JOURNAL OF VIROLOGY, May 1992, p. 3172-3178
0022-538X/92/053172-07$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 66, No. 5

Human Papillomavirus Type 33 in a Tonsillar Carcinoma Generates Its Putative E7 mRNA via Two E6* Transcript Species Which Are Terminated at Different Early Region Poly(A) Sites

PETER J. F. SNIJDERS,* ADRIAAN J. C. VAN DEN BRULE, HENRI F. J. SCHRIJNEMAKERS, PETRA M. C. RAAPHORST, CHRIS J. L. M. MEIJER, AND JAN M. M. WALBOOMERS

Department of Pathology, Section of Molecular Pathology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

Received 30 October 1991/Accepted 22 January 1992

Human papillomavirus type 33 (HPV-33)-specific early region transcripts in a tonsillar carcinoma were analyzed by using the RNA polymerase chain reaction method. A total of five cDNA species including species with potential to encode E6*I, E6*II, and E6*III, could be identified. As determined by 3′ cDNA end mapping, one E6*I cDNA species was found to utilize a novel early region poly(A) site and was polyadenylated at or near the putative initiation codon of the E1 open reading frame (ORF). Compared with the HPV-16 and HPV-18 E6* mRNAs, the HPV-33 E6*I and E6*II species utilize different splice acceptor sites, the latter being localized within the E7 ORF. Furthermore, HPV-33 E6* mRNAs were found to contain a short overlapping ORF resulting in alternative coding potentials if translation were to start at an internal AUG codon within the E6 region. These results indicate that like HPV-16 and HPV-18, HPV-33 generates E6* mRNAs which may serve as efficient mRNAs for E7. However, HPV-33 has the ability to generate its putative E7 mRNAs by the utilization of two early region poly(A) sites, which offers the possibility of expressing E7 in different ways.

At least 60 different human papillomavirus (HPV) types have been identified, a subset of which is associated with genital-mucosal lesions (7). Of these types, HPV-6 and HPV-11 are mainly found in benign condylomas and low-grade dysplasias and are considered low-risk HPV types, whereas the high-risk types HPV-16 and HPV-18 are the major types increasingly associated with high-grade dysplasias and carcinomas of the uterine cervix (43, 46). Additional high-risk HPVs such as HPV-31, HPV-33, and HPV-35 have been found in only a minority of cervical carcinomas. In contrast, we have detected HPV-33 in a substantial proportion (5 of 10 cases) of tonsillar carcinomas (39a). Moreover, the presence of HPV-33-specific transcripts has been demonstrated in neoplastic cells by RNA in situ hybridization (39a). These findings indicate that HPV-33 can be of greater importance in carcinogenesis than previously has been suggested on the basis of its association with cervical cancer.

Transformation and immortalization functions of HPV-16 and HPV-18 (9, 21, 26, 27, 41) have been mapped to the E6 and E7 open reading frames (ORFs) (2, 16, 22), regions which are consistently transcribed in cervical carcinomas and carcinoma-derived cell lines (1, 32, 33, 39). The importance of these genes has further been strengthened by the abilities of their products to bind the p53 and retinoblastoma tumor suppressor gene products, respectively (10, 23, 45). Comparative studies have revealed differences in biological activities between the E6 and E7 genes of the low-risk type HPV-6 and of the high- risk types HPV-16 and HPV-18 (3, 13, 24), which may account for certain in vitro immortalization and transformation properties being limited to the high-risk HPVs (26, 40). The in vivo oncogenic potential may also depend on the mechanism by which the E6- and E7-encoding transcripts are generated and the expression of these genes is regulated (3, 38). Putative E7 mRNAs of HPV-16 and HPV-18 are bi- or polycistronic and contain an intron within the E6 ORF (E6* mRNAs) (32, 39). Such spliced E6/E7 transcripts, two of which have been identified for HPV-16 (E6*I and E6*II), were not present in HPV-6- and HPV-11-containing condylomas (4, 38). Moreover, HPV-16 and HPV-18 can generate E6/E7-encoding viral-cellular fusion transcripts (17, 29, 32, 37), a phenomenon which is uncommon among the low-risk HPVs. Knowledge about the mechanism by which E6/E7 mRNAs of additional high-risk HPVs such as HPV-33 are generated is necessary to determine which viral features are most critical in determining the in vivo oncogenic potential. In this study, we applied the RNA polymerase chain reaction (PCR) to analyze HPV-33 early region transcripts present in a tonsillar carcinoma containing both extrachromosomal and integrated HPV-33 DNA (not shown).

Primer selection and analysis of amplified cDNAs. Three sense primers (E6, E7a, and E1a) and three antisense primers (E7b, E1b, and E2/E4) were chosen in such a way as to flank putative splice sites, as deduced from transcriptional mapping data of HPV-11 (31) and HPV-16 (8, 39). Primer locations in relation to the HPV-33 early region genome structure are depicted in Fig. 1A, and primer sequences are shown in Table 1. Although we did not investigate the 5′ mRNA ends, studies of HPV-16 have established a major cap site (P97) for E6/E7 transcripts at or near nucleotide (nt) position 97 (29, 37, 39); together with the high sequence homology between HPV-16 (36) and HPV-33 (5) around this region (including TATA box sequences), this finding suggests the existence of an equivalent major cap site for HPV-33. Indeed, all transcripts detectable with the E6 sense primer also could be detected with a sense primer (nt 95, 5′-GGTACTGCACGACTATGTTT-3′) specific for this putative cap region (not shown). Although the presence of cap sites further upstream of this region cannot be excluded, this finding justifies the inclusion of the putative E6 initiation

* Corresponding author.

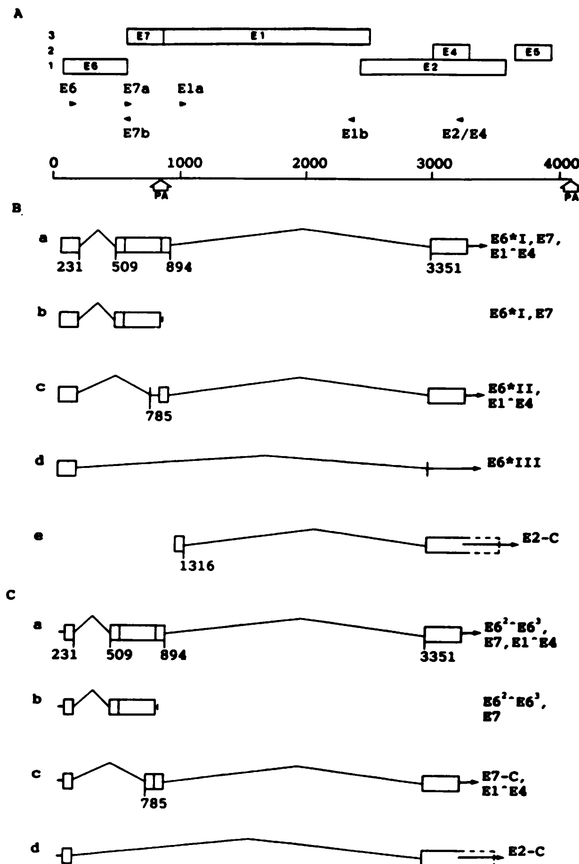

FIG. 1. Structures and coding potentials of HPV-33-specific cDNA species identified in the tonsillar carcinoma. (A) Primer positions in relation to the HPV-33 early region genome structure (5) are indicated by arrowheads, which point in the 5'-to-3' direction. Putative poly(A) sites are indicated by open arrows. Reading frames are numbered according to Cole and Streeck (5). (B) Structures and probable coding potentials of the five cDNA species (a to e) identified, depicted in relation to the genome structure shown in panel A. Numbers indicate the splice donor and acceptor sites. Open boxes represent potential coding regions. Coding potentials of the cDNA species are shown on the right. For species a to d, the potential to encode E6* proteins depends on the utilization of the E6 AUG codon at nt position 109. (C) Alternative coding potentials of cDNA species a to d, depending on the utilization of the second out-of-frame AUG codon within the E6 region mapping to nt 146. $E6^{2}$ˆ$E6^{3}$ indicates a putative fusion protein resulting from the in-frame connection of E6 sequences of reading frame 2 with E6 sequences of reading frame 3 (see panel A). E7-C indicates a putative protein consisting of a C terminus encoded by the E7 ORF, joined via splicing to N-terminal sequences encoded by E6 reading frame 2.

codon which maps to nt 109 when the coding potentials of these transcripts are determined.

Following the identification of HPV-16 and HPV-18 E6/E7 mRNAs, containing an intron within the E6 ORF (32, 39), sequence comparison data have revealed the presence of putative splice signals at equivalent positions within the E6 ORF of the remaining sequenced high-risk mucosotropic HPVs (34). For HPV-33, the predicted splice donor and acceptor sites of a putative E6* mRNA have been mapped to nt 231 and 414, respectively. The use of primers E6 and E7a, which flank these sites and span a region of 513 bp at the DNA level, revealed a single amplified cDNA fragment of 236 bp, whereas DNA PCR yielded the expected 513-bp fragment (Fig. 2A). DNA sequence analysis of the cDNA product allowed the identification of a splice junction connecting nt 231 to nt 509 (Fig. 3A), indicating that for this splice, the donor site is identical but the acceptor site is different from the predicted site. Also, the acceptor site is not equivalent to the HPV-16 E6*II acceptor site (38); thus, it involves a novel splice junction. RNA PCR of an additional tonsillar carcinoma (not shown) and a cervical carcinoma (Fig. 2A) yielded the same 236-bp fragment, suggesting that these sites are natural splice sites common to HPV-33. Since we failed to detect a full-length 513-bp RNA PCR product, it is assumed that unspliced E6/E7 mRNA, if present, must exist at very low levels within the cytoplasm.

Four cDNA species could be identified by using the different HPV-33-specific primer combinations. Primer pair E6-E2/E4 allowed the detection of three cDNA species, as reflected by the production of PCR fragments of 714, 438, and 328 bp (Fig. 2B). The 714-bp fragment was clearly the major amplified product (not shown), indicating that the mRNA species represented by this fragment is abundant compared with the 438- and 328-bp species. Moreover, 498- and 430-bp amplified cDNA fragments were obtained with primer combinations E7a-E2/E4 and E1a-E2/E4, respectively (Fig. 2B). DNA sequence determination revealed that the 714-bp E6-E2/E4 fragment and most likely also the 498-bp E7a-E2/E4 fragment represent cDNA species a. The remaining 438-bp (E6-E2/E4), 328-bp (E6-E2/E4), and 430-bp (E1a-E2/E4) fragments were found to represent cDNA species c, d, and e, respectively. The splice patterns and resulting cDNA structures are shown in Fig. 1B and C. Species a and c were found to contain at least three exons, the first two of which are joined via nt 231 to nt 509 (species a; Fig. 3A) and via nt 231 to nt 785 (species c; Fig. 3B). In both species, the second and third exons are joined by the same splice via nt 894 to nt 3351 (Fig. 3D). Species d and e consist of at least two exons joined via nt 231 to nt 3351 (species d; Fig. 3C) or via nt 1316 to nt 3351 (species e; Fig. 3E). Except for the first splice junctions of species a and c, equivalent splice junctions have been mapped for HPV-16 (8). cDNA species e could be detected only with the E1a sense primer and failed to be amplified with the E6 or E7a sense primer. This finding together with transcriptional mapping data for HPV-11 and HPV-16 (8, 31) suggests the presence of a cap site for this transcript which maps to the 5' portion of the E1 ORF.

Identification of a novel poly(A) site by 3' cDNA end mapping. 3' cDNA ends were analyzed by the method of Frohman et al. (11), combining one of the HPV-33-specific sense primers with an oligo(dT)-adaptor primer set. The E6-adaptor primer combination revealed three hybridizing cDNA fragments, approximately 1,300, 900, and 500 bp in size (Fig. 2C). Restriction enzyme analysis using *Rsa*I and *Sau*3A (not shown) suggests that the 1,300- and 900-bp fragments likely represent cDNA species a and d, respectively, uninterrupted downstream of the E4 ORF and utilizing the early region poly(A) motif which maps to nt 4176. Similar full-length cDNA species have been mapped for HPV-16 (8, 29). The 500-bp fragment, however, is aberrant in size and could not be related to any of the transcripts identified thus far. DNA sequence analysis of this product revealed that it involved a new transcript species (species b; Fig. 1B and C) consisting of two exons, joined via the same splice that was mapped for the first exons of species a and terminated at a cleavage/poly(A) addition site 14 or 17 nt

TABLE 1. Primers used for RNA PCR

Primer	Sequence[a]	Position
E6	5'-ggaattcGCATGATTTTGTGCCAAGCAT-3'	144–164
E7a	5'-ggaattcTATACTGCTATGAGCAATTA-3'	636–656
E7b	5'-ggaattcTAATTGCTCATAGCAGTATAG-3'	656–636
E1a	5'-ggaattcTGCACTAAAACGAAAGTTTGC-3'	1127–1147
E1b	5'-ggaattcATTTGCACCACGTCCTTGAGA-3'	2715–2695
E2/E4	5'-cgggatccCTTTTAAATGCACTATAGG-3'	3588–3569
$(dT)_{17}$[b]	5'-GACTCGAGGATCCTGCAGCCTTTTTTTTTTTTTTTTT-3'	
Adaptor[b]	5'-GACTCGAGGATCCTGCAGC-3'	

[a] Lowercase letters indicate sequences containing *Eco*RI or *Bam*HI recognition sites.
[b] Primers described by Frohman et al. (11).

downstream of the poly(A) signal at nt position 862 (Fig. 4). This poly(A) signal completely overlaps the putative termination codon of the E7 ORF (5).

Application of the method with a sense primer (nt 3569, 5'-ACCTATAGTGCATTTAAAAG-3') which is complementary to the E2/E4 primer and consequently anneals with cDNA species a, c, d, and e revealed a hybridizing cDNA fragment of approximately 650 bp (not shown). The size of this product once again suggests the utilization of the poly(A) signal at nt 4176, and no indications for the presence of viral-cellular fusion transcripts were obtained.

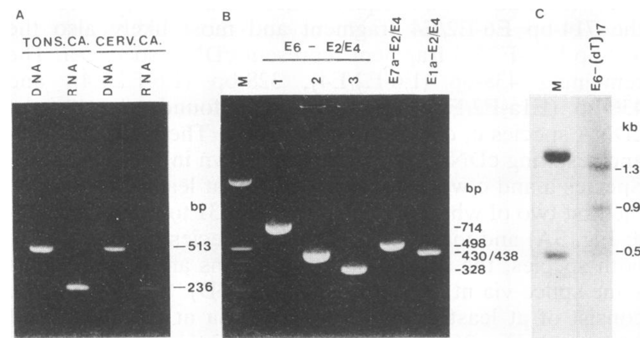

FIG. 2. HPV-33 RNA PCR analysis of a primary poorly differentiated tonsillar squamous cell carcinoma. Cytoplasmic RNA was isolated according to Gough (14). One strand of cDNA was synthesized in a final reaction volume of 50 μl containing 200 ng of cytoplasmic RNA, 25 pmol of antisense primer, 50 mM Tris-HCl (pH 8.3), 60 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each deoxynucleoside triphosphate, 40 U of RNasin (Promega), and 7 U of avian myeloblastosis virus reverse transcriptase (Promega). The mixture was incubated at 42°C for 45 min. In addition, reactions were performed without reverse transcriptase added to allow distinction between final RNA PCR products and products derived from eventually copurified DNA. Five microliters of the cDNA mixture was used for PCR. PCR on both cDNA and genomic DNA was performed under identical conditions as previously described for type-specific PCR analysis (42). To determine 3' cDNA ends, the method described by Frohman et al. (11) was applied. PCR products were analyzed on 1.5% agarose gels. Southern blot analysis was carried out (44) by using the cloned HPV-33 DNA as probe. (A) Comparison of E6-E7b-directed PCR products derived from DNA and RNA of the tonsillar carcinoma (TONS.CA.) and a cervical carcinoma (CERV.CA.) containing HPV-33. The agarose gel pattern is shown after ethidium bromide staining. (B) Detection of RNA PCR products of the tonsillar carcinoma obtained with the E2/E4 antisense primer. Three PCR fragments originally obtained in a single PCR assay with the E6-E2/E4 primer pair were electrophoretically separated, and isolated fractions were reamplified to facilitate subsequent sequencing (lanes 1 to 3). Lane M, pBR322 DNA digested with *Hin*fI. (C) Southern blot analysis of the 3'-end RNA PCR product generated by the E6-oligo(dT)-adaptor primer combination. Lane M, pBR322 DNA digested with *Hin*fI.

Coding potentials of cDNAs. The probable coding potentials of the identified cDNA species are shown in Fig. 1B. Species a and b may encode an E6*I protein (39). If translation started from the E6 initiation codon at nt 109 localized in reading frame 1 (5), the first splice would shift translation to reading frame 2 of the E6 region, resulting in the termination at the UAA codon which maps to nt 563. This codon lies 7 nt downstream of the termination codon of the full-length E6 ORF and 8 nt upstream of the putative initiation codon of the E7 ORF. The second splice junction of species a and c is equivalent to splice junctions previously mapped for HPV-1 (25), HPV-11 (31), HPV-16 (8), and HPV-18 (30) and allows the ability to encode an E1^E4 fusion protein. However, the presence of an additional E1^E4-encoding message which, similar to that of other HPVs (8, 25, 31), contains a cap site within the E1 ORF cannot be excluded. Additional 5' cDNA end mapping is necessary to resolve the presence of this putative mRNA. Species c differs from species a by the utilization of a different splice acceptor site to join the first and second exon and has the potential to encode a novel E6*II protein. Starting from the E6 AUG codon at nt 109, the splice would shift translation to out-of-frame sequences of the E7 ORF, resulting in the addition of three amino acids to the E6 C terminus before reaching a termination codon at nt 794. cDNA species d is equivalent to an HPV-16 message (8) and has coding potential for an E6*III protein. cDNA species e contains AUG codons at nt positions 1276 and 1305, both of which are in frame for initiation of an E2-C protein (carboxy-terminal domain of E2) consisting of N-terminal sequences derived from the E1 region. Therefore, if this species were to proceed without any interruption beyond the E2 ORF, it could encode an E2-C protein.

Within the E6 region of the HPV-33 genome, a second short overlapping ORF is present in reading frame 2 which proceeds past the E6 splice donor site (5). Consequently, removal of the first intron of species a to d generates new ORFs in addition to E6*, resulting in alternative coding potentials if translation were to start at the second AUG codon (nt 146) within the E6 region (Fig. 1C). For species a and b, this offers the ability to encode a protein containing N-terminal sequences encoded by E6 region reading frame 2 and a C terminus consisting of 10 amino acids encoded by E6 region reading frame 3 ($E6^{2}$^$E6^{3}$). Similarly, species c has additional coding potential for a protein containing a C terminus of 27 amino acids encoded by the E7 ORF (E7-C). Homology comparison with HPV-16 sequences revealed

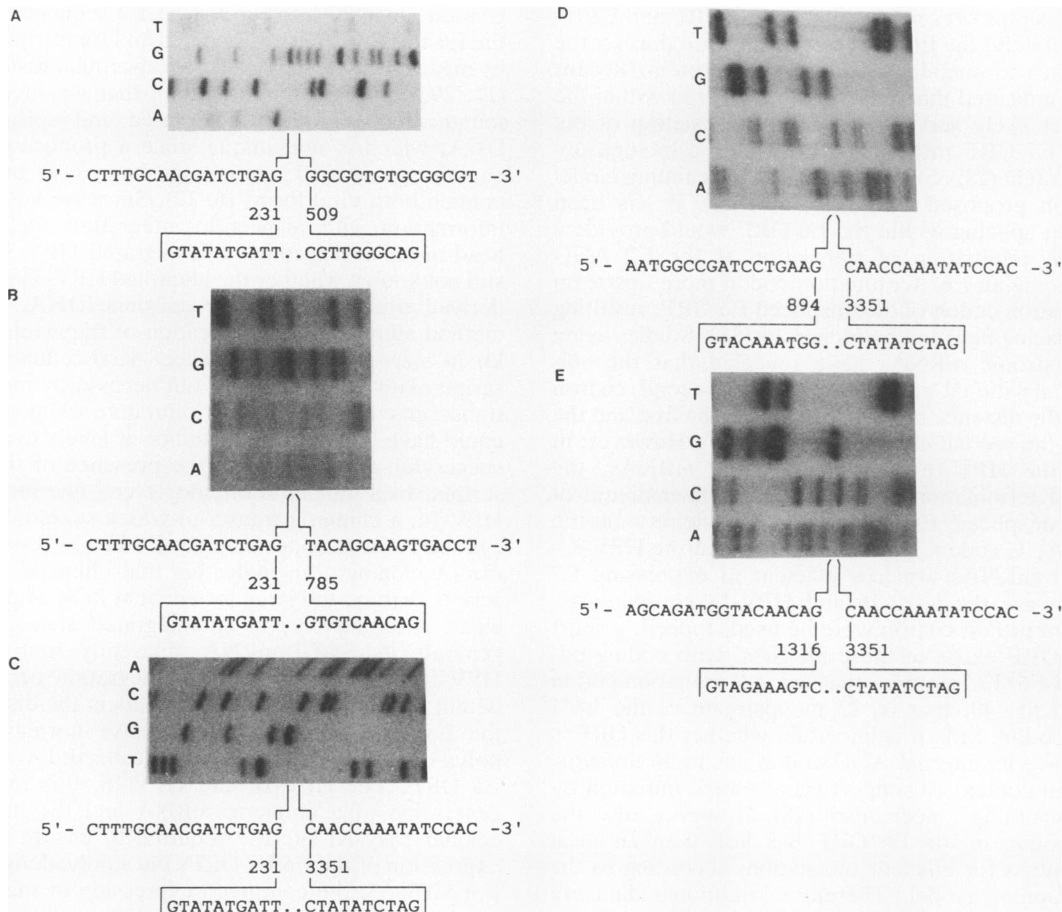

FIG. 3. cDNA sequences which span the splice junctions. Sequencing of the amplified cDNA fragments was carried out either directly after asymmetric PCR (15) or after cloning of the product into the *Eco*RI or *Eco*RI-*Bam*HI site of M13mp18/mp19 vector DNA. Sequencing was performed by using the dideoxy-chain termination reaction with a T7 polymerase sequencing kit (Pharmacia) according to instructions of the manufacturer. Nucleotide positions of splice donor and acceptor sites are indicated. Intron sequences around the splice junctions are shown beneath and between sequences of both exons. (A) E6*I splice junction (species a and b); (B) E6*II splice junction (species c); (C) E6*III splice junction (species d); (D) E1/E4 splice junction (species a and c); (E) E2-C splice junction (species e).

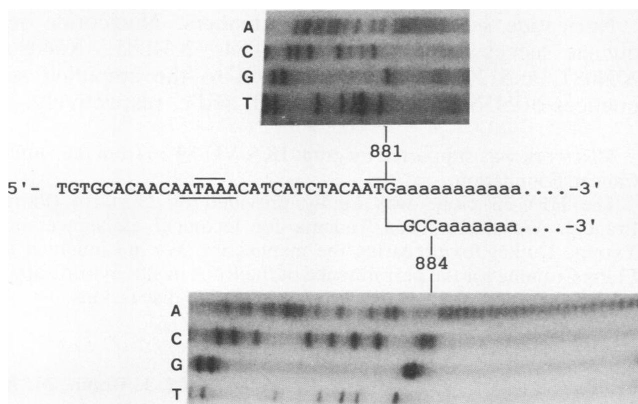

FIG. 4. 3′ end analysis of cDNA species b. Two cleavage/poly(A) addition sites were found, as shown for two M13 clones. The putative E7 termination codon (TAA), the putative E1 initiation codon (ATG), and the poly(A) signal (AATAAA) are indicated. Sequences of the poly(A) tail are shown by lowercase characters.

that this putative protein does not contain sequences that are thought to be involved in transformation and in the interaction with the retinoblastoma gene product (24). However, this putative protein contains a CXXH motif at the N terminus and a CXXC motif at the C terminus and consequently has the potential to form a hybrid zinc loop drawing the polypeptide ends together. Species d could have additional coding potential for an alternative E2-C protein if not interrupted within the 3′ part of the E2 ORF. This putative protein would presumably initiate from the second out-of-frame AUG codon of the E6 region, at nt position 146, and therefore its N terminus consists of E6 instead of E1 sequences.

If no further splicing or interruption occurred downstream of the E4 ORF, utilization of the putative early region poly(A) site at nt 4176 would provide species a, c, d, and/or e, with additional coding potential for an E5 protein.

Although HPV-33 is associated with only a minority of cervical carcinomas, it belongs to the group of high-risk HPV types, and in vitro transforming activities similar to those of HPV-16 and HPV-18 have been demonstrated (40). Three HPV-33-specific E6* transcripts could be demonstrated in the tonsillar carcinoma studied. Because the E6*II

and E6*III acceptor sites map within the E7 ORF and E2/E4 ORFs, respectively, the E6*I species a and b are thus far the only candidates to encode an HPV-33 E7 protein. Recent studies have indicated that splicing out an intron within the E6 ORF most likely serves to facilitate translation of the downstream E7 ORF rather than producing a biologically active E6* protein (28, 35). According to the scanning model for translation proposed by Kozak (18, 20), it has been suggested that splicing within the E6 ORF would provide a more efficient reinitiation of translation at the E7 AUG codon by placing an E6* termination codon more upstream of the termination codon of the unspliced E6 ORF, resulting in a larger spacing between both cistrons (38). Studies using different bicistronic mRNAs have revealed that the efficiency of translational reinitiation at the second cistron improves as the distance between the end of the first and the beginning of the second cistron increases (19). However, in contrast to the HPV-16 and HPV-18 E6* mRNAs, the HPV-33 E6*I termination codon is placed downstream of that of the unspliced E6 ORF, in close vicinity of the putative E7 AUG codon. This could mean that the HPV-33-specific E6*I mRNAs are less efficient in expressing E7 proteins than are the HPV-16 and HPV-18 counterparts, unless a different first cistron were be used. Indeed, a short overlapping ORF exists in these mRNAs, with coding potential for an $E6^{2}$^$E6^{3}$ protein, that has a termination codon positioned at nt 540, that is, 23 nt upstream of the E6*I termination codon. Still, it is uncertain whether this ORF is expressed since its internal AUG codon lies in an unfavorable sequence context to support translational initiation by the "leaky scanning" mechanism (18). However, also the first AUG codon in the E6 ORF has less than an ideal sequence context for efficient translation, according to the proposed scanning model. Therefore, additional data are required to determine how expression of the HPV-33 E7 ORF is regulated and whether the alternative ORFs would be expressed. If they are expressed, for species a, b, and c this most likely would serve to affect the translation of downstream ORFs rather than producing functional proteins, since there are no indications that other HPVs can encode similar proteins. In this context, only cDNA species d may encode a novel functional E2-C protein. cDNA species c contains a splice that places the termination codon for the E6*II ORF immediately beyond the splice acceptor site and in this way creates an optimal target for the production of an E1^E4 fusion protein, since the putative E1 AUG codon lies in a favorable sequence context for translational reinitiation (18).

Striking is the identification of an E6*I/E7 transcript that utilizes a novel poly(A) site at the 3′ end of the E7 ORF. Sequence analysis indicated that HPV-6b, HPV-11, HPV-16, HPV-18, and HPV-31 do not contain putative poly(A) signals in the equivalent region, suggesting that the presence of a second early region poly(A) site is not a general feature. Consequently, HPV-33 can express E7 via at least two early region mRNAs.

In general, in HPV-16- and HPV-18-containing carcinomas and cell lines derived from carcinomas, transcripts encoding a full-length E6 protein have been demonstrated (17, 33, 37, 39). However, quantities of such transcripts have been found to be relatively low (37, 39). Low amounts of HPV-33-specific unspliced E6 mRNAs could have been missed because the method certainly favors the amplification of the shorter E6* cDNAs. Moreover, in cervical carcinomas and carcinoma-derived cell lines containing HPV-16 or HPV-18, the viral genome is often integrated into the host genome, usually interrupting E1/E2 sequences and leaving the E6 and E7 ORFs undisrupted and transcriptionally active by means of the generation of viral-cellular fusion transcripts (17, 29, 32, 37). The carcinoma that we investigated was found to contain both integrated and episomal HPV-33 DNA, which is not unique since a proportion of HPV-16 containing cervical carcinomas have also been found to contain both viral forms (6, 12). Since we have no detailed information with respect to integration site and possible head-to-tail arrangement of integrated HPV-33 DNA, it is still not known whether the identified HPV-33 transcripts are derived from integrated or episomal DNA. Although the method allowed the amplification of fragments more than 2 kb in size, we failed to detect viral-cellular fusion transcripts. However, this does not necessarily mean that these transcripts are not present. Putative chimeric transcripts could have been too large and/or at levels too low to allow successful amplification in the presence of the other transcripts. In a human keratinocyte cell line immortalized by HPV-16, a chimeric transcript which contained a stretch of cellular RNA of approximately 2.8 kb has been identified by cDNA cloning (29), indicating that chimeric HPV-33 transcripts that are too large for efficient PCR amplification may exist. Alternatively, in an integrated state, HPV-33 may generate its E6/E7 mRNA differently from HPV-16 and HPV-18. If single-copy HPV integration occurs, typically within the E1/E2 region, this results in the disconnection of the E6/E7 region from the putative normal early region poly(A) site which is generally localized downstream of the E5 ORF. For HPV-16 and HPV-18, this means that the generation of a chimeric mRNA and the utilization of a cellular poly(A) site are required to ensure the continued expression of the E6/E7 ORFs via a polyadenylated mRNA. For HPV-33, the continued expression of the E6/E7 ORFs would not necessarily depend on a cellular poly(A) site, since the E6/E7 region provides its own poly(A) site.

In this study, we demonstrated that the HPV-33 E7 ORF, in addition to HPV-16 and HPV-18 E7 ORFs, is encoded by spliced E6* messages. The E6*I splice junction was also found in an additional tonsillar carcinoma and a cervical carcinoma, suggesting that this mechanism for generating E7 mRNA is common to HPV-33 regardless of the site of infection. The property of generating E7 mRNAs by the utilization of two early region poly(A) sites thus far seems specific for HPV-33 and may offer the possibility of expressing E7 in different ways.

Nucleotide sequence accession numbers. Nucleotide sequence accession numbers X64084, X64085, X64086, X64087, and X64088 were assigned to the amplified sequences of cDNA species a, b, c, d, and e, respectively.

This work was supported by grant IKA-VU-89-16 from the Dutch Cancer Foundation.

The HPV-33 clone was kindly provided by G. Orth (Paris, France). We thank Thea Tadema for technical assistance and Yvonne Duiker for preparing the manuscript. We are indebted to Frans Cromme for the performance of the RNA in situ hybridization experiments and to G. B. Snow for stimulating discussions.

REFERENCES

1. **Baker, C. C., W. C. Phelps, U. Lindgren, M. J. Braun, M. A. Gouda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61:**962–971.
2. **Barbosa, M. S., and R. Schlegel.** 1989. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. Oncogene **4:**1529–1532.

3. **Barbosa, M. S., W. C. Vass, D. R. Lowy, and J. T. Schiller.** 1991. In vitro biological activities of the E6 and E7 genes vary among human papillomaviruses of different oncogenic potential. J. Virol. **65:**292–298.
4. **Chow, L. T., M. Nasseri, S. M. Wolinsky, and T. R. Broker.** 1987. Human papillomavirus type 6 and 11 mRNAs from genital condylomata acuminata. J. Virol. **61:**2581–2588.
5. **Cole, S. T., and R. E. Streeck.** 1986. Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. J. Virol. **58:**991–995.
6. **Cullen, A. P., R. Reid, M. Campion, and A. T. Lörincz.** 1991. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. J. Virol. **65:**606–612.
7. **de Villiers, E.-M.** 1989. Heterogeneity of the human papillomavirus group. J. Virol. **63:**4898–4903.
8. **Doorbar, J., A. Parton, K. Hartley, L. Banks, T. Crook, M. Stanley, and L. Crawford.** 1990. Detection of novel splicing patterns in a HPV16-containing keratinocyte cell line. Virology **178:**254–262.
9. **Dürst, M., T. Dzarlieva-Petrusevska, P. Boukamp, N. E. Fusenig, and L. Gissmann.** 1987. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene **1:**251–256.
10. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934–936.
11. **Frohman, M. A., M. K. Dush, and G. R. Martin.** 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85:**8998–9002.
12. **Fukushima, M., Y. Yamakawa, S. Shimano, M. Hashimoto, Y. Sawada, and K. Fujinaga.** 1990. The physical state of human papillomavirus 16 DNA in cervical carcinoma and cervical intraepithelial neoplasia. Cancer **66:**2155–2161.
13. **Gage, J. R., C. Meijers, and F. O. Wettstein.** 1990. The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV-6b) and of the oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. J. Virol. **64:**723–730.
14. **Gough, N. M.** 1988. Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. Anal. Biochem. **173:**93–95.
15. **Gyllensten, U. B., and H. A. Ehrlich.** 1988. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc. Natl. Acad. Sci. USA **85:**7652–7656.
16. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV16 E6 and E7 proteins cooperate to immortalize primary human foreskin keratinocytes. EMBO J. **8:**3905–3910.
17. **Inagaki, Y., Y. Tsunokawa, N. Takebe, H. Nawa, S. Nakanishi, M. Terada, and T. Sugimura.** 1988. Nucleotide sequences of cDNAs for human papillomavirus type 18 transcripts in HeLa cells. J. Virol. **62:**1640–1646.
18. **Kozak, M.** 1986. Bifunctional messenger RNAs in eukaryotes. Cell **47:**481–483.
19. **Kozak, M.** 1987. Effects of intercistronic length on the efficiency of reinitiation by eukaryotic ribosomes. Mol. Cell. Biol. **7:**3438–3445.
20. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.
21. **Matlashewski, G. J., J. Schneider, L. Banks, N. Jones, A. Murray, and L. Crawford.** 1987. Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. EMBO J. **6:**1741–1746.
22. **Münger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63:**4417–4421.
23. **Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. **8:**4099–4105.
24. **Münger, K., C. L. Yee, W. C. Phelps, J. A. Pietenbol, H. L. Moses, and P. M. Howley.** 1991. Biochemical and biological differences between E7 oncoproteins of the high- and low-risk human papillomavirus types are determined by amino-terminal sequences. J. Virol. **65:**3943–3948.
25. **Palermo-Dilts, D. A., T. R. Broker, and L. T. Chow.** 1990. Human papillomavirus type 1 produces redundant as well as polycistronic mRNAs in plantar warts. J. Virol. **64:**3144–3149.
26. **Pecoraro, G., D. Morgan, and V. Defendi.** 1989. Differential effects of human papillomavirus type 6, 16, and 18 DNAs on immortalization and transformation of human cervical epithelial cells. Proc. Natl. Acad. Sci. USA **86:**563–567.
27. **Pirisi, L., S. Yasumoto, M. Feller, J. Doniger, and J. A. DiPaolo.** 1987. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J. Virol. **61:**1061–1066.
28. **Roggenbuck, B., P. Mose Larsen, S. J. Fey, D. Bartsch, L. Gissmann, and E. Schwarz.** 1991. Human papillomavirus type 18 E6*, E6, and E7 protein synthesis in cell-free translation systems and comparison of E6 and E7 in vitro translation products to proteins immunoprecipitated from human epithelial cells. J. Virol. **65:**5068–5072.
29. **Rohlfs, M., S. Winkenbach, S. Meijer, T. Rupp, and M. Dürst.** 1991. Viral transcription in human keratinocyte cell lines immortalized by human papillomavirus type-16. Virology **183:**331–342.
30. **Rotenberg, M. O., C. M. Chiang, M. L. Ho, T. R. Broker, and L. T. Chow.** 1989. Characterization of cDNAs of spliced HPV-11 E2 mRNA and other HPV mRNAs recovered via retrovirus mediated gene transfer. Virology **172:**468–477.
31. **Rotenberg, M. O., L. T. Chow, and T. R. Broker.** 1989. Characterization of rare human papillomavirus type 11 mRNAs coding for regulatory and structural proteins by the polymerase chain reaction. Virology **172:**489–497.
32. **Schneider-Gädicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5:**2285–2292.
33. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314:**111–114.
34. **Schwarz, E., A. Schneider-Gädicke, and H. zur Hausen.** 1987. Human papillomavirus type-18 transcription in cervical carcinoma cell lines and in human cell hybrids. Cancer Cells **5:**47–53.
35. **Sedman, S. A., M. S. Barbosa, W. C. Vass, N. L. Hubbert, J. A. Haas, D. R. Lowy, and J. T. Schiller.** 1991. The full-length E6 protein of human papillomavirus type 16 has transforming and trans-activating activities and cooperates with E7 to immortalize keratinocytes in culture. J. Virol. **65:**4860–4866.
36. **Seedorf, K., G. Krämmer, M. Dürst, S. Suhai, and W. G. Röwekamp.** 1985. Human papillomavirus type 16 DNA sequence. Virology **145:**181–185.
37. **Smits, H. L., M. T. E. Cornelissen, M. F. Jebbink, J. G. van den Tweel, A. P. H. B. Struyk, M. Briët, and J. ter Schegget.** 1991. Human papillomavirus type 16 transcripts expressed from viral-cellular junctions and full-length viral copies in CaSki cells and in a cervical carcinoma. Virology **182:**870–873.
38. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. **63:**1441–1447.
39. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83:**4680–4684.

39a. **Snijders, P. J. F., et al.** Unpublished data.

40. **Storey, A., D. Pim, A. Murray, K. Osborn, L. Banks, and L. Crawford.** 1988. Comparison of the in vitro transforming activities of human papillomavirus types. EMBO J. **7:**1815–1820.
41. **Tsunokawa, Y., N. Takebe, T. Kasamatu, M. Terada, and T. Sugimura.** 1986. Transforming activity of human papillomavirus type 16 DNA sequences in a cervical cancer. Proc. Natl. Acad.

Sci. USA **83:**2200–2203.

42. **van den Brule, A. J. C., P. J. F. Snijders, R. L. J. Gordijn, O. P. Bleker, C. J. L. M. Meijer, and J. M. M. Walboomers.** 1990. General primer-mediated polymerase chain reaction permits the detection of sequenced and still unsequenced human papillomavirus genotypes in cervical scrapes and carcinomas. Int. J. Cancer **45:**644–649.
43. **van den Brule, A. J. C., J. M. M. Walboomers, M. du Maine, P. Kenemans, and C. J. L. M. Meijer.** 1991. Difference in prevalence of human papillomavirus genotypes in cytomorphologically normal cervical smears is associated with a history of cervical intraepithelial neoplasia. Int. J. Cancer **48:**404–408.
44. **Walboomers, J. M. M., W. J. G. Melchers, H. Mullink, C. J. L. M. Meijer, A. Struyk, W. Quint, J. van der Noordaa, and J. ter Schegget.** 1988. Sensitivity of in situ detection with biotinylated probes of human papilloma virus type 16 DNA in frozen tissue sections of squamous cell carcinomas of the cervix. Am. J. Pathol. **131:**587–594.
45. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248:**76–79.
46. **Wettstein, F. O., and J. G. Stevens.** 1982. Variable-sized free episomes of shope papilloma virus DNA are present in all non-virus-producing neoplasms and integrated episomes are detected in some. Proc. Natl. Acad. Sci. USA **79:**790–794.
47. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomavirus in anogenital cancer, p. 245–263. *In* N. P. Salzman and P. M. Howley (ed.), The papillomaviruses. Plenum Publishing Corp., New York.