Analysis of Human Papillomavirus Type 16 Late mRNA 3' Processing Signals In Vitro and In Vivo

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In the human papillomavirus type 16 genome, three late mRNA putative 3' processing signals, designated LP1, LP2, and LP3, are located downstream of the late coding region. Our results show, both in vitro and in vivo, that in HeLa cells, the LP2 signal functions. Thus, the restriction in human papillomavirus type 16 late-gene expression observed in HeLa cells and other nondifferentiated epithelial cells is not achieved by regulation of late mRNA poly(A) site usage. Interestingly, alteration of three nucleotides in the GU-rich downstream sequence element converts the nonfunctional LP1 to an efficient 3' processing site, suggesting that LP1 may function in cell types other than HeLa, such as differentiated keratinocytes. Our transfection studies have identified a negative regulatory element located immediately upstream of the late mRNA 3' processing signals; this element was not associated with any alteration in 3' processing and may act as an mRNA instability element.

Papillomaviruses, containing double-stranded circular DNA genomes of approximately 8 kilobases, are the etiological agents of warts or papillomas and infect humans and animals with strict host and tissue specificity (reviewed in reference 2). The human papillomaviruses (HPV) are diverse, consisting of at least 60 distinct types, some of which have been associated with malignancy; HPV-5, -8, and -14 are linked with malignant skin lesions in patients with epidermodysplasia verruciformis (14), and HPV-16, -18, -31, -33, -35, -39, -45, -51, and -52 are linked with cervical cancer and other ano-genital cancers (reviewed in reference 3).

Research on papillomavirus-host cell interactions is hindered by the lack of a tissue culture system capable of supporting virus growth; virus capsid antigens and virus particles are observed only in cells that are in the process of keratinization or are already keratinized (21). Virus DNA replication and capsid protein synthesis have not been detected in basal keratinocytes. The nature of this restriction in undifferentiated cells is not known and is of considerable importance in understanding transmission of these viruses. Here we have utilized HeLa cells, a human cervical carcinoma cell line, which probably most closely resemble basal keratinocytes and which are nonpermissive for late-gene expression. We describe the use of in vitro polyadenylation assays and in vivo transfection assays to examine the HPV-16 sequences involved in late mRNA 3' end formation and to investigate the possibility that regulation of HPV-16 late-gene expression may be achieved through differential usage of the late poly(A) site.

Analysis of the published HPV-16 DNA sequence (26) indicates that the prototype HPV-16 noncoding region (NCR) contains three putative late mRNA 3' processing sites (LP1, LP2, and LP3) which, in RNA, consist of a conserved AAUAAA signal (22) and downstream sequences. For many 3' processing sites, a GU-rich downstream element is essential and a consensus YGUGUYYY sequence (where Y is a pyrimidine) has been described (16). Two of these sites (LP1 and LP2) are located within 210 base pairs (bp) of the L1 open reading frame stop codon (at 7152), whereas LP3 is

approximately 550 bp downstream of the stop codon (Fig. 1). In addition, we have a naturally occurring variant, HPV-16del (12), with a deletion of 122 bp in the NCR, having endpoints in a direct repeat, UGUUUU, at positions 7278 and 7400; this deletion removes a portion of the downstream sequence of LP1 and the whole of LP2 but leaves LP3 intact (Fig. 1).

LP2 functions efficiently in extracts from HeLa cells and is the only functional late mRNA 3' processing site in vitro. The putative late 3' processing signals from the prototype and HPV-16del genomes were examined by using 3' processing in vitro (19). Precursor RNAs, synthesized from recombinant pGEM plasmids by using SP6 or T7 RNA polymerase (18) and containing the appropriate HPV-16 sequences, were incubated with HeLa cell nuclear extracts (4, 19) in the presence of ATP or 3' dATP (17, 19). In reactions containing ATP, precursor RNA PD753, which contained HPV-16 sequences from the *Pst*I site at 7008 to the *Dde*I site at 7761 (Fig. 2B) and in which all three signals, LP1, LP2, and LP3, were present, was efficiently polyadenylated (Fig. 3A). In the presence of 3' dATP, which inhibits polyadenylation but does not prevent cleavage, with PD753 RNA, a prominent cleavage product (CP2) of approximately 355 nucleotides was detected; this corresponded to cleavage at the LP2 site (Fig. 3A). A second precursor RNA, PD753del, which lacks LP2, was not cleaved at either the LP1 or the LP3 site (Fig. 3A); the faint band at a size slightly larger than CP1 does not represent LP1 cleavage, since it is also present in the ATP reaction.

To eliminate any potential effects of the upstream LP1 and LP2 signals on LP3, we examined the use of LP3 in isolation by preparing a precursor RNA, ED308, which contained HPV-16 sequences from the *Eco*RI site at 7453 to the *Dde*I site at 7761 (Fig. 2B). ED308 RNA was not cleaved at the LP3 site (Fig. 3A), indicating that this site is inactive on its own. Lack of LP3 utilization may be due to the absence of a downstream element which closely resembles the GU-rich consensus.

Unlike the LP3 putative site, LP1 contains sequences with strong homology to both the AAUAAA and GU-rich elements required for RNA processing. To investigate the

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P T T S S T S T T A K R K K R K L
1101 CCCACCCT CATCTACCTC TACAAGTCT AAACGCAAAA AACGTAAGCT
L1 stop
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1151 GTAAGTATTG TATGTATGTT GAATTAGTGT TGTTTGTGT GTATATGTTT
1201 GTATGTGCTT GTATGTGCTT GTAAATATTA AGTTGTATGT GTGTTTGTAT
pA signal (LP1) YGTGTTY
1251 GTATGGTATA ATAAACACGT GTGTATGTGT TTTTAAATGC TTGTGTAAC
pA signal (LP2) YG
1301 ATTGTGTCAT GCAACATAAA TAAACTTATT GTTCAACAC CTACTAATTG
TGTTY
1351 TGTGTGGTT ATTCATTGTA TATAACTAT ATTTGCTACA TCCTGTTTT
1401 GTTTTATATA TACTATATT TGTAGCGCCA GGCCCATTTT GTAGC....
1451 .....TTACA CTGCACTATG TGCAACTACT GAATCACTAT GTACATTGTG
pA signal (LP3) YGTGTTY
1501 TCATATAAAA TAAATCACTA TCGGCCAACG CCTTACATAC CGCTGTTAGG

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FIG. 1. Sequence of HPV-16 DNA in the genome region containing the putative late mRNA 3' processing signals. The HPV-16 DNA sequence is taken from Seedorf et al. (26). Positions of the L1 stop codon, poly(A) signals, and YGTGTTY consensus for LP1, LP2, and LP3 are indicated. Arrows indicate the approximate position of the endpoints, within the direct repeat TGTTTT, for the 122-bp deletion in HPV-16del.

preferential utilization of LP2 over LP1, we examined poly(A) site use in isolation. Double-stranded oligonucleotides, consisting of sequences from LP1 (a 73-mer containing HPV-16 sequences from positions 7248 to 7315) and LP2 (a 67-mer containing HPV-16 sequences from positions 7305 to 7367), were synthesized and cloned into pGEM-1, and precursor RNAs produced from these plasmids were analyzed in vitro. Results (Fig. 3B) show that LP1 RNA gave a barely detectable CP1 cleavage product and was poorly polyadenylated. By contrast, LP2 RNA was efficiently cleaved and polyadenylated to a level comparable to that observed with the complete sequence (compare with Fig. 3A). Constructs in which the LP1 and LP2 synthetic sequences were linked in tandem were made. Precursor LP1/LP2 RNA was preferentially cleaved at the LP2 site (Fig. 3B). Similar results were obtained for an RNA precursor with the order LP2/LP1 (data not shown). These data clearly indicate that LP2 functions efficiently with extracts from HeLa cells and is the only functional late mRNA 3' processing site in vitro. The preferential usage of LP2 over LP1 is a feature of RNA sequences at these sites and is independent of their order in a precursor.

Lack of LP1 utilization is due to a poorly functional downstream GU-rich element. Lack of LP1 utilization was intrinsic to the LP1 site, and one possibility considered was that a poorly functional downstream GU-rich element was responsible. Comparison of the GU-rich elements of LP1 and LP2 indicated that they differed at the last three nucleotides. Substitution of an AAC with UGU would convert the LP1 GU-rich element to a sequence identical to a GU-rich element (UGUGUUGU) present within LP2 at a similar spacing from the poly(A) signal. A double-stranded LP1mut oligonucleotide with these base changes was cloned into pGEM-1. Precursor LP1mut RNA from this plasmid was efficiently cleaved at approximately 15 to 20 nucleotides from the AAUAAA signal, which is similar to that observed with LP2 RNA (Fig. 3B). These results indicate that the lack of LP1 utilization is due to a poorly functional downstream GU-rich element.

Transfection studies indicate the presence of a negative

FIG. 2. HPV-16 sequences analyzed by transfection or 3' processing studies. (A) Organization of the HPV-16 genome, with the positions of the early region, the late region, and the NCR indicated. Marked are the *Pst*I sites, and arrows indicate the endpoints of the PP1775 fragment (positions 7008 to 879). Below is shown an enlargement of the HPV-16 PP1775 fragment with the positions of the L1, E6, E7, and E1 open reading frame sequences and NCR indicated. Marked are the positions of the putative late mRNA 3' processing sequences LP1, LP2, and LP3 and various restriction enzyme cleavage sites (P = *Pst*I, S = *Ssp*I, E = *Eco*RI, and D = *Dde*I). Brackets locate the position of the 122-bp deletion in the HPV-16del genome between positions 7278 and 7400. (B) Relative positions of PP1775, PP1775del, and respective subfragments which have been analyzed by transfection or 3' processing studies.

regulatory element upstream of the functional LP2 site. These putative late mRNA 3' processing signals were examined further in vivo by insertion of appropriate HPV-16 DNA sequences, as shown in Fig. 2, into the CAT expression vector pLW1 (6), which lacks poly(A) site sequences. CAT levels were determined (8, 25) following transfection of these recombinants into HeLa cells (9). Recombinants CAT PP1775 and CAT PP1775del containing prototype and HPV-16del sequences between the *Pst*I sites at positions 7008 and 879 were analyzed; CAT PP1775 contains LP1, LP2, and LP3, while CAT PP1775del contains LP1 and LP3. The

FIG. 3. In vitro 3' processing with precursor RNAs containing putative late mRNA 3' processing signals. Precursor RNAs examined were as follows. (A) PD753, which contains all three signals, LP1, LP2, and LP3; PD753del, which contains LP1 and LP3; and ED308, which contains LP3 only. (B) LP1; LP2; LP1mut, which has a substitution of three nucleotides (UGU instead of AAC) in the downstream GU-rich element; and LP1/LP2, which has tandem LP1 and LP2 sequences. (C) PE445 and PE445del, which contain the negative regulatory sequences, and SE227 and SE227del, which lack these sequences. Analysis of RNA products was performed on 8% denaturing polyacrylamide gels. PRE, Unincubated precursor RNA; ATP, polyadenylation reaction containing ATP; 3' dATP, polyadenylation reaction containing 3' dATP; M, RNA size markers. Positions of polyadenylated RNA (pA), the full-length precursor RNA (P), and the 5' portion of the RNA cleaved at the LP1 poly(A) site (CP1), the LP2 poly(A) site (CP2), and the LP3 poly(A) site (CP3) are indicated.

HPV-16 fragments used also contain additional late, early, and NCR sequences. Surprisingly, both of these constructs failed to produce CAT activity (Fig. 4, lanes 1 and 2), even though CAT PP1775 contained LP2, which functions in vitro. This suggested the presence of inhibitory sequences within this *Pst*I fragment which prevented CAT expression. To identify any inhibitory sequences, subfragments (Fig. 2B) of the *Pst*I fragment were inserted into pLW1 and the CAT activities from these constructs were determined. Removal of HPV-16 sequences downstream of the *Eco*RI site at position 7453 (CAT PE445 and CAT PE445del), which takes

FIG. 4. CAT levels in HeLa cell extracts transfected with the HPV-16/CAT plasmid series. Transfections, containing 10 μ g of test plasmid, were performed as described previously (9). Cell extracts were prepared, and CAT activity was assayed by the procedure of Gorman et al. (8). Lanes: 1, CAT PP1775; 2, CAT PP1775del; 3, CAT PE445; 4, CAT PE445del; 5, CAT SE227; 6, CAT SE227del; 7, pLW1; 8, pTER5; and 9, no DNA. pTER5 is essentially pLW1 with the HSV-2 IE 5 mRNA 3' processing signal inserted downstream of the CAT gene (16).

away a large portion of the NCR, including LP3 and the E6, E7, and E1 open reading frame sequences, did not increase CAT activity (Fig. 4, lanes 3 and 4, respectively). By contrast, subsequent removal of sequences upstream of the *Ssp*I site at position 7226, which removes the L1 open reading frame sequences and most of the 3' untranslated region (plasmid CAT SE227), conferred high-level CAT activity to this recombinant containing prototype HPV-16 sequences. However, the recombinant CAT SE227del, which contains HPV-16del sequences and lacks LP2 sequences, also produced CAT activity, but levels were considerably lower than with CAT SE227 (Fig. 4, compare lanes 5 and 6). These data, summarized in Table 1, indicate that sequences between the *Pst*I site at 7008 and the *Ssp*I site at 7226 exert a negative regulatory effect on mRNA expression from the CAT constructs. Similar results were obtained with Cos1 cells, implying that the HPV-18 genomes present in HeLa cells (24) are not involved in the negative regulatory effect of the HPV-16 sequences.

LP2 appears to be the functional poly(A) site in vivo, since CAT SE227del, which lacks LP2, gave CAT levels only 2.7% of those given by CAT SE227, which contains LP2. To confirm this, the synthetic LP1, LP2, and LP1mut sequences were inserted separately into pLW1 downstream of the CAT gene and were transfected into HeLa cells. Results (Table 1) indicate that recombinant CAT LP2 gave high CAT activity, and levels obtained were very similar to those obtained with CAT SE227, which contains LP1 and LP2 but lacks the negative regulatory element. Recombinant CAT LP1 also gave some CAT activity, and levels obtained were 11.4% of those with the CAT SE227 recombinant, whereas the in vitro analysis of LP1 RNA gave negligible cleavage. The recombinant containing LP1mut sequences gave CAT activity

TABLE 1. CAT activities obtained with the HPV-16/CAT plasmid series^a

Plasmid	CAT levels					% SE227
	1	2	3	4	Mean	
CAT PP1775	0.2	3.6	0.5	0.3	1.1	0.6
CAT PP1775del	0.1	0.7	0.6	0.2	0.4	0.2
CAT PE445	0.6	0.3	1.2	1.2	0.8	0.4
CAT PE445del	0.0	0.0	0.0	0.0	0.0	0.0
CAT SE227	152.2	216.4	132.8	254.1	188.9	100.0
CAT SE227del	9.0	2.3	5.1	4.1	5.1	2.7
CAT LP1	12.2	36.5	29.8	7.4	21.5	11.4
CAT LP2	172.2	168.4	182.3	252.7	193.9	102.6
CAT LP1mut	177.1	108.6	159.9	166.0	152.9	80.9
pLW1	0.1	0.0	0.0	0.0	0.0	0.0
pTER5	481.4	566.7	107.4	135.6	326.8	173.0
HeLa	0.0	0.0	0.0	0.0	0.0	0.0

^a Procedures were carried out as described in the legend to Fig. 4, except that CAT assays were performed by the method of Seed and Sheen (25). Assays of four independent experiments are shown, and values for each experiment are expressed as picomoles of chloramphenicol acylated per microgram of protein per hour. Mean values were used to indicate percent CAT levels relative to that of CAT SE227. The positive control plasmid pTER5 is essentially pLW1 with the HSV-2 IE 5 mRNA 3' processing signal inserted downstream of the CAT gene (16).

similar to that observed with recombinants CAT LP2 and CAT SE227, and levels were approximately eightfold greater than those of CAT LP1. These data confirm that LP2 functions *in vivo* and indicate that alteration of three nucleotides in the LP1 downstream GU-rich element makes this a much more efficient poly(A) site *in vivo*.

Negative regulatory elements not associated with any alteration in RNA 3' processing. To investigate the effects of the negative regulatory element on RNA 3' processing, we compared levels of 3' processing *in vitro* by using precursor RNAs containing (PE445) or lacking (SE227) the negative regulatory sequences. Both RNAs are efficiently cleaved and polyadenylated at the LP2 site (Fig. 3C). The corresponding RNAs containing HPV-16del sequences both failed to give detectable cleavage in 3' dATP reactions, although some end polyadenylation of precursor RNA was detected by using ATP. These data indicate that the negative regulatory element does not alter the ability to process RNA *in vitro*.

Our studies both *in vitro* and *in vivo* indicate that by using HeLa cells, LP2 functions efficiently as a late HPV-16 mRNA 3' processing site. This suggests that the restriction in late-gene expression, observed in HeLa cells and other nondifferentiated epithelial cells, is unlikely to be due to the inability to process at a late poly(A) site. In contrast, LP1 functions inefficiently due to a poorly functional downstream GU-rich element; the LP3 signal does not function, perhaps for the same reason as LP1. This highlights the importance of the downstream GU-rich element in the functioning of HPV-16 late poly(A) sites. The GU-rich element of LP1 differs from the YGUGUYYY consensus sequence described by McLauchlan et al. (16) at positions six and seven; replacement of the last three nucleotides of this element with those of the LP2 GU-rich element converts LP1 to an efficient processing site. A similar result was obtained by Ryner et al. (23), who replaced the whole downstream sequences from a nonfunctional poly(A) site located within the coding region of the simian virus 40 large T antigen with those from the simian virus 40 late poly(A) site, thus generating a functional poly(A) site. McDevitt et al. (15) mutated the GU-rich element of the simian virus 40 early

poly(A) site and identified certain positions crucial for efficient functioning *in vivo*; an A residue, also present in the LP1 GU-rich sequence (UGUGUΔAC), was particularly unfavorable.

The reason for HPV-16 having two apparently nonfunctional poly(A) signals downstream of the late genes is unclear. One interesting possibility relates to the differential use of mRNA 3' processing signals in particular cell types. Differentiated keratinocytes are the only cells known to express papillomavirus late genes, and these cells could utilize 3' processing sites which are nonfunctional in other cell types. Since alteration of three nucleotides in the downstream GU-rich element greatly increases the efficiency of LP1, this suggests that any cell type specificity may reside in this 3' processing element, which does show sequence variability, rather than in the relatively invariant AAUAAA sequence. In some other systems in which alternative polyadenylation sites are utilized, alternative splicing pathways also are observed (1, 7, 13).

Our transfection studies indicate the presence of a negative regulatory element located immediately upstream of the late mRNA polyadenylation signals. *In vitro* polyadenylation analysis indicates that this element does not alter mRNA 3' processing. Studies in which an *in vitro* mRNA decay assay was used indicate that the negative element probably acts as an mRNA instability sequence (I. M. Kennedy, J. K. Haddow, and J. B. Clements, unpublished data). The high AU content and position of the negative element within the 3' untranslated region are consistent with instability elements found in other mRNAs (5, 10, 11, 20, 27).

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