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Human Papillomavirus Type 1 Produces Redundant as well as Polycistronic mRNAs in Plantar Warts

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Human papillomavirus type 1 (HPV-1) causes plantar warts. On the basis of previously mapped mRNAs and sequence homologies of HPV-1 to other papillomaviruses, we designed oligonucleotide primers and employed the polymerase chain reaction to recover HPV-1 cDNAs from plantar warts. Seven spliced RNA species were characterized, including three not previously detected, and the coding potentials of each were deduced. The most abundant viral mRNA encodes an E1iˆE4 protein. One new species is predicted to encode the full-length E2 protein, and another can, theoretically, encode the E2-C or E1-M proteins, three products that regulate mRNA transcription and DNA replication. One RNA species originating from a novel HPV promoter in the upstream regulatory region has the potential to encode the minor capsid protein L2. A newly recognized E5a open reading frame (ORF) is contained in all mRNAs that are polyadenylated at the E-region poly(A) site and also in a putative L2 mRNA. Three distinct species, two of which are derived from the upstream regulatory region promoter, have the potential to encode the L1 protein; the third species also contains the entire coding region of the E1iˆE4 protein 5′ to the L1 ORF. Both the E1iˆE4 mRNA and the potentially bicistronic L1 mRNA are derived from a promoter located in the E7 ORF. We uncovered no evidence of alternatively spliced mRNAs that could account for the multiple, abundant E4 proteins in plantar warts, suggesting that posttranslational modification is mainly responsible for the observed protein heterogeneity.

Human papillomavirus (HPV) infection can cause hyperproliferation of epithelial tissues at body sites that are characteristic for each known HPV type (14). None has been successfully propagated in cell cultures or in tissues of laboratory animals. The basic genetic organizations of all human and other animal papillomaviruses are highly conserved despite substantial sequence divergence (Fig. 1) (for a review, see reference 6). Production of virions in the different types of warts varies over a considerable range. For instance, HPV type 6 (HPV-6) and HPV-11 are tropic for anogenital and oropharyngeal mucosal epithelia and cause benign condylomata in which relatively few virions are produced, in spite of the replication of viral DNA to reasonably high copy numbers in the more differentiated cells of infected epithelium (37). HPV-1, on the other hand, typically causes plantar warts in highly keratinized cutaneous epithelium from which abundant virions can be recovered. Such tissue tropism and different degrees of permissiveness presumably stem in part from quantitative differences in viral RNA synthesis and in promoter usage regulated by viral proteins and by cellular transcription factors specific to different tissues. In addition, variations in precise positions of the coding sequences in the mRNAs may lead to different efficiencies of translation.

HPV-6 and HPV-11 mRNAs recovered from condylomata and from infected human foreskin xenografts in nude mice have been studied extensively by various methods (8, 11, 31, 34, 38). Families of overlapping mRNAs are generated by using alternative promoters and mRNA splice sites and one of two polyadenylation sites. Many of the HPV mRNAs appear to be polycistronic (30, 31). The other well-characterized wart virus is bovine papillomavirus type 1 (BPV-1), which causes massive fibropapillomas in cattle and yields large quantities of virions. BPV-1 uses additional promoters and mRNA splice sites not found in HPV-11 to access many of its open reading frames (ORFs) (2, 10, 36a, 39).

mRNAs recovered from plantar warts and from primary human foreskin keratinocytes infected with HPV-1 have been examined by the electron microscopic R-loop method (12). There are three putative promoters, as reflected by the reproducible 5′ ends of the mRNAs (12). The major promoter is located within the E7 ORF upstream of the E1 ORF, a minor initiation site precedes the E6 ORF, and the least active promoter is in the upstream regulatory region (URR). Fewer mRNA species have been identified for HPV-1 than for HPV-6, HPV-11, or BPV-1. In particular, mRNAs with the potential to encode the E2 and E2-C (carboxyl-terminal domain) proteins that regulate the viral enhancer in the URR (8, 9, 20, 24, 35) and E1-M, which modulates extrachromosomal DNA replication (4, 29), were not detected. In contrast, there appears to be more than one mRNA for the major capsid protein and for the E4 protein. In this study, we used the polymerase chain reaction (PCR) (31, 32) and DNA sequencing to define the structures of seven HPV-1 mRNAs recovered from plantar warts (Fig. 1). The oligonucleotide primers (Table 1) were designed to amplify selectively the mRNA species that originate from each of the apparent promoters and that utilize putative splice sites previously mapped or predicted on the basis of sequence homologies identified among many HPV types (11, 12). The authenticity of the recovered cDNAs is supported by the consensus sequences at the deduced splice sites and by their coding potentials, which were comparable to those of messages produced by other papillomaviruses (Table 2). The structures of the transcripts, in the context of their coding potentials, are discussed below.

Symbols for representing mRNA splicing are as follows. The ˆ indicates an mRNA splice which connects donor and

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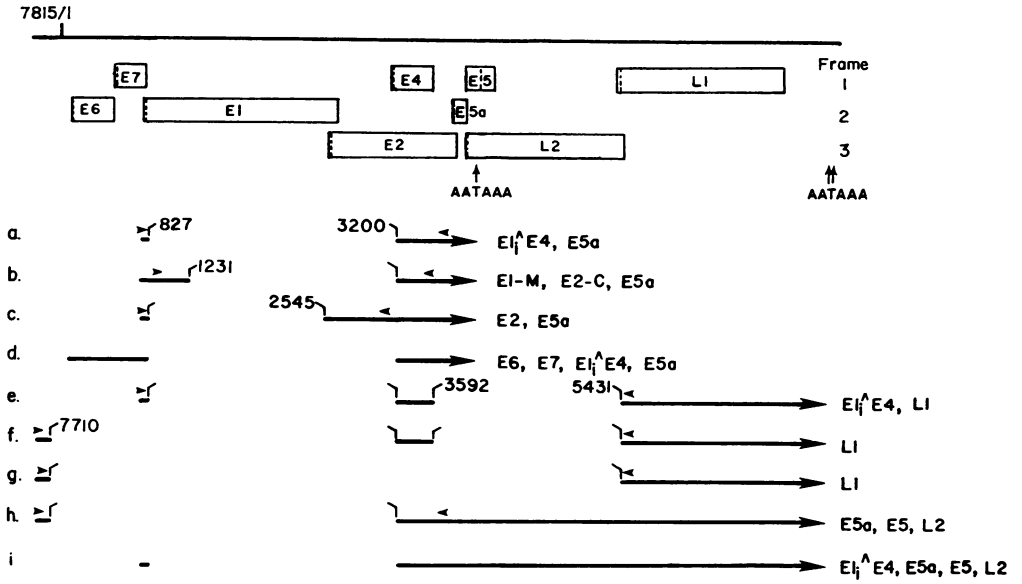

FIG. 1. Genetic organization and mRNAs of HPV-1 from plantar warts. The circular genome of 7,815 base pairs is represented in a linear fashion. ORFs (▭) deduced from the genomic DNA sequence are present in one of the DNA strands; the position of the first AUG codon in each ORF is shown (⋮). On the basis of results with HPV-1 and of homology to BPV-1 and other HPVs, the URR located between the end of L1 and the beginning of E6 contains transcriptional enhancers, promoters, RNA exons, and the origin of DNA replication; the E region encodes functions for episomal DNA replication and regulation (E1 ORF), enhancer regulation (E2 ORF), and cellular transformation (E6, E7, and E5 ORFs); and the L region encodes viral capsid proteins (L2 and L1 ORFs). Locations of the putative polyadenylation signal AATAAA in the genomic DNA are marked (↑). mRNAs are depicted (➡) in the 5′-to-3′ direction. Gaps in the arrows represent introns absent from the mature RNA. Species a, d, e, f, h, and i have previously been mapped by the R-loop method. The possible 5′ ends of species b and c are discussed in the text, but for simplicity, only one of the hypothetical ends is depicted. The coding potentials of the mRNAs, as deduced from the cDNAs, are given to their right. Positions and orientations of the oligonucleotide primers (►) for PCR amplification of cDNAs are shown. Nucleotide positions of the splice donor and acceptor sites are listed only once but are marked (⌜ , ⌝) when also determined in other mRNA species.

acceptor nucleotides or ORFS, and the / and the \ indicate mRNA splice donor and acceptor sites, respectively.

E4 cDNA. Proteins containing E4 epitopes are the most abundant HPV-1 proteins in plantar warts and are located predominantly in the cytoplasm of the spinous, granular, and superficial keratinocytes (5, 15, 16). Over 95% of the viral RNA in plantar warts contains a single splice predicted to fuse the beginning of the E1 ORF to the E4 ORF (Fig. 1, species a), as has been reported for HPV-11 (11, 28). It was transcribed from a promoter in the E7 ORF and terminated at the E-region poly(A) addition site near nucleotide (nt) 4000. Indeed, this cDNA was recovered after only 30 cycles of amplification by using primer pair 2-6 or 2-5. DNA sequence determination of several clones demonstrated a splice connecting nt 827 to nt 3200 (Table 2). Translation initiation would start from the AUG codon at nts 812 to 814 in the E1 ORF. After five and one-third codons, the mRNA splice shifts translation into the E4 ORF, which terminates at nt 3559. The only AUG codon in the genomic E4 ORF at nts 3181 to 3183 was spliced out of the mature mRNA. We now introduce a nomenclature whereby the short E1 peptide resulting from the use of this E1 splice donor, universal for all papillomaviruses characterized, is designated E1i, for E1 initiation, to distinguish it from other proteins encoded by different regions of the E1 ORF. The size of the E1i^E4 protein is estimated to be 14.3 kilodaltons (kDa).

TABLE 1. Oligonucleotide primers for PCR

Primer	Nucleotide position	Orientation[a]
1	110–130	S
2	734–753	S
3	925–956	S
4	3036–3055	AS
5	3556–3575	AS
6	3627–3646	AS
7	5484–5503	AS
8	7552–7571	S
9	7630–7656	S
10	7665–7684	S

[a] Sense (S) orientation is defined as that of the mRNA and is therefore complementary to the first-strand cDNA, whereas the antisense (AS) orientation is complementary to the mRNA or to the second-strand cDNA. Pairs of opposing primers, one in each orientation, were used to amplify the cDNAs.

L2 cDNA. Antibodies raised against bacterially expressed HPV-1 E4 ORF detect protein doublets of 10 and 11, 16 and 17, 21 and 23, and 32 and 34 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5, 15, 16). Some forms were hypothesized to be dimers, whereas others were attributed to posttranslational modification or to translation from different mRNAs. We previously detected a rare RNA in which a URR exon is spliced to the E4/E2 overlapping region (12). To determine whether this RNA could encode a variant E4 protein, we used primer pairs 9-6 and 8-6 to recover the corresponding cDNA. The downstream primer was positioned beyond the second splice donor (nt 3592) of L1 mRNA species f (described below) so as to exclude the amplification of the latter mRNA. The cloned cDNA contained a splice connecting URR nt 7710 to nt 3200 (Table 2). To locate the 5′ end of this URR exon, we performed primer extension experiments and identified a cluster of 5′ ends, or

TABLE 2. HPV-1 mRNA splice sites and their predicted coding potentials[a]

mRNA species	Splice donor (nucleotide)	Junction sequences	Splice acceptor (nucleotide)	Coding potential(s)
a	827	AAAG/GUAC..CUAG\CUCC	3200	E1i^E4, E5a
b	1231	ACAG/GUAG..CUAG\CUCC	3200	E1-M, E2-C, E5a
c	827	AAAG/GUAC..AAAG\GUUU	2545	E2, E5a
e	827	AAAG/GUAC..CUAG\CUCC	3200	E1i^E4, L1
	3592	UCAG/GUAC..UCAG\AUGG	5431	
f	7710	AACG/GUAG..CUAG\CUCC	3200	L1
	3592	UCAG/GUAC..UCAG\AUGG	5431	
g	7710	AACG/GUAG..UCAG\AUGG	5431	L1[b]
h	7710	AACG/GUAG..CUAG\CUCC	3200	E5a, E5, L2

[a] First-strand cDNA was synthesized from 5 to 10 μg of poly(A)-selected plantar wart RNA (12) with oligo(dT)$_{12-18}$ primer and a cDNA synthesis kit (Bethesda Research Laboratories). Portions of first-strand cDNA (2 to 10 ng) were then used as templates to initiate 30 cycles of PCR pairs of HPV-1-specific oligonucleotide primers (200 μM each) (Table 1) and reagents in a PCR kit (Perkin-Elmer/Cetus Corp.), as described previously (31). The reaction mix was analyzed by electrophoresis in a 1% agarose or 4% polyacrylamide gel for the presence of discrete reaction products. Except for the E1i^E4 cDNA, which was prominent after only one round of 30 cycles of amplification, a second round of 30 cycles seeded with a portion of the first reaction cocktail was necessary to produce clearly visible bands for cloning of all other cDNAs. Gel-purified PCR products were treated by T4 DNA polymerase to ensure blunt ends and were then phosphorylated with T4 DNA kinase. Portions of the DNA were blunt-end ligated to pUC9 at the *Sma*I site. The ligation mixtures were transformed into competent *Escherichia coli* HB101 or DH5α. Appropriate clones were sequenced by the chain termination method (33) with double-stranded templates (7) and M13 or PCR primers to determine the mRNA splice sites. The locations of the ORFs are depicted in Fig. 1. / and \, mRNA splice donor and acceptor junctions, respectively. The positions of the last nucleotide preceding the intron and the first nucleotide following the intron are given. As discussed in the text, our data do not permit determination of whether the same or separate mRNAs with the splice in species b encode E1-M, E2-C, or both.

[b] Up to three forms (56.9, 61.6, and 63.5 kDa), depending on whether URR initiation codons were to be used.

strong stops, between nts 7490 and 7524, the most prominent of which were at nts 7509, 7510, and 7511 (data not shown). There are two AUG codons in a weak initiator sequence context (22) at nts 7534 to 7536 and 7582 to 7584. Neither AUG codon is in frame with the E2 or E4 ORF in the second exon. Should translation initiate from either AUG codon, it would terminate 20 amino acids after the splice, resulting in a peptide of 79 or 63 amino acids. An analysis of this putative peptide did not indicate biased codon selection, which is normally found in expressed genes (36) such as the agnoprotein encoded by the late simian virus 40 mRNA leader (21). There is no AUG codon in the middle exon to initiate translation in the E2 or E4 ORF. Analysis of several independent clones revealed neither additional RNA species nor sequence variation relative to the published genomic DNA sequence (13) that would allow translation of either the E2 or the E4 ORF. Therefore, we uncovered no evidence for the existence of multiple abundant mRNA species capable of coding for different E4 proteins. Posttranslational events and dimerization are likely the main causes for the observed protein heterogeneity. These results also suggest that the RNA of interest is most likely a fragment of species h (12), which has also been described previously (12). Species h might be a splicing intermediate for L1 mRNA f (Fig. 1), in which the second intron has not been excised. We favor an attractive alternative explanation that species h is a message for the minor capsid protein L2 because the only ORF upstream of L2 is E5a (see below, under E5 and E5a ORFs). A transcript comparable to species h has not been reported in any other papillomavirus.

L1 cDNAs. The major virus capsid protein L1 is detected by immunocytochemistry in only the most differentiated, superficial cells of plantar warts (17). Two L1 mRNA species have been identified by R-loop analysis (12). Species e, with a 5′ end in the E7 ORF, is much more abundant than species f, which has an exon in the URR (Fig. 1). We have recovered the two corresponding cDNAs and have identified a new, third transcript which also has the potential to encode the L1 protein. We did not attempt to recover previously detected species i (Fig. 1) but presume that it has a splice identical to that of species e; it could be a processing intermediate for species e or a message for one or more of the ORFs spanned, E1i^E4, E5a, E5, L2, and L1. A transcript similar to species i has also been reported for HPV-6 and HPV-11 (11).

A cDNA of mRNA e was generated from primer pair 2-7. The mRNA contained two splices, linking nt 827 to nt 3200 and nt 3592 to nt 5431 (Table 2). The first splice is identical to that described above for species a. Together, the first two exons encode the entire E1i^E4 protein. The acceptor site of the third exon is located precisely at the AUG initiation codon for the L1 ORF. Therefore, theoretically, this mRNA could encode both the E1i^E4 and L1 proteins. The promoter location, splicing patterns, and coding potential are highly analogous to those of HPV-6 and HPV-11 (11, 31). With primer pair 9-7, we obtained a cDNA corresponding to L1 mRNA f. Sequence analysis showed that the first splice connected a donor at URR nt 7710 to an acceptor at nt 3200 (Table 2). The second splice was identical to that in species e (Table 2). As discussed above, the first two exons could not encode a protein in either the E2 or the E4 ORF. We conclude that this message could encode only the L1 capsid protein from the third exon, as has been concluded for the twice-spliced BPV-1 L1 mRNA (2). The third L1 cDNA (Fig. 1, species g), obtained with primer pair 9-7 or 10-7, contained one splice which connected the same URR exon as in species f from nt 7710 directly to nt 5431 at the L1 AUG codon (Table 2). This L1 transcript has not been detected previously but was preferentially amplified over species f by PCR, probably because of the selective advantage of its shorter length. The weak initiation codons at nts 7534 to 7536 and 7582 to 7584 in the first exon are in frame with the L1 ORF in the second exon. Should translation occasionally initiate from these URR AUG codons, the message would encode a 63.5- or 61.6-kDa variant capsid protein in addition to the major 56.9-kDa L1 protein.

Genomic DNA comparisons indicate that sequences surrounding the donor and acceptor splice sites connecting the second and third exons are conserved in all HPVs, suggesting that their L1 mRNAs are similarly spliced. We note that the confirmed or putative donor sites in all the mucosal HPVs are 12 nts downstream of the E4 termination codon, whereas in the cutaneous HPV-1, the separation is 31 nts. Whether this difference could affect the translation efficiency of the messages (23), thereby contributing to the varied

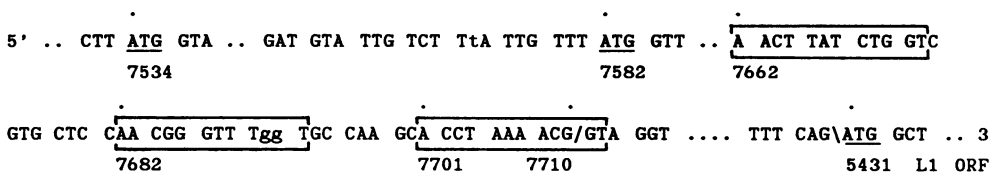

FIG. 2. Sequence features of the URR exon for L-region mRNAs f and g. Boxes mark the three adjacent potential E2 binding sites; one is the strong consensus site $ACCN_6GGT$ and two are variant $AACN_6GGT$ sites. The first nucleotide positions of the putative E2 binding sites, the URR ATG triplets (underlined) in frame with the first L1 ATG, the mRNA splice site donor (/), and the acceptor (\) at the L1 exon are indicated (.). A middle exon (nts 3200 to 3592) present in species f is not shown. The 5' ends of the transcripts are located between nts 7490 and 7524, as deduced from primer extension experiments. Single-base changes from C to T and two-base changes from CC to GG are indicated with lowercase letters.

abundances of virions produced by these two groups of viruses, remains to be tested. E1iˆE4 and L1 mRNA e are apparently derived from the same promoter immediately upstream of the E1 ORF, but they vary in abundance by at least 20-fold (12). Regulation of L1 mRNA synthesis must then be a function of differentiation-dependent transcription through the entire L region and utilization of the distal polyadenylation site. Similar conclusions have been reached for HPV-6, HPV-11, and BPV-1 (3, 11, 37).

Consistent with findings in our previous R-loop studies (12), the structures of the recovered L1 and L2 cDNAs and the results of primer extension experiments described above show that HPV-1, unique among HPVs, has a promoter in the URR upstream of nt 7490. Near nt 7470, there is an AT-rich region that might serve as a promoter. It is located within 70 nts of the second L-region polyadenylation signal AATAAA at nts 7426 to 7431, suggesting that transcription traverses practically the entire genome. This promoter, active in plantar warts but not in primary keratinocytes abortively infected with HPV-1 (12), is therefore differentiation specific.

During sequence analysis of several independent L1 and L2 cDNAs derived from the URR promoter, we detected three differences from the published sequence (13). There was a T rather than a C at nt 7574 and two Gs rather than two Cs at nts 7691 and 7692 in the sense strand (Fig. 2). The latter revisions result in a putative weak E2 protein binding site, $AACN_6GGT$ ($ACCN_6GTT$ in the reverse orientation) (25), 8 base pairs downstream of another potential weak E2 protein binding site (Fig. 2). A third, consensus E2 protein binding site, $ACCN_6GGT$, spans the splice donor of the URR exon at nt 7710 (Fig. 2) (1, 9, 18, 20, 26, 27). The three sites are spaced by 20 and 19 base pairs, two helical turns apart, and are about the size of an E2 or E2-C protein footprint (9, 20). Because of the proximity of the URR promoter to these potential E2 binding sites, we speculate that transcription from it might be regulated in part by the binding of the E2 or E2-C protein. We note that the BPV-1 splice donors at nts 864 and 7905 also overlap a variant or a consensus E2 binding sequence.

E2 cDNA. The E2 protein of HPV-1 has URR enhancer-stimulating activity similar to that of BPV-1 and other HPVs (19, 35). The E2 mRNA was not detected by R-loop studies. With primer pair 2-4 flanking the predicted splice sites, a cDNA fragment of the putative E2 mRNA was recovered (Fig. 1, species c). A splice connected a donor at nt 827 to an acceptor at nt 2545 (Table 2). This sequence arrangement is reminiscent of the HPV-11 E2 mRNA (30, 31). The initiation codon of the E2 protein is preceded by a small ORF defined by the strong E1 AUG codon at nts 812 to 814 and by in-frame termination codons at nts 2559 to 2561 and 2565 to 2567 in the second exon which precede the E2 initiation codon at nts 2592 to 2594. Internal reinitiation would be essential for the translation of the E2 protein, irrespective of whether the mRNA also contains E6 and E7 ORFs, as is the case in HPV-11 (30, 31). By analogy to HPV-11 (11, 30, 31, 34), the E2 mRNA could potentially initiate from the same promoter preceding the E1 ORF, as does the E1iˆE4 mRNA (species a), and also from a promoter preceding or in the E6 ORF. We were unsuccessful in our attempts to recover such a long E2 cDNA or the cDNA of a previously mapped putative E1iˆE4 mRNA with a longer first exon (species d) by placing the sense-strand primer 2 in the E6 ORF because of annealing to incorrect sites.

E2-C or E1-M cDNAs. We predicted the existence of one or two HPV-1 mRNAs that could encode E1-M (modulator) or E2-C (carboxyl-terminal) regulatory proteins (Fig. 1, species b) (11). With primer pair 3-5 in the PCR, we recovered the anticipated cDNA. The corresponding mRNA contained a splice connecting nt 1231 to nt 3200 (Table 2). To encode the E1-M protein, the message would necessarily originate from the major promoter in the E7 ORF or further upstream. Translation in the E1 ORF would initiate from the AUG codon at nts 812 to 814 and terminate shortly after the splice to the second exon. Translation of the E2-C protein would require initiation from AUG codons at nts 1200 to 1202 or 1218 to 1220 and continue in the E2 ORF after the splice. For lack of additional structural information and because the putative E2-C mRNA is completely contained within the E1-M transcript, we were unable to determine whether these two proteins are encoded by the same mRNA. Since the proposed initiation codon for the E2-C protein precedes the termination codon of E1-M, potentially decreasing E2-C translation efficiency, we suggest that the E2-C protein is specified by a separate mRNA initiated in the E1 ORF shortly upstream of the splice donor site at nt 1231, analogous to a known HPV-11 transcript (11, 31).

E5 and E5a ORFs. The identification of a coding region for an E5 protein and its mRNA remains problematic. We note that the conventional E5 ORF (Fig. 1), the designation of which was based on size, spans the putative E-region poly(A) site near nt 4000 and that its only initiation codon is beyond the poly(A) signal and downstream of the L2 initiation codon (13). However, we recognized a small ORF (nts 3788 to 3910) (Fig. 1, E5a) located between the E2 ORF and the putative E-region polyadenylation signal. Starting from an AUG codon in its respective ORF, E5a or E5 could encode a peptide of 36 or 42 amino acids. Aside from RNA species h or i, E5 could not be translated from any of the mRNAs that we have identified. On the other hand, all E-region mRNAs as well as the putative L2 mRNA species h and i have the potential to encode the E5a peptide. It remains to be determined whether HPV-1 indeed encodes one or both of the E5 peptides.

In summary, the HPV-1 mRNAs produced in keratinizing epithelium are highly analogous to those of mucosotropic

HPV-11 and HPV-6 in splice site usage, major promoter locations, and the synthesis of two E1iˆE4 mRNAs, one of which also has the potential to encode the major capsid protein. The only exception is the existence of a URR promoter from which two additional L1 mRNAs and a putative L2 mRNA were derived. The reason for the redundancy in mRNAs is not clear. Many of the messages apparently are polycistronic in that translation of the major ORF clearly requires internal reinitiation after termination of an upstream peptide. In comparison, to access many of its ORFs, BPV-1 in cutaneous warts uses additional promoters and mRNA splice sites that have so far not been found in HPV mRNAs (2, 10, 36a, 39). Thus it appears that species specificity is a more important determinant than are factors related to tissue site tropism in the overall transcriptional programs of these viruses. It is tempting to speculate that the monocistronic L1 and L2 mRNAs unique to HPV-1 and the potentially bicistronic L1 mRNA are translated much more efficiently than the HPV-11 L1 and L2 mRNAs and contribute to the difference in virion production between these viruses.

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