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Development and validation of a novel reporter assay for human papillomavirus type 16 late gene expression

Beatrice Orrù, Ciaran Cunniffe, Fergus Ryan*, Stefan Schwartz[1]

Dublin Institute of Technology, Kevin Street, Dublin 8, Ireland

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To facilitate the investigations of HPV-16 late gene expression HPV-16 reporter plasmids were generated using previously described sub-genomic HPV-16 plasmids, named pBEL and pBELM, that, similar to the full viral genome, produce primarily HPV-16 early mRNAs and very little, if any, late mRNAs in cervical cancer cells. The HPV-16 late L1 gene was replaced by the chloramphenicol acetyltransferase (CAT) reporter gene, or green fluorescent protein (GFP), preceded by the poliovirus internal ribosome entry site (IRES). Results show that the reporter genes mimic the expression of L1 from these plasmids. For example, overexpression of adenovirus E4orf4 protein (E4orf4), polypyrimidine tract binding protein (PTB), arginine/serine-rich SRp30c protein (SRp30c) or alternative splicing factor/splicing factor 2 (ASF/SF2) induced an increased expression of CAT or GFP. Stable cell lines with reporter plasmids pBELCAT and pBELMCAT were also generated. An induction of CAT was observed in HPV-16 reporter cell lines in the presence of the small molecule phorbol 12-myristate 13-acetate (TPA). Further experiments identified the TPA-inducible, hnRNP A2/B1 protein as a regulator of HPV-16 late gene expression. In conclusion, the HPV-16 reporter plasmids and reporter cell lines described herein can be used to identify small molecules and cellular factors that regulate HPV-16 gene expression.

1. Introduction

Cervical cancer is the second leading cause of death by cancer in women worldwide after breast cancer (WHO, 2006). Persistence of human papillomaviruses (HPVs) infection is a prerequisite for development of cervical cancer or pre-malignant lesions that could progress to cancer (Lowy and Howley, 2001; Zur Hausen, 2002). HPVs are subdivided into high risk and low risk groups and HPV type 16 (HPV-16) is the most common high-risk type that is present in approximately 50% of all cervical cancers (Munoz et al., 2003). HPV-16 gene expression is strictly dependent on cellular differentiation with late HPV-16 genes expressed only in differentiating cells. HPV-16 late gene expression is not detected in cervical cancer cells containing HPV-16 DNA (Doorbar, 2005; Longworth and Laimins, 2004; Ozbun et al., 2007). Late genes encode the highly immunogenic structural proteins, L1 and L2, and their production in the lower layers of the infected epithelium is strongly suppressed, possibly to prevent detection by the host immune system (Schwartz et al., 2007). It has been shown that processing of HPV-16 mRNAs is highly regulated (Graham, 2008; Schwartz, 2008; Zheng and Baker, 2006). HPV-16 splice donor SD3632 and splice acceptor SA5639 (Fig. 1A and B) are used exclusively by the late mRNAs and splicing silencers suppress the use of these splice sites in cervical cancer cells (Rush et al., 2005; Zhao et al., 2004, 2007). HPV-16 late gene expression is also indirectly inhibited by RNA elements that stimulate early mRNA splicing (Rush et al., 2005) and polyadenylation (Oberg et al., 2005, 2003; Terhune et al., 1999; Zhao et al., 2005). It was speculated that premature induction of HPV-16 late gene expression in the persistently infected cells would alert the immune system of the presence of the virus and that this immune activation could clear the infection. It is therefore of interest to understand how HPV-16 late gene expression is regulated.

In previous studies two subgenomic HPV-16 expression plasmids were described carrying the viral early and late genes, except E6 and E7, driven by the strong human cytomegalovirus (CMV) immediate early promoter, called pBEL and pBELM (Zhao et al., 2004) (Fig. 1B). When these plasmids are transfected into HeLa cells, they express HPV-16 early, but not late genes. It has been shown that overexpression of a number of viral and cellular proteins, such as adenovirus E4orf4 protein (E4orf4), cellular polypyrimidine tract binding protein (PTB) and members of the serine-arginine rich protein family, induce late gene expression in both pBEL and pBELM (Somberg et al., 2008, 2009, 2011; Somberg and Schwartz, 2010).

In order to facilitate the identification of cellular factors that regulate HPV-16 late gene expression, pBEL-derived plasmids were

* Corresponding author at: School of Biological Sciences, Dublin Institute of technology, Kevin Street, Dublin 8, Ireland. Tel.: +353 1 4024564, fax: +353 1 4024834.
E-mail address: fergus.x.ryan@dit.ie (F. Ryan).
[1] Present address: Department of Laboratory Medicine, Section of Medical Microbiology, Lund University, BMC-B13, room B1314c, 221 84 Lund, Sweden.

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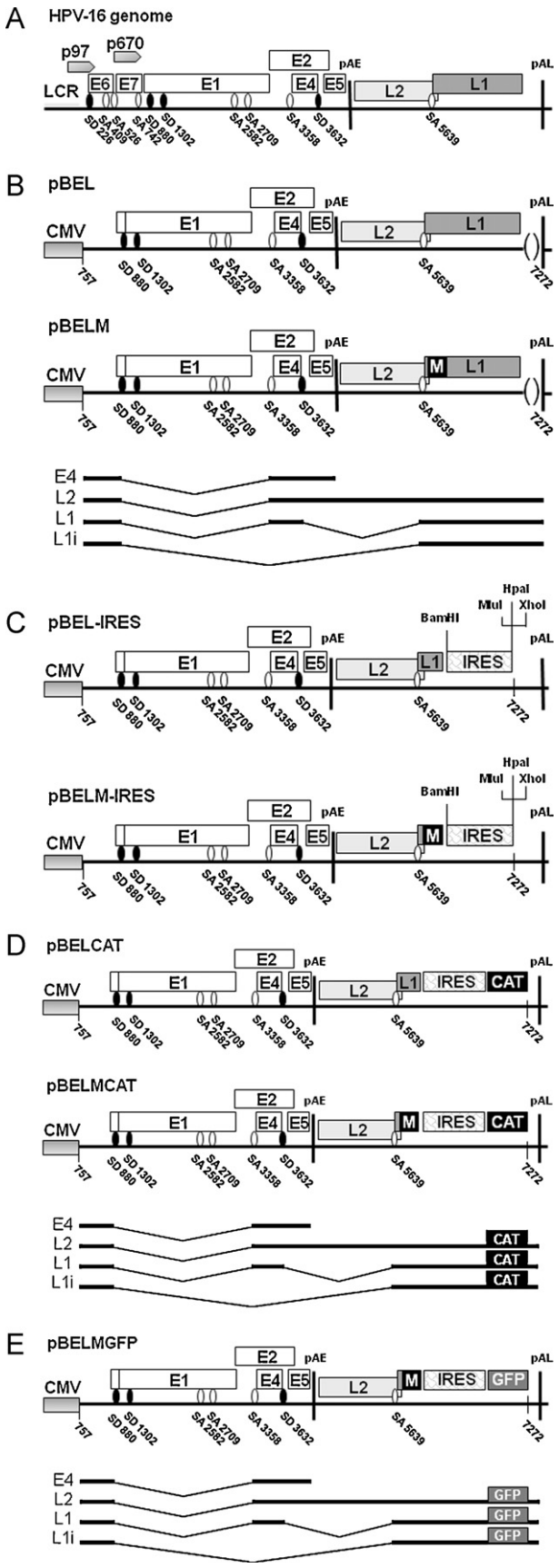

Fig. 1. (A) Schematic representation of the HPV-16 genome and (B, C, D, E) the subgenomic HPV-16 reporter plasmids used to study late gene expression. The early and late viral promoters, p97 and p670, and the early and late polyadenylation signals pAE and pAL are shown. The position of the human cytomegalovirus immediate early (CMV) promoter in the reporter plasmids is shown. Boxes indicate protein-coding regions. Black circles indicate splice donors (5′ splice sites) and white circles indicate splice acceptor (3′ splice sites). Numbers refer to nucleotide positions in the HPV-16 sequence. Major potential mRNAs that can be produced are shown. M indicates previously described mutations that inactivate splicing silencers downstream of SA5639 (Zhao et al., 2004). IRES: poliovirus 2A internal ribosome entry site. CAT: chloramphenicol acetyltransferase. GFP: humanised green fluorescent protein.

Table 1
Sequences of oligonucleotides used in this study.

Oligonucleotide	Sequence (5′-3′)
IRESs	GGGATCCTTAAAACAGCTCTGGGGTTG
IRESa	CCTCGAGTTAACACGCGTAGGTAATTCCAATAGGTGTGAGTG
CATs	GACGCGTACCATGAGTAAAGGAGAAGAACTTTTCACTGGA
CATa	CCTCGAGCTATTTGTATAGTTCATCCATGCC
757s	GTCGACGGTATCGATCGGTTGTGCGTACAAAGCACACACG
E4a	CCGCGCGCTGCCTAATAATTTCAGGAGAGG
L1a	CCGTGCTTACAACCTTAGATACTGGGACAG
L1Ma	CGCTGGGCAGCCACAGGC

generated by us containing easily detectable reporter genes, such as CAT and green fluorescent protein (GFP), in place of HPV-16 L1 (Fig. 1C–E). Stable cell lines harbouring these plasmids were generated and characterised. As proof of concept, it was demonstrated that the small molecule TPA could activate CAT expression from these reporter cell lines, and hnRNP A2/B1 protein was identified as a novel regulator of HPV-16 late gene expression.

2. Materials and methods

2.1. Plasmid construction

pBEL, pBELM, have been described previously (Zhao et al., 2004). In order to construct the reporter plasmids pBELCAT and pBELMCAT, PCR was first performed with oligonucleotides IRESs and IRESa (Table 1) to amplify the IRES sequence of poliovirus 2A. The PCR fragment was subcloned into pCR2.1-TOPO (Invitrogen, Paisley, Scotland) and then transferred as a BamHI-XhoI fragment into pBEL and pBELM, generating pBEL-IRES and pBELM-IRES (Fig. 1C), respectively, with the IRES sequence replacing all but the first 514-nucleotides of the L1 sequence in pBEL and pBELM. The chloramphenicol acetyltransferase (CAT) sequence was first PCR amplified with primers CATs and CATa (Table 1) and cloned into pCR2.1-TOPO. The CAT sequence was excised with MluI and XhoI and inserted downstream the IRES sequence in pBEL-IRES and pBELM-IRES to generate pBELCAT and pBELMCAT (Fig. 1D). pBspliceCAT and pBspliceMCAT were generated by digestion of pBELCAT and pBELMCAT, respectively with BssHII-SalI followed by filling in of overhangs with T4 DNA polymerase and relegation (Fig. 4A). To construct pMT1sdCAT, a HindIII-ApaI fragment from plasmid pMT1sd (Somberg et al., 2008) was inserted into pBELMCAT (Fig. 4A). To generate pBSPtatLTRCAT and pBSPMtatLTRCAT a DNA sequence encoding a modified HIV-1 tat gene in which the 3′-splice sites of rev and nef exons 4a, 4b, 4c and 5 had been mutationally inactivated, followed by the HIV-1 HXB2R LTR sequence and a codon optimised GFP coding was purchased from GenScript (Piscataway, USA). The DNA sequence was delivered as a pUC57 plasmid with the ordered insert that was named pUC57/tat/LTR/GFP. This sequence was inserted into pBspliceCAT and pBspliceMCAT using BssHII and XhoI. The GFP gene was replaced by CAT using MluI and XhoI, resulting in pBSPtatLTRCAT and pBSPMtatLTRCAT (Fig. 4A).

The plasmid pCMVCAT was constructed by cleaving pBELCAT with SalI and BamHI, filling in of overhangs and religation. In order to construct pBELMGFP (Fig. 1E), the GFP sequence from pUC57/tat/LTR/GFP was transferred to pBELM-IRES as a MluI-XhoI