

Purification and Characterization of Human Papillomavirus Type 16 E7 Protein with Preferential Binding Capacity to the Underphosphorylated Form of Retinoblastoma Gene Product

YOSHINORI IMAI,* YOUKO MATSUSHIMA, TAKASHI SUGIMURA,
AND MASAOKI TERADA

Genetics Division, National Cancer Center Research Institute,
1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

Received 22 January 1991/Accepted 14 June 1991

Human papillomavirus type 16 E7 is considered to be a major viral oncoprotein playing an important role(s) in cervical cancers. E7 protein was shown to bind to the protein product of the retinoblastoma gene (RB), while simian virus 40 large T and adenovirus E1A were also shown to possess binding activity to RB protein. The RB protein is a cell cycle regulator that is highly phosphorylated specifically in S, G₂, and M, whereas it is underphosphorylated in G₀ and G₁. Recently, large T was demonstrated to bind preferentially to the underphosphorylated RB protein, which is considered to be an active form restricting cell proliferation. However, it is not known whether E7 can bind to phosphorylated RB protein. We successfully purified large quantities of unfused human papillomavirus type 16 E7 protein expressed in *Escherichia coli* by using a T7 promoter-T7 RNA polymerase system. The purified E7 protein was demonstrated to bind preferentially to the underphosphorylated RB protein.

More than 60 types of human papillomaviruses (HPVs) have been reported. Among them, HPV types 16 (HPV-16), 18, 31, 33, 35, 39, 45, 51, 52, and 56 are frequently found in cervical cancer tissues or cell lines (5, 13, 14). HPV-16 or -18 DNA has the ability to transform established rodent cell lines (47, 52) and to immortalize primary rodent or human keratinocytes (39, 41). The HPVs are therefore believed to be involved in the development of cervical cancers. The viral DNA integrated into the genome of cancer cells is truncated to various degrees. However, E6 and E7 open reading frames are consistently retained and expressed as mRNA or proteins (2, 25, 43, 44, 46). Molecular genetic studies to localize the transforming activities of HPV revealed that the E7 region is the major transforming gene (38). Recent studies suggest that not only the E7 but also the E6 region is necessary for the transformation of primary human keratinocytes (35).

The retinoblastoma gene (*RB*) was deleted or mutated in many types of tumor cells, including retinoblastomas (17, 18, 30), osteosarcomas (17), small-cell lung carcinomas (21, 53), breast cancers (29), bladder carcinomas (22), prostate carcinomas (4), and leukemias (9). The *RB* gene was reported to restrict cell proliferation (4, 23). The loss of normal activity of the *RB* gene is consequently believed to play a key role in the generation of the cancers. It was reported that E7 proteins of HPVs could bind to the *RB* gene product, the RB protein (3, 15, 19, 36). The transforming proteins of adenovirus and simian virus 40 (SV40), E1A and large T, respectively, also associated with the RB protein (11, 50). These observations suggest that the formation of complexes of the RB protein and the transforming proteins of the DNA tumor viruses may cause the loss or alteration of the biological function of the *RB* gene product and allow the cells to proliferate uncontrollably (20).

To test this hypothesis and understand the other biological

significances of E7 protein, large quantities of pure E7 protein are required. However, HPV cannot propagate in cultures, and cancer cells contain too little E7 protein to be purified. Some investigators have reported the synthesis of recombinant E7 proteins; however, all of the E7 proteins reported so far were fused forms of E7 and prokaryotic proteins (3, 26, 43, 44). We report here the successful production of unfused HPV-16 E7 protein in *Escherichia coli* and the purification of the protein to homogeneity. Using E7-coupled Sepharose gels, we examine the ability of E7 protein to bind to the highly phosphorylated and the underphosphorylated forms of the RB protein.

MATERIALS AND METHODS

Construction of expression vector. An *Nde*I-*Hind*III fragment of T7 promoter expression vector pGEMEXTM-1 (Promega) was substituted for an *Eco*T22I-*Nco*I fragment of HPV-16 (nucleotide positions 563 to 867) (42) by using a synthetic single-strand oligonucleotide linker, 5'-TATGCA-3', which connected the translation initiation site of the vector and the first ATG codon of the E7 open reading frame in frame (Fig. 1). The resulting vector, pMX-E7, was used to transform *E. coli* JM109 λ DE3 lysogen carrying the T7 RNA polymerase gene under the control of the *lac* promoter.

Purification of HPV-16 E7 protein. *E. coli* JM109 λ DE3 lysogen harboring pMX-E7 was grown in 2 liters of Luria-Bertani broth containing 50 μ g of ampicillin per ml. To induce E7 protein, isopropyl- β -D-thiogalactopyranoside (IPTG) and zinc acetate were added to the medium at final concentrations of 0.5 and 1 mM, respectively. After 3 h of incubation, the cells were pelleted and stored at -80°C.

Approximately 6 g of the cell pellet was suspended in 5 volumes of 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5% glycerol and lysed by the addition of lysozyme at a final concentration of 0.5 mg/ml. After a 30-min incubation on ice, the lysate was sonicated briefly

* Corresponding author.

and ultracentrifuged at $100,000 \times g$ for 1 h. The resulting supernatant was subjected to a DE-52 (Whatman) column (25 by 80 mm) equilibrated in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH, pH 7.6, containing 1 mM dithiothreitol and 5% glycerol (buffer A). The column was washed with buffer A containing 200 mM NaCl, and then E7 protein was eluted with 280 ml of buffer A containing 400 mM NaCl. Solid ammonium sulfate was added to the eluate to give 40% saturation, and the eluate was incubated while being stirred on ice for 30 min. Precipitated E7 protein was collected by centrifugation, dissolved in 2 ml of buffer A, and dialyzed against buffer A. Finally, the dialysate was passed through a Sephadex G-75 (Pharmacia) column (15 by 710 mm) equilibrated in buffer A. Eluted fractions containing E7 protein were stored at -80°C and used as a source of the purified E7 protein. All of these procedures were performed at 0 to 4°C .

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (28), and the gel was silver stained with a kit from Daiichi Kagaku (Tokyo, Japan).

Cell extract. SKG-IIIa cells (37) were cultured in Ham's F12 medium supplemented with 10% heat-inactivated fetal calf serum. T601 cells, a HPV-16 primary transformant of NIH 3T3 (47), and NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium with 5 and 10% calf serum, respectively. HL60 cells were grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum to a semiconfluent state, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was added to the medium at a final concentration of 50 nM in order to induce the cells to differentiate, and the culture was continued for 40 h before harvesting.

The cell pellet was washed three times with phosphate-buffered saline and lysed with 10 volumes of 50 mM HEPES-NaOH, pH 7.0, containing 0.1% Nonidet P-40, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM dithiothreitol, 50 μg of aprotinin per ml, and 1 mM PMSF. After a 30-min incubation with stirring on ice, the lysate was clarified by centrifugation at $50,000 \times g$ for 10 min and stored at -80°C .

Immunoblot analysis. Proteins fractionated by SDS-PAGE were electrotransferred onto Durapore membrane (Millipore) as described previously (24). The membrane was blocked at 4°C for 12 h with a solution containing 4% nonfat dry milk, 25 mM Tris-HCl (pH 8.0), 125 mM NaCl, 0.1% Tween 20, and 0.1% NaN_3 . It was incubated at room temperature for 2 h with anti-HPV-16 E7 rabbit serum prepared in our laboratory (unpublished data) or anti-RB monoclonal antibody PMG3-245 (Pharmingen) in the blocking mixture and washed three times with the blocking mixture. It was then incubated at room temperature for 1 h with alkaline phosphatase-conjugated anti-rabbit or anti-mouse goat immunoglobulin G (Cappel) diluted in the blocking mixture. After being washed twice with the blocking mixture and three times with 25 mM Tris-HCl (pH 8.0)-125 mM NaCl-0.025% Tween 20, the membrane was stained with 0.4 mM Nitro Blue Tetrazolium and 0.4 mM 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt in 0.1 M Tris-HCl (pH 9.5)-0.1 M NaCl-5 mM MgCl_2 .

Immunoprecipitation of RB protein. HL60 cells cultured in the absence or presence of TPA were labeled for 3 h in methionine-free and phosphate-free Dulbecco's modified Eagle medium containing 100 μCi of [^{35}S]methionine per ml and 300 μCi of [^{32}P]phosphate per ml, respectively. The cells were lysed in 25 mM Tris-HCl (pH 7.4)-50 mM NaCl-0.5% sodium deoxycholate-2% Nonidet P-40-0.2% SDS-1 mM PMSF-50 μg of aprotinin per ml-50 μM leupeptin. After

being incubated at room temperature for 5 min and on ice for 30 min, the lysate was centrifuged at $20,000 \times g$ for 15 min. To 50 μl of the resulting supernatant was added 1.5 μg of anti-RB monoclonal antibody PMG3-245, and the mixture was incubated on ice for 30 min before the addition of 30 μl of a 50% suspension of Protein A-Sepharose CL-4B (Pharmacia) to which 6 μg of anti-mouse immunoglobulin G₁ rabbit immunoglobulin G (Pharmingen) had been adsorbed. After a 30-min incubation at 4°C with shaking, the Sepharose gel was precipitated and washed five times with 0.3 ml of 25 mM Tris-HCl (pH 7.4)-50 mM NaCl-0.5% sodium deoxycholate-0.2% Nonidet P-40-1 mM PMSF. The precipitated proteins were boiled for 5 min in 60 μl of Laemmli's SDS-PAGE sample mixture (28), resolved by 8% SDS-PAGE, and autoradiographed.

E7-Sepharose. E7-Sepharose was prepared essentially according to the instructions described in a manual from Pharmacia. CNBr-activated Sepharose 4B (bed volume, 1 ml) (Pharmacia) was washed with 1 mM HCl and coupling buffer containing 0.1 M NaHCO_3 -NaOH, pH 8.3, and 0.5 M NaCl. The Sepharose gel was then suspended in 2 ml of coupling buffer containing 5 mg of purified HPV-16 E7 protein or 5 mg of leucine 13-substituted E7 protein. After rotation at 4°C for 24 h, the remaining active groups were blocked with 0.1 M Tris-HCl, pH 8.0. Approximately 90% of the E7 protein was coupled with the Sepharose.

RB-binding assay. The cell extract (25 μl) was incubated with 5 μl of a 50% suspension of E7-Sepharose gel in binding buffer containing 50 mM HEPES-NaOH, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, and 1 mM dithiothreitol. After a 30-min incubation at 4°C with shaking, the gel was precipitated and washed three times with 0.3 ml of the binding buffer. The proteins that bound to E7-Sepharose were then eluted by boiling for 5 min in 60 μl of Laemmli's SDS-PAGE sample mixture and fractionated by 8% SDS-PAGE before being analyzed by immunoblotting with anti-RB monoclonal antibody PMG3-245.

Site-directed mutagenesis. Site-directed mutagenesis was done by the uracil incorporation method of Kunkel et al. (27). Mutations were introduced into E7 sequence cloned into M13mp18 phage vector with 21-mer synthetic oligonucleotides, and the generated mutations were examined by the dideoxy-chain termination sequencing method (40). Leucine 13-substituted E7 protein was synthesized and purified exactly by the method described for wild-type E7 protein.

Other methods. Protein was determined by the method of Bradford (6) with a kit from Bio-Rad. The first 20 amino-terminal residues of the E7 protein were determined by Edman degradation with an Applied Biosystems ABI 477A/120A protein sequencer. Oligonucleotides were synthesized with an Applied Biosystems ABI 391A DNA synthesizer.

RESULTS

Expression and purification of unfused HPV-16 E7 protein. In order to express unfused HPV-16 E7 protein in *E. coli*, we chose a T7 promoter-T7 RNA polymerase system. Downstream of the T7 promoter, a genomic fragment of HPV-16 E7 was ligated to express E7 protein from the first ATG codon (Fig. 1). The resulting vector was used to transform the bacteria carrying the T7 RNA polymerase gene under the control of the *lac* promoter. After 3 h of induction with 0.5 mM IPTG, a protein band of about 19,000 Da was accumulated in the cells (Fig. 2, lanes 1 and 2). The molecular mass of the HPV-16 E7 protein as deduced from its nucleotide sequence is about 11,000 Da. However, the E7 protein

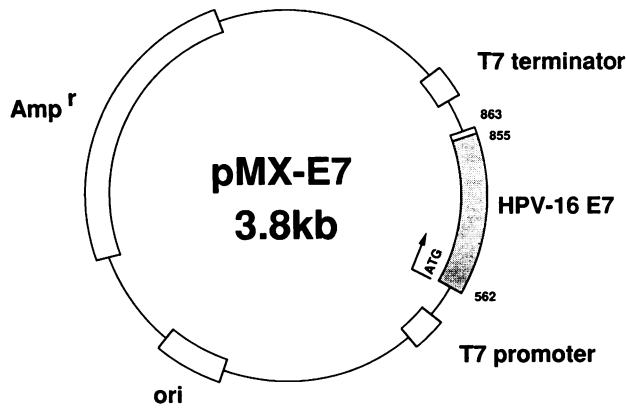


FIG. 1. Construction of expression vector pMX-E7. The method for constructing the plasmid is described in the text. The shaded box indicates the HPV-16 genome DNA fragment containing the E7 open reading frame, and numbers beside the box refer to nucleotide positions in HPV-16 (42).

expressed in cancer cell lines was reported to migrate to a position corresponding to a molecular mass greater than 11,000 Da by SDS-PAGE (15, 43-45). The bacterially expressed E7 protein described here also showed this unusual electrophoretic behavior.

Our E7 protein was soluble in the cells, although all of the reported E7 protein expressed in *E. coli* was insoluble fused protein forming inclusion bodies (3, 43, 44). The protein in the supernatant resulting from a $100,000 \times g$ centrifugation was purified to homogeneity by DEAE-cellulose chromatog-

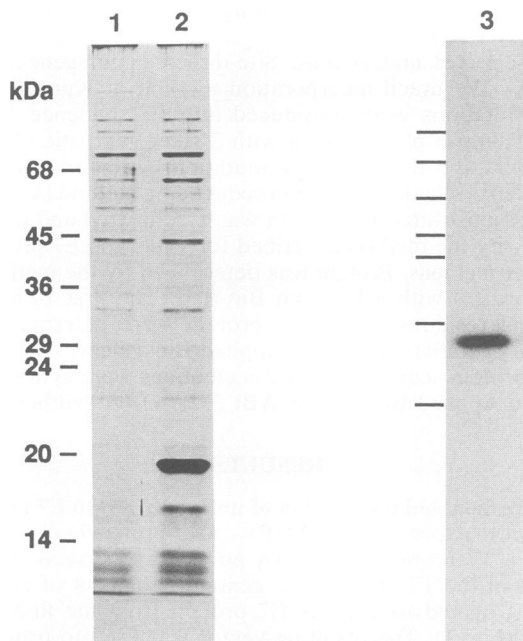


FIG. 2. Induction and purification of HPV-16 E7 protein. Total cell lysate from *E. coli* harboring pMX-E7 was analyzed by 12.5% SDS-PAGE before (lane 1) or after (lane 2) a 3-h induction with IPTG. Purified E7 protein was examined by 15% SDS-PAGE (lane 3). The migrations of standard proteins are indicated on the left along with their molecular masses.

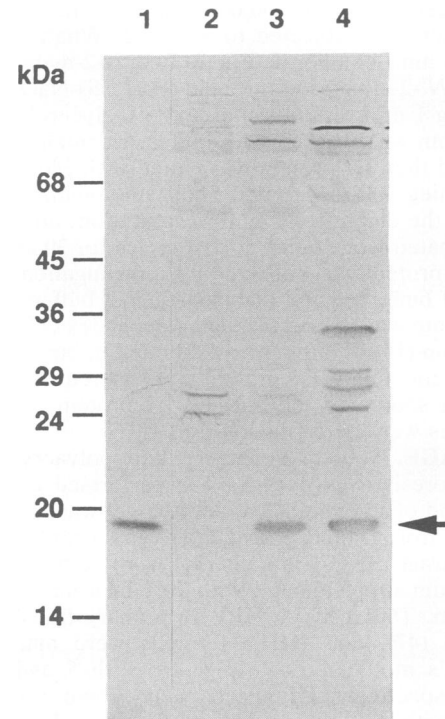


FIG. 3. Immunoblot analysis of E7 protein expressed in HPV-16 transformant or cancer cells. Extraction of cells and immunoblotting were performed as described in the text. Purified E7 protein (0.1 ng of protein; lane 1) or cell extract from NIH 3T3 (80 μ g of protein; lane 2), from T601, an HPV-16 transformant of NIH 3T3 (lane 3), or from cervical cancer cell line SKG-IIIa (lane 4) was fractionated by 12.5% SDS-PAGE and immunoblotted with anti-E7 rabbit serum. The arrow indicates the position of E7 protein.

raphy, ammonium sulfate precipitation, and Sephadex G-75 chromatography (Fig. 2, lane 3). Its first 20 amino-terminal residues, determined by Edman degradation, were identical with those of the E7 protein predicted by the nucleotide sequence (42).

Immunoblot analysis of E7 protein expressed in HPV-16 transformant or cancer cells. The extract from T601 cells, a HPV-16 transformant of NIH 3T3 (47), was examined by immunoblot analysis in which anti-E7 rabbit serum directed against the purified E7 protein recognized a discrete protein band migrating around 19,000 Da (Fig. 3, lane 3). A similar band was also detected in the extract from SKG-IIIa cells (lane 4) but not in the extract from NIH 3T3 cells (lane 2). SKG-IIIa is a cervical cancer cell line that contains a single copy of HPV-16 DNA per cell (48). The mobility of the purified E7 protein (lane 1) was the same as those of the bands that were effectively recognized by the anti-recombinant E7 serum, and the intensities of these bands markedly decreased when the serum had been preincubated with excessive amounts of the purified E7 protein. On the basis of these findings, we conclude that the 19,000-Da protein was HPV-16 E7 protein. Both the purified and the SKG-IIIa-derived E7 proteins showed similar intensities in the immunoblot analysis (Fig. 3, lanes 1 and 4), in which the total amounts of loaded proteins were 0.1 ng and 80 μ g, respectively. The content of E7 protein in SKG-IIIa cells is consequently estimated as about 0.0001% of the total cellular protein if there is no significant difference in the affinity of the anti-E7 serum to both of the E7 proteins.

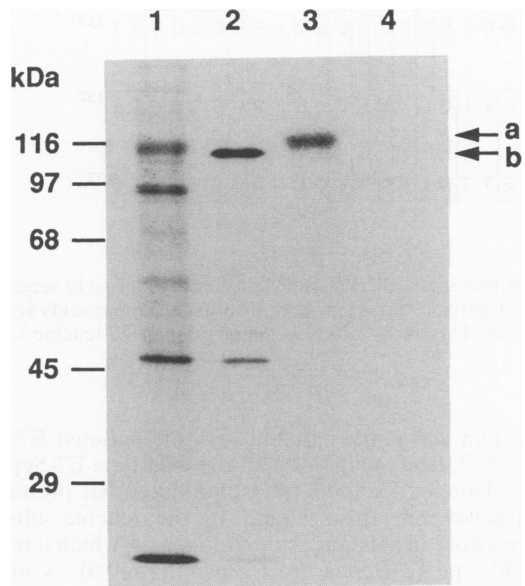


FIG. 4. TPA-induced dephosphorylation of the RB protein in HL60 cells. The RB protein in HL60 cells growing without TPA (lanes 1 and 3) or treated with 25 nM TPA for 42 h (lanes 2 and 4) was labeled for 3 h with [^{35}S]methionine (lanes 1 and 2) or [^{32}P]phosphate (lanes 3 and 4) and immunoprecipitated with anti-RB monoclonal antibody PMG3-245 as described in the text. Arrows indicate the positions of highly phosphorylated (a, pp110-RB) and underphosphorylated (b, p105-RB) forms of RB protein.

We could not detect any DNA-binding activities for the purified E7 protein when it was examined by DNA-cellulose chromatography or by a gel retardation assay using a DNA fragment corresponding to the HPV-16 long control region or the adenovirus E2 promoter.

RB-binding properties of HPV-16 E7 protein. The purified E7 protein was chemically coupled with Sepharose 4B gel beads and used for a novel assay to determine the E7-binding activity of cellular proteins. During incubation with E7-Sepharose, E7-binding proteins were associated with E7 and were therefore easily precipitated together with the gel. For the source of RB protein, we chose HL60 cells, a human leukemia cell line in which RB protein was reported to be highly phosphorylated under the usual culture conditions, whereas it was converted to the underphosphorylated form when cells were cultured in the presence of TPA or dimethyl sulfoxide (1, 8, 34).

The extract from HL60 cells cultured in the presence or absence of TPA was incubated with E7-Sepharose prior to the analysis of coprecipitated proteins by immunoblotting with PMG3-245, an anti-RB monoclonal antibody that could recognize both highly phosphorylated and underphosphorylated forms of RB protein (32). The phosphorylation status of RB protein could be easily examined by SDS-PAGE, in which the mobility of the RB protein shifted according to the extent of phosphorylation (7, 8, 12). We performed ^{35}S - and ^{32}P -labeling experiments showing that RB proteins in HL60 cells before and after the cells were cultured in the presence of TPA were really highly phosphorylated and underphosphorylated, respectively, under culture conditions essentially equal to those for the E7-Sepharose precipitation assay (Fig. 4). In the absence of TPA, the RB protein in the cells was highly phosphorylated to various degrees and showed

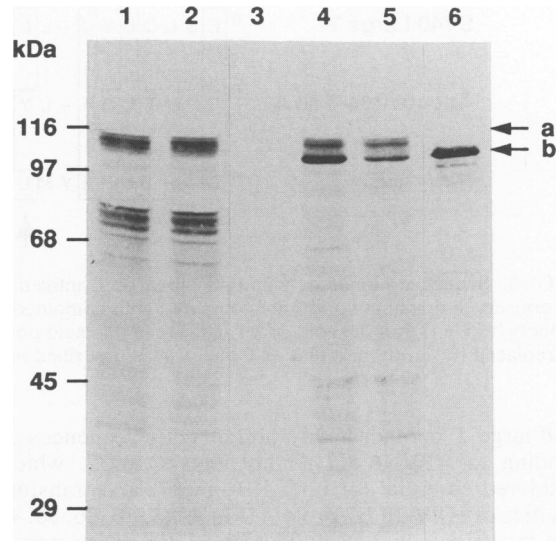


FIG. 5. RB-binding properties of HPV-16 E7 protein. An RB-binding assay was performed as described in the text. Extract from HL60 cells cultured in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of TPA was incubated with E7-Sepharose. The cell extracts before the incubation (lanes 1 and 4), E7-Sepharose unbound fractions (lanes 2 and 5), and bound fractions (lanes 3 and 6) were immunoblotted with anti-RB monoclonal antibody PMG3-245. Arrows indicate the positions of highly phosphorylated (a, pp110-RB) and underphosphorylated (b, p105-RB) forms of RB protein.

broad and slowly migrating bands (pp110-RB) (Fig. 4, lanes 1 and 3 and Fig. 5, lane 1). In the presence of TPA, by contrast, most of the RB protein was converted to the most rapidly migrating form (p105-RB) or the underphosphorylated form (Fig. 4, lanes 2 and 4 and Fig. 5, lane 4). When E7-Sepharose was incubated with the extract from proliferating HL60 cells, most of the RB protein, which was the highly phosphorylated form, could not bind to the gel (Fig. 5, lane 3) and remained in the liquid phase (Fig. 5, lane 2). On the other hand, when the cells were treated with TPA, most of the RB protein was converted to the underphosphorylated form, which was preferentially and stably associated with the E7-Sepharose and not released from the gel until boiled under denaturing conditions (Fig. 5, lane 6). The residual highly phosphorylated RB protein did not bind to the gel (Fig. 5, lane 5) as was shown to be the case for the TPA-untreated cells. The molecular masses (105 to 110 kDa) of the bands recognized by PMG3-245 anti-RB antibody in our experiments were identical to those demonstrated for RB proteins by several other laboratories (7, 8, 11, 12, 32) and compatible with the molecular mass deduced from its nucleotide sequence. In our preliminary experiments with PMG3-245 antibody, corresponding bands were not detected in the retinoblastoma cell line Y79, which expresses no normal RB protein (31) (data not shown). The nature of the additional faster migrating bands shown in Fig. 4, lanes 1 and 2 and in Fig. 5, lanes 1 and 2 is unknown. The E7-Sepharose also efficiently recognized the underphosphorylated RB protein from HL60 cells cultured in the presence of dimethyl sulfoxide (data not shown). These results indicate that only the underphosphorylated form of RB protein bore a higher affinity to E7 protein.

RB-binding activity of mutated E7 protein. Not only the E7 of the other types of HPVs but also adenovirus E1As and

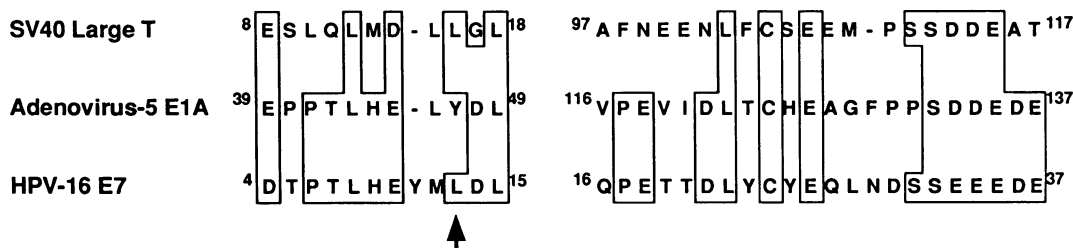


FIG. 6. Sequence homology among SV40 large T antigen, adenovirus-5 E1A protein, and HPV-16 E7 protein. Amino acid sequences of E1A conserved domains 1 and 2 are compared with homologous regions of large T antigen and E7 protein. Conserved amino acids are boxed. Numbers beside the amino acid codes indicate amino acid positions in the proteins. The arrow indicates the position of E7 leucine 13, which was replaced by serine in mutational analysis as described in the text.

SV40 large T contain highly homologous sequences corresponding to HPV-16 E7 amino acids 4 to 37, which are considered essential for both RB-binding and transforming activities of HPV-16 E7 protein (Fig. 6) (3, 16, 26, 36, 49). A point mutation substituting leucine 13 for serine was introduced into HPV-16 E7, and the expressed protein was purified and immobilized to Sepharose. The same quantities of wild-type protein and leucine-substituted E7 protein were coupled to the gel under the same conditions. Both proteins had the same solubility, SDS-PAGE mobility, and reactivity against anti-E7 antiserum. The resulting gel was incubated with the extract from HL60 cells cultured in the absence of TPA prior to the analysis of coprecipitated proteins by immunoblotting with anti-RB monoclonal antibody PMG3-245 (Fig. 7). About half of the highly phosphorylated form of

RB protein was coprecipitated with the mutated E7-Sepharose (Fig. 7, lane 5) but not with the wild-type E7-Sepharose (Fig. 7, lane 3). The underphosphorylated RB protein from TPA-treated cells also bound to the leucine-substituted E7-Sepharose in a manner similar to that in which it bound to the wild-type E7-Sepharose (data not shown). A mutation substituting cysteine 24 in conserved domain 2 was reported to reduce the binding affinity to RB protein (3, 26, 36). We prepared cysteine 24-substituted E7 for glycine and coupled it to Sepharose. The underphosphorylated RB protein bound less efficiently to the cysteine-mutated E7-Sepharose (unpublished data).

DISCUSSION

We have described here the purification and the RB-binding activity of the unfused HPV-16 E7 protein expressed in *E. coli*. This is the first report describing the purification of E7 protein in unfused soluble forms. In SDS-PAGE analysis, both cell line-derived and bacterially expressed unfused E7 proteins migrated to the same position, corresponding to 19,000 Da (Fig. 3), which is discrepant with the molecular mass of 11,000 Da deduced from its amino acid sequence. The reasons for this aberrant mobility are not clear.

E7 protein was reported to be a small nuclear phosphoprotein containing one possible zinc finger structure (19, 45, 49) and to be capable of transactivating the adenovirus E2 promoter (38). The cellular content of the E7 protein was only 0.0001% (Fig. 3), which was very low, like the cellular content reported for most of the transcription factors. However, we could not demonstrate any DNA-binding activity for the purified E7 protein by DNA-cellulose chromatography or by a gel retardation assay using the HPV-16 long control region and adenovirus E2 promoter DNA fragments as a probe. In contrast to E6 protein (24), E7 protein is an acidic protein and apparently does not have DNA-binding ability. The E7 protein is likely to exert its biological functions through protein-protein interactions, including those with RB protein, a nuclear phosphoprotein that is a possible cell cycle regulator (10). In fact, HPV E7 proteins which were fused or translated *in vitro* were reported to be capable of binding to RB protein in a manner similar to that of adenovirus E1A or SV40 large T (3, 15, 19, 36). However, it was not known whether the phosphorylation state of the RB protein affected its affinity to E7 protein. It is of great interest to solve this question because large T was reported to bind preferentially to underphosphorylated RB protein (32, 33), whereas E1A was suggested to bind both highly phosphorylated and underphosphorylated RB protein (51).

We have demonstrated here that the purified HPV-16 E7

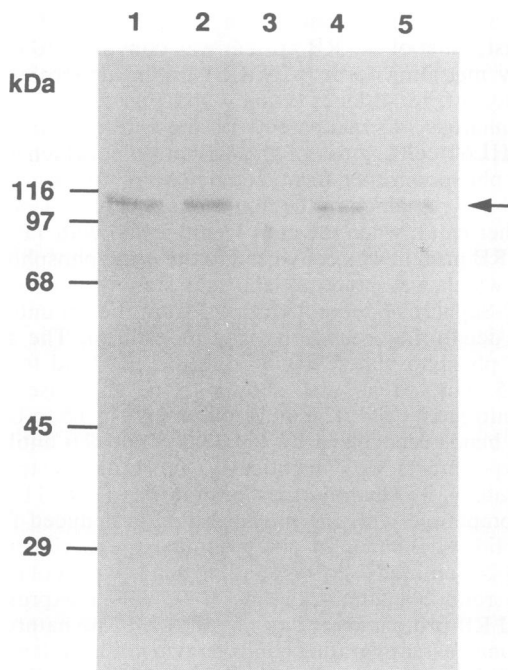


FIG. 7. RB-binding activity of E7 protein in which leucine 13 is substituted for serine. The cell extract from normally growing HL60 cells (lane 1) was incubated with wild-type (lanes 2 and 3) or mutated (lanes 4 and 5) E7-Sepharose as described in the text. After the incubation, both unbound (lanes 2 and 4) and bound (lanes 3 and 5) proteins were analyzed by immunoblotting with anti-RB monoclonal antibody PMG3-245. The arrow indicates the position of highly phosphorylated RB protein.

protein could bind preferentially to the underphosphorylated RB protein but not to the highly phosphorylated form (Fig. 5). It was shown that all of the RB protein was underphosphorylated in G₀ or G₁ phase (7, 8, 12). At the G₁/S boundary, the RB protein was converted to the highly phosphorylated form, possibly by cdc2 kinase (10). In M and at the M/G₁ boundary, the RB protein completely lost its phosphate groups. HL60 cells induced to differentiate showed a striking conversion of RB protein from the highly phosphorylated to the underphosphorylated form (Fig. 4) (1, 8, 34). These observations suggest that E7 protein functions to inactivate or modulate the underphosphorylated RB protein that shuts down the entrance of cells in the cycle at the G₁/S boundary.

We introduced a novel assay using E7-Sepharose gel as a ligand for the binding of RB protein. Our single-step precipitation method has some advantages over the commonly used procedures with in vitro-translated E7 and/or RB proteins (3, 15, 19, 26, 36); it is nonradioisotopic, easy and rapid, and free of the problems associated with the use of antibodies that may carry low affinity or cross-reactivity. As is well known, the RB protein translated in vitro is smaller than the RB protein expressed in cells, and therefore some of its natural properties may be lost or altered.

HPV-16 E7 amino acids 4 to 15 and 17 to 37 share significant homology with adenovirus type 5 E1A amino acids 39 to 49 and 117 to 137, which are designated E1A conserved domains 1 and 2, and SV40 large T amino acids 8 to 18 and 103 to 115, respectively (Fig. 6). These sequences are well conserved among not only the oncoproteins of these DNA tumor viruses but also E7 proteins of both malignant and benign types of HPV, suggesting that these structures carry very important functions common in the viruses. Indeed, RB protein-binding activity was located in these regions of E7, E1A, or large T (3, 11, 36, 51).

Both E1A conserved domains 1 and 2 were shown to be required for the RB binding of E1A protein (51), whereas no significant decrease in RB-binding affinity was observed with E7 protein that contained a mutation in the region homologous to domain 1 (36). Within the corresponding region of HPV-16 E7 protein, however, transforming activity for human fibroblasts was mapped at an amino acid, leucine 13 (49), which is conserved even among all of the other sequenced HPVs, including types 1a, 5c, 6b, 8, 11, 18, 31, and 33. We have shown here that this leucine of E7 protein plays an important role in distinguishing the underphosphorylated RB from the highly phosphorylated RB protein under the conditions we used. The leucine corresponding to leucine 13 of HPV-16 E7 is conserved in large T but not in E1A proteins. It is quite interesting that E1A protein but not large T was reported to bind to both the highly phosphorylated and the underphosphorylated RB protein (51). The relationship between the transforming activities of E7 protein and the ability to bind to highly phosphorylated and/or underphosphorylated RB protein remains to be clarified.

It was reported that cysteine 24 in domain 2 plays an important role in the binding of E7 to RB protein (3, 26, 36). With the cysteine 24-substituted E7-Sepharose, we confirmed the observation showing decreased affinity of RB protein to this type of mutated E7 protein. In any event, E7-coupled Sepharose will be a valuable tool to identify other cellular proteins that interact with E7 protein and also to understand the molecular interaction between E7 and RB proteins.

ACKNOWLEDGMENTS

We thank S. Nozawa for providing us with cervical cancer cell line SKG-IIIa.

This study was supported in part by a grant-in-aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control in Japan, and a grant-in-aid from the Ministry of Education, Science and Culture of Japan. Y.I. is an awardee of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research of Japan.

REFERENCES

1. Akiyama, T., and K. Toyoshima. 1990. Marked alteration in phosphorylation of the RB protein during differentiation of human promyelocytic HL60 cells. *Oncogene* 5:179-183.
2. Banks, L., P. Spence, E. Androphy, N. Hubbert, G. Matlashewski, A. Murray, and L. Crawford. 1987. Identification of human papillomavirus type 18 E6 polypeptide in cells derived from human cervical carcinomas. *J. Gen. Virol.* 68:1351-1359.
3. 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 II phosphorylation. *EMBO J.* 9:153-160.
4. Bookstein, R., J.-Y. Shew, P.-L. Chen, P. Scully, and W.-H. Lee. 1990. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. *Science* 247:712-715.
5. Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen. 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J.* 3:1151-1157.
6. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
7. Buchkovich, K., L. A. Duffy, and E. Harlow. 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* 58:1097-1105.
8. Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. J. Wang, and W.-H. Lee. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* 58:1193-1198.
9. Cheng, J., P. Scully, J.-Y. Shew, W.-H. Lee, V. Vila, and M. Haas. 1990. Homozygous deletion of the retinoblastoma gene in an acute lymphoblastic leukemia (T) cell line. *Blood* 75:730-735.
10. Cooper, J. A., and P. Whyte. 1989. RB and the cell cycle: entrance or exit? *Cell* 58:1009-1011.
11. 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.
12. DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C.-M. Huang, and D. M. Livingston. 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* 58:1085-1095.
13. de Villiers, E.-M. 1989. Heterogeneity of the human papillomavirus group. *J. Virol.* 63:4898-4903.
14. Dürst, M., L. Gissmann, 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.
15. 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.
16. Edmonds, C., and K. H. Vousden. 1989. A point mutational analysis of human papillomavirus type 16 E7 protein. *J. Virol.* 63:2650-2656.
17. Friend, S. H., R. Bernards, S. Rogelj, R. A. Weinberg, J. M. Rapaport, D. M. Albert, and T. P. Dryja. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature (London)* 323:643-646.
18. Fung, Y.-K. T., A. L. Murphree, A. T'Ang, J. Qian, S. H. Hinrichs, and W. F. Benedict. 1987. Structural evidence for the

- authenticity of the human retinoblastoma gene. *Science* **236**:1657-1661.
19. Gage, J. R., C. Meyers, and F. O. Wettstein. 1990. The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV-6b) and of oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. *J. Virol.* **64**:723-730.
 20. Green, M. R. 1989. When the products of oncogenes and anti-oncogenes meet. *Cell* **56**:1-3.
 21. 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.
 22. 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.
 23. Huang, H.-J. S., J.-K. Yee, J.-Y. Shew, P.-L. Chen, R. Bookstein, T. Friedmann, E. Y.-H. P. Lee, and W.-H. Lee. 1988. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science* **242**:1563-1566.
 24. Imai, Y., Y. Tsunokawa, T. Sugimura, and M. Terada. 1989. Purification and DNA-binding properties of human papillomavirus type 16 E6 protein expressed in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **164**:1402-1410.
 25. Inagaki, Y., Y. Tsunokawa, N. Takebe, H. Nawa, S. Nakanishi, M. Terada, and T. Sugimura. 1988. Nucleotide sequences of cDNAs for human papillomavirus type 18 transcripts in HeLa cells. *J. Virol.* **62**:1640-1646.
 26. Jones, R. E., R. J. Wegrzyn, D. R. Patrick, N. L. Balishin, G. A. Vuocolo, M. W. Riemen, D. Defeo-Jones, V. M. Garsky, D. C. Heimbrook, and A. Oliff. 1990. Identification of HPV-16 E7 peptides that are potent antagonists of E7 binding to the retinoblastoma suppressor protein. *J. Biol. Chem.* **265**:12782-12785.
 27. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367-382.
 28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
 29. 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.
 30. 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.
 31. 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.
 32. 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.
 33. Ludlow, J. W., J. Shon, J. M. Pipas, D. M. Livingston, and J. A. DeCaprio. 1990. The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. *Cell* **60**:387-396.
 34. Mihara, K., X.-R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A. T'Ang, and Y.-K. T. Fung. 1989. Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. *Science* **246**:1300-1303.
 35. Münger, K., W. C. Phelps, V. Bub, P. M. Howley, and R. Schlegel. 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J. Virol.* **63**:4417-4421.
 36. Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley. 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J.* **8**:4099-4105.
 37. Nozawa, S., Y. Udagawa, H. Ohta, S. Kurihara, and W. H. Fishman. 1983. Newly established uterine cervical cancer cell line (SKG-III) with Regan isoenzyme, human chorionic gonadotropin β -subunit, and pregnancy-specific β_1 -glycoprotein phenotypes. *Cancer Res.* **43**:1748-1760.
 38. 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.
 39. Pirisi, L., S. Yasumoto, M. Feller, J. Doniger, and J. A. DiPaolo. 1987. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J. Virol.* **61**:1061-1066.
 40. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
 41. Schlegel, R., W. C. Phelps, Y.-L. Zhang, and M. Barbosa. 1988. Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. *EMBO J.* **7**:3181-3187.
 42. Seedorf, K., G. Krämmer, M. Dürst, S. Suhai, and W. G. Röwekamp. 1985. Human papillomavirus type 16 DNA sequence. *Virology* **145**:181-185.
 43. Seedorf, K., T. Oltersdorf, G. Krämmer, and W. Röwekamp. 1987. Identification of early proteins of the human papillomavirus type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. *EMBO J.* **6**:139-144.
 44. 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.
 45. Smotkin, D., and F. O. Wettstein. 1987. The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. *J. Virol.* **61**:1686-1689.
 46. Takebe, N., Y. Tsunokawa, S. Nozawa, M. Terada, and T. Sugimura. 1987. Conservation of E6 and E7 regions of human papillomavirus types 16 and 18 present in cervical cancers. *Biochem. Biophys. Res. Commun.* **143**:837-844.
 47. Tsunokawa, Y., N. Takebe, T. Kasamatsu, M. Terada, and T. Sugimura. 1986. Transforming activity of human papillomavirus type 16 DNA sequences in a cervical cancer. *Proc. Natl. Acad. Sci. USA* **83**:2200-2203.
 48. Tsunokawa, Y., N. Takebe, S. Nozawa, T. Kasamatsu, L. Gissmann, H. zur Hausen, M. Terada, and T. Sugimura. 1986. Presence of human papillomavirus type-16 and type-18 DNA sequences and their expression in cervical cancers and cell lines from Japanese patients. *Int. J. Cancer* **37**:499-503.
 49. Watanabe, S., T. Kanda, H. Sato, A. Furuno, and K. Yoshiike. 1990. Mutational analysis of human papillomavirus type 16 E7 functions. *J. Virol.* **64**:207-214.
 50. 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.
 51. Whyte, P., N. M. Williamson, and E. Harlow. 1989. Cellular targets for transformation by the adenovirus E1A proteins. *Cell* **56**:67-75.
 52. Yasumoto, S., A. L. Burkhardt, J. Doniger, and J. A. DiPaolo. 1986. Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. *J. Virol.* **57**:572-577.
 53. Yokota, J., T. Akiyama, Y.-K. T. Fung, W. F. Benedict, Y. Namba, M. Hanaoka, M. Wada, T. Terasaki, Y. Shimosato, T. Sugimura, and M. Terada. 1988. Altered expression of the retinoblastoma (RB) gene in small-cell carcinoma of the lung. *Oncogene* **3**:471-475.