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Characterization of Human Papillomavirus Type 11 E1 and E2 Proteins Expressed in Insect Cells

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The study of human papillomavirus replication has been hampered by the lack of an in vitro system which reliably supports virus replication. Recent results from the bovine papillomavirus (BPV) system indicate that the E1 and E2 proteins are the only viral gene products required for replication. By analogy with simian virus 40 large T antigen, E1 is thought to possess ATPase and helicase activity, which may play a direct role in viral DNA replication. The precise role of E2 is unclear, but it may function in part to help localize E1 to the replication origin. We have initiated a study of replication in the human papillomavirus type 11 system which, by analogy to BPV, has focused on the E1 and E2 proteins of this virus. We have expressed the full-length E1 and E2 proteins in Sf9 insect cells by using a baculovirus expression vector. Both the 80-kDa E1 protein and the 42.5-kDa E2 protein are nuclear phosphoproteins. The E1 and E2 proteins form a heteromeric complex within the insect cells, and both proteins localize to a DNA fragment which contains the viral origin of replication. In addition, we have detected an E1-associated ATPase and GTPase activity, which is likely part of an energy-generating system for the helicase activity which is predicted for this protein. The human papillomavirus type 11 E1 and E2 proteins possess the same replication-associated activities exhibited by the corresponding BPV proteins, suggesting that the replication activities of these viruses are tightly conserved.

Human papillomaviruses (HPVs) are a family of small DNA viruses which induce benign hyperproliferative lesions of the cutaneous and mucosal epithelia. Of the 70 different virus types which have been identified, more than 20 are associated with anogenital lesions (11). Two groups of genital-associated viruses are recognized: those including HPV types 6 and 11 (HPV-6 and HPV-11) which induce primarily benign lesions, including condyloma acuminata, and those such as HPV-16 and HPV-18, which are associated with intraepithelial neoplasia and are considered to be at high risk for progression to cervical cancer (45). Greater than 85% of cervical carcinomas examined are associated with infection by the high-risk HPVs (35).

The PV life cycle is exquisitely attuned to the progressive vertical differentiation which accompanies maturation of the keratinocytes in the epidermis. The actively dividing basal cells maintain the virus as a low-copy-number nuclear plasmid. Vegetative viral replication occurs only in the suprabasal daughter cells, which are nondividing and committed to terminal differentiation. Replication of HPV DNA in cell culture has met with only limited success. Cell lines derived from genital lesions containing HPV-31 (2) and HPV-16 (37) have been shown to maintain the extrachromosomal viral replicon with continued passage in culture, and recently, transfection of HPV plasmid DNAs has been shown to permit transient replication of PV DNA if cotransfected with expression plasmids for the E1 and E2 proteins (7). It is presumably our inability to sufficiently mimic all facets of keratinocyte differentiation in vitro which has precluded the establishment of a productive system for PV infections in cell culture and has limited the genetic analysis of the HPV replication functions.

Bovine PV (BPV) has a genomic organization similar to that of the HPVs (Fig. 1A) (1), and because it can stably replicate as an extrachromosomal element in some established mouse cell lines, BPV has been the model of choice for the study of PV replication functions (12). Both genetic and biochemical evidence have shown that BPV replication is dependent on the virus-encoded E1 and E2 proteins. A variety of deletion, insertion, and frameshift mutations in both the E1 and E2 open reading frames (ORFs) leads to integration of the viral DNA into the host cell chromosome (12, 20). In transient-replication assays, as well as in a cell-free in vitro replication system, E1 and E2 are the only viral proteins required for replication of an origin-containing plasmid (40, 43).

The E1 protein is a nuclear, ATP-binding phosphoprotein which binds to the viral origin of replication (3, 36, 39–41, 43). On the basis of amino acid sequence homologies with the simian virus 40 (SV40) large T-antigen (T-Ag), it has been suggested that E1 possesses ATPase and helicase activities (8, 36, 39). Mutation of a conserved proline (Fig. 1B, *) in the ATP-binding domain of T-Ag makes SV40 temperature sensitive for viral DNA replication (9). This proline residue is well conserved among the PVs, including HPV-11 (Pro-479). Mutation of this residue in BPV E1 inhibits viral DNA replication (39). HPV-11 E1 also contains a basic tract of amino acids (119 to 127) similar to that of BPV E1 and T-Ag (Fig. 1B) which may function as a nuclear localization signal (8, 36, 39).

The E2 protein is a sequence-specific DNA-binding transcriptional modulator encoding both activating and repressing functions (for a review, see reference 20). Computer analysis predicts extensive similarities in secondary structure among the sequenced PV E2 proteins (34). Furthermore, the E2 transactivation activities are conserved as the PV proteins are interchangeable in their ability to activate an enhancer containing the canonical DNA-binding site for the E2 protein, $ACCN_6GGT$ (18, 19, 32). A BPV DNA containing an E2 temperature sensitivity mutation integrates into the host cell chromosome when grown at the nonpermissive

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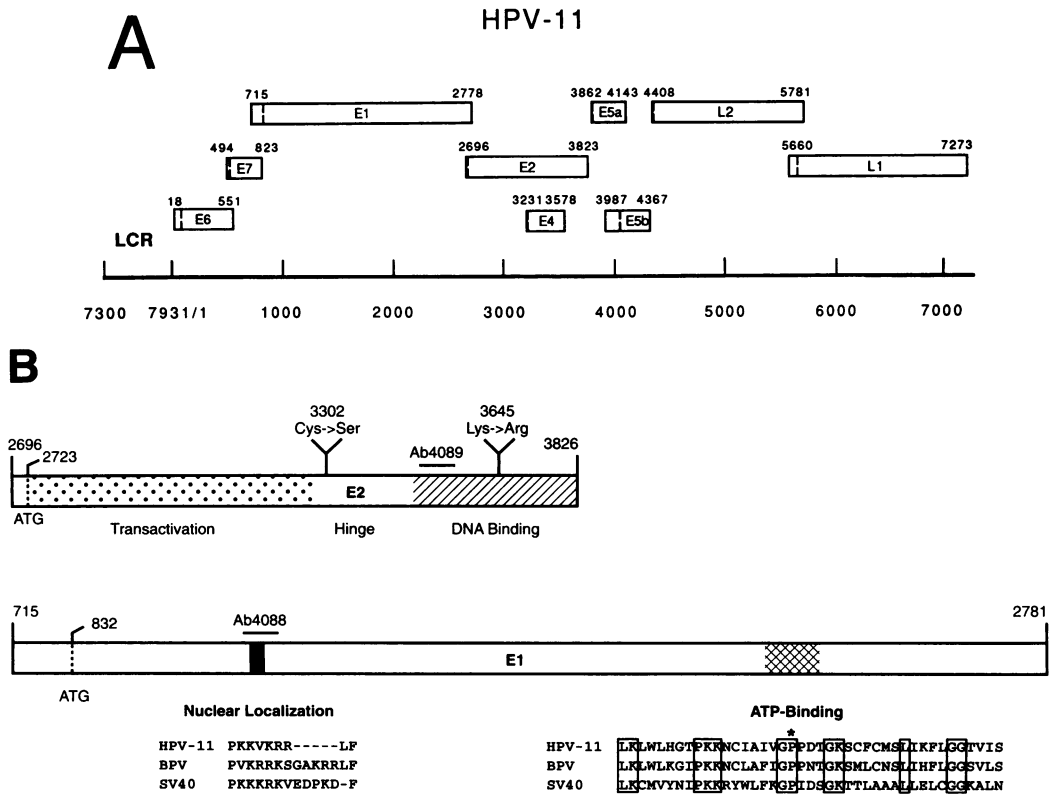
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HPV-11
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2778
E1
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5781
E5a
L2
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E7
2696 3823
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L1
18 551
E6
3231 3578 3987 4367
E4
E5b
LCR
7300 7931/1 1000 2000 3000 4000 5000 6000 7000
B
3302
Cys->Ser
3645
Lys->Arg
2696
2723
Ab4089
3826
E2
ATG
Transactivation
Hinge
DNA Binding
715
832
Ab4088
2781
E1
ATG
Nuclear Localization
HPV-11 PKKVKRR-----LF
BPV PVKRRKSGAKRRLF
SV40 PKKKRKVEDPKD-F
ATP-Binding
HPV-11 LKLWLHGTPKKNCIAIVGPPDTGKSCFCMSLIKFLGGTVIS
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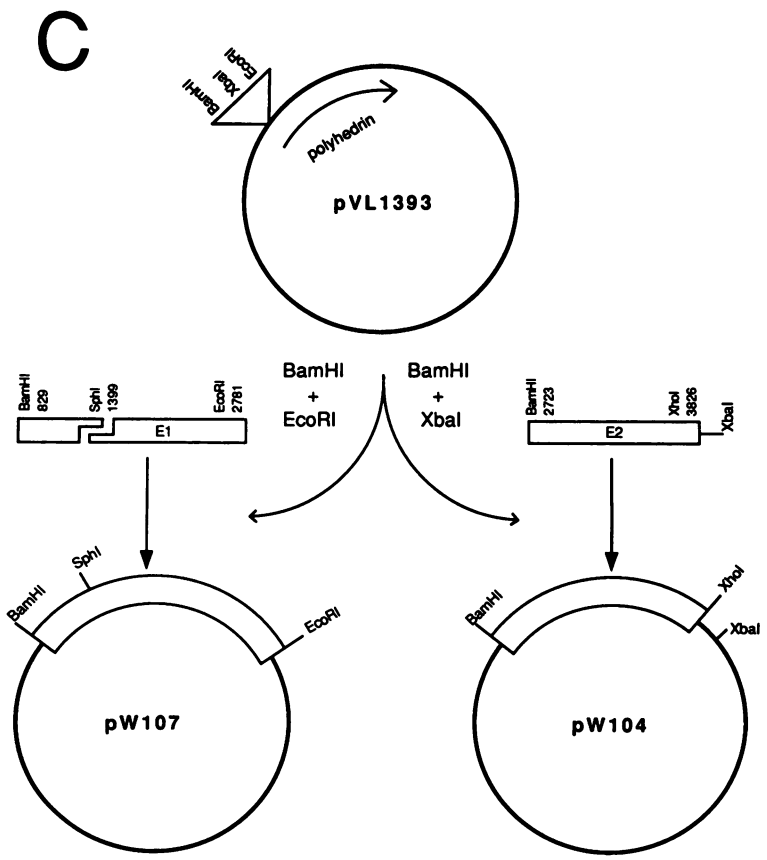
C
BamHI
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XbaI
BamHI
2723
XhoI
3826
XbaI
E2
SphI
BamHI
EcoRI
pW107
BamHI
XhoI
XbaI
pW104

temperature (13), indicating that E2 is required for viral replication; however, the precise role of E2 in replication of the viral genome is unclear.

Recent experiments have shown that at least a fraction of the BPV type 1 (BPV-1) E1 and E2 proteins exist as a heteromeric complex within insect cells (3, 27). Furthermore, this complex is localized to the viral origin of replication (*ori*), which contains the sequences that regulate the initiation of replication (40, 43). In addition, the complex binds to a region ~200 bp upstream of the *ori* that is associated with the onset of bidirectional replication (27, 42). The *ori* maps to a 60-nucleotide DNA fragment centered on the unique *Hpa*I site of BPV (7911 to 22) and contains binding sites for both the E1 and E2 proteins as well as an A+T-rich sequence similar to that found in the origins of other papovaviruses (40, 43). Nucleotide sequence comparison of the HPVs indicates that these viruses contain a similarly organized region spanning nucleotide 1 (7, 40).

In order that we might study HPV DNA replication in more detail and to circumvent the lack of an in vitro tissue culture system, we have undertaken a biochemical study similar to those recently initiated for BPV. The considerable sequence similarities between the HPV and BPV E1 and E2 proteins suggest that the replication and transcriptional modulatory functions may be tightly conserved. In this paper we describe the construction of baculovirus-based plasmids to overexpress the E1 and E2 polypeptides of HPV-11. We have conducted a preliminary analysis of the biochemical activities of the partially purified proteins and demonstrate that both proteins exhibit the replication-associated activities possessed by the counterpart BPV proteins. It is anticipated that these purified proteins can be used to study viral replication in a cell-free system.

MATERIALS AND METHODS

Construction of baculovirus E1 and E2 expression plasmids. The sequences encoding the HPV-11 E1 and E2 proteins were isolated by polymerase chain reaction (PCR) amplification from the HPV-11 clone pW79 (15). The sequence coding for E1 was cloned into baculovirus transfer vector pVL1393 (generously provided by Max Summers, Texas A&M University, College Station) as two fragments which were recombined at the unique, internal *Sph*I site. PCR primers for the 5′ portion of E1 were 5′-CGCGGATCCAG GATGGCGGACGATTCACG-3′ and 5′-GTCTGCATGCTC TCGGGTGC-3′. For the 3′ portion of the E1 fragment, we used the primers 5′-CGAGAGCATGCAGACACATCAGG-3′ and 5′-CGCGAATTCTCATAAAGTTCTAACAACTGA TCCTGG-3′. The 5′ *Bam*HI → *Sph*I PCR product and the 3′ *Sph*I → *Eco*RI fragment were ligated into pVL1393 restricted at the *Bam*HI and *Eco*RI sites (pW107; Fig. 1C). The E2 ORF was amplified as a *Bam*HI and *Xho*I fragment by using the primers 5′-GGCCGGATCCATGGAAGCAATAG CCAAGCG-3′ and 5′-GCGCCTCGAGGGTTACAATAAA TGTAATGAC-3′. The PCR product was ligated into the vector pW85, resulting in the plasmid pW103. pW103 was restricted with *Bam*HI and *Xba*I, which cuts outside *Xho*I in the polylinker sequences. This fragment was ligated into pVL1393, which had also been restricted with *Bam*HI and *Xba*I (pW104; Fig. 1C). The integrity of each plasmid was determined by double-stranded-DNA sequencing using standard techniques.

Expression of E1 and E2 in baculovirus-infected cells. The continuous insect cell line Sf9 was maintained in TMN-FH medium as described previously (38). The E1 and E2 transfer vectors were cotransfected into Sf9 cells with *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) DNA (38). Positive recombinant virus was identified and purified by four rounds of limiting dilution and dot blot hybridization using either the E1 or E2 DNA as a probe (31). Recombinant viral stocks were deemed pure on the basis of the absence of polyhedrin in Sf9 cells following infection and by PCR analysis of viral DNA using primers which flank the plasmid cloning sites. Subsequent infections for E1 and E2 expression were performed at a multiplicity of infection of 2. Sf9 cells were incubated for 72 h postinfection.

Generation of E1 and E2 peptide antisera. Antibody (Ab) to the E1 protein (Ab4088) was generated against the peptide TTQPKKVKRRLFETRELTDSGYGYS (amino acids 116 to 140), and E2 antibody (Ab4089) was generated against the peptide NIVTDNYNKHQRRNNCHSAATPI (amino acids 264 to 286). Both peptides were conjugated to bovine serum albumin (16) before injection into rabbits. Immunization was conducted by standard techniques (16).

Metabolic labeling of cells. At approximately 72 h postinfection, cells were starved in methionine-free Grace's insect cell medium for 2 h at room temperature (RT) and labelled with [^{35}S]Translabel (0.05 mCi/35-mm well) (ICN) for 2 h at RT. Phosphate labeling was in complete medium containing 1.5 mCi of $^{32}P_i$ per 35-mm well for 4 h at RT. After being labeled, cells were washed twice in ice-cold phosphate-buffered saline and suspended in 25 mM Tris (pH 8.0)–1 mM EDTA–1% Nonidet P-40–1 mM dithiothreitol–100 μg of aprotinin and leupeptin per ml. All subsequent steps were performed at 4°C. The cells were incubated on ice for 30 min. Nuclei were pelleted in an Eppendorf model 5402 centrifuge at 4,000 rpm for 10 min and suspended in lysis buffer containing 300 mM NaCl. Following a 30-min incubation, cellular debris was pelleted at 14,000 rpm for 15 min. The supernatant was transferred to new tubes.

Immunoprecipitations. Immunoprecipitations were performed as described previously (33). Peptide antisera were used at a dilution of 1:50. Immune complexes were washed four times with lysis buffer containing 300 mM NaCl, suspended in sample buffer, and analyzed by polyacrylamide gel electrophoresis.

FIG. 1. (A) Physical map of the 8-kb HPV-11 genome. The ORFs are designated as early (E) or late (L) on the basis of their expression during the virus life cycle. The first ATG codon within each ORF is designated by the dashed vertical line. The long control region (LCR) contains transcriptional control elements as well as the viral origin of replication (7). (B) Expanded view of the E2 and E1 ORFs. The nucleotide number of the first ATG is represented by a dashed vertical line. Regions of the E2 protein conserved among the PVs are represented by shading and are listed underneath each ORF. An amino acid sequence comparison for the nuclear localization signal sequence and the ATP-binding domain is included for the HPV-11 and BPV-1 E1 proteins and the SV40 T-Ag. Amino acid differences between the E2 protein used in this study and the prototype HPV-11 are indicated above the E2 ORF. Also above each ORF are indicated the peptides which were synthesized to raise the E2 (Ab4089) and E1 (Ab4088) antisera used in this study. (C) Flow chart of the construction of baculovirus transfer DNAs used for recombination into Ac*M*NPV. PV fragments were generated by PCR. E1 was generated as two pieces which were recombined at the unique internal *Sph*I site. These fragments were cloned into baculovirus transfer vector pVL1393.

Western blot analysis. Protein samples were separated on acrylamide gels, electroblotted to nitrocellulose, and subjected to Western immunoblot analysis as described previously (33), with minor modifications. Blocking solution was incubated for 30 min, and then fresh blocking solution containing either E1 or E2 peptide antibody was added and then incubated for 1 h, followed by three 5-min washes in Tris-buffered saline plus 0.1% Tween 20. Goat anti-rabbit serum–alkaline phosphatase conjugate was incubated in Tris-buffered saline–1% nonfat dry milk plus 0.1% Tween 20 for 30 min, followed by three 5-min washes. The final two washes were without Tween 20. Detection was with the alkaline phosphatase conjugate substrate kit (Bio-Rad).

Immuno-DNA binding assay. Extracts from E1-, E2-, E1- and E2-, or wild type (wt)-infected cells were prepared from 25-cm^2 flasks and immunoprecipitated as described above. Immune complexes attached to protein A-Sepharose beads were mixed with pW79 DNA which had been restricted with *Kpn*I, *Xba*I, and *Nde*I and 5′ end labelled with ^{32}P. This mixture was incubated for 2 h at room temperature in gel shift buffer (10 mM Tris [pH 7.0], 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 100 mM KCl) containing 100 ng of salmon sperm DNA per μl. Following incubation, the beads were washed four times in gel shift buffer. Protein was eluted in 1% sodium dodecyl sulfate–25 mM EDTA at 65°C for 15 min, brought to 20 mM Tris, and incubated with 50 μg of proteinase K per ml for 1 h at 37°C (27). This mixture was extracted twice with phenol and once with phenol-chloroform and ethanol precipitated. Samples were separated on a 5% nondenaturing polyacrylamide gel and exposed to film.

ATPase assay. Extracts from E1- or E2-infected cells were prepared from 150-cm^2 flasks and immunoprecipitated as described above. Immune complexes attached to protein A-Sepharose beads were mixed with 0.01 mCi of either [γ-^{32}P]ATP or [γ-^{32}P]GTP (Amersham) in a buffer containing 0.05 M Tris (pH 7.8), 5 mM $MgCl_2$, and 0.1% Nonidet P-40 (4) with 1 μg of single-stranded salmon sperm DNA. These immune complexes were allowed to react for 30 min at 37°C. One microliter of each sample was spotted onto a polyethyleneimine-cellulose thin-layer chromatography plate, which was developed for ~2 h in 0.75 M sodium phosphate buffer at pH 3.5. The plates were dried and visualized after exposure to Kodak X-Omat film for 30 min or quantitated on a Molecular Dynamics model 400 series Phosphorimager.

RESULTS

Construction of plasmids and determination of coding sequence. The sequences coding for the HPV-11 E1 and E2 proteins (Fig. 1A) were cloned into the baculovirus vector pVL1393 by PCR amplification from a clone of HPV-11. The viral DNA for this clone was isolated from the lesion of an adolescent patient with anogenital warts which contained exclusively episomal copies of HPV-11 (15). The plasmid pW107 contains PV sequence from nucleotides 829 to 2781; pW104 spans nucleotides 2723 to 3826 (Fig. 1C). Both are expected to initiate translation at the authentic PV ATGs for E1 and E2. Recombinant baculoviruses were established, and viral stocks (vE1 and vE2) were purified by standard techniques (31, 38).

The integrity of the PCR-amplified DNAs was confirmed by DNA sequencing of the entire coding regions. Eleven nucleotide differences compared with prototype HPV-11 (10) were identified in this new isolate and are listed in Table 1 along with the predicted change in the amino acid sequence.

TABLE 1. Nucleotide differences between pW79 and prototype HPV-11

Nucleotide position	Nucleotide change	ORF(s) affected	Amino acid change
1107	A → C	E1	NC[a]
2358	C → T	E1	NC
2580	A → G	E1	NC
2884	C → T	E2	NC
2888	T → C	E2	NC
3302[b]	T → A	E2	Cys → Ser
		E4	Tyr → Term
3391	A → G	E2	NC
		E4	Gln → Arg
3436	G → A	E2	NC
		E4	Gly → Glu
3487	C → T	E2	NC
		E4	Ser → Leu
3626	A → C	E2	NC
3645	A → G	E2	Lys → Arg

[a] NC, no change.
[b] Not present in the original isolate.

Nine of the changes occur in the wobble position of the respective codons and do not result in an amino acid change. The remaining two could be considered conservative amino acid substitutions (E2: Cys-194→Ser, Lys-308→Arg). Sequencing of the HPV-11 clone demonstrated that the Cys-194 substitution was not present in the original isolate. This change apparently resulted from misincorporation during the amplification step.

E1 and E2 proteins are expressed and phosphorylated in Sf9 cells. To determine whether E1 and E2 proteins were being expressed from the recombinant baculovirus, Sf9 insect cells were infected with either recombinant or wt (Ac*M*NPV) virus. At approximately 72 h postinfection, nuclear extracts were prepared, mixed with 2× Laemmli sample buffer, and separated by PAGE. Additional 42.5- and 80-kDa proteins are visible by Coomassie blue staining in the respective nuclear extracts of vE2- and vE1-infected cells which are not present in the lane containing wt virus (Fig. 2). The predicted molecular sizes for HPV-11 E2 and E1 are 41.7 and 73.5 kDa, respectively. The decreased mobility of E1 may be indicative of posttranslational modifications to the protein and/or a specific primary sequence which results in aberrant migration.

The identity of these proteins was confirmed by Western blot analysis using antisera generated against E1 (Ab4088) or E2 (Ab4089) peptides (Fig. 1B). αE1 readily detects an E1 protein of 80 kDa in nuclear extracts from cells infected with the vE1 recombinant baculovirus (Fig. 3B) which is not present in cells infected with either vE2 or wt baculovirus. Likewise, αE2 detects a 42.5-kDa protein only in cells infected with the vE2 baculovirus. Coinfections with the vE1 and vE2 viruses (Fig. 3) lead to expression of both proteins. Approximately 30% of the total E1 and E2 protein is soluble, most of which is present in the nucleus and can be recovered in a 300 mM NaCl nuclear wash.

One of the potential advantages of the baculovirus system relative to *Escherichia coli* is that large quantities of posttranslationally modified protein can be isolated. The BPV E1 and E2 proteins are both phosphorylated (24, 36, 39), and phosphorylation may be an important regulator of the activities of both of these proteins (22, 25). To determine whether the HPV-11 E1 and E2 proteins are phosphorylated in Sf9 cells, we incubated virus-infected cells in complete media

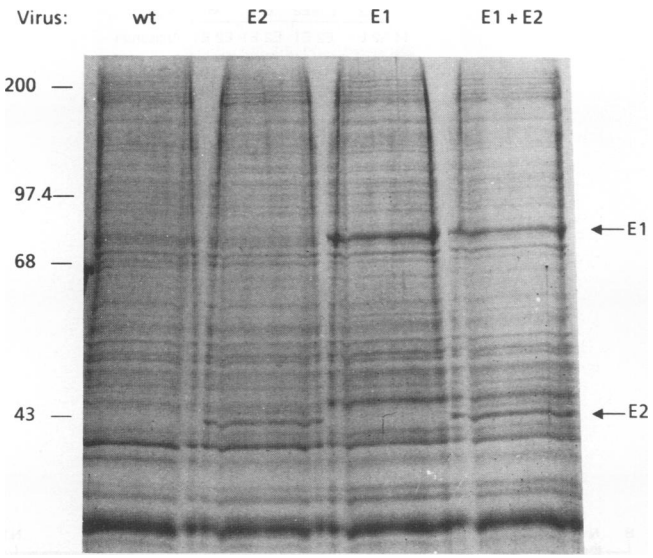

FIG. 2. Coomassie blue stain visualization of the E1 and E2 proteins in virus-infected Sf9 cells. Nuclear extracts of either wt virus (Ac*M*NPV)-, vE2-, vE1-, or vE1- and vE2-infected cells were separated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel. The positions of the 42.5-kDa E2 protein and the 80-kDa E1 protein are indicated by arrows. Molecular size markers (in kilodaltons) are shown on the left.

containing $^{32}P_i$. Nuclear extracts of the vE1- and vE2-infected cells were each immunoprecipitated with either αE1 or αE2 (Fig. 4). Proteins of the expected molecular sizes for E1 and E2 were immunoprecipitated in the presence of the appropriate Ab and detected by autoradiography, demonstrating that HPV-11 E1 and E2 proteins are phosphorylated. The relatively low level of phosphorylation of HPV-11 E2 is consistent with that observed for BPV E2, which is modified primarily at only two Ser residues (Ser-298 and Ser-301 [24]).

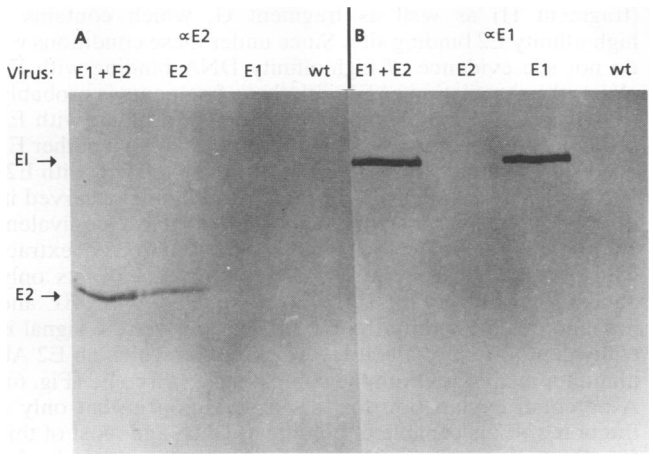

FIG. 3. Identification of the E1 and E2 proteins by Western blot analysis. Extracts were prepared and separated in duplicate as described in the legend to Fig. 2. Each blot was incubated with Ab to either E1 (Ab4088) or E2 (Ab4089). Goat anti-rabbit antibody conjugated to alkaline phosphatase was used to visualize the proteins. The positions of the E1 and E2 proteins are indicated. (A) αE2 (Ab4089) was used as the primary antibody. (B) αE1 (Ab4088) was used as the primary antibody.

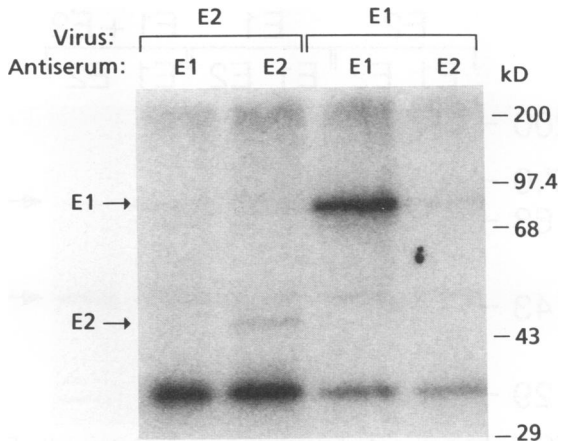

FIG. 4. Phosphorylation of the E1 and E2 proteins in Sf9 cells. $^{32}P_i$-labelled nuclear extracts from Sf9 cells infected with either vE2 or vE1 were immunoprecipitated with αE2 (Ab4089) or αE1 (Ab4088). Proteins bound in immune complexes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography. The E1 and E2 proteins are indicated. Molecular size markers (in kilodaltons) are shown on the right.

While the phosphorylated residues on the HPV-11 E2 protein have not been identified, the protein does not contain phosphorylation sites similar to those of the BPV E2 protein. The closest homology is the sequence SPAS (195 to 198) in HPV-11, compared to SPDS (298 to 301) for BPV. Although this HPV-11 sequence is also located in the hinge region, it is not embedded within an acidic region and is therefore not likely to be a substrate for the CKII kinase, as has been suggested for BPV E2 (25). Regulation of HPV-11 E2 phosphorylation may therefore be under the control of a different cellular kinase, which could have different functional consequences for this protein.

Biochemical activities. Genetic and biochemical data from a number of laboratories have shown that the E1 and E2 proteins exhibit a number of functional activities, which include DNA binding and transactivation for E2 and DNA binding, ATP binding, and ATPase for E1. In addition, these proteins have been shown to associate with each other in heteromeric complexes which are thought to play a regulatory role in replication. To confirm that the HPV-11 proteins expressed in insect cells are biologically active, we assayed protein from crude nuclear extracts for a number of the activities which the HPV-11 proteins are known to possess (18) or predicted to possess on the basis of their homology to the BPV proteins (8, 27, 39–41, 43).

(i) The E1 and E2 proteins form a molecular complex. The ability of the BPV E1 and E2 proteins to physically associate is thought to be a prerequisite for their participation in viral replication (27, 43). The strength of the homology between BPV and the HPVs suggests that E1-E2 complexation will also be important for replication of the human viruses. As is shown in Fig. 5, either the E1 or E2 protein is specifically immunoprecipitated from a ^{35}S-labelled cell extract by the complementary Ab in single-virus infections. In addition, immunoprecipitates from lysates of coinfected cells contain both the 80-kDa E1 and the 42.5-kDa E2 proteins when either the E1 or E2 Ab is used alone, indicating that at least a fraction of E1 and E2 expressed in insect cells is present as a complex. Analysis of these gels on a Phosphorimager indicates that between 5 and 10% of the E1 and E2 proteins

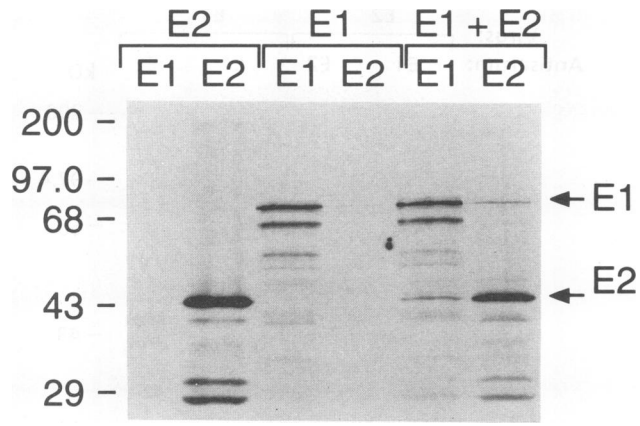

FIG. 5. Formation of a heteromeric complex between the E1 and E2 proteins. Nuclear extracts from Sf9 cells incubated with [^{35}S] Translabel (ICN) and infected with vE2, vE1, or vE1 and vE2 were immunoprecipitated with either αE1 or αE2, subjected to polyacrylamide gel electrophoresis, and visualized by autoradiography. The E1 and E2 proteins are indicated. Molecular size markers (in kilodaltons) are shown on the right.

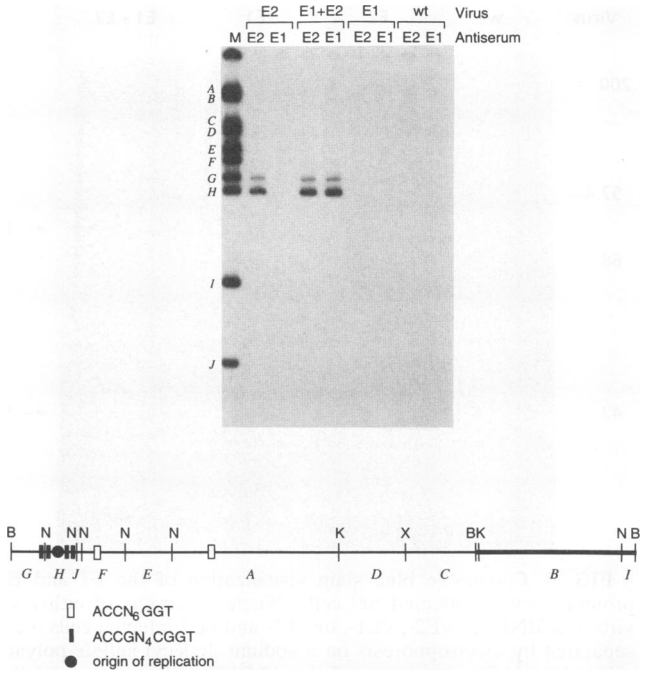

FIG. 6. The E1 protein localizes to a DNA fragment containing the viral replication origin. Extracts from cells infected by vE2, vE2 and vE1, vE1, or wt virus (Ac*M*NPV) were immunoprecipitated with either αE1 or αE2 and the protein A-Sepharose beads containing the Ab-Ag complex mixed with ^{32}P-labelled HPV-11 DNA restricted with *Kpn*I, *Xba*I, and *Nde*I. After extensive washing, the protein was removed and the bound DNA was recovered and separated on a polyacrylamide gel. The lane labelled M represents the input DNA; fragments are labelled A to J. At the bottom is a linearized map of the HPV-11 plasmid. Restriction sites are as follows: B, *Bam*HI; N, *Nde*I; K, *Kpn*I; and X, *Xba*I. pUC18 sequence is represented as a boldface line. The positions of E2 binding sites and the viral *ori* are indicated.

in the cell are in complex. When E1 and E2 are cotranslated in vitro, complexation occurs in the absence of nucleic acids, suggesting that the association is a genuine protein-protein interaction (5).

(ii) The E1 and E2 proteins are localized to the viral *ori*. Sequence-specific DNA binding by the BPV E1 and E2 proteins is an important part of the functions of both proteins in the virus life cycle. Binding to the sequence $ACCN_6GGT$ is required by the E2 protein for its functions as both a replication protein (6) and a transcriptional regulator (17, 19, 21, 26, 28). BPV E1 exhibits site-specific DNA binding to the viral *ori* (43). In addition, the E1 protein can be localized to DNA by virtue of its complexation with E2 (27). To determine whether the HPV proteins encode any or all of these properties, we performed an immuno-DNA binding assay. Extracts from cells infected with vE2, vE1, both vE2 and vE1, or wt baculovirus were immunoprecipitated with either αE1 or αE2, and the protein-beads were allowed to bind to a mixture of end-labelled restriction fragments from a clone of HPV-11. After unbound DNA was washed away, bound fragments were resolved on a nondenaturing polyacrylamide gel. An E2-containing extract immunoprecipitated with an E2 Ab binds to two DNA fragments, both of which contain at least one $ACCGN_4CGGT$ motif (Fig. 6, fragments G and H), which is believed to represent the high-affinity E2 binding sequence (21). No specific fragments are immunoprecipitated when the E2 extract is mixed with the E1 Ab. Interestingly, two fragments contain E2 binding sites of the sequence $ACCN_6GGT$ (Fig. 6, fragments A and F). Neither of these fragments is bound by an E2 extract, suggesting that we are able to detect only high-affinity DNA binding by this assay or that the HPV-11 E2 protein has a more stringent requirement for its binding site than the BPV E2 protein. When an E1 extract is used, no specific retention of any DNA fragments is observed, regardless of whether the E1 or E2 Ab is used. In addition, we have tested a second, epitopically distinct, E1 antipeptide antiserum as well as E1 prepared from *E. coli* and have been unable to detect high-affinity DNA binding by E1 in either case (data not shown). When the E1 Ab is used to immunoprecipitate from a coinfected extract, however, E1 is observed to be associated with the DNA fragment which contains the viral *ori* (fragment H) as well as fragment G, which contains a high-affinity E2 binding site. Since under these conditions we do not see evidence of high-affinity DNA binding with E1 alone, the association of E1 with these fragments is probably by virtue of its complexation with the E2 protein, with E2 providing the binding specificity. It is not known whether E1 makes any contact with the DNA when complexed with E2. If E2 is solely responsible for the DNA binding observed in this assay, it is somewhat surprising that an equivalent amount of fragment is retained by the coinfected extract when the E1 Ab is used. In the coinfected extracts only about 10% of the total E2 is complexed with E1 and immunoprecipitated by the E1 Ab (Fig. 5), yet the signal is equivalent to that of a coinfected extract in which an E2 Ab immunoprecipitates both the complexed and free E2 (Fig. 6). A potential explanation for this observation is that only a fraction of E2 is capable of binding to DNA and most of this fraction will complex with the E1 protein. Alternatively, E1 and E2 together may have enhanced DNA binding capabilities similar to that already described for BPV (43).

(iii) The E1 protein exhibits ATPase and GTPase activity. The E1 protein from BPV expressed in either COS or 3T6 cells binds ATP (36, 39), and recent data indicate that the protein also possesses ATPase activity (23). Since HPV-11 E1 contains an ATP-binding motif similar to those of BPV

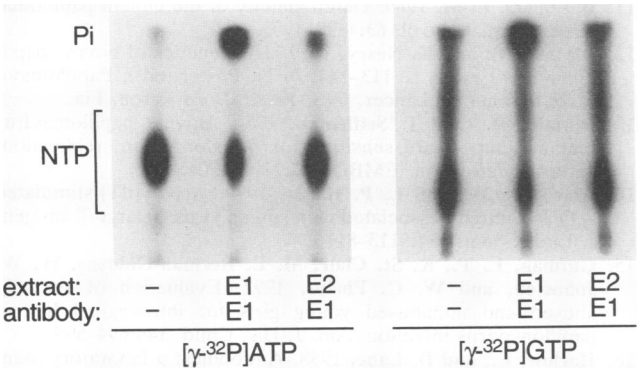

FIG. 7. The E1 protein has nucleoside triphosphatase activity. vE1 or vE2 extracts immunoprecipitated with αE1 were mixed with either [γ-^{32}P]ATP or [γ-^{32}P]GTP and incubated at 37°C. The position of the labelled nucleoside triphosphate or free phosphate is indicated. The percentages of free phosphate hydrolyzed from the nucleoside triphosphate in a 30-min reaction for ATP are as follows: no extract, 5.4%; E1 extract, 54%; E2 extract, 13%. The percentages for GTP are as follows: no extract, 16%; E1 extract, 70%; E2 extract, 21%.

E1 and SV40 T-Ag (Fig. 1B), we tested HPV-11 E1 for ATPase activity. Ab4088 (E1) was used to immunoprecipitate lysates of either vE1- or vE2-infected cells. The protein A-Sepharose resin containing the E1 Ab plus immunoprecipitated protein was incubated with [γ-^{32}P]ATP, and the products were separated by thin-layer chromatography on a polyethyleneimine-cellulose plate. An active ATPase will convert the radiolabelled ATP to free $^{32}P_i$ and unlabelled ADP. The [γ-^{32}P]ATP, when incubated in buffer alone, contains 5.4% of the label in the form of free phosphate. To determine the contaminating ATPase activity bound by the E1 Ab, a vE2-infected control extract was immunoprecipitated with the E1 Ab; 13% of the label was released as free phosphate when incubated with these beads. In contrast, the Ab complex from an E1-containing extract released 54% of the label as free phosphate (Fig. 7), which, after correction for nucleotide alone, is sixfold greater than the background level from the E2 extract. This result demonstrates that an ATPase activity can be immunoprecipitated from an E1-infected-cell extract with an antibody directed against the HPV-11 E1 protein. This assay, however, does not distinguish the activity of an ATPase which specifically associates with E1 from endogenous E1 activity. The ATPase activity is stimulated slightly by the presence of DNA, although there seems to be no difference between single-stranded DNA and double-stranded DNA, including double-stranded HPV-11 DNA (data not shown), which is similar to observations with SV40 T-Ag. For T-Ag, natural single-stranded or double-stranded DNA templates gave only minimal stimulation of ATPase activities; synthetic single-stranded homopolymers provided the greatest level of stimulation (14). It has been suggested that GTP is also a substrate for the BPV E1 ATPase (23). The ability to hydrolyze non-ATP nucleotides may be an important property of this protein since in vitro replication assays lacking ATP still retain 18% of their replication activities (43). When we tested the HPV-11 E1 protein for GTPase activity using the beads described above, the level of E1-specific activity was even greater than that of the ATPase activity. In this instance, the corrected E1-specific GTPase activity was almost 11 times greater than that of an E2-containing extract (Fig. 7).

DISCUSSION

In this study, we describe the expression and biochemical characterization of the HPV-11 E1 and E2 proteins. As suggested by amino acid sequence homology, these proteins exhibit the same replication-associated activities possessed by their counterpart BPV proteins. These observations support several recent reports which suggest that the replication functions are conserved among all of the PVs. Yang et al. (44) have shown that the E1 and E2 proteins of HPV-6b will associate when coexpressed in insect cells. They go on to show that the E1 or E2 protein of HPV-6b can associate with the E2 or E1 protein of BPV-1, respectively, indicating that the domains responsible for E1-E2 association are well conserved. Chiang-Ming et al. (7) showed that in transient assays both the E1 and E2 replication proteins, as well as the viral origins of replication from several different animal and human PVs, are essentially interchangeable.

The HPV-11 E1 and E2 proteins produced in insect cells have molecular sizes of 80 and 42.5 kDa, respectively, which agree well with the sizes determined for the same proteins expressed in COS-7 cells (E1, 82 kDa; E2, 43 kDa [7]). By immunoprecipitation, we detected a second major form of E1 with a molecular size of ~73 kDa (Fig. 6) which may represent unmodified E1 since it approximates the molecular size predicted for E1, 73.5 kDa. A similar observation has been made for BPV E1. While most of the 68-kDa BPV E1 expressed in Sf9 or COS cells runs near the predicted molecular size, a minor fraction shows retarded migration near 75 kDa and may represent a modified form of this protein. Our data indicate that both the E1 and E2 proteins are phosphorylated. This could account in part for the reduced mobility of the E1 protein. It has been suggested that the E1 protein of BPV associates only with the non- or hypophosphorylated forms of E2 (22). The low level of E2 phosphorylation, as well as the limited amounts of E2 which coprecipitate with the E1 Ab, have made it difficult to unequivocally confirm this observation for the HPV-11 proteins; however, our results thus far are consistent with this conclusion (data not shown). It is clear, however, that phosphorylated forms of E1 are present in complex with E2 (data not shown).

We show that the HPV-11 E1 and E2 proteins form a molecular complex and that both proteins can be localized to a DNA fragment containing the viral origin of replication. Under these conditions, localization of E1 to the origin is most likely by virtue of its association with E2, since we have thus far been unable to detect specific origin binding by the E1 protein. Active E1 protein is present on the αE1 beads since these beads exhibit an ATPase activity 13-fold greater than that of a vE2-infected-cell extract immunoprecipitated with the E1 Ab (data not shown), but the E1 is apparently incapable of binding to DNA with high affinity. While not expected, it remains formally possible that E1 of HPV-11 or of the HPVs in general, in contrast to BPV, does not bind DNA in a site-specific manner in the absence of E2 or binds with a substantially lower affinity.

In addition to the 80- and 73-kDa E1 proteins, several smaller forms of the E1 protein are immunoprecipitated with an amino-terminal E1 Ab (Fig. 5), which suggests that these forms represent COOH-terminal truncations of E1. Since an E2 Ab also immunoprecipitates these smaller forms of E1 from coinfected extracts, it suggests that the C terminus is dispensable for complexation with E2. This is in contrast to a study which mapped the sequences required for complexation of the BPV E1 and E2 proteins to the COOH terminus

of E1 (22). Our preliminary data indicate that in HPV-11 important E1 complexation sequences are located upstream of amino acid 251 (5).

Like the BPV E1 protein, the HPV-11 protein is associated with an ATPase and GTPase activity. The nucleoside triphosphatase activity is probably coupled to the postulated helicase activity, which is required for unwinding of the viral DNA at the replication fork. Thus far we have been unable to detect a helicase activity from the crude preparations of E1. We are in the process of purifying the E1 protein with the expectation that a more purified preparation will allow detection of this activity.

Purified E1 and E2 proteins will be useful in the development of a cell-free replication system for the study of the molecular events which occur during initiation of viral replication. Unlike the polyomavirus replicons, in which interaction of the specific viral T-Ag protein with the cellular DNA polymerase represents an important determinant in the species specificity of viral replication (29, 30), replication of the PV replicon seems rather promiscuous. While replication of the virus is restricted to differentiating keratinocytes, many different cell lines will support replication of PV origins of replication when the replication proteins are expressed from heterologous promoters (6, 40). Expression of the replication proteins, not the interaction of the viral proteins with the cellular machinery, seems to be the limiting factor for virus replication in the different cell types. Therefore, the information gathered from the study of viral DNA replication using HPV-11 should be applicable to most of the other mucosal and cutaneous PV systems as well.

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