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A Bovine Papillomavirus E1-Related Protein Binds Specifically to Bovine Papillomavirus DNA

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The E1 open reading frame of bovine papillomavirus (BPV) was expressed as a RecA-E1 fusion protein in *Escherichia coli*. The bacterially expressed RecA-E1 protein exhibited sequence-specific DNA binding activity; strong binding to the region from nucleotides 7819 to 93 on the BPV genome (designated region A) and weak binding to the adjacent region from nucleotides 7457 to 7818 (region B) were observed. The interaction between the BPV-derived RecA-E1 protein and region A appeared to be highly specific for BPV DNA, as no comparable binding was detected with heterologous papillomavirus DNAs. Binding to region A was eliminated by digestion of region A at the unique *HpaI* site, which suggests that the RecA-E1 binding site(s) was at or near the *HpaI* recognition sequence. Binding to region B but not region A was observed when nuclear extracts from ID13 cells were used as a source of E1 proteins. The absence of region A binding by ID13 extracts may reflect a negative regulation of E1 DNA binding activity.

The papillomavirus family comprises a large group of both human and animal viruses. A characteristic feature of these viruses is their ability to remain stably associated with the host cell in an extrachromosomal fashion (7, 14). After an initial period of amplification, replication of the viral genome is tightly regulated with the cell cycle so that viral copy number remains relatively constant (17, 35). This coordinate replication of the viral and cellular genomes implies that the virus is responsive to normal cellular control mechanisms. Furthermore, the small size of papillomavirus genomes, approximately 8,000 bp, suggests that these viruses will be dependent on the cellular DNA replication machinery for much of the actual enzymology of DNA synthesis. Work with bovine papillomavirus (BPV) has established that the viral genome can be manipulated in vitro and reintroduced into mouse cells to assay both transient amplification (3, 17, 18, 36) and stable replication (2, 19, 20). All of these features make papillomaviruses a valuable system for studying the processes and regulation of eukaryotic DNA replication.

An essential step in understanding papillomavirus DNA replication will be to identify and characterize viral gene products critical for replication. Initial examination of the BPV genome revealed that the E1 open reading frame (ORF) shares homology with the simian virus 40 (SV40) large T antigen, a known DNA binding protein involved in initiation of viral DNA synthesis (6). Mutational analysis of the E1 ORF confirmed that the E1 gene is essential for DNA replication and suggests that the E1 ORF encodes at least two products, a 5′ product termed M and a 3′ product termed R (20). An E1 M protein which is consistent with the genetic data has been identified (34), but a 3′-derived E1 R has not yet been observed. More recently, a full-length E1 product was detected as a nuclear protein and was shown to possess ATP binding activity (31). Further work will be required to determine whether or not there are additional E1 products and to define the properties of the various E1 ORF proteins.

Elucidation of the functional roles of the E1 ORF products will require biochemical characterization of the proteins themselves. Several groups have expressed the E1 ORF in eukaryotic viral vectors in order to produce sufficient amounts of protein for in vitro analysis (4, 22, 25). We have now expressed the E1 ORF as a soluble RecA-E1 fusion protein in *Escherichia coli*. By using an anti-E1 antiserum to precipitate RecA-E1–DNA complexes, we have demonstrated specific binding of the RecA-E1 fusion protein to two adjacent regions on the BPV genome. Strong binding to a region which contains a minimal origin of DNA replication (29) as well as known transcriptional regulatory sequences (1, 16) occurred. Weak binding to the 5′ adjacent region from nucleotides 7457 to 7819 occurred. The detection of RecA-E1 binding to functionally critical regions on the BPV genome is consistent with a direct role for E1 in regulatory activities via protein-DNA interactions.

MATERIALS AND METHODS

Plasmids, cells, and antisera. Plasmids pdBPV-1 (26), pDPV (10), pHPV1a (11), and pHPV16 (8) were the sources of BPV and of deer, human type 1a, and human type 16 papillomavirus (DPV, HPV1a, and HPV16, respectively) DNAs, respectively. Plasmid pGE374 contains the *recA* promoter and the first 35 codons of the RecA sequence fused out of frame to *lacZ* (15). pGE372 is a derivative of pGE374 in which the truncated *recA* gene is fused in frame with *lacZ* to produce a RecA–β-galactosidase fusion protein (15). pORF1 is similar in design to pGE374 and contains the *ompF* promoter and the first 33 codons of the OmpF sequence fused out of frame to *lacZ* (37). ID13 and C127 cells were obtained from P. Howley and were maintained in Dulbecco modified Eagle medium–10% heat-inactivated fetal bovine serum with 10 U of penicillin, 10 μg of streptomycin, and 2 μg of amphotericin B (Fungizone) per ml. Rabbit antisera against RecA and β-galactosidase were obtained from J. Leibowitz and 5 Prime→3 Prime, Inc., respectively.

Production of anti-E1 antisera. In order to generate proteins for antiserum production, portions of the E1 ORF were expressed by using the pORF1 vector system as described by Weinstock et al. (37). Plasmid $pE1_{249}$ was constructed by ligating a fragment containing BPV sequences from nucleotides 850 through 1073 into the *SmaI* site of the vector. This plasmid expressed a tribrid fusion protein of the form

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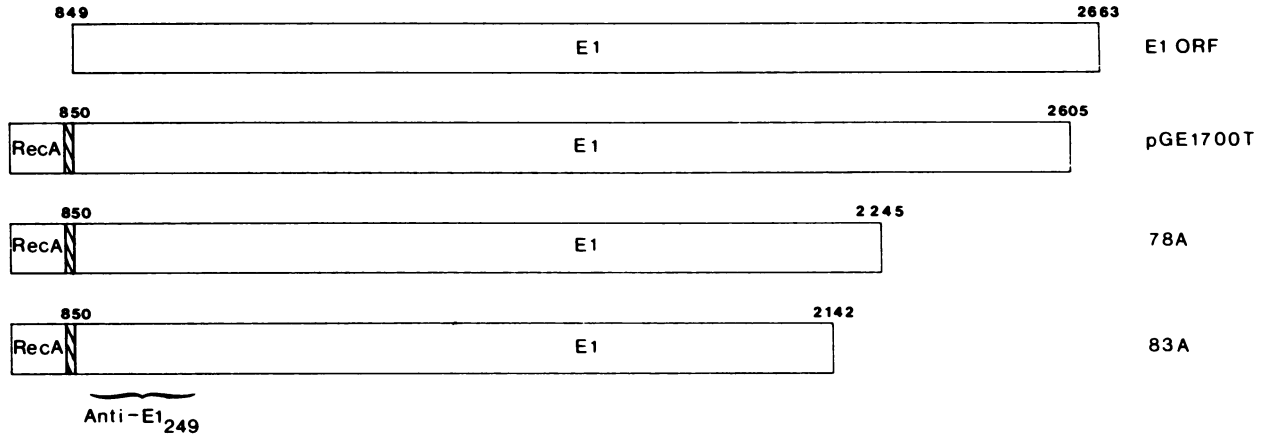

FIG. 1. Structures of RecA-E1 fusion proteins. The E1 ORF is shown from the first methionine codon beginning at nucleotide 849 to the end of the coding region at nucleotide 2663. This ORF encodes a 605-amino-acid protein. Below the E1 ORF are the predicted structures of the RecA-E1 fusion proteins expressed by plasmids pGE1700T, 78A, and 83A. RecA-E1 has an amino terminus composed of 35 amino acids encoded by the 5′ end of the *recA* gene and 5 amino acids encoded by plasmid polylinker sequences (cross-hatched region). The E1 portion of RecA-E1 includes nucleotides 850 to 2605 of the E1 sequence and encodes 585 of the 605 amino acids in the complete E1 ORF. The resulting RecA-E1 protein has a predicted molecular size of 75,000 Da. Mutants 78A and 83A have translation termination linkers inserted at nucleotides 2245 and 2142, respectively, in the E1 sequence. The resultant truncated RecA-E1 fusion proteins should have molecular sizes of 61,000 and 57,000 Da, respectively. Also shown is the location on the E1 protein to which the 5997 antiserum is directed (Anti-$E1_{249}$).

OmpF–E1–β-galactosidase, where the E1 portion should consist of amino acids 2 through 75 of the E1 protein. As a control plasmid, a similar construct was created in which the insert was a fragment from the E1 ORF inserted in the incorrect orientation. This fragment had no termination codons in this orientation, so it could express a tribrid fusion protein, but the fusion protein would have no E1 ORF-encoded amino acids. The control plasmid was designated pNE.

Cultures of $pE1_{249}$ and pNE were grown to an optical density at 600 nm of 0.3 at room temperature, and then plasmid protein expression was induced by shifting the cultures to 42°C for 90 min (37). Whole-cell extracts were prepared by suspending the cell pellets in sodium dodecyl sulfate (SDS)-gel sample buffer (75 mM Tris-sulfate [pH 8.3], 15% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.01% bromophenol blue) at a ratio of 5 ml of buffer per g of wet cell pellet and boiling the mixture for 5 min. Fusion proteins were purified from the extracts by preparative gel electrophoresis (33) followed by elution with an Elutrap electroeluter (Schleicher & Schuell). After dialysis against 5% (wt/vol) ammonium bicarbonate and lyophilization, the fusion proteins were dissolved in phosphate-buffered saline (PBS) to 200 μg/ml. Rabbits were bled for preimmune sera and then inoculated subcutaneously with 100 μg of purified fusion protein emulsified in complete Freund's adjuvant. Boosts were performed at 3-week intervals with 100 μg of protein emulsified in incomplete Freund's adjuvant. Sera were collected 7 to 10 days following each boost and stored at −20°C. Serum from the rabbit inoculated with the protein expressed by $pE1_{249}$ was designated 5997, and serum from the rabbit inoculated with the protein expressed by pNE was designated 5998.

Construction of RecA-E1 fusion proteins. The nearly full-length E1 ORF was expressed by using the pGE374 vector system (15). A 1,778-bp fragment derived from the E1 ORF was obtained by digestion of pdBPV-1 with *Fnu*DII followed by partial digestion with *Sph*I. The gel-purified fragment was treated with Bal 31 to randomize the fragment length. The resulting population of molecules was ligated into the *Sma*I site of pGE374. Restriction analysis and DNA sequencing of Lac^+ clones identified a recombinant that had the BPV E1 ORF inserted in the proper orientation with both ends in frame with vector sequences. This clone was designated pGE1700.

To produce a RecA-E1 fusion protein without the β-galactosidase sequences, an *Nhe*I linker (New England BioLabs) containing multiple translation termination codons was ligated to pGE1700 DNA (13), which had been linearized by partial *Bam*HI digestion. Lac^- colonies were picked, and plasmid DNA was screened by restriction digestion for the presence of a termination linker at the E1-*lacZ* junction. A clone with the linker in the appropriate position was designated pGE1700T (Fig. 1). Sequencing of the E1-*lacZ* junction in pGE1700T confirmed the location of the linker.

Truncated forms of RecA-E1 were created by ligation of the *Nhe*I linker into pGE1700 DNA linearized by random DNase I digestion. Plasmid DNA from white colonies was screened by restriction digestion for the presence of an *Nhe*I linker inserted in the E1 ORF. The exact location of the linker in each positive clone was determined by DNA sequencing. Two mutants, 78A and 83A, had the *Nhe*I linker inserted at BPV nucleotides 2245 and 2142, respectively, in the E1 sequence of pGE1700.

Preparation of RecA-E1 extracts. *E. coli* MC1061 containing pGE374 or derivative plasmids was grown at 37°C to an optical density at 600 nm of 0.4 in Luria-Bertani medium with ampicillin at 50 μg/ml. Mitomycin was added to a final concentration of 1.0 μg/ml, and the incubation continued for 2 h at 37°C to induce fusion protein production (15). Induced cultures were chilled on ice for 10 min and then harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Cell pellets were washed once with ice-cold PBS and suspended in ice-cold TNE (13 mM Tris HCl [pH 7.0], 0.1 mM EDTA, 10 mM NaCl) with 1 mM phenylmethylsulfonyl fluoride (PMSF) at 1 ml of buffer per g of wet cell pellet. Cells were lysed by two passages through a French pressure cell at 16,000 lb/in^2. Lysates were collected on ice, and cell debris

was removed by centrifugation at 27,000 × *g* for 10 min at 4°C. The soluble extract was made 50% with sterile glycerol and stored at −20°C. DNA binding activity was stable for several months at this temperature. For longer storage, small aliquots were frozen at −70°C and used immediately upon thawing. Total protein in the extracts was quantitated by the Bradford assay (5) and was typically 3 to 5 μg/μl.

Immunoprecipitation of RecA fusion proteins. For immunoprecipitation, 40 μl of extract prepared as described above was incubated with 10 μl of the appropriate antiserum for 60 min on ice. Antibody-antigen complexes were collected by the addition of 100 μl of 10% (vol/vol) protein A-Sepharose (Pharmacia, Inc.). After incubation for 60 min at 4°C with gentle agitation, the protein A-Sepharose was collected by centrifugation for 1 min at 12,000 × *g*. The beads were washed three times with 500 μl of cold 50 mM Tris HCl (pH 8.0)–150 mM NaCl–1% Nonidet P-40–1 mM PMSF. The final pellets were boiled for 5 min in 10 μl of SDS sample buffer and spun for 1 min at 12,000 × *g*, and the supernatants were electrophoresed on 15% SDS-polyacrylamide gels. The RecA fusion proteins were visualized by Western blotting (immunoblotting) with appropriate antisera.

Western blots of RecA fusion proteins. Immunoprecipitates or direct samples of extracts were electrophoresed on 15% SDS-polyacrylamide gels, and the proteins were transferred from the SDS gels to Optibind membranes (Schleicher & Schuell) by electrophoresis at 2.5 mA/cm^2 of gel in a Polyblot unit (American Bionetics, Inc.). Immunological detection of proteins on the filters was performed with an Auroprobe BLplus kit followed by IntenSE BL silver enhancement (Janssen) as described by the manufacturer. Primary antisera were as indicated in the figure legends and were used at a 1:1,000 dilution.

Preparation of ID13 and C127 extracts. ID13 or C127 cells were grown to near confluency in 150-cm^2 flasks. Prior to harvesting of cells, each flask was washed once with 10 ml of ice-cold PBS and once with 10 ml of ice-cold hypotonic buffer (20 mM Tris HCl [pH 7.6], 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dithiothreitol). Cells were scraped into cold hypotonic buffer (10 ml per flask) and pelleted by centrifugation at 1,200 × *g* for 10 min at 4°C. Cell pellets from each flask were suspended in 500 μl of hypotonic buffer and then lysed by the addition of 500 μl of hypotonic buffer supplemented with 1% Triton X-100 and 2 mM PMSF. Samples were incubated in the lysis buffer for 1 min on ice, and then nuclei were pelleted by centrifugation for 5 min at 4°C in a microfuge. Nuclei were extracted by resuspension in 50 μl of hypotonic buffer supplemented with 350 mM NaCl and 1 mM PMSF. Resuspended nuclei were incubated for 20 min on ice and then pelleted for 10 min at 4°C in a microfuge. The supernatants were made 50% with sterile glycerol and stored in small aliquots at −70°C. The total protein concentration in the nuclear extracts was generally 3 to 6 μg/μl.

Digestion and labeling of DNA. All plasmid DNAs were isolated by standard procedures (24) and were CsCl gradient purified. SV40 DNA was prepared from virus-infected cells as previously described (32). For end labeling, 1 μg of DNA was digested with *Sty*I or *Ava*II to generate 5′ overhangs. The overhangs were filled by extension of the 3′ strands with 6 U of Sequenase (USB Corp.) in a 30-μl reaction mixture containing 1 μg of restriction enzyme-digested DNA; 19 mM Tris HCl (pH 7.8); 38 mM NaCl; 7.6 mM $MgCl_2$; 5 mM dithiothreitol; 0.72 μM (each) dATP, dCTP, and dTTP; and 0.32 μM [^{32}P]dGTP (3,000 Ci/mmol; ICN, Inc.). Extension reaction mixtures were incubated for 10 min at room temperature, stopped by the addition of 10 μl of 200 mM EDTA, and diluted to 1.0 ml with TNE buffer. Unincorporated label was removed by centrifugation of the DNA twice in a Centricon-30 (Amicon) as specified by the manufacturer. Labeled DNA was adjusted to 10 ng/μl with TNE and stored at −20°C. For subdigestion of *Sty*I-cut pdBPV-1, labeled DNA was incubated with various restriction enzymes under the appropriate reaction conditions. After digestion, the DNA was phenol extracted, alcohol precipitated, and redissolved in TNE to the original concentration of 10 ng/μl.

DNA binding assay. To assay DNA binding, 15 to 30 μg of total protein (from bacterial or ID13 extracts) was incubated with 50 ng of labeled DNA in 25 μl of TNE containing 150 mM NaCl and 1,500 ng of unlabeled sheared salmon sperm DNA (5 Prime→3 Prime, Inc.). Binding reaction mixtures were incubated for 30 min at 25°C and then returned to an ice bath. Appropriate serum (10 μl) was added, incubation was continued for 60 min on ice, and then 100 μl of a protein A-Sepharose (Pharmacia) suspension (10% vol/vol in PBS) was added. After addition of the protein A-Sepharose, samples were incubated for 60 min at 4°C with gentle agitation. The Sepharose beads were collected by centrifugation for 1 min at 4°C; washed three times with 1 ml of TNE containing 200 mM NaCl, 0.25% Nonidet P-40, and 5 μg of sheared salmon sperm DNA per ml; and washed once with 1 ml of TNE. Washed beads were extracted for 15 min at 37°C with 10 μl of TBE sample buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA, 10% glycerol, 2.7% xylene cyanol, 2.4% bromophenol blue, 1% SDS) and pelleted by centrifugation for 1 min at room temperature in a microfuge, and the supernatants were electrophoresed on either 1% agarose gels or 8% nondenaturing polyacrylamide gels by using standard Tris-borate buffers (24). After electrophoresis, agarose gels were fixed for 30 min in 7.0% trichloroacetic acid, rinsed twice for 5 min with distilled water, and dried. Acrylamide gels were dried directly without fixing. Dried gels were exposed for autoradiography with intensifying screens.

RESULTS

Expression of the RecA-E1 fusion protein. To produce a BPV E1-related protein for biochemical analysis, the E1 gene was first cloned into the bacterial expression vector pGE374. Restriction analysis and partial sequencing of the resulting plasmid, pGE1700, confirmed that the cloned E1 gene fragment was present in the correct reading frame with respect to the vector *recA* and *lacZ* sequences (not shown). pGE1700 expressed a 188,000-molecular-weight protein (188K protein) that was immunoreactive with both anti-RecA and anti-β-galactosidase sera (Fig. 2); no such protein was expressed in cells containing the parental plasmid, pGE374. As expected for this vector system, expression of the 188K protein was induced by mitomycin (not shown). The size and immunoreactivity pattern of the 188K protein, along with its induction properties, confirmed that it was a RecA–E1–β-galactosidase fusion protein expressed from the constructed plasmid.

To eliminate the β-galactosidase portion of the RecA–E1–β-galactosidase fusion protein, a translation termination linker was inserted at the E1-*lacZ* junction of pGE1700 to generate pGE1700T. Sequencing of the plasmid-insert junctions indicated that pGE1700T should encode a 75K RecA-E1 fusion protein consisting of an amino terminus derived from both *recA* (35 amino acids) and vector sequences (5 amino acids) followed by amino acids 2 to 585 of the E1 ORF (Fig. 1). pGE1700T no longer expressed the

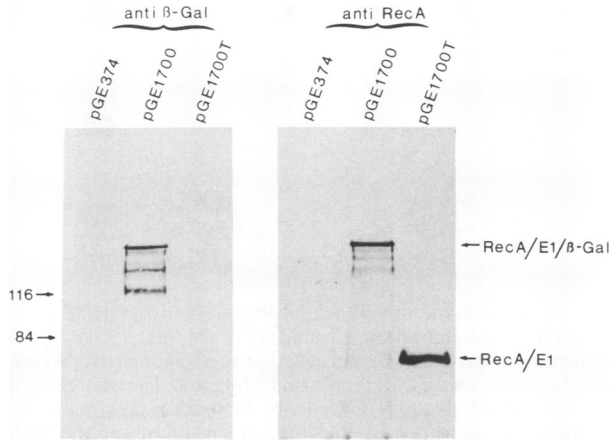

FIG. 2. Immunological identification of RecA fusion proteins. Whole-cell extracts were prepared from mitomycin-induced cells harboring plasmids pGE374, pGE1700, and pGE1700T. Samples of each extract were electrophoresed on 15% SDS gels and analyzed by Western blotting with either anti-β-galactosidase or anti-RecA as indicated. The positions of molecular size markers are shown on the left (in kilodaltons), and the RecA fusion proteins are indicated on the right.

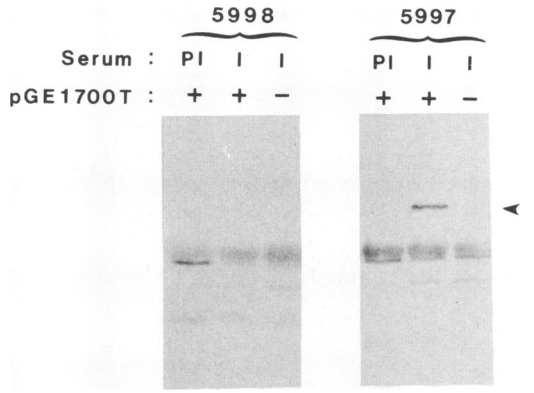

FIG. 3. Detection of RecA-E1 with an anti-E1 antiserum. Whole-cell extracts were prepared from mitomycin-induced cultures of MC1061 harboring pGE374 (−) or pGE1700T (+). Extracts were immunoprecipitated with preimmune (PI) or postimmune (I) sera as indicated. The immunoprecipitates were electrophoresed on 15% SDS-polyacrylamide gels, and the RecA-E1 fusion protein was visualized by Western blotting with anti-RecA serum. The position of RecA-E1 is indicated by the arrowhead.

188K protein observed with pGE1700 but did express a new 73K protein reactive with anti-RecA and not reactive with anti-β-galactosidase (Fig. 2). Again, the size and immunoreactivity pattern confirmed that the 73K protein was the predicted RecA-E1 fusion protein. The RecA-E1 fusion protein could be extracted in a soluble form from mitomycin-induced cultures and was therefore suitable for biochemical characterizations of E1 activities in vitro.

Production of an anti-E1 antiserum. To facilitate the biochemical analysis of E1 proteins in vitro, rabbit anti-E1 antisera were raised against five different subregions of E1 (38). Production of one of the antisera, 5997, is described in Materials and Methods; production and characterization of the remaining antisera will be reported elsewhere. All of the DNA binding studies presented in Fig. 4 through 8 utilized E1 antiserum 5997, though identical results have been obtained with the other sera (not shown).

Antiserum 5997 was raised against an OmpF–E1–β-galactosidase fusion protein containing E1 amino acids 2 through 75. Control antiserum 5998 was obtained from a rabbit inoculated with a similar fusion protein except that the insert did not encode E1 amino acid sequences. To evaluate these antisera, 5997 and 5998 were tested for immunoreactivity with RecA-E1. Since the only sequences in common between RecA-E1 and the immunogen were the E1 sequences, recognition of RecA-E1 by 5997 would indicate the presence of anti-E1 antibodies in this serum. As shown in Fig. 3, 5997 postimmune serum immunoprecipitated RecA-E1, while both of the preimmune sera and the 5998 postimmune serum failed to precipitate this fusion protein. Further analysis of these sera by direct Western blotting again demonstrated that only the 5997 postimmune serum detected the RecA-E1 protein (not shown). Failure of antiserum 5998 to detect the RecA-E1 protein indicated that antibodies against the OmpF and β-galactosidase portions of the immunogen were not cross-reacting with E1 sequences. Consequently, the 5997 antiserum must contain antibodies directed specifically against E1 sequences.

RecA-E1 protein specifically binds BPV DNA. Genetic analysis has confirmed that the E1 ORF product(s) is required for BPV DNA replication (19, 20, 36). In addition, there is sequence homology between the E1 protein and the SV40 large T antigen (6), suggesting that E1 may possess biochemical and functional activities similar to those of T antigen. Since T antigen mediates its replicative function via direct binding to the SV40 origin region (22, 28, 30), we attempted to determine if E1 also possessed a site-specific DNA binding activity. To investigate the DNA binding capability of RecA-E1, whole-cell extracts were prepared from cells expressing RecA-E1 (pGE1700T), a RecA–β-galactosidase fusion protein (pGE372), or no fusion protein (pGE374). Extracts were incubated with a radiolabeled *Sty*I digest of cloned BPV DNA and then were immunoprecipitated with the various sera (Fig. 4). For the extract prepared from cells expressing the parental plasmid, no specific precipitation of DNA fragments was detected when anti-RecA or the 5997 preimmune or immune sera were used (lanes 4 to 6). Some precipitation of the *Sty*I D fragment was observed, but the amount of fragment D precipitated was inconsistent and was detected even in the absence of serum, indicating that it was nonspecific background. In contrast, when the same precipitations were performed with extracts containing RecA-E1, both anti-RecA and 5997 immune serum but not 5997 preimmune serum precipitated the *Sty*I B fragment (lanes 7 to 9). The diminished precipitation of fragment B by anti-RecA (lane 8) compared with that by 5997 immune serum (lane 9) correlated with the less-effective precipitation of RecA-E1 by anti-RecA relative to that elicited by 5997 serum (38). The observation that fragment B was precipitated only when RecA-E1 was both present and immunoprecipitated strongly implicated the RecA-E1 protein in binding to this region of the BPV genome.

Further evidence that RecA-E1 was binding to the *Sty*I B fragment came from binding studies with two truncated forms of RecA-E1. Mutants 78A and 83A had translation termination linkers inserted at nucleotides 2245 and 2142, respectively, in the E1 sequences of plasmid pGE1700 (Fig. 1). These mutants should produce RecA-E1 proteins lacking the carboxyl-terminal 139 and 173 amino acids, respectively, of the E1 ORF. Western blot analysis of extracts from cells

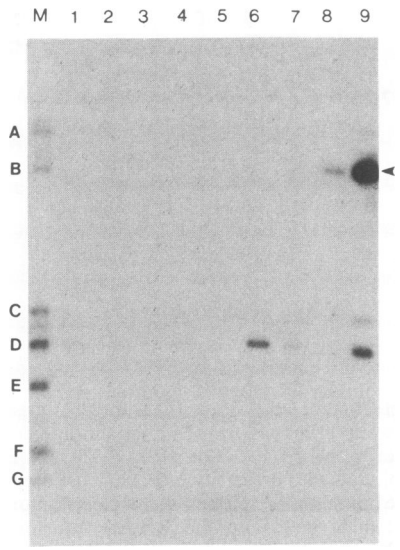

FIG. 4. Binding to the *Sty*I B fragment of pdBPV-1 is dependent on the presence of RecA-E1. Binding to *Sty*I-digested pdBPV-1 was performed with extracts from pGE372 (lanes 1 to 3), pGE374 (lanes 4 to 6), and pGE1700T (lanes 7 to 9). pGE372, pGE374, and pGE1700T expressed a RecA–β-galactosidase fusion protein, no RecA fusion protein, and the RecA-E1 fusion protein, respectively. The immunoprecipitation step was performed with preimmune serum (lanes 1, 4, and 7), anti-RecA serum (lanes 2, 5, and 8), anti-β-galactosidase serum (5 Prime→3 Prime, Inc.) (lane 3), or 5997 serum (lanes 6 and 9). Precipitated fragments were electrophoresed on a 1% agarose gel and detected by autoradiography. Lane M consists of a portion of the input *Sty*I digest of pdBPV-1, with the digestion fragments labeled A to G. The specifically bound *Sty*I B fragment is indicated with an arrowhead.

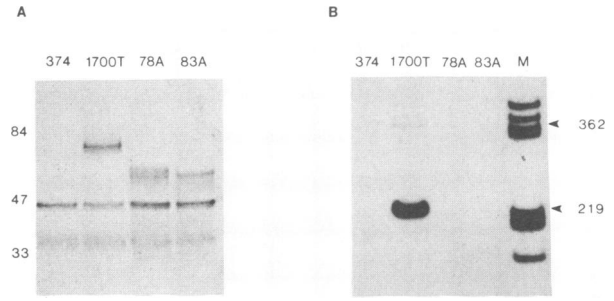

FIG. 5. Examination of truncated RecA-E1 fusion proteins. (A) Extracts from cells harboring plasmids pGE374, pGE1700T, and the termination constructs 78A and 83A were examined by Western blotting as described in Materials and Methods. The source of the extract is indicated above each lane. Molecular size markers in kilodaltons are indicated at the left, and positions of the fusion proteins are indicated on the right. Mutants 78A and 83A expressed truncated RecA-E1 proteins of the predicted molecular sizes. (B) Extracts from pGE374, pGE1700T, 78A, and 83A were tested for DNA binding as described in Materials and Methods. For this experiment, the pdBPV-1 DNA was digested with *Ava*II prior to end labeling. For each extract, precipitation of protein-DNA complexes was performed with 5997 serum. Precipitated DNA fragments were analyzed on an 8% polyacrylamide gel, a portion of which is shown. The source of the protein extract used for the binding reaction is indicated above each lane. Lane M is a sample of the input *Ava*II-digested pdBPV-1 DNA. Only the extract containing full-length RecA-E1 (lane 1700T) exhibited DNA binding. Strong binding to a 219-bp fragment and weak binding to a 362-bp fragment occurred as indicated.

containing 78A and 83A confirmed that each plasmid expressed a fusion protein of the correct predicted molecular weight (Fig. 5A). DNA binding studies revealed that extracts containing either of the truncated RecA-E1 proteins had no significant DNA binding activity compared with extracts containing full-length RecA-E1 (Fig. 5B). When the same conditions as for the DNA binding assay were used, 5997 immune serum effectively immunoprecipitated each truncated fusion protein, demonstrating that the failure to detect DNA binding was not due to inadequate immunoprecipitation (not shown). The elimination of DNA binding activity by mutations which caused truncation of the RecA-E1 protein confirmed that the binding to the *Sty*I B fragment was in fact due to the RecA-E1 protein. In addition, the lack of DNA binding activity by the truncated fusion proteins indicated that carboxyl-terminal sequences in E1 were required for the observed DNA binding activity.

While the results described above indicated that the E1 portion of the RecA-E1 fusion protein was critical for DNA binding activity, these data did not address the contribution of the RecA portion of the fusion protein. To ensure that the observed DNA binding activity was not intrinsic to the short RecA sequence of the fusion protein, a RecA–β-galactosidase fusion protein was assayed for binding to BPV DNA. The RecA–β-galactosidase fusion protein had the same RecA portion as RecA-E1, yet no binding to BPV DNA was detected when RecA–β-galactosidase was precipitated with either anti-RecA or anti-β-galactosidase (Fig. 4, lanes 2 and 3). As for the truncated fusion proteins, failure to detect DNA binding by the RecA–β-galactosidase protein was not due to lack of precipitation, since both anti-RecA and anti-β-galactosidase precipitated this fusion protein (not shown). The absence of binding activity by the RecA–β-galactosidase fusion protein confirmed that the mere presence of RecA sequences on a fusion protein was insufficient to confer binding to BPV DNA. In addition, it should be noted that all of the extracts utilized in these studies contained endogenous RecA protein, yet anti-RecA precipitated the *Sty*I B fragment only from extracts containing the full-length RecA-E1 fusion protein. Clearly, this established that neither endogenous RecA nor the RecA portion of the fusion proteins had any specific affinity for the BPV *Sty*I B fragment.

The specificity of RecA-E1 binding to the *Sty*I B fragment was examined by a competition experiment wherein increasing amounts of unlabeled, nonspecific DNA were added to the binding reactions (Fig. 6). In the absence of competitor, RecA-E1 showed a general DNA binding capacity. However, with increasing amounts of competitor DNA, binding was greatly reduced for all fragments except B. Binding to fragment B was reduced only 50% in the presence of 30-fold-excess unlabeled DNA, while precipitation of other fragments was reduced by 85 to 90%. Furthermore, at least a portion of the apparent residual binding to other fragments, especially fragment D, represented nonspecific precipitation, since it occurred in the absence of RecA-E1 as well as with preimmune serum (see Fig. 4, for example). These competition results indicated that the observed precipitation of the *Sty*I B fragment reflected a specific interaction between RecA-E1 and sequences within this fragment.

Localization of RecA-E1 binding on the BPV genome. The studies described above demonstrated specific binding of RecA-E1 to the *Sty*I B fragment of the BPV genome. However, this *Sty*I fragment spanned nucleotides 7657 to

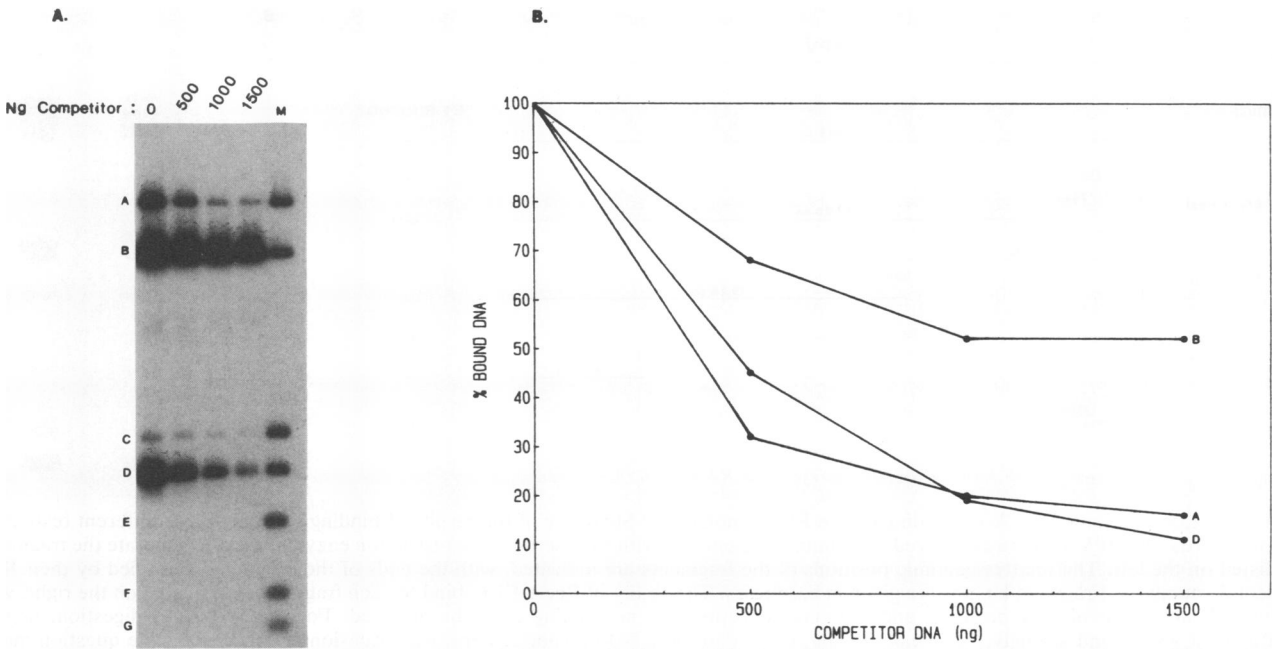

FIG. 6. Effect of competitor DNA on binding of RecA-E1 to BPV sequences. (A) RecA-E1 binding to pdBPV-1 DNA was assayed by immunoprecipitation of protein-DNA complexes with 5997 serum as described in Materials and Methods. The binding reactions contained 50 ng of a radiolabeled *Sty*I digest of pdBPV-1 DNA and increasing amounts of unlabeled competitor DNA as indicated above the lanes. The competitor DNA shown here was sheared salmon sperm DNA, but identical results have been obtained with poly(dI · dC) (Pharmacia). Precipitated DNA fragments were electrophoresed on a 1% agarose gel and detected by autoradiography. Lane M contains a portion of the input *Sty*I-digested pdBPV-1 DNA. *Sty*I fragments A to G are indicated. (B) Fragments A, B, and D were excised from each lane in panel A and quantitated by Cerenkov counting of the radioactivity present in each gel slice. The quantity of each fragment bound in the presence of increasing competitor was plotted as a percentage of the amount bound in the absence of competitor (panel A, lane 0).

2767 on the BPV map, a large region containing numerous *cis* elements. To localize binding within this large region, the *Sty*I fragment was subdivided with *Eco*RI, *Nru*I, or *Hpa*I prior to the immunoprecipitation binding assay (summarized in Fig. 7A). Each of these enzymes cut once within the *Sty*I B fragment to generate two labeled subfragments. For both the *Eco*RI and *Nru*I digestions, binding was to the fragment derived from the 5′ end of the *Sty*I B fragment. For the *Hpa*I digestion, however, neither of the two fragments generated by subdigestion were bound well by RecA-E1. To confirm this localization, binding of RecA-E1 to *Ava*II-digested BPV DNA was tested (Fig. 7B). Binding was primarily to a 219-bp *Ava*II fragment derived from nucleotides 7819 to 93 on the BPV map. The observed strong binding to the 219-bp fragment, which contained the *Hpa*I site, was consistent with the results from subdigestion of the *Sty*I B fragment. As with the *Sty*I B fragment, digestion of the 219-bp *Ava*II fragment with *Hpa*I resulted in no binding to either subfragment, suggesting that the binding site(s) may reside at or near the *Hpa*I recognition site.

In addition to the 219-bp fragment, a small amount of the 362-bp *Ava*II fragment was consistently precipitated with anti-E1 sera (see Fig. 5 and 8, for example). Precipitation of the 362-bp fragment was not observed with preimmune sera or in the absence of RecA-E1 (not shown). Weak precipitation of other fragments was observed inconsistently, occurred with preimmune sera as well as anti-E1 sera, and occurred in the absence of RecA-E1 (not shown). These results indicated that there was specific, albeit weak, binding by RecA-E1 to the 362-bp fragment, while other fragments occasionally observed were apparently the result of nonspecific precipitation. The 362-bp *Ava*II fragment consisted of nucleotides 7466 to 7818 on the BPV genome map, which was the region adjacent to the sequences present in the 219-bp fragment. From the results of binding to either *Sty*I- or *Ava*II-digested BPV DNA, it was apparent that the RecA-E1 fusion protein bound specifically to a very limited portion of the BPV genome toward the early end of the upstream regulatory region.

RecA-E1 binding to other papillomavirus genomes. To investigate whether the RecA-E1 fusion protein derived from BPV E1 could bind to other papillomavirus genomes, the immunoprecipitation binding assay was performed with *Ava*II-digested DPV, HPV1a, and HPV16 DNAs (not shown). As controls, binding to pBR322 and SV40 DNA was tested also (not shown). Binding comparable to that observed for the BPV 219-bp *Ava*II fragment was not detected with any of the heterologous DNAs, even the closely related DPV, suggesting a high degree of specificity between BPV E1 and its cognate DNA. There were, however, some weakly precipitated fragments reproducibly observed with the other papillomavirus DNAs. These fragments were not detected when the immunoprecipitations were performed with control serum or in the absence of RecA-E1, indicating that they represented specific binding by RecA-E1. The weak binding to these heterologous fragments was similar in degree to the binding observed for the 362-bp BPV fragment, but the significance of this observation has not yet been determined.

DNA binding by E1 from ID13 cells. The DNA binding studies described above were conducted with a bacterially expressed E1-related protein. To compare DNA binding by

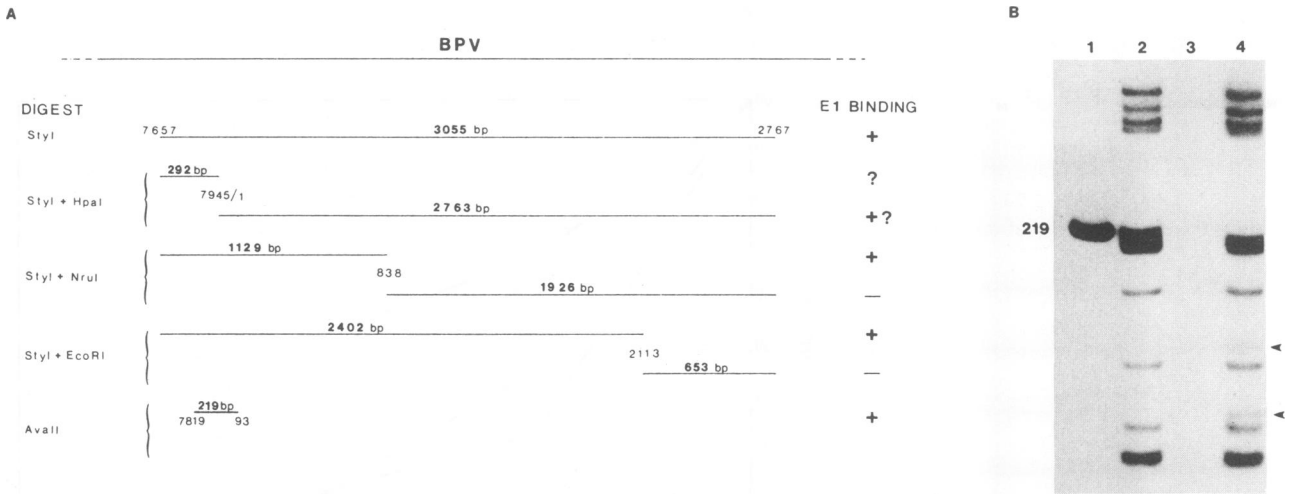

FIG. 7. Localization of RecA-E1 binding on the BPV genome. (A) Summary of the results of binding of RecA-E1 to different restriction fragments from the BPV genome as assayed by immunoprecipitation with 5997 serum. The restriction enzymes used to generate the fragments are listed on the left. The relative genomic positions of the fragments are indicated, with the ends of the fragments identified by their BPV nucleotide numbers. Fragment lengths are given in base pairs. The ability of RecA-E1 to bind to each fragment is indicated on the right, with a plus indicating that binding occurred and a minus indicating that no binding could be detected. For the *Sty*I-*Hpa*I digestion, neither subfragment was bound strongly, but weak binding, usually to the 2,763-bp subfragment, was occasionally observed. The question marks denote uncertainty about the binding results with these two *Sty*I fragments. (B) pGE1700T extracts were incubated with *Ava*II (lane 1)- or *Ava*II-*Hpa*I (lane 3)-digested pdBPV-1 DNA and precipitated with 5997 serum as described in Materials and Methods. Lanes 2 and 4 contain portions of the input *Ava*II- and *Ava*II-*Hpa*I-digested DNAs, respectively. The two subfragments produced by *Hpa*I digestion of the 219-bp *Ava*II fragment are marked with arrowheads.

RecA-E1 with DNA binding by authentic E1 protein(s), nuclear extracts from ID13 cells were prepared as a source of E1 protein(s) expressed in eukaryotic cells. ID13 cells are a BPV-transformed derivative of C127 mouse cells that express full-length BPV E1 protein (31). Nuclear extracts from ID13 cells and the parental C127 cells were tested for in vitro DNA binding under the same conditions as were used for RecA-E1 (Fig. 8). Immunoprecipitation of the ID13 and C127 extracts with 5997 anti-E1 serum demonstrated no significant binding to the 219-bp fragment, but the ID13 extract did exhibit binding to the 362-bp fragment. No binding to the 362-bp fragment was detected with preimmune serum or with the control C127 extract, indicating that the binding observed with the ID13 extract was E1 dependent. Binding of the ID13 extract to the 362-bp fragment in the absence of binding to the 219-bp fragment is in contrast to binding of RecA-E1, which binds strongly to the 219-bp fragment and only weakly to the 362-bp fragment.

DISCUSSION

The functional role of the BPV E1 ORF in DNA replication (19, 20, 36) and transcriptional control (12, 27) suggests the possibility that E1 proteins have sequence-specific DNA binding ability. To examine this question directly, we expressed the BPV E1 ORF in the form of a RecA-E1 fusion protein in *E. coli*. Using an in vitro immunoprecipitation assay, RecA-E1 was shown to bind specifically to a limited region of the BPV genome. Binding was primarily to a 219-bp *Ava*II restriction fragment spanning nucleotides 7819 to 93 on the BPV genome map. Digestion of this fragment with *Hpa*I, which cuts between nucleotides 3 and 4, destroyed the ability of RecA-E1 to bind to this region, suggesting that the binding site was at or near the *Hpa*I recognition sequence. No strong binding to heterologous papillomavirus DNAs was detected, even though there was significant sequence conservation in the *Hpa*I region. Consequently, the interaction of the BPV E1 protein with its cognate DNA must be extremely specific.

The biological significance of the interaction of RecA-E1 and the BPV *Hpa*I region has not been established, but the location of binding correlated well with the functional origin

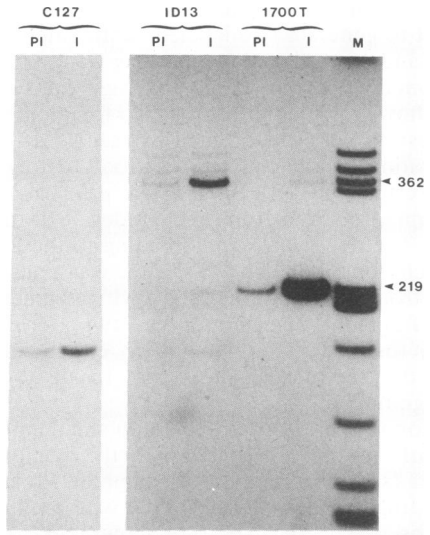

FIG. 8. Comparison of DNA binding by authentic E1 protein and RecA-E1. DNA binding was performed under standard conditions using pGE1700T extracts (lanes 1700T) or nuclear extracts from ID13 cells or C127 cells. The input DNA was *Ava*II-digested pdBPV-1, as shown in lane M. Precipitation of protein-DNA complexes was with preimmune serum (PI) or 5997 serum (I). The positions of the 219- and 362-bp fragments are indicated.

of BPV replication recently defined by Stenlund and Ustav (29). Using a transient replication assay, they mapped a BPV E1-dependent functional origin to a 100-bp region which includes the *Hpa*I site. Linker insertions at the *Hpa*I site inhibited replication, indicating that critical *cis* elements are located in this region. The failure of RecA-E1 to bind to *Hpa*I-cut DNA suggests that the replication-minus phenotype of Stenlund's *Hpa*I mutants may be due to an inability to bind E1 protein. Consistent with this is their observation that the analogous *Hpa*I-containing fragment from DPV cannot substitute for the BPV fragment in the BPV replication assay (29) and our observation that RecA-E1 did not bind well to this region from DPV (not shown).

The juxtaposition of an E1 binding region with the replication origin is consistent with a direct role for E1 in the DNA replication process such as has been determined for the SV40 T antigen (22, 28, 30). Precise mapping of the binding site(s) for RecA-E1 along with mutational analysis of the binding region should help clarify the relationship between binding and replication activity. Furthermore, the structural homologies between E1 and T antigen suggest that some of the functions of E1 in DNA replication will be similar to those defined for the well-studied T antigen. Biochemical characterization of the RecA-E1 protein should be useful for determining such activities.

In addition to RecA-E1 binding to the 219-bp fragment, weak binding to a 362-bp *Ava*II fragment containing the adjacent region from nucleotides 7458 to 7818 was detected. Recently, Mohr et al. observed weak binding of a baculovirus-expressed E1 protein to a similar fragment containing nucleotides 7477 to 7796 (21). This weak binding to the 7477-to-7796 fragment was enhanced by the addition of E2 protein, apparently through the formation of E1-E2 complexes (21). These results with both RecA-E1 and the baculovirus-expressed E1 protein suggest that in the absence of E2 protein, E1 has an intrinsic but weak interaction with BPV sequences in the region from approximately nucleotides 7450 to 7800. Furthermore, our observation that the 362-bp fragment was bound by ID13 extracts precipitated with anti-E1 serum is consistent with an interaction between an authentic E1 protein and this region of the BPV genome. However, as our ID13 extracts likely contain E2 as well as E1 protein, the binding we observed may reflect E1 plus E2 activity. Further experiments to address the effect of E1-E2 interactions on E1 DNA binding activity are in progress.

The significance of binding by RecA-E1 and the baculovirus-expressed E1 protein to the region from nucleotides 7457 to 7818 is unclear. While two-dimensional analysis of replicating DNA structures indicates that initiation of BPV replication occurs in this region (39), these sequences are not required in the functional replication assay (29). Consequently, the role of sequences in this region for DNA replication and the functional significance of E1 binding to these sequences remain to be determined.

While this paper was in preparation, two additional groups reported on the DNA binding properties of E1 proteins expressed from vaccinia virus (25) or baculovirus vectors (4). Neither group was able to demonstrate specific DNA binding by the expressed E1 proteins. The limited (21) or inapparent (4, 25) site-specific DNA binding ability of E1 protein expressed in eukaryotic systems is in sharp contrast to the binding exhibited by the bacterially expressed RecA-E1 protein. RecA-E1 bound specifically to the 219-bp *Ava*II fragment of BPV which contains the functional origin of replication, while none of the previously reported eukaryotically expressed E1 proteins have demonstrated this activity. However, if E1 binding to the origin is a requirement for replication, why then do the E1 proteins expressed in eukaryotic systems (from ID13 cells or from baculovirus or vaccinia virus vectors) fail to exhibit significant origin DNA binding activity? While differences in experimental conditions for DNA binding or in the functional stability of the examined E1 proteins could explain this discrepancy, more biologically interesting possibilities exist. Given the coordinated replication of BPV and cellular DNA during the cell cycle (9), there must be mechanisms which normally limit BPV replication. One mechanism may be to regulate E1 such that E1 is able to bind to the origin region only for a transient period during the cell cycle. This might be accomplished by reversible posttranslational modification (e.g., phosphorylation-dephosphorylation) of E1 such has been observed for SV40 T antigen (23), by use of cellular proteins which interact with E1 and/or the E1 binding site(s) to regulate E1 binding, or by ligand-induced changes in E1 binding activity. Consequently, E1 expressed from eukaryotic vectors may already be subjected to the negative regulatory mechanism(s) and therefore have limited origin DNA binding activity. In contrast, the bacterially expressed RecA-E1 would have escaped the normal control(s) imposed in eukaryotic cells and could exhibit the DNA binding activity intrinsic to this protein. Experiments to address these possibilities are in progress.

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