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Hierarchy of Polyadenylation Site Usage by Bovine Papillomavirus in Transformed Mouse Cells

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The great majority of viral mRNAs in mouse C127 cells transformed by bovine papillomavirus type 1 (BPV) have a common 3′ end at the early polyadenylation site which is 23 nucleotides (nt) downstream of a canonical poly(A) consensus signal. Twenty percent of BPV mRNA from productively infected cells bypasses the early polyadenylation site and uses the late polyadenylation site approximately 3,000 nt downstream. To inactivate the BPV early polyadenylation site, the early poly(A) consensus signal was mutated from AAUAAA to UGUAAA. Surprisingly, this mutation did not result in significant read-through expression of downstream RNA. Rather, RNA mapping and cDNA cloning experiments demonstrate that virtually all of the mutant RNA is cleaved and polyadenylated at heterogeneous sites approximately 100 nt upstream of the wild-type early polyadenylation site. In addition, cells transformed by wild-type BPV harbor a small population of mRNAs with 3′ ends located in this upstream region. These experiments demonstrate that inactivation of the major poly(A) signal induces preferential use of otherwise very minor upstream poly(A) sites. Mutational analysis suggests that polyadenylation at the minor sites is controlled, at least in part, by UAUAUA, an unusual variant of the poly(A) consensus signal approximately 25 nt upstream of the minor polyadenylation sites. These experiments indicate that inactivation of the major early polyadenylation signal is not sufficient to induce expression of the BPV late genes in transformed mouse cells.

Eukaryotic RNA polymerase II transcription units are typically transcribed past the mature mRNA 3′ end. These transcripts are then cleaved and a poly(A) tract of 200 to 300 nucleotides (nt) is added to generate the 3′ end of the mature mRNA (for reviews, see references 30 and 37). Eighty to ninety percent of animal cell mRNAs contain the sequence AAUAAA 10 to 30 nt upstream of the poly(A) tail. Another 10% have the variant AUUAAA; other variants are rare (38). These consensus sequences have been shown to be required for efficient and accurate cleavage and polyadenylation both in vivo and in vitro (17, 27, 29). Generally, when this sequence is mutated, polyadenylation occurs at a downstream site, often with reduced efficiency (17). The region upstream and downstream of the AAUAAA consensus signal, including GU-rich downstream sequences, has also been identified as playing a role in the cleavage and polyadenylation of some transcripts (30, 37).

Bovine papillomavirus type 1 (BPV) induces fibropapillomas in cattle and transforms a number of cultured rodent fibroblast cell lines to tumorigenicity. The papillomaviruses are unable to propagate in such transformed cells, in part because the early polyadenylation site used by essentially all BPV transcripts in transformed cells is located between the transcriptional promoters and L1 and L2, the two genes which encode the virion proteins (Fig. 1A) (16, 23, 39). Similarly, in BPV-induced skin fibropapillomas, usage of this early polyadenylation site precludes expression of the capsid protein genes in transformed dermal fibroblasts and presumably in the basal keratinocytes as well (4, 5, 35). In terminally differentiating keratinocytes which express the capsid proteins and produce virus, about 20% of the viral mRNA reads through the early polyadenylation site and is instead polyadenylated approximately 3,000 nt downstream at the late polyadenylation site (5). Thus, regulation of polyadenylation at the early site appears to be crucial for viral late gene expression.

To study signals that control polyadenylation in BPV-transformed mouse C127 cells, we mutated the early poly(A) consensus signal AAUAAA, located 23 nt upstream of the early poly(A) site. It was expected that mutant transcripts would now bypass the early poly(A) site and that late region sequences would be included in stable RNA. RNA mapping experiments instead demonstrated that mutant transcripts were polyadenylated at heterogeneous sites approximately 100 nt upstream of the early polyadenylation site used in cells transformed by wild-type BPV. Evidence is presented which suggests that an unusual variant of the poly(A) consensus sequence, UAUAUA, plays a role in the regulation of polyadenylation at the upstream polyadenylation sites.

Construction and preliminary characterization of the poly(A) consensus mutant. To disrupt polyadenylation at the BPV major early polyadenylation site at nt 4203, oligonucleotide-directed mutagenesis was used to mutate the poly(A) consensus signal at nt 4180 from AAUAAA to UGUAAA (Fig. 1B), thereby creating a new *Pvu*II cleavage site (23a). The resulting mutation on a *Bst*XI-to-*Sal*I fragment was reconstructed into the full-length wild-type BPV genome (clone pBPV-142-6 [33]) to generate mutant pBPV-EPA1. Nucleotide sequence analysis of the fragment replaced in generating pBPV-EPA1 (nt 3849 and 4450) demonstrated that no extraneous mutations were introduced during mutagenesis.

The ability of three isolates of pBPV-EPA1 to transform C127 cells was assayed by determining the efficiency of focus formation after *Bam*HI digestion to release the viral DNA from the plasmid vector and transfection as described previously (14). All three mutant isolates transformed cells with approximately the same efficiency as wild-type BPV DNA (data not shown). Cell lines were derived from pools of foci induced by pBPV-EPA1 (EPA1p) and by wild-type BPV DNA (142-6p). ID13 cells, a C127 cell line transformed by infection with BPV, were used as an additional wild-type control.

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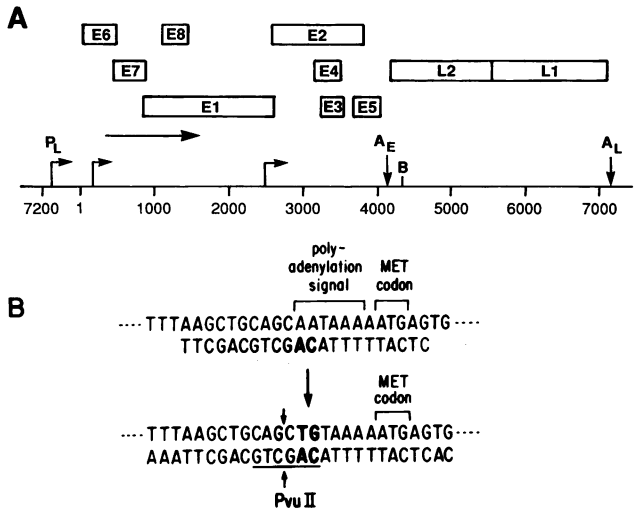

FIG. 1. The BPV genome and design of the EPA1 mutation. (A) The BPV genome linearized at the 3′ end of the late transcription unit is shown. Open boxes indicate translational open reading frames. The long horizontal arrow indicates the direction of transcription. The short horizontal arrows indicate the positions of major promoters. The late promoter is designated P_L. A_E and A_L denote the early and late polyadenylation sites, respectively. B indicates the position of the unique *Bam*HI site. Nucleotide numbers are shown at the bottom. (B) The EPA1 mutation. The top line shows the wild-type BPV DNA sequence around the early polyadenylation signal. The sequence of the mutagenic oligonucleotide L1 is shown directly below it, with the base substitutions shown in boldface. The template was the small *Bam*HI to *Eco*RI fragment of BPV-1 DNA cloned in M13mp8 (13). The sequence at the bottom shows the mutation with the new *Pvu*II site indicated. The open reading frame L2 initiation codon is designated the MET codon.

Southern blot analysis of viral DNA from transformed cells demonstrated that the mutant viral DNA was maintained in transformed cells as a multicopy plasmid without gross rearrangement and with restoration of the *Bam*HI site used to excise the viral DNA from the plasmid vector (data not shown).

Mapping the 3′ end of the mutant mRNA. The mutation in pBPV-EPA1 was designed to eliminate polyadenylation at the wild-type early polyadenylation site immediately upstream of the late open reading frames. Extensive Northern (RNA) blot analysis and RNA protection experiments failed to detect significant amounts of RNA extending past the polyadenylation site into the late region, but these experiments did not exclude the presence of low levels of read-through RNA (data not shown). There was severalfold more stable viral RNA in cells transformed by wild-type BPV than in those transformed by the polyadenylation site mutant (Fig. 2).

RNase protection experiments were performed to map the 3′ ends of the mutant transcripts. ID13 and EPA1p RNAs were assayed for protection of an antisense EPA1 RNA probe spanning the early polyadenylation site at nt 4203 (Fig. 2, left panel). The size of the fragment protected by RNA from ID13 cells indicates that, as expected, the wild-type viral RNA extends past nt 4180, the site of the mutation in the probe (lane c). In contrast, EPA1p RNA protected several fragments approximately 100 nt shorter than those protected by wild-type RNA, suggesting that the mutant RNA is polyadenylated upstream of the normal position (lanes a and b). The difference in the pattern of protected bands between the two EPA1p lanes, a and b, is due to the different cleavage specificities of the two RNases used in these reactions. The same result was obtained with oligo(dT)-selected EPA1p RNA (data not shown), indicating that these shorter species are polyadenylated, a conclusion confirmed by cDNA cloning (see below). There was no evidence of significant polyadenylation of EPA1p RNA at the usual position, nor were prominent shorter novel bands protected in the ID13 sample. RNA from two additional cell lines generated with the original isolate of the mutant and two additional cell lines generated with independent isolates of the mutant showed the protection pattern characteristic of the mutant (data not shown). These results suggested that sequences downstream of nt 4100 were absent from mutant RNA, an interpretation supported by the results of protection experiments with additional antisense probes and the results of Northern blot hybridization experiments with oligonucleotide probes (data not shown). These results are interpreted in the right panel of Fig. 2.

cDNA cloning and sequencing. The results presented above suggest that new heterogeneous polyadenylation sites near nt 4100 are utilized in EPA1p RNA. To confirm this interpretation, the 3′ ends of both wild-type and mutant RNAs were cloned and sequenced. Oligod(T)-selected (3) 142-6p and EPA1p RNAs were reverse transcribed with oligo(dT) as primer and the reagents and protocol of a cDNA synthesis kit (Amersham). The resulting first-strand cDNAs were amplified by the polymerase chain reaction (PCR) method with the primers diagrammed in Fig. 3A (18, 32, 34). To specifically amplify BPV sequences, the upstream PCR primer PCR5 corresponded to BPV nt 3998 to 4031. To selectively amplify polyadenylated molecules, the downstream PCR primer PCRT was 5′ d($GGGGATCCT_{25}$) 3′, which hybridized to any product containing a poly(A) tract. Annealing was carried out at 25°C, because PCRT has a calculated T_m of 38.9°C in PCR buffer conditions (32). The products of each amplification reaction were cloned into pUC18, and colonies containing an insert were identified by colony hybridization (22) with an oligonucleotide probe PCR1 complementary to a region (nt 4063 to 4089) between the upstream primer and the proposed 3′ end of mutant RNA.

The results of sequence analysis of the cDNA clones are summarized in Fig. 3B. Sites of polyadenylation were identified as junctions between BPV DNA sequence and tracts of poly(A). Six of the 11 clones derived from cells transformed by wild-type BPV were polyadenylated after nt 4203, the previously described early polyadenylation site (39), thus validating this strategy of identifying polyadenylation sites. In contrast, none of the clones derived from mutant RNA displayed the wild-type polyadenylation site. Instead, seven of the nine EPA1p clones contain a stretch of poly(A) immediately after BPV nt 4107, and the other two clones contain poly(A) after nt 4101 and 4092. These results are consistent with the RNase protection and Northern blot results which indicate the existence of heterogeneous 3′ ends near nt 4100 in mutant RNA and demonstrate that these new 3′ ends are in fact new sites of polyadenylation. Interestingly, the anomalous clones (almost half) derived from wild-type RNA showed polyadenylation at heterogeneous sites similar to those found with the mutant RNA. These results indicate that there is a population of mRNAs with heterogeneous polyadenylation sites around nt 4100 in cells transformed by wild-type BPV. The preferential amplification of shorter PCR products may explain the relatively frequent isolation of these shorter cDNAs from cells transformed by wild-type BPV. We have occasionally observed faint bands in protection experiments with ID13 RNA which

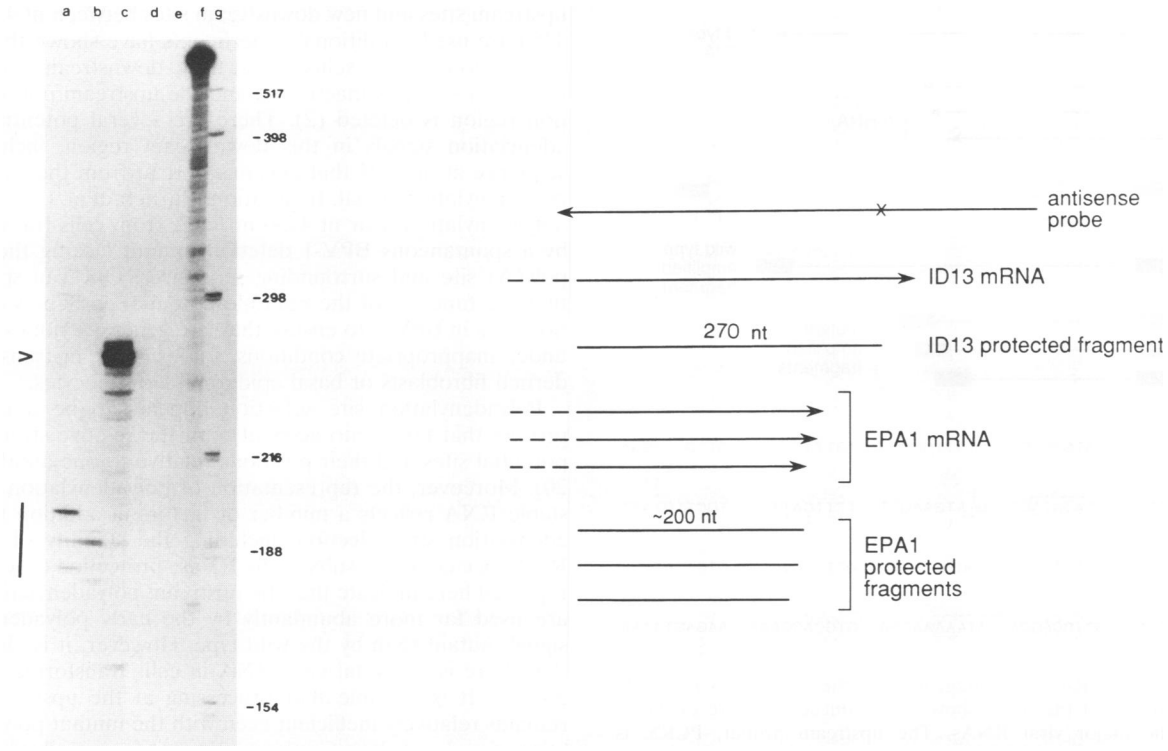

FIG. 2. RNase protection analysis of viral early region RNA in transformed cells. (Left panel) Ten micrograms of total cellular RNA (9, 21) from EPA1p (lanes a and b), ID13 (lane c), and C127 (lane d) cells was hybridized to an antisense EPA1 RNA probe (28) complementary to BPV nt 3912 to 4450, spanning the position of the normal early polyadenylation site but containing the mutation at the polyadenylation signal. Hybrids were digested with either 2 μg of RNase T1 per ml (lane a), 40 μg of RNase A per ml (lane b), or a mixture of both RNases (lanes c to e), and protected fragments were detected by autoradiography after electrophoresis through a 4% polyacrylamide–50% urea gel. The sample in lane e was the probe digested after mock hybridization; the sample in lane f is undigested probe. The nucleotide lengths of size markers in lane g are indicated. The arrowhead indicates the predicted position of a 270-nt fragment extending from the 3′ end of the probe to the site of the mutation, which is generated by cleavage at the mismatch between the wild-type RNA and the mutation in the probe. The vertical line on the left indicates the small cluster of bands protected by mutant RNA. (Right panel) Schematic representation of the probe, protected fragments generated by RNase digestion, and the deduced structure of viral RNA species. Arrows indicate the direction of transcription, with the arrowheads representing the 3′ end of each transcript. The X indicates the position of the mutation in the probe.

are consistent with minor sites of polyadenylation at these upstream positions (data not shown).

Identification of a signal controlling polyadenylation at the upstream sites. There is no poly(A) consensus sequence or previously described functional variant within 100 bp upstream of nt 4100. However, the sequence UAUAUA is present at nt 4073, approximately 30 nt 5′ to the poly(A) sites in EPA1p RNA (Fig. 3B). It is the closest match to the consensus sequence in the region, and it appears to be in the appropriate position to specify cleavage at the sites detected in mutant RNA. To test the role of this sequence in specifying polyadenylation in the absence of the wild-type signal, it was mutated from UAUAUA to GAUAUC by using the mutagenic primer 5′ d(AACTTCATAC AGGATATCAA ACAAATCA)3′, corresponding to BPV sequence from nt 4063 to 4090, and single-stranded EPA1 DNA as a template. The resulting mutant, pBPV-EPA2 (see Fig. 5) therefore contained both the original mutation at the poly(A) consensus signal and the new mutations in the putative variant signal. This mutant transformed C127 cells with approximately wild-type efficiency, and RNA from a pooled cell line transformed by EPA2 DNA was mapped by using RNase protection and an antisense EPA2 probe (Fig. 4). RNA from ID13 cells protected the fragment sizes predicted if cleavage occurred at the sites of mismatch between wild-type RNA and the probe (which contains mutations at nt 4073, 4078, 4180, and 4181) (lane b). EPA2 RNA protected two major size classes of fragments (lane a). One was a set of probe fragments approximately 190 to 200 nt long, corresponding to polyadenylation near nt 4100 as in EPA1p RNA. These protected fragments comigrate with the fragments protected by EPA1 RNA (data not shown) and are the size predicted if polyadenylation occurred at nt 4107, the mutant site mapped by cDNA cloning. In addition, EPA2p RNA protected several longer fragments corresponding to heterogeneous RNA 3′ ends between nt 4200 and 4450. The EPA2 mutation thus reduced the efficiency with which the upstream polyadenylation sites are used, but it did not appear to affect the position of poly(A) addition for those transcripts that are successfully polyadenylated in this region. These results indicate that the UAUAUA plays a role in specifying the new upstream sites of polyadenylation in EPA1p RNA.

Discussion. These experiments were designed to study polyadenylation site usage in BPV-transformed mouse cells. A point mutation in the poly(A) consensus signal disrupted polyadenylation at that site both in vivo, as demonstrated here, and in an in vitro polyadenylation system (24). RNase protection, Northern blotting, and cDNA cloning and sequencing established that stable mutant transcripts utilized heteroge-

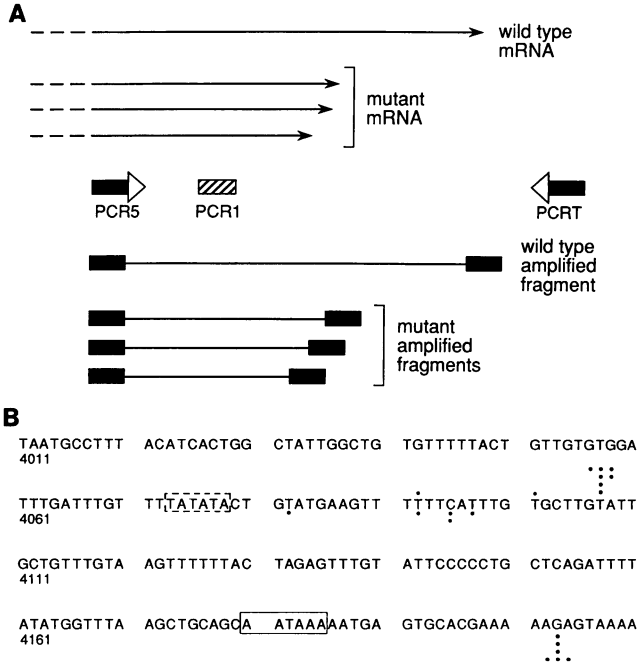

FIG. 3. (A) PCR-based strategy to clone the 3′ ends of viral RNA. The top portion of the panel shows the deduced structure of the 3′ ends of the major viral RNAs. The upstream primer, PCR5, is complementary to all known wild-type and mutant viral early RNAs. The downstream primer, PCRT, consists of oligo(dT) and a cloning site but no BPV-specific sequences. After amplification with cDNA reverse transcribed from polyadenylated RNA as a template, BPV cDNAs were cloned into pUC18, identified by hybridization to PCR1, and sequenced. (B) cDNA clone sequences. The sense strand BPV sequence from nt 4011 to 4210 is shown. The normal poly(A) consensus signal is enclosed in the solid line, and the putative upstream poly(A) signal is enclosed in the dashed line. Each dot indicates the position of a junction between BPV DNA and the poly(A) tract in a cDNA clone. Clones derived by amplification of wild-type RNA are represented by dots below the sequence, and those derived from mutant RNA are represented by dots above the sequence.

neous polyadenylation sites approximately 100 nt upstream of the wild-type polyadenylation site. The results of the cDNA cloning also demonstrated that some wild-type transcripts have heterogeneous 3′ ends in the region used by the mutant RNAs, indicating that this is a minor polyadenylation site in cells transformed by wild-type BPV. In a related system, Doniger et al. (15) found usage of upstream polyadenylation sites by human papillomavirus type 16 transcripts in an immortalized human exocervical epithelial cell line harboring a human papillomavirus type 16 genome with an extensive deletion immediately downstream of a wild-type early poly(A) signal. The 3′ ends of viral RNA from these cells mapped to both the normal site and to a heterogeneous region 400 to 500 nt upstream of that site.

The results described here suggest a hierarchy of polyadenylation site usage in BPV-transformed cells, as is summarized in Fig. 5. Wild-type BPV mRNA is polyadenylated at the major polyadenylation site at nt 4203, with a small fraction of transcripts being polyadenylated at minor upstream sites around nt 4100. When the major signal is disrupted (as in EPA1), the sites around nt 4100 become the predominant sites of polyadenylation. When the major signal is inactivated and the minor signal is partially disrupted (as in EPA2), both the upstream sites and new downstream sites between nt 4200 and 4450 are used. Additional experiments have shown that polyadenylation occurs exclusively at these downstream sites when the major signal is inactivated and the upstream polyadenylation region is deleted (2). There are several potential polyadenylation signals in this downstream region, including a sequence at nt 4304 that deviates by 1 nt from the consensus polyadenylation signal. In addition, Burnett et al. (8) observed polyadenylation near nt 4450 in RNA from cells transformed by a spontaneous BPV-1 deletion mutant lacking the major poly(A) site and surrounding sequences. One can speculate that the function of the multiple potential early polyadenylation sites in BPV is to ensure that late genes are not expressed under inappropriate conditions, for example in transformed dermal fibroblasts or basal epidermal keratinocytes.

Polyadenylation site selection appears to be a complex process that takes into account both the relative strengths of potential sites and their positions relative to one another (12, 20). Moreover, the representation of polyadenylation sites in stable RNA reflects a number of factors in addition to polyadenylation site selection, including the stability of various RNA species. The results of the RNase protection experiments reported here indicate that the upstream polyadenylation sites are used far more abundantly by the early polyadenylation signal mutant than by the wild type. However, it is also clear that there is less total viral RNA in cells transformed by the mutant. It is possible that processing at the upstream sites remains relatively inefficient even with the mutant polyadenylation signal, resulting in the synthesis of a rapidly degraded pool of unprocessed RNA extending into the late region. In fact, Furth and Baker (19) have described a sequence element in the BPV late region which prevents the accumulation of stable viral RNA in transformed cells.

The closest match to a poly(A) consensus signal in the vicinity of the minor upstream polyadenylation sites is UAUAUA, approximately 25 nt upstream of the new RNA 3′ ends. RNA from cells containing mutations of both the original poly(A) signal and this putative upstream signal contains heterogeneous 3′ ends at both the upstream sites and at additional positions downstream of the normal site. This result suggests that the UAUAUA plays a role in directing polyadenylation at the upstream polyadenylation sites and that the mutation did not fully disrupt the function of the UAUAUA sequence. We are not aware of a precedent for UAUAUA acting as a poly(A) signal in mammalian cells, although it can direct mRNA 3′ end formation and polyadenylation in *Saccharomyes cerevisiae* (31). However, we note that the region around the upstream cleavage sites contains numerous oligo(dT) tracks and GT dinucleotides, sequence motifs found near some bona fide mammalian poly(A) signals.

The wild-type poly(A) consensus signal appears to suppress utilization of the variant signal located approximately 100 nt upstream. Such suppression may be rather general. Connelly and Manley (10) studied the simian virus 40 early polyadenylation region, which contains two closely spaced AAUAAA signals. In the wild-type situation, only the 3′ site is efficiently utilized. However, if this preferred site was inactivated by mutation, increased usage of the 5′ site was observed. In addition, Denome and Cole (11) showed that addition of tandemly arranged polyadenylation signals decreased usage of the upstream site. These findings imply that genomes may contain numerous potential sites of polyadenylation whose activity is suppressed by the relatively close apposition of another polyadenylation signal, which perhaps competes more efficiently for a limiting polyadenylation factor. Therefore, alternative polyadenylation, which is a well-documented con-

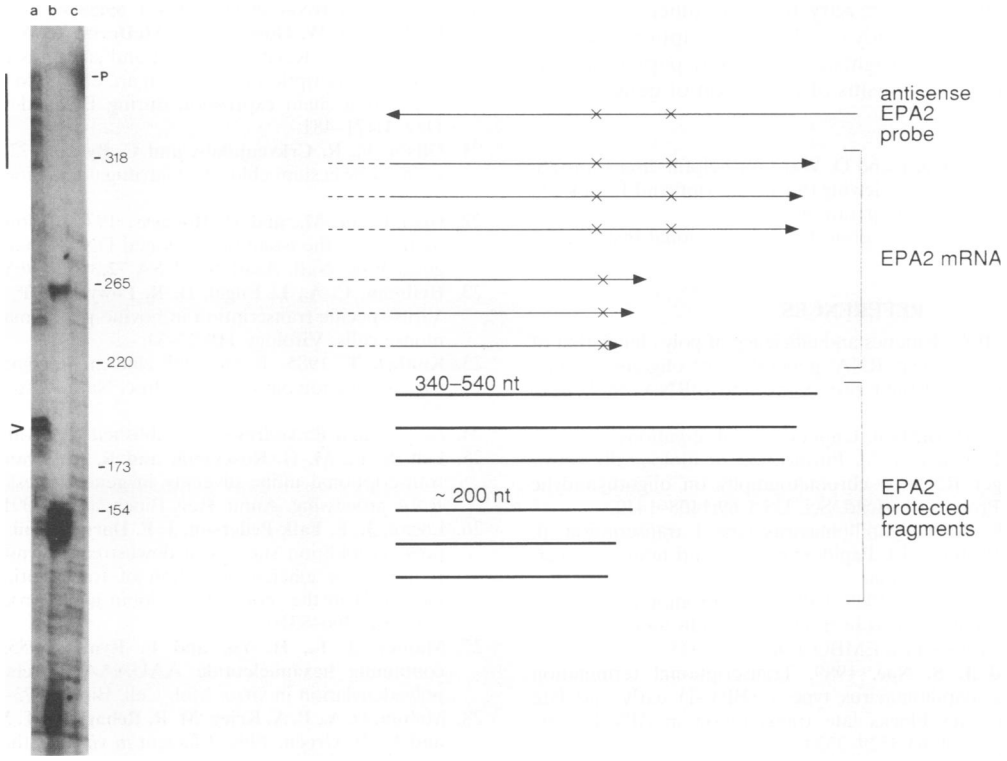

FIG. 4. Evidence that UAUAUA at nt 4073 plays a role in poly(A) site selection. (Left panel) Radiolabelled antisense RNA probe extending from BPV nt 4450 to 3912 was transcribed in vitro from EPA2, hybridized to 10 μg of cellular RNA isolated from EPA2p (lane a), ID13 (lane b), or C127 (lane c) cells, and digested with a mixture of RNases A and T1. Protected fragments were subjected to polyacrylamide gel electrophoresis and detected by autoradiography. The arrowhead on the left indicates the position of approximately 190- to 200-nt probe fragments extending from the 3′ end of the probe to the upstream sites of polyadenylation around BPV nt 4100. The vertical line on the left indicates the position of the larger fragments also protected by mutant RNA. The approximately 265-base fragment in lane b appears to be derived from partially digested hybrids. The lengths (in nucleotides) of coelectrophoresed size markers are shown. P indicates the position of undigested probe (538 nt). (Right panel) Schematic representation of the antisense probe, sizes of the protected fragments, and deduced structures of viral RNA species. The X's show the positions of the mutations at the upstream and downstream polyadenylation signals in the probe and in RNA isolated from cells transformed by the double mutant.

trol point for regulating gene expression (25), may result in some cases from inactivation of a preferred polyadenylation signal rather than by direct activation of a suboptimal one.

The mechanism by which BPV prevents expression of viral late genes in transformed cells but allows their expression in differentiated keratinocytes is central to an understanding of papillomavirus biology. One level of restriction in transformed cells is clearly at the level of stable mRNA accumulation, because little or no BPV mRNA from the late region is present in cultured fibroblasts. Analysis of nascent RNA from ID13 cells indicates that at least 90% of BPV transcripts terminate between the early and late poly(A) sites and therefore never reach the late poly(A) signal (6). The mechanism(s) allowing production of late RNAs during natural infection may act primarily at the level of polyadenylation site selection, or it may act at some other steps in mRNA biogenesis, such as alterations in promoter usage, splicing patterns, or transcription termination, which secondarily affect cleavage and polyadenylation (for examples, see references 1, 7, 26, and 36). However, the results presented here indicate that specific suppression of the major early polyadenylation signal is unlikely to be the sole step in releasing the block to BPV late gene expression, because inhibition of polyadenylation at additional potential early sites must also occur. The mechanism involved in late gene expression must coordinately suppress cleavage and polyadenylation at multiple potential sites near the 3′ end of the early region in some of the transcripts, while many transcripts are still polyadenylated at the early polyadenylation site. Regulation of BPV late gene expression is clearly a complex process and bypass of the early major

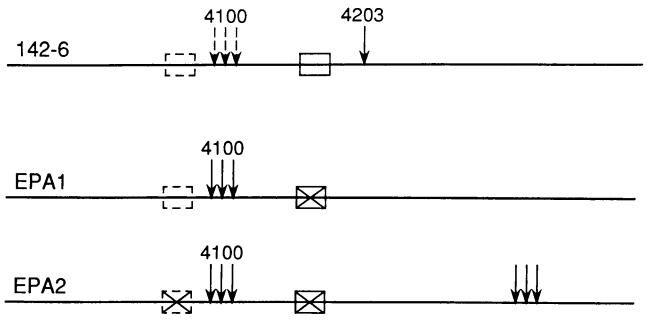

FIG. 5. Usage of early region polyadenylation sites. The horizontal lines represent the region of the BPV genome around the early polyadenylation site for wild-type BPV DNA (142-6) and the indicated mutants. Transcription proceeds from left to right. The unbroken box represents the normal early polyadenylation consensus signal at nt 4180, and the dashed boxes represent the putative upstream polyadenylation signal at nt 4073. The vertical arrows indicate the positions of poly(A) addition, and triple arrows indicate heterogeneous polyadenylation sites, with minor sites represented as dashed arrows. Boxes containing an X indicate a mutant polyadenylation signal.

polyadenylation site is a necessary but not sufficient component of that process. The study of BPV transcriptional regulation promises to provide insights into not only papillomavirus biology but also the mechanisms of regulation of gene expression in general.

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