JOURNAL OF VIROLOGY, Apr. 1991, p. 2093–2097
0022-538X/91/042093-05$02.00/0

Vol. 65, No. 4

A Negative Regulatory Element in the Human Papillomavirus Type 16 Genome Acts at the Level of Late mRNA Stability

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Received 1 October 1990/Accepted 17 December 1990

A negative regulatory element present in the human papillomavirus type 16 genome has been characterized. Deletion analysis has localized the 5′ end of the element to the late region of the genome at the extreme 3′ end of the coding region of the L1 open reading frame, around the L1 stop codon, with the element extending into the L1 3′ untranslated region. For the cell lines used, the element's function was independent of cell type, tissue, or species of origin, unlike papillomavirus infection, which is very dependent on such factors. By using an mRNA decay assay, we have determined that polyadenylated RNA containing the element is much less stable than polyadenylated RNA lacking the element. This indicates that the element acts as an mRNA instability element. The significance of A-rich, GU-rich, and AUG-rich sequences for the functioning of this human papillomavirus type 16 instability element is discussed.

Papillomaviruses contain double-stranded circular genomes of approximately 8 kb and are the etiological agents of warts, or papillomas. In situ hybridization of a section of a papilloma indicates that late gene expression is restricted to the fully differentiated keratinocytes in the granular layer (34). Thus, papillomavirus late gene expression appears to be tightly linked to differentiation of keratinocytes. The mechanisms which regulate papillomavirus late gene expression are not clearly understood. Although control of late gene expression may be achieved by the regulation of a wart-specific late promoter, this is unlikely to be the sole mechanism since the late region lies immediately downstream of the early region and in the same transcriptional orientation. Thus, additional mechanisms such as selective poly(A) site usage (21), transcriptional termination (2), and mRNA turnover may prevent the production of stable transcripts of the late region from early promoters which are active in nonproductively infected cells (3, 28, 34).

We have previously described a negative regulatory element in the human papillomavirus type 16 (HPV16) genome (21) which is located immediately upstream of the late mRNA 3′ processing signals. Here, we further characterize this element and show that it acts as a late mRNA instability element.

Localization of the negative regulatory element within the HPV16 genome. Our previous transfection studies used a chloramphenicol acetyltransferase (CAT) expression vector, pLW1, which contains the bacterial CAT gene linked downstream of a herpes simplex virus type 2 immediate early (IE) 5 gene promoter and lacks a polyadenylation site (15). An HPV16 DNA region containing the late mRNA 3′ processing signals was inserted downstream of the CAT gene, and an element was identified as an inhibitor of CAT expression (21). Deletion analysis using available restriction endonuclease cleavage sites located this element between the *Pst*I site at position 7008 and the *Ssp*I site at position 7226 in the HPV16 genome, immediately upstream of the late mRNA 3′ processing signals. Here, we present a detailed analysis of the negative element's location within the HPV16 insert by using a series of deletions generated by *Bal* 31 nuclease or exonuclease III digestion. We assayed for the release of the negative effect by measuring CAT levels following the transfection of these plasmids into HeLa cells. We used a previously described plasmid, CAT PE445, containing an HPV16 *Pst*I-*Eco*RI fragment (positions 7008 to 7453) inserted at the unique *Hin*dIII site in pLW1 by the use of *Hin*dIII linkers (21). For the present study, this plasmid was modified by the removal of the downstream *Hin*dIII site at the 3′ HPV16-plasmid junction, and deletion commenced from the now unique *Hin*dIII site located immediately adjacent to the *Pst*I site in the L1 open reading frame (Fig. 1A) at the 5′ plasmid-HPV16 junction (position 7008 in HPV16). Following *Bal* 31 or exonuclease III digestion, the HPV16 sequences were excised and recloned into the original CAT parent plasmid, pLW1; this procedure restored any deletion which may have occurred within the CAT sequences. A series of CAT PE445 plasmid derivatives which had different deletions within the 5′ portion of the HPV16 insert was produced, and the deletion endpoints were determined by dideoxynucleoside triphosphate sequencing. The positions of selected deletion endpoints are shown in Fig. 1B. CAT activity was measured for each plasmid following transfection into HeLa cells. Results (Fig. 2) indicate that the deletion of HPV16 DNA sequences from positions 7008 to 7128 (CAT PE445Δ73 and CAT PE445Δ121) did not unblock CAT expression. Plasmids CAT PE445Δ141 and CAT PE445Δ148, which delete HPV16 sequences from positions 7008 to 7147 and from positions 7008 to 7155, respectively, did show some CAT activity. Further deletion of HPV16 DNA sequences released the block and gave high levels of CAT activity (CAT PE445Δ172, -Δ183, -Δ210, -Δ287, and -Δ295). Plasmid CAT SE227, which contains an *Ssp*I-*Eco*RI fragment (positions 7226 to 7453) (21) also shows high CAT levels. These data indicate that the 5′ extent of the negative regulatory element was very close to the L1 stop codon (position 7152) and that the element extended into the L1 3′ untranslated region (3′ UTR).

The negative regulatory element is neither species, tissue, nor cell-type specific. HPVs show strict specificity for species, tissue, and cell type. We have determined the specificity of the HPV16 negative regulatory element by measuring the CAT activity of the CAT PE445 derivatives in a variety of different cell types. Results, summarized in Table

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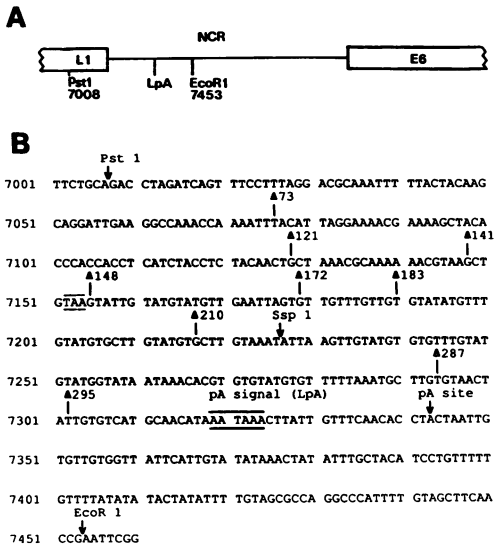

FIG. 1. Sequence of HPV16 DNA in the genome region containing the negative regulatory element. (A) Portion of the HPV16 genome with the positions of the L1 and E6 open reading frame sequences and the noncoding region indicated. The positions of the late mRNA poly(A) site (LpA) and of the *Pst*I and *Eco*RI restriction enzyme cleavage sites are indicated. (B) Portion of HPV16 DNA sequence, in agreement with the work of Seedorf et al. (31). The positions of selected deletion endpoints are indicated (vertical bars). Also indicated are the positions of the L1 stop codon (broken line above and below sequence); the late poly(A) signal (solid line above and below sequence); the late poly(A) site (vertical arrow); and the *Pst*I, *Ssp*I, and *Eco*RI restriction enzyme cleavage sites (vertical arrows).

1, indicate that the negative element was effective in all the cells tested, including human cervical carcinoma cells (HeLa), simian virus 40-transformed monkey kidney fibroblasts (Cos-1), and hamster kidney fibroblasts (BHK-21/C13). In addition, the pattern of CAT activity obtained with the deletions was similar in each cell type tested. This indicates that the negative regulatory element functions independently of cell type, tissue, and species of origin.

The negative regulatory element acts to destabilize late mRNA. We investigated the possibility that the negative regulatory element acts as an mRNA instability sequence, since similar elements are located in the 3′ UTR in other gene systems (7, 32, 35). The stability of two RNAs which have different 3′ portions of HPV16 late mRNA (PE445 and SE227) and which contain and lack the negative element, respectively, were tested in an mRNA decay assay similar to that used to investigate the stability of c-*myc* mRNA (7). In this study, PE445 and SE227 RNAs, produced by in vitro

TABLE 1. CAT activities obtained with plasmid CAT PE445 and derivatives in various cell types[a]

Plasmid	CAT level[b]		
	HeLa	Cos-1	BHK-21/C13
CAT PE445	2.3	1.0	3.5
CAT PE445Δ73	1.1	0.9	3.4
CAT PE445Δ121	2.9	3.3	2.2
CAT PE445Δ141	28.3	32.2	22.1
CAT PE445Δ148	28.6	18.9	41.0
CAT PE445Δ172	95.9	191.6	506.4
CAT PE445Δ183	163.1	104.3	426.3
CAT PE445Δ210	154.0	113.6	310.3
CAT SE227	200.3	479.1	651.4
CAT PE445Δ287	249.1	664.3	736.5
CAT PE445Δ295	132.4	636.1	492.3
pLW1	2.4	0.5	6.0
pTER5[c]	137.4	149.7	514.4
None[d]	0.6	0.4	0.1

[a] Procedures were carried out as for Fig. 2, except that CAT assays were performed as described by Seed and Sheen (30).
[b] Expressed as picomoles of acylated chloramphenicol per microgram of protein per hour.
[c] Positive control plasmid pTER5 is essentially pLW1 with the herpes simplex virus type 2 IE 5 mRNA 3′ processing signals inserted downstream of the CAT gene (25).
[d] Negative control.

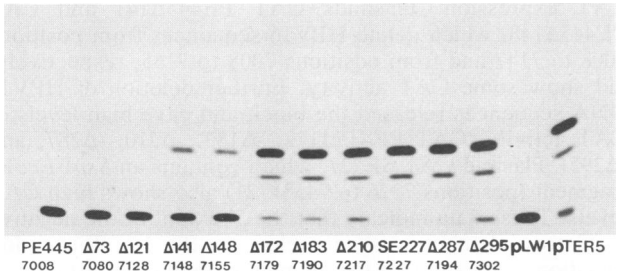

FIG. 2. CAT levels in HeLa cell extracts transfected with plasmid CAT PE445 and derivatives. Transfections, each containing 10 μg of test plasmid, were performed as described elsewhere (18). Cell extracts were prepared and CAT activities were assayed as described by Gorman et al. (17). The HPV16 5′ deletion endpoint is shown below the appropriate plasmid name. pTER5 is essentially pLW1 with the herpes simplex virus type 2 IE 5 mRNA 3′ processing signal inserted downstream of the CAT gene (25).

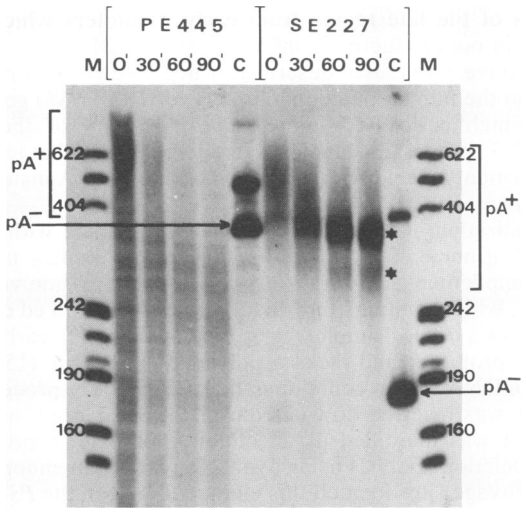

FIG. 3. mRNA decay assay of polyadenylated RNAs containing or lacking the negative regulatory element. RNAs analyzed were polyadenylated PE445 RNA, which contains the negative regulatory element, and polyadenylated SE227 RNA, which lacks these sequences. M, Size markers (pAT153 *Hpa*II digest); C, control track showing the position of the precursor RNA (upper band) and the cleaved unpolyadenylated RNA (pA^-). The size distribution of the poly(A)$^+$ RNA (pA^+; bracket) is shown. Two RNA products estimated to contain 200 and 100 A residues are shown (*).

TABLE 2. Comparison of AUG-rich sequences from L1 3′ UTRs of various human genital papillomaviruses[a]

HPV type	Sequence	Position	Stop codon	AATAAA
HPV16	UUGUAUGUAUGUU	7158–7170	7152–7154	7319–7324
	UUGUAUGUAUGGU	7245–7257		
	GUGUAUAUGUU	7189–7199		
	UUGUAUGU	7199–7206		
		7209–7116		
		7233–7240		
HPV6B	GUGUAUGUACUGUU	7323–7336	7289–7991	7407–7412
	AUGUAUAUGUG	7337–7347		
	GUGUAUAUGUA	7297–7307		
HPV11	GUGUAUGUAUGUU	7437–7450	7274–7276	7457–7462
	AUGUUAUGUAUGUU	7373–7386		
	UUGUUAUGUAUGUU	7390–7403		
	GUGUAUAUGUU	7319–7331		
		7349–7358		
	GUGUAUAUGUG	7341–7351		
		7361–7371		
HPV18	UUGUAUGGUAUGUA	7095–7108	7016–7018	7160–7166
	UUGUAUGU	7058–7065		
		7118–7125		
		7125–7132		
	UUGUAUGA	7082–7089		
	UGGUAUGU	7145–7152		
HPV31	GUGUAUGUAUGCU	7205–7217	7064–7066	7227–7232
	UUGUAUAUGUG	7117–7127		
	UGGUAUAUGUA	7147–7157		
	GUGUAUGU	7072–7079		
	UAGUAUGU	7192–7199		
	AUGUAUGU	7218–7225		
HPV33	AUGUAUGUUAUGUU	7190–7203	7091–7093	7286–7292
	GUGUAUAUGUU	7275–7285		
	UUGUAUGU	7202–7209		
	GUGUAUGU	7176–7183		

[a] Information derived from published DNA sequences for HPV16 (31), HPV6b (29), HPV11 (12), HPV18 (9), HPV31 (16), and HPV33 (10).

transcription from recombinant pGEM plasmids (21), were first polyadenylated in vitro by incubation with a HeLa cell nuclear extract (26, 27). In this reaction, the precursor RNAs were cleaved at the late mRNA poly(A) site (Fig. 1B) (referred to as LP2 in reference 21), followed rapidly by the addition of a poly(A) tail of approximately 250 to 300 A residues. The length of the poly(A) tail was estimated by the difference in size between poly(A)$^-$ and poly(A)$^+$ RNA species (Fig. 3) and is consistent with that found for newly synthesized mRNA in the nucleus (33). The polyadenylated RNAs were then incubated in an mRNA decay assay mixture containing HeLa cell polyribosomes, and we assayed for the stability or degradation of the poly(A)$^+$ RNA fraction following analysis by denaturing polyacrylamide gel electrophoresis. Results (Fig. 3) show that polyadenylated PE445 RNA, which contains the negative element, is rapidly degraded, with a half-life of approximately 30 min. In contrast, polyadenylated SE227 RNA, which lacks the negative element, is much more stable, with a prominent RNA species [estimated to contain a poly(A) tail of approximately 200 A residues] as well as a minor RNA species (containing approximately 100 A residues) still present at the end of the 90-min assay. This slight poly(A) shortening is consistent with that found for relatively stable mRNAs (7). These data indicate that the negative regulatory element acts to destabilize polyadenylated RNA.

In this study, we have localized a negative regulatory element present in the HPV16 genome to around the position of the late gene L1 stop codon and extending to a location within the L1 3′ UTR. We have not yet determined the precise 3′ extent of the element, but its total length cannot be greater than 190 to 200 nucleotides (i.e., extending to the 3′ terminus of the L1 mRNA at around position 7342). By using an mRNA decay assay, we have shown that the negative element acts to destabilize polyadenylated RNA. The location of the HPV16 late mRNA instability (negative) element in the L1 3′ UTR is consistent with the positions of instability elements found in short-lived mRNAs in other systems (7, 8, 32, 35). In these systems, the instability sequences were localized to AU-rich sequences, with the consensus sequence being UUAUUUAU (8, 20, 35); the HPV16 instability region lacks this element, although a repeated GU-rich sequence is present just downstream of the essential region affected by our deletions.

Important candidate sequences identified by our deletion analysis which may be part of the instability element include an A-rich tract immediately 5′ to the L1 stop codon and a AUG-rich sequence (UUGUAUGUAUGUU, positions 7158 to 7170) (Fig. 1B) located immediately 3′ to the stop codon. In addition, a related sequence (GUGUAUAUGUU) and several half sequences (UUGUAUGU) are present downstream of the AUG-rich sequence. In general, the late 3′

UTR has a high alternating purine-pyrimidine content. Interestingly, many other papillomaviruses have an A-rich tract at the 3′ end of the L1 coding region; however, the AUG-rich sequences and the high alternating purine-pyrimidine content are present in the L1 3′ UTR of other genital papillomaviruses (Table 2) but are absent from human cutaneous and animal papillomaviruses. A negative regulatory element has been localized to the L1 3′ UTR of BPV1 (1), indicating that late mRNA instability may be a general feature of papillomavirus late gene expression.

In short-lived mRNAs, the AU-rich instability element promotes the progressive shortening of the poly(A) tail as the first step in the degradation of the mRNA (for reviews, see references 5 and 19). This shortening may be achieved by the loss of binding of poly(A)-binding protein (PABP) to the poly(A) tail, thus leaving the poly(A) tail open to attack by nucleases present in the cell (4). The AU-rich sequence may be a binding site either for PABP itself or for a regulatory protein which destabilizes the binding of the PABP to the poly(A) tail (7). Alternatively, the AU-rich sequences may base pair with the poly(A) tail, displacing the PABP and allowing nuclease attack at the mismatched regions (35). The HPV16 instability sequences also act by promoting the rapid loss of a poly(A) tail from polyadenylated RNA. Hence, either the PABP or some regulatory protein, present in a variety of cells, should bind to the HPV16 instability sequences or, in the alternative hypothesis, the poly(A) tail should base pair with the instability sequence.

In addition to their role in mRNA turnover, AU-rich instability sequences present in the 3′ UTR of human beta interferon mRNA (22) act to inhibit mRNA translation; the inhibition is dependent to some extent on beta interferon coding sequences (23). In fertilized *Xenopus* oocytes, in which poly(A) shortening is normal, an AU-rich sequence (UUUUUAU) present in the 3′ UTR of certain genes actually stabilizes mRNA by promoting the lengthening of the poly(A) tail (14).

It is apparent that 3′ UTR sequences are important for the regulation of certain genes. How, then, can we explain the possible involvement of these sequences in the differential expression of papillomavirus late genes? This could be achieved if a putative instability sequence, described here for HPV16, is functional in immature cells but not in differentiated cells, a condition similar to the differential stability of c-*myc* mRNA which occurs during different conditions of growth or differentiation (6, 11, 13, 24).

We thank John H. Subak-Sharpe for his continuing interest.

This work was supported by Cancer Research Campaign grant SP1341.

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