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Analysis of the bacteriophage T4 DNA polymerase holoenzyme and Dda helicase

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Analysis of the Bacteriophage T4 DNA Polymerase Holoenzyme and Dda Helicase

by

Kevin J. Hacker

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Biophysics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

To my parents and my great aunt Ruth Craig

Acknowledgements

I would like to express my gratitude to Bruce Alberts for his advise and patience as I developed as a scientist.

I wish to thank the Alberts' laboratory for their support and friendship. I would especially like to thank Jack Barry for teaching me protein purification, and Barry Selick for teaching me DNA cloning techniques and phage biology. I also would especially like to thank Bin Liu for lively discussions and critical reading of chapters 4 and 5, and Karen Oegema for moral support, deriving kinetic equations, and critical reading of chapters 4 and 5. Finally, I would especially like to thank Chris Field for looking out for me and managing the laboratory so efficiently and fairly.

Acknowledgements of co-author contributions to published material

I am writing in reference to Kevin Hacker's dissertation to clarify what part of the work was done by the student.

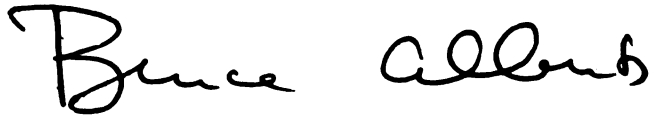
Chapter 1: Kevin Hacker and Bruce Alberts. 1992. Overexpression, purification, sequence analysis, and characterization of the T4 bacteriophage dda DNA helicase. J. Biol. Chem. 267: 20674-20681.

The work reported in this chapter was performed by Kevin, and the paper was written by himself. I acted as the advisor.

Chapter 2: P. Gauss, K. Park, T. E. Spencer, and K. Hacker. 1994. DNA helicase requirements for DNA replication during bacteriophage T4 infection. J. Bact. 176, in press.

This chapter was written entirely by Kevin. The dda deletion mutant was generated by Kevin. Peter Gauss and colleagues used the dda deletion mutant to generate the double dda59 mutant; they also assayed for DNA synthesis after infection.

Bruce Alberts



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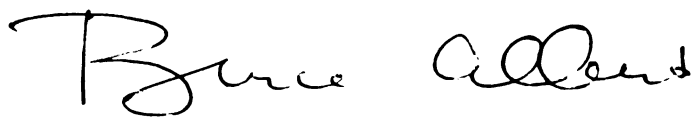
ANALYSIS OF THE BACTERIOPHAGE T4 DNA POLYMERASE HOLOENZYME AND DDA HELICASE
by Kevin Hacker

ABSTRACT

Two questions are addressed: 1) What is the role of the bacteriophage T4 Dda DNA helicase during T4 infection? 2) What is the mechansim that enables the T4 DNA polymerase holoenzyme to synthesize DNA processively on the leading strand of the replication fork for many minutes, while allowing the an identical holoenzyme on the lagging strand to recycle from finishing one Okazaki fragment to beginning the next in a few seconds?

The Dda protein is a 5'-3' DNA helicase that has been found to stimulate DNA replication and recombination reactions *in vitro.* Herein, I report the overexpression, purification, sequence analysis of the Dda protein. In addition, the T4 insertion-substitution system was used to create a deletion in the T4 *dda* gene. The deletion phage showed a delay in DNA synthesis during early times of infection, suggesting that the Dda protein is involved in the initiation of origin-dependent DNA synthesis. The gene 59 protein has been shown to load the gene 41 helicase onto DNA *in vitro* and to be important for recombination-dependent DNA synthesis *in vivo.* No DNA was synthesized during infection by double dda59 mutants. This suggests that the Dda protein is important for starting DNA replication *in vivo,* but that the gene 59-41 complex can partially substitute for it in this role.

To address the second question, I developed assays to monitor the dissociation of the T4 DNA polymerase holoenzyme—both when it is stalled by nucleotide omission and when it is stalled at a helical region of the DNA template. The dissociation of the holoenzyme stalled by nucleotide omission is a first order decay process with a half-life of 2.5 min. This half-life resembles that expected for the holoenzyme processively synthesizing DNA on the leading strand of the replication fork. In contrast, when the holoenzyme is stalled at a helical region, it dissociates with a half-life of 1 sec. From this data, a duplex DNA sensor model is presented, which explains the dissimilar behavior of the identical holoenzymes on the leading and lagging strands of the replication fork.



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CHAPTER ONE

INTRODUCTION

This thesis is divided into two parts. The first part (chapters 2 and 3) examines the properties of the T4 dda DNA helicase and the role of this helicase during T4 infection. The second part (chapters 4 and 5) investigates the dissociation of the T4 DNA polymerase holoenzyme from the DNA template. In the second part, I specifically wanted to answer the question: what is the mechanism that enables the holoenzyme to remain on the template for many minutes when synthesizing DNA, while allowing rapid dissociation at the end of each Okazaki fragment?

Characterization of the T4 Dda helicase:

The Dda DNA helicase -one of the two distinct DNA helicases encoded by the T4 phage- has been found to be important for DNA replication and recombination reactions *in vitro*. The Dda protein was originally identified and isolated from protein lysates of T4 infected cells by monitoring its high DNA-dependent ATPase activity(Behme and Ebisuzaki, 1975; Debreceni, et al., 1970). The Dda protein moves distributively (continuously dissociating and reassociating from the DNA) in the 5'-3' direction along single-stranded DNA(Jongeneel, et al., 1984). It is the T4 gene 41 DNA helicase that interacts with the gene 61 primase to form the primosome, which makes RNA primers for Okazaki fragment synthesis, and normally moves the replication fork. However, in absence of the gene 41 helicase, Dda protein stimulates the rate of DNA strand-displacement DNA synthesis *in vitro* (Jongeneel, et al., 1984). In addition, the Dda helicase stimulates the rate of T4 uvs-X (a recA analog) catalyzed DNA branch migration

4-fold *in vitro*(Kodadek and Alberts, 1987). The Dda protein binds tightly to the gene 32 protein and is retained when T4 infected cell lysates are passed over a T4 uvs-X protein agarose column(Formosa and Alberts, 1984; Jongeneel, et al., 1984).

In chapter 2, I report the overexpression, purification, and sequence analysis of the dda helicase. These studies were initiated so that the dda helicase could be purified in large quantities more easily than can be obtained from T4 infected cells lysates. Larger quantities were desired for protein affinity-chromatography studies. Affinity-chromatography studies have been very useful in isolating important T4 DNA replication and recombination proteins and in identifying their protein-protein interactions (Formosa and Alberts, 1984; Formosa, et al., 1983). Identifying the proteins that interact with the dda protein should aid in understanding the role of the dda protein during T4 infection. Identifying proteins in addition to the gene 32 protein and the UvsX protein that interact with the dda protein was of particular interest because of the dda protein's suggested role in origin-dependent initiation of DNA replication(Kreuzer and Morrical, 1994); I reasoned that by virtue of their affinity with the dda protein, perhaps these unidentified proteins could be isolated.

Unfortunately, while the overexpression of the dda protein did allow us to simplify its purification, the protein was completely inactivated when it was coupled to a agarose matrix as judged by its loss of affinity for the gene 32 protein. However, as a new finding the overexpressed and purified dda protein was passed directly over a uvsX column and the retention of the dda protein by the column

confirmed that the two proteins - previously shown to interact in a crude extract - in fact interact directly.

What is the role of the dda helicase *in vivo*? Whereas the T4 gene 41 helicase is essential for DNA synthesis during T4 infection, which is consistant with its biochemical properties *in vitro*, the dda protein is nonessential(Little, 1973). *In vitro*, the Dda protein is important in DNA recombination reactions, but no UV sensitivity or defects in recombination have been detected in *dda* mutants (Behme and Ebisuzaki, 1975). Mutant *dda* phage show a delay in DNA synthesis at early times of infection, but near normal amounts of DNA are eventually produced during later times (Little, 1973). Since initiation of DNA replication is predominantly replication origin-dependent at early times of infection and predominantly recombination-dependent at later times, the dda mutant phenotype suggests that the Dda protein plays a role initiation of DNA synthesis at origins (Kreuzer and Morrical, 1994).

Unfortunately, the *dda* mutant phage strains used in those studies carried either extensive deletions that also removed genes flanking the *dda* gene or contained mutations in additional genes (Little, 1973). As a result, when I began my work it was uncertain that the phenotype of these phage was due only to a *dda* deficiency.

These same strains had been used to construct a *dda59* double mutant. No DNA synthesis was observed during infections with the double mutants (Doherty and Gauss, 1982; Gauss, et al., 1983). Phage carrying only a T4 gene *59* mutation synthesize DNA at early times of infection, but DNA synthesis arrests at late times of infection (Cunningham and Berger, 1977; Gauss, et al., 1983; Wu and Yeh,

1972) . Biochemical characterization of the gene 59 protein has shown that it loads the T4 gene 41 helicase onto single-stranded DNA covered with gene 32 protein (Jack Barry and Bruce Alberts, manuscript submitted).

In **chapter 3**, T4 insertion-substitution system (Selick, et al., 1988) was used to create a deletion in the dda gene. This allowed me to analyze the phenotype of dda defective phage free of other mutations. From this dda defective phage, strains carrying mutations in both the *dda* and *59* genes were constructed and analyzed. Like the previously characterized *dda* mutant phage, the *dda* deletion phage showed a delay in T4 DNA synthesis at early times of infection, suggesting that the Dda protein plays a role in the initiation of DNA replication at origins. In addition, a double *dda59* mutant constructed from the dda deletion mutant showed no detectable DNA synthesis, suggesting that the dda helicase plays an important role in DNA replication and that its its function can be partially replaced by the gene59-41 protein complex.

Mechanism of T4 DNA polymerase holoenzyme dissociation from the DNA:

The five-protein T4 DNA polymerase holoenzyme contains an accessory protein with DNA-dependent ATPase activity (the T4 gene 44/62 protein complex) that is stimulated by an additional accessory protein (T4 gene 45 protein) (O'Donnell, et al., 1992; Young, et al., 1992). The DNA polymerase accessory proteins form a sliding clamp in a reaction requiring ATP hydrolysis that greatly increases the

processivity of the T4 gene 43 DNA polymerase (reviewed by (Alberts, 1987; Nossal, 1992; Young, et al., 1992).

In vitro studies have shown that both the leading and lagging strand DNA polymerase holoenzyme molecules remain bound to the fork at all times (Alberts, et al., 1983). To accomplish this, the leading strand holoenzyme must remain associated with the template, while the lagging strand holoenzyme functions in a less processive and more dynamic manner. In vivo, to the achieve the observed replication fork rates of 500 nucleotides/sec at the low nanomolar concentrations of DNA polymerase holoenzyme molecules present, the leading-strand polymerase must remain associated with the template for minutes before dissociating (McCarthy, et al., 1976; Young, et al., 1992). In contrast, on the lagging-strand of the replication fork, the holoenzyme must rapidly dissociate from the template within one to two seconds after it stops at the end of a previously synthesized Okazaki fragment and then reassociate with a newly synthesized primer to begin synthesis of the next Okazaki fragment (Selick, et al., 1987). In other replication systems, these requirements may be fulfilled in part by having structurally different holoenzymes on the leading and the lagging strands (Kornberg and Baker, 1991). However, in the T4 replication system, the holoenzymes on both strands of the replication fork seem to be composed of the same T4 gene 43, 45, and 44/62 polypeptides. What mechanism enables the holoenzyme to switch from highly processive DNA synthesis to rapid dissociation when it stops at the end of a previously synthesized Okazaki fragment?

One potential mechanism, originally put forward by Munn and Alberts, arose from their footprinting studies of the DNA polymerase and accessory proteins at a primer-template junction. Their "clock" model suggested a timing based mechanism for holoenzyme dissociation. Munn and Alberts could only detect the accessory protein complex at the primer-template junction in the presence of the nonhydrolyzable ATP analog, ATPγS (Munn and Alberts, 1991). Instead of facilitating the association of the T4 DNA polymerase with the primer-template junction, however, the accessory protein-ATPγS complex blocked the subsequent association of the polymerase (Munn and Alberts, 1991). In the presence of ATP, neither an accessory protein complex nor increased binding of the polymerase to the primer-template junction could be detected, even at protein concentrations in large excess of those necessary for maximal DNA synthesis by the holoenzyme. Since it is not possible to footprint a moving complex, nucleotide omission was used to stall the DNA polymerase during the footprinting experiments. Munn and Alberts proposed a timing based mechanism to explain the instability of the stalled holoenzyme. They suggested that ATP hydrolysis places the accessory proteins in a high energy state, which binds tightly to the template. In the presence of the DNA polymerase, the accessory proteins associate with the DNA polymerase to form the holoenzyme and the translocation of the polymerase manintains the high energy state of the accessory proteins. In the absence of the DNA polymerase, or when the polymerase stops moving, the accessory proteins rapidly decay through a series of states to a weak DNA binding ground state and dissociate from the template. The

dissociation of the accessory proteins then allows the polymerase itself to dissociate. The dissociation would not, therefore, follow first order kinetics; the decay would, instead, generate a lag-time between pausing of the holoenzyme and its dissociation. In principle, this would allow the leading strand holoenzyme to remain tightly associated even during transient pauses that might occur during replication while allowing the lagging-strand holoenzyme to rapidly dissociate after pausing for some small threshold time at after encountering the end of a previously synthesized Okazaki fragment (Munn and Alberts 1991).

In chapter 4, I report the development of an assay to measure the kinetics of dissociation of a holoenzyme stalled by nucleotide omission to test the major prediction of the clock model that rapid dissociation of a stalled holoenzyme from the DNA occurs only after a determined threshold time. The holoenzyme was allowed to assemble and incorporate 8 nucleotides before it was paused at a site consisiting of three consective nucleotides by the omission of dCTP. A free polymerase trap consisting of unlabelled excess DNA was added to prevent the association of another polymerase molecule with the labeled primer-template after the first molecule dissociates. After varied amounts of time, dCTP was added to allow the template-associated holoenzymes to continue DNA synthesis.

The dissociation of the holoenzyme was found to follow a first order decay and did not show a time-lag as predicted by the clock model. The half-life of the holoenzyme-DNA complex was 2.5 min, which is in the range expected for the leading-strand polymerase,

but much too long to allow recycling of the polymerase holoenzyme on the lagging-strand of the replication fork(Selick, et al., 1987; Young, et al., 1992).

The above assay has also allowed me to perform a related study of the effects of DNA template linearization on T4 DNA polymerase holoenzyme dissociation. Previous work on the DNA polymerase holoenzyme suggested that a component of the T4 holoenzyme, the gene 45 protein, forms a ring like structure around the DNA template similar to that formed by the β–subunit of the *E. coli* DNA polymerase holoenzyme (Gogol, et al., 1992; Kong, et al., 1992). Studies of the β–subunit showed that linearization of a circular DNA template greatly destabilizes the β–subunit-DNA complex; the likely explanation is that the β–subunit can slide off the end of a DNA molecule (Stukenberg, et al., 1991).

Like the results obtained with the β–subunit of the *E. coli* DNA polymerase holoenzyme, the rate of holoenzyme dissociation was greatly increased when the circular DNA template was converted to a linear form.

In **chapter 5,** I report a experimental system that measures the dissociation of the holoenzyme from the DNA under conditions that closely mimic an encounter of a holoenzyme with the end of a previously synthesized Okazaki fragment on the lagging-strand of the replication fork. In this assay, the holoenzyme is assembled in the absence of dCTP; then dCTP is added to allow the holoenzyme to encounter a hairpin helix where the holoenzyme stalls. After varying lengths of time, excess gene 32 single-stranded DNA binding protein

is added to melt the hairpin, thereby enabling associated holoenzymes to continue DNA synthesis.

This assay was developed after the rate dissociation of a holoenzyme stalled by nucleotide omission was determined to be much slower than that required for Okazaki fragment synthesis on the lagging-strand of the replication fork. The first assay aided greatly in the development of the new assay; the salt conditions, reaction temperature, and free DNA polymerase trap are the same for both assays. In addition the template used was a derivative of the M13mp7 template used in the first assay. The template was select for use in this new assay for the following reasons: 1) The template is circular. The linearization experiments mention above showed that the holoenzyme is not stable on a linear DNA template; thus, oligonucleotide templates were not used. 2) The template has a DNA hairpin helix that stops the movement of the DNA polymerase holoenzyme. A hairpin offers several advantages over an actual end of an Okazaki fragment or an oligonucleotide annealed to single-stranded DNA. The template containing the hairpin helix was easy to obtain since it was the product of M13 infection. Moreover, unlike annealing an oligonucleotide to a single-stranded DNA, which is not totally efficient (especially when small oligonucleotides are used), 100% of the DNA template molecules contained the hairpin. Thus the problem of holoenzymes synthesizing DNA when they were supposed to be stopped was avoided. 3) The hairpin block was rapidly melted by the addition of excess gene 32 protein.

In the assay, after encountering the hairpin, the holoenzyme rapidly dissociated following the kinetics of a first order reaction

with a half-life of 1 sec. The 1 sec half-life is similar to that required for polymerase recycling during Okazaki fragment synthesis in vivo (Selick, et al., 1987), and is much shorter than the half-life of holoenzyme dissociation when the holoenzyme is stalled by nucleotide omission. Thus, the holoenzyme must be able to sense hitting duplex DNA and switch to a state that rapidly dissociates.

In chapter 6, the thesis concludes with a reflection on the major discoveries that have occured in the field of DNA replication since my studies where initiated eight years ago; also discussed are the changes that my research has brought about in the way that we view the T4 DNA replication apparatus.

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CHAPTER TWO

OVEREXPRESSION, PURIFICATION, SEQUENCE ANALYSIS, AND CHARACTERIZATION OF THE T4 BACTERIOPHAGE DDA DNA HELICASE

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Overexpression, Purification, Sequence Analysis, and Characterization of the T4 Bacteriophage dda DNA Helicase*

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The bacteriophage T4 dda protein is a 5′-3′ DNA helicase that stimulates DNA replication and recombination reactions *in vitro* and seems to play a role in the initiation of T4 DNA replication *in vivo*. Oligonucleotide probes based on NH_2-terminal amino acid sequence were used to precisely map the location of the dda gene on the T4 chromosome. Using polymerase chain reaction techniques, the *dda* gene was then cloned into an expression vector, and the overproduced protein was purified in two chromatography steps. Both the genomic and cloned *dda* genes were sequenced and found to be identical, encoding a protein of 439 amino acids. The dda protein contains amino acid sequences resembling those of other known helicases, and is most homologous to the *Escherichia coli* recD protein. Protein affinity chromatography was used to show a direct interaction between the dda protein and the T4 uvsX protein (a rec A-type DNA recombinase).

Helicases have been isolated from a wide variety of eukaryotic and prokaryotic cells (reviewed by Matson and Kaiser-Rogers, 1990). Exact physiological roles have yet to be determined for many of these enzymes. However, they vary in a broad range of biochemical properties, including substrate unwound (RNA or DNA helices), direction of strand movement (5′ to 3′ or 3′ to 5′), and nucleotide cofactor hydrolyzed, reflecting their variety of functions inside the cell.

Bacteriophage T4 has been found to encode all of its own replication proteins. These include two distinct DNA helicases, the gene 41 protein and the dda protein, which appear to be important in both DNA replication and recombination reactions (Krell *et al.*, 1979; Alberts *et al.*, 1980; Jongeneel *et al.*, 1984b; Kodadek and Alberts, 1987). The gene 41 protein, which is essential for T4 DNA replication, is a highly processive DNA helicase that moves along a single-stranded DNA template in the 5′ to 3′ direction (*i.e.* along the lagging strand of a replication fork) (Liu and Alberts, 1981a; Venkatesan *et al.*, 1982). The gene 61 protein (the DNA primase that makes the RNA primers for Okazaki fragment synthesis) and the gene 41 protein interact to form the T4 primosome (Liu and Alberts, 1980, 1981b; Nossal, 1980).

The dda protein was originally isolated as a DNA-dependent ATPase by Ebisuzaki and co-workers (Debreceni *et al.*, 1970; Behme and Ebisuzaki, 1975). Mutant dda⁻ phage show a substantial delay in DNA synthesis, but because near normal amounts of DNA are eventually produced phage burst size is reduced only slightly (Little, 1973).[1] No UV sensitivity or defects in recombination have been detected in dda mutant infections (Behme and Ebisuzaki, 1975).

The dda protein and the gene 41 protein share some properties at a biochemical level. Both DNA helicases run in the 5′ to 3′ direction along single-stranded DNA. In the absence of the gene 41 protein, the dda protein stimulates the rate of DNA strand-displacement DNA synthesis at an *in vitro* replication fork (Jongeneel *et al.*, 1984b). Since no increase in this rate is observed when the dda protein is added to reactions that have been stimulated by the gene 41 protein, the two DNA helicases do not appear to act synergistically at the fork (Jongeneel *et al.*, 1984b).

The dda protein differs from the gene 41 protein in acting distributively (continuously dissociating and reassociating with the DNA molecule being unwound) rather than processively (Jongeneel *et al.*, 1984a). In addition, the dda helicase does not form a primosome with the 61 protein. Unlike the 41 protein, the dda protein binds tightly to the T4 gene 32 protein (helix-destabilizing or single-stranded DNA-binding protein), and it is retained when T4 infected cell lysates are passed over a uvsX protein agarose column (uvsX is a recA analog with a central role in T4 genetic recombination) (Jongeneel *et al.*, 1984a; Formosa and Alberts, 1984). A role in recombination is further suggested by dda protein's 4-fold acceleration of the rate of uvsX protein-catalyzed DNA branch migration in *in vitro* reactions (Kodadek and Alberts, 1987).

In general, one suspects that the two T4 DNA helicases can partially substitute for each other for some of the helicase functions inside the T4 bacteriophage-infected cell. Evidence for this assertion comes from studies on the T4 gene 59 protein. A T4 gene 59 amber mutant alone on a nonsuppressing strain shows normal DNA synthesis early in infection, followed by DNA synthesis arrest at late times of infection (Cunningham and Berger, 1977). However, if the phage is also dda⁻, almost no DNA is made.[2] Thus, without the 59 gene product, the dda protein is essential for any phage growth. Recent biochemical characterization of the gene 59 protein has shown that it loads the T4 gene 41 helicase onto single-stranded DNA.[3] Thus, the combined biochemical and genetic data suggest that the dda protein plays an important role in DNA metabolism *in vivo* but that its function can be partly

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M93048.

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[1] P. Gauss, personal communication.
[2] P. Gauss, unpublished observation.
[3] J. Barry, personal communication.

replaced with that of the gene 59–41 protein complex.

In order to further characterize the dda helicase and its interaction with other proteins, we precisely mapped its location within the T4 chromosome, sequenced and cloned the *dda* gene, and overexpressed and purified the dda protein. In addition, the purified dda protein was chromatographed on a uvsX protein affinity column to test for a direct uvsX-dda protein interaction.

MATERIALS AND METHODS

Reagents and Enzymes—All restriction and DNA modifying enzymes (including the Taq DNA polymerase) were purchased from New England Biolabs unless otherwise noted. Polynucleotide kinase and dideoxyribonucleoside triphosphates were obtained from Pharmacia LKB Biotechnology Inc., avian myeloblastosis virus reverse transcriptase from Life Sciences (St. Petersburg, FL). Sequenase was from United States Biochemical Corp., ampicillin from Roerig/Pfizer, lysozyme from Worthington, formamide from Fluka, and agarose from FMC Bioproducts. Dimethyl sulfate, piperidine, and hydrazine were from Aldrich, formic acid from Fisher Scientific, and [γ-^{32}P]ATP from Amersham Corp. The sequence of the 25 NH_2-terminal amino acids of the dda protein that we purified from T4-infected cells by published procedures (Jongeneel *et al.*, 1984a) was determined by Ken Williams (Yale University). Oligonucleotide primers were synthesized by the Biomolecular Resource Center at the University of California, San Francisco. The uvsX protein affinity column was prepared by Scott Morrical in this laboratory.

Plasmids—The plasmid vector pTL18xwd was obtained from Dr. T.-C. Lin (Yale University). This vector contains the large *Eco*RI-*Bam*HI fragment from the pBR322 derivative, pUC19, ligated to the *Eco*RI-*Bam*HI fragment from a pGW7 derivative that contains the λ late promoter control region carrying the gene encoding the repressor cI^{857} (Lin *et al.*, 1987). The λ-DNA has the rexA and rexB genes (map position 37,000–36,110) deleted, since they inhibit T4 infection when contained on a multicopy plasmid (Shinedling *et al.*, 1987). The plasmids Bluescript M13$^+$ and M13$^-$, which contain the M13 bacteriophage origin, were purchased from Stratagene.

Bacteria and Phage—The *E. coli* strain SG934 (*F-lac$^-$ trp$^-$ pho$^-$ sup^0 rpsL mal$^-$ tsX:3tn10 htpr^{am}*) was kindly provided by Susan Masters (University of California, San Francisco). The *Escherichia coli* strain TAB32-4 (B_E *hsd M_s^+ hsd R_shsdS_s mal$^+$(lambda^s) rg(thi) tabUa2 tabUb2*) (Nelson and Gold, 1982)) and T4 *ts75sud1* double-mutant phage (gene 32 temperature-sensitive, *sud* deletion) were provided by Peter Gauss (Western State College of Colorado). *E. coli* strain DG98 (F factor-containing) was obtained from Charles Craik (University of California, San Francisco).

Isolation of DNA and RNA—T4 cytosine-containing DNA (T4cDNA)[4] was obtained from laboratory stocks prepared according the Pribnow method (Pribnow *et al.*, 1981). RNA from T4 phage-infected *E. coli* B_E cells was prepared as described by McPheeters *et al.* (1986), with the modifications of Selick *et al.*[5]

Chemical Sequencing of DNA—To obtain the T4 *ClaI-HindIII* DNA fragment (map position 10.608–10.295) for sequencing, T4cDNA was digested with *Eco*RV. The DNA fragments were separated by electrophoresis on a 0.8% agarose, 10 mM Tris-borate, pH 7.4, 1 mM Na_2EDTA (TBE) gel. The 3.6-kb DNA fragment (map position 7.3–10.78) was cut out of the gel, and the DNA was recovered by electroelution onto a dialysis membrane followed by phenol extraction and ethanol precipitation. To purify the DNA from contaminants that inhibit the calf intestinal phosphatase, the DNA was purified on a NAC column (Bethesda Research Laboratories), following the manufacturer's instructions. The *Eco*RV fragment was then digested with *Hin*dIII and the 5′ phosphates removed with calf intestinal phosphatase. The phosphatase was inactivated by SDS and phenol treatments, and the 5′ ends of the DNA fragment were then labeled with ^{32}P by standard methods (Maniatis *et al.*, 1982). The labeled fragments were digested with *ClaI* and separated by electrophoresis on a 1.7% low temperature gelling agarose, 10 mM Tris-acetate, pH 7.4, 1 mM Na_2EDTA (TAE) gel. The DNA was recovered by melting the agarose at 65 °C, followed by phenol extraction and ethanol precipitation. The 300-base pair *ClaI-HindIII* fragment, labeled at its *HindIII* end with ^{32}P, was then sequenced according to the Maxam-Gilbert method (Maxam and Gilbert, 1980).

Enzymatic Sequencing of T4 RNA and DNA—Sequencing from RNA templates by primer extension with avian myeloblastosis virus reverse transcriptase and termination by dideoxyribonucleoside triphosphates was performed by the method of Inoue and Cech (1985) as modified by McPheeters *et al.* (1986) and by Selick *et al.*[5]

Sequencing from DNA templates was carried out as described previously for RNA templates,[5] with the following changes: 1) 56 fmol of T4cDNA (as molecules) was used per 12 μl of reaction; 2) the molar ratio of labeled primer to T4cDNA was 30:1; 3) T4cDNA and primer were heated for 3 min at 90 °C and quickly chilled in a dry-ice/ethanol bath just prior to the enzymatic reaction.

Southern Hybridization—The 0.7-μg samples of T4cDNA were digested individually with 64 units of either *ClaI*, *Eco*RV, *Eco*RI, *HindIII*, *NdeI*, or *XbaI*. After electrophoresis on a 0.75% agarose/TBE gel, Southern transfer and hybridization were performed as described in Maniatis *et al.* (1982).

Sequencing of Plasmid DNA—Plasmid pKHdda was digested with *Bam*HI and *SalI*. The fragments were separated by electrophoresis on a 1% low temperature gelling agarose/TAE gel and the 1.3-kb *Bam*HI-*SalI* fragment containing the *dda* gene was recovered by melting the agarose at 65 °C followed by phenol extraction. The DNA was ethanol precipitated, resuspended in digest buffer, and cut with *HindIII*. The 165-base pair *Bam*HI-*HindIII* and 1.1-kb *HindIII-SalI* fragments were ligated into separate Bluescript M13 origin-containing plasmids. After the constructs were transformed into *E. coli* DG98 cells, ssDNA was isolated and sequenced using the Sequenase protocol based on the Sanger method (Sanger *et al.*, 1977).

Amplification and Modification of the dda Gene by Exploiting the Polymerase Chain Reaction (PCR)—The PCR amplification reaction was based on the protocol of Kogan *et al.* (1987). Reactions were performed in 1.5-ml screw-capped Sarstedt tubes. The reaction mixture had a final volume of 100 μl and contained 250 pmol (as molecules) of each primer, 1.0×10^{-4} pmol of T4cDNA molecules, 1.5 mM each of dATP, dCTP, dGTP, TTP, 10% dimethyl sulfoxide, and Taq polymerase buffer (16.6 mM ammonium sulfate, 67 mM Tris-HCl, pH 8.8, at 25 °C, 6.7 mM $MgCl_2$, 6.7 μM Na_2EDTA, 170 μg/ml bovine serum albumin, and 10 mM β-mercaptoethanol). The DNA was denatured by incubation at 95 °C for 5 min, and the tube was spun briefly. Two units of Taq DNA polymerase were added, and the mixture was layered with mineral oil. The primers were annealed at 42 °C for 24 s, DNA synthesis was allowed at 65 °C for 4 min, and the DNA was denatured at 95 °C for 1 min. This cycle was repeated 30 times and stopped by placing the tubes in an ice-cold water bath. The amplified DNA was electrophoresed on a 1% low temperature gelling/TAE gel. The predominant 1.3-kb band was cut from the gel, and the DNA was recovered from the gel piece by melting the agarose at 65 °C followed by extraction with phenol. The DNA was ethanol precipitated, resuspended in digest buffer, and cut with *Bam*HI and *SalI*. After deproteinization, the 1.3-kb fragment with *Bam*HI and *SalI* ends was ligated into the pTL18xwd plasmid following standard methods (Maniatis *et al.*, 1982).

Plating of Phage—E. coli Tab32-4 cells containing either plasmid pKHdda or pTL19xwd in LB media were incubated at 30 °C. For complementation at 35 °C, samples were switched to a water bath at 35 °C 15 min prior to infection with a T4*ts75*(gene 32)*sud*1 double mutant. Cells were infected, plated in T4 soft agar, and incubated at the appropriate temperature (30 or 35 °C).

Overproduction and Purification of the Cloned dda Gene Product—An *E. coli* SG934 culture containing plasmid pKHdda was grown to 6×10^8 cells/ml in LB media containing 50 μg/ml ampicillin. The temperature was then quickly switched to 38 °C, and the incubation was continued for 3 h. After harvesting, the cells were stored at −20 °C.

Cells were lysed using a procedure (Alberts and Frey, 1970) modified by J. Barry in this laboratory.[3] Cells (28 g) were thawed and resuspended in 136 ml of buffer containing 20 mM Tris-HCl, pH 8.1, 1 mM Na_2EDTA, 1 mM β-mercaptoethanol, 10 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride. T4 lysozyme was added to a final concentration of 200 μg/ml and the suspension was incubated until viscous. The DNA was degraded by adding DNase I to 10 μg/ml and adjusting the extract to 10 mM $MgCl_2$, 1 mM $CaCl_2$. The suspension was incubated in ice water, gently mixed until the viscosity decreased, and then sonicated with repeated 1-min blasts from the 0.5-inch horn of a Branson sonifer (40% duty) until the OD_{600} had dropped to 15% of its original value. Another 10 μg/ml of DNase I

[4] The abbreviations used are: T4cDNA, T4 cytosine-containing DNA; cv, column volume; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; kb, kilobase(s).

[5] H. E. Selick, G. D. Stormo, R. L. Dyson, and B. M. Alberts, manuscript submitted

was added, and the extract was incubated at 15 °C for 20 min. The extract was centrifuged at 20,000 rpm (48,200 × g) in a Sorvall 34 rotor for 20 min to remove cell debris and then further clarified by centrifugation at 35,000 rpm (111,000 × g) in a Beckman vTi 50.2 rotor for 3 h. The supernatant was dialyzed against 4 two-liter changes of buffer A (20 mM Tris-HCl, pH 8.1, 5 mM Na_2EDTA, 1 mM β-mercaptoethanol, 2 mM benzamidine-HCl, and 10% (v/v) glycerol) to remove the divalent cations necessary for DNase I activity.

A 55-ml single-stranded DNA-cellulose column was constructed (Alberts and Herrick, 1971), containing 1.5 mg of DNA/packed ml. The dialyzed extract (fraction I, Table II) was pumped at 1 column volume (cv)/h through the column, washed with 1 cv of buffer A containing 0.10 M NaCl, followed by 1.5 cv of buffer A containing 0.25 M NaCl, and then eluted with a 2-cv linear gradient of 0.24–2 M NaCl in buffer A. Fractions of 4 ml were collected. ATPase activity was determined using a charcoal adsorption assay, as described previously (Liu and Alberts, 1981).

The fractions containing the dda protein were pooled and dialyzed against buffer B (20 mM Tris-HCl, pH 8.1, 20 mM NaCl, 1 mM Na_2EDTA, 2 mM β-mercaptoethanol, and 10% (v/v) glycerol). A 5-ml DEAE-cellulose column was constructed and equilibrated with buffer B. The pooled, dialyzed DNA cellulose fractions (fraction II, see Table II) were loaded at 1 cv/h onto the column, and then eluted with 2 cv of buffer B containing 60 mM NaCl. Fractions of 1.2 ml were collected. The fractions containing dda protein were pooled and concentrated in a Centricon 30 ultrafiltration device (Amicon Corp., Danvers, MA), and adjusted to storage buffer (20 mM Tris-HCl, pH 7.7, 50 mM NaCl, 1 mM β-mercaptoethanol, 0.2 mM Na_2EDTA, and 45% (v/v) glycerol. The dda preparation was free of detectable nuclease activities as evaluated by sensitive DNA gel electrophoresis assays (Morris *et al.*, 1979).

uvsX Protein Affinity Chromatography—Highly purified T4 uvsX protein was covalently linked to Affi-Gel-10 (Bio-Rad) to produce a matrix containing 1.7 mg uvsX protein/packed ml (Formosa *et al.*, 1983). A 0.5-ml uvsX-Affi-Gel column was prewashed with 2 cv of column buffer (20 mM Tris-Cl, pH 8.1, 1 mM Na_2EDTA, 1 mM β-mercaptoethanol, and 10% (v/v) glycerol) containing 2 M NaCl and then equilibrated with column buffer containing 25 mM NaCl at 4 °C. A mixture of 25 μg of purified dda protein and 37 μg of albumin in 0.2 ml of column buffer containing 25 mM NaCl was applied to the column at 1 cv/h. The column was washed with 2 cv of column buffer containing 25 mM NaCl and eluted successively with 50 mM, 100 mM, and 2 M NaCl in column buffer (1 cv each). Fractions of 100 μl were collected, subjected to electrophoresis on a 13.5% SDS-polyacrylamide gel, and visualized by Coomassie Blue staining.

RESULTS

The dda Gene Is Located at the 10.5–9.0-kb Position on the T4 Chromosome—Behme and Ebisuzaki (1975) mapped the dda gene by showing that its DNA-dependent ATPase activity was present in cells infected with the T4 *del(39–56)1* mutant (deletion map position 6.15–10.34 kb), but was missing in cells infected with the T4 *del(39–56)10* mutant (deletion map position 6.3–10.7 kb).

We used two 20 nucleotide-long probes derived from the NH_2-terminal amino acid sequence that we determined for the purified dda protein to further define the location of the *dda* gene in the T4 chromosome. Oligonucleotide 1 was derived from the first 6 amino acids of dda and was 128-fold degenerate. Oligonucleotide 2 was derived from amino acids 8–13 and was 192-fold degenerate.

Blot hybridization to restriction nuclease-digested T4cDNA was performed separately with oligonucleotides 1 and 2 according to standard methods (Maniatis *et al.*, 1982). Each probe hybridized to multiple restriction fragments, but only restriction fragments that hybridized with both probes were judged to contain the *dda* gene. Both oligonucleotides hybridized to a 2.0-kb *Hin*dIII fragment at map position 10.3–12.35 on the T4 linear chromosome and with a 1.7-kb *Eco*RI fragment at map position 8.94–10.59; thus the 5′ end of the dda gene must lie between map position 10.3–10.6. Assuming that dda is transcribed in the counter-clockwise direction on the T4 circular map like other early and middle T4 genes (Kutter and Ruger, 1983), the *dda* gene should lie somewhere between map positions 10.6 and 8.8.

The *Cla*I-*Hin*dIII DNA fragment, encompassing map positions 10.7–10.4, was isolated from a digest of T4 cytosine-containing DNA (T4cDNA). This fragment was sequenced using the Maxam-Gilbert technique to determine the precise location of the *dda* gene's 5′ end and confirm the expected orientation of the gene on the T4 chromosome. The results showed that the 5′ end of the *dda* gene lies at the 10.5 position on the chromosome with the predicted orientation; therefore, the 3′ end must lie at position 9.0 (assuming that the gene encodes a 50-kDa protein and does not contain an intron).

Sequence Analysis of the dda Gene—Repeated attempts to clone restriction fragments encompassing the entire *dda* gene by conventional methods failed, suggesting that the *dda* gene product is deleterious to *E. coli* when under the control of its own promoter. Fortunately, both T4cDNA and T4 mRNA are easily obtained from T4-infected cells, and by employing these nucleic acids as single-stranded templates we were able to use the dideoxy method to determine the sequence of the *dda* gene. The strategy used for sequencing is presented in Fig. 1. The *dda* gene sequence is shown in Fig. 2.

Sequence analysis predicts a dda protein sequence of 439 amino acids. The calculated mass of 49,947 daltons agrees with the estimated mass from SDS-polyacrylamide gel electrophoresis of 56,000 (Krell *et al.*, 1979), 50,000 (Purkey and Ebisuzaki, 1977), or 48,000 daltons (Jongeneel *et al.*, 1984a). The *dda* sequence contains a consensus nucleoside triphosphate-binding site and six regions of homology with other helicases (see "Discussion").

Amplification, Modification, and Cloning of the dda Gene for Overproduction of the Gene Product—We used the PCR technique to amplify the *dda* gene while simultaneously changing its Shine-Dalgarno sequence in preparation for cloning in a tightly regulated expression vector. The sequences of the two primers used for PCR are shown in Fig. 2. T4cDNA was used as the template, and the PCR reaction was carried out as described by Kogan *et al.* (1987), with the following modifications: the time and temperature used for polymerase incubation was increased to 4 min at 65 °C, primers were allowed to anneal at 42 °C for 24 s, and no additional Taq DNA polymerase was added during the cycles. On a 1% agarose gel, more than 90% of the DNA product migrated at 1.3 kb, the length of the *dda* gene; 30 rounds of synthesis yielded a 13,000-fold amplication (1.3 μg of DNA).

The 1.3-kb DNA fragment containing the *dda* gene was placed in an expression vector, pTL19xwd, downstream of the λ late promoter. This promoter is controlled by the cI^{857} repressor produced by the vector. The plasmid contains the 39,178–34,500 region of bacteriophage λ with the *rexA* and *B* genes deleted; the *rex* gene products inhibit T4 growth when contained on a plasmid (Shinedling *et al.*, 1987), which would complicate genetic studies.

DNA strands from the cloned *dda* gene, complementary to those sequenced from T4 genomic nucleic acids (see Fig. 1*A*), were sequenced to confirm the sequence obtained from the T4 genomic nucleic acids and to determine if any mutations were introduced during the cloning of the *dda* gene. The approach used to sequence the cloned *dda* gene, pKHdda, is shown in Fig. 1*B*. The sequence of both the genomic and the cloned *dda* gene were found to be identical, and the sequence of the first 25 amino acids encoded by the gene is the same as that determined for the dda protein isolated from phage T4 infected cells.

The production of functional dda protein was tested genetically by assaying the ability of the cloned *dda* gene to com-

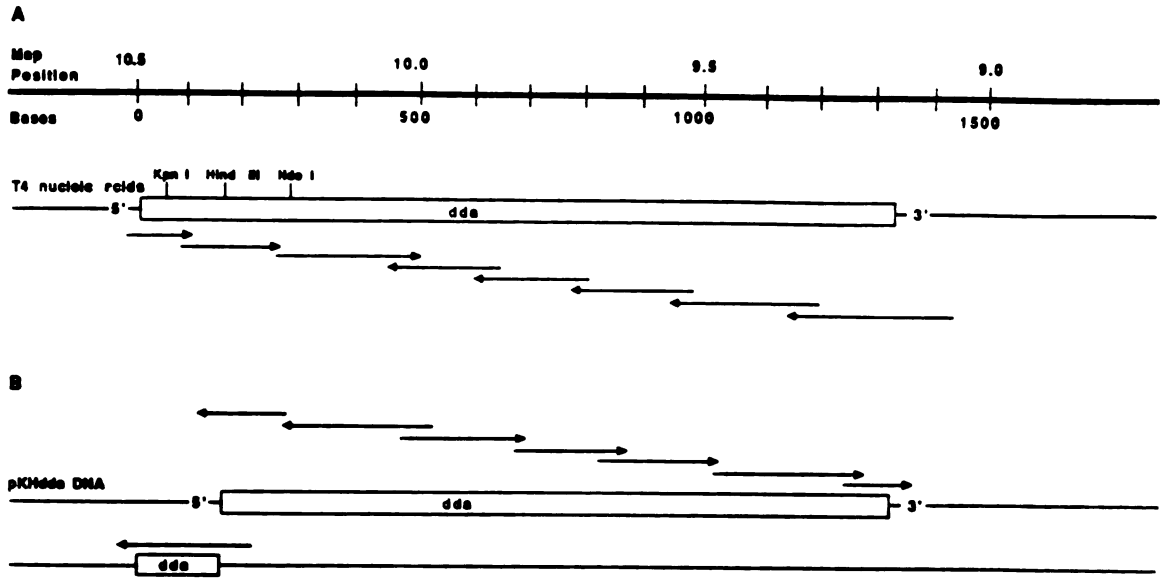

FIG. 1. **The strategy used to sequence the *dda* gene.** *A*, a linear map of the T4 chromosome from position 10.5 to 9.0 is shown at the *top* of the figure, with the *dda* gene shown below by an *open box*. Some restriction sites that occur once within the *dda* gene are shown above the *box*. Each *arrow* represents the sequence information obtained from an individual oligonucleotide primer with the *arrow* pointing in the direction of sequencing from the primer. The sequence information required to prepare the most 5′ primer was determined by Maxam-Gilbert sequencing of the T4 *Cla*I-*Hin*dIII DNA fragment, map position 10.608–10.295 (see "Materials and Methods"). The information required for the primer used at the 3′ end of the gene was obtained by dideoxy sequencing, starting from the published *Eco*RI-*Eco*RI fragment sequence at map position 7.6–8.942 (Gauss *et al.*, 1987). Denatured double-stranded T4 genomic DNA was the template for dideoxy sequencing at the start of the *dda* gene (*arrows pointing to the right*), whereas both this T4 DNA and total RNA from T4-infected *E. coli* cells were used as templates for dideoxy sequencing for *arrows pointing to the left*. *B*, the plasmid containing the *dda* gene, pKHdda, was cut with *Bam*HI, *Hin*dIII, and *Sal*I. The *Bam*HI-*Hin*dIII and *Hin*dIII-*Sal*I fragments were cloned separately into M13 origin-containing Bluescript plasmids as shown by the separate *open boxes*. Single-stranded DNA was isolated from M13-infected cells containing these plasmids, and the sequence of all DNA strands complementary to those sequenced from the T4 genome-derived nucleic acids shown in *A* were determined by the dideoxy sequencing method (thus, *arrows* in *B* point opposite to *arrows* in *A* for each part of the gene).

plement a bacteriophage T4 gene 32 temperature-sensitive, *sud* deletion double mutant (T4*ts*75*sud*1). The *sud* and *dda* genes are believed to be the same (Jongeneel *et al.*, 1984; Doherty *et al.*, 1982). Whereas either the T4 *ts75* (gene 32) or *sud*1 mutant alone will grow at both 30 and 35 °C, the T4*ts*75(gene 32)*sud*1 double mutant phage will not grow at either temperature on a Tab32–4 *E. coli* strain (Doherty *et al.*, 1982); this strain restricts the growth of many gene 32 temperature-sensitive mutants at normally permissive temperatures, without affecting wild-type T4 phage (Nelson and Gold, 1982). As shown in Table I, the T4*ts*75(gene 32)*sud*1 mutant phage grows on Tab32–4 containing pKHdda but not on Tab32–4 containing only the vector (pTL19xwd), at both 30 and 35 °C. This result shows that the *dda* gene product encoded by the plasmid is active *in vivo*, and it further supports the previous evidence that *sud* and *dda* are the same gene.

Overproduction and Purification of the Cloned dda Gene Product—The dda protein was overproduced from plasmid pKHdda in *E. coli SG934* cells, which contain a mutation in the *htpR* gene. The *htpR* gene is essential for the transcription of heat shock genes, and proteases normally induced upon heat shock are not expressed (for review, see Neidhardt *et al.*, 1984). The expression of the dda protein was induced at 38 °C for 3 h. After induction, the dda protein represented 1% of the total soluble protein, which is 10-fold more than obtained from T4-infected cells. Further overexpression of the dda protein could be obtained at higher temperatures, but this resulted in the formation of insoluble dda protein aggregates and a reduced final yield.

Purification of the Overexpressed dda Protein—The overexpression of the dda protein allowed us to simplify our previous procedure for dda purification from T4-infected cells, which involved five columns and resulted in a 5% yield of pure dda protein (Jongeneel *et al.*, 1984a). From our induced cells, the dda protein can be purified free of nucleases after only two columns with a yield of 60%, as detailed in Table II.

In the new procedure, the crude lysate is passed through a single-stranded DNA-cellulose column, from which the dda protein is eluted with a steep NaCl gradient. The fractions that contain the highly purified dda protein are pooled and chromatographed over a DEAE-cellulose column under conditions in which the dda protein flows through. The results of an SDS-polyacrylamide gel analysis at each stage of the purification are shown in Fig. 3.

The Overexpressed dda Protein Has DNA Helicase Activity—An assay was carried out to determine whether the overexpressed dda protein purified from T4-uninfected cells has DNA helicase activity, since it is conceivable that the dda protein requires post-translational modification or some other component picked up during T4 infection to become an active helicase.

To assay the dda protein for helicase activity, we constructed a DNA substrate that contains a fully complementary 5′ end-labeled 393-nucleotide DNA fragment annealed to single-stranded genomic M13 DNA. The unwinding of this substrate by a helicase changes the mobility of the labeled DNA fragment on a non-denaturing agarose gel and is readily detected by autoradiography (Jongeneel *et al.*, 1984a).

The unwinding of the DNA substrate by the overexpressed

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-26     gatcctactttaaggagatatacatATG ACA TTT GAT GAT TTG ACC GAA GGC CAG AAA AAT GCC
                                Met Thr Phe Asp Asp Leu Thr Glu Gly Gln Lys Asn Ala

 40  TTT AAC ATT GTT ATG AAG GCT ATT AAA GAA AAG AAA CAT CAT GTA ACT ATT AAT GGA CCT
     Phe Asn Ile Val Met Lys Ala Ile Lys Glu Lys Lys His His Val Thr Ile Asn Gly Pro

101  GCT GGT ACC GGT AAG ACT ACT CTT ACT AAG TTC ATC ATT GAA GCT TTA ATA TCT ACG GGT
     Ala Gly Thr Gly Lys Thr Thr Leu Thr Lys Phe Ile Ile Glu Ala Leu Ile Ser Thr Gly

161  GAA ACT GGT ATT ATT TTA GCA GCT CCT ACA CAT GCA GCT AAA AAG ATT CTT TCA AAA CTA
     Glu Thr Gly Ile Ile Leu Ala Ala Pro Thr His Ala Ala Lys Lys Ile Leu Ser Lys Leu

221  TCA GGG AAA GAA GCG AGT ACT ATT CAT AGT ATT CTT AAA ATT AAC CCA GTA ACA TAT GAA
     Ser Gly Lys Glu Ala Ser Thr Ile His Ser Ile Leu Lys Ile Asn Pro Val Thr Tyr Glu

281  GAA AAT GTT CTT TTT GAA CAA AAA GAA GTA CCT GAT TTA GCC AAA TGC AGA GTA TTA ATC
     Glu Asn Val Leu Phe Glu Gln Lys Glu Val Pro Asp Leu Ala Lys Cys Arg Val Leu Ile

341  TGC GAC GAA GTG TCA ATG TAT GAT AGA AAG CTA TTT AAA ATT CTG CTT TCA ACT ATT CCA
     Cys Asp Glu Val Ser Met Tyr Asp Arg Lys Leu Phe Lys Ile Leu Leu Ser Thr Ile Pro

401  CCT TGG TGT ACT ATA ATT GGA ATA GGG GAT AAT AAG CAA ATC AGA CCT GTT GAC CCA GGA
     Pro Trp Cys Thr Ile Ile Gly Ile Gly Asp Asn Lys Gln Ile Arg Pro Val Asp Pro Gly

461  GAA AAT ACT GCT TAT ATC AGT CCA TTC TTT ACA CAT AAA GAT TTT TAT CAG TGT GAA CTC
     Glu Asn Thr Ala Tyr Ile Ser Pro Phe Phe Thr His Lys Asp Phe Tyr Gln Cys Glu Leu

521  ACT GAA GTT AAA CGC AGT AAT GCT CCT ATT ATT GAT GTA GCT ACT GAC GTT CGC AAC GGT
     Thr Glu Val Lys Arg Ser Asn Ala Pro Ile Ile Asp Val Ala Thr Asp Val Arg Asn Gly

581  AAG TGG ATT TAT GAT AAA GTT GTT GAC GGG CAT GGA GTA CGT GGA TTT ACT GGT GAT ACC
     Lys Trp Ile Tyr Asp Lys Val Val Asp Gly His Gly Val Arg Gly Phe Thr Gly Asp Thr

641  GCT TTA CGC GAT TTT ATG GTA AAT TAT TTT TCA ATC GTC AAA TCA CTA GAT GAT TTG TTT
     Ala Leu Arg Asp Phe Met Val Asn Tyr Phe Ser Ile Val Lys Ser Leu Asp Asp Leu Phe

701  GAA AAT CGC GTA ATG GCA TTT ACG AAT AAA TCT GTT GAC AAG TTA AAT AGC ATT ATT CGT
     Glu Asn Arg Val Met Ala Phe Thr Asn Lys Ser Val Asp Lys Leu Asn Ser Ile Ile Arg

761  AAA AAG ATT TTT GAA ACT GAT AAA GAT TTT ATT GTC GGT GAA ATT ATT GTA ATG CAG GAA
     Lys Lys Ile Phe Glu Thr Asp Lys Asp Phe Ile Val Gly Glu Ile Ile Val Met Gln Glu

821  CCA TTA TTT AAA ACA TAT AAA ATT GAT GGA AAG CCT GTG TCA GAA ATT ATT TTT AAT AAC
     Pro Leu Phe Lys Thr Tyr Lys Ile Asp Gly Lys Pro Val Ser Glu Ile Ile Phe Asn Asn

881  GGA CAA TTA GTT CGT ATT ATA GAA GCA GAG TAT ACA TCA ACG TTT GTT AAA GCC CGT GGT
     Gly Gln Leu Val Arg Ile Ile Glu Ala Glu Tyr Thr Ser Thr Phe Val Lys Ala Arg Gly

941  GTT CCT GGA GAA TAT CTA ATT CGT CAT TGG GAT TTA ACA GTA GAA ACT TAT GGC GAT GAT
     Val Pro Gly Glu Tyr Leu Ile Arg His Trp Asp Leu Thr Val Glu Thr Tyr Gly Asp Asp

1001 GAA TAT TAT CGT GAA AAG ATT AAA ATA ATT TCA TCT GAC GAA GAA TTG TAT AAG TTT AAC
     Glu Tyr Tyr Arg Glu Lys Ile Lys Ile Ile Ser Ser Asp Glu Glu Leu Tyr Lys Phe Asn

1061 CTA TTT TTA GGT AAA ACA GCA GAA ACT TAT AAA AAT TGG AAC AAA GGC GGA AAA GCT CCG
     Leu Phe Leu Gly Lys Thr Ala Glu Thr Tyr Lys Asn Trp Asn Lys Gly Gly Lys Ala Pro

1121 TGG AGT GAT TTT TGG GAT GCT AAA TCA CAG TTT AGT AAA GTG AAA GCA CTT CCT GCA TCA
     Trp Ser Asp Phe Trp Asp Ala Lys Ser Gln Phe Ser Lys Val Lys Ala Leu Pro Ala Ser

1181 ACA TTC CAT AAA GCG CAG GGT ATG TCT GTA GAC CGT GCT TTC ATT TAT ACG CCT TGT ATT
     Thr Phe His Lys Ala Gln Gly Met Ser Val Asp Arg Ala Phe Ile Tyr Thr Pro Cys Ile

1241 CAT TAT GCA GAT GTT GAA TTA GCT CAA CAA CTT CTT TAT GTT GGT GTC ACC CGT GGT CGT
     His Tyr Ala Asp Val Glu Leu Ala Gln Gln Leu Leu Tyr Val Gly Val Thr Arg Gly Arg

1301 TAT GAT GTG TTT TAT GTA tgagtcgac
     Tyr Asp Val Phe Tyr Val
```

FIG. 2. **The nucleotide sequence of the *dda* gene.** The *lowercase letters* represent nucleotides intentionally added to each end of the *dda* gene as the 5′ portion of the two PCR primers used for the gene's amplification. The primer at the start of the gene contained 20 nucleotides complementary to the 5′ end of the *dda* gene, preceded by 21 nucleotides containing a near consensus Shine-Dalgarno sequence and a 6 nucleotide restriction site recognized by *Bam*HI. The primer complementary to the other end of the *dda* gene contained 20 nucleotides homologous to the 3′ end of the *dda* gene preceded by a 9-base *Sal*I restriction site. The translated amino acids are shown below the DNA sequence. The encoded dda peptide has 439 amino acids and a calculated mass of 49,947 Da. The sequence of the cloned (Fig. 1*B*) and wild-type (Fig. 1*A*) *dda* gene were identical. This result was unexpected since the clone was obtained using DNA amplified by the PCR technique with the Taq polymerase which (under slightly different reaction conditions) has a reported error rate of 0.25% (Saiki *et al.*, 1988). At this error rate, we would have expected to find three to four nucleotide changes in the clone compared to the wild-type sequence.

purified dda protein is presented in Fig. 4. The dda protein does not require post-translation modification by phage T4 proteins for helicase activity. The percentage of DNA unwound is a nonlinear function of dda protein concentration, as also observed for the dda protein purified from phage T4-infected cells (Jongeneel *et al.*, 1984a). The percentage of the DNA unwound greatly increases when the dda concentration is raised from 4 to 8 μg/ml, which increases the ratio of dda protein molecules to DNA nucleotides from 1:2 to 1:1. Three dda protein molecules/nucleotide are required for the unwinding of all of the DNA molecules. One dda protein molecule from phage T4-infected cells/three DNA nucleotides was needed to unwind 84% of the somewhat different DNA molecules used in our earlier study (Jongeneel *et al.*, 1984a).

The dda Gene Product Binds Directly to the T4 uvsX Protein—Overexpression of the *dda* gene product made it possible to obtain large enough quantities of the protein to produce a dda protein affinity column. Affinity chromatography with other T4 proteins involved in DNA metabolism attached to an agarose matrix showed a tight interaction of the dda protein with the gene 32 protein (Formosa *et al.*, 1983). To extend this analysis, the dda protein was covalently coupled to an agarose matrix, Affi-Gel 10, as described by Formosa *et al.* (1983). Although a column containing 2 mg of dda protein/

packed ml was prepared, we did not detect any binding of the purified gene 32 protein to this column (data not shown). This result suggests that the dda protein is inactivated during its attachment to the column. Interestingly, of the eight other T4 proteins that have previously been attached to this agarose matrix, the only one that was similarly inactivated was the other DNA helicase, the T4 gene 41 protein.[3]

The interaction of the dda protein with the T4 uvsX protein (a recA protein analogue) was previously suggested when the dda protein in an extract of T4-infected cells was retained on a uvsX protein-agarose column. Since the dda protein coeluted with the gene 32 protein from this column, the interaction of the dda protein with the uvsX protein could have been indirect (Formosa and Alberts, 1984). To determine if the uvsX and dda proteins bind directly to one another, we chromatographed a mixture of the pure dda protein with albumin on a uvsX protein affinity column. As shown in Fig. 5, the albumin is not retained by the column, whereas the dda protein binds to the column and is eluted by 50 mM NaCl. The dda protein and the albumin behaved identically on an agarose control column, and the dda protein was not retained on an albumin-agarose control column (data not shown). These results demonstrate that there is a direct, albeit weak, interaction between the uvsX and dda proteins. Similar weak interactions have been observed between the protein subunits of the T4 DNA polymerase holoenzyme. On protein affinity columns both the interactions between T4 DNA polymerase accessory proteins (the gene 45 protein and the 44/62 protein complex) and the interaction of the T4 DNA polymerase with the gene 45 protein are disrupted by washing with 50 mM NaCl (Formosa and Alberts, 1984; Formosa, 1985).

DISCUSSION

Direct sequencing of nucleic acids (DNA and RNA) produced from bacteriophage T4-infected cells has allowed us to use the PCR technique to engineer an appropriate vector to produce the dda protein in *E. coli*. The *dda* gene product was thereby overexpressed to approximately 1% of the total cellular protein in *E. coli htpR* mutant cells. The cloning and overexpression of the dda protein greatly simplified its purification, since its association with nucleases in the infected cell and with the tightly binding T4 gene 32 protein was avoided. Thus, the dda protein purified from overexpressing cells is more than 99% pure and free of nucleases after only two chromatography steps. The yield is greatly increased such that 7 mg of dda protein is obtained from 28 g of cells. The overexpressed dda protein has DNA helicase activity, and a direct interaction of the this protein with the T4 uvsX protein (a recA analogue essential for T4 genetic recombination) was detected on a uvsX protein-agarose column.

Both the genomic and cloned *dda* genes were sequenced using the dideoxy method. The dda amino acid sequence shares six conserved motifs with a superfamily of ATPases identified by Gorbalenya *et al.* (1988) and independently by Hodgman (1988). The consensus sequences for the superfamily and the alignment of dda with the family of *E. coli* helicases within the six regions is shown in Fig. 6. Motif I contains the sequence common to many GTP- and ATP-binding domains, originally described by Walker *et al.* (1982). This motif forms a loop that binds the ATP phosphate (La Cour *et al.*, 1985; Jurnack, 1985; Fry *et al.*, 1986). Motif II most likely binds the ATP phosphate indirectly via a magnesium ion (Jurnack, 1985). Motif III is also conserved among many DNA and RNA polymerases (Hodgman, 1986). Motif VI is believed to be involved in DNA binding because of its occurrence in putative DNA-binding proteins (Hodgman, 1988). Neither the structure nor function of motifs IV and V is known.

Among this superfamily of more than 20 proteins, the dda protein has the greatest homology with the *E. coli* recD protein. The recD protein is the most diverged member of the *E. coli* helicase family, which is composed additionally of the recB, rep, and uvrD proteins (Hodgman, 1988). recD is a subunit of the recBCD (exonuclease V) complex which plays a central role in homologous genetic recombination (for review, see Telander-Muskavitch *et al.*, 1981; Taylor, 1988). Exonuclease V moves along the DNA creating looped structures that are periodically cut by the enzyme (Taylor, 1988). The dda and recD protein share 38% amino acid identity within the six conserved sequence motifs and a Monte Carlo score of 4.5 when the amino acids NH_2-terminal to the first motif and COOH-terminal to the last motif are deleted from the analysis. For comparison, recD shares a 26, 31.5, and 30% amino acid identity within the conserved motifs with recB, rep, and uvrD, respectively, and Monte Carlo scores throughout the region encompassing all of the conserved motifs of 6.8, 4.4, 2.9, respectively.

The Monte Carlo scores give a statistical evaluation of the homology of the motifs and the regions between them, encompassing a larger part of the gene than just the conserved motifs. It is calculated by aligning the sequences using the SS2 algorithm to produce a similarity score, subtracting from the original similarity score the mean scores from compari-

TABLE I

The protein produced by the cloned dda gene is biologically active

Plating efficiencies of T4 ts75 (gene 32) *sud1* double mutant on *E. coli* strain Tab32-4, with and without the cloned *dda* gene. Plating efficiency is expressed as the number of plaques observed divided by the number of plaques produced on *E. coli* Tab32-4 containing the pKHdda plasmid at 35 °C. The plasmid used for the "vector only" control is identical to pKHdda, except that it lacks the dda gene insert.

Plasmid	Temperature	Plating efficiency
	°C	
pKHdda	35	(1.00)
pKHdda	30	0.28
"Vector only" control	35	<0.007
"Vector only" control	30	<0.007

TABLE II

Summary of dda protein purification

Fraction	Step	Volume	Protein	Units ATPase/ml[a]	Total units	Yield	Units/mg protein	Enzyme purification
		ml	*mg*			%		*fold*
I	Cleared lysate	135	1039	ND[b]	ND	(100)		(1.0)
II	DNA-cellulose	44	11.9	43.2	1901	88[c]	160	80
III	DEAE-cellulose	35	7.0	37.2	1302	60	186	100

[a] One unit of ATPase activity is defined as the amount of enzyme required to hydrolyse 1 μmole of ATP in 1 min at 37 °C.
[b] Not possible to determine due to high ATPase activity by proteins other than dda; ND, not determined.
[c] Calculated by determining the amount of dda protein in fractions I and II by SDS-polyacrylamide gel analysis.

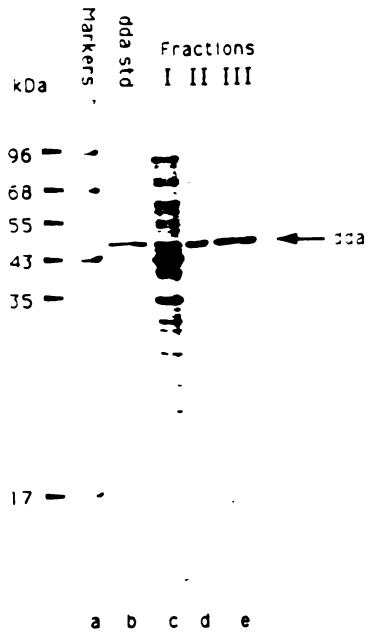

FIG. 3. **Analysis of the state of dda protein purification by SDS-polyacrylamide gel electrophoresis.** The gel was stained with Coomassie Blue: *a*, molecular weight standards; *b*, 2 μg of dda protein purified from T4-infected cells; *c*, 16 μg of Fraction I; *d*, 2 μg of Fraction II; *e*, 4 μg of Fraction III. (For a description of these fractions, see Table II.)

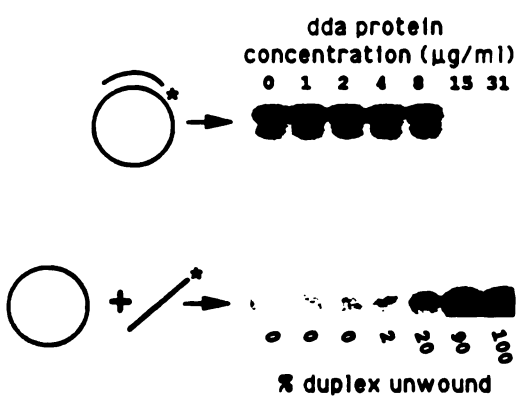

FIG. 4. **The helicase activity of the dda protein as a function of its concentration.** A DNA helicase assay was carried out as described by Jongeneel *et al.* (1989a). Reactions contained dda protein as indicated, 65 ng/ml DNA substrate constructed by annealing the fully complementary 5′ end-labeled 393 nucleotide fragment from pJMC110 to single-stranded M13 DNA, 1 mM ATP, 33 mM Tris acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, and 100 μg/ml human serum albumin. The reactions were incubated at 30 °C for 3 min, stopped by addition of sodium dodecyl sulfate and Na_2EDTA to 1% and 10 mM, respectively, and electrophoresed on a 1.2% agarose/TBE gel. The gel was then dried and autoradiographed.

sons with the randomized sequence, and then dividing by the standard deviation. A Monte Carlo score of between 3 and 6 indicates possible homology, whereas scores greater than 6 indicate a probable homology (Barker and Dayhoff, 1972; Argos and Vingron, 1990).

The overall homology between the recD and dda proteins

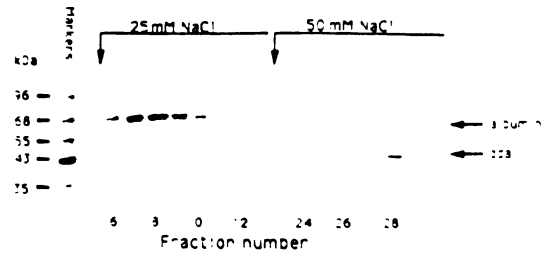

FIG. 5. **Binding of the dda protein to the T4 uvsX protein.** A mixture of 25 μg of purified dda protein and 37 μg of albumin in column buffer containing 25 mM NaCl was applied to a 0.5-ml uvsX protein-Affi-Gel column containing 0.85 mg of covalently bound uvsX protein. The matrix had been previously equilibrated with the same buffer. The column was washed with 1 ml of column buffer containing 25 mM NaCl and then eluted with column buffer containing 50 mM NaCl. Fractions of 100 μl were collected, and 20 μl of each fraction found to contain protein by Bradford assay (Bradford, 1872) was loaded on to a 13.5% SDS-polyacrylamide gel, electrophoresed, and visualized by Coomassie Blue staining. Note that the fractions at the end of the 25 mM NaCl wash contained no protein and are not shown.

is not enough to suggest a strong structural or functional homology. It is nevertheless worth pointing that their genetic phenotypes display some similarities. The dda⁻ mutant phage show a DNA delay phenotype, but they eventually attain a phage burst size that is close to normal (Little, 1973; P. Gauss, personal communication). Only in a T4 gene 59⁻ background is the *dda* gene essential for DNA synthesis.[1] The complex of recBC, missing recD, lacks exonuclease V activity *in vitro*. But recD mutant cells show a hyper-recombination phenotype and are viable (Chaudhury and Smith, 1984). Thus, like the *dda* gene, *recD* is an nonessential gene. However, in a recJ mutant (*recF* pathway gene) background, the *recD* gene is required for chromosome recombination and UV resistance; this suggests that the recBCD and recF pathways are somewhat redundant (Lovett *et al.*, 1988). Similarly, the dda DNA helicase seems to be partially replaceable by the gene 41 DNA helicase, providing that an accessory protein for 41 protein function, the gene 59 protein is present.

What are the physiological roles of the dda protein? The DNA delay phenotype of *dda* mutant phage, which is extended to a severe block in early DNA synthesis when the 59 protein is absent,[1] suggests an important function for the dda protein in the initiation of T4 DNA replication. Determination of its exact role in initiation is likely to require the reconstitution of the initiation process in a purified *in vitro* system containing a T4 replication origin (Kreuzer and Alberts, 1985; Kreuzer *et al.*, 1988; Menkens and Kreuzer, 1988). In addition, the direct interaction of the dda and uvsX proteins reported here suggests that the dda protein serves as a specific accessory factor in T4 uvsX protein-catalyzed genetic recombination. A role in DNA recombination is also suggested by the dda protein's 4-fold stimulation of uvsX-catalyzed DNA branch migration rates (Kodadek and Alberts, 1987). However, the observed binding of the dda protein to both 32 protein and uvsX protein could have the alternative function of promoting access of the dda protein to single-stranded DNA, which is believed to be completely covered by one or the other of these proteins in a T4-infected cell.

In summary, we have cloned and sequenced the *dda* gene and used the sequence to overproduce the dda protein. The overproduced dda protein has allowed us to demonstrate its direct interaction with the uvsX protein. This result supports a role of the dda protein in DNA recombination, but many questions remain. The availability of the clone and large

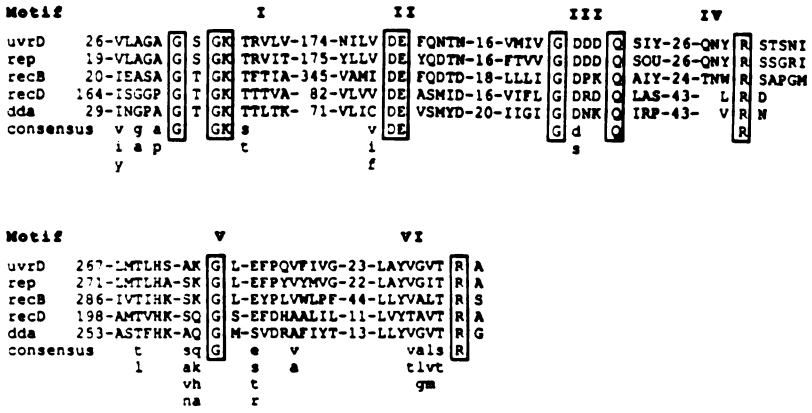

FIG. 6. **Alignment of the dda protein with motifs conserved among a family of *E. coli* helicases.** These motifs were independently identified by Gorbalenya *et al.* (1988) and by Hodgman (1988). The dda protein was aligned using the SS2 algorithm of Altschul and Erickson (1986). Residues placed in *boxes* are absolutely conserved among six protein families of putative ATPases (Gorbalenya *et al.*, 1988; Hodgman, 1988). *Small letters* are used in the consensus sequence to denote the range of observed amino acids at those positions where four or less alternatives exist among all members of the families. The *numbers* indicate the length of the gap between conserved motifs.

amounts of the dda protein should facilitate the further characterization of this DNA helicase's roles during T4 bacteriophage infection.

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CHAPTER THREE

DNA HELICASE REQUIREMENTS FOR DNA REPLICATION DURING BACTERIOPHAGE T4 INFECTION

ABSTRACT

The lytic bacteriophage T4 uses multiple mechanisms to initiate the replication of its DNA. Initiation occurs predominantly at replication origins at early times of infection, but there is a switch to genetic recombination-dependent initiation at late times of infection. The T4 insertion/substitution system was used to create a deletion in the T4 *dda* gene, which encodes a 5'-3' DNA helicase that stimulates both DNA replication and recombination reactions *in vitro*. The deletion caused a delay in T4 DNA synthesis at early times of infection, suggesting that the Dda protein is involved in the initiation of origin-dependent DNA synthesis. However, DNA synthesis eventually reached near wild-type levels, and the final number of phages produced per bacterium was similar to wild-type. When the *dda*$^-$ phage was also mutant in the T4 gene *59* (a gene normally required only for recombination-dependent DNA replication) essentially no DNA was synthesized. Recent *in vitro* studies have shown that the gene 59 protein loads a component of the primosome, the T4 gene 41 DNA helicase, on to DNA. A molecular model for replication initiation is presented that is based on our genetic data.

INTRODUCTION

One of the key events in the initiation of bacteriophage T4 DNA replication is likely to be the loading of the T4 gene 41 encoded DNA helicase onto the DNA template. The 41 protein is essential for DNA synthesis during T4 infection (11). *In vitro*, the gene 41 DNA helicase processively unwinds DNA in the 5' to 3' direction (i.e., moving along the lagging strand at the replication fork) (25, 31) and greatly stimulates the rate of DNA strand-displacement DNA synthesis at a replication fork. It also interacts with the T4 gene 61 protein (the DNA primase that makes the RNA primers for Okazaki fragment synthesis) to form the T4 primosome (25, 29).

The molecular events that lead to the loading of the gene 41 protein at replication origins during early times of infection are not known, but the phenotype of T4 *dda* helicase mutants suggests that Dda protein could play a role. Little (24) found that infections with phage carrying deletions that remove a significant fraction of the DNA between genes *39* and *56* (which delete the *dda* gene) show a substantial delay in DNA synthesis at early times of infection, but—because near normal amounts of DNA are eventually produced—phage burst size is reduced only slightly. We have previously shown that the *dda* gene is only essential when the phage is also mutant in the T4 gene *59* (10, 13; this gene is discussed below). Unfortunately, the *dda*$^-$ phage and *dda*$^-$*59*$^-$ double mutant phage strains used in those studies carried either extensive deletions that also removed genes flanking the *dda* gene or contained mutations in additional

genes. As a result, it was uncertain that the phenotype of these phage was due only to a *dda* deficiency.

Although the physiological role of the Dda helicase is not clear, a great deal has been learned about its biochemical properties from *in vitro* studies. The Dda protein was originally isolated as a DNA-dependent ATPase by Ebisuzaki and co-workers (2, 7). Like the gene 41 helicase, the Dda protein unwinds DNA in the 5' to 3' direction, stimulates the rate of DNA strand-displacement DNA synthesis at an *in vitro* replication fork, and removes DNA binding proteins that block replication fork movement (1, 17). Since no increase in the rate of replication fork movement is observed when the Dda protein is added to *in vitro* reactions that have been stimulated by the gene 41 protein, the two DNA helicases do not appear to act synergistically at the fork (17). The Dda protein differs from the gene 41 protein: i) by acting distributively (continuously dissociating and reassociating with the DNA molecule being unwound) rather than processively and ii) by not forming a primosome with the gene 61 protein (17).

Although no UV sensitivity or genetic recombination deficiencies have been detected during growth of *dda*$^-$ phage, an involvement of the Dda protein in DNA recombination has been suggested by results of *in vitro* studies. The Dda protein stimulates the rate of uvsX protein-catalyzed DNA branch migration four-fold and binds to the uvsX protein (a T4-encoded RecA analog) (12, 15, 18).

Recent studies suggest that during late times of infection, which are dominated by recombination-mediated initiation of DNA

replication, the gene 41 protein is loaded onto the DNA by the gene 59 protein. Gene *59*⁻ phage are unable to synthesize DNA at late times of infection and therefore, display a DNA arrest phenotype (5, 11, 34). Biochemical characterization of the gene 59 protein by Jack Barry and Bruce Alberts has shown that it loads the gene 41 helicase onto single-stranded DNA *in vitro* (manuscript submitted). The role of the gene 59 protein in the earlier origin-dependent replication is not clear. Removal of gene *59* does not affect the replication of plasmids containing a T4 replication origin (22) but replication intermediates in *59*⁻ infections are abnormal (5, 34).

Mutations in gene 59, and other genes involved in DNA recombination, are suppressed by a deficiency in the T4 encoded uvsW protein (5, 33-35). The suppression is not due to restoration of recombination-dependent DNA replication, but rather to the mutant allowing some other mode of DNA replication (possibly origin-dependent replication) to occur (9). The uvsW protein is thought to govern the switch from origin-dependent to recombination-dependent initiation of DNA replication by inhibiting initiation at replication origins. Although the uvsW protein has not been characterized biochemically, *in vivo* experiments have led some researchers to suggest that it may have RNAase H or RNA-DNA helicase activity (9, 23).

To further examine the role of the *dda* and *59* genes during T4 DNA replication, the T4 insertion/substitution system (30) was used to construct a deletion that only removes DNA sequences within the *dda* gene, and DNA synthesis was monitored during infection by the mutant. Phage strains carrying mutations in both the *59* and *dda*

genes were also constructed and analyzed. In addition, *59⁻dda⁻* pseudorevertants were isolated and their sensitivity to production of the *uvsW* protein was examined.

MATERIALS AND METHODS

Bacterial and Phage Strains. *E. coli* CR63(*supD*), NapIV(*sup^o*) and NapIV (*sup^o*) *optA*1 stocks are maintained in our laboratories and have been described (14). The MH1 strain of *E. coli*, which can be made competent for high frequency transformation, was obtained from Dr. Michael Hall, University of Basel, Basel Switzerland. T4 phage strains L148 (*dexA*, *dda* point mutations), sud1 (*dexA*, *dda* deletion), amHL628 (*59⁻*), and amB22 (*43⁻*) have been previously described (13, 14). T4 I/S(*38⁻*, *51⁻*, *denA*, *denB*) (30) was from stocks in the laboratory of Dr. Bruce Alberts. Construction of some phage strains required genetic crosses and the screening of progeny for the recombinant phenotype (see below).

Chemicals. [*methyl*-^{3}H]thymidine was purchased from New England Nuclear, thymidine and deoxyadenosine from Sigma Biochemicals. Whatmann GFC glass fiber filters from VWR Scientific. Agarose was purchased from FMC Bioproducts, nitrocellulose filters from Schleicher and Schuell, and [γ-^{32}P]ATP from Amersham. Oligonucleotides were synthesized by the Biomolecular Resource Center at UCSF.

Enzymes. All restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs unless otherwise noted. T4 polynucleotide kinase was obtained from Pharmacia LKB

Biotechnology Inc., and the T4 DNA polymerase was purified in the laboratory of Bruce Alberts according to published procedures (27).

Plasmids. Plasmid pKHdda contains the *dda* gene downstream of the λ leftward promoter, P_L, controlled by the $c\mathit{I}^{857}$ repressor encoded by the plasmid; this plasmid has been previously described (15). Plasmid pBSPLO+ is a pBR322 derivative containing a fusion of the T4 gene 23 promoter to the *supF* gene (30). Plasmid pLD-del6, which expresses the *uvsW* gene, and plasmid pLD-del4, which does not express the *uvsW* gene, were the generous gifts of Drs. Leslie Derr and Ken Kreuzer.

Growth of Phage and Bacteria. Procedures and media for genetic crosses have been described (11). Phage and bacteria were grown in M9 media (14) for measurement of DNA synthesis. Equal inputs of parental phage were used for crosses during strain constructions (10).

Measurement of DNA synthesis. DNA synthesis was measured as the incorporation of [*methyl* -^{3}H]thymidine into trichloracetic acid (TCA) insoluble material as described by Gauss et al. (14).

Generation of the dda deletion. Plasmid pKHdda was digested with KpnI and XmnI. After deproteinization, the 3'-overhang generated by KpnI was removed by incubation with 0.8 μg of T4 DNA polymerase in replication buffer (60 mM each of dATP, dCTP, dGTP, and dTTP; 66 mM potassium acetate, 33 mM Tris Acetate, pH 7.8, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 100 μg/ml human serum albumin) for 1 min at 37°C. The DNA polymerase was inactivated by incubation at 65°C for 5 min. The

DNA was further digested with BamHI and SalI and the DNA fragments were electrophoresed on a 2% low melting agarose gel. The 103 bp BamH1-KpnI, and the 1041 bp XmnI-SalI fragments were recovered by melting the agarose at 65°C, followed by phenol extraction and ethanol precipitation. The above *dda* gene fragments (minus the KpnI-XmnI fragment, which contains nucleotides 104-284 of the *dda* gene (15)) were ligated into the pBSPLO+ plasmid. This creates a *dda* gene with an internal 181 bp deletion, which removes the Walker consensus sequence for ATP binding (32). After transformation into the bacterial strain MH1 by standard methods (26), the plasmid construct called pKHddadel1 was verified by restriction enzyme mapping.

Generation of T4 KH1 phage. T4 I/S phage containing the deletion in the dda gene were generated by substituting the modified *dda* gene from the plasmid pKHddadel1 for the wild type *dda* gene in the T4 I/S phage using the T4 insertion/vector protocol as described by Selick et al. (30). To screen for T4 I/S phage containing the deletion in the *dda* gene, phage were plated on host CR63 on NZCYM media plates (26) transferred to nitrocellulose filters, and after prehybridization, hybridized with 1 ng/ml of the 5' end labelled oligonucleotide 5'-CATATCAGGGAAAGAAG-CGAG-3' (which is complementary to the deleted region of the *dda* gene). Hybridization was carried out in 6X SSC (0.9 M sodium chloride, and 90 mM sodium citrate, pH 7), 1X Denhardt's reagent (8), 100 µg/ml yeast tRNA, and 0.05% sodium pyrophosphate for 15 hr at 35°C. The filters were washed 4 times for 5 min with 6X SSC and 0.05% sodium pyrophosphate at 25°C, once for 30 min at 45°C, and

autoradiographed (26). Plaques that did not hybridize with the oligonucleotide were isolated, added to 5 ml of CR63 in LB media, and incubated for 2 hr at 37°C. Cells were removed by centrifugation, and phage DNA was isolated and digested with EcoRV and with both EcoRV and SspI as described by Kreuzer and Alberts (21). Southern blotting with the above ^{32}P-labeled oligonucleotide as a probe was used to confirm the identity of the phage called T4 I/S KH1, whose DNA did not hybridize with this oligonucleotide.

The phage T4 I/S KH1 carries the new deletion mutation in the dda gene as well as the mutations in genes *r*II, *37* and *51* of the original I/S phage (30). To remove these mutations, a cross was performed with the T4 phage *sud1*, which contains a large deletion in the gene *39-56* interval that encompasses the *dda* gene and the *dexA* gene. The progeny of the cross were plated on the bacterial host LG1900 (NapIV optA1) which restricts the growth of *dexA*$^-$, *37*$^-$, or *51*$^-$ phage. Only phage carrying the constructed *dda* mutation will form plaques. The progeny phage were then screened for the presence of an *rII* mutation by plating them on the host CR63(λ) (*rII* mutants are restricted on a host with a λ lysogen). One of the nonrestricted phages, designated T4 KH1, was crossed to amHL628 (5, 11, 34) to construct the *dda*$^-$*59*$^-$ double mutants described in this paper.

RESULTS

Deletion of the *dda* gene results in a delay in early DNA synthesis during T4 infection. To determine if the

previously reported delay in DNA synthesis during infection by *dda*$^-$ phage containing multiple mutations was solely due to a *dda* gene mutation, the T4 insertion/substitution system was used to construct the mutant KH1 that is wild-type except for the deletion of DNA sequences within the *dda* gene (15, 30). To insure that KH1 phage had not picked up a *dexA*$^-$ mutation found in many previously characterized *dda*$^-$ phage strains, the phage were plated on an *optA*$^-$ host, LG1900. Because, as shown in Table 1, no restriction of KH1 phage growth was observed, the KH1 phage is dexA$^+$ (14).

The DNA synthesis during infection by wild-type and various *dda*$^-$ phage is shown in Fig. 1. Like the dda$^-$ mutants previously characterized (sud1 and L148), the KH1 *dda*$^-$ phage shows a DNA delay phenotype. The recovery by the sud1 and L148 mutants from the delay appears to be more rapid than the recovery by KH1. Since sud1 and L148 are known to carry additional mutations in other genes, it is possible that those mutations partially enhance this recovery period.

Either the 59 or the dda protein is necessary to obtain a significant amount of DNA synthesis during infection. A gene *59* amber mutant, *am*HL628, was crossed with the KH1 *dda*$^-$ phage to generate a *dda*$^-$*59*$^-$ double mutant. This was done in order to analyze the phenotype of a *dda*$^-$*59*$^-$ double mutant in the absence of other deficiencies that complicated the previous analyses (10). DNA synthesis during infection by the resulting *dda*$^-$*59*$^-$ phage, PG20, is shown in Fig. 2A. Like previously studied *dda*$^-$*59*$^-$ strains (Fig. 2B), the PG20 mutant displays a "DNA zero" phenotype (Fig 2A). No DNA synthesis at early or late times of infection is observed and

no phage are produced (Table 1). The defect in early DNA synthesis is therefore more severe in the *dda⁻59⁻* double mutant than in either of the single mutants (Figs. 2A & 2B).

Mutations in the uvsW gene partially suppress the DNA synthesis defect in *59⁻dda⁻* double mutant infections.

When growing the *dda⁻59⁻* double mutant phage, a significant number of apparent revertants capable of making plaques on nonsuppressing bacterial strains were observed. In previously reported studies, pseudorevertants of gene *59* mutants have been found to map to the T4 encoded *uvsW* gene (5, 33, 35). To determine if the suppressing mutation in our *dda⁻59⁻* double mutant was a deficiency in the *uvsW* gene, hosts containing a plasmid that expresses the *uvsW* gene, pLD-del6, were infected with our revertants (9). If a deficiency in the *uvsW* gene allows our *dda⁻59⁻* mutant to grow, then expression of the wild-type *uvsW* gene from a plasmid should stop phage growth. A plasmid identical to pLD-del6 except for a deletion of the promoter and Shine-Dalgarno sequences necessary for *uvsW* gene expression was used as a control (9).

The *dda⁻59⁻* pseudorevertant, PG21, like many other similar revertant mutants that we tested, did not produce plaques on hosts harboring a plasmid expressing the *uvsW* gene (Table II). This result is not simply due to inappropriate expression of the *uvsW* gene since wild-type phage growth is only reduced 2-fold in this host (Table II). When hosts containing the expression vector alone are infected by the *59⁻dda⁻* pseudorevertant, PG21, there is a delay in DNA synthesis compared to wild-type phage, like that seen with *dda⁻* phage (Fig. 3). These results suggest that the gene *59* deficiency but

not the dda deficiency is suppressed in this pseudorevertant and that the suppression is due to a deficiency in the *uvsW* gene.

DISCUSSION

Although *dda*$^-$ mutant phage show a DNA delay phenotype, *dda* is a nonessential gene because the amount of DNA synthesized eventually reaches nearly wild-type levels during late times of infection and the phage burst size is only slightly reduced (if at all). However, when a phage carries both *dda*$^-$ and *59*$^-$ mutations, little or no DNA synthesis is observed, and no phage are produced. A deficiency in gene *59* alone results in an arrest of DNA synthesis at late times of infection, but because DNA synthesis is normal at early times (34), some phage are produced (5, 11, 34). The defect in early DNA synthesis for the double mutant is more severe than observed in either *dda*$^-$ or *59*$^-$ mutants alone suggesting that the two gene products may interact synergistically during origin-dependent replication. In summary, either the gene *59* or the *dda* gene is necessary for phage growth but not both.

A molecular model to explain the genetic results is presented in Figure 4. This model is based on the properties of the 59 and Dda proteins *in vitro*: the gene 59 protein loads the gene 41 DNA helicase onto single-stranded DNA (J. Barry and B. Alberts, manuscript submitted); whereas the dda protein is a DNA helicase that, unlike the 41 DNA helicase, loads easily onto single-stranded DNA by itself (16). Thus, we propose that the Dda protein facilitates the loading of the gene 59-41 protein complex at replication origins by increasing the length of single-stranded DNA available for binding of the complex. In *dda*$^-$ phage, less DNA is unwound at the origin and the

binding of the gene 59-41 protein complex is inefficient, although the gene 41 protein alone is eventually loaded onto the template to form a primosome at the replication fork. In *59*$^-$ phage, the Dda protein helps the gene 41 protein load onto the template by generating a long region of single-stranded DNA at the origin to serve as a loading zone for the 41 protein (J. Barry and B. Alberts, manuscript submitted). In *dda*$^-$*59*$^-$ phage, the length of DNA unwound at the origin may be too short for binding by gene 41 protein alone, so few if any normal replication forks are formed. We have proposed in the model in Figure 4 that the first steps in both origin unwinding (early) and genetic recombination (late) are normal in the *dda*$^-$*59*$^-$ mutant. These initial steps could involve some DNA synthesis, which escapes detection when [H^3]thymidine incorporation is measured.

The *uvsW* mutation suppresses the gene 59 but not the Dda protein deficiency. In the scheme diagrammed in Figure 4, an additional mutation in the *uvsW* gene would allow the double *59*$^-$ *dda*$^-$ mutant to produce phage by enabling DNA synthesis to occur during late times of infection - possibly by allowing an alternate form of replication initiation (9, 28) that does not require either Dda or 59 gene products.

The model in Figure 4 suggests that there is a synergistic interaction of the Dda and gene 59 proteins during origin-dependent DNA replication. However, the gene 59 protein may not normally play a role in loading the gene 41 protein onto the DNA at early times after infection with wild-type phage, being needed only when the phage is deficient in the Dda protein. This view remains viable, in as much as *59* mutants alone display little (if any) defects in DNA

synthesis at early times after infection (Fig 2 and references 5, 34), or in the replication of plasmids containing a T4 origin (22).

Another possible alternative to the model presented in Figure 4 is that early in a *dda*$^-$ phage infection, the gene 59 protein loads the gene 41 protein at D-loops created by genetic recombination. But, the presence of recombination intermediates at early times is a matter of controversy. Using a density shift technique, Dannenberg and Mosig detected a change in phage DNA density that they intrepreted to be a result of genetic recombination 5 to 6.5 minutes after phage infection (one minute after the onset of DNA replication) (6). However, others have detected genetic recombination only 15 to 20 minutes (4) and 11 minutes (19, 20) after infection. The arrest of DNA synthesis 20 to 25 minutes after infection with phage carrying mutations in genes involved in DNA recombination (5) may mean that DNA replication is initiated from recombinational intermediates only after several rounds of origin-dependent replication have occured.

In summary, we have generated a T4 *dda*$^-$ phage and used this phage to construct *dda*$^-$*59*$^-$ double mutants. The *dda* mutant phage have a DNA delay phenotype, which is extended to a severe block in early DNA synthesis when the phage is also mutant in the *59* gene. This result suggests that both the dda and gene 59 proteins may play a role in the origin-dependent DNA replication, which occurs at early times of infection. A model for the roles of the Dda and gene 59 proteins based on our genetic results can be derived that is based on their in vitro biochemical properties. Verifying the suggested roles these two proteins is likely to require reconstitution of the T4 DNA

replication initiation process in a purified in vitro system containing a T4 replication origin (3, 21, 22).

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TABLE 1. The T4 dda deletion phage, KH1, grows normally on *E. coli optA*$^-$ hosts

Phage	Plating efficiency[a] Host	
	optA$^+$	*optA*$^-$
wild-type T4	(1.0)	0.92
kh1 (dda$^-$)	(1.0)	0.71
sud1 (dda$^-$dexA$^-$)	(1.0)	$< 1.1 \times 10^{-4}$

[a]The host cells were grown at 30°C in H-broth. Plating efficiency is expressed as the number of plaques observed divided by the number of plaques on an *E. coli.* optA$^+$ strain.

TABLE 2. Extrachromosomal expression of T4 uvsW restricts the growth of a dda^{-}59^{-} pseudorevertant

Phage	Burst size[a] control plasmid (pLD-del4)	uvsW expression plasmid (pLD-del6)
T4	210	100
PG20 (dda^{-}59^{-})	0.04	0.02
PG21 (dda^{-}59^{-}revertant)	31	0.7

[a]Host cells were grown at 30°C in M9 media to a concentration of 2 x 10^8 cells per ml. The cell titer was determined immediately before infection. At 90 min post infection, the cells were lysed with chloroform and the number of phage was determined by plating the lysate on CR63 cells at 30°C. Burst size is expressed as the number of viable plaques produced per cell.

FIGURE LEGENDS

FIG. 1. DNA synthesis after infections of *E. coli* (strain NapIV) in M9 medium at 30°C with wild-type and *dda*$^-$ T4 bacteriophage. Incorporation of [*methyl*-^{3}H]thymidine in trichloracetic acid-insoluble DNA was measured. Infections with sudI and L148 (dotted lines) were preformed in experiments separate from those with KH1, and the amount of [*methyl*-^{3}H]thymidine incorporated in these experiments was normalized by reference to wild-type.

FIG. 2. DNA synthesis after infections with *59*$^-$, *dda*$^-$, and double mutant T4 bacteriophage. Incorporation of [*methyl*-^{3}H]thymidine in trichloracetic acid-insoluble DNA was measured. (A) The *dda*$^-$ mutant used was KH1, and the *59*$^-$*dda*$^-$ double mutant phage was PG20. (B) Infections were carried out as in (A), except that different mutant phage were used: the *59*$^-$ mutant was *am*HL628, and the *59*$^-$*dda*$^-$ double mutant phage was *am*HL628sud1 (10, 34).

FIG. 3. DNA synthesis after infection with *59*$^-$*dda*$^-$ double mutant phage carrying a supressor mutation. All hosts contained a plasmid. As a control, hosts containing pLD-del4 (vector only) were infected with wild-type T4, PG20 (*59*$^-$*dda*$^-$), and PG21 (*59*$^-$*dda*$^-$ pseudoreverant) phage. The same bacteria containing pLD-del6 (uvsW expression vector) were infected with PG21. Incorporation of [*methyl*-^{3}H]thymidine in trichloracetic acid-insoluble DNA was measured.

FIG. 4. A molecular model for the roles of the bacteriophage T4 dda and gene 59 proteins during infection. (A) At early times of infection by wild-type phage, the replication origin binding proteins unwind the origin, and this single-stranded DNA is extended by the dda helicase. The gene 59 protein efficiently loads the gene 41 DNA helicase on to the unwound region, which leads to the formation of a complete DNA replication fork. At late times of infection, the gene 41 DNA helicase is loaded by the gene 59 protein at the D-loop formed by strand invasion, following the synapsis step in genetic recombination.

(B) During infection by *dda*$^-$ phage, the origin is unwound but the length of single-stranded DNA is not extended since the dda protein is absent. After a period of time, the gene 59 protein loads enough molecules of the gene 41 DNA helicase to give efficient DNA synthesis, but the start of this synthesis is delayed. Recombination-dependent DNA synthesis at late times of infection is uneffected by the dda helicase deficiency.

(C) During infection by *59*$^-$ phage, the length of single-stranded DNA at the origin is extended by the dda helicase and serves as a loading zone for the gene 41 protein. DNA synthesis occurs with no noticeable delay (5, 34). At late times of infection, the gene 41 protein is not loaded onto the D-loop due to the gene *59* deficiency, and thus no DNA synthesis occurs.

Figure 1

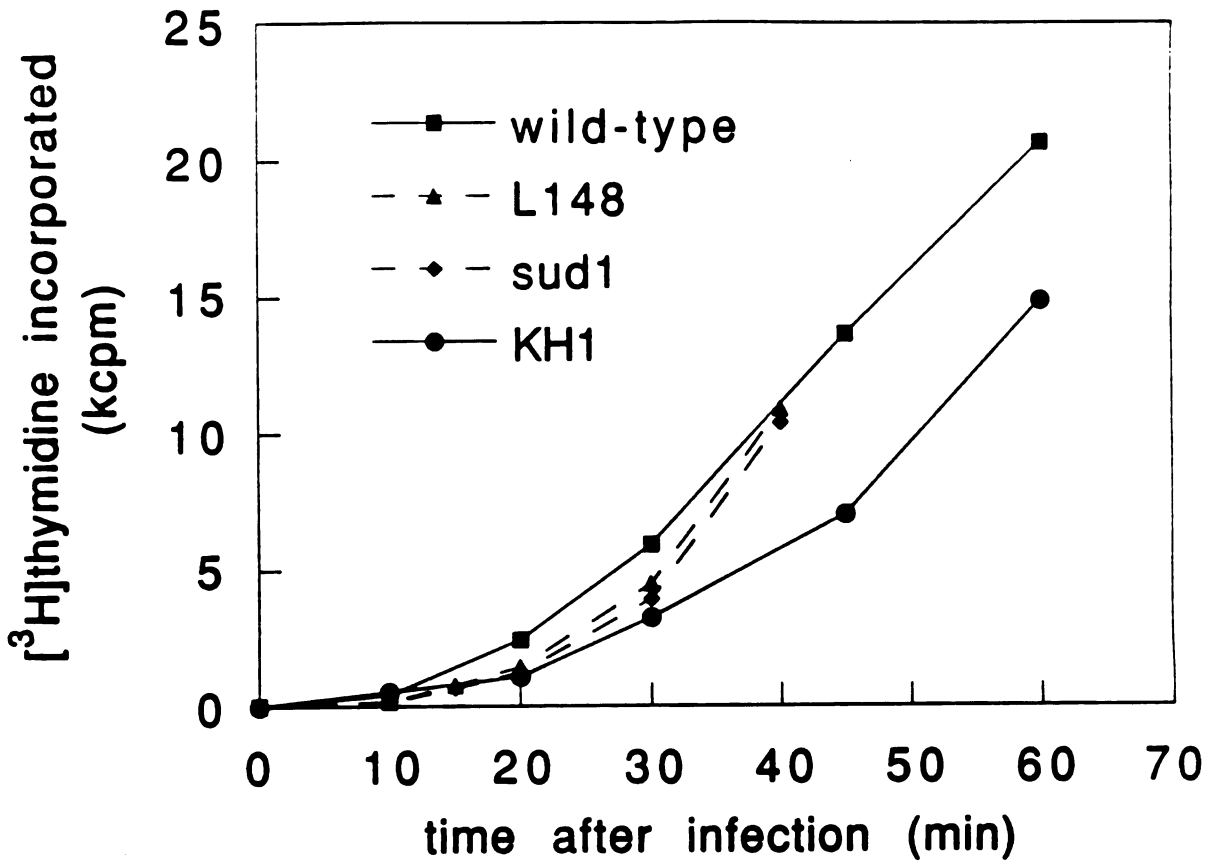


Figure 2A

A.

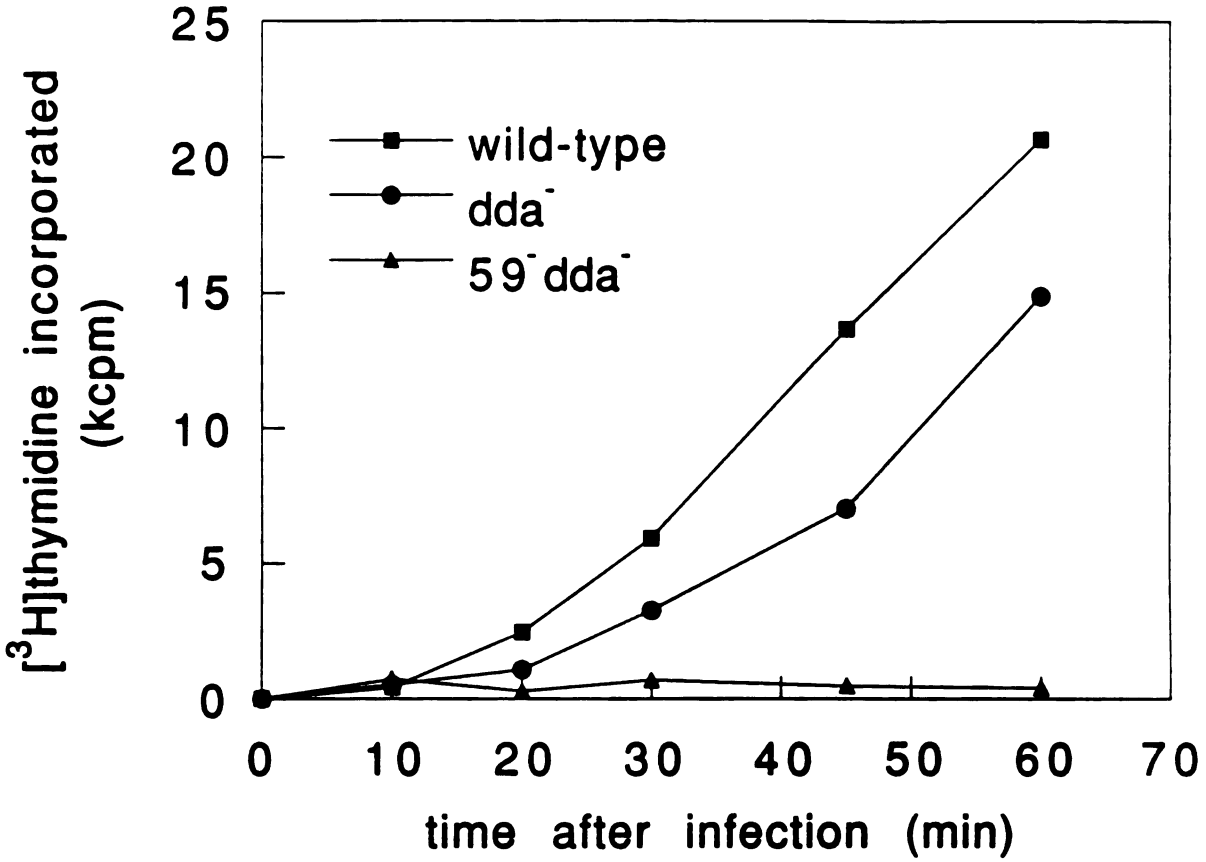


Figure 2B

B.

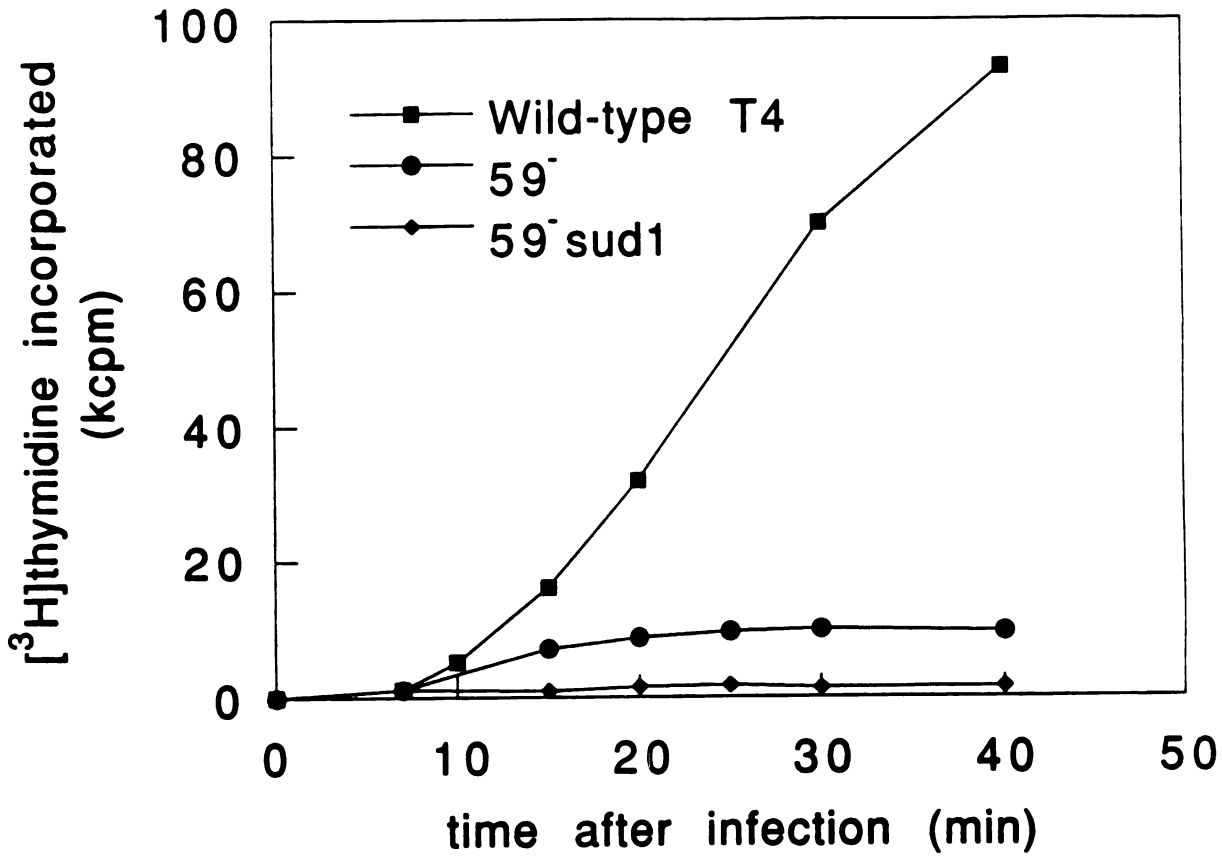

100
80
60
40
20
0
[3H]thymidine incorporated (kcpm)
Wild-type T4
59-
59- sud1
0
10
20
30
40
50
time after infection (min)

Figure 3

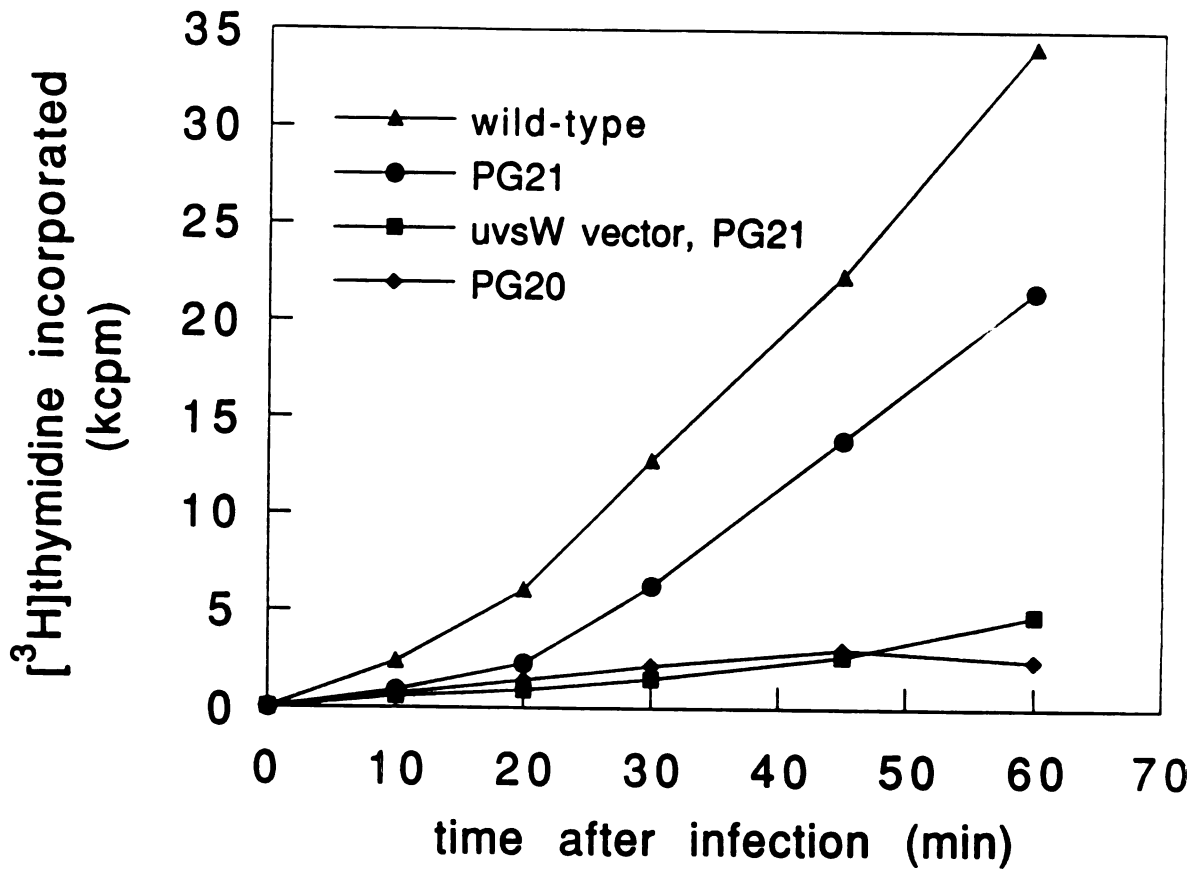
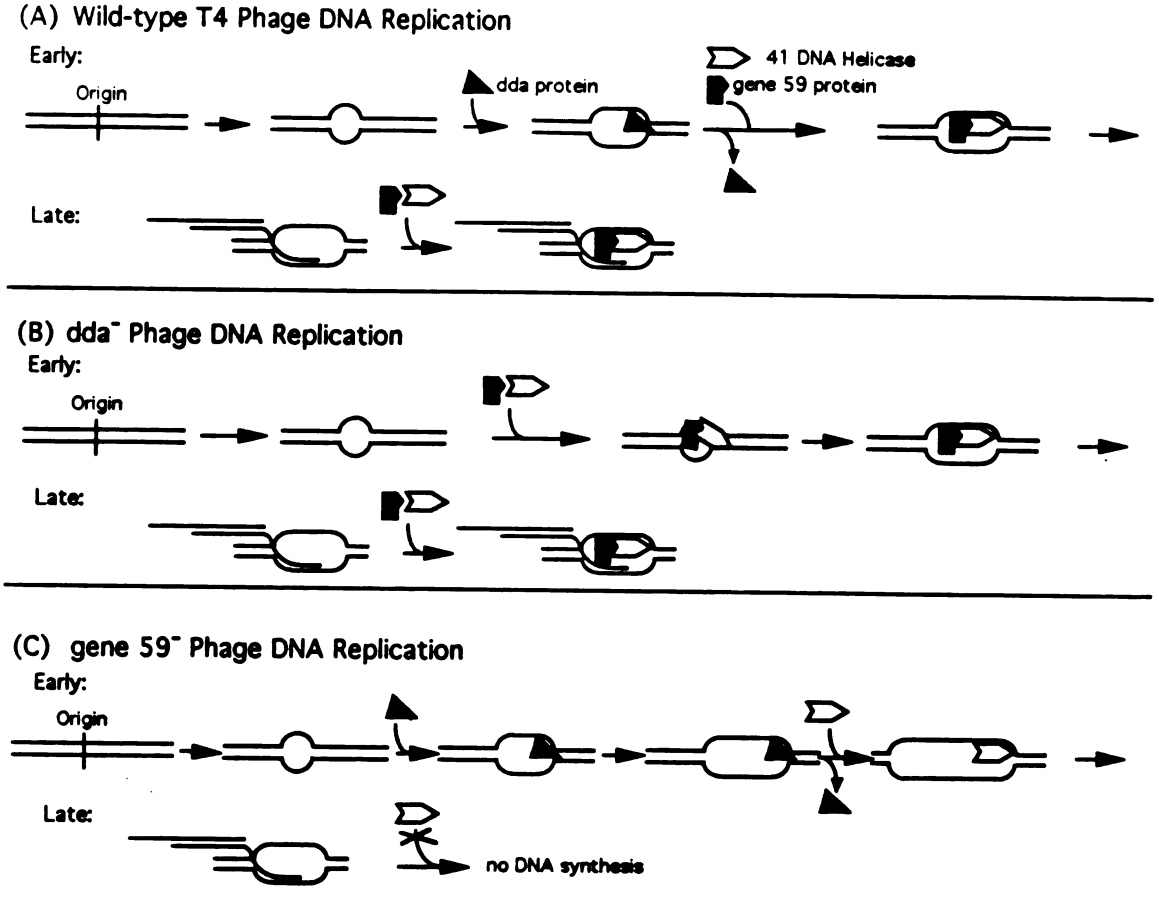

[3H]thymidine incorporated (kcpm)
35
30
25
20
15
10
5
0
wild-type
PG21
uvsW vector, PG21
PG20
0
10
20
30
40
50
60
70
time after infection (min)

Figure 4


(A) Wild-type T4 Phage DNA Replication
Early:
Origin
dda protein
41 DNA Helicase
gene 59 protein
Late:
(B) dda⁻ Phage DNA Replication
Early:
Origin
Late:
(C) gene 59⁻ Phage DNA Replication
Early:
Origin
Late:
no DNA synthesis

CHAPTER 4

KINETICS OF T4 DNA POLYMERASE HOLOENZYME DISSOCIATION WHEN STALLED BY NUCLEOTIDE OMISSION

ABSTRACT

We have developed an experimental assay to monitor the rate of dissociation of the T4 DNA polymerase holoenzyme (polymerase plus gene 44/62 and 45 proteins), once it has been stalled by nucleotide omission. Using this assay, we determined that the dissociation of the DNA polymerase holoenzyme follows a first order decay with a half-life of 2.5 min. The long half-life resembles that expected for the holoenzyme processively synthesizing DNA on the leading-strand of the replication fork. The holoenzyme dissociation rate is independent of the concentration of the DNA polymerase accessory proteins (44/62 and 45 proteins) and independent of ATP hydrolysis. The rate of holoenzyme dissociation is increased if the gene 32 protein is omitted, or if the primer-template region is shortened from 46 to 28 base pairs. But the rate of holoenzyme dissociation is most strikingly increased when the circular DNA template is converted to a linear form.

By analogy with other well studied prokaryotic and eukaryotic systems, these results support a model where ATP hydrolysis by the T4 gene 44/62 proteins serves to assemble a ring-like 45 accessory protein onto the DNA. Once assembled onto the DNA, the 45 protein—possibly along with the 44/62 complex—acts as a sliding clamp that tethers the DNA polymerase to the template. In the

accompanying paper, we examine the mechanism that regulates the switch of the holoenzyme from this relatively tight template bound state to a weakly bound state. This switch is essential for recycling of the holoenzyme during Okazaki fragment synthesis on the lagging-strand of the replication fork.

INTRODUCTION

The T4 DNA polymerase holoenzyme is analogous to the DNA polymerase holoenzymes of *E. coli* and eukaryotic replication systems and can be divided into three functional components: the gene 43 DNA polymerase, the gene 45 DNA polymerase accessory protein, and the gene 44/62 DNA polymerase accessory protein complex.

The 104 kDa gene 43 polymerase binds to itself and may exist as a dimer in solution (Alberts *et al.*, 1983; Munn and Alberts, 1991b). It has both a 5'-3' polymerase and a 3'-5' exonuclease activity, and it moves with a low degree of processivity under physiological salt conditions—frequently dissociating from the DNA. However, both its DNA polymerase and its exonuclease activities become highly processive in the presence of the DNA polymerase accessory proteins (Alberts, 1987; Nossal, 1992; Young *et al.*, Bedringer *et al.*, 1992).

It has been reported that the 77 kDa gene 45 protein trimer alone can increase the processivity of the 43 protein on a primed-single-stranded M13 molecule when a molecular crowding agent, polyethylene glycol, is included in the reaction (Jarvis *et al.*, 1989a; Reddy *et al.*, 1993). This effect is inefficient: only a few of the primers are elongated to an extent greater than that observed with the DNA polymerase alone. In the *E. coli* and yeast replication systems, very similar results are obtained when high concentrations of the β-subunit of the polymerase III DNA holoenzyme or the yeast PCNA polymerase accessory protein are added to reactions, respectively (LaDuca *et al.*, 1986; Burgers and Yoder, 1993). The X-

ray analysis of the *E. coli* β-subunit has shown that it is shaped like a ring with a center large enough to accommodate duplex DNA (Kong *et al.*, 1992). Elegant biochemical studies support the view that the β-subunit encircles the DNA template, tethering the pol III core to the template like a "sliding clamp" (Stukenberg *et al.*, 1991). The T4 45 gene protein, the *E. coli* β-subunit and the eukaryotic PCNA protein are believed to be analogs (O'Donnell *et al.*, 1992).

The 164 kDa gene 44/62 polymerase accessory protein complex is formed by the tight association of the proteins encoded by T4 genes 44 and 62; this association can only be broken by treatments that denature proteins. The gene 44/62 complex consists of four gene 44 protein molecules and one gene 62 protein molecule (Jarvis *et al.*, 1989a). The gene 44 protein alone has DNA-dependent ATPase activity, whereas the gene 62 protein does not (Rush *et al.*, 1989; Jarvis, *et al.*, 1991). Since each gene 44/62 complex contains four gene 44 molecules, this suggests that each complex has the potential to bind and hydrolyze four ATP molecules in concert. In the presence of the 45 protein, the DNA-dependent ATPase activity of the gene 44/62 protein complex becomes maximal when the DNA substrate is a primer-template junction with the primer being at least 18 nucleotides long (Jarvis *et al.*, 1989b). The gene 44/62 complex is analogous to the five subunit γ complex in the *E. coli* DNA replication system and to the five subunit RF-C complex in the yeast and human DNA replication systems (O'Donnell *et al.*, 1992; O'Donnell *et al.*, 1993). The 45 protein increases the ATPase activity of the 44/62 protein complex, and analogous ATPase stimulations are observed in the bacterial and mammalian systems (Piperno and

Alberts, 1978; Mace and Alberts, 1984; Jarvis *et al.*, 1989; Hurwitz *et al.*, 1990; Tsurimoto and Stillman, 1990; Onrust *et al.*, 1991; Tsurimoto and Stillman, 1991).

Although the 44/62 complex alone does not seem to interact with the 43 DNA polymerase, when it is added to reactions containing both the 45 and the 43 proteins, the DNA polymerase holoenzyme is formed in an ATP dependent manner. The holoenzyme performs DNA synthesis and nucleotide excision with much more processivity than does the DNA polymerase alone (Sinha *et al.*, 1980; Huang *et al.*, 1981; Venkatesan and Nossal, 1982; Alberts *et al.*, 1983; Bedinger and Alberts, 1983; Mace and Alberts, 1984; Jarvis *et al.*, 1991).

Addition of the T4 gene 32 single-stranded DNA binding protein (a helix-destabilizing protein) further increases the processivity of the DNA polymerase holoenzyme and enables the holoenzyme to carry-out strand-displacement DNA synthesis (Alberts *et al.*, 1983; Cha and Alberts, 1988). The gene 32 protein affects holoenzyme processivity by destabilizing short DNA helices that impede DNA synthesis; it also binds to each protein component of the holoenzyme and may thereby help stabilize their protein-DNA interactions (Huang *et al.*, 1981; Alberts *et al.*, 1983; Formosa *et al.*, 1983; Capson *et al.*, 1991; Munn and Alberts, 1991).

Addition of the primosome (composed of the gene 41 helicase and the gene 61 primase) to *in vitro* reactions containing the DNA polymerase holoenzyme, the gene 32 protein, and a nicked DNA template reconstitutes a fork with both leading- and lagging-strand DNA sythesis. The replication fork closely resembles forks found *in*

vivo in terms of protein requirements, fidelity, RNA primer synthesis, size of Okazaki fragments and rate of movement (Epstein et al., 1964; McCarthy et al., 1976; Hibner and Alberts, 1980; Liu and Alberts, 1980; Nossal, 1980, Sinha *et al.*, 1980). In the reconstituted replication system, DNA synthesis on the leading strand of the replication fork is processive for at least 165,000 nucleotides: an acheivement that requires a half-life of 4 minutes or more for the association of the DNA polymerase holoenzyme with the DNA. Theoretical calculations suggest that the holoenzyme-DNA complex in vivo will also have a half-life of minutes (Young *et al.*, 1992). In contrast, on the lagging-strand of the replication fork the half-life of the holoenzyme-DNA complex is expected to be 1-2 seconds, both *in vitro* and *in vivo* (Selick *et al.*,1987).

In this report, we examine the dissociation of the T4 DNA polymerase holoenzyme from the DNA template when the polymerase holoenzyme is stalled by omission of a nucleoside triphosphate. We find that the half-life of the stalled holoenzyme—DNA complex resembles that expected for a holoenzyme processively replicating DNA on the leading-strand of a replication fork.

MATERIALS AND METHODS

Reagents and Enzymes—All restriction and DNA modifying enzymes were purchased from New England Biolabs unless otherwise noted. EcoRI enzyme was purchased from Boehringer Mannheim, and E. *coli* DNA polymerase I Klenow fragment from Bethesda Research Laboratories. Polynucleotide kinase, deoxyribonucleoside triphosphates (ultrapure) and adenosine triphosphate were obtained

from Pharmacia LKB Biotechnology Inc., ampicillin from Roerig/Pfizer, and agarose from FMC Bioproducts. Radioisotopes were purchased from Amersham. Poly dT_{1500} and poly dA_{50} were from Supertechs, Bethesda, MD. Other chemicals (including ATPγS) were from Sigma unless specifically noted. Oligonucleotide primers were synthesized using an 8400 Cyclone Plus DNA synthesizer (MilliGen/Biosearch, MA).

The T4 DNA replication proteins (the gene 32, 43, 44/62, and 45 proteins) were purified by published protocols (Bittner *et al.*, 1979; Morris *et al.*, 1979) with modifications by Jack Barry and Bruce Alberts (manuscript submitted).

Purification of nucleoside triphosphates— dATP, dGTP and dTTP were individually purified to remove minute traces of contaminating dCTP by chromatography at 4°C on an 8 ml Mono Q HR 10/10 column (Pharmacia), with a mobile phase of ammonium carbonate as described for ribonucleotides triphosphates by Herpich and Krauss (1992). 10 μmol of nucleotide in 30 mM ammonium carbonate buffer, pH 8.95, was loaded onto the column at a rate of 3 ml/min (separately, 0.1 μmol dCTP was purified as a control). The column was washed with 4 column volumes (cv) of 30 mM ammonium carbonate, followed by 4 cv of 165 mM ammonium carbonate, and the nucleotide was eluted with an 18 cv linear gradient of 165 to 300 mM ammonium carbonate. The column was then washed with 6 cv of 300 mM ammonium carbonate. In the linear gradient of ammonium carbonate the control dCTP eluted first (186-203 mM) followed by dTTP (190-230 mM), dATP (210-240 mM), and dGTP (263-300 mM), respectively. Main peak fractions

clearly separated from the control dCTP were pooled and evaporated to dryness in a speedvac concentrator (Savant Instruments, NY). To remove the remaining traces of ammonium carbonate, the fractions were resuspended in water and taken to dryness; this was repeated until the dried fractions no longer contained a visible precipitate. The nucleotides were then resuspended in water.

Nucleic Acids— Circular single-stranded bacteriophage M13mp7 DNA was isolated by extraction from purified phage particles, and its concentration was determined by absorbance at 260 nm using 36 μg/ml/A_{260} (Sambrook *et al.*, 1989).

Polynucleotide kinase was used for 5' end labeling of oligonucleotides with ^{32}P under standard conditions (Sambrook *et al.*, 1989). After labeling, unincorporated radioisotopes were removed by passage through a Biospin-6 column (Bio-Rad Laboratories, CA), following the manufacturer's instructions.

For 3' end labeling of oligonucleotides, 0.08 μM (as molecules) of oligonucleotide annealed to single-stranded DNA was incubated with 2 units E. *coli* DNA polymerase I Klenow fragment and 10 μM each of [α-^{32}P]dGTP and [α-^{32}P]dTTP (specific activity > 3000 Ci/mmol), in 25 μl modified Klenow buffer (47 mM potassium acetate, 50 mM Tris acetate, pH 7.8, 10 mM magnesium acetate, 1 mM dithiothreitol, 200 mg/ml human serum albumin) for 15 min at 23°C. The labeling reaction was stopped by phenol extraction and the unincorporated nucleotides were removed by serial passage of the sample through two Biospin-30 columns (Bio-Rad Laboratories, CA).

Labeled oligonucleotides were annealed to single-stranded DNA templates in annealing buffer (66 mM potassium acetate and 33 mM Tris-HCl, pH 7.8) by incubation at 75°C for 4 min followed by slow cooling to 37°C.

Construction of plasmid pKH32G— All cloning and DNA isolation procedures were performed according to Sambrook *et al.* (1989) unless specified otherwise. The insert in pKH32G was a 1600 nucleotide DNA fragment comprised of 32 repeats, of which each contained only one G nucleotide (see Figure 9A). The insert was generated starting from the oligonucleotide template 5'-CGCGGATCCN_{44} AGATCTGGGGCTGCAG-3' (N= random C, A, or T), which contains BamHI and BglII restriction sites (underlined). A total of 7 μg of the oligonucleotide primer 5'-ATTCCTGCAGCCCCAGATC-3' was annealed to 7 μg of the above oligonucleotide template in AB buffer (40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 50 mM NaCl) and extended by incubation with 12.5 units E. *coli* DNA polymerase I Klenow fragment in 100 μl reaction buffer (40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl, 1 mM dithiothreitol, 100 μg/ml human serum albumin and 120 μM each of dATP, dCTP, dGTP, and dTTP) for 15 min at 23°C and then for 15 min at 37°C. The polymerase was inactivated in 2% SDS, 20 mM Na_3EDTA, 130 mM NaCl by heating for 10 min at 65°C. After slow cooling, the mixture was passed through a P-30 spin column to remove the polymerase and nucleotides. The DNA product was digested with BamHI and BglII, purified by electrophoresis on a 12% polyacrylamide, 10 mM Tris-borate, pH 7.4, 1 mM Na_3EDTA (TBE) gel. The repeat unit of 50 base-pairs with BamHI/BglII ends (5μg)

was recovered using the crush-soak method and further purified on a NAC52 column (Bethesda Research Laboratories, Md), following the manufacturer's instructions.

While DNA digested with BamHI or BglII have complementary 5' ends, ligation of an end created by BamHI with an end created by BglII generates a site that cannot be recut by either enzyme. Therefore, this DNA could be ligated into a series of direct repeats by incubation with BamHI and BglII in addition to ligase, following the protocol of Rosenfeld and Kelly (1986). After the ligation, the samples were electrophoresed on a 2% low melting agarose, 10 mM Tris-acetate, pH 7.4, 1 mM Na_3EDTA (TAE) gel. DNA bands representing more than two ligated repeats were cut out of the gel and cloned into the BamHI/BglII site of the plasmid BlueScript (pBS+) (Stratagene, CA). After the plasmid constructs were transformed into E. *coli* DG98 cells, double-stranded DNA was isolated from a number of clones. Sequencing by the procedure of La Baer (1989) showed that all inserts contained a single G nucleotide per fifty nucleotide repeat, as expected. Two of nine plasmids containing inserts (each with 4 repeats) lacked sites that slowed movement of the T4 DNA polymerase holoenzyme. These two inserts were ligated together to form one 400 nucleotide insert with 8 repeats using the two-fold amplification technique of Rosenfeld and Kelly (1986), and transformed into E. *coli* HB101 ($recA^-$) cells. By continuing this type of process, we obtained the pKH32G construct, which contains 32 repeats. The pKH32G construct was transformed into E. *coli* JM109 (F factor-containing, $recA^-$) cells, and single-stranded pKH32G DNA was obtained using the Stratagene protocol (Stratagene, CA).

T4 DNA polymerase holoenzyme dissociation assay—The DNA replication proteins (7.5 ng/ml 43 protein, 7.5 μg/ml 45 protein, 7.5 μg/ml 44/62 protein complex, and 3.6 μg/ml gene 32 protein) were mixed with 0.8 μg/ml DNA template (single-stranded M13 mp4 DNA annealed with a 5'-^{32}P-end labeled oligonucleotide of 38 nucleotides, 1.25 mM ATP, dGTP, and dTTP (0.625 μM each) in 24 μl of replication buffer (160 mM potassium acetate, 33 mM Tris acetate, pH 7.8, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 200 μg/ml human serum albumin). After a 10 sec incubation at 23°C, 20 μl of the sample was added to 5 μl of "free polymerase trap" consisting of 164 μg/ml (as nucleotides) poly dT_{1500} · oligo dA_{50} (1:3 molar ratio) and 177 μg/ml gene 32 protein in replication buffer. The reactions were incubated for varying times at 23°C. Primer elongation to the hairpin was then initiated by adding 20 μl of reaction mixture to 4 μl containing of dATP, dCTP, dGTP, and dTTP (0.72 mM each), plus 1 mM ATP in replication buffer. Elongation of the primer was stopped after 10 sec. by the addition of 50 μl 95% formamide/20 mM Na_3EDTA, and the samples were immediately placed in an ice water bath. Just prior to electrophoresis, the samples were heated for 4 min at 100°C and then immediately cooled to 0°C.

After samples were electrophoresed through an denaturing 8% polyacrylamide gel (Sambrook *et al.*, 1989), the radioactivity in each band on the gel was quantitated using a PhosphorImager (Molecular Dynamics). For each time point, the percentage of holoenzymes associated with the template was determined as $[(A\text{-}bk_A)/((A\text{-}bk_A) + (D\text{-}bk_D))] \times 100$, where A is the amount of radioactivity in the band at the hairpin, D is the amount of radioactivity in the band at the dG

site, and bk_A and bk_D are background on the gel in a control lane. The results were fitted by the least squares method to an exponential equation.

Assay for the effect of template linearization on the dissociation of the DNA polymerase holoenzyme-DNA complex— All reactions were performed at 37°C. The DNA replication proteins (14 ng/ml 43 protein, 7.0 μg/ml 45 protein, and 7.0 μg/ml 44/62 protein complex, and 4.6 μg/ml gene 32 protein) were mixed with 0.46 μg/ml primed pKH32G single-stranded DNA, 1.0 mM ATP; and either 0.63 μM or 120 μM each of dATP, dGTP, and dTTP in 50 μl of replication buffer. After a 20 sec incubation, 40 μl of the sample was added to 10 μl of "free DNA polymerase trap" consisting of 22μg/ml M13 single-stranded DNA annealed with 5 primers (29 nucleotide average length) equally spaced around the molecule, 224 μg/ml gene 32 protein, and 1 mM ATP in replication buffer. The incubation was continued for 20 sec and then 36 μl of sample was added to either 4 μl of 50 units/μl EcoRI in storage buffer (10 mM Tris-HCl, pH 7 at 4°C, 200 mM NaCl, 1 mM Na_3EDTA, 0.5 mM dithiothreitol, 0.2% Triton X-100, and 50% (v/v) glycerol as described by Boehringer Mannheim Biochemica) or 4 μl of storage buffer. The samples were incubated for various times before a 18 μl aliquot was removed and added to another tube containing 2 μl of 1.2 mM each of dATP, dCTP, dGTP, and dTTP, and 1 mM ATP in replication buffer. At the same time, 40 μl 95% formamide/20 mM Na_3EDTA was added to the remaining 22 μl of sample to stop its reaction. After 20 sec (or 30 sec in reactions containing 120 μM each of dATP, dGTP, and dTTP), the reactions containing dCTP were stopped by the addition of 95%

formamide/20 mM Na3EDTA. Just prior to electrophoresis the samples were heated for 4 min at 100°C and then immediately placed in an ice water bath. Samples were electrophoresed through a denaturing 8% polyacrylamide gel (Sambrook *et al.*, 1989). The radioactivity in each DNA band was quantitated with a PhosphorImager (Molecular Dynamics).

RESULTS

The design of a DNA polymerase holoenzyme dissociation assay–

The DNA template we used is diagrammed in Figure 1. Its most important features are the following: (1) The template is circular. The holoenzyme is less stable on linear DNA templates, as shown below. (2) The holoenzyme stop site, in the absence of dCTP, consists of three consecutive G nucleotides; a triple nucleotide stop site is much less likely than a single nucleotide stop site to be traversed by the holoenzyme in the presence of the low concentrations of dCTP that contaminate dATP, dGTP and dTTP stocks (see below and unpublished results). (3) The primer is 5'-^{32}P- end labeled; therefore, the amount of radioactivity we observed in the various products of DNA synthesis is directly proportional to the number of holoenzyme molecules that have elongated a primer. (4) A holoenzyme molecule that has been allowed to traverse the G nucleotide stop site will quickly encounter a DNA hairpin helix of 22 base-pairs that acts to block DNA synthesis. This block occurs after forty nucleotides have been incorporated. Those primers that are elongated past the G nucleotide stop site therefore produce a single

DNA band on a 8% polyacrylamide denaturing gel of 78 nucleotides (40 + 38 nucleotide primer), whereas those that stop at the first G produce a DNA band of 46 nucleotides.

The assay that we designed with this template is diagrammed in Figure 2. The holoenzyme was stalled by omission of one of the four deoxyribonucleoside triphosphates, dCTP. After varying times of stalling, dCTP was added to test for those holoenzymes that remained associated. A trap for any free DNA polymerase was present in these reactions, which allowed new holoenzymes to form only during a very short incubation period before the trap is added (10 sec in most assays; Fig. 2). The trap also prevents the association of a second holoenzyme with the labeled primer-template after the first DNA polymerase holoenzyme molecule dissociates.

The trap generally consisted of poly dT_{1500}·oligo dA_{50} (1:3 molar ratio) covered with the T4 gene 32 protein (a single-stranded DNA binding protein, SSB), and it was used at 8,300 3' -OH ends of trap per 5'-^{32}P- end labeled primer.

During development of the assay, other traps were tested-including sonicated single-stranded DNA from calf thymus and circular single-stranded DNA from wild-type M13 annealed with multiple primers. Judged by the blockage of labeled-primer elongation when added to the reaction mixture before the replication proteins, the dT_{1500}·oligo dA_{50} was found to be the most efficient per nucleotide at preventing DNA polymerase association with the labeled primer-template.

The polymerase holoenzyme dissociates slowly when it is paused by nucleoside triphosphate omission—

An autoradiograph of a denaturing polyacrylamide gel used to analyze the DNA products obtained from the Figure 2 assay is shown in Figure 3. Assay controls are on the left side of the autoradiograph, in lanes 1-8. Lanes 1-5 show that, in the absence of the DNA polymerase accessory proteins (the proteins encoded by genes 45, 44 and 62, which are components of the DNA polymerase holoenzyme), none of the primers are elongated to the first stop site under our experimental conditions of salt and temperature. This means that the products of reactions due to DNA polymerase molecules that are not incorporated into a holoenzyme are eliminated. Achieving this result required raising the concentration of potassium acetate from our standard 66 mM to 160 mM, and dropping the temperature to 23°C. In addition, the concentrations of dATP, dGTP and dTTP were kept well below the K_m, so that only tightly bound holoenzymes would remain associated long enough to elongate the primer to the G stop site.

A second control experiment is displayed in lanes 6-8, which demonstrates that, during incubations of 2, 4, and 8 minutes in the absence of dCTP, a single DNA product of 78 nucleotides is obtained, corresponding to molecules where the holoenzyme has paused before the run of three G nucleotides (see Figure 2). Thus, the holoenzyme molecules remain stalled at this site throughout an incubation in the absence of dCTP.

The results of the actual experiment are shown in lanes 9-13, where we have assayed for the continued presence of the

holoenzyme for the indicated stalling times before addition of dCTP. As expected, the number of primers elongated to the hairpin, which represent holoenzymes that have remained associated with the DNA template during the incubation in the absence of dCTP, decreases with increasing incubation times. Conversely, the intensity of the band corresponding to primers elongated to the G site, which represents holoenzymes that have dissociated from the DNA template, increases with increasing incubation times.

We can be sure that the DNA polymerase trap present in the Figure 3 experiment is effective: even though 99 percent of the primers remained at their initial length at the time of trap addition, new primers were not elongated during the subsequent incubations. Each elongated primer therefore is created by the association-dissociation of a single holoenzyme molecule under our conditions.

The percentage of holoenzymes that remained associated with the template in the Figure 3 experiment was quantitated, as plotted in Figure 4. The holoenzyme dissociation is seen to follow a simple exponential decay with a half-life of 2.5 min for the holoenzyme-DNA template complex.

To insure that the observed results represent only one holoenzyme association and dissociation event per labeled primer-template, the Figure 3 experiment was repeated with a 2 fold lower DNA polymerase concentration and, separately, with a 2 fold higher DNA trap concentration. Each of these changes should decrease DNA polymerase reassociation, if it is occurring. The results were not altered by these changes (Figure A-1). As a further control we repeated the Figure 3 experiment with a different DNA trap: one that

consisted of 5 primers annealed at equal distances around a wild-type M13 DNA molecule that had been preincubated with a high concentration of DNA polymerase accessory proteins. The rate of holoenzyme dissociation was not increased in the presence of this trap, and omitting the preincubation of the M13 DNA molecules with the accessory proteins did not alter the results (Figure A-1).

The rate of dissociation of the holoenzyme is faster from a shorter primer/template region—

The results in Figures 3 and 4 were obtained with a labeled primer that was 46 nucleotides long after its elongation to the G site (38 plus 8 nucleotides). In a separate experiment on a different DNA template that was designed to determine holoenzyme dissociation rates, the holoenzyme was started on a primer of 20 nucleotides and stopped by severely limiting dGTP or dTTP. We observed a more rapid dissociation for short products (primer elongated by 7 nucleotides or less) than for longer products (Fig. A-2).

To examine this primer/template length affect more carefully, the dissociation assay was carried-out using a 20 nucleotide primer whose the 3' end was complementary to the same site on M13mp7 as the original 38 nucleotide primer (see Figure 1). Elongation of these two primers to the G site on the template results in a 28 and 46 base-pair primer-template region, respectively. Figure 5 summarizes the results of polymerase dissociation experiments of the Figure 2 type. It can be seen that, for both the 28 base-pair and 46 base-pair primer-template region, the polymerase holoenzyme displays the kinetics of dissociation expected for a first-order

reaction. However, the rate of holoenzyme dissociation from the 28 base-paired region is more than 2 times faster than from the 46 base-pair region (half-life = 55 sec versus 2.5 min).

The rate of holoenzyme dissociation is independent of accessory protein concentration—

The rate of dissociation of a simple protein-DNA complex is expected to be independent of its concentration. However, in the case of the multi-protein T4 DNA polymerase holoenzyme, it is conceivable that different complexes could form at different DNA polymerase accessory protein concentrations and that these complexes could dissociate at different rates. Alternatively, since the polymerase is held in place by separate "sliding clamp" components, dissociation could be a multistep process that can be rescued by clamp reassembly before the polymerase leaves. To investigate these possibilities, we varied the concentration of the accessory proteins over a 64-fold range.

The results are presented in Figure 6. Under the reaction conditions used in these experiments, the number of holoenzymes initially formed was at a maximum when the accessory proteins from genes 45 and 44/62 were added at 5μg/ml each, and 50% maximum when they were added at 0.625 μg/ml each. However, when we vary the concentrations of each of these two proteins from 0.7 to 20 μg/ml, the rate of holoenzyme dissociation is independent of accessory protein concentration. This is the result expected for a simple dissociation reaction that involves a single species of enzyme

The gene 32 protein has an effect on the rate of polymerase holoenzyme dissociation—

When the DNA polymerase holoenzyme on the lagging-strand of the replication fork encounters the end of a previously synthesized Okazaki fragment, the 32 protein is likely to be displaced from the DNA ahead of the holoenzyme. To examine the effect of removal of the 32 protein from the template on polymerase stability, the gene 32 protein was omitted from the assay during the entire stall period in the absence of dCTP (see Figure 2 for experimental outline). The results are shown in Figure 7. In the absence of the 32 protein, the dissociation of the holoenzyme-DNA complex displays the kinetics of a first-order reaction, but the rate of dissociation is 1.4 times faster than in the presence of 32 protein, decreasing the half-life of the holoenzyme-DNA complex from 156 to 107 sec.

In both the plus and minus 32 protein reactions in Figure 7, we omitted the 32 protein that is normally added along with our DNA trap (see Figure 3 experiment for example). The observed rate of holoenzyme dissociation for the plus 32 protein reaction was not affected significantly by this change (half-time of 156 sec, compared to 168 sec in Figure 4). It seems therefore that the 32 protein does not move to the trap from the DNA template in the time allowed in our assay.

The addition of excess ATPγS does not affect holoenzyme dissociation—

Many studies have shown that ATP hydrolysis is required for holoenzyme formation and that an excess of a nonhydrolyzable ATP

analog, ATPγS, can block holoenzyme formation by preventing ATP hydrolysis (Piperno and Alberts, 1978; Huang *et al.*, 1981; Jarvis *et al.*, 1991; Munn and Alberts, 1991; Capson *et al.*, 1991). However, it is not known if ATP hydrolysis is required continuously to maintain the holoenzyme-DNA complex or to trigger its disassembly. To investigate these possibilities, we repeated the Figure 2 experiment with excess ATPγS added along with the free DNA polymerase trap. Controls showed that the ATPγS concentration used in these studies (1.5 mM in the presence of 0.1 mM ATP) blocks all assembly of the holoenzyme at a primer-template junction under our assay conditions (Figure A-3). We therefore reasoned that, if ATP hydrolysis is required for maintenance of the holoenzyme, the addition of ATPγS to the dissociation assay should accelerate the rate at which active polymerase molecules disappear. However, as shown in Figure 8, neither the addition of ATPγS along with the DNA trap nor major changes in the concentration of ATP affect the dissociation rate of the holoenzyme-DNA complex. We conclude that neither maintenance nor the dissociation of the holoenzyme-DNA complex requires ATP hydrolysis.

Linearization of the circular template increases the rate of holoenzyme dissociation—

The template and the experimental strategy that we used to test whether the topology of the DNA template effects holoenzyme dissociation is shown schematically in Figure 9. The initial steps in the assay (Fig. 9B, steps a & b) were the same as described previously for the dissociation assay, although a different DNA

template was used that was specially constructed (see Methods) to contain one G nucleotide every 50 nucleotides (Figure 9A). In addition, two different concentrations of dATP, dGTP, and dTTP were used (an increased concentration increases the number of G sites traversed by the holoenzyme due to contaminating dCTP). We could thereby vary the distance of the stopped DNA polymerase holoenzyme from a fixed EcoRI site located behind the polymerase over a wide range (Figure 9B).

The most important new feature of the assay in Figure 9B is the addition of an optional restriction nuclease cutting step (step c), which linearizes most of the DNA molecules during the first six seconds of the time that the polymerase holoenzyme is stalled by dCTP omission. As a control, the addition of buffer in place of the EcoRI enzyme leaves the template circular.

We first consider the results of the control experiment, where the DNA template remains circular. Results for pause sites close to the polymerase start site are illustrated in lanes 1 and 2 of Figure 10A. In lane 1, the elongated primers that have stopped at successive G sites create a display of discrete bands just prior to dCTP addition. All of those primers from which the polymerase have dissociated will be unaffected by the dCTP-containing "chase" incubation, whereas those that contain a polymerase at the moment of dCTP addition will disappear into products that fail to enter the gel. Thus, the dCTP chase results shown in lane 2 reveal that most of the polymerase holoenzymes are still in place after a 26 sec stall, since most of the labeled bands (60-90%) disappear. A similar result is obtained for pause sites located much further away from the point

where DNA synthesis begins (lanes 1 and 2 in Figure 10B). These are the results expected from the stability of the stalled complex, as measured in the Figure 2 assay (e.g., see Figures 3 and 4).

Now, we examine the effect of cutting the DNA template to create a linear DNA molecule to which the stalled DNA polymerase is bound (see Figure 9). These results are shown in lanes 3 and 4 of Figures 10A and 10B (that the DNA has been completely cut is evident from the decreased size of each DNA band in these lanes compared to the uncut DNA in lanes 1 and 2). The major result is that the dCTP chase (lane 4) has much less effect on DNA band intensities here than it does on a circular template. Thus, the creation of a linear DNA molecule destabilizes the T4 DNA polymerase holoenzyme, as expected if the polymerase accessory clamp is formed as a donut through which the DNA double helix is threaded (Stukenberg *et al.*, 1991; Kong *et al.*, 1992).

The results for a series of experiments in which the holoenzyme was stalled at sites close to the 5' end of the primer are plotted in Figure 11, whereas experiments where the holoenzyme was stalled at sites further from the 5' end of the primer are summarized in Table I. As expected if the components of the holoenzyme are sliding off the cut end of the DNA template, the holoenzyme dissociates more rapidly from those stall sites near the cut site than it does from stall sites further away (Figure 11 and Table I). As an example, for reactions incubated for 18 sec after the addition of EcoRI, only 26 percent of the holoenzymes remained associated at a site 170 nucleotides from the end of the template,

compared to 74 percent at a site 400 nucleotides from the end (Table I).

The EcoRI enzyme produces a 3' recessed end at its cut site that could in principle serve as a binding site for the DNA polymerase or its accessory proteins, retarding their exit from a DNA end. As a control, these experiments were repeated using EcoRV as the restriction enzyme, which generates blunt ends (and cuts just behind the EcoRI site on the primer). The results were unaffected by the this change (Table A-I).

DISCUSSION

We have developed an assay to measure the dissociation rate of the T4 DNA polymerase holoenzyme while it is stalled by omission of one deoxynucleoside triphosphate. Using this assay, the dissociation of the DNA polymerase component of the holoenzyme from the DNA was found to follow a simple exponential decay with a half-life of 2.5 min. We believe that this dissociation rate reflects that of the entire holoenzyme, since the polymerase dissociates with a half-life of about 0.12 sec in the absence of its accessory proteins (Capson *et al.*, 1992; Kaboord and Benkovic, 1993); consequently, the results of the assay are refered to as "the dissociation of the holoenzyme". However, in the assay, we were not able to also monitor the dissociation from the DNA of the polymerase accessory proteins (the holoenzyme components from T4 genes 45 and 44/62) from the DNA; thus, the possibility exists that they dissociate at an even slower rate than the polymerase.

We further characterized the properties of the stalled holoenzyme by showing that its dissociation rate from the DNA template is independent of the concentration of the polymerase accessory proteins and independent of ATP hydrolysis. Although, the rate of holoenzyme dissociation is increased by the omission of the gene 32 protein, this is only about a two fold effect. The rate of holoenzyme dissociation is increased by shortening the length of the primer-template region from 46 to 28 nucleotides, implying that the holoenzyme makes contact with an extended region of the DNA behind the 3' end at which synthesis occurs.

The affinity of the stalled holoenzyme for the template may be comparable to that of the holoenzyme processively synthesizing DNA on the leading-strand of a replication fork. In contrast, the half-life of 2.5 min for dissociation is two orders of magnitude greater than the half-life of one to two seconds required for holoenzyme recycling during Okazaki fragment synthesis on the lagging-strand of the replication fork (Selick et al., 1987). This suggests that merely preventing processive nucleotide incorporation by omitting a nucleotide does not trigger the holoenzyme to switch from a relatively tight DNA binding mode to the rapid dissociation mode. Thus, the holoenzyme apparently can sense a difference between being stopped or idled in place by omission of a nucleotide and being stopped by the end of an Okazaki fragment. This sensing mechanism is examined in detail in the accompanying paper (Hacker and Alberts, 1994).

Our experimental results have a number of implications; in the following subsections, first we consider their minor implications: the

size of the holoenzyme binding site and the role of the gene 32 single-stranded binding protein. Second, we discuss major insights that our data enables us to make concerning the mechanism of holoenzyme dissociation and the properties of the holoenzyme sliding clamp .

The holoenzyme binding site is larger than 28 base-pairs—

In our assays the holoenzyme dissociates more slowly from a 46 base-pair primer-template region than from a 28 base-pair region. This suggests that the DNA polymerase holoenzyme contacts more than 28 bases of duplexed DNA. A 46 base-pair binding site is in the range expected for a holoenzyme composed of the DNA polymerase and the three accessory proteins (gene 45 protein and the gene 44/62 complex) or even for the DNA polymerase and just the gene 45 protein (Capson *et al.*, 1991; Munn and Alberts, 1991b). Whether or not the assembled holoenzyme contains the gene 44/62 complex is still a viable question. The finding by Reddy *et al.* (1993) that the gene 45 protein alone can markedly increase the processivity of the gene 43 DNA polymerase (albeit inefficiently) lends support to the idea that the assembled holoenzyme could be composed of only the gene 45 and 43 proteins.

To test the possibility that the difference in the dissociation rate of the holoenzyme found with a 28 nucleotide verses a 46 nucleotide duplex DNA region is due to a 28 nucleotide duplex being too short for stable association of the gene 44/62 complex, we have tried to assemble a gene 45 protein - polymerase - DNA complex

using the conditions described by Reddy *et al.* (1993) on the two templates. To date, we have been unsuccessful in forming gene 45 protein-polymerase complexes in the absence of the gene 44/62 complex (data not shown).

Role of the gene 32 protein at the lagging strand of the replication fork—

When the DNA polymerase holoenzyme collides with the end of a previously synthesized Okazaki fragment at the lagging-strand of the replication fork, one would expect the 32 protein to be displaced from the DNA ahead of the holoenzyme. This removal of the gene 32 protein could help trigger a rapid dissociation of the holoenzyme from the template, since the gene 32 protein binds to components of the holoenzyme and increases the processivity of the holoenzyme (Huang *et al.*, 1981; Formosa *et al.*, 1983). When the gene 32 protein was omitted from our holoenzyme dissociation assay, the half-life of holoenzyme dissociation decreased from 2.5 min to 1.25 min. The 1.25 min half-life is still much longer than the 1-2 sec half-life required for recycling of the polymerase at the lagging strand of the replication fork. Therefore, the displacement of the gene 32 protein when the holoenzyme completes an Okazaki fragment is not sufficient to explain the rapid dissociation of the holoenzyme on the lagging strand of the replication fork. However, the results do support the view that the gene 32 protein increases the processivity of the holoenzyme by a direct protein interaction in addition to destabilizing DNA secondary structures that impede nucleotide incorporation.

Mechanism of dissociation of a stalled T4 DNA polymerase holoenzyme from the DNA—

The simple first-order decay of the T4 DNA polymerase holoenzyme-DNA complex argues against a "timing" or "clock" mechanism for the regulation of its dissociation. Newport and colleagues proposed that holoenzyme dissociation could involve a "timing" or "clock" mechanism based on the observation that ATP hydrolysis was required for formation of the holoenzyme (Newport, 1980; Newport *et al.*, 1981). They suggested that the energy of ATP hydrolysis by the T4 gene 44/62 polymerase accessory protein complex could be used to drive conformational changes in the complex, which would generate a tight DNA binding complex. For dissociation of the holoenzyme to occur, the DNA polymerase accessory protein complex would first have to decay from the tight to the weak DNA binding conformations. This decay would generate a lag-time before rapid dissociation of the complex, and the dissociation of the holoenzyme would <u>not</u> follow the kinetics of a first-order reaction.

A clock-like mechanism of holoenzyme disassembly was also proposed by Munn and Alberts to explain their results obtained from footprint analysis of proteins of the DNA polymerase holoenzyme (Munn and Alberts, 1991). Munn and Alberts only detected primer-template binding by the accessory protein complex when the nonhydrolyzable ATP analog, ATPγS, was present in the reaction. This accessory protein-ATPγS complex did not associate with the T4 DNA polymerase. When ATP was present, no accessory protein

complex or holoenzyme binding to the primer-template was detected, even at protein concentrations in large excess over those necessary for maximal DNA synthesis by the holoenzyme.

In the footprinting experiments, the DNA polymerase was stalled by the omission of a nucleotide, since it is not possible to footprint a moving complex . Munn and Alberts therefore suggested that DNA polymerase translocation was necessary for maintenance of a stable holoenzyme. They proposed that ATP hydrolysis places the accessory protein complex in a high energy state, which binds tightly to the template. In the absence of the DNA polymerase, the accessory proteins quickly decay through a series of states to the weak DNA binding ground state and dissociate from the template. In reactions containing the DNA polymerase, the accessory proteins in the high energy state associate with the DNA polymerase to form the holoenzyme, and the high energy state of the accessory proteins is maintained by the movement of the DNA polymerase. Once the polymerase stops moving, the accessory protein complex would start to decay through multiple energy states. When the ground state is reached the accessory protein complex allows the polymerase to dissociate from the template. Like the "clock" mechanism proposed by Newport and colleagues, this mechanism predicts a time delay before the rapid dissociation of the polymerase from the template (Newport, 1980; Newport *et al.*, 1980; Munn and Alberts, 1991). In our assays, no time delays were observed during the dissociation of the holoenzyme complex and thus, our results argue against a "clock-like" mechanism for holoenzyme dissociation.

In addition, the properties of the holoenzyme revealed by these studies suggest an alternative explanation for the failure to detect a footprint of the holoenzyme complex than that offered by the clock model. The clock model predicted that the holoenzyme was unstable under footprinting conditions where it is stalled by omission of a nucleotide because the polymerase must translate to keep the accessory proteins in a tight template binding state. In contrast to the footprinting results, we found that the stalled holoenzyme-DNA complex is quite stable. One of the major differences between the two studies is that the dissociation assay measures the affinity of the holoenzyme for the DNA template, whereas the footprinting technique measures the affinity of the holoenzyme for the primer-template junction, a specific site on the template. The second major difference between the studies is that our experiments were performed with a circular template whereas the footprinting experiments were conducted with a short linear template. Our template linearization experiments, which are discussed below, suggest that although a holoenzyme stalled by nucleotide omission has a tight affinity for a circular template, its components (or possibly the entire holoenzyme) do not have a tight affinity for the primer-template junction, but rather slide along the template. Thus, the holoenzyme would be unstable on the linear template used the footprinting studies (Munn and Alberts, 1991a; Munn and Alberts, 1991b) because its components would slide off the template ends. This idea is strongly supported by the finding that efficient assembly of the T4 DNA polymerase holoenzyme on short linear templates

requires the addition of streptavidin molecules to the DNA molecule's ends to serve as "bumpers" (Kaboord and Benkovic, 1993).

If "clock" models fail to explain why ATP is utilized is during holoenzyme formation, the role of ATP hydrolysis during holoenzyme formation needs to be explained in some other way. Studies of the *E. coli* polymerase III holoenzyme, which requires ATP for assembly, suggest a possibility. During the assembly of the *E. coli* pol III holoenzyme, the γ-complex hydrolyzes ATP in the act of transferring the ring-like β subunit onto the DNA template (O'Donnell *et al.*, 1992). The T4 gene 44/62 complex has sequence homology with the δ' and γ subunits of the γ complex, as well as homology with the functional analogs in the human replication system, RF-C or A1 (O'Donnell *et al.*, 1993). By analogy with the *E. coli* system, the gene 44/62 complex could utilize ATP to transfer the gene 45 protein onto the DNA template during T4 holoenzyme assembly. Therefore, the role of ATP hydrolysis during holoenzyme formation would be to overcome the energy barrier for breaking apart the gene 45 protein trimer and reassembling it around the DNA - rather than to drive allosteric changes in the gene 44/62 protein that must be relaxed before the disassembly of the holoenzyme can occur (as in the clock model).

Although we found that ATP hydrolysis was not required for holoenzyme disassembly, the results in this paper leave open the possibility that an ATP-driven mechanism of holoenzyme disassembly could play a role in the rapid dissociation of the holoenzyme during Okazaki fragment synthesis on the lagging-strand of the replication fork. This possibility is examined and eliminated in the accompanying paper (Hacker and Alberts, 1994).

The proteins of the holoenzyme behave like a sliding clamp—

As predicted for a holoenzyme composed of a sliding clamp that tethers the DNA polymerase to the DNA template, the rate of holoenzyme dissociation from a circular DNA template increases dramatically when template is cut by an restriction enzyme (Figure 10, and Table I). Moreover, holoenzymes stalled at sites on the template close to the cut site dissociate more rapidly than holoenzymes stalled at sites farther away from the cut site. This result is expected for a sliding clamp, since the closer the clamp is to the end of the DNA template the greater its chances of sliding off the end by a random diffusion process.

Our template linearization studies were modeled after similar experiments performed with the *E. coli* DNA pol III β-subunit, which has a ring-like structure (Kong *et al.*, 1992). When circular DNA molecules containing the β-subunit were cut, the subunit rapidly dissociated from the linear molecule; presumably because the subunit slid off the ends of the molecule, like a ring washer off the end of a rod (Stukenberg *et al.*, 1991). The similarity of this result with ours suggests that one of the proteins of the T4 holoenzyme has a ring-like structure resembling that of the β-subunit. Further support for this view comes from the detection of cylinder-like structures with DNA passing through their center in samples of T4 DNA polymerase accessory proteins and DNA using cryoelectron microscopy (Gogol *et al.*, 1992). The dimensions of 90Å by 30Å for the these cylinders are very similar to the dimensions of 80Å by 35Å

determined by X-ray analysis of the *E. coli* β-subunit (Kong *et al.*, 1992). The β-subunit homologue in the T4 DNA replication system is almost certainly the gene 45 protein. The *E. coli* β-subunit and the gene 45 protein have similar functional properties: both proteins lack catalytic activity, but stimulate the ATPase activity of their respective accessory protein complexes. In addition, both proteins bind directly to their respective DNA polymerases and have been found to increase their DNA polymerase's processivity (for review see O'Donnell *et al.*, 1992; Young *et al.*, 1992).

Although the results of template linearization studies reported here and those reported for the *E. coli* replication system are similar, there is a noteworthy difference. When the *E. coli* DNA polymerase core is present in reactions along with the β-subunit, and the DNA polymerase is idled by the omission of 2 nucleoside triphosphates, neither the dissociation of the β-subunit nor the dissociation of the polymerase core is affected by the template being linear (Stukenberg *et al.*, 1991). This difference with our results might be explained by the protein-protein and protein-DNA interactions within the T4 DNA polymerase holoenzyme-DNA complex being much weaker than those found within the *E. coli* DNA polymerase III holoenzyme-DNA complex. A T4 DNA holoenzyme-DNA, or DNA polymerase-polymerase accessory protein complex, has never been physically isolated. In contrast, the entire *E. coli* holoenzyme, composed of at least 10 subunits, as well as holoenzyme subassemblies can be isolated by gel filtration (Wickner, 1976; O'Donnell, 1987; Burgers and Yoder, 1993).

We do not know whether the entire T4 DNA polymerase holoenzyme, or just the accessory protein components slide away from the primer-template junction in our template linearization experiments.

Interactions between the DNA polymerase accessory proteins and the highly processive T4 gene 41 DNA helicase may further increase the stability of the holoenzyme at a replication fork *in vivo*. The DNA polymerase accessory proteins stimulate the activity of the gene 41 helicase *in vitro* suggesting that the two interact (Venkatesan *et al.*, 1982; Richardson and Nossal, 1989a; Richardson and Nossal, 1989b; Spacciapoli and Nossal, 1994). An interaction between the holoenzyme and helicase has been detected in many other replication systems (Kornberg and Baker, 1991), and this interaction may also play in role in keeping those holoenzymes tightly associated with the replication fork.

In summary, the dissociation of the holoenzyme from the DNA follows the behavior of a simple first-order reaction with a half-life of 2.5 minutes. The slow rate of dissociation of the stalled holoenzyme is similar to the slow rate expected for the holoenzyme on the leading-strand of the replication fork. The fact that the dissociation of the holoenzyme follows a simple exponential decay and the lack of a requirement for ATP hydrolysis argues strongly against "clock-like" and other ATP-driven mechanisms of dissociation. The results of our DNA template linearization studies resemble the results of similar experiments performed with the ring-like β-subunit of E. coli DNA polymerase III. By analogy with the *E. coli* replication system, the most likely role for ATP during

holoenzyme assembly is to overcome an energy barrier for opening the putative ring-like gene 45 protein and chaperoning it onto the DNA.

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Table I. The linearization of a circular DNA template with EcoRI rapidly destablizes the DNA polymerase holoenzyme. These results are from the linearization assay performed at 120 μM each of dATP, dGTP, and dTTP (as in Figure 10(B)).

	percentage of DNA polymerase holoenzymes associated with the template[1]															
nucleo-	seconds incubated with buffer (B) or EcoRI (RI)															
tides from	12		15		18		22		26		30		45		60	
EcoRI site	B	RI	B	RI	B	RI	B	RI	B	RI	B	RI	B	RI	B	RI
170	77	38	77	35	62	26	63	14	58	9	46	5	39	5	26	0
220	64	28	67	33	51	24	52	8	45	4	33	2	25	5	7	0
270	88	52	86	44	75	35	70	28	66	8	64	16	44	13	22	0
320	86	62	83	58	74	52	79	34	69	24	66	25	53	4	31	0
370	84	66	88	79	75	61	84	35	72	27	74	29	58	8	45	0
400	83	79	85	74	72	74	80	50	63	34	68	29	58	17	45	10
470	100	56	95	69	96	58	90	51	85	39	100	40	73	13	54	3
520	53	100	77	100	52	81	71	66	58	57	60	69	51	26	34	21

[1]The percentage of DNA polymerase holoenzymes associated with the DNA template was determined as described in Figure 11.

FIGURE LEGENDS

Figure 1. **A diagram of the primed circular M13mp7 single-stranded DNA template used in polymerase dissociation experiments.** The oval with a hairpin represents the M13mp7 single-stranded DNA template. The 5'-^{32}P-labeled oligonucleotide primer for DNA synthesis is 38 nucleotides in length and complementary to map position 6291-6329 of M13mp7. Downstream from this primer are 8 C, A, T nucleotides followed by 3 consecutive G nucleotides ("GGG"). The DNA polymerase holoenzyme will stop just before the GGG if dCTP is omitted from the replication reactions. Further downstream, 40 nucleotides from the 3'-end of the primer is the start of a 22 base-pair hairpin helix (located at the polycloning site), this helix blocks DNA polymerase movement under our conditions.

Figure 2. **The experimental strategy used to determine the kinetics of dissociation of a stalled DNA polymerase holoenzyme.**
(a) The replication proteins are incubated with the M13mp7 single-stranded DNA template annealed with the 5'-^{32}P -end labeled primer (shown in Fig. 1) in the presence of dATP, dGTP, dTTP and the absence of dCTP. The DNA polymerase holoenzyme forms on the primed template, and after incorporating 8 nucleotides, it stalls at the 3 consecutive G nucleotides on the template. (b) A free DNA polymerase trap consisting of excess DNA is added to prevent new

polymerase starts as well as to prevent the polymerase from reassociating with a labeled primer once it dissociates. (c) The incubation time is varied to vary the amount of time the holoenzyme remains stalled. (d) dCTP is added to allow those holoenzyme molecules that have remained associated with the labeled primer-template to continue DNA synthesis to the hairpin.

For analysis of the results, formamide and Na3EDTA are added, the samples electrophoresed through a denaturing gel, and the dried gel is autoradiographed. Two bands are seen on the autoradiograph: the first resulting from the holoenzyme elongating the primer to the GGG site, and the second resulting from the holoenzyme elongating the primer to the hairpin. The first band represents holoenzyme molecules that have dissociated during the incubation period, whereas the second band represents holoenzyme molecules that have remained associated with the DNA template throughout the incubation.

Figure 3. **An analysis of the dissociation of a stalled DNA polymerase holoenzyme.** Samples from the DNA polymerase holoenzyme dissociation assay illustrated in Figure 2 (and described in detail in Methods) were electrophoresed through a denaturing 8% polyacrylamide gel, and an autoradiograph of this gel is shown. DNA bands on the gel corresponding to the primer, the primer elongated to the first G nucleotide and the primer elongated to the hairpin on the M13mp7 template after the addition of dCTP ("products of chase to hairpin") are marked with arrows. Lanes 1-5: reactions contained the T4 gene 43 DNA polymerase and the gene 32 helix-destabilizing

protein, and were stopped prior to the addition of dCTP. Synthesis is extremely limited because the DNA polymerase accessory proteins (the gene 45 protein, and the gene 44/62 protein complex) required for holoenzyme formation were omitted from these reactions. Lanes 6-8: reactions contained complete holoenzyme (the proteins from genes 32, 43, 45 and 44/62) and were stopped at the indicated times without any addition of dCTP. Lanes 9-13: reactions contained the complete holoenzyme and were stopped after incubation with dCTP. The minutes indicated at the bottom of the autoradiograph denote the time the DNA polymerase holoenzyme was incubated with the free DNA polymerase trap in the absence of dCTP (step c in Figure 2).

Figure 4. **Plot of the percentage of DNA polymerase holoenzyme molecules associated with the DNA template as a function of the duration of stalling by dCTP omission.** The percentage of holoenzymes associated with the template was determined as described in Methods. The vertical-axis uses a logarithmic scale to plot remaining polymerase association, and the time on the horizontal-axis represents elapsed time from the addition of the free polymerase trap to the addition of dCTP (see Figure 2). The values for polymerase association were fitted to the exponential equation expected for a first-order dissociation reaction, and the best fit is shown.

Figure 5. **Plot of the percentage of DNA polymerase holoenzyme molecules associated with a 28 and 48 base-paired primer/template region as a function of the duration**

of stalling by dCTP omission. The percentage of DNA polymerase holoenzymes associated with the DNA template with time was determined as for Figure 4. For the short primer, a primer of 20 nucleotides was used, which formed a 28 nucleotide primer/template region when elongated 8 nucleotides to the first G. The primer used in the reactions in Figure 3 forms the 46 nucleotide primer/template region. The values for each polymerase association were fitted to the exponential equation expected for a first-order reaction, and the best fit is shown.

Figure 6. **There is no effect of DNA polymerase accessory protein concentration on the rate of DNA polymerase holoenzyme dissociation.** Each DNA polymerase accessory protein (gene 45 protein and gene 44/62 complex) was added at the same concentration, and the final concentration of each is indicated. The reactions were performed as for Figure 4 except that the primed template was incubated with the replication proteins for twice as long (20 sec) before addition of the free DNA polymerase trap, and 0.625 μM dATP and additional gene 32 protein (8 μg/ml, sufficient to cover 100% of the primed template) were present in this incubation. The percentage of DNA polymerase holoenzymes associated with the DNA template at each time is plotted as described for Figure 4.

Figure 7. **The gene 32 protein has a stabilizing effect on a stalled DNA polymerase holoenzyme.** Reactions were carried out as schematized in Figure 2 and described in Methods with the following exceptions: During formation of the holoenzyme, both

reactions contained 0.625 μM dATP, and reactions without gene 32 protein contained 15 ng/ml 43 protein in place of 7.5 ng/ml to raise the amount of holoenzymes formed to the level found in reactions containing gene 32 protein. In addition, gene 32 protein was omitted from the free polymerase trap, which consisted of 245 μg/ml poly dT_{1500} · oligo dA_{50}. For the dCTP chase both dCTP and 1.8 mg/ml gene 32 protein were added. The percentage of DNA polymerase holoenzymes associated with the DNA template with time was determined as described in Methods and plotted as described for Figure 4.

Figure 8. **There is no effect of ATPγS on the rate of dissociation of stalled DNA polymerase holoenzyme molecules.** Reactions were carried out as schematized in Figure 2, with ATPγS added with the free DNA polymerase trap (see step b) where indicated. The specific reaction steps and concentrations of reactants were as described in Methods with the following modifications: dAMPPNP was substituted for dATP because, like ATP, dATP can be utilized as an energy source for holoenzyme formation whereas dAMPPNP can not. During formation of the holoenzyme, reactions contained 20 μM dAMPPNP, and ATP at the indicated concentrations. The reactions indicated as containing ATPγS contained 0.1 mM ATP (and no ATPγS) during the formation of the holoenzyme-DNA complex, and the 0.1 mM ATP was maintained throughout the course of the experiment. The free DNA polymerase trap contained either 1.5 mM ATPγS or the indicated concentration of ATP, plus 20 μM dAMPPNP. The dCTP chase contained 0.36 mM

each of dCTP, dGTP, and dTTP, 0.62 mM dAMPPNP, plus 1.5 mM ATPγS (where indicated) or the indicated concentration of ATP. The percentage of DNA polymerase holoenzymes associated with the DNA template with time was calculated as described in Figure 4.

Figure 9. **An experimental strategy for examining the effect of template linearization on the dissociation of the DNA polymerase holoenzyme.** (A) A diagram of the primed circular single-stranded DNA substrate used in the template linearization experiments. The oval represents the pKH32G single-stranded DNA molecule constructed by placing an 1600 nucleotide insert (comprised of 32 fifty nucleotide repeats, with each repeat containing only one G nucleotide) into an M13 origin-containing plasmid (see Methods). The region with the annealed, 3' end-labeled oligonucleotide primer and the first of the 32 repeats is bordered by a box and shown in greater detail below. The primer contains an EcoRI restriction site 10 nucleotides downstream from its 5' end and four ^{32}P-labeled G and T nucleotides at its 3' end, which are represented by asterisks.

(B) Outline of the experiment. The replication proteins are incubated with the primed circular single-stranded DNA template in (A) with everything needed for DNA synthesis except dCTP. The DNA polymerase holoenzyme forms and stops at the G nucleotide sites on the template. Although no dCTP has been added for this step in the reaction, the dATP, dGTP, and dTTP nucleotides contain enough contaminating dCTP so that some of the holoenzymes are able to elongate the primer past several of the G nucleotide sites. (b) Excess

DNA is added as a free DNA polymerase trap that prevents DNA polymerase from reassociating with the labeled primer after dissociation. (c) EcoRI restriction enzyme is added (or the enzyme's storage buffer, as a control). (d) The samples are incubated for varied times. (e) An aliquot of sample is added to a tube containing dCTP, and 95% formamide/20 mM Na3EDTA is simultaneously added to the remaining sample to stop its reaction. The samples with dCTP are incubated long enough to allow the holoenzymes that remain associated with the labeled primer-template to continue DNA synthesis through all of the repeats (20 or 30 sec) and then this reaction is stopped as above.

For analysis of the results, formamide and Na3EDTA were added, the samples electrophoresed through a denaturing polyacrylamide gel, the gel was dried, and the radioactivity in each band was determined using a phosphor imager. The percentage of holoenzymes that remain associated at each G site was quantitated in two steps. First, the intensity of the bands at each G site in reactions stopped *before* a dCTP chase was quantitated to determine the *total* amount of holoenzyme that stopped at each G site (both those which dissociated during the incubation and those which remained associated are measured). Second, the intensity of the bands at each G site in reactions stopped after the dCTP chase were quantitated; the amount of holoenzyme which had dissociated at each G site during the incubation was then determined by subtraction.

Figure 10. **The effect of template linearization on the dissociation of DNA polymerase holoenzyme-DNA complex.**

Samples from the template linearization assay schematized in Figure 9B were electrophoresed through a denaturing 8% polyacrylamide gel, and autoradiographs of these gels are shown. The reaction conditions are described in detail in Methods. Unlike the previous experiments, which were conducted at 22°C, this experiment was performed at 37°C to facilitate rapid cutting of the DNA template by the EcoRI enzyme. At 37°C the poly dT·oligo dA DNA used in the previous experiments was not an efficient free polymerase trap, so that the wild-type M13 annealed with 5 primers was substituted. This primed M13 DNA, like the poly dT·oligo dA DNA, was covered with gene 32 protein. The effectiveness of the primed M13 DNA trap was rigorously tested in control experiments similar to those described earlier for the poly dT·oligo dA trap at 23°C (data not shown). (A) Data for stall sites close to the primer terminus. These reactions contained 0.5 μM each of dATP, dGTP, and dTTP before the addition of the dCTP chase. (B) Data for stall sites far from the primer terminus. These reactions contained 120 μM each of dATP, dGTP, and dTTP before the addition of the dCTP chase.

The samples were incubated with either EcoRI ((+) lanes 3 and 4) or buffer only as a control ((-) lanes 1 and 2) for 26 sec before being divided and either stopped ((-) lanes 1 and 3) or incubated with dCTP ((+), lanes 2 and 4).

In Figure A-5, we determined that 60% of the DNA molecules were cut at 6 seconds after the addition of EcoRI and that 90% were cut by 10 sec.

Figure 11. **Plot of the percentage of DNA polymerase holoenzyme molecules associated with the DNA template cut with EcoRI as a function of the duration of stalling by dCTP omission.** The percentage of DNA polymerase holoenzymes associated in samples from reactions identical to those described in Figure 10 (A) are shown. Note the unusually rapid dissociation of the polymerase from all of the linear DNA molecules (solid lines). The percentage of DNA polymerase holoenzymes associated with the DNA template was determined as (1-(D-bk)/(A-bk)) x 100, where D is radioactivity at each G nucleotide after the addition of dCTP, A is the amount of radioactivity at each G nucleotide site before the addition of dCTP, and bk is the background on the gel measured as close as possible to the G nucleotide site.

Figure 1

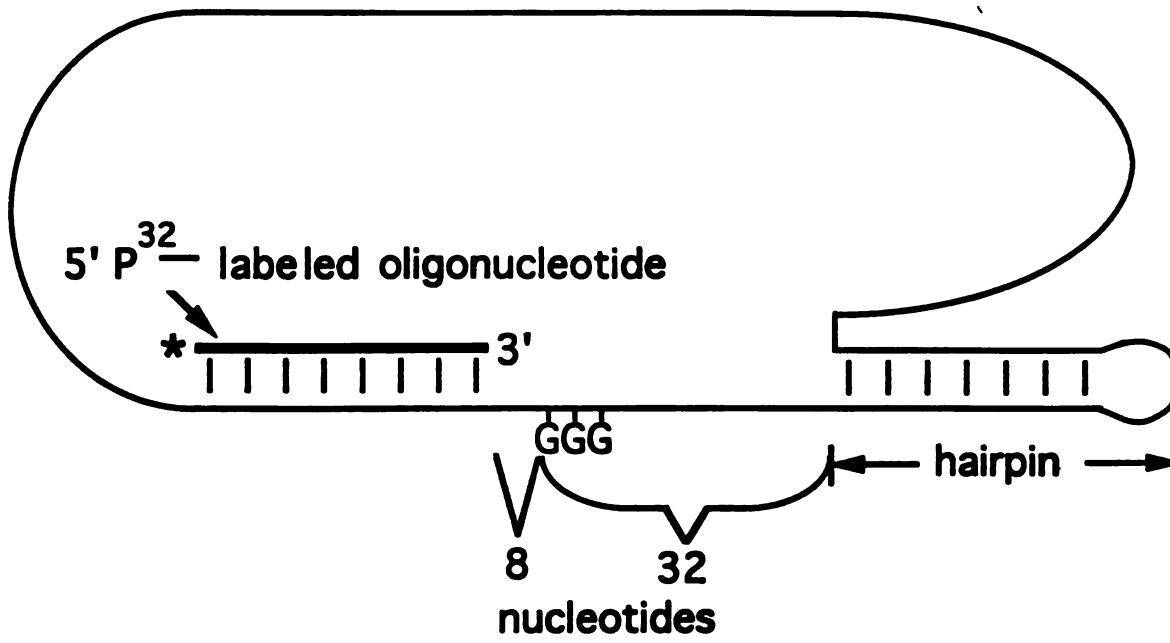

5' P32 labeled oligonucleotide
*
3'
GGG
hairpin
8
32
nucleotides

Figure 2

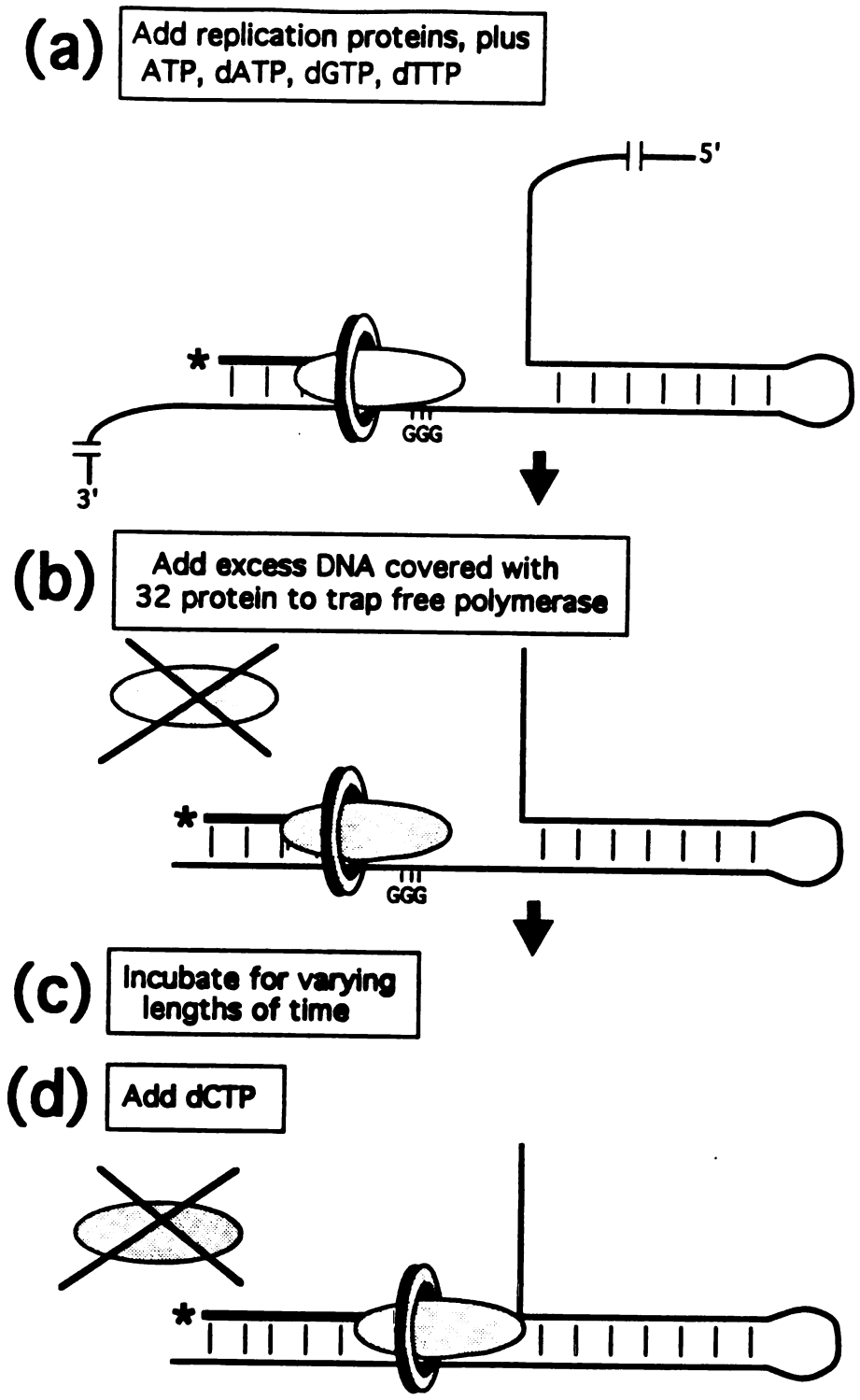

(a)
Add replication proteins, plus ATP, dATP, dGTP, dTTP
5'
*
GGG
3'
(b)
Add excess DNA covered with 32 protein to trap free polymerase
*
GGG
(c)
Incubate for varying lengths of time
(d)
Add dCTP
*

Figure 3

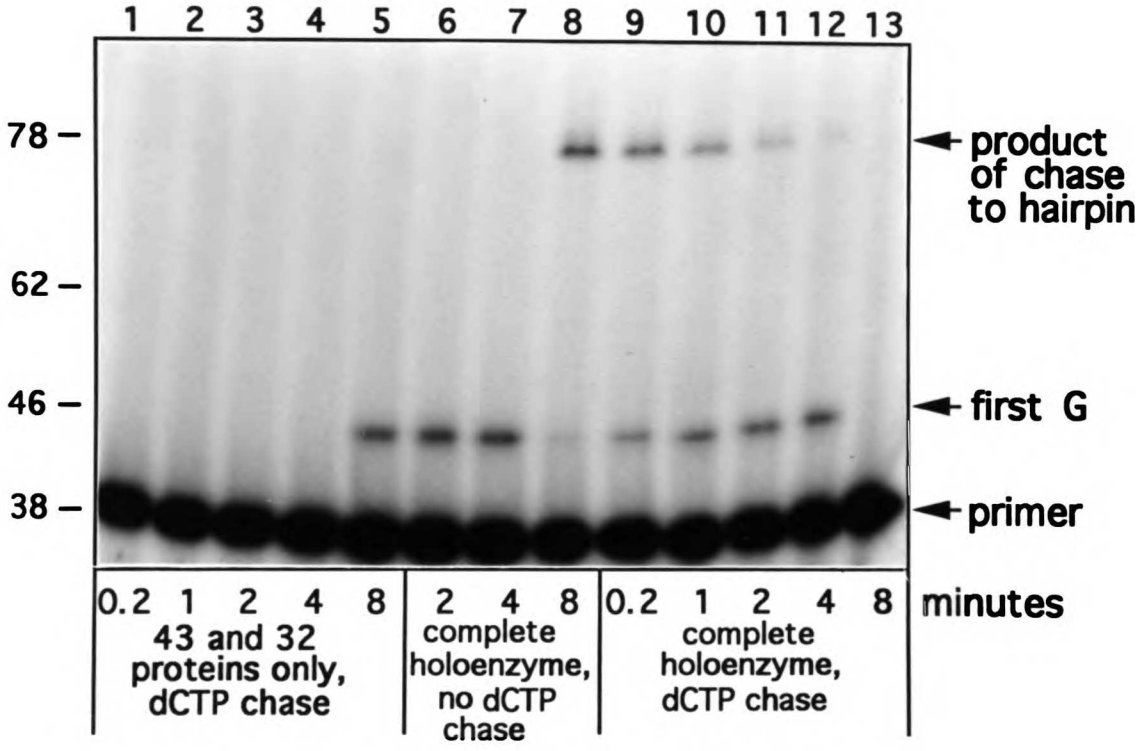

1 2 3 4 5 6 7 8 9 10 11 12 13
78 –
62 –
46 –
38 –
product of chase to hairpin
first G
primer
0.2 1 2 4 8
43 and 32 proteins only, dCTP chase
2 4 8
complete holoenzyme, no dCTP chase
0.2 1 2 4 8
complete holoenzyme, dCTP chase
minutes

Figure 4

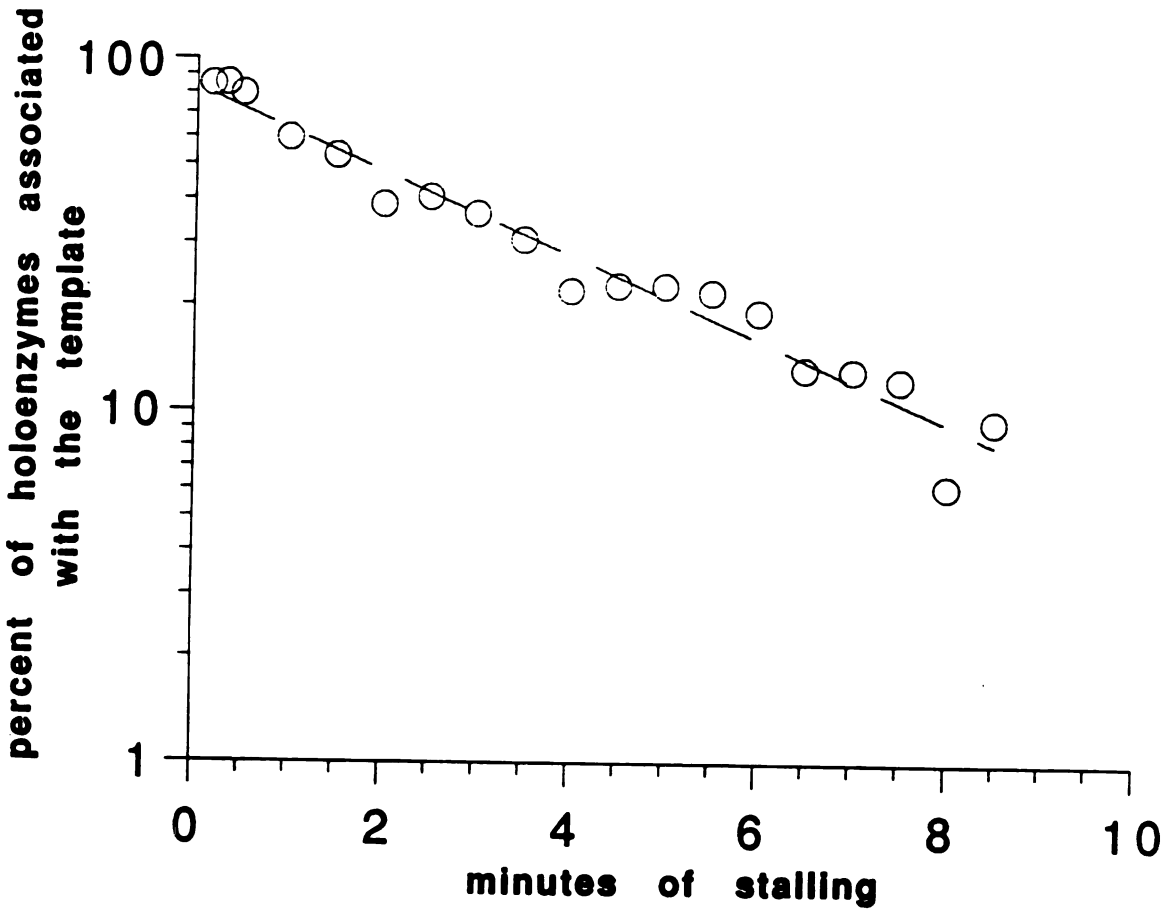

percent of holoenzymes associated with the template
100
10
1
0
2
4
6
8
10
minutes of stalling

Figure 5

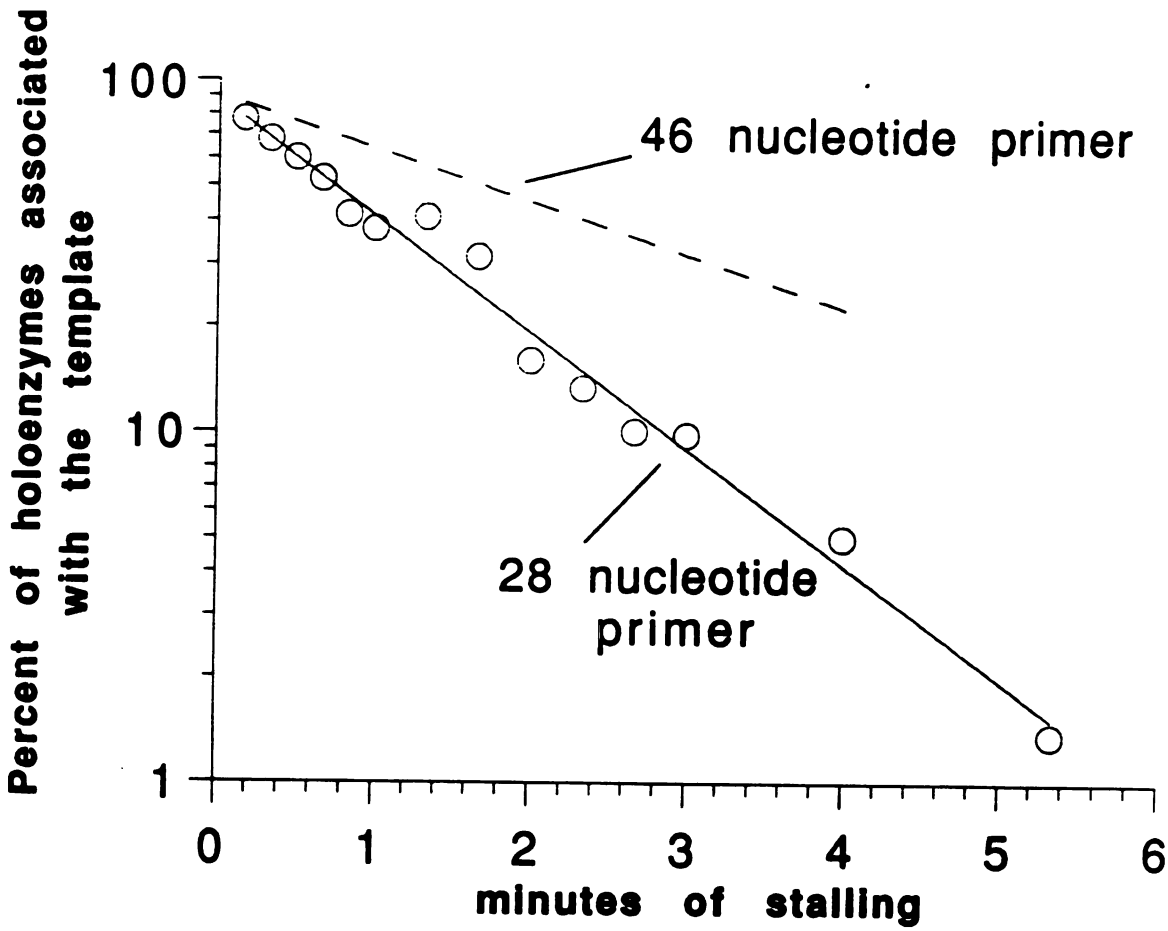

Percent of holoenzymes associated with the template
100
10
1
0
1
2
3
4
5
6
minutes of stalling
46 nucleotide primer
28 nucleotide primer

Figure 6

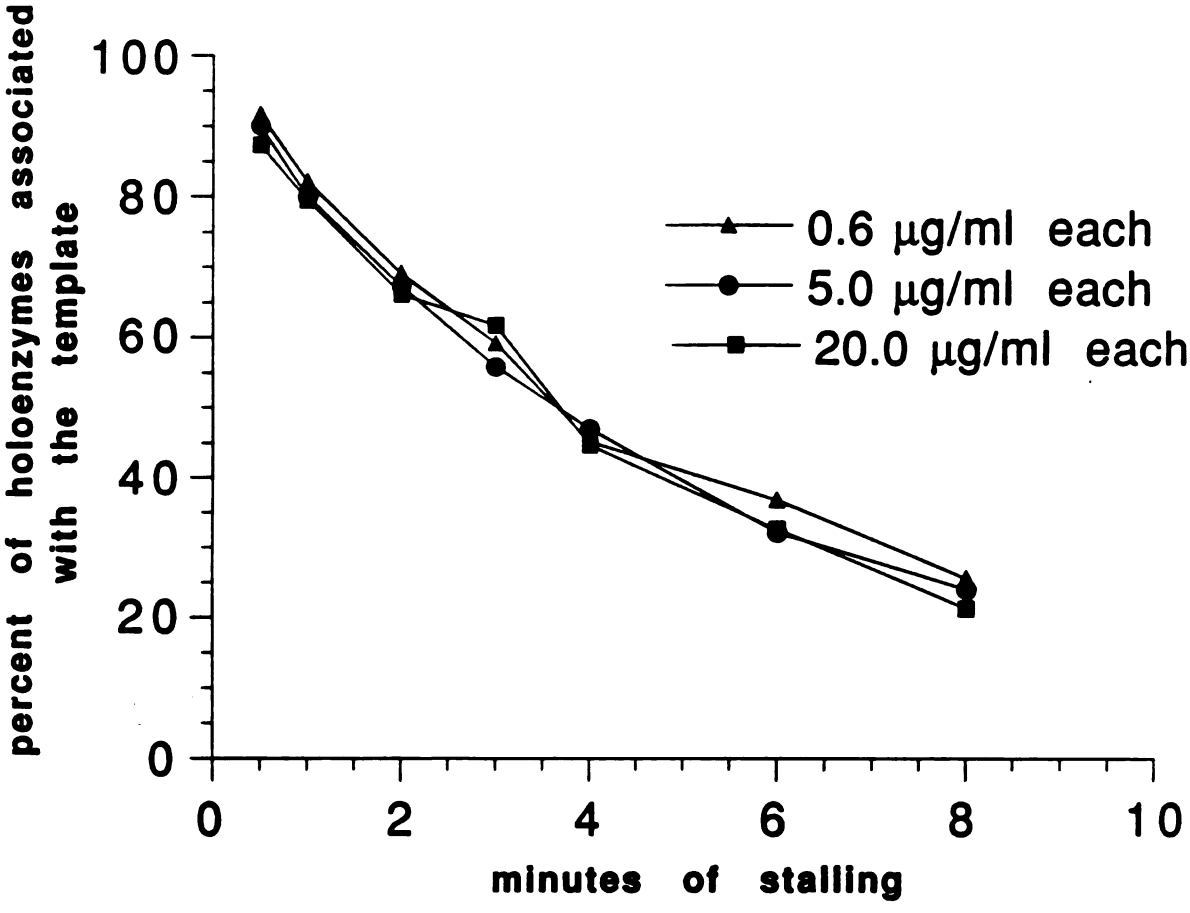

percent of holoenzymes associated with the template
100
80
60
40
20
0
0
2
4
6
8
10
minutes of stalling
0.6 µg/ml each
5.0 µg/ml each
20.0 µg/ml each

Figure 7

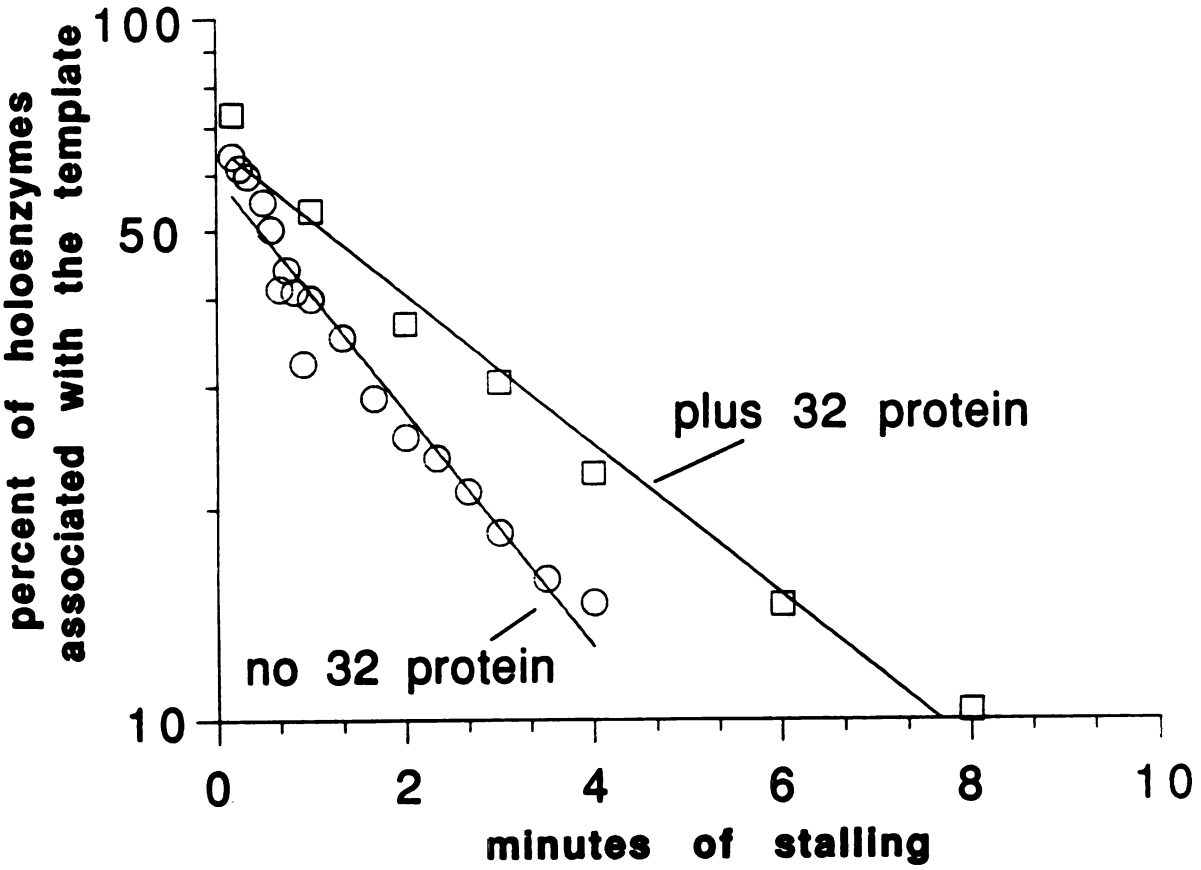

percent of holoenzymes associated with the template
100
50
10
0
2
4
6
8
10
minutes of stalling
plus 32 protein
no 32 protein

Figure 8

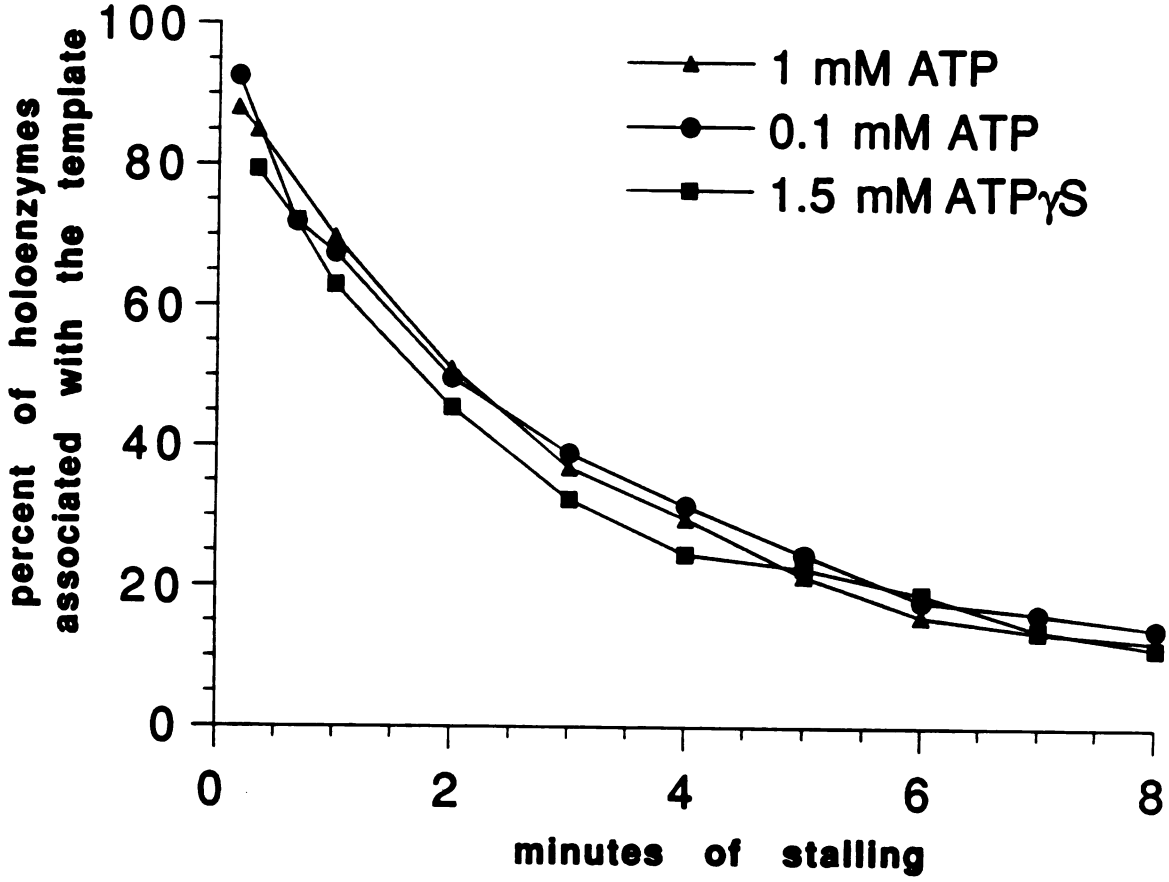

percent of holoenzymes associated with the template
100
80
60
40
20
0
0
2
4
6
8
minutes of stalling
1 mM ATP
0.1 mM ATP
1.5 mM ATPγS

Figure 9A

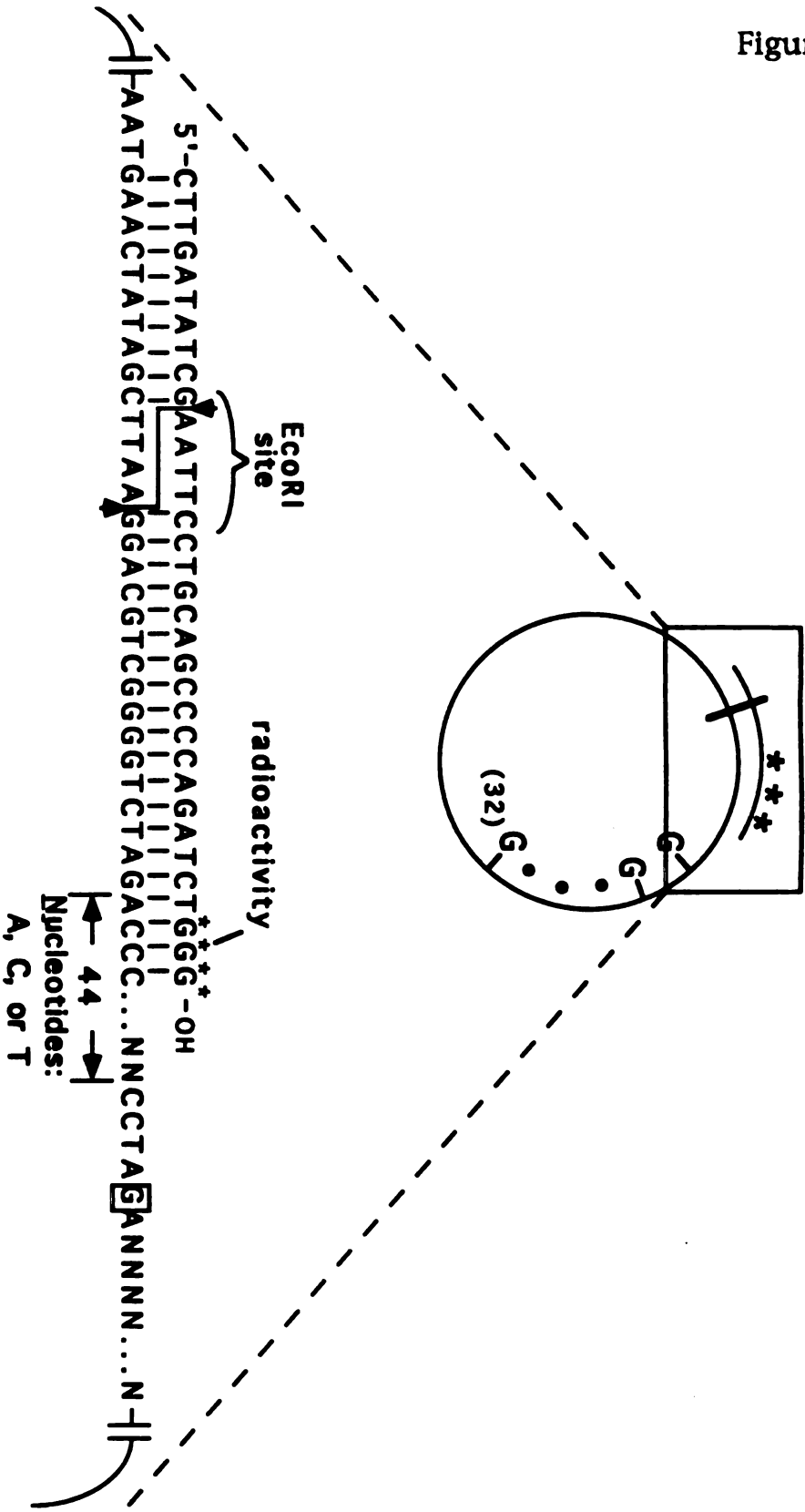

5'-CTTGATATCGAATTCCTGCAGCCCCAGATCTGGG*-OH
AATGAACTATAGCTTAAGGACGTCGGGGTCTAGACCC...NNCCTAGANNNN...N
EcoRI site
radioactivity
44 Nucleotides: A, C, or T
(32)
G
G
G
*
*
*

Figure 9B

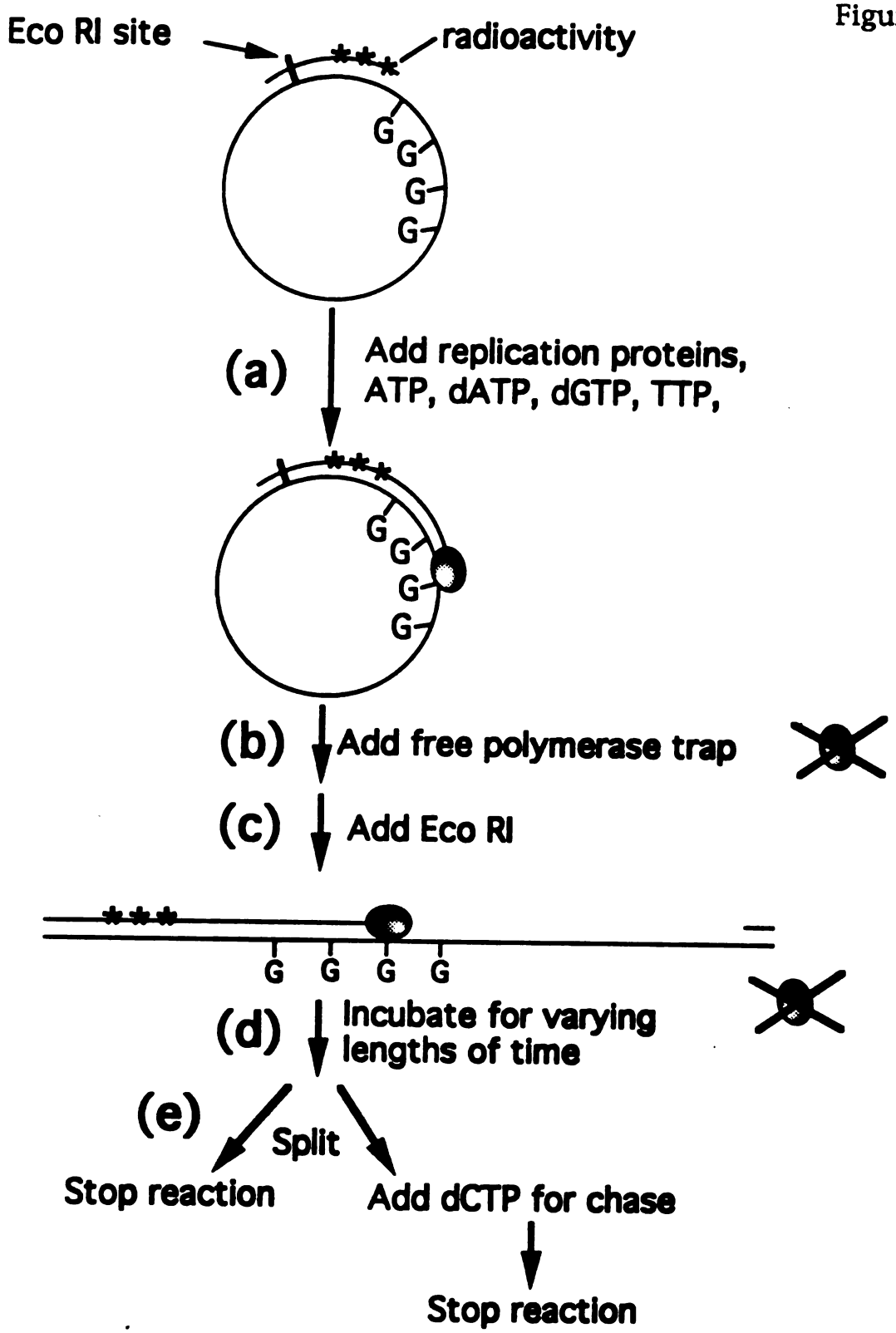

Eco RI site
radioactivity
G
G
G
G
(a)
Add replication proteins,
ATP, dATP, dGTP, TTP,
G
G
G
G
(b)
Add free polymerase trap
(c)
Add Eco RI
G G G G
(d)
Incubate for varying
lengths of time
(e)
Split
Stop reaction
Add dCTP for chase
Stop reaction

Figure 10A

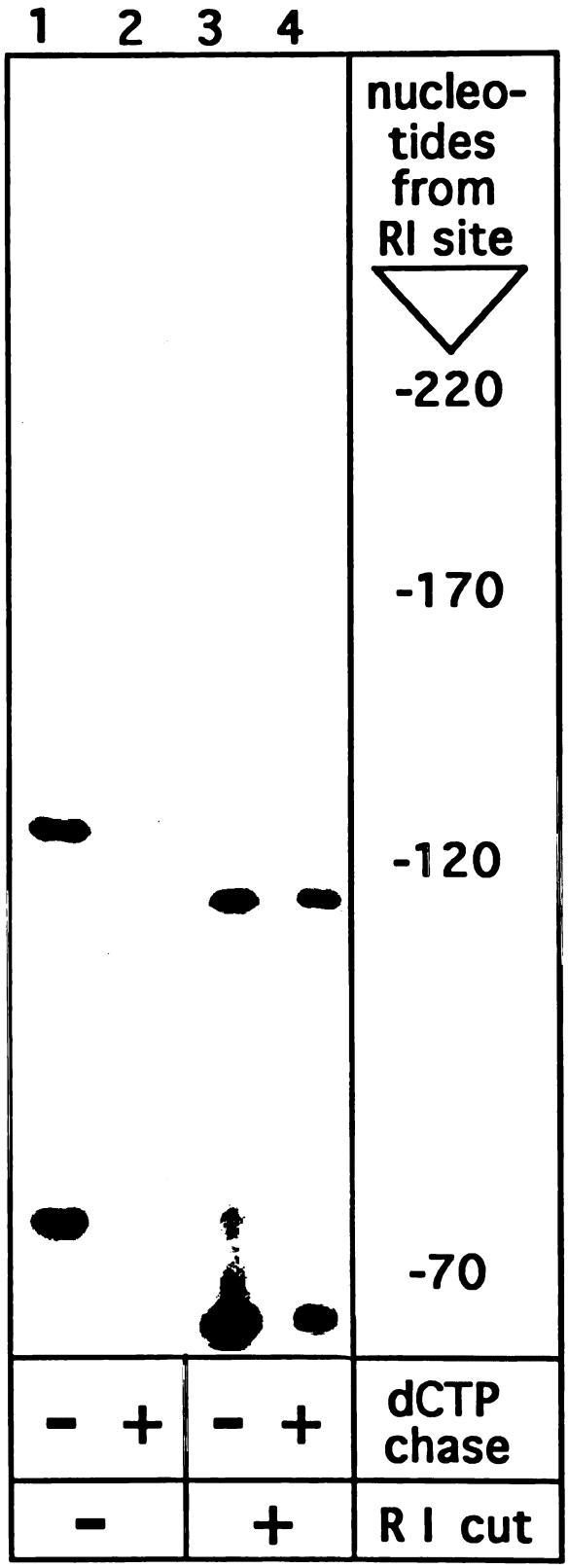

1
2
3
4
nucleo-
tides
from
RI site
-220
-170
-120
-70
- + - +
dCTP
chase
-
+
R I cut

Figure 10B

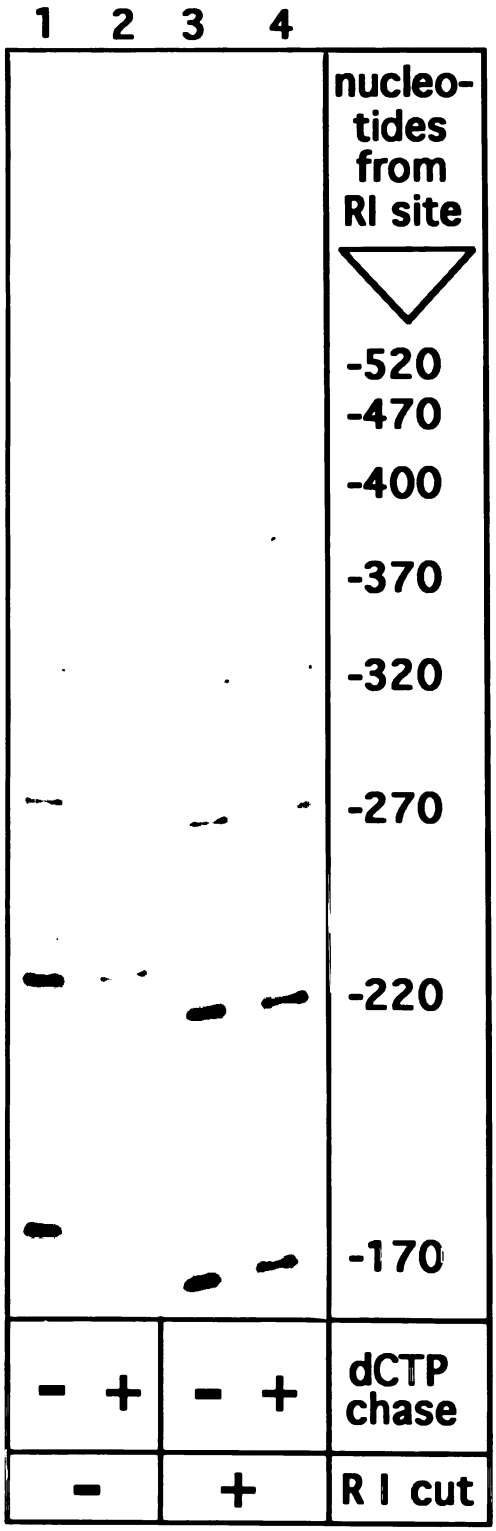
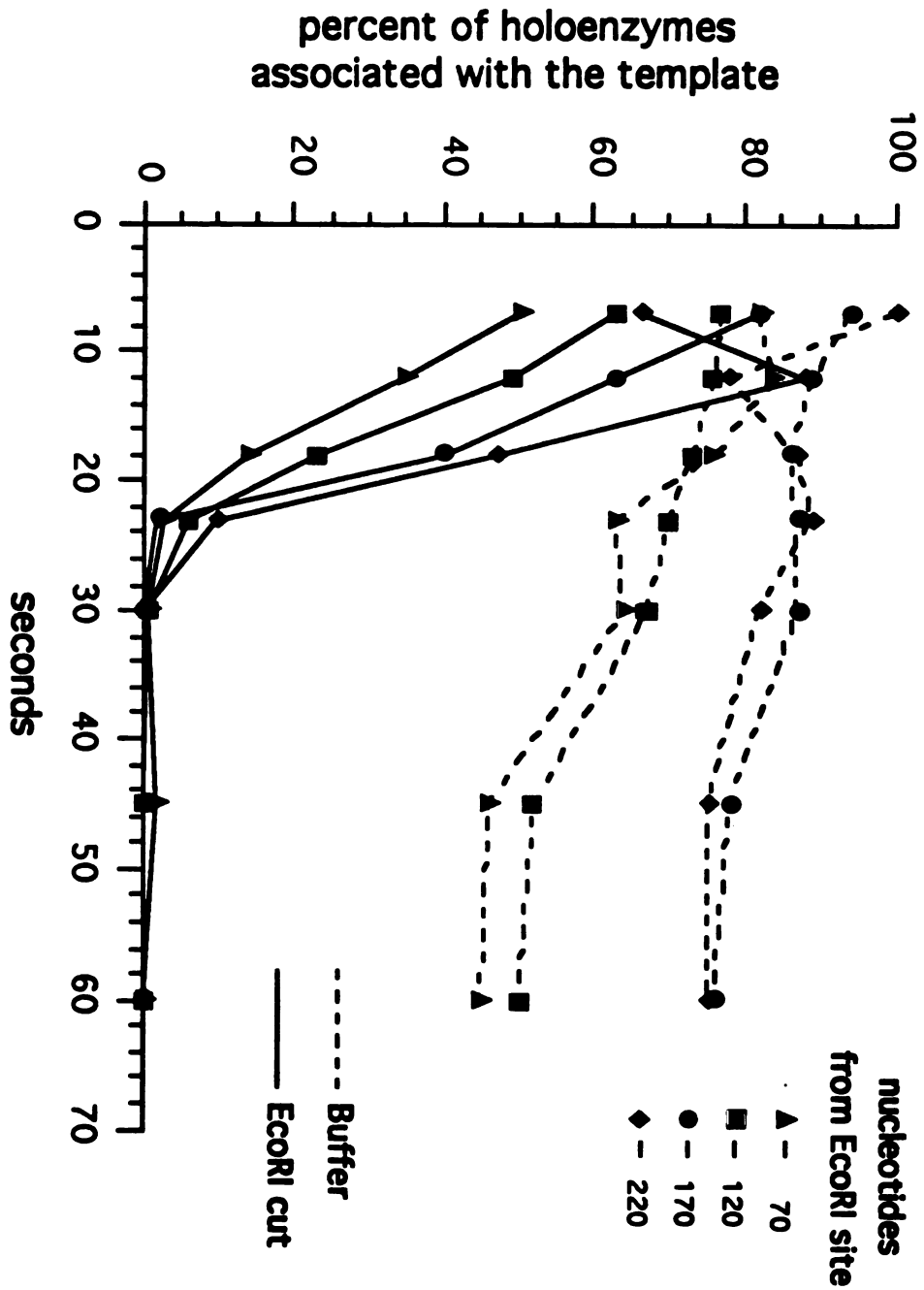

1
2
3
4
nucleo-
tides
from
RI site
-520
-470
-400
-370
-320
-270
-220
-170
- + - +
dCTP
chase
-
+
R I cut

Figure 11


percent of holoenzymes
associated with the template
0
20
40
60
80
100
0
10
20
30
40
50
60
70
seconds
nucleotides
from EcoRI site
▲ – 70
■ – 120
● – 170
◆ – 220
Buffer
EcoRI cut

CHAPTER FIVE

THE RAPID DISSOCIATION OF THE T4 DNA POLYMERASE HOLOENZYME DISSOCIATION WHEN STOPPED AT A DNA HELIX

ABSTRACT

We have examined the molecular mechanism that enables the T4 bacteriophage DNA polymerase holoenzyme to synthesize DNA processively on the leading strand of the replication fork for many minutes, while allowing an identical holoenzyme on the lagging strand to recycle from one Okazaki fragment to the next in less than four seconds. Our experimental system measures the dissociation rate of the holoenzyme under conditions that mimic those at the lagging strand of the replication fork. The holoenzyme is assembled at a primer and, after synthesizing 8 nucleotides, pauses due to dCTP omission. All non-engaged DNA polymerase molecules are then disabled by the addition of excess competitor DNA and dCTP is added. The moving polymerase holoenzyme almost immediately encounters a perfect hairpin helix of 15 base-pairs and stalls. Polymerase dissociation is monitored during the stall by the delayed addition at intervals of excess T4 gene 32 single-stranded DNA binding protein, which rapidly melts out the hairpin helix and allows the polymerase molecules that have not dissociated to continue synthesis to the end of the template.

In the accompanying paper, we show that polymerase holoenzyme dissociation is slow (half-life of 2.5 min) when this enzyme is stalled by nucleotide omission (Hacker and Alberts, 1994). In contrast, the holoenzyme dissociates with

a half-life of 1 sec after hitting the hairpin helix, a rate sufficient to allow efficient polymerase recycling on the lagging strand *in vivo*. We conclude that the holoenzyme senses an encounter with duplex DNA, and then switches to a state that rapidly dissociates.

INTRODUCTION

The four protein T4 DNA polymerase holoenzyme, like the mammalian delta DNA polymerase and *E. coli* DNA polymerase III holoenzyme, has an accessory protein with DNA-dependent ATPase activity (the T4 gene 44/62 protein complex) that is stimulated by a second accessory protein (T4 gene 45 protein) (O'Donnell *et al.*, 1992; Young *et al.*, 1992). In a reaction that requires ATP hydrolysis, these accessory proteins form a sliding clamp that increases the processivity of the DNA polymerase (The T4 gene 43 protein: Alberts, 1987; Nossal, 1992; Young *et al.*, 1992).

In the accompanying paper, we show that the rate of T4 DNA polymerase holoenzyme-DNA complex dissociation is slow (2.5 min half-life) when the holoenzyme is stopped by nucleotide omission (Hacker and Alberts, 1994). This rate is much too slow to explain the recycling of the DNA polymerase holoenzyme observed during Okazaki fragment synthesis on the lagging strand of the replication fork, (Alberts *et al.*, 1983). Where the DNA polymerase holoenzyme must dissociate and move to another primer within 1 to 2 sec (Selick *et al.*, 1987).

What is the mechanism that enables the same holoenzyme to remain on the DNA template for many minutes when synthesizing DNA (or when stopped by nucleotide omission), while permitting rapid dissociation at the end of each Okazaki fragment? To approach this question, we have developed an assay that measures the dissociation of the holoenzyme-DNA complex under conditions that mimic the encounter of the holoenzyme on the lagging strand with the 5'-end of the previous Okazaki fragment. Using this assay, we

find that the DNA polymerase holoenzyme dissociates in a first-order reaction with a half-life of 1 sec, which is comparable to the rate expected for holoenzyme dissociation at the lagging-strand of the DNA replication fork *in vivo*. We have also been able to demonstrate that this dissociation occurs independently of ATP hydrolysis.

MATERIALS AND METHODS

Reagents and Enzymes— Sequenase was purchased from United States Biochemical Corporation. All other enzymes and chemicals are described in the accompanying paper (Hacker and Alberts, 1994).

Construction of a DNA Template That Contains a Hairpin Helix— All cloning and DNA isolation procedures were performed according to standard procedures (Sambrook *et al.*, 1989), unless specified otherwise. The nucleotide sequence of the 22 base-pair hairpin that forms at the polycloning site of M13mp7 is illustrated in Figure 3A below; a DNA template with a hairpin of 13 base-pairs, M13mp5, was constructed by first digesting M13mp7 double-stranded DNA with BamHI, and then separating the DNA fragments by electrophoresis on a 0.8% low-melting-temperature agarose, 10 mM Tris-acetate, pH 7.4, 1 mM Na_3EDTA (TAE) gel. The large M13mp7 DNA fragment was cut out of the gel, and its BamHI ends were ligated. The ligation products were transformed into *E. coli* strain DG98 (F-factor containing), phage placques were isolated, and double-stranded DNA was extracted from the M13-infected cultures. The M13 DNA constructs containing the shortened hairpin were verified by restriction enzyme mapping, and single-stranded M13mp5 DNA was purified.

A DNA template with a 15 base-pair hairpin, M13mp4, was constructed by ligating an annealed nonphosphorylated oligonucleotide adapter (5'-GATCG$(T)_{18}$C/3'-C$(A)_{18}$GCTAG) to the BamHI-generated ends of double-stranded DNA from M13mp7. To confirm the presence of the insert and to determine its orientation, single-stranded DNA phage was isolated and sequenced using the Sequenase protocol (United States Biochemical Corp.) based on the Sanger method (Sanger *et al.*, 1977). In our construct labeled as M13mp4, the single-stranded DNA isolated from phage contains a 15 base-pair hairpin with a 18 deoxyadenosine nucleotide loop (see Figure 3A).

Assay for holoenzyme dissociation at a hairpin helix— The DNA replication proteins (0.075 μg/ml 43 protein, 4 μg/ml 45 protein, and 8.8 μg/ml 44/62 protein complex (no 32 protein was present)) were mixed by hand with 1.59 μg/ml single-stranded DNA template (M13mp4) annealed with the 5'-^{32}P- end labeled 20 nucleotide primer, 1 mM ATP, 60 μM each of dATP, dGTP, and dTTP in 60 μl of replication buffer (160 mM potassium acetate, 33 mM Tris-acetate, pH 7.8, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 200 μg/ml human serum albumin). Samples were incubated for 1 min at 23°C. Primer elongation to the hairpin was initiated by the addition of 40 μl of the above replication protein/DNA template solution to an equal volume of replication buffer containing 240 μM dCTP, 180 μM each of dATP, dGTP, and dTTP, 1 mM ATP, 55 μg/ml poly dT_{1500}·oligo dA_{50} (1:3 molar ratio) in a 0.3 ml Reacti-vial mixing rapidly with a spin vane (Pierce). At various intervals thereafter, 40 μl of 1.2 mg/ml gene 32 protein (nearly 3-fold more than needed to cover all

of the DNA present) in replication buffer was added. The reaction was stopped after 3 sec by the addition of an equal volume (120 μl) of 0.3 M Na_3EDTA, and the samples were immediately placed in an ice water bath.

To prepare the DNA for digestion with HhaI, the DNA polymerase was inactivated by adjusting the solution to 1% sodium dodecyl sulfate and heating for 15 min at 65°C. The replication proteins were then removed by extraction with an equal volume of phenol:chloroform (1:1). The DNA template was transferred into restriction enzyme buffer by passing the sample over a Bio-spin 6 column (BioRad Laboratories, CA) equilibrated with 50 mM potassium acetate, 20 mM Tris-acetate, pH 7.8, 10 mM magnesium acetate, and 1 mM dithiothreitol. The DNA was digested with HhaI, and 2.5 volumes of a 95% formamide/20 mM Na_3EDTA solution was added. Just prior to electrophoresis, the DNA template was denatured by heating for 4 min at 100°C and then immediately placed in a ice water bath.

After electrophoresis, the radioactivity on the gel was quantitated using a Phosphor Imager (Molecular Dynamics, CA). The percentage of holoenzymes associated with the template was determined as $[(A\text{-}bk_A)/((A\text{-}bk_A) + (D\text{-}bk_D))] \times 100$, where A is radioactivity in the DNA band representing "chased product" (see Figure 6), bk_A is background on the gel measured immediately below this band, D is radioactivity at the hairpin, and bk_D is background on the gel measured immediately above this band.

Assay for holoenzyme dissociation using a rapid quench technique— The DNA replication proteins (0.1 μg/ml 43 protein, 5.3

μg/ml 45 protein, and 11.7 μg/ml 44/62 protein complex (no 32 protein was present)) were pre-mixed with 2.1 μg/ml single-stranded M13mp4 DNA annealed with the 5'-^{32}P- end labeled 20 nucleotide primer, 240 μM dATP, 60 μM dGTP, 60 μM dTTP and 100 μM ATP in 80 μl of replication buffer. This sample was loaded in one loop (45 μl) of a Kintek Instruments model RGF-3 quench-flow apparatus (University Park, PA). A solution containing 80 μg/ml poly dT_{1500}·oligo dA_{50} (1:3 molar ratio), 3 mM ATPγS, 180 μM dGTP, 180 μM dTTP, and 240 μM dCTP in replication buffer was loaded in the second loop (45 μl). After the samples were incubated in the loops for 30 sec at 23°C, DNA synthesis was initiated by rapid mixing of equal volumes of the reactants. To melt the hairpin and allow template associated holoenzymes to continue DNA synthesis, a solution containing 0.8 mg/ml gene 32 protein, 1.5 mM ATPγS, 120 μM each of dATP, dCTP, dGTP, and dTTP in replication buffer was added with the "quench" syringe. After 3 sec, the reactions were stopped by adding 160 μl of 0.3 M Na_3EDTA with a hand-held syringe, and the samples were immediately placed in an ice water bath. The DNA template was then digested with HhaI, and the products were analyzed as described above.

An assay to measure the dissociation of a DNA polymerase holoenzyme stopped at a DNA helix— Our experimental scheme uses a DNA hairpin helix to mimic the 5'-end of an Okazaki fragment. The salt conditions, reaction temperature, and free DNA polymerase trap are the same as in the assay reported in the preceding paper (Hacker and Alberts, 1994). The primed single-stranded DNA template used was a derivative of M13mp7 called M13mp4. Downstream from a 5'-^{32}P-labeled 20 nucleotide primer are 8 C, A, T nucleotides followed by 3 consecutive G nucleotides, so that the DNA polymerase holoenzyme can be stopped just before the three G nucleotides by omitting dCTP (see Hacker and Alberts, 1994). Located 40 nucleotides from the 3'-end of the primer is the start of a 15 base-pair hairpin that was generated for use in this assay (Figure 1).

The time that the holoenzyme remains stopped at the hairpin is dependent not only on the time of incubation before adding the gene 32 protein (see Figure 1, steps c and d), but also on the rate of hairpin melting. Therefore, having a DNA helix that could be rapidly melted by the gene 32 protein was essential. Finding a hairpin that could stop holoenzyme movement in the absence of 32 protein but also allow holoenzyme movement shortly after the addition of high gene 32 protein concentrations was one of the most difficult steps in developing this assay.

A hairpin helix in single-stranded DNA that blocks DNA polymerase holoenzyme movement— Since some sites along single-stranded DNA not thought to form hairpin helices have

been found to arrest or stop DNA synthesis by the DNA polymerase holoenzyme (Huang *et al.*, 1981; Fairfield *et al.*, 1983; Charette *et al.*, 1986), it was important to determine that the block to holoenzyme movement at the polycloning site of M13mp7 single-stranded DNA (map position 6370 to 6417) was due to the presence of a hairpin helix at that site. Two predictions of such a block to holoenzyme movement were tested.

One prediction was that increasing the gene 32 protein concentration should lead to increased melting of the hairpin, which should in turn increase the fraction of holoenzymes that traverse the hairpin at the polycloning site. The effect of increasing the concentration of gene 32 protein on the number of holoenzymes that elongate a primer past the hairpin is shown in Figure 2. Different amounts of the gene 32 protein were preincubated with the template to allow it to destabilize the hairpin helix. The holoenzyme was then allowed to encounter the hairpin, with a free DNA polymerase trap present to prevent multiple cycles of primer elongation. As predicted for a DNA helix block of holoenzyme movement, increasing the concentration of the free gene 32 protein increases holoenzyme elongation past the hairpin (Figure 2).

A second prediction for a hairpin helix that blocks holoenzyme movement is that decreasing the length of the hairpin helix (and therefore its strength or stability) should reduce the block to holoenzyme movement. Two hairpin helices of smaller size than the original 22 base-pair hairpin of M13mp7 were generated (Figure 3A). Since these hairpins were constructed by altering the M13mp7 hairpin near its loop, the base pairs initially encountered by the

holoenzyme at the hairpin are the same for all three hairpin helices. Primer elongation by the holoenzyme through the region of the DNA template containing each hairpin as a function time is shown in Figure 3B. As predicted, the length of time that the DNA polymerase stalls at the DNA helix increases as the stability of the helix increases. Increasing the concentration of the DNA polymerase three-fold did not change the results, suggesting that - while movement through the hairpin could occur by multiple rounds of polymerase dissociation and reassociation (no DNA trap was present - the DNA polymerase reassociation rate was not limiting in these reactions (data not shown).

In summary, our data demonstrates that the hairpin formed at the polycloning site of M13mp7 single-stranded DNA causes a block to DNA polymerase holoenzyme movement under our conditions.

The DNA polymerase holoenzyme cannot pass a 15 base-pair hairpin helix in the absence of the gene 32 protein, but 32 protein addition rapidly removes the block— To measure a dissociation rate for the holoenzyme when stopped at a hairpin, we sought a hairpin that not only completely blocks holoenzyme movement but also melts quickly after addition of high concentrations of gene 32 protein. We found that the 15 base-pair hairpin on M13mp4 has these qualities. This hairpin completely blocks holoenzyme movement when the gene 32 protein is absent (Figure 4). At 0.08 sec after the addition of dCTP to initiate DNA synthesis, the holoenzyme has elongated the primer up to the first base-paired nucleotide in the stem of the hairpin (map position 6369), and at 0.15 sec, an equal distribution of bands (spanning from

one nucleotide before the stem of the hairpin to two nucleotides inside the stem of the hairpin), are observed. The distribution of the bands does not change even after longer incubations without dCTP (Figure 4) and no primer elongation past the hairpin is observed (data not shown). This shows that the holoenzyme is stopped at the hairpin, and is unable to elongate slowly through it.

In the presence of 400 μg/ml gene 32 protein (50 μg/ml was enough to cover all of the single-stranded DNA present), the holoenzyme rapidly elongates the labeled primer past the 15 base-pair hairpin on M13mp4. As shown in Figure 5, 50 percent of the holoenzymes have moved past the hairpin in 0.24 sec, and 100 percent of those still associated have done so in 0.4 sec.

The presence of a band at the hairpin at 0.5 sec indicates that an appreciable proportion of holoenzymes dissociate when they reach the hairpin, even with excess 32 protein present. This result is explained later (see Figure 9, below). Primer elongation through the region of the template containing the hairpin is an all-or-none process: the holoenzyme does not appear to inch its way through the region one nucleotide at a time. The conditions used in this experiment were the same as those used for the assay for holoenzyme dissociation (see Figure 1 and Methods). The concentration of T4 DNA polymerase was limiting and a free DNA polymerase trap was present during the melting of the hairpin by 400 μg/ml of gene 32 protein. Consequently, unlike the results shown in Figure 3, only the movement through the hairpin of holoenzymes that never dissociated from the M13mp4 template is observable.

The DNA polymerase holoenzyme-DNA complex rapidly dissociates when the holoenzyme stalls at a hairpin helix— The results of the holoenzyme dissociation assay in Figure 1 are shown in Figure 6. As expected, the intensity of the chase product band, which represents holoenzymes that have remained associated with the template, decreases with increasing incubation times. Conversely, the intensity of the band corresponding to primers elongated to the hairpin, which represents holoenzymes that have dissociated from the DNA template, increases with increasing incubation times. Under the conditions of very low DNA polymerase concentration, no primers were elongated to the hairpin when any of the three DNA polymerase accessory proteins (proteins from T4 genes 45, 44, and 62) was omitted from the reaction (data not shown). Therefore, the dissociation of the intact holoenzyme is measured in the assay, rather than the dissociation of a mixture of free DNA polymerase and holoenzyme molecules.

The percent of DNA polymerase holoenzyme molecules associated with the template as a function of time is shown in Figure 7. The dissociation when stopped at a hairpin helix is seen to be very rapid, with an apparent half-time of 1 to 2 seconds. However, a plateau is reached when 80 to 90% of the molecules have left the template. This apparent plateau appears to be an artifact caused by polymerase holoenzyme reassociation with the template after 32 protein addition for the following reasons. In control experiments where the free polymerase trap was mixed with the labeled primer-template and the replication proteins then added, very little labeled primer elongation was detected. However, primer elongation

increased if excess 32 protein was also added (Figure B-1). (The trap consists of poly dT·oligo dA which has a lower melting temperature (T_m) than the primer-template; thus, the presence of excess 32 protein may reduce the effectiveness of the trap by melting it.) Since the control experiments suggest that holoenzyme reassociation is occurring only during the 3 sec incubation with excess 32 protein and not during the time when the polymerase is stalled at the hairpin, this reassociation should add a constant background to all of the samples, independent of the time of holoenzyme stalling.

To test the above explanation for the plateau, the dissociation assay was repeated under conditions that should reduce holoenzyme reassociation. The concentration of the T4 DNA polymerase was reduced 2.5 fold, and, separately, a nonhydrolyzable ATP analog, ATPγS, was added with the poly dT·oligo dA trap to reduce holoenzyme reformation by competitively inhibiting ATP hydrolysis. Figure 8 shows that these changes in assay conditions reduce the percent of holoenzymes associated at the late time points, as expected. Thus, the failure of the percent of holoenzymes associated with the template to approach zero at long times is most likely a result of holoenzyme recycling during the incubation with excess 32 protein. In addition, this experiment shows that inhibiting ATP hydrolysis with ATPγS while the holoenzyme is stopped at the hairpin does not alter the holoenzyme dissociation rate.

Since the rate of holoenzyme dissociation at the hairpin is so fast, we have used a rapid-quench apparatus to precisely initiate and stop the reactions after very short incubations. The results of a dissociation assay using this apparatus are shown in Figure 9A. (To

minimize DNA polymerase holoenzyme reassociation with the labeled primer-template during the hairpin melting step, ATPγS was added with the free DNA polymerase trap [poly dT·oligo dA DNA].) The dissociation of the holoenzyme-DNA complex follows a simple exponential decay with a half-life of 0.78 -/+ 0.1 sec (Figure 9).

The time on the horizontal axis in Figure 9 is that between the addition of dCTP (which allows the holoenzyme to encounter the hairpin) and the addition of excess 32 protein. The actual time the holoenzyme remains stalled at the hairpin will include the time required to melt the hairpin after 32 protein addition. When the results from the shortest incubations are replotted on a logarithmic scale vertical-axis (Figure 9B), the straight line obtained extrapolates back to 100 percent associated holoenzymes at -0.36 sec, which is within experimental error of the observed half-time of 0.24 sec for the holoenzyme to traverse the hairpin (Figure 5) minus the travel time to the hairpin of 0.08 sec (Figure 4). We conclude that the rapid dissociation of the holoenzyme is a result of its encountering the hairpin and being forced to stall. In addition, the success of the back-extrapolation suggests that the dissociation of the first 30% of holoenzyme molecules, which could not be measured directly under our conditions, also follows first-order kinetics.

DISCUSSION

We have investigated the mechanism that enables the DNA polymerase holoenzyme to remain on the DNA template for many minutes when synthesizing DNA or stopped by nucleotide omission, while allowing it to dissociate from the DNA template rapidly when

stopped after encountering the 5'-end of a previously synthesized Okazaki fragment. Our assay measures the dissociation rate of the polymerase component of the holoenzyme from the DNA template when the holoenzyme encounters a DNA hairpin helix. We find that the DNA polymerase holoenzyme-DNA complex dissociates with a half-life of about 1 sec when the holoenzyme encounters this helix . The dissociation follows first-order reaction kinetics, and it occurs independently of ATP hydrolysis. This measured rate is fast enough to account for the holoenzyme dissociation required for polymerase recycling on the lagging-strand of the DNA replication fork(Selick *et al.*, 1987).

Mechanism of fast melting of a DNA hairpin by the gene 32 single-stranded DNA binding protein—

In developing the dissociation assay we sought a hairpin that would stop the polymerase holoenzyme in the absence of the gene 32 protein (a single-stranded DNA binding, or helix destabilizing protein), while melting very quickly after addition of excess gene 32 protein. We found that in the absence of the gene 32 protein, both a 15 and 22 base-pair hairpin completely blocks primer elongation by the holoenzyme, but that a 13 base-pair hairpin is not enough. The addition of excess gene 32 protein enables 50% of the holoenzyme molecules to traverse the 13, 15, and 22 base-pair DNA hairpins in 0.05, 0.24 and 0.5 sec, respectively (Figure 3).

How does the gene 32 protein melt the DNA hairpin helix so rapidly? The gene 32 protein single-stranded DNA binding site is reported to be seven nucleotides (Jensen *et al.*, 1976; Kelly *et al.*, 1976). In an intact helix, the simultaneous generation of seven

unpaired-nucleotides by helix breathing is expected to be rare (Porschke, 1974), and gene 32 protein alone is kinetically blocked from melting completely duplexed natural DNA (Jensen *et al.*, 1976; Newport, 1980). However, when the DNA substrate is mostly single-stranded (as is the case in our studies), gene 32 protein molecules could slide along the template and bind to any unpaired nucleotides generated by breathing of the DNA. In our system, sliding by the 32 protein is limited to the single-stranded DNA on 5' side of the hairpin, since the DNA polymerase holoenzyme has elongated a primer to the hairpin, thereby generating duplex DNA on its 3' side of the hairpin.

The association of the T4 DNA polymerase holoenzyme with the gene 32 protein could facilitate hairpin melting. Our experience with the 32I* protein can be interpreted as supporting this view. The 32I* protein, which is generated by the proteolytic removal of 60 amino acids from the COOH end of the intact gene 32 protein, binds to single-stranded DNA with 2 to 4 times greater affinity than the intact protein and can melt duplexed DNA (Greve *et al.*, 1978; Lonberg *et al.*, 1981). However, unlike the intact protein, 32I* protein does not interact with the T4 DNA polymerase (Burke *et al.*, 1980). In the simplest view, substitution of the 32 protein with the 32I* protein should greatly increase the percent holoenzymes associated with the template under conditions where the time the polymerase remains stalled at the hairpin is primarily determined by the speed of hairpin melting. However, we find that substituting gene 32 protein preparations that contain less than 5% 32I* with preparations containing 50% 32I* protein does not increase the percentage of

holoenzymes associated with the template when the omitted nucleotide and 32 protein are added simultaneously creating the shortest possible time of stalling (Table I-B). This might suggest that the interaction of the T4 DNA polymerase and the gene 32 protein facilitates hairpin melting and that the increased ability of the 32I* protein to melt duplexed DNA exactly offsets this loss.

The idea that the T4 DNA polymerase aids the gene 32 protein is also consistent with the previous finding that increasing the concentration of gene 32 protein, even above levels necessary to cover all of the single-stranded DNA present, increases the rate of DNA strand-displacement DNA synthesis by the polymerase holoenzyme, since neither protein alone can separate the duplexed helix (Alberts *et al.*, 1983). The interaction of the gene 32 protein with DNA polymerase could either enhance hairpin melting by increasing the local concentration of the gene 32 protein at the hairpin or by increasing the affinity of the gene 32 protein for the DNA by altering its conformation.

What is the trigger for rapid holoenzyme dissociation on the lagging-strand of the replication fork? —

In the accompanying paper, we showed that a holoenzyme stalled by nucleotide omission dissociated from the DNA with a half-life of 2.5 min (Hacker and Alberts, 1994). In contrast, we find that a holoenzyme stopped at a DNA hairpin helix dissociates from the template with a half-life of 1 sec. Clearly, the holoenzyme is sensing a difference between being stalled by nucleotide omission and being stalled by DNA secondary structure. This regulation of holoenzyme dissociation makes sense. *In vivo*, on the leading strand of the

replication fork, the holoenzyme is processive for more than 165,000 bases: an acheivement that requires the holoenzyme to remain associated with the template for more than 5 minutes (Sinha *et al.*, 1980). However, on the lagging-strand of the replication fork, the holoenzyme must dissociate from the template within 1 to 2 sec once the end of a previously synthesized Okazaki fragment is encountered (Selick *et al.*, 1987). Our studies suggest that the holoenzyme on the lagging strand senses being stopped by template structure, and that this triggers its rapid dissociation from the template. Since a DNA hairpin helix triggers rates of holoenzyme dissociation similar to those in vivo, the 5' terminal phosphate and RNA-DNA hybrid (which has an A-form nucleic acid structure) found at the end of an actual Okazaki fragment cannot be what is sensed in regulating the switch from slow to rapid dissociation.

In the *E. coli* replication system, rapid dissociation of the polymerase III holoenzyme only occurs when a challenging DNA template containing a complex of the DNA polymerase III accessory proteins, called the preinitiation complex, is present (O'Donnell, 1987; O'Donnell and Studwell, 1990). In accord with those findings and the results of E. *coli* replication protein dilution experiments, a model has been invoked where the primer for the next Okazaki fragment that contains the preinitiation complex triggers the rapid dissociation of the holoenzyme on the lagging strand of the replication fork (Marians, 1992; Wu *et al.*, 1992a; Wu *et al.*, 1992b; Wu *et al.*, 1992c; Zechner *et al.*, 1992a; Zechner *et al.*, 1992b). In our studies, preincubation of the challenging template or free DNA polymerase trap with T4 DNA polymerase accessory proteins was not required to

trigger rapid dissociation of the holoenzyme when it is stopped at a hairpin. Furthermore, the slow rate of dissociation of a holoenzyme stalled by nucleotide omission are not increased by preincubating the DNA polymerase trap with the DNA polymerase accessory proteins (Hacker, 1994). Our studies imply that the trigger for dissociation of the T4 DNA polymerase at the completion of each Okazaki fragment is different than that suggesed for the *E. coli* replication system.

A number of possible models can be proposed for regulation of holoenzyme disassembly and its mechanism. One possibility is that the DNA polymerase adopts a conformation that does not interact with the polymerase accessory proteins. A DNA polymerase molecule that is not associated with its accessory proteins dissociates rapidly, following a simple exponential decay with a half-life of 0.12 sec (Capson *et al.*, 1992).

A second possibility is that the accessory proteins are continually sliding along the template to associate with the DNA polymerase and disassociate from the DNA polymerase with reassociation is faster than dissociation rate of the polymerase from the template. However, when the polymerase encounters the DNA hairpin the polymerase now dissociates from the template faster than the accessory proteins can reassociate with it. This model differs from the first one in that encountering a hairpin changes the polymerase's affinity for the DNA and <u>not</u> its affinity for the accessory proteins. Evidence suggesting that the components of the holoenzyme slide on the template was reported in the preceding paper (Hacker and Alberts, 1994) and is supported by other studies (Gogol *et al.*, 1992; Herendeen *et al.*, 1992).

A third possibility is that the DNA polymerase accessory proteins sense the double helical region ahead of the polymerase and adopt a conformation that has little affinity for the DNA template.

Previous studies of the properties of the T4 replication protein suggested "clock-like" mechanisms for rapid holoenzyme dissociation (Newport, 1980; Newport *et al.*, 1980; Selick *et al.*, 1987; Jarvis *et al.*, 1991; Munn and Alberts, 1991). The "clock-like" mechanisms predicted a lag-time before the onset of rapid holoenzyme dissociation. The simple first-order decay for the dissociation of the holoenzyme observed here (Figure 5) argues against "clock-like" mechanisms for rapid holoenzyme dissociation. In addition, the absence of an ATP requirement during holoenzyme dissociation eliminates ATP-driven models for rapid holoenzyme disassembly.

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FIGURE LEGENDS

Figure 1. The experimental strategy used to determine the kinetics of DNA polymerase holoenzyme dissociation when stopped at a region of DNA double helix. (a) The replication proteins are incubated in the absence of dCTP with a single-stranded M13mp7 DNA template containing a 5'-^{32}P-end-labeled oligonucleotide primer. The DNA polymerase holoenzyme forms on this primed-template and stops at the 3 consecutive G nucleotides on the template after incorporating 8 nucleotides. (b) dCTP is added to enable the holoenzyme to proceed to the hairpin helix. A trap for free DNA polymerase molecules (excess cold DNA) is added with the dCTP to prevent new DNA polymerase molecules from associating with the labeled DNA molecule. (c) The incubation time is varied to determine the amount of time that the holoenzyme remains stalled at the hairpin helix, and then (d) excess 32 protein is added to melt the hairpin helix rapidly and allow each holoenzyme molecule that has remained associated with the DNA to continue its DNA synthesis.

For analysis of the results, the samples are phenol extracted, digested with HhaI, and electrophoresed through a polyacrylamide gel under denaturing conditions. The radioactivity on the dried gel is quantitated using a Phosphor Imager (Molecular Dynamics), and the gel is autoradiographed. By comparing the number of DNA strands that have been elongated past the hairpin helix with the total number of elongated primers for each time point, the percent of dissociated holoenzyme is determined as a function stalling time.

Figure 2. **The fraction of DNA polymerase molecules that remain stopped at a hairpin helix decreases with increasing 32 protein concentration.** The reactions analyzed in lanes 1-3 contained 45, 150, and 300 μg/ml of gene 32 protein, respectively (100μg/ml 32 protein is required to cover all of the single-stranded DNA present). The replication proteins (25 ng/ml 43 protein, 20 μg/ml 45 protein, 20 μg/ml 44/62 protein and 32 protein) were incubated with 0.53 μg/ml M13 mp7 single-stranded DNA (22 base-pair hairpin) annealed with a 5'-^{32}P-end-labeled 20 nucleotide primer, 1.0 mM ATP, 60 μM each of dATP, dGTP, and dTTP in replication buffer for 30 sec at 37°C. After the holoenzyme was allowed to assemble for 30 sec, a free polymerase trap (9.35 μg/ml single-stranded DNA plasmid, pKH8G, with two 20 nucleotide primers annealed per molecule) was added for 30 sec at 37°C and then 120 μM dCTP was added for 30 sec. The reactions were stopped by adding two volumes of a 95% formamide/20 mM Na_3EDTA solution. The samples were electrophoresed on a denaturing 8% polyacrylamide gel and an autoradiograph is shown. Note that, even at the highest concentration of 32 protein, some holoenzyme molecules dissociate when they encounter this hairpin helix (lane 3).

Figure 3. **Measuring the rate of movement of the DNA polymerase holoenzyme through three different hairpin helices in the presence of excess 32 protein.** (A) A diagram of the nucleotide sequence of the hairpin formed at the polycloning site of M13mp7 single-stranded DNA, and the sequence of 2 other hairpins derived from this M13mp7 hairpin. (a) The mp5 hairpin with its stem of 13 base-pairs and a 4 nucleotide loop has a calculated ΔG=24.0 kcal and a Tm=85.0°C. (b) The mp4 hairpin with its stem of 15 base pairs and an 18 nucleotide poly A loop has a calculated ΔG=27.8 kcal and a Tm=85.0°C. (c) The mp7 hairpin with a 22 base-pair stem and 4 nucleotide loop has a calculated ΔG=49.3 kcal and a Tm=123.6°C. The ΔG at 37°C and Tm for these hairpins was calculated using the nearest-neighbor method of Breslauer *et al.* (1986) and the values for the free energies of loops reported by Turner *et al.* (1988). Note that the stem of the mp5 and mp4 hairpins is formed from the same base pairs as the mp7 hairpin.

(B) The rate of movement past each hairpin helix, as determined with a rapid quench apparatus. Samples were electrophoresed on a denaturing 8% polyacrylamide gel, and a autoradiograph of the gel is shown. The seconds on the horizontal-axis indicate the length of time between initiating DNA synthesis at 37°C and stopping the reaction with Na3EDTA in a rapid quench apparatus (see Methods). The DNA replication proteins (20 μg/ml 43 protein, 40 μg/ml 45 protein, 40 μg/ml 44/62 protein complex, and 10 μg/ml gene 32 protein) were pre-mixed with 1.06 μg/ml single-stranded DNA template, as designated (each containing a 5'-^{32}P- end

labeled 20 nucleotide primer), in replication buffer containing 60 μM each of dATP, dGTP, and dTTP and 1 mM ATP; 45 μl was loaded in one loop of the apparatus. Into the second loop was loaded 45 μl of 800 μg/ml gene 32 protein, 40 μg/ml poly dT_{1500}·oligo dA_{50} (1:3 moles), and 120 μM dCTP, 60 μM each of dATP, dGTP, and dTTP in replication buffer. After the samples were incubated in the loops for 90 sec at 37°C, DNA synthesis was initiated by rapid mixing of equal volumes of the reactants. The reactions were quenched at the indicated times by the addition of 80 μl of 0.3 M Na_3EDTA. As DNA size markers, pBR322 DNA-digested with MspI and pBR322-digested with BstNI were run in lanes 12 and 13, respectively.

Figure 4. **Measuring the time required for the DNA polymerase holoenzyme to elongate the labeled primer to the hairpin of M13mp4.** A plot of the results in the inset is shown. The radioactivity in the DNA band representing primers elongated to the hairpin was quantitated using a PhosphorImager.

(inset) Samples were electrophoresed on a denaturing 8% polyacrylamide gel, and a autoradiograph of the gel is shown. The seconds indicate the length of time at 23°C between initiating DNA synthesis by the addition of dCTP and quenching the reaction with Na_3EDTA. The DNA replication proteins (0.1 μg/ml 43 protein, 5.3 μg/ml 45 protein, and 11.7 μg/ml 44/62 protein complex (no gene 32 protein was present)) were pre-mixed with 2.1 μg/ml single-stranded DNA mp4 template containing the 5'-^{32}P- end labeled 20 nucleotide primer, 240 μM dATP, 60 μM dGTP, 60 μM dTTP and 100 μM ATP in replication buffer; 45 μl was loaded in one loop of a

quench-flow apparatus (Kintek, University Park, PA). Into the second loop was loaded 45 µl of 180 µM dGTP, 180 µM dTTP, and 240 µM dCTP in replication buffer, plus 80 µg/ml poly dT_{1500}·oligo dA_{50} (1:3 moles) and 3 mM ATPγS to prevent further holoenzyme formation. After the samples were incubated in the loops for 90 sec at 23°C, DNA synthesis was initiated by rapid mixing of equal volumes of the reactants. The reactions were quenched at the indicated times by the addition of 80 µl of 0.3 M Na3EDTA.

Figure 5. **The DNA polymerase holoenzyme rapidly traverses the 15 base-pair hairpin helix of M13mp4 in the presence of excess gene 32 protein.** Plot of the rate of primer elongation past the hairpin of M13mp4 (in the experiment analyzed in the inset). The radioactivity above the DNA band representing primers elongated to the hairpin (marked with an arrow in the inset) was quantitated using a Phosphor Imager.

(inset) Samples were electrophoresed on a denaturing 8% polyacrylamide gel, and an autoradiograph of the gel is shown. Reactions were carried out with a rapid quench apparatus essentially as described in Figure 4, except that 0.8 mg/ml gene 32 protein was added to the mixture of the free polymerase trap with dCTP in the second loop of the rapid quench apparatus. The seconds indicate the length of time between initiating DNA synthesis by rapid mixing of the reactants and quenching the reaction with Na3EDTA.

Figure 6. **An analysis of the dissociation of the DNA polymerase holoenzyme when stopped a DNA hairpin helix.** Samples from the DNA polymerase holoenzyme dissociation assay in Figure 1 were electrophoresed on an denaturing 8% polyacrylamide gel, and an autoradiograph of this gel is shown. The seconds at the bottom of the autoradiograph refers to the time the DNA polymerase holoenzyme was stalled at the hairpin helix with no 32 protein present (the time between the addition of dCTP and the addition of excess gene 32 protein). All reactions were carried out at 23°C in 0.3 ml Reacti-vials (Pierce), which were rapidly mixing with a micro spin vane (Pierce). For reactions of less than 10 sec, timing was accomplished with the aid of a metronome. The reaction conditions are described in detail in Methods.

Figure 7. **Plot of the percentage of DNA polymerase holoenzyme molecules that remain associated with the DNA template when stalled at a DNA hairpin helix.** The percentage of DNA polymerase holoenzymes associated with the DNA template was determined from the gel in Figure 7 as described in Methods. The seconds indicated on the horizontal axis are the time between the addition of dCTP, which allows the holoenzyme to encounter the hairpin almost immediately (see Figure 5), and the addition of excess 32 protein, which initiates melting of the hairpin and thereby enables a template-associated holoenzyme to continue its DNA synthesis.

Figure 8. **Reducing the DNA polymerase concentration or adding a nonhydrolyzable analogue of ATP (ATPγS) reduces polymerase holoenzyme reassociation without altering the rapid rate of holoenzyme dissociation.** Reactions were carried out as illustrated in Figure 1 and described in Methods. Where indicated, 1.5 mM ATPγS (final concentration) was added with the solution containing dCTP. The reactions containing 25 ng/ml 43 protein contained 60 μM dATP and 1 mM ATP during the pre-mix step, whereas all other reactions contained 240 μM dATP and 100 μM ATP.

Figure 9. **Plot of the results of a DNA polymerase holoenzyme dissociation assay performed using a rapid-quench technique.** The seconds indicated on the horizontal-axis was the time between the addition of dCTP, which allows the holoenzyme to encounter the hairpin and the addition of excess 32 protein, which initiates melting of the hairpin and thereby enables the template associated holoenzymes to continue DNA synthesis. Rapid-quench experiments were performed by mixing a solution containing the replication proteins, labeled primed M13mp4 DNA template, ATP, dATP, and dGTP in replication buffer with a second solution containing dCTP, ATPγS, and DNA polymerase. At the indicated times (seconds on horizontal-axis), a high concentration of gene 32 protein was added with the "quench" syringe. After 3 sec, the reactions were stopped by adding an equal volume of 0.3 M Na3EDTA with a hand-held syringe. Incubations were at 23°C. For a detailed description of

the procedure, see Methods. (A) The data was fit to the equation $y = ae^{-kt} + b$, and the best fit is shown. (B) Back extrapolation of the results in (A) to 100 percent associated DNA polymerase holoenzymes. Only the results from the shortest reaction incubation times are shown. The vertical-axis is logarithmic scale. On the horizontal-axis, values to the right of the zero point indicate the seconds between the addition of dCTP and the addition of excess 32 protein (see Figure 9); minus values for seconds to the left of the zero point show the time needed to extrapolate back to the vertical-axis.

Figure 1

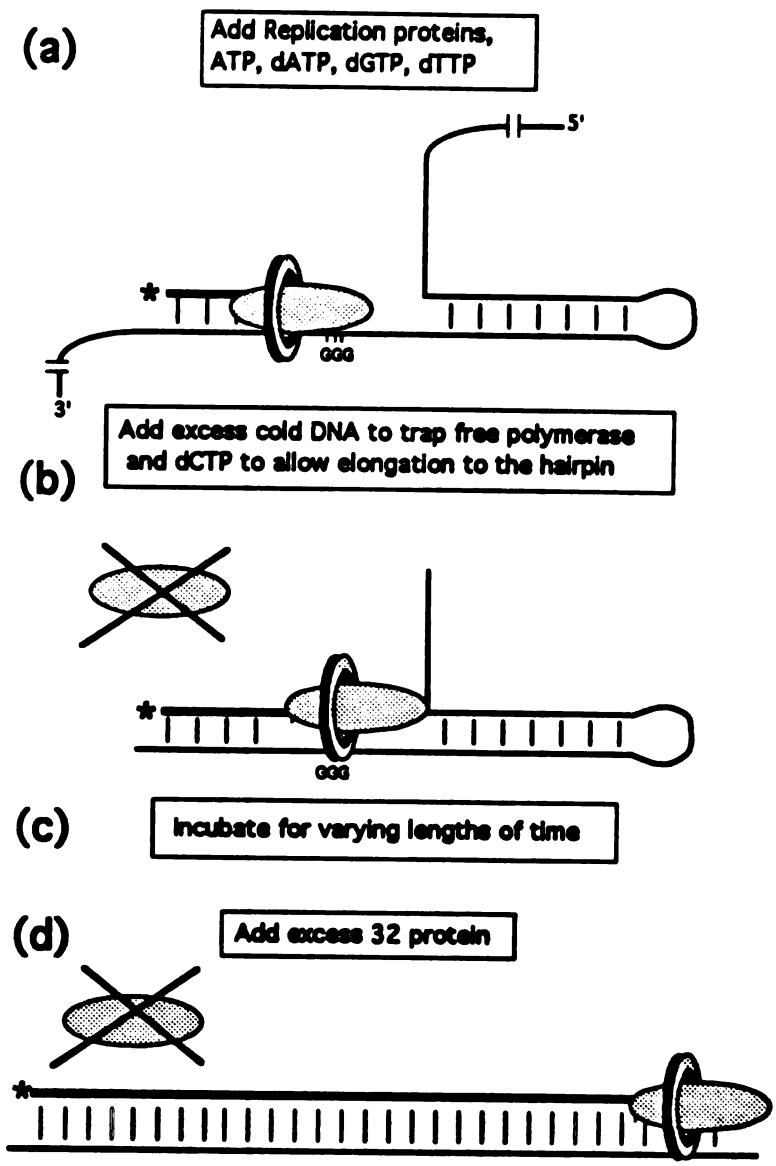

(a)
Add Replication proteins, ATP, dATP, dGTP, dTTP
5'
3'
GGG
(b)
Add excess cold DNA to trap free polymerase and dCTP to allow elongation to the hairpin
GGG
(c)
Incubate for varying lengths of time
(d)
Add excess 32 protein

Figure 2

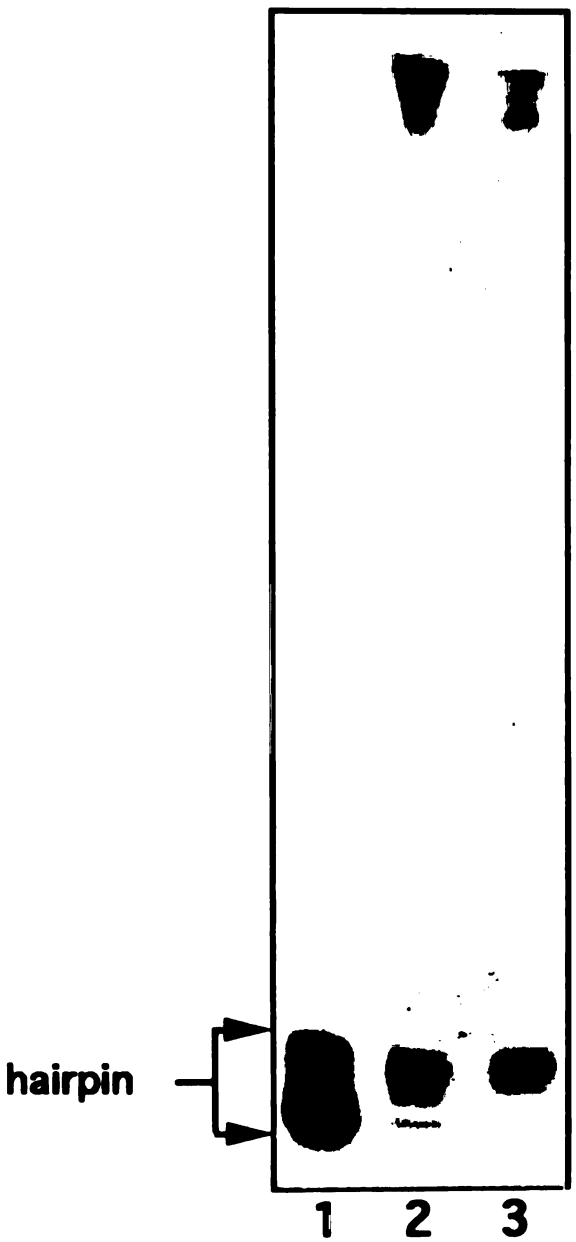


Figure 3A

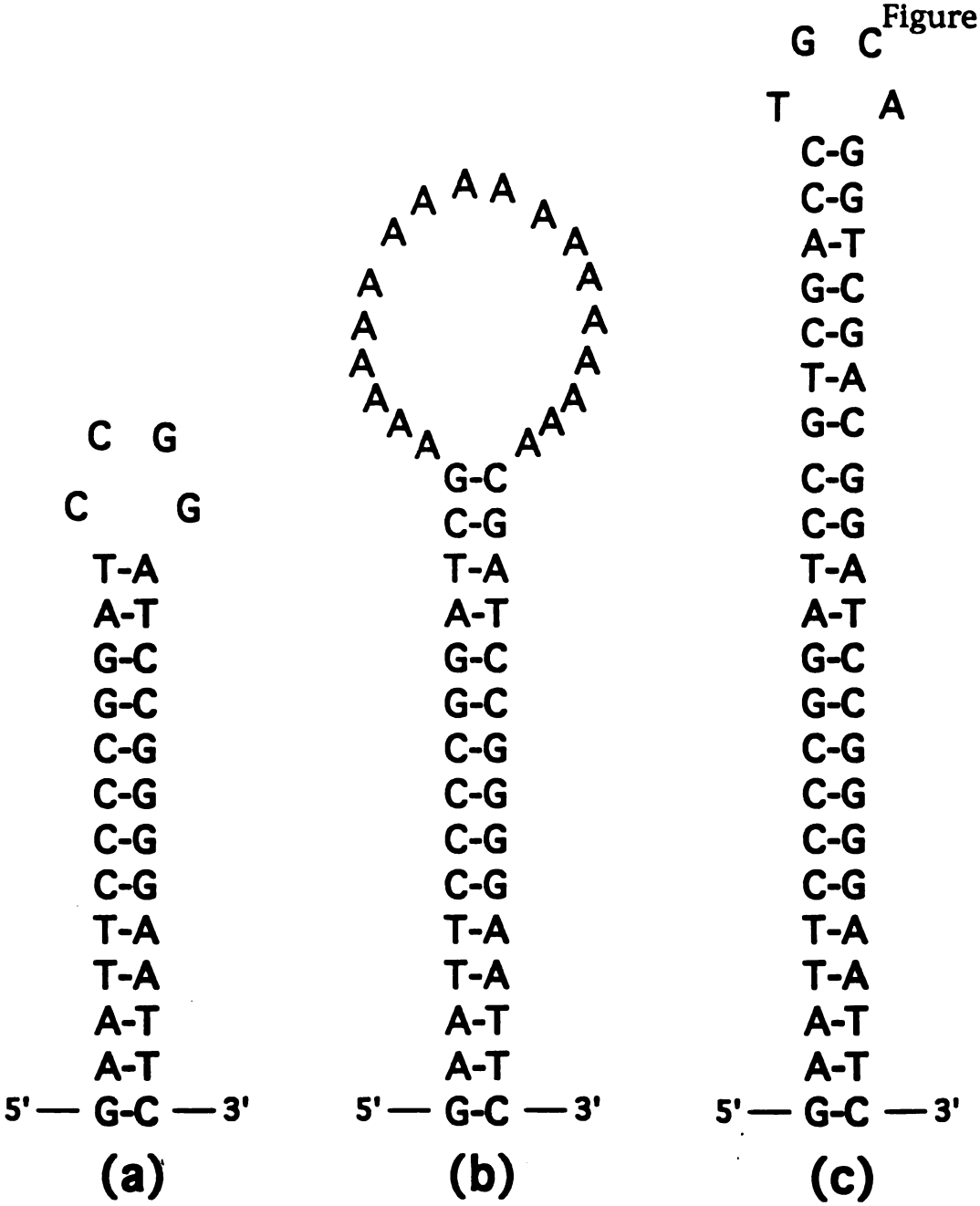

C G
C G
T-A
A-T
G-C
G-C
C-G
C-G
C-G
C-G
T-A
T-A
A-T
A-T
5' — G-C — 3'
(a)
A A A A A A A A A A A A A A
G-C
C-G
T-A
A-T
G-C
G-C
C-G
C-G
C-G
C-G
T-A
T-A
A-T
A-T
5' — G-C — 3'
(b)
G C
T A
C-G
C-G
A-T
G-C
C-G
T-A
G-C
C-G
C-G
T-A
A-T
G-C
C-G
C-G
C-G
C-G
T-A
T-A
A-T
A-T
5' — G-C — 3'
(c)

Figure 3B

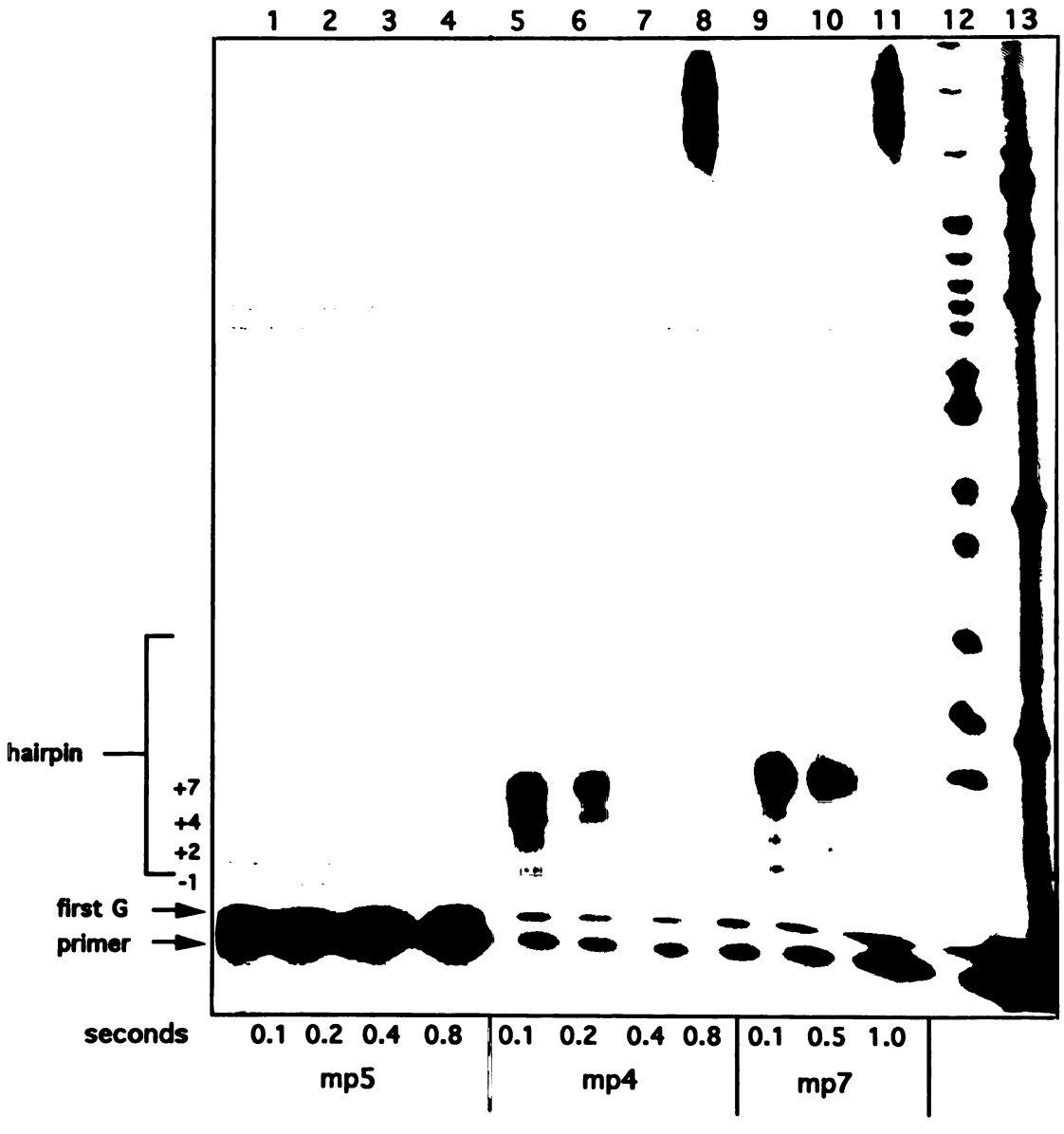

1 2 3 4 5 6 7 8 9 10 11 12 13
hairpin
+7
+4
+2
-1
first G
primer
seconds
0.1 0.2 0.4 0.8
0.1 0.2 0.4 0.8
0.1 0.5 1.0
mp5
mp4
mp7

Figure 4

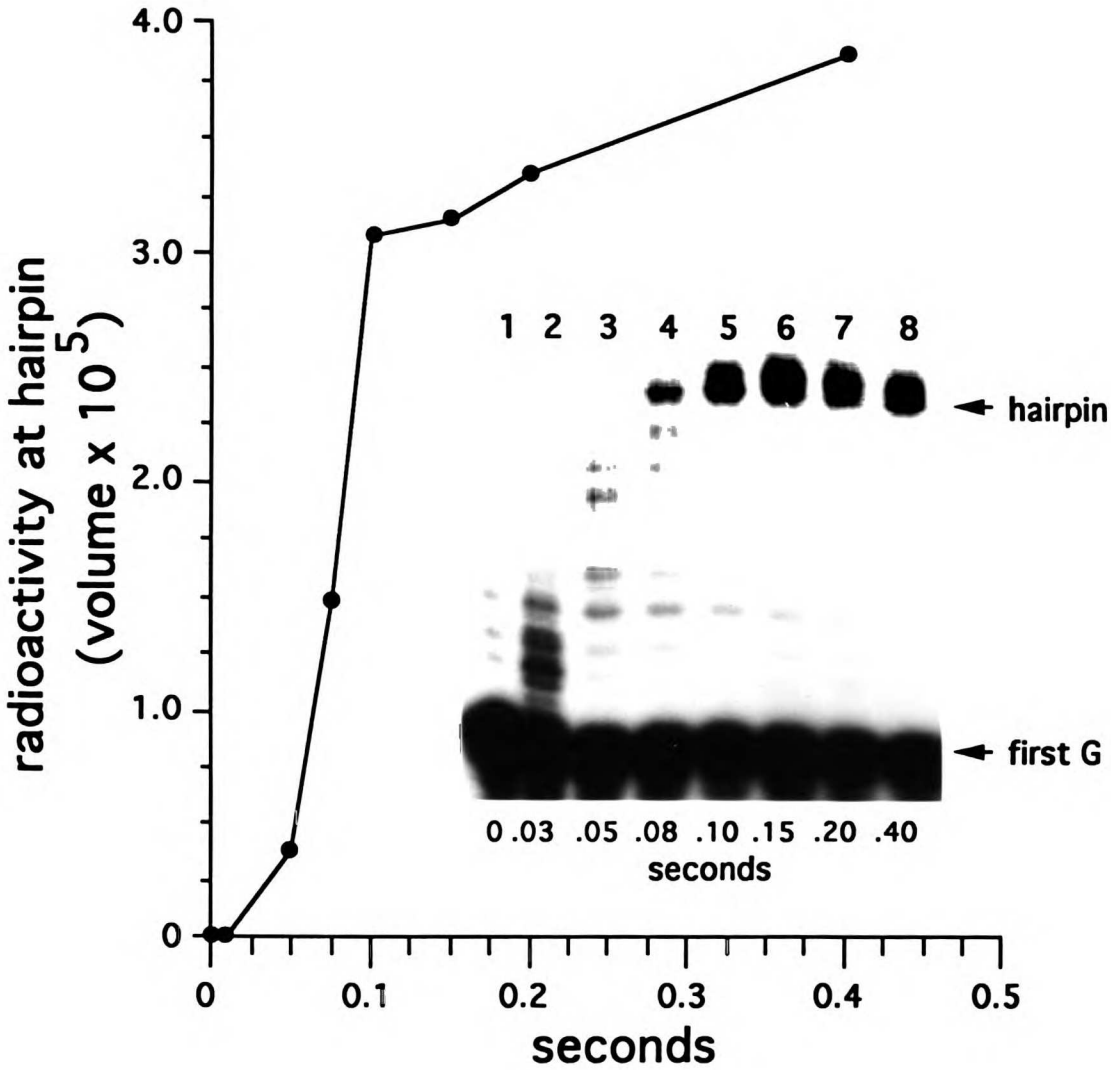

4.0
3.0
2.0
1.0
0
radioactivity at hairpin
(volume x 10^5)
0
0.1
0.2
0.3
0.4
0.5
seconds
1 2 3 4 5 6 7 8
hairpin
first G
0 .03 .05 .08 .10 .15 .20 .40
seconds

Figure 5

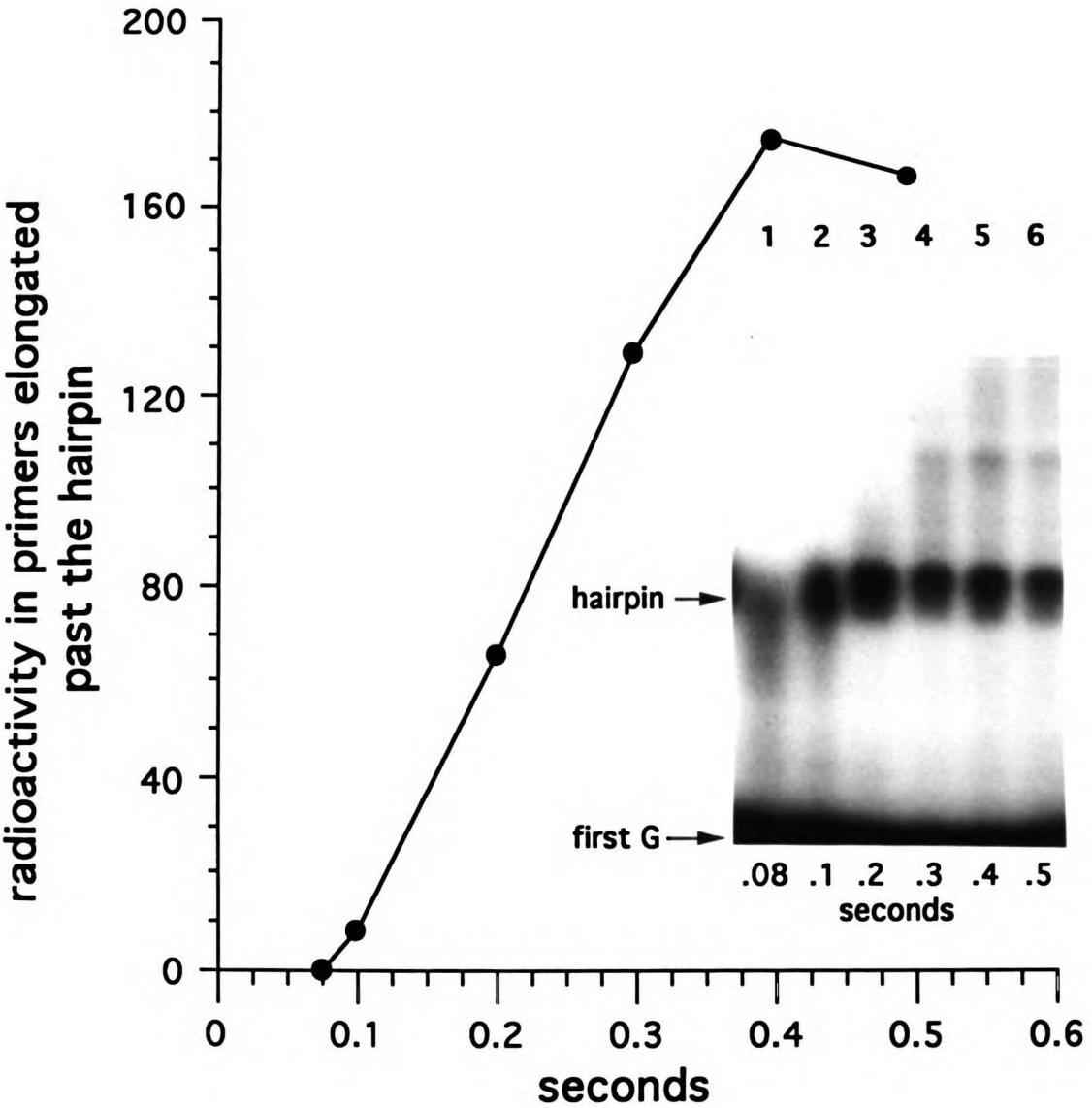

radioactivity in primers elongated past the hairpin
200
160
120
80
40
0
0
0.1
0.2
0.3
0.4
0.5
0.6
seconds
1 2 3 4 5 6
hairpin
first G
.08 .1 .2 .3 .4 .5
seconds

Figure 6

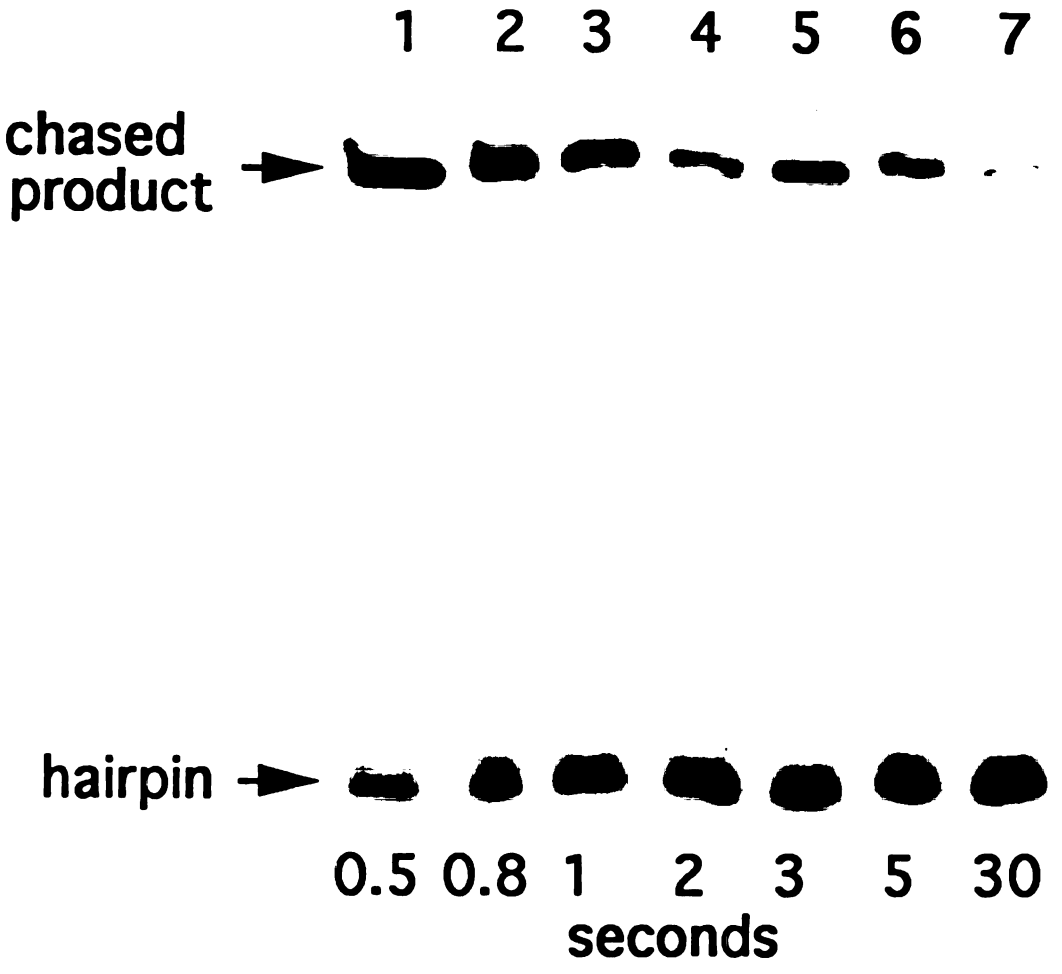


Figure 7

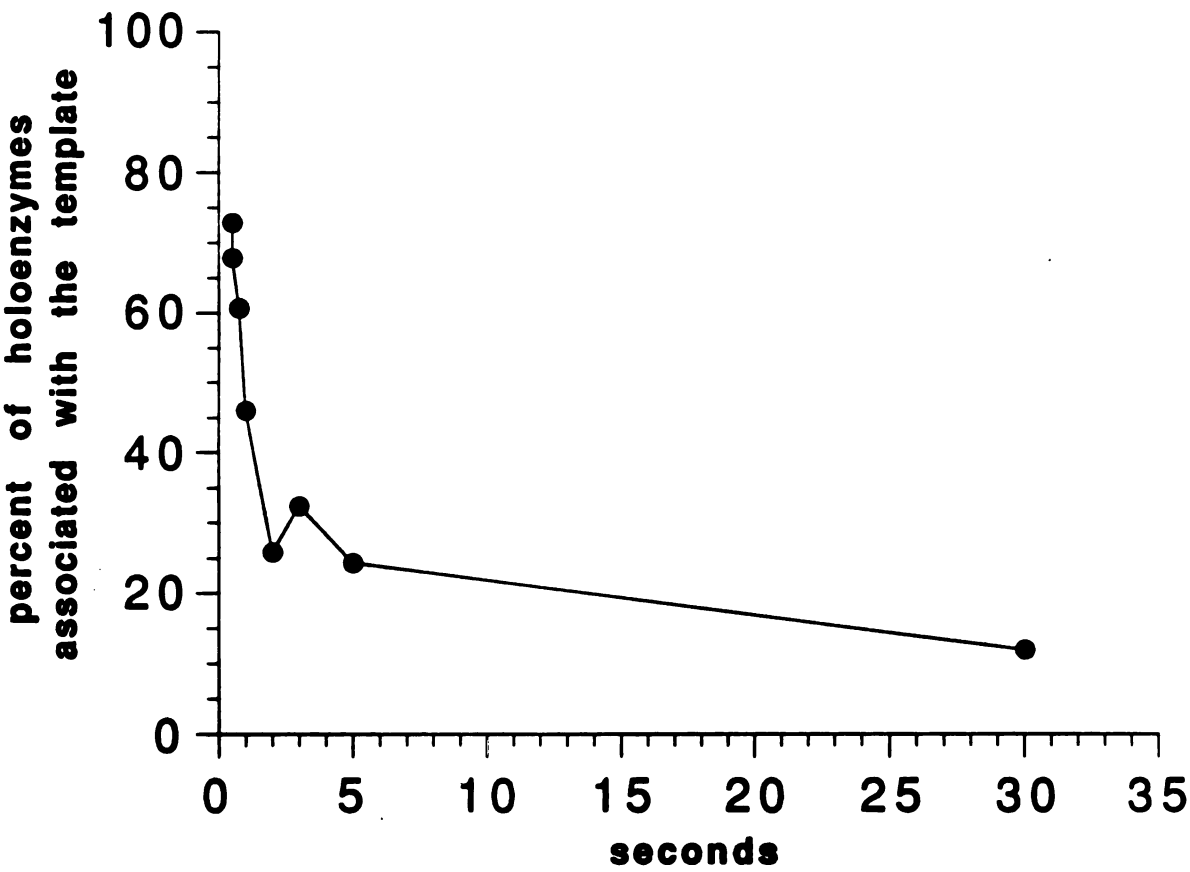

percent of holoenzymes associated with the template
100
80
60
40
20
0
0
5
10
15
20
25
30
35
seconds

Figure 8

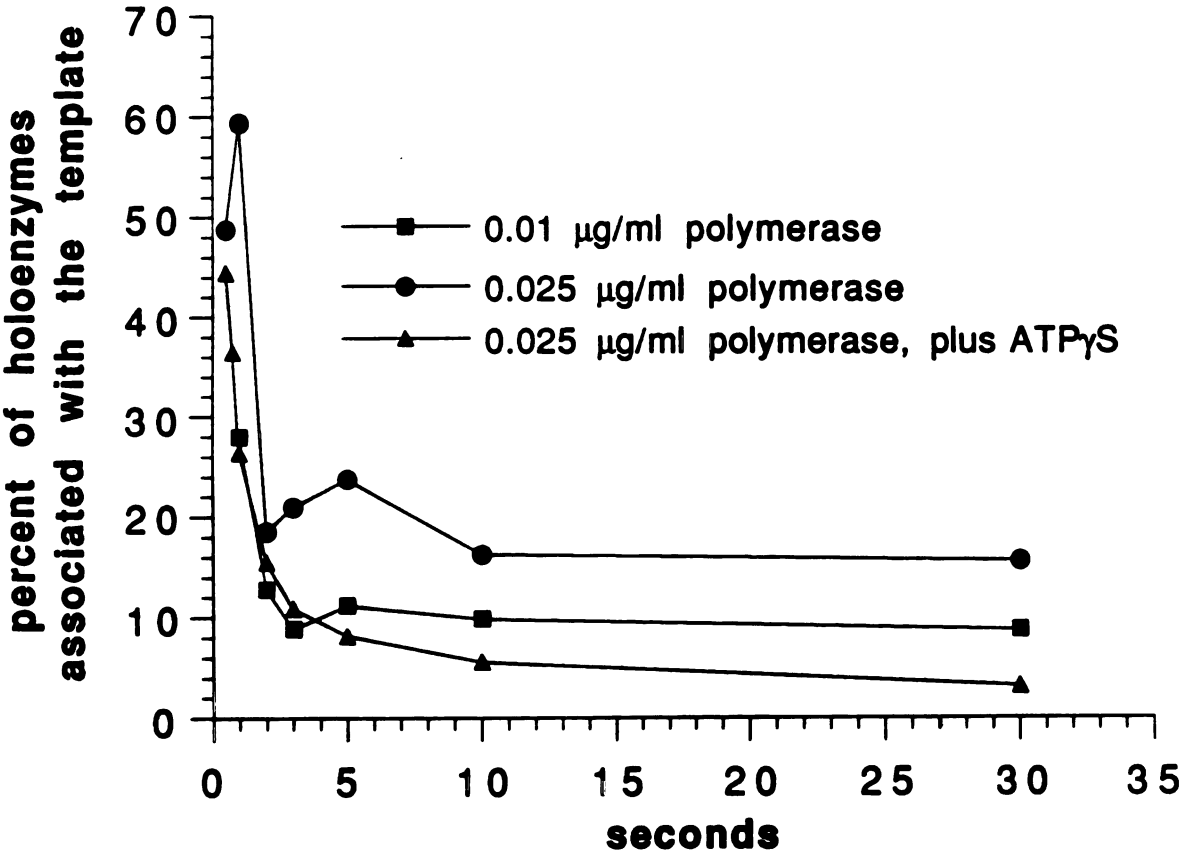

percent of holoenzymes associated with the template
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
seconds
0.01 µg/ml polymerase
0.025 µg/ml polymerase
0.025 µg/ml polymerase, plus ATPγS

Figure 9A

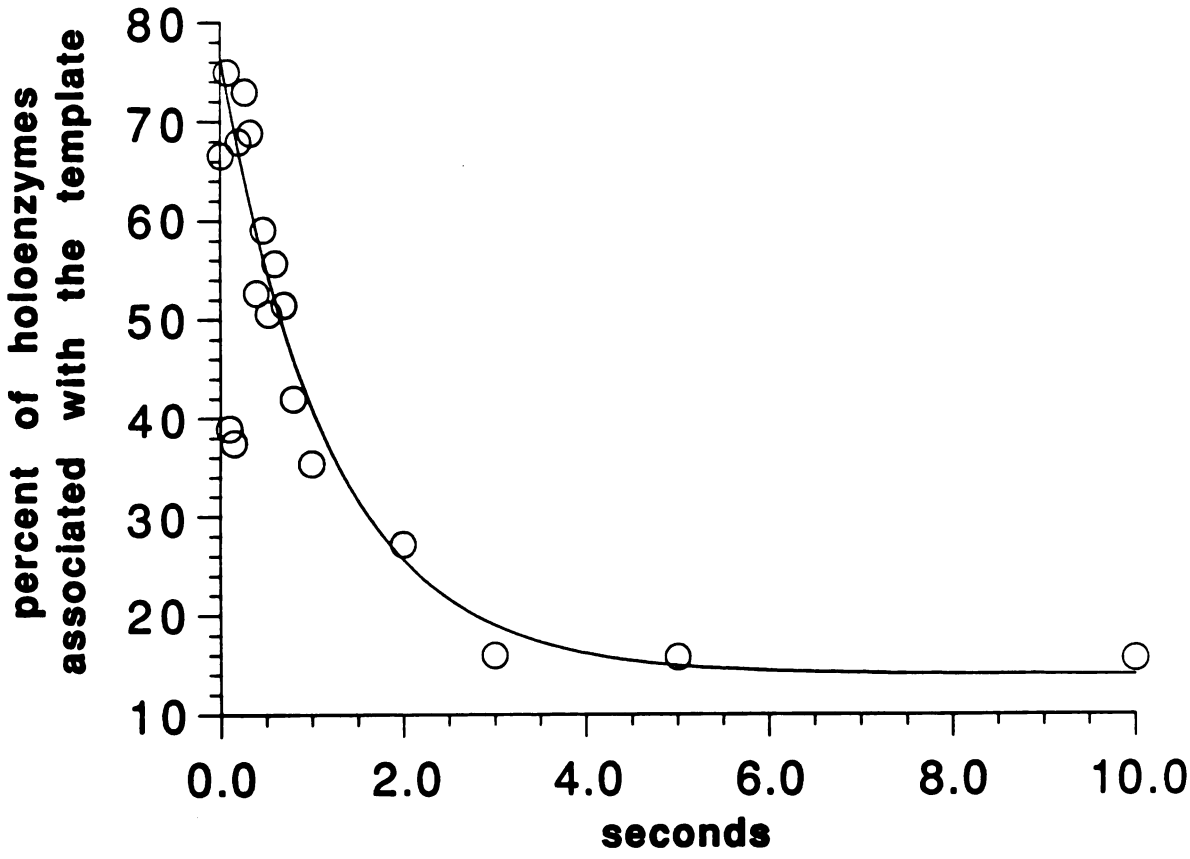
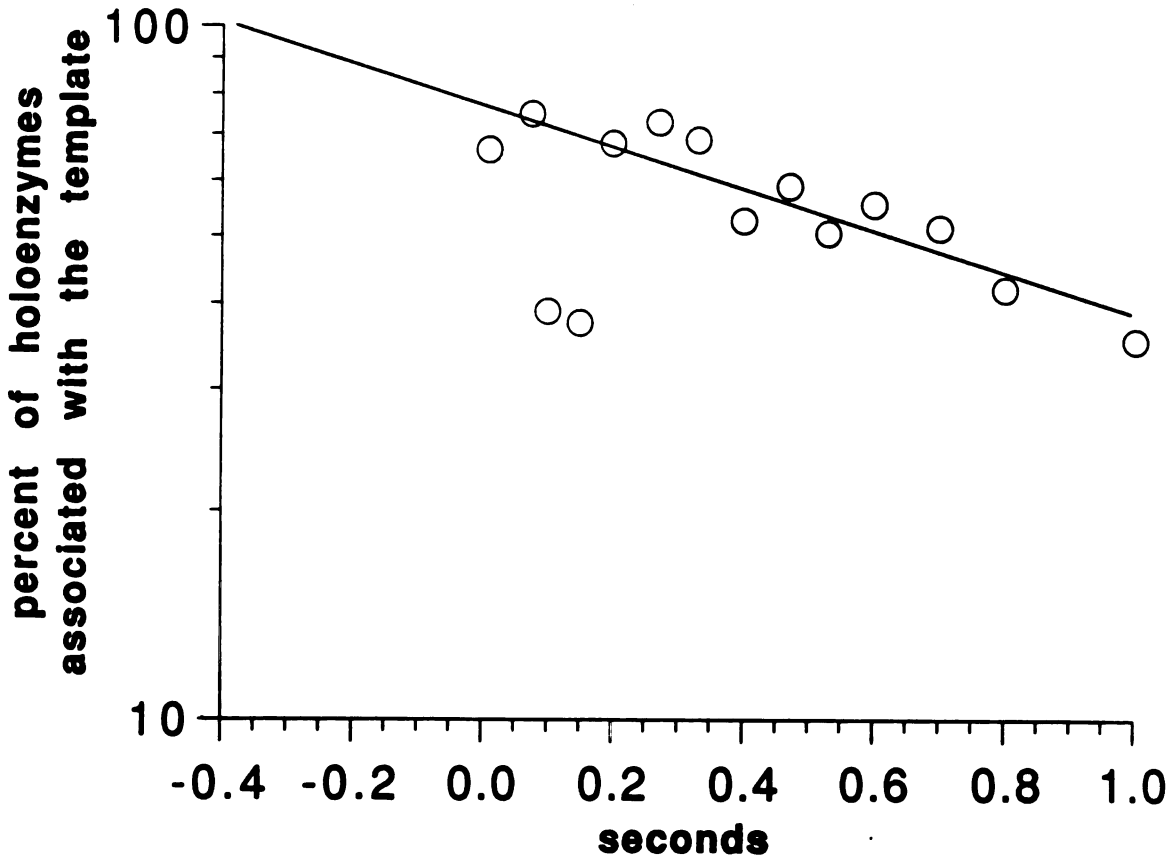

percent of holoenzymes associated with the template
80
70
60
50
40
30
20
10
0.0
2.0
4.0
6.0
8.0
10.0
seconds

Figure 9B


percent of holoenzymes associated with the template
100
10
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
seconds

Appendix A

Our DNA polymerase traps are efficient—

In chapter 4, the kinetics of T4 DNA polymerase holoenzyme dissociation from the DNA when the holoenzyme is stalled by nucleotide omission was studied. The assay used in these studies (Figure 2) employed a polymerase trap to limit the formation of holoenzymes to a very short incubation period and to prevent a second holoenzyme from associating with the labeled primer-template after the first holoenzyme dissociates. It was critical that the polymerase molecules be efficiently trapped since polymerase recycling would complicate determining the kinetics of holoenzyme dissociation and its parameters. The efficiency of the standard polymerase trap, which consisted of poly dT·oligo dA covered with the T4 gene 32 protein, was examined in three ways (Figure A-1): (1) The poly dT·oligo dA concentration was increased 2-fold . If recycling was occurring during the assay this increase should reduce it, and thereby, increase in the observed rate of holoenzyme dissociation. (2) The polymerase concentration was reduced 2-fold. With less polymerase to recycle, polymerase reassociation should be reduced if it was occurring. (3) The results of the dissociation assay using a different DNA polymerase trap consisting of 5 primers annealed at equal distances around a wild-type M13 molecule (50% of which was covered with gene 32 protein) were compared with the same trap preincubated with a high concentration of DNA polymerase accessory proteins. Although a stable DNA polymerase accessory protein-DNA complex has never been isolated, the

possibility exists that such a complex could be present on the labeled primer-M13mp7 template and preferred over our normal poly dT·oligo dA trap by the DNA polymerase (Polymerase accessory protein preincubation experiments were performed with the primed-wild-type M13 DNA instead of the standard trap because like the labeled primer-M13mp7 template, the wild-type M13 DNA molecule is circular, whereas poly dT·oligo dA DNA molecule is linear. Our findings (chapter 4: Figure 10, Figure 11 and Table I) and those of Gogol *et al.* (1992) suggest that the accessory protein complex is more stable on circular than linear DNA molecules)

Figure A-1 shows that increasing the standard polymerase trap concentration or, separately, decreasing the polymerase concentration did not change the rate of holoenzyme dissociation. Furthermore, in the dissociation assay performed using the primed-wild-type M13 DNA polymerase trap, omitting the preincubation of the trap with the accessory proteins did not change the results. All of these tests of the standard trap's efficiency suggest that it sufficiently prevents polymerase reassociation with the labeled primer-template after dissociation.

In the linearization of the DNA template studies (chapter 4: Figure 10, Figure 11 and Table I), an efficient polymerase trap was also important and its efficiency was tested. Although the trap consisting of 5 primers annealed to wild-type M13 (50% which was covered with gene 32 protein) did not appear to be efficient in the dissociation assay (Figure A-1), this trap is sufficient in the linearization assay. Figure A-4 shows that changes in the assay, which should reduce polymerase recycling if it was occurring

(increasing the concentration of the trap DNA or decreasing the polymerase concentration), did not alter the results in the longest incubation (60 sec) with EcoRI buffer (the template remains circular) with the holoenzyme stalled in the linearization assay. This difference in trap effectiveness compared to that of dissociation assay (Figure A-1) is mostly likely due to the slightly different conditions of the two assays (37°C verses 23°C, and 100% of the single-stranded DNA covered with 32 protein verses 50%) and the much shorter incubation times used in the linearization assay. The activity of the replication protein preparations may also have been different since the assays were performed months apart.

In control experiment on the circular template (Figure A-4), 73-78 percent of holonenzymes are associated at G sites 170 and 230 nucleotides away from the original primer, whereas only 45-55 percent of percent of holoenzymes are associated at G sites 70 and 120 nucleotides away. The most likely explaination is less stable holoenzymes dissociate before reaching the 170 and 230 G sites, while the stable holoenzymes remain associated and reach the 170 and 230 G sites. Differences in intrinsic holoenzyme stability could be due to heterogenity in the proteins that constitute the holoenzyme; some proteins are probably damaged during purification and storage.

In the linearization assay, the DNA template is rapidly cut with EcoRI or EcoRV—

In the linearization assay (chapter 4: Figure 10, Figure 11, and Table I), it was also important that all of the DNA template cut quickly with EcoRI enzyme and, in a separate experiment, with

EcoRV so that complexities associated with studying a mixture of linear and circular molecules could be avoided. Figures A-5 and A-6 show that under our linearization assay conditions the DNA template is cut rapidly with EcoRI. The rate of cutting with EcoRV, although slower than with EcoRI, is much faster than the rate of holoenzyme dissociation from a circular DNA molecule (half-life 2.5 min) and therefore also satisfactory.

The effect of template linearization on the dissociation of the DNA polymerase holoenzyme-DNA complex when the DNA template is cut with EcoRV—

The EcoRI enzyme was preferred over EcoRV in the linearization assay since EcoRI cut the DNA template nearly 2-times faster than the most active preparation of EcoRV available (compare Figure A-5 with A-6). However, EcoRI enzyme produces a 3' recessed end at its cut site that could possibly serve as a binding site for the DNA polymerase or its accessory proteins, retarding their exit from a DNA end. Therefore, as a control, the linearization assay was repeated with EcoRV, which generates blunt ends (and cuts just behind the EcoRI site on the primer). The results of these experiments are summarized in Table A-1, and they are the same as when EcoRI was used in the assay (chapter 4: Figure 11 and Table 1).

The holoenzyme dissociates more quickly from sites close to the original primer than from sites further away—

Initially, our holoenzyme dissociation assay employed as a template the circular single-stranded DNA of plasmid pKH8G (which

was used to derive the pKH32 used in the linearization studies (chapter 4, Figure 9B)). Using this template and omitting dCTP, the polymerase holoenzyme paused at a series of G sites, which contained a region of six identical nucleotides in common, located 50 nucleotides apart. The half-life of holoenzyme dissociation from these sites was greater than 2 minutes -a totally unexpected result. [A half-life of seconds was expected from the detailed footprint analysis of the T4 replication proteins performed by another student in the laboratory, Maureen Munn (Munn and Alberts, 1991a; Munn and Alberts, 1991b)]. The assay was repeated using a synthetic oligonucleotide substrate of identical sequence to the DNA template with a hairpin used in the footprint studies. Unlike the results with the pKH8G template, the rate of holoenzyme dissociation from the hairpined DNA template had a half-life of less than 30 seconds, when the holoenzyme was stalled by nucleotide omission (data not shown).

There are many differences between the two templates that could possibly explain the results. The two templates differ in the DNA sequence at the pause sites, the distance of the original primer from the pause site and the topology of the DNA molecules (pKH32 is circular, whereas the hairpined DNA template was linear). To examine the effect of DNA sequence and distance of the original primer from the pause site, dGTP or dTTP was limited instead of omitting dCTP in the dissociation assay (Figure A-2). This allowed the polymerase holoenzyme to elongate a primer to a variety of lengths along the pKH8G template before pausing and caused the polymerase to pause at sites other than the original G sites. An aliquot of the sample was taken immediately before a non-limiting concentration

of nucleotide (dGTP or dTTP) was added, which allows associated holoenzymes to continue DNA synthesis, so that the total number of holoenzymes paused at the various sites along the template could be determined.

An autoradiograph of the results of this altered assay is shown in Figure A-2. When a non-limiting concentration of nucleotide is added after a 30 sec incubation with limiting nucleotide (lanes 2 and 4), most of the labeled bands are extended by the associated holoenzymes to long DNA molecules that migrate at the top of the gel. In contrast, only a few bands, which represent dissociated holoenzymes, remain on the lower portion of the gel. This result suggests that the holoenzyme-DNA complex has a half-life of at least 30 sec at many sites on the DNA and therefore suggests that the original G site is not a uniquely stable one. It should also be noted that in lanes 2 and 4, the lower bands mostly represent DNA molecules less than 30 nucleotides long. This suggests that a region of duplexed DNA longer than 30 nucleotides is important for holoenzyme-DNA complex stability. This idea is investigated further in Figure 5, chapter 4. The synthetic oligonucleotide DNA template with a hairpin contained a duplexed DNA region of less than 30 nucleotides, and this may have contributed to the low stability of the holoenzyme-DNA complex on this template. Ultimately, however, a circular topology of the DNA was found to be critical for the stability of the holoenzyme-DNA complex; this probably explains why the holoenzyme was so much more stable on pKH8G than on the linear DNA template used for footprinting (see discussion chapter 4).

Excess ATPγS, a nonhydrolyzable ATP analog, prevents holoenzyme formation–

To examine whether ATP hydrolysis is required continuously to maintain the holoenzyme-DNA complex or trigger its assembly, it was necessary to find a concentration of ATPγS that inhibits holoenzyme assembly under our dissociation assay conditions. Figure A-3 shows that 1.5 mM ATPγS is sufficient to completely block holoenzyme formation when dAMPPNP is substituted for dATP in reactions (the dATP can otherwise be utilized as an energy source for holoenzyme formation) and 0.1mM ATP is present. A free polymerase trap was not included in these reactions, and therefore some primers were elongated to the G pause site by multiple rounds of synthesis by the polymerase alone. In contrast, when reactions contained 1.5 mM ATPγS and the polymerase accessory proteins, essentially no DNA synthesis was observed because the ATPγS bound accessory proteins inhibit binding of the polymerase to the primer-template junction (Munn and Alberts, 1991b) and because the holoenzyme can not form without ATP hydrolysis (Piperno and Alberts, 1978; Jarvis *et al.*, 1991; Munn and Alberts, 1991).

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Munn, M. M. and Alberts, B. M. (1991) DNA Footprinting Studies of the Complex Formed by the T4 DNA Polymerase Holoenzyme at a Primer-Template Junction *J. Biol. Chem.* **266**, 20034-20044.

Munn, M. M. and Alberts, B. M. (1991) The T4 DNA Polymerase Accessory Proteins Form an ATP-dependent Complex on a Primer-Template Junction *J. Biol. Chem.* **266**, 20024-2033.

Piperno, J. R. and Alberts, B. M. (1978) An ATP stimulation of T4 DNA polymerase mediated via T4 gene 44/62 and 45 proteins *J. Biol. Chem.* **253**, 5174-5179.

Table A-1(a). The linearization of a circular DNA template with EcoRV rapidly destablizes the DNA polymerase holoenzyme. Results from the linearization assay performed at 0.5 μM each of dATP, dGTP, and dTTP.

percent of DNA polymerase holoenzymes associated with the template[1]				
	seconds incubated with buffer or EcoRV [2]			
nucleotides from EcoRV site	25		33	
	buffer	EcoRV	buffer	EcoRV
74	80	15	70	15
124	76	20	74	19
174	82	43	74	39

(b). The linearization of a circular DNA template with EcoRV rapidly destablizes the DNA polymerase holoenzyme. Results from the linearization assay preformed at 120 μM each of dATP, dGTP, and dTTP

percent of DNA polymerase holoenzymes associated with the template				
	seconds incubated with buffer or EcoRV			
nucleotides from EcoRV site	25		30	
	buffer	EcoRV	buffer	EcoRV
174	47	21	28	11
224	44	23	25	10
274	63	46	62	22
324	70	44	65	34
374	62	57	49	47
424	62	82	68	48

[1]The percentage of DNA polymerase holoenzymes associated with the DNA template was determined as described in Figure 11.

[2]EcoRV cuts more slowly than EcoRI (compare Figures A-4 and A-5). At 20 seconds after the addition of EcoRV, 60% of the DNA molecules were cut, with 80% cut after 55 seconds as shown in Figure A-5.

FIGURE LEGENDS

Figure A-1. **During the dissociation assay, the standard free DNA polymerase trap is sufficient to prevent polymerase reassociation with the labelled primer-template after dissociation.** These control reactions were carried out as schematized in Figure 2 under the conditions described in Methods unless otherwise noted.

(A) "normal" reactions contained 5 ng/ml T4 DNA polymerase and the standard DNA polymerase trap consisting of 133 μg/ml poly dT·oligo dA (1:3 molar ratio) 50% of which was covered with 32 protein (150 μg/ml). The "2x DNA polymerase trap" reactions contained 266 μg/ml poly dT·oligo dA, 25% of which was covered with 32 protein. The "1/2 polymerase" reactions contained 2.5 ng/ml T4 DNA polymerase (DNA polymerase trap concentration was normal).

(B) The results with the poly dT· oligo dA DNA polymerase trap at the normal concentration is shown for comparison with the results using a primed M13 DNA polymerase trap. The primed M13 DNA polymerase trap consisted of 40 μg/ml single-stranded M13 DNA annealed with 5, 28 nucleotide-long primers; the single-stranded DNA was 50% covered with 32 protein (200 μg/ml). At the concentrations of DNA used, the poly dT·oligo dA trap has 100 times more 3' OH ends for binding by the polymerase than the M13 trap. The difference in the results using the two traps is most likely due the poly dT·oligo dA trap being much more efficient at preventing polymerase reassociation than the M13 trap.

(C) The primed M13 DNA used in (B) was preincubated with 1.2 44/62 protein molecules (20 μg/ml) and 1.35 45 protein molecules (10 μg/ml) per primer molecule in the presence of ATP. The results from (B) are shown for comparison.

Figure A-2. **The holoenzyme dissociates more quickly from sites close to the original primer than from sites further away.** The experimental scheme was the same as for the other experiments (Figure 2), although reaction conditions were different. These reactions were performed at 37°C in replication buffer containing 66 mM potassium acetate, 33 mM Tris acetate, pH 7.8, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 200 μg/ml human serum albumin, while the other experiments were performed at 23°C and in replication buffer containing 160 mM potassium acetate. The replication proteins (71 ng/ml T4 DNA polymerase, 0.8 μg/ml 45 protein, 1 μg/ml 44/62 protein and 6.67 μg/ml 32 protein) were incubated for 30 sec at 37°C with 0.34 μg/ml single-stranded circular DNA of plasmid pKH8G (see Methods) annealed with a 5'-^{32}P- end labeled 20 neucleotide-long oligonucleotide, and1mM ATP. The reactions also contained 60 μM each of three nucleotides (dATP, dCTP, and dGTP or dTTP), and 17.5 nM of the fourth nucleotide as indicated with a "-". A free polymerase trap (28 μg/ml unlabeled primed pKH8G single-stranded DNA preincubated with 280 μg/ml 32 protein, 35 μg/ml 45 protein, and 40 μg/ml 44/62 protein) was added to prevent DNA polymerase reassociation with the labeled primer after dissociation, and the incubation was continued for 25 sec. A small aliquot was removed and its reaction stopped with 2

volumes of a 95% formamide / 20mM Na_3EDTA solution (lanes 1 and 3) to determine the total number of holoenzymes paused at each site. 5 sec later, 60 μM dGTP (lane 2) or 60 μM dTTP (lane 4) was added for 20 sec to allow associated holoenzymes to continue DNA synthesis. The reactions were stopped by adding 2 volumes of a 95% formamide / 20mM Na_3EDTA solution. The samples were electrophoresed on a denaturing 8% polyacrylamide gel and a autoradiograph is shown. DNA molecule length in nucleotides is designated on the right of the gel.

Figure A-3. **Excess ATPγS, a nonhydrolyzable ATP analogue, prevents holoenzyme formation.** The reactions were performed as described in Figure 8 (chaper 4) except the free DNA polymerase trap was <u>not</u> included and no dCTP was added. The "polymerase only" reactions contained 7 ng/ml gene 43 polymerase and 2.4 μg/ml gene 32 protein. The "holoenzyme" reactions contained 7 ng/ml T4 DNA polymerase, 5 μg/ml 45 protein, 5 μg/ml 44/62 protein complex and 2.4 μg/ml gene 32 protein. Reactions contained 0.8 μg/ml single-stranded M13mp7 DNA annealed with the 5'-^{32}P- end-labeled 38 nucleotide oligonucleotide, 1.5 mM ATPγS where indicated, 0.1 mM ATP, all four deoxyribonucleoside triphosphates except dCTP (0.625 μM each of dGTP and dTTP, and 20 μM dAMPPNP) in replication buffer. The reactions were performed at 23°C, initiated by the addition of the replication proteins, and stopped at the indicated times with 2 volumes of 95% formamide/20 mM Na_3EDTA solution. The radioactivity in the band at the G site was quantitated using a PhosphorImager (Molecular Dynamics).

Figure A-4. **In the linearization studies, the free DNA polymerase trap prevents polymerase reassociation with the labelled primer-template after dissociation.** These control reactions were carried out as schematized in Figure 9B under the conditions described in Methods, unless otherwise noted. Results from the longest incubation (60 sec) at 37°C with EcoRI buffer is shown. The "normal reactions" contained 10 ng/ml T4 DNA polymerase and DNA polymerase trap consisting of 20 μg/ml single-stranded M13 DNA annealed with five 28 nucleotide-long primers (average length), 100% of which was covered with 32 protein (200 μg/ml). The "2x DNA polymerase trap" reactions contained 40 μg/ml of the multi-primed single-stranded M13 DNA covered with 32 protein (400 μg/ml). The "1/2x polymerase reactions" contained 5 ng/ml 43 polymerase (the DNA polymerase trap concentration was normal).

Figure A-5. **Rapid EcoRI cutting of the DNA template under linearization assay conditions.** Reaction conditions were the same as those described in chapter 4 Methods for the linearization assay, except that the T4 DNA polymerase and its accessory proteins were omitted. Seconds on the x-axis indicates the length of the EcoRI restriction enzyme incubation with the DNA template, which consisted of a labelled primer, which contains the EcoRI recognition site, annealed to plasmid pKH32 single-stranded DNA covered with 32 protein. Digestion by EcoRI (200 units) was stopped by the addition of 2 volumes of 98% formamide/20 mM Na_3EDTA solution;

no digestion of the DNA template was observed if the formamide solution was added to the reaction prior to addition of EcoRI enzyme. Samples were electrophoresed through a denaturing 12% polyacrylamide gel, and the radioactivity in the bands representing the uncut and cut primers was determined using a Phosphor Imager (Molecular Dynamics). The percent of DNA template cut was determined by dividing the radioactivity corresponding to cut primer by the total radioactivity and mutliplying by 100.

Figure A-6. **Rapid EcoRV cutting of the DNA template under linearization assay conditions.** The reactions were performed as described in Figure A-5 except that EcoRV enzyme (200 units) was substituted for the EcoRI enzyme.

Figure A-1a

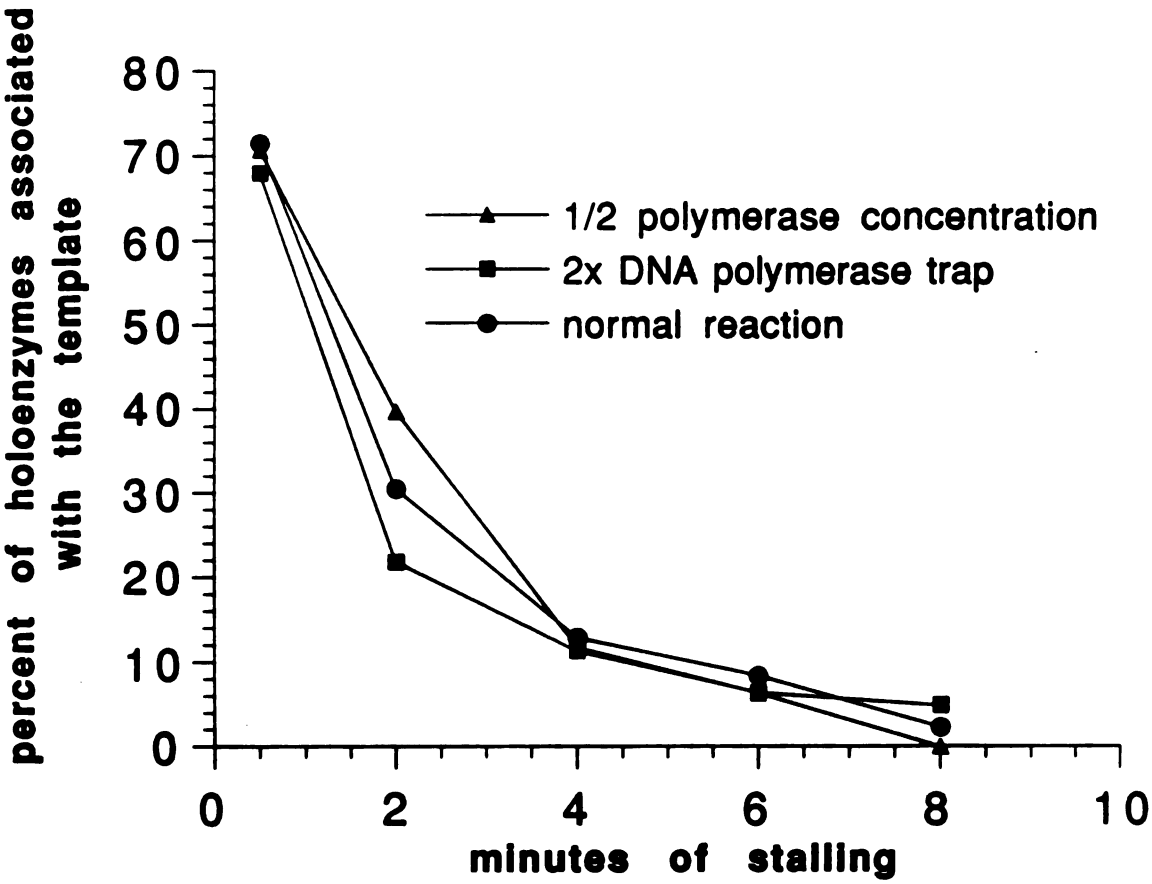

percent of holoenzymes associated with the template
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
minutes of stalling
1/2 polymerase concentration
2x DNA polymerase trap
normal reaction

Figure A-1b

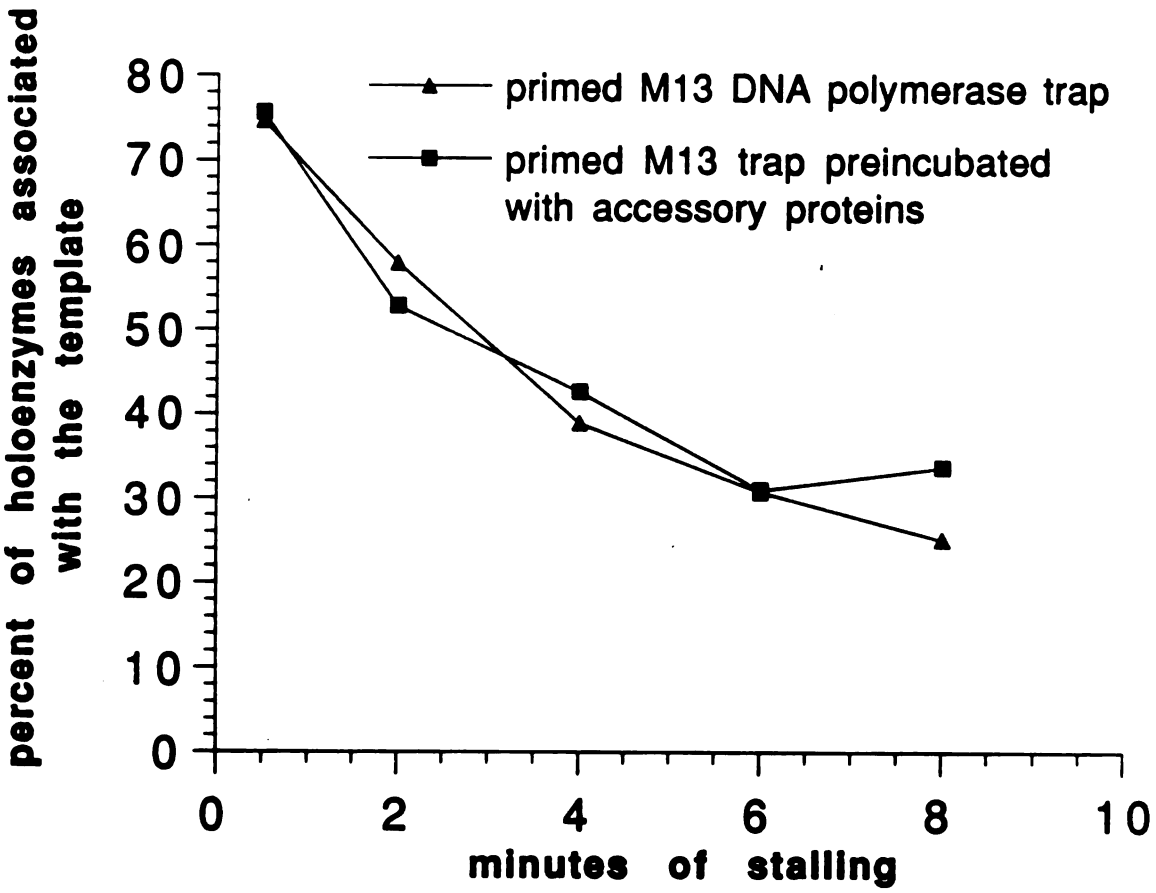

percent of holoenzymes associated with the template
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
minutes of stalling
primed M13 DNA polymerase trap
primed M13 trap preincubated with accessory proteins

Figure A-1c

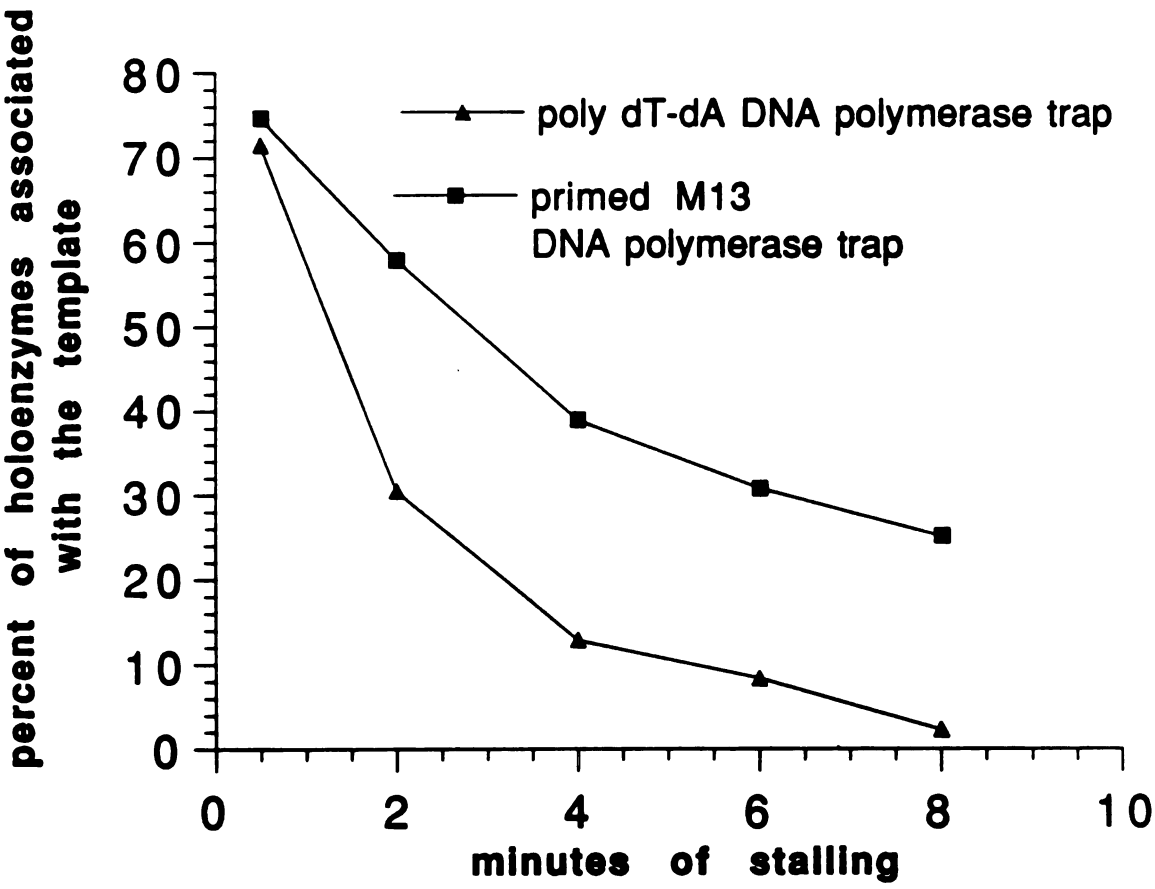

percent of holoenzymes associated with the template
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
minutes of stalling
poly dT-dA DNA polymerase trap
primed M13
DNA polymerase trap

Figure A-2

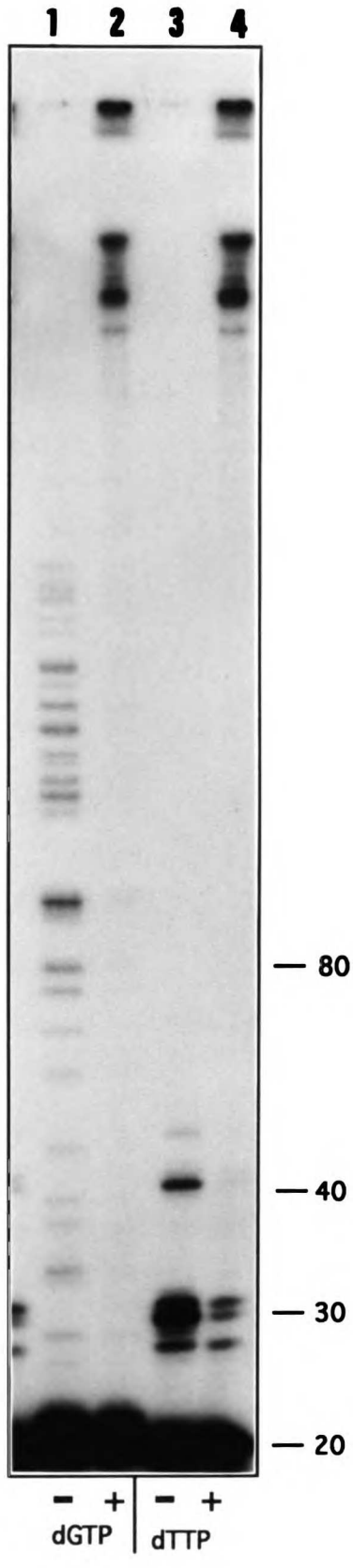


Figure A-3

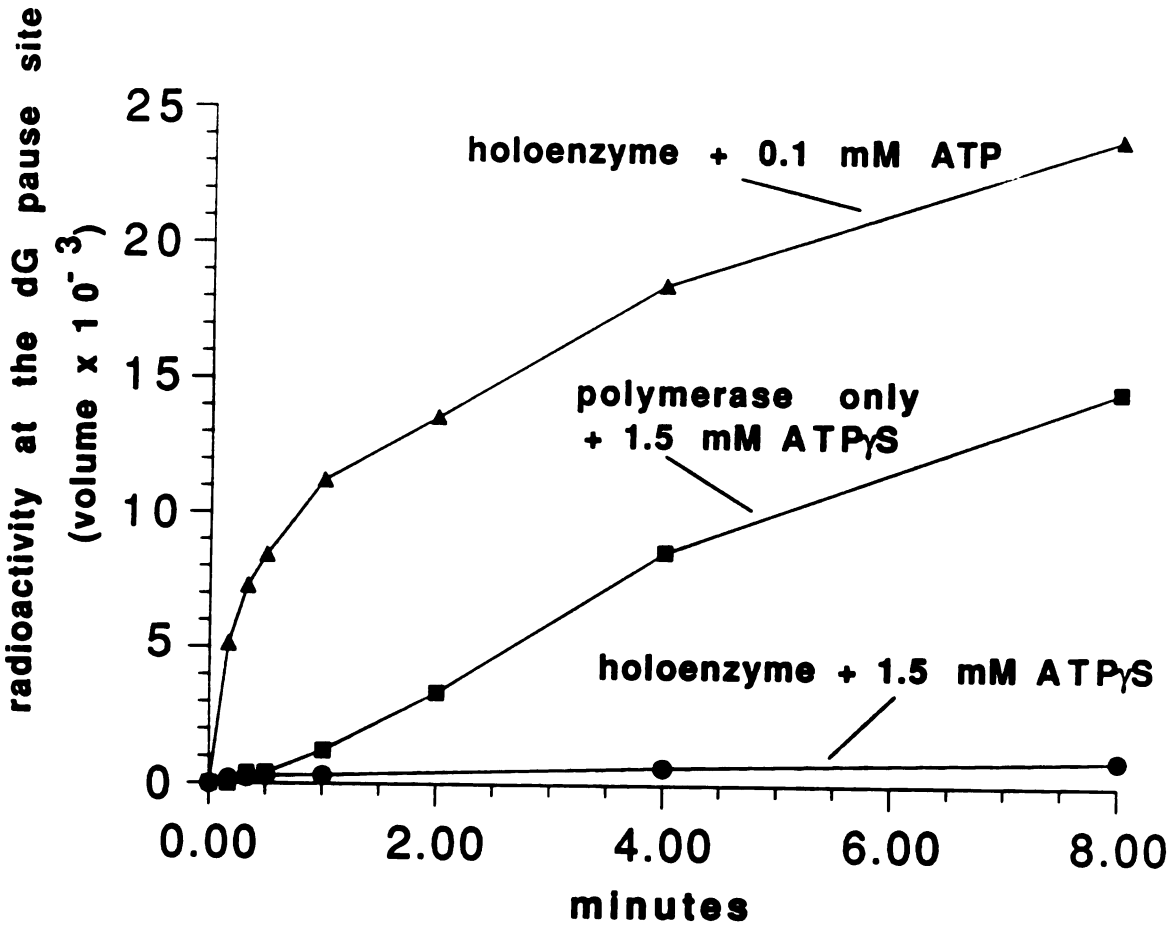

radioactivity at the dG pause site
(volume x 10^-3)
25
20
15
10
5
0
0.00
2.00
4.00
6.00
8.00
minutes
holoenzyme + 0.1 mM ATP
polymerase only
+ 1.5 mM ATPγS
holoenzyme + 1.5 mM ATPγS

Figure A-4

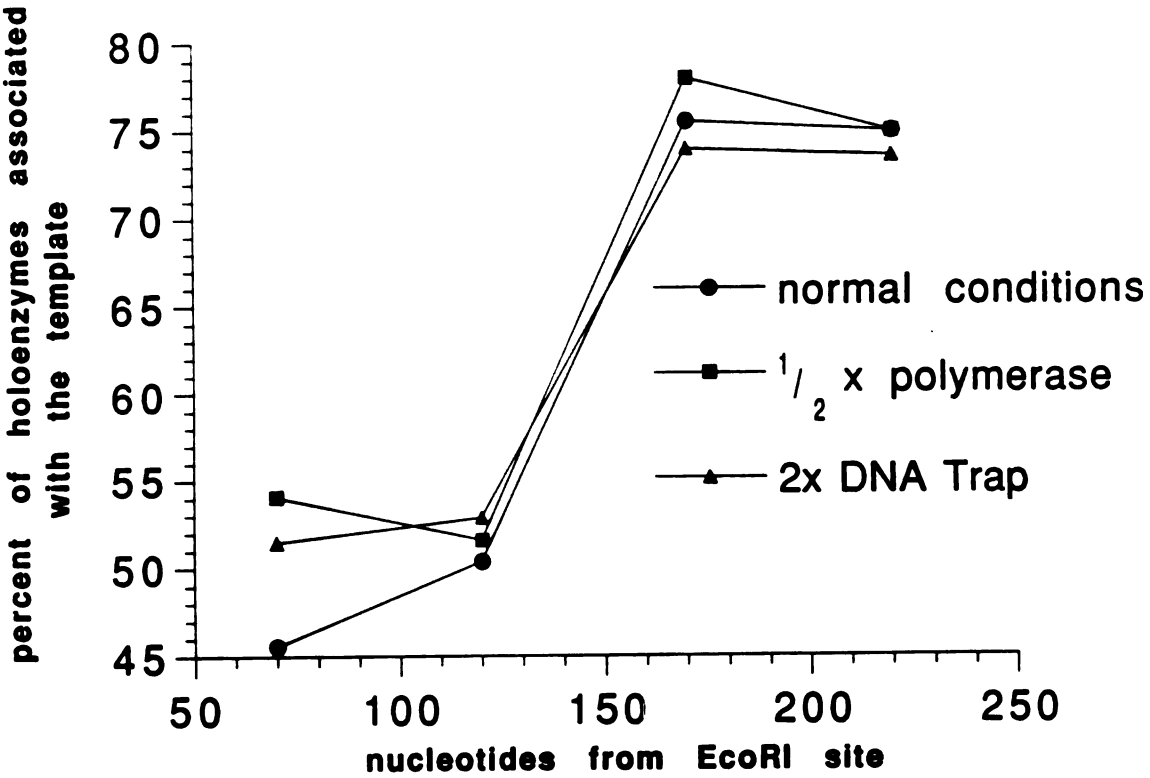

percent of holoenzymes associated with the template
80
75
70
65
60
55
50
45
50
100
150
200
250
nucleotides from EcoRI site
normal conditions
1/2 x polymerase
2x DNA Trap

Figure A-5

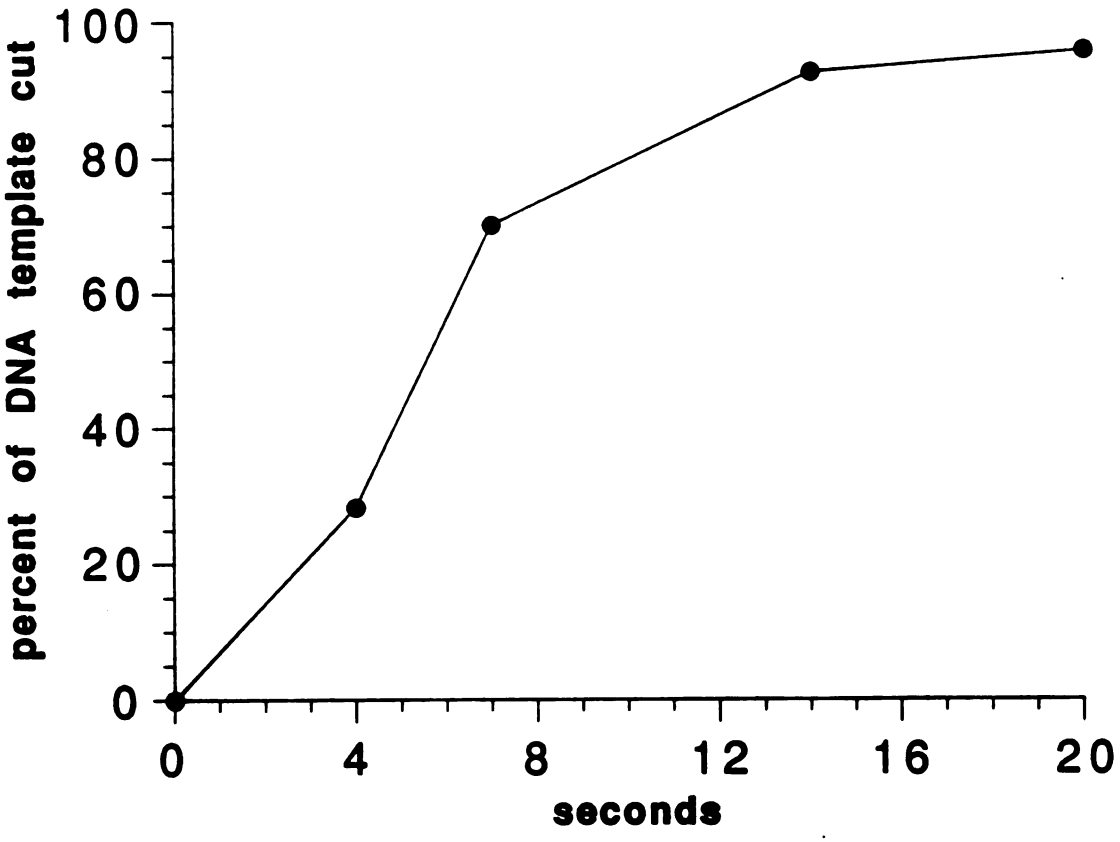
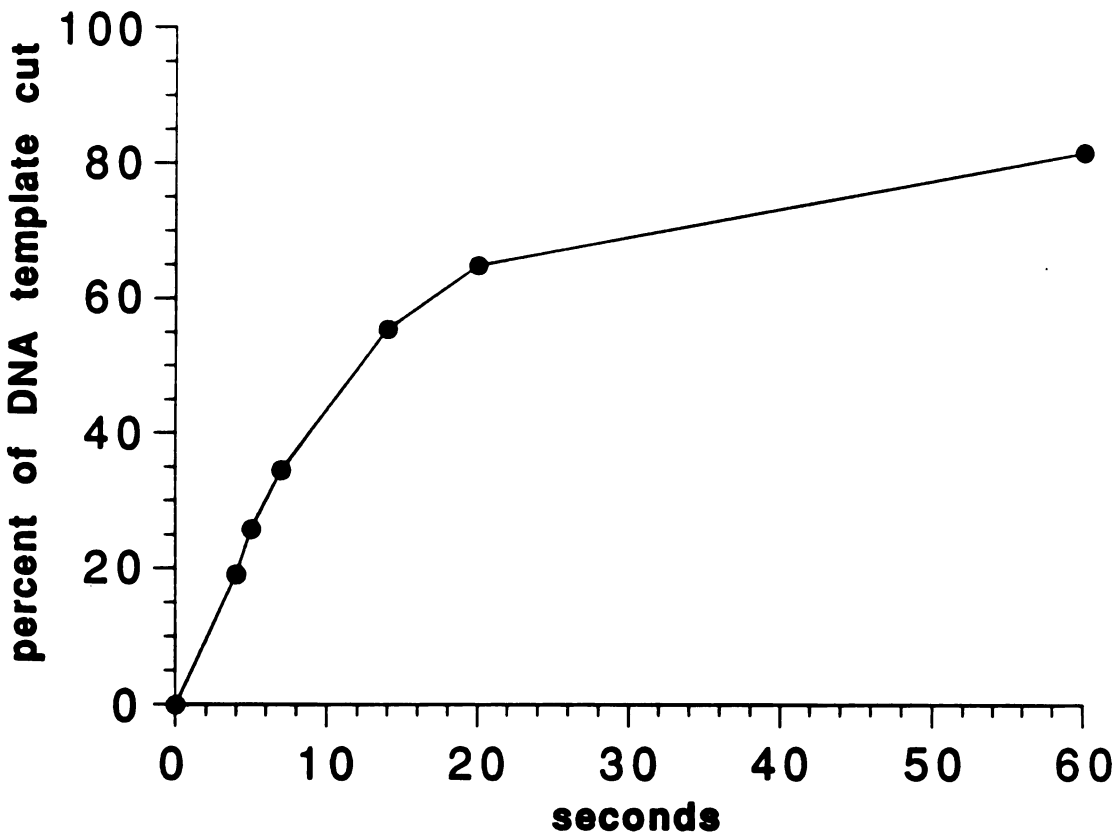

percent of DNA template cut
100
80
60
40
20
0
0
4
8
12
16
20
seconds

Figure A-6


percent of DNA template cut
100
80
60
40
20
0
0
10
20
30
40
50
60
seconds

APPENDIX B

In the assay for holoenzyme dissociation at a hairpin helix, the free DNA polymerase trap is rendered less efficient when excess gene 32 protein is added.

In chapter 4, the kinetics of T4 DNA polymerase holoenzyme dissociation from the DNA when the holoenzyme is stalled at a hairpin helix was studied. The assay used in these studies (Chapter 4, Figure 1) employed a polymerase trap to prevent the formation of holoenzymes after the synthesis to the hairpin was initiated by the addition of dCTP. The trap also was used to prevent a second holoenzyme from associating with the labeled primer-template after the first holoenzyme dissociates. It was critical that the polymerase molecules be efficiently trapped, since polymerase recycling would complicate determining the kinetics of holoenzyme dissociation and its parameters. The polymerase trap, which consisted of poly dT·oligo dA used at 8,300 3'-OH end of trap per 5'-^{32}P-end labeled primer, was efficient in preventing polymerase recycling to the labeled primer-template in the other holoenzyme dissociation assay (polymerase stalled by nucleotide ommission) (Figure A-1).

Both holoenzyme dissociation assays where performed at the same salt conditions and reaction temperature, but only the assay where the holoenzyme was stalled at a hairpin had a step where an 8-fold excess gene 32 single-stranded binding protein was added. The effect of excess gene 32 protein on the efficiency of the poly dT·oligo dA polymerase trap is shown in Figure B-1. Prior to addition of the replication proteins, the labeled primer-template was mixed

with the polymerase trap, and thus, once added, the holoenzymes must escape the large excess of unlabelled poly dT·oligo dA in order to elongate the labeled primer. The more efficient the DNA trap the less labeled primer will be elongated during the incubation with the replication proteins. A comparison of Lanes 1 and 2 (Figure B-1) shows that many more primers are elongated after an incubation with the gene 32 protein. This is most likely due to the gene 32 protein melting the poly dT·oligo dA polymerase trap, which has a lower melting temperature (T_m) than the primer-template. Therefore, to reduce holoenzyme recycling during the dissociation assay, a nonhydrolyzable ATP analog (ATPγS) was added with poly dT·oligo dA polymerase trap to competitively inhibit ATP hydrolysis required for holoenzyme formation.

Substituting normal gene 32 protein preparations with preparations containing 50% 32I* protein did not increase the percentage of holoenzymes associated with the template, even though the time that the polymerase remained at the hairpin was dependent on the rate of hairpin melting—

The time the polymerase holoenzyme remains stalled at the hairpin (chapter 5, Figure 1) is determined by the time between the addition of dCTP (which allows the holoenzyme to encounter the hairpin) and the addition of excess 32 protein, plus the time required by the gene 32 protein to melt the hairpin. Finding conditions where the hairpin would melt quickly was desirable so that the kinetics of holoenzyme dissociation could be accurately observed.

The properties of the 32I* protein led us to examine whether it could melt the hairpin helix more rapidly than the normal 32 protein in the dissociation assay. The 32I* protein, which is generated by the proteolytic removal of 60 amino acids from the COOH end of the intact gene 32 protein, binds to single-stranded DNA with 2 to 4 times greater affinity than the intact protein and can melt duplexed DNA (Greve *et al.*, 1978; Lonberg *et al.*, 1981). However, unlike the intact protein, 32I* protein does not bind to the T4 DNA polymerase (Burke *et al.*, 1980). Preparations of gene 32 protein containing 50 percent of 32I* were compared with normal preparations containing less than 5% 32I* protein in a dissociation assay in which the dCTP, DNA polymerase trap and excess 32 protein were added simultaneously. Under these conditions, the time that the polymerase remains stalled the hairpin should be determined by the time required to melt the hairpin, and the faster the hairpin melts the more holoenzymes should remain on the DNA through the hairpin. Table B-1 shows that the percentage of holoenzymes associated with the template is similar for both 32I* protein enriched and normal gene 32 preparations. This implies that 32I* protein under the conditions of assay does not melt the hairpin more rapidly than the normal gene 32 protein. What this result implies concerning the mechanism of hairpin melting by the gene 32 protein in these experiments is discussed in chapter 5.

Table A-1. In the assay for holoenzyme dissociation at a DNA hairpin helix in Figure 1 (chapter 5), substitution of the normal 32 protein preparation with 32I* protein does not increase the percentage of holoenzymes associated with the DNA when the dCTP, excess 32I* protein, and the DNA trap are added together (this provides the shortest possible time of stalling the polymerase at the hairpin). The replication proteins (17 ng/ml 43 protein, 20 μg/ml 45 protein, 20 μg/ml 44/62 protein, and 5 μg/ml 32 protein) were incubated with 0.53 μg/ml single-stranded DNA (as indicated) annealed with a 5'-^{32}P-end-labeled 20 nucleotide primer, 1.0 mM ATP, 60 μM each of dATP, dGTP, and dTTP in replication buffer (160 mM potassium acetate, 33 mM Tris-acetate, pH 7.8, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 200 μg/ml human serum albumin) for 30 sec at the designated temperature. After the holoenzyme was allowed to assemble for 30 sec, 400 μg/ml 32 or 32I* protein as indicated, a free polymerase trap (20 μg/ml poly dT·oligo dA), and 120 μM dCTP were added for 10 sec, and then reactions were stopped by adding two volumes of a 95% formamide/20 mM Na_3EDTA solution. The samples were electrophoresed on a denaturing 8% polyacrylamide gel and the radioactivity quantitated using a PhosphorImager.

DNA template	temperature (°C)	percent of holoenzymes associated with DNA[1]	
		normal 32 protein	32I* protein
mp4 (15 bp hairpin)	23	90.4	88.4
mp7 (22 bp hairpin)	23	79.6	75.3
mp4	37	95.9	94.4
mp7	37	81.5	75.3

[1]The percentage of DNA polymerase holoenzyme molecules associated with the DNA template was determined as described in chapter 5 Methods.

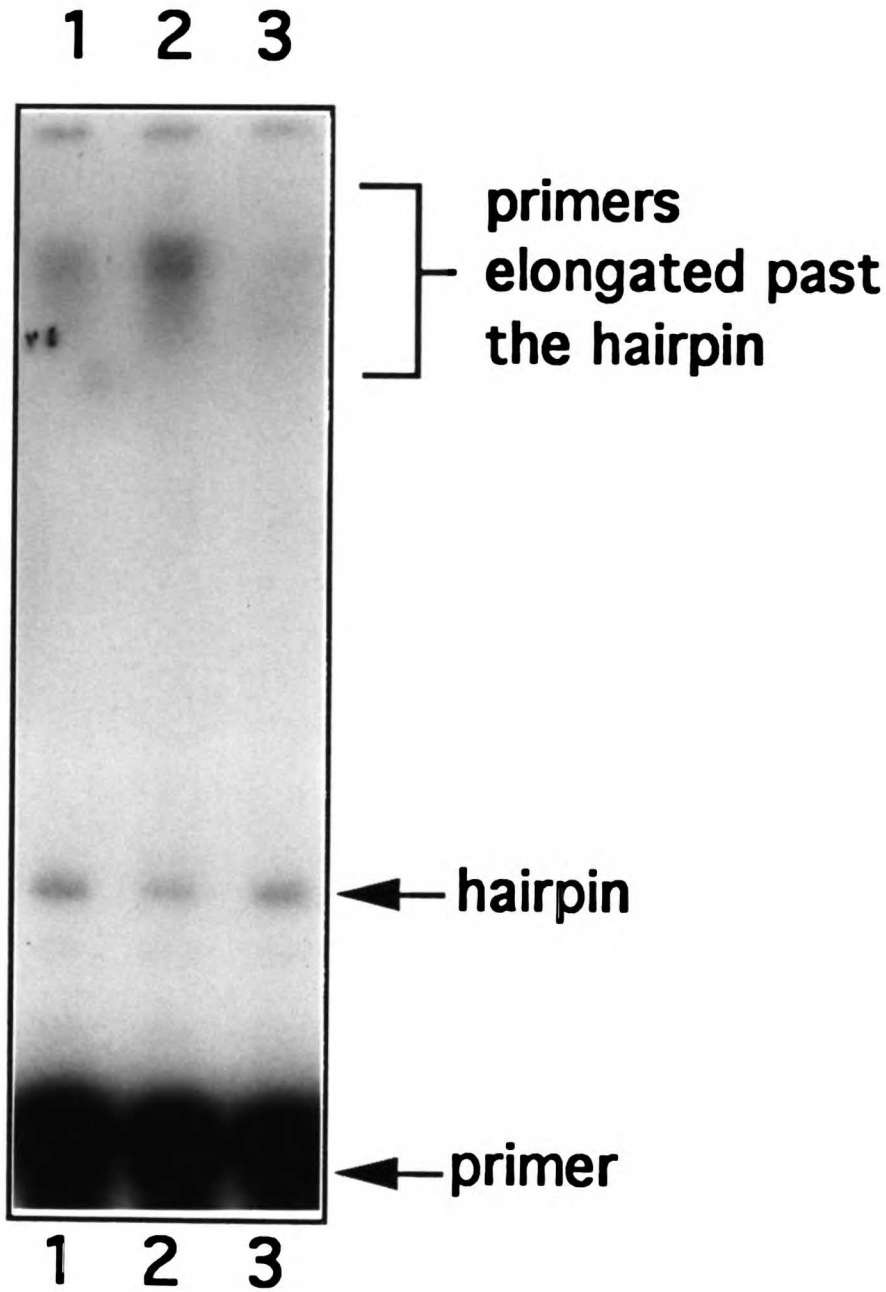
FIGURE LEGENDS

Figure B-1. **The DNA polymerase readily escapes the DNA polymerase trap during incubations with excess gene 32 protein.** Prior to addition of the replication proteins, the labeled primer-template (0.53 μg/ml single-stranded M13mp4 DNA annealed with a 5'-^{32}P-end labeled 20 nucleotide primer) was mixed with the polymerase trap consisting of 20 μg/ml poly dT·oligo dA in replication buffer (160 mM potassium acetate, 33 mM Tris-acetate, pH 7.8, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 200 μg/ml human serum albumin) containing 1.0 mM ATP, 60 μM each of dATP, dCTP, dGTP, and dTTP. The replication proteins (10 ng/ml 43 protein, 20 μg/ml 45 protein, 20 μg/ml 44/62 protein, and 5 μg/ml 32 protein) were incubated with the DNA mixture at 37°C for the designated times, then 400 μg/ml 32 protein was added and the incubation was continued as indicated. The reactions were stopped by adding of 2 volumes of a 95% formamide/20 mM Na_3EDTA. Prior to electrophoresis the DNA template was denatured by heating for 4 min at 100°C and then immediately placed in a ice water bath. The samples were electrophoresed on a denaturing 8% polyacrylamide gel and a autoradiograph of the gel is shown.

Lane 1: the sample was incubated for 30 sec with replication proteins alone plus 10 sec more with excess 32 protein, and lane 2: the sample incubation times were reversed, 10 sec with replication proteins alone plus 30 sec more with excess 32 protein. Thus, the total sample incubation time with the replication proteins in Lanes 1

and 2 was 40 sec. Lane 3: the sample was incubated with the replication proteins for 30 sec, as in the lane 1 sample, but the incubation with excess 32 protein was continued for only 3 more seconds. Therefore, unlike Lanes 1 and 2, the total sample incubation time with replication proteins was only 33 sec.

Figure B-1



CHAPTER SIX

CONCLUSION

What is the role of the Dda protein during T4 infection?

I think that the confusion over determining the role of the Dda protein during T4 infection illustrates how careful one has to be when guessing an *in vivo* function from results obtained *in vitro*. *In vitro* studies by Bedinger *et al.* (1983) suggested that Dda DNA helicase is important for removing DNA-bound proteins, like the *E. coli* RNA polymerase, that block replication fork movement and can not be removed by the replicative T4 gene 41 DNA helicase. However, recent in vitro experiments performed by Jack Barry (personal communiation) have shown that the gene 41 protein can remove RNA polymerase molecules from the template— both when it is efficiently loaded onto the DNA by the T4 gene 59 protein or when the gene 41 protein is provided with a very long region of single-stranded DNA for binding. The earlier studies utilized a double stranded DNA template with a nick for the initiation of DNA synthesis 173 nucleotides downstream of a RNA polymerase bound to a promoter in the absence of three of the four ribonucleosides (triphosphates Bedinger, et al., 1983). The 153 nucleotides of single-stranded DNA generated during DNA-strand displacement synthesis by the DNA polymerase holoenzyme before it encountered the RNA polymerase was too short for efficient binding by the gene 41 protein, and subsequently, gene 43 protein catalyzed DNA unwinding did not occur in these reactions.

Similarly, a role for the Dda protein in DNA recombination was suggested by Kodadek and Alberts (Kodadek and Alberts, 1987) from the finding that the Dda protein stimulates T4 UvsX protein catalyzed

branch migration 4-fold *in vitro*, whereas the gene 41 protein does not. But here again, addition of the gene 59 protein enabled the gene 41 protein to bind to the DNA in these reactions and increase the rate of branch migration (Morrical and Alberts, personal communication). This gene 59-41 complex activity is consistent with the phenotype of phage that are mutant in either the gene 59 or the gene 41 proteins. Mutations in either the gene 59 protein or the gene 41 protein result in defects in DNA recombination-mediated DNA synthesis during T4 infection. In contrast, an *in vivo* role for the Dda protein in DNA recombination is not supported by the phenotype of mutant in the *dda* gene; these mutants are not defective in DNA recombination and do not show UV sensitivity (Cunningham and Berger, 1977).

To determine the role of the dda protein during T4 infection, (chapters 4 and 5), I applied the approaches of protein affinity chromatography and genetic analysis that have been very effective in identifying proteins involved in DNA metabolism and their roles during T4 infection in the past. Unfortunately, the dda protein was inactivated when coupled to a Affi-Gel 10 agarose column (BioRad Laboratories, CA) and therefore no new interactions of the dda proteins with other proteins was identified. It is interesting that of the eight other T4 proteins that have been coupled to the Affi-Gel matrix, only the other DNA helicase, the gene 41 protein, was similarly inactivated (J. Barry, per. comm.).

The analysis of dda mutants in chapter 3 was more productive in determining the function of the Dda protein *in vivo*. Phage mutant in the Dda protein were defective in DNA synthesis at early times of infection where DNA replication is initiated predominantly at fixed

replication origins (reviewed by Kreuzer and Morrical, 1994). This suggests that the Dda protein may play a role in initiation of DNA replication at origins. The failure to detect DNA synthesis during infection with dda, 59 double mutant phage (chapter 3) supports the idea the Dda protein normally plays an important role in DNA synthesis in vivo, but is nonessential due to the ability of the gene 59-41 complex to substitute partially for its function.

Confirming that the Dda protein plays a role in initiation of DNA replication at origins is likely to require the development of an *in vitro* system of origin-dependent initiation that faithfully mimics the requirements for initiation *in vivo*. Such a system is currently being developed by Kreuzer and colleagues (Kreuzer, *et al.*, 1988; Benson and Kreuzer, 1992). Benson and Kreuzer have shown that DNA replication of a recombinant pBR332 plasmid containing a T4 origin during T4 infection requires all of the components of the holoenzyme complex (Benson and Kreuzer, 1992). Deletion analysis of two T4 replication origins that confer pBR322 with the ability to replicate during T4 infection have defined 100 nucleotides as a minimal origin. The minimal origin contains a T4 middle-mode promoter sequence and an A-T rich region 50 nucleotides downstream of the promoter (Menkens and Kreuzer, 1988). The precise roles of these sequences in replication initiation are unknown.

Mechanism of T4 DNA polymerase holoenzyme dissociation—

When I started my studies of the T4 DNA polymerase holoenzyme, it appeared to be very different from the *E. coli* DNA

polymerase III holoenzyme, but over the course of my graduate work the similarities between the two holoenzymes became evident. In the introduction to chapter 4 these similarities are outlined. Both holoenzymes are composed of a DNA polymerase with low processivity, a mutliprotein accessory protein complex that has DNA-dependent ATPase activity, and a single-subunit accessory protein, which stimulates the ATPase activity of the accessory protein complex. In the case of the *E. coli* replication system the single-subunit (the β-subunit) is shaped like a ring that encircles the DNA (Kong, *et al.*, 1992). The mutliprotein γ complex has been shown to load the ring-like β-subunit onto the DNA template in an ATP-requiring reaction (Stukenberg, *et al.*, 1991). The DNA linearization experiments in chapter 4 were modeled after studies of the β-subunit (Stukenberg, et al., 1991), and they demonstrate that the gene 45 protein is also likely to be ring-shaped.

This work suggests an alternative interpretation for the footprinting results obtained by Munn and Alberts (1991a;1991b). They proposed that the polymerase holoenzyme is unstable when stalled by omission of a nucleotide. The clock model then arose from the idea that the polymerase holoenzyme is stable while moving but quickly dissociates upon stalling. My work in chapter 4 shows that, on a circular template, the holoenzyme dissociates slowly with a half-life of 2.5 minutes when stalled by nucleotide omission. The failure of Munn and Alberts to obtain a footprint can be explained in light of the functional homology between the β-subunit and the gene 45 protein. It is likely that the holoenzyme rapidly slid off the end of the linear template used in the footprinting studies.

The clock model was also invoked to explain the role of ATP hydrolysis during formation of the holoenzyme. This role can also be explained by a requirement for ATP during the loading of the gene 45 protein onto the DNA template. Cumulatively, these results undermine the evidence that was the original basis for the clock model.

Despite the lack of prior evidence to support a clock model, a timing-based mechanism remained a plausible one to explain the dissimilar behavior of the identical holoenzymes on the leading and lagging strands of the replication fork. The results obtained in chapters 3 and 4, however, rule out a timing based mechanism for the dissociation of a holoenzyme stalled by nucleotide omission or at a hairpin helix.

The slow rate of dissociation of the holoenzyme when stalled by nucleotide omission (half-life 2.5 in) is similar to that expected for a holoenzyme actively synthesizing DNA. In sharp contrast, the rate of dissociation of the holoenzyme when stalled at a hairpin is very rapid (1 sec) and is the same as that expected for a holoenzyme completing synthesis of an Okazaki fragment on the lagging-strand. This suggests that the holoenzyme is designed to sense an encounter with duplex DNA like that found at the end of a previously synthesized Okazaki fragment.

A sensing mechanism can explain the ability of the DNA polymerase holoenzyme to remain associated with the template for many minutes when synthesizing DNA on the leading strand, while rapidly dissociating from the template when the end of a previously synthesized Okazaki fragment is encountered. On the leading-strand

of the replication fork the DNA polymerase holoenzyme is most likely to travel immediately behind the helicase, so that it never directly encounters duplexed DNA. (The removal of gene 32 protein, does not increase the pausing of the leading-strand polymerase (Cha and Alberts, 1989) suggesting that the polymerase travels so closely behind the gene 41 helicase that no single-stranded DNA generated by the helicase has time to form a duplexed structure before the complementary strand is synthesized.) On the lagging-strand, single-stranded DNA generated by the advancing helicase is likely to be immediately bound by gene 32 protein, so that it does not form a duplex structure. Thus, the only time that the polymerase is likely to encounter duplex DNA is at the completion of an Okazaki fragment.

What component of the holoenzyme is sensing the DNA helix ahead ? Three models are presented in the chapter 5 discussion. Distinguishing between models where the changes in polymerase conformation trigger dissociation of the accessory proteins and models where the accessory proteins instead sense the duplex DNA directly will require additional studies. A system that allows the kinetics of accessory protein dissociation from the DNA template to be measured is required. Given the fast dissociation of the polymerase component of the holoenzyme observed in my studies, this system would have to be capable of measuring dissociation rates of 1 sec^{-1}. The most obvious way to measure such rates would be by using a stopped-flow machine. Fluorescent probes on the accessory proteins that change fluorescence when the accessory proteins dissociate from the DNA could alternatively be used, but developing such a system would be difficult. Designing tests for some of the

polymerase sensing models would be easier, since measurements of the behavior of the polymerase in the absence of the accessory proteins could to be used to evaluate these models. In particular, the salt concentrations in the assays used in chapters 4 and 5 could be lowered to increase the processivity of the polymerase alone, and allowing measurement of its relative rates of dissociation when stalled by nucleotide omission versus when stalled at a hairpin helix. Finding similar dissociation rates under both conditions would favor a model that does not invoke a change in the affinity of the polymerase itself for the DNA when a helix is encountered.

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