Selection of the Bovine Papillomavirus Type 1 Nucleotide 3225 3' Splice Site Is Regulated through an Exonic Splicing Enhancer and Its Juxtaposed Exonic Splicing Suppressor

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Alternative splicing is an important mechanism for the regulation of bovine papillomavirus type 1 (BPV-1) gene expression during the virus life cycle. However, one 3' splice site, located at nucleotide (nt) 3225, is used for the processing of most BPV-1 pre-mRNAs in BPV-1-transformed C127 cells and at early to intermediate times in productively infected warts. At late stages of the viral life cycle, an alternative 3' splice site at nt 3605 is used for the processing of the late pre-mRNA. In this study, we used in vitro splicing in HeLa cell nuclear extracts to identify *cis* elements which regulate BPV-1 3' splice site selection. Two purine-rich exonic splicing enhancers were identified downstream of nt 3225. These sequences, designated SE1 (nt 3256 to 3305) and SE2 (nt 3477 to 3526), were shown to strongly stimulate the splicing of a chimeric *Drosophila* doublesex pre-mRNA, which contains a weak 3' splice site. A BPV-1 late pre-mRNA containing the nt 3225 3' splice site but lacking both SE1 and SE2 was spliced poorly, indicating that this 3' splice site is inherently weak. Analysis of the 3' splice site suggested that this feature is due to both a nonconsensus branch point sequence and a suboptimal polypyrimidine tract. Addition of SE1 to the late pre-mRNA dramatically stimulated splicing, indicating that SE1 also functions as an exonic splicing enhancer in its normal context. However, a late pre-mRNA containing both SE1 and SE2 as well as the sequence in between was spliced inefficiently. Further mapping studies demonstrated that a 48-nt pyrimidine-rich region immediately downstream of SE1 was responsible for this suppression of splicing. Thus, these data suggest that selection of the BPV-1 nt 3225 3' splice site is regulated by both positive and negative exonic sequences.

The papillomaviruses are epitheliotropic viruses which require the environment of a differentiating squamous epithelium for their life cycle. Bovine papillomavirus type 1 (BPV-1) has served as the prototype for studies on the molecular biology of the papillomaviruses because it also transforms rodent cells in culture and the viral DNA replicates as an extrachromosomal plasmid in these cells (for reviews, see references 23 and 28). Although the BPV-1 genome is small (ca. 8,000 bp in length), it expresses at least 10 proteins. These proteins are expressed from numerous mRNAs which are generated by the use of multiple promoters as well as by alternative splicing and alternative polyadenylation. The structures of BPV-1 mRNAs both in BPV-1-transformed C127 cells and in productively infected warts or fibropapillomas have been extensively studied (for reviews, see references 3 and 4). The majority of spliced mRNAs in transformed cells and at early stages of the viral life cycle in warts utilize a common 3' splice site at nucleotide (nt) 3225 even though alternative 3' splice sites are present both upstream and downstream of this site. The reason for the preferential selection of the nt 3225 3' splice site has not been elucidated. However, the utilization of this 3' splice site appears to be regulated during keratinocyte differentiation. Late pre-mRNAs in the intermediately differentiated keratinocytes of the spinous layer of the skin are processed by using the nt 3225 3' splice site. In contrast, an alternative 3' splice site at nt

3605 is used only in the fully differentiated keratinocytes of the granular layer of the skin (7).

Splicing is a major step in the maturation of a pre-mRNA to form an mRNA. It involves first the recruitment of various splicing factors to both the 5' and 3' splice sites to form a spliceosome. Subsequently, the actual chemical reactions which remove the intron(s) and ligate the exons take place (for reviews, see references 21, 34, and 41). The initial step in spliceosome assembly in vertebrates involves the recognition of the 5' splice site by the U1 small nuclear ribonucleoprotein (snRNP) and the 3' splice site by binding of U2AF to the polypyrimidine tract (25, 32, 39, 46, 61). U2AF subsequently facilitates binding of the U2 snRNP to the branch point (BP) sequence (39, 61). The binding affinity of U2AF for the polypyrimidine tract is a partial determinant of the strength of the 3' splice site and depends on the length of the polypyrimidine tract as well as its composition (38, 62). Weak 3' splice sites require splicing enhancers for efficient splicing. In the past few years, a class of purine-rich splicing enhancers known as exonic splicing enhancers (ESEs) or exon recognition sequences have been identified in a number of pre-mRNAs. These include the mouse immunoglobulin M exon M2 (56), human fibronectin ED1 exon (10, 29), bovine growth hormone exon 5 (12, 48), avian sarcoma-leukosis virus *env* 3' exon (14, 26, 51), human immunodeficiency virus type 1 (HIV-1) *tat/rev* exon 3 (44), rat β -tropomyosin exon 8 (19), chicken cardiac troponin T exon 5 (37, 59), and human hypoxanthine-guanine phosphoribosyltransferase exon 3 (47). ESEs are thought to function by binding SR proteins, which promote the binding of U2AF to the 3' splice site (29, 45). Although most ESEs work on upstream

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FIG. 1. Potential BPV-1 purine-rich ESEs. (A) The sequences of potential BPV-1 purine-rich ESEs (SE1 to SE4) are shown along with their relative positions in the BPV-1 genome. Numbers are nucleotide positions in the BPV-1 genome. The nt 3225 and nt 3605 3' splice sites and the nt 3764 5' splice site are indicated on the map. (B) Schematic representation of the *Drosophila dsx* chimeric pre-mRNA used to test potential splicing enhancer sequences. Exon sequences (boxes) and intron sequences (lines) are shown. The lengths (in nucleotides) of the exons and introns are indicated below the respective regions of the pre-mRNA. The 3' exon contains 30 nt of the *dsx* fourth exon and 14 nt of linker sequence derived from pSP72. The shaded box indicates the location where putative SE sequences were cloned. (C) Synthetic oligonucleotides (AAG)₈ and Py3 were cloned into the *dsx* vector shown in panel B and served as positive and negative controls, respectively, for splicing enhancement (51).

suboptimal 3' splice sites, there are a few examples of ESEs which act on 5' splice sites. The ESE in exon 5 of the bovine growth hormone pre-mRNA complements a weak 5' splice site in the preceding exon (12). Another example is an ESE in exon 5 of the caldesmon pre-mRNA, which stimulates the use of an upstream 5' splice site, regulating competition between two 5' splice sites within a differential internal exon (24).

In this study, we investigated exonic *cis* elements which influence utilization of the BPV-1 nt 3225 3' splice site. Two purine-rich sequences, SE1 and SE2, were identified downstream of the nt 3225 3' splice site and shown to enhance splicing of a heterologous *Drosophila doublesex* (*dsx*) pre-mRNA in HeLa cell nuclear extracts. SE1, which lies immediately downstream of the nt 3225 3' splice site, was also shown to be required for efficient splicing of a BPV-1 late pre-mRNA in vitro. Finally, we have also identified a pyrimidine-rich ex-

onic splicing suppressor (ESS) sequence immediately downstream of SE1.

MATERIALS AND METHODS

Plasmid construction. To construct the template plasmids for chimeric *dsx* pre-mRNA preparation, plasmid *pdsx*-Sa (51, 56), containing the entire third exon, the third intron, and 30 nt of the fourth exon of the *Drosophila dsx* gene, was cut with *Xba*I and *Hind*III. Annealed synthetic oligonucleotides corresponding to the BPV-1 putative splicing enhancer sequences shown in Fig. 1A and containing *Xba*I- and *Hind*III-compatible ends were ligated into these sites. All constructs were made by using standard cloning procedures and confirmed by dideoxy sequencing (40).

To construct the template plasmid pZMZ19-1 for the generation of BPV-1 late pre-mRNAs, two fragments of BPV-1 DNA (nt 7254 to 7563 and nt 3073 to 4454) were joined by PCR overlap extension with an intervening *Xho*I site and then cloned downstream of the T7 promoter at the *Sac*I and *Bam*HI sites in the pSP72 vector (Promega, Madison, Wis.).

Pre-mRNA synthesis. For the chimeric *dsx* pre-mRNA preparation, the template plasmids were linearized with *Hind*III and transcribed in vitro with T7 RNA polymerase in the presence of the cap analog m⁷GpppG (33) and [α -³²P] GTP (51). Full-length transcripts were separated on 6% denaturing polyacrylamide gels and eluted from gel slices by overnight incubation in elution buffer (0.5 M ammonium acetate, 2 mM EDTA, 0.2% sodium dodecyl sulfate). The eluted RNAs were then phenol extracted, ethanol precipitated, and resuspended in diethylpyrocarbonate-treated water.

For BPV-1 late pre-mRNA preparation, BPV-1 PCR products containing exon 1, intron, and various lengths of exon 2 were generated from plasmid pZMZ19-1 with a common 5' T7 promoter primer (5'-ATTAATACGACTCAC TATAG-3') combined with appropriately positioned BPV-1 antisense primers (Table 1). These PCR products were then gel purified and used as templates for capped RNA transcription with T7 RNA polymerase as described above.

In vitro splicing reactions. In vitro splicing reactions were performed at 30°C, using the Promega RNA splicing system with 4 ng of ³²P-labeled pre-mRNAs and HeLa cell nuclear extract as recommended by the manufacturer. The reaction products were extracted with phenol, precipitated with ethanol, and analyzed by electrophoresis on denaturing polyacrylamide gels followed by quantitation with a Molecular Dynamics PhosphorImager or autoradiography with Kodak XAR-5 film. Approximately half as much pre-mRNA as was used in the in vitro splicing reactions was electrophoresed without prior splicing (zero time point) to assess the state of the pre-mRNA. The splicing efficiency for each pre-mRNA was calculated as the percentage of total splicing products (intermediates and fully spliced) divided by the sum of the total splicing products plus the remaining pre-mRNA.

Identification of the spliced intermediates was performed by electrophoresis of the splicing products on both 5 and 8% polyacrylamide gels containing 8 M urea. The spliced products that exhibit different relative mobilities on these gels were identified as lariat or lariat-containing products (17, 20, 41).

To confirm the splice junction, the in vitro splicing products from unlabeled BPV-1 late pre-mRNA H were amplified by reverse transcription-PCR (RT-PCR) as described previously (65) with the 5' primer in exon 1 (oCCB47; 20-mer with 5' end at BPV-1 nt 7250) and the exon 2 antisense primer oMD8 (Table 1) end labeled with ³²P. The amplified PCR products were run on 8% denaturing polyacrylamide gels and detected with a PhosphorImager. Alternatively, the in vitro splicing products from unlabeled BPV-1 late pre-mRNA H were amplified by RT-PCR with unlabeled primers (oCCB47 and oMD8, described above). The amplified PCR product corresponding to the spliced mRNA was then gel purified and sequenced by using a chain termination method (U.S. Biochemical Sequenase kit) with added dimethyl sulfoxide (57).

Mapping of the BP in BPV-1 late pre-mRNAs. To map the BP of the BPV-1 late pre-mRNA, the lariat-containing intron and exon 2 product from in vitro splicing of unlabeled BPV-1 pre-mRNA H was gel purified and analyzed by

TABLE 1. Antisense 3' primers used to generate DNA templates for BPV-1 late pre-mRNA synthesis

Oligonucleotide	Sequence ^a	nt position	Product ^b
oMD6	CAGACTCCGTCTGGGCGATC	3245–3226	G
oMD8	GCAAAGAAGAGACAGGCTGG	3319–3300	H
oCCB70	TTTAACAGGTGGAATCCATC	3572–3553	I
oZMZ27	GATGCCAAGTCCACCGGTACCGTG	3476–3453	J
oZMZ50	GCCCCCGAGCCTGCAGGAAAATT	3417–3394	K
oZMZ52	GTAGGGGTGCGAGCGAGGACCGTC	3393–3370	M
oZMZ53	CACCGGGTGGAGGAAGAGCG	3447–3427	L
oZMZ54 ^c	CCTGCTCTcTGcagCGCAaGcActGcAcGAGCAAAGAAGAGACAGGCTG	3353–3301	O

^a All primers were synthesized and gel purified by Synthetic Genetics, Inc. (San Diego, Calif.).

^b Names corresponds with the pre-mRNAs shown in Fig. 3, 6, and 7.

^c A mutagenic primer. Lowercase indicates nucleotide substitutions in the BPV-1 wild-type sequence as described in Fig. 7.

FIG. 2. Enhancement of *dsx* pre-mRNA splicing by BPV-1 exon sequences. Four nanograms of chimeric *dsx* pre-mRNAs containing individual BPV-1 splicing enhancer sequences as indicated at the top was mixed with HeLa cell nuclear extracts under splicing conditions as described in Materials and Methods. The mixtures were incubated at 30°C for 0, 0.5, 1, or 2 h, as indicated. Electrophoresis was performed on a 5% polyacrylamide gel containing 8 M urea. Positions of fragments from a 100-bp ladder are shown on the left. The identities of splicing products are shown at the right. The top band in each lane is the unspliced pre-mRNA. The mobilities of the fully spliced products and lariat-exon 4 intermediates differ among the various templates as a result of the different sizes of the SE sequences downstream of exon 4.

primer extension (20, 48). The ³²P-labeled primer oMD8 (Table 1) was used for both primer extension and DNA sequencing to determine where the extended products were terminated. The primer extension products were analyzed along with sequencing reactions on an 8% denaturing polyacrylamide gel.

Image analysis. Autoradiographic data were captured with a Molecular Dynamics PhosphorImager or with Kodak XAR-5 film. The exposure of PhosphorImager data was adjusted with ImageQuant and exported as a TIFF file. X-ray films were scanned with a Microtek ScanMaker 600Z scanner. Bitmapped images were cropped in Aldus Photostyler and imported into Micrografix Designer, with which they were annotated and printed on a Tektronix Phaser IIstd dye sublimation printer.

RESULTS

Identification of potential ESEs. To determine why the nt 3225 3' splice site is strongly preferred for the processing of both early and late BPV-1 pre-mRNAs in undifferentiated and intermediately differentiated keratinocytes, we first examined the sequences of the nt 3225 (UCUCAUCCACCUCUUCU GAUUUUAG/A) and nt 3605 (UCAUGCUUUGCUCUAA UUUCAG/G) 3' splice sites. Although the nt 3225 3' splice site has a longer polypyrimidine tract, both polypyrimidine tracts are interrupted by purines (underlined). This feature would be expected to decrease the inherent strength or efficiency of both 3' splice sites. This is not unexpected, since regulated splicing requires suboptimal splice sites. We next examined the exon sequences downstream of both 3' splice sites for potential purine-rich ESE elements, since these elements have previously been shown to enhance the utilization of suboptimal splice sites in other pre-mRNAs. Two purine-rich sequences, each about 50 nt long, were identified between the two 3' splice sites and have been designated SE1 and SE2. SE1

and SE2 lie 31 and 252 nt downstream of the nt 3225 3' splice junction, respectively. Additional, much shorter purine-rich sequences designated SE3 and SE4 were identified 23 nt upstream and 107 nt downstream of the nt 3605 3' splice junction, respectively. The sequences and locations of these potential splicing enhancer elements are shown in Fig. 1A.

Functional analysis of potential BPV-1 ESEs using a *Drosophila dsx* pre-mRNA. To test if the purine-rich sequences described above can function as splicing enhancers, these sequences were analyzed for their stimulatory effects on the splicing of a *Drosophila dsx* pre-mRNA. The *dsx* pre-mRNA is expressed from a *Drosophila dsx* minigene-containing vector and consists of exon 3, intron 3, and a portion of exon 4 (Fig. 1B). The 3' splice site in exon 4 has a suboptimal polypyrimidine tract, and therefore this pre-mRNA is not spliced significantly in the absence of a splicing enhancer in exon 4 (22, 52). This system has been used for the study of other splicing enhancers (51, 56). The BPV-1 purine-rich sequences shown in Fig. 1A were inserted downstream of exon 4 in the *Drosophila dsx* minigene-containing vector, and chimeric pre-mRNAs were expressed in vitro and assayed for splicing in HeLa nuclear extracts. A *dsx* pre-mRNA containing the strong synthetic splicing enhancer (AAG)₈ was used as a positive control (Fig. 1C) (51). A mutated nonfunctional splicing enhancer (Py3) served as the negative control (Fig. 1C) (51).

Consistent with the results of others (51, 56), the *dsx* pre-mRNA was not significantly spliced in a 2-h reaction in the absence of a splicing enhancer (Fig. 2, lanes Py3). Addition of the synthetic splicing enhancer (AAG)₈ stimulated splicing significantly such that 11% of the pre-mRNA was spliced

FIG. 3. Effects of SE1 and SE2 on BPV-1 late pre-mRNA splicing in vitro. (A) Maps of BPV-1 late pre-mRNAs. Numbers above the pre-mRNAs indicate nucleotide positions in the BPV-1 genome. Numbers below species indicate the exon or intron length in nucleotides. All of the pre-mRNAs, prepared as described in Materials and Methods, contain exon 1, a truncated intron 1, and a variable length of exon 2 as indicated. Pre-mRNA G contains no SE1 in exon 2, pre-mRNA H contains SE1, and pre-mRNA I contains both SE1 and SE2. (B and C) In vitro splicing of BPV-1 late pre-mRNAs. Pre-mRNAs (4 ng of each) shown in panel A were incubated at 30°C for 0 or 2 h in HeLa cell nuclear extracts. Electrophoresis of splicing products was carried out on an 8% (B) or 5% (C) polyacrylamide gel containing 8 M urea. The 100-bp ladder DNA size marker is shown on the left. Identification of splicing products is shown at the right. Lariats and lariat-containing products were identified by comparison of the product mobilities in both 5 and 8% denaturing polyacrylamide gels, in which lariats and lariat-containing intermediates migrate with different relative mobilities compared with linear RNAs. The lariat intermediates run above the pre-mRNA in the 8% gel (B) and below the unspliced pre-mRNA in the 5% gel (C). Since the introns in the different pre-mRNAs are identical, the lariats derived from the introns of the different BPV-1 late pre-mRNAs run at the same position. However, the lariat-containing exon 2 intermediates generated from different pre-mRNAs have different mobilities as a result of the different sizes of exon 2 in different pre-mRNAs.

within 2 h. Under the same conditions, *dsx* pre-mRNAs containing BPV-1 SE1 and SE2 were 23 and 25% spliced, respectively (Fig. 2). Thus, both SE1 and SE2 stimulated splicing even better than the synthetic splicing enhancer (AAG)₈. The 5' and 3' portions of SE2 were also assayed separately. Most of the splicing enhancer activity of SE2 was localized to the 5' part (SE2A; nt 3477 to 3498), although the 3' portion of SE2 appears to contribute to the overall splicing enhancer activity of SE2. SE3 and SE4 were also tested and shown to have minimal splicing enhancer activity in the *dsx* pre-mRNA system under these experimental conditions (Fig. 2). SE3 and SE4

were therefore not investigated further. Thus, we concluded from these experiments that BPV-1 SE1 and SE2 have strong splicing enhancer activity in the heterologous *dsx* system.

Functional analysis of ESEs in BPV-1 late pre-mRNAs. To determine if SE1 and/or SE2 are required for the efficient splicing of the homologous BPV-1 pre-mRNA, various BPV-1 late pre-mRNAs with or without SE1 and/or SE2 (Fig. 3A) were generated and tested for splicing in vitro, using HeLa cell nuclear extracts. The pre-mRNA containing the nt 3225 3' splice site but lacking both SE1 and SE2 was spliced very inefficiently, as predicted from the sequence of this 3' splice

site (Fig. 3B, pre-mRNA G). In contrast, the late pre-mRNA containing SE1 (pre-mRNA H) was spliced efficiently (64.8% spliced) (Fig. 3B). These data indicate that the ESE SE1 is required for the efficient utilization of the suboptimal BPV-1 nt 3225 3' splice site.

The splicing intermediates and products were characterized further to verify that the correct splice sites were being used for splicing *in vitro*. Lariats and lariat-containing products generated from the BPV-1 late pre-mRNA were identified by comparison of the product mobilities in both 5 and 8% denaturing polyacrylamide gels, in which lariats and lariat-containing intermediates migrated differently (Fig. 3B and C). The splice junctions were identified by RT-PCR analysis of the fully spliced product of pre-mRNA H. RT-PCR with primers oCCB47 and oMD8 (Table 1) gave two bands corresponding to the unspliced pre-mRNA and the spliced mRNA (Fig. 4A). The size of the lower band matches the size predicted for a PCR product amplified from the correctly spliced mRNA. Comparison of the sequence of this PCR product with the BPV-1 genomic sequence identified the splice junction of exon 1 at nt 7385 and that of exon 2 at nt 3225. This result indicates that *in vitro* splicing of BPV-1 late pre-mRNAs utilizes the same 5' and 3' splice sites as previously identified for *in vivo*-spliced BPV-1 late mRNAs (5, 6).

Mapping of the BP in BPV-1 late pre-mRNAs. The *in vitro* splicing reactions described above demonstrate that the BPV-1 nt 3225 3' splice site in the absence of SE1 is utilized inefficiently in HeLa nuclear extracts and is therefore suboptimal. As discussed above, this is likely due in part to a suboptimal polypyrimidine tract. However, inefficiency of this 3' splice site

could also be due to a nonconsensus BP sequence. Regulation of pre-mRNA splicing by ESEs has been associated with a weak 3' splice site containing a nonconsensus BP and/or a suboptimal polypyrimidine tract in eukaryotic cells and viruses (8, 12, 14, 26, 27, 29, 35). To localize the BP which is used in the splicing of BPV-1 late pre-mRNA H, unlabeled splicing intermediates containing lariat and exon 2 were generated in *in vitro* splicing reactions and gel purified. Primer extension was performed on these intermediates with ³²P-labeled primer oMD8 (Table 1), which is complementary to exon 2 sequences. The same primer was used in both primer extension and dideoxy DNA sequencing reactions. This allowed us to determine exactly where the extended products were terminated. Since primer extension stops at one nucleotide before the BP, the BP could be mapped by comparing the extended products with the DNA sequencing ladder (20). The results shown in Fig. 5 demonstrate that primer extension stopped at the same adenosine nucleotide for both the gel-purified lariat-containing exon 2 RNA (lane 1) and an unfractionated splicing reaction (lane 3). This extension product was not seen when unlabeled pre-mRNA was used as the template (lane 2), although a few other bands were visible on the gel. These are most likely due to premature termination because of RNA secondary structure or pre-mRNA degradation. Since primer extension stops at one nucleotide before the BP, these results mapped the BP to an A residue on the sense strand at nt 3204. The BP is located 21 nt upstream of the nt 3225 3' splice junction and is therefore typical in its location. In addition, adenosine is the most common residue at BPs. However, the BP sequence (GUCUCAU; the BP is underlined) deviates from the mam-

FIG. 5. Mapping of the BP by primer extension analysis of the lariet intermediates. Primer extension was performed as described in Materials and Methods, using antisense ^{32}P -labeled primer oMD8 (Table 1). Substrates for the reactions were as follows: lane 1, gel-purified lariet-containing intron and exon 2 products generated from *in vitro* splicing reactions containing unlabeled BPV-1 late pre-mRNA H (Fig. 3A); lane 2, unlabeled unspliced pre-mRNA H; and lane 3, unspliced spliced products from pre-mRNA H. The same primer was also used for dideoxy DNA sequencing on pZMZ19-1 DNA (left). Primer extension and sequencing products were separated by electrophoresis on an 8% denaturing polyacrylamide gel. The sequence of the antisense strand is shown at the right along with the positions of the intron/exon boundary and the BP. 3'ss, 3' splice site.

malian BP consensus sequence (YNYURAC) at three positions. Thus, a nonconsensus BP sequence probably also contributes to the inefficient utilization of the BPV-1 nt 3225 3' splice site in vitro. The purine-rich splicing enhancer SE1 is required to compensate for these suboptimal splicing signals and therefore allow splicing to take place.

Identification of an ESS in the BPV-1 late pre-mRNA. Surprisingly, a BPV-1 late pre-mRNA containing both SE1 and SE2 as well as the sequences in between showed very little detectable splicing (Fig. 3B, pre-mRNA I). This finding suggests that sequences downstream of SE1 contain a splicing suppressor. To map this splicing suppressor further, BPV-1 late pre-mRNAs containing SE1 followed by various lengths of the sequence downstream of SE1 were prepared and tested for splicing in vitro (Fig. 6). This approach was also used to determine if SE2 influences the utilization of the nt 3225 3' splice site. As shown before, the BPV-1 late pre-mRNA G was spliced inefficiently since it contains no splicing enhancer sequences downstream of the 3225 3' splice site, whereas the late pre-mRNA H, which contains SE1, was spliced efficiently. However, the addition of even 48 nt immediately downstream of BPV-1 SE1 suppressed splicing approximately twofold (Fig. 6, pre-mRNA *StuI*). The addition of sequences further downstream of SE1 had little additional effect (Fig. 6, pre-mRNAs M, K, L, and J). These results indicate that an ESS is located upstream of nt 3353.

The sequence of the 48-nt region between SE1 and nt 3353 is most notable for its pyrimidine-rich composition. This includes a U-rich (67%) region between nt 3306 and 3317 and a C-rich (56%) region between nt 3319 and 3345. The U-rich region is contained within pre-mRNA H, which was spliced efficiently in vitro (Fig. 3 and 6). Confirmation that the C-rich region is required for suppression of splicing was further demonstrated by a mutational analysis. This region was randomly mutated by using the mutagenic 3' primer oZMZ54 (Table 1). Transcription of the resulting wild-type and mutant templates generated wild-type and mutant pre-mRNAs *StuI* and O, respectively, which are the same length (Fig. 7A and B). These pre-mRNAs were then compared for their abilities to be spliced in vitro. As shown in Fig. 7B and C, only 20% of pre-mRNA *StuI* was converted to lariat-containing intermediates or fully spliced product in a 2-h reaction. This represents threefold suppression compared with the 63% splicing seen for pre-mRNA H, which lacks the suppressor. In contrast, pre-mRNA O, which contains mutations in the suppressor region, was spliced as efficiently as pre-mRNA H. Thus, the C-rich region is required for the suppression of splicing. However, mutation of the C-rich region affected predominantly the accumulation of lariat-containing splicing intermediates. Very little increase in fully spliced product was seen. This finding suggests that although mutation of the suppressor sequence could fully restore the first step of splicing of the BPV-1 late mRNA, these mutations could only partially restore the second step of splicing (41). In summary, these results demonstrate that a 128-nt region immediately downstream of the nt 3225 3' splice site contains both a purine-rich splicing enhancer and a pyrimidine-rich splicing suppressor.

DISCUSSION

Eukaryotic alternative splicing is an important mechanism for increasing the diversity of gene expression. Regulated alternative splicing can lead to the production of different proteins from a single pre-mRNA or can function as an on-off switch in different cell types or at different times during development (18, 21). The life cycle of animal viruses is totally

FIG. 6. Sequences immediately downstream of SE1 suppress splicing of the BPV-1 late pre-mRNA. (A) Maps of BPV-1 late pre-mRNAs with various lengths of exon 2. Numbers above the pre-mRNAs indicate nucleotide positions in the BPV genome. Numbers below each species indicate the exon or intron length in nucleotides. Pre-mRNAs G and H are the same as in Fig. 3. Pre-mRNA *StuI* was synthesized from the *StuI*-linearized plasmid pZMZ19-1. All other pre-mRNAs were prepared from PCR-generated templates as described in Materials and Methods. (B) The sequences immediately downstream of SE1 inhibit splicing of BPV-1 late mRNAs. A splicing reaction with each pre-mRNA shown in panel A was performed as described in Materials and Methods. The spliced products of each pre-mRNA were separated by electrophoresis on an 8% polyacrylamide gel containing 8 M urea. Splicing products are identified at the right. The 100-bp ladder DNA size marker is shown on the left.

dependent on mammalian cells, and viral pre-mRNA splicing generally utilizes the cellular splicing machinery. As a result, viral systems still serve as valuable model systems for the study of the regulation of splicing. Alternative splicing is partially responsible for the large number of viral mRNA species which are expressed in BPV-1-infected cells. In spite of the diversity of spliced mRNA species, almost all spliced BPV-1 mRNAs which are expressed in BPV-1-transformed mouse cells are spliced by using a common 3' splice site at nt 3225. In infected bovine skin, alternative splicing of the BPV-1 late pre-mRNA

FIG. 7. Mutation of the ESS immediately downstream of SE1 partially restores splicing of a BPV-1 late pre-mRNA. (A) Map of BPV-1 pre-mRNAs. Numbers above the pre-mRNAs indicate nucleotide positions in the BPV-1 genome. Numbers below each species indicate the exon or intron length in nucleotides. Pre-mRNAs H and *Stul* were prepared as for Fig. 6. Pre-mRNA O was transcribed from a template generated by PCR using plasmid pZMZ19-1, the 5' T7 primer, and a 3' antisense primer (oZMZ54) which contains mutations in the ESS (Table 1). (B) Sequences immediately downstream of SE1 in the three BPV-1 pre-mRNAs shown in panel A. Nucleotide substitutions in the pre-mRNA O sequence are indicated in lowercase. The splicing efficiency of each pre-mRNA was calculated from the data shown in panel C as the percentage of total splicing products divided by the sum of the total splicing products plus the remaining pre-mRNA. (C) In vitro splicing analysis of pre-mRNA O containing ESS mutations in comparison with the pre-mRNAs with (pre-mRNA *Stul*) or without (pre-mRNA H) a wild-type ESS. Conditions for the splicing reactions and detection of the spliced products were the same as in Fig. 3B.

is regulated by keratinocyte differentiation (7). In the intermediately differentiated keratinocytes of the spinous layer of the epidermis, the majority of late viral pre-mRNAs are spliced by using the common 3' splice site at nt 3225. In the fully differentiated keratinocytes of the granular cell layer, splicing of the late viral pre-mRNA uses both the nt 3225 3' splice site and a

differentiation-dependent 3' splice site at nt 3605 to produce late mRNAs which encode the late proteins essential for viral capsid assembly. However, the mechanisms by which alternative splice site selection is regulated in BPV-1 pre-mRNA processing are not well understood.

In this study, we have investigated the regulation of splice site selection in BPV-1. Two issues were of particular interest to us. First, what *cis* elements are responsible for the preferential utilization of the nt 3225 3' splice site in BPV-1-transformed mouse cells and at early to intermediate stages of keratinocyte differentiation. Second, what *cis* elements are involved in the regulation of alternative splicing of the BPV-1 late pre-mRNA during keratinocyte differentiation. Although the experiments presented here directly address only the default pattern of splicing, the results give insight into signals which may regulate alternative splicing. The nt 3225 3' splice site was used inefficiently for the splicing of a BPV-1 late pre-mRNA in vitro, indicating that it is suboptimal. This is not surprising since regulated splice sites are usually suboptimal. Since efficient utilization of suboptimal splice sites requires additional signals, the sequences downstream of nt 3225 were searched for potential purine-rich splicing enhancer sequences. Two candidate elements were identified and named SE1 (nt 3256 to 3305) and SE2 (nt 3477 to 3526). These sequences were tested for the ability to enhance splicing of a *Drosophila dsx* pre-mRNA which contains a suboptimal 3' splice site and were shown to function as potent splicing enhancers. In addition, SE1, but not SE2, was shown to be required for the efficient utilization of the suboptimal nt 3225 3' splice site in the BPV-1 late pre-mRNA. Finally, we demonstrate that the enhancement of splicing by SE1 is balanced by the action of an ESS which is located immediately downstream of SE1.

The suboptimal nature of the BPV-1 nt 3225 3' splice site is most likely due to both a nonconsensus BP sequence as well as a suboptimal polypyrimidine tract. The BP was mapped to 21 nt upstream of the 3' splice site. The BP sequence is thus GUCUCAU (the BP is underlined) and deviates from the mammalian BP consensus sequence (YNYURAC) at three positions. In the yeast *Saccharomyces cerevisiae*, the BP sequence is highly conserved (UACUAAAC), while in mammals, the BP sequence is much less conserved. However, both yeast and mammalian BP sequences have been shown to base pair with the U2 snRNA (36, 58, 66). Nonconsensus BP sequences have been identified in several suboptimal 3' splice sites, and mutation of these sequences to or replacement with the consensus sequence restores efficient splicing (14, 27, 35, 43, 55). Binding of the U2 snRNP to nonconsensus mammalian BP sequences requires the polypyrimidine tract, and this binding is facilitated by the splicing factor U2AF (39, 61). Interaction of U2AF with the polypyrimidine tract determines the strength of a 3' splice site and is influenced by the length and composition of the polypyrimidine tract (13, 35, 38, 43, 55, 62). Random purine insertions or even a single purine insertion in a short polypyrimidine tract can be detrimental to splicing. Moreover, uridines and cytosines are not functionally equivalent in the polypyrimidine tract, since U2AF binds preferentially to runs of uridines. Although the polypyrimidine tract (UCCACCU CUUCUGAUUUU) associated with the BPV-1 nt 3225 3' splice site is 18 nt long, it also contains three purines and has no run of uridines longer than four. Such suboptimal polypyrimidine tracts are typical of regulated splice sites and have been seen in several viral systems, including adenovirus L1 pre-mRNA (27), Rous sarcoma virus *src* pre-mRNA (31, 63), HIV-1 *tat/rev* pre-mRNA (43), simian virus 40 (16), and the P4-generated pre-mRNA of the parvovirus minute virus of

mice (64). The polypyrimidine tract may also play a role in splicing in addition to the facilitation of U2 snRNP binding through U2AF. A single U-to-C transition in the avian sarcoma virus *env* polypyrimidine tract has been shown to inhibit primarily the second step of splicing (9).

Recognition of suboptimal 3' splice sites requires additional *cis*-acting positive elements. The *in vitro* data presented here suggest that this role is played at least in part by the ESE SE1. The characteristics of SE1 and SE2 are typical of other ESEs. Each is capable of enhancing the splicing of a heterologous pre-mRNA containing a suboptimal splice site (51, 56). In addition, both sequences are purine rich (64% purines for SE1 and 72% for SE2) and contain several clusters of the purine repeat GAR (R = G or A) which has been identified in other ESEs (37, 59). The SR protein family of splicing factors has been shown to bind several ESEs, including those in the human fibronectin alternative exon ED1, bovine growth hormone exon 5, avian sarcoma-leukosis virus *env* 3' exon, chicken cardiac troponin T exon 5, and *Drosophila dsx* exon 4 (29, 30, 37, 45, 49, 53, 54). The SR proteins are essential for the function of the ESE and appear to recruit U2AF to the 3' splice site (37, 45). Recently the binding specificities of two SR proteins were determined by using an *in vitro* selection procedure (50). A comparison of the binding sites of ASF/SF2 (SRp30a) and SC35 (SRp30b) with the sequences of SE1 and SE2 revealed potential binding sites for ASF/SF2, but not SC35, in both SE1 and SE2. In addition, we have preliminary data indicating that SE1 and SE2 bind SR proteins and that mutations in SE1 and SE2 that reduce splicing enhancer activity also result in a loss of SR protein binding capacity (64a).

One surprising finding in this study was the identification of a pyrimidine-rich ESS immediately downstream of the ESE SE1. The combination of these two elements thus forms a bipartite splicing element which modulates the activity of the BPV-1 nt 3225 3' splice site. The studies presented here do not address whether the ESS functions by blocking the activity of the ESE or by acting directly on the nt 3225 3' splice site. The pyrimidine-rich nature of the ESS suggests that it could function as a decoy polypyrimidine tract and block assembly of the spliceosome at the authentic 3' splice site. This type of mechanism has been shown for other splicing repressors. For example, the P-element pseudo-5' splice site acts as a decoy (42). Identification of the factors which bind the BPV-1 ESS element should help elucidate the mechanism by which the ESS suppresses splicing.

There are several other examples of negative-acting exonic splicing elements which affect utilization of upstream 3' splice sites (1, 2, 10, 11, 44). One common feature of these elements is that they all require suboptimal splice sites for their function. Another interesting feature which the ESS elements in the HIV-1 *tat/rev* exon 3 (2, 44) and the human fibronectin EDA exon (10) have in common with the BPV-1 ESS is that they also lie immediately downstream of an ESE element. However, the sequences of these ESS elements have a diverse composition and show no similarity to the BPV-1 ESS. The sequence of the human fibronectin EDA exon ESS is CAAGG (10). In the fibroblast growth factor receptor 2 K-SAM exon, an ESS sequence consists of TAGGCAGGC (11), whereas the sequence motif AGATCC is conserved in the ESS elements within *tat* exon 2 (1, 2) and *tat-rev* exon 3 (2, 44) in HIV-1. In contrast, the BPV-1 ESS consists predominantly of pyrimidines. It is likely that different cellular factors are required for the function of different ESS elements. To date, the cellular factors interacting with these ESS elements have not been identified.

It is not yet known what role SE1, SE2, and the ESS play in the regulation of BPV-1 splicing *in vivo*. The *in vitro* splicing data presented here suggest that SE1 is responsible for the

predominant utilization of the nt 3225 3' splice site in mouse cells and undifferentiated keratinocytes. It is impossible to determine from the *in vitro* studies if SE2 also plays a positive role in the selection of the nt 3225 3' splice site because the pre-mRNAs which contained SE2 also contained the ESS which inhibited splicing of the pre-mRNAs. SE2 is located 252 nt downstream of the nt 3225 3' splice site and 80 nt upstream of the nt 3605 3' splice site. Lavigne and coworkers (29) investigated the effect of position on the human fibronectin ED1 exon ESE and found that it could still function 293 nt downstream of the 3' splice site on which it functions. The *dsx* repeat element functions as a constitutive ESE when positioned 100 nt away from the 3' splice site but not when positioned 300 nt away (54). In the presence of Tra and Tra-2, which enhance the binding of SR proteins to the element, the *dsx* repeat element can function when located 500 nt away. The effect of distance on the function of an ESE may depend on the strength of the ESE. SE2 is a strong ESE, so it is possible that it also contributes *in vivo* to selection of the nt 3225 3' splice site. Another possibility role for SE2 is suggested by the fact that it occupies an intronic location with respect to the nt 3605 3' splice site. Depending on the position of the nt 3605 BP, factors bound to SE2 could block the binding of the U2 snRNP at the BP and inhibit utilization of the nt 3605 3' splice site. Thus, SR proteins could simultaneously enhance use of the nt 3225 3' splice site and inhibit use of the nt 3605 3' splice site by binding to both SE1 and SE2. The relative amounts of different SR proteins are known to vary in a tissue- and developmental stage-specific manner (15, 60). Thus, a change in SR protein levels or a biochemical modification of SR proteins during keratinocyte differentiation could be responsible for the shift in 3' splice site utilization in terminally differentiated keratinocytes. The role of the pyrimidine-rich ESS *in vivo* is also unknown. Data in this report suggest that the ESS may play an important role by balancing the strength of the nt 3225 3' splice site or by switching different alternative splice sites at various times in the BPV-1 life cycle and in keratinocyte differentiation. Experiments are currently in progress to determine the role of these elements in the regulation of BPV-1 alternative splicing *in vivo*.

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