2007

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Recommended Citation

Zhao, X., Fay, J., Lambkin, H., Schwartz, S. (2007) Identification of a 17-nucleotide splicing enhancer in HPV-16 L1 that counteracts the effect of multiple hnRNP A1-binding splicing silencers. *Virology* 369 (2007) 351–363. doi:10.1016/j.virol.2007.08.002

Available online at www.sciencedirect.com
ScienceDirect
Virology 369 (2007) 351–363
VIROLOGY
www.elsevier.com/locate/yviro

Identification of a 17-nucleotide splicing enhancer in HPV-16 L1 that counteracts the effect of multiple hnRNP A1-binding splicing silencers

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Received 23 May 2007; returned to author for revision 13 July 2007; accepted 1 August 2007
Available online 14 September 2007

Abstract

Human papillomavirus type 16 (HPV-16) infections can in rare cases persist and cause lesions that may progress to cervical cancer. Cells in the lesions are not permissive for virus production, nor are cervical cancer cells. The intracellular environment is such that it prevents production of the highly immunogenic, viral structural proteins L1 and L2. One may speculate that inhibition of L1 and L2 expression is a prerequisite for persistence and cancer progression. We have therefore investigated how expression of HPV-16 L1 is regulated. We found that the only splice site in the HPV-16 late region, which is used to produce L1 mRNAs, is under control of a splicing enhancer located in the 17 nucleotides immediately downstream of the splice site. However, the function of this enhancer in cervical cancer cells is largely overshadowed by multiple splicing silencers in the late region which bind to hnRNP A1. High levels of hnRNP A1 therefore inhibit HPV-16 L1 expression. Immunohistological analysis of cervical epithelia revealed that hnRNP A1 is expressed primarily in the lower layers of the epithelium. hnRNP A1 is undetectable in terminally differentiated cells that can express HPV-16 late genes, which supports the conclusion that high levels of hnRNP A1 inhibit HPV-16 L1 expression.

Keywords: HPV-16; Cervical cancer; Splicing; Silencer; Enhancer; RNA processing; hnRNP A1

Introduction

Human papillomaviruses (HPVs) are a group of small DNA tumor viruses with strict tropism for epithelial cells (Howley, 1996; Shah and Howley, 1996; zur Hausen, 1999). HPV-16 is the most prevalent genotype in cervical cancer, the second most common cancer worldwide in women. While most HPV-16 infections are cleared by the host immune system less than 1 year after appearance, the infected cells can in rare cases persist for decades and progress to cancer. The cancer cells lack the ability to terminally differentiate and thus do not express the immunogenic, late L1 and L2 capsid proteins (Ho et al., 1995). We speculate that inhibition of L1 and L2 late gene expression is a prerequisite for persistence and progression to cancer.

It has been shown that papillomavirus L1 and L2 mRNAs contain inhibitory RNA elements that reduce late mRNA levels in proliferating cells. These elements have been found in the late 3′UTR of BPV-1 (Furth and Baker, 1991), HPV-1 (Tan and Schwartz, 1995), HPV-16 (Kennedy et al., 1991) and HPV-31 (Cumming et al., 2002) and the L1 and L2 coding regions (Collier et al., 2002; Schwartz, 1998; Sokolowski et al., 1998; Tan et al., 1995). Efficient polyadenylation of the early poly(A) signal (pAE) also inhibits late gene expression. Our laboratory has reported that this pAE is regulated by upstream elements in the early 3′UTR and by downstream elements in the L2 coding region (Oberg et al., 2005; Zhao et al., 2005). The early UTR element interacts with Fip1, CstF-64, PTB and hnRNP C1/C2 and the downstream elements in the L2 coding region interact with CstF-64 and hnRNP H (Oberg et al., 2003; Zhao et al., 2005). We have shown that the levels of hnRNP H are reduced in the layers with differentiated cell in normal cervical epithelium in vivo, supporting a regulatory role for hnRNP H in early polyadenylation and late gene expression of HPV-16

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doi:10.1016/j.virol.2007.08.002

(Oberg et al., 2005; Zhao et al., 2005). Terhune et al. have identified a polyadenylation element in HPV-31 L2 that interacts with CstF-64 (Terhune et al., 2001, 1999). The levels of CstF-64 decrease as primary keratinocytes are induced to differentiate in vitro, suggesting a role for CstF-64 in the regulation of HPV-31 early mRNA polyadenylation (Terhune et al., 2001, 1999).

HPVs make extensive use of alternative splicing. Alternatively spliced mRNAs have been mapped in several HPV types and in particular in HPV-16 and HPV-31 (Baker and Calef, 1997; Billakanti et al., 1996; Klumpp and Laimins, 1999; Milligan et al., 2007; Ozbun and Meyers, 1997). Many of the splice sites in this region are suboptimal and are, or are likely to be, under control of potent splicing silencers and enhancers. Splicing regulatory elements have been identified in bovine papillomavirus type 1 (BPV-1) (Baker, 1997; Zheng, 2004; Zheng et al., 1999, 1998, 2000a,b) and in HPV-16 (Rush et al., 2005; Zhao et al., 2004). Previous results from our laboratory identified a 48-nucleotide splicing silencer located 178 nucleotides downstream of the late 3′ splice site and showed that it interacted with hnRNP A1 (Zhao et al., 2004). The results presented herein are a continuation of previously reported work in which we sought to identify the additional regulatory RNA elements in L1 and determine their function. We show that the first 17 nucleotides of the HPV-16 L1 coding region contain a splicing enhancer interacting with a 55-kDa protein. In dividing cells, this enhancer is overshadowed by multiple hnRNP A1-binding splicing silencers located further down in L1.

Results

Positive and negative splicing elements in the first 22 nucleotides of the HPV-16 L1 coding region

To investigate the effect of HPV-16 L1 RNA sequences on late HPV-16 mRNA splicing, we introduced deletions in the L1 region of the CMV driven, subgenomic HPV-16 expression plasmid named pBELDPU (Fig. 1) (Zhao et al., 2005). This plasmid contains the strong CMV promoter immediately upstream of the E1 gene in the early region for high expression of the HPV genes and lacks the early polyadenylation signal to allow read-through into the late region for production of detectable levels of late mRNAs. Northern blots on RNA extracted from transfected HeLa cells were hybridised to the L1 probe that specifically detects late mRNAs (Fig. 1). As can be seen in Fig. 2B, deletion of all L1 sequences between the AG of the 3′ splice site and position 514 in L1 as in p0, or all sequences except the first 3 nucleotides as in p1–3, resulted in very inefficient splicing. These results suggested that the 3′ splice site required a splicing enhancer or that the L1 sequence downstream of position 514 (which was present in all plasmids in Fig. 2A) also inhibited splicing of the late mRNAs. In contrast, there was a dramatic increase in splicing efficiency as the 17 first nucleotides of L1 were inserted downstream of the splice acceptor (Fig. 2B), suggesting that the first 17 nucleotides contain a splicing enhancer. Percentage splicing was calculated with results from at least three independent transfections and mean values with standard deviations are shown in the graphs (Fig.

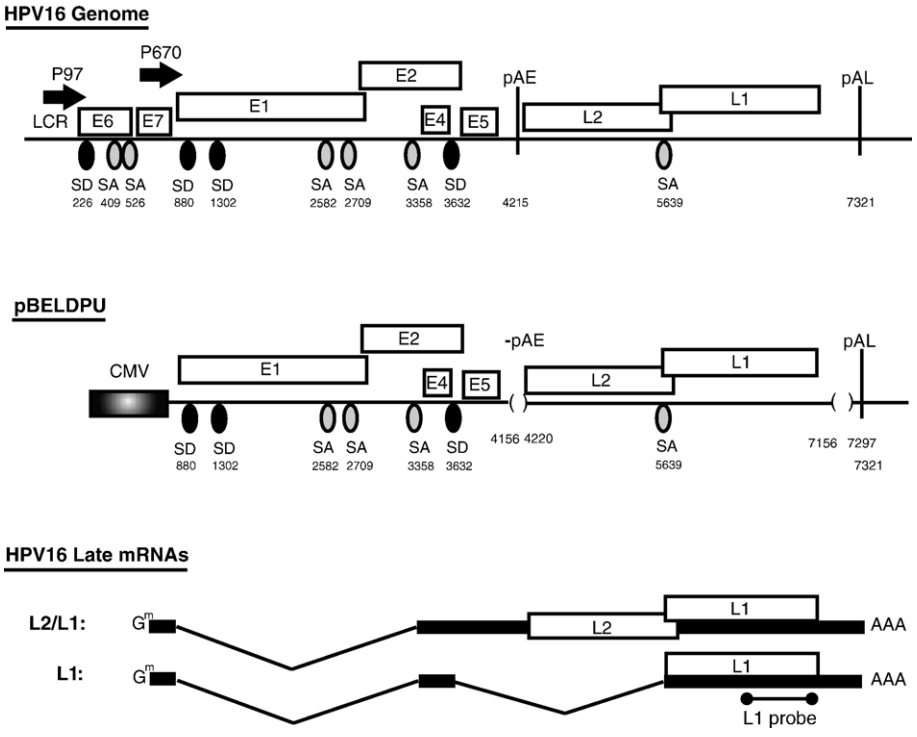

Fig. 1. Schematic representation of the HPV-16 genome. Boxes indicate the protein coding regions. Numbers refer to nucleotide positions in the HPV-16R sequence (Billakanti et al., 1996). The major p97 promoter and the differentiation dependent promoter p670 are indicated. Splice sites and polyadenylation signals are shown. The structure of the pBELDPU (Zhao et al., 2005) expression plasmid is shown and the predicted late mRNAs are displayed. The L1 probe used in Northern blotting is indicated. pAE, early polyA signal; pAL, late polyA signal, CMV, human cytomegalovirus immediate-early promoter; SD, 5′ss; SA, 3′ss.

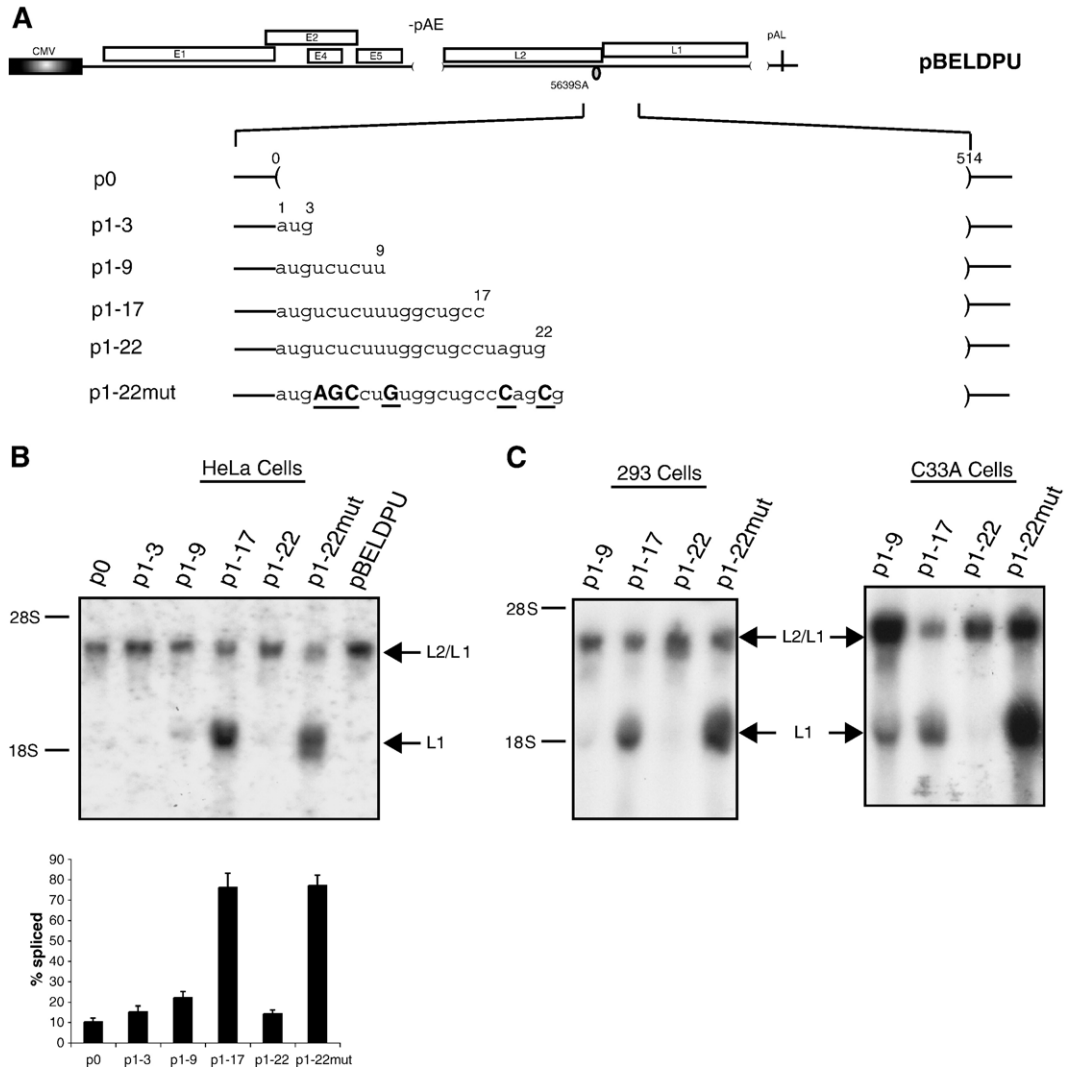

Fig. 2. (A) Schematic representation of pBELDPU-derived (Zhao et al., 2005) plasmids. The deletions introduced in L1 are indicated. Plasmid names are on the left. Numbers refer to nucleotide positions in HPV-16 L1, position 1 being A in the AUG. Sequences of the 5-end of the L1 sequences present in plasmids p1–3, p1–9, p1–17, p1–22 and p1–22mut are displayed. Introduced mutations are bold, underlined and capitalised. (B) Northern blots of total RNA extracted from HeLa cells transfected with the indicated plasmids and hybridised to the L1 probe (Fig. 1). Spliced mRNA as a percentage of total late RNA is shown in the graph below the gel. Numbers represent mean values with standard deviations from at least three independent transfections with the same plasmid. (C) Northern blots of total RNA extracted from HeLa cells transfected with the indicated plasmids in 293 cells and C33A cells.

2B). Extending the 17 nucleotides to 22 instead caused a strong inhibition of L1 splicing (Fig. 2B), demonstrating that splicing inhibitory elements that counteracted the effect of the first 17 nucleotides had been included. The corresponding mutant 22-nucleotide L1 sequence, derived from a previously described L1 mutant sequence (Collier et al., 2002; Zhao et al., 2004), did not inhibit splicing (Fig. 2B), which is in line with our previous results which showed that these mutations destroyed splicing inhibitory elements but did not negatively affecting splicing (Zhao et al., 2004). The first 17 nucleotides of L1 are pyrimidine rich, whereas the 18–22-sequence has a more balanced nucleotide composition (Fig. 2A). Interestingly, mutations at positions 4, 5, 6 and 9 in p1–22mut did not affect the positive splicing elements in the first 17 nucleotides, indicating that a splicing enhancer was located in the UGGCUGCC sequence, between position 10 and 17 (Fig. 2A). We also transfected plasmids p1–17, p1–22 and p1–22mut into C33A cells and 293 cells with similar results (Fig. 2C). In conclusion, positive splicing elements were located in the first 17 nucleotides of L1 and negative splicing elements were located in the first 22 nucleotides of L1.

Sequence specific activation of HPV-16 late mRNA splicing by a splicing enhancer in the first 17 nucleotides of L1

To study the 17-nucleotide enhancing sequence further, pairwise nucleotide substitutions were introduced in p1–17 (Fig. 3A). As can be seen in Fig. 3B, the enhancer was not affected by the substitutions in mut3, but the substitutions in mut5 and mut6, which had the strongest effect, caused a reduction in splicing from 79% to 34% and 55%, respectively (Fig. 3B). These results support the conclusion above that the enhancer is located between nucleotides positions 10 and 17 of L1. However, since

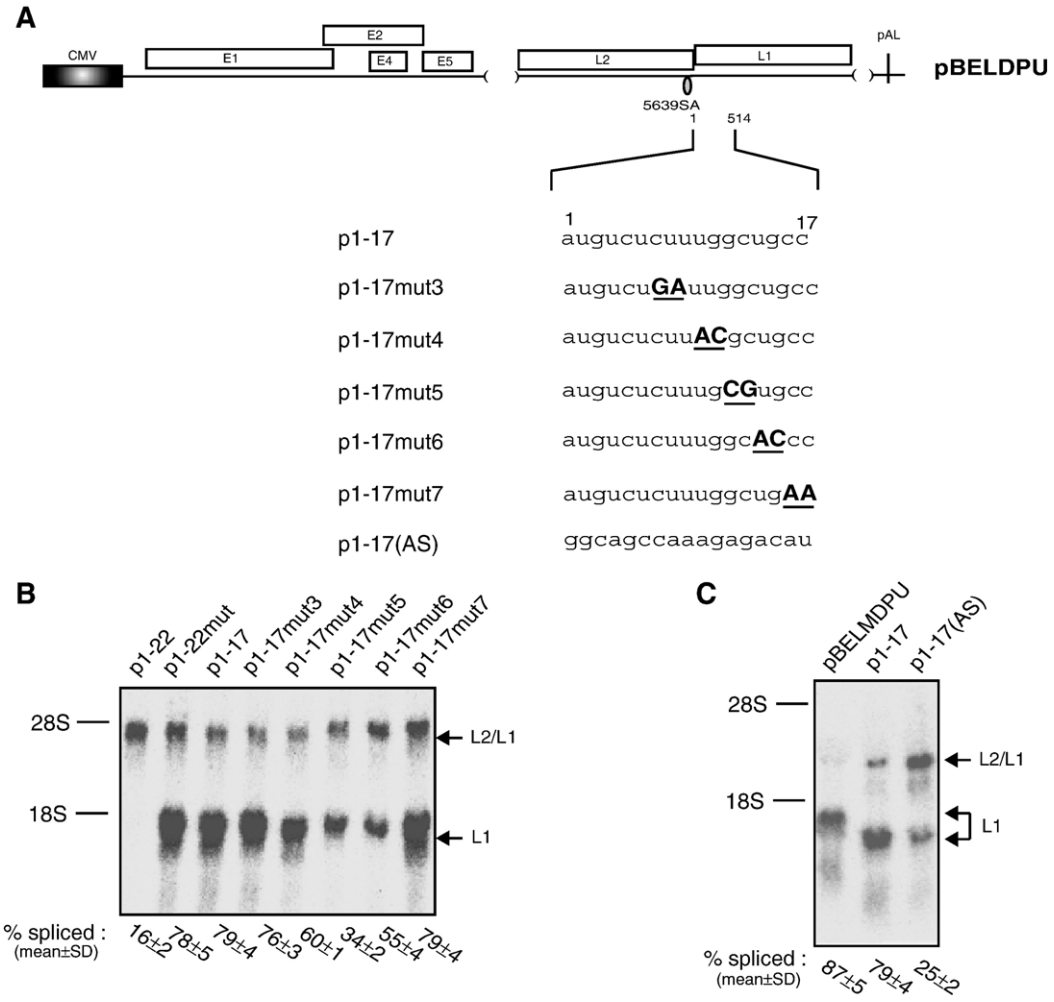

Fig. 3. (A) Schematic representation of plasmid pBELDPU (Zhao et al., 2005). The sequences inserted immediately downstream of the AG of the L1 3′ splice site are displayed. Numbers refer to nucleotide positions in L1, starting at the A in the AUG. Plasmid names are on the left. (B and C) Northern blots of total RNA extracted from HeLa cells transfected with the indicated plasmids. Blots were hybridised to the L1 probe (Fig. 1). Spliced mRNA as a percentage of total late RNA in each lane is indicated at the bottom of the gel. Numbers represent mean values with standard deviations from at least three independent transfections with the same plasmid.

none of the mutants completely inhibited splicing, we could not exclude that the 17-nucleotide L1 sequence stimulated splicing both through a sequence specific mechanism and by increasing the distance between the 3′ splice site and potential downstream splicing inhibitory elements in L1. To provide further evidence for a sequence specific activation of splicing by the first 17 nucleotides of L1, we replaced these 17 L1 nucleotides with the same sequence in antisense orientation, resulting in p1–17(AS) (Fig. 3A). In contrast to p1–17, this plasmid produced primarily unspliced late mRNAs (Fig. 3C), confirming that an RNA element in the first 17 nucleotides of L1 specifically enhanced splicing to the late 3′ splice site (Tables 1 and 2).

To confirm the presence of positive splicing elements in the HPV-16 L1 coding region, we inserted a larger, 1–177 L1 wt fragment in sense and antisense orientation upstream of the 178–366 sequence in the previously described plasmid pPL178–366 (Zhao et al., 2004) (Fig. 4A), resulting in pPL1–177–366 (Fig. 4A). Plasmid pPL178–366 produces primarily unspliced late mRNAs as a result of a splicing silencer located between L1 positions 178 and 226, as reported earlier (Zhao et al., 2004) (Fig. 4B). However, insertion of sequence 1–177 in sense orientation as in pPL1–177–366 (Fig. 4A) caused a significant increase in mRNA splicing (Fig. 4B), confirming that the first 177 nucleotides of L1 contained elements that enhanced splicing, whereas insertion of the same sequence in antisense orientation as in pPL1–177(AS)–366 (Fig. 4A) failed to promote splicing (Fig. 4B). We also inserted a mutant 1–177 sequence derived from the previously described L1 mutant (Zhao et al., 2004), in which splicing inhibitory sequences had been destroyed by point mutations, into pPL178–366, resulting in p1–177(mut)–366 (Fig. 4A). The mutant 1–177 also induced late mRNA splicing as expected (Fig. 4C). Taken together, these results demonstrated that the 5′-end of L1 contains elements that enhanced splicing of HPV-16 L1 mRNAs in a sequence specific manner.

The 17-nucleotide HPV-16 L1 splicing enhancer stimulates splicing in vitro

To test if the 17-nucleotide sequence could stimulate splicing in vitro, the first 17 nucleotides of L1 were inserted immediately

Table 1
Sequences of PCR oligonucleotides used to make pBEL-derived plasmids

Oligonucleotide	Sequence (5′→3′)
L2R	GGGCCCTTCTGATCCTTCTATAG
#L1AS	GGATCCCTGAAAAAAAATATGGTAAAC
16L1(3)AS	GGATCCCATCTGAAAAAAAATATGGTAAACG
16L1(9)AS	GGATCCAAGAGACATCTGAAAAAAAATATGG
16L1(17)AS	GGATCCGGCAGCCAAAGAGACATCTGAAAAAAAATATGG
16L1(22)AS	AAGCTTGGATCCCACTAGGCAGCCAAAGAG
16L1(22)ASmut	AAGCTTGGATCCCGCTGGGCAGCCACAG
16L1(129)AS	AAGCTTGGATCCCAAGTAGTCTGGATG
16L1M(129)AS	AAGCTTGGATCCCAGCAG
16L1(22)ASmut1	AAGCTTGGATCCCACTAGGCAGCCAAAGGCTCAT
16L1(22)ASmut2	AAGCTTGGATCCCGCTGGGCAGCCACAGAGACAT
16L1(22)mut3AS	GGATCCCACTAGGCAGCCAATCAGA
16L1(22)mut4AS	GGATCCCACTAGGCAGCGTAAGAGA
16L1(22)mut5AS	GGATCCCACTAGGCACGCAAAGAGA
16L1(22)mut6AS	GGATCCCACTAGGGTGCCAAAGAGA
16L1(22)mut7AS	GGATCCCACTATTCAGCCAAAGAGA
16L1(22)mut8AS	GGATCCCAGAAGGCAGCCAAAGAGA
16L1(22)mut9AS	GGATCCGACTAGGCAGCCAAAGAGA
16L1(22)mut10AS	GGATCCCACTAGGCAGCCACAGAGA
16L1(22)mut11AS	GGATCCCACTGGGCAGCCAAAGAGA
16L1(22)mut12AS	GGATCCCGCTAGGCAGCCAAAGAGA
16L1(17)mut3AS	GGATCCGGCAGCCAATCAGACATCTGAAAA
16L1(17)mut4AS	GGATCCGGCAGCGTAAGAGACATCTGAAAA
16L1(17)mut5AS	GGATCCGGCACGCAAAGAGACATCTGAAAA
16L1(17)mut6AS	GGATCCGGGTGCCAAAGAGACATCTGAAAA
16L1(17)mut7AS	GGATCCTTCAGCCAAAGAGACATCTGAAAA
L1START(*Sal*I)	GTCGACCAGCGCGCCAAGATGTC
16L1(1/*Cla*I)S	ATCGATATGTCTCTTTGGCTGCC
16L1(177/*Cla*I)AS	ATCGATTTTGTTATTGTTAGG
16L1(177/*Sal*I)AS	GTCGACTTTGTTATTGTTAGG
16L1M(177AS)*Cla*I	ATCGATCTTGTTGTTGTTAGGCTTC
17AS(AS)	GGATCCATGTCTCTTTGGCTGCCCTGAAAAAAAAATGGTAAAC
16L1(366)AS	AAGCTTGGATCCTAAAGGATGGCCACTAATGC

downstream of the 3′ splice site in the previously described, adenovirus-derived pTA plasmid (Collier et al., 2002; Zhao et al., 2004) (Fig. 5A). The pTA plasmid produces an mRNA that can be spliced in vitro to a certain extent (Figs. 5A and B). Insertion of the first 17 nucleotides of HPV-16 L1 enhanced splicing in vitro (Fig. 5B), demonstrating that this region contains a splicing enhancer that could enhance splicing of a heterologous RNA in vitro. Surprisingly, the 22-nucleotide sequence also promoted splicing (Fig. 5B). The reason for this is that it contains the positive element in the first 17 nucleotides of L1 and that the inhibitory element located in the 22-nucleotide sequence is dependent on downstream L1 sequences that are

Table 2
Sequences of the oligonucleotides used to make in vitro RNA synthesis plasmids

Oligonucleotide	Sequence (5′→3′)
L1 start (s)	GTCGACATGTCTCTTTGGCTGCCTAGTG
L1Mstart(*Sal*I)	GTCGACATGAGCCTGTGGCTGCCCAGCG
16L1–17(S)	cATGTCTTTTGGCTGCCg
16L1–17(AS)	gatccGGCAGCCAAAGACATggtac
16L1–22(S)	cATGTCTCTTTGGCTGCCTAGTGg
16L1–22mut(AS)	gatccCGCTGGGCAGCCACAGGCTCATggatc
WTsilencer1×4(S)	cTAGTGATAGTGATAGTGATAGTGAg
WTsilencer1×4(AS)	gatccTCACTATCACTATCACTATCACTAggtac
MUTsilencer1×4(S)	cCAGCGGCAGCGGCAGCGGCAGCGGg
MUTsilencer1×4(AS)	gatccCCGCTGCCGCTGCCGCTGCCGCTGggtac
PThnRNPA1(S)	cTATGATAGGGACTTAGGGTGa
PThnRNPA1(AS)	agcttCACCCTAAGTCCCTATCATAggtac
(Ad)AG17(AS)	ACGCGTGGCAGCCAAAGAGACATCTGTGGAAAAAAAAG
(Ad)AG22(AS)	ACGCGTCACTAGGCAGCCAAAGAGACATCTGTGGAAAAA

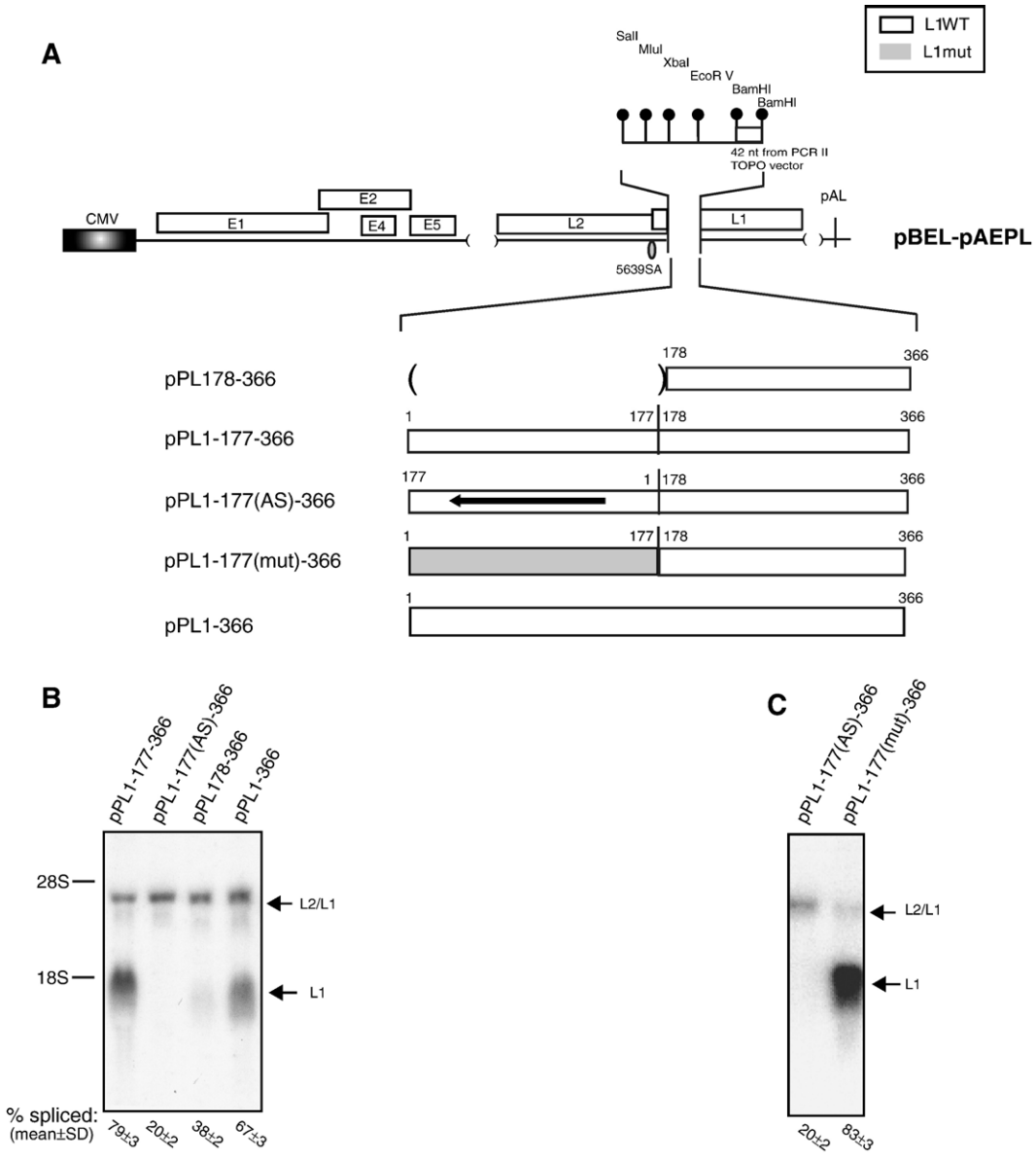

Fig. 4. (A) Schematic diagram of the pBEL-pAEPL plasmid (Zhao et al., 2004). A polylinker including a small sequence from the pCRII-TOPO cloning vector (Invitrogen) was inserted into L1, thereby replacing nucleotides 23 to 513 of L1 (numbering starts at the A in the AUG of L1). The indicated wt and mutant L1 sequences (Zhao et al., 2004) of various length were inserted into the polylinker as *Mlu*I–*Bam*HI or *Sal*I–*Mlu*I fragments. (B and C) Northern blots on total RNA extracted from HeLa cells transfected with the indicated plasmids. The blots are probed with the L1 probe (Fig. 1). Spliced mRNA as a percentage of total late RNA in each lane is indicated at the bottom of each gel. Numbers represent mean values with standard deviations from at least three independent transfections with the same plasmid.

absent in this plasmid (see below Fig. 6). Since these downstream L1 sequences were absent in pTA22, only the enhancer element was operational. We concluded that the first 17 nucleotides of L1 contain a splicing enhancer that promotes splicing of heterologous 3′ splice sites in vitro.

The identified splicing enhancer is not required for utilisation of the HPV-16 L1 3′ splice site in the absence of silencers

Although the results presented above established that the first 17 nucleotides of L1 contain a splicing enhancer, sequence analysis of the L1 3′ splice site shows that it conforms well to the consensus 3′ splice site. We therefore wished to test if the L1 3′ splice site was functional in the absence of the enhancer. The L1 sequence from 514 to 1512 was inserted in the antisense orientation in p1–3, resulting in p3A (Fig. 6A). In contrast to p1–3, plasmid p3A produced primarily spliced mRNAs (Fig. 6B), suggesting that the L1 3′ splice site could function in the absence of enhancer elements in L1, if only the downstream L1 sequences were altered as well, as in p3A (Fig. 6A). Similar results were obtained if the L1 sequence downstream of position 3 in p1–3 was replaced by the CAT sequence, as in p1–3CAT (Fig. 6C). The control plasmid p1–514CAT (Fig. 6A) produced primarily unspliced mRNA as expected, due to the presence of a longer L1 sequence (Fig. 6C).

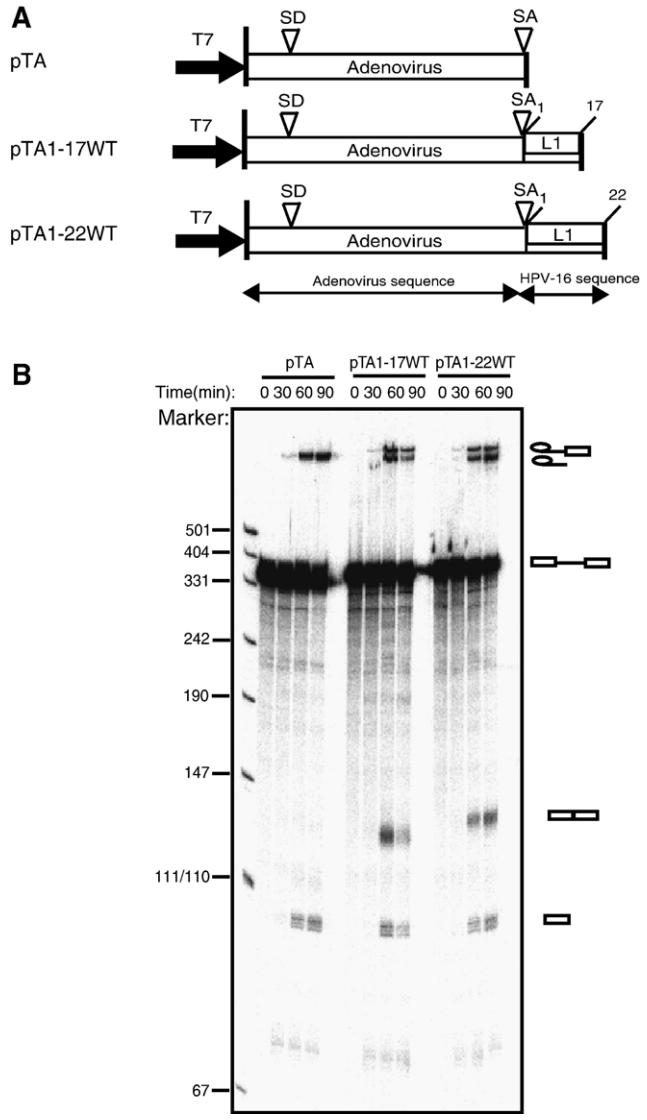

Fig. 5. (A) Schematic representation of the adenovirus derived pTA plasmid (Zhao et al., 2004), pTA1–17WT and pTA1–22WT that were used for in vitro synthesis of preRNA substrates for the in vitro splicing reactions. The numbering of the inserted HPV-16 L1 fragments starts at the A in the L1 AUG. T7, T7 RNA polymerase promoter; SD, adenovirus 5′ splice site; SA, adenovirus 3′ splice site. (B) In vitro splicing reactions using RNA derived from the indicated plasmids. The splicing products are indicated.

To test the idea that the splicing silencer in the first 22 nucleotides of L1 was functionally dependent on downstream L1 sequences, L1 sequences downstream of positions 17 or 22 were replaced by CAT, resulting in p17CAT and p22CAT, respectively (Fig. 6A). The p17CAT plasmid produced primarily spliced mRNAs, as expected (Fig. 6C). The efficient splicing of p22CAT (Fig. 6C) demonstrated that the inhibitory sequences in the first 22 nucleotides of L1 were unable to inhibit splicing in isolation (Fig. 6C). These results therefore established that the splicing inhibitory element located in the first 22 nucleotides of L1 was functionally dependent on downstream L1 sequences. In addition, the HPV-16 3′ splice site was only dependent on the enhancer when the splicing silencers in L1 were present, or active, indicating that the role of the enhancer is to counteract the effect of downstream splicing silencers in L1.

Sequence specific inhibition of HPV-16 late mRNA splicing by a splicing inhibitor in the first 22 nucleotides of L1

The results presented above demonstrated that a splicing silencer was located in the first 22 nucleotides of L1. Although it was dependent on downstream L1 sequences, it dominated over the splicing enhancer in the first 17 nucleotides of L1. We therefore wished to characterise the splicing inhibitor in the first 22 nucleotides of L1 further. Plasmid p1–22 and p1–22mut differ on 6 positions (Fig. 7A). These substitutions were introduced three by three in the wt 22-sequence, resulting in p1–22mut1 and p1–22mut2 (Fig. 7A). The results showed that the 3′-most mutations inactivated the silencer whereas the 5′-most three did not (Fig. 7B). Introduction of the three inactivating mutations one by one as in p1–22mut10, p1–22mut11 and p1–22mut12 (Fig. 7A) revealed that all three substitutions negatively affected the silencer (Fig. 7B). In addition, the negative effect on the silencer by the substitution at position 9 in p1–22mut10 (Fig. 7A) indicated that the silencer in the 3′-end of the 22-nucleotide sequence extended upstream of position 17 in L1 (Fig. 7B). We concluded that the 18–22-sequence was an important part of the splicing silencer in HPV-16 L1.

Next, pair-wise substitutions were introduced in the 22-nucleotide sequence (Fig. 7A). The majority of the mutations that caused an increase in splicing clustered to the 3′-end of the 22 sequence (Fig. 7B). However, the increase in splicing caused by the dinucleotide substitution at positions 7 and 8 in p1–22mut3 (Fig. 7B), together with the data obtained with p1–22mut10, which demonstrated the importance of nucleotide position 9 (Fig. 7B), indicated that sequences in this upstream region also contributed to the function of the splicing silencer. In conclusion, these results demonstrated that the splicing inhibitory element in the first 22 nucleotides of L1 acted in a sequence specific manner.

UV cross-linking of a 35- and 65-kDa protein to the splicing silencer and a 55-kDa protein to the splicing enhancer sequence in the HPV-16 L1 RNA

Having established that the enhancer and the silencer in the first 22 nucleotides of L1 both acted in a sequence specific manner, we wished to identify factors that interacted specifically with the two RNA elements. However, UV cross-linking of radiolabelled 1–17WT, 1–22WT and 1–22mut L1 RNAs (Fig. 8A) to nuclear extract revealed that all RNAs interacted with the same 55-kDa protein (Fig. 8B). Since we had shown above that the splicing silencer in the first 22 nucleotides of L1 was dependent on downstream sequences, we extended the L1 probe to L1 position 129 and performed UV cross-linking with RNAs 1–17WT and 1–129WT (Fig. 8A). We identified two factors (p35 and p65) that bound to 1–129WT L1 sequence, but not to the 1–129mut or 1–17WT RNAs (Fig. 8C), suggesting that they were involved in splicing inhibition. The 129WT L1 sequence inhibits splicing in transient transfection experiments,

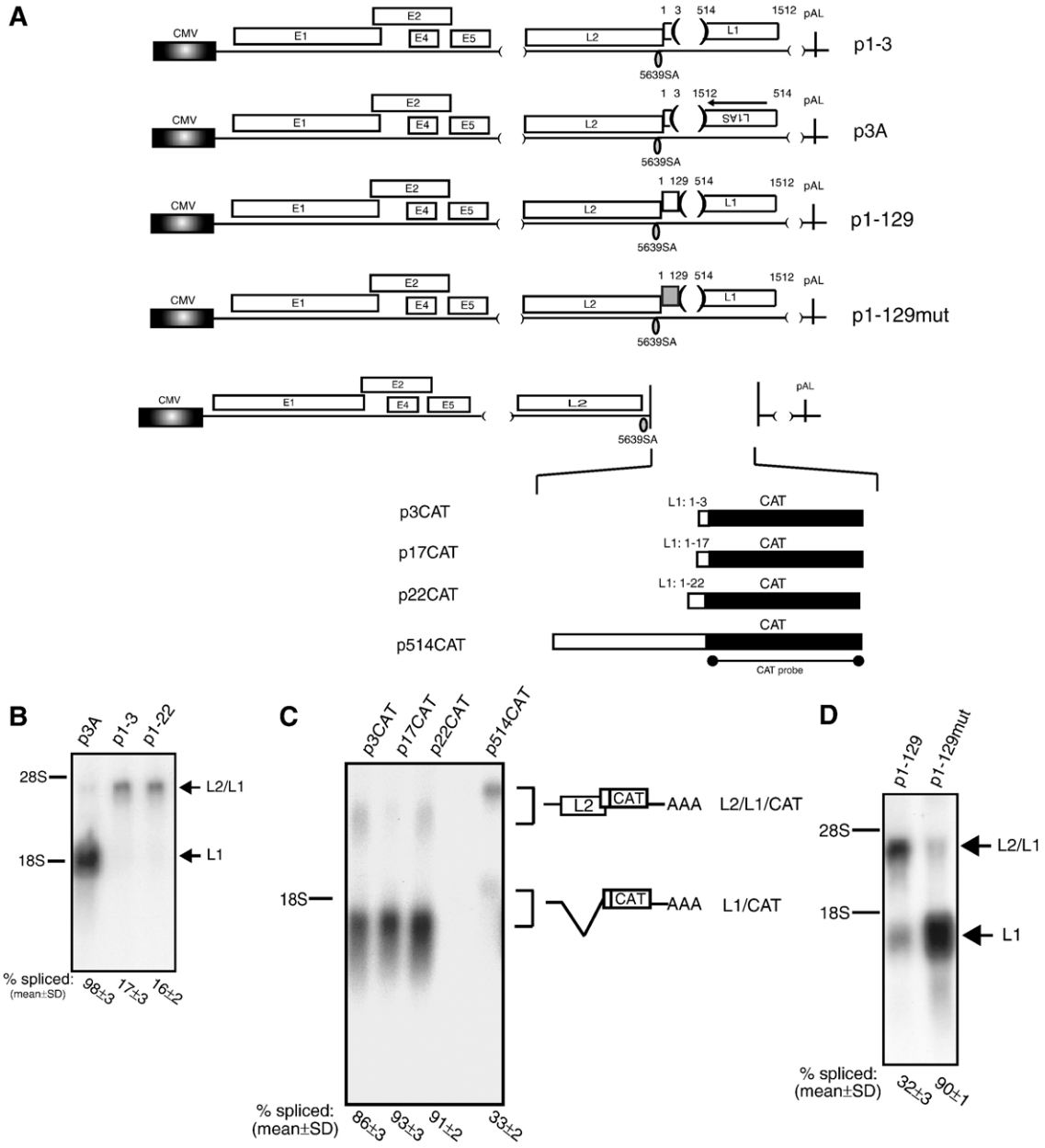

Fig. 6. (A) Schematic representation of plasmids p1–3, p3A, p1–129, p1–129mut, p3CAT, p17CAT, p22CAT and p514CAT. (B–D) Northern blots of total RNA extracted from HeLa cells transfected with the indicated plasmids. Blots were hybridised to the L1 probe (Fig. 1) in panels B and D, and the CAT probe (A) in panel C. Spliced mRNA as a percentage of total late RNA in each lane is indicated at the bottom of each gel. Numbers represent mean values with standard deviations from at least three independent transfections with the same plasmid.

whereas the 1–129mut L1 sequence does not (Fig. 6D). In conclusion, binding of the 35-kDa and 65-kDa proteins correlated with splicing inhibition, whereas binding of the 55-kDa protein correlated with splicing enhancement.

UV cross-linking of the 35-kDa protein to a multimer of the 18–22 L1 mRNA sequence

To explain the results of the mutational analysis in Fig. 7, which identified a splicing inhibitory sequence between nucleotide position 18–22 in L1 and the lack of specific UV cross-linking to the 1–22WT sequence compared to 1–17WT, we reasoned that a weak binding site for an inhibitory factor was present in the 3′-end of the first 22 nucleotides of HPV-16 L1, between nucleotide position 18 and 22, and that multiple binding sites for this factors were needed to obtain efficient binding. To test this idea, a multimer of the 18–22 L1 sequence 4×(18–22WT) and a multimer of the 18–22 mutant L1 sequence 4×(18–22mut) were generated and UV cross-linked to nuclear extract (Fig. 8A). As can be seen, a protein of the same size as the 35-kDa protein that interacted specifically with the 1–129WT L1 sequence also interacted with the 18–22WT L1 multimer 4×(18–22WT), but not with the 18–22 mutant L1 multimer 4×(18–22mut) (Fig. 8D). These results suggested that

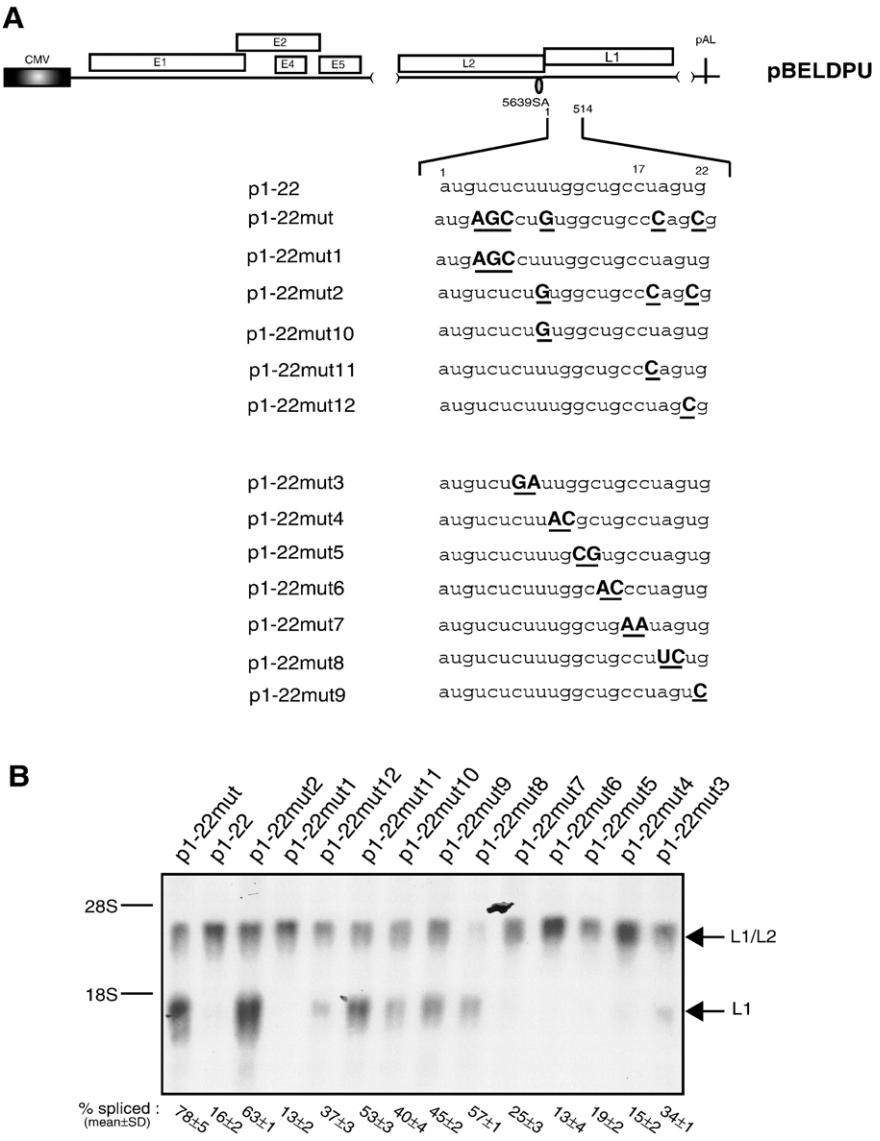

Fig. 7. (A) Schematic representation of plasmid pBELDPU (Zhao et al., 2005). The sequences inserted immediately downstream of the AG in the 3′ splice site in pBELDPU are displayed. Numbers refer to nucleotide positions in L1, starting at the A in the AUG. Plasmid names are on the left. (B) Northern blot of total RNA extracted from HeLa cells transfected with the indicated plasmids. Blots were hybridised to the L1 probe (Fig. 1). Spliced mRNA as a percentage of total late RNA in each lane is indicated at the bottom of the gel. Numbers represent mean values with standard deviations from at least three independent transfections with the same plasmid.

it was the 35 kDa factor that inhibited splicing. To confirm that the 35-kDa protein binding to the 4×(18–22WT) and the 1–129WT RNA was the same protein, a competition experiment was performed. The results revealed that the 4×(18–22WT) multimer competed efficiently with the 1–129WT RNA for the 35 kDa factor, whereas the 18–22 mutant multimer 4×(18–22mut) did not (Fig. 8E). This competition occurred with efficiency comparable to that of the 1–129WT RNA competitor (Fig. 8E), demonstrating that the 4×(18–22WT) and the 1–129WT RNA interacted with the same 35 kDa factor.

UV cross-linking of hnRNP A1 to all HPV-16 RNA sequences that bind the 35-kDa protein

To investigate if the 35-kDa protein that interacted with the 4×(18–22WT) and the 1–129WT RNA was the 35 kDa hnRNP A1 protein previously shown to bind to the 178–226 L1 silencer (Zhao et al., 2004), UV cross-linking reactions to 1–129WT RNA were subjected to competition with 178–226WT or 178–226mut competitors (Zhao et al., 2004). As can be seen, the 178–226WT sequence competed efficiently with the 1–129WT probe for the 35-kDa protein, whereas the 178–226mut RNA did not (Fig. 8E). These results indicated that the 35-kDa protein was hnRNP A1. Recombinant GST-hnRNP A1 also cross-linked to the 1–129WT and 4×(18–22WT) RNAs, but not to the 1–129mut or 4×(18–22mut) RNAs (Fig. 8F). The 178–226WT and 178–226mut RNAs (Zhao et al., 2004) served as controls (Fig. 8F). The results revealed that all HPV-16 wt L1 sequences that cross-linked to the 35 kDa factor in nuclear extracts also cross-linked to GST-hnRNP A1, whereas the mutant L1 sequences did not (Fig. 8F).

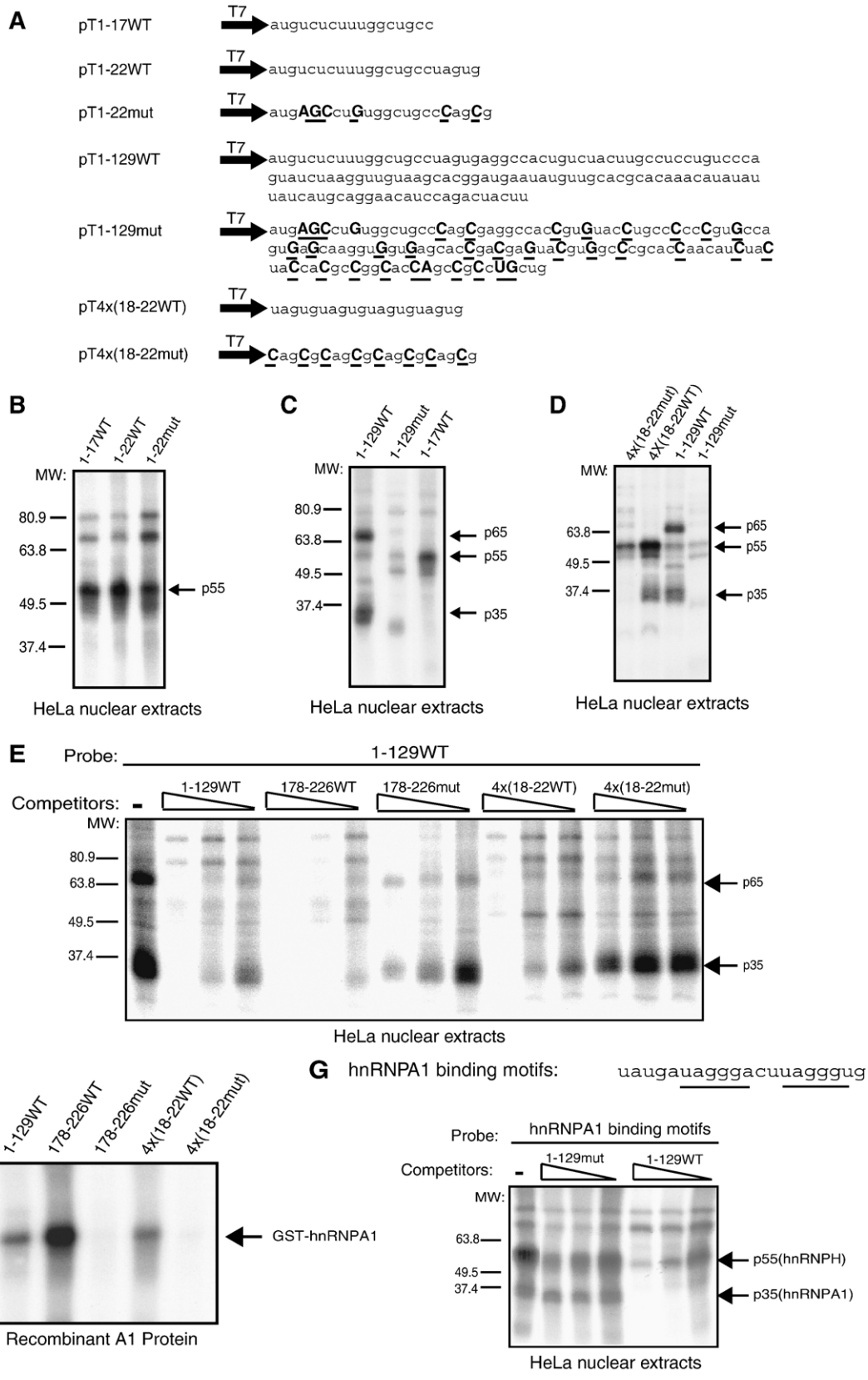

Fig. 8. (A) Sequences of the in vitro transcribed HPV-16 wild type and mutant L1 RNAs from the indicated plasmids. The mutant nucleotide positions are capitalised and underlined. The T7 promoter is indicated. (B–D) UV cross-linking of nuclear extract to the indicated radiolabelled RNAs. The p35, p55 and p65 proteins cross-linking to the HPV-16WT L1 sequence are indicated to the right. (E) UV cross-linking of nuclear extract to radiolabelled 1–129WT RNA in the absence or presence of the indicated, serially diluted unlabelled competitor RNAs. (F) UV cross-linking of recombinant GST-hnRNP A1 to the indicated radiolabelled RNAs. (G) UV cross-linking of nuclear extract to an optimal hnRNP A1-binding site in the absence or presence of serially diluted, unlabelled competitor RNAs.

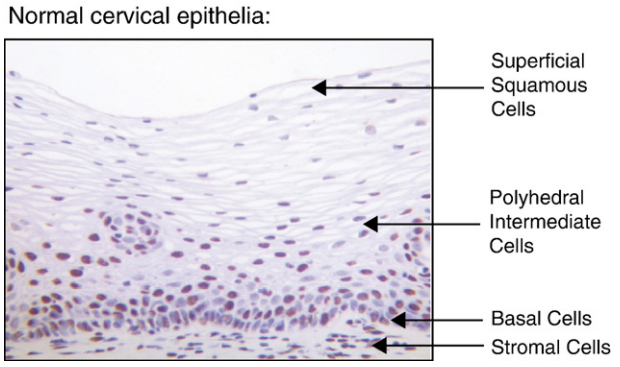

Fig. 9. Immunohistochemical detection of hnRNP A1 in normal cervical epithelium. The epithelial sections were stained with mouse monoclonal antibody specific for hnRNP A1 (clone 9H10, Abcam).

We also cross-linked nuclear extract to a high affinity hnRNP A1-binding site (Fig. 8G) and investigated if the 1–129WT RNA competed with the hnRNP A1-binding site for the 35 kDa, hnRNP A1 protein. As expected, the 129WT L1, but not the 1–129mut L1, sequence competed for hnRNP A1 (Fig. 8G). Note that hnRNP H that has been shown previously to bind to the hnRNP A1 high affinity site (5) is also detected here (Fig. 8G). Although some competition for p55 was seen with 1–129WT RNA, it is less efficient than competition for the 35 kDa hnRNP A1 (Fig. 8G). In conclusion, these results confirmed that hnRNP A1 binds specifically to multiple HPV-16 L1 RNA sequences with splicing inhibitory activity.

Expression of hnRNP A1 is cell differentiation dependent in cervical squamous epithelia

The results described above indicated that hnRNP A1 inhibits splicing into the L1 region by binding to multiple splicing silencers that override the effect of the splicing enhancer in the first 17 nucleotides of L1. These results also suggested that high levels of hnRNP A1 inhibit the use of the L1 3′ splice site and prevent L1 expression and predicted that hnRNP A1 levels should be high in cervical epithelial cells that are not permissive for HPV-16 L1 production, for example cells in the lower layer of the cervical epithelium. In contrast, cells in the superficial levels of the epithelium that are permissive for HPV-16 L1 expression and virus production should not express hnRNP A1. Staining of sections from normal cervical epithelium with antiserum against hnRNP A1 revealed that high levels of hnRNP A1 were found in the lower layers of the epithelium, whereas hnRNP A1 was undetectable in the superficial layers (Fig. 9). These results supported the conclusion that hnRNP A1 is a negative regulator of HPV-16 L1 expression.

Discussion

hnRNP A1 has been shown to be involved in regulation of splicing of a multitude of cellular and viral mRNAs (Cartegni et al., 2002; Zheng, 2004). In general hnRNP A1 acts by inhibiting the use of a certain splice site. This effect is antagonised by SR proteins, primarily ASF/SF2 (Mayeda and Krainer, 1992). Several models for the action of hnRNP A1 have been proposed. Binding of hnRNP A1 to a high affinity site on HIV-1 pre-mRNAs has been shown to cause multimerisation and filling up of multiple low affinity hnRNP A1 biding sites on the mRNA (Damgaard et al., 2002). In one case, multimerisation is occurring in combination with binding of hnRNP A1 to an intronic splicing silencer overlapping the branch point of the 3′ splice site (Damgaard et al., 2002; Tange et al., 2001). These multiple hnRNP A1 interactions with the pre-mRNA prevented binding of U2 snRNP to the branch point, thereby inhibiting splicing (Tange et al., 2001). This effect was counteracted by ASF/SF2 and to some extent by SC35. Another HIV-1 splice site was shown to be under control of an exonic splicing silencer overlapping and enhancer. These elements were binding to hnRNP A1 and SC35, respectively (Zahler et al., 2004). In this case, a direct competition between the two factors determined the effect on splicing (Zahler et al., 2004). Binding of hnRNP A1 to HIV-1 mRNAs has also been shown to prevent binding of U2AF65 to the upstream polypyrimidine tract of a 3′ splice site, either by inhibiting U2AF65 directly or indirectly by inhibiting binding of U2AF35 (Domsic et al., 2003). The results shown here with binding of hnRNP A1 to multiple sites within HPV-16 L1 combined with the previously published results demonstrating that binding of hnRNP A1 to at least one of these sequences causes inhibition of splicing in vitro allow us to speculate that multimerisation of hnRNP A1 and binding to multiple sites on the mRNA may result in inhibition of splicing by inhibiting binding of either U2 snRNP or U2AF to the HPV-16 mRNA.

The first 17 nucleotides of L1 is relatively pyrimidine rich and contains a potential binding site (UCUU) for the splicing regulatory factor named polypyrimidine tract binding protein (PTB) (Perez et al., 1997). However, mutations that altered the potential PTB binding site UCUU did not affect mRNA splicing and we have been unable to show that the 55-kDa protein is PTB. Another interesting feature of this sequence is that it displays a significant homology with an HIV-1 sequence present in an ESS element termed ESS2p (Jacquenet et al., 2001). While the HPV-16 sequence in the first 17 nucleotides of L1 is UUGGCUGCC, HIV-1 M isolates has the consensus of UUGGGUGUC, HIV-1 N has UUGGCUGCC and HIV-1 O has UUGGAUGCC. It was shown that hnRNP H binds to the HIV-1 M (Jacquenet et al., 2001). However, it is unclear if hnRNP H binds to the other HIV-1 sequences since they all lack the triple G motif that appears to be crucial for hnRNP H binding to an RNA substrate (Caputi and Zahler, 2001). Similarly, the HPV-16 RNA sequence lacks the triple G motif and we were unable to show specific binding to hnRNP H. The HPV-16 sequence and the HIV-1 M sequence differ in only two positions, one of which is the third G in the triple G motif. This position is not conserved amongst the three HIV-1 M, N and O groups either. The sequence similarity and the close proximity of the sequences to 3′ splice site argue for a conserved role of these sequences in gene expression. This is further supported by the relatively high conservation of this sequence amongst HPV types in different genera. It will be of interest to determine the identity of the factor that interacts with the first 17 nucleotides of the HPV-16 L1 sequence. The enhancer appears to be located

between nucleotide positions 10 and 17 in L1. In HPV-16, this sequence is UGGCUGGCC, whereas in many other HPV types a remarkably well conserved sequence is UGGCGGCC (Billakanti et al., 1996).

Although we show that the HPV-16 L1 3′ splice site functions perfectly well in the absence of downstream L1 sequences, probably due to its relatively long uninterrupted polypyrimidine tract (9 consecutive pyrimidines), we cannot rule out that hnRNP A1 competes with SR proteins binding to the late HPV-16 mRNAs. In this respect it is of interest to point out that ASF/SF2, which can antagonise the effect of hnRNP A1, has been shown to interact with the UTR of late HPV-16 mRNAs (McPhillips et al., 2004; Mole et al., 2006). Although the functional significance of this interaction between ASF/SF2 and the viral mRNA remains unknown, ASF/SF2 could potentially affect binding of hnRNP A1 negatively, thereby promoting HPV-16 late gene expression. Interestingly, differentiating murine erythroblasts exhibit activation of exon 16 splicing of the cytoskeletal protein 4.1R mRNA as the hnRNP A/B levels decrease in a differentiation dependent manner (Hou et al., 2002). It would be interesting to investigate the expression of hnRNP A1 in relation to HPV-16 genes in a differentiating environment to determine the role of hnRNP A1 in the HPV-16 life cycle.

Materials and methods

Plasmid constructions

Plasmid constructions are described in a supplementary section.

Transfections, RNA extraction and Northern blotting

Transfections were performed in HeLa cells according to the Fugene 6 method (Roche Molecular Biochemicals). Total RNAs were extracted from the transfected cells at 24 h post transfection according to the RNeasy Mini protocol (Qiagen). All plasmids were analysed in a minimum of three independent transfections. All quantitations represent mean values from at least three independent transfections. The data variation in each transfection experiment was less than 20%. Northern blot analysis was performed as described previously (Collier et al., 2002). The L1 and CAT probes are indicated in Figs. 1 and 3. All Northern blots were quantified in a Bio-Rad phosphorimager (GS-250).

UV cross-linking and preparation of cellular extracts and recombinant hnRNP A1

UV cross-linking and synthesis of radiolabelled RNA were performed as previously described (Spångberg et al., 2000). HeLa cell nuclear extracts were prepared according to the method of Dignam et al. (1983). GST-hnRNP A1 was prepared as described previously (Sokolowski et al., 1999). Twenty micrograms of nuclear extract or fifty nanograms of recombinant hnRNP A1 was used for each UV cross-linking.

In vitro splicing

In vitro splicing experiment was performed as previously described (Zhao et al., 2004). 70,000 cpm radiolabelled capped pre-mRNA substrate and 8 μg of HeLa nuclear extract were used in the in vitro splicing reaction.

Immunohistochemical detection of hnRNP A1

Immunohistochemical detection was performed as described previously (Oberg et al., 2005) except that 100 μl of mouse monoclonal antibody specific for hnRNP A1 (clone 9H10, Abcam) or anti-hnRNP A1 antiserum Y-15, Santa Cruz Biotechnologies, was used at a dilution of 1:25. Antigen retrieval was carried out using Trilogy pressure cooker method (Cell Marque) as described by the manufacturer.

Acknowledgments

We thank Margaret Rush, Daniel Öberg, Monika Somberg, Anna Tranell and members of Göran Magnussons, Catharina Svenssons and Göran Akusjärvis laboratories for discussions. We are also grateful to Christina Tobin for contributing to some experiments.

Research supported by the Swedish Cancer Society, by the Swedish Research Council-Medicine, by Linneus Support from the Swedish Research Council to Uppsala RNA Research Center, by Science Foundation Ireland, by Technological Sector Research Study I, Ireland, and by the Dublin Institute of Technology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.08.002.

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