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The E7 Proteins of the Nononcogenic Human Papillomavirus Type 6b (HPV-6b) and of the Oncogenic HPV-16 Differ in Retinoblastoma Protein Binding and Other Properties

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The E7 early viral protein of the oncogenic human papillomavirus type 16 (HPV-16) has been strongly implicated in the maintenance of the malignant phenotype in cervical cancers and cancer-derived cell lines. HPV-16 E7 is a nuclear phosphoprotein that can cooperate with *ras* to transform baby rat kidney cells, transactivates the adenovirus E2 promoter, and binds to the retinoblastoma (RB) protein. The E7 phosphoprotein of the nononcogenic HPV-6b, which is generally associated with benign genital warts, is similar to the HPV-16 E7 in amino acid sequence but differs dramatically in migration in sodium dodecyl sulfate-polyacrylamide gels, sedimentation in nondenaturing glycerol gradients, and the ability to bind the RB protein. Our results indicate that the RB protein preferentially binds the phosphorylated form of HPV-6b E7, which comprises a minor fraction of the total E7 expressed in transiently transfected COS-7 cells. These characteristics may help to explain the difference in the oncogenic potential of the oncogenic and nononcogenic types of genital papillomaviruses.

The human papillomaviruses (HPV) that are found in genital lesions can be divided into two categories: the oncogenic types, such as HPV type 16 (HPV-16), -18, -31, and -33, which are associated with a high percentage of cervical cancers, and the nononcogenic types, such as HPV-6b and -11, which generally cause benign tumors (warts) and rarely progress to malignancy (24). The E7 early viral proteins of HPV-16 (1, 33–35) and HPV-18 (29, 32) have been strongly implicated in the maintenance of the malignant state, as it has been shown to be the major viral protein in cervical cancers and cancer-derived cell lines containing HPV-16 or HPV-18 DNA, and in some cervical cancers only the E7 DNA is retained (39). Furthermore, the inhibition of HPV-18 E7 expression by antisense RNA to the E6 and E7 open reading frames in C4-1 cells decreases cell growth (37). E7 mRNA in the oncogenic types is derived by splicing the major E6-E7 transcript, which may be more efficient at producing high levels of E7 than are the nononcogenic types, which transcribe the E7 mRNA from a promoter located within the E6 open reading frame (29, 33). It is possible, therefore, that quantitative or biochemical differences in the E7 proteins in the oncogenic and nononcogenic types may account for the oncogenic potential of each type.

HPV-16 E7 shares some functional characteristics, such as transformation and transactivation, with the viral proteins E1A of adenovirus (25) and large T antigen (LgT) of simian virus 40 (SV40) (6; for a review, see reference 22). In addition, it has been shown that HPV-16 E7 (11), E1A (38), and LgT (8, 23) all bind to a recently identified product of an antioncogene, the retinoblastoma (RB) protein, which apparently functions to restrict cell proliferation. It has been postulated that defects in the expression or function of the RB protein may play a significant role in various cancers, such as retinoblastoma (18, 19), mesenchymal tumors (13), breast cancers (17), small cell lung cancers (14), and bladder carcinoma cells (15). The observation that the transforming viral proteins E1A, LgT, and E7 of HPV-16 can bind to the RB protein has led to the hypothesis that these interactions may functionally eliminate RB protein from the cell and allow uncontrolled proliferation (21).

The experiments described here demonstrate some of the differences and similarities in the E7 proteins of the oncogenic HPV-16 and the nononcogenic HPV-6b. We now report that the two E7 proteins differ in migration in sodium dodecyl sulfate (SDS)-acrylamide gels, sedimentation in glycerol gradients, and ability to bind the RB protein.

MATERIALS AND METHODS

Antisera. The HPV-6b E7-TrpE fusion protein plasmid was constructed by cloning the 1.1-kilobase-pair *Nsi*I fragment between nucleotides 534 and 1644 of HPV-6b into the *Pst*I site of the bacterial TrpE fusion protein vector, pATH 11 (generously provided by T. J. Korner and A. Tzagoloff). The E7-TrpE fusion protein plasmid was expressed in *Escherichia coli* HB101, and the fusion protein was extracted and purified on a 10% SDS-polyacrylamide gel as previously described (3). Gel fragments containing approximately 0.5 mg of fusion protein were homogenized with Freund complete adjuvant (Difco Laboratories) and used to immunize rabbits. The animals were boosted four times with an equivalent amount of protein in Freund incomplete adjuvant at 3- to 4-week intervals.

Antiserum to the HPV-16 E7-TrpE fusion protein has been previously described (34). Antiserum to the RB protein, raised against the synthetic RB peptide 5 (38) containing amino acid residues 248 to 261 of the RB protein (19), was kindly provided by J. Horowitz.

Construction of HPV-16 and HPV-6b E7 expression vectors. HPV-16 DNA (10, 31) and HPV-6b DNA (9, 30) cloned into pBR322 or pUC19 have been described and were generously provided by H. zur Hausen, E. M. deVillier, L. Gissman, and M. Durst.

To construct HPV-6b E7/pSVLA4, genomic HPV-6b DNA was cleaved at nucleotides 473 and 4722 with *Tha*I and

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*Bam*HI, respectively, and blunt-ended with Klenow fragment (Bethesda Research Laboratories, Inc.). *Xho*I linkers (Pharmacia, Inc.) were ligated to the 4.2-kilobase-pair fragment, which was then cloned into the *Xho*I site of the eucaryotic expression vector pSVLA4 (3), into which an *Xho*I site had been introduced next to the *Kpn*I site.

The HPV-16 E7 expression vector was constructed by recloning HPV-16 at the *Sph*I site (nucleotide 7467). The *Sph*I-cloned HPV-16 DNA linearized by *Sph*I was digested with BAL 31, provided with *Xho*I linkers (Pharmacia), and inserted into the *Xho*I-containing pSVLA4.

Cells and transfections. COS-7 cells (American Type Culture Collection) were grown in 60-mm-diameter tissue culture dishes in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 100 μg of penicillin per ml, 100 μg of streptomycin per ml, and 25 μg of amphotericin B (Fungizone; GIBCO Laboratories) per ml. DNA transfections were performed with Lipofectin (Bethesda Research Laboratories), using 3 μg of DNA per 60-mm-diameter plate. Cells were labeled and harvested 72 h after transfection.

SiHa, CaSki, and HT-3 cell lines (American Type Culture Collection) were grown in RPMI 1640 medium supplemented with 10 or 15% fetal bovine serum, penicillin, streptomycin, and amphotericin B at the concentrations indicated above.

Metabolic labeling and immunoprecipitations. Cells were labeled with [^{35}S]trans (ICN Pharmaceuticals Inc.), [^{35}S]cysteine (Amersham Corp.), or [^{32}P]H_3PO_4 (ICN) as previously described (3), and each 60-mm-diameter plate was harvested in 0.5 ml of ELB buffer (250 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 5 mM EDTA, 0.5 mM dithiothreitol, 0.1% Nonidet P-40) containing 0.2 mM phenylmethylsulfonyl fluoride and 0.005% aprotinin. Cell extracts were incubated at 4°C overnight with a 1:100 dilution of antiserum. Immune complexes were bound to protein A-Sepharose CL-4B beads (Sigma Chemical Co.) for 2 h at 21°C. The beads were then washed four times in ELB buffer and once in 62.5 mM Tris hydrochloride (pH 8) and suspended in final sample buffer (62 mM Tris hydrochloride [pH 6.8], 10% glycerol, 0.0015% bromphenol blue, 3% SDS, 5% 2-mercaptoethanol). Proteins were eluted from the beads by boiling for 3 min. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The gels were fixed in 50% ethanol–7% glacial acetic acid, soaked in Autoflour (National Diagnostics), and autoradiographed.

Phosphatase treatments. Calf intestinal alkaline phosphatase (CIAP) treatments were performed as described by others (23). Briefly, immunoprecipitated proteins bound to protein A beads were washed four times in ELB buffer and equilibrated in CIAP buffer (100 mM Tris hydrochloride [pH 8], 5 mM $MgCl_2$, 100 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.005% aprotinin). Samples were then incubated for 15 min at 37°C in 40 μl of CIAP buffer containing 24 U of CIAP (Boehringer Mannheim Biochemicals). The reaction was stopped by washing the beads in NET-N (20 mM Tris hydrochloride [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Conditions for potato acid phosphatase (PAP) treatment were as described by others (7). Samples were washed in PAP buffer (40 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid [PIPES; pH 6.0], 1 mM dithiothreitol, 0.005% aprotinin, 0.2 mM phenylmethylsulfonyl fluoride) and incubated for 10 min at 30°C in 40 μl of PAP buffer containing 1 U of PAP (Sigma). Samples with phosphatase inhibitors contained 200 μM sodium orthovanadate and 100 μM NaF during phosphatase treatments. Mock reactions contained only phosphatase buffers. Reactions were stopped by washing in NET-N, and all samples were washed with 62.5 mM Tris hydrochloride (pH 8) before suspension in final sample buffer.

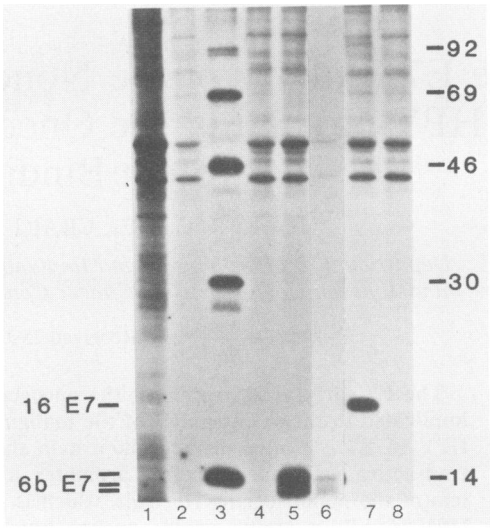

FIG. 1. Immunoprecipitation of HPV-16 and HPV-6b E7 proteins expressed in COS-7 cells. Autoradiogram of 12% SDS-polyacrylamide gel of cells transfected with HPV-6b E7/pSVLA4 (lanes 1, 2, 5, and 6), the parental vector pSVLA4 (lanes 4 and 8), HPV-16 E7/pSVLA4 (lane 7). Transfected cells were labeled with [^{35}S]trans and immunoprecipitated as follows: preimmune anti-HPV-6b E7 serum (lane 1), anti-HPV-6b E7 serum blocked with HPV-6b E7-TrpE fusion protein (lane 2), anti-HPV-6b E7 serum (lanes 4 to 6), or anti-HPV-16 E7 serum (lanes 7 and 8). Lanes 5 and 6 are 8- and 1-day exposures, respectively, of the same immunoprecipitation to more clearly demonstrate the multiple species of HPV-6b E7. The positions of the HPV-6b E7 species and HPV-16 E7 protein are indicated on the left. Lane 3, Molecular size markers (indicated in kilodaltons on the right).

Phosphoamino acid analysis. COS-7 cells were transfected with HPV-6b E7/pSVLA4 and labeled with 1 mCi of [^{32}P]H_3PO_4 per 60-mm-diameter plate in phosphate-free medium for 1 h. The E7 protein was immunoprecipitated and purified by SDS-PAGE. The protein was extracted from the gel and hydrolyzed in 6 N HCl at 110°C for 1.25 h. The phosphoamino acids were analyzed by high-voltage paper electrophoresis as previously described (35).

Glycerol gradients. ^{35}S-labeled cell extracts were layered onto glycerol gradients (10 to 25% glycerol in phosphate-buffered saline with 0.1% Nonidet P-40) and centrifuged at 4°C for 18 h at 190,000 × *g* in an SW50.1 rotor (Beckman Instruments, Inc.). Fifteen 0.33-ml fractions were collected from the top of the gradient and immunoprecipitated with antisera at 4°C overnight.

RESULTS

Expression of HPV-16 and HPV-6b E7 proteins. To examine and compare the characteristics of the HPV-16 and HPV-6b E7 proteins, we used a transient transfection system to express the E7 proteins in COS-7 cells. The E7 proteins were detected by immunoprecipitation of labeled cell extracts with the appropriate antiserum, separated by SDS-PAGE, and visualized by autoradiography. Figure 1 shows the characteristic migration patterns of the HPV-6b E7 (lanes 5 and 6) and HPV-16 E7 (lane 7) proteins. HPV-6b E7 was resolved into two major and one minor species by

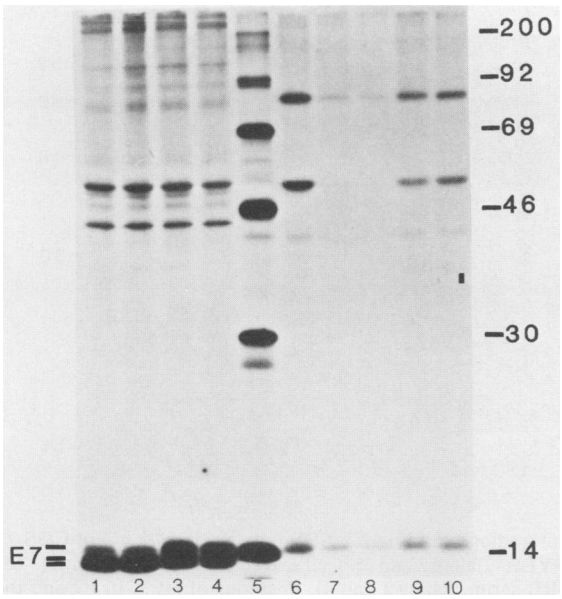

FIG. 2. Identification of the phosphorylated species of HPV-6b E7. COS-7 cells were transfected with HPV-6b E7/pSVLA4, and cell extracts were labeled with [^{35}S]trans (lanes 1 to 4) or [^{32}P]H_3PO_4 (ICN) (lanes 6 to 10). All samples were immunoprecipitated with anti-HPV-6b E7 serum and treated as follows: CIAP alone (lanes 1 and 7), CIAP followed by PAP (lanes 2 and 8), CIAP followed by PAP with phosphatase inhibitors sodium orthovanadate and NaF (lanes 3 and 9), mock reactions without phosphatases (lanes 4 and 10), or no treatment (lane 6). Proteins were separated in a 12% SDS-polyacrylamide gel and autoradiographed. Lane 5, Molecular size markers (indicated in kilodaltons on the right). Multiple species of HPV-6b E7 are indicated on the left.

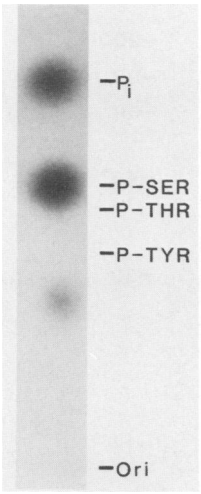

FIG. 3. Demonstration that HPV-6b E7 contains phosphoserine residues. COS-7 cells were transfected with HPV-6b E7/pSVLA4 and labeled with [^{32}P]H_3PO_4, and proteins were immunoprecipitated with anti-HPV-6b E7 sera. The proteins were separated by SDS-PAGE, the HPV-6b E7 protein was extracted from the gel, and phosphoamino acid analysis was carried out by high-voltage paper electrophoresis. The positions of P_i, phosphoserine (P-SER), phosphothreonine (P-THR), phosphotyrosine (P-TYR), and the origin of migration (Ori) are indicated.

SDS-PAGE; in contrast, the HPV-16 E7 protein migrated as a single species. In the short exposure of HPV-6b E7 protein (lane 6), the three species of this protein can be clearly recognized. Both E7 proteins were abundantly expressed in COS-7 cells when cloned into the pSVLA4 vector. Preimmune serum and serum blocked with the HPV-6b E7-TrpE fusion protein (Fig. 1) failed to precipitate the E7 proteins, confirming that the multiple species migrating at 12 to 15 kilodaltons (kDa) were HPV-6b E7 proteins.

Phosphatase treatments of E7 proteins. The appearance of multiple species suggested the presence of modified forms of HPV-6b E7. Since E7 of HPV-16 was shown to be phosphorylated (35), we first determined whether the multiple bands represented species differing in the extent of phosphorylation. A phosphorylated protein (Fig. 2, lane 6) comigrated with the slowest-moving ^{35}S-labeled E7 band (lane 4). This band was confirmed to be a species of E7, since it was absent in immunoprecipitates of ^{32}P-labeled extracts from COS-7 cells transfected with the parental plasmid, pSVLA4 (data not shown). If the slow migration of this E7 species was due to phosphorylation, it should have been possible to convert it into a faster-migrating species by phosphatase treatment. This was indeed accomplished (Fig. 2; compare lanes 1, 2, 7, and 8 with lanes 3, 4, 9, and 10). The treatment may not have completely eliminated the slowest-moving band, consistent with the phosphatase treatment of the ^{32}P-labeled extracts, where a faint band was still detectable after the treatment. The reason for the incomplete conversion and removal of phosphate is not known, since twofold differences in the enzyme levels used produced the same result. The observation that the addition of phosphatase inhibitors prevented the elimination of the slowest-moving species indicated that the removal of the phosphate groups, rather than nonspecific proteolytic cleavage, caused the shift in migration. Finally, when E7 of HPV-16 was treated in the same fashion, no change in mobility could be observed (data not shown), leaving open the question of whether all or only some of HPV-16 E7 was phosphorylated.

Phosphoamino acid analysis of HPV-6b E7. Protein phosphorylation is a major mechanism by which activity and specificity of proteins are altered. Identification of the amino acid(s) that is phosphorylated in E7 could provide preliminary information concerning the protein kinases involved. To identify the phosphorylated amino acid(s), COS-7 cells transfected with HPV-6b E7/pSVLA4 were labeled with [^{32}P]H_3PO_4, the labeled HPV-6b E7 protein was gel purified and hydrolyzed, and the phosphoamino acids were separated by paper electrophoresis. The results (Fig. 3) indicated that HPV-6b E7 contained phosphoserine. The identity of the small labeled spot that migrated more slowly than phosphotyrosine is unknown. Thus, the E7 proteins of both HPV-6b and HPV-16 (35) are phosphorylated at serine residues.

Binding of HPV-16 E7 and HPV-6b E7 to the RB protein. Genetic analysis of LgT of SV40 and E1A of adenovirus showed a close linkage between transformation and binding to the RB protein. The recent finding that E7 of the oncogenic HPV-16 can bind to in vitro-synthesized fragments of the RB protein could indicate that this interaction is also closely linked to the oncogenic potential of the papillomaviruses. To investigate this possibility, the in vivo interaction of the HPV-16 E7 with the RB protein was compared with that of E7 of HPV-6b, which is rarely associated with cancer. First, it had to be established that HPV-16 E7 forms complexes with the intact RB protein in vivo. To accomplish this, extracts from ^{35}S-labeled cells transfected with HPV-16 E7/pSVLA4 or with pSVLA4 were immunoprecipitated with

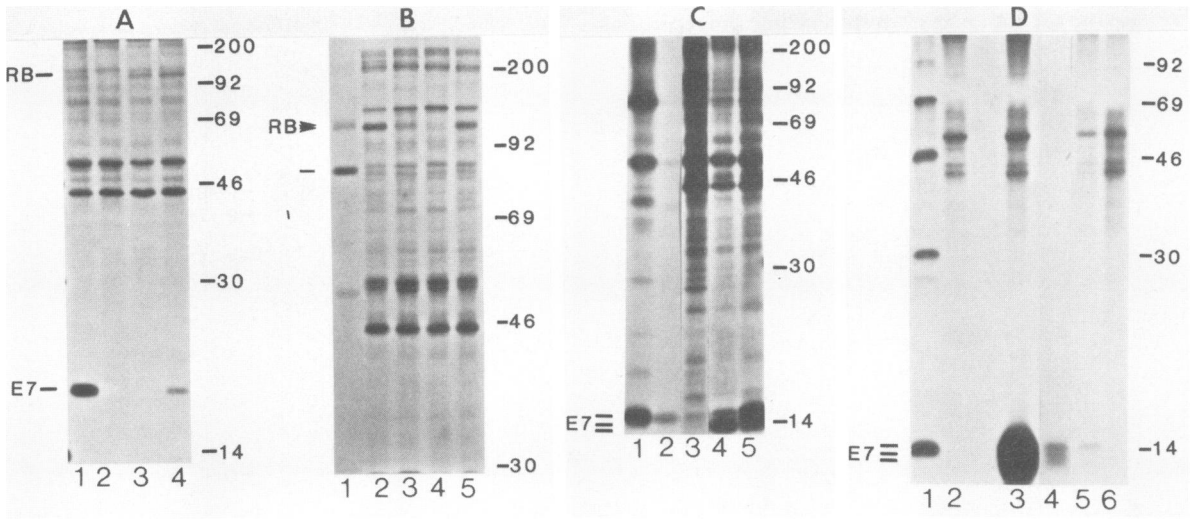

FIG. 4. Binding of HPV-16 E7 and HPV-6b E7 to the RB protein. (A) Autoradiogram of a 12% SDS-polyacrylamide gel of COS-7 cells transfected with HPV-16 E7/pSVLA4 (lanes 1 and 4) or the parental vector pSVLA4 (lanes 2 and 3). Cell extracts were labeled with [^{35}S]trans and immunoprecipitated with anti-HPV-16 E7 serum (lanes 1 and 2) or anti-RB serum (lanes 3 and 4). Positions of HPV-16 E7 and the RB protein are indicated on the left. Molecular sizes (in kilodaltons) of size markers are indicated on the right of each panel. (B) Autoradiogram of the immunoprecipitates shown in panel A when analyzed by 7.5% SDS-PAGE. The analysis of a ^{32}P-labeled extract is included (lane 1). The position of the RB protein is shown on the left, as is the position of the 81-kDa SV40 LgT protein (——). (C) Autoradiogram of a 12% SDS-polyacrylamide gel of cells transfected with HPV-6b E7/pSVLA4. Cells were labeled with [^{32}P]H_3PO_4 (lanes 1 and 2) or [^{35}S]trans (lanes 3 to 5). Cell extracts were immunoprecipitated with anti-HPV-6b E7 serum (lanes 1, 2, 4, and 5) or anti-RB serum (lane 3). Samples in lanes 2 and 4 were treated with CIAP to determine the position of the slowest-moving phosphorylated species of HPV-6b E7 in these gross overexposures. The positions of the multiple species of HPV-6b E7 are indicated on the left. (D) Autoradiogram of a 15% SDS-polyacrylamide gel of cells transfected with HPV-6b E7/pSVLA4 (lanes 3 to 5) or pSVLA4 (lanes 2 and 6). The transfected cells were labeled with [^{35}S]cysteine, and cell extracts were immunoprecipitated with anti-HPV-6b serum (lanes 2 to 4) or anti-RB serum (lanes 5 and 6). All lanes represent 6-day autoradiographs except for lane 4, which shows a 5-h exposure of lane 3 to permit better identification of the individual HPV-6b E7 species. Lane 1, Molecular size markers. Positions of the multiple HPV-6b E7 species are indicated on the left.

anti-HPV-16 E7 or with anti-RB antisera (Fig. 4A). The anti-RB serum precipitated HPV-16 E7, but it precipitated only a fraction of that precipitated with the anti-HPV-16 E7 serum. Conversely, the anti-HPV-16 E7 serum precipitated a portion of the RB protein (Fig. 4A; compare the RB and E7 bands in lanes 1 and 4). The finding that no RB protein was precipitated with anti-HPV-16 E7 in COS-7 cells that did not express the HPV-16 E7 (Fig. 4A, lane 2) indicated that the coprecipitation from the E7-expressing cells was not due to an immunologic cross-reactivity. These results thus show that a complex was formed in vivo between the RB protein and HPV-16 E7.

Since it has been reported that LgT of SV40 preferentially binds to the underphosphorylated form of the RB protein (23), it was of interest to see whether the same was true for HPV-16 E7. In our analysis, the slower-moving phosphorylated RB protein was only marginally separated from the bulk ^{35}S-labeled RB protein (Fig. 4B, lanes 1 and 2). Furthermore, the width of the ^{35}S band seemed not to cover the position of the ^{32}P band, which suggested that in these cells, the phosphorylated RB protein represented a minor species. Since the RB protein that coprecipitated with anti-HPV-16 E7 migrated exactly as did that precipitated with anti-RB, we can only conclude that the phosphorylated species was not preferentially bound. As expected, immunoprecipitation with anti-HPV-16 E7 from ^{32}P-labeled cells did not result in a ^{32}P-labeled RB band (data not shown).

By far the strongest band immunoprecipitated with the anti-RB serum from ^{32}P-labeled COS-7 cells expressing HPV-16 E7 (Fig. 4B, lane 1) was the 81-kDa LgT protein of SV40 (22). In contrast to immunoprecipitation with anti-RB serum, precipitates with anti-HPV-16 E7 serum did not precipitate this protein (data not shown). This finding indicates that immunoprecipitation of HPV-16 E7 from COS-7 cells by anti-RB serum was the result of binding of HPV-16 E7 to the RB protein rather than to LgT of SV40. The absence of an SV40 LgT band in immunoprecipitates with anti-RB serum of ^{35}S-labeled extracts reflects the fact that during a 1-h pulse, LgT of SV40 was much more efficiently labeled with ^{32}P than with ^{35}S.

A potential interaction of the RB protein with E7 of HPV-6b was analyzed in the same manner as that with HPV-16 E7. Since HPV-6b E7 could be resolved into three species, it was of particular interest to see whether there was preferential binding to any particular species. The results presented in Fig. 4C were obtained with cells labeled with [^{35}S]trans (ICN) containing mostly [^{35}S]methionine. In comparison with COS-7 cells expressing HPV-16 E7, only a faint band could be seen in precipitates with anti-RB serum (Fig. 4C, lane 3). The mobility of the band suggested that it was the phosphorylated species of HPV-6b E7 that was coprecipitated. Because of the low intensity of the E7 band relative to the background, a similar experiment was performed by using [^{35}S]cysteine, which preferentially labels E7; to resolve the E7 species more clearly, SDS-PAGE analysis was performed on a 15% gel (Fig. 4D). Because of the low background, the results clearly show that the band immunoprecipitated with anti-RB and comigrating with the phosphorylated form of E7 was actually an E7 species, since it was not present in COS-7 cells that did not express E7 (Fig. 4D, lane 6). Not surprisingly, in none of the precipitates with anti-HPV-6b E7 could the RB protein band be recognized. These results clearly show that E7 of HPV-6b bound much less extensively to the RB protein than did E7 of

HPV-16; interestingly, the binding was predominantly to the phosphorylated species of HPV-6b E7.

Glycerol gradient analysis of HPV-16 E7, HPV-6b E7, and the RB protein. In previous experiments, it was shown that HPV-16 E7 from CaSki cells exhibits a heterogeneous and unexpectedly high sedimentation rate in nondenaturing glycerol gradients (35). The evidence shown here for a HPV-16 E7–RB protein complex formation in vivo and by others for a complex formation in vitro (11) suggested that this complex formation may be at least in part responsible for the unusual sedimentation properties. Since we showed that HPV-6b E7 bound the RB protein to a much lower extent than did HPV-16 E7, a difference in sedimentation between HPV-16 E7 and HPV-6b E7 would be expected. HPV-16 E7 was present throughout most of the gradient, and the band intensities were similar from fractions 3 to 13 (Fig. 5C). In contrast, HPV-6b E7 was predominantly present in fractions 3 to 6, and there was a clear indication of a biphasic distribution with peaks in fractions 3 and 6. A direct indication that some of the fast-sedimenting HPV-16 E7 was complexed to the RB protein is provided in Fig. 5A and B. In fractions 6 to 13, which contained RB protein (Fig. 5A), a major portion of the E7 protein was precipitated with anti-RB serum (Fig. 5B). The results thus show that binding of HPV-16 E7 to the RB protein was in part responsible for the unusual sedimentation properties of HPV-16 E7. In addition, the biphasic distribution of HPV-6b E7 may indicate the interaction with yet another cellular protein(s) or the formation of oligomeric complexes.

Demonstration of the RB protein in SiHa, CaSki, HT-3, and COS-7 cells. When the sedimentation of HPV-16 E7 in COS-7 cells (Fig. 5C) is compared to that in CaSki cells shown previously (35), it is clear that the fast sedimentation of the E7 protein in COS-7 cells was much more pronounced; as indicated, this phenomenon can be attributed in part to HPV-16 E7–RB protein complex formation. It was possible, therefore, that the relatively slow sedimentation of E7 from CaSki cells was due to a low content of RB protein in these cells, which would limit complex formation. Furthermore, the finding that some cancers are associated with a defect in RB protein structure or expression (13–15, 17) and the theory that interaction of the viral proteins SV40 LgT and adenovirus E1A with the RB protein may be critical for the transforming function could indicate that relative levels of E7 and RB protein were an important factor in the development of HPV-associated genital cancers. To test this, we immunoprecipitated the RB protein from three cervical cancer-derived cell lines. One, HT-3, does not contain any known HPV DNA, whereas CaSki and SiHa cells contain HPV-16 DNA. The level of HPV-16 E7 expression is high in CaSki cells and low in SiHa cells. The results of the anti-RB precipitations (Fig. 6) showed that SiHa, HT-3, and COS-7 cells expressed similar levels of RB protein, whereas the RB band in CaSki cells was much less prominent. The high level of RB protein in COS-7 cells relative to RB protein levels in the CaSki cell line may very likely explain the differences in sedimentation of E7 observed between the two types of cells. However, high levels of RB protein expression are not necessarily paralleled by high levels of E7 in cervical cancers, since SiHa cells express a low level of E7 and a high level of RB protein, and the reverse situation is seen in CaSki cells. This suggests that besides the interaction of the RB protein with the E7 proteins, other factors play an important role in the development of HPV-associated genital cancers.

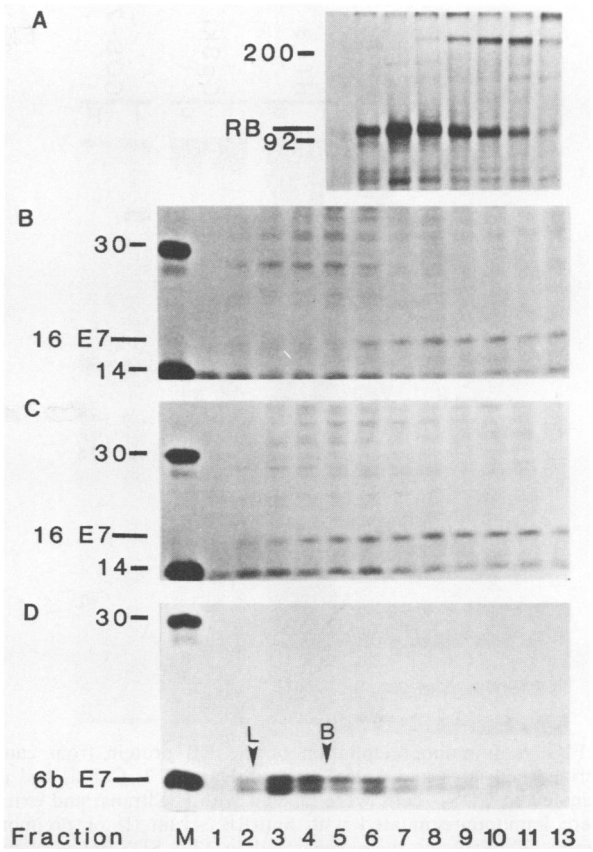

FIG. 5. Sedimentation of RB, HPV-16 E7, and HPV-6b E7 in nondenaturing glycerol gradients. COS-7 cells were transfected with HPV-16 E7/pSVLA4 (A to C) or HPV-6b E7/pSVLA4 (D). The cells were labeled with [^{35}S]trans and sedimented in glycerol gradients. Fractions were individually immunoprecipitated as follows: anti-RB serum (A and B), anti-HPV-16 E7 serum (C), and anti-HPV-6b E7 serum (D). Fraction numbers at the bottom correspond to all panels. Fraction 11 contained half of pooled fractions 11 and 12, and fraction 13 contained half of fractions 13 and 14. Positions and sizes (in kilodaltons) of molecular size markers, positions of the RB, HPV-16 E7, and HPV-6b E7 proteins are indicated to the left of each panel. Sedimentation of the glycerol gradients was from left to right, and the positions of lysozyme (L) (13 kDa) and bovine serum albumin (B) (68 kDa) are indicated. The bands migrating at 14 kDa in panels B and C represent labeled material migrating with the tracking dye band. The gel in panel D was electrophoresed longer. The proteins in panel A were separated on a 7.5% SDS-polyacrylamide gel, whereas those in the other panels were separated in 12% SDS-polyacrylamide gels.

DISCUSSION

We have used transient transfections of COS-7 cells to characterize and compare the properties of the E7 proteins of the nononcogenic HPV-6b and oncogenic HPV-16. The E7 proteins were shown to differ in several properties and particularly in the extent of their binding to the RB protein. Although both proteins contain 99 amino acids and have the same calculated molecular size of 11 kDa, their electrophoretic mobilities in SDS-PAGE are quite different. The mobility of HPV-16 E7 corresponds to a protein of about 20 kDa and this, together with the very slow sedimentation on denaturing glycerol gradients (35), suggested that the protein had an unusual extended conformation similar to that of E1A (36). HPV-6b E7 differs in this respect from HPV-16 E7; its

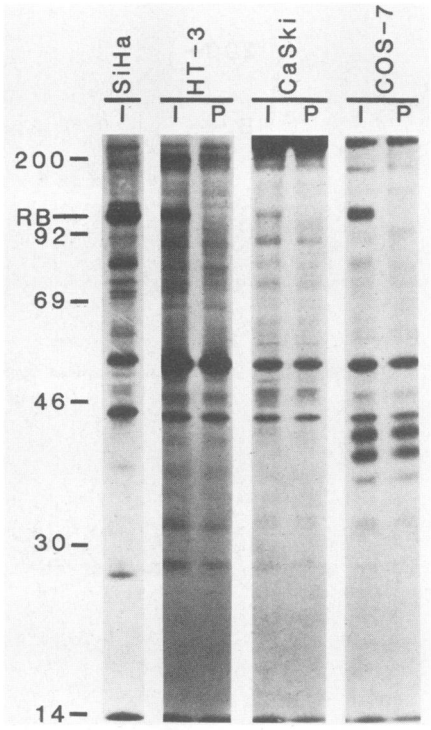

FIG. 6. Immunoprecipitation of the RB protein from cancer-derived cell lines and COS-7 cells. SiHa, HT-3, CaSki, and non-transfected COS-7 cells were labeled with [^{35}S]trans, and extracts were immunoprecipitated with anti-RB serum (I) or preimmune serum (P). Proteins were separated in 12% SDS-polyacrylamide gels. Position and sizes (in kilodaltons) of molecular size markers and the position of the RB protein are indicated on the left.

actual mobility in SDS-PAGE is only slightly lower than that expected from the calculated molecular weight, and in this regard it is similar to E7 of cottontail rabbit papillomavirus (3) and to E7 of HPV-18 (4). A second property in which E7 of HPV-6b differs from other E7 proteins is that it is resolved into three species. Labeling with ^{32}P and treatment with phosphatases clearly showed that the slowest-moving species was phosphorylated and that phosphorylation was responsible for the slower migration. On the basis of relative intensities of the three ^{35}S-labeled E7 bands, less than half of the E7 protein appears to be phosphorylated. The molecular basis for the different mobilities of the other two forms is not known. Both HPV-16 E7 and HPV-6b E7 are phosphorylated at serine residues. In contrast, E7 of cottontail rabbit papillomavirus is not phosphorylated (3). The finding that HPV-16 E7 is phosphorylated in cancer-derived CaSki cells as well as in COS-7 cells makes it less likely that the extent of phosphorylation of HPV-6b E7 is a state peculiar to COS-7 cells.

The E7 proteins of HPV-16 and HPV-6b demonstrate further differences in sedimentation properties in nondenaturing glycerol gradients. Although both proteins have a relatively high heterogeneous sedimentation rate, the overall sedimentation rate of HPV-6b E7 is lower than that of HPV-16 E7. The multiple species of HPV-6b E7 exhibit a biphasic sedimentation profile, similar to the E7 protein of cottontail rabbit papillomavirus (3), and the calculated molecular masses for the two peaks are 22 and 82 kDa. Although the measurement of the sedimentation rates, particularly the slower peak, is not very accurate, it may suggest that the HPV-6b E7 protein forms tetramer structures or complexes with some other cellular protein. Clearly, the biphasic distribution is not dependent on the level of phosphorylation, since all species appear in the faster-sedimenting peak. Since E7 has been shown to be a zinc-binding protein (2), we have considered the possibility that this binding may affect its sedimentation. Pretreatment of an extract with 10 mM EDTA and sedimentation through an EDTA-containing gradient did not affect the sedimentation of HPV-6b E7 (data not shown), suggesting that zinc was not directly involved.

The first evidence for the binding of HPV-16 E7 to the RB protein has been provided by an in vitro binding assay in which a cDNA clone of the RB protein was transcribed in vitro and translated in a cell-free system (11). Although no intact RB protein was synthesized in this system, nevertheless some of the RB protein fragments were shown to bind to HPV-16 E7 (11). To provide evidence for the binding of intact RB protein to HPV-16 E7, we expressed HPV-16 E7 in COS-7 cells. In this system, only full-length RB protein was present, and immunoprecipitation with anti-RB serum resulted in coprecipitation of a portion of the HPV-16 E7 protein. The previous reports that E7 is located in the cytoplasm (34) and that the RB protein is located in the nucleus (19) could indicate that the complex was formed after cell disruption. More recently, it has been shown by using immunohistochemical methods rather than cell fractionation that HPV-16 E7 is located in the nucleus (28).

The COS-7/pSVLA4 expression system used in this study allows high levels of expression of the HPV-16 and HPV-6b E7 proteins against an identical spectrum of background proteins. Importantly, the level of RB protein is not affected by the expression of HPV-16 E7 (Fig. 4A and B); similarly, no effect was noted in cells expressing HPV-6b E7 when analyzed on 7.5 or 12% gels (data not shown). It is possible that LgT of SV40 present in the COS-7 cells competes to some extent with the E7 proteins for binding to the RB protein; however, on the basis of the results of others (8, 23), only a fraction of the RB protein is immunoprecipitated with anti-LgT serum from these cells. Furthermore, the coprecip-

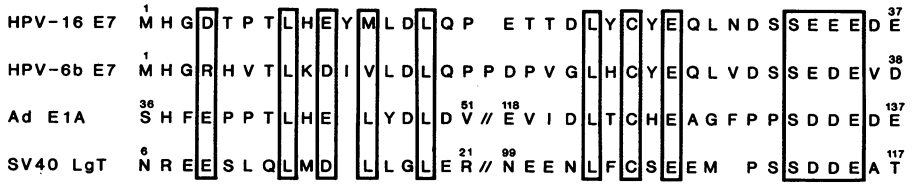

FIG. 7. Amino acid sequence homologies between HPV-16 E7, HPV-6b E7, adenovirus E1A, and SV40 LgT proteins. Only a portion of the homologous amino acid sequences are shown. Minimum regions of E1A required for binding to the RB protein (11) and the corresponding residues in HPV-16 E7, HPV-6b E7, and SV40 LgT are boxed. Numbers above each sequence indicate the amino acid position of each protein. / /, Break in the amino acid sequence.

itation of HPV-16 E7 with anti-RB serum is not mediated by LgT of SV40, since anti-HPV-16 E7 serum coprecipitates RB protein but not SV40 LgT (Fig. 4A and B). Finally, in immunoprecipitates from SiHa cells with anti-RB serum, a weak band of E7 protein could be detected after much longer exposures of X-ray films than those shown in Fig. 6.

Although the role of RB-HPV E7 complexes in malignancy is unknown, the differences in binding by the oncogenic HPV-16 E7 and nononcogenic HPV-6b E7 to the RB protein support the current hypothesis that the E7 proteins may effectively remove functional RB protein from the cell, allowing uncontrolled growth (11). The difficulty in detecting the interaction of HPV-6b E7 with the RB protein could imply that the interaction is very weak and many complexes do not survive the immunoprecipitation procedures or that the RB protein binds only a subset of the E7 proteins. Our data strongly suggest that the RB protein binds only to the phosphorylated species of HPV-6b E7. Therefore, the level of phosphorylation of the E7 protein may play a critical role in binding to the RB protein. However, even when only the phosphorylated species of HPV-6b E7 is considered, much less of the RB protein is bound by this subset of HPV-6b E7 than by HPV-16 E7.

The E7 protein of HPV-16 has functional and structural characteristics similar to those of the adenovirus E1A protein and SV40 LgT. Phelps et al. (25) have demonstrated that, like E1A, HPV-16 E7 can transactivate the adenovirus E2 promoter and can cooperate with the oncogene *ras* to transform primary baby rat kidney cells. E1A (38), LgT (8, 23), and HPV-16 E7 (11) have all been shown to bind to the RB protein. Furthermore, mutational analysis of E1A and LgT have shown that there is a close linkage between transforming ability and binding to the RB protein. To determine whether sequence differences between HPV-16 E7 and HPV-6b E7 could explain the difference in RB protein binding and the apparent inability of HPV-6b E7 to cooperate with *ras* in cell transformation, the regions required for binding to the RB protein by E1A (11) were compared with the homologous sequences in HPV-16 E7, HPV-6b E7, and LgT (Fig. 7). The minimal regions, 12 amino acid residues, of E1A required for binding to the RB protein are enclosed in boxes, along with the corresponding amino acids in HPV-16 E7, HPV-6b E7, and LgT. Of these 12 amino acid positions, HPV-6b is identical to HPV-16 at 8 positions. Of those that differ, three (Asp-10, Val-12, and Asp-35 in HPV-6b) represent conservative changes. HPV-16 E7 differs from E1A in four of the boxed positions, and all of the differences are conservative. It is interesting to note that mutations in Cys-124, Glu-126, Ser-132, and Glu-135 of E1A eliminate the ability of this protein to cooperate with *ras* in transformation (20, 40). In the corresponding amino acids of HPV-16 E7, mutations in Cys-24 and Glu-26 also abolish transformation and severely inhibit transactivation (12). HPV-6b E7 is identical to HPV-16 E7 at these amino acid residues. DeCaprio et al. (8) have reported that mutations in Glu-107, Glu-108, and Ser-112 abolish transformation and RB-binding in SV40 LgT and that mutations in both Cys-105 and Asp-114 eliminate binding to the RB protein. In these apparently critical positions, HPV-16 E7 and HPV-6b E7 are identical. The only nonconservative difference exists at position 4, where in HPV-6b E7 a basic amino acid, arginine, is located, and the corresponding residue in HPV-16 E7 is an acidic amino acid, aspartic acid. At present it is not known whether this nonconservative difference or conservative differences are linked to the difference in RB protein binding or to the difference in cooperation with *ras*.

Finally, our finding that the phosphorylated HPV-6b E7 binds preferentially to the RB protein could provide an additional explanation for why HPV-6b is associated with cancer in rare cases. The first explanation is that in some of these cancers, alteration in the noncoding region (5, 16, 26) resulted in a more active enhancer (27), which may lead to a higher level of E7 expression and binding of RB protein. The second explanation would be that changes in the expression of cellular kinases may lead to a higher level of E7 phosphorylation and stronger binding of the RB protein. This situation may exist in those HPV-6-associated cancers in which no alterations in the noncoding region of the resident HPV-6 were noted (5).

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LITERATURE CITED

1. **Androphy, E. J., N. L. Hubbert, J. T. Schiller, and D. R. Lowy.** 1987. Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. EMBO J. **6**:989–992.
2. **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.
3. **Barbosa, M. S., and F. O. Wettstein.** 1988. Identification and characterization of the CRPV E7 protein expressed in COS-7 cells. Virology **165**:134–140.
4. **Bernard, H.-U., T. Oltersdorf, and K. Seedorf.** 1987. Expression of the human papillomavirus type 18 E7 gene by a cassette-vector system for the transcription and translation of open reading frames in eukaryotic cells. EMBO J. **6**:133–138.
5. **Boshart, M., and H. zur Hausen.** 1986. Human papillomaviruses in Buschke-Lowenstein tumors: physical state of the DNA and identification of a tandem duplication in the noncoding region of a human papillomavirus 6 subtype. J. Virol. **58**:963–966.
6. **Brown, M., M. McCormack, K. G. Zinn, M. P. Farrell, I. Bikel, and D. M. Livingston.** 1986. A recombinant murine retrovirus for simian virus 40 large T cDNA transforms mouse fibroblasts to anchorage-independent growth. J. Virol. **60**:290–293.
7. **Cooper, J. A., and C. S. King.** 1986. Dephosphorylation or antibody binding to the carboxy terminus stimulates pp60$^{c\text{-}src}$. Mol. Cell. Biol. **6**:4467–4477.
8. **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.
9. **de Villiers, E.-M., L. Gissmann, and H. zur Hausen.** 1981. Molecular cloning of viral DNA from human genital warts. J. Virol. **40**:932–935.
10. **Dürst, M., L. Gissman, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA **80**:3812–3815.
11. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–937.
12. **Edmonds, C., and K. H. Vousden.** 1989. A point mutational analysis of human papillomavirus type 16 E7 protein. J. Virol. **63**:2650–2656.
13. **Friend, S. H., J. M. Horowitz, M. R. Gerber, X.-F. Wang, E. Bogenmann, F. P. Li, and R. A. Weinberg.** 1987. Deletions of a DNA sequence in retinoblastomas and mesenchymal tumors: organization of the sequence and its encoded protein. Proc. Natl. Acad. Sci. USA **84**:9059–9063.
14. **Harbour, J. W., S.-L. Lai, J. Whang-Peng, A. F. Gazdar, J. D. Minna, and F. J. Kaye.** 1988. Abnormalities in structure and

expression of the human retinoblastoma gene in SCLC. Science **241**:353–357.

15. **Horowitz, J. M., D. W. Yandell, S.-H. Park, S. Canning, P. Whyte, K. Buchkovich, E. Harlow, R. A. Weinberg, and T. P. Dryja.** 1989. Point mutational inactivation of the retinoblastoma antioncogene. Science **243**:937–940.
16. **Kasher, M. S., and A. Roman.** 1988. Characterization of human papillomavirus type 6b DNA isolated from an invasive squamous carcinoma of the vulva. Virology **165**:225–233.
17. **Lee, E. Y.-H. P., H. To, J.-Y. Shew, R. Bookstein, P. Scully, and W.-H. Lee.** 1988. Inactivation of the retinoblastoma susceptibility gene in human breast cancers. Science **241**:218–221.
18. **Lee, W.-H., R. Bookstein, F. Hong, L.-J. Young, J.-Y. Shew, and E. Y.-H. P. Lee.** 1987. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science **235**:1394–1399.
19. **Lee, W.-H., J.-Y. Shew, F. D. Hong, T. W. Sery, L. A. Donoso, L.-J. Young, R. Bookstein, and E. Y.-H. P. Lee.** 1987. The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature (London) **329**:642–645.
20. **Lillie, J. W., M. Green, and M. R. Green.** 1986. An adenovirus E1a protein region required for transformation and transcriptional repression. Cell **46**:1043–1051.
21. **Linzer, D. I. H.** 1988. The marriage of oncogenes and anti-oncogenes. Trends Genet. **4**:245–247.
22. **Livingston, D. M., and M. K. Bradley.** 1987. Review: the simian virus 40 large T antigen. A lot packed into a little. Mol. Biol. Med. **4**:63–80.
23. **Ludlow, J. W., J. A. DeCaprio, C.-M. Huang, W.-H. Lee, E. Paucha, and D. M. Livingston.** 1989. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell **56**:57–65.
24. **Peto, R., and H. zur Hausen (ed.).** Banbury report, vol. 21. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Phelps, W. C., C. L. Yee, K. Münger, 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.
26. **Rando, R. F., D. E. Groff, J. G. Chirikjian, and W. D. Lancaster.** 1986. Isolation and characterization of a novel human papillomavirus type 6 DNA from an invasive vulvar carcinoma. J. Virol. **57**:353–356.
27. **Rando, R. F., W. D. Lancaster, P. Han, and C. Lopez.** 1986. The noncoding region of HPV-6vc contains two distinct transcriptional enhancing elements. Virology **155**:545–556.
28. **Sato, H., S. Watanabe, A. Furuno, and K. Yoshiike.** 1989. Human papillomavirus type 16 E7 protein expressed in *Escherichia coli* and monkey COS-1 cells: immunofluorescence detection of the nuclear E7 protein. Virology **170**:311–315.
29. **Schneider-Gaedicke, A., and E. Schwartz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**: 2285–2292.
30. **Schwartz, E., M. Dürst, C. Demankowski, O. Latterman, R. Zech, E. Wolfsperger, S. Suhai, and H. zur Hausen.** 1983. DNA sequence and genome organization of genital human papillomavirus type 6b. EMBO J. **2**:2341–2348.
31. **Seedorf, K., G. Krammer, M. Dürst, S. Suhai, and W. G. Rowekamp.** 1985. Human papillomavirus type 16 DNA sequence. Virology **145**:181–185.
32. **Seedorf, K., T. Oltersdorf, G. Krammer, and W. Rowekamp.** 1987. Identification of early proteins of the human papilloma viruses type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. EMBO J. **6**:139–144.
33. **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.
34. **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.
35. **Smotkin, D., and F. O. Wettstein.** 1987. The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. J. Virol. **61**:1686–1689.
36. **Spindler, K. R., D. S. E. Rosser, and A. J. Berk.** 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. J. Virol. **49**:132–141.
37. **von Knebel Doeberitz, M., T. Oltersdorf, E. Schwartz, and L. Gissmann.** 1988. Correlation of modified human papilloma virus early gene expression with altered growth properties in C4-1 cervical carcinoma cells. Cancer Res. **48**:3780–3786.
38. **Whyte, P., K. J. Buchkovich, 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.
39. **Wilczynski, S. P., L. Pearlman, and J. Walker.** 1988. Identification of HPV 16 early genes retained in cervical carcinomas. Virology **166**:624–627.
40. **Zerler, B., R. J. Roberts, M. B. Mathews, and E. Moran.** 1987. Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. Mol. Cell. Biol. **7**:821–829.