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Rapid assembly of the bacteriophage T4 core replication complex on a linear primer/template construct

(accessory proteins/processivity/biotin/streptavidin)

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ABSTRACT **DNA synthesis on a primed DNA substrate by bacteriophage T4 requires the assembly of a core replication complex consisting of the T4 DNA polymerase, a single-stranded binding protein (32 protein), and the accessory proteins 44/62 and 45. In this paper, we demonstrate the successful assembly of this core complex on a short linear primer/template system at levels of accessory proteins equivalent to the concentration of primer 3′ ends. The key to this assembly is the presence of streptavidin molecules bound at each end of the DNA substrate via biotin moieties incorporated into the template strand. Streptavidin serves to block the ends of the primer/template, thus preventing translocation of the accessory proteins away from the site of assembly and their subsequent dissociation from the ends of the primer/template. Complex assembly on this substrate requires ATP and the presence of both the 44/62 and 45 proteins. The time required for assembly of a full enzyme equivalent of complex in our system is ≈2 s.**

Replication of the T4 genome is catalyzed by a phage-encoded multienzyme complex. Lagging-strand DNA synthesis on a primed DNA substrate can be reconstituted *in vitro* by a core subassembly (holoenzyme) consisting of the T4 DNA polymerase (product of gene *43*), the 44/62 and 45 accessory proteins, and the single-stranded binding protein (32 protein) (reviewed in refs. 1–3). The T4 DNA polymerase is capable of both 5′ → 3′ DNA synthesis and 3′ → 5′ exonuclease activity. While these activities are intrinsic to the polymerase itself, the presence of the accessory proteins greatly enhances the processivity of the polymerization reaction. The 44/62 protein complex possesses a DNA-dependent ATPase activity that is stimulated by the presence of the 45 protein (4). One or both of these accessory proteins are believed to act as a protein clamp that serves to stabilize the polymerase on the DNA, thus enabling processive DNA synthesis. This sliding clamp in conjunction with the 32 protein has been shown to facilitate synthesis through secondary structure in the DNA template (5) and thereby attain overall polymerization rates comparable to those observed *in vivo* (6, 7).

The functional roles played by the T4 replication core subunits parallel those observed in the *Escherichia coli* pol III and the eukaryotic pol δ systems. Common to each complex is a multisubunit, DNA-dependent ATPase (T4 44/62 protein, *E. coli* γ, eukaryotic RF-C) that is stimulated by a polymerase processivity factor [T4 45 protein, *E. coli* β, eukaryotic PCNA (proliferating cell nuclear antigen)] (8–11). The T4 polymerase with the 44/62 and 45 accessory proteins can functionally substitute for their eukaryotic counterparts in an *in vitro* simian virus 40 replication system (12). In addition, the T4 gene *44* protein (ATPase) shares common structural epitopes with the ATPase subunit of the calf thymus RF-C complex (13). For these reasons, the T4 replication system serves as a good general model in which to conduct mechanistic studies of complex assembly.

Numerous physical studies have been conducted to map the dimensions of the T4 replication complex bound to DNA. Crosslinking (14) and footprinting (15) experiments have shown that 44/62 protein and 45 protein are both found at the primer/template junction in the presence of ATP. However, if the 45 protein is present in excess along with gene *43* polymerase (45/43 protein, >100:1; 45/DNA, 5–10:1), both can bind to DNA in the absence of the 44/62 protein (ref. 16; N. G. Nossal, personal communication reported in ref. 16) suggesting an active replication complex may consist primarily of the 43, 45, and 32 protein with 44/62 protein acting to catalyze its formation. Cryoelectron microscopy revealed distinct structures are bound to DNA in the presence of the 44/62 and 45 proteins, but the size of the structure was equivalent to either the 44/62 or the 45 protein (17).

In general, the processivity assays and the physical studies described above utilized large primed, single-stranded circular DNA templates (M13 or φX174) and used accessory proteins at levels that were not conducive to finding the number or stoichiometry of active replicative complexes. Consequently, there is a need to develop short, defined primer/template substrates that facilitate stoichiometric assembly of the complex (minimize nonspecific binding to DNA distant from the primer/template junction) in order to conduct detailed kinetic studies of the formation and dynamic properties of the replicative complex analogous to those done with the polymerase alone (18). Curiously, the hash-mark structures seen by electron microscopy (17) were not visualized on linear DNA. Several properties of the accessory proteins may contribute to this phenomenon. The crystal structure of the *E. coli* β subunit indicates that this protein exists as a dimer that completely encircles the DNA (19). This topology provides a means by which the β subunit can physically clamp itself to the DNA while still allowing for its translocation along the growing duplex. Indeed, the β subunit was found to slide freely along duplex DNA and dissociate from the ends of linearized plasmids (20). Analogously, the 44/62 and 45 proteins were capable of translocation along duplex DNA in their role as transcriptional activators (21). Taken together, these observations suggest that replication complexes might not successfully assemble on linear DNA substrates owing to the movement and loss of one or more of the accessory proteins from the primer/template junction before the complete complex can be assembled.

As a solution to this problem, we describe the construction and properties of a linear primer/template system that contains a biotin moiety at each end of the template strand. The high affinity between biotin and streptavidin (22) was exploited to set up protein "bumpers" to prevent the loss of the

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Abbreviation: BioP/T, biotin-labeled primer/template.

accessory proteins off the primer/template ends. By confining the accessory proteins to the primer/template junction we can successfully demonstrate the assembly of the T4 core replication complex on a linear DNA substrate at levels of polymerase and accessory proteins equivalent to the primer/template junction, thus permitting measurement of its time of formation.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP was purchased from New England Nuclear. All unlabeled dNTPs were obtained from Pharmacia (ultrapure). Oligonucleotides, including those containing biotin derivatives, were synthesized by Operon Technologies (Alameda, CA) and purified as described by Capson *et al.* (18). The biotin phosphoramidite derivatives used by Operon Technologies were the BioTEG derivatives synthesized by Glen Research. The T4 exonuclease-deficient polymerase D219A (Asp-219 to Ala mutation) (23) was a generous gift of Nancy G. Nossal (National Institutes of Health) and Michelle West Frey (Pennsylvania State University). The T4 accessory proteins 44/62 and 45 were a gift from M. W. Frey and were purified from overproducing strains obtained from William Konigsberg (Yale University). The concentrations of the 44/62 and 45 proteins are reported as units of 4:1 complex and trimer, respectively, in agreement with the stoichiometry determined by Jarvis *et al.* (24).

Primer/Template Construction. The template strands of the biotin-labeled and control primer/templates (see Fig. 1) were constructed from two shorter oligonucleotides (23-mer plus 27-mer). Purified 23-mer was 5'-phosphorylated by T4 polynucleotide kinase (United States Biochemical) according to the manufacturer's protocol. The two template oligonucleotides were then ligated by T4 DNA ligase (Boehringer-Mannheim) with the hybridized primer strand (34-mer) serving to bridge the two substrate oligonucleotides. Completion of the ligation reaction was assessed by 5' labeling an aliquot of the mixture with [γ-^{32}P]ATP and analyzing it on a 16% acrylamide/8 M urea/90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3, sequencing gel to look for formation of 50-mer. The duplex was then purified as described by Capson *et al.* (18) and quantitated as described by Kuchta *et al.* (25).

Complex Formation/DNA Synthesis Assays. All reactions were carried out at 20°C in a buffer consisting of 25 mM Tris·OAc (pH 7.5), 150 mM KOAc, 10 mM $Mg(OAc)_2$, and 10 mM 2-mercaptoethanol. All concentrations given are final reaction concentrations. Reactions were initiated by mixing 50 nM primer/template, 100 nM streptavidin [when the biotin-labeled primer/template (BioP/T) was used], 10 μM dCTP, and 1 mM ATP in assay buffer with 10 nM T4 D219A polymerase, 55 nM 44/62 protein, and 55 nM 45 protein in assay buffer. This mixture was allowed to incubate for 15 s and then the remaining dNTPs (10 μM each) and single-stranded salmon sperm DNA trap (1 mg/ml) were added. At the indicated time, aliquots were removed and quenched in 0.25 M EDTA. The polymerization products were analyzed on 16% sequencing gels as described by Mizrahi *et al.* (26). Gel images were obtained with a Molecular Dynamics PhosphorImager.

Determination of Complex Assembly Time. Assembly of the T4 DNA replication complex was monitored by incubating 500 nM primer/template, 1 μM streptavidin (when BioP/T was used), 10 μM dCTP, and 1 mM ATP in assay buffer with 100 nM T4 D219A polymerase, 550 nM 44/62 protein, and 550 nM 45 protein in assay buffer for variable times (0.01–15 s) in the rapid quench instrument described by Johnson (27). DNA synthesis by the assembled complex was then initiated by introduction of the remaining dNTPs (10 μM each) and single-stranded salmon sperm DNA trap (1 mg/ml) via the third syringe. The reactions were quenched after 10 s by addition of 1 M HCl. Samples were immediately extracted with phenol/$CHCl_3$ (1:1), neutralized with 3 M NaOH in 1 M Trizma base, and analyzed as indicated above. Kinetic simulations were done with the program KINSIM as described (18).

RESULTS AND DISCUSSION

Assembly of the T4 Replication Complex on BioP/T. The DNA sequence used in our primer/template system was derived from a sequence of the ϕX174 genome used in previous crosslinking studies (14) with a slight modification in the template strand to allow incorporation of each of the four dNTPs before becoming degenerate. In addition, a biotin moiety was strategically placed at the 3' end and in the 5' penultimate position of the template strand. Since the binding of streptavidin to biotin is essentially irreversible ($K_d = 10^{-15}$ M) (22), each end of the template strand is occupied by bound protein. Because streptavidin is tetrameric, there is the potential for intramolecular circularization of the primer/template. However, if this population exists, it is indistinguishable in our assays from the linear population. Both primer/templates (with and without biotin) are shown in Fig. 1.

The hallmark of an intact replication complex is its processivity. However, the degree of processivity of the complex on a short primer/template may be difficult to differentiate from that of the polymerase. To augment differences in polymerase versus complex activity, a salt concentration of 150 mM was chosen, since at this physiological level the polymerase becomes more distributive (28). In addition, the D219A exonuclease-deficient mutant of the T4 DNA polymerase was used in these studies to eliminate any potential complications imposed by the presence of the exonuclease activity. The polymerase activity of the mutant is identical to that of the wild-type enzyme (23). Moreover, the D219A polymerase can interact with the accessory proteins to form a replication complex in a manner thus far indistinguishable from that of the wild-type 43 protein (B.F.K., unpublished data).

To test for complex formation on both primer/templates, polymerase and accessory proteins were incubated with either 34/50-mer or BioP/T plus streptavidin and dCTP (the first nucleotide to be incorporated) for 15 s to allow enough time for the assembly of any protein complexes on the DNA. After incubation, the full complement of nucleotides was added simultaneously with single-stranded DNA at a level sufficient to trap all unbound polymerase and accessory protein molecules (data not shown). Only those complexes that endured the 15-s incubation or those that assembled just before addition of the trap would support DNA synthesis.

Fig. 2 shows that there was little or no extension (<0.5 nM) past the 35-mer position by the polymerase (*Left*) or the polymerase and accessory proteins (*Middle*) on the 34/50-

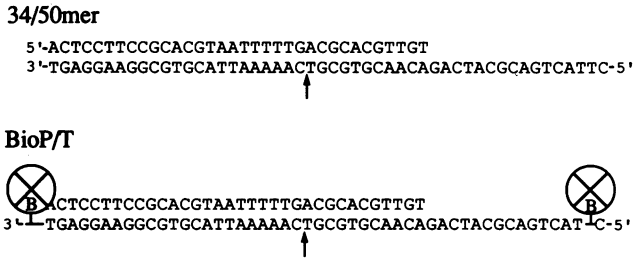


FIG. 1. Sequences of DNA substrates used in the complex assembly experiments. Arrows denote sites at which the template strands were ligated. (*Upper*) Control sequence (no biotin). (*Lower*) BioP/T. B, locations of biotin derivatives in the sequence. Quartered circles represent bound streptavidin tetramers.

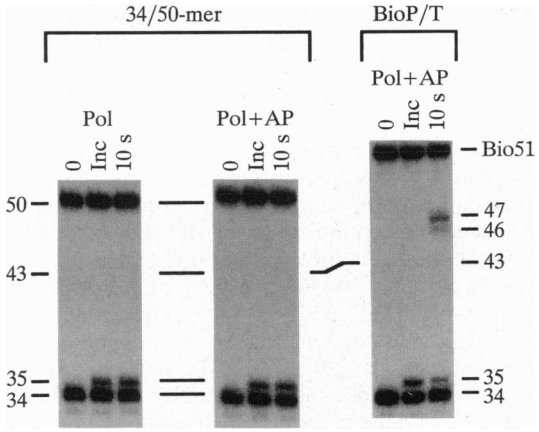


FIG. 2. Demonstration of complex assembly on BioP/T. Polymerase (10 nM) with or without accessory proteins (AP) (55 nM 44/62 protein, 55 nM 45 protein) was incubated with the indicated 5′ ^{32}P-labeled primer/template (50 nM), ATP (1 mM), and dCTP (10 μM) for 15 s (lanes Inc) and then chased with the remaining dNTPs (10 μM each) and single-stranded DNA trap (1 mg/ml) for 10 s. Lanes 0, unextended primer/template only.

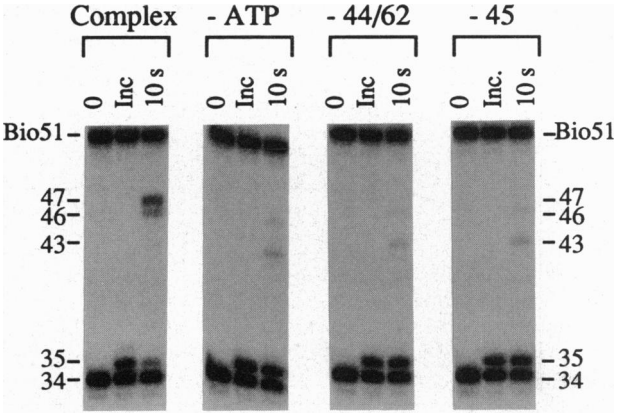


FIG. 3. Subtractive experiment to demonstrate the requirement for all complex components. (*Left*) Polymerase (10 nM), accessory proteins (55 nM 44/62 protein, 55 nM 45 protein), and BioP/T (50 nM) plus streptavidin (100 nM) were incubated and chased as described in Fig. 2. (*Middle* and *Right*) Extent of elongation when the indicated component was eliminated from the reaction is shown.

mer. However, it is clear that a significant quantity (7.9 nM) of 35-mer was generated during the initial incubation and, as will be shown later, may be attributed to multiple binding events by the polymerase during that period. The amount of 35-mer produced in these reactions was less than the quantity predicted from the off rate of the polymerase. This discrepancy most likely reflects a significant amount of nonproductive binding of the polymerase to other regions of the primer/template. A similar quantity of 35-mer was produced in the initial 15-s incubation when polymerase and accessory proteins were mixed with BioP/T. In contrast, however, the 35-mer (4.8 nM) was extended to a mixture of 46- and 47-mer upon addition of the full complement of nucleotides plus single-stranded DNA trap, thereby demonstrating successful complex formation capable of processive DNA synthesis with the biotin/streptavidin system. Using the polymerase alone or eliminating streptavidin gave results similar to either the polymerase or the complex on the 34/50-mer (data not shown). The addition of streptavidin to the 34/50-mer reaction mixture had no effect on complex formation, demonstrating that it was not simply an increase in protein concentration or a contaminant in the streptavidin that was responsible for the extension seen in the BioP/T reactions (data not shown).

Another indication of replicative complex formation is the position to which the primer is extended on each template. The small amount of primer that was extended past the 35-mer on the 34/50-mer was only elongated to a 43-mer, which apparently is a pause site. In contrast, the primer of BioP/T was extended past this pause site by the complex to create a distribution of 46- and 47-mer. Although maximum elongation should result in a 48-mer, subsequent incorporation is probably impeded by the complex abutting the bound streptavidin.

ATP and 44/62 and 45 Proteins Are Required for Complex Formation. To verify that the synthesis observed in Fig. 2 was due to an intact complex, each of the components required for complex assembly was systematically eliminated from the assay. Fig. 3 (*Left*) shows the 46- and 47-mer products formed with BioP/T in the presence of polymerase, 44/62 and 45 proteins, ATP, and streptavidin. The remainder of the data shows that excluding either ATP, 44/62 protein, or 45 protein from the reaction mixture nearly abolishes all extension past the 43-mer position (<0.5 nM). There is a trace of 46-mer present that may be the product of partial complexes in these reactions. Therefore, at the accessory protein concentrations used here, the 45 protein cannot bind and confer processivity to the polymerase without 44/62 protein and ATP. The addition of 32 protein to the complete complex reaction mixture had no effect on the assembly of the complex or the length of the products when used at a concentration providing 50% coverage of the single-stranded DNA (data not shown). Therefore, it appears that while 32 protein is essential for strand displacement synthesis or to replicate long, circular plasmids, it is not required for DNA synthesis on this short primer/template.

Time of Complex Assembly. In the previous experiments, a time frame of 15 s was allowed for binding and assembly of the replication proteins onto the primer/template. However, it remains unclear how much time is actually needed for the complex to assemble correctly. To investigate this question, the rapid quench experiment outlined in Fig. 4*A* was designed. Polymerase and 44/62 and 45 proteins were mixed in the instrument with BioP/T, streptavidin, ATP, and dCTP for 0.01–15 s to span the range of the original incubation time. At this point, the full complement of dNTPs was introduced along with a single-stranded DNA trap. DNA synthesis by the assembled complexes was allowed to proceed for 10 s and then the reaction was quenched with 1 M HCl.

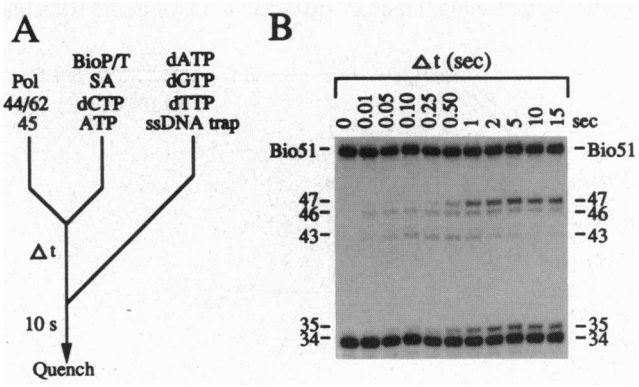


FIG. 4. Assembly time of the replication complex. (*A*) Schematic of the rapid quench experiment. (*B*) Polymerase (100 nM) and accessory proteins (550 nM 44/62 protein, 550 nM 45 protein) were incubated with BioP/T (500 nM), streptavidin (SA) (1 μM), ATP (1 mM), and dCTP (10 μM) for the indicated times and then chased with the remaining dNTPs (10 μM each) and single-stranded (ss) DNA trap (1 mg/ml) for 10 s. Autoradiograph of the extension products is shown.

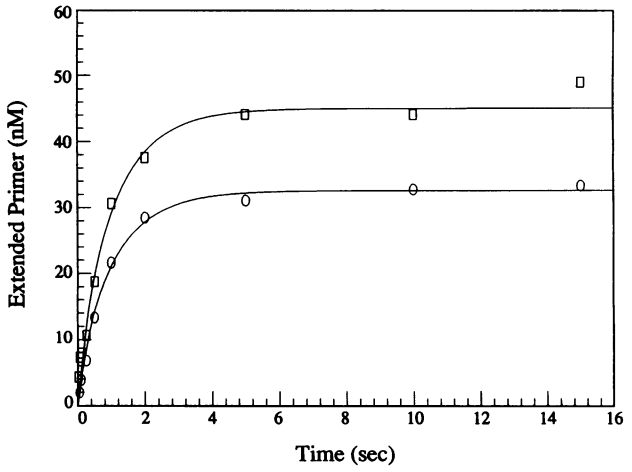
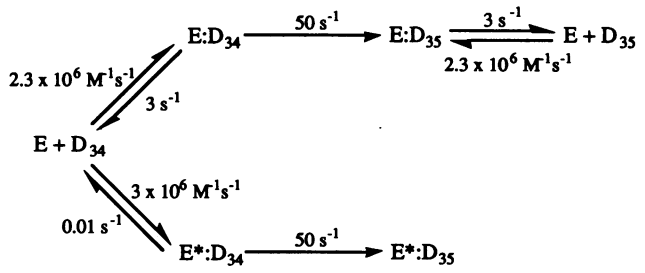


FIG. 5. Plot of complex formation as a function of incubation time. Data from Fig. 4 were quantitated and the amounts of 46- plus 47-mer (□) and 35-mer (○) products were plotted. Solid curves were generated by computer simulation using the KINSIM program and the kinetic sequence shown in Scheme I.

From the results shown in Fig. 4*B*, it is evident that there is primer elongation occurring after only 10 ms, the first time point taken. Throughout the time course there is a notable shift in products from 43- and 46-mer to primarily 46- and 47-mer in the later time points. From an identical control reaction in which only polymerase was incubated with BioP/T plus streptavidin (data not shown) and from Figs. 2 and 3, we were able to assign the 43-mer band as an elongation product of the polymerase alone. Fig. 4*B* shows that there is an increase in the amount of 43-mer produced up to 0.25 s, after which 47-mer is produced at the expense of 43-mer. This reflects the shift from polymerase binding to productive complex formation. Since the same trend was not observed with the 46-mer, we have assigned this band, together with the 47-mer band, as products of the complex. Also of note is the significant amount of 35-mer generated at time points > 0.5 s. However, the amount of 35-mer produced in the complex reaction (30 nM) is 6- to 7-fold less than the amount generated in the absence of accessory proteins (data not shown).

The amounts of 46- plus 47-mer and 35-mer in Fig. 4*B* were quantitated and plotted as a function of assembly time (Fig. 5). The data were then fit by computer simulation to the simple partitioning mechanism shown in Scheme I. This



Scheme I

mechanism sets up a competition between the polymerase alone (or an incomplete complex) and a processive (intact) complex (denoted E*) for elongation of the primer. Whereas some of the DNA is bound initially by the polymerase alone, which then incorporates dCTP to form 35-mer, at sufficiently longer incubation times the enzyme dissociates and eventually becomes incorporated into a complex on another DNA substrate. Once assembled, the complex ($E^*:D_{35}$) processively synthesizes DNA upon introduction of the full complement of nucleotides without E*:D dissociation. In the absence of accessory proteins, the amount of 35-mer formed in excess of active polymerase sites† during the initial incubation in Fig. 2 and in the polymerase control mentioned above illustrates the distributive behavior (i.e., faster k_{off}) of the polymerase alone. From Fig. 5 we conclude that one enzyme equivalent of complex is assembled within 2 s and, in the absence of continuing 35-mer formation, must remain intact for at least 15 s on the longest time point.

Since the levels of 44/62 and 45 proteins approximately match that of the 3′ ends of the primer, the replicative complex may have a unitary stoichiometry in agreement with previous observations (29, 30). Furthermore, in this case it is not necessary to add a macromolecular "crowding" agent such as polyethylene glycol (16, 30), although the action of such an agent affords another interpretation of the role of streptavidin in our system.

Complex formation on the biotin primer/template required ATP, the 44/62 protein, and the 45 protein consistent with the formation of a physiologically relevant complex. It is not known, however, as noted earlier, whether the 44/62 protein remains a part of the complex or dissociates once the 45 protein is loaded. Munn and Alberts (15, 31) were able to obtain a footprint for the polymerase and 45 proteins but could not detect the presence of the 44/62 protein. Crosslinking experiments show that 44/62 is no longer efficiently crosslinked to the DNA once ATP is hydrolyzed and the polymerase binds (14). This may be attributed to a conformational change in the complex or to dissociation of 44/62 protein. Others have noted that the 45 protein can bind without the 44/62 protein when present in high enough concentrations (ref. 16; N. G. Nossal, personal communication reported in ref. 16). While collectively these data suggest that the 44/62 protein is dispensable, others have measured periodic ATP hydrolysis during elongation (4, 28), implying that 44/62 remains or rebinds when secondary structure or pause sites are encountered. Although the 32 protein was shown to be necessary for strand displacement synthesis even on short primer/templates (16), our experiments suggest that 32 protein does not participate in conferring processivity on the polymerase, but rather the 44/62 and 45 proteins are alone responsible for forming the sliding clamp.

The kinetics of replicative complex assembly (Scheme I) feature a partitioning between binding to DNA by polymerase alone or in combination with accessory proteins. A precedence for this competition between accessory proteins and polymerase for the primer/template junction is found in the footprinting data of Munn and Alberts (31), who obtained a print for the 44/62 plus 45 complex and for the polymerase with 32 protein but not for the entire complex. The slower than diffusion-controlled rates (2–3 × 10^6 $M^{-1}\cdot s^{-1}$) for binding required by the simulation may reflect nonproductive combinatorial events in the complex assembly (possible ordering) or an artificially slow binding imposed by the presence of streptavidin. Other linear systems terminated with biotin will be examined to differentiate these effects. However, whereas the two pathways compete kinetically, formation of the complex ultimately predominates because of the difference between off rates (0.01 s^{-1} for the complex; 3 s^{-1} for the polymerase). The simulated rate constant values used in Scheme I also satisfy the kinetics observed with polymerase alone except that k_{on} is at the diffusion limit (10^8 $M^{-1}\cdot s^{-1}$) (data not shown).

†Although the stoichiometric concentration of polymerase is 100 nM, under these conditions the active site titration is ≈50 nM (data not shown). Therefore, one "active" enzyme equivalent for the data in Fig. 2 is 5 nM and in Fig. 5 is 50 nM.

The presence of the accessory proteins makes the T4 DNA polymerase a highly efficient enzyme. The T4 accessory proteins 44/62 and 45 are not only functionally similar to the analogous proteins in the *E. coli* and eukaryotic pol δ systems but were recently found to share a significant level of sequence homology (32, 33). The parallels drawn between the proteins in these diverse systems reinforce the concept that the basic mechanism of DNA replication has been conserved, so that information regarding the mechanism and kinetics of complex assembly in the T4 system should prove useful in understanding other multienzyme replication systems.

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