HB101 E.*Coli* and Leu pACTII plasmid recovered by leucine selection on M9 minimal agar plates for miniprep DNA preparation and sequencing.

solid medium lacking histidine, leucine and tryptophan supplemented with 5mM 3-Aminotriazole (3-AT) (section 2.2.1.3) (Figure 3.5). Thus, yeast harbouring "true" positive cDNA prey constructs grew on histidine-deficient medium when coexpressed with VDR, but not with GAL4DBD empty vector. Conversely, yeast harbouring "false" positive cDNA constructs grew when co-expressed with empty vector but not the VDR bait, the latter being consistent with a non-specific activation of the histidine reporter gene.

From this analysis, 124 "true" positive prey constructs were identified whilst 25 "false" positives were excluded from further analysis (Figure 3.7). Of the 124 "true" positives, 105 were retested for the effects of ligand on growth of the yeast on replica CSM-histidine, -leucine and -tryptophan solid media. Of these clones, 78 were ligand-independent (36 from non-ligand and 42 from ligand screen) and 27 exhibited ligand-responsive effects on growth. Of this latter group, growth of 7 were augmented by ligand, whilst ligand inhibited the growth of the remaining 20 clones. Only 2 of the 7 that showed augmented growth in the presence of ligand were isolated originally from the non-ligand screen, whereas 15 of the 20 colonies whose growth was inhibited by ligand were originally isolated from that non-ligand screen. These positive clones and the remainder of the strong β-gal positive clones not directly sequenced were not analysed further because subsequent studies concentrated on the first set of characterised interacting proteins; thus these positive clones remain to be further characterised.

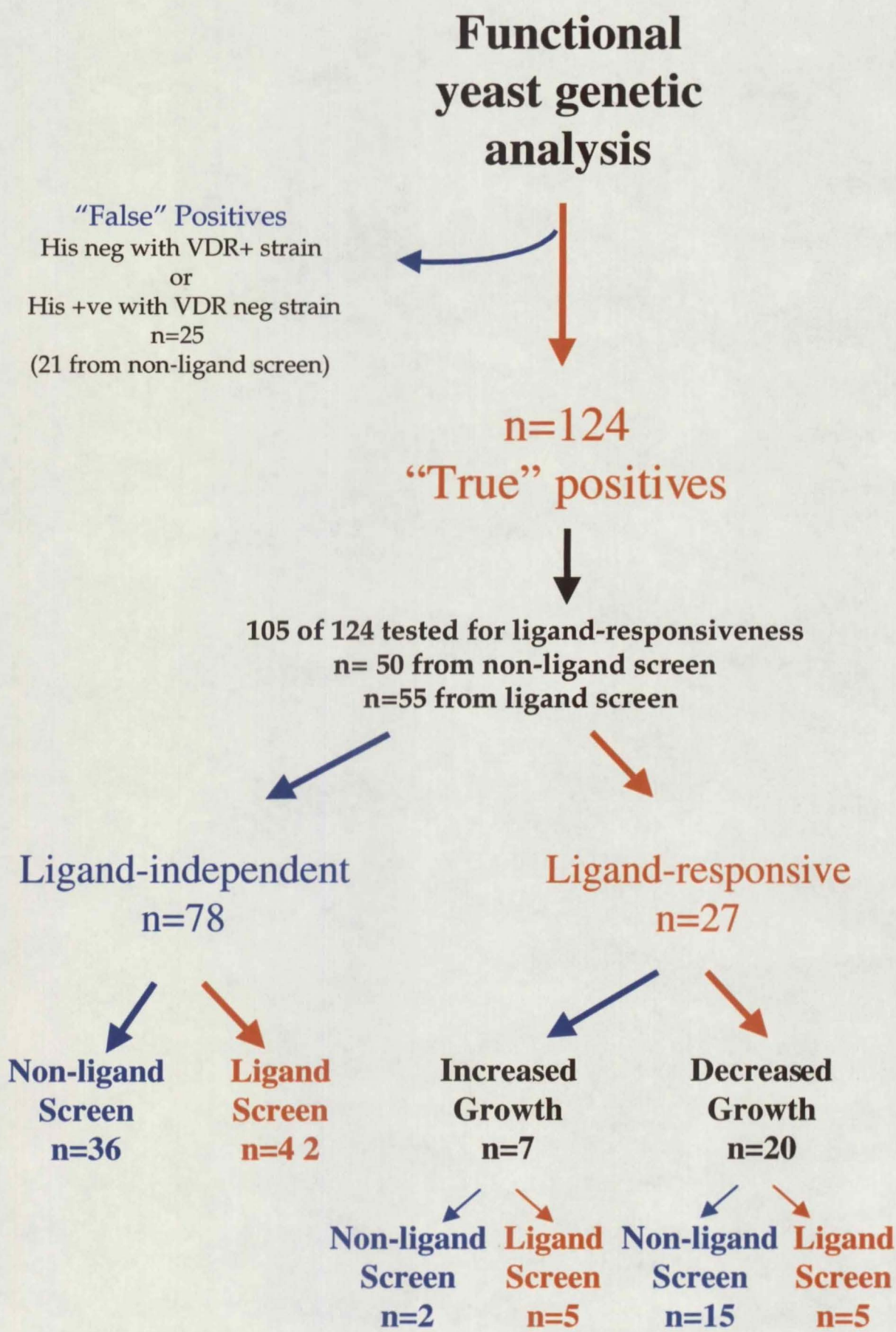


Fig. 3.7. Functional yeast genetic analysis.

3.3 RESULTS

3.3.1 Identification of VDR-interacting cofactors

Sequencing of a subset of the strong positive clones (66 of 131) revealed a large proportion as definite and probable "false" positives (Table 3.1). Fifty-eight clones were confirmed to be "false" positives either because they had previously been associated with false positive results published in the yeast two-hybrid literature (Golemis, 2001), or because they were negative after remating with the VDR-GAL4DBD strain and GAL4DBD yeast strains. Thus, only 8 of the 66 sequenced clones remained. From the non-ligand screen, they were Ski-interacting protein (SKIP) (3 separate clones) (Dahl *et al.*, 1998b), a putative LXXLL-containing protein and a protein with high homology to mN-CoR (RIP13) (Seol *et al.*, 1995). From the ligand screen, only 2 cDNAs were identified: human retinoid X receptor (RXR-α) (2 separate clones) and a cytoplasmic protein apoplipoprotein E3 precursor (McLean *et al.*, 1984).

Four of these encoded proteins appeared to have clear relevance to VDR function: RXRα, SKIP, a clone which had partial homology to mN-CoR (RIP13), and another that contained a LXXLL-coactivator motif. Apolipoprotein E3 precursor, a cytoplasmic protein, did not immediately suggest a physiologically relevant role for VDR action. Of the 4 proteins that appeared likely to be nuclear, only two had 100% homology with previously published proteins, hRXRα and SKIP, while the N-CoR/RIP13-like and the LXXLL-containing protein were possibly novel. As mN-CoR/RIP13 had previously been identified through its interaction with RXR and was known to interact with VDR, this corepressor-like protein was not chosen for further investigation. SKIP and the LXXLL- motif containing protein thus were thought to be more likely to be novel VDR cofactors.

Table 3.1 Results of sequencing putative strong β-gal and Histidine "positive" clones

Potentially "physiologically relevant interacting proteins (n=7)		
Name of clone	**Genbank or Swiss-Pro Accession No.**	**Number of clones**
Ski-interacting protein (SKIP)	U51432	3
hRXRα	X52773	2
?mN-CoR or RXR-interacting protein-13 (RIP-13)	Q60974	1
?LXXLL protein	CAC38678	1
Probable "Spurious" negative proteins based on cytoplasmic nature and doubtful functional significance, includes the following:		
Name of clone(s)	**Genbank or Swiss-Pro Accession No.**	**Number of clones**
Apolipoprotein E3 precursor	P02649	1
IGFBP-4	U20982	2
Ca2+ antagonist binding protein	B56122	1
Uridine kinase	P52623	6
Ubiquinone proteins	L32917;M32246;P03905	5
Hypothetical proteins	S61185;P38182	4
Collagen proteins	A42426;S23298;P78421	2
Albumin	M12523	2
Haptoglobin	X00637	2
Antithrombin	E00116	1
Cytochrome B	M21186	1
ATF4 related protein	G245896	1
Complement	J03507	1
"False" positive proteins as failed to activate reporter genes when retransformed into VDR strain, includes the following proteins:		
Name of clone(s)	**Genbank or Swiss-Pro Accession No.**	**Number of identical clones**
Ribosomal proteins	U13369;M77233	4
Fibrinogen	J00128	1
p68 DNA helicase	X52104	1
ATP synthase	P00846	1
EBNA-1	P03211	1
Hepatitis B	CAA53358	1

3.3.2 Ski-interacting protein (SKIP)

SKIP was originally cloned using the proto-oncogene c-Ski as a "bait" in a two-hybrid screen (Dahl *et al.*, 1998b). It is a nuclear protein with high homology to a *Drosophila melanogaster* protein, Bx42, which is upregulated by treatment with the steroid hormone 20-OH-ecdysone, a ligand for the NHR Ecdysone receptor (ER). The ER heterodimerises with the ultraspiracle receptor, the *Drosophila* homologue of RXR. Furthermore, Bx42 is tightly associated with chromatin, is expressed throughout *Drosophila* development and appears to have a role in active transcription (Wieland *et al.*, 1992). Based on what little was known about SKIP at the time and the probable role of SKIP in NHR-dependent action, it was decided that further studies would focus on this protein. As for most genetic screens, the decision to pursue one line of enquiry left a number of interesting avenues for future investigation. Thus, in addition to the sequenced LXXLL-containing cDNA, there remain a number of other strong and weak positive clones that may represent other unknown or uncharacterised VDR-interacting factors.

3.3.3 Confirmation of Ski-interacting protein (SKIP) as a VDR-interacting protein.

Three identical partial SKIP cDNA clones encoding aa 145 to 536 and including 490 base pairs of the 3′ UTR (Figure 3.8) were isolated. SKIP-GAL4AD interaction with VDR-GAL4DBD and the converse combination of VDR-GAL4AD and SKIP-GAL4DBD confirmed a strong interaction between VDR and SKIP (Figure 3.9). Although ligand decreased interaction between SKIP-GAL4AD and VDR-GAL4DBD by 75%, it had no effect on the converse combination. Furthermore, the VDR-SKIP interaction appeared to be specific, as SKIP-GAL4AD failed to interact with irrelevant GAL4DBD fusion proteins including lamin, p53, Grb14 (Lyons *et al.*, 2001), TC4 and RANGAP1 (Deane *et al.*, 1997).

A)

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  1  AGGAAGAAGA AGCGGTAGAA GATGGCGCTC ACCAGCTTTT TACCTGCACC
 51  TACTCAGCTA TCTCAGGACC AGCTTGAGGC TGAAGAAAAG GCAAGATCCC
101  AGAGATCACG GCAGACCTCA CTGGTCTCCT CCCGAAGAGA ACCTCCCCCG
151  TACGGATACC GGAAAGGCTG GATACCTCGG TTATTAGAGG ATTTTGGAGA
201  TGGAGGTGCT TTTCCAGAGA TCCATGTGGC CCAGTATCCA CTGGATATGG
251  GACGAAAGAA AAAAATGTCG AATGCGCTGG CCATTCAGGT GGATTCTGAA
301  GGAAAAATTA AATATGATGC AATTGCTCGA CAAGGACAGT CAAAAGACAA
351  GGTCATTTAT AGCAAATACA CTGACCTGGT TCCAAAGGAG GTTATGAATG
401  CAGATGATCC AGACCTGCAA AGGCCCGATG AAGAAGCTAT TAAAGAGATA
451  ACAGAAAAGA CAAGAGTAGC CTTAGAAAAA TCTGTATCAC AGAAGGTCGC
501  CGCAGCCATG CCAGTTCGAG CAGCTGACAA ATTGGCTCCT GCTCAGTATA
551  TCCGATACAC ACCATCTCAG CAAGGAGTGG CATTCAACTC TGGAGCTAAA
601  CAGAGGGTTA TTCGGATGGT AGAAATGCAG AAAGATCCAA TGGAGCCTCC
651  AAGGTTCAAG ATTAATAAGA AAATTCCCCG GGGACCACCT TCTCCTCCTG
701  CGCCTGTCAT GCATTCTCCT AGCCGAAAGA TGACTGTAAA GGAACAACAA
751  GAGTGGAAGA TTCCTCCTTG TATTTCTAAC TGGAAAAATG CAAAGGGTTA
801  TACAATTCCA TTAGACAAAC GTCTGGCTGC TGATGGAAGA GGACTACAGA
851  CAGTACACAT AAATGAAAAT TTCGCCAAAT TGGCAGAAGC CCTCTACATT
901  GCTGATCGGA AGGCTCGTGA AGCTGTGGAA ATGCGTGCCC AAGTAGAGAG
951  AAAAATGGCT CAGAAAGAAA AGGAAAAACA TGAAGAGAAA CTTAGAGAAA
1001 TGGCCCAGAA AGCCAGGGAA AGAAGAGCTG GGATCAAAAC TCATGTGGAA
1051 AAAGAGGATG GGGAGGCACG TGAGAGGGAT GAAATCCGGC ATGACAGGCG
1101 AAAAGAGAGA CAGCATGACC GGAATCTTTC CAGGGCAGCT CCTGATAAGA
1151 GGTCGAAACT TCAGAGAAAT GAAAATCGGG ATATCAGTGA AGTTATTGCT
1201 CTCGGTGTTC CTAATCCTCG GACTTCCAAT GAAGTTCAGT ATGACCAAAG
1251 GCTCTTCAAC CAATCCAAGG GTATGGACAG TGGATTTGCA GGTGGAGAAG
1301 ATGAAATTTA TAATGTTTAT GATCAAGCCT GGAGAGGTGG TAAAGATATG
1351 GCCCAGAGTA TTTATAGGCC CAGTAAAAAT CTGGACAAGG ACATGTATGG
1401 TGATGACCTA GAAGCCAGAA TAAAGACCAA CAGATTTGTT CCCGACAAGG
1451 AGTTTTCTGG TTCAGACCGT AGACAGAGAG GCCGAGAAGG ACCAGTTCAG
1501 TTTGAGGAAG ATCCTTTTGG TTTGGACAAG TTTTTGGAAG AAGCCAAACA
1551 GCATGGTGGC TCTAAAAGAC CCTCAGATAG CAGCCGCCCC AAGGAACACG
1601 AGCATGAAGG CAAGAAGAGG AGGAAGGAAT AGGCACAGGT CTCTCCAAAG
1651 TGAATGAACT CTTACCCATA ACCCTAATGA TGCAAGTCAT ATGGGGGAAC
1701 ACTTTGTAAA TGGTCAGGAT AAAAACCAAA TCTGGGTGCC AGATCCCAGC
1751 ACTACTTTTT ATTACTGGAG AAATGGGGGG GATAGAAAAT TCTACTTTGA
1801 ATTATTTAGT TTTTTTTAAA GAGTGGGTTG TGTTTGTGCT TCTCCCACCT
1851 TTCAGCATTT ATAGAACATG CTGCCCCACA TACAAAGTCA AGACCACTTA
1901 CTTTTATGTG ACACTAGTAG TTTGGGGTTA ATGTTTTGTG TAAGAACAGC
1951 TGCATATGAG TAAAGTTACC CCAACCACAG TGAGGAGGAA GATGTTCACA
2001 TACTGGAACT GTCCTGCCAA ATAAATTTTG CCCCTATTGT GCTCTGTTTT
2051 AATTTGGAGT GGGCAAAGTA ACCTCTTGCT TGGTGCAACT ATTTGTTTCA
2101 AATAAAAACA TTTAGACAAA AA
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B)

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  1  MALTSFLPAP TQLSQDQLEA EEKARSQRSR QTSLVSSRRE PPPYGYRKGW

 51  IPRLLEDFGD GGAFPEIHVA QYPLDMGRKK KMSNALAIQV DSEGKIKYDA

101  IARQGQSKDK VIYSKYTDLV PKEVMNADDP DLQRPDEEAI KEITEKTRVA

151  LEKSVSQKVA AAMPVRAADK LAPAQYIRYT PSQQGVAFNS GAKQRVIRMV

201  EMQKDPMEPP RFKINKKIPR GPPSPPAPVM HSPSRKMTVK EQQEWKIPPC

251  ISNWKNAKGY TIPLDKRLAA DGRGLQTVHI NENFAKLAEA LYIADRKARE

301  AVEMRAQVER KMAQKEKEKH EEKLREMAQK ARERRAGIKT HVEKEDGEAR

351  ERDEIRHDRR KERQHDRNLS RAAPDKRSKL QRNENRDISE VIALGVPNPR

401  TSNEVQYDQR LFNQSKGMDS GFAGGEDEIY NVYDQAWRGG KDMAQSIYRP

451  SKNLDKDMYG DDLEARIKTN RFVPDKEFSG SDRRQRGREG PVQFEEDPFG

501  LDKFLEEAKQ HGGSKRPSDS SRPKEHEHEG KKRRKE*
```

Fig.3.8. SKIP cDNA sequence (Genbank accession number U51432)
A) SKIP cDNA sequence: ATG initiation and TAG stop codons are shown in blue. Sequence in red corresponds to partial cDNA isolated from yeast two-hybrid screen from base pair 454 to 2122 including protein coding sequence from aa145 to 536 and the 3′ UTR.
B) SKIP protein sequence: encoded protein is 536aa long, 61.5kD. Note the proline-rich (aa 219-233) (indicated by blue shading), SNW (aa174-339aa) (yellow shading) and SH2-like (373-453aa) (black shading) domains.

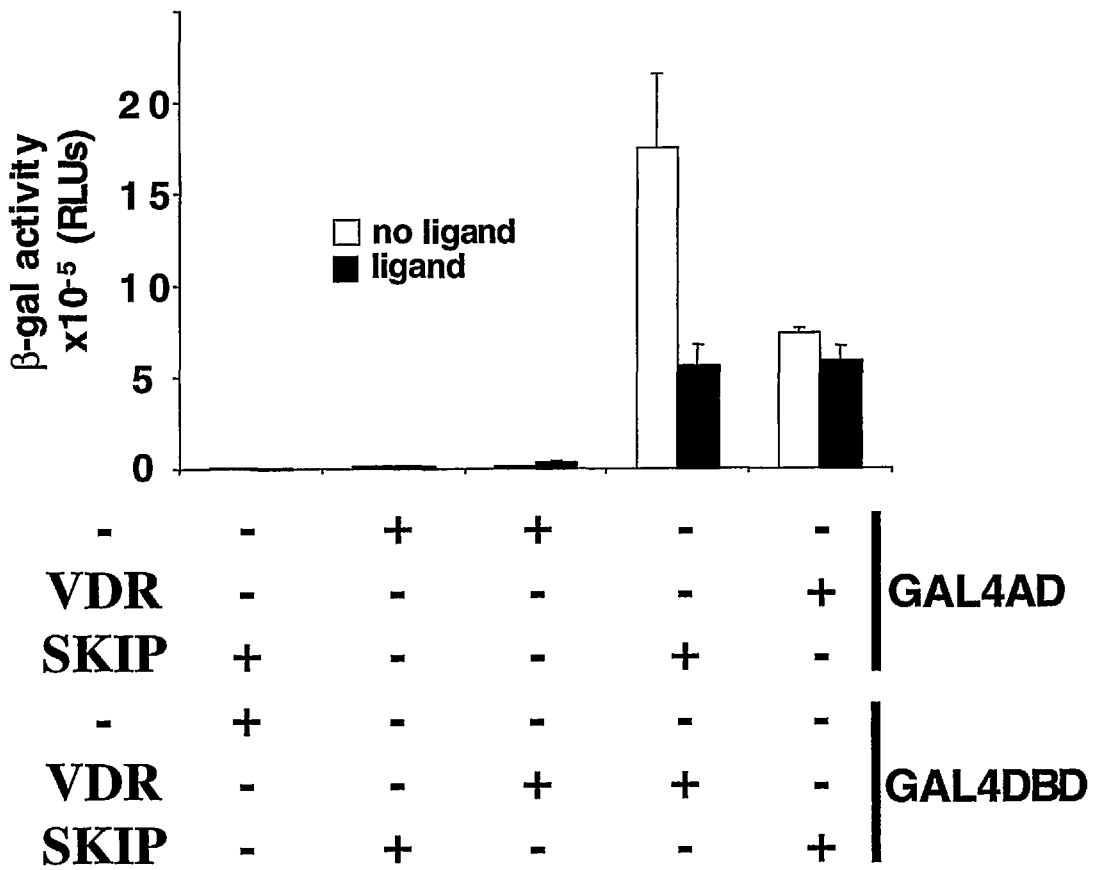

Fig. 3.9 SKIP VDR interaction and fusion protein orientation. Yeast two-hybrid interaction analysis showing SKIP-VDR interaction and effect of ligand $1,25(OH)_2D_3$ (10^{-8} M) on this interaction.

3.4 DISCUSSION

From its inception, the aim of this project was to gain understanding of how the variety of physiological functions of vitamin D might be mediated by the VDR. To this end, a screen was undertaken to identify and characterise VDR-interacting proteins using the yeast two-hybrid system. Using the VDR as a bait protein various putative VDR-interacting proteins were isolated from a human liver cDNA library. A large number of probable false positive clones were also isolated which made eventual identification of "true" positives more difficult. Use of a second reporter interaction assay (i.e. after the initial selection for growth in His-deficient medium and expression of β-galactosidase) reduced the number of original histidine positive clones by almost 50%. Further genetic screening of a subset of these clones identified a significant proportion of colonies (25 of 149 or 17%) which, when co-expressed with the original VDR, either failed to activate the reporter genes or non-specifically activated the reporter in the absence of VDR co-expression.

A decision was made to sequence strong positives without further genetic screening, based on the likelihood that false positives would be significantly fewer in this group than the moderate or weaker β-gal positives. This did not necessarily appear to be the case, as only 8 of 66 clones directly sequenced revealed potentially interesting sequences, while the reminder contained sequences associated with well-known "spurious" positives (Golemis, 2001). For this reason the direct sequencing approach was not continued for all the 131 strong β-gal positives. Whether the clones that remain to be analysed will reveal a similar proportion of "spurious" positives remains to be determined (Table 3.1). Amongst these are 27 clones that showed a ligand-dependent effect on activation of the histidine reporter. Presumably these 27 may include interacting proteins with higher degrees of physiological relevance. In this regard it was surprising that of the sequenced strong β-gal positive clones identified from the ligand screen, only two identical clones contained sequences of known biologically relevance to VDR physiology i.e. hRXRα. As expected because of the selection bias for and against yeast growth with ligand, a greater number of those clones in which ligand

decreased growth were identified in the non-ligand screen (15 of 20 or 75%). Whereas those that had increased growth (5 of 7 or 71%) were identified in the ligand screen. Thus, in retrospect, the ligand-responsive clones should have had higher priority for analysis and all clones, including strong positives, should have been screened by the yeast mating strategy to identify "false" positives. Because of the problem of non-specific protein-protein interactions with the system, use of the subsequently developed dual-bait system may have helped to minimise isolation of false positives (Serebriiskii *et al.*, 1999).

After several rounds of selection and screening, four highly likely candidates emerged. These clones included SKIP, hRXR, a putative protein with partial protein homology to the corepressor mN-CoR/RIP13 and a putative LXXLL containing coactivator protein. As such, Ski-interacting protein (SKIP) appeared to be have the highest probability of being a "true" VDR-interacting factor. Firstly, SKIP was originally identified as a protein-partner of the c-ski proto-oncogene through a yeast two-hybrid screen (Dahl *et al.*, 1998b). Secondly, VDR and SKIP were known nuclear proteins, whilst the nuclear localisation of the two other putative VDR-interacting proteins identified remained to be established. Based on the previously established nuclear localisation of SKIP and its strong interaction with VDR in the initial yeast interaction studies, it was thus decided that for this thesis further studies would focus on this protein.

The partial SKIP clone isolated from the two-hybrid screen, which lackied the first 144aa, was validated by testing against a series of other GAL4DBD fusion proteins. These included a number of nuclear (e.g. p53, lamin), and membrane type proteins (e.g. TC4, RANGAP (Deane *et al.*, 1997) and Grb14 (Lyons *et al.*, 2001). Consistent with expectations, these proteins did not interact with SKIP, thus indicating that the SKIP-VDR interaction was specific. Experiments in which the GAL4DBD and GAL4AD fusions with SKIP and VDR were reversed further supported this finding. Interestingly, ligand repressed VDR-SKIP interaction when the VDR was expressed as a GAL4DBD fusion and SKIP as a GAL4AD fusion, but not vice versa. The reason for this difference is unclear, but may relate to whether the VDR is

DNA-bound. As such, DNA binding may create a VDR-LBD conformation distinct from that of the VDR-GAL4AD fusion protein, in some way allowing a ligand-induced repression of the interaction. Further studies will be required to address the mechanisms for this observation.

In summary, SKIP was isolated from a human liver library as a strongly interacting protein with full-length hVDR. At the time of its identification in these studies, full-length SKIP identified through its interaction with the Ski oncogene, was listed in the protein database (Dahl *et al.*, 1998b). Given the strength of the SKIP-VDR interaction further studies were undertaken to define the role of SKIP in VDR-dependent gene transcription. These included studies which tested the role of SKIP in modulation of VDR- and RXR-dependent transcription in mammalian cells (Chapter 4), its interaction with cofactors involved in histone acetylation (corepressors and coactivators, Chapter 5), and its potential role in modulation of transforming growth factor (TGF)–β signalling through its interaction with Smad proteins and c-Ski (Chapter 6).

Chapter Four

SKIP modulation of NHR-Dependent Transcription

4.1 INTRODUCTION

Coactivators bind NHRs either directly or via other intermediary proteins to modulate target gene transcription (Glass and Rosenfeld, 2000). These interactions can be mediated via the AF-1 domain in a ligand-independent manner, or more commonly via the AF-2 domain in a ligand-dependent manner (McKenna *et al.*, 1999). Interactions between the AF-1 and AF-2 domains of NHRs can also be facilitated by various cofactors, such as SRC-1 or p300 (Kobayashi *et al.*, 2000; Onate *et al.*, 1998). The mechanisms for these effects are not well understood, but may involve histone acetylation and/or phosphorylation, or interaction with other cofactor complexes involved in each or many of these functions (Chen *et al.*, 2001; Cheung *et al.*, 2000a; Jacobson *et al.*, 2000; Kremer and Kremer, 2001; Kuo and Allis, 1998; Lemon and Tjian, 2000; Lemon and Freedman, 1999).

Numerous coactivators have been demonstrated to interact with a diverse range of NHRs (McKenna *et al.*, 1999) (Table 1.2). Few coactivators have been shown to display any cell-line-specific actions, although this, may in part reflect a lack of investigation for such effects. However, various coactivators and corepressors appear to be expressed in tissue-, cell-line- and development stage-specific patterns (Misiti *et al.*, 1999; Misiti *et al.*, 1998). Additionally, many, if not all, of these cofactors exist in large protein complexes associated with other regulatory proteins (Glass and Rosenfeld, 2000) (sections 1.7 and 1.9). Thus, differences in patterns of cofactor expression may be involved in differential modulation of NHR target gene transcription (Lavinsky *et al.*, 1998; Misiti *et al.*, 1999; Misiti *et al.*, 1998).

The focus of the present studies, Ski-interacting protein (hSKIP), or NCoA-62 (mSKIP) was originally cloned using the v-ski proto-oncogene as a bait in a yeast two-hybrid screen (Dahl *et al.*, 1998b). Subsequently, SKIP was identified as a VDR-interacting factor in a yeast two-hybrid screen (section 3.3.3) (Leong *et al.*, 1998a). During the course of these studies, SKIP was also identified independently by other groups as a VDR coactivator (Baudino *et al.*, 1998) and then as a CBF-1-interacting factor involved in Notch signalling (Zhou *et al.*, 1999; Zhou and Hayward, 2001; Zhou *et al.*, 2000b) and more recently as a poly(A)-binding 2-

(PABP2)-interacting protein in association with MyoD, a key transcription factor involved in muscle differentiation (Kim *et al.*, 2001).

As SKIP action as a VDR coactivator was examined in only one cell line (COS7 cells) (Baudino *et al.*, 1998), neither its potential cell-specific effects nor its modulation of RXR-dependent transcription had been investigated. Therefore, SKIP modulation of NHR-dependent transcription was examined in two mammalian cell-lines.

4.1.1 Aims

The primary aims of this chapter were to determine if SKIP modulation of NHR action was cell-line and receptor-specific. The corollary of this hypothesis was that cell-line specific factors may modulate SKIP action and its interactions with different NHRs.

Initial studies of SKIP interactions with VDR and RXR were performed *in vivo* using the yeast two-hybrid system. Subsequent studies examined the effect of SKIP overexpression on various hormone-responsive reporter constructs in two cells line, the terminally-differentiated CV-1 (African green monkey kidney) and undifferentiated P19 (murine embryonal carcinoma) cell lines. These studies investigated whether SKIP behaved as a cell line-specific modulator of NHR-dependent transcription. The effects of SKIP on VDR- and RXR-dependent transcription were compared to its effects on thyroid and glucocorticoid receptor-dependent reporters.

Direct physical interactions between SKIP and VDR and RXR *in vitro* were examined by GST-binding studies. Finally, to investigate whether specific domains within SKIP were required for activation or repression function, a series of SKIP deletion mutants were tested in mammalian one-hybrid and RXR transactivation assays.

4.2 METHODS

4.2.1 Construction and cloning of SKIP mutants in yeast and mammalian expression plasmids

4.2.1.1 PCR amplification of SKIP mutant cDNAs

The plasmid SKIP-pCGN (Dahl *et al.*, 1998b) was used as a template for amplification of the wild-type and mutant SKIP cDNAs. For C-terminal deletion SKIP mutants (1-200aa, 1-333aa) a common forward primer (GL51) incorporating both an *EcoR*1 and *Xba*1 at the 5'end site was used, while for N-terminal deletion mutants (145-536aa, 201-536aa and 334-536aa) a common reverse primer (GL50) incorpartaing a *BamH*1 3' site was used (Table 4.1). Other deletion contructs in which different regions of the N- and C-terminal of SKIP were deleted (145-333aa and 201-333aa) required the use of specific forward and reverse primers as listed in Table 4.1. PCR products were generated by using *Taq* polymerase (Perkin Elmer) (section 2.2.7) and cloned by T/A cloning into the pGEMTeasy™ vector (Promega) as per the manufacturer's instructions. Ligation reactions were transformed into XL1-Blue (section 2.1.8) and recombinants diagnosed by blue white selection on X-gal LB ampicillin plates (section 2.2.8.4).

4.2.1.2 Cloning of SKIP mutants into yeast and mammalin expression vectors

Wild-type and deletion mutant recombinant SKIP-pGEMTeasy™ plasmid DNAs were then purified and amplified (section 2.2.4.2) after diagnostic restriction analysis (section 2.2.8.1). Large scale DNA preparations were then used for restriction digestion (2.2.8.1) from which cDNA inserts were purified (section 2.2.5.2) and cloned into the following expression vectors: *EcoR*1/*BamH*1 inserts into pACTIIb (yeast two-hybrid, Issa *et al.*, 2001); pM and VP16 (mammalian two-hybrid, Clontech) and pSG5 (mammalian expression, Stratagene) vectors; *Xba*1/*BamH*1 SKIP cDNA inserts were cloned into pCGN (HA-tagged mammalian expression) vector (Dahl *et al.*, 1998b). All constructs were confirmed to be correct and in frame by automatic sequencing (section 2.2.9.2).

Table 4.1 Primers used for cloning SKIP mutant constructs in pACTIIB, pSG5, pcGN, pM and VP16 expresion vectors

Primer Name	Sequence and description
GL51	5′-GGGAATTCCCGGGGTCTAGAACCACCATGGCGCTCACCAG CTTTTA-3′ Corresponds to SKIP cDNA sequence encoding first 7 aa where +1 (underlined) is the A of the first methionine-encoding ATG (Dahl *et al.*, 2001)
GL50	Reverse primer 5′-GCGGGATCCCTATTCCTTCCTCCTCTT-3′ Corresponds to nucleotides 1632 to 1615 of SKIP coding sequence encoding C-terminal 5aa and TAG stop codon (aa532-536aa)
GL52	5′-GGGAATTCCCGGGGTCTAGAACCACCATGGGGAGAAGAGC TGGGATCAAA-3′ corresponds to nucleotides 1027 to 1044 of SKIP coding sequence encoding aa334-339
GL53	5′-GGGAATTCCCGGGGTCTAGAACCACCATGGAAATGCAGAA AGATCCAATG-3′ corresponds to nucleotides 628 to 648 of SKIP coding sequence encoding aa201-207)
GL54	Reverse primer 5′-GCGGGATCCTACCATCCGAATAACCCTCTG-3′ corresponds to nucleotides 627 to 607 of SKIP coding sequence encoding aa200-194
GL55	Reverse primer 5′-GCGGGATCCTTCCCTGGCTTTCTGGGCCAT-3′ corresponds to 1025 to 1006 of SKIP coding sequence encoding aa333-297
GL81	Forward primer 5′-GGGCCCGAATTCTCTAGAATGGAAAAGACA AGAGTAGCC-3′ corresponds to 460 to 477 of SKIP coding sequence encoding aa145-150

4.2.1.3 Cloning of RXR-pLEXA yeast expression plasmid

To create mRXRβ-pLEXA yeast expression plasmid for yeast three-hybrid studies (section 4.4.1) (Issa *et al.*, 2001), an *Eco*RI/*Sal*I insert from mRXRβ–pAS1-CYH2 (Leong *et al.*, 1998b) was excised (section 2.2.8.1), purified (section 2.2.5.2) and cloned (section 2.2.8.2) into pLEXA vector (Clontech).

4.2.1.4 Cloning of VDR-GAL4DBD mammalian expression plasmid

The mammalian expression plasmid VDR-pM expressing a wild-type VDR-GAL4DBD fusion protein was cloned by ligating the *Bam*H1 insert from VDR-pAS2-1 (section 3.3.4) into pM (Clontech).

4.3. RESULTS

4.3.1. SKIP INTERACTION WITH VDR AND RXR

4.3.1.1. SKIP interaction with VDR and RXR in yeast two hybrid system

The original SKIP clone isolated in the yeast two hybrid screen (SKIP 145-536aa) was re-tested for interaction with VDR in a mated diploid yeast strain (CG1945/Y187) in the absence and presence of 1,25-$(OH)_2D_3$ (Figure 4.1A). In the absence of 1,25-$(OH)_2D_3$ there was a strong interaction between SKIP and VDR with a high β-galactosidase reporter activity, which decreased by about 30% in the presence of 1,25-$(OH)_2D_3$. There was no significant 1,25-$(OH)_2D_3$ effect on reporter activity when either protein was expressed alone. SKIP interaction with RXR, the heterodimer partner of VDR, was tested in a diploid yeast strain in the absence or presence of 9-*cis*-RA. In contrast to SKIP interaction with VDR, its interaction with mRXRβ occurred only in the presence of its ligand, 9-*cis*-retinoic acid (9-*cis*-RA) (Figure 4.1A).

To determine whether ligand-dependency of these interactions was modulated by yeast-specific factors, a similar two-hybrid interaction analysis was performed in the haploid yeast strain, SFY526 (Figure 4.1B). In this strain, in contrast to the YRG2 strain and the CG1945/Y187 (Figure 3.9), VDR interaction with SKIP was ligand-dependent, whilst RXR interaction with SKIP remained ligand-dependent. Thus, yeast-strain specific factors modulated SKIP interaction with VDR, whereas its interaction with RXR appeared primarily ligand-dependent in all yeast strains tested.

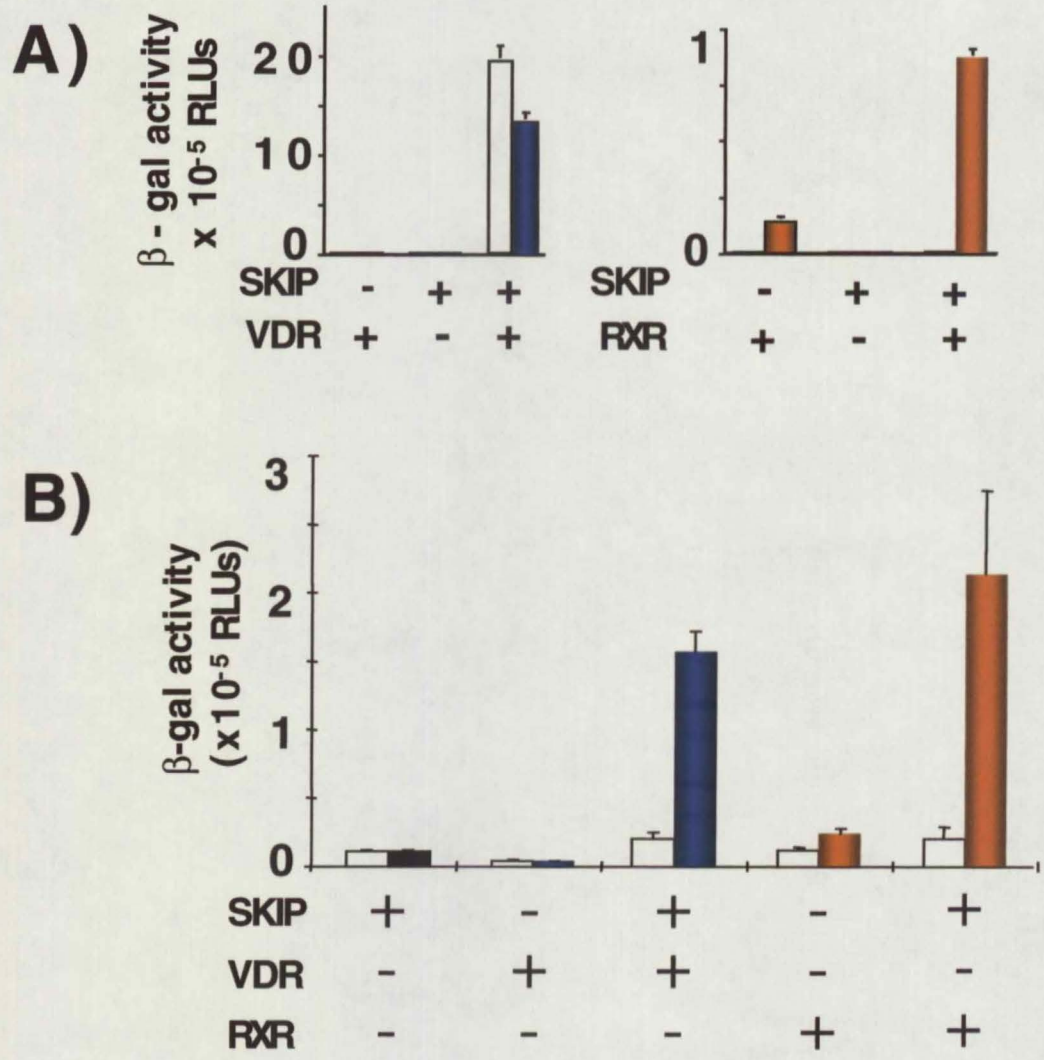

Fig. 4.1 SKIP interaction with RXR and VDR in yeast two-hybrid system.
A) SKIP interaction with VDR and RXR show ligand-specific differences. SKIP (aa145-536)-GAL4AD interaction with wild-type VDR-GAL4DBD or mRXRβ-GAL4DBD in haploid mated yeast strain CG1945/Y187.
B) Ligand-dependency of SKIP interaction with VDR, but not RXR, is dependent on yeast-specific factors. Yeast two-hybrid interaction analysis as in A), except performed in haploid SFY526 yeast strain in absence (open columns) or presence of ligand (closed columns $1,25(OH)_2D_3$ (blue) or 9-*cis*-RA (red), or both ligands added to SKIP only strain, black, in B).

4.3.1.2 RXR Deletion Analysis in Yeast Two-Hybrid Assay

Although the large majority of coactivators interact with NHRs through the AF-2 domain, SKIP interaction with VDR had previously been shown to be AF-2-independent (Baudino *et al.*, 1998; McKenna *et al.*, 1999). As RXR is an obligate heterodimer partner of VDR and thus important for modulating VDR function *in vivo* (Li *et al.*, 2000b), it was next determined whether the AF-2 domain of RXR was required for SKIP interaction in a RXR-deletion analysis in yeast.

Deletion of the AF-2 (ΔAF-2) and/or the LBD (ΔLBD) of RXR abrogated interaction with SKIP (Fig 4.2A). Immunoblot analysis of lysates from yeast used in two hybrid studies using antibodies directed against GAL4DBD and GAL4AD showed 3 fold decreased expression levels of mutant versus wild-type mRXRβ-GAL4DBD proteins, but comparable levels of SKIP-GAL4AD fusion proteins (Figure 4.2). As the level of reporter activity was abrogated by co-expression of the mutant RXR proteins with SKIP these changes in expression may partially explain the lack of interaction between the mutant RXR proteins and SKIP. However, there was still measurable mutant RXR protein expression but no detectable interaction with SKIP. Thus, in contrast to VDR interaction with SKIP which is AF-2-independent (Baudino *et al.*, 1998), SKIP interaction with RXR was AF-2- and ligand-dependent.

4.3.1.3 SKIP Deletion Analysis in Yeast Two-Hybrid Assay

To determine which domains of SKIP are required for interaction with RXR, yeast two-hybrid assays with mutant SKIP proteins were performed. Only the 1-333aa mutant SKIP protein interacted with RXR in a ligand-dependent manner comparable to wild-type SKIP (Figure 4.2B). The 1-200aa and 201-536aa mutants had weaker interaction and the C-terminus (aa334-536) failed to interact with RXR. In contrast, interaction of the SKIP (145-536aa) with RXR was about 5-7 fold greater than that of wild-type SKIP.

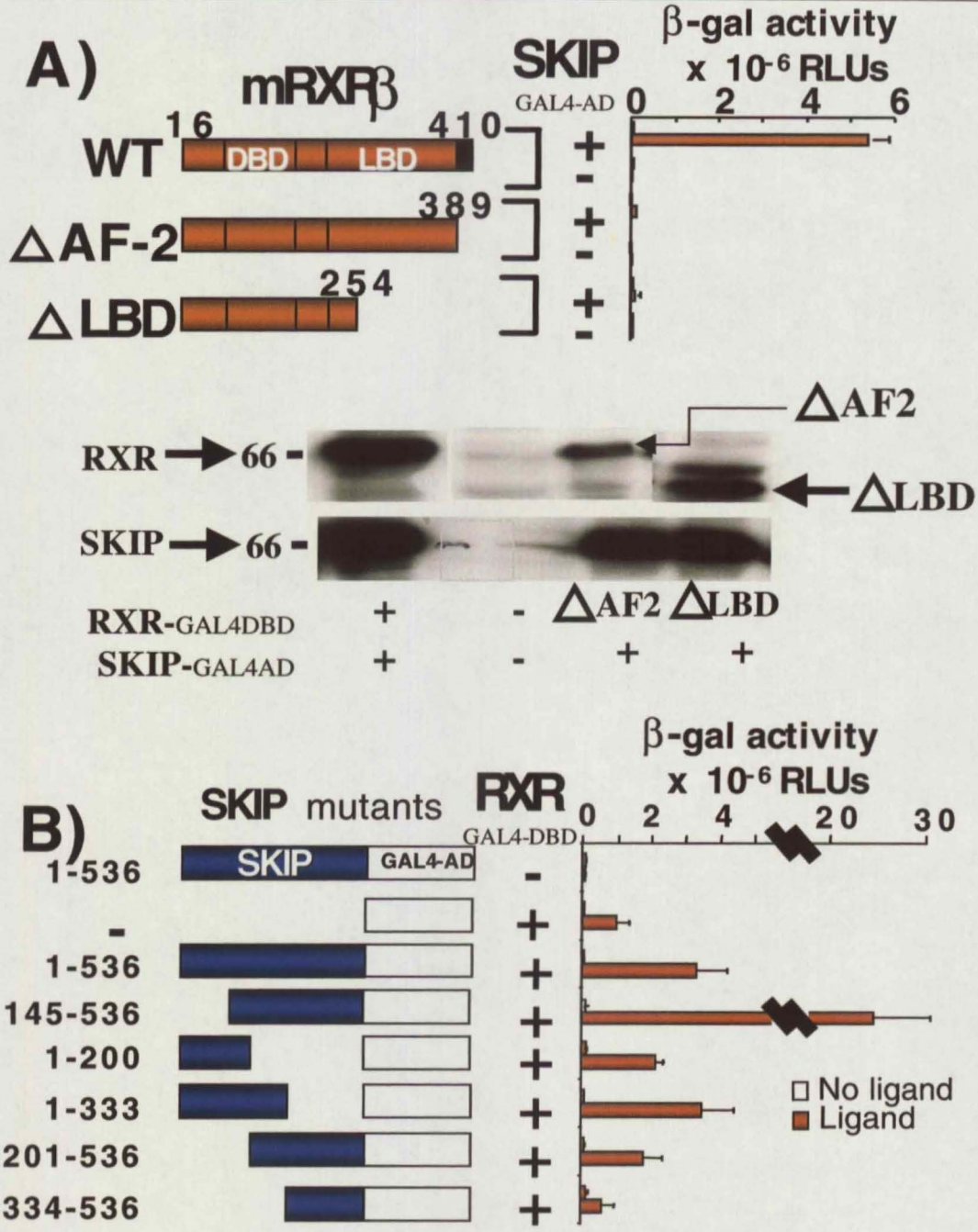

Fig. 4.2 RXR and SKIP regions required for interaction in yeast two-hybrid system.

A) AF-2 domain within RXR required for interaction with SKIP. Deletion of mRXRβ AF-2 domain (black box from aa390-410) (ΔAF-2) or ligand-binding domain (ΔLBD) abrogates interaction between RXR and SKIP. Immunoblot analysis of yeast extracts using GAL4DBD (upper) and GAL4AD (lower) antibodies (Santa Cruz). 66 =66kD MW of fusion proteins.

B) Region between 145-333aa within SKIP sufficient for RXR interaction. Interaction analysis with SKIP-GAL4AD wild-type and mutants, as indicated with wild-type mRXRβ-GAL4DBD.

For both studies diploid yeast strain CG1945/Y187 was used. β-galactosidase reporter activity was measured using liquid cultures of at least 6 individual colonies from 3 independent experiments with results shown as mean ±SEM. (vehicle, open column), (9-*cis*-RA, filled column).

4.3.1.4 SKIP interaction with RXR and VDR in vitro

To determine whether SKIP interacted directly with RXR and VDR *in vitro*, GST-binding experiments were performed (Figure 4.3). *In vitro* translated [^{35}S]-labelled wild-type SKIP interacted with wild-type GST-RXR. Similarly, [^{35}S]-labelled RXR and VDR interacted with wild-type GST-SKIP protein. In contrast to the *in vivo* effects, ligand had no effect on SKIP binding to RXR or VDR *in vitro*.

4.4 SKIP MODULATION OF NUCLEAR RECEPTOR-DEPENDENT TRANSCRIPTION IN MAMMALIAN CELLS

4.4.1. NHR-transactivation Assays in CV-1 and P19 cells

The 24 hydroxylase promoter contains two highly responsive VDREs, whilst the RXRE-tk-luciferase promoter binds RXR homodimers and thus allows assessment of the RXR signalling pathway without associated heterodimer effects (Dwivedi *et al.*, 1998; Mangelsdorf *et al.*, 1991; Zierold *et al.*, 1995). The CV-1 and P19 cell lines were chosen for these initial studies as both cell lines express low amounts of endogenous VDR. In contrast, RXR is ubiquitously expressed being 5-7 fold more highly expressed in P19 than CV-1 cells (Issa *et al.*, 2001). Additionally, P19 cells are undifferentiated, in contrast to CV-1 cells, thus allowing comparison in two cell-types at vary different stages of differentiation. Furthermore, the capacity to induce P19 cell differentiation were valuable for later studies (Chapter 5) (Bain *et al.*, 1994). Undifferentiated P19 cells have also exhibited cell-line specific effects with transcription factors e.g. TFIIB (Blanco *et al.*, 1995), and thus were thought a suitable cellular model in which to examine the potential cell-specific actions of SKIP.

In the CV-1 cell line, SKIP augmented ligand-dependent RXRE and VDRE reporter activity between 4- and 10-fold, whereas basal reporter activities of the control tk-luciferase and RXRE and VDRE reporters were increased about 2-3 fold (Figure 4.4A). In contrast, in the P19 cell line, completely opposite effects of SKIP were observed (Figure 4.4A). SKIP decreased ligand-dependent reporter activity for both the RXRE and VDRE-containing reporters by about 2 fold without altering basal reporter activity.

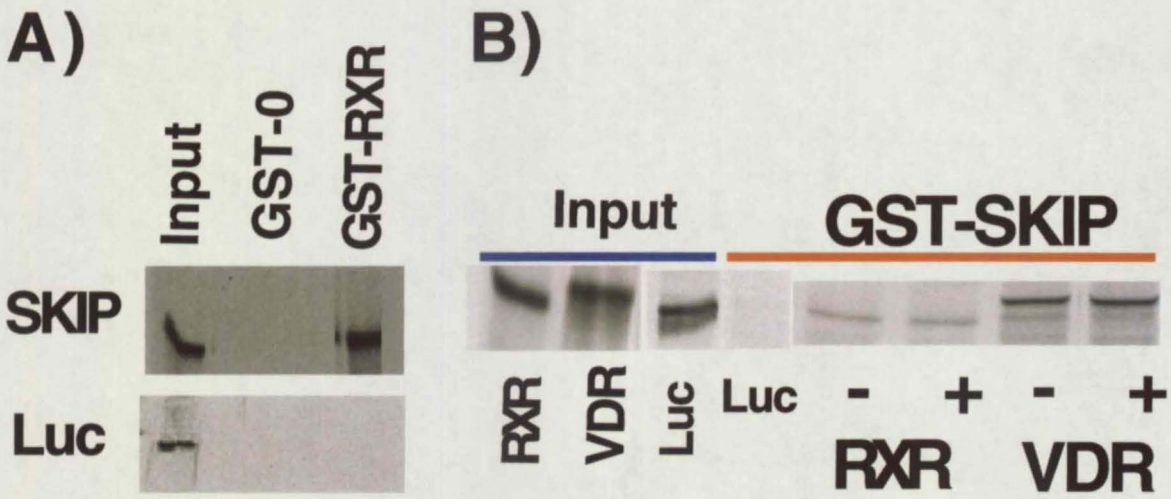

Fig. 4.3 SKIP interaction with RXR and VDR *in vitro*.
A) In contrast to SKIP interaction with RXR *in vivo* in yeast, its interaction with RXR is ligand-independent *in vitro*..
Glutathione sepharose transferase (GST) binding of wild-type RXR or GST-O control incubated with equal amounts of *in vitro* translated full-length [^{35}S]-labelled SKIP (upper panel) or luciferase (luc) as a negative control (lower panel).
B) SKIP interaction with VDR is ligand-independent*in vitro*.
Similar GST-SKIP binding studies was performed with full-length [^{35}S]-labelled RXR and VDR in the absence (-) or presence of respective ligand (+). Input proteins represent one-tenth of loaded lysate. Results are representative of one of 3 independent repeat experiments. No binding of lysates was observed to GST-O control (data not shown).

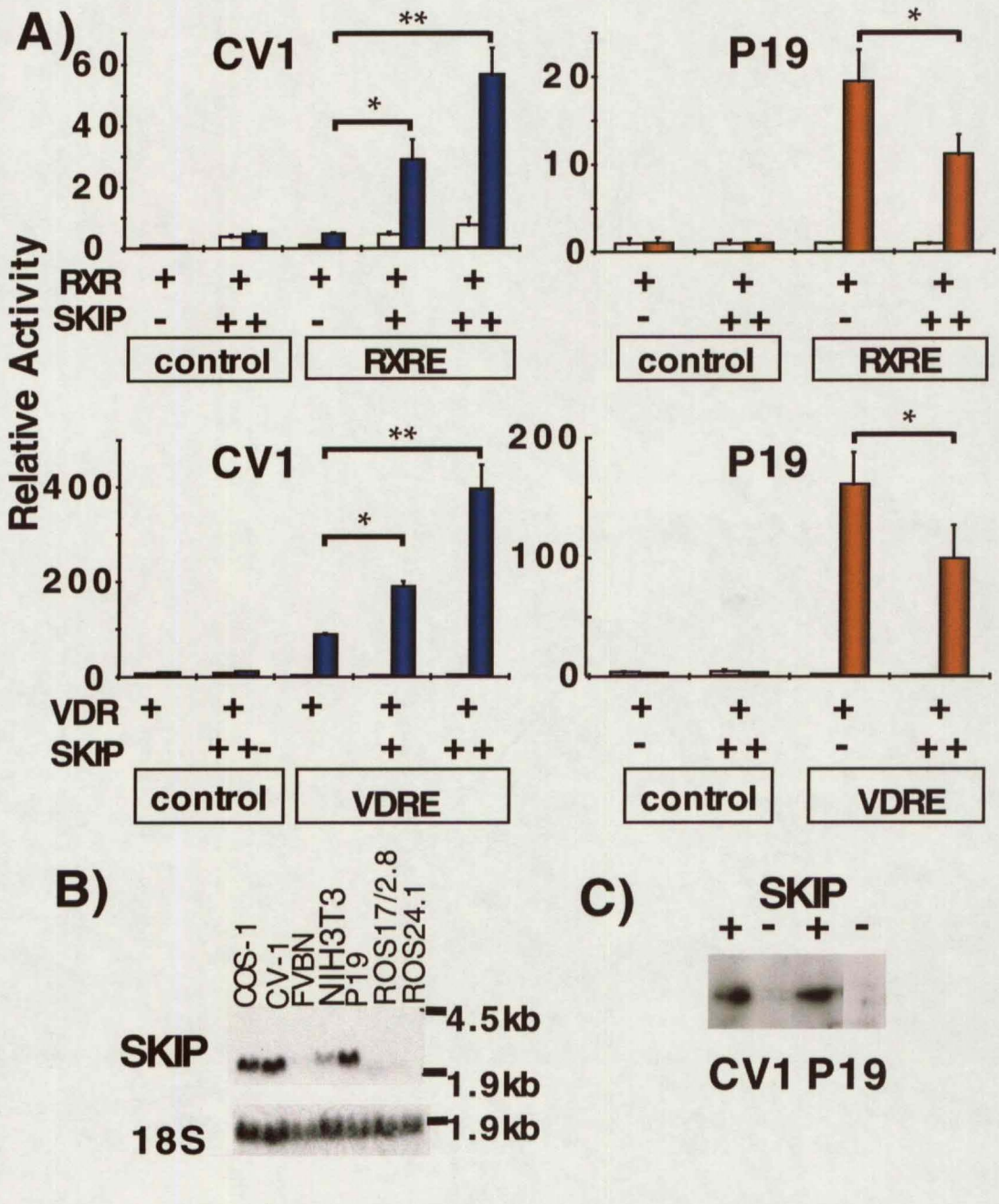
A)
CV1
P19
Relative Activity
RXR
SKIP
control
RXRE
VDR
VDRE
B)
COS-1
CV-1
FVBN
NIH3T3
P19
ROS17/2.8
ROS24.1
SKIP
18S
4.5kb
1.9kb
1.9kb
C)
SKIP
CV1 P19

Fig. 4.4. Cell-specific effects of SKIP on VDR- and RXR-dependent reporters.

A) SKIP activates VDR- and RXR-dependent reporter gene activity in CV-1, but represses these same activities in P19 cells. In CV1 or P19 cells mCRABPII RXRE-tk-luc reporter or control tk-luc vector (upper panel) or r24-hydroxylase-VDRE-luciferase reporter (lower panel) or control pGL-3 basic vector (each 2.5μg), as indicated, was co-transfected with pExpress-mRXRβ or pM-hVDR (each 2.5μg) with increasing amounts of wild-type SKIP-pCGN expression plasmid [2.5 μg (+), or 5 μg (++)] or empty pCGN vector. Cells were treated overnight with vehicle (open columns) or ligand (filled columns 9-cis-RA or 1,25 $(OH)_2D_3$), as indicated. Reporter activities are expressed as luciferase activity relative to non-SKIP unliganded transfection control which was set at 1. Results are shown as mean ± SEM and represent the results from 6 independent transfections each performed in triplicate. * indicates $P<0.05$ or ** $P< 0.002$ between ligand-induced reporter activities

B) SKIP mRNA levels are similar in COS-1, CV-1 and P19 cells, but lower in other cell-lines. Northern blot analysis of SKIP expression in various cell types with 18S control

C) SKIP protein levels are similar in transfected CV-1 or P19 cells. Western analysis of nuclear extracts prepared from CV1 or P19 cells transfected with Skip-pCGN or pCGN plasmids using anti-HA antibody.

To evaluate whether differences in expression of endogenous SKIP could explain these cell-specific effects, SKIP RNA levels were examined (Figure 4.4B). SKIP was expressed at similar high levels in P19, CV-1 and COS-1 cells, and low levels in NIH3T3, primary mouse osteoblast cells (FVB/N) and rat osteosarcoma cell lines (ROS 17/2.8 and ROS 24.1).

As differences in SKIP expression following transient transfection may also potentially explain the opposite cell-line specific effects observed, a Western analysis was performed on nuclear extracts prepared from cells transfected with the HA-SKIP expression construct (Figure 4.4C). There was equal expression of transfected full-length HA-tagged SKIP in both cell lines. These analyses indicate that the cell-line specific effects of SKIP were not secondary to differences in endogenous or transient expression levels of SKIP.

4.4.2 Receptor-Specific actions of SKIP

To test whether SKIP could influence the activity of other NHRs, and whether there were cell line differences in any SKIP effects, transient transfections were then performed using reporters containing a thyroid response element (TRE, IR0-tk-luciferase) and a glucocorticoid response element (GRE-tk-luciferase) (Schule *et al.*, 1988) (Figure 4.5A and B).

In the CV-1 cell line, SKIP augmented both basal and ligand-dependent reporter activities by 3-4 fold with the RXRE reporter such that there was no change in the 4-fold induction with ligand. With the TRE reporter there was a 5-fold increase in basal and 16-fold increase in ligand-dependent reporter activity and hence a concomitant enhancement of fold-induction with ligand by SKIP from 7 to 24-fold, whereas with the GRE reporter, SKIP augmented ligand-dependent reporter activity by 4-fold.

In contrast in P19 cells, SKIP in these studies failed to activate rather than frankly repressing RXR-dependent transcription. With the TRE reporter, SKIP increased ligand-dependent reporter activity by 2-fold, such that there was an increase in fold-induction from 98 to 137 i.e. 1.4 fold, whereas with the GRE reporter, SKIP augmented ligand-dependent reporter activity to a similar degree as in CV-1 cells i.e. 4-fold.

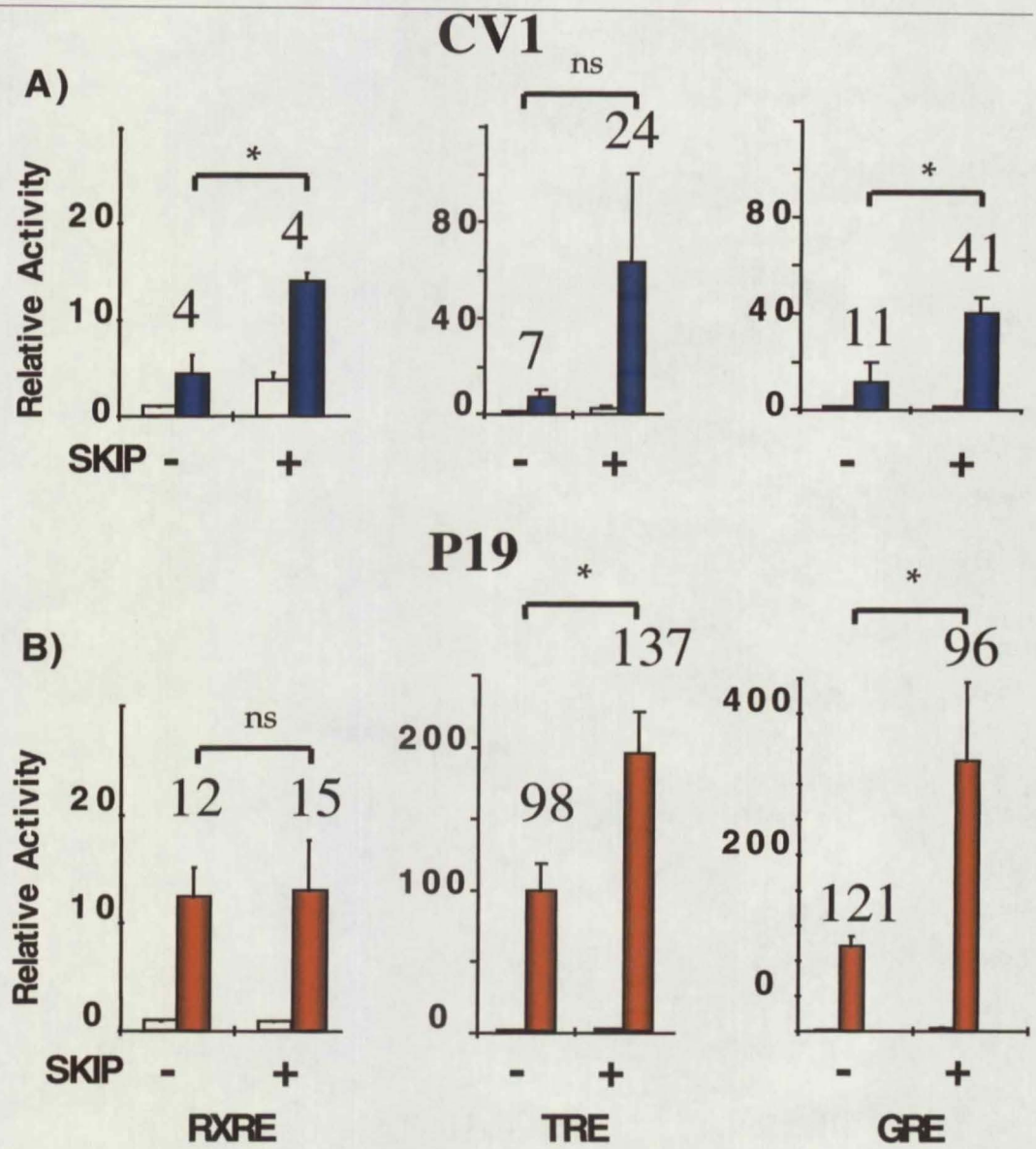

Fig. 4.5. SKIP modulation of RXR-, TR- and GR-dependent reporters.
A) In CV1 cells, SKIP activates all three reporters.
B) In P19 cells, SKIP action is receptor-specific, as SKIP activates only the TR- and GR-dependent reporters, but fails to activate the RXR-dependent reporter.
CV1 cells (A) and P19 cells (B) were transiently transfected in 24 well plates with luciferase reporter genes containing a RXRE (CRABPII-tk-luc), TRE (IR-0-tk-luc) and GRE (GRE_2)-tk-luc (250ng) with expression plasmids for the wildtype mRXRβ (mRXRβ–pEXpress, TRβ1 (TRβ1–pEXpress) and GR (GR1-pSV) (each 100 ng), respectively, with empty vector pCGN (-) or wildtype SKIP expression plasmid (SKIP-pCGN), (+) (450ng) as indicated. Cells were treated with ligand (9-*cis*-RA, T3 or dexamethasone (1μM) (filled columns) or vehicle (open columns). The figure represents the activity relative to cells transfected with pCGN in absence of ligand set at 1 and are results from at least 3 independent experiments performed in triplicate and luciferase reporter activity is shown as mean ± SEM. Numbers above columns represents mean fold induction with ligand. * indicates $P<0.05$ between ligand-induced reporter activities. ns non-significant.

4.4.3 SKIP and RXR Modulation of Vitamin-D-Dependent Transactivation of the 24-hydroxylase promoter reporter

The receptor-specific effects of SKIP in the transient transfection studies (Figure 4.6) suggested a correlation between the degree of SKIP repression and the RXR-binding properties of the various reporters i.e. RXR homodimer (RXRE) versus RXR heterodimer (TRE) and non-RXR binding (GRE) (Dahl *et al.*, 1998a; Delaunay *et al.*, 1996; Leong *et al.*, 1998b; Schule *et al.*, 1988).

While VDR and TR expression does not vary between P19 and CV-1 cells, there is about a 5-7 fold greater expression of RXR in P19 than CV-1 cells (Issa *et al.*, 2001). Thus differences in expression of RXR or a RXR-associated protein could in part have contributed to the divergent effects of SKIP action in the two cell types.

To test this hypothesis, transient RXR and VDR levels were varied in cotransfections with the rat 24-hydroxylase promoter luciferase reporter into CV-1 cells, where SKIP previously activated reporter activity in a dose-dependent manner (Figure 4.4). This promoter contains two functional VDREs and a functional RXRE (Arbour *et al.*, 1998; Zou *et al.*, 1997).

Co-transfection of SKIP with VDR led to approximately an 8-fold increase in vitamin-D-induced reporter activity, whereas co-treatment with 1,25-$(OH)_2D_3$ and 9-*cis*-RA caused a 40% decrease in this activity (Figure 4.6). By comparison, when SKIP was co-transfected with both VDR and RXR, there was a 2-fold repression of vitamin D-induced reporter activity ($P<0.05$). The addition of 9-*cis*-RA with 1,25$(OH)_2D_3$ led to a 60% decrease in this reporter activity. Co-transfection of SKIP with RXR induced only a slight increase in reporter activity after ligand treatment, indicating that RXR:SKIP was not a powerful combination on the RXRE in this promoter. Thus in this promoter context, functional interaction between RXR and SKIP was readily detected only by its negative impact on VDR-mediated transcription. Whether this was as a consequence of a RXR interaction with SKIP and "squelching" of SKIP away from the VDR transcriptional complex, or whether

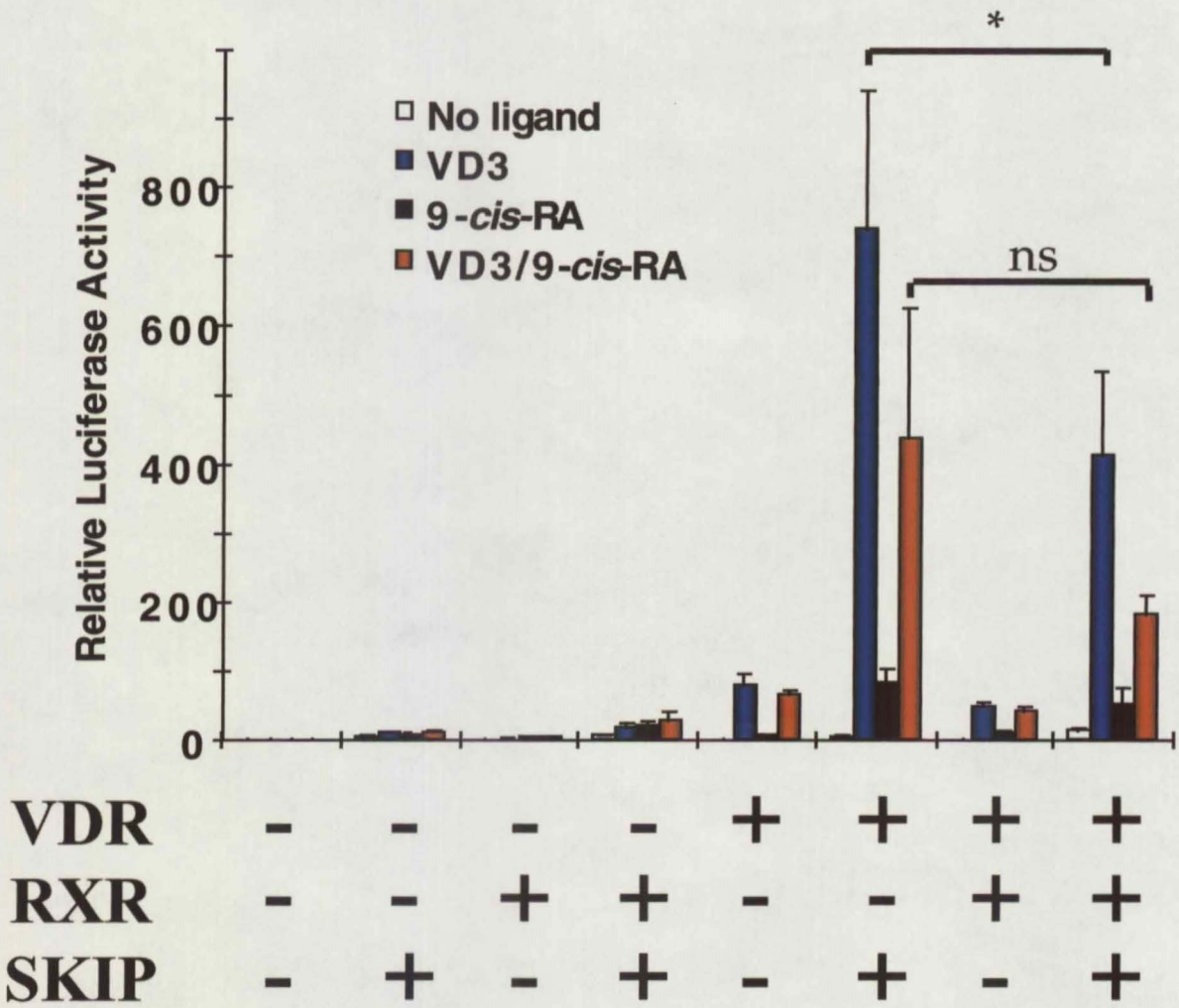

Fig. 4.6. SKIP modulation of RXR/VDR effects on the 24-hydroxylase promoter.

RXR with SKIP represses VDR-dependent transcription in CV1 cells. Transient transfections were performed with the 24-OH-promoter-luc reporter (250ng) with VDR-pM (125ng), mRXRβ-pExpress (125ng)and SKIP-pCGN (450ng) as indicated. Cells were treated overnight with respective ligands. Results shown are relative to activity associated with non-ligand empty vector control set at 1 and are mean ± SEM of triplicate samples from 6 independent transfections. * $P<0.05$, ns not significant.

SKIP and RXR in combination, but not individually, associated with repressor molecules, such as N-CoR and HDACs, are two possible explanations for these observations. These possibilities are addressed further by studies outlined in the next section and by studies discussed in Chapter 5.

4.5 INTERACTIONS BETWEEN SKIP, VDR AND RXR IN YEAST

4.5.1. Yeast Three-Hybrid System

One major advantage of using yeast as a cell model to study NHR action is that yeast do not express NHRs or the majority of associated corepressors and coactivators (McEwan, 2001). Thus an interaction between exogenously overexpressed NHRs in yeast is less likely to be complicated by endogenous factors present in the yeast host, as compared to mammalian cells.

Therefore to investigate whether co-expression of RXR could modulate SKIP interaction with VDR *in vivo*, a yeast three hybrid analysis was performed (Figure 4.7) using a diploid, mated yeast strain expressing mRXRβ as LEXADBD, VDR as GAL4DBD and SKIP as GAL4AD fusion proteins. By employing a single yeast diploid strain thus may minimise the impact of strain-specific effects on NHR function as observed earlier (Figure 4.1).

4.5.2 SKIP, VDR, RXR interaction *in vivo* in Yeast Three Hybrid Assay

An analysis was made of a mRXRβ-LEXADBD strain coexpressing VDR and SKIP (Figure 4.7). In the VDR-SKIP positive and RXR-negative strain, as in the two-hybrid analysis, there was a high level of ligand-independent reporter activity; in the present case however, this activity was unaltered by ligand (Figure 4.7). When RXR was co-expressed with SKIP in the absence of VDR there was a moderate level of ligand-independent reporter activity. This suggested that SKIP and RXR were able to interact and alter transcription despite the lack of a DNA-binding domain protein intermediate; this presumably occurred via an interaction with the basal transcription machinery. This activity appeared to be dependent on the presence of both SKIP and RXR, as no such activity was observed when SKIP was

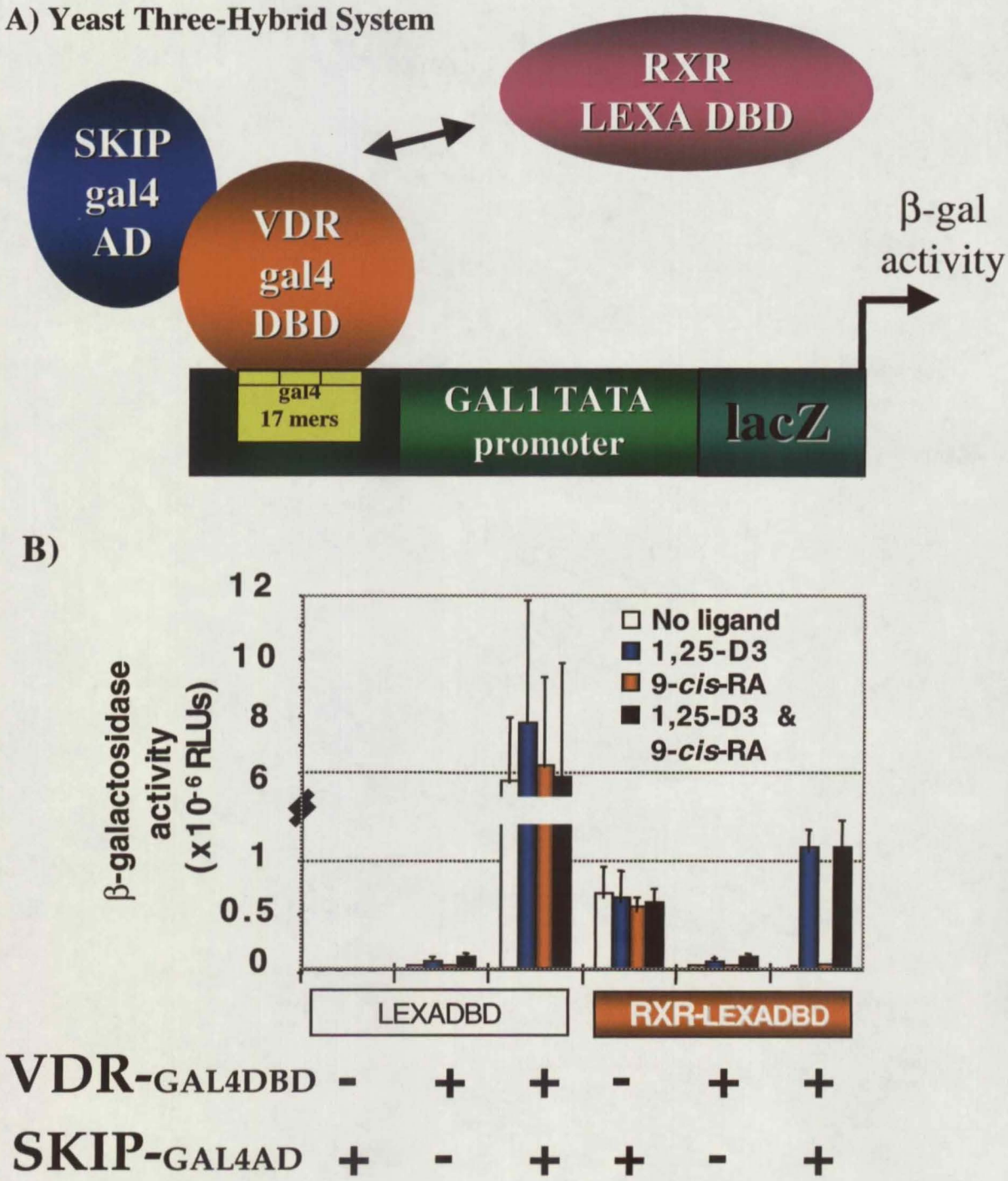

Fig. 4.7. VDR/RXR and SKIP interactions in yeast three-hybrid analysis. A) Schematic diagram of three-hybrid system showing hVDR-GAL4DBD fusion, SKIP-GAL4AD fusion and mRXRβ-LeXADBD fusion protein expressed in SFY526/Y187 diploid yeast strain containing an integrated GAL4 binding sites upstream of GAL1TATA promoter and lacZ reporter gene.
B) RXR coexpression with SKIP represses VDR-SKIP interaction. β-gal reporter activity shown as mean ± SEM of at least 6 individual colonies performed in at least three independent experiments.

expressed alone, or when VDR and RXR were co-expressed without SKIP. Furthermore, when RXR was co-expressed with SKIP and VDR there was complete abrogation of the ligand-independent activity observed previously between VDR and SKIP (Figure 4.7). The addition of 1,25-$(OH)_2D_3$, either alone or with 9-*cis*-RA, partially restored (~20%) the VDR-SKIP interaction. Western blot analysis of yeast strains tested in this analysis using antibodies against hVDR, GAL4DBD, GAL4AD and LEXADBD confirmed appropriate and equal expression of the various fusion proteins (Figure 4.8).

These results suggest that SKIP can interact with RXR and the basal transcription machinery possibly through a non-DNA-binding intermediary protein. Also RXR co-expression abrogated SKIP interaction with VDR *in vivo*. These data do not exclude the possibility that these three proteins form a complex in mammalian cells, nor do they confirm whether other factors recruited by SKIP, possibly in association with RXR, mediate the observed repression.

4.6. SKIP REPRESSION IN MAMMALIAN CELLS

4.6.1 Mammalian One-Hybrid Assay.

To investigate SKIP function in mammalian cells, a mammalian one-hybrid analysis was performed (Figure 4.9). In this assay, constructs expressing SKIP wild-type and deletion mutants in GAL4DBD fusion proteins were co-transfected in COS-1 cells with a luciferase reporter containing 5xGAL4-DNA-binding elements in front of the minimal adenovirus E1b promoter (Figure 4.9) (Rubino *et al.*, 1998). Thus, any changes in basal reporter activity (activation or repression) related to SKIP co-transfection compared to GAL4DBD alone are related to a transcriptional effect on this reporter through interaction with the basal transcription machinery.

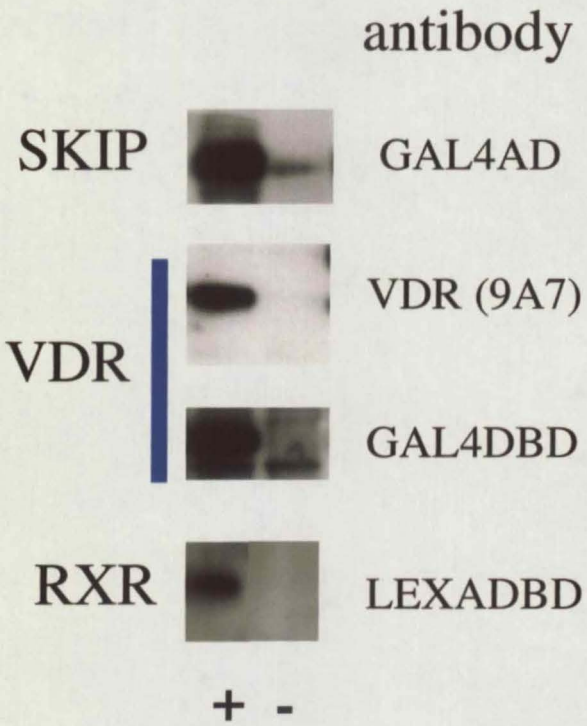

Fig. 4.8 Immunoblot analysis of three-hybrid yeast Strains.
Yeast lysates prepared from yeast strains used in 3 hybrid analysis in Fig. 4.7B) expressing either SKIP(145-536aa)-GAL4AD, VDR-GAL4DBD or mRXRβ-LEXADBD (indicated by + or empty vector controls -).

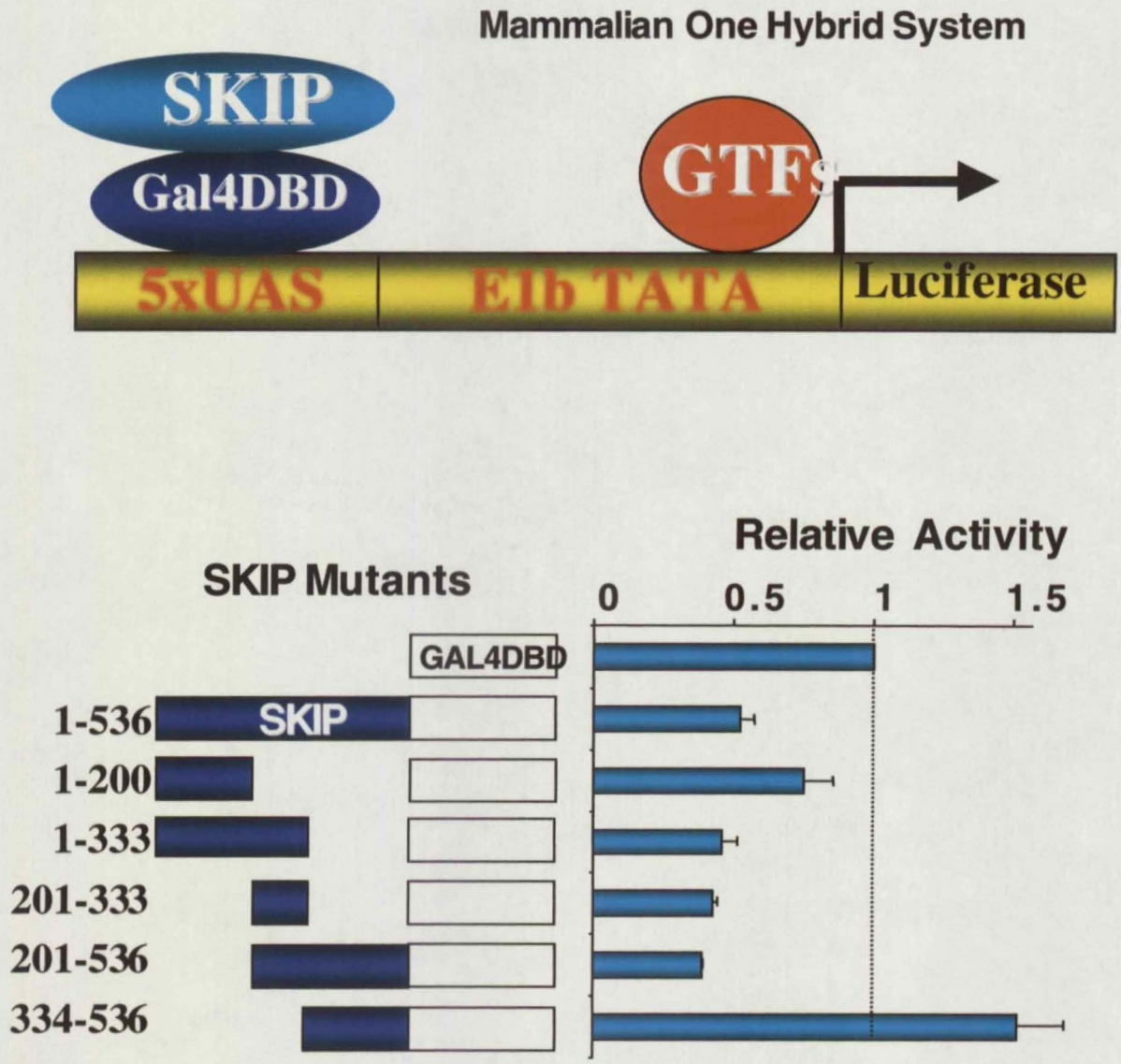

Fig. 4.9. SKIP repressor activity in mammalian cells
SKIP modestly represses basal reporter activity through a region between 200-333aa.
Mammalian one-hybrid assay was performed in COS1 cells co-transfected with wild-type or mutant SKIP-GAL4DBD deletion mutants (500ng) and the $GAL4_5$E1bTATA-luciferase reporter (500ng) as schematically shown above with general transcription factors (GTFs). Data are expressed relative to GAL4DBD alone (set at 1) and are mean ± SEM of at least 4 independent experiments performed in triplicate.

4.6.1.1 SKIP Repression Domains

In the mammalian one-hybrid assay, transfection of wild-type SKIP repressed reporter activity by about 50%, as did the 1-333aa, 201-333aa and 201-536aa truncated proteins (Figure 4.9). In contrast the N-terminal 1-200aa construct was weakly repressive, while the C-terminal 334-536aa construct weakly activated reporter activity (1.5-fold). These results thus suggest that SKIP has a modest intrinsic repression domain between 201-333aa.

4.6.2 SKIP deletion mutants and repression

To determine whether specific domains within SKIP mediate its hormone-dependent repression function, the SKIP mutants were tested in a RXRE-dependent transactivation assay in P19 cells (Figure 4.10). Strikingly, the 1-333aa and 145-333aa mutants increased both basal and ligand-dependent reporter activities by about 4- and 10-fold, respectively compared to wild-type SKIP or the other mutants that either repressed or failed to activate RXR-ligand-dependent reporter activity. Thus, the central region of SKIP, which appears to be mediate interaction with RXR in yeast, may also be involved in enhancement of RXR-dependent transactivation in mammalian cells. Importantly, deletion of the C-terminal domain of SKIP (334-536aa) relieved SKIP-mediated repression in P19 cells and converted SKIP into an activator in these cells, suggesting that this region of SKIP may mediate cell-specific repression or suppression of activation in P19 cells.

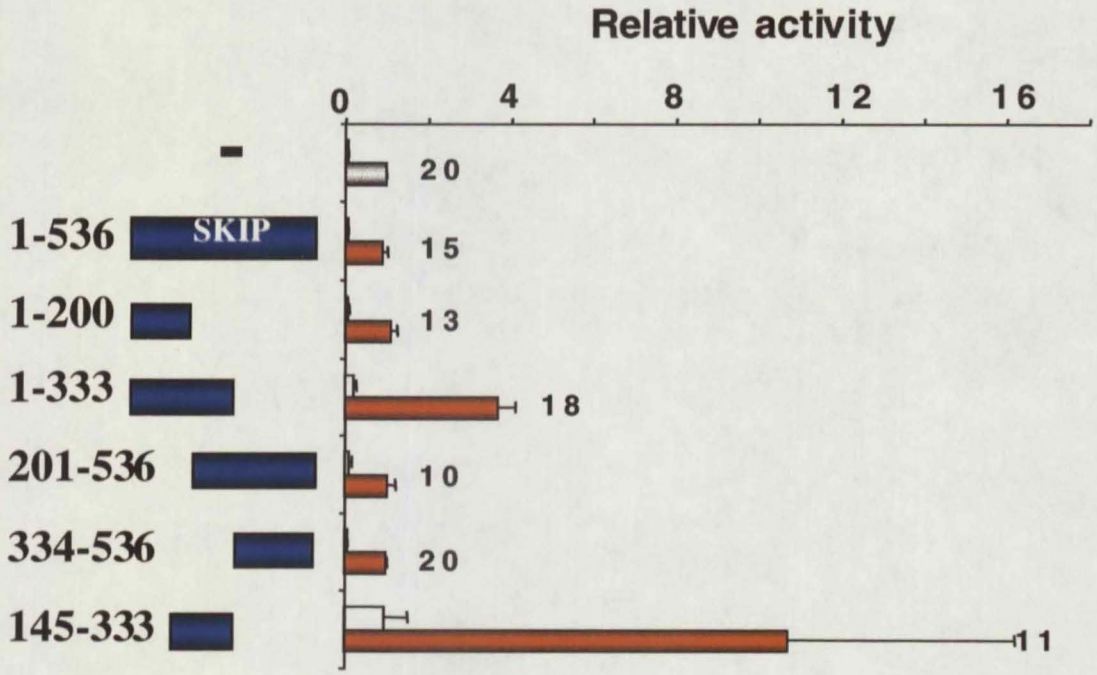

Fig. 4.10. The effects of SKIP mutants on RXR-dependent transactivation in P19 cells.
Both the N- and C-terminal of SKIP appear to act as repression domains.
Transient transfections were performed using the RXRE-tk-luciferase reporter (250ng), mRXRβ-pExpress (100ng) and SKIP-pCGN wild-type and mutants (100ng). Cells were treated with 9-*cis*-RA ligand (red columns) or vehicle (open columns). Results are mean ± SEM activity relative to empty vector pCGN control of triplicate wells and are representative of 3 independent experiments. Numbers above columns represent fold induction with ligand.

4.7 DISCUSSION

Studies presented in this chapter suggest that SKIP acts as a cell line-specific transcriptional modulator of NHR-dependent transcription. Specifically, SKIP augmented RXR and VDR-dependent transcription in CV-1 cells, but repressed or failed to activate these transcriptional activities in P19 cells. These effects were also receptor and promoter-specific as SKIP activated to various extents, GR-, TR- as well as Hox-dependent transcription (data not shown) in these same cells. Deletion of the N- and C-terminal regions of SKIP also converted SKIP into a strong transcriptional activator of RXR-dependent transcription in P19 cells. These latter results strongly suggest that SKIP may require interactions with cell-specific repressor proteins to modulate its transcriptional function.

An examination of SKIP putative functional domains was made in a yeast two-hybrid analysis with RXR, in a mammalian one-hybrid assay and in RXR transactivation assays in P19 cells (Figure 4.9 and 4.10). These studies revealed distinct regions within SKIP which may interact with RXR (aa145-333), act as an intrinsic basal repression domain (201-333aa), or mediate cell-specific activation or repression of RXR activities in P19 cells. In the latter RXR transactivation study, the greatest basal and ligand-dependent reporter transactivation was observed with the 145-333aa SKIP mutant, with almost 10-fold elevation compared to wild-type SKIP; in contrast, the 1-333aa SKIP mutant transactivated by only 4-fold. Thus, the N-terminal 1-145aa and C-terminal 334-536aa regions may both contain P19 cell-specific repression domains (Figure 4.10).

It is a concern that in the second and later series of SKIP transactivation studies in P19 cells, a lack of activation rather than frank repression was observed. Whether there were subtle differences in the P19 cells from experiment to experiment and/or changes in other experimental conditions critical for SKIP-mediated repression to be observed is not known. In this context it is important to note that although not visibly altered in these experiments, the undifferentiated state of P19 cells is subject to changes in culture conditions (Bain *et al.*, 1994). Nevertheless, the transcriptional effects of SKIP in P19 cells clearly contrasted with its action as

coactivator in all other cell lines tested, including HepG2, QT6 cells and COS-1 cells (R.Dahl, M.Hayman, personal communication).

The possibility the observed repression in P19 cells was mediated by SKIP in association with RXR or a RXR-associated protein is supported by the differences in RXR expression between the P19 and CV-1 cells (Issa *et al.*, 2001) and several other lines of evidence presented in this chapter. These include the results of the yeast three-hybrid analysis that suggests that RXR co-expression with SKIP and VDR markedly reduced VDR-SKIP interaction (Figure 4.7). However, another possible explanation for this result could be that RXR "squelched" or titrated away SKIP from an active VDR-SKIP transcriptional complex. Thus, possibly SKIP is limiting in the cell, so when both RXR and VDR are coexpressed with SKIP, the amount of SKIP available for interaction with either receptor is decreased and hence available for transcription. Further studies will be required to address these various possibilities and potential tripartite interactions.

Furthermore, these yeast studies suggested that SKIP with RXR interacted with the basal transcription machinery to activate the reporter gene, either directly or indirectly via endogenous yeast intermediary proteins. As RXR interacts with TFIIB and TAFs, this may be one mechanism by which RXR with SKIP was able to activate gene transcription in this system in the absence of a DNA binding intermediate (Leong *et al.*, 1998b). In support of this proposed mechanism SKIP also appears to be to interact with TFIIB *in vitro* (data not shown).

SKIP interacted directly with both VDR and RXR in GST binding studies *in vitro*. No effect of ligand was observed in these interactions, in contrast to *in vivo* observations in yeast. This difference in the ligand-dependency of interactions between yeast *in vivo* and *in vitro* studies has also been observed with RXR and TFIIB, as well as ER and SRCs (Hall *et al.*, 2000; Leong *et al.*, 1998b). It is thus possible that factors present *in vivo* but not *in vitro* modulate the ligand-dependency of these interactions.

The RXR AF-2 domain appears to be critical for regulation of coactivator interaction and corepressor binding to NHRs (Westin *et al.*, 1998; Zhang *et al.*, 1999a), preventing coactivator binding to VDR/RXR and RAR/RXR heterodimers (Leo *et al.*, 2000; Leo *et al.*, 2001; Westin *et al.*, 1998). As SKIP interacts with the AF-2 domain of RXR, but not that of VDR, it is possible that within the context of the VDR/RXR heterodimer, SKIP may modulate heterodimer-cofactor interactions. The observation that on the 24-hydroxylase promoter, SKIP co-transfection with liganded RXR modestly repressed VDR/RXR-dependent transcription (Figure 4.6) is consistent with this suggestion.

Based on these results SKIP appears to belong to a small group of recently described bifunctional proteins which may modulate tissue-specific actions of the NHR superfamily (Section 1.9.7). These include factors such as CIA (corepressor independent of AF-2 function), an ER coactivator which has an overlapping NHR coactivator and CoRNR repressor motifs; mZac1, which in a cell-specific manner activates or represses AR-dependent transcription; and RIP140, which originally was isolated as an ER coactivator, but also represses GR-dependent transcription mediated in part by an interaction with HDAC-2 (Huang and Stallcup, 2000; Suave *et al.*, 2001; Subramaniam *et al.*, 1999; Wei *et al.*, 2001; Wei *et al.*, 2000).

In summary, the studies in this chapter have addressed the role of SKIP in modulation of NHR-dependent transcription. These results suggest that SKIP acts as both a transcriptional repressor and an activator depending on the cell and/or receptor context. Furthermore, it appears that SKIP contains distinct domains that may be involved in cell-specific actions, presumably through interaction with other transcriptional cofactors. The next chapter (Chapter 5) describes further studies that address whether these putative functional regions within SKIP differentially interact with coactivators, such as the p160/SRC family, and with corepressors such as N-CoR/SMRT. An understanding of the range of SKIP-interacting factors may provide further insight into the divergent cell-specific actions of SKIP observed in the studies presented to date.

Chapter Five

SKIP interaction with Coactivators and Corepressors

5.1 INTRODUCTION

Gene expression is controlled by a diverse and prolific range of factors and cellular enzymatic activities (Lemon and Tjian, 2000). These include corepressors, coactivators and factors that modulate histone acetylation, phosphorylation and/or methylation, or target regulatory proteins for proteosomal degradation (Cheung *et al.*, 2000a; Glass and Rosenfeld, 2000; Weissman, 2001; Zhang *et al.*, 1998a). Thus, one potential mechanism for the diversity of eukaryotic gene expression may relate to differential expression of these various cofactors within large protein complexes that modulate the transcriptional end-point (Lavinsky *et al.*, 1998; Park *et al.*, 1999).

The corepressors N-CoR and SMRT associate with a subset of NHRs and a diverse range of histone deacetylase enzymes (HDACs) (Guenther *et al.*, 2000; Huang *et al.*, 2000; Humphrey *et al.*, 2001; Jones *et al.*, 2001; Kao *et al.*, 2000; Li *et al.*, 2000a; Wen *et al.*, 2000). This suggests distinct roles for different repressor complexes in NHR-mediated repression. In an analogous fashion, there is a large range of coactivator protein complexes, some of which contain HAT activities e.g. CBP/p300, SRCs oand p/CAF, and others that mediate communication with the basal transcription machinery e.g Med220/TRAP220/DRIP205 interaction with RNA Pol II (Chen *et al.*, 2001; Glass and Rosenfeld, 2000; Lemon and Tjian, 2000; Rachez and Freedman, 2001).

Though the NHR ligand "switch" acts as a fundamental mechanism that determines the balance of transcriptional effects between the repressed deacetylated and activated acetylated chromatin state, other factors also appear to be necessary to coordinate the complexity of cellular roles ascribed to NHRs (Glass and Rosenfeld, 2000). Thus alteration in cellular expression levels of histone modifying cofactors such as CBP/p300 or N-CoR may play a role in control of gene expression during development and disease states (Martinez de Arrieta *et al.*, 2000; Misiti *et al.*, 1998). In support of this possibility, decreasing levels of N-CoR, or inhibition of corepressor binding to the receptor, may account for the ability of anti-oestrogens to induce activation in specific cell types and in late-stage breast

cancers (Lavinsky *et al.*, 1998; Soderstrom *et al.*, 1997). Furthermore, changes in N-CoR levels may be altered by interaction with mSiah2, the mammalian homologue *of Drosophila Seven* in absentia (*sina*), which targets N-CoR for ubiquitin-mediated proteosomal degradation (Della *et al.*, 1993; Weissman, 2001; Zhang *et al.*, 1998b). This mechanism appears to be cell-specific, as unlike N-CoR, mSiah2 expression is restricted primarily to cells of the nervous system (Zhang *et al.*, 1998b). Other examples of altered states of histone acetylation in human disease occur in various leukaemias that arise from chromosomal translocations that involve key regulatory proteins, such as CBP/p300 and RAR (section 1.10) (Minucci and Pelicci, 1999). Furthermore, in Rubinstein-Taybi syndrome, a genetic disorder which occurs secondary to CBP haploinsufficiency, there is an associated defect in HAT activity (Murata *et al.*, 2001).

The nuclear cofactor SKIP appears to act as a bifunctional protein with both activating and repressing functions in different cellular and signalling contexts. It is thus possible that SKIP may function in part to coordinate the opposing transcriptional actions of various cofactor complexes within a given cell. Thus, it was hypothesised that SKIP may interact with different factors that modulate its bifunctional effects (activation or repression) on NHR-mediated transcription.

5.1.1. Aims

Previously, SKIP had only been described as a coactivator of NHR-dependent transcription (Baudino *et al.*, 1998). Studies presented in the previous chapter suggested that SKIP may act as a cell line- and receptor-specific transcriptional coregulator. Studies in this chapter address the possible molecular mechanisms for these effects. A role for N-CoR in SKIP-mediated repression or lack of activation in P19 cells was suggested by studies that demonstrated a direct *in vitro* and *in vivo* interaction between SKIP and N-CoR and the related repressor SMRT. To test whether alteration of N-CoR protein levels may modulate SKIP repression, N-CoR expression was down-regulated by all-*trans*-retinoic acid (RA) treatment in a P19 cell differentiation model (Bain *et al.*, 1994) and its effect on SKIP-mediated transcription observed (Soderstrom *et al.*, 1997). To determine whether SKIP also

interacted with coactivators, the interaction between SKIP and the major classes of coactivators were examined in mammalian two-hybrid assays. The results from studies in this chapter suggest that SKIP acts as a bifunctional (activator and repressor) regulatory protein possibly via its differential interactions with corepressors, such as N-CoR, and with coactivators, such as SRC-1. As such, changes in cell-specific expression of these SKIP-interacting factors may modulate its action.

5.2. METHODS

5.2.1. Cloning of RXR, SRC-1 and N-CoR mammalian expression plasmids

mRXRβ-VP16 and mRXRβ-pM was cloned from *EcoR*1/*Sal*1 insert from mRXRβ-pASCYH2 and cloned into the *EcoR*1/*Xho*1 sites of pM and VP16 vectors. SRC-1-pACT VP16AD and SRC-1-pBIND GAL4DBD plasmids constructs were cloned as follows: Bstx1/Xba1 fragment was excised from SRC-1-pCR3.1 (Spencer *et al.*, 1997) and blunted by Klenow DNA polymerase (section 2.2.6.1) and ligated (section 2.2.8.2) into *Bam*H1 cut pACT (VP16AD) and pBIND (GAL4DBD) mammalian two-hybrid vectors (Promega) (section 2.1.11). N-CoR-pSG5β was cloned by inserting *EcoR*1 insert from N-CoR-VP16AD plasmid into pSG5β (Tagami, *et al.*, 1998, Issa *et al.*, 2001). All plasmid constructs were confirmed to be correct and in frame by automated fluorescent DNA sequencing (Perkin-Elmer).

5.3 RESULTS

5.3.1 SKIP INTERACTION WITH COREPRESSOR PROTEINS

5.3.1.1 SKIP interaction with N-CoR and SMRT

As SKIP has been shown previously to interact directly with the corepressor SMRT, but not with N-CoR, SKIP interaction with both corepressors were tested and the domains in SKIP required for these interactions were defined in a mammalian two-hybrid analysis (Figure 5.1) (Tagami *et al.*, 1998; Zhou *et al.*, 1999). Wild-type SKIP, 1-200aa and 1-333aa SKIP mutant constructs interacted with both

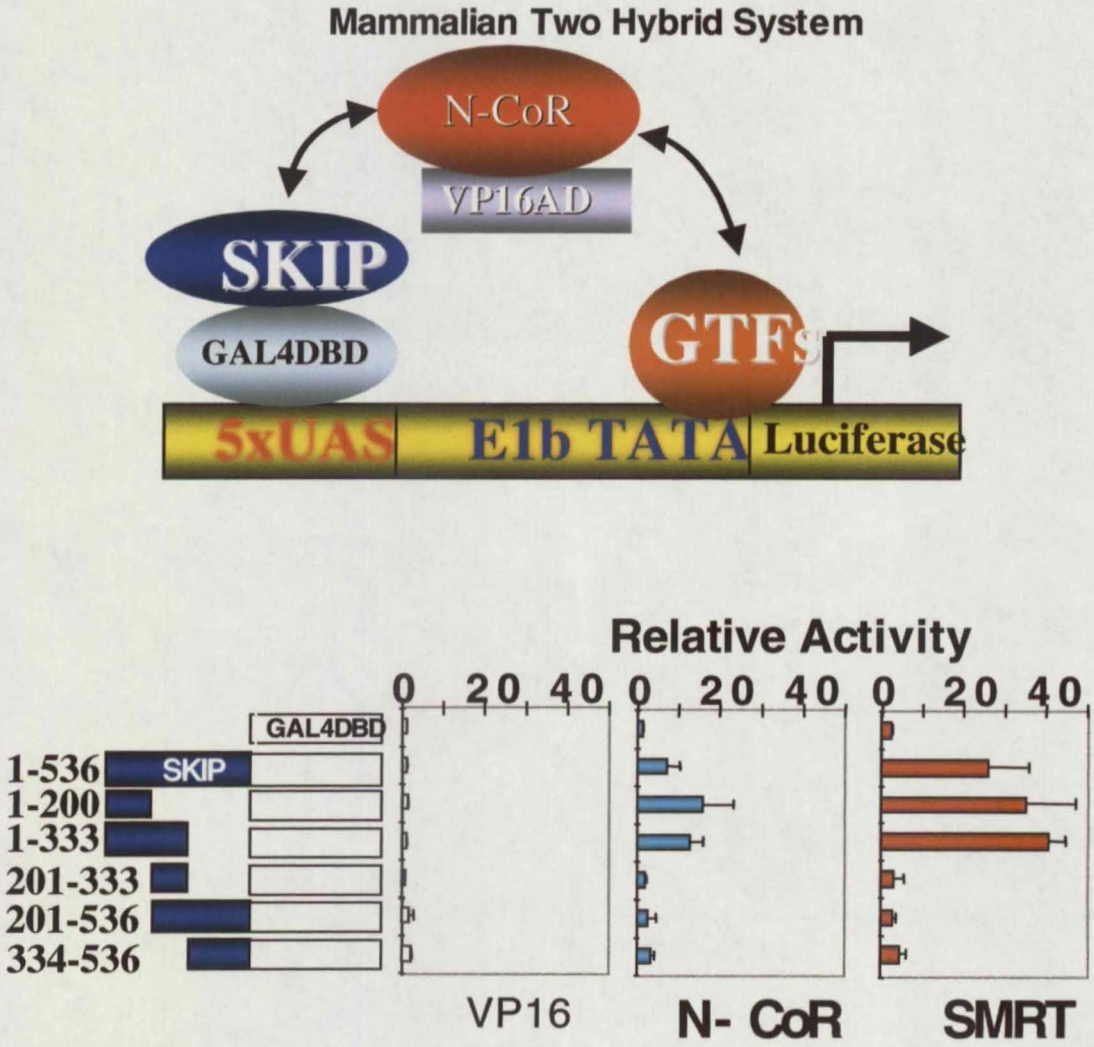

Fig. 5.1 SKIP interaction with Corepressors in Mammalian Two-Hybrid System.
Mammalian two-hybrid analysis using SKIP-GAL4DBD wild-type and mutant constructs (125ng) co-transfected with N-CoR-VP16AD or SMRT-VP16AD (250ng) (Tagami *et al.*, 1998). Data are expressed relative to GAL4DBD alone (set at 1) and are mean ± SEM of at least 4 independent experiments performed in triplicate.

N-CoR and SMRT, whereas no interaction was observed between the 201-333aa, 201-536aa or 334-536aa deletion constructs (Figure 5.1). Thus SKIP appears to interact with N-CoR and SMRT through a domain (1-200aa) distinct from the central (201-333aa) domain involved in repression of basal activity (Figure 4.9).

5.3.1.2 SKIP and a putative-HDAC repressor complex

To determine whether the repressive effect of SKIP on NHR-dependent transcription may involve interaction with a HDAC-repressor complex, transient transfections were performed in CV-1 and P19 cells using the RXRE-reporter and the HDAC inhibitor trichostatin (TSA; Figure 5.2). Though TSA is a global inhibitor of type 1 HDACs, it has been used in a number of studies to indirectly indicate recruitment of class 1 HDACs by various proteins (Minucci *et al.*, 1997).

In these studies, TSA treatment led to a doubling (from 13-24-fold) of reporter activity above ligand alone in SKIP-transfected P19 cells, in which ligand-dependent reporter activity had been repressed by 50%, consistent with previous observations (Figure 4.4). When both TSA and ligand were added to P19 cells transfected with SKIP or empty vector, there was a 7- to 10-fold synergistic increase in reporter activity above ligand alone. These results suggest that TSA relieved SKIP repression in the P19 cells and hence are consistent with SKIP association with a putative HDAC-repressor complex.

In contrast, in CV-1 cells SKIP caused a 7-fold induction in reporter activity, consistent with earlier observations. TSA treatment increased reporter activity in SKIP-transfected cells to levels comparable to treatment with ligand alone, and had no effect on cells transfected with empty vector. When both TSA and ligand were added to CV-1 cells transfected with SKIP or empty vector there was only an additive effect on ligand-dependent reporter activity, in comparison to the synergistic effect observed in P19 cells.

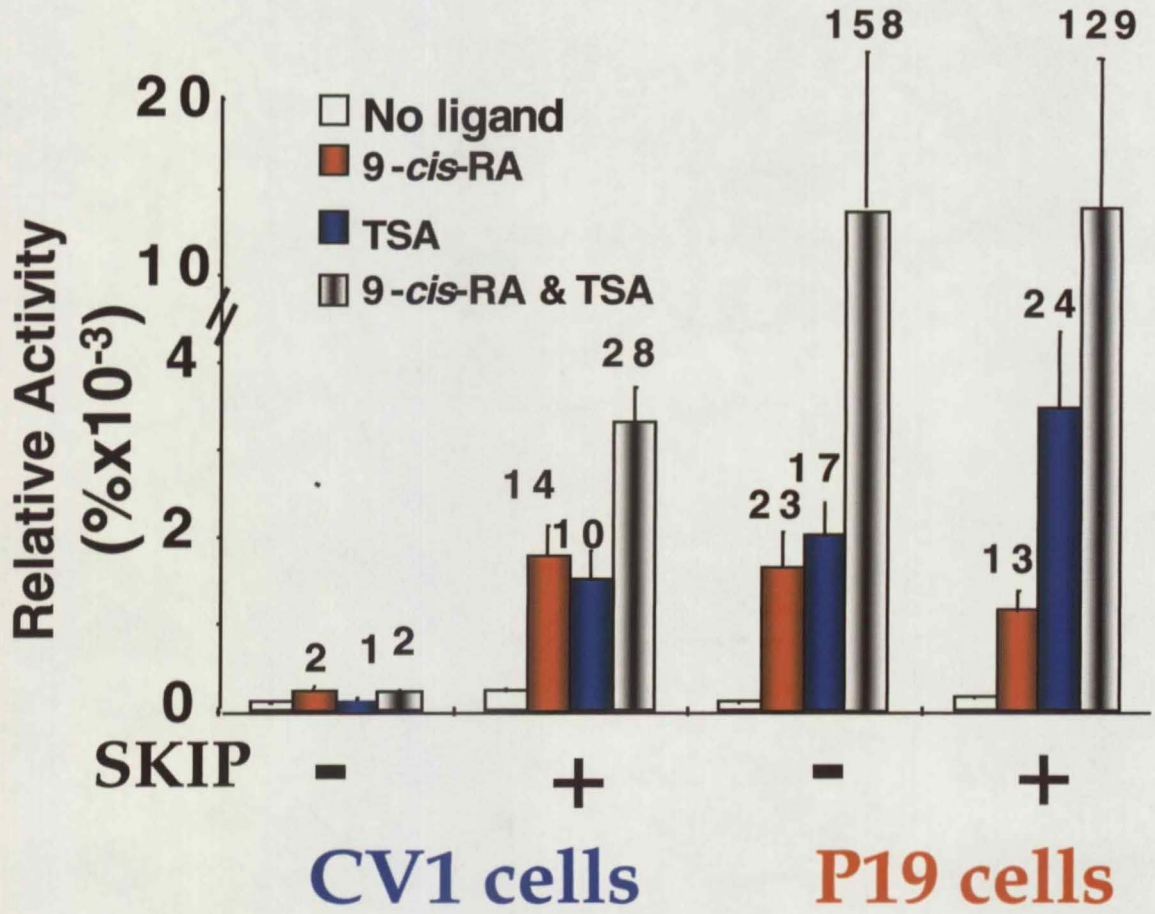

Fig. 5.2. SKIP repression of RXR-dependent reporter activity is reversed by Trichostatin (TSA) in P19 cells. Transient transfections were performed in P19 and CV1 cells with the RXRE-tk-luciferase reporter (125 ng) or empty vector tk-luciferase, co-transfected with mRXRβ-pExpress (65 ng), Skip-pCGN (450ng). Cells were treated with vehicle, 9-*cis*-RA, TSA or both, as indicated. Luciferase reporter activity is shown as relative to non-ligand-treated cells transfected with RXRE-tk-luciferase reporter with pCGN, and are corrected for non-specific effects of the different treatments on the basal tk-luciferase reporter. Values are mean ± SEM of 3 independent experiments performed each in triplicate. Values above columns represent relative fold induction above respective non-ligand controls.

Overall these studies are consistent with previous observations that SKIP interacts directly with HDAC proteins (Zhou *et al.*, 1999). Furthermore, they suggest TSA induced a greater transcriptional activity in P19 than CV-1 cells, implying there may be greater HDAC activity in P19 than CV-1 cells.

5.4 DIFFERENTIATION AND SKIP TRANSCRIPITIONAL EFFECTS

5.4.1 RA-induced differentiation converts SKIP into a transcriptional activator in P19 cells

The differences observed in SKIP action between CV-1 and P19 cells suggested that its activity might be modulated by the state of cellular differentiation. SKIP activity was therefore examined without and with ATRA treatment to induce P19 cell differentiation into a neuronal cell phenotype (Bain *et al.*, 1994) (Figure 5.3).

In SKIP-transfected undifferentiated P19 cells, as before, SKIP did not activate RXR-dependent transcription, TSA alone increased reporter activity to levels equivalent to that achieved with ligand, while TSA with ligand led to a synergistic increase in reporter activity.

By contrast, in the cells treated with ATRA for 3 days, SKIP had no effect on ligand-dependent reporter activity and TSA had minimal effects on reporter activity. However TSA in combination with 9-*cis*-RA decreased reporter activity by 60% in both the SKIP and non-SKIP transfected cells. In the P19 cells differentiated by 7 days of RA treatment, SKIP co-transfection led to a 3-fold increase in ligand-dependent reporter activity, with or without TSA, while TSA alone had only a minimal effect on reporter activity in the SKIP transfected cells.

It was possible that differences in the expression of coregulators involved in histone acetylation, such as N-CoR, HDAC-1, CBP and p300, may correlate with changes in SKIP transactivation between the CV-1 and the P19 cells prior to and

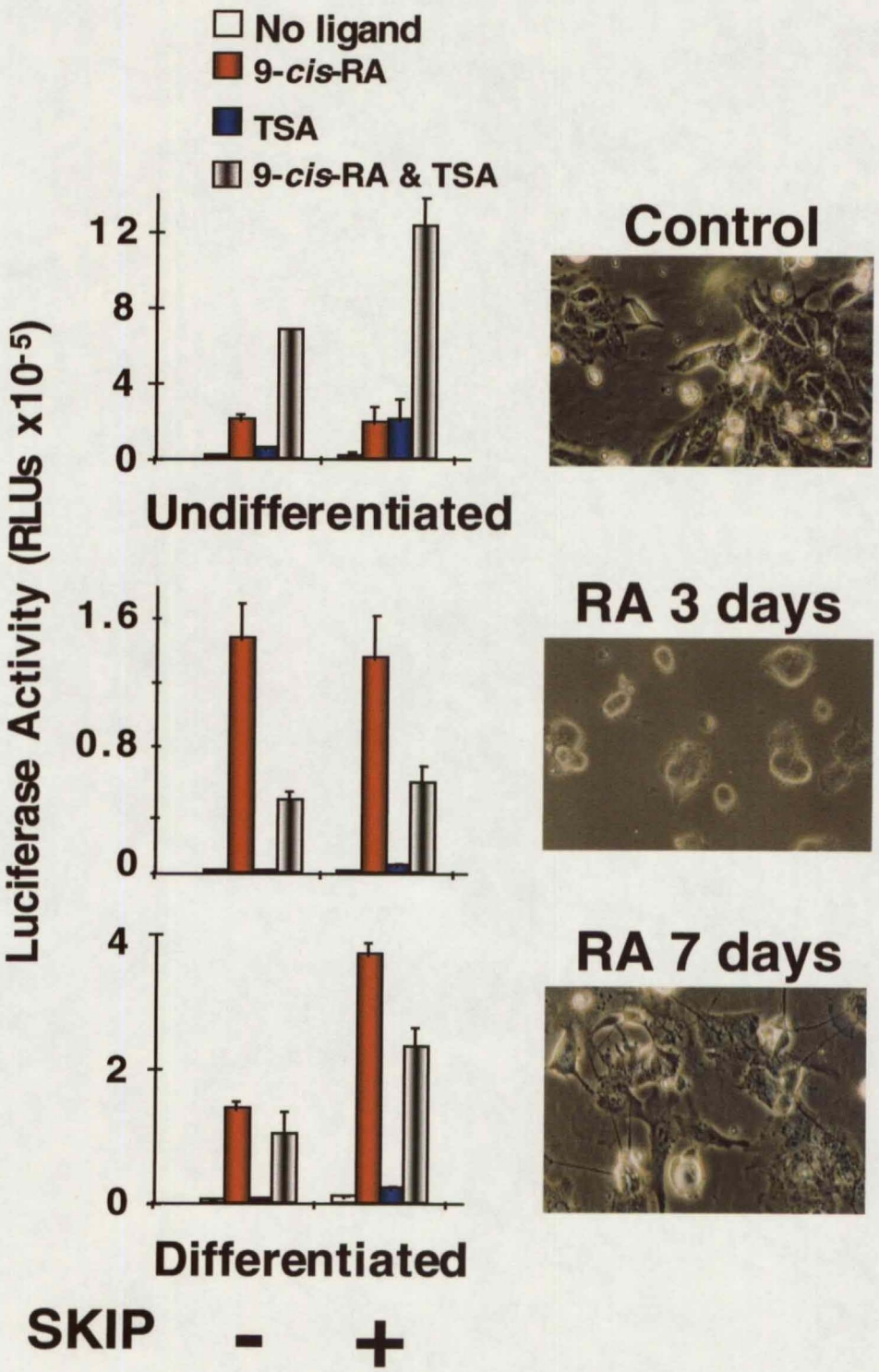
No ligand
9-cis-RA
TSA
9-cis-RA & TSA
Luciferase Activity (RLUs x10-5)
12
8
4
0
Undifferentiated
1.6
0.8
0
4
2
0
Differentiated
SKIP - +
Control
RA 3 days
RA 7 days

Fig. 5.3. Effects of cellular differentiation of P19 cells on SKIP transactivation of RXR-dependent reporter.

Transient transfections were performed in undifferentiated P19 cells and P19 cells after 3 and 7 days of 1uM RA treatment with the RXRE-tk-luciferase (125ng) and mRXRβ-pExpress (60ng) and SKIP-pCGN (450ng). Cells were treated with vehicle (0.1%DMSO), 9-*cis*-RA (1μM) and/or TSA (10ng/mL). Luciferase reporter activity is shown relative to cells transfected with pCGN in absence of ligand and is corrected for non-specific effects of the different treatments on the basal tk-luciferase reporter. Data are mean luciferase activity (± SEM) of triplicate samples and are representative of experiment repeated three times. The effect of Morphological changes following differentiation of the parent P19 cells (right panels) after 3 days of RA treatment when cells became small and rounded and by 7 days of treatment when neurite formation is evident.

during RA treatment. Hence, immunoblot anlyssis was performed on nuclear extracts prepared from these cell cultures (Figure 5.4).

N-CoR protein levels were undetectable in CV-1 and COS-1 cells. By contrast, in the undifferentiated P19 cells there were moderate levels of expression which decreased during RA treatment, such that by 7 days there was no detectable expression (Soderstrom *et al.*, 1997). There were no major differences in HDAC-1 expression between the various cell-lines though after 7 days of RA treatment in P19 cells appeared to lead to a small increase in its expression.

Expression of p300 was higher in CV-1 than undifferentiated P19 cells, while the reverse was true for CBP. Furthermore, whilst RA treatment in the P19 cells increased p300 expression, it had the opposite effect on CBP expression.

The differences in N-CoR and possibly p300 expression between CV-1 and undifferentiated P19 cells, and the changes during RA differentiation of the latter cell line, appeared consistent with the differences in SKIP transactivation between these cell lines. However, the opposite changes in CBP expression and the lack of major differences in HDAC-1 expression, suggest that other factors may also be involved in modulating SKIP action. Nevertheless, the cell-line specific expression of N-CoR and/or p300 and their alteration of expression in differentiated P19 cells may play a role in determining the direction of SKIP-mediated transcriptional action (Kawasaki *et al.*, 1998).

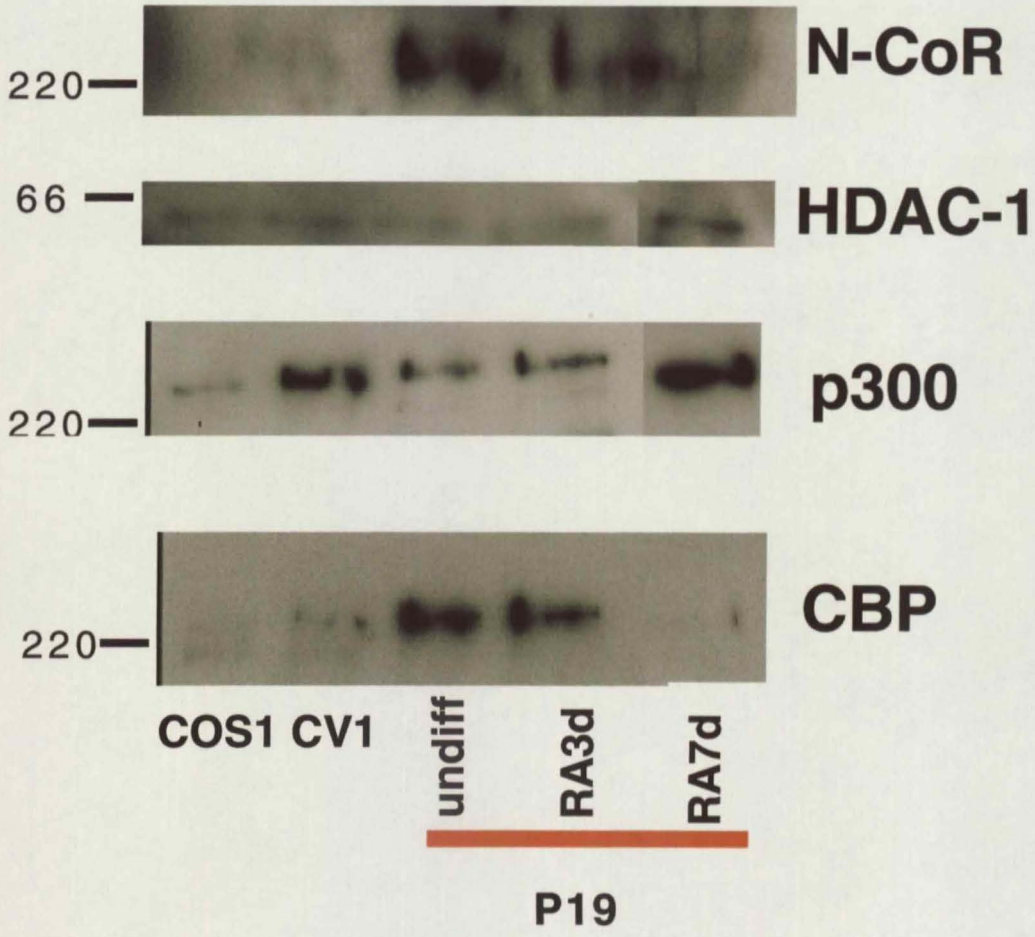

Fig. 5.4. Endogenous Cofactor Expression in CV1, COS1 and P19 cells during differentiation. P19 cell differentiation is associated with downregulation of N-CoR and CBP expression, but upregulation of p300 expression.
Immunoblot analysis of nuclear extracts prepared from COS-1, CV-1 and undifferentiated and RA-treated (3 and 7 days) P19 cells using antibodies against N-CoR, HDAC-1, p300 and CBP (Santa Cruz). The extracts tested in the far right lane of the blot (P19 RA7d) were probed and exposed at the same time as the remainder of the blot, but during SD-PAGE were run in a different order to that shown in this figure, thus necessitating "cutting and pasting" for presentation.

5.5 SKIP INTERACTION WITH COACTIVATORS *IN VIVO* AND *IN VITRO*

5.5.1 SKIP interaction with SRC-1

The possibility that the cell-specific effects of SKIP may in part be related to an interaction between SKIP and the p160 coactivator SRC-1 was explored in a mammalian two-hybrid analysis (Figure 5.5).

The SRC-1-VP16AD construct was associated with high autonomous transcriptional activity presumably due to the interaction of SRC-1 with basal transcription factors (Ikeda *et al.*, 1999). Wild-type SKIP-GAL4DBD repressed this SRC-1-VP16AD activity by about 80%. This repression appeared to be mediated largely though the central (201-333aa) domain of SKIP, although the greater repression occurring with the 201-536aa mutant suggested that the C-terminus of SKIP may also modulate this activity. The repression by SKIP of SRC-1 autonomous activity was also detected in a converse experiment with SRC-1-GAL4DBD and SKIP-VP16AD (Figure 5.5B). As a positive control both SRC-1-VP16AD interacted interacted in a ligand-dependent manner with VDR-GAL4DBD (Figure 5.5C).

5.5.2. Physical interaction between SKIP and N-CoR, SMRT and SRC-1 *in vitro*.

To determine if SKIP can physically associate with both corepressors and coactivators, a GST-SKIP binding assay was performed. The results were consistent with a physical interaction *in vitro* between SKIP and *in vitro* translated [^{35}S]-labelled SRC-1 and N-CoR proteins, and between GST-N-CoR and *in vitro* translated [^{35}S]-labelled SKIP (Figure 5.6).

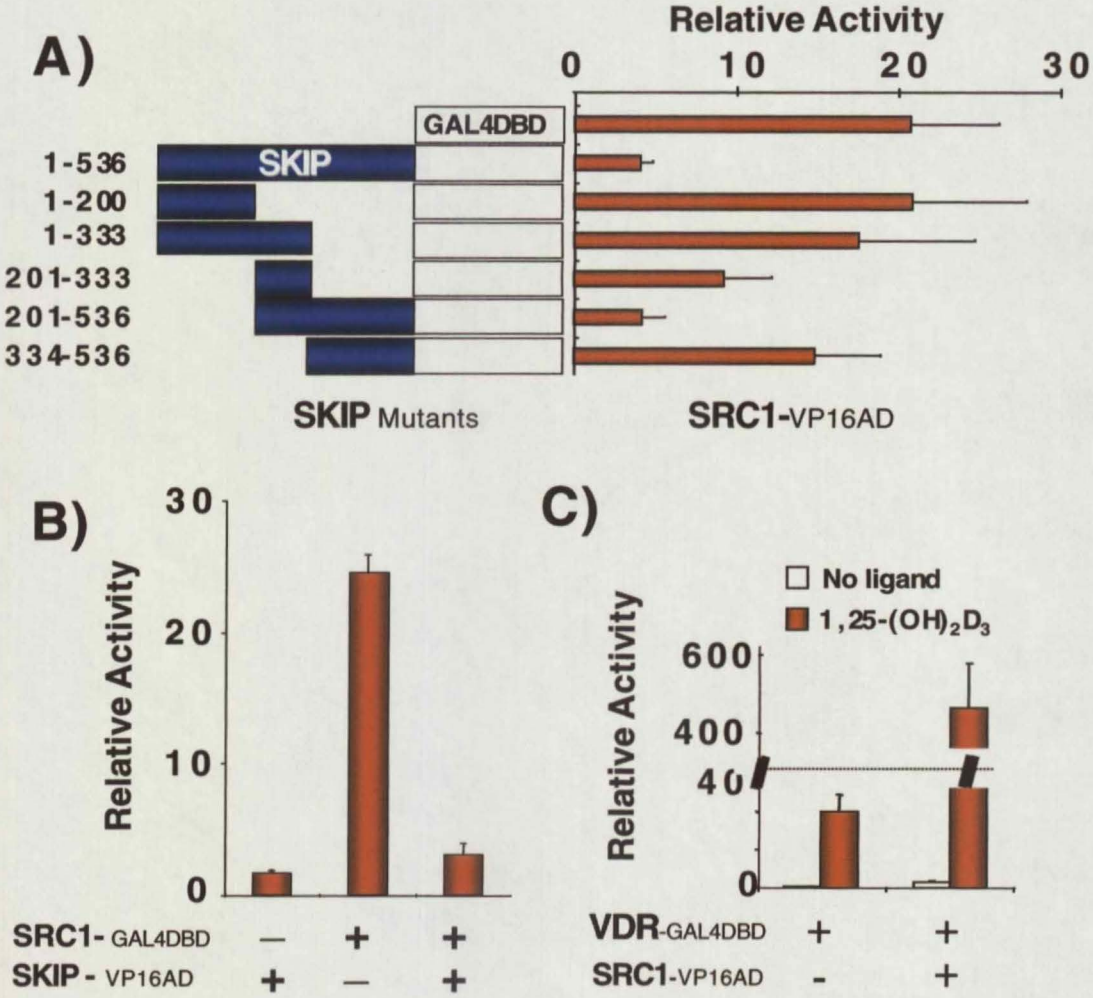

Fig. 5.5 SKIP interaction with SRC/p160 coactivators.
A) SKIP represses SRC-1 autonomous transcriptional activity largely through a region between 201-333aa.
Mammalian two-hybrid analysis was performed in 24 well plates with COS-1 cells co-transfected with SRC1-VP16AD and wild-type or mutant SKIP-GAL4DBD constructs (125ng), as indicated with $GAL4_5$E1bTATA-luciferase reporter (250ng). Data are expressed relative to GAL4DBD alone (set at 1) and are mean ± SEM of at least 4 independent experiments performed in triplicate.
B) SKIP represses SRC-1-GAL4DBD autonomous transcriptional activity.
Mammalian two-hybrid assays in COS1 cells transfected with SRC-1-GAL4DBD and SKIP-VP16AD constructs were performed as in A).
C) SRC-1 interacts with VDR in a ligand-dependent manner.
Mammalian two-hybrid assays as in A) and C) in COS-1 cells transfected with VDR-GAL4DBD and SRC-1-VP16AD.

Fig. 5.6. SKIP interacts physically with SRC-1 and N-CoR *in vitro*. *In vitro* binding of wild-type GST-SKIP with [^{35}S]-labelled SRC-1, N-CoR or luciferase (Luc) control, and GST-N-CoR with [^{35}S]-labelled SKIP. Input proteins represent 20% of loaded lysate. Results are representative of 3 independent experiments.

5.5.3 SKIP interaction of other coactivator proteins in mammalian two-hybrid assay.

The specificity of the repressive effects of SKIP was examined with other classes of coactivators including the p160 family member SRC-2, the HAT and bromodomain proteins p300 and p/CAF, and TRAP220, a component of the Mediator complex, using mammalian two-hybrid assays (Figure 5.7) (Chen *et al.*, 2000b; Kobayashi *et al.*, 2000; Kurooka and Honjo, 2000).

All of the cofactor-VP16AD constructs were associated with 2-3 fold increase in basal reporter activity, except for SRC-2 which was higher (about 7-fold) and p/CAF and the C-terminal portion of p300 (p300C) which had lower activities not significantly greater than the empty vector. Co-transfection of SKIP-GAL4DBD repressed the transcriptional activities of SRC-2 and TRAP220 by 5-7 fold, and the central portion of p300 and p/CAF by 2-3 fold, whereas no or minimal effects were observed on the N- and C-termini of p300. Thus, SKIP appears to interact with various classes of coactivators, including SRC-1 and SRC-2, p300, TRAP220 and possibly p/CAF.

5.5.4 SKIP and SRC transactivation of RXRE-reporter

The investigate whether SKIP modulated SRC coactivation in a cell line-specific manner, transient transfections of the RXRE-reporter construct were performed with SKIP and SRC-1-3 expression plasmids (Figure 5.8). As previously, SKIP had cell line-specific effects with activation of ligand-dependent reporter activity in CV-1 and repression in P19 cells. Co-transfection of SRC-1 and SRC-3 alone resulted in a 1.5- to 2–fold augmentation of ligand-dependent reporter activity in both cell lines, whereas SRC-2 repressed reporter activity by 2-3 fold in CV-1 cells but augmented these same activities by 1.5 fold in P19 cells. Co-transfection of SKIP with SRC-1 in the CV-1 cells led to a 30% increase in response to ligand, whereas SKIP had no effects on ligand response with SRC-2 or SRC-3 in this cell line. In P19 cells, SKIP co-transfection repressed by 40-60% the augmentation of ligand-dependent reporter activity with each SRC. Overall, these results suggest that SKIP effects on SRC transactivation may also be cell-line specific.

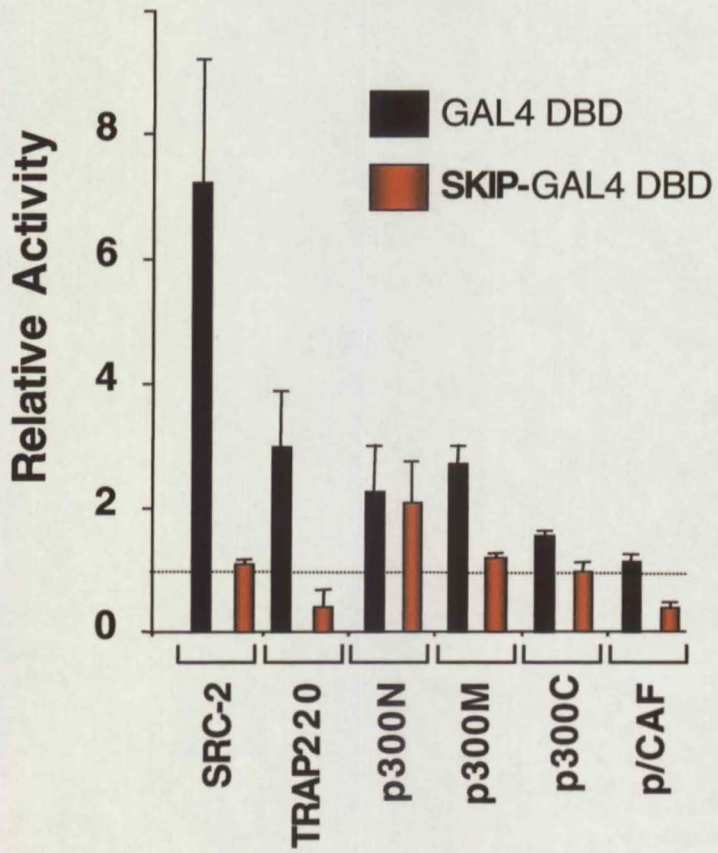

Fig. 5.7, SKIP interaction with other coactivator classes.
SKIP represses autonomous transcriptional activities of SRC-2 and TRAP220 and to a lesser extent p/CAF and the central domain of p300.
Mammalian two-hybrid analysis was performed as in Fig. 5.5., using wild-type SKIP-GAL4DBD co-transfected with SRC-2- , TRAP220-, p300 [N-, Middle (aa610-1570 which largely encompasses the p300 HAT domain) and C-termini] and p/CAF-VP16AD plasmids (250ng).

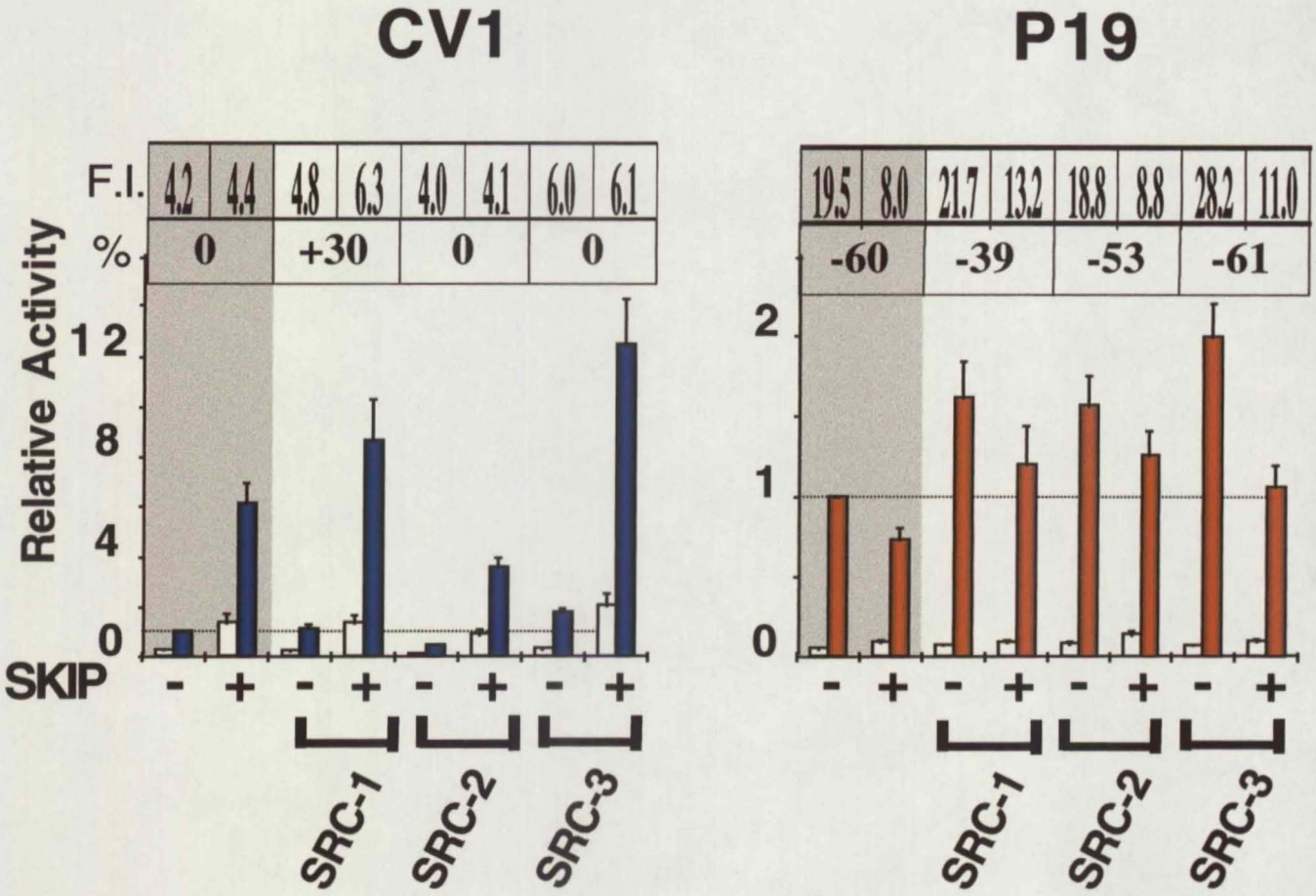

FIG. 5.8 Cell-specific effects of SKIP with SRC on RXR-dependent reporter.

SKIP modulates SRC transactivation of RXR-dependent reporter activity in a cell line-specific manner.

Transient transfections performed in 24 well plates in CV1 and P19 cells with the RXRE-tk-luciferase reporter (250ng), mRXRβ-pExpress (100ng) and SKIP-pCGN (450ng) and SRC1-3-CMV expression plasmids (450ng each). Cells were treated with 9-*cis*-RA ligand (filled columns) or vehicle (open columns). Results are mean ± SEM activity relative to ligand treated vector pCGN control (indicated by dashed line) of 4 independent experiments. Shaded zones indicate similar data presented in Figure 4.4. Shown above figure in box is fold-induction (F.I.) with ligand (above) and % change in SKIP-associated fold-induction (below).

5.5.5 SKIP facilitates NHR-SRC-1 interaction

To determine if SKIP facilitates RXR and VDR interaction with coactivators, a mammalian three-hybrid assay was performed in COS-1 cells (Fig 5.9). Although both RXR and VDR interacted strongly with SRC-1, SRC-1 interaction with RXR was ligand-independent, whereas its interaction with VDR was ligand-dependent (as previously, Figure 5.5C). Co-transfection of wild-type SKIP augmented RXR-SRC-1 interaction by about 3-fold and VDR-SRC-1 interaction by about 2-fold. These data suggest that SKIP facilitated interactions between SRC-1 with RXR and VDR. Further studies will be required to address the complex interactions occuring between these various proteins. Nevertheless, these and other data (section 5.3 and 5.5) are consistent with a role for SKIP interaction with both coactivators and corepressors.

5.6 DISCUSSION

Several potential mechanisms may exist to explain the bifunctional effects of SKIP on NHR-dependent transcription. These include the ability of SKIP to interact with both corepressors and coactivators in a cell-specific manner. In this model, SKIP may interact with corepressors such as N-CoR and SMRT and act to derepress transcription by facilitating removal of corepressors from receptor transcriptional complexes, and so allow coactivator interactions. SKIP interaction with coactivators may also facilitate transcriptional activity through recuitment of factors with HAT activity. SKIP may modulate histone acetylation or phosphorylation either directly or indirectly through interaction with HATs or HDAC protein complexes. Finally, SKIP may itself recruit other cofactors and transcriptional modulators via protein-protein interactions to form enhanceosomes that may determine the transcriptional outcome of a target gene. In this chapter studies addressed whether SKIP may act as a bifunctional protein via interactions with both corepressors and coactivators in various cellular systems.

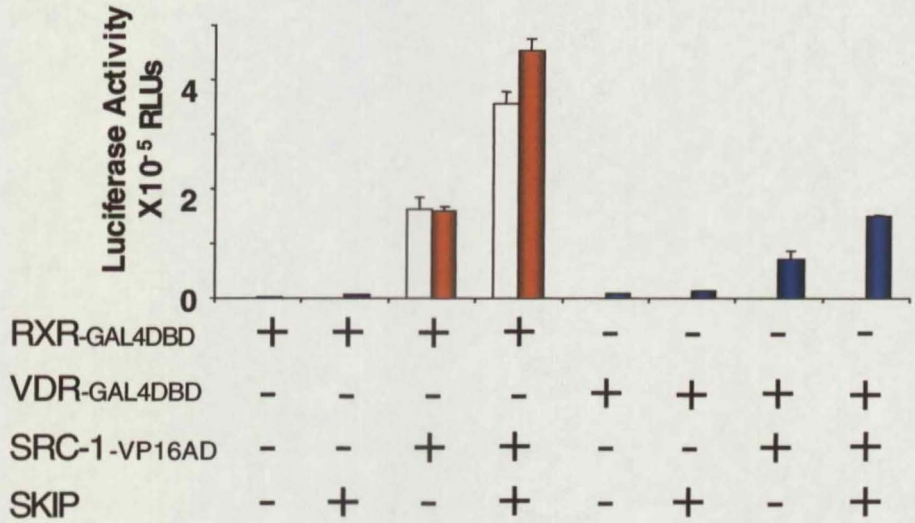

Fig. 5.9 VDR/RXR interactions with SRC-1 in Mammalian Three-Hybrid System.
SKIP augments SRC-1 interaction with VDR and RXR.
Transient transfections were performed in COS1 cells with GAL4-E1b-luciferase reporter and VDR or RXR-GAL4DBD (125ng) with SRC-1-VP16AD (250ng) co-transfected with SKIP-pCGN (100ng) in presence of vehicle (open columns) or ligand (filled columns: red, 9-cis-RA or blue, $1,25(OH)_2D_3$). Results and mean ± SEM of triplicate wells and are representative of duplicate experiments.

In the studies presented in this chapter, SKIP appeared to modulate NHR transcriptional activities through a putative RXR-associated repressor protein in P19 cells, as SKIP repression of a RXR-dependent reporter was reversed by the HDAC-inhibitor TSA. Furthermore, ATRA differentiation of P19 cells converted SKIP into a modest activator and was associated with decreased N-CoR protein levels, consistent with a role for this regulator in SKIP repression. SKIP repressed basal GAL4-dependent reporter activity through a region between 201-333 aa, which also mediated transcriptional repression of SRC-1 autonomous activity but was distinct from a N-CoR/SMRT N-terminal interaction domain (1-200aa) (Figure 5.1). Overall, these data suggest that the actions of SKIP depend on cell-specific interactions with both coactivators and corepressors and support an emerging concept that several types of transcriptional coregulators are targets of SKIP action.

The effects of TSA on the RXRE-dependent reporter implicate a HDAC-containing complex in SKIP repression. Differentiation of P19 cells led to abrogation of TSA-inducible reporter activity associated with SKIP co-transfection, suggesting that regulation by SKIP may relate to endogenous levels of HDAC complexes or their activities (Minucci *et al.*, 1997). This possibility was also consistent with the down-regulation of N-CoR protein level coincident with all-*trans*-retinoic-acid-induced differentiation of the P19 cells (Soderstrom *et al.*, 1997). The identification of a N-CoR-interacting region in the N-terminus of SKIP further supports such a mechanism. Thus, the data suggest that differential expression of N-CoR and possibly other protein complexes involved in histone acetylation and deactylation in the P19 cells may play a role in the modulation of SKIP action as an activator or repressor of NHR-dependent transcription. This possibility is consistent with the observation that SKIP interacts directly with components of a SMRT/HDAC-2/mSIN3 repressor complex associated with the DNA binding protein CBF1 (Zhou *et al.*, 1999). In this model, SKIP has been proposed to convert CBF1 from a transcriptional repressor to an activator through switching its interaction between the corepressor SMRT and the transactivator Notch 1C (Zhou *et al.*, 2000b). This is therefore another context in which SKIP may function as a transcriptional repressor or as an activator. Our recent finding that SKIP interacts with Smad2 and

Smad3 to activate the TGF-β-signalling pathway, possibly as a competitor for the Smad repressors c-Ski and Sno, supports this concept (Leong *et al.*, 2001) (Chapter 6).

SKIP antagonism of the p160/SRC function suggests SKIP may act in some instances as an "anti-coactivator". SKIP interacted directly with SRC-1 *in vitro* and repressed autonomous SRC-1 activity. This repression was mediated by the same central region of SKIP (i.e. 201-333aa) that repressed basal GAL4-dependent reporter activity. In addition, SKIP also repressed SRC-2 and TRAP220 transcriptional activities, although it had only modest repressive effects on p300 and p/CAF (Figure 5.8). Thus SKIP may in some circumstances compete for interaction with coactivators and sequester them from transcriptionally active complexes, a model analogous to that described for repression of hepatocyte nuclear factor 4 (HNF-4)-dependent transcription by the orphan receptor SHP (Lee *et al.*, 2000). In contrast, however, SHP unlike SKIP, does not interact with corepressors such as N-CoR or associated HDACs. In this regard, the bifunctional effects of SKIP on NHR-dependent transcription appear to be similar to the repressor protein, TGIF, which has been suggested to displace coactivators, such as p300, as well as recruit HDACs to modulate Smad-dependent transcription (Wotton *et al.*, 1999).

Studies in this chapter demonstrated an interaction between SKIP and SRC-1 *in vitro*, though no studies were performed to address whether a similar physical interaction occurs between SKIP and SRC-2, TRAP220, p300 or p/CAF. Thus, it is not yet established whether SKIP repression of the transcripitonal activities of these factors is direct or via an indirect mechanism. On chromatin templates the transcriptional activities of proteins such as CBP/p300 is dependent on its HAT activity, though this is not the case on naked DNA templates (Kraus *et al.*, 1999; Vo and Goodman, 2001). Though the mechanism for this difference is unclear, the repressive effect of various coactivators by SKIP in the mammalian two-hybrid assay suggests that SKIP may in some manner be acting as an "anti-coactivator".

Whether this involves inhibition of the intrinsic HAT activity of SRC or other mechanisms remains to be determined (Seo *et al.*, 2001).

In summary, SKIP appears to contain several putative regions that may mediate repressor activity: an N-terminal N-CoR-interacting domain and a central repression domain with apparent anti-coactivator activity. These data provide the first evidence for SKIP as a cell line- and differentiation-specific bi-functional coregulator of NHR-dependent gene transcription. The differences in cell line-specific effects of SKIP may reflect the balance of cellular coactivator (SRCs and others) and/or corepressor complexes (N-CoR/HDACs) available for SKIP interaction. This model of repression and activation by the same cofactor in different cellular environments may also apply to other transcriptional complexes involving SKIP, such as in Notch signaling and in TGF-β-mediated transcription, as described in the next chapter (Glass and Rosenfeld, 2000; Leong *et al.*, 2001).

Chapter Six

SKIP modulation of the Transforming Growth Factor-β Signalling Pathway

6.1 INTRODUCTION

Transforming growth factor-β (TGF-β) superfamily members are multifunctional intracellular signalling proteins which include the TGF-βs, bone-morphogenetic proteins (BMPs), activins and inhibins, mullerian-inhibiting substance and growth differentiation factors (Massague and Wotton, 2000). Members of this superfamily mediate many key cellular events in growth and development and are evolutionarily conserved from Drosophila to mammals (Whitman, 1998). TGF-β signalling requires the action of a family of DNA-binding proteins, Smads, which include TGF-β-specific (Smad2 and Smad3), BMP-specific (Smad1, Smad5 and Smad8), a common Smad4 and "anti-Smads" (Smad6 and Smad7). TGF-β signals through sequential activation of two cell surface receptor serine-threonine kinases which phosphorylate Smad2 and/or Smad3. Phosphorylated Smad2 or Smad3, together with Smad4, translocates into the nucleus where the Smad heterodimer binds Smad-binding elements (SBEs) in association with other nuclear factors in promoters of target genes (Massague and Wotton, 2000; Nakao *et al.*, 1997; Wrana, 2000) (Figure 6.1).

Smad proteins also interact with other nuclear factors such as c-Ski and the Ski-related novel (Sno) protein and nuclear hormone receptors (NHRs), including the VDR and GR, to modulate TGF-β signalling (Akiyoshi *et al.*, 1999; Song *et al.*, 1999; Stroschein *et al.*, 1999; Subramaniam *et al.*, 2001; Sun *et al.*, 1999a; Xu *et al.*, 2000b; Yanagisawa *et al.*, 1999). Ski and Sno have been implicated in oncogenic transformation and enhancement of muscle differentiation by blocking TGF-β signalling (Berk *et al.*, 1997; Colmenares and Stavnezer, 1989; Colmenares *et al.*, 1991; Sutrave *et al.*, 1990). The mechanism of Ski/Sno repression of TGF-β signalling appears to involve an interaction with a complex consisting of N-CoR and HDAC-1 (Luo *et al.*, 1999; Nomura *et al.*, 1999). Interestingly, the Ski-interacting protein (SKIP) was initially identified in a two hybrid screen using v-Ski as a bait, and was later independently identified by our group and others as a VDR- and a CBF1-interacting factor, the latter involved in Notch signalling (Baudino *et al.*, 1998; Dahl *et al.*, 1998b; Leong *et al.*, 1998a; Zhou *et al.*, 1999). Thus, the observation that SKIP modulates both NHR- and Notch-dependent signalling

suggests that SKIP may play a role in the regulation of a number of different and distinct cellular signalling pathways (Zhou *et al.*, 2000b).

6.1.1 Aims

As Ski and Sno can modulate TGF-β-dependent signalling, it was of interest to determine whether the Ski-associated protein, SKIP, could also modulate the TGF-β-signalling pathway through interaction with the Smad proteins. Thus, the primary aim of the studies in this chapter was to address whether SKIP interacts with the Smad proteins and, if so, whether it modulates Ski and/or Sno-dependent gene repression. The well-characterised TGF-β-responsive promoter reporter 3TP-lux from the plasminogen activator inhibitor gene-1 was tested in mammalian cells co-transfected with SKIP, Ski and Sno and the Smad2 and Smad3 proteins. Furthermore, protein-protein interactions between SKIP and Smad proteins were tested *in vivo* in the yeast two-hybrid system and *in vitro* in GST binding and far western assays.

6.2. METHODS

6.2.1 Cloning of Smad yeast two-hybrid plasmids

The Smad-pBridge yeast two hybrid constructs were made by *EcoR*1/*Xho*1 digestion of Smad2-, Smad3- and Smad4-pCDNA3 plasmids (Nakao *et al.*, 1997) and sub-cloning of Smad cDNAs into the *Eco*R1/Sal1 sites of pBridge (Clontech).

6.2.2 Cloning of Sno mammalian expression plasmid

The Sno cDNA was amplified by PCR (section 2.2.7) using the forward primer 5′-GCAATCTAGAGAAAGCCCACAAGCAAATTTCCC-3′ and reverse primer 5′-GCAAGGATCCCTATTTTCCATTTCCATTTTTG-3′ and the PCR product ligated (section 2.2.8.1) into the *Xba*1/*Bam*H1 site of pCGN (Dahl *et al.*, 1998b).

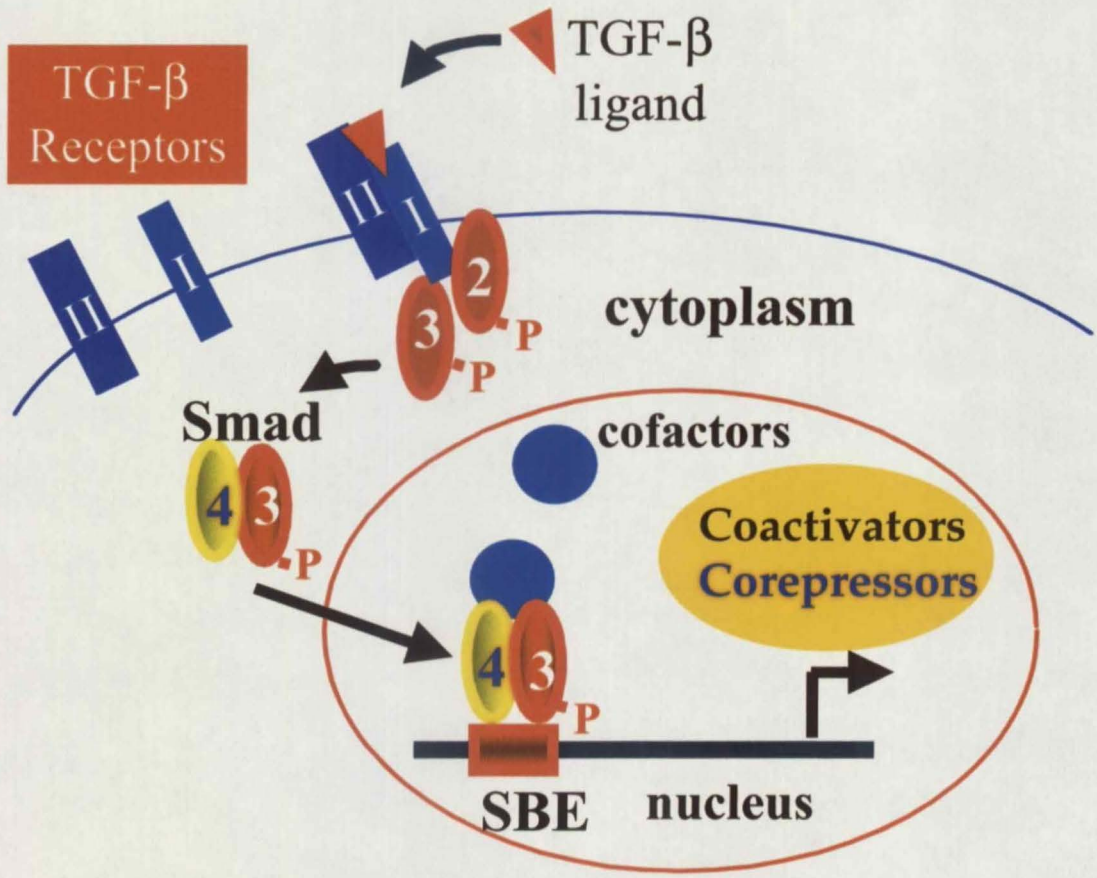

Fig. 6.1 The TGF-β signalling pathway.
In response to ligand the TGF-β receptor complex is assembled consisting of two serine-threonine kinase receptors (type I and II). This complex phosphorylates Smad2 or Smad3, which form heterodimers with the common Smad4 and translocate to the nucleus to bind Smad-binding elements (SBEs) within target gene promoters. This Smad complex associates with cell-specific cofactors such as c-Ski, but also with many other cofactors, some of which are unknown. These cofactors recruit coactivators, such as CBP/p300 and corepressors, such as N-CoR, that determine the transcriptional outcome.

6.3 RESULTS

6.3.1 SKIP augments TGF-β–dependent transcription

As Ski and Sno interact directly with Smad proteins (Smad2 and Smad3) to repress TGF-β dependent transcription, the effects of SKIP on the TGF-β-responsive 3TP-lux reporter construct were tested (Carcamo *et al.*, 1995). In COS-1 cells, the 3TP-lux reporter responded to TGF-β with a 4-fold increase in reporter activity (Figure 6.2). Smad3 alone, or Smad2 and Smad4 together (but neither alone) augmented both basal (2-fold) and TGF-β responses (10-fold) of this reporter activity. Smad3 co-transfection with Smad4 led to a 6-fold increase in basal and a 30-fold increase in ligand-dependent reporter activity. This augmentation was similar to that of SKIP alone on ligand-dependent reporter activity (Figure 6.2A). An interaction between SKIP and Smads was suggested in co-transfection studies with the fold increase of basal activity progressively increasing when SKIP was co-transfected with Smad2 alone (8-fold), Smad2 and 4 (20-fold), Smad3 alone (53-fold) and Smad3 and Smad4 (164-fold). The comparable increases in TGF-β induced activity were 39-fold, 116-fold, 96-fold and 323-fold, respectively. These data are consistent with a functional interaction primarily occurring between SKIP and Smad3 without exogenous Smad4, and enhanced by increased levels of the common mediator Smad.

6.3.2 Mapping of SKIP-Smad interaction domains

SKIP interaction with Smad proteins was investigated by yeast two-hybrid interaction analysis. SKIP interacted with both Smad2 and Smad3 (Figure 6.2B). Smad4 had a high level of reporter activity, which was unaltered by co-expression of SKIP. However, Smad4, as expected, interacted strongly with v-Ski-GAL4AD in yeast (Figure 6. 2C).

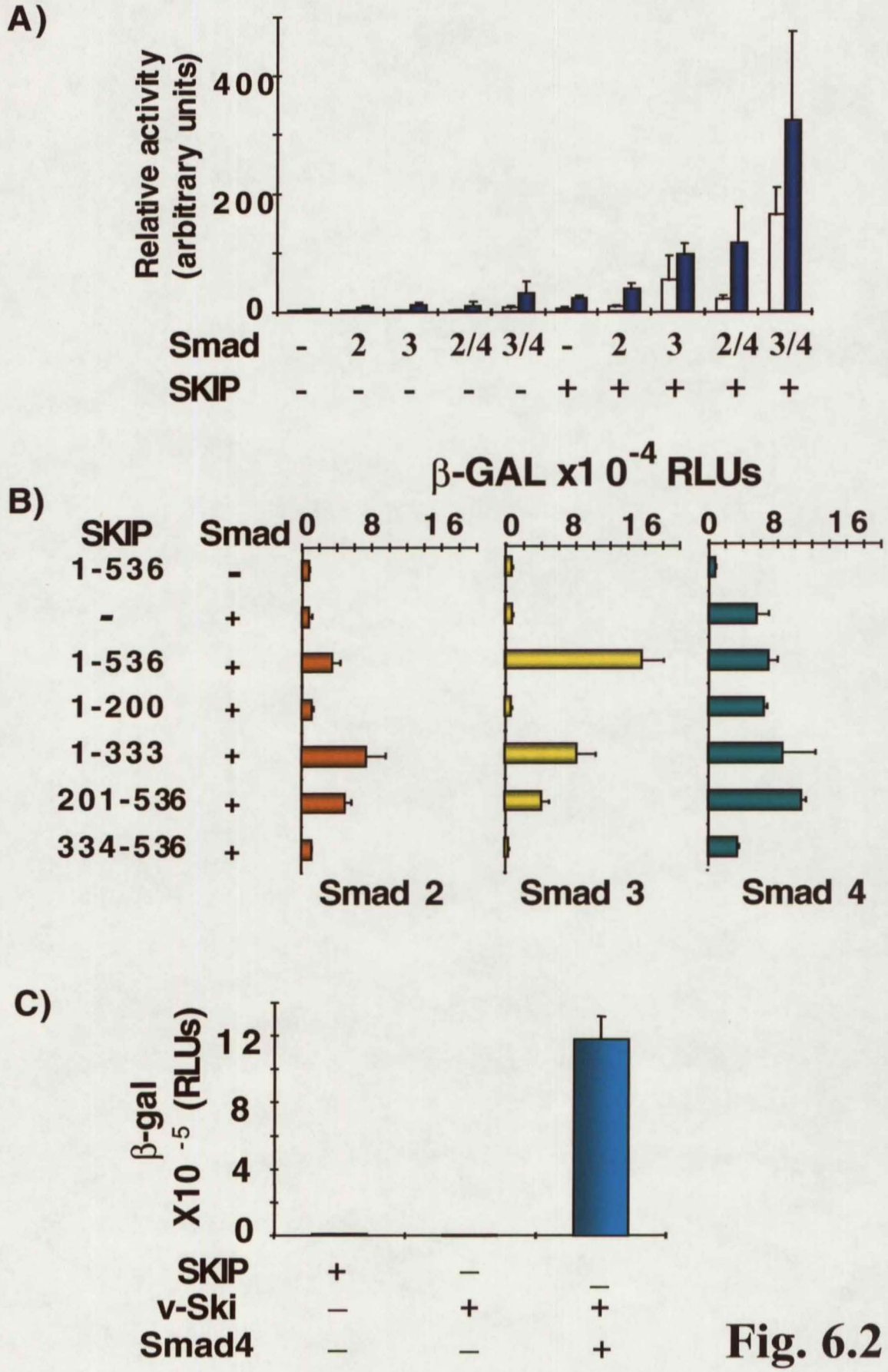
A)
Relative activity (arbitrary units)
400
200
0
Smad - 2 3 2/4 3/4 - 2 3 2/4 3/4
SKIP - - - - - + + + + +
B)
β-GAL x1 0-4 RLUs
SKIP
Smad
1-536 -
- +
1-536 +
1-200 +
1-333 +
201-536 +
334-536 +
0 8 16
Smad 2
Smad 3
Smad 4
C)
β-gal X10 -5 (RLUs)
12
8
4
0
SKIP + – –
v-Ski – + +
Smad4 – – +

Fig. 6.2

Fig. 6.2. SKIP augmentation of TGF-β dependent transactivation in mammalian cells.

A) SKIP activates Smad-dependent transcription. Transient transfections of COS-1 cells were performed with the 3TP-lux reporter (250ng per well) and expression plasmids: SKIP-pCGN and Smad2-, Smad3- or Smad4-pCDNA3. Cells were treated with vehicle □ or TGF-*β* (1ng/mL) ■. The values are mean luciferase activity ± SEM of three independent experiments, each performed in triplicate.

B) SKIP interacts *in vivo* with Smad2 and 3 through a domain between aa 201-333. Yeast two-hybrid interaction analysis of wild-type and mutant SKIP-GAL4AD proteins co-expressed with wild-type Smad2-, Smad3- or Smad4-GAL4DBD. Values are mean ± SEM from 3 independent colonies obtained in at least duplicate experiments.

C) Smad4 interaction with v-Ski.

Yeast two-hybrid interaction analysis of v-Ski-GAL4AD co-expressed with Smad4-GAL4DBD. Values are mean ± SEM from 3 independent colonies obtained in at least duplicate experiments.

Domains of SKIP required for Smad interaction were examined using deletion constructs of SKIP. The C-terminally deleted 1-333aa and N-terminally deleted 201-536aa SKIP mutants were comparable to wild-type SKIP in their interaction with Smad2 (Figure 6.2). However, their interactions with Smad3 were about 50% and 25% of wild-type SKIP, respectively. There was no evidence of interaction of the SKIP N-terminal (1-200aa) or C-terminal (334-536aa) domains with Smad2 or Smad3. Thus, these results suggest that the region of SKIP between aa201-333 is required for its interaction with Smad2 and Smad3.

The same SKIP deletion constructs were tested with the TGF-β responsive reporter in the COS-1 mammalian cell line (Figure 6.3). Co-expression of wild-type SKIP (1-536aa) with Smad3 caused a 3-fold increase in reporter activity above SKIP or Smad3 alone. The N-terminal domain of SKIP (1-200aa) had no effect on reporter activity, while the other SKIP constructs had comparable transactivation to that of wild-type SKIP, though the N-terminal SKIP (1-333aa) construct exhibited 2-fold greater transactivation than wild-type. Western analysis of COS-1 cells transfected with the wild-type SKIP and deletion mutant expression plasmids showed comparable expression, except for the 1-200aa construct which, despite being expressed at approximately twice the level of wild-type SKIP had no apparent transactivation activity (Figure 6.3). Thus, these transfection data were consistent with the yeast interaction data and suggest that 201-333aa region of SKIP with Smad3 is sufficient for near maximal transactivation of the TGF-β responsive reporter.

Surprisingly, the 334-536aa SKIP construct was able to activate the TGF-β responsive reporter with Smad3, even though no interaction with this mutant by Smad3 was observed in yeast. This suggests that an additional C-terminal domain, which recruits other Smad-interacting co-factors present in mammalian cells but not in yeast, may be transcriptionally functional.

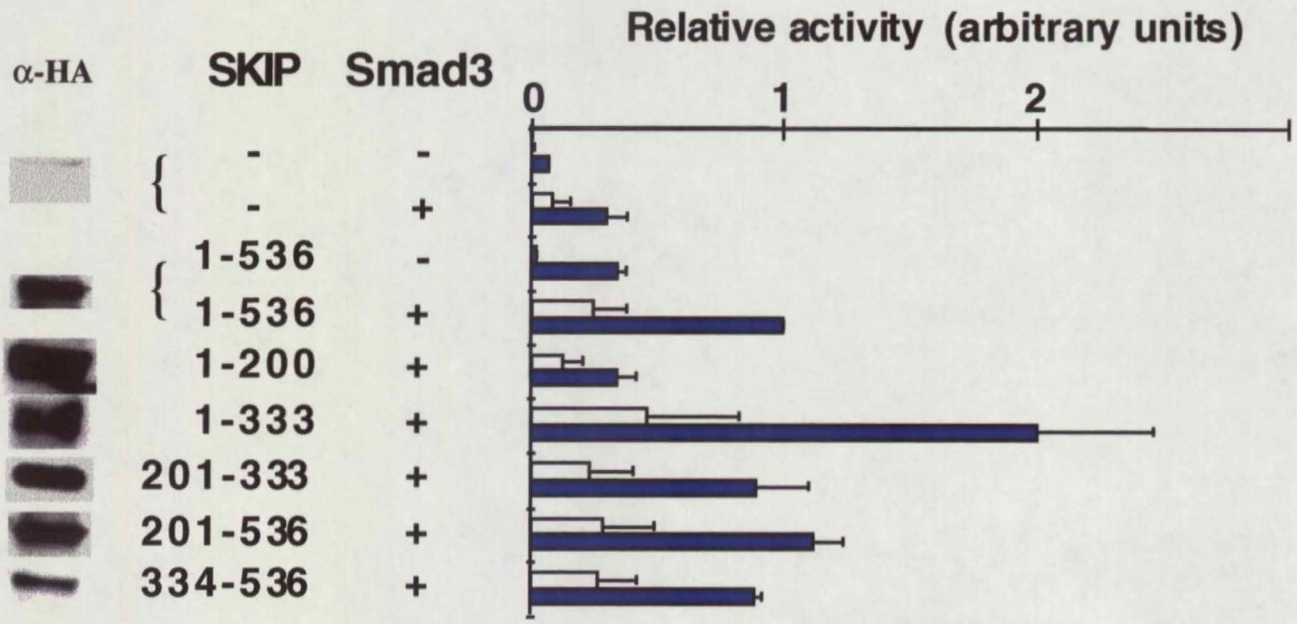

Fig. 6.3 SKIP domains and TGF-β transactivation in mammalian cells. Transient transfections of COS1 cells were performed as described in Fig. 6.2 with the 3TP-lux reporter and the Smad3-pcDNA3 expression plasmid (100ng) and wildtype SKIP-pCGN or deletion constructs as indicated (50ng). Cells were treated with vehicle □ or TGF-β ligand (1ng/mL) ■ . The results are shown as the mean luciferase activity ± SEM of two independent experiments performed in triplicate. On left panel are shown the western analysis with α-HA antibody of COS1 cell nuclear extracts transfected with wild-type SKIP and various deletion mutants as indicated. All SKIP wild-type and mutant protein extracts were run with a molecular weight marker and were appropriate in size.

6.3.3 SKIP interaction with Smad2 and Smad3 *in vitro*

The potential direct physical interaction between the Smad proteins and SKIP was explored using a GST binding assay. GST-SKIP bound both Smad2 and Smad3 (Figure 6.4A). In the controls with GST-0, there was minimal, if any binding of Smad2 or Smad3; similarly there was no binding of the negative control lysate, luciferase, to GST-SKIP.

To determine which domains of Smad3 may be involved in SKIP interaction, a GST-Smad3 binding assay was performed with [^{35}S]-labelled *in vitro* translated SKIP (Figure 6.4B). GST-wild-type Smad3 bound SKIP and the positive control VDR (Yanagisawa *et al.*, 1999). Deletion of the MH1 domain of Smad3 (aa199-427aa) had no effect on SKIP binding, but as expected, its VDR binding was abolished. Both SKIP and VDR binding were lost when the MH1 and MH2 domains of Smad3 were both deleted (GST-Smad3 199-405aa). However, no binding of SKIP was observed to a GST-MH2 construct that expressed only the last 26 aa of hSmad3. These results indicate, that while VDR binds to the N-terminal MH1 domain of Smad3, the MH1 domain is not required for SKIP interaction, whereas the MH2 domain is necessary, but alone is not sufficient for SKIP interaction.

To further support the existence of a direct protein-protein interaction *in vitro*, a far western assay was performed using mammalian cell nuclear extracts overexpressing HA-SKIP or Smad3 (Figure 6.4C). Smad3 co-localised with HA-SKIP, but not with the empty vector control extracts. These results with the GST-binding studies thus strongly support a direct protein-protein interaction *in vitro* between SKIP and Smad3.

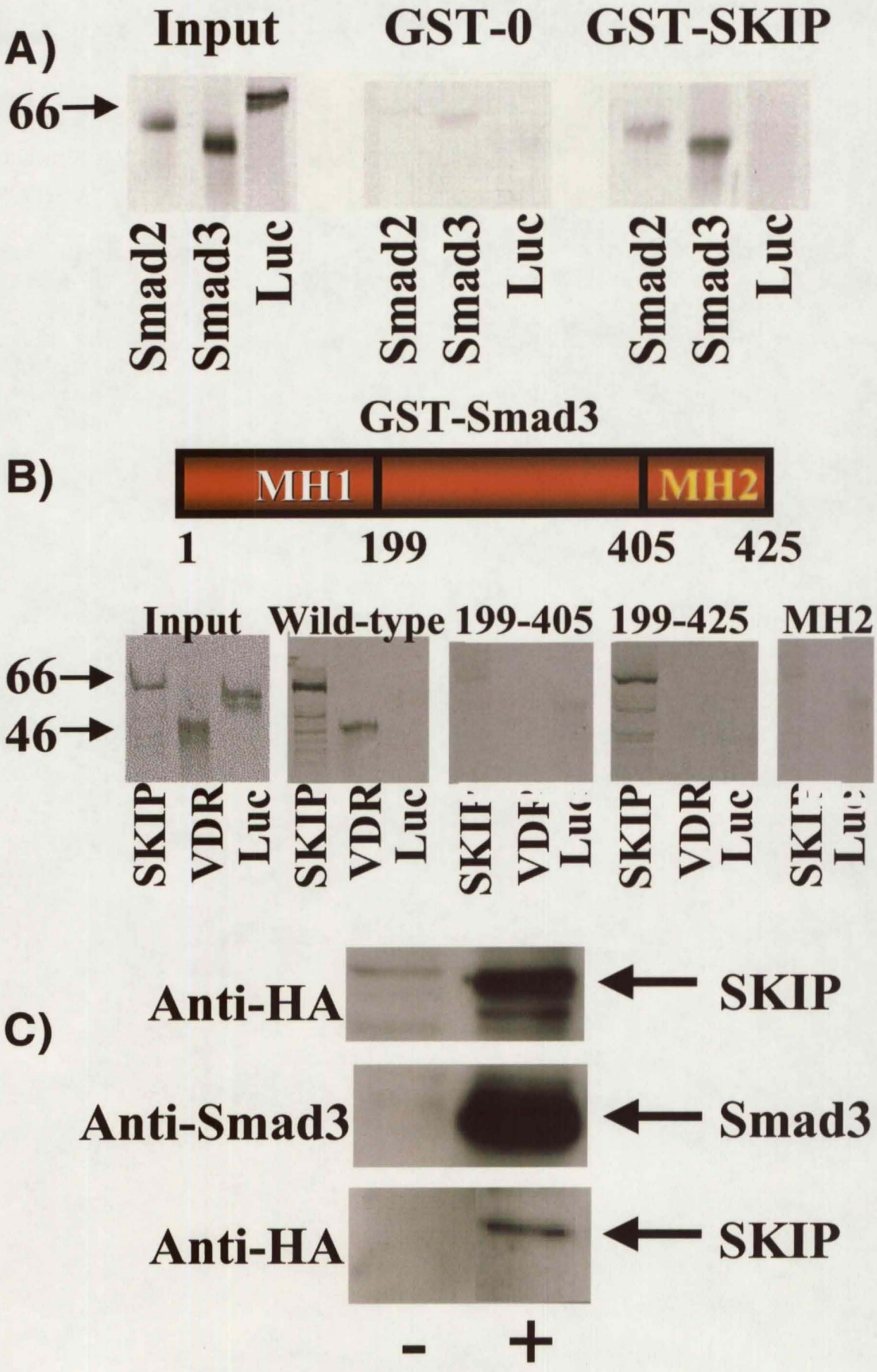
A)
Input
GST-0
GST-SKIP
66
Smad2
Smad3
Luc
Smad2
Smad3
Luc
Smad2
Smad3
Luc
B)
GST-Smad3
MH1
MH2
1
199
405
425
Input
Wild-type
199-405
199-425
MH2
66
46
SKIP
VDR
Luc
SKIP
VDR
Luc
SKIP
VDR
Luc
SKIP
VDR
Luc
C)
Anti-HA
SKIP
Anti-Smad3
Smad3
Anti-HA
SKIP
-
+

Fig. 6.4 SKIP interacts with Smad2 and Smad3 *in vitro*.

A) Smads interact directly with SKIP *in vitro*.

GST-SKIP binding assay using with equal amounts of [^{35}S]-labelled wild-type Smad3 and luciferase. Input proteins are one-fifth of loaded lysate.

B) Smad MH2 domain required, but not sufficient, for SKIP interaction *in vitro*. GST-Smad3 binding assay includes wild-type and mutant GST-Smad3 proteins, a MH1 and MH2 domain double mutant (aa199-405), a phosphorylated MH1 domain deletion mutant which retains the MH2 (aa199-425) and the core MH2 domain consisting of last 26aa of the C-terminus of Smad3 (aa400-425). Beads were incubated with equal amounts of [^{35}S]-labelled wild-type SKIP, and hVDR (Yanagisawa *et al.*, 1999) and luciferase as positive and negative controls, respectively.

C) SKIP interacts with Smad3 in Far Western Analysis.

Twenty μg of nuclear extracts containing HA-SKIP (upper panel) or Smad3 (middle panel) was detected by western analysis. COS-1 nuclear extracts were prepared from cells transfected with the empty vector pCDNA3 or pCGN (indicated by -) or Smad3-pCDNA3 or HA-SKIP-pCGN expression plasmid (indicated by +). Proteins in the Smad3 containing membrane were resolved on SDS-PAGE, electoblotted to a PVDF membrane, denatured, and renatured with serial dilutions of 6M guanidine hydrochloride and then probed with an anti-HA antibody in a far western analysis (lower panel). SKIP comigrated with Smad3, but not the negative control extract, consistent with direct physical interaction between SKIP and Smad3.

6.3.4 Ski and Sno competitively inhibit SKIP-dependent activation

The Smad3 transcriptional repressors, Ski and its related protein Sno, are known to bind to the MH2 domain of Smad3 (Sun *et al.*, 1999b). Since SKIP also interacts with Ski and Sno, SKIP modulation of Ski/Sno repression of Smad3-dependent transcription was tested. As before, SKIP increased basal and TGF-β-dependent transactivation, particularly in the presence of Smad3 (Figure 6.5). Both Ski and Sno attenuated this SKIP-dependent transactivation by about 80% and 40%, respectively (Figure 6.6). SKIP transactivation, either alone or with Smad3, was repressed in a dose-dependent manner by co-transfection with Ski. Sno had a similar but weaker effect (Figure 6.5). These data suggest that SKIP and Ski/Sno may act as competing regulators of the TGF-β transcriptional response.

6.3.5 SKIP interactions with Ski/Sno and Smads

As Ski/Sno interact with both Smad3 and SKIP, one alternative possibility other than a competitive interaction between these proteins is that they form a ternary complex. To address this question a gel shift analysis was performed using the PE-2 cDNA probe from the PAI-1 promoter which binds a Smad3/4 heterodimer (Hua *et al.*, 1999) (Figure 6. 7), The addition of increasing amounts of SKIP nuclear extracts was associated with increased binding of a second higher molecular weight complex which presumably contained SKIP and Smad3/4 (lanes 3 to 6). The addition of Sno nuclear extracts alone led to augmentation of Smad3/4 binding (lanes 12-13). However, in the presence of both SKIP and increasing Sno, no ternary complex was observed (lanes 7-10), though a further enhancement of Sno binding to Smad3/4 was observed. This complex was specific as it was abrogated by addition of cold probe (lane 11).

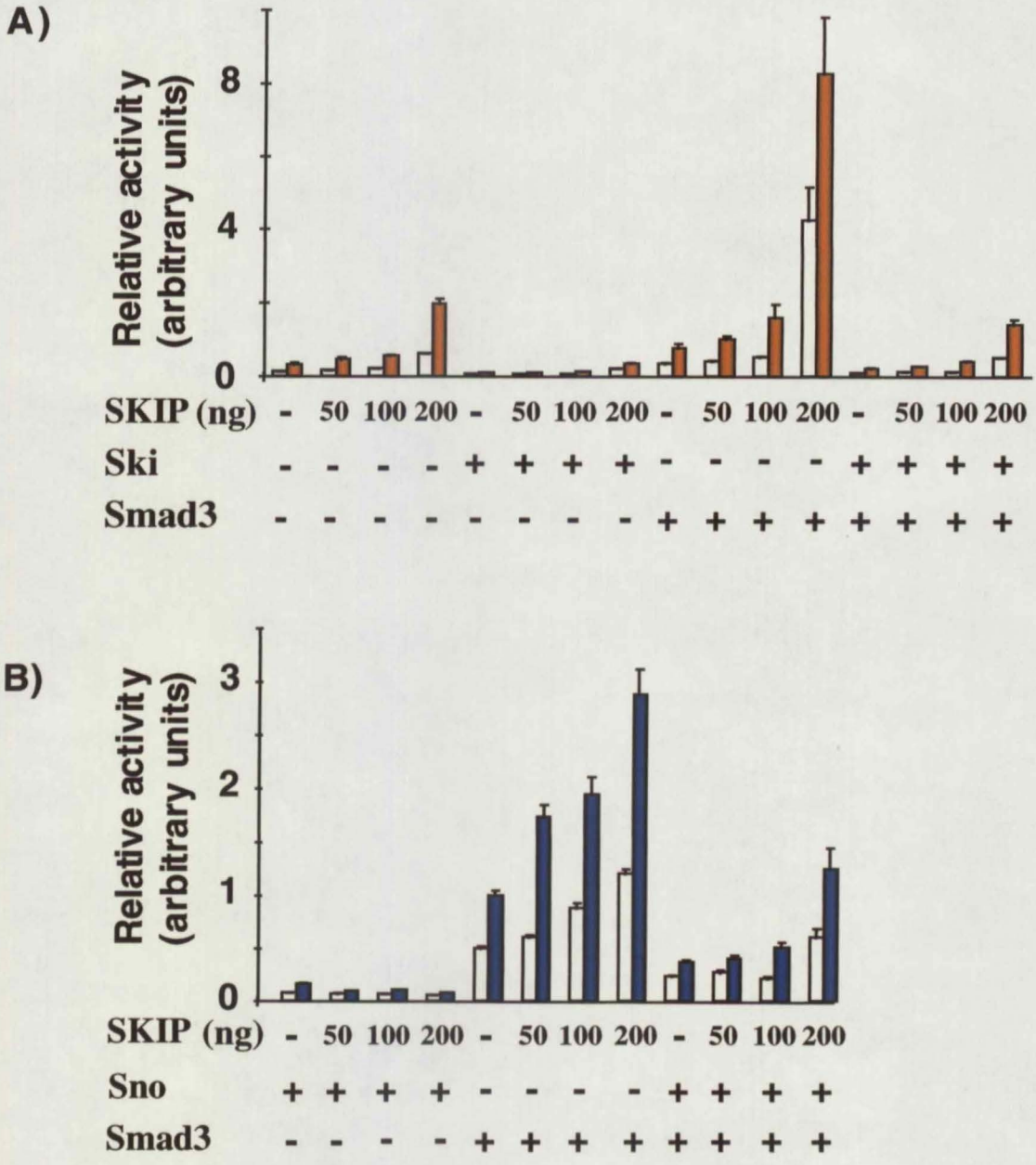

Fig. 6.5 SKIP augments whereas Ski/Sno repress TGF-β–dependent transcription. Transient transfections of COS1 cells were performed with the 3TP-lux reporter (250ng), Smad3-pcDNA3 (100ng), and increasing amounts of wildtype SKIP-pCGN as indicated, with either A) c-Ski-pMT2 (100ng) or B) Sno-pCGN (100ng) expression plasmids. Cells were treated with vehicle (open columns) or TGF-β ligand (1ng/ml) (filled columns). The results are shown as the mean luciferase activity ± SEM of triplicate wells relative to ligand-dependent reporter activity of Smad3 transfection alone set at 1, and are representative of three independent experiments.

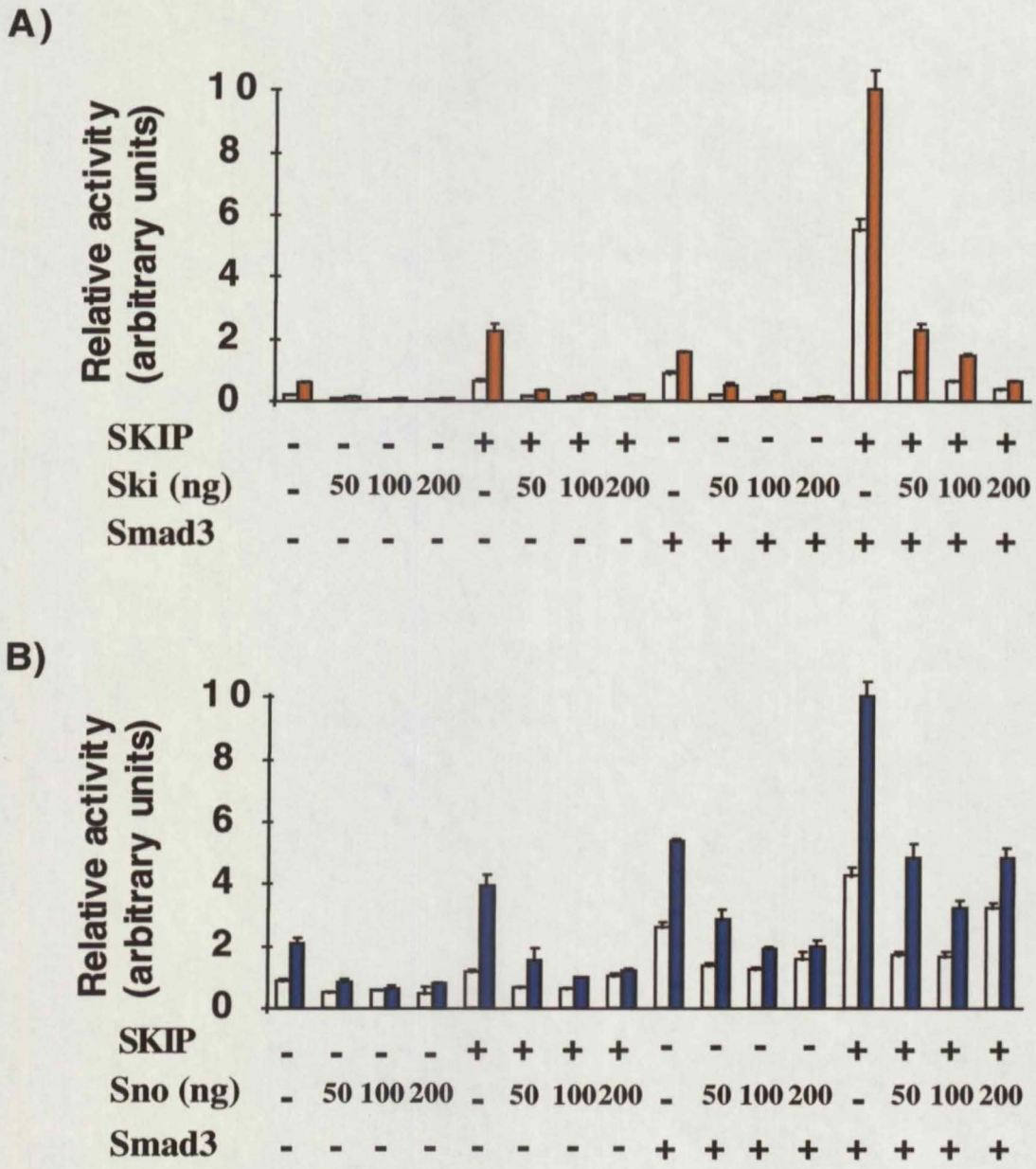

Fig. 6.6. Ski and Sno attenuation of SKIP augmentation of TGF-β–dependent transcription. Transient transfections of COS1 cells were performed with the 3TP-lux reporter (250ng) and Smad3-pcDNA3 (100ng), wildtype SKIP-pCGN (50ng) and increasing amounts of either A) c-Ski-pMT2 or B) Sno-pCGN expression plasmids as indicated. Cells were treated with vehicle (open columns) or TGF-β ligand (1ng/ml) (closed columns). The results are shown as the mean luciferase activity ± SEM of triplicate wells relative to maximal ligand-dependent reporter activity with SKIP and Smad3 co-transfection set at 10, and are representative of three independent experiments.

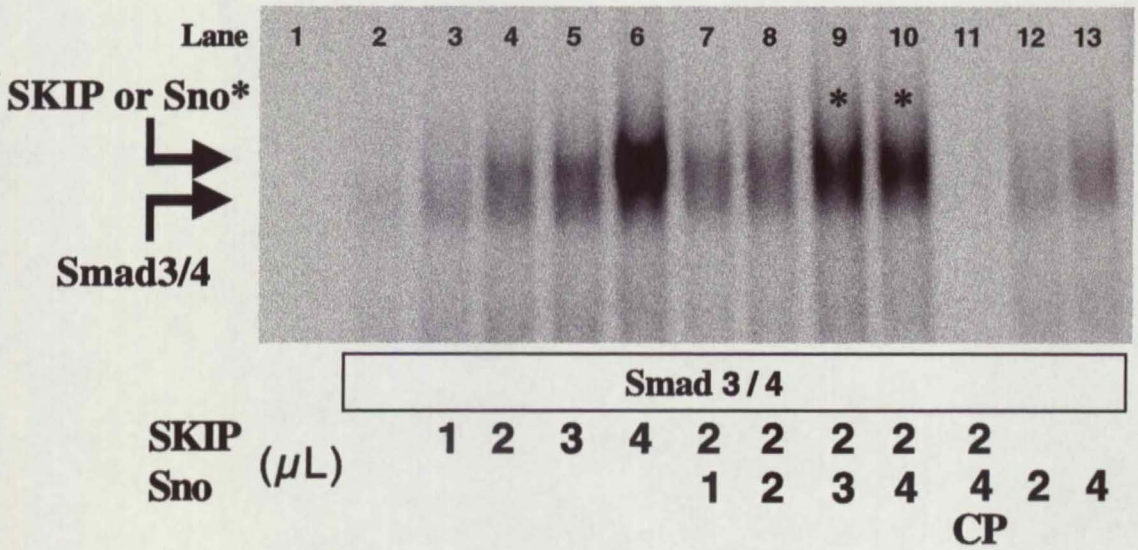

Fig. 6.7. SKIP and Sno independently augment Smad3/4 DNA binding.

In vitro [^{35}S]-labelled Smad3 and Smad4 (2μL each) were added with COS-1 nuclear extracts of SKIP and Sno, (as indicated in μL), with radiolabelled PE2 probe, a fragment of the PAI-1 promoter (Hua *et al.*, 1999) and analysed by gel shift assay. Lane 1 contains untranslated lysate and lane 2 Smad3/4 lysate alone (lower arrow). The presumed SKIP/Smad3/4 (lanes 3-6) and Sno/Smad3/4 complexes (lanes 9-10 & 12-13) migrate as higher molecular weight complexes as indicated by the upper arrow.

6.3.6 SKIP modulation of the Human Osteocalcin Promoter

One key mechanism for increasing the range and diversity of transcriptional responses under control of various gene families, including the NHR superfamily, is the occurrence of overlapping and/or distinct DNA binding elements within the same gene which function cooperatively or competitively to amplify or repress gene transcription. A number of examples exist for the presence of such enhanceosomes within natural promoters, including various hormone- or cytokine-responsive genes (Merika and Thanos, 2001). In other work from our group (Subramaniam *et al.*, 2001), the human osteocalcin promoter was shown to be TGF-β responsive through the binding of a Smad3/4 heterodimer to two functional Smad-binding elements positioned 5′ and 3′ to the consensus VDRE (Figure 6.8). Smad3 and VDR with their respective ligands synergistically augmented transcription of this promoter. As SKIP appears to be both a VDR- and Smad3-interacting protein, it was hypothesised that SKIP may facilitate this transcriptional response and facilitate crosstalk between these two signalling pathways.

In transient transfections in COS-1 cells, SKIP or Smad3 alone with VDR each augmented basal and $1,25(OH)_2D_2$-induced reporter activity by about 2 fold (Figure 6.8). Smad3 had the greatest effect on basal activity, as previously observed (Subramaniam *et al.*, 2001; Sun *et al.*, 1999a). However, when Smad3 was co-transfected with SKIP and VDR there was a 4-fold augmentation of both basal and ligand-induced reporter activities, suggesting that Smad3 and SKIP synergistically activated the human osteocalcin promoter.

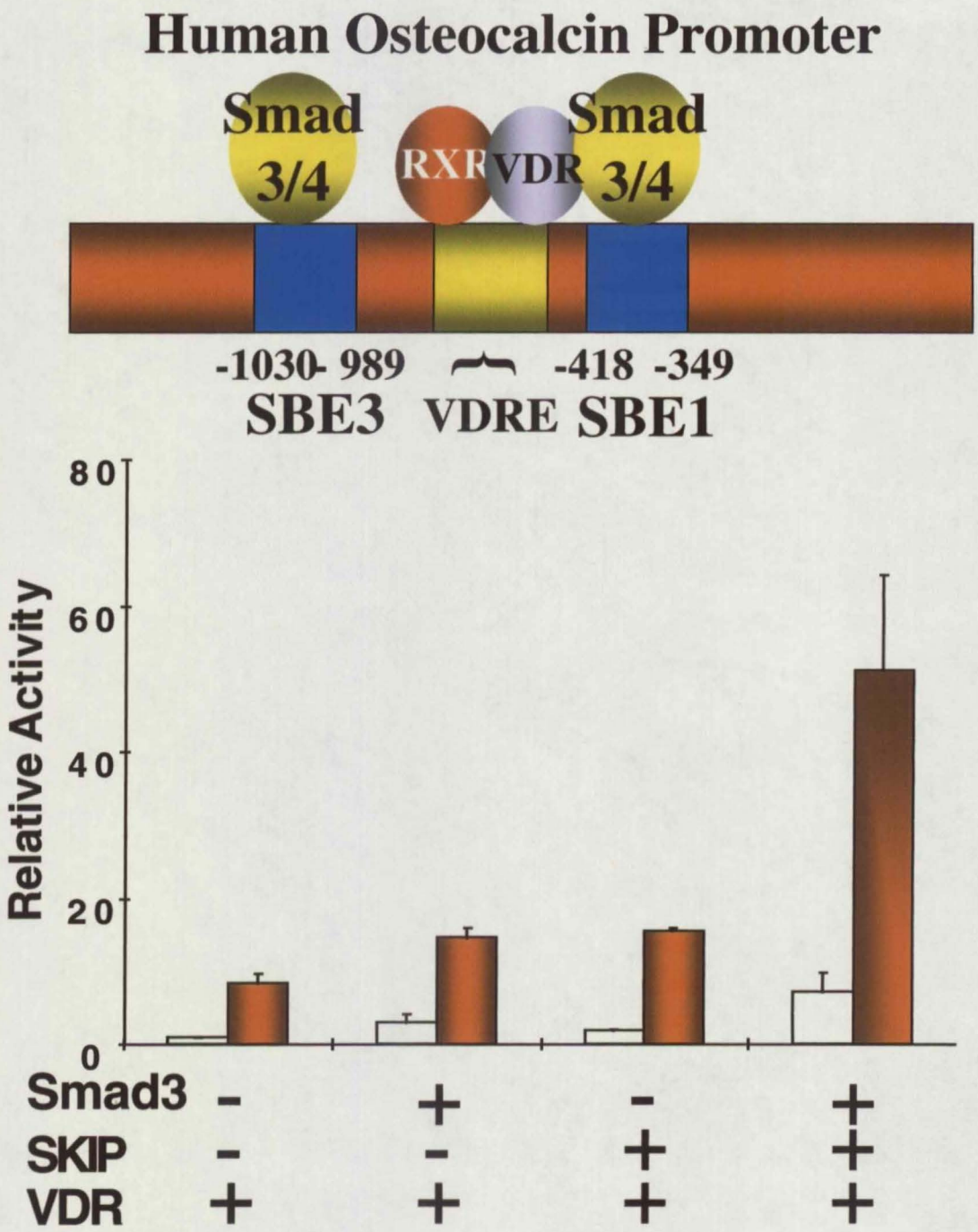

Fig. 6.8 SKIP with Smad3 synergistically activates the vitamin-D-responsiveness of the hOsteocalcin promoter.
Shown schematically in upper figure are the two functional Smad binding elements (SBE3 and SBE1) within this promoter in relationship to the VDRE (Subramaniam *et al.*, 2001). Transient transfections were performed in COS1 cells with the hOS2-luc promoter reporter (250ng) co-transfected with mVDR-pSG5 (50ng), SKIP-pCGN (50ng) and Smad3-pCDNA3 (100ng) expression plasmids. Cells were treated overnight with 10^{-8} M $1,25(OH)_2D_3$ ■ or vehicle □ . Results are mean ± SEM of triplicate wells relative to unliganded control transfected with VDR alone and are representative of 3 repeat experiments.

6.4. DISCUSSION

SKIP, a NHR-interacting cofactor, also associates with both Ski and Sno, which have been shown to negatively modulate TGF-β signalling through an interaction with a N-CoR repressor complex (Luo *et al.*, 1999). Thus, studies in this chapter were undertaken to determine the potential role of SKIP in TGF-β signalling. SKIP augmented TGF-β–dependent transcription and exhibited a direct interaction with Smad proteins. This SKIP-Smad interaction was apparent both *in vitro* and *in vivo*, as demonstrated by GST "pulldown" assays, far western analysis and yeast two-hybrid protein-protein studies. The region between aa201-333 within SKIP appeared to act as the Smad-interacting domain. Though deletion of the MH2 domain of Smad3 abrogated its interaction with SKIP, this domain alone was insufficient for interaction with SKIP. Moreover, Ski and Sno appeared to attenuate SKIP transactivation, while SKIP partially counteracted Sno-, if not Ski-mediated transcriptional repression.

The C-terminal MH2 domain of Smad2 and Smad3 has been reported to be a key region involved in multiple protein-protein interactions, including those with the coregulators CBP/p300 and the Smad repressors Ski and Sno (Massague and Wotton, 2000). Although the N-terminal MH1 domain of the Smads confers only low affinity DNA binding to a consensus Smad-binding element (SBE) (Massague and Wotton, 2000), it appeared to be necessary and sufficient for VDR binding and was required as well as the MH2 domain for SKIP binding.

Natural TGF-β-responsive promoters contain functional clusters of SBEs. Other DNA-binding factors, such as FAST-1, TFE3 and AP-1, as well as non-DNA binding factors are involved in determining the specificity and direction of Smad target gene action (Chen *et al.*, 1996; Hua *et al.*, 1999; Zhang *et al.*, 1998c). As such, SKIP appears to play a role in augmentation of TGF-β-specific Smad transcriptional activity via an interaction with the MH2 domain of Smad3.

However the present data also suggest that the MH1 domain may be required for a full interaction.

Though SKIP was able to interact with Smad2 and Smad3 in yeast, SKIP co-transfection with Smad3 with or without Smad4, led to the greatest increases in reporter activity in mammalian cells, presumably because the 3TP-lux TGF-β-responsive reporter is Smad3-selective (Carcamo *et al.*, 1995). Thus, as SKIP interacted with Smad2 *in vivo* and *in vitro*, it is also possible that SKIP may be able to modulate TGF-β signalling through Smad2 in mammalian cells (Labbe *et al.*, 1998). Furthermore, in the transient transfections Smad3 augmented basal reporter activity, as has been previously described for the 3TP promoter (Sun *et al.*, 1999a), and this activity was further increased by SKIP. Additional studies will be required to address the specific reasons for this effect of SKIP, though is consistent with the observation that SKIP interacts *in vitro* with the general transcription factor, TFIIB (data not shown).

The deletional analysis of SKIP in yeast and mammalian cells suggested that the aa201-333 region of SKIP is required for Smad interactions *in vivo*. However some functional differences were observed between yeast and mammalian cells. Specifically, while the C-terminal SKIP construct (aa334-536) did not interact with Smad2 or Smad3 in yeast, its transactivation activity in mammalian cells was comparable to wild-type SKIP. These differences were not related to expression levels of the various deletion mutants as assessed by western analysis (Figure 6.3B). A C-terminal transactivation domain of SKIP that functions in mammalian cells, distinct from the Smad interaction domain, is consistent with involvement of a domain C-terminal to aa437 of murine SKIP (NcoA-62) in vitamin-D-dependent transactivation (Baudino *et al.*, 1998).

As SKIP interacts with Ski/Sno and Smad3 and in turn Ski/Sno interact with Smad3, the possible formation of a ternary complex was analysed in gel shift using the PE2 cDNA probe from the PAI-1 promoter. This revealed that both SKIP and

Ski/Sno formed a higher mobility complex with Smad3/4. However, there was no evidence of formation of a ternary complex in the presence of all three proteins. Thus these data are consistent with the transient transfection results which suggest competition rather than formation of a ternary complex between SKIP and Ski/Sno for Smad3 transactivation.

Studies were then undertaken to examine whether SKIP may facilitate crosstalk between the TGF-β and VDR-signalling pathways using the TGF-β and vitamin-D-responsive human osteocalcin promoter (Subramaniam *et al.*, 2001). As SKIP is both a Smad3- and VDR-interacting factor, transient transfections were performed with the human osteocalcin-luciferase reporter with or without SKIP or Smad3 in the presence of vitamin D (Figure 6.8). SKIP or Smad3 alone modestly activated this reporter, but SKIP and Smad3 synergistically activated both basal and ligand-dependent reporter activities. Thus, in the osteocalcin promoter SKIP appears to facilitate crosstalk between these two signalling pathways (Figure 6.8 and Figure 6.9). Whether this effect is cell line- and/or promoter-specific, whether it is dependent on other factors, and the role of TGF-β ligand in these responses remain to be determined. Nevertheless, these and other data suggest SKIP modulates multiple transcriptional pathways which may be fundamental to its role in gene regulation.

In these studies SKIP acted as a coactivator of TGF-β-dependent transcription. SKIP similarly acts as a coactivator of NHR-dependent transcription, but also as a repressor of Notch signalling through its interaction with SMRT and associated HDAC proteins (Baudino *et al.*, 1998; Zhou *et al.*, 2000a). These divergent effects of SKIP may depend on interaction of SKIP with other, possibly cell-specific, nuclear factors. For example, SKIP converts CBF1 from a transcriptional repressor to activator through switching its interaction between the corepressor SMRT and Notch 1C (Zhou *et al.*, 2000a). In the present studies, SKIP and Ski/Sno modulated each other's opposing transcriptional activities, raising the intriguing possibility that the relative cellular expression of SKIP versus Ski or Sno may play a regulatory role on TGF-β–dependent transcription and hence its effects on cell

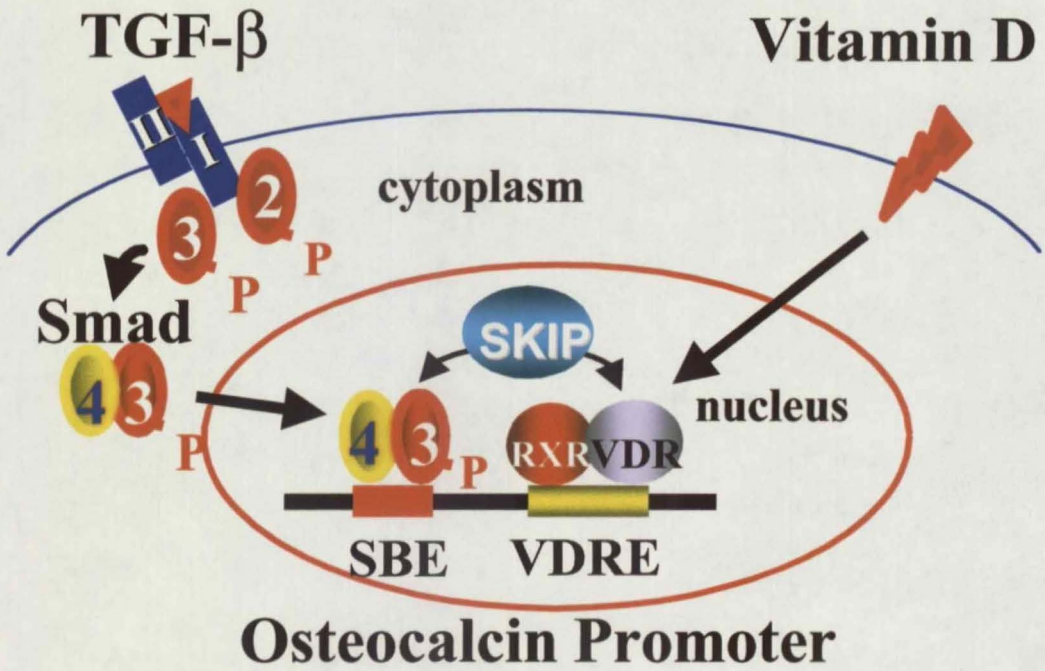

Fig. 6.9 Model of Skip-VDR transcriptional crosstalk on human Osteocalcin promoter.
Hypothesis: "As SKIP is both a Smad- and VDR-interacting protein it may modulate crosstalk between the TGF-β and VDR signalling pathways. Shown schematically is translocation of the Smad3/4 heterodimer from the cytoplasm to nucleus following TGF- β stimulation and phosphorylation (P). Within the nucleus the Smad3/4 heterodimer binds the SBEs and with the VDR/RXR heterodimer and respective ligands synergistically activates the osteocalcin gene promoter (Subramaniam *et al.*, 2001).

growth and differentiation. Interestingly, the Smad-interacting domain of SKIP (201-333aa) appears to be also involved in interaction with Ski and Sno (Prathapam *et al.*, 2001). These results suggest that the opposing transcriptional effects of SKIP and Ski/Sno may involve competition for Smad3 binding between SKIP and c-Ski/Sno, and/or other Smad3 MH2-interacting factors, such as with CBP/p300 (Akiyoshi *et al.*, 1999; Feng *et al.*, 1998b; Janknecht *et al.*, 1998). Thus, the modulatory effects of SKIP through the MH2 domain potentially increase the complexity and diversity of Smad-dependent transcriptional effects. Furthermore, as SKIP and Ski/Sno interact with each other and also with the related corepressors N-CoR/SMRT, an additional mechanism could involve SKIP-mediated derepression (Dahl *et al.*, 1998b; Luo *et al.*, 1999; Massague and Wotton, 2000; Zhou *et al.*, 2000a). This may possibly occur via SKIP sequestration of corepressors such as SMRT or N-CoR from the Ski/Sno repressor complex, a mechanism similar to that suggested for Hoxc-8 and Smad1 (Shi *et al.*, 1999). Whatever the molecular mechanism(s) of SKIP action, it is nevertheless clear that SKIP plays a role in modulation of this important cellular and signalling pathway.

In summary, the present results support a model in which SKIP positively modulates TGF-β-dependent-transcription and competes with other Smad MH2-interacting factors, such as c-Ski and Sno, to determine the transcriptional outcome of a TGF-β responsive target gene. These data suggest a potential role for SKIP in the regulation of TGF-β effects on cell growth and differentiation.

CHAPTER 7

General Discussion

Multiple regulatory steps are required for precise and efficient tissue- and development stage-specific actions of NHRs (Chen *et al.*, 2001; Glass and Rosenfeld, 2000; Lemon and Tjian, 2000; Rachez and Freedman, 2001). The complexity and range of factors that have been described to interact and modulate NHR action is in one sense staggering, but perhaps not surprising in the light of the complex transcriptional outcomes required for maintenance of normal eukaryotic homeostasis and mammalian development. The studies in this thesis set out to determine some of the molecular mechanism(s) in relation to VDR action. In order to achieve this primary aim, VDR-interacting factors were identified by the yeast two-hybrid system. From these studies a nuclear cofactor, Ski-interacting protein (SKIP), was identified and characterised as a VDR- and RXR-interacting cofactor and shown to behave in a bifunctional transcriptional manner, activating NHR-dependent transcription in CV-1 cells, but repressing these same activities in undifferentiated P19 cells.

To explore the molecular mechanism(s) of these actions of SKIP, an analysis was undertaken of its interactions with key cofactors involved in transcriptional repression and activation, such as N-CoR and SMRT, and SRC-1, respectively. This analysis led to functional domains being defined within SKIP (Figure 7.1) which suggest that it, in part, functions by interacting with various opposing cofactor complexes, such as N-CoR/SMRT/HDAC- and SRC/HAT-containing complexes. Thus a model for SKIP action may involve its cell-specific interactions with different cofactor complexes which in turn presumably determines whether SKIP act as a positive, or a negative transcriptional coregulator in different cellular and promoter contexts (Figure 7.2). This model was further supported by studies presented in this thesis on the role of SKIP in TGF-β-dependent transcription, which suggest that it plays an additional role in mediating crosstalk between the NHR- and TGF-β signalling pathways.

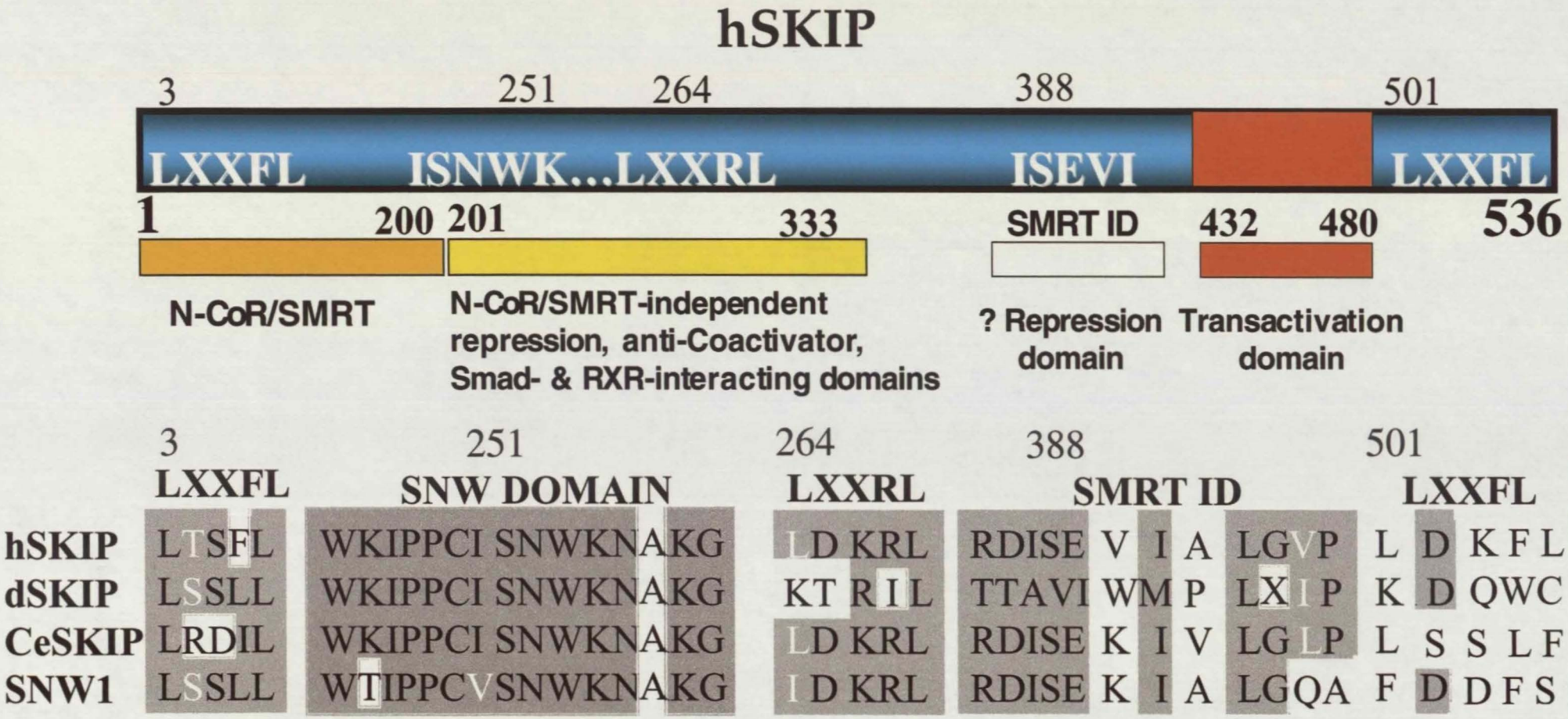

Fig.7.1. Putative functional domains and motifs in hSKIP showing sequence alignment with dSKIP(Bx42), CeSKIP and SNW1.

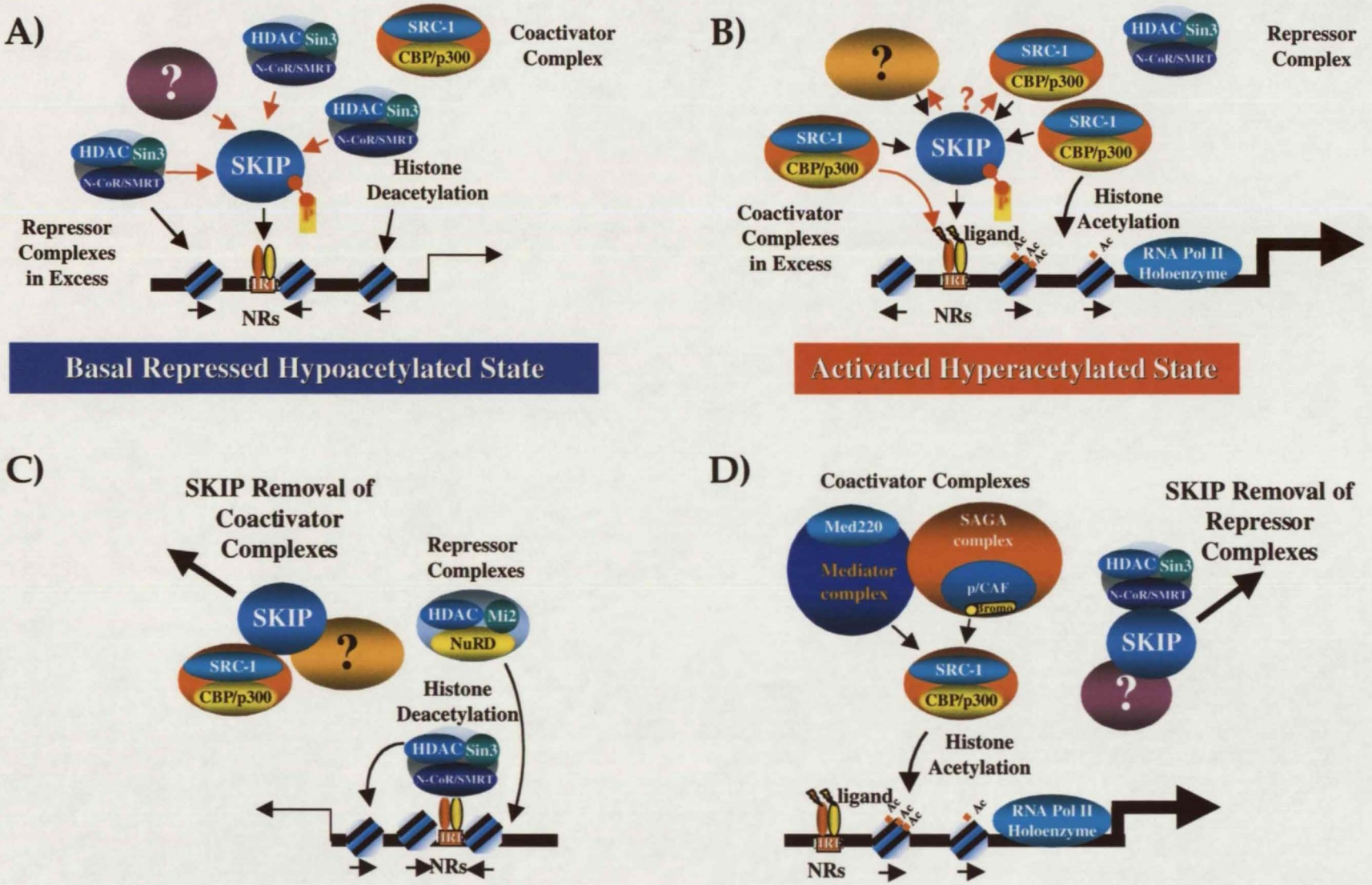

Fig. 7.2 Model for SKIP action in different cellular contexts.

Fig. 7.2 Model for SKIP action in different cellular contexts.

In cells in which corepressors are in excess SKIP preferentially recruits repressor complexes which leads to deacetylation and repression of target gene promoters, whilt B) in cells in which coactivators are in excess, SKIP recuits coaactivator complexes which acetylate and activate target gene promoters. Alternatively, SKIP may sequester either coactivator (C) or corepressor complexes (D) from target gene promoters and lead to gene repression, or derepression, respectively.

Modifications of the N-terminal tails of core histones, including acetylation and phosphorylation, are two fundamental mechanisms involved in modulation of transcription by NHRs and other signalling pathways. As SKIP shares no protein homology with other known HATs or HDACs it does not itself appear to be possess these enzymatic activities intrinsically (Jonine Figueroa and Michael Hayman, personal communication). The present studies nevertheless indicate that SKIP interacts with both these classes of proteins (Zhang *et al.*, 2001a; Zhou *et al.*, 1999). As such, SKIP modulation of the histone modifying actions of these proteins may play a role in its action. The coregulator p300, for instance, acetylates the HAT coactivator SRC-3 to terminate transcription and disrupt its interaction with ER (Chen *et al.*, 1999b), whilst a HAT "masking" protein called INHAT has been suggested to act as an inhibitor of CBP/p300 and p/CAF HAT activity (Seo *et al.*, 2001). Thus, the observation that SKIP acted as an "anti-coactivator" repressing transactivation mediated by the HAT protein SRC-1 may involve these or similar mechanisms. As SKIP also repressed the transactivation associated with TRAP220 or SRC-2, neither of which has which known HAT function, suggests other mechanisms may also be involved in the actions of SKIP. The available data suggest that SKIP may modulate gene transcription through effects on the function of HDACs and/or HAT proteins. Thus SKIP may, like other coregulator proteins such as CBP/p300 or N-CoR, participate in a large protein complex, the function of which is dependent on its various components. To this end, studies identifying other novel SKIP-interacting factors, either through yeast two-hybrid screening or *in vitro* SKIP binding assays, will be two further essential approaches for defining SKIP function.

The studies presented in this thesis suggest that the bifunctional effects of SKIP may, in part, be related to cellular differences in N-CoR expression. Cell-specific expression of N-CoR has been associated with tamoxifen resistance in breast cancer cell models and alteration of N-CoR expression in these and other models have been associated with differential effects on NHR-dependent transcription (Lavinsky *et al.*, 1998; Soderstrom *et al.*, 1997). Thus, it would be of interest using other cellular models of altered N-CoR expression to determine if a similar relationship between N-CoR levels and SKIP repression is also observed. The use of N-CoR knockout fibroblasts and re-expression of N-CoR in such a cell model may provide more insights in these areas of investigation (Jepsen *et al.*, 2000).

The present studies also addressed the question whether the differentiation-state of the cell, or the endogenous levels of other cofactors such as CBP/p300 or HDACs may play a role in modulating SKIP action as either an activator or repressor. SKIP transactivation in the undifferentiated P19 cells was absent or frankly repressive, and SKIP conversion to a modest activator was associated with downregulation of N-CoR expression in differentiated P19 cells. This suggests involement of the HDAC enzymes, but other mechanisms could possibly explain these effects. Expression of other unidentified factors may have been also altered by the differentiation process, including effects on expression of SRCs, p/CAF or other HDACs. As SKIP interacts with a number of factors that play roles in differentiation, such as c-Ski, Smads, Notch and MyoD, examination of various differentiation models, e.g muscle differentiation in C2C12 cells, may be one means to address some of these key questions (Kim *et al.*, 2001; Zhou *et al.*, 2000b).

SKIP has several conserved functional motifs within its protein sequence which may function as coactivator or corepressor motifs similar to those present in SRCs and CBP/p300 (LXXLL-like) and SMRT (CoRNR box) (Figure 7.1). Mutagenesis studies will be required to ascertain whether any of these motifs are required for SKIP coactivator or repressor function. It is intriguing that the LXXFL motif in the SNW "anti-coactivator"domain of SKIP is essentially the same motif found in the

ERα-LBD bound to tamoxifen, that assumes a conformation that mimics but prevents coactivator binding (Shiau *et al.*, 1998). Thus, analysis of this and the other putative LXXLL-like and CoRNR box motifs within SKIP are a high priority for further study.

Examination of the SKIP protein sequence also suggests that SKIP may be a phosphoprotein (Figure 7.3). Thus, one potential mechanism of SKIP modulation and crosstalk with other signaling pathways may involve modulation of its interaction with other cofactors by phosphorylation-dependent mechanisms. This modification may potentially play roles in nuclear cytoplasmic shuttling, as has been shown for the corepressor SMRT; transactivation function, as has been shown for SRC-1 interaction with the ER AF-1 domain; and targeting of activators for degradation by the proteasosme ubiquitin pathways (Chi *et al.*, 2001; Hong and Privalsky, 2000; Tremblay *et al.*, 1999; Weissman, 2001).

The present studies support the conclusion that SKIP is involved in modulation of a number of key signalling pathways. In particular, SKIP interacts with the TGF-β-specific Smad proteins, Smad2 and Smad3 and appears to partially counteract the Smad repressors, c-Ski and related Sno proteins. Like SKIP, c-Ski also interacts with a N-CoR/HDAC repressor complex, but in contrast to SKIP, c-Ski has not been shown to interact with HAT coactivator proteins. As SKIP interacts with c-Ski it is reasonable to hypothesise that SKIP may also modulate c-Ski repression of Mad- and TR-dependent transcription (Nomura *et al.*, 1999). Thus, further studies examining the role *in vivo* of SKIP modulation of c-Ski mediated repression would be of interest. Use of c-Ski knockout fibroblast cells may assist in such studies (Berk *et al.*, 1997).

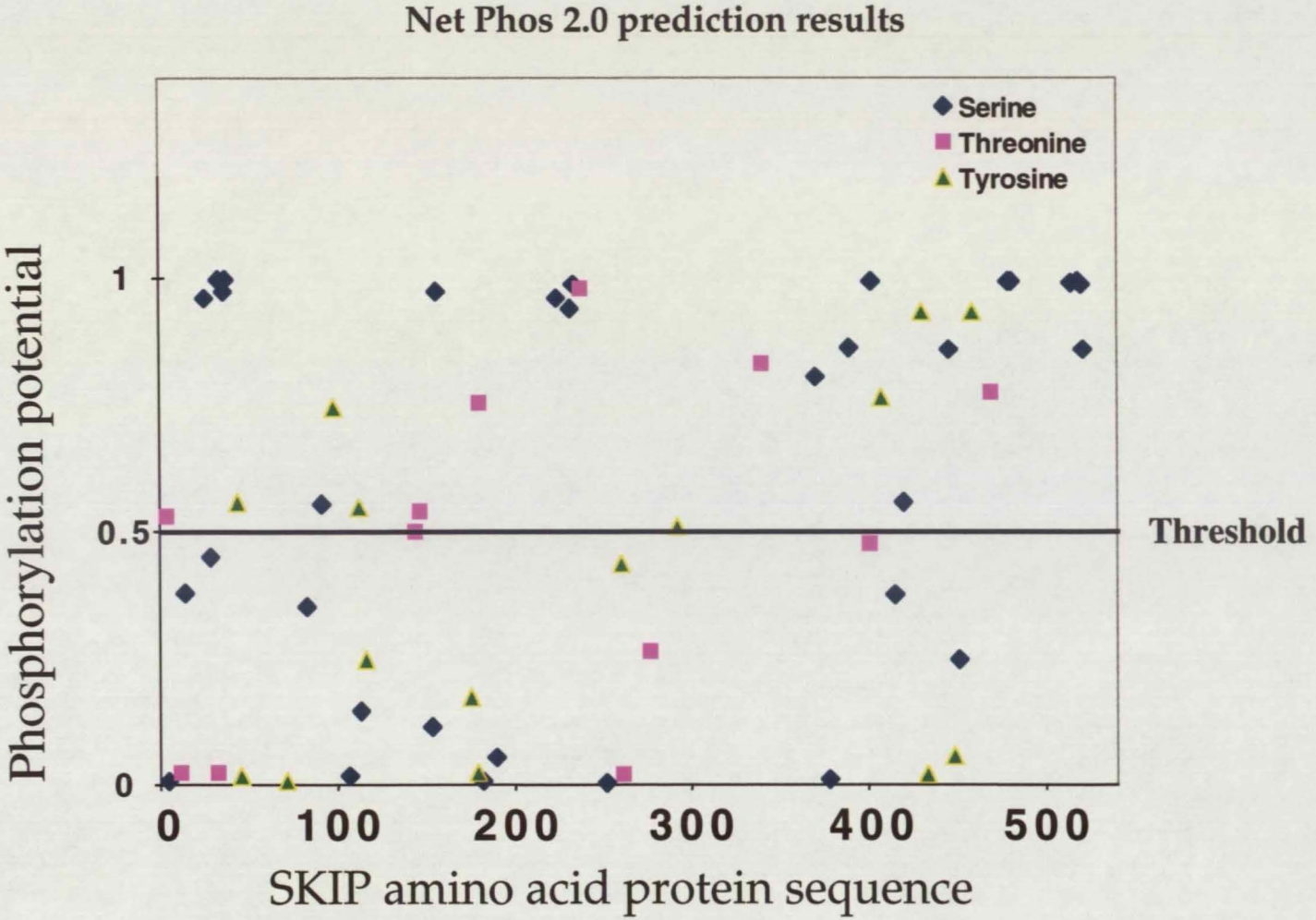

Fig. 7.3. Putative SKIP phosphorylation sites.
Phopshorylation sites as predicted by NetPhos 2.0 programme (http:www.cbs.dtu.uk/srevices/NetPhos) (Kreegipuu *et al.,* 1998). All points above threshold line have greater than 50% chance of being a phosphorylation site based on sequence homology of phosphorylation sites from a database of known phosphoproteins. Those values approaching 1.0 thus have the greatest likelihood of being true phosphorylation sites.

The effects of SKIP on other gene promoters that are TGF-β-responsive and involved in control of cell proliferation such as the p21 or $p15^{INK4b}$ promoter would also be of interest (Claassen and Hann, 2000; Liu *et al.*, 1996a; Liu *et al.*, 1996c; Pardali *et al.*, 2000; Seoane *et al.*, 2001; Staller *et al.*, 2001). In studies presented in this thesis, SKIP appeared to activate the PAI-1 promoter reporter largely though its interaction with Smad3, rather than Smad2. This, however, may be related to this reporter being Smad3-specific. Thus, the effects of SKIP on Smad-2 responsive reporters, such as the goosecoid promoter, (Labbe *et al.*, 1998), in addition to studies with Smad2- and Smad3-null fibroblast cells, may provide further insights into the role of SKIP modulation of TGF-β-signalling via these two distinct Smads (Piek *et al.*, 2001). Furthermore, as c-Ski has been shown to repress BMP signalling, it is possible that SKIP may play an analogous activation role in this pathway, as it does in TGF-β signalling (Leong *et al.*, 2001; Wang *et al.*, 2000).

Recent evidence suggests that gene regulation targeted by NHRs is a dynamic process involving the actions of many cofactors that act either sequentially or in concert and are recycled for repeated rounds of transcriptional initiation (Shang *et al.*, 2000; Stenoien *et al.*, 2001a). Thus, the exact pattern and repertoire of cofactors required for regulation of different promoters, some of which may have tissue-restricted patterns of expression, may differ significantly from promoter to promoter. Recently described methods such as the chromatin immunoprecipitation (ChiP) assay which can identify promoter-specific assembly of cofactors in the context of the chromatin environment is an essential tool in investigating gene regulation *in vivo* (Lee and Kraus, 2001). Other studies imaging SKIP interaction with different NHRs using fluorescent tagged proteins and FRET (fluorescence resonance energy transfer) would also be useful. Thus, the *in vitro* studies presented in this thesis need to be complemented by these and other *in vivo* studies of SKIP function.

Ultimately, understanding of the *in vivo* role for SKIP may require generation of a SKIP transgenic knock-out mouse. However, it would not be surprising, based on the many key developmental pathways that appear to involve SKIP, that such a mouse model may have an embryonic lethal phenotype. Though such a phenotype would prevent study of post-natal function of SKIP, heterozygous mice may be associated with subtle developmental defects, such as occurs with CBP haploinsufficiency in Rubinstein-Taybi syndrome (Murata *et al.*, 2001; Petrij *et al.*, 1995). Nevertheless, examination of the effects of SKIP deficiency in embryonic development may still provide novel insights into its role in development (Jepsen *et al.*, 2000), whilst, the generation of SKIP null fibroblasts would be a valuable *ex vivo* tool for analysis of SKIP action.

Finally, it is useful to consider what role, if any, SKIP could have in human disease? Studies of its modulation of both the TGF-β and NHR-signalling pathways suggest that SKIP is likely to play a role in cell proliferation and/or differentiation. Though the present studies have not specifically addressed these potential roles for SKIP, other groups have shown that downregulation of SKIP expression by an anti-sense approach interferes with muscle differentiation (Zhou *et al.*, 2000b). This latter effect may possibly occur via attenuation of SKIP activation of MyoD-dependent transcription, or modulation of SKIP effects on Notch1c (Kim *et al.*, 2001; Zhou *et al.*, 2000b). Thus, it would be of interest to determine in other cellular models, such as in breast cancer or bone cells, whether changes in SKIP expression may modulate the proliferation and differentiation of these cells in response to various mitogens and hormonal agents, such as oestrogen or vitamin D. Studies investigating the role of SKIP in ERα- and ER-β-dependent transcription would further support a role for SKIP in such models.

In summary, the studies from this thesis have identified the Ski-interacting protein (SKIP) as a transcriptional coregulator for the NHR- and TGF-β signalling pathways. SKIP appears to act as a cell- and receptor-specific transcriptional regulator that interacts with both corepressors and associated HDACs, as well as

interacting with HAT coactivators. Furthermore, SKIP appears to mediate crosstalk between the VDR and TGF-β signalling pathways, and partially counteracts the Smad repressors Ski and Sno to positively modulate TGF-β signalling. These transcriptional effects of SKIP strongly suggest that SKIP may play a role in control of cell proliferation and differentiation. Future studies addressing these potential roles of SKIP action may provide novel insights into the apparent divergent and diverse effects of this transcriptional coregulator, thus providing clues about its possible roles in human development and disease.

REFERENCES

Adachi, M., Takayanagi, R., Tomura, A., Imasaki, K., Kato, S., Goto, K., Yanase, T., Ikuyama, S., Nawata, H., Schapira, M., Raaka, B.M., Samuels, H.H. and Abagyan, R. (2000) Androgen-insensitivity syndrome as a possible coactivator disease. *New England Journal of Medicine*, 343, 856-862.

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T. and Thanos, D. (2000) Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. *Cell*, 103, 667-678.

Ait-Si-Ali, S., Carlisi, D., Ramirez, S., Upegui-Gonzalez, L.C., Duquet, A., Robin, P., Rudkin, B., Harel-Bellan, A. and Trouche, D. (1999) Phosphorylation by p44 MAP Kinase/ERK1 stimulates CBP histone acetyl transferase activity in vitro. *Biochemical & Biophysical Research Communications*, 262, 157-162.

Akiyoshi, S., Inoue, H., Hanai, J., Kusanagi, K., Nemoto, N., Miyazono, K. and M., K. (1999) c-Ski acts as a Trasncriptional Co-repressor in Transforming Growth Factor-B Signalling through interaction with Smads. *The Journal of Biological Chemistry*, 274, 35269-35277.

Alfred, J. (2001) Cracking conditional transgenesis. *Nature Reviews Genetics*, 2.

Alfrey, V.G., Faulkner, R. and Mirsky, A.E. (1964) Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, 51, 786-794.

Alland, L., Muhle, R., Hou, H., Potes, J., Chin, L., Schreiber-Agus, N. and DePinho, R.A. (1997) Role for N-CoR and histone deactylase in Sin3-mediated trasncriptional repression. *Nature*, 387, 49-55.

Altincicek, B., Tenbaum, S.P., Dressel, U., Thormeyer, D., Renkawitz, R. and Baniahmad, A. (2000) Interaction of the corepressor Alien with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita. *Journal of Biological Chemistry*, 275, 7662-7667.

Andrews, N.C. and Faller, D.V. (1991) A rapid microcentrifugation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acis Research*, 19, 2499.

Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Guan, X.Y., Sauter, G., Kallioniemi, O.P., Trent, J.M. and Meltzer, P.S. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*, 277, 965-968.

Arany, Z., Newsome, D., Oldread, E., Livingston, D.M. and Eckner, R. (1995) A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. *Nature*, 374, 81-84.

Arbour, N.C., Ross, T.K., Zierold, C., Prahl, J.M. and DeLuca, H.F. (1998) A highly sensitive method for large-scale measurements of 1,25-dihydroxyvitamin D. *Analytical Biochemistry*, 255, 148-154.

Archer, S.Y. and Hodin, R.A. (1999) Histone acetylation and cancer. *Current Opinion in Genetics & Development*, 9, 171-174.

Arents, G., Burlingame, R.W., Wang, B.C., Love, W.E. and Moudrianakis, E.N. (1991) The nucleosomal core histone octamer at 3.1 A resolution: a tripartite protein assembly and a left-handed superhelix. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 10148-10152.

Atkins, G.B., Hu, X., Guenther, M.G., Rachez, C., Freedman, L.P. and Lazar, M.A. (1999) Coactivators for the orphan nuclear receptor RORalpha. *Molecular Endocrinology*, 13, 1550-1557.

Auger, A.P., Tetel, M.J. and McCarthy, M.M. (2000) Steroid receptor coactivator-1 (SRC-1) mediates the development of sex-specific brain morphology and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 7551-7555.

Ayer, D.E., Lawrence, Q.A. and Eisenman, R.N. (1995) Mad Max Transcriptional Repression Is Mediated By Ternary Complex Formation With Mammalian Homologs of Yeast Repressor Sin3. *Cell*, 80, 767-776.

Bain, G., Ray, W.J., Yao, M. and Gottlieb, D.I. (1994) From embryonal carcinoma cells to neurons - the P19 pathway. *Bioessays*, 16, 343-348.

Baker, A.R., McDonnell, D.P., Hughes, M., Crisp, T.M., Mangelsdorf, D.J., Haussler, M.R., Pike, J.W., Shine, J. and O'Malley, B.W. (1988) Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 3294-3298.

Baniahmad, A., Ha, I., Reinberg, D., Tsai, S., Tsai, M.J. and O'Malley, B.W. (1993) Interaction of human thyroid hormone receptor beta with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 8832-8836.

Bannister, A.J. and Kouzarides, T. (1996) The CBP co-activator is a histone acetyltransferase. *Nature*, 384, 641-643.

Baudino, T.A., Kraichely, D.M., Jefcoat, S.C., Jr., Winchester, S.K., Partridge, N.C. and MacDonald, P.N. (1998) Isolation and characterization of a novel coactivator protein, NCoA-62, involved in vitamin D-mediated transcription. *Journal of Biological Chemistry*, 273, 16434-16441.

Benecke, A., Chambon, P. and Gronemeyer, H. (2000) Synergy between estrogen receptor alpha activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2. *EMBO Reports*, 1, 151-157.

Berk, M., Desai, S.Y., Heyman, H.C. and Colmenares, C. (1997) Mice lacking the ski proto-oncogene have defects in neurulation, craniofacial, patterning, and skeletal muscle development. *Genes & Development*, 11, 2029-2039.

Bevan, C.L., Hoare, S., Claessens, F., Heery, D.M. and Parker, M.G. (1999) The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. *Molecular & Cellular Biology.*, 19, 8383-8392.

Blanco, J.C., Minucci, S., Lu, J., Yang, X.J., Walker, K.K., Chen, H., Evans, R.M., Nakatani, Y. and Ozato, K. (1998) The histone acetylase PCAF is a nuclear receptor coactivator. *Genes & Development*, 12, 1638-1651.

Blanco, J.C., Wang, I.M., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., Jurutka, P.W., Haussler, M.R. and Ozato, K. (1995) Transcription factor TFIIB and the vitamin D

receptor cooperatively activate ligand-dependent transcription. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 1535-1539.

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H. and Moras, D. (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature*, 375, 377-382.

Boyd, J.M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S. and Chinnadurai, G. (1993) A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. *EMBO Journal*, 12, 469-478.

Braselmann, S., Graninger, P. and Busslinger, M. (1993) A selective transcriptional induction system for mammalian cells based on Gal4-estrogen receptor fusion proteins. *Proceedings of the National Academy of Sciences of United States of America*, 90, 1657-1661.

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y. and Allis, C.D. (1996) Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell*, 84, 843-851.

Brzozowski, A.M., Pike, A.C.W., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A. and Carlquist, M. (1997) Molecular Basis of Agonism and Antagonism in the Oestrogen Receptor. *Nature*, 389, 753-758.

Burke, L.J. and Baniahmad, A. (2000) Co-repressors 2000. *FASEB Journal*, 14, 1876-1888.

Burris, T.P., Nawaz, Z., Tsai, M.J. and O'Malley, B.W. (1995) A nuclear hormone receptor-associated protein that inhibits transactivation by the thyroid hormone and retinoic acid receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 9525-9529.

Caira, F., Antonson, P., Pelto-Huikko, M., Treuter, E. and Gustafsson, J.-A. (2000) Cloning and Characterization of RAP250, a Novel Nuclear Receptor Coactivator. *J. Biol. Chem.*, 275, 5308-5317.

Cairns, B.R., Lorch, Y., Li, Y., Zhang, M., Lacomis, L., Erdjument-Bromage, H., Tempst, P., Du, J., Laurent, B. and Kornberg, R.D. (1996) RSC, an essential, abundant chromatin-remodeling complex. *Cell*, 87, 1249-1260.

Carcamo, J., Zentella, A. and Massague, J. (1995) Disruption Of Transforming Growth Factor Beta Signaling By a Mutation That Prevents Transphosphorylation Within the Receptor Complex. *Molecular & Cellular Biology*, 15, 1573-1581.

Cavailles, V., Dauvois, S., Danielian, P.S. and Parker, M.G. (1994) Interaction of proteins with transcriptionally active estrogen receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 10009-10013.

Cavailles, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P.J. and Parker, M.G. (1995) Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *Embo Journal*, 14, 3741-3751.

Chakravarti, D., LaMorte, V.J., Nelson, M.C., Nakajima, T., Schulman, I.G., Juguilon, H., Montminy, M. and Evans, R.M. (1996) Role of CBP/P300 in nuclear receptor signalling. *Nature*, 383, 99-103.

Chakravarti, D., Ogryzko, V., Kao, H.Y., Nash, A., Chen, H., Nakatani, Y. and Evans, R.M. (1999) A viral mechanism for inhibition of p300 and PCAF acetyltransferase activity. *Cell*, 96, 393-403.

Chang, C.Y., Norris, J.D., Gron, H., Paige, L.A., Hamilton, P.T., Kenan, D.J., Fowlkes, D. and McDonnell, D.P. (1999) Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: Discovery of peptide antagonists of estrogen receptors alpha and beta. *Molecular & Cellular Biology*, 19, 8226-8239.

Chen, D.G., Huang, S.M. and Stallcup, M.R. (2000a) Synergistic, p160 coactivator-dependent enhancement of estrogen receptor function by CARM1 and p300. *Journal of Biological Chemistry*, 275, 40810-40816.

Chen, D.G., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W. and Stallcup, M.R. (1999a) Regulation of transcription by a protein methyltransferase. *Science.*, 284, 2174-2177.

Chen, H., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y. and Evans, R.M. (1997a) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell*, 90, 569-580.

Chen, H., Lin, R.J., Xie, W., Wilpitz, D. and Evans, R.M. (1999b) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell*, 98, 675-686.

Chen, H., Tini, M. and Evans, R.M. (2001) HATS on and beyond chromatin. *Current Opinion in Cell Biology*, 13, 218-224.

Chen, H.W., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y. and Evans, R.M. (1997b) Nuclear Receptor Coactivator Actr Is a Novel Histone Acetyltransferase and Forms a Multimeric Activation Complex With P/Caf and Cbp/P300. *Cell*, 90, 569-580.

Chen, H.W., Lin, R.J., Xie, W., Wilpitz, D. and Evans, R.M. (1999c) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell.*, 98, 675-686.

Chen, J.D. and Evans, R.M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*, 377, 454-457.

Chen, S.L., Dowhan, D.H., Hosking, B.M. and Muscat, G.E.O. (2000b) The steroid receptor coactivator, GRIP-1, is necessary for MEF-2C-dependent gene expression and skeletal muscle differentiation. *Genes & Development*, 14, 1209-1228.

Chen, X., Rubock, M.J. and Whitman, M. (1996) A Transcriptional Partner For Mad Proteins in Tgf-Beta Signalling. *Nature*, 383, 691-696.

Cheung, P., Allis, C.D. and Sassone-Corsi, P. (2000a) Signaling to chromatin through histone modifications. *Cell*, 103, 263-271.

Cheung, W.L., Briggs, S.D. and Allis, C.D. (2000b) Acetylation and chromosomal functions. *Current Opinion in Cell Biology*, 12, 326-333.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A. and Deshaies, R.J. (2001) Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes and Development*, 15, 1078-1092.

Chirgin, J.M., Przybyla, A.E., MacDonald, R.J. and W.J., R. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*, 18, 5294-5299.

Claassen, G.F. and Hann, S.R. (2000) A role for transcriptional repression of p21(CIP1) by c-Myc in overcoming transforming growth factor beta-induced cell-cycle arrest. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 9498-9503.

Clark, J., Lu, Y.J., Sidhar, S.K., Parker, C., Gill, S., Smedley, D., Hamoudi, R., Linehan, W.M., Shipley, J. and Cooper, C.S. (1997) Fusion of splicing factor genes PSF and NonO (p54nrb) to the TFE3 gene in papillary renal cell carcinoma. *Oncogene*, 15, 2233-2239.

Clements, A., Rojas, J.R., Trievel, R.C., Wang, L., Berger, S.L. and Marmorstein, R. (1999) Crystal structure of the histone acetyltransferase domain of the human PCAF transcriptional regulator bound to coenzyme A. *EMBO Journal*, 18, 3521-3532.

Cohen, R.N., Wondisford, F.E. and Hollenberg, A.N. (1998) Two separate NCoR (nuclear receptor corepressor) interaction domains mediate corepressor action on thyroid hormone response elements. *Molecular Endocrinology*, 12, 1567-1581.

Colmenares, C. and Stavnezer, E. (1989) The ski oncogene induces muscle differentiation in quail embryo cells. *Cell*, 59, 293-303.

Colmenares, C., Teumer, J.K. and Stavnezer, E. (1991) Transformation-defective v-ski induces MyoD and myogenin expression but not myotube formation. *Molecular & Cellular Biology*, 11, 1167-1170.

Conneely, O.M. and Lydon, J.P. (2000) Progesterone receptors in reproduction: functional impact of the A and B isoforms. *Steroids*, 65, 571-577.

Cosma, M.P., Tanaka, T. and Nasmyth, K. (1999) Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. *Cell*, 97, 299-311.

Couse, J.F. and Korach, K.S. (1999) Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrine Reviews*, 20, 358-417.

Cramer, P., Bushnell, D.A. and Kornberg, R.D. (2001) Structural basis of transcription: RNA polymerase II at 2.8 A resolution. *Science*, 292, 1863-1875.

Crawford, P.A., Dorn, C., Sadovsky, Y. and Milbrandt, J. (1998) Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. *Molecular & Cellular Biology*, 18, 2949-2956.

Cress, W.D. and Seto, E. (2000) Histone deacetylases, transcriptional control, and cancer. *Journal of Cellular Physiology*, 184, 1-16.

Dahl, R., Kieslinger, M., Beug, H. and Hayman, M.J. (1998a) Transformation of hematopoietic cells by the Ski oncoprotein involves repression of retinoic acid receptor signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 11187-11192.

Dahl, R., Wani, B. and Hayman, M.J. (1998b) The Ski oncoprotein interacts with Skip, the human homolog of Drosophila Bx42. *Oncogene*, 16, 1579-1586.

Das, S.K., Taylor, J.A., Korach, K.S., Paria, B. C., Dey, S. K. and Lubahn, D.B. (1997) Estrogenic responses in estrogen receptor-deficient mice reveal a distinct estrogen signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 12786-12791.

Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., Fletterick, R.J. and Yamamoto, K.R. (1998) Structure and specificity of nuclear receptor-coactivator interactions. *Genes & Development*, 12, 3343-3356.

de Caestecker, M.P., Piek, E. and Roberts, A.B. (2000) Role of transforming growth factor-beta signaling in cancer. *Journal of the National Cancer Institute*, 92, 1388-1402.

Deane, R., Schafer, W., Zimmermann, H.P., Mueller, L., Gorlich, D., Prehn, S., Ponstingl, H. and Bischoff, F.R. (1997) Ran-Binding Protein 5 (Ranbp5) Is Related to the Nuclear Transport Factor Importin-Beta But Interacts Differently With Ranbp1. *Molecular & Cellular Biology*, 17, 5087-5096.

Delage-Mourroux, R., Martini, P.G.V., Choi, I., Kraichely, D.M., Hoeksema, J. and Katzenellenbogen, B.S. (2000) Analysis of estrogen receptor interaction with a repressor of estrogen receptor activity (REA) and the regulation of estrogen

receptor transcriptional activity by REA. *Journal of Biological Chemistry*, 275, 35848-35856.

Delaunay, F., Liden, J., Gustafsson, J.A. and Okret, S. (1996) Glucocorticoid receptor lacking the tau 1 transactivation domain is a gene-specific regulator of the wild-type glucocorticoid-receptor activity. *European Journal of Biochemistry*, 242, 839-845.

Della, N.G., Senior, P.V. and Bowtell, D.D. (1993) Isolation and characterisation of murine homologues of the Drosophila seven in absentia gene (sina). *Development*, 117, 1333-1343.

Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K. and Zhou, M.M. (1999) Structure and ligand of a histone acetyltransferase bromodomain. *Nature*, 399, 491-496.

Di Croce, L., Koop, R., Venditti, P., Westphal, H.M., Nightingale, K.P., Corona, D.F., Becker, P.B. and Beato, M. (1999) Two-step synergism between the progesterone receptor and the DNA-binding domain of nuclear factor 1 on MMTV minichromosomes. *Molecular Cell*, 4, 45-54.

Dilworth, F.J., Fromental-Ramain, C., Yamamoto, K. and Chambon, P. (2000) ATP-driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR in vitro. *Molecular Cell*, 6, 1049-1058.

Dressel, U. Bailey P.J., Wang S.C., Downes M., Evans R.M. and Muscat G.E. (2001) A dynamic role for HDAC-7 in MEF2-mediated muscle differentiation. *Journal of Biological Chemistry*, 276, 17007-17013.

Dressel, U., Thormeyer, D., Altincicek, B., Paululat, A., Eggert, M., Schneider, S., Tenbaum, S.P., Renkawitz, R. and Baniahmad, A. (1999) Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. *Molecular & Cellular Biology*, 19, 3383-3394.

Dunphy, E.L., Johnson, T., Auerbach, S.S. and Wang, E.H. (2000) Requirement for TAF(II)250 acetyltransferase activity in cell cycle progression. *Molecular & Cellular Biology*, 20, 1134-1139.

Dwivedi, P.P., Muscat, G.E., Bailey, P.J., Omdahl, J.L. and May, B.K. (1998) Repression of basal transcription by vitamin D receptor: evidence for interaction of unliganded vitamin D receptor with two receptor interaction domains in RIP13delta1. *Journal of Molecular Endocrinology*, 20, 327-335.

Eckner, R., Arany, Z., Ewen, M., Sellers, W. and Livingston, D.M. (1994) The adenovirus E1A-associated 300-kD protein exhibits properties of a transcriptional coactivator and belongs to an evolutionarily conserved family. *Cold Spring Harbor Symposia on Quantitative Biology*, 59, 85-95.

Egea, P.F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P. and Moras, D. (2000) Crystal structure of the human RXRalpha ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. *EMBO Journal*, 19, 2592-2601.

Eisman, J.A. (1994) Vitamin D and cancer: new insight into vitamin D physiology and potential for cancer therapy. *Bone and Mineral Research*, 8, 45-76.

Endoh, H., Maruyama, K., Masuhiro, Y., Kobayashi, Y., Goto, M., Tai, H., Yanagisawa, J., Metzger, D., Hashimoto, S. and Kato, S. (1999) Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. *Molecular & Cellular Biology*, 19, 5363-5372.

Ericsson, J., Usheva, A. and Edwards, P.A. (1999) YY1 is a negative regulator of transcription of three sterol regulatory element-binding protein-responsive genes. *Journal of Biological Chemistry*, 274, 14508-14513.

Evans, R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science*, 240, 889-895.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D. and Chambon, P. (1996) Ligand-activated site-specific recombination in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 10887-10890.

Feng, W., Ribeiro, R.C., Wagner, R.L., Nguyen, H., Apriletti, J.W., Fletterick, R.J., Baxter, J.D., Kushner, P.J. and West, B.L. (1998a) Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science*, 280, 1747-1749.

Feng, X.H., Zhang, Y., Wu, R.Y. and Derynck, R. (1998b) The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. *Genes & Development*, 12, 2153-2163.

Fields, S. (1997) The future is function. *Nature Genetics*, 15, 325-327.

Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature*, 340, 245-246.

Fields, S. and Sternglanz, R. (1994) The two-hybrid system: an assay for protein-protein interactions. *Trends in Genetics*, 10, 286-292.

Fondell, J.D., Ge, H. and Roeder, R.G. (1996) Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 8329-8333.

Fondell, J.D., Roy, A.L. and Roeder, R.G. (1993) Unliganded thyroid hormone receptor inhibits formation of a functional preinitiation complex: implications for active repression. *Genes & Development*, 7, 1400-1410.

Font de Mora, J. and Brown, M. (2000) AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Molecular & Cellular Biology*, 20, 5041-5047.

Freedman, L.P., Anderson, E., Bromleigh, V.C., Cheskis, B., Lemon, B.D., Liu, M., Rachez, C., Rots, N.Y., Sukldan, Z. and Towers, T.L. (1997) Transcriptional targets of the vitamin D3 receptor mediating cell cycle arrest and differentiation. In Norman, A.W., Bouillon, R. and Thomasset, M. (eds.), *10th Workshop on Vitamin D. Chemistry, biology and clinical applications of the steroid hormone.* University of California-Riverside, Strasbourg, France, pp. 188-194.

Freedman, L.P., Luisi, B.F., Korszun, Z.R., Basavappa, R., Sigler, P.B. and Yamamoto, K.R. (1988) The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature*, 334, 543-546.

Fryer, C.J. and Archer, T.K. (1998) Chromatin Remodelling By the Glucocorticoid Receptor Requires the Brg1 Complex. *Nature*, 393, 88-91.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. and Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature*, 389, 251-260.

Luo, K.X., Stroschein, S.L., Wang, W., Chen, D., Martens, E., Zhou, S. and Zhou, Q. (1999) The Ski oncoprotein interacts with the Smad proteins to repress TGF beta signaling. *Genes & Development.*, 13, 2196-2206.

Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery, C., Jr., Shyamala, G., Conneely, O.M. and O'Malley, B.W. (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes & Development*, 9, 2266-2278.

Lyons, R.J., Deane, R., Lynch, D.K., Ye, Z.S.J., Sanderson, G.M., Eyre, H.J., Sutherland, G.R. and Daly, R.J. (2001) Identification of a novel human tankyrase through its interaction with the adaptor protein Grb14. *Journal of Biological Chemistry*, 276, 17172-17180.

Ma, H., Hong, H., Huang, S.M., Irvine, R.A., Webb, P., Kushner, P.J., Coetzee, G.A. and Stallcup, M.R. (1999) Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. *Molecular & Cellular Biology*, 19, 6164-6173.

MacDonald, P.N. (1998) A two-hybrid protein interaction system to identify factors that interact with retinoid and vitamin D receptors. *Methods in Molecular Biology*, 89, 359-375.

MacDonald, P.N., Shermann, D.R., Dowd, D.R., S. C. Jefcoat, J. and DeLisle, R.K. (1995) The vitamin D receptor interacts with general transcription factor IIB. *Journal of Biological Chemistry*, 270, 4748-4752.

Mahajan, M.A. and Samuels, H.H. (2000) A new family of nuclear receptor coregulators that integrate nuclear receptor signaling through CREB-binding protein. *Molecular & Cellular Biology*, 20, 5048-5063.

Mak, H.Y., Hoare, S., Henttu, P.M.A. and Parker, M.G. (1999) Molecular determinants of the estrogen receptor-coactivator interface. *Molecular & Cellular Biology.*, 19, 3895-3903.

Malik, S., Gu, W., Wu, W.Z., Qin, J. and Roeder, R.G. (2000) The USA-derived transcriptional coactivator PC2 is a submodule of TRAP/SMCC and acts synergistically with other PCs. *Molecular Cell*, 5, 753-760.

Mangelsdorf, D.J. and Evans, R.M. (1995) The RXR heterodimers and orphan receptors. *Cell*, 83, 841-850.

Mangelsdorf, D.J., Umesono, K., Kliewer, S.A., Borgmeyer, U., Ong, E.S. and Evans, R.M. (1991) A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell*, 66, 555-561.

Martinez de Arrieta, C., Koibuchi, N. and Chin, W.W. (2000) Coactivator and corepressor gene expression in rat cerebellum during postnatal development and the effect of altered thyroid status. *Endocrinology*, 141, 1693-1698.

Massague, J., Blain, S.W. and Lo, R.S. (2000) TGF beta signaling in growth control, cancer, and heritable disorders. *Cell*, 103, 295-309.

Massague, J. and Wotton, D. (2000) Transcriptional control by the TGF-beta/Smad signaling system. *EMBO Journal*, 19, 1745-1754.

Masuda, N., Yasumo, H., Furusawa, T., Tsukamoto, T., Sadano, H. and Osumi, T. (1998) Nuclear receptor binding factor-1 (NRBF-1), a protein interacting with a wide spectrum of nuclear hormone receptors. *Gene*, 221, 225-233.

Masuyama, H., Brownfield, C.M., St-Arnaud, R. and MacDonald, P.N. (1997) Evidence for ligand-dependent intramolecular folding of the AF-2 domain in vitamin D receptor-activated transcription and coactivator interaction. *Molecular Endocrinology*, 11, 1507-1517.

Masuyama, H. and MacDonald, P.N. (1998) Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF-2 domain of VDR. *Journal of Cellular Biochemistry*, 71, 429-440.

Mathur, M., Tucker, P.W. and Samuels, H.H. (2001) PSF Is a Novel Corepressor That Mediates Its Effect through Sin3A and the DNA Binding Domain of Nuclear Hormone Receptors. *Molecular & Cellular Biology*, 21, 2298-2311.

Maurer, U., Jehan, F., Englert, C., Hubinger, G., Weidmann, E., DeLuca, H.F. and Bergmann, L. (2001) The Wilms' tumor gene product (WT1) modulates the

response to 1,25-dihydroxyvitamin D-3 by induction of the vitamin D receptor. *Journal of Biological Chemistry*, 276, 3727-3732.

McEwan, I.J. (2001) Baker's yeast rises to the challenge: reconstitution of mammalian steroid receptor signalling in S.Cerevisiae. *Trends in Genetics*, 17, 239-243.

McInerney, E.M., Rose, D.W., Flynn, S.E., Westin, S., Mullen, T.M., Krones, A., Inostroza, J., Torchia, J., Nolte, R.T., Assa-Munt, N., Milburn, M.V., Glass, C.K. and Rosenfeld, M.G. (1998) Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. *Genes & Development*, 12, 3357-3368.

McKenna, N.J., Lanz, R.B. and O'Malley, B.W. (1999) Nuclear receptor coregulators: Cellular and molecular biology. *Endocrine Reviews.*, 20, 321-344.

McKinsey, T.A., Zhang, C.L., Lu, J.R. and Olson, E.N. (2000) Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature*, 408, 106-111.

McLean, J.W., Elshourbagy, N.A., Chang, D.J., Mahley, R.W. and Taylor, J.M. (1984) Human apolipoprotein E mRNA, cDNA cloning and nucleotiode sequencing of a new variant. *Journal of Biological Chemistry*, 259, 6498-6504.

McNally, J.G., Muller, W.G., Walker, D., Wolford, R. and Hager, G.L. (2000) The glucocorticoid receptor: Rapid exchange with regulatory sites in living cells. *Science*, 287, 1262-1265.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P. and Mark, M. (1994) Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development*, 120, 2749-2771.

Mengus, G., May, M., Carre, L., Chambon, P. and Davidson, I. (1997) Human TAF(II)135 potentiates transcriptional activation by the AF-2s of the retinoic acid, vitamin D3, and thyroid hormone receptors in mammalian cells. *Genes & Development*, 11, 1381-1395.

Mengus, G., May, M., Jacq, X., Staub, A., Tora, L., Chambon, P. and Davidson, I. (1995) Cloning and characterization of hTAFII18, hTAFII20 and hTAFII28: three subunits of the human transcription factor TFIID. *Embo Journal*, 14, 1520-1531.

Merika, M. and Thanos, D. (2001) Enhanceosomes. *Current Opinion in Genetics & Development*, 11, 205-208.

Meyer, M.E., Gronemeyer, H., Turcotte, B., Bocquel, M.T., Tasset, D. and Chambon, P. (1989) Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell*, 57, 433-442.

Minucci, S., Horn, V., Bhattacharyya, N., Russanova, V., Ogryzko, V.V., Gabriele, L., Howard, B.H. and Ozato, K. (1997) A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 11295-11300.

Minucci, S., Maccarana, M., Cioce, M., De Luca, P., Gelmetti, V., Segalla, S., Di Croce, L., Giavara, S., Matteucci, C., Gobbi, A., Bianchini, A., Colombo, E., Schiavoni, I., Badaracco, G., Hu, X., Lazar, M.A., Landsberger, N., Nervi, C. and Pelicci, P.G. (2000) Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. *Molecular Cell*, 5, 811-820.

Minucci, S. and Pelicci, P.G. (1999) Retinoid receptors in health and disease: co-regulators and the chromatin connection. *Seminars in Cell & Developmental Biology*, 10, 215-225.

Misiti, S., Koibuchi, N., Bei, M., Farsetti, A. and Chin, W.W. (1999) Expression of steroid receptor coactivator-1 mRNA in the developing mouse embryo: a possible role in olfactory epithelium development. *Endocrinology*, 140, 1957-1960.

Misiti, S., Schomburg, L., Yen, P.M. and Chin, W.W. (1998) Expression and hormonal regulation of coactivator and corepressor genes. *Endocrinology*, 139, 2493-2500.

Miyamoto, H., Yeh, S., Wilding, G. and Chang, C. (1998) Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 7379-7384.

Mizuguchi, G., Vassilev, A., Tsukiyama, T., Nakatani, Y. and Wu, C. (2001) ATP-dependent nuclesome remodeling and histone hyperacteylation synergistically facilitate transcription of chromatin. *Journal of Biological Chemistry*, 276, 14773-14783.

Monden, T., Kishi, M., Hosoya, T., Satoh, T., Wondisford, F.E., Hollenberg, A.N., Yamada, M. and Mori, M. (1999) p120 acts as a specific coactivator for 9-cis-retinoic acid receptor (RXR) on peroxisome proliferator-activated receptor-gamma/RXR heterodimers. *Molecular Endocrinology*, 13, 1695-1703.

Monden, T., Wondisford, F.E. and Hollenberg, A.N. (1997) Isolation and characterization of a novel ligand-dependent thyroid hormone receptor-coactivating protein. *Journal of Biological Chemistry*, 272, 29834-29841.

Montano, M.M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P. and Katzenellenbogen, B.S. (1999) An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 6947-6952.

Muchardt, C. and Yaniv, M. (1993) A Human Homologue of Saccharomyces-Cerevisiae Snf2/Swi2 and Drosophila-Brm Genes Potentiates Transcriptional Activation By the Glucocorticoid Receptor. *EMBO Journal*, 12, 4279-4290.

Murata, T., Kurokawa, R., Krones, A., Tatsumi, K., Ishii, M., Taki, T., Masuno, M., Ohashi, H., Yanagisawa, M., Rosenfeld, M.G., Glass, C.K. and Hayashi, Y. (2001) Defect of histone aceryltransferase activity of the nuclear transcriptional coactivator CBP in Rubinstein-Taybi sydnrome. *Human Molecular Genetics*, 10, 1071-1076.

Muscat, G.E.O., Burke, L.J. and Downes, M. (1998) The Corepressor N-Cor and Its Wariants Rip13a and Rip13-Delta-1 Directly Interact With the Basal Transcription Factors Tfiib, Taf(Ii)32 and Taf(Ii)7o. *Nucleic Acids Research*, 26, 2899-2907.

Myers, L.C. and Kornberg, R.D. (2000) Mediator of transcriptional regulation. *Annual Review of Biochemistry*, 69, 729-749.

Na, S.Y., Lee, S.K., Han, S.J., Choi, H.S., Im, S.Y. and Lee, J.W. (1998) Steroid receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor kappaB-mediated transactivations. *Journal of Biological Chemistry*, 273, 10831-10834.

Naar, A.M., Beaurang, P.A., Zhou, S., Abraham, S., Solomon, W. and Tjian, R. (1999) Composite co-activator ARC mediates chromatin-directed transcriptional activation. *Nature*, 398, 828-832.

Nagy, L., Kao, H.Y., Love, J.D., Li, C., Banayo, E., Gooch, J.T., Krishna, V., Chatterjee, K., Evans, R.M. and Schwabe, J.W. (1999) Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes & Development*, 13, 3209-3216.

Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C.H., Miyazono, K. and ten Dijke, P. (1997) TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO Journal*, 16, 5353-5362.

Nawaz, Z., Lonard, D.H., Smith, C.L., Lev-Lehman, E., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1999) The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Molecular & Cellular Biology*, 19, 1182-1189.

Nolte, R.T., Wisely, G.B., Westin, S., Cobb, J.E., Lambert, M.H., Kurokawa, R., Rosenfeld, M.G., Willson, T.M., Glass, C.K. and Milburn, M.V. (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature*, 395, 137-143.

Nomura, T., Khan, M.M., Kaul, S.C., Dong, H.D., Wadhwa, R., Colmenares, C., Kohno, I. and Ishii, S. (1999) Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. *Genes & Development*, 13, 412-423.

Nuclear Receptor Subcommitee. (1999) A unified nonmenclature system for the nuclear receptor superfamily. *Cell*, 97.

O'Brien, T. and Tjian, R. (1998) Functional analysis of the human TAFII250 N-terminal kinase domain. *Molecular Cell*, 1, 905-911.

Ogawa, S., Chester, A.E., Hewitt, S.C., Walker, V.R., Gustafsson, J.A., Smithies, O., Korach, K.S. and Pfaff, D.W. (2000) Abolition of male sexual behaviors in mice lacking estrogen receptors alpha and beta (alpha beta ERKO). *Proceedings of the National Academy of Sciences of the United States of America*, 97, 14737-14741.

Ogawa, S., Lubahn, D.B., Korach, K.S. and Pfaff, D.W. (1997) Behavioral effects of estrogen receptor gene disruption in male mice. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 1476-1481.

Ogryzko, V.V., Kotani, T., Zhang, X., Schiltz, R.L., Howard, T., Yang, X.J., Howard, B.H., Qin, J. and Nakatani, Y. (1998) Histone-like TAFs within the p/CAF histone acetylase complex. *Cell*, 94, 35-44.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H. and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell*, 87, 953-959.

Onate, S.A., Boonyaratanakornkit, V., Spencer, T.E., Tsai, S.Y., Tsai, M.J., Edwards, D.P. and Omalley, B.W. (1998) The Steroid Receptor Coactivator-1 Contains Multiple Receptor Interacting and Activation Domains That Cooperatively Enhance the Activation Function 1 (Af1) and Af2 Domains Of Steroid Receptors. *Journal of Biological Chemistry*, 273, 12101-12108.

Onate, S.A., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science*, 270, 1354-1357.

Ordentlich, P., Downes, M., Xie, W., Genin, A., Spinner, N.B. and Evans, R.M. (1999) Unique forms of human and mouse nuclear receptor corepressor SMRT. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 2639-2644.

Owen-Hughes, T., Utley, R.T., Steger, D.J., West, J.M., John, S., Cote, J., Havas, K.M. and Workman, J.L. (1999) Analysis of nucleosome disruption by ATP-driven chromatin remodeling complexes. *Methods in Molecular Biology*, 119, 319-331.

Pardali, K., Kurisaki, A., Moren, A., ten Dijke, P., Kardassis, D. and Moustakas, A. (2000) Role of smad proteins and transcription factor Sp1 in p21(Wafl/Cip1)

regulation by transforming growth factor-beta. *Journal of Biological Chemistry*, 275, 29244-29256.

Park, E.J., Schroen, D.J., Yang, M., Li, H., Li, L. and Chen, J.D. (1999) SMRTe, a silencing mediator for retinoid and thyroid hormone receptors-extended isoform that is more related to the nuclear receptor corepressor. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 3519-3524.

Perissi, V., Staszewski, L.M., McInerney, E.M., Kurokawa, R., Krones, A., Rose, D.W., Lambert, M.H., Milburn, M.V., Glass, C.K. and Rosenfeld, M.G. (1999) Molecular determinants of nuclear receptor-corepressor interaction. *Genes & Development*, 13, 3198-3208.

Petrij, F., Giles, R.H., Dauwerse, H.G., Saris, J.J., Hennekam, R.C., Masuno, M., Tommerup, N., van Ommen, G.J., Goodman, R.H., Peters, D.J. and et, a. (1995) Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature*, 376, 348-351.

Petty, K.J., Krimkevich, Y.I. and Thomas, D. (1996) A TATA binding protein-associated factor functions as a coactivator for thyroid hormone receptors. *Molecular Endocrinology*, 10, 1632-1645.

Piek, E., Ju, W., Heyer, J., Escalante-Alcade, D., Stewart, C.L., weinstein, M., Deng, C., Kucherlapati, R., Bottinger, E.P. and Roberts, A.B. (2001) Functional characterisation of TGF-b signaling in Smad2- and Smad3-deficient fibroblasts. *Journal of Biological Chemistry*, 276, 19945-19953.

Pollard, K.J. and Peterson, C.L. (1998) Chromatin Remodeling - a Marriage Between Two Families. *Bioessays*, 20, 771-780.

Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T. and Carlberg, C. (2000) VDR-Alien: a novel, DNA-selective vitamin D(3) receptor-corepressor partnership. *FASEB Journal*, 14, 1455-1463.

Prathapam, T., Kuhne, C., Hayman, M. and Banks, L. (2001) Ski interacts with the evolutionarily conserved SNW domain of SKIP. *Nucleic Acids Research*, 29, 3469-3476.

Puigserver, P., Adelmant, G., Wu, Z., Fan, M., Xu, J., O'Malley, B. and Spiegelman, B.M. (1999) Activation of PPARgamma coactivator-1 through transcription factor docking. *Science*, 286, 1368-1371.

Qi, C., Zhu, Y., Pan, J., Yeldandi, A.V., Rao, M.S., Maeda, N., Subbarao, V., Pulikuri, S., Hashimoto, T. and Reddy, J.K. (1999) Mouse steroid receptor coactivator-1 is not essential for peroxisome proliferator-activated receptor alpha-regulated gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 1585-1590.

Rachez, C. and Freedman, L.P. (2001) Mediator complexes and transcription. *Current Opinion in Cell Biology*, 13, 274-280.

Rachez, C., Gamble, M., Chang, C.P., Atkins, G.B., Lazar, M.A. and Freedman, L.P. (2000) The DRIP complex and SRC-1/p160 coactivators share similar nuclear receptor binding determinants but constitute functionally distinct complexes. *Molecular & Cellular Biology*, 20, 2718-2726.

Rachez, C., Lemon, B.D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A.M., Erdjument-Bromage, H., Tempst, P. and Freedman, L.P. (1999) Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature*, 398, 824-828.

Rachez, C., Sautiere, P., Formstecher, P. and Lefebvre, P. (1996) Identification of amino acids critical for the DNA binding and dimerisation properties of the human retinoic acid receptor α. *J. Biol. Chem.*, 271, 17996-18006.

Rachez, C., Suldan, Z., Ward, J., Chang, C.B., Burakov, D., Erdjument-Bromage, H., Tempst, P. and Freedman, L.P. (1998) A novel protein complex that interacts with the vitamin D3 receptor in a ligand-dependent manner enhances VDR transactivation in a cell-free system. *Genes & Development*, 12, 1787-1800.

Reinke, H., Gregory, P.D. and Horz, W. (2001) A transient histone hyperacetylation signal marks nucelsomes for remodelling at the PHO8 promoter in vivo. *Molecular Cell*, 7, 529-538.

Renaud, J.P., Harris, J.M., Downes, M., Burke, L.J. and Muscat, G.E.O. (2000) Structure-function analysis of the Rev-erbA and RVR ligand-binding domains

reveals a large hydrophobic surface that mediates corepressor binding and a ligand cavity occupied by side chains. *Molecular Endocrinology*, 14, 700-717.

Renaud, J.P. and Moras, D. (2000) Structural studies on nuclear receptors. *Cellular & Molecular Life Sciences*, 57, 1748-1769.

Renaud, J.P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H. and Moras, D. (1995) Crystal structure of the RAR-gamma ligand-binding domain bound to all-trans retinoic acid. *Nature*, 378, 681-689.

Rochel, N., Wurtz, J.M., Mitschler, A., Klaholz, B. and Moras, D. (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Molecular Cell*, 5, 173-179.

Ross, S.A., McCaffery, P.J., Drager, U.C. and De Luca, L.M. (2000) Retinoids in embryonal development. *Physiological Reviews*, 80, 1021-1054.

Rowan, B.G., Garrison, N., Weigel, N.L. and O'Malley, B.W. (2000a) 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. *Molecular & Cellular Biology*, 20, 8720-8730.

Rowan, B.G., Weigel, N.L. and O'Malley, B.W. (2000b) Phosphorylation of steroid receptor coactivator-1 - Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. *Journal of Biological Chemistry*, 275, 4475-4483.

Rubino, D., Driggers, P., Arbit, D., Kemp, L., Miller, B., Coso, O., Pagliai, K., Gray, K., Gutkind, S. and Segars, J. (1998) Characterization of Brx, a novel Dbl family member that modulates estrogen receptor action. *Oncogene*, 16, 2513-2526.

Ryu, S., Zhou, S., Ladurner, A.G. and Tjian, R. (1999) The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1. *Nature*, 397, 446-450.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual, second edition*. Cold Spring Har. Lab. Press, Plainview.

Sande, S. and Privalsky, M.L. (1996) Identification of TRACs (T3 receptor-associating cofactors), a family of cofactors that associate with, and modulate the activity of, nuclear hormone receptors. *Molecular Endocrinology*, 10, 813-825.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, 74, 5463-5467.

Sasaki, S., Lesoon-Wood, L.A., Dey, A., Kuwata, T., Weintraub, B.D., Humphrey, G., Yang, W.M., Seto, E., Yen, P.M., Howard, B.H. and Ozato, K. (1999) Ligand-induced recruitment of a histone deacetylase in the negative-feedback regulation of the thyrotropin beta gene. *EMBO Journal.*, 18, 5389-5398.

Schule, R., Muller, M., Kaltschmidt, C. and Renkawitz, R. (1988) Many transcription factors interact synergistically with steroid receptors. *Science*, 242, 1418-1420.

Schulman, I.G., Chakravarti, D., Juguilon, H., Romo, A. and Evans, R.M. (1995) Interactions between the retinoid X receptor and a conserved region of the TATA-binding protein mediate hormone-dependent transactivation. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 8288-8292.

Schulman, I.G., Juguilon, H. and Evans, R.M. (1996) Activation and repression by nuclear hormone receptors: hormone modulates an equilibrium between active and repressive states. *Molecular & Cellular Biology*, 16, 3807-3813.

Seo, S.B., McNamara, P., Heo, S., Turner, A., Lane, W.S. and Chakravarti, D. (2001) Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell*, 104, 119-130.

Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M. and Massague, J. (2001) TGFB influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nature cell Biology*, 3, 400-408.

Seol, W., Choi, H.S. and Moore, D.D. (1995) Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. *Molecular Endocrinology*, 9, 72-85.

Serebriiskii, I., Khasak, V. and Golemis, E.A. (1999) A two-hybrid dual bait system to discriminate specificity of protein interactions. *The Journal of Biologcal Chemistry*, 274, 17080-17087.

Shang, Y.F., Hu, X., DiRenzo, J., Lazar, M.A. and Brown, M. (2000) Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell*, 103, 843-852.

Shao, G., Heyman, R.A. and Schulman, I.G. (2000a) Three amino acids specify coactivator choice by retinoid X receptors. *Molecular Endocrinology*, 14, 1198-1209.

Shao, W.L., Rosenauer, A., Mann, K., Chang, C.P.B., Rachez, C., Freedman, L.P. and Miller, W.H. (2000b) Ligand-inducible interaction of the DRTP/TRAP coactivator complex with retinoid receptors in retinoic acid-sensitive and -resistant acute promyelocytic leukemia cells. *Blood*, 96, 2233-2239.

Sharma, D. and Fondell, J.D. (2001) Temporal formation of distinct thyroid hormone receptor coactivator complexes in HeLa cells. *Molecular Endocrinology*, 14, 2001-2009.

Sheppard, H.M., Harries, J.C., Hussain, S., Bevan, C. and Heery, D.M. (2001) Analysis of the steroid receptor coactivator 1 (SRC1)-CREB binding protein interaction interface and its importance for the function of SRC1. *Molecular & Cellular Biology*, 21, 39-50.

Shi, X., Yang, X., Chen, D., Chang, Z. and Cao, X. (1999) Smad1 interacts with homeobox DNA-binding proteins in bone morphogenetic protein signaling. *Journal of Biological Chemistry*, 274, 13711-13717.

Shi, Y., Downres, M., Xie, W., Kao, H.Y., Ordentlich, P., Tsai, C.C., Hon, M. and Evans, R.M. (2001) Sharp, an inducible cofactor that integrates nuclear receptor repression and activation. *Gene and Development*, 5, 1140-1151.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A. and Greene, G.L. (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*, 95, 927-937.

Shih, H.M., Goldman, P.S., DeMaggio, A.J., Hollenberg, S.M., Goodman, R.H. and Hoekstra, M.F. (1996) A positive genetic selection for disrupting protein-protein

interactions: identification of CREB mutations that prevent association with the coactivator CBP. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 13896-13901.

Smith, C.L., Nawaz, Z. and O'Malley, B.W. (1997) Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Molecular Endocrinology*, 11, 657-666.

Soderstrom, M., Vo, A., Heinzel, T., Lavinsky, R.M., Yang, W.M., Seto, E., Peterson, D.A., Rosenfeld, M.G. and Glass, C.K. (1997) Differential effects of nuclear receptor corepressor (N-CoR) expression levels on retinoic acid receptor-mediated repression support the existence of dynamically regulated corepressor complexes. *Molecular Endocrinology*, 11, 682-692.

Song, C.Z., Tian, X. and Gelehrter, T.D. (1999) Glucocorticoid receptor inhibits transforming growth factor-beta signaling by directly targeting the transcriptional activation function of Smad3. *Proceedings of the National Academy of Sciences of the United States of America.*, 96, 11776-11781.

Soutoglou, E. Viollet, B., Vaxillaire, M. Yaniv, M. Pontoglio, M. Talianidis I. (2001) Transcription factor-dependent regulation of CBP and p/CAF histone acetyltransferase activity. *EMBO J.*

Soutoglou, E., Katrakili, N. and Talianidis, I. (2000) Acetylation regulates transcription factor activity at multiple levels. *Molecular Cell*, 5, 745-751.

Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J., Mizzen, C.A., McKenna, N.J., Onate, S.A., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1997) Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature*, 389, 194-198.

Staller, P., Puekert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massague, J., Hanel, F. and Eilers, M. (2001) Repression of p15INK4b expression by Myc through association with Miz-1. *Nature Cell Biology*, 3, 392-399.

Stenoien, D.L., Nye, A.C., Mancini, M.G., Patel, K., Mancini, M.G., Dutertre, M., O'Malley, B.W., Smith, C.L., Belmont, A.S. and Mancini, M.A. (2001a) Ligand-mediated assembly and real-time cellular dymanics of estrogen receptor-alpa-coactivator complexes in living cells. *Molecular and Cellular Biology*, 21, 4404-4412.

Stenoien, D.L., Patel, K., Mancini, M.G., Dutertre, M., Smith, C.L., O'Malley, B.W. and Mancini, M.A. (2001b) FRAP reveals that mobility of oestrogen receptor-alpha is ligand- and proteasome-dependent. *Nature Cell Biology*, 3, 15-23.

Stroschein, S.L., Wang, W., Zhou, S.L., Zhou, Q. and Luo, K.X. (1999) Negative feedback regulation of TGF-beta signaling by the SnoN oncoprotein. *Science.*, 286, 771-774.

Struhl, K. (1998) Histone acetylation and transcriptional regulatory mechanisms. *Genes & Development*, 12, 599-606.

Struhl, K., Kadosh, D., Keaveney, M., Kuras, L. and Moqtaderi, Z. (1998) Activation and repression mechanisms in yeast. *Cold Spring Harbor Symposia on Quantitative Biology*, 63, 413-421.

Suave, F., McBroom, L., Gallant, J., Moraitis, A., Labrie, F. and Giguere, V. (2001) CIA, a novel estrogen receptor coactivator with a bifunctional nuclear receptor interacting determinant. *Molecular & Cellular Biology*, 21, 343-353.

Subramaniam, N., Leong, G.M., Flanagan, J., Cock, T., Eisman, J.A. and Kouzmenko, A.P. (2001) Cross-talk between 1.25-dihydroxyvitamin D and transforming growth factor-beta signalling requires binding of VDR and Smad3 to their cognate DNA recognition elements. *Journal of Biological Chemistry*, 276, 15741-15746.

Subramaniam, N., Treuter, E. and Okret, S. (1999) Receptor interacting protein RIP140 inhibits both positive and negative gene regulation by glucocorticoids. *Journal of Biological Chemistry*, 274, 18121-18127.

Sucov, H.M., Dyson, E., Gumeringer, C.L., Price, J., Chien, K.R. and Evans, R.M. (1994) RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes & Development*, 8, 1007-1018.

Sucov, H.M., Izpisua-Belmonte, J.C., Ganan, Y. and Evans, R.M. (1995) Mouse embryos lacking RXR alpha are resistant to retinoic-acid-induced limb defects. *Development*, 121, 3997-4003.

Suen, C.S., Berrodin, T.J., Mastroeni, R., Cheskis, B.J., Lyttle, C.R. and Frail, D.E. (1998) A transcriptional coactivator, steroid receptor coactivator-3, selectively

augments steroid receptor transcriptional activity. *Journal of Biological Chemistry*, 273, 27645-27653.

Sun, X., Zhang, Y., Cho, H., Rickert, P., Lees, E., Lane, W. and Reinberg, D. (1998) NAT, a human complex containing Srb polypeptides that functions as a negative regulator of activated transcription. *Molecular Cell*, 2, 213-222.

Sun, Y., Liu, X.D., Eaton, E.N., Lane, W.S., Lodish, H.F. and Weinberg, R.A. (1999a) Interaction of the Ski oncoprotein with Smad3 regulates TGF-beta signaling. *Molecular Cell.*, 4, 499-509.

Sun, Y., Liu, X.D., Ng-Eaton, E., Lodish, H.F. and Weinberg, R.A. (1999b) SnoN and Ski protooncoproteins ave rapidly degraded in response to transforming growth factor beta signaling. *Proceedings of the National Academy of Sciences of the United States of America.*, 96, 12442-12447.

Suto, R.K., Clarkson, M.J., Tremethick, D.J. and Luger, K. (2000) Crystal structure of a nucleosome core particle containing the variant histone H2A.Z. *Nature Structural Biology*, 7, 1121-1124.

Sutrave, P., Kelly, A.M. and Hughes, S.H. (1990) Ski can cause selective growth of skeletal muscle in transgenic mice. *Genes & Development*, 4, 1462-1472.

Tagami, T., Lutz, W.H., Kumar, R. and Jameson, J.L. (1998) The interaction of the vitamin D receptor with nuclear receptor corepressors and coactivators. *Biochemical & Biophysical Research Communications*, 253, 358-363.

Tagami, T., Madison, L.D., Nagaya, T. and Jameson, J.L. (1997) Nuclear receptor corepressors activate rather than suppress basal transcription of genes that are negatively regulated by thyroid hormone. *Molecular & Cellular Biology*, 17, 2642-2648.

Tagami, T., Park, Y. and Jameson, J.L. (1999) Mechanisms that mediate negative regulation of the thyroid-stimulating hormone alpha gene by the thyroid hormone receptor. *Journal of Biological Chemistry*, 274, 22345-22353.

Takeshita, A., Cardona, G.R., Koibuchi, N., Suen, C.S. and Chin, W.W. (1997) TRAM-1, A novel 160-kDa thyroid hormone receptor activator molecule, exhibits

distinct properties from steroid receptor coactivator-1. *Journal of Biological Chemistry*, 272, 27629-27634.

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992) brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell*, 68, 561-572.

Tansey, W.P. (2001) Transcriptional activation: risky business. *Genes and Development*, 15, 1045-1050.

Tcherepanova, I., Puigserver, P., Norris, J.D., Spiegelman, B.M. and McDonnell, D.P. (2000) Modulation of estrogen receptor-alpha transcriptional activity by the coactivator PGC-1. *Journal of Biological Chemistry*, 275, 16302-16308.

Thomas, G.P., Bourne, A., Eisman, J.A. and Gardiner, E.M. (2000) Species-divergent regulation of human and mouse osteocalcin genes by calcitropic hormones. *Experimental Cell Research*, 258, 395-402.

Tocchini-Valentini, G., Rochel, N., Wurtz, J.M., Mitschler, A. and Moras, D. (2001) Crystal structures of the vitamin D receptor complexed to superagonist 20-epi ligands. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 5491-5496.

Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K. and Rosenfeld, M.G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature*, 387, 677-684.

Tremblay, A., Tremblay, G.B., Labrie, F. and Giguere, V. (1999) Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. *Molecular Cell*, 3, 513-519.

Treuter, E., Albrektsen, T., Johansson, L., Leers, J. and Gustaffson, J.A. (1998) A regualtory role for RIP140 in nuclear receptor activation. *Molecular Endocrinology*, 12, 864-881.

Treuter, E., Johansson, L., Thomsen, J.S., Warnmark, A., Leers, J., Pelto-Huikko, M., Sjoberg, M., Wright, A.P.H., Spyrou, G. and Gustafsson, J.A. (1999) Competition between thyroid hormone receptor-associated protein (TRAP) 220

and transcriptional intermediary factor (TIF) 2 for binding to nuclear receptors - Implications for the recruitment of trap and P160 coactivator complexes. *Journal of Biological Chemistry*, 274, 6667-6677.

Trievel, R.C., Rojas, J.R., Sterner, D.E., Venkataramani, R.N., Wang, L., Zhou, J., Allis, C.D., Berger, S.L. and Marmorstein, R. (1999) Crystal structure and mechanism of histone acetylation of the yeast GCN5 transcriptional coactivator. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 8931-8936.

Tsai, C.C., Kao, H.Y., Yao, T.P., McKeown, M. and Evans, R.M. (1999) SMRTER, a Drosophila nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. *Molecular Cell*, 4, 175-186.

Umesono, K., Murakami, K.K., Thompson, C.C. and Evans, R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. *Cell*, 65, 1255-1266.

Underhill, C., Qutob, M.S., Yee, S. and Torchia, J. (2000) A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. *J. Biol. Chem.*, 275, 40463-40470.

Uppenberg, J., Svensson, C., Jaki, M., Bertilsson, G., Jendeberg, L. and Berkenstam, A. (1998) Crystal Structure of the Ligand Binding Domain of the Human Nuclear Receptor Ppar-Gamma. *Journal of Biological Chemistry*, 273, 31108-31112.

Varga-Weisz, P.D., Wilm, M., Bonte, E., Dumas, K., Mann, M. and Becker, P.B. (1997) Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II. *Nature*, 388, 598-602.

Vassilev, A., Yamauchi, J., Kotani, T., Prives, C., Avantaggiati, M.L., Qin, J. and Nakatani, Y. (1998) The 400 kDa subunit of the PCAF histone acetylase complex belongs to the ATM superfamily. *Molecular Cell*, 2, 869-875.

Vidal, M., Brachmann, R.K., Fattaey, A., Harlow, E. and Boeke, J.D. (1996) Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 10315-10320.

Vo, N. and Goodman, R.H. (2001) CBP and p300 in transcriptional regulation. *Journal of Biological Chemistry*, 276, 13505-13508.

Voegel, J.J., Heine, M.J., Tini, M., Vivat, V., Chambon, P. and Gronemeyer, H. (1998) The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. *EMBO Journal*, 17, 507-519.

Voegel, J.J., Heine, M.J.S., Zechel, C., Chambon, P. and Gronemeyer, H. (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.*, 15, 3667-3675.

Wagner, R.L., Apriletti, J.W., McGrath, M.E., West, B.L., Baxter, J.D. and Fletterick, R.J. (1995) A structural role for hormone in the thyroid hormone receptor. *Nature*, 378, 690-697.

Wagner, R.L., Huber, B.R., Shiau, A.K., Kelly, A., Cunha Lima, S.T., Scanlan, T.S., Apriletti, J.W., Baxter, J.D., Wset, B.L. and Fletterick, R.J. (2001) Hormone selctivity in thyroid hormone receptors. *Molecular Endocrinology*, 25, 398-410.

Wahlstrom, G.M., Vennstrom, B. and Bolin, M.B. (1999) The adenovirus E1A protein is a potent coactivator for thyroid hormone receptors. *Molecular Endocrinology*, 13, 1119-1129.

Waltzer, L. and Bienz, M. (1998) Drosophila Cbp Represses the Transcription Factor Tcf to Antagonize Wingless Signalling. *Nature*, 395, 521-525.

Wan, Y.J.Y., An, D.S., Cai, Y., Repa, J.J., Chen, T.H.P., Flores, M., Postic, C., Magnuson, M.A., Chen, J., Chien, K.R., French, S., Mangelsdorf, D.J. and Sucov, H.M. (2000) Hepatocyte-specific mutation establishes retinoid X receptor alpha as a heterodimeric integrator of multiple physiological processes in the liver. *Molecular & Cellular Biology*, 20, 4436-4444.

Wang, J.C., Stafford, J.M. and Granner, D.K. (1998) Src-1 and Grip1 Coactivate Transcription With Hepatocyte Nuclear Factor 4. *Journal of Biological Chemistry*, 273, 30847-30850.

Wang, L., Andersson, S., Warner, M. and Gustafsson, J.A. (2001) Morphological abnormalities in the brains of estrogen receptor beta knockout mice. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 2792-2796.

Wang, W., Mariani, F.V., Harland, R.M. and Luo, K.X. (2000) Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 14394-14399.

Watanabe, M., Yanagisawa, J., Kitagawa, H., Takeyama, K., Ogawa, S., Arao, Y., Suzawa, M., Kobayashi, Y., Yano, T., Yoshikawa, H., Masuhiro, Y. and Kato, S. (2001) A subfamily of RNA-binding DEAD-box proteins acts as an estrogen receptor coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA. *The EMBO Journal*, 20, 1341-1352,.

Webb, P., Anderson, C.M., Valentine, C., Nguyen, P., Marimuthu, A., West, B.L., Baxter, J.D. and Kushner, P.J. (2000) The nuclear receptor corepressor (N-CoR) contains three isoleucine motifs (I/LXXII) that serve as receptor interaction domains (IDs). *Molecular Endocrinology*, 14, 1976-1985.

Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M.P., Chen, D., Huang, S.M., Subramanian, S., McKinerney, E., Katzenellenbogen, B.S., Stallcup, M.R. and Kushner, P.J. (1998) Estrogen receptor activation function 1 works by binding p160 coactivator proteins. *Molecular Endocrinology*, 12, 1605-1618.

Wei, L.N., Farooqui, M. and Hu, X. (2001) Ligand-dependent formation of retinoid receptors, receptor-interacting protein (RIP140), and histone deacetylase complex is mediated by a novel receptor-interacting motif of RIP140. *Journal of Biological Chemistry*, 276, 16107-16112.

Wei, L.N., Hu, X., Chandra, D., Seto, E. and Farooqui, M. (2000) Receptor-interacting protein 140 directly recruits histone deacetylases for gene silencing. *Journal of Biological Chemistry*, 275, 40782-40787.

Weiss, R.E., Xu, J.M., Ning, G., Pohlenz, J., O'Malley, B.W. and Refetoff, S. (1999) Mice deficient in the steroid receptor co-activator 1 (SRC-1) are resistant to thyroid hormone. *EMBO Journal.*, 18, 1900-1904.

Weissman, A.M. (2001) Themes and variations on ubiquitylation. *Nature Reviews Molecuar Cell Biology*, 2, 169-178.

Wen, Y.D., Perissi, V., Staszewski, L.M., Yang, W.M., Krones, A., Glass, C.K., Rosenfeld, M.G. and Seto, E. (2000) The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 7202-7207.

Westin, S., Kurokawa, R., Nolte, R.T., Wisely, G.B., McInerney, E.M., Rose, D.W., Milburn, M.V., Rosenfeld, M.G. and Glass, C.K. (1998) Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature*, 395, 199-202.

Whitfield, G.K., Jurutka, P.W., Haussler, C.A. and Haussler, M.R. (1999) Steroid hormone receptors: evolution, ligands, and molecular basis of biologic function. *Journal of Cellular Biochemistry*, Suppl, 110-122.

Whitman, M. (1998) Smads and early developmental signaling by the TGFbeta superfamily. *Genes & Development*, 12, 2445-2462.

Wieland, C., Mann, S., von Besser, H. and Saumweber, H. (1992) The Drosophila nuclear protein Bx42, which is found in many puffs on polytene chromosomes, is highly charged. *Chromosoma*, 101, 517-525.

Williams, S.P. and Sigler, P.B. (1998) Atomic Structure of Progesterone Complexed With Its Receptor. *Nature*, 393, 392-396.

Winston, F. and Allis, C.D. (1999) The bromodomain: a chromatin-targeting module? *Nature Structural Biology*, 6, 601-604.

Wolffe, A.P., Jones, P.L. and Wade, P.A. (1999) DNA demethylation. *Proceedings of the National Academy of Sciences of the United States of America.*, 96, 5894-5896.

Wolffe, A.P., Urnov, F.D. and Guschin, D. (2000) Co-repressor complexes and remodelling chromatin for repression. *Biochemical Society Transactions*, 28, 379-386.

Wong, C.W. and Privalsky, M.L. (1998) Transcriptional repression by the SMRT-mSin3 corepressor: multiple interactions, multiple mechanisms, and a potential role for TFIIB. *Molecular & Cellular Biology*, 18, 5500-5510.

Wotton, D., Lo, R.S., Lee, S. and Massague, J. (1999) A Smad transcriptional corepressor. *Cell*, 97, 29-39.

Wrana, J.L. (2000) Regulation of Smad activity. *Cell*, 100, 189-192.

Wurtz, J.M., Bourguet, W., Renaud, J.P., Vivat, V., Chambon, P., Moras, D. and Gronemeyer, H. (1996) A canonical structure for the ligand-binding domain of nuclear receptors. *Nature Structural Biology*, 3, 206.

Xu, H.E., Lambert, M.H., Montana, V.G., Parks, D.J., Blanchard, S.G., Brown, P.J., Sternbach, D.D., Lehmann, J.M., Wisely, G.B., Willson, T.M., Kliewer, S.A. and Milburn, M.V. (1999) Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Molecular Cell*, 3, 397-403.

Xu, J.M., Liao, L., Ning, C., Yoshida-Komiya, H., Deng, C.X. and O'Malley, B.W. (2000a) The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 6379-6384.

Xu, J.M., Qiu, Y.H., Demayo, F.J., Tsai, S.Y., Tsai, M.J. and Omalley, B.W. (1998) Partial Hormone Resistance in Mice With Disruption of the Steroid Receptor Coactivator-1 (Src-1) Gene. *Science*, 279, 1922-1925.

Xu, W.D., Angelis, K., Danielpour, D., Haddad, M.M., Bischof, O., Campisi, J., Stavnezer, E. and Medrano, E.E. (2000b) Ski acts as a co-repressor with Smad2 and Smad3 to regulate the response to type beta transforming growth factor. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 5924-5929.

Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawabata, M., Miyazono, K. and Kato, S. (1999) Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science*, 283, 1317-1321.

Yang, W., Rachez, C. and Freedman, L.P. (2000) Discrete roles for peroxisome proliferator-activated receptor gamma and retinoid X receptor in recruiting nuclear receptor coactivators. *Molecular & Cellular Biology*, 20, 8008-8017.

Yang, X.-J., Ogryzko, V.V., Nishikawa, J.-I., Howard, B.H. and Nakatani, Y. (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature*, 382, 319-324.

Yeh, S. and Chang, C. (1996) Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 5517-5521.

Yoh, S.M., Chatterjee, V.K. and Privalsky, M.L. (1997) Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T3 receptors and transcriptional corepressors. *Molecular Endocrinology*, 11, 470-480.

Yoshinaga, S.K., Peterson, C.L., Herskowitz, I. and Yamamoto, K.R. (1992) Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors. *Science*, 258, 1598-1604.

Yoshizawa, T., Handa, Y., Uematsu, Y., Takeda, S., Sekine, K., Yoshihara, Y., Kawakami, T., Arioka, K., Sato, H., Uchiyama, Y., Masushige, S., Fukamizu, A., Matsumoto, T. and Kato, S. (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nature Genetics*, 16, 391-396.

Yuan, C.W., Ito, M., Fondell, J.D., Fu, Z.Y. and Roeder, R.G. (1998) The TRAP220 component of a thyroid hormone receptor-associated (TRAP) coactivator interacts directly with nuclear receptors in a ligand-dependent fashion. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 7939-7944.

Zamir, I., Dawson, J., Lavinsky, R.M., Glass, C.K., Rosenfeld, M.G. and Lazar, M.A. (1997a) Cloning and characterization of a corepressor and potential component of the nuclear hormone receptor repression complex. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 14400-14405.

Zamir, I., Harding, H.P., Atkins, G.B., Horlein, A., Glass, C.K., Rosenfeld, M.G. and Lazar, M.A. (1996) A nuclear hormone receptor corepressor mediates transcriptional silencing by receptors with distinct repression domains. *Molecular & Cellular Biology*, 16, 5458-5465.

Zamir, I., Zhang, J. and Lazar, M.A. (1997b) Stoichiometric and steric principles governing repression by nuclear hormone receptors. *Genes & Development*, 11, 835-846.

Zanger, K. Radovick, S., Wondisford, F.E. (2001) CREB binding protein recuitment to the transcription complex requires growth factor-dependent phosphorylation of its GF box. *Molecular Cell*, 7, 551-558.

Zhang, C., Baudino, T.A., Dowd, D.R., Tokumaru, H., Wang, W. and MacDonald, P.N. (2001a) Ternary complexes and cooperative interplay between NCoA-62/SKIP and SRC coactivators in VDR-mediated transcription. *The Journal of Biologcial Chemistry*, In press.

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D.D., Luo, R.X., Harbour, J.W. and Dean, D.C. (2000a) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell*, 101, 79-89.

Zhang, J., Guenther, M.G., Carthew, R.W. and Lazar, M.A. (1998a) Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Genes & Development*, 12, 1775-1780.

Zhang, J., Hu, X. and Lazar, M.A. (1999a) A novel role for helix 12 of retinoid X receptor in regulating repression. *Molecular & Cellular Biology*, 19, 6448-6457.

Zhang, J.S., Guenther, M.G., Carthew, R.W. and Lazar, M.A. (1998b) Proteasomal Regulation of Nuclear Receptor Corepressor-Mediated Repression. *Genes & Development*, 12, 1775-1780.

Zhang, J.S., Hu, X. and Lazar, M.A. (1999b) A novel role for helix 12 of retinoid X receptor in regulating repression. *Molecular & Cellular Biology.*, 19, 6448-6457.

Zhang, J.S., Hug, B.A., Huang, E.Y., Chen, C.W., Gelmetti, V., Maccarana, M., Minucci, S., Pelicci, P.G. and Lazar, M.A. (2001b) Oligomerization of ETO is obligatory for corepressor interaction. *Molecular & Cellular Biology*, 21, 156-163.

Zhang, Q.H., Yao, H., Vo, N. and Goodman, R.H. (2000b) Acetylation of adenovirus E1A regulates binding of the transcriptional corepressor CtBP.

Proceedings of the National Academy of Sciences of the United States of America, 97, 14323-14328.

Zhang, Y., Feng, X.H. and Derynck, R. (1998c) Smad3 and Smad4 Cooperate With C-Jun/C-Fos to Mediate Tgf-Beta-Induced Transcription. *Nature*, 394, 909-913.

Zhang, Y., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Bird, A. and Reinberg, D. (1999c) Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. *Genes & Development*, 13, 1924-1935.

Zhou, G., Cummings, R., Li, Y., Mitra, S., Wilkinson, H.A., Elbrecht, A., Hermes, J.D., Schaeffer, J.M., Smith, R.G. and Moller, D.E. (1998) Nuclear receptors have distinct affinities for coactivators: characetrisation by fluorescence resonance energy transfer. *Molecular Endocrinology*, 12, 1594-1604.

Zhou, S., Fujimoro, M., Hsieh, J.J., Chen, L. and Hayward, S.D. (1999) A role for SKIP in EBNA2 activation of CBF1-Repressed Promoters. *Journal of Virology*, 74, 1939-1947.

Zhou, S. and Hayward, S.D. (2001) Nuclear localisation of CBF1 is regulated by interactions with the SMRT corepressor complex. *Molecular and Cellular Biology*, 21, 6222-6232.

Zhou, S.F., Fujimuro, M., Hsieh, J.J.D., Chen, L. and Hayward, S.D. (2000a) A role for SKIP in EBNA2 activation of CBF1-repressed promoters. *Journal of Virology*, 74, 1939-1947.

Zhou, S.F., Fujimuro, M., Hsieh, J.J.D., Chen, L., Miyamoto, A., Weinmaster, G. and Hayward, S.D. (2000b) SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC to facilitate NotchIC function. *Molecular & Cellular Biology*, 20, 2400-2410.

Zhu, Y., Kan, L., Qi, C., Kanwar, Y.S., Yeldandi, A.V., Rao, M.S. and Reddy, J.K. (2000) Isolation and characterization of peroxisome proliferator-activated receptor (PPAR) interacting protein (PRIP) as a coactivator for PPAR. *Journal of Biological Chemistry*, 275, 13510-13516.

Zhu, Y., Qi, C., Jain, S., Rao, M.S. and Reddy, J.K. (1997) Isolation and characterization of PBP, a protein that interacts with peroxisome proliferator-activated receptor. *Journal of Biological Chemistry*, 272, 25500-25506.

Zierold, C., Darwish, H.M. and DeLuca, H.F. (1995) Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. *Journal of Biological Chemistry*, 270, 1675-1678.

Zilliacus, J., Holter, E., Wakui, H., Tazawa, H., Treuter, E. and Gustafsson, J.A. (2001) Regulation of glucocorticoid receptor activity by 14-3-3-dependent intracellular relocalization of the corepressor RIP140. *Molecular Endocrinology*, 15, 501-511.

Zou, A.H., Elgort, M.G. and Allegretto, E.A. (1997) Retinoid X Receptor (Rxr) Ligands Activate the Human 25-Hydroxyvitamin D-3-24-Hydroxylase Promoter Via Rxr Heterodimer Binding to Two Vitamin D-Responsive Elements and Elicit Additive Effects With 1,25-Dihydroxyvitamn D-3. *Journal of Biological Chemistry*, 272, 19027-19034.

Zwijsen, R.M.L., Buckle, R.S., Hijmans, E.M., Loomans, C.J.M. and Bernards, R. (1998) Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1. *Genes & Development*, 12, 3488-3498.

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Journal: The Journal of Biological Chemistry

Title: Cross-talk between 1,25-Dihydroxyvitamin D3 and Transforming Growth Factor-B Signalling Requires Binding of VDR and Smad3 Proteins to Their Cognate DNA Recognition Elements
Authors: Nanthakumar Subramaniam, Gary M. Leong, Terrie-Anne Cock, Judith L. Flanagan, Colette Fong, John A. Eisman, and Alexander P. Kouzmenko
Journal: The Journal of Biological Chemistry

Title: Glucocorticoid Excess During Adolescence Leads to a Major Persistent Deficit in Bone Mass and an Increase in Central Body Fat
Authors: Veronica Abad, George P. Chrousos, James C. Reynolds, Lynnette K. Nieman Suvimol C. Hill, Robert S. Weinstein, and Gary M. Leong
Journal: Journal of Bone and Mineral Research

ADDENDUM

Page 42, paragraph 3, line 4. "The evidence . . ."
Page 60, paragraph 1, line 1. ". . . media in the absence or presence . . ."
Page 60, paragraph 2, line 4. ". . .vortexing cell pellets . . ."
Page 60, paragraph 2 & 3. Page 6, paragraph 1. Change ul to µl.
Page 60, paragraph 3, line 2. "One µl of plasmid DNA was . . ."
Page 61, paragraph 3, line 8. "All media were adjusted . . ."
Page 64, paragraph 3, last sentence. "the DNA was ethanol precipitated"
Page 67, paragraph 5, line 6. ". . .100 µl of LB and . . ."
Page 75, paragraph 4, line 1. ". . . transformed into BL21- . . ."
Page 97, paragraph 2, line 14. ". . .Known biological relevance . . ."
Page 98, paragraph 3, line 1. ". . . which lacked the . . ."
Page 102, paragraph 4, line 4. ". . cell lines . . ."
Page 107 Section 4.3.1.2 paragraph 2, line 2 after "SKIP", insert "though no LBD construct retaining the AF-2 domain was specifically tested."
Page 115, paragraph 1, line 1. ". . . (Figure 4.5)."
Page 115, paragraph 4, line 9, change sentence beginning "Thus in this promoter..." to "Thus in this promoter context, functional interaction between RXR and SKIP was detected both by a modest enhancement of RXR-mediated transcription and by its negative impact on VDR-mediated transcription."
Page 120, Section 4.5.2, insert in Fig.4.8 legend "Western analysis for expression of RXR, VDR and SKIP fusion proteins using equal amounts of whole cell yeast lysates were re-blotted with appropriate antibodies after re-stripping of the original membrane. This ensured that a comparison of expression of the 3 different fusion proteins could be made within and between the different yeast strains".
Page 122, paragraph 2, line 7. ". . . appears to mediate . . ."
Page 125, Section 4.7, paragraph 1, line 2. " In addition to, or as a cause or consequence of the differences in cell-type and differentiation state between the CV-1 and P19 cell-lines, differences in VDR/RXR function in these two cell-lines may have also modulated SKIP action".
Page 125, paragraph 2, line 1. ". . . possibility that the . . ."
Page 125, paragraph 3, line 7. ". . . appears to interact . . ."
Page 126, Section 4.7 paragraph 2,line 1. Change sentence beginning "Based on these results..." to "Based on these results SKIP appears to belong to a small group of recently described bifunctional proteins which may modulate the cell- and possibly tissue-specific actions of NHR superfamily (Section 1.9.7). "
Page 134, Section 5.4.1, paragraph 3, line 6 after "with or without TSA", insert " and a 2-fold increase in basal reporter activity, "
Page 137, paragraph 1, line 1. ". . . analysis . . ."
Page 143, Figure 5.7 included as missing from one examiner's copy of thesis.
Page 177, Chapter 7, end of page insert " A better understanding of the tissue-specific expression of SKIP and its developmental expression will also provide further insights into its potential role *in vivo*."
Page 182, Chapter 7, Line 1 insert " The key question of the physiological function of SKIP remains incompletely answered by these studies."

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