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Remodeling of the Human Papillomavirus Type 11 Replication Origin into Discrete Nucleoprotein Particles and Looped Structures by the E2 Protein

Jeonggu Sim[*,%,1], **Sezgin Ozgur**[1,*], **Biing Yuan Lin**[2], **Jei-Hwa Yu**[2], **Thomas R. Broker**[2], **Louise T. Chow**[2], and **Jack Griffith**[1,#]

[1] Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill NC 27599-7295

[2] Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005

Abstract

The human papillomavirus (HPV) origin (*ori*) of DNA replication shares a common theme with many DNA control elements in having multiple binding sites for one or more proteins spaced over several hundred base pairs. The HPV type-11 *ori* spans 103 bp and contains three palindromic binding sites (E2BS-2, E2BS-3, and E2BS-4) for the dimeric E2 origin binding protein. These sites are separated by 64 bp and 3 bp. E2BS-1 is located 288 bp upstream of E2BS-2 and is not required for efficient transient or cell-free replication. In this study, electron microscopy was used to visualize complexes of HPV-11 *ori* DNA bound by purified E2 protein. DNA containing only E2BS-2 showed a single E2 dimer bound. DNA containing E2BS-3 and E2BS-4 showed two side-by-side E2 dimers, while DNA containing E2BS-2, E2BS-3, and E2BS-4 exhibited a large disk/ring-shaped protein particle bound indicating that the DNA had been remodeled into a discrete complex, likely containing an E2 hexamer. With all four binding sites present, up to 27% of the DNA molecules were arranged into loops by E2, the majority of which spanned E2BS-1 and one of the other three sites. Studies of the dependence of looping on salt, ATP, and DTT using full length E2 and an E2 protein containing only the carboxyl-terminal DNA binding and protein dimerization domain suggest that looping is dependent on the N terminal domain as well as factors which may affect the manner in which E2 scans DNA for binding sites. The role of these structures in the modeling and regulation of the HPV-11 *ori* is discussed.

Keywords

HPV; E2; EM; DNA binding; DNA looping

#To whom correspondence should be addressed. Phone: (919) 966-2151, e-mail: jdg@med.unc.edu.
*These two authors contributed equally to the work
%current address: Department of Oriental Medicinal Material and Processing, Kyunghee University, Republic of Korea

Introduction

A common structural theme of eukaryotic replication and transcriptional promoters is the presence of arrays of binding sites for DNA binding proteins. These sites may be close together or separated by up to hundreds of base pairs, suggesting that the DNA provides a scaffold upon which complex three dimensional nucleoprotein structures are sculpted. There are many ways in which this can occur, including the formation of DNA loops and condensed nucleoprotein particles. The binding of human papillomavirus (HPV) type 11 E2 protein to the homologous *ori* offers an excellent model for understanding how such DNA-protein remodeling may occur and, in turn, regulate DNA function.

HPVs are a family of small DNA viruses which infect human squamous epithelia at mucosal or cutaneous sites, causing benign hyperproliferative, warty lesions. HPV has a double-stranded (ds) circular DNA genome of approximately 7.9 kb and replicates as multi-copy nuclear plasmids. Viral DNA replication requires the viral *ori* and the cellular DNA replication machinery. The virus contributes two replication factors, the *ori*-binding protein E2 and the replicative DNA helicase E1.[1–4] The papillomaviral protein E1 is required throughout initiation and elongation[5], as it is an ATP-dependent helicase, equivalent to the cellular replicative DNA helicase (the minichromosome maintenance complex). In contrast, the *ori*-binding E2 protein is only essential for the assembly of the pre-initiation complex and is absent during the elongation phase of papillomavirus DNA replication.[5] In addition, E2 is also responsible for equitable viral genome segregation into daughter cells.[6–11]

The HPV-11 *ori* spans 103 bp and lies at the 3′ end of the upstream regulatory region (URR), a 700 bp long non-coding region which also controls transcription from the adjacent E6 promoter. The *ori* is comprised of three E2 binding sites (E2BSs) flanking an array of E1 BSs where E1 assembles into a di-hexameric helicase. The three E2BSs are separated by 64 and 3 bp from one another. E2BS-2 lies immediately upstream of the E1BS whereas the E2BS-3 and E2BS-4 are located just downstream. E2BS-4 is situated 3 bp upstream of the TATA sequence of the E6 promoter. By binding to the overlapping *ori*/promoter elements, E2 also functions as a transcription factor and modulates the activity of this promoter. The E1 protein is recruited to the *ori* by the E2 protein.[12,13] Studies of HPV-11 E1 and E2 binding to the HPV *ori* using electron microscopy (EM) have shown that the cellular heat shock/chaperone proteins hsp40 and hsp70 facilitate the formation of the E1 di-hexamer at the *ori*, with the release of E2.[14,15] In addition, there is another E2 binding site, E2BS-1, which lies 288 bp upstream of E2BS-2 and is not required for efficient transient replication in transfected cells or for cell-free replication.[3,4,16] This sequence arrangement in the HPV-11 *ori* is highly conserved in many HPV genotypes.

The E2 proteins vary in size among the papillomavirus types including the bovine papillomavirus (BPV), ranging from 42–48 kDa, but their functions are highly conserved. The amino terminal half of the protein is the *trans*-acting domain which interacts with the E1 helicase, recruiting it to the *ori*, and with transcription factors.[12,13,17] A central hinge region is poorly conserved in length and in sequence. However, for HPV-11 E2 and perhaps additional mucosotropic HPV genotypes, this region contains a nuclear localization sequence and also associates with the nuclear matrix.[18] The carboxyl-terminal domain promotes E2 protein dimerization, and the dimer then binds in the major groove of the DNA at a 12-base pair palindromic sequence (5′-ACCGN4CGGT-3′).[19] All papilloma virus E2 proteins recognize this 12 bp sequence although the 4 base pair spacer may vary among viruses.

The BPV *ori*/promoter contains a cluster of 12 E2 binding sites, and Knight et al.[20] showed that BPV E2 binds DNA as a dimer and also forms larger protein particles when two E2 binding sites are spaced close together. Using EM, loops were occasionally noted when the binding

sites were artificially spaced by a larger distance. This study also implicated the N terminal domain of BPV E2 in the loop formation. In an X-ray crystallographic study[21], E2N dimers were observed with HPV-16 E2, but not with HPV-11 E2[22] nor with HPV-18 E2 truncated of residues 1–65.[21] These studies suggested that an EM examination of the binding of HPV E2 to the natural HPV *ori* might reveal multiple levels of protein-mediated folding and remodeling of the DNA central to the control of HPV replication and transcription.

In this work we have carried out such a study using purified functional HPV-E2 protein and HPV-11 *ori*-spanning fragments containing from one to all four of the E2 binding sites. HPV-11 was selected for this study, as the structures and functions of the E1 and E2 proteins of this virus have been examined intensively, including collaborative EM work between the two laboratories involved in this present work. The 4 binding sites provide ample opportunity for remodeling events in the *ori* but without the complexity of the much larger number of E2 sites found in the BPV genome. We found that the cluster of three closely spaced sites generated a trimer of dimers arranged into a disk/ring-shaped hexamer. Moreover, when the DNA fragment contained all four binding sites, frequent DNA loops were observed between the distal E2BS-1 and one of the cluster of three adjacent sites some 288 bp downstream. E2C, which contains only the protein dimerization and DNA binding domain, abolished looping, indicating the amino terminal portion of the protein is necessary for DNA looping. On the basis of these structures, we discuss the implications on the assembly and regulation of functional replication complexes.

Results

E2 and E2C proteins bind to E2BS in EMSA

The functionality of the proteins used in this study was tested by electrophoretic mobility shift assays (EMSA) using probes containing E2BS-3 and E2BS-4. As shown in Fig. 1, a slow migrating band was observed when either full length E2 or E2C containing only the carboxyl terminal 83 amino acids (a.a.) were present. As expected, E2C formed a much faster migrating complex relative to the complex formed with the full-length E2 protein. The specificity of these protein-DNA complexes was demonstrated by the inability of E2 C298S, which is mutated in the DNA binding motif, to retard the probe. This mutant form of E2 did not support transient or cell-free replication, whereas the purified wild type full-length E2 protein did (data not shown). The native E2 and epitope tagged E2 functioned similarly in cell free replication assays when the same levels of the two E2 proteins are used (data not shown).

E2 binds E2BS-2 alone as a single protein dimer

Several linear DNA templates containing portions of the HPV-11 URR were generated and used as substrates for E2 binding (Fig. 2). The short fragments each contains E2BS-2, E2BS-3, 4, or E2BS-2, 3, 4. One long fragment contains all 4 sites. The optimal amounts of DNA and protein for EM visualization were empirically determined to be 100 ng and 150 ng, respectively, and were used in all the experiments unless otherwise stated. When less E2 protein was added, fewer DNAs showed bound protein, whereas more E2 led to more complexes but also DNA-protein aggregation.

To visualize the binding of E2 protein to HPV-11 *ori* DNA when only a single site is present, a 403 bp DNA fragment containing just E2BS-2 was cleaved from a pUC19-based plasmid, p7874-20 (Fig. 2B). E2BS-2 is located 229 bp from one end and 162 bp from the other end. Examination of fields of DNA revealed several structural forms. Scoring 962 molecules, 26 % of the DNA fragments were bound by protein, with the remainder appearing protein-free. Further analysis of the E2 bound DNA molecules demonstrated that 210 of the complexes consisted of a DNA molecule bound by a single protein particle located slightly off center,

roughly corresponding to the location of E2BS-2 (Fig. 3A, B). The remainder of the complexes (40) consisted of DNAs with a large protein particle of variable size (presumably an E2 multimer) bound at the center or near one end (not shown) (Table 1A).

The majority of the E2 protein bound at E2BS-2 had a size consistent with E2 dimers (84 kDa dimer mass) based on comparison with other proteins prepared by these EM methods in previous studies. To measure the mass of the E2 particles, E2-DNA complexes were formed, fixed with glutaraldehyde and just prior to mounting on the EM substrates, creatine phosphokinase (CPK, 81 KDa) was added and the samples prepared for EM. This protein is roughly spherical and is present in solution as a monomer making it an ideal size marker in the range of 80 KDa. An example of apoferritin used in this way is shown later in Fig. 4. In the same fields, the projected areas of several CPK particles were measured and compared to the projected area of the E2 particle bound to the DNA. From the ratio of the projected areas, an estimate of the mass of the E2 particle could be calculated (Materials and Methods). The resulting value, 95 KDa, (n=14), is in good agreement with the mass of an E2 dimer plus a short segment of DNA. In this and the subsequent experiments in which a single binding site was present, there was no noticeable shortening of the DNA following protein binding.

E2 binds E2BS-3 and E2BS-4 as two side-by-side dimers

The plasmid p7902-99 containing E2BS-3 and E2BS-4 was cleaved with *Pvu II* to generate a 482 bp fragment with the two sites centrally located (Fig. 2C). Of 355 DNAs scored, 56% were protein-bound and were divided into three classes. In the first class (46% of the E2-DNA complexes scored) the DNAs showed a single E2 particle bound near the center (not shown). In the second class (42% of the E2-DNA complexes scored) there were two side-by-side particles located near the center of the DNA (Fig. 3C, D) with no indication of any organization of the two dimers into a compact E2 tetramer following DNA binding. The size of the individual particles was indistinguishable from the dimeric particles present when the DNA contained only E2BS-2. In the third class (12% of the E2-DNA complexes scored), a large E2 multimer of variable size was centrally bound along the DNA (not shown) (Table 1B).

A disk-shaped complex of E2 forms on DNA containing E2BS-2, 3, and 4

The plasmid p7730-99 was cleaved with *Pvu II* to generate a 623 bp DNA fragment containing E2BS-2, E2BS-3 and E2BS-4 (Fig. 2D). E2BS-2 and E2BS-3 are separated by 64 bp while E2BS-3 and E2BS-4 are separated by 3 bp (see above). The three sites span the central 103 bp in this fragment, with 266 bp between E2BS-2 and the nearest DNA end and 254 bp from E2BS-4 to the nearest DNA end. Incubation of this DNA fragment with E2 generated a series of DNA-protein complexes. Two complexes recapitulated what was observed with DNA containing only E2BS-3 and E2BS-4: a single centrally bound E2 dimer particle, or two side-by-side E2 dimers near the center of the DNA. In addition, a new DNA-protein structure was observed which was the most abundant of the E2-DNA complexes. These molecules consisted of a DNA fragment containing a large round particle close to the center of the DNA (Fig. 4A, D–I) flanked by DNA arms, each of roughly 250 bp in length. This large complex frequently showed a hole or depression in the center, giving it a disk or ring shape. The remaining (minor) E2-DNA species consisted of larger multimers of E2 on the DNA or aggregates of the E2-DNA complexes (not shown). Titration of E2 from 40 to 320 ng relative to a constant amount of DNA (100 ng) revealed a relatively constant number of single E2 dimers on the DNA (17–24%), a low amount of side-by-side dimers (3.7 to 8%), and a frequency of disk-shaped particles that increased from 11% to 25 % (Table 1C–F). It was of interest to examine the binding of E2 to a circular DNA containing the HPV-11 *ori* to determine the specificity on a much larger DNA. Incubation of the 3.3 kb circular p7874-99 plasmid DNA (100 ng), which also contains E2BS-2, -3, and 4, with E2 (150 ng) led to primarily a single disk-shaped particle bound to the DNA (Fig. 4B,C). This illustrated the high specificity of E2 binding. The majority

of the E2-bound DNA was in the supercoiled form. But for clear visualization of the E2 disk, complexes formed on open circular DNA are shown. This latter observation suggests that this structural remodeling of the three binding sites by E2 may be facilitated by negative supercoiling.

The disk-shaped particles contacted the DNA along one side (Fig. 4A, D–I), in contrast to E2 binding at E2BS-3 and E2BS-4 where the DNA took a straight path through the center of the side-by-side dimers (Fig. 3C,D). These results suggest that the addition of the third E2 dimer induced a conformational transition from a linear chain of 3 dimers to a ring or disk-shaped particle. The 64 bp segment between E2BS-2 and E2BS-3 would be looped out of the E2 complex but it was too small to be easily observed by these EM methods.

Comparison with the size of apoferritin (443 KDa) provided an estimate of the mass of the E2 disk on DNA. A single disk-shaped E2 particle on DNA with four apoferritin molecules in close proximity is shown in Fig. 4I. The free apoferritin particles in the background cannot be free E2 disks since they only form on long DNA, and further the apoferritin particles appear identical to apoferritin mounted on parallel grids alone. Finally the size of the apoferritin particles is in good agreement with the known diameter of 184A. The projected area of each E2 particle was measured and divided by the average projected area of the several apoferritin molecules lying nearby. From 50 such measurements, the mean projected area of the E2 complexes was 1.16 times that of apoferritin. Calculation of the masses (Materials and Methods) revealed that a spherical protein particle with this projected area would have a mass of 560 KDa. However the E2-DNA particles contain 103 bp of DNA equivalent to 70 KDa which must be subtracted. Thus, if the E2-DNA particles are spherical they would contain 5 E2 dimers; but if they are disk shaped as suggested by the images, they would have a substantially lower mass. We propose that this complex is most likely a particle containing three E2 dimers which has rearranged into a disk shaped particle with a central hole. An illustration of the proposed structure is shown in Figure 8. Interestingly, the BPV-1 E2 has also been thought to form a hexameric complex.[20] In that study, a DNA bow formed by E2 proteins bound to three E2 BSs that were artificially separated from one another by 500 bp each.

DNA forms a loop when E2 and all 4 binding sites are present

The full HPV upstream regulatory region (long control region) contains 4 E2 binding sites, with E2BS-1 located 288 bp upstream from E2BS-2 (Fig. 2). The role of this E2 BS in viral DNA replication has not been examined. We asked whether E2 binding at E2BS-1 would form a complex with E2 bound at the other 3 sites to generate a new higher order complex. A1244 bp fragment was generated which contains all 4 sites (Fig. 2A) (Materials and Methods). The distances to the nearest ends are 162 bp and 640 bp. The DNA fragment was incubated with E2 protein (150 ng of E2 and 100 ng of DNA) under the same buffer conditions used above and prepared for EM. Inspection of fields of molecules revealed a variety of E2-DNA species. Several recapitulated the forms observed for DNAs containing 1 to 3 E2 binding sites, including 1 or 2 E2 dimers bound at positions consistent with the known locations of the E2 binding sites and the disk-shaped particles bound along the DNA. In addition, a new E2-dependent species was commonly observed in which the DNA was arranged into a loop with E2 protein at the node of the loop (Fig. 5A–F). Scoring 301 DNA molecules, 84% of the DNA was bound by E2 protein. Furthermore, 27% of theDNA molecules scored were arranged into a loop by the protein. The rest of the complexes were in one of the forms described in previous sections.

Using the 1244 bp DNA (Fig. 2A) with 162 and 640 bp arms, the fraction of the total DNA length comprising the loop and the fractional length of each arm were measured in 89 looped molecules. This analysis revealed that, in 65% of the molecules, the size of the loop was consistent with looping between E2BS-1 and the cluster of E2BS-2, E2BS-3 and E2BS-4 sites, constraining the intervening DNA into a loop of 250 to 400 bp in length as shown in a histogram

(Fig. 6). However, in ~1/3rd of the molecules the loop length was greater than 400 bp (Fig. 5F), with some as long as 800 bp. One possible explanation is described in the Discussion.

Inspection of the E2 particles in the looped species revealed a range of sizes and structures. Frequently there were 2 distinct equal-sized lobes (Fig. 5A, B, F, H, I) suggestive of 2 E2 dimers. In some cases (Fig. 5G) it appeared that 3 E2 dimers were arranged side-by-side in a linear manner with the DNA folding back to bind along the outside of the chain of dimers. In rare cases (Fig. 5J) there were 2 domains with one significantly larger, possibly a hexameric disk while the other was smaller likely a single E2 dimer. Overall this suggested that loop formation most commonly occurred by the binding of E2 dimers at E2BS-1 and one of the 3 downstream binding sites followed by interaction between the 2 dimers to generate a loop.

Salt, DTT, and ATP regulate DNA loop formation

To determine whether DNA looping is regulated by co-factors, E2 was incubated with the 1244 bp DNA template containing all 4 E2 binding sites (Fig. 2A). In the earlier studies using DNAs with 1 to 3 E2 binding sites, we noted that inclusion of 2 mM ATP in the absence of Mg^{++} ions provided more consistent results and somewhat higher levels of binding than when it was left out and thus ATP was included in the work above. ATP is essential for the loading of E1 in an E2-dependent fashion, although any direct effect of ATP on E2 is not known.[23] Incubation of the full-length E2 with the DNA containing 4 E2 sites as described above (Fig. 5) in the presence of 2 mM ATP resulted in 27% of the total molecules scored being arranged into E2-mediated loops (Table 2). When ATP was omitted from the incubation, the level of looping dropped to 11%. If however 1 mM DTT was included (in the absence of ATP), then the level of looping was restored (Table 2). Looping was almost completely abolished (3% loops) by raising the salt in the incubation to 50 mM KCl (Table 2). In the latter case, while looping was abolished, 60% of the DNA showed E2 particles bound along the linear DNA. Further inspection of these results (Table 2) revealed that, in the presence of ATP or DTT, there were fewer DNA molecules with no protein bound (16% and 7% respectively) with a corresponding higher level of looping. In contrast, in the absence of ATP or in the presence of salt, there were more protein-free DNAs (48% and 38% respectively) and fewer loops. These observations point to complex ionic and co-factor requirements for loop formation. A model, which may help explain these observations, is described in the Discussion.

The E2 amino terminal *trans*-acting domain is required for DNA looping

The 83 a.a. E2 carboxyl-terminal fragment is ~9 KDa which is too small to be detected by the EM methods used here bound to DNA. Based on previous work in this laboratory with DNA binding proteins of increasing size, a tetramer of the E2C fragment would be the smallest oligomeric form that we would expect to be visualizable bound to DNA by these shadow-casting methods. When E2C was incubated with the 1244 bp DNA containing all 4 E2 binding sites in a buffer containing 50 mM KCl and no ATP, tiny protein particles were observed bound to 81% of the DNA molecules (arrows Fig. 7A–C) and their positions corresponded roughly to the E2 BSs. When the incubations contained 2 mM ATP and no salt, these tiny particles were also present on the DNA (84%) (Fig. 7E) but larger particles were also observed (Fig. 7D,F). These large particles must represent some higher multimers of the E2C fragment. Under either condition, only a few DNAs were arranged into loops, ranging from 1 to 6% of the total DNA. In the cases of looping the protein complex at the node of the loop was large, such as the particles in Fig. 7D,F. These observations suggest that DNA looping could be attributable to an interaction between the amino terminal *trans*-acting domain of pairs of E2 dimers bound to E2BS-1 and one of the downstream sites, in agreement with the report for BPV-1 E2.[20] This interpretation also agrees with the amino-terminal interaction model based on the X ray crystal structures of HPV-16 E2.[24]

Discussion

In this study we employed transmission electron microscopy to examine the remodeling of the HPV-11 replication origin by the E2 protein. This involves local and long range interactions between E2 and DNA over a distance of ~500 bp, and we observed the generation of both small and large DNA-protein particles and looped species. While the HPV *ori* has been known to bind both E2 and the replicative helicase E1, no previous studies have been carried out to determine how E2 interacts globally with the URR segment containing all 4 binding sites. We can summarize observations of several thousand individual molecules as follows: E2 binds to each of the 4 sites as single protein dimers at low protein concentration. When just the 2 closely spaced sites are present, a pair of E2 dimers is arranged side-by-side on the DNA. With the addition of the third member of the cluster of three sites, a rapid conversion of the E2-DNA complex into a disk-shaped particle occurs, suggestive of a ring-like trimer of E2 dimers (or hexamer) with DNA wound around the ring. Finally upon inclusion of the distal E2BS-1, DNA loops 250 to 400 bp in size formed between E2BS-1 and one of 3 closely spaced sites. Looping frequently involves 2 dimers, one at E2BS-1 and another at one of the downstream sites. These studies revealed two DNA remodeling events upon binding by E2: the generation of a ring trimer of E2 dimers and the formation of long range DNA loops. These structures likely function to control the binding of the E1 helicase and access of other proteins to the *ori*.

Hexameric E2 ring formation on the origin

The formation of the disk-shaped E2 oligomers must be rapid, as we never observed 3 side-by-side dimers under the standard binding conditions. Mass analysis and modeling suggested that the disk or ring is composed of 3 E2 dimers and ~100 bp of DNA. The observation that the DNA enters and exits from a single point on the side of the particle suggests that the linear chain of 3 E2 dimers coils into a ring with the DNA along the outside of the ring. A trimer of dimers has been observed in the crystal structure of the carboxyl terminal DNA binding domain of the BPV-1 E2 protein.[19,25] The 3 E2C dimers are cross-linked by 3 disulfide bonds between neighboring E2 dimers. We have no information as to whether the HPV-11 E2 ring complex described in this study also involves disulfide bonds between neighboring dimers. However we note that addition of DTT to the binding buffer did not abolish E2 disk formation, suggesting disulfide bond formation is not essential. The validity of this argument was further confirmed by the fact that the DNA binding activity of E2 increased in the presence of DTT. The crystal structure of BPV-1 E2 revealed that one of the cysteine residues involved in trimer formation of E2C is also essential for the DNA binding activity. Thus, disulfide formation could inhibit the DNA binding of the enzyme as well. Within this hexameric ring is a 64 bp DNA segment containing the binding site for the E1 replicative helicase, and this segment would not be expected to be bound by E2. Rather it may either be looped into the center of the E2 ring or perhaps looped out on the surface presenting the 64 bp segment to E1 (Fig. 8). The strain of bending the DNA helix into a tight 64 bp loop would be expected to facilitate E1 loading and denaturing the *ori* sequence. Negative supercoiling of the DNA template should and did promote the wrapping of the 3 E2 dimers into the compact hexameric particle, as a toroidal loop is topologically equivalent to a supercoil in covalently closed DNA molecules. This compact E2-*ori* particle may have similarities in the DnaA complex that forms at the E. coli *ori*[26] or the lambda O-some formed by the binding of the O protein at the lambda replication *ori*.[27]

Examination of the binding of E2 when there were two adjacent sites (Table 1) revealed an apparent lack of cooperativity in binding. DNAs were seen with one or two dimers bound rather than only two dimers or none (cooperative binding). This is in agreement with the work of Tan and colleagues[28] who noted a lack of cooperativity for HPV16 E2 and in contrast to the work of Monini and colleagues[29] who observed cooperative binding for BPV1 E2. The observation

that inclusion of 2 mM ATP led to a 2-fold enhancement in E2 binding seemed curious as magnesium was not added and no ATPase activity has been detected or reported for E2 protein. It remains possible however that E2 weakly binds ATP and that upon entry of E1 (Fig. 8) E2 acts as an ATP donor presenting a required substrate to E1 for its ensuing unwinding of the DNA.

DNA looping by E2

Protein-mediated DNA looping has been visualized previously, beginning with the demonstration of loops generated by lambda repressor.[30] The activation of the *ori* of the *E. coli* R6K plasmid has been shown to involve long range DNA looping mediated by the *ori* binding protein RepA.[31,32], DNA looping also occurs within the latency replication *ori* (*ori*P) of the human Epstein-Barr virus (EBV).[33,34] *Ori*P contains two clusters of binding sites for the *ori* binding protein EBNA-1 separated by ~1 kb, and looping between the two clusters mediated by EBNA-1 may serve to generate a large DNA-protein complex that controls switching the virus from latent to lytic states. DNA looping has been observed with BPV-1 E2 protein bound to BPV-1 *ori* fragments.[20] In that study, a tiny loop was occasionally observed between two sites spaced very close together, and larger loops generated by BPV-1 E2 were also noted but these involved an artificial template that did not reflect the spacing of sites in the BPV *ori*.

DNAs containing E2-mediated loops were the most frequently observed species when the *ori* DNA contains all 4 E2BS's. Because looping was reduced 5–7 fold with E2C which lacks the amino-terminal *trans*-acting domain, looping may be attributed to interactions between the amino termini between pairs of dimers, one bound at site 1, the other at one of the three downstream sites. Loop formation in which a ring/disk shaped hexamer of E2 encompassing E2BS-2,-3, and 4 interacted with an E2 dimer bound at E2BS-1 was very uncommon, suggesting the long distance interaction involving one of E2 dimers at the downstream site may have prevented its incorporation into a hexameric ring with two E2 dimers bound at neighboring sites.

The HPV-11 protein E1 binds to the *ori* as a hexameric or dihexameric ring.[14,15] It is tempting to speculate that the hexameric E2 protein bound on HPV-11 *ori* at E2BS-2, -3 and -4 plays an important role in recruitment of the HPV-11 E1 protein. The 64 bp segment between E2BS-2 and E2BS-3 may undergo a great change in torsion when the linear chain of E2 dimers bound at E2BS-2,-3-, and -4 coils around to form the disk-shaped particle with the E1 binding site pinched out, as we illustrate in Fig. 8. This would facilitate the assembly of the E1 dihexamer, whereupon E2 would be released from the *ori*.[17] Further high resolution EM studies will be focused on examination of the recruitment of E1 to the *ori* in the presence of E2. In this model, the DNA looping observed when E2 was also bound to E2BS-1 DNA might provide a mechanism to regulate DNA replication by competing for the E2 hexamer disk formation on the *ori*, thereby inhibiting the recruitment of E1 to the *ori* and initiation of replication. The large DNA loop does not induce steric strain as the small DNA loop does and hence may not be able to facilitate E1 binding and DNA melting. It is conceivable that E2 protein may increase along with viral DNA copy number in the differentiated keratinocytes. Upon achieving certain high protein level, E2 would cause viral DNA looping, thereby inhibiting further viral DNA amplification. This would then permit the expression of late gene encoding capsid proteins for virion assembly.

A model for E2 binding to DNA

The observation of a minor but significant fraction (1/3rd) of the looped molecules in which the size of the loop was greater than the distance between E2BS-1 and the cluster of 3 adjacent binding sites may reflect the mode by which E2 scans DNA for its binding sites. E2 dimers

may bind loosely to DNA and then scan along the DNA until a high affinity binding site is located. In this model, a transitory loop might form between an E2 dimer bound at, for example E2BS-4 and another E2 dimer bound in a scanning mode beyond E2BS-1 near the far terminus of the DNA fragment generating a very large loop. In this model, loop formation serves to stabilize the E2 dimer bound in the scanning mode at a non-consensus binding site until it is finally captured by the specific binding sites in a diffusion driven reaction. This model might also explain the larger protein complexes observed on some looped molecules if loading of additional E2 within the loop followed by scanning resulted in the trapping of more E2 dimers at the node of the loop.

Materials and Methods

Plasmids and protein

HPV-11 E2 expression construct and purification has been described previously.[35] The E2 protein from pRSET-11E2 was tagged at the amino terminus with an 8-amino acid epitope derived from a cytomegalovirus-encoded protein, pp65 (a phosphor-protein encoded by the UL83 gene of cytomegalovirus). The replication activities by the epitope tagged E2 and the native E2 proteins were similar (Data not shown). Construction of the HPV-11 E2 C298S mutant and the E2C protein used the same vector. Both clones were constructed by PCR mutagenesis and cloned into the pRSET expression vector. E2C spans the carboxyl terminus of E2 (residues 285–367). Purified proteins were tested in electrophoretic mobility shift assays (EMSA) as described.[36] The probes were end-labeled double-stranded synthetic oligonucleotides spanning nucleotides 7902-7933/1-92 containing E2 BS-3 and BS-4. A DNA substrate containing all four E2 BS's was generated by PCR using the following set of primers: a 1244 bp long substrate with the 5′-TACGCCAGCTGGCGAAAGG-3′ and 5′-GCTTTACACTTTATGCTTCCGG-3′. The primers for this substrate were designed to regions of the pUC19 plasmid flanking the HPV-11 URR fragment in the p7072-99 plasmid. Plasmid p7072-99 contains the HPV-11 URR fragment (nucleotides [nt] 7072 to 7933/1 to 99 between the *Hind III* and *Bam HI* sites of pUC 19.[37] The reactions were performed with a Qiagen Thermocycler using standard Taq Polymerase reaction conditions according to the manufacturer's protocol (Invitrogen). The PCR product was separated on 0.8 % agarose gels run in 1x Tris-Borate buffer (TBE) for 1.5 hours at 125 Volts. The correct DNA band was excised from the gel and purified using a gel purification kit (Qiagen). The final concentration of the DNA was adjusted to 50 ng/μl. Plasmidp7730-99, p7874-20, and p7902-99 contain HPV-11 nucleotides 7730 to 7933/1 to 99, 7874 to 7933/1 to 20, and 7902 to 7933/1 to 99, respectively.[5] These sequences contain E2 BS-2, -3, -4; E2 BS-2; and E2 BS-3, -4 respectively and are illustrated in Fig. 2.

DNA binding reactions

Binding assays were conducted with 100 ng of linear DNA and, unless otherwise noted, 150 ng of E2 protein in 20 μl of buffer containing 20 mM HEPES, pH 7.5, 2 mM ATP. The mixture of DNA and E2 protein was incubated for 20 min at 37°C, and then treated with glutaraldehyde (0.6 % (v/v) final concentration) for 5 min at room temperature. DNA-E2 complexes were separated through 2 ml columns of 6% agarose beads (Agarose Bead Technologies Inc.) equilibrated with 10 mM Tris-HCl, pH8.0, 0.1 mM EDTA.

Electron microscopy

Thin carbon foils supported by 400 mesh copper grids were treated with a glow discharge for 45 seconds as described previously.[38] Aliquots from the agarose bead column fractions were adsorbed for 3 min to the grids in a buffer containing 2 mM spermidine. The grids were washed sequentially with water and graded water/ethanol solutions to 100 % ethanol, followed by air-drying and rotary shadow-casting with tungsten at 1×10^{-6} torr. Philips CM12 and Tecnai 12

instruments were used at 40 KV, and images were recorded on film or digitally with a GATAN 794 slow scan camera (CM12) and GATAN Ultrascan 4000SP (Tecnai 12). The images were analyzed with Gatan software, and contrast was adjusted with Adobe Photoshop software.

The masses of E2-DNA particles were determined as described.[39] Briefly, internal size markers consisting of proteins of known molecular weight were added to the DNA-E2 complexes prior to preparation for EM. Fields containing the E2-DNA complexes with internal size marker proteins lying nearby were photographed using the GATAN 794 camera. Using the NIH Image software, the relative projected areas of the standard proteins and the E2 complex bound to the DNA were determined. The ratio of the projected areas was converted to relative masses using the formula [(Mass1/Mass2)=(Area1/Area2)[39]

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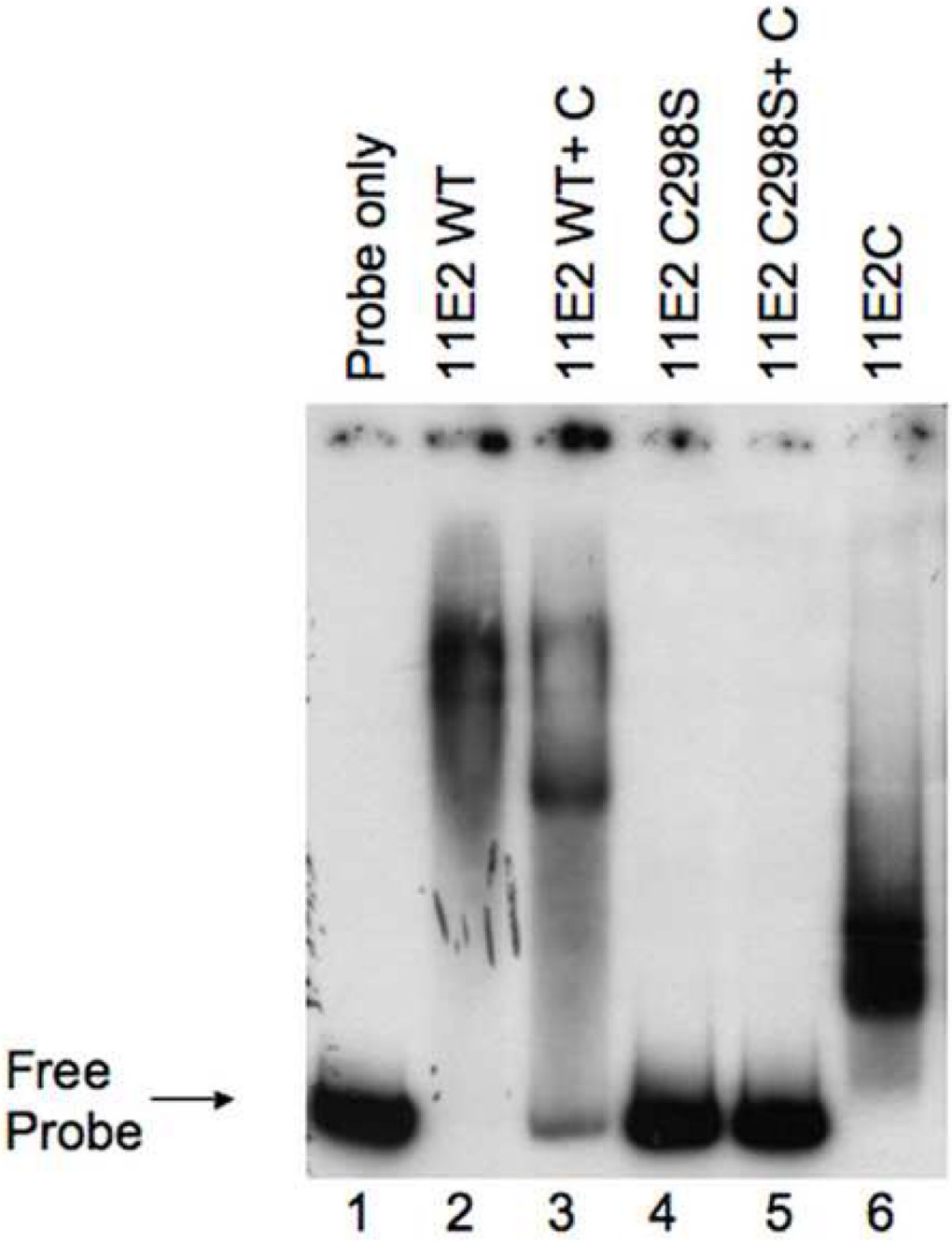

Figure 1. Gel shift analysis of the binding of HPV E2 proteins to the HPV ori

DNA probes containing E2BS-3 and E2BS-4 were incubated with HPV E2 proteins and separated by gel electrophoresis on a non-denaturing polyacrylamide gel. Lane 1, DNA substrate only, lane 2, full length wild type E2 protein. In lane 3, cold DNA substrate was included in the same reaction as in lane 2. Lane 4, E2 C298S, E2 protein containing a point mutation in the DNA binding domain which causes the protein to fail to bind DNA. Lane 5, E2 C298S binding in the presence of cold DNA substrate and lane 6, E2C protein, which contains only the protein dimerization and DNA binding domain.

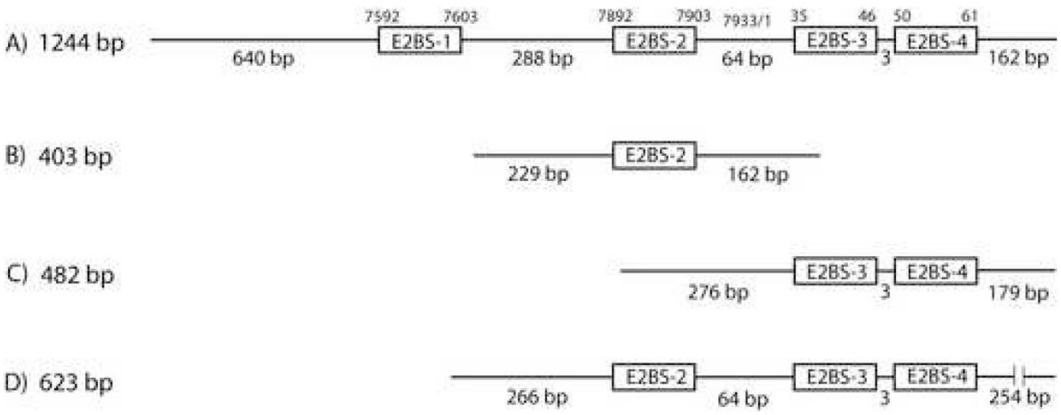

Figure 2. Map of the DNA fragments used to examine E2 binding

(A) HPV ori fragment containing all four E2 binding sites. Base pair separation between the sites is noted as well as the plasmid sequence numbers (see Materials and Methods for details of plasmids and fragment construction). (B–D) Fragments containing one, two, or three E2 binding sites.

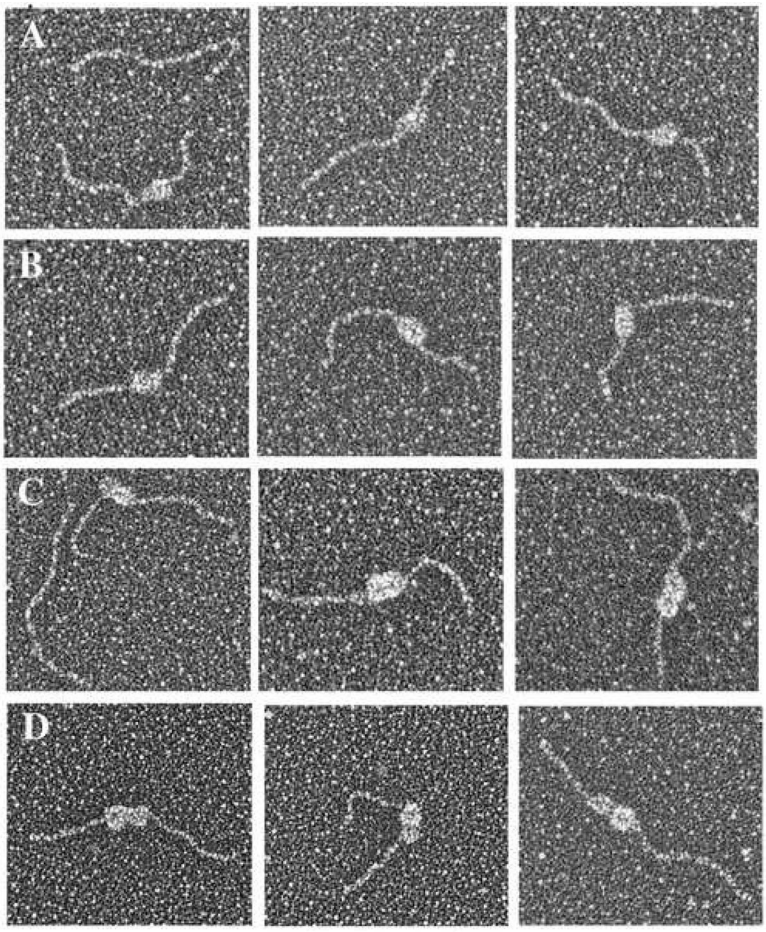

Figure 3. E2 protein forms dimers and paired dimers on DNAs containing one and two E2 binding sites

DNA fragments (100 ng) containing one E2 binding site (A,B) or two binding sites (C,D) (see Fig. 2) were incubated with 100 ng of E2 protein and prepared for EM by fixation with glutaraldehyde, dehydration, and rotary shadow-casting with tungsten (Materials and Methods). Side-by-side dimers of E2 can be seen in C and D. Since the examples shown here were taken from different experiments and EM grids, the variation of metal coating from one grid to the next results in some inevitable differences in apparent size. Nonetheless, single dimers were very different in appearance from two side by side dimers. The bar is equivalent to 300 bp. Shown in reverse contrast.

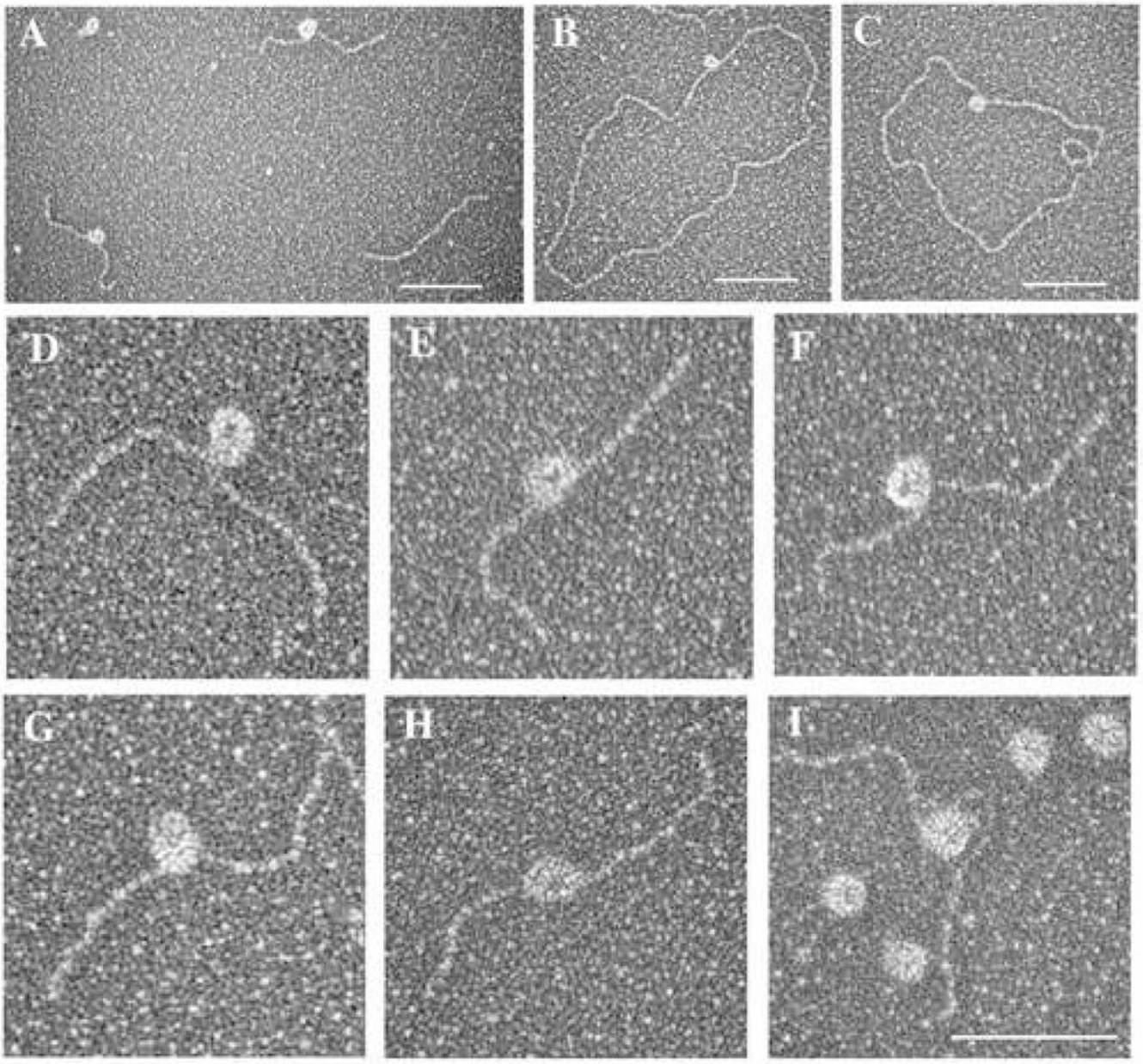

Figure 4. E2 protein forms a hexameric particle on DNA containing three E2 binding sites

A DNA fragment containing E2BS-2, -3, and 4 (A, D–I) or a 3.3 kb circular plasmid with the three sites (B, C) was incubated with E2 protein and prepared for EM as in Fig. 3. In B and C, relaxed circles show E2 bound more clearly than when E2 was bound on supercoiled DNAs, although supercoiled DNA bound with E2 predominated. On the linear DNA fragment, a large E2 complex is bound near the center. The complex appears as a round particle, often with a central hole and usually lying along one side of the DNA. This particle is similar in size to apoferritin added to the sample just before preparation for EM (I). Analysis suggested that this particle is an E2 hexamer (see text). Images are shown in reverse contrast. The bar equals to 350 bp in A–C and 250 bp in D–I.

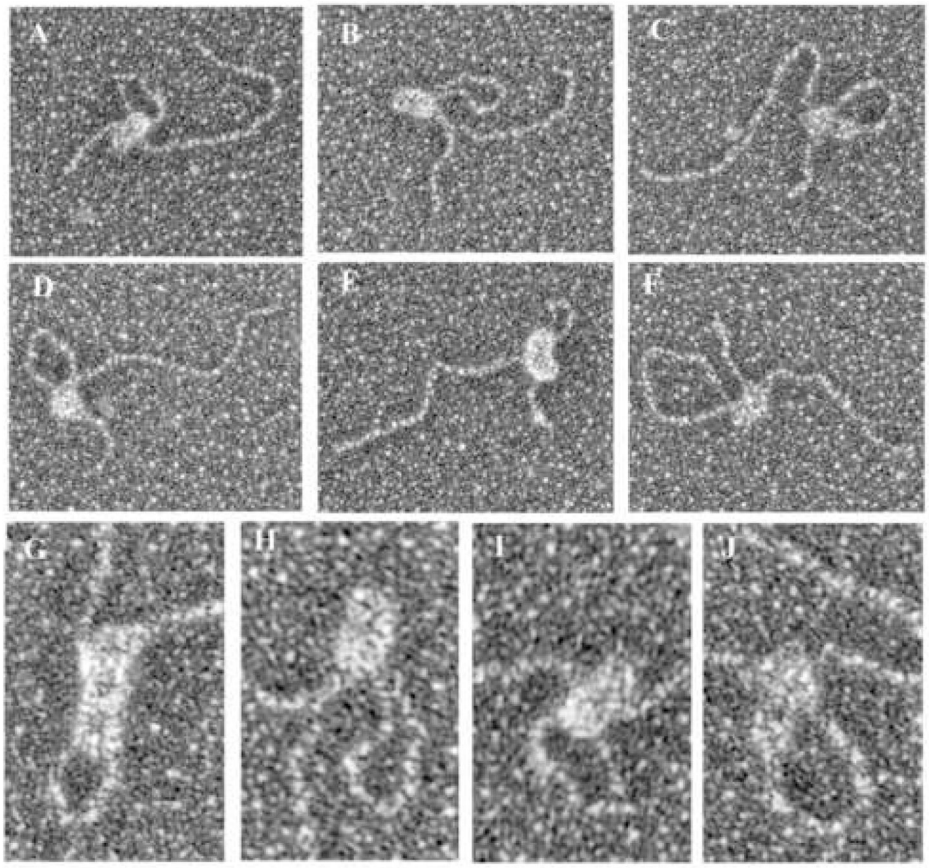

Figure 5. E2 protein forms loops on DNA containing all four natural E2 binding sites

Full length E2 protein was incubated with the 1244 bp DNA containing all four E2 binding sites (Fig. 2) in which E2BS-1 is spaced by 287 bp from E2BS-2. The incubation mixtures contained ATP, but not DTT or KCl. Preparation for EM was as described in Fig. 3 and 4. The loop sizes for the molecules in A–E are consistent with interactions between E2BS-1 and the cluster of sites containing E2BS-2,-3, and -4. The loop in F is larger than predicted for this interaction. Examples in the enlargements, G–F show molecules with loops formed by 3 E2 dimers (G), 2 E2 dimers (H, I) or possibly one dimer and one E2 hexamer (J). Images are shown in reverse contrast. The bar equals to 1 kbp (A–F) or 2 kbp (G–J).

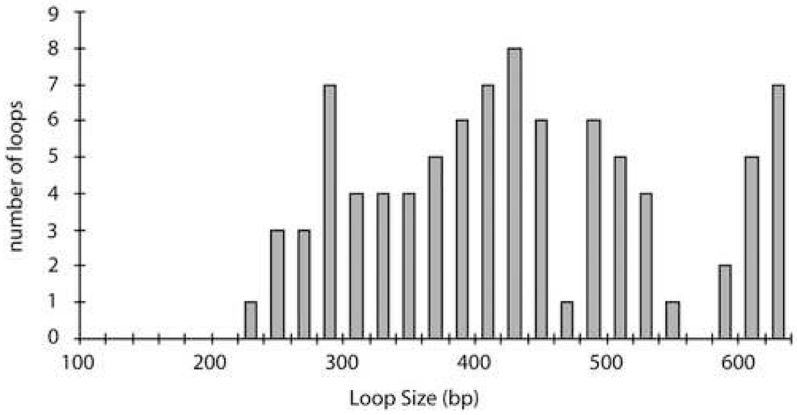

Figure 6. Statistical analysis of loop formation by E2 on 4BS

The loop sizes were measured from micrographs such as those in Fig. 5. The DNA loop lengths were translated into base pairs by calculating the fraction of the loop compared to the full length of the DNA molecule. Even though these are approximate numbers, they give a clear estimate of the size of the DNA loop. The majority of the loops fall in the range of 250–400 bp, which is close to the 288 bp distance between E2BS-1 and E2BS-2. The graph represents the number of complexes having the indicated loop size. The histogram of the loops was generated using Microsoft Excel Data Analysis tools.

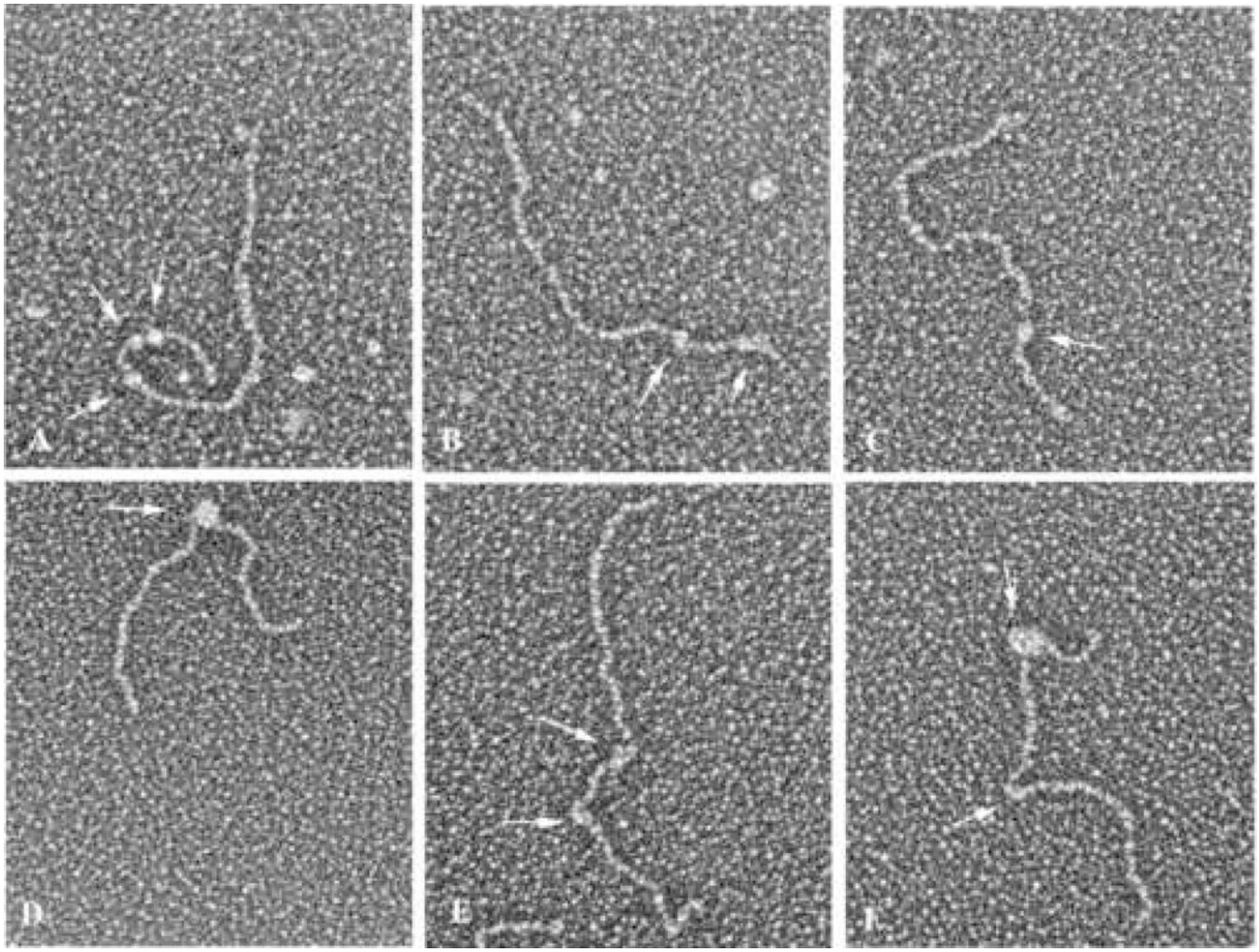

Figure 7. Visualization of E2C bound to DNA containing all binding sites

The 83 a.a. C-terminal fragment of E2 was incubated with the 1244 bp DNA fragment containing all four E2 binding sites and prepared for EM as in Fig. 3, 4. Arrows indicate protein complexes on the DNAs. The reaction buffers contained either 50 mM KCl (A–C) or 2 mM ATP (D–F). The large complexes on the DNAs in D and F likely represent multimerization of E2C bound to E2 binding sites. Images are shown in reverse contrast. The bar is equal to 150 bp.

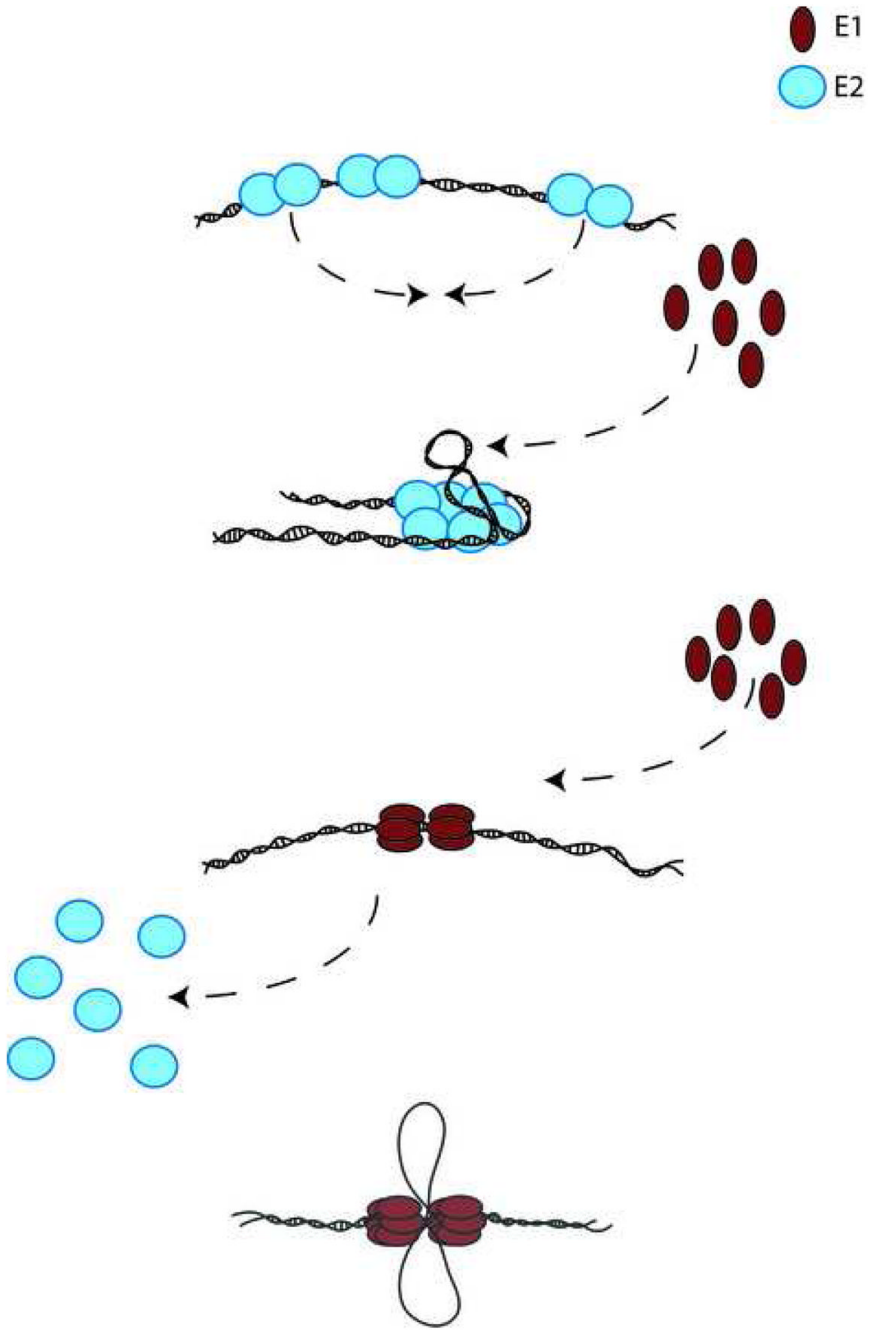

Figure 8. Model of the binding of E2 and E1 to the HPV ori

E2 dimers interact with the cluster of 3 adjacent E2 binding sites in the HPV *ori.* E2BS-1 is distant and is not shown. Once bound, the linear chain of three E2 dimers at E2BS-2, E2BS-3 and E2BS4 remodel the *ori* into a hexameric disk-ring shaped structure in which the 65 bp segment between E2BS-3 and E2BS-4 is pinched off into a tight loop. Interactions between the 3 dimers to generate this particle is facilitated by interactions between their N-terminal domains. This structure with the E1 binding site under torsional strain facilitates the entry of the E1 helicase complex which then excludes E2, and initiates unwinding of the DNA.

ited (first column). After
A with a single E2 dimer