VIROLOGY 211, 418–433 (1995)

Characterization of Human Papillomavirus Type 16 E2 Protein and Subdomains Expressed in Insect Cells

CYRIL M. SANDERS,* PETER L. STERN,† and NORMAN J. MAITLAND*,[1]

*Cancer Research Unit, Department of Biology, University of York, Heslington, York, YO15DD, United Kingdom; and †CRC Department of Immunology, Paterson Institute for Cancer Research, Christie Hospital, Wilmslow Road, Manchester, M20 4BX, United Kingdom

Received January 31, 1995; accepted June 5, 1995

The E2 open reading frame of human papillomavirus type 16 (HPV-16) encodes a DNA-binding protein which modulates papillomavirus transcription and replication. To investigate the biological and biochemical properties of the HPV-16 E2 protein, we have constructed recombinant baculoviruses which express the full-length molecule and individual N- and C-terminal domains in Sf21 insect cells. In this system the full-length E2 protein was phosphorylated and targeted to the insect cell nucleus. A 93 amino acid C-terminal fragment encompassing the DNA binding and dimerization functions of E2 was also translocated to the nucleus but was not modified by phosphorylation. The E2 N-terminal protein accumulated in the insect cell cytoplasm but was not efficiently phosphorylated. The formation of heterodimers between full-length and N-terminally truncated E2 species was observed when Sf21 cells were co-infected with recombinant viruses and when homodimers were mixed *in vitro*, suggesting that the dimer interface is not sufficiently stable to prevent subunit exchange *in vivo*. Both homo- and heterodimeric E2 species were able to bind specifically and in any combination to tandem E2 binding sites from the HPV-16 regulatory region. Furthermore, the HPV-16 E2 protein bound to DNA exhibited a distinct susceptibility profile to pronase digestion, potentially contrasting with that reported for BPV-1 E2. These observations suggest that significant structural and functional differences may exist between the BPV/HPV E2 proteins and have implications for understanding E2-dependent regulation of transcription and replication.

INTRODUCTION

The papillomaviruses are a related group of epitheliotropic DNA viruses that are established in many hosts, including man, where over 70 types have been characterized (De Villiers, 1994). The human papillomaviruses (HPVs) have been grouped for convenience into those that predominantly associate with either cutaneous or mucosal sites (De Villiers, 1989). Included in the latter group are the genital HPVs which have been implicated in the aetiology of benign and malignant lesions of the anogenital tract (zur Hausen, 1991a and 1991b). The so called "low risk" types, which include HPV 6 and 11, are commonly linked with condylomas and intraepithelial neoplasia which rarely progress to malignant disease. By contrast, approximately 80% of frank cervical carcinomas are associated with one or other of the "high risk" viral types 16, 18, 31, and 33, but most commonly HPV-16 (Cullen *et al.*, 1991; reviewed in Lowy *et al.*, 1994). Distinct changes in the physical state of the viral chromosome and transcriptional activity accompany the transformation process. Integration of viral DNA into the host genome often leads to disruption of the viral E1 and E2 open reading frames (ORFs), and selective retention and expression of ORFs E6 and E7 (Schwarz *et al.*, 1985; Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986; Baker *et al.*, 1987; Shirasawa *et al.*, 1988). The E6 and E7 proteins are considered to be the principal viral contributors to the oncogenic process (Hawley-Nelson *et al.*, 1989; Chen *et al.*, 1993; Lambert *et al.*, 1993), since they associate with the cellular tumor suppressers p53 and Rb, respectively (Werness *et al.*, 1990; Dyson *et al.*, 1989), and have *in vitro* cell transforming activity.

The genomes of all papillomaviruses are circular molecules of approximately 8 kb, composed of early and late gene loci, separated by an 800-bp control region, the upstream regulatory region (URR), which contains many of the sequences regulating transcription and replication (Chen *et al.*, 1982). The URR of HPV-16 has a single major transcription initiation site, P97, at its 5′ end, adjacent to the E6 and E7 ORFs (Smotkin and Wettstein, 1986). Within the (HPV-16) URR a keratinocyte-dependent enhancer is positioned upstream of a promoter proximal E2 responsive region, which contains three recognition sites for the viral E2 DNA binding protein (Cripe *et al.*, 1987; Bedrosian and Bastia, 1990). Two binding sites are in a tandem array and overlap essential promoter elements, an SP-1 motif and the TATA-box (Gloss and Bernard, 1990). Together with a third motif they encompass, or are within, a putative origin of replication, characterized

[1] To whom correspondence and reprint requests should be addressed. Fax: (0904) 432615.

0042-6822/95 $12.00

for the closely related virus HPV-18 (Remm *et al.*, 1992). A fourth E2 binding site marks the distal end of the cell-specific enhancer.

The papillomavirus E2 protein is a modulator of papillomavirus transcription and replication. E2 binds to the general palindromic consensus ACC(N)$_6$GGT (Androphy *et al.*, 1987; Moskaluk and Bastia, 1987), repeated a number of times in the genomes of all papillomaviruses sequenced to date. The arrangement of E2 sites is conserved between viruses within a particular subgroup, but differs for distantly related viral types (Dartmann *et al.*, 1986; Guido *et al.*, 1992). Conservation of the papillomavirus E2 proteins appears low at the primary sequence level, but becomes more marked and apparent at the level of secondary structure and function. Three distinct functional domains have been defined: A C-terminal DNA-binding and dimerization domain is separated from the N-terminal "transactivation" domain by a flexible proline rich "hinge." The N-terminal domain is more extensively conserved than the C-terminus, and the hinge shows little conservation overall, being highly variable in both sequence and length (Giri and Yaniv, 1988).

The bovine papillomavirus type 1 (BPV-1) protein has been well characterized structurally and functionally. The protein is a 48-kDa nuclear phosphoprotein (Meneguzzi *et al.*, 1989; McBride *et al.*, 1989a), which dimerizes spontaneously in the absence of DNA (McBride *et al.*, 1989b). Dimers are stable and highly resistant to urea denaturation (Prakash *et al.*, 1992; Corina *et al.*, 1993), and can be reduced to a small protease-resistant core which retains the ability to bind DNA (Dostatni *et al.*, 1988). The full-length BPV-1 E2 protein purified from insect cells infected with a recombinant baculovirus binds to a single E2 palindrome with a K_d of 2×10^{-11} *M*, and two full-length dimers bind cooperatively to two adjacent motifs (Monini *et al.*, 1991). The crystal structure of the C-terminal DNA-binding/dimerization domain of BPV-1 E2 has been solved to 1.7 Å and reveals a novel β-barrel structure with many individual contacts contributing to stability at the dimer interface. Upon DNA-binding, the target sequence is distorted to accommodate multiple protein–DNA interactions within a novel DNA-binding motif (Hegde *et al.*, 1992).

The functional role of the BPV-1 E2 protein in the control of the viral life cycle is comparatively well understood relative to its HPV counterparts. It is capable of strong synergistic activation of chimeric promoters bearing E2 recognition motifs in transient transfection assays (Harrison *et al.*, 1987; Hawley-Nelson *et al.*, 1988; Spalholz *et al.*, 1988; Thierry *et al.*, 1990), and will activate transcription *in vitro* of minimal promoters bearing E2 sites (Ushikai *et al.*, 1994). These properties are shared with the E2 proteins of some HPV types (Phelps and Howley, 1987; Ushikai *et al.*, 1994). The full-length BPV-1 E2 protein is an activator of the BPV E2 conditional enhancer (Spalholz *et al.*, 1985; Haugen *et al.*, 1987, 1988; Hermonat *et al.*, 1988; Spalholz *et al.*, 1991). Several BPV promoters are influenced by E2 activity, and specific regulation possibly results from the antagonizing activity of the full-length activator and truncated C-terminal repressor (E2-C) species which lack the N-terminal activation domain (Lambert *et al.*, 1987; Choe *et al.*, 1989). The existence of naturally occurring E2 repressor proteins, analogous to those of the bovine system, is as yet undefined for the human papillomavirus group. However, rare mRNA species with the potential to encode E2-C repressors have been isolated, and the activity of their products determined in heterologous systems, for the low risk genital virus HPV-11 (Rotenberg *et al.*, 1989; Chiang *et al.*, 1991), as well as HPV-16 (Doorbar *et al.*, 1990; Bouvard *et al.*, 1994). In addition to its ability to interact with the host transcription apparatus, BPV-1 E2 is able to complex with the viral E1 protein, a nuclear ATPase/helicase (Santucci *et al.*, 1990; Blitz and Laimins, 1991; Yang *et al.*, 1993), and to stimulate papillomavirus DNA replication (Yang *et al.*, 1991, Ustav and Stenlund, 1991). The E1–E2 complex of BPV-1, as well as that of HPV-11, associates with the viral origin of replication *in vitro* (Mohr *et al.*, 1990; Bream *et al.*, 1993).

Although sequence and structural homology predictions for HPV-16 E2, the model BPV-1 E2, and related human papillomaviruses suggest that replication and transcription functions are conserved, the differences between the human and bovine viruses are sufficiently pronounced to warrant an independent definition of biological and biochemical E2 activity in each native viral system. Indeed many of the properties of the HPV-16 E2 protein are inferred or predicted based on studies with BPV E2.

In this study, the baculovirus expression system has been used to produce full-length HPV-16 E2 and subdomains in insect cells for biological and biochemical characterization. We have analyzed protein phosphorylation and intracellular protein targeting by immunofluorescence. We show that E2/C-terminal heterodimers will form both *in vivo* and *in vitro*, and that all homo- and heterodimeric E2 species retain the ability to bind tandem E2 recognition sites from the HPV-16 enhancer. Also, the HPV-16 E2 protein shows a distinct protease sensitivity profile, potentially contrasting that of the BPV-1 protein.

MATERIALS AND METHODS

Plasmid constructions

The nucleic acid sequences encoding the full-length HPV-16 E2 protein and subdomains were amplified from the reference viral clone (Seedorf *et al.*, 1985), kindly supplied by H. zur Hausen and E-M. de Villiers (German Cancer Research Center, Heidelberg), by polymerase

chain reaction (PCR) and inserted directly into the TA-cloning vector pT7Blue (Novagen). PCR primers incorporated restriction enzyme sites for nucleic acid manipulations, and in frame start and stop codons for translation of appropriate subdomains. PCR primers for the full-length E2 protein were 5′-CAAGACGTGCGCTAGCAT-ATGGAGACTCTTTGCCA-3′ and 5′-AGCAGCAAGTGGATCCGCTAGC-AGCACGCCAGTAATGTT-3′. For the N-terminal domain construct the downstream primer was 5′-CAGCAAGTGGATCCGCTAGCTAA-CTTAGTGGTGTGGCAGG-3. For the C-terminal constructs upstream primers were 5′-CAAGACGTGCGCTAGCATATG-TGCCACACCACTAAGTT-3′ (long C-terminus) and 5′-TAAGACTCCATGGCACATATG-GGACGGATTAACTGTAATAGT-3′ (short C-terminus). Appropriate restriction enzyme fragments were ligated into the baculovirus transfer vector pVL941 which had previously been modified by insertion of a polylinker in both orientations at the unique *Bam*HI site (5′-GATCGCTAGCCTGCAGTCTAGAGGATCCCCCGGGGAGCTC-3′ upper strand, 5′-GATCGAGCTCCCCGGGGGATCCTCTAGACTGCAGGCTAGC-3′ lower strand) and cleaved with the restriction enzymes *Nhe*I and *Bam*HI. The restriction fragments containing full-length E2, N-terminal E2, and the long C-terminal ORFs were the products of *Bam*HI digestion and limited *Nhe*I digestion of the appropriate pT7Blue clone. Manipulations resulted in the generation of plasmids pVLE2 (full-length molecule), pVLE2NT (N-terminus), pVLE2LCT (long C-terminus), and pVLE2SCT (short C-terminus), which contained the polyhedrin promoter sequences upstream of the translation initiation codons (*Fig. 1*). The entire nucleic acid sequence of the open reading frames was verified by *Taq* cycle sequencing (USB Corp.).

Cell lines, production of recombinant baculoviruses, viral infection, and propagation

Spodoptera frugiperda Sf21 cells were grown in Grace's insect medium supplemented with 10% fetal calf serum (Gibco BRL) and maintained at 27°. The cells were also adapted to grow in the serum free medium EX-CELL 401 (Sera Lab) and were routinely transferred from monolayer to spinner culture. Recombinant baculoviruses were generated by cotransfection of recombinant plasmids with linearized BaculoGold AcMNPV DNA (PharMingen), according to the manufacturers' instructions. General techniques for the propagation and characterization of baculoviruses were taken from Summers and Smith (1987) and King and Possee (1992). Clonal viral stocks were generated by two rounds of plaque purification. Viral nucleic acid was characterized by restriction enzyme digestion to verify the nature of the allelic replacement reaction. Recombinant viral stocks were designated rvE2 for the virus containing full-length E2, rvE2NT for E2 N-terminus, and rvE2LCT and rvE2SCT for the long and short C-terminal ORF's, respectively. For protein production, Sf21 cells were infected at a multiplicity of infection (m.o.i.) of 10 or greater and maintained at 27° for 48 hr.

Subcellular fractionation and protein analysis

Forty-eight hours postinfection, cells (typically 1×10^8) were pelleted, washed in ice-cold phosphate-buffered saline (PBS), pelleted, and resuspended in five packed cell volumes (PCVs) of hypotonic buffer (20 m*M* Tris–HCl, 5 m*M* KCl, 5 m*M* $MgCl_2$, 2 m*M* DTT, 1 m*M* EDTA, 0.5 m*M* PMSF, 10 m*M* leupeptin, pH 7.9, at 4°). Following a 10-min incubation at 4°, cells were pelleted (300 *g*, 4°, 10 min), resuspended in 2.5 times the original PCV of hypotonic buffer, and lysed by homogenization (B pestle, 10 strokes). The homogenate was centrifuged for 2 min at 15 000 *g*, 4°. The pellet of membrane bound material (referred to as the nuclear fraction) was washed with 2.5 PCVs of hypotonic buffer and sedimented as above. The supernatant from the high speed spin (referred to as the cytoplasmic fraction) and wash were pooled and adjusted to 300 m*M* NaCl. The nuclear fraction was evenly resuspended in three PCVs of nuclear extraction buffer (NEB; 25 m*M* HEPES, 300 m*M* NaCl, 1 m*M* EDTA, 5 m*M* DTT, 1% NP-40, 10% glycerol, 0.5 m*M* PMSF, 10 m*M* leupeptin, 1 m*M* pepstatin, pH 7.9, 4°) and incubated on ice for 30 min. Extracts (cytoplasmic and nuclear extract) were spun for 90 min at 150,000 *g*, 4°. Supernatants were dialyzed overnight against NEB containing 250 m*M* NaCl and 20% glycerol. Protein concentrations were determined using the Bio-Rad assay.

Protein samples were electrophoresed on 10 or 12% (E2 and N-terminus) and 15% (C-terminal domains) SDS–polyacrylamide gels containing 5.5 or 5 *M* urea and visualized by staining with Coomassie brilliant blue.

Antisera and immunoblot analysis

The N-terminal and short C-terminal domains of E2 were expressed in *Escherichia coli* using a recombinant bacteriophage T7 polymerase system and purified to homogeneity by standard chromatographic techniques. The E2 domain amino acid coordinates were identical to those described above for the baculovirus constructs. Expression and purification of the C-terminal domain has been described (Sanders and Maitland, 1994), procedures for the N-terminal domain will be reported elsewhere. N- and C-terminal specific antisera were prepared by injecting New Zealand White rabbits subcutaneously with 800 μg of protein in PBS emulsified in an equal volume of Freund's incomplete adjuvant and subsequently boosted once or twice at monthly intervals with 800 μg of antigen in PBS. The animals were bled 10 days after immunization.

For Western blot analysis, protein samples corre-

sponding to 5×10^4 cells from each of the subcellular fractions prepared as described above were separated on acrylamide gels and transferred to nitrocellulose membranes (Hybond-ECL, Amersham Life Science). Filters were blocked with 10% nonfat dried milk (Marvel) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) overnight at 4° prior to incubation with anti-E2 polyclonal antisera diluted 1:2500 in TBS-T 5% dried milk. (1 hr at room temperature). Membranes were then washed for a total of 30 min in a large excess of TBS-T with several changes of buffer. Blots were then incubated as described above with secondary peroxidase-labeled anti-rabbit antibody (1:2000 dilution), washed, and developed using a chemiluminescent detection system (ECL, Amersham Life Sciences), as described by the manufacturers.

Metabolic labeling of cells

At 38 hr postinfection mock-infected, wild-type Ac-MNPV, and recombinant virus-infected cells (2.5×10^6 cells grown in serum-free medium) were incubated for 90 min in phosphate free medium (EX-CELL 401 minus phosphate) prior to a 6 to 7 hr incubation in phosphate-free medium supplemented with [^{32}P]orthophosphoric acid (NEN; 8500–9120 Ci/mmol) at 100 mCi/ml. After metabolic labeling, cells were washed twice in ice-cold phosphate-buffered saline and resuspended in 200 μl of NEB buffer, described above, containing EDTA to 10 m*M*, 10% glycerol, and the protease inhibitor E64 to 10 m*M*. After a 30-min incubation at 4°, cell debris was pelleted (20 min, 20,000 *g* at 4°). Supernatants were recovered and proteins analyzed on polyacrylamide gels as crude lysates and specific immunoprecipitates.

Immunoprecipitations

All procedures were carried out at 4°. Preimmune sera and anti-HPV-16 E2 N- and C-terminal specific antisera were bound to protein A sepharose beads (Sigma) for use in immunoprecipitations. Protein extracts from metabolically labeled cells were diluted with an equal volume of 25 m*M* HEPES (pH 7.9 at 4°), 10 m*M* EDTA, with protease inhibitors, to adjust the final salt concentration of the extract to 150 m*M*. Lysates were cleared with preimmune serum prior to addition of the protein A sepharose-bound antisera. After a 4-hr incubation, immobilized immune complexes were recovered by centrifugation, washed twice with modified NEB buffer (25 m*M* HEPES, 150 m*M* NaCl, 10 m*M* EDTA, 2.5 m*M* DTT, 0.5% NP-40, and 5% glycerol, pH 7.9, at 4°) and once with 25 m*M* HEPES (pH 7.9 at 4°), 10 m*M* EDTA, 0.1% NP-40 with protease inhibitors. Bound proteins were eluted into sample buffer and analyzed on polyacrylamide gels as described above.

Immunofluorescence

At 48 hr postinfection, cells were pelleted, washed twice in serum-free medium, and 2.5×10^4 cells were spotted onto poly-L-lysine-coated slides. After attachment, slides were immersed in periodate-lysine-paraformaldehyde (PLP) fixative (McLean and Nakane, 1974) for 50 min at room temperature and washed extensively in PBS. Membranes were permeabilized by incubation in PBS containing 0.5% Triton X-100 for 2 min. Primary anti-E2 antibodies were diluted 1:400 in PBS 0.01% Triton X-100 and incubated with fixed cells for 1 hr. Washes were at room temperature and antibody incubations at 37°. Unbound antibody was removed by washing extensively in PBS 0.01% Triton X-100, prior to reaction with secondary anti-rabbit FITC-conjugated antibody (Sigma). Cells were washed as above, mounted in Vectashield mounting medium (Vector Laboratories), and viewed by phase contrast and fluorescence microscopy.

DNA binding assays and pronase treatment

Binding and electrophoresis conditions for gel shift assays were carried out as previously described (Sanders and Maitland, 1994). Binding reactions contained nuclear protein extract at a final concentration of 2.5 to 5 ng/μl and a 30-base pair ^{32}P-labeled probe with a single E2 binding site from the HPV-16 enhancer at a final concentration of 1×10^{-10} *M* (5′-ACCGAAATCGGT core motif). A second nucleic acid probe bearing tandem E2 binding sites was also derived from the HPV-16 promoter. A 77-base pair fragment from nucleotides 24 to 100 was amplified by PCR using genome-specific primers and cloned into the vector pT7Blue (Novagen). Probe was released from the resulting clone by digestion at the flanking vector restriction endonuclease sites (*Bam*HI and *Nde*I) and labeled to high specific activity by incorporation of [α-^{32}P]dCTP via the Klenow fill in reaction.

To investigate subunit mixing between full-length E2 and N-terminally truncated E2 homodimers, nuclear lysates were mixed in equal proportions in a buffer of final composition 20 m*M* HEPES, 125 m*M* NaCl, 50 m*M* KCl, 0.6 m*M* EDTA, 0.75% NP-40, 0.5 m*M* PMSF, 10 m*M* pepstatin (protease inhibitors added fresh), pH 7.6, and incubated at 37° for 30 or 60 min. As controls, unmixed lysates were incubated similarly.

For pronase digestion experiments, nuclear protein extracts were diluted to 50 ng/μl in 1× DNA-binding buffer and digested with an equivalent mass of pronase E (Sigma) at 37° for various times before or after DNA binding. Reactions were analyzed by electrophoresis.

RESULTS

Expression of HPV-16 E2 and subdomains in insect cells

Sequences encoding the entire E2 protein and subdomains were amplified by polymerase chain reaction and

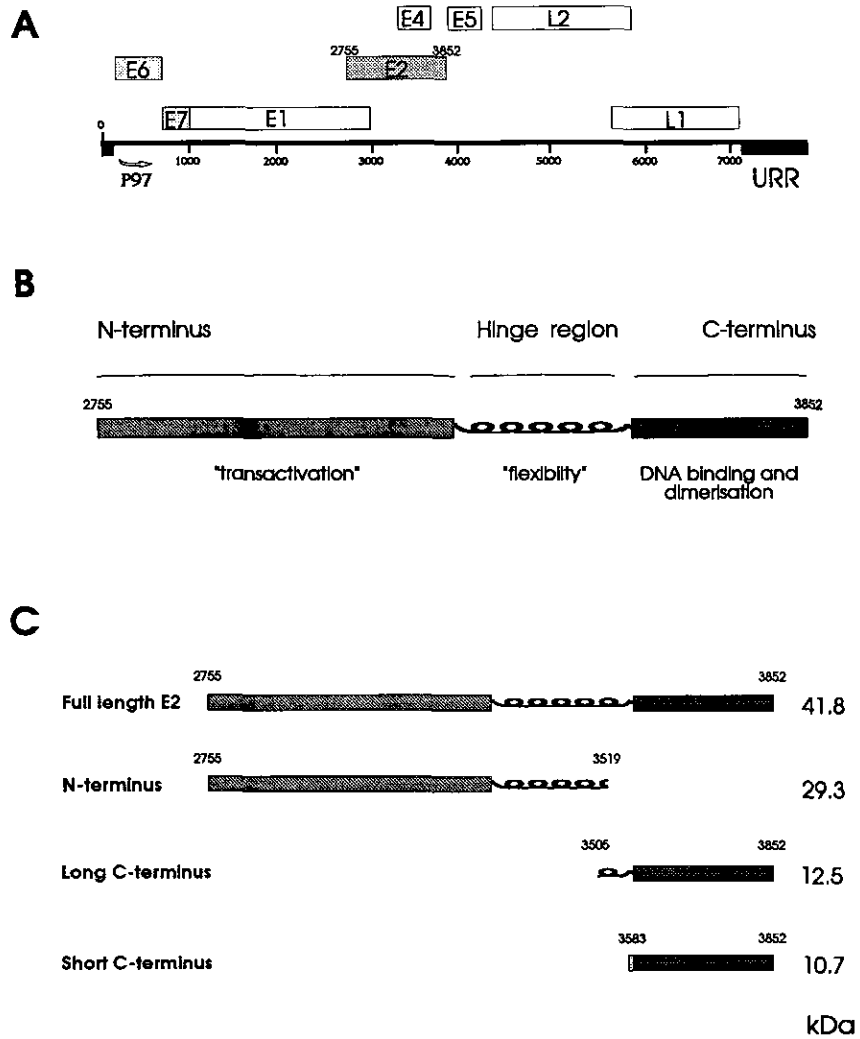


FIG. 1. Structure and derivation of E2 open reading frames. (A) Physical map of the HPV-16 genome showing the location of the major ORFs and the viral promoter/enhancer. The position and coordinates of the E2 ORF are indicated. (B) Structure and function of the E2 protein. By analogy with its BPV-1 counterpart, the HPV-16 E2 protein is shown to be composed of three distinct domains. An N-terminal "transactivation" domain, a central flexible hinge, and a C-terminal DNA-binding/dimerization domain. (C) Nucleotide coordinates and the relationship between the E2 ORF and subdomains expressed in this study. The predicted molecular mass of each protein is also given. The long C-terminus and N-terminal ORFs were constructed so that the entire E2 ORF could be represented as two distinct molecules.

inserted directly into the TA-cloning vector pT7Blue. The nucleotide coordinates of each ORF are given in *Fig. 1.* The full-length E2 protein, the core DNA-binding/dimerization domain, and the entire protein as two overlapping polypeptides (E2NT and E2LCT) were represented in the molecular constructions. ORFs were subcloned into the modified baculovirus transfer vector pVL941 and the entire sequence of the coding regions was verified by nucleic acid sequencing. Only clones with 100% sequence identity (at the predicted amino acid level) to the reference clone were chosen for viral construction. Recombinant viruses were generated by cotransfection, the allelic replacement reaction resulting in viruses containing E2 ORFs under the control of the polyhedrin promoter. Clonal isolates were generated, and the structures of the modified viral genomes were verified by PCR and restriction enzyme analysis coupled with Southern blot hybridization of viral DNA to E2-specific probes (data not shown).

Viral isolates were screened for protein production by SDS–PAGE and staining with Coomassie blue. Protein samples were prepared by cell fractionation, and cells infected with recombinant viruses were compared to wild-type and mock-infected cells. A 45-kDa protein was readily detected in the nuclear protein fraction from cells infected with rvE2 and a 35-kDa protein in the cytoplasmic fraction from cells infected with rvE2NT. These values are close to the predicted molecular mass of the E2 and E2 N-terminal proteins (*Fig. 2*). Both E2 and the N-terminal domain appeared to be entirely soluble. A

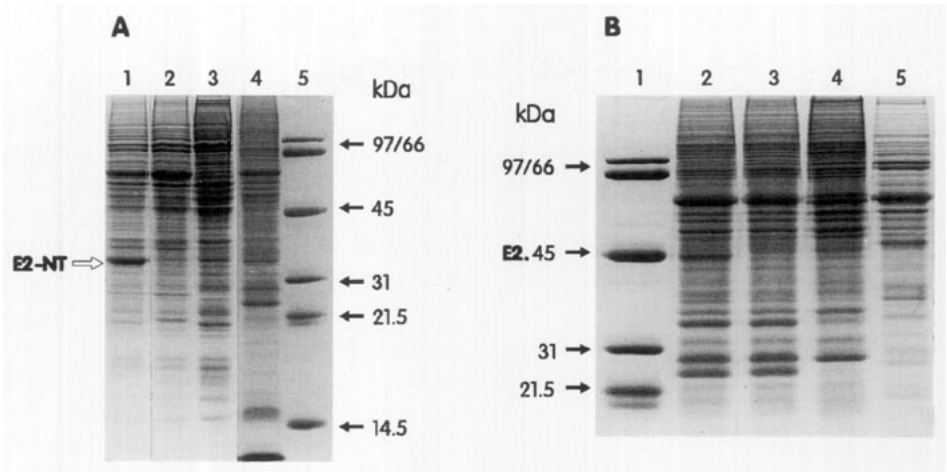


FIG. 2. Visualization of E2 and N-terminal E2 proteins from cells infected with recombinant baculoviruses rvE2 and rvE2NT. Sf21 insect cells were infected with virus, and nuclear and cytoplasmic fractions were prepared 48 hr postinfection. Proteins were separated on 12% SDS–polyacrylamide gels containing 5 *M* urea and stained with Coomassie blue. (A) E2 N-terminal expression. Lane 1, Cytoplasm, rvE2NT infected; Lane 2, cytoplasm, wild-type AcMNPV infection; Lane 3, cytoplasm, mock-infected cells; Lane 4, nuclear fraction, rvE2NT infected; Lane 5, molecular weight marker. The position of the 35-kDa N-terminal product is indicated. (B) Full-length E2 expression. Lane 1, molecular weight marker; Lane 2, rvE2 infection, nuclear fraction; Lane 3, wild-type AcMNPV infection, nuclear fraction; Lane 4, mock-infected cells, nuclear fraction; Lane 5, cytoplasmic fraction, rvE2 infection. The 45-kDa E2 protein was present in the nuclear fraction of infected cells.

product corresponding to the short C-terminal protein could not be easily detected in denaturing gels stained with Coomassie blue, and the long C-terminal protein was undetectable.

Confirmation of expression and protein identity was achieved by Western blot analysis using polyclonal antisera (*Fig. 3*). An N-terminal 35-kDa protein and the short C-terminal DNA-binding and dimerization domain were expressed in *E. coli*, purified to homogeneity by conventional nondenaturing chromatography, and used to raise anti-N-terminal (αNT) and anti-C-terminal (αCT) E2 domain-specific antisera. αNT confirmed the identity of the putative E2 and N-terminal expression products detected in Coomassie blue-stained gels. αCT antiserum readily detected E2 and the short C-terminal expression product in nuclear protein fractions from cells infected with recombinant viruses, but not controls. No expression product corresponding to the long C-terminal protein could be detected by αCT in the representative soluble nuclear protein fraction. No cross reactivity for E2 species was observed between the domain-specific antisera or with host or viral proteins. Preimmune sera were unreactive.

Phosphorylation of the E2 protein

Both the E2 proteins of BPV-1 and HPV-11 have been shown to be phosphorylated in the insect cell expression system. However, there seems to be no conservation of potential phosphorylation sites (Bream *et al.*, 1993). To determine whether the E2 protein of HPV-16 is phosphorylated in Sf21 cells, we metabolically labeled cells with [^{32}P]orthophosphoric acid following infection with recombinant viruses rvE2, rvE2NT, and rvE2SCT. After labeling, soluble whole cell protein extracts were prepared and specific E2 products immunopurified using antisera coupled to protein A sepharose beads. Whole cell protein extracts and immunopurified proteins were analyzed by SDS–PAGE and Coomassie blue staining to confirm expression and recovery. Gels were dried and subjected to autoradiography (*Fig. 4*).

The full-length E2 protein was clearly a target for kinase activity and labeled protein corresponding to the expected E2 product could be clearly distinguished when whole cell protein samples were analyzed by gel electrophoresis and autoradiography (*Fig. 4A*). However, phosphorylation of the individual N- and C-terminal domains was undetectable under the same conditions. Results were confirmed by analysis of the immunopurified products, where the incorporation of ^{32}P could be compared to the recovery of protein. Phosphorylation of the full-length E2 protein could be detected in short exposures to autoradiography film, and evidence of minor phosphorylation of the N-terminal domain was apparent upon extended exposure. Based on densitometry measurements, we estimate that the full-length E2 protein had incorporated approximately 20 times more label than a similar quantity of the N-terminal domain alone. The short C-terminal E2 protein was not phosphorylated as judged by extended autoradiography of immunopurified protein resolved by electrophoresis (*Fig. 4B*). The long C-terminal domain was not tested on account of the low expression achieved in this system.

Intracellular localization of the E2 protein

Cell fractionation experiments showed that only the E2 N-terminal domain could be recovered from the cyto-

plasm of virally infected cells, whereas the full-length E2 and C-terminal DNA-binding/dimerization domains were located in the nuclear components of the cell. To confirm the expected nuclear localization of E2 and the C-terminus, cells were fixed 48 hr postinfection using PLP fixative, permeabilized with Triton X-100, and incubated with E2-specific antisera, followed by FITC-conjugated secondary antibody. Cells were analyzed by phase contrast (data not shown) and immunofluorescence microscopy *(Fig. 5).* Specific nuclear fluorescence could be detected for rvE2SCT- and rvE2-infected cells, but intense fluorescence of the entire cell for rvE2NT infections. The latter result would be expected for an abundant cytoplasmic antigen where cells are fixed by a nondehydrating technique that preserves cellular architecture.

DNA-binding and dimerization

To assess the ability of the full-length HPV-16 E2 protein to form heterodimers with the N-terminal-truncated DNA-binding/dimerization domain, insect cells were co-infected with viruses rvE2 and rvE2SCT and protein extracts prepared at 48 hr postinfection. When subjected to electrophoretic mobility shift analysis (EMSA), with ^{32}P-labeled E2-specific binding site oligonucleotides, complexes of low or high mobility were observed for nuclear extracts prepared from cells infected with rvE2 and rvE2SCT, respectively. For co-infected cells extracts, a complex of intermediate mobility, indicating heterodimer formation, was observed *(Fig. 6A).* All three complexes could be supershifted with αCT E2 C-terminal-specific antiserum, but only the complexes of low and intermediate mobility with αNT antiserum (*Fig. 6B*). All three complexes competed for unlabeled E2 binding site oligonucleotide, but not an oligonucleotide containing a mutated E2 core palindrome (data not shown). When nuclear protein extracts prepared from cells infected with rvE2 and rvE2SCT were mixed at a final concentration of 5 mg/ml and incubated at 27 or 37°, formation of heterodimers was observed. The rate of formation of heterodimers was temperature-dependent and was unaffected by the addition of ATP or nonhydrolyzable ATP analogues (AMP-PNP (Adenylyl-imidodiphosphate), AMP-PCP (Adenylyl (β,γ-methylene)-diphosphonate), ATP-γ-S (Adenosine-5′-O-(3-triphosphate)); data not shown).

The promoter of HPV-16 contains a pair of E2 binding sites, separated by only three nucleotides, adjacent to promoter P97. When this tandem E2 binding site was assayed by EMSA, nuclear protein extracts from cells infected with rvE2 and rvE2SCT gave rise to a number of complexes of discrete mobility (*Fig. 7*), indicating that adjacent P97 binding sites could be occupied simultaneously by two full-length E2 homodimers or two C-terminal homodimers. When the tandem binding site arrangement was assayed in conjunction with a nuclear extract prepared from cells co-infected with rvE2 and

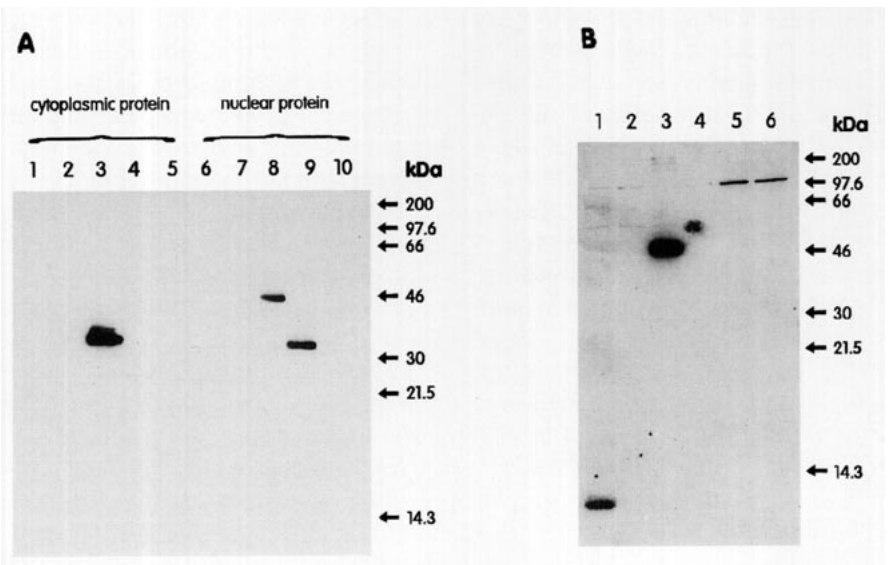


FIG. 3. Confirmation of E2, E2 N-terminal, and E2 C-terminal expression by Western blot analysis. Protein samples from subcellular fractionations prepared from infected and mock-infected cells were separated on 12% SDS–polyacrylamide gels containing 5 *M* urea, transferred to nitrocellulose membranes, and probed with αNT and αCT specific antisera. Visualization was achieved by chemiluminescence following reactivity with a secondary peroxidase-labeled anti-rabbit antibody. (A) Reactivity with anti-N-terminal antiserum. Lanes 1 to 5, cytoplasmic fractions and Lanes 6 to 10 nuclear fractions. Lanes 1 and 10, rvE2SCT-infected cells; Lanes 2 and 8, rvE2-infected cells; Lanes 3 and 9, rvE2NT-infected cells; Lanes 4 and 7, wild-type AcMNPV infection; Lanes 5 and 6, mock-infected cells. The small amount of E2 N-terminal protein in lane 9 is likely to result from the presence of unbroken cells in the nuclear fraction. (B) Reactivity with anti-C-terminal antiserum. Lane 1, rvE2SCT-infected cells, nuclear fraction; Lane 2, rvE2LCT-infected cells, nuclear fraction; Lane 3, rvE2-infected cells, nuclear fraction; Lane 4, rvE2NT-infected cells, cytoplasmic fraction; Lanes 5 and 6, total protein, mock-infected and wild-type AcMNPV-infected cells, respectively.

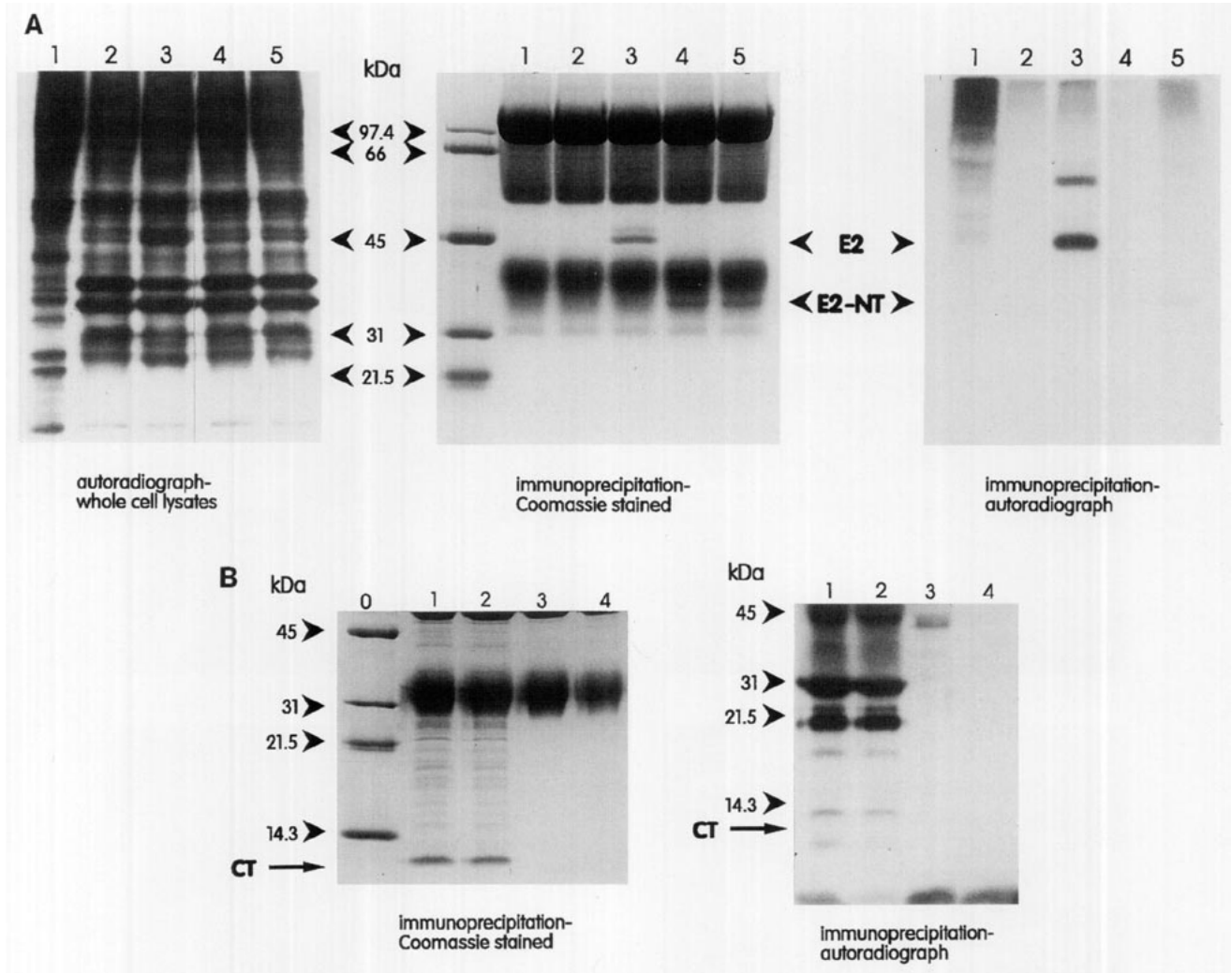
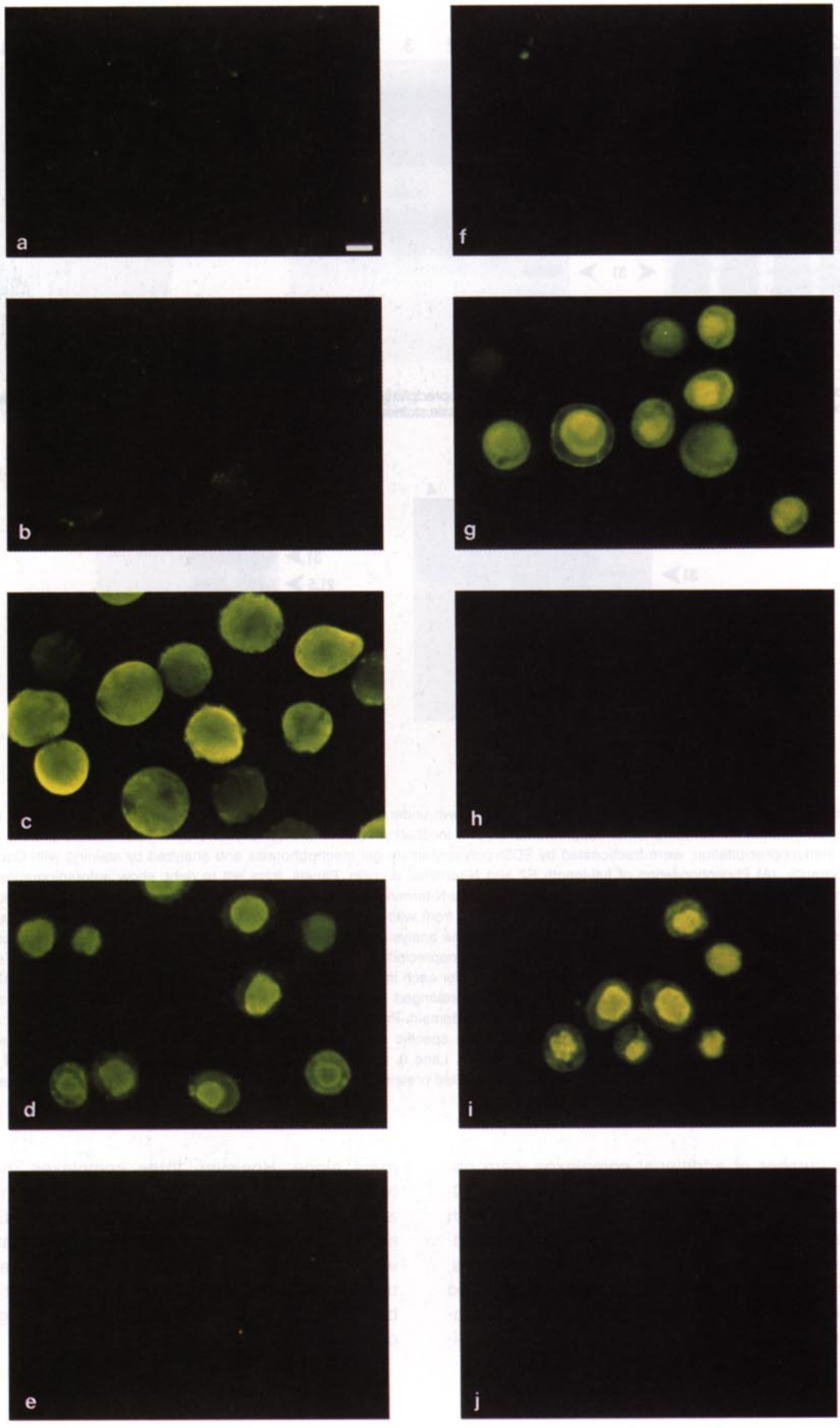


FIG. 4. Phosphorylation of the HPV-16 E2 protein in Sf21 cells grown under serum free conditions. At 38 hours postinfection, cells were starved in phosphate-free medium for 90 min prior to supplementation and incubation with ^{32}P orthophosphoric acid. Total soluble protein, and proteins recovered by immunoprecipitation, were fractionated by SDS–polyacrylamide gel electrophoresis and analyzed by staining with Coomassie blue and autoradiography. (A) Phosphorylation of full-length E2 and N-terminal domain. Panels, from left to right, show autoradiography of total cell protein, Coomassie staining of immunoprecipitated protein (using anti-N-terminal antiserum), and autoradiography of proteins recovered by immunoprecipitation (10% gel). Lane 1, mock-infected cells; Lane 2, protein from wild-type AcMNPV infection; Lane 3, rvE2-infected cells; Lanes 4 and 5, rvE2NT-infected cells. Labeled E2 protein can be distinguished in the analysis of whole cell protein, but not E2NT. However, expression of E2NT was lower in the serum free system. Recovery of protein by immunoprecipitation was evaluated by staining gels with Coomassie blue prior to autoradiography. Total soluble protein from 2×10^6 cells was used for each immunoprecipitation reaction, except lane 5 where 4×10^6 cells were used. Phosphorylation of the N-terminus was only detected upon prolonged exposure. The identity of the ~58-kDa band coimmunoprecipitating with E2 is unknown. (B) Analysis of the E2 C-terminal DNA-binding domain. Protein was efficiently recovered by immunoprecipitation using anti-C-terminal antiserum, but no modification by phosphorylation of the specific product was detected, even after prolonged exposure. Left panel, immunoprecipitation, Coomassie stain; right panel, autoradiograph. Lane 0, molecular weight marker; Lane 1, immunoprecipitated protein from rvE2SCT-infected cells (4×10^6 cells); Lanes 2 to 4, immunoprecipitated protein from 2×10^6 cells, rvE2SCT, wild-type AcMNPV-infected and mock-infected cells, respectively.

rvE2SCT, a number of additional complexes were observed. If the protein–DNA complexes formed by simultaneous binding of two E2 heterodimers or a full-length E2 homodimer with a C-terminal homodimer are considered identical on the basis of similar charge and mass, we observe the additional four complexes expected when homo- and heterodimers are probed with a tandem binding site, compared to preparations of homodimers alone. However, three complexes migrate between the position of a single bound E2 homodimer and two C-terminal homodimers. On the basis of mass alone, only two such complexes of intermediate mobility would have been expected. The electrophoretic mobilities of the E2-binding site complexes do not appear to be directly proportional to the molecular weight of their constituent proteins.


a
f
b
g
c
h
d
i
e
j

Protease susceptibility of the E2 DNA-binding domain

Previous analysis has shown that the DNA-binding/dimerization domain of BPV-1 E2 can be reduced to a small protease-resistant core, since the same limited digestion product was obtained whether the protein was treated with protease either before or after binding to DNA. To test whether the E2 protein of HPV-16 would display a similar resistance, nuclear protein extracts containing full-length or C-terminal HPV-16 E2 were treated with pronase E before or after binding DNA. Both forms of E2 protein bound to DNA were not totally resistant to proteolysis and exhibited a biphasic susceptibility profile: The protein was rapidly reduced to a core domain bound to DNA which was slowly degraded by prolonged incubation with protease (*Fig. 8*), at a rate greatly reduced compared to the molecule not bound to its DNA target. Rapid loss of E2 binding activity upon exposure to protease in nuclear protein extracts from rvE2- and rvE2SCT-infected cells (<5 min) in the absence of specific binding site DNA was observed. Loss of DNA binding activity also occurred in control extracts not supplemented with pronase E, suggesting that the core DNA binding domain was sensitive to endogenous insect cell proteases (data not shown).

DISCUSSION

The ability to precisely define the viral factors and mechanisms that regulate the papillomavirus life cycle has been severely restricted for several reasons: First, systems that reproduce the life cycle *in vitro* are poorly developed, a problem mainly associated with the inability to easily mimic keratinocyte differentiation to which vegetative viral replication is tightly linked. Second, transformed cells that maintain viral genomes contain low levels of viral transcripts (Heilman *et al.*, 1982) and proteins. Alternative strategies are therefore required to study viral transcription and replication. The E2 protein of HPV-16 is thought to encode a modulator of viral transcription and, by analogy and sequence comparison, would also be presumed to have a role in viral replication. The precise role of HPV-16 E2 in regulating both of these processes in the native viral system is unclear and controversial. To initiate a biochemical study of HPV-16 E2, the full-length protein and subdomains that are predicted to have distinct biological activities have been expressed in insect cells using recombinant baculovirus vectors.

We constructed four recombinant viruses that contained the ORF's illustrated in *Fig. 1*. In the original experimental design we set out to express the full-length protein and the whole molecule represented as two overlapping functional subdomains, E2NT and E2LCT. The expected products of the full-length and C-terminally truncated (E2NT) ORF were successfully expressed in insect cells and identity confirmed by immunoblot analysis using E2-specific antisera. The apparent molecular weights of the two proteins under reducing and denaturing conditions, 45 kDa for full-length E2 and 35 kDa for the N-terminal protein, were close to the predicted values (41.8 and 29.3 kDa, respectively). It is unlikely that the minor discrepancies are due to extensive posttranslational modifications in the N-terminal domain since similar proteins expressed in bacteria migrate with the same aberrant mobility (C. M. Sanders, unpublished observations). However, we failed to detect the predicted E2LCT product when proteins were analyzed by SDS–PAGE, and only detected minimal specific E2 binding activity by EMSA in nuclear extracts from cells infected with rvE2LCT (data not shown). The reasons for poor expression of the E2LCT domain are unclear. We have not analyzed RNA from infected cells, but have confirmed the integrity of the allelic replacement event. Poor expression does not appear to be a consequence of the functional activity of the E2 DNA binding domain expressed in isolation, since the active subunit was successfully expressed from recombinant virus rvE2SCT. E2SCT differs from E2LCT in that 26 E2 codons have been removed from the N-terminus and are replaced with four heterologous amino acids as a result of the cloning procedure (Met Ala His Met). Specific DNA binding activities were similar for E2 and E2SCT cell extracts. It is possible that the particular combination of 5′ sequences was not conducive to efficient expression. Alternatively, in the absence of the N-terminal domain, sequences in the hinge region of E2 may mark truncated proteins for rapid turnover. The naturally occurring N-terminally truncated E2 repressor species of BPV-1 have shorter half lives than their full-length counterpart (Hubbert *et al.*, 1988). The E2SCT DNA-binding domain migrated with its predicted molecular mass of 10.7 kDa, under denaturing/reducing conditions, and its identity was confirmed by immunoblot analysis.

Heterologous proteins expressed in insect cells have been shown to be targeted to the correct subcellular compartment. This has been demonstrated for SV40 large T antigen (Rice *et al.*, 1987), and the nonviral pro-

FIG. 5. Analysis of protein compartmentalization by indirect immunofluorescence. Sf21 cells were infected with wild-type baculovirus (AcMNPV), recombinant viruses expressing E2 and subdomains or mock infected. At 48 hr postinfection, cells were fixed, stained with anti-E2 N-terminal, anti-C-terminal, or preimmune antisera (data not shown) followed by FITC-conjugated anti-rabbit secondary antibody. Cells were examined by phase contrast (data not shown) and fluorescence microscopy: (a–e) staining with anti-N-terminal primary antibody; (f–j) Staining with anti-C-terminal primary antibody. (a and f) Wild-type AcMNPV-infected cells; (b and g) rvE2SCT-infected cells; (c and h) rvE2NT-infected cells; (d and i) rvE2 infected cells; (e and j) mock-infected cells. The scale bar in a represents 7 μm.

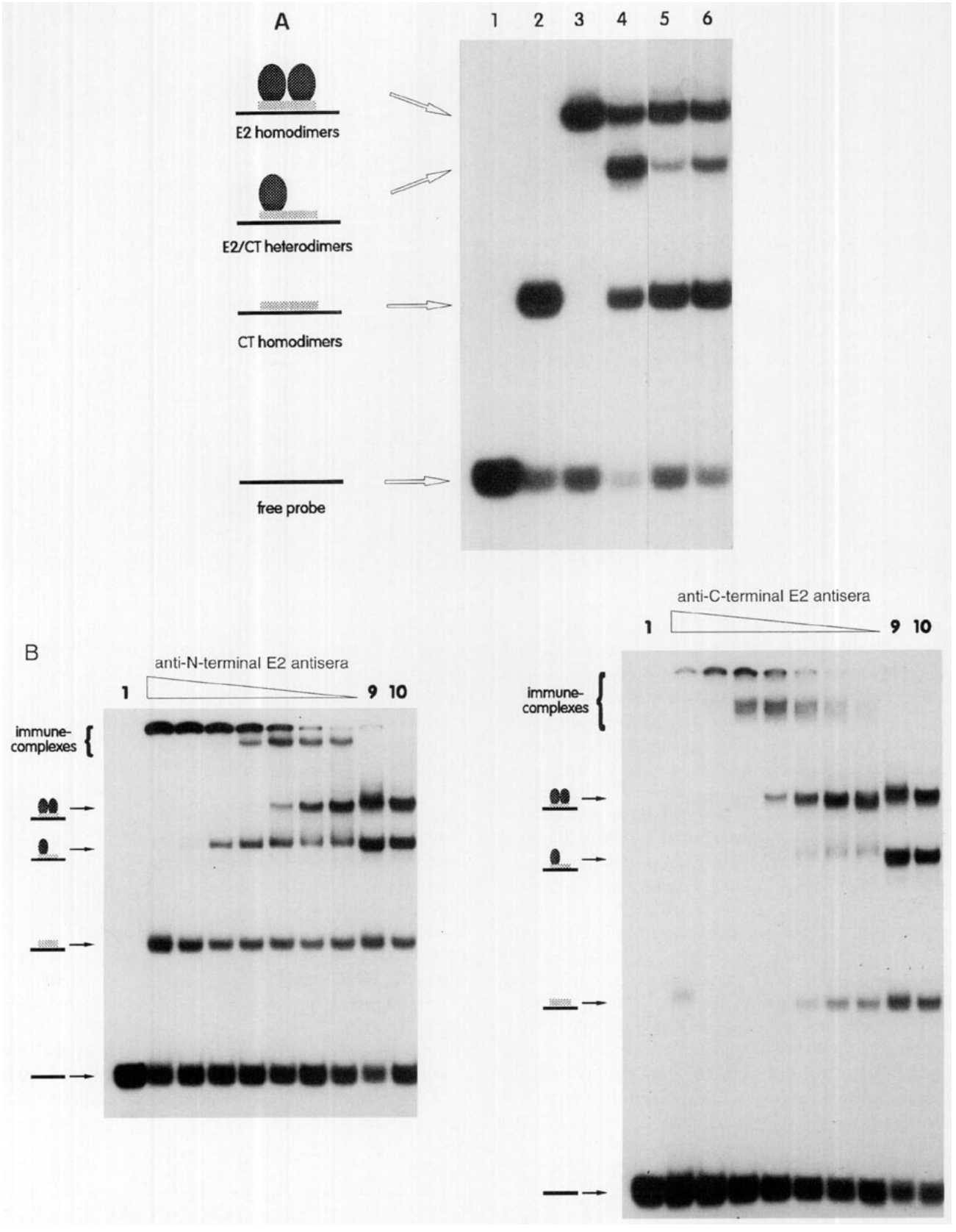


FIG. 6. DNA binding and dimerization of the HPV-16 E2 protein. Extracts were prepared from wild-type AcMNPV, recombinant virus-infected cells and mock-infected cells at 48 hr postinfection and proteins from the nuclear fraction were analyzed by EMSA. The probe was a 30-base pair ^{32}P-labeled double-stranded E2 binding site oligonucleotide, derived from the HPV-16 promoter. (A) Heterodimeric complexes of full-length E2 and its

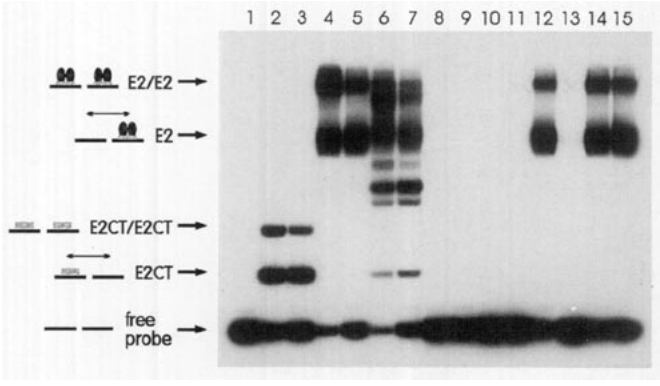


FIG. 7. Binding of E2, E2SCT and heterodimeric complexes to tandem E2 binding sites derived from the HPV-16 promoter. Lane 1, free probe; Lanes 2 and 3, E2SCT protein extract, 5 and 2.5 ng total per reaction, respectively; Lanes 4 and 5, E2 extract, 5 and 2.5 ng; Lanes 6 and 7, protein extract from cells co-infected with rvE2 and rvE2SCT, 5 and 2.5 ng; Lanes 8 and 9, protein from wild-type AcMNPV-infected cells, 5 and 2.5 ng; Lanes 10 and 11, protein from mock-infected cells, 5 and 2.5 ng; For lanes 12 to 15, all reactions contained 2.5 ng of E2 extract with the addition of a 50-fold excess of specific cold competitor to 13 and a 50- and 100-fold excess of mutant E2 binding site oligonucleotide competitor to 14 and 15, respectively.

teins c-myc (Miyamoto *et al.,* 1985) and the product of the *Drosophila* Kruppel gene (Ollo and Maniatis, 1987). BPV-1 expresses three forms of the E2 protein in transformed cells. All have the central hinge region and C-terminal DNA-binding/dimerization domain in common and have been localized to the nucleus by subcellular fractionation and immunofluorescence (Hubbert *et al.,* 1988; Burnett *et al.,* 1990). However, the sequences responsible for nuclear localization have not been formally identified. In this study, it has been demonstrated by cell-fractionation and immunofluorescence that the nuclear localization signals reside in the C-terminal 89 amino acids of the HPV-16 E2 protein. Therefore, any truncated HPV-16 E2 proteins analogous to those of BPV-1 should have the capacity to exert their effect in the nucleus of the cell.

Recombinant baculovirus-infected insect cells are capable of faithfully reproducing many modifications of heterologous eucaryotic proteins as present in their native environment, with the probable exception of glycosylation. The full-length E2 proteins of cottontail rabbit papillomavirus (CRPV), BPV-1, and HPV-11 are phosphoproteins (Barbosa and Wettstein, 1988; McBride *et al.,* 1989a; Meneguzzi *et al.,* 1989; Bream *et al.,* 1993). Phosphorylation of BPV-1 and HPV-11 E2 was demonstrated in insect cells and the major phosphorylation sites of the BPV-1 protein were subsequently mapped to two serine residues in the hinge domain. The same amino acids were phosphorylated in cos cells. The target residues are buried within an acidic region and are potential casein kinase II (CK II) recognition sites (McBride *et al.,* 1989a). The phosphorylated amino acids of the HPV-11 protein were not identified and nor were any potential sites with similarities to the BPV-1 motifs located. Moreover, the hinge region within which the BPV phosphoserines are found is unconserved, even between more closely related viruses (McBride *et al.,* 1989a).

The protein phosphorylation studies of HPV-16 E2 and subdomains reported here have demonstrated that the full-length E2 protein was an efficient substrate for kinase activity, that the isolated N-terminal domain (amino acids 1–255) was a poor substrate, and that the C-terminal 89 amino acids (277–365) of E2 are not phosphorylated. Protein phosphorylation has been shown to be a key element in the regulation of the function of many transcription factors. The phosphorylation of a single serine residue of the serum response factor (SRF) will enhance its rate of association with and affinity for its binding site (Rivera *et al.,* 1993). Phosphorylation of a single tyrosine residue of the 98-kDa protein Stat91 in response to IFNγ, controls nuclear localization, DNA binding, and gene activation (Shuai *et al.,* 1993). From our results, E2 phosphorylation is not obviously involved in dimerization, DNA binding, or nuclear translocation, although it may affect the rate or extent to which these processes occur. In addition, efficient phosphorylation of the E2 protein is not due to cytoplasmic kinase activity targeting phosphorylation sites in residues 1–255. We cannot exclude the possibility that the 20 amino acid stretch (LLHRDSVDSAPILTAFNSSHK, which includes four serines), not represented in either of the domains E2NT or E2SCT, contains the major targets for phosphorylation. However, this region is not highly acidic and bears little resemblance to the CKII target sequence in BPV-1 (QEEEEQSPDSTEEEPVTLPR) (McBride *et al.,* 1989a). Also, since the hinge region is unconserved, and conservation of structurally/functionally important sites appears as a recurring theme in the papillomaviruses, we consider that relevant HPV target sites may occur elsewhere in the E2 molecule. It would be interesting to speculate that N-terminal phos-

N-terminally truncated DNA-binding/dimerization domain were formed when cells were co-infected with rvE2 and rvE2SCT or when protein extracts were mixed and incubated *in vitro:* Lane 1, free probe; Lane 2, E2SCT protein extract; Lane 3, full-length E2 protein extract; Lane 4, protein extract from cells co-infected with rvE2 and rvE2SCT. Protein extracts for reactions 2, 3, and 4 were preincubated at 37° for 60 min; Lanes 5 and 6, mixing of proteins from rvE2- and rvE2SCT-infected cells. Binding reactions were performed after preincubation of mixed extracts for 30 (Lane 5) and 60 (Lane 6) minutes at 37°. (B) EMSA immunological supershift assay. Protein extracts from cells co-infected with rvE2 and rvE2SCT were incubated with labeled E2 binding site probe followed by preimmune and anti-E2 domain specific antisera. Left panel, anti-N-terminal antiserum, right panel, anti-C-terminal antiserum. Lanes 1, free probe; Lanes 2 to 8, serial dilution of antisera to 1/800; Lanes 9, no antibody control; Lanes 10, addition of 1/10th volume of preimmune serum.

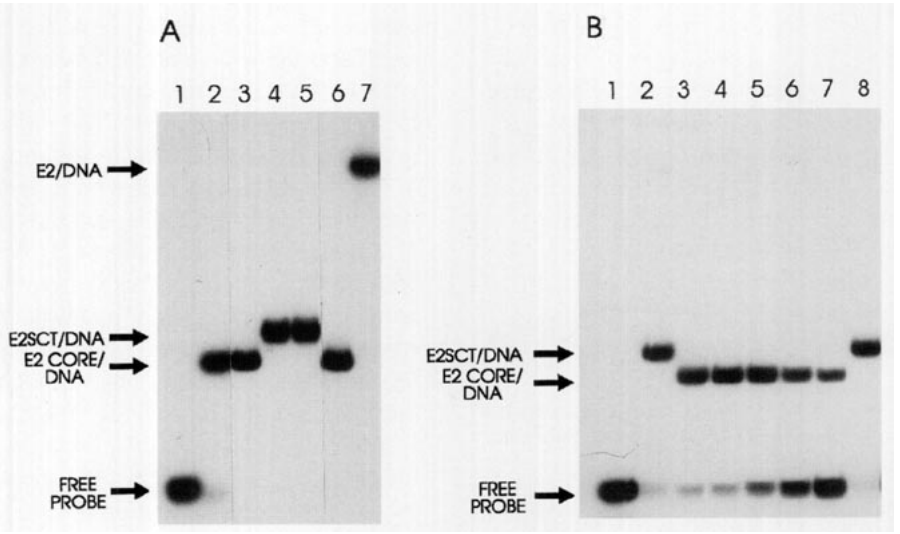


FIG. 8. Biphasic susceptibility of the E2 protein to proteolysis. (A) Protein was bound to DNA before treatment with an equivalent mass of pronase E. Lane 1, free probe (single E2 binding site, 30-base pair oligonucleotide); Lane 2, E2SCT extract, 10-min incubation; Lane 3, E2SCT extract, 5-min incubation; lanes 4 and 5, 5- and 10-min incubations without the addition of pronase E; Lane 6, E2 protein extract, 5-min incubation with pronase addition; Lane 7, E2 extract, no protease treatment. (B) Time course digestion of E2SCT-binding site complex. Lane 1, free probe; Lane 2, time (t) zero, undigested; Lanes 3 to 7, 5, 10, 25, 35, and 60-min digestion with pronase E. Lane 8, protein maintained at 37° for 60 min following the addition of pronase dilution buffer.

phorylation by nuclear kinases may have important implications for the regulation of E2-dependent activity. Indeed, BPV-1 replication factor E1 has been shown to preferentially bind to the non- or hypophosphorylated species of E2 expressed in Sf21 cells. The phosphorylation of E2 may therefore control E1–E2 interactions and, in turn, E2-dependent regulation of transcription and replication (Lusky and Fontane, 1991).

HPV-16 E2 and E2NT were immunoprecipitated with polyclonal anti E2 N-terminal domain-specific antiserum when performing metabolic labeling experiments. A discrete 58-kDa phosphoprotein was immunoprecipitated from lysates of cells infected with rvE2 but not rvE2NT-infected cells and control extracts (*Fig. 4A*). The identity of this protein is unknown but is unlikely to be a product derived from the E2 ORF since the protein was not detected in Western blots or its presence revealed in mobility shift assays. It is possible that the protein is a member of the conserved eucaryotic transcription or replication apparatus which associates specifically with E2, possibly, at least in part, via determinants in the C-terminal domain. Multiple proteins detected in Coomassie blue-stained gels and by autoradiography when the core DNA binding domain was precipitated from rvE2SCT-infected cells with αCT antiserum are also unlikely to be derived from the E2SCT ORF for the same reasons. Since they were not detected when full-length E2 was immunoprecipitated with αNT antiserum, they are most likely the result of interactions with the sepharose immobilized *Staphylococcal* protein A-immunoglobulin–E2SCT complex.

When translated in a reticulocyte system (McBride *et al.*, 1989b), or expressed in transient transfection assays (Haugen *et al.*, 1988), the BPV-1 E2 protein and its core DNA binding domain dimerize spontaneously. There is no requirement for DNA containing the E2 recognition motif. Dimers are stable and only denature at urea concentrations in excess of 5 *M*. The formation of heterodimers between BPV-1 E2 and truncated species was only observed when proteins were cotranslated or coexpressed, whereas mixing of preformed full-length and C-terminal dimers apparently did not result in inter subunit exchange. Similar results have recently been obtained for the HPV-16 E2 protein translated in a wheat germ lysate system (Bouvard *et al.*, 1994). In our study, dimerization was possible by simply mixing baculovirus-infected cell extracts.

Sensitivity to proteolysis and urea denaturation have been directly linked to dimer stability: UV cross-linked dimers displayed dramatically increased stability when exposed to proteases (chymotrypsin and trypsin) compared to unlinked dimers, and DNA binding notably shifted the point at which dimers dissociated from 5 to 7.2 *M* urea (Corina *et al.*, 1993). Here we have analyzed the rate of digestion when HPV-16 E2 was exposed to a mixture of aspartate and serine proteases (pronase E). Unlike the data originally reported for BPV-1 E2 expressed in bacteria (Dostatni *et al.*, 1988), DNA-bound HPV-16 E2 dimers were susceptible to proteolysis, however, at a very reduced rate compared to non-DNA-bound dimers. E2 or E2SCT bound to DNA were rapidly reduced to a resistant core, which was further degraded, with

loss of binding activity, on prolonged exposure. This disparity with data for the E2 protein of BPV-1 could be explained, at least in part, by the fact that we exposed E2 to protease over an extended time couse. In addition, differences in specific activity of the protease, and the differing systems in which the two viral E2 proteins were generated, may also contribute. However, the most accurate assessment regarding the protease sensitivity of the two papillomavirus E2 proteins can only be made by comparing purified preparations of both proteins in a parallel experiment.

In the course of analyzing E2 dimerization, we were able to demonstrate formation of heterodimers when insect cells were co-infected with recombinant viruses expressing full-length E2 and N-terminally truncated forms (E2SCT). All E2 species were able to bind specifically to an oligonucleotide probe derived from the HPV-16 promoter. When nuclear extracts from cells singly infected with rvE2 and rvE2SCT were mixed *in vitro* we observed the accumulation of heterodimeric species over a period of time, the rate of which was enhanced at elevated temperature and unaffected by the addition of ATP or a number of nonhydrolyzable analogues. These observations appear to contrast recent results for HPV-16 E2 (Bouvard *et al.,* 1994) and those for BPV-1 E2 (Haugen *et al.,* 1988; McBride *et al.,* 1989b), and could be related to the results of protease sensitivity experiments: HPV-16 E2 dimers may be inherently less stable than their BPV-1 counterparts. This may have implications for HPV-16 E2 dependent regulation of transcription and replication, suggesting that subunits may be free to interchange between dimers *in vivo.* When drawing these conclusions from the *in vitro* data we have assumed that the equilibrium between E2 and E2SCT homodimers and respective monomers in lysates from rvE2 and rvE2SCT infected cells has already been reached prior to mixing, and the extent to which all dimers form (homo- and heterodimers) is the same. These assumptions do not preclude a direct comparison with published data using *in vitro* translation systems or mammalian cell extracts. However, we cannot rule out the involvement of molecular chaperones, present in baculovirus-infected cells but absent in systems previously used to characterize BPV-1 and HPV E2 dimerization, that can affect the rate of E2 monomer association and/or dissociation. The activity of this putative class of molecular chaperone is unaffected by a number of ATPase inhibitors.

The full-length E2 protein of BPV-1 purified from insect cells has been shown to bind cooperatively to two adjacent palindromic recognition sequences separated by four nucleotides (Monini *et al.,* 1991). However, when two sites are separated by two base pairs, occupancy is restricted to a single palindrome due to steric hindrance (Gauthier *et al.,* 1991). On account of the differences in primary structure between the BPV-1 and HPV-16 E2 proteins, and the observations that the contact points made with DNA upon binding of the two proteins are qualitatively different, (Bedrosian and Bastia, 1990) we analyzed binding of HPV-16 E2 to the tandem E2 binding sites from the native promoter, where they are separated by only three nucleotides. The results demonstrate that the occupancy of both sites is sterically unrestricted. In addition, both homo- and heterodimeric E2 species may occupy a binding site with any neighboring dimer. Full-length BPV-1 E2 and N-terminally truncated dimers have been shown to bind cooperatively to two adjacent palindromic sites (the cooperative binding parameter for two full-length E2 dimers being several fold greater than that for two dimers of the core DNA binding domain), and participate in heterocooperative interactions (Monini *et al.,* 1991, 1993). In addition, Tan *et al.* (1994) have recently measured minimal cooperativity of binding for two recombinant HPV-16 E2 dimers purified from *E. coli* when interacting with this HPV-16 binding site configuration (Tan *et al.,* 1994). Although it appears from our experiments that certain binding configurations appear to be favored, we are at present unable to speculate on HPV-16 E2 cooperativity since the relative proportions of the various E2 dimers in our crude nuclear lysates, and their relative DNA binding affinities, are unknown.

Interestingly, E2:DNA complexes formed by binding of homo- and heterodimeric E2 species to the tandem binding site probe probably do not migrate strictly according to molecular weight. This could be due to the differences in charge between the N- and C-terminal domains, the predicted isoelectric points of which are and 6.099 and 9.65, respectively, or possibly the magnitude or direction of HPV-16 E2-induced DNA bending (Bedrosian and Bastia, 1990).

In conclusion, we have successfully expressed the full-length E2 protein and its subdomains using a recombinant baculovirus system, and have shown distinct biochemical differences between the HPV-16 E2 and the "prototype" E2 from BPV-1. *Ex vivo* studies of viral protein function may therefore provide much useful information when large scale *in vitro* culture of viruses such as HPV is difficult. These biochemical differences may be helpful in interpreting the action of the HPV E2 protein, whose role is still poorly understood, and is in some respects controversial.

ACKNOWLEDGMENTS

We thank David Roper and James Brannigan in the Department of Chemistry at the University of York for oligonucleotide synthesis and Peter Crosby and Meg Stark for photography. We are also grateful to Leticia Rocha-Zavaleta for initial screening of the antisera and Leslie Ramsden and Mike Anderson for expert technical support. C.M.S. was in receipt of a Medical Research Council Studentship, and the research was supported by a program grant to N.J.M. from the Yorkshire Cancer Research Campaign. P.L.S. is supported by the Cancer Research Campaign.

REFERENCES

Androphy, E. J., Lowy, D. R., and Schiller, J. T. (1987). Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. *Nature* **325,** 70–73.

Baker, C. C., Phelps, W. C., Lindgren, V., Braun, M. J., Gonda, M. A., and Howley, P. M. (1987). Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J. Virol.* **61,** 962–971.

Barbosa, M. S., and Wettstein, F. O. (1988). E2 of cottontail rabbit papillomavirus is a nuclear phosphoprotein translated from an mRNA encoding multiple open reading frames. *J. Virol.* **62,** 3242–3249.

Bedrosian, C. L., and Bastia, D. (1990). The DNA-binding domain of HPV-16 E2 protein interaction with the viral enhancer: Protein-induced DNA bending and role of the nonconserved core sequence in binding site affinity. *Virology* **174,** 557–575.

Blitz, I. L., and Laimins, L. A. (1991). The 68-kilodalton E1 protein of bovine papillomavirus is a DNA binding phosphoprotein which associates with the E2 transcriptional activator in vitro. *J. Virol.* **65,** 649–656.

Bouvard, V., Storey, A., Pim, D., and Banks, L. (1994). Characterization of the human papillomavirus E2 protein: Evidence of trans-activation and trans-repression in cervical keratinocytes. *EMBO J.* **13,** 5451–5459.

Bream, G. L., Ohmstede, C., and Phelps, W. C. (1993). Characterization of human papillomavirus type 11 E1 and E2 proteins expressed in insect cells. *J. Virol.* **67,** 2655–2663.

Burnett, S., Ström, A.-C., Jareborg, N., Alderborn, A., Dillner, J., Moreno-Lopez, J., Pettersson, U., and Kiessling, U. (1990). Induction of bovine papillomavirus E2 gene expression and early region transcription by cell growth arrest: Correlation with viral DNA amplification and evidence for differential promoter induction. *J. Virol.* **64,** 5529–5541.

Chen, E. Y., Howley, P. M., Levinson, A. D., and Seeburg, P. H. (1982). The primary structure and genetic organization of the bovine papillomavirus type 1 genome. *Nature* **299,** 529–534.

Chen, L., Ashe, S., Singhal, M. C., Galloway, D. A., Hellström, I., and Hellström, K. E. (1993). Metastatic conversion of cells by expression of human papillomavirus type 16 E6 and E7 genes. *Proc. Natl. Acad. Sci. USA* **90,** 6523–6527.

Chiang, C.-M., Broker, T. R., and Chow, L. T. (1991). An E1M*E2C fusion protein encoded by human papillomavirus type 11 is a sequence-specific transcription repressor. *J. Virol.* **65,** 3317–3329.

Choe, J., Vaillancourt, P., Stenlund, A., and Botchan, M. (1989). Bovine papillomavirus type 1 encodes two forms of a transcriptional repressor: Structural and functional analysis of new viral cDNAs. *J. Virol.* **63,** 1743–1755.

Corina, K., Grossman, S. R., Barsoum, J., Prakash, S. S., Androphy, E. J., and Pepinsky, R. B. (1993). The tryptophan bridge is a critical feature of the papillomavirus E2 DNA binding domain. *Virology* **197,** 391–396.

Cripe, T. P., Haugen, T. H., Turk, J. P., Tabatabai, F., Schmid, P. G., Durst, M., Gissmann, L., Roman, A., and Turek, L. P. (1987). Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 transactivator and repressor gene products: implications for cervical carcinogenesis. *EMBO J.* **6,** 3745–3753.

Cullen, A. P., Reid, R., Campion, M., and Lorincz, A. T. (1991). Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *J. Virol.* **65,** 606–612.

Dartmann, K., Schwarz, E., Gissmann, L., and zur Hausen, H. (1986). The nucleotide sequence and genome organisation of human papillomavirus type 11. *Virology* **151,** 124–130.

De Villiers, E.-M. (1989). Minireview: Heterogeneity of the human papillomavirus group. *J. Virol.* **63,** 4898–4903.

De Villiers, E.-M. (1994). Human pathogenic papillomavirus types: An update. *Curr. Top. Microbiol. Immunol.* **186,** 1–12.

Doorbar, J., Parton, A., Hartley, K., Banks, L., Crook, T., Stanley, M., and Crawford, L. (1990). Detection of novel splicing patterns in a HPV 16 containing keratinocyte cell line. *Virology* **178,** 254–262.

Dostatni, N., Thierry, F., and Yaniv, M. (1988). A dimer of BPV-1 E2 containing a protease resistant core interacts with its DNA target. *EMBO J.* **7,** 3807–3816.

Dyson, N., Howley, P. M., Munger, K., and Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243,** 934–937.

Gauthier, J.-M., Dostatni, N., Lusky, M., and Yaniv, M. (1991). Two DNA-bound E2 dimers are required for strong transcriptional activation and for cooperation with cellular factors in most cells. *New Biol.* **3,** 498–509.

Giri, I., and Yaniv, M. (1988). Structural and mutational analysis of E2 trans-activating proteins of papillomaviruses reveals three distinct functional domains. *EMBO J.* **7,** 2823–2829.

Gloss, B., and Bernard, H. (1990). The E6/E7 promoter of human papillomavirus-16 is activated in the absence of E2 proteins by a sequence-aberrant Sp1 distal element. *J. Virol.* **64,** 5577–5584.

Guido, M. C., Zamorano, R., Garrido-Guerrero, E., Gariglio, P., and Garcia-Carranca, A. (1992). Early promoters of genital and cutaneous human papillomaviruses are differentially regulated by the bovine papillomavirus type 1 E2 gene product. *J. Gen. Virol.* **73,** 1395–1400.

Harrison, N. M., Gearing, K. L., Kim, S.-Y., Kingsman, A. J., and Kingsman, S. M. (1987). Multiple cis-active elements in the long control region of bovine papillomavirus type 1 (BPV-1). *Nucleic Acids Res.* **15,** 10267–10283.

Haugen, T. H., Cripe, T. P., Ginder, G. D., Karin, M., and Turek, L. P. (1987). Trans-activation of an upstream early gene promoter of bovine papilloma virus-1 by a product of the viral E2 gene. *EMBO J.* **6,** 145–152.

Haugen, T. H., Turek, L. P., Mercurio, F. M., Cripe, T. P., Olson, B. J., Anderson, R. D., Seidl, D., Karin, M., and Schiller, J. (1988). Sequence-specific and general transcriptional activation by the bovine papillomavirus-1 E2 trans-activator require an N-terminal amphipathic helix-containing E2 domain. *EMBO J.* **7,** 4245–4253.

Hawley-Nelson, P., Androphy, E. J., Lowy, D. R., and Schiller, J. T. (1988). The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. *EMBO J.* **7,** 525–531.

Hawley-Nelson, P., Vousden, K. H., Hubbert, N. L., Lowy, D. R., and Schiller, J. T. (1989). HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J.* **8,** 3905–3910.

Hegde, R. S., Grosmann, S. R., Laimins, L. A., and Sigler, P. B. (1992). Crystal structure at 1.7 A of the bovine papillomavirus-1 E2 DNA-binding domain bound to its DNA target. *Nature* **359,** 505–512.

Heilman, C. A., Engel, L., Lowy, D. R., and Howley, P. M. (1982). Virus specific transcription in bovine papillomavirus-tranformed cells. *Virology* **119,** 22–34.

Hermonant, P. L., Spalholz, B. A., and Howley, P. M. (1988). The bovine papillomavirus P2443 promoter is E2 trans-responsive: Evidence for E2 autoregulation. *EMBO J.* **7,** 2815–2822.

Hubbert, N. L., Schiller, J. T., Lowy, D. R., and Androphy, E. J. (1988). Bovine papilloma virus-transformed cells contain multiple E2 proteins. *Proc. Natl. Acad. Sci. USA* **85,** 5864–5868.

King, L. A., and Possee, R. D. (1992). "The Baculovirus Expression System: A Laboratory Guide." Chapman and Hall, London, UK.

Lambert, P. F., Spalholz, B. A., and Howley, P. M. (1987). A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. *Cell* **50,** 69–78.

Lambert, P. F., Pan, H., Pitot, H. C., Liem, A., Jackson, M., and Griep, A. E. (1993). Epidermal cancer associated with expression of human papillomavirus type 16 E6 and E7 oncogenes in the skin of transgenic mice. *Proc. Natl. Acad. Sci. USA* **90,** 5583–5587.

Lowy, D. R., Kirmbauer, R., and Schiller, J. T. (1994). Genital human papillomavirus infection. *Proc. Natl. Acad. Sci. USA* **91,** 2463–2440.

Lusky, M., and Fontane, E. (1991). Formation of the complex of bovine

papillomavirus E1 and E2 proteins is modulated by E2 phosphorylation and depends upon sequences within the carboxyl terminus of E1. *Proc. Natl. Acad. Sci. USA* **88,** 6363–6367.

McBride, A. A., Bolen, J. B., and Howley, P. M. (1989a). Phosphorylation sites of the E2 transcriptional regulatory proteins of bovine papillomavirus type 1. *J. Virol.* **63,** 5076–5085.

McBride, A. A., Byrne, J. C., and Howley, P. M. (1989b). E2 polypeptides encoded by bovine papillomavirus type 1 form dimers through the common carboxyl-terminal domain: Transactivation is mediated by the conserved amino-terminal domain. *Proc. Natl. Acad. Sci. USA* **86,** 510–514.

McLean, I. W., and Nakane, P. K. (1974). Periodate-lysine-paraformaldehyde fixative: A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* **22,** 1077–1083.

Meneguzzi, G., Lathe, R., Kieny, M. P., and Vogt, N. (1989). The E2 transacting protein of bovine papillomavirus type 1 (BPV-1) is serine-phosphorylated *in vivo. Oncogene* **4,** 1285–1290.

Miyamoto, C., Smith, G. E., Farrell-Towt, J., Chizzonite, R., Summers, M. D., and Ju, G. (1985). Production of human c-myc protein in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **5,** 2860–2865.

Mohr, I. J., Clark, R., Sun, S., Androphy, E. J., MacPherson, P., and Botchan, M. R. (1990). Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science* **250,** 1694–1699.

Monini, P., Grossman, S. R., Pepinsky, B., Androphy, E. J., and Laimins, L. A. (1991). Cooperative binding of the E2 protein of bovine papillomavirus to adjacent E2-responsive sequences. *J. Virol.* **65,** 2124–2130.

Monini, P., Blitz, I. L., and Cassai, E. (1993). Cooperative DNA binding of the bovine papillomavirus E2 transcriptional activator is antagonized by truncated E2 polypeptides. *J. Virol.* **67,** 5668–5676.

Moskaluk, C., and Bastia, D. (1987). The E2 "gene" of bovine papillomavirus encodes an enhancer binding protein. *Proc. Natl. Acad. Sci. USA* **84,** 1215–1218.

Ollo, R., and Maniatis, T. (1987). Drosophila Krüppel gene product produced in a baculovirus expression system is a nuclear phosphoprotein that binds to DNA. *Proc. Natl. Acad. Sci. USA* **84,** 5700–5704.

Phelps, W. C., and Howley, P. M. (1987). Transcriptional transactivation by the human papillomavirus type 16 E2 gene product. *J. Virol.* **61,** 1630–1638.

Prakash, S. S., Grossman, S. R., Pepinsky, R. B., Laimins, L. A., and Androphy, E. J. (1992). Amino acids necessary for DNA contact and dimerization imply novel motifs in the papillomavirus E2 trans-activator. *Genes Dev.* **6,** 105–116.

Remm, M., Brain, R., and Jenkins, J. R. (1992). The E2 binding sites determine the efficiency of replication for the origin of human papillomavirus type 18. *Nucleic Acids Res.* **20,** 6015–6021.

Rice, W. C., Lorimer, H. E., Prives, C., and Miller, L. K. (1987). Expression of polyomavirus large T antigen by using a baculovirus vector. *J. Virol.* **61,** 1712–1716.

Rivera, V. M., Miranti, C. K., Misra, R. P., Ginty, D. D., Chen, R. -H., Blenis, J., and Greenberg, M. E. (1993). A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. *Mol. Cell. Biol.* **13,** 6260–6273.

Rotenberg, M. O., Chow, L. T., and Broker, T. R. (1989). Characterization of rare human papillomavirus type 11 mRNAs coding for regulatory and structural proteins, using the polymerase chain reaction. *Virology* **172,** 489–497.

Sanders, C. M., and Maitland, N. J. (1994). Kinetic and equilibrium binding studies of the human papillomavirus type-16 transcription regulatory protein E2 interacting with core enhancer elements. *Nucleic Acids Res.* **22,** 4890–4897.

Santucci, S., Androphy, E. J., Bonne-Andrèa, C., and Clertant, P. (1990). Proteins encoded by the bovine papillomavirus E1 open reading frame: Expression in heterologous systems and in virally transformed cells. *J. Virol.* **64,** 6027–6039.

Schneider-Gaedicke, A., and Schwarz, E. (1986). Different human cervical carcinoma lines show similar transcription patterns of human papillomavirus type 18 early genes. *EMBO J.* **5,** 2285–2292.

Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A., and zur Hausen, H. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314,** 111–113.

Seedorf, K., Krammer, G., Durst, M., Suhai, S., and Rowekamp, W. G. (1985). Human papillomavirus type 16 DNA sequence. *Virology* **145,** 181–185.

Shirasawa, H., Tomita, Y., Kubota, K., Kasai, T., Sekiya, S., Takamizawa, H., and Simizu, B. (1988). Transcriptional differences of the human papillomavirus type 16 genome between precancerous lesions and invasive carcinomas. *J. Virol.* **62,** 1022–1027.

Shuai, K., Stark, G. R., Kerr, I. M., and Darnell, J. E., Jr. (1993). A single phosphotyrosine residue of Stat91 required for gene activation by interferon-γ. *Science* **261,** 1744–1746.

Smotkin, D., and Wettstein, F. O. (1986). Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA* **83,** 4680–4684.

Spalholz, B. A., Yang, Y.-C., and Howley, P. M. (1985). Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. *Cell* **42,** 183–191.

Spalholz, B. A., Byrne, J. C., and Howley, P. M. (1988). Evidence of cooperativity between E2 binding sites in E2 trans-regulation of bovine papillomavirus type 1. *J. Virol.* **62,** 3143–3150.

Spalholz, B. A., van de Pol, S. B., and Howley, P. M. (1991). Characterisation of the cis elements involved in basal and E2-transactivated expression of the bovine papillomavirus P2443 promoter. *J. Virol.* **65,** 743–753.

Summers, M. D., and Smith, G. E. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. Texas A and M University College Station and Texas Agricultural Experiment Station Bulletin No. 1555.

Tan, S-Y., Leong, E.-C., Walker, P. A., and Bernard, H,-U. (1994). The human papillomavirus type 16 E2 transcription factor binds with low cooperativity to two flanking sites and represses the E6 promoter through displacement of Sp-1 and TFIID. *J. Virol.* **68,** 6411–6420.

Thierry, F., Dostatni, N., Arnos, F., and Yaniv, M. (1990). Cooperative activation of transcription by bovine papillomavirus type 1 E2 can occur over a large distance. *Mol. Cell. Biol.* **10,** 4431–4437.

Ushikai, M., Lace, M. J., Yamakawa, Y., Kono, M., Anson, J., Ishiji, T., Parkkinen, S., Wicker, N., Valentine, M. -E., Davidson, I., Turek, L. P., and Haugen, T. H. (1994). Trans activation by the full-length E2 proteins of human papillomavirus type 16 and bovine papillomavirus type 1 in vitro and *in vivo:* Cooperation with activation domains of cellular transcription factors. *J. Virol.* **68,** 6655–6666.

Ustav, M., and Stenlund, A. (1991). Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. *EMBO J.* **10,** 449–457.

Werness, B. A., Levine, A. J., and Howley, P. M. (1990). Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248,** 76–79.

Yang, L., Li, R., Mohr, I. J., Clark, R., and Botchan, M. R. (1991). Activation of BPV-1 replication *in vitro* by the transcription factor E2. *Nature* **353,** 628–632.

Yang, L., Mohr, I., Fouts, E., Lim, D. A., Nohaile, M., and Botchan, M. (1993). The E1 protein of bovine papilloma virus 1 is an ATP-dependent DNA helicase. *Proc. Natl. Acad. Sci. USA* **90,** 5086–5090.

zur Hausen, H. (1991a). Viruses in human cancers. *Science* **254,** 1167–1173.

zur Hausen, H. (1991b). Minireview: Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184,** 9–13.