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Mutational Inactivation of Two Distinct Negative RNA Elements in the Human Papillomavirus Type 16 L2 Coding Region Induces Production of High Levels of L2 in Human Cells

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Here we show that the 5′ end and the middle region of the L2 coding sequence of human papillomavirus type 16 contain strong inhibitory RNA sequences termed inhibitory regions I and II. This is in contrast to L1, which contains one inhibitory region in the 5′ end of the coding region. Inhibitory regions I and II acted in *cis* to reduce L2 mRNA levels and to inhibit the use of the mRNA. In tandem, the two regions reduced L2 mRNA production to undetectable levels. Specific mutational inactivation of the two inhibitory elements in the 5′ end and in the middle region of L2 by the introduction of nucleotide substitutions that changed the nucleotide sequence but not the protein sequence resulted in production of high levels of L2 mRNA and protein. In contrast to L2, a partial L1 mutant in which only the first one third of L1 was mutated produced levels of L1 mRNA and protein similar to those in a full L1 mutant. In addition, the constitutive transport element of simian retrovirus type 1 overcomes the effect of the inhibitory sequences of L1 but not L2.

Human papillomaviruses (HPVs) are a group of small, double-stranded DNA tumor viruses (27). The HPV genome is approximately 8 kb in length and can be divided into an early (E) and late (L) region (Fig. 1) (16). The late region codes for the two structural proteins L1 and L2, which are the major and minor capsid proteins, respectively. Together they form the icosahedral capsid that contains the viral genomic DNA.

The HPV life cycle is tightly linked to the differentiation stage of the infected cell (16). Upon entry into the basal epithelial cells, only early genes are expressed. As the cell progresses towards terminal differentiation, induction of viral DNA replication occurs to high levels (30). This is followed by activation of viral late gene expression and assembly of infectious viral particles at the uppermost layers of the epithelium. The requirement for terminal cell differentiation has hampered the study of the viral life cycle in vitro. However, propagation of papillomavirus has been successful in a xenograft model and in the organotypic (raft) culture systems that induce terminal cell differentiation (10, 21, 29). Propagation of HPV-containing cell lines in these systems leads to the completion of the differentiation-dependent life cycle of HPVs in vitro. The L1 and L2 proteins were detected only after culturing of the HPV DNA-containing cells in raft cultures and then only in the superficial layers of the terminally differentiated cells (12–14, 21–23). L1 and L2 proteins were not seen in the proliferating keratinocytes, demonstrating that expression of L1 and L2 is efficiently suppressed in proliferating cells.

We are interested in the regulation of HPV late gene expression, and we and others have identified inhibitory sequences on late HPV mRNAs (1, 24–26). These sequences are located in the late 3′ untranslated region (6, 15, 17, 18, 33) and in the L1 and L2 coding regions (4, 5, 28, 31). Our previous work on HPV-16 L1 expression demonstrated the presence of *cis*-acting negative elements in the L1 coding region (5, 31). The HPV-16 L1 coding region was shown to act in *cis* to inhibit chloramphenicol acetyltransferase (CAT) protein production in an orientation-dependent manner when placed downstream of the *CAT* reporter gene (28, 31). We found that negative elements are located in the first 514 nucleotides of L1, but no inhibitory sequences were present between nucleotide positions 514 (genomic position 6152) and 1518 (7156) in the L1 open reading frame (5, 31). We successfully inactivated the inhibitory elements in the first 514 nucleotides of L1 by mutagenesis and generated a synthetic HPV-16 L1 gene that produced high levels of L1 mRNA and L1 protein in HeLa cells. Interestingly, the human immunodeficiency virus type 1 nuclear export factors Rev and Rev-responsive element (RRE) or simian retrovirus type 1 constitutive transport element (CTE) could overcome inhibition and induce expression of the L1 protein, indicating that the inhibitory sequences were active in the cell nucleus (31).

We have previously shown that sequences in the HPV-16 L2 coding region inhibit expression of the *CAT* reporter gene in *cis* in an orientation-dependent manner (4, 28). This is partly due to a reduction of the mRNA half-life by the L2 sequences (28). In other experiments we found that translation of the L2 mRNA was suppressed (4). However, the elusive nature of the inhibitory elements in L2 has prevented mapping and localization of these elements.

MATERIALS AND METHODS

Plasmid constructions. (i) L2EIAV hybrids. PCL250EIAV50 was generated by PCR amplification of nucleotides 1 to 724 of HPV-16 L2 with oligonucleotides L2start (5′-CC*GTCGACGCGCGC*GAAATGCGACACAAACGTTCTGC-3′) and L2wt(50%)AS (5′-CC*ACGCGT*GGGTCTACAACTTTAACCTG-3′) and cloning into the Topo TA vector followed by insertion into pC16L150EIAV50 (5), thereby replacing the L1 sequences with L2 sequences. Restriction sites are in italics. pCEIAV50L250 was generated by PCR amplification of nucleotides 1

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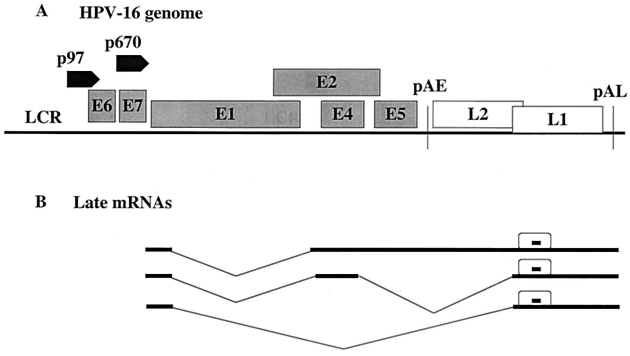

FIG. 1. (A) Structure of the HPV-16 genome. The early promoter p97 and the late promoter p670 are indicated. LCR, long control region; pAE, early polyadenylation signal; pAL, late polyadenylation signal. (B) Schematic structures of the late mRNAs encoding L1 and L2. The previously identified negative elements in the L1 coding region are indicated on the mRNAs (5, 31).

to 746 of equine infectious anemia virus (EIAV) *gag* with oligonucleotides EIA Vstart (5′-CC*GTCGACGCGCGC*GAAATGGGAGACCCTTTGACATGG-3′) and EIAV(50%)AS (5′-GCCTAAACTGA*ACGCGT*GCAGGCTCC-3′) and amplification of nucleotides 718 to 1422 of the HPV-16 L2 gene with oligonucleotides L2wt(50%)sense (5′-*ACGCGT*CCTGCTTTTGTAACCACTCCC-3′) and L2stop (5′-CC*CTCGAG*CTAGGCAGCCAAAGAGACATC-3′). The PCR fragments were first subcloned in the Topo TA PCR cloning kit (Invitrogen), excised, and inserted into pCL0806 (5).

pCL225EIAV75 was generated by PCR amplification of nucleotides 1 to 378 of the HPV-16 L2 gene with oligonucleotides L2start and L2wt(nt378)AS (5′-C CTGATACATC*ACGCGT*AATGGAAGGTACAG-3′), cloning into the Topo TA vector (Invitrogen), followed by transfer into pC16L125EIAV75 (5), thereby replacing the L1 sequence with the L2 sequence. PCEIAV25L275 was generated by PCR amplification of nucleotides 343 to 1422 of the HPV-16 L2 gene with oligonucleotides L2wt(nt343)sense (5′-GGAAGAAACT*ACGCGT*ATTGATG CTGGTGC-3′) and L2stop, cloning into the Topo TA vector, followed by transfer into pC16EIAV25L175 (5), thereby replacing L1 sequences with L2 sequences.

(ii) L2L1 hybrids. pCL2L1 was generated by PCR amplification of nucleotides 1 to 533 of the HPV-16 L2 gene with oligonucleotides L2start and L2wt(nt533)AS (5′-C*GGATCCATGGCCA*CCAGTTTCTGCAGGTGTTGG-3′), cloning into Topo TA vector, followed by transfer into pCL1MUT (5), thereby replacing the mutant L1 sequence with L2 sequences. pCL1ML2 was generated by PCR amplification of nucleotides 534 to 1422 of the HPV-16 L2 gene with oligonucleotides L2wt(nt541)sense (5′-*GGATCC*GG*TGGCCA*TTTT ACACTTTCATCATC-3′) and L2stop. The PCR fragment was subcloned into a Topo TA vector (Invitrogen), excised, and inserted into pCL1MUT (5), thereby replacing L1 sequences with L2 sequences. To generate pCL2ML1, mutant L2 sequences were first PCR amplified from pCL2MUT with oligonucleotides L2mstart and L2m(nt537asBamHI) (5′-C*GGATCC*ATGGCCACCGGTCTCG GCGG-3′), followed by subcloning into a Topo TA vector (Invitrogen). The mutant L2 sequences were excised with *Sal*I and *Bam*HI and inserted into pCL1MUT (5), thereby replacing mutant L1 sequences with mutant L2 sequences. In all plasmids, L1 and L2 were fused in frame.

(iii) L2 mutant. pC16L2MUT was generated by changing adenosines and thymidines to cytosines and guanidines in HPV-16 L2, neither changing the protein sequence nor introducing rare codons. The L2 gene was divided into ten (1 to 10) segments. Segment 1 (5′-ATGCGCCACAAGCGCAGCGCCAAGCG CACCAAGCGCGCCAGCGCCACCCAGCTGTACAAGACCTGCAAGCA GGCCGGCACCTGCCCCCCCCGACATCATCCCCAAGGTGGAGGGCAA GACCATCGCCGAGCAGATCCTGCAG-3′) was used as the template in generating the L2 M1-2 fragment with oligonucleotides L2mstart (5′-*GTCGAC* CAATGCGCCACAAGCGCAGC-3′) as the sense primer and oligonucleotide 2 (5′-*GGGCCC*ACGGGGTCCACGGTCAGGGGGGGCCTCACGGGGGCCA GGGTGTCGGTGGCGGTGGGGGGCCTGGTGCCCAGGGGGATGTAGC CGGTGCGGCCGCCGGTGCCGCTGCCGGTGCCGATGCCCAGGCCGC CAAAAAACACGCCCATGCTGCCGTACTGCAGGATCTGCTCGGCGAT G-3′) as the antisense primer. The restriction enzyme sites used for cloning are in italics.

The L2 M1-2 fragment was used as the template in PCR, generating the L2 M1-3 fragment with oligonucleotides L2mstart as the sense primer and oligonucleotide 3 (5′-*ACTAG*TGGTGATGCTAAAGCCGGACACGTCGGGGGGG ATGCTGGGCACGCTGGTGGGGGCGCCGGCGTCGATAAAGCTGGTC TCCTCCACCAGGCTCACGATGGAGGGGTCGCTGGGGCCCACGGGG TCCACGGTC-3′) as the antisense primer. The L2 M1-3 fragment was used as the template in PCR, generating the L2 M1-4 fragment with oligonucleotide L2mstart as the sense primer and oligonucleotide 4 (5′-G*TGGCCA*CCGGTCT CGGCGGGGGTGGGGGGCTGCAGCACGCTGGGGTCGGTAAAGGTG GGGTTGTTGTGGGTGGTCACGGTGGTCACGGTGTTGTTGATGTCCA GGATGGCGGGGGTGGTGTCGGTACTAGTGGTGATGCTAAAGCC-3′) as the antisense primer.

Segment 5 (5′-*TGGCCA*CTTTACCCTGAGCAGCAGCACCATCAGCACC CACAACTACGAGGAGATCCCCATGGACACCTTTATCGTGAGCACCA ACCCCAACACCGTGACCAGCAGCACCCCCATCCCCGGCAGCCGCCC CGTGGCCCGCCTGGGCCTGTACAGCCGCACCACCCAGCAGGTGAA GG-3′) was used as the template in PCR to generate L2 M5-6 with oligonucleotide 5shortsense (5′-*TGGCCA*CTTTACCCTGAGCAG-3′) as the sense primer and oligonucleotide 6 (5′-CAC*GGATCC*GATATCCAGAAAGTCGGGGTCGGGGGC GATGTTGATGCTGTTGTCGTTGCTGCTAAAGTACAGGGTGTTGTCCAC GTCGATGCCCTCGTAGGCGGGGTTGTCGTATGTGATGAGCTTGGTGG GGGTGGTCACAAAGGCGGGGTCCACCACCTTCACCTGCTGGGTGGTG CGGC-3′) as the antisense primer.

Segment 7 (5′-*GATATC*GTGGCCCTGCACCGCCCCGCCCTGACCAGCC GCCGCACCGGCATCCGCTACAGCCGCATCGGCAACAAGCAGACCCT GCGCACCCGCAGCGGCAAGAGCATCGGCGCCAAGGTGCACTACTA CTACGACCTGAGCACCATCGACCCCGCCGAGGAGATCGAGC-3′) was used as the template in PCR to generate L2 M7-8 with oligonucleotide 7shortsense (5′-*GATATC*GTGGCCCTGCACC-3′) as the sense primer and oligonucleotide 8 (5′-GCTG*CTCGAG*CTGGGTACCGGGGTGGTGCTGGTGTCGGTGATAAA GTCGTCGGCGTAGATGTCGTACAGGCCGTTGTTGATGCTGTGGGGCT GGCGGCGTGGCTGGTGGTGGTGTAGGTGCTGGGGGTGATGGTCTGC AGCTCGATCTCCTCGGCGGGGTCG-3′) as the antisense primer.

Segment 9 (5′-*GGTACC*CAGCGTGCCCAGCACCAGCCTGAGCGGCTA CATCCCCGCCAACACCACCATCCCCTTTGGCGGCGCCTACAACATCC CCCTGGTGAGCGGCCCCGACATCCCCATCAACATCACCGACCAGGC CCCCAGCCTGATCCCCATCGTGCCCGGCAGC-3′) was used as the template in PCR amplification to generate L2 M9-10 with oligonucleotide 9shortsense (5′-*G GTACC*CAGCGTGCCCAG-3′) as the sense primer and oligonucleotide 10 (5′-C TG*ACTAGT*GCGGCCGCCTCGAGCTAGGCGGCCAGGCTCACGTCGCTA AAAAAGTAGGGCAGGCGCTTGCGGCGCTTGCGCAGCATGTAATAGCT GGGGTGCAGGTAAAAGTCGCCGGCGTCGGCGATGATGGTGTACTGG GGGCTGCCGGGCACGATGGGGATCAG-3′) as the antisense primer. The PCR fragments were subcloned into either *Eco*RV-digested pBluescript KS(−) (Stratagene) or into the Topo TA vector (Invitrogen). Clones with the insert were identified and subjected to sequencing. Mutant L2 inserts with the correct sequence were transferred into pCL0806 (5) to generate pCL2MUT. For the location of restriction sites in the sequence, see Fig. 4A.

(iv) L2 mutant/wild-type hybrids. pC12 was generated by excision of L2 wild-type sequences from pCL2L1 with *Sal*I and *Apa*I, followed by transfer into pCL2MUT. pC34 was generated by excision of the wild-type L2 sequence from pCL2L with *Apa*I and *Msc*I, followed by transfer into pCL2MUT. pC3-6 was generated by PCR amplification of the wild-type L2 sequence with oligonucleotides L2start and L2(nt853AS) (5′-*GATATC*CAAAAAGTCAGGATCTG-3′), followed by subcloning into a Topo TA vector, excision with *Apa*I and *Eco*RV, and transfer into pCL2MUT. pC56 was generated by PCR amplification of wild-type L2 sequences with oligonucleotides L2(nt537sense) (5′-*TGGCCA*CT TTACACTTTCATCATCCACTAT-3′) and L2(nt853AS), subcloning into a Topo TA vector, and transfer into pCL2MUT.

pC5-8 was generated by PCR amplification of wild-type L2 sequences with oligonucleotides L2(nt537sense) and L2stop, subcloning into a Topo TA vector, excision with *Msc*I and *Kpn*I, and transfer into pCL2MUT. pC78 was generated by PCR amplification of wild-type L2 sequences with oligonucleotides L2(nt853sense) (5′-G*GATATC*GTTGCTTTACATAGGCCAGC-3′) and L2stop, subcloning into a Topo TA vector, excision with *Eco*RV and *Kpn*I, and transfer into pCL2MUT. pC3-10 was generated by excision of mutant L2 sequences from pCL2MUT with *Sal*I and *Apa*I, followed by transfer into pC16L2. pC5-10 was generated by excision of mutant L2 sequences from pCL2MUT with *Sal*I and *Msc*I, followed by transfer into pCL1ML2.

pC4-10 was generated by first PCR amplifying wild-type L2 sequences from pC16L2 with oligonucleotides L2(nt410sense) (5′-GATTTAGTATTACT*ACTA GT*ACTGATACCACAC-3′) and L2stop. This fragment was digested with *Spe*I and *Xho*I and inserted into pC5-10. pC1-6 was generated by transfer of a *Sal*I-

*Eco*RV fragment of wild-type L2 sequences to pCL2MUT, and pC1-8 was generated by transferring a *Sal*I-*Kpn*I fragment of wild-type L2 sequences from pC16L2 to pC16L2MUT. pC7-10 was generated by transfer of an *Eco*RV-*Xho*I fragment encoding wild-type L2 sequences into pC78. pC910 was generated by excising the wild-type L2 sequence with *Kpn*I and *Xho*I from pC7-10, followed by transfer into pCL2MUT.

p16L2CTE was constructed by replacing the *Bss*HII-*Xho*I L1 sequence encoding the L1 open reading frame in p16L1CTE (31) with the L2 sequence from pC16L2.

(v) L1 mutant. pNLL1(1-9) was generated by dividing the HPV-16 L1 sequence from +1 to +1518 into nine sections; then, where possible, codons were changed, taking care not to introduce rare codons. This was achieved with long oligonucleotides corresponding to the new L1 sequence, which were used as PCR templates for the construction of the L1 mutant gene. The first section from nucleotides +1 to +188 was generated by PCR on the long oligonucleotide (L1Mut1; 5′-ATGAGCCTGTGGCTGCCCAGCGAGGCCACCGTGTACCT GCCCCCCGTGCCCGTGAGCAAGGTGGTGAGCACCGACGAGTACGT GGCCCGCACCAACATCTACTACCACGCCGGCACCAGCCGCCTGCTG GCCGTGGGCCACCCCTACTTCCCCATCAAGAAGCCTAACAACAACA AGATCCTGGTGCCCAAGG-3′) with L15′-MUTANT1 (5′-CC*GTCGAC*GC GCGCCAAGATGAGCCTGTGGCTGCCCAGCG-3′) and L13′-MUTANT1 (5′-*AGGCCT*CCTTGGGCACCAGGATCTTG-3′). The second section from nucleotides +188 to +356 was generated by PCR on the long oligonucleotide (L1Mut2; 5′-CCCAAGGTGAGCGGCCTGCAGTACCGCGTGTTCCGCAT CCACCTGCCCGACCCCAACAAGTTCGGCTTCCCCGACACCAGCTTCT ACAACCCCGACACCCAGCGCTGGTGTGGGCCTGCGTGGGCGTGGA GGTGGGCCGCGGCCAGCCCCTGGGCGTGGGCATCTCTGGCCACCG GATCCGTCGAC-3′) with L15′-MUTANT2 (5′-CCCAAGGTGAGCGGCCT GCAGTACCGC-3′) and L13′-MUTANT2 (5′-*GTCGAC*GGATCCGGTGGC CAGAGATGCCCACGCCCAGGGG-3′).

The third section from nucleotides +356 to +514 was generated by PCR on the long oligonucleotide (L1Mut3): (5′-AGCGGCCACCCCCTGCTGAACAA GCTGGACGACACCGAGAACGCCAGCGCCTACGCCGCCAACGCCGG CGTGGACAACCGCGAGTGCATCAGCATGGACTACAAGCAGACCCA GCTGTGCCTGATCGGCTGCAAGCCCCCCATCGGCGAGCACTGGGGC AAGGGATCCCTCGAG-3′) with L15′-MUTANT3 (5′-AG*TGGCCA*CCCCC TGCTGAACAAGCTGG-3′) and L13′-MUTANT3 (5′-*CTCGAG*GGATCCCT TGCCCCAGTGCTCGCC-3′). The fourth section from nucleotides +514 to +667 was generated by PCR on the long oligonucleotide (L1Mut4; 5′-GGATC CCCCTGCACCAACGTGGCCGTGAACCCCGGCGACTGCCCCCCCCTG GAGCTGATCAACACCGTGATCCAGGACGGCGACATGGTGGACACC GGCTTCGGCGCCATGGACTTCACCACCCTGCAGGCCAACAAGAGCG AGGTGCCCCTGGATATC-3′) with L15′-MUTANT4 (5′-*GGATCC*CCCCTGC ACCAACGTGGCC-3′) and L13′-MUTANT4 (5′-*GATATC*CAGGGGCACCT CGCTCTTGTTGGCC-3′).

The fifth section from nucleotides +667 to +832 was generated by PCR on the long oligonucleotide (L1Mut5; 5′-GATATCTGCACCAGCATCTGCAAGTAC CCCGACTACATCAAGATGGTGAGCGAGCCCTACGGCGACAGCCTGT TCTTCTACCTGCGCCGCGAGCAGATGTTCGTGCGCCACTTGTTCAAC CGCGCCGGCACCGTGGGCGAGAACGTGCCCGACGACCTGTACATC AAGCTTAGC-3′) with L15′-MUTANT5 (5′-*GATATC*TGCACCAGCATCTG C-3′) and L13′-MUTANT5 (5′-*CTCGAG*GCTAAGCTTGATGTACAGGTC-3′) The sixth section from nucleotides +832 to +1000 was generated by PCR on the long oligonucleotide (L1Mut6; 5′-AAGCTTAGCGGCAGCACCGCCAAC CTGGCCAGCAGCAACTACTTCCCCACCCCCAGCGGCAGCATGGTTA CCAGCGACGCCCAGATCTTCAACAAGCCCTACTGGCTGCAGAGGGC CCAGGGCCACAACAACGGCATCTGCTGGGGCAACCAGCTGTTCGTG ACCGTGGTCGAC-3′) with L15′-MUTANT6 (5′-*AAGCTT*AGCGGCAGCA CCGCC-3′) and L13′-MUTANT6 (5′-*CTCGAG*GTCGACCACGGTCACGAA CAG-3′).

The seventh section from nucleotides +1000 to +1182 was generated by PCR on the long oligonucleotide (L1Mut7; 5′-GTCGACACCACCCGCAGCACCA ACATGAGCCTGTGCGCCGCCATCAGCACCAGCGAGACCACCTACAA GAACACCAACTTCAAGGAGTACCTGCGCCACGGCGAGGAGTACGA CCTGCAGTTCATCTTCCAGCTGTGCAAGATCACCCTGACCGCCGAC GTGATGACCTACATCCACAGCATGAATTC-3′) with L15′-MUTANT7 (5′-*G AATTC*GTCGACACCACCCGCAGCACC-3′) and L13′-MUTANT7 (5′-*CTCGA G*GAATTCATGCTGTGGATGTAGG-3′). The eighth section from nucleotides +1182 to +1345 was generated by PCR on the long oligonucleotide (L1Mut8; 5′-ATGAATTCCACCATCCTGGAGGACTGGAACTTCGGCCTGCAGCCCC CCCCCGGCGGCACCCTGGAGGACACCTACCGCTTCGTGACCCAGGCC ATCGCCTGCCAGAAGCACACCCCCCCCGCCCCCAAGGAGGACGACCC CCTGAAGAAGTACACCTTCTGGGAGGTTAAC-3′) with L15′-MUTANT8 (5′-AT*GAATTC*CACCATCCTGGAGG-3′) and L13′-MUTANT8 (5′-*CTCGAG* GTTAACCTCCCAGAAGGTGTAC-3′).

The ninth section from nucleotides +1345 to +1518 was generated by PCR on the long oligonucleotide (L1Mut9; 5′-GTTAACCTGAAGGAGAAGTTCAGC GCCGACCTGGACCAGTTCCCCCTGGGCCGCAAGTTCCTGCTGCAGG CCGGCCTGAAGGCCAAGCCCAAGTTCACCCTGGGCAAGCGCAAGG CCACCCCCACCACCAGCAGCACCAGCACCACCGCCAAGCGCAAGA 3′) with L15′-MUTANT9 (5′-*GTTAAC*CTGAAGGAGAAGTTCAGCG-3′) and L13′-MUTANT9 (5′-AGCGCAAGCTGTAACTCGAG-3′). The PCR fragments were subcloned into *Eco*RV-digested pBluescript KS(−) (Stratagene) or Topo TA vectors (Invitrogen). Fragments 1 to 6 were assembled into pBluescript KS(−) and transferred into the pEGR (32) expression plasmid with *Bss*HII and *Sal*I, generating pEGRL1Mut1-6. Fragment L1Mut7 was transferred to pEGRL1Mut1-6 with *Sal*I and *Eco*RI, generating pEGRL1Mut1-7. Fragments 8 and 9 were transferred to pEGRL1Mut1-7 with *Eco*RI and *Xho*I, generating pNLL1(1-9). pNLL1(1-3) was generated by digestion of pNLL1 (31) and pNLL1(1-9) with *Bam*HI and *Xho*I, followed by replacement of the mutant L1 sequence with wild-type L1 sequence. The mutant L1 sequences were transferred into pBluescript KS(−) as *Sal*I-*Xho*I fragments, resulting in T7L1(1-3) and pT7L1(1-9), which contain the mutant L1 genes after the bacteriophage T7 promoter.

The following plasmids have been described previously: pCL125EIAV75 (5), pCEIAV25L175 (5), pCEIAV (5), pT7CAT (4), pCTAT (5), and pC16L1 (5), pCL1MUT was previously described as pC16L1MUTANT123 (5), p16L1CTE (31), p16L1 (31), and pH16L2 (28).

Cells and transfections. Transfections were performed with HeLa or HeLa-tat cells according to the Fugene 6 method (Roche Molecular Biochemicals). Briefly, 1 μg of DNA was transfected in combination with 3 μl of Fugene 6 and added in 200-μl aliquots consisting of DNA, Fugene 6, and medium to 60-mm plates containing subconfluent HeLa or HeLa-tat cells. Then 1 μg of pCTAT (5) or pDKX (32) was included as an internal control for transfection efficiency. The transfected cells were harvested at 24 h posttransfection. All plasmids shown in the figures have been transfected at least three times at different occasions and have produced similar results.

RNA extraction and Northern blotting. Cytoplasmic RNA extraction was performed as previously described (34), and total RNA was prepared according to the RNeasy Mini protocol (Qiagen). Northern blot analysis was performed by the separation of 5 μg of total or cytoplasmic RNA on a 1% agarose gel containing 2.2 M formaldehyde, followed by transfer to a nitrocellulose or nylon filter and hybridization. Random priming of the DNA probe was performed with a Decaprime kit (Ambion) according to the manufacturer's instructions. The DNA template used was generated by digestion of the pCL086 vector with *Sac*I and *Sal*I, thereby releasing a 133-nucleotide cytomegalovirus sequence (5).

3′ rapid amplification of cDNA ends. Total RNA was reverse transcribed at 42°C for 1 h in a total volume of 25 μl with oligodTGC (5′-GCGAGCTCCGC GGCCGCGTTTTTTTTTTTT-3′), as previously described (33). The reaction without reverse transcriptase was performed in parallel and served as a control for the absence of plasmid DNA contamination. A 5-μl aliquot of cDNA product was PCR amplified in a 100-μl reaction volume with oligonucleotides oligodTGC and L1start (5), which hybridized to the 5′ end of the L1 coding sequence. The amplified products were cloned into a Topo TA vector (Invitrogen) and subjected to sequencing.

In vitro translation, Western blotting, immunoprecipitation, and indirect immunofluorescence. In vitro translation was performed in the coupled transcription-translation rabbit reticulocyte lysate system (Promega) according to the manufacturer's instructions. Labeling and radioimmunoprecipitation of transfected cells were performed as described previously (31). Western blot analysis was performed as described previously (33). Guinea pig and rabbit anti-L2 peptide antisera and rabbit anti-L1 antiserum were generously provided by J. Dillner (8). The guinea pig anti-L2 peptide serum was used at a dilution of 1:2,000 in immunoblots, and the rabbit anti-L2 peptide serum was used at a dilution of 1:50 in indirect immunofluorescence and in radioimmunoprecipitation. Indirect immunofluorescence was performed on transfected formaldehyde fixed cells.

RESULTS

Localization of two inhibitory regions in HPV-16 L2 designated inhibitory regions I and II. We are interested in the posttranscriptional regulation of HPV-16 late gene expression, and we have previously mapped inhibitory, regulatory RNA

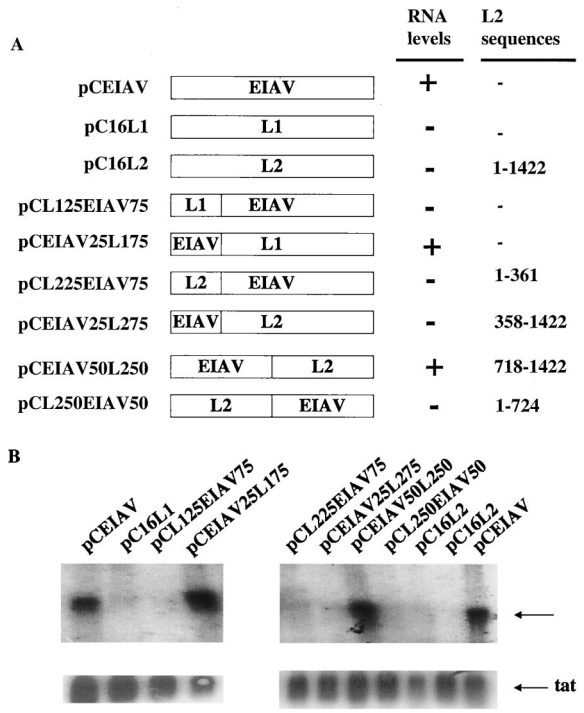

FIG. 2. (A) Structures of the hybrids between the HPV-16 L2 and the EIAV *gag* genes expressed from the cytomegalovirus promoter-driven expression plasmids. A summary of the RNA levels is shown in B. The L2 sequences present in the plasmids are shown to the right. Numbering starts at the A in the L2 ATG. (B) Northern blotting of RNA extracted from HeLa cells transfected with the indicated hybrid genes shown in panel A under the control of the cytomegalovirus promoter. The probe spans the first 72 transcribed nucleotides of the cytomegalovirus promoter that are included in all mRNAs. tat, *tat* mRNAs produced by the internal control pCTAT (5).

sequences in the 5′ end of the L1 coding sequence (Fig. 1) (5). We used hybrids of the efficiently expressed equine infectious anemia virus (EIAV) *gag* gene and HPV-16 L1 to map RNA elements in the HPV-16 L1 coding region (5). The EIAV *gag* gene was chosen because it expresses high mRNA levels in transfected cells and, like the late HPV-16 genes, encodes a viral capsid protein with a predicted molecular mass of 55 kDa.

Here we made hybrids between EIAV *gag* and HPV-16 L2 to map negative elements in the L2 open reading frame (Fig. 2A). These hybrids were inserted downstream of the cytomegalovirus promoter as described in Materials and Methods section and transfected into HeLa cells. Cytoplasmic RNA was extracted and analyzed by Northern blotting. As expected, plasmid pCEIAV expressed high mRNA levels, whereas the HPV-16 L1- and L2-expressing plasmids pC16L1 and pC16L2 failed to produce detectable mRNA levels (Fig. 2A and B) (5). All plasmids shown in the figures have been transfected at least three times on different occasions and have produced similar results.

The results revealed that plasmid pCL225EIAV75 produced undetectable mRNA levels (Fig. 2A and B). We concluded that the first 361 nucleotides of the HPV-16 L2 gene contained inhibitory sequences, designated inhibitory region I. These results were similar to those we obtained previously with HPV-16 L1, which demonstrated the presence of inhibitory sequences in the first 367 nucleotides of L1 (see pCL125EIAV75) (Fig. 2A and B) (5). However, when the 3′ end (nucleotides 358 to 1422, genomic positions 4594 to 5658) of the L2 gene was fused to EIAV, the plasmid (pCEIAV25L275) (Fig. 2A) produced undetectable mRNA levels, demonstrating the presence of inhibitory sequences in the 3′ end too (Fig. 2B). The inhibitory sequences in the 3′ end of L2 were termed inhibitory region II. The presence of inhibitory sequences in the 3′ end of the coding sequence is in contrast to the results we obtained with the L1 gene of HPV-16 (5).

The L1 coding sequence did not contain negative elements in the 3′ end (nucleotides 367 to 1518, genomic positions 6005 to 7156) of the gene (see pCEIAV25L175) (Fig. 2B) (5). Plasmid pCEIAV50L250 (Fig. 2A), encoding the hybrid between the 5′ half of the EIAV gene and the 3′ half (718 to 1422, genomic positions 4954 to 5658) of the L2 gene, expressed high mRNA levels (Fig. 2B), suggesting that the major negative elements in HPV-16 L2 were located in the 5′ 718 nucleotides of the L2 coding sequence. This was confirmed by the observance of undetectable mRNA levels by pCL250EIAV50 (Fig. 2A and B). Alternatively, the inhibitory region spans the junction between L2 and EIAV and was therefore inactivated as a result of the fusion. In conclusion, the L2 and L1 coding sequences are different in that multiple inhibitory elements were present in L2. Inhibitory region I was located in the first 361 nucleotides of L2, and inhibitory region II was located between nucleotide positions 358 and 1422.

Mutational inactivation of inhibitory region I. We have shown in earlier experiments that introduction of point mutations that altered the RNA sequence but not the protein sequence in the first 514 nucleotides of HPV-16 L1 induced production of L1 protein (5). Therefore, the first 533 nucleotides of L2 were mutated in a similar manner (for the exact mutant sequence of the first 533 nucleotides of L2, see Fig. 4A). Nucleotides 1 to 533 (genomic positions 4237 to 4769) of both the wild-type and mutant L2 sequences were fused to the 3′ end of HPV-16 L1 (nucleotides 514 to 1518, genomic positions 6152 to 7156), which lacks inhibitory activity, resulting in pCL2L1 and pCL2ML1, respectively (Fig. 3A). Plasmid pCL2ML1 produced high mRNA levels, whereas mRNAs produced from pCL2L1 were undetectable (Fig. 3B), confirming the presence of inhibitory elements in the 5′ end of the L2 coding sequence and demonstrating that these were functionally inactivated by the introduced mutations. The additional lower band seen in cells transfected with pCL2ML1 and pCL1MUT is the product of the use of a cryptic polyadenylation signal 50 nucleotides downstream of nucleotide position 520 (genomic position 6158) in the HPV-16 L1 gene (for a detailed description, see Fig. 7).

Fusion of the first 520 nucleotides of the L1 mutant sequence to the wild-type L2 sequence (nucleotides 534 to 1422, genomic positions 4770 to 5658) (Fig. 3A) resulted in production of detectable mRNA levels, although lower levels than those observed with the L1 mutant pCL1MUT (Fig. 3B), demonstrating that the L2 sequences between 534 and 1422 (genomic positions 4770 to 5658) displayed weak inhibitory activity (Fig. 3B). Similar results were obtained when the mutant first 533 nucleotides of L2 were fused to wild-type L2 sequences spanning 534 to 1422 (genomic positions 4770 to 5658), as in pC5-10 (Fig. 3A and C). Previous experiments with

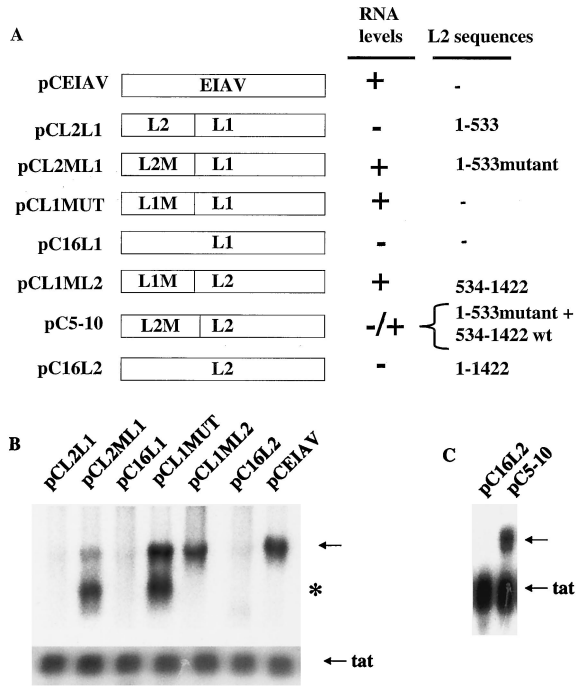

FIG. 3. (A) Structures of the hybrids between the HPV-16 L2 and L1 wild-type and mutant genes expressed from the cytomegalovirus promoter-driven expression plasmids. A summary of the RNA levels is shown to the right. The L2 sequences present in the plasmids are indicated. Numbering starts at the A in the L2 ATG. (B) Northern blotting of RNA extracted from HeLa cells transfected with the indicated hybrid genes shown in panel A under control of the cytomegalovirus promoter. tat, *tat* mRNAs produced by the internal control pCTAT (5). * indicates the position of a short transcript that was prematurely polyadenylated in the L1 coding sequence (see also Fig. 7). (C) Northern blotting of RNA extracted from HeLa cells transfected with the indicated plasmids.

the L2-EAIV hybrids showed that the 367-to-1422 (genomic positions 4603 to 5658) sequence of L2 efficiently reduced mRNA levels when fused to EIAV (Fig. 2B). We concluded that the 5′ boundary of inhibitory region II is located between nucleotide positions 367 and 533 (genomic positions 4603 to 4769). Further hybrids between wild-type and mutant L2 sequences were required to map the location of the inhibitory regions.

Generation and analysis of a complete mutant HPV-16 L2 sequence. In order to study the multiple L2 elements further, we first generated a full L2 mutant with long, overlapping oligonucleotides (see Materials and Methods). The mutations did not change the protein coding sequence but replaced 85% of the codons and lowered the AU content of the L2 mRNA (Fig. 4A). Care was taken not to introduce rare codons. Plasmid pCL2MUT, encoding the full L2 mutant, produced high levels of L2 mRNA and protein in transfected HeLa cells (Fig. 4B and C). Fractionation of transfected cells and immunofluorescence revealed that the majority of the L2 protein was found in the nucleus (Fig. 4D and E). These results demonstrated that the negative elements had been inactivated. The L1 protein produced from mutant L1 mRNA (5) expressed from the cytomegalovirus promoter was found in the nucleus (Fig. 4E). L2 localized to punctate regions in the nucleus and induced relocation of L1 to the same regions (Fig. 4E), demonstrating that the L1 and L2 sequences interacted in the cellular nuclei. These results are in agreement with the results of Day et al., who demonstrated that bovine papillomavirus type 1 L2 induced a relocation of L1 to promonocytic leukemia protein oncogenic domains (PODs) (7).

Mapping and functional inactivation of inhibitory regions I and II in HPV-16 L2. Next, the full mutant was used to map and characterize the inhibitory RNA elements in the L2 coding sequence with the help of hybrids between the wild-type and mutant L2 sequences. The wild-type and mutant L2 sequences were divided into 10 different regions, each separated by unique restriction sites (Fig. 5A and 6E). To map the inhibitory regions, hybrids between the highly expressed mutant and wild-type L2 were constructed (Fig. 5A and 6A).

The sequences in fragments 1 and 2 were replaced with mutant sequences, resulting in pC3-10 (Fig. 5A). This plasmid produced higher mRNA levels than pC16L2 (Fig. 5B), demonstrating that inhibitory sequences had been inactivated. However, the mRNA levels were lower than those produced by pCL2MUT (Fig. 5B), indicating that not all inhibitory sequences were affected. Plasmid pC4-10 produced slightly higher mRNA levels than pC3-10, whereas pC5-10 and pC7-10 produced mRNA levels similar to those produced by pCL2MUT (Fig. 5A and B). Therefore, an inhibitory element was spanning fragments 1 and 2 and a second inhibitory element was spanning fragments 3 to 6. The quantified mRNA levels are shown in Fig. 5C. These results also excluded the presence of inhibitory elements that reduced mRNA levels in fragments 7 to 10.

Plasmid pC12 (Fig. 5A), which contained wild-type sequences in fragments 1 and 2 and mutant sequences in fragments 3 to 10, produced lower mRNA levels than the full mutant pCL2MUT (Fig. 5B and C). These results confirmed the presence of inhibitory sequences in fragments 1 and 2 and the presence of additional inhibitory sequences downstream of fragment 2. Plasmid pC1-6, which contained wild-type L2 sequences in fragments 1 to 6 (Fig. 5A), produced undetectable mRNA levels (Fig. 5B and C). Plasmid pC1-8 produced undetectable mRNA levels, as expected (Fig. 5B and C). Since the mRNA levels produced by pC1-6 and pC1-8 were lower than those produced by pC12 (Fig. 5B and C), the results confirmed the presence of two inhibitory elements in the first six fragments of L2. In addition, the presence of both elements was required for the dramatic reduction in L2 mRNAs levels seen with the wild-type L2 sequence and with pC1-6 and pC1-8.

The production of L2 protein from the various hybrids was also monitored in the transfected cells. Plasmids pC7-10 and pC910, in which fragments 1 to 6 and 1 to 8 were mutated, respectively, produced high levels of L2 protein (Fig. 6C), whereas the remaining plasmids failed to produce detectable levels of L2 protein (data not shown). These results confirmed the presence of inhibitory sequences in fragments 1 to 6 of L2 and the absence of major inhibitory sequences in fragments 7 to 10 in the 3′ end of L2. Furthermore, the results also demonstrated that the specific mutational inactivation of the inhibitory elements in the first six fragments of L2 resulted in the production of L2 protein in human cells.

A second set of hybrids with overlapping, internal regions of

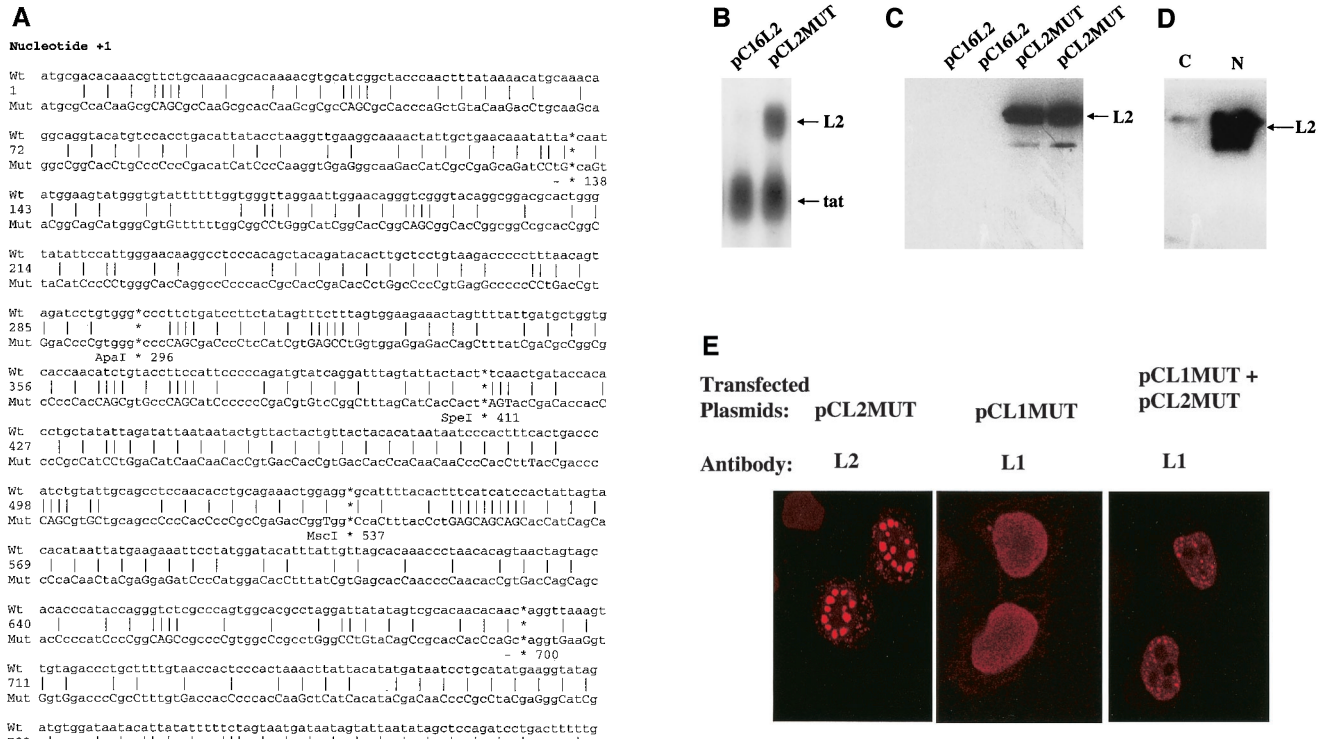

FIG. 4. (A) Sequence of the wild-type and mutant L2 coding regions. * indicates the borders of the segments shown in Fig. 6E. Restriction sites used for subcloning are shown. (B) Northern blotting of RNA extracted from HeLa cells transfected with pC16L2 and pCL2MUT, which encode the wild-type and mutant HPV-16 L2, respectively, under control of the cytomegalovirus promoter. tat, *tat* mRNAs produced by the internal control pCTAT (5). (C) Western immunoblot of cell extracts from HeLa cells transfected with pC16L2 or pCL2MUT in duplicate. (D) Western immunoblot of cytoplasmic (C) and nuclear (N) extracts from HeLa cells transfected with pCL2MUT. (E) Immunofluorescence of HeLa cells transfected with plasmids pCL2MUT and pCL1MUT (5). Fixed cells were stained with antibodies against HPV-16 L1 or L2, as described in Materials and Methods.

wild-type sequences were generated (Fig. 6A). Analysis of these hybrids by Northern blotting revealed that pC12 produced lower levels of L2 mRNA than the full mutant L2 sequence in pCL2MUT (Fig. 6A and B), as expected. Surprisingly, the remaining hybrids in this series all produced high mRNA levels (Fig. 6A and B), supporting the idea that the presence of two inhibitory RNA elements was required in order to cause a substantial reduction of L2 mRNA levels. In addition, these results support a role of the inhibitory L2 sequences in translation. To investigate this further, we monitored production of L2 protein by the same hybrids in transfected cells.

Plasmid pC12, in which the first two fragments were wild-type L2 sequences, produced very low levels of L2 protein (Fig. 6C). The CAT-producing plasmid pDKX (32) was included as an internal control (data not shown). Fragments 3 and 4 in pC34 (Fig. 6A) did not display inhibitory activity and produced high levels of L2 protein (Fig. 6C). A second element was mapped to fragments 5 and 6 because none of the plasmids pC3-6, pC5-8, and pC56 produced detectable levels of L2 (Fig. 6C). In contrast, pC78, pC7-10, and pC910 produced high levels of L2 and therefore did not contain intact negative elements (Fig. 6C). Quantitation of L2 mRNA and protein levels, shown in Fig. 6B and C, showed that the mRNA levels of hybrids that contained sections 5 and 6 of the wild-type L2 sequence were reduced less than 2-fold, whereas L2 protein levels were reduced 20-fold (Fig. 6D). We concluded that pC12 contained the upstream inhibitory element inhibitory region I and that pC56 contained the second, downstream negative element termed inhibitory region II (Fig. 6E). In addition,

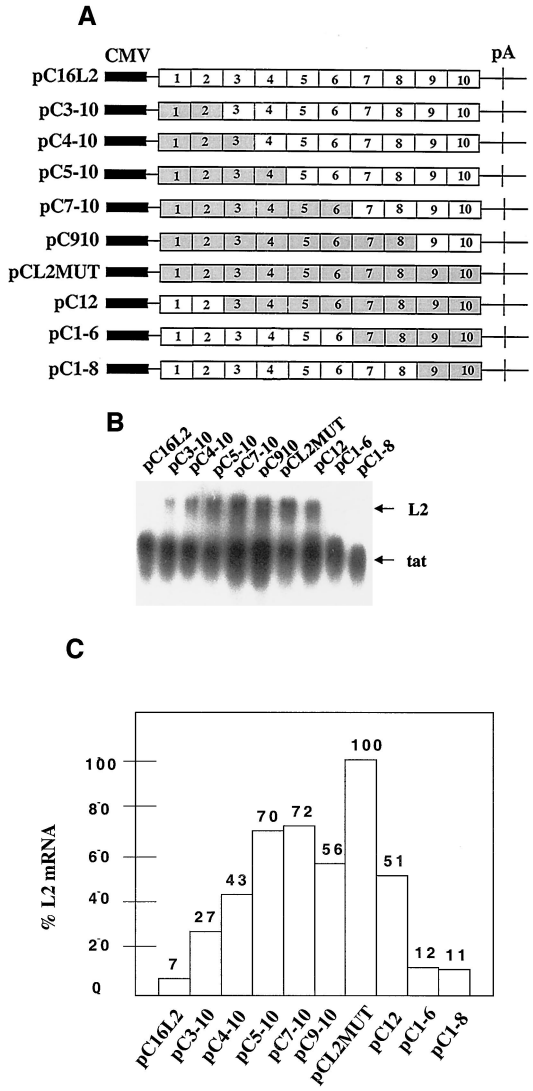

FIG. 5. (A) Schematic structures of the hybrids between the HPV-16 L2 wild-type and mutant genes. Plasmid names are shown to the left. White boxes indicate wild-type L2 sequences, and grey boxes indicate mutant L2 sequences. (B) Northern blotting of RNA extracted from HeLa cells transfected with the indicated plasmids. tat, *tat* mRNAs produced by the internal control pCTAT (5). (C) The levels of the L2 mRNAs were quantified and normalized to the internal control mRNA. The normalized L2 mRNA level produced by pCL2MUT was set at 100%.

strong negative elements were absent from fragments 7 to 10 in the 3′ end of L2.

A comparison of the RNA data in Fig. 6B and the protein data in Fig. 6C revealed that the element in fragments 1 and 2 had a small but significant effect on mRNA levels (compare pC12 and pCL2MUT [Fig. 6B]). The second element spanning fragments 5 and 6 also displayed a small but significant effect on mRNA levels, whereas the two elements combined resulted in dramatically reduced L2 mRNA levels (see pC1-6 [Fig. 5B]). This is in agreement with our previous observations that the first 800 nucleotides of the L2 sequence had an RNA-destabilizing function (28). In addition, the inhibitory sequences in fragments 1 and 2 and fragments 5 and 6 also efficiently inhibited translation of the L2 mRNAs. For example, compare the difference in mRNA and protein levels between pC12 and pC56 on one hand and pC7-10 and pC78 on the other hand (Fig. 6B and C). The effect on mRNA use confirmed the inhibitory effect on translation by intragenic L2 elements previously reported by our laboratory (4). All L2 hybrids were in vitro translated in rabbit reticulocytes to confirm that they had the potential to express a full-length L2 protein. Although the L2 coding sequence is more complex than the L1 coding sequence in that it contains multiple inhibitory sequences, the specific inactivation of the two negative elements, such as in pC7-10, resulted in efficient L2 protein production and clearly demonstrated that distinct RNA elements inhibit expression of L2 in mammalian cells.

Partial and full mutants of the HPV-16 L1 capsid gene produce similar levels of L1 protein, demonstrating that specific inactivation of negative RNA elements alleviates inhibition and results in high expression of L1. Our data showed that both L1 and L2 contained negative elements in the first 520 and 533 nucleotides, respectively, of the coding sequence. Introduction of point mutations that altered the RNA but not the protein sequence in the first 520 nucleotides of L1 resulted in high levels of L1 protein (5), whereas similar mutations in the first 533 nucleotides in L2 did not induce production of detectable levels of L2. Mutational inactivation of inhibitory regions I and II in tandem resulted in the production of high levels of L2 protein.

In the next experiment we wished to investigate if the introduction of point mutations that altered the RNA but not the protein sequence of the L1 sequence downstream of nucleotide position 520 further increased L1 production from pNLL1(1-3), in which L1 sequences up to position 520 were mutated (Fig. 7B). The entire L1 sequence was mutated without altering the L1 protein sequence (Fig. 7A). The full L1 mutant was named pNLL1(1-9) (Fig. 7B), and the sequence is shown in Fig. 7A. The results revealed that this mutant produced higher levels of L1 protein than pNLL1(1-3) (Fig. 7C), which was only mutated up to position 520. However, looking at the mRNA levels produced from the two mutants, we found that the major species produced from pNLL1(1-3) was substantially shorter than the full-length L1 mRNA (Fig. 7D).

Inspection of the sequence revealed a potential polyadenylation signal (AAUAAA) downstream of position 520. With the 3′-rapid amplification of cDNA ends (RACE) method, we showed that this polyadenylation signal was used in cells transfected with pNLL1(1-3) but not with pNLL1(1-9) (Fig. 7E). Cloning and sequencing of the p16L1MUT-specific reverse transcription-PCR product mapped the cleavage site (Fig. 7F). A quantitative analysis of L1 mRNA and protein levels revealed that the mRNAs produced by pNLL1(1-3) and pNLL1(1-9) were used with the same efficiency to produce L1 protein. This was further supported by the results obtained with an in vitro translation system, in which both mutant mRNAs were subjected to in vitro translation and shown to produce similar protein levels (Fig. 7G). We concluded that the mutations introduced in the first 520 nucleotides of L1 inactivated regulatory RNA sequences, which resulted in higher RNA levels and as a result higher L1 protein levels.

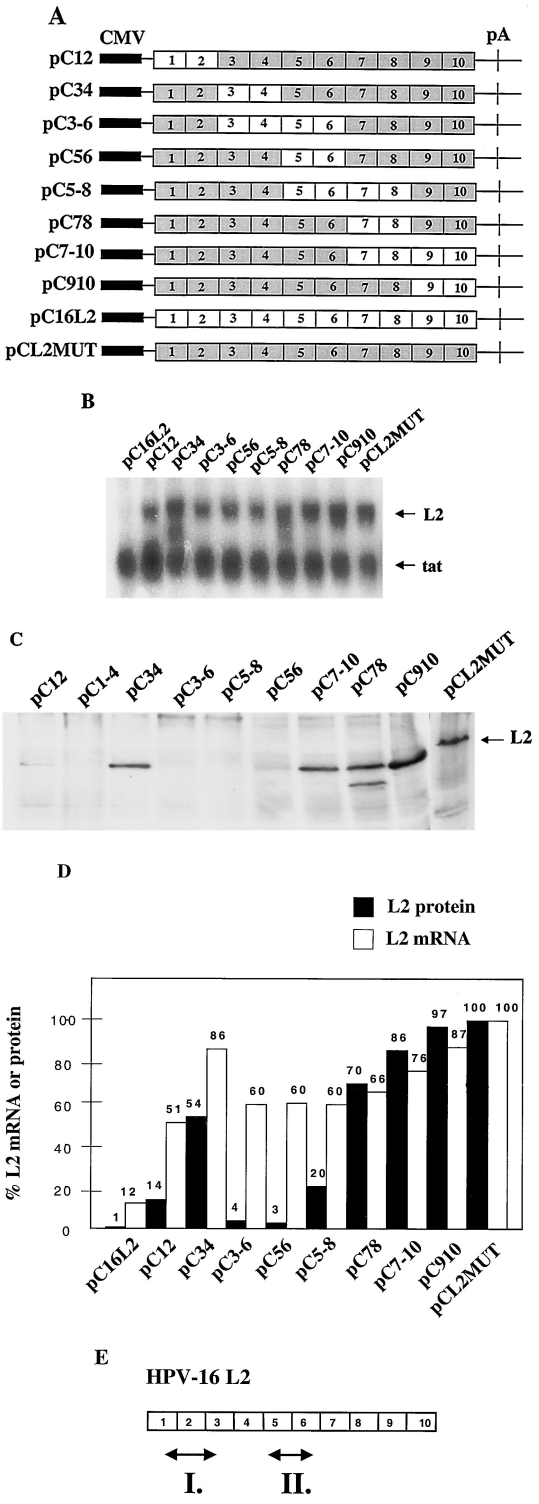

FIG. 6. (A) Schematic structures of the hybrids between the HPV-16 L2 wild-type and mutant genes. Plasmid names are shown to the left. White boxes indicate wild-type L2 sequences, and grey boxes indicate mutant L2 sequences. (B) Northern blotting of RNA extracted from HeLa cells transfected with the indicated plasmids. tat, *tat* mRNAs produced by the internal control pCTAT (5). (C) Western immunoblot of cell extracts from HeLa cells transfected with the indicated plasmids. (D) The levels of the L2 mRNAs were quantified and normalized to the internal control mRNA. The normalized L2 mRNA level produced by pCL2MUT was set at 100%. The normalized L2 protein levels were quantified, and the L2 protein level produced by pCL2MUT was set at 100%. White bars, L2 mRNA levels; black bars, L2 protein levels. (E) Two inhibitory regions termed inhibitory regions I and II were identified with the L2wt/L2MUT hybrids.

CTE of simian retrovirus type 1 overcomes the effect of the negative elements in HPV-16 L1 but not L2. We have previously shown that human immunodeficiency virus type 1 (HIV-1) Rev and RRE and simian retrovirus type 1 CTE induced HPV-16 L1 production in transfected cells (31) (Fig. 8B). The CTE was inserted downstream of the L2 gene driven by the HIV-1 long terminal repeat promoter (Fig. 8A), and the plasmids were transfected into HeLa-tat cells. However, CTE did not overcome the inhibition exerted by the negative elements in L2 (Fig. 8B), whereas high levels of L1 were produced, as expected (Fig. 8B), indicating that the negative elements in L1 and L2 act by different mechanisms.

DISCUSSION

Some regulatory sequences are DNA sequences, whereas many of the sequences that regulate late gene expression must be RNA sequences that induce or inhibit various RNA processing steps to ensure an ordered expression of the L1 and L2 proteins in the viral life cycle. Since all sequences outside of the long control region with the exception of the early untranslated region, are protein coding, many regulatory RNA sequences are likely to overlap protein coding sequences. We have previously shown that the L1 and L2 sequences contain RNA elements that mediate RNA degradation. This is consistent with the results obtained here, which showed that the wild-type L2 expression plasmid produced undetectable mRNA levels, whereas the mutant L2 sequence produced high mRNA levels. RNA instability elements in the L1 and L2 ORFs may function to reduce the late mRNA half-lives to prevent untimely production of the L1 and L2 in infected proliferating cells. The mRNAs would then be stabilized in terminally differentiated cells, and as a result, L1 and L2 proteins and virions would be produced in the superficial layers of the epithelium, as discussed in detail previously (5, 24, 26, 31).

No data are presented to suggest that the inhibitory sequences described in the paper are involved in the differentiation-dependent regulation of L2 expression. The effect of the L2 inhibitory sequences may be a result of reduced mRNA stability. The RNA elements in L2 may form secondary structures that may affect the RNA polymerase or the processing of the RNA. Alternatively, inhibition of the transcribing RNA polymerase or binding and sequestering of essential transcription factors by the L2 RNA elements may occur. Alternatively, the sequences may mediate RNA instability as a result of the interaction of the L2 RNA sequences with cellular factors that cannot execute their functions when the L2 mRNAs are not expressed in the context of the whole genome, for example, splicing and polyadenylation factors.

The Rev-RRE and TAP-CTE interactions may compete with the factors binding to the HPV-16 regulatory RNA sequences. HIV-1 Rev and RRE can overcome the effect of the inhibitory RNA elements in the L1 coding region (31). Since it has been speculated that HIV-1 Rev and RRE inhibit splicing

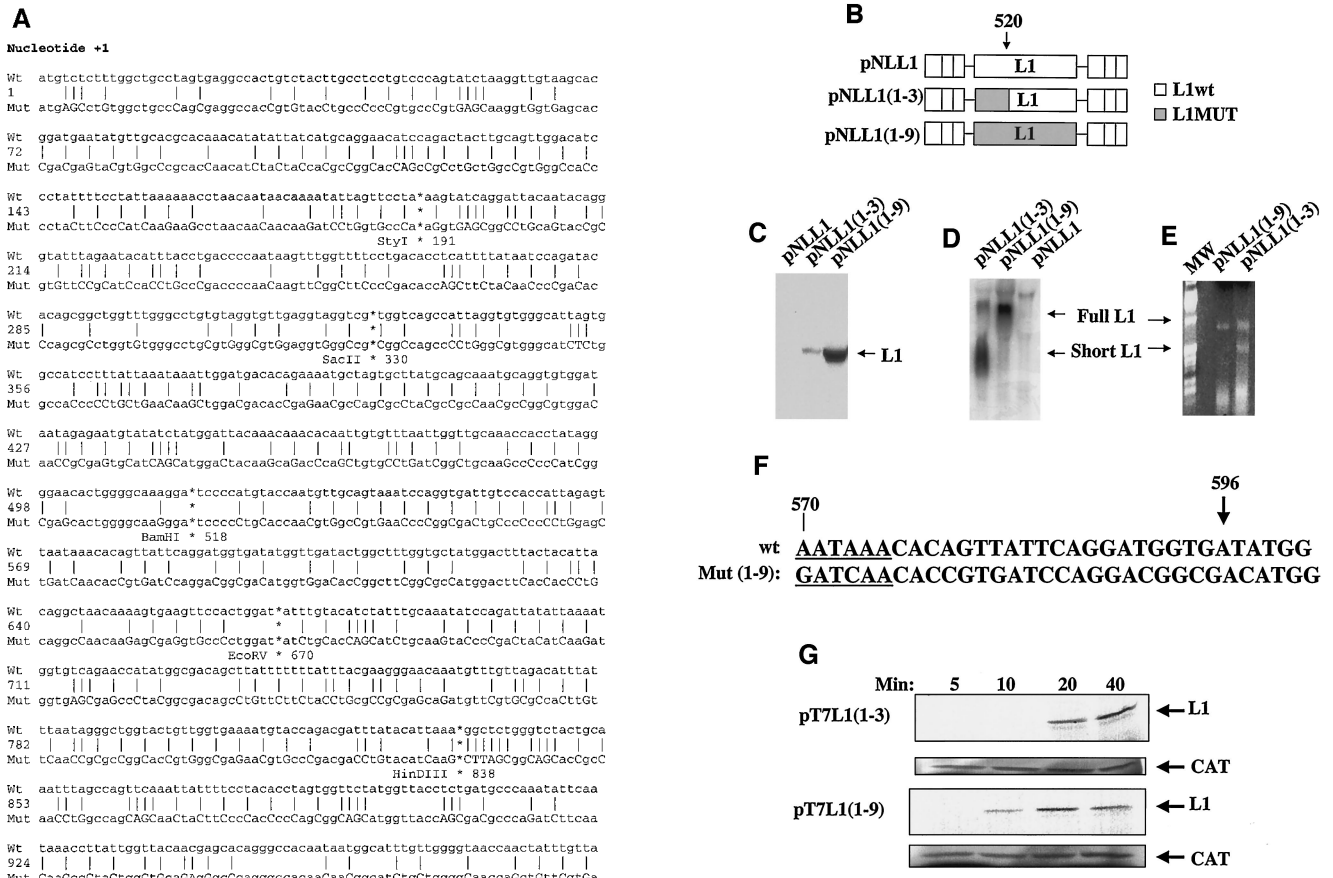

FIG. 7. (A) Sequence of the wild-type L1 gene and the full HPV-16 L1 mutant. * indicates the borders of the nine long oligonucleotides used for the generation of the L1 mutant gene. Restriction sites used for subcloning are shown. (B) Schematic structures of the eukaryotic expression plasmids containing the HPV-16 wild-type L1 gene (31), the L1 mutant 1-3 (5), and the L1 mutant 1-9 gene. White bars indicate wild-type and grey bars indicate mutant L1 sequences. The various L1 genes were under the control of the HIV-1 long terminal repeat promoter. (C) Western immunoblot of cell extracts from HeLa-tat cells transfected with the indicated plasmids. (D) Northern blotting of RNA extracted from HeLa-tat cells transfected with the indicated plasmids. The additional short L1 mRNA is indicated. (E) 3′-RACE on mRNA extracted from HeLa-tat cells transfected with pNLL1(1-9) or pNLL1(1-3). The amplification product of the short, prematurely polyadenylated mRNA and the full-length L1 mRNA is indicated. Lane MW, size markers. (F) The 3′-RACE product representing the short, prematurely polyadenylated mRNA shown in E was cloned and sequenced. This resulted in the mapping of the cleavage site to position 596 (numbering starts at the A of the L1 ATG). Both wild-type and mutant L1 sequences are shown. (G) In vitro translation of the mutant L1 genes pT7L1(1-3) and pT7L1(1-9) under control of the bacteriophage T7 promoter. Aliquots were taken at different time points and loaded on an acrylamide gel. A T7-CAT plasmid was included as an internal control (4). The CAT levels are shown below. Min, minutes of in vitro translation.

and promote nuclear export of mRNAs that would normally be spliced (3), one may speculate the inhibitory sequences in the HPV-16 L1 coding region interact with splicing factors. However, Rev and RRE also stabilize HIV-1 mRNAs encoding the HIV-1 capsid proteins (11), and it is therefore equally plausible that Rev-RRE acts by overcoming the destabilizing effects of the intragenic L1 RNA instability elements. HIV-1 Rev and RRE can override the effect of papillomavirus negative regulatory RNA sequences, as has been shown previously for HPV-1 (33), HPV-16 (31), and bovine papillomavirus type 1 (2). However, others have failed to see an effect of Rev/RRE or CTE (19). Although in the latter case, the complete absence of RNA analysis of the expression levels prevented a more detailed analysis of the data, it is likely that the failure to detect L1 protein may be a result of variations in transfection efficiencies or expression levels of L1 or Rev.

In our experiments, production of L2 could not be induced

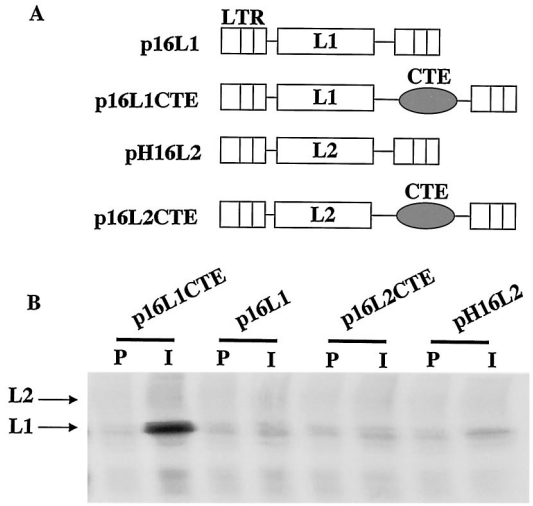

FIG. 8. (A) Schematic structures of the eukaryotic expression plasmids containing the HPV-16 wild-type L1 or L2 gene in the absence and presence of the simian retrovirus type 1 CTE. The L1 and L2 genes were under control of the HIV-1 long terminal repeat promoter. Plasmid names are shown to the left. (B) Immunoprecipitation of HPV-16 L1 and L2 protein in metabolically [^{35}S]methionine-labeled HeLa-tat cells transfected with the indicated plasmids. P, preimmune serum; I, HPV-16 L1- or L2-specific antipeptide antiserum.

by Rev/RRE or CTE, indicating that the inhibitory RNA sequences in the L2 coding region interact with factors other than the L1 inhibitory sequences, as mentioned earlier, and suggesting that the elements in L1 and L2 may function by different mechanisms and have different functions in the viral life cycle. This is supported by the observed differences between the L1 and L2 elements. In L1, one major inhibitory element was mapped to the immediate 5′ end of L1 (5, 31), whereas in L2, multiple elements spanning a relatively large part of L2 were identified. Furthermore, the inhibitory effect of the element in L1 correlated with a decrease in mRNA levels, while the L2 elements affected both mRNA levels and translation. The effect on translation is in agreement with previous results reported on bovine papillomavirus type 1 L2 (35). Therefore, the distantly related bovine papillomavirus type 1 and HPV-16 L2 sequences contain RNA elements that inhibit translation. Both HPV-16 and bovine papillomavirus type 1 have evolved to encode L2 mRNAs with low translation efficiency. However, the mechanism of inhibition of translation is different in the two distantly related papillomaviruses. In addition, the HPV-16 L2 coding sequence contains RNA elements that reduce the mRNA half-life (28).

The introduction of the point mutations in HPV-16 L1 that inactivated the inhibitory elements and induced high expression levels of L1 mRNA also revealed the existence of a functional polyadenylation signal in the L1 open reading frame. The polyadenylation signal is used relatively efficiently (approximately 70%). A TGGTT and a TGGCTTTGGTG sequence are located downstream of the polyadenylation signal and the cleavage sites and may serve as a G/U-rich downstream element. In the viral life cycle, the polyadenylation signal could possibly be used to generate L2 mRNAs that lack most of the L1 sequences and are not polyadenylated at the late polyadenylation signals downstream of L1. To our knowledge, polyadenylation signals have not been identified in L1 in any other HPV type. However, we do not know if this newly identified polyadenylation signal in L1 is used in infected cells.

The results presented here and in a previous article by our group (5) demonstrated that the introduction of point mutations that affect the function of the inhibitory RNA elements induces production of L1 and L2. The presence of these distinct, inhibitory RNA elements explains the lack of L1 and L2 production from the HPV-16 L1 and L2 coding sequences. Others have changed rare codons in the HPV-16 L1 and L2 genes and obtained production of L1 and L2 protein in human cells (19). These authors speculated that the induction of L1 and L2 production was the result of an increase in translation due to an "optimization" of the mRNA coding sequence (19). However, since no RNA results were presented in their article, the results were inconclusive regarding the function of the elements.

Since the tropism of HPV-16 limits infection to humans and HPV-16 is one of the most common sexually transmitted HPV types, it appears reasonable to assume that the late papillomavirus genes are optimized for expression in human cells. In terminally differentiated cells, L1 and L2 mRNAs are expressed and are efficiently translated into high levels of L1 and L2 protein. The HPV-16 L1 and L2 mRNAs contain rare codons, but so do many cellular mRNAs. From an evolutionary point of view, it is also clear that HPV-16 has been optimized for replication and expression in human cells, since no other host is known for HPV-16. A more likely explanation for the low expression levels of the HPV-16 L1 and L2 genes from subgenomic expression plasmids is therefore that HPV-16 has evolved to contain distinct regulatory RNA elements that are necessary for an ordered and highly regulated late gene expression, RNA sequences that probably contributed to the successful establishment of HPV-16 in the human population (24–26). Interestingly, "codon-optimized" early papillomavirus genes also display enhanced expression levels (9, 20), and it would be interesting to investigate the mechanisms behind the improved protein production from these mutants. The elucidation of the functions of the regulatory RNA elements in L1 and L2 will be important, and a comparison of the elements and their function in various HPV types may be informative.

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REFERENCES

1. **Baker, C. C.** 1997. Posttranscriptional regulation of papillomavirus gene expression, p. 11–16. *In* S. R. Billakanti, C. E. Calef, A. D. Farmer, A. L. Halpern, and G. L. Myers (ed.), Human papillomaviruses: a compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, N.Mex.
2. **Barksdale, S. K., and C. C. Baker.** 1995. The human immunodeficiency virus type 1 Rev protein and the Rev-responsive element counteract the effect of an inhibitory 5′ splice site in a 3′ untranslated region. Mol. Cell. Biol. **15:**2962–2971.
3. **Chang, D. D., and P. A. Sharp.** 1990. Messenger RNA transport and HIV rev regulation. Science **249:**614–615.
4. **Collier, B., L. Goobar-Larsson, M. Sokolowski, and S. Schwartz.** 1998.

Translational inhibition in vitro of human papillomavirus type 16 L2 mRNA mediated through interaction with heterogenous ribonucleoprotein K and poly(rC)-binding proteins 1 and 2. J. Biol. Chem. **273:**22648–22656.
5. **Collier, B., D. Öberg, X. Zhao, and S. Schwartz.** 2002. Specific inactivation of inhibitory sequences in the 5′ end of the human papillomavirus type 16 L1 open reading frame results in production of high levels of L1 protein in human epithelial cells. J. Virol. **76:**2739–2752.
6. **Cumming, S. A., C. E. Repellin, M. McPhilips, J. C. Redford, J. B. Clements, and S. V. Graham.** 2002. The human papillomavirus type 31 untranslated region contains a complex bipartite negative regulatory element. J. Virol. **76:**5993–6003.
7. **Day, P. M., R. B. S. Roden, D. R. Lowy, and J. T. Schiller.** 1998. The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. J. Virol. **72:**142–150.
8. **Dillner, L., P. Heino, J. Moreno-Lopez, and J. Dillner.** 1991. Antigenic and immunogenic epitopes shared by human papillomavirus type 16, bovine, canine, and avian papillomaviruses. J. Virol. **65:**6862–6871.
9. **Disbrow, G. L., I. Sunitha, C. C. Baker, J. Hanover, and R. Schlegel.** 2003. Codon optimisation of the HPV-16 E5 gene enhances protein expression. Virology **311:**105–114.
10. **Dollard, S. C., J. L. Wilson, L. M. Demeter, W. Bonnez, R. C. Reichman, T. R. Broker, and L. T. Chow.** 1992. Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. Genes Dev. **6:**1131–1142.
11. **Felber, B. K., M. Hadzopoulou-Cladaras, C. M. Cladaras, T. Copeland, and G. N. Pavlakis.** 1989. The Rev protein of HIV-1 affects the stability and transport of the viral mRNA. Proc. Natl. Acad. Sci. USA **86:**1495–1499.
12. **Flores, E. R., B. L. Allen-Hoffmann, D. Lee, C. A. Sattler, and P. F. Lambert.** 1999. Establishment of the human papillomavirus type 16 (HPV-16) life cycle in an immortalized human foreskin keratinocyte cell line. Virology **262:**344–354.
13. **Frattini, M. G., H. B. Lim, J. Doorbar, and L. A. Laimins.** 1997. Induction of human papillomavirus type 18 late gene expression and genomic amplification in organotypic cultures from transfected DNA templates. J. Virol. **71:**7068–7072.
14. **Frattini, M. G., H. B. Lim, and L. A. Laimins.** 1996. In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. Proc. Natl. Acad. Sci. USA **93:**3062–3067.
15. **Furth, P. A., and C. C. Baker.** 1991. An element in the bovine papillomavirus late 3′ untranslated region reduces polyadenylated cytoplasmic RNA levels. J. Virol. **65:**5806–5812.
16. **Howley, P. M.** 1996. *Papillomavirinae*: the viruses and their replication, p. 2045–2076. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
17. **Kennedy, I. M., J. K. Haddow, and J. B. Clements.** 1990. Analysis of human papillomavirus type 16 late mRNA 3′ processing signals in vitro and in vivo. J. Virol. **64:**1825–1829.
18. **Kennedy, I. M., J. K. Haddow, and J. B. Clements.** 1991. A negative regulatory element in the human papillomavirus type 16 genome acts at the level of late mRNA stability. J. Virol. **65:**2093–2097.
19. **Leder, C., J. A. Kleinschmidt, C. Wiethe, and M. Müller.** 2001. Enhancement of capsid gene expression: preparing the human papillomavirus type 16 major structural gene L1 for DNA vaccination purposes. J. Virol. **75:**9201–9209.
20. **Liu, W. J., F. Gao, K. N. Zhao, W. Zhao, G. J. Fernando, R. Thomas, and I. H. Frazer.** 2002. Codon modified human papillomavirus 16 E7 DNA vaccine enhances cytotoxic T-lymphocyte induction and anti-tumour activity. Virology **301:**43–52.
21. **Meyers, C., T. J. Mayer, and M. A. Ozbun.** 1997. Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. J. Virol. **71:**7381–7386.
22. **Ozbun, M. A.** 2002. Human papillomavirus type 31b infection of human keratinocytes and the onset of early transcription. J. Virol. **76:**11291–11300.
23. **Ozbun, M. A., and C. Meyers.** 1998. Temporal usage of multiple promoters during the life cycle of human papillomavirus type 31b. J. Virol. **72:**2715–2722.
24. **Schwartz, S.** 1998. *cis*-Acting negative RNA elements on papillomavirus late mRNAs. Semin. Virol. **8:**291–300.
25. **Schwartz, S.** 2000. Regulation of human papillomavirus late gene expression Ups. J. Med. Sci. **105:**171–192.
26. **Schwartz, S., M. Sokolowski, B. Collier, A. Carlsson, and L. Goobar-Larsson.** 1999. *cis*-Acting regulatory sequences on papillomavirus late mRNAs. Recent Res. Dev. Virol. **1:**53–74.
27. **Shah, K. V., and P. M. Howley.** 1996. Papillomaviruses, p. 2077–2109. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
28. **Sokolowski, M., W. Tan, M. Jellne, and S. Schwartz.** 1998. mRNA instability elements in the human papillomavirus type 16 L2 coding region. J. Virol. **72:**1504–1515.
29. **Stoler, M. H., A. Whitbeck, S. M. Wolinsky, T. R. Broker, L. T. Chow, M. K. Howett, and J. W. Kreider.** 1990. Infectious cycle of human papillomavirus type 11 in human foreskin xenografts in nude mice. J. Virol. **64:**3310–3318.
30. **Stubenrauch, F., and L. A. Laimins.** 1999. Human papillomavirus life cycle: active and latent phases. Semin. Cancer Biol. **9:**379–386.
31. **Tan, W., B. K. Felber, A. S. Zolotukhin, G. N. Pavlakis, and S. Schwartz.** 1995. Efficient expression of the human papillomavirus type 16 L1 protein in epithelial cells by using Rev and the Rev-responsive element of human immunodeficiency virus or the *cis*-acting transactivation element of simian retrovirus type 1. J. Virol. **69:**5607–5620.
32. **Tan, W., M. Schalling, C. Zhao, M. Luukkonen, M. Nilsson, E. M. Fenyö, G. N. Pavlakis, and S. Schwartz.** 1996. Inhibitory activity of the equine infectious anemia virus major 5′ splice site in the absence of Rev. J. Virol. **70:**3645–3658.
33. **Tan, W., and S. Schwartz.** 1995. The Rev protein of human immunodeficiency virus type 1 counteracts the effect of an AU-rich negative element in the human papillomavirus type 1 late 3′ untranslated region. J. Virol. **69:**2932–2945.
34. **Wiklund, L., M. Sokolowski, A. Carlsson, M. Rush, and S. Schwartz.** 2002. Inhibition of translation by UAUUUAU and UAUUUUUAU motifs of the AU-rich RNA instability in the HPV-1 late 3′ untranslated region. J. Biol. Chem. **277:**40462–40471.
35. **Zhou, J., W. J. Liu, S. W. Peng, X. Y. Sun, and I. Frazer.** 1999. Papillomavirus capsid protein expression level depends on the match between codon usage and tRNA availability. J. Virol. **73:**4972–4982.