Papillomavirus E7 Protein Binding to the Retinoblastoma Protein Is Not Required for Viral Induction of Warts

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Human papillomaviruses (HPVs) are the etiologic agents responsible for benign epithelial proliferative disorders including genital warts and are a contributory factor in the pathogenesis of cervical cancer. HPVs demonstrate strict species and cell-type specificity, which is manifested by the inability of these viruses to induce disease in any species other than humans. The natural history of HPV infection in humans is closely mimicked by cottontail rabbit papillomavirus (CRPV) infection in domestic laboratory rabbits. The CRPV E7 gene is known to play an essential role in virus-mediated induction of papillomas. We now show by mutational analysis that the CRPV E7 protein's biochemical and biological properties, including binding to the retinoblastoma suppressor protein (pRB), transcription factor E2F transactivation of the adenovirus E2 promoter, disruption of pRB-E2F complexes, and cellular transformation as measured by growth in soft agar, mimic those of the HPV E7 protein. Intradermal injection of CRPV DNA lacking E7 gene sequences critical for the binding of the CRPV E7 protein to pRB induced papillomas in rabbits. These studies indicate that E7 protein binding to pRB is not required in the molecular pathogenesis of virally induced warts and suggest that other properties intrinsic to the E7 protein are necessary for papilloma formation.

Human papillomaviruses (HPVs) are the etiologic agents responsible for several benign epithelial proliferative lesions, including genital warts, respiratory papillomatosis, and epidermodysplasia verruciformis, and are contributing factors in the pathogenesis of cervical carcinoma (19, 77). Approximately 20 types of HPVs infect the epithelium of the genital mucosa (19). This subset of HPVs can be further subdivided into two groups. The first group, composed of viruses such as HPV type 6 (HPV-6) and HPV-11, is associated with the majority of condylomas (benign warts) which are at low risk for malignant conversion. The second group, composed of viruses such as HPV-16 and HPV-18, is associated with lesions which are at high risk for progression to cancer (25, 27, 38). The genomes of papillomaviruses are relatively small, containing approximately 8,000 nucleotides that are known to encode eight gene products (77). Two of these genes, E6 and E7, have been implicated in the disruption of normal growth regulation that occurs in HPV-infected cells. The HPV-6 and HPV-11 E6 and E7 genes are invariably present and transcriptionally active in wart tissues (58, 61, 77). Similarly, the E6 and E7 genes from HPV-16, HPV-18, and HPV-33 are present and transcriptionally active in HPV-infected cervical-cancer cells (3, 58, 62). The HPV-16 E7 gene by itself is capable of transforming NIH 3T3 cells and can cooperate with an activated *ras* or *fos* oncogene in transforming primary baby rat kidney cells (17, 40, 52, 67). The HPV-16 E7 gene is also capable of transactivating the adenovirus E2 (AdE2) promoter through the E2F transcription factor (52). The HPV-16 E6 gene is capable of inducing anchorage-independent growth of NIH 3T3 cells and transactivating the AdE2 promoter (59). In human keratinocytes, which are the natural host cells for HPVs, the HPV-16 E6

and E7 genes are necessary and sufficient for transformation (43, 72). The E6 and E7 genes of other HPV strains are also capable of cellular transformation, immortalization, and AdE2 promoter transactivation, although the first two functions invariably occur to a lesser degree than is seen with the HPV-16 E6 and E7 genes (9, 15, 28, 31, 33, 45, 59, 67). These studies suggest that the HPV E6 and E7 genes, whether derived from high- or low-risk virus types, play a major role in the pathophysiology of HPV-induced proliferative disorders.

The HPV-16 E7 gene encodes a 21-kDa phosphoprotein containing 98 amino acids (62, 63). No enzymatic activity for E7 has been defined, but the amino terminus of E7 shares amino acid sequence similarity with two noncontiguous portions of the AdE1A and simian virus 40 large T proteins. The regions of sequence similarity between these heterologous oncoproteins have been shown by genetic studies to be required for cooperation with *ras* in cell transformation, transactivation of the AdE2 promoter, and attenuation of transforming growth factor β -induced transcriptional repression of the *c-myc* promoter (52, 53). One of these conserved regions (CR2) was shown to be essential in mediating the direct binding of these proteins to the 105-kDa retinoblastoma suppressor gene product (pRB) (5, 6, 18, 21, 35, 36, 44, 75). Recent studies have indicated that the E2F transcription factor is capable of forming heteromeric complexes with pRB and with cyclin A, p33^{cdk2}, and p107, a cell cycle-regulated protein related to pRB (2, 4, 12, 13, 16). Activation of the AdE2 promoter is at least partially mediated by disruption of these heteromeric E2F protein complexes (1). Transcriptional transactivation of the AdE2 promoter by HPV-16 E7 occurs through the E2F transcription factor, and in a manner similar to that of AdE1A, HPV-16 E7 is capable of disrupting cellular protein complexes containing E2F (50). More recently, it has been reported that E7-mediated trans-

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formation and transcriptional transactivation require overlapping sequences in the E7 domain that are required for binding to pRB (51). It has also been shown that mutations in HPV-16 E7 which reduce binding to pRB do not affect HPV-mediated immortalization of primary human keratinocytes but do eliminate the ability of the E7 protein to cooperate with an activated *ras* gene in transforming primary rat embryo fibroblasts (34). These structural and functional similarities between E7, large T antigen, and AdE1A strongly suggest that the ability of E7 proteins to bind to the retinoblastoma protein is a critical biochemical property which may participate in the disruption of normal cell growth regulation by papillomaviruses. An important implication of these studies is that a pharmacologic agent that could antagonize E7 binding to pRB might prove to be a useful inhibitor of HPV-induced diseases in humans.

We sought to test this hypothesis in an animal model of papillomavirus-induced disease in order to define which biochemical properties of the E7 protein are responsible for generating clinically recognizable pathology. Although introduction of a variety of papillomavirus genes into cultured cells can cause diverse abnormalities of growth and differentiation (8, 10, 31, 33, 37, 40, 52, 57, 65, 70, 71, 73), true papilloma formation occurs only in infected animals. The natural history of HPV infection in humans, including frequent progression to cancer (69), is closely mimicked by cottontail rabbit papillomavirus (CRPV) infection in domestic rabbits (39). By utilizing the CRPV animal model, it has been demonstrated that the CRPV E7 gene is essential for the induction of papillomas (11, 41). In addition, it has been shown that the CRPV E6 and E7 genes can transform NIH 3T3 cells (41). It has also been reported that CRPV DNA can immortalize human cervical keratinocytes (46). In this report, we show that the CRPV E7 protein as characterized by pRB binding, disruption of pRB-E2F complexes, transactivation of the AdE2 promoter, and cellular transformation mimics the biochemical and biological properties of the HPV E7 protein. Utilizing CRPV variants that code for E7 proteins defective in pRB binding, we also show that E7-pRB association is not required for the induction of papillomas in vivo. These results indicate that neither the transforming activity of the E7 protein nor the E7-pRB-mediated regulation of promoters containing E2F DNA-binding elements is required for the induction of papillomas.

MATERIALS AND METHODS

Plasmid constructions. Mutant CRPV E7 genes were constructed as follows. CRPV-pLAII DNA (47) (Fig. 1B) was digested with *Eco*RI and *Sac*I, and the 817-bp fragment containing the wild-type E7 gene was isolated by gel electrophoresis. This fragment was subcloned into the *Eco*RI-*Sac*I sites of pGEM 4Z (Promega, Madison, Wis.). This plasmid was then used both as a source of wild-type CRPV E7 and as a base construct for the derivation of the various E7 mutant genes. Individual mutants were made by polymerase chain reaction (PCR) procedures (56) in a Perkin Elmer thermal cycler. The oligonucleotides used to make the CRPV E7 C-24→A-24 and L-22→A-22, C-24→A-24 mutants were 5'-GCTGAAGCGCTTAGTTGCCATGCGACGAAG CATTAGAG-3' and 5'-GCTGAAGCGCTTAGTGCGCA TGCCGACGAAGCATTAGAGAATTAAAG-3'. Underlining indicates nucleotides that were changed from the wild-type sequence. Construction of the CRPV E7 p9Leu mutant has been previously described (11). All mutants were sequenced by the dideoxy method (USB, Cleveland, Ohio). For

FIG. 1. Comparison of CRPV E7 and HPV-16 E7 amino acid sequences. (A) Amino acid sequences were aligned according to the algorithm of Needleman and Wunsch (48). Identical residues are indicated by vertical bars. Substitution (residues 22 and 24) and insertion (residue 9) of amino acids are shown above the sequence. (B) Genetic structure of CRPV-pLAII. pLAII is a "poisonless" pBR322 derivative (47) that was cloned into the unique *Sal*I site in the CRPV viral genome. E6, E7, and E1 are the early open reading frames as deduced from DNA sequence studies. MET, initiating methionine residue for the E7 protein. (C) Genetic structure of the eukaryotic expression vector pCMV^{neo}. CMVIE, cytomegalovirus immediate-early promoter; BGH T.T., bovine growth hormone transcription terminator sequences (74).

mammalian cell experiments, wild-type and mutant E7 genes were cloned into the unique *Bgl*II site in the eukaryotic expression vector pCMV^{neo} (74) (Fig. 1C).

The HPV-16 E7 gene was a kind gift from P. Howley. The pEC113 and pAdE1A plasmids were a kind gift from J. Nevins. CRPV-pLAII DNA was generously provided by F. O. Wettstein.

Synthesis of GST fusion proteins. The AdE1A, HPV-16 E7, CRPV E7, and CRPV E7 mutant genes were subcloned into the pGEX 3X bacterial expression plasmid (Pharmacia, Piscataway, N.J.) by conventional methods. Plasmid DNA was transfected into DH5αF'IQ cells (GIBCO BRL, Gaithersburg, Md.). Overnight cultures were diluted 1/10 into

Luria broth containing 50 µg of ampicillin per ml. Cultures were grown to a A_{600} of 0.9, and isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.) was added to a final concentration of 1 mM. After an additional 90 min, cells were collected by centrifugation and suspended (1/100 of the culture volume) in 20 mM Tris-HCl (pH 8.0)–100 mM NaCl–1 mM EDTA–0.5% Nonidet P-40 (vol/vol). Cells were lysed by sonication, and the lysates were cleared by centrifugation at $10,000 \times g$ for 10 min. Glutathione *S*-transferase (GST) fusion proteins were captured by batch absorption to glutathione-Sepharose 4B beads (Pharmacia). The beads were washed and equilibrated in 50 mM Tris-HCl (pH 8.0), and the proteins were eluted with 10 mM glutathione (Sigma). The eluate was dialyzed overnight against phosphate-buffered saline.

Antisera and immunoprecipitations. Antibodies to human pRB were raised by injecting rabbits with affinity-purified pRB60 (23) with complete Freund's adjuvant as described by Harlow and Lane (29). Rabbit anti- β -galactosidase antibody was purchased from 5'-3' Inc., West Chester, Pa. Murine monoclonal antibody directed against human pRB, PMG3-245.11, was purchased from Pharmingen, San Diego, Calif. Additional anti-human pRB monoclonal antibodies (XZ 104 and XZ 133) were a gift from E. Harlow. pGEM 4Z transcription vectors (Promega) containing the CRPV E7 and CRPV E7 mutant genes were transcribed in vitro with T7 polymerase. These RNAs were used to program nuclease-treated rabbit reticulocyte lysates containing [35 S]cysteine as described by the manufacturer (Promega). Labeled proteins were characterized by polyacrylamide gel electrophoresis (PAGE). An equivalent nonsaturating amount of each E7 protein was used in each immunoprecipitation assay. Immunoprecipitations were performed as previously described (21) and analyzed by autoradiography after PAGE in Tris-glycine-sodium dodecyl sulfate (SDS) buffer. The HPV E7 plate binding assay was performed as previously described (35).

Cell culture. U-937 human lymphoma cells (ATCC CRL 1593) were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS). African green monkey kidney CV-1 cells (ATCC CCL 70) and Cos-7 cells (ATCC CRL 1651) were maintained in Dulbecco's modified Eagle medium with 10% FBS. Weri-27 cells were obtained from L. Donoso (Wills Eye Institute, Philadelphia, Pa.) and maintained in RPMI 1640 medium with 15% FBS. NIH 3T3 cells were obtained from D. Lowy (National Institutes of Health, Bethesda, Md.) and maintained in minimal essential medium alpha (MEM α) plus 10% FBS.

Mammalian cell transfections. DNA transfections for transient chloramphenicol acetyltransferase (CAT) assays were performed with Lipofectin (GIBCO BRL) on CV-1 cells. Cells were seeded at 7.5×10^5 per 100-mm dish 24 h prior to transfection. pSV- β -galactosidase (2 µg) and 5 µg each of the AdE2CAT- and E7-containing plasmids were cotransfected with 85% Lipofectin at 37°C in Opti-mem medium (GIBCO BRL). After 6 h, an equal volume of MEM α with 20% FBS was added, and the transfection reaction was continued overnight. The following day, cells were refed with MEM α plus 10% FBS and incubated for an additional 48 h before being assayed for CAT activity. Cellular transformation assays were performed with NIH 3T3 cells seeded at 5×10^5 cells per 100-mm dish. The cells were cotransfected with 2 µg of pCMV^{neo} plasmid and 10 to 20 µg of the respective CRPV-pLAI plasmids with 100% Lipofectin. After 6 h, an equal volume of MEM α plus 20% FBS was added, and the transfection continued overnight. The following day, plates

were refed with MEM α plus 10% FBS. Cells were started on G418 selection medium (MEM α containing 10% FBS and 500 µg of G418 per ml) at 48 h after transfection.

Colony formation in soft agar. Approximately 2 weeks after the addition of G418 selection medium, drug-resistant colonies were pooled and seeded in triplicate into 0.3% low-gelling-temperature agarose at a density of 3×10^4 cells per 35-mm dish. Individual transfections were scored for colony formation after 4 weeks.

CAT assays. CAT assays were performed as described by Gorman et al. (27). CV-1 cell extracts were incubated with [14 C]chloramphenicol (68 mCi/mmol; ICN, Irvine, Calif.) and 4 mM acetyl coenzyme A (Pharmacia) in 250 mM Tris-HCl (pH 7.8) at 37°C for 1 h. The products of the acetylation reaction were separated by ascending thin-layer chromatography followed by autoradiography. Quantitation of reaction products was performed by liquid scintillation counting as described elsewhere (26). Protein contents of cell lysates were determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). β -Galactosidase activity was determined with the Promega β -galactosidase enzyme assay kit. Each CAT reaction mixture contained an equivalent amount of β -galactosidase activity per microgram of protein to normalize for individual transfection efficiencies.

E2F-binding assay. Whole-cell lysates were prepared as described elsewhere with some modification (20). All steps were performed at 4°C. U-937 cells were washed in phosphate-buffered saline and collected by centrifugation. Hypotonic buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.4 mM Na₃VO₄, 0.4 mM NaF, 0.5 mM dithiothreitol) containing 50 µg of AEBSF (Calbiochem, La Jolla, Calif.) per ml as a protease inhibitor was added to the cell pellet (1.5 packed cell volumes). After 20 min, the cell suspension was subjected to 35 strokes in a Dounce homogenizer. High-salt buffer (10 mM HEPES [pH 7.9], 1.6 M KCl, 4 mM EDTA, 0.4 mM Na₃VO₄, 0.4 mM NaF, 1 mM dithiothreitol, 20% glycerol [vol/vol], 5 µg of AEBSF per ml) was added dropwise (1.7 packed cell volumes) to the cell lysate with stirring. The lysate was mixed gently for 30 min. Microsomal debris and lipid were removed by centrifugation at $100,000 \times g$ for 60 min. The supernatant was dialyzed against 20 mM HEPES (pH 7.9)–50 mM KCl–0.2 mM EDTA–0.4 mM Na₃VO₄–0.4 mM NaF–1 mM dithiothreitol–10% glycerol (vol/vol) containing 5 µg of AEBSF per ml and cleared again by centrifugation at $10,000 \times g$ for 10 min. The lysate was frozen and stored at –70°C.

The E2F-binding assay was performed as previously described (76). Each reaction mixture contained 4 µg of whole-cell lysate protein and 50,000 cpm of 32 P-end-labeled AdE2 promoter double-stranded DNA (5'-TAGTTTTCG CGCTTAAATTTGAGAAAGGGCGCGAACTAG-3').

Extraction and analysis of papilloma DNA. A portion of each papilloma was removed and homogenized in 10 mM NaCl–10 mM EDTA–10 mM Tris-HCl (pH 8.0) by using a Mikro-Dismembrator (B. Braun Instruments, Burlingame, Calif.). SDS (0.5%) and proteinase K (50 µg/ml) were added to the tissue homogenate and incubated for 1 h at 37°C. Two successive phenol-chloroform extractions were performed, and DNA was precipitated by the addition of 2.5 volumes of ethanol.

PCR was performed with a sense oligonucleotide (5'-GATCCAGTCTGTTTCTGTGAG-3') and an antisense oligonucleotide (5'-CACAGTCATCTAAAGCCAGAC-3') which encompassed the E7 region of the CRPV genome. PCR

conditions were 35 1.5-min cycles at 94, 56, and then 72°C. PCR products were run on a 2% agarose gel to ensure size and integrity. The products were then denatured and spotted onto nitrocellulose filters. Oligonucleotides specific for wild-type CRPV E7 (5'-GTCGCAATGCAGACTAAGC-3'), E7 C-24→A-24, (5'-GCTTCGTCGGCATGCAA-3'), and E7 L-22→A-22, C-24→A-24 (5'-GTCGGCATGCGCACTAAGC-3') were end labeled with ³²P and utilized as hybridization probes. Each nitrocellulose filter was incubated individually with one of the oligonucleotide probes listed above. Hybridizations were performed with Quik-Hyb solution (Stratagene, La Jolla, Calif.) under stringent conditions as specified by the manufacturer. Filters were mounted and exposed to Kodak X-Omat AR film for 2 h.

RESULTS

CRPV E7 interacts with pRB. The amino acid sequences of the Washington B strain CRPV E7 protein are compared with those of the HPV-16 E7 protein in Fig. 1. Using the algorithm of Needleman and Wunsch (48) and allowing for conservative amino acid substitutions, the two proteins are approximately 56% similar, with 34% of the residues being identical. The CRPV E7 protein contains sequences similar to those in HPV-16 E7 which have been shown to be critical for pRB binding (residues 22 to 26) (5, 6, 21, 36, 44) and casein kinase II phosphorylation (residues 32 to 37) (6). Further similarity to HPV E7 is evident in the fact that CRPV E7 also contains two -Cys-X-X-Cys- motifs (residues 56 to 59 and 89 to 92) which are believed to be involved in the coordinate binding of a zinc metal ion (7, 49).

Analysis of DNAs from rabbit tissues by Southern blot hybridization indicates the presence of sequences homologous to the human pRB gene (data not shown). However, the rabbit pRB gene has not yet been cloned. Thus, direct binding of CRPV E7 protein to the rabbit pRB has not been tested. In other studies, we demonstrated that crude rabbit reticulocyte lysates contain a protein(s) that mimics the biochemical activities of human pRB (30). In those studies, rabbit reticulocyte lysates were spiked with [³⁵S]cysteine-labeled HPV-16 E7 or CRPV E7 proteins and incubated with a rabbit polyclonal antibody directed against human pRB. Immune complexes were captured on protein A-Sepharose beads and examined by autoradiography after SDS-PAGE. Both CRPV E7 and HPV-16 E7 proteins were indirectly precipitated by this method. CRPV E7 and HPV-16 E7 proteins were not precipitated when preimmune or rabbit polyclonal antibodies directed against β-galactosidase were used. CRPV E7 association with these immunocomplexes is specific, since a decapeptide, E7(20-29), that contains sequences from HPV-16 E7 which are essential for HPV-16 E7-pRB binding (36) was able to completely inhibit binding. A peptide with the same amino acid composition scrambled into a different sequence did not block E7 binding. In addition, we demonstrated that CRPV E7 binds to the same COOH-terminal portion of human pRB as does HPV E7. Both CRPV and HPV-16 E7 proteins interact with human pRB through similar if not identical contact points, since the HPV-16 E7(20-29) peptide which binds to pRB can block 100% of the CRPV E7 interaction with human pRB. These data demonstrate that rabbit reticulocytes contain a protein(s) highly related to human pRB.

In order to evaluate whether CRPV E7 binding to pRB is required for papilloma induction, we constructed a number of mutant CRPV E7 genes which should encode proteins that exhibit altered pRB binding. The specific mutations created

FIG. 2. Rabbit and human pRB proteins bind to CRPV E7 proteins in a homologous manner. Rabbit reticulocyte cell lysate (lanes 1 and 2) and human bladder carcinoma cell (T24) lysate (lanes 3 and 4) were used as sources of pRB. Lysates were mixed with equivalent amounts of in vitro-synthesized [³⁵S]cysteine-labeled CRPV E7 proteins. Rabbit polyclonal anti-human pRB serum was added to lanes 1 and 3. Preimmune serum was added to lanes 2 and 4. Immunoprecipitates were analyzed by autoradiography after SDS-PAGE. Identical results were obtained with murine anti-human pRB monoclonal antibodies. A faint band is detectable on the original autoradiograph of CRPV E7 C-24→A-24 (lane 1). Numbers at left indicate molecular sizes in kilodaltons.

are shown in Fig. 1. The CRPV E7 p9Leu represents an in-frame insertion of a leucine residue at position 9. This residue lies outside of the predicted pRB-binding domain for E7 proteins. CRPV E7 p9Leu was previously described and has been shown to be incapable of inducing papillomas in the context of the complete viral genome (11). The CRPV E7 C-24→A-24 and CRPV E7 L-22→A-22, C-24→A-24 mutations were selected, since these residues have been shown to be critical for HPV-16 E7 binding to pRB (5, 35, 36, 44). In order to minimize any conformational changes in the CRPV E7 protein, we utilized alanine rather than glycine. Substitution of glycine for amino acids that possess a side chain significantly increases flexibility and rotation at the substituted site and facilitates formation of β turns (60). Substitution of alanine also allows increased flexibility but limits rotation at the substituted site.

The abilities of these mutant CRPV E7 proteins to bind to human and rabbit pRBs was tested by in vitro coimmunoprecipitation. Radiolabeled CRPV E7 proteins were mixed with either human bladder carcinoma cell line (T24) lysate as a source of full-length 105-kDa pRB or rabbit reticulocyte lysate, and the E7 proteins were indirectly precipitated with either a mouse monoclonal antibody specific for pRB (PMG3-245.11; PharMingen) or the anti-human pRB polyclonal sera described above. As shown in Fig. 2, all of the CRPV E7 proteins except the E7 L-22→A-22, C-24→A-24 mutant were capable of binding to both rabbit and human pRBs. The CRPV E7 p9Leu mutant appeared to bind both rabbit and human pRB proteins with less avidity than wild-type CRPV E7 (Fig. 2B). As expected, the CRPV E7 C-24→A-24 mutant protein exhibited a profound loss in binding activity for both species of pRB. These results suggest that the rabbit and human pRB proteins are functionally conserved, since pRB association with the HPV and CRPV E7 proteins was similar.

A more quantitative comparison of the binding of CRPV E7 proteins was performed by determining their abilities to compete with recombinant HPV-16 E7 protein for binding to human pRB. The competition experiments were performed by using a plate binding assay as previously described (35). Purified recombinant HPV-16 E7 protein was bound to 96-well plates and incubated with an affinity-purified COOH-terminal fragment of pRB that is known to be fully competent for HPV-16 E7 binding (23). The incubation was performed in the presence or absence of various amounts of the E7 competitors. Purified GST-E7 fusion proteins were generated for use as competitors in this assay. The HPV E7 protein was sixfold better than the CRPV E7

FIG. 3. Inhibition of human pRB binding to immobilized HPV-16 E7 protein by recombinant HPV-16 E7 and CRPV E7 proteins. Values represent means from at least two experiments. Each experiment was conducted in triplicate. $\circ$, HPV-16 E7; $\bullet$, CRPV E7; ∇ , CRPV E7 p9Leu; $\blacktriangledown$, CRPV E7 C-24 $\rightarrow$ A-24; $\square$, CRPV E7 L-22 $\rightarrow$ A-22, C-24 $\rightarrow$ A-24.

protein (50% inhibitory concentration [IC_{50}], 10 versus 60 nM) at blocking E7-pRB binding in this assay (Fig. 3). The CRPV E7 p9Leu insertion mutant demonstrated a slight loss in pRB binding. In three separate experiments, the CRPV E7 p9Leu protein always bound less well than the wild-type CRPV E7 protein (IC_{50} , 60 versus 100 nM). On the other hand, both the CRPV E7 C-24 $\rightarrow$ A-24 and CRPV E7 L-22 $\rightarrow$ A-22, C-24 $\rightarrow$ A-24 proteins showed a dramatic loss in pRB-binding activity. The substitution of alanine for cysteine at position 24 resulted in an approximately 85 fold loss in pRB binding (IC_{50} , 60 versus 5,200 nM). The double mutant resulted in a greater than 1,600-fold loss in their respective IC_{50} s for pRB binding (IC_{50} , 60 versus >100,000 nM). Similar results are obtained when mutations in the pRB-binding domain of the HPV-16 E7 protein were examined in this assay. These data strongly suggest that CRPV E7 associates in a similar manner with both rabbit and human

pRBs. The plate binding studies indicate that under physiological conditions, the CRPV E7 L-22 $\rightarrow$ A-22, C-24 $\rightarrow$ A-24 mutant protein does not possess any significant pRB-binding activity.

CRPV E7 antagonizes E2F-pRB binding. The E7 proteins from HPV types 6, 11, 16, and 18 have been shown to be multifunctional, possessing both transcriptional regulatory and immortalizing activities. Similar to the AdE1A 12S product, the HPV-16 E7 protein has been shown to modify the interaction of the E2F transcription factor with pRB, cyclin A, and p107 (50). The E2F in these heteromeric protein complexes is believed to be nonfunctional, since E2 gene transcription can be stimulated under conditions that cause E2F to dissociate from these cellular proteins (2, 32, 50). The ability of HPV-16 E7 to affect these protein-protein interactions was also shown to depend on the E7 sequences required for pRB binding (50).

We analyzed the ability of CRPV E7 to affect E2F-containing complexes. AdE1A, HPV-16 E7, and CRPV E7 proteins were produced in *Escherichia coli* as GST fusion proteins. These proteins were affinity purified from *E. coli* lysates by using glutathione-Sepharose beads. Protein purity was determined after gel electrophoresis by Coomassie staining. A single protein species of greater than 95% purity was obtained for each sample. Whole-cell extracts from human U-937 lymphoma (68) cells were prepared as previously described (54). The lysate was mixed with buffer alone or buffer containing the various protein inhibitors (76). After a 10-min incubation, a 32 P-labeled oligonucleotide representing the E2F-binding site from the promoter of the AdE2 gene was added, and E2F-DNA complexes were analyzed in gel retardation assays. The identities of the E2F-DNA and pRB-E2F-DNA complexes were determined by the addition of deoxycholate, which disrupts protein-protein interactions, or by the addition of a monoclonal antibody specific for pRB (data not shown). As shown in Fig. 4A through C, the addition of increasing amounts of AdE1A, HPV-16 E7, and CRPV E7 proteins results in the loss of pRB-E2F-DNA complexes and an increase in the amount of the E2F-DNA complexes. The great increase in the amount of E2F-DNA complex most likely results from dissociation of the pRB-E2F-DNA complex and other heteromeric E2F protein-containing complexes that do not bind DNA. Alternatively, this result may be the consequence of E2F having a low DNA-binding activity while complexed with pRB. Loss of the pRB-E2F-DNA complexes in these assays is dependent on the added protein-possessing sequences that are known

FIG. 4. CRPV E7-binding activity for pRB is required for CRPV E7-mediated disruption of E2F complexes. E2F DNA-binding activity in U-937 cell extracts was assayed by gel retardation assay of E2F alone (lane 1) or after incubation with recombinant GST fusion proteins. Lanes 2 through 6 represent reaction mixtures that contained 11, 43, 170, 700, and 2,700 nM concentrations, respectively, of each protein.

FIG. 5. CRPV E7-binding activity for pRB is required for activation of the AdE2 promoter. a, Cytomegalovirus immediate-early promoter-driven AdE1A, HPV-16 E7, CRPV E7, and CRPV E7 mutant genes were transfected along with an AdE2 promoter-driven CAT gene into CV-1 cells. b, Percent transactivations were normalized to that of CRPV E7 and are the averages of three experiments. A trace amount of acetylated chloramphenicol is detectable on the original autoradiograph in the CRPV E7 L-22→A-22, C-24→A-24 lane. c, ND, not detectable.

to be essential for the direct binding of the CRPV E7 protein to pRB (Fig. 2 and 3). As shown in Fig. 4E, the addition of CRPV E7 C-24→A-24 at a concentration greater than 170 nM was required to substantially disrupt the pRB-E2F-DNA complexes and generate the formation of E2F-DNA complexes, whereas significantly less wild-type CRPV E7 protein was required to generate these results. This requirement for CRPV E7-pRB binding is more dramatically demonstrated in Fig. 4F, where CRPV E7 L-22→A-22, C-24→A-24 was used as the inhibitor. A protein concentration of 2,700 nM failed to eliminate the preformed pRB-E2F complexes or generate an increase in the concentration of E2F-DNA complexes. The CRPV p9Leu mutant, which was shown to have only a slight loss in pRB-binding activity (Fig. 2 and 3), was indistinguishable from the wild-type CRPV E7 protein in this assay (Fig. 4D). The biochemical effects demonstrated in these gel retardation assays mimic the pRB-binding data shown in Fig. 3 and clearly establish the importance of CRPV E7 binding to pRB for the disruption of E2F-pRB complexes.

These CRPV E7 mutants were further characterized with regard to their transactivating activities in cell culture. Cytomegalovirus immediate-early promoter-driven CRPV E7 and HPV-16 E7 genes (Fig. 1C) were transfected into monkey CV-1 cells along with a plasmid containing an AdE2 promoter-driven CAT gene (pEC113). As shown in Fig. 5, cotransfection of plasmids encoding HPV-16 E7 or CRPV E7 led to activation of the AdE2 promoter. In multiple experiments, the CRPV E7 gene was more potent than the HPV-16 E7 gene at inducing CAT activity. Cotransfection of the CRPV E7 p9Leu gene also resulted in activation of the AdE2 promoter but to a slightly lower level. The CRPV E7 C-24→A-24 gene, on the other hand, demonstrated a substantial loss in transactivation activity, while the CRPV E7 L-22→A-22, C-24→A-24 gene exhibited barely detectable transactivation activity. These results suggest that the CRPV E7 protein is functionally similar to HPV-16 E7 with respect to its ability to modify E2F complexes and transactivate the AdE2 promoter. In addition, the region of the CRPV E7 protein which has been shown to be required for pRB binding is also necessary for these activities.

CRPV E7 can induce anchorage-independent growth. The ability of HPV-16 E7 to induce anchorage-independent growth of NIH 3T3 cells correlates with pRB-binding activ-

TABLE 1. Biological activity of CRPV DNA containing wild-type and mutant E7 genes

Plasmid	Anchorage-independent growth (avg no. of colonies/plate) ^a
CRPV-pLAII	360 (4)
CRPV-p9Leu ^b	0 (4)
CRPV-E7 C-24→A-24 ^b	0 (2)
CRPV-E7 L-22→A-22, C-24→A-24 ^b	0 (4)

^a Number of experiments is given in parentheses.

^b The wild-type E7 gene was removed from the CRPV-pLAII plasmid by *EcoRI-SacI* digestion and replaced with the CRPV E7 mutant gene as indicated. NIH 3T3 cells (5×10^5 /100-mm dish) were cotransfected with 2 μ g of pCMV^{neo} and 10 to 20 μ g of the CRPV-pLAII plasmid. G418-resistant colonies were pooled, and 3×10^4 cells in 0.3% low-gelling-temperature agarose were seeded in triplicate into 35-mm dishes. See Materials and Methods for additional details.

ity. It has also been demonstrated that transfection of full-length CRPV genomic DNA (CRPV-pLAII) into NIH 3T3 cells enables these cells to form colonies in soft agar and that the CRPV E6 and E7 genes are necessary for this activity (41, 42). On the basis of these observations, we tested the abilities of our mutant CRPV E7 genes, which are defective for pRB binding, to induce anchorage-independent growth of NIH 3T3 cells. Recombinant CRPV-pLAII DNAs, which contained wild-type or E7 p9Leu, E7 C-24→A-24, or E7 L-22→A-22, C-24→A-24 mutations in the E7 gene, were prepared as previously described (11). NIH 3T3 cells were cotransfected with pSV2neo along with each of these DNAs. Cells were selected with G418, and colonies were pooled and plated in soft agar. The data in Table 1 show that in multiple experiments, only the CRPV genome containing a wild-type E7 gene was able to induce anchorage-independent growth in NIH 3T3 cells.

CRPV E7-pRB binding is not required for papilloma formation. It was previously demonstrated that the CRPV E7 gene product is required for papilloma formation (11, 41). However, these studies did not determine whether the ability of E7 to bind pRB was required for this aspect of the disease process. The CRPV E7 p9Leu mutant, which we have found to possess near-wild-type pRB-binding activity, was shown to be incapable of inducing papillomas (11). This result suggests that pRB binding may be necessary but is not sufficient for papilloma formation. We therefore tested the abilities of genomic CRPV DNAs containing our mutant CRPV E7 genes to induce papillomas in rabbits.

Recombinant CRPV-pLAII DNAs containing wild-type, C-24→A-24, or L-22→A-22, C-24→A-24 mutations in their E7 genes were prepared as previously described (11). The biological effects of these mutant DNAs were analyzed in vivo by inoculation into the skin on the backs of rabbits according to published procedures (11). Each inoculation site was monitored for the presence of a proliferative lesion. After 4 weeks, lesions were detectable at sites inoculated with both wild-type and mutant DNAs. All lesions exhibited typical signs of rabbit papillomas, including gross pathologic and microscopic histological architecture. In two experiments (Table 2), CRPV DNA containing a wild-type E7 gene produced 40 papillomas from 247 inoculation sites (16.2% positive). However, in contrast to the CRPV E7 p9Leu DNA, which produced no papillomas from 290 inoculation sites (11), CRPV DNA containing the mutant E7 L-22→A-22, C-24→A-24 gene produced eight papillomas from 233 inoculation sites (3.4% positive). Similarly, the CRPV DNA

TABLE 2. Induction of papillomas by CRPV DNAs containing mutations in E7 gene

Type of DNA and expt	No. of papillomas/no. of inoculated sites in rabbit no.:								Total (%)
	1	2	3	4	5	6	7	8	
Expt 1									
pLAII ^a	4/17	2/20			0/20		5/20	1/20	12/97 (12.4)
E7-459 ^b					0/24	1/24	1/23		2/71 (2.8)
E7-479 ^c		0/24	1/24	2/35					3/83 (3.6)
Expt 2									
pLAII	9/50	12/50		7/50					28/150 (18.7)
E7-479		2/50	3/100						5/150 (3.3)

^a Wild-type CRPV.^b CRPV DNA containing a mutant E7 C-24→A-24 gene.^c CRPV DNA containing a mutant E7 L-22→A-22, C-24→A-24 gene.

containing the E7 C-24→A-24 mutant gene produced two papillomas from 71 inoculation sites (2.8% positive).

To verify that papillomas that formed following inoculation of CRPV E7 mutants still harbored the mutant input DNA and that revertants or contamination with wild-type CRPV DNA had not occurred, tissue DNAs were analyzed. DNAs were extracted from a portion of each lesion at 8 weeks after inoculation, and the CRPV E7 region was amplified by PCR. The PCR products were blotted onto nitrocellulose filters and incubated with ³²P-labeled oligonucleotide probes specific for the mutated portion of each E7 gene. The filters were stringently washed and exposed to X-ray film. As shown in Fig. 6, the only DNA detected in each papilloma was the genetically engineered species used in the inoculation. These results demonstrate that the input DNA and not wild-type DNA was responsible for induction of each papilloma.

Our data demonstrate that papillomas can be induced by CRPV DNAs that contain an E7 gene lacking the sequences critical for binding the E7 protein product to rabbit pRB. The observed rate at which papillomas formed with the two E7 mutant CRPV DNAs was approximately fivefold lower than the rate with wild-type CRPV DNA, a difference that was statistically significant. Nevertheless, it is clear that in the context of a complete viral genome, these mutant E7 genes did not preclude formation of typical epithelial papillomas. These findings clearly demonstrate that this aspect of the pathogenesis of disease does not require direct interaction between the CRPV E7 protein and pRB.

DISCUSSION

The E7 gene product of HPV is capable of disrupting normal cell growth regulation in a variety of mammalian cell culture systems and inducing cell transformation in some cell

types. The biochemical mechanisms underlying the growth stimulatory activities of E7 are poorly understood. Similarly, E7 is presumed to play a critical role in inducing and maintaining HPV-induced epithelial-cell proliferative disorders in humans, although the molecular basis for E7's contribution to these disorders is unclear. The HPV E7 protein is known to possess several biochemical properties, including specific binding to pRB, the ability to disrupt pRB binding to DNA, transactivation of the AdE2 promoter via the E2F transcription factor, and coordinate binding of divalent cations such as zinc via multiple cysteine residues located in the carboxy-terminal half of the protein (7, 44, 49, 52, 64). Three of these biochemical properties, including disruption of pRB binding to DNA and transactivation of the AdE2 promoter, are dependent on E7's ability to bind to pRB. Moreover, E7's ability to transform cells in vitro is also dependent on E7's pRB-binding activity (5, 22, 44). These observations suggest that E7's ability to bind pRB is responsible for at least some of E7's stimulatory effects on cell growth and may be a crucial biochemical mechanism that contributes to the pathogenesis of HPV-induced proliferative disorders. If this hypothesis could be proven valid, it would imply that pharmacologic agents that inhibit E7 binding to pRB could be effective therapeutics in the treatment of HPV-induced diseases. Unfortunately, the establishment of a correlation between E7 binding to pRB and HPV-induced disease in vivo is precluded because of the lack of an HPV animal model. However, the natural history of HPV infection in humans is closely mimicked by CRPV infection in domestic laboratory rabbits. We have demonstrated that rabbit tissue contains a protein immunologically related to human pRB which can bind both CRPV E7 and HPV-16 E7 proteins (30; this report). This interaction with CRPV E7 is specific and depends on the same residues known to be critical for HPV-16 E7 binding to human pRB. This result is not unexpected, since the amino acid sequence of pRB is highly conserved in evolution. Analysis of published DNA sequences shows that the pRB-binding domain for E7, AdE1A, and simian virus 40 large T antigen is more than 95% identical between the murine and human pRB proteins at the amino acid sequence level. The similarity of CRPV E7- and HPV E7-binding activities for human pRB strongly argues for the conservation of functional activity as well. We have demonstrated by genetic and biochemical analysis that the CRPV E7 protein mimics the known biochemical and biological activities of HPV E7. These results make a compelling argument supporting the hypothesis that in CRPV-induced proliferative disorders, the CRPV E7 protein functionally mimics the role played by the HPV E7 protein in

FIG. 6. CRPV E7 genes deficient for pRB binding are found in experimentally induced papillomas. DNA was extracted from papillomas induced by CRPV-pLAII DNAs containing either E7, E7 C-24→A-24 (E7-459), or E7 L-22→A-22, C-24→A-24 (E7-479) genes and bound to nitrocellulose filters. Oligonucleotide probes specific for the E7 (A), E7 C-24→A-24 (B), and E7 L-22→A-22, C-24→A-24 (C) genes were hybridized to the filters.

human disease. Therefore, it appears reasonable to extrapolate the data from our studies of CRPV E7 activity in rabbits to the probable behavior of HPV E7 function in humans.

The current study demonstrates that CRPV E7 protein binding to pRB is not essential for CRPV-mediated induction of warts in rabbits. The mutant CRPV E7 genes C-24→A-24 and L-22→A-22, C-24→A-24 encode proteins with either dramatically reduced or undetectable pRB-binding properties. Nonetheless, genomic CRPV DNAs harboring these mutant E7 genes induced rabbit papillomas that were pathologically and histologically indistinguishable from lesions produced by wild-type CRPV DNA. While these DNAs were clearly capable of inducing warts, they did so with an approximately fivefold-reduced frequency relative to that of wild-type CRPV DNA (16.2, 2.8, and 3.4% for wild-type, C-24→A-24, and L-22→A-22, C-24→A-24 DNAs, respectively). One possible explanation for this result is that the presence of a functionally active pRB presents a partial barrier which the remaining viral functions must overcome in deregulating cellular growth controls leading to papilloma formation. Another possible explanation is that the mutations we made may have resulted in subtle changes in the three-dimensional conformation of the E7 protein. These changes may result in altered interactions between E7 and other cellular proteins which participate in cell growth regulation. This possibility is intriguing in light of the recent report demonstrating that the transforming and immortalizing functions of E7 map to different E7 domains (34). Mutations that reduced E7 binding to pRB were shown to inhibit E7-mediated transformation of rodent fibroblasts but had little or no effect on E7-mediated immortalization of primary human keratinocytes (34). A similar result was observed with mutations in the pRB-binding region of simian virus 40 large T antigen (14, 65).

Our findings allow, for the first time, the biological and biochemical behavior of a papillomavirus gene product, previously characterized *in vitro*, to be correlated with its pathogenicity in the intact animal. We have demonstrated that E7's ability to bind to pRB is not required for the induction of papillomas. This result indicates that neither the transforming activity of the E7 protein nor the E7-pRB-mediated regulation of promoters containing E2F DNA-binding elements is crucial for the induction of papillomas. Therefore, one or more of E7's other biochemical properties must be responsible for E7's contribution to the pathogenesis of virally induced warts. At present, it is unclear which of these biochemical functions is most important for induction of papillomas. In this regard, it is noteworthy that the -Cys-X-X-Cys- motifs, which are presumed to coordinately bind zinc to E7, are required for E7 to be capable of transforming fibroblasts *in vitro* (22, 34, 51, 65). Alternatively, an as-yet-uncharacterized biochemical property may be associated with E7 and may be responsible for E7's role in papilloma formation. One such property is hinted at in the results obtained with the CRPV E7 p9Leu mutant. As shown in Fig. 2 and 3, the mutant CRPV E7 p9Leu protein exhibits a minimal loss in pRB-binding activity. In addition, the CRPV E7 p9Leu protein possesses nearly normal E2F-mediated transactivating activity in CV-1 cells. These results suggest that the inability of CRPV genomic DNA containing this E7 mutation to generate papillomas is not the result of a gross alteration in the tertiary structure or stability of this protein. The ability of viral DNA containing the CRPV E7 L-22→A-22, C-24→A-24 gene to induce papillomas coupled with the inability of viral DNA containing the CRPV E7 p9Leu mutation to do so (11) implies that a critical interac-

tion essential for papilloma formation is localized to the amino terminus of E7. This conclusion is in keeping with our study of CRPV E7 shown in Table 1 and another study of HPV-16 E7 (51) which showed that an insertion of a leucine at position 9 in CRPV or the deletion of E7 residues 6 through 10 in HPV-16 E7 resulted in a loss of transforming activity while maintaining pRB-binding and E2F-transactivating activities. Genetic analysis of the AdE1A protein has shown that sequences in the extreme amino terminus and in the CR1 domain are essential for binding the cellular protein p300 (24). AdE1A-p300 association correlates with E1A's ability to induce cellular DNA synthesis, mediate enhancer repressor activity, and transform primary cells in cooperation with an activated *ras* oncogene (see reference 55 and references therein). HPV-16 E7 and CRPV E7 possess some amino acid sequence homology to AdE1A CR1 and may therefore associate with p300 or a related protein in a fashion analogous to that of the E1A protein.

While demonstrating that E7 binding to pRB is not required for induction of warts *in vivo*, the present study does not address the relevance of E7 binding to pRB for the subsequent formation of carcinomas within these papillomas. If wart formation is deemed analogous to the cell culture phenomenon of immortalization and if formation of carcinomas is considered analogous to transformation of cells in culture, then our findings are not entirely unexpected. R. J. Jewers and his colleagues have shown that E7 binding to pRB is not required for E7-dependent immortalization of human keratinocytes. However, E7 must be capable of binding pRB to transform rodent cells (5, 22, 34, 44). If these cell culture studies are predictive of E7's activity *in vivo*, then we should anticipate that the papillomas caused by the mutated E7-containing CRPV DNAs used in our studies will not progress to carcinomas. It is too early to definitively measure the frequency of carcinoma formation in these lesions, although at present, we have not seen any carcinomas up to 8 months following inoculation with these DNAs.

Finally, it is apparent from our studies that drugs designed to interfere with E7's binding to pRB are unlikely to be useful agents in the treatment of virally induced warts. However, the potential utility of such agents in the treatment of cancers that arise in papilloma virus-infected tissues remains to be established. For now, it seems prudent to continue to examine the other biochemical properties of the E7 protein in order to identify additional methods of antagonizing the biological effects caused by this multifunctional oncoprotein.

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REFERENCES

1. Bagchi, S., P. Raychaudhuri, and J. R. Nevins. 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A transactivation. *Cell* 62:4352-4356.
2. Bagchi, S., R. Weinmann, and P. Raychaudhuri. 1991. The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. *Cell* 65:1063-1072.
3. Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley. 1987. Structural and transcriptional

- analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J. Virol.* **61**:962-971.
4. Bandara, L. R., and N. B. LaThangue. 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature (London)* **351**:494-497.
 5. Banks, L., C. Edmonds, and K. H. Vousden. 1990. Ability of the HPV-16 E7 protein to bind rb and induce DNA synthesis is not sufficient for efficient transforming activity in NIH 3T3 cells. *Oncogene* **5**:1383-1389.
 6. Barbosa, M. S., C. Edmonds, C. Fisher, J. T. Schiller, D. R. Lowy, and K. H. Vousden. 1990. The region of the HPV E7 oncoprotein homologous to adenovirus E1A and SV40 large T antigen contains separate domains for rb binding and casein kinase 2 phosphorylation. *EMBO J.* **9**:153-160.
 7. Barbosa, M. S., D. R. Lowy, and J. T. Schiller. 1989. Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *J. Virol.* **63**:1404-1407.
 8. Barbosa, M. S., W. C. Vass, D. R. Lowy, and J. T. Schiller. 1991. In vitro biological activities of the E6 and E7 genes vary among human papillomaviruses of different oncogenic potential. *J. Virol.* **65**:292-298.
 9. Bedell, M. A., K. H. Jones, S. R. Grossman, and L. A. Laimins. 1989. The identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. *J. Virol.* **63**:1247-1255.
 10. Bedell, M. A., K. H. Jones, and L. A. Laimins. 1987. The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and Rat-1 cells. *J. Virol.* **61**:3635-3640.
 11. Brandsma, J. L., Z.-H. Yang, S. W. Barthold, and E. A. Johnson. 1991. Use of a rapid efficient inoculation method to induce papillomas by cottontail rabbit papillomavirus DNA shows that the E7 gene is required. *Proc. Natl. Acad. Sci. USA* **88**:4816-4820.
 12. Cao, L., B. Faha, M. Dembski, L.-H. Tsai, E. Harlow, and N. Dyson. 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. *Nature (London)* **355**:176-179.
 13. Chellappan, S. P., S. Heibert, M. Mudryj, J. M. Horowitz, and J. R. Nevins. 1991. The E2F transcription factor is a cellular target for the rb protein. *Cell* **65**:1053-1061.
 14. Chen, S., and E. Paucha. 1990. Identification of a region of simian virus 40 large T antigen required for cell transformation. *J. Virol.* **64**:3350-3357.
 15. Chesters, P. J., and D. J. McCance. 1989. Human papillomavirus types 6 and 16 in cooperation with Ha-ras transform secondary rat embryo fibroblasts. *J. Gen. Virol.* **70**:353-365.
 16. Chittenden, T., D. M. Livingston, and W. G. Kaelin. 1991. The T/E1A-binding domains of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. *Cell* **65**:1073-1082.
 17. Crook, T., A. Story, N. Almond, K. Osborn, and L. Crawford. 1988. Human papillomavirus type 16 cooperates with activated *ras* and *fos* oncogenes in the hormone-dependent transformation of primary mouse cells. *Proc. Natl. Acad. Sci. USA* **85**:8820-8824.
 18. DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**:275-283.
 19. de Villiers, E.-M. 1989. Heterogeneity of the human papillomavirus group. *J. Virol.* **63**:4898-4903.
 20. Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475-1489.
 21. Dyson, N., P. M. Howley, K. Munger, and E. Harlow. 1989. The human papillomavirus 16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**:934-937.
 22. Edmonds, C., and K. H. Vousden. 1989. A point mutational analysis of human papillomavirus type 16 E7 protein. *J. Virol.* **63**:2650-2656.
 23. Edwards, G. M., H. E. Huber, D. Defeo-Jones, G. Vuocolo, P. J. Goodhart, R. Z. Maigetter, G. Sanyal, A. Oliff, and D. C. Heimbroom. 1992. Purification and characterization of a functionally homogeneous 60 kDa species of the retinoblastoma gene product. *J. Biol. Chem.* **267**:7971-7974.
 24. Egan, C., T. N. Jelsma, J. A. Howe, S. T. Bayley, B. Ferguson, and P. E. Branton. 1988. Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. *Mol. Cell Biol.* **8**:3955-3959.
 25. Gissmann, L., L. Wolnik, H. Ikenberg, U. Koldvsky, G. Schnurch, and H. zur Hausen. 1983. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical biopsies. *Proc. Natl. Acad. Sci. USA* **80**:560-563.
 26. Gormann, 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.
 27. Gown, A., and A. Vogel. 1984. Monoclonal antibodies to human intermediate filament proteins. II. Distribution of filament proteins in normal tissues. *Am. J. Pathol.* **114**:309-321.
 28. Halbert, C. L., G. W. Demers, and D. A. Galloway. 1992. The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells. *J. Virol.* **66**:2125-2134.
 29. Harlow, E., and D. Lane. 1988. *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 30. Haskell, K. M., G. A. Vuocolo, D. Defeo-Jones, R. E. Jones, and M. Ivey-Hoyle. *J. Gen. Virol.*, in press.
 31. Hawley-Nelson, P., K. H. Vousden, N. L. Hubbard, D. R. Lowy, and J. T. Schiller. 1989. HPV 16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J.* **8**:3905-3910.
 32. Hubert, S. W., M. Blake, J. Azizkhan, and J. R. Nevins. 1991. Role of E2F transcription factor in E1A-mediated transactivation of cellular genes. *J. Virol.* **65**:3547-3552.
 33. Hudson, J. B., M. A. Bedell, D. J. McCance, and L. A. Laimins. 1990. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. *J. Virol.* **64**:519-526.
 34. Jewers, R. J., P. Hildebrandt, J. W. Ludlow, B. Kell, and D. J. McCance. 1992. Regions of human papillomavirus type 16 E7 oncoprotein required for immortalization of human keratinocytes. *J. Virol.* **66**:1329-1335.
 35. Jones, R. E., D. C. Heimbroom, H. Huber, R. J. Wegrzyn, N. S. Rotberg, K. Stauffer, P. K. Lumma, V. M. Garsky, and A. O. Oliff. 1992. Specific N-methylations of HPV-16 E7 peptides alter binding to the retinoblastoma suppressor protein. *J. Biol. Chem.* **267**:908-912.
 36. Jones, R. E., R. J. Wegrzyn, D. Patrick, N. L. Balishin, G. Vuocolo, M. W. Riemen, D. Defeo-Jones, V. M. Garsky, D. C. Heimbroom, and A. O. Oliff. 1990. Identification of peptides that are potent antagonists of E7 binding to the retinoblastoma suppressor protein. *J. Biol. Chem.* **265**:12782-12785.
 37. Kanda, T., A. Furuno, and K. Yoshiike. 1988. Human papillomavirus type 16 open reading frame E7 encodes a transforming gene for rat 3Y1 cells. *J. Virol.* **62**:610-613.
 38. Kapan, R., G. Trask, and E. Fuchs. 1987. Retinoids as important regulators of terminal differentiation in individual epidermal cells at various stages of keratinization. *J. Cell Biol.* **105**:427-440.
 39. Kreider, J. W., and G. L. Bartlett. 1981. The Shope papilloma-carcinoma complex of rabbits: a model system of neoplastic progression and spontaneous regression. *Adv. Cancer Res.* **35**:81-110.
 40. Matlashewski, G., J. Schneider, L. Banks, D. Jones, A. Murray, and L. Crawford. 1987. Human papillomavirus type 16 DNA cooperates with activated *ras* in transforming primary cells. *EMBO J.* **6**:1741-1746.
 41. Meyers, C., J. Harry, Y.-L. Lin, and F. O. Wettstein. 1992. Identification of three transforming proteins encoded by cottontail rabbit papillomavirus. *J. Virol.* **66**:1655-1664.
 42. Meyers, C., and F. O. Wettstein. 1991. The late region differentially regulates the *in vitro* transformation by cottontail rabbit

- papillomavirus DNA in different cell types. *Virology* **181**:637-646.
43. Munger, K., W. C. Phelps, V. Bubbs, P. M. Howley, and R. Schlegel. 1989. The E6 and E7 genes of human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J. Virol.* **63**:4417-4421.
 44. Munger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley. 1989. Complex formation of human papilloma virus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J.* **8**:4099-4105.
 45. Munger, K., C. Yee, W. C. Phelps, J. A. Pietenpol, H. L. Moses, and P. M. Howley. 1991. Biochemical and biological differences between E7 oncoproteins of the high- and low-risk human papillomavirus types are determined by amino-terminal sequences. *J. Virol.* **65**:3943-3948.
 46. Nasser, M., J. R. Gage, A. Lorincz, and F. O. Wettstein. 1991. Human papillomavirus type 16 immortalized cervical keratinocytes contain transcripts encoding E6, E7, and E2 initiated at the p97 promoter and express high levels of E7. *Virology* **184**:131-140.
 47. Nasser, M., C. Meyers, and F. O. Wettstein. 1989. Genetic analysis of CRPV pathogenesis: The L1 open reading frame is dispensable for cellular transformation but is required for papilloma formation. *Virology* **170**:321-325.
 48. Needleman, S. B., and C. D. Wunsch. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* **48**:443-453.
 49. Patrick, D. R., K. Zhang, D. Defeo-Jones, G. R. Vuocolo, R. Z. Maigetter, M. K. Sardana, A. Oliff, and D. C. Heimbrook. 1992. Characterization of functional HPV-16 E7 protein produced in *Escherichia coli*. *J. Biol. Chem.* **267**:6910-6915.
 50. Phelps, W. C., S. Bagchi, J. A. Barnes, P. Raychaudhuri, V. Kraus, K. Munger, P. M. Howley, and J. R. Nevins. 1991. Analysis of *trans* activation by human papillomavirus type 16 E7 and adenovirus 12S E1A suggests a common mechanism. *J. Virol.* **65**:6922-6930.
 51. Phelps, W. C., K. Munger, C. Yee, J. A. Barnes, and P. M. Howley. 1992. Structure-function analysis of the human papillomavirus type 16 E7 oncoprotein. *J. Virol.* **66**:2418-2427.
 52. Phelps, W. C., C. L. Yee, K. Munger, and P. M. Howley. 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell* **53**:539-547.
 53. Pietenpol, J. A., R. W. Stein, E. Moran, P. Yaculik, R. Schlegel, M. Lyons, M. R. Pittelkow, K. Munger, P. M. Howley, and H. L. Moses. 1990. TGF- β inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* **61**:777-785.
 54. Raychaudhuri, P., R. Roomey, and J. R. Nevins. 1987. Identification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter. *EMBO J.* **6**:4073-4081.
 55. Rikitake, Y., and E. Moran. 1992. DNA-binding properties of the E1A-associated 300-kilodalton proteins. *Mol. Cell. Biol.* **12**:2826-2836.
 56. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487-491.
 57. Sato, H., A. Furuno, and K. Yoshiike. 1989. Expression of human papillomavirus type 16 E7 gene induces DNA synthesis of rat 3Y1 cells. *Virology* **168**:195-199.
 58. Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen. 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature (London)* **314**:111-114.
 59. Sedman, S. A., M. S. Barbosa, W. C. Vass, N. L. Hubbert, J. A. Hass, D. R. Lowy, and J. T. Schiller. 1991. The full-length E6 protein of human papillomavirus type 16 has transforming and *trans*-activating activities and cooperates with E7 to immortalize keratinocytes in culture. *J. Virol.* **65**:4860-4866.
 60. Smith, S. A., and L. G. Pease. 1980. Reverse turns in peptides and proteins. *Crit. Rev. Biochem.* **8**:315-397.
 61. 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.
 62. Smotkin, D., and F. O. Wettstein. 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA* **83**:4680-4684.
 63. Smotkin, D., and F. O. Wettstein. 1987. The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. *J. Virol.* **61**:1686-1689.
 64. Stirdivant, S. M., H. E. Huber, D. R. Patrick, D. Defeo-Jones, E. M. McAvoy, V. M. Garsky, A. Oliff, and D. C. Heimbrook. 1992. Human papillomavirus type 16 E7 protein inhibits DNA binding by the retinoblastoma gene product. *Mol. Cell. Biol.* **12**:1905-1914.
 65. Storey, A., N. Almond, K. Osborn, and L. V. Crawford. 1990. Mutations of human papillomavirus type 16 E7 gene that affect transformation, transactivation and phosphorylation by the E7 protein. *J. Gen. Virol.* **71**:965-970.
 66. Storey, A., K. Osborn, and L. Crawford. 1990. Co-transformation by human papillomavirus types 6 and 11. *J. Gen. Virol.* **71**:165-171.
 67. Storey, A., D. Pim, A. Murray, K. Osborn, L. Banks, and L. Crawford. 1988. Comparison of the *in vitro* transforming activities of human papillomavirus types. *EMBO J.* **7**:1815-1820.
 68. Sundstrom, C., and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* **17**:565-577.
 69. Syverton, J. T. 1952. The pathogenesis of rabbit papilloma to carcinoma sequence. *Ann. N.Y. Acad. Sci.* **54**:1126-1140.
 70. Tanaka, A., T. Noda, H. Yajima, M. Hatanaka, and Y. Ito. 1989. Identification of a transforming gene of human papillomavirus type 16. *J. Virol.* **63**:1465-1469.
 71. Voudsen, K. H., J. Doniger, J. A. DiPaolo, and D. R. Lowy. 1988. The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. *Oncogene Res.* **3**:167-175.
 72. Watanabe, S., T. Kanda, and K. Yoshiike. 1989. Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. *J. Virol.* **63**:965-969.
 73. Watanabe, S., and K. Yoshiike. 1988. Transformation of rat 3Y1 cells by human papillomavirus type 18 DNA. *Int. J. Cancer* **41**:896-900.
 74. Whang, Y., M. Silberklang, A. Morgan, S. Munshi, A. B. Lenny, R. W. Ellis, and E. Kieff. 1987. Expression of the Epstein-Barr virus gp350/220 gene in rodent and primate cells. *J. Virol.* **61**:1796-1807.
 75. Whyte, P., K. S. Buchkovick, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow. 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature (London)* **334**:124-129.
 76. Yee, A. S., P. Raychaudhuri, L. Jakoi, and J. R. Nevins. 1989. The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. *Mol. Cell. Biol.* **9**:578-585.
 77. zur Hausen, H., and A. Schneider. 1987. The role of papillomaviruses in human anogenital cancer, p. 245-263. *In* N. P. Salzman and P. M. Howley (ed.), *The Papovaviridae*, vol. 2. Plenum Press, New York.