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Oncogenic and Nononcogenic Human Genital Papillomaviruses Generate the E7 mRNA by Different Mechanisms

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A new promoter located within E6 was mapped in human papillomavirus type 6b (HPV6b)- and HPV11-containing benign genital condylomata (genital warts). The RNA transcribed from this promoter represented the major RNA species colinear with open reading frames E6 and E7 and can encode the E7 protein. No equivalent promoter was active in HPV16-containing cancers and cancer-derived cell lines. In those, the major transcripts contained one of two different introns within E6 and the RNAs could encode two different E6* proteins and E7.

The presence of the DNAs of certain human papillomaviruses (HPV) (oncogenic human papillomaviruses) in most cervical cancers has suggested a role for these viruses in the development of cervical neoplasia (reviewed in reference 40). This is further supported by the continued transcription of some viral sequences. S1 mapping (32) and cDNA sequencing (25) have indicated that mRNAs coding for the E6 and E7 proteins are present. Furthermore, when the viral DNA is integrated, integration often interrupts sequences downstream of the E6 and E7 open reading frames (ORF) (3, 7, 17, 26, 40), strengthening the notion that the E6 and E7 proteins play a critical role in cancer development.

The DNAs of nononcogenic papillomaviruses are almost never found in invasive cervical cancers. The reason for this nononcogenicity is not known but must be due to some difference in the biologies of the different virus types. Insight into the differences between oncogenic and nononcogenic HPVs might be revealed by a comparison of the viral transcripts in cancers versus condylomata (benign genital warts). Previously, we showed that the major and two minor RNAs in the cancer-derived CaSki cell line were transcribed from the same promoter, P97. The major transcript had an intron within E6 and probably represented the mRNA for E7 which is synthesized by these cells (1, 30, 32, 33). An equivalent HPV18 transcript is also present in HeLa cells (25) which also express E7 (30). In an HPV16-containing cancer analyzed in parallel with CaSki cells, only the major transcript was identified (32). Sequence data indicate that oncogenic HPV16, HPV18, HPV31, and HPV33, but not nononcogenic HPV6b and HPV11, contain equivalent signals to splice out an intron within E6 (28), and no alternate introns were detected by R-loop mapping of RNAs isolated from HPV6b and HPV11 condylomata (8). This difference could indicate that only oncogenic HPV types synthesize a transcript which could serve as efficient mRNA for E7 (4, 5).

Here we show that the major E6-E7 colinear transcripts in HPV16-containing cervical cancers were generated by splicing out one of two introns within the E6 sequences, thus creating an mRNA that potentially codes for a full-length E7 protein, as well as one of two different short E6 peptides, referred to as E6* (25). All E6-E7 transcripts in the tumors originated from a common promoter, P97, located upstream of the second ATG of E6. In contrast, in HPV6b- or HPV11-containing condylomata, the major E6-E7 colinear mRNA was transcribed from a promoter located downstream of the E6 ATG and this RNA encoded the entire E7 protein. A less prominent transcript in both the cancers and the benign genital warts contained the entire coding sequences for E6.

Previous mapping by S1 and *Exo*VII digestion and primer extension of HPV16 transcripts in the cervical cancer-derived CaSki cells indicated that all three transcripts colinear with E6 and E7 have a common cap site at nucleotide (nt) 97 located between ATGs 1 and 2 of E6 and 30 nts downstream of a TATA box. The major transcript contained an intron within E6 equivalent to that mapped for HPV18 in HeLa cells (25). A minor transcript contained a different intron within E6, utilizing different splice signals, and the third transcript contained an exon extending uninterrupted from nt 97 to a common splice donor just downstream of the end of E7. In a cancer analyzed in parallel, only the major splice acceptor within E6 could be identified because of limiting material available. The mapping data presented in Fig. 1 characterize the 5′ ends of E6-E7 colinear transcripts isolated from (i) the HPV16-containing cancer-derived SiHa cell line (3, 23, 32, 39) obtained from the American Type Culture Collection and (ii) two cervical cancers. *Exo*VII digestion, performed as described elsewhere (22, 32), resulted in essentially a single band of about 770 nts, corresponding to a 5′ end at nt 97. This end has been shown by primer extension with mRNA from CaSki cells to represent the cap site for these transcripts (32). S1 digestion (6) resulted in three bands of 770, 455, and 345 nts of various intensities. Bands of the same sizes have previously been identified in CaSki cells. In SiHa cells and cancer BF, the 455-nt band was by far the strongest, as it is in CaSki cells (32). The 5′ end of the band maps to a splice donor for which an analogous site is also used in HPV18 in HeLa cells (12, 25). In cancer BC, the 455- and 345-nt bands are about equal in intensity. The 5′ end of the 345-nt band also maps to a splice acceptor. The third S1 band, which maps to the cap site, is very weak in both cancers and more pronounced in SiHa cells. Since the intensity of this band reflects the level of E6 mRNA, it can be concluded that expression of E6 in

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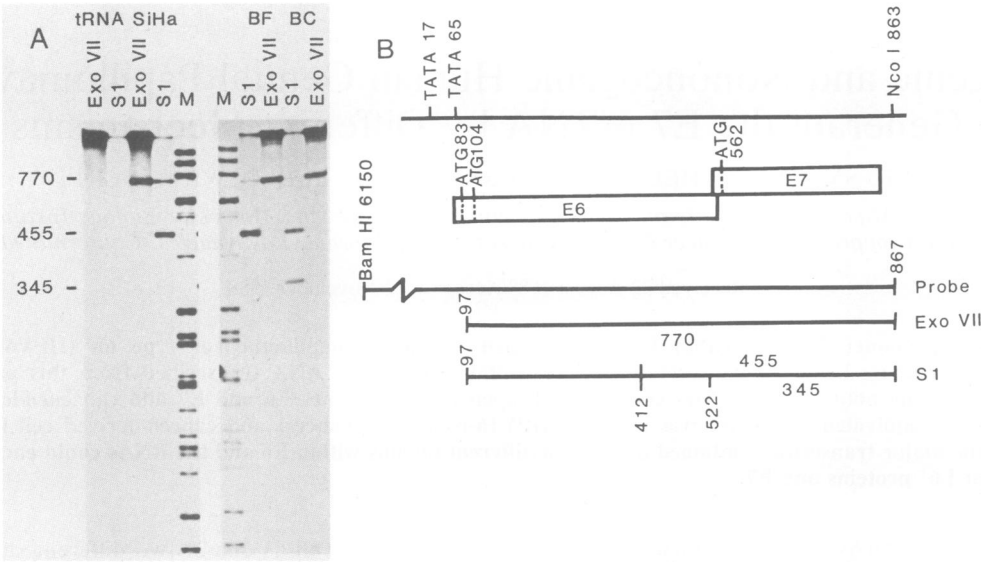

FIG. 1. Mapping of the 5′ ends of HPV16 E6-E7 transcripts in SiHa cells and two cervical cancers. Poly(A)$^+$ RNA isolated from SiHa cells, cancer BF or BC, or tRNA was hybridized to an *Nco*I-*Bam*HI probe labeled 5′ at nt 867, and the hybrids were digested with *Exo*VII or S1 nuclease. The DNA fragments were analyzed on 5% denaturing acrylamide gels, and the gels were autoradiographed with amplifying screens. (A) Autoradiograms. The sizes of bands are indicated in nucleotides on the left. The size markers (M) were 5′-end-labeled fragments of ϕX replicative form DNA *Hae*III digested and pBR322 *Msp*I digested. The sizes of the markers, in nucleotides, from the top are 1,353, 1,078, 872, 603 (622), 527, 403, 310 (309), 281, 271, 243, 234 (238), 217, 201, 194, 190, 180, 160, and 147. (B, top) Map of the E6-E7 region of HPV16 derived from the sequence (29) and indicating the positions of TATA boxes, ORFs E6 and E7, and the ATG codons. (B, bottom) Map of the probe and the *Exo*VII and S1 digestion products. The sizes of the segments, in nucleotides, are given by the horizontal numbers, and the map positions of the 5′ ends are indicated by vertical numbers.

the two cancers was indeed very low. Both 5′ ends of the exons represented by the 455- and 355-nt bands are located upstream of the E7 ATG, and this suggested that the parent transcripts represented the mRNAs for the E7 protein, which has been identified in CaSki and SiHa cells (1, 30, 32, 33). These results thus indicate that in cancer-derived cell lines and particularly in cancers, the abundance of the mRNA for E7 is much higher than that of the mRNA for E6.

Since the 5′ ends of all E6-E7 transcripts are located upstream of the second ATG of E6, initiation of translation could start at this position; however, an incomplete E6, which has been designated E6* (25), would result from translation of mRNAs with an intron within E6. To define precisely the nature of the E6 introns and the E6* protein, the 3′ end of the leader exon was mapped with a probe labeled 3′ at the *Eco*0109 site (nt 112) only a few nucleotides from the 5′ end of the transcripts. With SiHa RNA, two bands resulted after S1 digestion. A major band of 117 nts mapped the 3′ end of the leader exon to a splice donor located within E6 and a minor band of about 770 nts representing the 3′ end of the uninterrupted E6-E7 exon. In the cancers, the 117-nt band was also prominent, but the 770-nt band was barely detectable, in agreement with the data obtained with the 5′-labeled *Nco*I probe. Finding one splice donor and two splice acceptors within E6 in both cancers indicated that two E6* proteins (E6*I and E6*II) were encoded (Fig. 2, bottom diagrams; Table 1). In SiHa cells and cancer BF, E6*I would be synthesized predominantly, while in cancer BC both E6* proteins would be present at comparable levels.

Three findings suggest that a major difference between the oncogenic and nononcogenic genital human papillomaviruses could be a major difference in the level of functional E7 mRNA, and this could be particularly significant since E7 of HPV16 appears to be critical for transformation of cells in culture (16, 24). (i) The sequences of HPV6b (27) and HPV11 (11) do not contain the splice donor and acceptor present within E6 of the oncogenic viruses (25). (ii) R-loop mapping of RNA from HPV6b- and HPV11-containing condylomata indicate that the transcripts do not contain alternate introns within E6 (8). (iii) When cottontail rabbit papillomavirus early proteins were expressed under the control of the late simian virus 40 promoter in Cos-7 cells, it was found that from transcripts containing the sequences of ORFs E6 and E7 uninterrupted, only E6, but not E7, was synthesized (4, 5). To determine exactly the location of the cap sites for E6-E7 transcripts and to determine whether alternate, small introns not detectable by R-loop mapping might be present within E6, S1 and *Exo*VII digestion and primer extension experiments were performed with RNA isolated from an HPV6b-containing condyloma by using probes 5′ end labeled at the *Acc*I site located within E7 (Fig. 3). The results showed that the bands obtained after S1 or *Exo*VII digestion or primer extension were identical, indicating that no introns were located within E6 and that the ends mapped represented the cap sites. The 5′ end of the longest segment (530 nts) mapped to position nt 90, located 26 nts downstream of a TATA box and 12 nts upstream of the ATG codon of E6. This most likely represented the mRNA coding for E6. By far the most intense band was 350 nts in size. Its 5′ end was located at nt 270, well downstream of the ATG of E6 and 27 nts downstream from a second TATA box. In addition, two minor, slightly larger bands were also present. The results thus indicated that in this HPV6b-containing condyloma, a transcript, probably the mRNA for E7, was much more abundant than that for E6, as found in HPV16-containing cancers and cancer-derived cell lines.

In addition to the HPV6b condyloma, we also analyzed

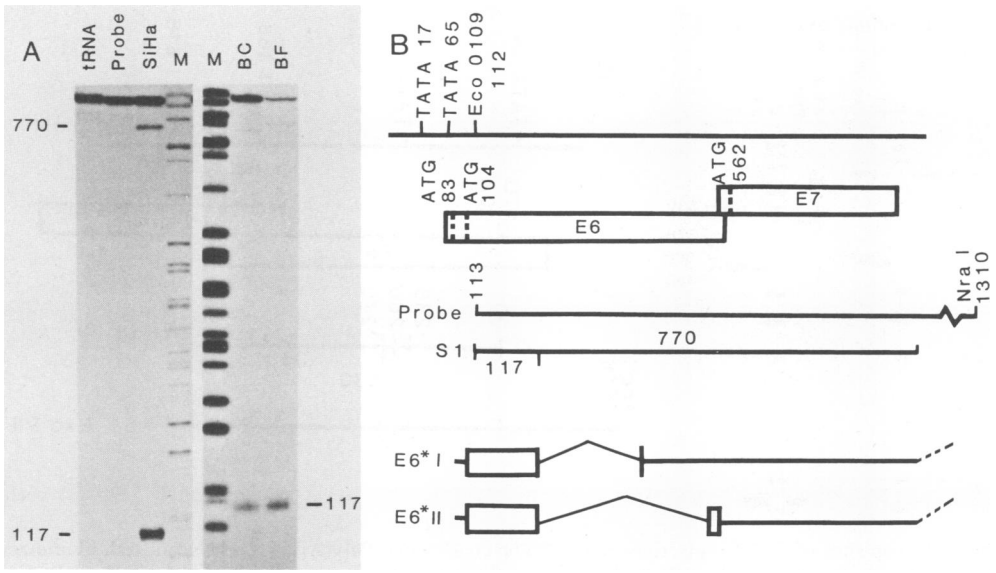

FIG. 2. Identification of HPV16 transcripts encoding two different E6* proteins. Poly(A)$^+$ RNA isolated from SiHa cells, cancer BF or BC, or tRNA was hybridized to an *Eco*0109-*Nra*I probe, 3′ labeled at nt 131, digested with S1, and analyzed as described in the legend to Fig. 1. (A) Autoradiograms. The sizes of the bands and the sizes of the markers (M) are as indicated in the legend to Fig. 1. Three additional marker bands are shown with sizes of 122 (double band), 118 (weak band), and 110 nts. (B, top) A map and ORFs of the E6-E7 region are shown as in Fig. 1. (B, bottom) Maps of the probe and the S1 digestion products are shown, as indicated in the legend to Fig. 1, along with a map of the transcripts with introns within E6. The sequences encoding the two E6* proteins E6*I and E6*II are indicated by the boxes.

one containing HPV11 (Fig. 4). The probe used to map the 5′ ends of the E6-E7 transcripts was 5′ end labeled at the *Hin*fI site at nt 841. Two bands, identical in relative intensity and representing segments of identical sizes of about 780 and 580 nts, were present in S1 and *Exo*VII digests. This showed that the parent transcripts had 5′ ends near the TATA box located at nt 62 and at nt 264, 34 nts downstream of a second TATA box. It also indicated that there were no introns within E6 or E7. The suggestion that the TATA box at nt 62 was part of the promoter for the longer transcript was further ascertained by mapping the 5′ end more accurately with a probe labeled 5′ at nt 301, a *Bbv*I site. S1 digestion resulted in a 211-nt-long fragment, mapping the end of the minor E6-E7 transcript to nt 90 (data not shown), and this clearly indicated that this TATA box was part of the promoter for the E6-encoding transcript. Thus, in the HPV11 condyloma, as well as in the HPV6b condyloma, a major transcript could encode E7 and a minor one could encode E6. These data indicated that in both condylomata and in HPV16-associated cancers the major transcript from the E6-E7 region encoded E7.

In addition to the 5′ ends of the E6 and E7 mRNAs, S1 digestion of the RNA hybridization with the 5′-labeled *Hin*fI probe revealed a series of bands representing sizes between 130 and 170 nts (Fig. 4). *Exo*VII digestion of the same hybrids resulted in a similar but not identical series of bands. The average size difference was about 5 nts. Most likely, the differences reflect different capabilities of the two enzymes to efficiently cleave the last probe nucleotides extending past the RNA ends. No similar transcript ends could be detected in the two HPV16-containing cancers, although the probe used was labeled at an equivalent site downstream of the 3′ end of E7 and close but upstream to the major splice donor located nearby.

The downstream E4-E2 exons were also mapped. As reported by others (8, 21), the 3′ ends of these exons map to an AGTAAA sequence which presumably functions as a polyadenylation signal. The 5′ end of the major exon mapped to a major splice acceptor within E4, and a minor exon had its 5′ end a short distance upstream of E2. However, neither in this condyloma nor in CaSki cells before (32) could an exon end be detected that is equivalent to that of the bovine

TABLE 1. Splice signals and amino acid sequences of HPV16 E6*I and E6*II[a]

HPV16 consensus sequence	Nucleotide and amino acid sequences of the:	
	Donor ($^{A}_{C}$AG/GT$^{A}_{G}$AGT)	Acceptor ([Py])$_6$XCAG/G$^{G}_{T}$)
E6*I	GAG/GTATAT (nts 224–232) Glu	TGTTAATTAG/GTGTATTAA (nts 399–417) ValTyr
E6*II	GAG/GTATAT (nts 224–232) Glu	CTTGTTGCAG/ATCATCAAGAACACGTAG (nts 516–543) IleIleLysAsnThr

[a] The exact locations of the splice signals were determined from the sequence (28). The mapped position of the common splice donor was nt 230, and the sequence-derived one was nt 226; for the acceptors, the mapped positions were nts 412 and 522 and the sequence derived ones were nts 409 and 526, respectively. The minor differences resulted from S1 digestion, which may leave 1 or 2 nts or cleave 1 or 2 extra nts into the hybrid. For small bands, there is an effect of the nucleotide composition on the mobility demonstrated by the resolution of the 122-nt marker into two bands (Fig. 1, *Hin*fI panel), and for larger bands, there is a limit to the accuracy of the size determination. For none of the splice signals were there alternative sites close to the mapped positions.

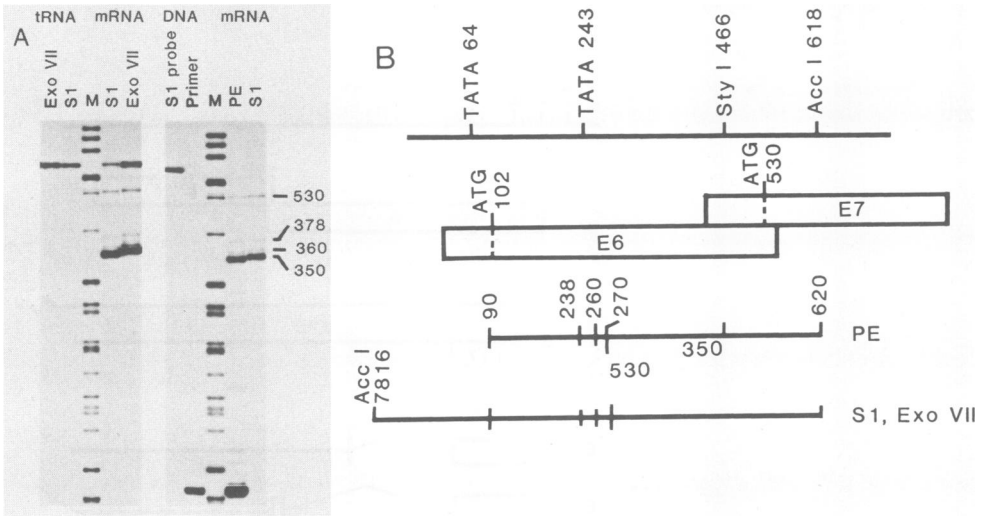

FIG. 3. Mapping of the cap sites of E6-E7 transcripts of an HPV6b condyloma. Poly(A)$^+$ RNA was isolated, hybridized to an *Acc*I probe 5′ labeled at nt 620, and digested with S1 or *Exo*VII as described in the legend to Fig. 1 or hybridized to an *Acc*I-*Sty*I primer 5′ labeled at nt 620, and the primer was extended with reverse transcriptase (PE). (A) Autoradiograms. The sizes of the bands are indicated, and the sizes of the markers (M) are as indicated in the legend to Fig. 1. (B, top) Map of the E6-E7 region of HPV6b derived from the sequence (27) as described for HPV16 in the legend to Fig. 1. (B, bottom) Results of primer extension (PE) and S1 and *Exo*VII digestion. The sizes of the segments and their 5′ ends are as indicated in the legend to Fig. 1.

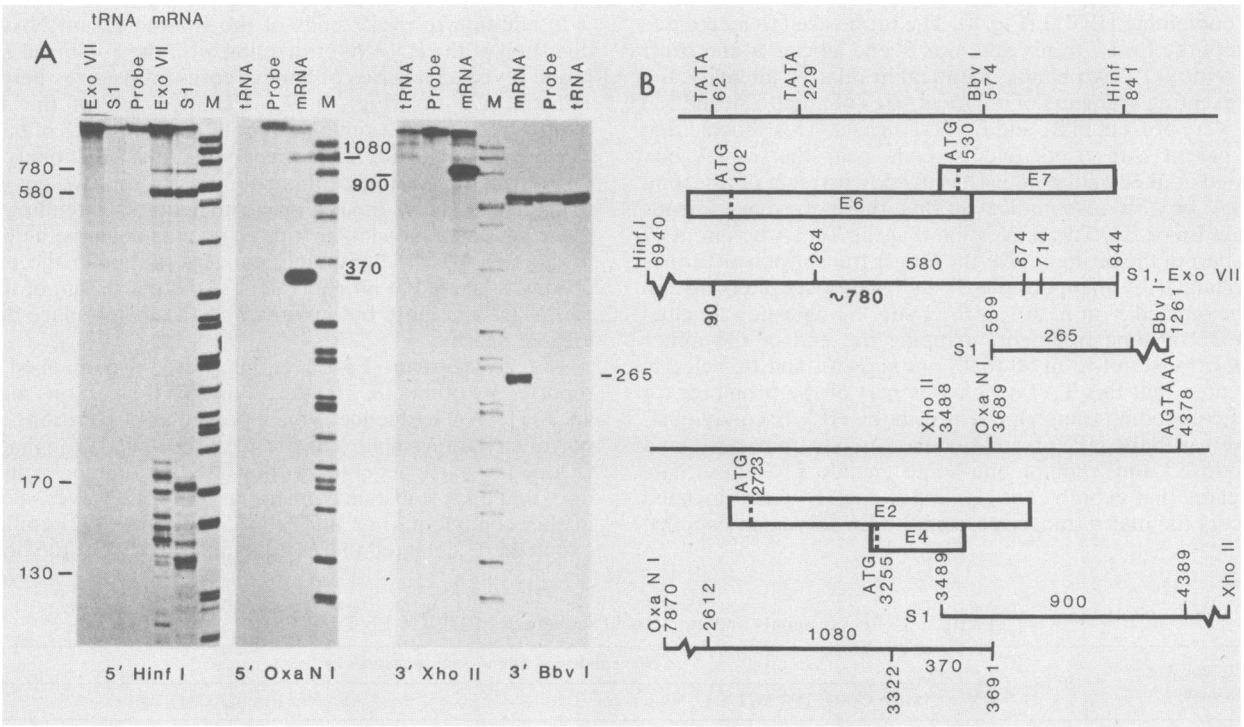

FIG. 4. Early transcripts in an HPV11 condyloma. Poly(A)$^+$ RNA was isolated from an HPV11-containing condyloma. The 5′ ends of the E6-E7 colinear exons were mapped with a *Hin*fI probe 5′ end labeled at nt 844 and 3′ end labeled with a *Bbv*I probe at nt 589. The 5′ ends of the E2-E4 colinear exon were mapped with an *Oxa*NI probe 5′ end labeled at nt 3691 and 3′ end labeled with an *Xho*II probe at nt 3489. The 5′ ends of the E6-E7 exons were identified by *Exo*VII and S1 nuclease digestion. (A) Autoradiograms. The probes used are indicated below the panels. The sizes of the markers (M) for the *Hin*fI panel are as indicated in the legend to Fig. 2, and those for the other two panels and the sizes of the S1 and *Exo*VII bands are as indicated in the legend to Fig. 1. (B, top) Map of the E6-E7 region derived from the sequence (11) and results of S1 and *Exo*VII mapping of the 5′ and 3′ ends. The end of the exon ending near the 5′ end of E6 was more accurately mapped with a *Bbv*I probe 5′ end labeled at nt 301 (the autoradiogram of the panel is not shown). For the series of 5′ ends represented by bands with sizes of 130 to 170 nts, only the range of the map positions from nt 714 to nt 674 is indicated. (B, bottom) Map of the E2-E4 region and S1 mapping of the 5′ and 3′ ends.

papillomavirus type 1 cDNA (34, 38) coding for the repressor function of E2 (15, 18–20).

We mapped E6-E7 colinear HPV16 transcripts in two cancers and the cancer-derived SiHa cell line. The major transcripts had one of two different introns within E6 which permitted expression of two different E6* proteins and the E7 protein. In the condylomata analyzed, the major HPV6b and HPV11 E6-E7 colinear transcripts can also serve as mRNA for E7, but they were generated by initiation of transcription from a promoter located downstream of the E6 ATG.

The major transcript in cancer BF and the Sitta cell line had an intron within E6 and was identical to the major HPV16 transcript of the CaSki cell line (32) and equivalent to that identified by cDNA sequencing of HPV18 RNA in HeLa cells (25). This transcript can code for an E6* protein and probably represents the mRNA for E7 which has been identified in CaSki and SiHa cells (1, 30, 32, 33). A minor transcript encoded the entire E6-E7 region and was of particularly low abundance in the two cancers but was relatively prominent in SiHa cells. A third transcript that was characterized had an intron in E6 that used the same splice donor in E6 as in the major transcript but used a different splice acceptor. This transcript was barely detectable in SiHa cells and cancer BF but was rather prominent in cancer BC. This indicated that in cancer BC, two different E6* proteins, E6*I and E6*II, could be synthesized at comparable levels. The two E6* proteins would be identical in their first 41 amino acids. The splicings would shift the reading frame and for E6*I would add two amino acids to the carboxy terminus, while five amino acids would be added to E6*II (Table 1). The function of E6* is not known, and therefore it is not possible to know whether the difference between the carboxy-terminal ends of the two proteins is functionally significant. The alternate splicing in the E6*II-encoding mRNA could, however, have an effect on its efficiency as an mRNA for E7. This is because this splicing places the termination codon for E6*II closer (36 nts) to the initiation codon for E7. In a synthetic bicistronic message, the observation has been made that the efficiency of translation of a second ORF increases with the distance between the termination codon of the first ORF and the initiation codon of the second (14). The E6*I-encoding mRNA has a much larger spacing of 153 nts between the ORFs and therefore may provide for more efficient translation of an E7 protein.

The minor mRNA, which contains the entire E6-E7-coding region, may allow translation of an intact E6 protein but not of the E7 protein. The spacing between the E6- and E7-coding sequences in this transcript is only a few nucleotides and may not permit efficient reinitiation of translation. In fact, with cottontail rabbit papillomavirus, a transcript encoding the full E6 and E7 ORFs serves as an mRNA only for E6 (4, 5).

It has previously been pointed out that the splicing signals for the major E6 intron that are found in the sequences of the oncogenic viruses HPV16, HPV18, HPV31, and HPV33 are absent in homologous positions in the sequences of the nononcogenic viruses HPV6b and HPV11 (28). In addition, previous characterization of viral RNA by R-loop mapping in HPV6- and HPV11-containing condylomata detected no introns within E6 (8). We used three methods, S1 and *Exo*VII digestion and primer extension, to identify the 5′ ends of E6-E7 colinear mRNAs in an HPV6b-containing genital wart. Analyses by all three methods resulted in the same bands with the same relative intensities. The same results were obtained with an HPV11-containing wart analyzed by S1 and *Exo*VII mapping. On the basis of these results, we conclude that the ends mapped are true ends and, in agreement with the R-loop mapping (8), no E6 internal introns existed. Our mapping data did reveal the existence in HPV6b and HPV11 of a new promoter within the E6 sequences which controls the expression of an E7 mRNA. The transcription pattern is similar to that of cottontail rabbit papillomavirus, in which separate promoters are used to generate the E6 and E7 mRNAs (10, 22, 37). This separate E7 promoter was not detected in the HPV16-containing cancers and cell lines by our mapping here or in a previous mapping study (32).

Interestingly, the TATA boxes for the promoters for the E7 mRNAs in the HPV6b and HPV11 condylomata are not located at equivalent map positions. The TATA box used in the HPV6b condyloma starts at nt 243 and has the sequence TATAAA; in the HPV11 condyloma, the TATA box starts at nt 229 with the sequence TATATG. The identical TATATG sequence is present at an equivalent site in HPV16, but it is not part of an active promoter in cancer cells. In the HPV11 condyloma, the sequence TATAAG at nt 243 does not appear to be part of an active promoter.

Mapping of the HPV11 condyloma also revealed abundant transcripts whose 5′ ends mapped to a heterogeneous cluster of sites around nts 674 to 714 and for which there is no TATA box roughly 30 nts upstream. These transcripts most likely represented the E1-E4 mRNA previously characterized by R-loop mapping (8) and cDNA sequencing (21). The absence of a TATA box may indicate that this promoter is equivalent to the late promoter of cottontail rabbit papillomavirus (37) and bovine papillomavirus type 1 (2), neither of which contains the TATA sequence. Furthermore, at least for bovine papillomavirus type 1, it was shown that a transcript encoding E4 was transcribed from the late promoter (2). Similar transcripts were not detected in the HPV16-containing cancers, since the 5′ ends would have been detected by the *Nco*I probe (Fig. 1), and it may mean that such a promoter is active only in differentiating keratinocytes and not in cancer cells. This is in agreement with findings of subgenomic probe hybridizations which suggest the presence of E4-specific RNA in preinvasive lesions but not in cancers (9, 31, 35).

In the HPV11 condyloma, the E2-E4 exons were mapped. As reported by others (8, 21), the 3′ end mapped to an AGTAAA sequence, which is a presumed polyadenylation signal. The most common 5′ end was located within E4 at a splice acceptor used by all papillomaviruses for which transcripts have been mapped in detail. The 5′ end of a second exon was mapped to the 5′ end of E2, and this may represent an exon of an mRNA that codes for the E2 protein.

The expression of transcripts for E6*, E6, and E7 in cervical cancers and cancer-derived cell lines suggests a possible role for one or more of these proteins in oncogenesis. In addition, recent experiments (24) have shown that the E7 of HPV16 can cooperate with the activated *ras* oncogene to transform primary rat kidney epithelial cells. Furthermore, the early region of all oncogenic papillomaviruses (HPV16, HPV18, HPV31, and HPV33) but not that of the nononcogenic types (HPV6 and HPV11) can cooperate with *ras* (36). In the rare HPV6b- and HPV11-associated cancers which contain extrachromosomal DNA only, the untranslated regulatory region appears to differ from those of HPV6b and HPV11 isolated from condylomata. This suggests that the properties of E7 and E6 of HPV6b and HPV11 are such that they could support a development toward

malignancy, provided that their expression is maintained at an appropriate level. This notion is further supported by transformation experiments with NIH 3T3 cells (13). In these experiments, it was shown that HPV6b with alterations in the upper regulatory region does transform NIH 3T3 cells but wild-type HPV6b DNA does not.

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