Mutations in the human papillomavirus type 16 E2 protein identify multiple regions of the protein involved in binding to E1

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Human papillomavirus type 16 (HPV-16) DNA replicates episomally and requires two virally expressed proteins, E1 and E2. The E1 protein has both helicase and ATPase activities and is absolutely required for viral DNA replication. The E2 protein is a potent transcriptional activator and greatly increases viral DNA replication by colocalizing E1 to the origin of replication. Recently, we characterized a region of the E2 protein essential for the binding to E1. In this study we have analyzed in further detail the nature of the association between E1 and E2. Using an extensive set of E2 mutant proteins we have identified two widely separate regions of the E2 protein which are essential for binding to E1. Interestingly, two E2 mutants which fail to bind E1 also fail to activate gene expression, indicating the existence of multifunctional domains on the E2 protein. In addition, cotransfection of E1 with E2 significantly increases E2 transcriptional activity on an heterologous promoter.

Human papillomaviruses (HPVs) are a family of small DNA viruses, strictly epitheliotropic, which infect a wide variety of anatomical sites. At present, about 74 genotypes of HPVs have been characterized, and of these, approximately 25 types are associated with anogenital lesions (de Villiers, 1989). Anogenital HPV infections can be subdivided into high and low risk infections: in particular, types 16, 18, 31, 33, and 45 are linked to the development of cervical intraepithelial neoplasias (CIN) which may progress to malignancy (zur Hausen & Schneider, 1987). These CINs usually harbour many copies of episomal viral DNA; in contrast, invasive carcinomas often possess mainly integrated HPV DNA sequences (Schwarz et al., 1985; Baker et al., 1987; Matsukura et al., 1989).

The HPV life cycle takes place in differentiating keratinocytes where the actively dividing basal cells maintain the virus as a low-copy-number nuclear plasmid. Vegetative viral replication occurs only in the suprabasal daughter cells which are non-dividing and committed to terminal differentiation. It has been shown that two viral proteins are unequivocally required for supporting transient replication of plasmids containing the viral origin: the full-length E1 open reading frame protein and the full-length E2 open reading frame protein (Yang et al., 1991; Ustav & Stenlund, 1991; Chiang et al., 1992). The E1 protein is a nuclear, ATP-binding phosphoprotein of about 68 kDa which binds to the origin of replication (Sun et al., 1990; Blitz & Laimins, 1991; Yang et al., 1991; Wilson & Ludes-Meyers, 1991). In addition, it has been shown that E1 has both ATPase and helicase activity (Seo et al., 1993; Hughes & Romanos, 1993; Bream et al., 1993).

The viral origin of replication consists of a short segment containing an E1 binding site, a short A + T-rich segment and one or more sequences which are recognized by the E2 protein (Yang et al., 1991; Chiang et al., 1992; Holt et al., 1994; Svedrup & Kahn, 1994). The E2 protein is a well characterized major regulator of viral gene expression (Phelps & Howley, 1987; Cripe et al., 1987; Bouvard et al., 1987; Bouvard et al., 1994). It binds as a dimer to the viral upstream regulatory region (URR) at ACCN₆GGT motifs which are found repeated in the viral enhancer (Androphy et al., 1987). The structure of the full-length E2 protein can be functionally divided into three different domains: an N-terminal region of the protein which includes the transcriptional activation domain, a central hinge region, and a C-terminal domain involved in specific DNA binding to the viral URR as well as in E2 dimerization (Giri & Yaniv, 1988).

Using mutant forms of the E2 protein, we recently identified a small region in the activation domain which appears to be required for efficient binding to the HPV-16 E1 protein (Storey et al., 1995). This region (amino acids 156–159) is highly conserved amongst a large number of HPV genotypes which suggests a common mechanism of interaction between E1 and E2. However,
Fig. 1. Schematic representation of the HPV-16 E2 mutants used in this study. Numbers indicate the amino acid residue mutated together with the new residue at that position.

(a) (b)

Fig. 2. Association between GST–E1 and HPV-16 E2 mutant proteins. The GST–E1 fusion protein was tested for binding to six different in vitro translated E2 mutants and to the wild-type (wt) E2 as indicated in (a) and (b). Protein inputs were equalized throughout prior to the binding assay. The autoradiogram was exposed for 16 h at −80 °C with intensifying screens.

in a recent study using anti-E2 monoclonal antibodies to block the E1–E2 association it appeared that the region of E2 important for this interaction was within the extreme amino-terminal portion of the protein (Hibma et al., 1995). This suggested that more than one region of the E2 molecule may contribute to E1 binding. To further characterize the mechanism of interaction between these proteins we generated additional mutants of E2 throughout the amino-terminal domain of the protein at residues conserved between the different HPV types. These mutants were assigned the numbers 3, 4, 5, 13, 14 and 15 and are depicted schematically in Fig. 1. We then proceeded to investigate the ability of these mutant E2 proteins to complex with the E1 protein. The mutant proteins were in vitro translated using the TNT coupled reticulocyte lysate system (Promega) in the presence of radiolabelled cysteine and assessed for their ability to bind to purified glutathione S-transferase (GST)–E1 protein (Storey et al., 1995). Prior to incubation with E1, translation efficiencies were monitored by PAGE and autoradiography. Equal amounts of the wild-type and mutant E2 proteins were then added to the GST–E1 fusion protein, and following extensive washing bound proteins were monitored by PAGE and autoradiography. The results obtained are shown in Fig. 2. In agreement with our previous observations, M9 (Δ156–159) was completely defective for binding E1 (Storey et al., 1995). However, it is clear from Fig. 2 that three additional mutants of E2 are also defective for E1 binding. M3 (Arg → Pro-47) and M4 (Glu → Pro-74) are clearly reduced in their ability to bind E1 while M13 (Δ23–26) is completely negative. Mutant 5 (Leu → Pro-94) and the double point mutant 14 (Glu → Ala-100; Tyr → Ala-102), however, retain wild-type levels of E1 binding. It is noteworthy that a truncated form of E2 is retained in the wild-type and M14 lanes but is absent in the M3, M4 and M5 lanes. This lower band lacks the carboxy-terminal sequences of the E2 protein (data not shown). The reason for the loss of binding of this protein in M3, M4 and M5 is unclear but may be a reflection of decreased conformational stability of the truncated protein in combination with the particular mutation. Nevertheless, in terms of the full-length E2 protein, these results define an additional region within the amino-terminal domain of E2 which is essential for complex formation with E1. This is in agreement with recent observations which also indicate that the region of E2 spanning amino acids 18–41 is involved in complex formation with the E1 protein (Hibma et al., 1995).

In the above analysis the protein–protein interaction between E1 and E2 was assayed using GST–E1 beads and in vitro translated E2. In order to monitor the
specificity of these associations the same experiment was also performed by mixing GST–E2 beads with radio-labelled E1. In addition, only the new E2 mutant with a deletion of amino acids 23 to 26 in the full-length E2 protein seemed to abolish completely the binding to E1. Since the leucine residue at position 26 is conserved between all genital HPVs we were interested in determining if this was essential for the E1–E2 interaction. Therefore, an additional point mutation was made within E2 replacing the leucine at position 26 with proline (M15). To monitor binding with E1 the wild-type E2 and M15 were cloned into pGEX2T. The GSTE2 fusion proteins were purified as described previously (Storey et al., 1995) and the protein profiles are shown in Fig. 3(a). Lower molecular mass proteins are predominantly degradation products as determined by Western blot analysis (data not shown). These purified proteins were then incubated with radiolabelled E1 and the results obtained are shown in Fig. 3(b). Clearly, wild-type E2 retains a large proportion of the input E1 protein; however, M15 only binds to the E1 protein very weakly. Luciferase was also included as an additional control and, as can be seen, none was retained on the E2 column. These results demonstrate that the region of E2 around amino acid 26 is also essential for complex formation with E1.

One of the major functions of E2 is the regulation of viral gene expression (Phelps & Howley, 1987; Cripe et al., 1987). Recent studies have also shown that BPV-1 E1 can modulate the transcriptional activity of the BPV-1 E2 protein (Le Moal et al., 1994). We were therefore interested in determining whether the HPV-16 E1 protein possessed a similar activity and, secondly, whether the E2 mutants defective in E1 binding could activate viral gene expression. To investigate this, the full-length E2 expression plasmid, pJ4Ω16E2 (Lees et al., 1990) together with increasing amounts of the E1 expression plasmid pJ4Ω16E1 (containing nucleotides 864–2809 of HPV-16 cloned into pJ4Ω) were cotransfected with the HPV-16 URR::TKCAT reporter plasmid (Bouvard et al., 1994) into HT1080 cells. After 48 h the cells were harvested and CAT assays performed as described previously (Bouvard et al., 1994). The results obtained are shown in Fig. 4(a). It is clear that cotransfection of E1 results in a significant increase in HPV-16 E2 transcriptional transactivation. These results indicate a similar pattern of interaction between the HPV-16 E1 and E2 proteins to that reported for the BPV-1 E1 and E2 proteins (Le Moal et al., 1994).

We then proceeded to investigate the ability of the E2 mutants defective for complex formation with E1 to activate viral gene expression. Cells were transfected with two of the E2 mutants together with the HPV-16 URR::TKCAT reporter plasmid. A parallel series of transfections was also performed with a β-galactosidase expressing plasmid (pCH110, Pharmacia) in order to correct for transformation efficiency. After 48 h the cells were harvested and CAT assays performed. Typical results obtained are shown in Fig. 4(b). The results from three independent experiments indicate that wild-type protein induced approximately 3-fold activation of enhancer activity, similar to that reported previously (Bouvard et al., 1994). In contrast, both M9 (1-6-fold activation) and M15 (1.3-fold activation) had minimal effect upon enhancer activity. These results demonstrate the existence of multifunctional domains on the E2 protein which are necessary both for binding E1 and for transcriptional activation.
Two viral proteins, E1 and E2, are found to be essential for papillomavirus DNA replication in vivo and in vitro (Ustav & Stenlund, 1991; Yang et al., 1991; Chiang et al., 1992). In view of this it is important to understand in detail the mechanism of association between E1 and E2. The results presented in this paper describe two widely spaced regions within the N-terminal transactivation domain of E2 which are essential for binding E1. Previously, we identified a stretch of amino acids between residues 156 to 159 as being essential for complex formation between E1 and E2 (Storey et al., 1995). In the present study we have extended this mutational analysis to the entire amino-terminal half of E2. We found that both a point mutation at amino acid 26, where a leucine is changed to a proline, and a deletion of amino acids 23 to 26 completely abolish the E1–E2 protein complex. This binding site may in fact be more extensive since reduced E1 binding was obtained with the point mutations of arginine to proline at position 47 and isoleucine to proline at position 73. Mutations at residues 94 and 100/102 had no effect on the ability of E2 to bind E1. These results are in agreement with a recent report which used monoclonal antibodies directed against E2 to probe the interaction between E1 and E2 complexes. In this case an antibody which mapped to amino acids 18 to 41 inhibited the E1–E2 interaction whereas an antibody mapping to amino acids 2 to 17 had minimal effect (Hibma et al., 1995). Taken together, these results define two widely spaced regions, both within highly conserved stretches of the E2 protein, which are essential for the binding of E2 with E1.

Previous studies with BPV-1 E1 and E2 indicated that E1 could stimulate the transcriptional activity of the E2 protein (Le Moal et al., 1994). The results presented here indicate that a similar situation holds true for the HPV-16 E1 and E2 proteins. Co-transfection of progressively increasing amounts of E1 expression plasmid with a constant input of E2 expression plasmid produced a marked increase in the ability of E2 to activate the HPV-16 enhancer. The multifunctional nature of the E2 protein was also highlighted by the analysis of the mutant E2 proteins in transcriptional assays. Both of the E2 mutants which failed to complex with the E1 protein were also seriously defective in their ability to activate transcription. These results provide a further demonstration of the close association between the mechanisms which regulate DNA replication and gene expression.

In conclusion, we have performed an extensive analysis of the mechanism of association between the HPV-16 E1 and E2 proteins and have defined two regions of E2 which are essential for this association. These results provide vital information in the search for potential therapeutic compounds directed towards the inhibition of the E1–E2 association.

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