JOURNAL OF VIROLOGY, Oct. 1993, p. 6000–6014
0022-538X/93/106000-15$02.00/0

Vol. 67, No. 10

DNA-Binding Domain of Bovine Papillomavirus Type 1 E1 Helicase: Structural and Functional Aspects

LAUREN K. THORNER, DANIEL A. LIM, AND MICHAEL R. BOTCHAN*

Department of Molecular and Cell Biology, 401 Barker Hall, University of California, Berkeley, California 94720

Received 10 May 1993/Accepted 30 June 1993

The E1 protein of bovine papillomavirus type 1 is a multifunctional enzyme required for papillomaviral DNA replication. It assists in the initiation of replication both as a site-specific DNA-binding protein and as a DNA helicase. Previous work has indicated that at limiting E1 concentrations, the E2 protein is required for efficient E1 binding to the replication origin. In this study, we have defined the domain of the E1 protein required for site-specific DNA binding. Experiments with a series of truncated proteins have shown that the first amino-terminal 299 amino acids contain the DNA-binding domain; however, the coterminal M protein, which is homologous to E1 for the first 129 amino acids, does not bind origin DNA. A series of small internal deletions and substitution mutations in the DNA-binding domain of E1 show that specific basic residues in this region of the protein, which are conserved in all E1 proteins of the papillomavirus family, likely play a direct role in binding DNA and that a flanking conserved hydrophobic subdomain is also important for DNA binding. A region of E1 that interacts with E2 for cooperative DNA binding is also retained in carboxy-terminal truncated proteins, and we show that the ability of full-length E1 to complex with E2 is sensitive to cold. The E1 substitution mutant proteins were expressed from mammalian expression vectors to ascertain whether site-specific DNA binding by E1 is required for transient DNA replication in the cell. These E1 proteins display a range of mutant phenotypes, consistent with the suggestion that site-specific binding by E1 is important. Interestingly, one E1 mutant which is defective for origin binding but can be rescued for such activity by E2 supports significant replication in the cell.

The initiation of DNA replication involves a concerted assembly of proteins at specific genetic loci termed origins of replication. Such intricate assembly of replication factors at origins has been described for many prokaryotic systems. Among the eukaryotes, small DNA viruses (simian virus 40 [SV40] and polyomavirus) have been the model for understanding how the cellular polymerases and their ancillary factors become engaged in DNA synthesis (20). The general principles that can be applied across the range of a diverse group of eukaryotic replication systems will require detailed study of other replicons.

Bovine papillomavirus type 1 (BPV-1) presents one such system with which to study the assembly of a eukaryotic replication machine. In transformed mouse cells, BPV-1 maintains itself in the nucleus as multicopy circular episomes replicating in synchrony with the host cell. Only two viral proteins, E1 and E2, are necessary and sufficient to support this in vivo replication. The DNA polymerase and all other factors are provided by the host (43, 47). E1 is a 68-kDa nuclear phosphoprotein that has been recently demonstrated to be an ATP-dependent DNA helicase and unwinding enzyme (34, 48). Whereas prokaryotic helicases often require the assistance of auxiliary proteins for efficient loading onto DNA, the E1 protein itself is equipped to bind the origin of replication (36, 44, 45, 47, 49). This origin-binding property of E1 is analogous to that of other virally encoded helicases, such as the large T antigen of SV40 (27, 41) and the UL9 helicase of herpes simplex virus type 1 (7, 13, 14), both of which bind their respective origins. Hence, it appears that certain viral helicases play a dual role in replication, as origin recognition factors and as helicases. At present, it is not clear whether the DNA-binding properties of E1 by itself give the protein enough specificity to initiate assembly at the unique origin site (48). For example, other proteins (such as E2) may assist in providing additional specificity.

E2, a 48-kDa transcriptional activator, can markedly enhance the affinity of E1 for origin-containing DNA (33, 36, 47, 49). The BPV-1 origin of replication contains an inverted repeat which lies within a region protected by E1 from DNase I digestion (44, 47, 49), and there is an A:T-rich region immediately upstream and a proximal E2 binding site downstream (42). E1 and E2 have been observed to form a tight complex in solution (4, 26, 29), and E1 is necessary for E2 interaction with origin DNA lacking an intact E2 binding site (47). Thus, it is reasonable to believe that the mutual benefits of E2 and E1 for DNA binding are at least partly attributable to protein-protein interactions. Recent findings show that E2 can function in DNA replication from sites far removed from the E1 binding site, consistent with the idea that protein-protein interactions between E1 and E2 are pivotal in the assembly of a replication complex (42).

There are two mechanisms that adequately describe the cooperative assembly of a ternary complex involving E1, E2, and DNA. One thought is that E2 allosterically affects the conformation of E1 by direct protein-protein contact, which changes the shape of the E1 DNA binding domain and thus increases the affinity of E1 for DNA. Another notion is that, unrelated to favorable shape changes, protein-protein contact between E1 and E2 contributes to an overall favorable ΔG for E2-E1-DNA assembly (that E1 tethers E2 to DNA and vice versa). Of course, a subtle combination of these two possibilities may prove to be the actual case. In addition, the DNA sequence may be an active component of the assembly reaction, in accord with the induced-fit model

* Corresponding author.

of protein-ligand binding. In other words, the specific sequence itself might effect a structural change in the DNA-binding domain that is propitious for binding.

To explore these possibilities requires a detailed analysis of the structure of E1, and clearly, more knowledge about its DNA-binding domain will be critical. To begin this analysis, we have asked where in the protein the DNA-binding region lies and what peptide sequences are necessary and sufficient for specific origin recognition. A difficulty inherent in mapping the DNA-binding domain of a helicase is that the same domain may be capable of binding different DNA substrates (i.e., specific versus nonspecific sequences and double versus single strands). For instance, studies of T antigen have revealed separable origin-specific and nonspecific DNA-binding activities; however, it does not appear that these represent separate domains (23). E1 can nonspecifically displace a single-stranded helicase substrate from a template and can processively unwind circular duplex DNA (34, 48). In the latter reaction, the helicase must make non-specific contacts with the DNA as it unwinds. These nonspecific interactions may be essential for elongation during DNA synthesis if E1 is the only helicase important in papillomavirus replication. Given these considerations, it is clear that assay protocols must be adopted which preferentially optimize site-specific binding and reduce nonspecific binding. However, despite this caveat, we believe that the standard approach of optimization for one activity is the most direct and simple way to initiate a structure-function analysis of a multifunctional protein such as E1.

Helicases probably have their DNA-binding affinities modulated through ATP binding and hydrolysis (24). A recently elucidated example is the *Escherichia coli* dimeric Rep helicase, for which ATP and ADP serve as allosteric effectors of DNA binding. The hydrolysis of ATP serves as a switch in the helicase's affinity for DNA—each subunit demonstrates a preference for either double- or single-stranded DNA—which provides the basis for a model of helicase action (46). Since E1 requires ATP for its helicase activity, we examined the effect of mutations of ATPase activity on origin DNA binding to determine whether this region can be found (by genetic means) to influence the site-specific DNA-binding domain.

If, as we have suggested, one of the major ways by which E2 enhances papillomavirus replication is through assisting and directing E1 binding to the origin site, then certain E1 mutants should be recovered that are defective for site-specific DNA binding yet do not have severe replication phenotypes. That is, a particular mutant may be severely crippled for binding, but in the presence of E2, its binding may be sufficient to effect some replication activity. We have generated six E1 mutants with different substitution mutations in the sequences encoding the DNA-binding domain of E1 and used these mutants to explore this question.

MATERIALS AND METHODS

Plasmids and recombinant baculoviruses. The expression vector used for in vitro translation of E1, pSPE1, contained the *Nru*I-*Acc*I fragment (nucleotides [nt] 849 to 3004) of BPV-1 inserted into the *Xba*I site of pSP65 (Promega) so that transcription ensued from the SP6 promoter. Point mutations between nt 1385 and 1674 in the DNA-binding region of E1 were generated by oligonucleotide-directed mutagenesis with the modifications of Kunkel, as described elsewhere (31). The generation of the point mutations in E1 that affect ATP binding and hydrolysis (nt 2148 to 2369) will be described elsewhere (26a) except for the 434P-S mutation at nt 2148, which has been described previously (39). Carboxy-terminal and internal deletion mutations in E1 were generated by polymerase chain reaction by standard protocols. Carboxy-terminal deletion mutants were constructed with a translation termination codon inserted at the 3′ end of the coding sequence. Internal deletion mutants contained an in-frame insertion of two amino acids (Gly-Thr) encoded by the *Kpn*I site used to fuse the two ends of nondeleted sequence. All of these mutations were transferred to pSPE1; in addition, the point mutations in the DNA-binding region were transferred to the E1 open reading frame (ORF) in pCGEag, which is located downstream of the cytomegalovirus promoter (43). The presence of all mutations was confirmed by DNA sequencing. Plasmid pSS3 contains the entire BPV-1 genome linearized at the unique *Bam*HI site and inserted into the *Bam*HI site of pUC18 (38a). The construction of plasmids which contain linker insertions of 10, 32, and 47 bp in the unique *Hpa*I site of pSS3 will be described elsewhere (28a). Plasmids pMLBPV (25), pKSO (47), and pCGE2 (43) have been described previously.

The construction of recombinant baculoviruses that express the E1 (29), GE1 (47), or E2 (19) protein has been described previously. A recombinant baculovirus expressing the GE1Δ424 protein was constructed by cleaving pAcGE1 (47) at the *Eco*RI site (nt 2113) in the E1 ORF, filling in the overhangs with the Klenow fragment of DNA polymerase I (USB Corp.), and inserting an *Xba*I linker (New England BioLabs; catalog no. 1062) with termination codons in all three reading frames between the blunt ends of the recircularized plasmid. The construction of the recombinant baculovirus expressing the M protein will be described elsewhere (39a). Both the GE1Δ424 and M coding sequences were recombined into the *Autographa californica* nuclear polyhedrosis virus baculovirus via these transfer vectors as described by Mohr et al. (29).

Cell lines and antisera. CHO cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 10% CO_2. *Spodoptera frugiperda* cells (Sf9 cells) were grown in suspension cultures at 24°C as described before (38) in the presence of 0.1% Pluronic F-68 (GIBCO) to prevent cell clumping. Pluronic F-68 was omitted when the cells were plated on tissue culture dishes for virus infection.

Anti-M polyclonal antiserum, raised against a 204-amino-acid (aa) segment from the 5′ portion of the E1 ORF (nt 860 to 1471) fused to the *E. coli* TrpE protein, has been described previously (40). Anti-E2 monoclonal antiserum (B202) was from E. Androphy (Tufts University). The EE monoclonal antiserum, which recognizes the 9-aa tag at the amino terminus of GE1, was from G. Walter (15).

In vitro transcription and translation. RNAs to be translated were synthesized in vitro with the pSP65 plasmid system (Promega); 4 μg of pSPE1 wild-type and mutant plasmid DNAs linearized at the unique *Hin*dIII site just downstream of the E1 ORF were transcribed by the SP6 phage RNA polymerase in a final volume of 50 μl with the Riboprobe System II transcription system (Promega) as instructed by the manufacturer. Capping the transcripts with $m^7G(5')ppp(5')G$ (catalog no. 1404; New England BioLabs) increased the protein yield only for pSPE1Δ7 and so was omitted from all other synthesis reactions. For in vitro translation, one-fourth of the RNA synthesized as described above was added to 35 μl of nuclease-treated rabbit reticulocyte lysate, 1 μl of a mixture of amino acids minus methionine (both from Promega), and 4 μl (60 μCi) of L-[^{35}S]methionine (>1,000 Ci/mmol; Amersham) in a final

reaction volume of 50 μl and incubated at 30°C for 90 min. After the reaction was complete, RNase A was added to a final concentration of 20 μg/ml, and incubation was continued for another 10 min. Samples were centrifuged for 10 min, and the supernatants were used in binding assays. Proteins were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels as described previously (40).

Purification of BPV-1 proteins from cells infected with recombinant baculoviruses. The E2 protein was purified by specific oligonucleotide affinity chromatography as described before (22). The GE1 protein was purified by immunoaffinity chromatography with the EE antibody as described elsewhere (47).

DNA-protein immunoprecipitation assays. DNA fragments used in binding assays were generated from restriction enzyme digests of one of the following plasmids: pSS3 digested with *Ava*II, pMLBPV digested with *Ava*II alone or *Ava*II plus *Hpa*I, or pKSO digested with *Bam*HI and *Eco*RI. Fragments were end labeled with [^{32}P]dGTP, [^{32}P]dATP, [^{32}P]TTP, [^{32}P]dCTP, and the Klenow fragment of DNA polymerase I. Labeled DNA was precipitated with ethanol and resuspended in Tris-EDTA buffer at a final concentration of 50 ng/μl.

(i) Purified GE1 protein expressed from baculovirus. DNA-binding assays with purified GE1 were done essentially as described before (45) with the following exceptions. GE1 (140 ng) was used in place of bacterial or ID13 extracts, and anti-M antibody was used (1.5 μl) to precipitate the GE1-DNA complexes. After the final wash, the pellets were resuspended in 190 μl of a solution containing 10 mM Tris-hydrochloride (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.2% SDS, and 200 μg of proteinase K per ml and incubated at 50°C for 1 h. Five micrograms of yeast tRNA was added to each sample before they were extracted once with phenol-chloroform, and the DNA in the aqueous phase was precipitated with ethanol. DNA precipitates were resuspended in 12 μl of 1× sucrose DNA loading dye (31) and electrophoresed on 8% nondenaturing polyacrylamide gels with standard Tris-borate buffers (31). Dried gels were exposed for autoradiography with intensifying screens.

(ii) Baculovirus-expressed M and E1 proteins from radiolabeled Sf9 cell extracts. Extracts used in DNA-binding assays were subjected to Western immunoblotting analysis to quantitate the amount of M and E1 proteins present (method described in reference 40), and an amount of extract corresponding to 10 μg of M or E1 protein was used. To rid the extracts of contaminating nucleic acids, extracts were rocked with 5 μl of protein A-Sepharose (PAS; Pharmacia) (no antibody) at 4°C for 1 h in 250 μl (final volume) of extract buffer. The samples were centrifuged, and the supernatants were transferred to tubes containing PAS (5 μl) bound to anti-M antibody (1.5 μl) plus 50 μl of extract buffer and rocked for a further 1.5 h. Immune complexes were pelleted and washed once with buffer D (13 mM Tris-hydrochloride [pH 7.0], 200 mM NaCl, 0.1 mM EDTA, 0.25% Nonidet P-40) and once with buffer E (13 mM Tris-hydrochloride [pH 7.0], 150 mM NaCl, 0.1 mM EDTA). Pellets were incubated with 200 ng of labeled DNA and 6 μg of unlabeled sheared salmon sperm DNA in 100 μl of buffer E containing 0.35% Nonidet P-40 with gentle agitation at room temperature for 2 h. The PAS beads were pelleted by centrifugation and washed three times with buffer D containing 5 μg of sheared salmon sperm DNA per ml and once with buffer E. The beads were resuspended in 200 μl of buffer E, and 20 μl was removed for analysis by SDS-polyacrylamide gel electrophoresis (PAGE) to monitor the efficiency of protein binding to the PAS. The remaining 180 μl was extracted once with phenol-chloroform and once with chloroform-isoamyl alcohol, and the DNA in the aqueous phase was precipitated with ethanol. DNA precipitates were washed with 70% ethanol, dried, and resuspended in 10 μl of 1× sucrose DNA loading dye before being run on an 8% polyacrylamide gel and exposed for autoradiography as described above.

(iii) In vitro-translated E1 proteins. For each protein tested, 100 μl of in vitro translation mixture (two reactions) was added to 200 μl of buffer E containing PAS (10 μl) bound to anti-M antibody (1.5 μl) and rocked at 4°C for 1 to 2 h. Immune complexes were pelleted and washed once with buffer E containing 0.5 M LiCl and twice with the same buffer minus LiCl. Pellets were incubated with 50 ng of labeled DNA and 1.5 μg of unlabeled sheared salmon sperm DNA in 130 μl of buffer E containing 0.35% Nonidet P-40 with gentle agitation at room temperature for 1 h. The PAS beads were pelleted by centrifugation and washed three times with buffer D containing 5 μg of sheared salmon sperm DNA per ml and once with buffer E. The beads were resuspended in 200 μl of buffer E, and 20 μl was removed for analysis by SDS-PAGE to monitor the efficiency of protein binding to the PAS. The remaining 180 μl was made 0.2% SDS and 200 μg of in proteinase K per ml and incubated at 50°C for 1 h. Five to 10 μg of yeast tRNA was added to each sample before they were extracted once with phenol-chloroform, and the DNA in the aqueous phase was precipitated with ethanol. Precipitated DNAs were washed with 70% ethanol, dried, and resuspended in 12 μl of 1× sucrose DNA loading dye before they were electrophoresed on 5 or 8% polyacrylamide gels and exposed for autoradiography as described above. In some experiments, the proteinase K step was omitted and instead, samples were extracted twice with phenol-chloroform and once with chloroform-isoamyl alcohol before precipitation with ethanol. Both methods yielded the same results.

(iv) In vitro-translated E1 proteins together with purified E2 expressed from baculovirus. In a typical experiment, 150 μl (three reactions) of each E1 protein was synthesized in vitro, combined, and rocked with PAS (6 μl) bound to anti-M antibody (4.5 μl) at 4°C for 2 h. Immune complexes were pelleted and washed once with buffer E containing 0.5 M LiCl and twice with the same buffer minus LiCl. After the E1-PAS beads were resuspended in 430 μl of buffer E containing 5 μg of unlabeled sheared salmon sperm DNA and 0.35% Nonidet P-40, they were divided into three equal portions, and 0, 10, or 100 ng of E2 plus 50 ng of labeled DNA was added to the samples. Binding was performed at room temperature for 1 h with gentle rocking. The PAS beads were pelleted by centrifugation and washed three times with buffer D containing 5 μg of sheared salmon sperm DNA per ml and once with buffer E. DNA was released from the complexes by resuspending the pellets in 100 μl of a solution containing 200 mM NaCl, 20 mM EDTA, 1% SDS, and 250 μg of yeast tRNA per ml and then extracting once with phenol-chloroform. DNA was precipitated from the aqueous phase with ethanol and the pellets were washed twice with 80% ethanol before being dried and resuspended in 10 μl of 1× sucrose DNA loading dye. Samples were electrophoresed on 5% polyacrylamide gels and exposed for autoradiography as described above.

Footprint analysis. DNase I footprint analysis was carried out as described elsewhere (47). The probe used for footprinting was generated by cleaving pKSO with *Bam*HI and *Eco*RI to release a fragment containing the BPV-1 sequence

from nt 7805 to 100, which was then labeled with ^{32}P at the 5′ end of the top strand (*Bam*HI site).

Preparation of radiolabeled baculovirus-infected Sf9 extracts. Sf9 cells (10^7 cells per 10-cm dish) were infected with either E1, GE1, E2, M, or GE1Δ424 recombinant baculoviruses at a multiplicity of infection of approximately 10. At 48 h after infection, the infected cells were starved for 1 h in Grace's medium containing 10% dialyzed fetal calf serum lacking methionine and cysteine (5 ml). The cells were then incubated for 5 h in the same medium (3 ml) containing 1 mCi of Tran^{35}S-label (>1,000 Ci/mmol; ICN) and 0.5 mCi of L-[^{35}S]-cysteine (>600 Ci/mmol; NEN/DuPont). Extracts were prepared by adding 1 ml of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4)–300 mM NaCl–1 mM EDTA–10% glycerol–0.5% Nonidet P-40–10 μg of leupeptin per ml–1 mM phenylmethylsulfonyl fluoride per dish. The dishes were incubated at 4°C for 15 to 30 min, after which the contents were scraped into a microcentrifuge tube and centrifuged for 15 min at 4°C before the supernatant was transferred to a new tube. Extracts were stored at −70°C.

Immunoprecipitation from radiolabeled baculovirus-infected Sf9 extracts. Immunoprecipitation from radiolabeled baculovirus-infected Sf9 extracts was done as previously described (29). Briefly, 25 or 50 μl of each extract was mixed with a solution of 50 mM HEPES (pH 7.6)–200 mM NaCl–1 mM EDTA–5% glycerol–0.125% Nonidet P-40 (final volume, 200 or 400 μl, respectively) and rotated at the indicated temperature for 2 to 3 h. PAS (5 or 10 μl) bound to either anti-M or B202 antibodies was also included in the reaction. The beads were pelleted and washed four times with buffer B (50 mM HEPES [pH 7.6], 200 mM NaCl, 1 mM EDTA, 0.05% Nonidet P-40) at the temperature indicated, and the samples were analyzed by SDS-PAGE.

Immunoprecipitations followed by Western blotting analyses. (i) Reactions containing pure baculovirus-expressed GE1 and E2. Reaction mixes containing 80 ng of purified GE1 and 40 ng of purified E2 in 100 μl of buffer A (50 mM HEPES [pH 7.6], 200 mM NaCl, 1 mM EDTA, 10% glycerol) were incubated at the temperatures indicated for 1 h. Ten microliters of protein G-Sepharose (Pharmacia) bound to anti-E2 antibody (B202) was added, and incubation was continued for another hour at the same temperature with gentle agitation. Immune complexes were washed twice with buffer A to which 0.1% Nonidet P-40 and 1 M LiCl had been added and twice with the same buffer without LiCl. All washes were performed at room temperature. Pellets were boiled for 5 min in 12 μl of 4× Laemmli sample buffer and subjected to electrophoresis on 10% polyacrylamide–SDS gels. Western blotting of gels was performed as described previously (49) except that the blots were incubated with anti-M antibody (1:5,000) in addition to B202 antibody (1:100) before being incubated with 1 μCi of ^{125}I-protein A (10 μCi/ml; ICN) per ml.

(ii) CHO cells transfected with E1 expression plasmids. Analysis of E1 proteins was performed essentially as has been described for the E2 protein (49). Three days following electroporation, three dishes of cells from each transfection were combined and lysed, and their protein concentrations were determined by the bicinchoninic acid assay (Pierce). Equal amounts of total protein (1.7 mg) from each sample were incubated with 1.5 μl of anti-M antibody for 1 h at 4°C with gentle agitation before the immune complexes were collected, washed, and fractionated by electrophoresis as described elsewhere (49). Western blotting of gels was performed as described previously (49) except that the blots were blocked overnight at 4°C in Tris-saline (13.2 mM Tris-hydrochloride [pH 7.4], 169 mM NaCl) containing 0.5% Nonidet P-40, 0.1% Tween 20, 0.5% bovine serum albumin, and 2.3% nonfat dry milk. The blots were incubated with anti-M antibody (1:5,000), washed, and then incubated with protein A-horseradish peroxidase (1:1,000; Amersham) before being developed by electrochemiluminescence (Amersham).

Electroporation and replication assays. Electroporations were performed as described before (43) except that CHO cells were used, the voltage was 250 V, and the cells were plated onto six dishes following transfection. One microgram of pKSO and 0.5 μg of pCGE2 were used in each electroporation together with 0.5 or 2.5 μg of either wild-type or mutant pCGEag. Plasmid KSO was transfected uncut, but the expression vectors were linearized with *Xmn*I, which cleaves only once, in a region of the pCG vector that is nonessential for E1 or E2 expression. Replication assays were modified from that in Ustav and Stenlund (43) in that low-molecular-weight DNA was isolated by the method of Hirt (17), extracted once with phenol-chloroform and once with chloroform-isoamyl alcohol, and precipitated with isopropanol before being treated with RNase A and processed for Southern blots as described elsewhere (43).

RESULTS

DNA-binding domain of E1 is contained within the first 299 amino acids. To define the region of E1 that is both necessary and sufficient to bind the origin, we first synthesized a series of progressively shorter E1 proteins by in vitro transcription and translation. Then, using a modification of the DNA-protein immunoprecipitation assay of McKay (27), we tested each protein's ability to bind a BPV-1-origin-containing fragment. Figure 1A shows the carboxy-terminal deletion endpoints of the seven mutants tested (Δ1 through Δ7) and the full-length E1 protein (E1 ORF). The constructs were made so that all proteins initiated translation from the bona fide E1 ATG at nt 849. Each construct produced one predominant protein of the predicted molecular mass (Fig. 1B). The E1 and Δ1 through Δ5 translation products also contained several additional smaller polypeptides. These less abundant polypeptides were all immunoprecipitated by the anti-M antibody (data not shown), which recognizes only E1 sequences encoded by nt 860 to 1471. Because the antibody would not precipitate proteins initiating from the next available ATG, at nt 1506, these short polypeptides probably represent premature translational termination products or carboxy-terminal degradation products. The Δ1 through Δ7 polypeptides were translated more efficiently than the full-length E1 protein, as judged from the signal intensities in Fig. 1B, adjusting for the number of methionines present in each sequence.

When the full-length E1 protein was incubated with radiolabeled *Ava*II DNA fragments of cloned BPV-1 DNA, a specific DNA-protein complex could be immunoprecipitated with anti-M antibodies (Figure 1C, lane E1). A retained fragment of 219 bp was detected, and this fragment corresponds in length to the fragment spanning the origin site. This finding confirms the observations of Wilson and Ludes-Meyers (45), who also used a DNA-protein immunoprecipitation assay and *Ava*II fragments to demonstrate specific E1-DNA interactions. The other conspicuous band in the E1 lane (and some of the other lanes in Fig. 1C) that migrates at a mobility of 680 bp emanates from the 219-bp fragment (perhaps a denatured form; data not shown) and is an artifact

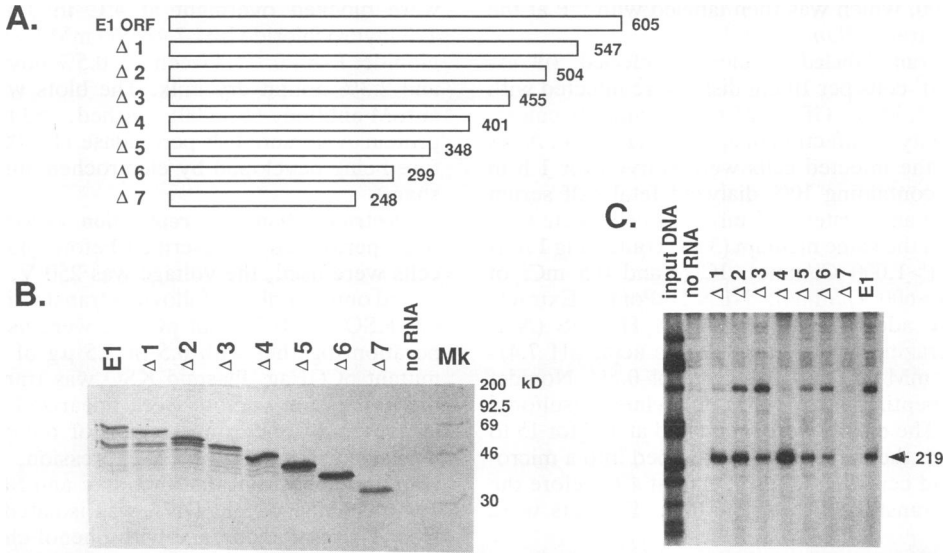

FIG. 1. DNA-binding domain of E1 maps to within the N-terminal 299 aa. (A) Diagrammatic representation of the truncated E1 proteins used to map the DNA-binding domain of E1. At the top is the full-length E1 ORF, which encodes a 605-aa protein. Below are diagrammed seven E1 deletion mutants, with the number corresponding to the C-terminal amino acid of each. All polypeptides initiate translation from the E1 ATG at nt 849. (B) The full-length E1 ORF and the seven E1 deletion mutants produce proteins of the predicted sizes. The DNAs described above were transcribed and translated in vitro, and equivalent amounts (1 μl) of the ^{35}S-labeled proteins were analyzed by SDS-PAGE. A translation reaction mix containing everything except RNA showed no detectable proteins (lane no RNA). Lane Mk, size markers. (C) Binding to the 219-bp *Ava*II fragment of pMLBPV is dependent on sequences present in the full-length E1 protein and mutants Δ1 through Δ6. Binding to end-labeled, *Ava*II-digested pMLBPV was performed by in vitro translation reactions to which RNA from either E1 or Δ1 through Δ7 or no RNA had been added, as indicated above the lanes. DNA-protein complexes were immunoprecipitated with anti-M antibody, and precipitated DNA fragments were analyzed on an 8% polyacrylamide gel. A portion of the input fragment mixture is shown on the left (input DNA). The position of the 219-bp BPV-1 origin fragment is indicated by an arrow. The additional, larger "fragment" seen in lanes Δ1 through Δ6 and E1 is a slower-migrating form of the 219-bp fragment (see Results).

that can be eliminated by careful ethanol washing of the precipitated DNA pellet before gel loading. An identical reaction in which no RNA had been added to the translation mix did not yield a protein which could retain the 219-bp fragment or any other specific pMLBPV fragment (Fig. 1C), showing that the polypeptide responsible for binding to this fragment was indeed the in vitro-translated E1.

To confirm that the 219-bp fragment contains the BPV-1 origin, a series of DNA-protein immunoprecipitation assays were performed with purified BPV-1 E1 protein (GE1) and *Ava*II digests of BPV-1 templates containing insertions of linkers of increasing size at the unique *Hpa*I site in the viral genome. This site defines the center of the E1 recognition sequences in the origin of replication. GE1 is the BPV-1 E1 protein with a 9-aa peptide at its N terminus (47). As shown in Fig. 2, the linkers diminish or eliminate the extent of complex formed, as measured by retention of the 219-bp fragment. An *Ava*II plus *Hpa*I digest of pMLBPV destroyed the 219-bp fragment, generating two new fragments (Fig. 2, lane 6), neither of which bound E1, as detected in this assay (Fig. 2, lane 12). Immunoprecipitation assays with in vitro-translated E1 as the source of protein with the same DNA templates and restriction enzyme digests yielded parallel results (data not shown).

When we tested the truncated E1 proteins in the same binding assay, Δ1 through Δ6 also bound the 219-bp *Ava*II fragment (Fig. 1C). However, Δ7 did not bind this fragment, even though comparable amounts of the Δ7 and full-length E1 proteins were immunoprecipitated (data not shown), suggesting that sequences required for binding to the origin were located between aa 248 and 299. In summary, the data

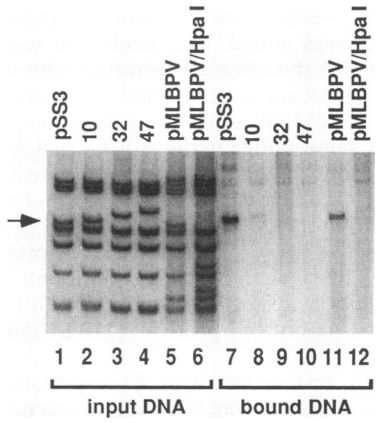

FIG. 2. The 219-bp *Ava*II fragment bound by GE1 contains the BPV-1 origin. Lanes 1 to 6 show radiolabeled input DNAs: *Ava*II digests of pSS3 and derivatives of pSS3 containing 10-, 32-, and 47-bp insertions into the *Hpa*I site at the origin (lanes 1 to 4, respectively) plus *Ava*II and *Ava*II-*Hpa*I digests of pMLBPV (lanes 5 and 6, respectively). The arrow points to the 219-bp origin fragment with no insertion. Purified GE1 (140 ng) was incubated with the same input DNAs, and the DNA-protein complexes were immunoprecipitated with anti-M antibody (lanes 7 to 12). Bound and input DNA fragments were analyzed on an 8% polyacrylamide gel.

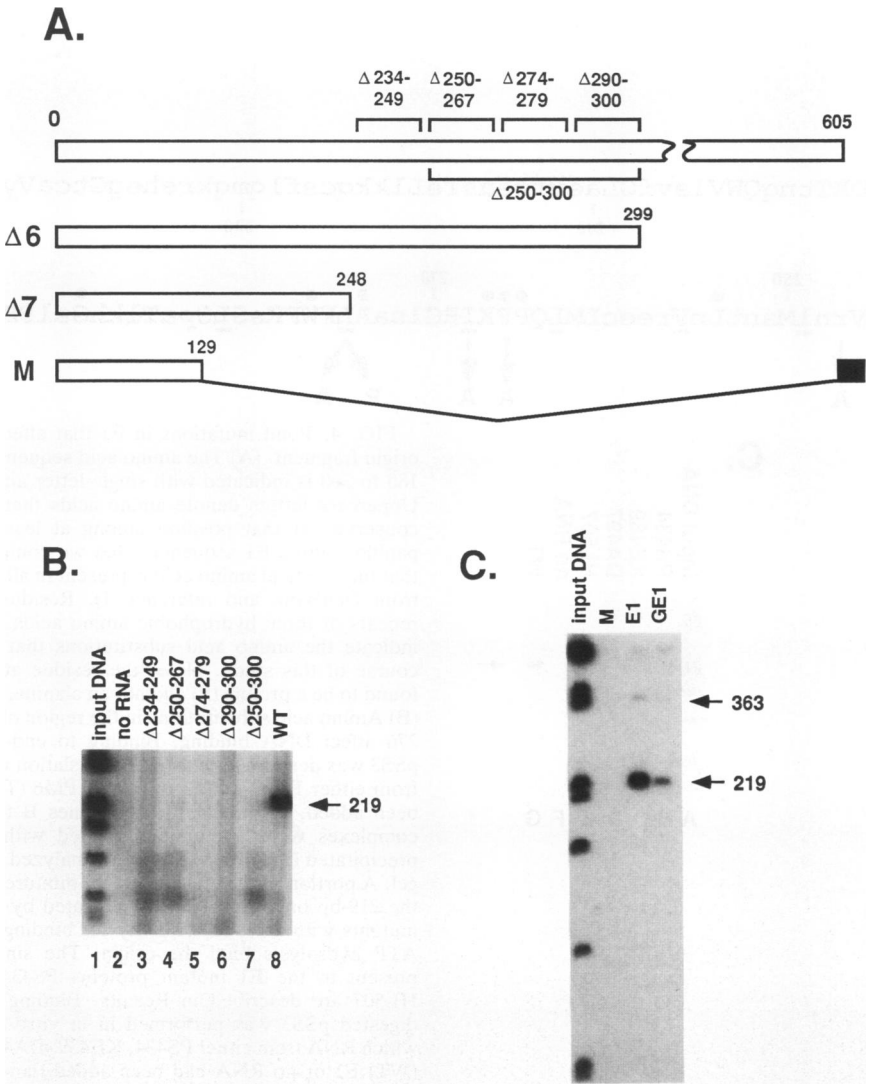

FIG. 3. Binding to the BPV-1 origin requires amino acids between aa 233 and 300 of E1. (A) Diagrammatic representation of the five different internal deletions (brackets) created in the full-length E1 protein (top), the C-terminal deletion mutant proteins Δ6 and Δ7, and the M protein (not to scale). Sequences present in the E1 proteins are depicted by open boxes. The 13 aa encoded by downstream sequences that are spliced to M are depicted as a black box. (B) Small deletions in the region of E1 between aa 233 and 301 abolish origin binding by E1. Binding to end-labeled, *Ava*II-digested pSS3 was performed by in vitro translation reactions to which either wild-type E1 RNA, RNA made from the E1 internal deletion mutants shown above, or no RNA was added (as indicated above the lanes). DNA-protein complexes were immunoprecipitated with anti-M antibody, and precipitated DNA fragments were analyzed on an 8% polyacrylamide gel. A portion of the input fragment mixture is shown in lane 1, and the position of the 219-bp origin fragment is indicated by an arrow. (C) M does not bind the origin fragment. Binding to end-labeled, *Ava*II-digested pSS3 was performed with either baculovirus extracts containing the M or E1 protein (10 μg) or purified GE1 (100 ng), as indicated above the lanes. DNA-protein complexes were immunoprecipitated with anti-M antibody, and precipitated DNA fragments were analyzed on an 8% polyacrylamide gel. A portion of the input fragment mixture is shown in the left lane (input DNA), and the 219-bp origin and 363-bp E2RE1 fragments are indicated by arrows.

obtained with the deletion proteins show that aa 1 through 299 of the E1 ORF define sequences sufficient for origin DNA recognition.

Amino acids between 233 and 300 of E1 are necessary for DNA binding. Examination of the predicted 51-aa sequence that was present in the shortest E1 peptide still able to bind DNA (Δ6) but absent in the one that could not (Δ7) revealed several clusters of amino acids that are highly conserved among all papillomavirus E1 proteins (1, 6). A simple extrapolation from the data thus far presented is that these conserved residues play some role in site-specific DNA binding. However, the difference detected between Δ6 and Δ7 for DNA binding could perhaps be attributed to an unfortunate choice of endpoint for Δ7, which created a misfolded protein. To further analyze the role of these amino acids in DNA binding, we synthesized five E1 proteins, each containing a different small deletion in this region within the context of the full-length E1 protein (Fig. 3A), and tested their abilities to precipitate the origin fragment from a radiolabeled *Ava*II digest of pSS3. In contrast to wild-type E1, none of the mutants tested bound detectable levels of the origin fragment or any other fragment of the input DNA specifically (Fig. 3B,

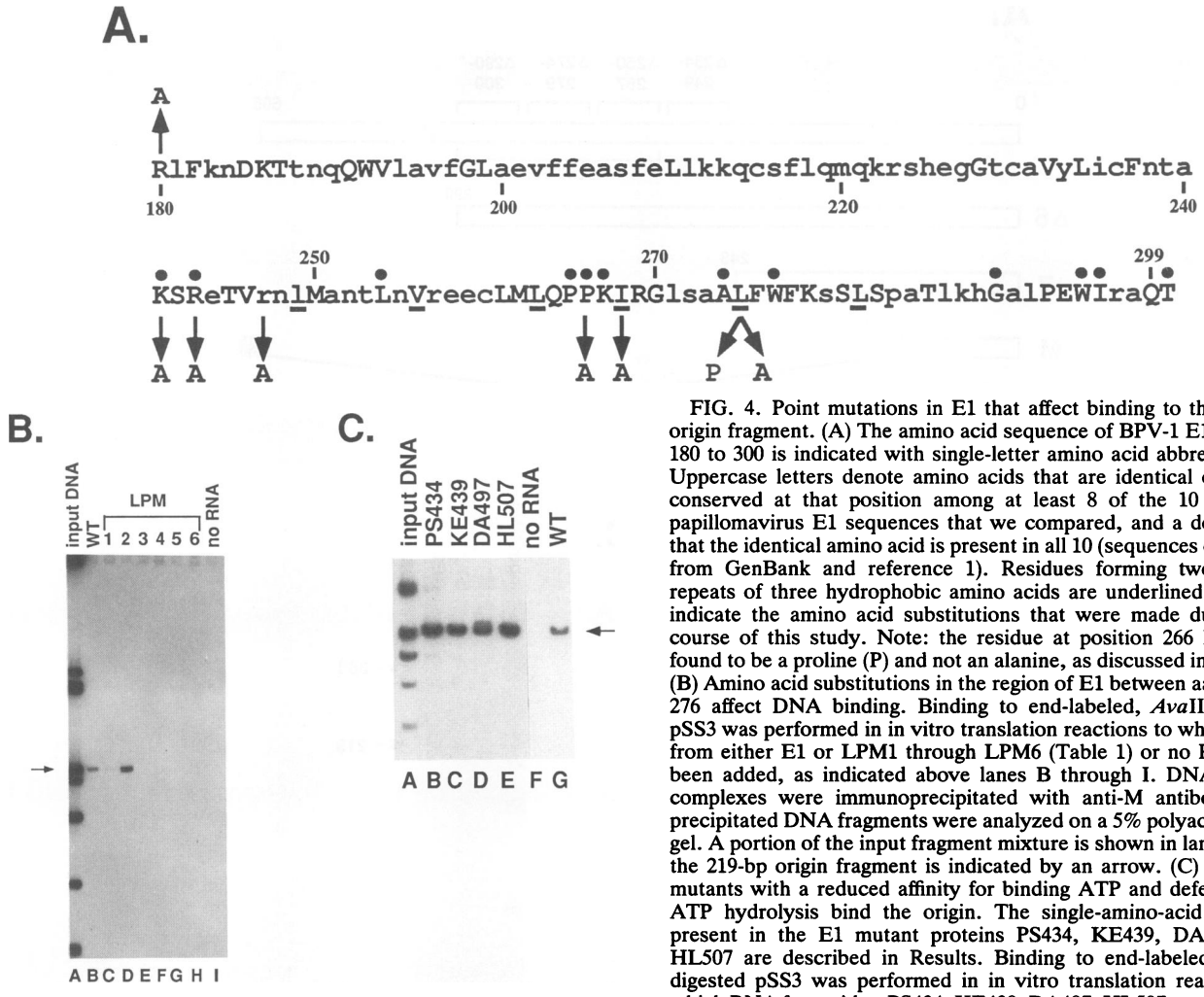

FIG. 4. Point mutations in E1 that affect binding to the 219-bp origin fragment. (A) The amino acid sequence of BPV-1 E1 from aa 180 to 300 is indicated with single-letter amino acid abbreviations. Uppercase letters denote amino acids that are identical or highly conserved at that position among at least 8 of the 10 different papillomavirus E1 sequences that we compared, and a dot means that the identical amino acid is present in all 10 (sequences compiled from GenBank and reference 1). Residues forming two heptad repeats of three hydrophobic amino acids are underlined. Arrows indicate the amino acid substitutions that were made during the course of this study. Note: the residue at position 266 has been found to be a proline (P) and not an alanine, as discussed in Results. (B) Amino acid substitutions in the region of E1 between aa 179 and 276 affect DNA binding. Binding to end-labeled, *Ava*II-digested pSS3 was performed in in vitro translation reactions to which RNA from either E1 or LPM1 through LPM6 (Table 1) or no RNA had been added, as indicated above lanes B through I. DNA-protein complexes were immunoprecipitated with anti-M antibody, and precipitated DNA fragments were analyzed on a 5% polyacrylamide gel. A portion of the input fragment mixture is shown in lane A, and the 219-bp origin fragment is indicated by an arrow. (C) E1 point mutants with a reduced affinity for binding ATP and defective for ATP hydrolysis bind the origin. The single-amino-acid changes present in the E1 mutant proteins PS434, KE439, DA497, and HL507 are described in Results. Binding to end-labeled, *Ava*II-digested pSS3 was performed in in vitro translation reactions to which RNA from either PS434, KE439, DA497, HL507, or wild-type (WT) E1 or no RNA had been added (lanes B through G). DNA-protein complexes were immunoprecipitated with anti-M antibody, and precipitated DNA fragments were analyzed on an 8% polyacrylamide gel. A portion of the input fragment mixture is shown in lane A, and the 219-bp origin fragment is indicated by an arrow.

lanes 3 to 7), despite the fact that they were precipitated as well as wild-type E1 (data not shown). Although we could not exclude the possibility that these results were due to aberrant folding of the mutant proteins, it seemed unlikely that all five would be crippled for this reason, especially as four of them had deletions of nonoverlapping sequences. Thus, it appears that sequences throughout the region between aa 233 and 300 are indeed important for binding the BPV-1 origin of DNA replication.

The results described above predict that the M protein by itself cannot bind DNA. The 23-kDa M protein is a bona fide BPV-1 protein, comprising the first 129 aa of E1 joined to 13 aa encoded from sequences downstream (Fig. 3A). Because the M protein was translated poorly in vitro, we compared the binding of M and E1 proteins expressed from recombinant baculoviruses. When Sf9 cell extract containing E1 was used in our DNA-protein immunoprecipitation assay, E1 bound the origin fragment, as expected, as did purified GE1. However, Sf9 extract containing an equivalent amount of M did not bind this or any other fragment of the input DNA (Fig. 3C), despite the fact that M and E1 were immunoprecipitated with equal efficiency (data not shown).

This result leads us to conclude that although the M region may influence the structure of the site-specific DNA-binding domain of E1 (see below), by itself it does not include sequences sufficient for DNA recognition. We attempted to identify the amino-terminal boundary of the DNA-binding domain of E1 by testing a short E1 polypeptide containing sequences from aa 167 to 348. This polypeptide starts just downstream of the M domain and contains a highly conserved region of E1 that extends from aa 167 to 198. Although this polypeptide was efficiently precipitated by the antibody, it did not bind the origin fragment significantly above background levels (data not shown). Therefore, the amino-terminal boundary of the E1 binding domain is undetermined by our studies.

Interestingly, in the experiment shown in Fig. 3C, E1 also specifically bound another *Ava*II fragment of 363 bp, although much more weakly than the 219-bp fragment. This larger fragment corresponds to nt 7457 to 7820 of BPV-1, which contains the E2-dependent enhancer E2RE1 (35). Wilson and Ludes-Meyers also observed that, in addition to the origin fragment, a small amount of this 363-bp *Ava*II fragment was consistently and specifically precipitated in

their immunoprecipitation assays under certain conditions (45). Most likely, significant binding to the 363-bp fragment was detected only in this experiment because we used 10 μg of E1, much more than the amounts of in vitro-translated E1 or purified GE1 used in the other experiments. In a separate study, we have shown that E1 binds to this region (49), and a more extensive comparison of E1's affinity for the origin region and the E2RE1 region will be presented elsewhere.

Substitution mutations in E1 that affect binding to the origin. Our results suggesting that multiple amino acids scattered throughout aa 233 to 300 were involved in DNA binding led us to examine this region in greater detail. Of the 67 aa in this stretch of sequence, 12 are absolutely identical in the 10 papillomavirus E1 proteins that we compared, and another 25 are highly conserved among at least 80% of the E1 proteins (Fig. 4A). One striking feature of this region is the presence of two highly conserved short heptad repeats of hydrophobic amino acids. These hydrophobic residues are underlined in Fig. 4A, and they are interrupted by 4 aa that include two prolines. Secondary-structure prediction indicates that this hydrophobic repeat motif region in E1 may possess some α-helical structure interrupted by a turn or loop (see Discussion). Immediately N-terminal to the heptad repeats are three highly conserved basic amino acids, Lys-241, Arg-243, and Arg-247, and these positively charged residues may form part of a "basic region" responsible for contacting DNA.

To determine whether any of these amino acids are important for binding DNA, we made the substitution mutants shown in Table 1. We intended to make only single and double substitution mutants, but in the course of this work, we discovered that our wild-type E1 DNA and that in pCGEag (43) contained the same single nucleotide difference from the published sequence, a C instead of a G at nt 1644. This difference changes Ala-266 to Pro, and because every E1 ORF of the nine different papillomaviruses that we compared has a Pro at this position, it is likely that there is an error in the published sequence for BPV-1 (9). We will therefore define codon 266 as a Pro in the wild-type protein. As we were not initially aware of this error in the E1 sequence, in the course of generating mutations in LPM3 by site-directed oligonucleotide mutagenesis, we changed Pro-266 to Ala.

Of the six mutant E1 proteins tested, only LPM1 and LPM2 retained the ability to bind the 219-bp origin fragment from a radiolabeled *Ava*II digest of pSS3 (Fig. 4B, lanes C to H). In repeated experiments, LPM2 consistently bound more origin fragment than wild-type E1, whereas LPM1 consistently bound significantly less, and these amounts did not reflect the amounts of protein immunoprecipitated. In some experiments, LPM5 bound a barely detectable amount of origin fragment (see Table 1). Mutant proteins unable to bind the origin fragment were precipitated by the antibody as well as or better than the wild-type protein was (data not shown). The behavior of these point mutants provides convincing evidence that residues in this region of E1 are critical for binding DNA. Interestingly, LPM6, with a single-amino-acid change 60 residues N-terminal to the putative basic region, was also unable to bind the origin fragment (Fig. 4B, lane H). The Arg-180 that was changed to Ala in LPM6 falls in another cluster of highly conserved amino acids (aa 167 to 198) containing other conserved basic amino acids. This result, together with those presented earlier, indicates that the region of E1 critical for DNA binding extends minimally from aa 180 to sequences between aa 289 and 300.

A previous study suggests that ATP together with $MgCl_2$ significantly stimulates E1 binding to DNA containing the BPV-1 origin in fragment retardation and nitrocellulose binding assays (34). Even though we have not yet determined whether ATP stimulates E1-DNA binding in our immunoprecipitation assay, we do believe that ATP is not essential for two reasons. First, neither ATP nor Mg^{2+} was included in our binding reactions. Although both are present in the rabbit reticulocyte lysate used for in vitro translation, they were undoubtedly significantly reduced in concentration when the proteins were bound to PAS and washed extensively before the addition of DNA. Furthermore, we tested four mutant E1 proteins containing different single-amino-acid changes: Pro-434 to Ser (PS434), Lys-439 to Glu (KE439), Asp-497 to Ala (DA497), and His-507 to Leu (HL507). All of these mutant proteins display a reduced affinity for ATP compared with the wild-type protein, and all except PS434 are completely defective for ATP hydrolysis (26a, 39). We observed that all of the ATP-binding mutant proteins bind the origin fragment specifically (Fig. 4C, lanes B to E), and interestingly, they reproducibly bound more of this fragment than did an equivalent amount of precipitated wild-type protein (lane G). From this, we conclude that neither ATP binding nor its hydrolysis is required for E1 to bind the origin fragment in our immunoprecipitation assay. This result was predicted from our previous observation that all of the E1 sequences necessary for origin binding mapped N-terminal to aa 300; that is, the ATP binding domain could be deleted.

TABLE 1. Relative DNA binding of E1 mutants[a]

E1	Amino acid change(s)	Relative binding	Relative binding with E2
Wild type	None	1	1
LPM1	L275P	0.14	1.6
LPM2	L275A	2.7	1.6
LPM3[b]	P266A, I268A, L275A	0	0.02
LPM4	K241A, R243A	0	0
LPM5	R247A	0.05	0.3
LPM6	R180A	0[c]	0[c]

[a] Values are from a single experiment. This experiment has been repeated twice, and similar values were obtained. Quantitation was done from densitometric tracings of autoradiographs (within a linear exposure range) with a Microtek ScanMaker and the Adobe Photoshop program (Macintosh). In the repeated experiments, quantitation was done by PhosphorImager. Binding values are relative to that for wild-type E1.

[b] The P266A substitution was made as a result of an error in the published DNA sequence for BPV-1 (see Results).

[c] In one experiment, LPM6 showed relative binding values of 0.001 without E2 and 0.007 with E2.

N-terminal 423 aa of E1 are sufficient for origin-binding enhancement by E2. It has been shown that E1 forms a stable complex with the E2 transcriptional activator protein of BPV-1 (4, 26, 29) and that E2 enhances the ability of E1 to bind the origin sequence (33, 36, 47, 49). That the enhancement or cooperativity in DNA binding requires protein-protein interaction is implied by several indirect pieces of evidence, yet it has not been conclusively proven. Because of our interest in the DNA-binding characteristics of E1, we wished to identify the sequence(s) of the E1 protein that is necessary for E2 to mediate cooperative binding of E1 to DNA.

Lusky and Fontane (26) reported that an E1 protein truncated at aa 423 was severely impaired for interaction with E2 and that an E1 polypeptide containing aa 220 to 605 efficiently associated with E2. In agreement with these

findings, mapping experiments performed in our laboratory by mixing truncated E1 proteins translated in vitro with purified baculovirus-expressed E2 indicated that E1 sequences from aa 458 to 605 were both necessary and sufficient for complex formation when the proteins were incubated at 4°C (unpublished results). We therefore wanted to determine whether these particular C-terminal sequences which could contact E2 were also necessary for E2 stimulation of E1 DNA binding. We tested a truncated GE1 protein lacking these sequences for cooperative binding with E2 in a DNase I footprint assay. GE1Δ424, which contains E1 sequences from aa 1 to 423, protects the origin sequence in the absence of E2, as does wild-type GE1 (Fig. 5, lanes 2 to 4 and 10 to 12). The stimulatory effect of E2 in the binding reaction was measured with an amount of GE1 or GE1Δ424 protein which could by itself initially protect the origin sequence from DNase I digestion but, when diluted, could no longer afford such protection. Figure 5 shows that E2 enhanced protection by both the GE1 and GE1Δ424 proteins to about the same extent (Fig. 5, compare lanes 6 to 8 with 14 to 16). These experiments are not strictly quantitative, as we do not have the appropriate endpoints to measure the extent of cooperativity provided by E2. That is, for all concentrations of GE1 tested in the presence of E2, complete protection of the E1 binding site was found. Nevertheless, we conclude that the fragment of GE1 used, GE1Δ424, clearly retains DNA-binding activity and cooperative interaction with E2. This conclusion suggests that the protein-protein interactions reported previously are not essential for cooperative binding with E2 and that E1 may contain other sequences able to interact with E2 which were not identified by the earlier mapping studies.

E1 binding to E2 is cold sensitive. All previous studies on the protein-protein interactions between E1 and E2 were performed at 4°C or at least included incubation of the immune complexes at 4°C for a significant length of time (4, 26, 29, 49). However, we have observed that the cooperativity between E1 and E2 for binding DNA is cold sensitive. That is, when DNase I protection experiments such as that shown in Fig. 5 are performed at 4°C instead of 37°C (with wild-type E1 protein), no cooperative binding between E1 and E2 to DNA can be detected (21a). This implies that the potential contacts between E1 and E2 that are critical for cooperativity do not form effectively at the lower temperature. It is known that hydrophobic effects, which are a common driving force in the formation of protein complexes, are reduced at low temperature (11). To explore this question further, we purified full-length GE1 and E2 proteins, mixed them together at three different temperatures, and precipitated E2 with monoclonal antibody B202. GE1 that coprecipitated with E2 was measured after SDS-PAGE separation of the proteins. As shown previously, the E2 antibody does not cross-react with GE1 (Fig. 6A, lane 2) and precipitates E2 at all temperatures tested (lanes 3 to 5). However, only at room temperature and 37°C is complex formation between E2 and GE1 detected. We assume that 4°C is below the association constant required for nanogram amounts of GE1 and E2 to complex at this temperature. These results indicate that the interactions between the proteins are indeed cold sensitive. With this information, we then asked whether conditions could be found that would demonstrate protein-protein interaction between the truncated E1 protein, GE1Δ424, and E2.

Extracts from Sf9 cells infected with different baculovirus expression vectors were prepared, and the total protein composition of these extracts, as measured by metabolically

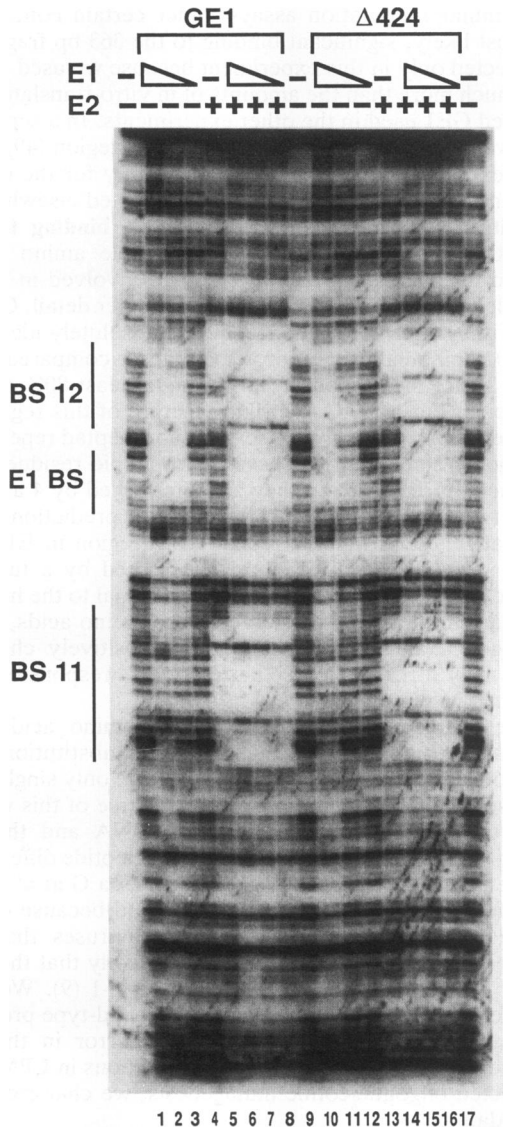

FIG. 5. Amino acids 1 to 423 of GE1 are sufficient for origin-binding enhancement by the BPV-1 E2 protein. DNA fragments containing BPV-1 sequences from nt 7805 to 100 were labeled with ^{32}P at the 5′ end of the top strand. The binding reaction was carried out at 37°C for 15 min and followed by standard DNase I digestion as described by Yang et al. (47). GE1 concentrations: lanes 2 and 6, 360 ng; lanes 3 and 7, 120 ng; lanes 4 and 8, 40 ng. GE1Δ424 (Δ424) concentrations: lanes 10 and 14, 80 ng; lanes 11 and 15, 27 ng; lanes 12 and 16, 9 ng. E2 concentrations: lanes 5 to 8 and 13 to 16, 60 ng. Lanes 1, 9, and 17 show DNA treated with DNase I in the absence of any added BPV-1 proteins. BS11 and BS12 are E2 binding sites 11 and 12, which flank the E1 binding site (E1 BS).

labeling proteins in vivo with [^{35}S]methionine and [^{35}S]cysteine, is shown in Fig. 6C. As reported previously (29), when equal volumes of the E1 and E2 extracts are incubated together at room temperature and then immune complexes formed in the presence of anti-E2 antiserum are washed at 4°C, the E1 protein as well as E2 can be specifically precipitated (Fig. 6D, lane 18). It should be noted that the amounts of E1 and E2 in these extracts are at least 10-fold higher than those used in the preceding experiment (Fig.

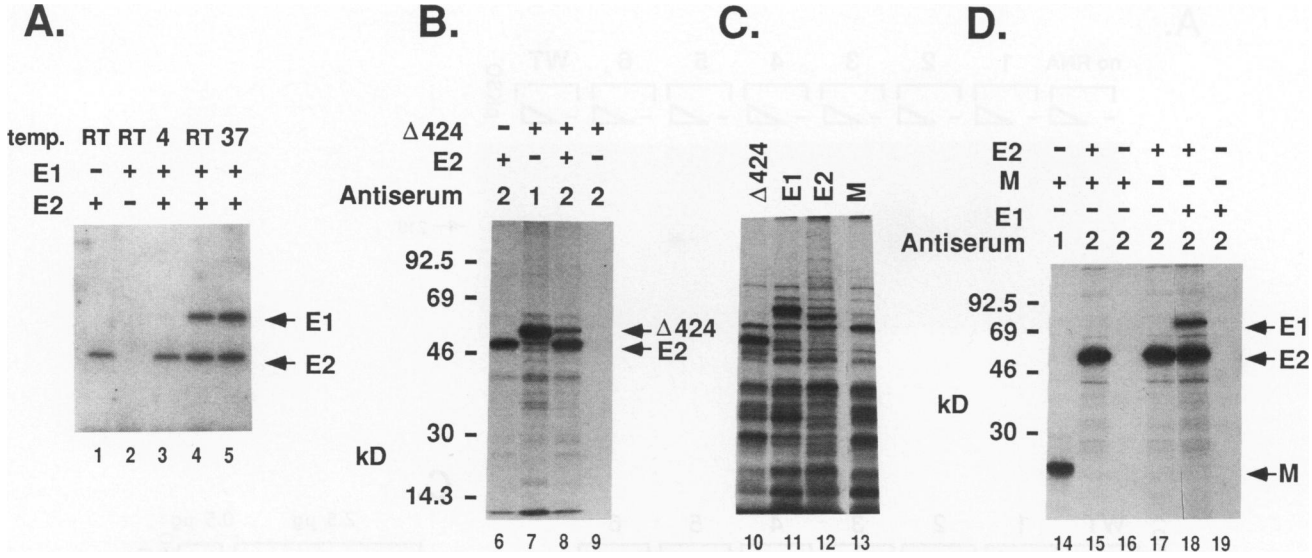

FIG. 6. Temperature affects the binding of E1 and E1-related proteins to E2. (A) GE1 binds E2 better at higher temperatures. Purified GE1 (80 ng) and purified E2 (40 ng) were incubated together (lanes 3 to 5) or alone (lanes 1 and 2) for 2 h at the temperatures indicated in the presence of anti-E2 antiserum. After the immune complexes were washed at room temperature (RT), proteins were subjected to SDS-PAGE and Western blotting. The blot was incubated with both anti-M and anti-E2 antisera and then visualized by using ^{125}I-protein A. The ratios of GE1 to E2 (arrows) at the different temperatures were 0 (4°C), 0.7 (room temperature), and 1.4 (37°C). (B) Amino acids 1 to 423 of GE1 (Δ424) are sufficient for binding E2 at 30°C. Equivalent amounts (25 μl) of radiolabeled Sf9 extracts containing the Δ424 or E2 protein expressed from baculoviruses were incubated at 30°C for 2.5 h alone or in the combinations indicated (lanes 6 to 9). Protein complexes were immunoprecipitated with either anti-M (lane 1) or anti-E2 (lanes 2) antiserum and washed at 30°C, and proteins were analyzed by SDS-PAGE. The Δ424 and E2 proteins are indicated by arrows. (C) A portion of each radiolabeled baculovirus-infected Sf9 extract (1/120) was analyzed by SDS-PAGE for comparison (lanes 10 to 13). (D) E2 does not bind M under conditions previously used to demonstrate binding to E1. Equivalent amounts (50 μl) of radiolabeled Sf9 extracts containing either the M, E1, or E2 protein expressed from baculoviruses were incubated at room temperature for 2 h alone or in the combinations indicated (lanes 14 to 19). Protein complexes were immunoprecipitated with either anti-M (1) or anti-E2 (2) antiserum and washed at 4°C, and proteins were analyzed by SDS-PAGE. The M, E1, and E2 proteins are indicated by arrows.

6A). The E2 protein, however, does not complex with the M protein (Fig. 6D, lanes 14 to 16). Similarly, as anticipated from previous studies, we could not detect an interaction between the GE1Δ424 protein and E2 under these conditions (data not shown). However, when the GE1Δ424 and E2 extracts were mixed and incubated at 30°C, complex formation was detected (Fig. 6B). These experiments are consistent with the view that direct protein-protein interaction between E1 and E2 plays a role in their cooperative binding to DNA and that at least some of this interaction occurs via E1 surfaces retained in aa 1 to 423.

Cooperative binding interactions between mutant E1 and E2 proteins. In a further attempt to map the sequences in the E1 protein required for cooperativity with E2, we took advantage of the fact that E2 enhances E1-DNA binding in a DNA-protein immunoprecipitation assay (29, 36). Interestingly, origin binding by the truncated E1 protein Δ6 (Fig. 1A) was enhanced by E2 in this assay (data not shown). This suggests that residues in E1 which are involved in contacting E2 map N-terminal to aa 300 and is consistent with the cooperativity observed for GE1Δ424 (see above). We used this assay to test whether the point mutations in those E1 proteins that could still bind DNA (e.g., LPM1 and LPM2) affected E1 enhancement by E2. We were also interested to know whether E2 could "rescue" any of the mutants that did not bind detectable levels of DNA (LPM3, LPM4, and LPM6).

As expected, addition of purified E2 protein significantly increased the amount of origin-containing fragment that was retained by wild-type E1 in the complex immunoprecipitated with anti-M serum (Fig. 7A, lanes WT). This enhancement was clearly E1 dependent, as addition of E2 to rabbit reticulocyte extract alone precipitated only a barely detectable amount of the origin fragment at the highest concentration of E2 (Fig. 7A, lanes no RNA). The mutants that were able to bind the origin fragment in the absence of E2 (LPM1 and LPM2) bound significantly more fragment in the presence of E2 (Fig. 7A). In addition, LPM3 and LPM5, which bound undetectable and barely detectable amounts of the origin fragment alone, respectively, reproducibly bound an amount of this fragment that was above background in the presence of E2 (Fig. 7A).

The absolute amounts of fragment detected in complex with the different mutants in the presence of E2 varied significantly (Table 1). In the presence of 100 ng of E2, LPM1 and LPM2 reproducibly retained the most fragment, more than 1.6 times the amount precipitated by wild-type E1. With E2, wild-type E1 precipitated approximately 3.3 and 50 times more fragment than LPM5 and LPM3, respectively, while LPM4 and LPM6 did not precipitate detectable amounts of the origin fragment alone or in the presence of E2 (Fig. 7A). From these results, we conclude that the mutations contained in LPM1, LPM2, LPM3, and LPM5 probably do not deleteriously affect E1-E2 interactions, at least not significantly. The E2 enhancement measured by this assay relies on the ability of E1 to bind DNA. Consequently, mutant proteins unable to bind the origin fragment alone or

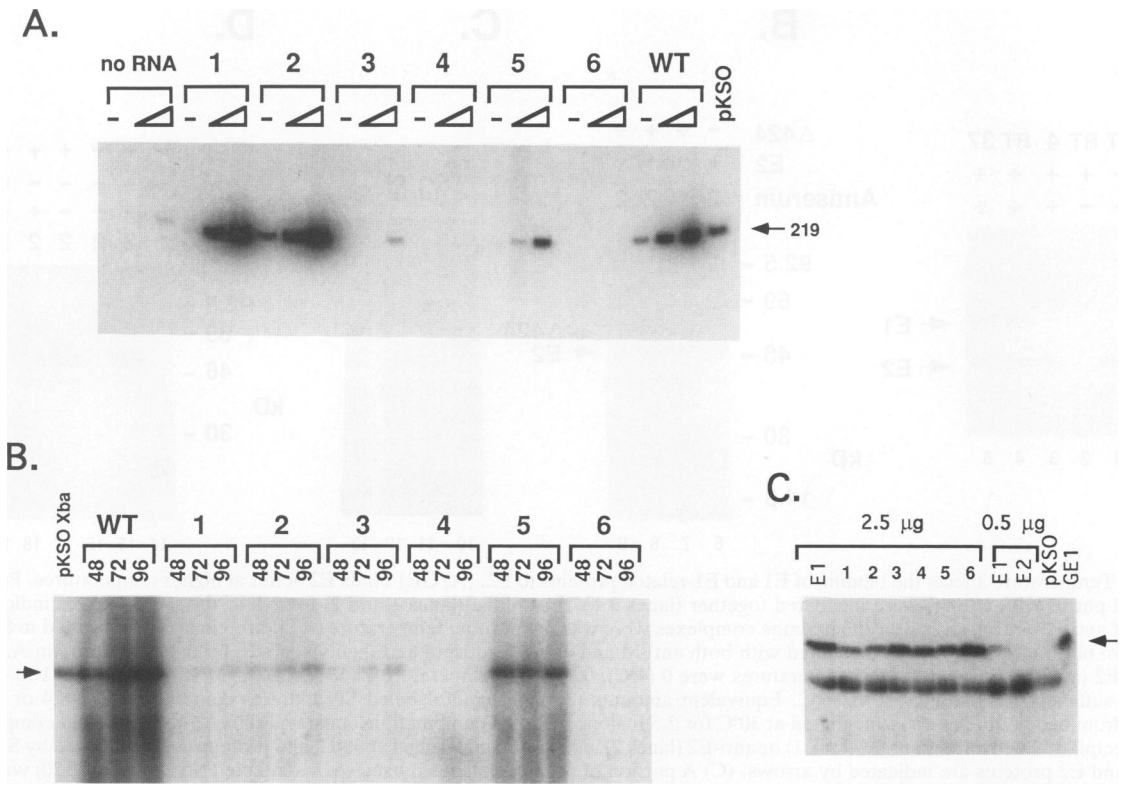

FIG. 7. Behavior of E1 DNA-binding mutants in vitro is predictive of some but not all mutant phenotypes in tissue culture cells. The numbers 1 to 6 correspond to mutants LPM1 to LPM6 (Table 1). (A) Addition of E2 increases the amount of origin fragment precipitated by E1 (wild type [WT]) and certain DNA-binding mutants. Binding to the 219-bp origin fragment from *Bam*HI- plus *Eco*RI-digested pKSO (arrow) was performed in in vitro translation reactions to which RNA from either E1 or LPM1 through LPM6 or no RNA had been added (as indicated), either without E2 (—) or with increasing amounts of purified E2 (10 or 100 ng). DNA-protein complexes were immunoprecipitated with anti-M antibody, and precipitated DNA fragments were analyzed on a 5% polyacrylamide gel. (B) Southern blot showing that E1 DNA-binding mutants are crippled for transient replication of a BPV-1 origin-containing plasmid. Plasmid pKSO (1 μg) was transfected together with expression vectors producing E2 (0.5 μg) plus either wild-type or mutant E1 (2.5 μg) into hamster CHO cells. At the indicated time points (hours), low-molecular-weight DNA was harvested, digested with *Dpn*I, and linearized with *Xba*I. The lane on the left shows the position of linearized pKSO (arrow). (C) Western blot showing that the amounts of the mutant E1 proteins in cells do not explain their replication phenotypes. The amounts of wild-type or mutant E1 in the cells used for the transient-replication assay shown above were measured at 72 h postelectroporation. Proteins were immunoprecipitated with anti-M antiserum, subjected to SDS-PAGE, and immunoblotted with anti-M antiserum. Cells were transfected with 2.5 or 0.5 μg of a wild-type or mutant E1 expression vector, as indicated, plus 0.5 μg of an E2 expression vector and 1 μg of pKSO. In addition, pKSO was transfected alone (pKSO) and with E2 (E2). Purified GE1 protein (GE1, 42 ng) was included as a marker for the E1 protein (arrow). The faster-migrating protein present in all lanes except GE1 is the immunoglobulin G heavy chain.

with E2 (LPM4 and LPM6) tell us nothing about how their mutations affect interaction with E2.

Characterization of the replication defects of the E1 DNA-binding mutants. To measure the effects of these substitution mutations in the DNA-binding domain on transient replication in vivo, we transferred these mutations to the E1 expression vector pCGEag (43). We transfected these mutant E1 vectors into CHO cells together with the E2 expression vector pCGE2 (43) and the BPV-1-origin-containing plasmid pKSO (47). As anticipated, plasmid pKSO transfected alone or together with either pCGEag or pCGE2 gave no signal (data not shown). Mutants LPM1, LPM2, and LPM3 all supported poor but reproducible replication of pKSO, approximately 5 to 10% of the amount seen with wild-type E1 (Fig. 7B). LPM4 did not support any detectable replication of pKSO, and similarly, in two independent experiments, LPM6 showed only a barely detectable signal at 72 h that was not significantly above background (Fig. 7B and data not shown). Neither of these proteins bound the origin fragment in our immunoprecipitation assays, with or without E2 (see Table 1). Perhaps most interesting was the behavior of LPM5, which replicated pKSO to approximately 35% of the amount seen with wild-type E1. In the immunoprecipitation assays, this protein bound only a barely detectable amount of the origin fragment by itself, although its binding was greatly enhanced (50-fold) by the addition of 100 ng of E2.

To address the possibility that these mutations crippled pKSO replication simply by lowering the levels of E1, we measured the E1 concentration in the cells during the transient-replication assay. All of the cell extracts contained detectable E1, but the amounts varied between the different mutant and wild-type proteins (Fig. 7C). The LPM3, LPM4, and LPM6 proteins were present in amounts that were nearly the same or greater than those of wild-type E1, and yet they replicated pKSO poorly or not at all. Likewise, although the amounts of these three mutant proteins was greater than that of LPM5, it supported much better pKSO

replication. To address the significance of this sort of quantitative variance in E1 levels, we transfected cells with 0.5 μg of wild-type pCGEag instead of 2.5 μg. Even though there was less E1 in the cells transfected with 0.5 μg, they exhibited slightly better pKSO replication (data not shown). The levels of E1 detected in the cells with this amount of vector are shown in Fig. 7C (compare E1 [0.5 μg] with E1 [2.5 μg]). The amounts of LPM1 and LPM2 present in the transfected cells were low but not as low as the amount of wild-type E1 in cells transfected with 0.5 μg of pCGEag; therefore, we conclude that these amounts are sufficient to support high-level replication. We conclude that the amounts of E1 produced by all of the mutants is saturating and, therefore, that the different phenotypes can be attributed to altered activities of the proteins.

DISCUSSION

The E1 protein can bind to specific sequences in the BPV-1 origin (34, 44, 45, 47, 49). In the present study, we have identified the region of E1 that is required for DNA binding and the specific amino acids in this region that are important for this activity. A truncated E1 protein consisting of only the first 299 aa of E1 contains everything necessary and sufficient to bind the origin of BPV-1 replication. In contrast, a truncated protein consisting of the first 248 aa cannot bind the origin. As predicted from these mapping data, the BPV-1 M protein does not specifically bind origin DNA; therefore, any role that M plays in viral replication probably occurs through other means (2, 18, 40). Six mutant proteins containing one, two, or three amino acid substitutions between aa 179 and 276 altered origin binding by E1 dramatically. Wild-type and mutant E1 proteins could be stimulated to bind the origin by the addition of E2, and this stimulation might explain the ability of certain mutants with greatly diminished binding ability to support replication in the cell.

The results reported here differ from those of Wilson and Ludes-Meyers, who showed, using RecA-E1 fusion proteins expressed in *E. coli*, that proteins consisting of the N-terminal 465 or 431 aa failed to bind the viral origin fragment in similar assays (45). The inference from their work was that residues downstream of aa 465 are directly important for DNA binding. From the data presented here, we believe that this is mistaken. All of our truncated proteins larger than Δ7 bound DNA, although we did not test any that terminated exactly at aa 465 or aa 431, nor were our proteins fused to any heterologous sequences. At this time, we cannot explain the basis for this discrepancy; it may reflect interesting differences between proteins synthesized in bacteria versus eukaryotic systems, or may merely be the result of improper folding of these particular E1 proteins truncated near the ATP-binding domain. For SV40 large T antigen, it has been observed that certain truncations decrease DNA binding but that further deletion actually restores activity (28, 37). Perhaps a similar phenomenon may have obscured the mapping of the E1 DNA-binding domain. We have used truncated E1 proteins produced both by in vitro translation and by baculovirus expression vectors, and proteins from both sources show specific DNA binding. Moreover, the fact that certain point mutations engineered in the E1 ORF between aa 179 and 276 have significant effects on DNA binding substantiates our conclusion that the DNA-binding domain of the protein resides in the first 300 aa.

The mutant carrying the single amino-terminal deletion, which starts from aa 167 and extends to aa 348, was defective for DNA binding. This implies that binding requires certain sequences N-terminal to aa 167 and that these may affect the folding or stability of the DNA-binding domain. However, it is clear that further mutational analysis in this region is required to define the amino-terminal border of the DNA-binding domain. Several indirect lines of reasoning lead us to conclude that the residues that contact DNA and constitute the core of the DNA-binding domain lie within a stretch of amino acids starting at approximately aa 170 and continuing to aa 299. All papillomaviruses are likely to have diverged from a common ancestral form, and the E1 ORF is the most highly conserved gene among the viral coding sequences (8). Although some patches of homology exist within the first 167 residues of the various E1 ORFs, this region of the protein is the most divergent. Moreover, many of the highly conserved amino acids in this region have already been shown to be part of nuclear localization motifs (21). The BPV-4 genome contains a truncated E1 ORF that is deleted entirely of sequences analogous to the first 167 aa in the BPV-1 E1 protein. While BPV-4 lies in a branch of the papillomavirus family that is most distally related to all of the others (8), it seems unlikely to us that this group would have emerged with an entirely new DNA-binding structure. Thus, it is likely that the DNA-binding domain of BPV-4 is homologous to those of the other papillomaviruses. Furthermore, the notion that the DNA-binding domain of E1 is C-terminal to aa 167 is consistent with the idea that the splice donor at nt 1235 (aa 129) in the E1 ORF of BPV-1 (and at similar sites in the human papillomaviruses [HPVs]) marks the end of a distinct domain of E1 which encodes the M protein, and M does not bind BPV-1 DNA. Therefore, we propose that the core of the DNA-binding domain of all papillomavirus E1 proteins is contained within the conserved residues highlighted in Fig. 4A. Consistent with this speculation is the finding that the HPV type 6b (HPV-6b) E1 protein binds the origin of DNA replication of both HPV-6b and BPV-1, as measured by DNase I protection experiments. Moreover, a point mutation in HPV-6b E1 that changes a leucine residue to a proline at the position, analogous to Leu-275 of BPV-1 E1, renders the HPV-6b protein incapable of site-specific DNA binding (46a). In a recent report, Bream et al. (5) detected no binding by the HPV-11 E1 protein alone to the HPV-11 origin sequence, yet E1 binding was observed in the presence of E2. The HPV-11 origin and E1 protein are highly homologous to those of HPV-6b; therefore, the results from our lab and those of Bream et al. may appear superficially to differ. However, Bream et al. used only DNA-protein immunoprecipitation assays, and differences between their findings and our unpublished results with respect to HPV E1 DNA-binding ability may be due to the greater stringency of the immunoprecipitation protocol than of DNase I protection. From our work, it seems that E1 proteins possess a biochemical conservation of DNA-binding activity, as predicted by their interchangeability in vivo (10, 12).

The highest concentration of conserved amino acids in the DNA-binding domain of E1 occurs from aa 234 to 299. When we tested E1 proteins with small, nonoverlapping internal deletions in sequences between aa 233 and 301, none of them could complex with the origin fragment, suggesting that multiple residues scattered throughout this sequence are involved in the binding or the folding of the domain. Inspection of these residues revealed a regular array of very highly conserved hydrophobic amino acids from aa 249 to 296. The residues from aa 249 to 282 can be arranged into two heptad repeats of three hydrophobic amino acids separated by four amino acids (two of which are prolines). Robson-Garnier

protein structure analysis predicts that this sequence has the potential to form an α-helix that is interrupted by the prolines (Mac Vector; IBI). Immediately N-terminal to these repeats are three highly conserved basic amino acids—Lys-241, Arg-243, and Arg-247. Two of these, Lys-241 and Arg-243, are present at this position in every one of the 10 E1 protein sequences we examined; Arg-247 is not as well conserved, but every E1 protein has an Arg or Lys at this position or within 1 aa. Our hypothesis, that these positively charged residues form part of a basic region that contacts the DNA, is supported by the Robson-Garnier prediction that this sequence can also form an α-helix. The ability to form α-helices is a common feature of basic regions that bind to DNA (16). Thus, the portion of E1 known to be required for binding DNA possesses some characteristics in common with basic helix-loop-helix DNA-binding domains (reference 3 and references therein).

Table 1 summarizes the DNA-binding properties of the six E1 substitution mutants that were analyzed in our study. We would like to emphasize in our discussion of these mutants that the quantitative aspects of this study may not reflect the absolute binding affinities of these proteins for DNA. The DNA-protein immunoprecipitation assay is particularly sensitive to off-rates in a protein-DNA complex because of the extensive washing of the complexes and because the antibody itself puts a constraint on the complex which can lead to reduced binding. Nevertheless, this assay is convenient and obviates the need to purify large amounts of protein; also, differences in the amount of fragment retained by mutants do measure a qualitative change in binding affinities. We therefore used this method to screen for relative binding differences which may provide useful information for subsequent studies.

LPM1 and LPM2, in both of which Leu-275 is affected, are interesting DNA-binding mutants for two reasons. It would seem that this leucine does not contribute directly to specific DNA binding, as changing the amino acid to an alanine (LPM2) actually increases the apparent affinity for origin DNA. However, because LPM2 is deficient for DNA replication, it is likely that this residue participates in other functions of E1. Consistent with our hypothesis that hydrophobic residues in the region including residue 275 might form an α-helix with structural or functional significance is the observation that changing this codon to a proline—a helix breaker—leads to a defective protein (LPM1) that can barely bind DNA. Along these lines, it is also interesting that an alanine is usually more favorable for α-helix formation than a leucine, and this may account for the relative binding differences between LPM1, LPM2, and wild-type E1. These two mutations are also of special interest because, of the six substitution mutants generated, LPM1 and LPM2 are the only ones whose activities within the cell are not predicted by their ability to bind origin DNA in the presence of the E2 protein. Both LPM1 and LPM2, together with the E2 protein, retain even more origin DNA fragment than does wild-type E1 protein under identical conditions. However, the level of replication detected is, at best, 10% of the wild-type level. This type of mutation is to be expected for a multifunctional protein such as E1, and we conclude that this subdomain is also important for functions other than specific DNA binding, such as helicase activity or protein-protein interactions. The multiple substitution mutant LPM3 cannot bind DNA by itself, but it reproducibly yields some complex with the E2 protein and origin DNA. This DNA binding in the presence of E2 presumably explains why LPM3 is able to support a low level of replication in cells, as replication is absent in mutants that are unable to bind DNA either alone or with E2 (e.g., LPM4 and LPM6).

We have suggested that the basic residues within the E1 DNA-binding domain contact DNA and play a direct role in origin DNA recognition. This suggestion was initially based on analogies with other motifs found in DNA-binding proteins but was later also supported by the behavior of substitution mutants LPM4, LPM5, and LPM6, all of which carry mutations in basic residues found in conserved basic clusters. LPM4 and LPM6 do not bind the origin DNA, either alone or in the presence of E2, and support no transient replication in cells. LPM5 barely binds DNA on its own, but E2 rescues its ability to bind DNA. The amount of origin fragment retained by LPM5 in the presence of 100 ng of E2 is 30% of the amount retained by wild-type E1 under identical conditions. Similarly, the level of pKSO replication supported by this mutant in the cell is approximately 35% of the wild-type level, implying that its phenotype is roughly measured by its ability to be tethered to the origin site. To reiterate a point made above, for all mutations except those affecting Leu-275, the level of replication measured in the cell is in proportion to the ability of E1 and E2 to be tethered to the origin site (Table 1 and Fig. 7). Thus, we conclude that the ability to bind DNA in a site-specific manner is critical for BPV-1 DNA replication.

E1 performs multiple functions in papillomavirus replication: it is known to be a helicase (34, 48), to interact productively with E2 (33, 36, 47, 49), and to bind DNA polymerase α (29a), which is presumed to be the polymerase involved in initiation (47). Another function of the E1 protein may be its ability to interact with itself, and this interaction may be relevant to DNA binding. Because we do not know whether E1 binds to DNA as a monomer or as an oligomer under the conditions of our immunoprecipitation assay, it is possible that some of our mutations affect binding by influencing the formation of E1 multimers. E1-E1 interactions may be important for helicase or other activities of E1 required for replication. The possibility that E1-DNA complexes involve multimeric E1 molecules is intriguing with regard to the binding observed with the ATP-binding mutants. SV40 large T antigen can form hexamers in the presence of ATP without DNA, but such hexamers do not efficiently bind DNA (30). In the study reported here, the E1 ATP-binding mutants reproducibly retained more of the origin fragment than did equivalent amounts of wild-type E1. If E1, synthesized in a reticulocyte extract containing ATP, also forms less active multimers in an ATP-dependent manner, we would predict that mutants defective for ATP binding might form more stable complexes with DNA. This observation raises several questions which must be answered in order to unravel the mechanism of action of E1—or any helicase, for that matter. Namely, how do ATP and ADP affect the shape of the protein and how does the binding of these effectors change the protein's interaction with DNA substrates (both double and single stranded)? Our experiments have simply shown that E1 can bind to the origin site in some form that does not require ATP.

Yang et al. (47) first proposed a model which suggested that E2 enhances BPV-1 replication by increasing the affinity of E1 for the origin. Thus, at limiting E1 and E2 protein concentrations in the cell, this cooperativity would be critical to target E1 to the origin site. Additional support for this model came from biochemical studies showing that E1 and E2 can physically bind to each other and form a stable complex (29). These in vitro results have been confirmed and extended by others (4, 26, 33, 36). Three lines of evidence

now support the model that protein-protein interactions between E1 and E2 are critical for papillomavirus DNA replication in vivo. Spalholz et al. (36) recently reported that viral DNAs containing mutations in the origin that impaired E1 binding could be rescued for replication, at least partially, but only if intact E2 binding sites were retained near the E1 binding site. The same conclusion about the ability of protein-protein (E1-E2) interactions to stabilize weak protein-DNA interactions was reached by Ustav et al. (42). These authors used this explanation to account for why low-affinity E2 binding sites were functional only when positioned close to the E1 binding site, whereas high-affinity sites could function from a distance. In our study, we have interpreted the data obtained with mutants LPM3 and LPM5 according to this model. Specifically, these proteins alone are either unable to bind DNA (LPM3) or severely crippled for binding (LPM5), yet in cells they are capable of supporting replication when helped by E2. Indeed, LPM5 is 30% as efficient as wild-type E1.

All of the transient-replication experiments are, however, indirect and leave room for alternative interpretations. For example, we do not know definitively whether, in the presence of artificially high levels of E1, the protein can initiate replication at sites other than the origin (48), or whether or not E2 activates replication from such hypothetical sites. Although one can interpret the results of Ustav et al. (42) in terms of a protein-protein interaction between E1 and E2, it is also worth noting that cooperative binding to DNA by proteins spaced far apart could still occur through distortion of the DNA template (32).

In view of the above points, it is clearly essential to know more about how E1 and E2 physically interact with each other and how these interactions affect cooperative binding and other biochemical functions critical for replication. It seems highly likely to us that both proteins together present a surface important for DNA replication and that this can occur even when the E2 and E1 sites are placed far apart, via DNA looping. For this reason, we are interested in identifying E1 mutants which can bind as well as the wild type but are not enhanced for binding by E2. We predict that such mutants will not bind E2 protein in the absence of DNA and will be defective for DNA replication. Perhaps such mutants will be difficult to find because of the potentially large number of residues involved in protein-protein binding. We have shown that the interaction between the two replication proteins (E1 and E2) is cold sensitive and that, at low temperatures, the interaction is greatly reduced. We would then suggest that some of the important interactions between E1 and E2 for cooperative DNA binding are also cold sensitive. Fragments of E1 deleted for C-terminal sequences shown to bind E2 at 4°C are still capable of cooperative DNA binding with E2. This brings into question the significance of the protein-protein interactions in this C-terminal region for cooperative binding. It is of course possible to envision two distinct surfaces of E1 which interact with E2, one important for initiation and one having another role. We suspect that protein-protein interaction surfaces between E1 and E2 important for initiation are included in the Δ6 protein, as it too can be assisted for binding to DNA by E2. That the Δ424 protein can physically complex with the E2 protein at 30°C is consistent with this speculation and suggests that further mutational analysis of the region of E1 contained by Δ6 is warranted.

ACKNOWLEDGMENTS

We thank Rong Li for his many suggestions throughout the course of this work and Jim Litts and Jon Driller for producing the GE1Δ424 baculovirus.

This work was supported by funds from the NIH through grant CA42414.

REFERENCES

1. **Baker, C. C.** 1987. Sequence analysis of papillomavirus genomes, p. 321–385. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae, vol. 2: the papilloma virus. Plenum Publishing Corp., New York.
2. **Berg, L., M. Lusky, A. Stenlund, and M. R. Botchan.** 1986. Repression of bovine papilloma virus replication is mediated by a virally encoded trans-acting factor. Cell **46**:753–762.
3. **Blanar, M. A., and W. J. Rutter.** 1992. Interaction cloning: identification of a helix-loop-helix zipper protein that interacts with c-fos. Science **256**:1014–1018.
4. **Blitz, I. L., and L. A. Laimins.** 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.
5. **Bream, G. L., C.-A. Ohmstede, and W. C. Phelps.** 1993. Characterization of human papillomavirus type 11 E1 and E2 proteins expressed in insect cells. J. Virol. **67**:2655–2663.
6. **Broker, T. R., and M. Botchan.** 1986. Papillomaviruses: retrospectives and prospectives, p. 17–36. *In* M. Botchan, T. Grodzicker, and P. A. Sharp (ed.), Cancer Cells 4: DNA tumor viruses: control of gene expression and replication, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Bruckner, R. C., J. C. Crute, M. S. Dodson, and I. R. Lehman.** 1991. The herpes simplex virus 1 origin binding protein: a DNA helicase. J. Biol. Chem. **266**:2669–2674.
8. **Chan, S.-Y., H.-U. Bernard, C.-K. Ong, S.-P. Chan, B. Hofmann, and H. Delius.** 1992. Phylogenetic analysis of 48 papillomavirus types and 28 subtypes and variants: a showcase for the molecular evolution of DNA viruses. J. Virol. **66**:5714–5725.
9. **Chen, E. Y., P. M. Howley, A. D. Levinson, and P. H. Seeburg.** 1982. The primary structure and genetic organization of the bovine papillomavirus type 1 genome. Nature (London) **299**: 529–534.
10. **Chiang, C.-M., M. Ustav, A. Stenlund, T. F. Ho, T. R. Broker, and L. T. Chow.** 1992. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. Proc. Natl. Acad. Sci. USA **89**:5799–5803.
11. **Creighton, T. E.** 1993. Proteins: structures and molecular properties, p. 160. W. H. Freeman and Co., New York.
12. **Del Vecchio, A., H. Romanczuk, P. Howley, and C. Baker.** 1992. Transient DNA replication of human papillomavirus. J. Virol. **66**:5949–5958.
13. **Elias, P., and I. R. Lehman.** 1988. Interaction of origin binding protein with an origin of replication of herpes simplex virus 1. Proc. Natl. Acad. Sci. USA **85**:2959–2963.
14. **Elias, P., M. E. O'Donnell, E. S. Mocarski, and I. R. Lehman.** 1986. A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **83**:6322–6326.
15. **Grussenmeyer, T., K. Scheidtmann, M. A. Hutchinson, W. Eckhart, and G. Walter.** 1985. Complexes of polyoma virus medium T antigen and cellular proteins. Proc. Natl. Acad. Sci. USA **82**:7952–7954.
16. **Harrison, S. C.** 1991. A structural taxonomy of DNA-binding domains. Nature (London) **353**:715–719.
17. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
18. **Hubert, W. G., and P. F. Lambert.** 1993. The 23-kilodalton E1 phosphoprotein of bovine papillomavirus type 1 is nonessential for stable plasmid replication in murine C127 cells. J. Virol. **67**:2932–2937.
19. **Knight, J., R. Li, and M. Botchan.** 1991. The activation domain of the bovine papillomavirus E2 protein mediates association of DNA bound dimers to form DNA loops. Proc. Natl. Acad. Sci. USA **88**:3204–3208.

20. **Kornberg, A., and T. A. Baker.** 1992. DNA replication. W. H. Freeman and Co., New York.
21. **Lentz, M. R., D. Pak, I. Mohr, and M. R. Botchan.** 1993. The E1 replication protein of bovine papillomavirus type 1 contains an extended nuclear localization signal that includes a $p34^{cdc2}$ phosphorylation site. J. Virol. **67:**1414–1423.
21a. **Li, R., and M. Botchan.** Unpublished data.
22. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. Botchan.** 1989. Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev. **3:**510–526.
23. **Lin, H. L., R. H. Upson, and D. T. Simmons.** 1992. Nonspecific DNA-binding activity of simian virus 40 large T antigen: evidence for the cooperation of two regions for full activity. J. Virol. **66:**5443–5452.
24. **Lohman, T. M.** 1992. Escherichia coli DNA helicases: mechanisms of DNA unwinding. Mol. Microbiol. **6:**5–14.
25. **Lusky, M., and M. R. Botchan.** 1984. Characterization of the bovine papilloma virus plasmid maintenance sequences. Cell **36:**391–401.
26. **Lusky, M., and E. Fontane.** 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.
26a. **MacPherson, P., and L. Thorner.** Unpublished data.
27. **McKay, R. D. G.** 1981. Binding of a simian virus 40 T antigen-related protein to DNA. J. Mol. Biol. **145:**471–488.
28. **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:**5525–5536.
28a. **Mendoza, R., and M. Botchan.** Unpublished data.
29. **Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science **250:**1694–1699.
29a. **Park, P., I. Mohr, W. Copeland, T. Wang, and M. Botchan.** Unpublished data.
30. **Parsons, R. E., J. E. Stenger, S. Ray, R. Welker, M. E. Anderson, and P. Tegtmeyer.** 1991. Cooperative assembly of simian virus 40 T-antigen hexamers on functional halves of the replication origin. J. Virol. **65:**2798–2806.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Schultz, S. C., G. C. Shields, and T. A. Steitz.** 1991. Crystal structure of a CAP-DNA complex: the DNA is bent by 90°. Science **253:**1001–1007.
33. **Seo, Y.-S., F. Müller, M. Lusky, E. Gibbs, H.-Y. Kim, B. Phillips, and J. Hurwitz.** 1993. Bovine papilloma virus (BPV)-encoded E2 protein enhances binding of E1 protein to the BPV replication origin. Proc. Natl. Acad. Sci. USA **90:**2865–2869.
34. **Seo, Y.-S., F. Müller, M. Lusky, and J. Hurwitz.** 1993. Bovine papilloma virus (BPV)-encoded E1 protein contains multiple activities required for BPV DNA replication. Proc. Natl. Acad. Sci. USA **90:**702–706.
35. **Spalholz, B. A., P. F. Lambert, C. L. Yee, and P. M. Howley.** 1987. Bovine papillomavirus transcriptional regulation: localization of the E2-responsive elements of the long control region. J. Virol. **61:**2128–2137.
36. **Spalholz, B. A., A. A. McBride, T. Sarafi, and J. Quintero.** 1993. Binding of bovine papillomavirus E1 to the origin is not sufficient for DNA replication. Virology **193:**201–212.
37. **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.
38. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vector and insect cell culture procedures. Texas Agricultural Experiment Station bulletin no. 1555. Texas Agricultural Experiment Station, College Station, Tex.
38a. **Sun, S., and M. Botchan.** Unpublished data.
39. **Sun, S., L. Thorner, M. Lentz, P. MacPherson, and M. Botchan.** 1990. Identification of a 68-kDa nuclear ATP-binding phosphoprotein encoded by bovine papillomavirus type 1. J. Virol. **64:**5093–5105.
39a. **Thorner, L., and M. Botchan.** Unpublished data.
40. **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.
41. **Tjian, R.** 1981. T antigen binding and the control of SV40 gene expression. Cell **26:**1–2.
42. **Ustav, E., M. Ustav, P. Szymanski, and A. Stenlund.** 1993. The bovine papillomavirus origin of replication requires a binding site for the E2 transcriptional activator. Proc. Natl. Acad. Sci. USA **90:**898–902.
43. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. **10:**449–457.
44. **Ustav, M., E. Ustav, P. Szymanski, and A. Stenlund.** 1991. Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1. EMBO J. **10:**4321–4329.
45. **Wilson, V. G., and J. Ludes-Meyers.** 1991. A bovine papillomavirus E1-related protein binds specifically to bovine papillomavirus DNA. J. Virol. **65:**5314–5322.
46. **Wong, I., and T. M. Lohman.** 1992. Allosteric effects of nucleotide cofactors on *Escherichia coli* Rep helicase-DNA binding. Science **256:**350–355.
46a. **Yang, L., R. Li, and M. Botchan.** Unpublished data.
47. **Yang, L., R. Li, I. J. Mohr, R. Clark, and M. R. Botchan.** 1991. Activation of BPV-1 replication *in vitro* by the transcription factor E2. Nature (London) **353:**628–633.
48. **Yang, L., I. Mohr, E. Fouts, D. A. Lim, M. Nohaile, and M. Botchan.** 1993. The E1 protein of the papillomavirus BPV-1 is an ATP dependent DNA helicase. Proc. Natl. Acad. Sci. USA **90:**5086–5090.
49. **Yang, L., I. Mohr, R. Li, T. Nottoli, S. Sun, and M. Botchan.** 1991. Transcription factor E2 regulates BPV-1 DNA replication *in vitro* by direct protein-protein interaction. Cold Spring Harbor Symp. Quant. Biol. **56:**335–346.