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Structure and Function of the Bacteriophage T4 DNA Polymerase Holoenzyme†

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Progress in understanding DNA[1] replication over the past two decades has been impressive. Isolation and identification of key DNA intermediates have led to an overall picture of the DNA structures involved in the replication fork (Ogawa & Okazaki, 1980). For many organisms, most of the essential DNA replication proteins have been identified, purified, and reconstituted in vitro into functional assemblies that display most of the properties of the in vivo systems (Alberts et al., 1975, 1977; Nossal & Peterlin, 1979; Richardson et al., 1987; Kornberg, 1988; Kelly, 1991). In addition, the functions of many of the proteins of the replication complexes of a number of organisms have been elucidated. The current challenge in the replication field is to clarify the detailed mechanisms by which the component proteins discharge their functions and to determine how these mechanisms are modified by integration into functional DNA replication complexes.

Studies of DNA replication using systems obtained from a variety of organisms have revealed that the faithful and rapid replication of the genome of an organism requires a defined set of enzymatic activities (Kornberg & Baker, 1992). This set of activities appears to be the same in organisms as disparate as mammals and bacteriophages, although the organization of these activities, and their distribution between protein subunits, varies from one species to another (Kornberg, 1988). Figure 1 shows schematically the minimum functional requirements of a DNA replication complex.

The replication complex of the *Escherichia coli* bacteriophage T4 was one of the first systems to be reconstituted successfully in vitro (Morris et al., 1975; Alberts et al., 1975, 1977). Building on the elegant genetic studies of Epstein and his colleagues (Epstein et al., 1964), the laboratories of Bruce Alberts and Nancy Nossal have been instrumental in identifying and characterizing the seven T4-coded gene products that function together in the elongation phase of T4 DNA replication (Barry & Alberts, 1972; Alberts et al., 1975, 1977; Nossal, 1979; Nossal & Peterlin, 1979). Five of these proteins comprise a subassembly of the T4 complex that is capable of efficient, rapid, and high-fidelity DNA synthesis on primed single-stranded DNA substrates under physiological conditions. This subassembly contains the T4 DNA polymerase, three T4-coded polymerase accessory proteins, and the T4-coded single-stranded DNA binding protein. The combination of the DNA polymerase and the three accessory proteins has been termed the T4 DNA polymerase holoenzyme (Munn & Alberts, 1991a,b). In this Perspectives, we expand this definition to include the T4 single-stranded binding protein, because it is the five-protein system that manifests an efficiency superior to that of the polymerase alone.

The T4 DNA polymerase is itself capable of synthesis on such substrates, but with lower rate, processivity, and fidelity than are characteristic of the five-protein holoenzyme. The mechanisms by which T4 DNA polymerase is converted to the holoenzyme form, and the biochemical differences between the two forms, have been the subject of intense scrutiny over the past decade. The T4 holoenzyme is of particular interest because it contains a small number of protein components that are readily separable. This makes structure and function studies with these component proteins and subassemblies particularly accessible. The T4 holoenzyme may also be reconstituted from its separated parts, making the holoenzyme an ideal substrate for mechanistic studies and for developing molecular interpretations of the interactions between components that give the holoenzyme its unique functional

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[1] Abbreviations: DNA, deoxyribonucleic acid; gp43, product of T4 gene 43 (DNA polymerase); gp44/62, complex of products of T4 genes 44 and 62; gp45, product of T4 gene 45; gp32, product of T4 gene 32 (single-stranded DNA binding protein); ATP, adenosine 5′-triphosphate; ATPase, adenosinetriphosphatase or ATP phosphohydrolase; cryoEM, cryoelectron microscopy; dGTP, deoxyguanosine 5′-triphosphate; dCTP, deoxycytosine 5′-triphosphate; dNTP, deoxynucleoside 5′-triphosphate; ADP, adenosine 5′-diphosphate; dADP, deoxyadenosine 5′-diphosphate; HPLC, high-performance liquid chromatography.

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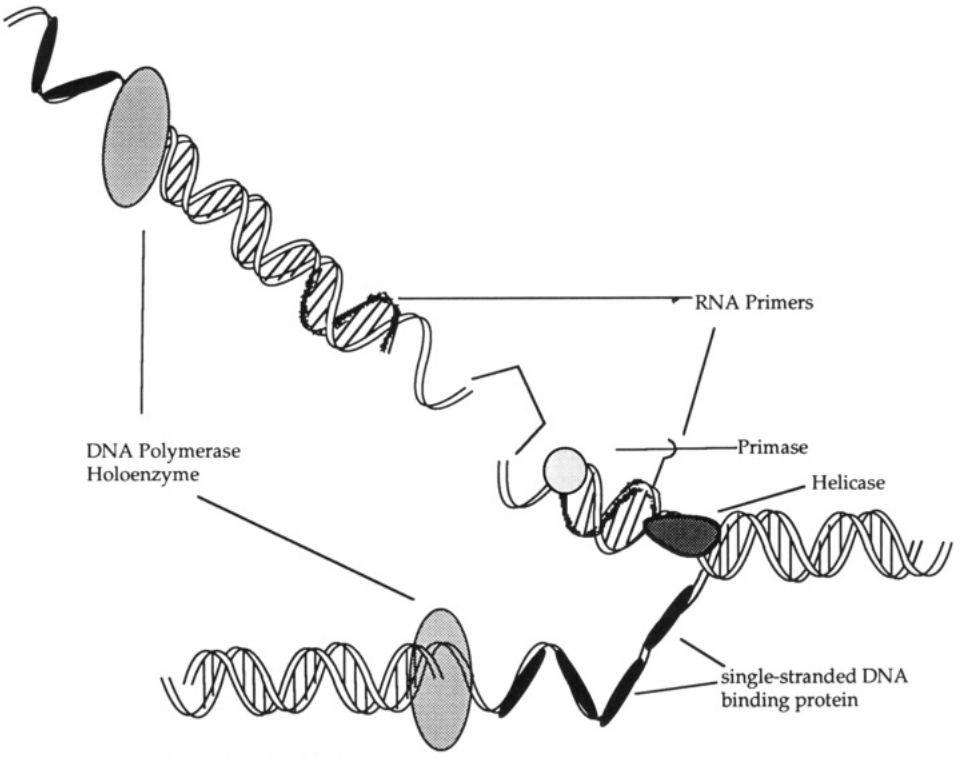


FIGURE 1: General representation of a replication fork, showing the activities necessary for fork elongation. For ease of presentation, each activity is shown separately. In reality, the arrangement of proteins at the replication fork is likely to form a complex three-dimensional structure. A schematic replication fork is shown, with the lagging strand above and the leading strand below, each with one DNA polymerase holoenzyme at the primer-template junction. At the fork itself, a helicase unwinds the parent strands. In preparation for new DNA synthesis on the lagging strand, a primase is shown producing RNA primers. A single-stranded binding protein binds to single-stranded regions of DNA. The bracket on the lagging strand indicates intervening single-stranded DNA covered with single-stranded DNA binding protein.

characteristics.

In this Perspectives we describe our current understanding of the structure and assembly of the high-efficiency, high-fidelity DNA polymerase holoenzyme of bacteriophage T4, stressing both the integrating interactions that are now quite well understood and those that remain unclear. Since the proteins of the T4 holoenzyme all have analogues in more complicated replication systems, it is likely that experiments with the T4 system can be interpreted in more general terms. There are a number of excellent reviews that deal with the overall replication process and describe, in part, the involvement of each of the classes of functional components listed above in particular systems [e.g., Nossal and Alberts (1983), Nossal (1983, 1992), Alberts (1987), Richardson et al. (1987), Hurwitz et al. (1990), Kelly (1991), and Wang (1991)].

For convenience, we divide our discussion of the T4 holoenzyme into three sections that reflect its functional organization. These are (i) the T4 DNA polymerase (product of gene 43, gp43), (ii) the T4 single-stranded DNA binding protein (gp32), and (iii) the T4 DNA polymerase accessory protein complex (gp44, gp62, and gp45). Each of these components is discussed first in isolation and then in the context of the reconstituted holoenzyme.

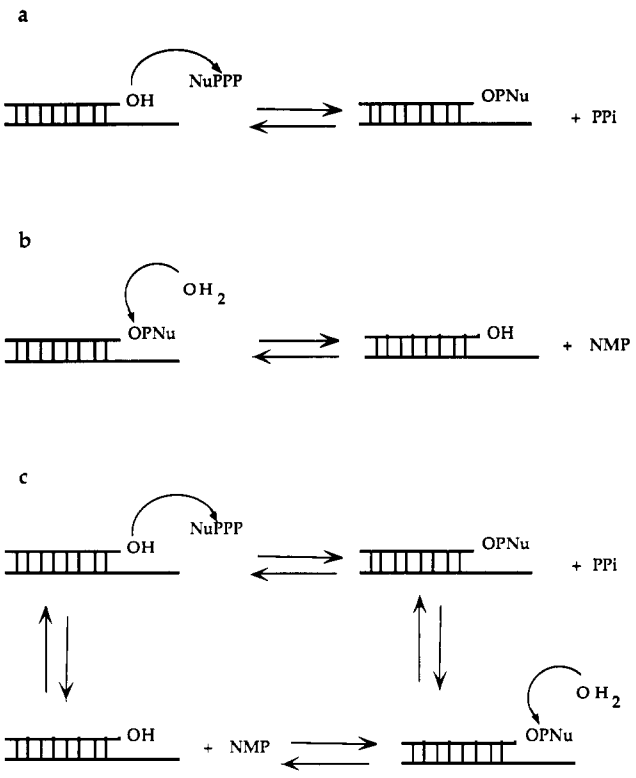
T4 DNA POLYMERASE

Although the T4 DNA polymerase is "improved" by incorporation into a multiprotein holoenzyme, the polymerase lies at the heart of the replication process. No understanding of holoenzyme function can be complete without a thorough examination of the capabilities and shortcomings of the isolated polymerase. In this way we can ask how the other components of the holoenzyme utilize the capabilities, and obviate the shortcomings, of the isolated polymerase to build the functionally improved complex that is required for efficient DNA replication.

Structure and Activities of the T4 DNA Polymerase. The T4 DNA polymerase is a monomeric protein with a calculated molecular weight of 103 572 (Spicer et al., 1988) and carries two enzymatic activities that permit it to catalyze the synthesis of DNA with high efficiency and accuracy. The first of these activities is the template-directed DNA synthesis (polymerase) activity, shown in Scheme Ia. The deoxynucleoside monophosphate moiety of a deoxynucleoside triphosphate is added to the 3′-hydroxyl of a DNA primer strand annealed to a DNA template, with concomitant production of inorganic pyrophosphate. The chemistry of this reaction has been investigated by Romaniuk and Eckstein (1982) and appears to involve attack of the 3′-hydroxyl group of the primer strand on the α-phosphorus of the nucleotide triphosphate, resulting in a stereochemical inversion at the phosphorus without the formation of a covalent intermediate on the enzyme. The mechanism of this reaction has been extensively studied in other DNA polymerases (Carroll & Benkovic, 1990).

In addition to its synthesis capability, T4 DNA polymerase displays a 3′→5′ exonuclease activity in which the 3′-terminal primer nucleotide is removed by hydrolysis, generating a nucleoside monophosphate (Scheme Ib). The chemistry of the reaction involves the nucleophilic attack of water on the 3′-phosphate ester, with inversion of the phosphorus stereochemistry (Gupta et al., 1982). This exonuclease is much more active with single-stranded DNA as substrate than it is on the paired 3′-terminus of a template-bound primer. This observation suggested that the 3′→5′ exonuclease activity might be more active in excising terminal mispaired bases than in removing those that are properly base-paired, since such mispaired bases might be thought to resemble more closely the

Scheme I



preferred single-stranded substrate (Brutlag & Kornberg, 1972; Muzyczka et al., 1972).

The combined action of the polymerase and exonuclease activities results in the so-called idling–turnover reaction (Scheme Ic). Here the 3′-terminal nucleotide is added and removed in a futile cycle (Nossal & Hershfield, 1973; Das & Fujimura, 1980; Gupta et al., 1982). Although a clear division is not possible, mutational studies of T4 polymerase suggest that the polymerase activity resides near the carboxy terminus of the protein (Nossal & Hershfield, 1971; Reha-Krantz, 1988) and that the active site of the exonuclease is situated near the amino terminus. Reha-Krantz and her colleagues have also mutationally identified residues important for exonuclease activity (Reha-Krantz et al., 1986, 1991). To date, unlike other prokaryotic polymerases, *single* amino acid substitutions of proposed metal binding residues (Bernad et al., 1989; Blanco et al., 1992) have been ineffective in eliminating the exonuclease activity of T4 polymerase.

Mechanism of the T4 DNA Polymerase. Complete kinetic descriptions of the polymerase and 3′→5′ exonuclease activities of the Klenow fragment of *E. coli* polymerase I (Kuchta et al., 1987, 1988; Eger et al., 1991; Dahlberg & Benkovic, 1991) and of the bacteriophage T7 DNA polymerase (Patel et al., 1991; Wong et al., 1991; Donlin et al., 1991) are available. Although intensive studies of the kinetics of the T4 polymerase have recently been completed (Capson et al., 1992), few mechanistic details are available in published form on either of the enzymic activities of T4 DNA polymerase. The unpublished results suggest, however, that the T4 enzyme will share many of the properties of the two polymerases that have been studied in detail (Carroll & Benkovic, 1990). Most studies to date have described polymerase mechanisms in terms of two functional parameters: processivity and fidelity.

Processivity. One measure of the suitability of a DNA polymerase for replication is that it must be able to catalyze replication of the entire genome on the time scale of the cell cycle. The suitability of a polymerase in this regard may be measured in terms of its catalytic efficiency, which describes the rate at which new DNA is synthesized. This parameter may be broken down into two parts. These are (i) the intrinsic rate of polymerization, which is a measure of the rate of addition of a single nucleotide residue, and (ii) the processivity, which is a measure of the number of nucleotide residues added per polymerase–DNA binding event.

Although rigorous definitions of processivity exist (McClure & Chow, 1980; Fairfield et al., 1983), processivity is often expressed simply as the average number of bases incorporated per polymerase–DNA association event [for example, see Jarvis et al. (1990a)]. This number may be roughly calculated by first estimating the mean time of association as $1/k_d$. The mean number of bases polymerized is then the single nucleotide polymerization rate constant times the mean association time, or k_p/k_d.[2]

The processivity of isolated T4 polymerase has been found to be quite dependent on the type of DNA template used to measure this parameter. Das and Fujimura (1979), Newport et al. (1980), Newport (1980), and Fairfield et al. (1983) showed that T4 DNA polymerase on homopolymer templates will add only ~20 nucleotide residues per association event, even at low salt concentrations (~35 mM KCl and 5 mM Mg^{2+}), and that this polymerase becomes totally nonprocessive, or dispersive, at salt concentrations in the physiological range (~150 mM KCl and 5 mM Mg^{2+}). These authors employed the so-called "single-hit" protocol to measure processivity. In this approach, a high ratio of DNA primer-template to DNA polymerase is maintained, so that the probability of any given primer being extended more than once is very low. Newport et al. (1980) were able to conclude from an analysis of product distributions that the probability of dissociation from a homopolynucleotide template is, as expected, constant at each template position.

In contrast, the processivity on natural DNA templates is less well characterized. Mace and Alberts (1984b) applied a different measurement technique in their studies of the processivity of T4 polymerase on "activated" natural DNA. These authors used a very low concentration of DNA primer 3′ ends and collected data at short times to ensure that each primer was extended only once; measurements of the processivity of T4 polymerase were then made by determining the product length distribution as a function of time. Mace and Alberts (1984b) concluded that, in 25 mM KCl and 2 mM Mg^{2+}, T4 DNA polymerase synthesizes an average of ~800 nucleotide residues before dissociating. They also

[2] The interrelation between intrinsic rate, processivity, and catalytic efficiency may be seen by comparing the time required to replicate a genome using two polymerases that display the same intrinsic rate constant of polymerization, k_p, but differ in processivity. Let us assume that one of these polymerases is highly processive and replicates the entire genome following polymerase binding, while the other polymerase is characterized by low processivity and dissociates frequently. The time t required to replicate a genome of length N with the highly processive polymerase with the low-processivity polymerase will be $t = N/k_p + T$, where T is the extra time taken for all polymerase dissociation and rebinding events over the length of the genome. T may be estimated as $T = n/k_a'$, where n is the number of dissociations per genome equivalent of DNA synthesized and k_a is the pseudo-first-order net rebinding rate constant: $k_a' = k_a[\text{Pol}]/[k_p/(k_d + k_p)]$. The number of dissociations per genome may be written as $n = Nk_d/k_p$, which gives the expression for T as $k_d(k_d + k_p)N/k_p^2k_a[\text{Pol}]$. Therefore, $t = N/k_p + k_d(k_d + k_p)N/k_p^2k_a[\text{Pol}]$. When the processivity is low and the genome is large, T may be large as well. Thus DNA replication will be most rapid when the polymerase is infinitely processive and does not dissociate from the DNA template during the replication cycle.

estimated the average intrinsic polymerization rate to be ~250/s and the average DNA dissociation rate to be 0.3/s.

Jarvis et al. (1991) measured a very low processivity for T4 polymerase (<100 nucleotide residues per binding event) on a primed single-stranded M13 template. Earlier, Charette et al. (1986) had demonstrated a similarly low processivity on a similar substrate. The major difference between these two sets of studies is that Mace and Alberts (1984b) used polymerase in 20-fold excess over 3′-hydroxyl ends, while Charette et al. (1986) and Jarvis et al. (1991) maintained the concentration of 3′-hydroxyl ends in excess. Ideally, both protocols should give accurate and equivalent processivities, although the experiments with polymerase in excess are more difficult to control since very rapid measurements are necessary. Mace and Alberts (1984b) noted a very low primer usage, which they suggested might result from nonspecific binding of the polymerase to single-stranded regions of DNA. If nonspecifically bound polymerase can move to primer ends (e.g., by sliding) more rapidly than can polymerase free in solution, the processivity of DNA polymerase may have been overestimated in this study.

Fidelity. The term fidelity refers to the propensity of a DNA polymerase to incorporate "incorrect" nucleotide residues that cannot form proper Watson–Crick hydrogen bonds with the coding template base. In enzymatic terms, fidelity can be defined as substrate specificity (Jencks, 1975; Fersht, 1985). Therefore, the fidelity of DNA polymerase is a particularly fascinating example of the general problem of how enzymes differentiate between substrates.

The replication of bacteriophage T4 in vivo exhibits an error frequency of one nucleotide residue misincorporated per 10^8 residues polymerized (Drake, 1969). In contrast, T4 DNA polymerase by itself misincorporates an average of one nucleotide residue for every 10^4–10^5 residues polymerized on homopolymer primer-template systems (Hall & Lehman, 1968; Loeb & Kunkel, 1982; Topal & Sinha, 1983). However, this fidelity may improve to an average of one residue misincorporated per 10^7 residues correctly incorporated on natural DNA templates (Kunkel et al., 1984). The latter number gives T4 polymerase the distinction of being among the most accurate polymerases known (Kunkel, 1988).

The discovery of a 3′→5′ exonuclease activity of *E. coli* DNA polymerase I (Lehman & Richardson, 1964) and of T4 DNA polymerase (Goulian et al., 1968) led Kornberg (1969) to hypothesize that this activity acts as a "proofreader" by preferentially removing newly incorporated mispaired bases. Support for this hypothesis was soon forthcoming from the studies of Brutlag and Kornberg (1972). Further confirmation was provided by experiments from Bessman and his colleagues on T4 DNA polymerase mutants in exonuclease activity (Muzyczka et al., 1972, 1973; Bessman et al., 1974a,b). The accuracies of such mutants were in rough proportion to the exonuclease activity of these enzymes (Muzyczka et al., 1972), suggesting that the exonuclease reaction is important to high fidelity. Studies on other polymerases have led to the same conclusion (Das & Fujimura, 1980; Loeb & Kunkel, 1982).

The magnitude of the contribution of the exonuclease to the maintainence of high fidelity, and its specific role in fidelity mechanisms, has been addressed by several authors (Hopfield, 1974, 1980; Ninio, 1975; Galas & Branscomb, 1978; Bernardi et al., 1979). The specificity of the 3′→5′ exonuclease activity of T4 polymerase for different mismatches has been investigated by Sinha (1987). His data show that the efficiency of mismatch editing is context dependent. This author also estimated that exonuclease editing increases the overall fidelity of T4 polymerase 10^3–10^6-fold. A recent study with an exonuclease-deficient T4 DNA polymerase indicates a 10^3-fold contribution by exonucleolytic proofreading (Reha-Krantz et al., 1991). This contribution for bacteriophage T7 DNA polymerase has also recently been determined to be approximately 10^3 (Donlin et al., 1991).

Coupling of the Exonuclease and Polymerase Activities. The notion that the 3′→5′ exonuclease is a proofreading activity that efficiently hydrolyzes mispaired residues from the 3′-terminus of the primer strand, combined with the view of the transition between the polymerase and exonuclease active sites as a fraying–unfraying of these terminal residues, has motivated experiments designed to investigate the interaction between the polymerase and exonuclease active sites. Since a processive mode of action is required for efficient genome replication, it is reasonable that efficient error repair should be promoted by concerted polymerase and exonuclease events without dissociation of T4 polymerase from the DNA template. Although this switching between catalytic modes is very inefficient in the repair enzyme Pol I (Joyce, 1989), it is in fact the most likely pathway for the replicative enzymes of bacteriophages T5 (Das & Fujimura, 1980), T7 (Donlin et al., 1991), and T4 (Reddy et al., 1992).

In the latter study, utilizing primer-template substrates with differing numbers of 3′ mismatches on the primer strand and a heparin trapping protocol, Reddy et al. (1992) have shown that T4 polymerase is able to perform sequential exonuclease and polymerization reactions without dissociating from the DNA. The authors observed that this process is most efficient when the two residues at the 3′-terminus of the primer strand are mismatched. This result is consistent with a previous observation (Cowart et al., 1989) that T4 polymerase frays two bases in the transition from polymerase to exonuclease modes. In this study, employing primer-templates that had been chemically cross-linked within the double-stranded region, it was shown that a molecule with a cross-link two bases upstream from the primer terminus is a substrate for the 3′→5′ exonuclease, whereas a cross-link at the primer terminus, or one base upstream, renders a DNA molecule inactive toward hydrolysis (Cowart et al., 1989). This suggested that T4 polymerase requires some fraying of the primer terminus in order to hydrolyze the 3′-terminal residue from the primer strand. A similar process has been suggested for *E. coli* DNA polymerase I on the basis of X-ray analysis of cocrystals of the Klenow fragment with double-stranded DNA oligomers (Freemont et al., 1988). However, the extent to which this sliding–fraying model applies to the T4 enzyme is not known.

Polymerase as a Replication Enzyme. Clearly, T4 DNA polymerase has all the qualitative features that are required for successful DNA replication. These features include a certain level of processivity and fidelity and functional coordination of the polymerase and exonuclease activities of the enzyme. In some respects (e.g., fidelity) the polymerase alone compares favorably with the properties of the in vivo replication complex. However, the isolated polymerase has other features that makes it less than optimal for the replication of natural DNA.

The maximum reported processivity of the T4 polymerase in isolation is ~800 nucleotide residues, corresponding to more than 150 dissociation events per T4 genome replicated. At nanomolar concentrations of T4 polymerase, it would be difficult to support the observed in vivo replication rate of 500 nucleotides polymerized/s (McCarthy et al., 1976).[2] In fact, we may estimate that replication of a T4 genome would require

~8.5 h under these conditions, as opposed to the 2–5 min that replication requires in T4 infection.

The presence of many secondary-structure-related dissociation sites on natural DNA templates (Huang & Hearst, 1980; Huang et al., 1981; Roth et al., 1982) suggests that T4 DNA polymerase may also have difficulty synthesizing through template DNA containing such structures. In addition, certain sequences without noticeable secondary structure also seem to induce dissociation of the polymerase (Fairfield et al., 1983; Bedinger et al., 1989). These observations suggest that high processivity, including resistance to the effects of secondary structure and other types of sequences on processivity, represents a major difference between T4 polymerase operating in isolation and as part of the T4 polymerase holoenzyme.

T4 GENE 32 PROTEIN

Gene 32 protein (gp32) is the single-stranded DNA binding protein of bacteriophage T4 (Williams & Konigsberg, 1983; Chase & Williams, 1986) and is analogous in function to the SSB protein of *E. coli* (Meyer & Laine, 1990). Gp32, like SSB, takes part in replication, recombination, and repair functions. It is an essential protein for replication since, under nonpermissive conditions, conditional mutations in gp32 cause T4 DNA replication to arrest (Riva et al., 1970; Curtis & Alberts, 1976) and the T4 genome to be extensively nucleolytically degraded (Curtis & Alberts, 1976). Studies by Alberts (1970) and Snustad (1968) have shown that gp32 is required continually and stoichiometrically during replication. Thus gp32 appears to play at least a dual role in DNA replication: (i) it participates in maintaining a viable DNA replication complex, and (ii) it protects from nucleases the transient single-stranded DNA sequences that form at the replication fork. It is (i) that we will examine here.

Structure and Properties of Gene 32 Protein. Gp32 is a 33-kDa protein that binds cooperatively to single-stranded DNA (Alberts & Frey, 1970; Delius et al., 1972). Proteolysis studies have shown that the protein may be cleaved into three domains, which segregate various DNA binding aspects of gp32 (Hosoda et al., 1974; Greve et al., 1978; Lonberg et al., 1981; Shamoo et al., 1988). Gp32 contains an intrinsic zinc ion (Giedroc et al., 1986), which is necessary both for cooperative binding to single-stranded DNA and for the maintainence of gp32 stability (Giedroc et al., 1987; Keating et al., 1988).

Gp32 binds cooperatively to single-stranded DNA with an overall binding constant of $\sim 10^7$ M^{-1} and a binding site size of ~7.5 nucleotide residues (Jensen et al., 1976; Kelly et al., 1976), although estimates of this site size have ranged from 5 (Kelly et al., 1976; at 0.1 M NaCl) to 11 (Bobst et al., 1982) nucleotide residues. The overall binding affinity of gp32 for single-stranded DNA consists of an intrinsic binding constant of about 10^4 M^{-1} and a cooperativity parameter (ω) of about 10^3 (Kowalczykowski et al., 1981; Newport et al., 1981) at 0.1 M NaCl. The mechanisms of single-stranded DNA binding and the kinetic parameters governing cooperativity have been investigated (Lohman & Kowalczykowski, 1981; Kowalczykowski et al., 1981; Newport et al., 1981; Lohman, 1984a,b).

It has been shown that gp32 can destabilize poly[d(A–T)] but that it lacks the ability to destabilize DNAs of natural sequence (Jensen et al., 1976). This observation led to the proposal that the melting of double-stranded DNA by gp32 is kinetically blocked. The fact that proteolytic removal of the carboxyl terminus allows gp32 to melt natural sequence DNA (Hosoda et al., 1974; Greve et al., 1978) suggests that this kinetic blocking activity may require the maintenance of a specific conformation of gp32.

Role of Gene 32 Protein in Replication. Clues to the role of gp32 in replication come from biochemical studies spanning two decades. Huberman et al. (1971) studied rates of T4 polymerase-catalyzed DNA synthesis on "nuclease-activated" natural DNA in the presence of gp32. These workers found that gp32 stimulates the rate of DNA synthesis 5–10-fold. After studying this effect as a function of temperature and salt concentration, these authors concluded that one way in which gp32 might exert its effect is by destabilizing DNA secondary structure ahead of the polymerase. This would reduce the propensity of the polymerase to dissociate from the DNA, corresponding to an increase in apparent processivity. As an extreme example of this property of gp32, Nossal (1974) observed that T4 polymers can perform so-called strand displacement synthesis on nicked, double-stranded DNA at low salt concentration and high concentrations of gp32. Presumably in this process gp32 takes advantage of the fraying of the ends of double-stranded DNA to trap transient single-stranded regions, since the protein is kinetically blocked from melting double-stranded DNA directly. Although gp32 does reduce the inhibitory effect of secondary structure, it appears that this reduction is insufficient to suppress totally the activity of polymerase dissociation sites on natural DNA. Roth et al. (1982) observed an approximately 3-fold stimulation and Charette et al. (1986) observed a 10–15-fold stimulation of T4 polymerase on primed single-stranded templates at optimal gp32 concentrations, in rough agreement with the results of Huberman et al. (1971). However, the pattern of dissociation, or "pause" sites observed, was not changed by gp32 in the 1986 results, while Roth et al. (1982) observed an almost complete elimination of hairpin-induced pause sites.

There are also several lines of evidence for a direct protein–protein interaction between the T4 polymerase and gp32, and we base our inclusion of gp32 in a functional definition of the T4 holoenzyme primarily on these observations. First, substitution of gp32 by *E. coli* SSB has been shown to abolish the stimulatory effect of this protein on DNA synthesis by the T4 enzyme (Sigal et al., 1972; Burke et al., 1980). This result suggested that a specific interaction takes place between the T4 polymerase and gp32 and that destabilization of DNA secondary structure by a nonspecific single-stranded DNA binding protein is insufficient to account for the stimulatory effect.

Furthermore, Huberman et al. (1971) were able to isolate a complex of gp32 with T4 polymerase by sucrose gradient centrifugation in the presence of high concentrations of gp32. Binding of T4 polymerase to a gp32 affinity column in up to 200 mM NaCl has also been observed (Formosa et al., 1983). The studies of Burke et al. (1980) also show that gp32*I (a gp32 species from which the carboxyl terminus has been proteolytically cleaved) actually inhibits DNA synthesis by DNA polymerase and will not bind to polymerase, suggesting a role for the carboxyl terminus of gp32 in mediating interactions with other replication proteins.

Gp32 has also been observed to have an effect on polymerase fidelity when DNA synthesis is carried out on homopolymer templates. Gillen and Nossal (1976) measured incorporation of dAMP into poly[d(A-T)] and conversion of dGTP, dCTP, and dTTP to the corresponding monophosphates by mutator, antimutator, and wild-type T4 DNA polymerases in the presence and absence of gp32. Their results were the first to indicate that gp32 exerts an effect on T4 polymerase fidelity.

Gp32 increased the incorporation of correct nucleotide by 15–20-fold, while turnover of both correct and incorrect nucleotides was suppressed or left unaffected.

Topol and Sinha (1983) measured misincorporation of dGTP and dCTP into poly[d(A-T)] as a function of gp32 concentration. They found that gp32 can inhibit misincorporation by up to 100-fold under optimal conditions. They also found that this inhibition depends on the amount of DNA bound by gp32 and not on the ratio of gp32 to T4 polymerase. On the basis of these results, these authors concluded that gp32 affects the fidelity of T4 polymerase by binding DNA and not by direct protein–protein interaction. Interestingly, since gp32 suppresses the conversion of incorrect dNTPs to dNMPs (Topol & Sinha, 1983) and increases correct incorporation (Gillen & Nossal, 1976), these data support the conclusion that higher fidelity in the polymerase–gp32 system is accomplished by an increase in substrate specificity, rather than by an increase in 3′→5′ exonuclease activity (proofreading).

The molecular mechanism by which gp32 promotes higher fidelity and processivity is not clear. The observation that gp32 affects the structure of single-stranded DNA, possibly by extending the sugar–phosphate backbone (Scheerhagen et al., 1989; van Amerongen et al., 1990) may be related to these activities of this protein.

T4 DNA POLYMERASE ACCESSORY PROTEINS

Three proteins coded by bacteriophage T4, when added to T4 DNA polymerase, permit the augmented polymerase to synthesize DNA at an accelerated rate. These are the products of T4 genes 44, 62, and 45; collectively these entities are known as the T4 DNA polymerase accessory proteins. It is through interaction with these accessory proteins, as well as with gp32, that T4 polymerase is transformed into its high-efficiency holoenzyme form. The accessory proteins also carry a DNA-dependent ATPase activity that is required for the stimulatory effect of these proteins on the DNA polymerase in DNA synthesis (Piperno & Alberts, 1978). However, manifestation of this ATPase activity does not require other protein factors. The three polymerase accessory proteins can thus be considered to comprise a functional subassembly of the holoenzyme and of the replication complex.

Structure of the Accessory Proteins and of the Accessory Protein Complex. The amino acid sequences of the T4 DNA polymerase accessory proteins have all been determined from the DNA sequences, and the monomer molecular weights calculated from the primary sequences in the Swiss-Prot database are as follows: gp44, 35 779 (Spicer et al., 1984); gp62, 21 363 (Rush et al., 1989); gp45, 24 832 (Spicer et al., 1982). The T4 polymerase accessory protein complex is made up of two subassemblies, one consisting of gp44 and gp62 and the other comprising a multimer of gp45.

That gp44 and gp62 exist as a tightly bonded complex was first suggested by the consistent copurification of their protein constituents through several preparatory steps (Barry & Alberts, 1972; Nossal, 1979). The subunit stoichiometry of the complex has been studied by a number of groups. Barry et al. (1973) estimated a stoichiometry of four gp44 subunits per two gp62 monomers from sucrose gradient sedimentation, gel filtration, and protein determination from bands eluted from denaturing polyacrylamide gels (Johns, 1967). Nossal (1979) postulated a gp44 to gp62 stoichiometry of 5–6:1 on the basis of scanning densitometry of Coomassie blue-stained denaturing polyacrylamide gels. Spicer et al. (1984) suggested a gp44 to gp62 stoichiometry of 3.6 (±0.6):1 on the basis of Edman degradation of gp44/62 complexes. Rush et al. (1989) used gel filtration and sucrose gradient sedimentation experiments (Siegel & Monty, 1966) to estimate a gp44 to gp62 stoichiometry of 3:2. Finally, Jarvis et al. (1989a) utilized two molecular weight measurements, one by sedimentation equilibrium and the other by combination of sedimentation velocity and diffusion coefficient determination with dynamic light scattering, to arrive at a total mass of 163 700 Da for the gp44/62 complex. The latter workers also used reverse-phase HPLC separation with UV absorbance detection of subunits in the gp44/62 complex to arrive at a 4:1 stoichiometry of gp44 to gp62, in good agreement with the earlier estimate of Spicer et al. (1984). Therefore, the best estimate of the size and stoichiometry of the gp44/62 complex is 4 gp44 subunits to 1 gp62 subunit, resulting in a complex of 164 kDa. Jarvis et al. (1989a) also concluded from diffusion coefficient mesurements that the 4:1 complex has a very asymmetric shape, corresponding in frictional properties to a prolate ellipsoid with an axial ratio of 5:1. This result is consistent with an earlier hydrodynamic shape estimate by Barry and Alberts (1972).

Recently it has been shown that the gp44/62 complex may be further subdivided. Rush et al. (1989) cloned gene 44 into a separate overexpression vector. The apparent molecular weight of the isolated expressed gp44 in solution is close to that of the gp44/62 complex, suggesting that gp44 subunits can oligomerize in the absence of gp62.

The association state of gp45 has also been determined by Jarvis et al. (1989a). Sedimentation equilibrium experiments, as well as chemical cross-linking studies, showed that the apparent molecular weights of gp45 in solution is ~77 000, suggesting (on the basis of the calculated molecular weight of the gp45 monomer) that this protein exists in solution as a trimer. From a separate sedimentation velocity determination, Jarvis et al. (1989a) calculated that, like the gp44/62 complex, the gp45 trimer can be modeled as an asymmetric prolate ellipsoid, in this case with an axial ratio of 6:1. Rush et al. (1989), using a method involving gel filtration and sucrose gradient sedimentation (Siegel & Monty, 1966), have suggested that gp45 exists in solution as a dimer. The reasons for this discrepancy are not readily apparent, but the buffer and temperature conditions used for the two determinations were very different, making comparisons between results difficult.

DNA-Dependent ATPase Activity of the Accessory Proteins. As mentioned above, the accessory proteins show a DNA-dependent ATPase activity that appears to play a major role in the in vitro function of these proteins in DNA synthesis. As a consequence, considerable effort has been expended in establishing how ATP hydrolysis by the accessory protein complex is involved in holoenzyme function. Early studies of this ATPase activity showed that the reaction involves the hydrolysis of the β,γ-phosphate anhydride bond of ATP (or dATP), producing ADP (or dADP) and inorganic phosphate (Piperno & Alberts, 1978; Mace & Alberts, 1984a). More recent studies have focused on the DNA cofactor specificity of the hydrolysis reaction and on the roles of each of the accessory proteins in modulating the enzymic properties of the complex.

An overview of the parameters characterizing the ATPase activity of the various accessory protein subassemblies is presented in Table I (Mace & Alberts, 1984a; Jarvis et al., 1989b). The gp44/62 subassembly alone has a low, but detectable, basal level of activity, which is stimulated ~20-fold by the addition of primer-template DNA. The addition

Table I: ATPase Turnover Numbers and K_m for ATP for Various Components of the T4 DNA Polymerase Accessory Protein Complex[a]

reaction components[b]	k_{cat}[c]	K_m for ATP[c]
44/62	<1	ND[e]
44/62 + 45	20 ± 4	8 ± 1
44/62 + DNA[d]	20 ± 4	260 ± 40
44/62 + DNA + 45	1800 ± 300	180 ± 40

[a] Adapted from Jarvis et al. (1989b). Reactions were carried out in buffer containing 6 mM $Mg(OAc)_2$, 60 mM KOAc, 5 mM 2-mercaptoethanol, and 25 mM Tris–OAc, pH 7.5. [b] Proteins are listed by gene number. [c] K_m values are reported as μM nucleotide of the template strand; k_{cat} values are reported in min^{-1}. [d] The DNA used is a primer-template hybrid of 5′-GCG$(A)_{35}$ and 5′-$(T)_{20}$CGC. The base-paired GCG and CGC ends assure a defined alignment of the primer-template structure. [e] Cannot be determined due to the low ATPase activity of gp44/62 alone.

of gp45 also stimulates the basal ATPase activity of gp44/62 approximately 20-fold, but this three-protein complex is further stimulated about 100-fold by primer-template DNA addition. As a result, the ATPase activity of the full accessory protein–DNA complex is nearly 2000-fold more active than is the gp44/62 complex alone. An investigation of the DNA cofactor preference of the ATPase activity of the accessory proteins (Mace & Alberts, 1984a; Jarvis et al., 1989b; Table II) has shown that the addition of gp45 to the gp44/62 subassembly provides binding specificity for primer-template DNA cofactors over purely single- or double-stranded DNA sequences. It was also found that the ATPase activity of the accessory protein complex bound to DNA primer-template junctions is much more resistant to inhibition by added salt or added gp32 than that of the complex bound to either single-or double-stranded DNA (Jarvis et al., 1989b).

The ATPase active sites of the accessory protein complex has recently been localized to the gp44 subunits. Rush et al. (1989) showed that gp44 purified from a plasmid carrying this gene in isolation shows a DNA-dependent ATPase activity that is equal to that of the intact gp44/62 complex. Jarvis et al. (1989b) came to the same conclusion by showing that reaction of the accessory protein complex with the ATP photoaffinity analogue 8-azido-ATP results in labeling only the gp44 subunits of the complex. Effects on the ATPase activity have also been used to assign functional roles to the proteins within the complex. The DNA-dependent ATPase of gp44 subunits in isolation is only slightly stimulated by gp45, whereas the gp44/62 DNA-dependent ATPase is stimulated ~100-fold (Rush et al., 1989). This suggests that one of the functions of gp62 may be to modulate interactions between the gp44/62 complex and gp45.

The stimulation of the ATPase activity of gp44/62 complexes by gp45 trimers suggests that these two accessory subassemblies interact physically. Measurements of the binding affinity and stoichiometry of the gp44/62 and gp45 subassemblies within the complex have been made by monitoring the stimulation of the ATPase activity of the gp44/62 complex as a function of gp45 concentration (Jarvis et al., 1990b). Using this approach, these workers estimated a binding constant of 7×10^6 M^{-1} for the formation of the accessory protein complex, consistent with the observation that, under the conditions of most kinetic experiments (gp44/62 concentrations in the 10 nM range), a large excess of gp45 is needed to obtain a maximal stimulation of the ATPase activity (Mace & Alberts, 1984a; Jarvis et al., 1989b, 1990b). This weak binding interaction makes estimates of subunit stoichiometry within the complex difficult.

This situation has been alleviated by the addition of poly(ethylene glycol) (PEG), as a macromolecular crowding agent (Zimmerman & Pheiffer, 1983), to gp44/62–gp45 ATPase assay solutions. This solvent additive has been shown to increase the binding affinity between macromolecules in a number of systems (Zimmerman & Pheiffer, 1983; Forterre et al., 1985; Harrison & Zimmerman, 1986; Zimmerman & Harrison, 1987; Zimmerman & Trach, 1988). The mechanism of this effect has been extensively studied (Minton, 1981, 1983; Berg, 1990); current views suggest that it may reflect a decrease in the configurational degrees of freedom of individual macromolecules in such solutions.

Jarvis et al. (1990b) showed that the gp44/62 and gp45 subassemblies interact much more strongly in solutions containing significant (~7.5% w/v) concentrations of 12-kDa PEG. Under optimal conditions, these workers determined a gp44/62–gp45 subassembly binding constant of $\sim 3.5 \times 10^8$ M^{-1}, about 50-fold larger than the value of K_a measured in the absence of PEG. This measurement allows the stoichiometry of the accessory protein complex to be estimated as one gp44/62 4:1 complex per gp45 trimer.

Structure–Function Studies of the Accessory Protein Complex. Insights into the structure of the accessory protein complex have also been gained by the use of UV laser protein–nucleic acid cross-linking studies and cryoelectron microscopy.

We have applied nanosecond UV laser cross-linking to map protein–nucleic acid contacts within the accessory protein complex. This technique makes use of the well-known fact that protein and nucleic acid species can be cross-linked by irradiation with UV light (Shetlar, 1980) to form a "zero-length" covalent cross-link. The laser provides a high-intensity monochromatic light pulse of short (nanosecond) duration, ensuring that only initially excited species interact after the pulse and that side reactions are minimized. The short pulse duration offers the possibility of studying transient states of temporally unstable protein–nucleic acid complexes. Hockensmith et al. (1986, 1991) have extensively characterized protein–nucleic acid oligomer cross-linking by 8-ns pulses at 266 nm and have found that a wide variety of nucleic acid binding proteins can be cross-linked with efficiencies ranging from 0.01% to 16%.

The nucleic acid portion of the cross-links formed involve primarily pyrimidine residues, with thymidine exhibiting a 25-fold higher cross-linking quantum yield than cytidine and uridine. Proteins of the accessory protein complex were shown to cross-link to $(dT)_{10}$ with efficiencies of ~0.3% for T4 polymerase, ~0.2% for gp45, and ~0.01% for gp44/62 in one-pulse experiments (Hockensmith et al., 1986). Ongoing work in this laboratory has shown that the effective lifetime of photochemically produced reactive states after pulsing is approximately 0.5 μs (E. M. Evertsz, D. T. Kuninger, and P. H. von Hippel, in preparation), indicating that only protein–nucleic acid interfaces that are actually in contact at the time of the pulse will be cross-linked.

In cross-linking studies of the functional accessory protein complex in the presence of ATP (or of the nonhydrolyzable analogue ATP-γ-S), Hockensmith, et al. (1987) showed that gp45 produces an extraordinary change in the cross-linking pattern of gp44/62 with primer-template DNA complexes. Although the identity of cross-linked proteins is tentative, their results are quite striking. In the absence of gp45, the gp44 subunits cross-link preferentially to the thymine-containing template strand. As gp45 is added, the gp62 subunit becomes the predominant cross-linked species, suggesting that a significant subunit rearrangement has taken place on formation of the full accessory protein complex. Addition of ADP to the complex on the primer-template DNA results in

Table II: Influence of gp45 on DNA Cofactor Preference[a]

		single-stranded DNA		primer-template DNA		double-stranded DNA	
[45] (nM)	[44/62] (nM)	K_m[b]	V_{max}	K_m	V_{max}	K_m	V_{max}
0	43	60 (2100)	0.2	50 (1300)	0.2	500	0.1
110	3	6 (230)	1.4	0.3 (7)	2.2	ND[c]	ND
200	3	6 (210)	2.4	0.3 (9)	4.2	ND	ND
420	3	7 (250)	3.4	0.4 (11)	5.6	50	0.1

[a] Adapted from Jarvis et al. (1989b). Reaction conditions were as described in Table I. [b] K_m values are reported as μM nucleotide of the template strand, with K_m values in nM 3′ ends reported in parentheses for comparison. V_{max} values are reported in nmol min^{-1} (μg of gp44/62)$^{-1}$ to facilitate comparison of the results with and without gp45. [c] ND, not determined.

the abolition of all cross-linking, perhaps due to a second conformational change that occurs following ATP hydrolysis (Hockensmith et al., 1987; Kubasek et al., 1989). The extent of cross-linking of gp62 in the presence of the nonhydrolyzable ATP-γ-S is much greater than in the presence of ATP. On the basis of these observations, along with the fact that the ADP-bound accessory protein complex does not cross-link, it was concluded that the ATP-bound complex cross-links with high efficiency and that, in the steady state, only a small proportion of the complex is present in the ATP-bound state.

Cryoelectron microscopy (cryoEM; Dubochet et al., 1988) has also been used to examine the solution structure of the accessory protein complex (Gogol et al., 1992) in its active state. Under appropriate conditions, striking structures are seen that resemble bars positioned perpendicular to the DNA axis (Gogol et al., 1992). These structures, which we have termed "hash marks", are only seen under the solution conditions that are used to demonstrate optimal ATPase activity of the accessory protein complex. Gp44/62, gp45, ATP, and a nicked or gapped DNA cofactor structure that resembles a primer-template junction are required. Gp32 is not absolutely required, but its presence stimulates hash mark formation. ATPase activity of the accessory protein complex is also required, since cessation of ATP hydrolysis as a consequence of ATP depletion or the addition of EDTA abolishes the hash marks, and the addition of the nonhydrolyzable ATP analogue ATP-γ-S does not promote their formation. These observations are consistent with a continuous requirement for ATP hydrolysis to maintain these structures.

A further feature of the hash mark structures is that they most frequently occur in clusters of ten or more. We have suggested that this "cooperative" aspect of hash mark formation results from a mechanism whereby the structures are "injected" into DNA at the primer-template junction and then translocate along the DNA (Gogol et al., 1992; see Figure 2). This "injection–translocation" mechanism for hash mark formation is particularly attractive in light of studies from the Geiduschek laboratory on the transcription enhancer activity of the same T4 accessory protein complex on DNA substrates containing a nick distal to late T4 promoters (Herendeen et al., 1989, 1990). In these studies, transcriptional enhancement by the accessory protein complex shows the same requirements as those for hash mark formation. In addition, further experiments suggest that the accessory protein complex exerts its enhancement effect by a tracking mechanism (Herendeen et al., 1992) analogous to the proposed translocation of the hash marks.

At present, there is uncertainty about the protein composition of the hash mark structures. Measurement of the size of the structures suggests that they have a molecular weight of ~150 000, which is insufficient to account for the presence of all three accessory proteins if they maintain the 1:1 stoichiometry of the gp44/62 and gp45 subassemblies described above. The fact that the hash marks have all the characteristics of the specific, functional accessory protein complex, but do not display the size expected, presents an interesting conundrum. There are several possible solutions. First, the structures may represent only one of the accessory protein subassemblies (with the solution stoichiometry). This would relegate one of the subassemblies to the role of a transiently bound "helper" protein. Correspondence analysis

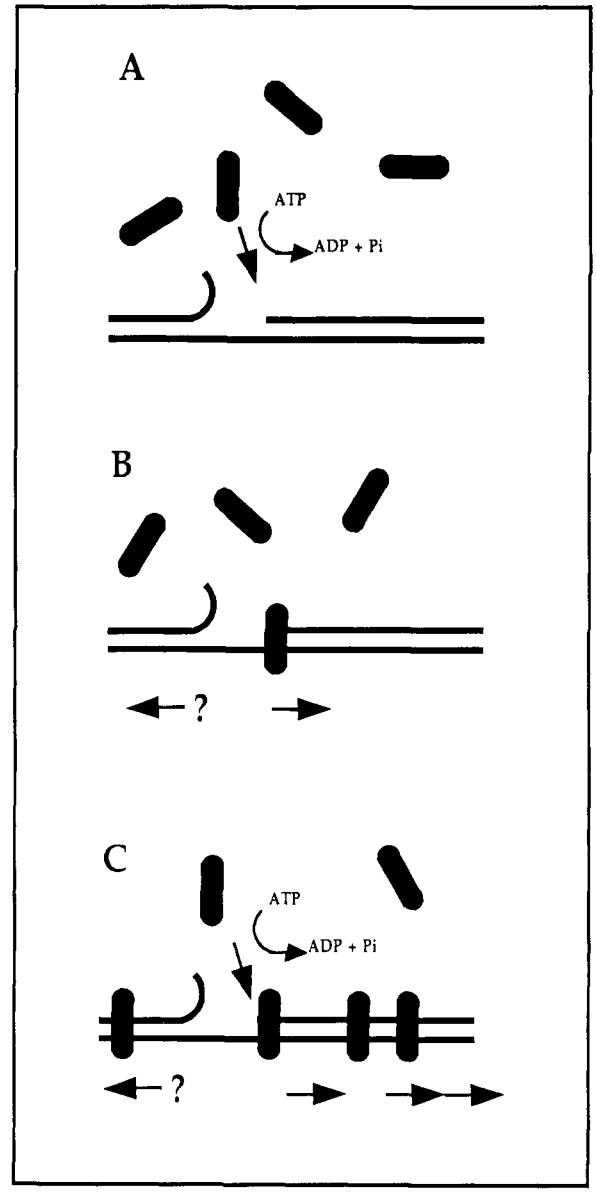


FIGURE 2: Schematic illustration of a model for the assembly of hash mark structures at nicks or gaps in double-stranded DNA. This assembly requires gp44/62 and gp45, in addition to ATP. Migration of the assemblies away from their points of initial association with DNA leads to clusters on one or perhaps both sides of their assembly points. These processes are further explained in the text. Adapted from Gogol et al. (1992).

of the hash mark images (van Heel & Frank, 1981; Bretaudiere & Frank, 1986) reveals features of internal structure that appear to be three or four large subunits arranged in a triangular or square configuration with the DNA at its center, giving an ellipsoidal shape with axial ratio ~4–5:1. These size and shape estimates are consistent with the properties of either accessory protein complex (Gogol et al., 1992). The cross-linking results discussed above would indicate the gp44/62 model, since in fully assembled accessory complexes gp62 cross-links maximally to the primer-template DNA (Hockensmith et al., 1987; Kubasek et al., 1989). Another possible interpretation of the hash marks is that they represent gp45 trimers. An additional interpretation that cannot presently be ruled out is that subunit disproportionation occurs on formation of the functional accessory protein complex and that the hash marks consist of subunits from both subassemblies in stoichiometries different from those seen in solution. Work is currently underway to clarify these issues (E. Gogol and M. Young, unpublished results).

Overview of the Accessory Protein Complex. The picture of the structure and function of the accessory protein complex that emerges, though incomplete, is suggestive. A schematic summary of proposed steps in the formation of the accessory protein complex is presented in Figure 3. In brief, we believe that the gp44/62 and gp45 subassemblies combine with ATP, in a reaction that hydrolyzes ATP and seems to involve protein conformational changes (and/or subunit disporportionation), to induce the formation of a subassembly that then interacts with DNA polymerase to form the functional holoenzyme. This species is most probably what is being visualized in the cryoEM studies of the accessory protein complex, and it may be represented roughly as a torus around the DNA axis, as shown.

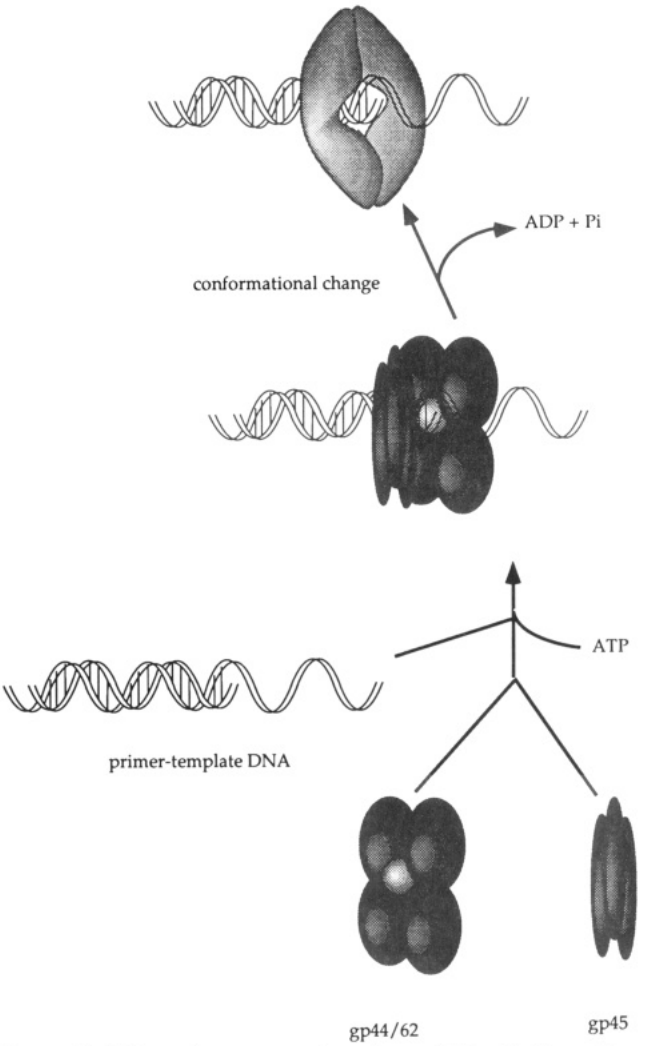


FIGURE 3: Schematic representation of a model for the formation of the active DNA-bound accessory protein complex, incorporating the results of the solution structure studies of gp45 and the gp44/62 complex (Jarvis et al., 1989a), the UV laser cross-linking studies (Hockensmith et al., 1987; Kubasek et al., 1989), and the cryoEM results (Gogol et al., 1992). One gp44/62 4:1 complex and one gp45 trimer combine with ATP and primer-template DNA to form an uncharacterized DNA-bound protein complex that undergoes a conformational change upon ATP hydrolysis to form the disk-shaped object observed as a hash mark in the cryoEM experiments.

HOLOENZYME ASSEMBLY: THE FOUR- AND FIVE-PROTEIN SYSTEMS

The first detailed studies of the reconstitution of the T4 DNA polymerase holoenzyme were carried out in the laboratory of Bruce Alberts (Piperno & Alberts, 1978; Piperno et al., 1978; Mace, 1975; Huang et al., 1981). From these experiments it quickly became apparent that T4 polymerase, gp44/62, and gp45, in combination, form an autonomous subcomplex of the T4 DNA replication apparatus. The defining characteristic of this subcomplex is ATP-dependent synthesis of DNA on primed, single-stranded templates that is severalfold more efficient than synthesis catalyzed by the isolated DNA polymerase. Addition of gp32 to the four-protein system results in a further increase in efficiency (Huang et al., 1981) and allows the resulting five-protein complex to utilize the 3′-hydroxyl group of nicked, double-stranded DNA as a primer to initiate strand-displacement, or rolling circle, DNA synthesis (Nossal, 1974; Nossal & Peterlin, 1979).

Effects of the Accessory Proteins and gp32 on the Activity of the DNA Polymerase. The intrinsic rate, processivity, and fidelity of T4 DNA polymerase have all been shown to be affected by the accessory protein complex or by the accessory protein complex together with gp32.

(*A*) *Processivity.* Newport et al. (1980) performed experiments on the processivity of DNA synthesis catalyzed on homopolymer templates by T4 polymerase alone and by polymerase plus accessory proteins. Their results showed that under low salt conditions (25 mM NaCl, 4 mM $MgCl_2$) the accessory proteins (plus 300 μM ATP) alone did not stimulate the processivity of the DNA polymerase, although they did produce a 4-fold stimulation in the association rate of DNA to the polymerase. The further addition of gp32 caused an increase in the apparent processivity to at least 300 nucleotide residues added per binding event.

Huang et al. (1981) came to a similar conclusion based on the observation that the propensity of DNA polymerase to traverse regions of secondary structure in primed single-stranded templates of natural DNA was increased 4-fold by the accessory proteins plus ATP. These studies led to the hypothesis that the accessory protein complex might act as a "sliding clamp" for the polymerase, increasing its residence time on the DNA and allowing it more time to synthesize DNA and to progress through hairpins. Although the authors interpreted this result in terms of an increase in the processivity of the polymerase, this finding can be also accommodated by the commensurate increase in the polymerase–DNA association rate observed by Newport et al. (1980). Again, the presence of gp32 vastly increased the rate of hairpin traversal by polymerase plus the accessory protein complex; a 40-fold increase was observed by Huang et al. (1981).

The observed increase of the association rate of the polymerase to the DNA substrate upon accessory protein

addition may also explain the apparent 4-fold increase in processivity observed by Mace and Alberts (1984c). These processivity measurements were carried out under the same conditions as those previously used by these authors (see above; Mace & Alberts, 1984b). If the observed large products do result from multiple synthetic rounds from nonspecifically bound T4 polymerase, an increase in the transfer rate from nonspecific DNA sites to primer-end-bound sites could explain the increase in primer length observed upon addition of the accessory proteins.

Mace and Alberts (1984c) also attempted to determine the intrinsic rate of polymerization from analysis of DNA product lengths at short reaction times. Their data show a 2–3-fold stimulation in the intrinsic rate of the polymerase from 250/s (Mace & Alberts, 1984b) to 600/s, the latter rate being comparable to the ~500/s rate of synthesis observed in vivo for each nascent DNA chain at the replication fork (McCarthy et al., 1976).

The most recent study of processivity has been carried out by Jarvis et al. (1991), using primed single-stranded M13 templates. These workers observed a large effect on the processivity of T4 polymerase in the presence of the accessory protein complex and small amounts of gp32 (~10% coverage of single-stranded DNA) and concluded that the lifetime of the holoenzyme (polymerase, accessory proteins, and gp32) at the DNA primer-template junction is 10 times longer than that of the T4 polymerase alone. Using the intrinsic rate difference measured by Mace and Alberts (1984c), this is equivalent to a 20–30-fold increase in processivity in going from polymerase to holoenzyme. Jarvis et al. (1991) also observed an increase in the rate at which polymerization proceeded through secondary structure, as shown previously by Huang et al. (1981).

Although the T4 holoenzyme is much more processive than DNA polymerase alone, it still responds to kinetic barriers in the template. Charette et al. (1986) found that the pattern of polymerase pausing did not change upon combination with the accessory protein complex and with gp32 but that the duration of the pauses was decreased. These conclusions were also confirmed by Jarvis et al. (1991). As with polymerase alone, both DNA sequences that should form secondary structure, and sequences for which no structure is expected cause the holoenzyme to "pause" or dissociate (Huang et al., 1981; Roth et al., 1982; Fairfield et al., 1983; Charette et al., 1986; Bedinger et al., 1989; Jarvis et al., 1991).

(*B*) *Fidelity and the 3′→5′ Exonuclease.* As discussed previously, the T4 enzyme appears to be a very accurate polymerase in its own right on natural DNA; error estimates for synthesis by this polymerase are one misincorporation per 10^6–10^7 residues of DNA polymerized (Kunkel, 1988; Loeb & Kunkel, 1982), which is close to the observed in vivo error rate for DNA replication (Drake, 1969). Liu et al. (1978) and Sinha and Haimes (1980) have measured an error rate very close to this for the T4 DNA polymerase holoenzyme, carrying out synthesis on natural templates. On homopolymer templates, however, error rates for the T4 DNA polymerase alone are about 10–100-fold higher (Loeb & Kunkel, 1982; Sinha & Goodman, 1983), suggesting that incorporation into the holoenzyme may be necessary to improve polymerase fidelity on templates of this kind.

The 3′→5′ exonuclease activity of T4 polymerase is stimulated 3–4-fold by the accessory protein complex (Venkatesan & Nossal, 1982; Bedinger & Alberts, 1983). This stimulation is dependent on ATP and requires a primer-template DNA substrate. As expected from its ability to bind single-stranded DNA, the accessory protein complex inhibits the 3′→5′ exonuclease activity on this substrate in an ATP-dependent manner. The same DNA substrate specificity is observed for the further stimulation by gp32 of the exonuclease activity of the holoenzyme complex. Bedinger and Alberts (1983) observed that this stimulation is dependent on gp32 concentration. Gp32 at high concentrations appears to inhibit exonuclease activity, even on primer-template DNA.

A Direct Interaction between gp45 and T4 DNA Polymerase (gp43)? Review of the literature reveals that there have been several significant pieces of evidence that point to a direct interaction between gp45, gp43, and primer-template DNA. We will briefly enumerate evidence garnered from various laboratories that supports the notion of a direct and functionally significant interaction between these components.

(*A*) *Structural and Functional Observations.* Two sets of results from the laboratory of Bruce Alberts, obtained using an affinity column technique to probe protein–protein interactions, implied a direct interaction between gp45 and gp43. The first observation was indirect, in that purified gp45 did not bind to a gp32 affinity column, but it did bind to such a column when applied in crude lysates. The authors (Formosa et al., 1983) put forth a possible explanation for this observation, suggesting that the binding of gp45 to gp32 is mediated via an interaction with gp43. This interpretation was based on the fact that gp43 was present in the lysate and was known to bind to a gp32 affinity column, but the interaction has yet to be demonstrated directly. More direct evidence was provided by the finding that gp43 and gp45 affinity columns each will quantitatively remove the other protein from a lysate (Alberts et al., 1983).

DNA footprinting results, again from the Alberts laboratory, show an "enlargement" of the footprint of gp43 that is caused by the addition of gp45. This alteration of the observed footprint was shown to be dependent on gp45 concentration. Furthermore, no change in the footprint was observed upon addition of gp44/62 and/or ATP (Munn & Alberts, 1991b).

Topal and Sinha (1983) reported that gp45 enhances fidelity of synthesis by gp43. These authors interpreted their results in terms of a direct interaction between gp45 and T4 DNA polymerase, as opposed to an interaction of gp45 with the DNA itself. Our laboratory has investigated the effects of macromolecular crowding by poly(ethylene glycol) on protein–protein interactions in the holoenzyme complex (Jarvis et al., 1990b). One important conclusion was that the primary effect of crowding was to increase the affinity of the holoenzyme–DNA assembly for gp45.

(*B*) *Analogy.* The first "argument by analogy" is based on the observation that gp45 is required for late transcription in T4 (Wu et al., 1975; Coppo et al., 1975), which appears to be a manifestation of a direct interaction between gp45 and T4-modified *E. coli* RNA polymerase (Ratner, 1974). Perhaps it is possible that the gp44/62 complex modifies T4 DNA polymerase in an unknown manner, which causes the postulated interaction between gp45 and gp43 to occur.

Previous work on the *E. coli* DNA polymerase III holoenzyme from the Bambara laboratory (Crute et al., 1983) demonstrated that very large amounts of β protein can bypass to some extent the requirement for ATP and other accessory proteins of the *E. coli* DNA polymerase III holoenzyme. Unpublished observations from our laboratory (M. K. Reddy, S. E. Weitzel, and P. H. von Hippel, in preparation) also suggest that under certain conditions the addition of large amounts of gp45 alone to defined primer-template substrates (30/50-mer) results in an increase in processivity of gp43,

both in its exonuclease and in its synthesis mode. This observed alteration of gp43 is independent of the addition of either ATP and/or gp44/62. Therefore, as suggested previously by O'Donnell and Studwell (1990), the β protein is very possibly an analogue to gp45.

The analogies between the T4 system and the *E. coli* system can be carried even further, in that the formation of the high-processivity *E. coli* DNA polymerase III holoenzyme is also dependent on the action of two accessory protein complexes. The γ-complex (composed of five different subunits) contains an ATPase active site and utilizes the hydrolysis of ATP to assemble the β protein onto primed DNA (O'Donnell, 1987; Maki & Kornberg, 1988). After assembly on DNA the β protein, which has been shown to be a dimer of identical subunits arranged in a toroidal shape (Kong et al., 1992), is capable of sliding along duplex DNA in an ATP-independent manner (Stukenberg et al., 1991). Kong et al. have also demonstrated that each monomer of the β dimer is composed of three domains with similar structure. Finally, their analysis of the amino acid sequences revealed that monomers of T4 gp45 and eukaryotic PCNA may each have two structural domains similar to the three in β.

These analogies further suggest that the hash marks observed in our cryoEM results (see discussion on cryoEM, above) may be composed of gp45 and that the function of the gp44/62 complex is to place gp45 on DNA at the primer-template junction. The sliding of the β protein on duplex DNA is also reminiscent of the injection–translocation mechanism proposed for the hash mark structures (Gogol et al., 1992).

Structure–Function Studies of the Holoenzyme. Recently the dynamics of protein–DNA contacts in the T4 holoenzyme have been investigated by incorporating photoactivatable nucleotide analogues into the primer strand of a primed M13 template (Capson et al., 1991). In this system, the position of the cross-linker can be varied by subsequent incubation with one, two, or three dNTPs, causing extension of the 3′ end of the primer to different defined extents. Two different length primers were used in this study to generate a wide variety of distances between the primer 3′ end and the cross-linking group; in one the cross-linking group was located four nucleotides upstream from the 3′ end, and in the other the cross-linker was 20 nucleotides upstream. Other primer–cross-linker distances were obtained by the addition of one, two, or three nucleotide residues to extend the primer strand.

In the absence of T4 polymerase, with the photoactivatable group situated four nucleotide residues from the primer end, the authors obtained results qualitatively similar to those described previously by Hockensmith et al. (1987), i.e., that gp62 was found to be most highly cross-linked to the primer strand. The efficiency of cross-linking of gp62 in this experiment was also found to be greater in the presence of ATP-γ-S than in the presence of ATP. Upon the addition of polymerase, gp62 was still found to be the most highly cross-linked of the accessory proteins.

In contrast, when the primer was extended to place the cross-linker 14 nucleotides upstream from the primer terminus, gp45 became the major cross-linked species, and very little cross-linking of gp62 was seen. This suggests that gp62 is situated near the 3′ end of the primer. Positioning the photoactivatable group 20 nucleotide residues upstream caused the cross-linking of gp45 to increase still more. Finally, when the cross-linker was placed 25 or 34 nucleotides upstream of the 3′ end of the primer, cross-linking of gp45 was greatly reduced.

These results place gp45 at the upstream end of the holoenzyme. Cross-linking was observed to gp44 only in the presence of ATP-γ-S. In these same experiments T4 polymerase was shown to cross-link relatively efficiently at position –4 (on the single-stranded side of the primer-template junction) but not at positions –9 or –14. Not surprisingly, this places the polymerase near the 3′-hydroxyl group of the primer. These cross-linking data are also consistent with previously determined site sizes for the polymerase activity (Newport et al., 1980; Dolejsi, 1988). Capson et al. (1991) also suggested that cross-linking of the polymerase requires ATP hydrolysis, since no cross-linking was seen in the presence of ATP-γ-S. They postulate that one consequence of ATP hydrolysis is a conformational change that brings about movement of the accessory protein complex, perhaps unmasking the 3′ end of the primer for polymerase binding.

These data define the DNA site size requirements of the holoenzyme on the double-stranded side of the primer-template junction at 20–25 base pairs, in good accord with earlier DNA footprinting results (Munn & Alberts, 1991b). These results are also consistent with a double-stranded DNA site size of ~20 base pairs determined for the accessory protein complex by Jarvis et al. (1989b) and by Munn and Alberts (1991a). The length of single-stranded DNA required near the primer-template junction has not been defined for either the accessory protein complex or the holoenzyme.

Role of ATP and Its Hydrolysis. Clearly, ATP hydrolysis is required for holoenzyme function; however, the exact role of ATP is still not known. Nevertheless, much has been learned through careful analysis of the stimulation of the processivity of the polymerase brought about by the accessory protein complex. Piperno and Alberts (1978) showed that the holoenzyme engaged in replication hydrolyzes less than one ATP molecule per ten nucleotides incorporated into the nascent DNA. This observation showed that the accessory protein complex need not hydrolyze an ATP molecule for every template base traversed by the holoenzyme but instead suggested that ATP hydrolysis might be required for the assembly of the accessory protein complex and the holoenzyme.

This outcome argues against a model in which the accessory protein complex acts as a "translocase", driving holoenzyme movement after each polymerization step in a mechanochemical process fueled by ATP. It is important to remember, however, that the energy requirements of such a translocase are not intuitively obvious and that there may exist mechanisms whereby ATP hydrolysis can drive holoenzyme translocation in steps of more than one template position at a time.

The periodic requirement for ATP hydrolysis in holoenzyme function led Newport et al. (1980) and Jarvis et al. (1991) to attempt to discriminate between the translocase model discussed above, in which ATP hydrolysis drives the movement of holoenzyme along the template, and the assembly model, in which ATP hydrolysis causes the assembly of a highly processive holoenzyme which must then be periodically reassembled. These workers reasoned that if the translocation model were correct, then processivity should be a function of ATP concentration. In fact, holoenzyme processivity is independent of ATP concentration for synthesis of both primed homopolymer (Newport et al., 1980) and primed, single-stranded, natural (Jarvis et al., 1991) templates, while primer usage increases with ATP concentration. These data provide evidence for a role for ATP in holoenzyme assembly but not in elongation.

Further evidence for the lack of involvement of ATP in elongation comes from studies of holoenzyme processivity in

the presence of the nonhydrolyzable ATP analogue ATP-γ-S. Huang et al. (1981) found that, although addition of ATP-γ-S to an elongating holoenzyme halted DNA synthesis eventually, synthesis continued for several seconds after such addition. Jarvis et al. (1991) also observed holoenzyme elongation with ATP-γ-S present and further concluded that ATP-γ-S had no effect on processivity when added after preincubation of the holoenzyme protein components with ATP in the putative holoenzyme assembly step. Both groups reported that the presence of ATP-γ-S during or before assembly of the complex completely suppresses the stimulation of the processivity of DNA polymerase by the accessory proteins (Huang et al., 1981; Jarvis et al., 1991).

Both of the above studies were performed during DNA synthesis on primed single-stranded templates. The situation may be more complex during strand-displacement synthesis. Alberts et al. (1980) found that addition of ATP-γ-S to strand-displacement synthesis reactions halted synthesis within 1 min, in contrast to the above results obtained using primed single-stranded DNA substrates, indicating that ATP hydrolysis is required more frequently when the holoenzyme is invading a leading double strand. Therefore, as has been previously observed (Alberts et al., 1980; Venkatesan & Nossal, 1982), it is necessary to interpret these results in the context of the DNA substrate used.

The above experiments place the hydrolysis of ATP at the very beginning of what may be termed a "processive cycle" on primed single-stranded DNA, a process that begins with the ATP-hydrolysis-dependent assembly of the T4 holoenzyme and ends when the hydrolysis of an additional ATP molecule is required. The early stages of this cycle seem clear in outline; holoenzyme components combine in an ATP-dependent reaction and begin synthesizing DNA in a processive fashion. Several models for this process have been proposed. The most popular of these are versions of the so-called "clocking" or "timing" mechanism first proposed by Newport (Newport, 1980; Newport et al., 1980) and refined by Jarvis et al. (1990, 1991). In this view, ATP hydrolysis produces an ADP and P_i-bound form of the holoenzyme that is conformationally different from the unbound or the ATP-bound form and is characterized by a slower dissociation rate from the DNA template. This form then slowly relaxes to a "fast-dissociating" form and is released from the DNA. In this model, the "clock" is started as soon as ATP is hydrolyzed and is independent of holoenzyme translocation. Pauses simply provide more time for the holoenzyme to dissociate and "use up time on the clock" (Jarvis et al., 1991). Further ATP hydrolysis reassembles the components for another round of processive synthesis.

A more complex version of the timing mechanism was postulated by Selick et al. (1987). In this model, the ATP-bound accessory protein complex binds to the primer-template junction, and the ATPase is activated. Holoenzyme is formed when DNA polymerase joins the complex, perhaps partially displacing the accessory protein subassembly from the 3′ end of the primer. Activation of the ATPase forms ADP and inorganic phosphate on the holoenzyme. This ADP/P_i-bound species is postulated to be the "sliding clamp" form of the accessory protein complex that results in the formation of a holoenzyme that is characterized by high processivity. This species is thought to be in a "high-energy" state and to be prone to decay at pause sites on the template. To prevent decay of the high-energy state, holoenzyme translocation as a consequence of DNA synthesis is proposed to provide the energy needed for its reactivation. The pausing of the holoenzyme then starts the "decay clock", since the high-energy state cannot be maintained in the absence of translocation. The sliding clamp holding the polymerase to the template DNA is then reactivated by the hydrolysis of a new ATP molecule.

An alternative proposal for the signal that ends the processivity cycle might be that dissociation of one or both of the products of ATP hydrolysis (ADP or P_i) could signal the dissociation of the holoenzyme from the DNA template. No data are yet available on ATPase product dissociation rates from the holoenzyme.

Another possibility is that the high-processivity state may correspond to a phosphorylated enzyme intermediate, characterized by a hydrolysis rate that governs the processivity. To date, no phosphorylated holoenzyme protein has been discovered (Piperno & Alberts, 1978; Huang et al., 1981). However, the steady-state concentration of such an intermediate might be too low to detect by the methods employed to date.

OVERVIEW AND FUTURE DIRECTIONS

DNA Polymerase Holoenzyme. As discussed above, a major reason for the existence of the DNA polymerase holoenzyme may be to increase the efficiency of DNA synthesis by increasing the processivity characteristic of the isolated polymerase under physiological conditions. The emergent picture of holoenzyme assembly and function on a natural primer-template DNA is presented in Figure 4. Components of the accessory protein complex (the gp44/62 4:1 complex and the gp45 trimer) assemble on the primer-template upon binding ATP. Since gp44 carries the ATP binding site, and a gp44/62 complex has four gp44 subunits, it is possible that as many as four ATP molecules could be bound per gp44/62 complex. After assembly of the accessory protein complex on the primer-template, ATP hydrolysis occurs, causing some sort of conformational change. The cross-linking results of Capson et al. (1991) suggest that this reaction then allows the T4 DNA polymerase to bind, perhaps by movement of the accessory protein complex away from the 3′ end of the primer. However, the exact order of these events is unclear. With the addition of DNA polymerase, holoenzyme assembly is complete and DNA processive synthesis begins. After ~10 s (though this time may be much longer in vivo), there is a further requirement for ATP binding and hydrolysis, presumably reflecting the fact that the holoenzyme has dissociated from the template DNA and that the processivity cycle must be initiated again (Jarvis et al., 1991). The precise role of gp32 in the assembly of the holoenzyme is also not known, but it seems clear that one of its major functions is to make single-stranded DNA binding sites at the replication fork inaccessible to the other protein components of the holoenzyme. Beyond that, the evidence already discussed suggests that at least one gp32 molecule interacts directly with the polymerase.

The above scheme is based on data reviewed in this Perspectives and is less than complete. A complicating factor is the fact that all the partial reactions described here, including the binding of the accessory protein complex, the binding of polymerase, the binding of gp32, and even ATP hydrolysis, can proceed partially or completely independent of the other events. This makes the T4 replication a wonderful system for the reductionist molecular biology and biochemistry but also makes the delineation of a unique assembly pathway difficult. Therefore, at present, the order of events in holoenzyme assembly is not precisely known, particularly in the early stages of the reaction.

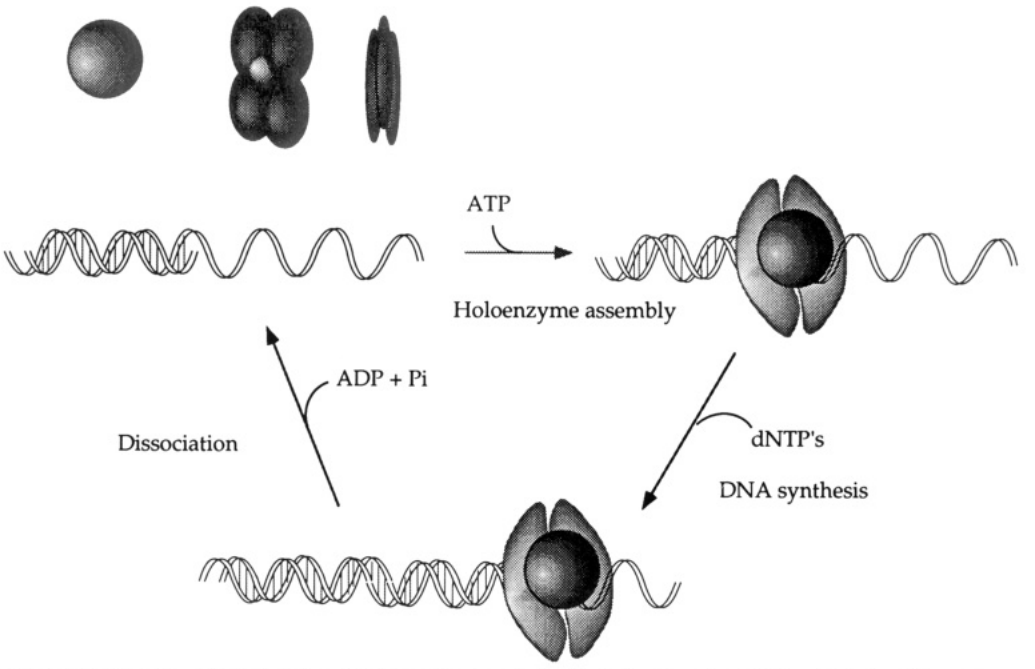


FIGURE 4: Processive cycle of T4 DNA polymerase holoenzyme on a primed single-stranded DNA substrate. The sphere represents DNA polymerase; other symbols are as given in Figure 3. ATP binding and hydrolysis are used to assemble a DNA polymerase holoenzyme that processively synthesizes DNA. After a certain number of base residues have been incorporated, a dissociation event begins the cycle anew. The release of ATP hydrolysis products is placed at the dissociation step to indicate that product release must occur to complete cycle. The exact placement of these reactions is not known.

The success of pre-steady-state kinetic investigations in sorting out mechanistic questions of this kind in single-enzyme systems (Carrol & Benkovic, 1990; Anderson & Johnson, 1990) suggests that this technique may be useful for problems in the holoenzyme system. Therefore, we, and others, have begun to study the pre-steady-state kinetics of T4 holoenzyme assembly and function (T. Capson and M. Young, unpublished results). Experiments using these techniques are likely to folllow two directions. One attractive area is the study of the effect of holoenzyme formation on the kinetics of the polymerase. On the basis of the emerging data that are being gathered by this technique on the T4 DNA polymerase (Carroll & Benkovic, 1990; Capson et al., 1992), the kinetic consequences of its incorporation into the holoenzyme will provide information on the mechanism of processivity stimulation by the accessory protein complex and permit tests of the sliding clamp hypothesis.

The second area that will be pursued by these means is the molecular mechanism of the ATPase of the accessory protein complex studied in isolation and as a part of the holoenzyme. Virtually no pre-steady-state information on the mechanism of ATP hydrolysis by the accessory protein complex is currently available. In order to understand how ATP hydrolysis is coupled to holoenzyme assembly and how the length of the processivity cycle is determined, rate constants for events in the ATPase cycle must be measured. Such information will help in evaluating the various models for the role of ATP hydrolysis in determining the lifetime of the DNA-bound holoenzyme.

Leading and Lagging Strands: Approaches to the Seven-Protein System. Two T4 DNA replication proteins that we have not discussed, the T4 replicative helicase (gp41) and the T4 primase (gp61), complete the T4 DNA replication complex. From the point of view of the holoenzyme, the helicase appears to increase polymerization rates during strand-displacement synthesis and greatly reduces the requirement for gp32 in this reaction (Cha & Alberts, 1989). The primase, however, injects a qualitatively new ingredient into the system: the ability to perform discontinuous DNA synthesis on the displaced, or lagging, strand (Liu et al., 1978; Nossal, 1980) by elongating from RNA primers spaced approximately 1200 nucleotides apart along the lagging-strand template. An apparent conundrum exists, since discontinous strand DNA synthesis necessitates periodic DNA polymerase dissociation, while continuous, or leading-strand, synthesis is most efficient in the limit of no polymerase dissociation. This conundrum is resolved in the *E. coli* DNA replication system by the existence of an apparently asymmetric holoenzyme dimer, the monomers of which may have different processivities (Johanson & McHenry, 1984; McHenry, 1991; Maki & Kornberg, 1988). There is no evidence for such a species in the T4 system, although the presence of two polymerase molecules at the T4 replication fork has been hypothesized (Alberts et al., 1975; Sinha et al., 1980).

Consideration of this question has lead us to believe that, although some studies using more reasonable forked models have been performed (Cha & Alberts, 1989, 1990; Richardson et al., 1990), to date, most studies of holoenzyme function should be thought of as models for lagging-strand DNA synthesis, rather than as models for leading-strand synthesis. This argument is based on two main lines of reasoning. First, the primed, single-stranded DNA structures used for most processivity studies resemble lagging-strand DNA more closely than the forked "leading–lagging junction" that moves ahead of the leading-strand holoenzyme. In addition, calculation of the minimum time necessary to replicate the T4 genome,[2] using the holoenzyme lifetime of <10 s determined by Jarvis et al. (1991), yields a value of 34 min, which is longer than the latent period of wild-type T4 phage. This suggests that a further processivity increase is needed, at least for the leading-strand polymerase. On the lagging strand, however, dissociation of the holoenzyme is required from time to time. Furthermore, the length of newly synthesized DNA produced during the suggested holoenzyme lifetime of <10 s is consistent with the median length of T4 Okazaki fragments in vivo

[~1200 bases; see Alberts et al. (1983) and Selick et al. (1987)].

Interaction of the leading-strand holoenzyme with T4 helicase may provide the necessary increase in processivity. Support for this idea was recently obtained by Richardson and Nossal (1989). Their studies with proteolytic fragments of the helicase indicate a direct protein–protein interaction between the protein and elements of the T4 leading-strand holoenzyme. This combination could result in an asymmetry between the leading and lagging holoenzymes analogous to that found in the *E. coli* system. Further studies of the strand-displacement synthesis reaction are needed to elucidate the leading-strand holoenzyme model.

The T4 DNA replication system offers unique opportunities for the study of replication mechanisms. Because of the apparently loose interactions between most of its components, it has been possible to examine in depth the properties of each separate part. In this sense, the T4 system has been the "reductionists' dream". The reconstructionists' task is initially to explain the action of partially reconstituted systems in terms of the structure and function of the separate components and finally to integrate the knowledge gained into an explanation of the entire system. Work from many laboratories has progressed to a point roughly midway in a major, although partial, mechanistic reconstitution effort of the apparatus responsible for the elongation phase of T4 DNA replication. Further investigation should lead to an increasingly complete understanding of this elegant and fascinating "protein machine" (Alberts, 1984).

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