JOURNAL OF VIROLOGY, Feb. 1991, p. 649–656
0022-538X/91/020649-08$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 65, No. 2

The 68-Kilodalton E1 Protein of Bovine Papillomavirus Is a DNA Binding Phosphoprotein Which Associates with the E2 Transcriptional Activator In Vitro

IRA L. BLITZ[1] AND LAIMONIS A. LAIMINS[1,2]*

Department of Molecular Genetics and Cell Biology, University of Chicago,[1] and Howard Hughes Medical Institute,[2] Chicago, Illinois 60637

Received 22 August 1990/Accepted 22 October 1990

The E1 open reading frame of bovine papillomavirus type 1 encodes factors necessary for extrachromosomal maintenance of the viral genome in transformed cells. To facilitate biochemical characterization of the gene products encoded by this open reading frame, we have expressed the full-length E1 protein in a baculovirus-insect cell system. This protein was found to be phosphorylated and localized to the nucleus of infected cells. The E1 protein alone has affinity for DNA but appears to lack specificity for viral sequences. In addition, we present evidence that the E1 protein interacts with the virally encoded transcriptional activator E2 in vitro. These results are consistent with a model in which the E1 protein, as part of a complex with E2, interacts with specific DNA sequences in the viral genome.

Cells transformed in vitro by bovine papillomavirus type 1 (BPV-1) maintain the viral genome as autonomously replicating extrachromosomal elements (4). Mutational analysis has demonstrated that several viral early-gene products are required for extrachromosomal maintenance. Of primary importance is the E1 open reading frame (ORF), since mutations throughout this ORF result in the integration of the viral genome into the host chromosomes (3, 13, 21, 22, 32, 36). The E1 ORF is the largest and most conserved papillomavirus ORF (6), and genetic studies have suggested that it encodes at least two distinct replication functions, a positive replication factor, R, and a negative replication modulator, M (3, 34). Recently a 68-kDa phosphoprotein has been identified in BPV-1-transformed cells (34a, 47), which may correspond to the R function. In addition to its role in viral DNA replication, the 68-kDa protein may possess transcriptional repressor activity, since viral genomes with mutations throughout the E1 ORF are derepressed for transcription (17, 37). A 23-kDa polypeptide encoded by the 5′ portion of the E1 ORF, which may correspond to the M function, has also been identified in transformed cells (48). This protein is encoded by a spliced transcript, resulting in fusion of the 5′ region of the E1 ORF to a short downstream exon, adding 14 amino acids to the carboxy terminus (48).

Information regarding the E1 proteins and their role in replication can be inferred from comparative sequence analysis. The polyomavirus large T antigens and the full-length papillomavirus E1 proteins have significant amino acid sequence similarity at their carboxy termini (5, 38). This region of T antigen has been shown to be important for ATPase activity (50) and helicase activity (7, 43, 53), but the E1 protein has not yet been shown to possess these activities. A short region of sequence similarity exists between BPV-1 E1 and simian virus 40 (SV40) T antigen at the amino terminus (5, 38), but this similarity does not extend to the E1 proteins of other papillomaviruses and does not lie within the minimal sequence required for sequence-specific DNA binding of T antigen to the SV40 origin (2, 27, 30, 40, 41, 45). A biochemical characterization of the E1 protein would significantly expand our knowledge of the mechanisms of BPV-1 DNA replication.

A second papillomavirus ORF, E2, has also been implicated in viral DNA replication, as mutations in this ORF result in the integration of the BPV-1 genome into the host chromosomes (9, 13, 32, 36). In addition, a temperature-sensitive mutant of E2 replicates as a stable plasmid at the permissive temperature but is found integrated when shifted to the nonpermissive temperature (10). The full-length E2 ORF encodes a transcriptional activator (42) which specifically interacts with the sequence motif $ACCGN_4CGGT$, where N is any nucleotide, that is present in multiple copies in the genomes of all papillomaviruses. The role of E2 in viral DNA replication is unclear, because it may act directly in replication or indirectly as a transcriptional activator of papillomavirus genes.

In order to characterize the biochemical properties of the full-length E1 protein, we have synthesized large amounts of E1, using a baculovirus vector. The E1 protein was found to be a nuclear phosphoprotein which by itself binds DNA in a nonspecific manner. It has been demonstrated that E1 and E2 proteins synthesized in insect cells associate in vitro (3a). In this study, we have confirmed that E1 complexes with other viral proteins such as E2 in vitro and may thereby bind specific regions of the BPV-1 genome.

MATERIALS AND METHODS

Plasmids. The baculovirus transfer vector pVL941 contains the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) flanked on either side by 3 kb of viral sequences (20). Following a Klenow fill-in reaction of the *Avr*II site at position 2766, synthetic *Bam*HI linkers (New England BioLabs) were ligated to the ends of the *Nru*I (position 838)–*Avr*II (position 2766) fragment containing the BPV-1 E1 ORF. This fragment was then ligated into the unique *Bam*HI site immediately downstream of the polyhedrin promoter in pVL941 to produce plasmid pVL-BPVE1 (Fig. 1). The plasmid pEX-BPVE1 was constructed by inserting the E1-containing *Nru*I-*Avr*II fragment of

* Corresponding author.

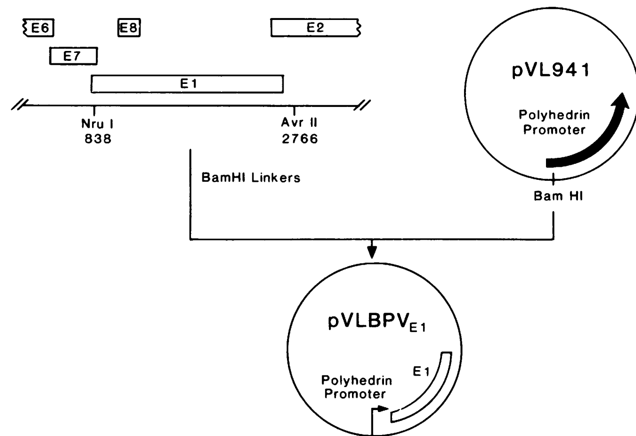

FIG. 1. Construction of an E1-expressing recombinant baculovirus. The E1 ORF of BPV-1 was cloned into a unique *Bam*HI site located downstream of the polyhedrin promoter in the baculovirus transfer vector pVL941 to produce plasmid pVLBPVE1. This plasmid was then used in in vivo homologous recombination with the wild-type baculovirus genome to generate recombinant virus.

BPV-1 between the *Sma*I and *Xba*I sites of pEX3 (44), resulting in an in-frame fusion of the E1 sequences to the *lacZ'* gene.

Recombinant baculovirus construction and infections. Sf9 cells, a cell line derived from the fall armyworm *Spodoptera frugiperda*, were cotransfected (46) with 2 μg of pVL-BPVE1 and 1 μg of wild-type AcNPV DNA. Supernatants containing virus were harvested, and recombinant virus was identified as occlusion-minus plaques in a standard plaque assay (46). Recombinant virus isolates were then purified following multiple rounds of plaque assays. At 60 h postinfection, Sf9 cells were incubated for 1 h in Grace's insect medium (GIBCO) lacking methionine and then incubated in the presence of [^{35}S]methionine (200 μCi/ml) for 4 h. Cells were lysed in sample buffer (62 mM Tris, 10% [vol/vol] glycerol, 5% [vol/vol] β-mercaptoethanol, 2.3% [wt/vol] sodium dodecyl sulfate [SDS], 0.005% [wt/vol] bromophenol blue, pH 6.8), and total cell protein was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. One viral isolate, vE1, was chosen for use in this study. The recombinant baculovirus vFE2 expresses the E2 protein of BPV-1 under the control of the polyhedrin promoter, and its construction will be described elsewhere (28b).

Antibody production and Western immunoblotting. A Cro–β-galactosidase–E1 fusion protein was synthesized from the plasmid pEX-BPVE1 in *Escherichia coli* POP2316 cells. This fusion protein was purified from inclusion bodies by gel filtration chromatography on a Sepharose-CL4B (Pharmacia) column (100 cm by 5.3 cm^2) in the presence of 5% SDS (11). Partially purified E1 fusion protein was injected subcutaneously into two female New Zealand White rabbits with complete Freund's adjuvant (Sigma). Rabbits were boosted with E1 fusion protein in incomplete Freund's adjuvant and sera were collected 14 days after each boost. Sera from one of these rabbits were designated P-951 and I-951 for preimmune and postimmune sera, respectively. The following antibody preparations were provided by E. Androphy (unpublished data): antisera 502-1 and 502-2 were prepared by immunization of two rabbits with the carboxy-terminal E1 decapeptide CSARNTNAVD; polyclonal antiserum P24 was prepared against a fusion protein containing amino-terminal E1 amino acids 34 to 246; monoclonal antibodies B202, B203, B204, and B205 were generated against the BPV-1 E2 protein and recognize uncharacterized epitopes in the E2 carboxy terminus; and polyclonal antiserum II-1 was prepared against a fusion protein containing E2 amino acids 114 to 410 (1). For Western blot analysis, infected Sf9 cells at 60 h postinfection were lysed in sample buffer, and total cell protein was analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose (51) and separately probed with P-951 and I-951 sera. Bound antibody was visualized with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and 4-chloro-1-naphthol according to the manufacturer's instructions (Bio-Rad Laboratories).

Phosphorylation. At 48 h postinfection, Sf9 cells infected with wild-type AcNPV or vE1 were incubated for 1 h at 27°C in phosphate-free Grace's medium, followed by the addition of $^{32}P_i$ (500 μCi/ml). After additional incubations for 1 to 4 h, cells were lysed in sample buffer, and the lysate was treated with RNase A. ^{32}P-labeled proteins were analyzed by SDS-PAGE and visualized by autoradiography.

Subcellular fractionation and immunofluorescence. At 40 h postinfection, Sf9 cells were resuspended in STM buffer (250 mM sucrose, 10 mM Tris hydrochloride [Tris-HCl, pH 8], 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg of aprotinin per ml) and lysed with a Potter-Elvejem homogenizer, and nuclei were pelleted by centrifugation at 500 × *g* for 10 min at 4°C. The resultant supernatant was collected and centrifuged at 150,000 × *g* for 30 min at 4°C to separate cytoplasmic membranes (pellet) from soluble cytoplasmic protein (supernatant). Nuclei were washed once with ice-cold STM and incubated in 100 mM NaCl–10 mM Tris-HCl (pH 8)–1% Nonidet P-40–1 mM dithiothreitol–10 μg of aprotinin per ml for 30 min at 4°C. Extracted nuclei were then centrifuged at 150,000 × *g* for 90 min at 4°C to separate insoluble nuclear material (pellet) from soluble nucleoplasmic proteins (supernatant). Material from each fraction was analyzed by 7.5% SDS-PAGE, followed by Western blot analysis with anti-E1 antiserum I-951. For immunofluorescence, cells were infected with wild-type AcNPV or vE1 and, at 48 h postinfection, fixed in 37 mM sodium phosphate (pH 7.4)–10 mM sodium *m*-periodate–75 mM lysine–2% paraformaldehyde (26). Fixed cells were then treated with 0.5% Triton X-100 in phosphate-buffered saline and incubated with anti-E1 antiserum I-951. Bound antibody was reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Jackson Labs). Cells were examined by both phase contrast and fluorescence microscopy.

McKay immunoprecipitation DNA binding assay. A modification of the DNA binding assay of McKay (25) was used. Antiserum 502-2 was first incubated with protein A-Sepharose (Sigma), and then antibody-protein A complexes were incubated with nuclear extracts (8, 39) of vE1-infected Sf9 cells in buffer A (50 mM Tris-HCl [pH 7.2], 0.5% Nonidet P-40, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml) containing 300 mM NaCl. The beads were then washed and resuspended in buffer A containing 150 mM NaCl. The plasmid pdBPV-1, containing the viral genome cloned into pML2d (35), was digested with *Sau*3A and end-labeled with Klenow and [α-^{32}P]dCTP (23). E1-antibody-protein A-Sepharose complexes were incubated with probe for 20 min at 20°C, and the protein-DNA complexes were centrifuged at 10,000 × *g* at 4°C for 5 s. The beads were then washed once in ice-cold buffer A containing 150 mM NaCl. Bound DNA was eluted in 10 mM Tris-HCl (pH 8)–1 mM EDTA–1%

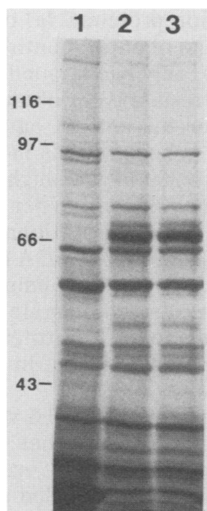

FIG. 2. Expression of E1 protein by a recombinant baculovirus. Sf9 cells were infected with the wild-type baculovirus (lane 1) or recombinant viral isolates (lanes 2 and 3). Cells were incubated with [^{35}S]methionine, and protein was analyzed by 7.5% SDS-PAGE and autoradiography. Apparent sizes are shown to the left (in kilodaltons). The E1 protein migrates at 68 kDa (lanes 2 and 3).

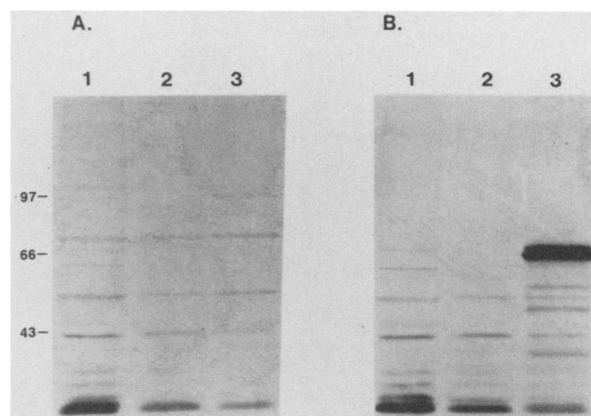

FIG. 3. Western blot of E1 protein expressed in insect cells. Proteins from uninfected Sf9 cells (lanes 1), wild-type baculovirus-infected cells (lanes 2), and vE1-infected cells (lanes 3) were run on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with preimmune serum P-951 (A) or anti-E1 immune serum I-951 (B). Bound antibody was visualized with horseradish peroxidase-conjugated secondary antibody. Apparent sizes are shown to the left (in kilodaltons).

SDS. DNA was then extracted with phenol-$CHCl_3$, ethanol precipitated, and analyzed on a 50-cm-long nondenaturing 5% polyacrylamide gel. The gels were dried, and radiolabeled fragments were visualized by autoradiography.

Immunoprecipitations. At 44 h postinfection, Sf9 cells were incubated in cysteine-free Grace's insect medium for 1 h at 27°C. [^{35}S]cysteine (900 Ci/mmol) was added (200 μCi of [^{35}S]cysteine per ml of medium), and the cells were further incubated for 4 h. Cells were then pelleted and resuspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM dithiothreitol, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine hydrochloride, 1 μg of pepstatin per ml, 1 μg of leupeptin per ml). Cells were incubated for 20 to 30 min at 4°C with gentle agitation, and cellular debris was pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was removed, and antibody-complexed protein A-Sepharose was added. Following incubation for 1 h at 4°C, the Sepharose beads were pelleted and washed three times with ice-cold lysis buffer. Bound proteins were eluted with sample buffer and analyzed by SDS-PAGE. Following electrophoresis, the gels were treated with fluor (En^{3}Hance; NEN) and dried. ^{35}S-labeled proteins were visualized by fluorography.

RESULTS

Construction of a recombinant baculovirus expressing E1 protein. A recombinant baculovirus expressing the entire E1 ORF of BPV-1 was constructed by homologous recombination (46) between the wild-type baculovirus genome and a plasmid containing the E1 ORF cloned downstream of the polyhedrin promoter (Fig. 1). Recombinant viruses were isolated, and single isolates were purified by multiple rounds of plaque assays (46). In order to screen recombinant viral isolates for E1 expression, Sf9 cells were infected with each of the isolates or wild-type virus, and at 60 h postinfection, proteins were metabolically labeled with [^{35}S]methionine. Analysis of labeled proteins with SDS-PAGE revealed a 68-kDa polypeptide which was present in recombinant-virus infections (Fig. 2) but absent in wild-type-virus infections. Western blot analysis with rabbit polyclonal antiserum I-951, directed against a β-galactosidase–E1 fusion protein, confirmed that this 68-kDa protein was the E1 protein (Fig. 3). Lower-molecular-weight proteins present in vE1-infected cells reacted with the I-951 antiserum but not the preimmune serum and so are probably minor degradation products of E1. One viral isolate, vE1, was chosen for further analysis.

E1 protein is a phosphoprotein. We first investigated whether the E1 protein, like T antigen of SV40, was phosphorylated. At 48 h postinfection, vE1-infected cells were incubated with $^{32}P_i$ for 1 to 4 h. Cells were lysed in sample buffer, and total cell protein was analyzed by SDS-PAGE and autoradiography. As shown in Fig. 4, the 68-kDa E1 protein was prominently labeled with $^{32}P_i$, demonstrating that E1 is a phosphoprotein.

E1 protein resides in the nucleus. To determine the intracellular location of E1, we performed cellular fractionation studies on vE1-infected cells. Studies with many proteins have shown that insect cells accurately target a number of expressed proteins to the same subcellular locations as found in mammalian cells (19, 28). At 48 h postinfection, vE1-infected insect cells were fractionated into subcellular components (12) and analyzed on an SDS-polyacrylamide gel. Electrophoretically separated proteins were subsequently transferred to nitrocellulose and probed with anti-E1 polyclonal serum I-951 (Fig. 5). Nearly all of the detectable E1 protein was found in nuclear fractions (lanes 1 and 2). A majority of this protein was associated with the insoluble fraction of the nucleus, while a smaller portion was found in the nucleoplasm. Since the E1 protein aggregates at low salt concentrations (below 250 mM; data not shown), a substantial proportion of E1 protein found in the insoluble fraction may have resulted from this property. In similar experiments, no proteins of molecular weight comparable to E1 were detected by the preimmune serum in any fraction (data not shown).

In order to confirm that E1 resides in the nucleus, we performed immunofluorescence studies. Sf9 cells were in-

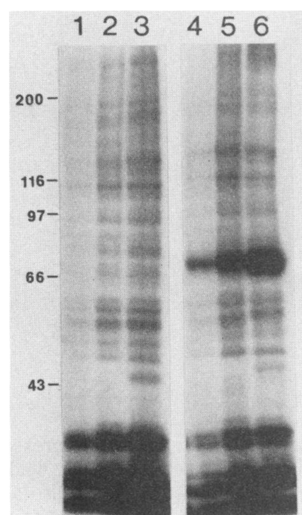

FIG. 4. E1 is a phosphoprotein. Wild-type baculovirus-infected (lanes 1, 2, and 3) and vE1-infected (lanes 4, 5, and 6) cells were incubated with $^{32}P_i$ for 1 h (lanes 1 and 4), 2 h (lanes 2 and 5), or 4 h (lanes 3 and 6), and total protein was analyzed on a 7.5% SDS–polyacrylamide gel, followed by autoradiography. Apparent sizes are shown to the left (in kilodaltons).

fected with vE1 virus or wild-type baculovirus. At 48 h postinfection, the fixed cells were treated with anti-E1 polyclonal serum I-951, incubated with a FITC-conjugated secondary antibody, and examined by phase contrast and immunofluorescence microscopy. Only cells infected with the vE1 recombinant baculovirus and treated with anti-E1 immune serum exhibited intense fluorescence (Fig. 6). The majority of the fluorescence was localized to the nucleus, consistent with the localization found by subcellular fractionation.

E1 protein by itself binds DNA in a nonspecific manner. Since it seemed likely that a protein required for viral DNA replication would interact with the viral genome, we investigated whether the E1 protein possesses DNA-binding activity. Because of the limited solubility of the E1 protein in NaCl concentrations below 250 mM, DNA-cellulose chromatography and gel mobility shift analyses are problematic since they are typically performed at NaCl concentrations in the 100 to 150 mM range. Instead, we used a modification of the McKay immunoprecipitation DNA binding assay (25). This assay circumvented the solubility problem, since the E1 protein in nuclear extracts of vE1-infected Sf9 cells was first complexed to protein A-Sepharose-bound anti-E1 antibodies in 300 mM NaCl. The bound complex was then washed and subsequently resuspended in a 150 mM NaCl buffer for the DNA binding reaction.

In this protocol, E1-antibody-protein A-Sepharose complexes were incubated with an end-labeled *Sau*3A digest of pdBPV-1 DNA. *Sau*3A digestion of this plasmid, which contains the entire BPV-1 genome cloned into pML2d, generates 35 fragments. Bound DNA fragments were sedimented, eluted, and visualized by autoradiography following electrophoresis on nondenaturing polyacrylamide gels (Fig. 7). vE1-infected cell extracts bound 5- to 10-fold more labeled DNA (determined by Cerenkov radiation) than did identical extracts prepared from wild-type baculovirus-infected insect cells (Fig. 7A, compare lanes 2 and 3). When polyclonal antiserum directed against a Cro–β-galactosidase–HPV-18 E7 fusion protein (24a) or nonimmune rabbit serum was used instead of anti-E1 antiserum (Fig. 7A, lanes 4 and 5, respectively), only background levels of DNA were precipitated. Similar results were obtained when either the E1 extract (lane 6) or the anti-E1 antiserum (lane 7) was omitted. When NaCl concentrations in the binding reaction mix were increased to 300 mM or higher, all DNA binding was abolished (data not shown). When similar experiments were performed with anti-E2 polyclonal antiserum II-1 and vFE2-infected Sf9 cell extracts, *Sau*3A fragments containing E2 binding sites were specifically immunoprecipitated (data not shown). These results demonstrate that the DNA-binding activity was specific to the E1 protein, but no preference for any of the 35 fragments present in the binding reaction mix was observed.

Since all of the fragments bound E1 with apparently equal efficiency, the possibility existed that specific binding to a fragment(s) could be observed by saturating nonspecific binding with the addition of unlabeled nonspecific competitor DNA. Therefore, we added increasing amounts of unlabeled nonspecific competitors, such as poly(dI · dC) (data not shown) and salmon sperm DNA (Fig. 7B), to DNA-binding reaction mixes. We found that binding to all of the fragments were reduced with approximately equal efficiency with increasing amounts of competitor DNA. We can conclude from these data that E1 protein by itself has nonspecific DNA-binding activity.

E1 protein associates with the E2 transcriptional activator in vitro. Since E1 alone did not bind specifically to any sequences in the BPV-1 genome, the possibility existed that E1 interacts with another protein and that together the complex could bind DNA with specificity. We mixed extracts from BPV-transformed C127 cells with extracts of vE1-infected SFg cells and attempted McKay assays as before; however, no changes in DNA binding were observed. Mohr et al. have presented evidence that the E1 protein interacts with the full-length E2 gene product in vitro (28a). Genetic evidence suggests that the E2 protein is a good candidate to form a complex with E1, since E2 mutants are defective for extrachromosomal maintenance of BPV genomes (9, 10, 13, 32, 36). To examine whether E1 and E2 interact, we made use of

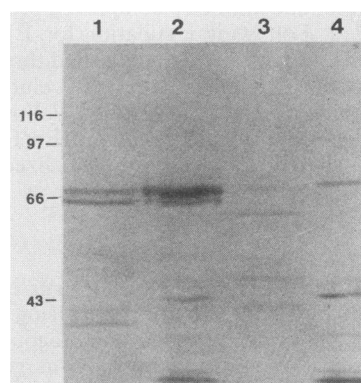

FIG. 5. Subcellular fractionation of vE1-infected cells. Cells were disrupted and fractionated as described in the text, and subcellular fractions were analyzed on a 7.5% SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose and visualized with anti-E1 antiserum I-951 and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Lane 1, Nucleoplasm; lane 2, insoluble nuclear material; lane 3, postnuclear cytoplasmic fraction; lane 4, nonnuclear membranes. Sizes are shown in kilodaltons.

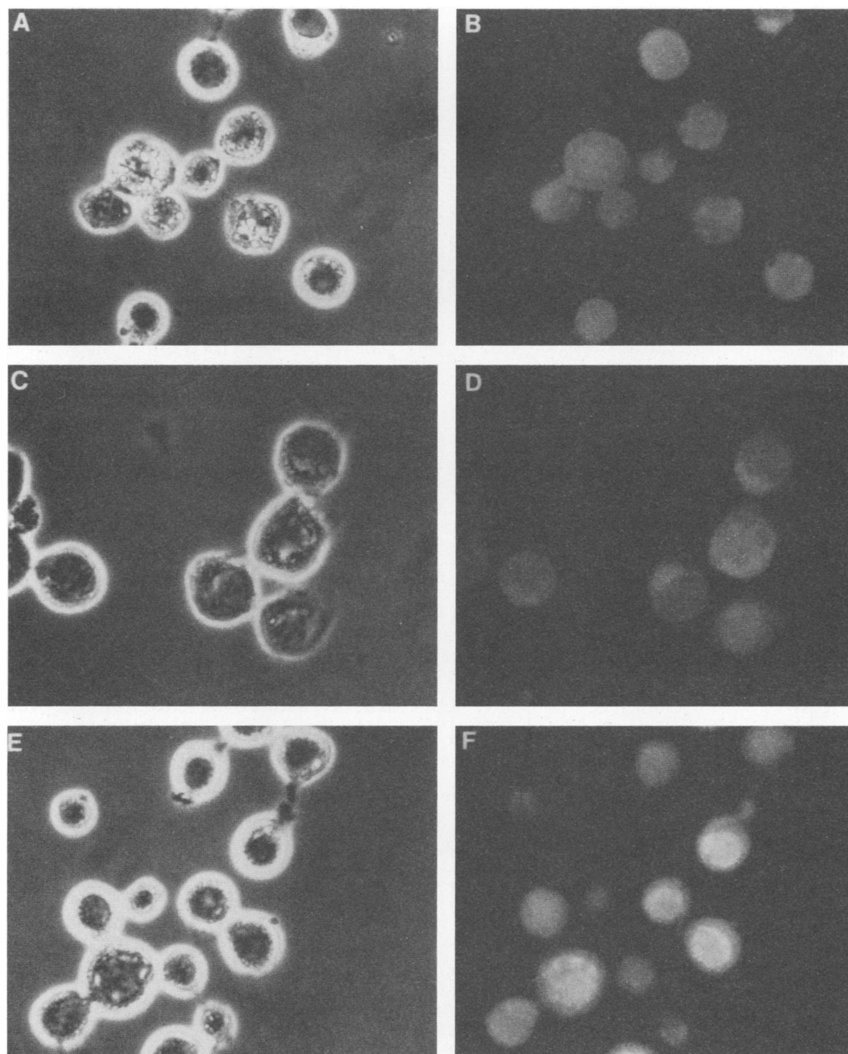

FIG. 6. Immunofluorescence of vE1-infected cells. Sf9 cells were infected with wild-type baculovirus (AcNPV) or vE1, and at 48 h postinfection cells were fixed and stained with anti-E1 antiserum I-951 or preimmune serum P-951 and FITC-conjugated secondary antibody. (A, C, and E) Phase contrast micrographs. (B, D, and F) Same fields visualized with fluorescence microscopy. (A and B) AcNPV-infected cells treated with immune serum. (C and D) vE1-infected cells treated with preimmune serum. (E and F) vE1-infected cells treated with immune serum.

a recombinant baculovirus, vFE2, which contains the entire E2 ORF under the control of the polyhedrin promoter. Sf9 cells were either infected singly with baculovirus expressing BPV-1 E1 or the full-length BPV-1 E2 or doubly infected with both viruses. At 44 h postinfection, cells were incubated in the presence of [^{35}S]cysteine for 4 h, and whole-cell extracts were prepared. The extracts were then incubated with protein A-Sepharose-immobilized antibodies directed against E1 or E2, and pelleted immune complexes were analyzed by SDS-PAGE. Efficient immunoprecipitation of baculovirus-synthesized E2 protein was observed with five different antibody preparations directed against the E2 protein (four monoclonal antibodies [B202, B203, B204, and B205] and one polyclonal antiserum [II-1]). Three representative immunoprecipitations are shown in Fig. 8 (lanes 5, 8, and 11). Little or no precipitation of E1 protein from vE1-infected cells was observed with the E2 antibodies (lanes 4, 7, and 10). In contrast, when doubly infected (vE1 plus vFE2) cell extracts were incubated with these antibodies, both E1 and E2 were precipitated (lanes 6, 9, and 12). While numerous other baculovirus proteins are labeled in this protocol, only the E1 protein in the presence of E2 was precipitated by E2-specific antibodies, further suggesting that a specific interaction exists. The precipitation of an E1-E2 complex was observed even at NaCl concentrations up to 1 M, suggesting a strong interaction between the two proteins. In addition, mixing singly infected cell extracts followed by immunoprecipitation with anti-E2 monoclonal antibody B202 gave results indistinguishable from that seen in vE1 plus vFE2 coinfections (data not shown), demonstrating that cotranslation of E1 and E2 is not required for complex formation.

We next examined the ability of E1 antibodies to precipitate an E1-E2 complex. Polyclonal antisera directed against either a Cro–β-galactosidase–E1 fusion protein (I-951), an amino-terminal E1 fragment (P24), or a 10-amino-acid synthetic peptide from the carboxy terminus of E1 (502-2) were incubated with whole-cell extracts prepared from Sf9 cells

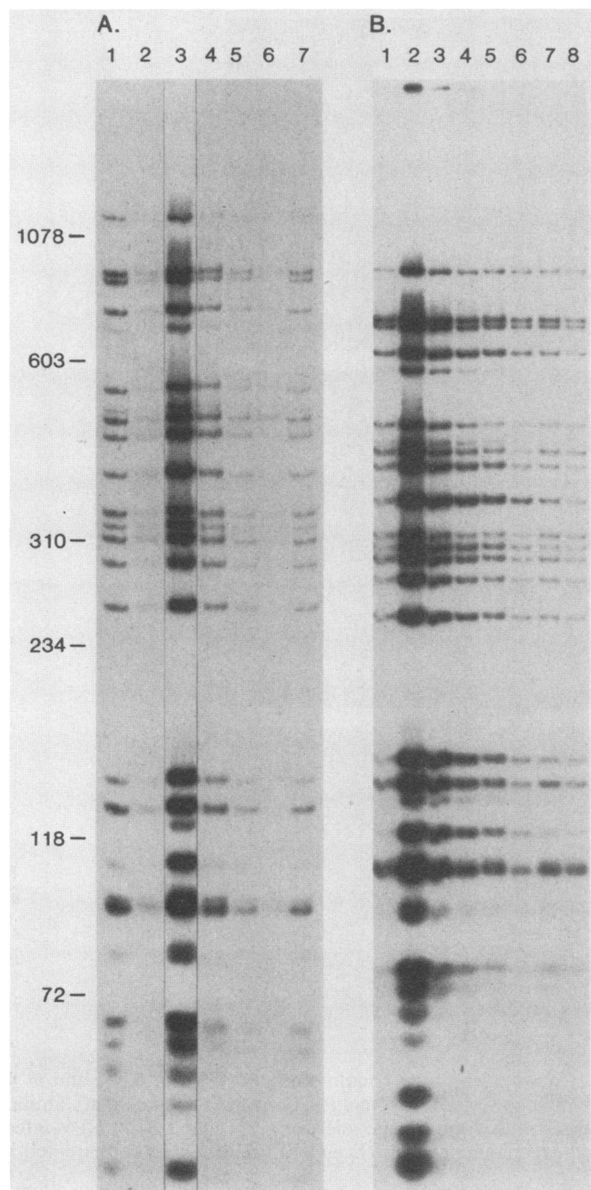

FIG. 7. McKay DNA-binding assays. Radiolabeled *Sau*3A fragments from the BPV-1 genome (in pdBPV-1) were incubated with antibody-protein A-Sepharose complexes and various nuclear extracts of baculovirus-infected cells as described in Materials and Methods. Bound DNA fragments were immunoprecipitated, eluted, and resolved on 5% polyacrylamide gels. Separate experiments are presented in panels A and B. (A) Lane 1, free probe; lanes 2 to 7, fragments immunoprecipitated with a wild-type baculovirus extract (lane 2), with a vE1-infected cell extract (lane 3), with an anti-E7 antiserum (lane 4), with a nonimmune serum (lane 5), without extract (lane 6), and without antiserum (lane 7). (B) Lane 1, free probe; lane 2, DNA bound by protein in a vE1-infected cell extract; lanes 3 to 8, labeled DNA bound in the presence of 2, 5, 10, 20, 50, and 100 μg of unlabeled salmon sperm DNA, respectively. Sizes are shown in kilodaltons.

infected with vE1 or vE2 recombinant baculoviruses. All three antisera precipitated a 68-kDa E1 protein from vE1-infected cells (Fig. 8, lanes 13 and 16), and little or no E2 protein from vFE2-infected cells (lanes 14 and 17). Incuba-

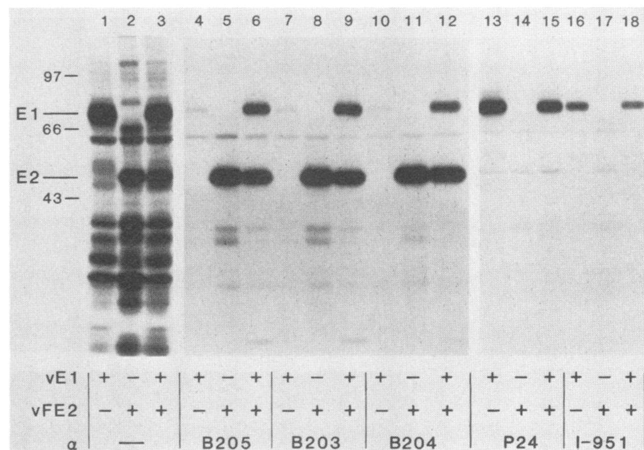

FIG. 8. Immunoprecipitations. Infected Sf9 cell proteins were radiolabeled with [^{35}S]cysteine, and proteins were analyzed on a 9% SDS–polyacrylamide gel. Sf9 cells were infected with vE1 and vFE2 alone or in combination, as indicated below the photograph. The α refers to the antibody preparation used in each immunoprecipitation as described in Materials and Methods.

tion of the antisera with doubly infected (vE1 plus vFE2) cell extracts resulted in the immunoprecipitation of only E1 protein (lanes 15 and 18). We suspect that this failure to precipitate a complex may be due either to the masking of E1 epitopes in an E1-E2 complex or to disruption of the E1-E2 interaction by anti-E1 antibodies.

DISCUSSION

In this study, we describe a biochemical characterization of the 68-kDa E1 protein of BPV-1. Since the abundance of the E1 protein in transformed cells is extremely low, we have overexpressed E1 in insect cells, using a baculovirus expression vector. This system has the advantage that proteins synthesized in insect cells are posttranslationally modified in a manner similar to that observed in mammalian cells (19, 28). In addition, the baculovirus-insect cell expression system has also been shown to localize recombinant proteins to the same intracellular locations as observed in mammalian cells (19, 28).

We have shown that the 68-kDa E1 protein produced in insect cells is phosphorylated similar to its state in transformed mouse cells (34a, 47). Several nuclear phosphoproteins produced in insect cells, including SV40 T antigen (14), BPV-1 E2 (24), and c-Fos (52), are phosphorylated at the same sites as their counterparts produced in mammalian cells. It has been demonstrated that phosphorylation affects the DNA binding and origin-unwinding activities of SV40 T antigen and is thought to play a role in modulating SV40 DNA replication (reviewed in reference 31). While it is likely that E1 has a similar role in BPV-1 replication, the function of E1 phosphorylation is presently unknown.

We have also demonstrated that the E1 protein resides in the nucleus. Since papillomavirus DNA replication occurs in the nucleus of infected cells (29), it is likely that E1 is a nuclear protein, but this has not yet been demonstrated in papillomavirus-infected cells in vivo. The nuclear localization of E1 is consistent with a direct role in viral DNA replication.

Significant similarities exist between the T antigens and the E1 proteins (5, 38) in the carboxy terminus, which is

required for T antigen's helicase activity (7, 43, 53). In contrast, only a limited sequence similarity between the T antigens and the E1 proteins is found in the amino terminus, and this sequence lies outside the region of SV40 T antigen which has been shown to contain its minimal DNA binding domain (2, 27, 30, 40, 41, 45). T antigen binds to sequences in the SV40 origin of replication (49), but site-specific DNA binding of E1 protein to papillomavirus DNA sequences was not detected in this study. This inability to demonstrate site-specific DNA binding may either reflect a deficiency of the in vitro DNA binding assays used in this study or suggest that other proteins complex with E1 to confer DNA binding ability.

In an attempt to identify potential auxiliary proteins which might facilitate the binding of an E1 protein to DNA, we investigated a role for the E2 protein. In this study, we used coinfection of two recombinant baculoviruses expressing the E1 and E2 proteins to demonstrate that E2 is capable of forming a complex with E1. This methodology is similar to that used previously to demonstrate that the E1 protein interacts with the full-length E2 gene product in vitro (28a). Five different E2-specific antibody preparations (four monoclonal antibodies and one polyclonal antiserum) were capable of precipitating an E1-E2 complex from doubly infected cells. No E1 protein was precipitated with E2-specific antibodies from vE1-infected cells, demonstrating that the coimmunoprecipitation observed from doubly infected cells is not due to cross-reactivity of these antibodies with E1. In contrast, none of the three E1-specific antisera we examined were capable of precipitating an E1-E2 complex from doubly infected cells. This may be due to the masking of epitopes on the E1 protein by complex formation with E2, or high-affinity binding of antibodies to E1 may compete for interaction with E2, thereby disrupting the complex. Since five different E2-specific antibodies were capable of specifically coprecipitating E1 and E2, we believe that this is strong evidence for complex formation in vitro. The interaction appears to be specific, since other labeled proteins also present in the extracts are not precipitated by the E2 antibody. Furthermore, the interaction appears to be of high affinity, as the interaction is stable in high salt concentrations.

Complex formation between E1 and E2 was observed in insect cells, which produce high levels of these proteins, and it is unclear whether the interaction between E1 and E2 represents a physiologically relevant phenomenon. However, results of genetic analyses are consistent with a role for E2 in replication. Mutants with alterations in the E2 ORF are unable to maintain themselves as extrachromosomal elements (9, 10, 13, 32, 36). Whether this is due to an indirect effect of E2 on transcription or a direct role in viral DNA replication will require further investigation.

It is intriguing to speculate that the E1-E2 interaction reflects an in vivo association and that this complex may bind to the viral genome to initiate DNA replication. Numerous examples exist of multicomponent factors in which the individual subunits cooperate to bind DNA specifically, including the herpes simplex virus VP16/Oct-1 (15, 16), the *c-fos*/*c-jun* (33), and the UBF/SL1 (18) complexes. It is of great interest to determine the DNA binding character of the E1-E2 complex because there are multiple E2 binding sites in the BPV-1 genome which could serve as the target for E1-E2 binding. We have not performed McKay DNA binding experiments with the E1-E2 complex because none of the E1 antibodies we have tested immunoprecipitated E2 in association with E1. The use of E2 antibodies in these experiments will probably be complicated by the specific DNA-binding activity of the uncomplexed E2 protein.

In this study we have identified E1 as a nuclear phosphoprotein which binds DNA nonspecifically. Our studies show that the full-length E1 protein forms a complex with E2 which may then interact with DNA. Additional studies are in progress to characterize the DNA-binding properties and biochemical activities of the E1-E2 complex.

ACKNOWLEDGMENTS

I. B. was supported by a predoctoral fellowship from the Lucille P. Markey Charitable Trust and a gift from the Searle Foundation. This work was supported by the Howard Hughes Medical Institute.

We acknowledge Dan DiMaio, Steve Grossman, David Mack, Jeremy Paul, Harinder Singh, and Vikas Sukatme for critical reading of the manuscript, expert advice, and many stimulating conversations. We thank Max Summers and Peter Howley for providing the plasmids pVL941 and pdBPV-1, respectively. We are especially grateful to Terry Martin for assistance with immunofluorescence and Elliot Androphy for providing antibodies.

REFERENCES

1. **Androphy, E. J., D. R. Lowy, and J. T. Schiller.** 1987. Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. Nature (London) **325**:70–73.
2. **Arthur, A. K., A. Hoss, and E. Fanning.** 1988. Expression of simian virus 40 T antigen in *Escherichia coli*: localization of T-antigen-origin DNA-binding domain to within 129 amino acids. J. Virol. **62**:1999–2006.
3. **Berg, L., M. Lusky, A. Stenlund, and M. R. Botchan.** 1986. Repression of bovine papilloma virus replication is mediated by a virally encoded transacting factor. Cell **46**:753–762.

3a. **Botchan, M.** 1990. Abstr. Papillomavirus Workshop, 1990. Heidelberg, Federal Republic of Germany.

4. **Broker, T. R., and M. Botchan.** 1986. Papillomaviruses: retrospectives and prospectives. Cancer Cells **4**:17–36.
5. **Clertant, P., and I. Seif.** 1984. A common function for polyoma virus large-T and papillomavirus E1 proteins? Nature (London) **311**:276–279.
6. **Danos, O., I. Giri, F. Thierry, and M. Yaniv.** 1984. Papillomavirus genomes: sequences and consequences. J. Invest. Dermatol. **83**:7s–11s.
7. **Dean, F. B., P. Bullock, Y. Murakami, C. R. Wobbe, L. Weissbach, and J. Hurwitz.** 1987. Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **84**:16–20.
8. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
9. **DiMaio, D.** 1986. Nonsense mutation in open reading frame E2 of bovine papillomavirus. J. Virol. **57**:475–480.
10. **DiMaio, D., and J. Settleman.** 1988. Bovine papillomavirus mutant temperature sensitive for transformation, replication, and transactivation. EMBO J. **7**:1197–1204.
11. **Doorbar, J., D. Cambell, R. J. A. Grand, and P. H. Gallimore.** 1986. Identification of the human papillomavirus-1a E4 gene products. EMBO J. **7**:355–362.
12. **Graham, J.** 1984. Isolation of subcellular organelles and membranes, p. 161–182. *In* D. Rickwood (ed.), Centrifugation—a practical approach, 2nd ed. IRL Press, Oxford.
13. **Groff, D. E., and W. D. Lancaster.** 1986. Genetic analysis of the 3′ early region transformation and replication functions of bovine papillomavirus type 1. Virology **150**:221–230.
14. **Hoss, A., I. Moarefi, K.-H. Scheidtmann, L. J. Cisek, J. L. Corden, I. Dornreiter, A. Arthur, and E. Fanning.** 1990. Altered phosphorylation pattern of simian virus 40 T antigen expressed in insect cells by using a baculovirus vector. J. Virol. **64**:4799–4807.
15. **Kristie, T. M., J. H. LeBowitz, and P. A. Sharp.** 1989. The

octamer-binding proteins form multi-protein-DNA complexes with the HSV αTIF regulatory protein. EMBO J. **8**:4229–4238.

16. **Kristie, T. M., and B. Roizman.** 1987. Host cell proteins bind to the *cis*-acting site required for virion-mediated induction of herpes simplex virus 1 α genes. Proc. Natl. Acad. Sci. USA **84**:71–75.
17. **Lambert, P. F., and P. M. Howley.** 1988. Bovine papillomavirus type 1 E1 replication-defective mutants are altered in their transcriptional regulation. J. Virol. **62**:4009–4015.
18. **Learned, R. M., T. K. Learned, M. M. Haltiner, and R. T. Tjian.** 1986. Human rRNA transcription is modulated by the coordinate binding of two factors to an upstream control element. Cell **45**:847–857.
19. **Luckow, V. A., and M. D. Summers.** 1987. Trends in the development of baculovirus expression vectors. Bio/Technology **6**:47–55.
20. **Luckow, V. A., and M. D. Summers.** 1989. High level expression of nonfused foreign genes with *Autographa californica* nuclear polyhedrosis virus expression vectors. Virology **170**:31–39.
21. **Lusky, M., and M. R. Botchan.** 1985. Genetic analysis of bovine papillomavirus type 1 *trans*-acting replication factors. J. Virol. **53**:955–965.
22. **Lusky, M., and M. R. Botchan.** 1986. Transient replication of bovine papillomavirus type 1 plasmids: *cis* and *trans* requirements. Proc. Natl. Acad. Sci. USA **83**:3609–3613.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 115–116. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **McBride, A. A., J. B. Bolen, and P. M. Howley.** 1989. Phosphorylation sites of the E2 transcriptional regulatory protein of bovine papillomavirus type 1. J. Virol. **63**:5076–5085.

24a. **McIntyre, M., and L. A. Laimins.** Unpublished data.

25. **McKay, R. D. G.** 1981. Binding of a simian virus 40 T antigen-related protein to DNA. J. Mol. Biol. **145**:471–488.
26. **McLean, I. W., and P. H. Nakahe.** 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. J. Histochem. Cytochem. **22**:1077–1083.
27. **McVey, D., M. Strauss, and Y. Gluzman.** 1989. Properties of the DNA-binding domain of the simian virus 40 large T antigen. Mol. Cell. Biol. **9**:5225–5536.
28. **Miller, L. K.** 1988. Baculoviruses as gene expression vectors. Annu. Rev. Microbiol. **42**:177–199.

28a. **Mohr, I., R. Clark, S. Sun, E. Androphy, P. MacPherson, and M. Botchan.** Science, in press.

28b. **Monini, P., S. G. Grossman, E. J. Androphy, and L. A. Laimins.** Unpublished data.

29. **Orth, G., P. Jeanteur, and O. Croissant.** 1971. Evidence for and localization of vegetative viral DNA replication by autoradiographic detection of RNA · DNA hybrids in sections of tumors induced by Shope papilloma virus. Proc. Natl. Acad. Sci. USA **68**:1876–1880.
30. **Paucha, E., D. Kalderon, R. W. Harvey, and A. E. Smith.** 1986. Simian virus 40 origin DNA-binding domain on large T antigen. J. Virol. **57**:50–64.
31. **Prives, C.** 1990. The replication functions of SV40 T antigen are regulated by phosphorylation. Cell **61**:735–738.
32. **Rabson, M. S., C. Yee, Y.-C. Yang, and P. M. Howley.** 1986. Bovine papillomavirus type 1 3′ early region transformation and plasmid maintenance functions. J. Virol. **60**:626–634.
33. **Rauscher, F. J., III, P. J. Voulalas, B. R. Franza, Jr., and T. Curran.** 1990. Fos and Jun bind cooperatively to the AP-1 site: reconstitution in vitro. Genes Dev. **2**:1687–1699.
34. **Roberts, J. M., and H. Weintraub.** 1986. Negative control of DNA replication in composite SV40-bovine papilloma virus plasmids. Cell **46**:741–752.

34a. **Santucci, S., E. J. Androphy, C. Bonne-Andréa, and P. Clertant.** 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.

35. **Sarver, N., J. C. Byrne, and P. M. Howley.** 1982. Transformation and replication in mouse cells of bovine papillomavirus/pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. USA **79**:7147–7151.
36. **Sarver, N., M. S. Rabson, Y.-C. Yang, J. C. Byrne, and P. M. Howley.** 1984. Localization and analysis of bovine papillomavirus type 1 transforming functions. J. Virol. **52**:377–388.
37. **Schiller, J. T., E. Kleiner, E. J. Androphy, D. R. Lowy, and H. Pfister.** 1989. Identification of bovine papillomavirus E1 mutants with increased transforming and transcriptional activity. J. Virol. **63**:1775–1782.
38. **Seif, I.** 1984. Sequence homology between the large tumor antigen of polyoma viruses and the putative E1 protein of papilloma viruses. Virology **138**:347–352.
39. **Shapiro, D. J., P. A. Sharp, W. W. Wahli, and M. J. Keller.** 1988. A high-efficiency HeLa cell nuclear transcription extract. DNA **7**:47–55.
40. **Simmons, D. T.** 1986. DNA-binding region of the simian virus 40 tumor antigen. J. Virol. **57**:776–785.
41. **Simmons, D. T.** 1988. Geometry of the simian virus 40 large tumor antigen-DNA complex as probed by protease digestion. Proc. Natl. Acad. Sci. USA **85**:2086–2090.
42. **Spalholz, B. A., Y.-C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.
43. **Stahl, H., P. Droge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5**:1939–1944.
44. **Stanley, K. K., and J. P. Luzio.** 1984. Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. EMBO J. **3**:1429–1434.
45. **Strauss, M., P. Argani, I. J. Mohr, and Y. Gluzman.** 1987. Studies on the origin-specific DNA-binding domain of simian virus 40 large T antigen. J. Virol. **61**:3326–3330.
46. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin No. 1555.
47. **Sun, S., L. Thorner, M. Lentz, P. MacPherson, and M. Botchan.** 1990. Identification of a 68-kilodalton nuclear ATP-binding phosphoprotein encoded by bovine papillomavirus type 1. J. Virol. **64**:5093–5105.
48. **Thorner, L., N. Bucay, J. Choe, and M. Botchan.** 1988. The product of the bovine papillomavirus type 1 modulator gene (M) is a phosphoprotein. J. Virol. **62**:2474–2482.
49. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13**:165–179.
50. **Tjian, R., and A. Robbins.** 1979. Enzymatic activities associated with a purified SV40 T-antigen-related protein. Proc. Natl. Acad. Sci. USA **76**:610–615.
51. **Towbin, H., T. Staehlin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
52. **Tratner, I., P. De Togni, P. Sassone-Corsi, and I. M. Verma.** 1990. Characterization and purification of human *fos* protein generated in insect cells with a baculoviral expression vector. J. Virol. **64**:499–508.
53. **Wun-Kim, K., and D. T. Simmons.** 1990. Mapping of the helicase and helicase substrate-binding domains on simian virus 40 large T antigen. J. Virol. **64**:2014–2020.