Effects of mutations within two hydrophilic regions of the bovine papillomavirus type 1 E1 DNA-binding domain on E1–E2 interaction

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The interaction between papillomavirus E1 and E2 proteins is essential for viral genome replication. Using both in vivo and in vitro assays to evaluate the regions of the two proteins necessary for the E1–E2 interaction, three independent interactions were identified for bovine papillomavirus E1: the N terminus of E1 (E1N, residues 1–311) interacts with the E2 transactivation domain (E2TAD) and the E2 DNA-binding domain (E2DBD) and the C terminus of E1 (E1C, residues 315–605) interacts with E2. Nine mutations within E1N were evaluated for their effects on E2 interaction. Five mutations eliminated interaction with the E2TAD; four of these were located within two previously identified conserved, hydrophilic regions, HR1 and HR3. Since HR1 and HR3 residues appear to comprise the origin of replication recognition element for E1, simultaneous interaction with the E2TAD during initiation complex formation would seem unlikely. Consistent with this inference is the fact that three of the five mutants defective for E2TAD binding exhibited wild-type levels of replication. The replication-positive phenotype of these mutants suggests that the E1N–E2TAD interaction is not essential for replication function and is probably involved in some other E1–E2 function, such as regulating transcription. Only one of the five mutations defective for E2TAD binding also prevented E2DBD interaction, indicating that the regions of E1N that interact with the E2TAD and the E2DBD are not identical. The ability of E1N to cooperatively interact with E2 bound to E2-binding site (E2BS) 11 versus E2BS12 was also examined, and cooperative binding was only observed when E2 was bound to E2BS12.

Introduction

Bovine papillomavirus type 1 (BPV-1) is an attractive model of metazoan DNA replication because of its relatively simple genome and the ability of the viral genome to replicate along with the host genome during the S phase of the cell cycle (Chow & Broker, 1994). Only two viral proteins, E1 and E2, are required; all other replication factors are supplied by the host cell (Ustav & Stenlund, 1991). E1 is the protein that recognizes the origin of replication (ori) and recruits cellular proteins, such as the DNA polymerase α-primase (Bonne-Andrea et al., 1995; Park et al., 1994) and the replication protein A (Han et al., 1999), to the ori to form the replisome. Recognition of the ori is mediated by the E1 DNA-binding domain (E1DBD), which is approximately located between amino acids 150 and 300 (Fig. 1) (Chen & Stenlund, 1998; Enemark et al., 2000; Leng et al., 1997; Sarafi & McBride, 1995). Mutational analysis of the E1DBD DNA sequence revealed two hydrophilic domains, HR1 and HR3, which are critical for DNA binding (Gonzalez et al., 2000; Thorner et al., 1993). Resolution of the E1DBD crystal structure indicates that HR1 and HR3 form a juxtaposed surface that probably interacts directly with the E1-binding site (E1BS) (Enemark et al., 2000). Although the 68 kDa E1 protein is seen as a monomer when in solution (Sedman & Stenlund, 1996), it forms large oligomeric complexes of trimers and...
HR3, which are critical for DNA binding (Gonzalez et al. 1997). Within the DBD are two hydrophilic regions, HR1 and HR3, which are critical for DNA binding (Gonzalez et al., 2000). Note that the precise minimal boundaries of a functional DBD have not been defined, although polypeptides slightly smaller than the 121–311 peptide are functional as an independent polypeptide consisting of E1121–311 amino acids. DBD refers to the DNA-binding domain, which we have defined, although polypeptides slightly smaller than the 121–311 peptide sequence. The three constructs, E1N (E11–311), E1DBD (E1121–311) and E1C (E1315–605), analysed in this study are also indicated.

**Methods**

**Mutagenesis and plasmid constructs.** For the construction of pGEX2TAD and pGAD424E2TAD, a cloned E2 gene was amplified using the primers 5’TAD, 5’ ATGGAGAAGCATGGAACTGT 3’, and 3’ E2TAD, 5’ CTCACACTCTGCTCCATCTTACAG 3’. The PCR product was ligated into pCRBLUNT (Invitrogen) and transformed into TOP10 cells. E2DBD clones were made in a similar manner using the primers 5’ E2DBD, 5’ CAGTGGTCTGCTCATAATTCG 3’, and 3’ E2DBD, 5’ TACAGAGTCCAGCCTGGTCTA 3’. E2DBD and E2DBD fragments from pCRBLUNT were excised using EcoRI and ligated into pGAD424 (Clontech) and pGEX4T-1 (Pharmacia), digested previously with the same enzyme. Ligation mixtures were transformed into TOP10 cells and correct clones were identified by PCR. pGEX1DBD was constructed as described previously (Gonzalez et al., 2000). pGEX2E2 was constructed by first amplifying the E2 open reading frame from pDBPV.1 and unidirectionally cloning the PCR product into pGEX-4T-1 (Pharmacia), digested previously with EcoRI/SfiI. pSET, used for in vitro translation, and pGBT9E1, used for yeast two hybrid assays, have been described elsewhere (McShan & Wilson, 2000; Wang & Wilson, 1999; Sedman & Wilson, 2000; Wilson & Ludes-Meyers, 1991). pG8T9E1, 5’–3’ was constructed by inserting a stop codon at amino acid 312 in pGBTE1. pG8T9E1, 5’–3’ was made by inserting a BamHI site in front of amino acid 315 by site-directed mutagenesis using the QuickChange Mutagenesis kit (Strategene) and primers E1N1, 5’–3’. DNA was digested with BamHI to excise the DNA fragment, gel-purified, and ligated into pG8T9 digested with BamHI and transformed into TOP10 cells. pG8T9E1, 5’–3’ mutants were made by site-directed mutagenesis using the QuickChange Mutagenesis kit. pCGEagE1 and pCGE2, provided by A. Stenlund, were described previously (Ustav & Stenlund, 1991). Mutant pCGEagE1 constructs were made as described above using the QuickChange Mutagenesis kit. pBOR, previously designated ORI 105, has been described by Holt et al. (1994). All constructs and mutants were verified by sequencing.

**Protein purification.** For the expression of GSTE2 (glutathione S-transferase fused to E2), 2 × YT medium containing 2% glucose and 50 μg/ml ampicillin was inoculated with an overnight culture of pGEXE2 and grown for either 3 h at room temperature or 1 h at 18 °C. Protein expression was induced with IPTG at a final concentration of 1 mM and the culture was incubated for a further 2 h at room temperature or 12–18 h at 18 °C. Cells were then centrifuged at 10,000 g for 15 min and the pellet was frozen at −70 °C for at least 1 h. B-per reagent (Pierce) containing 5 mM DTT and 1 mM PMSF was used for cell resuspension. Lysozyme was added to a concentration of 100 μg/ml and the suspension
was incubated on ice for 1 h. The sample was then sonicated twice for 15 s using an Ultrasonics sonicator with the micropipet at maximum power and then centrifuged for 30 min at 4°C. Glutathione-Sepharose beads were added to the supernatant and rotated overnight at 4°C. Beads were washed twice with GST-C buffer (50 mM Tris–HCl, pH 7.9, 250 mM NaCl, 5 mM EDTA, 10% glycerol) plus 5 mM DTT and 10 mM PMSF, once with GST-E buffer (50 mM Tris–HCl, pH 8.0, 1 M NaCl, 5 mM EDTA, 10% glycerol) plus 5 mM DTT and 10 mM PMSF, and finally in GST-C buffer. Protein was eluted using 10 mM glutathione in GST-C buffer. Human thrombin (5 units) was added directly to 100 µg of eluted GSTE2 and incubated for 4 h at 20°C. PMSF was added to a final concentration of 1 mM in order to inhibit the thrombin and the cleaved GST plus uncleaved GSTE2 were removed using glutathione-Sepharose beads. Conversely, GSTE2 bound to glutathione-Sepharose beads were cleaved using 10 units of thrombin overnight at room temperature. Beads were centrifuged at low speed and supernatant containing cleaved E2 was removed and stored at −20°C. Protein quality was assessed by SDS–PAGE and protein concentration was determined using the Bradford method. GSTE2DBD was expressed and purified as above. GSTE2TAD was purified as above except that the cell pellet was resuspended in PBS and French-pressed at 16000 PSI before centrifugation and the addition of glutathione–Sepharose beads to the supernatant.

**Electromobility shift assays (EMSAs).** Gel shift assays were performed as described previously (Gonzalez et al., 2000). Briefly, each 10 µl reaction mixture contained EMSA buffer (20 mM potassium phosphate, pH 7.0, 100 mM NaCl, 1 mM EDTA, 0.1% NP-40, 10% glycerol, 5 mM DTT, 0.07% BSA), 2.5 fmol of radiolabelled oligonucleotide, 20 ng pUC18 DNA and purified GSTE1DBD and E2. Oligonucleotide E1BS 1-4 consists of BPV-1 nucleotides 7926–29 (designated substrate B) and contains the authentic BPV-1 18 bp E1 binding element (E1BE) and the 12 bp E2BS12. Oligonucleotide E1BS 1-4 BS11 consists of nucleotides 7891–29 (designated substrate A) and includes the authentic BPV-1 EBS11 and the 18 bp E1BE. BPV-1 nucleotides 16–18 (ACC) in this sequence were changed to TAG to destroy the 5′ portion of E2BS12. Samples were incubated for 30 min at 25°C and then electrophoresed by 8% PAGE in 0.5× Tris-borate–EDTA (TBE) buffer (pH 7.5). Protein quantification was carried out using a Molecular Dynamics PhosphorImager.

**Yeast two-hybrid assay.** Yeast transformations and liquid β-galactosidase assays were performed as described by McShan & Wilson (2000). Alternatively, competent Saccharomyces cerevisiae strain SFY526 was co-transformed with pGBT9E1, pGBT9E1_1–311 or pGBT9E1_311–605 and pGAD424E2 using the lithium–acetate method. Three separate clones from each transformation experiment were isolated and assayed for β-galactosidase activity using chloramphenicol red-β-galactopyranoside as the substrate. Yeast co-transformants with E1N versus the E2 subdomains were mapped sequentially by first transforming yeast strain SFY526 with the pGBT9E1_1–311 Plasmid DNA. Competent pGBT9E1_311 SFY526 cells were then transformed with DNA from either pGAD424E2TAD or pGAD424E2DBD. All E1N mutant co-transformants were made by first making competent pGAD424E2, pGAD424E2TAD and pGAD424E2DBD-transformed SFY526 cells. These cells were then re-transformed with pGBT9E1_1–311 mutant plasmids. Fold stimulation was calculated from the β-galactosidase activities of individual co-transformants using the equation (pGBT9X + pGAD424Y)−(pGBT9X + pGAD424Y)/pGBT9X + pGAD424), where X is the E1 protein and Y is the E2 protein.

**GST pulldown assay.** GSTE2, GSTE2TAD, GSTE2DBD or GST alone (10 µg) were incubated with 30 µl glutathione–Sepharose beads in 500 µl of binding buffer (10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 2% BSA). Samples were incubated for 5 h at 4°C and then centrifuged at 500 g for 4 min. In vitro translated 35S-labelled E1, E1_{1–311} or E1_{311–605} was added to the beads in 500 µl binding buffer with 1% BSA and rocked for 20 h at 4°C. Beads were washed first with TSA (10 mM Tris–HCl, pH 8.0, 140 mM NaCl, 0.025% NaN3) and then three times with TSA containing 0.1% Triton X-100. Further washing steps were carried out with TSA containing 0.2% Triton X-100, then with TSA alone and finally with 50 mM Tris–HCl, pH 6.8, containing 1 mM PMSF. After resuspension in each wash buffer, samples were vortexed for 5 s and incubated on ice for 2–3 min before centrifuging at 500 g. After washing, pellets were resuspended in 25 µl 2× SDS sample buffer and heated at 75°C for 4 min. Half of each sample was electrophoresed by 10% SDS–PAGE. Gels were then dried and visualized using a Molecular Dynamics PhosphorImager.

**Transient replication assays.** pCGEagE1 (1 µg) or mutant pCGEagE1 (1 µg), and pCGE2 (0.1 µg) and either pBOR (1 µg; containing the ori) or pUC (1 µg; not containing the ori) were mixed with 12 µl PLUS reagent (Gibco) and incubated at room temperature for 20 min in 250 µl of Ham’s media with non-essential amino acids. LipofectAMINE reagent (Gibco) (12 µl) was mixed with 250 µl of Ham’s media containing non-essential amino acids and incubated for 10 min. LipofectAMINE solution was then added to the DNA/PLUS reagent mixture and incubated for an additional 20 min. Samples were added directly onto CHO cells, which had been seeded at a density of 1×10^6 cells/ml 12–24 h earlier. Transfection was allowed to proceed for 3 h at 37°C, after which the cells were trypsinized, split into three 60 mm plates and incubated for an additional 48, 72 or 96 h. DNA from each transfection experiment was harvested, digested with DpnI/HindIII and analysed by Southern blotting, as described previously (McShan & Wilson, 1997).

**Results**

E2 interacts with both E1N and E1C

It is well-established that papillomavirus E1 and E2 proteins are both required for virus replication and transcription and that cooperative interaction between these proteins is essential for the assembly of the replication initiation complex at the viral ori in vitro (Sanders & Stenlund, 1998, 2000; Sedman & Stenlund, 1995). Various reports have identified different, and sometimes conflicting, subregions of the two proteins to be responsible for mediating an interaction (Benson & Howley, 1995; Berg & Stenlund, 1997; Chen & Stenlund, 1998, 2000; Gillitzer et al., 2000; Lusky & Fontane, 1991; Lusky & Fontane, 1991; Mohr et al., 1990; Moscufo et al., 1999; Sarafi & McBride, 1995; Thorner et al., 1993). In order to re-evaluate and better define the BPV-1 E1–E2 complex, we used a combination of an in vivo yeast two-hybrid system and an in vitro binding assay to examine the domains of E1–E2 interaction. Consistent with published reports (Blitz & Laimins, 1991; Lusky & Fontane, 1991; Mohr et al., 1990), full-length E1 and E2 interacted effectively and, in the two-hybrid assay, produced a 10-fold stimulation of reporter activity compared to when protein was expressed alone (Fig. 2A); this interaction was detected at similar levels by the in vitro binding assay (Fig. 2B). Since prior studies have demonstrated an interaction...
between E2 and both the N-terminal and the C-terminal regions of E1 (Benson & Howley, 1995; Leng et al., 1997; Moscufo et al., 1999; Sarafi & McBride, 1995; Thorner et al., 1993), we initially tested each half of E1 separately against E2. As reported previously by Moscufo et al. (1999) who used similar E1 constructs, we found that both the N (E1N = E1(1–311)) and the C (E1C = E1(315–693)) terminus of E1 interacted independently with full-length E2 both in vivo and in vitro (Fig. 2A, B). The remainder of this report will focus on the E1N–E2 interaction; the E1C–E2 interaction is still under investigation.

To further investigate the E1N–E2 interaction, we separated E2 into the E2TAD domain (E2(1–194)) and the E2DBD domain (E2(236–410)) and tested each subregion separately for E1N interaction. Although fold stimulation was weak in multiple assays with independent co-transformants, each separate E2 domain reproducibly enhanced reporter activation in the presence of E1N, indicating an interaction with the N-terminal region of E1 (Fig. 2A). To confirm these in vivo results and further strengthen support for an E1N interaction with both the E2TAD and the E2DBD, identical domains were tested in a GST pulldown assay (Fig. 2C). Each separate E2 domain bound GST(E1N at levels significantly higher than background binding to GST, again indicating a specific interaction. The combined results in Fig. 2 (A–C) define at least three separate E1–E2 interactions: E1C–E2, E1N–E2TAD and E1N–E2DBD.

**E2 is unable to rescue the HR1 and HR3 E1DBD mutants to wild-type levels**

The above results, in combination with previous reports (Benson & Howley, 1995; Leng et al., 1997; Moscufo et al., 1999; Thorner et al., 1993), confirm that E2 interacts with the N-terminal region of E1, a region that includes the functional E1DBD (Chen & Stenlund, 1998; Enemark et al., 2000; Leng et al., 1997). Consistent with these direct interaction results is the observation that origin binding of an isolated wild-type E1DBD polypeptide is cooperatively enhanced by full-length E2 in an EMSA (Chen & Stenlund, 1998). Although indirect, the EMSA results probably indicate that the E1DBD region itself makes contact with E2. To begin to define the E1DBD residues critical for this interaction, we examined substitution mutants in three E1DBD hydrophilic domains, designated HR1, HR2 and HR3 (Fig. 3A) (Gonzalez et al., 2000). The E1DBD proteins were tested for cooperative binding with E2 in the EMSA using substrate B (BPV-1 nucleotides 7926–29), which contains the authentic BPV-1 18 bp E1BE and the 12 bp E2BS12. Wild-type GST(E1DBD protein, in the absence of E2, formed two discrete complexes on the DNA (Fig. 3B, lanes 3 and 17). E2 alone formed a predominant protein–DNA complex that migrated faster than the E1–DNA complexes (Fig. 3B, lanes 2 and 16). Combination of E2 with the wild-type E1DBD protein resulted in a new E1DBD–E2–DNA complex that migrated to a position in-between the two E1–DNA complexes (Fig. 3B, lanes 4 and 18). In the absence of E2, all mutations in the E1DBD, with the exception of the K557A mutant, decreased E1–DNA binding to some degree. As reported previously (Gonzalez et al., 2000), origin binding by HR1 and HR3 mutants (K183A, K186A, T187A and K241A) was less than 10% of that seen for the wild-type (Fig. 3B, lanes 7, 9, 11 and 21; Fig. 3C), while non-HR mutants bound between 45 and 130% of wild-type (Fig. 3B, lanes 5, 19, 23 and 25; Fig. 3C). In the presence of E2, all the E1DBD proteins with mutations outside of HR1 and HR3 were rescued at levels similar to those seen for the wild-type (Fig. 3B, lanes 14, 20, 24 and 26; Fig. 3C). In contrast, no protein with a mutation falling...
within the two conserved hydrophilic domains was com-
parably rescued (Fig. 3B, lanes 8, 10, 12 and 22; Fig. 3C), with
the exception a nonconserved amino acid mutant, T188A (Fig.
3B, lane 14; Fig. 3C). These results indicate that the interac-
tion of full-length E2 with the E1DBD is able to compensate for
reduced DNA binding by the E1DBD non-HR1 or -HR3
mutants and restore wild-type levels of E1DBD–E2–DNA
complex formation. In contrast, the E1DBD mutants with
amino acid changes at conserved residues in HR1 and HR3,
which are likely to be involved in direct DNA contact, were
only partially rescued by E2. These data support and extend
previous work by Thorner et al. (1993), which demonstrated
that E2 was unable to rescue DNA binding by the HR3
mutants and a single mutation in HR1.

**E1DBD mutants are capable of E2 interaction**

The inability of the HR1 and HR3 mutants to be completely
rescued by E2 could be due to a change in the E1DBD structure
that prevents interaction with E2. To investigate this issue, we
used the yeast two-hybrid system to determine interaction
between E2 and the E1DBD mutants in the context of E1N
(Fig. 4A). Although quantitative comparisons are somewhat
uncertain due to potential expression differences between
mutant co-transformants, the assay can at least be used in a
qualitative manner to assess whether or not any interaction is
occurring. All the E1N mutant proteins, with the possible
exception of the K241A and K267A mutants, showed stimulation
of reporter activity by full-length E2, consistent with the
retention of E1–E2 interaction. While the stimulation of both
the K241A mutant and the K267A mutant was very weak, the
K267A mutant exhibited wild-type levels of cooperative E2
binding in vitro (Fig. 3), so even a weak response in the two-
hybrid assay presumably reflects some intrinsic E1–E2 in-
teraction. Therefore, the similar two-hybrid response by the
K241A mutant suggests that this mutant also retains E2
interaction capability. Overall, the two-hybrid results suggest
that the reduced cooperative DNA binding by the HR1 and

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Fig. 3. (A) Diagram of the BPV-1 E1DBD. Three hydrophilic regions HR1, HR2 and HR3 are indicated in grey. The dark grey
blocks indicate conserved regions among the papillomaviruses. The light grey block indicates a nonconserved domain. The
hatched block indicates an E1DBD C-terminal hydrophobic domain. Numbers shown above the diagram represent amino acid
boundaries of specific regions. Residues mutated in this study are indicated below. Underlined residues signify amino acids that
are conserved among the papillomavirus family. (B) E2 rescue of the E1DBD and the E1DBD mutant proteins. EMSAs were
performed with GSTE1DBD (80 ng) or mutant GSTE1DBD (80 ng) and E2 (60 ng). Proteins were incubated with 32P-labelled
E1BE/BS12 probe (substrate B) for 30 min at 25 °C. Samples were analysed by 8% TBE–PAGE. (C) Quantification of binding
results from (B). The amounts of E1–DNA and E1–E2–DNA complexes were quantified using a Molecular Dynamics
PhosphorImager. Amounts are expressed as percentage binding relative to wild-type.
HR3 mutants was not a result of a complete loss of E2 interaction.

HR1 and HR3 mutants lack E2TAD binding ability

Based on the previous experiment, none of the E1DBD mutants was completely defective for E2 interaction. However, as E1N interacts with both the E2TAD and the E2DBD (Fig. 2A, C), it is possible that interaction with one of the E2 domains could result in the positive response seen in Fig. 4(A), thus masking a defect in interaction with the other E2 domain. Consequently, all E1DBD mutations were tested in the yeast two-hybrid assay for separate interaction with the E2TAD and the E2DBD domains (Fig. 4B). As in Fig. 2, these assays were repeated at least three times with separate co-transformants, so although stimulation values were weak, all were highly reproducible. All the E1DBD mutant constructs showed stimulation by E2DBD, except the T187A mutant. In contrast, E1N with mutations at the conserved amino acids within the HR1 and HR3 domains, as well as residue 267, exhibited no stimulation by E2TAD. These data indicate that the E1DBD amino acids involved in E2TAD and E2DBD interaction are not the same, since only the T187A mutant was impaired for both interactions, while four other mutants were defective for E2TAD interaction but still functional for E2DBD binding.

Furthermore, since three of the four HR1/HR3 mutants with reduced cooperative binding still interacted with the E2DBD, it appears that reduced rescue by E2 reflects the intrinsic binding defects of the E1 mutants rather than defective E1–E2 interaction.

E2–E1 cooperative binding is not sufficient for in vivo replication

To relate the biochemical properties of the HR1 and HR3 domains to biological activity, the E1DBD mutations were transferred to full-length E1 to evaluate replication capacity using an in vivo triple plasmid transient replication assay (Fig. 5). All mutants were able to support replication of pBOR at levels comparable to those seen for wild-type E1, except for the T187A, K241A and K279A mutants. Surprisingly, the K186A mutant replicated quite well, even though its cooperative E2 binding in vitro was only marginally better than the T187A mutant (Fig. 3). Conversely, the K279A mutant exhibited wild-type origin binding and E2 cooperativity, yet was completely defective for in vivo replication. Consequently, it appears that while cooperative E1–E2 binding is required for in vivo replication capacity, it is not necessarily sufficient. All replication defective mutants were assayed by Western blot and found to express E1 at least as well as the wild-type, ensuring

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**Fig. 4. In vivo yeast two-hybrid mutant E1N–E2 interaction.** As described in Fig. 2, GAL-4 BD–E1N mutants were expressed along with GAL-4 AD–E2, –E2TAD or –E2DBD fusion proteins in SFY526 yeast cells. Clones were assayed at least three times with separately transformed colonies. β-galactosidase units were determined and converted to fold stimulation as explained in Methods. The horizontal line designates a fold stimulation of one, such that anything above the line is considered a positive interaction. (A) E1N–E2 yeast two-hybrid interaction. (B) E1N–E2TAD and E1N–E2DBD yeast two-hybrid interaction.
that the lack of replicative ability was not due to a lack of protein expression (data not shown).

No cooperative binding between E1DBD and E2 bound to BS11

As shown in Fig. 3(B), the E1DBD is sufficient for cooperative binding when E2 is bound to a substrate containing the authentic E1BE and E2BS12 (designated substrate B in this study). It has been shown previously that full-length E1 and E2 can also cooperatively interact when E2 is bound upstream to E2BS11 (Gillette & Borowiec, 1998). Since E2BS11 and E2BS12 differ both in relative affinity for E2 and in spacing with regard to the E1BE, it is unclear if E1 interaction with E2 bound to these sites involves the same regions of E1. We were interested primarily in determining if the isolated E1DBD was sufficient for mediating cooperative binding with upstream E2. To investigate this, we tested the E1DBD for cooperative binding with E2 on substrate B versus a substrate containing both the E2BS11 and the E1BE (designated substrate A). In Fig. 6, low concentrations of the E1DBD were used to emphasize cooperative binding, and the E1DBD complexes in the absence of E2 (Fig. 6, lanes 1 and 3) were difficult to detect; at higher concentrations, the wild-type E1DBD bound to both substrates equally well in the absence of E2 (data not shown). In the presence of E2, cooperative binding was observed on substrate B (Fig. 6, lane 2), but not substrate A (Fig. 6, lane 4). E2 itself bound more effectively to substrate A than substrate B, as expected, since E2BS11 is a higher affinity site than E1BS12. This assay was also repeated using the larger GSTE1N protein with the same results (data not shown). These results indicate that the interaction between E1 and E2 is different depending on which E2BS is occupied. More specifically, it indicates that while the E1DBD is sufficient for cooperative binding with E2 bound to BS12, the E1DBD (or E1N) is neither involved nor sufficient for interaction with E2 bound to BS11. These results are consistent with those of Chen & Stenlund (1998), which showed that spacing between the E1BS and E2BS12 was critical for cooperative interaction between the E1DBD and the E2DBD. The increased spacing between E2BS11 and E1BS probably precludes an effective interaction between the E2DBD and the E1DBD, suggesting that the cooperativity seen with the full-length proteins bound to these sites is mediated via sequences outside the E1DBD.
Fig. 6. E1DBD–E2 cooperative binding on both substrate A and substrate B. EMSA was performed using GSTE1DBD (15 ng), E2 (10 ng) and either 32P-labelled substrate A or substrate B. Proteins were incubated with either substrate A or substrate B for 30 min at 25°C and analysed by 8% TBE–PAGE. Lanes 1 and 3 contain GSTE1DBD protein only. Lanes 2 and 4 contain both GSTE1DBD and E2 protein.

Discussion

BPV-1 protein E1 is the initiator of replication, interacting with viral protein E2 as well as numerous cellular proteins. These protein–protein interactions, along with the intrinsic ATPase and helicase activity of E1, are vital for the replication of the viral genome. Within the literature, there are conflicting reports on the number and location of the E1–E2 interactions. Thorner et al. (1993) first showed an E1Tad interaction with full-length E2 in an immunoprecipitation assay, but only at 30°C. Later studies showed that smaller N-terminal E1 domains can also interact with E2 by immunoprecipitation, as well as yeast two-hybrid and GST pulldown assays (Benson & Howley, 1995; Leng et al., 1997; Moscufo et al., 1999). Both in vitro and in vivo data presented here support this E1N–E2 interaction. E1N (E1Tad) effectively interacted with full-length E2 and showed a weaker interaction with both the E2Tad and the E2DBD. Other reports have shown that E1E2 and E1 DBD are not sufficient for an interaction with E2 (Benson & Howley, 1995; Moscufo et al., 1999). This is consistent with our data showing that residues within HR1 (residues 179–192) affect the interaction of both the E2Tad and the E2DBD and it is likely that E1 polypeptides lacking this region would be completely defective for E2 interaction. In the current study, we also demonstrated an E1E2 interaction, verifying previous reports of an E1Tad and E1 DBD interaction with full-length E2 (Moscufo et al., 1999; Sarafi & McBride, 1995). Although further mapping was not carried out in our study, one could speculate that E1 amino acids 340–424 are vital for E1–E2 interaction, since Benson & Howley (1995) reported that E1424–605 could not interact with E2.

Our present study defined further the E1N–E2 interaction by demonstrating that the N-terminal E1 domain is sufficient for interaction with the E2Tad as well as the E2DBD. Our E1N–E2Tad results correlate well with an earlier study by Benson & Howley (1995), which showed an E1 N-terminal domain interaction with E2Tad. However, several previous studies using immunoprecipitation or GST pulldown assays were unable to detect either a full-length E1–E2DBD interaction or an E1N–E2DBD interaction (Benson & Howley, 1995; Mohr et al., 1990; Moscufo et al., 1999). The only previous reports of an E1N–E2DBD interaction have been in assays using a DNA probe containing either the E1BE or the E1BE and the E2BS (Chen & Stenlund, 1998, 2000; Gillitzer et al., 2000). Due to the apparently weak nature of this interaction, it may be that it is often difficult to detect E1–E2DBD complexes in the absence of DNA. Nevertheless, we have now confirmed this interaction directly in both yeast two-hybrid and GST pulldown assays. Therefore, our combined results clarify some of the previous discrepancies and define three distinct E1–E2 interactions: E1C–E2, E1N–E2Tad and E1N–E2DBD.

Using a series of substitution mutations in the E1DBD, we were able to evaluate the contribution of this region to E2Tad and E2DBD interaction and replication function (summarized in Table 1). Mutants with mutations at conserved residues in HR1 (K183, K186 and T187) and HR3 (K241), as well as at residue K287, were unable to bind to the E2Tad. From the E1DBD crystal structure (Enemark et al., 2000), these five amino acids form a confluent accessible surface that could be available for interaction with the E2Tad. However, it does not appear that the interaction defined by these mutations is required for viral genome replication, as three of the five mutants are wide-type for DNA replication activity. Also, it seems unlikely that the E2Tad could be interacting with this E1 region to facilitate replication, since this region is apparently involved in direct contact with origin DNA (Enemark et al., 2000). Using a different set of mutants, the same approximate region of the E1DBD was shown recently to be required for mediating E1 modulation of E2 transcriptional activity (Parker et al., 2000). Since the E1 transcriptional effect requires the E1BS (Parker et al., 2000), there would be no conflict between DNA binding and E2Tad binding in this context. Taken together, our results and those of Parker et al. (2000) strongly suggest that the E1DBD–E2Tad interaction is relevant for transcription, rather than playing a role in replication. Therefore, the E1–E2Tad requirement for replication (Berg & Stenlund, 1997; Chen & Stenlund, 1998) is probably mediated through sequences downstream of the E1DBD in the C-terminal region of E1.
Table 1. E2DBD mutant phenotypes

<table>
<thead>
<tr>
<th>Mutant</th>
<th>In vitro binding assay</th>
<th>Yeast two-hybrid assay</th>
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<tbody>
<tr>
<td></td>
<td>DNA binding</td>
<td>E2 rescue</td>
</tr>
<tr>
<td>K157A</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>K160A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>K187A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>T188A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K214A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K218A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K279A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wild-type</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

While five mutations were capable of abrogating the E1DBD–E2TAD interaction, only mutation of the E1 residue T187 abolished E2DBD interaction as well. The lack of exact coincidence between mutations affecting E2TAD versus E2DBD binding indicates that the regions of the E1DBD required for these interactions are different, although they may overlap somewhat. Interestingly, the T187A mutant was the only HR1 mutant that was unable to support replication and was the most defective for cooperative origin binding in vitro. It has been shown previously that cooperative origin binding is mediated through E2DBD–E1DBD interaction (Chen & Stenlund, 1998). Consequently, the abrogation of the E1DBD–E2DBD interaction by the T187A mutation may prevent effective formation of the initiation complex in vitro and account for the complete replication defect of this mutant. In contrast, the other HR1 mutants all exhibited higher levels of cooperative binding and were capable of normal levels of replication. Even the K186A mutant, which was only slightly more proficient than the T187A mutant for cooperative binding, apparently was beyond a threshold required for efficient replication. This critical E1DBD–E2DBD interaction can apparently only be mediated through E2 bound to E2BS12, as no cooperative binding was noted at the E1DBD (or E1N) when E2 was bound solely to E2BS11 (Fig. 6). These results are consistent with an earlier study that showed that cooperative interaction of the E1DBD and E2 required close spacing between E1BS and E2BS12 (Chen & Stenlund, 1998). The increased distance between E2BS11 and E1BS apparently does not allow effective interaction of E2 with the E1DBD.

The explanation for the replication defects of the K214A and K279A mutants is less clear. The K214A mutant interacted with the E2DBD and exhibited more cooperative origin binding activity in vitro than the K186A mutant; total inability to form the origin initiation complex seems unlikely. However, this mutation could clearly affect some more subtle aspect of initiation complex assembly or could affect post-assembly processes, such as DNA unwinding (Gillette et al., 1994) and interaction with host cell proteins. Likewise, the K279A mutant was not impaired in any tested function, yet was unable to replicate. Based on a hydropathy plot of the E1DBD (Gonzalez et al., 2000), amino acid 279 falls within a 12 amino acid hydrophobic sequence (aa 272–283) (Fig. 3A). Another mutation in this hydrophobic region, L275A, has the same phenotype and is functional for origin recognition and E2 cooperative binding, but is extremely impaired for replication (Thorner et al., 1993). From the crystal structure (Enemark et al., 2000), this hydrophobic region contains the α-helix 5 and, consistent with the wild-type DNA binding properties of the K279A mutant, does not appear to contribute directly to the DNA contact region formed by HR1 and HR3. Consequently, while their precise role in replication function remains obscure, mutants L275A and K279A appear to define a new functional subregion for the E1DBD that is critical for replication but does not directly involve origin recognition activity.

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References


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