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Induction of structural changes in the bovine papillomavirus type 1 origin of replication by the viral E1 and E2 proteins

(DNA–protein interaction/DNA structure/DNA replication/potassium permanganate)

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ABSTRACT **Chemical and enzymatic probing techniques were used to examine the interaction of the bovine papillomavirus type 1 E1 and E2 proteins with the viral origin of replication (*ori*). E1 was found to generate significant distortions to the structure of *ori*, as assayed by $KMnO_4$ oxidation of DNA. The primary site of *ori* distortion was located within and adjacent to the AT-element of the core replicator sequence, although a number of minor structural transitions were also detected. The induction of these structural changes required ATP and appeared to require ATP hydrolysis. E2 was found to decrease the amount of E1 required for *ori* distortion but did not significantly alter the pattern of structural distortion. In contrast, the presence of E2 resulted in a biphasic mechanism for E1 binding to *ori*, as assayed by nuclease protection. Under these conditions, E1 bound preferentially to the dyad symmetry region containing the conserved *Hpa* I site. Higher levels of E1 were required for binding to the adjacent *ori* AT-rich region. Thus, these data suggest that E2 can order the stepwise binding of E1 to *ori*.**

The initiation of DNA replication is a key step of DNA replication, yet molecular analysis is difficult in higher eukaryotes because of the absence of defined cell-free systems. Animal viruses represent valuable alternatives because the viral replication mechanisms reflect primary aspects of chromosomal DNA replication. Members of the papillomavirus family, for example, use known components of the host replication machinery to replicate the viral DNA. As the replication of bovine papillomavirus has been reproduced *in vitro* (1), biochemical investigation of this process is possible.

Bovine papillomavirus type 1 (BPV) can transform bovine and rodent cells *in vivo*. In these cells, BPV generally exists as a stable episome with a constant copy number (2) and replicates during S phase along with the host chromosomal DNA (3). The maintenance of a constant copy number proceeds through a random-choice mechanism similar to the copy number control of bacterial plasmids (3, 4).

Viral replication *in vivo* requires the expression of two viral gene products termed E1 and E2 (5), although only E1 is mandatory *in vitro* (1, 6). The 68-kDa E1 protein binds specifically to the BPV origin of replication [*ori* (1, 7)] in a reaction stimulated by ATP (8, 9). Similar to the simian virus 40 large tumor antigen (10), E1 is an NTP-dependent DNA helicase that can catalyze the gross unwinding of plasmids containing the cognate origin (6, 11). The E2 open reading frame can encode three site-specific DNA binding proteins by differential splicing, including a full-length 48-kDa transcriptional transactivator (E2; for review, see ref. 12). Specific complexes between E1 and E2 form, with affinities influenced by the phosphorylation state of E2 (13, 14).

Examination of the BPV *ori* region reveals two binding sites for the E2 protein (BS11 and BS12) flanking a central region that includes two overlapping inverted repeat elements (IR1 and IR2) and an AT-rich region (toward the BS11 side). Deletion studies indicate that BPV replication *in vitro* requires the ≈50-bp central region (15), while replication *in vivo* also requires the BS12 element (16). E1 appears to recognize IR1 in a reaction stimulated by E2 binding to BS12 (1, 8, 9) or to high-affinity E2 binding sites placed adjacent to *ori* (16).

The binding of an initiator protein to its cognate origin is a critical step in the mechanism of DNA replication and serves two main functions. (*i*) The initiator–origin complex acts as a target for the binding of other replication factors to a specific site on the DNA. (*ii*) Formation of this complex generates significant DNA structural changes that are required for gross denaturation of the origin. We have probed initiation complexes formed by the BPV E1 or E1 and E2 proteins on *ori*. We have found that the E1 protein distorts discrete regions of *ori* in a reaction that requires ATP and, likely, ATP hydrolysis. E2 does not change the pattern of distorted DNA but appears to orchestrate the binding of E1 to two regions within the core *ori* sequence.

MATERIALS AND METHODS

E1 and E2 Proteins. Recombinant baculovirus containing the E1 and E2 genes (14) and the purification of E1 (6) and E2 (8) have been described.

***ori* DNA Fragment.** The *ori*-containing DNA used was an ≈240-bp fragment generated by digestion of pKSO (1) with *Bam*HI and *Eco*RI. The DNA fragments were singly 5′-^{32}P-labeled with T4 polynucleotide kinase (Boehringer Mannheim) to a specific activity of 1–2 × 10^6 cpm/pmol. The top strand contains the run of thymines between BPV positions 7925 and 7930.

$KMnO_4$ Modification of DNA. Reaction mixtures (30 μl), containing 25 mM potassium phosphate (pH 7.5), 0.1 M potassium glutamate, 7 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, 10% (vol/vol) glycerol, 300 ng of pBluescript KS+ (as a nonspecific competitor), E1 and E2 proteins (as indicated), and 100 fmol of *ori* fragment, were incubated at 37°C for 20 min. When indicated, the reaction mixtures included 4 mM ATP, 4 mM ADP, or 4 mM adenosine 5′-[β,γ-imido]triphosphate (p[NH]ppA). $KMnO_4$ was added to 6 mM and the mixture was incubated for an additional 3 min. The reaction was quenched by the addition of 5 μl of 2-mercaptoethanol and extracted once with phenol/chloroform (1:1), and DNA was precipitated with ethanol.

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Abbreviations: BPV, bovine papillomavirus type 1; DSR, dyad symmetry region; ATR, AT-rich region; IR, inverted repeat element; p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate.

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The modified DNA was cleaved at 90°C with piperidine for 30 min. The resulting cleavage products were separated by denaturing PAGE and autoradiographed.

DNase I Analysis of E1–*ori* or E1–E2–*ori* Complexes. E1 and E2 were incubated with the pKSO *ori* fragment as described above; 10 units of DNase I was added, and the mixture was incubated for 60 sec. The nuclease cleavage reaction was quenched by the addition of 5 μl of 0.5 M EDTA and phenol-extracted. The DNA was precipitated with ethanol and the cleavage products were separated by denaturing PAGE.

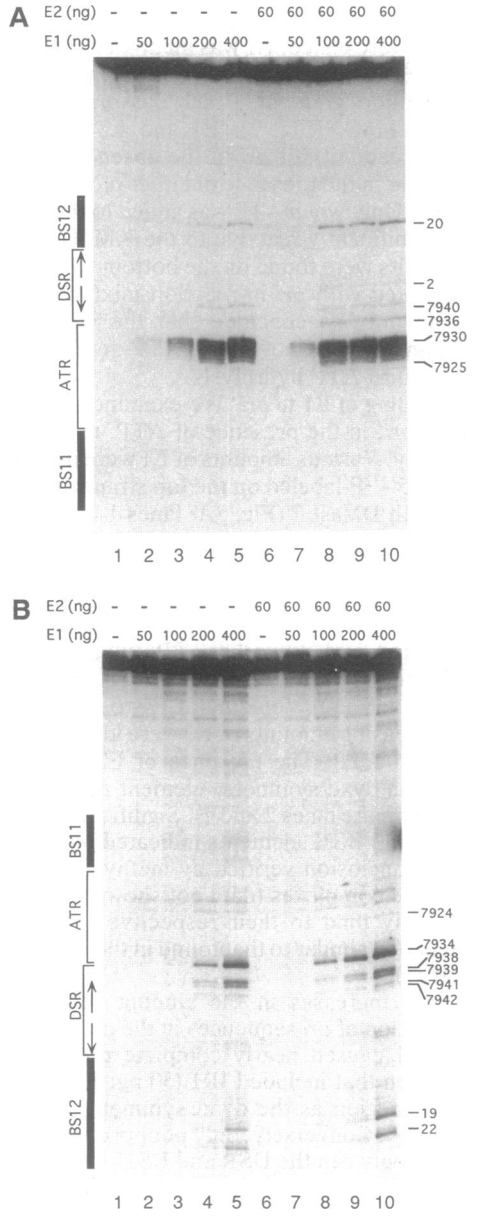

FIG. 1. Structural changes within *ori* generated by the BPV E1 and E2 proteins as detected with $KMnO_4$ oxidation. Increasing amounts of BPV E1 protein (as indicated) were incubated with the pKSO fragment [containing *ori* (1)] in the absence (lanes 1–5) or presence (lanes 6–10) of 60 ng of E2. The *ori* fragment was 5′-^{32}P-labeled on the top (*A*) or bottom (*B*) strand. The *ori* DNA was probed with $KMnO_4$ and chemically cleaved at the modified sites. An autoradiograph of the cleavage products, subjected to denaturing gel electrophoresis, is shown. E1- or E1- and E2-dependent modifications are indicated on the right. The relative locations of the E2 binding sites 11 and 12 (BS11 and BS12; from DNase I protection analysis in Fig. 3), the DSR (IR1 indicated by the two arrows), and the ATR are indicated on the left.

RESULTS

ATP-Dependent Induction of *ori* Structural Changes by E1. We examined the ability of the BPV E1 and E2 proteins to induce distortions in the structure of the BPV *ori*, by oxidation with $KMnO_4$ (17). The binding of E1 to *ori* has been shown recently to be stimulated significantly by ATP (8, 9). As ATP is required for the distortion of replicator elements for various model replication systems, we initially tested the induction of *ori* structural changes in its presence. An *ori*-containing DNA fragment containing BPV positions 7805–100 [the pKSO *ori* (1)] was incubated with various amounts of the E1 protein. The DNA was then modified with $KMnO_4$, and the sites of oxidation were determined.

A low (50 ng) amount of E1 resulted in a low level of $KMnO_4$ oxidation of the pKSO fragment (5′-^{32}P-labeled on the top strand; Fig. 1*A*, lane 2). Increasing the amount of E1 to 100 and 200 ng resulted in distinct regions in *ori* becoming highly reactive to the $KMnO_4$ probe (lanes 3 and 4). The primary region, contained within a 10-bp AT-tract in *ori*, includes six consecutive thymines at top-strand positions 7925–7930. We noted that the thymines closest to position 7930 had the highest reactivity. Three additional E1-dependent oxidation sites, each weaker in intensity than the major site within the AT-tract, were detected at positions 7936, 7940, and 20. An increase in the amount of added E1 protein above 200 ng (to 400 ng) did not cause any significant change in either the intensity or pattern of modification.

We examined the effect of E2 protein on the induction of *ori* structural changes by E1 (Fig. 1*A*, lanes 6–10). Increasing amounts of E1 were incubated with E2 (60 ng) and the pKSO *ori*, and the DNA structure was probed with $KMnO_4$. The most notable effect of E2 was to lower the amount of E1 protein required for *ori* distortion. While only minor levels of

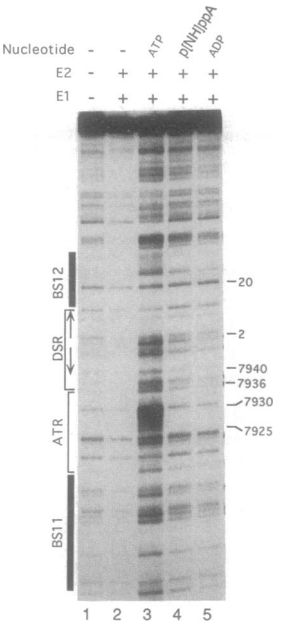

FIG. 2. Nucleotide requirements for the induction of structural changes in *ori* by E1. The BPV E1 (200 ng) and E2 (60 ng) proteins (as indicated) were incubated with a pKSO *ori* fragment that was 5′-^{32}P-labeled on the top strand. Also present in the reaction mixture was no nucleotide (lanes 1 and 2), ATP (lane 3), p[NH]ppA (lane 4), or ADP (lane 5). The samples were then treated with $KMnO_4$, and the DNA was chemically cleaved at the modified sites. An autoradiograph of the cleavage products, subjected to denaturing gel electrophoresis, is shown. Reactive thymines and critical elements within the *ori* region are as described in Fig. 1. Extra bands in lane 3 correspond to guanines that arise as artifacts from our piperidine cleavage reactions.

$KMnO_4$ oxidation were observed using 100 ng of E1 in reaction mixtures lacking E2, addition of E2 gave rise to $KMnO_4$ oxidation levels equivalent to the maximum detected (Fig. 1*A*; compare lanes 3 and 8). This effect was dependent upon a functional E2 binding site (data not shown). E2 did not greatly affect the $KMnO_4$ modification pattern as only a single additional thymine (at position 2) became weakly reactive to $KMnO_4$ attack when E2 was added to E1. E2 did not induce this structural transition in the absence of E1 (lane 6), even using 10-fold higher levels of E2, showing that this *ori* distortion was E1-dependent.

The generation of structural changes within the bottom strand of the pKSO *ori* was similarly examined (Fig. 1*B*). The presence of 200–400 ng of E1 caused the distortion of all bottom-strand thymines between positions 7935 and 7942. As detected with the top strand, positions of the most reactive thymines were skewed to be closest to position 7935. A number of minor E1-dependent modifications were also noted (Fig. 1*B* and see Fig. 5). The addition of E2 again decreased the amount of E1 required to detect these structural transitions (compare Fig. 1*B*, lanes 3 and 8). Interestingly, while E2 stimulated the generation of *ori* distortion in the central region, no simulation was observed for induction of structural changes within the BS12 element (positions 19 and 22). Moreover, no new distortions were detected, on this bottom strand, when E2 was added to E1. Thus E2 can stimulate the induction of *ori* structural changes by E1 yet does not significantly alter the pattern of distortion.

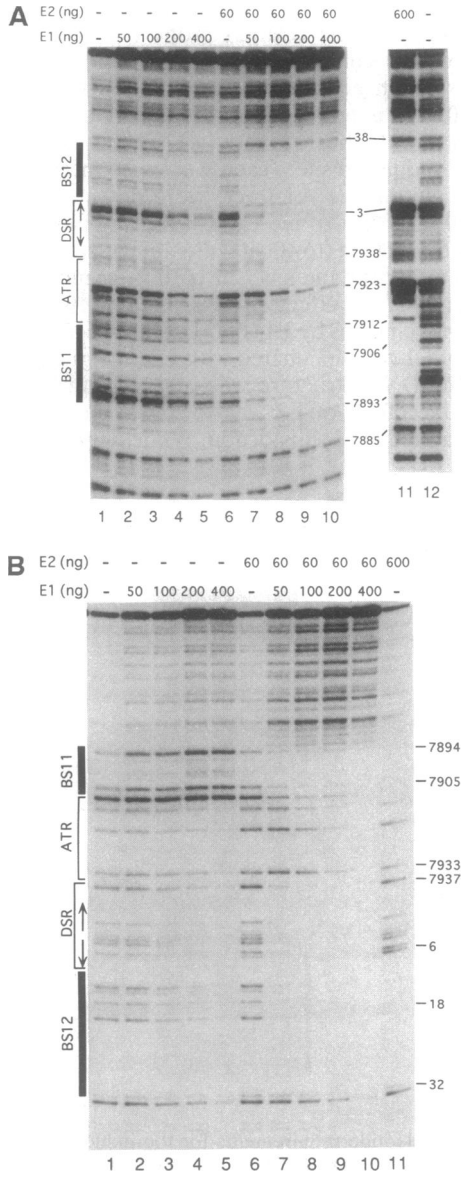

FIG. 3. DNase I footprint of E1 binding to *ori* in the presence and absence of E2 protein. Increasing amounts of BPV E1 protein (as indicated) were incubated with the pKSO *ori* fragment in the absence (lanes 1–5 and 12) or presence (lanes 6–10) of 60 ng of E2. Lane 11 contained 600 ng of E2. The *ori* fragment was 5′-^{32}P-labeled on either the top strand (*A*) or bottom strand (*B*). Each reaction mixture also contained 4 mM ATP. The *ori* DNA was then briefly digested with DNase I, and an autoradiograph of the cleavage products subjected to denaturing gel electrophoresis is shown.

The nucleotide requirements for the induction of structural changes by E1 were tested. E1 was incubated with E2 and the pKSO *ori* (5′-^{32}P-labeled on the top strand) in the presence of various nucleotide cofactors, and the *ori* structure was probed with $KMnO_4$ (Fig. 2). E1 and E2 did not induce $KMnO_4$ hyperreactivity of *ori* in the absence of nucleotide (lanes 1 and 2) or in the presence of either p[NH]ppA or ADP (lanes 4 and 5). Only when ATP was added to the reaction did *ori* become significantly reactive to the $KMnO_4$ probe (lane 3). Similar results were found on the bottom strand and when E1 was incubated with *ori* in reaction mixtures lacking E2 (data not shown). We conclude that the induction of *ori* structural changes by E1 not only is ATP-dependent but also appears to require ATP hydrolysis.

Biphasic Binding of E1 to *ori*. We examined the binding of E1 and E2 to *ori*, in the presence of ATP, using a nuclease protection assay. Various amounts of E1 were incubated with the pKSO *ori*, 5′-^{32}P-labeled on the top strand, and the DNA was probed with DNase I (Fig. 3*A*; lanes 1–5). Intermediate to high levels (200–400 ng) of E1 significantly protected IR1 (containing the conserved *Hpa* I site; indicated by double-headed arrow) and, to a lesser extent, flanking regions. Similar findings have been noted by Botchan and coworkers (1) and Stenlund and coworkers (7), who examined the binding of E1 to *ori* in the absence of ATP.

The effect of E2 on the binding of E1 to *ori* was similarly examined. Increasing amounts of E1 were incubated with *ori*, E2 (60 ng), and ATP. The presence of E2 resulted in the protection of the dyad symmetry element at lower levels of E1 (Fig. 3*A*; compare lanes 2 and 7). Significant protection of both the BS11 and BS12 elements indicated binding of E2 to these sites, a conclusion verified by methylation protection analysis of these complexes (data not shown). Thus, E1 and E2 cooperatively bind to their respective recognition elements in a reaction similar to that found in the absence of ATP (e.g., see ref. 1).

Significantly, increases in the amount of E1 caused a biphasic protection of *ori* sequences in the presence of E2. A low level of E1 caused nearly complete protection of the central *ori* region that included IR1 (50 ng; Fig. 3*A*, lane 7). We define this region as the dyad symmetry region (DSR; positions 7936–9). Conversely, only poor protection of the *ori* AT-rich region between the DSR and BS11 (ATR, defined as positions 7910–7935) occurred under these conditions. The effect of E2 is most clearly visualized by comparing the relative intensity of cleavage products at positions 3 and 7923 in lanes 4 and 8. Increasing E1 to 400 ng protected the ATR from nuclease cleavage (lanes 8–10). As shown above, E1 protected the *ori* region between BS11 and BS12, whereas E2 protected only the actual E2 binding sites. The ≈20-bp separation between the DSR and the BS11 makes it unlikely that E1 and E2 binding to these elements sterically inhibits DNase I cleavage of the ATR. Moreover, the E2 effect was observed on *ori* constructs lacking BS11 (data not shown). Therefore, these data strongly suggest that the biphasic protection pattern is a result of E1 binding to *ori* in two stages, first to the DSR and then to the ATR.

The binding of E1 and E2 to the pKSO *ori*, labeled on the bottom strand, was also examined (Fig. 3*B*). In the absence of E2, increasing levels of E1 led to a general protection of *ori* sequences bordered by the distal edge of BS12 and the proximal border of BS11 (lanes 1–5). In the presence of E2, a small amount of E1 completely protected the DSR and BS12 (lane 7). As the level of E1 was increased, a second phase of protection to the ATR was noted, indicating that the biphasic binding of E1 to *ori* occurs on both strands of *ori* (see the protection of the two bands labeled 7933 and 7937). Densitometric analysis of the autoradiographs revealed that, in the presence of E2, the amount of E1 leading to 50% occupancy of the DSR and ATR was ≈35 ng and ≈155 ng, respectively (data not shown). In the absence of E2, the amount of E1 required to fill 50% of each region was <2-fold different (155 vs. 235 ng, respectively). This quantitation indicates that E2 preferentially facilitates E1 binding to the DSR but has only minor effects on E1 binding to the ATR.

The effect of ATP on the binding of E1 and E2 to *ori* was directly examined (Fig. 4). Saturating amounts of E1 and E2 were added to the pKSO *ori* (5′-^{32}P-labeled on the top strand), in the presence or absence of ATP or ATP analogs, and the *ori* DNA was probed with DNase I. In the absence of ATP, a residual DNase I cleavage site was observed at position 7923 in the ATR (lane 2; indicated by arrow). In the presence of ATP, this residual DNase I cleavage site was lost (lane 3). The lack of accessibility of this cleavage site at position 7923 appears to be dependent upon ATP hydrolysis as neither p[NH]ppA nor ADP could substitute for ATP in this reaction (lanes 4 and 5). We did not detect any significant effects of ATP on subsaturating levels of these proteins, nor did ATP alter the boundaries of the *ori* protected by these two proteins. The presence of ATP also did not affect the biphasic binding of E1 to *ori* in the presence of E2. The cleavage site at position 7923 was located within 2 nt of the region of primary distortion in *ori*. This result indicates that ATP induces conformational changes in the E1–E2–*ori* complex that are associated with the generation of structural changes within *ori*.

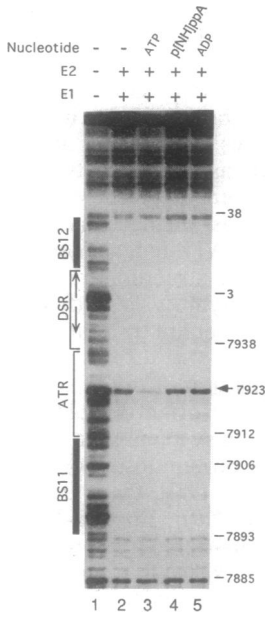

FIG. 4. Effect of nucleotides on the DNase I cleavage pattern of E1 and E2 bound to *ori*. BPV E1 (200 ng) and E2 (60 ng) proteins (as indicated) were incubated with a pKSO *ori* fragment that was 5′-^{32}P-labeled on the top strand. The reaction mixture contained no nucleotide (lanes 1 and 2), ATP (lane 3), p[NH]ppA (lane 4), or ADP (lane 5). The samples were then probed with DNase I, and an autoradiograph of the cleavage products subjected to denaturing gel electrophoresis is shown. The single DNase I cleavage site affected by ATP is indicated by an arrow. Critical elements within the *ori* region are as described in Fig. 1.

DISCUSSION

The E1–*ori* Complex. The nuclease protection and $KMnO_4$ oxidation patterns of E1 and E2 bound to *ori* were compiled (Fig. 5). In the absence of E2 (Fig. 5*A*), E1 distorted distinct regions of *ori* that encompassed nearly 50 bp. As E1 protected ≈70 bp from nuclease digestion including the complete core *ori*, E1 bound to *ori* is capable of inducing structural transitions over nearly the entire length of covered DNA. While a broad region was distorted by E1, the primary site of structural transitions was located asymmetrically within the core *ori* sequence at positions 7925–7942. This primary site could be bifurcated into right (positions 7925–7930) and left (positions 7934–7939) halves in which the top and bottom strands, respectively, were hyperreactive to the $KMnO_4$ probe. The most reactive thymines were at positions 7929,

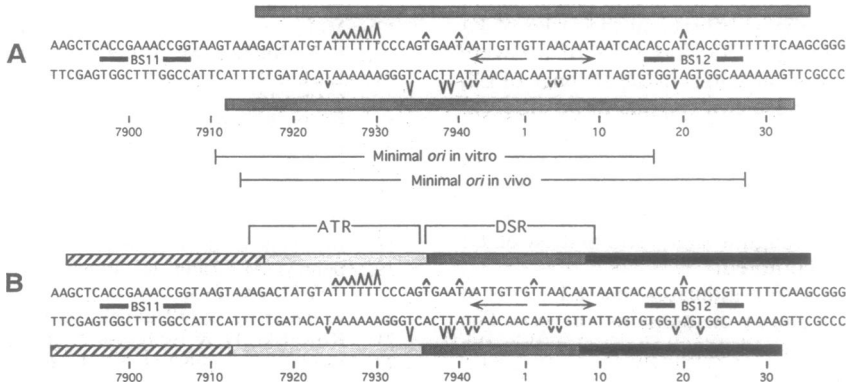

FIG. 5. Compilation of $KMnO_4$ oxidation and DNase I protection patterns of E1 and E2 bound to *ori*. Results were obtained with E1 alone (*A*) or with E1 and E2 (*B*). Thymines hyperreactive to $KMnO_4$ are indicated by carets, with the intensity of modification indicated by the relative size of the carets. (*A*) Top and bottom strand regions protected by E1 from DNase I cleavage are indicated by solid boxes above and below, respectively, the sequence. (*B*) The region of DNA protected using low levels of E1 is shown by a darkly stippled bar. The region requiring higher levels of E1 for protection is indicated by a lightly stippled bar. The BS11 region protected by E2 is indicated by a cross-hatched bar, and the region to which E2 and E1 bind to the BS12 overlap is indicated by a solid bar. The positions of the DSR and ATR of *ori* are shown. The recognition elements for E2 binding, BS11 and BS12, are indicated between the two strands of the sequence. The IR1 is indicated by a double-headed arrow. Sequence positions are indicated by numbers below each panel. The minimal *ori* sequences required *in vitro* (positions 7911–16; ref. 15) and *in vivo* (positions 7914–27; ref. 16) are shown.

7930, and 7934, suggesting that a focal point for the structural transition is located at the center of this primary site.

The generation of $KMnO_4$ hypersensitivity in *ori* requires ATP. As p[NH]ppA could not substitute for ATP, ATP hydrolysis appears to be required to induce these distortions. While ATP stimulates the binding of E1 to *ori* (8, 9), our nuclease protection assays indicated that ATP had only subtle effects on the general placement of E1 on *ori*. Because ATP did not cause gross changes in the binding of E1 to *ori*, we believe that the ATP-independent E1–*ori* complex is predisposed to undergo structural transitions. With the input of energy from ATP hydrolysis, sufficient changes occur in the E1 conformation that are translated into distortion of the bound *ori*.

Significant deformation of origin structure caused by the formation of an initiator–origin complex has been observed in virtually all replication systems examined. These include the binding of the *dnaA* protein to the origin of the *Escherichia coli* chromosome [*oriC* (18)], the λ O protein to the bacteriophage λ origin [*oriλ* (19)], Epstein–Barr nuclear antigen 1 protein to the Epstein–Barr virus origin (20), and the tumor antigen to the simian virus 40 origin (10). Where examined (e.g., refs. 21 and 22), the induction of these distortions has been found to be an integral step in the initiation of DNA replication. With the simian virus 40 origin, one of two distorted regions is significantly melted (23) and appears to act as a binding site for human replication protein A (hRPA) during complete denaturation of the origin (24). Although the nature of the structural distortions in the BPV *ori* is still under investigation, a likely possibility is that the primary distortion in *ori* acts as an entry site for other essential replication factors, such as hRPA, onto the *ori* DNA.

The E1–E2–*ori* Complex. E2 did not significantly alter the pattern of *ori* structural changes but rather lowered the amount of E1 needed to generate the structural transitions. As we (above) and others (1, 8, 9) have shown that E1 and E2 bind cooperatively to *ori*, the stimulation in structural changes is most easily explained by E2 increasing the affinity of E1 for *ori*. We note, however, that E2 stimulated structural changes within the ATR and DSR of *ori* but had no effect on distortions within the bottom strand of the E2 BS12 (Fig. 1*B*). The lack of effect within BS12 is somewhat surprising as E1 and E2 binding overlap in this region. Thus, a secondary explanation is that E2 induces conformational changes within the E1–*ori* complex that facilitate the generation of structural changes within *ori*.

Although E2 did not greatly alter the general placement of E1 on *ori*, E2 did have significant effects on the order of E1 binding to *ori*. While both the DSR and ATR of *ori* were bound by E1 with nearly equal affinities in the absence of E2, the presence of E2 caused these two regions to be differentially occupied by E1. Low levels of E1 preferentially bound to the DSR, whereas binding of the ATR was dependent on roughly 4- to 5-fold higher levels of E1. Our quantitative analysis (Fig. 3*C*) indicates that E2 preferentially stimulated E1 binding to the DSR, although a slight stimulation of E1 binding to the ATR was also noted.

This biphasic binding reaction indicates that E1 forms at least two distinct substructures or domains on *ori*. The domain over the DSR appears to use sequences within the dyad symmetry element for recognition of the *ori*, a conclusion supported by the deleterious effects of mutations within this element on E1 binding (7–9, 25, 26). The ATR does not significantly overlap this element, and there is little apparent sequence homology between the ATR and DSR. Although it is possible that E1 binding to the ATR utilizes cryptic recognition elements, we believe it more likely that E1 binding to the DSR stabilizes the binding of E1 to the adjacent ATR.

The border separating the DSR and the ATR was most clearly distinguished on the bottom strand of *ori* where it was found approximately at positions 7935–7936. Intriguingly, the thymines most reactive to the $KMnO_4$ probe were detected at positions 7929–7934. The primary site of structural transitions, therefore, closely coincides with the boundary region, separating the two E1 domains bound to *ori*. These transitions were located within the ATR and DSR of *ori*, suggesting E1 must form a substructure on each region to induce structural changes within that region. Distortion of the DSR and ATR occurred concomitantly at all levels of E1, even in the presence of E2. Thus, we suggest that the induction of structural changes within *ori* requires both the DSR and ATR be bound by E1. This model makes testable predictions concerning the correlation of E1 binding to the DSR and ATR and the distortion of *ori* structure.

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