JOURNAL OF VIROLOGY, June 1991, p. 3317–3329 Vol. 65, No. 6
0022-538X/91/063317-13$02.00/0
Copyright © 1991, American Society for Microbiology

An E1M^E2C Fusion Protein Encoded by Human Papillomavirus Type 11 Is a Sequence-Specific Transcription Repressor

CHENG-MING CHIANG, THOMAS R. BROKER, AND LOUISE T. CHOW*

Department of Biochemistry, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, New York 14642

Received 27 November 1990/Accepted 17 March 1991

We have isolated a putative, spliced E5 cDNA of human papillomavirus type 11 (HPV-11) by polymerase chain reaction amplification of cDNAs from an experimental condyloma. Using retrovirus-mediated gene transfer, we isolated two novel HPV-11 cDNAs, one of which had a splice linking nucleotides 1272 and 3377. This transcript also existed in experimental condylomata and in cervical carcinoma cells transfected with cloned genomic HPV-11 DNAs. The 5′ end of the transcript in transfected cells originated upstream of the initiation codon of the E1 open reading frame (ORF). It could conceptually encode a fusion protein consisting of the amino-terminal 23% of the E1 ORF and the carboxy-terminal 40% of the E2 ORF. This E1M^E2C fusion protein contained both the DNA replication modulator domain E1M, as defined in the bovine papillomavirus system, and the DNA binding domain of the E2 protein, which regulates viral transcriptional activities. Indirect immunofluorescence with polyclonal antibodies raised against the bacterially expressed TrpE-HPV-11 E2 protein demonstrated nuclear localization of the E1M^E2C protein in cells transiently transfected with an expression plasmid. Immunoprecipitation revealed a specific protein with an apparent molecular weight of 42,000 in transfected cells. The chloramphenicol acetyltransferase assay established that the putative E1M^E2C protein was a potent transcriptional repressor of both E2-dependent and E2-independent HPV-11 enhancer/promoter activities. Northern (RNA) blot hybridization indicated the repression was on the transcriptional level. Mutational analysis suggested that the E1M^E2C protein is an E2-binding site-specific repressor. The fusion protein also repressed bovine papillomavirus type 1 (BPV-1) E2 protein-dependent BPV-1 enhancer activity. When constitutively expressed in mouse C127 cells, the E1M^E2C protein inhibited BPV-1 transformation and episomal DNA replication, consistent with a role in the modulation of replication.

Human papillomaviruses (HPV) infect only human epithelial tissues and normally cause benign hyperproliferation at anatomic sites preferred by each of the individual HPV types. One of the most studied HPV is type 11 (HPV-11), which causes genital warts (condylomata) and laryngeal papillomatosis. Although no papillomavirus has been propagated in cell cultures or in animal tissues, laboratory production of HPV-11 (Hershey isolate) has been achieved by implanting infected neonatal human foreskin chips under the renal capsules of nude mice (34). HPV-11 mRNAs from such experimental condylomata and from patient biopsy specimens have been characterized by a variety of methods (16, 46, 55, 58, 69). Additionally, the pattern of viral DNA replication and gene expression in patient specimens and the time course of infection in experimental condylomata have been studied by in situ hybridization with mRNA exon-specific riboprobes (62, 63). The copy numbers of both viral mRNAs and DNA are extremely low in basal epithelial cells, which serve as reservoirs of infection and as stem cells for the differentiating epithelium. Viral activities increase with cellular differentiation, and virion assembly takes place only in some of the superficial, terminally differentiated keratinocytes.

Transcriptional regulation of HPV and bovine papillomavirus (BPV) has been investigated in cultured cells by transient transfection of the chloramphenicol acetyltransferase (CAT) reporter gene linked to viral enhancer and promoter elements. The E2 open reading frame (ORF) encodes several enhancer and promoter regulatory proteins that are translated from mRNAs generated by alternative splicing of primary transcripts derived from different promoters (14, 54, 55, 67, 72). These E2 proteins bind to a consensus responsive sequence, ACCN$_6$GGT (E2-RS), present in multiple copies in the upstream transcription regulatory region (URR; also called the long control region [LCR]) of all papillomaviruses (1, 12, 18, 24, 28, 39, 43, 45). The full-length E2 protein can *trans*-activate (18, 23–25, 27, 50, 59, 65, 67) or repress (5, 11, 53, 60, 65, 68) homologous or heterologous papillomavirus promoters. Proteins consisting predominantly of the carboxyl half of the E2 ORF (E2-C for HPV-11, E2-tr and E8/E2 for BPV type 1 [BPV-1]) retain the protein dimerization and DNA binding domain, are competitive repressors of E2-dependent transcription, and also can repress E2-independent enhancer/promoter activities, probably by interfering with the assembly of transcription complexes (11, 12, 14, 37).

Control of papillomavirus DNA replication is less clear, and virtually all studies have been carried out with BPV-1 transformed mouse cell lines. In these cells, the BPV-1 DNA persists as extrachromosomal plasmids at a constant copy number (38) and replicates in synchrony with host chromosomes once per cell cycle (6, 52). In contrast, most HPV DNA is found integrated in transfected, immortalized, or transformed cells in culture. The origin of BPV-1 DNA replication in transformed cells has been localized to the enhancer region by using two-dimensional gel electrophoresis (71). However, in cutaneous warts of cows, both Cairns (θ) and rolling circle forms of replication intermediates have been detected (8). The inferred replication origins were widely distributed in the viral genome. It is possible that the virus uses different origins of replication during the mainte-

* Corresponding author.

nance state in the transformed cells and during vegetative propagation.

A *trans*-acting factor which modulates BPV-1 replication has been mapped to the 5′ region of the E1 ORF and is termed E1M (4, 40, 51). The replication modulator(s) is required to maintain a constant copy number of BPV-1 DNA in transformed mouse C127 cells and to prevent runaway replication of a simian virus 40 (SV40)/BPV-1 dual replicon in transfected COS cells (monkey kidney cells constitutively expressing the SV40 T antigen). A 23,000 M_r protein in transformed mouse cells is postulated to be BPV-1 E1M and its putative cDNA has been described (14, 66), but no function has been demonstrated directly. An approximately 70,000 M_r protein corresponding to the full-length E1 ORF has been identified and postulated to be the viral replication factor (56, 64).

Using retrovirus-mediated gene transfer (9), we recovered two extremely rare and novel HPV-11 cDNAs. We demonstrated that both species existed in experimentally induced condylomata of human tissues and in human cervical carcinoma C-33A cells transfected with cloned HPV-11 whole genomic DNA. In this report, we describe the characterization of one of these mRNAs, which contained a splice linking viral nucleotides (nt) 1272 and 3377. It had the potential to encode an E1M^E2C fusion protein consisting of the putative E1M replication modulator domain and the E2-C DNA binding domain. Functional analysis suggested that the protein is an E2-responsive site specific transcriptional repressor and that binding of this protein to one or more copies of the E2-RS located in the URR directly or indirectly contributed to the control of papillomavirus DNA replication. In the course of this study, we also recovered a spliced putative E5 mRNA directly from an experimental condyloma by using the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Plasmid constructions. Plasmid W1-29 (see Fig. 2A) is a Moloney murine leukemia virus vector pLJ (31) containing the HPV-11 E-region subgenomic sequence spanning nt 951 to 4402 in the sense orientation. The 5′ end of the HPV insertion was generated by BAL 31 exonuclease digestion from the *Bam*HI site at HPV-11 nt 812 in plasmid pLJX/11E6A$^+$ (54), followed by a *Bam*HI linker addition. The fragment containing nt 951 to 4402 of the HPV-11 genome together with the SV40 sequence was excised by *Bam*HI and *Hin*dIII digestion and recloned into the *Bam*HI and *Hin*dIII sites of the pLJ vector.

The protein initiation codon of the HPV-11 E1 ORF is at nt 832 to 834. To reconstruct the 5′ end of the retrovirus-recovered cDNA, clone C32 DNA spanning nt 951-1272^3377-4402 was restricted with *Nde*I and *Hin*dIII. The fragment spanning HPV-11 nt 1102-1272^3377-4402 and the downstream SV40 early promoter was ligated to the *Bam*HI-*Nde*I restriction fragment of pRS/11E6A$^+$ containing HPV-11 nt 812 to 1101 and cloned into the *Bam*HI and *Hin*dIII sites of pGEM-7Zf(+) (Promega) to generate pCM40-7. The SV40 early promoter-enhancer sequence at the 3′ end of the cDNA was removed to create pCM-7, as follows: the *Pst*I-*Hin*dIII fragment of pCM40-7 was replaced with a corresponding fragment from pUC19/MOR10 which contains HPV-11 nt 3900 to 4402 but no SV40 sequences (54). This latter fragment contains both the HPV polyadenylation signal and the poly(A) addition site (46). The cDNA (nt 812-1272^3377-4402) was then excised from pCM-7 with *Bam*HI and *Hin*dIII digestions and recloned into pr779, an expression vector using the Rous sarcoma virus (RSV) long terminal repeat (LTR) modified to contain a polylinker (12), to generate pRS-CM. The cDNA was also transferred into pLJX (54), a derivative of the Moloney murine leukemia virus vector pLJ lacking the 3′ LTR and the polyomavirus sequence, to generate pLJX(SV$^-$)-CM.

Two mutations in the E1M^E2C cDNA were first introduced into pCM-7 and then recloned into eucaryotic expression vectors. To generate the *N*-terminal deletion cDNA clone pRS-CM-*dl*951, the *Bam*HI-*Nde*I fragment of pCM-7 was replaced with the corresponding fragment from C32 to generate pCM-7-*dl*951. The frameshift mutation plasmid pRS-CM-*Nar*I was made by restricting and then filling in the *Nar*I site at nucleotide 3425 and religating the blunt ends. The cDNA from pCM40-7 was also recloned into pLJX at the *Bam*HI and *Hin*dIII sites to generate pLJX-CM, which was used to establish the C127 cell lines containing E1M^E2C (see below). Mutations were confirmed by restriction enzyme digestion or by DNA sequencing.

pMT2-CM was constructed for overexpression of the E1M^E2C fusion protein as follows. pCM-7 DNA was cleaved with *Bam*HI and *Hin*dIII. *Eco*RI linkers were added at both ends of the cDNA insertion after filling in the staggered ends with the Klenow fragment of *Escherichia coli* DNA polymerase I. The cDNA was then cloned into the *Eco*RI site of pMT2 vector (30) in the orientation for transcription from the adenovirus major late promoter, giving a fusion transcript with the bulk of the tripartite leader sequences at the 5′ end. The vector also contains the adenovirus-associated RNA I gene and the SV40 DNA replication origin and enhancer sequences. The deletion clone, pMT2-CM-*dl*951, was similarly constructed by moving the *Bam*HI and *Hin*dIII fragment from pCM-7-*dl*951 into pMT2.

Additional CAT plasmids used in this study included pUR23-3 (12), pUR27, pUR61, pCAT-A (27), and pSV2CAT (20), which express the bacterial CAT gene from the HPV-11 URR-E6 promoter, the HPV-11 URR-SV40 minimal early promoter, the BPV-1 LCR-SV40 minimal early promoter, the SV40 minimal early promoter, and the SV40 enhancer-early promoter, respectively.

Retrovirus-mediated gene transfer. Two retroviral systems were used to recover cDNAs. In the first method, 20 μg of W1-29 was transfected into psi-2 helper cells (42) by calcium phosphate coprecipitation, as described by Graham and van der Eb (21) and modified by Parker and Stark (49). Viral particles were harvested 18 h after transfection and used to infect mouse C127 cells. After G418 selection (1 mg/ml), individual drug-resistant colonies were expanded into cell lines and fused separately with COS cells, as previously described (9). Low-molecular-weight plasmid DNA in the Hirt lysates that contained the excised cDNA was rescued by transformation into *E. coli* DH5-α cells selected with kanamycin (50 μg/ml). Minipreparations of DNA were then made by the LiCl-boiling method (70) and used for restriction enzyme analyses. In the second method, 10 μg of W1-29 was cotransfected with 10 μg of pMOV-psi$^-$ (42) into the human cervical carcinoma cell line C-33A (American Type Culture Collection). Viral particles were harvested 21 and 42 h after DNA transfection and used to infect mouse C127 cells. The remaining procedures were the same as described for the packaging cell line. Both procedures produced the same 1272^3377 and 1459^3325 splices.

Amplification of HPV-11 cDNAs by the PCR. cDNAs were generated by PCR amplification of poly(A)$^+$ RNA isolated from experimental condylomata (34). In addition, cDNAs

were obtained from RNAs recovered from human C-33A cervical carcinoma cells transfected with whole HPV-11 genomic DNA cloned in either orientation at the *Bam*HI site (nt 7072) in vector pSVO10, which contains the minimal SV40 early promoter and no enhancer. A sense strand primer (nt 5′ 1093-1112 3′) and an antisense strand primer (nt 3′ 3388-3407 5′) flanking the mRNA splice junction were used for PCR amplification of the first-strand cDNAs by using *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer/Cetus), as described (55). One percent of the first-round PCR products served to seed a second round of amplification.

To localize selectively the 5′ end of the E1M^E2C transcript in samples containing overlapping message species, a splice junction-spanning primer (3′ 1263-1272^3377-3386 5′) in the antisense orientation was used in combination with one of several upstream, sense strand primers. The anticipated cDNA fragment was first recovered by using the sense strand primer 5′ 748-767 3′ under modified reaction conditions. Briefly, a 100-μl reaction mixture containing all ingredients under mineral oil was preheated to 90°C. The Vent DNA polymerase (New England BioLabs), rather than the *Taq* DNA polymerase, was then added. PCR was performed for 30 cycles, each cycle consisting of 1 min of denaturation at 94°C, 90 s of annealing at 63°C, and 2 min of elongation at 72°C, with the final cycle completed with a 7-min extension at 72°C. The reaction products were then loaded on a 1.2% agarose gel for electrophoresis. Only one band, which was of the anticipated length, was visible after ethidium bromide staining. It was eluted and reamplified with the *Taq* DNA polymerase by using the same splice junction antisense primer prepared for the initial isolation in combination with one of three sense strand primers: 5′ 748-767 3′, 5′ 812-831 3′, or 5′ 1093-1112 3′. As visualized in a 3.5% polyacrylamide gel, a partial cDNA of the appropriate size was generated with each primer pair, in addition to a nonspecific band which appeared in each reaction.

DNA sequence determination. Miniprep DNA for sequencing was purified as described (33), with some minor modifications. The purified DNA was used directly for double-stranded DNA sequencing with the T7 DNA polymerase (Sequenase), as described by the manufacturer (US Biochemicals). Direct sequencing of the PCR-amplified DNA eluted from gels was performed as described (26) by using one of the amplification primers or an internal primer from nt 5′ 1147-1166 3′.

Transient transfection and CAT assays. C-33A cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells in each confluent 100-mm plate were divided among 21 60-mm plates and transfected 24 h later with a high-efficiency protocol (10) which we modified for transient transfection by including 5 mM sodium butyrate (19) in the medium. Briefly, 4 μg of CAT plasmid and 8 μg of cDNA expression plasmid or cloning vector were diluted into 0.5 ml of 2× BES (*N*,*N*-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid)-buffered saline containing 50 mM BES (pH 6.95), 280 mM NaCl, and 1.5 mM Na_2HPO_4. A total of 0.5 ml of 0.25 M $CaCl_2$ was then added into the solution dropwise during vortexing. The DNA-calcium phosphate coprecipitate formed in 10 to 20 min at room temperature was dispensed onto duplicate 60-mm plates. Cells were incubated at 35°C with 3% CO_2 for 15 to 24 h. The medium was removed, and the cells were rinsed twice, each time with 2 ml of DMEM. Five milliliters of DMEM supplemented with 10% fetal bovine serum and 5 mM sodium butyrate was added to each plate, and the cells were incubated at 37°C with 5% CO_2 for an additional 24 h. All transfection experiments for CAT assays were performed independently at least twice, each time in duplicate.

To prepare lysates for the CAT assays, the cells were harvested after trypsinization and resuspended in 50 μl of 0.25 M Tris-HCl, pH 7.8. Three cycles of freezing and thawing were conducted at −70 and 37°C. The lysates were heated at 70°C for 10 min to inactivate any endogenous deacetylase. After centrifugation at room temperature for 5 min, 15 or 5 μl of supernatant was used for the CAT assay monitored by the fluor-diffusion method (47). Samples of pure CAT enzyme (0.5, 1.0, and 4.0 U, as well as 0 U in the negative control) were assayed in parallel as the standard curve and for calculating total counts per minute (cpm) used in the reaction. Conversion was defined as the ratio (cpm of products minus background cpm)/(input cpm).

Northern analysis of CAT transcripts. For physical analysis of CAT gene transcripts, 30 μg of CAT reporter plasmid and 60 μg of one of the cDNA expression plasmids or cloning vector were transfected into six 100-mm plates of C-33A cells as just described. Forty-eight hours after transfection, two plates were harvested for CAT assay and the remaining four plates were used for harvesting total RNA by the guanidinium isothiocyanate-cesium chloride method (13). Northern (RNA) blotting was performed by using 30 μg of total RNA per lane in a 1% formaldehyde agarose gel (41). After electrophoretic transfer onto a Nytran membrane (Schleicher & Schuell), the blots were probed with either the 552-bp *Nco*I-*Hin*dIII fragment of the CAT gene from pSV2CAT or a mouse α-actin cDNA probe (44) as an internal standard. Both probes were prepared by the random hexamer labeling method according to the instructions of the manufacturer (Amersham). Additional Northern blots of HPV-11 RNAs isolated from C127-derived cell lines were probed with whole genomic HPV-11 DNA.

Transformation assays. Five micrograms of a wild-type BPV-1 clone, pdBPV-1(142-6) (57), obtained from P. M. Howley, was cotransfected with 10 μg of various cDNA expression plasmids or cloning vector into mouse C127 cells (also from P. M. Howley) in 100-mm plates by the protocol of Chen and Okayama (10). Two days after transfection, cells were divided among three 100-mm plates. The media were changed every 4 days. Two weeks later, foci either were counted following fixation in methanol and staining with Giemsa solution or were individually isolated and expanded into cell lines for Southern blotting analysis.

For transformation assays in the C127-derived cell lines that were previously transduced with vector or cDNA expression vectors, only 5 μg of wild-type BPV-1 DNA was used. The cDNA-containing cell lines or pooled cells were established by retrovirus-mediated gene transfer (9) and were confirmed by the successful rescue of the cDNAs after fusion with COS cells and in some cases by Northern blot hybridization, which demonstrated a low level of expression of the HPV-11 cDNAs.

Southern blotting. Ten micrograms of total DNA isolated from individual cell lines expanded from foci induced by BPV-1 in C127 cells or in C127-derived, HPV-11 cDNA-containing cells was sheared by 10 passages through a 22-gauge needle before being loaded onto a 0.8% agarose gel for one-dimensional electrophoretic analysis. The BPV-1 DNA released from the pML2d vector by restriction enzyme digestion was labeled by the random hexamer method.

Indirect immunofluorescence. A confluent 60-mm plate of C-33A cells was resuspended in 50 ml of DMEM supplemented with 10% fetal bovine serum, and 1 ml of cell

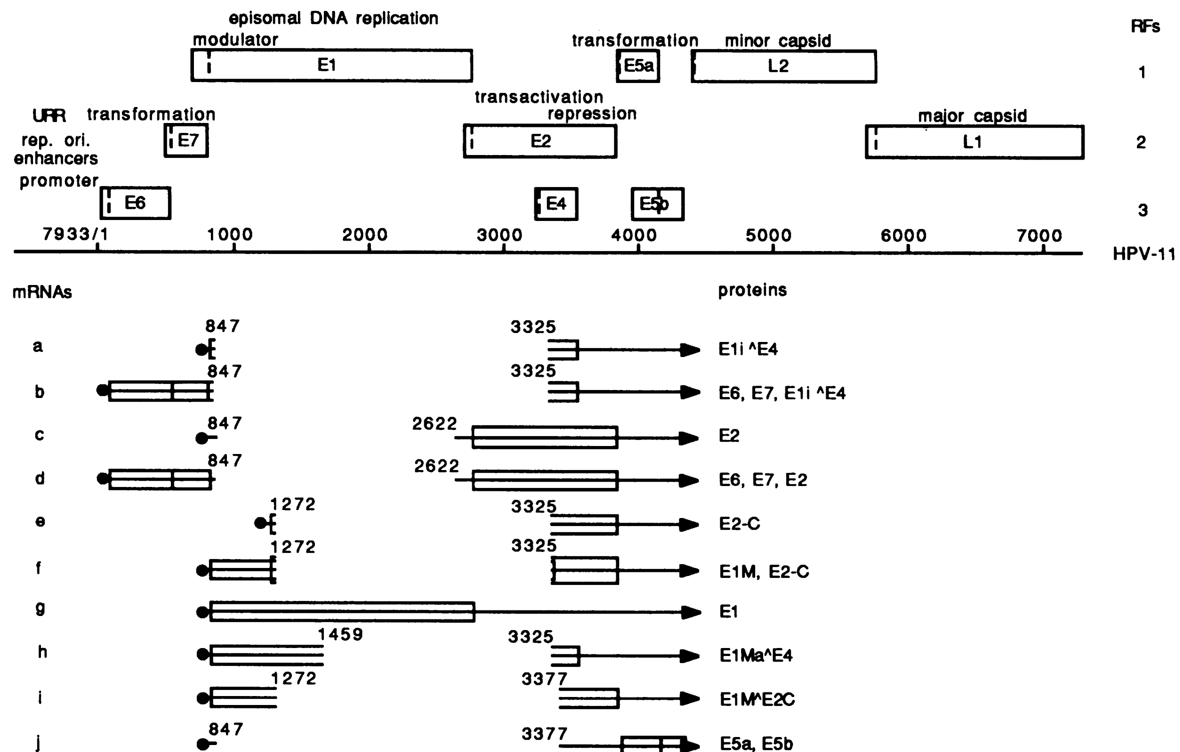

FIG. 1. Coding potentials of the HPV-11 E-region transcripts. The circular genome is represented in a linear form with the ORFs (open boxes) and their possible functions indicated above. Vertical dashed lines inside each ORF mark the location of the first AUG codon. All viral E-region transcripts are depicted as arrows in the 5′-to-3′ direction, with gaps representing introns spliced out of the transcripts and numbers indicating the nucleotide positions of exon boundaries adjacent to splice donors and acceptors. The closed circle at the 5′ end of each message represents the proven or putative promoter, and the arrowheads at the 3′ ends designate the polyadenylation sites. Coding potentials, as deduced from the cDNA sequences, are drawn as open boxes superimposed on each mRNA arrow, and the encoded proteins are named at the 3′ end of each transcript. Viral RNA species a through g have been described previously (16, 46, 54, 55).

suspension was then dispensed into each well of two-well chambered slides the day before DNA transfection. The transfection procedures were performed as described for the CAT assays, except that only 0.1 ml of the DNA-calcium phosphate coprecipitate was added to each chamber. Forty-eight hours later, cells were probed with rabbit polyclonal antibodies raised against the bacterially expressed TrpE-HPV-11 E2 fusion protein or E1i^E4 control protein expressed in *E. coli* (15, 28a) by using the procedure described by Labow et al. (36). Biotinylated goat anti-rabbit immunoglobulin G antibodies were purchased from Vector Laboratories, and avidin-fluorescein isothiocyanate conjugate was purchased from Sigma Chemical Co.

Immunoprecipitation from transfected cells. Transient transfection in COS-7 cells at 25% confluency (100-mm plates with 20 μg of DNA in each) was performed by the procedure described above, except that no sodium butyrate was added. Forty-eight hours later, cells were washed once in methionine-free DMEM and labeled for 3 h with 200 μCi of Tran-^{35}S label (ICN) per ml in methionine-free DMEM supplemented with 5% normal DMEM, 2 mM glutamine, and 10% fetal bovine serum. Cell extracts were prepared by adding 1 ml of RIPA buffer (17) containing 0.25 mM phenylmethylsulfonyl fluoride by the procedure described by Harlow and Lane (22). The lysates from each plate were divided into halves, which were then immunoprecipitated with rabbit polyclonal antibodies raised against the bacterially expressed TrpE-HPV-11 E2 fusion protein or HPV-11 E1i^E4 protein. Protein A Sepharose CL-4B (Pharmacia) was used for immunoadsorption, and one-third of the recovered proteins were analyzed in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel.

RESULTS

Recovery of novel cDNAs following retrovirus-mediated gene transfer. Families of alternatively spliced HPV-11 mRNA species in genital condylomata have been mapped by the electron microscopic R-loop method, and the resulting reading frame fusions were characterized by direct sequence determination after PCR amplification of cDNAs from an experimental condyloma (16, 55) (Fig. 1). These particular cDNA splice junction fragments could not easily be reconstructed for functional tests. A complementary method of retrovirus-mediated gene transfer has been employed to obtain intact cDNAs (54). However, rare HPV-11 mRNAs could not be recovered because the predominant splice donor site at nt 847 in the E1 ORF is also used preferentially in the helper cell line. Only the most abundant E1i^E4 mRNA with the nt 847^3325 splice and the E2 mRNA with the nt 847^2622 splice were generated. To recover additional and rare viral mRNAs by using the retrovirus system, a subgenomic DNA clone W1-29 which spanned HPV-11 nt 951 to 4402 was cloned into pLJ, a Moloney murine leukemia virus vector (Fig. 2A). It was introduced into the psi-2 packaging cell line or cotransfected with a helper plasmid

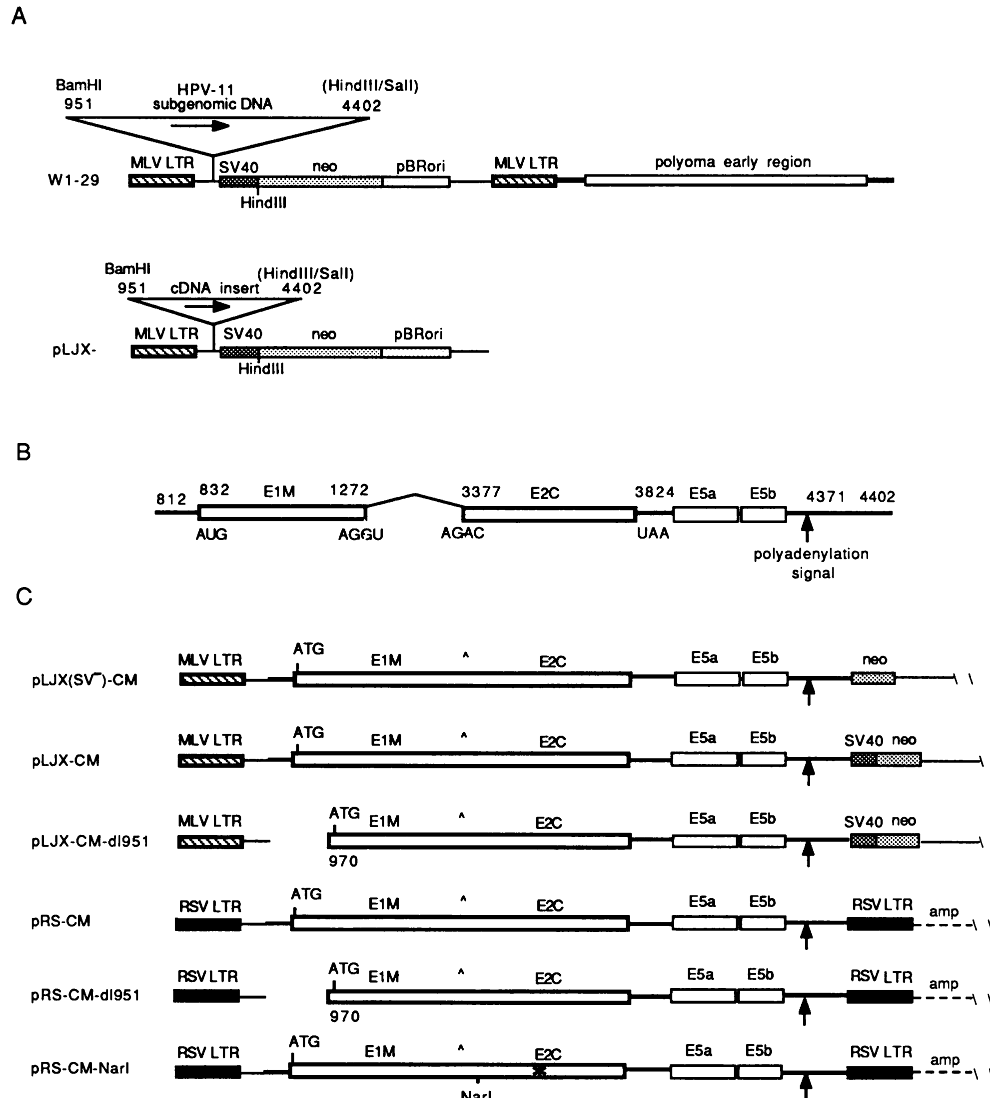

FIG. 2. Recovery of HPV-11 cDNAs via retrovirus-mediated gene transfer, and expression vectors for wild-type and mutated E1M^E2C cDNAs. (A) Retrovirus-mediated gene transfer. W1-29, the progenitor clone used to recover novel cDNAs, contained the HPV-11 subgenomic sequence spanning nt 951 to 4402 in the pLJ vector. The cDNAs, with the introns removed, were rescued after homologous recombination between the flanking Moloney murine leukemia virus (MLV) LTRs and recovered as plasmids in the pLJX vector, which contains only one MLV LTR and lacks the polyomavirus early region (54). (B) Reconstructed E1M^E2C cDNA. The retrovirus-recovered E1M^E2C cDNA was reconstructed by restoring its 5′ end to nt 812, thus including the initiation codon of E1 ORF at nt 832 to 834. (C) Expression vectors for E1M^E2C cDNA and mutations. The vertical bar at the beginning of each cDNA coding sequence indicates the initiation codon. Carets mark the fusion of two ORFs by mRNA splicing. The asterisk designates the site of translation termination by a frameshift mutation at the *Nar*I site. Gaps represent deletions. \\ indicates additional vector sequences.

pMOV-psi$^-$ (42) into the human cervical carcinoma cell line C-33A (see Materials and Methods). Two cDNAs previously unknown in any papillomavirus were recovered by both methods (Fig. 1, species h and i).

The first cDNA originates from a transcript containing a splice between nt 1272 and 3377 (Fig. 3, right panel). The splice donor site at nt 1272 is highly conserved among papillomaviruses (16, 48) and has been found joined to a highly conserved acceptor at nt 3325 in an HPV-11 mRNA recovered from an experimental condyloma (55). The relatively rare mRNAs with the splice 1272^3325 have the potential to encode the E1M protein, the E2-C protein, or both, depending on the locations of the 5′ ends and the possibility of internal reinitiation of translation. The alternative splice acceptor site at nt 3377 or its equivalent has not been reported in any other papillomavirus. If the 5′ end of the new transcript extends upstream to include the E1 AUG codon at nt 832-834, the 1272^3377 splice would generate an in-frame fusion between the 23% amino-terminal portion of the E1 ORF and the 40% carboxy-terminal portion of the E2 ORF (Fig. 1, species i). The second cDNA corresponds to a transcript with a splice between nt 1459 and 3325. This alternative nt 1459 donor site is also novel. If translation initiated from the E1 AUG codon at nt 832-834, this splice would connect the amino-terminal 32% of the E1 ORF in frame with the same E4 coding region as in the E1i^E4

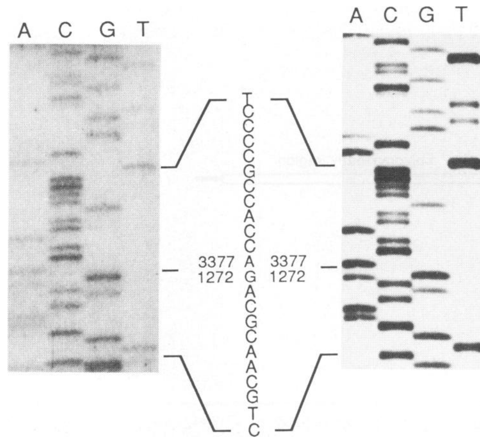

FIG. 3. HPV-11 E1M^E2C cDNA splice junction. Left panel, direct sequencing of PCR-amplified E1M^E2C cDNA (see Fig. 4A, lanes 1 and 2) of poly(A)$^+$ RNA recovered from an experimental condyloma (34); right panel, miniprep DNA sequencing of the retrovirus-recovered cDNA (C32).

message, thereby generating an alternative E1Ma^E4 fusion protein (Fig. 1, species h). The characterization of this cDNA and the encoded protein is the subject of a separate report. As is the case with all E-region transcripts, both new cDNAs could potentially encode E5a and E5b proteins should internal reinitiation occur during translation.

Confirmation of the novel HPV-11 mRNAs in vivo and in transfected cells by PCR amplification and direct DNA sequence determination. The new splice combinations for both the putative E1M^E2C and E2Ma^E4 transcripts conformed to the mRNA splice junction consensus sequence /GU...AG\. Their authenticity was confirmed by direct sequencing of PCR-amplified cDNA generated from two experimental condylomata by using the primer pair 5′ 1093-1112 3′ and 3′ 3388-3407 5′ flanking the splice sites (Fig. 3, left panel, and Fig. 4A, lanes 1 and 2) (data not shown). Both new transcripts were much less abundant than the previously described rare E2-C RNA with a splice of nt 1272 to 3325 (Fig. 4A, lanes 1 and 2). All three RNAs were also generated in C-33A cells transfected with whole genomic HPV-11 DNA linearized at the *Bam*HI site (nt 7072) in the L1 ORF and cloned in either orientation into the vector pSVO10, which contains the minimal SV40 early promoter with no enhancer (Fig. 4A, lanes 3 and 4). In the transfected cells, both new RNAs were more abundant than the E2-C transcript.

5′-end mapping of the E1M^E2C transcript. To determine whether the 5′ end of the transcript with the 1272^3377 splice was located upstream of the E1 initiation codon at nt 832-834, a necessary condition for encoding the putative E1M^E2C fusion protein, we initially used sense strand PCR primers spanning nt 5′ 748-767 3′ or 5′ 800-819 3′ and an antisense strand primer from nt 3′ 3388-3407 5′ to amplify cDNAs prepared from the experimental condylomata. However, these primer pairs always preferentially amplified more abundant overlapping viral cDNAs, especially the predominant E1i^E4 species (data not shown). In an attempt to

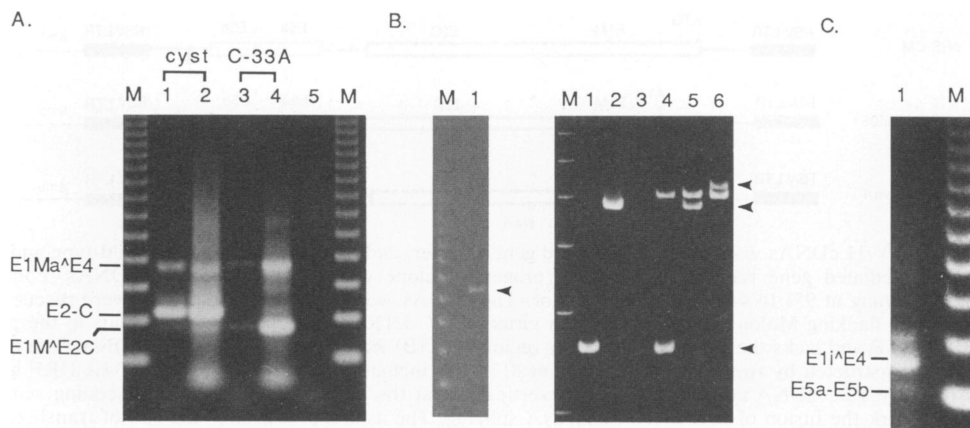

FIG. 4. Identification of novel HPV-11 transcripts by PCR. (A) Confirmation of the splice junctions of the newly identified E1M^E2C and E1Ma^E4 cDNAs. Poly(A)$^+$ RNA was isolated from an experimental condyloma (cyst) or from HPV-11 transfected human C-33A cells. The first round of 30 cycles of PCR was performed with the sense strand primer (nt 5′ 1093-1112 3′) and the antisense strand primer (nt 3′ 3388-3407 5′) to amplify the first-strand cDNA (lanes 1 and 3). One percent of the first-round PCR products was subjected to amplification by a second round of 30 cycles (lanes 2 and 4). A negative control without the cDNA template is displayed in lane 5. PCR products were separated in a 1.5% agarose gel. The amplification products of the E1M^E2C (nt 1272^3377), E2-C (nt 1272^3325), and E1Ma^E4 (nt 1459^3325) DNAs were confirmed by direct sequencing of each individual band (data not shown). (B) 5′-end mapping of the E1M^E2C transcript in HPV-11 transfected C-33A cells. Left panel, first round of PCR amplification of poly(A)$^+$ RNA isolated from transfected C-33A cells by using Vent DNA polymerase with the sense strand primer 5′ 748-767 3′ and the antisense strand splice junction-spanning primer of the E1M^E2C transcript (nt 3′ 1263-1272^3377-3386 5′). The product was displayed in a 1.2% agarose gel. Right panel, second round of PCR amplification from eluted first round E1M^E2C cDNA (lanes 4 to 6) by using *Taq* DNA polymerase with various upstream primers in combination with the E1M^E2C splice junction-spanning primer. pRS-CM DNA (0.5 ng) was included as positive control (lanes 1 to 3). Upstream primers are as follows: lanes 1 and 4, 5′ 1093-1112 3′; lanes 2 and 5, 5′ 812-831 3′; lanes 3 and 6, 5′ 748-767 3′. The products were separated in a 3.5% polyacrylamide gel. Arrowheads point to the correct cDNA fragments. (C) Identification of the novel E5 cDNA in an experimental condyloma. PCR was performed with the sense strand (nt 5′ 748-767 3′) and antisense strand (nt 3′ 3388-3407 5′) primers to amplify the first-strand cDNA from an experimental condyloma. The amplification products of the E1i^E4 (nt 847^3325) and E5a-E5b (nt 847^3377) cDNAs were confirmed after cloning and sequencing the individual bands (data not shown). The PCR products were separated in a 1.5% agarose gel. M, Length markers consisting of a 123-bp ladder.

confer hybridization specificity, a primer spanning the splice junction of the E1M^E2C transcript (3′ 1263-1272^3377-3386 5′) was used with the expectation that stable hybridization over the 20 base pairs would occur only to the cDNA with the particular splice targeted, whereas alternative splicing of the upstream or downstream exon would offer only 10 base pairs of hybrid. However, only nonspecific products were generated under standard PCR conditions (data not shown). Elongation by the *Taq* DNA polymerase from mismatched 3′ ends has also recently been observed by others (35). This phenomenon appears to be caused by the lack of proofreading activity in the *Taq* polymerase. "Vent" DNA polymerase isolated from archaebacteria that colonize undersea thermal vents possesses 3′→5′ proofreading activity and is extremely heat stable. When Vent polymerase was employed under the more stringent hybridization conditions (63°C rather than the standard 55°C; see Materials and Methods), we were successful in obtaining the correct E1M^E2C cDNA, along with nonspecific products, from RNAs isolated from transfected cells, using the sense strand primer 5′ 748-767 3′ and the antisense splice junction primer (Fig. 4B, left panel). Upon a second round of amplification with the *Taq* DNA polymerase by using other internal sense strand primers in combination with the same junction primer, products of expected sizes as well as a nonspecific band were observed (Fig. 4B, right panel). Direct-sequence determination of the second-round PCR products demonstrated that each was indeed the correct cDNA (data not shown). We concluded that the 5′ end of the E1M^E2C transcript extended at least as far upstream as nt 748, where the 5′-most sense strand primer was placed. Therefore, the transcript indeed has the potential to encode a fusion protein derived from the strong initiation codon at the beginning of the E1 ORF, with splicing at the end of the E1M domain into the E2 ORF to create an E1M^E2C fusion protein. The nature of the nonspecific PCR products was not pursued.

Recovery of a novel E5 cDNA from an experimental condyloma by PCR. In the course of our attempts to determine the 5′ end of the E1M^E2C transcript by using PCR on first-strand cDNAs generated from either experimental condylomata or transfected cells, a faint band directly below the E1i^E4 transcript was reproducibly observed (Fig. 4C). It was cloned after PCR amplification by using the sense strand primer 5′ 748-767 3′ and the antisense strand primer 3′ 3388-3407 5′ or 3′ 4147-4166 5′. Sequence determination indicated that it contained a splice from nt 847 to 3377. The ORF starting from the E1 initiation codon at nt 832 closes at nt 3433 in the second exon. The only significant ORFs present downstream were the E5a (nt 3871 to 4143) and E5b (nt 4146 to 4367) coding sequences. Therefore, this new transcript has the potential to encode the E5a and E5b proteins only (Fig. 1, species j). It displays a similarity to the BPV-1 E5 transcript (72) in that an approximately 500-nt 5′ untranslated region precedes the ORFs, possibly providing a region for translational regulation.

Transcriptional repression of the E2-independent HPV-11 enhancer/E6 promoter and E2-dependent enhancers by cotransfection with the E1M^E2C cDNA in C-33A cells. To test the function of the putative E1M^E2C protein, the 5′ portion of the retrovirus-recovered cDNA was restored to nt 812 to include the E1 initiation codon (nt 832-834). The SV40 early promoter-enhancer sequence at the 3′ end of the cDNA was removed to prevent any complication during the functional assays. The reconstructed cDNA spanning nt 812-1272^3377-4402 (Fig. 2B) was then recloned into the pLJX vector for expression from the Moloney murine leukemia virus LTR [pLJX(SV⁻)-CM] or into pr779 for expression from the RSV LTR (pRS-CM) (Fig. 2C). The E2C domain is slightly shorter than that in the previously characterized HPV-11 E2-C protein (encoded by nt 3325 to 3823) but still retains the DNA binding domain which resides within the C-terminal 107 amino acids (29a), similar to the binding domain in BPV-1 E2 proteins (43, 45). To assay effects on homologous and heterologous promoters, plasmids pUR23-3 and pSV2CAT were separately transfected into human cervical carcinoma C-33A cells together with various expression plasmids or with the vector without an insertion. pUR23-3 and pSV2CAT express the bacterial CAT gene from the HPV-11 enhancer-E6 promoter and the SV40 early promoter, respectively. The results showed that the SV40 early promoter was not affected by the expression of any HPV-11 protein (Table 1). In contrast, cotransfection with the E1M^E2C cDNA (pRS-CM) repressed pUR23-3 by 50-fold. For comparison, the repression of pUR23-3 by the previously characterized E2-C protein expressed from the same RSV vector (pRSE2-C) was only sixfold, and cotransfection of the E1i^E4 cDNA expression vector had little, if any, effect. Repression of E2-independent viral E6 promoter activity by E2 or E2-C has been postulated to stem from interference of the assembly of transcription complexes around the viral TATA motif by the binding of the E2 proteins to E2-RS adjacent to the TATA motif (5, 11, 12, 53, 65). When expressed from the Moloney murine leukemia virus LTR pLJX(SV⁻)-CM, the E1M^E2C protein also repressed the HPV-11 enhancer activity to a similar extent and had no effect on the SV40 promoter (Table 1). pRS-CM did not repress pRSVCAT, which expresses CAT from the RSV LTR (data not shown).

The effects of various expression vectors on the E2-dependent enhancer activity were also compared by cotransfection of pUR27 (HPV-11 URR-SV40 minimal early promoter-CAT) or pUR61 (BPV-1 LCR-SV40 minimal early promoter-CAT) together with a BPV-1 E2 expression plasmid, pRSE2BP (27). Both CAT plasmids have high basal activity in C-33A cells and were induced by the BPV-1 E2 protein by only 2.3- or 4.5-fold (Table 2). None of the

TABLE 1. Repression of the HPV-11 enhancer-E6 promoter by vectors expressing E1M^E2C or mutated cDNAs[a]

Plasmid	pUR23-3		pSV2CAT	
	% Total substrate converted to products	Relative activity	% Total substrate converted to products	Relative activity
pr779	12.5	1.00	24.4	1.00
pRSE4	11.3	0.90	31.4	1.29
pRSE2-C	2.1	0.17	24.1	0.99
pRS-CM	0.3	0.02	21.9	0.90
pRS-CM-*dl*951	1.5	0.12	31.3	1.28
pRS-CM-*Nar*I	9.2	0.74	22.4	0.92
pLJX	34.6	1.00	55.0	1.00
pLJX(SV⁻)-CM	1.3	0.04	56.0	1.02

[a] Transfections were performed in C-33A cells by using 2 μg of CAT plasmid and 4 μg of cDNA expression plasmid or cloning vector. CAT assays were conducted at 37°C for 1 h with 30% (for pUR23-3) or 10% (for pSV2CAT) total cell lysate from a 60-mm plate 48 h after transfection. pr779 is the cloning vector containing the RSV LTR. pRSE4 and pRSE2-C express HPV-11 E1i^E4 and E2-C cDNAs, respectively (12). Clones expressing E1M^E2C or mutated versions are described in Fig. 2C. In pUR23-3 and pSV2CAT, the CAT gene was linked to the HPV-11 enhancer-E6 promoter and the SV40 enhancer-early promoter, respectively. The results are the averages of two experiments, each performed in duplicate.

TABLE 2. Repression of HPV-11 and BPV-1 E2-dependent enhancer activities by E1M^E2C protein[a]

Plasmid(s)	pr779		pRSE4		pRSE2-C		pRS-CM	
	% Total substrate converted to products	Relative activity	% Total substrate converted to products	Relative activity	% Total substrate converted to products	Relative activity	% Total substrate converted to products	Relative activity
pUR27	30.8	1.00						
pUR27 + pRSE2BP	70.2	2.28	76.0	2.47	54.3	1.76	41.7	1.35
pUR61	4.6	1.00	6.0	1.30	4.3	0.93	4.1	0.89
pUR61 + pRSE2BP	20.7	4.50	17.1	3.71	8.7	1.89	8.8	1.91

[a] CAT assays for E2-dependent enhancer activity were performed with 2 μg of pUR27 or pUR61, 0.2 μg of pRSE2BP, and 3.8 μg of HPV-11 cDNA expression plasmids or cloning vector. CAT assays for the E2-independent enhancer activity were performed as described in Table 1. In pUR27 and pUR61, the CAT gene is expressed from the minimal SV40 early promoter linked to the HPV-11 URR and the BPV-1 LCR, respectively (27). pRSE2BP is the expression vector for BPV-1 E2 protein (27).

cotransfected control plasmids affected the E2-independent activity. Cotransfection of pRSE2-C or pRS-CM repressed much of the BPV E2 activation, whereas the other plasmids had little effect. The inability to repress to basal level (relative activity, 1.0) may be due to a higher affinity of the BPV E2 protein than HPV-11 E2 proteins for E2-RS. Alternatively, the BPV E2 amino terminus is known to have a general transcription activation function (23a). Together with the data presented in Table 1, these results demonstrated that the E1M^E2C expression vector specifically repressed enhancers and promoters that contain E2 protein responsive sites but not heterologous promoters which do not contain an E2-RS.

To determine the mechanism of repression, we performed Northern blot hybridization to quantitate the CAT transcripts present in total RNA isolated from transfected C-33A cells. Figure 5 shows that the repression of pUR23-3 occurred at the transcriptional level. The 1.8- and 1.4-kb bands, corresponding to the correctly or aberrantly spliced CAT transcripts, respectively (29), were both greatly reduced by the E1M^E2C and E2-C proteins relative to the levels present when the control cloning vector or E1i^E4 cDNA were cotransfected.

Determination of the functional domains of the E1M^E2C protein by mutational analysis. To localize the functional domains of the protein encoded by the novel cDNA, two mutation clones (Fig. 2C) were constructed and tested by CAT assays following transient transfection into C-33A cells. The amino-terminal deletion mutation pRS-CM-*dl*951 still retains an in-frame initiation codon at nt 970-972 which has the sequence context of a good initiator (32). This mutation therefore should encode an amino-terminal truncated E1M^E2C protein. pRS-CM-*Nar*I, a two-base insertion at nt 3425, resulted in a frameshift into the E4 ORF and translation termination at nt 3579-3581, and thus the DNA binding domain of the E1M^E2C protein was lost. The results of cotransfection with pUR23-3 are shown in Table 1. An amino-terminal truncation in pRS-CM-*dl*951 reduced the ability to repress compared with the wild-type E1M^E2C expression vector. The degree of residual repression was similar to that achieved with the simple E2-C protein. In contrast, the frameshift mutation pRS-CM-*Nar*I completely eliminated the repressor function of E1M^E2C. These studies clearly demonstrated that the intact E1M^E2C protein was responsible for the highly efficient E2-RS-specific transcriptional repressor activity. They further indicated that the DNA binding domain was essential for the repressor function and that the presence of the E1M domain appeared to contribute to the more effective repressor activity compared with the previously described E2-C protein (see also Discussion).

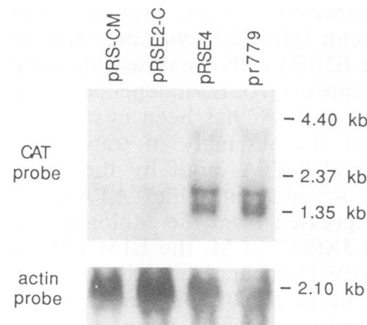

FIG. 5. Transcriptional repression revealed by Northern blot hybridization of CAT transcripts. Hybridization was performed with 30 μg of total RNA harvested from C-33A cells transfected with pUR23-3 and various cDNA expression plasmids or the vector. The blots were probed with either the 552-bp *Nco*I-*Hin*dIII fragment of the CAT gene from pSV2CAT or with the mouse α-actin cDNA as an internal control. The 1.8-kb CAT RNA corresponds to the correctly spliced transcripts, and the 1.4-kb band represents aberrantly spliced transcripts (29). RNA size markers (Bethesda Research Laboratories) are indicated.

Nuclear localization of the E1M^E2C protein in transfected cells. Subcellular localizations of the E2-C and E1M^E2C proteins were determined in C-33A cells transiently transfected with expression vectors by indirect immunofluorescence with rabbit serum raised against the TrpE-HPV-11 E2 fusion protein expressed in *E. coli* (28a). As shown in Fig. 6A and C, both E1M^E2C and E2-C proteins were localized to the nucleus. No signal was detected when the primary rabbit sera were raised against the E1i^E4 protein expressed in *E. coli* (Fig. 6B) (15, 28a). The nuclear localization was consistent with the presence of two possible nuclear localization signals in the E1M^E2C protein. One putative signal (PKKVKRR) is located in the E1M domain (encoded by nt 1186 to 1206), while the other (PPRKRAR) is in the E2C domain (encoded by nt 3428 to 3448).

Immunoprecipitation of the E1M^E2C protein in transfected cells. The E1M^E2C fusion protein in transfected cells was further characterized in COS-7 cells. The E1M^E2C cDNA was expressed from pMT2-CM by using the adenovirus major late promoter, placing the majority of the tripartite leader sequence at the 5′ end of the transcript. A protein of approximately 42,000 M_r was immunoprecipitated specifically when antibodies raised against TrpE-HPV-11 E2 pro-

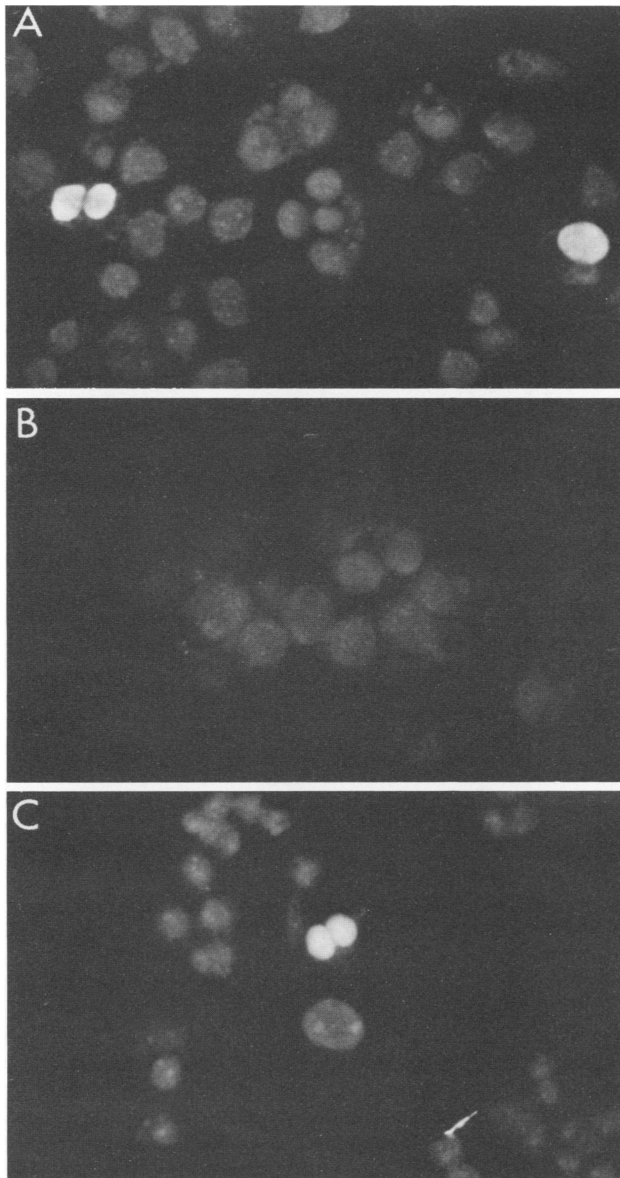

FIG. 6. Immunofluorescence staining of C-33A cells transiently transfected with HPV-11 cDNA expression plasmids. C-33A cells were cotransfected with pUR23-3 together with pRS-CM (E1M^E2C) (A and B) or pRSE2-C (C), as described in Materials and Methods. Polyclonal antibodies raised against the TrpE-HPV-11 E2 fusion protein (A and C) or E1i^E4 protein (B) were used as the primary antibodies for indirect immunofluorescence staining.

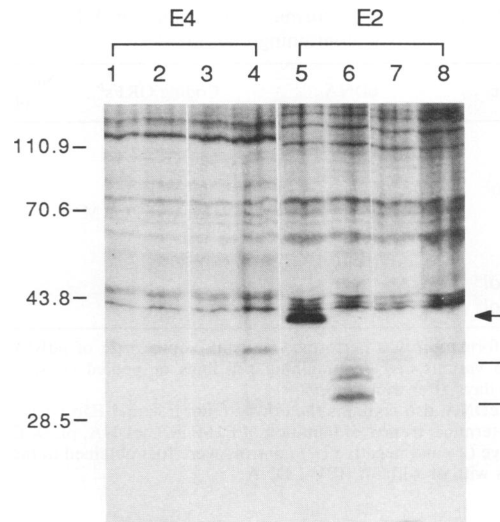

FIG. 7. Immunoprecipitation of E1M^E2C fusion protein in transiently transfected COS-7 cells. COS-7 cells were transfected with pMT2-CM (lanes 1 and 5), pMT2-CM-*dl*951 (lanes 2 and 6), pMT2 (lanes 3 and 7), and pLJX-CM (lanes 4 and 8). Anti-HPV-11 E4 (lanes 1 to 4) or anti-TrpE-HPV-11 E2 (lanes 5 to 8) antibodies were used for immunoprecipitation, and the recovered proteins were analyzed in an SDS–10% polyacrylamide gel. The arrow points to the E1M^E2C fusion protein, and the bracket indicates various forms of *N*-terminal truncated proteins. Protein size markers (in kilodaltons) were also included. Each lane contained about one-sixth of the immunoprecipitated lysate from a 100-mm plate. The autoradiograph shown was exposed for 13 h.

tein, but not those raised against E4, were used (Fig. 7, lanes 1 and 5). The size was somewhat larger than the anticipated 33,600 M_r because of either posttranslational modification or unusual migration associated with the high negative charge (−17) in the E1M domain. Protein species of approximately 31,000, 35,000, and 36,000 M_r were precipitated by sera directed against TrpE-HPV-11 E2 when the *N*-terminal truncated cDNA, pMT2-CM-*dl*951, was expressed, and the E4 antisera did not react (Fig. 7, lanes 2 and 6). The heterogeneity might be due to modification or lower stability of this truncated protein. No specific protein was detected when cells were transfected with the vector pMT2 (Fig. 7, lanes 3 and 7). The expression of this protein from pLJX-CM was too weak to be detected (Fig. 7, lanes 4 and 8).

Repression of BPV-1 transformation and episomal DNA replication in C127 cells. Because of its ability to repress BPV-1 E2-dependent BPV-1 enhancer activity and its similarity to the BPV-1 E1M protein, we tested whether the HPV-11 E1M^E2C protein could also repress BPV-1 transformation, as does the BPV-1 E2-transcriptional repressor E2-tr (37). We cotransfected BPV-1 DNA with E1M^E2C expression plasmid pRS-CM into C127 cells. The frequency of BPV-1 transformation was not altered. Southern blot hybridizations of individual transformants indicated that all BPV-1 DNA remained episomal in the transformants (data not shown). However, when BPV-1 DNA was transfected into C127-derived clones or pooled cells that were previously transduced with recombinant retrovirus and stably expressed the E1M^E2C cDNA from a Moloney murine leukemia virus LTR, the BPV-1 transformation frequency was reduced 60-fold (Table 3). The inhibition was largely alleviated in 29-1, a C127-derived cell line transduced with the amino-terminal truncated E1M^E2C cDNA pLJX-CM-*dl*951 (i.e., C32). No inhibition was observed in the C127-derived cell line 1-2′, which was transduced with the vector-containing virus, or in other C127-derived cell lines transduced with viruses containing HPV-11 E6-E7-E1i^E4 or E6-E7-E2 control cDNAs (Table 3 and data not shown). Line 3-3′ transduced with the HPV-11 E2 cDNA had a slightly increased transformation efficiency. Examination of the physical state of the BPV-1 DNA in the E1M^E2C-containing C127 cells revealed that it was integrated into cellular chromosomes at low copy number in about half of the expansion clones, while in the other half of the transfor-

TABLE 3. BPV-1 transformation frequency in HPV-11 cDNA-containing cell lines[a]

Cell line	cDNA	Coding ORFs[b]	No. of foci/μg of DNA
CM1-1	812-1272^3377-4402	E1M^E2C	6
CM1-6	812-1272^3377-4402	E1M^E2C	5
CM1-pool	812-1272^3377-4402	E1M^E2C	5
29-1	951-1272^3377-4402	E1M^E2C (M)[c]	139
3-3′	812-847^2622-4402	E2	401
1-2′	pLJX	None	218
+ Control[d]			314
− Control[d]			2

[a] Transformation was performed by transfecting 5 μg of pdBPV-1(142-6) DNA into various cDNA-containing cell lines or pooled cells. Foci were scored 16 days after transfection.

[b] Each cDNA also contains the downstream E5a and E5b ORFs.

[c] M, *N*-terminal truncated mutation of E1M^E2C cDNA, pLJX-CM-*dl*951.

[d] Positive (+) and negative (−) controls were foci obtained in the parental C127 cells with or without BPV-1 DNA.

mants both episomal plasmids and integrated BPV-1 DNA were observed (Fig. 8A). In the truncated mutant E1M^E2C cell line 29-1 as well as in C127-derived cell lines transduced with the other HPV-11 cDNA expression vectors, BPV-1 DNA remained episomal (Fig. 8B and data not shown). It should be pointed out that we consistently observed rearrangements or deletions in the episomal BPV-1 DNA in most of the BPV-1 transformants generated in control cell lines, including those derived from transfection of the parental C127 cells with BPV-1 DNA alone (data not shown). We speculate that the rearrangements might be related to the method of transfection.

DISCUSSION

In previous experiments using retrovirus-mediated gene transfer with HPV-11 subgenomic segments spanning the entire E region, only transcripts utilizing the dominant splicing donor site at nt 847 were recovered (54). By modifying the input HPV-11 DNA to a subgenomic fragment from which the predominant splice donor was deleted, we were able to recover two rare and novel cDNAs. One cDNA contained a splice from nt 1272 to 3377 and had a potential to encode an E1M^E2C fusion protein. The other contained a splice between nt 1459 and 3325 and could encode an E1Ma^E4 fusion protein. The alternative splice donor site at nt 1459 and acceptor site at nt 3377 were both unexpected because no equivalent transcripts have been reported for any other papillomavirus. Both splice combinations existed in transfected cells and in experimental condylomata, although neither species has previously been detected by electron microscopic R-loop or PCR studies using RNAs isolated from patient lesions or from experimental condylomata induced by HPV-11, attesting to their rarity. We postulate that both mRNAs might exist only in the basal cells to exert negative regulation on viral DNA replication and RNA transcription in a low-copy maintenance state. Because these cells are only a small fraction of a condyloma, the relative and absolute abundances of these mRNAs would be extremely low. Although we could not define their 5′ ends by using RNA recovered from an experimental condyloma, several lines of evidence suggested that these mRNAs existed in vivo and were most likely initiated from the E1 promoter or other promoters farther upstream. First, we have demonstrated that both novel RNA splice combinations existed in experimental condylomata by using PCR amplification with primers flanking the splice sites and direct DNA sequencing (Fig. 3 and data not shown). The resulting splice junction fragments were much less abundant than the previously characterized rare E1M and E2-C mRNAs (Fig. 4A, lanes 1 and 2). Second, we showed by PCR that the 5′ ends of both E1M^E2C (Fig. 4B) and E1Ma^E4 (our unpublished data) transcripts extended at least as far upstream as nt 748 in human cervical carcinoma cells transfected with whole genomic HPV-11 DNA cloned in either orientation. Third, several possible splice acceptor sites functionally equivalent to the HPV-11 nt 3377 can be found in BPV-1 as well as in other HPV specific for the genital tract. Fourth, the splice acceptor at nt 3377 was also used in the putative E5 mRNA recovered from the same experimental condyloma.

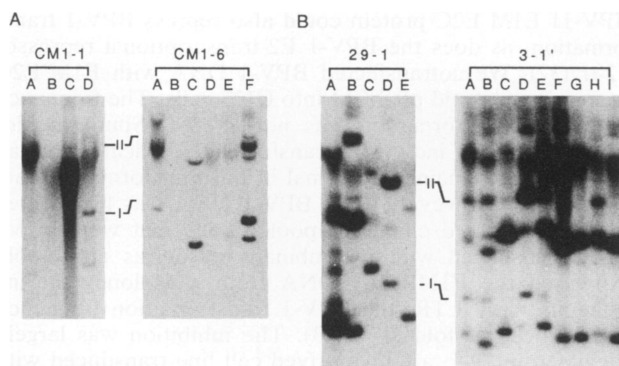

FIG. 8. Southern blot hybridization of BPV-1 DNA in various HPV-11 cDNA-transduced C127 cell lines. (A) Repression of BPV-1 extrachromosomal DNA replication in E1M^E2C cDNA-containing cell lines. CM1-1 and CM1-6 were independent C127-derived cell lines that were transduced with HPV-11 E1M^E2C cDNA via retrovirus-mediated gene transfer. (B) BPV-1 DNA in a C127-derived cell line (29-1) previously transduced with the amino-terminal truncated E1M^E2C cDNA (pLJX-CM-*dl*951) and an E2 cDNA-containing cell line (3-1′). Each lane represents an individual BPV-1 transformant. The migration positions of closed (I) and open (II) circular forms of the input pdBPV-1(142-6) DNA were indicated. Most of the episomal BPV-1 DNA suffered deletions or rearrangements.

Because of the similarities among all papillomaviruses, we are inclined to think that other papillomaviruses also encode this novel E1M^E2C fusion protein. It most likely exists in small amounts and thus so far has escaped detection. Alternatively, it might only be produced in homologous host systems. Indeed, differences in gene expression between BPV-1-transformed rodent cells and bovine fibropapillomas or BPV-1-infected bovine fibroblasts have been reported. More species of E1 transcripts were detected in transformed bovine cells than in the transformed murine cells (2, 7, 14, 61, 72). Accordingly, identification of an analogous E1M^E2C transcript from other papillomaviruses might require natural lesions or homologous host cells.

We demonstrated that the E1M^E2C protein was localized to the nucleus and had an apparent molecular weight of 42,000 (Fig. 6 and 7). It was a potent sequence-specific transcription repressor. It repressed both E2-dependent and E2-independent papillomavirus enhancer/promoter regions that contain E2-responsive sites. It did not affect the SV40 early promoter or the RSV LTR, which do not contain an E2-RS. On the basis that the mutation removing the E2C DNA binding domain completely eliminated the repressor

function, we conclude that the E1M^E2C protein functions by binding to the E2-RS, as do the E2 and E2-C proteins (12, 28). Mutational analysis also suggested that the E1M domain might contribute to the high repressor activity of the E1M^E2C protein relative to that of the E2-C protein, which contains no E1M domain (12) (Table 1). An intact E1M domain was also required for suppressing BPV-1 transformation and episomal DNA replication in C127-derived cell lines (Table 3, Fig. 8). The role of E1M could be explained by several hypotheses that are not mutually exclusive. First, the E1M domain might play an active role in transcription repression. Second, the E1M^E2C mRNA might be more efficiently translated than the E2-C mRNA. Third, either the E1M^E2C mRNA or protein might be more stable than E2-C.

The E1M^E2C protein repressed BPV-1 episomal replication when it was constitutively expressed in the cell prior to the introduction of the BPV-1 DNA (Table 3). If the expression vector was cotransfected with the BPV-1 DNA into the recipient cells, no effect was detected, unlike the results obtained with BPV-1 E2-tr (37). We interpret these observations to mean that the E1M^E2C protein did not repress the BPV-1 enhancer as strongly as BPV-1 E2-tr. There are several possible explanations. The DNA binding domains of BPV-1 E2 and HPV-11 E2 proteins are not identical, despite the identical consensus binding site $ACCN_6GGT$, and the binding affinities might be expected to differ. The internal 3 or 4 bp of the four copies of HPV-11 E2-RS are all A:T, whereas those of BPV-1 are not. It has been shown that BPV-1 and HPV-16 E2 proteins have different contact points on the DNA strand and that their affinities are differentially affected by these internal nonconsensus nucleotides (3, 45). Therefore, it is conceivable that the HPV-11 E1M^E2C protein may bind the BPV-1 E2-RS less efficiently than does the BPV-1 transcriptional repressor. Since the E1M^E2C expression plasmid could not replicate autonomously in transfected C127 cells, it would be lost quickly. Thus, the BPV-1 DNA was able to sustain episomal replication long enough to achieve a normal transformation efficiency. However, when the recipient cells expressed the HPV-11 E1M^E2C protein constitutively prior to the introduction of BPV-1, the BPV-1 transcription and replication (see below) were under continuous suppression, resulting in the loss of BPV-1 episomes, the integration of BPV-1 DNA, and a greatly reduced transformation efficiency (Table 3, Fig. 8A). Possible mechanisms for suppressing BPV-1 transformation and hindering episomal replication involve (i) an indirect effect of transcriptional repression, as just discussed, and (ii) binding of the fusion protein to the BPV-1 origin of replication at E2-responsive sequences (71). The E1M domain of the HPV-11 fusion protein exhibits a great deal of homology to that of BPV-1. Interactions between the E1M domain of the bound fusion protein and other positive replication factors such as the intact E1 protein and other viral and host proteins might then lead to the suppression of autonomous BPV-1 replication. Consistent with this possibility is the observation that, in COS cells containing stable SV40/BPV-1 composite plasmids, the SV40 large T antigen is sequestered, possibly by interaction with the BPV-1 E1M protein(s) (52), and is not available to participate in the replication of other incoming plasmids that contain the SV40 origin of replication.

The putative E1Ma^E4 fusion protein also represses transcription of both homologous and heterologous promoters by mechanisms yet to be defined. When constitutively expressed in C127-derived cells, E1Ma^E4 also strongly inhibited autonomous BPV-1 replication (our unpublished results). There is every reason to suspect that viral replication control in vivo is very complicated, considering the variety of cellular conditions under which the virus must survive. In a productive infection, the basal cells maintain a low copy number of the viral DNA and express viral genes minimally. As the keratinocytes differentiate, both DNA replication and mRNA transcription become derepressed, culminating in the assembly of progeny virions in the most differentiated superficial cells of the epithelium (62, 63). In some patients, the virus regresses into a latent state about which virtually nothing is known except that warts might recur months or years later. We postulate that these new E1M proteins play important roles in controlling transcription and DNA replication in vivo. Confirmation of this hypothesis awaits the development of a system for viral propagation in vitro.

ACKNOWLEDGMENTS

We thank D. G. Strike for providing experimental condylomata, P. M. Howley for providing C127 cells and pdBPV-1(142-6) DNA, and Thau Ho for the polyclonal antisera used in the study.

This research was supported by USPHS grant CA 36200.

REFERENCES

1. **Androphy, E. J., D. R. Lowy, and J. T. Schiller.** 1987. Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. Nature (London) **325:**70–73.
2. **Baker, C. C., and P. M. Howley.** 1987. Differential promoter utilization by the bovine papillomavirus in transformed cells and productively infected wart tissues. EMBO J. **6:**1027–1035.
3. **Bedrosian, C. L., and D. Bastia.** 1990. The DNA-binding domain of HPV-16 E2 protein interaction with the viral enhancer: protein-induced DNA bending and role of the nonconserved core sequence in binding site affinity. Virology **174:**557–575.
4. **Berg, L. J., M. Lusky, A. Stenlund, and M. R. Botchan.** 1986. Repression of bovine papilloma virus replication is mediated by a virally encoded trans-acting factor. Cell **46:**753–762.
5. **Bernard, B. A., C. Bailly, M.-C. Lenoir, M. Darmon, F. Thierry, and M. Yaniv.** 1989. The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J. Virol. **63:**4317–4324.
6. **Botchan, M. R., L. Berg, J. Reynolds, and M. Lusky.** 1986. The bovine papillomavirus replicon. CIBA Found. Symp. **120:**53–67.
7. **Burnett, S., J. Moreno-Lopez, and U. Pettersson.** 1987. Messenger RNAs from the E1 region of bovine papillomavirus type 1 detected in virus-infected bovine cells. Nucleic Acids Res. **15:**8607–8620.
8. **Burnett, S., J. Zabielski, J. Moreno-Lopez, and U. Pettersson.** 1989. Evidence for multiple vegetative DNA replication origins and alternative replication mechanisms of bovine papillomavirus type 1. J. Mol. Biol. **206:**239–244.
9. **Cepko, C. L., B. E. Roberts, and R. C. Mulligan.** 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell **37:**1053–1062.
10. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
11. **Chin, M. T., T. R. Broker, and L. T. Chow.** 1989. Identification of a novel constitutive enhancer element and an associated binding protein: implications for human papillomavirus type 11 enhancer regulation. J. Virol. **63:**2967–2976.
12. **Chin, M. T., R. Hirochika, H. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Regulation of human papillomavirus type 11 enhancer and E6 promoter by activating and repressing proteins from the E2 open reading frame: functional and biochemical studies. J. Virol. **62:**2994–3002.
13. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid

from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.

14. **Choe, J., P. Vaillancourt, A. Stenlund, and M. Botchan.** 1989. Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: structural and functional analysis of new viral cDNAs. J. Virol. **63**:1743–1755.
15. **Chow, L. T., H. Hirochika, M. Nasseri, M. H. Stoler, S. M. Wolinsky, M. T. Chin, R. Hirochika, D. S. Arvan, and T. R. Broker.** 1987. Human papillomavirus gene expression. Cancer Cells **5**:55–72.
16. **Chow, L. T., M. Nasseri, S. M. Wolinsky, and T. R. Broker.** 1987. Human papillomavirus types 6 and 11 mRNAs from genital condylomata acuminata. J. Virol. **61**:2581–2588.
17. **Cullen, B.** 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. Methods Enzymol. **152**: 684–704.
18. **Giri, I., and M. Yaniv.** 1988. Structural and mutational analysis of E2 trans-activating proteins of papillomaviruses reveals three distinct functional domains. EMBO J. **7**:2823–2829.
19. **Gorman, C. M., B. H. Howard, and R. Reeves.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res. **11**:7631–7648.
20. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
21. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
22. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Haugen, T. H., T. P. Cripe, G. D. Ginder, M. Karin, and L. P. Turek.** 1987. Trans-activation of an upstream early gene promoter of bovine papilloma virus-1 by a product of the viral E2 gene. EMBO J. **6**:145–152.

23a. **Haugen, T. H., L. P. Turek, F. M. Mercurio, T. P. Cripe, B. J. Olson, R. D. Anderson, D. Seidl, M. Karin, and J. Schiller.** 1988. Sequence-specific and general transcriptional activation by the bovine papillomavirus-1 E2 trans-activator require an N-terminal amphipathic helix-containing E2-domain. EMBO J. **7**:4245–4253.

24. **Hawley-Nelson, P., E. J. Androphy, D. R. Lowy, and J. T. Schiller.** 1988. The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. EMBO J. **7**:525–531.
25. **Hermonat, P. L., B. A. Spalholz, and P. M. Howley.** 1988. The bovine papillomavirus P2443 promoter is E2 *trans*-responsive: evidence for E2 autoregulation. EMBO J. **7**:2815–2822.
26. **Higuchi, R., C. H. von Beroldingen, G. F. Sensabah, and H. A. Erlich.** 1988. DNA typing from single hairs. Nature (London) **332**:543–546.
27. **Hirochika, H., T. R. Broker, and L. T. Chow.** 1987. Enhancers and *trans*-acting E2 transcriptional factors of papillomaviruses. J. Virol. **61**:2599–2606.
28. **Hirochika, H., R. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Functional mapping of the human papillomavirus type 11 transcriptional enhancer and its interaction with the transacting E2 proteins. Genes Dev. **2**:54–67.

28a. **Ho, T., D. G. Strike, M. T. Chin, T. Murant, L. T. Chow, and T. R. Broker.** Unpublished data.

29. **Huang, M. T. F., and C. M. Gorman.** 1990. The simian virus 40 small-t intron, present in many common expression vectors, leads to aberrant splicing. Mol. Cell. Biol. **10**:1805–1810.

29a. **Kaplitt, M., L. T. Chow, and T. R. Broker.** Unpublished data.

30. **Kaufman, R. J., M. V. Davies, V. K. Pathak, and J. W. B. Hershey.** 1989. The phosphorylation state of eukaryotic initiation factor 2 alters translational efficiency of specific mRNAs. Mol. Cell. Biol. **9**:946–958.
31. **Korman, A. J., J. D. Frantz, J. L. Strominger, and R. C. Mulligan.** 1987. Expression of human class II major histocompatibility complex antigens using retrovirus vectors. Proc. Natl. Acad. Sci. USA **84**:2150–2154.
32. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
33. **Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand.** 1988. Using mini-prep plasmid DNA for sequencing double stranded templates with Sequenase. BioTechniques **6**:544–547.
34. **Kreider, J. W., M. K. Howett, A. E. Leure-Dupree, R. J. Zaino, and J. A. Weber.** 1987. Laboratory production in vivo of infectious human papillomavirus type 11. J. Virol. **61**:590–593.
35. **Kwok, S., D. E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky.** 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. **18**: 999–1005.
36. **Labow, M. A., S. B. Baim, T. Shenk, and A. J. Levine.** 1990. Conversion of the *lac* repressor into an allosterically regulated transcriptional activator for mammalian cells. Mol. Cell. Biol. **10**:3343–3355.
37. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. Cell **50**:69–78.
38. **Law, M.-F., D. R. Lowy, I. Dvoretzky, and P. M. Howley.** 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. Proc. Natl. Acad. Sci. USA **78**:2727–2731.
39. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. Botchan.** 1989. Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev. **3**:510–526.
40. **Lusky, M., and M. R. Botchan.** 1986. A bovine papillomavirus type 1-encoded modulator function is dispensable for transient viral replication but is required for establishment of the stable plasmid state. J. Virol. **60**:729–742.
41. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
43. **McBride, A. A., R. Schlegel, and P. M. Howley.** 1988. The carboxy-terminal domain shared by the bovine papillomavirus E2 transactivator and repressor proteins contains a specific DNA binding activity. EMBO J. **7**:533–539.
44. **Minty, A. J., M. Caravatti, B. Robert, A. Cohen, P. Daubas, A. Weydert, F. Gros, and M. E. Buckingham.** 1981. Mouse actin messenger RNAs: construction and characterization of a recombinant plasmid molecule containing a complementary DNA transcript of mouse α-actin mRNA. J. Biol. Chem. **256**:1008–1014.
45. **Moskaluk, C. A., and D. Bastia.** 1988. Interaction of the bovine papillomavirus type 1 E2 transcriptional control protein with the viral enhancer: purification of the DNA-binding domain and analysis of its contact points with DNA. J. Virol. **62**:1925–1931.
46. **Nasseri, M., R. Hirochika, T. R. Broker, and L. T. Chow.** 1987. A human papilloma virus type 11 transcript encoding an E1^E4 protein. Virology **159**:433–439.
47. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5**:444–447.
48. **Palermo-Dilts, D. A., T. R. Broker, and L. T. Chow.** 1990. Human papillomavirus type 1 produces redundant as well as polycistronic mRNAs in plantar warts. J. Virol. **64**:3144–3149.
49. **Parker, B. A., and G. R. Stark.** 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. **31**:360–369.
50. **Phelps, W. C., and P. M. Howley.** 1987. Transcriptional *trans*-activation by the human papillomavirus type 16 E2 gene product. J. Virol. **61**:1630–1638.
51. **Roberts, J. M., and H. Weintraub.** 1986. Negative control of DNA replication in composite SV40-bovine papilloma virus plasmids. Cell **46**:741–752.
52. **Roberts, J. M., and H. Weintraub.** 1988. Cis-acting negative control of DNA replication in eukaryotic cells. Cell **52**:397–404.
53. **Romanczuk, H., F. Thierry, and P. M. Howley.** 1990. Mutational

analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. J. Virol. 64:2849–2859.
54. **Rotenberg, M. O., C.-M. Chiang, M. L. Ho, T. R. Broker, and L. T. Chow.** 1989. Characterization of cDNAs of spliced HPV-11 E2 mRNA and other HPV mRNAs recovered via retrovirus-mediated gene transfer. Virology **172**:468–477.
55. **Rotenberg, M. O., L. T. Chow, and T. R. Broker.** 1989. Characterization of rare human papillomavirus type 11 mRNAs coding for regulatory and structural proteins, using the polymerase chain reaction. Virology **172**:489–497.
56. **Santucci, S., E. J. Androphy, C. Bonne-Andrea, and P. Clertant.** 1990. Proteins encoded by the bovine papillomavirus E1 open reading frame: expression in heterologous systems and in virally transformed cells. J. Virol. **64**:6027–6039.
57. **Sarver, N., J. C. Byrne, and P. M. Howley.** 1982. Transformation and replication in mouse cells of a bovine papillomavirus-pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. USA **79**:7147–7151.
58. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. **63**:1441–1447.
59. **Spalholz, B. A., Y.-C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.
60. **Stenlund, A., and M. R. Botchan.** 1990. The E2 trans-activator can act as a repressor by interfering with a cellular transcription factor. Genes Dev. **4**:123–136.
61. **Stenlund, A., J. Zabielski, H. Ahola, J. Moreno-Lopez, and U. Pettersson.** 1985. Messenger RNAs from the transforming region of bovine papilloma virus type 1. J. Mol. Biol. **182**:541–554.
62. **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.
63. **Stoler, M. H., S. M. Wolinsky, A. Whitbeck, T. R. Broker, and L. T. Chow.** 1989. Differentiation-linked human papillomavirus types 6 and 11 transcription in genital condylomata revealed by *in situ* hybridization with message-specific RNA probes. Virology **174**:331–340.
64. **Sun, S., L. Thorner, M. Lentz, P. MacPherson, and M. Botchan.** 1990. Identification of a 68-kilodalton nuclear ATP-binding phosphoprotein encoded by bovine papillomavirus type 1. J. Virol. **64**:5093–5105.
65. **Thierry, F., and M. Yaniv.** 1987. The BPV1-E2 transacting protein can be either an activator or a repressor of the HPV18 regulatory region. EMBO J. **6**:3391–3397.
66. **Thorner, L., N. Bucay, J. Choe, and M. Botchan.** 1988. The product of the bovine papillomavirus type I modulator gene (M) is a phosphoprotein. J. Virol. **62**:2474–2482.
67. **Vaillancourt, P., T. Nottoli, J. Choe, and M. R. Botchan.** 1990. The E2 transactivator of bovine papillomavirus type 1 is expressed from multiple promoters. J. Virol. **64**:3927–3937.
68. **Vande Pol, S. B., and P. M. Howley.** 1990. A bovine papillomavirus constitutive enhancer is negatively regulated by the E2 repressor through competitive binding for a cellular factor. J. Virol. **64**:5420–5429.
69. **Ward, P., and P. Mounts.** 1989. Heterogeneity in mRNA of human papillomavirus type-6 subtypes in respiratory tract lesions. Virology **168**:1–12.
70. **Wilimzig, M.** 1985. LiCl-boiling method for plasmid mini-preps. Trends Genet. **1**:158.
71. **Yang, L., and M. Botchan.** 1990. Replication of bovine papillomavirus type 1 DNA initiates within an E2-responsive enhancer element. J. Virol. **64**:5903–5911.
72. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82**:1030–1034.