Characterization of the DNA-binding activity of the E1 and E2 proteins and the E1/E2 complex of human papillomavirus type 33

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The E1 and E2 proteins of papillomaviruses are essential for the initiation of viral DNA replication. We have purified the E2 protein of human papillomavirus type 33 (HPV-33) by immunoaffinity chromatography. The purified E2 protein bound with high affinity to all four consensus binding sites of HPV-33 ($K_d \approx 2 \times 10^{-10}$ M). A putative E2 binding site differing at one position in the second stem of the palindrome was not bound by E2. The E1 protein of HPV-33 purified by affinity chromatography using glutathione S-transferase as tag displayed specific DNA-binding activity in footprint analyses protecting HPV-33 nucleotides 7896 to 7909/1 to 18 from DNaseI digestion. Hypersensitive sites at position 6 on the sense and position 1 on the antisense strand were observed in the middle of the protected region. An E1/E2 complex protected the E1 binding site and E2 binding sites from DNaseI digestion suggesting that both proteins retain DNA-binding activity in the complex.

Papillomaviruses (PV) comprise a heterogeneous group of small double-stranded DNA viruses infecting the basal layer of epithelia. They are associated with benign or malignant hyperproliferations which have a wide variety of clinical manifestations. The E1 and E2 proteins of PV have been shown to be essential for papillomaviral DNA replication by genetic studies (Lusky & Botchan, 1985), by transient replication assays (Ustav & Stenlund, 1991) and by the establishment of cell-free replication assays (Yang et al., 1991). The E1 protein contains an intrinsic NTPase, a DNA helicase and a DNA-binding activity (Seo et al., 1993). The E2 protein was initially identified as a transcriptional modulator. It binds with high affinity to a consensus sequence, ACC(N)$_n$GGT, which is found several times in the long control region (LCR) of all PV (Hawley Nelson et al., 1988). The E1 and E2 proteins interact in solution to form heteromeric complexes (Mohr et al., 1990).

The complex is able to interact with DNA and was shown to be essential for supporting DNA replication (Sedman & Stenlund, 1995). The requirements of cis- and trans-factors for...
Fig. 2. E2 binds with similar affinity to all the E2BS. Increasing amounts of purified E2 (lanes 2 to 6) were incubated with 20 fmol of a DNA fragment spanning HPV-33 nt 7773 to 80, which had been radioactively labelled at the sense strand, for 30 min at room temperature. Each assay contained 50 ng of poly(dI–dC) as competitor DNA. The DNA was digested with 3 U of DNaseI for 1 min after addition of MgCl₂ (1 mM) and CaCl₂ (0.5 mM) and processed for analysis on a denaturing polyacrylamide gel. Lanes 1 and 7, controls without protein; lanes 2 to 6, 48, 95, 190, 380 and 760 fmol of purified E2 dimer, respectively.

Fig. 3. Footprint of G33E1 and of the G33E1/E2 complex. G33E1 and G33E1/E2 complexes were coupled to glutathione–Sepharose and incubated with 20 fmol of a DNA fragment encompassing HPV-33 nt 7773 to 80, which had previously been labelled on the sense (lanes 1 to 4) and antisense strands (lanes 5 to 9), respectively. Unbound DNA was removed and DNA bound to Sepharose was digested with 9 U of DNaseI. The probes were processed for analysis on a denaturing polyacrylamide gel. Lanes 1, 2, 6 and 7, G33E1; lanes 3 and 8, G33E1/E2 complexes; lanes 4, 5 and 9: control lanes without protein. HSS, hypersensitive site; numbers in parentheses indicate the nucleotide positions at which the HSS occurs. G+A, G/A sequencing reaction according to Maxam & Gilbert (1980). Numbers indicate the nucleotide positions in the HPV-33 genome.
viral DNA replication differ between PV types to some extent. Whereas E1 is absolutely necessary for all PV tested, E2, although always stimulating, is, for example, dispensable for human papillomavirus (HPV)-1a replication assays in vivo (Gopalakrishnan & Khan, 1994) and at high concentrations of E1 for bovine papillomavirus (BPV)-1 replication in vivo (Yang et al., 1991). In addition, depending on the PV type studied, the E1 or the E2 binding sites can be mutated or deleted without fully impairing the function of the origin of replication (ori) (Sverdrup & Khan, 1995; Lu et al., 1993; Gopalakrishnan & Khan, 1994). It was recently suggested that different affinities between E1 and E2 themselves and different affinities between the proteins and their respective DNA-binding sites might explain the differences regarding the requirements for viral DNA replication of the various PV types (Sedman & Stenlund, 1995). We have recently shown that the E1 and E2 proteins of HPV-33 interact in solution and have mapped regions of E1 involved in binding E2 (Müller & Sapp, 1996). To further our understanding of the forces governing the initiation of HPV-33 DNA replication, we have now purified both proteins and analysed by quantitative and qualitative analyses their DNA-binding activity alone and after E1/E2 complex formation.

The E2 protein was purified by immunoaffinity chromatography using monoclonal antibody (MAb) 33E2-2 from crude nuclear extracts obtained from insect cells infected with the recombinant baculovirus bac33E2 (Müller & Sapp, 1986). This antibody was raised against recombinant E2 protein produced in E. coli and recognizes a conformational epitope in the hinge region of the HPV-33 E2 protein (amino acids 210 to 250; data not shown). A single protein with an apparent molecular mass of 43 kDa was eluted with 4 M LiCl from protein A–G-Sepharose columns to which MAb 33E2-2 was covalently cross-linked (Fig. 1A, lanes 1 and 2) (Harlow & Lane, 1988). An immunoblot using the E2-specific MAb 33E2-Ste3 (Mueller & Sapp, 1996) confirmed that this protein is indeed E2 (lanes 3 and 4). The purified E2 protein retained its specific DNA-binding activity, since a radioactively labelled oligonucleotide containing E2 binding site 3 [E2BS3; HPV-33 nucleotides (nt) 39 to 50] but not an unspecific oligonucleotide was retarded by E2 in electromobility shift assays (EMSA) (Fried & Crothers, 1981; Garner & Revzin, 1981) (data not shown). Since nucleotides within and outside the ACC(N)GGT palindrome influence E2 binding in addition to the consensus nucleotides, the affinity between E2 and various E2BS cannot be predicted with certainty (Li et al., 1989; Bedrosian & Bastia, 1990). We therefore determined the dissociation constant between E2 protein and E2BS3. To this end, increasing amounts of E2 protein were added to a fixed amount of DNA. The resulting protein–DNA complexes were analysed by EMSA (Fig. 1B) and the fraction of shifted and free DNA was quantified on a phosphoimager and plotted against the amount of E2 used in the assays. The amount of E2 needed to shift 50% of the input DNA allowed us to calculate the dissociation constant to be approximately $2 \times 10^{-10}$ M (mean value from three independent experiments). This is within the range of the $K_d$ values determined for BPV-1 and HPV-16 E2 (Tan et al., 1994; Monini et al., 1991).

The LCR of HPV-33 contains four consensus E2BS in addition to a putative weak binding site which has one mismatch in the second stem of the palindrome (ACCA GGTG TGGGA, HPV-33 nt 7851 to 7862). A similar E2BS (E2BS12 of BPV-1; ACCA TACCCA GGT) is part of the BPV-1 ori. Competition experiments using single E2BS in EMSA suggested that E2BS1 and 2 and 4 (nt 7502 to 7513, 7866 to 7877 and 54 to 65, respectively) were recognized by E2 as efficiently as E2BS3 whereas the putative E2BS behaved like an unspecific competitor (data not shown). The binding of purified E2 to the E2BS in the context of the putative wild-type ori was qualitatively analysed by footprint analysis using a DNA fragment encompassing HPV-33 nucleotides 7773 to 80 (Fig. 2). Even at high amounts of E2, binding to the putative E2BS was never observed. In contrast, addition of increasing amounts of purified E2 resulted in essentially the simultaneous protection of E2BS2, 3 and 4, which were present in the construct. Taken together, the affinity of E2 for all E2BS of the putative ori seems to be higher than that between E2 and E2BS12 of BPV-1 to which BPV-1 E2 binds with 100-fold lower affinity than to the consensus E2BS10 (Li et al., 1989). It was previously reported for HPV-16 that E2 binds with low cooperativity to E2BS3 and 4 (Tan et al., 1994). We have not seen a significant preferential protection of the adjacent E2BS3 and 4 as a result of cooperative binding of E2 under our conditions. We cannot exclude, though, that low cooperativity exists which could possibly be measured with assay systems not applied by us.

It was recently proposed that the interaction between E1 and E2 targets E1 to the origin of replication. The E1 proteins of BPV-1, HPV-11 and HPV-31b were shown to display DNA-binding activity with a weak specificity for the origin. To analyse the DNA-binding activity of the E1 protein of HPV-33, we constructed a fusion between the full-length E1 and the glutathione S-transferase (GST) gene and expressed it in insect cells using the baculovirus expression system. The GST tag was then utilized to purify the GST–E1 fusion (G33E1) via glutathione–Sepharose. G33E1 coupled to glutathione–Sepharose beads was incubated with an ori fragment encompassing E2BS2 to 4 and also including the E1BS, which was radioactively labelled either at the sense or at the antisense strand. Unbound DNA was removed and the DNA bound to Sepharose via E1 was digested with DNasel. As shown in Fig. 3, the G33E1 fusion protein specifically binds to a 32 bp region spanning nucleotides 7896 to 18 (lanes 1, 2, 6 and 7). A similar extensive footprint was previously reported for E1 proteins encoded by other PV types (Frattini & Laimins, 1994; Sun et al., 1996). Like others, we never obtained a complete footprint. This might be in part due to the presence of divergent cations during DNasel digestion, which strongly reduced the DNA-binding activity of E1 (our unpublished results). The core
region of the E1BS was better protected than the edges of the binding site. Our footprints differ from others in that binding of E1 induced one hypersensitive site on the sense strand at nucleotide position 6 and one hypersensitive site on the antisense strand at nucleotide position 1. It was previously reported that E1 distorts DNA upon binding (Gillette et al., 1994). The appearance of the hypersensitive sites might be the result of such a distortion. We have noticed that, similar to BPV-1 and HPV-11, the E1 protein of HPV-33 binds the ori with fairly low specificity (data not shown) (Sedman & Stenlund, 1995; Liu et al., 1995).

It was recently demonstrated that the E1/E2 complex of HPV-31b binds in the presence of excess E2 to high-affinity E2BS whereas E1 alone binds to the E1BS (Frattini & Laimins, 1994). We studied the binding activity of the E1/E2 complex of HPV-33 under similar conditions. After mixing E1- and E2-containing nuclear extracts using an excess of E2, the E1/E2 complex was bound to glutathione–Sepharose and subsequently used for footprint analysis as described above. To our surprise, all three E2BS were protected from DNaseI digestion in addition to the E1BS (Fig. 3, lanes 3 and 8). Because of our assay design only DNA molecules which were bound to E1/E2 complexes, which themselves were bound to Sepharose via the GST tag of E1, were analysed. This suggests that three E2 dimers are forming a complex with an unknown number of E1 molecules. An alternative explanation is that some of the E1/E2 complexes may undergo a rearrangement resulting in free E2 dimers which can then protect the unoccupied E2BS of DNA molecules already attached to the Sepharose via the E1BS or/and one of the E2BS. But DNA binding does not automatically result in the disintegration of the E1/E2 complex, since the latter can bind oligonucleotides comprising the E2BS and the E1BS, respectively, in modified McKay assays (data not shown). The footprint induced by E1 was sometimes reduced in the presence of E2, even though the hypersensitive sites were always still clearly visible (e.g. Fig. 3, lane 8). We conclude that, in contrast to HPV-31b, the E2 protein of HPV-33 does not recruit E1 to the E2BS in the presence of excess E2. We feel rather that the E1/E2 complex binds both the E1BS and the E2BS, even though the complex can bind to the E2BS in the absence of E1BS and vice versa. Similar observations have been made by Sun et al. (1996) who found that the E1 and E2 proteins of HPV-11 bind to their binding sites and that complex formation increases the binding activities of both proteins.

Taken together, we have shown that E2 of HPV-33 binds with high affinity to all four consensus E2BS present in the LCR and that a putative E2BS with one mismatch is not bound by E2. We have furthermore demonstrated that E1 specifically protects HPV-33 nt 7896 to 18 from DNaseI digestion and that the E1/E2 complex protects the E1BS as well as the E2BS in the ori. Further experiments will hopefully help to unravel whether the E1/E2 complex binds cooperatively to ori and which of the E2BS are more important for binding the complex.

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References


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