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The Product of the Bovine Papillomavirus Type 1 Modulator Gene (*M*) Is a Phosphoprotein

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The *M* gene of bovine papillomavirus type 1 has been genetically defined as encoding a *trans*-acting product which negatively regulates bovine papillomavirus type 1 replication and is important for establishment of stable plasmids in transformed cells. The gene for this regulatory protein has been mapped in part to the 5′ portion of the largest open reading frame (E1) in the virus. We constructed a *trpE*-E1 fusion gene and expressed this gene in *Escherichia coli*. Rabbits were immunized with purified fusion protein, and antisera directed against the product were used to identify the *M* gene product in virus-transformed cells. In this way a polypeptide with an apparent molecular mass of 23 kilodaltons was detected. The virus-encoded product is phosphorylated and can be readily detected by immunoprecipitation assays from cells transformed by the virus. Cells that harbor viral DNA without *M* as integrated copies do not produce this protein, whereas cells that harbor integrated viral genomes which are defective for another E1 viral gene important for plasmid replication, *R*, do produce this protein. The protein has an anomalously low electrophoretic mobility. An in vitro translation product of an SP6 RNA product of a sequenced cDNA predicts a molecular mass of 16 kilodaltons for the protein, and this in vitro translation product has an electrophoretic mobility identical to that of the in vivo immunoprecipitated protein. The results of these studies confirm our previous genetic studies which indicated that part of the E1 open reading frame defined a discrete gene product distinct from other putative products which may be encoded by this open reading frame.

Many papillomaviruses from a diverse group of organisms have been isolated, and their genomes have been cloned and entirely sequenced. The conservation of genome organization is notable if one considers the number of open reading frames (ORFs) encoded by this family of viruses and the homologies that exist between comparable ORFs in the different isolates (2). The E1 ORF is the largest stretch of continuous coding sequence, and conceptual translation of this DNA region shows that amino acid sequences over the carboxy terminus of the putative products are highly homologous. In addition, Clertant and Seif (8) have found homologies between the putative bovine papillomavirus (BPV) E1 protein from this region and the ATP-binding domains of the simian virus 40 and polyomavirus large-T antigens. We have been particularly interested in the putative products from this ORF, since deletion, insertion, or frameshift mutations sustained in the central or terminal part of the coding sequences of E1 either abolish or severely limit the extent of BPV type 1 (BPV-1) replication detected in transient-replication assays (21, 22). Since these transient-replication assays do not depend upon stable plasmid maintenance or selection for a particular phenotype, it seems likely that E1 coding sequences play some direct role in viral replication, although this has not been proven. Indeed, the E1 ORF provides the only target coding sequences for viral factors which absolutely abolish transient viral amplification.

That the E1 ORF encodes for *trans*-acting factors has been established by complementation assays, which demonstrate that either the wild-type virus or mutants in other genes can complement the E1 mutants for transient and stable plasmid replication (22). A surprising feature of the genetic analysis of the E1 ORF was that the coding sequence appears to define two separate genes rather than the one gene that was suggested by the conservation of a single large ORF in all known papillomaviruses. Frameshift insertions or deletions in the 5′ portion of the ORF define a negative regulator of replication and permit transient replication of these BPV mutants in cells wherein the wild-type virus cannot replicate (3, 22, 27). That is, mutations in this region of E1 could replicate upon transfection into BPV-1-transformed cells that are normally immune to amplification by wild-type DNA, but cotransfection of the wild type with these mutants resulted in repression of both mutant and wild-type DNA replication (3, 22). In contrast, mutations in the middle or 3′ end of the E1 ORF are negative for transient replication in BPV-infected or uninfected cells; although these mutants can be complemented by the 5′ frameshift mutations in a variety of transient or stable assays. Whereas these complementation tests do not rule out some form of intracistronic complementation between complementing fragments of different domains of mutant proteins, we reasoned that this was unlikely and that two separate genes with different functions were indicated. This was based upon the fact that the amino-terminal frameshift mutations by themselves replicated in a manner identical to that of the wild type in untransformed cells yet could not produce carboxy-terminal fragments without internal translational reinitiation at an ATG far distal to the frameshift, a process believed to be very inefficient in eucaryotes (15). Thus, we defined two separate genes by conventional *cis* and *trans* tests. The carboxy portion of the E1 ORF was called the *R* gene, since it was important for detecting replication, and the amino portion was called *M*, since it appeared to modulate replication of the viral DNA negatively. Both genes are important for stable plasmid maintenance, since only integrated viral DNA is detected in cell lines transformed by the respective single mutants. We speculated that the *M* gene is required for either transition to or maintenance of regulated plasmid replication, whereas the *R* gene is required to drive viral amplification (3, 22). Accordingly, mutations in the *M* gene

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would lead to integration, since free plasmids could not be maintained stably in any selection for a stable cell line and mutations in the *R* gene would lead to integration of the transforming DNA because the positive factor(s) required for viral replication would be missing (3, 22).

A direct prediction of the genetic model described above is that a small polypeptide encoded in part by the 5′ portion of the E1 ORF should be detectable in BPV-1-transformed cells. This protein would be the product of the *M* gene and should be distinct from a full-length E1 product or any other E1 product, such as *R*. To test this point critically, we created a BPV-1 fusion protein in *Escherichia coli* and raised polyclonal antisera to this bacterial protein. The antisera do recognize a bona fide BPV-1-encoded protein in transformed cells, and we report below upon the detection and structure of this protein.

MATERIALS AND METHODS

Construction of recombinant plasmids. A mutant of plasmid pMLBPV-5, D10 (22), which has a *Bam*HI linker inserted at BPV-1 position 860 (see Fig. 1), was the source of the E1 DNA sequence for the construction of pATH1-5′E1. The *trpE* fusion vector pATH1 was generously provided by T. J. Koerner and A. Tzagaloff (Columbia University). The *lacIZ* fusion vectors pHK410 and pHK411 were a gift of Molecular Genetics Inc. (Minnetonka, Minn.); pHK411-E1 contains a fragment of the wild-type BPV sequence, from positions 841 to 2410, cloned into the *Bam*HI site of pHK411 (provided by M. Naruto, this laboratory). cDNA N7-1 (see Fig. 1) will be described in another report. The sequences from this cDNA between nucleotides 838 and 3351 were cloned into the *Bam*HI site of pSP65 (Promega). Transformation of *E. coli* HB101 (5) with pATH1, pATH1-5′E1, or pSP65-5′E1 DNA and *E. coli* NF1829 (*lacI*^{q}) (31) with pHK410 or pHK411-E1 DNA was done by selecting for ampicillin resistance by standard methods (23).

Preparation of bacterial cell extracts. Induction of the *trp* promoters in HB101 strains that contain pATH1 and pATH1-5′E1 plasmids was done essentially as described by Spindler et al. (35). Strains were grown overnight at 37°C in M9 plus 0.5% Casamino Acids, 10 μg of thiamine per ml, 20 μg of tryptophan per ml, and 50 μg of ampicillin per ml. Cultures were diluted 1:10 into the same medium lacking tryptophan and grown for 1 h at 30°C with vigorous aeration before addition of a 1:200 dilution of a 1-mg/ml stock of indoleacrylic acid (Sigma Chemical Co.) dissolved in 100% ethanol. Growth was continued for 2 h at 30°C, and the cells were harvested by centrifugation. We prepared whole-cell lysates by suspending the cell pellet in 1/20 of the original volume of urea-sodium dodecyl sulfate (SDS) cracking buffer (0.01 M sodium phosphate [pH 7.2], 1% β-mercaptoethanol, 1% SDS, 6 M urea) and incubating this suspension at 37°C for 0.5 to 3 h before boiling it in 2× Laemmli sample buffer (17). The insoluble protein fraction was isolated by lysing the cells and treating them with DNase I exactly as described by Kleid et al. (14). We then suspended the washed insoluble pellet in 1/20 of the original volume of urea-SDS cracking buffer and incubated it at 37°C for approximately 0.5 h before boiling it in 2× Laemmli sample buffer.

NF1829 strains containing pHK410 and pHK411-E1 were grown overnight at 30°C in M9 plus 0.5% Casamino Acids–0.5% glycerol–10 μg of thiamine per ml–50 μg of ampicillin per ml. Cultures were diluted 1:10 into the same medium lacking glucose and grown for 1 h at 30°C with vigorous aeration before addition of a 1:100 dilution of a 100 mM stock of isopropyl-β-D-thiogalactopyranoside (Bethesda Research Laboratories, Inc.). Growth was continued for 1 h at 30°C, and cells were harvested by centrifugation; whole-cell lysates and insoluble protein fractions were prepared as described above.

Preparation of rabbit antisera against the *trpE*-5′E1 bacterial fusion protein. Two separate preparations of the same *trpE*-5′E1 fusion protein were used to raise polyclonal antisera in New Zealand White rabbits by injecting the antigen directly into the popliteal lymph nodes (32). Subsequent booster injections were administered intramuscularly or subcutaneously into the backs and legs of the animals. Group 1, consisting of three female animals, was injected with the partially purified fusion protein. Injections were given every 2 to 4 weeks with 30 to 300 μg of protein per rabbit in complete Freund adjuvant (Difco Laboratories) for the initial immunization and incomplete adjuvant for subsequent boosts. Group 2, consisting of two male animals, was injected with the fusion protein extracted from SDS-polyacrylamide gels. Injections were given every 2 to 3 weeks with 60 to 200 μg of protein per rabbit in complete (primary injection) or incomplete (booster injections) Freund adjuvant.

The partially purified antigen was prepared by lysing the induced bacteria and applying the crude extract to a phosphocellulose column as described by Rawlins et al. (25), except that the extreme insolubility of the *trpE*-5′E1 fusion protein required that it be extracted from the pelleted cell debris. This extraction was done by thoroughly suspending the pellet in 1/50 of the original volume of 8 M urea–0.5 M NaCl–0.5 M Tris hydrochloride (pH 7.9)–1 mM EDTA, 30 mM β-mercaptoethanol–100 μg of phenylmethylsulfonyl fluoride (PMSF; Sigma) by using a tissue homogenizer and then incubating the mixture on ice for 1 h. To encourage renaturation of the extracted protein, this suspension was dialyzed in 50 mM Tris hydrochloride (pH 7.5)–10% glycerol–1 mM PMSF that contained sequentially decreasing amounts of urea (i.e., 4, 2, 1, and 0 M), with several hours between each buffer change. Insoluble material was removed by centrifugation at 10,000 × *g* for 20 min before the supernatant was applied to a phosphocellulose column (Whatman P11) equilibrated in 50 mM Tris hydrochloride (pH 7.5)–10% glycerol–1 mM PMSF. The column was washed in the same buffer, and the *trpE*-5′E1 fusion protein was eluted with a linear gradient from 0 to 1 M NaCl in 50 mM Tris hydrochloride (pH 7.5)–10% glycerol–1 mM PMSF. The *trpE*-5′E1 protein eluted as a broad peak centered at around 200 mM NaCl, and fractions containing significant amounts of this protein, as determined by analysis on SDS-polyacrylamide gels, were pooled and concentrated with an Amicon Microconcentrator 30 to obtain the small volume required for popliteal injections (<0.2 ml per lymph node).

To prepare the second antigen, the insoluble material recovered after dialysis (see above) was boiled in 2× Laemmli sample buffer and fractionated by electrophoresis in a preparative SDS-polyacrylamide gel (4% stacking gel and 8% resolving gel, cast in 1-mm-thick slabs). The protein position was determined by staining with Coomassie brilliant blue, gel strips were excised, and the protein was eluted as previously described (10). The eluted protein was precipitated by adding trichloroacetic acid to a final concentration of 10% and suspended in 0.2 ml of phosphate-buffered saline–1% SDS.

Antibodies that specifically recognize the *trpE*-5′E1 fusion protein were purified from rabbit B antiserum (native anti-

gen) by first diluting the serum with 4 volumes of 0.15 M NaCl and precipitating it by addition of 0.282 g of ammonium sulfate [$(NH_4)_2SO_4$] per ml; $(NH_4)_2SO_4$ crystals were added slowly over a period of 30 min with constant stirring at 4°C and stirred for an additional 30 to 60 min. After this suspension was centrifuged at 12,000 × *g* for 10 min at 4°C, the protein pellet was redissolved in a minimum volume of 20 mM potassium phosphate buffer (pH 7.15) and dialyzed overnight at 4°C against 200 volumes of the same buffer. The dialysate was cleared by centrifugation at 12,000 × *g* for 20 min, passed through a DEAE–Affi-Gel Blue column (Bio-Rad Laboratories) exactly as previously described (7) and eluted by the stepwise elution scheme of Bruck et al. (7) with 20 mM Tris hydrochloride (pH 7.2)–50 mM NaCl. The eluted antibodies were next applied to a 1-ml Affi-Gel 15 (Bio-Rad) column to which approximately 20 mg of partially purified native *trpE*-5′E1 protein (see above) had been coupled. The column was washed and developed as previously described (26), except that Tris-buffered saline was used in place of phosphate-buffered saline and the antibodies were passed through the column only once. Protein concentrations of the column fractions were measured (6), and those having greater than 0.1 mg/ml were pooled.

Cells and radiolabeling procedure. Mouse C127 cells (9) and derived cell lines (E1-Sma and i2113), ID13 cells (19), and VI216 cells (J. Reynolds, this laboratory) were grown in Dulbecco modified Eagle medium (GIBCO Laboratories)–10% fetal bovine serum (Irvine Scientific) supplemented with penicillin-streptomycin. E1-Sma and i2113 cell lines were made by transfecting C127 cells (13) with BPV DNAs containing mutations at either the *Sma*I (position 945) or the *Eco*RI (position 2113) site, respectively (see Fig. 1 and references 3 and 21) and were shown to contain the correct mutant DNAs by Southern blotting. In both cases, the BPV-1 DNA was found to be integrated into the cellular genome. Digestion of the cellular DNA with the enzyme *Bgl*II and subsequent blotting analysis showed that the DNA sequences spanning the E1 frame were intact in these cells.

Proteins were labeled with $^{32}PO_4$ (ICN Pharmaceuticals, Inc.) in vivo by using cells seeded into 100-mm-diameter petri dishes 24 to 48 h earlier so that they were 70 to 80% confluent at the time of labeling. The medium was removed, and the monolayers were washed twice with Tris-buffered saline. Cells were starved for 2 to 3 h in 3 ml of phosphate-minus medium (Flow Laboratories, Inc.) per dish plus 10% dialyzed fetal bovine serum. Postincubation, the medium was removed and replaced with 1 ml of the same medium containing ≥1 mCi of $^{32}PO_4$ per dish. Dishes were rocked periodically for 3 h before the medium was removed, and the monolayer was washed twice with Tris-buffered saline. Tris-buffered saline (1 ml) was added to each dish, and the cells were removed with a rubber policeman and centrifuged at low speed. The cell pellet was solubilized in 1% Triton X-100–0.5% deoxycholate–5 mM EDTA–250 mM NaCl–25 mM Tris hydrochloride (pH 7.5) (24) by using 0.25 ml per dish. Proteins were labeled with ^{35}S in exactly the same manner except that medium lacking cysteine and methionine (GIBCO) was used, and 1.5 ml of this medium containing 0.5 mCi each of [^{35}S]cysteine and [^{35}S]methionine (Amersham) per ml was used to label each dish for 14 h. If extracts were not used immediately, they were stored at −70°C before use in immunoprecipitation reactions.

Immunoprecipitations. In vivo labeled cell extracts were cleared by pelleting the insoluble debris. Incorporation of [^{35}S]cysteine and [^{35}S]methionine into extracts was quantitated by measuring trichloroacetic acid-precipitable counts, and all samples in a given experiment contained equivalent amounts of incorporated radioactivity. ^{32}P-labeled extracts were simply divided into equal volumes for immunoprecipitation. Proteins were immunoprecipitated from these extracts by a simplified version of the two-step immunoadsorption procedure of Platt et al. (24). Samples were brought to a final volume of 1 ml with solubilization buffer plus 1 mM PMSF before either 1 μl of preimmune or immune serum or 5 μl of affinity-purified immune serum was added. After the mixtures were incubated at room temperature for 1 h, 25 μl of a 10% (wt/vol) suspension of Pansorbin (Calbiochem-Behring) pretreated with 1 mg of bovine serum albumin (radioimmunoassay grade; Sigma) per ml was added and the mixtures were incubated for 15 min futher. Immune complexes were isolated by centrifugation, washed twice with solubilization buffer and once with 10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA. Antigen-antibody complexes were released from the Pansorbin with 30 μl of 1% SDS in phosphate-buffered saline by incubation for 10 min at room temperature, followed by centrifugation for 3 min. The supernatant was added to 500 μl of solubilization buffer containing 10 mg of bovine serum albumin per ml and the same amount of the appropriate immune or preimmune serum. Incubation, Pansorbin addition, and washes were done exactly as described above. Immunoprecipitated proteins were removed from the final pellet by 5 min of boiling in 25 μl of 2× Laemmli sample buffer and electrophoresed in 12% SDS-polyacrylamide resolving gels (4% stacking gels). Proteins were fixed in the gel by 30 min of incubation in 10% acetic acid–10% methanol, dried under vacuum, and exposed to Kodak X-Omat film (Eastman Kodak Co.) at −70°C. Gels containing ^{35}S-labeled proteins were impregnated with Enlightning (New England Nuclear Corp.) for 30 min before drying, and gels containing ^{32}P-labeled proteins were exposed to film in the presence of intensifying screens. ^{14}C-labeled protein standards were from New England Nuclear.

The in vitro translated M protein was immunoprecipitated by using a one-step version of the method described above, with the following changes. The 200-μl rabbit reticulocyte extract was divided into two equal portions, and each was added to 0.9 ml of solubilization buffer plus PMSF. Twice as much antiserum was used, and incubations were for 1 h at 4°C before 50 μl of Pansorbin was added, and incubations were continued for 30 min at 4°C with occasional mixing. Immune complexes were washed three times with buffer B (120 mM NaCl, 50 mM Tris hydrochloride [pH 7.6], 0.5% Nonidet P-40)–1 M $LiCl_2$–1 mM PMSF and one time with buffer B–1 mM PMSF (no $LiCl_2$) (16). Pellets were boiled for 5 min in 25 μl of 2× Laemmli sample buffer and electrophoresed in 12% resolving gels (4% stacking gels).

In vitro transcription and translation. The portion of the N7-1 cDNA from 838 to 3351 was inserted into pSP65, and a large amount of the recombinant plasmid DNA was isolated from two successive CsCl gradients and dialyzed. Plasmid DNA (40 μg) linearized with *Xba*I was added to the Riboprobe System II transcription system (Promega) as instructed by the manufacturer. One-fifth of the in vitro synthesized transcripts were added to each 200-μl in vitro translation cocktail consisting of rabbit reticulocyte lysate, a mixture of all the amino acids minus methionine, [^{35}S]methionine, and RNasin as recommended by Promega.

Western blotting (immunoblotting). Proteins separated on SDS-polyacrylamide gels were electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Inc.) by using an electroblot apparatus (Bio-Rad). Transfer was done at 55

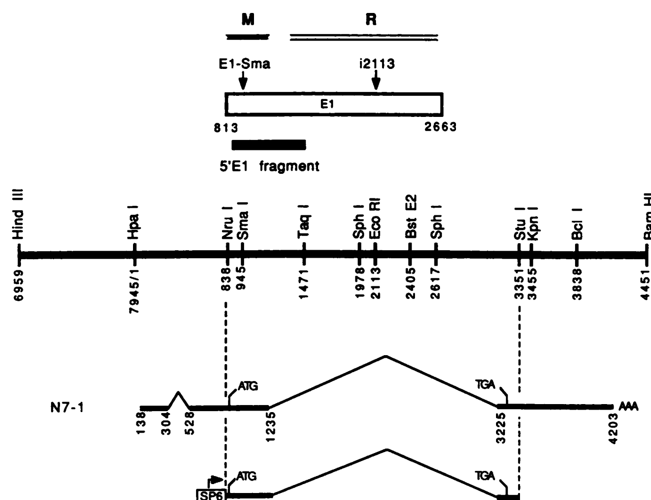
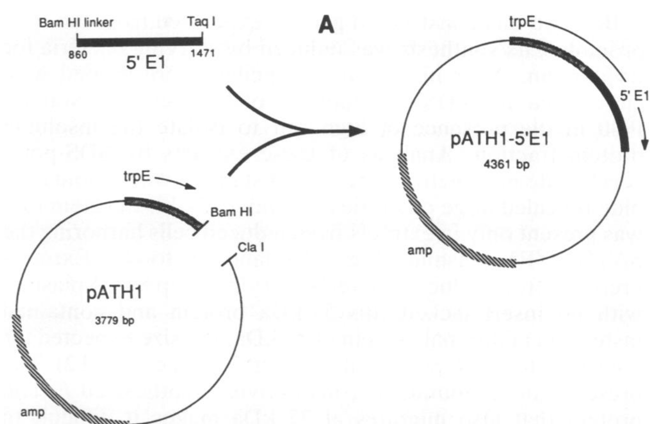


FIG. 1. Restriction map of the transforming region of BPV-1 DNA showing features of the E1 ORF. The extents of the *M* and *R* genes, as defined by previous genetic analyses, are shown at the top of the figure. Underneath, the locations of the M^- E1-Sma mutation and R^- i2113 mutations are shown by arrows. The 5'E1 fragment used to construct the *trpE*-5'E1 gene fusion is shown by a stippled box. The entire N7-1 cDNA and that portion inserted into pSP65 are shown at the bottom of the figure. Exons are represented by heavy lines, and introns are shown by thin lines. The SP6 sequences necessary for transcription are represented by a box with an arrow above it to indicate the direction of transcription.

V for 12 to 16 h with buffer consisting of 20 mM Tris, 150 mM glycine, and 20% methanol. To assign marker positions and monitor transfer efficiency, the filter was stained with the reversible dye Ponceau S (Sigma Chemical Co.). Blocking of the nitrocellulose was for 1 h at room temperature with TBSM (50 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 5% nonfat dry milk, 0.1% Nonidet P-40). Filters were reacted for 2 to 3 h at room temperature with rabbit antiserum against *trpE*-5'E1 diluted 1:2,000 in TBSM with gentle shaking and washed three times for 15 min each time with TBSM. Next, filters were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (TAGO, Inc.) diluted 1:2,000 in TBSM for 1 to 1.5 h at room temperature and washed three times for 15 min each time with TBSM. Transferred antigens were detected by placing the filter in a solution of 100 mM Tris hydrochloride (pH 9.5)–100 mM NaCl–5 mM $MgCl_2$ containing 0.33 mg of Nitro Blue Tetrazolium (Sigma) per ml and 0.165 mg of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) per ml. Color development was stopped by addition of 20 mM Tris hydrochloride (pH 8.0)–5 mM EDTA.

RESULTS

Construction of an inducible gene fusion expressing a 5' segment of the E1 ORF. To obtain sufficient quantities of the protein encoded by the 5' portion of the BPV E1 ORF for use as an immunogen, we expressed this portion of E1 as a fusion protein in *E. coli*. A 611-base-pair fragment, extending from the *Bam*HI site at 860 to the *Taq*I site at 1471, was isolated from the pMLBPV-5 mutant D10 (Fig. 1). This fragment was inserted into the pATH1 vector at the *Bam*HI-*Cla*I sites of the polylinker, located downstream of a portion of the *trpE* gene (T. J. Koerner, unpublished data) (Fig. 2A).



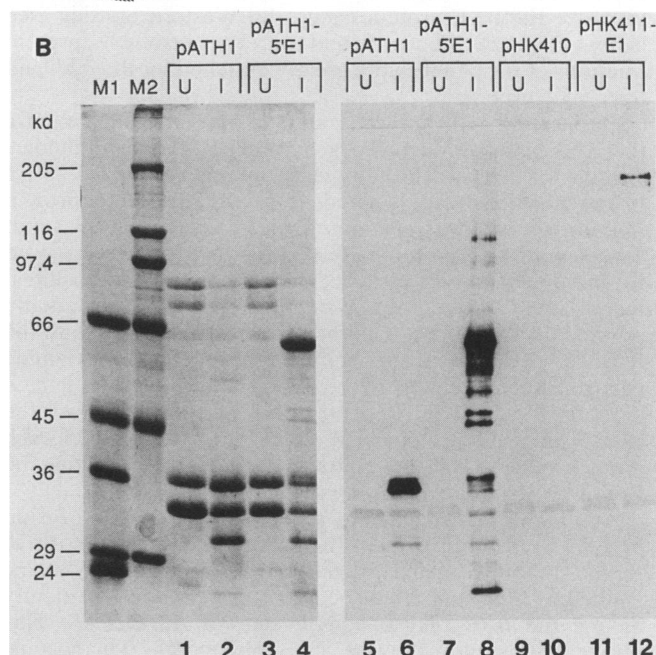


FIG. 2. (A) Construction of pATH1-5'E1. The 611-nucleotide portion of the E1 ORF, from 860 to 1471, was inserted into pATH1 distal to a portion of the *E. coli trpE* gene. The resulting plasmid, pATH1-5'E1, encodes a hybrid *trpE*-5'E1 fusion protein. bp, Base pair. (B) Induction of pATH1-5'E1 and characterization of antiserum raised against the *trpE*-5'E1 fusion protein. Lanes 1 to 4 are a Coomassie blue-stained SDS-polyacrylamide gel, and lanes 5 to 12 are an alkaline phosphatase-reacted Western blot. M1 and M2 are molecular mass standards, which are the same for both the gel and the blot. Protein was extracted from uninduced (U) and induced (I) bacteria carrying the fusion plasmid, pATH1-5'E1, and the parental plasmid, pATH1. For the blot, protein was also extracted from uninduced (U) and induced (I) bacteria carrying pHK411-E1, which consists of almost the entire E1 ORF fused to β-galactosidase (producing a protein with an expected molecular mass of 184 kDa), and the parental plasmid, pHK410. The induced 60-kDa fusion protein is clearly visible on the Coomassie blue-stained gel (lane 4) and on the Western blot (lane 8). The induced *trpE* protein from the pATH1 vector alone is visible only on the Western blot (lane 6), because it is obscured by an *E. coli* protein on the stained gel (lane 2). kd, Kilodaltons.

The resulting plasmid, pATH1-5'E1, should express a hybrid protein of approximately 59 kilodaltons (kDa), consisting of 22 kDa of E1 coding sequence appended to 37 kDa of the *trpE* protein.

Because this constructed gene is expressed from the *trpE* promoter, its synthesis was induced by starving bacteria for tryptophan. After induction, cell pellets were treated with either urea and SDS to solubilize total proteins or Nonidet P-40 in the presence of high salt to isolate the insoluble protein fraction. Analysis of these extracts by SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue revealed large quantities of a novel 57-kDa protein that was present only in extracts from induced cells harboring the pATH1-5′E1 plasmid (Fig. 2B, lanes 1 to 4). Extracts prepared from induced bacteria carrying the pATH1 plasmid with no insert lacked this 57-kDa protein and contained instead an additional protein of 37 kDa, the size expected for the truncated *trpE* protein alone (Fig. 2B, lanes 1 and 2). The presence of an abundant, constitutively synthesized *E. coli* protein that also migrates at 37 kDa makes it difficult to visualize the *trpE* protein except by Western blotting (see below; Fig. 2B, lanes 5 and 6). The insoluble protein fractions of the pellets were greatly enriched for the *trpE* and *trpE*-5′E1 proteins.

Preparation and characterization of antisera that recognize the 5′E1 gene product. To generate polyclonal antibodies specific for the 5′ E1 protein, large amounts of the *trpE*-5′E1 fusion protein were isolated from the insoluble protein fraction of bacteria containing the pATH1-5′E1 plasmid. Two separate preparations of this fusion protein were used to immunize rabbits by direct injection into the popliteal lymph node. One group was injected with the partially purified fusion protein. Another group was injected with the fusion protein extracted from preparative polyacrylamide gels run under denaturing conditions. Sera were collected periodically and checked for their reactivity against the *trpE*-5′E1 fusion protein by Western blots; rabbits from each group produced high-titer antisera capable of recognizing the fusion protein (data not shown).

Antibodies that specifically recognize the *trpE*-5′E1 fusion protein were purified from the serum of one such positive rabbit, rabbit B (partially purified immunogen) by being applied to a protein affinity column prepared by covalently linking the *trpE*-5′E1 fusion protein to Affi-Gel 15. This purified antiserum showed a 90-fold increase in specific activity as measured by its ability to immunoprecipitate radiolabeled *trpE*-5′E1 fusion protein (data not shown).

To determine whether the reactive antisera could recognize both the 5′E1 and *trpE* domains of the immunogen, the sera were tested by Western blots for their ability to identify proteins from various bacterial extracts. The affinity-purified antiserum from rabbit B not only recognized the fusion protein used as the immunogen (Fig. 2B, lane 8) but also reacted strongly against the truncated *trpE* protein encoded by pATH1 (lane 6). The ability of this serum to recognize the E1 moiety was tested by using an extract containing another fusion protein, pHK411-E1, which consists of the E1 ORF from positions 841 to 2410 fused to a portion of the *E. coli lac* operon encoding β-galactosidase. Because the rabbit B antiserum recognized the E1–β-galactosidase fusion protein (Fig. 2B, lane 12) but not the β-galactosidase protein alone (lane 10), this recognition must be by virtue of the E1 domain. Additional larger and smaller proteins were also recognized to a lesser extent by this serum, and these may be products of proteolysis or could be proteins that have antigenic epitopes in common with the *trpE*-5′E1 fusion protein. The same results were obtained with the antiserum from rabbit C (data not shown).

Identification of a bonafide BPV-1 protein. To identify a virus-encoded protein present in transformed cells, the affinity-purified antiserum from rabbit B was used to immunoprecipitate proteins from extracts of BPV-positive (VI216) and -negative (C127) cells that had been labeled overnight with [^{35}S]cysteine and [^{35}S]methionine. VI216 cells are a recently established cloned line of virus-infected and -transformed C127 cells. Analysis of immunoprecipitated proteins by SDS-polyacrylamide gel electrophoresis followed by autoradiography showed that a protein with an apparent molecular mass of approximately 23 kDa was precipitated from VI216 cell extracts with the immune serum (Fig. 3, lane 2) but not from C127 cell extracts (Fig. 3, lane 1). The same results were obtained when immunoprecipitation experiments were done with antiserum from rabbit C (data not shown).

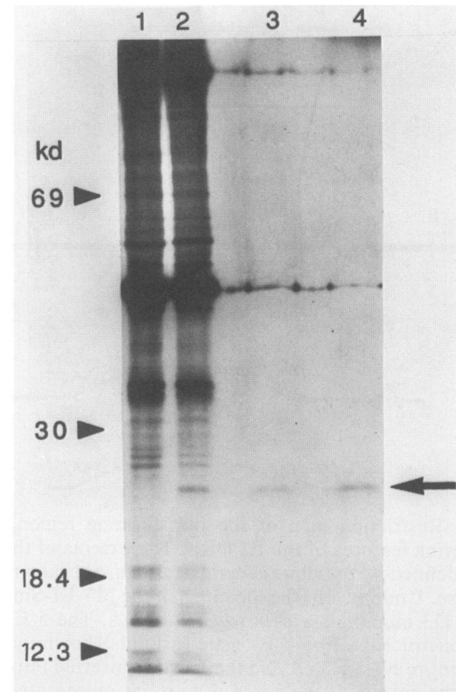


FIG. 3. Autoradiogram showing the M protein after immunoprecipitation from BPV-1-infected and uninfected mouse cells labeled with either ^{35}S or ^{32}P. C127 (lane 1) and VI216 (lane 2) cells were labeled with ^{35}S, and VI216 cells were labeled with ^{32}P (lanes 3 and 4) as described in the text. The amount of ^{32}P extract loaded on the gel was approximately 1/10 the amount of ^{35}S extract loaded so that their signals for M (arrow) would be comparable after 2 weeks of exposure to film. kd, Kilodaltons.

To detect this protein by immunoprecipitation from ^{35}S-labeled extracts, the gels needed to be exposed to film for 2 weeks, suggesting that the polypeptide is present at low levels in BPV-infected cells. Even though we used a two-step immunoadsorption procedure specifically developed to analyze low-abundance polypeptides at low background levels (24), it was still difficult to distinguish this protein band from others that were precipitated nonspecifically (Fig. 3). For this reason and because many virus regulatory proteins are known to be phosphorylated (28, 37), we decided to test whether the protein detected is a phosphoprotein and whether this labeling protocol would increase the sensitivity of the assay. This was done by immunoprecipitating proteins from extracts of cells that had been labeled with $^{32}PO_4$ for 3 h and analyzing the precipitated proteins by SDS-polyacrylamide gel electrophoresis and autoradiography. Examination of the immunoprecipitated proteins revealed only a

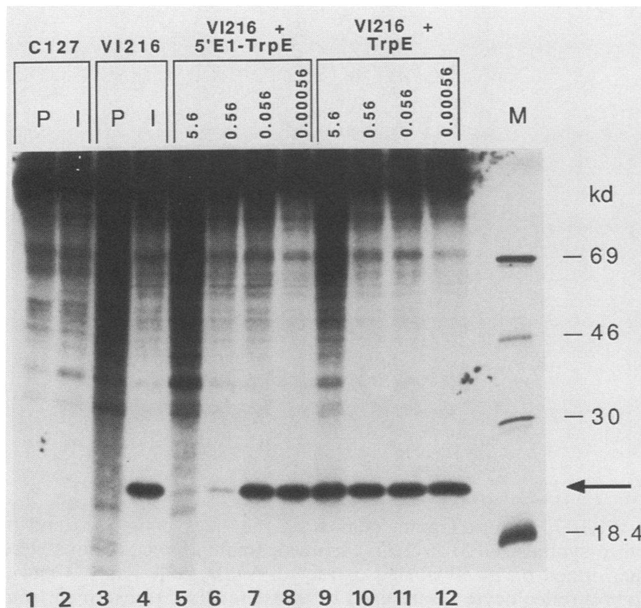


FIG. 4. Autoradiogram showing that M protein immunoprecipitation from BPV-1-infected mouse cells is blocked by preincubation of anti-M serum with the *trpE*-5′E1 fusion protein. Cells were labeled with ^{32}P as described in the text, with one 100-mm-diameter dish of cells which were approximately 80% confluent for each immunoprecipitation. Extracts from C127 (lanes 1 and 2) and VI216 (lanes 3 and 4) cells were immunoprecipitated with preimmune (P) or affinity-purified immune (I) serum from rabbit B. The arrow points to the position of the M protein. Extracts from VI216 cells were immunoprecipitated with this antiserum after it was preincubated with either the *trpE*-5′E1 fusion protein (lanes 5 to 8) or the *trpE* protein alone (lanes 9 to 12). The amounts of *E. coli* protein used in the preincubation are given in micrograms above the corresponding lanes. kd, Kilodaltons.

single major phosphoprotein of approximately 23 kDa present in VI216 cell extracts (Fig. 3, lanes 3 and 4). The presence of the band was not sensitive to RNase treatment but was abolished by predigestion of the extracts with proteinase K (data not shown). This protein was not precipitated with preimmune serum (see below for an example; Fig. 4, lane 3), nor was it precipitated from C127 cell extracts (see below for an example; Fig. 4, lanes 1 and 2). In addition, this phosphoprotein comigrated with the ^{35}S-labeled protein (Fig. 3). With the ^{32}P-labeling protocol, we were able to detect this polypeptide with overnight exposures of the autoradiogram. Thus, the amounts of extracts and immunoprecipitates loaded had to be adjusted to compare the ^{35}S-labeled protein side by side with the ^{32}P-labeled protein (Fig. 3). Data from all subsequent experiments were derived with ^{32}P-labeled protein.

To demonstrate that the antiserum did indeed recognize a BPV-1-encoded product related antigenically to the *trpE*-5′E1 fusion protein, a blocking experiment was performed. This was done by incubating the affinity-purified antiserum from rabbit B with partially purified *trpE* or *trpE*-5′E1 protein before it was used in immunoprecipitation experiments. Blocking with the *trpE*-5′E1 fusion protein inhibited precipitation of the 23-kDa phosphoprotein (Fig. 4, lanes 5 and 6), whereas blocking with the same amount of the *trpE* protein alone had no effect on its precipitation (lanes 9 and 10), demonstrating that the phosphoprotein was specifically recognized by antibodies to the 5′E1 gene product.

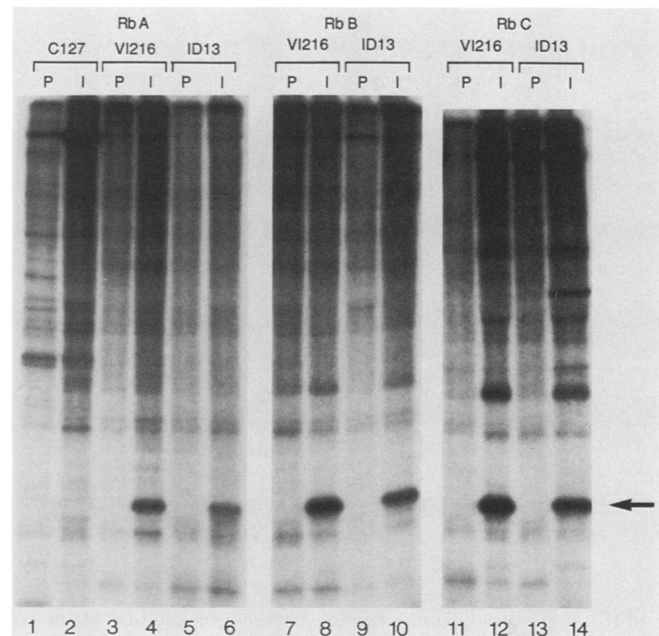


FIG. 5. Autoradiogram showing immunoprecipitation of the M protein from BPV-1-infected mouse cells with anti-M antisera obtained from three different rabbits. ^{32}P-labeled cell extracts from C127 cells (lanes 1 and 2), VI216 cells (lanes 3, 4, 7, 8, 11, and 12), and ID13 cells (lanes 5, 6, 9, 10, 13, and 14) were immunoprecipitated with preimmune (P) and immune (I) sera from rabbits (Rb) A, B, and C. The arrow shows the location of the M protein.

Antisera from two additional rabbits were tested for their ability to precipitate the same 23-kDa phosphoprotein. Rabbit A had been immunized with the *trpE*-5′E1 fusion protein extracted from SDS-polyacrylamide gels. Rabbit C, like rabbit B (whose serum was affinity purified), had been immunized with the partially purified trpE-5′E1 fusion protein. The immune sera from all three rabbits precipitated the 23-kDa phosphoprotein from ^{32}P-labeled extracts of BPV-containing VI216 and ID13 cells (Fig. 5, lanes 4, 6, 8, 10, 12, and 14) but not from extracts of uninfected C127 cells (lane 2 and data not shown). None of the preimmune sera from these rabbits reacted with this phosphoprotein (lanes 3, 5, 7, 9, 11, and 13).

Detection of the 23-kDa phosphoprotein correlates with the genetics of the E1 ORF. The results described above show that antiserum raised against an engineered protein which carries amino acids from the 5′ portion of the E1 ORF recognizes a BPV-1 encoded protein of 23 kDa. This result is consistent with our earlier genetic data which implied that the E1 ORF must contain at least two separate genes (3, 22), since a single protein from the entire ORF would have a minimal apparent molecular mass of 68 kDa. To probe this point further, we transfected cells with BPV frameshift mutants defective in either the *M* gene (5′E1) or the *R* gene (3′E1). We expected that cells infected with BPV plasmids carrying a mutation at the *Eco*RI site in the 3′ portion of E1 (i2113) (22) would still synthesize M, whereas those infected with plasmids carrying a mutation at the *Sma*I site in the 5′ portion of the gene (E1-Sma) (3) would not. Indeed, the affinity-purified antiserum from rabbit B precipitated the 23-kDa phosphoprotein from ^{32}P-labeled extracts of VI216 cells and two different i2113 cell lines, i2113-B and i2113-D (Fig. 6, lanes 4, 8, and 10), but not from extracts of C127

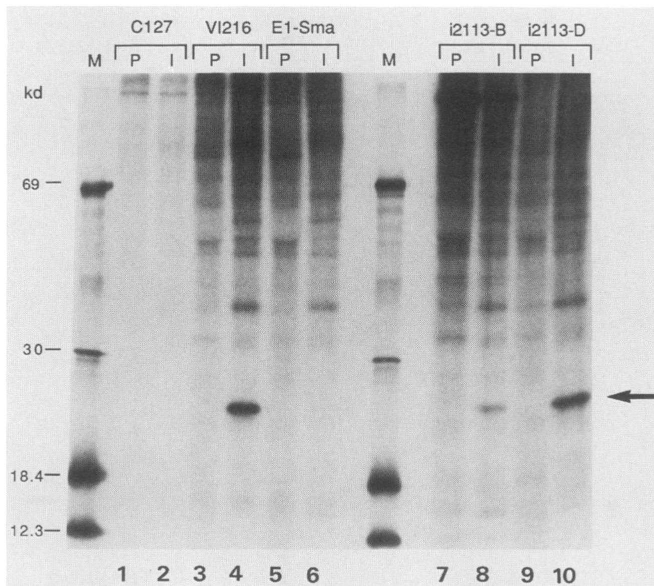


FIG. 6. Autoradiogram showing immunoprecipitation of the M protein from ^{32}P-labeled mouse cells infected with BPV-1 DNAs mutated in the E1 ORF. The lanes marked M contained molecular mass standards. Extracts from C127 (lanes 1 and 2) and VI216 (lanes 3 and 4) cells were immunoprecipitated with preimmune (P) or affinity-purified immune (I) serum from rabbit B. Extracts from C127 cells infected with M^- (E1-Sma; lanes 5 and 6) or R^- (i2113-B and i2113-D; lanes 7 to 10) mutant BPV-1 DNA were immunoprecipitated. The arrow shows the position of the M protein found in the R^- but not the M^- mutants. kd, Kilodaltons.

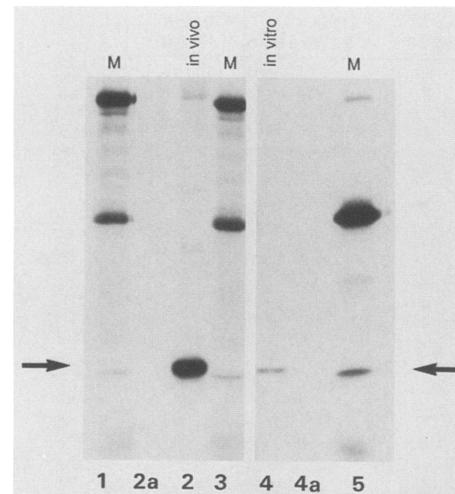


FIG. 7. Autoradiogram comparing the sizes of in vivo and in vitro synthesized M proteins (arrows). Immunoprecipitations were performed on a ^{32}P-labeled extract of VI216 cells (lane 2) and a rabbit reticulocyte lysate used to translate RNA transcribed from the SP6 vector containing the N7-1 cDNA (lane 4). The SP6 vector provides only a short leader, and the first ATG in the RNA occurs at nucleotide 849 in the E1 ORF (Fig. 1). The in vitro product was labeled with [^{35}S]methionine. The samples were electrophoresed on the same SDS-polyacrylamide gel, and the gel was then cut so that the two halves could be treated separately before being exposed to film overnight (the ^{32}P half was exposed with two intensifying screens, and the ^{35}S half was impregnated with Enlightning). The lanes marked M contained molecular mass standards. Preimmune serum was also used in immunoprecipitation reactions with the ^{32}P-labeled extract of VI216 cells (lane 2a) and the reticulocyte lysate containing the ^{35}S-labeled N7-1 translation product (lane 4a).

cells or the E1-Sma cell line (lanes 2 and 6). The corresponding preimmune serum did not recognize this protein in any of the extracts (Fig. 6, lanes 1, 3, 5, 7, and 9). The 23-kDa protein band from i2113-B cells was much fainter than the same band from i2113-D cells, even though comparable amounts of the labeled extracts were immunoprecipitated (for this ^{32}P experiment only, the radioactivity of the samples was measured and equivalent counts of each radioactive sample were immunoprecipitated). This may be because the BPV plasmids in these two cell lines integrated differently and that this has an effect on the expression of *M*.

Translation of the M protein in vitro. Prior genetic analysis indicated that the *M* gene might contain a major exon that started at the beginning of the E1 ORF and that this exon would end at a previously mapped donor site at nucleotide 1235 (36). We have recently obtained a series of different cDNAs which contain this exon as part of what might be polycistronic mRNAs. One such cDNA is indicated in Fig. 1 and is named N7-1. The predicted molecular mass of a protein initiating at the first ATG in the E1 ORF and translating through to the chain terminator just past the acceptor at 3225 (Fig. 1) would be approximately 16 kDa. We were therfore interested to know how the apparent molecular mass of the 23-kDa band described above migrated relative to a protein whose structure would be predicted by N7-1.

N7-1 was cleaved with the restriction enzymes *Nru*I and *Stu*I, and the fragment was ligated into the pSP65 vector (Fig. 1). This placed the first ATG in the E1 ORF just 42 base pairs downstream (3′) of the start site of RNA synthesis by the SP6 polymerase in this vector. Furthermore, the synthetic RNA contains no AUG codons 5′ to the putative E1 initiator codon. Immunoprecipitation experiments were then performed on both the extract containing the in vitro synthesized protein labeled with [^{35}S]methionine and an extract from ^{32}P-labeled VI216 cells. The precipitates were analyzed side by side on the same gel, and the synthetic protein comigrated with the in vivo detected protein (Fig. 7, lanes 2 and 4). We therefore conclude that the protein has an abnormally high apparent molecular mass, since the predicted molecular mass of the synthetic polypeptide is only 16 rather than 23 kDa. Furthermore, it seems that mRNAs with the coding capacity of N7-1 are reasonable candidates for the *M* message. We point out that this mRNA would have a multiple coding capacity.

DISCUSSION

Detection of the regulatory proteins encoded by papillomaviruses has not been possible by technologies successfully used for other transforming viruses (18). This is presumably because the genes for these proteins (important for either transformation or replication) are expressed at very low levels (11) and these proteins may have short half-lives (29). However, in the past few years several of these gene products have been serologically detected by using viral ORFs as the genetic material for production of proteins in *E. coli*, anticipating that viral proteins would share epitopes in common with the synthesized fusion products. Thus, Androphy et al. (1) first used this technique to identify the BPV-1 E6 protein, Schlegel and Wade-Glass (30) identified the BPV-1 E5 protein, and Smotkin and Wettstein (33) identified the human papillomavirus type 16-encoded E7 protein.

Here, we report using this method for detection of a BPV-1-encoded phosphoprotein with an apparent molecular

mass of 23 kDa. Antisera raised against the *trpE* fusion protein precipitated this 23-kDa protein from mouse cells transformed by either BPV-1 virions (ID13 or VI216 cells) or viral DNA. The protein was not detected by antisera in uninfected parental cells (C127 cells) or by preimmune sera. Moreover, precipitation of this phosphoprotein was blocked when the antisera were preadsorbed with the *E. coli*-synthesized *trpE*-5′E1 fusion protein. The molecular mass of the protein was apparently not accurately measured by SDS-polyacrylamide gel electrophoresis. This was deduced from the fact that synthetic RNA transcribed from a potential cDNA for the protein translated efficiently in vitro produced a protein identical in apparent molecular mass to the 23-kDa protein when fractionated by SDS-polyacrylamide gel electrophoresis side by side. However, the actual molecular mass of this in vitro product must have been 16 kDa, as deduced from the known sequence of the cDNA. Furthermore, when a T7 fusion protein made with this cDNA construct was induced in *E. coli* and the protein was analyzed, a protein which also migrated in an anomalously slow manner was detected (data not shown). Finally, we noted that the protein detected by ^{35}S ran as a discrete species and aligned precisely with the ^{32}P product; thus, we view it unlikely that phosphorylation alters the electrophoretic mobility of the primary translation product.

The DNA restriction fragment used to generate the *trpE* fusion protein spanned the region of the E1 ORF that was previously defined as a gene encoding a modulator or repressor of BPV-1 replication: the *M* gene. Antibodies that recognize the amino-terminal domains of the putative E1 protein(s) should, in principle, detect all proteins coterminal with the *M* gene. Our ability to detect a small polypeptide and not a large (≥68-kDa) protein does not preclude the existence of such a protein, since it may be much less abundant than the 23-kDa protein. Another, perhaps more likely, possibility is that such a protein is not expressed in cells harboring viral DNA in a latent state. For example, such a protein may be transiently expressed during productive vegetative replication of the virus.

The genetic data relevant to the E1 ORF show that the ORF can be divided into at least two different complementation groups, both of which are required for establishment or maintenance (or both) of stable plasmid DNAs in transformed cells. However, only the *R* gene or the 3′ two-thirds of the ORF is required for transient plasmid replication. The first methionine in the E1 ORF occurs at nucleotide 849, yet Lusky and Botchan (22) showed that frameshift mutations as far into the E1 ORF as position 1132 had the M^-R^+ phenotype. The results presented in this study are consistent with these genetics. For example, cell lines transformed with M^+R^- mutants express a 23-kDa phosphoprotein, whereas cells transformed by M^-R^+ DNA do not produce this protein. Most importantly, the in vitro translation product of E1 encoded by the cDNA N7-1 comigrates with this 23-kDa protein. The N7-1 cDNA splices from E1 position 1235 to the BPV-1 common acceptor at 3225 and terminates after 13 amino acids. This finding implies that most of the *M* gene is encoded by the 5′ end of the E1 ORF but does not necessarily define the precise amino- or carboxy-terminal amino acids of the protein. This is because we cannot conclude that the N7-1 cDNA represents the only class of RNAs which encode the M protein. For example, we know that the donor at 1235 can splice to E1 acceptors at 1866 or 2558 (J. Choe, P. Vaillancourt, and M. Botchan, manuscript in preparation). These two cDNAs may also represent polycistronic RNAs and could encode for a small protein of almost precisely the same predicted size as that encoded by N7-1. Both of these cDNAs are exceedingly rare compared with the type represented by N7-1. Nevertheless, these RNAs could be used to produce an M protein, particularly in mutant DNAs in which the acceptor at 3225 is destroyed as in the mutant 1039-1 (12) or deleted as in the BAL-15 and BAL-26 mutants (20). In this regard, it is interesting that Roberts and Weintraub (27) defined a *trans*-acting BPV-1 gene that negatively regulated hybrid BPV-1–simian virus 40 origins of replication in COS-7 cells. In that study, it was reported that deletions of all BPV-1 sequences 3′ to the *Eco*RI site at 2113 still encoded a functional negative regulator, yet deletions to the *Bgl*II site at 1515 (which eliminates both acceptors at 1866 and 2558) destroyed such activity. Taken together, these genetic studies imply that the functional domain of the *M* gene lies 5′ to the donor at 1235 and that one simply must have an acceptor 3′ to the donor which can provide a translation terminator in the RNA to produce the functional polypeptide. This, of course, predicts that a mutant with a chain termination mutation at position 1235 in the E1 frame would be M^+. This is currently being tested.

An ambiguity also exists with respect to the 5′-most coding sequences for the *M* gene. Our previous genetic studies identified a region in the upstream regulatory region of BPV-1 as being part of the *M* gene. This genetic analysis could not distinguish between sequences required for protein coding and those required for expression of the functional gene. It was striking that these mutations all fell within a very short coding stretch, and we called this the E9 ORF. This ORF, however, would encode an additional 4 kDa of polypeptide. The in vitro protein product encoded by the N7-1 cDNA is identical to the M product by SDS-polyacrylamide gel electrophoretic analysis. Therefore, given the high resolution of proteins in this region of the gel (we can detect separation between the M protein and fusion proteins made from the N7-1 cDNA containing as little as 1 kDa of protein added to their amino termini), we consider the mutations in the E9 ORF as defining *cis*-acting regulatory elements. This is certainly consistent with the results of Spalholz et al. (34), who have shown that this region of the upstream regulatory region is a principal target for the E2 transactivation of transcription. Nevertheless, the results presented here do not preclude a very short leader exon for the M protein.

Whereas our detection of a small E1-encoded protein substantiates one prediction of the genetic analysis previously reported, many paradoxes remain to be explained. We are primarily concerned with understanding why the large E1 ORF is maintained as it is in all papillomaviruses. It is possible, as suggested above (see also the discussion in reference 4), that the *M* gene represents a fragment of a potent replicator gene which is never expressed in C127 cells. Indeed, expression of *M* is indicated as essential for stable plasmid maintenance and may be part of the apparatus which helps to maintain viral latency; however, this does not preclude the possibility that a larger protein is expressed in vegetative replication. Similarly, the putative R protein encoded by the *R* gene may be a weak or truncated version of another replication protein. The action of the R protein has been shown genetically to be required for initial viral amplification upon entry into cells, but its expression is exceedingly low as measured by RNA levels in stably transformed cells. Finally, the structural and biochemical relationships between the M and R proteins need elucidation. Is M a site-specific DNA-binding protein that competes with the amplification functions of the R protein? We assume

that the serological tools described here will help to answer these questions.

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