Cooperative Assembly of the Bovine Papilloma Virus E1 and E2 Proteins on the Replication Origin Requires an Intact E2 Binding Site*

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Using quantitative gel retardation assays the properties of the bovine papilloma virus (BPV) origin recognition protein E1 and the effect of the viral E2 protein on the binding of E1 to BPV origin DNA were examined. As reported previously (Seo, Y. S., Mueller, F., Lusky, M., Gibbs, E., Kim, H.-Y., Phillips, B. and J. Hurwitz (1993) Proc. Natl. Acad. Sci. U. S. A., 90, 2865-2869), the E1 protein binds specifically to DNA sequences within the BPV origin (ori*) of replication. We also show that the presence of MgCl₂ and ATP could stabilize the E1-ori* DNA complex. At low levels of E1, ori* DNA binding was greatly stimulated by the viral E2 protein when the intact E2 binding site 12 was present on the DNA. In addition DNA-protein complexes formed in the presence of both E1 and E2 were more stable than those formed with E1 alone. In the absence of an E2 binding site the E2 protein inhibited the binding of E1 to the BPV origin. Spacing of 0 or 9 base pairs between the E1 binding site and the E2 binding site 12 abolished the stimulation of E1-DNA binding by E2, whereas spacing of 6 base pairs between the two binding sites allowed for efficient stimulation. The data presented account for a direct role of E2 in BPV DNA replication. We propose that the cooperative binding of both the E1 and E2 proteins to BPV ori* DNA is mediated by protein-protein interactions and by protein-DNA interactions, which include the formation of specific contacts of E2 with DNA.

Bovine papilloma virus (BPV)† provides an interesting system to study the control of replication because the proviral DNA is maintained as a freely replicating plasmid at a constant copy number in BPV-transformed rodent cells (1). Only two viral proteins, E1 and the viral transactivator E2, are required for BPV DNA replication in vitro (2). In vivo and in vitro studies (3, 4) also identified a minimal BPV origin (ori*) sequence (nt 7911-22 of BPV DNA) that contains a binding site for E1 (E1 BS) centered around the unique HpaI site of the BPV DNA and half of an E2 binding site (E2 BS12). A cell-free in vitro replication system has been described (4) in which DNA synthesis was absolutely dependent on the BPV minimal ori* and on the E1 protein. It was also reported that the E2 protein markedly stimulated the replication reaction in the presence of E1, and at low levels of E1, E2 was absolutely required (4).

Recently we described the overexpression and purification of the BPV E1 protein (5) and confirmed that E1 supported the synthesis of BPV ori* DNA in vitro. These studies also showed that E1 shared a number of biochemical characteristics with the SV40 T antigen (6-8) including a DNA-dependent ATPase activity, DNA helicase activity, ori* DNA binding activity, and the ability to unwind superhelical ori* DNA, leading to the production of highly unwound DNA. These results clearly established E1 as an initiator protein for BPV replication.

The role of the E2 transactivator protein in BPV replication is less clear. The E2 protein could indirectly affect BPV DNA synthesis by regulating the expression of viral and cellular genes required for replication. However, in vivo studies showed that the absolute requirement for E2 remained even when the expression of the E1 and E2 genes was driven from heterologous promoters and thus was uncoupled from each other (2). Moreover, it was shown that the transactivation function of E2 is not required for its role in replication (9). Together, these results indicate that E2 might play a more direct role in BPV replication. The full-length E2 protein can form a complex with the E1 protein in solution (10, 11), suggesting that protein-protein interactions between E1 and E2 might be important for some stages of BPV DNA synthesis.

In order to gain insight into the initial steps of BPV DNA synthesis we have investigated in more detail the binding of the E1 protein to the BPV origin DNA. Using gel retardation assays the influence of E2 on the binding of E1 to ori* DNA was examined. Furthermore, the effect of spacing between the E1 BS and the E2 BS12 within the origin was probed. Our results and those described elsewhere (12) demonstrate that the presence of the complete E2 BS12, next to the E1 BS, is crucial for the enhancement of E1 binding and for the stabilization of E1 at the origin by the E2 protein.

MATERIALS AND METHODS

E1 and E2 Proteins—The generation of recombinant baculoviruses containing the wild type E1 and E2 genes were described previously (11) as was the purification of E1 (5). The E2 protein was purified by DNA affinity chromatography (13) with the modifications described by Seo et al. (12).

Antisera—The use of polyclonal E1 antisera (anti-E1) was described previously (11), whereas polyclonal E2 antisera (anti-E2) were a generous gift from Dr. E. Androphy (Tufts University, Boston).

DNA Probes—The BPV sequences of the plasmid DNAs used in these studies are shown in Fig. 1. Plasmid pKSO (nt 7806-100; Ref.
**Results**

**Specific Binding of Purified E1 Protein to BPV ori+ DNA**—Previous studies have shown that the BPV E1 protein binds to BPV ori+ DNA (2, 4, 14). In order to gain further insight into the properties of E1 binding and to examine the effect of the BPV E2 protein on E1 binding, we sought to establish a quantitative gel retardation assay. Fig. 2 demonstrates that the binding of E1 to ori+ DNA, but not to ori- DNA (see also Fig. 9), resulted in a DNA-protein complex with retarded mobility compared with that of free DNA. Several points are
were assembled and processed as described under “Materials and the ori’ DNA (pKSO) fragment under the conditions indicated above the lanes. After incubation, reactions were terminated with glutaraldehyde and DNA-protein complex formation was analyzed by electrophoresis through a 1.8% horizontal agarose gel in 0.5 to ori’ DNA. DNA binding reactions containing 100 ng of El protein or addition of the indicated components: which were normally between 1 and 4, omission of nonspecific competitor DNA and the addition of 600 fmol (20-fold molar excess) of unlabeled ori’ DNA; lane 7, with the level of MgCl₂ used (Fig. 4, A and B, lanes 11 and 12). Increasing amounts of El in this assay reduced the stimulation effect to between 3- and 4-fold (Fig. 4, A and B, lane 14). The data presented also show that the DNA-protein complexes formed in the presence of El plus E2 migrated on the gels as broad bands and included species that migrated faster than those obtained with El alone (see also Figs. 6 and 8). We did not observe DNA-protein complexes that migrated significantly slower than those obtained with El alone.

Under these assay conditions, stable E2 binding to BS11 and BS12 contained within pKSO DNA, in the absence of E1, was not observed above background levels (Fig. 4, A and B, lanes 8) with the level of E2 used (60 ng). Using the same assay conditions, binding of E2 to BS10 was observed (see Fig. 11). It was reported previously that E2 binding to BS11 and BS12 was 11- and 100-fold lower than the binding to BS10 (15). In our studies we observed weak E2 binding to omission of nonspecific competitor DNA and the addition of 600 fmol of ori’ (pUC19) DNA. Numbers at the bottom indicate the amount of complex formation.
Origin Recognition by the BPV E1 and E2 Proteins

Bound DNA → Free DNA

A.

- MgCl₂ - ATP
+ MgCl₂ + ATP

Time (min)

0 2 4 8 16

0 2 4 8 16

Bound DNA

Free DNA

B.

BS11 and BS12 only in McKay assays (data not shown).

To ascertain that the stimulatory effect was due to both the E1 and E2 proteins present in the DNA-protein complexes, E1- and E2-specific antibodies were included in the binding reactions, and supershift assays were performed. Addition of either antibody to DNA in the absence of proteins did not alter the migration of free DNA (Fig. 5, lanes 9 and 15). However, the addition of anti-E1 to reactions containing either E1 alone (lanes 10 and 11) or E1 plus E2 (lanes 12 and 13) further retarded the migration of the DNA-protein complexes. In the presence of E2 antibodies, the migration and amount of E1-DNA complex formed in the absence of E2 was not changed (lanes 16 and 17), confirming the specificity of the antibody preparation. However, the addition of anti-E2 to reactions containing both E1 and E2 proteins resulted in a supershift of DNA-protein complexes, indicating the presence of E2 in the respective complexes. In all reactions where the addition of either E1 or E2 antibodies resulted in supershifted DNA-protein complexes, the amount of complex formed was also increased. We do not believe that this activation is specific as recently shown for the activation of p53 binding by certain monoclonal antibodies (16), since the antibodies used here were polyclonal. It is likely that in our case the antibodies stabilized the complexes during the electrophoretic separation and thus increased the amount of complex formed.

Taken together, the results presented in Figs. 4 and 5 suggest that the interplay between E1 and E2 mediates cooperative binding of E1 to the BPV origin DNA in the presence of E2 BS11 and E2 BS12.
Bound after tions contained MgCl₂ and ATP. In the panels marked the conditions described under “Materials and Methods.” All reactions contained MgCl₂ and ATP. Quantitation of these data (Fig. 6) showed that the half-life of the DNA-protein complex formed in the presence of El plus E2 was extended approximately 2-fold over the half-life of the complex formed with E1 alone. These results suggest that E2 not only enhances E1 binding to ori⁺ DNA but also stabilizes the DNA-protein complex.

Stimulation of E1 Binding by E2 Requires the Presence of the Intact E2 BS12—Previous reports on the physical association between the E1 and E2 proteins (10, 11) suggested that the interaction between E1 and E2 might play a role in the recognition of the origin by E1. Because of this, we determined whether the presence of an E2 BS (and thus E2 binding) was required for the stimulatory effect of E2 on the binding of E1 to ori⁺ DNA. The binding of E1 in the absence and presence of E2 was monitored using the DNA substrate pUCOMBS12, which contains the BPV sequences nt 7911–16, including the E1 BS, but has both the E2 BS11 and BS12 deleted (Fig. 1). Fig. 7 demonstrates that BPV sequences beyond nt 16 were not required for efficient E1 binding (lanes 2–4). However, the presence of E2 did not stimulate E1 binding to pUCOMBS12 DNA (lanes 5–7). The addition of E1 antibodies resulted in a supershift of the complex, both in the absence and presence of E2 in reactions containing E1 (lanes 10–13). The addition of E2 antibodies did not alter the mobility of the DNA-protein complexes in reactions where E1 plus E2 were present (lanes 18 and 19). We conclude from these results that E1-E2 protein interactions alone are not sufficient to account for the stimulatory effect observed with pKSO DNA.

The results shown in Fig. 7 (lanes 2–7) also suggest that in the absence of an E2 BS the binding of E1 was inhibited by E2. This was examined by adding increasing amounts of E2 to reactions that contained 50 ng of E1 and comparing complex formation with pKSO DNA and with pUCOMBS12. Fig. 8 shows that with pKSO DNA E1-dependent DNA-protein complex formation increased with increasing amounts of E2 (lanes 3–5). In contrast, using pUCOMBS12 DNA, the amount of DNA-protein complex was reduced by increasing amounts of E2 (lanes 9–13). At the highest levels of E2 used (60 and 120 ng), the amount of complex formed was reduced 5-fold. These results confirm those described in Fig. 7 and indicate that E2 inhibits E1 binding in the absence of an E2 BS. One possible interpretation is that at high levels of E2 complex formation between E1 and E2 is favored over complex formation between E1 and DNA and that a preformed E1-E2 protein complex is not active in binding to the E1 binding site in the absence of an E2 BS.
Origin Recognition by the BPV E1 and E2 Proteins

The effect of E2 on E1 binding was also examined using pUCOM DNA (nt 7911–22) containing the entire E1 BS and half of the E2 BS12 (see Fig. 1). Under the conditions described in the experiments shown in Figs. 6 and 8, E2 stimulated E1 binding to pUCOM DNA only 2-fold (data not shown; Ref. 12). Together, the results obtained with pUCOM\(\Delta\)BS12 and pUCOM DNAs suggest that for the cooperative binding observed with E1 plus E2 on pKSO DNA, binding of E2 to either E2 BS11 or BS12 is required.

In vivo (3) and in vitro (4) studies indicated that E2 BS11 was dispensable for origin function. Thus, the stimulatory effect of E2 on E1 binding to wild type pKSO DNA is most likely mediated through protein-DNA interactions which involve E2 BS12 (see below).

The binding of E1 in the absence and presence of E2 was also measured using an ori\(^+\)-DNA substrate, pUC0-Xho. This DNA contains an intact E2 BS12 and a XhoI linker inserted in the center of the E1 BS (Fig. 1). In vivo studies have shown that this DNA template does not support DNA replication in the presence of E1 and E2 (3). In addition, a similar linker insertion did not support El- and E2-dependent DNA synthesis in vitro (4). Fig. 9 shows that hardly any complex formation occurred with increasing amounts of E1 in the absence of E2 (lanes 2–4). However, E2 addition stimulated the complex formation (lanes 5–7), although the amount of complex formed was considerably lower than that observed with pKSO DNA. The data presented in Fig. 9 establish that the presence of the intact E2 BS12 is crucial for the stimulatory effect of E2 on E1 binding to ori\(^+\) DNA and for the stability of ori\(^+\) DNA-protein complexes.

Influence of Spacing between the E1 BS and E2 BS12 on

**Fig. 7.** Lack of stimulation of E1 binding to the E1 BS in the absence of an E2 BS. Increasing amounts of E1, in the absence (−) or presence (+) of 60 ng E2 protein, with or without antibodies (as indicated), were incubated with 30 fmol of \(^{32}\)P-labeled pUCOM\(\Delta\)BS12 DNA fragment (see Fig. 1). Reactions containing MgCl\(_2\) and ATP were assembled and processed as described under “Materials and Methods” and in the legend to Fig. 5. The amounts of complex formed (femtomoles) are presented at the bottom of the figure.

**Fig. 8.** E2 inhibits the binding of E1 to BPV ori\(^+\) DNA in the absence of an E2 BS. A, 50 ng of E1 and increasing amounts of E2 (as indicated) were incubated with labeled pKSO DNA (lanes 1–7) or pUCOM\(\Delta\)BS12 DNA fragments (lanes 8–14) and the formation of DNA-protein complexes was analyzed. B, quantitation of the data shown in A. Complex formation (expressed in femtomoles) was measured in the absence (0) or presence (100) of 60 ng of E2 protein.

**Fig. 9.** Influence of E2 on the binding of E1 to BPV ori\(^+\) DNA. A, increasing amounts of E1 in the absence (−) or presence (+) of 60 ng of E2 protein were incubated with the \(^{32}\)P-labeled pUCO-Xho DNA fragment (see Fig. 1). Complex formation was analyzed as described. B, quantitation of the data shown in A. Complex formation (expressed in femtomoles) was measured in the absence (○) or presence (●) of E2.
the Effect of E2 on E1 Binding—To gain further insight into the mechanisms underlying the enhancement effect of E2, we asked whether the distance between the two binding sites, E1BS and E2BS12, was important. Three mutant substrates were generated and compared with pKSO DNA. pUCOM-BS12-0 has the 3 bp (CAC) (nt 13-15) between the E1BS and E2BS12 deleted, whereas pUCOM-BS12-6 and pUCOM-BS12-9 contain 6 and 9 bp, respectively, between the E1 BS and the E2 BS (see Fig. 1). The binding of E1 in the absence and presence of E2, as well as in the absence and presence of E1 and E2 antibodies was examined. The results are presented in Fig. 10. E1 alone interacted with all three substrates with equal efficiency (Fig. 10, A and B, lanes 2-4, and data not shown), indicating that the 3 bp 3' to the “E1 palindrome” did not contribute to the E1 BS. The presence of E2 neither stimulated nor decreased the amount of E1-DNA complex formed when pUCOM-BS12-0 (Fig. 10A, lanes 5-7) and pUCOM-BS12-9 (data not shown) were used as substrates. Addition of E1 antibodies supershifted the DNA-protein complexes formed both in the absence and presence of E2 (Fig. 10 A and B, lanes 10-13). However, the addition of E2 antibodies had no effect on the mobility of the DNA-protein complexes (Fig. 10A, lanes 18 and 19). We conclude from these results that deletion of the 3 bp between the E1 BS and the E2 BS (pUCOM-BS12-0) or extending the distance to 9 bp (pUCOM-BS12-9) interfered with efficient E1-E2 protein interactions and protein-DNA interactions.

Surprisingly, the results obtained with pUCOM-BS12-6 DNA (Fig. 10B) which contains a total of 6 bp (CACCAC) between the E1 and E2 binding sites were similar to those obtained with wild type pKSO DNA (Fig. 5). Maximal stimulation of DNA-protein complex formation (16-fold) in the presence of E2 was observed at low levels of E1 (25 ng), whereas the stimulation decreased (2.3-fold) at the highest amount of E1 used (100 ng). The addition of E2 antibodies clearly caused a supershift in the DNA-protein complex when both E1 and E2 were present (lanes 18 and 19), whereas the migration of complexes formed with E1 alone was unaffected by anti-E2 (lanes 16 and 17). These data, together with those shown in Fig. 5, demonstrate that efficient E1-E2 protein and protein-DNA interactions occur at a spacing of either 3 bp (pUCOM-BS12-0) or 6 bp (pUCOM-BS12-6) between the two binding sites, but not when the binding sites are too close together (pUCOM-BS12-0) or too far apart (pUCOM-BS12-9). Experiments to test whether the separation of the two binding sites by 1 or 2 and 7 or 8 bp influences the stimulatory role of E2 on E1 binding are currently under investigation. When the E2 BS12 was placed more distal, e.g. 30 bp 3' or 35 bp 5' to the E1 BS, E2 had no effect on E1 binding (data not shown).

Effect of the Strength of the E2 BS on the Influence of E2 on E1 Binding—It was of interest to ask whether the strength of the E2 BS next to the E1 BS in wild type BPV ori+ DNA was important. For this purpose E2 BS12 was replaced with the strong E2 BS10 (15) generating pUCOM-BS10 DNA (Fig. 1). With this substrate, significant binding with E2 alone was observed (Fig. 11, lane 8). This result shows that under the conditions described here for binding and gel retardation E2 binding to a strong binding site such as BS10 can indeed be monitored; however, these conditions do not lead to stable binding of E2 to the weak E2 BS12.

The addition of E2 to reactions containing E1 resulted in a marked stimulation of DNA-protein complex formation (Fig. 11, lanes 5-7). However, when the amount of complex formed with E2 alone was subtracted from the amount of complex formed with E1 plus E2, it was apparent that complex formation was proportional to the amount of E1 added. Thus a strong E2 binding site, such as BS10, used in place of the weak E2 BS12, resulted in a stimulation of E1 binding by E2 but did not give rise to the sigmoidal response observed with wild type BPV ori+ DNA. Similar results were obtained when BS10 was placed 30 bp distal to the E1 BS (data not shown).

**DISCUSSION**

Using quantitative gel retardation assays, we have demonstrated that the BPV E1 protein specifically recognizes and binds to BPV ori+ DNA, but not to ori- DNA (Figs. 2 and 9). Efficient E1-ori+ DNA complex formation was observed in the absence and presence of MgCl2 and ATP (Figs. 2 and 4). Interestingly, the amount of complex formed showed a sigmoidal response to the amount of E1 added, regardless whether the binding was carried out in the absence or presence of MgCl2 and ATP or in the presence of MgCl2 alone. This cooperative binding suggests that E1 forms multimers at the two half-sites of the BPV origin.

The ability of E1 to bind ATP (17, 18) appears to be important for its replication functions, since a mutant in the ATP binding domain was shown to be replication defective in vivo (11, 17) and in vitro.2 Thus, ATP binding to E1 might induce conformational changes or, alternatively, might mediate the formation of multimeric structures of defined size, analogous to what has been observed with SV40 T antigen (19, 20). In addition ATP-induced structural changes of the E1 protein could affect the stability of E1-ori+ DNA com-

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2 M. Lusky, unpublished observations.
plexes. In support of this we observed that complexes formed without E2 was measured in the absence and is also indicated (○).

ATP on the structure of El in the absence and presence of the E2 BS10 in place of BS12. Moreover, increasing amounts of E2 seemed to interfere with the binding of E1 to this DNA, but not to wild type pKSO DNA (Fig. 8). These results indicate that maximal E2-mediated stimulation of E1 binding to the BPV origin requires the presence of an intact E2 BS. We conclude from these results that E1-E2 protein-protein interactions alone are not sufficient for the E2 enhancement of E1 ori recognition. In fact, the results presented in Fig. 8 suggest that formation of a productive E1-E2 protein complex on the origin DNA is regulated by and requires specific contacts of both proteins with the DNA. Furthermore, these data are consistent with the idea that a preformed E1-E2 protein complex might be in a nonproductive conformation which prevents or interferes with origin recognition at least in the absence of an E2 binding site. This notion is not without precedent; it was shown that preformed hexameric structures of SV40 T antigen rendered the protein inactive for binding to the site II core origin (19).

Interestingly, the results shown in Fig. 9, together with those described previously (12), indicate that E1 DNA complex formation can be stimulated by E2 in the presence of E2 BS12 and one half-site of the E1 palindrome (pUCO-Xho), whereas E1 binding or E2 binding alone to this DNA was not observed. These results further indicate that E2 plays a direct role in targeting E1 to its binding site. We have speculated that E1 might be assembled on two half-sites of the E1 palindrome, and one multimer interacting with E2 complexed with BS12 may facilitate the assembly of the other multimer on the “far” side of the E1 palindrome (12).

Taken together, our results imply that E2 binding to BS12, located next to the E1 BS, is required for the stimulation and stabilization of E1 origin recognition and binding. Our data are entirely consistent with the requirements observed for the in vivo replication of BPV in transient transfection assays (3). Those studies (3) also reported that a deletion of the most carboxyl-terminal 33 amino acids, including the DNA binding and dimerization domains of E2, rendered the resulting E2 mutant defective in supporting BPV replication in vivo. In addition, E2 mutants only affecting the DNA binding, but not the dimerization domain, failed to support BPV DNA synthesis, thus indicating that the DNA binding activity of E2 is required for replication (3). In a different study (9), however, a more complex set of phenotypes was observed with carboxyl-terminally truncated E2 mutants. In those studies (9) the same mutation as described by Ustav et al. (Ref. 3; deletion of 33 amino acids at the carboxyl terminus) also failed to support BPV replication. In contrast, certain E2 mutants missing more than 33 amino acids from the carboxyl terminus could support replication although at reduced levels. It is possible that the latter mutations lead to certain conformational changes in the E2 protein that could influence protein-protein and or protein-DNA interactions. Thus, it will be of interest to determine the effect of these E2 mutants on the binding of E1 to BPV ori DNA in the absence and presence of E2 BS12.

Mutant BPV origin DNA substrates were used to further investigate the influence of E2 on E1 binding. The results presented in Fig. 10 showed that the spacing between the E1 BS and E2 BS12 within the BPV origin appears crucial for the stimulatory effect of E2. Deletion of the 3 bp (nt 13-15)
between the E1BS and E2 BS12 (Fig. 10A) or insertion of a total of 9 bp (data not shown) between the two binding sites completely repressed the stimulatory function of E2. These data were further substantiated by the results that the DNA-protein complexes formed in the presence of E1 and E2 were not supershifted by E2 antibodies. This indicates that E2 was not stably engaged in active E1 containing DNA-protein complexes.

Surprisingly, when the E1 and E2 binding sites were spaced apart from each other by a total of 6 bp (pUCOM-BS12-6) E2 stimulated the binding of E1 (Fig. 10B) as well as it stimulated the binding of E1 to wild type BPV ori+ DNA (pKSO). The antibody supershift experiments demonstrated that E2 was part of the DNA-protein complexes formed in the presence of both the E1 and E2 proteins. Together, these data indicate that a rather precise spacing is required between the two binding sites in order to engage the E1 and E2 proteins into a complex which results in cooperative binding.

Interestingly, it seems important that the E2 BS12 within the BPV origin is one of the weak E2 binding sites. As shown in Fig. 11 the E2 stimulation of E1 binding to BPV ori+ DNA which contained the E2 BS10 instead of BS12 at the wild type position (pUCOM-BS10) did not reflect cooperative binding. It appeared that the E1 and E2 proteins bound independently and did not communicate with each other.

The data described in this study support a direct role of E2 in BPV DNA replication and indicate that E1-E2 protein-protein and E1-E2/DNA interactions are a critical feature in the initial stages of BPV DNA synthesis. It will be important to investigate the subunit structure of E1-E2 protein complexes in the absence and in the presence of BPV origin-containing DNA to gain insight into the mechanism of assembly of the origin recognition complex.

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