JOURNAL OF VIROLOGY, Jan. 1989, p. 366–374
0022-538X/89/010366-09$02.00/0

Vol. 63, No. 1

Intracellular Localization and DNA-Binding Properties of Human Papillomavirus Type 18 E6 Protein Expressed with a Baculovirus Vector

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Received 11 July 1988/Accepted 21 September 1988

The E6 protein of human papillomavirus type 18 (HPV-18) is a putative zinc-finger protein that is expressed in HPV-18-induced genital neoplasias. We have studied the biochemical properties of E6 protein synthesized in large amounts with a baculovirus expression vector. When E6 protein was synthesized in insect cells infected with an E6-expressing baculovirus, the protein was localized to both nuclear and membrane fractions, with half-lives of 4 and 2 h, respectively. Changing the first five amino acids of E6 did not alter the pattern of cellular localization of the protein but dramatically increased the half-life of the nuclear component to longer than 30 h and increased the half-life of the membrane component to 8 h. Although the baculovirus-expressed E6 protein bound to double-stranded DNA with high affinity, no sequence specificity for HPV-18 DNA was detected.

The E6 protein of human papillomavirus type 18 (HPV-18) is one of two papillomavirus proteins expressed in cervical tumor cell lines containing integrated copies of HPV-18 (4, 31, 32). The HPV-18 E6 protein is a small, lysine-arginine-rich polypeptide containing a potential metal-binding motif (Cys-x-x-Cys) (6, 18) repeated four times at regularly spaced intervals (Fig. 1). These sequences, often referred to as zinc fingers, have been implicated as important structural features of many nucleic acid-binding proteins, including TFIII-A, steroid receptor proteins, GAL4, and Sp-1 (6, 12, 17, 18, 24). In contrast to other zinc-finger proteins which contain 10 to 15 amino acids between each metal-binding motif (6, 12), the HPV-18 E6 motifs are separated by 29 amino acids.

Except for the pattern of regularly spaced cysteine doublets (11), the E6 proteins of all papillomaviruses are poorly conserved. However, the similar genomic positions of the E6 open reading frames (ORFs) in all papillomaviruses, as well as the conserved pattern of cysteine doublet motifs, suggest that E6 proteins may share common functions. The E6 ORFs of bovine papillomavirus type 1 (BPV-1) and HPV-18 are capable of transforming immortalized rodent fibroblasts to anchorage independence (30; M. Bedell, K. Jones, S. Grossman, and L. A. Laimins, submitted for publication). In addition, the BPV-1 E6 gene product has been implicated in the control of BPV-1 episomal copy number (7), whereas recent evidence suggests that the HPV-18 E6 gene product may transactivate an enhancer in the HPV-18 upstream regulatory region (13). Study of the E6 protein and the role it plays in the development of genital neoplasias is thus an area of significant interest.

The low abundance of E6 protein in tumor cell lines, or even in cell lines that express the E6 ORF from strong heterologous promoters (1, 2, 4, 32), has hampered study of the biochemical properties of E6 protein. The HPV-6 E6 protein has been overexpressed in bacteria and found to bind DNA nonspecifically (21), although this protein may lack posttranslational modifications necessary for sequence-specific binding. Several eucaryotic expression systems exist which allow for the synthesis of large amounts of correctly processed proteins. We have used one of these, the baculovirus expression system, to produce large amounts of HPV-18 E6 protein.

Baculoviruses are large DNA viruses that infect insects of the family *Lepidoptera* (20). A nonessential protein for growth in tissue culture, polyhedrin, is synthesized in large quantities, accounting for up to 50% of total cellular protein synthesized late in infection (20). Recombinant viruses can be constructed in which a gene of interest has replaced the polyhedrin-coding sequences, allowing high-level expression of foreign genes in insect cells grown in tissue culture. Eucaryotic proteins expressed in insect cells with these recombinant baculoviruses are targeted to their proper subcellular compartments and undergo normal eucaryotic posttranslational modifications (16, 20, 26, 27, 29). We report here that E6 protein overexpressed in insect cells has an apparent molecular mass of 17 kilodaltons (kDa), binds nonspecifically to double-stranded DNA, and is localized to both the nuclear matrix and nonnuclear membranes. The in vivo stabilities of the two E6 subpopulations are different and are controlled in part by amino acids localized to the N terminus.

MATERIALS AND METHODS

Plasmids. The pAc373 baculovirus transfer vector (20) contains a *Bam*HI cloning site downstream of the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV), along with 3 kilobases of flanking virus sequence on either side of the promoter. pVL106 is a similar transfer vector except that it is used to generate fusion proteins that use the polyhedrin ATG (20). Genes are cloned downstream of the polyhedrin ATG at the *Bam*HI or *Sma*I site. pAc18E6 contains the E6 gene cloned into pAc373 on a 540-base-pair *Ava*II fragment that was made blunt with Klenow enzyme, ligated to *Bgl*II linkers, cut with *Bgl*II, and ligated into the *Bam*HI site of pAc373 (Fig. 2). pVL18E6 contains the C-terminal 180 nucleotides of E6 cloned into the *Bam*HI site of pVL106 as an *Xho*II fragment from pAc18E6.

Cell and virus culture. The Sf-9 cell line is derived from the fall armyworm, *Spodoptera frugiperda*, and is permissive for baculovirus propagation. Cells were maintained and infected

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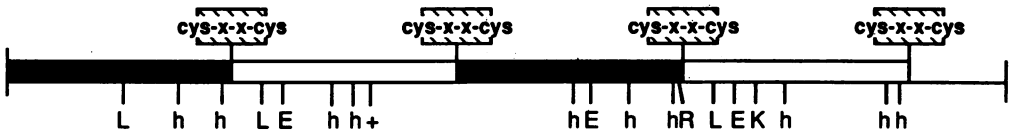

FIG. 1. Conserved domains and amino acid residues in the HPV-18 E6 predicted amino acid sequence. Symbols: ▭ and ▬, 30- to 40-amino acid domains that are conserved in size and the position of a few key residues in all papillomavirus E6 proteins (11); ▨, positions of the repeating Cys-x-x-Cys metal-binding motifs. Capital letters indicate residues that are conserved in most papillomavirus E6 proteins; h, hydrophobic; +, basic.

with baculovirus at a multiplicity of infection of 10 to 100 PFU per cell as previously described (M. D. Summers and G. E. Smith, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experiment Station bulletin no. 1555, 1987). Infected cells were harvested 40 to 48 h postinfection and either processed immediately or metabolically labeled in Grace medium with 100 μCi to 1 mCi of [^{35}S]cysteine per ml for 2 to 4 h at 27°C or 2.5 mCi of $^{32}PO_4$ for 30 min at 27°C.

Construction of an HPV-18 E6 recombinant baculovirus. Sf-9 cells (2.5×10^6) were cotransfected with 1 μg of AcNPV DNA and 2 μg of either pAc18E6 or pVL18E6 by calcium phosphate precipitation as described previously (Summers and Smith, 1987). The cell supernatant was harvested after 6 days, and recombinant viruses were purified by two to three rounds of plaque assays. Recombinant plaques were identified either by a ^{32}P-labeled HPV-18-specific probe as described previously (Summers and Smith, 1987) or by visual screening for plaques lacking the occlusion-forming polyhedrin protein. Two plaque-purified recombinant viral isolates, designated vAcE6 (full-length E6) and vFE6 (E6-polyhedrin fusion), were used for all further experiments.

Preparation of E6 antipeptide antibodies. A 14-amino-acid peptide, consisting of residues 5 through 17 of the E6 ORF (CEDPTRRPYKLPDL) and an additional amino-terminal cysteine necessary for attachment of the peptide to carrier protein, was synthesized with a peptide synthesizer (model 430A; Applied Biosystems, Foster City, Calif.). After cleavage from the resin with hydrogen fluoride, the peptide was shown by C_{18} reversed-phase high-performance liquid chro-

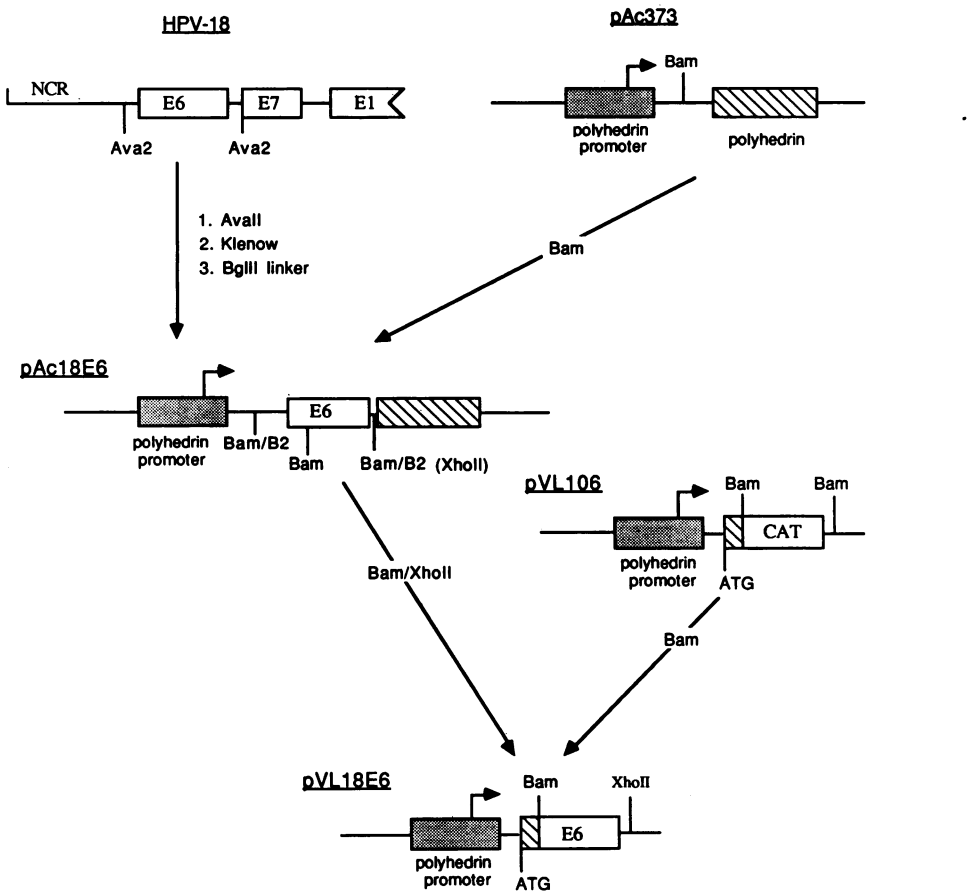

FIG. 2. Construction of transfer vectors for the production of E6-expressing recombinant baculoviruses. pAc373 and pVL106 are baculovirus transfer plasmids that allow the expression of foreign genes by the polyhedrin promoter. Genes expressed in pAc373 utilize their natural ATG, whereas genes expressed with pVL106 are fusion proteins that utilize the polyhedrin ATG. In the E6-polyhedrin protein, the first five amino acids of the E6 protein (Met-Ala-Arg-Phe-Glu) are replaced by four amino acids derived from the vector (Met-Pro-Ala-Arg). Restriction enzyme cleavage sites are indicated; B2, *Bgl*II.

matography analysis to be 75% pure. The peptide was conjugated to keyhole limpet hemocyanin as described elsewhere (15) and used to immunize three rabbits. Sera from all three rabbits reacted with the peptide in an enzyme-linked immunosorbent assay, and serum from one rabbit (P1297) was used in all further experiments.

Protein analysis and immunoblotting. Infected or uninfected Sf-9 cells were suspended in medium, pelleted, and washed twice with phosphate-buffered saline (PBS). Whole-cell lysates were prepared by suspending the pellet in water (10^7 cells per ml) and adding an equal volume of 2× Laemmli sample buffer (19). Electrophoresis through 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) was performed as previously described (19). Immunoblotting with P1297 anti-E6 antibody (1:300 dilution) and visualization with ^{125}I-protein A was performed as previously described (9) except that blocking and incubations were performed in 50 mM Tris (pH 7.5)–150 mM NaCl–0.1% Tween 20.

Immunoprecipitation of E6 protein from Sf-9 cells. Approximately 10^7 Sf-9 cells were infected with either AcNPV or vAcE6 and labeled 48 h postinfection with either [^{35}S] cysteine or $^{32}PO_4$. Preparation of cell lysates and immunoprecipitations were performed as described by Androphy et al. (1). A 40- to 50-μl amount of preimmune, immune, or peptide-blocked immune serum was used per immunoprecipitation. Immune serum blocked with peptide was prepared by incubation of 40 μl of immune serum with 10 μg of peptide for 1 h at 4°C. Immunoprecipitated proteins were separated by electrophoresis through 15% SDS-PAGE gels. Gels were treated with fluor (Amplify; Amersham Corp., Arlington Heights, Ill.) and autoradiographed at −70°C with intensifying screens (X-Omat; Eastman Kodak Co., Rochester, N.Y.).

Half-life determination. Sf-9 cells infected with either vAcE6 or vFE6 were labeled at 40 h postinfection with [^{35}S]cysteine. Labeled cells were pelleted and incubated in cold complete medium for various amounts of time. At each time point, a sample of cells was pelleted, washed in PBS, and lysed in Laemmli sample buffer. A portion of the lysate from each time point was analyzed by electrophoresis through 15% SDS-PAGE gels, followed by fluorography. The band corresponding to E6 was quantitated with a densitometer (LKB Instruments, Inc., Rockville, Md.). The half-lives of the membrane and nuclear fractions of E6 proteins were determined in a similar manner except that at each time point cells were lysed in buffer containing 10 mM Tris (pH 8.0)–1% Nonidet P-40 to solubilize membrane proteins, and the pellet, containing the nuclei, was solubilized in Laemmli sample buffer. Equal portions of each fraction were separated by 15% SDS-PAGE, and the autoradiograms were analyzed as described above.

Subcellular fractionation. Subcellular fractions of Sf-9 cells were prepared by a minor modification of standard techniques (8, 15a). At 40 h postinfection, 10^7 vFE6-infected cells were harvested and washed twice with PBS. The cells were split into two equal portions and suspended in either H buffer (10 mM Tris [pH 8.0], 1 mM dithiothreitol [DTT], 1 mM EDTA, 10 μg of aprotinin per ml) or STM buffer (250 mM sucrose, 10 mM Tris [pH 8.0], 10 mM $MgCl_2$, 10 μg of aprotinin per ml). The cells in H buffer were incubated for 10 min on ice and disrupted with 25 strokes in a Dounce homogenizer. The cells in STM buffer were disrupted similarly, and the nuclei from both lysates were pelleted at 500 × *g* for 10 min. Supernatants from both portions were separated into membrane and soluble cytoplasmic fractions by centrifugation at 100,000 × *g* for 30 min. Membranes were solubilized from the high-speed pellets with H buffer containing 1% Triton X-100. The nuclei from cells disrupted in hypotonic medium were washed in STM buffer and pelleted, and nucleoplasm was extracted with 100 mM NaCl–1% Nonidet P-40–aprotinin (10 μg/ml) for 15 min on ice. Nuclei were pelleted at 500 × *g* for 15 min, suspended in nuclease digestion buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 μg of aprotinin per ml), and 200 U of DNase and 150 U of micrococcal nuclease were added. After 10 min on ice and 10 min at room temperature, an equal volume of 4 M NaCl was added to solubilize chromatin, and the remaining insoluble material was pelleted for 15 min at 12,000 × *g* and solubilized by being boiled for 3 min in 2× Laemmli sample buffer. Subcellular fractions were analyzed for E6 protein by 15% SDS-PAGE of 1/20 of each fraction and immunoblotting with P1297 anti-E6 antibody.

Immunofluorescence. Sf-9 cells were plated onto acid-washed glass cover slips in 60-mm dishes, infected with AcNPV or vFE6, and incubated for 40 h at 27°C. Cells were washed with PBS, fixed for 15 min in periodate-lysine-paraformaldehyde fixative (22), washed for 30 min in PBS, and incubated in 50 μl of a 1:40 dilution of monoclonal antibody C1P5 ascites fluid (gift of L. Banks and L. Crawford [4]) for 30 min at 25°C. Cover slips were then washed for 30 min in PBS, incubated in fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin for 30 min at 25°C, and washed for another 30 min in PBS before viewing.

DNA-binding assays. DNA-binding assays were performed by protein (Southwestern) blotting with ^{32}P-labeled DNA probes (23, 25). Sf-9 cells infected with AcNPV or vFE6 were harvested at 40 h postinfection and washed in PBS. Cells were lysed in 10 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM DTT, 10 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride. NaCl was added to a concentration of 2 M, and insoluble material was pelleted at 12,000 × *g* for 10 min. The pellet was suspended in 2× Laemmli sample buffer, electrophoresed on 15% SDS-PAGE gels, and transferred to nitrocellulose as described above. Blotted proteins were renatured by incubation in three changes of 10 mM Tris (pH 7.5)–5% nonfat dry milk–10% glycerol–150 mM NaCl–2.5% Nonidet P-40–1 mM DTT–100 μM $ZnCl_2$ for 6 h at room temperature. After a brief rinse in binding buffer (10 mM Tris [pH 7.2], 50 mM NaCl, 1 mM DTT, 0.25% nonfat dry milk), nitrocellulose strips were incubated for 2 to 12 h at room temperature in binding buffer with 10^6 cpm of ^{32}P-end-labeled pUC-19 or HPV-18 restriction fragments. The strips were washed in three changes of 10 mM Tris (pH 7.5)–1 mM DTT with varying amounts of NaCl (150 to 750 mM) for 1 to 2 h at room temperature and then dried and autoradiographed. After autoradiography, the strips were incubated with P1297 anti-E6 antibody and E6 protein was visualized with protein A-gold (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Identification of E6 protein in vAcE6- and vFE6-infected Sf-9 cells. A recombinant baculovirus containing the HPV-18 E6 ORF expressed from the polyhedrin promoter was constructed by in vivo recombination between wild-type AcNPV DNA and the E6 transfer plasmid pAc18E6. pAc18E6 contains the entire HPV-18 E6-coding sequences placed downstream of the polyhedrin promoter (Fig. 2). pAc18E6 was cotransfected with AcNPV DNA into Sf-9 cells, and recombinant virus was purified from the cell supernatant after several rounds of plaque assays. Sf-9 cells

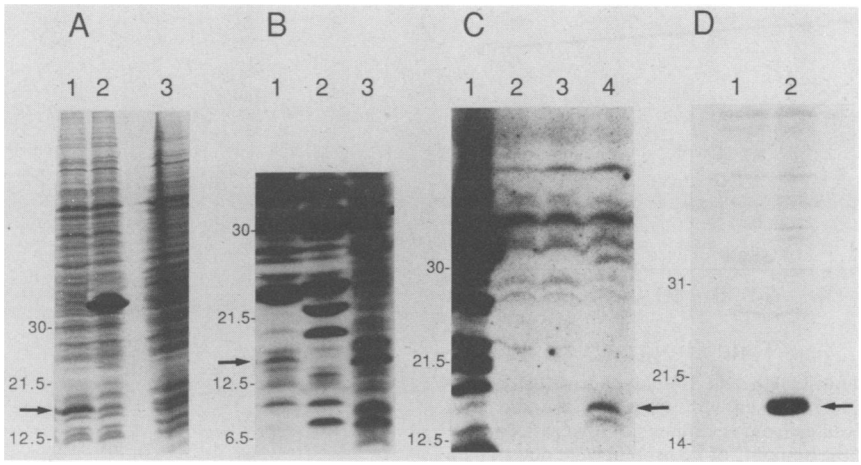

FIG. 3. Production of E6 protein in recombinant baculovirus-infected cells. (A) Coomassie blue-stained 15% SDS-PAGE of whole-cell lysates. Lanes: 1, vFE6-infected cells; 2, AcNPV-infected cells; 3, vAcE6-infected cells. (B) Autoradiogram of 15% SDS-PAGE of whole-cell lysates from infected cells labeled with [^{35}S]cysteine. Lanes: 1, vAcE6-infected cells; 2, AcNPV-infected cells; 3, mock-infected cells. (C) Immunoprecipitation of whole-cell lysates from AcNPV-infected (lane 1) or vAcE6-infected (lanes 2 through 4) Sf-9 cells. Lanes: 1, anti-E6 immune serum (P1297); 2, preimmune serum; 3, P1297 antiserum preincubated with the 14-amino-acid E6 peptide; 4, P1297 antiserum. (D) Western blot of whole-cell lysates from AcNPV-infected (lane 1) or vFE6-infected (lane 2) Sf-9 cells, using P1297 anti-E6 antibody. Arrows indicate the position of E6 protein.

infected with this virus (vAcE6) were analyzed for the presence of E6 protein by 15% SDS-PAGE of whole-cell lysates and staining with Coomassie blue. The most notable difference between the protein profiles of cells infected with AcNPV (Fig. 3A, lane 2) and with vAcE6 (lane 3) was the lack of polyhedrin synthesis (molecular mass, 33 kDa) in cells infected with vAcE6. There was no evidence of overproduction of a protein with a molecular mass close to that of the 16-kDa E6 protein (4) in vAcE6-infected cells. In contrast, when cells were metabolically labeled with [^{35}S]cysteine, a novel band with an apparent molecular mass of 17 kDa was detected in vAcE6-infected cells (Fig. 3B, lane 1) but not in AcNPV-infected (lane 2) or mock-infected (lane 3) cells.

The identification of this band as E6 protein was confirmed by immunoprecipitating whole-cell extracts of [^{35}S]cysteine-labeled AcNPV- or vAcE6-infected cells with a polyclonal antibody (P1297) raised against an N-terminal 13-amino-acid peptide. A band migrating at the same apparent molecular weight as the novel band observed in whole-cell lysates of [^{35}S]cysteine-labeled vAcE6-infected cells was specifically precipitated by P1297 anti-E6 antiserum (Fig. 3C, lane 4). This band was not observed when lysates of AcNPV-infected cells were immunoprecipitated with anti-E6 antibody or when lysates from vAcE6-infected cells were immunoprecipitated with preimmune serum (Fig. 3C, lanes 1 and 2). Preincubation of immune serum with the original peptide immunogen completely blocked immunoprecipitation of E6 from vAcE6-infected cells (Fig. 3C, lane 3), which indicated that E6 protein was precipitated specifically by the antipeptide antibodies. The specifically precipitated band appearing just below E6 in Fig. 3C, lane 4, was not reproducible and probably represents a proteolytic degradation product.

Since the level of E6 protein accumulation in vAcE6-infected cells was low, a second recombinant baculovirus was constructed from a vector (pVL106) that has been used to express foreign proteins in infected insect cells at higher levels than are attainable with the original pAc373 transfer vector (20). The higher level of expression from this vector has been attributed to the presence of the normal polyhedrin ATG as well as the normal 5′ mRNA leader sequence, 8 base pairs of which are deleted in pAc373 (20). These sequences confer both higher translatability and higher steady-state mRNA levels (20). The E6 protein produced with this vector is actually a fusion protein in which four amino acids encoded by vector sequences replace the first five residues of E6. Protein profiles of cells infected with the E6-polyhedrin fusion protein-expressing virus (vFE6) displayed a novel, intensely stained band on Coomassie blue-stained gels that comigrated with the 17-kDa E6 protein produced in vAcE6-infected cells (Fig. 3A, lane 1). Western blot (immunoblot) analysis of total infected-cell proteins with P1297 anti-E6 antibody (Fig. 3D) identified this novel band as E6 protein.

Stability of the E6 fusion protein. The first five amino acids of the E6 protein (Met-Ala-Arg-Phe-Glu) are replaced by a four-amino-acid leader (Met-Pro-Ala-Arg) in the polyhedrin-E6 fusion protein. To determine whether there was any difference in protein stability between the natural and fusion E6 proteins, we performed a pulse-chase experiment with vAcE6- and vFE6-infected cells to determine the in vivo half-lives of the two E6 proteins in Sf-9 cells. After a 2-h pulse with [^{35}S]cysteine at 40 h postinfection, infected Sf-9 cells were chased with cold medium. Samples of cells from each time point were lysed and analyzed by SDS-PAGE. The band corresponding to E6 polypeptide was quantitated with a densitometer, and the amount of E6 protein was plotted versus time on a log scale. E6 protein in vAcE6-infected Sf-9 cells had a half-life of approximately 3 h (Fig. 4), consistent with results reported for HPV-16 E6 protein in Caski cells, in which the half-life of the major portion of E6 protein is 4 h (1). In vFE6-infected cells, however, there was little decrease in the levels of ^{35}S-labeled E6 fusion protein after a 17-h chase, which suggested that the protein had increased stability. By extrapolation of the curve in Fig. 4, we conservatively estimate that the half-life of E6-polyhedrin in these cells may be longer than 30 h.

Subcellular localization. Numerous investigators have shown that mammalian proteins expressed in insect cells are localized to their appropriate subcellular compartments (16,

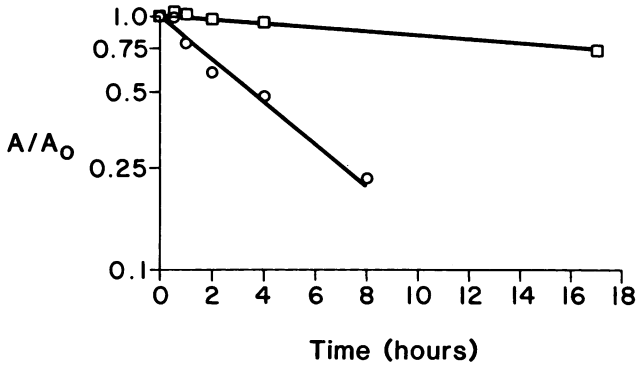

FIG. 4. Half-lives of full-length and E6-polyhedrin fusion proteins. Infected Sf-9 cells were pulsed with [^{35}S]cysteine and chased with cold medium. Cell lysates from each time point were separated by 15% SDS-PAGE, and the amounts of E6 protein were determined by densitometric scanning. The amount of E6 protein at each time point is normalized to the amount present at time zero. The half-life of the full-length E6 is 3 to 4 h, whereas the half-life of the E6 fusion protein is at least 30 h.

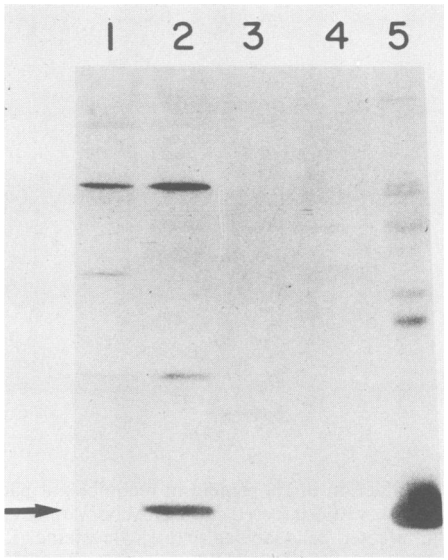

FIG. 5. Subcellular fractionation of vFE6-infected cells. Infected Sf-9 cells were disrupted by homogenization in isotonic (cytoplasmic fraction) or hypotonic (nuclear fractions) buffers, and subcellular fractions were prepared as described in Materials and Methods. Equal portions of each fraction were analyzed by 15% SDS-PAGE, followed by Western blotting with P1297 anti-E6 antibody. Lanes: 1, cytoplasm; 2, nonnuclear membranes; 3, nucleoplasm; 4, chromatin; 5, nuclear matrix. Arrow indicates the position of E6 protein.

20, 26, 27, 29). In mammalian cells, BPV-1 E6 protein is localized in equal proportions to both the nucleus and nonnuclear membranes (2), whereas the majority of cottontail rabbit papillomavirus (CRPV) E6 (long form) is associated with the nuclear matrix fraction, with minor membrane and cytoplasmic fractions (5). We investigated whether the HPV-18 E6 protein exhibited a similar multicompartmental distribution in Sf-9 cells.

Cells infected with vFE6 were divided into two equal portions and disrupted in either hypotonic medium without Mg^{2+} (for all noncytoplasmic fractions) or isotonic medium with 10 mM $MgCl_2$ (for the cytoplasmic fraction). Disruption of cells in isotonic buffer containing Mg^{2+} stabilizes membranes and limits the leakage of proteins from other compartments, especially the nucleoplasm, into the cytoplasm during fractionation (14). Nuclear fractions were prepared without Mg^{2+} to limit contamination of nuclei with adhering nonnuclear membranes (14). Each portion was treated as described in Materials and Methods, and equal portions of each fraction were examined by Western blot analysis. Essentially all E6 protein was found in nonnuclear membranes or associated with the insoluble nuclear matrix fraction (Fig. 5, lanes 2 and 5). Densitometric scanning of the gel revealed that approximately 90% of E6 protein was associated with the nuclear matrix and that 10% was localized to nonnuclear membranes. Fractionations performed on vAcE6-infected cells gave similar results (data not shown). The subcellular fractionation of the E6 protein was performed by standard techniques. The E6 protein was not observed in soluble fractions, which demonstrated that leakage was not a problem in the extraction protocol. Given the distribution of E6 in membrane and nuclear matrix fractions, the possibility of cross-contamination was minimal since one fraction was Triton X-100 soluble and the other was Triton X-100 insoluble. We therefore believe that our subcellular fractionation accurately represents the distribution of E6 seen in infected insect cells.

The subcellular localization of E6 was confirmed by immunofluorescent staining of vFE6-infected Sf-9 cells with C1P5 anti-E6 monoclonal antibody (4). Sf-9 cells were plated on glass cover slips, infected with AcNPV or vFE6 or mock infected, and incubated for 40 h. Cells were then fixed and stained with C1P5 antibody and fluorescein isothiocyanate-conjugated secondary antibody. Only background fluorescence was seen in mock-infected (data not shown) or AcNPV-infected (Fig. 6A) cells, whereas vFE6-infected cells displayed intense fluorescence in a fairly diffuse distribution (Fig. 6B). In a subset of cells, distinct nuclear staining was evident, along with a bright halo around the nucleus near the plasma membrane (Fig. 6C) that may represent the membrane-associated form of E6 seen in the fractionation studies.

Different stabilities of different subcellular fractions of E6. We measured the half-lives of both subcellular fractions of E6 to determine whether the membrane fraction of E6 in Sf-9 cells represented a transient fraction of protein in transit to the nucleus. Cells infected with vAcE6 or vFE6 were pulsed with [^{35}S]cysteine for 30 min and chased with cold medium. At each time point, membrane and nuclear fractions were prepared and analyzed by SDS-PAGE, autoradiography, and scanning densitometry as before. The membrane fraction of E6-polyhedrin fusion protein had a half-life of 8 h, whereas the nuclear matrix fraction retained a half-life of longer than 30 h. In vAcE6-infected cells, the membrane fraction of E6 had a half-life of 2 h, whereas the matrix-associated E6 had a half-life of 4 h. We therefore conclude that the membrane fraction of E6 in insect cells is not a transient fraction of protein involved in nuclear transport, since nuclear proteins are normally transported into the nucleus within minutes of synthesis (28). Surprisingly, the different subcellular populations of E6 protein exhibited distinct rates of turnover. In addition, the half-lives of both the nuclear and membrane components of E6 were affected by the sequence of the first few N-terminal amino acids.

Evidence that E6 is not phosphorylated. Both vFE6- and vAcE6-infected cells were metabolically labeled with $^{32}PO_4$ to determine whether E6 protein is phosphorylated in these cells. A number of other eucaryotic proteins have been

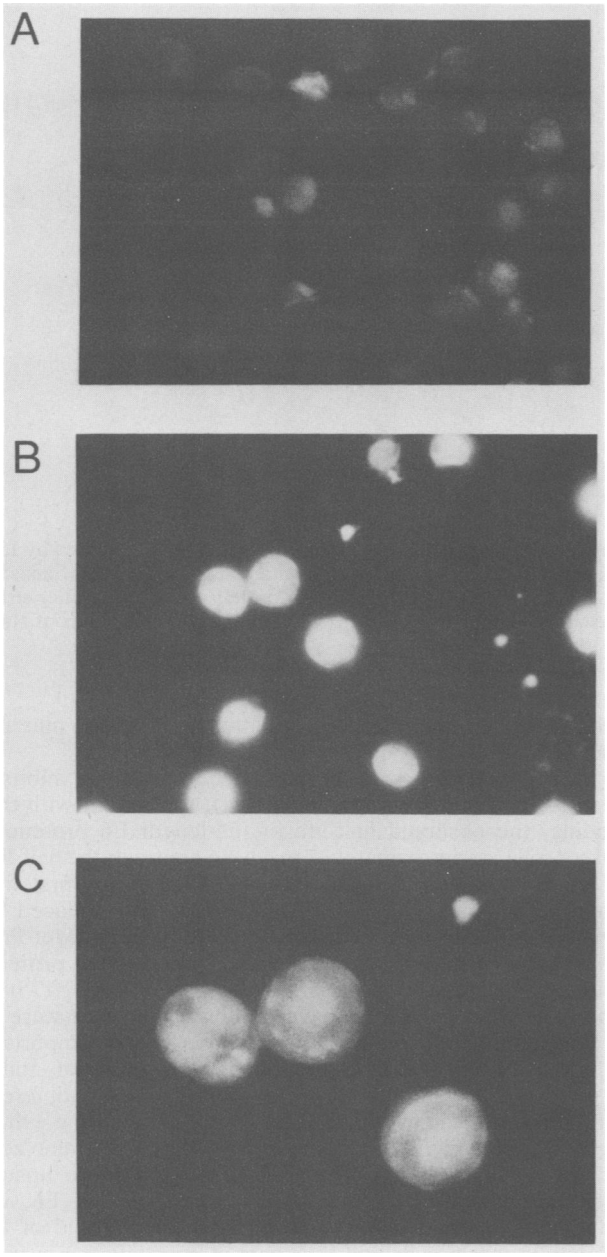

FIG. 6. Immunofluorescent staining of vFE6-infected Sf-9 cells with anti-E6 monoclonal antibody. Sf-9 cells infected with either AcNPV or vFE6 were fixed and incubated with C1P5 anti-E6 monoclonal antibody (4) followed by fluorescein isothiocyanate-conjugated secondary antibody. (A) AcNPV-infected cells; (B) vFE6-infected cells; (C) higher magnification of cells in panel B showing dense nuclear staining with a halo around the perimeter of the cell.

shown to undergo phosphorylation in this system (16, 26, 27). However, examination of $^{32}PO_4$-labeled proteins from vAcE6- or vFE6-infected cells versus AcNPV-infected cells by SDS-PAGE or by immunoprecipitation with P1297 antiserum followed by SDS-PAGE revealed no differences in the patterns of labeled proteins (data not shown), suggesting that at least a majority of E6 polypeptides are not phosphorylated in Sf-9 cells.

DNA-binding activity. To determine whether HPV-18 E6 protein expressed in insect cells could bind DNA, we used a protein-blotting technique (often referred to as Southwestern blotting [23, 25]) in which proteins are separated by SDS-PAGE, transferred to nitrocellulose, renatured, and incubated with ^{32}P-labeled probe DNA. When the nuclear matrix fraction of AcNPV- or vFE6-infected Sf-9 cells was blotted in this manner and probed with a ^{32}P-labeled *Sau*3A-*Acc*I restriction digest of pUC DNA (Fig. 7A), a strong signal at 17 kDa was detected with vFE6 (lane 2) but not with AcNPV (lane 1). The identity of this strongly labeled band as E6 was confirmed by reaction of the blot with P1297 antibody, followed by protein A-gold (Fig. 7B). To determine the strength of this binding, strips containing identical amounts of blotted E6 protein were reacted with ^{32}P-labeled *Sau*3A-*Acc*I-digested pUC DNA and washed with increasing amounts of salt. DNA remained bound after washes containing greater than 500 mM salt (Fig. 7C), which indicated that E6 has a high affinity for DNA.

We next examined whether HPV-18 E6 protein exhibited HPV-specific DNA-binding properties. Filters containing blotted E6 were probed with a ^{32}P-labeled *Bam*HI or *Ava*II restriction digest of HPV-18 DNA. After a wash with 300 mM NaCl, the bound DNA was eluted from the filters and separated on agarose gels. All fragments (including those containing only vector sequences) were bound equally (data not shown), which indicated that in this assay HPV-18 E6 protein, even when produced in a eucaryotic system, does not have a specific affinity for HPV-18 DNA.

DISCUSSION

The E6 protein is one of two HPV-specific proteins identified in cervical tumor cell lines (1, 2, 4, 32) and, as such, is of significant interest in the study of human papillomavirus-induced malignancies. The E6 proteins of BPV-1 and HPV-18 have been shown to be involved in cellular transformation (30; Bedell et al., submitted) and may also play a role in the control of plasmid copy number (7) and the activation of early-region transcription (13). We have shown that the E6 protein of HPV-18 can be produced in large quantities in a baculovirus insect cell expression system, in contrast to the trace amounts of E6 normally produced in cervical tumor cell lines.

The HPV-18 E6 protein expressed in insect cells manifests many of the properties of CRPV and BPV-1 E6 proteins observed in mammalian cell lines. A large portion is associated with an insoluble nuclear matrix fraction, being fully released only after treatment with SDS, whereas a smaller portion fractionates with nonnuclear membranes. This distribution is similar in many respects to the distribution observed for both the CRPV (long form) and BPV-1 E6 proteins (2, 5). BPV-1 E6 protein in mammalian tissue culture is not believed to be phosphorylated (E. Androphy, unpublished observations), whereas the long form of CRPV E6 is phosphorylated at an extremely low level (5). In our studies, the HPV-18 E6 protein is also not phosphorylated. The CRPV E6 protein is not glycosylated (5), and the BPV-1 and HPV-16 E6 proteins are also probably not glycosylated since their observed molecular weights are consistent with their predicted molecular weights (1, 2), similar to what was observed in our studies with HPV-18 E6 protein.

The E6 protein contains a pattern of regularly spaced cysteine doublets (Cys-x-x-Cys) that is also found in many zinc-finger nucleic acid-binding proteins (6, 12). However, the 29-amino-acid separation of Cys-x-x-Cys motifs in E6 differs from the 10- to 15-amino-acid separation found in all other known zinc-finger proteins. The implications of this

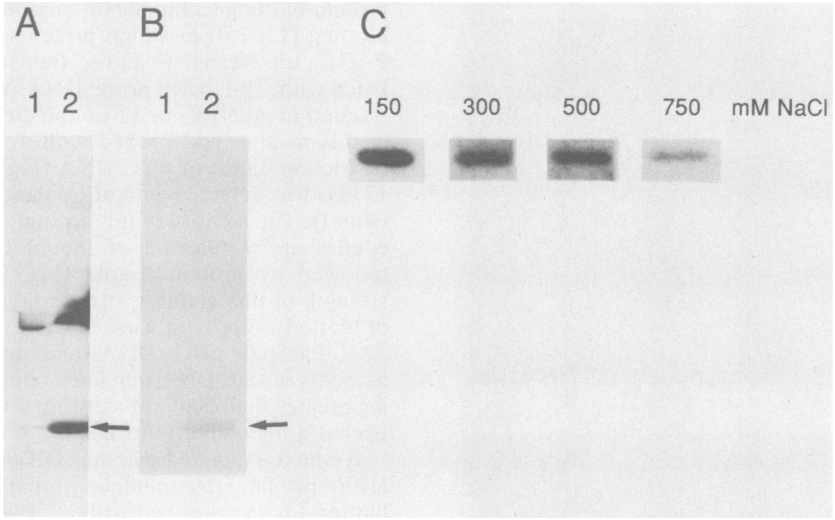

FIG. 7. DNA-binding activity of E6 protein blotted to nitrocellulose filters. (A) Nuclear matrix fractions of infected cells separated by 15% SDS-PAGE, transferred to nitrocellulose, renatured, and probed with ^{32}P-labeled pUC DNA. Final wash contained 150 mM NaCl. Lanes: 1, AcNPV; 2, vFE6. (B) Western blot of the filter shown in panel A reacted with P1297 anti-E6 antiserum and protein A-gold. (C) Filter strips containing equal amounts of blotted E6-polyhedrin fusion protein probed as described for panel A but with different concentrations of NaCl in the final wash.

difference for the proposed nucleic acid-binding function of E6 is unclear. In this study, E6 protein bound to double-stranded DNA with high affinity, although specific interactions with HPV-18 genomic DNA were not observed. This result is in contrast to those of studies with a bacterially synthesized E6 protein, wherein binding was much less stringent (21). We believe that our eucaryotically synthesized E6 protein may more accurately reflect the nucleic acid-binding properties of E6 in vivo.

The absence of specific binding to the HPV-18 genome was surprising, since the E6 ORF appears to transactivate an enhancer located at the 5′ end of the HPV-18 upstream regulatory region (13). The possibility exists that E6 must either dimerize or interact with other cellular proteins to specifically bind papillomavirus DNA and activate transcription. This hypothesis cannot be ruled out by the results of our DNA-binding assays because of the denaturation of protein complexes in SDS reducing gels. It is, however, unlikely that E6 is a nonspecific nucleic acid-binding protein, since it is normally found in such low abundance in HPV-infected cells. Alternatively, E6 may not interact with HPV-18 DNA at all and may act by transactivating a cellular gene which in turn could transactivate the E6-inducible enhancer. Furthermore, the possibility that E6 interacts with RNA rather than DNA is currently under investigation.

The identification of a significant portion of E6 protein in the membrane fraction of eucaryotic cells suggests that E6 may have pleiotropic properties. It is intriguing that a protein such as E6, which has such a high affinity for DNA, has both a membrane and nuclear component. By analogy, simian virus 40 T antigen is a nuclear transcription- and replication-regulatory protein that also has a minor plasma membrane component essential for transformation (10). Whether the membrane-bound forms of E6 and T antigen retain their nucleic acid-binding properties is a question of particular interest. The membrane-bound form of either protein may be involved in interactions with cytoplasmic RNA or may perform a completely separate function unrelated to nucleic acid binding. This question is currently under study, along with examination of the transformation ability of E6 mutants that are defective for membrane localization.

In mammalian fibroblasts, HPV-16 E6 protein exhibits a bimodal half-life of 30 min and 4 h (1). Consistent with this finding, the observed half-life of full-length E6 protein in insect cells is approximately 3 to 4 h. In contrast, the half-life of an E6-polyhedrin fusion protein, wherein the first five amino acids of E6 (Met-Ala-Arg-Phe-Glu) are replaced by four amino acids encoded by vector sequences (Met-Pro-Ala-Arg), is much longer than that of the original protein, with little turnover (<10%) of labeled proteins after 17 h of chase. Bachmair et al. (3) have proposed that the nature of the N-terminal amino acid of a protein has an important effect on protein stability in eucaryotic cells. In that study, β-galactosidase–ubiquitin fusion proteins with different amino acids at the ubiquitin–β-galactosidase junction exhibited different stabilities, depending on the type of amino acid at the N terminus of β-galactosidase. As we are unsure whether processing occurs at the N terminus of E6, we cannot be sure of the exact N-terminal amino acid of E6 protein in insect cells and thus cannot correlate the in vivo stability of the protein with the N-terminal amino acid. However, there is a striking difference in the half-lives of the two proteins (3 to 4 h versus >30 h), and the only discernible difference in the two proteins lies in the first four or five amino acids.

One possible explanation for the increased stability of the E6-polyhedrin fusion protein is that the protein is expressed at such a high level that the turnover system of the cell is overwhelmed and simply cannot degrade the E6 protein as quickly as it is produced. If this were true, however, one would expect the half-lives of other cellular proteins to be similarly increased because of the overload of the proteolytic system by the overexpressed E6 protein. This explanation, of course, assumes a single or at least rate-limiting pathway for protein degradation. Since the half-lives of other short-lived ^{35}S-labeled proteins from vAcE6- or vFE6-infected Sf-9 cells are comparable (data not shown), this is probably not the case. A second explanation for the increased half-life

of the E6-polyhedrin fusion protein might be that the protein forms insoluble inclusion bodies inaccessible to cellular proteases. Electron microscopy of glutaraldehyde-fixed, osmium-stained sections of vFE6-infected Sf-9 cells revealed no such inclusion bodies at any time during infection (S. Grossman and T. Martin, unpublished observations).

The observation of different stabilities of the membrane and nuclear fractions of E6-polyhedrin protein is intriguing. Different subcellular fractions of simian virus 40 large-T antigen have also been reported to have different stabilities (33). Specifically, when simian virus 40-transformed cells were pulse-labeled with ^{14}C-amino acids, the chromatin-associated fraction of T antigen was decreased 10-fold after a 2-h chase, whereas the nuclear-matrix-associated fraction remained stable after a 4-h chase. The observation of distinct turnover rates for protein species from different subcellular fractions suggests that different protein degradation systems may exist for individual cellular compartments.

We estimate the yield of E6-polyhedrin fusion protein to be on the order of 10 to 50 mg/liter of cells. Purification of large amounts of properly processed E6 protein will allow further biochemical characterization as well as microinjection studies that might aid in the elucidation of the in vivo function of E6 protein. The determination of E6 function will be an important step in the elucidation of the role of human papillomaviruses in genital malignancies.

ACKNOWLEDGMENTS

This work was supported by the Howard Hughes Medical Institute. Immunocytochemistry was supported in part by University of Chicago CRC grant CA 14599. S.R.G. was supported by Public Health Service training grant 5T32 GM07821 from the National Institutes of Health, and R.M. was supported by Public Health Service grant 2T32HD-07009-09 from the National Institute of Child Health and Human Development.

We thank V. Luckow and M. Summers for providing us with baculovirus transfer plasmids and for valuable assistance with using the baculovirus expression system; L. Banks and L. Crawford for C1P5 monoclonal antibody; T. Martin for assistance with immunofluorescence; S. Meredith, J. Paul, and members of the Laimins laboratory for helpful discussions; and L. B. Rothman-Denes and E. Androphy for critical reading of the manuscript.

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