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THE USE OF HUMAN PAPILLOMAVIRUS PROMOTER TO TARGET CERVICAL CANCER CELLS

by

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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ABBREVIATIONS

Amp^r	ampicillin resistance gene
AP1	active promoter factor 1
ATP	adenosine triphosphate
bp	base pair
BPV	bovine papillomavirus 1
CIN	cervical intraepithelial neoplasia
CMV	cytomegalovirus
dATP	deoxyadenosine 5′-triphosphate
DTT	dithiothreitol
DMEM	Dulbecco's Modified Eagle Media
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleotide 5′-triphosphate
E	early
EDTA	ethylenediaminetetra acetic acid
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
FBS	foetal bovine serum
FDA	Food and Drug Administration
FSC	forward scatter
GFP	green fluorescence protein
GRE	glucocorticoid response element
HPV	human papillomavirus
HSV	herpes simplex virus
Kan^r	kanamycin resistance gene
kb	kilo base pair
KOAc	potassium acetate
kPa	kilo Pascal
KRF-1	keratinocyte response factor
L	late

LB	Luria-Bertani
LCR	long control region
luc+	luciferase gene
MCS	multiple cloning site
NaOAc	sodium acetate
Neo^r	neomycin resistance gene
NCR	non-coding region
NF1	nuclear factor 1
nt	nucleotide
Oct-1	Octamer 1
ONPG	*o*-nitrophenyl β-D-galactopyranoside
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pRb	retinoblastoma protein
RLB	reporter lysis buffer
RLU	relative light unit
RNase	ribonuclease
SDS	sodium dodecylsulphate
SEM	standard error of the mean
Sp1	specific promoter factor
SSC	side scatter
STD	sexually transmitted disease
SV40	Simian Virus 40
TBE	Tris/boric acid/EDTA
TE	Tris-HCl/EDTA
TGA	Therapeutic Goods Administration
tk	thymidine kinase
URR	upstream regulatory region
UV	ultraviolet
VLP	virus-like particle
YY1	*Ying Yang* 1

ABSTRACT

Human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease worldwide. Infections by high-risk HPVs, such as HPV-18, have been associated etiologically with cervical cancer. The successful development of HPV vaccines may be beneficial to the HPV-naïve population, but women that have already been exposed to the virus are still at risk of developing HPV-associated malignancies. A need for a systemic cure for HPV-infection therefore still exists. Gene therapies using tissue-specific promoters have been reported to be a promising tool for treating cancers; however, few studies have explored this possibility for cervical cancer.

The aim of this project is to construct a gene expression vector that can specifically target HPV-infected cervical cancer cells, by making use of the activity and selectivity of the P_{105} promoter which is determined by transcription control elements within the HPV-18 long control region (LCR). The first part of this study involved the construction of LCR deletion plasmids, and examining the subsequent level of gene expression induced within different mammalian cell lines. The results suggest the LCR to be capable in achieving cervical cancer-specific gene expression. The 3′-end of the viral L1 gene upstream of the LCR appeared to have a repressive effect on the promoter and therefore should be excluded for maximum LCR promoter activity. The second part of the project involved site-directed mutagenesis studies performed on selected transcription factor binding sites with an attempt to further increase the level of LCR promoter activity and specificity towards HPV-infected cervical cancer cells. The results suggest that a GRE/YY1 mutation may significantly enhance promoter activity. In terms of promoter regulation, the E2BSs appeared to be responsible for promoter activation in the absence of viral E2 proteins.

The findings of this study suggest a possible gene therapy approach towards the treatment of cervical cancer. By making use of the activity and specificity of the HPV-18 P_{105} promoter to induce cervical carcinoma-specific expression of appropriate therapeutic genes, suicidal phenotypes can be introduced selectively within HPV-positive cervical cancer cells while normal HPV-negative cells are unaffected.

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Dedication

This thesis is lovingly dedicated to my dearest Mum, Dad and Zhe.

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 Human papillomavirus and cervical cancer

Papillomavirus is a genus under the *Papovaviridae* family of viruses (ICTVdB, 2002). They are highly host-specific and each species is named after its natural host. All presently known human and animal papillomaviruses together form 16 genera, 5 of which are composed exclusively of human papillomaviruses (HPV) and other papillomaviruses isolated from some apes and monkeys (de Villiers *et al.*, 2004). An HPV type is defined as a complete genome whose L1 gene sequence (details on different HPV genes discussed in Section 1.2.2) varies at least 10% to that of any other HPV type (Bernard, 2005). To date, over 200 types of HPV have been recognised on the basis of DNA sequence data (Shillitoe, 2006). The whole genome of about 100 HPV types have been isolated and completely sequenced (Bernard, 2005), while the remaining are potential new variants that are partially characterized. Table 1.1 is a table extracted from Bernard (2005) which is a summarised list of human papillomavirus types and their taxonomy that are relevant to understand most clinical, epidemiological or molecular publications.

Table 1.1 The most frequently studied papillomavirus types and their biological and clinical properties.

Family: papillomaviruses (*Papillomaviridae*)

Genus	Species	Type (s)	Properties
Alpha-papillomaviruses	4	HPV-2, HPV-27, HPV-57	Common skin warts, frequently in genital warts of children
	5	HPV-26, HPV-51, HPV-69, HPV82	High-risk malignant and benign mucosal lesions
	6	HPV-53, HPV-30, HPV-56, HPV-66	High-risk malignant and benign mucosal lesions
	7	HPV-18, HPV-39, HPV-45, HPV-59, HPV-68, HPV-70	High-risk malignant mucosal lesions, some (esp. HPV-18) more frequent in adeno- than in squamous carcinoma of the cervix
	8	HPV-7, HPV-40, HPV-43	Low-risk mucosal and cutaneous lesions, HPV-7 known as butcher's wart virus, often in lesions of HIV infected patients
	9	HPV-16, HPV-31, HPV-33, HPV-35, HPV-52, HPV-58, HPV-67	High-risk malignant mucosal lesions, some (esp. HPV-16) more frequent in squamous than in adenocarcinoma of the cervix, HPV-16 most prevalent HPV type in cervical malignancies
	10	HPV-6, HPV-11, HPV-13, HPV-44, HPV-74	Benign mucosal lesions. HPV-6 and HPV-11 in male and female genital warts, condylomata acuminata of cervix, laryngeal papillomas. Some of these lesions can progress malignantly
Beta-papillomaviruses	1	HPV-5, HPV-8 (selected from a very type-rich genus)	Cutaneous benign and malignant lesions in EV and immune-suppressed patients
Gamma-papillomaviruses	1	HPV-4, HPV-65 (selected from a very type-rich genus)	Cutaneous benign lesions
Delta-papillomaviruses	4	Bovine papillomavirus-1 (BPV-1) (selected from a type-rich genus)	Fibropapillomas in cattle, sarcoids in horses. An important cell culture model.
Kappa-papillomaviruses	1	Cottontail rabbit papillomavirus (CRPV)	Cutaneous lesions. An important animal model
Mu-papillomaviruses	1, 2	HPV-1, HPV-63	Cutaneous lesions, frequently in footwarts
Nu-papillomaviruses	1	HPV-41 (unrelated to any other HPV type)	Cutaneous lesions

Table reproduced from Bernard (2005).

1.1.1 Worldwide prevalence

HPV is the one of the most common causes of sexually transmitted disease (STD) in the world (Vandepapeliere *et al.*, 2005; Villa, 2006). Epidemiological estimates suggest that the world prevalence of HPV infection is about 9-13% which equates to about 630 million infected people, and approximately 70% of the sexually active adult population are infected with HPV (WHO, 2001). About 70% of genital HPV infections are subclinical and regress spontaneously without progressing to disease, presumably because the host eventually mounts a successful immune response (Lowy and Schiller, 2006; Meijer *et al.*, 2000). Chronic infection of HPV which may possibly lead to cervical cancer in women develops only in a small proportion of infected individuals. Cervical cancer is the second leading cause of female cancer mortality

worldwide (Jo and Kim, 2005), with approximately 288,000 deaths and 510,000 new cases reported each year (Saslow *et al.*, 2007), and the DNA of HPV is found in virtually all cervical cancers (>99.7%) (Doorbar, 2006).

Like many STDs, genital HPV infections often do not have any visible signs and symptoms (Markowitz *et al.*, 2007). The best ways to prevent the development of cervical cancer is through early detection and treatment. Cervical cancer can be detected and diagnosed by cytology-based screening programs such as Papanicolaou (Pap) smear, colposcopy, biopsy, pelvic exam and endocervical curettage (NCI, 2006). However, due to the lack of knowledge and accessible resources, HPV-induced cervical cancer is particularly prevalent in developing countries, where cervical cancer is the most common cancer in women and accounts for about 80% of cases worldwide (WHO, 2006). In developed countries, a large and significant reduction in deaths from cervical cancer has been attributed to advances in technology, where organised cervical screening programmes are generally accepted to be responsible for a substantial fall in cervical cancer-associated deaths (Cuschieri and Cubie, 2005). In the United States for example, the number of deaths from cervical cancer has declined by over 80% in the last 50 years coincidently with the implementation of Pap smear as a diagnostic (Longworth and Laimins, 2004). However, even today, Pap smears are not regularly performed on approximately 33% of eligible women worldwide (NCI, 2006).

1.1.2 Pathogenesis

All known HPVs are greatly restricted in tissue tropism, infecting human cells of epithelial origin only. HPVs are often found in the differentiating human keratinocytes of the stratified cutaneous and secretory mucosal epidermis (Dybikowska *et al.*, 2002). There are 40 different genotypes of HPV that can infect the anogenital region of men and women, including the skin of the penis, vulva and anus, and the lining of the vagina, cervix and rectum (WHO, 2006; zur Hausen, 1998).

HPV infects in the basal layer of epithelial cells, where the cells are least differentiated and are still able to proliferate, via microtraumas to the overlying suprabasal epidermal cells (Lowy and Schiller, 2006; Schwartz, 2000). At the basal epithelial cell level, which is referred to as the nonproductive stage of HPV infection (Jo and Kim, 2005), the viral genome remains as a low copy number episome within host cells and the viral genes are poorly expressed. During the productive stage of the viral life cycle as the basal cell layer differentiates, the expression of late genes is initiated. Viral replication takes place and structural proteins are formed. As the suprabasal layers continue to migrate upwards, they eventually become enucleated and form a flattened protein barrier known as squames, where complete viral particles are assembled. The terminal stage of differentiation when virion-laden squames are shed from the skin is referred to as the 'productive' phase of HPV infection. At the same time, 'persistent' infection occurs within the suprabasal cell layer where viral plasmids are being replicated and continually passed on to the newly formed daughter cells (see Figure 1.1).

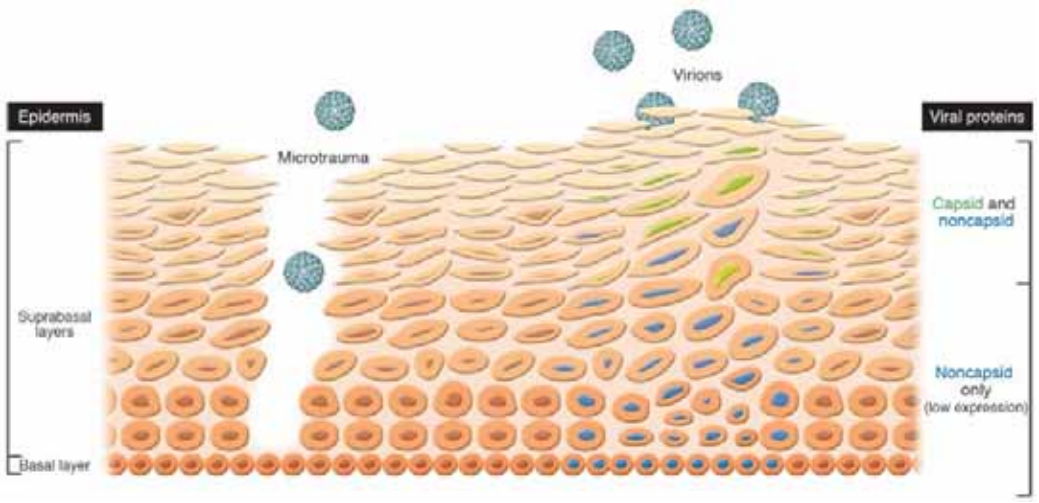

Figure 1.1 Viral production in the differentiating epithelium of HPV-infected cells.

The epidermis of the skin contains layers of keratinocytes. To establish infection, HPV must infect the basal layer of epithelial cells which are least differentiated and therefore able to divide, via microtraumas in the upper layers of the epithelium (suprabasal layers). Viral genes are expressed at low levels in the basal layer. Viral replication takes place as the keratinocytes migrate towards the top and become increasingly differentiated. At the terminal stage of differentiation, viral progenies are released as the squames are shed from the skin and a new cycle of infection can then begin. Figure reproduced from Lowy and Schiller (2006).

HPVs are generally divided into two categories – the high-risk and low-risk types, depending on their malignant properties within human cells. Infections with low-risk HPV may lead to the formation of benign lesions, which includes non-genital and anogenital skin warts, oral and laryngeal papillomas, and anogenital mucosal condylomata (Lowy and Schiller, 2006). Genital warts often regress spontaneously over time. In all benign lesions, the viral genome replicates autonomously as an extra-chromosomal episome within the nucleus of the infected cells. These benign lesions generally do not progress to cancer and the vegetative viral life cycle is closely related to the differentiation state of the host keratinocytes (Steger *et al.*, 2001). Two of the

most common low-risk genotypes are HPV-6 and 11, which account for a substantial proportion of low-grade cervical dysplasia detected in screening programs and more than 90% of genital warts (Chan and Berek, 2007). Some of the other low-risk HPV types are HPV-40, 42, 43, 44, 54, 61, 70, 72 and 81 (Munoz *et al.*, 2003).

Persistent infections with high-risk HPVs are responsible for the majority of HPV-related cancers of the cervix, vagina, vulva, anus and penis (Basta *et al.*, 1999; Bosch *et al.*, 1995; Cuschieri *et al.*, 2005; Munoz *et al.*, 1992; WHO, 2006; zur Hausen, 1996). There are also studies suggesting the involvement of HPV in oral cancer (Miller and Johnstone, 2001; Shillitoe and Noonan, 2000). The association of papillomavirus and human cancers is particularly strong for cancers of the uterine cervix as more than 90% of cervical cancer lesions are found to be HPV-positive (Bosch *et al.*, 2002; Castellsague *et al.*, 2006; Munoz *et al.*, 2003; Nakagawa *et al.*, 2000; Pater and Pater, 1985; Walboomers *et al.*, 1999), and cervical cancer accounts for about 70% of all the cancer cases linked etiologically to HPV (Lowy and Schiller, 2006). In general, HPV infection may lead to two types of cervical carcinoma. Squamous cell carcinoma refers to the malignancy derived from epidermal cells and comprises approximately 90% of cervical cancers, and the remainder being adenocarcinomas which originate from the glandular tissue and are relatively rare. Primary sarcomas of the cervix have been described occasionally, and malignant lymphomas of the cervix have also been reported (Frey *et al.*, 2006; Garavaglia *et al.*, 2005). In most malignant lesions the viral DNA is frequently integrated into the genome of the host cell (Kanodia *et al.*, 2007), resulting in cellular transformation and the formation of cervical intraepithelial neoplasias (CIN) which are considered to be putative precancerous lesions (Meijer *et al.*, 2000). CIN has the potential for progression to invasive cervical carcinoma due to the expression of viral oncogenes leading to unscheduled proliferation (see Figure 1.2).

High-risk HPV types include HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. Case-control studies performed by Munoz *et al.* (2003) have shown that these high-risk HPV types account for over 95% of HPV-positive squamous cell carcinomas. Amongst HPV-associated cervical cancer, about 70% of cervical carcinoma contain the DNA of either HPV-16 or 18 (Chan and Berek, 2007; Saslow *et al.*, 2007) (see Figure 1.3) and have been studied most intensively. Most high-risk HPVs are also phylogenetically related to either HPV-16 (31, 33, 35, 52 and 58) or HPV-18 (39, 45, 59 and 68) (Chan *et al.*, 1995).

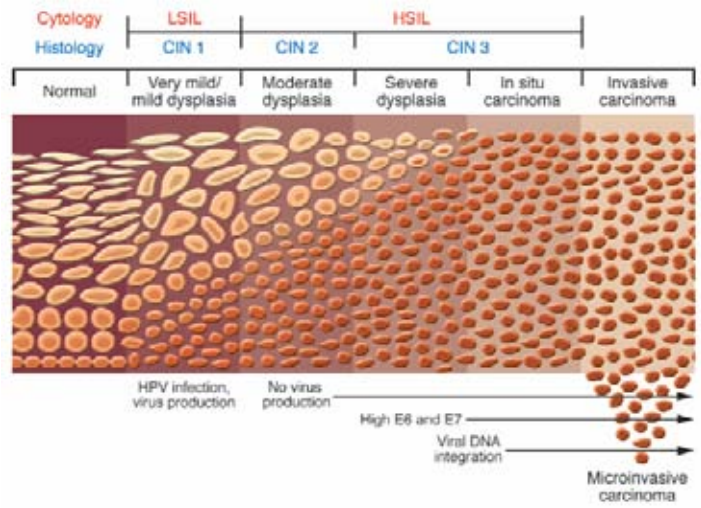

Figure 1.2 Progression from a benign cervical lesion to invasive cervical cancer. LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CIN: cervical intraepithelial neoplasia. Figure reproduced from Lowy and Schiller (2006).

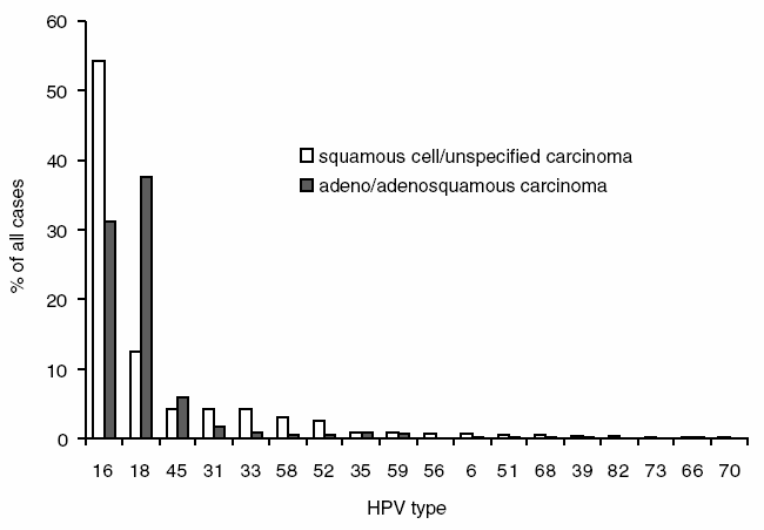

Figure 1.3 Type-specific worldwide prevalence of HPV in invasive cervical cancer.

About 70% of cervical carcinomas contain the DNA of HPV-16 and 18; with HPV-16 of the highest prevalence in squamous cell carcinomas (55%) and the highest prevalence of HPV-18 in adenocarcinomas (38%). Figure reproduced from Clifford *et al.* (2003).

Although the most prevalent type of HPV leading to cervical cancer is HPV-16, there are studies suggesting that HPV-18 is associated with a greater risk of progression or a more rapid transition to malignancy, leading to the development of more clinically aggressive disease (Arends *et al.*, 1993; Burger *et al.*, 1996; Kitagawa *et al.*, 1996). Previous studies also showed that HPV-18 is about 10- to 50-fold more efficient in its immortalisation potential when compared with HPV-16 (Barbosa and Schlegel, 1989; Schlegel *et al.*, 1988; Villa and Schlegel, 1991). This may be associated with the fact that the DNA of HPV-18 is nearly always integrated into the host genome, whereas HPV-16 DNA can often be found both episomally and in an integrated form within the host cells (Bosch *et al.*, 2002). Sichero *et al.* (2005) have also tested 6 variants of HPV-18 and showed that all their P_{105} promoters were more active than the P_{97} HPV-16 prototype promoter. In addition, HPV-18 related cervical cancers are

shown to be associated with higher mortality rate than those associated with HPV-16 (Ault *et al.*, 2004), because they are more often present in adenocarcinomas and small cell carcinomas of the uterine cervix (Andersson *et al.*, 2001; Burger *et al.*, 1996; Madeleine *et al.*, 2001); which are lesions that have a particularly poor prognosis (Cid *et al.*, 1993; Liu *et al.*, 2001). Approximately half of the HPV-positive adenocarcinomas are attributed to HPV-18 (Goto *et al.*, 2005). Due to the above reasons, this study was focused on HPV-18 in particular.

1.1.3 Treatment

At early stages, cervical cancer precursors can be treated with local measures such as cryotherapy, electrocautery and surgical excision (Lacey, 2005). However, while these surgical procedures remove the neoplasia, growth usually recurs due to persistence of the virus in the healthy tissue (Bernard, 2004). At advance stages of the disease, cervical cancer has to be treated with chemotherapy or radiotherapy. Previous studies have also shown that cisplatin-based chemotherapy given concurrently with radiation therapy provide improved treatment for cervical cancer (Morris *et al.*, 1999; Rose *et al.*, 1999; Thomas, 1999). Unfortunately improvements in therapeutic treatments did not manage to significantly decrease the mortality rate of cervical cancer (Rein and Kurbacher, 2001), and patients with advanced, recurrent or metastatic diseases still have poor prognosis (Rein *et al.*, 2004). Moreover, these methods of treatment can be physically exhausting for the patients with side effects such as hair loss, nausea and vomiting, or to a more severe extent, depression of the immune system.

In June 2006, the first vaccine against cervical cancer, Gardasil® produced by Merck, was approved by the Food and Drug Administration (FDA) in the United States

(FDA, 2006). The vaccine protects against the four most common strains of HPV leading to genital warts (HPV-6 and 11) and cervical cancer (HPV-16 and 18), and has been approved by the Therapeutic Goods Administration (TGA) of Australia for use in females aged 9 to 26 and males aged 9 to 15. The Australian government has also added Gardasil® to the National Immunisation Program and made it available for free to eligible women from July 2007 onwards. Another vaccine, Cervarix™ produced by GlaxoSmithKline, protects against HPV-16 and 18. It has also been approved by TGA for use in females aged 10 to 45 in April 2007, and is currently available on the Australian market. Both vaccines work similarly by inducing immune responses against different HPV types by making use of the viral L1 proteins which self-assemble into virus like particles (VLP) when injected into the human body.

The successful development of the vaccines for cervical cancer is expected to greatly reduce the incidence of HPV infections and subsequent cervical abnormalities in the long run. However, issues remain as protection by the vaccines is restricted to a few oncogenic HPV types. Moreover, the vaccines only manage to efficiently protect women who are HPV-naïve, and may not be beneficial to the current HPV-infected population who are at risk of developing HPV-associated malignancies. On average, it takes 12 to 15 years before a persistent high-risk HPV infection may ultimately, via consecutive premalignant stages, lead to an overt cervical carcinoma (Snijders *et al.*, 2006). Since there is generally a delay between the acquisition of HPV infection and the development of precancerous lesions, cytology-based screening methods may not effectively identify the possible risk of developing cervical cancer at early stages, and the positive effect of the HPV vaccine on the immunised population will not be apparent in at least a decade's time (see Figure 1.4). The development of an HPV vaccine therefore is effective only on the preventative aspect for a limited population

group, and promising novel treatment options such as DNA-based therapeutics for HPV infection and HPV-associated cancer are still needed.

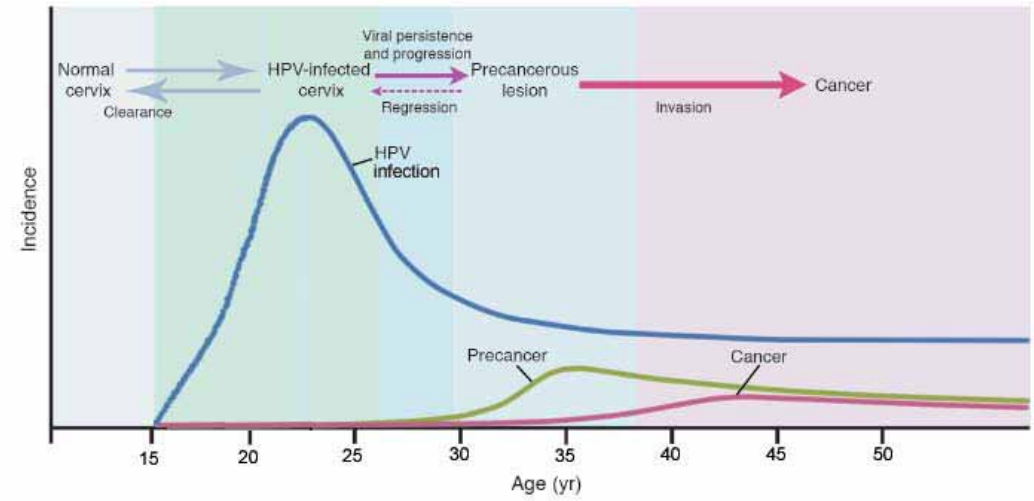

Figure 1.4 Relationship among incidences of cervical HPV infection, precancerous lesions and cervical cancer by age of women.

The incidence of HPV infection (blue curve) develops soon after women initiate sexual activity during their teens and 20s, with the highest prevalence in females aged 20-24 (Dunne *et al.*, 2007). The subsequently lower incidence is because infections can be self-limited. There is generally a delay between the acquisition of HPV infection and the development of precancer lesions, approximately 10 years later, and only a subset of infected women develop precancers, hence a much lower peak for precancer incidence (green curve). The incidence of invasive cancer (purple curve) reaches its peak as women approach 40 years of age. Approximately 20 million women in the U.S. are currently infected with HPV (CDC, 2008); 500,000 women are diagnosed with high-grade cervical dysplasia each year and the American Cancer Society (2008) estimates that 11,070 women will be diagnosed with cervical cancer in 2008 (The peaks of the curves are not drawn to scale). Figure modified from Schiffman and Castle (2005) using the updated information from Dunne *et al.* (2008).

1.2 The human papillomavirus type 18

1.2.1 Virion structure

HPV-18 belongs to the *Alphapapillomavirus* genus, species 7 (de Villiers *et al.*, 2004). The HPV-18 virion contains a covalently closed circular; double stranded deoxyribonucleic acid (DNA) genome of 7,857 base pairs (bp). The viral DNA is packed inside a capsid made up of 72 capsomers or subunits, arranged in an icosahedral structure (Bishop *et al.*, 2007). It is a relatively small virus of about 55 nm in diameter. Unlike some of the larger DNA viruses, papillomaviruses have no lipid envelopes surrounding the capsids (see Figure 1.5).

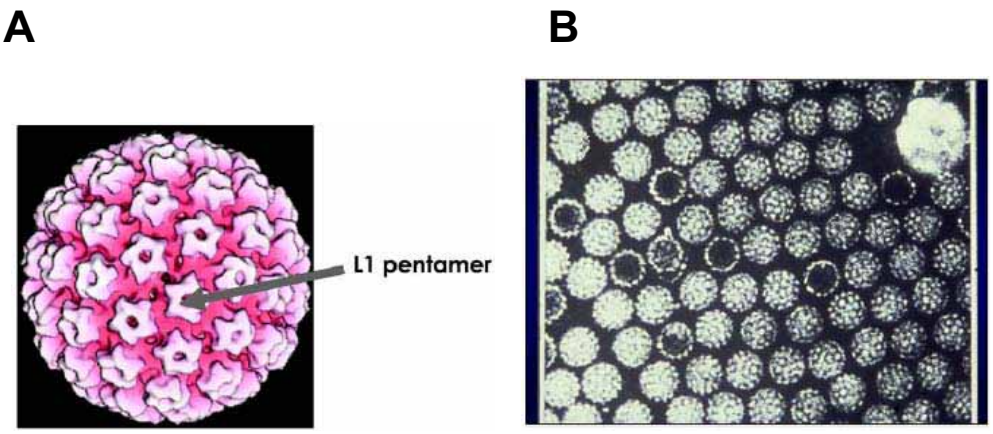

Figure 1.5 Viral structure of HPV.

A: Model of the papillomavirus capsid. The rosette-like surface structures (arrowed) are pentamers each consisting of 5 molecules of L1; 1 molecule of L2 fits into the central dimple of each pentamer. B: Transmission electronphotomicrograph of HPV particles. Both full (contain DNA) and empty (no DNA) viral particles can be seen. Figures reproduced from Stanley *et al.* (2006).

1.2.2 Genome organization

The genome of HPV-18 can be divided into three functional sections. The early (E) region contains the E1, E2 and E4 to E7 open reading frames (ORF), and the late (L) region contains the L1 and L2 ORFs. The long control region (LCR), also known as the upstream regulatory region (URR) or the non-coding region (NCR); lies between the L1 and E6 ORFs (see Figure 1.6). The LCR regulates transcription from the early and late regions, and contains enhancer and promoter elements that are responsive to cellular and virally encoded transcription regulatory factors (discussed in detail in Section 1.4).

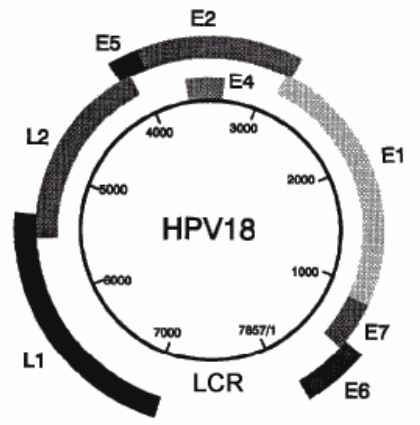

Figure 1.6 Circular map of the HPV-18 genome.
The localization of the open reading frames (E1, E2, E4 to E7 for early genes, L1 and L2 for late genes) and the long control region (LCR) of HPV-18. The numbers (1 to 7,857) on the inside of the circle refer to the DNA base pairs starting from start of DNA replication start site. Figure modified from zur Hausen (1996).

The late region is made up of the two ORFs L1 and L2, which encode for the major and minor capsid proteins respectively. These are structural proteins required during the packaging of viral DNA produced by replication during the productive stage

of viral life cycle, forming infectious viral progenies. These proteins are expressed during the later stages of viral life cycle, following the expression of the early viral proteins (Jo and Kim, 2005) once viral genome amplification has been completed (Doorbar, 2005). The L1 and L2 proteins are not found to be expressed in precancerous and malignant cells (zur Hausen, 2002), hence they do not appear to be involved in the immortalisation of HPV-infected cervical cancer cells.

The early region is made up of the E1, E2 and E4 to E7 ORFs, encoding the early HPV proteins controlling viral DNA transcription and replication, and also proteins that are responsible for cellular transformation. These genes are the first group of genes to be expressed immediately after viral infection.

The E1 ORF is the largest of the HPV ORFs. E1 protein has been shown to play an important role in the extra-chromosomal regulation of DNA replication (Winkler and Richart, 1989) by binding to and unwinding the viral origin of replication, an action which is facilitated by the E2 and chaperone proteins (Wilson *et al.*, 2002). E1 then recruits replication protein A, DNA polymerase α and primase from host cells (Conger *et al.*, 1999) to initiate viral replication. Apart from origin binding activity, E1 also possesses helicase and ATPase activity (Auster and Joshua-Tor, 2004; Hughes and Romanos, 1993). It is also believed that the E1 and E2 proteins are expressed in order to maintain the viral DNA as an episome during the non-productive stage of the viral life cycle (Wilson *et al.*, 2002), and to facilitate the correct segregation of genomes during cell division (You *et al.*, 2004).

The main function of E2 protein is to enhance viral DNA replication by recruiting E1, the viral replication factor, to the origin of replication (Bechtold *et al.*, 2003; Wilson *et al.*, 2002). E2 has also been suggested to assist the segregation of HPV DNA as minichromosomes by association with mitotic spindles (Van Tine *et al.*, 2004).

The expression of HPV E2 has also been reported to be related to the induction of apoptosis through p53-dependent and –independent mechanisms (Massimi *et al.*, 1999; Webster *et al.*, 2000). Additionally E2 protein has been suggested to be responsible for the transcriptional regulation of the viral early genes, including the E6 and E7 oncogenes, by binding to the LCR (Hines *et al.*, 1998; Jo and Kim, 2005; Swindle *et al.*, 1999; Tan *et al.*, 1992). The involvement of E2 protein in cellular transformation is discussed in more detail in Section 1.3 and 1.5.1

The E4 ORF is entirely contained within the E2-encoding sequences, and is translated from spliced transcripts as a fusion with the first 5 amino acids of E1 to generate E1^E4 fusion proteins, which are expressed in the late phase of the viral life cycle (Longworth and Laimins, 2004). The E4 ORF itself lacks an initiator AUG codon and uses the E1 sequence for translation initiation (Howley, 1996). The expression of E1^E4 is important for viral genome amplification and prevents premature transcription of the late genes (Rush *et al.*, 2005; Wilson *et al.*, 2005), disrupts the cytokeratin network (Doorbar *et al.*, 1991) and facilitates the release of viral particles (Longworth and Laimins, 2004).

The HPV E5 protein is a membrane-associated protein found in the perinuclear region, associated with the Golgi body and endoplasmic reticulum (Conrad *et al.*, 1993; Disbrow *et al.*, 2005; Gieswein *et al.*, 2003). It is very important for the amplification of viral genome, and the initiation of E1^E4 expression and other viral late genes (Fehrmann *et al.*, 2003; Genther *et al.*, 2003). The E5 protein associates with vacuolar ATPase (Conrad *et al.*, 1993; Gieswein *et al.*, 2003), which delays endosomal acidification (Straight *et al.*, 1995), slows down the degradation of epidermal growth factor receptor (EGFR) and increases EGFR recycling to the cell surface (Straight *et al.*, 1995; Straight *et al.*, 1993).

A common feature of high-risk HPV is that their E5, E6 and E7 proteins all possess growth stimulating and transforming properties. The expression of E5 protein has been shown to enhance E6/E7 immortalisation efficiency in human keratinocytes (Stoppler et al., 1996). The expression of HPV-16 E5 in particular have also displayed the ability to transform rodent fibroblasts (Straight *et al.*, 1993), and its overexpression in transgenic mice resulted in the development of spontaneous skin tumours (Genther Williams et al. 2005). However, the role of E5 protein in the development of uterine cervical cancer is controversial as disruption of the E5 gene upon viral integration into host genome has been observed (Scheffner *et al.*, 1994; Schneider-Gadicke and Schwarz, 1986; Schwarz *et al.*, 1985). The E6 and E7 ORFs encode the two important oncogenic proteins that are involved in the process of cellular immortalisation. During natural infection, the activity of E6 and E7 allows the small number of infected cells to expand, increasing the number of cells that subsequently go on to produce infectious virions. The ability of E6 and E7 to drive cells into *S*-phase is also necessary, along with E1 and E2, for viral replication and maintaining the viral DNA as an episome (Doorbar, 2006; Lee *et al.*, 2006; Oh *et al.*, 2004; Thomas *et al.*, 1999). However, in HPV-infected keratinocytes, the E6 and E7 proteins degrade and inactivate the tumour suppressor proteins p53 and the retinoblastoma protein (pRb) respectively resulting in immortalisation. The precise functions and transforming properties of E6 and E7 are discussed in detail in Section 1.4.

Table 1.2 Summary of the functions of the products of the HPV early region open reading frames.

Early region	Protein functions
E1	Unwinds the DNA strands working with E2 protein Modulate the transcription activity of the E2 protein
E2	Enables E1 protein to bind to the viral origin of replication located within the LCR Encodes a LCR-binding protein that regulates transcription of the early region
E4	Encodes a protein that interacts with cytokeratin Expressed in later stages of infection, when complete virions are being assembled
E5	Augment cellular proliferation and DNA synthesis in a context of cell membrane receptors, such as EGF and PDGF Induces an increase in mitogen-activated protein kinase activity
E6	Binds to p53 and targets it for rapid degradation via a cellular ubiquitin ligase Induces telomerase activation
E7	Binds to the hypophosphorylated Rb proteins and liberate E2F, which results in S phase entry Interacts with inhibitors of cyclin dependent kinases Induces abnormal centrosome duplication resulting in aneuploidy

Table reproduced from Jo and Kim (2005).

1.3 The HPV-18 long control region

1.3.1 Components of the HPV-18 long control region

The LCR of HPV-18 is a 825 bp long non-coding region which lies between the L1 and E6 ORF, from nucleotide (nt) 7,137 to 104, with the number of nucleotides corresponding to the published sequence by Cole and Danos (1987). It is contained within a 1,050 bp *Bam*HI fragment which comprises three functional domains separated by *Rsa*I recognition sites (Bauknecht *et al.*, 1992; Garcia-Carranca *et al.*, 1988; Gius *et al.*, 1988) (see Figure 1.7). At the 3′-end of the LCR lies a promoter of the early viral genes which is known as the P_{105} promoter.

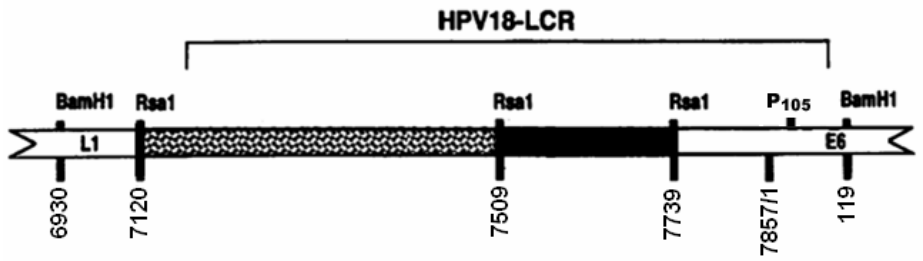

Figure 1.7 Schematic representation of the HPV-18 LCR.
The HPV-18 LCR can be divided into different functional domains. The distal *Bam*HI-*Rsa*I fragment (nt 6,930 to 7,119); the distal long *Rsa*I-*Rsa*I fragment (nt 7,120 to 7,508); the central *Rsa*I-*Rsa*I fragment (nt 7,509 to 7,738) and the proximal *Rsa*I-*Bam*HI fragment (nt 7,739 to 119). Figure modified from Bernard *et al*. (1989).

The distal *Bam*HI-*Rsa*I fragment (nt 6,930 to 7,119), which is 189 bp in size, is entirely contained within the L1 ORF. No studies have been performed to identify its significance in regards to P_{105} promoter regulation. The long *Rsa*I-*Rsa*I fragment (nt 7,120 to 7,508), which is 388 bp in size, is known as the distal enhancer but the precise function of this region is unclear (Cid *et al.*, 1993). There were previous studies

suggesting that it contributes about 25% of the basal level of P_{105} promoter activity induced by the full-length *Bam*HI LCR fragment (Garcia-Carranca *et al.*, 1988), and some other studies suggested it to be E6-responsive (Gius *et al.*, 1988; Hoppe-Seyler *et al.*, 1991).

The central *Rsa*I-*Rsa*I fragment (nt 7,509 to 7,738), which is 229 bp in size, is commonly known as the constitutive enhancer. Studies suggested that this enhancer element is active in cell lines of epithelial origin only (Garcia-Carranca *et al.*, 1988; Nakshatri *et al.*, 1990). Since there are no E2 binding sites (E2BS) present in the constitutive enhancer region, it was also suggested to be independent of regulation by the viral E2 protein (Bernard *et al.*, 1989). In fact previous studies have showed that the constitutive enhancer is active in both HPV-positive HeLa cells, as well as in SW13 cells, which is a human adrenocortical cells that is not associated with HPV (Garcia-Carranca *et al.*, 1988; Thierry *et al.*, 1987), thus supporting the idea that the activity of the constitutive enhancer is not dependent on the presence of viral DNA.

The proximal *Rsa*I-*Bam*HI fragment of 237 bp (nt 7,739 to 119) is known as the promoter proximal fragment, which was identified as E2-responsive (Gius *et al.*, 1988) and appeared to be highly repressed in the presence of the bovine papillomavirus type 1 (BPV-1) E2 gene product (Garcia-Carranca *et al.*, 1988). All three distinct regions of HPV-18 LCR are capable of independent enhancer function when tested with an enhancerless reporter plasmid containing a heterologous SV40 promoter (Gius *et al.*, 1988), and they work in a cooperative manner in the regulation of the P_{105} promoter downstream at the 3′-end of the LCR.

1.3.2 The P_{105} promoter

The promoter element of the LCR is found to initiate transcription at nt 105, which is the ATG transcription start codon of the downstream E6 ORF. For this reason it is generally known as the P_{105} promoter. The P_{105} promoter appears to be responsible for directing the expression of the E6 and E7 oncogenes (Romanczuk *et al.*, 1991; Thierry *et al.*, 1987). The mapping of transcripts encoding E6 and E7 in a range of cervical carcinoma cell lines harbouring the integrated HPV-18, all showed the initiation of transcription to be around nt 105 (Schneider-Gadicke and Schwarz, 1986; Thierry *et al.*, 1987). There have also been studies suggesting the presence of another potential promoter of the early viral proteins initiating transcription at position 56 within the LCR of the HPV-18 (Steger *et al.*, 2001). However, the P_{105} promoter is still considered to be the major early promoter responsible for the transcription of early viral genes.

The transcriptional activity of the P_{105} promoter is tightly regulated by a complex interplay between viral and cellular proteins which act as transcription factors binding to sites along the sequence of the LCR. The LCR contains binding sites for many known cellular transcription factors and four recognition sequences for the viral E2 protein.

There were many studies performed attempting to identify factors that determine the activity of transcription control elements acting on their corresponding promoter. Studies on the SV40 enhancer region suggested that proper spacing between different *cis*-regulatory elements could be highly important for their functional cooperation (Fromental *et al.*, 1988). Butz and Hoppe-Seyler (1993) later suggested that the activity of a given regulatory element within the enhancer region may also be strongly dependent on the overall composition of a transcriptional control region, i.e. on

the nature of potentially cooperating transcription factors. Other studies suggested that the activity of transcription control elements is dependent on their proximity to the promoter, similar to the E2-binding motifs within the LCR. The precise function of these transcription regulatory elements is discussed in Section 1.5.

1.4 Cellular transformation by early gene expression

With infections by the low-risk types of HPV, the viral DNA is often transcribed and replicated separately from the host chromosome as an episome. The viral regulatory protein E2 is being expressed (Hudelist *et al.*, 2004; Park *et al.*, 1997), which leads to the repression of the P_{105} promoter (regulation of the P_{105} promoter described in detail in Section 1.5) and thus the expression of the E6 and E7 genes are being suppressed and carcinogenic progression do not occur. There have also been study groups suggesting that the E6 protein expressed from low-risk HPV types have low binding affinity to p53 and does not lead to its degradation (Crook *et al.*, 1991; Foster *et al.*, 1994; Scheffner *et al.*, 1990).

However, cell transformation often results from an infection caused by high-risk types of HPV, where part of the viral genome has been integrated into the chromosomal DNA of the host cells (Cullen *et al.*, 1991; Pirami *et al.*, 1997). The integration of the viral DNA preferentially occurs within the E1 and E2 ORF resulting in their disruption (Bednarek *et al.*, 1998; Corden *et al.*, 1999; Kitagawa *et al.*, 1996; Rosales *et al.*, 2001) (see Figure 1.8). There are also reports suggesting that the E1 and E2 ORF are absent in all tumours positive for HPV-18 (Berumen *et al.*, 1995). In contrast the LCR, E6 and E7 ORF are found to be invariably intact in the integrated viral genome within malignant cells (Butz *et al.*, 2000; Steger *et al.*, 2001), and the E6 and E7 genes are found to be expressed within cervical cancer cell lines (Schwarz *et al.*, 1985). Disruption of the E2 ORF results in the absence of viral E2 protein expression, hence leading to the de-repression of the P_{105} promoter responsible for the expression of the viral E6 and E7 proteins (Yee *et al.*, 1985). According to the studies carried out by Rosales *et al*. (2001), the E6 and E7 proteins are expressed immediately after the

disruption or inactivation of the E2 gene. This suggests that the expression of E6 and E7 genes are a consequence of the downregulation of E2 proteins within HPV-induced carcinomas.

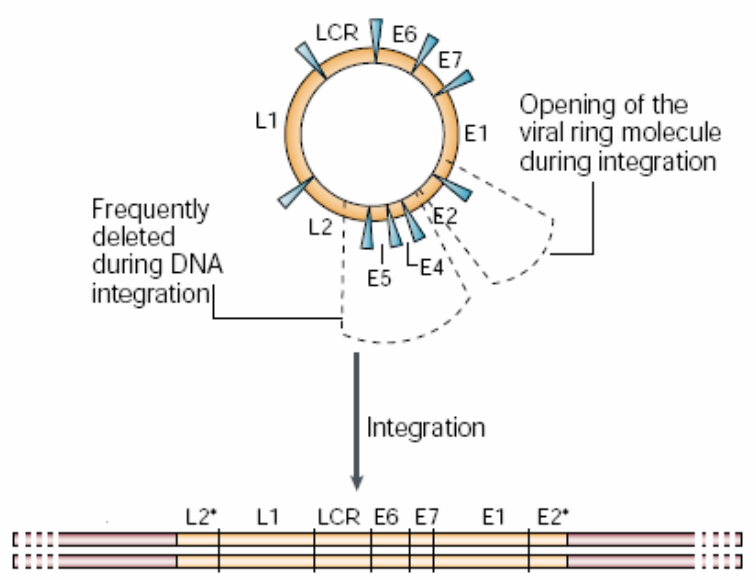

Figure 1.8 The integration of HPV DNA into the genome of the host cell.
In the course of cancer development, part of the HPV DNA frequently integrates into the genome of the host cell. The circular HPV DNA is often opened within the E2 ORF, resulting in the partial deletion of the E2 and L2 ORF (partial genes represented by an asterisk) and complete removal of the E4 and E5 ORF. The blue triangles indicate the approximate start/end of the labelled genes and the LCR within the HPV genome. Figure modified from zur Hausen (2002).

E6 proteins expressed from high risk HPV types interacts with the tumour suppressor protein p53 via the cellular ubiquitin-dependent proteolytic pathway resulting in p53 degradation (Scheffner *et al.*, 1993; Scheffner and Whitaker, 2003). The ubiquitin-dependent proteolytic pathway plays a major role in selective protein degradation. The HPV E6 oncoprotein binds to a cellular protein termed E6-associated

protein (E6-AP). The E6-E6-AP complex interacts with p53, resulting in the rapid ubiquitin-dependent degradation of p53 (Werness *et al.*, 1990). p53 functions in response to DNA damage. When cells are under genotoxic stress, the half life of p53 protein is significantly extended (Alarcon *et al.*, 1999; Geyer *et al.*, 2000; Shin *et al.*, 1996), resulting in an accumulation of p53 protein in the cells (Alarcon *et al.*, 1999; Clarke *et al.*, 1993; Inoue *et al.*, 2001; Kapoor and Lozano, 1998; Kastan *et al.*, 1991; Shin *et al.*, 1996). Upon stabilisation, p53 is activated which leads to cell cycle arrest (Geyer *et al.*, 2000; Kastan *et al.*, 1991; Shin *et al.*, 1996; Yin *et al.*, 1992) or apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Shaw *et al.*, 1992), depending on the severity of the DNA damage, cell type and cellular environment (Bates and Vousden, 1996; Vousden and Lu, 2002). When cellular DNA is damaged or mutated, the cell cycle is normally arrested at *G1* and p53 activates the expression of cellular genes involved in DNA repair. Once damaged DNA is repaired, the cell cycle resumes. If the extent of DNA damage is too great, the cell undergoes apoptosis. However, in HPV-infected cancer cells, p53 is often non-functional and degraded due to the interaction with the E6-E6-AP complex. This allows the accumulation of genetic mutations and will eventually lead to deregulated cell growth and malignant tumour formation (Scheffner *et al.*, 1990). In addition, E6 expressed from high risk HPVs is also associated with the induction of telomerase activity (Damania, 2007; Kim *et al.*, 2007; Liu *et al.*, 2007), an enzyme that synthesises the telomere repeat sequences. Activating this enzyme leads to malignancy as the mutant cells continue to reproduce without control (Reddel, 2003).

E7 interacts with the retinoblastoma protein (pRb), which is also a tumour suppressor protein. pRb binds to the transcription factors necessary for the progression through the cell cycle, preventing the cells from dividing until it has bound sufficient transcription factors for cell division. The important protein to which pRb binds is E2F,

forming the pRb-E2F complex. The E2F proteins control the transcription of a number of cellular genes necessary for *S*-phase entry and progression (Chien *et al.*, 2002). The pRb-E2F complex acts as a transcriptional repressor, while free E2F activates transcription from promoters containing E2F binding sites. During HPV infection E7 can bind to and inactivate pRb, disrupting the pRb-E2F complex and releasing free E2F, thus deregulating the repressive function of pRb in cell cycle progression (Hwang *et al.*, 2002). This results in a cycle of uncontrolled cell proliferation leading to malignant diseases.

The differences regarding the oncogenic potential of the high-risk and low-risk types of HPV appear to correlate with the functional differences between their oncoproteins. E6 and E7 proteins from high-risk types of HPV possess higher binding affinity to p53 and pRb tumour suppressor proteins respectively (Hwang *et al.*, 2002). Both E6 and E7 (from high risk HPV) can immortalise human cells independently, but their co-expression strongly increases their transforming potential, indicating their functional cooperativity (Hawley-Nelson *et al.*, 1989; McDougall, 1994; Munger *et al.*, 1989). The two oncoproteins work cooperatively to induce cell cycling/cell division and to overcome the *G1/S* and G2 checkpoints in the DNA-damaged cells (zur Hausen, 2000), resulting in an anti-apoptotic effect and increased chance of accumulation of genetic mutations, which can result eventually in the progression to full malignancy (Nishimura *et al.*, 2000). Virtually all HPV-positive cervical neoplasia specimens (Nakagawa *et al.*, 2000; Rosales *et al.*, 2001; Schwarz *et al.*, 1985) contain the E6 and E7 proteins. The expression of both viral proteins are necessary for the efficient immortalisation of human squamous epithelial cells (Hawley-Nelson *et al.*, 1989; Hudson *et al.*, 1990; Kaur *et al.*, 1989; Munger *et al.*, 1989), as well as for the

maintenance of the transformed phenotype of cervical cancer cells (Crook *et al.*, 1989; von Knebel Doeberitz *et al.*, 1992).

1.5 Transcriptional control of the long control region

The activity of the P_{105} promoter of HPV-18 is tightly regulated by a complex interplay between viral and cellular proteins which act as transcription regulatory elements binding to recognition motifs along the sequence of the LCR (Figure 1.9).

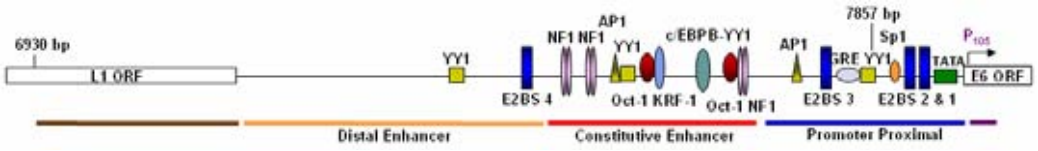

Figure 1.9 Binding sites for viral and cellular transcription factors within the LCR.

The diagram shows the 1,050 bp (nt 6,930 to 119) *Bam*HI fragment of HPV-18, which contains the LCR fragment in between the L1 and E6 ORF. The crooked arrow represents the transcription start site of the P_{105} promoter upstream of the E6 ORF (blue line representing the promoter proximal region).

1.5.1 E2 binding sites

The viral E2 protein plays an important regulatory role in the activity of the P_{105} promoter. It binds as a dimer to a 12-bp palindromic sequence, $ACCN_6GGT$, which is found four times in the LCR of all genital HPV (Rapp *et al.*, 1997). Three of the E2BSs are located within the promoter proximal fragment of the LCR (E2BS#1 to 3), while the fourth one lies within the distal enhancer fragment (E2BS#4) (see Figure 1.9). These E2BS appeared to be highly conserved in their relative positions among different types of HPV (Demeret *et al.*, 1994; Rapp *et al.*, 1997).

The E2 protein is the only viral product thought to regulate HPV transcription, and is crucial in determining the level of expression of the E6 and E7 oncogenes (Bednarek *et al.*, 1998; Schwarz *et al.*, 1985). Extensive studies have been performed on

the E2BSs in an attempt to understand the precise mechanism of E2 modulation of the P_{105} promoter. It has been well established that E2 can repress the expression of the E6 and E7 oncoproteins from their promoter (Bernard *et al.*, 1989; Hirochika *et al.*, 1987; Hirochika *et al.*, 1988; McBride *et al.*, 1998; Thierry and Howley, 1991). Recent studies performed on the LCR of HPV-16 by Soeda *et al*. (2006) confirmed that when expressed from the viral genome, E2 is primarily a repressor of the P_{97} promoter; this repression involves the E2BS#1, 2 and 3, and both the DNA binding and transactivation functions of E2. They also found no evidence that P_{97} is strongly activated by E2.

Similarly, results from previous mutation studies performed on HPV-18 suggest that, upon the induction of E2 expression, all three E2BSs within the promoter proximal region (E2BS#1, 2 and 3) are responsible for full repression of LCR promoter activity. Repression appeared to be mediated mainly though E2 binding to the promoter proximal E2BS#1 and E2BS#2, while the E2BS#3 also contributed to maximal transcription repression (Demeret *et al.*, 1997). In contrast, the binding of E2 to the distal E2BS#4 only weakly affects transcription activity of the P_{105} promoter (Demeret *et al.*, 1994). E2BS#2 and #1 form a tandem repeat located just 3 bp upstream of the TATA box of the P_{105} promoter, and was found to induce E2-mediated repression through steric hindrance with the proteins binding to the TATA box and the Sp1 binding site (Demeret *et al.*, 1994; Dostatni *et al.*, 1988). This is due to the fact that E2BS#1 functionally overlaps with the TATA box downstream, while E2BS#2 functionally overlaps with the Sp1 binding site upstream. The binding of E2 to these E2BSs displaces the TATA box-binding proteins and Sp1 from their recognition motifs, thus repressing transcription from the P_{105} promoter (Dong *et al.*, 1994; Dostatni *et al.*, 1991; Tan *et al.*, 1992). This correlates very well with the situation of infection by low-risk types of HPV, when the viral DNA exists within the host cell in the form of an

episome, the viral E2 protein is continually expressed thus repressing the activity of the P_{105} promoter and the expression of the E6 and E7 oncogenes. Low-risk HPV infections are therefore not associated with malignancy.

However, some studies also suggested that E2 can function both as a transcriptional activator at low concentrations or repressor at high concentrations, depending on the differential occupancy of the four E2BSs within the LCR (Bernard *et al.*, 1989; Bouvard *et al.*, 1994; Dell *et al.*, 2003; Doorbar, 2006; Grm *et al.*, 2005), which is directly related to the affinity of E2-binding for the individual E2BS (Hou *et al.*, 2002; Moskaluk and Bastia, 1988). However, observations regarding the affinity of protein binding to different E2BSs have not been consistent. The studies performed by Romanczuk *et al*. (1990) showed that the E2BS#3 has a much lower affinity for protein binding when compared to the other three E2BSs within the LCR of HPV-16. Several studies have then suggested the promoter proximal E2BSs have lower affinity for the E2 protein than those located further upstream within the LCR (Jackson and Campo, 1995; Sanders and Maitland, 1994; Steger *et al.*, 1995). Later studies performed by Demeret *et al*. (1997) then showed that E2 proteins are associated with the four E2BSs in the HPV-18 LCR with similar affinities.

That aside, the HPV-16 P_{97} promoter and the HPV-18 P_{105} promoter have always been thought to be regulated by similar mechanisms by viral E2 proteins (Romanczuk *et al.*, 1990), because a comparison of the sequences upstream showed a similar spatial arrangement of the four E2BSs, the TATA boxes and the transcription start sites (Figure 1.10).

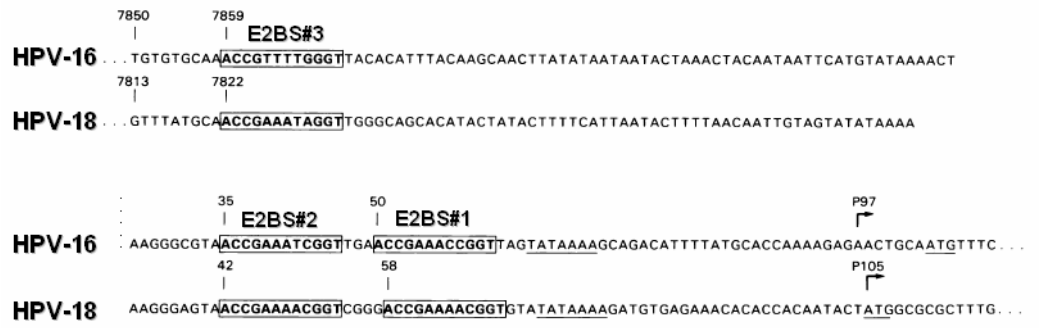

Figure 1.10 Comparison of the HPV-16 and 18 sequences upstream of their respective P_{97} and P_{105} promoters.

The positions of E2BS are boxed, and the TATA boxes relative to the early promoter downstream are underlined. The P_{97} and P_{105} promoters of HPV-16 and 18 respectively are indicated by the crooked arrows, and their ATG transcription start codons are underlined. Similarity in their spatial arrangement suggests that the promoters of HPV-16 and 18 may be regulated by similar mechanisms by the E2BS. Figure modified from Romanczuk *et al*. (1990).

In general the sequences and spatial arrangement of the E2BS, in their relative positions to the early promoter downstream, were found to be very similar in between the two major types of low-risk (HPV-6 and 11) and high-risk (HPV-16 and 18) HPV. The only exemption was that the E2BS#3 of HPV-16 and 18 did not appear to be a perfect palindrome, when compared to HPV-6 and 11 which have all four E2BS in perfect palindromic sequences of $ACCGN_4CGGT$ (Garcia-Carranca *et al.*, 1988) (see Figure 1.11). It was suggested that this corresponds to the differences in E2-mediated transcriptional regulations in between the two groups of genital HPV. This may also be an explanation to the lower binding affinity of the E2BS#3 as observed by Romanczuk *et al*. (1990) as mentioned previously.

	E2BS#4	E2BS#3	E2BS#2	E2BS#1
HPV-6b (7,902 bp)	**ACCG**ttttCGGT…**ACCG**gtttCGGT…**ACCG**aaaaCGGTTca.			**ACCG**aaaa**CGGT**
	(-456)	(-143)	(-67)	(-52)
HPV-11 (7,931 bp)	**ACCG**ttttCGGT…**ACCG**gtttCGGT…**ACCG**aaaaCGGTtca.			**ACCG**aaaa**CGGT**
	(-441)	(-143)	(-67)	(-52)
HPV-16 (7,904 bp)	**ACCG**aattCGGT…**ACCG**attttGGg…**ACCG**aaatCGGTtga.			**ACCG**aaac**CGGT**
	(-558)	(-151)	(-69)	(-54)
HPV-18 (7,857 bp)	**ACCG**atttCGGT…**ACCG**ttttaGGT…**ACCG**aaagCGGTcggg			**ACCG**aaaa**CGGT**
	(-504)	(-140)	(-63)	(-47)

Figure 1.11 Comparison of the sequences of E2 palindromes in the LCR of HPV-6, 11, 16 and 18.

HPV-6 and 11 are the two major low-risk HPV associated with benign lesions, whereas HPV-16 and 18 are high-risk HPV associated with cancer. All four E2BS within these genital HPVs are perfect palindromes of $ACCGN_4CGGT$, apart from the two E2BS#3 of HPV-16 and 18. This may account for the differences in E2-regulation of the two groups of HPV, as well as a possible explanation to the lower binding affinity observed at the E2BS#3 of HPV-16. In bold and caps are the bases matching with the perfect palindromic sequence. Numbers in brackets represent relative positions of the E2BS to the promoter downstream, assuming the start of transcription occurs at nt 1. Figure modified from Garcia-Carranca *et al*. (1988).

As mentioned previously in Section 1.4, the E2 gene is often found to be disrupted upon the integration of high-risk HPV into the host genome. In this case early gene promoter activity appeared to be regulated entirely by cellular transcription factors. The precise role of E2 in the regulation of the P_{105} promoter in LCR, in the context of HPV-infected cervical cancer cells, is difficult to be determined. However, studies performed on BPV-1 suggested that the binding of full-length E2 protein to various E2BSs may transactivate the HPV promoter, whereas truncated E2 proteins may act as a repressor instead (Androphy *et al.*, 1987; Choe *et al.*, 1989; Lambert *et al.*, 1987; Moskaluk and Bastia, 1987). Demeret *et al*. (1997) also proposed that a N-terminally truncated form of HPV-18 E2 protein repressed transcription more efficiently than the

full-length protein. In the context of genital HPV infection, although truncated forms of E2 protein has not been reported, it is theoretically possible for a truncated E2 protein to be expressed, as E2 ORF is often disrupted during integration of the viral DNA. In fact a recent review article by Shillitoe (2006) also suggested that in tumour cells from HPV-associated cancers, the viral sequence integrated into the host cell chromosome preserves the LCR, E6 and E7 genes, as well as the 5′-end of the E2 ORF. A truncated form of E2 protein is therefore suggested to be expressed and together with other cellular transcription factors, they act on the viral promoter in the LCR to control the expression of E6, E7 and E2. It should also be noted that there have been studies reporting the presence of intact E2 protein present in pre-malignant lesions (Durst *et al.*, 1992; Matsukura *et al.*, 1989), which suggests that the disruption of the E2 ORF is often a late event in cancer formation and the possible role of E2 in the regulation of oncogene expression.

It is therefore logical to propose that the E2BSs within the LCR do not only play an important role in the viral DNA replication, but may also be involved in the regulation of oncogene expression from the P_{105} promoter within HPV-infected cervical cancer cells. It is, however, important to take into account the fact that E2-mediated promoter regulation can be modulated by other *cis*-regulatory elements present in the HPV-18 LCR. Conclusions on the precise mechanism of P_{105} promoter regulation by E2 within HPV-infected cells cannot be drawn without understanding the nature of transcription factors binding to the LCR and their possible interaction with the viral E2 proteins.

1.5.2 Various transcription factor binding sites

Apart from the viral E2 protein, cellular transcription factors also play a very important role in the regulation of the HPV-18 P_{105} promoter activity, and they bind to recognition sequences which are commonly known as the *cis*-regulatory elements. The locations of various transcription factor binding sites are shown in Figure 1.9. Most of the sites identified to date are located within the constitutive enhancer and promoter proximal fragment of the LCR. These include binding sites for nuclear factor 1 (NF1), activator protein 1 (AP1), *Ying Yang* 1 (YY1), octamer-binding protein (Oct-1), keratinocyte response factor (KRF-1), specific promoter factor (Sp1) and also a glucocorticoid response element (GRE). Previous studies have identified some of the properties of these transcription factor binding sites in regards to their contribution towards the P_{105} promoter activity and specificity. However, the overall promoter regulation is a very complex interplay of ubiquitous and cell-type specific transcription factors, which leads to the tissue and differentiation specific activation of the HPV-18 promoter. It is impossible to mention every study for each individual transcription factor binding site within the LCR of HPV-18. Below is a summary of significant findings regarding the contribution of each protein binding elements towards the activity and specificity of the P_{105} promoter.

(i) Nuclear factor 1

There are three NF1 binding sites identified within the constitutive enhancer of the LCR by footprinting studies performed by Gloss *et al*. (1989). Although the NF1 consensus motif was identified to be 5′-TTGGCTN_3AGCCAA-3′ (Jones *et al.*, 1987), the two distal NF1 binding sites (nt 7,513 to 7,527 and nt 7,569 to 7,583) did not display the properties of such consensus sequence, while the third NF1 site at the proximal end

of the constitutive enhancer only contains half of the recognition motif (Butz and Hoppe-Seyler, 1993). Studies performed on the three NF1 sites within the HPV-18 LCR suggested that both individual and combined mutations resulted in only a slight decrease of the P_{105} promoter activity induced within HeLa cells (HPV-18 positive human cervical carcinoma cell line) (Butz and Hoppe-Seyler, 1993). These results suggested that NF1 does not play a crucial role in the regulation of the P_{105} promoter in the context of HPV-infected cervical cancer cells.

(ii) Activator protein 1

The two AP1 binding sites within the LCR of HPV-18 were first identified by Garcia-Carranca *et al*. (1988) by DNase I footprinting assay, one within the constitutive enhancer region (nt 7,608 to 7,614) and the another one within the promoter proximal region (nt 7,792 to 7,798). It contains a recognition sequence of 5′-TGACTAA-3′. Mutation studies performed individually on the two sites significantly reduced the level of P_{105} promoter activity within HeLa cells (Butz and Hoppe-Seyler, 1993), suggesting that the AP1 binding sites are very strong transcriptional activators of the P_{105} promoter in the context of HPV-infected cervical cancer cells. The two AP1 sites were also suggested to functionally cooperate (Mack and Laimins, 1991), since mutation of one of the AP1 binding sites located within the promoter proximal fragment resulted in a downregulation of enhancer activity. This downregulation was suggested to be a result of the loss of cooperatively with the other AP1 binding site located within the constitutive enhancer region (Bauknecht et al., 1992). The enhancer activity induced by AP1 was also reported to be directly repressed by silencer elements which bind the YY1 proteins within the LCR of HPV-18 (Bauknecht *et al.*, 1992).

In terms of promoter specificity, AP1 was shown to be a key regulator for epithelial cell-specificity of the promoter (Mack and Laimins, 1991). Higher binding affinity of AP1 was observed in cervical carcinoma cell lines both positive (HeLa) and negative (C33A) for HPV-18, as well as in human keratinocytes positive for HPV-16 (Angel and Karin, 1991; Prusty and Das, 2005).

(iii) Ying Yang 1

The name of YY1 protein relates to the ability of YY1 being able to repress or activate transcription depending on the context of the promoter. There are three YY1 binding sites identified within the HPV-18 LCR, one within the distal enhancer region (nt 7,441 to 7,449), one within the constitutive enhancer region (nt 7,610 to 7,618) which overlaps the 3′-end of a AP1 binding site (Bauknecht and Shi, 1998), and one within the promoter proximal region (nt 7,847 to 12) which overlaps with the 3′-end of a GRE binding site (Bauknecht *et al.*, 1992).

Initial studies of the YY1 site in the promoter proximal fragment suggested it to be a strong repressor on the P_{105} promoter activity by repressing the activation induced by AP1 binding to the constitutive enhancer region. Mutation on the promoter proximal YY1 site resulted in resulted in enhanced promoter activity (Bauknecht *et al.*, 1992; Shi *et al.*, 1991). Later studies discovered that the activity of the promoter proximal YY1 is determined by a C/EBPβ-YY1 switch region located upstream within the constitutive enhancer (nt 7,710 to 7,718) (Bauknecht *et al.*, 1995). In the absence of an intact switch region, YY1 acts as a repressor of the LCR. A double mutation of the C/EBPβ-YY1 switch region and the promoter proximal YY1 site, however, completely abolished P_{105} promoter activity induced from the LCR. Mutations performed on the other two YY1 sites showed no effect on the P_{105} promoter activity.

An important point to note is that two of the YY1 sites in the LCR appeared to be overlapping with AP1 and GRE binding sites in the distal enhancer and promoter proximal region respectively, hence possible interference by YY1 on the binding of AP1 and GRE to these sites may also occur.

(iv) Octamer-binding protein

A recognition motif for an octamer-binding protein, Oct-1, was first found located close to the half-palindromic NF1 recognition sequence within HPV-16 LCR by Chong *et al*. (1991). The site was found to contribute significantly towards enhancer function in HPV-16, for upon deletion promoter activity was strongly reduced (Chong *et al.*, 1991; Morris *et al.*, 1993). In the context of HPV-18, a corresponding sequence of 5′-AATTGCAT-3′ (nt 7,721 to 7,728) was found just 2 bp upstream of the half NF1 motif within the constitutive enhancer. Mutational analysis on this site, however, resulted only in a slight decrease of P_{105} promoter activity within HeLa cells (Butz and Hoppe-Seyler, 1993), indicating this Oct-1 binding site is not crucial for promoter activation in the physiological context of cells infected by HPV-18, and the binding of Oct-1 proteins appeared to be positively regulating the promoter downstream.

Oct-1 was also found to bind at low affinity to another footprint mapped by Garcia-Carranca *et al*. (1988) from nt 7,644 to 7,657, which overlaps with the 5’ portion of a KRF-1 footprint identified further downstream but also within the constitutive enhancer. Since the binding of Oct-1 and KRF-1 to this site appeared to be mutually exclusive, competitive binding was suggested to contribute to the cell-type specific promoter activation of HPV-18 (Mack and Laimins, 1991).

(v) Keratinocyte response factor

A KRF-1 footprint (nt 7,648 to 7,669) has been identified within the constitutive enhancer region of the LCR, which slightly overlaps with a low affinity Oct-1 binding site. The binding of KRF-1 to this site has shown to contribute significantly to the epithelial cell-type specificity of the constitutive enhancer (Mack and Laimins, 1991). Studies performed by Butz and Hoppe-Seyler (1993) further defined that the activity of KRF-1 can vary significantly between different epithelial cell types, with a mutation at this binding site resulting in the strongest reduction of P_{105} promoter activity in primary keratinocytes, and the least reduction in HeLa cells. This suggested that by abolishing KRF binding the LCR resulted in increased specificity towards HPV-positive cervical cancer cells.

(vi) Glucocorticoid response element

The GRE motif 5′-AGCACAT ACTATACT-3′ (nt 7,839 to 7,853) within the promoter proximal region of the LCR can positively regulate promoter activity when induced by glucocorticoids such as dexamethasone or progesterone. This responsiveness to progesterone was also suggested to be a possible explanation for the higher incidence of malignant HPV lesions in women than in men, for oncogene expression from HPV would go through recurrent boosts during part of the ovulation cycle and also during pregnancy when the level of progesterone in the female body increases (Chan *et al.*, 1989).

Mutation studies performed on the GRE, however, significantly increased the basal level of P_{105} promoter activity by up to 2-fold in HeLa cells, while at the same time completely abolished the hormonal response of the promoter when tested with increasing doses of dexamethasone (Butz and Hoppe-Seyler, 1993). This suggested that

the GRE within the LCR induces significant repression on the P_{105} promoter which appeared to be independent of the binding of glucocorticoid or other hormonal elements.

(vii) Specific promoter factor

Sp1 binds to a G-rich recognition motif 5′-GGAGT-3′ (nt 35 to 40) within the promoter proximal region of the LCR, which contains two mismatches to the Sp1 consensus recognition sequence 5′-GGGCGG-3′ (Hoppe-Seyler and Butz, 1992). Mutation lead to a strong reduction in the P_{105} promoter activity from the LCR in HeLa cells (Butz and Hoppe-Seyler, 1993), indicating that the Sp1 element contributes significantly to strong promoter activation. The promoter proximal region, however, was shown to induce only weak promoter activity (Hoppe-Seyler *et al.*, 1991; Thierry *et al.*, 1987). This suggested that the Sp1 binding site has to functionally cooperate with other *cis*-regulatory elements upstream in the distal and constitutive enhancer regions of the LCR (Butz and Hoppe-Seyler, 1993). However, studies performed by Rose *et al*. (1998) also suggested that naturally occurring mutations in the Sp1 motif may result in an elevated level of binding affinity by the Sp1 protein, thus enhanced transcription by up to 4-fold in HeLa cells.

The binding of Sp1 to its promoter proximal recognition motif has been reported to be sterically hindered by the binding of viral E2 proteins to the promoter proximal E2BS#1 as well as the TATA box-binding protein. (Dostatni *et al.*, 1988; Tan *et al.*, 1992). However, it was suggested by Demeret *et al*. (1994) that this repression could be compensated and transcription restored by E2 binding to the E2BS#2 which is located only 1 bp downstream and functionally overlaps the Sp1 recognition motif. This was in agreement with earlier studies suggesting that Sp1 can functionally cooperate with viral E2 protein to induce transactivation of the HPV promoter (Ham *et al.*, 1991;

Li *et al.*, 1991). This result also suggested the possibility of the viral E2 protein binding to other *cis*-regulatory elements within the LCR, resulting in P_{105} promoter activation.

By gathering the results from previous studies, it is therefore possible to identify some of the *cis*-regulatory elements which possess positive or negative regulatory effects on the transcriptional activity induced from the P_{105} promoter within the LCR. The sequences and exact locations of the previously mentioned transcription control elements are shown in Figure 1.12.

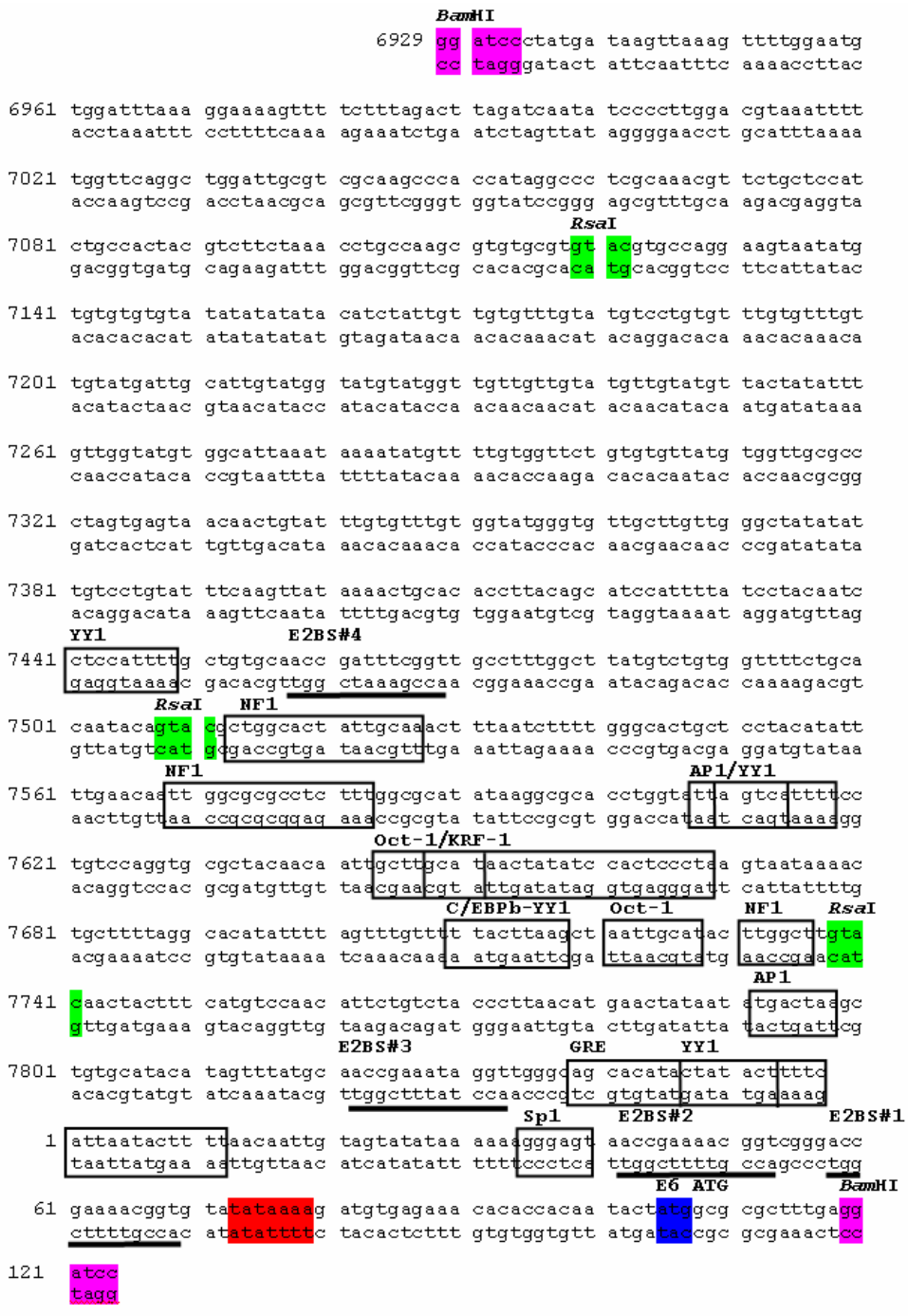

Figure 1.12 Sequences of transcription factor binding sites within the LCR.

The 1,050 bp *Bam*HI (highlighted in purple) fragment contains the entire LCR separated into functional domains by *Ras*I sites (highlighted in green). Transcription factor binding sites mentioned in Section 1.5.2 are labelled and boxed. The TATA box is highlighted in red and the ATG start codon of the E6 ORF is highlighted in blue.

1.6 Gene therapy for HPV-associated cervical cancer

1.6.1 Different approaches of gene therapy for cervical cancer

With advances in the understanding of the pathogenesis of cervical cancer and the regulation of oncogene expression within HPV-infected cells, it is possible to develop gene therapies which may selectively target the virally-infected cells. Possible approaches for the gene therapy of HPV-associated cervical cancer has been reviewed by Shillitoe (2006) (Figure 1.13).

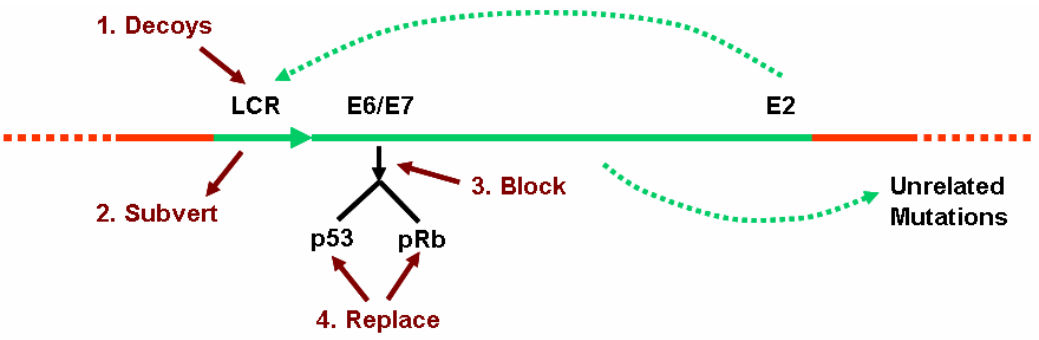

Figure 1.13 Gene therapy approaches to HPV-associated cervical cancer.
In cervical cancer cells, part of the HPV DNA sequences (green) are integrated into the host cell chromosome (orange), with the LCR, the E6 and E7 genes and the 5′-end of the E2 gene being preserved. The truncated E2 protein, together with cellular transcription factors, act on the LCR to promote the expression of E6, E7 and E2. The E6 and E7 genes inhibit the function of the p53 and pRb genes respectively. Approaches to gene therapy (brown) include: 1. Decoy proteins to inhibit the function of the enhancer/promoter elements of the LCR; 2. subversion by the use of the LCR to control the expression of antitumour genes; 3. blocking of the expression of antisense, ribozymes or siRNA, and 4. replacement of the missing p53 and pRb proteins. Figure reproduced from Shillitoe (2006).

Various gene therapy approaches to HPV-associated cervical cancer can be generally classified into four main groups. As mentioned in Section 1.5, the activity of the P_{105} promoter of HPV-18 is regulated by viral proteins and cellular transcription factors binding to recognition motifs along the sequences of the LCR. It is therefore possible to make use of decoy factors that binds to the LCR as a substitution of specific transcription factors, to produce an adverse effect on promoter activation/expression of oncogenes. Previous studies performed by Hwang *et al.* (1993) and Hwang *et al.* (1996) have demonstrated that the expression of the BPV-1 E2 protein in HeLa cells resulted in an acute and profound decrease in cellular proliferation and a dramatic inhibition of HPV-18 E6/E7 expression. However, the implication of these findings in the development of a gene therapy for cervical cancer has remained unexplored.

The second approach to cervical cancer gene therapy is subversion by the use of the HPV LCR, which is to make use of cell-type specificity of the LCR to direct cervical cancer-specific expression of therapeutic or suicide genes. Promoter elements that have been proposed and studied for similar use in oral cancer gene therapy include the promoters of human cytomegalovirus (CMV), Simian Virus 40 (SV40), mouse mammary tumour virus (MMTV), HPV-16 and 18, and the multi-drug-resistance gene (*mdr*1) (Shillitoe and Noonan, 2000). Tissue-specific promoters are therefore ideal elements to be used for the selective targeting of cervical cancer cells. However, few studies have explored this possibility for cervical cancer (Lim *et al.*, 2004). This project was set out to explore the possibility of using the LCR of HPV-18 as a tool to induce selective gene expression within HPV-associated cervical cancer cells, with more details in regards to this approach in Section 1.7.

As the development of cervical cancer is associated with the expression of the E6 and E7 oncogenes, the third approach is to block the expression of E6 and E7 by

making use of DNA-based therapeutics such as antisense RNA, ribozymes or small-interfering RNAs (siRNAs) (Patil *et al.*, 2005). Antisense RNA molecules bind to the RNA transcripts of the target genes resulting in duplex formation and degradation. The use of antisense RNA has been tested by von Knebel Doeberitz *et al*. (1988) in C-4 I cervical cancer cells (HPV-18 positive and expresses HPV-18 RNA) and by Steele *et al*. (1992) in other HPV-positive cervical and oral cancer cells, which resulted in the elimination of many malignant phenotype of the HPV-associated cancer cells. This approach has also been shown in a mouse model by He and Huang (1997) to result in the inhibition of tumour growth by downregulating the expression of E6 and E7. Ribozymes work in a similar fashion which compromises antisense molecules with secondary structures that provide enzymatic ability to cleave the target molecules. Anti-HPV ribozymes have been demonstrated by Chen *et al*. (1996) and Alvarez-Salas *et al*. (1998) to successfully inhibit the transformed phenotype of HeLa cells and prevent immortalisation of cells by HPV-16, respectively. The advances on the use of antisense RNA and ribozymes led to the development of siRNAs, which are typically 21 to 23-nucleotide double-stranded RNA segments, designed to be used for the downregulation of the oncogenes through RNA interference (RNAi). The silencing of E6 by siRNAs has been shown to induce apoptosis of HeLa cells (Butz *et al.*, 2003) and increase the sensitivity of cancer cells to chemotherapy (Koivusalo *et al.*, 2005). Overall, the use of siRNAs in gene therapy is a relatively new invention with many possibilities yet to be explored.

Since the expression of E6 and E7 in cervical cancer cells results in the loss of function of the tumour suppressor proteins p53 and pRb respectively, another approach of gene therapy is to restore the expression of functional p53 and pRb within the cervical cancer cells. However, this method tends to be difficult to control in terms of

the expression level and specificity, as overexpression of p53 is toxic to cells and independent to the presence of HPV DNA (Ahn *et al.*, 2002). The potential use of p53 and pRb as gene therapy has been examined for many types of cancers, quite apart from those that are associated with HPV, and has progressed to the stage of multiple human trials but with limited success (McNeish *et al.*, 2004). Results from clinical trials have not mirrored the preclinical studies.

1.6.2 Delivery of anti-HPV gene therapy

Apart from the different approaches to target HPV-associated cervical cancer cells, another significant problem associated with gene therapy is the delivery of therapeutic molecules into the tumour of a patient. DNA delivery methods can be classified into physical application using electrical and mechanical techniques, and viral and non-viral vector-assisted delivery systems.

Since HPV-associated cervical cancers develop in the basal layer of epithelial cells, the most obvious and direct way to transfer DNA to the site of a tumour would be by physical application. Examples of electrical and mechanical strategies include microinjection which is highly efficient but time-consuming as it targets one cell at a time (McAllister *et al.*, 2000). Particle bombardment of DNA-coated gold beads can also be achieved using gene guns, which has, however, not shown sufficient efficiency in previous study using oral cancer cells (Shillitoe *et al.*, 1998). The Helios® gene gun from Bio-Rad Laboratories has been designed to assist the delivery of therapeutic plasmids for gene therapy in animal models, and is yet to be tested for its efficacy in the treatment of human cancers. Mechanical transfection by electroporation uses high-

voltage electrical current to facilitate DNA transfer, which results in high cell mortality and is not suitable for clinical use (Patil *et al.*, 2005).

Most of the studies performed in regards to gene therapy for cervical cancer have been relied on the utility of viral vector delivery systems (Green et al., 2006; Lim et al., 2004; Song et al., 2003). Adenoviral vectors have been suggested to be capable of inducing transgene expression in a wide range of tissues for a relatively long period of time. The use of adenoviral vectors in gene therapy has also shown promising results in preclinical studies and Phase I clinical trials ranging from cystic fibrosis to Parkinson's disease (Li et al., 2005). In the context of cervical cancer, however, problems such as low infection efficiency and lack of tissue specificity were observed (Kawakami *et al.*, 2004; Kawakami *et al.*, 2005), thus limiting the efficacy of adenovirus-mediated gene therapy for the treatment of cervical cancer. Moreover, the use of viruses to deliver and integrate DNA into host cells in gene therapy is unavoidably associated with potential dangers such as triggering undesirable cell-mediated immune responses (Ferber, 2001; Glover *et al.*, 2005). Hence the development of safer, non-viral gene delivery approaches for cervical cancer gene therapy would be ideal.

Non-viral technologies consist of plasmid-based expression systems containing a gene encoding a therapeutic protein and synthetic gene delivery systems (Rolland, 1998). Commonly used non-viral gene vectors are in forms of DNA-polymer complexes and DNA entrapped in and/or complexed to liposomes (Patil *et al.*, 2005). These systems do not carry the risk of developing adverse immune responses, and are easy to formulate and assemble (Merdan *et al.*, 2002). Non-viral vectors, however, are generally lower in transfection efficiencies when compared to viral vectors. Further advances in the development of safe and efficient DNA delivery platforms will be

required to assist the successful implementation of gene therapy to target HPV-associated cervical cancer cells.

1.7 Aims of the project

Efforts to improve gene therapy strategies over the past years were mainly aimed at solving the problem of delivery, without paying much attention to the optimisation of the expression cassette (van Gaal *et al.*, 2006). With the current understanding of the eukaryotic transcription machinery and advanced molecular biology techniques at our disposal, it is possible to create custom-made transgene expression cassettes optimised for gene therapy applications.

In this study the focus was on the P_{105} promoter of the HPV-18 LCR and cervical cancer. The aim of this project is to investigate the regulation of the P_{105} promoter within the HPV-18 LCR, with an attempt to develop a strategy to induce selective gene expression within cervical cancer cells infected by HPV, which could be a possible approach for gene therapy. It has already been proven that a continuous expression of the HPV E6 and E7 genes are necessary factors for the malignant phenotype of HPV-positive cervical cancers (von Knebel Doeberitz *et al.*, 1992), and the HPV-18 LCR contains enhancer and promoter elements responsible for driving the expression of the E6 and E7 oncogenes (Cid *et al.*, 1993; Romanczuk *et al.*, 1991; Thierry *et al.*, 1987). It was therefore logical to hypothesise that the promoter element of the HPV-18 LCR is active within HPV-infected cervical cancer cells, and could be used to direct carcinoma-specific expression of appropriate therapeutic genes. The HPV-18 LCR may not appear to be a very potent promoter element for the induction of a high level of gene expression in eukaryotic cells. However, the HPV promoter possesses unique specificity for squamous cells which was not observed from the other more potent promoters such as the SV40 and CMV promoter (Shillitoe and Noonan, 2000).

The first part of the project involved studies performed on the wild-type HPV-18 LCR to determine its suitability to be used as a candidate promoter. The full-length LCR was cloned into an expression vector for promoter analysis, and transiently transfected into HPV-positive and HPV-negative cervical cancer cells as well as other control mammalian cell lines. The level of gene expression induced from the promoter element of the LCR was utilised to determine the reference level of promoter activity and specificity. A series of LCR deletion constructs were then produced to identify the locations of important transcription control elements, and to examine the effect of removing different functional regions from the LCR in different cell types.

The second part of the project was an attempt to further increase the level of promoter activity and enhance promoter specificity within HPV-infected cervical cancer cells. This involved the construction of plasmids containing mutations at important transcription control elements, with attempts to abolish possible transcriptional repression and increase promoter specificity. These LCR mutation constructs were again tested for their abilities to induce gene expression in different mammalian cells. By means of creating a HPV-18 LCR expression vector with maximised promoter activity and specificity, which can be selectivity expressed within HPV-infected cervical cancer cells but remains silent or under-expressed in other cell types, will thus provide the basis of possible gene therapy for targeting cervical cancer.

The ultimate aim of the project is to develop a tool for a gene therapy treatment of cervical cancer. This can be accomplished by substituting the reporter gene in the HPV-18 LCR expression vector with a suicide gene. A suicide gene can be any gene that confers a suicidal phenotype in the target cells upon its expression. By selective expression of the suicide gene induced by the HPV-18 LCR, cervical cancer cells can be selectively destroyed

CHAPTER 2

MATERIALS AND METHODS

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2.1 Materials

2.1.1 General materials and chemicals

Ammonium persulfate, ethylaminediaminetetra acetic acid (EDTA), mineral oil, *o*-nitrophenyl β-D-galactopyranoside (ONPG), phenol and sodium dodecyl sulfate (SDS) were all purchased from Sigma-Aldrich, USA. Adenosine triphosphate (ATP) was obtained from Progen, Australia. Calcium chloride, potassium acetate, sodium acetate, sodium chloride and tris (hydroxymethyl) aminomethane were purchased from BDH Chemicals, Australia. Chloroform, ethanol, glycerol, isopropanol, and sodium hydroxide were purchased from APS Ajax Finechem, Australia. 100mM solutions of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP) (referred to as dNTPs) were obtained from Quantum Scientific, Australia. Lipofectamine reagent was purchased from Invitrogen Australia Pty. Ltd., Australia, and the luciferase assay system with reporter lysis buffer was from Promega Corporation, Australia. Sodium bicarbonate was obtained from May & Baker Ltd.

Polymerase chain reactions (PCR) were performed using the RoboCycler® gradient 96 temperature cycler with hot top assembly from Stratagene, Australia. Medium scale plasmid purification was performed using the plasmid purification midi kit from Qiagen Pty. Ltd., Australia. The concentration and purity of DNA was measured by the NanoDrop® ND-1000 spectrophotometer from Biosciences Biolab, Australia. Images of GFP-expressing mammalian cells were captured using the Olympus BX60 system microscope and Olympus U-RFL-T camera from Olympus

Australia Pty. Ltd., Australia. Computer software used for imaging was IPLab alias version 3.2.3 from BD Biosciences Bioimaging, USA. Flow cytometry was performed by a MoFlo™ high-performance cell sorter and the results were analysed by the Summit software, both from Dako Cytomation, Dako Australia Pty. Ltd., Australia. The luciferase assay system was purchased from Promega, Australia. Luciferase activity was measured using the Turner Biosystems Model 20/20 single tube luminometer from Quantum Scientific, Australia. The SpectraMax® 340 microplate spectrophotometer was used to read absorbance of samples for β- galactosidase activity.

2.1.2 Agarose gel electrophoresis

DNA-grade agarose powder was from Quantum Scientific, Australia, and ethidium bromide (EtBr) from Sigma-Aldrich, USA. Scanning and analysis of agarose gels were performed using a UV transilluminator and The Discovery Series: Quantity One software from Bio-Rad Laboratories Pty. Ltd., Australia. DNA bands were purified using the QIAquick Gel Extraction Kit from Qiagen Pty. Ltd., Australia.

2.1.3 Bacterial cell culture

The *Escherichia coli* (*E. coli*) host strain used was DH5α: *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R, Δ (lacZYA-argF), U169. Antibiotics used for selection were ampicillin from Sigma-Aldrich, USA and kanamycin from Roche, Australia. Agar and yeast extract were from Oxoid, Australia. Tryptone was from Sigma-Aldrich, USA and glycerol from APS Ajax Finechem, Australia.

2.1.4 Enzymes

Ampli*Taq* DNA polymerase was from Perkin Elmer Cetus, Australia. Restriction enzymes *Bam*HI, *Hind*III and *Xho*I, and T4 DNA ligase were purchased from Promega, Australia. Pancreatic RNase was from Sigma-Aldrich, USA. All enzyme units were specified by manufacturers.

2.1.5 Mammalian cell culture

The HeLa, C33A, H1299 and MRC-5 cell lines were prepared from frozen stocks maintained by Dr. Noel Whitaker in the School of Biotechnology and Biomolecular Sciences, the University of New South Wales, Sydney, Australia. The SiHa cell line was a kind gift from Dr. Murray Cairns from the Johnson and Johnson Research Laboratories, Sydney, Australia. The cell lines used are listed in Table 2.1.

Table 2.1 Description of mammalian cell lines used.

Cell Line	Description
HeLa	HPV-18 positive human cervical carcinoma
SiHa	HPV-16 positive human cervical carcinoma
C33A	HPV negative human cervical carcinoma
H1299	Human fetal non-small cell lung carcinoma
MRC-5	Human fetal lung fibroblast

Dulbecco's Modified Eagle Media (DMEM) powder, fetal bovine serum (FBS) and EDTA containing 0.25% (w/v) trypsin were all purchased from Invitrogen Australia Pty. Ltd., Australia. Phosphate buffered saline (PBS) tablets were from MP Biomedicals, Australia. Hibitane concentrate used as disinfectant was from ICI Australia. All tissue culture flasks and plates, pipettes, polypropylene cryogenic vials and centrifuge tubes were purchased from Interpath Services Pty. Ltd., Australia. Trypan blue solution (0.4%

w/v) and dimethyl sulfoxide (DMSO) (≥99.5%(GC)) was from Sigma-Aldrich, USA. Filters (Sterivex-GP: SVGPB1010) and the filtering pump (XX8020230) used for preparing media were purchased from Millipore, Australia. Coating buffer used to attach adhesive cells to coverslips was prepared by courtesy of a postgraduate colleague at work Miss Flora Kan, which contained BSA solution, fibronectin and MCDB 153 medium from Sigma, and collagen from Dr. Ken Moon at the University of New South Wales, Sydney, Australia.

2.1.6 Oligonucleotides

All oligonucleotides used were purchased from Invitrogen Australia Pty. Ltd., Australia, at 50 nmole scale of synthesis, desalted and unmodified. Details of all oligonucleotides designed for this project are listed in Table 2.2. Bases in bold indicates restriction sites added to assist cloning or mutations introduced to the target sequences. Oligonucleotides were designed with the help of various computer softwares such as the Oligo Calculator version 3.08 available online (Kibbe, 2006) and DNA Strider 1.2 for restriction mapping.

Table 2.2 List of oligonucleotides used in the EGFP constructs.

Primer	Purpose	Sequence (5'-3')	bp	F/R	RE site
174	pLCR874-EGFP	GTATCA**AAGCTT**TGCGTGTACGTGCCAGGAAG	32	F	HindIII
176	pLCR874-EGFP	GTATCA**AAGCTT**ATAGTATTGTGGTGTGTTTCTC	34	R	HindIII
241	sequencing	GCTCACCTGTTCTTTCCTGCGT	22	F	/
242	sequencing	CGGTGGTGCAGATGAACTTC	20	R	/
243	sequencing	CGAAATAGGTTGGGCAGCACA	21	F	/
244	sequencing	CGCAACCACATAACACACAGA	21	R	/
277	pLCR1000-EGFP	GGCTGAAAAT**AAGCTT**CCCTATG	23	F	HindIII
278	pLCR1000-EGFP	CTCAAAG**GGATCC**ATAGTATTGTGGT	26	R	BamHI
279	pSV40-EGFP	TGGAATGTGTG**AAGCTT**AGGGTGT	24	F	HindIII
280	pSV40-EGFP	CATCTTGT**GGATCC**ATGCGAAAC	23	R	BamHI

Table 2.3 List of oligonucleotides used in the luciferase deletion constructs.

Primer	Purpose	Sequence (5'-3')	bp	F/R	RE site
285	sequencing	CTAGCAAAATAGGCTGTCCC	20	F	/
286	sequencing	GCTCTCCAGCGGTTCCATCTTCCA	24	R	/
287	pGL3-LCR1000	CGAGCTCAA**CTCGAG**CTATGATAAGTT	27	F	XhoI
288	pGL3-LCR800	GTGCGT**CTCGAG**GCCAGGAAGTAATAT	27	F	XhoI
289	pGL3-LCR400	GCACAATACA**CTCGAG**TGGCACTAT	25	F	XhoI
290	pGL3-LCR200	GGCTT**CTCGAG**CTACTTTCATGT	23	F	XhoI
291	pGL3-LCR	GTGGCGA**AAGCTT**GCGCCATAGTATTGTG	29	R	HindIII
292	pGL3-SV40	TGGAATGTGTG**CTCGAG**AGGGTGT	24	F	XhoI
293	pGL3-SV40	GCAATCCAT**AAGCTT**CAATCATGCGAAAC	29	R	HindIII

Table 2.4 List of oligonucleotides used in site-directed mutagenesis experiments.

Primer	Target	Sequence (5'-3')	bp	F/R
263	pGL3-ESBS#1	CCGAAAACGGTCGGGA**TT**GAAAAC**CC**TGTATATAAAAGATGT	42	F
265	pGL3-ESBS#2	AAGGGAGTAA**TT**GAAAAC**CC**TCGGGACCGAA	31	F
267	pGL3-ESBS#3	GTTTATGCAA**TT**GAAATA**CC**TTGGGCAGCAC	31	F
269	pGL3-ESBS#4	TTGCTGTGCAA**TT**GATTTC**CC**TTGCCTTTGG	31	F
273	pGL3-ESBS#1	CACATCTTTTATATACA**GG**GTTTTC**AA**TCCCGACCGTTTTCGG	43	R
274	pGL3-ESBS#2	TTCGGTCCCGA**GG**GTTTTC**AA**TTACTCCCTT	31	R
275	pGL3-ESBS#3	GTGCTGCCCAA**GG**TATTTC**AA**TTGCATAAAC	31	R
276	pGL3-ESBS#4	GCCAAAGGCAA**GG**GAAATC**AA**TTGCACAGCA	31	R
294	pGL3-AP1/YY1	ACCTGGTATTAGTCA**CCGCGG**TGTCCAGGTG	31	F
295	pGL3-AP1/YY1	CACCTGGACA**CCGCGG**TGACTAATACCAGGT	31	R
296	pGL3-GRE/YY1	TAGGTTGGGCAGCA**AT**TACTATA**AG**TTTCATTAATA	36	F
297	pGL3-GRE/YY1	TATTAATGAAA**CT**TATAGTA**AT**TGCTGCCCAACCTA	36	R
298	pGL3-Sp1	ATATAAAAAAA**ACT**AGTAACCGAAAAC	26	F
299	pGL3-Sp1	GTTTTCGGTTACT**AGT**TTTTTTATAT	26	R
300	pGL3-KRF-1	TGCTT**AACG**AACTATATCCACT**AAA**TA**T**GT	30	F
301	pGL3-KRF-1	AC**A**TA**TTT**AGTGGATATAGTT**CGTT**AAGCA	30	R

2.1.7 Vectors

A plasmid vector containing the HPV-18 genome was a gift from Dr. Harald zur Hausen, German Cancer Research Centre, Heidelberg, Germany.

The promoterless enhanced green fluorescent protein (EGFP) vector used was pEGFP-1 purchased from Clontech, BD Australia. The vector diagram and restriction sites within the multiple cloning site (MCS) of pEGFP-1 are shown in Figure 2.1.

The pCMVβ from Clontech, BD Australia, and the promoterless luciferase vector pGL3-Basic from Promega, Australia, were kind gifts from Miss Jodie Stephenson of the University of Technology Sydney, Sydney, NSW. The vector diagram and restriction sites of pCMVβ and pGL3-Basic are shown in Figure 2.2 and Figure 2.3 respectively.

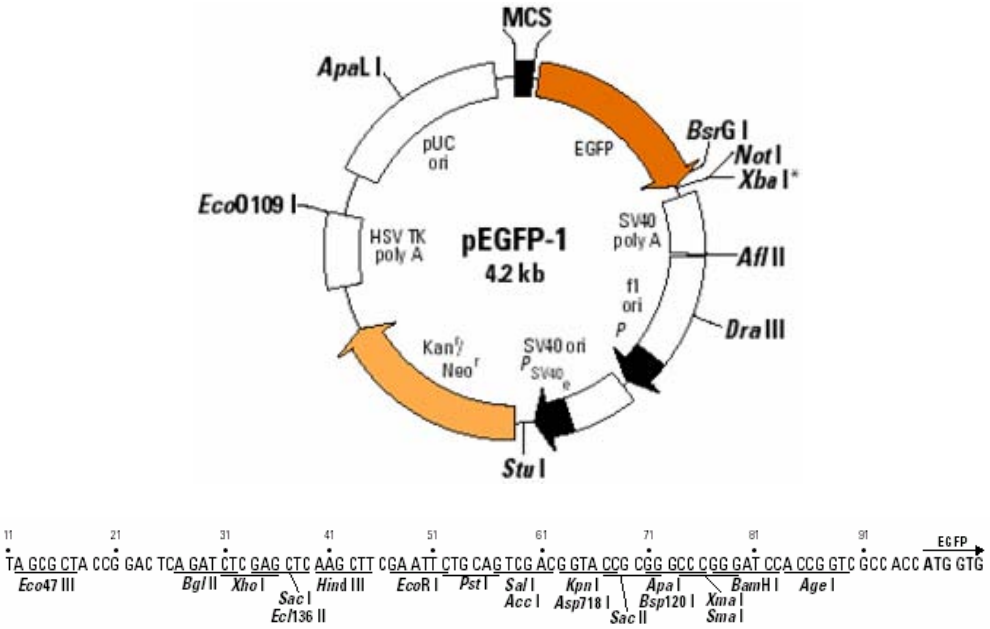


Figure 2.1 Vector diagram and multiple cloning site of pEGFP-1.

The promoterless pEGFP-1 consists of a MCS upstream of an EGFP gene, which can be used to induce the expression of EGFP upon the insertion of a functional enhancer and promoter elements into the MCS. Further downstream contains an antibiotic resistance cassette (Kan^r/Neo^r) in the same orientation which confers kanamycin resistance in *E.coli*, and neomycin selection in eukaryotic cells which is driven by an early SV40 promoter. Figure reproduced from Clontech Technical Manual (Clontech, 2002).

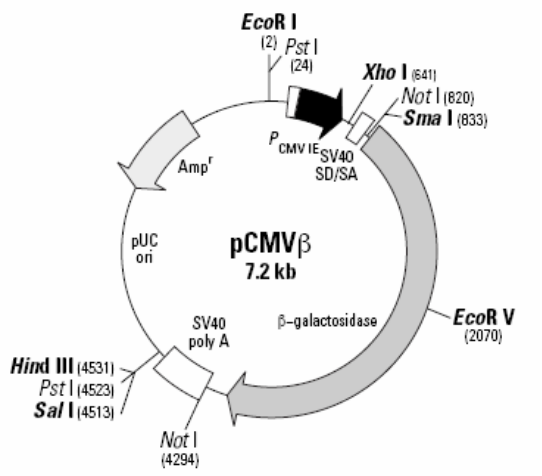


Figure 2.2 Vector diagram of pCMVβ.

The pCMVβ vector contains a CMV promoter which is used to drive the expression of a β-galactosidase gene within eukaryotic cells, and an antibiotic resistance gene (Amp^r) in the opposite orientation which confers ampicillin resistance in *E. coli*. Figure reproduced from Clontech Technical Manual (Clontech, 2003).

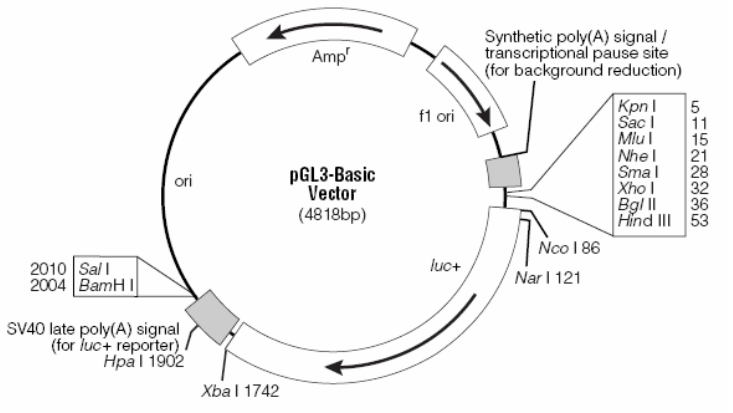


Figure 2.3 Vector diagram of pGL3-Basic.

The promoterless pGL3-Basic vector consists of a MCS upstream of a luciferase (*luc*+) gene, which can be used to induce the expression of luciferase upon the insertion of a functional enhancer and promoter elements into the MCS, and an antibiotic resistance gene (Amp^r) in the opposite orientation which confers ampicillin resistance in *E. coli*. Figure reproduced from Promega Technical Manual (Promega, 2007).

2.2 Methods

2.2.1 Sterilization of solutions and disposables

Disposable pipettes tips, glassware, heat-stable solutions and microcentrifuge tubes were sterilized for 20 minutes at 120°C and 125 kilopascal (kPa) in an autoclave. Filter-sterilised Milli-Q water was prepared by the Milli-Q purification system. Other solutions were sterilized by filtration through disposable 0.22 μm cellulose acetate filters from Millipore, Australia, when necessary.

All scientific wastes were disposed into designated bins, and biological discards were autoclaved prior to disposal as scientific wastes.

2.2.2 Mammalian cell culture

(i) General cell culture techniques

All cell cultures were maintained at 37°C with humidified air containing 5% (v/v) CO_2. All procedures were performed aseptically in a Biohazard Class II Hood and all materials being placed into the hood were sprayed with 70% (v/v) ethanol. Gloved hands were sprayed with ethanol before handling materials that were to be placed into the hood. Bottle and tube rims were wiped with alcohol wipes before and after pouring. Cells were allowed to grow in 175 cm^2 tissue culture flasks with 50 mL of DMEM with 10% (v/v) FBS. Exhausted media was discarded into a beaker containing 10% Hibitane diluted in 70% (v/v) ethanol.

(ii) Preparing DMEM media

To make 1 L medium, DMEM powder and 3.7 g $NaHC0_3$ were stirred in 810 mL Milli-Q water with a magnetic stirrer until completely dissolved. The pH of the medium was adjusted to 7.4 using 1 M HCl or 1 M NaOH before making up to the final volume of 900 mL. The media was then filtered into sterile 1 L bottles under sterile conditions. Prepared medium were stored at 4^oC and 100 mL FBS was added prior to use.

(iii) Preparing cells from frozen stocks

Frozen stocks were kept under liquid nitrogen in 1 mL aliquots, in 2 mL polypropylene cryogenic vials. Freezing medium for cell stocks contained 10% FBS and 10% DMSO in DMEM. The 2 mL tubes were thawed in a 37°C water bath, and then transferred into 25 cm^2 tissue culture flasks containing 9 mL of medium. After 12 hours of incubation in a 37°C incubator the media was poured off, and 10 mL fresh medium was added. The cells in the flask were allowed to grow till confluent then transferred into a 175 cm^2 flask.

(iv) Feeding mammalian cells

The medium was changed about two times per week depending on the growth of the cells. Exhausted medium was discarded, and the flask was then filled with 10 mL fresh medium. Cells were subcultured when they reached approximately 80-90% confluence.

(v) Mammalian cell passaging

Cells were detached by trypsin-EDTA as they approach 90% confluence. Exhausted medium was discarded; the flask was then washed twice with 1x PBS as the

FBS supplement in DMEM inhibits the reaction of enzyme. 3 mL of trypsin-EDTA was added to the flask and incubated at 37°C for approximately 3 minutes or until most the cells have been detached from the tissue culture flask, which can be observed under a light microscope. Prolonged incubation may kill the cells. When most of the cells were detached from the bottom of the flask, DMEM with FBS was added to stop the enzymatic reaction, making up the final volume to 10 mL. The mixture was then transferred into a 50 mL tube and centrifuged for 5 minutes at 1,300 x g. The cells were then washed twice with 10 mL of 1x PBS. A 10 µl aliquot was stained with an equal volume of 0.4% trypan blue solution and the concentration of cells in the cell suspension was calculated by counting cells using a haemocytometer under a light microscope. Approximately 10^5 cells were added to a new 175cm^2 flask containing 50 mL fresh DMEM with FBS. The cells were then passaged again when the culture flask reached approximately 90-100% confluence.

2.2.3 Preparation of HPV-18 LCR inserts

(i) Polymerase chain reaction

PCR was performed to amplify the desired LCR sequences from a plasmid containing the HPV-18 DNA, as well as to attach restriction sites to the two ends of the viral inserts to assist cloning. 20 µl of PCR reaction mixture contained approximately 50 ng of template DNA, 20 pmole forward primer and 20 pmole reverse primer, 0.3 mM dNTPs, 16.6 mM $(NH_4)SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 1.74 units of *Taq* DNA polymerase (stock concentration of 5 units/µl). Sterile water was used to make up the final volume to 20 µl.

The PCR mixtures were first denatured at 95°C for 4 minutes, followed by 25 cycles of 95°C for 30 seconds (denaturing), 55°C to 60°C for 1 minute (annealing of primers to DNA templates) and 72°C for 1.5 minutes (chain extension by *Taq* DNA polymerase), then finishing off with an extra extension time of 10 minutes at 72°C. PCR samples were then combined with gel loading buffer (0.1% (w/v) bromophenol blue/glycerol) and electrophoresed on 1% or 2% (w/v) agarose gels to detect for the presence of appropriate sized PCR products.

(ii) Site-directed mutagenesis

Site-directed mutagenesis (SDM) is the *in vitro* synthesis of mutant DNA. The method used was by overlap extension using PCR, as described by (Ho *et al.*, 1989) illustrated in Figure 2.4.

For each of the mutations performed, a pair of oligonucleotides was designed to cover and introduce the mutation into the wild-type HPV-18 LCR. Together with a pair of primers flanking the entire LCR, two separate PCR reactions were performed under the conditions as described in Section 2.2.3(i), generating two halves of the LCR containing the desired mutations. The two aliquots of PCR products were then electrophoresed on a 2% (w/v) agarose gel (see Section 2.2.3(iii)), visualised briefly by a hand-held UV transilluminator and the bands of the appropriate sizes were cut out. The DNA from the gel pieces was then extracted by the QIAquick gel extraction kit from Qiagen, as per instruction by the manufacturer.

A second round of PCR was then performed by making use of both aliquots of gel-purified DNA as templates and the pair of outer primers flanking the entire LCR. During the reaction the overlapping ends anneal, allowing the 3′ overlap of each strand

to serve as a primer for the 3′ extension of the complementary strand. The resulting fusion product is amplified further by PCR (Ho *et al.*, 1989).

Since the pair of flanking primers was designed to contain appropriate restriction sites, the final PCR product was then again gel purified before being digested by restriction enzymes and ligated into an expression vector for promoter analysis.

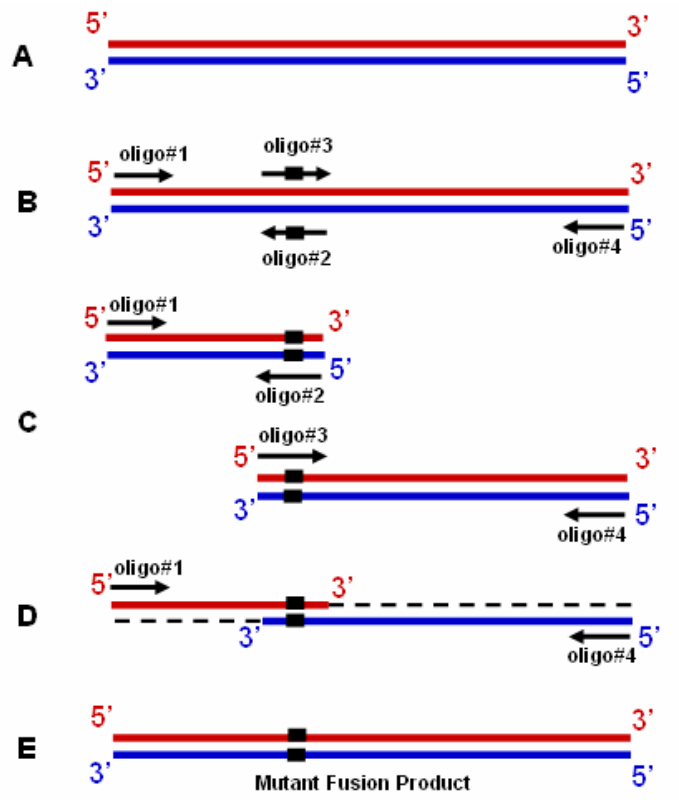


Figure 2.4 Schematic diagram of site-directed mutagenesis by overlap extension. A: Original ds DNA template; B: The site of mutagenesis indicated by the small black rectangle. Oligos #1 and #4 are primers flanking the region of interest, while oligos #2 and #3 cover and introduce the mutation into the region amplified by PCR; C: Two separate first-round PCRs, to obtain two halves of the final PCR product; D: The denatured fragments anneal at the overlap and are extended by DNA polymerase (dotted line) to form the product containing the site of mutagenesis; E: Combine the two PCR products and run a second-round PCR using the pair of flanking oligos #1 and #4 to further amplify the mutant fusion product by PCR.

(iii) Agarose gel electrophoresis

DNA fragments were separated and visualised on either a 1% or 2% (w/v) agarose gel containing 1x TBE buffer (Tris-HCl, pH 8.3, 89 mM boric acid, 2 mM EDTA), with the addition of 1 μg/mL ethidium bromide. The gel containing DNA samples was electrophoresed at 100 volts for 30 to 45 minutes, depending on the size of the DNA fragments to be analysed. 1x TBE buffer was used as a running buffer. The gel was visualized using a UV transilluminator and photographed.

(iv) Restriction enzyme digestion

Oligonucleotides flanking the region of interest within the LCR were designed to incorporate corresponding restriction enzyme digestion sites. HPV LCR inserts produced by PCR and promoterless vectors were digested with restriction enzymes so as to produce adhesive ends to assist the cloning process. Approximately 2 μg of PCR products or vector DNA was digested in a 20 μl digestion mixture with 5 units of restriction enzyme(s), 1x final concentration of the corresponding buffer and the rest of the volume was made up with sterile water. Digestion was carried out at for at least 2 hours at 37°C.

2.2.4 Construction of HPV-18 promoter plasmids

(i) Preparation of calcium chloride competent bacterial cells

Competent *E. coli* cells were prepared using $CaCl_2$ prior to plasmid transformation experiments. The method used was based on that described by (Maniatis et al., 1982). A fresh overnight culture of DH5α was prepared and 1 mL of it was used to inoculate 100 mL of Luria-Bertani (LB) broth (10 g tryptone, 5 f yeast extract and 10

g NaCl in 1 L Milli-Q water, autoclaved). The media was allowed to grow to A_{600} = 0.5-0.7, with vigorous shaking at 37°C for approximately 3-4 hours. Cultures were transferred into two sterile capped 50 mL tubes and chilled on ice for 10-15 minutes, then centrifuged at 2,000 x g for 10 minutes at 4°C. The supernatant was discarded; the cell pellet in each tube was resuspended in 25 mL of cold 100 mM $CaCl_2$ and incubated on ice for 20 minutes. The suspension was again centrifuged at 2,000 x g for 10 minutes at 4°C, and the supernatant was removed. The cell pellet in each tube was then resuspended in 5 mL of cold 100 mM $CaCl_2$ and dispensed in 850 µl aliquots into 2 mL polypropylene cryogenic vials containing 150 µl of 100% glycerol, making the final glycerol concentration 15%. Competent cells prepared were stored at -80°C for no longer than 2 months prior to use.

(ii) Ligation reaction

Ligation experiments were carried out to clone the HPV LCR inserts into promoterless expression vectors using T4 DNA ligase. The vector to insert molar ratio used was 1:5. Appropriate amount of restriction enzyme digested inserts and linearised vectors were combined in a 20 µl ligation mixture containing 1x T4 DNA ligase buffer, 1 mM ATP, 5 units of T4 DNA ligase (5 U/µl) and the rest of the volume made up by sterile Milli-Q water. The mixture was either incubated at room temperature for 2 hours or at 4°C overnight. Half of the ligated plasmid was then transformed into fresh competent *E. coli*.

(iii) Plasmid transformation into competent bacterial cells

Ligation mixtures were subsequently transfected into $CaCl_2$ competent DH5α cells to obtain transformants of bacterial cells containing the desired recombinant clone.

250 µl of competent DH5α cells was mixed with 10 µl of ligation mixture in a pre-chilled 5 mL capped culture tube and incubated on ice for 30 minutes. The mixture was then heat-shocked in a 42°C water bath with gentle shaking for 2 minutes. 1 mL of LB broth was then added and the suspension was incubated on a shaking platform at 37°C for 1 hour before being transferred to a 1.5 mL microcentrifuge tube and centrifuged for 10 seconds. After the removal of the bulk supernatant the cell pellet was resuspended in the remaining solution. The suspension was then spread onto a LB agar plate (15 g agar in 1 L LB broth) containing appropriate antibiotic with a hockey stick and incubated for overnight at 37°C.

2.2.5 Screening of transformed colonies

The screening for successfully transformed colonies were either carried out by performing colony PCR, which is a relatively fast method but more inaccurate; or by performing a small scale plasmid purification, known as miniprep, followed by a series of experiments such as restriction enzyme digestion and agarose gel electrophoresis.

(i) Colony PCR

Transformed bacterial colonies were selected and colony PCRs were performed to confirm the presence of HPV LCR inserts within the recombinant clones. PCR was carried out under similar conditions as described in Section 2.2.3(i) but the DNA template used was from the transformed bacterial colonies instead. This was performed by gently touching the selected bacterial colony grown on the agar plate with a pipette tip, then mixing the cells through the PCR mixture by pipetting. The initial denaturing step at 95°C for 4 minutes was sufficient to lyse the cells in the suspension

and release their DNA content. Primers used for colony PCR usually consist of one insert primer and one vector primer, so as to ensure that the colony was successfully transformed with the recombinant plasmid containing the insert. PCR products were then separated by a 1% (w/v) agarose gel electrophoresis.

(ii) Small scale plasmid purification (miniprep)

This method was based on that described by Ish-Horowicz and Burke (1981). A recombinant colony was picked up by toothpick using aseptic technique, and was grown in 2 mL of LB broth or Superbroth (12 g tryptone, 14 g yeast extract and 6.3 g glycerol in 900 mL Milli-Q) containing the appropriate antibiotic at 37°C with vigorous shaking overnight. The bacterial cells were collected by centrifugation at 16,100 x g for 20 seconds. The cell pellet was then resuspended with 100 µl GTE (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA) and was lysed with 200 µl of freshly made lysis solution containing 0.2 M NaOH and 1 % (w/v) sodium dodecylsulphate (SDS). Lysis was allowed to proceed for 10 minutes on ice. The cell suspension was then mixed with 150 µl of cold 5 M potassium acetate (KOAc) at pH 5.0 to precipitate cellular protein and lipid in the cells. Precipitation was allowed to proceed for another 10 minutes on ice and was then centrifuged at 16,100 x g for 10 minutes. The supernatant was transferred into new microcentrifuge tube. An equal volume of isopropanol was mixed with the supernatant to precipitate the nucleic acids. Precipitation was allowed to proceed for 10 minutes at room temperature. The nucleic acid was collected by centrifugation at 16,100 x g for 10 minutes and the supernatant was removed. The pellet was then washed with 80% (v/v) ethanol to remove excess salt. After removal of all the ethanol, the pellet was allowed to air dry for 10 minutes and was then resuspended in the 40 µl of 1x TE buffer (10 mM Tris-HCl/0.1 mM EDTA, pH 8.8).

(iii) Ribonuclease treatment

DNA extracted from bacterial cells; such as those from miniprep and colony PCR, contained a significant amount of RNA. Since both DNA and RNA are visible when samples are electrophoresed and exposed under a UV transilluminator, the presence of RNA would be a problem if the bands of interest were covered up by the RNA patches on a gel photo. Another problem associated with RNA is that its absorbance can be detected at 260 nm (A_{260}) together with DNA. Thus when the concentration of a plasmid DNA sample is measured by a spectrophotometer (see Section 2.2.5(vi)), the presence of RNA will lead to an over-estimated concentration reading (indicated by A_{260}/A_{280} ratio of 1.9 or higher). To remove RNA prior to gel electrophoresis, approximately 4 μg of RNase was added to each 20 μl DNA sample, and incubated at 37°C for 30 minutes to allow the digestion of RNA by RNase. Treated samples were then phenol/chloroform extracted and ethanol precipitated before being loaded onto agarose gel for analysis or to be used in other experiments.

(iv) Phenol-chloroform extraction

This was an extraction method used to remove all the protein impurities within a DNA sample, such as enzymes and cellular proteins. The volume of DNA sample to be purified was made up to 100 μl. 50 μl of phenol was added and the solution was mixed by vigorous vortexing, followed by 20 seconds of centrifugation which separated the mixture into two phases. The upper aqueous layer contained the DNA extracted and the lower phenol layer contained all the impurities. The upper DNA layer was then transferred to a new microcentrifuge tube. 150 μl of chloroform was added and again vortexed vigorously, followed by 30 seconds of centrifugation. The upper layer was DNA and the lower layer contained the chloroform. Impurities of some

insoluble proteins showed up as a thin layer between the two phases. The upper aqueous DNA layer was transferred to a new tube and chloroform extraction was repeated. The sample was then subjected to ethanol precipitation to further purify and concentrate (see Section 2.2.5(v)).

(v) Ethanol precipitation

Ethanol precipitation was often performed as a clean-up procedure for DNA samples, or sometimes when it was necessary to concentrate the DNA samples into a lower volume. About 1/10 volume of 3 M sodium acetate (NaOAc) was added to the DNA sample to be purified, followed by the addition of 3 volumes of cold 95% (v/v) ethanol. The solution was mixed thoroughly then allowed to precipitate for 30 minutes to 2 hours at -80°C or overnight at -20°C. The DNA pellet was then collected by centrifugation at 16,100 x g for 10 minutes at 4°C and washed twice with 70% (v/v) ethanol. Supernatant was removed and the pellet was allowed to dry completely before being dissolved in a desired volume of 1x TE buffer. Purified DNA samples were stored at -20°C.

(vi) Measurement of DNA concentration and purity

The concentration of DNA samples were calculated by measuring its absorbance at A_{260}, given that A_{260} of 1 corresponds to 50 µg/mL of double stranded-DNA. To estimate the purity of DNA with respect to contaminants that absorb in the UV, such as protein, the ratio of spectrophotometric readings at 260 nm and 280 nm (A_{260}/A_{280}) was determined. The DNA sample was considered as pure if the ratio of A_{260}/A_{280} was close to 1.8. Since the A_{260}/A_{280} ratio is known to be influenced by pH (Wilfinger *et al.*, 1997), DNA samples to be measured were all diluted in 1x TE to

produce a buffered environment, and the same buffer was also for calibrating the spectrophotometer prior to any measurements.

(vii) Automated DNA sequencing

In automated sequencing a different fluorescent label is attached to each of the four dideoxy nucleotides ddA, ddC, ddG and ddT. Sequencing was essential on all the purified plasmids preparations to ensure the mutations of interest were successfully created by site-directed mutagenesis and no random mutations had occurred during the cloning processes. For the sequencing of one strand, a sequencing mixture containing 1 µl Big Dye terminator, 3.2 pmol of primer, 1.5 µl of 5x sequencing buffer, and 100 ng to 500 ng plasmid DNA was made up to 20 µl. Sequencing cycles were carried out with the following parameters for 25 cycles: 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The samples were then ethanol precipitated (Section 2.2.5(v)), dried thoroughly, and run on the ABI 3730 capillary sequencer. Results were analysed using the ABI Prism Sequencing Analysis software (version 3.3), and other computer programs available online such as WebAngis from the Australian National Genomic Information Services (ANGIS, 2005) and ClustalW from the European Bioinformatics Institute (EMBL-EBI, 2006) to assist sequence alignment.

2.2.6 Plasmid DNA purification

Following DNA sequencing which confirmed that the recombinant plasmids contained the correct inserts, a larger scale of plasmid purification was performed to obtain a sufficient quantity for the various experiments. This was achieved by either using the Qiagen Plasmid Midi Kits (instructions as per manufacturer) which produced

a maximum of 100 µg per preparation, or by a large scale plasmid purification method (maxiprep) which could yield up to 500 µg per preparation.

(i) Large scale plasmid purification (maxiprep)

This method was based on the one described by (Ish-Horowicz and Burke, 1981). A recombinant colony was picked up by a toothpick aseptically. It was grown in 500 mL of superbroth containing 100 µg/mL ampicillin in a 37°C shaker set at 200 rpm overnight. Bacterial cells were collected by centrifugation at 10,750 to 16,800 x g for 10 minutes or until the superbroth was clear. The cell pellet was then suspended in 4 mL GTE, and was lysed in 8 mL of freshly prepared lysis solution containing 0.2 M NaCl and 1% (w/v) SDS. The lysis was allowed to proceed for 10 minutes on ice. The cell suspension was mixed with 6 mL of cold 5 M KOAc at pH 5.0 to precipitate cellular protein and lipid. Precipitation was allowed to proceed for 10 minutes on ice and was then centrifuged at 12,300 x g for 5 minutes. The supernatant was transferred into a new SS34 centrifuge tube. Equal volume of isopropanol was mixed with the supernatant to precipitate nucleic acids. Precipitation was allowed to proceed for 20 minutes at room temperature. The nucleic acid was collected by centrifugation at 12,300 x g for 15 minutes. After removal of all isopropanol, the pellet was allowed to air dry for 10 minutes, and was then resuspended in 3 mL of 1x TE buffer.

A CsCl density gradient was created by adding 4.1 g of CsCl into the resuspended pellet and the mixture was vigorously vortexed until all the CsCl were dissolved. 200 µl of 100 mg/mL EtBr was added to the mixture to precipitate cellular protein and lipid, as well as intercalating into DNA. This mixture was centrifuged at 2,000 x g for 10 minutes and the supernatant was transferred into 5 mL ultracentrifuge tube. The ultracentrifuge tube was then filled up with 1x TE buffer. This was then

centrifuged at 287,600 x g in a Beckman VT_i65 rotor at 20°C overnight. During centrifugation a different density was created between chromosomal DNA and plasmid DNA, which allowed the isolation of plasmid DNA. The plasmid sample was collected by a 2 mL disposable syringe and 22-gauge needle. The EtBr content was then removed by extracting the plasmid sample with equal volume of CsCl/water saturated isopropanol. The plasmid sample was then dialysed at 4°C against 1x TE buffer for 6 hours with a change of buffer every 2 hours. This was carried out to remove CsCl from plasmid sample. The plasmid sample was transferred to a clean microcentrifuge tube and stored at -20°C. Measurement of plasmid DNA concentration and purity was again performed by spectrophotometry (see Section 2.2.5(vi)).

2.2.7 Plasmid transformation in mammalian cells by lipofection

Recombinant plasmids containing the HPV-18 LCR insert, or together with the transfection control plasmid pCMVβ, were transfected into different mammalian cell lines using lipofectamine as a transfection reagent. Transfection experiments were performed in either 6-well or 24-well tissue culture plates. The day before transfection, cells were seeded into a tissue culture plate together with complete growth medium. The cells were incubated at 37°C in a CO_2 incubator until they were 50-80% confluent. The plasmid DNA and lipofectamine reagent were then diluted with serum-free growth medium separately before being mixed together and incubated at room temperature for 45 minutes, allowing DNA-liposome complexes to form. While complexes were forming, the complete growth medium on the cells were rinsed thoroughly and replaced by serum-free growth medium. The DNA-liposome complexes were then further diluted with serum-free medium and gently overlayed onto the rinsed cells. The cells with the

complexes were incubated for 5 hours at 37°C in a CO_2 incubator, before the transfection medium was replaced by complete growth medium containing serum. The transfected cells were continually incubated at 37°C in a CO_2 incubator for 48 hours from the start of transfection, before cells were harvested and assayed for transient gene expression. All transfection experiments were repeated at least 3 times with each experiment performed in triplicate.

2.2.8 Fluorescence microscopy

In order to detect the level of promoter activity by making use of a GFP reporter plasmid, the method of fluorescence microscopy was employed to examine fluorescing cells by the naked eye. A piece of coverslip was placed in each well of a new 6-well tissue culture plate and was covered with 200 μl of coating buffer (5 mg fibronectin, 1% collagen and 10 mg BSA in 100 mL of MCDB153 medium). This treatment enables adhesive cells to attach and grow on the coverslips. During the incubation time, transfected cells (48 hours post-transfection) in plates were rinsed 3 times by 1x PBS. About 400 μl of trypsin-EDTA, just enough to cover up the cells, was added to each of the wells and the plates were incubated at 37°C for approximately 1 minute or until most of the cells have been detached from the base of the well by observation under the microscope. Harvested cells were then centrifuged and washed thoroughly by 1x PBS and the number of cells was counted by using a haemocytometer (see Section 2.2.2(v)).

Excess coating buffer was then removed from the incubated plates containing the coverslips. Approximately 10^5 cells were plated onto the treated coverslips in each of the wells, with the addition of 2 mL of complete growth medium, and allowed to

recover for a 24-hour period at 37°C in a CO_2 incubator. The coverslips were then carefully lifted out of the wells by using a cell scraper and placed upside down on a glass slide for observation under an upright fluorescence microscope. The number of GFP-expressing whole cells was counted by randomly picking a field of view through the 20x magnification objective. This was repeated three times per coverslip and the average numbers were calculated. Photos of the transfected cells were taken by a camera connected to the fluorescent microscope.

2.2.9 Flow cytometry analysis

Flow cytometry is a more accurate and precise detection method for fluorescence when compared to fluorescence microscopy, enabling us to quantify both the number of fluorescing cells in a sample as well as the level of fluorescence induced from each individual cell particle. Approximately 10^5 transfected cells harvested 48 hours post-transfection were collected and made up to 200 µl with 1x PBS in disposable plastic culture tubes, the cell suspension was then ran through a MoFlo™ cell sorter and the amount of fluorescence emitted was measured. Fluorescence induced from the expression of EGFP protein has an excitation maximum at 488 nm and emission maximum at 507 nm (Clontech, 2002). Results obtained were plotted into graphs against different parameters using the Summit software.

2.2.10 Luciferase assay

Another method to detect promoter activity was the use of a luciferase reporter plasmid. Luciferase assay works by measuring the level of luminescence from a sample of cell lysate, therefore it was not necessary to keep the transfected cells in the form of

whole cells. Instead of treating the transfected cells attached on the tissue culture plates with trypsin, cells were treated with lysis buffer directly. Cells in 24-well tissue culture plates that had been incubated for 48 hours post-transfection were rinsed 3 times with 1x PBS, then 110 μL of 1x reporter lysis buffer (RLB) was added to each well. A single freeze-thaw cycle at -80^{o}C was performed to ensure complete lysis of cells. Lysed cells were scraped off from the plate and transferred to a microcentrifuge tube and placed on ice. The tubes were vortexed for 10-15 sec, then centrifuged at 12,000 x g for 2 min at 4^{o}C. The supernatant was transferred to a clean microcentrifuge tube. Cell supernatants were stored at -80^{o}C until ready for assay.

The luciferase assay reagent was prepared by reconstituting the luciferase assay substrate with 10 mL of luciferase assay buffer. 1 mL aliquots of the luciferase assay reagent were stored in microcentrifuge tubes at -80^{o}C. Prior to the luciferase assay, cell lysates and luciferase assay reagents prepared previously were thawed at room temperature since luciferase activity is optimum at room temperature. The luminometer was programmed to perform a 2-second delay followed by a 10-second measurement for luciferase activity. Samples were assayed by adding 20 μL of cell lysate to 100 μL of luciferase assay reagent. The tube was vortexed briefly then placed in the luminometer. Each sample was measured three times and the average was calculated.

2.2.11 β-galactosidase assay

A pCMVβ (see Figure 2.2 for details) plasmid was used as a transfection control plasmid in co-transfection experiments to allow for variations in transfection efficiencies between individual experiments. β-galactosidase assay reagents are able to work in the cell lysates prepared in the reporter lysis buffer for the luciferase assays.

Prior to the measurement of β-galactosidase activity, the cell lysates and ONPG solution (3 mM ONPG, 50 mM Tris, 50 mM KCl, pH 7) were equilibrated at 37^{o}C. 50 µl of ONPG solution was added to aliquots of 50 µL cell lysates in a 96-well microtitre plate and mixed well by pipetting. The plate was incubated at 37^{o}C for 3 hours during which the substrate β-galactosidase hydrolyses the colourless ONPG to *o*-nitrophenol, which has a yellow colouration. The absorbances of the samples were then measured at 405 nm in a microplate spectrophotometer.

2.2.12 Statistical analysis

For graphical results of the mean of experiments repeated for two times (n=2), error bars were added to represent the range of the results obtained from the two individual experiments. For graphical results of the mean of experiments repeated for three times (n=3) or more, error bars were added to represent the standard error of the mean (SEM) of results obtained from the repeated experiments. Since all the experiments were performed with less than ten repeats, the errors were quoted to one significant figure. Mean results represented by numerical values were quoted to the number of significant figures so that the last digit was the same order of magnitude as the error (Hase and Hughes, 2004).

CHAPTER 3

THE HPV-18 LCR AND DELETION CONSTRUCTS

CHAPTER 3 THE LCR AND DELETION CONSTRUCTS

3.1 Introduction

The first part of the project was to determine the reference level of promoter activity induced from the full-length HPV-18 LCR. Preliminary work was performed by isolating the LCR region from a HPV-18 plasmid, which lies between the 3′-end of the L1 ORF and 5′-end of the E6 ORF from nt 7,114 to 105 (numbering according to Cole and Danos (1987). Primers used to amplify this region were designed by similar methods as described by Villa and Schlegel (1991) with minor alterations to accommodate the appropriate restriction enzyme recognition sequences (Figure 3.1). The initial reporter plasmid utilised was pEGFP-1. The plasmid pLCR874-EGFP was produced which, as its name suggests, contained a LCR fragment from HPV-18 which was 874 bp in size (Figure 3.2) cloned upstream of an EGFP gene. Preliminary results obtained by transiently transfecting the pLCR874-EGFP plasmid into mammalian cell lines suggested that the HPV-18 P_{105} promoter could be selectively activated within HPV-positive cervical cancer cells, since promoter activity could be observed in HeLa cells but not in H1299 and MRC-5 cells (Lung, 2002). Further work was required to confirm these previously obtained preliminary results.

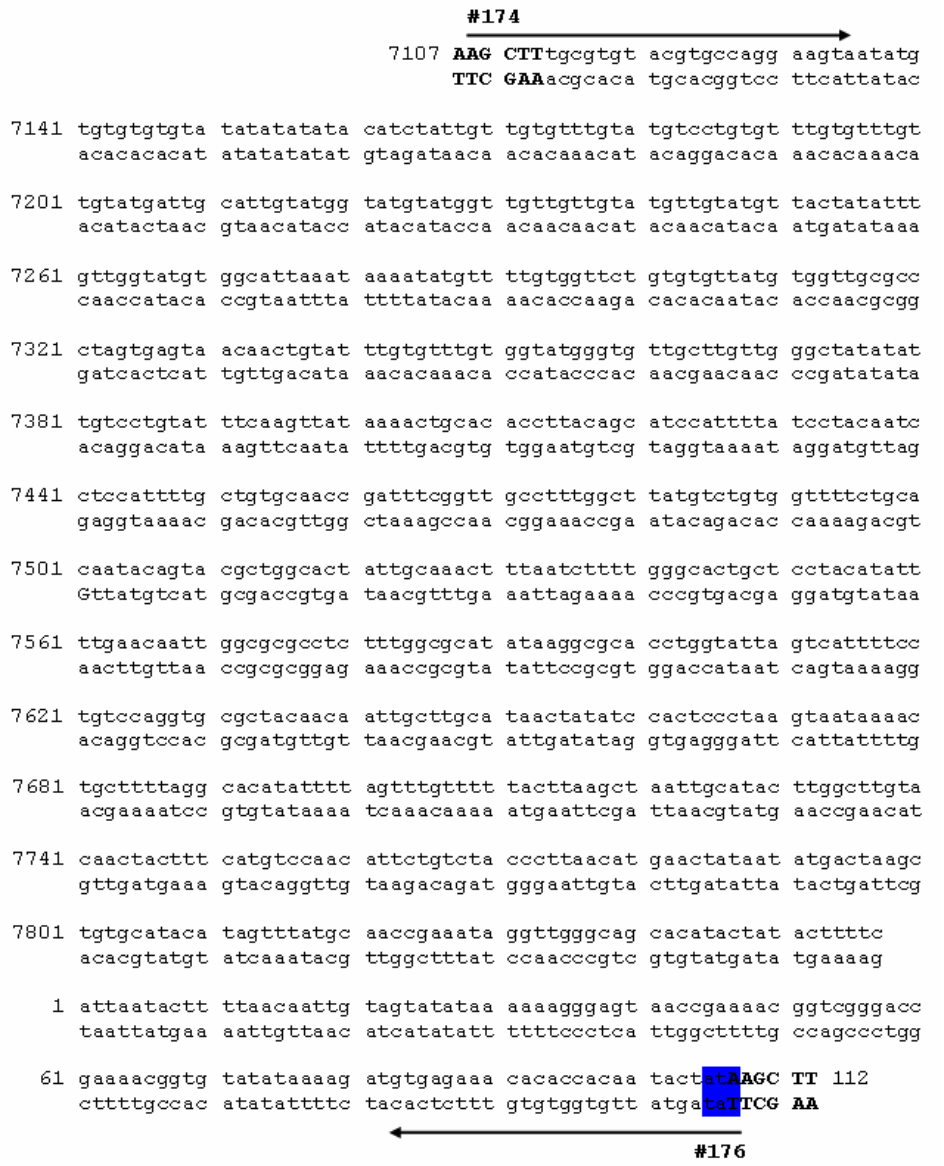


Figure 3.1 DNA sequence of LCR insert of pLCR874-EGFP.

The 874 bp LCR insert of pLCR874-EGFP was amplified by oligonucleotides #174 and #176 (see Table 2.2 for details). The region indicated by the arrows represents the exact length of the amplified LCR insert after being digested by *Hind*III and cloned into the pEGFP-1 vector. nt 1 represents the beginning of the HPV-18 genome, as established by Cole and Danos (1987). Letters in capital and bold are base changes to the wild-type sequence, so as to accommodate a restriction enzyme recognition sequence for *Hind*III (AAGCTT) to assist cloning. Highlighted in blue is the position of the ATG start codon for early gene transcription in the original wild-type sequence.

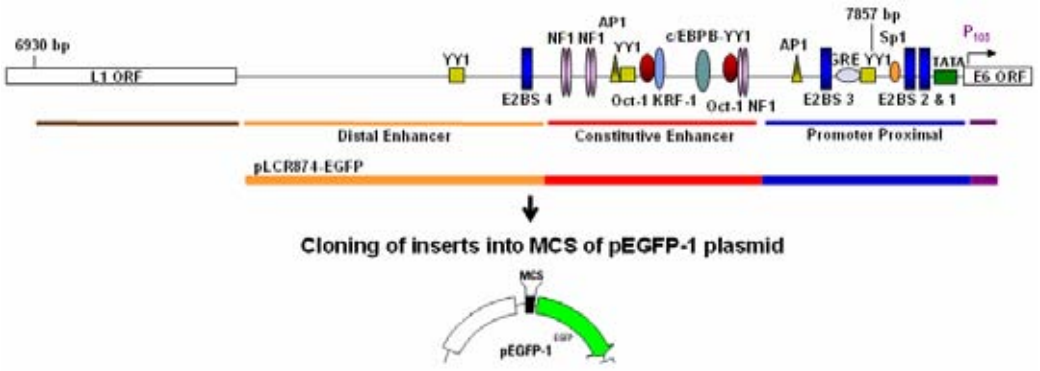


Figure 3.2 Schematic diagram of preliminary study of the HPV-18 LCR.

A 874 bp fragment of the HPV-18 LCR was amplified by PCR and cloned into the MCS upstream of the EGFP coding sequence of the pEGFP-1 plasmid, to produce the pLCR874-EGFP plasmid.

3.2 Results and discussion

3.2.1 Continuation from preliminary studies using a GFP reporter plasmid

The pEGFP-1 vector is a promoterless EGFP plasmid which can be used to monitor transcription from different enhancer and promoter elements inserted into the MCS located upstream of the EGFP coding sequence (see Figure 2.1 for vector diagram). It encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression levels in mammalian cells. Without the addition of a functional promoter, this vector is not expected to express EGFP. A bacterial promoter is located upstream of a neomycin/kanamycin resistance gene which confers kanamycin resistance in *E. coli* (Clontech, 2002).

The pLCR874-EGFP plasmid was sequenced (see Section 2.2.5(vii)) to ensure it contained the correct insert with no random mutations. The primers designed to be used for sequencing purpose were oligonucleotides #241, #242, #243 and #244 (see Table 2.2 for details).

(i) Determination of optimal transfection conditions

Careful optimization of transfection conditions was essential for higher efficiency transfections and lower toxicity. Optimization of transfection was performed on HeLa cells, in which the HPV promoter activity was of our main interest. Experiments were initially performed in 6-well tissue culture plates, so as to accommodate the coverslips required for observation of transfected cells under an upright fluorescent microscope.

The plasmid was transfected into mammalian cells using lipofectamine. Lipofectamine works at its best in cells at 50-80% confluence according to the

manufacturer's instructions. In order to determine the optimal seeding density, HeLa cells were plated into the 35 mm wells of 6-well tissue culture plates, in a range from 0.5 to $3.0x10^5$ cells per well, then incubated overnight with 3 mL of DMEM containing 10% (v/v) FBS. The percentage confluence of cells in each individual well was recorded 24 hours later. Results are represented in Figure 3.1 below. The seeding density was chosen to be $3.0x10^5$ cells which resulted in about 60% confluence after a 24-hour incubation.

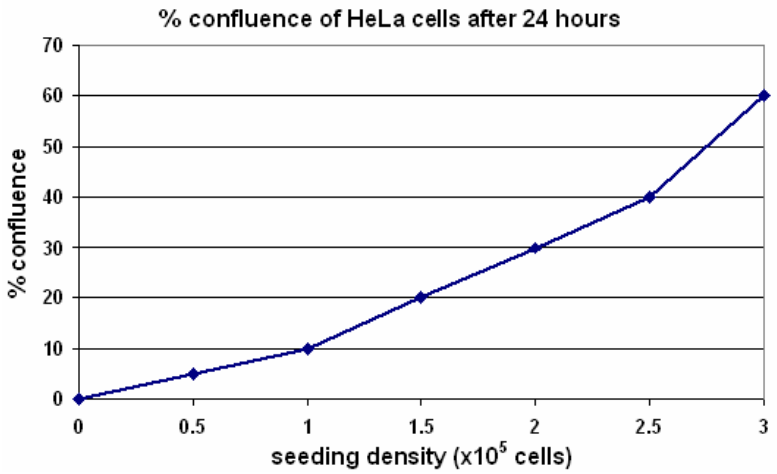


Figure 3.3 Seeding density for transfection in HeLa cells (6-well plate).
HeLa cells ranging from 0.5 to $3x10^5$ cells per well were seeded into a 6-well tissue culture plate, and percentage confluence of individual wells were recorded after 24 hours. The % confluence shown for different seeding densities are averages of triplicate wells. The seeding density of $3x10^5$ cells per well resulted in about 60% confluence and was utilised as the seeding density for HeLa cells.

Since cellular toxicity is often associated with the use of lipofectamine in transfection experiments (Ahrens *et al.*, 2005; Rasmussen *et al.*, 2006), the next step was to determine the optimal combination of lipofectamine and plasmid DNA concentrations to be used. All optimisation experiments were again performed on HeLa

cells in 6-well tissue culture plates. The range of pLCR874-EGFP plasmid DNA tested was from 0.5 to 5.0 μg with an addition of 3, 6 and 12 μl of lipofectamine (2 mg/mL). Transfected cells were examined under an upright fluorescent microscope 48 hours post-transfection.

The numbers of EGFP-expressing whole cells were counted by randomly picking a field of view through the 20x magnification eyepiece. This procedure was repeated three times per coverslip. The average numbers of fluorescing cells of each transfection condition were calculated and expressed as the mean of three counts ± SEM, and the % fluorescence were calculated by dividing the average numbers of fluorescing cells by the estimation of total cell count visible under a field of view of 20x magnification. The data results are shown in Table 3.1. Pictures of HeLa cells transfected by different amounts of lipofectamine captured under fluorescent light are shown in Figure 3.4, while Figure 3.5 are pictures captured by phase contrast which shows the relative cell integrity and density of the transfected cells.

Table 3.1 Optimal amount of plasmid DNA and lipofectamine for transfection in HeLa cells (6-well plate).

3µL lipofectamine – EGFP-expressing whole cell count (~250 cells/field of view)						
DNA (µg)	**0.5**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**
r#1	4	2	3	0	0	0
r#2	4	2	2	0	0	0
r#3	3	3	2	0	0	0
mean	3.7 ± 0.3	2.3 ± 0.3	2.3 ± 0.3	0	0	0
% fluorescence	1.5	0.93	0.93	0	0	0
6µL lipofectamine – EGFP-expressing whole cell count (~150 cells/field of view)						
DNA (µg)	**0.5**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**
r#1	7	8	2	2	2	3
r#2	11	12	2	1	1	0
r#3	6	11	1	1	0	2
mean	8 ± 2	10 ± 1	1.7 ± 0.3	1.3 ± 0.3	1.0 ± 0.6	1.7 ± 0.9
% fluorescence	5.3	6.9	1.1	0.89	0.67	1.1
12µL lipofectamine – EGFP-expressing whole cell count (~80 cells/field of view)						
DNA (µg)	**0.5**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**
r#1	7	6	6	4	2	0
r#2	9	5	7	3	3	0
r#3	6	5	5	3	3	0
mean	7 ± 1	5.3 ± 0.3	6.0 ± 0.6	3.3 ± 0.3	2.7 ± 0.3	0
% fluorescence	9.2	6.7	7.5	4.2	3.3	0

The number of EGEP-expressing whole cells per field of view was counted. Three random field of views were chosen and the mean number of fluorescing cells, expressed as the mean ± SEM of the three counts, were used to calculate the percentage fluorescence. Note that for different amounts of lipofectamine used, the number of cells per field of view were different, since as the amount of lipofectamine increased the level of cellular toxicity increased resulting in a lower cell density (3 µL lipofectamine: 250 cells/field of view; 6 µL lipofectamine: 150 cells/field of view; 12 µL lipofectamine: 80 cells/field of view). The highest EGFP-expressing cell count is boxed in red; while the highest percentage fluorescence is boxed in blue. The % fluorescence is rounded off to two significant figures.

Although the highest transfection efficiency was obtained when 12 µL of lipofectamine was used (see Table 3.1), that was not an ideal transfection condition since toxicity was induced by the high level of lipofectamine. Cells were less healthy and a significant number of dead cells were observed resulting in lower cell density.

The percentage cell growth was highest when the least amount of lipofectamine was used (see Figure 3.5(A)). In contrast, cells were relatively unhealthy, as indicated by detached cells and debris of dead cells in the background, when the lipofectamine concentration was too high (see Figure 3.5(C)). The optimal conditions chosen for transfection in a 6-well tissue culture plate were at a seeding density of $3.0x10^5$ HeLa cells, transfected with 1 μg of plasmid DNA by 6 μL of lipofectamine. All subsequent experiments with the EGFP constructs were performed under these optimal conditions.

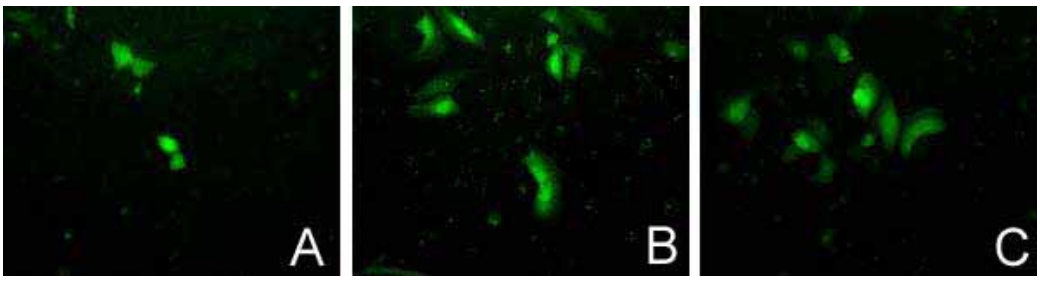


Figure 3.4 EGFP-expressing cells under the fluorescent microscope.
Photomicrographs of HeLa cells captured under an upright fluorescent microscope exposed to fluorescent light, all transfected with 0.5 μg of pLCR874-EGFP plasmid but using varying amounts of lipofectamine. A: 3 μL; B: 6 μL and C: 12 μL of lipofectamine.

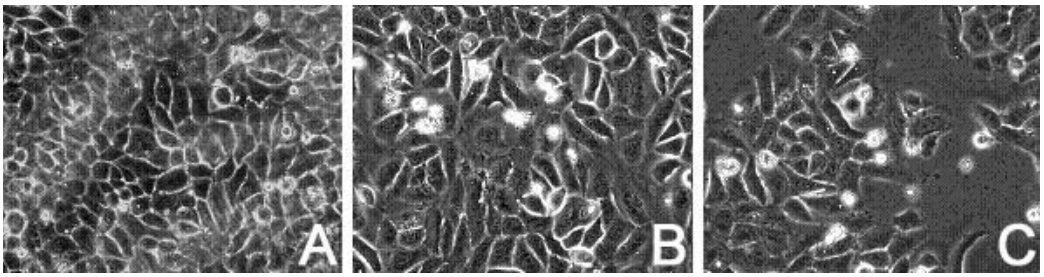


Figure 3.5 Cellular toxicity induced by lipofectamine.
Photomicrographs of HeLa cells grown on coverslips captured under an upright phase contrast microscope, all transfected with 0.5 μg of pLCR874-EGFP plasmid but varying amount of lipofectamine. A: 3 μL; B: 6 μL and C: 12 μL of lipofectamine.

(ii) HPV-18 P_{105} promoter activity from the GFP LCR constructs

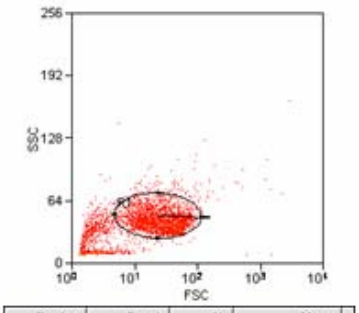
In order to quantify the level of promoter activity induced by the wild-type LCR, it was necessary to have negative and positive control plasmids. The promoterless pEGFP-1 vector was used as the negative control; without the addition of a functional promoter, EGFP was not expected to be induced. As for the positive control, the SV40 early promoter and enhancer elements from the pEGFP-1 vector (nt 1,689 to 2,049), which was initially present to assist neomycin selection in mammalian cells, was amplified by oligonucleotides #279 and #280 (see Table 2.2 for details) and cloned into the MCS of the promoterless pEGFP-1 vector. The pSV40-EGFP plasmid was produced and its sequence was confirmed by automated sequencing using oligonucleotides #241 and #242 (see Table 2.2 for details) prior to experiments.

Transfection experiments were then repeated in HeLa cells under the pre-determined optimal conditions, and aliquots of transfected cells were subjected to flow cytometry analysis (see Section 2.2.9 for details). The experiment was repeated three times and results from experiment #2 are shown in Figure 3.6. In each experiment five transfections were performed. Forward (FCS) and side scatter (SSC) signals were used to restrict the analysis to viable cells only (grouped as "R1"). Fluorescence induced from non-expressing cells were measured by a sample containing HeLa cells only (Figure 3.6(A)) and a mock transfection sample containing HeLa cells treated with lipofectamine but no plasmid DNA (Figure 3.6(B)), which was set as background fluorescence (gated as "R2"). The level of fluorescence induced from the viable cell population (R1) excluding background fluorescence (R2) was gated as "R3". Transfection of the promoterless pEGFP-1 (Figure 3.6(C)) resulted in expression levels which was about 15-fold over background (mean of R2 = 7.06; mean of R3 = 104.02). Similar results were obtained from the EGFP constructs with inserts containing

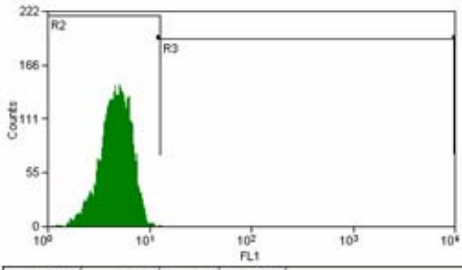
functional enhancer/promoter elements from HPV-18 (Figure 3.6(D)) and SV40 (Figure 3.6(E)).

The average results from three repeats of the experiment (represented graphically in Figure 3.7) showed that samples of cells only and mock transfection resulted in percentage fluorescence of less than 1% with a mean fluorescence of less than 14, which was identified as the background fluorescence from non-expressing cells. The negative control plasmid pEGFP-1 which was a promoterless vector resulted in a significant increase both in the percentage and intensity of fluorescence. Addition of a functional HPV promoter in the LCR insert of pLCR874-EGFP and the SV40 promoter in the positive control plasmid pSV40-EGFP did not affect the percentage of fluorescing cell population, indicating the consistency in transfection efficiency, and the intensity of mean fluorescence induced by both plasmids were only increased by less than 2-fold.

A

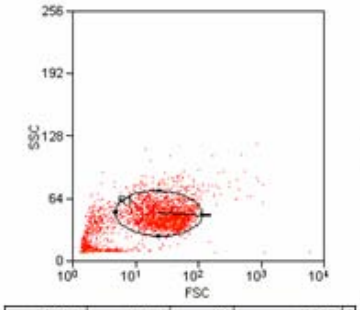


Region	Count	%	Mean
Total	5735	100.00	28.87, 35.82
R1	3566	62.18	30.69, 43.18

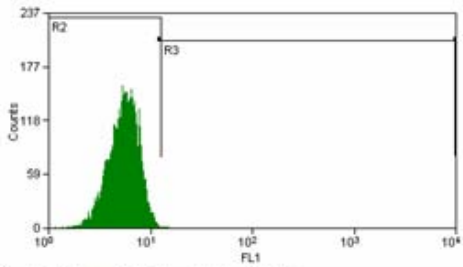


Region	Count	%	Mean
Total	3566	100.00	4.87
R2	3564	99.94	4.86
R3	3	0.08	12.71

B

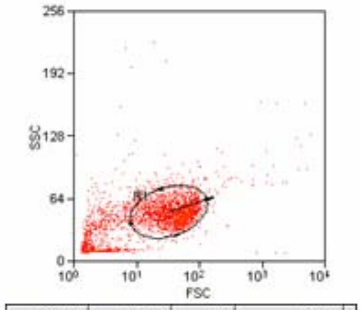


Region	Count	%	Mean
Total	5461	100.00	29.00, 37.72
R1	3520	64.46	33.10, 44.97

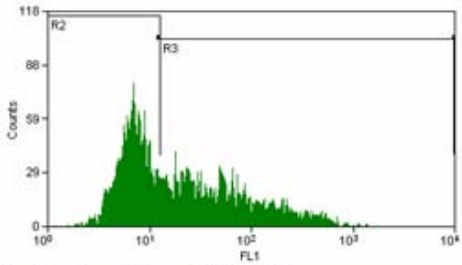


Region	Count	%	Mean
Total	3520	100.00	5.70
R2	3510	99.72	5.67
R3	12	0.34	14.45

C



Region	Count	%	Mean
Total	5234	100.00	47.63, 41.58
R1	3364	64.27	46.32, 48.12



Region	Count	%	Mean
Total	3364	100.00	60.42
R2	1539	45.75	7.06
R3	1853	55.08	104.02

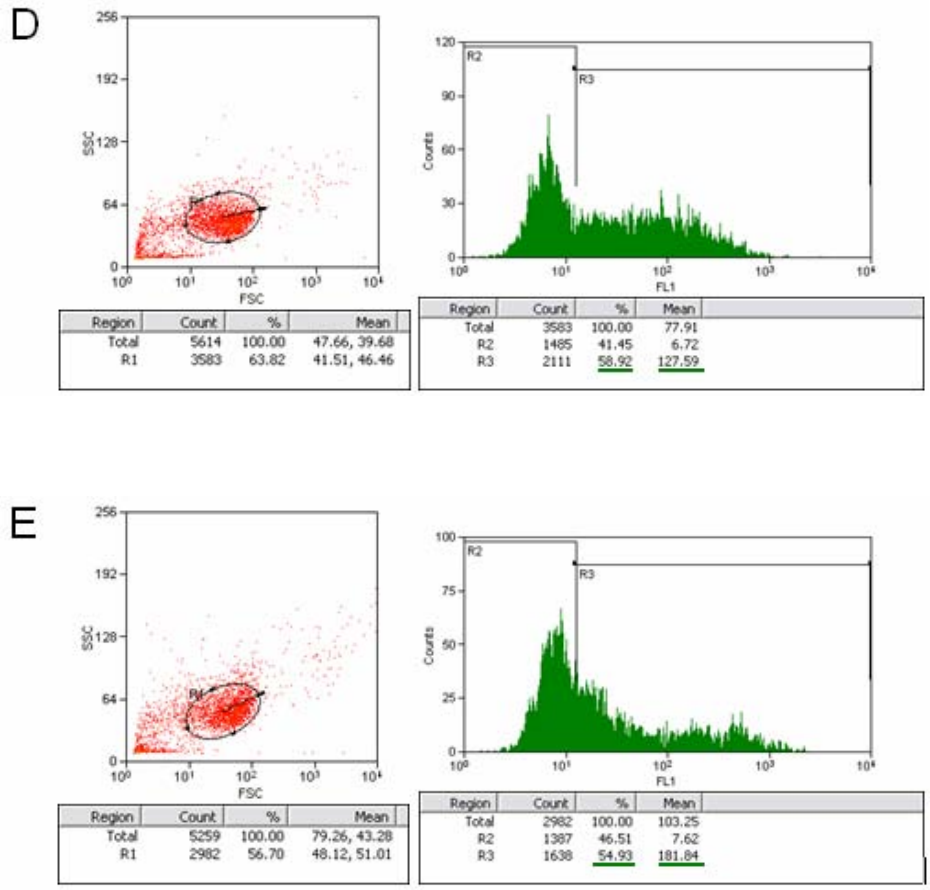


Figure 3.6 Flow cytometry analysis of transfection of GFP constructs in HeLa. Histograms showing flow cytometry results of experiment #2 in (left; in red) side scatter (SSC) against forward scatter signals (FSC), and (right; in green) cell counts against fluorescence intensity (FL1). A: HeLa cells only; B: mock transfection with lipofectamine and no plasmid DNA; C: pEGFP-1; D: pLCR874-EGFP; E: pSV40-EGFP. Analysis was restricted to viable cells only (grouped as R1). Background fluorescence of non-expressing cells (fluorescence induced from A and B) was gated as R2. The percentages of cell population expressing EGFP and mean fluorescence figures are shown in the prints underlined in green.

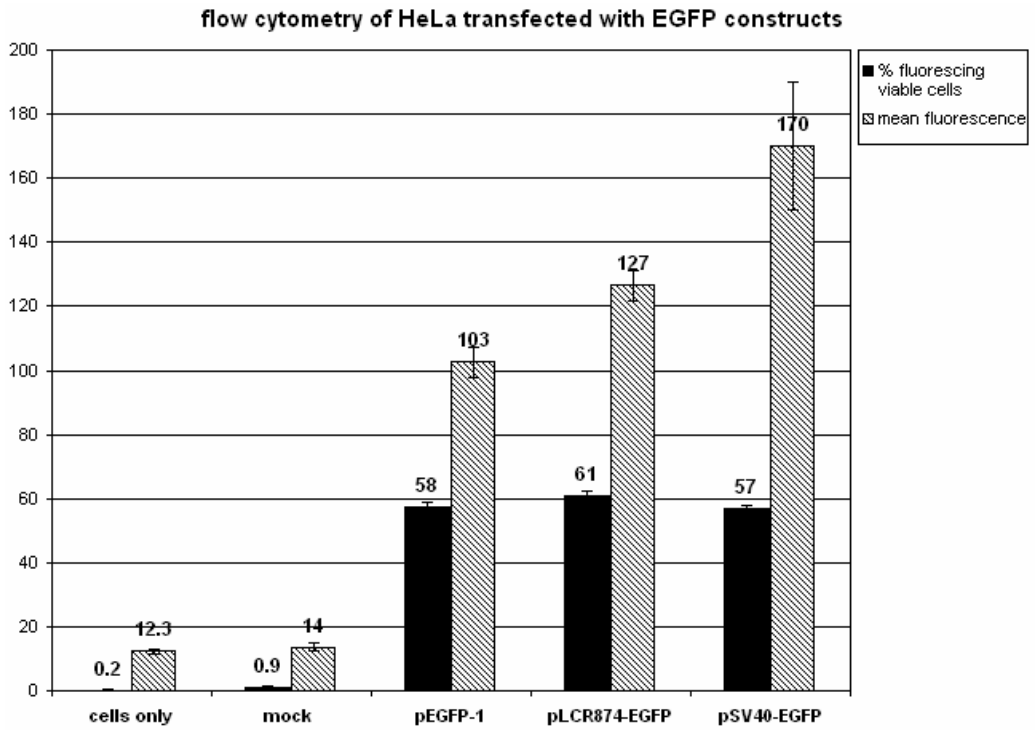


Figure 3.7 Graphical representation of the average flow cytometry result from the EGFP constructs in HeLa cells.

The pLCR874-EGFP plasmid was transfected into HeLa cells together with positive and negative control plasmids for comparison. The solid black bar represents the percentage of viable cell population that was fluorescing. The striped bar represents the level of mean fluorescence of the fluorescing population. The SEM of the three independent experimental repeats are represented by the error bars on the graph.

From results obtained it was concluded that the pEGFP-1 vector failed to serve as an efficient tool to monitor transcription for promoter analysis purpose in mammalian cells. The level of self-induced fluorescence from the promoterless pEGFP-1 vector itself was very high, which did not make it sensitive enough for the study of weak enhancer/promoter elements. In this case the addition of the HPV-18 promoter as well as the positive control SV40 promoter, only managed to induce a slightly higher level of transcription when compared to the promoterless pEFP-1 which served as a negative

control. It was therefore necessarily find a more suitable reporter plasmid and the pGL-3 luciferase reporter vector was chosen for this purpose.

3.2.2 Introduction to the luciferase reporter plasmid

The pGL3-Basic vector is similar to the pEGFP-1 vector used previously, as it is a promoterless plasmid for the quantitative analysis of enhancer and promoter elements that potentially regulate mammalian gene expression (see Figure 2.3 for vector diagram). Instead of a gene that encodes for the EGFP protein in pEGFP-1, the pGL3-Basic vector contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimised for monitoring transcriptional activity in transfected eukaryotic cells (Promega, 2007). Without the addition of a functional promoter into the MCS, the *luc*+ gene downstream will not be expressed. The vector also contains an Amp^r gene conferring ampicillin resistance in *E. coli.*

(i) Design and construction of the LCR deletion constructs

Instead of the exact 874 bp of the HPV-18 LCR, most of the research groups studying the transcriptional control of the HPV-18 LCR (Bauknecht *et al.*, 1992; Cid *et al.*, 1993; Demeret *et al.*, 1994; Gius *et al.*, 1988; Hoppe-Seyler *et al.*, 1991; Thierry *et al.*, 1987) make use of a 1,050 bp *Bam*HI fragment (as described in Section 1.4.1) which, in addition to the entire length of the LCR, also contained 208 bp at its 5′-end corresponding to the end of the L1 ORF and the 20 bp at its 3′-end corresponding to the beginning of the E6 ORF (Thierry *et al.*, 1987). However, no explanation has been given on why this *Bam*HI fragment was preferably chosen.

Since the aim of the project is to induce the maximum level of gene expression in HPV-positive cervical cancer cells by making use of the HPV-18 LCR, to avoid

excluding important elements which may possibly influence the level of promoter activity, new primers were designed to include this longer *Bam*HI fragment. Transcription activities induced from the 1,050 bp *Bam*HI fragment were compared with those from the 874 bp LCR fragment.

Fragments containing different components of the HPV-18 LCR as described in Section 1.41 were also amplified from the HPV-18 plasmid by PCR (see Section 2.2.3(i) for details) and cloned into the pGL3-Basic vector (see Figure 3.8). The template used for these deletion fragments was a pLCR1000-EGFP plasmid which was constructed previously, containing the LCR of HPV-18 from nt 6,928 to 107 amplified by oligonucleotides #277 and #278 (see Table 2.2 for details). All the primers were designed based on the studies performed by Cid *et al.* (1993) and Kim and Taylor (2003), with appropriate alterations to include the restriction enzyme recognition sequences of choice.

The full-length *Bam*HI fragment of HPV-18 LCR was amplified by oligonucleotides #287 and #291 to produce the insert for the pGL3-LCR1000 plasmid (nt 6,930 to 112); the distal enhancer/constitutive enhancer/promoter fragment was amplified by oligonucleotides #288 and #291 to produce the insert for the pGL3-LCR800 plasmid (nt 7,120 to 112); the constitutive enhancer/promoter fragment was amplified by #289 and #291 to produce the insert for the pGL3-LCR400 plasmid (nt 7,509 to 112) and the promoter proximal fragment was amplified by #290 and #291 to produce the insert for the pGL3-LCR200 plasmid (nt 7,739 to 112) (see Table 2.3 for details of oligonucleotides used). All the forward primers used contained a *Xho*I restriction enzyme recognition sequence and the same reversed primer was used for all the fragments which contained a *Hind*III restriction enzyme recognition sequence. The positions of oligonucleotides binding to the target sequence are shown in Figure 3.9. A

positive control plasmid pGL3-SV40 was also made by amplifying the early promoter and enhancer elements from pEGFP-1 (nt 1,689 to 2,049) by oligonucleotides #292 and #293 (see Table 2.3 for details of oligonucleotides used) and cloned into the pGL3-Basic vector. Figure 3.10 is a photo of a 2% agarose gel which shows the different length of LCR fragments and the SV40 promoter fragment obtained by PCR.

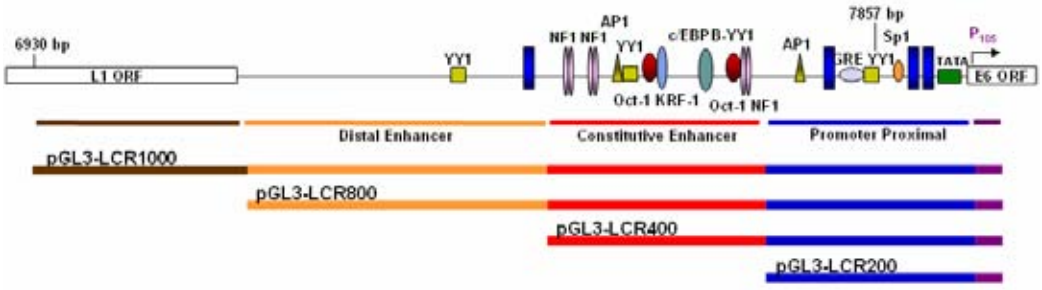
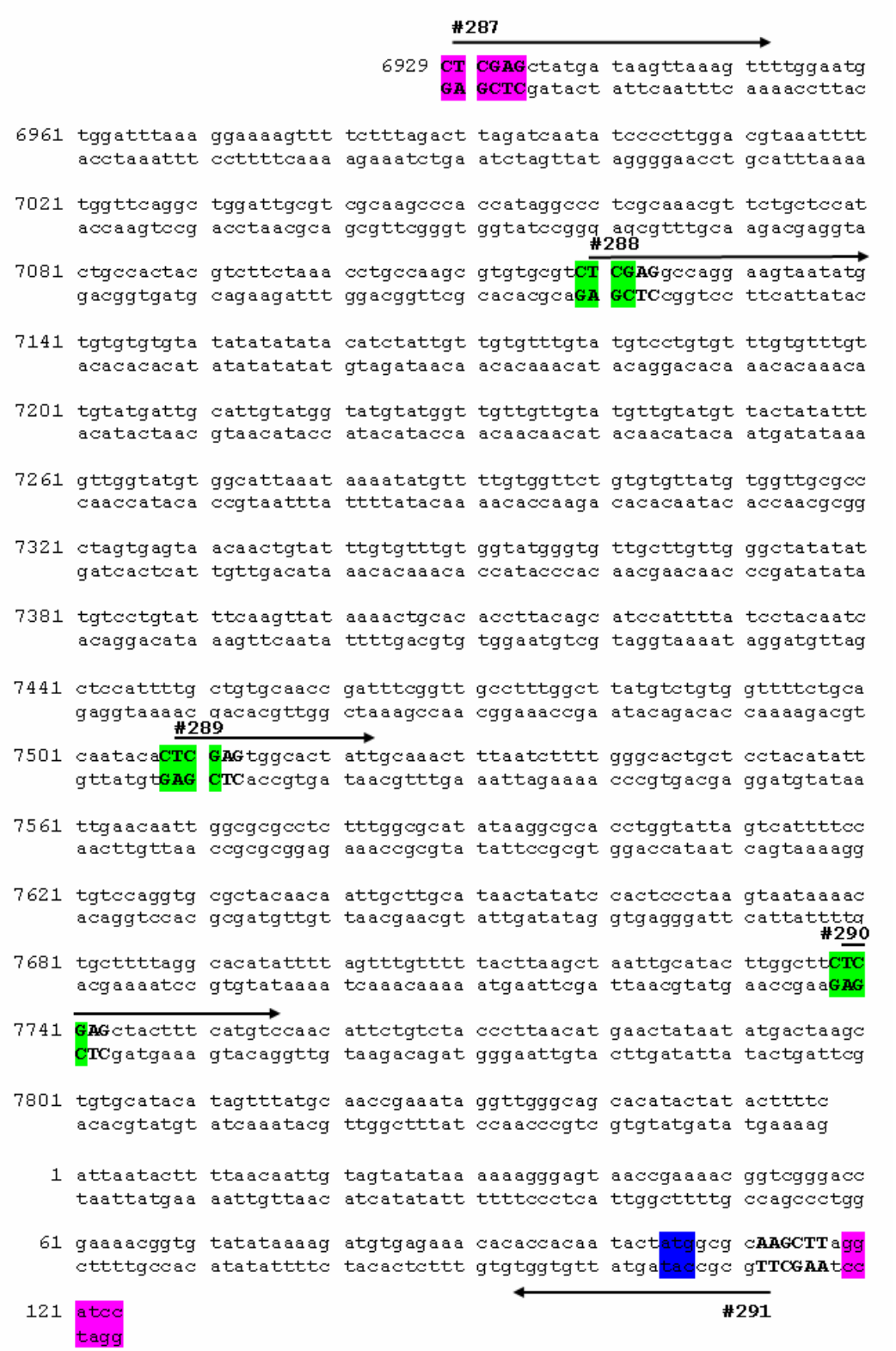


Figure 3.8 Schematic diagram of fragments of the HPV-18 LCR being cloned into the pGL3-Basic vector.

The pGL3-LCR1000 plasmid contained the 1 kb *Bam*HI LCR fragment cloned into the pGL3-Basic vector; removal of the distal 200 bp resulted in the pGL3-LCR800 plasmid; further removal of the distal enhancer region resulted in the pGL3-LCR400 plasmid and removal of the constitutive enhancer region resulted in the pGL3-200.

Figure 3.9 DNA sequences of the inserts of luciferase LCR deletion constructs.
The complete HPV-18 LCR comprises a *Bam*HI fragment of 1,050 bp (nt 6,930 to 123) (positions of *Bam*HI sites in wild-type sequence highlighted in purple), with three functional domains separated by *Rsa*I recognition sites as described in Section 1.4.1 (positions of *Rsa*I sites in wild-type sequence highlighted in green). The region indicated by the arrows represents the exact length of the amplified LCR fragment after being digested by restriction enzymes and cloned into the pGL3-Basic vector. pGL3-LCR1000 (nt 6,930 to 112; 1,040 bp): #287/#291; pGL3-LCR800 (nt 7,120 to 112; 850 bp): #288/#291; pGL3-LCR400 (nt 7,509 to 112; 461 bp): #289/#291 and pGL3-LCR200 (nt 7,739 to 112; 231 bp): #290/#291 (see Table 2.3 for details of oligonucleotides used). nt 1 represents the beginning of the HPV-18 genome, as established by Cole and Danos (1987). Letters in capital and bold are base changes to the wild-type sequence to accommodate restriction enzyme recognition sequences (CTCGAG for *Xho*I in the forward primers; AAGCCT for *Hind*III in the reverse primer) to assist cloning. Highlighted in blue is the start codon ATG for early gene transcription from the P_{105} promoter.


#287
6929 CT CGAGctatga taagttaaag ttttggaatg
GA GCTCgatact attcaatttc aaaaccttac
6961 tggatttaaa ggaaaagttt tctttagact tagatcaata tccccttgga cgtaaatttt
acctaaattt ccttttcaaa agaaatctga atctagttat aggggaacct gcatttaaaa
7021 tggttcaggc tggattgcgt cgcaagccca ccataggccc tcgcaaacgt tctgctccat
accaagtccg acctaacgca gcgttcgggt ggtatccggg agcgtttgca agacgaggta
#288
7081 ctgccactac gtcttctaaa cctgccaagc gtgtgcgtCT CGAGgccagg aagtaatatg
gacggtgatg cagaagattt ggacggttcg cacacgcaGA GCTCcggtcc ttcattatac
7141 tgtgtgtgta tatatatata catctattgt tgtgtttgta tgtcctgtgt ttgtgtttgt
acacacacat atatatatat gtagataaca acacaaacat acaggacaca aacacaaaca
7201 tgtatgattg cattgtatgg tatgtatggt tgttgttgta tgttgtatgt tactatattt
acatactaac gtaacatacc atacatacca acaacaacat acaacataca atgatataaa
7261 gttggtatgt ggcattaaat aaaatatgtt ttgtggttct gtgtgttatg tggttgcgcc
caaccataca ccgtaattta ttttatacaa aacaccaaga cacacaatac accaacgcgg
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#289
7501 caatacaCTC GAGtggcact attgcaaact ttaatctttt gggcactgct cctacatatt
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7561 ttgaacaatt ggcgcgcctc tttggcgcat ataaggcgca cctggtatta gtcattttcc
aacttgttaa ccgcgcggag aaaccgcgta tattccgcgt ggaccataat cagtaaaagg
7621 tgtccaggtg cgctacaaca attgcttgca taactatatc cactccctaa gtaataaaac
acaggtccac gcgatgttgt taacgaacgt attgatatag gtgagggatt cattattttg
#290
7681 tgcttttagg cacatatttt agtttgtttt tacttaagct aattgcatac ttggcttCTC
acgaaaatcc gtgtataaaa tcaaacaaaa atgaattcga ttaacgtatg aaccgaaGAG
7741 GAGctacttt catgtccaac attctgtcta cccttaacat gaactataat atgactaagc
CTCgatgaaa gtacaggttg taagacagat gggaattgta cttgatatta tactgattcg
7801 tgtgcataca tagtttatgc aaccgaaata ggttgggcag cacatactat acttttc
acacgtatgt atcaaatacg ttggctttat ccaacccgtc gtgtatgata tgaaaag
1 attaatactt ttaacaattg tagtatataa aaaagggagt aaccgaaaac ggtcgggacc
taattatgaa aattgttaac atcatatatt ttttccctca ttggcttttg ccagccctgg
61 gaaaacggtg tatataaaag atgtgagaaa cacaccacaa tactatggcg cAAGCTTagg
cttttgccac atatattttc tacactcttt gtgtggtgtt atgatacCGC gTTCGAAtcc
#291
121 atcc
tagg

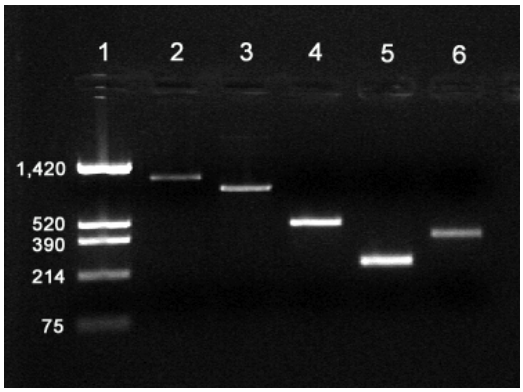


Figure 3.10 PCR products for luciferase LCR deletion constructs.
A 2% (w/v) agarose gel ran at 90V for 25 minutes, showing all the PCR products of the LCR deletion constructs as described in Figure 3.8. Lane 1 was 500 ng of pUC/*Hinf*I DNA marker. Lane 2 was the PCR product from oligonucleotides #287/#291 for pGL3-LCR1000 (1,062 bp). Lane 3 was the PCR product from oligonucleotides #288/#291 for pGL3-LCR800 (869 bp). Lane 4 was the PCR product from oligonucleotides #289/#291 for pGL3-LCR400 (484 bp). Lane 5 was the PCR product from oligonucleotides #290/#291 for pGL3-LCR200 (249 bp). Lane 6 was the PCR product from oligonucleotides #292/#293 for pGL3-SV40 (392 bp).

The PCR products obtained were gel-purified and digested by restrictions enzymes *Xho*I and *Hind*III (see Section 2.2.3(iv) for details). The pGL3-Basic vector was also linearised by the same pair of restriction enzymes. All the digested products were again gel-purified prior to ligation to form the pGL3-LCR constructs.

The LCR deletion constructs were designed to contain both the ATG start codon from the insert sequence as well as that from the pGL3-Basic vector for the luciferase gene, and two ATG start codons were ensured to be in frame. Figure 3.11 shows the joining ends of the LCR inserts for the deletion constructs as well as the SV40 promoter insert for the positive control plasmid to the pGL3-Basic vector.

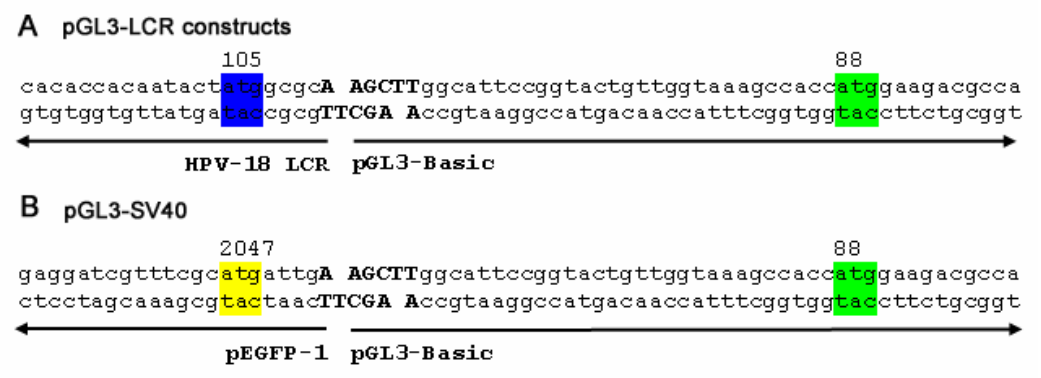


Figure 3.11 Joining ends of the luciferase LCR deletion inserts and the SV40 promoter insert to the pGL3-Basic vector.

Both the inserts and the pGL3-Basic vector were double-digested by *Xho*I and *Hind*III to obtain sticky ends for cloning purpose. The diagram shows the 3′-ends of the inserts (left) and 5′-ends of the pGL3-Basic vector, which were joined to each other by the *Hind*III restriction enzyme recognition sequence (AAGCTT) in caps and bold. (A) shows the joining ends of all the LCR deletion constructs, containing the ATG start codon of early gene transcription initiated by the P_{105} promoter (highlighted in blue) at nt 105 of HPV-18. The ATG start codon of the luciferase gene is located 39 bp downstream (highlighted in green) at nt 88 of pGL3-Basic. (B) shows the joining end of the positive control plasmid pGL3-SV40, containing the ATG start codon of the kanamycin/neomycin resistance gene initiated by the SV40 early promoter (highlighted in yellow) at nt 2,047 of pEGFP-1. The ATG start codon of the luciferase gene is again located 39 bp downstream (highlighted in green) at nt 88 of pGL3-Basic.

Ligation of the inserts to the linearised pGL3-Basic vector was performed as described in Section 2.2.4(ii); the ligation mixture was then transformed into $CaCl_2$ competent *E. coli* (see Section 2.2.4(iii) for details) and selected on LB plates containing ampicillin. Minipreps were performed on selected colonies (see Section 2.2.5(ii) for details) and 5 µL of the miniprep samples were digested again with *Xho*I and *Hind*III and analysed on an agarose gel to check for the presence of the original insert. Clones containing the inserts of the right sizes were then subjected to automated

sequencing (see Section 2.2.5(vii) for details) using oligonucleotides #285 and #286 (see Table 2.3 for details oligonucleotides used). Plasmid constructs containing the correct sequences were purified in large scale either by maxiprep (see Section 2.2.6(i) for details) or using the Qiagen Midi kit. The concentration and purity of plasmid preparations were measured by the NanoDrop® as described in Section 2.2.5(vi). A flow diagram to illustrate the procedures involved in the construction of the luciferase plasmids containing the LCR inserts is shown in Figure 3.12.

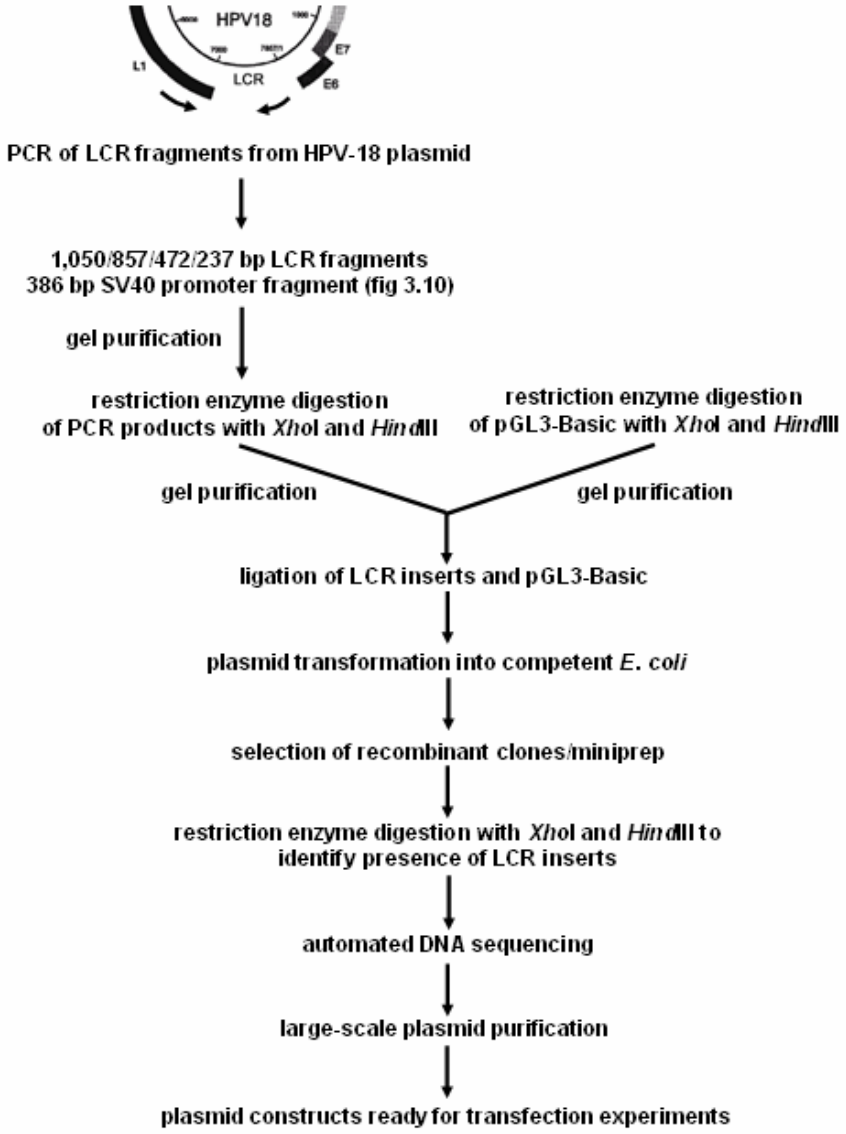


Figure 3.12 Flow diagram of the construction of the LCR deletion constructs.

Different length fragments of the LCR and the SV40 promoter fragment were amplified by PCR and gel purified prior to restriction enzyme digestion by *Xho*I and *Hind*III to produce the adhesive ends for cloning purpose. Similarly, the pGL3-Basic vector was also digested by the same restriction enzymes. Both digested inserts and vectors were again gel purified prior to ligation, and transformed into competent *E. coli*. Small-scale plasmid purification was performed on selected recombinant clones, and again digested by *Xho*I and *Hind*III to identify the presence of the inserts. Successfully ligated plasmids were then subjected to sequencing prior to large-scale plasmid purification to produce sufficient amount of plasmids for subsequent transfection experiments.

(ii) Determination of optimal transfection conditions

As mentioned previously in Section 2.2.10, for luciferase assays it was not necessary to maintain transfected cells in the form of whole cells since luciferase assays were performed on cell lysates. Furthermore it was no longer necessary to attach transfected cells to coverslips for no microscopy procedures were involved. Transfection experiments could therefore be performed in a much smaller scale in order to conserve resources. Instead of performing transfections in 6-well tissue culture plates for the pEGFP-1 constructs, 24-well tissue culture plates were being used for the transfection of luciferase plasmid constructs into different cell lines.

The seeding density of cells was the first parameter to be optimised. An ideal seeding density should result in approximately 60% confluence in 24 hours, which is the optimal cell density for transfection with lipofectamine. HeLa cells were seeded in triplicate into the wells of 24-well tissue culture plates in a range from 2 to $6x10^4$ cells per well, and the cell densities were observed and recorded after 24 hours. Results are graphically represented in Figure 3.13. A seeding density of $3x10^4$ cells per well was chosen for HeLa cells in a 24-well tissue culture plate. Similar experiments were performed on all the other cell lines used and the optimal seeding densities determined are shown in Table 3.2.

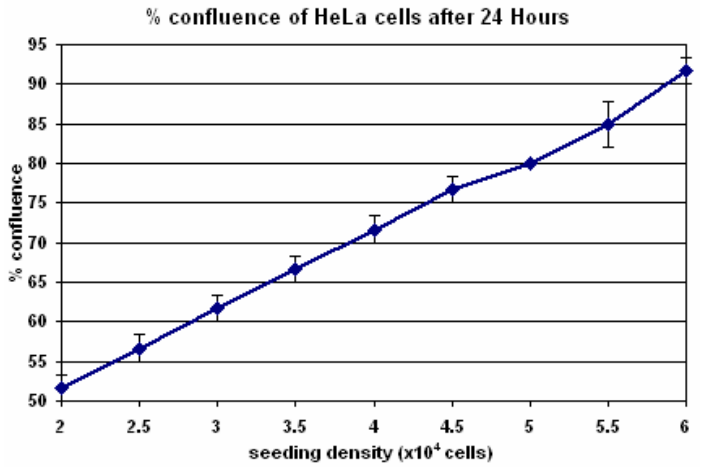


Figure 3.13 Optimal seeding density for transfection in HeLa cells (24-well plate). HeLa cells ranging from 2 to 6x10^4 cells per well were seeded into triplicate wells of 24-well tissue culture plates, and percentage confluence of individual wells were recorded after 24 hours. The standard error of the mean of the triplicate wells are represented by error bars.

Table 3.2 Optimal seeding densities for transfection in various cell lines.

cell line	optimal seeding densities (x10^4 cells per well)
HeLa	**3.0**
SiHa	**3.0**
C33A	**3.5**
H1299	**5.5**
MRC-5	**4.0**

The optimal seeding densities of different cell lines were determined by seeding 2 to 6x10^4 cells per well in a 24-well tissue culture plate and recording the percentage confluence of individual wells after 24 hours. Seeding densities which resulted in approximately 60% confluence were chosen to be optimal for each cell line.

To ensure that the self-expressing problem of the pEGFP-1 vector did not exist in the pGL3-Basic vector, a preliminary experiment was carried out by transfecting the pGL3-LCR800 construct into HeLa cells together with the negative and positive control plasmids, pGL3-Basic and pGL3-SV40. The luciferase activities were expressed in

relative light units (RLU) measured by the luminometer. Results showed that the promoterless pGL3-Basic vector induced a relatively low level of level of luciferase activity. With the addition of the HPV promoter in the LCR insert of pGL3-LCR800 the level of luciferase activity increased by almost 10-fold, and with the positive control SV40 promoter by almost 40-fold when compared to the empty vector (Figure 3.14). This result showed that the problem of promoterless expression, as seen in the previously used pEGFP-1 vector, is not apparent in the pGL3-Basic luciferase reporter system. This low background luciferase activity detected from the promoterless vector, pGL3-Basic, facilitated accurate promoter analysis.

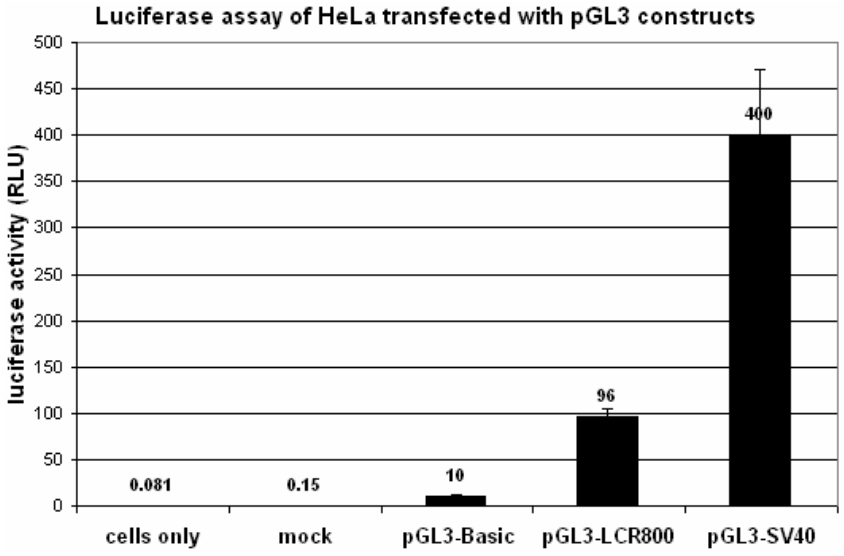


Figure 3.14 Preliminary testing of the pGL3-Basic vector in HeLa cells.
The pGL3-LCR800 plasmid was transfected into HeLa cells together with positive and negative control plasmids for comparison. The levels of luciferase activities detected were expressed in RLU, represented by the solid black bars in the graph. The error bars represent the range of results obtained from two experimental repeats.

The next step was to determine the optimal amount of plasmid DNA to be used. The pGL3-SV40 plasmid was transfected into HeLa cells in a range of 75 to 350

ng per well in a 24-well tissue culture plate with 3 μL of lipofectamine (see Section 2.2.7 for details). Transfected cells were harvested after 48 hours and cell lysates were prepared for luciferase assay (see Section 2.2.10 for details). The relative light units of luciferase activity induced per ng of plasmid DNA were calculated. The amount of plasmid DNA resulting in the highest RLU per ng DNA was determined as the optimal amount. Results are shown in Figure 3.15, and the optimal amount of plasmid DNA chosen for HeLa cells was 300 ng.

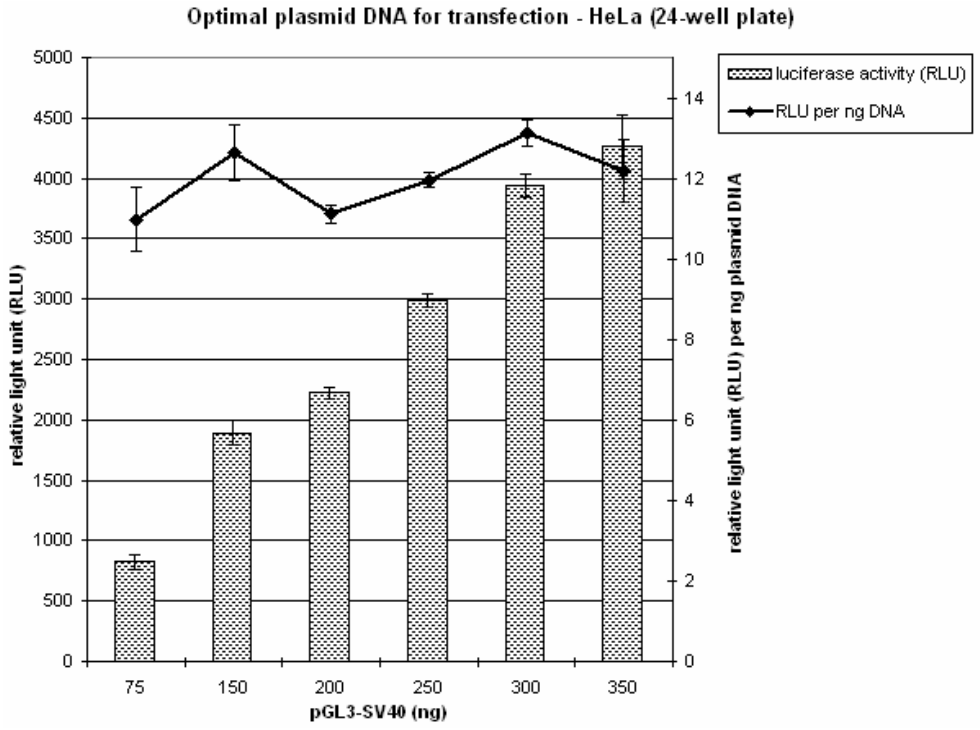


Figure 3.15 Optimal amount of DNA for transfection in HeLa (24-well plate).
HeLa cells were transfected by the positive control plasmid pGL3-SV40 in different amounts ranging from 75 to 350 ng per well in a 24-well tissue culture plate. Luminescence detected was expressed in RLU represented by the bar graph (primary y-axis on the right); RLU induced per ng DNA was calculated and represented by the line graph (secondary y-axis on the left). The optimal amount was determined by the highest RLU per ng DNA used. The error bars represent the range of results obtained from two experimental repeats.

(iii) Co-transfection with the β-galactosidase vector

A reference plasmid, pCMVβ, was employed to be co-transfected into the cells together with the test plasmids. pCMVβ is a vector containing a β-galactosidase gene linked to the CMV promoter and expresses high level of β-galactosidase in eukaryotic cells (Clontech, 2003) (see Figure 2.2 for vector diagram). The luciferase activities measured with the test plasmids were divided by the β-galactosidase activity expressed from the reference plasmid to obtain a normalised luciferase reading which represents gene expression induced from the population of cells that were successfully transfected by the plasmids only. A schematic diagram demonstrating the principle of co-transfection is shown in Figure 3.16.

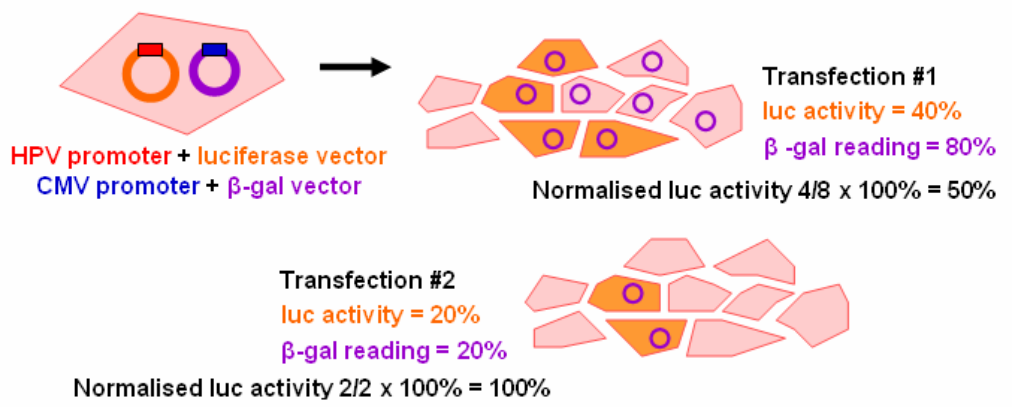


Figure 3.16 Schematic diagram demonstrating the principle of co-transfection. The test plasmid (HPV promoter in luciferase vector) was co-transfected with a reference plasmid (CMV promoter in β-gal vector) into mammalian cell lines. By performing luciferase assay alone transfection #1 (40%) appeared to have a higher level of gene expression induced when compared to transfection #2 (20%). However, when β-galactosidase readings induced from the reference plasmid was also measured and used to normalise the luciferase activity detected, the relative luciferase activity of transfection #1 (50%) was in fact lower than that of transfection #2 (100%), which is a more accurate indication of luciferase expression restricted to the actual transfected population only.

Since the two plasmids to be co-transfected contained different promoters, the CMV promoter and the HPV-18 P_{105} promoter, that are known to be functional within mammalian cell lines, it was necessary to determine the optimal reference plasmid to test plasmid ratio; in order to minimise the interference from possible promoter competition in co-transfection experiments. The pCMVβ and pGL3-LCR800 plasmids were transfected into HeLa cells in ratios of 1:1, 1:2, 1:4 and 1:9 in a total of 500 ng of plasmid DNA (see Section 2.2.7 for details), and luciferase assays were performed on the cell lysates of the transfected cells at 48 hours post-transfection (see Section 2.2.10 for details). The relative light units of luciferase activity induced per ng of pGL3-SV40 were calculated. The CMV to P_{105} promoter ratio resulting in the highest RLU per ng of pGL3-LCR800 DNA was determined as the optimal ratio. Results are shown in Figure 3.17, and the optimal CMV to P_{105} promoter ratio for co-transfection in HeLa was found to be 1:4. A point to note is that the competition in between two promoter elements is dependent on their relative molar ratio in the co-transfection experiment, and the molar ratio stays the same regardless of the total amount of plasmid DNA used (in ng) as long as the ratio (in ng) remains to be 1:4. Hence, although the competition experiments were performed in a total amount of 500 ng DNA, the subsequent co-transfection experiments were performed using the optimal DNA amount determined previously (as shown in Figure 3.15).

All the subsequent co-transfection experiments were performed in 24-well tissue culture plates, transfected with the pCMVβ and pGL3 plasmids in 1:4 reference plasmid to test plasmid ratio (in a total of 300 ng of plasmid DNA) using 3 μL of lipofectamine. Although optimisation experiments were all performed on only HeLa cells, the same parameters were used for all the other cell lines used. This is to allow

more accurate comparison of promoter activity in between different cell lines by limiting variable factors.

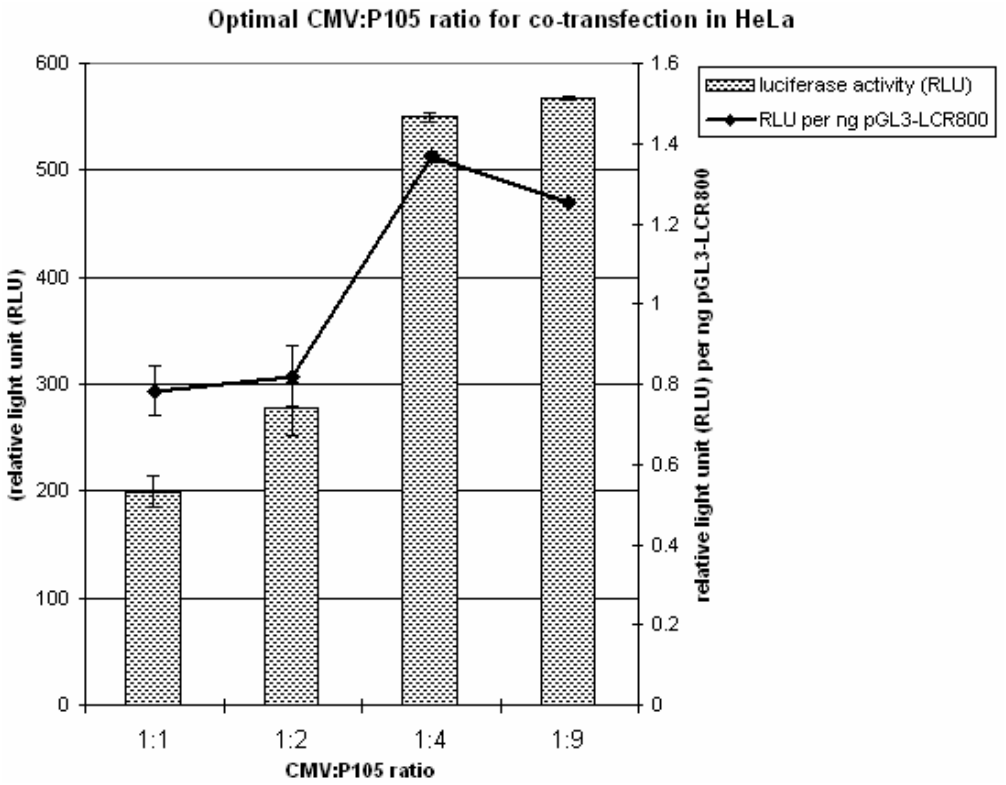


Figure 3.17 Optimal CMV to P_{105} promoter ratio for co-transfection in HeLa.
The optimal CMV to P_{105} promoter ratio to be used in co-transfection experiments in HeLa cells was determined by transfecting the pCMVβ and the pGL3-LCR800 plasmids in ratios of 1:1, 1:2, 1:4 and 1:9 (total plasmid DNA = 500 ng). Luminescence detected in RLU were represented by the bar graph (primary y-axis on the right); RLU induced per ng pGL3-LCR800 was calculated and represented by the line graph (secondary y-axis on the left). The optimal amount was determined by the highest RLU per ng pGL3-LCR800 DNA used. The error bars represent the range of results obtained from two experimental repeats.

3.2.3 HPV-18 LCR promoter studies

The prepared luciferase constructs containing different sized fragments of the HPV-18 LCR were subsequently transfected into the five mammalian cell lines as described in Table 2.1, together with the positive control plasmid pGL3-SV40. Transfected cells were harvested 48 hours after transfection and cell lysates were collected for luciferase and β-galactosidase assays as described in Sections 2.2.10 and 2.2.11. An independent experiment on each cell line was repeated at least three times and each independent experiment was carried out in triplicate. A sample of data results from HeLa cells is shown in Table 3.3 to show how the results were processed, and results from all the other cell lines were calculated by the same method. A summary of results from the transfection experiments of the LCR deletion constructs in all five cell lines is graphically represented in Figure 3.18. The raw data results are shown in the appendix chapter.

Table 3.3 Interpretation of data results from transfection of luciferase LCR deletion constructs into HeLa cells (experiment #1 and summary).

	A	B	C	D	E	F
1	**HeLa experiment #1**					
2	**b-galactosidase assay**	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	pGL3-SV40
3	repeat #1	0.945	1.018	0.998	0.775	0.729
4	repeat #2	0.945	0.937	1.023	0.714	0.702
5	repeat #3	0.981	0.936	0.994	0.889	0.700
6						
7	**luciferase assay**	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	pGL3-SV40
8	repeat #1	283.2	584.7	126.0	4.68	6680
9	repeat #2	314.9	588.0	125.0	4.69	6940
10	repeat #3	396.1	622.4	126.9	4.74	9600
11						
12	**Normalised data (luc/b-gal)**	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	pGL3-SV40
13	repeat #1	300	570	130	6.00	9400
14	repeat #2	330	630	120	6.60	8800
15	repeat #3	400	670	130	5.30	11000
16	average	350	620	130	6.00	9900
17	SV40 as 100%	**3.5**	**6.3**	**1.3**	**0.061**	**100**
18						
19	**HeLa SUMMARY**					
20		pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	pGL3-SV40
21	**HeLa experiment #1**					
22	average luc activity	350	620	130	6.00	9900
23	SV40 as 100%	**3.5**	**6.3**	**1.3**	**0.061**	**100**
24	**HeLa experiment #2**					
25	average luc activity	490	820	250	2.2	15000
26	SV40 as 100%	**3.2**	**5.4**	**1.6**	**0.015**	**100**
27	**HeLa experiment #3**					
28	average luc activity	620	770	180	1.2	14000
29	SV40 as 100%	**4.5**	**5.6**	**1.3**	**0.0089**	**100**
30	**Average HeLa experiment #1-3**					
31	average luc activity	480	740	180	3.1	13000
32	average SV40 as 100%	**3.7**	**5.8**	**1.4**	**0.028**	**100**
33	stardard error of mean	0.38	0.29	0.12	0.016	

The upper part of the table (rows 1 to 17) is results from experiment #1 of HeLa cells. Cells were transfected with the same plasmid in triplicates (repeats #1 to 3) in three separate wells. Rows 3 to 5 are the β-galactosidase readings of each well, which were used to normalise their relative luciferase readings (rows 8 to 10) to control for transfection efficiency. For example, the luciferase reading of pGL3-LCR1000 in repeat #1 (B8) was divided by its relative β-galactosidase reading (B3) to obtain a normalised luciferase reading (B13) of repeat #1. The averages of the three repeats of normalised luciferase readings for each plasmid construct were calculated (row 16). Results were then calculated as a percentage relative to the normalised luciferase reading of the positive control pGL3-SV40 (row 17). By repeating the same procedure for experiment #2 (row 26) and experiment #3 (row 29), an average of relative luciferase activity to the positive control of the three individual experiments was calculated (row 32), which was taken as the final reading for HeLa cells. The standard error of the average values of the three repeats of the experiment were calculated (row 33). Numbers shown in the table were all rounded off to two significant figures.

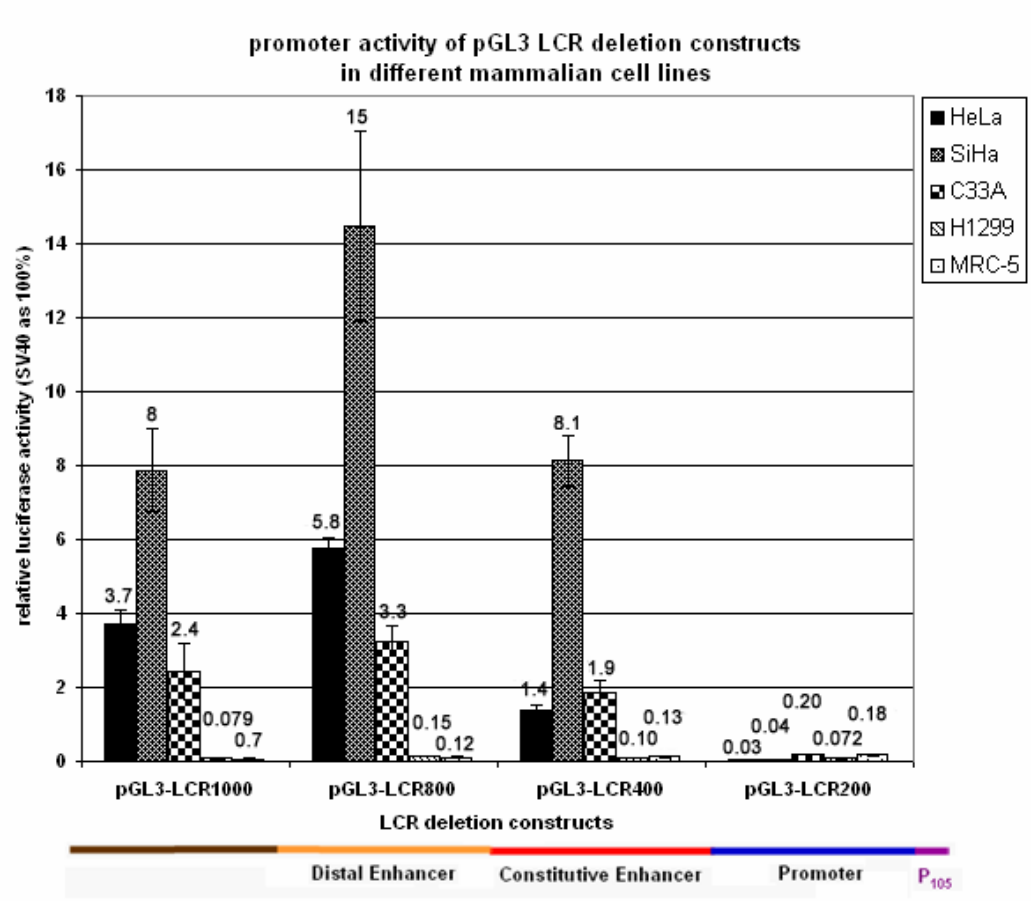


Figure 3.18 Luciferase activity from LCR deletion constructs in different cell lines.

The graph represents results obtained from transfection experiments using five different cell lines. Luciferase activities detected from various LCR deletion constructs were plotted as percentages relative to the luciferase readings from the pGL3-SV40 plasmid which served as a positive control. The standard error of the mean of three experimental repeats in each cell line are represented by error bars.

(i) Overall P_{105} promoter activity and specificity

The results showed a significant level of luciferase activity induced from the LCR deletion constructs in the three cervical cancer cell lines tested (HeLa, SiHa and C33A), with the luciferase reading detected in SiHa cells being the highest (8%),

followed by HeLa (3.7%) and C33A (2.4%). This shows that the P_{105} promoter is highly active within cervical carcinoma cell lines in the context of the cell lines tested. Similar results were reported by Hoppe-Seyler and Butz, (1992) when the HPV-18 LCR promoter activity was tested by a different reporter system, suggesting the HPV-18 LCR to be highly active in SiHa, HeLa and C33A cells. The level of luciferase readings detected in H1299 (0.079%) and MRC-5 cells (0.07%) were extremely low, indicating that the P_{105} promoter is essentially inactive in the carcinoma cell lines tested that are not of a cervical origin, and also in the non-cancerous cell line tested. These results are very similar to previously reported findings by Hoppe-Seyler *et al*. (1991) which studied the HPV-18 LCR promoter activity in different mammalian cell lines using different reporter systems, suggesting the HPV-18 LCR could be strongly stimulated within HeLa and C33A cells, weakly stimulated in primary human fibroblast and almost completely inactive in HepG2 cells (human hepatoma cell line).

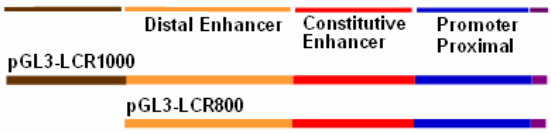
It is important to note that since the level of normalised luciferase activities detected from H1299 and MRC-5 cells were extremely low, the differences in promoter activity induced by the LCR deletion constructs may not be significant and accurate enough for comparison purpose, due to the overlapping error bars. For the above reason, contribution from different LCR components towards promoter activation in H1299 and MRC-5 cells has only been briefly discussed in this section, but not included as major findings of this thesis. The summary of this chapter has been focused on the three cervical cancer cell lines tested instead.

In terms of promoter specificity, in the context of limited cell lines used in this project, the results indicate that the P_{105} promoter of the LCR is not only specific towards epithelial cells in general, but more precisely towards cervical epithelial cells in particular. This observation is in agreement with Butz and Hoppe-Seyler (1993)

suggesting that although a number of transcription factors binding to the LCR have been implicated as contributing to the tissue-specificity of the HPV-18 LCR, the regulatory mechanisms resulting in the transcriptional activation of the HPV E6 and E7 genes can significantly differ among epithelial cells. It seems unlikely that epithelial cell-specific activity of the HPV-18 LCR observed was simply the effect of common, epithelial transactivating factors but rather a result from alternate regulatory pathways in different epithelial cells.

(ii) Comparison of promoter activity from pGL3-LCR1000 and pGL3-LCR800

In order to examine the effect of removing the distal 200 bp from the LCR on the P_{105} promoter strength, the level of luciferase activity detected from pGL3-LCR1000 was set as 1 and activities from pGL3-LCR800 were calculated as a ratio relative to the undeleted LCR fragment (Figure 3.19).



HeLa	SiHa	C33A	H1299	MRC-5
1	1	1	1	1
1.5	1.7	1.3	1.8	2.1

Figure 3.19 Relative promoter activity from pGL3-LCR800 in different cell lines. The level of luciferase activity detected from the full length LCR insert in the pGL3-LCR1000 plasmid was set at 1, and the ratios of luciferase readings from the pGL3-LCR800 plasmid were calculated. Numbers were all rounded off to two significant figures.

Removal of the distal 200 bp fragment (pGL3-LCR800) resulted in a significantly increased level of luciferase activity, indicated by a ratio above 1 (increased by 50% in HeLa, 70% in SiHa, 30% in C33A, 80% in H1299 and 110% in MRC-5). This suggests that the shorter 800 bp LCR fragment in the pGL3-LCR800

plasmid has the ability to induce stronger promoter activation when compared with the full-length LCR fragment in the pGL3-LCR1000 plasmid. This result has not been reported previously, as earlier studies have been focused on promoter activity induced by the entire *Bam*HI LCR fragment and no attempts have been made in identifying the possible contribution of the extra 208 bp from the L1 ORF at the 5′-end of the *Bam*HI LCR fragment towards promoter activation. The results suggest the existence of possible transcriptional repressor elements present within the distal *Bam*HI-*Rsa*I fragment, which contains about 200 bp from the 3′-end of the L1 ORF.

In order to identify whether the promoter element within the LCR may possibly possess selectivity towards HPV-positive cervical cancer cells in particular, the ratios of luciferase activity from HeLa and SiHa cells against the other control cell lines were calculated and shown in Table 3.4.

Table 3.4 Comparison of luciferase activity from the LCR deletion constructs in HeLa and SiHa with other cell lines.

Selectivity towards HeLa (HPV-18 +ve cervical cancer cells)	LCR1000	LCR800
HeLa (HPV-18) : SiHa (HPV-16)	0.47	0.40
HeLa (HPV +ve) : C33A (HPV -ve)	1.6	1.8
HeLa (cervix) : H1299 (lung)	48	39
HeLa (cancerous epithelial) : MRC-5 (normal fibroblast)	54	50

Selectivity towards SiHa (HPV-16 +ve cervical cancer cells)	LCR1000	LCR800
SiHa (HPV-16) : HeLa (HPV -18)	2.1	2.5
SiHa (HPV +ve) : C33A (HPV -ve)	3.3	4.5
SiHa (cervix) : H1299 (lung)	100	98
SiHa (cancerous epithelial) : MRC-5 (normal fibroblast)	110	130

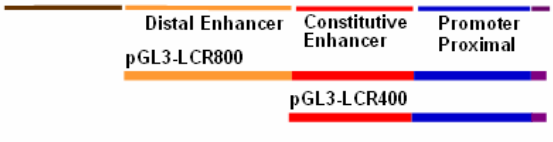
A ratio higher than 1 represents selectivity towards HeLa cells or SiHa and *vice versa*. A green square on the top right corner of each box in the columns of the pGL3-LCR800 plasmid denotes an increase in specificity towards HeLa cells or SiHa upon the deletion of the distal 200 bp fragment. A red square denotes a decrease in specificity towards HeLa or SiHa cells instead. All numbers were rounded off to two significant figures.

The level of promoter activity has already shown to be significantly higher within the three cervical cancer cell lines tested (see Figure 3.18). Moreover ratios calculated in Table 3.4 showed that the P_{105} promoter appeared to have higher activity level within the two HPV-positive cervical cancer cells tested in particular. Promoter activities detected from the pGL3-LCR1000 plasmid in HeLa and SiHa cells were 1.6-fold and 3-fold higher than that in C33A cells respectively. Deletion of the distal 200 bp *Bam*HI-*Rsa*I fragment (pGL3-LCR800) resulted in a further increase in the selectivity of the P_{105} promoter towards the two HPV-positive cervical cancer cells tested was observed. Promoter activities detected from the pGL3-LCR800 plasmid in HeLa and SiHa cells were almost 1.8-fold and 4.5-fold higher than that in C33A cells respectively. These results suggest the presence of HPV-dependent promoter repressors acting on the distal 200 bp fragment. The shorter fragment of the LCR within the pGL3-LCR800 plasmid therefore appeared to be a better option to be used as a tool for selecting HPV-positive cervical cancer cells when compared to the *Bam*HI LCR fragment within the pGL3-LCR1000 plasmid, both in terms of promoter strength and specificity.

(iii) Significance of the distal enhancer region

As mentioned in Section 1.5, since most of the transcription control elements identified within the HPV-18 LCR are located within the constitutive enhancer and proximal promoter region, and the precise function of the distal enhancer region contributing to the LCR promoter activation is unclear (Cid *et al.*, 1993). From the results obtained from the LCR deletion constructs, further deletion of the distal enhancer region (pGL3-LCR400) resulted in a decreased level of luciferase activity in both HeLa and C33A cells, showing only 38% and 77% promoter activity of the full-length LCR respectively. In SiHa cells, however, upon the corresponding deletion

luciferase activity detected was still 12% higher than that from the full-length LCR. It is difficult to draw a conclusion regarding the contribution of the distal enhancer to promoter activity based on these results. However, when another set of ratios were calculated by setting the luciferase activity from the pGL3-LCR800 as 1 instead, the effect upon the deletion of the distal enhancer became more apparent (Figure 3.20).



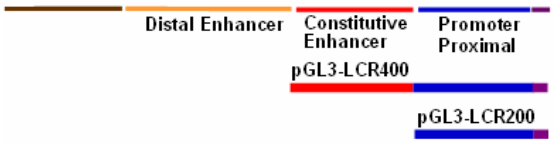
HeLa	SiHa	C33A	H1299	MRC-5
1	1	1	1	1
0.25	0.64	0.57	0.65	1.2

Figure 3.20 Ratios of changes in promoter activity upon removal of the distal enhancer region in different cell lines.
The level of luciferase activity detected from the pGL3-LCR800 plasmid was set at 1, and the ratios against the luciferase readings from the pGL3-LCR400 plasmid were calculated. Numbers were all rounded off to two significant figures.

The results showed that the removal of the distal enhancer region has the most significant impact in the downregulation of promoter activity in HeLa cells, which retained only 25% of promoter activity of that prior to deletion. The other three epithelial cell lines tested showed similar results, retaining about 60% of promoter activity of that prior to deletion. A higher impact on HeLa cells suggested that the distal enhancer region may contain *cis*-regulatory elements that can be more specifically activated by the presence of HPV-18 DNA in HeLa cells, which may not be found in the other cell lines tested. These results differ slightly from those obtained by Hoppe-Seyler *et al*. (1991) which suggested that the deletion of the distal enhancer resulted in a 20% decrease in the promoter activity induced from the HPV-18 LCR. However, it was not mentioned which cell line was tested. In addition the HPV-18 LCR was not examined in a range of carcinoma cell lines.

(iv) The constitutive enhancer and proximal promoter region

It was difficult to determine the contribution of the constitutive enhancer region towards promoter activation, since further removal of the constitutive enhancer region (pGL3-LCR200) almost completely abolished the luciferase activity in all cervical cancer cell lines tested. Similar to Figure 3.20, the effect upon the deletion of the constitutive enhancer was clearer when another set of ratios were calculated by setting the luciferase activity from the pGL3-LCR400 as 1 (Figure 3.21).



HeLa	SiHa	C33A	H1299	MRC-5
1	1	1	1	1
0.017	0.0019	0.11	0.76	1.4

Figure 3.21 Ratios of changes in promoter activity upon removal of the constitutive enhancer region in different cell lines.
The level of luciferase activity detected from the pGL3-LCR400 plasmid was set at 1, and the ratios against the luciferase readings from the pGL3-LCR200 plasmid were calculated. Numbers were all rounded off to two significant figures.

The results showed that the removal of the constitutive enhancer region has the most significant impact in the downregulation of promoter activity in HeLa and SiHa cells, down to 2% and 0.2% of promoter activity prior to the deletion respectively. This was then followed by C33A cells, which retained about 11% of promoter activity. The impact of the deletion was not as apparent in H1299 cells, which still managed to retain 76% of activity upon deletion, and in contrast MRC-5 cells showed an increase in promoter activation upon the deletion. These results correlates well with previous studies by Garcia-Carranca *et al.* (1988) and Nakshatri *et al.* (1990) that the constitutive enhancer region is active in cell lines of epithelial origin only (see Section 1.3.1). The more significant decrease in promoter activity in the three cervical cell lines tested also

suggests that the constitutive enhancer region may contain *cis*-regulatory elements that may be more specifically activated within cervical cancer cells and in particular in those that were transformed by HPV.

The proximal promoter region alone did not manage to induce significant luciferase activity in all the cell lines tested. These results correlates with previous deletion studies performed by Hoppe-Seyler *et al*. (1991) suggesting that the promoter proximal region by itself exhibits only weak *cis*-stimulatory activity and requires the cooperative interaction with transcriptional elements contained within other parts of the LCR (Hoppe-Seyler and Butz, 1992). This abolishment of promoter activity, however, does not exist in the two non-cervical carcinoma cell lines tested, H1299 and MRC-5 cells. One of the possible explanations is that most of the *cis*-regulatory elements within the HPV LCR are highly specific towards cervical carcinoma cells, thus the impact of removing majority of the enhancer elements from the LCR was not as high in H1299 and MRC-5 cells; or the very low level of luciferase activity detected from H1299 and MRC-5 cells were in fact not induced from the P_{105} promoter but background activity independent on the P_{105} promoter activity, thus promoter activity in these two cell lines were not affected by the removal of important components of the LCR.

3.3 General discussion

3.3.1 The choice of vector for promoter analysis - pEGFP-1 versus pGL3-Basic

GFP is widely used as a marker for gene expression. It allows *in vivo* quantification of promoter activity because GFP is able form a fluorophore in the absence of exogenous substrates and cofactors other than oxygen (Chalfie *et al.*, 1994; Scholz *et al.*, 2000). GFP can also be used as a trace of cell lineage and as a fusion tag to monitor protein localization within living organisms (Chalfie *et al.*, 1994; Cubitt *et al.*, 1995).

Due to the above reasons, a GFP expression vector was initially chosen to be used as a tool for promoter analysis. It was hoped that, since transfected cells can be analysed at single-cell level and in the same cells, the expression of a reference plasmid can be monitored by two-colour fluorescence (Ducrest *et al.*, 2002), variations in transfection efficiencies in between cell lines tested would not be a problem, particularly within primary human cell lines such as MRC-5 CELLS which are commonly known to be difficult to transfect (Ducrest *et al.*, 2002). The problem encountered was, however, a significant level of autofluorescence detected from the promoterless pEGFP-1 vector in HeLa cells. This can be problematic particularly when the strength of the promoter of interest is not strong enough to induce a significantly higher level of fluorescence when compared to that induced by the empty vector, resulting in an inaccurate estimation of promoter activity.

Enquires to the manufacturer suggested that there were previous reports of the promoterless pEGFP-1 vector expressing EGFP, but it was emphasised that the problem appeared to be cell-type dependent. It was then decided that instead of continuing to work with the pEGFP-1 vector with the need of locating another source of HPV-18

positive cervical cancer cell line, and at the same time be at risk of detecting self-induced fluorescence from the promoterless vector, it was more convenient to adopt a new reporter plasmid for promoter analysis.

The pGL3-Basic luciferase reporter vector was then utilised since there are previous studies suggesting that the sensitivities of the GFP and luciferase reporter systems are very similar (Ducrest *et al.*, 2002). Although transfected cells could no longer be analysed at single-cell level, and the pGL3-Basic vector does not contain any antibiotic resistance gene to assist selection in eukaryotic cells, this, however, did not cause any inconvenience in the aspect of this project since only transient transfection was involved. In fact the luciferase assay allows more flexibility because cell lysates could be stored for postponed assays. Our results showed that the promoterless pGL3-Basic vector produced a very low level of background luciferase activity which was significantly better than the pEGFP-1 vector chosen previously.

There are several ways to explain the level of fluorescence detected from pEGFP-1 without a functional promoter. As seen in the vector diagram of pEGFP-1 (Figure 2.1), it contains a functional SV40 early promoter which is responsible driving the expression of the neomycin resistance gene within successfully transfected eukaryotic cells. Although the SV40 promoter is located about 2 kb downstream from the MCS, but since the promoter is in the same orientation as the EGFP gene within the vector, there is a chance of SV40-induced EGFP expression from the pEGFP-1 vector without the addition of functional enhancer and promoter elements. Another possible explanation of the problem could be random integration of the pEGFP-1 vector into the host cell genome upon transfection, resulting in transcription initiation signals upstream of EGFP gene leading to non-specific transcription. However, in the context of this

project only transient transfection was involved, and thus the integration of transfected plasmid into the host genome is not like to be involved (Wang *et al.*, 2004).

3.3.2 The choice of mammalian cell lines tested

Due to the availability of resources and time constraints, the testing of the promoter constructs was performed in a limited number of cell lines. As discussed earlier in Section 1.5, the activity of the HPV-18 P_{105} promoter is regulated by viral E2 proteins and various cellular transcription factors that bind to recognition sequences within the LCR. Attempts were made when choosing cell lines to be tested, so as to cover both positive and negative controls for most of the known crucial factors contributing towards P_{105} promoter activity. Table 3.5 is a summary table which explains the choice of cell lines used for the testing of the LCR promoter constructs.

Table 3.5 The choice of mammalian cell lines tested.

	HeLa	SiHa	C33A	H1299	MRC-5
HPV type	18	16	Nil	Nil	Nil
Presence of HPV	Yes	Yes	No	No	No
Origin from cervix	Yes	Yes	Yes	No	No
Cancerous	Yes	Yes	Yes	Yes	No

The differences in characteristics of the five mammalian cell lines chosen are summarised in the above table, illustrating the significant characteristics of each cell line that contribute towards the promoter activity of P_{105} in the LCR promoter constructs.

The choice of cell lines provided general representations of different cell types and comparison pairs for various contributing factors, which may not be sufficient to draw solid conclusions from results of the experiments performed, but good enough to

support the hypothesis of the mode of action and idea behind this project, which is to activate a foreign gene within cervical cancer cells selectivity with the use of the HPV-18 promoter element for gene therapy purpose.

3.3.3 Transfection efficiencies in different cell lines

It is known that transfection efficiency of plasmid DNA into eukaryotic cells is dependent on many factors such as the type of transfection reagent used, the reagent/DNA ratio, cell passage number and most importantly, on the cell type used (Kiefer *et al.*, 2004). Viral vectors have proven to be efficient in the delivery of genetic materials into a wide range of cell types (Imai *et al.*, 1998) but in the context of this project, a non-viral delivery system was employed. In this project five different mammalian cell lines were used, however, all transfection experiments were performed under the optimal conditions determined for HeLa cells. Optimisation experiments have not been performed on each individual cell lines, for transfection conditions had to be consistent to allow direct comparison of results across all five cell lines. For this reason, variations in between the transfection efficiencies of the different cell lines used were expected. Transfection efficiencies achieved by using lipofectamine were very high in HeLa, C33A and H1299 cells, as indicated by the high level of β-galactosidase expression induced in the co-transfection experiments. However, transfection efficiencies were markedly lower in SiHa and MRC-5 cells (tables of raw data results in the appendix chapter).

This was expected for MRC-5 cells, which is a primary human fibroblast cell line, since it is known that transfection efficiency is particularly low in primary cells (Martin and Murray, 2000; Sipehia and Martucci, 1995; Teifel *et al.*, 1997). In regards

to SiHa cells, however, a possible explanation of the problem of low transfection efficiency could be, since the SiHa cell stock was not cultured in our own laboratory, the number of passages that the given cell stock had gone through was unknown. Although the levels of luciferase activity detected have been normalised by making use of a transfection control plasmid (pCMVβ) and the results showed significant promoter activation within SiHa cells, but one cannot rule out the possibility of inaccuracy associated with normalisation using extremely low level of β-galactosidase results. It would therefore be ideal to confirm results in SiHa cells by obtaining another cell line that can be transfected with higher efficiency which is more comparable with the other cell lines used.

3.3.4 Specificity of the HPV-18 P_{105} promoter

(i) Overall cell-type specificity

One of the most important aspects of a successful gene therapy approach is the ability to induce selective activation within the target cells. In our results the HPV-18 LCR has shown significant selectivity towards two HPV-induced cervical carcinoma cell lines tested (HeLa and SiHa), and almost negligible activity in a lung carcinoma cell line (H1299) and a primary human fibroblast cell line (MRC-5) tested (see Section 3.2.3(i)). This cell-type specificity observed is in agreement with most of the studies performed the past, with the promoter activity of the HPV-18 LCR often reported to be inactive or very weakly activated in primary or transformed human fibroblast (Hoppe-Seyler and Butz, 1992; Hoppe-Seyler *et al.*, 1991; Romanczuk *et al.*, 1990; Thierry *et al.*, 1987).

In terms of epithelial cell-type specificity, as discussed in Section 3.2.3(i), although a number of transcription factors binding to the LCR of HPV-18 have been suggested to contribute to the epithelial cell-type specificity of the P_{105} promoter, it seems unlikely that the epithelial cell-specific activity of the HPV-18 LCR is induced from a common, epithelial transactivating factor, and alternate regulatory pathways may exist in different epithelial cell types (Butz and Hoppe-Seyler, 1993). In agreement with this idea, apart from the results obtained from the non-cervical epithelial cell line used in this project (H1299), which showed negligible level of promoter activity, a number of studies performed in the past have made use of a human hepatocellular carcinoma cell line HepG2, in which the HPV-18 LCR has constantly been reported to be inactive (Hoppe-Seyler and Butz, 1992; Hoppe-Seyler *et al.*, 1991; Thierry *et al.*, 1987). In contrast, the HPV-18 LCR has often been reported to be active within cervical carcinoma cell lines (Gius *et al.*, 1988; Hoppe-Seyler and Butz, 1992; Hoppe-Seyler *et al.*, 1991; Thierry *et al.*, 1987). These observations show that the HPV-18 LCR appeared to be selectivity activated not only within epithelial cells, but in particular within epithelial carcinomas of a cervical origin, which makes it an ideal promoter element to be used for gene therapy to selectively target cervical cancer cells.

(ii) HPV-positive versus HPV-negative cervical cancer cells

Promoter activities from the Bam*HI* fragment containing the HPV-18 LCR observed in SiHa and HeLa cells was about 3 and 1.5 times stronger than that in C33A cells respectively, indicating that the HPV-18 LCR possesses selectivity for HPV-positive cervical cancer cells in the context of the limited number of cell lines tested. This observation is in agreement with previous work performed by Hoppe-Seyler and Butz (1992) who tested the HPV-18 LCR in six different cervical carcinoma cell lines,

and the level of gene expression induced was lower in two of the cell lines that were not transformed by HPV. The results in Section 3.2.3(ii) identified the distal 200 bp of the *Bam*HI fragment of HPV-18 to possess stronger repressor activity in HeLa and SiHa cells, results in Section 3.2.3(iii) showed that the removal of the distal enhancer region has the most significant impact in the downregulation of promoter activity in HeLa cells, and results in Section 3.2.3(iv) also showed that upon the removal of the constitutive enhancer region from the LCR, a larger percentage reduction in promoter activity was observed in HeLa and SiHa cells when compared to the other cell lines tested. These results suggest possible differences in P_{105} promoter regulation within HPV-positive and HPV-negative cell lines tested, and the involvement of HPV viral gene products in promoter regulation within the context of HPV-transformed cells. It was hoped that the mutational studies performed in the second part of the project would be able to identify transcription control elements responsible for the promoter regulation of the LCR within HPV-positive cervical cancer cells.

(iii) HPV-18 versus HPV-16 infected cervical cancer cells

Results from the estimation of the HPV-18 P_{105} promoter activity in HeLa and SiHa cells suggested that the promoter may be able to induce stronger activity in the presence of the HPV-16 DNA when compared to HPV-18, with the promoter being about two times stronger in SiHa (HPV-16 positive) than in HeLa cells (HPV-18 positive). This result correlates with previous findings by Hoppe-Seyler and Butz (1992) which detected an almost 2-fold higher promoter activity of the HPV-18 LCR from SiHa compared with HeLa cells. This was unexpected for, by making use of the novel early promoter of HPV-18 in the LCR reporter constructs, the strongest level of promoter activity was expected to be detected in HeLa instead of SiHa cells. The results

obtained also identified a larger percentage reduction in promoter activity upon the deletion of the distal enhancer fragment from the LCR in HeLa cells when compared to the other cell lines tested, suggesting that the distal enhancer may be more dependent on HPV-18 viral gene products for promoter activation (see Section 3.2.3(iii)).

However, it has been well documented that the HPV-18 P_{105} promoter is functionally equivalent to the HPV-16 P_{97} promoter (Romanczuk *et al.*, 1990; Thierry *et al.*, 1987), and the LCR of the two types of HPV can be regulated by similar mechanisms due to the similarities in their composition of transcriptional control elements (Butz and Hoppe-Seyler, 1993; Chong *et al.*, 1990). Moreover, many common cellular transcription factors appear to interact with both the HPV-18 and HPV-16 LCR (Bednarek *et al.*, 1998). Romanczuk *et al*. (1990) have also suggested that the two viral promoters may be similarly regulated by E2, since a comparison of the sequences upstream showed a similar spatial arrangement of the four E2BS, the TATA boxes and the transcription start sites (see Figure 1.10). Due to the above reasons it is therefore not surprising that the promoter element within the HPV-18 LCR constructs produced could be activated not only within HPV-18 positive HeLa cells, but also within HPV-16 infected SiHa cells. This result is favourable in the context of gene therapy, since it permits the HPV-18 P_{105} promoter to be used to target cervical cancer cells transformed by both HPV-18 and 16. Further experiments will be required to confirm the higher level of promoter activation observed within SiHa cells when compared to HeLa cells. These experiments could be mutational studies of transcription control elements and examination of their effects on promoter activation within the two cell types.

3.3.5 Possible unidentified transcription factor binding sites

As mentioned in Section 3.2.2(i), the luciferase reporter constructs produced contained a 1,050 bp *Bam*HI fragment (as described in Section 1.4.1) which, in addition to the entire length of the LCR, also contained the 208 bp at its 5′-end corresponding to the end of the L1 ORF and the 20 bp at its 3′-end corresponding to the beginning of the E6 ORF (Thierry *et al.*, 1987). Results from the LCR deletion constructs showed that upon the removal of the distal 200 bp *Bam*HI-*Rsa*I fragment, promoter activity detected from the LCR significantly increased within the three cervical cancer cell lines tested, and in particular towards the HPV-positive cervical cancer cell lines HeLa and SiHa cells. This observation suggested the presence of both cellular and HPV-dependent transcription control elements within the distal 200 bp fragment that may be repressive on the P_{105} promoter activity and has not been identified to date.

Most of the identified transcription factor binding sites are located within the constitutive enhancer and promoter proximal region of the HPV-18 LCR (see Section 1.5), and the precise contribution of the distal enhancer region towards the P_{105} promoter activity was suggested to be unclear (Cid *et al.*, 1993). There have also been previous studies suggesting that the removal of the distal enhancer region from the LCR diminished transcription only slightly (Bednarek *et al.*, 1998; Hoppe-Seyler *et al.*, 1991). The results obtained from the LCR deletion constructs as described in Section 3.2.3(iii), however, detected a significant drop in promoter activity upon the removal of the distal enhancer region, and transcription control elements present in the region appeared to be strongly activated by HPV-18 viral gene products. In correlation to these results, DNase I footprinting experiments of the HPV-18 LCR performed by Garcia-Carranca *et al.* (1988) have identified four footprints within the distal enhancer region caused by cellular factors not yet known (footprints I, II, III and IV) (see Figure 3.22). Attempts

have been made in identifying the functional significance of these footprints, and footprint III has been suggested by Hoppe-Seyler and Butz (1993) to contain recognition sites for the cellular transcription factor Sp1, which contributes to the activation of the HPV-18 LCR in driving gene expression from a HSV thymidine kinase (*tk*) promoter. These observations may explain for the significant decrease in promoter activity detected from the LCR deletion constructs, since Sp1 has been shown to be a strong transcriptional activator of the P_{105} promoter (see Section 1.5.2).

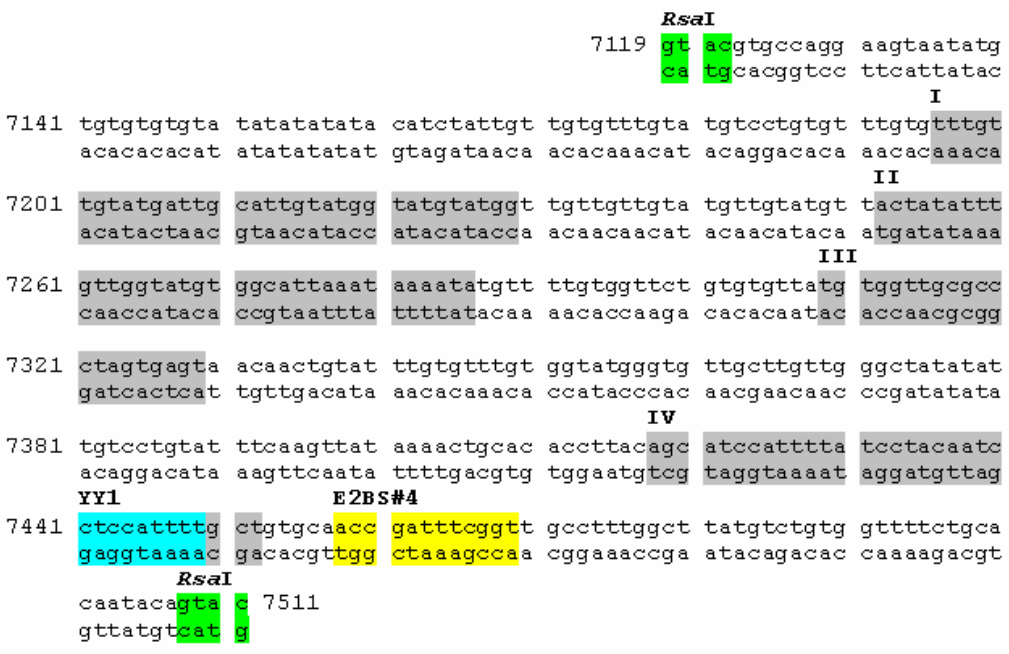


Figure 3.22 Footprint analysis in the distal enhancer region of HPV-18 LCR.
DNase I footprinting studies performed by Garcia-Carranca *et al*. (1988) have identified four sites of protein-DNA interactions within the distal enhancer region caused by unidentified cellular transcription factors (footprints I, II, III and IV highlighted in grey). The *Rsa*I recognition sequences (GTAC) which defines the distal enhancer region are highlighted in green. The only transcription factors binding to this region that has been identified to date are the YY1 binding site (highlighted in blue) and the E2BS#4 (highlighted in yellow).

3.4 Summary

The first part of the project was focused on determining the suitability of the P_{105} promoter within HPV-18 LCR to be used as a tool for gene therapy in targeting selective foreign gene expression within HPV-positive cervical cancer cells. The P_{105} promoter activity induced from the HPV-18 LCR was examined by making use of a promoterless luciferase expression vector, pGL3-Basic. The LCR reporter constructs produced were transiently transfected into five different mammalian cell lines to examine the level of promoter activity and specificity. The results showed that while the 1,050 bp *Bam*HI fragment has been most commonly used in the context of studying the promoter activity induced from the HPV-18 LCR, the shorter 850 bp *Rsa*I-*Bam*HI fragment resulted in a stronger level of promoter activity and increased promoter specificity towards the two HPV-positive cervical cancer cells HeLa and SiHa cells. Hence it appeared to be a better option to be used as a LCR fragment for gene therapy purpose.

The overall promoter activity induced from the HPV-18 LCR appeared to be highly specific towards the three cervical carcinoma cell lines tested (HeLa, SiHa and C33A), and in particular towards the two cervical carcinoma cell lines that were transformed by HPV. The results from the LCR deletion constructs also showed that the distal 200 bp of the *Bam*HI LCR fragment, which was derived from the 3′ of the L1 ORF, appeared to be repressive on the P_{105} promoter activity in all the cell lines tested. On the other hand, selective promoter activation in the two HPV-positive cervical cancer cells tested appeared to be determined by the constitutive enhancer region, whereas selectivity towards the HPV-18 positive HeLa cells in particular was observed from the distal enhancer region. It is, however, problematic to draw conclusions on the

regulation of the HPV-18 promoter simply by examining the level of activity induced or suppressed by different Sections of the LCR. The next attempt was to further enhance the level of promoter activity and increase specificity of the HPV-18 LCR towards HPV-infected cervical cancer cells for gene therapy purpose, thus leading to the mutation studies performed in Chapter 4.

3.5 Future directions

In future experiments, the specificity of the HPV-18 LCR can be confirmed in a wider range of mammalian cell lines. To test for the promoter selectivity in different types of high-risk HPV, other HPV-transformed cervical cancer cell lines can be used such as C-4 I (HPV-18), MS751 (HPV-18), CaSki (HPV-16) and ME-180 (HPV-39) (ATCC, 2007). The results from SiHa cells can also be confirmed by obtaining another stock of the same cell line which is known to have a low passage number. To test the promoter specificity in different epithelial cell types, other human carcinoma cell lines may be used such as breast cancer cells (MCF7), prostate cancer cells (PC-3), ovarian cancer (A2780) (Godwin *et al.*, 1992) and colorectal cancer (SW948) (ATCC, 2007). In the context of this project, an ideal negative control cell line to be used is a normal cervical cell line, which is, however, extremely difficult to obtain due to ethical issues. Therefore it would only be possible to confirm inactivity of the P_{105} promoter in normal cells by using a wider range of primary human keratinocytes and fibroblasts.

In regards to the promoter regulation of the P_{105} promoter, attempts can be made to identify the repressive transcription control elements within the distal 200 bp of the *Bam*HI fragment containing the LCR, which is part of the 3′-end of the L1 ORF. This can be performed by DNase footprinting studies (Galas and Schmitz, 1978) or more conveniently, by making use of computer softwares which may assist in the localisation of transcription factor binding sites in sequences of unlimited length, such as the *MatInspector* software (Genomatix, 2007). Gel retardation assays can also be performed on the footprints discovered by Garcia-Carranca *et al*. (1988) within the distal enhancer region, to confirm the identity of the transcription factors binding to these sites, and their contribution towards the P_{105} promoter activity.

CHAPTER 4

HPV-18 LCR MUTATION CONSTRUCTS

CHAPTER 4 MUTATION STUDIES ON HPV-18 LCR

4.1 Introduction

As discussed in the previous chapter, the HPV-18 P_{105} promoter within the LCR showed selective activation within cervical cancer cells, and in particular towards cells that were infected by HPV. The next series of experiments was to identify transcription control elements within the LCR that might contribute to the observed selectivity of the promoter, and to further increase the level of promoter strength and specificity within cervical cancer cells. There are a number of transcription factor binding sites within the LCR of HPV-18 that have been identified to date, along with four binding sites for the HPV-18 E2 protein which is known to be involved in controlling viral gene expression. In the second part of this project transcriptional control elements that were previously reported to possess repressor effect on the HPV-18 LCR promoter activity and specificity were chosen to be mutated, which was an attempt to further elevate the level of promoter activity and selectivity towards HPV-infected cervical cancer cells in particular.

Transcription factor binding sites that were chosen to be mutated along the LCR were the four E2BSs which are recognition motifs for the viral E2 protein and the AP1/YY1, KRF-1, GRE/YY1 and Sp1 binding sites. The mutations were introduced into the LCR fragment by site-directed mutagenesis (see Section 2.2.3(ii)) and again cloned into the promoterless luciferase vector pGL3-Basic (Section 3.2.2(i)). The sequences of all the plasmid constructs were confirmed by automated sequencing (see Section 2.2.5(vii)) to ensure they contained the designated mutations. The mutation constructs and the positive control plasmid pGL3-SV40 were then co-transfected into

the five mammalian cell lines together with the transfection control plasmid pCMVβ as described in Section 3.2.2(iii). The levels of luciferase activity were then normalised with respect to the corresponding β-galactosidase values, and promoter activities were either calculated as percentages relative to the positive control SV40 promoter, or as ratios relative to the wild-type LCR promoter activity.

4.2 Results and discussion

4.2.1 The E2BS mutation constructs

Previous studies have found the E2 proteins to be repressive in the regulation of the P_{105} promoter (Bernard *et al.*, 1989; Bouvard *et al.*, 1994; Dong *et al.*, 1994; Jackson and Campo, 1995; Romanczuk *et al.*, 1990; Thierry and Howley, 1991). In the context of HPV-infected cervical cancer cells, the E2 ORF is disrupted upon integration of the viral DNA into the host genome (Berumen *et al.*, 1994; Schwarz *et al.*, 1985), and E2 protein expression has been reported to be lost within these cells (Demeret *et al.*, 1997). Hence E2-mediated response of the LCR within cervical cancer cells has always been studied by co-transfecting the LCR constructs with an E2-expressing plasmid (Bernard *et al.*, 1989; Demeret *et al.*, 1997). Experiments performed in the first part of the project, however, have managed to detect a higher level of LCR promoter activity within the two HPV-positive cell lines tested when compared to the HPV-negative cell line (see Section 3.3.3(ii)), which suggested the possible involvement of viral gene products in promoter regulation. In correlation to these findings, there have been studies suggesting the possibility of a truncated form of E2 protein being expressed (Bernard *et al.*, 1989; Shillitoe, 2006). In addition, components of the LCR have also been reported to be responsive to other viral products such as the E6 protein (Gius *et al.*, 1988), which is known to be expressed within HPV-infected cervical cancer cells (Demeret *et al.*, 1997). Since the only transcription factor binding sites within the LCR which are known to be recognised by viral proteins are the four E2BSs, these sites may possibly be targeted by other viral gene products.

In the second part of the project, the four E2BSs within the LCR were chosen to be mutated, with an attempt to identify possible repression induced by protein

binding at these sites, which may either be a form of truncated E2 protein, or other viral proteins that work in similar mechanisms as the binding of the homologous viral E2 protein. By mutating the E2BSs and preventing protein binding, repression at the promoter can be abolished, thus further increasing the basal level of promoter activity from the LCR. It was also hoped to identify possible promoter regulation by viral proteins within cells HPV-positive cervical cancer cells, which can be used as a selective marker to identify cells infected by HPV.

(i) Design and construction of the E2BS mutation constructs

There are four E2BSs within the LCR of HPV-18. E2BS#1-3 are located within the promoter proximal region of the LCR, whereas E2BS#1 is in the distal enhancer region. The E2BS is a 12-bp palindromic $ACCN_6GGT$ motif, where N_6 represents the six base pairs that differ between the four sites (Boner and Morgan, 2002; Demeret *et al.*, 1994; McBride *et al.*, 1991; Sverdrup and Khan, 1995). The E2 protein consists of a C-terminal DNA binding domain linked to an N-terminal transactivation domain by a non-conserved hinge region (McBride and Myers, 1997). The C-terminal domain of E2 binds specifically to the recognition motifs as a dimer, hence resulting in two contact points on the motif with a spacer sequence in between (see Figure 4.1(A)). Previous studies performed by McBride *et al.* (1988) with *in vitro* binding assays suggest that synthetic polypeptides corresponding to the full-length E2 protein binds to the original E2 motif $ACCN_6GGT$ but not to a mutated motif of $ATTN_6CCT$.

In order to examine the contribution of each of the four E2BSs on P_{105} promoter regulation, site-directed mutagenesis was performed on the pGL3-LCR800 plasmid by the method described in Section 2.2.3(ii). Primers were designed to contain mutations to convert the recognition motifs from $ACCN_6GGT$ to $ATTN_6CCT$ (see

Figure 4.1(B)). Four LCR fragments containing individually mutated E2 binding sites were produced (Figure 4.2). Mutations were introduced to E2BS#4 by oligonucleotides #269 and #276 to produce the LCR insert for the pGL3-E2BS#4 plasmid; to E2BS#3 by oligonucleotides #267 and #275 to produce the LCR insert for the pGL3-E2BS#3 plasmid; to E2BS#2 by oligonucleotides #265 and #274 to produce the LCR insert for the pGL3-E2BS#2 plasmid and to E2BS#1 by oligonucleotides #263 and #273 to produce the LCR insert for the pGL3-E2BS#1 plasmid (see Table 2.4 for details of the oligonucleotides used). The outer primers used in the second-round PCR for site-directed mutagenesis were oligonucleotides #288 and #291, which were the same primers used for the construction of the pGL3-LCR800 plasmid construct as described in Section 3.2.2(i) (see Table 2.3 for details of oligonucleotides used). The positions of oligonucleotides binding to the LCR are shown in Figure 4.3.

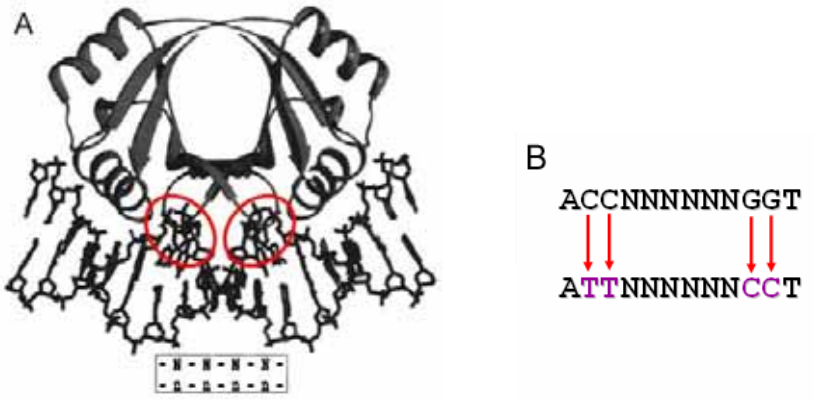


Figure 4.1 The E2 binding site.

A: X-ray crystal structure of the E2 DNA binding domain-DNA complex. The region of the E2BSs where the E2 protein contacts the DNA upon binding are circled in red (Hines *et al.*, 1998). B: The E2 binding domain consists of a consensus sequence of $ACCN_6GGT$. By mutating the two contact points of E2 protein as shown (CC mutated to TT; GG mutated to CC), the E2 protein would not be able to recognize its binding domain.

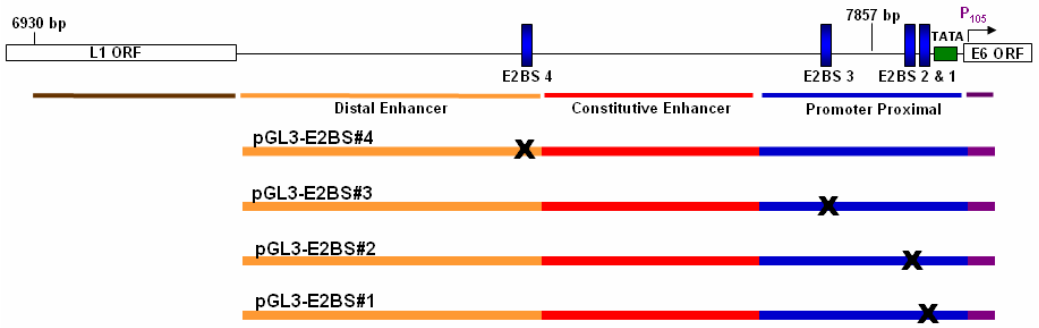
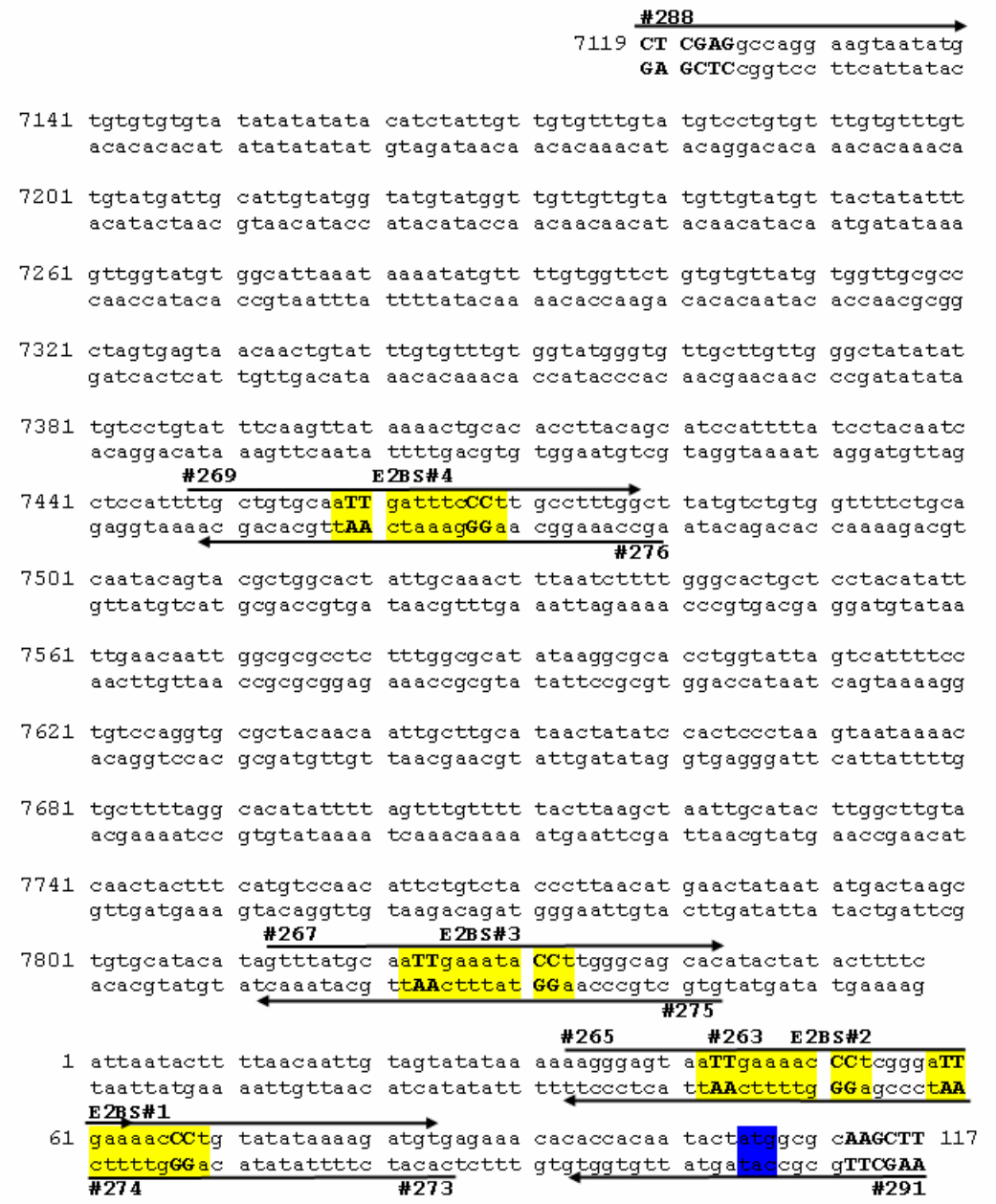


Figure 4.2 Schematic diagram of the pGL3-LCR800 plasmids containing the four individually mutated E2BSs.

E2BS mutation constructs were produced by making use of the pGL3-LCR800 plasmid as a template. The pGL3-E2BS#4 plasmid contained a mutation at the E2BS#4 within the distal enhancer region; the pGL3-E2BS#3, pGL3-E2BS#2 and pGL3-E2BS#1 contained mutations at the E2BS#3, E2BS#2 and E2BS#1 respectively within the promoter proximal fragment of the LCR

Figure 4.3 Binding positions of oligonucleotides used for site-directed mutagenesis of E2BSs within the LCR.

For the site-directed mutagenesis performed on each individual E2BS, a pair of oligonucleotides was needed to introduce the designated mutations into both strands during the first round of PCR. Oligonucleotides #269/#276 was used for mutation on E2BS#4; #267/#275 for E2BS#3; #265/#274 for E2BS#2 and #263/#273 for E2BS#1. The E2BS were highlighted in yellow, with the mutations introduced in bold and caps. The outer pair of oligonucleotides #288/#291 were used to generate the 850 bp *Rsa*I-*Bam*HI fragment containing the mutations in the second round of PCR, with the restriction enzyme recognition sequences added to assist cloning (*Xho*I in #288 and *Hind*III in #291; in bold and caps). The TATA box and ATG start codon of the E6 ORF were highlighted in red and blue respectively.


#288
7119 CT CGAGgccagg aagtaatatg
GA GCTCcggtcc ttcattatac
7141 tgtgtgtgta tatatatata catctattgt tgtgtttgta tgtcctgtgt ttgtgtttgt
acacacacat atatatatat gtagataaca acacaaacat acaggacaca aacacaaaca
7201 tgtatgattg cattgtatgg tatgtatggt tgttgttgta tgttgtatgt tactatattt
acatactaac gtaacatacc atacatacca acaacaacat acaacataca atgatataaa
7261 gttggtatgt ggcattaaat aaaatatgtt ttgtggttct gtgtgttatg tggttgcgcc
caaccataca ccgtaattta ttttatacaa aacaccaaga cacacaatac accaacgcgg
7321 ctagtgagta acaactgtat ttgtgtttgt ggtatgggtg ttgcttgttg ggctatatat
gatcactcat tgttgacata aacacaaaca ccatacccac aacgaacaac ccgatatata
7381 tgtcctgtat ttcaagttat aaaactgcac accttacagc atccatttta tcctacaatc
acaggacata aagttcaata ttttgacgtg tggaatgtcg taggtaaaat aggatgttag
#269
E2BS#4
7441 ctccattttg ctgtgcaaTT gatttcCCtt gcctttggct tatgtctgtg gttttctgca
gaggtaaaac gacacgttAA ctaaagGGaa cggaaaccga atacagacac caaaagacgt
#276
7501 caatacagta cgctggcact attgcaaact ttaatctttt gggcactgct cctacatatt
gttatgtcat gcgaccgtga taacgtttga aattagaaaa cccgtgacga ggatgtataa
7561 ttgaacaatt ggcgcgcctc tttggcgcat ataaggcgca cctggtatta gtcatttcc
aacttgttaa ccgcgcggag aaaccgcgta tattccgcgt ggaccataat cagtaaaagg
7621 tgtccaggtg cgctacaaca attgcttgca taactatatc cactccctaa gtaataaaac
acaggtccac gcgatgttgt taacgaacgt attgatatag gtgagggatt cattattttg
7681 tgcttttagg cacatatttt agtttgtttt tacttaagct aattgcatac ttggcttgta
acgaaaatcc gtgtataaaa tcaaacaaaa atgaattcga ttaacgtatg aaccgaacat
7741 caactacttt catgtccaac attctgtcta cccttaacat gaactataat atgactaagc
gttgatgaaa gtacaggttg taagacagat gggaattgta cttgatatta tactgattcg
#267
E2BS#3
7801 tgtgcataca tagtttatgc aaTTgaaata CCttgggcag cacatactat acttttc
acacgtatgt atcaaatacg ttAActttat GGaacccgtc gtgtatgata tgaaaag
#275
#265
#263
E2BS#2
1 attaatactt ttaacaattg tagtatataa aaaagggagt aaTTgaaaac CCtcgggaTT
taattatgaa aattgttaac atcatatatt ttttccctca ttAActtttg GGagccctAA
E2BS#1
61 gaaaacCCtg tatataaaag atgtgagaaa cacaccacaa tactatggcg cAAGCTT 117
cttttgGGac atatattttc tacactcttt gtgtggtgtt atgatacCgc gTTCGAA
#274
#273
#291

(ii) Effect of E2BS mutations on P_{105} promoter activity and specificity

Similar to the transfection experiments described in Chapter 3, plasmid constructs containing the wild-type LCR and LCR fragments carrying mutations at individual E2BSs were co-transfected into the five mammalian cell lines as described in Table 2.1, together with the positive control plasmid pGL3-SV40. Transfected cells were harvested 48 hours after transfection and cell lysates were collected for luciferase and β-galactosidase assays as described in Sections 2.2.10 and 2.2.11. Experiment on each cell line was repeated at least three times and each individual experiment was performed in triplicate. Results were analysed as described in Table 3.3 and calculated into percentage luciferase activity relative to the activity detected from the positive control plasmid pGL3-SV40. A summary of the results is graphically represented in Figure 4.4. The raw data results are shown in the appendix chapter.

As discussed earlier in Section 1.5.1, the HPV-18 E2 protein may function as either an activator or repressor of viral gene transcription depending on many possible factors such as the concentration and length of E2 protein expressed, the binding affinity of E2 protein to different E2BSs and the location of the E2BSs within the LCR. However, it is generally accepted that the E2 region of HPV normally represses the transcription of early viral genes from the promoter element of the LCR (Bernard *et al.*, 1989; Finzer *et al.*, 2002; Jo and Kim, 2005). In contrast to the expected outcome, the mutations introduced to the E2BSs did not result in an elevated level of promoter activity. The luciferase activities detected from the four E2BS mutation constructs were all lower than that from the LCR before the mutations were introduced in the three cervical cancer cell lines tested, suggesting that the P_{105} promoter is not repressed by protein binding to the E2BSs in the context of this project. Results of the E2BS mutations are graphically represented in Figure 4.4.

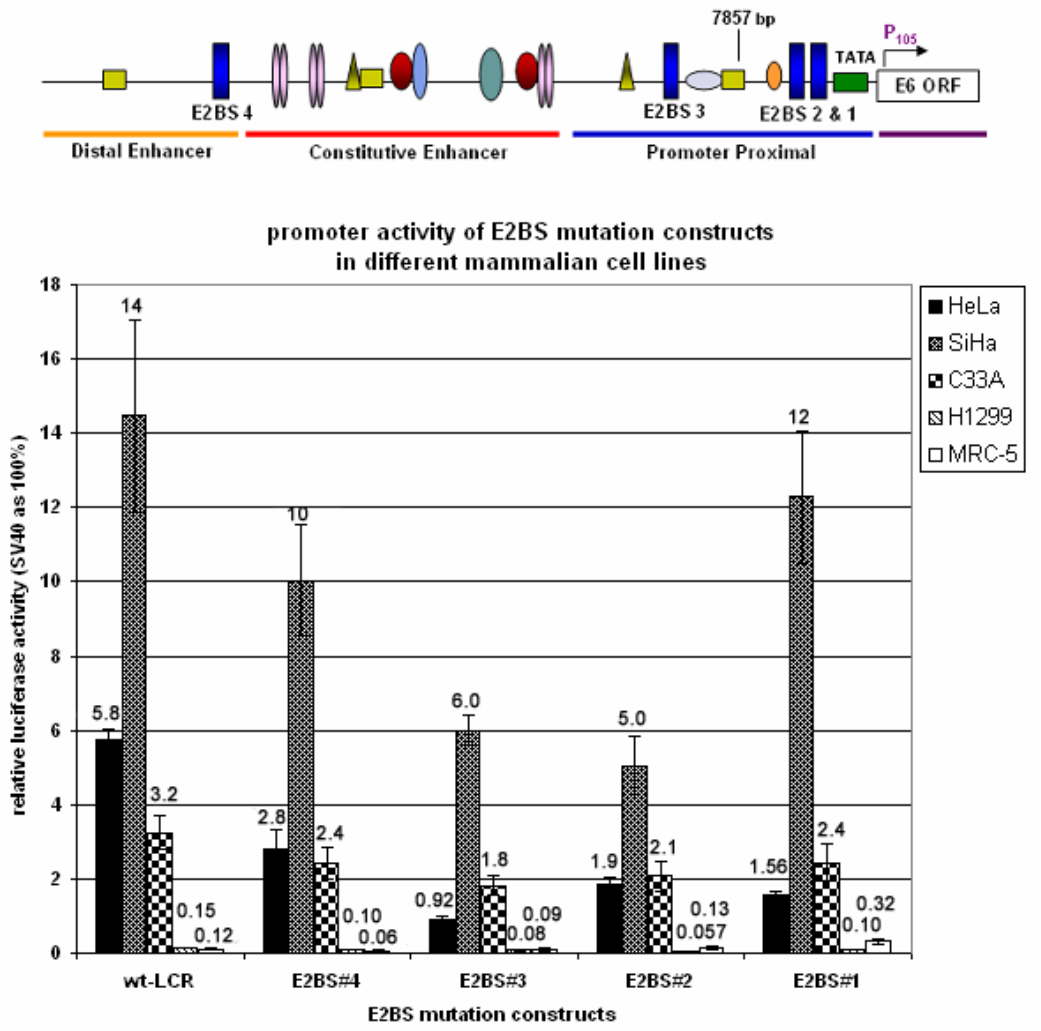


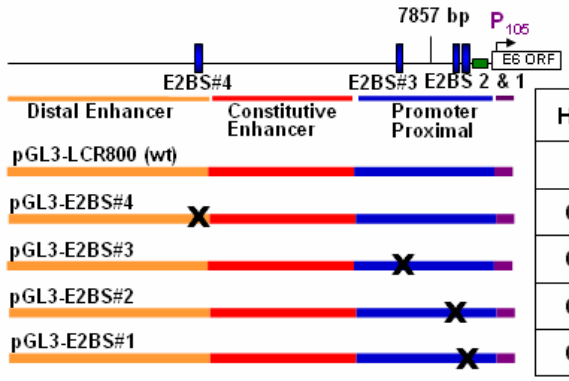
Figure 4.4 Promoter activity of E2BS mutation constructs in different mammalian cell lines.

The graph represents results obtained from transfection experiments using five different cell lines. Relative luciferase activities from various E2BS mutation constructs were plotted as percentages relative to the luciferase activity from the pGL3-SV40 plasmid which served as a positive control. The error bars indicate the SEM from the three experimental repeats performed in each cell line.

Instead of a repressive effect on the P_{105} promoter induced by viral E2 proteins binding to the E2BSs, the decreased level of promoter activity detected upon the E2BS mutations suggested the presence of promoter activation from protein binding at the

E2BSs. A similar pattern of activities were observed for all three cervical cancer cell lines tested, including both HPV-positive and negative cell lines, suggesting the possible presence of cellular transcription factors that are commonly found within cervical cancer cells which bind to the E2BSs leading to the activation of the P_{105} promoter within the LCR. However, E2-mediated promoter repression was suggested to be a result of E2-binding to the two promoter proximal E2BSs which displaces Sp1 and TATA box-binding protein from their recognition sequences (Demeret *et al.*, 1994; Dong *et al.*, 1994; Dostatni *et al.*, 1991; Tan *et al.*, 1992). Hence if the binding of other cellular transcription factors occur at these E2BSs within cervical cancer cells, subsequent mutations at E2BS#2 and E2BS#1 should also result in an increased level of promoter activation due to the same steric hindrance effect. In contrast if the E2BS only recognises the viral E2 proteins and E2 is not expressed within cervical cancer cells, mutations introduced to the E2BSs should not affect the activity of the promoter. The performed experiments were not sufficiently detailed enough to explain for the decreased promoter activation upon the E2BS mutations. Additional DNA footprinting assays would be required to identify possible transcription factors binding to the E2 recognition sequences within the LCR. It is, however, not within the context of this project to examine the precise mechanism of promoter regulation in the HPV-18 LCR.

In order to compare the effect of the individual E2BS mutations on the P_{105} promoter strength in different cell lines, the level of luciferase activity detected from pGL3-LCR800 was set as 1 and activities from E2BS mutation constructs were calculated as a ratio relative to the wild-type LCR fragment (Figure 4.5).



HeLa	SiHa	C33A	H1299	MRC-5
1	1	1	1	1
0.47	0.81	0.75	0.69	0.39
0.16	0.44	0.56	0.50	0.68
0.32	0.34	0.64	0.38	1.2
0.28	0.91	0.76	0.60	3.3

Figure 4.5 Relative promoter activity from the E2BS mutation constructs in different cell lines.

The level of luciferase activity detected from the wild-type LCR insert in the pGL3-LCR800 plasmid was set at 1, and the ratios of luciferase readings from various E2BS mutation constructs were calculated. A green square on the top right corner denotes an increase in promoter activity and a red square denotes a decrease in promoter activity when compared to the wild-type LCR. Numbers were all rounded off to two significant figures.

The strongest reduction of promoter activity occurred within HeLa cells, with an over 50% decrease for all the E2BS mutations. The reduction in promoter activity was particularly larger within HeLa cells upon the mutations of E2BS#4, E2BS#3 and E2BS#1. This suggests the possible involvement of HPV-18 viral gene products in promoter activation, and the differences in promoter regulation within cervical cancer cells infected by different HPV types. A larger decrease in promoter activity observed in HeLa and SiHa cells upon the mutation at E2BS#2 (decreased by about 65%) when compared to C33A cells (decreased by 35%) also suggested the possible presence of viral gene products from HPV-18 and HPV-16 which works in similarly towards P_{105} promoter activation. In general, these results suggest the involvement of both cervical cancer cell-type specific transcription factors and viral gene products in promoter activation at the four E2BSs. Similar to the results from the LCR deletion constructs

discussed in Chapter 3, luciferase activity detected from H1299 and MRC-5 cells were extremely low with overlapping error bars, and hence the differences in promoter activity induced from the E2BS mutation constructs were difficult to determine. Overall in terms of promoter activity, the E2BS mutations did not lead to an increased level of promoter activation within the three cervical cancer cell lines tested; hence the results were not favourable towards the aim of the project, which was to increase promoter activity and specificity towards cervical cancer cells.

The effect of E2BS mutations in regards to the promoter specificity towards HPV-positive cervical cancer cells in particular can be examined more precisely by calculating the ratios of luciferase activity from HeLa and SiHa cells against the other control cell lines (Tables 4.1 and 4.2).

Table 4.1 Ratios of luciferase activity from E2BS mutation constructs in HeLa against other cell lines.

Selectivity towards HeLa (HPV-18 +ve cervical cancer cells)	wt-LCR	E2BS#4	E2BS#3	E2BS#2	E2BS#1
HeLa (HPV-18) : SiHa (HPV-16)	0.40	0.28	0.15	0.37	0.13
HeLa (HPV +ve) : C33A (HPV -ve)	1.8	1.2	0.51	0.90	0.64
HeLa (cervix) : H1299 (lung)	39	28	12	33	16
HeLa (cancerous epithelial) : MRC-5 (normal fibroblast)	50	46	11	14	4.9

A ratio higher than 1 represents selectivity towards HeLa cells and *vice versa*. A red square at the top right corner denotes a decrease in promoter specificity towards HeLa cells upon mutations on the specified E2 binding sites of the LCR. All numbers shown were rounded off to two significant figures.

Table 4.2 Ratios of luciferase activity from E2BS mutation constructs in SiHa against other cell lines.

Selectivity towards SiHa (HPV-16 +ve cervical cancer cells)	wt-LCR	E2BS#4	E2BS#3	E2BS#2	E2BS#1
SiHa (HPV-16) : HeLa (HPV -18)	2.5	6.6	6.5	2.7	7.9
SiHa (HPV +ve) : C33A (HPV -ve)	4.5	4.2	3.3	2.4	5.0
SiHa (cervix) : H1299 (lung)	98	100	77	87	130
SiHa (cancerous epithelial) : MRC-5 (normal fibroblast)	130	170	69	38	39

A ratio higher than 1 represents selectivity towards SiHa cells and *vice versa*. A green square at the top right corner denotes an increase in promoter specificity towards SiHa cells upon mutations on the specified E2 binding sites of the LCR. A red square denotes a decrease in promoter specificity towards SiHa cells instead. All numbers shown were rounded off to two significant figures.

The E2BS#4 and E2BS#1 mutations slightly increased promoter specificity for SiHa cells compared with other cell lines, but the same effect was not observed in HeLa cells. Overall none of the E2BS mutations resulted in an increased level of promoter specificity towards both HPV-positive cervical cancer cells tested. These results suggest HPV viral gene products are not likely to be involved in the regulation of the LCR promoter specificity at the four E2BSs, and also supported previous observation suggesting that the P_{105} promoter may be regulated by different mechanisms within cervical cancer cells that were infected by different types of HPV.

4.2.2 Transcription factor binding sites mutation constructs

AP1/YY1: As mentioned earlier in Section 1.5.2(ii), AP1 is a very strong transcription activator of the P_{105} promoter (Butz and Hoppe-Seyler, 1993), and also appeared to be a

key regulator for epithelial cell-type specificity (Mack and Laimins, 1991). Since AP1 is such an important transcription control element of the P_{105} promoter, one of the possible ways to further enhance promoter activity and specificity would be to eliminate existing competition for AP1 at its binding sites. There have been two AP1 sites identified within the LCR of HPV-18, and one of which is located within the constitutive enhancer region and is overlapped by a YY1 site at its 3′-end. It is therefore possible for AP1 binding to be interfered by the binding of YY1 due to their proximity, and hence limiting the level of promoter activation induced by AP1. Electrophoretic mobility shift assays (EMSA) performed by Bauknecht *et al*. (1995) has determined that mutation introduced at the YY1 binding site, while managed to abolish YY1 binding, did not affect AP1 binding activity. The experiment, however, was performed on oligonucleotides corresponding to the constitutive enhancer fragment of the LCR and the same regulation may not apply in the context of the HPV-18 LCR. The LCR carrying the same YY1 mutation has also been tested by Bauknecht *et al*. (1995) in HeLa and HepG2 cells, and was reported to have no effect on promoter activity. Hence suggesting it does not play a major role in regulating the activity of the LCR. The results, however, have not been confirmed in other cervical cancer cell lines apart from HeLa cells. This YY1 site within the constitutive enhancer region was therefore chosen to be mutated to observe possible repression on the P_{105} promoter activity and specificity.

KRF-1: A KRF-1 binding site is located further downstream within the constitutive enhancer, which overlaps with an Oct-1 binding site at its 5′-end, and is known to be an important determinant of the cell-type specificity of the LCR promoter activity. Mutation studies performed by Butz and Hoppe-Seyler (1993) on the KRF-1 site

showed that promoter activity was only decreased by 20% and 25% in HeLa and C33A cells respectively, while a significant decrease was observed from HaCaT, a human immortalised keratinocyte cell line (Boukamp *et al.*, 1988), and in primary keratinocytes. A KRF-1 mutation is therefore an ideal approach to enhance promoter selectivity against cervical cancer cells in particular. The slight decrease in promoter activity resulted from the KRF-1 mutation observed by Butz and Hoppe-Seyler (1993) was likely to be due to the effect of base changes introduced to the overlapping region with the Oct-1 binding site. Although Oct-1 is known to be a promoter activator of the LCR, the binding affinity of Oct-1 to this overlapping site with KRF-1 is relatively low (Butz and Hoppe-Seyler, 1993). Hence, the corresponding mutation would still be in favour towards the aim of the project by increasing the selectivity ratio of the P_{105} promoter towards cervical cancer cells.

GRE/YY1: The most direct approach to enhance promoter activity is to identify a repressor element which when mutated may lead to significant increase in transcription. A GRE binding site is located in the promoter proximal region which overlaps with a YY1 site at its 3′-end. Mutation studies performed by Butz and Hoppe-Seyler (1993) suggested that a GRE mutation resulted in 2 to 3-fold increase of the basal activity of the HPV-18 LCR in HeLa and HaCaT cells. In addition the overlapping YY1 site has also been demonstrated to increase promoter activity upon mutation when the promoter proximal fragment was isolated and tested for its ability to induce gene expression (Bauknecht *et al.*, 1992; Shi *et al.*, 1991). Ideally mutation of both sites at their overlapping region will result in a further increase in promoter activity when compared to the two individual mutations. As seen in Figure 4.6, when comparing the GRE recognition sequences of the most common types of HPV, a similar pattern was

observed which appeared to be crucial in determining the binding affinity of the GRE proteins. Hence these bases should be chosen to be mutated in the attempt to abolish GRE-binding. As for the YY1 recognition sequence, Bauknecht *et al*. (1995) suggested that the TTTT motif is important for YY1-binding and hence was taken into account when oligonucleotides to be used for site-directed mutagenesis were designed. Selected bases were mutated by substituting purines with pyrimidines and *vice versa*.

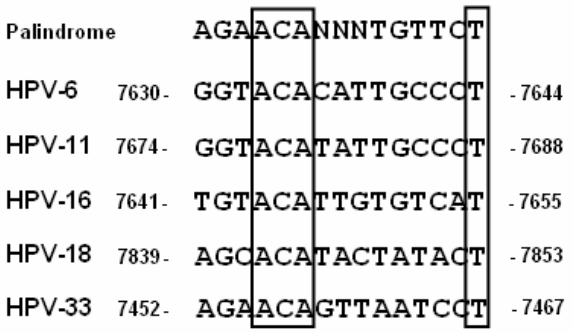


Figure 4.6 Comparison of GRE recognition sequences in common HPV types. GRE recognition sequences of the major low-risk (HPV-6 and 11) and high-risk (HPV-16, 18 and 33) HPV. Bases identical to the functionally analysed palindrome (Strahle *et al.*, 1987) are boxed, suggesting that they are crucial for binding. The numbers represent the relative positions of the GRE recognition sequences in the genome of the corresponding HPV type. Figure modified from Chan *et al*. (1989).

Sp1: The transcription factor Sp1 has been identified to be an activator of the HPV-18 LCR (Butz and Hoppe-Seyler, 1993; Demeret *et al.*, 1994; Hoppe-Seyler and Butz, 1992). A Sp1 recognition sequence is located downstream of the GRE/YY1 site in the promoter proximal region. Studies performed by Butz and Hoppe-Seyler (1993) suggested that a mutation of this element resulted in a strong reduction of transcription activity from the P_{105} promoter both in HeLa and HaCaT cells. However, Rose *et al*. (1998) has also reported that point mutations in the Sp1 motif of the HPV-18 LCR may

lead to an increase in promoter activity by 2 to 3-fold. In addition Demeret *et al*. (1994) suggested the transcriptional repression induced by mutation on the Sp1 binding motif can be compensated by the binding of E2 to the E2BS#2, which is located immediately downstream of the Sp1 binding site. Based on these observations, the Sp1 mutation may possibly be used to induce selective promoter activation within HPV-positive cells. Hence a LCR construct carrying a Sp1 mutation was produced and tested for promoter activation in different cell lines.

Double mutation of AP1/YY1 and GRE/YY1: In addition to all the individual mutations, an extra double mutation construct was also produced by combining the mutations on the AP1/YY1 and GRE/YY1 sites. It was produced to examine whether a combined effect of two individual mutation plasmids would result from a plasmid containing the corresponding double mutation. It was also hoped that by combining the effect of mutations on three of the potential transcriptional repressors, a maximum increase in the P_{105} promoter activity could be induced.

The five LCR fragments containing mutations at different transcription factor binding sites were produced and represented by a schematic diagram shown in Figure 4.7.

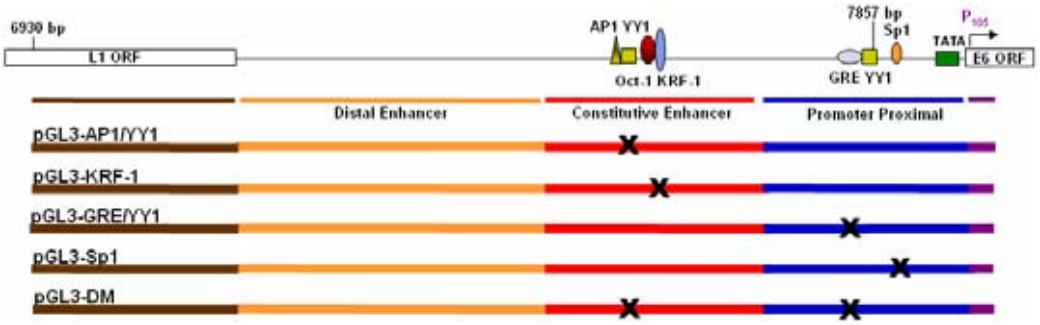


Figure 4.7 Schematic diagram of pGL3-LCR1000 plasmids containing mutations on various transcription factor binding sites.

The transcription factor binding sites mutation constructs were produced by making use of the pGL3-LCR1000 plasmid as a template. The pGL3-AP1/YY1 plasmid and the pGL3-KRF-1 plasmid contained mutations at the YY1 and KRF-1 binding sites respectively within the constitutive enhancer region; the pGL3-GRE/YY1 and pGL3-Sp1 plasmids contained mutations at the GRE/YY1 and Sp1 binding sites respectively within the promoter proximal region of the LCR. The pGL3-DM plasmid contained both mutations of the pGL3-AP1/YY1 and pGL3-GRE/YY1 plasmids.

(i) Design and construction of the transcription factor binding sites mutation constructs

The oligonucleotides #294 (5′-ACCTGGTATTAGTCACCGCGGTGTCCAG GTG-3′; underlined CCGCGG replaces TTTTCC in wild-type sequence) and #295 (5′-CACCTGGACACCGCGGTGACTAATACCAGGT-3′); underlined CCGCGG replaces GGAAAA in wild-type sequence) were designed to introduce mutations to the AP1/YY1 site as described by Bauknecht *et al*. (1995), which showed no binding of YY1, to produce the pGL3-AP1/YY1 plasmid. The oligonucleotides #300 (5′-TGCTTA ACGAACTATATCCACTAAATATGT-3′; underlined replaces GCAT, CCC and A in wild-type sequence) and #301 (5′-ACATATTTAGTGGATATAGTTCGTTAAGCA-3′; underlined replaces T, GGG and ATGC in wild-type sequence) were designed to

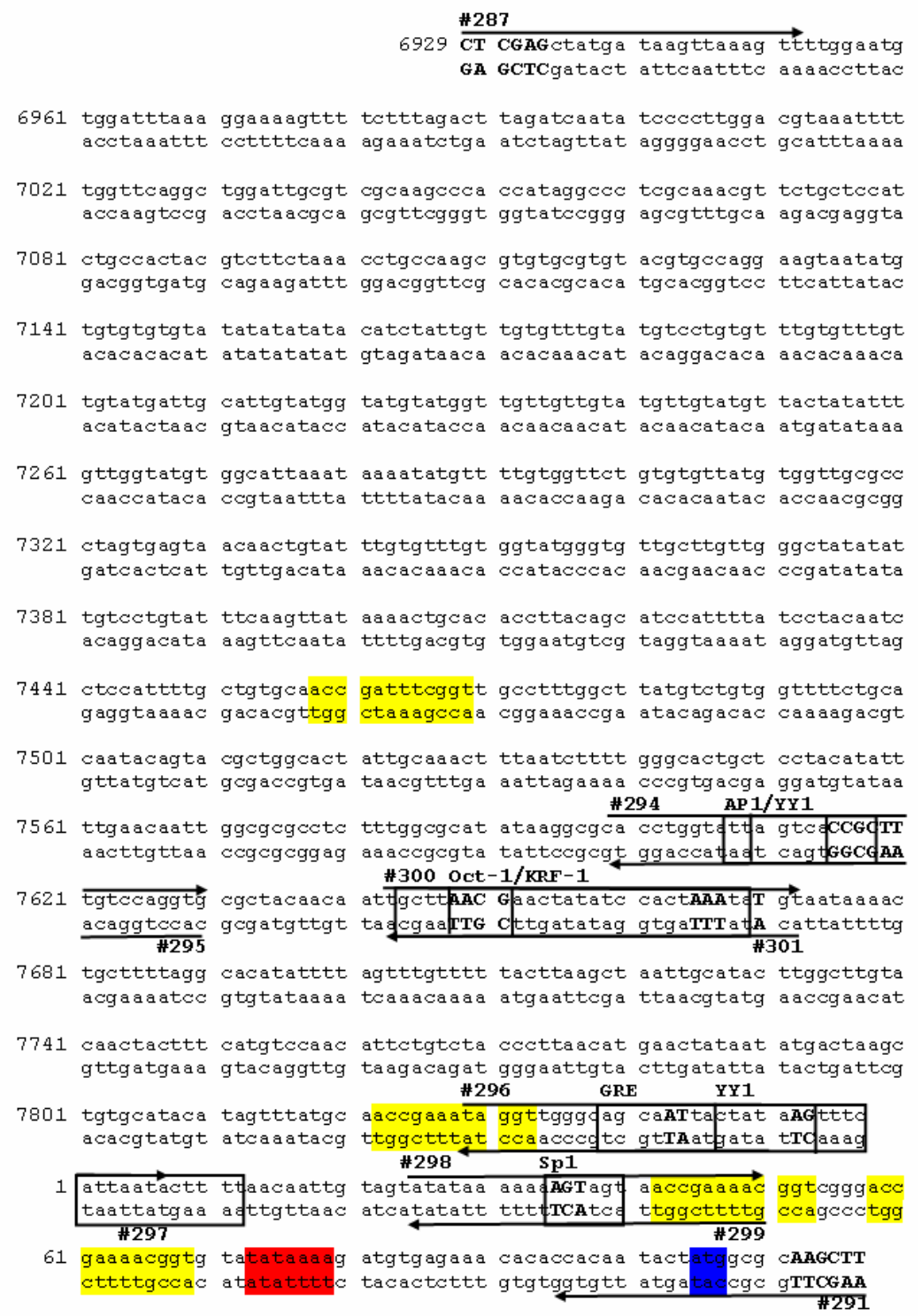
introduce mutations to the KRF-1 site as described by Butz and Hoppe-Seyler (1993), to produce the pGL3-KRF-1 plasmid. The oligonucleotides #296 (5′-TAGGTTGGGCAG CAATTACTATAAGTTTCATTAATA-3′; underlined replaces CA and CT in wild-type sequence) and #297 (5′-TATTAATGAAACTTATAGTAATTGCTGCCCAACCTA-3′; underlined replaces AG and TG in wild-type sequence) were designed to introduce mutations to the GRE/YY1 binding site as described in Section 4.2.2(iii), to produce the pGL3-GRE/YY1 plasmid. The oligonucleotides #299 (5′-ATATAAAAAAACTAGTA ACCGAAAAC-3′; underlined replaces GGG in wild-type sequence) AND #300 (5′-GT TTTCGGTTACTAGTTTTTTTATAT-3′; underlined replaces CCC in wild-type sequence) were designed to introduce mutations to the Sp1 binding site as described by Hoppe-Seyler and Butz (1992), which has been shown to abolish Sp1 binding to the site, to produce the pGL3-Sp1 plasmid. The pGL3-DM plasmid containing the double mutation of AP1/YY1 and GRE/YY1 was produced by performing a second-round site-directed mutagenesis on the pGL3-AP1/YY1 plasmid with the GRE/YY1 mutation oligonucleotides #296 and #297. The pair of outer primers used for the second-round PCR of site-directed mutagenesis was the same pair of primers used to produce the LCR insert of the pGL3-LCR1000 plasmid, oligonucleotides #287 and #291 (see Table 2.3 for details of oligonucleotides used).

In contrast to the E2BS mutation constructs as described in Section 4.2.1, the pGL3-LCR1000 plasmid was used as the template for the mutations performed on the transcription factor binding sites instead. This is due to the fact that it was not appreciated that, the 850 bp *Rsa*I-*Bam*HI fragment was stronger in inducing promoter activity when these experiments were designed. However, as results were interpreted by calculating the promoter activity induced as a relative percentage compared to the positive control plasmid pGL3-SV40, the different length of LCR inserts in the two sets

of mutation constructs will not interfere with the results. The positions of oligonucleotides binding to the LCR are shown in Figure 4.8.

Figure 4.8 Binding positions of oligonucleotides used for site-directed mutagenesis of transcription factor binding sites within the LCR.

For site-directed mutagenesis performed on the chosen transcription factor binding sites, a pair of oligonucleotides was needed to introduce the designated mutations into both strands during the first round of PCR. Oligonucleotides #294/#295 was used for mutation on AP1/YY1; #300/#301 for KRF-1; #296/#297 for GRE-YY1 and #298/#299 for Sp1. The E2BSs were highlighted in yellow and the transcription factor binding sites chosen to be mutated are boxed. The outer pair of oligonucleotides #287/#291 was used to generate the 1,040 bp *Bam*HI fragment containing the mutations in the second round of PCR, with the restriction enzyme recognition sequences added to assist cloning (*Xho*I in #287 and *Hind*III in #291; in bold and caps). The TATA box and ATG start codon of the E6 ORF was highlighted in red and blue respectively.


#287
6929 CT CGAGctatga taagttaaag ttttggaatg
GA GCTCgatact attcaatttc aaaaccttac
6961 tggatttaaa ggaaaagttt tctttagact tagatcaata tccccttgga cgtaaatttt
acctaaattt ccttttcaaa agaaatctga atctagttat aggggaacct gcatttaaaa
7021 tggttcaggc tggattgcgt cgcaagccca ccataggccc tcgcaaacgt tctgctccat
accaagtccg acctaacgca gcgttcgggt ggtatccggg agcgtttgca agacgaggta
7081 ctgccactac gtcttctaaa cctgccaagc gtgtgcgtgt acgtgccagg aagtaatatg
gacggtgatg cagaagattt ggacggttcg cacacgcaca tgcacggtcc ttcattatac
7141 tgtgtgtgta tatatatata catctattgt tgtgtttgta tgtcctgtgt ttgtgtttgt
acacacacat atatatatat gtagataaca acacaaacat acaggacaca aacacaaaca
7201 tgtatgattg cattgtatgg tatgtatggt tgttgttgta tgttgtatgt tactatattt
acatactaac gtaacatacc atacatacca acaacaacat acaacataca atgatataaa
7261 gttggtatgt ggcattaaat aaaatatgtt ttgtggttct gtgtgttatg tggttgcgcc
caaccataca ccgtaattta ttttatacaa aacaccaaga cacacaatac accaacgcgg
7321 ctagtgagta acaactgtat ttgtgtttgt ggtatgggtg ttgcttgttg ggctatatat
gatcactcat tgttgacata aacacaaaca ccatacccac aacgaacaac ccgatatata
7381 tgtcctgtat ttcaagttat aaaactgcac accttacagc atccatttta tcctacaatc
acaggacata aagttcaata ttttgacgtg tggaatgtcg taggtaaaat aggatgttag
7441 ctccattttg ctgtgcaacc gatttcggtt gcctttggct tatgtctgtg gttttctgca
gaggtaaaac gacacgttgg ctaaagccaa cggaaaccga atacagacac caaaagacgt
7501 caatacagta cgctggcact attgcaaact ttaatctttt gggcactgct cctacatatt
gttatgtcat gcgaccgtga taacgtttga aattagaaaa cccgtgacga ggatgtataa
#294
AP1/YY1
7561 ttgaacaatt ggcgcgcctc tttggcgcat ataaggcgca cctggtatta gtcaCCGCTT
aacttgttaa ccgcgcggag aaaccgcgta tattccgcgt ggaccataat cagtGGCGAA
#300 Oct-1/KRF-1
7621 tgtccaggtg cgctacaaca attgcttAAC GAactatatc cactAAATaT gtaataaaac
acaggtccac gcgatgttgt taacgaaTTG CTtgatatag gtgaTTTatA cattattttg
#295
#301
7681 tgcttttagg cacatatttt agtttgtttt tacttaagct aattgcatac ttggcttgta
acgaaaatcc gtgtataaaa tcaaacaaaa atgaattcga ttaacgtatg aaccgaacat
7741 caactacttt catgtccaac attctgtcta cccttaacat gaactataat atgactaagc
gttgatgaaa gtacaggttg taagacagat gggaattgta cttgatatta tactgattcg
#296
GRE
YY1
7801 tgtgcataca tagtttatgc aaccgaaata ggttgggcag caATtactat aAGtttc
acacgtatgt atcaaatacg ttggctttat ccaacccgtc gtTAatgata tTCaaag
#298
Sp1
1 attaatactt ttaacaattg tagtatataa aaaaAGTagt aaccgaaaac ggtcgggacc
taattatgaa aattgttaac atcatatatt ttttTCAtca ttggcttttg ccagccctgg
#297
#299
61 gaaaacggtg tatataaaag atgtgagaaa cacaccacaa tactatggcg cAAGCTT
cttttgccac atatattttc tacactcttt gtgtggtgtt atgataccgc gTTCGAA
#291

The steps involved in the construction of the pGL3-KRF-1 plasmid are shown below as an example of typical results obtained. The pGL3-KRF-1 plasmid contains the 1,040 bp *Bam*HI LCR fragment carrying the mutations introduced at the KRF-1 binding site as an insert. The oligonucleotides designed to introduce the mutations at the KRF-1 binding site by site-directed mutagenesis were #300 (forward) and #301 (reverse) (see Figure 4.8 for details). A first-round PCR was performed using the pGL3-LCR1000 plasmid as the template with oligonucleotide pairs #287/#301 and #300/291 (see Table 2.3 for details of oligonucleotides used), producing two separate PCR products containing partial fragments of the insert (Figure 4.9). The two PCR products obtained from the first-round PCR were then mixed and used as the template for the second-round PCR, using the outer oligonucleotide pair #287/#291 as primers (Figure 4.10). The resulting PCR product carrying mutation at the KRF-1 binding site was digested by restriction enzymes *Xho*I and *Hind*III, and ligated to the pGL3-Basic vector which was linearised by the same restriction enzymes. The ligation mixture was then transformed into competent DH5α cells and the clones obtained were screened by colony PCR to ensure the successful cloning of the pGL3-KRF-1 plasmid (Figure 4.11).

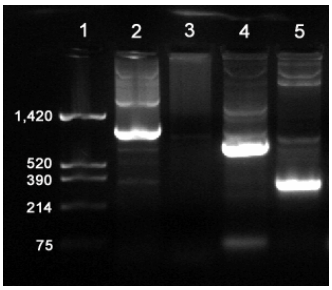


Figure 4.9 Construction of the pGL3-KRF-1 plasmid – first-round PCR.
A 2% (w/v) agarose gel ran at 90V for 25 minutes, showing the PCR products of the first-round PCR during the construction of the pGL3-KRF-1 plasmid by site-directed mutagenesis. Lane 1 was 500 ng of pUC/*Hinf*I DNA marker. Lane 2 was PCR product from the positive control reaction using the outer oligonucleotide pair #287/#291 as the primers and the pGL3-LCR1000 plasmid as the template (1,062 bp). Lane 3 was the negative control reaction with no DNA template. Lane 4 was the PCR product from oligonucleotides #287/#301 (753 bp). Lane 5 was the PCR product from oligonucleotides #300/#291 (339 bp).

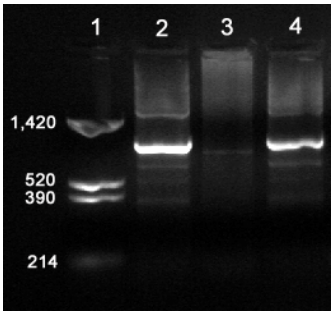


Figure 4.10 Construction of the pGL3-KRF-1 plasmid – second-round PCR.
A 2% (w/v) agarose gel ran at 90V for 25 minutes, showing the PCR products of the second-round PCR during the construction of the pGL3-KRF-1 plasmid by site-directed mutagenesis. Lane 1 was 500 ng of pUC/*Hinf*I DNA marker. Lane 2 was PCR product from the positive control reaction using the outer oligonucleotide pair #287/#291 as the primers and the pGL3-LCR1000 plasmid as the template (1,062 bp). Lane 3 was the negative control reaction with no DNA template. Lane 4 was the PCR product from oligonucleotides #287/#301 by using a mixture of the two PCR products obtained from the first-round PCR previously (1,062 bp), which is also the final PCR product of the LCR1000 fragment carrying the mutation at the KRF-1 binding site.

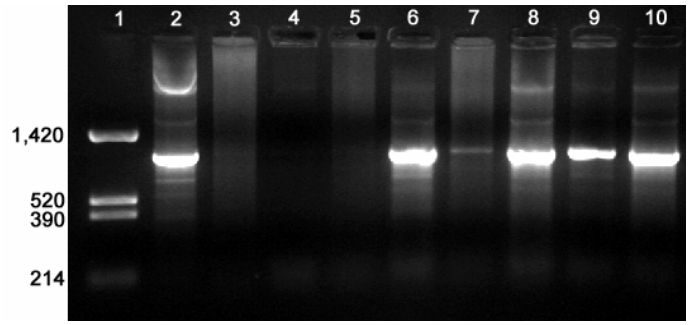


Figure 4.11 Construction of the pGL3-KRF-1 plasmid – colony PCR.

A 2% (w/v) agarose gel ran at 90V for 30 minutes, showing the PCR products of the colony PCR performed on selected clones from the transformation of the pGL3-KRF-1 plasmid into DH5α. Lane 1 was 500 ng of pUC/*Hinf*I DNA marker. Lane 2 was PCR product from the positive control reaction using the outer oligonucleotide pair #287/#291 as the primers and the pGL3-LCR1000 plasmid as the template (1,062 bp). Lane 3 was the negative control reaction with no DNA template. Lane 4 to 10 were PCR products of colony PCR performed on seven of the selected clones using the outer oligonucleotides pair #287/#301, which should produce a product size of 1,062 bp if an insert was present. As shown in the gel lane 6, 8, 9 and 10 represent 4 clones which have been successfully transformed with the pGL3-KRF-1 plasmid.

(ii) Effect of transcription factor binding sites mutations on P_{105} promoter activity and specificity

Similar to transfection experiments performed in Chapter 3, plasmid constructs containing the wild-type LCR and LCR fragments carrying mutations at selected transcription factor binding sites were co-transfected into the five mammalian cell lines in Table 2.1, together with the positive control plasmid pGL3-SV40. Transfected cells were harvested 48 hours after transfection and cell lysates were collected for luciferase and β-galactosidase assays as described in Sections 2.2.10 and 2.2.11. Experiments on each cell line were repeated for at least three times and each individual experiment was performed in triplicates. Results were analysed as described in Table 3.3 and calculated

into percentage luciferase activity relative to the activity detected from the positive control plasmid pGL3-SV40. A summary of the results is graphically represented in Figure 4.12. The raw data results are shown in the appendix chapter.

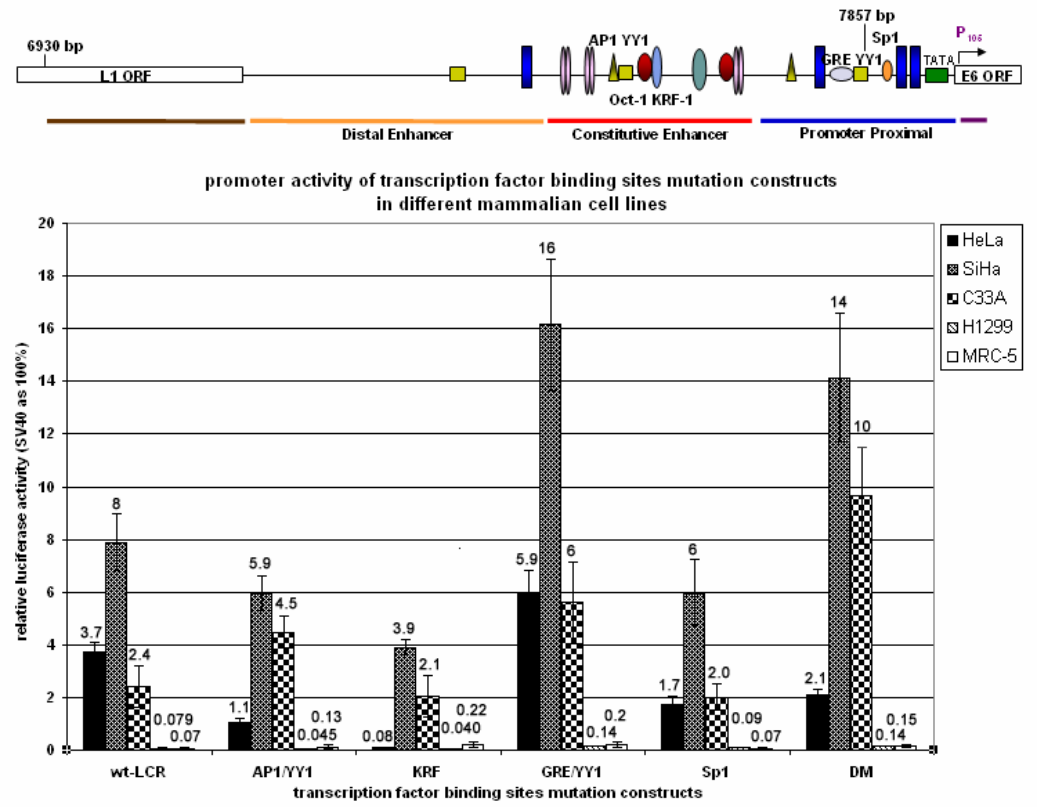


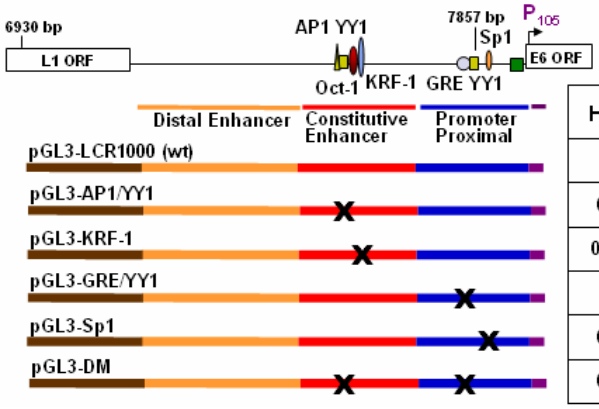
Figure 4.12 Promoter activities of transcription factor binding sites mutation constructs in different mammalian cell lines.

The graph represents results obtained from transfection experiments using five different cell lines. Relative luciferase activities detected from various transcription factor binding sites mutation constructs were plotted as percentages relative to the luciferase activity from the pGL3-SV40 plasmid which served as a positive control. The error bars indicate the SEM from the three experimental repeats performed in each cell line.

As seen in Figure 4.12, not all the transcription factor binding sites chosen to be mutated resulted in an increased level of promoter activity. In all three cervical

cancer cell lines tested, an increase in luciferase activity was observed from the mutation performed at the GRE/YY1 binding site. In contrast, mutation at the AP1/YY1 site resulted in increased activity only in the HPV-negative cervical cancer cell line tested. The double mutation of the AP1/YY1 and GRE/YY1 binding sites appeared to have an additive effect of the two individual mutations, which resulted in significant increase of luciferase activity in both of the HPV-16 positive and HPV-negative cervical cancer cell lines tested. Mutations of the KRF-1 and Sp1 binding sites resulted in a decreased level of luciferase activity from all three cervical cancer cell lines tested.

In order to compare the effect of the individual transcription factor binding sites mutations on the P_{105} promoter strength in different cell lines, the level of luciferase activity detected from pGL3-LCR1000 was set as 1 and activities from the mutation constructs were calculated as ratios relative to the wild-type LCR fragment (Figure 4.13).



HeLa	SiHa	C33A	H1299	MRC-5
1	1	1	1	1
0.27	0.77	1.9	0.54	2.4
0.020	0.53	0.82	0.49	4.3
1.5	1.9	2.4	4.9	4.6
0.44	0.85	0.84	1.1	1.0
0.56	1.6	4.0	1.6	2.8

Figure 4.13 Relative promoter activity from the transcription factor binding sites mutation constructs in different cell lines.

The level of luciferase activity detected from the wild-type LCR insert in the pGL3-LCR1000 plasmid was set at 1, and the ratios of luciferase readings from various transcription factor binding sites mutation constructs were calculated. A green square on the top right corner denotes an increase in promoter activity and a red square denotes a

decrease in promoter activity when compared to the wild-type LCR. Numbers were all rounded off to two significant figures.

The effect of the mutations was analysed individually for their contribution towards the P_{105} promoter activity. The ratios calculated in Figure 4.13 showed that a mutation at the YY1 site adjacent to an AP1 binding site within the constitutive enhancer region resulted in a significant decrease in the P_{105} promoter activity within HeLa cells, retaining only 27% of activity from the wild-type LCR, as opposed to observations by Bauknecht *et al*. (1995) suggesting the corresponding mutation did not affect promoter activity in HeLa cells. The same mutation also led to a decrease in promoter activity within SiHa cells but to a lesser extent, retaining about 77% of wild-type LCR promoter activity. These results suggest that protein binding to this YY1 recognition motif enhances the P_{105} promoter activity within the HPV-positive cervical cancer cells tested in the context of this project. In contrast, the mutation resulted in an almost two-fold increase in promoter activity within the HPV-negative C33A cells, suggesting the possibility of HPV-dependent promoter activation which appeared to overcome the repressive effect of common cervical cancer cell-specific transcription factors binding to the YY1 site.

Previous results obtained by Butz and Hoppe-Seyler (1993) suggested that a mutation at the KRF-1 binding site within the constitutive enhancer region had a significantly stronger effect in reducing the level of LCR promoter activity in non-cervical cancer cell lines HaCaT and primary keratinocytes (about 75% decrease), when compared to cervical cancer cell lines HeLa and C33A cells (about 25% decrease). It was initially hoped that a mutation introduced to this KRF-1 binding site would lead to an increase in promoter specificity towards the cervical cancer cells tested, which

contributes towards the aim of the project. Although the KRF-1 mutation construct carrying exactly the same mutations resulted in decreased promoter activity in all three cervical cancer cell lines tested, which correlates with previous observations by Butz and Hoppe-Seyler (1993), however, the extent of reduction in promoter activity appeared to be different. Promoter activity was almost completely abolished within HeLa cells; about 47% decrease in SiHa cells and about 18% decrease C33A cells when compared to the wild-type LCR promoter activity. These results suggest possible binding of common cervical cancer cell-specific transcription activators to the KRF-1 recognition motif. The more significant decrease in promoter activity upon the mutation in HeLa and SiHa cells suggested the possible involvement of HPV viral gene products in promoter activation at the KRF-1 binding site, which is particularly significant in the presence of HPV-18. The mutation of the KRF-1 binding site, however, is not beneficial towards the aim of the project for it completely abolished the activity of the P_{105} promoter in HeLa cells.

The mutation introduced at the GRE/YY1 site within the promoter proximal region was the only mutation that resulted in an increased promoter activity in all three cervical cancer cell lines tested. A 1.5-fold increase in promoter activity was observed in HeLa cells and almost 2-fold and 2.5-fold increase in SiHa and C33A cells respectively. Although the pGL3-GRE/YY1 plasmid contained mutations at both the GRE and YY1 binding sites, it did not appear to have a combined effect of the two individual mutations performed in previous studies. Butz and Hoppe-Seyler (1993) reported that a mutation at the GRE binding site resulted in a 2 to 3-fold increase in promoter activity in both HeLa and HaCaT cells, whereas Bauknecht *et al*. (1995) suggested that the mutation of the adjacent YY1 binding site in the context of the LCR resulted in a decrease in promoter activity in HeLa cells by 3 to 4-fold. The elevated

level of promoter activity all three cervical cancer cell lines suggested the possible existence of cellular transcriptional repressors, which may be commonly found in cervical cancer cells, binding to the GRE/YY1 site. The less significant increase in the level of promoter activation observed in HPV-positive HeLa and SiHa cells suggested possible HPV-dependent promoter activation which compensates for the repressive effect of common cellular transcription factors found in all three cervical cancer cell lines.

Similar to the mutation on the KRF-1 binding site, mutation of the Sp1 binding site within the promoter proximal region resulted in decreased level of promoter activity in all three cervical cancer cell lines tested, which suggested the possible existence of common cellular transcription activators acting on the Sp1 binding site. The decrease was most significant in HeLa cells, retaining only about 44% of wild-type LCR promoter activity, whereas in SiHa and C33A promoter activities were both decreased slightly by about 15%. The results were dissimilar to those reported by Butz and Hoppe-Seyler (1993), which suggested the same mutation reduced the level of promoter activity in HeLa cells by almost 75%. A more significant decrease in promoter activity upon mutation was observed in HeLa cells, whereas the mutation exerted a similar effect in SiHa and C33A cells, suggesting that promoter activation at the Sp1 binding site may possibly be related to the expression of viral gene products from HPV-18 in particular.

The pGL3-DM plasmid contained both mutations of the AP1/YY1 and the GRE/YY1 binding sites within the LCR, and appeared to display an additive effect of the two individual mutations. A reduction in promoter activity was still observed in HeLa cells, retaining about 56% of wild-type LCR promoter activity. In contrast an

increase in promoter activity was observed in both SiHa and C33A cells, by over 1.5-fold and almost 4-fold respectively.

The effect of transcription factor binding sites mutations in regards to the promoter specificity towards HPV-positive cervical cancer cells can be examined more precisely by calculating the ratios of luciferase activity from HeLa and SiHa cells against the other control cell lines (Tables 4.3 and 4.4).

Table 4.3 Ratios of luciferase activity from transcription factor binding sites mutation constructs in HeLa against other cell lines.

Selectivity towards HeLa (HPV-18 +ve cervical cancer cells)	wt-LCR	AP1/ YY1	KRF-1	GRE/ YY1	Sp1	DM
HeLa (HPV-18) : SiHa (HPV-16)	0.47	0.18	0.021	0.37	0.29	0.15
HeLa (HPV +ve) : C33A (HPV -ve)	1.6	0.24	0.040	1.1	0.86	0.22
HeLa (cervix) : H1299 (lung)	48	23	2.1	43	20	16
HeLa (cancerous epithelial) : MRC-5 (normal fibroblast)	54	8.1	0.38	28	24	14

A ratio higher than 1 represents selectivity towards HeLa cells and *vice versa*. A red square at the top right corner denotes a decrease in promoter specificity towards HeLa cells upon mutations on the specified transcription factor binding sites of the LCR. All numbers shown were rounded off to two significant figures.

Table 4.4 Ratios of luciferase activity from transcription factor binding sites mutation constructs in SiHa against other cell lines.

Selectivity towards SiHa (HPV-16 +ve cervical cancer cells)	wt-LCR	AP1/ YY1	KRF-1	GRE/ YY1	Sp1	DM
SiHa (HPV-16) : HeLa (HPV -18)	2.1	5.6	47	2.7	3.5	6.7
SiHa (HPV +ve) : C33A (HPV -ve)	3.3	1.3	1.9	2.9	3.0	1.5
SiHa (cervix) : H1299 (lung)	100	130	97	120	68	100
SiHa (cancerous epithelial) : MRC-5 (normal fibroblast)	110	46	18	75	82	95

A ratio higher than 1 represents selectivity towards SiHa cells and *vice versa*. A green square at the top right corner denotes an increase in promoter specificity towards SiHa cells upon mutations on the specified transcription factor binding sites of the LCR. A red square denotes a decrease in promoter specificity towards SiHa cells instead. All numbers shown were rounded off to two significant figures.

As shown in Tables 4.3 and 4.4, the wild-type LCR promoter activity is 1.6-fold stronger in HeLa cells, and over 3-fold stronger in SiHa cells, when compared to C33A cells. None of the mutations of transcription factor binding sites introduced resulted in an increased level of promoter specificity towards both of the HPV-positive cervical cancer cell lines tested when compared to HPV-negative ones, suggesting that HPV-dependent repression of the P_{105} promoter may not be involved at the transcription factor binding sites chosen to be studied. Although the mutations at the AP1/YY1 and GRE/YY1 binding sites resulted in increased P_{105} promoter specificity towards SiHa cells when compared to H1299 cells, which may represent the presence of HPV-dependent and cervical cancer cell type-specific promoter repression; but since the same effect of the mutations was not observed in HeLa cells, hence these mutations may not be ideal to be used to further increase promoter specificity within HPV-positive cervical cancer cells.

4.3 General discussion

As discussed in Section 3.3.3, the HPV-18 LCR can be selectively activated within cervical cancer cells, in the context of the limited number of cell lines used in this project, suggested the involvement of transcription factors that are specifically found within cervical cancer cells in the regulation of the P_{105} promoter activity. Moreover, a higher level of promoter activity was observed within two cervical cancer cells tested that are HPV-positive, and in particular, within the HPV-16-positive SiHa cells when compared to the HPV-18-positive HeLa cells. These results also suggested the possible involvement of HPV viral gene products in the regulation of P_{105} promoter activity. Hence the second part of the project was aimed at identifying transcription control elements which may responsible for the observed promoter specificity, and also to further increase promoter activity and specificity towards cervical cancer cells by performing mutations at transcription factor binding sites which were suggested by previous studies to be transcriptional repressor on the HPV-18 LCR.

4.3.1 Promoter regulation by cervical cancer cell type-specific transcription factors

It has been suggested that the epithelial cell-specific activity of the HPV-18 LCR is unlikely to be induced from common, epithelial transactivating factors, and alternate regulatory pathways may exist in different epithelial cell types (Butz and Hoppe-Seyler, 1993). In the context of the five mammalian cell lines tested in this project, the results obtained from the LCR deletion constructs also suggested that the HPV-18 LCR promoter activity is not only specific towards epithelial cells, but more precisely towards epithelial carcinomas of a cervical origin. In addition, the mutations at

the four E2BSs, as well as the KRF-1 and Sp1 binding sites, resulted in decreased promoter activities within all three cervical cancer cell lines tested. These results suggest the presence of cervical cancer cell type-specific transcriptional activators acting on these transcription factor binding sites prior to mutation, which may also explain the promoter specificity observed from the LCR. Alternatively, the introduced mutations may have caused conformational changes in the promoter which could be expected to affect the binding of transcription factors and regulators. In contrast, mutations at the GRE/YY1 binding site resulted in increased promoter activities in all three cervical cancer cell lines tested, while the promoter activities detected from both non-cervical cancer cell lines H1299 and MRC-5 cells remained very low. This result suggests the presence of cellular transcriptional repressors, which are commonly found in cervical cancer cells, binding to the GRE/YY1 site prior to mutation.

4.3.2 Promoter regulation by HPV viral gene products

(i) HPV-positive versus HPV-negative cervical cancer cells

One of the main issues in the context of the project was the involvement of HPV viral gene products in the regulation of the P_{105} promoter. As mentioned previously that the E2 protein is the only viral gene product known to be responsible in the regulation of HPV transcription and is crucial in determining the level of expression of the E6 and E7 oncogenes (Bednarek *et al.*, 1998; Schwarz *et al.*, 1985), and only recognition sequences for the E2 protein are found to be present within the LCR. Upon the integration of high-risk HPV into the host genome during the cellular transformation process, the E2 ORF has been reported to be always disrupted (Bednarek *et al.*, 1998; Corden *et al.*, 1999; Kitagawa *et al.*, 1996; Rosales *et al.*, 2001) with the viral E2

protein not being expressed. Hence promoter regulation in the context of HPV-infected cervical cancer cells is thought to be dependent solely on cellular transcription factors present. However, other studies suggest the involvement of truncated forms of E2 proteins in the regulation of the P_{105} promoter within HPV-infected cervical cancer cells, which may in turn influence early promoter activation and the expression of E6 and E7 oncogenes (see Section 1.5.1). Moreover, studies performed by Gius *et al*. (1988) suggest that the distal enhancer region of the LCR is induced by E6. It is therefore reasonable to suggest that the presence of the integrated part of viral DNA within HPV-infected cervical cancer cells does contribute to promoter activation within the LCR, and attempted were made to identify the difference in promoter regulation in between HPV-positive and HPV-negative cervical cancer cell lines.

Results obtained from the LCR deletion constructs as discussed in Chapter 3 suggested that the P_{105} promoter is not only specific towards the three cervical cancer cells tested, but more precisely towards the two cervical cancer cells that are infected by HPV. In addition, results obtained from the mutation studies identified several transcription factor binding sites to be particularly responsive within the HPV-positive cervical cancer cell lines tested. The mutation at the AP1/YY1 resulted in decreased promoter activity only within the two HPV-positive cervical cancer cell lines, as opposed to increased activity observed in the HPV-negative cervical cancer cell line tested. Moreover, mutations at E2BS#3, E2BS#2, and the KRF-1 binding sites all resulted in a stronger reduction in promoter activity within the HPV-positive cervical cancer cell lines when compared to the HPV-negative cell line tested. These results suggest the presence of HPV-dependent promoter activation. In contrast to promoter regulation by cervical cancer cell type-specific transcription factors, HPV-dependent promoter repression was not observed at the chosen transcription factor binding sites.

The results obtained have therefore suggest the possible involvement of HPV viral gene products in the regulation of the P_{105} promoter in the context of HPV-infected cervical cancer cells. The precise mechanism of promoter regulation, however, remains unclear as experiments performed were not designed for detailed studies on that aspect.

(ii) HPV-18 versus HPV-16 infected cervical cancer cells

In addition to differences observed in promoter regulation between HPV-positive and HPV-negative cervical cancer cell lines, differences were also observed with respect to cervical cancer cells infected by different types of HPV. Results obtained from the LCR deletion constructs as mentioned in Chapter 3 identified a larger percentage reduction in promoter activity upon the deletion of the distal enhancer fragment from the LCR in HeLa cells when compared to the other cell lines tested, suggesting the involvement of HPV-18 viral gene products in promoter activation at transcriptional control elements present within the distal enhancer region (see Section 3.2.3(iii) for details). In addition, the mutations introduced at the four E2BSs, the AP1/YY1 and Sp1 binding sites all resulted in a more significant reduction of promoter activity in HeLa cells when compared to SiHa cells, which suggested promoter regulation to be more dependent on activation by HPV-18 viral gene products when compared to HPV-16 at the selected transcription factor binding sites. This may be due to the fact that the P_{105} promoter was originally derived from HPV-18; hence the binding of HPV-18 viral gene products to the LCR is crucial in determining the level of P_{105} promoter activity induced in the context of HeLa cells.

Ideally, a lower level of P_{105} promoter activity should also be observed from other cell lines that do not contain the DNA of HPV-18 if the above assumption on HPV-dependent promoter regulation is true. Results obtained from the LCR deletion

constructs, however, showed that P_{105} promoter activity within cervical cancer cell line infected by HPV-18 (HeLa) was significantly lower than that infected by HPV-16 (SiHa), and only slightly higher than promoter activity from HPV-negative cervical cancer cells tested (C33A). Moreover, none of the transcription factor binding sites chosen to be studied appeared to be responsible for stronger promoter activation by HPV-16 viral gene products when compared to HPV-18. The higher level of promoter activity observed within SiHa cells when compared to HeLa cells, and the less significant decrease in promoter activity upon mutations at corresponding transcription factor binding sites, may possibly be due to a higher binding affinity of HPV-16 viral proteins to the transcriptional control elements of the HPV-18 LCR, since viral proteins from different HPV types are known to possess different binding affinities to their target recognition sites (Hwang *et al.*, 2002).

4.4 Conclusion

In conclusion, there are three novel observations arising from this project. Firstly, the HPV-18 P_{105} promoter is shown to possess selective activity within cervical cancer cells in the context of the five mammalian cell lines tested, and therefore may serve as a novel tool to achieve cervical cancer-specific gene expression. Upon linkage to a suicide gene, the HPV-18 LCR may be used as a tissue-specific promoter element for gene therapy targeting cervical cancer cells. Secondly, in regards to promoter regulation of the HPV-18 LCR, the 3′-end of the HPV L1 gene (distal *Bam*HI-*Rsa*I fragment) appeared to possess repressive properties on the activity of the P_{105} promoter. In contrast, the distal enhancer fragment (distal *Rsa*I-*Rsa*I fragment) appeared to contain important transcription control elements responsible for strong promoter activation, which may not have been identified to date. Thirdly, results from the mutation studies performed suggest that the E2BSs are not responsible for promoter repression within three cervical cancer cell lines tested, as opposed to the E2-mediated promoter repression during the normal viral life cycle of HPV. Instead, the E2BSs appeared to be involved in P_{105} promoter activation by possible interaction with cellular transcription factors.

In regards to the specificity of the LCR towards HPV-associated cervical cancer cells in particular, most of the transcription control elements chosen to be studied in this project induced a more significant decrease in promoter activity upon mutation within the two cervical cancer cells that are HPV-positive, and in particular, in HeLa cells which is infected by HPV-18. These results suggest the presence of transcription control elements involved in promoter specificity towards HPV-positive cervical cancer

cells, and the P_{105} promoter may be regulated by the same transcription factors to a different extent in cervical cancer cells infected by different HPV types.

Overall, the findings of this project support the use of the HPV-18 LCR in cervical cancer gene therapy. The P_{105} promoter of HPV-18 appeared to be an ideal promoter element to be used for directing cervical carcinoma-specific expression of therapeutic or suicide genes. Optimal activity and specificity from the P_{105} promoter can be achieved by making use of the 850 bp *Rsa*I-*Bam*HI fragment of the LCR carrying a mutation at the GRE/YY1 binding site within the promoter proximal region (proximal *Rsa*I-*Bam*HI fragment). From the results obtained in this project, the introduced GRE/YY1 mutation did not induce a significantly higher level of P_{105} promoter activity within the HPV-18-positive cervical cancer cell line (HeLa) when compared to the HPV-negative cervical cancer cell line (C33A). However, since virtually all cervical cancer cases are linked to genital infection with HPV (WHO, 2006) and HPV-negative cervical cancer cells are extremely rare (Walboomers and Meijer, 1997), it is in theory unnecessary for the candidate promoter element to distinguish cervical cancer cells that are HPV-positive in particular. However, this would be an ideal outcome in the aspect of retaining promoter activation by the candidate promoter element within a highly specific population of target cells, hence minimising the chances of promoter activation in normal non-cancerous cells that are HPV-negative.

4.5 Future directions

In terms of promoter regulation of the HPV-18 LCR, in addition to future experiments as described in Section 3.5, it would be beneficial to further investigate the precise mechanism of the P_{105} promoter regulation. The mutations introduced at the selected transcription factor binding sites may have resulted in the formation of recognition sequences for other unknown transcription factors, which may affect the level of promoter activity induced in unexpected ways. Hence DNase footprinting studies can be performed on the mutated sequences to identify possible binding of other transcription factors, and their contribution towards the P_{105} promoter activity. Attempts can also be made to identify transcription factors responsible for the HPV-dependent promoter regulation observed. Since the viral gene products expressed in the context of HPV-infected cervical cancer cells are limited, gel retardation assays can be performed to identify the viral proteins binding to the transcription factor binding sites studied. A deeper understanding of the HPV-dependent promoter regulation may also contribute towards the improvement of the HPV-18 LCR promoter selectivity towards HPV-positive cervical cancer cells in particular, which would be beneficial towards the development of a highly specific HPV promoter to be used for gene therapy.

The ultimate aim of the project is to develop a tool for gene therapy in the treatment of cervical cancer. This can be accomplished by substituting the *luc* gene in the LCR constructs produced with a suicide gene. A suicide gene can be any gene that confers a suicidal phenotype in target cells upon its expression. By selective expression of the suicide gene induced by the HPV-18 LCR, cervical cancer cells can be preferentially destroyed. A schematic diagram illustrating the ultimate aim of the project is shown in Figure 4.14. Examples of a suicide gene may be a gene that encodes for an

endonuclease such as DNase I, or a cytotoxin such as saporin. Saporin is a ribosome-inactivating protein from seeds of the plant *Saponaria officinalis*, and a recent study by Zarovni *et al*. (2007) have demonstrated the efficacy of making use of a saporin gene in cancer gene therapy via a non-viral gene delivery approach. The HPV-18 LCR constructs can first be tested *in vitro* by transfecting into different mammalian cell lines and performing cytotoxicity assays to determine the efficiency of the promoter constructs in inducing apoptosis within cervical cancer cell lines. In the application of cervical cancer gene therapy *in vivo*, however, additional studies will be required regarding the efficient delivery of the therapeutic plasmids into the targeted cervical carcinomas cells (as discussed in Section 1.6). Despite the efficacy of the use of the HPV-18 LCR to target cervical cancer cells, which has been explored in this project; a safe and effective delivery system of the anti-HPV therapeutic genes will be required before the activity and specificity of the HPV-18 promoter element can be fully utilised for the gene therapy of HPV-associated cervical cancer.

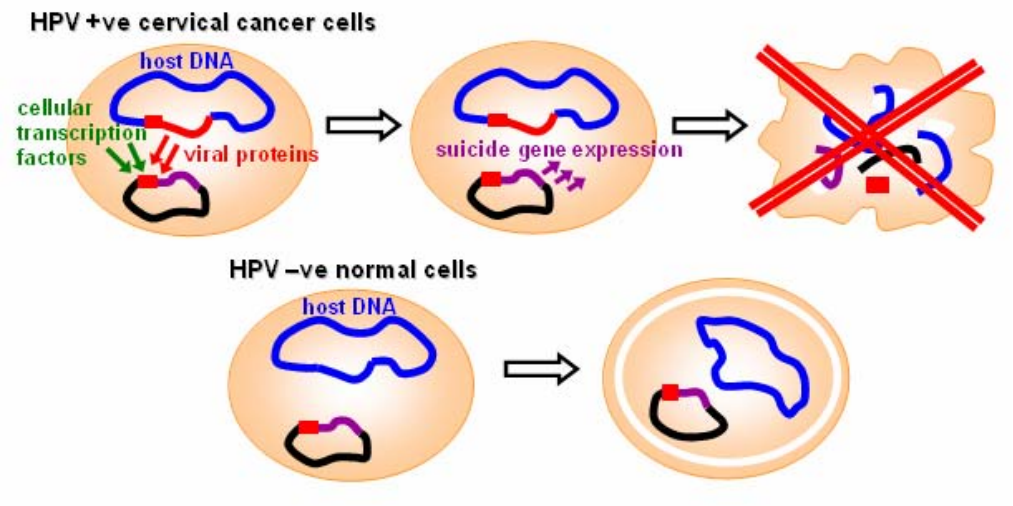


Figure 4.14 Schematic diagram illustrating the ultimate aim of the project.

The ultimate aim of this project is to develop a gene therapy which can specifically target cervical cancer cells. By constructing a suicide gene expression vector containing the LCR of HPV-18, and by increasing its promoter activity and specificity by performing appropriate mutations at selected transcription factor binding sites, suicide gene expression can be induced selectivity within HPV-infected cervical cancer cells, leading to the self-destruction of the target cells. In normal cells that are HPV-negative, the promoter element within the HPV-18 LCR will remain inactive and the suicide gene will not be expressed thus the cells remain healthy and unaffected.

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APPENDICES

Appendix I: Raw data results from HeLa cells

HeLa experiment #1															
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.945	1.018	0.998	0.775	0.934	0.972	1.031	1.012	0.997	1.023	0.982	1.080	0.912	0.711
repeat #2	0.945	0.937	1.023	0.714	0.921	0.992	0.936	0.998	1.024	0.991	0.936	1.126	0.853	0.788
repeat #3	0.961	0.936	0.994	0.889	0.946	0.948	1.124	1.024	0.964	1.066	1.026	1.046	0.976	0.847
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	283	585	126	4.68	355	96.3	226	148	128	7.02	742	246	219	6680
repeat #2	315	588	125	4.69	355	95.4	219	145	127	20.3	734	245	219	6940
repeat #3	396	622	127	4.74	355	97.1	232	151	129	7.40	750	247	219	9600
Normalised data luc/b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	300	574	126	6.04	380	99.1	219	147	128	6.86	795	228	240	9400
repeat #2	333	627	122	6.57	385	96.2	234	146	124	20.5	784	218	256	8800
repeat #3	404	665	128	5.33	375	102	206	148	133	6.94	731	236	225	11300
average luc activity	346	622	125	5.98	380	99.2	220	147	128	11.4	757	227	240	9650
SV40 as 100%	**3.51**	**6.32**	**1.273**	**0.0608**	**3.86**	**1.01**	**2.23**	**1.49**	**1.30**	**0.12**	**7.69**	**2.31**	**2.44**	**100**
HeLa experiment #2														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.937	0.924	0.903	0.939	0.942	0.916	0.975	0.973	1.010	1.047	1.005	0.932	0.967	1.060
repeat #2	0.886	0.796	0.784	1.232	0.909	0.739	1.024	1.246	1.175	0.991	0.990	0.982	0.873	0.803
repeat #3	0.992	0.936	0.612	1.248	0.998	0.948	0.936	0.985	1.024	1.066	1.026	0.927	0.976	1.085
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	456	757	225	2.07	352	107	233	219	120	2.25	751	244	300	16000
repeat #2	437	688	228	2.69	366	96.4	236	245	127	2.03	751	251	219	16500
repeat #3	476	722	127	2.77	355	97.1	232	251	130	2.40	756	247	358	14800
Normalised data luc/b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	487	819	249	2.20	374	117	239	225	119	2.15	747	261	310	15135
repeat #2	494	864	291	2.19	391	130	231	197	108	2.05	758	256	250	16784
repeat #3	480	772	207	2.22	356	102	248	255	127	2.25	737	266	367	13644
average luc activity	487	818	249	2.20	374	116	239	226	118	2.15	748	261	309	15188
SV40 as 100%	**3.21**	**5.39**	**1.64**	**0.0145**	**2.46**	**0.767**	**1.57**	**1.49**	**0.778**	**0.0141**	**4.92**	**1.72**	**2.04**	**100**
HeLa experiment #3														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.833	0.821	0.819	0.762	0.856	0.902	0.942	0.853	0.904	0.810	0.884	0.880	0.827	0.729
repeat #2	0.845	0.790	0.839	1.294	0.824	0.773	0.946	0.735	0.938	0.991	0.936	0.998	0.784	0.702
repeat #3	0.893	0.782	0.825	0.663	0.965	0.948	0.946	0.879	0.882	1.066	0.877	0.927	0.912	0.700
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	516	632	144	0.942	246	123	232	203	136	13.1	638	137	214	10100
repeat #2	515	588	148	1.69	256	116	233	172	137	15.3	651	151	219	9600
repeat #3	563	622	144	0.775	255	117	232	211	135	17.6	666	147	218	9800
Normalised data luc/b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	620	769	176	1.24	287	137	246	238	151	16.2	721	156	259	13834
repeat #2	609	744	176	1.31	310	151	246	234	147	15.4	695	152	279	13671
repeat #3	630	796	174	1.17	264	123	245	240	154	16.5	748	158	239	14005
average luc activity	620	770	175	1.24	287	137	246	237	150	16.1	722	155	259	13837
SV40 as 100%	**4.48**	**5.56**	**1.27**	**0.00894**	**2.08**	**0.989**	**1.77**	**1.71**	**1.09**	**0.116**	**5.22**	**1.12**	**1.87**	**100**
HeLa SUMMARY														
Average HeLa experiment #1-3	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
average luc activity	484	737	182	3.14	347	118	235	203	132	9.88	742	214	270	13000
average SV40 as 100%	**3.73**	**5.76**	**1.39**	**0.0281**	**2.80**	**0.92**	**1.86**	**1.56**	**1.06**	**0.0821**	**5.94**	**1.72**	**2.12**	**100**
standard error of mean	0.384	0.286	0.123	0.0164	0.542	0.0774	0.195	0.0754	0.153	0.0340	0.877	0.342	0.169	

Appendix II: Raw data results from SiHa cells

SiHa experiment #1															
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.083	0.080	0.078	0.080	0.077	0.087	0.094	0.064	0.095	0.142	0.074	0.196	0.140	0.097
repeat #2	0.075	0.075	0.082	0.077	0.091	0.082	0.085	0.062	0.093	0.194	0.069	0.206	0.168	0.109
repeat #3	0.090	0.069	0.064	0.076	0.062	0.091	0.076	0.064	0.083	0.179	0.082	0.224	0.154	0.099
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	12.0	22.1	10.5	0.0147	13.8	7.54	6.24	11.3	9.91	9.08	20.7	9.58	26.8	140
repeat #2	11.8	18.8	10.3	0.0177	11.5	7.15	5.33	11.5	9.96	12.31	20.4	9.93	31.6	156
repeat #3	11.9	20.8	9.11	0.0113	14.5	7.79	5.39	10.7	8.5	11.37	21.8	10.74	30.0	143
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	145	276	135	0.183	179	86.7	66.3	134	104	63.9	280	48.4	191	1436
repeat #2	158	250	125	0.229	127	87.2	62.7	140	107	63.5	295	48.2	188	1434
repeat #3	132	302	142	0.149	233	85.6	70.9	128	102	63.5	265	47.9	195	1440
average luc activity	145	276	134	0.187	180	86.5	66.6	134	105	63.6	280	48.2	191	1437
SV40 as 100%	**10.1**	**19.2**	**9.33**	**0.0130**	**12.5**	**6.02**	**4.64**	**9.3**	**7.27**	**4.43**	**19.5**	**3.35**	**13.3**	**100**
SiHa experiment #2														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.078	0.077	0.073	0.068	0.104	0.073	0.077	0.066	0.118	0.076	0.087	0.081	0.074	0.068
repeat #2	0.069	0.067	0.084	0.084	0.099	0.084	0.089	0.087	0.097	0.067	0.086	0.091	0.082	0.072
repeat #3	0.088	0.094	0.069	0.086	0.110	0.069	0.067	0.077	0.127	0.087	0.105	0.101	0.068	0.066
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	3.91	7.48	3.57	0.0473	5.43	3.44	3.55	7.23	4.35	1.76	10.9	4.21	10	48.0
repeat #2	3.15	7.34	3.43	0.0340	5.45	3.51	3.60	9.32	3.83	1.69	11.1	4.21	10	50.0
repeat #3	4.75	8.17	3.86	0.0647	5.40	3.63	3.48	8.62	4.28	1.89	12.6	5.62	10	47.2
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	50.1	97.2	48.9	0.696	52.2	47.1	46.1	110	36.9	23.1	125	52.0	131	706
repeat #2	45.7	110	40.9	0.405	55.1	41.8	40.4	107.1	39.5	25.2	129	46.3	116	695
repeat #3	53.9	86.9	56.0	0.752	49.1	52.6	52.0	111.9	33.7	21.8	119.7	57.6	148	715
average luc activity	49.9	97.9	48.6	0.618	52.1	47.1	46.2	109.5	36.7	23.3	124	51.9	132	705
SV40 as 100%	**7.08**	**13.9**	**6.89**	**0.0876**	**7.39**	**6.68**	**6.54**	**15.5**	**5.20**	**3.31**	**17.6**	**7.37**	**18.7**	**100**
SiHa experiment #3														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.062	0.051	0.065	0.058	0.061	0.052	0.055	0.053	0.062	0.066	0.063	0.052	0.056	0.053
repeat #2	0.068	0.058	0.061	0.049	0.073	0.048	0.068	0.055	0.077	0.075	0.072	0.048	0.061	0.061
repeat #3	0.056	0.044	0.069	0.057	0.069	0.067	0.051	0.049	0.063	0.067	0.069	0.059	0.072	0.059
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	15.4	20.2	20.3	0.0547	23.9	10.6	8.27	24.4	12.4	8.30	27.3	14.2	22.1	203
repeat #2	14.6	18.8	20.3	0.0527	32.5	11.7	8.68	24.8	13.5	11.23	35.3	13.6	29.7	252
repeat #3	15.6	20.6	20.2	0.0473	23.3	10.9	8.91	22.9	14.3	9.96	25.9	15.6	21.7	209
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	248	396	312	0.94	392	203	150	460	200	148.2	434	274	394	3825
repeat #2	215	324	333	1.07	446	244	128	450	175	149.7	490	263	487	4123
repeat #3	279	467	292	0.83	337	162	175	467	228	148.7	375	264	302	3537
average luc activity	247	396	312	0.95	392	203	151	459	201	148.9	433	274	394	3828
SV40 as 100%	**6.46**	**10.3**	**8.16**	**0.0248**	**10.23**	**5.30**	**3.94**	**12.0**	**5.25**	**3.89**	**11.3**	**7.15**	**10.30**	**100**
SiHa SUMMARY														
Average SiHa experiment #1-3	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
average luc activity	147	256	165	0.585	208	112	87.9	234	114	78.6	279	125	239	1990
average SV40 as 100%	**7.87**	**14.5**	**8.13**	**0.0418**	**10.0**	**6.00**	**5.04**	**12.3**	**5.91**	**3.88**	**16.2**	**5.96**	**14.1**	**100**
standard error of mean	1.12	2.57	0.71	0.0231	1.48	0.400	0.778	1.80	0.682	0.323	2.48	1.30	2.46	0.000

Appendix III: Raw data results from C33A cells

C33A experiment #1														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	1.160	1.156	1.168	1.188	1.144	1.158	1.070	1.037	1.060	1.132	1.091	1.051	1.134	1.101
repeat #2	1.331	1.145	1.259	1.298	1.247	1.184	1.201	1.058	1.171	1.198	1.081	1.021	1.012	1.009
repeat #3	1.298	1.089	1.189	1.173	1.185	1.164	0.998	1.012	1.012	1.099	1.102	1.126	1.058	1.249
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	161	319	196	23.8	283	231	245	264	473	54.1	685	232	1300	9530
repeat #2	190	316	210	25.1	246	238	255	263	479	53.6	701	235	1190	9470
repeat #3	176	300	199	24.2	355	234	245	264	473	56.5	669	237	1180	9840
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	136	276	168	20.0	247	199	229	255	438	47.8	628	220	1143	8660
repeat #2	142	276	167	19.3	197	201	212	248	409	44.8	648	230	1172	9380
repeat #3	135	276	167	20.6	300	198	246	261	467	51.4	607	210	1117	7880
average luc activity	139	276	167	20.0	248	199	229	255	438	48.0	628	220	1144	8640
SV40 as 100%	**1.61**	**3.19**	**1.94**	**0.231**	**2.87**	**2.31**	**2.65**	**2.95**	**5.07**	**0.556**	**7.27**	**2.55**	**13.2**	**100**
C33A experiment #2														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.847	0.930	0.943	0.898	0.867	1.011	1.050	1.034	1.130	1.080	1.125	1.124	1.101	0.974
repeat #2	0.824	1.002	0.950	0.950	0.845	1.024	1.012	1.030	1.079	1.143	1.024	1.127	1.040	0.980
repeat #3	0.869	0.870	0.926	0.836	0.892	0.998	1.110	1.043	1.025	1.021	1.068	1.054	1.105	0.991
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	293	323	191	18.5	212	146	210	264	473	226	655	232	799	8400
repeat #2	297	336	196	19.9	207	146	217	264	477	229	642	232	753	8520
repeat #3	288	314	186	17.6	216	145	203	264	469	223	648	232	804	8490
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	346	348	202	20.58	244	144	200	255	419	209	582	206	725	8620
repeat #2	361	334	206	20.91	244	143	214	256	442	201	627	206	724	8690
repeat #3	331	361	201	21.11	242	146	183	253	458	218	607	220	728	8560
average luc activity	346	348	203	20.87	244	144	199	255	440	209	605	211	726	8630
SV40 as 100%	**4.01**	**4.03**	**2.35**	**0.242**	**2.82**	**1.67**	**2.31**	**2.96**	**5.10**	**2.43**	**7.02**	**2.44**	**8.42**	**100**
C33A experiment #3														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.773	0.781	0.821	0.810	0.779	0.860	0.840	0.832	0.777	0.753	1.116	0.817	0.786	0.737
repeat #2	0.803	0.699	0.841	0.902	0.815	0.752	0.810	0.785	0.743	0.734	1.124	0.728	0.841	0.706
repeat #3	0.811	0.647	0.926	0.836	0.821	0.998	0.928	0.891	0.802	0.788	1.127	0.893	0.738	0.724
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	93.3	148	78.6	8.54	90.2	94.7	80.1	88.9	189	180	210	61.0	429	5530
repeat #2	95.4	138	82.8	8.36	93.2	95.1	83.9	90.9	186	188	213	60.7	425	5320
repeat #3	99.5	117	85.9	9.65	96.0	94.3	80.7	86.9	191	176	210	59.8	431	5410
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	121	189	95.8	10.5	116	110	95.3	107	243	240	188	74.6	546	7500
repeat #2	119	197	98.5	9.27	114	126	104	116	250	256	190	83.4	506	7530
repeat #3	123	181	92.7	11.5	117	94.5	86.9	97.6	238	223	186	67.0	584	7480
average luc activity	121	189	95.7	10.5	116	110	95.3	107	244	240	188	75.0	545	7500
SV40 as 100%	**1.61**	**2.52**	**1.27**	**0.139**	**1.54**	**1.47**	**1.27**	**1.42**	**3.25**	**3.19**	**2.50**	**1.00**	**7.26**	**100**
C33A SUMMARY														
Average C33A experiment #1-3	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
average luc activity	202	271	155	17.1	202	151	174	205	374	166	474	169	805	8260
average SV40 as 100%	**2.41**	**3.25**	**1.85**	**0.204**	**2.41**	**1.82**	**2.08**	**2.44**	**4.47**	**2.06**	**5.60**	**2.00**	**9.64**	**100**
standard error of mean	0.801	0.437	0.314	0.0326	0.435	0.252	0.415	0.510	0.612	0.783	1.55	0.499	1.83	

Appendix IV: Raw data results from H1299 cells

H1299 experiment #1														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.701	0.673	0.643	0.639	0.774	0.786	0.817	0.862	0.838	0.832	0.841	1.097	0.812	0.911
repeat #2	0.660	0.675	0.526	0.642	0.757	0.869	0.808	0.920	0.885	0.782	0.875	1.133	0.789	0.962
repeat #3	0.774	0.663	0.536	0.728	0.723	0.792	0.602	0.860	0.857	0.821	0.857	1.114	0.792	0.868
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	2.34	5.50	3.53	2.61	3.53	3.14	1.90	4.95	1.72	1.80	5.91	3.67	6.34	4280
repeat #2	2.26	5.50	3.41	2.65	3.43	3.29	1.88	5.10	1.90	1.84	5.97	3.92	6.36	4280
repeat #3	2.48	5.45	3.50	2.57	3.40	3.60	1.56	5.06	1.71	1.80	5.99	3.69	6.37	4300
Normalised data lucb-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	3.34	8.16	5.50	4.08	4.56	3.99	2.32	5.61	2.06	2.17	7.02	3.34	7.81	4696
repeat #2	3.42	8.15	6.48	4.12	4.53	3.79	2.32	5.54	2.14	2.35	6.83	3.46	8.06	4450
repeat #3	3.21	8.22	6.53	3.53	4.71	4.55	2.59	5.89	2.00	2.20	6.99	3.31	8.04	4951
average luc activity	3.32	8.18	6.17	3.91	4.60	4.11	2.41	5.68	2.06	2.24	6.95	3.37	7.97	4699
SV40 as 100%	**0.0707**	**0.174**	**0.131**	**0.0832**	**0.0979**	**0.0874**	**0.0513**	**0.121**	**0.0439**	**0.0476**	**0.148**	**0.0718**	**0.170**	**100**
H1299 experiment #2														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.890	0.845	0.831	0.799	0.753	0.800	0.802	0.785	0.867	0.887	0.820	0.806	0.854	0.798
repeat #2	0.860	0.875	0.836	0.842	0.857	0.819	0.798	0.856	1.080	0.982	0.875	0.733	0.889	0.821
repeat #3	0.974	0.863	0.993	0.689	0.703	0.872	0.693	0.735	0.874	0.855	0.807	0.814	0.852	0.792
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	3.32	5.33	4.05	2.72	3.32	4.02	2.89	4.78	2.55	1.25	7.00	5.49	7.26	4400
repeat #2	3.26	5.50	4.41	2.65	3.43	4.41	2.88	4.83	2.90	1.34	7.31	4.92	7.38	4480
repeat #3	3.48	5.45	4.50	2.57	3.40	3.95	2.56	4.83	2.71	1.24	6.99	5.69	7.37	4400
Normalised data lucb-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	3.73	6.31	4.87	3.40	4.40	5.02	3.60	6.09	2.94	1.41	8.54	6.81	8.51	5510
repeat #2	3.79	6.29	5.27	3.14	4.00	5.38	3.61	5.65	2.88	1.36	8.35	6.71	8.30	5468
repeat #3	3.57	6.32	4.53	3.73	4.84	4.53	3.70	6.57	3.10	1.45	8.67	6.99	8.65	5552
average luc activity	3.70	6.31	4.89	3.42	4.41	4.98	3.63	6.10	2.91	1.41	8.52	6.84	8.49	5507
SV40 as 100%	**0.0671**	**0.114**	**0.0888**	**0.0622**	**0.0802**	**0.0904**	**0.0660**	**0.111**	**0.0528**	**0.0256**	**0.155**	**0.124**	**0.154**	**100**
H1299 experiment #3														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.801	0.851	0.805	0.783	0.682	0.823	0.886	0.949	0.817	0.890	0.796	0.868	0.820	0.879
repeat #2	0.775	0.860	0.836	0.842	0.720	0.739	0.685	0.920	0.955	0.748	0.840	0.933	0.819	0.886
repeat #3	0.850	0.863	0.934	0.768	0.645	0.802	0.802	0.948	0.857	1.021	0.867	0.864	0.792	0.898
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	6.30	10.4	5.06	4.52	6.43	3.62	3.70	4.16	2.58	3.34	7.53	4.67	5.42	7062
repeat #2	6.26	10.5	5.41	4.65	6.43	3.29	2.88	4.10	2.90	2.84	7.97	4.92	5.34	7281
repeat #3	6.48	10.5	5.50	4.57	6.40	3.60	3.56	4.06	2.71	3.80	7.99	4.69	5.37	6997
Normalised data lucb-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	7.87	12.2	6.28	5.78	9.42	4.40	4.17	4.38	3.15	3.75	9.46	5.38	6.61	8034
repeat #2	8.08	12.4	6.47	5.52	8.93	4.45	4.20	4.46	3.03	3.80	9.49	5.27	6.52	8218
repeat #3	7.62	12.1	5.89	5.95	9.93	4.49	4.44	4.28	3.16	3.73	9.22	5.43	6.78	7792
average luc activity	7.86	12.2	6.21	5.75	9.43	4.45	4.27	4.37	3.12	3.76	9.39	5.36	6.64	8015
SV40 as 100%	**0.0980**	**0.153**	**0.0775**	**0.0717**	**0.118**	**0.0555**	**0.0533**	**0.0546**	**0.0389**	**0.0469**	**0.117**	**0.0669**	**0.0828**	**100**
H1299 SUMMARY														
Average H1299 experiment #1-3	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
average luc activity	4.96	8.91	5.76	4.36	6.15	4.51	3.44	5.39	2.7	2.47	8.29	5.19	7.7	6070
average SV40 as 100%	**0.0786**	**0.147**	**0.0992**	**0.0724**	**0.0985**	**0.0778**	**0.0569**	**0.0954**	**0.0452**	**0.0400**	**0.140**	**0.0876**	**0.136**	**100**
standard error of mean	0.00976	0.0174	0.0163	0.00608	0.0108	0.0112	0.00459	0.0206	0.0041	0.0072	0.0115	0.0183	0.0267	

Appendix V: Raw data results from MRC-5 cells

MRC-5 experiment #1														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.057	0.061	0.055	0.057	0.062	0.059	0.063	0.055	0.104	0.087	0.059	0.065	0.054	0.063
repeat #2	0.047	0.058	0.074	0.062	0.063	0.057	0.071	0.049	0.110	0.091	0.062	0.065	0.030	0.061
repeat #3	0.068	0.062	0.043	0.059	0.055	0.058	0.059	0.062	0.103	0.079	0.048	0.065	0.077	0.060
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.0103	0.0267	0.0253	0.0290	0.00967	0.00833	0.0123	0.0590	0.00067	0.0210	0.0480	0	0.0170	30.7
repeat #2	0.0132	0.0350	0.0233	0.0407	0.0117	0.00733	0.0137	0.0210	0.00433	0.0303	0.0150	0.00900	0.00967	23.5
repeat #3	0.00767	0.0183	0.0300	0.0170	0.00733	0.00867	0.0120	0.0973	0.00700	0.0117	0.0817	0.0100	0.0247	37.7
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.181	0.437	0.461	0.509	0.156	0.141	0.196	1.07	0.0064	0.241	0.814	0	0.315	488
repeat #2	0.282	0.603	0.315	0.656	0.185	0.129	0.192	0.429	0.0394	0.333	0.242	0.138	0.322	386
repeat #3	0.113	0.296	0.698	0.288	0.133	0.149	0.203	1.570	0.0680	0.148	1.70	0.154	0.320	628
average luc activity	0.192	0.445	0.491	0.484	0.158	0.140	0.197	1.02	0.0379	0.241	0.919	0.097	0.319	500
SV40 as 100%	**0.0383**	**0.0890**	**0.0982**	**0.0968**	**0.0316**	**0.0279**	**0.0394**	**0.205**	**0.00758**	**0.0481**	**0.184**	**0.0195**	**0.0638**	**100**
MRC-5 experiment #2														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.072	0.066	0.066	0.067	0.069	0.070	0.064	0.066	0.068	0.093	0.062	0.068	0.063	0.060
repeat #2	0.062	0.055	0.073	0.072	0.086	0.068	0.089	0.069	0.067	0.091	0.069	0.073	0.071	0.072
repeat #3	0.098	0.069	0.053	0.059	0.077	0.070	0.072	0.085	0.063	0.092	0.083	0.092	0.082	0.059
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.0333	0.0440	0.0233	0.0707	0.0217	0.0130	0.0463	0.0773	0.0163	0.0927	0.0110	0.0380	0.0390	23.5
repeat #2	0.0297	0.0510	0.0297	0.0647	0.0220	0.0130	0.0647	0.0973	0.0170	0.130	0.0140	0.0287	0.0597	21.2
repeat #3	0.0303	0.0373	0.0253	0.0747	0.0227	0.0137	0.0123	0.0590	0.0147	0.0563	0.0063	0.0500	0.0170	25.9
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.463	0.667	0.364	1.05	0.314	0.186	0.724	1.17	0.240	1.00	0.177	0.559	0.619	392
repeat #2	0.478	0.927	0.406	0.898	0.256	0.192	0.727	1.41	0.254	1.42	0.203	0.393	0.840	294
repeat #3	0.310	0.541	0.478	1.27	0.294	0.195	0.171	0.694	0.233	0.612	0.0643	0.543	0.207	439
average luc activity	0.417	0.712	0.413	1.07	0.288	0.191	0.541	1.092	0.242	1.01	0.148	0.498	0.556	375
SV40 as 100%	**0.111**	**0.190**	**0.110**	**0.286**	**0.0768**	**0.0509**	**0.144**	**0.291**	**0.0646**	**0.270**	**0.0395**	**0.133**	**0.148**	**100**
MRC-5 experiment #3														
b-galactosidase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.067	0.064	0.068	0.069	0.068	0.075	0.071	0.069	0.069	0.065	0.068	0.072	0.071	0.066
repeat #2	0.059	0.069	0.073	0.093	0.064	0.072	0.064	0.065	0.054	0.068	0.076	0.063	0.068	0.079
repeat #3	0.061	0.059	0.059	0.074	0.064	0.068	0.075	0.076	0.076	0.072	0.065	0.087	0.079	0.069
luciferase assay	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.0143	0.0183	0.0533	0.0403	0.0220	0.0520	0.0647	0.131	0.0633	0.0917	0.114	0.0253	0.0693	28.8
repeat #2	0.0103	0.0267	0.0483	0.0290	0.0197	0.0497	0.0277	0.124	0.0783	0.0913	0.0717	0	0.0533	30.7
repeat #3	0.0187	0.00933	0.0483	0.0707	0.0217	0.0567	0.103	0.137	0.0910	0.0927	0.161	0.0380	0.0647	26.9
Normalised data luc:b-gal	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
repeat #1	0.214	0.286	0.784	0.585	0.324	0.693	0.911	1.89	1.21	1.41	1.68	0.352	0.977	437
repeat #2	0.175	0.386	0.662	0.312	0.234	0.690	0.329	1.90	1.45	1.34	0.943	0	0.784	389
repeat #3	0.306	0.158	0.819	0.955	0.339	0.833	1.37	1.80	1.20	1.29	2.48	0.437	1.07	390
average luc activity	0.232	0.277	0.755	0.617	0.299	0.739	0.870	1.86	1.29	1.35	1.70	0.263	0.944	405
SV40 as 100%	**0.0572**	**0.0684**	**0.186**	**0.152**	**0.0737**	**0.182**	**0.215**	**0.460**	**0.317**	**0.332**	**0.420**	**0.065**	**0.233**	**100**
MRC-5 SUMMARY														
Average MRC-5 experiment #1-3	pGL3-LCR1000	pGL3-LCR800	pGL3-LCR400	pGL3-LCR200	E2BS#4	E2BS#3	E2BS#2	E2BS#1	AP1/YY1	KRF	GRE/YY1	Sp1	DM	pGL3-SV40
average luc activity	0.280	0.478	0.553	0.725	0.248	0.356	0.536	1.33	0.522	0.866	0.923	0.286	0.606	426.8183284
average SV40 as 100%	**0.0689**	**0.116**	**0.132**	**0.178**	**0.0607**	**0.0871**	**0.133**	**0.319**	**0.130**	**0.217**	**0.214**	**0.0724**	**0.148**	**100**
standard error of mean	0.0218	0.0375	0.0276	0.0562	0.0146	0.0481	0.0509	0.0751	0.0951	0.0862	0.111	0.0330	0.0488	