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Multiple ASF/SF2 Sites in the Human Papillomavirus Type 16 (HPV-16) E4-Coding Region Promote Splicing to the Most Commonly Used 3′-Splice Site on the HPV-16 Genome▽

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Our results presented here demonstrate that the most abundant human papillomavirus type 16 (HPV-16) mRNAs expressing the viral oncogenes E6 and E7 are regulated by cellular ASF/SF2, itself defined as a proto-oncogene and overexpressed in cervical cancer cells. We show that the most frequently used 3′-splice site on the HPV-16 genome, site SA3358, which is used to produce primarily E4, E6, and E7 mRNAs, is regulated by ASF/SF2. Splice site SA3358 is immediately followed by 15 potential binding sites for the splicing factor ASF/SF2. Recombinant ASF/SF2 binds to the cluster of ASF/SF2 sites. Mutational inactivation of all 15 sites abolished splicing to SA3358 and redirected splicing to the downstream-located, late 3′-splice site SA5639. Overexpression of a mutant ASF/SF2 protein that lacks the RS domain, also totally inhibited the usage of SA3358 and redirected splicing to the late 3′-splice site SA5639. The 15 ASF/SF2 binding sites could be replaced by an ASF/SF2-dependent, HIV-1-derived splicing enhancer named GAR. This enhancer was also inhibited by the mutant ASF/SF2 protein that lacks the RS domain. Finally, silencer RNA (siRNA)-mediated knockdown of ASF/SF2 caused a reduction in spliced HPV-16 mRNA levels. Taken together, our results demonstrate that the major HPV-16 3′-splice site SA3358 is dependent on ASF/SF2. SA3358 is used by the most abundantly expressed HPV-16 mRNAs, including those encoding E6 and E7. High levels of ASF/SF2 may therefore be a requirement for progression to cervical cancer. This is supported by our earlier findings that ASF/SF2 is overexpressed in high-grade cervical lesions and cervical cancer.

Human papillomavirus type 16 (HPV-16) is the foremost cause of cervical cancer, which is one of the most common cancers in women globally (10, 37). Persistence of high-risk HPV types, such as HPV-16, is the highest risk factor for the development of cervical cancer. The majority of all DNA viruses that establish persistence have evolved a highly organized gene expression program, often divided into clear early and late phases. The HPV-16 genome contains an early promoter that could potentially express mRNAs encoding all viral gene products, and a late differentiation-dependent promoter that specifically excludes expression of E6 and E7 (21). The switch from early to late gene expression includes a promoter switch as well as derepression and activation of the late poly(A) signal and late splice sites (16). To activate late splice sites and the late poly(A) signal, many early splice sites and the early poly(A) signal must be downregulated to allow for competition from mutually exclusive late splice sites and poly(A) signal (8, 26, 36). Other HPV-16 splice sites are used by both early and late mRNAs and should function well in both mitotic cells and terminally differentiated cells. One of the major splice sites used by both early and late mRNAs is SA3358 (Fig. 1A). This splice site is outstanding in that it is used to produce the majority of all HPV-16 mRNAs, including the mRNAs of the oncogenes E6 and E7 and the E4, E5, L1, and perhaps L2 proteins. In contrast, efficient usage of SA3358 specifically prevents expression of HPV-16 E1 and E2.

Many, if not all, HPV types contain a 3′-splice site in the E4 open reading frame (orf) that is spliced to an upstream 5′-splice site that joins the E1 AUG with the E4 orf. In HPV-16, these splice sites are named SA3358 and SD880 (Fig. 1A), whereas they are named SD847 and SA3325 in HPV-11 and SD877 and SA3295 in HPV-31 (1). Splicing between HPV-16 SD880 and SA3358 (6, 9, 27), or the corresponding sites in HPV-11 (5, 20, 23) and HPV-31 (11, 12), occurs on the most-common early mRNAs encoding E6 and E7, as well as on the most-abundant late mRNA encoding E4. In addition, the most-common L1 mRNA is also spliced between SD880 and SA3358 (17), or the corresponding sites in HPV-11 (23) and HPV-31 (12, 22). Analysis of HPV-16 splicing in cervical scrape samples revealed that splicing between SD880 and SA3358 was the most-common splicing event in both low- and high-grade cervical lesions (25). *In vitro* transfection experiments demonstrated that splicing to SA3358 was required for efficient expression of E6 and E7 (2). As a matter of fact, splicing between SD880 and SA3358 was required for production of E6 and E7 quantities that were needed for transformation of cells by these HPV proteins. In HPV-31, SA3295 corresponds to HPV-16 SA3358. Mutational inactivation of HPV-31 SA3295 in an infectious molecular clone of HPV-31 immediately caused splicing to a cryptic 3′-splice site located three nucleotides further down (15). These results indicated

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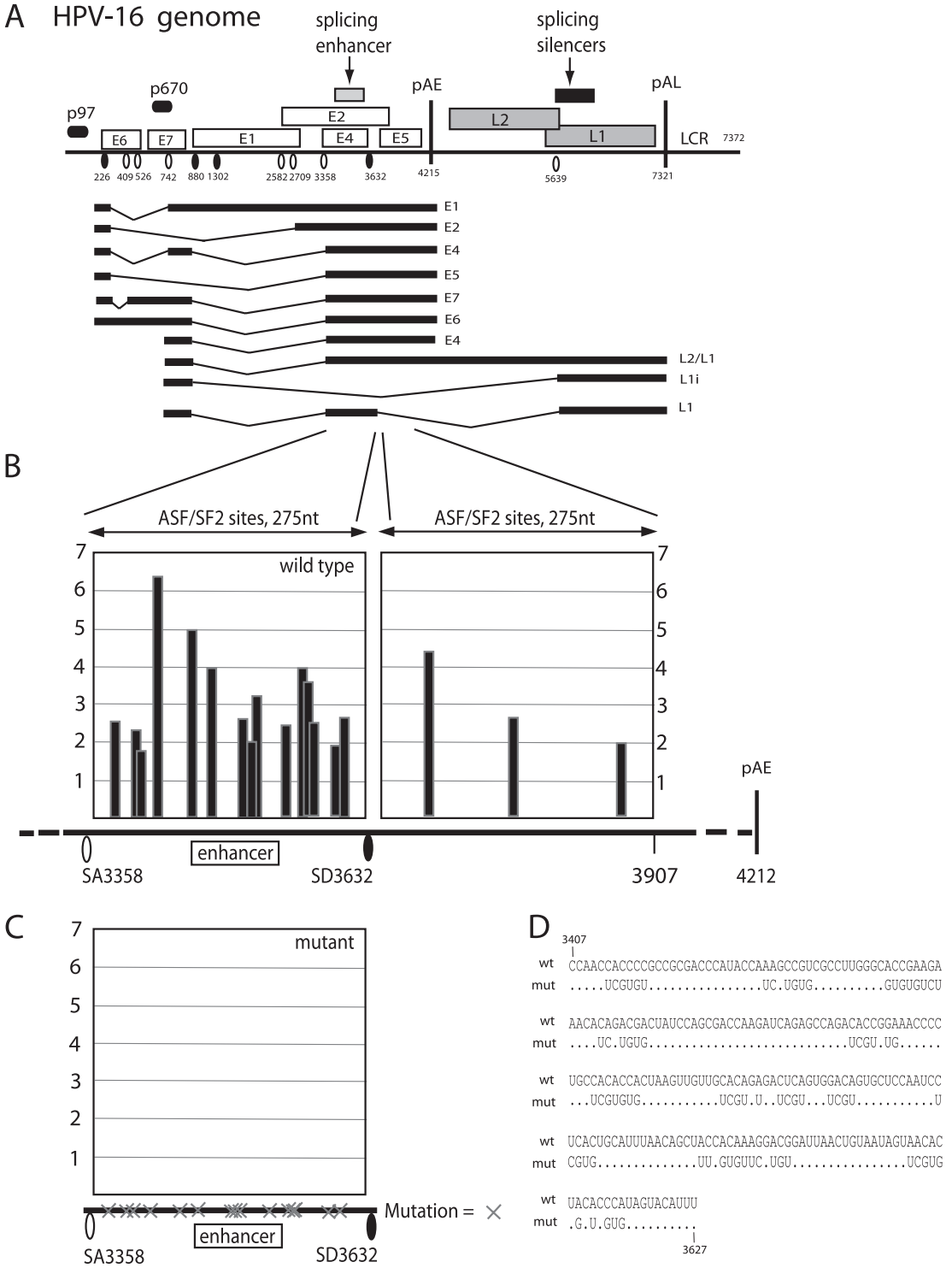

FIG. 1. (A) Schematic representation of the HPV-16 genome. Early and late viral promoters p97 and p670 are indicated. Numbers indicate nucleotide positions of 5′-splice sites (filled circles), 3′-splice sites (open circles), or early and late poly(A) signals pAE and pAL, respectively. LCR, long control region. A few selected early and late mRNAs are shown (1). Previously described splicing silencers and enhancers are indicated (24, 34, 35). (B) Diagram with potential ASF/SF2 sites upstream and downstream of SD3632 predicted by ESEfinder (4). Heights of the bars represent degrees of similarity to ASF/SF2 binding sites according to ESEfinder. HPV-16 splice sites SA3358 and SD3632 are indicated. Numbers indicate nucleotide positions in the HPV-16 genome. The position of a previously described enhancer is indicated (24). (C) ASF/SF2 sites in the mutant HPV-16 sequence in which the ASF/SF2 sites had been inactivated, as predicted by ESEfinder (4). (D) Exact sequences of the wt and mutant (mut) HPV-16 Predicted sequences between nucleotide positions 3407 and 3627 in the HPV-16 genome. Dots represent identical nucleotides.

that HPV-31 SA3295 is under the control of strong splicing enhancer elements and that there is a strong pressure on the virus to maintain a 3′-splice site in that exact region.

We have previously reported that HPV-16 SA3358 has an exceptionally poor 3′-splice site sequence compared to a consensus 3′-splice site (24). This is due primarily to an almost complete absence of an upstream row of uninterrupted pyrimidines that normally characterize an efficiently utilized 3′-splice site. However, SA3358 is one of the most efficiently used splice sites on the HPV-16 genome (24, 33). We have previously shown that utilization of HPV-16 SA3358 is totally dependent on exonic sequences downstream of SA3358, and we concluded that a splicing enhancer was located downstream of SA3358 (24). Here, we have followed up these findings; we demonstrate that the enhancer elements downstream of HPV-16 SA3358 are binding sites for ASF/SF2, and we show that ASF/SF2 enhances splicing to SA3358.

MATERIALS AND METHODS

Plasmids. pMT1SD was constructed by cleaving pT1SD (24) with SalI and BssHII, filling in overhangs, and religation. pTEx4m was created by first PCR amplifying an HPV-16 sequence with oligonucleotides exon4mutS (5′-GGCGC GCCCAACTCGTGTGCCGCGACCCATACCATCGTGTGCGCCTTGGGT CGTGTGTCTAACATCGTGTGCTATCCAGCGACCAAGATCAGAGCCA GATCGTGTGAACCCCTGCTCGTGTGCTAAGTTGTTGTCGTGTG-3′) and exon4mutAS (5′-TTCTAGAAAATGTACTCACACGAGCACACGAACT ATTACAGTTAATCACACGAACACGAAGCTGTTAAATGCACACGAGA TTGGAGCACACGACACACGATCACACGACAACAACTTAGCACACGA GCAGGGGTTCACACGATCT-3′) (Scandinavian Gene Synthesis AB). The PCR fragment was cleaved with XbaI and BssHII and subcloned into pT1SD (24), resulting in pTEx4m. pMTEx4m was constructed by cleaving pTEx4m with SalI and BssHII, filling in overhangs, and religation. Plasmid pT7wtexon4 was created by PCR amplifying a sequence from pBEL (35) with oligonucleotides exon4wtSKpnI (5′-GGGTACCCCAACCACCCCGCCGCGACCCATAC-3′) and exon4wtASXbaI (5′-TTCTAGAAAATGTACTATGGGTGTAGTGTTA C-3′). The PCR fragment was cleaved with KpnI and XbaI and subcloned into pUC19T7 (32), resulting in pT7wtexon4. pT7mutexon4 was created by PCR amplifying a sequence from pTEx4m with oligonucleotides exon4mutSKpnI (5′-CGGTACCCCAACTCGTGTGCCGCGACCCATAC-3′) and M3A (5′-GGGC CCAGGCCTCGACACTGCAGTATACAATGTACAATGCT-3′). The PCR fragment was cleaved with KpnI and XbaI and subcloned into pUC19T7 (32), generating pT7mutexon4. Glutathione *S*-transferase (GST), GST-ASF/SF2, and GST-ASFDRS were generated using pGEX-GST (GE Healthcare).

The following plasmids have been described previously: pBEL (35), pBELM (35), pBEL-OPSA (35), pBSplice and pBSpliceM (30), pT1SD (24), pMT198 (29), pBearly97 (33), and pT1-5/GAR (A. Tranell, E. M. Fenyö, and S. Schwartz, submitted). pASF/SF2, pASFDRS, and pAdE4orf4 are kind gifts from Göran Akusjärvi, IMBIM, Uppsala University, Sweden.

Transfection and cell culture. Maintenance and transfections of HeLa cells with Fugene 6 (Roche Molecular Biochemicals) have been described previously (30).

RNA extraction, Northern blotting and radiolabeled DNA probe synthesis, and RT-PCR. Cytoplasmic RNA was extracted using isoB/NP-40 buffer as described previously (32). Northern blot analysis was carried out as described previously (30). Reverse transcription-PCR (RT-PCR) with cytoplasmic RNA was performed as previously described using oligonucleotides 757s (35), p97s (33), and L1aM (35), E4A (35), or 135 (5′-CCTCGAGCTACTTATCGTCGTC ATCCTTGTAATCTGGTTTCTGAGAACAGATGGGGCACAC-3′), as indicated in the figures. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified as control using previously described primers (13).

siRNA-mediated knockdown of ASF/SF2. Transfections of HeLa cells with the ASF/SF2 siGenome Smartpool-designed silencer RNA (siRNA) (Dharmacon) was performed using Oligofectamine (Invitrogen) according to the manufacturer's protocol. The sequences of the sense and antisense strands of the four siRNA duplexes were as follows: ASF/SF2-1 sense, 5′-GAAAGAAGAUAUG ACCUAUUU-3′; ASF/SF2-1 antisense, 5′-AUAGGUCAUAUCUUCUUUCU U-3′; ASF/SF2-2 sense, 5′-UAACUUACCUCCAGACAUCUU-3′; ASF/SF2-2 antisense, 5′-GAUGUCUGGAGGUAAGUUAUU-3′; ASF/SF2-3 sense, 5′-U GAAGCAGGUGAUGUAUGUUU-3′; ASF/SF2-3 antisense, 5′-ACAUACAU CACCUGCUUCAUU-3′; ASF/SF2-4 sense, 5′-CGACGGCUAUGAUUACG AUUU-3′; ASF/SF2-4 antisense, 5′-AUCGUAAUCAUAGCCGUCGUU-3′.

Briefly, HeLa cells were plated in regular growth medium in a six-well tissue culture dish 24 h prior to transfection. The next day, cells were transfected with 133 nM siRNA mix in a total volume of 1.8 ml of serum- and antibiotic-free Dulbecco's modified Eagle's medium (DMEM) with 4 μl Oligofectamine. After incubation for 4 h, 0.5 ml DMEM containing three times the normal concentration of antibiotics and fetal bovine serum was added to the transfected HeLa cells without removal of the transfection mixture. At 24 h after siRNA transfection, cells were transfected with 0.5 μg of subgenomic HPV-16 plasmid pBELM (35). At 72 h after the initial siRNA transfection, cytoplasmic RNA was extracted and RT-PCR was performed. A total cell extract was used for Western blotting with antibody mab65 (a kind gift of Göran Akusjärvi, Uppsala University, Sweden) against ASF/SF2 to ascertain efficient ASF/SF2 knockdown.

Purification of recombinant GST proteins and UV cross-linking. Purification of recombinant GST fusion proteins using glutathione-Sepharose (GS) was performed as specified by the manufacturer (GE Healthcare). Plasmids pT7mutexon4 and pT7wtexon4 were individually linearized with XbaI and subjected to *in vitro* transcription with T7 RNA polymerase in the presence of with [γ-^{32}P]UTP. Each RNA probe (10^5 cpm) was UV cross-linked to GST-ASF/SF2 or GST-ASFDRS as previously described (28, 31).

RESULTS

Multiple ASF/SF2 binding sites downstream of SA3358. SA3358 is one of the major 3′-splice sites on the HPV-16 genome, and it is required for the production of all early mRNAs, except E1 and E2 mRNAs, as well as L1 and L2 mRNAs (Fig. 1A). We have previously shown that SA3358 is suboptimal and totally dependent on a downstream splicing enhancer (24). However, splicing factors that regulate SA3358 have not been identified. We have used the ESEfinder program (4) to search for potential SR-protein binding sites downstream of SA3358. Interestingly we found 15 potential ASF/SF2 binding sites in the 275-nucleotide exon located between SA3358 and SD3632, overlapping the previously identified splicing enhancer (Fig. 1A and B) (24). In contrast, analysis of the adjacent 275 nucleotides located downstream of SD3632 identified only three potential ASF/SF2 binding sites (Fig. 1B). This analysis suggested that ASF/SF2 may play a role in the regulation of SA3358. If these sites regulated SA3358, mutational inactivation of the predicted ASF/SF2 sites would impair splicing to SA3358. To test this idea, we mutationally inactivated all 15 ASF/SF2 sites located between SA3358 and SD3632. Analysis of the mutant sequence in ESEfinder (4) revealed that the predicted ASF/SF2 sites were no longer recognized by ESEfinder (Fig. 1C). The exact nucleotide changes are shown in the alignment of wild-type (wt) and mutant HPV-16 sequence in Fig. 1D.

Mutational inactivation of all predicted ASF/SF2 sites downstream of SA3358 inhibits the use of SA3358 and redirects splicing to late splice acceptor SA5639. In order to investigate if mutational inactivation of the predicted ASF/SF2 binding sites downstream of SA3358 affected HPV-16 mRNA splicing, we inserted all 15 mutations in subgenomic HPV-16 plasmid pBELM (Fig. 2A) (35), resulting in plasmid pTEx4m (Fig. 2A). The pBELM plasmid has been described by us previously (35). It produces high levels of E4 mRNA spliced from SD880 to SA3358 and low levels of the L1 mRNAs as a result of mutationally inactivated splicing silencers at the late 3′-splice site SA5639 (35). Analysis of mRNA produced from pBELM and pTEx4m in transfected HeLa cells confirmed that pBELM produced high levels of E4 mRNA and low levels of

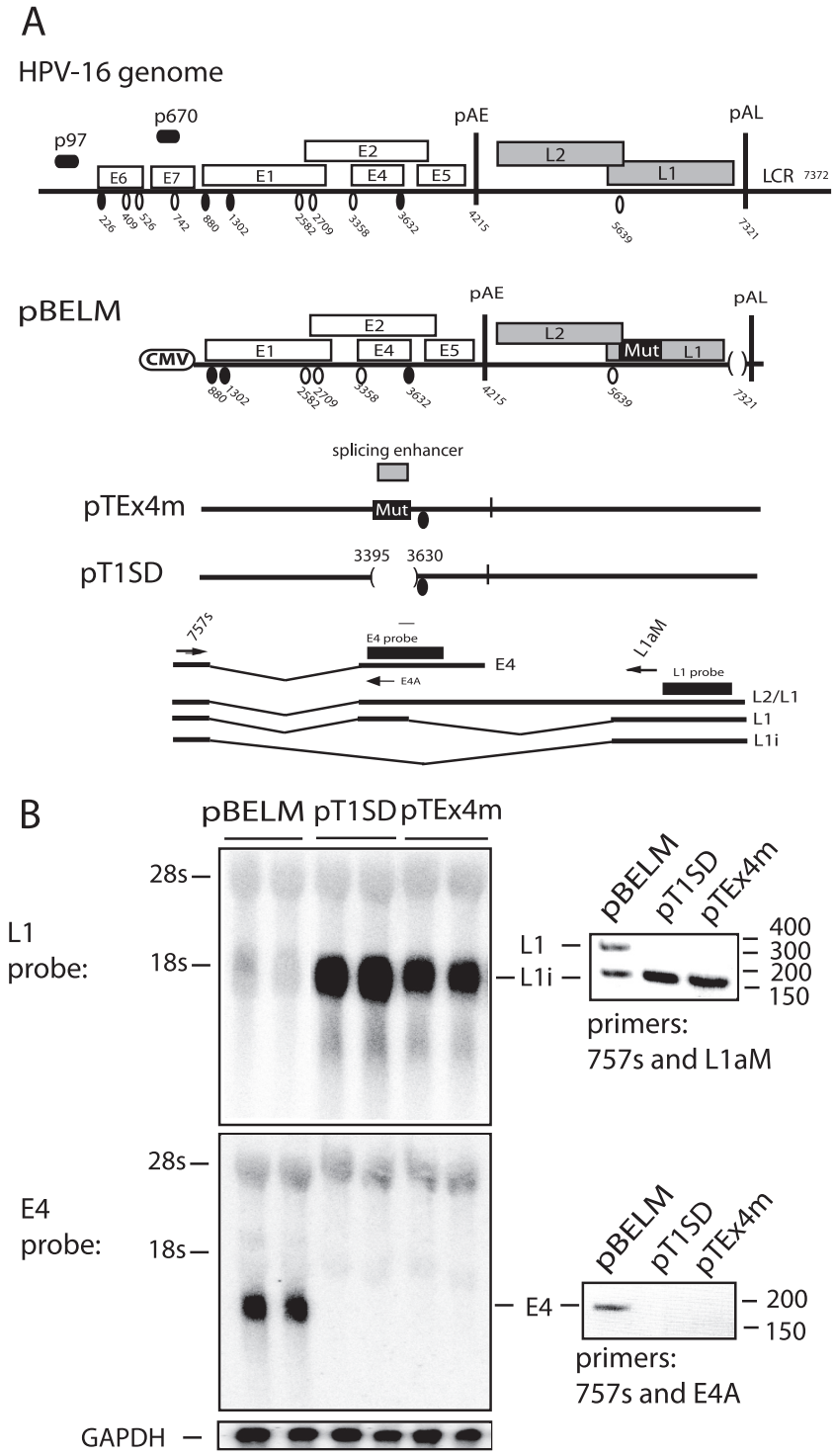

FIG. 2. (A) Schematic representation of the HPV-16 genome and the subgenomic HPV-16 expression plasmids pBELM (35), pTEx4m, and pT1SD (24). Numbers indicate nucleotide positions of 5′-splice sites (filled circles) and 3′-splice sites (open circles) or poly(A) sites pAE and pAL. Structures of selected HPV-16 mRNAs produced by pBELM (35) are indicated below plasmids. Black bars represent E4 and L1 probes used for Northern blotting. RT-PCR primers 757s, E4A, and L1aM are indicated. (B) Left panels, Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with pBELM, pT1SD (24), or pTEx4m. Blots were probed with L1 or E4 probe, as indicated. Gels were also probed for GAPDH. Right panels, RT-PCR on the same RNA analyzed by RT-PCR using primers 757s and L1aM or 757s and E4A (35). The E4A primer hybridizes to a sequence that is present in all three plasmids and that is unaffected by the nucleotide substitutions in pTEx4m.

L1 mRNAs, as expected (Fig. 2B). In contrast, pTEx4m did not produce detectable levels of E4 mRNAs (Fig. 2B), suggesting that usage of SA3358 was totally dependent on the ASF/SF2 sites. Interestingly, the decrease in E4 mRNA production for pTEx4m was paralleled by an increase in L1i mRNA production (Fig. 2A and B). Northern blot and RT-PCR analysis showed that mutational inactivation of the potential ASF/SF2 binding sites redirected splicing from SA3358 to late splice acceptor SA5639 (Fig. 2A and B). Similar results were obtained when the sequences downstream of SA3358 were deleted as in plasmid pT1SD (Fig. 2A and B) (24). Endogenous HPV-18 sequences in HeLa cells do not interfere with the assays described here, as HPV-16 primers and probes are specific for HPV-16. In addition, some of the experiments have been reproduced with HPV-negative cell lines, such as 293T cells, with similar results. Taken together, our results indicated that the potential ASF/SF2 sites were required for splicing to SA3358.

ASF/SF2 binds specifically to exonic sequences located between SA3358 and SD3632. To determine if ASF/SF2 binds directly to the predicted ASF/SF2 sites in the sequence between SA3358 and SD3632, GST-ASF was UV cross-linked to radiolabeled RNA representing the wt or mutant sequences shown in Fig. 1D. The results revealed that GST-ASF UV cross-linked efficiently to this HPV-16 sequence, whereas GST did not (Fig. 3A). GST-ASF did not UV cross-link to an RNA sequence derived from the HPV-1 late untranslated region (UTR) (data not shown). Cross-linking to the mutant RNA was less efficient, as expected (Fig. 3A). However, UV cross-linking of ASF/SF2 to the mutant RNA is probably overestimated by the gel, as the mutant RNA contains 72 radiolabeled U nucleotides, whereas the wt RNA contains only 36 (Fig. 1D). We concluded that ASF/SF2 binds directly to the sequence located between HPV-16 SA3358 and SD3632.

Overexpression of the ASF/SF2 RS mutant inhibits usage of SA3358. To confirm that ASF/SF2 acts via the ASF/SF2 sites in the enhancer region, we used an ASF/SF2 mutant that lacks the RS-domain (ASFDRS) and therefore binds RNA but fails to activate splicing. The ASFDRS *trans*-dominant mutant should compete with endogenous ASF/SF2 for the ASF/SF2 binding sites and inhibit splicing to SA3358. UV cross-linking of serially diluted GST-ASF or GST-ASFDRS to radiolabeled wt HPV-16 RNA, shown in Fig. 1D, revealed that the two proteins cross-linked to this RNA sequence with similar efficiency (Fig. 3B and C). In addition, overexpression of ASFDRS in transfected cells inhibited splicing to SA3358 in plasmid pBELM, thereby inhibiting production of E4 mRNA (Fig. 4B). Overexpression of ASFDRS also caused a redirection of splicing from SA3358 to SA5639, thereby inducing L1i mRNA production (Fig. 4C). Overexpression of ASFDRS therefore causes the same phenotype as mutational inactivation of ASF/SF2 sites at SA3358 in pTEX4m (compare Fig. 2B and 4C). This result is seen only when SA5639 is optimized either by mutational inactivation of splicing silencers located downstream of SA5639 or by direct optimization of the 5639 polypyrimidine tract as in pBEL-OPSA (Fig. 4C and D). Therefore, ASFDRS cannot induce splicing to wt SA5639 in pBEL since it is suppressed by previously identified splicing silencers (Fig. 4C). RT-PCR confirmed that ASFDRS induced L1i by exon skipping (Fig. 4E). This effect is clearly distinct from the induction of L1 mRNA production by the adenovirus E4orf4 protein, as previously described (Fig. 4E) (29).

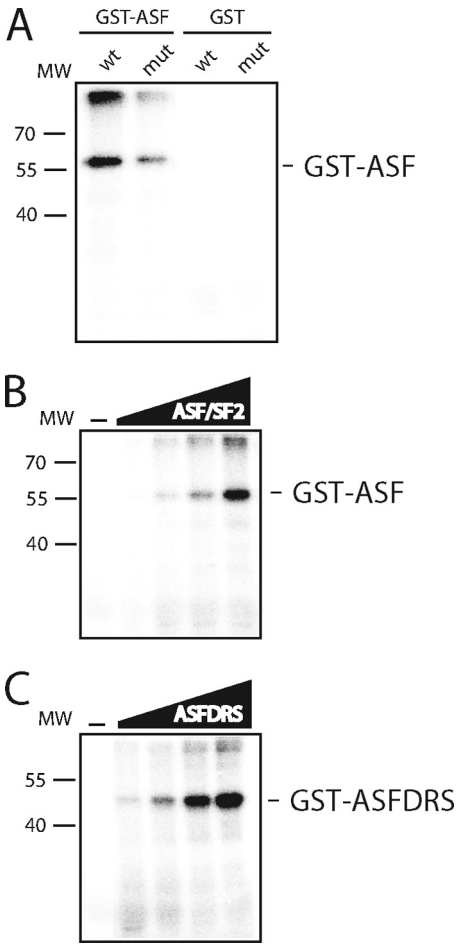

FIG. 3. (A) UV cross-linking of GST or GSTASF to the *in vitro* synthesized, radiolabeled wt and mutant HPV-16 RNAs shown in Fig. 1D. UV cross-linking of serially diluted GST-ASF (B) or GST-ASFDRS (C) to radiolabeled wt HPV-16 RNA shown in Fig. 1D. Molecular weights (MW; in thousands) are indicated on the left.

Overexpression of ASFDRS did not induce L1 production from pT1SD or pTEx4m (Fig. 4F), in which the ASF/SF2 binding sites had been either deleted or mutationally inactivated. These results indicated that redirection of splicing to SA5639 by ASFDRS was dependent on the ASF/SF2 binding sites in the enhancer region downstream of SA3358. In contrast, a small inhibitory effect on L1i mRNA levels was seen when ASFDRS was overexpressed (Fig. 4G). This result suggested that ASFDRS inhibited SA5639 and will be discussed below. We concluded that ASFDRS inhibits the ability of endogenous ASF/SF2 to enhance splicing to SA3358 and that this inhibition was dependent on intact ASF/SF2 sites in the enhancer region downstream of SA3358.

Our results suggested that E6/E7 mRNAs are also regulated by ASF/SF2, as it has been shown previously that the most-common E6 and E7 mRNAs that are spliced between SD226 and SA409 or SA526 are also spliced between SD880 and SA3358. As such, they would be dependent on ASF/SF2. To investigate if this was the

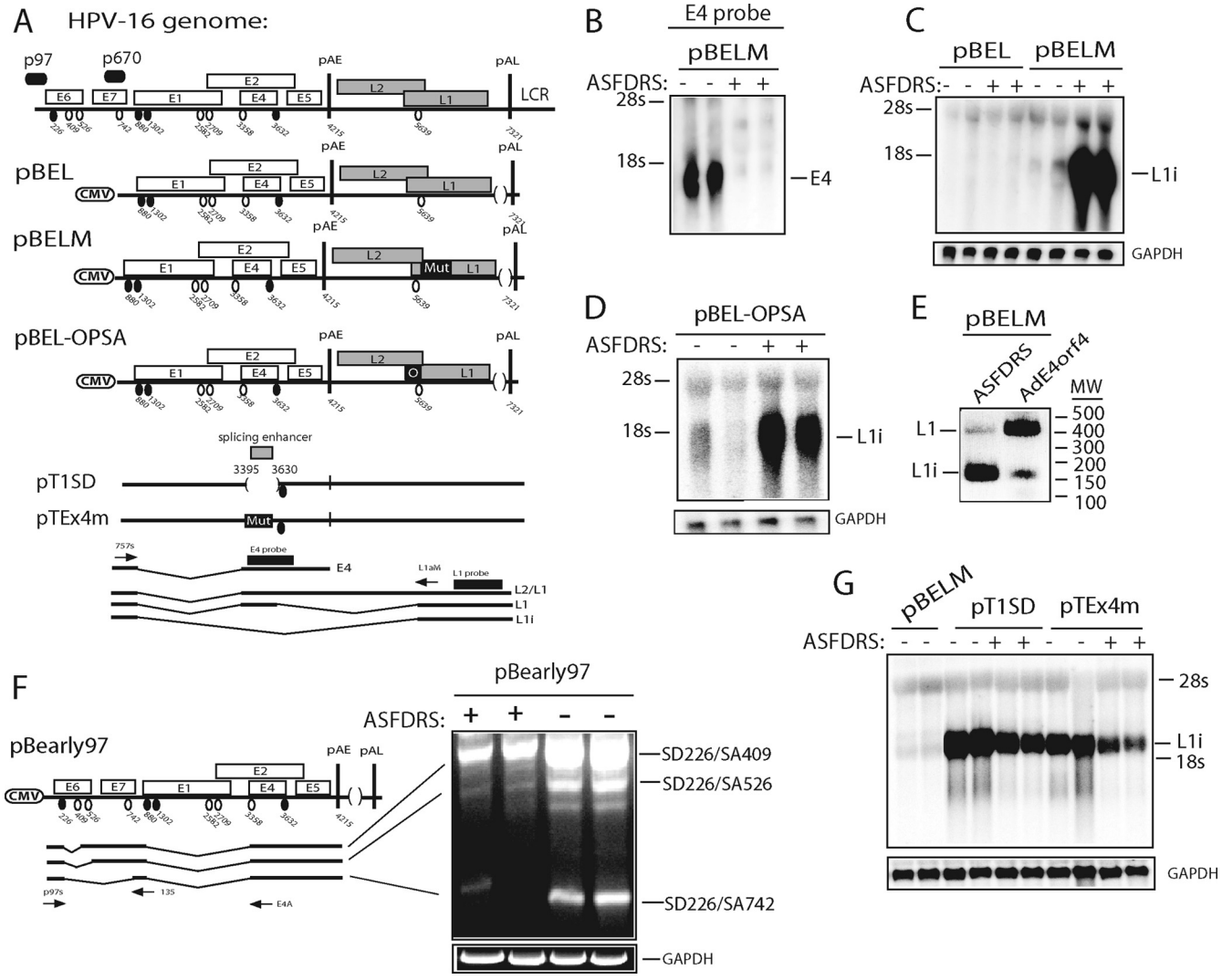

FIG. 4. (A) Schematic representation of the HPV-16 genome and the subgenomic HPV-16 expression plasmids pBEL (35), pBELM (35), pBEL-OPSA (35), pT1SD (24), and pTEx4m. Numbers indicate nucleotide positions of 5′-splice sites (filled circles) and 3′-splice sites (open circles) or poly(A) sites pAE and pAL. Structures of selected HPV-16 mRNAs produced by pBELM (35) are indicated below plasmids. Black bars represent E4 and L1 probes used for Northern blotting. RT-PCR primers 757s and L1aM are indicated (35). CMV, cytomegalovirus. (B to D and G) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with the indicated plasmids in the absence or presence of ASFDRS-expressing plasmid. Blots are probed with E4 or L1 probe as indicated. Gels were also probed for GAPDH. Positions of the E4 and L1 or L1i mRNAs are indicated. (E) RT-PCR on cytoplasmic RNA from HeLa cells transfected with pBELM in the presence of ASFDRS expression plasmid or adenovirus E4orf4 expression plasmid. The RNA samples were analyzed by RT-PCR using primers 757s and L1aM (35). (F) Left panel, schematic drawing of plasmid pBeary97 (33). Right panel, RT-PCR on cytoplasmic RNA from HeLa cells transfected with pBeary97 in the absence or presence of ASFDRS expression plasmid. The RNA samples were analyzed by RT-PCR using primers p97s and 135 (35).

case, plasmid pBearly97 (Fig. 4F) was transfected into HeLa cells in the absence or presence of the mutant ASF protein named ASFDRS. If the E6/E7 mRNAs are regulated by ASF/SF2, overexpression of ASFDRS should inhibit the production of these mRNAs. As can be seen in Fig. 4F, E6/E7 mRNAs that are spliced between SD226 and SA409 or SA526 were negatively affected by ASFDRS but not by transfection with an empty expression plasmid (Fig. 4F). We concluded that expression of E6 and E7 mRNAs is dependent on ASF/SF2.

The ASF/SF2-dependent HIV-1 splicing enhancer termed GAR can substitute for the HPV-16 enhancer at SA3358. To provide further support for a role of ASF/SF2 in the regulation of HPV-16 SA3358, we inserted the ASF/SF2-dependent GAR enhancer from HIV-1 (3), into the enhancerless HPV-16 plasmid pT1SD (Fig. 5A) (24). As can be seen in Fig. 5B and C, pT1SD produced only L1i mRNA, as SA3358 was not used, whereas pT1-5/GAR, in which the ASF/SF2-dependent GAR enhancer had been inserted, produced only L1 mRNA as a result of efficient usage of SA3358 (Fig. 5B and C). We concluded that splicing to HPV-16 SA3358 could be restored by a heterologous but ASF/SF2-dependent enhancer, supporting the conclusion that ASF/SF2 regulates HPV-16 SA3358. Overexpression of ASFDRS with pT1-5/GAR induced production of L1i mRNA (Fig. 5B and C), indicating that

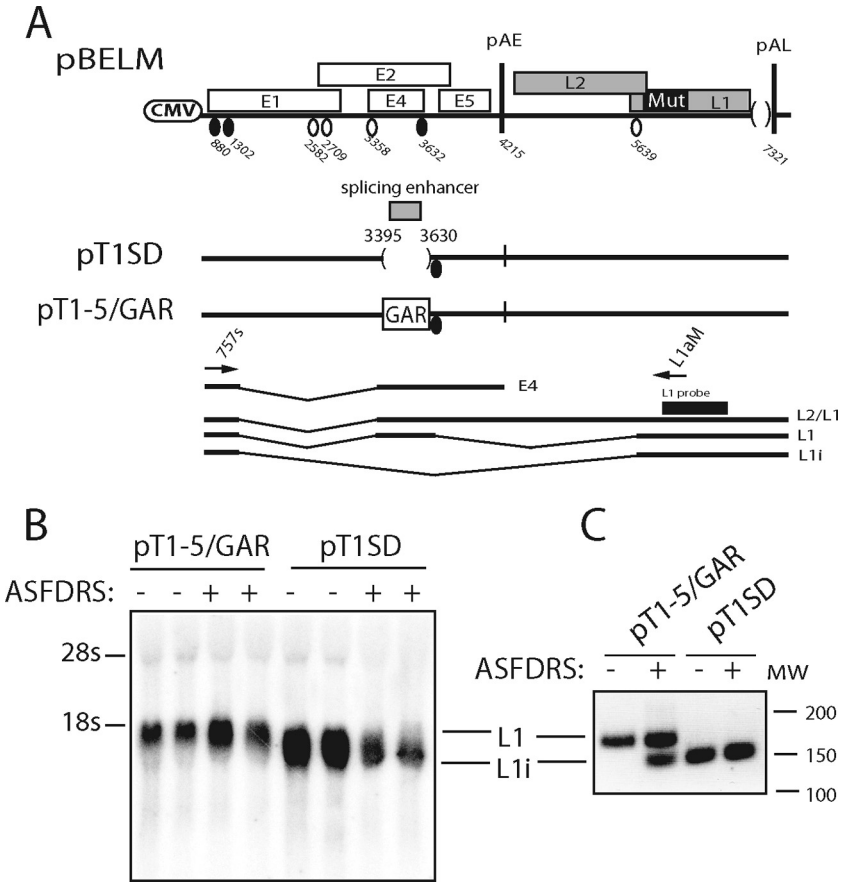

FIG. 5. (A) Schematic representation of the HPV-16 genome and the subgenomic HPV-16 expression plasmids pBELM (35), pT1SD (24), and pT1-5/GAR. Numbers indicate nucleotide positions of 5′-splice sites (filled circles) and 3′-splice sites (open circles) or poly(A) sites pAE and pAL. The location of a previously identified splicing enhancer in the HPV-16 genome is indicated (24). The inserted HIV-1 sequence encompassing GAR, a previously identified splicing enhancer in the HIV-1 genome (3), is indicated. Structures of selected HPV-16 mRNAs produced by pBELM are indicated below plasmids. Black bar represents the L1 probe used for Northern blotting. RT-PCR primers 757s and L1aM are indicated (35). (B) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pT1-5/GAR or pT1SD (24) in the absence or presence of ASFDRS-expressing plasmid. Blot was probed with L1 probe. (C) RT-PCR on the same RNA analyzed by RT-PCR using primers 757s and L1aM. L1 and L1i mRNAs are indicated.

ASFDRS interferes with the enhancer function of GAR. In contrast, ASFDRS did not alter splicing in pT1SD (Fig. 5C) as expected, since ASF/SF2 sites had been deleted. These data strengthened the conclusion that ASF/SF2 enhances splicing to SA3358.

ASF/SF2 contributes to the suppression of splice donor SD3632. To investigate if the ASF/SF2 binding sites located between SA3358 and SD3632 contribute to suppression of SD3632, we constructed pMT1SD and pMTEx4m from pBSpliceM (Fig. 6A) (30). pBSpliceM does not produce detectable levels of E4 or L1 mRNA in the absence of transacting factors, e.g., PTB or AdE4orf4 (Fig. 6B) (29, 30). These plasmids contain a large deletion between nucleotide position 757 and 3395 in the early region of the HPV-16 genome which removes all HPV-16 splice sites except late splice sites SD3632 and SA5639 (Fig. 6A). In addition, the ASF/SF2 binding sites upstream of SD3632 were either deleted (pMT1SD) or mutationally inactivated (pMTEx4m) (Fig. 6B). Transfection of pMT1SD and pMTEx4m into HeLa cells and analysis of mRNA production showed that pMT1SD produced high L1 mRNA levels, whereas L1 mRNA production from pMTEx4m was less elevated than that from pBSpliceM (Fig. 6B). These results indicated that sequences located between nucleotide positions 3395 and SD3632 strongly suppressed SD3632. However, mutations of the potential ASF/SF2 binding sites upstream of SD3632 only partially alleviated inhibition, indicating that ASF/SF2 contributes to inhibition of SD3632, although additional factors must be involved as well.

To investigate if ASF/SF2 indeed inhibits SD3632, we monitored the effect of ASFDRS on HPV-16 plasmids pBSplice and pBSpliceM (Fig. 6A) (30). Transfection of pBSplice or pBSpliceM in the absence or presence of ASFDRS showed that ASFDRS induced L1 mRNA from pBSpliceM but not from pBSplice (Fig. 6C). These results suggested that either wt ASF/SF2 inhibits SD3632 or ASF/SF2 can activate SD3632 in an RS domain-independent manner. To differentiate between these two possibilities, we overexpressed a similar level of ASF/SF2 or ASFDRS with pBSpliceM. The results revealed that ASFDRS induced high levels of L1, whereas L1 mRNA was undetectable in the presence of ASF/SF2 (Fig. 6D). ASFDRS

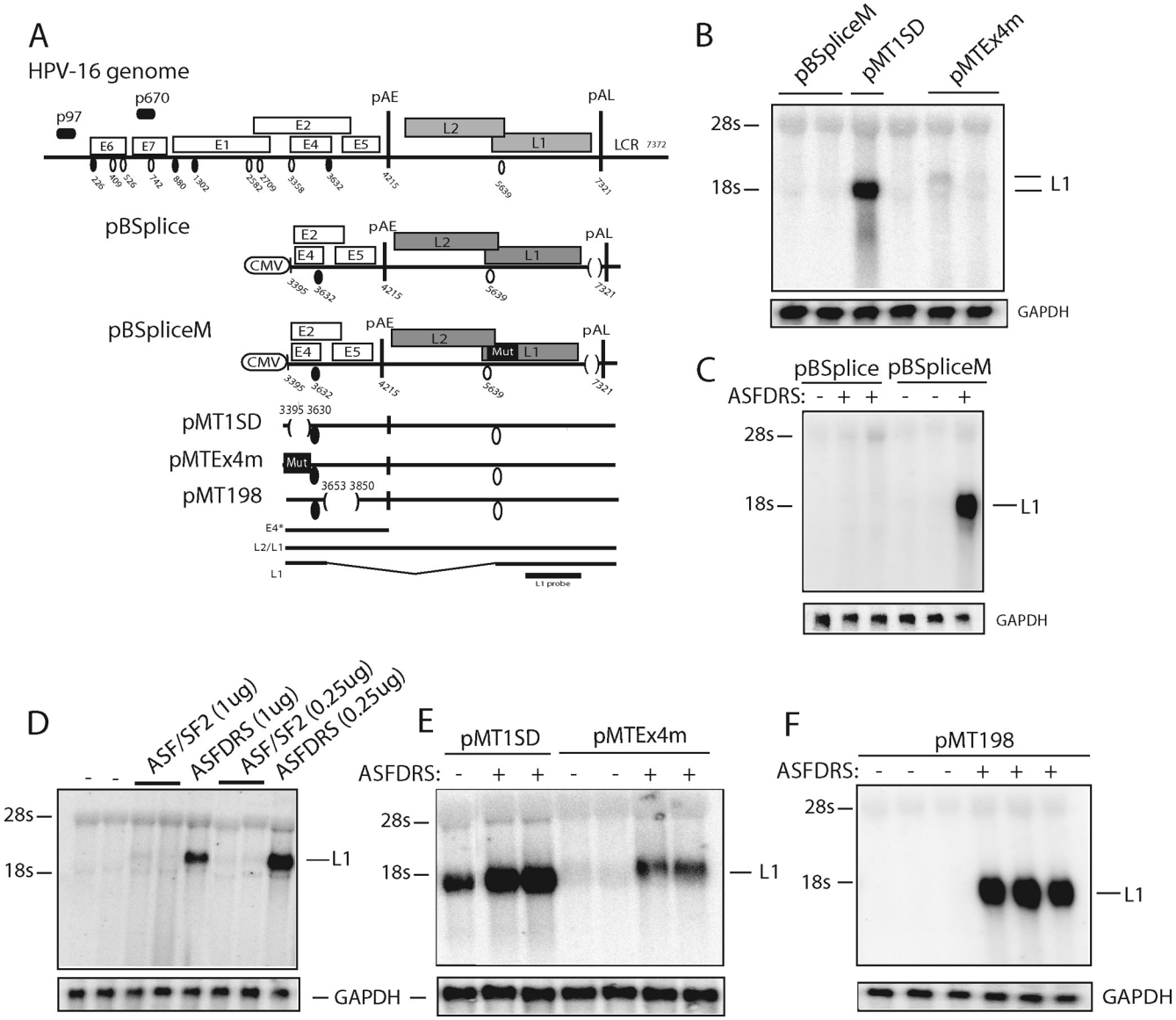

FIG. 6. (A) Schematic representation of the HPV-16 genome and the subgenomic HPV-16 expression plasmids pBSplice (30), pBSpliceM (30), pMT1SD, pMTEx4m, and pMT198. Numbers indicate nucleotide positions of 5′-splice sites (filled circles) and 3′-splice sites (open circles) or poly(A) sites pAE and pAL. Structures of selected HPV-16 mRNAs produced by pBELM (35) are indicated below plasmids. Black bar represents L1 probe used for Northern blotting. (B to F) Northern blots of cytoplasmic RNA extracted from HeLa cells transfected with the indicated plasmids in the absence or presence of ASFDRS-expressing plasmid. Blots are probed with L1 probe. Gels were also probed for GAPDH. Position of the L1 mRNA is indicated.

also caused an increase in L1 expression from pMTEx4m (Fig. 6E). Therefore, ASFDRS either inhibited independently of the ASF/SF2 binding sites upstream of SD3632, or ASFDRS could still bind to the sequences upstream of SD3632. Nevertheless, ASFDRS induced splicing from SD3632, indicating that ASF/SF2 therefore inhibits SD3632. To investigate if inhibition of SD3632 was mediated by the potential ASF/SF2 sites located downstream of SD3632 (Fig. 1B), two of the three sites were deleted in pM198 (Fig. 6A). This plasmid did not produce detectable levels of L1, and overexpression of ASFDRS resulted in induction of L1 (Fig. 6F). We concluded that sequences between SD3632 and pAE did not inhibit SD3632, nor were they targeted by ASF/SF2. We concluded that ASF/SF2 exerts an inhibitory effect on SD3632, but other factors are major inhibitors of SD3632.

ASF/SF2 can induce splicing to SA5639 but less efficiently than to SA3358. As we described previously, mutations or deletions of the ASF/SF2 binding sites downstream of SA3358 resulted in redirection of splicing to SA5639 (Fig. 2B and 4G). However, these plasmids were not indifferent to ASFDRS overexpression, although the ASF/SF2 sites at SA3358 had been inactivated (Fig. 4G and 5B). Careful quantitations of L1 mRNA levels produced by these mutants in the absence or presence of ASFDRS revealed that ASFDRS actually reduced L1 mRNA production from pTEx4m (Fig. 4F) and pT1SD

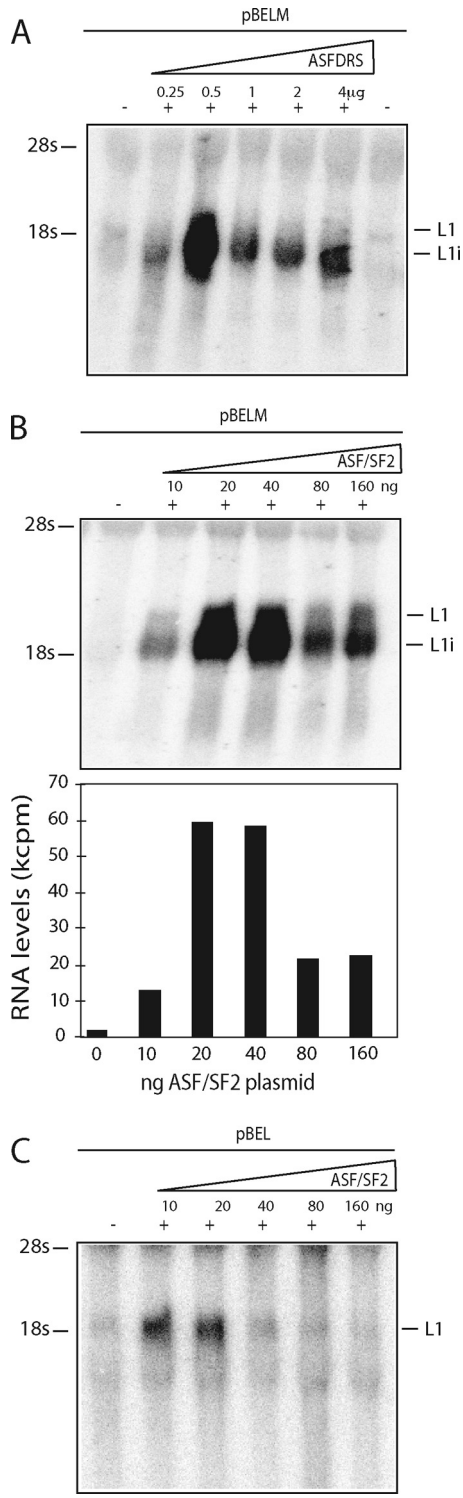

FIG. 7. (A) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBELM (35) in the absence or presence of serially diluted ASFDRS-expressing plasmid (0.25 to 4 μg). Blot was probed with L1 probe. (B) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBELM (35) in the absence or presence of serially diluted ASF/SF2-expressing plasmid (10 to 160 ng). Blot was probed with L1 probe. Numbers indicate nanograms of ASF/SF2 plasmid used in the transfection. Blot was probed with L1 probe. Positions of the L1 and L1i mRNAs are indicated. Lower panel, quantitation of the L1 bands in the gel using phosphorimager. (C) Northern blot of cytoplasmic RNA extracted from HeLa cells transfected with pBEL (35) in the absence or presence of serially diluted ASF/SF2-expressing plasmid (10 to 160 ng). Blot was probed with L1 probe. Numbers indicate nanograms of ASF/SF2 plasmid used in the transfection. Blot was probed with L1 probe. Positions of the L1 mRNA are indicated.

(Fig. 4G and 5B). We concluded that splicing to SA5639 was inhibited by ASFDRS, suggesting that SA5639 also was affected by ASF/SF2.

If ASFDRS inhibits splicing from SD880 to both SA3358 and SA5639, the net effect of ASFDRS should be dependent on the concentration of ASFDRS. pBELM (35) was transfected with a serial dilution of ASFDRS plasmid. The results revealed that induction of L1 expression was dependent upon the amount of ASFDRS expression plasmid transfected (Fig. 7A). Optimal induction by inhibition of SA3358 occurred at a concentration of 0.5 μg of ASFDRS plasmid, whereas higher concentrations of ASFDRS reduced L1 mRNA levels, presumably by inhibiting SA5639 (Fig. 7A). Similar results were seen with two different concentrations of ASFDRS (Fig. 6D). We concluded that ASFDRS inhibits both SA3358 and SA5639. As a consequence, ASF/SF2 should have a positive effect on splicing to SA5639. To investigate if ASF/SF2 could induce L1 mRNA production, we overexpressed ASF/SF2 with plasmids pBEL and pBELM. Transfection of a 2-fold serial dilution of ASF/SF2 plasmid, ranging from 4 μg to 0.25 μg, induced L1 expression from neither pBELM nor pBEL (data not shown). In contrast, submicrogram levels of ASF/SF2 plasmid induced L1 mRNA from both pBELM and pBEL (Fig. 7B and C). The induced L1 mRNA levels were higher from pBELM (Fig. 7B) than from pBEL (Fig. 7C), demonstrating that ASF/SF2 could not overcome suppression of SA5639 very efficiently. The failure of high levels of ASF/SF2 to induce L1 mRNA production was expected, since ASF/SF2 also stimulates splicing to the competing 3′-splice site SA3358 and has an inhibitory effect on SD3632. We concluded that ASF/SF2 could stimulate splicing to SA5639, albeit with low efficiency.

Knockdown of ASF/SF2 reduces splicing to SA3358 and SA5639. We also wished to determine if ASF/SF2 was needed for splicing to SA3358 and SA5639. We therefore knocked down endogenous ASF/SF2 using siRNAs against ASF/SF2. We reasoned that siRNA-mediated knockdown of ASF/SF2 should inhibit the production of E4, L1, and L1i mRNAs. As can be seen in Fig. 8A, a Western blot shows successful knockdown of ASF/SF2 in cells transfected with pBELM. As expected, E4, L1, and L1i mRNA levels were reduced when ASF/SF2 was knocked down (Fig. 8B). The E4 mRNA is suppressed to a lower extent, probably because E4 mRNAs are more abundantly expressed from pBELM than is the L1 mRNA (Fig. 8B). These results confirmed that splicing to both SA3358 and SA5639 is enhanced by ASF/SF2.

DISCUSSION

It has been shown previously that ASF/SF2 is a proto-oncogene that can transform rodent cells *in vitro* and that it is overexpressed in various cancers (14). We have shown that a number of RNA-binding proteins, including splicing factor

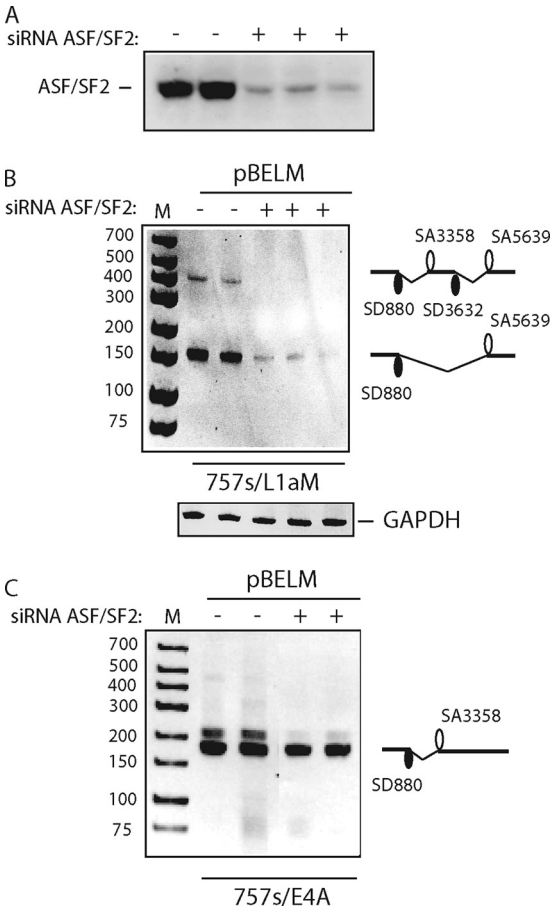

FIG. 8. (A) Western blot of cell extracts from HeLa cells transfected with pBELM in the absence or presence of siRNAs against ASF/SF2, as described in Materials and Methods. (B and C) RT-PCR on cytoplasmic RNA extracted from HeLa cells transfected with pBELM (35) in the absence or presence of siRNA against ASF/SF2. RT-PCR was performed with primers 757s and L1aM (B) or 757s and E4A (C) (35). RT-PCR was also performed with GAPDH mRNA (C) (13). Structures of the L1 and L1i mRNAs, or the E4 mRNA, are shown to the right of the gels. Numbers indicate positions of splice sites on the mRNAs. M, molecular size marker.

ASF/SF2, are overexpressed in cervical cancers (7). As a matter of fact, ASF/SF2 expression increased with the grade of severity of the cervical lesion induced by HPV, as did expression of most RNA binding proteins (7). Relatively few cells expressed ASF/SF2 in normal HPV-negative cervical epithelium (7). These cells were found in the basal cell layers as well as in suprabasal layers but not in the superficial layers of the epithelium. In contrast, ASF/SF2 was abundantly expressed in the entire epithelium for high-grade, CIN-III cervical intraepithelial lesions and all cervical cancers (7). Mole et al. also observed higher levels of expression of ASF/SF2 in high-grade lesions compared to those in low-grade lesions (18). Taken together, these results suggest that cancer cells, as well as some HPV-16 genes, benefit from a high expression level of ASF/SF2. As ASF/SF2 is a key regulator of RNA processing, high expression in cancer cells may reflect the high metabolic activity of these cells and a need for high and efficient expression of many genes. In case of HPV-16, ASF/SF2 is required primarily for splicing to SA3358. This splicing event removes the intron between SD880 and SA3358 or that between SD226 and SA3358, resulting in the production of HPV-16 mRNAs that are efficiently expressed in HPV-16-infected cells (Fig. 1). HPV-16 E6 and E7 mRNAs that are spliced between SD880 and SA3358 are vastly more common than those that retain the intron between SD880 and SA3358 (9, 17, 24, 35). In general, intron-containing mRNAs are retained and degraded in the nucleus to a higher degree than spliced mRNAs, which may explain the low levels of HPV-16 mRNAs that contain sequences between SD880 and SA3358. One may therefore conclude that mRNAs that are spliced between SD880 and SA3358 benefit from high levels of ASF/SF2 (Fig. 9). Such mRNAs encode E6, E7, or E4 proteins, and the shorter forms of E6 named E6*-I and E6*-II, depending on the upstream splicing events on these mRNAs (Fig. 1A). Similarly, mRNAs that are spliced between SD226 and SA3358, and have the potential to produce E5 protein (Fig. 1A), would benefit from high levels of ASF/SF2. Of the early HPV-16 mRNAs that are selectively stimulated by high levels of ASF/SF2, there is an overrepresentation of mRNAs that express viral cell growth-stimulatory proteins, including E5, E6, and E7. This deduction fits well with the previously observed high expression levels of ASF/SF2 in high-grade cervical lesions and cervical cancers (7). It was previously reported that HPV-16 E2 enhances ASF/SF2 expression by transcriptional activation of the ASF/SF2 promoter (19). Here, we report that ASF/SF2 induces splicing to SA3358 on the HPV-16 genome. Interestingly, splicing to SA2709, the splicing event that generates E2 mRNAs, and splicing to SA3358 are two mutually exclusive events (Fig. 1A). High levels of ASF/SF2 may therefore downregulate expression of E2, presumably in a negative feedback manner, perhaps marking the entry into the late phase of the viral life cycle with L1 and L2 expression.

As HPV-16 enters the late stage of the viral life cycle and the infected cell differentiates terminally, suppression of the exclusively late splice sites SD3632 and SA5639 is released and efficiency of early polyadenylation reduced. These events cause induction of L1 and L2 mRNA production. However, SA3358 must be efficiently used also in these cells, as it is used by late L1 mRNAs. This would require the presence of ASF/SF2 also in terminally differentiated cells. Alternatively, the role of ASF/SF2 is taken over by another, yet-unidentified splicing factor that is present in terminally differentiated cells. As ASF/SF2 is normally not detected in the upper layers of uninfected cervical epithelium, this may indeed be the case (7). However, one may speculate that HPV-16 selectively enhances production of ASF/SF2, even in the terminally differentiated cells in which L1 and L2 protein can be detected. This could be performed by E2 or indirectly by another HPV protein. It would be interesting to compare expression of ASF/SF2 and HPV-16 L1 and L2 expression in individual cells in HPV-16-infected cervical epithelium to determine if ASF/SF2 is present in the cells that express L1 and/or L2 protein. In addition, it would be of interest to determine if the dependence on ASF/SF2 is conserved among different HPV types. We have found that the ESEfinder (4) predicts the presence of multiple ASF/SF2 sites in the corresponding position of both low-risk and high-risk HPV types and in mucosal and cutaneous HPV types. We

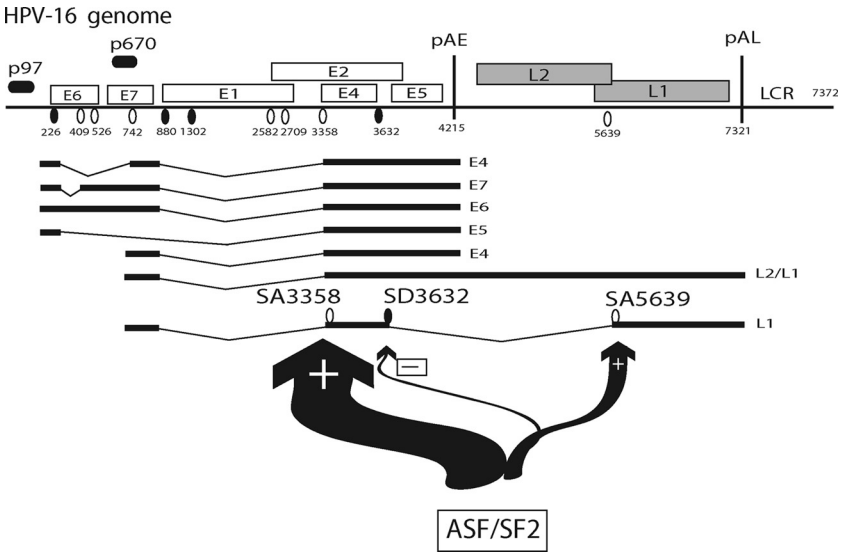

FIG. 9. Effect of ASF/SF2 on HPV-16 mRNA splicing in HeLa cells. ASF/SF2 is primarily stimulating splicing to SA3358 and at the same time weakly inhibiting late splice site SD3632. However, SD3632 may be more efficiently suppressed by another factor(s). ASF/SF2 can also stimulate splicing to SA5639, but this effect is seen primarily when SA5639 is optimized by inactivation of silencers downstream of SA5639 or by optimization of the SA5639 polypyrimidine tract. Taken together, these results show that ASF/SF2 strongly favors production of mRNAs that are using SA3358 and are polyadenylated at pAE. These mRNAs include the E4, E5, E6, and E7 mRNAs indicated in the figure.

therefore believe that ASF/SF2 regulates splicing of both low- and high-risk HPV types.

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