

Research article

**THE USE OF A HUMAN PAPILLOMAVIRUS 18 PROMOTER FOR
 TISSUE-SPECIFIC EXPRESSION IN CERVICAL CARCINOMA CELLS**

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Abstract: The use of tissue-specific promoter elements in the treatment of cervical cancer has been explored in this paper. The P₁₀₅ promoter of human papillomavirus 18 (HPV18) was utilised to direct tissue-specific expression in a number of cell types. Expression was examined in three cervical carcinoma cell lines: HeLa (HPV18 positive), SiHa (HPV16 positive), and C33A cells (HPV negative); the epithelial cell line, H1299; and the foetal fibroblast cell line, MRC5, utilising a luciferase expression vector. Expression was highest in the cervical cell lines by a factor of at least 80. The effect of a number of mutations in the P₁₀₅ promoter on expression levels was examined. Three deletion constructs of the long control region (LCR) were investigated: an 800 bp fragment (LCR800), a 400 bp fragment (LCR400), and a 200 bp fragment (LCR200), as well as the full length product LCR of HPV18 (LCR1000). The LCR800 construct of the HPV18 P₁₀₅ promoter had the highest level of expression in the cervical cell lines and was also highest in the HPV18-positive HeLa cell line. Site-directed mutagenesis was then employed on the LCR800 construct to create four further constructs that each had inactivating mutations in one of the four E2 binding sites (E2BSs). Overall, this study indicated that the LCR800 construct of the HPV18 P₁₀₅ promoter could be utilised as a tissue-restricted promoter in cervical cancer cells.

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Abbreviations used: E2BS – E2-binding site; LCR – long control region; ORF – opening reading frame

Key words: Cervical cancer; Long control region, HPV18, P₁₀₅, E2-binding site, Gene therapy

INTRODUCTION

Cervical cancer and human papillomavirus

Cervical cancer is the second leading cause of female cancer mortality worldwide [1], with about 288,000 deaths and 510,000 new cases reported each year [2]. Cervical cancer is treated with chemotherapy or radiotherapy. Studies have shown that cisplatin-based chemotherapy given concurrently with radiation therapy provides improved treatment for cervical cancer [3-5]. Unfortunately improvements in therapeutic treatments have not significantly decreased the mortality rate of cervical cancer [6], and patients with advanced, recurrent or metastatic diseases still have a poor prognosis [7]. Moreover, these methods of treatment have side effects such as hair loss, nausea, vomiting, and depression of the immune system. Thus there is a major requirement for a cure for cervical cancer and gene therapy could be an important approach in achieving that aim [8]. Infections with high-risk human papillomaviruses (HPVs), such as HPV18, have been associated etiologically with cervical cancer and the DNA of HPV is found in virtually all cervical cancers (> 99.7%) [2, 9-11]. The successful development of HPV vaccines may be beneficial to the HPV-naive population, but women who have already been exposed to the virus are still at risk of developing HPV-associated malignancies. A need for a systemic cure for HPV-related cancers therefore still exists.

Upon infection, a segment of the HPV genome is integrated into the DNA of the host cell and the long control region (LCR) is always preserved [12, 13]. The LCR of HPV18 contains the enhancer and promoter elements for the expression of two main oncogenes E6 and E7 [14-19]. The start of transcription and the ATG initiation codon of the E6 open reading frame (ORF) are located at nucleotide 105 and therefore this promoter element of HPV18 is also known as P₁₀₅ (Fig. 1). The transcriptional activity of P₁₀₅ is tightly regulated by a complex interplay between the viral E2 protein and cellular transcription factors binding to recognition sequences along the LCR.

Gene therapy using tissue-specific promoters has been reported to be a promising tool for treating cancers [8]. Few studies have explored this possibility for the treatment of cervical cancer; however, a SCCA2 promoter [20] and an HPV16 promoter [21] have been utilised to direct tissue-restricted gene expression in cervical carcinoma cells.

The malignant phenotype of HPV-positive cervical cancers is dependent on the continuous expression of E6 and E7 via the P₁₀₅ promoter [22]. Therefore it lead us to hypothesize that the P₁₀₅ promoter of HPV18 could be used to direct cervical carcinoma-specific expression of therapeutic genes, since cervical cancer cells clearly possess transcription factors essential for the activation of the P₁₀₅ promoter. Hence in this project we explored the proposal that the

HPV18 P₁₀₅ promoter could be utilised to direct tissue specific expression in cervical cancer cells.

LCR deletion constructs

The LCR of HPV18 is an 825 bp non-coding region which lies between the L1 and E6 ORF, from nucleotide 7,137 to 104, with the nucleotide number corresponding to the published sequence by Cole and Danos [23]. It is contained within a 1,050 bp *Bam*HI fragment which comprises three functional domains separated by *Rsa*I recognition sites – the distal enhancer, constitutive enhancer and promoter proximal region (Fig. 1) [24-26].

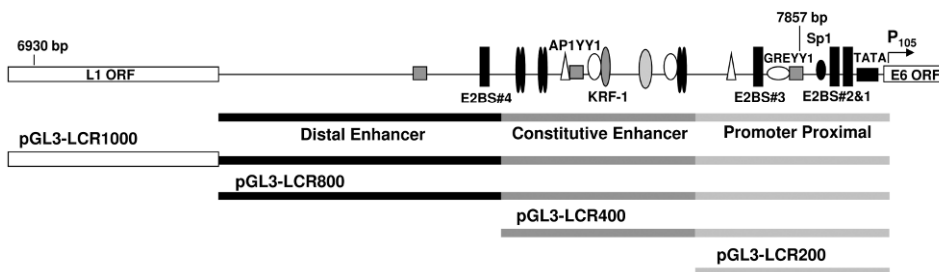


Fig. 1. LCR deletion constructs. The pGL3-LCR1000 plasmid contained the 1 kb *Bam*HI LCR fragment cloned into the pGL3-Basic vector; removal of the distal 200 bp resulted in the pGL3-LCR800 plasmid; further removal of the distal enhancer region resulted in the pGL3-LCR400 plasmid and removal of the constitutive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

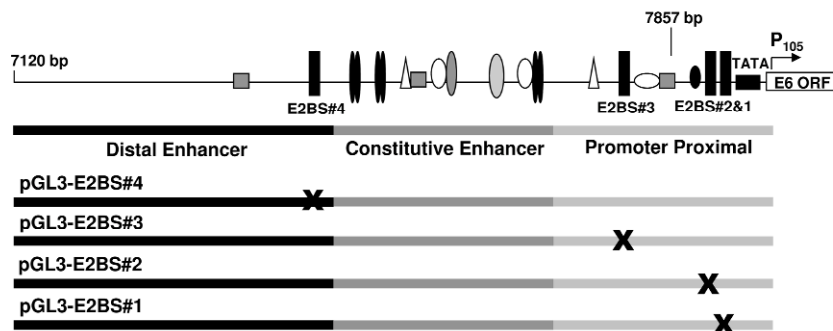


Fig. 2. Schematic diagram of the pGL3-LCR800 plasmids containing the four individually mutated E2BSs. E2BS mutation constructs were produced by making use of the pGL3-LCR800 plasmid as a template. The pGL3-E2BS#4 plasmid contained a mutation at the E2BS#4 within the distal enhancer region; the pGL3-E2BS#3, 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. The black crosses indicate the positions of the mutation within the LCR fragments.

In order to determine the location of important transcription control elements in the LCR promoter, a series of LCR deletion fragments were cloned into a promoter-less luciferase vector (Fig. 1). These deletion vector constructs were transiently transfected into five cell lines: 1) the HPV18-positive HeLa human cervical carcinoma cell line, 2) the HPV16-positive SiHa human cervical carcinoma cell line, 3) the HPV-negative C33A human cervical carcinoma cell line, 4) the non-cervical H1299 epithelial carcinoma cell line, and 5) the non-transformed MRC5 fibroblast cell line. The effect of these deletions on P₁₀₅ promoter activity was then assessed in the five human cell lines.

E2-binding sites

E2 protein has been suggested to be responsible for the transcriptional regulation of the E6 and E7 oncogenes, by binding in the LCR [1, 27-30]. There are four E2-binding sites (E2BS) along the LCR of HPV18 (Fig. 2). The E2BS is a 12-bp palindromic ACCN₆GGT motif, where N₆ represents the six base pairs that differ between the four sites [31-34]. The E2 protein consists of a C-terminal DNA-binding domain linked to an N-terminal transactivation domain by a non-conserved hinge region [35]. 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. Previous studies performed by McBride *et al.* [36] with *in vitro* binding assays suggest that synthetic polypeptides corresponding to the full-length E2 protein binds to the original E2 motif ACCN₆GGT but not to a mutated motif of ATTN₆CCT. In this study, we systematically inactivated each of the four E2BSs by site-directed mutagenesis and investigated their effect on tissue-specific gene expression.

MATERIALS AND METHODS

Plasmids

All plasmid constructs were generated from the promoterless luciferase plasmid pGL3-Basic (Promega, USA), by cloning different promoter fragments into the *Xho*I / *Hind*III restriction sites. A plasmid containing the complete genome of HPV18 was a kind gift from Dr. Harald zur Hausen at the German Cancer Research Centre, Heidelberg, Germany. A β -galactosidase expression vector driven by a cytomegalovirus promoter, pCMV β (Clontech, USA), was used as a transfection control plasmid in all co-transfection experiments.

LCR deletion constructs

The full-length LCR fragment (1,062 bp) was amplified from the HPV18 plasmid using the primers (F)5'-CGAGCTCAACTCGAGCTATGATAAGTT-3' and (R)5'-GTGGCGAAAGCTTGCGCCATAGTATTGTG-3', to produce the pGL3-LCR1000 plasmid (LCR1000) (Fig. 1). All the subsequent deletion fragments used the same reverse primer as above. The primer (F)5'-GTGCGTCTCGAGGCCAGGAAGTAATAT-3' was used to produce the LCR fragment (869 bp) for the pGL3-LCR800 plasmid (LCR800). The primer

(F)5'-GCACAATACACTCGAGTGGCACTAT-3' was used to produce the LCR fragment (484 bp) for the pGL3-LCR400 plasmids (LCR400). The primer (F)5'-GGCTTCTCGAGCTACTTTTCATGT-3' was used to produce the LCR fragment (249 bp) for the pGL3-LCR200 plasmid (LCR200). Underlined bases are restriction enzyme recognition sequences introduced to the primers to assist the cloning of LCR fragments at the *Xho*I and *Hind*III sites.

Site-directed mutagenesis of E2BS

All site-directed mutagenesis were performed using the pGL3-LCR800 plasmid as a template for amplification by polymerase chain reaction. The primers (F)5'-TTGCTG TGCAATTGATTTCCCTTGCCTTTGG-3' and (R)5'-GCCAAAGGCAAGGGAAATCA ATTGCACAGCA-3' were designed to introduce mutations to the E2BS#4 to produce the pGL3-E2BS#4 (E2BS#4) plasmid (Fig. 2). The primers (F)5'-GTTTATGCAATTGAAATACCTTGGGCAGCAC-3' and (R)5'-GTGCTGCCCAAGGTATTTCAATTGCATAAAC-3' were designed to introduce mutations to the E2BS#3 to produce the pGL3-E2BS#3 plasmid (E2BS#3). The primers (F)5'-AAGGGAGTAATTGAAAACCCCTCGGGACCGAA-3' and (R)5'-TTCGGTCCCGAGGGTTTTCAATTACTCCCTT-3' were designed to introduce mutations to the E2BS#2 to produce the pGL3-E2BS#2 plasmid (E2BS#2). The primers (F)5'-CCGAAAACGGTTCGGGATTGAAAACCCCTGTATATAAAAAGATGT-3' and (R)5'-CACA TCTTTTATATACAGGGTTTTCAATCCCGACCGTTTTTCGG-3' were designed to introduce mutations to the E2BS#1 to produce the pGL3-E2BS#1 plasmid (E2BS#1). Underlined bases represent the introduced mutations as previously described. The sequences of all the plasmid constructs were confirmed by automated sequencing to ensure the designated mutations were successfully introduced.

Polymerase chain reaction

A typical PCR reaction of 20 µl contained 50 ng of plasmid DNA template, 20 pmol of each primer, 0.3 mM dNTPs, 16.6 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl₂, 10 mM dithiothreitol (DTT) and 1.74 units of *Taq* DNA polymerase (Perkin Elmer, USA). Cycling parameters are initial denaturation at 95°C for 4 min, followed by 25 cycles of 95°C denaturing step for 30 s, 55°C to 60°C annealing step for 1 min and 72°C extension step for 90 s, with an extra extension step at 72°C for 10 min.

Human cell culture

All cell cultures were maintained in Dulbecco's Modified Eagle Media (DMEM) with 10% (v/v) foetal bovine serum (FBS) at 37°C with humidified air containing 5% (v/v) CO₂. Human cell lines used were HeLa (HPV18-positive cervical carcinoma), SiHa (HPV16-positive cervical carcinoma), C33A (HPV-negative cervical carcinoma), H1299 (non-small cell lung carcinoma) and MRC-5 (foetal lung fibroblast).

Plasmid transfection into human cell lines

Recombinant plasmids containing the LCR mutation insert, together with the transfection control plasmid pCMV β , were delivered into different human cell lines using Lipofectamine (Invitrogen, USA) following the manufacturer's instructions. All experiments were performed in 24-well tissue culture plates, and the cells were incubated for 48 hours post-transfection before being harvested and assayed for transient gene expression. All transfection experiments were repeated at least 3 times and each individual experiment was performed in triplicate.

Luciferase and β -galactosidase assays

All luciferase assays were performed using the Luciferase Assay System (Promega, USA) following the manufacturer's instructions. The single-tube luminometer (Turner Biosystems) was programmed to perform a 2 s delay followed by a 10 s measurement for luciferase activity. Samples were assayed by adding 20 μ l of cell lysate to 100 μ l of luciferase assay reagent. The tube was vortexed briefly before being placed into the luminometer for measurement. Each sample was measured three times for an averaged reading.

The β -galactosidase assay reagents used were compatible with the cell lysates prepared from the Luciferase Assay System. Prior to the measurement of β -galactosidase activity, the cell lysates and ortho-nitrophenyl- β -galactosidase (ONPG) solution (3 mM ONPG, 50 mM Tris-HCl (pH 7.0), 50 mM KCl) were equilibrated at 37°C. 50 μ l of ONPG solution was added to aliquots of 50 μ l cell lysates in a 96-well microtitre plate and mixed well by pipetting. The plate was incubated at 37°C for 3 h during which the substrate β -galactosidase hydrolyses the colourless ONPG to yellow *o*-nitrophenol. The absorbances of the samples were then measured at 405 nm in a microplate spectrophotometer (SpectraMax).

Statistics

The statistical analysis was performed using the GraphPad Prism 5 software version 5.03 (USA).

RESULTS

Strongest promoter activity in HPV18-positive cells

The plasmid promoter constructs LCR1000, LCR800, LCR400 and LCR200 were transfected into the human cell lines: HeLa, SiHa, C33A, H1299 and MRC5. The luciferase and β -galactosidase activities were determined for each of these promoter construct/human cell line combinations. In each individual experiment, the measured luciferase activities were normalised to the β -galactosidase readings to allow for variations in transfection efficiencies.

Our results with the full length P₁₀₅ promoter of HPV18 (LCR1000), showed strong luciferase activity in the HeLa, SiHa and C33A cervical carcinoma cell lines (Fig. 3). The HeLa, SiHa and C33A cervical cell lines had at least 80-fold

more activity than the non-cervical H1299 (epithelial) and the MRC5 (fibroblast) cell lines.

With the HPV18-LCR1000 construct, the level of luciferase activity was statistically significantly higher (One-Way ANOVA with Bonferroni's Multiple Comparison Test, $p < 0.05$) in the HPV18-positive HeLa cells, with 2.4-3.3 fold more activity than that observed in the HPV16-positive SiHa and the HPV-negative C33A cells.

The differences between activities measured with the SiHa and C33A cells were not significant (One-Way ANOVA with Bonferroni's Multiple Comparison Test, $p > 0.05$). Negligible activity was detected from the non-cervical cancer cell lines H1299 and MRC-5 (Fig. 3).

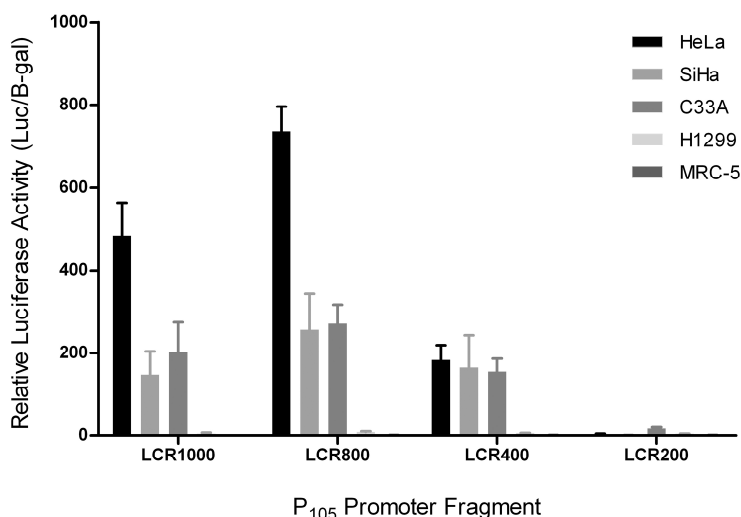


Fig. 3. Promoter activity of LCR deletion constructs in different mammalian cell lines. The graph represents results obtained from transformation experiments using five different cell lines. Luciferase activities relative to the β -galactosidase activity from the pCMV β plasmid were plotted for the deletion constructs. The standard error of the three repeated experiments in each cell line is represented by error bars.

Major differences in promoter activity in deletion constructs

There were major differences in relative luciferase activity detected with the LCR1000, LCR800, LCR400 and LCR200 constructs within the five cell lines (Fig. 3). Comparing the LCR1000 construct with the LCR800 construct, a large increase in luciferase activity occurred in HeLa cells, 250 relative luciferase activity units, compared with lesser amounts in SiHa and C33A cells. The relative level of HeLa cell luciferase activity compared with the non-HPV18 SiHa and C33A cell lines, was also maintained at 2.7-2.9 fold higher in the LCR800 construct.

Comparing the LCR800 construct with the LCR400 construct, a large decrease in luciferase activity occurred in HeLa cells, 500 relative luciferase activity units, compared with lesser amounts in SiHa and C33A cells. The relative level

of HeLa cell luciferase activity compared with the two HPV18-negative cell lines was also reduced to near equality at 1.1-1.2 fold in the LCR400 construct.

In contrasting the LCR400 construct with the LCR200 construct, a decrease in luciferase activity occurred in HeLa, SiHa and C33A cells, by more than 140 luciferase units. With this LCR200 construct the luciferase activities were reduced to almost background levels.

When analysed by One-Way ANOVA with the Bonferroni's Multiple Comparison Test, our results showed that the variation in luciferase activity measured with the deletion constructs were the most significant in HeLa cells. A 1.5-fold statistically significant increase in activity was detected with the LCR800 construct in HeLa cells upon the removal of the proximal 200 bp of L1 ORF at the distal end of the LCR fragment. In contrast, upon the removal of the distal enhancer region from the LCR to give the LCR400 construct, a 4.0-fold statistically significant decrease in activity was detected in HeLa cells. The differences in activity with the LCR400 plasmid are insignificant between HeLa, SiHa and C33A cells ($p > 0.05$) when results were analysed by One-Way ANOVA. The variations in luciferase activity measured from the deletion constructs was also insignificant in SiHa, H1299 and MRC-5 cells ($p > 0.05$).

E2BS mutations resulted in decreased promoter activity

The four E2BSs were individually mutated in the LCR800 construct to give four new constructs: E2BS#4, E2BS#3, E2BS#2 and E2BS#1 (Fig. 2). All of these constructs led to decreased expression in the HeLa, SiHa and C33A cell lines (Fig. 4).

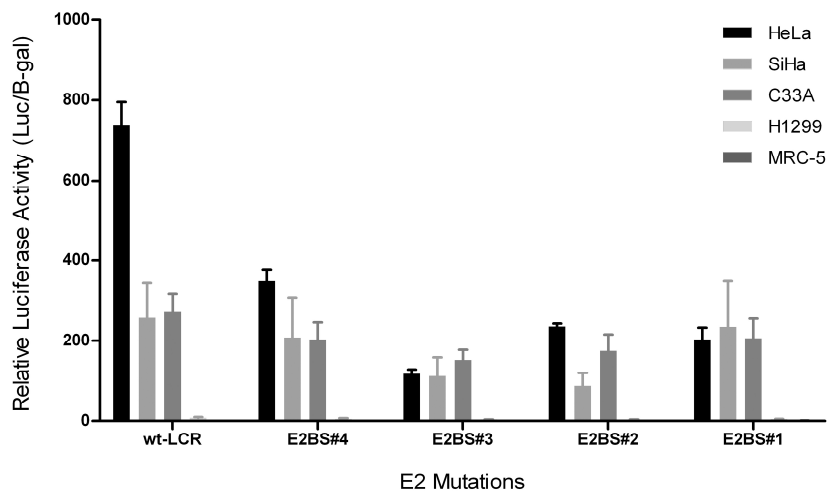


Fig. 4. Promoter activity of E2BS mutation constructs in different mammalian cell lines. The graph represents results obtained from transformation experiments using five different cell lines. Luciferase activities relative to the β -galactosidase activity from the pCMV β plasmid were plotted for the four E2BS mutation constructs. The standard error of the three repeated experiments in each cell line is represented by error bars.

In the cervical cell lines, the luciferase expression levels were reduced to the greatest extent with the E2BS#3 construct, followed by the E2BS#2 construct, E2BS#1 construct, while the E2BS#4 construct was the least affected. The HeLa cell line was affected to the greatest extent while the C33A cells were the least affected by the E2BS mutations.

The ratio of expression of the HPV18-positive HeLa cell line to the HPV18-negative cell lines was reduced from 2.7-2.9 fold to approximate equality in the E2BS#1 and E2BS#3 mutation constructs. With the E2BS#4 construct, the ratio was reduced to 1.7.

Results were analysed by One-Way ANOVA with the Dunnett's Multiple Comparison Test. A significant decrease ($p < 0.05$) in luciferase activity was observed in HeLa cells upon the mutation of the individual E2BSs. In contrast, the variation in luciferase activity measured from SiHa and C33A cells were insignificant ($p > 0.05$).

DISCUSSION

Cell-type specificity of P₁₀₅ promoter

One of the most important aspects of successful gene therapy is the ability to induce selective activation within the target cells. In our results the HPV18 P₁₀₅ promoter (LCR1000 and LCR800) has clearly shown significant selectivity towards the three cervical carcinoma cell lines tested (HeLa, SiHa and C33A). This observation is agreement with previous reports of the HPV18 LCR being active within certain carcinoma cell lines [14, 25, 37, 38]. Our results also showed that the strongest promoter activity was in HeLa cells. There was 2.4-3.3 fold more activity in the HPV18-positive HeLa cells compared with the HPV18-negative SiHa and C33A cell lines. This indicates the possible involvement of HPV18 viral protein(s) in P₁₀₅ promoter activation in the HeLa HPV18-positive cervical carcinoma cell line. With the non-cervical H1299 and MRC-5 cell lines, there was an over 80-fold reduction in activity compared with the cervical cell lines, HeLa, SiHa and C33A. This indicates that the HPV18 P₁₀₅ promoter could be utilised as a tissue-restricted promoter in cervical cells. This is the first occasion that a comprehensive study of HPV18 P₁₀₅ promoter activity has been evaluated in a variety of cell lines.

Significance of distal enhancer, constitutive enhancer and proximal promoter regions of LCR

Results from the LCR deletion constructs showed that upon the removal of the distal 200 bp *Bam*HI-*Rsa*I fragment to give the LCR800 construct, luciferase activity increased by 250 relative luciferase activity units in HeLa cells. There may therefore be transcription control elements within the distal enhancer region that are repressive of P₁₀₅ promoter activity and which have not been previously identified. This effect was not as pronounced in SiHa and C33A cells and hence

could be HPV18-specific. The LCR800 construct would appear to be a strong candidate for use as a tissue-specific promoter in cervical cells.

Contrasting the LCR1000 and LCR800 constructs with the LCR400 construct, a dramatic change occurred in the relative luciferase activities observed in HeLa cells compared with SiHa and C33A cells. In the LCR1000 and LCR800 constructs, the HPV-positive HeLa relative luciferase activities were 2.3-3.3 fold higher than the HPV18-negative cell lines, while in the LCR400 constructs the ratios were 1.1 to 1.2. This implies that there are HPV18-specific promoter elements in this 600bp distal enhancer region.

Most of the identified transcription factor binding sites are located within the constitutive enhancer and promoter proximal region of the HPV18 LCR, and the precise contribution of the distal enhancer region towards the P₁₀₅ promoter activity is unclear. Our results obtained from the LCR deletion constructs showed a significant drop in promoter activity upon the removal of the distal enhancer region in HeLa cells, which results in a reduction of HeLa cell luciferase activity to the same level as found in SiHa and C33A cells. This observation suggests the distal enhancer region contains HPV18-subtype specific transcription control elements.

Possible role of E2BS

Apart from cell-type specificity, another important aspect of successful gene therapy is the strength of the transcriptional activity induced from the promoter of choice. The viral E2 protein is generally accepted to repress transcriptional activity from the HPV18 P₁₀₅ promoter [1, 39, 40]. However, the viral E2 protein is generally disrupted upon HPV genome integration and the E2 protein occurs in a truncated form where it may act as a transcriptional activator [41, 42]. In our study, the E2BS mutation constructs in HeLa cells resulted in a decreased level of relative luciferase activity compared with the wild-type LCR800 P₁₀₅ promoter. This could indicate that a truncated viral E2 protein is acting as a transcriptional activator. Another explanation is that the viral E2 protein is absent from HeLa cells (due to disruption on HPV integration) and host cell transcription factors are responsible for transcriptional activation.

E2BS#2 and #1 form a tandem repeat located just 3 bp upstream of the TATA box of the P₁₀₅ promoter, and this repeat has been found to cause E2-mediated repression through steric hindrance with the proteins binding to the TATA box and the Sp1 binding site [33, 43]. 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 P₁₀₅ promoter [29, 44, 45]. However, in HeLa cells which lack E2 (due to integration disruption), there is no E2-mediated repression and host cell factors are able to stimulate transcription. On mutating the E2BSs, the expression levels would decrease under this scenario. However, this scenario does not fully explain the larger decrease in

HeLa cells where HPV18-specific factors must be invoked to fully explain the data. The HPV18 E6 and E7 proteins lead to the increased expression of a large number of cellular proteins that could interact with and activate the HPV18 P₁₀₅ promoter via the E2BSs [46-49].

The expression ratio of the HPV18-positive HeLa cells to the HPV18-negative SiHa and C33A cells was reduced from 2.7-fold to approximate equality in the E2BS#1, #2, and #3 mutation constructs. This indicates that these E2 binding sites involve an HPV18-specific component that is required for an HPV18-specific response. As mentioned above, this could be a truncated E2 protein that acts as a transcriptional activator at E2BSs. The HPV18 and HPV16 E2BSs have slightly different sequences that could also be mediating these differences [48, 50-54]. The EBS#4 mutation had a smaller reduction in the ratio. The EBS#4 is found in the distal enhancer region, rather than the promoter proximal region where the other three E2 binding sites are located. Overall, these results indicate that mutations in the four E2BSs would not be advantageous in the cervical cancer context.

Conclusion

The ultimate aim of the project is to develop a tool for gene therapy in the treatment of cervical cancer. This could be accomplished by substituting the luciferase gene in the LCR constructs with a gene that causes cell death on expression. Examples of cell death genes are endonucleases 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 study [55] has demonstrated the efficacy of using a saporin gene in cancer gene therapy via a non-viral gene delivery approach.

The HPV18-LCR800 luciferase promoter construct had approximately 17-fold lower activity than an SV40 (early promoter) pGL3 luciferase vector (average of three experiments) (data not shown) and this is in line with other studies [56]. However, this lower promoter activity should still be sufficiently strong to drive a suicide gene, such as the saporin cytotoxin gene, and result in cell death. The HPV promoter has been shown to have sufficient activity to be used in gene therapy studies [21, 56]. Extra E2BSs could be added to the promoter to increase activity [21]. Furthermore, if the HPV promoter is not sufficiently strong, a two-step transcriptional activation system can be utilized to substantially increase promoter activity [57].

In conclusion, the HPV18 LCR800 P₁₀₅ promoter could be an excellent starting point to begin the next stage of this gene therapy project. The use of site-directed mutagenesis in transcription factor binding sites may lead to further increases in tissue-restricted P₁₀₅ expression. Site-directed mutagenesis of transcription factor binding sites could also permit the residual activity of the promoter to be removed in non-HPV-containing cells. By careful combination of mutations in promoter transcription factor binding sites that are responsible for cell type-

specific gene expression in HPV-containing cells, a strong and HPV-specific promoter could be devised.

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