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

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Production of the human papillomavirus (HPV) late gene products L1 and L2 is limited to terminally differentiated keratinocytes. Here, we demonstrate that mRNA encoding the HPV-16 L1 capsid protein contains *cis*-acting RNA elements that inhibit expression at the posttranscriptional level. While cytoplasmic L1 mRNA is detectable in transfected HeLa cells, L1 protein is not produced. We have identified at least one major inhibitory element that is located within the L1 open reading frame, whereas another negative element had been reported to lie in the 3'-untranslated region of L1. The presence of these elements may explain the lack of HPV late gene expression in undifferentiated epithelial cells. Efficient production of HPV-16 L1 could be achieved with posttranscriptional regulatory elements of human immunodeficiency virus type 1 or simian retrovirus type 1. L1 protein was expressed in the presence of human immunodeficiency virus type 1 Rev from hybrid mRNAs containing the RNA binding site for Rev (Rev-responsive element). In addition, we have achieved efficient expression of L1 from hybrid mRNAs containing a *cis*-acting transactivation element from simian retrovirus type 1. Our data show that HPV-16 L1 protein production is regulated posttranscriptionally. This regulated expression may allow virus production in terminally differentiated epithelial cells and is probably a conserved and important mechanism for HPV expression.

Human papillomaviruses (HPVs) are nonenveloped, double-stranded circular DNA viruses, which infect human epithelia and mucosa in a tissue-specific manner (41). The HPV genome is approximately 8,000 bp and consists of early and late genes as well as a noncoding region (NCR) (Fig. 1). Early genes encode proteins involved in viral replication, transformation, and transcriptional regulation, while late genes encode the viral capsid proteins L1 and L2 (64). The NCR contains *cis*-acting transcriptional and replicative elements in addition to elements that respond to keratinocyte-specific factors. The most frequently found cancer-associated HPV type is HPV-16, which is present in approximately 60% of cervical cancers (77). At present, HPVs are linked to up to 10% of the worldwide cancer burden, mainly because of their involvement in anogenital cancers (77). Cervical carcinoma biopsies isolated from the majority of cervical cancer patients contain HPV DNA. Because of the clinical importance and the risk of malignant progression of lesions induced by HPV-16, a better understanding of the molecular mechanisms that regulate the life cycle of HPVs is of particular importance.

The mechanisms which control HPV late gene expression are still not fully elucidated. Studies of the interaction of HPV with host cells and on the control of HPV late gene expression

are hampered by the lack of an in vitro cell culture system for efficient propagation of virus. Analyses of basal cells in HPV-infected squamous epithelium have shown that HPV DNA is present at low copy numbers in these cells and that only early HPV genes are expressed (55). Concurrent with cell differentiation and migration of cells to suprabasal layers, HPV DNA copy number increases and expression of late genes is activated. In the differentiated cells, the HPV late proteins L1 and L2 are produced and virions are detected. The intimate association between HPV late gene expression and epithelial cell differentiation is likely to be regulated by *cis*-acting sequences on the viral genome or viral mRNAs and by transacting viral or cellular factors produced in infected cells. Studies of these regulatory mechanisms may provide valuable information for development of an efficient in vitro cell culture system for HPVs.

In situ hybridization studies of sections of HPV-33-infected tissue from a cervical condyloma accuminata (7) or from HPV-11-infected human xenografts in nude mice (71) revealed that L1 and L2 mRNAs produced in the middle layers of the infected epithelium were restricted to the nucleus, while high levels of L1 and L2 mRNAs were present in the cytoplasm in the superficial layers with differentiated cells. These observations suggested to us that expression of HPV late genes could be under posttranscriptional control. Expression of late genes of several retroviruses, such as human immunodeficiency virus type 1 (HIV-1), simian retrovirus type 1 (SRV-1), and Mason-Pfizer monkey virus, is posttranscriptionally regulated and has been well studied (9, 16, 17, 26, 33, 59, 61, 73, 76). Production

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FIG. 1. Schematic representation of the genomic organization of HPV-16. Open boxes represent ORFs coding for early proteins E1 to E7, stippled boxes indicate ORFs coding for late proteins L1 and L2, and the solid box indicates the NCR. The nucleotide positions of the L1 translational start and stop codons and the major late poly(A) signal LP2 are indicated (43, 60). Numbers refer to nucleotide positions in the HPV-16 genomic clone pHPV16 (68).

of the retrovirus structural proteins Gag, Pol, and Env is regulated by positive and negative *cis*- and *trans*-acting factors. Here, we have used positive regulatory elements from HIV-1 and SRV-1 to investigate if the presence of these elements on HPV-16 late mRNAs could induce production of L1 capsid protein in human epithelial cells.

HIV-1, the etiologic agent of AIDS, is the prototype of the complex retroviruses (16, 26, 59). These viruses are characterized by the presence on their genomes of several short open reading frames (ORFs) coding for regulatory and accessory proteins. Two of these proteins, Tat and Rev, are regulators of gene expression and are essential for viral replication (16, 17, 26, 33, 59, 61, 73). Tat is a transcriptional transactivator, while Rev acts posttranscriptionally to induce expression of HIV-1 late genes. The Rev protein binds to an RNA sequence termed the Rev-responsive element (RRE) (1, 3, 4, 15, 20, 21, 24, 34, 37–40, 47, 53, 58, 70) that is present on late viral mRNAs coding for the structural proteins Gag, Pol, and Env. Rev overcomes the effect of inhibitory RNA sequences present on these mRNAs (10, 14, 51, 57, 62, 65, 67) and acts by promoting nuclear export of RRE-containing mRNAs (24, 25, 35, 53) and by increasing their stability (25, 67) and translatability (2, 18, 48). Therefore, Rev acts as a switch between early and late phases of viral gene expression in the HIV-1 life cycle.

Mason-Pfizer monkey virus and SRV-1 are type D retroviruses causing persistent infections in macaque monkeys (30). It has recently been shown that a *cis*-acting transactivation element (CTE) present on Mason-Pfizer monkey virus and SRV-1 mRNAs can substitute for the Rev-RRE regulatory system (9, 76), suggesting that factors similar in function to HIV-1 Rev are present in eucaryotic cells.

In the present study, we show that the HPV-16 L1 coding region contains inhibitory sequences that act posttranscriptionally to repress production of L1 capsid protein in the human epithelial cell line HeLa. Production of HPV-16 L1 protein could be induced from hybrid mRNAs containing the HIV-1 RRE in the presence of the HIV-1 Rev protein. Similarly, hybrid mRNAs containing the SRV-1 CTE expressed L1 protein efficiently. We also show that inhibition exerted by a previously identified negative element in the HPV-16 late 3' untranslated region (UTR) [44] could be overcome by HIV-1 Rev and RRE or the SRV-1 CTE. Taken together, these results indicate that expression of HPV-16 late genes in nonkeratinized epithelial cells is likely to be regulated by the interaction of cellular factors with sequences present on viral late mRNAs. The results presented here favor a model for activation of HPV-16 late gene expression in infected squamous epithelium undergoing differentiation, which predicts either

upregulation of positive factors antagonizing the negative elements or downregulation or inactivation of a cellular factor(s) presumably interacting with viral inhibitory sequences. This would allow HPV-16 late gene expression and production of virions.

MATERIALS AND METHODS

Plasmid constructions. The complete HPV-16 L1 gene was PCR amplified from a previously described molecularly cloned HPV-16 genome (68) and was inserted into *EcoRV*-digested pBluescript, generating pT7-16L1. The HPV-16 L1 gene was transferred as a *Bss*HII-*Asp*718 fragment into *Bss*HII-*Asp*718-digested pNL17R (67), generating p16L1. p16L1RRE was generated by insertion of a 330-bp RRE-containing fragment (70) into *Asp*718-digested, calf intestinal alkaline phosphatase (CIAP)-treated p16L1. To generate p16L1CTE, the SRV-1 CTE-containing sequence was first PCR amplified from plasmid pS12 (76) with the oligonucleotides 12745 (5'-GCATCAACGCGTGTCTGACGGATCCAGAC CACCTCCCTGCGAG-3') and 13827 (5'-GCATCAGCGGCCGCTCGAG TCTAGACAAATCCCTCGGAAGCTGCG-3') and cloned into *EcoRV*-digested pBluescript. Restriction sites introduced in oligonucleotides are underlined. The SRV-1 CTE was excised with *Xho*I, treated with a large fragment of Klenow DNA polymerase, and inserted into p16L1 digested with *Asp*718 and treated with Klenow and CIAP. These cloning steps resulted in plasmid p16L1CTE. To generate p16L1UTRE, p16L1 was digested with *Eco*RI, treated with CIAP, and ligated to an *Eco*RI fragment, containing HPV-16 sequences from nucleotide (nt) 6818 to nt 7453 and the HIV-1 RRE inserted at position 7282.

To construct pNLCATW, the chloramphenicol acetyltransferase (CAT) gene was PCR amplified with the oligonucleotides CATS (5'-GCTAAGGAAGCTA AAATGGAG-3') and CATA (5'-CTATTAGGCCCCGCTCGCACTC-3') and subsequently cloned blunt ended into pNL17R (67), digested with *Sall* and *Asp*718, and treated with CIAP and Klenow DNA polymerase. This resulted in plasmid pNLCATW. pNLCATW was digested with *Asp*718, followed by CIAP treatment and filling in of the 5' overhang, and was used as a vector in the cloning steps described below. To construct CAT expression plasmids containing the complete HPV-16 L1 gene, the L1 coding sequence was PCR amplified and inserted into *Asp*718-digested pNLCATW. This generated pCATLIS and pCATLIA, containing the L1 gene in sense and antisense orientations, respectively. To generate derivatives of pCATLIS containing deletions in the L1 gene, various portions of the HPV-16 L1 gene were excised from pT7-16L1 (described above) with *Bss*HII in combination with *Eco*NI, *Pst*I, *Bst*XI, and *Bam*HI, filled in or trimmed, and cloned into *Asp*718-restricted pNLCATW, resulting in pCATLIAΔE, pCATLIAΔB, pCATLIAΔBS, and pCATLIAΔSS, respectively. pCATLIASS was constructed by direct cloning of the *Ssp*I fragment (nt 5813 to 6551) of the L1 gene into *Asp*718-digested pNLCATW. pCAT-209 and pCAT-347 were generated by insertion of PCR fragments of the HPV-16 L1 gene, amplified with the oligonucleotides L1START and 16A (5'-CGGATCCGTAT TGTAATCCTGATACTTTAGGAAC-3') or 16B (5'-CGGATCCGCCACAC CTAATGGCTGACCAGC-3'), respectively, into *Asp*718-digested pNLCATW. To generate derivatives of pCATLIS containing deletions in the 5' end of the L1 gene, portions of HPV-16 L1 were PCR amplified with oligonucleotide L1STOP in combination with oligonucleotide 16-711 (5'-TTGGATCCGGTGTCTCAG ACCATATGGCGACAGC-3') or 16C (5'-CGGATCCACAATAATGGCAT TTGTTGGGG-3') and cloned into *Asp*718-digested pNLCATW, resulting in pCAT-804 and pCAT-565, respectively.

To construct pCH16pA, an *Eco*RI fragment containing the HPV-16 late 3' UTR (nt 6818 to 7453) and late poly(A) signal was cloned into *Eco*RI-digested, CIAP-treated pBluescript. Uracil-containing single-stranded DNA was prepared as described previously (50) and used as the substrate for site-specific, oligonucleotide-directed mutagenesis to introduce a unique *Hpa*I site at nt 7282. The *Eco*RI fragment containing the mutated HPV-16 sequence was inserted into *Eco*RI-digested, CIAP-treated pCM1234, which contains a mutated HIV-1 p17^{gag} gene, p17M1234 (65). This resulted in plasmid pCH16pA, in which the HPV-16 3' UTR is located immediately downstream of p17M1234. p17M1234 contains multiple point mutations which altered the RNA sequence but maintained the original primary protein sequence of p17^{gag} (65, 67). This resulted in high p17^{gag} production independently of Rev (65, 67). p17M1234 is used as a reporter gene and is under control of the human cytomegalovirus (CMV) immediate early promoter (nt -671 to +74) (8). The HPV-16 late poly(A) signal LP2, located at nt 7319, has been shown to be efficiently utilized both *in vitro* and *in vivo* (43, 60) (Fig. 1 and 7). pCH16pA was generated by digestion of pCH16pA with *Eco*NI (nt 6858) and *Hpa*I (nt 7282) and filling in of the 5' overhang, followed by religation. pCH16RRE was generated by insertion of a 330-bp fragment spanning the HIV-1 RRE (70) into *Hpa*I-digested, CIAP-treated pCH16pA. To construct pCH16CS and pCH16CA, the SRV-1 CTE was PCR amplified from plasmid pS12 with oligonucleotides 12745 and 13827 (76) and subsequently cloned into *Hpa*I-digested, CIAP-treated pCH16pA, generating plasmids pCH16CS and pCH16CA, which contain the SRV-1 CTE in the sense and antisense orientations, respectively. pCM1234pA was constructed by insertion into an *Eco*RI-digested, CIAP-treated pCM1234 plasmid of an *Eco*RI

fragment containing the simian virus 40 (SV40) early poly(A) signal. To construct pT7-16pA and pT7-16ΔpA, sequences containing p17M1234 together with the HPV-16 3' UTR or its truncated form were excised with *Sall* and *EcoRI* from pCH16pA and pCH16ΔpA and cloned into pBluescript, resulting in plasmids pT7-16pA and pT7-16ΔpA, respectively.

Plasmid pCS1X was constructed by cloning of a DNA fragment (nt 7016 to 7525; numbers refer to the genomic HPV-1 sequence [19]) containing the HPV-1 late poly(A) signals into pHCMVSEAP (5) digested with *HpaI* and *EcoRI*. These cloning steps replaced the insulin poly(A)-containing sequence with the HPV-1 sequence. The Rev-producing plasmids pNL1.4A.7 (66) and pLsRev (54), the SRV-1 CTE-containing plasmid pS12(*BamHI-XhoI*) (76), and the HIV-1 *gag* plasmid pNLyagSty330 (70) have been described before.

Cells and transfections. HeLa cells or HLtat cells (66) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed as follows: 0.3×10^5 cells were plated onto 60-mm culture dishes 1 day before transfection. Transfections were carried out according to the calcium phosphate coprecipitation technique (32). The amount of plasmid used for transfection was adjusted with pBluescript DNA to 15 μ g in 0.5 ml of precipitate for 60-mm-diameter dishes or 30 μ g in 1 ml of precipitate for 100-mm-diameter dishes. Transfected cells were washed twice with phosphate-buffered saline (PBS) 6 h posttransfection and refed with fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and cultured overnight. Cells were harvested 24 h posttransfection. All plasmid DNAs used for transfection were purified on Qiagen columns (Diagen). One microgram of pCMVCAT, producing CAT enzyme, or pCS1X, producing secreted placental alkaline phosphatase (SEAP), was included as an internal control for transfection efficiency. Radioactive CAT assays were carried out by the method described by Gorman et al. (31). Production of CAT protein was quantitated with a CAT antigen capture enzyme-linked immunosorbent assay (ELISA; Boehringer Mannheim GmbH). To quantitate SEAP produced from the transfected cells, medium was collected and subjected to centrifugation at $13,000 \times g$ for 2 min. The supernatants were incubated at 65°C for 15 min and then cooled to room temperature and incubated with alkaline phosphatase reagent (Sigma Diagnostics) for kinetic determination of alkaline phosphatase activity according to the manufacturer's recommendation.

Cells transfected with plasmid pT7-16pA, pT7-16ΔpA, or pT7-16L1 were first infected with recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase (27, 28). One hour postinfection, cells were transfected by the calcium phosphate precipitation technique.

Western blot (immunoblot) analysis. Cells were lysed in 500 μ l of lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl [pH 7.5]), and lysates were cleared of insoluble material by centrifugation at $13,000 \times g$ for 15 min. Forty-microliter aliquots of cell extracts were loaded on sodium dodecyl sulfate (SDS)-12% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes by electroblotting in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) at constant current (250 mA) for 12 to 16 h. Membranes were blocked for 1 h at 37°C with 10% nonfat milk in PBS containing 0.3% Tween 20 (PBS-T). After three washings in PBS-T buffer, the blots were incubated for 1 h at 37°C with serum. To detect HIV-1 p17^{gag} or HPV-16 L1, HIV-1-positive patient serum or rabbit anti-HPV-16 L1 peptide serum (22) was used as the primary antibody, respectively, followed by incubation with horseradish peroxidase-conjugated anti-human or anti-rabbit immunoglobulin. Specific proteins were visualized with the enhanced chemiluminescence detection system (Amersham).

Radioimmunoprecipitation assay. Transfected cells were washed twice with prewarmed PBS 24 h posttransfection and then washed twice with methionine-free $1 \times$ Eagle's minimum essential medium. The cells were starved for 30 min at 37°C in 1.0 ml of Met-free medium containing 0.5% FCS, followed by metabolic labeling for 1 h with 200 μ Ci of [³⁵S]Met (Amersham). The cells were washed with cold PBS and harvested in 600 μ l of ice-cold 0.5% radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-Cl [pH 7.4], 75 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS). After three freeze-thawings, cell debris was removed by centrifugation at $15,000 \times g$ at 4°C for 15 min. The supernatants were mixed with preimmune rabbit serum and incubated at 4°C for 1 h, followed by addition of protein A-Sepharose (Pharmacia) suspension and incubation at 4°C for 30 min. Samples were centrifuged, and supernatants from each sample were collected and aliquoted into two Eppendorf tubes, to which either normal rabbit serum or rabbit anti-HPV-16 L1 peptide serum (22) was added. Incubation was kept at 4°C for 12 to 16 h with gentle rocking. Protein A-Sepharose was added, and samples were incubated for 3 h at 4°C. After centrifugation, the Sepharose beads were washed three times in $1 \times$ RIPA buffer and resuspended in $5 \times$ SDS-loading buffer (625 mM Tris [pH 6.8], 6.25% SDS, 50% glycerol, 20% 2-mercaptoethanol), boiled, and loaded on SDS-10% polyacrylamide gels under reducing conditions. Immunoprecipitated proteins were visualized by autoradiography (Kodak XAR film) at -70°C.

Cell fractionation and extraction of poly(A)⁺ mRNA. Nuclear and cytoplasmic poly(A)⁺ mRNA was isolated with Dynabeads oligo(dT)₂₅ (DynaL A.S.). Twenty-four hours posttransfection, cells from 100-mm-diameter dishes were washed twice with ice-cold PBS, followed by lysis in 500 μ l of lysis buffer D (10 mM Tris-HCl [pH 7.5], 0.14 M NaCl, 5 mM KCl, 1% Nonidet P-40). Nuclear and cytoplasmic fractions were separated by low-speed centrifugation at $8,000 \times g$ for 2 min. To extract cytoplasmic mRNA, supernatants were mixed with an equal

volume of $2 \times$ binding buffer (20 mM Tris-HCl [pH 7.5], 1.0 M LiCl, 2 mM EDTA, 0.5% SDS) containing 400 μ g of Dynabeads oligo(dT)₂₅. Hybridization was carried out at room temperature for 3 to 5 min, followed by washing of Dynabeads oligo(dT)₂₅ three times with 500 μ l of washing buffer (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 2 mM EDTA). The poly(A)⁺ mRNAs were eluted with 150 μ l of elution buffer (2 mM EDTA [pH 7.5]) at 65°C for 5 min and stored at -70°C until use. Nuclear fractions were washed once with lysis buffer D, and the pellets were resuspended in lysis buffer D without Nonidet P-40. An equal volume of lysis buffer D containing 1% SDS was added, and the samples were incubated on ice for 10 min, followed by freezing on dry ice. The samples were thawed at room temperature and centrifuged for 2 min at $8,000 \times g$. Supernatants were used for extraction of nuclear poly(A)⁺ mRNA with Dynabeads oligo(dT)₂₅ as described above.

RT-PCR. Twenty microliters of poly(A)⁺ mRNA or dilutions thereof were reverse transcribed at 42°C for 1 h in a total volume of 30 μ l, containing 4 U of avian myeloblastosis virus reverse transcriptase (RT; Promega); 19 U of RNA guard (Pharmacia); 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 4 mM MgCl₂; 10 mM Tris-HCl (pH 8.3); 50 mM KCl; and 50 μ g of random hexamer oligonucleotides per ml (Pharmacia). A reaction without RT was performed in parallel and served as a control for the absence of plasmid DNA contamination. Five microliters of the cDNA product was PCR amplified in a 100- μ l reaction volume with oligonucleotides 4704 (5'-GCCTGTAGAAACATCAGAAAGGC-3') and 5912 (5'-CTAGTAATTTGGCTGACCTG-3') detecting cDNA of p17^{gag} or p55^{gag} mRNA; oligonucleotides 16G (5'-CGATATCCCAGATACACAGCGGTGGTTTG-3') and 16M (5'-CGGATCCCACAATTGTTGTTTGTGTGTAATCC-3'), detecting cDNA of L1 mRNA; ACTINS (5'-TGAGCTGGCTGTGGCTCC-3') and ACTINA (5'-GGCATGGGGGAGGGCATAACC-3'), specifically amplifying cDNA of spliced actin mRNA; or ACTINS-1 (5'-CCAGTGGCTTCCCAGTG-3') and ACTINA, detecting cDNA of unspliced actin mRNA. To detect cDNA of CAT mRNA produced from the CAT expression plasmid used as an internal control for transfection efficiency, oligonucleotides CATS-2 (5'-CGTCTCAGCCAATCCCTGGGTG-3') and CATA (5'-CTATTAGGCCCGCCCTGCCACTC-3') were used. Oligonucleotides were purchased from Scandinavian Gene Synthesis.

A 250-bp p17^{gag} fragment was PCR amplified by oligonucleotides 4704 and 5912, a 199-bp product was amplified by 16G and 16M, a 247-bp product was amplified by ACTINS and ACTINA, a 203-bp product was amplified by ACTINS-1 and ACTINA, and a 229-bp fragment was amplified by CATS-2 and CATA. Oligonucleotides 5912, 16 M, ACTINA, and CATA were end labeled with [γ -³²P]ATP prior to use. PCR amplification was performed in a total volume of 100 μ l with 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) for 20 to 25 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s with a final extension at 72°C for 10 min. Ten microliters of each RT-PCR mixture was analyzed by electrophoresis on 6% polyacrylamide gels with or without urea. The gels were dried and exposed to X-ray film at -70°C.

Northern RNA blotting. At day 2 posttransfection, cytoplasmic RNA was extracted from duplicate plates (18) and Northern blots were prepared as described previously (34). The L1 mRNAs were detected with a probe spanning nt 8304 to 9008 of the HIV-1 molecular clone HXB2. Subsequently, the same blots were probed with a 2-kb *HindIII* fragment of β 2000 plasmid (12) to detect β -actin mRNAs.

RESULTS

Efficient production of the HPV-16 L1 protein with a vaccinia virus-T7 RNA polymerase-based expression system. The HPV-16 genome can be divided into early and late regions (Fig. 1). Expression of HPV-16 late genes (L1 and L2) is restricted to terminally differentiated epithelial cells in genital mucosa. To study expression of HPV-16 L1 protein in nonkeratinized cells, we introduced HPV-16 L1 gene expression vectors into HeLa cells, a human epithelial cell line that resembles cells in the basal layer of stratified epithelium. Two types of expression systems were employed: a vaccinia virus-T7 RNA polymerase-based expression system (27, 28), which allows transcription of the gene of interest in the cytoplasm, and eucaryotic expression plasmids containing promoters which depend on nuclear transcription factors. Since it has been demonstrated that an inhibitory element is present in the HPV-16 late 3' UTR (44), the HPV-16 late 3' UTR was excluded from the plasmids shown in Fig. 2A.

To circumvent nuclear regulatory mechanisms of gene expression, the vaccinia virus-T7 RNA polymerase-based expression system (27, 28) was utilized. The HPV-16 L1 gene was cloned downstream of the bacteriophage T7 promoter, generating plasmid pT716L1 (Fig. 2A). Infection of HeLa cells with

FIG. 2. HIV-1 Rev and RRE activate production of HPV-16 L1 protein. (A) Schematic structures of the HPV-16 L1 expression plasmids. The small open box at the top represents the bacteriophage T7 promoter, and the bent arrow indicates the direction of transcription driven by the T7 promoter. Large open boxes indicate HPV-16 sequences or the HIV-1 RRE as indicated. Numbers indicate nucleotide positions on the genomic HPV-16 clone (68). Light stippled boxes represent the HIV-1 LTR. The names of the plasmids are indicated on the left. (B) Western blot analysis of the HPV-16 L1 protein. HeLa cells were first infected with recombinant vaccinia virus vTF7-3 (27, 28) and then were transfected with pT7-16L1. Cells were harvested 24 h posttransfection, and proteins were separated on SDS-12% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. The L1 protein was detected with a rabbit anti-HPV-16 L1 peptide antiserum (22), followed by incubation with horseradish peroxidase-conjugated anti-human immunoglobulin G antiserum. The L1 protein was visualized with enhanced chemiluminescence detection reagents. The position of the L1 protein is indicated on the right. MW, molecular mass markers (in kilodaltons). (C) Radioimmunoprecipitation of HPV-16 L1 protein. HLTat cells were transfected with p16L1 or p16L1RRE in the absence or presence of Rev. Twenty-four hours posttransfection, cells were labeled with [³⁵S]methionine for 1 h. Immunoprecipitation was performed with either rabbit preimmune serum or rabbit anti-HPV-16 L1 peptide antiserum (22). Proteins were separated on SDS-12% polyacrylamide gels and visualized by autoradiography. P, rabbit preimmune serum; L, rabbit anti-HPV-16 L1 peptide antiserum. The position of the p55 L1 protein is indicated on the right. (D) Northern blot analysis of cytoplasmic RNA from HLTat cells, transiently transfected with the indicated plasmids in the presence or absence of an HIV-1 Rev expression plasmid, pLsRev. The positions of the rRNAs are indicated. (E) Analysis of subcellular distribution of HPV-16 L1 mRNAs. Nuclear and cytoplasmic poly(A)⁺ mRNAs were isolated from cells transfected with p16L1 or from cells transfected with p16L1RRE in the presence of Rev and were subsequently reverse transcribed and PCR amplified with oligonucleotides end labeled with [³²P]ATP. The PCR products were analyzed on 6% polyacrylamide gels, followed by autoradiography. The upper left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted nuclear poly(A)⁺ mRNAs isolated from cells transfected with p16L1 in the absence of HIV-1 Rev or from cells transfected with p16L1RRE in the presence of HIV-1 Rev. The positions of the amplified DNA fragments are indicated on the left. The upper right panel shows PCR amplification of cDNA from spliced or unspliced actin mRNA in the nuclear fraction of transfected cells to control for cell fractionation. The number of cycles used for PCR amplification of cDNA was adjusted to detect cDNA in the linear range of the assay. The lower left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)⁺ mRNA isolated from cells transfected with p16L1 in the absence of HIV-1 Rev or from cells transfected with p16L1RRE in the presence of HIV-1 Rev. The lower right panel shows PCR amplification of cDNA from spliced and unspliced actin mRNAs in the cytoplasmic fraction of transfected cells. (F) RT-PCR analysis of cytoplasmic poly(A)⁺ mRNAs. Serial fourfold dilutions of mRNA samples from cells transfected with pNLvgagSty330 (70) in the absence or presence of Rev were performed, and cDNAs were generated from each dilution. PCR was performed with oligonucleotides specific for the HIV-1 p55^{gag} mRNA produced by pNLvgagSty330 (left panel) or with oligonucleotides specific for spliced actin mRNA as a control for RNA levels (right panel). The positions of PCR products are indicated on the left.

the recombinant vaccinia virus vTF7-3 (27, 28), producing bacteriophage T7 RNA polymerase, is a prerequisite for expression of the L1 gene. The virally produced T7 RNA polymerase localizes to the cytoplasm and specifically recognizes the bacteriophage T7 promoter (27, 28). Since T7 RNA polymerase produced by vTF7-3 is a large protein lacking a nuclear localization signal, it does not enter the nucleus (23). Therefore, transcription directed by the T7 RNA polymerase takes place primarily in the cytoplasm. Figure 2B shows that transfection of pT7-16L1 into HeLa cells infected with recombinant vaccinia virus vTF7-3 gave rise to high levels of the HPV-16 L1 protein, as determined by Western blots with an HPV-16 L1-specific antipeptide antiserum (22).

Lack of L1 protein production with eucaryotic expression vectors. Expression of HPV-16 L1 protein was also examined with plasmids in which the L1 gene was under the control of a promoter that requires nuclear localization for function. We constructed plasmid p16L1, in which the HPV-16 L1 gene coding sequence is under the control of the HIV-1 long terminal repeat (LTR) promoter (Fig. 2A). Plasmid p16L1 was transfected into HLTat cells (66), a HeLa-derived cell line constitutively producing the HIV-1 Tat protein required for transcription from the HIV-1 LTR. We could not detect L1 protein expression in HLTat cells transfected with p16L1 by radioimmunoprecipitation with a rabbit anti-HPV-16 L1 peptide antiserum (Fig. 2C) (22). In conclusion, high levels of HPV-16 L1 protein were produced with the vaccinia virus-T7 RNA polymerase-based expression system, while L1 protein was undetectable in cells transfected with p16L1, which depends on nuclear factors. These observations suggested that inhibition of HPV-16 L1 gene expression may involve nuclear events.

HIV-1 Rev and RRE activate production of HPV-16 L1 protein. HIV-1 produces the posttranscriptional transactivator protein Rev, which binds to the RRE RNA site and is required for production of the HIV-1 structural proteins Gag, Pol, and Env. We wished to investigate whether the HIV-1 Rev protein could activate expression of HPV-16 L1. Therefore, a 330-nt fragment encompassing HIV-1 RRE was first introduced into plasmid p16L1, resulting in p16L1RRE (Fig. 2A). This plasmid was transfected into HLTat cells in the absence or presence of HIV-1 Rev-producing plasmid pNL1.4A.7 (66). Results of radioimmunoprecipitations showed that L1 protein was detected only in the presence of HIV-1 Rev and RRE (Fig. 2C). A CAT-expressing plasmid, pCMVCAT, was included as an internal control in each transfection experiment. The amount of CAT protein in each sample did not vary more than twofold among these transfections (data not shown). We concluded that HIV-1 Rev and RRE could activate expression of HPV-16 L1 in human HLTat cells of epithelial origin.

L1 mRNAs accumulate in the cytoplasm but are not efficiently translated. Since previous studies had established that HIV-1 Rev acts at least partially by promoting nuclear export of HIV-1 mRNAs (24, 25, 35, 53), thereby affecting subcellular distribution of RRE-containing mRNAs, we analyzed nuclear and cytoplasmic L1 mRNA levels in the absence or presence of HIV-1 Rev and RRE. Northern blot analysis revealed that intact L1 mRNA was readily detectable in the cytoplasmic fraction (Fig. 2D). The presence of Rev resulted in a small increase in mRNA produced from p16L1RRE. To quantitate the levels of mRNA, we subjected poly(A)⁺ mRNA to RT-PCR analysis. Nuclear and cytoplasmic poly(A)⁺ mRNAs were extracted from HLTat cells transfected with p16L1 in the absence of Rev or from HLTat cells transfected with p16L1RRE in the presence of Rev. Serial fourfold dilutions of the mRNA samples were subjected to random primed cDNA

synthesis followed by PCR with oligonucleotides specific for the HPV-16 L1 sequence. Figure 2E revealed that high levels of HPV-16 L1 mRNAs were present in the cytoplasm in the absence of Rev and RRE. An increase in cytoplasmic L1 mRNA levels estimated to be less than fourfold was observed in the presence of Rev (Fig. 2E). Similarly, L1 mRNA levels in the nucleus were also slightly increased in the presence of Rev (Fig. 2E). Levels of CAT protein produced from the internal control plasmid pCMVCAT were analyzed in the same transfections and varied less than twofold between the transfections (data not shown).

It could be argued that HPV-16 L1 mRNAs trapped in the nucleus leak out into the cytoplasm during cell fractionation. To control for the fractionation technique, we analyzed unspliced and spliced actin mRNAs by RT-PCR. While spliced actin mRNA was found in both nuclear and cytoplasmic fractions, unspliced actin mRNA was primarily found in the nuclear fraction (Fig. 2E), demonstrating that nuclei remained intact during fractionation. In parallel transfections, we analyzed cytoplasmic HIV-1 *gag* mRNA levels produced from pNLvgagSty330 (25, 70) in the absence or presence of Rev. The results showed a big increase in cytoplasmic p55^{gag} mRNA levels in the presence of Rev, while similar levels of cytoplasmic actin mRNA were detected (Fig. 2F). This is in agreement with previous studies that established that the majority of the HIV-1 *gag* mRNAs produced from this plasmid are retained in the nucleus in the absence of Rev (25). We concluded that inhibition of HPV-16 L1 production was not caused exclusively by entrapment of L1 mRNAs in the nucleus. Although Rev did increase the levels of L1 mRNAs in the cytoplasm, the increase in L1 protein levels appeared to be greater than that in L1 mRNA levels, suggesting that Rev acted by also increasing translation of the HPV-16 L1 mRNAs.

It has been shown that the HPV-16 late 3' UTR contains sequences that inhibit gene expression (44). Since such sequences are normally present on L1 mRNAs, we wished to determine whether Rev and RRE could activate L1 production from an L1 mRNA also containing the HPV-16 late 3' UTR. Therefore, RRE was inserted into a plasmid containing the entire HPV-16 L1 gene and 3' UTR sequence, resulting in plasmid p16L1UTRRE (Fig. 3A). L1 production from this plasmid in the absence or presence of Rev was assessed by radioimmunoprecipitation. Figure 3B shows that L1 protein was produced from p16L1UTRRE only in the presence of Rev. The levels of L1 protein were similar between p16L1UTRRE and p16L1RRE in the presence of Rev (Fig. 3B). These results demonstrated that Rev could also activate L1 expression when the previously described inhibitory sequences in the HPV-16 late 3' UTR (44) were present on the mRNAs.

The SRV-1 CTE acts in cis to induce HPV-16 L1 expression. It was recently reported that the 3' UTR of Mason-Pfizer monkey virus and SRV-1 contains an RNA sequence, CTE (9, 76), which is able to substitute for HIV-1 Rev and RRE and render HIV-1 structural gene expression Rev independent. It is thought that cellular factors analogous to Rev interact with CTE. It was therefore of interest to test whether CTE could activate expression of HPV-16 L1 protein. The SRV-1 CTE was inserted downstream of the L1 gene in plasmid p16L1, generating plasmid p16L1CTE (Fig. 3A). HLTat cells were transfected with p16L1CTE and metabolically labeled with [³⁵S]methionine. Radioimmunoprecipitation showed that high levels of L1 protein were produced from p16L1CTE, demonstrating that the presence of the SRV-1 CTE in a sense orientation induced production of L1 protein (Fig. 3B). Comparison of the HPV-16 L1 protein levels induced by Rev and RRE or

FIG. 3. The presence of HIV-1 Rev *in trans* and RRE *in cis* or the SRV-1 CTE element *in cis* activates expression of the HPV-16 L1 capsid protein. (A) Schematic structures of HPV-16 L1 expression plasmids. Lightly shaded boxes indicate HIV-1 LTRs. Open boxes indicate HPV-16 sequences (HIV-1 RRE or SRV-1 CTE) as indicated. The position of the translational stop codon at position 7154 is indicated. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). The small open triangle represents the major late poly(A) signal LP2 present in the HPV-16 3' UTR (43, 60). (B) Immunoprecipitation of the HPV-16 L1 protein. HLTat cells were transfected with the various plasmids shown in panel A. Cell lysates were subjected to immunoprecipitation with rabbit preimmune serum or rabbit anti-HPV-16 L1 peptide antiserum (22). Proteins were analyzed on SDS-12% polyacrylamide gels and visualized by autoradiography. The position of the HPV-16 L1 protein is indicated on the right. P, rabbit preimmune serum; L, rabbit anti-HPV-16 L1 peptide antiserum; MW, molecular mass marker (in kilodaltons). (C) SRV-1 CTE increases the HPV-16 L1 mRNA levels in the cytoplasm. HLTat cells were transfected with p16L1 or p16L1CTE. Cytoplasmic poly(A)⁺ mRNAs isolated from transfected cells were reverse transcribed and subjected to PCR amplification with oligonucleotides specific for HPV-16 L1 mRNA. Oligonucleotides were end labeled with [γ -³²P]ATP prior to PCR. The amplified products were analyzed on 6% polyacrylamide gels and examined by autoradiography. The left panel shows PCR amplification of cDNAs synthesized from serially fourfold-diluted cytoplasmic poly(A)⁺ mRNA isolated from cells transfected with p16L1 or p16L1CTE. The positions of the PCR products are indicated on the left. The right panel shows RT-PCR analysis of spliced actin mRNA used to control for the amount of mRNA used for RT-PCR.

by the CTE element revealed that L1 levels produced in the presence of CTE were significantly higher than those produced in the presence of Rev and RRE (Fig. 3B). This was consistently observed in three independent experiments. Therefore, in these cells, CTE was a better activator of L1 expression.

We next analyzed mRNA levels produced in the absence or presence of the CTE element *in cis*. poly(A)⁺ mRNA was extracted from HLTat cells transfected with p16L1 or p16L1CTE. The mRNA samples were serially diluted and subjected to cDNA synthesis and PCR amplification. Figure 3C shows that the cytoplasmic poly(A)⁺ mRNA levels produced from p16L1CTE were four- to eightfold higher than those produced from p16L1. The amounts of CAT produced from the internal control plasmid pCMVCAT varied less than twofold between the transfections (data not shown). Actin mRNA levels were similar in both RNA samples (Fig. 3C), demonstrating that equal amounts of poly(A)⁺ mRNA were analyzed in RT-PCR. We concluded that the CTE element acted by increasing both cytoplasmic L1 mRNA levels and L1 protein levels similar to what was described above for Rev and RRE. The increase appeared to be greater at the protein level than at the mRNA level, indicating that CTE acts by increasing both cytoplasmic L1 mRNA levels and mRNA translation.

Identification of *cis*-acting inhibitory sequences in the HPV-16 L1 coding region. Since HPV-16 L1 protein was produced only in the presence of the CTE or Rev and RRE, these results suggested that either L1 mRNAs lack *cis*-acting RNA sequences required for efficient mRNA processing and utiliza-

tion or that inhibitory sequences are present in the HPV-16 L1 coding sequence. To investigate if the L1 sequence contains an inhibitory element, we asked whether the presence of the L1 coding sequence downstream of the CAT reporter gene could inhibit CAT production. The entire L1 coding sequence was inserted in a sense or antisense orientation downstream of the CAT gene in plasmid pNLCATW (Fig. 4A), resulting in pCATL1S and pCATL1A, respectively (Fig. 4A). CAT activity or CAT protein levels in HLTat cells transfected with pCATL1S, pCATL1A, or pNLCATW were evaluated. Figure 4B shows that low levels of CAT activity were produced from pCATL1S, while pCATL1A and pNLCATW produced high levels of CAT, demonstrating that the L1 coding sequence inhibits CAT production in an orientation-dependent manner.

We also used a CAT ELISA specifically detecting CAT protein to quantitate the amount of CAT present in the cell extracts analyzed for Fig. 4B. The results revealed that plasmid pCATL1S produced 78-fold lower levels of CAT than did pNLCATW (Fig. 4C). The presence of the L1 gene in an antisense orientation, as in pCATL1A, only resulted in a two- to threefold reduction in CAT production (Fig. 4C). Similar results were obtained from three independent experiments. Plasmid pCS1X, producing SEAP, was included in these transfections and served as an internal control for transfection efficiency. SEAP production did not vary more than twofold between the different transfections (data not shown). The two- to threefold inhibitory effect on CAT production observed with the HPV-16 L1 sequence in the antisense orientation is be-

FIG. 4. HPV-16 inhibitory element inhibits CAT expression in an orientation-dependent manner. (A) Schematic structures of CAT expression plasmids. Lightly shaded boxes represent the HIV-1 LTRs. Darkly shaded boxes indicate the CAT gene. Open boxes represent HPV-16 L1 coding sequences. Numbers indicate nucleotide positions on the genomic HPV-16 clone (68). Arrows indicate orientation of the HPV-16 L1 gene in the plasmid. The names of the plasmids are indicated on the left. (B) The presence of the HPV-16 L1 gene in the sense orientation strongly inhibits CAT activity. HLTat cells transfected with pNLCATW, pCATL1S, and pCATL1A were harvested in lysis buffer (0.5% Triton X-100, 100 mM Tris-HCl [pH 7.8]). CAT activity in each cell lysate was analyzed in triplicate by the method described by Gorman et al. (31). Results from a representative experiment are shown. (C) CAT protein levels from the same samples used for measuring CAT activities in Fig. 4B were determined by a CAT-capture ELISA kit (Boehringer Mannheim GmbH). CAT protein levels are displayed as CAT units.

lied to be unspecific and is too low to account for the inhibitory effect on L1 production. We concluded that the HPV-16 L1 coding sequence contained *cis*-acting inhibitory elements that acted in an orientation-dependent manner to inhibit gene expression.

An inhibitory element located between nt 5813 and 6150 in the HPV-16 L1 coding sequence. To map the inhibitory sequences, we generated a series of deletions in the 3' end of the L1 coding sequence in pCATL1S (Fig. 5). Triplicate transfections were performed and CAT levels were determined with the CAT ELISA as described above. The CAT values were normalized to those of SEAP produced from plasmid pCS1X, included in each transfection as an internal control for transfection efficiency. Deletions in the 3' end of L1 in pCATL1S resulted in a gradual increase in CAT levels. The L1 fragment present in pCATL1ΔBS substantially inhibited CAT expression, while higher levels of CAT were produced from pCAT-

FIG. 5. Identification of inhibitory sequences present in the HPV-16 L1 coding region. Lightly shaded boxes represent the HIV-1 LTRs. The darkly shaded box indicates the CAT gene. Solid bars represent the HPV-16 L1 coding sequence. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). The names of the plasmids are shown on the left. Triplicate transfections were performed. The difference in transfection efficiency among CAT expression plasmids was corrected by being normalized to SEAP produced from pCS1X, included as an internal control. Arithmetic means and standard deviations were calculated by standard methods and are shown on the right.

347 (Fig. 5). These experiments mapped the 3' boundary of a negative element to sequences between nt 5984 and 6150. To investigate if inhibitory sequences were present in the 5' end of the L1 gene, 5' deletions were introduced in the L1 gene in plasmid pCATL1S. Deletion of sequences between nt 5637 and 6347 as in plasmid pCAT-804 resulted in increased levels of CAT protein. However, these levels were significantly lower than those produced from pNLCATW. Therefore, inhibitory sequences were present also between nt 6150 and 7154. Interestingly, plasmid pCAT-565 produced high levels of CAT, demonstrating that inhibitory sequences were entirely or partially deleted in this plasmid. Plasmid pCATL1SS, containing an L1 fragment spanning sequences between nt 5813 and 6551 near the 5' end of the L1 coding sequence, produced low levels of CAT. These results indicated that sequences between nt 5637 and 5813 were not required for strong inhibition. These results suggest that one inhibitory element is located between nt 5813 and 6150 and that another is located downstream of nt 6150.

To investigate the effect of the L1 sequence on CAT mRNA levels, we analyzed cytoplasmic poly(A)⁺ mRNA levels in HLTat cells transfected with pNLCATW or pCATL1SS (Fig. 6A). RT-PCR was performed on serially diluted RNA samples with oligonucleotides specifically detecting CAT mRNAs. The results showed that CAT mRNA levels generated from pCATL1SS were fourfold lower than those produced from pNLCATW (Fig. 6B). Levels of SEAP produced from pCS1X, included to serve as a control for transfection efficiency, varied

A

B

FIG. 6. HPV-16 L1 coding sequence (nt 5813 to 6551) is necessary and sufficient for inhibition. (A) Schematic structures of CAT expression plasmids. Lightly shaded boxes represent the HIV-1 LTRs. Darkly shaded boxes indicate the CAT gene. The open box represents the HPV-16 L1 coding sequence. Numbers indicate nucleotide positions on the genomic HPV-16 clone (68). Names of the plasmids are shown on the left. (B) Analysis of cytoplasmic poly(A)⁺ mRNA levels from HLTat cells transfected with pNLCATW or pCATL1SS. The left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)⁺ mRNAs isolated from cells transfected with pNLCATW or pCATL1SS. The positions of the amplified DNA fragments are indicated on the left. The oligonucleotides were end labeled with [γ -³²P]ATP prior to PCR. The PCR products were analyzed on 6% polyacrylamide gels, followed by autoradiography. The right panel shows PCR amplification of cDNA from spliced actin mRNA in the cytoplasmic fraction of transfected cells. The number of cycles for PCR amplification of cDNA was adjusted to detect the amplified products in the linear range of the assay.

less than twofold between transfections (data not shown). These results verified that the presence of inhibitory sequences in HPV-16 L1 decreased cytoplasmic poly(A)⁺ mRNA levels. However, the difference in CAT protein production between pNLCATW and pCATL1SS was approximately 140-fold (Fig. 5). Therefore, the inhibitory effect was greater at the protein level than at the mRNA level. These results demonstrated that mRNAs containing inhibitory HPV-16 sequences were present in the cytoplasm but were not efficiently translated. We concluded that inhibitory L1 sequences acted by reducing both cytoplasmic poly(A)⁺ mRNA levels and mRNA utilization.

Inhibitory sequences in the HPV-16 late 3' UTR act by reducing mRNA levels and mRNA utilization. It has been reported that the HPV-16 late 3' UTR contains inhibitory sequences that act in *cis* to reduce expression of a reporter gene in transient transfection experiments (44). To investigate whether these sequences were functionally similar to the negative element in the HPV-16 L1 coding sequence identified here, we inserted an HPV-16 sequence (nt 6818 to 7453), containing the HPV-16 late 3' UTR and late poly(A) signal, downstream of a CMV promoter and an HIV-1 p17^{gag} gene used here as a reporter gene (65) (see Materials and Methods). This resulted in plasmid pCH16pA (Fig. 7A). A deletion which removed the previously identified negative element was introduced into pCH16pA, resulting in pCH16ΔpA (Fig. 7A). Production of p17^{gag} from cells transfected with pCH16pA or pCH16ΔpA was assessed by Western immunoblotting. Figure

7B showed that pCH16ΔpA produced high levels of p17^{gag}, while pCH16pA produced only low levels of p17^{gag}. The levels of p17^{gag} produced from pCH16ΔpA were similar to those produced from pCM1234pA (Fig. 7B), in which the HPV-16 late poly(A) signal was replaced with the SV40 early poly(A) signal (Fig. 7A). Western blot analysis of serial 2-fold dilutions with extracts from cells transfected with pCH16ΔpA revealed that approximately 64-fold higher levels of p17^{gag} were produced from pCH16ΔpA compared with those produced from pCH16pA (Fig. 7C). This is in agreement with previous studies of the inhibitory effect of this region of HPV-16 (44). Plasmid pCMVCAT was included as an internal control for transfection efficiency, and CAT levels did not vary more than twofold between the different transfections (data not shown).

To determine whether the 64-fold difference in p17^{gag} production between pCH16ΔpA and pCH16pA was reflected at the mRNA level, we analyzed cytoplasmic poly(A)⁺ mRNA produced from pCH16ΔpA and pCH16pA, respectively. The cytoplasmic poly(A)⁺ mRNA samples were serially diluted and subjected to random hexamer oligonucleotide-primed reverse transcription followed by PCR amplification with oligonucleotides specific for p17^{gag} mRNA or oligonucleotides specific for CAT mRNA produced from the internal control plasmid pCMVCAT. The RT-PCR results revealed that pCH16pA produced approximately fourfold lower levels of cytoplasmic poly(A)⁺ mRNA than did pCH16ΔpA (Fig. 7D), while CAT mRNA levels did not vary substantially (Fig. 7D). Similar to the inhibitory sequences in the L1 coding region, the negative element in the HPV-16 late 3' UTR acted in *cis* to decrease both cytoplasmic mRNA levels and mRNA utilization.

HIV-1 Rev and RRE can overcome the effect of the inhibitory element in the HPV-16 late 3' UTR. Since the presence of HIV-1 Rev in *trans* and RRE in *cis* could overcome the effect of the inhibitory sequences in the L1 coding sequence, we next tested if Rev and RRE could also overcome the effect of the inhibitory element in the HPV-16 late 3' UTR. RRE was therefore inserted into plasmid pCH16pA, generating pCH16RRE (Fig. 8A).⁹ Analysis of p17^{gag} production from this plasmid in the absence or presence of Rev revealed that Rev could overcome the negative effects of the inhibitory HPV-16 element (Fig. 8B). The p17^{gag} levels produced from pCH16RRE in the presence of Rev were similar to those produced from pCM1234pA (Fig. 8B), demonstrating that HIV-1 Rev was able to efficiently restore levels of expression of p17^{gag} protein. A quantitative estimate of p17^{gag} protein levels produced in the absence or presence of Rev revealed that the presence of Rev increased p17^{gag} protein levels by approximately 30-fold (data not shown).

We next analyzed nuclear and cytoplasmic poly(A)⁺ p17^{gag} mRNA levels in HLTat cells transfected with pCH16RRE in the absence or presence of Rev. Serial dilutions of extracted mRNAs were subjected to RT-PCR. The results revealed that high levels of p17^{gag} mRNA were present in the cytoplasm in the absence of Rev (Fig. 8C). A two- to fourfold increase in both nuclear and cytoplasmic p17^{gag} mRNA levels was observed in the presence of Rev (Fig. 8C). The amount of CAT protein produced from pCMVCAT included as an internal control for transfection efficiency varied less than twofold between transfections. The levels of spliced actin mRNA were similar in the absence or presence of Rev, demonstrating that similar amounts of poly(A)⁺ mRNA were used for analysis (Fig. 8C). To control for the fractionation technique used to separate nuclei from cytosol, RT-PCR was performed with oligonucleotides specifically detecting unspliced actin mRNA. These results showed that unspliced actin RNA was found

FIG. 7. Inhibitory sequences in the HPV-16 late 3' UTR. (A) Schematic structures of p17^{gag} expression plasmids. Solid boxes indicate the human CMV immediate early promoter. Darkly shaded boxes indicate the mutated p17^{gag} gene named p17M1234 (65). Open boxes indicate the HPV-16 late 3' UTR-containing sequences. The lightly shaded box indicates the SV40 early poly(A) signal. Brackets mark the limits of a deletion between nt 6858 and 7282. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). LP2 is the major late poly(A) signal present in the HPV-16 3' UTR and is shown as an open triangle (43, 60). The names of the plasmids are indicated on the left. (B) Western blot analysis of p17^{gag} production from HeLa cells transfected with pCH16ΔpA, pCH16pA, or pCM1234pA. Proteins were resolved on SDS-15% polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes. The p17^{gag} protein was detected with an HIV-1-positive patient serum, followed by incubation with horseradish peroxidase-conjugated anti-human immunoglobulin G antiserum. p17^{gag} was visualized with enhanced chemiluminescence detection reagents. The position of the p17^{gag} protein is indicated on the left. (C) Western blot analysis of p17^{gag} production from cells transfected with pCH16ΔpA and pCH16pA, respectively. Cell lysate from pCHPV1ΔpA-transfected cells was serially twofold diluted, and p17^{gag} protein was determined by Western immunoblotting as described in the legend to Fig. 7B. The position of p17^{gag} protein is indicated on the left. (D) RT-PCR analysis of dilutions of cytoplasmic poly(A)⁺ mRNA. Serial fourfold dilutions of cytoplasmic poly(A)⁺ mRNAs from cells transfected with pCH16ΔpA or pCH16pA were reverse transcribed, followed by PCR. The upper panel shows PCR amplification of the cDNA samples with oligonucleotides specific for the p17^{gag} sequence present in pCH16ΔpA and pCH16pA. The lower panel shows PCR amplification of the same cDNA samples with oligonucleotides specific for the CAT sequence present in the CAT expression plasmid used here to control for transfection efficiency. Oligonucleotides were end labeled with [γ -³²P]ATP prior to PCR. The PCR products were analyzed on 6% polyacrylamide gels, followed by autoradiography. The positions of the amplified DNA fragments are shown on the left. RT (-) indicates that RT-PCR was performed in the absence of RT in the cDNA synthesis reaction mixture.

primarily in the nuclear fraction (Fig. 8C). In conclusion, Rev had only a moderate effect on p17^{gag} mRNA levels, while p17^{gag} protein levels were greatly increased in the presence of Rev. Therefore, Rev and RRE acted primarily by increasing translation of the mRNAs containing inhibitory HPV-16 sequences.

SRV-1 CTE counteracts the effect of inhibitory sequences in the HPV-16 late 3' UTR. As shown above, the SRV-1 CTE element could efficiently overcome inhibition exerted by the inhibitory sequences in the HPV-16 L1 coding region. We also investigated whether the SRV-1 CTE could overcome inhibition exerted by the inhibitory sequences in the HPV-16 late 3' UTR. The SRV-1 CTE was inserted into pCH16pA in sense and antisense orientations, resulting in pCH16CS and pCH16CA, respectively (Fig. 9A). Figure 9B shows that pCH16CA produced low levels of p17^{gag}, while pCH16CS produced high levels of p17^{gag}, similar to those produced from pCM1234pA. These results demonstrated that the SRV-1 CTE

element was able to counteract the negative effect of the HPV-16 late 3' UTR sequences.

We also analyzed cytoplasmic poly(A)⁺ mRNA levels in HLaT cells transfected with pCH16CS or pCH16CA. RT-PCR was performed on serially fourfold-diluted RNA samples with oligonucleotides specific for p17^{gag} mRNA. The results revealed that approximately four times more p17^{gag} mRNA was present in cells transfected with pCH16CS than in cells transfected with pCH16CA (Fig. 9C). Actin mRNA levels in these two samples were similar, demonstrating that similar amounts of poly(A)⁺ mRNA were used in the RT-PCR. Quantitation of serially diluted p17^{gag} protein by Western blot analysis revealed that pCH16CS produced approximately 64-fold higher levels of p17^{gag} than did pCH16CA (data not shown). Therefore, the presence of the CTE element on the mRNA resulted in increased cytoplasmic poly(A)⁺ mRNA levels and increased translation of mRNAs containing inhibitory HPV-16 sequences.

A

B

C

FIG. 8. Comparison of p17^{gag} protein levels and nuclear and cytoplasmic mRNA levels from cells transfected with pCH16RRE in the absence or presence of Rev protein. (A) Schematic structures of p17^{gag} expression plasmids. Solid boxes indicate the human CMV immediate early promoter (CMV), shaded boxes indicate the mutated p17^{gag} gene named p17M1234 (65), and open boxes represent the HPV-16 late 3' UTR-containing sequences or the HIV-1 RRE as indicated. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). LP2 is the major late poly(A) signal present in the HPV-16 3' UTR and is shown as an open triangle (43, 60). The names of the plasmids are displayed on the left. (B) Western blot analysis of extracts of cells transfected with pCH16pA, pCM1234, or pCH16RRE in the absence or presence (+) of Rev protein. p17^{gag} production was analyzed by Western immunoblotting as described in the

Transcription in the cytoplasm relieves inhibition exerted by sequences in the HPV-16 late 3' UTR. The results described above showed that inhibition exerted by the negative elements in the HPV-16 L1 coding sequence could be relieved if transcription of these sequences occurred in the cytoplasm. We examined if this was also true for the inhibitory sequences in the HPV-16 late 3' UTR. The CMV promoter present in pCH16pA and pCH16ΔpA was replaced with the bacteriophage T7 promoter, resulting in plasmids pT7-16pA and pT7-16ΔpA (Fig. 10A), respectively. HeLa cells were infected with the recombinant vaccinia virus vTF7-3 (27, 28), producing T7 RNA polymerase, followed by transfection with pT7-16pA or pT7-16ΔpA. Western blot analysis revealed that similar high levels of p17^{gag} protein were produced from both plasmids (Fig. 10B), demonstrating that transcription in the cytoplasm relieves inhibition exerted by the inhibitory element in the HPV-16 late 3' UTR. These results indicate that nuclear factors are required for inhibition.

DISCUSSION

In this study, we show that the HPV-16 major capsid protein L1 coding region contains intragenic inhibitory sequences that act in an orientation-dependent manner to posttranscriptionally inhibit L1 expression. We also confirmed previous findings (44) that an independently acting inhibitory element is present in the HPV-16 late 3' UTR. The presence of these inhibitory elements on HPV late mRNAs offers an explanation for previous observations demonstrating that expression of HPV-16 capsid proteins is restricted to terminally differentiated keratinocytes. Interestingly, production of HPV-16 L1 could be activated with HIV-1 Rev and RRE or by providing the SRV-1 CTE element. Alternatively, inhibition could be bypassed by transcription of the HPV-16 L1 sequence in the cytoplasm with the vaccinia virus-T7 RNA polymerase-based expression system (27, 28), suggesting that nuclear processes participate in the inhibition.

Similarly to the HPV-16 L1 mRNAs, HIV-1 mRNAs encoding Gag, Pol, and Env proteins contain inhibitory sequences in coding regions (10, 14, 51, 57, 62, 65, 67). These sequences prevent expression of viral structural proteins in the absence of Rev. Comparison of inhibitory sequences in coding regions of HIV-1 gag with those in HPV-16 L1 mRNAs revealed that they have a high AU content. One inhibitory element on the HIV-1 gag mRNA was characterized in detail in previous work as an AU-rich sequence with an AU content of 61.5% (65, 67). The AU content of the HPV-16 L1 region associated with inhibitory sequences (nt 5813 to 6150) is 59.6%. Similarly, the previously identified negative elements in the HPV-1 and HPV-16 late 3' UTRs were described as AU-rich sequences (44, 72). AU richness may be a general property of viral RNA se-

legend to Fig. 7B. The position of p17^{gag} protein is indicated on the left. (C) RT-PCR analysis of nuclear and cytoplasmic poly(A)⁺ mRNA. The upper left panel shows PCR amplification of the cDNA synthesized from poly(A)⁺ mRNAs isolated from nuclear fractions of cells transfected with pCH16RRE in the absence or presence of Rev protein. Oligonucleotides were end labeled with [γ -³²P]ATP prior to PCR. The upper right panel shows PCR amplification of cDNA from spliced or unspliced actin mRNAs in the nuclear fraction of cells transfected with pCH16RRE in the absence or presence of Rev protein. The positions of the amplified DNA fragments are indicated on the left. The lower left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)⁺ mRNAs isolated from cells transfected with pCH16RRE in the absence or presence of Rev protein. The lower right panel shows PCR amplification of cDNA from spliced or unspliced actin mRNAs in the cytoplasmic fraction of cells transfected with pCH16RRE in the absence or presence of Rev protein.

FIG. 9. Inhibition exerted by the HPV-16 late 3' UTR is relieved by the presence of the SRV-1 CTE in the sense orientation. (A) Schematic structures of p17^{gag} expression plasmids. The solid boxes indicate the human CMV immediate early promoter (CMV), shaded boxes indicate the mutated p17^{gag} gene named p17M1234 (65), and open boxes represent the HPV-16 late 3' UTR-containing sequences or the SRV-1 CTE-containing sequence as indicated. Arrows indicate the orientation of the CTE in the plasmids. Numbers refer to nucleotide positions on the genomic HPV-16 clone (68). LP2 is the major late poly(A) signal present in the HPV-16 3' UTR and is shown as an open triangle (43, 60). The names of the plasmids are displayed on the left. (B) Western blot analysis of extracts from cells transfected with pCH16CS, pCH16CA, pCH16pA, or pCM1234pA. The production of p17^{gag} was analyzed by Western immunoblotting as described in the legend to Fig. 7B. The position of p17^{gag} protein is indicated on the left. (C) RT-PCR analysis of dilutions of cytoplasmic poly(A)⁺ mRNA. The left panel shows PCR amplification of cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)⁺ mRNAs isolated from cells transfected with pCH16CS or pCH16CA. Oligonucleotides were end labeled with [γ -³²P]ATP prior to use. The right panel shows PCR amplification of cDNA synthesized from spliced actin mRNAs in the cytoplasmic fraction of cells transfected with pCH16CS or pCH16CA. The positions of the amplified DNA fragments are indicated on the left.

quences with inhibitory function. In addition, several cellular mRNAs that are under posttranscriptional regulation contain AU-rich inhibitory sequences in the 3' UTR. Such AU-rich sequences usually contain multiple copies of the AUUUA pentanucleotide (13, 63). This sequence motif is not present in the negative element in the HPV-16 late 3' UTR (44). However, the inhibitory region in the HPV-16 L1 coding sequence (nt 5813 to 6150) contains seven AUUUA pentanucleotides. Additional experiments are required to establish the role of AUUUA motifs in HPV-16 L1 posttranscriptional inhibition. The various sequence motifs proposed by Kennedy et al. to play a role in the inhibitory function of the negative element in the HPV-16 late 3' UTR (44) were not present in the L1

FIG. 10. Transcription in the cytoplasm could bypass inhibition exerted by the inhibitory element in the HPV-16 late 3' UTR. (A) Structures of the p17^{gag} expression plasmids. Small open boxes indicate the bacteriophage T7 promoter, and large open boxes indicate HPV-16 sequences. Shaded boxes indicate the mutated p17^{gag} gene named p17M1234 (65). Bent arrows indicate the direction of transcription driven by the T7 promoter. The brackets mark the limits of a deletion between nt 6858 and 7282. The numbers indicate nucleotide positions on the genomic HPV-16 clone (68). LP2 is the major late poly(A) signal present in the HPV-16 late 3' UTR and is shown as an open triangle (43, 60). The names of the plasmids are indicated on the left. (B) Western blot analysis of p17^{gag} production. HeLa cells were infected with recombinant vaccinia virus vTF7-3 (27, 28), followed by transfection with pT7-16pA or pT7-16ΔpA. Production of p17^{gag} was analyzed by Western immunoblotting as described in the legend to Fig. 7B. The position of p17^{gag} protein is indicated on the left.

inhibitory coding region. In bovine papillomavirus type 1, splice site-like sequences have been proposed to play a regulatory role in late gene expression (29). Sequences with substantial homology to the 5' splice site consensus sequence AAGGUAAGU could not be found in the HPV-16 L1 inhibitory region (nt 5813 to 6150), while the HPV-16 late 3' UTR contains 5' splice site-like sequences. It has been suggested that these sequences inhibit HPV-16 late gene expression (29). It has been reported that unutilized splice sites on HIV-1 mRNAs inhibit late gene expression in the absence of Rev (11, 49). This is not easy to reconcile with the fact that *gag* and *pol* mRNAs produced from simple retroviruses lacking *rev* genes also contain unutilized splice sites but are efficiently exported to the cytoplasm. There is also growing evidence that sequences distinct from splice sites inhibit expression of HIV-1 late genes in the absence of Rev (10, 57, 65). If such sequences are altered by mutagenesis, production of the HIV-1 late gene products occurs in the absence of Rev (65).

Although the function of HIV-1 Rev has been extensively studied, the exact mechanism of Rev regulation is not fully elucidated. It is generally accepted that Rev facilitates transport of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm (17, 25, 26, 36, 59). In addition, Rev has been proposed to increase mRNA stability (25, 52, 67) and translatability (2, 18, 48). Our results on HPV expression reported here revealed that high levels of L1 mRNA produced from plasmid p16L1 were present in the cytoplasm in the absence or

presence of Rev, while L1 protein was only detectable in the presence of Rev. When Rev protein was provided in *trans*, cytoplasmic L1 mRNA levels produced from p16L1RRE increased less than fourfold (Fig. 2E), while high levels of L1 protein were produced (Fig. 2C). This demonstrated that HPV-16 L1 mRNAs were produced and exported from the nucleus in the absence of Rev. However, these mRNAs were not efficiently translated, indicating that the interaction of Rev and RRE improves utilization of RRE-containing mRNAs. Similar results were obtained when expression of CAT mRNA and protein levels in cells transfected with pNLCATW or pCATLISS, which contains HPV-16 L1 inhibitory sequences, were compared. There is only a 4-fold difference between the levels of cytoplasmic CAT mRNA produced from these two plasmids, while the difference in CAT protein levels was greater than 140-fold. It is possible that in the absence of Rev protein, the HPV-16 L1 mRNAs are transported to a cellular compartment in which they are not accessible to the cellular translation machinery. Our results are consistent with those of previous studies showing that the Rev protein has a positive effect on translation efficiency by affecting mRNA loading on polysomes (2, 18, 48). To explain these results, we postulate that the route by which mRNAs are transported from the nucleus to the cytoplasm or the cellular factors associated with the mRNA may determine the fate of mRNAs in the cytoplasm. Rev may efficiently interact with RRE-containing mRNAs by helping them enter a productive nuclear export pathway used by cellular mRNAs, thereby directing them to the cellular translation machinery. Alternatively, Rev may compete with a nuclear factor that interacts with the inhibitory sequences and acts by inhibiting RNA processing or translation. Therefore, Rev may indirectly affect translation of RRE-containing mRNAs. It is reasonable to assume that events which decrease utilization of the HPV-16 L1 mRNA occur in the nucleus before the mRNA enters the cytoplasm, since transcription of HPV-16 L1 coding sequences in the cytoplasm with a vaccinia virus-T7 RNA polymerase-based expression system resulted in loss of inhibition.

In addition to the HIV-1 Rev-RRE system, the SRV-1 CTE could activate expression of HPV-16 L1. The CTE element does not require the presence of viral *trans*-acting factors for function, indicating that a Rev-like cellular factor interacts with this sequence to overcome the inhibitory effect of the negative elements on the HPV-16 L1 mRNAs. Since a positive element similar in function to the SRV-1 CTE has not been identified on the late HPV-16 mRNAs, it is conceivable that cellular factors which bind to the HPV-16 inhibitory element are present in basal cells of the epithelium, while expression or activity of such cellular factors is inhibited in terminally differentiated keratinocytes. Alternatively, positive cellular or viral regulatory factors able to compete with negative factors bound to inhibitory RNA sequences are activated in fully differentiated keratinocytes. Since a Rev-like protein has not been identified in any known HPV, we favor a model which predicts that cellular negative factors bound to inhibitory HPV sequences are inactivated as epithelial cells differentiate. This would allow expression of HPV late gene products.

Although inhibitory elements present in the HPV-16 L1 coding sequence and HPV-16 late 3' UTR are structurally different and functionally independent, they act in parallel to reduce polyadenylated mRNA levels and to inhibit translation of mRNA. The fact that multiple inhibitory elements are present on the HPV-16 late mRNAs indicates that the presence of these elements may be important to ensure that viral L1 protein is produced only when infected cells differentiate and reach the superficial layer of the epithelium. Production of

virus in terminally differentiated keratinocytes may be important for efficient spreading of virus or for the avoidance of an immune reaction against HPV-producing cells. Similarly, HIV-1 mRNAs contain multiple inhibitory elements that prevent expression in the absence of Rev. Also *c-myc* (6), *c-fos* (42, 69, 74), and urokinase-type plasminogen activator (56) mRNAs are found to contain multiple inhibitory elements, suggesting that this is a general feature of posttranscriptionally regulated mRNAs.

Although HPV-16 and HIV-1 are not related, the negative effect on gene expression exerted by the HPV-16 L1 inhibitory element could be overcome by HIV-1 Rev and RRE. This is a surprising finding which indicates that HIV and HPV may use a similar strategy to regulate expression of late genes. However, HIV-1 late mRNAs are located primarily in the nucleus in the absence of Rev, while the HPV-16 L1 mRNAs are located in the cytoplasm but are not translated. This may imply a different kind of regulation. The results presented here support the idea that Rev is a positive regulator able to correct different defects on viral or cellular mRNAs at a posttranscriptional level. In agreement with this, it was recently shown that the effect of a cellular mRNA instability element in the 3' UTR of the transferrin receptor mRNA could be overcome by the Rev-RRE regulatory system of HIV-1 or by the Rex-RexRE system of human T-cell leukemia virus type 1 (75).

The finding that CTE or Rev and RRE can activate HPV-16 L1 expression in human epithelial cells may have practical applications. HPVs cannot be propagated *in vitro*, presumably as a result of a block in late gene expression in cells other than terminally differentiated keratinocytes. A genomic HPV-16 clone containing CTE inserted in the late region may produce virus upon introduction into human cells. Furthermore, HPV-16 L1 protein has been shown to self-assemble into viruslike particles in insect cells with a baculovirus-based expression system (46). Viruslike particles have proved to be a more specific antigen than denatured particles in serological screenings of HPV-infected individuals (45). It will be of interest to investigate if HPV-16 L1 produced with the posttranscriptional regulatory systems of HIV-1 and SRV-1 assembles into viruslike particles in human cells and if such viruslike particles induce strong immune responses against HPV-16.

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