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Isolation of an Amino-Terminal Region of Bovine Papillomavirus Type 1 E1 Protein That Retains Origin Binding and E2 Interaction Capacity

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In vitro DNA binding results from a series of E1 proteins containing amino-terminal or carboxy-terminal truncations indicated that sequences between amino acids 121 and 284 were critical for origin binding. Additional binding experiments with E1 proteins containing internal, in-frame insertions or deletions confirmed the importance of the region defined by truncated E1 proteins and also demonstrated that downstream sequences were not required for binding activity in the context of the full-length E1 protein. On the basis of mapping results from the E1 mutants, a clone ($pE1_{121–311}$) was constructed that expressed E1 amino acids within the approximate boundaries of the critical sequences for DNA binding. The $E1_{121–311}$ protein retained origin-specific DNA binding, confirming that this region was not only necessary but was also sufficient for origin recognition. In addition to origin binding, $E1_{121–311}$ bound E2 protein in a cold-sensitive manner. Therefore, DNA binding and E2 binding activities colocalize to a 191-amino-acid functional domain derived from the amino-terminal half of the E1 protein. Finally, three E1 proteins with mutations in this region all lacked DNA binding activity and were all defective for in vivo replication. Two of these E1 mutants retained E2 binding capability, demonstrating that origin recognition by E1 is critical for replication and cannot necessarily be rescued by an interaction with E2 protein.

The bovine papillomavirus type 1 (BPV-1) E1 protein is an essential viral DNA replication factor that is both an origin recognition protein (5, 6, 13, 17, 18, 20) and an ATP-dependent helicase (14, 21). E1 binds to an 18-bp inverted repeat element (5) and presumably initiates DNA replication both by unwinding the origin duplex DNA and by recruiting cellular replication factors such as DNA polymerase α (3, 11) to the initiation complex. In vitro, E1 alone is sufficient to initiate replication (3), but in vivo the viral full-length E2 transcriptional activator protein is also absolutely required (16). E1 can form complexes with the E2 protein (2, 8, 15, 20), and both binding to and unwinding of origin DNA by E1 is enhanced by these cooperative interactions with E2 (4, 9, 13, 20). Consequently, the replication function of E1 appears dependent upon both protein-DNA and protein-protein contacts. Definition of the regions of E1 involved in each of these activities will facilitate our understanding of how protein-DNA complexes assemble to mediate replication initiation. Previous studies have begun the mapping of both the DNA binding domain (12, 15) and the E2 interaction domain(s) of E1 (1, 8, 12, 15) but have yielded partially conflicting results. We have now refined the mapping of the DNA binding domain and further demonstrate that this isolated domain is capable of both binding origin DNA and interacting with the E2 protein.

Construction of E1 deletion mutants. Previously, we showed that a bacterially expressed form of the E1 protein (RecA-E1) exhibited site-specific DNA binding to the BPV-1 origin (5, 18). The RecA-E1 protein (designated 1700T in this report) consists of E1 amino acids 1 to 586 with an amino-terminal tag of 35 amino acids derived from the amino terminus of the RecA protein (19). To localize the DNA binding domain of 1700T we constructed a set of clones expressing truncated forms of the 1700T protein. Carboxy-terminal truncations were created by insertion of a translation termination linker into the pGE1700T vector as previously described (7). Amino-terminal truncations were constructed from either the parental pGE1700T clone (clone N1) or from translation termination linker derivatives of pGE1700T (clones N2, N3, and N5) by removal of an appropriate restriction digestion fragment followed by religation. The last amino-terminal truncation, clone N4, was constructed by de novo cloning of a PCR-generated fragment of the E1 gene into the pGE vector. Protein production by each clone was assessed by Western blotting with anti-E1 sera directed against either the carboxy (serum 5996) or amino terminus (serum 5997) of E1. Each of the constructs expressed a soluble protein of the correct predicted size that was reactive with the appropriate anti-E1 serum (data not shown). The E1 amino acids expressed from each construct are summarized in Fig. 1C.

Evaluation of the DNA binding ability of E1 mutants. The origin binding capability of each truncated E1 protein was evaluated by an immunoprecipitation assay as previously described (5, 6). In brief, bacterial extracts lacking or containing E1 proteins were incubated with an equimolar mixture of an origin-positive (256-bp) and an origin-negative (151-bp) DNA fragment in 0.01 M Tris-HCl (pH 7.0), 150 mM NaCl, 0.01 mM EDTA, and 5 μg of sheared salmon sperm DNA per ml. The volume of each extract used was adjusted to provide equivalent amounts of E1 protein as detected by Western blotting. The 256- and 151-bp DNA fragments were generated by PCR amplification and radiolabeled by incorporation of [α-^{32}P]TTP during the amplification. After 30 min at 25°C, E1 proteins

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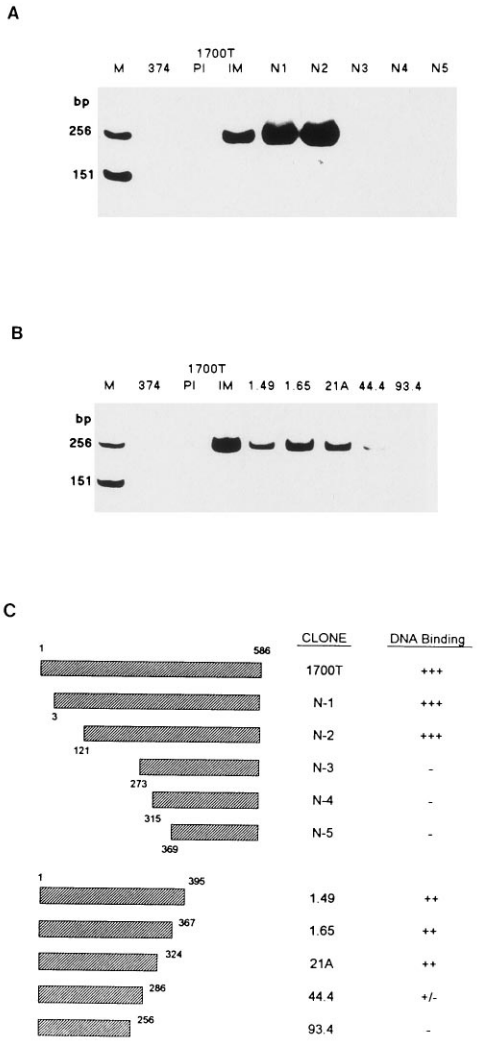

FIG. 1. DNA binding by E1 truncation mutants. French press extracts were prepared as previously described (18) from cells expressing the parental RecA-E1 fusion protein (1700T lanes), cells lacking E1 (374 lanes), or cells with the E1 truncation indicated above each lane. DNA binding with the extracts was performed as described in the text. Extracts containing truncated E1 proteins or no E1 protein were precipitated with anti-E1 serum 5996 (A) or anti-E1 serum 5997 (B). Extracts containing the 1700T E1 protein were precipitated with these same two antisera (IM lanes) or preimmune serum (PI lanes). Lanes marked M show the results for a sample of the input DNA consisting of a fragment containing the BPV origin (256 bp) and a fragment lacking the origin (151 bp). (C) A summary of the amino acids present in the various truncated proteins and of their DNA binding results.

were precipitated with the appropriate anti-E1 serum and protein A-Sepharose. Coprecipitated DNA fragments were extracted in Tris-borate-EDTA sample buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, 10% glycerol, 2.7% xylene cyanol, 2.4% bromophenol blue, 2% sodium dodecyl sulfate [SDS]) and analyzed on 8% polyacrylamide gels (Fig. 1A and B).

The specificity of the DNA binding assay is demonstrated by the observation that neither DNA fragment was bound either by an extract lacking E1 protein (374 lanes) or when the 1700T extract was precipitated with a preimmune serum (PI lanes). Furthermore, when the 1700T extract was precipitated with either anti-E1 serum 5996 or 5997, only the origin-containing fragment was bound (IM lanes in Figs. 1A and 1B). Under these same reaction conditions, deletion of amino acids 1 to 120 did not impair the origin-specific DNA binding ability of E1 (clone N2) but removal of amino acids 1 to 272 (clone N3) or more (clones N4 and N5) completely abolished binding activity (Fig. 1A). These results map the amino-terminal boundary of the DNA binding domain to between amino acids 121 and 273; this is consistent with a previous observation that an $E1_{162–422}$ protein retained origin binding activity (12).

Analysis of the carboxy-terminal E1 truncations revealed that $E1_{1–395}$ (clone 1.49), $E1_{1–367}$ (clone 1.65), and $E1_{1–324}$ (clone 21A) all retained origin-specific DNA binding activity. Truncation to amino acid 286 (clone 44.4) produced an E1 protein that bound very weakly but that was still origin specific, while further truncation to residue 256 (clone 93.4) eliminated DNA binding completely. From these results it is clear that sequences downstream from amino acid 324 are not absolutely necessary for origin binding. The dramatic reduction in binding that occurred between clones 21A and 44.4 also suggests that the functional carboxy-terminal boundary of this domain is between amino acids 286 (clone 44.4) and 324 (clone 21A). Note, however, that all the carboxy-terminal truncations bound less origin DNA than that bound by the 1700T protein. This may indicate that sequences in the carboxy terminus can contribute directly to origin binding or alternatively that they influence the overall functional stability of the E1 protein.

The carboxy-terminal boundary that we determined is consistent with the previous observation that an $E1_{1–299}$ protein retained origin binding activity (15) but is in contrast to the recent results of Sarafi and McBride that an $E1_{1–328}$ protein lacked origin binding activity (12). Furthermore, Sarafi and McBride showed that an $E1_{1–378}$ and an $E1_{162–422}$ protein bound the origin but that an $E1_{162–378}$ protein did not (12). These results imply that sequences beyond amino acid 328 are critical for origin binding but that the exact sequences required are somewhat dependent on the overall amino acid content of the E1 construct.

To address the contribution of downstream residues in the context of our 1700T protein, we constructed a series of internal, in-frame deletion and insertion mutations by the previously described procedure of Ludes-Meyers and Wilson (7). Thirteen mutants were constructed throughout the region of E1 from amino acid 197 to 487 (Fig. 2A), and their specific amino acid changes are listed in Table 1. Expression of each mutant E1 protein was verified by Western blotting (data not shown), and each was tested for DNA binding as was done for the truncation mutants. The three mutants with alterations in the E1 sequence upstream of amino acid 300 (clones 15.2-64, 26.7-1, and 44.4-29) were completely defective for origin binding, confirming the importance of the amino-terminal region of E1 for DNA binding. Conversely, all 10 of the downstream mutants retained origin-specific DNA binding ability, indicating that there was no absolute requirement for these sequences in the context of a full-length E1 protein.

The combined DNA binding results from the truncation mutations and the in-frame, internal mutations defined a region between, approximately, amino acids 121 and 300 as containing the E1 sequences necessary for origin binding. To determine if this region was also sufficient for origin binding we constructed clone $E1_{121–311}$. Truncation mutant N2 was digested with *Bst*BI plus *Bst*EII, and the 935-bp fragment containing BPV-1 nucleotides 1470 to 2405 was removed. This fragment was replaced with the corresponding fragment derived from a translation termination mutant (6a) containing stop codons after nucleotide 1783. The resultant construct was expected to express E1 amino acids 121 to 311, and a product with the correct molecular weight was detected by Western

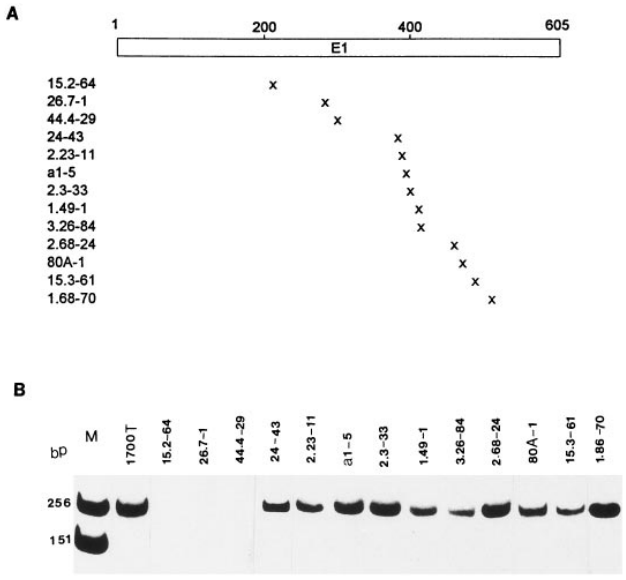

FIG. 2. DNA binding by E1 proteins with internal, in-frame deletions and insertions. (A) A diagram showing the relative locations of the 13 mutations in the E1 open reading frame. The actual sequence changes for each mutant are shown in Table 1. (B) For each E1 mutant shown in panel A extracts were prepared and assayed for DNA binding with serum 5996 as described in the legend for Fig. 1. Identical results were obtained with serum 5997 (data not shown). Lanes M and 1700T correspond to lanes M and 1700T IM in Fig. 1A.

blotting (Fig. 3A). $E1_{121–311}$ was fully active for origin-specific DNA binding (Fig. 3B), confirming that the critical E1 structural features for origin recognition reside in this 191-amino-acid region.

E2 binding by E1 proteins. In addition to its origin binding ability, E1 protein forms specific complexes with the E2 protein (1, 8, 12, 15). Since E2 enhances DNA binding by E1 (9, 13, 20), we asked whether or not our isolated DNA binding domain ($E1_{121–311}$) could still interact with the E2 protein in vitro. A fragment containing the E2 coding region was generated by PCR amplification of pdBPV-1 with primers containing a *Bam*HI site (primer 1, 5′-GTATGGATCCTTGACGA GGAGGAGGATAGTG-3′; primer 2, 5′-ATAGGGATCCTC AGAAGTCCAAGCTGGCTGT-3′). The amplified fragment containing BPV-1 nucleotides 2581 to 3837 was digested with *Bam*HI and cloned into the *Bam*HI site of the pRSET-A vector (InVitrogen). A resultant clone was designated pRSET-E2 and was confirmed by DNA sequencing. E2 RNA was produced by in vitro transcription of purified pRSET-E2 DNA in the Ribomax system (Promega, Inc.) under standard conditions specified by the manufacturer; control luciferase RNA was synthesized in parallel reactions. Radiolabeled E2 and luciferase proteins were produced by incorporation of [^{35}S]methionine (>1,000 Ci/mmol; New England Nuclear) during in vitro translation of their respective RNAs with the Promega rabbit reticulocyte lysate system. The in vitro-translated products were analyzed by electrophoresis on an 8% polyacrylamide-SDS gel and are shown in Fig. 3C (lanes 1 and 2). Each translation reaction yielded a predominant radiolabeled product of the predicted size for the full-length E2 or luciferase proteins, as well as several shorter products that are most likely premature termination products. The identity of the E2 product was further confirmed by immunoprecipitation with an anti-E2 monoclonal antibody (data not shown).

To assess E2 interactions with the amino terminus of E1, extracts containing $E1_{121–311}$ or the three amino-terminal region E1 mutants (15.26-1, 26.7-3, and 44.4-29) were immunoprecipitated with anti-RecA serum and protein A-Sepharose as previously described (18). The washed immunoprecipitates were resuspended in 15 μl of 50 mM HEPES (pH 7.6)–0.01% Nonidet P-40 containing either the in vitro-translated E2 or luciferase proteins. Reactions were incubated for 1 h at either room temperature or 4°C, and all subsequent wash steps were conducted at the same temperature as that of the initial incubation. After incubation the beads were collected by centrifugation for 1 min, washed three times with 1.0 ml of buffer A (50 mM HEPES [pH 7.6], 1.0 mM EDTA, 0.2 M NaCl, 0.5% Nonidet P-40), and washed once with 1.0 ml of 50 mM HEPES [pH 7.6]–1.0 mM EDTA. Bound proteins were extracted with 20 μl of SDS gel sample buffer at 100°C for 2 min, and the extracted material was analyzed on an 8% polyacrylamide-SDS gel (Fig. 3C). Under these conditions both bacterially expressed (1700T) and baculovirus-expressed E1 (Fig. 3C, lanes 6 and 7) bound E2 protein. The presence of E2 in the immunocomplex was E1 dependent since no associated E2 was observed when the initial immunoprecipitation step was performed with preimmune serum (data not shown), when an extract lacking E1 protein was used (data not shown), or when the 26.7-1 E1 mutant was used (see below). When incubated at room temperature, $E1_{121–311}$ was also capable of binding the E2 protein but not the control luciferase protein (Fig. 3C, lanes 4 and 5). Consequently, the isolated DNA binding domain does retain the ability to recognize and specifically bind the E2 protein. Interestingly, this interaction is cold sensitive, and the E1-E2 complex could not be detected at 4°C (lane 3). A similar observation that the amino-terminal E2 interaction domain is cold sensitive was reported previously by Thorner et al. (15) and Benson and Howley (1) for BPV-1 E1 and more recently for human papillomavirus type 33 E1 by Müller and Sapp (10).

To further analyze the interaction between E2 and E1, E2 binding by the three amino-terminal region mutants was tested. All three mutants lack origin binding ability, yet mutants 15.26-1 and 44.4-29 were competent for E2 binding while mutant 26.7-1 was completely defective (Fig. 3C, lanes 8 to 10). The E1 26.7-1 mutation is located at amino acid 277, and the failure of this mutant E1 to bind E2 is consistent with the boundaries of the E2 interaction domain defined by $E1_{121–311}$. However, the location of the 26.7-1 mutation is between the 15.26-1 and 44.4-29 mutations; this suggests that while the E2 binding and origin recognition activities of E1 overlap, the

TABLE 1. Amino acid sequence of in-frame E1 mutants

Clone	Sequence[a]
15.2-64	^{192}WVLAVF(lvav)GLAEVF^{203}
26.7-1	^{271}SAALFW(slfw)FKSSLS^{282}
44.4-29	^{280}SLSPAT-KHGALP^{292}
24-43	^{366}HYLRAE----SMPAYI^{382}
2.23-11	^{369}LRAETQ-----(e)AYIKAA^{385}
a1-5	^{371}AETQAL-------(a)ARCKL^{A389}
2.3-33	^{379}PAYIKA------(k)GEGSWK^{396}
1.49-1	^{392}EGSWKS------YQNIEL^{409}
3.26-84	^{406}NIELIT----LKLWLK^{421}
2.68-24	^{439}KSMLCN--(lc)IHFLGG^{452}
80A-1	^{455}LSFANH-------(lv)SLADTR^{473}
15.3-61	^{473}RAALVD--------YFDTYL^{492}
1.68-70	^{481}THACWR----------VSIDRK^{506}

[a] The wild-type E1 amino acid sequence is shown in uppercase letters. Inserted amino acids are shown in parentheses in lowercase. Each hyphen represents a deleted amino acid. Numbers at each end of the sequence refer to the original wild-type E1 amino acids.

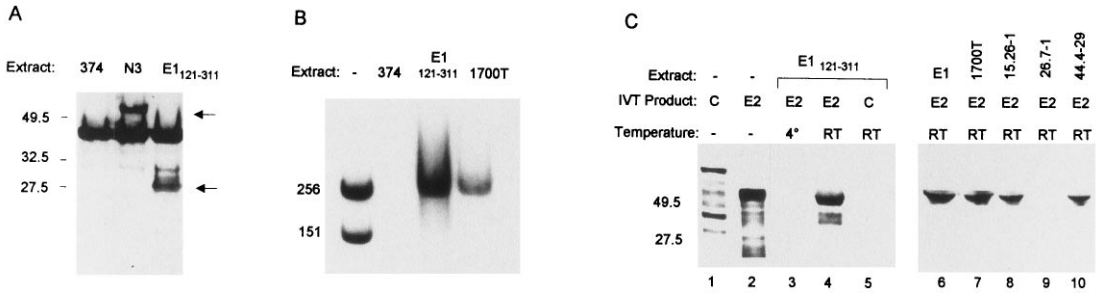

FIG. 3. Expression, DNA binding, and E2 binding by the various E1 proteins. (A) Western blot of extracts lacking E1 (374), expressing an amino-terminal truncation mutant (N3), and expressing the $E1_{121–311}$ clone. Anti-RecA was used as the primary serum for this blot. The N3 and the $E1_{121–311}$ proteins are indicated by arrows, and molecular mass markers (in kDa) are on the left. The prominent band present in all three lanes is the endogenous RecA protein. (B) $E1_{121–311}$ was assayed for DNA binding as described in the legend for Fig. 1. (C) Binding of E1 proteins to E2 in vitro was performed as described in the text with the bacterial extracts containing E1 proteins indicated above the lanes, except for lane 6 which represents baculovirus-expressed E1 protein purified from SF9 cells (generously provided by M. Lentz, Texas A&M University). Binding reactions included either the in vitro-translated (IVT) control luciferase protein (C lanes) or the pRSET-E2 protein (E2 lanes) and were performed at 4°C or room temperature (RT) as indicated (lanes 3 to 10). All precipitations were performed with either anti-RecA serum (lanes 3 to 5) or anti-E1 serum 5996 (lanes 6 to 10). Lanes 1 and 2 represent samples of the input IVT proteins that were not subjected to the binding assay. Molecular mass markers (in kDa) are on the left.

region required for E2 binding is smaller than that needed for origin recognition.

DNA replication activity of E1 mutants. The above studies confirmed the importance of the amino-terminal portion of E1 for origin recognition in vitro. To determine if origin binding is required for E1-dependent DNA replication, the mutations in clones 15.2-64, 26.7-1, and 44.4-29 were constructed back into the context of the viral genome (in clone pdBPV-1) and the mutant BPV-1 genomes were tested for transient in vivo replication as previously described (17). All three mutations rendered BPV-1 unable to replicate (Fig. 4 and data not shown). This replication defect was observed at 32, 37, and 39°C, in contrast to the wild-type pdBPV-1, which replicated efficiently at all three temperatures. The inability of 15.2-64 and 44.4-29 to replicate even though they could bind E2 indicates that E2 binding alone is insufficient for replication function.

The results presented in this study localize the boundaries of the functional DNA binding domain of the BPV-1 E1 protein to a 191-amino-acid region encompassing residues 121 to 311 of the full-length E1 protein. This is the smallest E1 polypeptide to date that still retains origin-specific binding activity, but the actual essential domain could be even smaller. The carboxy-terminal boundary of the functional domain is fairly well established and must be in the vicinity of amino acid 300 since no origin binding activity has been demonstrated for any E1 protein truncated upstream of amino acid 284 (12, 15). However, the amino-terminal boundary is less well defined. An $E1_{162–422}$ protein has been shown to retain origin binding activity (12), suggesting that sequences between amino acids 121 and 162 may not be critical, though this has not been directly tested. Additional deletion and internal mutations will be required to more completely define the amino-terminal boundary of the minimal DNA binding domain. Nonetheless, the results presented here indicate that it is possible to isolate the DNA binding domain of E1 as an independent polypeptide consisting of less than one-third of the total E1 sequence.

In addition to origin binding activity, the $E1_{121–311}$ protein retained the ability to complex with the E2 protein at room temperature but not at 4°C. This is consistent with earlier reports that an $E1_{1–423}$ protein (15) and an $E1_{1–222}$ protein (1) bound E2 in a cold-sensitive fashion. Surprisingly, our 26.7-1 mutant failed to bind E2 even though this mutation is at amino acid 277, which is not included in the active $E1_{1–222}$ protein. However, we have found that an $E1_{1–286}$ protein (clone 44.4) binds E2 while an $E1_{1–256}$ protein (clone 93.4) does not (data not shown), which is consistent with the results for the 26.7-1 mutant. The explanation for this difference in the apparent C-terminal boundary of the E2 interaction domain observed for the various E1 truncation mutants is unknown but may reflect conformational differences specific to the constructs being tested or differences in the sensitivities of assays used. In either case, the cold-sensitive character of this amino-terminal E2 interaction domain of E1 has now been observed by several groups and may explain the previously reported failure of an $E1_{1–378}$ protein to bind E2, since the assay appeared to include an immunoprecipitation step at 4°C (12). Therefore, the combined evidence from available studies is strongly supportive of a functional E2 interaction domain in the amino-terminal region of the E1 protein. The location of this E2 interaction domain is overlapping with but not coincident with the sequences required for origin-specific DNA binding activity. Note, however, that the presence of this amino-terminal E2 interaction domain does not preclude the existence of other

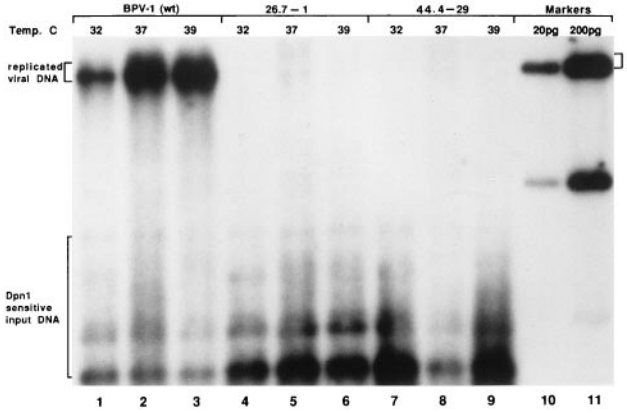

FIG. 4. In vivo transient replication of BPV-1 genomes with wild-type or mutant E1 genes. Shown are the in vivo replication results for wild-type (wt), pdBPV-1 (lanes 1 to 3), and the 26.7-1 and 44.4-29 mutants (lanes 4 to 9). Each DNA was *Bam*HI digested prior to electroporation to liberate the BPV-1 sequences from the vector sequences. After electroporation of each DNA (5 μg), recipient C127 cells were maintained at either 32, 37, or 39°C for 72 h. For all samples, low-molecular-weight DNAs were harvested and digested with *Bam*HI and *Dpn*I prior to blotting with radiolabeled pdBPV-1. Lanes 10 and 11 show results for 20 and 200 pg, respectively, of the *Bam*HI-digested pdBPV-1. The upper bands in these marker lanes represent the BPV-1 genome, and the lower bands represent the vector genome.

regions of E1 that could also interact with the E2 protein (8, 12, 15).

The studies presented here also demonstrate that origin binding is a critical requirement for E1 function in replication. Three E1 mutants lacking in vitro origin recognition activity were all completely defective for transient in vivo replication. Two of these mutants were still capable of binding E2 in vitro, indicating that E1-E2 interactions alone are not necessarily sufficient to rescue defective origin binding by E1.

X.L. and J.H.L. contributed equally to this study.

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