Identification of the Protein Encoded by the E6 Transforming Gene of Bovine Papillomavirus

Abstract. *Papillomaviruses (PV) contain several conserved genes that may encode nonstructural proteins; however, none of these predicted gene products have been identified. Papillomavirus E6 genes are retained and expressed as RNA in PV-associated human and animal carcinomas and cell lines. This suggests that the E6 gene product may be important in the maintenance of the malignant phenotype. The E6 open reading frame of the bovine papillomavirus (BPV) genome has been identified as one of two BPV genes that can independently transform mouse cells in vitro. A polypeptide encoded by this region of BPV was produced in a bacterial expression vector and used to raise antisera. The antisera specifically immunoprecipitated the predicted 15.5-kilodalton BPV E6 protein from cells transformed by the E6 gene. The E6 protein was identified in both the nuclear and membrane fractions of these transformed cells.*

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Papillomaviruses (PV's) are infectious agents that cause benign tumors (papillomas or warts) in many epithelia including skin, oropharynx, urethra, vagina, and cervix. Some animal PV's also induce malignant tumors in their natural host (*1*). Specific types of human papillomaviruses (HPV's) have been associated with malignant cutaneous neoplasms in epidermodysplasia verruciformis (widespread chronic PV infection) (*2*). Human papillomavirus DNA has now been identified in more than 80 percent of cervical dysplasias and cancers and approximately 40 percent of vulval and penile cancers (*3, 4*).

DNA sequence analysis of human and animal PV's has revealed that they have a similar genetic organization; comparison of their analogous open reading frames (ORF's, which represent potential protein coding sequences) suggests that most of their predicted proteins also share significant amino acid homology (*5*). The inability to obtain full replication of PV's in vitro has been a hindrance to the genetic analysis of these viruses. Therefore, PV genetics has been most thoroughly studied in vitro by bovine papillomavirus (BPV)–induced transformation, since BPV virions and BPV DNA readily induce tumorigenic transformation of certain established rodent cell lines (*6*).

The 8-kilobase (kb) BPV genome has been divided into early (E) and late (L) regions. The late region ORF's (L1 and L2), which encode virion structural proteins, are expressed in virus-producing papillomas but not in cultured cells and are not required for transformation by BPV. The early region ORF's (E1 to E8) are expressed as RNA in papillomas as well as in transformed cells (*7, 8*). Genetic analysis of BPV has begun to reveal specific functions of defined regions within its DNA genome. BPV contains two transforming genes, one of which is located in the E6 ORF (*9, 10*). The analogous E6 region in HPV DNA is retained and expressed as RNA in cervical carcinomas that contain PV DNA (*4*). Other genetic studies associated *trans*-acting functions with early region ORF's and suggested their involvement in viral replicative processes (*11*). However, no protein product encoded by an early-region ORF has been identified for any PV.

The failure to detect these putative gene products may be explained in part by the low levels of early messenger RNA (mRNA) found in PV-transformed cells and papillomas or the lack of appropriate antisera (or both). To identify the BPV E6 gene product, we used bacterially synthesized BPV E6 polypeptide to generate antisera to E6. Transformed mouse cell lines that expressed higher levels of E6 RNA than cells transformed with the full-length genome were developed. We now report the identification and preliminary characterization of the protein encoded by the E6 ORF of BPV in mammalian cells morphologically transformed by this gene.

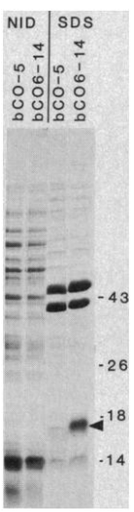

Fig. 1. Coomassie blue–stained polyacrylamide gel analysis of bacterial proteins from thermally induced control (bCO-5) and E6-containing (bCO6-14) bacteria (*12*). (Lanes 1 and 2) Proteins extracted in nonionic detergent (NID); (lanes 3 and 4) proteins extracted in 0.5 percent SDS. The arrow indicates the mobility of the 17.5-kD E6 fusion protein.

A prokaryotic expression vector that would produce large amounts of the E6 ORF polypeptide in *Escherichia coli* was first constructed. The vector included the phage lambda P_L promoter, ribosome binding sequence, and the amino terminal end of the phage CII protein (*12*). A segment of BPV DNA that included the entire E6 ORF was inserted in frame so that the plasmid would encode a 153–amino acid fusion protein composed of the first 13 amino acids of the CII gene followed by E6 coding sequences beginning three amino acids upstream from the predicted initiation AUG of E6 and extending past the stop codon of the ORF (*12*). This DNA was introduced into an *E. coli* strain that contained the lambda cI857ts gene, a temperature-sensitive repressor of P_L. After induction at 42°C, more than 10 percent of the proteins from these bacteria (bCO6-14) were the predicted 17.5-kilodalton (kD) E6 fusion protein (Fig. 1). Bacteria without an insert (bCO-5) or with the E6 gene in the negative orientation (not shown) did not produce this protein. The E6 protein was insoluble in nonionic detergents, as commonly occurs with proteins synthesized at high levels in bacteria; 0.5 percent sodium dodecyl sulfate (SDS) was required for its extraction. The 17.5-kD band was excised from SDS–polyacrylamide gels and used with Freund's adjuvant to immunize rabbits at 3- to 4-week intervals. This resulted in antisera that recognized both the reduced and nonreduced fusion protein by immunoprecipitation and immunoblotting.

Cells transformed by the entire BPV genome contain low levels of mRNA's [only 0.006 to 0.01 percent of total poly(A)$^+$ RNA] (*8*). The detection of a putative E6 protein product should be easier in a cell line that expresses the gene at high levels, and to be biologically relevant this cell line should be morphologically transformed by the E6 gene. The expression of E6 RNA was increased by transfecting susceptible mouse cells with plasmids in which the E6 ORF was driven by the long terminal repeat (LTR) of the Moloney sarcoma retrovirus; this LTR has signals for enhancement and promotion of RNA transcription. Multiple clones of C127 mouse cells morphologically transformed by such chimeric plasmids were generated and screened for E6 mRNA by dot-blot hybridization; clones with higher levels

were used for further studies. In the LTR-activated plasmid pXH800 (*9*) the BPV sequences were limited to the E6 and E7 ORF's and a small fragment that contains a BPV enhancer element (*13*) and the viral polyadenylation signal for the early RNA's.

The transformed C127 cells were assayed for synthesis of the E6 gene product by immunoprecipitation of [^{35}S]cysteine-labeled extracts with antiserum containing antibodies to the bacterial fusion protein. The antiserum specifically precipitated a 15.5-kD protein on reducing SDS–polyacrylamide gels from each of two cell lines transformed by pXH800 (Fig. 2A). The mobility of this protein corresponds to the predicted molecular weight of the 137–amino acid E6 ORF if the first AUG in the ORF represents the translation initiation codon (*5*), and the protein is unmodified. This protein band was not detected in C127 cells transformed by the v-*ras*^{H} gene of Harvey sarcoma virus (Ham Cl 8) (*14*) or by a plasmid that contains the 3′ 2.3-kb transforming segment of BPV (pHLB-1) but not the E6 or E7 ORF's (*15*). C127 cells transformed by BPV virions via infection (ID14) as well as C127 and NIH 3T3 lines transfected by the entire BPV genome also synthesized the 15.5-kD protein, but at less than one-tenth the quantity detected in pXH800-1. This low level of protein may in part explain the previous inability to detect early region proteins from BPV-transformed cells. With nonreducing SDS gels, the 15.5-kD band was still the only specifically immunoprecipitated protein. These results suggest that the 15.5-kD protein is the E6 protein and that it is not in disulfide linkage with other proteins.

To confirm that the 15.5-kD protein contained sequences encoded by the BPV E6 gene, we performed immunoprecipitation inhibition experiments. Identical [^{35}S]cysteine-labeled pXH800-1 lysates were added to antisera that had been incubated with nonradiolabeled SDS extracts from thermally induced bCO-5 (control) or bCO6-14 (which synthesizes the E6 fusion peptide). The immunoprecipitation of the 15.5-kD band was specifically blocked only by the extract that contained the E6 fusion protein (Fig. 2B). Unlabeled E6-containing bacterial lysate did not block precipitation of bacterially synthesized p21 *ras* protein by p21-specific antisera. These results strongly imply that the 15.5-kD protein is encoded by the viral E6 gene.

Since plasmid pXH800 contains both the E6 and E7 ORF's, cells transformed by pXH800 could potentially synthesize an E6-E7 fusion protein (called E67) from a spliced mRNA predicted by analysis of complementary DNA's (cDNA's) from BPV-transformed cells (*10*). Therefore, it was theoretically possible that the 15.5-kD band might represent this gene product, although the predicted size of E67 is approximately 20.5 kD. The 15.5-kD band was detected by immunoprecipitation of lysates from a C127 cell line transformed by pXH997 (*8*), a deletion mutant of pXH800 from which most of the E7 ORF was deleted (Fig. 2A). Since this mutant contains an intact E6 ORF but cannot synthesize an intact E67, we conclude that the 15.5-kD protein is the product of the E6 ORF.

Having demonstrated that the 15.5-kD molecule is BPV E6, we localized the protein by subcellular fractionation. Cells transformed with pXH800 were labeled with [^{35}S]cysteine and separated by two different procedures into nuclei, cytoplasm, and membranes (*16, 17*). Approximately equal quantities of the E6 protein were detected in the nuclear and membrane fractions by the two methods [Fig. 3A; data shown for the method of Hann and Eisenman (*16*)]. The efficiency of this fractionation was confirmed (Fig. 3, B and C) by examining the fractions for the presentation of c-*ras*, which is found in the plasma membrane (*14*), c-*myc*, which is a nuclear protein (*16*), and other markers. As expected, there was virtually no contamination of nuclei with c-*ras*, and the c-*myc* proteins were confined entirely in the nuclear fraction. As judged by an assay for the enzyme β-acetylglucosaminidase, there was only trace contamination of the nuclei by membranes. When cells incubated with [^{3}H]thymidine were fractionated, more than 95 percent of the trichloracetic acid–precipitable radioactivity was found in the low-speed nuclear pellet, confirming that this nuclear fraction contained virtually all of the DNA.

These data demonstrate the existence

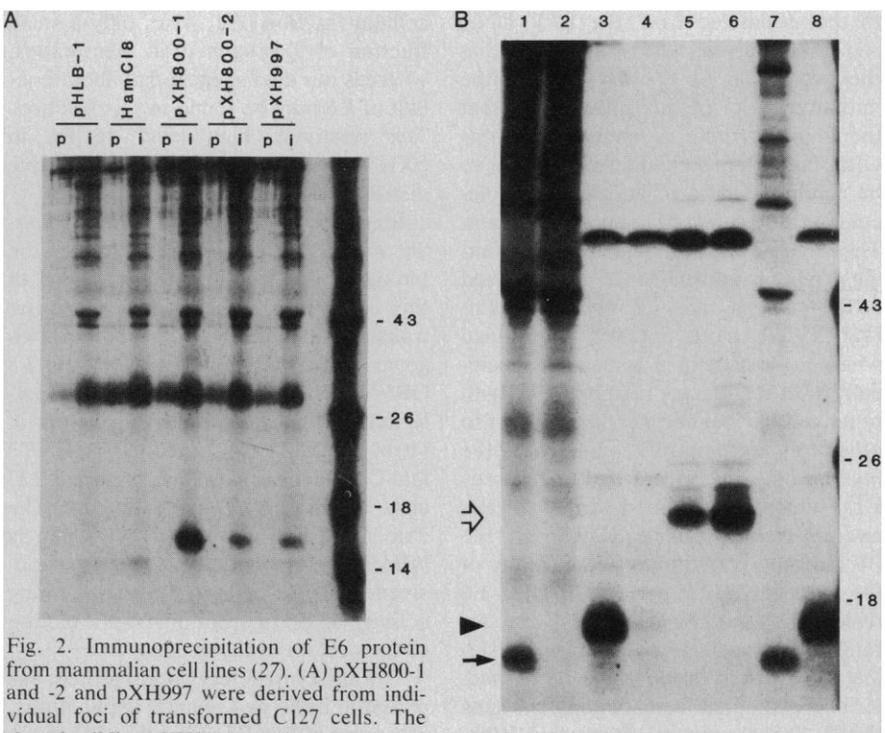

Fig. 2. Immunoprecipitation of E6 protein from mammalian cell lines (*27*). (A) pXH800-1 and -2 and pXH997 were derived from individual foci of transformed C127 cells. The cloned cell line pXH800-1 produced the greatest quantity of E6 mRNA, estimated to be at least ten times that of full-length BPV-transformed cells (ID14). pHLB-1 (*15*), a transformed C127 cell line that contains the 3′ 2.3-kb transforming segment of BPV, but not the E6 or E7 ORF's, and Ham Cl 8, a (nonproducer) C127 cell line transformed by the v-*ras*^{H} oncogene of the Harvey sarcoma virus (*14*) were used as control transformed cell lines. Abbreviations: p, preimmune sera; i, immune sera. Exposure, 13 days. (B) Immunoprecipitation inhibition experiment. Antisera to E6 (lanes 1 to 4, 7, and 8) were initially incubated for 1 hour with nonradiolabeled SDS extracts from temperature-induced bCO-5 (control; lanes 1, 3, and 5) or bCO6-14 (containing the E6 fusion protein; lanes 2, 4, and 6). In lanes 5 and 6, a rabbit antibody to bacterially produced v-*ras*^{H} (*28*) was preincubated with bacterial extracts as above. No nonlabeled bacterial extracts were added to the anti-E6 sera in lanes 7 and 8. After collection of the immune complexes with protein A–Sepharose and washing (*27*), [^{35}S]cysteine-labeled lysates from pXH800-1 cells (lanes 1, 2, and 7) or bacteria bCO6-14 (lanes 3, 4, and 8) or bacterial v-*ras*^{H} (lanes 5 and 6) were added. Immune complexes were collected after 1 hour and washed. The small arrow indicates the migration of E6 from transformed cells; the solid black carat indicates the bacterial E6 fusion protein; and the open arrow marks the size of the bacterial *ras* protein. Only the bacterial E6 fusion protein specifically inhibited immunoprecipitation of the E6 protein from transformed cells or bacteria. Exposure, 10 days.

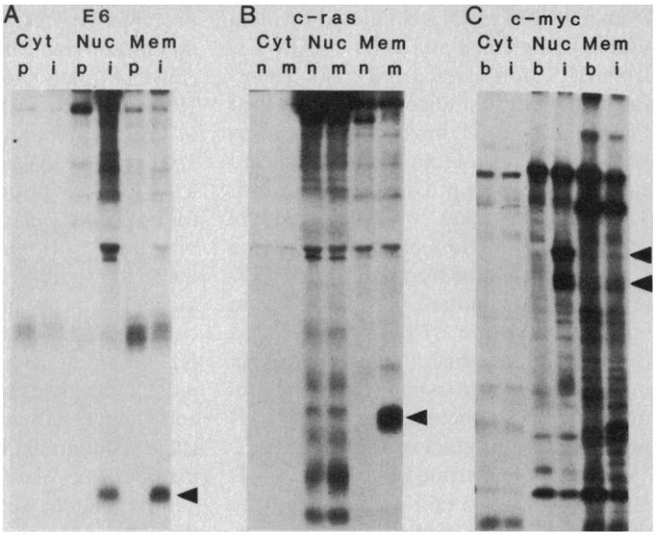

Fig. 3. Subcellular localization of the E6 protein. Approximately 8 × 10^7 pXH800-1 cells were labeled, fractionated, and immunoprecipitated (*16, 26*). (A) Nuclear (Nuc), cytoplasmic (Cyt), and membrane (Mem) fractions immunoprecipitated with preimmune (p) or immune (i) antisera to E6. Exposure, 9 days. (B) Immunoprecipitation with normal rat serum (n) or monoclonal antibody Y13-259 (m) to the 21-kD plasma membrane protein c-*ras* (*29*). Exposure, 3 days. (C) Immunoprecipitation with affinity-purified antisera to mouse c-*myc* peptide (*16*). Antisera preincubated and blocked with *myc* peptide (b) or unblocked antisera (i) to detect the 62- and 67-kD nuclear protein c-*myc*. Exposure, 3 days.

of the BPV E6 protein and show that its apparent 15.5-kD mobility corresponds to that predicted from the BPV DNA sequence analysis. The results imply that the first AUG of the E6 ORF is the initiation AUG of this protein and that the entire protein is encoded by this ORF. The E6 protein does not appear to be disulfide-linked to another E6 molecule (or molecules) or to another protein. The distribution of this small transforming protein is unusual since it is localized in the nucleus and in nonnuclear membranes. We have not yet determined whether the protein is associated specifically with the plasma membrane or with other cellular membranes. In contrast to other transforming proteins such as the *myc* and *fos* oncogenes and adenovirus E1a, which are confined to the nucleus and are rich in proline (*16, 18, 19*), the PV E6 gene is rich in cysteine. Many of the cysteines (10.9 percent of BPV E6 residues should be cysteines) are arranged in repeats of cysteine-x-x-cysteine, a sequence proposed to be characteristic of nucleic acid–binding proteins (NBP) (*20*). As is true of known NBP's, E6 has a high percentage of basic amino acids; 17 percent are arginine or lysine. The nuclear localization of some E6 molecules is consistent with the hypothesis that E6 may be a NBP. The Cys-x-x-Cys arrangement also occurs in SV40 T/t-antigens, polyoma t-antigen, and the glycoprotein hormone family (*21*). The localization of the BPV E6 protein may appear to be similar to that of SV40 T-antigen, which has a nucleotide binding domain and is found in the nucleus and plasma membrane. However, T-antigen is found in nonionic detergent extracts of nuclei (nucleoplasm) (*22*), whereas E6 is not immunoprecipitated from this subcellular fraction (*23*). Also, only a small fraction of T-antigen is in membranes, whereas our data suggest that about one-half of E6 may be found in this fraction. The relatively high level of E6 in pXH800 cells may affect the relative distribution of the protein.

Identification of the E6 protein product should allow characterization of the biological and biochemical functions of this protein, as is being done for other transforming proteins. BPV contains two genes, corresponding to the E6 and E5 ORF's, either of which can independently induce tumorigenic transformation in vitro. While E5 can transform NIH 3T3 and C127 cells (*24*), E6 transforms C127 cells but not NIH 3T3 cells (*8*). The inability of E6 to transform cells such as NIH 3T3 may result from the unresponsiveness of the cells to the transforming activity of E6 or from aberrant function of the protein in those cells.

The ability to detect the E6 protein has potential basic and clinical applications. The roles of E6, E5, and other PV genes in the development of warts and malignant tumors remain to be determined. Appropriate antisera to E6 and other PV early proteins should permit identification of the cells that express PV early proteins in warts and may enhance understanding of how PV proteins influence growth and differentiation of the epidermis.

The speculation that E6 may serve an important function in malignant tumors is supported by the finding that the E6 region is selectively retained and transcriptionally active in transplantable carcinomas induced by the cottontail rabbit or Shope PV (*25*). In addition, several human cervical carcinoma cell lines, including HeLa, contain integrated copies of the E6-E7 region of HPV 18 that are expressed as mRNA, but these cells lack the 3′ end of the early region and portions of the late region of HPV 18 (*4*). Messenger RNA corresponding to the E6 ORF has also been identified in carcinomas of the cervix associated with HPV 16 infection (*4*). The technique described here should be applicable to the identification of the analogous E6 protein encoded by other PV's, such as those associated with malignant tumors in humans and animals, as well as the putative gene products of other ORF's. Since most PV-associated dysplasias, carcinomas in situ, and invasive cancers do not produce virions or virion structural proteins, identification of early PV gene products in these clinical settings may be useful diagnostically and in the elucidation of the pathogenesis of these lesions.

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CII protein inserted into the Cla I site of pBR322. The prokaryotic expression vector pCO-5 is identical to vector pJL6 of Lautenberger and his co-workers (*26*); pJL6 was not made available by those authors despite its previous publication. The 589-bp Hpa II fragment of BPV (nucleotide numbers 80 to 669) was cloned into the regenerated Cla I site of pCO-5. The resultant construct, pCO6-14, encodes the first 13 amino acids of the phage CII gene fused to BPV coding sequences, which begin three amino acids upstream from the predicted AUG of E6 and continues past the stop codon of the ORF. This DNA was then introduced into bacteria N6405, a N^+ RR1 strain of *E. coli* that contains a lysogenic temperature-sensitive P_L repressor. Proteins were extracted by lysing thermally induced (90 minutes) bacteria bCO-5 (control) or bCO6-14 (E6 insert) in 50 m*M* tris, *p*H 8, 1 m*M* EDTA, and lysozyme (0.25 mg/ml) for 30 minutes at 4°C. $MgCl_2$ and NaCl were added to attain a final concentration of 6 m*M* and 0.2*M*, respectively, and DNase I was added to attain a final concentration of 0.1 mg/ml, for 30 minutes at 4°C. Bacterial proteins were extracted with nonionic detergents (0.5 percent NP-40 and 0.5 percent deoxycholate); insoluble proteins, including the E6 fusion protein, were subsequently solubilized with 0.5 percent SDS.

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A Pea Mutant for the Study of Hydrotropism in Roots

Abstract. *Plant roots grow in the direction of increasing soil moisture, but studies of hydrotropism have always been difficult to interpret because of the effect of gravity. In this study it was found that roots of the mutant pea 'Ageotropum' are neither gravitropic nor phototropic, but do respond tropically to a moisture gradient, making them an ideal subject for the study of hydrotropism. When the root caps were removed, elongation was not affected but hydrotropism was blocked, suggesting that the site of sensory perception resides in the root cap.*

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Roots grow in response to water gradients (*1*). Usually the gradient is vertical; that is, desiccation occurs by evaporation at the soil surface and gravity tends to pull water to lower levels. Garwood and Williams (*2*) showed that in a light alluvial soil beneath a pasture, the water potential decreased from −2 bars in April to −15 bars in July at a depth of 30 cm. However, at a depth of 60 cm, water potential decreased from −1 bar in April to only −4 bars in July. Thus, during the summer, a period of considerable root growth, there was a water gradient of 14 bars between 30 and 60 cm.

Roots appear to grow toward the region of greater water potential. Klepper *et al.* (*3*) monitored the growth of cotton roots during a period of soil desiccation. Before desiccation, the water potential was the same (about −0.5 bar) from 23 to 173 cm below the surface. After 13 days of desiccation, the water potential at 173 cm was still about −1 bar, but at 23 cm it was about −9 bars, establishing an 8-bar gradient. During the experiment the density distribution of the root system changed from being highest at about 20 cm deep and lowest at about 180 cm to the reverse. (Care must be taken in interpreting such results. They could indicate a greater amount of growth in moist areas or the death of roots in drier areas, instead of directed growth.)

The mechanism of hydrotropism [as Sachs (*4*) called the directed growth of roots in a moisture gradient] has been studied at least since the early 19th century. Dutrochet (*5*) suggested that roots may bend in response to moisture gradients in the root environment, but was unable to demonstrate such a response experimentally. Sachs (*4*) described a simple experiment in which he planted pea seeds in a freely hanging cylinder of damp sawdust held together by a zinc mesh screen. The roots penetrated and grew through the sawdust (positive gravitropism); however, when they grew out of the lower surface, they bent around and grew back toward the wet substratum, thus overcoming the force of gravity. Sachs observed that thigmotropism also plays a role in the directional growth of roots. If roots encounter a unilateral impediment or are rubbed on one side, they will bend in the direction of the mechanical perturbation (*6*).

It is likely that both thigmotropism and gravitropism interact with hydrotropism and therefore interfere with the study of hydrotropism. Mechanical stimuli can easily be avoided, but gravity is ubiquitous (at least on Earth). Therefore a root system lacking the gravitropic reaction would be an important tool in the study of root hydrotropism.

The roots and shoots of the pea mutant 'Ageotropum' are gravitropically unresponsive when the plant is grown in darkness (*6, 7*). However, when the plants are illuminated, the shoots become gravitropic, bending upward regardless of the direction of illumination. The photoactivation of gravitro-