Laser Cross-linking of Proteins to Nucleic Acids

II. INTERACTIONS OF THE BACTERIOPHAGE T4 DNA REPLICATION POLYMERASE ACCESSORY PROTEINS COMPLEX WITH DNA*

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In this paper we examine the interactions of the polymerase accessory proteins subassembly of the bacteriophage T4 DNA replication complex, using single-pulse ultraviolet laser excitation to induce protein-nucleic acid cross-links. The laser-induced cross-linking permits effective “freezing” of the instantaneous equilibrium state of the complex and thus provides a mechanism to dissect the individual protein-nucleic acid interactions involved in complex assembly. We find that the binding of the gene 44, 62, and 45 proteins is dependent not only on the presence of each of the other proteins, but also on the presence of adenine nucleotide cofactors. We find that the nonhydrolyzable analogs of ATP often behave more like ADP than ATP in these experiments.

Gene 45 protein is able to induce an increase in cross-linking of the gp44/62 complex to nucleic acids, and this increased cross-linking correlates with changes in the apparent K_m of the gp44/62 complex for polynucleotides and with changes in V_max during ATP hydrolysis. Our results suggest that the enhanced DNA binding is predominately through the gene 62 protein and not the ATPase catalytic subunit (gene 44 protein). Thus the gene 62 protein seems to play an integral role in gp45-mediated enhancement of the ATP hydrolytic activity of gp44. These results are summarized and integrated in the form of a model for the multiple interactions of

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The bacteriophage T4 DNA replication complex contains seven proteins, all of which have been identified and purified (Morris et al., 1979; Nossal and Peterlin, 1979). Five of these proteins, the gene 43, 44, 44/62, and 45 proteins, from the T4 DNA replication holoenzyme whose structure and function have recently been reviewed by Young et al. (1992). The replication rate, fidelity, and processivity of the T4 DNA polymerase (gene 43 protein) are markedly improved by the presence of the gene 44/62 and 45 proteins and ATP, which interact to form a polymerase accessory proteins complex displaying DNA-dependent ATPase activity. Under optimal conditions, the holoenzyme incorporates several hundred nucleotide residues per second into new DNA with both high processivity and fidelity (Albers et al., 1975).

Although the T4 DNA polymerase accessory proteins complex contains only three different gene products, the stoichiometry of the complex is more complicated. The solution properties of the accessory proteins have been investigated by Jarvis et al. (1989a) using hydrodynamic and equilibrium techniques. These workers showed that the gp44/62 complex is asymmetric and contains four molecules of the 35,584-Da gp44 and one molecule of the 21,342-Da gp62. Similar studies of the 24,710-Da gp45 reveal that it exists as an asymmetric trimer (Jarvis et al., 1989a). The accessory proteins complex is loosely associated in dilute buffer solution, although studies under “macromolecular crowding” conditions have suggested a 1:1 complex of the 4:1 gp44/62 proteins subassembly with a gp45 trimer (Jarvis et al., 1990).

The complex stoichiometry of the accessory proteins subassembly, coupled with the requirement that this assembly translocate relative to the DNA every few milliseconds, limits the application of many standard biophysical techniques. This difficulty led us to develop a laser cross-linking methodology for examination of protein-nucleic acid interactions on a microsecond time scale (Hockensmith et al., 1986, 1991, 1993), and we have used this technique to investigate the access to the accessory proteins with DNA and one another in the presence of mononucleotide cofactors and substrates.
interactions of the accessory proteins complex with various DNA templates. UV laser cross-linking is very useful in probing DNA studies because: (i) unlike methods such as footprinting, filter binding and gel shift assays, this technique can reveal which of the proteins of a multiprotein complex system interacts with the nucleic acid; (ii) it can detect transient contacts between DNA and proteins, such as during cycles of nucleoside triphosphate hydrolysis, which are not easily detected by conventional methods; (iii) it can reflect the "degree of intimacy" of the contact between protein components and DNA; and (iv) it can reveal the relative distribution of those proteins that interact with the DNA at various points within the single-nucleotide addition cycle. Thus the laser UV cross-linking approach is especially well suited for application to the T4 DNA replication system, since this system features protein-DNA interactions that cycle through the successive stages of single-nucleotide residue addition on a millisecond time scale.

Using this methodology we here provide quantitative structural data for these protein-DNA interactions, which have mostly been treated qualitatively in the past. In particular, we have been able to draw close parallels with the enzymological results of Jarvis et al. (1993), who carefully measured the steady-state ATPase activity parameters of the accessory proteins using defined DNA constructs to represent single-stranded DNA, double-stranded DNA, and primer-template DNA. It is evident from these studies that the accessory proteins interact most strongly with a DNA template construct that resembles a primer-template junction; such structures almost certainly exist at the center of an operating DNA replication complex.

MATERIALS AND METHODS

The materials and methods used in this study have been largely described in the companion paper (Hockensmith et al., 1993). Only differences from and additions to these descriptions are presented here. Unless otherwise indicated, the data shown herein represent the averages of experiments performed in triplicate.

Stock solutions of adenine nucleotides and their derivatives were prepared by dissolving the nucleotide in 10 mM Tris-OAc (pH 7.5) and 1 mM EDTA and subsequently titrating to a final pH of 7.5. Unless otherwise specified, all reactions were carried out in buffer B of Jarvis et al. (1993b), which contains 6 mM Mg(OAc)$_2$, 60 mM KOAc, 25 mM Tris-OAc (pH 7.5). Nucleotide cofactors were added to a final concentration of 0.5 mM. Homopolymer oligonucleotides were present at a 3 μM nucleotide residue concentration for 20-mers and at a 2.4 μM concentration for 16-mers, corresponding to an oligomer concentration of 150 nM in each case. "Tailed" homopolymers, carrying a GCG sequence at the 5'-end of one strand (5'-dCGG(A)$_3$) and a complementary CGC sequence at the 3'-end of the other strand (3'-dCGC(T)$_3$), were used in some experiments to provide a single-stranded region of defined length for oligo(dA)-oligo(dT) primer-template constructs. These molecules were generally used at an oligomer concentration of 200 nM. Reactions run in the absence of magnesium ion included 5 mM EDTA.

Unless otherwise specified, gp44/62 and gp45 were used in these experiments at a 1:3 weight ratio. Thus, based on the stoichiometries of Jarvis et al. (1993b), the gp44/62 complex was present at a molar concentration of 80 nM, and the gp45 trimer was present at a molar concentration of 540 nM. Samples were prepared at 4 °C and maintained on ice prior to irradiation. Samples were irradiated with a single 5-ns pulse of ultraviolet light (266 nm, 4.15 × 10$^4$ photons) (Hockensmith et al., 1991, 1993) after 1 min of incubation at 37 °C.

As indicated above, for each of the proteins studied here we find that most of the protein remains uncross-linked. Thus the uncross-linked protein can be silver stained and provides a reference to establish the position of the unliganded protein in the gel. In the companion paper we show that the apparent molecular weights of the cross-linked protein-DNA oligomer products, which migrate more slowly than the free protein, move approximately as the sum of the molecular weights of the nucleic acid and the protein species

RESULTS

The Accessory Proteins and Model DNA Systems—We have approached the study of the physical interactions that occur in the bacteriophage T4 DNA replication complex by examining subassemblies of the complex. We have cross-linked combinations of the three accesssory complex proteins to five different model DNA effector molecules, in the presence and in the absence of mononucleotide substrates/cofactors. The DNA molecules used as models included (dT)$_{16}$, (dT)$_{19}$, (dA)$_{16}$·(dA)$_{20}$, (dA)$_{16}$·(dT)$_{20}$, and (dA)$_{20}$·(dA)$_{20}$. In many cases, reactions were also run with oligo(dT) and oligo(dA) that had been tailied with alternating GC base pairs to fix the positions of these complementary oligomers in double-stranded structures, thus avoiding slippage (see "Materials and Methods"). Oligothymidylicate molecules were 5' radio-labeled with $^{32}$P, whereas the oligodeoxadenosine molecules were unlabeled. The formation of laser-induced protein-DNA cross-links only through thymidine residues, coupled with the selective labeling, provides a specific methodology for examination of protein contacts on single-stranded DNA ((dT)$_{16}$ or (dT)$_{19}$), the primer strand of a primer-template junction ((dA)$_{16}$·(dT)$_{20}$), the template strand of a primer-template junction ((dA)$_{16}$·(dA)$_{20}$), and double-stranded DNA ((dG)$^3$·(dA)$_{20}$).

Since we have previously established that the yield for laser cross-linking of protein-nucleic acid complexes is proportional to the amount of protein bound to the nucleic acid (Hockensmith et al., 1991) and since binding constants to DNA have not been measured for the individual accessory proteins, we present our data as relative cross-linking to expedite comparison of the protein-nucleic acid interactions for each protein under a variety of conditions. To normalize the data, we have designated the amount of radiolabeled with gp62 cross-linked to (dA)$_{16}$·(dT)$_{20}$ in the presence of MgATP as unity. Calibration of the single-pulse laser-induced cross-linking with gene 32 protein-oligonucleotide complexes results in yields of ~5% of the input oligonucleotide cross-linked or ~12% of the bound protein cross-linked using 266 nm irradiation (Hockensmith et al., 1986). Similar yields can be obtained when gp44/62 and/or gp45 is irradiated in the presence of DNA, although a protein titration is generally required to maximize the yield (~1% of the input DNA can be cross-linked at optimal gp45 concentrations).

The T4 accessory proteins were mixed with each of the five model nucleic acids in the presence of each of the following substrates/cofactors: ATP, MgATP, MgADP, MgATP-S, MgAMPPNP, or magnesium ion with no added nucleotide. We have used a concentration of 500 μM of each adenosine mononucleotide to facilitate comparison of the different systems. A brief discussion of the effects of "shielding" as a consequence of the absorbance of the incident laser light by mononucleotides is presented in the companion paper (Fockensmith et al., 1993).

A typical set of gel patterns and autoradiogram from cross-linking experiments using the gene 44/62 and 45 proteins are shown in Fig. 1. As indicated above, for each of the proteins studied here we find that most of the protein remains uncross-linked. Thus the uncross-linked protein can be silver stained and provides a reference to establish the position of the unliganded protein in the gel. In the companion paper we show that the apparent molecular weights of the cross-linked protein-DNA oligomer products, which migrate more slowly than the free protein, move approximately as the sum of the molecular weights of the nucleic acid and the protein species.
Laser Cross-linking of T4 Polymerase Accessory Proteins to DNA

Fig. 1. Silver-stained 12.5% sodium dodecyl sulfate-polyacrylamide gel (left) and autoradiogram of the gel (right). Reaction conditions were 6 mM Mg(OAc)₂, 60 mM KOAc, 5 mM β-mercaptoethanol, and 25 mM Tris-OAc (pH 7.5). ATP was added to a final concentration of 0.5 mM ATP at zero time. Homopolymer oligonucleotides were present at 3 μM (nucleotide residues) for 20-mers and 2.4 μM (nucleotide residues) for 16-mers (i.e., at 150 nM oligomer concentrations). The gp44/62 complex and gp45 were used at a 1:3 weight ratio.

Thus using the stoichiometries of Jarvis et al. (1989a), the gp44/62 complex (4:1) was present at a concentration of 80 nM, and the gp45 trimer was present at a concentration of 540 nM. Samples were mixed at 4 °C and held on ice prior to irradiation. Immediately prior to irradiation, the sample temperature was shifted to 37 °C for 1 min, and the sample was irradiated with a single 5-ns pulse of 266 nm UV light carrying 4.15 × 10⁸ photons/pulse (Hockensmith et al., 1991).

Fig. 2. Bacteriophage T4 gp44 cross-linking to various DNA effectors. Reaction and irradiation conditions were as specified in Fig. 1. Adenine nucleotides were added to a final concentration of 0.5 mM. The concentration of gene 44/62 proteins was 80 nM (4:1 complex); the nucleic acid was present at 150 nM (oligomers) for both single- and double-stranded DNA.

The interaction of gp45 with the five model DNA effectors has been examined. Unlike gp44 which cross-links to both

(Hockensmith et al., 1993). The autoradiogram shown in Fig. 1 demonstrates that the length of the nucleic acid affects the migration of each of the cross-linked complexes in the gel and also that this simple gel system affords sufficient resolution of the different cross-linked species (Hockensmith et al., 1993). We present data in a quantitative format since we have established previously that the pulsed laser cross-linking effectively "traps" the existing equilibrium and thus permits relative cross-linking to be directly related to equilibrium binding (Hockensmith et al., 1986, 1991, 1993; Mesner and Hockensmith, 1992).

Gp44/62 Interactions with Model DNA Effectors—A comparison of Figs. 2 and 3 reveals that the cross-linking responses of gp44 and gp62 are qualitatively indistinguishable, regardless of the DNA effector or added nucleotide. These qualitative similarities suggest that the two proteins are tightly coupled with respect to nucleic acid binding and hence yield qualitatively similar responses to changes in the adenine nucleotides present. Nevertheless, quantitative differences do exist between the cross-linking of the two proteins and generally reveal about 5-fold more cross-linking of DNA to gp44 than to gp62. This ~5:1 ratio of gp44 to gp62 cross-linking is consistent with the results of Jarvis et al. (1989a), who established the stoichiometry of the gp44/62 complex at 4:1, if we assume that the cross-linking efficiency of DNA to gp44 and gp62 is equivalent.
Laser Cross-linking of T4 Polymerase Accessory Proteins to DNA

FIG. 3. Bacteriophage T4 gp62 cross-linking to various DNA effectors. Reaction and irradiation conditions were as specified in Fig. 1. Adenine nucleotides were added to a final concentration of 0.5 mM. The gene 44/62 protein concentration was 80 nM (4:1 complex); the nucleic acid was present at 150 nM (oligomers) for both the single- and double-stranded DNAs.

(dT)16 and (dT)20 in a roughly equal fashion, gp44 and gp62 cross-link very poorly or undetectably to (dT)16 (Figs. 2 and 3). Except in the presence of AMPPNP, gp44 and gp62 demonstrate a higher degree of cross-linking when the oligonucleotide cofactor length is increased from 16 to 20 nucleotide residues. Since the amount of cross-linking obtained is not proportional to the increase in length of the oligonucleotide, we interpret these data to mean that the DNA binding site of this protein complex can make stable thermodynamic contact with a total single-stranded DNA length exceeding 16 nucleotide residues.

We have reported previously that ATP and ADP have differential effects on the cross-linking of gp45, gp44, and gp62 (Hockensmith et al., 1987). Other investigators have indicated that ATP and ATPγS also have differential effects on binding of these three proteins to DNA (Capson et al., 1991; Munn and Alberts, 1991a). Figs. 2 and 3 confirm these reports and demonstrate that for this system the binding of the nonhydrolyzable nucleotide analogs, ATPγS and AMPPNP, yield cross-linking results that are more comparable to those obtained in the presence of MgADP than in the presence of MgATP. For example, consider the interaction of gp44/62 with (dA)16, (dT)20. This complex in the presence of MgADP and MgATPγS results in 31- and 40-fold higher levels of cross-linking for gp44 and 28- and 37-fold for gp62 than does MgATP for either protein. For all three cases in which the oligo(dT) molecule is 20 nucleotides in length ((dT)20, (dA)16, (dT)20, and (dT)20, (dA)16), MgATPγS results in slightly more cross-linking than is observed with MgADP and much more than is observed with MgATP. In fact, the level of cross-linking of gp44/62 to these DNA molecules is so low in the presence of MgATP that it could be attributed to the presence of low concentrations of ADP contaminating the ATP stock solution or to an initial hydrolysis of the MgATP in the solution, yielding a small component of MgADP (see below). Competition experiments with various nucleotide substrates/cofactors, which will help to establish the role of each nucleotide in this system, are currently in progress. In the absence of these data, we cannot rule out the possibility of end binding to our short double-stranded oligomers.

Although Figs. 2 and 3 demonstrate that gp44 and gp62 can be cross-linked to (dT)16 and (dT)20, (dA)20 quite efficiently under the proper conditions, we were nevertheless concerned that: (i) (dT)16 may be too small to permit proper binding; (ii) the single-stranded region of the (dT)16, (dA)20 and (dA)16, (dT)20 may be too small to permit proper binding; and (iii) the strands of the double-stranded DNAs might "slide" relative to one another thereby yielding either larger or smaller regions of single-stranded DNA (Mesner and Hockensmith, 1992). For these reasons we synthesized the following additional deoxynucleotides: 3'-(dGCG(T))16, 5'-(dGCG(A))20, and 5'-dGCG(A)16. These longer oligonucleotides were then used as DNA effectors in the presence of MgATP, permitting us to examine cross-linking for single-stranded, primer-template and double-stranded DNAs. The qualitative responses were the same (data not shown) as those obtained with the homopolymer DNA. Quantitatively the cross-linking yields were similar, with none of the longer DNAs yielding dramatic changes in cross-linking (Kubasek, 1988) that might indicate that the untailored oligo(dT) oligo(dA) templates were smaller than the binding site size (Hockensmith et al., 1993). These results strongly support those of Munn and Alberts (1991a), which showed that gp44, gp62, and gp45 protect no more than 10 residues of duplex DNA from neocarzinostatin and bleomycin-Fe(II), whereas DNase I footprinting yielded a slightly larger footprint, which is generally recognized as an over-estimate of the protein binding site size (Suck and Oefner, 1986; Suck et al., 1988). Our data are also consistent with the gp44 and gp62 cross-linking data of Capson et al. (1991), in which specific points of contact were found at the 4th and 14th nucleotides from the 3'-end of the primer strand.

Model Template Interactions with the Three-protein Accessory Protein Complex—We mixed the gp44/62 complex with gp45 and added DNA to characterize the perturbation of the DNA effector binding of the individual proteins by the presence of the other accessory proteins in the complex. The cross-linking of each of the proteins was affected by the presence of the others.

Fig. 4 reveals a striking effect, which was demonstrated for each of the Mg2+-nucleotide complexes and shows that the cross-linking of gp45 is dramatically reduced by the addition of gp44/62 in every reaction, regardless of the type of DNA present. For the most part, the absolute levels of gp45 cross-linking were quite low and approach the limits of detection. In particular, the cross-linking of gp45 to double-stranded DNA becomes completely undetectable in the presence of MgADP, MgAMPPNP, and MgATPγS. The drop in cross-linking efficiency is also precipitous (more than 10-fold) for the MgATP reaction with gp44/62 added. This decrease in

4 We have shown previously (Hockensmith et al., 1986, 1993) that the amount of cross-linking observed for a given DNA-protein complex is directly proportional to the binding affinity; i.e. the binding equilibrium is "frozen" in these systems, although the cross-linking efficiency varies markedly from one type of complex to another. DNA-binding proteins can be cross-linked to nucleic acid oligomers that are not long enough to fill the complete nucleic binding site on the protein, but detection of such cross-linked species requires a high level of sensitivity since such oligomers will not be bound as tightly as oligomers that are long enough to fill the site and involve the maximal number of thermodynamically significant contacts. We have reported previously cross-linking of gp44 to (dT)16 (Hockensmith et al., 1986), but the amount of cross-linking observed was rather low since only a small fraction of the input gp44 was bound to this short oligomer.

5 The decrease in gp45 cross-linking upon addition of gp44/62 is in direct contrast to the findings of Capson et al. (1991). We attribute the discrepancy to the differences in the concentration of gp46 and adenine nucleotides used for the two sets of experiments. The cross-linking (binding) of gp45 to DNA does not follow typical binding isotherms and appears to be sensitive to changes in protein concentration and other parameters (see Footnote 3).
cross-linking to various DNA effectors in the absence (hatched bars) and presence (solid bars) of the bacteriophage gp44/62 complex. Reaction conditions were as described in Fig. 1. Adenine nucleotide was added to the reaction to a final concentration of 0.5 mM. Homopolymer oligonucleotides were present at 3 μM nucleotide residues for 20-mers and 2.4 μM nucleotide residues for 16-mers (i.e., 150 nM oligomers). The gp44/62 complex and gp45 were used at a 1:3 weight ratio. Thus, based on the stoichiometries of Jarvis et al. (1989a), the gp44/62 complex (4:1) was present at a concentration of 80 nM, and the gp45 trimer was present at 540 nM. Samples were mixed at 4 °C and held on ice prior to irradiation. Immediately prior to irradiation, the sample temperature was shifted to 37 °C for 1 min and the sample was irradiated with a single 5-ns pulse of 266 nm UV light at an intensity of 4.15 × 1016 photons/pulse (Hockensmith et al., 1991).

**Table I**

Gene 44/62 protein cross-linking

<table>
<thead>
<tr>
<th>DNA effector</th>
<th>gp44 cross-linking MgATP</th>
<th>gp44 cross-linking MgATP-S</th>
<th>gp62 cross-linking MgATP</th>
<th>gp62 cross-linking MgATP-S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No gp45</td>
<td>gp45 added</td>
<td>No gp45</td>
<td>gp45 added</td>
</tr>
<tr>
<td>(dT)10</td>
<td>3</td>
<td>2</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>(dT)10</td>
<td>20</td>
<td>5</td>
<td>142</td>
<td>2</td>
</tr>
<tr>
<td>(dT)10-(dA)10</td>
<td>25</td>
<td>1</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>(dA)10-(dT)10</td>
<td>6</td>
<td>0</td>
<td>240</td>
<td>1</td>
</tr>
<tr>
<td>(dT)10-(dA)10</td>
<td>5</td>
<td>2</td>
<td>156</td>
<td>0</td>
</tr>
</tbody>
</table>

Cross-linking makes the cross-linking level very similar to that observed in the presence of either magnesium ion or ATP alone. Based on the data shown in Fig. 4, we conclude that the addition of the gp44/62 complex causes gp45 to have some preference for single-stranded DNA ((dT)10, (dT)10, (dA)10, (dA)10) in the presence of MgADP. The cross-linking of gp45 to the primer strand of a primer-template junction ((dT)10-(dA)10) in the presence of either MgATP, MgAMPPNP or MgATP-S could be specific, or may merely reflect binding to the single-stranded dA sequence that is directly adjacent to the primer.

Cross-linking of gp44 and gp62 to DNA in the presence of gp45 contrasts strikingly with the cross-linking observed in the absence of this protein. The addition of gp45 induced dramatic changes in the cross-linking response of the accessory proteins, with the nature of the change being dependent on the cofactor present. In the presence of MgATP, gp45 causes gp44 cross-linking to decrease precipitously, whereas the cross-linking of gp62 increases dramatically (Table I and Fig. 5). Although it is possible to argue that the added gp45 is merely competing with gp44 for DNA binding sites, this cannot be true for gp62 cross-linking. Thus we conclude that these opposing trends for gp44 and gp62 negate any arguments that we are merely observing competition for binding sites, thereby giving us further confidence in our interpretation of these data. For the three-protein system, cross-linking is essentially undetectable for gp44 and gp62 in the presence of MgATP (no Mg"), MgADP, MgAMPPNP, or Mg" (no nucleotide).

Although the qualitative cross-linking patterns of gp44 and gp62 in the three-protein system are the same for MgATP and MgATP-S, quantitatively they are very different. The amount of gp62 that cross-links to single-stranded DNA is 10–15 times greater with MgATP than with MgATP-S in the presence of gp45. The addition of gp45 appears to induce a switch in the conformation of the gp44/62 accessory proteins.

# Of 117 experimental samples, 105 showed no detectable cross-linking of either gp44 or gp62. The remaining 12 samples showed barely detectable cross-linking, all with relative cross-linking values less than 5.6.
which results in a striking change in the cross-linking responses of the accessory proteins complex. Although gp45 induces this switch in the conformation of the accessory proteins complex, gp45 itself does not undergo such dramatic changes in cross-linking to the DNA while in these complexes. As before, these results have been confirmed using the longer tailed oligonucleotides described above (Kubasek, 1988).

As mentioned above, in the absence of gp45 the cross-linking of gp44 and gp62 to DNA appears to be tightly coupled, with the relative order of cross-linking to the different templates being qualitatively similar for each of the two proteins. Table I and Fig. 5 show that for the MgATP reaction, the presence of gp45 alters the binding of gp44 and gp62 such that the apparent cross-linking efficiencies change from approximately 5-fold more gp44 than gp62 cross-linking to approximately 20-fold more gp62 than gp44 cross-linking. However, the qualitative response with and without gp45 in the presence of MgATP is still similar, with binding for both gp44 and gp62 following the order single-stranded > double-stranded > primer-template. Binding in the presence of MgATPγS is also qualitatively similar for gp44 and gp62 in the presence and absence of gp45 with the relative order of binding differing from that of MgATP. With this nucleotide substrate/cofactor, the rank order of cross-linking efficiency for the DNA strand cross-linked is single-stranded > primer-template > double-stranded. Nevertheless, the 5:1 (44:62) cross-linking ratio revealed in Figs. 2 and 3 is no longer applicable for the three-protein system; in the presence of MgATP this ratio is closer to 1:20.

As shown in Table I for the three-protein system, gp62 in the presence of MgATP cross-links to a single-stranded DNA molecule with a much higher yield than to a primer-template junction, although gp62 does appear to demonstrate some preference for the template strand of primer-template DNA or one strand of a double-stranded DNA. Alternatively, in the presence of MgATPγS, gp62 cross-links much more efficiently to the template strand of a primer-template junction than to single-stranded DNA and does not cross-link to the primer strand of a primer-template junction or to double-stranded DNA. Again, these results were confirmed by use of the tailed oligonucleotides (Kubasek, 1988).

Under each cofactor condition (MgATP, MgATPγS, MgADP, MgAMPPNP, and no adenine nucleotide), gp45 induces a switch in the cross-linking pattern of the accessory proteins. In the presence of ATP or ATPγS, the nature of the change in the cross-linking pattern was, as expected, qualitatively the same; in each case we see a switch from gp44 to gp62 as the principal cross-linked species. In the presence of ADP, or in the absence of cofactor, the observed change was, as expected, from gp44 as the principal cross-linked species to no cross-linking of gp44 or gp62.

Fig. 5 shows cross-linking and ATP hydrolysis resulting from a gp45 titration curve of the gp44/62 complex in the presence of primer-template DNA and ATP. In this experiment the concentration of gp45 was varied up to 1 μM trimer. The autoradiogram (Fig. 5) depicts the ratio of gp44/62 to gp45 at which gp45 induces a "conformational switch" in the accessory protein complex, as indicated by a change in the cross-linking pattern of these proteins. Specifically, the switch occurs between 108 and 270 nM gp45 trimers, at which point gp44 cross-linking diminishes dramatically. We note that the decrease in gp44 cross-linking correlates inversely with gp45 concentration and gp62 cross-linking, which increases dramatically. This inverse relationship between gp44 and gp62 cross-linking in the presence of gp45 is in direct contrast to the proportional relationship that we noted in the absence of gp45 (Figs. 2 and 3). The abrupt transition in the binding of gp62 in the presence of gp45 and ATP appears to provide a direct correlation with the abrupt change in the apparent $K_m$ for the polynucleotide effector during ATP hydrolysis, whereas the continued gradual increase in gp62 binding as the gp45 ratio is increased thereafter appears to correlate with...
the gradual increase in the $V_{\text{max}}$ for ATP hydrolysis (Jarvis et al., 1989b). Therefore, we conclude that the values of $K_a$ and $V_{\text{max}}$ for the DNA effector-dependent ATP hydrolysis are directly linked to the binding of gp42 to the nucleic acid. Although the DNA dependence of adenosine triphosphatase activity has been reported for homogeneous gp44 (Rush et al., 1989), the regulation of DNA-dependent ATP hydrolysis appears to be directly coupled to gp62 binding to DNA. This observation is consistent with the observation that gp45 is a poor stimulator of gp44 alone (Rush et al., 1989).

During our studies we have noted that gp45 cross-linking may increase and gp62 cross-linking decrease under these conditions if the length of incubation or temperature is significantly altered. We have observed that gp45 cross-linking appears to be enhanced by the presence of ADP, and we propose that ATP hydrolysis yields an increasing ADP concentration which then promotes a conformational change in gp45, resulting in increased DNA binding and cross-linking. The presence of an unknown amount of ADP in our cross-linking experiments after a 1-min incubation, along with the complex effects possible if both ATP and ADP were present, led us to use ATPγS in a gp45 titration similar to that shown in Fig. 5. In this experiment the concentration of gp44/62 was held constant at 150 nM while the concentration of gp45 was varied from 0 up to 1 μM trimer. The gp45 again induced the conformational switch in the gp44/62 accessory protein complex, as indicated by a change in the cross-linking pattern of these proteins. The change occurred at a stoichiometric ratio of approximately one gp44/62 complex to one gp45 trimer (i.e. the switch occurred between 50 and 100 nM) on the primer-template DNA and also on single-stranded DNA (Kubasek, 1988). The transition in cross-linking is qualitatively complete at a 1:1 ratio of gp44/62 to gp45 complex, but the amount of gp62 induced to cross-link continues to increase as the concentration of gp45 increases, up to a ratio of one gp44/62 complex/seven gp45 timers. Thus the results in the presence of ATPγS also support the conclusion that the switch correlates with the abrupt change in $K_a$ and changes in $V_{\text{max}}$ reported by Jarvis et al. (1989b).

DISCUSSION

The relative advantages and disadvantages of the laser cross-linking methodology have been presented elsewhere (Hockensmith et al., 1993; Kubasek et al., 1993), and we have attempted here to focus on application of the technology to the specific problem of protein complexes which interact with nucleic acids. Many of our results parallel those of Capson et al. (1991), who used a photoactive nucleotide for their cross-linking experiments. Both methods are based on photoinduction of a covalent bond between protein and nucleic acid; the two methods yield similar results. The primary differences in the two methods is that the method of Capson et al. (1991) results in the introduction of a cross-link through a single specific nucleotide of the nucleic acid, whereas the laser cross-linking provides data based on a single rapid pulse. We presume that future kinetic studies of complex enzymatic systems will lead to the use of the rapid laser pulse to induce cross-links through specific photoactive nucleotides similar to those of Capson et al. (1991).

Our studies include only the proteins that comprise the bacteriophage T4 DNA-dependent ATPase, whereas Capson et al. (1991) also included the single-stranded DNA-binding protein (gp32) and DNA polymerase (gp43). We have limited our studies to these proteins in an attempt to correlate these studies of protein-nucleic acid interactions with our previous physical and kinetic studies of the DNA-dependent ATPase (Jarvis et al., 1989a, 1989b).

In a previous report we took a classical steady-state enzyme kinetic approach to the properties of the bacteriophage T4 DNA-dependent adenosine triphosphatase of the gp44/62 complex (Jarvis et al., 1989b). That analysis allowed us to characterize the consequences for the ATPase kinetics of the accessory proteins complex when adding DNA and/or gp45 to the complex. Although that study defined the kinetic consequences of effector binding, the primary goal of the current study has been to characterize the specific protein-nucleic acid interactions that accompany these functional changes.

The major conclusions that we have come to in this study are that: (i) gp45 is able to induce an increase in binding of the gp44/62 complex to nucleic acids; (ii) the increased binding of the gp44/62 complex results from a conformational change that provides more intimate contacts between the nucleic acid and gp62; (iii) the increased binding of gp62 to nucleic acids in the presence of gp45 directly correlates with changes in the apparent $K_a$ for polynucleotides and in $V_{\text{max}}$ during ATP hydrolysis; and (iv) nonhydrolyzable analogs may not be ideal cofactors for use in differentiating the effects of ATP binding and hydrolysis by laser cross-linking.

The Interaction of the Accessory Proteins with Nucleic Acids—With this information we have accordingly attempted further expansion of previously presented models (Capson et al., 1991; Jarvis et al., 1991; Munn and Alberts, 1991a, 1991b) of the interaction of the T4 polymerase accessory proteins complex with primer-template DNA. Fig. 6 summarizes, in pictorial style, a possible "model" interpretation of the information derived from our cross-linking studies. In the process of developing models that incorporate this information it is important to remember that an increase in the efficiency of UV laser cross-linking of proteins to DNA can potentially result from a change in several aspects of the interaction between the proteins and the DNA. These might include more intimate contacts at the protein-DNA interface, resulting in more reactive groups at the protein-DNA interface or tighter binding of the protein to the DNA. Such interactions might be the result of simple conformational changes or might reflect more complex multisubunit interactions.

The left side of Fig. 6 presents a qualitative picture of the gp45 interactions with a primer-template DNA. We have used a primer-template as a model since it has been established that this DNA structure is the most efficient DNA effector structure for ATP hydrolysis (Jarvis et al., 1989b). The preferential cross-linking of gp45 to the template strand of a primer-template [(dA) 10, (dT) 10 versus (dT) 10, (dA) 10] has led us to place gp45 at the primer-template junction, bound predominately to the template strand. Since gp45 may functionally resemble the β-subunit of the Escherichia coli DNA polymerase III holoenzyme, we have specifically employed a ring-shaped structure that encircles the DNA as shown for the β-subunit (Kong et al., 1992; Reddy et al., 1993). However, we note that cross-linking is nonsymmetric, preferentially occurring with the template strand, although some cross-linking does occur to single-stranded DNA and to the primer strand. Consequently, in Fig. 6, gp45 is placed in contact with both the single-stranded and double-stranded regions of the primer-template junction. The asymmetry of cross-linking could reflect the asymmetry of this structure that we have noted previously in solution studies of the gp45 trimer alone (Jarvis et al., 1989a).

As indicated previously, the binding of gp45 to the single-stranded DNA makes it difficult to interpret any cross-linking

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7 J. W. Hockensmith, unpublished results.
Fig. 6. Qualitative model for the DNA-protein interactions of the bacteriophage T4 gp44, gp62, and gp45 that form the bacteriophage T4 DNA-dependent ATPase. The model is descriptive of the gp44/62-45 system in only three states: (i) with Mg\(^{2+}\) alone; (ii) with MgATP; (iii) with MgADP. The primer strand of the DNA duplex is shaded. The protein stoichiometries represented throughout are those established by Jarvis et al. (1989a), although in some cases these stoichiometries may be protein concentration-dependent.

The Capson et al. (1991) studies examined only cross-linking to the primer strand of a primer-template junction and always included the single-stranded DNA-binding protein (gene 32 protein), making their complexes slightly more complicated than those we have considered here. Unlike previous studies, we have specifically sought to draw parallels between ATP hydrolysis and DNA binding.

Starting with the gp44/62 complex we have specifically noted that cross-linking of the two proteins to nucleic acids in the presence of mononucleotide cofactors appears to be coupled; that is, any mononucleotide cofactor that alters the binding of gp44 also alters the binding of gp62 in a coordinated fashion. As might be expected from the dependence of gp44 on a DNA effector for ATP hydrolysis, gp44 cross-links about five times better to each of the DNA effectors than does gp62. The right side of Fig. 6 shows the binding of these two proteins to a primer-template junction. In the absence of any nucleotide cofactor, gp44 does not appear to discriminate between single- and double-stranded DNAs. However, gp62 cross-links best to single-stranded DNA, albeit at low levels. Thus, Fig. 6 shows both proteins bound to the DNA. The addition of MgATP to the gp44/62 binding mixture results in very little change in the DNA strand preference for the two proteins. The addition of MgADP, however, results in quantitatively large changes in the cross-linking of both gp44 and gp62, with binding predominantly to the primer strand of the primer-template junction.

The combination of the three proteins, gp45 and gp44/62, results in dramatic changes in the binding of the individual proteins to the nucleic acids. These changes are schematically represented in Fig. 6. First, we note that gp45 shows some quantitative differences in its binding when compared with
binding in the absence of the gp44/62 complex but that the qualitative pattern of binding remains similar to that seen in the absence of the gp44/62 complex. Consequently, in Fig. 6 we locate gp45 near the primer-template junction throughout.

In the three-protein accessory proteins system, the absence of the mononucleotide cofactor appears to result in very poor binding of gp62 to any nucleic acid, whereas gp45 and gp44 both show significant binding. Again, we emphasize that this is a qualitative and not a quantitative model. Consequently, we depict gp62 as not interacting productively with either the gp45 trimer or the DNA to emphasize that this three-protein mixture is not effective at promoting DNA-protein interactions as a complex. However, upon the addition of MgATP to the three-protein system, we find qualitatively that gp45 is predominantly cross-linked and that this cross-linking is primarily to the template strand. Qualitatively, the change in accessory protein cross-linking pattern induced by the addition of gp45 is complete at a gp44/62 to gp45 ratio of one gp44/62 complex to one gp45 trimer, regardless of the nature of that change (i.e. no cross-linking of either gp44 or gp62 in the presence of ADP or no cofactor compared with cross-linking of gp62 instead of gp44 in the presence of ATP and ATPγS). Therefore, we suggest, in accord with direct measurements under macromolecular crowding conditions (Jarvis et al., 1990), that a 1:1 ratio of gp44/62 (complex) to gp45 (trimer) may represent the biologically relevant ratio of protein subunits in the accessory proteins complex. However, we note that although the transition in cross-linking responses is qualitatively complete at this ratio of proteins, the amount of gp62 that is induced to cross-link beyond this transition point continues to increase with increasing gp45 concentration, up to and beyond a ratio of one gp44/62 complex to seven gp45 trimers. The continuing increase occurs in the presence of either ATP or ATPγS, and, as indicated above, appears to track the abrupt change in the K_m of the ATPase activity of the gp44/62 complex induced by gp45 and the continued gradual increase in V_max, which were observed by Jarvis et al. (1989b).

We have invoked a conformational change in gp62 and not gp45 because the former undergoes enormous quantitative alterations in its cross-linking as gp45 is added to the reaction. The changes in gp45 binding are comparatively subtle. Nevertheless, the alterations in binding nucleic acids by gp45 when various mononucleotide cofactors are added may be indicative of changes in conformation. Thus, if one were to imagine gp45 sitting at a primer-template junction and gp44/62 translocating along the DNA, as gp62 comes in contact with the gp45 trimer a conformational change could take place such that gp45 would interact with gp62 subunit and form an asymmetric platform that could completely surround the DNA and provide a "sliding clamp" for assembly of the replication complex (Kong et al., 1992; Munn and Alberts, 1991a). Again, this model fits well with the data of Rush et al. (1988), which demonstrate that the ATPase activity of isolated gp44 is only slightly stimulated by gp45, even at saturating conditions. Thus one role of gp62 may be to facilitate a productive interaction with gp45. This model is also consistent with the work of Jarvis et al. (1989b), suggesting that the role of gp45 is primarily to confer DNA primer-template specificity onto the accessory proteins complex.

Finally, Fig. 6 attempts to detail the effect of ADP on the binding of the entire three-protein accessory complex to DNA. Simply stated, no detectable binding of gp44 or gp62 is seen in the presence of ADP. We conclude that the hydrolysis of the ATP, followed by the release of phosphate, results in an obligatory dissociation of the gp44/62 complex from the DNA (Jarvis et al., 1991). We specifically focus on phosphate release since gp44/62 (in the absence of gp45) is able to bind both ADP and DNA. Gp45 is left behind on the DNA and may then serve to identify the primer-template junction for the next assembly event. Since the product of the ATP hydrolysis reaction is ADP, once the gp44/62 complex has dissociated any ADP that is released may play a critical role in maintenance of gp45 at the primer-template junction. Assembly of ATPase-mediated holoenzyme complexes and possible clocking mechanisms have been discussed by Jarvis et al. (1991) and by Young et al. (1992) and are consistent with the results reported here. Furthermore, accessory protein subassemblies that have been visualized by cryoelectron microscopy (Gogol et al., 1992) behave very similarly to the model we have proposed. After extensive ATP hydrolysis or disruption of ATP hydrolysis by the addition of EDTA or ATPγS, the subassemblies then dissociate from the DNA in a fashion similar to that which we have reported for the gp44/62 complex in the presence of ADP.

Adenine Nucleotide Derivatives—Studies of E. coli DNA polymerase III holoenzyme have demonstrated that ATPγS can support the formation of complexes between the holoenzyme and primed DNA but that the extent of initiation is only one-half of that seen in the presence of ATP. This observation has led to the idea of a dimeric polymerase assembly that can replicate both leading and lagging strand DNA simultaneously (Johnson and McHenry, 1984). We have not incorporated the data for the cross-linking of the accessory proteins to DNA in the presence of nonhydrolyzable ATP analogs (ATPγS and AMPPNP) into our model (Fig. 6) at this time. ATPγS has been used as a competitive inhibitor of the gp44/62 ATPase activity, which in turn results in inhibition of DNA synthetic activity and leads to the conclusion that ATP hydrolysis is required for DNA synthesis (Bedinger and Alberts, 1983; Piperno and Alberts, 1978). Although this may well be the case, our data for these non-hydrolyzable analogs support the view that they may also mimic ADP binding, and thus the effects on DNA replication may be more complex than merely inhibiting the hydrolysis of ATP. It may be that ATPγS does not promote "normal" DNA binding and normal protein conformations and as a consequence may not be an acceptable model for the ATP binding step that occurs prior to hydrolysis.

Footprinting studies of the bacteriophage T4 accessory proteins complex in the presence of ATP have not detected a protein complex that is stable or long-lasting enough to protect the DNA. However, such stable complexes have been visualized in the presence of ATPγS (Munn and Alberts, 1991a, 1991b). Photochemical cross-linking studies (Capon et al., 1991) have confirmed that the three accessory proteins bind to the DNA in the presence of ATP and have definitively shown that replacement of ATP with ATPγS changes the positions and intensities of the cross-linking of each of the three accessory proteins. Our data further confirm these observations and also imply that under certain conditions MgATPγS may behave analogously to MgADP rather than MgATP. Thus the argument that ATP binding is sufficient for establishment of the three-protein complex and that binding of a nonhydrolyzable analog distorts this complex is consistent with the available data. Finally, Munn and Alberts (1991a) have also shown that an ADP challenge of a reaction containing ATPγS results in no change in their footprinting experiments. This result may support the idea that ATPγS and ADP behave similarly in this system.

Conclusions—The studies reported here help to lay some of the structural groundwork for the identification of specific
structural relationships between protein-DNA binding and ATP hydrolysis in the T4 DNA polymerase accessory proteins complex. Significantly, the changes in ATP hydrolysis effected by the gp44/62 complex in the presence of gp45 appear to be directly correlated with the binding of gp62 to DNA. Gp45 seems to provide a “regulatory switch” that ensures that the ATPase complex will assemble at the primer-template junction. Furthermore, the binding of gp45 may be regulated by ADP binding, and since ADP is the product of the ATP hydrolysis reaction these observations suggest a novel regulatory mechanism for the assembly and control of T4 DNA replication complexes.

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