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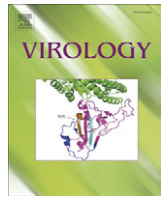
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## Adenovirus E4orf4 induces HPV-16 late L1 mRNA production

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### ABSTRACT

The adenovirus E4orf4 protein regulates the switch from early to late gene expression during the adenoviral replication cycle. Here we report that overexpression of adenovirus E4orf4 induces human papillomavirus type 16 (HPV-16) late gene expression from subgenomic expression plasmids. E4orf4 specifically overcomes the negative effects of two splicing silencers at the two late HPV-16 splice sites SD3632 and SA5639. This results in the production of HPV-16 spliced L1 mRNAs. We show that the interaction of E4orf4 with protein phosphatase 2A (PP2A) is necessary for induction of HPV-16 late gene expression. Also an E4orf4 mutant that fails to bind the cellular splicing factor ASF/SF2 fails to induce L1 mRNA production. Collectively, these results suggest that dephosphorylation of SR proteins by E4orf4 activates HPV-16 late gene expression. Indeed, a mutant ASF/SF2 protein in which the RS-domain had been deleted could itself induce HPV-16 late gene expression, whereas wild type ASF/SF2 could not.

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### Introduction

Human papillomavirus (HPV) type 16 is the most common cancer-associated HPV type in the human population. In rare cases, HPV-16 infections are not cleared by the immune system and persist for decades. Persistent HPV-16 infections constitute a risk factor for development of cervical cancer (zur Hausen, 2002). Progression to cancer requires expression of viral early gene products E6 and E7 to drive cell proliferation (Howley and Lowy, 2001). Expression of HPV-16 L1 and L2 is rarely, if ever, seen in mitotic cells in pre-malignant lesions or cervical cancer (Doorbar, 2005). Based on the well-established observation that HPV-16 late gene expression is cell-differentiation-dependent, we speculate that inhibition of the highly immunogenic L1 and L2 capsid proteins is a prerequisite for progression to cancer.

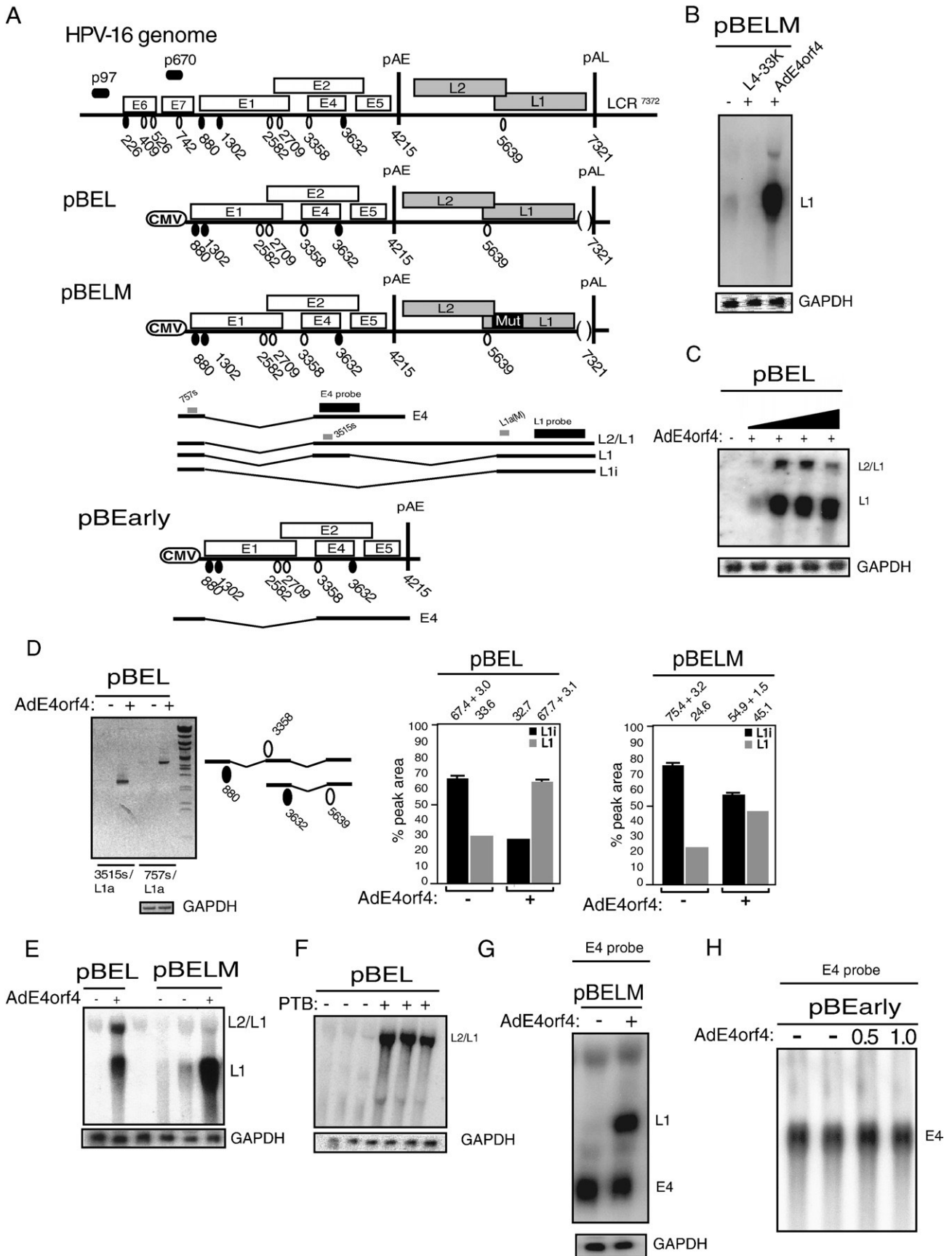
Papillomaviruses produce a large number of alternatively spliced mRNAs (Baker and Calef, 1997). At the RNA level, regulatory RNA elements, and the factors they interact with, must be present on the viral genome to prevent usage of late splice sites or polyadenylation signals at an early stage of the viral replication cycle (Mole et al., 2006; Schwartz, 2008; Schwartz et al., 2007; Zheng and Baker, 2006). On the other hand, other regulatory RNA elements are probably needed to induce late gene expression at the appropriate time during a productive HPV-16 infection

(Mole et al., 2006; Schwartz, 2008; Schwartz et al., 2007; Zheng and Baker, 2006). Previously published results from several groups have identified cis-acting regulatory RNA elements on HPV mRNAs that affect HPV mRNA stability (Kennedy et al., 1991; Sokolowski et al., 1997, 1998), polyadenylation (Öberg et al., 2005; Terhune et al., 1999, 2001; Zhao et al., 2005), splicing (Rush et al., 2005; Zhao et al., 2004, 2007; Zheng et al., 2004) and translation (Collier et al., 1998; Gu et al., 2004; Wiklund et al., 2002). Regulatory bovine papillomavirus RNA elements have also been identified and characterised (Baker, 1997; Zheng and Baker, 2006). Mutational inactivation of regulatory RNA elements in the HPV-16 late coding regions increased L1 and L2 gene expression levels (Collier et al., 2002; Öberg et al., 2003) to the extent that cellular and humoral immune responses were induced upon DNA immunisations of mice with these genes (Rollman et al., 2004). The increase in L1 and L2 expression correlated with less efficient binding of cellular proteins to the HPV-16 L1 and L2 mRNAs (Zhao and Schwartz, 2008). Although many cellular factors that bind to HPV and BPV mRNAs have been identified (Zhao and Schwartz, 2008), only polypyrimidine tract binding protein (PTB)/heterologous nuclear ribonucleoprotein I (hnRNP I) has been shown to induce HPV-16 late gene expression in human cells (Somberg et al., 2008).

Adenoviruses are bigger and more complex DNA viruses than the HPVs. Similarly to HPV, the replication cycle of adenoviruses is divided into an early and a late phase. Adenoviral genes are also expressed from a large number of alternatively spliced mRNAs and regulated alternative splicing is an essential step in the switch from early to late gene expression (Akusjärvi and Stevenin, 2003). Adenoviruses encode two viral proteins named E4orf4 and L4-33K that regulate adenovirus

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alternative splicing and that are key players in the induction of late gene expression during an adenovirus infection. While L4-33K appears to have properties similar to cellular splicing factors termed SR-proteins, and seems to be directly involved in splicing regulation (Törmänen et al., 2006), E4orf4 is indirectly involved in splicing regulation due to its ability to bind SR-proteins and PP2A, thereby altering the function of SR-proteins through dephosphorylation (Kanopka et al., 1996, 1998). Here we have investigated if the two key inducers of adenovirus late gene expression E4orf4 and L4-33K can also induce HPV-16 late gene expression.

## Results

### *Adenoviral protein E4orf4 but not L4-33K, induces HPV-16 late gene expression*

Adenoviral protein E4orf4 (AdE4orf4) is involved in many important steps in the adenoviral life cycle, including the switch from early to late gene expression. At the molecular level it induces apoptosis and has been shown to dephosphorylate SR-proteins by binding to PP2A (Kanopka et al., 1996, 1998). L4-33K is an adenoviral protein which functions as a virus-encoded splicing factor that specifically activates weak late adenoviral splice sites and induces early to late switch in adenovirus splicing (Törmänen et al., 2006). In order to investigate if these viral factors can activate HPV-16 late gene expression, we are using subgenomic HPV-16 plasmids named pBEL and pBELM (Fig. 1A) (Zhao et al., 2004), which encode viral early and late genes after a human cytomegalovirus immediate early (CMV) promoter placed at the position of the late HPV-16 promoter p670. These plasmids produce primarily HPV-16 early E4 mRNAs upon transfection of cervical cancer cells (Zhao et al., 2004) (see Fig. 1G below). The mutant version of pBEL, named pBELM, also produces low levels of L1 mRNAs as a result of the mutationally inactivated splicing silencers in the L1 region (Fig. 1A) (Zhao et al., 2004). Both pBEL and pBELM are used as reporter plasmids. Overexpression of AdE4orf4 induces HPV-16 late gene expression from pBEL and pBELM, whereas L4-33K does not (Figs. 1B and C).

### *AdE4orf4 counteracts splicing silencers in the HPV-16 L1 coding region*

Cotransfection of AdE4orf4 with pBEL showed, that AdE4orf4 induces mainly the spliced L1 mRNA in a dose dependent manner (Fig. 1C). Similar results were obtained with HPV-negative cell lines C33A and A459 (data not shown). Cloning and sequencing of RT-PCR products from this experiment (Fig. 1D) revealed a splicing pattern consistent with induction of L1 mRNA spliced from SD880 to SA3358 and further from SD3632 to SA5639 (Fig. 1A). Analysis of L1 mRNA production over a wider mRNA concentration range using real-time PCR allowed detection of both L1 and L1i mRNAs (Fig. 1A). Real-time PCR gave rise to two peaks representing the two differently spliced L1 mRNAs. Quantitation of L1 and L1i mRNAs produced from pBEL and pBELM in the absence or presence of AdE4orf4 revealed that AdE4orf4

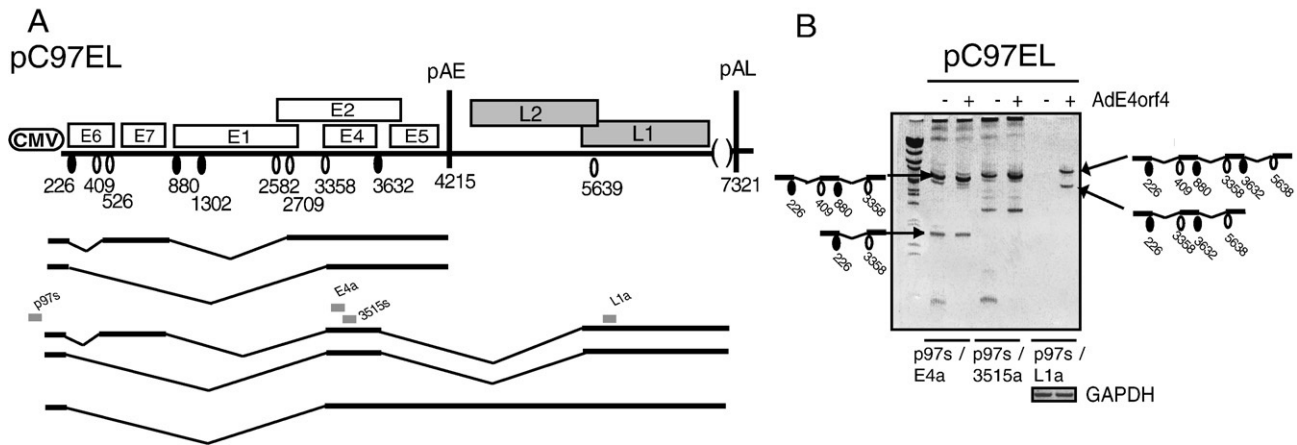
enhanced the production of L1 mRNAs, which contain the middle exon derived from the HPV-16 E4 coding region, while L1i mRNA levels were reduced (Fig. 1D). These results suggested that AdE4orf4 acts on SA3358 and/or SD3632 (Fig. 1A) or sequences in the exon located between these two splice sites.

A comparison of the late mRNAs that were induced from pBEL and pBELM, established that AdE4orf4 induces splicing of the late mRNAs, irrespectively of the absence or presence of splicing silencers in the L1 coding region (Fig. 1E). Induction of spliced L1 mRNA from pBEL by AdE4orf4 is in sharp contrast to the induction of L2/L1 mRNA by overexpression of PTB that we have reported previously (Fig. 1F) (Somberg et al., 2008). While PTB can only overcome the effect of splicing inhibitory sequences at SD3632, AdE4orf4 overcomes the effect of splicing inhibitory elements located both at SD3632 and in the HPV-16 L1 coding region downstream of SA5639 and therefore induces production of spliced L1 mRNA. Clearly, adenovirus E4orf4 and PTB induce HPV-16 late gene expression by different mechanisms. Analysis of Northern blots with HPV-16 E4 probe revealed that L1 mRNAs were induced 39-fold from pBELM, while early HPV-16 E4 mRNA levels were reduced only 1.6-fold (Fig. 1G). The small inhibitory effect on HPV-16 E4 mRNA could be a direct effect of AdE4orf4 on early mRNA splicing, or an indirect effect of increasing late mRNA splicing. To distinguish between the two, we cotransfected AdE4orf4 with pBEarly (Fig. 1A) (Zhao et al., 2005), which expresses only the early region of the HPV-16 genome and monitored E4 mRNA levels produced by pBEarly (Fig. 1H). AdE4orf4 did not affect HPV-16 early mRNAs directly. We also tested AdE4orf4 on plasmid pC97EL (Fig. 2A) (Somberg et al., 2008), which encodes the entire early region including E6 and E7 genes, to investigate if AdE4orf4 affected any of the early mRNAs encoding E6 and E7. Analysis of the early mRNAs in the absence or presence of AdE4orf4 did not reveal any qualitative or quantitative differences for any of the detectable early mRNAs (Fig. 2B). Late L1 mRNAs were induced from pC97EL in the presence of AdE4orf4, as expected (Fig. 2B). Therefore, the reduction of E4 mRNA levels from pBELM by AdE4orf4 is probably an indirect effect of the induction of late mRNA splicing by AdE4orf4. Taken together, our results demonstrate that overexpression of AdE4orf4 specifically induces HPV-16 L1 mRNA production.

### *AdE4orf4 enhances splicing from HPV-16 late splice site SD3632*

To confirm that AdE4orf4 acts directly on HPV-16 late mRNA splicing, a plasmid that contains only late HPV-16 splice sites (SD3632 and SA5639) was used. This plasmid is named pBSplice (Fig. 3A) (Somberg et al., 2008). Cotransfection of E4orf4 with pBSplice, or pBSpliceM (Somberg et al., 2008) in which silencers in L1 have been inactivated, revealed that spliced L1 mRNAs were induced by E4orf4 in both cases, although higher levels of L1 mRNAs were obtained from pBSpliceM than from pBSplice (Fig. 3B). These results confirmed that AdE4orf4 acts on late HPV-16 splice sites and that it overcomes the effect of previously described splicing silencer sequences (Rush et al., 2005; Zhao et al., 2004, 2007).

**Fig. 1.** (A) Schematic representation of the HPV-16 genome and CMV-promoter driven subgenomic HPV-16 expression plasmids pBEL, pBELM and pBEarly (Zhao et al., 2004, 2005). Numbers indicate nucleotide positions of 5'-splice sites (filled circles) and 3'-splice sites (open circles) or early and late polyA signals (pAE and pAL). p97 and p670 indicate early and late promoters, respectively. Major mRNAs that could be produced by pBEL and/or pBELM are indicated below plasmids. Grey bars represent RT-PCR primers and black bars represent E4 and L1 probes used for Northern blotting. (B) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBELM in the absence or presence of plasmids expressing adenovirus E4orf4 (AdE4orf4) or adenovirus L4-33K. The blot was probed with L1 probe and the position of the L1 mRNA is indicated. (C) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBEL in the absence or presence of increasing concentration of plasmid expressing AdE4orf4. The blot was probed with L1 probe that detects both L2/L1 and L1 mRNAs. (D) (left panel) RT-PCR on cytoplasmic RNA extracted from HeLa cells transfected with pBEL in the absence or presence of plasmid expressing AdE4orf4. The RT-PCR was performed either with primer combinations 3515s and L1a, or 757s and L1a (panel A) (Zhao et al., 2005). (right panel) Real time PCR on cytoplasmic RNA with primers 757s and L1a over a range that detects both L1 and L1i mRNAs as separate peaks. The areas under the peaks are shown as percentage of total and indicate that AdE4orf4 promotes splicing of L1 mRNA, whereas L1i mRNA production is inhibited. (E) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBEL or pBELM in the absence or presence of plasmids expressing AdE4orf4. Blot was probed with L1 probe and L2/L1 and L1 mRNAs are indicated. (F) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBEL in the absence or presence of plasmids expressing PTB. Triplicate experiments are shown. Blot was probed with L1 probe and L2/L1 mRNA is indicated. (G, H) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with pBELM or pBEarly in the absence or presence of AdE4orf4. Blots were probed with E4 probe and E4 and L1 mRNAs are indicated. Gels were also probed for GAPDH.

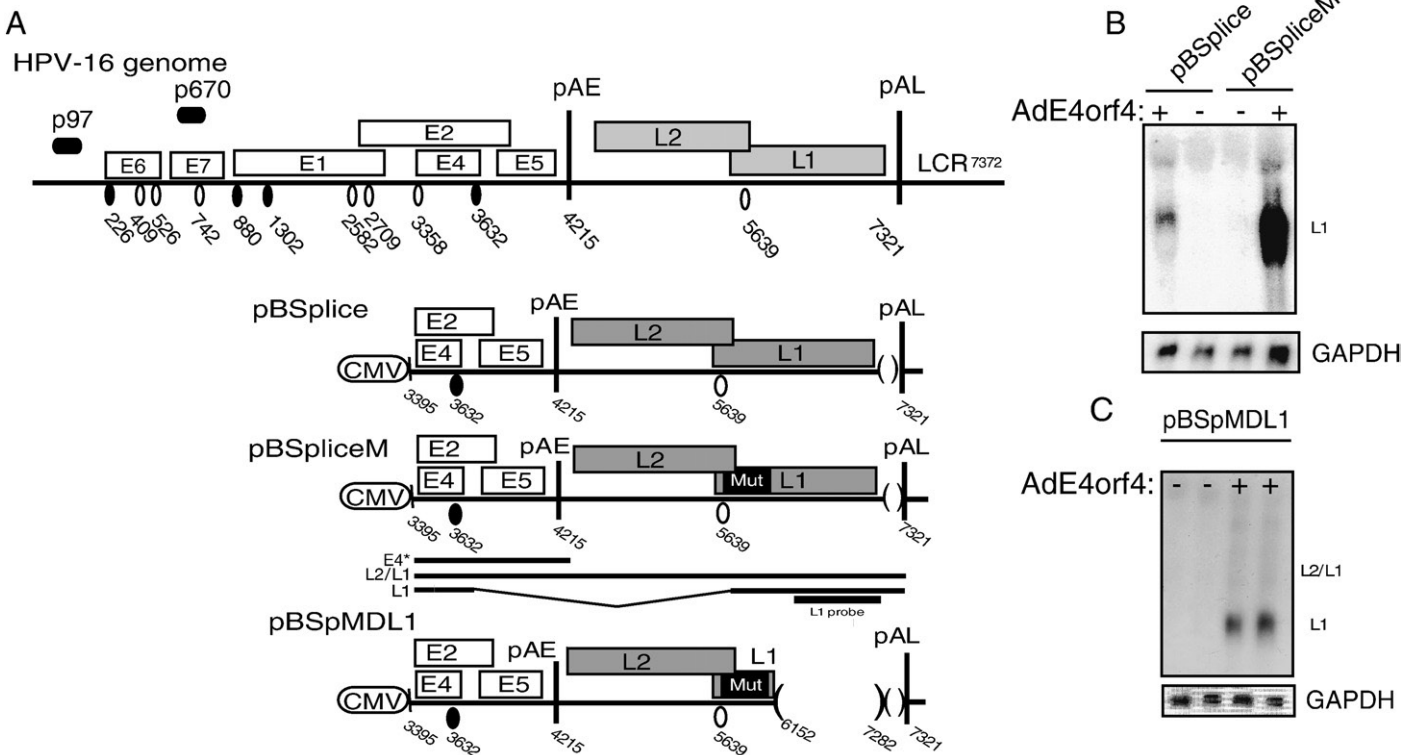


**Fig. 2.** (A) Schematic representation of subgenomic HPV-16 expression plasmid pC97EL (Somberg et al., 2008). Numbers indicate nucleotide positions of 5'-splice sites (filled circles), 3'-splice sites (open circles) or polyA sites (pAE and pAL). Major mRNAs produced by pC97EL are indicated below plasmid. Grey bars represent RT-PCR primers. Major mRNAs are indicated. (B) RT-PCR of cytoplasmic RNA extracted from HeLa cells transfected with pC97EL in the absence or presence of plasmids expressing AdE4orf4. The RT-PCR was performed with different primer-combinations p97s and E4a (early mRNAs), p97s and 3515a (early mRNAs) and p97s and L1a (late mRNAs) (Table 1). The structures of the detected mRNAs are shown on each side of the gel. RT-PCR for GAPDH was also performed.

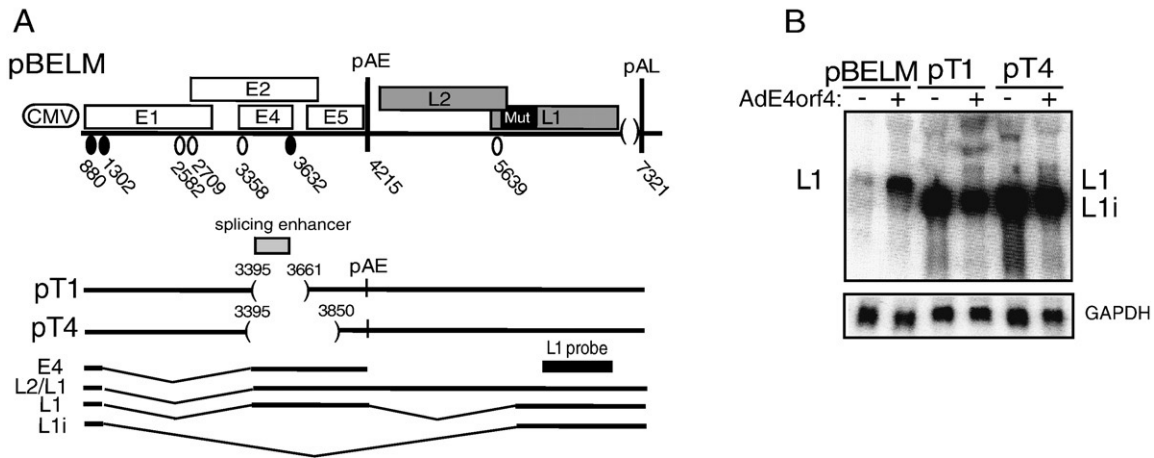
To provide evidence that AdE4orf4 acts on other sequences than the silencers in the L1 coding region, we tested AdE4orf4 on plasmid pBSpMDL1 (Fig. 3A). In this plasmid, splicing silencers in L1 that are located between nucleotide positions 5639 and 6152 (Zhao et al., 2004) are destroyed by point mutations, and wt L1 sequences with splicing inhibitory functions located downstream of position 6152 (Zhao et al., 2007) are deleted. Therefore, all previously identified splicing silencers in L1 were either deleted or destroyed. Late gene

expression from this plasmid is therefore primarily inhibited by splicing silencers at the 5-splice site SD3632. Since AdE4orf4 induced production of spliced L1 mRNAs from pBSpMDL1 (Fig. 3C), we concluded that AdE4orf4 activates splicing from SD3632.

To confirm that AdE4orf4 acts specifically on SD3632, we also tested pBELM-derived plasmids pT1, pT3 and pT4 (Fig. 4A), in which a key regulatory splicing enhancer (Rush et al., 2005) located downstream of SA3358 in the early region had been deleted. Since splicing



**Fig. 3.** (A) Schematic representation of the HPV-16 genome and CMV-promoter driven subgenomic HPV-16 expression plasmids. Numbers indicate nucleotide positions of 5'-splice sites (filled circles) and 3'-splice sites (open circles) or early and late polyA signals (pAE and pAL). p97 and p67 indicate early and late promoters, respectively. Major potential mRNAs produced by these plasmids are indicated below pBSpliceM. The E4\* mRNA indicates a short mRNA encoding E4 and E5 that is unspliced due to the absence of SD880 and other 5'-splice sites upstream of the major E4 3'-splice site SA3358 in the pBSplice-derived plasmids. The L1 probe used for Northern blotting is indicated. (B, C) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBSplice, pBSpliceM or pBSpMDL1 in the absence or presence of plasmid expressing AdE4orf4. Blots were probed with L1 probe and L2/L1 and L1 mRNA is indicated. Duplicates are shown in panel C. Gels were also probed for GAPDH.



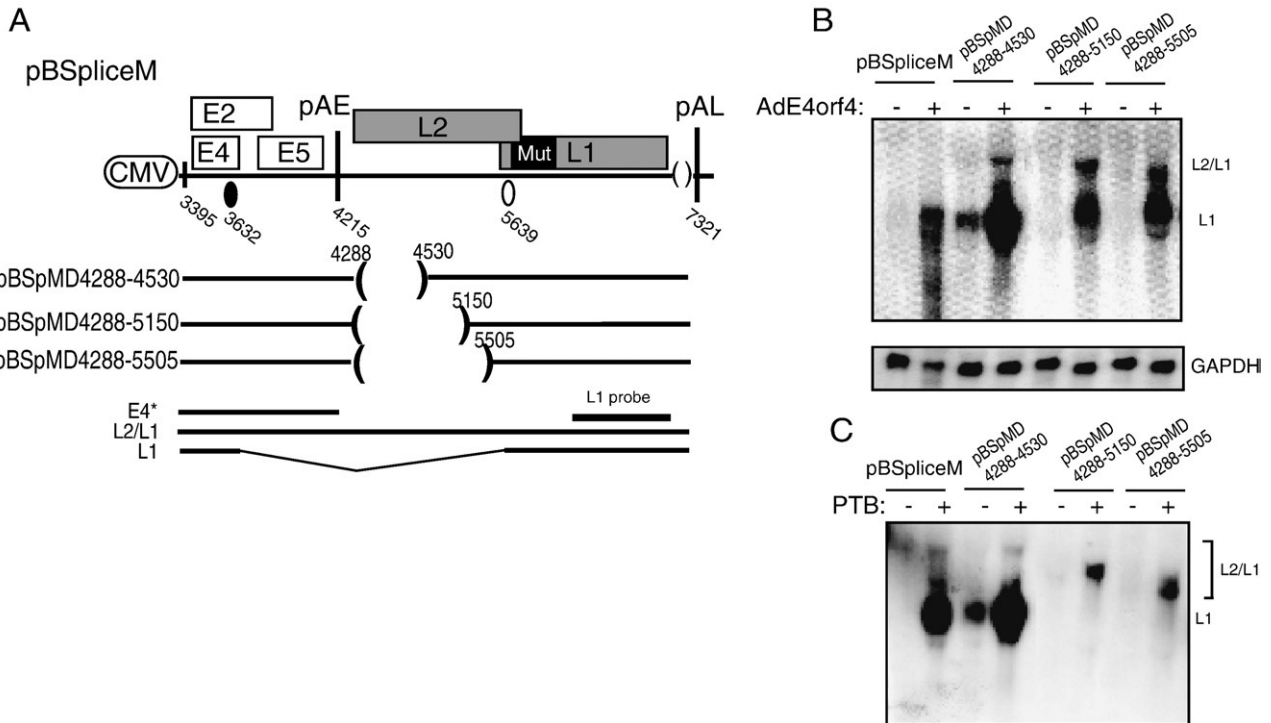
**Fig. 4.** (A) Schematic representation of subgenomic HPV-16 expression plasmid pBELM, pT1 and pT4 (Rush et al., 2005; Zhao et al., 2004). Numbers indicate nucleotide positions of 5'-splice sites (filled circles) and 3'-splice sites (open circles) or early and late polyA signals (pAE and pAL). p97 and p67 indicate early and late promoters, respectively. Mut indicates the presence of the L1 mutant sequence in which splicing silencers in L1 had been inactivated by multiple substitutions. The deletions present in the pT1 and pT4 plasmids are indicated. The major splicing enhancer located in the E4 coding region is indicated (Rush et al., 2005). Major potential mRNAs produced by these plasmids are indicated. The L1 probe used for Northern blotting is indicated. (B) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBELM, pT1 or pT4 in the absence or presence of plasmid expressing AdE4orf4. Blot was probed with L1 probe and L2/L1, L1 and L1i mRNAs are indicated and with GAPDH probe.

silencers in L1 had been inactivated by mutations as well, splicing occurs directly from SD880 to SA5639, thereby bypassing the central exon using SA3358–SD3632 (Rush et al., 2005). pT1 and pT4 produce primarily spliced L1i mRNA (Figs. 4A and B). Overexpression of AdE4orf4 did not increase L1 mRNA levels further (Fig. 4B). In contrast, AdE4orf4 induced expression of L1 mRNA from pBELM, as expected (Fig. 4B). RT-PCR results confirmed that SD880, and not SD3632, is used in pT1 and pT4 (data not shown). These results confirmed that AdE4orf4 acts specifically on the suppressed SD3632, and not on

SD880. Taken together, our results demonstrate that AdE4orf4 induces splicing from the suppressed splice sites SD3632 and SA5639 that are used exclusively by late HPV-16 mRNAs.

*Novel late mRNA splicing-enhancing sequence in HPV-16 L2 is needed for induction of L1 mRNA by PTB, but not by AdE4orf4*

In an effort to map the target sequence for AdE4orf4, we first introduced deletions from nucleotide position 4288 in L2 to various



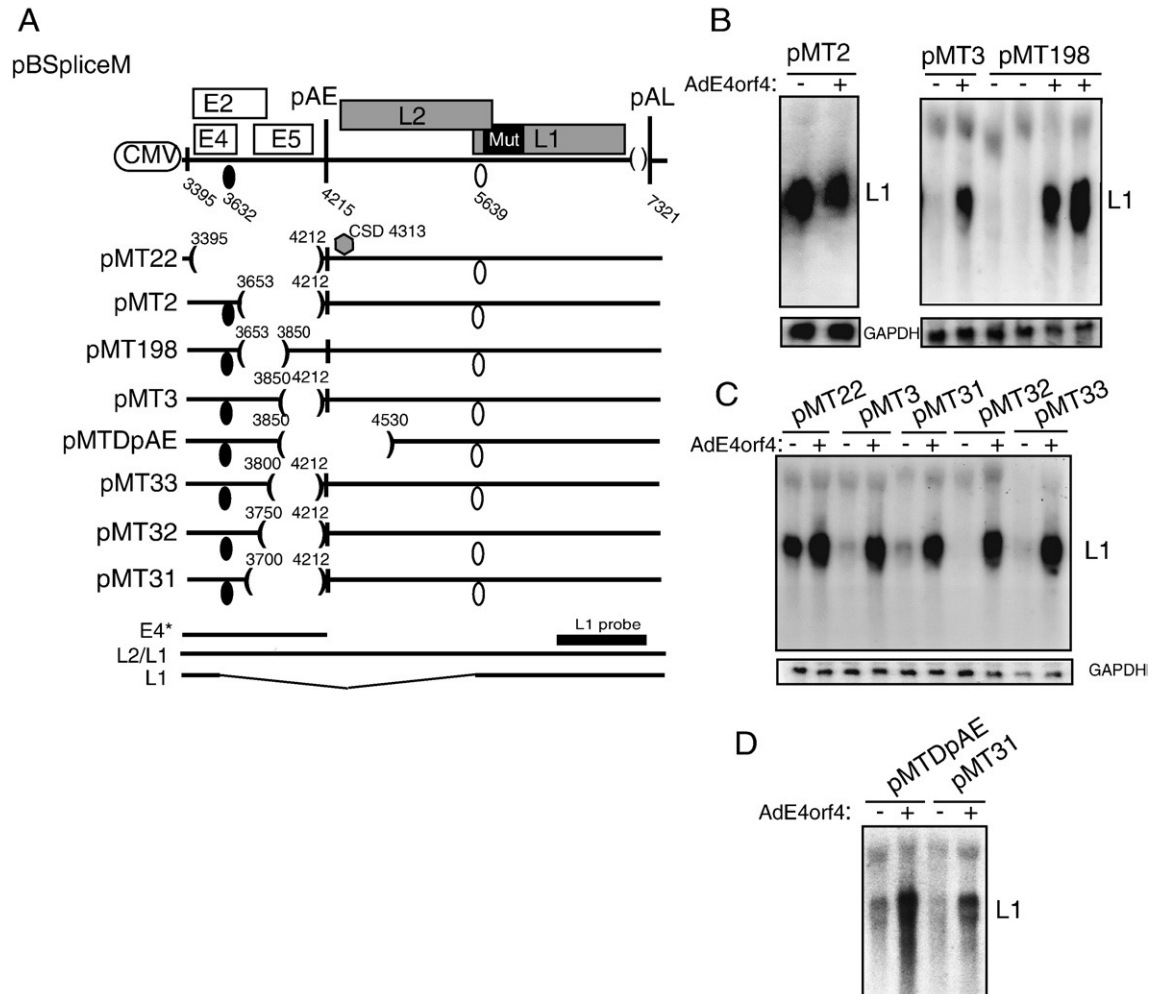
**Fig. 5.** (A) Schematic representation of subgenomic HPV-16 expression plasmids pBSpliceM, pBSpMD4288–4530, pBSpMD4288–5150 and pBSpMD4288–5505. Numbers indicate nucleotide positions of 5'-splice sites (filled circles) and 3'-splice sites (open circles), positions of pAE and pAL mark the borders of deletions. Major mRNAs produced by these plasmids are indicated. The E4\* mRNA indicates a short mRNA encoding E4 and E5 that is unspliced due to the absence of SD880 and other 5'-splice sites upstream of the major E4 3'-splice site SA3358 in the pBSplice-derived plasmids. The L1 probe used for Northern blotting is indicated. Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with indicated HPV-plasmids in the absence or presence of plasmid expressing AdE4orf4 (B) or PTB (C). Blots were probed with L1 probe and L2/L1 and L1 mRNAs are indicated. Gels were also probed for GAPDH.

positions further down in L2 in pBSpliceM (Fig. 5A). The results revealed that AdE4orf4 induced production of spliced L1 mRNA to a similar extent in all deletions, including pBSpMD4288–5505 in which 1218 nucleotides of the 1422 nucleotides of L2 had been deleted (Fig. 5B), indicating that AdE4orf4 acts independently of L2 sequences. Interestingly, the PTB protein that only induces spliced L1 mRNA if L1 silencers are destroyed, only induced L1 mRNA from the smallest L2-deletion (pBSpMD4288–4530). In contrast, only unspliced late mRNAs were induced from the larger L2 deletions by PTB (Fig. 5C). That these slower migrating bands represented unspliced mRNAs was confirmed by RT-PCR (data not shown). These results indicated that induction of spliced L1 mRNA by PTB is dependent on a sequence located downstream of nucleotide position 4530 in the HPV-16 L2 coding region (Fig. 5A). This region may therefore encode a conditional, splicing enhancer element. Since AdE4orf4 acted independently of this sequence, the simplest explanation is that AdE4orf4 counteracts the effect of multiple inhibitory RNA elements and therefore is independent of splicing enhancers in L2. These results further supported the conclusion that PTB and AdE4orf4 induce HPV-16 late gene expression by different mechanisms. We concluded that AdE4orf4 induces late mRNA splicing independently of sequences in the HPV-16 L2 coding region, whereas PTB does not.

#### AdE4orf4 alleviates inhibition from splicing silencer elements downstream of HPV-16 late splice site SD3632

To map the AdE4orf4 target sequences at SD3632, the effect of AdE4orf4 on plasmids with various deletions at SD3632 were analysed. The deletion mutants are shown in Fig. 6A. Both pMT2 and pMT22 produced high levels of L1 mRNA, both in the absence and presence of AdE4orf4, indicating that splicing silencer elements had been deleted (Figs. 6B and C). Note that SD3632 is also deleted in plasmid pMT22 and that a previously described cryptic splice donor CSD4313 is used in this plasmid (Somberg et al., 2008 and data not shown). Two non-overlapping deletions were introduced between SD3632 and pAE, resulting in pMT3 and pMTD198 (Fig. 6A). AdE4orf4 induced late mRNAs from both plasmids (Fig. 6B), suggesting that there are two splicing inhibitory elements between SD3632 and pAE. A series of deletions (pMT31, -32 and -33 (Fig. 6A)) were analysed and were all found to be responsive to AdE4orf4 (Fig. 6C). Therefore, one splicing inhibitory element is located between SD3632 and 3700.

Alternatively, the early polyA signal (pAE) that competes with SD3632, also directly suppresses SD3632. We therefore extended the deletion in pMT3 to include also pAE, resulting in pMTDpAE (Fig. 6A). L1 mRNA production from this plasmid is suppressed in the absence of



**Fig. 6.** (A) Schematic representation of subgenomic HPV-16 expression plasmids pBSpliceM and pBSpliceM-derived deletion mutants. Numbers indicate nucleotide positions of 5'-splice sites (filled circles) and 3'-splice sites (open circles), positions of pAE, pAL and cryptic 5'-splice site (CSD) mark the borders of deletions. Major mRNAs produced by these plasmids are indicated below the plasmids. The E4\* mRNA indicates a short mRNA encoding E4 and E5 that is unspliced due to the absence of SD880 and other 5'-splice sites upstream of the major E4 3'-splice site SA3358 in the pBSplice-derived plasmids. The L1 probe used for Northern blotting is indicated. (B, C, D and E) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with indicated plasmids in the absence or presence of AdE4orf4. Blots were probed with L1 probe and L1 mRNAs are indicated. Gels were also probed for GAPDH.



AdE4orf4 (Fig. 6D), but is induced by AdE4orf4. These results suggest that pAE per se is not needed for inhibition of late gene expression. However, we cannot exclude that a cryptic polyA signal at nucleotide position 3820, previously named CpA (Zhao et al., 2005), suppresses late gene expression in pMTDpAE. Cryptic polyA signals are activated upon inactivation of pAE also in BPV-1 (Andrew and DiMaio, 1993) and HPV-31 (Terhune et al., 1999, 2001). We concluded that AdE4orf4 activates the suppressed late splice site SD3632 by overcoming the effect of adjacent splicing inhibitory elements.

To further investigate if pAE was specifically targeted by AdE4orf4 and was required for AdE4orf4-mediated induction of HPV-16 late gene expression, we analysed the effect of AdE4orf4 on pBELDP (Fig. 7A) (Zhao et al., 2005), a plasmid in which the pAE had been mutationally inactivated by point mutations in the canonical AAUAAA motif, converting it to an MluI site (ACGCGU) (Zhao et al., 2005). However, AdE4orf4 efficiently induced late gene expression from pBELDP (Fig. 7B), demonstrating that pAE was not required for induction of HPV-16 late gene expression by AdE4orf4.

To confirm that pAE was not targeted by AdE4orf4, we replaced pAE by the HPV-16 late polyadenylation signal pAL in pBspliceM (plasmid p2pAL (Fig. 7A) (Somberg et al., 2008)). pAL is not negatively affected by AdE4orf4 since it is used efficiently by the HPV-16 L1 mRNAs that are induced by AdE4orf4. AdE4orf4 induced high levels of late mRNAs from plasmid p2xpAL that carries two late polyA signals (Fig. 7C). We concluded that AdE4orf4 does not induce HPV-16 late gene expression by specifically inhibiting the early polyA signal. Further experiments are needed to establish the exact mechanism of suppression of SD3632 in the absence of AdE4orf4.

#### Overexpression of proapoptotic proteins Bad, Bax and p53 or APC/C inhibitor Emi-1 does not induce HPV-16 late gene expression

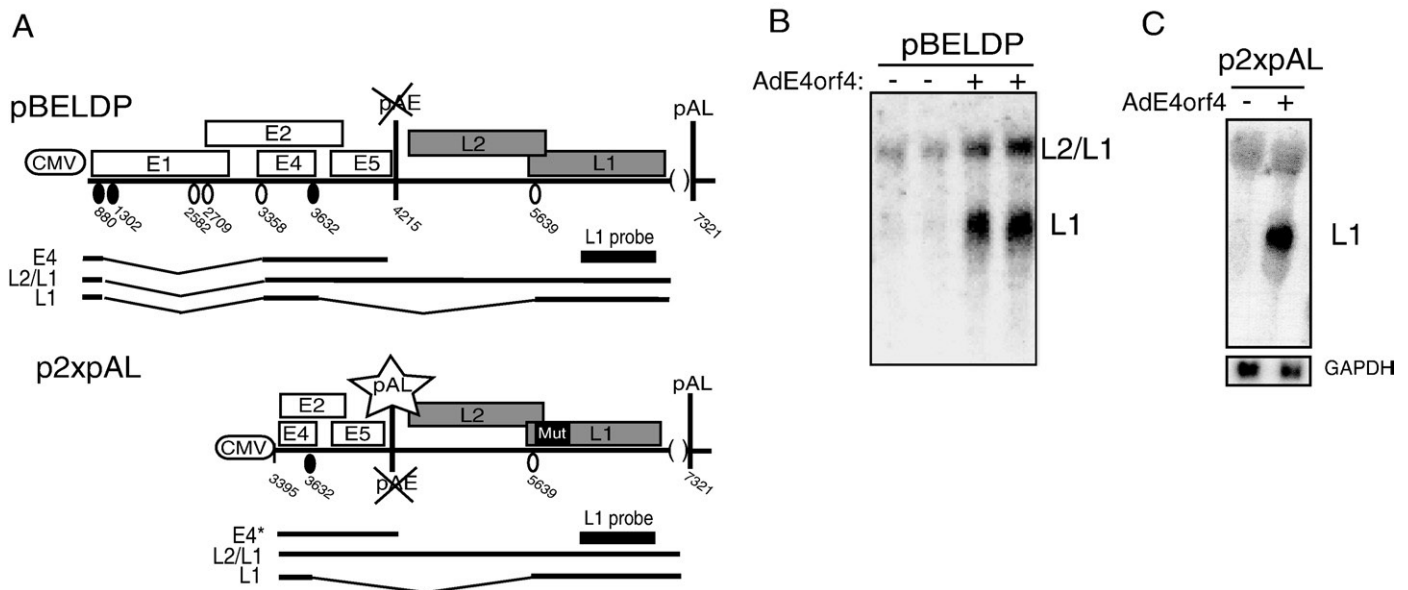
One of the most characteristic properties of AdE4orf4 is its ability to induce apoptosis (Branton and Roopchand, 2001; Kleinberger, 2000). During the HPV-16 life cycle, the late genes are expressed in fully differentiated epithelial cells. Induction of HPV-16 late genes by AdE4orf4 could therefore be a response to induction of apoptosis. In addition, p53 has been shown to enhance adenovirus late gene expression (Royds et al., 2006). We therefore tested if proapoptotic proteins p53, Bad and Bax

could induce HPV-16 late gene expression, but none of these proteins did (Figs. 8A–C). The results were confirmed in HPV negative cell lines (data not shown). In addition anti apoptotic protein Bcl-2 could neither induce HPV-16 late gene expression, nor counteract the effect of AdE4orf4 (Fig. 8D). We concluded that HPV-16 late gene expression could not be induced by these regulators of apoptosis.

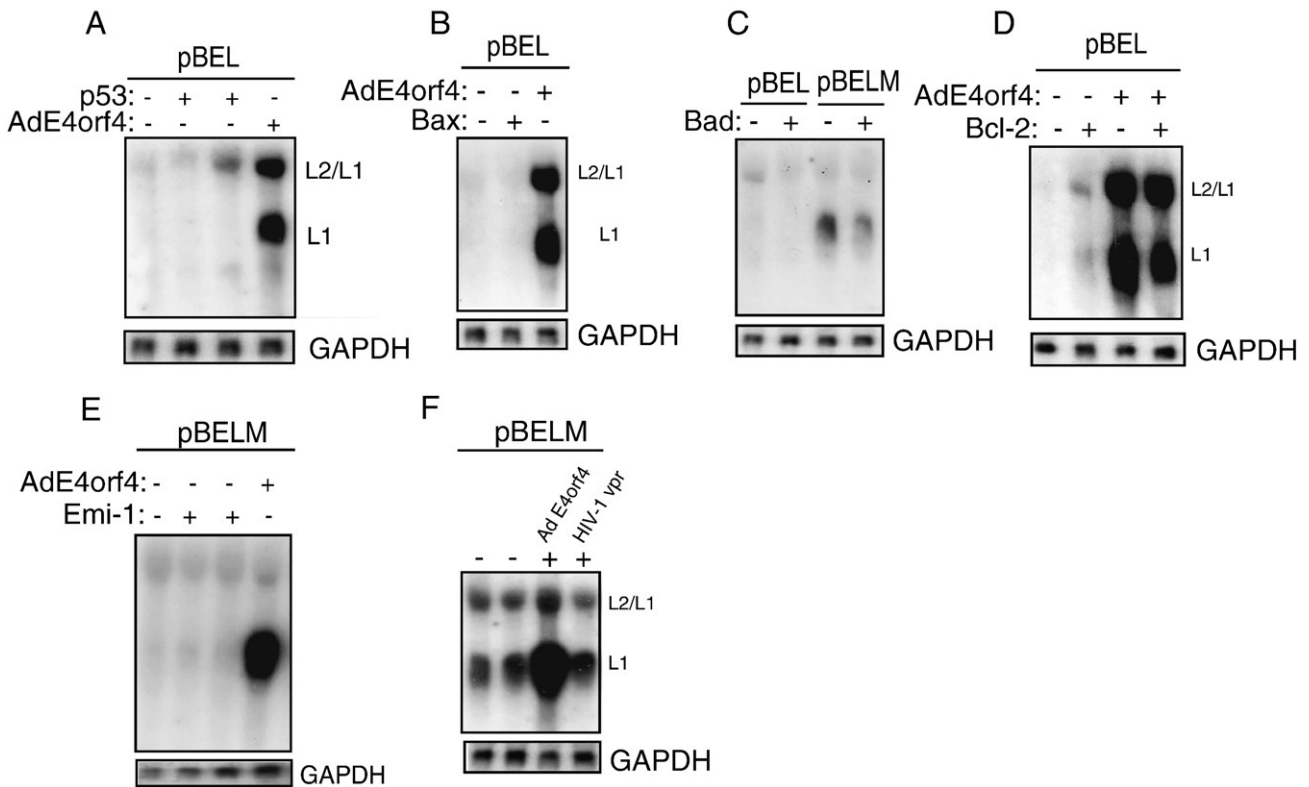
Adenovirus E4orf4 can induce apoptosis in a p53-independent manner, by inhibiting the anaphase promoting complex APC/C through dephosphorylation (Kornitzer et al., 2001). In uninfected cells, the APC/C is instead regulated by the cellular Emi-1 protein, which also inhibits APC/C through dephosphorylation (Reimann et al., 2001). The viral HIV-1 vpr protein also induces apoptosis and interferes with G2/M control (Stewart et al., 1999), but through another mechanism than AdE4orf4. Neither overexpression of Emi-1, nor HIV-1 vpr, induced HPV-16 late gene expression from pBEL or pBELM (Figs. 8E and F). Taken together, these results demonstrated that HPV-16 late gene expression is not induced by proapoptotic factors, nor by inhibition of the APC/C complex. We concluded that AdE4orf4 induced HPV-16 late gene expression through another pathway.

#### Binding of AdE4orf4 to PP2A is necessary for induction of HPV-16 late gene expression

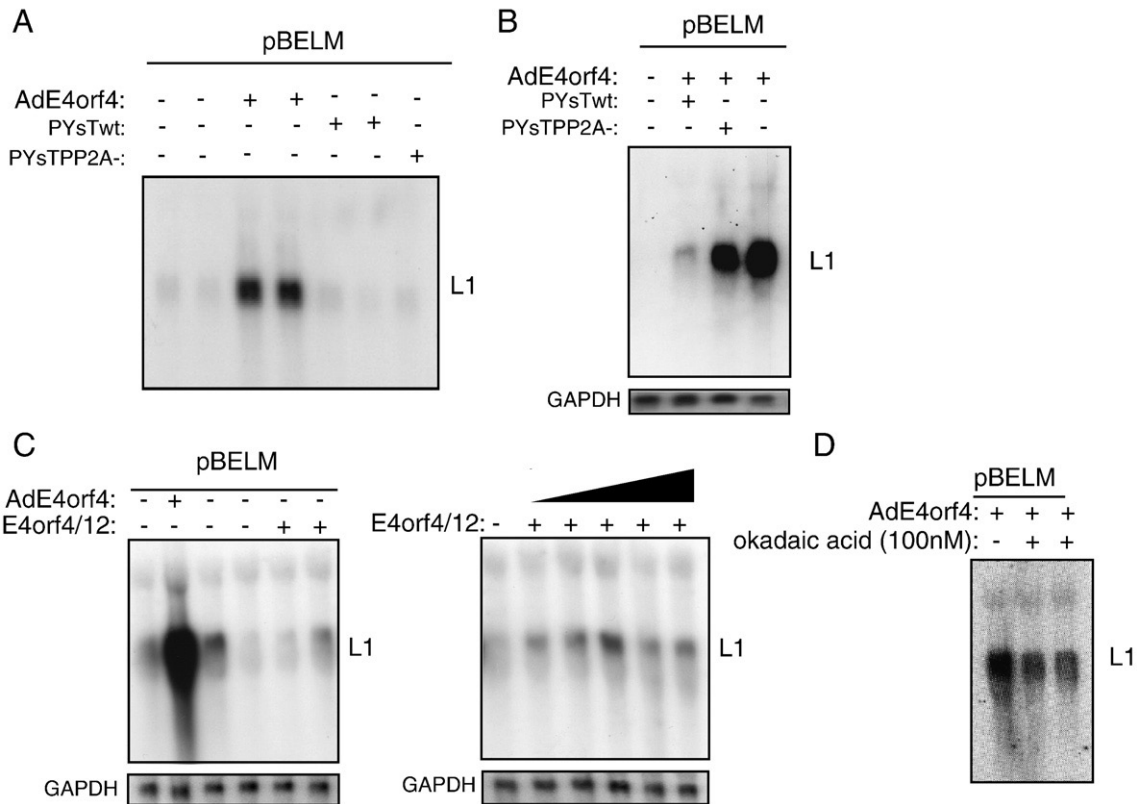
Interaction of AdE4orf4 with protein phosphatase type 2A (PP2A) is required for dephosphorylation of APC/C (Kornitzer et al., 2001) or SR proteins (Kanopka et al., 1996, 1998). HPV-16 E7 and polyomavirus small t protein also bind PP2A (Pallas et al., 1990; Pim et al., 2005), but they could not induce HPV-16 late gene expression (Figs. 9A, B and data not shown). However, when polyomavirus small t is co-expressed with AdE4orf4, it inhibits AdE4orf4-mediated induction of HPV-16 late gene expression (Fig. 9B). In contrast, a previously described mutant polyomavirus small t protein that does not bind to PP2A (Fan, 2001), had no effect on AdE4orf4-induction of HPV-16 late gene expression (Fig. 9B). The most likely explanation for these results is that polyomavirus small t, that binds to the A-subunit of PP2A and displaces the B-subunit, inhibits AdE4orf4 interaction with PP2A since AdE4orf4 is known to mediate its effect by interacting with the B-subunit to recruit PP2A (Branton and Roopchand, 2001; Kleinberger, 2000). Overexpression of polyomavirus small t therefore is highly likely to reduce the



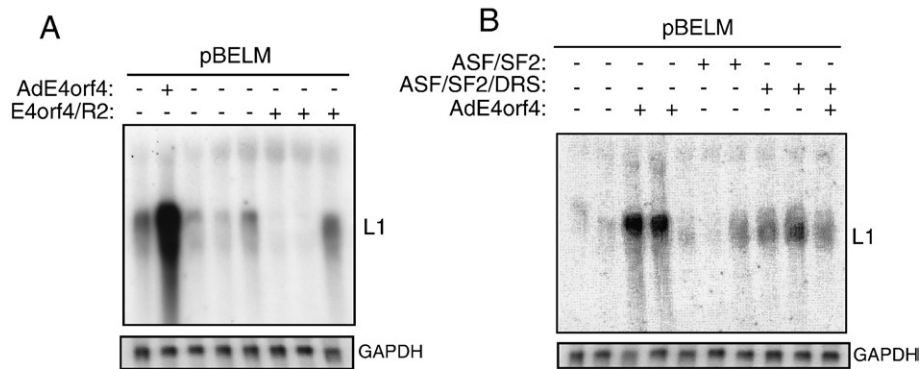
**Fig. 7.** (A) Schematic representations of subgenomic HPV-16 expression plasmids pBELDP (Zhao et al., 2005) and p2xpAL (Somberg et al., 2008). Numbers indicate nucleotide positions of 5'-splice sites (filled circles) and 3'-splice sites (open circles), or pAE and pAL. Crossed pAE indicates that pAE is mutationally inactivated in these plasmids by point mutations or deletions (Somberg et al., 2008; Zhao et al., 2005). (B, C) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with pBELDP or p2xpAL in the absence or presence of AdE4orf4. Duplicates are shown in panel B. Blots were probed with L1 probe and L2/L1 and L1 mRNAs are indicated. Gels were also probed for GAPDH.



**Fig. 8.** (A–F) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with pBEL or pBELM in the absence or presence of plasmids expressing AdE4orf4, p53, Bax, Bad, Bcl-2, Emi-1 or HIV-1 vpr. Blots were probed with L1 probe and L1 and L2/L1 mRNAs are indicated. Gels were also probed for GAPDH.



**Fig. 9.** (A–C) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with pBEL or pBELM in the absence or presence of plasmids expressing AdE4orf4, polyoma small T-wt (PYsTwt), polyoma small T-mut (PYsTPP2A-) (which fails to bind PP2A) or AdE4orf412 (Shtrichman et al., 1999) (which fails to bind PP2A). Duplicate transfections are shown in panels A and C. (D) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with pBELM and AdE4orf4 in the absence or presence of okadaic acid. Blots were probed with L1 probe and L1 and L2/L1 mRNAs are indicated. Gels were also probed for GAPDH.



**Fig. 10.** (A, B) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with pBELM in the absence or presence of plasmids expressing AdE4orf4, AdE4orf4R2 (which fails to bind SR-protein ASF/SF2 (Estmer Nilsson et al., 2001), but still interacts with PP2A (Shtrichman et al., 1999)), ASF/SF2 or ASFDRS, a deletion mutant of ASF/SF2 that lacks the RS-domain. Duplicate transfections are shown. Blots were probed with L1 probe and L1 mRNAs are indicated. Gels were also probed for GAPDH.

intracellular pool of PP2A that is available to AdE4orf4. These results are in line with the hypothesis that binding of AdE4orf4 to PP2A is necessary for induction of HPV-16 late gene expression.

To confirm that PP2A is needed for induction of HPV-16 late gene expression by AdE4orf4, we also tested a previously described AdE4orf4 mutant named E4orf4/R12 (Shtrichman et al., 1999), in which asparagine at amino acid position #60 had been replaced by serine, and therefore does not bind PP2A (Shtrichman et al., 1999). This mutant does not induce HPV-16 late gene expression, as expected (Fig. 9C). Finally, if PP2A is required for induction of HPV-16 late gene expression, okadaic acid should inhibit this effect of AdE4orf4, which is shown in Fig. 9D. We concluded that binding of AdE4orf4 to PP2A is required for induction of HPV-16 late gene expression by AdE4orf4.

#### ASF/SF2 lacking RS-domain efficiently induces HPV-16 late gene expression

It has previously been shown that AdE4orf4 binds ASF/SF2, and that it causes dephosphorylation of ASF/SF2 in vitro (Kanopka et al., 1996, 1998). A mutant AdE4orf4 protein that does not bind ASF/SF2 (E4orf4/R2) (Estmer Nilsson et al., 2001), but still interacts with PP2A (Shtrichman et al., 1999), failed to induce HPV-16 late gene expression (Fig. 10A), suggesting that binding of AdE4orf4 to ASF/SF2 is required for induction of HPV-16 late gene expression. We speculated that dephosphorylated ASF/SF2 could induce HPV-16 late gene expression and tested if overexpression of AdE4orf4 in HeLa cells caused dephosphorylation of ASF/SF2. However, we were unable to detect SR protein dephosphorylation in AdE4orf4-transfected cells (data not shown), suggesting that only a fraction of ASF/SF2 is dephosphorylated in these cells. In an effort to provide evidence that dephosphorylated ASF/SF2 could induce HPV-16 late gene expression, a mutant ASF/SF2 lacking the RS-domain (ASF/SF2/DRS) was tested. This mutant ASF/SF2 protein could no longer be phosphorylated. As can be seen in Fig. 10B, ASF/SF2/DRS, induced high levels of HPV-16 L1 mRNAs, while wt ASF/SF2 protein did not. Induction of HPV-16 late gene expression is less efficient with ASF/SF2/DRS than with AdE4orf4. We concluded that ASF/SF2 induced high levels of HPV-16 L1 mRNAs in its unphosphorylated state, independently of the RS-domain. Transfection with a combination of AdE4orf4 and ASF/SF2/DRS increased L1 mRNA expression further (Fig. 10B), as expected since AdE4orf4 targets endogenous ASF/SF2. We concluded that ASF/SF2 induces HPV-16 late gene expression only in the absence of RS-domain phosphorylation, supporting the idea that AdE4orf4 induces HPV-16 late gene expression by dephosphorylating ASF/SF2.

#### Discussion

AdE4orf4 apparently overcomes the activity of the splicing inhibitory sequences at both HPV-16 splice sites SD3632 and SA5639, which are the only splice sites that are used exclusively by

late HPV-16 mRNAs. Both splice sites are either suppressed by the same cellular factor(s), or AdE4orf4 interacts with multiple cellular RNA regulatory proteins. Splicing silencers at SA5639 have been shown to interact with hnRNP A1 (Zhao et al., 2004, 2007). It remains to be investigated if AdE4orf4 interferes with the function of hnRNP A1. AdE4orf4 has been shown to bind ASF/SF2 and it has been shown to dephosphorylate ASF/SF2 in vitro (Estmer Nilsson et al., 2001; Kanopka et al., 1998). Here we show that overexpression of a deletion mutant of ASF/SF2 that cannot be phosphorylated induces HPV-16 late gene expression. These results support the conclusion that AdE4orf4 acts by targeting ASF/SF2 for dephosphorylation. Overexpression of ASF/SF2 inhibits adenovirus late gene expression (Molin and Akusjärvi, 2000) and seems to have an inhibitory effect on HPV-16 gene expression as well (Fig. 10). One may speculate that phosphorylated ASF/SF2 inhibits HPV-16 late gene expression and that this inhibition is relieved by overexpression of AdE4orf4 or by deletion of the RS-domain of ASF/SF2, which prevents ASF/SF2 phosphorylation. The mechanism by which ASF/SF2 regulates HPV-16 late gene expression is unclear but is under investigation. ASF/SF2 has also been shown to bind to the late UTR of HPV-16 (McPhillips et al., 2004), but the presence or absence of the late UTR did not affect the response to ASF/SF2 in our experiments (data not shown).

The RS domain of ASF/SF2 interacts with U2AF35 (Wu and Maniatis, 1993), but has also been shown to bind RNA (Hertel and Graveley, 2005), in both cases to stimulate the splicing reaction. Overexpression of ASF/SF2 has been shown to inhibit late mRNA splicing during an adenovirus infection (Molin and Akusjärvi, 2000). Recent data demonstrate that ASF/SF2 is not simply inhibiting splicing by competing with another splicing factor, but actively inhibits splicing (Dauksaite and Akusjärvi, 2002). These results demonstrate that ASF/SF2 can play the role of either a splicing suppressor or activator, depending on context.

A comparison with the effect of PTB on HPV-16 late gene expression revealed that PTB induces expression of L2/L1 mRNAs (Somberg et al., 2008), unless splicing silencers at SA5639 were inactivated, demonstrating that PTB overcomes the splicing silencers at SD3632, but not those at SA5639. In contrast, E4orf4 overcomes the effect of splicing silencers at both late splice sites. In addition, induction of late mRNAs by PTB also required splicing enhancing elements in the L2 coding region, whereas induction of L1 mRNA by AdE4orf4 occurred independently of these elements. It is therefore reasonable to speculate that AdE4orf4 targets multiple proteins and that one of those also interacts with PTB. Proteins interacting with both AdE4orf4 and PTB have not been identified. There does not appear to be PTB binding sites in the splicing silencer at SD3632 and AdE4orf4 is not an RNA-binding protein, suggesting that both PTB and AdE4orf4 may target a cellular factor rather than HPV-16 RNA. Both proteins may interact with ASF/SF2 to prevent ASF/SF2 from inhibiting

HPV-16 late gene expression. However, ASF/SF2 binding sites cannot be detected in this region with the increased specificity score matrix for prediction of ASF/SF2 binding sites (Cartegni et al., 2003). Alternatively, PTB and AdE4orf4 simply interact with different proteins. It will be of interest to identify the cellular factors that bind to HPV-16 RNA and suppress SD3632.

One draw back with the experimental system used here is that HeLa cells are unable to differentiate. One can therefore not investigate the effect of AdE4orf4 on HPV-16 in a differentiating environment, such as the W12 cell lines, derivatives thereof (Lambert et al., 2005) or cell lines created by transfection with immortalising HPV types (Lee et al., 2004; McLaughlin-Drubin and Meyers, 2005). It would be informative to investigate the status of PTB and ASF/SF2 in cell lines that are permissive for HPV-16 virus production.

The more sophisticated adenoviruses encode proteins that induce a shift in viral RNA splicing, thereby initiating the late gene expression program (Akusjärvi and Stevenin, 2003). HPVs on the other hand, are less complex and encode relatively few proteins. One may speculate that HPVs simply sit in the epithelial cells and await terminal cell differentiation and a change in the intracellular environment that activates late gene expression. We have shown that SR proteins as a group are down-regulated in terminally differentiated cells and using a monospecific antibody against ASF/SF2 this was shown also for ASF/SF2 specifically (Fay et al., in press). We have also shown that SR proteins are strongly overexpressed in cervical cancers (Fay et al., in press), which support the recently published results that classify ASF/SF2 as an oncogene (Karni et al., 2007). The expression pattern of ASF/SF2 shows an inverse correlation with HPV-16 late gene expression in the epithelium, indicating that other factors than ASF/SF2 induce HPV-16 late gene expression, or that the few cells in the cervical epithelium that actually express HPV-16 L1 and L2 are also producing unphosphorylated ASF/SF2. Alternatively, the anti-ASF/SF2 antibody used detects unphosphorylated ASF/SF2 less efficiently than phosphorylated ASF/SF2. It would be of interest to determine the role of ASF/SF2 in the HPV-16 life cycle.

## Materials and methods

### Plasmid constructions

pBEL and pBELM, have been described previously (Zhao et al., 2004). pT1, pT2, pT3, pT4, pBSplice (pBSplice was referred to as pC16L1L2splice) and pBSpliceM have been described (Rush et al., 2005). pMT1, pMT2, pMT3, pMT22, p2xpAL, pC97EL, pBSpMDL1, pBSpMD4288–4530 and pCMVPTB1 have been described (Somberg et al., 2008). pBearly and pBELDP have been described previously (Zhao et al., 2005).

pBSpMD4288–5505 was created by first PCR amplifying a sequence from pBR-HPV-16 with oligonucleotides M4S and 16F (Table 1). The PCR fragment was subcloned into pCR2.1-TOPO, released with ApaI and BamHI and subcloned into pBSpMD4288–4530. pBSpMD4288–5150 was created by first PCR amplifying a sequence from pBR-HPV-16 with oligonucleotides M12S and 16F (Table 1). The PCR fragment was subcloned into pCR2.1-TOPO, released with ApaI and BamHI and subcloned into pBSpD4288–4530.

pMT31, pMT32 and pMT33 were created by PCR amplifying a sequence from pBR-HPV-16 with oligonucleotides 3455s and M21A, M22A and M23A respectively (Table 1). PCR fragments were digested with XbaI and MluI and cloned into pMT1.

pMT198 was created by PCR amplifying a sequence from pBR-HPV-16 with oligonucleotides M14S and M17A (Table 1), PCR fragment was digested with MluI and ApaI and cloned into pMT2. pMTDpAE was constructed by cutting of pMT3 with MluI and ApaI followed by filling in, trimming and religation.

HIV-1 vpr plasmid referred to as pNL13AE has been described previously (Schwartz, Felber, and Pavlakis, 1991). pASF/SF2, pASF/SF2DRS, pL4-33K, pE4orf4, pE4orf4R2 and pE4orf4R12 have been

**Table 1**  
Oligonucleotides

Oligo name	Oligo sequence
M3A	GGGCCAGGCCTGCACCTGACAGTATACAATGTAATGCT
M4S	AGGCCTGGGCGCCCTCCTTAATTCCTATAGTCCAGGG
M12S	AGGCCTGGGCCAATTGGTAATAAACAAACAC
M14S	GGACGCGTATGACAAATCTTGATACTGCATCC
M17A	CACTAAAGAAAACCTATAGAGAGGATCAG
M21A	GACGCGTGAGCAAAAATTGTCACAGTGGCCATT
M22A	GACGCGTATTGCACCTTTATGTTTTACATTATGT
M23A	GACGCGTCACTGCAGTATACAATGTCAAATGCTT
16F	CCTCGAGCATTTAATAATCTGGATAATTTGC
3455s	GGCGCTCTAGACCAACCCCGCCGCGACC

described previously (Shtrichman et al., 1999; Törmänen et al., 2006). Plasmids encoding HPV-16 E7, murine polyoma sT (wt (PYsTwT) and mutant (PYsTPP2A-) (Fan, 2001)), BAX, Bad, Bcl2 and Emi1 under CMV promoter were generously provided by Drs Lawrence Banks, Göran Magnusson, Alan Escher, John C. Reed, Strathford May and Peter Jackson, respectively.

### Transfection and cell culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat inactivated foetal bovine calf serum and Penicillin–Streptomycin. Transfections were carried out by using Fugene 6 according to manufacturer's protocol (Roche Molecular Biochemicals) as described previously (Somberg et al., 2008). All plasmids were transfected in a minimum of three independent experiments with similar results.

### RNA extraction, Northern blotting and radiolabelled DNA probe synthesis

Cytoplasmic RNA was extracted using NP40 buffer as described previously. The RNA was treated with DNaseI prior to Northern blotting. Northern blot analysis was carried out by size separation of 10 µg cytoplasmic RNA on a 1.2% agarose gels containing 2.2 M formaldehyde. The RNA was transferred over night to a nitrocellulose filter followed by hybridization with either L1, E4 or GAPDH probe as described previously (Somberg et al., 2008; Zhao et al., 2004). L1 probe was excised from pBEL with BamHI and XhoI whereas the E4 probe was generated by PCR using the following primers: E4S and K1. DNA probes were radiolabelled with [ $\alpha$ -<sup>32</sup>P]dCTP using a Decaprime kit (Ambion).

### Reverse Transcription (RT)-PCR

200 ng of cytoplasmic RNA was reverse transcribed at 42°C using Superscript II and random hexamers according to the manufacturer's protocol (Invitrogen). 2 µl of cDNA was amplified in a 100 µl PCR reaction using oligonucleotides indicated in the figures, as described previously. GAPDH cDNA was amplified as control using previously described primers (Somberg et al., 2008).

### Real-time PCR

Real-time PCR was carried out on random hexamer primed reverse transcribed cDNA products using primers GAPDHF (5'-AGTCCGAGTCAACGGATTGG-3') and GAPDHR (5'-ACAGTCTTCTGGTGGCAGTGATG-3') or 757s (5'-TCGGTTGTGCGTACAAAGCACACAG-3') and L1A-wt (5'-CCGTGCTTACAACCTTAGATACTGGGACAG-3') or L1A-mut (5'-AAGCTTGATCCCGCTGGGACGCCACAG-3'). All real-time PCRs were carried out using Lightcycler FastStart DNA Master SYBR Green I Kit. All real-time PCRs were analysed for specificity by melt curve analysis. GAPDH from each individual transfection was used to normalise the L1 mRNA values for each transfection. Peak area data was calculated using the Lightcycler 3 software and was used to

calculate L1 mRNA averages and peak area percentages for graphs and standard error for error bars.

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