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The bovine papillomavirus origin of replication requires a binding site for the E2 transcriptional activator

(DNA replication/transcription factor)

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ABSTRACT **The bovine papillomavirus type I transcriptional activator E2 is essential for replication of bovine papillomavirus DNA, yet most of the high-affinity binding sites for E2 are dispensable. Here we demonstrate an absolute requirement for a binding site for the E2 polypeptide as a cis-acting replication element, establishing that site-specific binding of E2 to the origin is a prerequisite for bovine papillomavirus replication *in vivo*. The position and distance of the E2 binding site relative to the other origin of replication components are flexible, but function at a distance requires high-affinity E2 binding sites. Thus, low-affinity binding sites function only when located close to the origin of replication, while activity at greater distances requires multimerized high-affinity E2 binding sites. The requirement for E2, although different in some respects, shows distinct similarities to what has been termed replication enhancers and may provide insight into the function of this class of DNA replication element.**

The process of initiation of DNA replication is well studied in only a few eukaryotic systems and studies have been largely restricted to lytic viruses due to the fact that the necessary cis-acting elements have not been available from other systems (1, 2). Bovine papillomavirus (BPV) is an interesting addition to this group, because the life cycle of the virus is significantly different from most well-studied viruses (reviewed in ref. 3). It has been suggested that BPV is a particularly good model for mammalian chromosomal DNA replication, since the viral DNA in transformed mouse cells is stably maintained at a constant copy number for many generations and appears to replicate in synchrony with the cellular DNA (4). However, in terms of replication properties—i.e., long-term stability, strict copy number control, and low frequency of loss—the systems that most closely resemble BPV are some prokaryotic plasmids.

We have previously demonstrated that a small noncoding region from the BPV genome contains all the sequences required in cis for DNA replication *in vivo* (5). This region is also necessary and sufficient for replication *in vitro* (6). As illustrated in Fig. 1, this short sequence contains three recognizable elements, an A+T-rich region, a binding site for the E1 polypeptide, and a binding site for the E2 polypeptide (E2 BS12). The E1 and E2 polypeptides are absolutely required in trans for replication of BPV DNA (7). The E1 polypeptide is a DNA binding protein, which appears to function as the origin of replication (Ori) recognition factor (5, 6, 37). E2 is a site-specific DNA binding protein with transcriptional activation properties (reviewed in ref. 8), which has been reported to facilitate binding of E1 to its binding site (6). Based on mutational analysis, we previously concluded that E2 BS12 was of little importance for replication since mutations that reduced the ability of E2 to bind to this site had little effect on DNA replication (5). As demonstrated in this paper, however, subsequent analysis of the E2 polypeptide has indicated that specific DNA binding was required for DNA replication and, furthermore, the ability of E2 to support DNA replication was proportional to its DNA binding activity. Therefore, we have reexamined the importance of the E2 binding site at the origin by a more thorough mutational analysis. The results demonstrate that an E2 binding site is absolutely required for replication from the BPV origin. When the E2 binding site is located close to the other Ori elements, crippled, very low-affinity E2 binding sites can be utilized. However, function at greater distances requires E2 binding sites with higher affinities. These results are consistent with a DNA-dependent interaction between the E1 and E2 polypeptides where binding sites for both proteins are required to form a functional initiation complex.

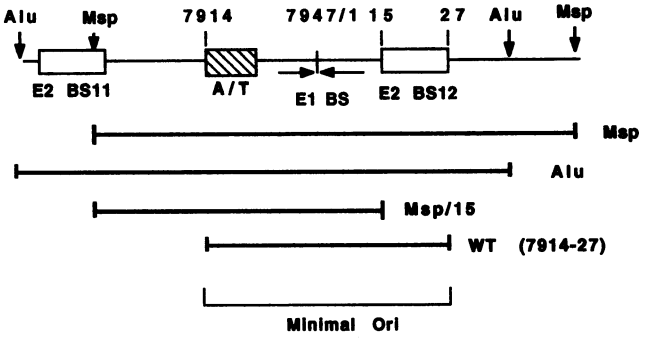

FIG. 1. Ori region of BPV is depicted schematically, including restriction sites and known elements of the Ori region. Coordinates given are nt numbers from the BPV genome. Regions involved in binding of the E1 and E2 polypeptides are indicated. Some of the Ori constructs used in this study are indicated below. Minimal Ori functional *in vivo* is also indicated. WT, wild type.

MATERIALS AND METHODS

Plasmids. The expression vectors for the different forms of E2 have been described (7). The point mutations in E2 were generated by oligonucleotide-directed mutagenesis as described (9). The HA epitope tag (10) in E2 was generated by inserting a sequence encoding 13 amino acids from the influenza hemagglutinin polypeptide into the E2 coding sequence at a *Stu* I site at nt 3351. All Ori constructs were cloned into pUC19 (Fig. 1). The *Msp* and *Alu* Ori constructs have been described (5). The mutations in E2 BS12 were generated in the context of the wild-type minimal Ori (nt 7914–7927), which was cloned between *Xba* I and *Hin*dIII in the poly linker of pUC19. The *Msp* L and *Alu* L plasmids, in addition to the respective Ori fragments, contain a fragment

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Abbreviations: BPV, bovine papillomavirus; EBV, Epstein–Barr virus.

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from the late region of BPV between nt 4450 and 7187 cloned into the *Bam*HI site of pUC19. *Msp*/15+BS9 contains an oligonucleotide (E2 BS9; see below) cloned into the *Bam*HI site immediately upstream of the *Msp*/15 Ori fragment. *Msp*/15+10×BS9i contains 10 copies of the same oligonucleotide cloned into the *Bam*HI site. Between the *Bam*HI and *Xba* I sites in the polylinker, a 1.0-kb spacer fragment from the late region (nt 6132–7187) of BPV was inserted. All point mutations and deletions in Ori were generated by PCR using standard protocols.

Cell Lines. Generation of the CHO cell lines 4.15 and 2.12 expressing E1 and E2 and the conditions for culture will be described elsewhere (M.U. and A.S., unpublished data). COS-7 cells were maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum.

Electroporation and Replication Assays. All electroporations and replication assays were carried out as described (7). Fifty nanograms of Ori-containing plasmid was used in each electroporation. Where an internal standard was cotransfected, 100 ng of this larger plasmid was also used, resulting in roughly equimolar quantities of the two plasmids. Samples were processed as described (7) except that the cells were treated with trypsin and resuspended at a density of 2×10^7 cells per ml. Cell suspension (0.25 ml) was mixed with DNA and pulsed at 960 μF, 230 V in a Bio-Rad gene pulser.

Dimerization Assay. Forty-eight hours after electroporation COS cells were labeled with Tran^{35}S-label (ICN) for 4 hr. Extracts were prepared by lysing the cells using a buffer containing 0.1% Nonidet P-40, 0.2 M KCl, 1 mM EDTA, 25 mM Hepes (pH 7.9). The extracts were divided into three equal portions. Two of these were denatured with 1% SDS diluted 1:10 and precipitated with polyclonal E2 antiserum and a monoclonal antibody directed against the HA epitope (12CA5) (10), respectively. The third portion was immunoprecipitated with 12CA5 under nondenaturing conditions. The samples were analyzed by SDS/PAGE according to Laemmli (11).

DNA Binding Assays. Gel-retardation assays were carried out using an end-labeled double-stranded synthetic oligonucleotide E2 BS9 (GATCTGTACCGTTGCCGGTCG). Extracts for gel retardation were prepared by lysis of the transfected cells as described above. DNase footprint analysis was carried out as described (12) except that the buffer used was 20 mM potassium phosphate pH 7.5/100 mM potassium glutamate/1 mM EDTA/5 mM dithiothreitol/10% (vol/vol) glycerol. The probes used for footprinting were generated by PCR amplification from the Ori plasmids with end-labeled universal sequencing primers. Expression and purification of the E2 polypeptide will be described elsewhere (A.S. and J. Sedman, unpublished data).

RESULTS

Mutations in E2 That Affect Specific DNA Binding but Not Dimerization Are Incapable of Supporting Replication. To determine whether the E2 sequence-specific DNA binding activity was required for replication of BPV-1 DNA, mutations were constructed in E2 that were defective for DNA binding but left the protein structurally intact, including the capability to form dimers. At the time, the DNA binding and dimerization domains had not been characterized in detail. We therefore generated substitution mutants in three short blocks of sequence that are conserved between E2s from different papillomaviruses (13). Subsequently, in a thorough

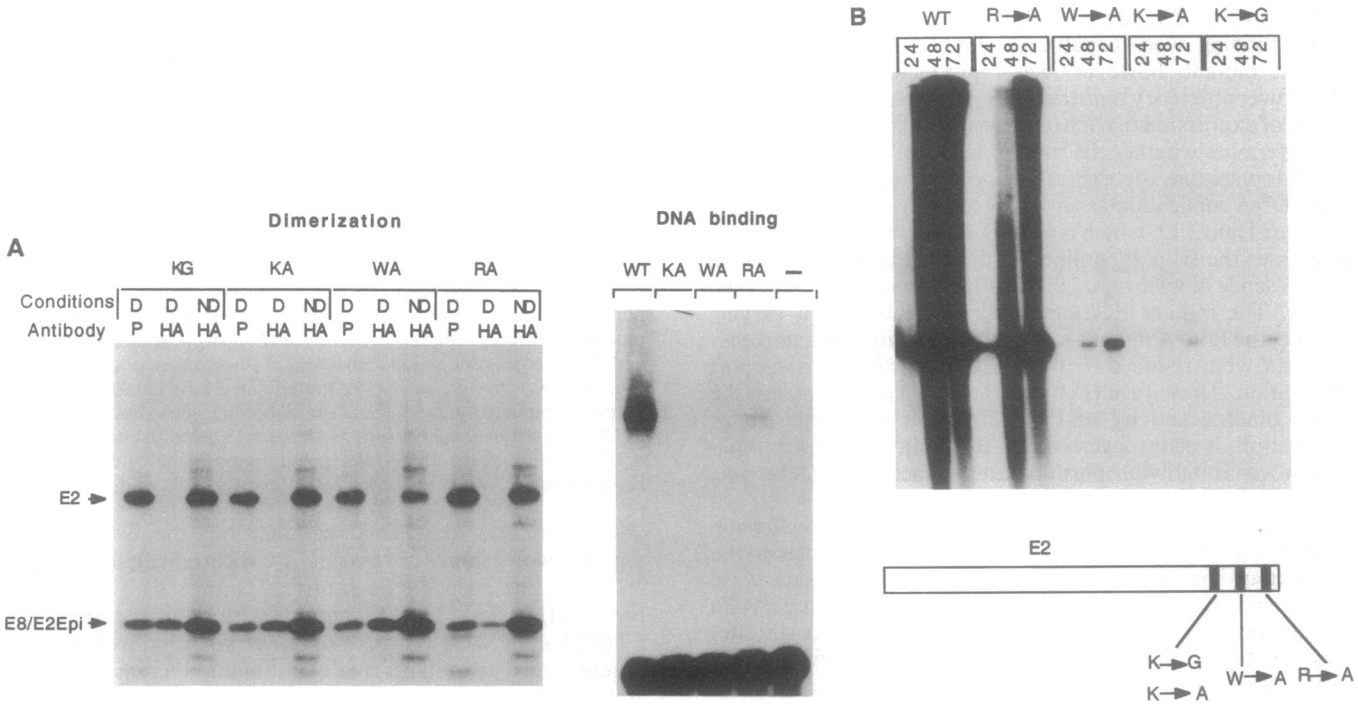

FIG. 2. (*A*) Mutants in the DNA binding domain of E2 are defective for DNA binding but can dimerize *in vivo*. Mutants were tested for dimerization by cotransfection of the expression vectors encoding the mutant forms of E2 together with a vector encoding a short version of E2 tagged with the HA epitope (E8/E2Epi). After transfection and metabolic labeling, cells were lysed under nondenaturing conditions. To detect dimerization (*Right*), samples were immunoprecipitated with a monoclonal antibody directed against the HA epitope (HA). Immunoprecipitations were performed under denaturing (D) or nondenaturing (ND) conditions. As a control, a portion of each sample was immunoprecipitated with a polyclonal antiserum (P) under denaturing conditions. (*Right*) Extracts from parallel transfections were prepared and used for gel-retardation assays with an oligonucleotide probe. (*B*) E2 mutants defective for DNA binding are defective for DNA replication. Ability of the E2 mutants to support replication of an Ori plasmid was tested by electroporation of the Ori plasmid together with expression vectors encoding the respective E2 mutants into a CHO cell line (2.12) stably expressing the E1 polypeptide. At the indicated times (hr), cells were harvested and replicated DNA was measured as described. Quantitation of the blots indicated that the R → A mutant was reduced ≈10-fold in its ability to support replication, while the other three mutants were reduced ≈100-fold in their ability to replicate. WT, wild type.

study using a random mutagenesis approach Prakash *et al.* (14) have identified a number of amino acids important for DNA binding and dimerization and these sequences correspond largely to the conserved regions. The mutants were tested for specific DNA binding in a gel-retardation assay with extracts from COS-7 cells transfected with expression vectors encoding either the mutant proteins or the wild-type form. As demonstrated in Fig. 2*A*, the three mutants K339A, W360A, and R386A were all reduced in their ability to bind to the oligonucleotide. The R386A mutant was reduced ≈10-fold compared to the wild type, while the other two were reduced at least 100-fold in their ability to bind DNA.

To determine whether these mutants were defective for DNA binding due to an inability to dimerize and also to determine that the proteins were expressed at similar levels, we designed an assay to measure dimerization *in vivo*. We have previously determined that shorter forms of E2 when coexpressed with the full-length form *in vivo* can dimerize to form heterodimers. Therefore, expression vectors encoding the full-length E2 and its mutant forms, together with a short form of E2, E8/E2Epi were cotransfected into COS cells. The short form of E2 was tagged with a short sequence encoding 13 amino acids from the influenza hemagglutinin protein, which is recognized by the monoclonal antibody 12CA5. After transfection and metabolic labeling, cells were lysed under nondenaturing conditions. To detect dimerization, samples were immunoprecipitated with the monoclonal antibody directed against the HA epitope. Under denaturing conditions, only the tagged short form was precipitated. In contrast, under nondenaturing conditions, the full-length form of E2 was efficiently coprecipitated. As a control, immunoprecipitations were carried out with a polyclonal antiserum that recognizes both forms of E2 under denaturing conditions. As demonstrated in Fig. 2*A*, the four mutants that were defective for DNA binding were capable of dimerization *in vivo*. The W360A mutant, however, was reproducibly coprecipitated with lower efficiency than the other mutants. Furthermore, the levels of expression of each of these mutants appeared similar. To determine whether the mutant forms of E2 could support DNA replication, the expression vectors were used in short-term DNA replication assays. These assays were carried out in the cell line 2.12, which is a CHO cell line that constitutively expresses the E1 polypeptide. As demonstrated in Fig. 2*B*, in the presence of wild-type E2, the origin replicated to very high levels. The mutant E2s supported replication to a varying degree, the R389A mutant was ≈10-fold down, while the other mutants were reduced ≈100-fold in their ability to support replication. These results gave a clear indication that specific DNA binding activity of the E2 protein was required for replication. It also suggested that the ability to support replication was roughly proportional to the specific DNA binding activity of E2.

The Affinity of E2 for BS12 Correlates with DNA Replication Activity. A set of mutants in E2 BS12 were constructed in the context of the minimal Ori. Two of these mutants were designed to increase the E2 binding affinity (PM19 and PM25) by generating a site more similar to a strong consensus site. One point mutation was designed to reduce the affinity of E2 (PM17) by generating a site less similar to a strong consensus site. In addition, a deletion mutant (D22) was generated that deleted half of the palindromic E2 BS12 binding site. When these mutants were tested for their ability to bind E2 in a DNase I footprint assay, it was found that the results were consistent with the predictions—i.e., mutants PM19 and PM25 had increased ability to bind E2, PM17 showed slightly reduced ability to bind E2, while for D22 binding could not be detected (Fig. 3*B*). When tested for their ability to replicate, all these Ori mutants replicated with only minor differences in efficiency (data not shown). To provide an internal standard in the replication assay and also possibly to enhance

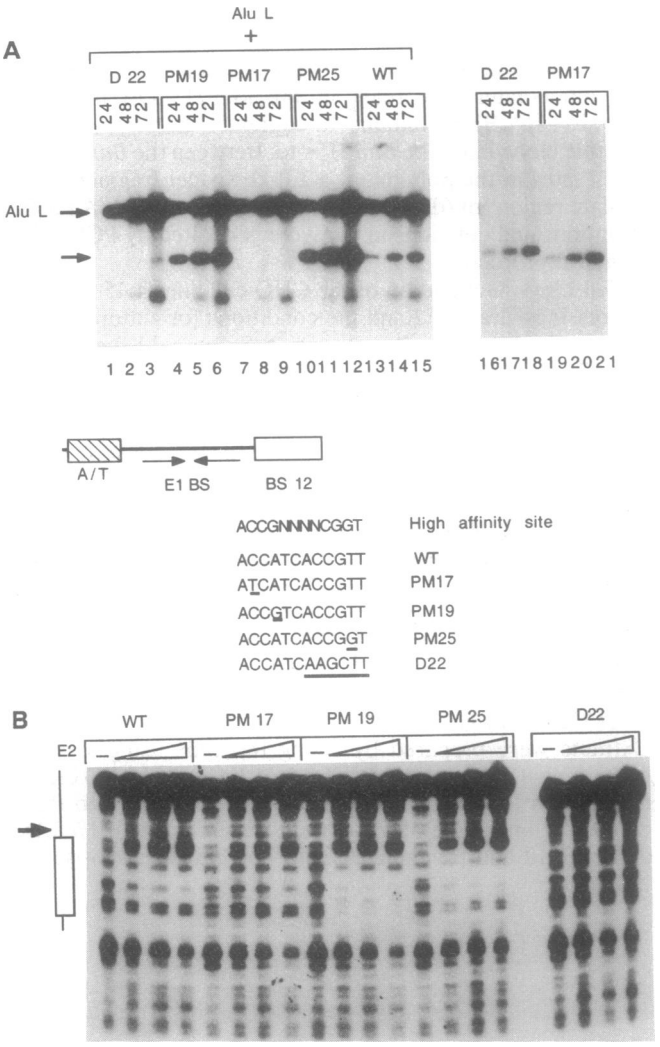

FIG. 3. A functional E2 binding site is essential for replication *in vivo*. (*A*) A series of mutants in E2 BS12 (*Lower*) were tested for replication. (*Left*) Constructs were cotransfected together with a larger Ori construct *Alu* L into 4.15 cells expressing both E1 and E2 polypeptides. Lower band (arrow) represents the test plasmid and upper band corresponds to the internal standard *Alu* L. (*Right*) Two mutants that were replication defective (*Left*) were transfected alone and shown to be replication competent in the absence of a competing Ori. (*B*) DNA binding activity of mutants in E2 BS12. Ability of the E2 polypeptide to bind to the mutant E2 binding sites was determined by DNase footprint analysis in the absence (−) or presence of 20, 40, or 80 ng of E2, respectively. Protected region is indicated by a bar. Hypersensitive site is indicated by an arrow. WT, wild type.

differences due to plasmid competition, the Ori mutants together with a larger Ori plasmid were cotransfected into the 4.15 cell line, which constitutively expresses E1 and E2 proteins (Fig. 3*A*). The Ori mutants with reduced affinity for E2 (D22 and PM17) in this competitive situation were unable to replicate. The mutants that were slightly increased in their ability to bind E2 (PM19 and PM25) had increased capacity to compete with the larger replicon compared to wild-type Ori. As shown in Fig. 3*A* (*Right*), the two mutants D22 and PM17 were replication competent when transfected individually in the absence of competitor.

An E2 Binding Site Is Absolutely Required for DNA Replication. These results clearly indicated that the 12 nt that constitute E2 BS12 were important for replication and it was suggestive that the replication competence appeared to be related to the affinity of the binding site for E2 protein. However, it did not prove that the E2 binding site *per se* was

important. To rule out that other sequences overlapping the BS12 were responsible for the importance of this region, we deleted the 12 nt that constitute E2 BS12 to generate the construct *Msp*/15. This plasmid was inactive for replication both by itself and in the presence of a competing Ori (Fig. 4). Into this construct, we inserted a different, strong E2 binding site from the E2-dependent enhancer (E2 BS9). As demonstrated in Fig. 4, insertion of BS9 into the polylinker sequence outside the minimal Ori sequence restored replication to wild-type levels (or better), demonstrating that the replication deficiency in *Msp*/15, caused by the loss of E2 BS12, could be restored by insertion of another E2 binding site with partly different DNA sequences. This strong E2 binding site was functional at a position different than BS12 relative to the E1 binding site.

Ability of the E2 Binding Site to Function from a Distance Correlates with Its Affinity for E2 Protein. These results demonstrated that an E2 binding site was an essential part of a functional BPV Ori. Furthermore, the position and distance of the E2 binding site relative to the E1 binding site did not appear to be critical. However, a peculiar fact apparent from the analysis of mutants in E2 BS12 was that the D22 mutant replicated, yet binding of E2 protein could not be detected (Fig. 3*A*). The sequence ACC, which is the only conserved part of the E2 binding motif remaining in this mutant, is obviously present in many other positions in the plasmid but is nonfunctional at these positions. A possible explanation for the activity of this crippled E2 binding site could be its close proximity to the E1 binding site. E2 BS9, which could function at some distance from the E1 binding site, has considerably higher affinity than BS12. To determine whether there was a relation between the affinity of the E2 binding site and the ability to function for replication at a distance, we generated Ori constructs where the distance between E2 BS12 and the E1 binding site was increased by inserting 3, 6, or 10 nt between the two sites. As demonstrated in Fig. 5 (lanes 1–9), this progressively increased distance resulted in a progressively reduced replication efficiency, and +10 shows hardly detectable replication. To determine whether increasing the affinity of the binding site for E2 protein would restore replication, we introduced the PM25 mutation in the +10 background. This mutation restored replication to close to wild-type levels, indicating that the increased distance could be compensated for by increased affinity of the E2 binding site. Thus, a low-affinity site appears to be functional only when located close to the E1 binding site, while for function at greater distances higher affinities are required. The most extreme case is the half E2 binding site that still functions for replication when located immediately adjacent to the E1 binding site.

To test whether these observations were true when the E2 binding site was moved considerably further away, we inserted a 1.0-kb DNA sequence between E2 BS9 and the Ori region in the *Msp*/15 construct. The inserted DNA fragment was derived from the late region of BPV and contains no known E2 binding sites. This construct failed to replicate, indicating that even a high-affinity site was limited in its capacity to function over large distances (data not shown). However, when BS9 was multimerized to 10 copies and placed at the same position, replication competence was again restored (Fig. 5, lanes 13–15).

DISCUSSION

From a number of examples it is clear that transcriptional elements play an important role in initiation of DNA replication. Such diverse replicons as ARS elements from *Saccharomyces cerevisiae* and the lytic polyomavirus require transcriptional activators for initiation of DNA replication (15–20). Polyomavirus is the best-characterized replicon in this regard. A variety of transcription factors can activate replication from the polyomavirus core origin. These factors range from *S. cerevisiae* Gal4 and mammalian AP1 and BPV E2 to various hybrid transactivators, such as Gal4/VP16 and Gal4/Jun (21–25). The presence of a transcriptional activation domain appears to be an absolute requirement; the DNA binding domain of these proteins is not sufficient (21, 25). In contrast to their function in transcription, the sites generally

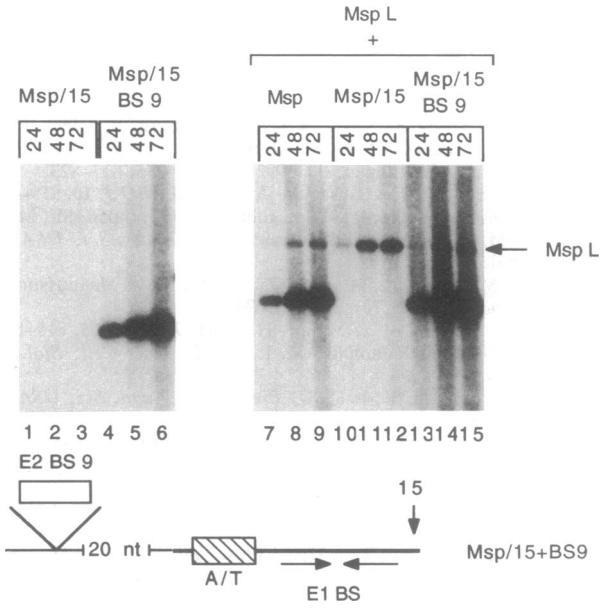

FIG. 4. A different E2 binding site at a different position can substitute for E2 BS12. An Ori construct where the entire E2 BS12 had been deleted (*Msp*/15) was tested for replication in parallel with the same construct where an oligonucleotide comprising E2 BS9 had been inserted into the polylinker sequence (*Msp*/15+BS9). These constructs were tested for replication either alone (*Left*) or together with a larger internal standard (*Msp*/L; *Right*).

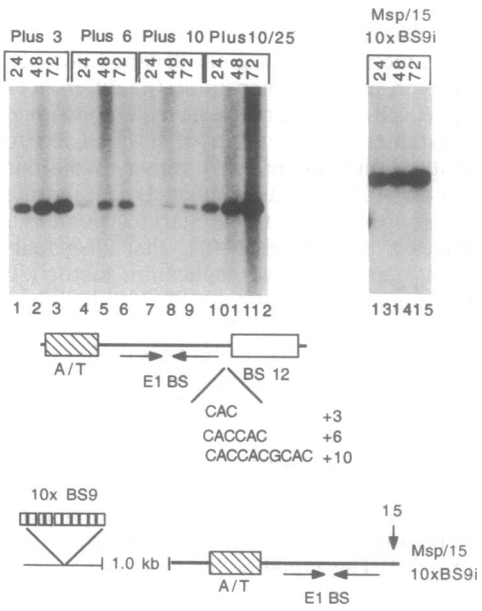

FIG. 5. Correlation between distance and required affinity of E2 for replication activity. (*Left*) To determine the effect of increased distance between the E1 and E2 binding sites 3, 6, or 10 nt were inserted between the two sites in the context of the minimal Ori as shown (*Lower*). In +10/25, a point mutation has been introduced at position 25 (see Fig. 3). (*Right*) Replication-defective construct *Msp*/15 was modified by insertion of 10 copies of E2 BS9 separated from the Ori by a 1.0-kb spacer sequence and tested for replication.

have to be located close to the Ori and are not functional at distances greater than ≈100 bp (22). In spite of this relatively detailed information, the direct function of the transcriptional component is not known.

It has been demonstrated that E2 can function to facilitate the binding of E1 to its binding site (6). In those experiments, no requirement for an E2 binding site was found and it was assumed that E2 could perform this function without specific binding to DNA. However, the E2 BS^- construct that was used in that study is the equivalent of the D22 mutant, which retains half of the E2 binding site, which clearly is sufficient for replication *in vivo*. This half palindrome is sufficient for cooperative binding of E1 and E2 *in vitro* (J. Sedman and A.S., unpublished data). A possible function of E2 therefore is that binding of E2 to its binding site changes the structure of the DNA locally, which in turn facilitates E1 binding. This type of mechanism has been suggested for cooperative binding of RepA and dnaA to the plasmid R1 origin (26). This model would be consistent with the sensitivity to slight increases in distance observed with the low-affinity BS12. The argument against this model is that E2 clearly can work from a distance and also that the DNA binding domain of E2 alone is not capable of supporting replication.

A more likely explanation is that the sensitivity to distance is a question of occupancy. If both E1 and E2 have low affinity for their respective sites, these sites may need to be close together to promote a protein–protein interaction that stabilizes the binding of both proteins. If, on the other hand, the E2 binding site has high affinity and is occupied a large fraction of the time, the interaction could still take place in spite of a greater distance between the two sites. This scenario requires interaction between E1 and E2. Such an interaction has been detected when the two proteins are overexpressed from baculovirus vectors (27–29).

Superficially, the properties of the E2 polypeptide in replication appear quite different from the classical replication enhancer discussed above. The requirement for E2 cannot be circumvented by addition of the polyomavirus enhancer to the minimal Ori (M.U. and A.S., unpublished data). The specific E2 transactivation domain is required and cannot be replaced with the VP16 transactivation domain (5), and E2 can function from a great distance in multimerized form. However, at closer inspection the differences are not as distinctive as it may seem. We have demonstrated that E2 can function either close to the Ori or at a distance from it and that the distinction is quantitative rather than a qualitative. For another class of replication enhancers represented by the Epstein–Barr virus (EBV), the similarities are more obvious. EBV encodes a protein, EBNA-1, that also doubles as a transcriptional activator and replication factor (30–32). A replication enhancer consisting of 20 binding sites for EBNA-1 is located 1 kb away from Ori. This enhancer is required for replication and cannot be replaced with other enhancers (30, 33, 34). This arrangement is virtually identical to the requirement for multimerized E2 binding sites at a distance for BPV replication.

An interesting way of viewing replication enhancers, based on the results obtained with E2, is that the distance dependence may reflect the strength of interaction between the enhancer and some component in core Ori (presumably the initiator protein). This could be the case irrespective of what purpose the interaction is serving, either to clear nucleosomes, which has been suggested in the case of simian virus 40 (35, 36), or to facilitate the interaction of the initiator protein with DNA, which appears to be the case for BPV, or a combination of the two. An intrinsically weak interaction would require multiple sites close by, while an intrinsically strong interaction could use either strong sites at a distance or a weak site close by. An interesting correlation is apparent. BPV and EBV, which both show a high degree of specificity (i.e., other activators cannot substitute for E2 and EBNA-1), also show high affinity based on the ability to function at a distance. Polyomavirus can utilize many different activation domains (low specificity) and the interactions are low affinity based on the inability to function at a distance. One important distinction between these two types is that while core Ori of polyomavirus has to be capable of interacting with various activation domains present in the cell, BPV and EBV need only interact with their own dedicated, virus-encoded activators E2 and EBNA-1, respectively. The need to preserve the capacity to interact with multiple activation domains may require a compromise in terms of affinity, while BPV and EBV with one specific partner could utilize a strong interaction.

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