Biochemical and Electron Microscopic Image Analysis of the Hexameric E1 Helicase*

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DNA replication initiator proteins bind site specifically to origin sites and in most cases participate in the early steps of unwinding the duplex. The papillomavirus preinitiation complex that assembles on the origin of replication is composed of proteins E1 and the activator protein E2. E2 is an ancillary factor that increases the affinity of E1 for the ori site through cooperative binding. Here we show that duplex DNA affects E1 (in the absence of E2) to assemble into an active hexameric structure. As a 10-base oligonucleotide can also induce this oligomerization, it seems likely that DNA binding allosterically induces a conformation that enhances hexamers. E1 assembles as a bi-lobed, presumably double hexameric structure on duplex DNA and can initiate bi-directional unwinding from an ori site. The DNA takes an apparent straight path through the double hexamers. Image analysis of E1 hexameric rings shows that the structures are heterogeneous and have either a 6- or 3-fold symmetry. The rings are about 40–50 Å thick and 125 Å in diameter. The density of the central cavity appears to be a variable and we speculate that a plugged center may represent a conformational flexibility of a subdomain of the monomer, to date unreported for other hexameric helicases.

The synthesis of duplex DNA is a complex enzymatic process that requires the coordination of large numbers of proteins. The mechanisms are elaborate in part because the enzymes that use the complementary template strand as a guide for nucleotide incorporation catalyze this synthesis only in the 5' to 3' direction. Given the antiparallel nature of the duplex this usually requires that two synthetic enzymes move in opposite polarities on the two strands. Synthesis of the so-called lagging strand is discontinuous and requires the cyclical association of the enzyme, while synthesis of the other strand is continuous. Nevertheless, in many prokaryote replication systems it is clear that coordination of these enzymes is achieved and maintained by a dimeric polymerase that creates a looped DNA structure in the lagging strand. This loop is mediated by multiple protein-protein interactions across the growing fork (Ref. 1, and references therein). Helicases are enzymes that can catalyze the unwinding of the template strands ahead of the fork, thus allowing for new complementary strand DNA synthesis. They were initially discovered as ancillary factors required for synthesis, but recently this view of the helicase activity has been characterized as “naive” or at least incomplete (2). Compelling evidence has been presented demonstrating that the helicase is an integral member of a large protein complex that serves as a molecular motor or pump for the replication apparatus empowering the polymerase and increasing the rate of DNA polymerase synthesis (3). The Escherichia coli dnaB helicase also plays a critical function in establishing the asymmetry at the growing fork. The helicase tracks on the lagging strand template but through interactions it holds the leading strand DNA polymerase while allowing for recycling of the other DNA polymerase (4). How helicases actually convert the binding and hydrolysis of ATP into mechanical energy resulting in DNA unwinding and can concomitantly achieve relative movement along the DNA is presently under intense investigation (5, 6). While many issues remain unresolved, it seems as if the well studied replication helicases of E. coli (and its phage encoded ones) engage DNA by encircling at least one of the DNA strands that have been prepared for this loading by other replication proteins (7–10). Thus, for example, the dnaB helicase is loaded onto DNA in complex with dnaC to a duplex structure at oriC already melted by the dnaA protein (11).

In eukaryotes, despite the ubiquitous presence of many DNA helicases (12), little is known about the relationship between such enzymes and the replication complex. However, the importance of such proteins in eukaryotic DNA replication is highlighted by the fact that many DNA viruses that replicate in the nucleus encode a helicase. One type of such viral helicase can initiate unwinding from within duplex structures not prepared for activity by prior melting. These helicases encoded by the herpes simplex virus, papillomaviruses or the SV40, and polyoma viruses can serve as DNA initiators by first recognizing small repeat motifs within the origin of replication. Thus, a particularly challenging structural problem exists in determining how these proteins convert from a site-specific DNA binding mode to a helicase. For the SV40 T antigen, monomers bind to pentameric base pair repeats utilizing specific nucleotide base information. After double hexamer formation on the DNA and ATP binding DNA-protein contacts shift toward sugar-phosphate interactions (13). A complex series of steps must therefore occur to change both the oligomeric state of the protein and the nature of its contacts with DNA. Presumably, both the DNA and ATP could be allosteric effectors of this change, but in the case of T antigen ATP is sufficient for hexamer formation. Similarly for the HSV-1 origin binding protein UL9 a pair of dimers interact with each other and bend the ori region as duplex DNA-binding proteins. In the presence of ATP the complex becomes an active unwinding enzyme that can extrude catenated single-stranded loops (14).

The papillomaviruses provide a unique system for analyzing this assembly and transition process. The bovine papilloma
E1 Hexameric Helicase

MATERIALS AND METHODS

Plasmid Construction and DNA Substrates—PKSO has been described previously (22). pSS3, pSS3-L15C, and pSS3-jopal are described by Mendoza et al. (16). The BPV-1 origin containing fragment generated by BamHI and HindIII restriction enzyme digest of pKSO was inserted into the pACYC177 vector linearized by BamHI and HindIII to give rise to pCLO. The 429-base pair BPV-1 origin containing fragment of pKSO was used in the transient unwinding assay and for DNA induced E1 oligomerization was generated by digesting pKSO with the EcoRI and PstI restriction endonucleases. The 242-base pair origin containing DNA fragment used in EM linear compaction studies was generated by digesting pKSO with EcoRI and BamHI restriction endonucleases. Both of the duplex fragments were purified from agarose gel using the QIAquick gel extraction kit. The sequence of the 10-base pair oligomer used for DNA induced E1 oligomerization is 5’-AACAAACATC-3’. The E1 construct used for overexpression in E. coli, pGEX-2TK-E1, was generated by cloning the E1 open reading frame from the pET11-GST-E1 plasmid (25) into the pGEX-2TK vector (Pharmacia number 27-4857-01, Genbank accession number U13851). The integrity of the boundaries for the E1 coding sequence was verified by DNA sequencing. 

Protein Purification—The BPV-1 E1 protein was purified from S9 cells infected with a recombinant baculovirus expression vector by immunoaffinity chromatography as described by Yang et al. (22). The E1 protein purified from E. coli began with transforming XA90 cells with the pGEX-2TK-E1 expression vector and proceeded according to methods described by Sedman et al. (25) as modified by C. Sanders. In brief, extracts from XA90 transformed with pGEX-2TK-E1 were prepared, cleared of nucleic acid by a Polylemin P (10% w/v) precipitation (0.5% w/v final) centrifuged by a 25,000 × g spin for 20 min. E1 protein in the supernatants was precipitated by 65% ammonium sulfate at 4°C. The recovered protein was purified by adsorption to glutathione-Sepharose beads and eluted in buffer containing 20 mM glutathione. The GST moiety was cleaved with thrombin and the E1 was further purified by chromotography on an S-Sepharose column (Pharmacia). The E1 containing fractions were pooled, dialyzed against E1 dialysis buffer (20 mM pKPO4, pH 7.5, 150 mM potassium glutamate, 1 mM EDTA, 1 mM DTT, and 10% glycerol aliquoted, and stored at −80°C.

Unwinding Assays—The unwinding reactions using either covalently closed circular DNA or duplex DNA fragment substrates were performed as described previously (19).

Electron Microscopy and Measurement of DNA Regions—Unwinding reactions using substrates indicated in the text were incubated at 32°C for 1 h. Micrographs of linearized DNA were prepared by adding 6 units of the indicated restriction enzyme and incubating for an additional 20 min. Reaction products were purified by the addition of glutaraldehyde to 1%, visualized by filtration through a 0.5-mLBio-Gel A5-M column (Bio-Rad) and applied to glow-discharged carbon grids coated with 2 mM merspin. The grids were then rotary shadowed with tungsten. Photographs were taken at ×30,000 with a JEOL 1200 EX electron microscope at an acceleration voltage of 80 kV (61). Micrographs of E1 complexes bound to a BPV-1 origin containing DNA fragment for linear compaction studies were prepared using the same method described above. Measurement of duplex regions was performed by projecting photographic negatives onto a Numonics digitizing tablet.

Image Analysis—The E1 protein (3 mM concentration, in 25 mM KPO4, 60 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5) was incubated with 18 μM of a 60-mer oligonucleotide for 30 min at 30°C, and then applied to glow-discharged grids and stained with 2% (w/v) uranyl acetate. Electron micrographs were recorded under minimal dose conditions (with no prior exposure to the high magnification electron beam prior to recording) at ×30,000 magnification, using a JEOL 1200 EXII microscope. Negatives were scanned with a Leaf 45 microdensitometer, with a sampling interval of 4 Å/pixel. Images of rings were masked into 44 × 44 pixel arrays (corresponding to 176 × 176 Å), band-pass filtered (bandwidth 1/180 and 1/120) to zero mean density, and the contrast was normalized. After applying a reference-free alignment (44), images were ranked by the strength of either the 3- or 6-fold power, as described in Yu et al. (39). For the 6-fold ranking, we excluded those images which contained a significant 3-fold power. After sorting based upon rotational symmetry, images were then sorted based upon the strength of the integrated density within a 16-Å radius area at the rotational axis of the ring.

Radiolabeling of E1—The 7 amino acid NH2-terminal tag on the E1 purified from overexpression in E. coli contains a recognition sequence as

2 The abbreviations used are: BPV, bovine papilloma virus; GST, glutathione S-transferase; DTT, dithiothreitol; SSB, single stranded-binding protein; PAGE, polyacrylamide gel electrophoresis.

2 C. Sanders, personal communication.
well as a serine that can be specifically phosphorylated by bovine heart muscle kinase. A typical 30-μl labeling reaction contains 2.5 μg of E1 (1.3 μM final), 10 units of bovine heart muscle kinase (Sigma P-2645), and 2 μl of [γ-32P]ATP at 6000 Ci/mM in 20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM MgCl2, and 0.1 mM DTT. The reaction is incubated for 30 min and stopped with the addition of EDTA containing an acrylamide:bis ratio of 80:1. A stacking phase was not used and electrophoresis was carried out in 25 mTris, 250 mM glycerine, 0.1% SDS with a final pH of 8.8. Current was held constant at 10 mA.

**Electrophoresis of E1 Complexes**—Native gel electrophoresis of E1 was performed as indicated in the text. For analysis of cross-linked E1 complexes, the E1 sample was boiled for 5 min in Laemmli SDS loading buffer (pH 8.8) + 100 mM β-mercaptoethanol. The samples were then loaded onto polyacrylamide gradient gels containing an acrylamide:bis ratio of 80:1. A stacking phase was not used and electrophoresis was carried out in 25 mTris, 250 mM glycerine, 0.1% SDS with a final pH of 8.8. Current was held constant at 10 mA.

**Glyceral Gradient Sedimentation**—E1 purified from Sf9 cells infected with recombinant baculovirus was centrifuged in a 15–35% glycerol gradient with 0.1 KCl-HEPG (pH 7.5), 5 mM EDTA, and 0.01% Nonidet P-40 was used.

## RESULTS

**E1 Unwinds DNA Bidirectionally from the ori Site**—BPV-1 replicates bidirectionally in *vivo* and in cell-free extracts (22, 34, 35). With purified replication components wherein E1 provided the only helicase activity (36), fully replicated circles were obtained. Furthermore, acting on covalent closed circles, E1 is capable of producing a highly unwound DNA (form U) consistent with complete denaturation of the circles (19, 23). It was therefore anticipated that E1 as a replicative helicase must be capable of processive unwinding that spreads bidirectionally from the BPV-1 origin region. Electron microscopy and an *in vitro* unwinding assay were employed to map both the location and extent of unwinding of BPV-1 genomic DNA by E1. Covalently closed circular DNA was relaxed with calf thymus topoisomerase I and then incubated with purified E1, ATP, topoisomerase I, and *E. coli* SSB protein. For this analysis, we used the plasmid pSS3 that contains an intact BPV-1 genome cloned into pUC18. The samples were fixed with glutaraldehyde, purified by gel filtration, and linearized by restriction endonuclease hydrolysis at a unique site. Representative images are shown in Fig. 1, A-D. The lengths of the unwound regions of DNA and total contour lengths were measured to determine both the size and position of the unwound regions with respect to the entire length. A compilation of data is presented in Fig. 1E. As each of the molecules could be aligned in either of two directions (and one such orientation chosen for each to make the alignment) it was necessary to repeat this analysis with a different unique single cutter. This separate set increases the statistical significance of the conclusions. Such data obtained with either *Afl*I or *SacI* defining the ends (Fig. 1E) show that unwinding initiates at the ori site and that denaturation spreads bidirectionally from that position.

These results are compatible with those presented previously by Seo et al. (18), who showed that E1-dependent formation of form U was dependent upon the integrity of ori. However, our earlier results (19) on form U production showed little dependence upon origin sequences, a result that might predict scattered bubble positions. With different preparations of the E1 protein we performed *in vitro* unwinding assays with various mutant termini templates. Mutations were engineered into the E1-binding site to determine whether the E1 DNA-binding site contributed quantitatively to the level of form U DNA produced. Two mutants were derived from the plasmid pSS3; LI5C contains a 5-bp pair linker insertion between the inverted repeat, and for ΔOPAL the entire palindrome is deleted. The reaction products for each were separated by agarose gel electrophoresis, blotted to filters, and probed with pUC18. PhosphorImage analysis of these Southern blots was used to determine the amount of form U DNA generated (Fig. 2). The data do show that while all substrates are capable of directing unwinding the wild type ori site is indeed preferred. It therefore seems likely that when a template contains a bona fide ori with E1 sites organized in such a way as to allow for helicase formation, such sites will be utilized *in vitro*. We do not understand why such preferences were not detected earlier, but perhaps this specificity is sensitive to monomer E1 concentration and variations in this regard might influence the data.

The E1 Protein Forms Hexamers and Oligomerization Correlates with Helicase Activity—To determine the oligomeric states of E1 we analyzed the sedimentation profiles of the baculovirus-purified E1 incubated in the presence and absence of ATP. Fractions from a glycerol gradient were collected, and the positions of the E1 protein determined by SDS-PAGE and Western blotting using polyclonal E1 antisera. The protein profiles of the gradients (Fig. 3) indicate that E1 purified by single step affinity chromatography sediments in a heterogeneous manner. In addition, it is clear from this analysis that ATP is not sufficient for the oligomerization of E1 monomers. The molecular mass markers suggest that the slowest migrating peak corresponds to monomeric E1 (68 kDa) and the second peak a hexameric form (408 kDa). Fractions at the bottom of the tube likely correspond to aggregates. Glycerol gradient fractions containing the putative hexamer fraction and monomer fractions were analyzed by electron microscopy. The images of the putative hexamer peak showed a typical 6-membered toroidal structure (Fig. 3) bearing striking similarity to the published micrographs of hexameric helicases (8, 10, 37–39). The monomeric peak showed no such structures (data not shown).

Protein preparations from baculovirus vectors showed a mixture of forms, and to study a more homogeneous population and to investigate the relationship between the monomer and hexamer forms in more detail, we purified E1 from *E. coli* cells using the methods described by Sedman et al. (25). Indications from spectrophotometric 280/260 absorption ratios for baculovirus E1 preparations were that the yields of hexameric peaks and aggregated material correlated with trace nucleic acid contaminations. We therefore explored the notion that DNA binding might be a factor in oligomerization. The *E. coli* E1 possesses an amino-terminal sequence which can be phosphorylated to high specific activity *in vitro*; such modification has no effect upon helicase activity or other biochemical tests described below (data not shown). The purified material sedimented as a homogeneous monomeric fraction (Fig. 4B). Moreover, this protein’s sedimentation behavior was not affected by ATP binding. The *E. coli* protein was found to be active in cell-free DNA replication and its activity was stimulated by E2 (data not shown).

The monomeric radiolabeled E1 was incubated with a 429-bp duplex DNA fragment in the absence of ATP and Mg2+. Reactions were fixed employing titrations of glutaraldehyde and subjected to denaturing polyacrylamide gradient electrophoresis (Fig. 4A). A ladder of cross-linked phosphorylase or commercial precast standards (Kaleidoscope, Bio-Rad) were used as gel standards. The results show that duplex DNA can promote oligomerization and the data confirm that hexameric forms of E1 predominate. In the absence of DNA no multimerization was detected at any concentration of cross-linking agent. These data were obtained both with nonspecific duplex and single-stranded DNA. Even very small oligonucleotides can catalyze this oligomerization. In the presence of a 10-base single strand oligomer (in the absence of ATP), E1 sediments as
a hexamer in a glycerol gradient (Fig. 4B). Analysis of the protein peaks collected from these gradients (after glutaraldehyde fixation) by denaturing acrylamide gel electrophoresis confirms that the high and low molecular weight peaks represent hexameric and monomeric E1 forms (Fig. 4C).

To explore the functional significance of this oligomerization we sought to correlate E1 unwinding activity with its multimeric state. To obtain such correlations we chose the duplex

**Fig. 1.** Electron micrographs of unwound BPV-1 origin containing plasmid DNA. Unwound pSS3 DNA molecules cleaved with either AflII (A and B) or SacI (C and D) restriction enzymes. The molecules were applied to carbon grids and rotary shadowed with tungsten, as described under “Materials and Methods.” The single-stranded DNA appears thicker than duplex DNA, because the single-stranded DNA is coated with E. coli SSB and/or E1. Bar in D = 200 nm. E, measurements of unwinding of BPV-1 origin containing DNA in vitro. To determine the extent, direction, and location of unwound regions, molecules were photographed, and the length of duplex DNA was measured by projecting negatives onto a Numonics digitizing tablet. The positions of unwound regions (black boxes) were plotted below a linear map of the plasmid. The relative position of the origin in base pairs is indicated at the bottom of the histograms. The standard of deviation for total length was 9%.
unwinding assay utilizing a duplex restriction fragment that contains the BPV-1 ori sequence. In previous experiments from our laboratory (19), unwinding in this assay was dependent upon ori sequences and absolutely required an SSB. In two parallel sets of reactions we either followed the oligomeric state of E1 across a range of protein concentrations (3.75 to 480 nM) (Fig. 5A) or the state of the duplex DNA (4.2 nm) over the same protein titration (Fig. 5B). In these side-by-side experiments reaction conditions were identical with the exception that SSB was not present in the data obtained for Fig. 5A. As E1 assembles on the DNA in the presence of ATP/Mg"2" and E. coli SSB, the duplex is melted and converted to single strands. The ability of E1 to act as a duplex unwinding enzyme is very cooperative (Fig. 5C) with respect to concentration and correlates with oligomerization. Hexamers and high forms (perhaps double hexamers) correlate with such activity. At 60 nM E1 some unwinding is first detected and at this concentration hexamers and notably higher forms are first detected.

DNA Takes an Apparent Linear Path Through a Bilobed E1 Complex—From the data in Fig. 5 there is a suggestion that complexes of higher order than hexamer may be the most efficient in unwinding duplex DNA. For SV40 T antigen direct unwinding in this assay was dependent on ori sequences and absolutely required an SSB. In two parallel sets of reactions we either followed the oligomeric state of E1 across a range of protein concentrations (3.75 to 480 nM) (Fig. 5A) or the state of the duplex DNA (4.2 nm) over the same protein titration (Fig. 5B). In these side-by-side experiments reaction conditions were identical with the exception that SSB was not present in the data obtained for Fig. 5A. As E1 assembles on the DNA in the presence of ATP/Mg"2" and E. coli SSB, the duplex is melted and converted to single strands. The ability of E1 to act as a duplex unwinding enzyme is very cooperative (Fig. 5C) with respect to concentration and correlates with oligomerization. Hexamers and high forms (perhaps double hexamers) correlate with such activity. At 60 nM E1 some unwinding is first detected and at this concentration hexamers and notably higher forms are first detected.

**Image Analysis of E1 Hexameric Ring**—To obtain a clearer analysis of the ring structures, minimal dose electron microscopy and image analysis were used to study the organization of the oligomeric state of the E1 protein. Fig. 7 shows electron micrographs where both top views of the rings (Fig. 7a, formed with an oligonucleotide) and side views of the rings stacked on double-stranded DNA (Fig. 7B) can be seen. Images of 968 top views of the rings were averaged together, using a reference-free alignment procedure (44). The resulting average (not shown) suggested a hexameric structure, but subsequent analysis indicated that the population of rings was non-homogeneous. First, a sorting by rotational power (as done for DnaB protein in (39)) indicated that a subset of the rings had a significant 3-fold rotational power, consistent with the asym-
metric unit in these rings being a dimer. Trimers of dimers has previously been observed for the hexameric rings formed by the DnaB protein (39, 45) and the RecA protein (46). Fig. 8, a and b, show the 6-fold symmetric averages (containing 678 rings), while Fig. 8, c and d, show the 3-fold symmetric average (containing 100 rings). The rotational power spectra for the 6- and 3-fold symmetric averages are shown in Fig. 8, i and j, respectively. The main difference between the 6-fold symmetric rings and the 3-fold symmetric ones is that there appears to be a modulation of the projected subunit density in the 3-fold symmetric averages, such that there are alternating “strong” and “weak” subunits. In addition, the outermost ends of the subunit arms appear to move in toward the center for the three weaker subunits in the 3-fold conformation. Both forms of the ring appear to be about 125 Å in diameter.

Second, the density within the central channel appeared to
be continuously variable. The images contained in the 6- and 3-fold averages were then sorted based upon the strength of this central density. Fig. 8a shows an average of 400 6-fold symmetric rings with a strong hole near the center, while Fig. 8b shows an average of 278 6-fold symmetric rings with a “plug” of density in the center. Similarly, Fig. 8c shows an average of 56 3-fold symmetric rings with a strong hole near the center, and Fig. 8d shows an average of 44 3-fold symmetric rings with a plug in the center. There did not appear to be any correlation between the strength of the 3- or 6-fold power and the strength of the central density. Furthermore, both the relative strength of the 3-fold rotational power and the relative strength of the central density appeared to be continuously variable parameters. Thus, the groupings that are shown in Fig. 8 represent arbitrary divisions. For example, averages could have been created in Fig. 8, b and d, showing a stronger central density by using fewer images, just as averages could have been generated in Fig. 8, c and d, showing a slightly stronger 3-fold power by using fewer images.

Symmetrized versions of the averages in Fig. 8, a-d, are shown in Fig. 8, e-h, respectively. One consequence of the symmetrization, which eliminates noise, is that asymmetric features disappear. However, this can also obscure real asymmetric features. The central holes in Fig. 8, a and c, are displaced from the central axis, while the symmetrization forces these holes to lie on the central axis in Fig. 8, e and g. It is likely that this displacement of the stain-filled hole from the central axis is due to the binding of the 60-nucleotide oligomer, since this mass might be expected to be bound within the central channel to only one or two of the subunits, as shown for the T7 gp4 hexameric helicase (10). Thus, the bound oligonucleotide would be filling some of the central channel (indicated by arrows in Fig. 8, a and c), leading to an asymmetric location of the stain-filled hole.

While the averages in Fig. 8 have a slight hand, the degree of chirality is much less than that observed for DnaB (39, 45), T7 gp4 (10), or SV40 large T antigen (37). We therefore checked to see if the lack of a strong hand arose from averaging together individual projection images that were related by mirror symmetry. This would occur if the rings were randomly oriented on the grid, as opposed to predominantly one side adsorbing to the grid. We used the highly chiral average of SV40 large T (37) as a reference to align all images, and then did the alignment against the mirror image of the reference. Images were ranked based upon the coefficient of correlation against the reference, and averages were then created from those with the strongest correlation using the reference-free alignment (44). The results of this procedure suggested that there was no strong chirality present, as no averages were generated with the opposite band to that shown in Fig. 8. Thus, the rings appear to predominantly adsorb to the grid by the same surface.

Conditions were found where “side” views of the E1 rings could be obtained by binding them to double-stranded DNA molecules in the presence of ATP (Fig. 7b). Fig. 7c shows an average generated by aligning 217 side view images, each image containing three rings. The spacing between adjacent rings is about 50 Å, and unless there is a large degree of interdigitation, this would be the thickness of each ring. Since there does not appear to be a large continuous density running between adjacent rings, a large degree of interdigitation appears unlikely. The spacings between the rings were observed to be quite variable, and a number of different characteristic side views appeared to be present, as well. We therefore sorted the images into subgroups using correspondence analysis (47) and Fig. 7, d and e, show subaverages generated from 44 and 52 different rings, respectively. Since the alignment method used to generate the averages in Fig. 7, d and e, only examined the density of the central ring (of the three contained in each image) the density of the central ring is averaged properly, while the density of the surrounding rings is smeared due to their variable spacing. Nevertheless, the spacing of about 80 Å seen in Fig. 7d between the two outer rings suggests that these rings may be as close together as 40 Å. These side views help establish that the E1 ring is about 40–50 Å in thickness.

**Fig. 5.** Oligomerization of E1 correlates with helicase activity. A, titration of radiolabeled E1 monomers (3.75, 7.5, 15, 30, 60, 120, 240, and 480 nM) into reactions containing 25 mM KPO4 (pH 7.5), 75 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mg/ml bovine serum albumin, and a 429-base pair BPV-1 origin containing DNA fragment (4.2 nM) for 20 min at room temperature. Glutaraldehyde was added to a 0.04% final concentration and incubated for 20 min at room temperature. Reactions were boiled in SDS sample buffer + β-mercaptoethanol and equal amounts of E1 protein loaded onto a denaturing acrylamide gradient gel (4–10%). Autoradiography was performed on the dried gel. The positions of prestained molecular weight markers are indicated. Cross-linked radiolabeled E1 hexamer (408 kDa) purified from a glycerol gradient of prestained molecular weight markers is plotted against E1 protein concentration.

B, boiled E1. C, the amount of denatured DNA was determined by PhosphorImager analysis and is plotted against E1 protein concentration.
Although the site-specific DNA-binding domains of the papillomavirus E1 have no homology to the SV-40/polyoma large T antigens, both of which are highly homologous to each other, and the nucleotide sequence motifs that serve as binding sites are distinct for these papillomaviruses; the initiator proteins assemble as helicases in remarkably similar ways. In both situations a double-ringed structure assembles at the origin site, and this helicase activity is capable of denaturing DNA bidirectionally from the assembly point. As we have shown here the toroidal rings formed by BPV-1 E1 are hexameric as are the T antigens.

It is also significant to point out that some differences have been uncovered between these helicases, particularly in the assembly pathways. Monomers of the T antigens can form hexameric complexes solely upon incubation with ATP and Mg$^{2+}$; in contrast, as we show here E1 must bind DNA in order to assemble as a hexamer. Similar observations have recently been reported by Sedman and Stenlund (48), who have shown that single strand DNA can initiate hexamer formation. That duplex DNA can also affect this multimerization fits nicely into a pathway through which the enhancer protein E2 helps target E1 to the ori site and once E2 frees itself from the preinitiation complex (27) double hexamers may readily follow at appropriate E1 concentrations.

These apparent biochemical differences may, however, be discussed in another way that brings the papillomaviruses' mode of DNA replication even closer to the SV40/polyoma family. For DNA replication in vivo both SV40 and polyoma large T antigens have enhancer sequences as cis-dominant elements and for polyoma virus these elements are absolutely required. Interestingly, the polyomavirus large T antigen can bind cooperatively with c-Jun, a factor naturally found to bind to polyoma DNA, and this targeting stimulates helicase activity (49). Furthermore, E2 can activate polyoma virus replication in the cell if E2 sites are engineered into viral vectors (50). Although it is perhaps too premature to speculate on the evolutionary pathway through which the genes encoding these viral initiators descend, at least in part because we do not know how eukaryote chromosomal replication origins engage or assemble active helicases, it seems possible that the special relationship that E2 and E1 have with each other mimics cellular processes captured by the SV40/polyomavirus family.

Image Analysis of the Rings Reveals an Unexpected Heterogeneity—Electron microscopy and image analysis have shown that the rings formed by the E1 protein are hexameric. However, the population of such rings formed in the presence of an oligonucleotide are not homogeneous, and two parameters of...
variability were observed. First, a subset of rings existed not as symmetric hexamers, but as trimers of dimers, generating a 3-fold rotational symmetry. This has previously been observed by electron microscopy for the hexameric rings formed by the dnaB protein (39, 45) and the RecA protein (51). It is likely that this structural dimerization correlates with the biochemical non-equivalence of subunits observed for other hexameric helicases. The T7 gp4 hexamer, for example, has been shown to contain only three, not six, high affinity ATP-binding sites (52), as has the hexameric rho protein (53) and DnaB (54).

Second, a large mass of density can exist in the central channel of the ring, and appears to not depend upon whether the ring has 6- or 3-fold symmetry. What gives rise to this density? We think it very unlikely that this density could arise from the oligonucleotide used to induce ring formation. One primary reason is that this mass appears too large to be due to the approximately 20-kDa mass of the oligonucleotide. The mass also appears to be continuously variable in its strength, an observation not compatible with it arising from the bound oligonucleotide. Also, since this oligonucleotide is required for ring formation, it is hard to explain the existence of rings without this density if the density is due to the oligonucleotide. Third, this mass appears to be found on the rotational axis of the rings (Fig. 8, b and d), while we would expect the density due to the oligonucleotide not to be much smaller but asymmetrically displaced from the central axis (10). Based upon our experience imaging the T7 gp4 helicase with a bound oligonucleotide (10), the weak asymmetric density that is found within the central channel in the E1 rings shown in Fig. 8, a and c, is consistent with the density that we would expect from the oligonucleotide.

Since the preparation is at least 95% pure (as judged by SDS-PAGE), and plugged centers were observed for E1 purified from both E. coli and SF9 cells, and the density is too great to be due to the oligonucleotide, the most likely explanation is that this central density arises from a portion of the E1 protein. This density may therefore arise from a disordered or highly mobile domain of the E1 protein, that can exist in multiple conformations. The recent crystal structure of an E. coli helicase, the Rep protein (55), provides a possible clue in this regard. Two copies of Rep were observed in the crystal, and the two differed by a rotation of the 2B subdomain by 130°. Since all helicases, including papilloma E1, are highly likely to have a conserved structure (55, 56), the highly mobile subdomain in Rep provides support for the possibility that the variable central density in E1 may be due to the large movement of such a domain.

Observations of the related SV40 large T antigen helicase have also shown a similar, variable central density. It is noteworthy that a three-dimensional reconstruction of large T antigen with a hole in the center (37) only accounted for about 60% of the expected molecular volume, perhaps due to the fact that a portion of the subunit is mobile or disordered and not seen in the averaged reconstruction. A recent study also used electron microscopy to address the multimeric state of E1. Liu et al. (57) estimated from molecular volumes that E1 complexes on DNA are either hexameric or dihexameric. We did not find such size heterogeneity on DNA templates and it is possible that the hexamers observed by Liu et al. were actually intermediates or aggregates not detected in our experiments.

The Path of DNA through the Double Rings—The electron microscopic images of E1 assembled on duplex DNA (Fig. 6) are consistent with the idea that the DNA somehow passes through the central cavities of the rings. Our end to end measurements of the DNA fragments so engaged with the helicase do not indicate a shortening and as shown in Fig. 9 this data by itself would be consistent with two sorts of models for strand passage. In one model both strands might pass through the centers of the double hexamers as shown in Fig. 9A. In the presence of a single strand binding (SSB) protein and ATP unwinding might proceed either with the helicase working as a molecular motor translocating along the DNA and unwinding in opposite directions, or as a molecular pump denaturing the duplex and forcing it out through a central port. This class of models is the one that seems to fit most of the data gathered for the SV40 large T antigen. The DNase I and chemical protection data (obtained prior to SSB addition) argues in any case that both strands are protected. Moreover Dean et al. (58) have shown that preformed hexamers of large T antigen are inactive for unwinding of circular duplex DNA. We have made similar observations for the BPV-1 E1 protein (data not shown). These results would be consistent with models that required a topo-

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3 X. Yu and E. H. Egelman, unpublished data.
logical link between the helicase ring and the circular DNA, and that stable hexamers once formed could not engage the circle.

Curiously, preformed hexamers can displace single-stranded oligonucleotides annealed to circular single-stranded molecules (58). This result perhaps suggests that a hexameric ring might engage the single strand circle from an external binding site, track along the DNA and upon engaging a duplex region pass the other strand through the center of the ring upon cycles of helicase action. Faced with duplex DNA the hexamer may have no such entry and therefore a complex assembly process starting from monomers would create such possibilities for engagement. Such a model might predict a strand passage situation for duplex DNA as depicted in Fig. 9B. The electron microscopic data presented here would also be consistent with this notion. Considerable variation in lengths for melted or single-stranded DNA have been found, and the channels created between subunits of the hexamers could space a strand as close to its complement in this arrangement as in the situation wherein both strands passed through the center of the rings. (Compare the positions of the black dots in the cross-sections shown in Fig. 9, A and B.) In the model shown in Fig. 9B both single strands might be protected from DNase protection by positing that the external one is buried or wrapped in the channel. Gillette et al. (59) have concluded from their results of DNA protection experiments that E1 binding does produce one type of complex resulting in DNA distortions even in the absence of ATP. Thus protein binding and assembly of the hexamer around DNA may provide enough energy to allow for one cycle of denaturation. It is also possible that the initial complexes depicted in Fig. 9, A and B, are in some equilibrium with each other and SSB or ATP might be expected to change this distribution.

Studies with the hexameric replication helicases from E. coli and their T phages have shown that a single subunit contacts the single strand (at a given time) and that the other strand passes outside of the ring (10, 60). In models for helicase action, this internal strand might be passed from one subunit to the next in cycles of ATP hydrolysis. Thus an attraction of the model in Fig. 9B is that it would lead to a conserved mode of action for the animal viral DNA helicases and their prokaryote relatives.

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4 E. Fouts and M. R. Botchan, unpublished observations.
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REFERENCES

1. Lee, J., Chastain, P. D., Kusakabe, T., Griffith, J. D., and Richardson, C. C. (1998) Mol. Cell 1, 1001–1010
2. West, S. C. (1996) Cell 86, 197–210
3. Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) Cell 84, 643–650
4. Yuzhakov, A., Turner, J., and O'Donnell, M. (1996) Cell 86, 877–886
5. Lohman, T. M., Thorn, K., and Vale, R. D. (1998) Cell 93, 9–12
6. Marians, K. J. (1997) Structure 5, 1129–1134
7. Bujalowski, W., and Jezewska, M. J. (1995) Biochemistry 34, 8513–8519
8. Egelman, E. H., Yu, X., Wild, R., Hingorani, M. M., and Patel, S. S. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 3869–3873
9. San Martin, C., Rudermacher, M., Welpensinger, B., Engel, A., Miles, C. S., Dixon, N. E., and Carazo, J. M. (1998) Structure 6, 501–509
10. Yu, X., Hingorani, M. M., Patel, S. S., and Egelman, E. H. (1996) Nat. Struct. Biol. 3, 740–743
11. Wahle, E., Lasken, R. S., and Kornberg, A. (1989) J. Biol. Chem. 264, 2469–2475
12. Lohman, T. M., and Bjornson, K. P. (1996) Annu. Rev. Biochem. 65, 169–214
13. Sen Gupta, D. J., and Borowiec, J. A. (1994) EMBO J. 13, 982–992
14. Makhotin, A., Boehmer, P. E., Lehman, I. B., and Griffith, J. D. (1996) EMBO J. 15, 1742–1750
15. Chen, G., and Stenlund, A. (1996) J. Biol. Chem. 261, 2469–2473
16. Lohman, T. M., and Bjornson, K. P. (1996) Annu. Rev. Biochem. 65, 169–214
17. Sen Gupta, D. J., and Borowiec, J. A. (1994) EMBO J. 13, 982–992
18. Makhotin, A., Boehmer, P. E., Lehman, I. B., and Griffith, J. D. (1996) EMBO J. 15, 1742–1750
19. Chen, G., and Stenlund, A. (1996) J. Biol. Chem. 261, 2469–2473
20. Lohman, T. M., and Bjornson, K. P. (1996) Annu. Rev. Biochem. 65, 169–214
21. Sen Gupta, D. J., and Borowiec, J. A. (1994) EMBO J. 13, 982–992
22. Makhotin, A., Boehmer, P. E., Lehman, I. B., and Griffith, J. D. (1996) EMBO J. 15, 1742–1750
23. Chen, G., and Stenlund, A. (1996) J. Biol. Chem. 261, 2469–2473
24. Lohman, T. M., and Bjornson, K. P. (1996) Annu. Rev. Biochem. 65, 169–214
25. Sen Gupta, D. J., and Borowiec, J. A. (1994) EMBO J. 13, 982–992
26. Makhotin, A., Boehmer, P. E., Lehman, I. B., and Griffith, J. D. (1996) EMBO J. 15, 1742–1750
27. Chen, G., and Stenlund, A. (1996) J. Biol. Chem. 261, 2469–2473
28. Lohman, T. M., and Bjornson, K. P. (1996) Annu. Rev. Biochem. 65, 169–214
29. Sen Gupta, D. J., and Borowiec, J. A. (1994) EMBO J. 13, 982–992
30. Makhotin, A., Boehmer, P. E., Lehman, I. B., and Griffith, J. D. (1996) EMBO J. 15, 1742–1750

Fig. 9. Models illustrating how hexameric helicases might engage DNA. A, two hexameric helicases assemble as rings topologically linked to DNA with both strands passing through their central cavities. Two possible modes of unwinding are shown. To the left, the helicases translocate away from each other along the DNA, leaving single-stranded DNA in their wake. On the right is a mode of unwinding in which the helicases remain in contact and the single strands are spooled out between the ring/ring interface. This arrangement has been proposed for SV40 large T antigen based on electron microscopy of T antigen-mediated DNA unwinding reactions and is analogous to the way in which E. coli RuvB assembles at Holliday junctions (38, 64, 65). For this case a mechanism for keeping the strands apart within the ring cavity must operate, for example, two binding sites for single strands (5' to 3' and 3' to 5'). B, an adaptation of the model mentioned above addressing the possibility that the helicases assemble on DNA with only a single strand passing through the central cavity of each ring. Note that in model B a shortening of the DNA may be anticipated, but this shortening is a function of the angle between the two rings, as well as the precise contour and width of the rings.
M. R. (1990) *Science* **250**, 1694–1699

25. Sedman, T., Sedman, J., and Stenlund, A. (1997) *J. Virol.* **71**, 2867–2896

26. Lusky, M., Hurwitz, J., and See, Y.-S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8895–8899

27. Sanders, C. M., and Stenlund, A. (1998) *EMBO J.* **17**, 7044–7056

28. Lehman, C. W., and Botchan, M. R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4338–4343

29. Skiadopoulos, M. H., and McBride, A. A. (1998) *J. Virol.* **72**, 2079–2088

30. Park, P., Yang, L., Wang, T., Botchan, M. R., and Mohr, I. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8700–8704

31. Lentz, M. R., Pak, D., Mohr, I., and Botchan, M. R. (1993) *J. Virol.* **67**, 1414–1423

32. Mansky, K. C., Batiza, A., and Lambert, P. F. (1997) *J. Virol.* **71**, 7600–7608

33. Gorbalenya, A. E., and Koonin, E. V. (1993) *Curr. Opin. Struct. Biol.* **3**, 419–429

34. Schvartzman, J., Adolph, S., Martin-Parras, L., and Schildkraut, C. (1990) *Mol. Cell. Biol.* **10**, 3078–3086

35. Yang, L., and Botchan, M. (1990) *J. Virol.* **64**, 5903–5911

36. Muller, F., See, Y.-S., and Hurwitz, J. (1994) *J. Mol. Biol.* **239**, 1078–1094

37. San Martin, M. C., Gruss, C., and Carazo, J. M. (1997) *J. Mol. Biol.* **268**, 15–20

38. Stasiak, A., Tsaneva, I. R., West, S. C., Benson, C. J., Yu, X., and Egelman, E. H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7618–7622

39. Yu, X., Jezewska, M. J., Bujalski, W., and Egelman, E. H. (1996) *J. Mol. Biol.* **259**, 7–14

40. Wessell, R. J., Schweizer, J., and Stahl, H. (1992) *J. Virol.* **66**, 804–815

41. Smelkova, N. V., and Borowiec, J. A. (1997) *J. Virol.* **71**, 8766–8773

42. Mastrangelo, I., Hough, P., Wall, J., Dodson, M., Dean, F., and Hurwitz, J. (1989) *Nature* **338**, 658–662

43. Dean, F. B., Borowiec, J. A., Eki, T., and Hurwitz, J. (1992) *J. Mol. Biol.* **267**, 14129–14137

44. Yu, X., West, S. C., and Egelman, E. H. (1997) *J. Mol. Biol.* **266**, 217–222

45. Frank, J., Radermacher, M., Wagenknecht, T., and Versehoor, A. (1988) *Methods Enzymol.* **164**, 3–35

46. Yu, X., and Egelman, E. H. (1997) *Nat. Struct. Biol.* **4**, 101–104

47. Hingorani, M. M., and Patel, S. S. (1996) *Biochemistry* **35**, 2218–2228

48. Stitt, B. L. (1988) *J. Biol. Chem.* **263**, 1130–1137

49. Biswas, E. E., Biswas, S. B., and Bishop, J. E. (1986) *Biochemistry* **25**, 7368–7374

50. Korolev, S., Heile, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997) *Cell* **90**, 635–647

51. Liu, J.-S., Kuo, S.-R., Makhatov, A. M., Cyl, D. M., Griffith, J. D., Broder, T. R., and Chow, L. T. (1998) *J. Biol. Chem.* **273**, 30704–30712

52. Hingorani, M. M., and Patel, S. S. (1996) *Biochemistry* **35**, 2218–2228

53. Stitt, B. L. (1988) *J. Biol. Chem.* **263**, 11130–11137

54. Biswas, E. E., Biswas, S. B., and Bishop, J. E. (1986) *Biochemistry* **25**, 7368–7374

55. Korolev, S., Heile, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997) *Cell* **90**, 635–647

56. Subramanya, H. S., Bird, L. E., Brannigan, J. A., and Wigley, D. B. (1996) *Nature* **384**, 379–383

57. Liu, J.-S., Kuo, S.-R., Makhatov, A. M., Cyl, D. M., Griffith, J. D., Broder, T. R., and Chow, L. T. (1998) *J. Biol. Chem.* **273**, 30704–30712

58. Dean, F. B., Borowiec, J. A., Eki, T., and Hurwitz, J. (1992) *J. Biol. Chem.* **267**, 14129–14137

59. Gillette, T. G., Lusky, M., and Borowiec, J. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8864–8865

60. Hacker, K. J., and Johnson, K. H. (1997) *Biochemistry* **36**, 14080–14087

61. Dodson, M., Echols, H., Wickner, S., Alfano, C., Messe-Wilmot, K., Gomes, B., Lebowitz, J., Roberts, J. D., and McMacken, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 7638–7642

62. Heberlein, U., and Tjian, R. (1988) *Nature* **331**, 410–415

63. Crowther, R. A., and Amos, L. A. (1971) *Nature* **331**, 635–647

64. Parsons, C. A., Stasiak, A., Bennett, R. J., and West, S. C. (1995) *Nature* **374**, 375–378

65. Stahl, H., Drüge, P., and Knippers, R. (1986) *EMBO J.* **5**, 1939–1944
