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Bovine papilloma virus-transformed cells contain multiple E2 proteins

(gene expression/viral proteins/trans-activation)

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ABSTRACT **Genetic evidence suggests that the bovine papilloma virus type 1 (BPV) E2 open reading frame may encode at least two gene products involved in the regulation of viral gene expression. One, which is probably the full-length product, trans-activates transcription via an enhancer in the viral regulatory region. A second, containing sequences from the 3′ end of the open reading frame, inhibits the trans-activating activity of the first product. We now report the identification and initial characterization of three E2-encoded proteins, with mobilities corresponding to 48, 31, and 28 kDa in cells transformed by the wild-type BPV. Pulse–chase experiments indicated that the 48-kDa protein had the longest half-life (40 min), but there was no indication that one species was the precursor of another. The 48-kDa species corresponds to the full-length trans-activating protein. The two smaller species contain only carboxyl-terminal determinants, and either or both could represent inhibitory E2 proteins. Subcellular fractionation localized all three E2 proteins to the nucleus. Consistent with the low rate of viral transcription in BPV-transformed cells, the 31-kDa presumptive repressor species was more abundant than the 48-kDa species.**

Bovine papilloma virus type 1 (BPV) has been used as a model for studying papillomavirus molecular biology because the virus readily transforms susceptible rodent cells (1, 2). Two genes can independently induce morphologic transformation (E5 and E6; see Fig. 1), and their protein products have been identified (3, 4). Gene products of the E2 open reading frame (ORF) influence BPV-induced transformation by a complex but probably indirect mechanism. Based on genetic analysis of full-length BPV mutants and activated cDNA clones, it has been reported that the E2 ORF encodes both a trans-activator of viral transcription that activates an enhancer in the upstream regulatory region (URR; also called the long control region) and a repressor that inhibits the activity of the E2 trans-activator (5, 6). An intact E2 ORF is required for expression of the trans-activating function but the expression of the repressor function only requires the 3′ half of the ORF (downstream of the methionine at nucleotide 3091). A third function affecting cellular transformation may also be encoded by the 3′ end of the ORF. This presumptive gene product may require coding sequences outside of the E2 ORF, since it cannot be complemented by the full-length E2 gene product and its activity is abolished by a mutation in the splice acceptor at nucleotide 3225 (ref. 7; Fig. 1).

Bacterial fusion proteins containing E2 peptides (9, 10), and also extracts from cultured cells expressing the E2 ORF (11), were recently shown to specifically bind fragments of the URR *in vitro*. Both the binding of E2 proteins to the URR and E2-dependent trans-activation have been correlated with the presence of a motif ($ACCN_6GGT$) that is found in repeated copies in the URRs of all papillomaviruses, suggesting that direct binding of E2 to these sequences may play an important role in E2-mediated enhancement (9, 12, 13). Functional analysis of E2 has indicated that only the 3′ one-third of the ORF is required for specific DNA binding (14) but that both amino- and carboxyl-terminal determinants are required for efficient trans-activation of the URR (T. Haugen, L. Turek, and J.T.S., unpublished data). The repressor function therefore maps to the DNA binding domain of the trans-activator, suggesting that it could repress trans-activation by competitively inhibiting the specific binding of the activator to the URR.

Since the E2 activator acts in trans to enhance BPV transcription and its activity is abolished by frameshift mutations in the ORF, it is likely that an E2-encoded protein is responsible for trans-activation. The fact that complexes between E2 antibodies and bacterial E2 fusion proteins or E2 extracts from cultured cells bind specific URR sequences strongly reinforces the genetic evidence that the trans-activator is a protein product of the E2 ORF. However, it is unclear whether the inhibitory activity detected when carboxyl-terminal E2 cDNAs were activated by heterologous promoters reflects the expression of an E2 inhibitor function by the wild-type viral genome or represents a laboratory artifact resulting from overexpression of a truncated form of E2 that contains a functional DNA binding domain. mRNAs that might encode protein products from the 3′ end of the E2 ORF have been identified (8, 15, 16). However, these messages also contain other ORFs.

Despite the apparent importance of E2 to the regulation of papillomavirus gene expression, no protein product encoded by the ORF has been identified in cells. We therefore sought to identify and characterize the E2-encoded proteins in cultured cells transformed by the wild-type virus. Our results indicate that E2-encoded proteins with the characteristics predicted for both an E2 trans-activator and repressor are present in BPV-transformed cells.

MATERIALS AND METHODS

E2 Antibodies. The generation of rabbit antisera to a bacterial fusion protein containing the carboxyl-terminal two-thirds of the E2 ORF has been described (9). To partially purify the E2-specific antibodies, the immunoglobulins were collected from the antisera in a 30–50% ammonium sulfate fraction, resuspended in water, and then loaded onto a Sepharose 4B column to which purified E2 fusion protein was coupled by using cyanogen bromide. E2 antibody was specifically eluted with 0.1 M acetic acid/0.5 M NaCl, pH 2.5, immediately neutralized, and concentrated in a Centricon 30 filter (Amicon). To generate peptide-specific E2 antibodies, the 12 residues from amino acids 343–353 (Arg-Phe-Arg-Val-Lys-Lys-Asn-His-Arg-His-Arg-Tyr) were synthesized and

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Abbreviations: BPV, bovine papilloma virus type 1; ORF, open reading frame; URR, upstream regulatory region.

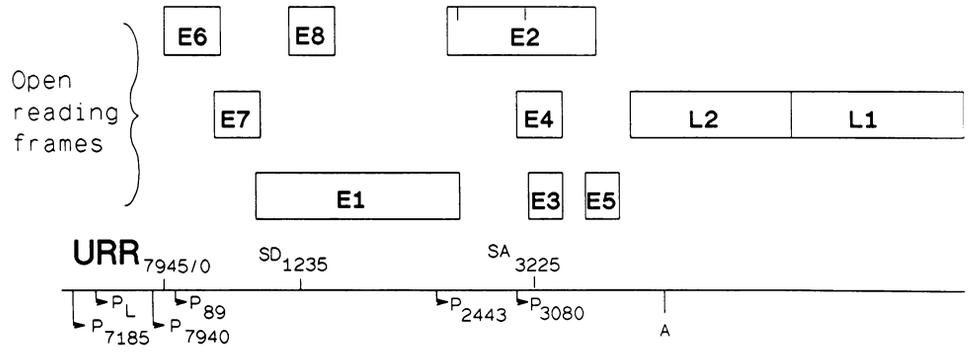

FIG. 1. The linearized BPV genome. E1–E8, early ORFs transcribed in cultured cells; L1 and L2, late ORFs, which are only transcribed in productively infected epidermis. The URR contains several promoters (P), both early (designated by the nucleotide number of the cap site; ref. 8) and late (designated "L"). "A" indicates the polyadenylylation signal used to process the early region transcripts. The splice donor (SD) and acceptor (SA) of the E8-E2 mRNA are shown. Vertical lines in the E2 ORF indicate the first ATG codon and the ATG at nucleotide 3091.

the peptide was coupled with glutaraldehyde to keyhole limpet hemocyanin (Calbiochem). New Zealand White rabbits were immunized by intradermal injection, and the peptide-specific antibodies were recovered by affinity purification as described above. Monoclonal antibodies were generated by immunizing BALB/c mice with purified bacterial E2 fusion protein. After detecting serum antibodies in mice, the splenic lymphocytes were fused to SP2 myeloma cells. The hybridomas were selected by standard procedures (17) and supernatants were screened for antibody production with an ELISA to the bacterial E2 protein (18). Positive clones were expanded and the E2-specific antibodies were recovered from the supernatants by affinity purification.

Immunoprecipitation. Subconfluent monolayers of cells were metabolically labeled with [^{35}S]cysteine (0.17 mCi/ml; 1 Ci = 37 GBq) from Amersham (600 Ci/mmol) for 20 min in cysteine-free medium supplemented with 2% dialyzed fetal calf serum after starving the cells for 30 min in the deficient medium. Lysates were prepared as described (3), with the following changes. The RIPA, RIPA-NS, and RIPA-HS buffers were adjusted to 20 μg of aprotinin per ml (Boehringer Mannheim)/5 mM $MgCl_2$/0.1 mM dithiothreitol. The lysates were precleared with 0.1 ml of protein A-Sepharose (20% slurry; Pharmacia) coated with rabbit serum antibodies to a heterologous bacterially derived fusion protein. The cleared lysates (5–7 $\times$ 10^6 cpm) were then incubated with the E2 antibodies for 45 min on ice. The complexes were collected on protein A-Sepharose for 45 min and then washed three times with RIPA, twice with RIPA-HS, and twice more with RIPA. The complexes were dissociated by boiling in 1% $NaDodSO_4$ and the proteins were subjected to electrophoresis in a 12% polyacrylamide gel. The relative amounts of the immunoprecipitated E2 proteins were determined by scanning the autoradiographs in a Hoefer GS300 densitometer with GS360 software.

Subcellular Fractionation. Cells were fractionated by a modification of a procedure used to determine the subcellular localization of the v-myb oncoprotein (19). All steps were performed on ice or at 4°C. Harvested cells, labeled as described above, were disrupted in hypotonic buffer A (10 mM Tris·HCl, pH 8.0/0.1 mM dithiothreitol/20 μg of aprotinin per ml) by 40 strokes in a glass Dounce homogenizer. After the low-speed centrifugation (600 $\times$ *g* for 10 min), the supernatant (containing the cytoplasm and nonnuclear membranes) was removed and subjected to high-speed centrifugation (100,000 $\times$ *g* for 30 min). Triton X-100 was added to 1% to the high-speed supernatant (cytoplasmic fraction), and the high-speed pellet was solubilized in buffer A containing 1% Triton X-100 (membrane fraction). The pellet from the low-speed centrifugation, containing the intact nuclei, was washed twice in STM (250 mM sucrose/10 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride/20 μg of aprotinin per ml/0.1 mM dithiothreitol), and resuspended in STM containing 0.5% Nonidet P-40 and 0.25% deoxycholate (STM/NID) for 15 min. After centrifugation at 600 $\times$ *g* for 15 min, the supernatant was removed (nucleoplasm fraction) and the pellet, containing the insoluble chromatin and matrix components, was solubilized in STM/NID containing 0.5% $NaDodSO_4$ (nuclear matrix fraction).

Pulse–Chase Experiment. After labeling for 20 min as described above, the cells were washed twice in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 2% dialyzed fetal calf serum and incubated in this medium until they were harvested and lysed as described above; 5–7 $\times$ 10^6 cpm of each lysate was used in the immunoprecipitation reactions.

RESULTS

Generation of E2 Antibodies. To begin the process of identifying E2 proteins in BPV-transformed cells, E2-specific antibodies were generated by three different procedures. First, we induced rabbit antisera directed against a bacterial fusion protein that encoded the carboxyl-terminal two-thirds of the 410-amino acid E2 ORF (counting the initial ATG as amino acid 1). These antibodies had been previously used to identify the specific DNA binding activity of E2 (9). Second, we generated rabbit antisera, designated SP20, against a carboxyl-terminal E2 peptide (amino acids 343–353) after coupling of the synthetic peptide to a hemocyanin carrier. Third, we developed a mouse monoclonal antibody to the bacterial fusion protein described above (see *Materials and Methods* for details).

Identification of E2 Proteins in BPV-Transformed Cells. To identify E2 proteins in cells, we used the three antibody preparations in immunoprecipitation assays of BPV-transformed cell (ID14) extracts after metabolic labeling with [^{35}S]cysteine. As a control, parallel immunoprecipitations of extracts of the parental C127 cells were also carried out. Three proteins were specifically immunoprecipitated from ID14 cells by all three antibodies (Fig. 2) but were not immunoprecipitated by the preimmune sera (data not shown). Based on their mobility in the gel, these proteins were 48, 31, and 28 kDa, and so are designated E2-48, E2-31, and E2-28, respectively. All three species probably contain determinants from the carboxyl-terminal part of the E2, since they were recognized by the antiserum raised against the carboxyl-terminal peptide. The relative amounts of the three proteins immunoprecipitated were similar with each of the three antibody preparations. Based on densitometric scans of the autoradiograph, the relative ratio (after corrections for probable cysteine content) of the E2 proteins was 1:10:3 for E2-48/E2-31/E2-28. The same three proteins were also immunoprecipitated from NIH 3T3 cells transformed by the

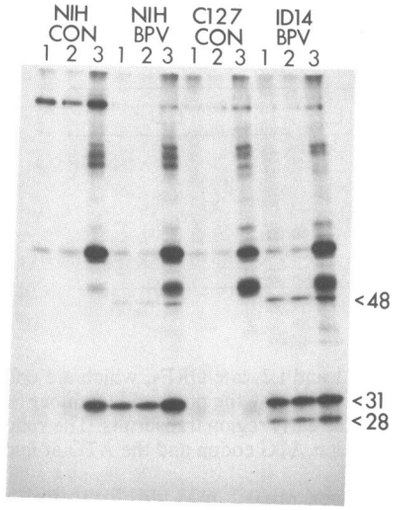

FIG. 2. Identification of E2 proteins in BPV-transformed cells. The proteins immunoprecipitated by three E2 antibody preparations, after separation by $NaDodSO_4$/PAGE, are shown. NIH CON, immunoprecipitation of an NIH 3T3 cell extract; NIH BPV, extract of a clone of NIH 3T3 that was transformed by the cloned BPV genome; C127 CON, extract of control C127 cells; ID14 BPV, extract of a clone of C127 cells that was transformed by infection with BPV. Lanes: 1, immunoprecipitations with rabbit anti-peptide antiserum SP20; 2, mouse monoclonal antibody ME10; 3, rabbit serum to an E2 bacterial fusion protein. Locations and apparent molecular masses of the three E2 specific proteins are indicated on the right (in kDa).

cloned viral genome (Fig. 2), but relative to the other two species, the 31-kDa protein was even more abundant, with a relative ratio of 1:19:1.5 for E2-48/E2-31/E2-28.

As expected for E2-encoded products, the immunoprecipitation of the three proteins by the SP20 peptide antiserum was blocked by preincubation of the antiserum with the E2 bacterial fusion protein but not by preincubation with a BPV E6 bacterial fusion protein (3) that contained the same bacterial leader peptide (Fig. 3). The immunoprecipitation of a fourth protein, with an apparent mass of 60 kDa, was also specifically blocked by the bacterial E2 protein. This protein does not appear to be virally encoded, since it was also immunoprecipitated from the

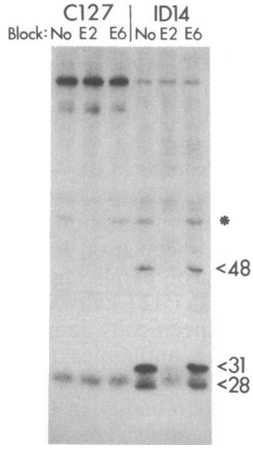

FIG. 3. Immunoprecipitation inhibition experiment. The immunoprecipitation of E2 proteins from control C127 cells and BPV-transformed C127 cells (ID14) after preincubation of the SP20 peptide antiserum without bacterial extract (no), with a bacterial extract containing the E2 fusion protein (E2), or with a bacterial extract containing a BPV E6 fusion protein (E6) is shown. The locations and apparent molecular masses of the three E2 proteins are indicated on the right (in kDa). Asterisk indicates the 60-kDa protein, present in both C127 and ID14 cells, that is specifically blocked by the E2 extract.

control C127 cells and because its migration was unaffected by mutations in the E2 ORF (see below).

To determine genetically whether the three proteins specifically recognized by the E2 antibodies were E2 encoded, we performed immunoprecipitation assays on cell lines transformed by two mutants of the full-length genome carrying alterations in the E2 ORF. Clone 717 was transformed by a mutant genome that has a linker insertion–deletion that is predicted to result in an in-frame deletion of 19 amino acids near the carboxyl terminus of the E2 protein (amino acids 358–377; ref. 20), while clone 112 was transformed by a mutant genome in which a linker insertion introduces a frameshift into the amino terminus of the ORF (at amino acid 121). Three proteins were specifically immunoprecipitated from the 717 cells. When compared with the proteins immunoprecipitated from the wild-type clone, all three species migrated more rapidly, as would be expected if they had the 19-amino acid deletion (Fig. 4; the diffuse background band between 28 and 31 kDa is more prominant in this figure because of increased length of exposure). These results confirm that all three proteins are E2 encoded and that they each contain carboxyl-terminal determinants. E2-48 was not detected in the 112 transformed cells, but both E2-31 and E2-28 were immunoprecipitated. This indicates that E2-48 has amino-terminal determinants but that the two smaller forms probably do not.

Subcellular Localization of E2 Proteins. To determine the location of the E2 proteins in BPV-transformed cells, metabolically labeled ID14 cells were fractionated into membrane, cytoplasmic, and nuclear fractions, and the fractions were assayed by immunoprecipitation with SP20, the antiserum to the carboxyl-terminal E2 peptide. As controls, the parental C127 cells were also fractionated. Nuclei were isolated by low-speed centrifugation after hypotonic lysis, and the supernatant was separated into cytoplasmic and membrane components by high-speed centrifugation (see *Materials and Methods* for details). The nucleoplasm fraction was extracted from the nuclei in isotonic buffer with Nonidet P-40 and

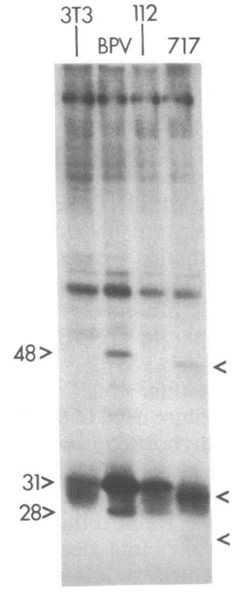

FIG. 4. Immunoprecipitation of E2 proteins from E2 mutant genomes. The immunoprecipitation assays, using the SP20 antiserum, of extracts from control NIH 3T3 cells (3T3), NIH 3T3 cells transformed by the cloned wild-type BPV (BPV), mutant genome 112, and mutant genome 717, are shown. Locations of the three E2 proteins extracted from the wild-type extract are indicated on the left and those from the 717 extract are indicated on the right. In the original autoradiogram, the 28-kDa species is clearly visible with mutant 112. Sizes (in kDa) are indicated on the left.

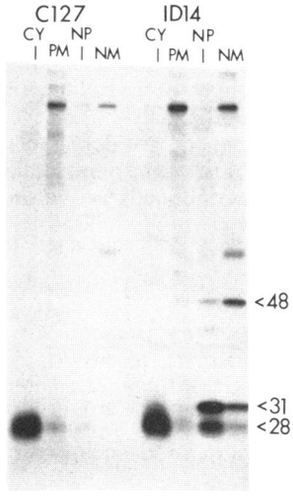

FIG. 5. Subcellular localization of the E2 proteins. The immunoprecipitations of extracts from control C127 and BPV-transformed C127 (ID14) cells, using the SP20 antiserum, are shown. The four fractions are designated cytoplasm (CY), plasma membrane (PM), nucleoplasm (NP), and nuclear matrix (NM). Locations and sizes (in kDa) of the three E2 proteins are indicated on the right.

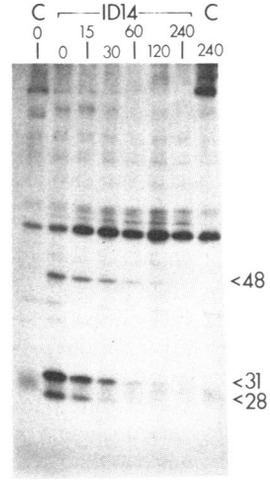

FIG. 6. Pulse–chase experiment. The immunoprecipitation assays, using the SP20 antiserum, of control C127 (C) and ID14 cell extracts are shown. Cells were harvested immediately after the 20-min labeling with [^{35}S]cysteine (0), or 15, 30, 60, 120, or 240 min after addition of medium containing unlabeled cysteine. Sizes (in kDa) are indicated on the right.

deoxycholate, and then the insoluble chromatin and nuclear matrix-associated components were solubilized in 0.5% $NaDodSO_4$. Almost all of the E2 protein in the ID14 cells was detected in the two nuclear fractions, but there was a quantitative difference in the solubility of the proteins (Fig. 5). Approximately three-quarters of E2-48 was associated with the nonionic detergent-insoluble fraction, and one-quarter was in the soluble nucleoplasm fraction. In contrast, only one-quarter of E2-31 and E2-28 was in the insoluble fraction, with the remainder found in the nucleoplasm fraction. A small amount of E2-48 was also detected in the nonnuclear membrane fraction (Fig. 5). The physiological significance of this observation is uncertain since, after a 10-min chase with unlabeled cysteine, this protein was no longer detected in the membrane fraction, perhaps suggesting that the E2-48 associated with this fraction is in transit to the nucleus (data not shown). In the assay shown in Fig. 5, a diffuse protein band that comigrates with E2-28 was immunoprecipitated from the cytoplasm of both the ID14 and control C127 cells, making it difficult to determine whether the 28-kDa protein was also present in the cytoplasmic fraction. In other assays, the comigrating band was diminished and better separated from E2-28, allowing us to determine that very little or no E2 was in the cytoplasmic fraction (data not shown).

***In Vivo* Stability of the E2 Proteins.** To determine the half-life of the E2 proteins in ID14 cells, pulse–chase experiments were carried out. The cells were labeled with [^{35}S]cysteine for 20 min and then chased with medium containing unlabeled cysteine. All three E2-encoded proteins had short half-lives (Fig. 6). The 48-kDa species was more stable than the two smaller species. From densitometric tracing of the autoradiograph, we estimate that E2-48 has a half-life of ≈40 min and E2-31 and E2-28 have half-lives of 10 and 15 min, respectively. This does not suggest, however, that E2-31 or E2-28 is processed into E2-48, since the genetic experiments described above showed that the 48-kDa protein has coding sequences that are not contained in the smaller species. Consistent with the hypothesis that each of the E2 proteins represents a primary translation product, we have observed the same ratio of the three proteins after labeling periods as short as 2.5 min (data not shown).

DISCUSSION

In collaboration with T. Haugen and L. Turek we have conducted a functional analysis of the E2 ORF (unpublished data). It appears that the full-length E2 protein contains at least two domains that are functionally independent: an amino-terminal domain with nonspecific trans-activating activity and a carboxyl-terminal domain with specific DNA binding activity. When linked by a peptide that is not required for either function, the two functional domains combine to generate an E2 enhancer-specific trans-activator.

In this study, we have identified three proteins encoded by the E2 ORF in BPV-transformed cells. We believe that E2-48, the 48-kDa protein, is the E2 trans-activator, since it has an apparent molecular mass close to that predicted for a full-length E2 protein (45 kDa, if the first ATG of the ORF is utilized), and it contains both carboxyl-terminal and amino-terminal determinants. As previously suggested, this protein could be translated from an unspliced message that has a cap site at nucleotide 2440 (14, 15).

We have also identified two smaller E2 species, E2-31 and E2-28, either or both of which could be a repressor protein. Both contain the carboxyl-terminal DNA binding domain but lack at least the central portion of the amino-terminal domain (as defined by the frameshift mutation 112 at nucleotide 2970) that is required for trans-activating activity. We suspect that E2-31 is translated from an unspliced mRNA with a cap site at nucleotide 3080. The fact that this mRNA is relatively abundant in BPV-transformed cells (16) is consistent with our observation that much more E2-31 was immunoprecipitated from the cells than were the other two forms of E2. The relative abundance of this presumptive repressor E2 protein in comparison to the full-length trans-activating E2 protein could, in part, explain why the rate of viral transcription is so low in BPV-transformed cells.

We do not know which mRNA encodes E2-28. It is unlikely that it is a processed product of E2-31, since there was relatively little change in the ratio of the two proteins in the pulse–chase experiment or when short labeling periods were used. It is possible that this less abundant protein is translated from an E8-E2 spliced message, which could encode a protein with 11 amino-terminal amino acids from the E8 ORF fused to the carboxyl-terminal amino acid of E2, starting at the splice acceptor at nucleotide 3225 (J. Choe and M. Botchan, personal communication). This mRNA would

therefore have the potential of encoding an E2 function that might not be complemented by a full-length E2 cDNA (7).

The 3080-nucleotide and E8-E2 spliced mRNAs are predicted to encode proteins of 27.4 and 23.7 kDa, respectively; as is true of the full-length E2, this is 3–4 kDa less than the apparent molecular masses of the two short E2 proteins. We have found that bacterial fusion proteins containing the carboxyl-terminal portion of E2 also migrate slower than predicted (data not shown), suggesting that the DNA binding domain of the E2 protein induces an anomolous migration rate. To show conclusively that the two short E2 proteins are translated from the RNAs described above, mutations that specifically disrupt the transcription or splicing of these mRNAs will have to be introduced into the viral genome. After this manuscript was submitted for review, it was reported that antibodies raised against a bacterial fusion protein encoded by the human papilloma virus type 6 E2 ORF identified the presumed full-length E2 protein product in genital papillomas infected with various human papilloma virus types (21). No shorter forms were detected in that study, and it was not possible to verify the identity of the protein by genetic analysis.

The identification of two potential E2 repressor proteins raises several questions. It seems unlikely to us that the virus would synthesize two different proteins that are functionally equivalent. We therefore speculate that the two proteins may have somewhat different spectrums of activity. Both are predicted to contain the domain that is required for specific binding *in vitro*. Whether they have differences in the relative affinity of binding remains to be determined. In addition to the minimum DNA binding domains, both proteins are predicted to contain an amino-terminal domain of at least 90 amino acids, part of which would be common to both and part of which would be unique to each. An analysis of mutations in the unique domains of these proteins may help determine whether functions in addition to specific DNA binding play a role in the activity of the proteins.

In contrast to the two smaller forms of E2, the majority of the larger, presumably trans-activating, E2 protein was found in the nonionic detergent-insoluble nuclear fraction. Since the amino-terminal trans-activating domain is unique to E2-48, it is likely that this domain is responsible for the difference in solubility. Using similar extraction procedures, others have shown that the nuclear matrix and chromatin, and proteins tightly associated with them, are the major components of the nonionic detergent-insoluble nuclear fraction (19). Therefore, the differential extraction of the full-length E2 may be due to its increased affinity for chromatin, which would suggest that amino-terminal sequences, in addition to the carboxyl-terminal domain required for sequence-specific DNA binding *in vitro*, may be involved in the association of E2 with chromatin. Alternatively, several transcription trans-activators, such as E1a (22), and presumptive trans-activating oncoproteins, such as v-myb (19), have been shown to be associated with the matrix, the nuclear structure thought to be the site of mRNA transcription (23). If the insolubility of the full-length E2 protein is due to its association with the matrix, then the possibility exists that the amino-terminal effector domain enhances transcription, at least in part, by directing or maintaining the E2-bound viral genome at transcriptionally active sites in the nucleus.

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