

Active domains of human papillomavirus type 11 E1 protein for origin replication

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Viral proteins E1 and E2 are essential for transient human papillomavirus (HPV) DNA replication. E1 is a multifunctional protein which can bind DNA and complex with E2, has ATPase and helicase activities, and interacts with DNA polymerase α -primase. E2 is a transactivator–repressor protein, playing an important role in replication and transcriptional regulation. A series of deletion mutants of HPV-11 E1 were constructed and tested in functional assays to define those domains of HPV-11 E1 which are important for binding to the origin DNA and E2. The domain of HPV-11 E1 involved in binding to the origin was located between aa 186 and 649, and

that for binding to E2 was between aa 346 and 649. Since E1 binds to the origin more efficiently in the presence of E2, we also mapped the DNA binding domain of E1 in the presence of E2, and found that when binding was enhanced, the region of E1 involved in binding was similar to that observed with E1 alone. The same deletion mutation constructs of E1 were subcloned into an expression vector for use in transient replication assays to study the effect of the deletions on the replication of the origin DNA *in vivo* and the data suggest that the C-terminal domain contains important functions for replication.

Introduction

Papillomaviruses, a group of small, non-enveloped, icosahedral DNA viruses, infect and induce proliferative lesions in many species of higher vertebrates including humans (Howley, 1990; Lowy *et al.*, 1994; McCance, 1994; zur Hausen & de Villiers, 1994). As human pathogens, some of the low-risk types of human papillomaviruses (HPV), represented by HPV-6 and -11, infect the lower genital tract producing genital warts, whereas the high-risk group of human papillomaviruses, such as HPV-16, -18, -31, -33 and -35, are associated with cervical cancer (Boshart *et al.*, 1984; Durst *et al.*, 1983; Howley, 1990; Lorincz *et al.*, 1992; McCance *et al.*, 1985). The

investigation of the virus life-cycle has been limited because of the difficulty of propagating the virus in cell culture. Transient replication assays provide a powerful tool to elucidate the replication of papillomaviruses and, from several studies, it is known that viral proteins E1 and E2 are essential for viral DNA replication (Lambert, 1991; Ustav & Stenlund, 1991; Ustav *et al.*, 1991); however, most of our knowledge about E1 and E2 is from research on bovine papillomavirus type 1 (BPV-1).

BPV-1 E2 is a transactivator–repressor protein (Androphy *et al.*, 1987; Choe *et al.*, 1989; Hubbert *et al.*, 1988; Lambert *et al.*, 1987, 1989*a, b*; McBride *et al.*, 1988) and the BPV-1 E2 open reading frame (ORF) encodes three proteins, a full-length 48 kDa protein (E2TA), which contains a transactivation domain at its N terminus and a DNA binding and dimerization domain at its C terminus, connected by a hinge region, a 31 kDa protein (E2-TR) encoded by the C terminus alone, and a 28 kDa protein (E8/E2), which is a spliced product combining the N terminus of E8 and the C terminus of E2. The E2 protein plays an important role in replication and transcriptional regulation and binds the E1 protein, disrupts chromatin, and interacts with cellular replication factors (Blitz & Laimins, 1991; Li & Botchan, 1994; Lorincz *et al.*, 1992; Ustav *et al.*, 1993; Yang *et al.*, 1991). Recently, it was shown that BPV-1 E2 and the TATA binding protein (TBP) bind cooperatively to

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DNA, thus enhancing the transcription of viral genes (Rank & Lambert, 1995). The E2 protein binds as a dimer to DNA at the E2 binding site (E2BS), which is composed of a consensus sequence ACCN₆GGT located as multiple copies in the upstream regulatory region of papillomaviruses (Li *et al.*, 1989). The full-length E2 is required for replication of the viral origin in transient replication assays. While it appears that, unlike the BPV-1 E2, the major function of HPV E2 is to down-regulate the major early promoter of the HPV genome (Chiang *et al.*, 1991), this may not always be true as activation by E2 of the HPV-16 and -18 promoter has been observed (Bouvard *et al.*, 1994). Studies on mutated BPV-1 E2 protein suggest that the transactivation and replication properties of E2 protein may partially overlap since many mutations in the N terminus of BPV-1 E2 affect both transcription and replication, but two mutated E2 proteins which are completely defective for transcription replicate viral DNA at very low levels (Brokaw *et al.*, 1996).

BPV-1 E1 is a 68 kDa phosphoprotein with multiple functions including DNA binding, E2 binding, ATPase and helicase activities, and it has the ability to interact with DNA polymerase α -primase (Blitz & Laimins, 1991; Gillette *et al.*, 1994; Sarafi & McBride, 1995; Seo *et al.*, 1993a; Wilson & Ludes-Meyers, 1991; Yang *et al.*, 1993). As a replication initiator, BPV-1 E1 specifically binds to the virus replication origin at the E1 DNA binding site (E1BS), which contains an *Hpa*I site at nt 1 flanked by two inverted repeats (Ustav *et al.*, 1991; Mendoza *et al.*, 1995). DNase I footprinting indicates that BPV-1 E1 protects a 28 bp region between nt 7932 and 15, and a 18 bp inverted repeat within this region is sufficient for specific E1 binding (Holt *et al.*, 1994; Wilson & Ludes-Meyers, 1991). The E1BS of HPV-11 and HPV-31 are located at a similar region (Frattini & Laimins, 1994a, b; Sun *et al.*, 1996) on the genome. Using protein affinity columns, it was shown that BPV-1 E1, in the form of a GST fusion protein, binds DNA polymerase α -primase (Bonne-Andrea *et al.*, 1995). Binding does not appear to be species-specific because the GST-E1 of BPV-1 binds DNA polymerase α -primase both from FM3A cell extracts (a mouse cell line) and COS cell extracts (a monkey cell line). However, unlike the SV40 T antigen, BPV-1 E1 does not bind to replication protein A.

E1 and E2 can form a complex when co-expressed in a baculovirus expression system (Blitz & Laimins, 1991; Mohr *et al.*, 1990), and in the presence of E2, E1 binds to the E1BS more efficiently (Lu *et al.*, 1993; Russell & Botchan, 1995; Sun *et al.*, 1996; Seo *et al.*, 1993b). One study has shown that aa 1–299 contain the DNA binding domain of BPV-1 E1 (Thorner *et al.*, 1993), while another has shown that aa 162–378 are important for DNA binding (Sarafi & McBride, 1995). The domain important for E2 interactions has been mapped in one study to aa 162–605 (Sarafi & McBride, 1995) and in another to the N-terminal 250 aa (Benson & Howley, 1995). The domain of E1 important for binding to E2 has been mapped for HPV-16 and -31 to the C-terminal region between aa 144 and 649 and

between aa 312 and 644, respectively, at physiological normal temperatures (Yasugi *et al.*, 1997; Muller & Sapp, 1996). It is not clear at present if the large regions required are due to multiple binding sites in E1, or because of the necessity of some structural constraint on the protein.

In this study, a series of deletion mutants of HPV-11 E1 were constructed and tested in functional assays to define those domains of HPV-11 E1 which are important for binding to the origin DNA and E2. The same deletion mutation constructs of E1 were subcloned into an expression vector for use in transient replication assays to determine for the first time the effect of the deletions on the replication of HPV-11 origin-containing DNA *in vivo*.

Methods

■ **GST-E1 fusion proteins.** GST fusion protein plasmids for HPV-11 E1 were constructed by subcloning the fragment containing the HPV-11 E1 ORF from pVL-11E1 (Lu *et al.*, 1993) into pGEX2T (Pharmacia) at the *Bam*HI and *Eco*RI sites at the 5' and 3' ends, respectively. C- and N-terminal deletion mutation constructs were produced by the exo III series deletion method. Sequencing was used to verify the positive clones of the GST deletion mutations and to determine the exact point of the N- and C-terminal deletion of the HPV-11 E1 gene. The GST fusion proteins were expressed and purified as described (Smith & Johnson, 1988; Sun *et al.*, 1996); the expression of GST fusion proteins was verified by SDS-PAGE and Coomassie blue staining. The concentration of the free GST fusion proteins was determined by a Bradford assay. Eight C-terminal and eleven N-terminal proteins were obtained and used in subsequent assays (Fig. 1a).

■ **Binding of E1 proteins to an origin-containing plasmid.** A ³²P-end-labelled DNA fragment from *Bam*HI digestion of plasmid N9 (Sun *et al.*, 1996), which contains the HPV-11 origin, and 1 μ g GST-E1 full-length protein or different deleted proteins bound to glutathione beads were incubated at 37 °C for 2 h in 200 μ l buffer C (50 mM Tris-HCl, pH 7.2, 0.5% NP40, 2 mM DTT, 1 mM PMSF, 1 μ g/ml leupeptin and 1 μ g/ml pepstatin) supplemented with 150 mM NaCl. The complexes that bound to glutathione beads were collected by centrifugation and washed four times with 0.5 ml buffer C containing 150 mM NaCl, incubated for 15 minutes at 65 °C in dissociation buffer (50 mM EDTA, pH 8.0 and 1% SDS), phenol-chloroform extracted and finally ethanol-precipitated. The precipitated DNA fragment was then resolved on 12% PAGE gel; the gel was dried and then subjected to autoradiography.

■ **Binding of E1 proteins to E2.** GST-E1 (1 μ g) proteins bound to beads were used to bind 1 μ l (100 000 c.p.m.) of *in vitro* translated E2 labelled with [³⁵S]Met using the TNT-coupled reticulocyte lysate system (Promega). The 200 μ l mixture containing GST-E1 and *in vitro* translated E2 in buffer C with 150 mM NaCl was rotated at room temperature for 2 h and washed four times with 500 μ l per wash of buffer C with salt. The bead-bound proteins were then boiled in 10 μ l 2 \times protein loading buffer and loaded onto 8% SDS-PAGE gel. After electrophoresis, the gel was dried and subjected to autoradiography.

■ **Replication of an origin-containing plasmid by E1 proteins *in vivo*.** The mutated E1 protein constructs, cloned into an expression vector containing the cytomegalovirus (CMV) immediate-early promoter (IE) (Lu *et al.*, 1993), were transfected into 293 cells together with a full-length E2 expression vector pCHE2 (E2 is also expressed from the CMV IE promoter) and the HPV-11 origin-containing vector N9. Sub-confluent

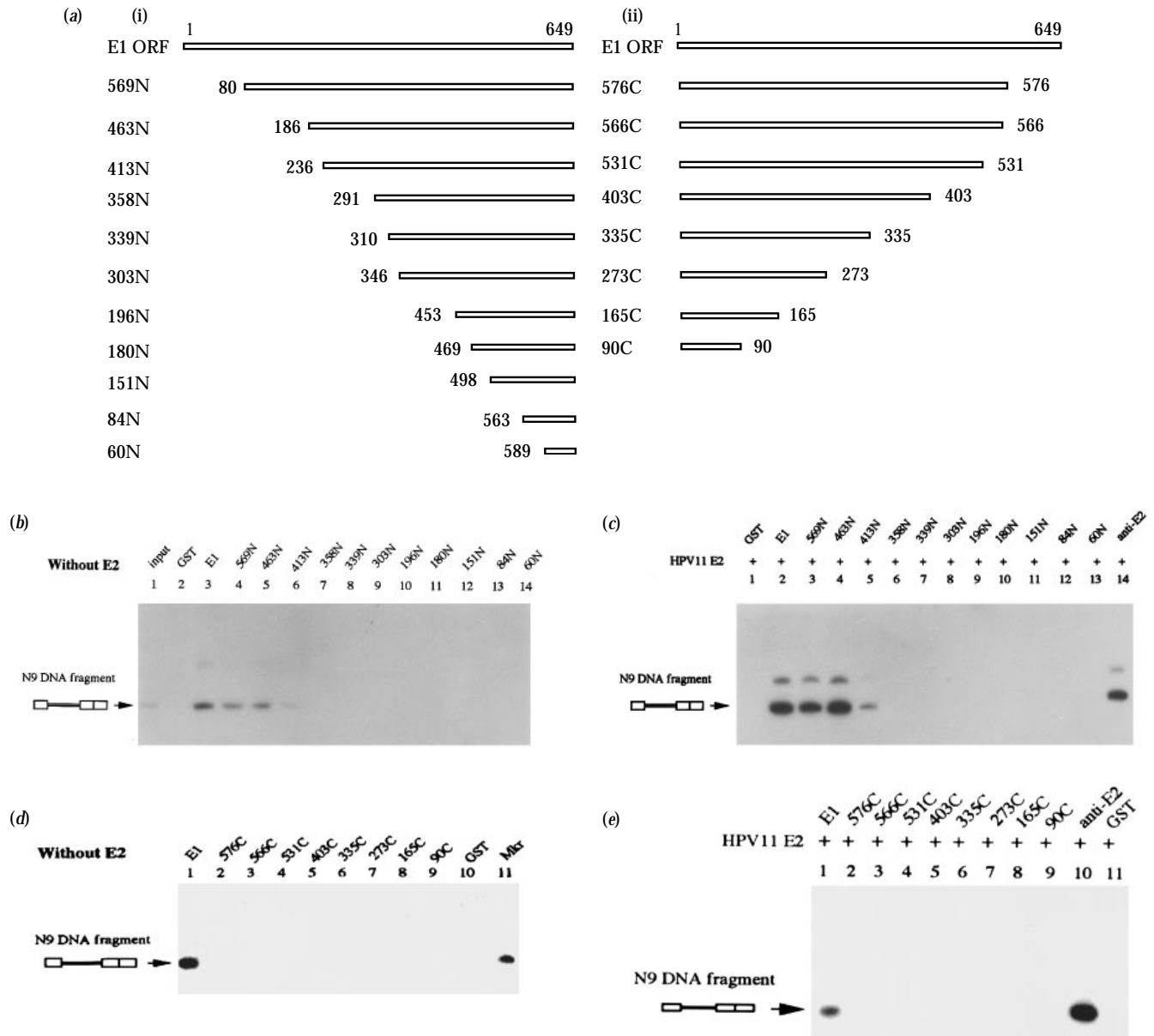


Fig. 1. Binding of E1 to a HPV-11 origin-containing fragment in the presence or absence of E2. (a) Eleven E1 N-terminal deletion mutations (i) and eight C-terminal mutations (ii) were used in the binding assays. The deletion mutations were named as xxxN or xxxC where xxx represents the size of the deletion mutation and the N or C represents the N- or C-terminal deletion mutation, respectively. The results of the binding of N-terminal truncated proteins to the 100 bp origin-containing fragment in the absence or presence of 0.25 μ g baculovirus-expressed E2 are shown in (b) and (d), respectively, while (c) and (e) show the DNA-binding results of C-terminal deleted proteins in the absence or presence of E2, respectively. In (b) and (d), the gels were exposed to X-ray film for 3 days, while in (c) and (e), the gels were exposed to X-ray film for 10 h. Lane 1 in (b), labelled input, shows the position of the 100 bp origin-containing fragment. Lane 11 in (d), labelled Mkr, shows the distance run by the N9 fragment. The anti-E2 lane is a positive control using anti-E2 antibody to immunoprecipitate the E2-DNA complex.

cultures of 293 cells were trypsinized and resuspended in growth medium (DMEM with 10% calf serum) at a density of 1×10^7 cells per ml. Common reagents were added to the cell mixture so that 0.25 ml of an aliquot contained 3 μ g each of pCHE1 (containing either wild-type or mutated E1 genes) and pCHE2, 50 μ g single stranded salmon sperm DNA and 2.5 μ l HEPES (0.5 M; pH 7.2). Origin plasmid N9 (0.5 μ g) was added to each 0.25 ml cell aliquot and transfected by electroporation

using a Bio-Rad Gene Pulser at 170 V and 960 μ F. After standing at room temperature for 10 min, cells were washed with 6 ml complete medium and then seeded onto a 100 mm plate. Transfected cells were harvested 72 h after electroporation. Low molecular mass DNA was extracted by the method of Hirt as previously described (Lu *et al.*, 1993). One-eighth of the isolated DNA was used for digestion with *DpnI* and *Bam*HI, run on an 0.8% agarose gel. The replicated origin-containing plasmid was

detected by Southern blotting using ^{32}P -labelled pSK(+) plasmid as a probe.

Results and Discussion

E1 has been shown to specifically bind to an AT-rich palindrome contained in the HPV-11 origin (Lu *et al.*, 1993; Sun *et al.*, 1996) though this binding sequence is not essential since replication occurs even in its absence. However, replication is enhanced by the presence of this sequence, indicating that the DNA binding function of E1 still plays a role in replication, so we carried out DNA-protein binding assays using both the N- and C-terminal deleted E1 proteins to locate the DNA binding domain of E1 and to address the importance of this DNA binding function of E1 to HPV-11 replication. When the full-length GST-E1 fusion protein was incubated with a radiolabelled 100 bp HPV-11 origin fragment, a specific DNA-protein complex (specificity determined in previous experiments, as described by Sun *et al.*, 1996) was produced as indicated by a retained 100 bp origin fragment (Fig. 1*b* and *c*, lane E1). When the truncated E1 proteins were tested in the same binding assay, deletions 569N and 463N of the N-terminal mutated proteins also bound the 100 bp origin fragment (Fig. 1*b*, lanes 4 and 5). Deletion 413N bound weakly (Fig. 1*b*, lane 6). However, proteins 358N to 60N and all the C-terminal mutated proteins (Fig. 1*c*) did not bind this fragment, suggesting that sequences required for binding to the origin

were located in a wide region of the C terminus between an 186 and 649. The relative binding of the mutated proteins is shown in Table 1.

Our previous work (Sun *et al.*, 1996) showed that there is a mutual enhancement for DNA binding between E1 and E2. E2 would facilitate the binding of E1 to its cognate site and this facilitation involved the binding of E1 to its E1BS and not to E2 alone, while E1 would also enhance the binding of E2 to its DNA binding site. We therefore carried out E1-DNA binding assays in the presence of E2 to see if E2 could modify the configuration of E1 deletion proteins and affect their DNA binding activity. The interaction between E1 and E2 detected by McKay DNA binding assay was performed as described above, except that 0.25 μg E2 (Lu *et al.*, 1993), which was obtained from *Spodoptera frugiperda* (Sf-9) cells 3 days after infection with baculovirus recombinants, was added to the mixture of the ^{32}P -end-labelled DNA fragments from *Bam*HI digestion of plasmid N9 and 1 μg GST-E1 bound to glutathione beads. As expected, E2 enhanced the association of wild-type E1 and mutated proteins 569N and 463N with the origin (Fig. 1*b* and *c*). Note that the exposure time in Fig. 1(*b* and *d*) is 3 days and in Fig. 1(*c* and *e*) is 10 h. In the presence of E2, the binding of deletion 413N was clearly visible, although it was weaker than that of 569N and 463N (compare Fig. 1*b* and *c*). Table 1 shows the relative levels of binding.

Since the binding of E1 to E2 is an important event in HPV-11 replication, we attempted to identify the domain of E1

Table 1. Functional assays of N- and C-terminal deletion proteins of GST-E1

Numbers in the table are the percentage activities of the mutated proteins compared to that of the wild-type (E1), as measured by scanning of X-ray films.

(a) N-terminal deletion proteins

Activity	E1	569N	463N	413N	358N	339N	303N	196N	180N	151N	84N	60N
Origin binding	100	31	37	1.7	0	0	0	0	0	0	0	0
E2 binding	100	112	76	79	82	98	77	< 2	< 2	< 2	14	9.8
Origin binding + E2*	100	73	119	4.4	0	0	0	0	0	0	0	0
Transient replication	100	4.6	6.2	< 0.5	< 0.5	< 0.5	0.0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5

(b) C-terminal deletion proteins

Activity	E1	576C	566C	531C	403C	335C	273C	165C	90C
Origin binding	100	0	0	0	0	0	0	0	0
E2 binding	100	< 1	< 1	< 1	< 2	< 2	< 2	< 2	3
Origin binding + E2*	100	0	0	0	0	0	0	0	0
Transient replication	100	0	0	0	0	0	0	0	0

* Origin binding of E1 mutations in the presence of E2. It should be noted that the relative activities of origin binding are based on scans of X-ray film exposed for 10 h in the presence of E2, compared to 3 days in the absence of E2.

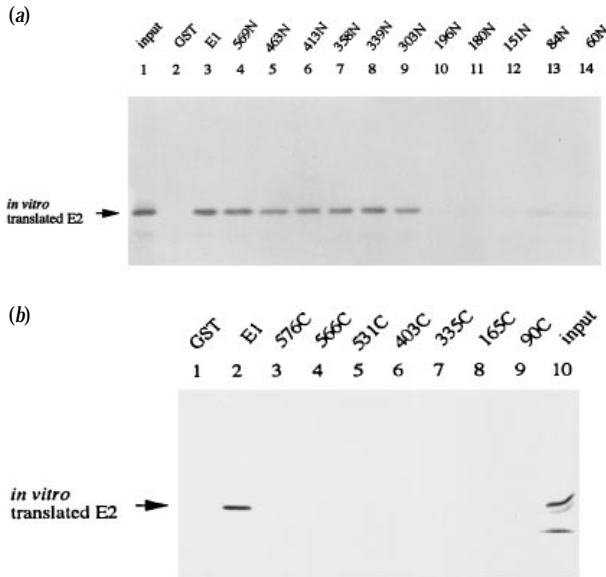


Fig. 2. E2 binding domain of E1 maps to within the C-terminal half of the protein. Binding of the N-terminal deletions is shown in (a) and binding of the C-terminal deletions is shown in (b). The lane marked input indicates the amount of E2 added to the GST fusion proteins.

responsible for binding to E2. Both the eleven N-terminal and eight C-terminal mutant proteins were tested for their ability to bind to radiolabelled *in vitro* translated E2; full-length E1 was used as a positive control and GST protein was a negative control. The TNT-coupled reticulocyte lysate system (Promega) was used to translate HPV-11 E2 protein *in vitro*. The full-length E1 co-precipitated a band of the same size of input E2; this was also observed with the N-terminal deletion proteins 569N to 303N (Fig. 2a). However, while no co-precipitated bands were detected above background (> 2%) with deletion proteins 196N to 151N, there was significant binding of 10% of wild-type with the C-terminal 84 aa (Fig. 2a; Table 1). All the C-terminal deletion proteins (Fig. 2b) were negative for E2 binding. Even the removal of 73 aa at the C-terminal end abolished the ability to bind E2. Therefore, the results appear to localize the domain of E1 responsible for binding E2 within the C-terminal half (aa 346–649).

To measure the effects of these truncated E1 proteins on transient DNA replication *in vivo*, we subcloned both N- and C-terminal mutants into an eukaryotic expression vector containing the IE promoter from CMV, and put an in-frame ATG start codon upstream of each of the N-terminal deletions. The mutated E1 protein constructs were transfected into 293 cells together with a full-length E2 expression vector pCHE2 and the HPV-11 origin-containing vector N9. As anticipated, N9 transfected with pCHE2 alone gave no signal (data not shown), whereas in the presence of full-length E1, efficient replication was observed (Fig. 3a, lane 1, and b, lane 2). N-terminal deletion proteins 569N and 463N, which retain the E2 binding and DNA binding activities of E1, also replicated,

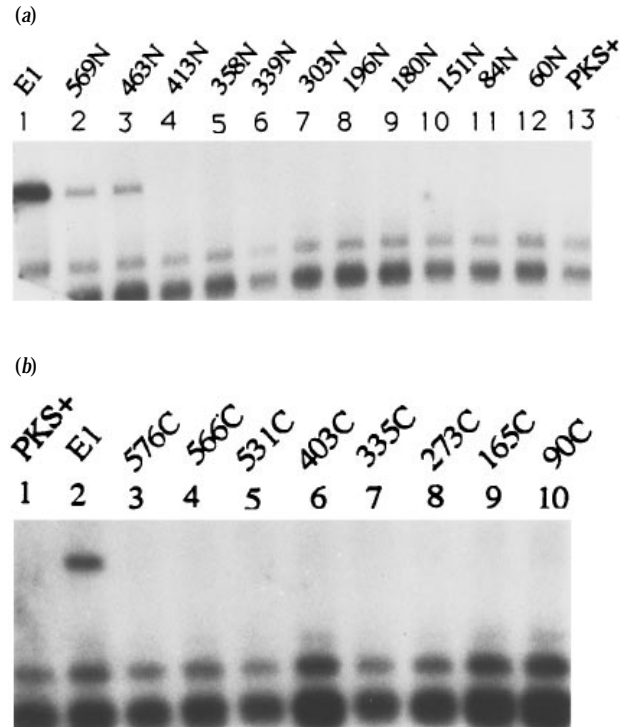


Fig. 3. Transient replication assay of an HPV-11 origin-containing plasmid using truncated E1 proteins and full-length E2. The results of using both N- and C-terminal E1 mutations are shown in (a) and (b), respectively, plus wild-type E2. The PKS+ lanes contain plasmid without HPV-11 origin sequences. Filters were exposed to X-ray film for 3–5 h.

although less well than the wild-type E1 (Fig. 3a; Table 1). However, N-terminal deletion proteins 303N, 358N and 339N, which still retain E2 binding activity but do not bind DNA, could not replicate HPV-11 origin DNA. The rest of the mutations retaining one of the above activities of E1 failed to replicate the origin plasmid. None of the C-terminal E1 mutated proteins were able to support origin replication (Fig. 3b). The results indicate that E2 binding and DNA binding activity of E1 are both necessary for the replication function of the E1 protein (Table 1), although since the domains overlap, site-directed mutations need to be created to confirm such a conclusion.

The E1 and E2 proteins of papillomaviruses are essential for genome replication. E1 is a multifunctional protein which binds to the origin region in a sequence-specific manner and to the E2 protein and has ATP-binding/ATPase and helicase activity. The aim of this study was to determine the active domains of E1 for E2 and DNA binding using deletions from both the N and C termini. In addition, we sought for the first time to determine the effects these mutated proteins have on replication of the HPV-11 origin *in vivo*. The results are summarized in Table 1. We have previously shown that HPV-11 E1 binds specifically to the origin region and that binding is enhanced in the presence of E2 (Sun *et al.*, 1996). The data presented here indicates that the DNA binding domain of E1 is located in a large area of the protein between aa 186 and

649, although removal of the last 73 aa alone abrogates binding. Previous studies on BPV-1 E1 showed that the DNA binding domain was between aa 162 and 378 (Saraf & McBride, 1995), or aa 121 and 284 (Leng *et al.*, 1997). The larger domain observed in the HPV-11 E1 protein may be explained by the differences in the affinity of the E1 proteins for DNA, since the binding of HPV-11 E1 to the origin is very weak compared to that of BPV-1 E1 (Sun *et al.*, 1996) and may therefore need a higher degree of integrity of the protein in order to exhibit binding. In addition, different analysis conditions or systems may also contribute to the observations, or there may be a real difference between bovine and human viruses since it has been shown that there are variances in protein activities between BPVs and HPVs. For example, the transcriptional activity of the E2 proteins from BPVs and HPVs differs depending on the arrangement of the E2 binding sites in the promoter region (Kovelman *et al.*, 1996).

Another property of E1 is its ability to bind to E2; the domain required for this is also in the C-terminal region between aa 346 and 649. Since the presence of E2 enhances the ability of E1 to bind to its recognition sequence (Sun *et al.*, 1996), we also tested the binding of E1 and E2 in the presence of an origin-containing fragment. Figs 1 and 2 show that the E1 domain important for origin and E2 binding is overlapping and between aa 186 and 649 and aa 346 and 649, respectively. As expected, there was also an increase in the efficiency of E1 binding to the origin in the presence of E2 (compare Fig. 1*b* with *c*). These results are similar to those of Saraf & McBride (1995) who mapped the E2 binding domain of BPV-1 E1 to between aa 162 and 605, and of Muller & Sapp (1996), who mapped the E2 binding domain of HPV-33 E1 to between aa 312 and 644, and of Yasugi *et al.* (1997), who mapped the E2 binding domain of HPV-16 E1 to between aa 412 and 649, but is different from other studies which showed the N-terminal of BPV-1 E1 was responsible for E2 binding (Benson & Howley, 1995; Leng *et al.*, 1997).

We had shown that HPV-11 E1 could replicate an origin without an E1 binding site (Lu *et al.*, 1993), and the activity was thought to be mediated by the ability of E1 to bind to E2 at the origin. The fact that the C-terminal mutations did not bind the origin or E2 and were negative for replication is in line with previously reported results. However, the results presented here (Table 1), show that N-terminal deletion proteins 569N and 463N bound to E2 at 100 and 76%, respectively, of the level of wild-type E1, but only bound the origin at approximately 33% of full-length E1. In the presence of E2, binding of both to the origin increased to 73–100% of full-length E1, yet the level of replication supported by both was 20-fold less than that of full-length E1 (Table 1). This suggests that either binding to the origin independently of E2 binding is important for efficient replication, or that a significant replicative function is contained in the N-terminal 186 aa. Similar observations were made by Leng *et al.* (1997), who showed that two independent BPV-1 E1 N-terminal deletions, which still bound

E2, would not support origin replication. However, contrary to our results with HPV-11 E1, they showed that the DNA and E2 binding domains of BPV-1 E1 were in the N-terminal half, between aa 121 and 284.

Therefore, in summary (Table 1), the origin binding domain of HPV-11 E1 has been mapped to between aa 186 and 649, and the E2 binding domain to between aa 346 and 649. The functions contained in the C-terminal region are essential for replication of an HPV-11 origin *in vivo*. In addition, with deletion of the N-terminal 186 aa of E1, binding to E2 and the origin is observed, but replication *in vivo* is reduced by 20-fold compared to full-length E1, suggesting that a replicative function is contained in these N-terminal amino acids.

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