Mechanism and Requirements for Bovine Papillomavirus, Type 1, E1 Initiator Complex Assembly Promoted by the E2 Transcription Factor Bound to Distal Sites*

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DNA replication of papillomavirus requires the viral initiator E1 and the transcription factor E2. Bovine papillomavirus, type 1 (BPV-1), E1, and E2 bind cooperatively as dimers to proximal sites in the viral replicator generating a sequence-specific E1E2-ori complex. This complex is critical for replication and can be converted to a multimeric E1-ori initiator complex by displacement of E2 in the presence of hydrolyzable ATP. However, E2 can function over extended distances, and E2 at a distal position 33 base pairs upstream of the E1-binding site also loads an E1 dimer onto ori. Under these conditions, neither displacement of E2 nor ATP hydrolysis are required for E1-ori formation, consistent with a need for ATP hydrolysis in E2 displacement from E1E2-ori. However, ATP is required for stabilization of the resulting E1-ori complex. These results indicate that BPV (with a proximal E2-binding site) and human papillomaviruses (with distal E2-binding sites) utilize the same general mechanism for E1 loading but suggest that E1E2-ori, which forms preferentially on ori, may perform an additional role in BPV replication.

The papillomaviruses are an extensive family of closely related DNA viruses. Infection with some human papillomaviruses (HPV)† is associated with a risk of developing certain cancers. For example, HPV-16 and -18 are strongly implicated in the etiology of cervical cancer. Many other HPV types infect the mucosal and cutaneous epithelia causing benign papillomas or warts (1, 2). The fact that papillomaviruses can exist in a latent state imparts an additional degree of complexity to the clinical infection. The papillomaviruses are also important models for eukaryotic gene expression and replication. An understanding of genome regulation in bovine papillomavirus type 1 (BPV-1), the prototype of the group, is eclipsed perhaps only by bacteriophage λ in prokaryotes and simian virus 40 in eukaryotes. However, BPV-1 offers several distinct advantages over other eukaryotic viral models in regard to these studies. In the latent state, the viral chromosome is maintained at a constant low copy number, directs a low level of gene expression, and replicates in synchrony with the host cell chromosome (3). Thus, in several important aspects, the viral genome behaves much like the host genome.

Replication of BPV-1 requires only two viral proteins, E1 and E2, that perform the early steps in replication initiation. E1 has all the activities of an initiator protein including DNA binding, origin melting, and perhaps the ability to act as a regulatory component of the initiation reaction (4–9). It is also a helicase, responsible for processive DNA unwinding (10–12). The E2 protein is a prototypic eukaryotic transcription factor and the major transactivator and regulator of BPV-1 transcription (reviewed in Ref. 13). The role of transcription factors in the initiation of DNA replication is a recurrent, yet poorly understood, theme in eukaryotes. Possible functions of transcription factors include chromatin remodeling to expose the DNA sequence determinant of origin specificity, recruitment of general replication factors, a role in origin melting by DNA looping or bending, and targeting and assembly of the initiator complex (reviewed in Ref. 14). For BPV-1, the latter is the focus of much investigation, since it appears to explain the absolute and specific requirement of E2 for papillomavirus replication in vivo. It is also intimately linked to the intriguing ability of E1 to perform multiple biochemical functions that can be assigned to different assembly states of E1. From these studies the concept of an assembly pathway for the E1 helicase, governed by transcription factor E2, has emerged (9, 15–17).

The minimal BPV-1 origin of replication (ori) consists of binding sites for E1 and E2 and an A/T-rich region (7, 18–21). We have characterized extensively a replication pre-initiation complex that forms on the origin with the E1- and E2-binding sites separated by 3 base pairs (16, 22–24). This configuration is found only in a subgroup of papillomaviruses that generate lesions with both a fibroblastic and epithelial cell component. The complex, E1E2-ori, is most likely a dimer of E1 bound cooperatively with a dimer of E2 (25). The physical interactions between the two proteins, which are critical for cooperativity, map to two distinct regions. An interaction between the DNA binding domains of each protein appears to facilitate a second interaction between the E2 activation domain and E1 helicase domain (25–32). Formation of this complex, which forms with high specificity and affinity, may be critical for the initiation of DNA replication since E1 is expressed at low levels in infected cells and binds DNA with low specificity and affinity (22). In vitro, E1E2-ori can be converted to a higher order E1-ori complex. Conversion is ATP-dependent and results in the displacement of E2 and the incorporation of additional E1 molecules (16). The function of E1-ori appears to be stable and specific E1 binding, and it also forms the core of a higher order origin melting complex. E2 bound to distant binding sites assists formation of this complex (17). Thus, E2 appears to have two distinct but linked roles in initiator complex assembly, acting...
as a specificity factor for E1 binding and a general assembly factor. A second E2-binding site of intermediate affinity (BS11) exists in close proximity to the BPV-1 minimal origin of replication, 35 bp upstream of the E1 site (Fig. 1). Constructs with only E2 BS11 replicate efficiently in vitro (15), and binding of E2 to this site readily stimulates formation of E1-ori and the origin melting complex in vitro (17). Here, we have explored how E2 bound to a distal E2 site recruits E1 and assembles E1-ori. The results reveal that E2 serves similar roles at proximal and distal sites and initially recruits an E1 dimer to ori. However, E1E2-ori formation at a proximal E2 site may have an additional role in replication, possibly as a regulatory complex. BPV-1 origins with distal E2-binding sites resemble the replication origins of HPV's, including those in the oncogenic group, which lack an E2 site proximal to the E1-binding site (3). Like BPV-1, HPV replication is E2-dependent, and an E1E2-ori-like complex appears to form on HPV origins (32–34). The results presented here describe further the early events in E1 complex assembly and may also be useful to model the early events in HPV replication, for which there is currently little available data.

MATERIALS AND METHODS

Viral Proteins, Antibodies, and Origin Probes—E1 and E2 proteins and anti-E2 and HA epitope antibodies have been described previously (16, 24). Origin probes were constructed from BPV-1 nucleotides 7894 to 27 cloned into pUC19. Nomenclature is based on the wild type template with E2-binding sites (BS) 11 and 12, denoted 11/12. Templates were modified to alter the affinity of E2-binding sites as described previously (17). E2 BS9 is a high affinity site; E2 BS11 is an intermediate affinity site, and E2 BS12 is a low affinity site (36); "X" indicates mutation of the E1-binding site. A single prominent complex, which was not detected with E1 alone, formed with E2 on the probe 11/X with distal E2 BS11 and A/T-rich region. The low affinity E2 BS12 is located 3 base pairs upstream of the E1-binding site; either E2 site alone supports replication in vivo. In HPV, the E1-binding site is positioned between two distal E2 sites, one of which is duplicated. In HPV-18, the up- and downstream sites are 26 and 23 base pairs, respectively, from the boundaries of the E1 palindrome. Only one E2 site is required for replication of chimeric HPV-18 origins, but replication efficiency improves with multiple sites (35). The nomenclature for the BPV-1 origins used in this study is as follows. The wild type probe with E2 BS11 and BS12 is referred to as ori 11/12. Origins with BS11 or BS12 only are referred to as 11/X (distal) and X/12 (proximal).

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Fig. 2. Complex formation with E1 and E2 bound to distal sites. A, binding reactions with the indicated probes were analyzed on polyacrylamide gels. The concentration of E1 used was 20 nM except lanes 21–23 (100 nM). The concentrations of E2 were from 1.25 to 3.75 nM and 3.75 nM without E1. For probes 11/X (distal E2 site) and X/12 (proximal E2 site), selected reactions were examined with anti-E1 and -E2 antibodies (lanes 7–10 and 16–19). Lanes 21–23 show complex formation at high E1 concentration without E2; six bands were observed. Lanes 24 and 25 indicate a small mobility difference between the E1-E2 origin complexes formed on probes 11/X or X/12. Lanes 26–43 demonstrate the dependence of E2E1-ori complex formation on E2 site affinity. With the probe 11/12 (proximal and distal E2 sites, lanes 46–49), the complex that formed at low E2 concentrations co-migrated with E1E2-ori (lane 24), and formed more efficiently than the complex on probe 11/X (lanes 3–5). B, E1E2-ori and E2E1-ori formation assessed by glutaraldehyde cross-linking and agarose gel electrophoresis, without (top panel) and with ethidium bromide (25 μg/ml, lower panel). The same reactions as in A were analyzed. Only E2E1-ori formed on probes with a high affinity E2 site were detected (lanes 10–12, BS9, compared with 16–18, BS 11). Instability due to cross-linking appears to account for this in part. All complexes were unstable in the presence of EtBr (lower panel).

(lanes 2–5). This complex, now termed E2E1-ori, migrated slower than E2-ori (lane 6). Curiously, less probe was shifted in the presence of E1 for reactions that contained the same concentration of E2 (compare lanes 5 and 6). Anti-E2 polyclonal antibodies supershifted E2E1-ori and E2-ori (lanes 4 and 6 compared with lanes 7 and 8), but only E2E1-ori was supershifted with anti-E1 antibody (lanes 9 and 10). A similar complex, which migrated slightly slower than E2E1-ori and was supershifted with anti-E1 and -E2 antibodies, formed with E1 and E2 on the probe with proximal E2 BS12 (X/12, lanes 12–20). This most likely corresponds to the E1E2-ori complex described previously (22). At high E1 concentrations, in the absence of E2, a series of protein-DNA complexes formed that bind anti-E1 but not anti-E2 antibodies (lanes 21–23). Six bands can be discerned, the largest of which is the most prominent form.

We also replaced the distal E2 BS11 with high and low affinity E2-binding sites (36). With the high affinity E2 BS9 in the distal position (probe 9/X, lanes 27–30), the E2E1-ori complex formed more efficiently than with probe 11/X (lane 25 compared with 30). The low affinity E2 BS12 at the distal position supported E2E1-ori formation to a lesser extent (lanes...
Fig. 3. E2 bound to a distal site recruits E1 to the E1 palindrome. Parallel binding reactions were assembled with each probe (E1, 35 nM; E2, 2.5–20 nM; 20 nM in reactions with E2 only). E2 bound to a distal binding site stabilizes E1 binding to its palindrome (lanes 1–7 and 15–21), and E1E2-ori forms more readily than E2E1-ori on probes with the wild type E2 sites (lanes 1–7 compared with 8–14). Footprints for E1 and E2 bound to DNA can be clearly discerned and suggest that E1 is bound in the same form in each complex. The proportion of unbound probe did not appear to be lower in reactions with E1 and the same concentration of E2, as observed by polyacrylamide gel electrophoresis. The former observation is likely to be an artifact of gel electrophoresis.

33–36), whereas a probe lacking an E2-binding site failed to form an E2E1-ori complex (lanes 39–42). As noted above for the probe 11/X, in the presence of E1 less probe was shifted than by E2 alone (compare lanes 36 and 37). On probe 11/12, which corresponds to the arrangement in the wild type ori, a complex that co-migrated with E1E2-ori formed at low E2 concentration (lanes 46 and 47 compared with 24). Upon addition of more E2 (lane 48) a larger complex of unknown composition was formed. We conclude, based on these results, that E2 bound to a distal E2 site can recruit E1 to ori. The complex that forms, E2E1-ori, resembles E1E2-ori, but complex formation from the distal E2-binding site is less efficient than from a proximal E2-binding site with similar affinity.

E1E2-ori, but not the multimeric E1-ori complexes, is unstable in the presence of low concentrations of ethidium bromide (EtBr, 25 μg/ml) (16, 17). The reactions with probes X/12, 9/X, and 11/X, examined above, were also examined by agarose gel-shift following glutaraldehyde cross-linking, with and without EtBr treatment. In Fig. 2B, top panel, lane 1 shows E1-ori formation. The extent of E1E2-ori formation on probe X/12, is similar to that seen by polyacrylamide gel-shift (compare lanes 2–7 with Fig. 2A, lanes 13–15). For probe 9/X, E2E1-ori was detected but only at 50% that observed by polyacrylamide gel electrophoresis (compare lanes 8–12 with Fig. 2A, lanes 28–30). E2 binding was not detected after cross-linking, as observed previously (lane 13). For probe 11/X, E2E1-ori was not detectable under these assay conditions. In the lower panel, EtBr (25 μg/ml) was added to samples 1–19. Like the E1E2-ori complex, E2E1-ori is unstable in the presence of EtBr (lanes 4–6 and 10–12 upper and lower panels). Thus, sensitivity to EtBr can be used to differentiate the E2E1-ori complex from the multimeric E1-ori complexes and suggests that E1 is bound to DNA in a similar low oligomeric form as in E1E2-ori.

High Resolution Footprinting Shows Occupancy of the E1- and E2-binding Site in E1E2-ori—To investigate the disposition of proteins on the DNA in E2E1-ori, complexes were footprinted with OP-Cu. By using probes with E2-binding sites of varying affinity and position, E2 was titrated at low concentration of E1. Reactions were treated with OP-Cu, quenched, and the DNA recovered for analysis on a sequencing gel (Fig. 3, top strand). On probe 11/X (lanes 1–7), E2 completely protected the 12-base E2 palindrome and one or two more bases up- and downstream (lane 7). A similar protection was observed on the bottom strand and for all E2 sites tested. With E1 and E2, the 18-base E1 palindrome and an additional 2 bases up- and downstream were also protected (lanes 3–6, compared with 2 and 7 as indicated in the annotations to the right). Cleavage of the DNA between the two binding sites was also slightly reduced. On probe 11/12 (lanes 8–14), E2 protected BS11, and there was a similar weak protection over BS12 (lane 14). With E1 and E2 (lanes 9–13), the E1-binding site was occupied at a lower concentration of E2 compared with probe 11/X, and E2 BS12 was completely protected (compare lanes 3–7 with 9–13). With probe 9X, the E1-binding site was clearly protected in the presence of E1 and E2 at the lowest concentrations of E2 where BS9 was fully occupied (lanes 17–20). Cleavage of all DNA between the two binding sites was reduced 15–30% depending on the position. For probe X/12 (lanes 22–28), the E1 and E2 sites were completely protected at low concentrations of E1 and E2. The E1E2-ori protection over E2 BS12 and the E1-binding site was identical to that observed for probe 11/12. Solution footprints and in-gel footprints obtained with OP-Cu were identical (data not shown). Therefore, the complexes characterized in polyacrylamide gels are likely to be identical to those characterized in solution, and the weak protection between the E1- and E2-binding sites in E2E1-ori is unlikely to be due to the binding of additional protein molecules in a sub-set of the complexes.

In summary, E2 bound to a distal site recruits E1 to the E1-binding site to form E2E1-ori, but E1E2-ori forms preferentially on the wild type sequence. E2 bound to BS11 does not impair E1E2-ori formation but may assist it (compare lanes 10–13 with 24–27). The protections suggest that E1 is bound to DNA in the same low oligomeric form in both E1E2-ori and E2E1-ori. Therefore, initiator complex assembly begins with recruitment of a low oligomeric form of E1 to ori. The E1-binding site is the ultimate determinant of where assembly begins, which is not altered by the position of the E2-binding site.

DEPC Interference Analysis of E2E1-ori—Diethyl pyrocarbonate carbamylates A and G residues and can be used to assess the importance of specific base contacts in DNA-protein complex formation (39). DEPC interference data was generated
for E2 alone and E1 and E2 bound on probes 11/X and X/12 (Fig. 4). Modified probe was incubated with protein, and complexes were resolved on and recovered from polyacrylamide gels. Modified residues were cleaved with piperidine, and the products analyzed on a sequencing gel. Results of several experiments were analyzed using a PhosphorImager. The size of the circles or triangles is an approximate relative measure of the changes in band intensity by interference or enhancement of DNA binding upon DEPC modification (large symbol, >70% change; intermediate size symbol 40–70% change; less than 40% discernible change, small symbol. No base positions gave greater than 70% change in band intensity as a result of enhancement of DNA binding). The positions of the DNA-binding sites are shown and the BPV-1 nucleotide coordinates are given.

**Fig. 4. DEPC interference analysis.**

A, DEPC interference was performed with probes 11/X and X/12 (both strands) for E2 and the E2E1-ori and E1E2-ori complexes resolved in acrylamide gels. Probes were recovered from the gel, cleaved with piperidine, and the products analyzed on a sequencing gel. B, the results of several experiments were analyzed using a PhosphorImager. The size of the circles or triangles is an approximate relative measure of the changes in band intensity by interference or enhancement of DNA binding upon DEPC modification (large symbol, >70% change; intermediate size symbol 40–70% change; less than 40% discernible change, small symbol. No base positions gave greater than 70% change in band intensity as a result of enhancement of DNA binding). The positions of the DNA-binding sites are shown and the BPV-1 nucleotide coordinates are given.

**Two Monomers of Full-length E1 Bind the E1 Palindrome Along with E2 at a Distal Site—**

Full-length E1 is a monomer in dilute solutions (12). The stoichiometry of E1 binding in E2E1-ori was determined with a mixing experiment using full-length E1 and E1 tagged at its N terminus with a hemagglutinin epitope (HAE1) (16). If E1 binds to ori as a monomer, only two types of E2E1-ori complex would form when E1 and the variant are mixed, one containing E1 and one containing HAE1. How-
ever, if full-length E1 binds as a dimer, we would expect a novel complex to form containing tagged and untagged E1, as well as those with the respective E1 homodimers. Any higher order binding configuration would result in a more complex mixture of species.

Binding reactions were assembled with probe 9/X, E2, and a mixture of E1 and HA-E1 at ratios from 100% E1 to 100% HA-E1 for polyacrylamide gel-shift analysis (Fig. 5). To resolve clearly the various complexes, excess anti-HA antibody (12CA5) was added. Fig. 5, lane 1, is free probe, and lanes 2–5 demonstrate that without E2 neither form of E1 binds the probe, with or without antibody. E2 binds the probe (lane 6) and is not recognized by the anti-HA antibody (lane 7). E2E1-ori forms with untagged E1 (lane 8) and is not supershifted with the anti-HA antibody (lane 9). The E2E1-ori complex also forms with epitope-tagged HA-E1 (E2HA-E1-ori) and is supershifted with excess anti-HA antibody (lanes 14 compared with 15). When an increasing proportion of tagged HA-E1 was mixed with E1 (lanes 10–13), a single complex of intermediate mobility formed along with the tagged and untagged complexes. A similar result was obtained without using anti-HA antibody (data not shown), since the mobility difference between all the complexes that form is just sufficient to allow resolution of all complexes (lanes 8 and 15). The complex that migrates between the E2-containing complex and E2E1-ori is most likely E2-bound with a monomer of E1, since the complex that forms with HA-E1 can be supershifted with anti-HA antibody (compare lanes 14 and 15). These results are consistent with the binding of full-length E1 as a dimer to its binding site in E2E1-ori.

Hydroxyl Radical Footprints of the E1 Dimer and E2 Bound to DNA—E2E1-ori formation offers a unique opportunity to study the E1 dimer-DNA interaction when E1 and E2 are not bound in close proximity. Since discrete hydroxyl radical footprints of E2E1-ori proved difficult to obtain in reactions without glycerol (a prerequisite for the hydroxyl radical cutting reaction), the following protocol was adopted. Complexes were formed using HA-tagged E1 and E2 on probe 9/X, in the standard reaction buffer containing glycerol. After 30 min of incubation, anti-HA antibody was added, the reaction incubated for a further 10 min, and the E2HA-E1-ori (or HA-E2-ori) complex recovered by passage over protein G-Sepharose. The Sepharose beads were then washed in reaction buffer without glycerol, and the bound complex was treated with the hydroxyl radical. Footprints were then revealed on a sequencing gel (Fig. 6).

Fig. 6A shows the hydroxyl radical footprints for E1E2-ori (probe X/9) and E2E1-ori (probe 9/X). Lane 1 is a G ladder. Lane 2 is the hydroxyl radical cleavage ladder for probe X/9. Lanes 3 and 4 are the solution footprints for E2 and HA-E2-ori, respectively. As indicated in the annotations to the left, three sets of protections are observed over and beyond proximal E2 BS9 when E2 is bound. The footprints of HA-E2-ori in solution and bound to protein G-Sepharose beads (lanes 4 and 5) are identical. There is a general protection over a region encompassing the E1- and E2-binding sites, within which a periodic set of specific protections can be discerned. As indicated in the annotations, the three 5′-most sets of protections are similar to those for E2 and were previously assigned to E2 (17). Over and beyond the E1-binding site there are two sets of strong protections and one weak 3′-set (dashed box) that defines the upstream boundary of E1 bound to DNA (upper arrow). For the E2HA-E2-ori complex (lane 6), there is an extended set of periodic protections and a stronger general protection over and beyond the E1-binding site compared with E1E2-ori. Three sets of periodic protections, indicated between the arrows, are similar to a subset in HA-E2-ori. Significant E1 protection is observed in the region corresponding to the proximal E2-binding site that is protected by E2 alone (lane 3). Three sets of protections (dark boxes) are clearly observed for E2 bound to distal E2 BS9 (lane 7), but one set (indicated with an *) has no counterpart in HA-E1E2-ori or E2 bound to DNA. A similar but weaker protection is found at a related position downstream of the E1-binding site (dashed box).

The protections on the top strand (lanes 9–16, right) are similar to those on the bottom strand. HA-E1E2-ori and E2HA-E1-ori share some related protections, indicated between the arrows. The two 3′-protections in E2HA-E1-ori overlap with protections observed with E2 alone (lane 10). The E2 protections over distal E2 BS9 (lane 14) are clearly recognizable in E2HA-E1-ori, and one group of protections (*) is not seen in HA-E1E2-ori or E2 bound to DNA (as above). The hydroxyl radical protections of the E1 dimer bound to DNA are shown in Fig. 6B. The protein-DNA contacts flanking the central pair of hydroxyl radical protections are qualitatively different and are not clearly revealed with OP-Cu (Fig. 3) or DNase I (boundaries indicated with the arrows; see Ref. 16). Although it is formally possible that the stronger upstream hydroxyl radical protections (*) are generated by E2, we consider this unlikely since a similar set is found at a related position downstream of the E1-binding site and at a related position in E1-ori.

3 C. M. Sanders, unpublished data.
**E2E1-ori Is the Precursor of a Higher Order E1-ori Complex**—E2 could load a pre-formed initiator complex on the DNA or assemble E1-ori in a stepwise fashion from monomers. Consistent with the latter, we demonstrated that a pre-formed E1E2-ori complex (formed on probe X/12) was a preferred substrate for E1-ori formation. ATP (ATP/Mg²⁺) was required for the transition to displace E2 from its binding site as additional E1 molecules were incorporated. The experimental protocol was based on molecule tagging (16). Briefly, HA-tagged E1 was used to form HAE1E2-ori in the absence of ATP, conditions inhibitory for E1-ori formation. This substrate was then diluted into a reaction containing ATP, a 100-fold excess of untagged E1, nonspecific competitor DNA, and an oligonucleotide E2-binding site competitor (the “assembly reaction”). Product was sampled for up to 25 min, cross-linked, and analyzed by gel-shift with and without specific antibodies (anti-HA and anti-E2). Under these conditions, if E1-ori is a hexamer, we would expect only 6% of the E1-ori complexes that form to contain HAE1, if E1-ori formed by random assortment of E1 molecules. However, the observed value deviates considerably from the one predicted for random assortment, indicating that E1E2-ori was a direct precursor for E1-ori. To test whether E2E1-ori is a
substrate for formation of a higher order E1-ori complex, a similar experiment was performed with E2HAE1-ori formed on probe 9/X (Fig. 7).

Fig. 7A shows the assembly of a multimeric E1-ori complex from a pre-formed E2HAE1-ori precursor. Reaction products were sampled for up to 25 min after dilution and addition of ATP, untagged E1 (100-fold excess), and competitor DNAs, cross-linked, and analyzed in agarose gels after addition of EtBr to 25 mg/ml. Lane 1 is free probe, and lane 2 demonstrates that no EtBr-resistant products form in the pre-incubation (E2HAE1-ori is unstable in the presence of EtBr, Fig. 2B, but was detected by polyacrylamide gel-shift). Lanes 3 and 4 show E1-ori formed at high concentrations of HAE1 and E1, and only HAE1-ori can be supershifted with anti-HA antibody (lanes 5 and 6). E1-ori is not supershifted with anti-E2 antibodies (lanes 7 and 8), but some of the probe is retained in the wells. Lanes 9–13 show rapid formation of an EtBr-resistant multimeric E1-ori complex upon dilution and addition of untagged E1 and ATP. Approximately 80% of the complex that forms at early time in the reaction (<5 min) can be supershifted with anti-HA antibody and therefore contains epitope-tagged HA E1. This proportion decreased slightly with time, possibly as a result of complex formation directly from free or E2-bound probe. The value deviates considerably from the value predicted for random assortment, even if E1-ori is composed of up to 12 E1 molecules (12%). Therefore, like HAE1E2-ori, E2HAE1-ori is a substrate for formation of the multimeric E1-ori complex. E2 is a component of the final E1-ori complex (lanes 19–23), demonstrat-
Therefore, the E2HAE1- is likely to be the maximum that can occur in the absence of E2, so all E2 is effectively bound by the E2-binding site competitor Finally, none of the complex contains E2 (sufficiently high to allow proper interpretation of the results.

14–18 lanes complex could be supershifted with anti-HA antibody (lanes 14–18). Therefore, the final ratio of tagged to untagged E1 is sufficiently high to allow proper interpretation of the results. Finally, none of the complex contains E2 (lanes 19–23, Fig. 7B), so all E2 is effectively bound by the E2-binding site competitor in the assembly reaction, and the extent of complex formation is likely to be the maximum that can occur in the absence of E2. Therefore, the E2HA-E1-ori complex is a better substrate for formation of the multimeric E1-ori complex than free probe.

In Fig. 7C, the ATP/Mg2+ requirement for formation of E1-ori from E2HAE1-ori was assessed. Lanes 1–8 are the controls described above. Lanes 9–13 are a time course after dilution of substrate E2HA-E1-ori and addition of untagged E1 and competitor DNA. Surprisingly, formation of a multimeric E1-ori complex was observed at early times in the reaction (2.5 min, lane 9), albeit at a lower extent (2-fold) compared with the reaction with ATP/Mg2+ (Fig. 7A, lane 9). However, the complex that formed was unstable with a half-life of ~11.5 min, which is close to the dissociation rate of E1-ori that forms without ATP/Mg2+ (16). The majority of the complex that formed could be supershifted with anti-HA antibodies, indicating that it was derived from the E2HAE1-ori precursor. Finally, lanes 19–23 demonstrate that E2 does not dissociate from the multimeric E1-ori complex during its formation. Therefore, additional E1 molecules can be recruited to E2HAE1-ori in the absence of ATP/Mg2+, but a stable E1-ori complex does not form.

In summary, like the E1E2-ori complex, E2E1-ori is a substrate for formation of a multimeric E1-ori complex, which appears to form by stepwise binding of E1 to the ori. However, displacement of E2 is not required for formation of the multimeric E1-ori complex. Unlike E1E2-ori, additional E1 molecules are readily recruited to E2E1-ori in the absence of ATP/Mg2+, consistent with a role for ATP in the displacement of E2 during E1-ori formation from E1E2-ori. However, the resulting complex is unstable relative to the complex that formed in the presence of ATP. Therefore, the requirement for ATP/Mg2+ in E1-ori complex formation is manifested at two levels. First, ATP/Mg2+ is required to displace E2 bound to a proximal E2-binding site; and second, it is required to stabilize the complex of E1 molecules recruited to the DNA.

**DISCUSSION**

BPV-1 is an important model for the initiation of DNA replication in eukaryotic cells. Although the viral components required for initiation are relatively simple (E1, E2, and a replicator with the cognate binding sites), a series of initiator complexes and a network of interactions of surprising complexity have been revealed. We have been intrigued by the difference in ori structure between BPV and HPVs, and we undertook these studies to determine how an initiator complex (E1-ori) is assembled by E2 from a distal site and how this assembly compares to E1-ori formation when E1 and E2 are bound to juxtaposed sites. The results are gratifying, indicating that the basic mechanism for stepwise assembly that we observe with the proximal site occurs also with a distal site. The results presented here indicate that E1-ori formation in HPV may proceed in a similar manner to that in BPV. Thus, the E1-binding site is the ultimate determinant of where complex assembly begins. The precision of the initial binding event (that of the E1 dimer) is therefore critical for replication. The E1 site may be required to correctly position, with respect to each other, the first E1 molecules that bind ori so that an active multimeric E1 initiator complex can successfully form. Alternatively, the positional relationship between the E1 site and other DNA sequences necessary for initiation may be critical.

**Binding of the Full-length E1 Dimer to ori**—Binding of an E1 dimer to the E1-binding site is stabilized by E2 bound at proximal or distal sites. By using a number of footprinting reagents (hydroxyl radical, OP-Cu, and DNase I), we have compared how full-length E1 interacts with DNA in the two complexes E1E2-ori (E1 and E2 bound to adjacent sites) and E2E1-ori (E2 and E1 bound to distal sites). From this compar-
ison, we can deduce the extent to which an E1 dimer can interact with DNA, a form that is not readily detected in solution. The E1 dimer appears to be capable of more extensive interactions with the DNA than determined previously. An extended set of periodic hydroxyl radical protections is observed in E2E1-ori, and the entire region where E1 interacts is more resistant to hydroxyl radical cleavage. The DNA between the E1 and distal E2 sites in E2E1-ori is also generally less reactive to OP-Cu and DNase I cleavage. However, no discrete protections are seen with these reagents compared with the hydroxyl radical. It therefore appears that the E1 interactions in this region are qualitatively different from those over the E1 palindrome, which appears to be the principal determinant of sequence-specific DNA recognition by the E1 DNA binding domain (25). It is possible that regions of E1, other than the DNA binding domain (DBD), can interact over this region. Models for helicase-catalyzed DNA unwinding predict that a minimum of two DNA-binding motifs, one each for single- and double-stranded DNA (40), are required for helical motor activity. It is possible that their existence in E1 is being revealed, even in the initial E1 dimer-DNA interaction.

The second interesting observation to emerge is that E2 clearly can modulate the way in which an E1 dimer interacts with DNA, depending on its positional relationship to E1. This also has functional consequences for the bound E1 molecules. First, the E1-DNA interactions are more extensive in E2E1-ori, compared with E1E2-ori. Second, hydroxyl radical protections by E1 and E2 alone overlap extensively at proximal sites, indicating that in E1E2-ori changes in the way the proteins interact with DNA may occur. It is possible that the proximal interaction of the two proteins can modulate individual protein domains so that either a new composite interaction surface is generated or binding of one protein precludes a DNA interaction made by the other. At this point it is unclear which of these possibilities prevails. However, the protections in this region in E1E2-ori resemble more closely those generated by E2, and in the absence of E2, E1 interacts extensively over the whole proximal E2 site. The concept of structural flexibility in DNA-binding proteins is not without precedent. The Oct-1 POU domain contains two separate DNA-binding modules joined by a flexible linker, and the relative position of these domains on DNA can vary depending on the DNA target (41).

Cooperative binding of E1 and E2 to juxtaposed sites involves two separate interactions between the individual E1 and E2 DNA binding domains and the activation domain of E2 and the helicase domain of E1 (25–32). The latter interaction can be readily detected in solution and on DNA. Indeed, it is the sole determinant of cooperative binding between E1 and E2 bound to distal sites, and a significant component of the forces that stabilize E1E2-ori (32). The DBD interaction, however, does not allow DNA replication by itself and is significant only when E1 and E2 are bound in close proximity (27, 42). Cooperative binding of the E1 and E2 DBDs in E1E2-ori generates a sharp bend in the DNA (29). E1 and E2 alone can bend DNA, but the combined bend in the E1E2-ori complex is much greater than the sum of the individual bends. It is possible that either the remodeling of binding domains or the exclusion of one specific interaction at the expense of another is a critical component of “bending cooperativity.” DNA bending is required for the second interaction between the E2 activation domain and E1 helicase domain to occur. In E1E2-ori that forms in the absence of ATP, this interaction may limit (in addition to mutual exclusion by E2 downstream of the E1 site) E1-DNA interactions, possibly by drawing an interaction surface away from the DNA. It is enticing to speculate that this could explain why the E1E2-ori complex, in the absence of ATP, is a poor substrate for binding of additional E1 molecules (16). This is clearly not the case when E1 interacts more extensively with DNA in E2E1-ori, which readily recruits E1 even in the absence of ATP (Fig. 7). Thus, E1 bound in a specific extended conformation along the DNA may be the template necessary for further E1 binding and assembly of a higher order E1-ori complex.

**Regulation of E1-ori Complex Formation**—The occurrence of an E2 site immediately adjacent to the E1 site, and as a consequence the E1-E2 DBD interaction, is unique to BPV-1 and other fibropapillomaviruses. Most papillomaviruses, including the HPVs, have an E2-binding site at a more distal position. An important question is what role this interaction plays in the BPV-1 life cycle. One distinct possibility is that it governs a regulatory switch critical for the regulation of BPV replication. Our data indicate that E1E2-ori forms preferentially on the BPV ori sequence with E2 BS11 and 12, regardless of E2 binding to BS11. In the absence of the nucleotide cofactor ATP, E1E2-ori hinders E1-ori formation and may negatively regulate its formation. The energy of ATP hydrolysis may induce a conformational change in E1, which alters the quality of the interaction between the E2 activation domain and E1 helicase domain, and may also be transmitted to the cooperative binding surface between the E1 and E2 DBDs destabilizing the DBD component of cooperativity that facilitated it. The consequences would then be 2-fold as follows: E2 would freely dissociate from its low affinity binding site, and the E1 dimer bound to DNA may be primed to recruit more E1 upon forming more extended interactions with DNA. Conceivably, E2 bound to BS11 could stabilize binding of the E1 dimer during the transition, promote the extended interactions with DNA, as well as recruit additional E1 molecules. The latter is critical for formation of a stable E1-ori complex that cannot form in the absence of ATP. The critical evidence required in support of this hypothesis would be the demonstration that E1 ATP binding is regulated in vivo. Why this type of negative regulation may be unique to the fibropapillomaviruses is unclear. The regulation of E1-ATP binding could also control replication without E1E2-ori formation, since stable E1-ori formation and the catalytic activities of the initiator are ATP-dependent. HPV replication may be controlled in such a way, or alternatively, the proposed regulatory role of E2 at proximal BS12 may be performed by a cellular DNA-binding protein.

Cooperative interactions are common in processes that involve assembly of specific multiprotein complexes on DNA. They serve to stabilize large protein DNA complexes through multiple weaker protein-protein interactions and can provide regulatory opportunities through allosteric control. A network of homo- and heterotypic interactions between the BPV E1 and E2 proteins are now well documented. E1 can assemble into higher order structures on DNA that are stabilized by ATP, presumably through a cofactor-driven conformational change. The heterotypic interactions between E1 and E2 are of two kinds. One (between the E2 activation domain and the E1 helicase domain) serves generally in recruitment and assembly of E1 at the origin of replication. A second, dependent on the proximity of the E1- and E2-binding sites, recruits E1 and may also cause a substantial re-modeling of the E1-DBD interaction. This has not been described before for cooperating DNA-binding interactions. We suggest that this phenomenon may be part of a sensitive switch governed by an ATP-induced conformational change that serves to regulate E1-ori formation.

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