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Separation of the transcriptional activation and replication functions of the bovine papillomavirus-1 E2 protein

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Replication of bovine papillomavirus-1 (BPV-1) DNA requires two viral gene products, the E1 protein and the full-length E2 protein. The 48 kDa E2 protein is a site-specific DNA-binding protein that binds to several sites which lie adjacent to the BPV-1 origin of replication. The 85 amino acid C-terminal domain contains the specific DNA binding and dimerization properties of the protein. The ~200 amino acid N-terminal domain is crucial for transcriptional activation. Both of these domains are highly conserved among different papillomaviruses. An internal hinge region separates the two functional domains. The region varies in amino acid sequence and length among the E2 proteins of different papillomaviruses. A series of mutations were constructed within the E2 open reading frame which delete various regions of the conserved DNA binding and transactivation domains as well as the internal hinge region. Two mutated E2 proteins that lack portions of the conserved DNA-binding domain but which support DNA replication were identified using transient replication assays. These mutated E2 proteins were unable to function as transcriptional activators. Conversely, two E2 proteins containing large deletions of the hinge region were able to activate transcription, but were defective for replication. Thus, the replication and transactivation functions of the E2 protein are separable.

Key words: DNA replication/papillomavirus/transcriptional activation

Introduction

The papillomaviruses are small, double-stranded circular DNA viruses which induce benign squamous epithelial tumors in a variety of vertebrate hosts including humans. In recent years papillomaviruses have attracted considerable attention because of their association with human anogenital carcinomas. However, the interaction between certain papillomaviruses and their host cells also provides an important model for the study of regulated DNA replication in mammalian cells. In transformed cells, the bovine papillomavirus type 1 (BPV-1) genome is maintained as a stable multi-copy nuclear plasmid which apparently replicates in synchrony with the host DNA (Law *et al.*, 1981). This highly regulated DNA replication contrasts markedly with the run-away viral DNA replication seen in lytic mammalian viruses such as Simian virus 40 (SV40) and adenovirus. Although the SV40 and adenovirus models have contributed significantly to our understanding of the initiation of replication, DNA chain elongation and many of the viral and cellular proteins involved in replication, BPV-1 may provide an important model for studying mechanisms involved in regulated DNA replication in which templates are replicated only once per cell cycle.

Only two viral proteins, E1 and E2, are required for BPV-1 DNA replication (Ustav and Stenlund, 1991). The 68 kDa E1 phosphoprotein shares amino acid homology with SV40 large T antigen which is the only virally encoded protein required for SV40 DNA replication. E1 shares a number of biochemical characteristics with T antigen including the ability to bind ATP (Sun *et al.*, 1990) and to bind specifically its cognate origin of replication (Ustav *et al.*, 1991; Wilson and Ludes-Meyers, 1991; Yang *et al.*, 1991). The E2 gene encodes several major regulatory proteins of BPV-1. Three proteins which share a C-terminal sequence-specific DNA-binding domain are encoded by the E2 open reading frame (ORF) (see Figure 1; Hubbert *et al.*, 1988; Lambert *et al.*, 1989). The E2 polypeptides bind as dimers to a 12 bp palindromic sequence, $ACCN_6GGT$ (Androphy *et al.*, 1987; Dostatni *et al.*, 1988; McBride *et al.*, 1989b; Moskaluk and Bastia, 1987, 1989). The 48 kDa full-length E2 protein is a strong transcriptional transactivator (Spalholz *et al.*, 1985) which interacts with specific transcriptional enhancer elements within the BPV-1 long control region (LCR) resulting in increased transcription from viral promoters P_{7940}, P_{89}, P_{2443} and P_{3080} (Haugen *et al.*, 1987; Spalholz *et al.*, 1987; Li *et al.*, 1991; Prakash *et al.*, 1988; Hermonat *et al.*, 1988). These transcriptional enhancer sequences are comprised of multiple copies of the DNA-binding motif which co-operate synergistically *in vivo* to form highly responsive enhancer elements (Spalholz *et al.*, 1988). The two shorter proteins act as transcriptional repressors by inhibiting E2-mediated transactivation (Choe *et al.*, 1989; Lambert *et al.*, 1987, 1989). The full-length E2 polypeptide is required for BPV-1 DNA replication in stably transformed cells (Rabson *et al.*, 1986; Di Maio and Settleman, 1988) and transient replication assays (Ustav and Stenlund, 1991). Although a BPV-1 mutant unable to express the E2-TR repressor protein replicates to a high copy number (Lambert *et al.*, 1990; Riese *et al.*, 1990), the role of the E2 repressors in replication is not clear.

The origin of replication in many viral and eukaryotic genomes consists of two important components. The core origin contains *cis* sequences which are required for initiation of replication. The second component consists of promoter or enhancer elements which augment replication *in vivo*. These enhancer elements often function as modulators of transcription as well as replication (DePamphilis, 1988). *Trans*-acting viral or cellular transcription factors also play an important role in replication though it is unclear how these factors actually modulate replication. One hypothesis suggests that transcriptional activators might direct replication factors to the DNA template or stabilize the interaction of replication factors with the template. Cheng

and Kelly have proposed an alternative model in which transcriptional activators function indirectly by binding DNA and disrupting the assembly of chromatin structure thus allowing initiation factors greater access to the origin (Cheng and Kelly, 1989; Cheng *et al.*, 1992).

There has been much speculation regarding the role that E2 plays in BPV DNA replication. The full-length BPV-1 E2 protein could indirectly affect replication by enhancing transcription of either viral or cellular genes necessary for replication. However, when the E1 and E2 proteins are expressed from heterologous promoters on separate expression vectors, E2 is still absolutely required for DNA replication (Ustav and Stenlund, 1991). Additional studies demonstrating transient BPV-1 DNA replication in cell-free extracts indicate that E2-mediated transactivation of cellular proteins is not required (Yang *et al.*, 1991). Alternatively, as described above, E2 could bind viral DNA and recruit replication factors to the origin or inhibit formation of nucleoprotein complexes. However, the BPV-1 minimal origin of replication does not require the adjacent E2-binding sites (Ustav *et al.*, 1991; Yang *et al.*, 1991). The full-length E2 protein forms a complex with the E1 protein and stimulates binding of E1 to the origin (Mohr *et al.*, 1990). This result suggests that protein−protein interactions between the E2 protein and other replication factors may be important for DNA replication.

The full-length E2 proteins of various papillomaviruses share two relatively conserved domains, a sequence-specific DNA-binding region which is encoded in the C-terminal 85 amino acids and a transactivating region encoded by an ~200 amino acid N-terminal domain (Giri and Yaniv, 1988; Haugen *et al.*, 1988; McBride *et al.*, 1988, 1989b). These two domains are separated by a non-conserved internal hinge region which varies in length and amino acid sequence. The N terminus of the E2 protein is likely to be required for DNA replication since the E2 repressor proteins, which share only the DNA-binding domain with the full-length protein, do not support viral DNA replication and are unable to complex with the E1 protein (Mohr *et al.*, 1990; Blitz and Laimins, 1991; Ustav and Stenlund, 1991).

To determine whether the transcriptional activation function of the full-length E2 protein could be separated from its replication function, the transactivation and replication properties of a number of mutant E2 proteins were analyzed. Mutations were constructed within the E2 ORF which deleted various regions of the conserved DNA-binding and transactivation domains and the hinge region. Two truncated E2 proteins that did not contain the DNA-binding domain but which supported DNA replication were identified using transient DNA replication assays. Thus, DNA binding by E2 is important for transcriptional regulation but is not absolutely essential for replication. Conversely, two mutated E2 proteins containing large deletions of the hinge region were able to activate transcription but were defective for replication suggesting that the transactivation function of the E2 protein is not sufficient for replication.

Results

The transactivation function of the E2 proteins

In many eukaryotic systems, transcription factors are frequently involved in replication functions. To determine whether the transcriptional activation function of the full-length E2 protein could be separated from its replication function, the transactivation and replication properties of a number of mutated E2 proteins were analyzed. The full-length E2 polypeptide consists of an N-terminal transactivation domain linked to a C-terminal DNA-binding/dimerization domain by a hinge region. Mutations which consisted of N-terminal truncations, C-terminal truncations, in-frame internal deletions and the replacement of internal regions by synthetic linker insertions were constructed in a plasmid which expresses the cDNA for the full-length E2 protein from the SV40 early promoter (Figure 1) (Yang *et al.*, 1985a). The C59-E2 mutants were assayed for their ability to transactivate an E2-responsive reporter plasmid, p964, which expresses the CAT gene from the enhancer-deleted SV40 early promoter linked to the E2-responsive element, $E2RE_1$ (Spalholz *et al.*, 1987).

Many of the C59-E2 mutants have been analyzed previously for transactivation function (McBride *et al.*, 1989b) and our findings agreed with the previous studies. Deletions of the hinge region between amino acids (aa) 194 and 310 had little effect on transactivation of the $E2RE_1$-CAT plasmid whereas larger deletions extending into the N terminus diminished transcriptional activation. E2 proteins containing deletions of the first 15 or 52 aa or of aa 157−283 or 91−162 were unable to transactivate the $E2RE_1$-CAT plasmid. We have previously shown that those constructs that were unable to activate transcription were able to repress E2-mediated transactivation, demonstrating that these mutant C59-E2 constructs expressed stable proteins (McBride *et al.*, 1989b). In this study we assayed some additional E2 proteins containing internal in-frame deletions and found that $E2_{\Delta 213-218}$, $E2_{\Delta 220-309}$ and $E2_{\Delta 285-309}$ transactivated as well as or better than the wild-type E2 protein. Proteins in which the hinge region had been replaced with the unrelated RNGS peptide activated transcription better than wild-type E2. The 20 aa RNGS epitope was derived from the lck tyrosine kinase (Veillette *et al.*, 1988). This epitope is not known to have a specific function though it is possible that insertion of this epitope helps maintain crucial spacing between the transactivation and DNA-binding domains or makes the mutated E2 proteins more stable. As expected, E2 polypeptides that contained truncations of the C-terminal DNA-binding region (Figure 1A) were unable to activate transcription (data not shown).

Analysis of transient replication supported by mutated E2 proteins

E2 proteins containing mutations in the DNA-binding, transactivation or hinge regions were analyzed for their ability to support transient DNA replication of a origin-containing plasmid. A 3.2 kb fragment of the BPV-1 genome containing sequences from the entire LCR and coding sequences for the two late proteins, L1 and L2 (nucleotides 4786−83) was used as a replicon. The 3.2 kb fragment was cleaved from the prokaryotic vector sequences and was recircularized prior to electroporation. C127 cells were electroporated with 2 μg of BPV-1 replicon DNA, 5 μg of E1 expression plasmid pCGEag-1235 and 5 μg of either wild-type or mutant C59-E2 DNAs. Low mol. wt DNA was extracted from cells after 5 days, digested with *Dpn*I and *Hin*dIII and analyzed by Southern blot hybridization with a BPV-1 DNA probe (p142-6). *Dpn*I cuts methylated (non-replicated) input DNA whereas the replicated non-methylated

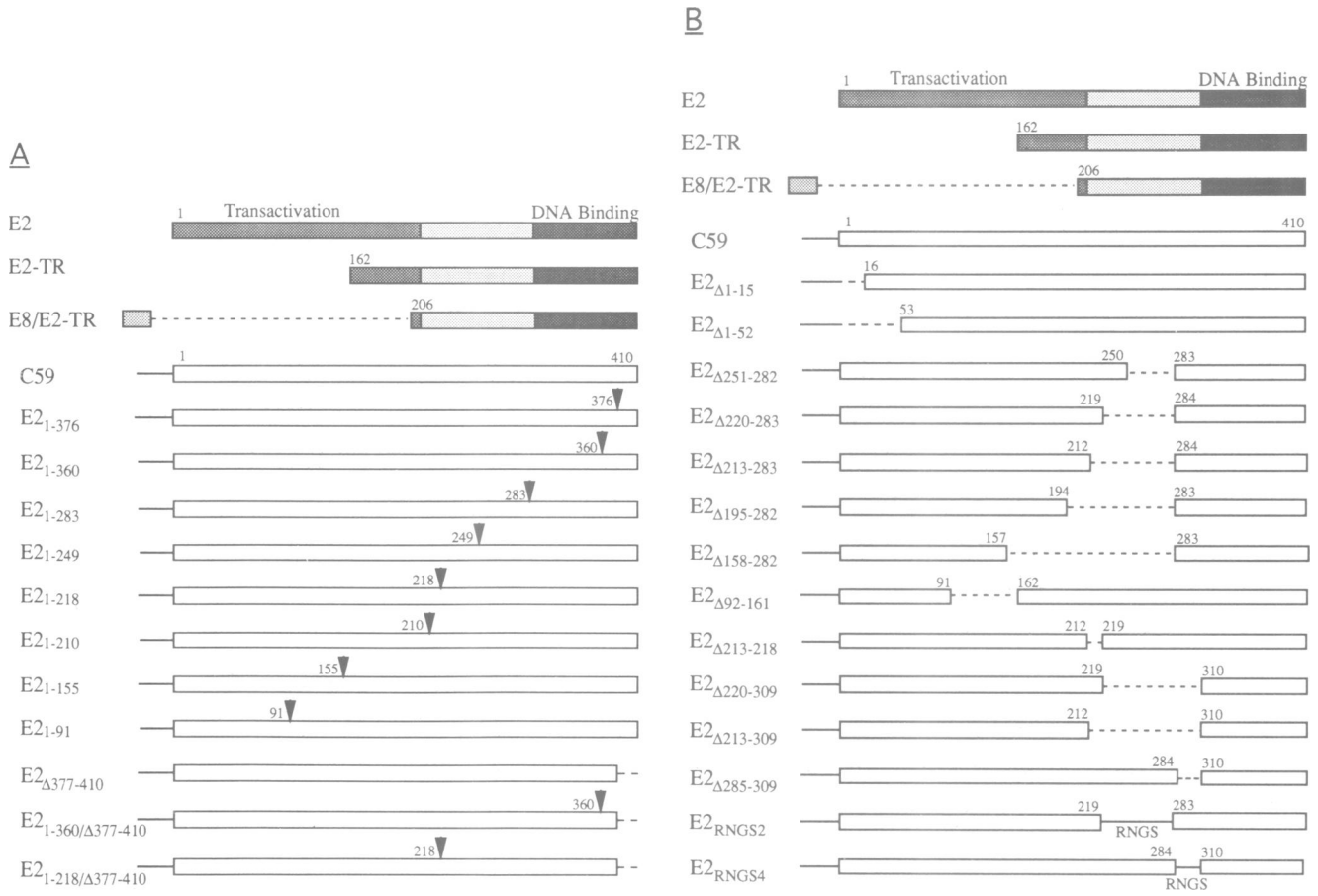

Fig. 1. Diagram of wild-type E2 and mutated E2 proteins. The 420 aa full-length E2 polypeptide contains an N-terminal domain of ~200 aa which is required for transcriptional activation. The C-terminal 85 aa comprise the DNA-binding and dimerization domain. The E2-TR and E8/E2-TR repressor proteins share the C-terminal domain. The structure of each mutated E2 protein is represented in the diagram below the wild-type E2 proteins. **A.** Proteins were expressed from templates which contained translation termination linkers (TTLs) inserted at various positions within the E2 gene indicated by the arrow heads. $E2_{\Delta 377-410}$ contains a deletion within the DNA-binding domain. $E2_{1-360/\Delta 377-410}$ and $E2_{1-218/\Delta 377-410}$ contain TTLs at aa 360 and 218 respectively, and contain a deletion between aa 377–410. All of the C-terminally truncated proteins were unable to activate transcription of the $E2RE_1$-CAT plasmid. **B.** Mutated E2 proteins are designated according to those amino acids that have been deleted. $E2_{RNGS2}$ and $E2_{RNGS4}$ contain a synthetic epitope, RNGS, which has been inserted between aa 219–283 or aa 284–310 respectively. The $E2_{RNGS2}$ and $E2_{RNGS4}$ proteins were expressed in a C59 vector which contained a Kozak initiation consensus sequence (see Materials and methods). With the exception of $E2_{\Delta 1-15}$, $E2_{\Delta 1-52}$, $E2_{\Delta 158-282}$ and $E2_{\Delta 92-161}$, all of the proteins shown were able to activate transcription with activities which were at least 30% of the wild-type E2 protein.

DNA is resistant to *Dpn*I cleavage and migrates as a 3.2 kb linear DNA fragment due to a single cleavage by *Hin*dIII. Each experiment included two negative control lanes in which cells were transfected with only BPV-1 replicon DNA or replicon plus pCGEag-1235 DNAs.

The results demonstrated that the conserved N-terminal domain of the E2 polypeptide, present in the full-length protein but not in the E2-TR and E8/E2-TR repressor forms of E2, was important for replication. E2 polypeptides with deletions within the first 194 aa were unable to support DNA replication in the transient assays (Figure 2B, proteins $E2_{\Delta 1-15}$, $E2_{\Delta 1-52}$, $E2_{\Delta 92-161}$). As previously mentioned, the N-terminal domain was also required for transactivation (Figure 1).

A series of E2 proteins with internal in-frame deletions which removed portions of the hinge region and linked the N-terminal domain to the intact C-terminal DNA-binding domain were assayed for DNA replication. Deletion of aa 195–282 reduced transient replication efficiency, but did not abolish the replication function (Figure 2B, lanes 6–9). However, proteins with deletions of aa 220–309 and 213–309 ($E2_{\Delta 220-309}$ and $E2_{\Delta 213-309}$) were unable to support BPV-1 DNA replication. Although $E2_{\Delta 220-309}$ and $E2_{\Delta 213-309}$ could not support DNA replication, both were able to activate transcription at least as well as the wild-type E2 protein. These results suggested that the 27 aa sequence between aa 283–310 might be important for replication. This region contains the major phosphorylation sites of the E2 protein (McBride *et al.*, 1989a). BPV-1 viral genomes which contain a point mutation in E2 that alters the phosphorylation site at aa 301 replicate to a high copy number (McBride and Howley, 1991) suggesting that this region of the E2 protein could have a direct role in DNA replication. However, an E2 protein containing a deletion of aa 285–309 functioned normally in transient replication assays (Figure 4, lane 8). Thus, this phosphorylation region does not appear to be critical for transient replication of a subgenomic BPV-1 DNA fragment.

To test whether there was an absolute requirement for any specific sequences in the hinge region for DNA replication, two deletion mutants were constructed in which each half of the hinge region was replaced with a 20 aa synthetic

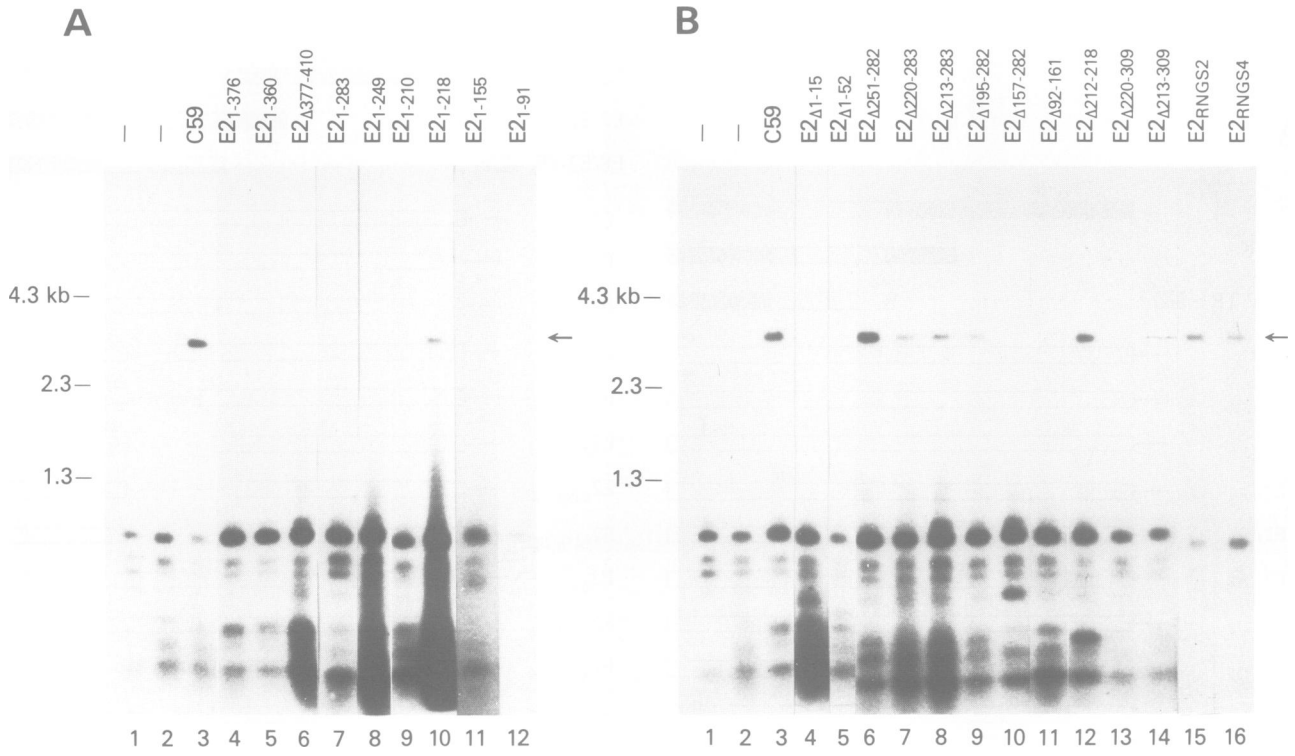

Fig. 2. Transient replication properties of the mutated E2 proteins in C127 cells. Controls included cells electroporated with 2 μg of a 3.2 kb fragment which contains the origin of replication (p716) alone (lane 1) or together with 5 μg of pCGEag-1235 which expresses the E1 protein (lane 2). In all other lanes, cells were electroporated with p716, pCGEag-1235 and either 5 μg of C59, which expresses wild-type E2, or 5 μg of mutated C59 DNA. Cells were harvested 5 days after electroporation and low mol. wt DNA was digested with *Hin*dIII and *Dpn*I. The *Dpn*I-resistant material (replicated DNA) formed a 3.2 kb band which was detected using a BPV-1 DNA probe (p142-6). **Panel A** depicts transient replication properties of the mutated E2 proteins which contain TTLs at various positions in the E2 genome. **Panel B** demonstrates replication properties of mutated E2 proteins containing in-frame deletions.

epitope, RNGS. In $E2_{kzRNGS2}$ the epitope replaced aa 219–283 and in $E2_{kzRNGS4}$, the epitope was inserted between aa 283 and 310. As mentioned above, mutant E2 proteins which contained deletions similar to those found in the RNGS mutants ($E2_{\Delta 219-283}$ and $E2_{\Delta 285-309}$), supported replication (Figure 2B, lane 8 and Figure 4, lane 8). The RNGS mutants also functioned like the wild-type E2 protein in replication assays. Thus, no particular sequence in the hinge appeared to be absolutely required for replication though the fact that a deletion that removed the entire hinge region ($E2_{\Delta 220-309}$) resulted in an E2 protein unable to support DNA replication suggested that an internal region might be important for maintaining the replication function.

Separation of the DNA-binding and replication functions of the E2 protein

The DNA-binding and dimerization functions of the BPV-1 E2 protein are encoded by the C-terminal 85 aa and this region is required for transactivation by the E2 protein (Figure 1; Androphy *et al.*, 1987; Moskaluk and Bastia, 1987, 1989; Dostatni *et al.*, 1988; McBride *et al.*, 1989b). To determine whether the DNA-binding region is required for transient replication, E2 ORF mutations were constructed which contained translation termination linkers (TTLs) throughout the E2 protein. The results from this study demonstrated that the majority of these C-terminally truncated E2 proteins were also unable to support replication in transient assays. However, $E2_{1-218}$, which contains a TTL after aa 218 in the hinge region maintained replication function (Figure 2A, lane 10 and Figure 3, lane 6). The mutant $E2_{1-360}$, which is missing the C-terminal 50 aa of the E2 protein, also supported replication though at a reduced efficiency compared with wild-type C59-E2. Replication of $E2_{1-360}$ was best detected with a labeled LCR probe (Figure 3, lane 9). It is noteworthy that C-terminally truncated proteins which contained TTLs several amino acids upstream or downstream from aa 218 or 360 were unable to support DNA replication.

The possibility that the $E2_{1-218}$ and $E2_{1-360}$ mutations might have induced new splice acceptor sites which created in-frame internal deletions similar to those described above was considered. To address this concern, the *Bcl*I fragment (aa 377–410) was deleted in both C59-$E2_{1-360}$ and C59-$E2_{1-218}$. As shown in Figure 3, the *Bcl*I deletion alone created a mutant E2 protein ($E2_{\Delta 377-410}$) which was unable to support replication. However, the double mutants $E2_{1-218/\Delta 377-410}$ and $E2_{1-360/\Delta 377-410}$ could support replication. Since the *Bcl*I deletion encodes a deletion of the C-terminal 33 aa of the E2 protein which fuses the short E5 protein to the truncated E2 protein, there was a slight possibility that the E5 protein could complement the dimerization functions of the mutant E2 protein. Therefore, an additional mutation was created in $E2_{1-218/\Delta 377-410}$ which inserted a second TTL at the site of the deletion. This mutant E2 protein could not be fused to E5 yet it continued to support transient DNA replication (data not shown).

To ensure that the *Dpn*I-resistant band seen with these mutants contained replicated DNA and was not partially

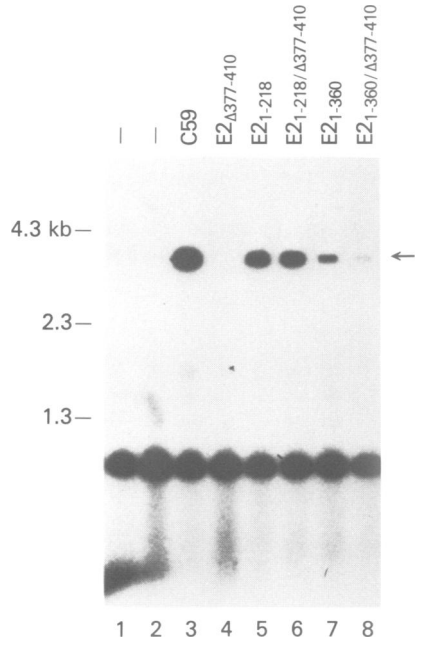

Fig. 3. The E2 DNA-binding domain is not required for replication. Lanes 1 and 2 contain DNA extracted from cells transfected with replicon DNA alone (lane 1), or replicon DNA and pCGEag-1235 (lane 2). Lanes 3–8 contain DNA extracted from cells co-transfected with the replicon, pCGEag-1235 and either wild-type C59 or mutated C59 DNA. Cells were transfected with C59 DNAs containing a TTL at aa 218 or aa 360 (lanes 5 and 7) or with C59 DNAs containing these TTLs and an additional deletion within the C-terminal domain, $E2_{1-218/\Delta377-410}$ and $E2_{1-360/\Delta377-410}$ (lanes 6 and 8). The replicated DNA was detected with a probe from the LCR region of BPV-1 (nt 6958–6936).

*Dpn*I-resistant input DNA, low mol. wt DNA samples were also digested with *Mbo*I and *Hin*dIII. *Mbo*I cleaves only replicated (non-methylated) DNA. The expected 769 bp fragment of replicated DNA was detected in cells co-transfected with $E2_{1-218}$ and $E2_{1-360}$ whereas mutant E2 proteins which did not support replication ($E2_{1-376}$) did not show this DNA fragment (Figure 4). These results demonstrate that the DNA-binding domain of the E2 protein is not absolutely required for replication activity.

This study demonstrates that the replication and transcriptional regulatory activities of the E2 protein are functionally distinct. The replication function of the full-length E2 protein is not absolutely dependent on the ability of this protein to bind DNA. The $E2_{1-218}$ and $E2_{1-360}$ proteins and the corresponding double mutants, $E2_{1-218/\Delta377-410}$ and $E2_{1-360/\Delta377-410}$ lack the conserved DNA-binding domain; these mutants did not function as transcriptional activators yet they did support transient DNA replication. Conversely, the $E2_{\Delta220-309}$ and $E2_{\Delta213-309}$ mutants were able to stimulate transcription better than the wild-type E2 protein, but were unable to function in transient replication assays. Therefore, the transactivation function of the E2 protein was not sufficient to support transient DNA replication.

As shown in Figure 5, each of the four mutated E2 proteins were able to function in either transactivation or replication assays suggesting that a stable protein product was expressed. However, the replication experiments were performed in rodent C127 cells and the transactivation experiments were performed in a primate cell line (CV-1). To address the question of differential protein stability between these cell lines, we assayed transactivation by this series of mutants (and $E2_{1-210}$, $E2_{1-249}$, $E2_{\Delta377-410}$, $E2_{\Delta220-283}$, $E2_{\Delta213-283}$) in C127 cells. These proteins had similar transcriptional activation properties in both C127 and CV-1 cells. In particular, $E2_{1-218}$ and $E2_{1-376}$ failed to transactivate the E2RE1-CAT construct in C127 cells. Additionally, $E2_{\Delta219-310}$ and $E2_{\Delta212-310}$ maintained the transactivation function in both cell lines (data not shown).

Discussion

Transcription factors frequently play a significant role in replication. The initial step of SV40 DNA replication requires binding of T antigen to the origin of replication (reviewed by Stillman, 1989); T antigen is also a regulator of viral transcription. Replication from the SV40 core origin of replication is stimulated by *cis*-acting auxiliary elements which have transcriptional enhancer and promoter functions (reviewed by DePamphilis, 1988). Similarly, the adenovirus-2 core origin contains both promoter and enhancer motifs and binding of an initiation complex to the ori core is stimulated by the cellular protein nuclear factor-1 (NF-1). NF-1 is indistinguishable from cellular transcription factor (CTF) which augments transcription from multiple promoters (DePamphilis, 1988). Similar to DNA replication in papillomaviruses, Epstein–Barr virus (EBV) replicates as a stable multi-copy nuclear plasmid. Two regions of the EBV genome act in *cis* to support plasmid replication in cells that express the EBV nuclear antigen-1 (EBNA-1). One component of the origin is made up of 20 copies of a 30 bp direct repeat which can function as a transcriptional enhancer that is stimulated in *trans* by EBNA-1 (Reisman and Sudgen, 1986). Thus *cis* elements and *trans* factors act together as important regulators of DNA replication and transcription. However, it has been difficult to separate the transcription and replication functions of these regulators so their exact role in DNA replication is unclear. In EBV, mutations in EBNA-1 which affect either one of these processes invariably affect the other (Yates and Camiolo, 1988). It is also important to consider that mutations which alter the transcription or replication properties of a virus *in vivo* may not have the same effect *in vitro*. In SV40, transcriptional enhancer sequences which flank the essential core origin stimulate DNA replication *in vivo* yet have no effect on SV40 replication in standard *in vitro* assays (Stillman *et al.*, 1985; DeLucia *et al.*, 1986; Li *et al.*, 1986; Smale and Tjian, 1986).

BPV-1 DNA replication has several unique features. Since two viral proteins, E1 and E2, are required for DNA replication it may be easier to separate regulation of transcription from replication. As mentioned previously, E1 binds specifically to a region of BPV-1 DNA surrounding the unique *Hpa*I site (Ustav *et al.*, 1991; Wilson and Ludes-Meyers, 1991). Although the E1-binding site is flanked by two E2-binding sites, they do not seem to be required for replication in transient assays (Ustav *et al.*, 1991; Yang *et al.*, 1991). However, E2 can form a complex with E1 and this complex binds more readily to DNA (Mohr *et al.*, 1990). Our results also indicate that DNA binding by E2 is not absolutely required for replication since certain mutants which lack portions of the C-terminal DNA-binding domain continue to support replication. This result also suggests that dimerization of the E2 protein is not necessary for replication

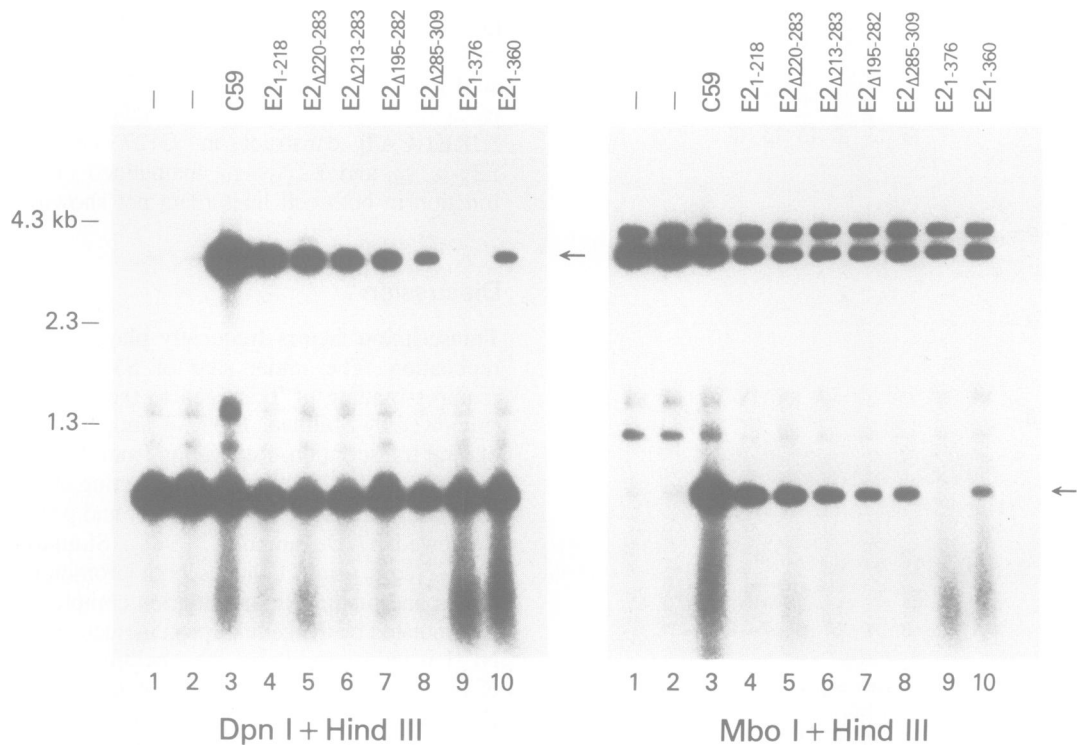

Fig. 4. Replicated BPV-1 DNA is *Dpn*I resistant and *Mbo*I sensitive. Transient replication assays were performed using mutated E2 expression vectors to determine whether *Dpn*I-resistant DNA represented undigested input replicon DNA. Low mol. wt DNA was harvested from transfected cells and digested with *Dpn*I and *Hin*dIII (left panel) or *Mbo*I and *Hin*dIII (right panel). Lane 1 from each panel contains DNA extracted from cells electroporated with p716 alone and lane 2 contains DNA from cells electroporated with p716 and pCGEag-1235. All other lanes contain DNA extracted from cells electroporated with replicon DNA, pCGEag-1235 and either wild-type C59 DNA or mutated C59 DNA. The replicated DNA was detected with a probe from the LCR region of BPV-1 (nt 6958–36). Replicated *Mbo*I-sensitive BPV-1 DNA ran as a 769 bp fragment as indicated by the arrow.

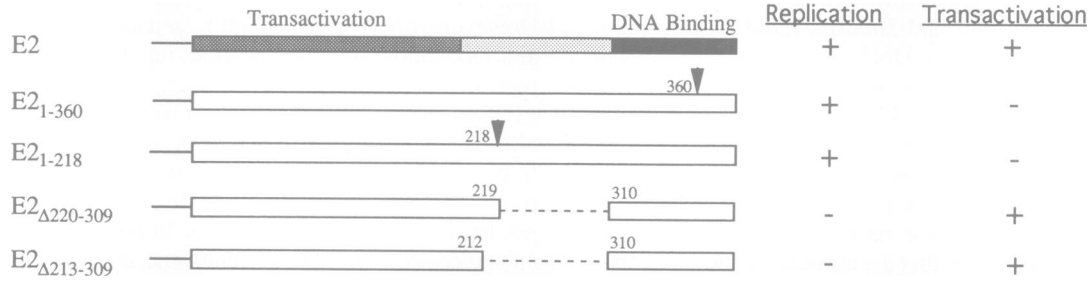

Fig. 5. Separation of the transactivation and replication properties of the E2 protein.

since dimerization is also mediated by the conserved C-terminal domain which has been deleted in two mutants that are replication competent. It is certainly possible though, that the DNA-binding and dimerization properties of the full-length E2 protein are required for replication of the entire viral genome *in vivo* or may affect the efficiency of viral DNA replication *in vivo*.

An unpredictable pattern of replication function was observed with the series of C-terminally truncated E2 proteins. Initially the possibility that the TTL inserted at the coding sequences for aa 219 and aa 360 might induce cryptic splice acceptor sites was considered. A second mutation in the C terminus which by itself destroyed DNA-binding, transactivation and replication functions was also added to these mutants. These double mutants could still support DNA replication suggesting that replication was an integral property of $E2_{1-218}$ and $E2_{1-360}$. However, TTLs inserted 21 bp upstream or 93 bp downstream from the TTL at aa 218 abolished the replication function. It is possible that these mutations influence the secondary structure of the E2 protein altering a crucial conformation required for complex formation with other replication factors. Studies have shown that E2 forms a complex with E1 and can stimulate the binding of E1 to the origin (Mohr *et al.*, 1990). The effects of these mutations on complex formation between the E2 and E1 proteins are under investigation. Alternatively, these mutations may affect stability of the E2 protein rendering the $E2_{1-218}$ and $E2_{1-360}$ proteins more stable than the other C-terminally truncated proteins. Using a recombinant SV40/BPV-1 vector, we have expressed $E2_{1-218}$, $E2_{1-360}$, $E2_{\Delta220-310}$, $E2_{\Delta213-309}$ and several of the other mutated E2 proteins in COS-1 cells. The mutated E2 proteins were of the expected mol. wts and stability of these mutant proteins was similar to that seen with wild-type E2 (data not shown).

These studies show that the transactivation function of the E2 protein is not sufficient for replication. Two proteins with in-frame deletions within the hinge region functioned as effective transactivators but were inactive in transient replication assays. This corroborates the finding that a VP16/E2 fusion protein which retains the site-specific transactivation properties of E2 was also unable to support replication (Ustav *et al.*, 1991). One possibility is that the transactivation and DNA-binding domains are linked so closely as to cause steric hindrance. For example, such a polypeptide may still be able to transactivate yet be unable to complex with the E1 protein. Though the exact mechanism by which E2 functions in DNA replication is still unclear our findings suggest that E2 has additional functions in replication which are independent of its function as a transactivator.

Materials and methods

Plasmid constructions

The plasmid used as a replicon, p716, was generated by synthesizing a DNA fragment from nucleotides 4786 to 83 using PCR. *Bam*HI recognition sites were incorporated into the ends of the fragment and used to clone the fragment into the *Bam*HI site of pTZ18R (United States Biochemicals). Before electroporation the BPV-1 fragment was cleaved from the vector and religated at a concentration of 5 μg/ml to favor intramolecular recircularization.

The E1 expression vector, pCGEag1235, was a generous gift from Dr Arne Stenlund (Ustav and Stenlund, 1991). The reporter plasmid, p964, in which the CAT gene is expressed from the enhancer-deleted SV40 early promoter linked to the E2-responsive element, $E2RE_1$ and the wild-type full-length BPV-1 genomic plasmid, p142-6 has been described previously (Sarver *et al.*, 1982; Spalholz *et al.*, 1987).

To generate $pTZE2_{sh}$, the *Bst*E2 to *Bam*HI fragment that encodes the E2 ORF was isolated from the E2 expression vector, C59, and was cloned into pTZ18R. In the series of C-terminally truncated E2 mutants a TTL with the sequence 5′ TTAGTTAACTAA-3′ (Phelps and Howley, 1987) was inserted at various sites within the E2 ORF in $pTZE2_{sh}$. The plasmids which expressed E2 polypeptides $E2_{1-91}$, $E2_{1-210}$, $E2_{1-218}$, $E2_{1-249}$ and $E2_{1-283}$ were constructed by inserting the TTL into the *Nco*I (nucleotide 2878), *Tth*31 (nucleotide 3238), *Dra*II (nucleotide 3259), *Stu*I (nucleotide 3353) and *Kpn*I (nucleotide 3460) sites respectively. The *Bst*E2 (nucleotide 2405) to *Bst*XI (nucleotide 3460) fragments from the mutants described above, which encoded the mutated E2 polypeptides, were then exchanged into the E2 expression vector, C59, which expresses a cDNA from the SV40 early promoter which encodes the full-length E2 protein (Yang *et al.*, 1985b). The plasmids which expressed E2 polypeptides $E2_{1-155}$, $E2_{1-360}$ and $E2_{1-376}$ were generated by inserting the TTL into *Ban*II (nucleotide 3077), *Pfl*MI (nucleotide 3683) and *Bcl*I (nucleotides 3737 and 3838) sites respectively, directly in C59. The plasmid C59 *Bcl*Δ was generated by deleting the *Bcl*I fragment between nucleotides 3737 and 3838 of BPV-1. This plasmid encodes a polypeptide that fuses 55 amino acids from the E5 ORF to the N-terminal 379 amino acids of E2. Plasmids C59-$E2_{1-219}$ and C59-$E2_{1-360}$ were further mutated by deleting the *Bcl*I fragment between nucleotides 3737–3838 to create C59–$E2_{1-218/\Delta 377-410}$ and C59-$E2_{1-360/\Delta 377-410}$.

The plasmid that expresses the N-terminally truncated E2 polypeptide, C59-$E2_{\Delta 1-15}$ and the plasmids containing E2 ORFs with internal in-frame deletions, C59-$E2_{\Delta 251-282}$, C59-$E2_{\Delta 213-283}$, C59-$E2_{\Delta 195-282}$, C59-$E2_{\Delta 92-161}$, C59-$E2_{\Delta 220-283}$ and C59-$E2_{\Delta 157-282}$ have been described previously (McBride *et al.*, 1989b). Synthetic oligonucleotides were used to generate in-frame deletions between convenient restriction sites in pTZ-$E2_{sh}$ and the resulting mutated E2 ORFs were exchanged into C59 to generate C59-$E2_{\Delta 212-218}$, C59-$E2_{\Delta 220-309}$, C59-$E2_{\Delta 213-309}$ and C59-$E2_{\Delta 285-309}$.

Plasmids $pTZE2_{kzRNGS2}$ and $pTZE2_{kzRNGS4}$ contain a 20 aa synthetic epitope between the *Dra*II (nt 3259) and *Kpn*I (nt 3460) sites or between the *Kpn*I (nt 3460) and *Sty*I (nt 3535) sites of the E2 polypeptide respectively, and have been described previously (McBride *et al.*, 1989a). These mutations were exchanged into C59 as described above to generate C59-$E2_{kzRNGS2}$ and C59-$E2_{kzRNGS4}$. pTZ-$E2_{kz}$ and C59-$E2_{kz}$ are very similar to pTZ-$E2_{sh}$ and C59, except that sequences from the *Bst*E2 (nt 2406) to *Sph*I (nt 2622) sites were replaced with synthetic oligonucleotides that provided a Kozak initiation consensus sequence (CCACCATG, Kozak, 1986) for the E2 ORF.

Transient replication assay

Transient replication assays were carried out as described by Ustav and Stenlund (1991). Briefly, mouse C127 cells were electroporated with 2 μg of replicon DNA, 5 μg of pCGEag-1235 and 5 μg of wild-type or mutant C59-E2 plasmid DNA. After 5 days cells were lysed and low mol. wt DNA was harvested. DNA samples were digested with *Dpn*I and *Hin*dIII or *Mbo*I and *Hin*dIII, separated on 0.8% agarose gels in TBE buffer and blotted to Nytran (Schleicher and Schuell). High specific activity ^{32}P-labeled probes were generated from p142-6 using a random primer kit (Boehringer Mannheim). Alternatively, a fragment from the LCR-containing nucleotides 6958–6936 was generated using PCR and was labeled using the random primer technique.

Transient expression assays

Monkey CV-1 cells were transfected using the calcium phosphate technique as previously described (Spalholz *et al.*, 1985). Briefly, duplicate 60 mm^2 dishes of CV-1 cells were transfected with 4 μg of the $E2RE_1$CAT plasmid (p964) and 0.3 μg of wild-type C59-E2 or mutant C59-E2 DNA. After 5 h cells were subjected to glycerol shock and grown for an additional 12 h in DMEM (Gibco–BRL) containing 3 mM sodium butyrate. Cell lysates were prepared after 48 h. CAT assays were carried out using equivalent quantities of protein from each dish and were performed in the linear range. Each plasmid was tested in two or more independent studies.

Cell culture

CV-1 and C127 cells were maintained in Dulbecco's MEM containing 10% heat inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco–BRL).

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