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DNA POLYMERASE α: THE EUKARYOTIC LAGGING STRAND POLYMERASE

BY

SCOTT KIRKNESS DAVEY

DEPARTMENT OF BIOCHEMISTRY

SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

FACULTY OF GRADUATE STUDIES

THE UNIVERSITY OF WESTERN ONTARIO

LONDON, ONTARIO

AUGUST 1989

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Abstract

This work is an *in vitro* study of the DNA polymerase α-DNA primase complex, which is thought to synthesize DNA on the lagging strand at replication forks. We have purified the complex, and studied two aspects of RNA primed DNA synthesis catalyzed by this enzyme complex *in vitro*.

DNA polymerase α has been purified from cultured mouse Ehrlich ascites tumour cells, by classical purification methods, to a specific activity of 60 000 units/mg total protein. The purified DNA polymerase α complex has four subunits, 182, 70, 55, and 47kDa. A 3'→5' exonuclease activity, detected after treatment of the complex with ethylene glycol, cosedimented with the DNA polymerase activity, on sedimentation through glycerol gradients. The sensitivity of DNA polymerase α to the nucleotide analogues butylphenyl-dGTP and butylanilino-dATP did not change when the 3'→5' exonuclease was active.

The effect of template DNA sequence on the rate of initiation of RNA primed DNA synthesis by the purified mouse DNA polymerase α-primase complex was examined. We have observed discontinuous DNA synthesis, which was dependent on ATP or GTP. RNA primed initiation is favoured 10 nucleotides upstream of 3' CCA and 3' CCC sequences, which we have called ψ. Alteration of the ψ domain led to changes in the rate of RNA primed DNA synthesis, and kinetic analysis showed that these changes were due to less efficient interaction with the DNA template. Changes in the K_m with respect to DNA concentration (from an optimal 6pM to 240pM) were observed when ψ was varied from the consensus sequences. Mutations in actual sites of priming led to an altered rate of synthesis

of RNA primed DNA chains, reflected in changes in V_{max} for the reaction (from 5.5-30pmol deoxynucleotides incorporated/hour).

DNA polymerase α was also studied in a direct gap filling assay. DNA polymerase α is able to fill gaps to completion. However, incorporation of the final nucleotide was biphasic. This was reflected in an increased K_m for *de novo* incorporation of one nucleotide at a single nucleotide gap (0.7μM), as opposed to the K_m for incorporation of one nucleotide into singly primed M13 DNA (0.18μM).

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List of abbreviations

A	adenine
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CTP	cytidine 5'-triphosphate
cpm	counts per minute
D. melanogaster	*Drosophila melanogaster*
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
ddH_2O	double distilled water
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5'-triphosphate
ds	double stranded
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EtdBr	ethidium bromide
g	gravity
G	guanine
HAP	hydroxylapatite
IPTG	isopropyl β-D-thiogalactopyranoside

kb	kilobase
kbp	kilobase pair
kDa	kilodalton
moi	multiplicity of infection
MVM	minute virus of mice
O/N	overnight
PEG	polyethylene glycol
pfu	plaque forming units
phenol	phenol equilibrated to pH 7.5 with 5x TE
PMSF	phenylmethylsulphonylflouride
RPM	revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
ss	single stranded
STE	100mM NaCl, 10mM tris·Cl, 1mM EDTA; pH 8.0
T	thymine
10x TBE	1M Tris·borate, 20mM EDTA; pH 8.3
TE (1x TE)	10mM Tris·Cl, 1mM EDTA; pH 7.5
UV	ultra-violet
w.t.	wild type

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Chapter 1

General introduction

1.1 DNA replication

1.1.1 DNA and DNA polymerization

The basic concepts underlying DNA replication were suggested by the very structure of DNA (Watson and Crick, 1953a/b). During replication, the strands of the double helix separate to expose the internal nitrogenous bases, which encode genetic information. The separated strands then serve as templates, each directing the synthesis of a new strand, resulting in the formation of two copies of the original DNA molecule. While the structure of DNA had been elucidated, the details of its replication had not. The nature of the polynucleotide precursors, the mechanism of double helix unwinding, how torque generated by unwinding the helix is relieved, and even whether enzymes played a role in these processes, were all unknown. These, and other details of DNA replication, have been studied ever since.

There is a fundamental asymmetry in both DNA and DNA polymerization. The sugar-phosphate backbones of DNA are antiparallel. If one strand runs 5'→3', then the complementary strand runs 3'→5'. Further, all known DNA polymerases work exclusively in a 5'→3' direction (Kornberg, 1980). Figure 1a illustrates the two forms of DNA polymerization at a replication fork. On the leading strand, DNA is synthesized continuously in the 5'→3' direction. On the lagging strand, short RNA primed DNA fragments, Okazaki fragments, are oriented away from the

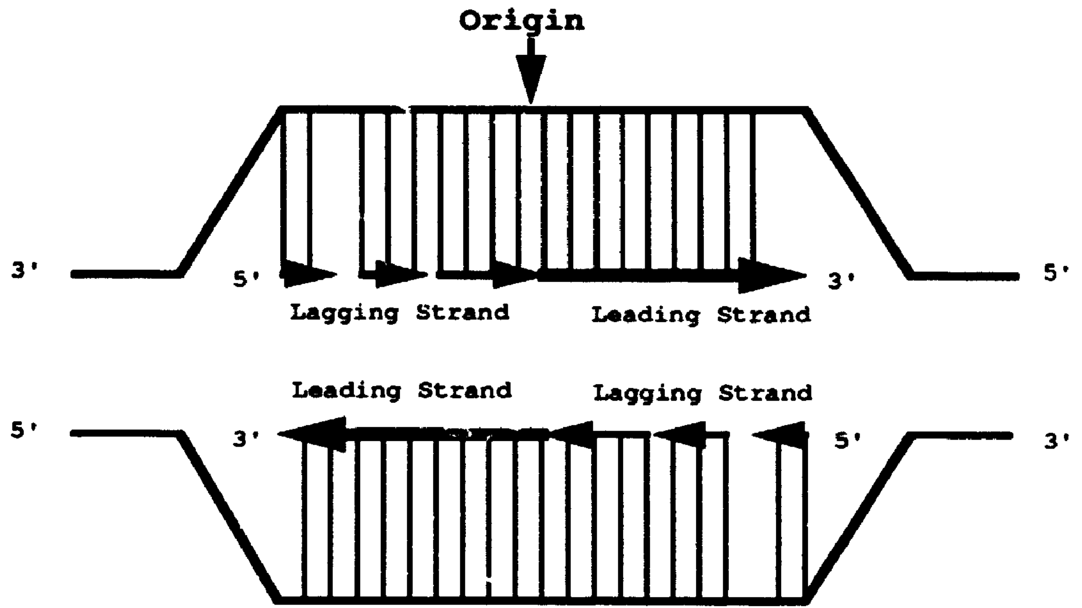
Figure 1

Schematic representation of a replication fork

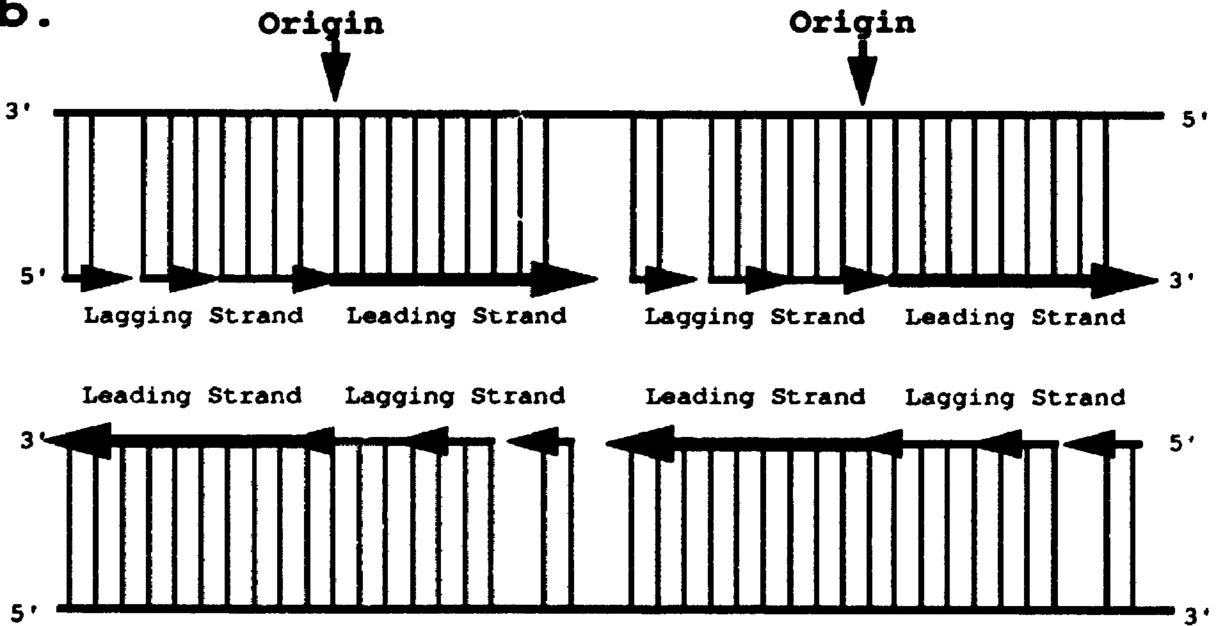
a. Replication begins at an origin, and proceeds bidirectionally. At any replication fork, there is both a leading and lagging strand.

b. As replication forks from two adjacent origins converge, the leading strands undergo gap filling and ligation, similar to what lagging strands do to join each Okazaki fragment.

a.



b.



replication fork (Okazaki and Okazaki, 1969). The work described in this thesis deals with the enzymology of DNA polymerase α, an enzyme which is adapted to synthesize DNA on the lagging strand at a replication fork.

1.1.2 Replication in *Escherichia coli*

The best characterized cellular replication system is that of *Escherichia coli*. And as such, replication in *E. coli* is a good model on which studies of eukaryotic DNA replication may be founded. The following simplified version points out the main features of *E. coli* DNA replication, and serves as an introduction to the concepts of cellular DNA replication.

Replication in *E. coli* initiates at a unique 245 nucleotide pair origin, *oriC*. The *dnaA* protein recognizes and binds *oriC* in a sequence dependent manner (Fuller and Kornberg, 1983; Fuller *et al.*, 1984). This interaction opens the double helix to allow entry of the *dnaB* and *dnaC* proteins. The *draB* protein is a helicase; it can catalytically unwind the double helix. Together, *oriC*, and the *dnaA*, *dnaB*, and *dnaC* proteins, form the prepriming complex.

A second group of proteins prevent the initiation of replication at sites other than *oriC*. These auxiliary, or specificity, proteins include RNase H (Ogawa *et al.*, 1984), topoisomerase I (Kaguni and Kornberg, 1984a), and HU protein (Kaguni and Kornberg, 1984b; Ogawa *et al.*, 1985; van der Ende *et al.*, 1985; Baker *et al.*, 1986).

Following assembly of the prepriming complex at *oriC*, the *dnaB* helicase begins unwinding the double helix. Extensive unwinding is dependent on the prepriming complex, single strand DNA binding protein,

and gyrase. Gyrase is a type II topoisomerase, which relieves the torque (supercoiling) generated by separation of the DNA strands. On addition of the *dnaG* protein to the prepriming complex, a priming complex, or primosome is formed. The *dnaG* protein is a primase; it synthesizes oligoribonucleotides which serve as primers for DNA synthesis.

The replicative DNA polymerase of *E. coli* is DNA polymerase III, a multifunctional complex, which forms an asymmetric dimer with two active DNA polymerase catalytic sites (Maki *et al.*, 1988). The DNA polymerase III α subunit (*dnaE* protein), contains the DNA polymerase catalytic activity (Maki and Kornberg, 1985). This activity, like all known DNA polymerases, synthesizes DNA from activated precursors, in a 5'→3' direction (Kornberg, 1980). The activated precursors are the deoxyribonucleoside triphosphates dATP, dCTP, dGTP, and dTTP. Nucleotides are added sequentially to pre-existing 3'-hydroxyl residues of either an RNA primer, or a nascent DNA chain. On the joining of a nucleotide to the growing chain, the α-phosphate of the nucleotide and the 3'-hydroxyl of the nascent chain form a phosphodiester bond, liberating the β and γ phosphates of the nucleotide as inorganic pyrophosphate. The ε subunit of DNA polymerase III (*dnaQ* protein) exhibits 3'→5' exonuclease activity which preferentially removes misincorporated nucleotides, and functions as an editor, proofreading the newly synthesized DNA (Scheuermann and Echols, 1984). This exonuclease acts cooperatively with DNA polymerase III α subunit to increase the fidelity of DNA replication (Maki and Kornberg, 1987). The third subunit of the DNA polymerase III core enzyme is the θ subunit, the function of which is unknown.

The form of DNA polymerase III that is responsible for the replication of *E. coli* DNA is the holoenzyme, which is comprised of the core enzyme in association with additional subunits. Binding of the τ subunit (*dnaZX* protein) by the core enzyme leads to DNA polymerase III dimerization. The γ subunit (*dnaZ* protein) is identical to the amino-terminal portion of the τ subunit, and may contribute, together with the τ subunit, to the asymmetry of the DNA polymerase III dimer (Hawker and McHenry, 1987). The β subunit of DNA polymerase III (*dnaN* protein) holoenzyme increases the processivity of the complex (Crute *et al.*, 1983; LaDuca *et al.*, 1986). In addition, the δ, δ', χ, and ψ subunits are likely components of the DNA polymerase III holoenzyme, though their functions, and the genes which encode them, have yet to be determined (Maki *et al.*, 1988).

1.1.3 Generalized DNA replication

The main features of *E. coli* DNA replication may be generalized into a scheme for all cellular DNA replication. Prepriming includes the processes of origin recognition, and partial unwinding of the DNA double helix. Priming encompasses two processes: the priming of the leading strand, which occurs primarily, if not exclusively, at replication origins; and priming along the lagging strand, at Okazaki fragment initiation sites. DNA polymerases use a single stranded DNA template, primed with RNA, in a process termed elongation. DNA synthesis along the lagging strand involves three additional steps: Removal of the RNA primers; gap filling, the synthesis of DNA up to the 5' end of the downstream Okazaki fragment; and ligation of adjacent Okazaki fragments. These final steps need only occur once on the

leading strand, as replication forks from adjacent origins converge (Figure 1b).

1.2 Overview of Eukaryotic DNA polymerases

The nomenclature currently used to describe the DNA polymerases of eukaryotic cells was proposed by Weissbach *et al.* (1975). DNA polymerase α was defined as a high molecular weight (>100kDa), acidic protein, that is particularly active on DNA substrates with many small gaps (activated DNA). DNA polymerase α is inhibited by sulfhydryl blocking reagents, and lacks associated exonuclease activity. DNA polymerase β was defined as a low molecular weight (<50kDa), basic protein, localized in the nucleus, and resistant to sulfhydryl blocking reagents. DNA polymerase γ was defined as a high molecular weight (>100kDa), acidic protein, with the ability to copy poly(rA)/oligo(dT), and requiring sulfhydryl containing compounds for maximum activity. Mitochondrial DNA polymerase was also proposed as a distinct polymerase by Weissbach *et al.* (1975), but has since been shown to be DNA polymerase γ (Bolden *et al.*, 1977).

DNA polymerase α was thought to be the replicative enzyme in eukaryotic cells, based on several lines of evidence. DNA polymerase α is localized in the nucleus, and is associated with the nuclear matrix, which is believed to be the site of DNA replication (Bensch *et al.*, 1982; Smith and Berezney, 1982; Matsukage *et al.*, 1983; Nakamura *et al.*, 1984). DNA polymerase α activity correlates with the proliferative activity of cells both in tissue culture, and *in situ*, while the activities of DNA polymerases β and γ do not (Chang *et al.*, 1973; Spadari and Weissbach, 1974; Chiu and Baril, 1975; Matsukage et

al., 1983; Delfini *et al.*, 1985; Philippe *et al.*, 1986; Kozu *et al.*, 1986). A temperature sensitive mutation in DNA polymerase α exhibits defective DNA replication (Murakami *et al.*, 1985; Eki *et al.*, 1986). DNA polymerase α and DNA replication are sensitive to aphidicolin, while DNA polymerases β and γ are not (Ikegami *et al.*, 1978; Pedrali-Noy and Spadari, 1979; Liu *et al.*, 1983). Butylanilinouracil and butylphenylguanine inhibit DNA replication and DNA polymerase α activity at similar concentrations (Wright *et al.*, 1980; Wright *et al.*, 1982). Monoclonal antibodies raised against DNA polymerase α inhibit both DNA replication and DNA polymerase α activity at similar concentrations (Miller *et al.*, 1985).

The identity of the DNA polymerases involved in DNA repair has been in question. Early reports implicated both DNA polymerases α and β (Miller and Chinault, 1982; Dresler *et al.*, 1982; Mattern *et al.*, 1982; Mosbaugh and Linn, 1983). However, DNA polymerase α was implicated mainly on data involving the inhibitor aphidicolin, which at that time was thought to be specific for DNA polymerase α. But aphidicolin is now known to be an inhibitor of DNA polymerase δ as well, casting doubt on the role of DNA polymerase α in repair. Recent studies of repair utilizing the DNA polymerase specific inhibitor BuPdGTP have suggested that DNA polymerase δ, and not α, is the aphidicolin sensitive DNA repair enzyme (Nishida *et al.*, 1988; Dresler and Frattini, 1988).

In addition to the cellular DNA polymerases, a number of viruses encode their own DNA polymerases. These include the herpesviruses (Keir and Gold, 1963; Keir *et al.*, 1966), poxviruses (Moss and Cooper, 1982), and adenoviruses (Lichy *et al.*, 1982). Other viruses use host DNA polymerases. The use of viral systems in deciphering eukaryotic

cellular replication machinery will be discussed below.

1.3 DNA polymerase α

1.3.1 Purification

DNA polymerase α has been the subject of purification attempts for almost 30 years (Bollum, 1960). Early attempts at purification yielded largely degraded products, with catalytic subunit sizes in the 50-90kDa range (Holmes *et al.*, 1974; Karkas *et al.*, 1975; Fisher and Korn *et al.*, 1977; Filpula *et al.*, 1982), or exhibiting multiple peaks of activity eluting after column chromatography (Matsukage *et al.*, 1976; Brakel and Blumenthal, 1977; Brakel and Blumenthal, 1978). Purification by classical procedures led to the isolation of DNA polymerase α with catalytic subunits of 120-150kDa (Holmes *et al.*, 1976; Matsukage *et al.*, 1976; Banks *et al.*, 1979; McKune and Holmes, 1979; Mechali *et al.*, 1980; Villani *et al.*, 1980; Grosse and Krauss, 1980; Hubscher *et al.*, 1981; Yamaguchi *et al.*, 1982; Masaki *et al.*, 1982), and eventually to highly purified preparations with catalytic subunits in the 180-185kDa range (Kaguni *et al.*, 1983a; Faust *et al.*, 1985; Vishwanatha *et al.*, 1986; Prussak and Tseng, 1987).

Immunoaffinity purification procedures are now used to purify DNA polymerase α, and to avoid the problems of degradation encountered by more time consuming methods of purification. Sixteen murine hybridomas producing monoclonal antibodies against KB cell DNA polymerase α have been established (Tanaka *et al.*, 1982). DNA polymerase α has been purified using two of these monoclonal antibodies (Wang *et al.*, 1984; Wahl *et al.*, 1984; Nasheuer and Grosse, 1987), as well as monoclonal

antibodies developed by others (Chang *et al.*, 1984; Karawya *et al.*, 1984). These protocols yield DNA polymerase α with catalytic subunits of 150-190kDa. However, there are unique problems associated with immunoaffinity purification protocols. Purification of KB cell DNA polymerase α using the monoclonal antibody SJK-237-71 requires 1M KCl/pH 5.5 to dissociate the antibody/polymerase complex from a column of protein A-Sepharose (Wang *et al.*, 1984), but separation of the antibody from the polymerase is not possible. This means that functional analysis must be done using the antibody/polymerase complex. Purification of DNA polymerase from calf thymus using monoclonal antibody SJK-287-38, allowed dissociation of the polymerase from the antibody, but resulted in only 1% recovery of activity (Wahl *et al.*, 1984). These problems may be avoided by the choice of an appropriate monoclonal antibody. Chang *et al.* (1984) purified calf thymus DNA polymerase α using an immunoadsorbent column, dissociating the polymerase from the antibody by treatment with 3.2M $MgCl_2$, which resulted in recovery of 40% of the DNA polymerase activity. Another purification scheme utilizing SJK-287-38 allowed recovery of 50% of the polymerase activity applied to the antibody column by elution with potassium phosphate pH 12.5-13, but more than half of the catalytic subunit was degraded to 148-155kDa (Nasheuer and Grosse, 1987). Even with immunoaffinity purification, the DNA polymerase catalytic subunits have been found to have a molecular weight of 150-180kDa, which is less than the 180-240kDa found when crude extracts are immunoprecipitated (Masaki *et al.*, 1984; Karawya *et al.*, 1984; Ottiger *et al.*, 1987), suggesting that degradation can occur even during an immunoaffinity based purification.

1.3.2 Primase activity

Primase activity was found to be associated with DNA polymerase α purified from mouse Ehrlich ascites cells and *Drosophila melanogaster* embryos (Yagura et *al.*, 1982; Conway and Lehman, 1982), and has since been found to copurify with DNA polymerase α from most sources (Shioda *et al.*, 1982; Hubscher, 1983; Yoshida *et al.*, 1983; Yagura *et al.*, 1983; Wang *et al.*, 1984; Chang *et al.*, 1984; Wahl *et al.*, 1984; Grosse and Krauss, 1985; Faust *et al.*, 1985; Vishwanatha *et al.*, 1986). One notable exception to this involves purification of separate DNA polymerase and primase components when purification is performed in the presence of ethylene glycol (Tseng and Ahlem, 1982; Tseng and Ahlem, 1983; Prussak and Tseng, 1987). However, ethylene glycol has been shown to dissociate the polymerase and primase subunits (Suzuki *et al.*, 1985; Murakami *et al.*, 1986; Yagura *et al.*, 1986), suggesting that the separation of polymerase and primase in the above cases may reflect a purification anomaly, as opposed to an actual difference in the organization of the DNA polymerase α complex.

The polymerase and primase subunits have also been resolved by treatment of the enzyme with 3.4M urea, followed by centrifugation through glycerol gradients containing 2.8M urea. This technique was used to identify the primase subunits as the 50 and 60kDa subunits of the intact enzyme (Kaguni *et al.*, 1983b). This is in agreement with the estimated molecular weights (46 and 56kDa) of the subunits of polymerase free primase purified in the presence of ethylene glycol (Murakami *et al.*, 1986).

Primase synthesizes oligoribonucleotide and mixed oligoribo-deoxyribonucleotide primers (Yagura *et al.*, 1982; Conway and Lehman,

1982; Tseng and Ahlem, 1982; Faust *et al.*, 1985). The RNA primers are typically 8-15 nucleotides in length (Hu *et al.*, 1984; Grosse and Krauss, 1985). Primase which has been dissociated from DNA polymerase α by treatment with ethylene glycol synthesized primers which are typically 26-30 nucleotides in length (Cotterill *et al.*, 1987a). Primase initiates RNA synthesis exclusively with the purine nucleotides ATP and GTP (Conway and Lehman, 1982; Grosse and Krauss, 1985), though CTP, UTP, and as noted above dNTPs, are substrates for elongation of the primers (Yagura *et al.*, 1982; Conway and Lehman, 1982).

In vivo, primase is thought to synthesize the RNA primers which initiate Okazaki fragments. The sequence specificity of the primase associated with DNA polymerase α will be considered in chapter 4.

1.3.3 Proofreading activity

A DNA polymerase responsible for replication must be very accurate. One way in which polymerases increase their accuracy is by removing misincorporated nucleotides via a 3'→5' exonuclease or proofreading activity. Actual proofreading can only be demonstrated by observing a number of other criteria: Excision of mispaired termini in preference to correctly paired termini; physical association with the polymerase as part of the same subunit, or as an associated subunit; coordinate action with the polymerase to enhance the fidelity of DNA synthesis (Kunkel, 1988). However, a 3'→5' exonuclease in association with a DNA polymerase would typically be expected to fulfill a proofreading function *in vivo*. The presence of a 3'→5' exonuclease, and at least one of the criteria for proofreading have been demonstrated *in vitro* for *E. coli* DNA polymerase I (Brutlag and Kornberg, 1972), *E. coli* DNA

polymerase III (Scheuermann *et al.*, 1983), T4 DNA polymerase (Brutlag and Kornberg, 1972), *Saccharomyces cerevisiae* DNA polymerase II (Chang, 1972), *S. cerevisiae* DNA polymerase III (Bauer *et al.*, 1988), adenovirus DNA polymerase (Field *et al.*, 1984), herpesvirus DNA polymerase (Knopf, 1979), poxvirus DNA polymerase (Challberg and Englund, 1979), and mammalian DNA polymerase δ (Byrnes *et al.*, 1976).

In contrast to these polymerases, purified DNA polymerase α typically lacks an associated 3'→5' exonuclease. Until recently, the only exceptions to this have been in partially purified preparations (Chen *et al.*, 1979; Ottiger and Hubscher, 1984), or in high molecular weight complexes (Vishwanatha *et al.*, 1986). However, the 182kDa DNA polymerase catalytic subunit, dissociated from the 70kDa polypeptide by treatment with ethylene glycol, exhibits a 3'→5' exonuclease activity which is consistent with a proofreading function (Cotterill *et al.*, 1987c). The exonuclease preferentially removes 3' terminally mismatched nucleotides as opposed to correctly base paired terminal nucleotides, and leads to increased accuracy in the ϕX174 am3 reversion assay by 100 fold over the intact DNA polymerase α (Cotterill *et al.*, 1987c).

In vivo, proofreading is an integral part of prokaryotic DNA replication systems, existing as a domain of the polymerase catalytic subunit, or as a separate subunit associated with the polymerase complex (Kornberg, 1980). In *E. coli*, mutations affecting the genes which code for the pol III 3'→5' exonuclease (*mutD* or *dnaQ*, Scheuermann *et al.*, 1983) led to a 10^{3}-10^{5} fold increase in mutation frequency (Degnen and Cox, 1974; Horiuchi *et al.*, 1978; Echols *et al.*, 1983; DiFrancesco, 1984). This is a strong indication of proofreading *in*

vivo. Eukaryotic cells are thought to have spontaneous mutation rates of 10^{-10}-10^{-12} errors per base pair per generation, equal to or lower than the spontaneous mutation rates found in prokaryotes (Drake, 1969). One would thus assume that a proofreading function would be one component of any eukaryotic replication system.

Two studies have examined eukaryotic proofreading *in vivo*. DNA synthesis performed in permeablized HeLa cells incorporate 2-aminopurine deoxyribonucleoside triphosphate at the same frequency as cell free systems utilizing calf thymus DNA polymerase α, or *E. coli* DNA polymerase I (Wang *et al.*, 1981). However, *E. coli* DNA polymerase I would have been expected to perform proofreading, so it is not clear whether the presence of proofreading is detectable at all in this system. In the second study, the spectrum of mutations induced by nucleotide pool imbalances in Chinese hamster ovary cells was studied. Mutations at the aprt locus exhibited next-nucleotide effects, characteristic of DNA polymerases with proofreading exonucleases (Phear *et al.*, 1987).

1.3.4 DNA polymerase δ

Since its discovery in 1976, DNA polymerase δ has been included as a subheading under the category of DNA polymerase α. This action was justified on the basis that DNA polymerases α and δ were very similar in many respects, and the possibility existed that DNA polymerase δ was a form of DNA polymerase associated with different subunits. This view is slowly changing.

DNA polymerase δ was purified, and considered distinct from DNA polymerase α, largely on the fact that it possessed an associated 3'→5'

exonuclease (Byrnes *et al.*, 1976). Since its discovery, DNA polymerase δ, like DNA polymerase α, has undergone a progressive increase in estimated size. The DNA polymerase δ catalytic subunit has was initially found to be about 60kDa (Lee *et al.*, 1981), then 125kDa (Goscin and Byrnes, 1982; Lee *et al.*, 1984), and recently 165-170kDa (Lee and Toomey, 1987). None of these forms of DNA polymerase δ was reported to be associated with a primase activity.

The action of DNA polymerase δ is modified by a protein which has been called variously the DNA polymerase δ auxiliary protein, cyclin, and proliferating cell nuclear antigen or PCNA (Tan *et al.*, 1986; Bravo *et al.*, 1987; Prelich *et al.*, 1987b). The term PCNA has been suggested as the most appropriate, and least confusing (Blow, 1987). PCNA is required for SV40 replication *in vitro* (Prelich, 1987a), and is involved in cellular replication (Zuber *et al.*, 1989). PCNA does not affect DNA polymerase α *in vitro* (Tan *et al.*, 1986).

Another form of DNA polymerase δ has been reported to function independently of PCNA *in vitro* (Crute *et al.*, 1986; Wahl *et al.*, 1986; Sabatino *et al.*, 1988; Focher *et al.*, 1988; Syvaoja and Linn, 1989; Bambara *et al.*, 1989). PCNA independent DNA polymerase δ (currently being renamed DNA polymerase ε by convention) exhibits a 3'→5' exonucleaseivity, and is capable of highly processive DNA synthesis in the absence of PCNA.

1.3.5 The replicative polymerase of eukaryotic cells

For many years, DNA polymerase α has been implicated as the replicative polymerase in eukaryotic cells by the lines of evidence presented above (section 1.2). However, most of this evidence either

did not consider DNA polymerase δ, or considered it to be just a form of DNA polymerase α. But with the availability of selective inhibitors of DNA polymerases α and δ, has come the ability to dissect the replication machinery further. Monoclonal antibodies against DNA polymerase α inhibit replication in permeablized cells (Miller *et al.*, 1985). DNA replication is less sensitive to the inhibitor BuPdGTP than is purified DNA polymerase α (Dresler and Frattini, 1986), but more sensitive than is purified DNA polymerase δ (Hammond *et al.*, 1987).

DNA polymerase α has been implicated in replication by the fact that antibodies raised against it inhibit replication. DNA polymerase δ is implicated as the aphidicolin sensitive, BuPdGTP resistant enzyme involved in DNA replication. Hammond *et al.* (1987) have estimated the relative contributions of DNA polymerases α and δ to cellular DNA replication are 50-80% and 20-50% respectively. What has not been conclusively shown is that DNA polymerase α and δ are truly different, and if so, what roles these DNA polymerases play in replication. The recent finding that DNA polymerase α possesses an intrinsic, though cryptic, 3'→5' exonuclease has cast doubt on the distinction. The differential inhibition by BuPdGTP may simply reflect the active exonuclease of DNA polymerase δ. This possibility will be considered in Chapter 3.

The asymmetric dimer theory, which was applied to the *E. coli* DNA polymerase III holoenzyme model, may be applicable here as well. DNA polymerase α has a low processivity *in vitro* (Hockensmith and Bambara, 1981; Cotterill *et al.*, 1987b), which may be increased by association with other factors (Hohn and Grosse, 1987), or ATP (Wierowski *et al.*, 1983; Riedel *et al.*, 1984). Also, the association of a DNA primase

activity with DNA polymerase α suggests that it is a candidate for the lagging strand DNA polymerase. DNA polymerase δ, is highly processive when associated with PCNA (Prelich *et al.*, 1987b), and may be the leading strand polymerase.

The first line of evidence supporting the action of a concerted DNA polymerase α/δ asymmetric dimer theory comes from SV40 DNA replication *in vitro*. The replication of SV40 DNA *in vitro* can lead to the production of two circular daughter molecules. But in the absence of PCNA, only lagging strand DNA synthesis is observed, replication intermediates accumulate, and circular daughter molecules are not observed (Prelich and Stillman, 1988). Since PCNA is a DNA polymerase δ accessory protein, which has no effect on DNA polymerase α, one can correlate the PCNA dependent synthesis on the leading strand with DNA polymerase δ, and PCNA independent synthesis of DNA on the lagging strand with DNA polymerase α. Further, the dependence of the completion of lagging strand DNA synthesis on concomitant leading strand DNA synthesis during SV40 DNA replication *in vitro*, suggests that DNA polymerases α and δ function as a single unit at the replication fork. By looping the lagging strand DNA, the DNA polymerase α/δ complex could move in one direction spatially, while synthesizing 5'→3' on both strands (Figure 2).

1.4 Model Systems

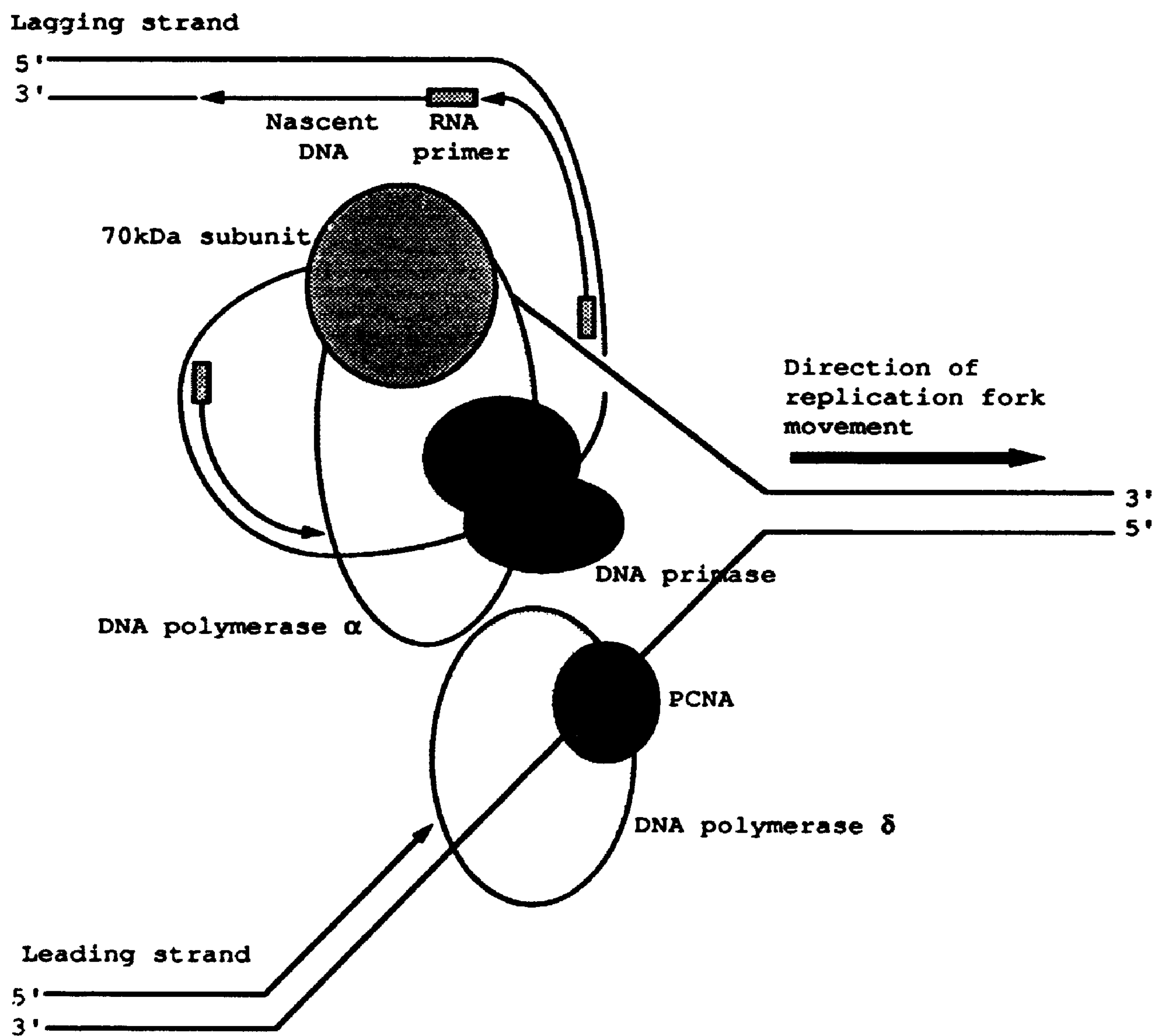
1.4.1 *In vitro* replication as a source of information

One approach to the study of replication in eukaryotes has been to study viruses which infect eukaryotic cells. Viruses rely on both

Figure 2

DNA polymerase α/δ: a concerted model

The leading and lagging strands may be copied by a single multienzyme complex involving both DNA polymerases α and δ. DNA polymerase δ synthesizes the leading strand in a continuous manner, aided by the DNA polymerase δ accessory protein PCNA. DNA polymerase α synthesizes the DNA on the lagging strand in a discontinuous manner.



Lagging strand
5'
3'
Nascent DNA
RNA primer
70kDa subunit
Direction of replication fork movement
3'
5'
DNA primase
DNA polymerase α
PCNA
DNA polymerase δ
Leading strand
5'
3'

viral and cellular proteins to achieve their replication. The extent to which any given viral system serves to elucidate cellular replication is a function of the extent to which the virus relies on the host cell's replication machinery.

The practical use of a viral system depends on one's ability to replicate the virus *in vitro*, allowing dissection of the components which play a role in the viral replication. Depletion and reconstitution of an *in vitro* replication system allows the purification its components. Adenovirus was the first virus which infects eukaryotic cells to be replicated *in vitro* (Challberg and Kelly, 1979). The adenovirus replication system is dependent on the cellular proteins NF-1, NF-2, and NF-3 (or ORP-3). NF-1 and NF-3 are cellular transcription factors which are sequence specific DNA binding proteins. Both NF-1 and NF-2 bind the adenovirus origin, and are required for initiation complex formation (Nagata *et al.*, 1982; Nagata *et al.*, 1983b; Pruijn *et al.*, 1986; Wides *et al.*, 1987; Rosenfeld *et al.*, 1987). NF-2 is a type I topoisomerase which is required for completion of viral DNA replication, but may be replaced by eukaryotic topoisomerase I (Nagata *et al.*, 1983a). Adenovirus relies on its own DNA polymerase, and priming is achieved through a terminal protein, as opposed to the 3' hydroxyl of nascent RNA, as occurs during cellular replication initiation and Okazaki fragment synthesis.

A more useful viral model is simian virus 40 (SV40), a papovavirus. SV40 DNA is assembled into a nucleoprotein complex reminiscent of chromatin (Griffith, 1975; Cremisi *et al.*, 1976). Initiation occurs at a unique origin, from which replication proceeds bidirectionally (Danna and Nathans, 1972; Fareed *et al.*, 1972). SV40 can be replicated *in*

vitro, and is dependent upon only one viral protein, the SV40 large tumour (T) antigen (Ariga and Sugano, 1983; Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). T antigen interacts with DNA polymerase α, and both DNA polymerase α and DNA primase are required for SV40 replication *in vitro* (Murakami *et al.*, 1986; Smale and Tjian, 1986). PCNA is also required for SV40 replication *in vitro*, which suggests that DNA polymerase δ is involved as well (Prelich and Stillman, 1988). However, comparisons of SV40 and cellular DNA replication are complicated by the action of the SV40 T antigen. SV40 T antigen binds both the SV40 origin and DNA polymerase α, suggesting that T antigen localizes DNA polymerase α to a specific region of the SV40 origin. Whether T antigen remains bound to DNA polymerase α during replication, and if so, the result of that interaction, have yet to be determined.

1.4.2 Minute Virus of Mice

Minute virus of mice, referred to throughout this work as MVM, is an autonomous parvovirus (Crawford, 1966). The sequence of both the prototype (fibrotropic) strain MVMp, and the lymphotropic immunosuppressive variant, MVMi, have been determined (Astell *et al.*, 1983; Sahli *et al.*, 1985; Astell *et al.*, 1986). MVM DNA replicating *in vivo* is complexed with proteins in a form which is distinct from chromatin (Doerig *et al.*, 1986). The DNA sequence at both the 3' and 5' ends of parvovirus DNA are inverted repeats, which form hairpin structures when in the base paired configuration (Astell *et al.*, 1979). In addition to the terminal hairpin, MVM has a base paired 3' hydroxyl terminus, which serves as a primer for DNA synthesis *in vitro* (Figure

3a). DNA polymerase α uses this 3' hydroxyl as a primer (Faust and Rankin, 1982) to convert MVM ssDNA to dsDNA *in vitro*. MVM ds DNA is distinct from MVM RF DNA, in that the 5' hairpin is elongated in the RF form, but not in the dsDNA form (Figure 3a).

In vivo, MVM replication is aphidicolin sensitive (Hardt *et al.*, 1983; Robertson *et al.*, 1984). Okazaki fragments have not been detected *in vivo*, and MVM infection leads to the production of primase free forms of DNA polymerase α, suggesting that a variant form of DNA polymerase α may be involved in MVM replication *in vivo* (Ho *et al.*, 1989). However, further studies are required to show conclusively which DNA polymerase, DNA polymerase α or δ, or whether both are required for parvovirus DNA replication *in vivo*.

1.4.3 Bacteriophage M13

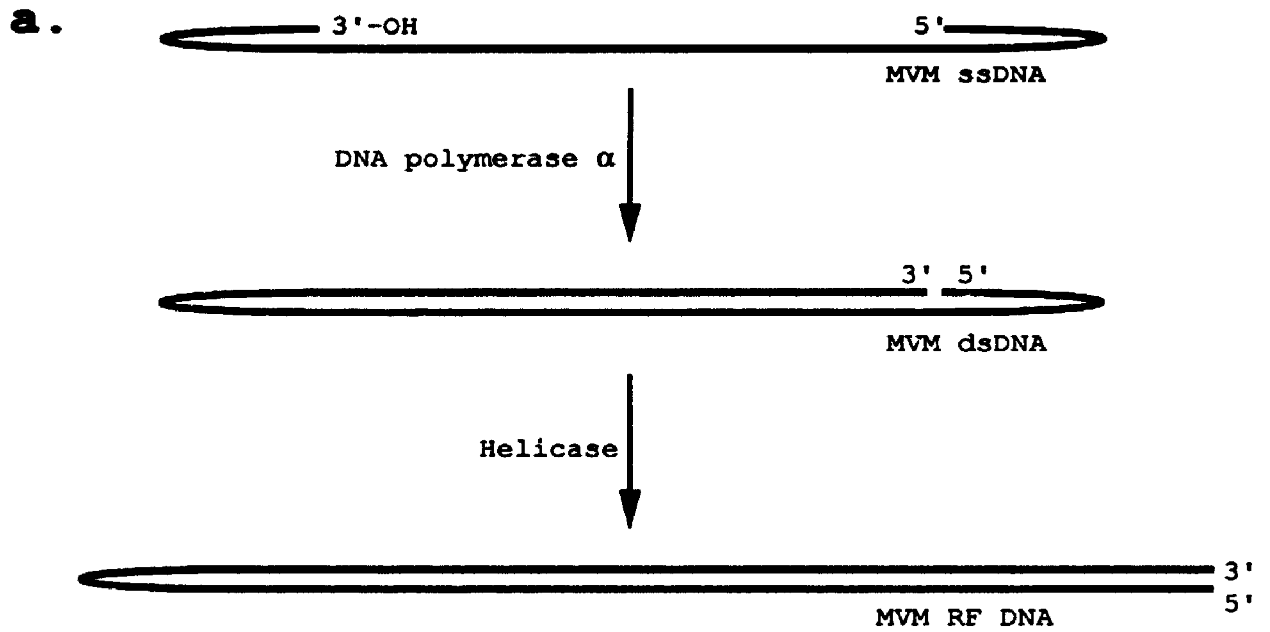
M13 is a small filamentous coliphage, with a single stranded circular DNA genome of about 6400 nucleotides. The principal variant of M13 used in this study is M13mp19, which has been modified for use as a cloning vector (Yanisch-Perron *et al.*, 1985). M13mp19 contains a polylinker region, where unique restriction sites are arranged in tandem. A synthetic oligonucleotide immediately upstream of the polylinker region allows priming of DNA synthesis. This primer can be utilized by a number of DNA polymerases, including E. coli DNA polymerase I Klenow fragment, T4 DNA polymerase, and DNA polymerase α (Figure 3b). DNA fragments may be inserted immediately downstream of the primer, into the polylinker region. M13 has been used in this work because it is uniquely suited to allow preparation of large quantities of single stranded DNA, which facilitate the study of sequence

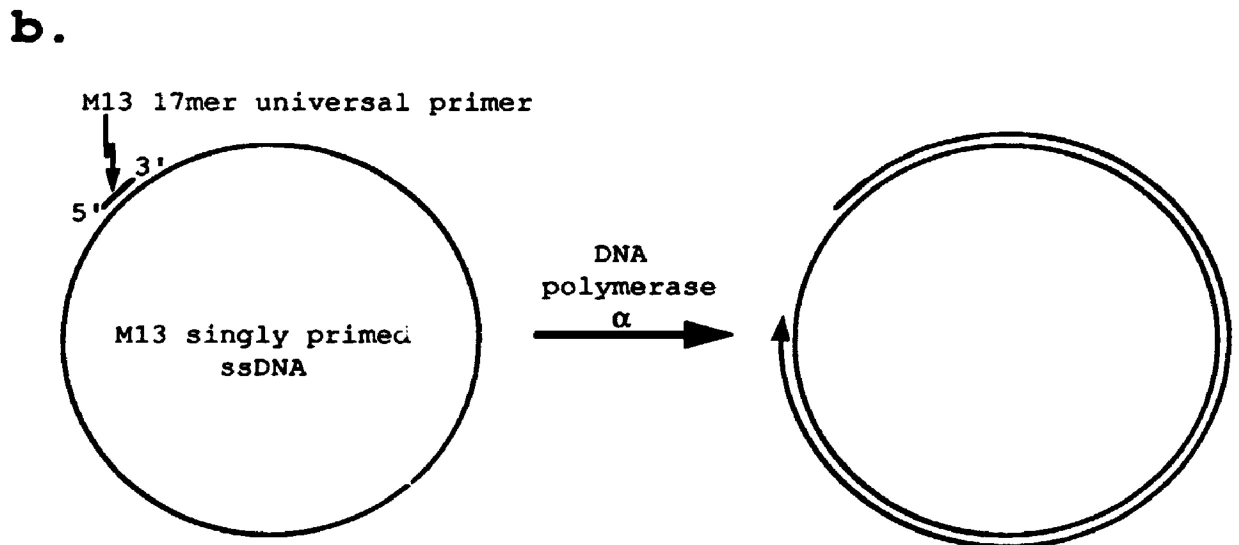
Figure 3

Model systems

a. The first stage of MVM replication *in vivo*, is the conversion of MVM ssDNA-RF DNA. *In vitro*, DNA polymerase α does not displace the 5' terminal hairpin, generating an intermediate that can be termed MVM dsDNA.

b. Singly primed M13 ssDNA can be copied by DNA polymerase α.


a.
3'-OH
5'
MVM ssDNA
DNA polymerase α
3' 5'
MVM dsDNA
Helicase
3'
5'
MVM RF DNA


b.
M13 17mer universal primer
3'
5'
M13 singly primed
ssDNA
DNA
polymerase
α

dependent effects during M13 DNA synthesis *in vitro*.

1.5 General statement of goals

This work is a consideration of the properties of DNA polymerase α that enable it to function as the lagging strand DNA polymerase at a replication fork. The first part of the work (chapter 3) deals with the purification protocol which we have developed to produce highly purified DNA polymerase α in a rapid and reproducible manner. The second section of the work (chapter 4) deals with sequence determinants which specify favoured sites of initiation of RNA primed DNA synthesis (ψ sequences). The final portion of the work (chapter 5) deals with the behaviour of the enzyme as it approaches a downstream double stranded region. Taken together, these studies provide an *in vitro* consideration of events corresponding to the initiation, elongation, and termination of Okazaki fragments, which are the main products of any lagging strand DNA polymerase *in vivo*.

Chapter 2

Materials and methods

Chemical and molecular biology reagents were purchased from commercial sources. Where the products of one company were used preferentially, or where reagents were only available from a limited number of sources, specific supliers are given in the text. All reagents were of enzyme or molecular biology grade where available.

The highest grade of nucleotides available were purchased from Pharmacia LKB Biotechnology, and used without further purification. Nucleotides were resuspended in TE, neutralized with NaOH, and assayed spectrophotometrically to accurately determine concentration.

DNA concentrations are given as molecular concentrations; when nucleotide concentrations were used, this was noted.

DNA polymerase α throughout the text refers to the DNA polymerase α-DNA primase complex fraction VI (see 3.3.1).

BuPdGTP and BuAdATP were gifts from Dr. George Wright. DNA polymerase α_2 was a gift from Dr. Earl Baril.

2.1 Growth and maintenance of cell lines, bacteria and viruses

2.1.1 Ehrlich ascites mouse tumour cells

DNA polymerase α was purified from Ehrlich ascites mouse tumour cells (van Venrooij *et al.*, 1970), which were grown in suspension culture in Joklik's media (Gibco), supplemented to 5% fetal bovine serum (Gibco or Boknek), and 2mM L-glutamine. The cells were diluted with fresh media

daily, to a concentration of 2×10^5 cells/ml. Excess cells were harvested by successive rounds of centrifugation in an IEC #981 rotor at 2 000 RPM for 15 minutes, or in an IEC #215 rotor at 1 500 RPM for 5 minutes. Cells typically grew from 2×10^5 cells/ml to 8×10^5 cells/ml, in 24 hours.

2.1.2 Minute virus of mice

Minute virus of mice prototype strain (MVM) DNA was prepared by sedimentation of purified MVM virions through alkaline sucrose gradients, as described (Faust and Ward, 1979).

2.1.3 *Escherichia coli*

E. coli strains utilized in the course of this study are summarized in Table 1. Long-term cultures of these strains were maintained as glycerol stocks (50% stationary-phase culture, 50% glycerol) at -70°C. Short term stock cultures of JM103 and JM109 were maintained for up to one month at 4°C on M9 plates (1.5% w/v agarose, 60mM K_2HPO_4, 33mM KH_2PO_4, 7.5mM $(NH_4)_2SO_4$, 1.7mM sodium citrate, pH 7.4) supplemented with 0.2% w/v glucose, 15nM thiamine HCl, and 1.0mM $MgCl_2$ (Maniatis *et al.*, 1982). Short term cultures of CJ236 were maintained on YT plates (1.5% w/v agar, 0.8% w/v Bacto tryptone, 0.5% w/v Bacto yeast extract, 0.5% w/v NaCl) supplemented with 34μg/ml chloramphenicol (Kunkel, 1987). These storage procedures exerted selective pressure to prevent the loss of the F' factor, which is required for subsequent M13 infections. DH5αF' cells do not require selection to maintain the F' factor, so were maintained on unsupplemented YT plates. Stationary phase cultures of all cells were prepared by growth in YT media, for 12-24

Table 1

Bacterial strains used in this work

The names of bacterial strains used in this work, and their genotypes have been summarized. All strains were derivatives of *E. coli* K12.

Bacterial Strains	Genotype	Reference
E. coli JM103	*Δ(lac-pro), supE, thi, strA, sbcB15, endA, hspR4, [F'traD36, proAB, lacI^{q}ZΔM15]*	Messing, 1983
E. coli JM109	*recA1,endA1, gyrA96, thi, hsdR17, supE44, relA1, λ$^-$, Δ(lac-proAB), [F'traD36, proAB, lacI^{q}ZΔM15]*	Yanisch-Perron et al., 1985
E. coli CJ236	*dut1, ung1, thi1, relA1,* pCJ105 (Cm^r)	Kunkel et al., 1987
E. coli DH5αF'	*F'φ80dlacZΔM15Δ(lacZYA-argF) U169, recA1, endA1, hsdR17 (rk$^-$, mk$^+$), supE44, λ$^-$, thi1, gyrA, relA1*	BRL, 1988

hours, at 37°C; logarithmic phase cultures were produced by diluting stationary phase cultures to 0.01-1% v/v in YT media, followed by incubation for 2-6 hours at 37°C with constant aeration.

2.1.4 Bacteriophage M13

Bacteriophage M13mp19 (Yanisch-Perron *et al.*, 1985) was propagated in the *E. coli* strains summarized in Table 1. Infections were carried out on logarithmic phase *E. coli* grown in unsupplemented YT media, except infection of *E. coli* CJ236 (see section 2.6).

2.2 Purification of DNA

2.2.1 M13 replicative form DNA

One litre of YT media was inoculated with 1ml late logarithmic phase JM103 cells, and 1ml (≈1×10^{12} pfu) M13 HTϕ, and incubated for 3 hours at 37°C. The cells were centrifuged in an IEC 981 rotor at 3 500 rpm, for 30 minutes, at 4°C. The cell pellet was resuspended in 20ml cold STE, and centrifuged in a Sorval HB-4 rotor at 8 000 rpm, for 15 minutes, at 4°C. The cell pellet was resuspended in 8ml plasmid lysis buffer (8% w/v sucrose, 0.5% v/v triton X-100, 50mM EDTA, 10mM Tris·Cl; pH 8.0), and 20mg fresh lysozyme (in 1ml 10mM Tris·Cl pH 8.0) was added. The cells were lysed by incubation in a boiling water bath for 1 minute with constant agitation, and cooled on ice for 5 minutes.

The lysate was centrifuged in a Beckman SW40 rotor at 35 000 rpm, for 20 minutes, at 4°C. The supernatant (cleared lysate) was extracted with 1 volume phenol:chloroform:iso-amyl alcohol (25:24:1), then with 1 volume chloroform:iso-amyl alcohol (24:1). One gram of CsCl was added

per ml of cleared lysate (to ρ=1.55g/ml). Ethidium bromide was added to the cleared lysate/CsCl solution; 80µl of a 10mg/ml EtdBr solution was added per millilitre of cleared lysate/CsCl solution. The lysate solution was centrifuged in a Beckman 70.1 Ti rotor in self-seal tubes at 60 000 rpm, for 17 hours, at 18°C. The DNA was visualized under UV light, and the lower band, containing supercoiled DNA, was removed through a side puncture. A second ultracentrifugation was performed if the supercoiled phage DNA and cellular DNA bands were not well resolved by the first centrifugation. Conditions for the second CsCl/EtdBr centrifugation were the same as for the first, except the centrifugation was performed in a Beckman 50 Ti rotor at 50 000 rpm, for 17-24 hours.

The supercoiled phage DNA was extracted 5 times with 1 volume of ddH_2O saturated 1-butanol, then dialyzed against 1 litre TE, for 17-24 hours, at 4°C. The buffer was changed, and dialysis performed for an additional 1-2 hours, at 4°C. The dialyzed DNA was treated with 40-50µg/ml RNase A, for 1 hour, at 37°C, extracted once with 1 volume of phenol, and once with 2 volumes of diethyl ether. The purified DNA was precipitated with ethanol, for 1 hour, at -70°C, and recovered after centrifugation in a Sorval HB-4 rotor at 10 000 rpm, for 30 minutes, at 4°C. The DNA pellet was lyophilized for 10 minutes, then resuspended in 200µL TE. Recoveries ranged from 0.2 to 0.6 milligrams of supercoiled DNA.

2.2.2 M13 single stranded DNA

Small scale preparations of M13 ssDNA were made from 2ml cultures of infected *E. coli*. Infections were performed at 37°C for 8 to 16 hours.

Most of the culture was transferred to an Eppendorf tube, and the *E. coli* cells were sedimented to a pellet by centrifugation in an Eppendorf microfuge, for 10 minutes. The supernatant was transferred to another Eppendorf tube, and 0.2 volumes of 20% w/v PEG 8000/2.5M NaCl were added. The phage particles were allowed to precipitate at room temperature (≈23°C) for 15 minutes, then on ice for 5 minutes. Samples were then centrifuged in an eppendorf microfuge for 15 minutes. The supernatant was removed, and the M13 phage pellet resuspended in 100μL T . Phage particles were disrupted by extraction with 1 volume of phenol. The aqueous phase, containing the M13 ssDNA, was adjusted to 0.3M sodium acetate, and the DNA precipitated by the addition of 2 volumes of ethanol. DNA was recovered by centrifugation for 15-30 minutes in an eppendorf microfuge. The supernatant was removed, and the DNA dried in a Savant speed vac for 5 minutes. The DNA was resuspended in 50μL TE, which generated concentrations of DNA in the 100-200 μg/ml range, and was used primarily for DNA sequencing.

Large scale M13 ssDNA preparations , which were used as substrates in DNA polymerase α reactions, were prepared by a modification of the above procedure. Infections were performed in 0.01-1 litre cultures, typically 100ml, for 8-16 hours. A series of purification steps were performed, each including a centrifugation to remove bacterial cells, precipitation of the M13 phage particles by addition of 0.2 volumes of 20% w/v PEG 8000/2.5M NaCl, and another centrifugation to recover the phage particles. Centrifugations were performed in a Beckman JA-14 or JS-13.1 rotor at 10 000 rpm for 1 hour, or as above, in an eppendorf microfuge, for 10-15 minutes, at 4°C. Typically, three rounds of purification, in progressively smaller volumes, were performed on large

scale M13 preparations.

2.2.3 Alkaline sucrose gradients

Alkaline sucrose gradients contained 0.15% w/v n-laurylsarkosine, 0.3N NaOH, 0.7N NaCl, 1mM EDTA, and sucrose as indicated. Linear 5-20% w/v sucrose gradients were formed in Beckman SW40 rotor tubes. Up to 0.5ml sample volume was layered onto 12ml gradients. Centrifugation was performed as indicated. Following centrifugation, fractions were collected from the bottom of the gradient, dialyzed against TE, extracted with phenol, then with ether, and precipitated with ethanol.

2.3 *In vitro* DNA manipulations

2.3.1 Restriction endonuclease digestions

Restriction endonucleases were used according to the manufacturers instructions. Samples of DNA were typically overdigested 2-10 fold (ie. 2-10 units/μg·hour) to ensure complete digestion.

2.3.2 5' end labeling with T4 polynucleotide kinase

M13 17mer universal primer was 5' end labeled by incubation in a 100μl volume containing 10pmol 17mer 5' ends, 70mM Tris·Cl pH 7.6, 10mM $MgCl_2$, 5mM DTT, 100μCi [γ-^{32}P]ATP (3 000Ci/mmol), and 5 units T4 polynucleotide kinase, for 4 hours, at 37°C. Radiolabeled oligonucleotides were purified from unincorporated mononucleotides by chromatography in TE, on a 7-10ml Sephadex G-50(fine) column. Purified oligonucleotides were precipitated with ethanol and dissolved in TE at 0.5pmol/μl. This method generates radiolabeled oligonucleotides with a

specific activity of approximately $1x10^6$ cpm/pmol 5'ends.

For lower specific activity of labeled oligonucleotides, or when no radiolabel was used, the reaction was supplemented with ATP to 10-100µM, and reaction times were decreased to 1 hour.

2.3.3 3' end labeling with terminal deoxynucleotidyl transferase

Terminal deoxynucleotidyl transferase was purchased from Pharmacia P-L Biochemicals. The M13 17mer universal primer was incubated in a 150µl volume containing 45pmol 17mer 3' ends, 100mM sodium cacodylate pH 7.0, 1mM $CoCl_2$, 0.1mM DTT, 50µg/ml BSA, 200µCi [α-^{32}P]dNTP (3 000Ci/mmol), and 20 units terminal deoxynucleotidyl transferase for 2 hours, at 37°C. dCTP and dGTP residues were incorporated in separate reactions. The goal of these reactions was to incorporate a single nucleotide at the majority of 3' ends. The nature of the products was confirmed by autoradiography, after denaturing polyarcylamide gel electrophoresis (see 2.4.2).

Blocking of 3' ends using terminal deoxynucleotidyl transferase to incorporate a single terminal ddTTP residue is considered in section 2.12.3.

2.3.4 Bacterial alkaline phosphatase

Bacterial alkaline phosphatase was purchased from Boehringer Mannheim. Fragments of DNA purified after restriction endonuclease digestion, or after DNA polymerase reactions, were incubated in 20-100µl volumes containing 50mM Tris·Cl pH 8.0, and 5 units bacterial alkaline phosphatase, for 10 minutes, at 45°C. These samples were extracted at least three times with phenol, and precipitated with

ethanol, before subsequent reactions were performed.

2.3.5 Tobacco acid pyrophosphatase

Tobacco acid pyrophosphatase was purchased from Boehringer Mannheim. DNA fragments purified after DNA polymerase α reactions were incubated in a 50μl volume containing 50mM sodium acetate pH 5.5, 10mM 2-mercaptoethanol, 1mM EDTA, and 2 units tobacco acid pyrophosphatase, for 1 hour, at 37°C.

2.3.6 Calf intestinal phosphatase

Calf intestinal phosphatase was purchased from Boehringer Mannheim. Restriction endonuclease digested DNA fragments were incubated in 20-100μl volumes containing either TE or 20mM Tris·Cl pH 8.0, 10mM $MgCl_2$, 1mM $ZnCl_2$, and 2 units calf intestinal phosphatase, for 1 hour, at 37°C. Calf intestinal phosphatase was heat inactivated for 10 minutes, at 75°C. The DNA was then extracted with phenol, and precipitated with ethanol, before subsequent reactions were performed.

2.3.7 T4 DNA ligase

T4 DNA ligase was purchased from Pharmacia LKB Biotechnology or New England Biolabs. If the DNA fragments generated by restriction endonuclease digestion had complementary protruding ends, ligation was performed by incubation in a 50μl volume containing 0.07-0.1pmol DNA fragment ends, 50mM Tris·Cl pH 7.6, 10mM $MgCl_2$, 5% w/v PEG 8000, 1mM ATP, 1mM DTT, and 1 unit T4 DNA ligase, for up to 4 hours, at 37°C. If the DNA fragments to be ligated had blunt ends, the amount of T4 DNA ligase was increased to 10 units.

2.4 Gel electrophoresis

2.4.1 Agarose gel electrophoresis

Native agarose gels contained 0.5-1.0% Ultra Pure agarose (Bethesda Research Laboratories) dissolved by boiling in 1x agarose running buffer (40mM Tris base, 5mM sodium acetate, 0.5mM EDTA, pH 8.0). Samples were loaded in 0.4M urea, 0.2M sucrose, 10mM EDTA, 0.1% w/v bromophenol blue. Electrophoresis was performed with the gel submerged in 1x running buffer, at 40-50V, for 12-24 hours.

Alkaline agarose gels contained 1.0% agarose, dissolved by boiling in 1x agarose running buffer (above), and were adjusted to 30mM NaOH, 1mM EDTA, after cooling to 42°C. Samples were loaded in 0.4M urea, 0.2M sucrose, 10mM EDTA, 0.1% w/v bromophenol blue, and 0.1N NaOH. Electrophoresis was performed with the gels submerged in 30mM NaOH, 1mM EDTA, at 50V, for 12-24 hours.

2.4.2 Denaturing polyacrylamide gel electrophoresis of DNA

Denaturing polyacrylamide gels contained 4-20% acrylamide (19:1 bis), 8M Ultra pure urea (Bethesda Research Laboratories or ICN), 1x TBE, and were polymerized by the addition of 0.45ml 10% w/v ammonium persulfate, and 10µl (per 50ml gel volume) TEMED. Gels were cast between siliconized glass plates, to a thickness of 0.4-1.5mm. After addition of ammonium persulfate, the gels were allowed to polymerize for at least 1 hour. Electrophoresis was performed with 1x TBE running buffer, for a sufficient length of time to allow the gel surface temperature to reach 55°C (typically 1 hour), before loading the

samples. Prior to loading, 2 volumes of formamide dye mix (95% deionized formamide, 10mM EDTA, 0.01% w/v bromophenol blue, 0.01% w/v xylene cyanole FF) were added, and the samples were incubated in a boiling water bath for 3 minutes. The gel surface temperature was maintained at 55°C throughout the electrophoresis. Gels were run at constant current; 35mA for a 40mm long 8% acrylamide/0.4mm gel.

2.4.3 Non-denaturing polyacrylamide gel electrophoresis of DNA

Non-denaturing polyacrylamide gels contained 8% acrylamide (19:1 bis), 1x Jeppesen buffer (40mM Tris·acetate, 20mM sodium acetate, 2mM EDTA; pH 8.3), and were polymerized as described for denaturing polyacrylamide gels. These gels were cast between siliconized glass plates, to a thickness of 0.7mm. Samples were loaded in 1x sample buffer supplemented with 0.01% w/v xylene cyanole FF. Non-denaturing gels were run with 1x Jeppesen as running buffer, at 10-20mA, gel surface temperature <35°C, for 6-20 hours.

2.4.4 Discontinuous polyacrylamide gel electrophoresis of proteins

Discontinuous SDS polyacrylamide gel electrophoresis was performed essentially according to the method of Laemmli (1970). The separating gel contained 0.37M Tris·Cl pH 8.8, 0.1% w/v SDS, 6.5% acrylamide (30:1 bis), 2mM EDTA, and was polymerized by the addition of 0.1ml 10% w/v ammonium persulfate, and 20μl TEMED. The stacking gel contained 0.125M Tris·Cl pH 6.8, 0.1% w/v SDS, 5.25% w/v acrylamide (30:1 bis), and was polymerized by the addition of 0.004 volumes 10% w/v ammonium persulfate, and 0.001 volumes TEMED. Discontinuous SDS polyacrylamide gels were cast to a thickness of 0.7-1.5mm.

Samples were adjusted to a volume of 50-100μl, by addition of 1-2 volumes of SDS sample buffer (0.25M Tris·Cl pH 6.8, 4% w/v SDS, 1.4M 2-mercaptoethanol, 10% glycerol, 0.002% w/v bromophenol blue). Immediately prior to loading, samples were incubated in a boiling water bath for 3 minutes, then transferred to ice.

The running buffer for discontinuous SDS polyacrylamide gels consisted of 50mM Tris·glycine pH 8.2, and 0.1% w/v SDS. Electrophoresis was performed at 45V for 3-4 hours.

2.5 *In vitro construction of recombinant M13/MVM DNA Sequences*

MVM ssDNA was converted to MVM dsDNA by incubation in a 50μl volume containing 0.75μg ssDNA, 1x premix (50mM Tris·Cl pH 7.5, 5mM $MgCl_2$, 10mM DTT, 5mM KCl, 10% v/v glycerol, 0.5mg/ml BSA, 2.5×10^{-5}M each dATP/dCTP/dGTP/dTTP), and 5 units Klenow polymerase, for 30 minutes, at 37°C. The fragments of interest were generated by double restriction endonuclease digestion of MVM dsDNA, either concurrently, or as sequential single digestions of the MVM dsDNA. M13mp19 RF DNA (Yanisch-Perron *et al.*, 1985) was used as the vector, and was digested with restriction endonucleases to generate ends compatible with the MVM DNA fragments to be inserted. The details of the digestions are summarized in Table 2. Figure 4 is a schematic representation of the construction of M13mp19·154. Following restriction endonuclease digestion, the DNA was extracted with phenol then ether, and precipitated with ethanol. The MVM and M13 DNAs were ligated, and used to transfect *E. coli*. In each case, the expected clone was found in 3 of 3 isolated plaques, as determined by DNA sequence analysis.

Table 2

Summary of cloned DNA fragments

Restriction endonuclease digestions performed on the insert (MVM) and vector (M13mp19) DNA are indicated. In cases were the restriction endonuclease buffers were compatible, digestions were performed simultaneously. In cases where restriction endonuclease buffers were incompatible, digestions were performed in two steps; digestion in the lower ionic strength buffer was performed first. Clones names indicate the length of MVM DNA inserted. MVM fragments are identified by the numbering scheme proposed by Astell *et al.* (1986).

Clone	Restriction Endonucleses	Fragment of Interest
M13mp19·94		
Insert	*Hin*dIII/*Mbo*I double digestion	2561-2654
Vector	*Hin*dIII followed by *Bam*H1 digestions	
M13mp19·154		
Insert	*Hae*III/*Hin*dIII double digestion	2501-2654
Vector	*Sma*I followed by *Hin*dIII digestions	
M13mp19·529		
Insert	*Hin*dIII followed by *Pst*I digestions	2126-2654
Vector	*Hin*dIII followed by *Pst*I digestions	

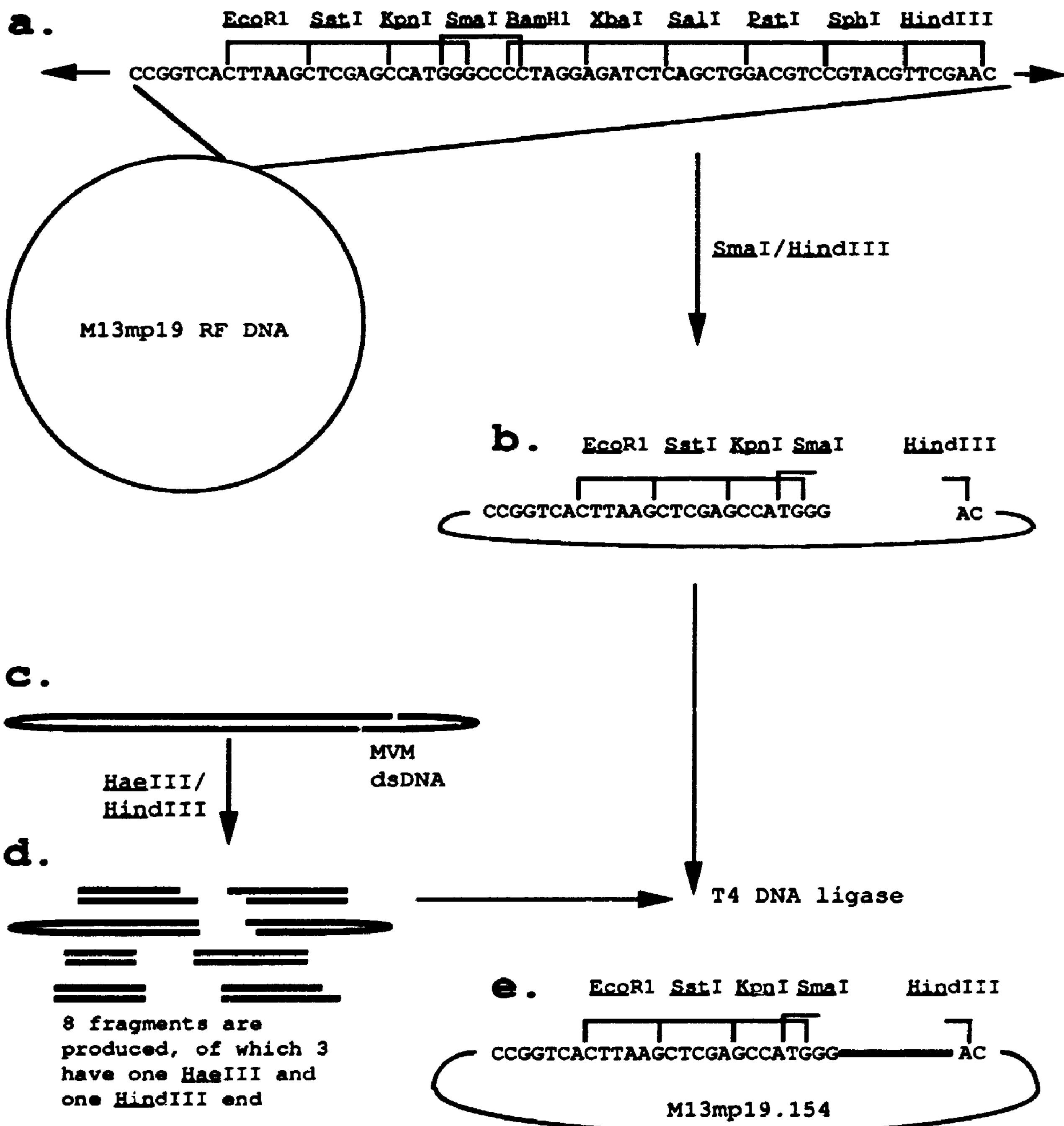
Figure 4

Construction of M13mp19·154

(a) M13mp19 with enlargement of polylinker region. The M13 RF DNA is digested with *Sma*I and *Hin*dIII, to generate the vector DNA (b).

(c) MVM RF DNA synthesized with Klenow polymerase is digested with *Hae*III and *Hin*dIII. This digestion of MVM DNA generates 8 fragments, of which three have one *Hae*III and one *Hin*dIII end, compatible with the vector. These three fragments are the insert DNA (d).

(e) Insert and vector DNA are mixed and then ligated by the action of T4 DNA ligase, to generate the recombinant DNA product.


a.
EcoR1 SstI KpnI SmaI BamH1 XbaI SalI PstI SphI HindIII
CCGGTCACTTAAGCTCGAGCCATGGGCCCCTAGGAGATCTCAGCTGGACGTCCGTACGTTCGAAC
M13mp19 RF DNA
SmaI/HindIII
b.
EcoR1 SstI KpnI SmaI HindIII
CCGGTCACTTAAGCTCGAGCCATGGG AC
c.
MVM dsDNA
HaeIII/ HindIII
d.
8 fragments are produced, of which 3 have one HaeIII and one HindIII end
T4 DNA ligase
e.
EcoR1 SstI KpnI SmaI HindIII
CCGGTCACTTAAGCTCGAGCCATGGG AC
M13mp19.154

2.6 Site directed oligonucleotide mutagenesis

Site directed mutagenesis was performed essentially according to Kunkel (1987). Logarithmic phase *E. coli* CJ236 were infected with M13 bacteriophage, at a multiplicity of infection of <0.001, in YT supplemented with 34µg/ml chloramphenicol, and 0.25µg/ml uridine. The cells were incubated 12-24 hours at 37°C, and ssDNA purified as described (2.2.2).

Oligonucleotides for mutagenesis were purchased from the Regional DNA Synthesis Laboratory in Calgary, Alberta. The sequences of the mutagenic oligonucleotides are summarized in Table 3. Prior to hybridization, the oligonucleotides were 5' phosphorylated using T4 polynuleotide kinase, in the presence of 66-100µM ATP. Hybridization of the mutagenic oligonucleotide to the target DNA was carried out in a 10µl volume containing 20mM Tris·Cl pH 7.5, 20mM $MgCl_2$, 100mM NaCl, at a two fold molar excess of DNA (0.5pmol ends oligonucleotide, 1.0pmol≈2.7µg M13 DNA) Theoretical melting temperatures (T_m) were calculated using the formula:

$$T_m=16.6\log[M]+0.41[P_{gc}]+81.5-P_m-B/L-0.65[P_f];$$

where M is the molar concentration of Na^+, P_{gc} is the percent G+C bases in the oligonucleotide, P_m is the percent mismatched bases, B is 675 for oligonucleotides up to 100 bases in length, L is the length of the oligonucleotide in bases, and P_f is the percent of formamide in the annealing buffer (Lathe, 1985). Annealing was performed at a temperature between 15 and 20°C below the calculated T_m (for an oligonucleotide without mismatches), for 2 hours. Polymerization and ligation were performed simultaneously, in a 50µl volume containing 10mM Tris·Cl pH 7.5, 10mM $MgCl_2$, 2mM DTT, 1mM ATP, 0.5mM each

Table 3

Sequences of Mutagenic Oligonucleotides

Melting temperatures have been estimated according to the formula presented in section 2.6. For degenerate oligonucleotides 3, 5, 6, 7, and 8, the G+C content assumes the oligonucleotide has the complementary sequence to wild type MVM. For oligonucleotide #4, the G+C content was calculated from the 18 non-degenerate nucleotides present in the sequence. For the deletion and insertional oligonucleotides 1, 2, and 4, the number of mismatched nucleotides were calculated as the length of the deletion/insertion, over the length of base pairing to the complementary MVM sequence.

Oligonucleotide		Values used in calculating T_m		
Number	Sequence	G+C content	Mismatch	T_m
#1	5' GGAGGCAAGGGGTCACTACT 3'	12/20=60%	2/29=9%	41.6°C
#2	5' GGCGAGGCAATCACTACTTT 3'	10/20=50%	6/26=23%	23.6
#3	5' dCCAAAGANNGGGGAGG 3'	10/16=63%	0	43.3
#4	5' dGGAGGCAANNTTGGTTGGTC 3'	10/18=56%	4/20=20%	28.8
#5	5' dGAGGCAANNTTGGTCA 3'	9/16=56%	0	40.8
#6	5' dGGCAAGGNNGGTCACT 3'	9/16=56%	0	40.8
#7	5' dCAAGGTTNNTCACTAC 3'	8/16=50%	0	38.2
#8	5' dAAAGACTNNGGAGGCA 3'	9/16=56%	0	40.8

dATP/dCTP/dGTP/dTTP, 5 units T4 DNA polymerase, and 5 units T4 DNA ligase. The samples were incubated for 30 minutes on ice, for 30 minutes at room temperature (≈23°C), and for 4 hours at 37°C. Reactions were terminated by incubation at 75°C for 10 minutes, and were used to transfect *E. coli* JM103. Mutant clones were found in approximately 20-50% of isolated plaques, as determined by DNA sequence analysis.

2.7 Transfection of *E. coli*

All transfections in this study involved an *E. coli* host, and M13 bacteriophage or recombinant derivatives with incorporated MVM sequences as vectors. The method of Cohen *et al.* (1972) was used exclusively. Late logarithmic phase cells were pelleted by mild centrifugation (Sorval HB-4 rotor, <5 000 rpm, <5 minutes, 4°C, or equivalent), resuspended in 0.5 volumes 50mM $CaCl_2$, and incubated on ice for 20 minutes. The cells were again centrifuged mildly, resuspended in 0.1 volume 50mM $CaCl_2$, and 1-20µl of ligation mixture (see 2.5 and 2.6) was added. The mixture was incubated on ice for 40 minutes, then heat shocked for 2 minutes at 42°C. Transfected cells were plated to YT, and incubated for 12-24 hours at 37°C. Purified plaques were prepared, and M13 ssDNA mini-preparations were utilized as templates in dideoxy chain termination sequencing.

2.8 Dideoxy chain termination sequencing

Dideoxy chain termination sequencing was performed essentially according to Sanger *et al.* (1977). Hybridization was performed in a 15µl volume containing 1µg=0.5pmol M13 ssDNA, 5ng=1.0pmol M13 17mer universal primer, and 1x annealing buffer (10mM Tris·Cl pH 7.5, 10mM

$MgCl_2$, 50mM NaCl), for 1 hour at 56°C. To the hybridized DNA, 5-10µCi [α-^{35}S]dATP (500Ci/mmol), and 1 unit Klenow polymerase, were added. Aliquots of this solution were added to the appropriate dNTP/ddNTP mixes to initiate polymerization of DNA chains. Samples were incubated for 20 minutes at room temperature (≈23°C), and chased with 0.5nmol dATP for an additional 20 minutes. Polymerization was terminated by the addition of 2 volumes of formamide dye mix. Samples were stored for up to 48 hours, at 4°C, before electrophoresis in denaturing polyacrylamide gels. After electrophoresis, the gels were fixed in 5% v/v methanol, 5% v/v acetic acid, for 30 minutes, washed with ddH_2O, and dried under vacuum. Autoradiography was performed using Kodak XAR-5 film, for 18-72 hours, at room temperature.

2.9 Bradford protein assay

Protein levels in samples were assayed essentially according to the method of Bradford (1976). Sample volumes of 1-100µl were adjusted to 100µl, and 1-5ml of reagent (0.01% w/v coomassie brilliant blue G-250, 4.7% w/v ethanol, 8.5% w/v phosphoric acid) was added. The samples were mixed by vortexing, and were allowed to develop for 10 minutes. Standard curves were generated by assaying dilutions of BSA, in buffer equivalent to DNA polymerase purification buffer B with 0.2M NaCl (see 3.3.1).

2.10 Staining SDS polyacrylamide gels

2.10.1 Coomassie brilliant blue staining

After electrophoresis, SDS polyacrylamide gels were fixed in 50% v/v

methanol, 10% v/v acetic acid, for 2 hours. The fixing solution was removed, and replaced with 0.05% w/v coomassie brilliant blue R-250, 50% v/v methanol, 10% v/v acetic acid. The gel was stained for 12-24 hours, at room temperature. Destaining was performed in 5% v/v methanol, 7% v/v acetic acid, with solution changes occasionally, until optimal resolution was obtained (6-24 hours).

2.10.2 Silver staining

After electrophoresis, SDS polyacrylamide gels were fixed for 12-18 hours in 50% methanol. Gels were washed sequentially with 70% methanol, ddH_2O, 70% methanol, ddH_2O, and 50% methanol, for 30 minutes to 1 hour each. This series of washes was performed to ensure that all glycine was removed from the gel, as glycine inhibits the silver staining reaction.

The silver stain was prepared by dissolving 1.6g $AgNO_3$ in 8ml ddH_2O (solution A), and mixing 42ml 0.36% w/v NaOH with 2.8ml fresh NH_4OH (solution B). Ammonium hydroxide was frozen at -70°C in aliquots immediately after purchase, until its use. Solution A was added dropwise to solution B, with constant vigorous agitation. The mixture was adjusted to 100ml with ddH_2O, and added to the gel immediately. Staining was performed for 15 minutes, with constant vigorous agitation, in a covered pyrex dish. The used stain was discarded into ddH_2O saturated with NaCl, and the gel rinsed with 50ml ddH_2O, and washed in 250ml ddH_2O for 5 minutes. The gel was treated with developer (0.01% w/v fresh citric acid, 0.38% w/v formaldehyde), with constant agitation. Stained proteins appear 5-15 minutes after the addition of developer. Developing was stopped when appropriate, by transfer of the

gel into 50% v/v methanol. If the gel was overdeveloped, the gel was destained with Kodak Rapid Fix, for 1-5 hours, or with 0.1x Rapid Fix overnight.

2.11 General Assays

2.11.1 DNA polymerase assay

DNA polymerase assays during enzyme purification were performed in a 20µl volume containing 10µl of 2x premix (100mM Tris·Cl pH 7.5, 10mM $MgCl_2$, 20mM DTT, 10mM KCl, 20% v/v glycerol, 1mg/ml BSA, and $5x10^{-5}$M each dATP/dCTP/dGTP/dTTP), 1µl=1µCi [^{3}H-8]dATP (22Ci/mmol), 2µl=0.1µg maximally activated calf thymus DNA, and 1µl of the column or gradient fraction, for 30 minutes, at 37°C. Reactions were terminated by addition of 0.5ml 10% w/v TCA, 0.025M sodium pyrophosphate. Samples were made up to 5ml with 5% w/v TCA, 0.025M sodium pyrophosphate, and precipitable material collected on Schleicher & Schuell #30 glass microfiber filters. The amount of radiolabel on the glass filters was determined by liquid scintillation counting, in the presence of Omnifluor (New England Nuclear), or Ecolume (ICN). One unit of DNA polymerase activity is defined as the amount of enzyme that incorporates 1nmol of deoxynucleotides into acid precipitable form on a maximally activated calf thymus DNA template, in 1 hour, at 37°C.

DNA polymerase assays conducted on ethylene glycol/glycerol gradient fractions (3.3.2, and 3.3.3) were performed as above, except 10µCi [α-^{32}P]dATP (3 000Ci/mmol) replaced [^{3}H-8]dATP.

2.11.2 DNA primase assay

Primase assays performed on ethylene glycol/glycerol gradient fractions were performed in a 20µl volume containing 10µl of primase mix (50mM Tris·Cl pH 7.5, 20mM $MgCl_2$, 10mM DTT, 5mM KCl, 1mg/ml BSA, 5mM ATP, 50µg/ml poly(dT), 1µl=10µCi [α-^{32}P]dATP, 0.5 units *E. coli* DNA polymerase I), and 10µl of the gradient fraction, for 30 minutes, at 37°C. Acid precipitable material was collected and the amount of radiolabel determined in the same manner as described for DNA polymerase assays (see 2.11.1). One unit of primase activity is defined as the amount of enzyme required for the incorporation of 1nmol of dATP into acid insoluble material, in 1 hour, at 37°C.

2.11.3 3'→5' exonuclease assay

The assay of 3'→5' exonuclease activity was performed using M13 17mer universal primer to which one of [α-^{32}P]dCTP, or [α-^{32}P]dGTP, had been added to the 3' end by the action of terminal deoxynucleotidyl transferase (see 2.3.3). These oligonucleotides were purified from denaturing polyacrylamide gels, generating a supply of M13 17mer to which a single nucleotide residue had been added. When annealed to M13 ssDNA, the oligonucleotide to which a 3' terminal dCTP residue had been added had mismatched 3' termini, while the oligonucleotide to which a 3' terminal dG had been added had all 18 nucleotides correctly base paired.

Oligonucleotide substrates for the 3'→5' exonuclease assay were hybridized to M13mp19·150 ssDNA in a volume of 150µl containing 1x annealing buffer (see 2.8), 20pmol (5' ends) oligonucleotide, and 40pmol DNA, for 1 hour, at 56°C. To the annealed substrate, 1 volume of 4x exonuclease buffer (0.2M Tris·Cl pH 7.5, 50mM $MgCl_2$, 10mM DTT,

0.2M KCl) was added, and this mix was aliquoted into 30μl samples. Exonuclease assays were initiated by addition of an equal volume of the appropriate ethylene glycol/glycerol gradient fraction (see 3.3.2). Reactions were incubated for 15 minutes to 1 hour, at 37°C.

The reactions were stopped by the addition of 1ml 10% TCA, 0.025M sodium pyrophosphate. Acid precipitable material was collected and the amount of radiolabel determined in the same manner as for DNA polymerase assays (see 2.11.1). One unit of 3'→5' exonuclease is defined as the amount of enzyme which causes the release of 1nmol of a mismatched nucleotide from the 3' end of the M13 17mer.

2.12 Sequence dependent DNA polymerase α reactions

2.12.1 Conversion of MVM ssDNA to dsDNA

MVM ssDNA was converted to duplex form (MVM dsDNA), by incubation in a 20μl volume containing 1x premix (see 2.5), 30ng MVM ssDNA, 0.5-1μCi[α-^{32}P]dTTP, and 0.1 units DNA polymerase α, for 2 hours, at 37°C. Ribonucleotides were present at concentrations of 0.01-4mM, as indicated. The reaction was scaled up as much as 100x, and the isotope was increased to as much as 0.6mCi (with [α-^{32}P]rGTP or [γ-^{32}P]rGTP) as indicated.

2.12.2 Sequence specific termination reactions

Primase activity was measured indirectly in M13 by termination of a DNA chain initiated upstream of an RNA priming site. The M13 17mer universal primer was radiolabeled with [γ-^{32}P]ATP by the action of T4 polynucleotide kinase, and hybridized to the indicated M13 ssDNA

template. Hybridization was performed in a 15µl volume containing 1x annealing buffer, and a 2 fold molar excess of 17mer primer to DNA, for 1 hour, at 56°C. Polymerization reactions were performed in a 20-30µl volume containing 1x premix, 30ng annealed template/primer, 0.01-4.0mM ribonucleotides (as indicated), and 0.1 unit DNA polymerase α, for 10 minutes to 4 hours, at 37°C. Reactions were terminated by extraction with phenol, then ether, and precipitated with ethanol. The samples were resuspended in TE, and 2 volumes of formamide dye mix was added prior to electrophoresis through denaturing polyacrylamide gels. Polyacrylamide gels were dried and autoradiographed.

2.12.3 Sequence specific RNA primed DNA synthes -

M13mp19·154 ssDNA with specific mutations, generated by oligonucleotide mutagenesis, was grown in large scale preparations. One O.D. 260nm unit of DNA (40µg=15pmol) was incubated in a 30µl volume containing 1x annealing buffer, and 20pmol M13 17mer universal primer, for 1 hour, at 56°C, then at room temperature for 15 minutes. To the annealed DNA, dNTPs (7.4nmol dATP, 4.8nmol dCTP, 5.1nmol dGTP, and 5.7nmol dTTP), and 5 units Klenow polymerase were added, and the mixture was incubated for 45 minutes, at 37°C. Nucleotides concentrations are indicative of nucleotide frequencies in M13 DNA, and allow for maximal utilization of added dNTPs. Since 23nmol of dNTPs were added, and there were 120nmol (nucleotides) available in the template DNA, all the dNTPs can be incorporated into nascent DNA. The DNA was digested with restriction endonucleases in a volume of 150µl, containing 10mM Tris·Cl pH 7.5, 10mM $MgCl_2$, 10mM NaCl, 1mM DTT, 50 units *Kpn*I, and 50 units *Hin*dIII, for 1 hour, at 37°C. The 3' hydroxyl

residues of the restriction fragments were blocked by the addition of 40µl 5x ddTdT buffer (500mM sodium cacodylate pH 7.0, 5mM CoCl2, 0.5mM DTT, 0.5mM ddTTP, 250µg/ml w/v BSA), and 20 units terminal deoxynucleotidyl transferase, and incubation for 30 minutes, at 37°C. This allows incorporation of one dideoxy nucleotide to each 3' end of the restriction fragments. The samples were extracted with phenol and ether, precipitated with ethanol, and resuspended in 50µl 65% formamide dye mix. The fragments of DNA containing MVM sequences generated in this procedure were approximately 160 nucleotides long, with the viral strand being 8 nucleotides longer than the nascent strand. This allowed the resolution of the nascent strand from the viral strand after electrophoresis through denaturing 8% polyacrylamide gels. The gels were soaked in 1mg/l EtdBr for 15-30 minutes at room temperature, washed in ddH_2O for 5 minutes, and the DNA fragments visualized under short wavelength UV light. The viral DNA fragment was excised from the gel, and soaked in 400µl TE for 12-24 hours, at 35-37°C. The TE was decanted, the DNA precipitated with ethanol and resuspended in 100µl ddH_2O. The recovery was quantified spectrophotometrically, and ranged from 50-85% for the entire procedure. Primase assay fragments purified this way are referred to as paDNA.

Primase assays were performed in a two step procedure. Priming was performed in a 20µl volume containing 1x SPA buffer (50mM Tris·Cl pH 7.5, 10mM $MgCl_2$, 5mM DTT, 1.5mM KCl, 0.1mg/ml w/v BSA), 1µCi [α-^{32}P]dATP, 0.01-0.1µg paDNA (as indicated), 0.1µM each ATP, CTP, GTP, and UTP, 0.1 unit DNA polymerase α, for 15 minutes, at 37°C. Priming was quenched, and the reactions chased by addition of 2µl dNTP chase solution (1.5×10^{-6} or 2.5×10^{-4}M each dATP, dCTP, dGTP, and dTTP; as

indicated), for 15 minutes at 37°C. Samples were either precipitated with acid and the amount of DNA synthesis determined (see 2.11.1), or precipitated with ethanol in preparation for electrophoresis through polyacrylamide gels.

All fragment purification and RNA primed DNA synthesis reactions described in this section (2.12.3) were performed under RNase free conditions.

2.13 Filling specific gaps generated in M13 DNA

2.13.1 The standard gap filling reaction

The M13 17mer (-20) universal primer (5' GTAAAACGACGGCCAGT 3') was purchased from New England Biolabs. The 20mer gap filling oligonucleotide (5' dACTGGGGAAGCTGGTTCCAA 3') is complementary to nucleotides 2142-2161 of the MVM genome (Astell *et al.*, 1986), and was purchased from the Oligonucleotide Synthesis Laboratory, Queen's University, Kingston, Ontario, Canada.

Gap filling assays were performed in two steps; oligonucleotides were hybridized to the template, followed by polymerization. The M13 17mer used in gap filling assays was 5' end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP to high specific activity (see 2.3.2). Hybridization of the M13 17mer, and where indicated the 20mer gap filling oligonucleotide, was performed in a 15µl volume containing 10mM Tris·Cl pH 7.5, 10mM $MgCl_2$, 50mM NaCl, 0.25pmol M13mp19·529 ssDNA, and 1pmol each of the indicated oligonucleotides, for 1 hour, at 56°C, then at room temperature, for 15 minutes. This method gave quantitative hybridization when oligonucleotides were present in at least a two fold

molar excess. Standard gap filling reactions were performed in a 20μl volume containing 30ng primed single stranded M13mp19·529 DNA, 1x premix (see 2.14.1), and 0.1 units DNA polymerase α, and were incubated for 5 minutes to 6 hours, at 37°C (as indicated). In experiments involving inhibitors, various concentrations of BuPdGTP or BuAdATP, 25μM ddCTP or ddTTP, or 0.5mM each of ddATP, ddCTP, ddGTP, and ddTTP, were present as indicated.

2.13.2 Determination of the K_m for incorporation of a single nucleotide at a gap

GF-1 (5' dCGACCTGCAGAAACCCAGAA 3') was purchased from the Regional DNA Synthesis Laboratory, Calgary, Alberta. DNA polymerization was performed, with DNA synthesis primed from GF-1. When the gap filling oligonucleotide was present, there was a one nucleotide gap between GF-1 and the gap filling oligonucleotide, and a two nucleotide gap between GF-2 and the gap filling oligonucleotide.

Hybridization of the GF-1, and where indicated the 20mer gap filling oligonucleotide, was performed in a 150μl volume containing 10mM Tris·Cl pH 7.5, 10mM $MgCl_2$, 50mM NaCl, 0.11nmol M13mp19·529 ssDNA, and 0.5nmol each of the indicated oligonucleotides, for 1 hour, at 56°C, then at room temperature, for 15 minutes. Gap filling reactions at single nucleotide gaps were performed in a 50μl volume containing 0.1-0.3μg primed single stranded M13mp19·529 DNA, 1x SPA buffer (see 2.12.3), 10μCi=0.25μM[α-^{32}P]dCTP (800Ci/mmol) and 0.5 units DNA polymerase α, and were incubated for 15 minutes, at 37°C. Reactions were terminated by acid precipitation (see 2.11.1), and the amount of acid precipitable ^{32}P determined.

Chapter 3

Purification and biochemical characterization of DNA polymerase α

3.1 Abstract

DNA polymerase α has been purified from cultured mouse Ehrlich ascites tumour cells, by classical purification methods. The purified DNA polymerase α complex has four subunits, 182, 70, 55, and 47kDa. The specific activity of purified DNA polymerase α is 65 000 units/mg total protein. The subunits of DNA polymerase α were dissociated with ethylene glycol, and separated by sedimentation through 0-10% linear glycerol gradients, in the presence of 50% ethylene glycol. The DNA polymerase and DNA primase activities of DNA polymerase α were resolved by this treatment. A 3'→5' exonuclease activity revealed by this treatment cosedimented with DNA polymerase activity. Half maximal inhibition of the 182kDa subunit by BuPdGTP and BuAdATP was observed at 7.9×10^{-8}M and 3.1×10^{-8}M respectively, indicating that the purified 182kDa subunit is as susceptible to these inhibitors as the DNA polymerase α-primase complex.

3.2 Introduction

The purification of DNA polymerase α has been attempted by numerous groups since the first attempts in the 1960s (Bollum, 1960; Ynieda and Bollum, 1965). Here, we detail the purification of DNA polymerase α from cultured murine cells by classical purification methods. These included ammonium sulfate precipitation and backwashes, ion exchange chromatography, hydroxylapatite chromatography, and sedimentation through glycerol gradients. The purpose of this work was to generate highly purified DNA polymerase α, in an efficient, reproducible manner. The purification scheme described below has been reproduced four times with equal success. From cell lysis, to the completion of the final dialysis, and storage of the purified enzyme at -70°C, the time required is 4 days.

Recently, a cryptic 3'→5' exonuclease has been discovered in the 182kDa DNA polymerase α catalytic subunit of *Drosophila melanogaster* embryos, which is only active after removal of the three other DNA polymerase α subunits (Cotterill et al., 1987c). We describe here the separation and subsequent resolution of the subunits of murine DNA polymerase α by treatment with ethylene glycol, and sedimentation through glycerol gradients. The goal of this work was to determine whether a cryptic 3'→5' exonuclease is present in DNA polymerase α from a mammalian source.

Native DNA polymerase α, that is the DNA polymerase α-primase complex is inhibited by low concentrations of both BuPdGTP and BuAdATP (Lee et al., 1985). DNA polymerase δ, the putative leading strand polymerase, is much more resistant to these analogues. DNA polymerase δ is also distinguished from DNA polymerase α by the presence of a 3'→5'

exonuclease (proofreading) function (Byrnes et al., 1976). One explanation of the differential sensitivity to the butylated nucleotides, is that the 3'→5' exonuclease activity of DNA polymerase δ is responsible for removal of incorporated BuPdGTP and BuAdATP, rendering these analogues less inhibitory to DNA synthesis (Lee et al., 1985). The final goal of this work has been to determine the sensitivity of purified DNA polymerase α catalytic subunit to BuPdGTP and BuAdATP. We have shown that unmasking of 3'→5' exonuclease activity in DNA polymerase α does not alter its sensitivity to BuPdGTP nor to BuAdATP.

3.3 Results

3.3.1 Purification of DNA polymerase α

Ehrlich ascites mouse tumour cells were grown in suspension culture, harvested daily, and frozen at -70°C. Enzyme preparations were made from 30-60g frozen cells. The cells were quick thawed, then placed on ice. Thawed cells were disrupted in a dounce homogenizer, in 3 volumes 1mM PMSF (added immediately before use) in buffer H (50mM Tris·Cl pH 7.5, 0.25M sucrose, 1mM MgCl2, 5mM KCl, 1mM 2-mercaptoethanol). The cell lysate was centrifuged in a Beckman SW28 rotor at 28 000 rpm, for 1.5 hours, generating an S-100 fraction (fraction I).

Proteins were precipitated by addition of 0.27g ammonium sulfate per millilitre of S-100. The mixture was incubated at 4°C for 30 minutes, with constant stirring. The precipitate was centrifuged in a Sorval HB-4 rotor at 10 000 rpm, for 30 minutes, at 4°C. The pellet was recovered, and resuspended in 1 S-100 volume of 0.22g $(NH_4)_2SO_4$/ml buffer A (50mM Tris·Cl pH 7.5, 5mM KCL, 0.1mM EDTA, 5mM 2-mercaptoethanol, 20% v/v glycerol), dounce homogenized, and incubated at 4°C for 30 minutes. The precipitate was collected as above, the pellet resuspended in 1 S-100 volume of 0.19g $(NH_4)_2SO_4$/ml buffer A, homogenized, incubated, and centrifuged. The 0.19g/ml pellet was resuspended in 1 S-100 volume of buffer A (fraction II), in preparation for loading onto DEAE-cellulose.

Fraction II was loaded onto a DE-52 DEAE cellulose column (Whatman), with a bed volume at least one half that of the S-100 fraction. The column was washed and eluted in a one step linear gradient; 5 column volumes of 0-0.2M NaCl in buffer B (50mM Tris·Cl pH 7.5, 5mM KCL, 0.1mM

EDTA, 5mM 2-mercaptoethanol, 30% v/v glycerol). DNA polymerase activity was eluted in a single major peak at 0.15M NaCl (Figure 5). The fractions were pooled as indicated, to generate fraction III.

Fraction III was loaded onto a P-11 phosphocellulose column (Whatman), with a bed volume at least one sixth that of the S-100 fraction. The column was washed and eluted in a one step linear gradient; 6.7-12.5 column volumes of 0.2-0.5M NaCl in buffer B. DNA polymerase activity was eluted in a major peak at 0.35M NaCl, with a trailing peak eluting at 0.4M NaCl (Figure 6). The fractions were pooled as indicated, to generate fraction IV.

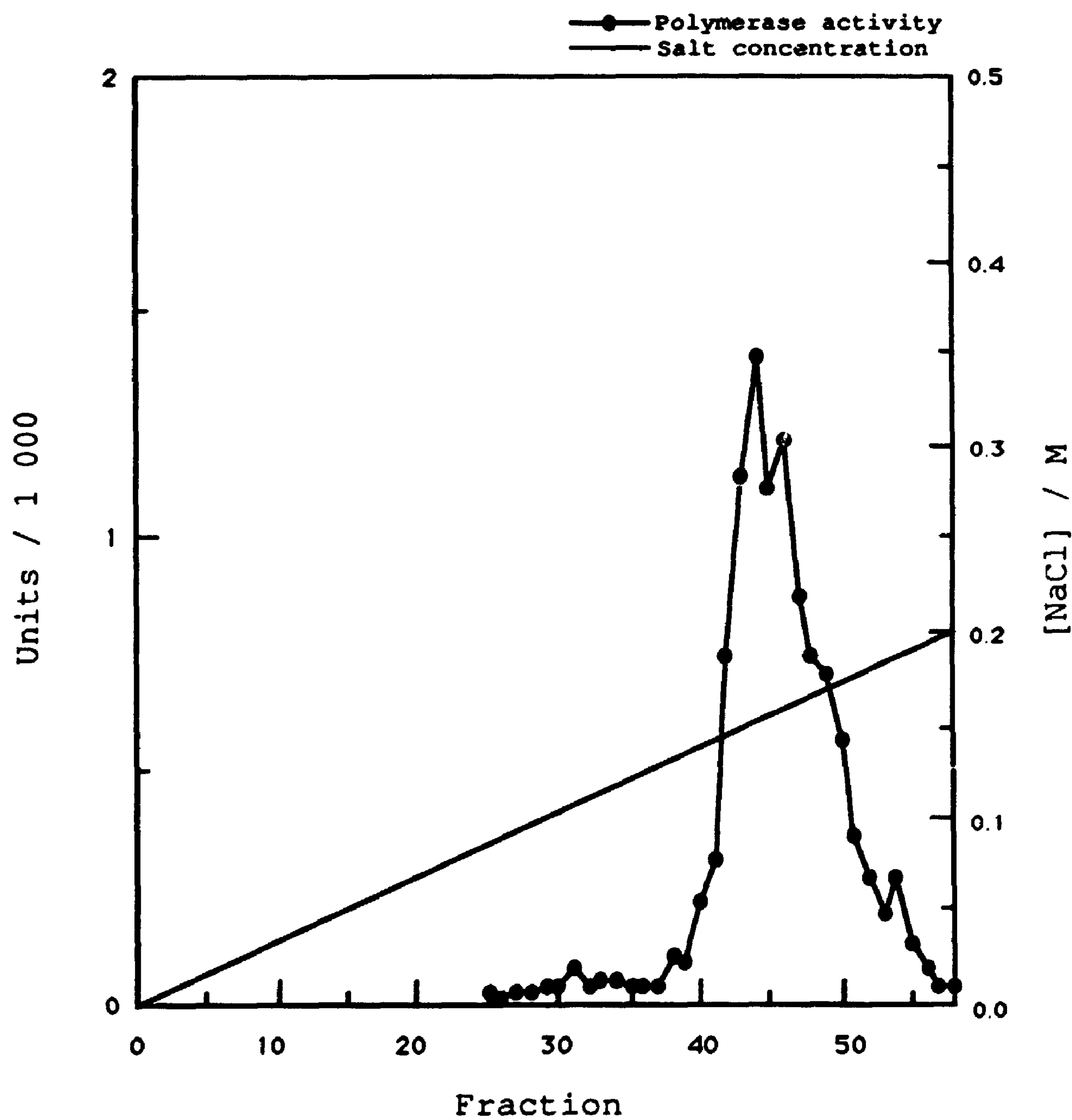
Fraction IV was loaded onto a hydroxyapatite column (Bio-Rad), with a bed volume at least one fiftieth that of the S-100 fraction. The column was washed with 5 column volumes of buffer C (30mM potassium phosphate pH 6.95, 5mM KCl, 0.5M NaCl, 0.1M EDTA, 5mM 2-mercaptoethanol, 40% v/v glycerol), and eluted with a 5 column volume linear gradient of 0.03-0.44M phosphate in buffer C. DNA polymerase activity eluted in a major peak at 0.2M phosphate (Figure 7). The fractions were pooled as indicated, to generate fraction V.

Fraction V was dialyzed against 200 volumes 0.5x buffer A, for 1.5 hours at 4°C. After dialysis, fraction V was layered onto 15-30% linear glycerol gradients (50mM Tris·Cl pH 7.5, 0.1M KCl, 0.1mM EDTA, 5mM 2-mercaptoethanol). Centrifugation was performed in a Beckman SW40 rotor at 35 000 rpm, for 40 hours, at 4°C (30g preparation), or in a Beckman SW28 rotor at 28 000 rpm, for 48 hours at 4°C. DNA polymerase activity sedimented in a single peak at about 20% v/v glycerol (Figure 8). The fractions were pooled as indicated, and dialyzed against 1 litre buffer D (50mM Tris·Cl pH 7.5, 5mM KCL, 0.1mM EDTA, 5mM

Figure 5

DEAE cellulose column profile

Fraction II (90ml) was loaded onto a DE-52 DEAE cellulose column (Whatman), with a bed volume of 150ml (30x210mm). The column was washed and eluted in a one step linear gradient; 500ml 0-0.2M NaCl in buffer B (50mM Tris·Cl pH 7.5, 5mM KCL, 0.1mM EDTA, 5mM 2-mercaptoethanol, 30% v/v glycerol). Fractions of approximately 10ml were collected and assayed for DNA polymerase activity by the standard procedure (see 2.11.1). The four peak fractions (43-46) were pooled, to generate fraction III.

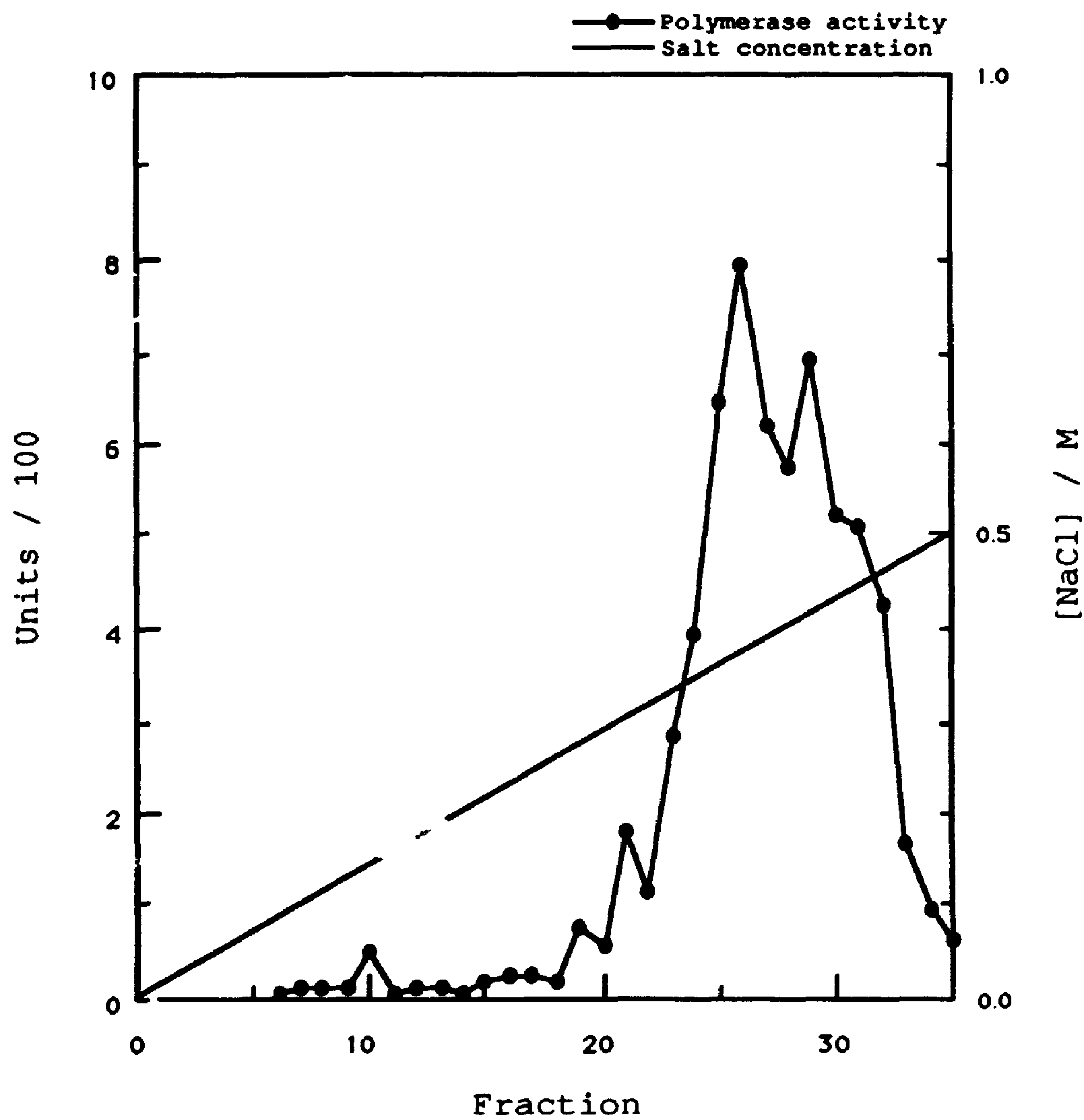


Polymerase activity
Salt concentration
Units / 1 000
[NaCl] / M
Fraction
0
1
2
0.0
0.1
0.2
0.3
0.4
0.5
10
20
30
40
50

Figure 6

Phosphocellulose column profile

Fraction III (40ml) was loaded onto a P-11 phosphocellulose column (Whatman), with a bed volume of 30ml (15x170mm). The column was washed and eluted in a one step linear gradient; 200ml 0.2-0.5M NaCl in buffer B. Fractions of approximately 5ml were collected and assayed for DNA polymerase activity by the standard procedure (see 2.11.1). Fractions 25-31 were pooled, to generate fraction IV.

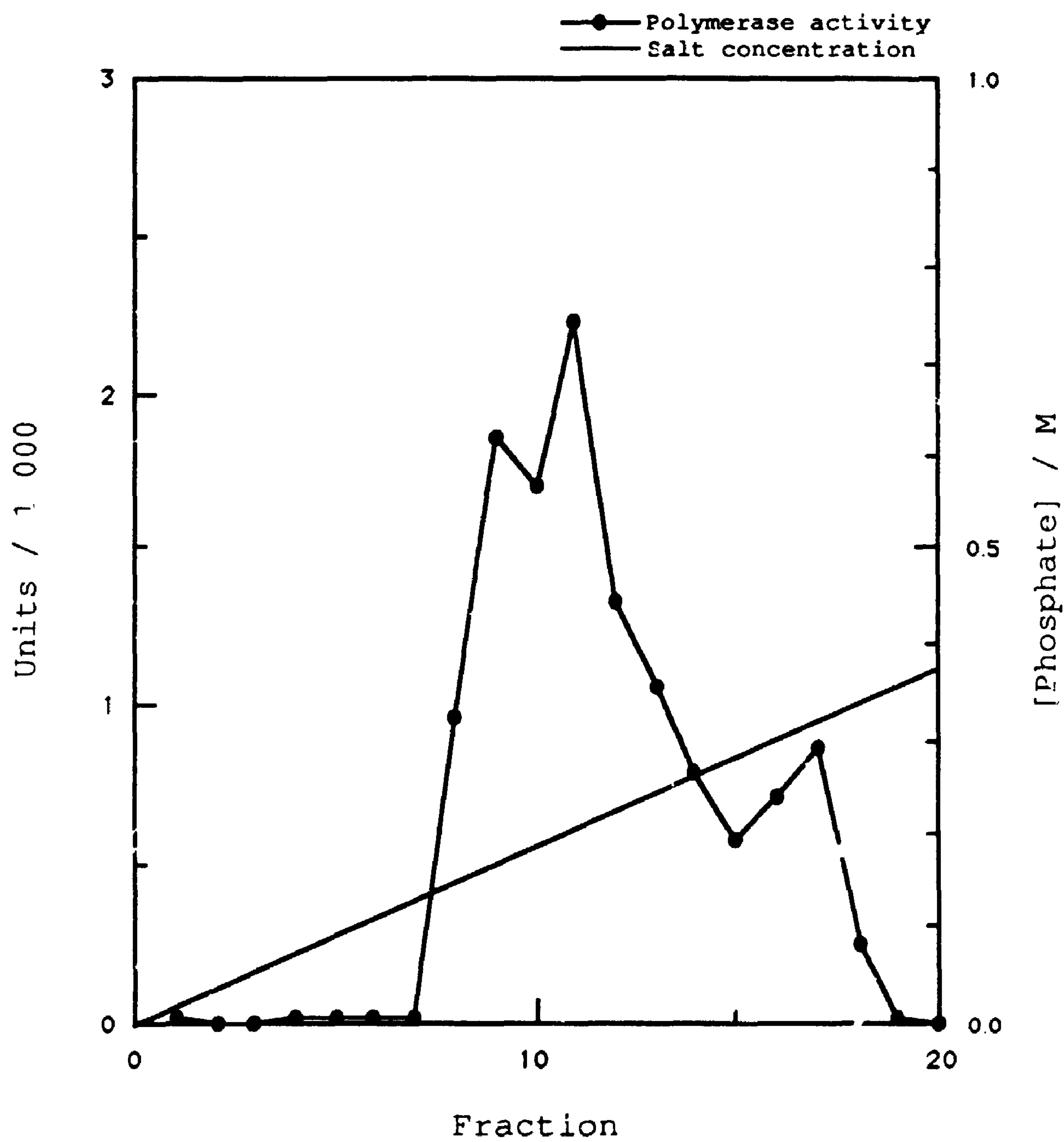


Polymerase activity
Salt concentration
Units / 100
[NaCl] / M
Fraction
10
8
6
4
2
0
1.0
0.5
0.0
0
10
20
30

Figure 7

Hydroxylapatite column profile

Fraction IV (40ml) was loaded onto a Bio-Gel HTP hydroxyapatite column (Bio-Rad), with a bed volume 4ml (8x80mm). The column was washed with 20ml buffer C (30mM potassium phosphate pH 6.95, 5mM KCl, 0.5M NaCl, 0.1M EDTA, 5mM 2-mercaptoethanol, 40% v/v glycerol), and eluted with a 10ml linear gradient of 0.03-0.44M phosphate in buffer C. Fractions of approximately 0.5ml were collected and assayed for DNA polymerase activity (see 2.11.1). Fractions 8-13 were pooled, to generate fraction V.

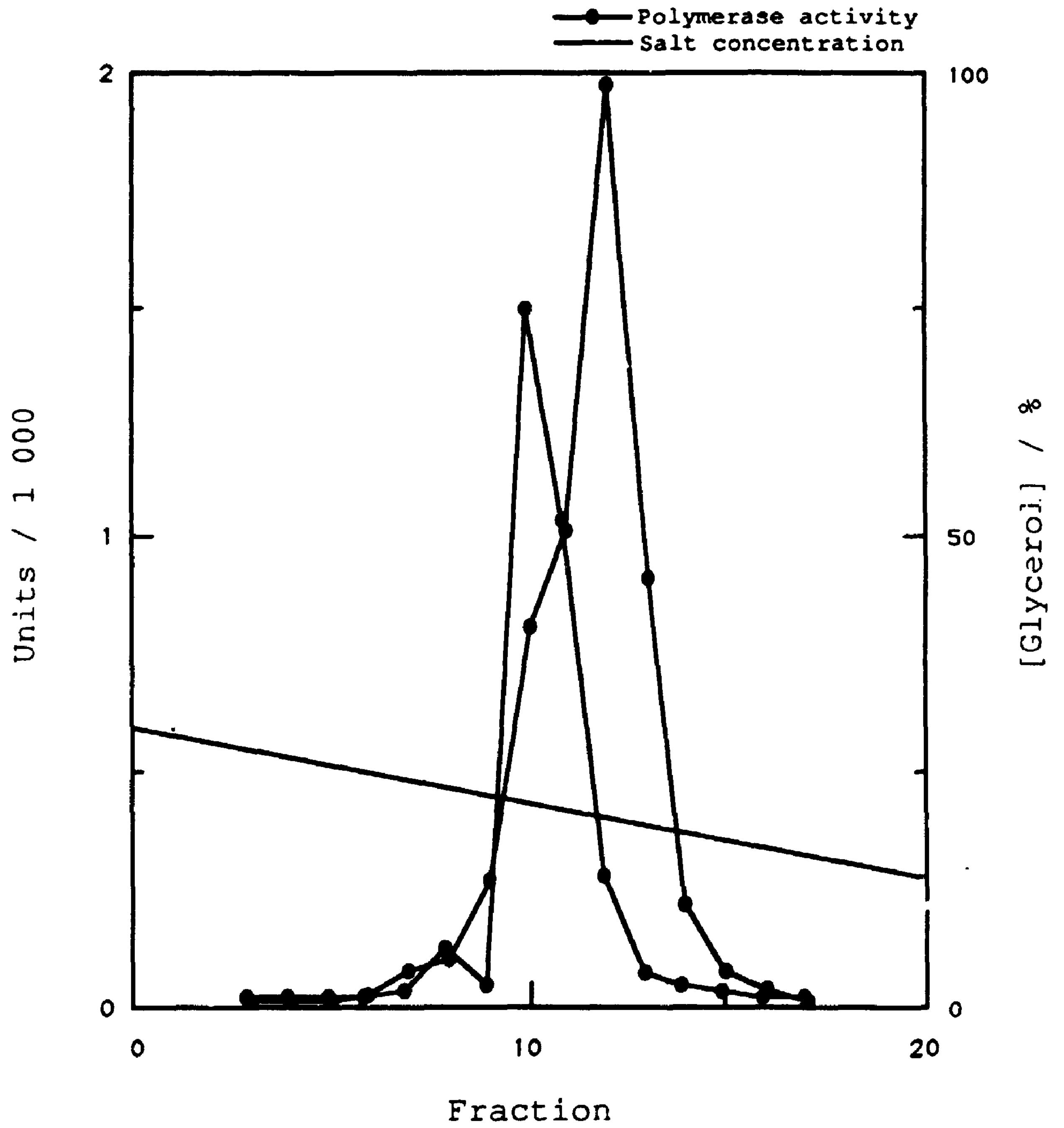


Polymerase activity
Salt concentration
Units / 1 000
[Phosphate] / M
Fraction
0
1
2
3
0
10
20
0.0
0.5
1.0

Figure 8

Glycerol gradient profile

Fraction V (3ml) was dialyzed against 1 litre 0.5x buffer A, for 1.5 hours at 4°C. After dialysis, fraction V was layered onto 15-30% linear glycerol gradients (50mM Tris·Cl pH 7.5, 0.1M KCl, 0.1mM EDTA, 5mM 2-mercaptoethanol). Centrifugation was performed in a Beckman SW28 rotor at 28 000 rpm, for 48 hours at 4°C. Fractions of approximately 2.0ml were collected from the bottom of the gradient, and assayed for DNA polymerase activity (see 2.11.1). Fractions were 10-11 (first gradient), and 11-13 (second gradient) were pooled, and dialyzed to generate fraction VI.


Polymerase activity
Salt concentration
2
1
0
100
50
0
0
10
20
Units / 1 000
[Glycerol] / %
Fraction

2-mercaptoethanol, 50% v/v glycerol) for 4 hours, with a buffer change after 2 hours. This was called fraction VI, purified DNA polymerase α, which was divided into 100μl aliquots and stored at -70°C.

The steps of purification of DNA polymerase α from a 60g cell mass, are presented in Table 4. The large loss of DNA polymerase activity through purification was due to the tight pooling required for optimal purification. Each of the six fractions were assayed for total protein content by the Bradford protein analysis method. Fraction VI did not contain sufficient protein for analysis by the Bradford method, so was analyzed by comparison to known concentrations of standard proteins in a coomassie brilliant blue stained gel. Fraction IV was quantitated both by Bradford and coomassie stained gel to ensure agreement between the two methods.

SDS polyacrylamide gel electrophoresis of the purified polymerase was performed in 6.5% acrylamide gels (Figure 9). The major bands have apparent molecular weights of 182kDa, 70kDa, 55kDa, and 47kDa. Another major component appears as a doublet, and has an apparent molecular weight of 220kDa. These polypeptides sediment away from the peak of polymerase activity during glycerol gradient centrifugation, and are not thought to be components of the polymerase complex. The 182kDa polymerase subunit has been shown to be particularly susceptible to degradation, generating fragments of 150 and 120kDa particularly. The amounts of these degradation products are somewhat variable, and increase with the length of time they are stored at -70°C. In freshly purified DNA polymerase α preparations, we have never detected the presence of catalytic subunit degraded to 120kDa, and the 150kDa subunit was less than 1/10 the concentration of the 182kDa subunit, as

Table 4

DNA polymerase α purification summary

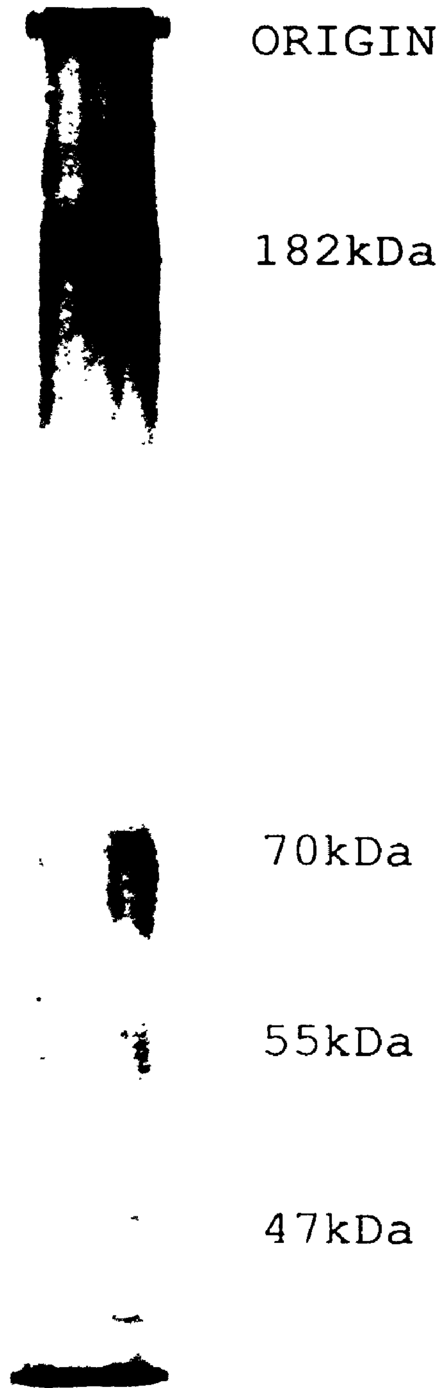
Summary of the steps of purification of DNA polymerase α from a 60g cell mass. Each of the fractions, except fractions V and VI, was assayed for total protein content by the Bradford protein analysis method. Fraction VI did not contain sufficient protein for analysis by the Bradford method, so was analyzed by comparison to known concentrations of standard proteins in a coomassie brilliant blue stained gel. Fraction IV was quantitated both by Bradford and coomassie stained gel to ensure agreement between the two methods.

Fraction	Polymerase Activity / units	Total Protein / mg	Specific Activity
S-100 (I)	14 000	13 500	1.1
Ammonium sulfate (II)	8 800	950	9.2
DEAEcellulose (III)	5 000	44	110
Phosphocellulose (IV)	2 500	2	1 200
Hydroxylapatite (V)	n/d	n/d	n/d
Glycerol Gradients (VI)	1 300	0.02	65 000

Figure 9

SDS polyacrylamide gel analysis of purified DNA polymerase α

SDS polyacrylamide gel electrophoresis of the purified polymerase was performed in 6.5% acrylamide gels (see 2.4.4). The gel was stained with silver (see 2.10.2). The major bands have apparent molecular weights of 182kDa, 70kDa, 55kDa, and 47kDa. The markers were myosin (200kDa), β-galactosidase (116kDa), phosphorylase B (97kDa), bovine serum albumin (66kDa), and ovalbumin (45kDa).


ORIGIN
182kDa
70kDa
55kDa
47kDa

judged by silver stain.

3.3.2 Ethylene glycol-glycerol gradient centrifugation

One half millilitre of DNA polymerase α fraction VI was dialyzed against 100ml 40% v/v ethylene glycol, 16mM potassium phosphate pH 7.5, 1.6mM DTT, 16mM $(NH_4)_2SO_4$, for 20 hours at 4°C. The dialyzed DNA polymerase α was then layered onto a linear 0-10% glycerol gradient (50% v/v ethylene glycol, 20mM potassium phosphate pH 7.5, 2mM DTT, 20mM $(NH_4)_2SO_4$). The gradient was centrifuged in a Beckman SW50.1 Ti rotor at 45 000 rpm for 72 hours, at 2°C. Fractions of approximately 300µl were collected from the bottom. These fractions were assayed immediately for DNA polymerase, primase, and 3'→5' exonuclease activity.

The results of DNA polymerase and primase assays performed on these fractions are presented in figure 10. Polymerase and primase activity peaks were observed in fractions 5 and 7 respectively. The polymerase peak fraction contained 2.4×10^{-1} units of DNA polymerase α activity, while the peak primase fraction contained 7.4×10^{-3} units. The trailing peak of polymerase activity may represent a small amount of dNTP incorporation catalyzed by DNA primase. There was not enough recovered material to be assayed by silver staining after SDS polyacrylamide gel electrophoresis (data not seen).

The fractions obtained after ethylene glycol-glycerol gradient centrifugation were also assayed for 3'→5' exonuclease activity. When the M13 17mer universal primer was 3' end labeled with [α-^{32}P]dGTP, and subsequently hybridized to M13mp19, all 18 nucleotides could base pair to the template. No conversion of the substrate to acid precipitable

^{32}P was detected (<5%) after a 1 hour incubation in the presence of any gradient fraction. When [α-^{32}P]dCTP was added to the 3' end, up to 35% of the ^{32}P in the sample was rendered acid soluble, by gradient fraction 5. By contrast, DNA polymerase α which had not been exposed to ethylene glycol did not lead to the production of acid soluble ^{32}P (<5%) after incubation for 1 hour in the presence of oligonucleotides labeled at the 3' end with either [α-^{32}P]dGTP or [α-^{32}P]dCTP. The relative activity of the exonuclease was determined across the ethylene glycol-glycerol gradient, and has been plotted in relation to the polymerase activity across the gradient, in figure 11. Both polymerase and exonuclease peak in fraction five. The peak of 3'→5' exonuclease activity represents 7.0×10^{-3} units.

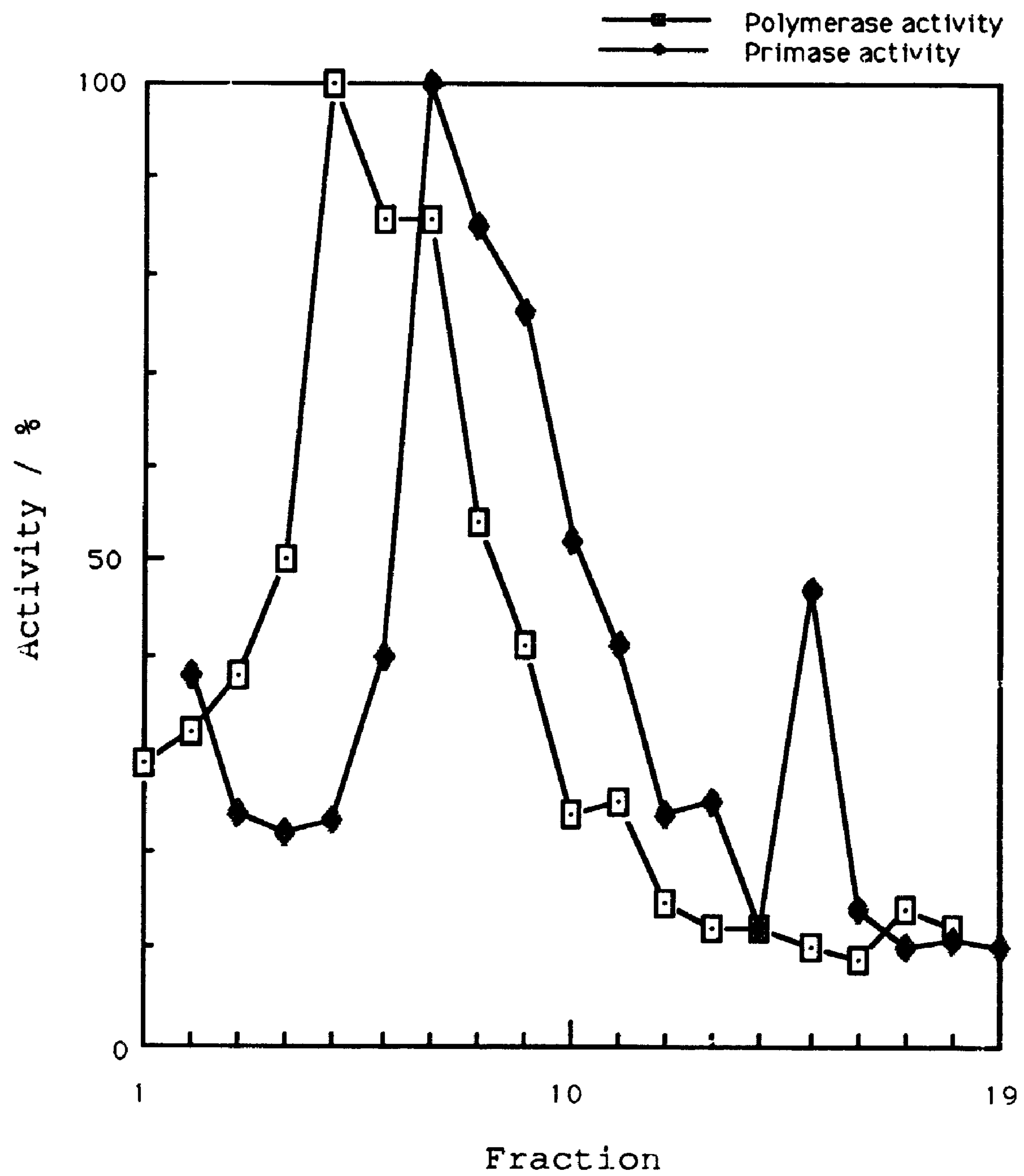
3.3.3 Inhibition of DNA polymerase activity by BuPdGTP and BuAdATP

The peak polymerase fraction obtained after ethylene glycol/glycerol gradient centrifugation was assayed for sensitivity to the inhibitors BuPdGTP and BuAdATP. Polymerase assays were performed under standard conditions, except 8×10^{-3} units of polymerase activity, 10μCi[α-^{32}P]dATP, and the indicated amount of inhibitor, were added per reaction. The inhibition of the polymerase activity by BuPdGTP and BuAdATP are summarized in Figures 12 and 13 respectively. Half maximal inhibition by BuPdGTP occurs at 7.9×10^{-8}M. Half maximal inhibition by BuAdATP occurs at 3.2×10^{-8}M. These values are similar to the inhibition of murine DNA polymerase α complex obtained in this lab (S.K.D., unpublished results), and these published for human DNA polymerase α (Lee et. al., 1985), but contrast the published K_i of human DNA polymerase δ by these compounds (Lee et al., 1985).

Figure 10

Ethylene glycol/glycerol gradient profiles: Resolution of DNA polymerase and primase activities

DNA polymerase α fraction VI (0.5ml), was dialyzed against 100ml 40% v/v ethylene glycol, 16mM potassium phosphate pH 7.5, 1.6mM DTT, 16mM $(NH_4)_2SO_4$, for 20 hours at 4°C. The dialyzed DNA polymerase α was then layered onto a linear 0-10% glycerol gradient (50% v/v ethylene glycol, 20mM potassium phosphate pH 7.5, 2mM DTT, 20mM $(NH_4)_2SO_4$). The gradient was centrifuged in a Beckman SW50.1 Ti rotor at 45 000 rpm for 72 hours, at 2°C. Fractions of approximately 300μl were collected from the bottom. These fractions were assayed immediately for DNA polymerase, primase. DNA polymerase activity was assayed as described (see 2.11.1), with 1μCi [α-^{32}P]dATP per reaction mixture. Primase activity was assayed as described (2.11.2). Activity levels wereconverted to represent the percent of maximal activity observed in each fraction. The polymerase and primase activity peaks were observed in fractions 5 and 7, and represented 2.4×10^{-1} units of DNA polymerase α activity, and 7.4×10^{-3} units primase activity.

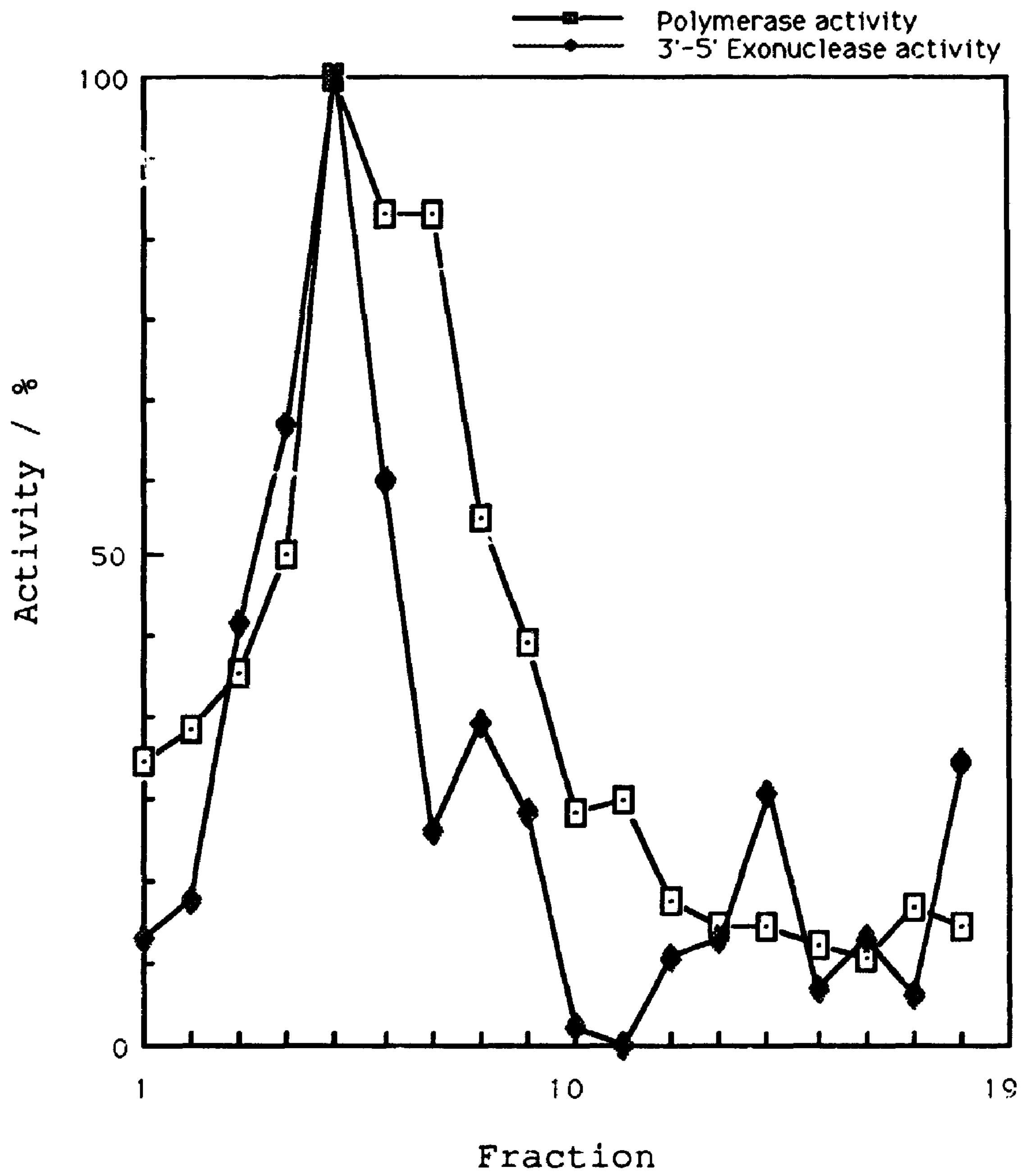


Polymerase activity
Primase activity
100
50
0
Activity / %
1
10
19
Fraction

Figure 11

Ethylene glycol/glycerol gradient profiles: Copurification of polymerase and 3'→5' exonuclease activity

DNA polymerase α fraction VI was subjected to centrifugation through glycerol gradients containing 50% ethylene glycol, as described in the legend to Figure 3.6. Fractions were collected from the bottom, and assayed for DNA polymerase (see Figure 10) and 3'→5' exonuclease (see 2.11.3) activity. The results have been converted to represent the percent of maximal activity which was observed in any fraction. The polymerase and primase activities peak in fraction 5, and represent 2.4×10^{-1} units of polymerase activity, and 7.0×10^{-3} units of 3'→5' exonuclease activity.


Polymerase activity
3'-5' Exonuclease activity
100
50
0
1
10
19
Activity / %
Fraction

2

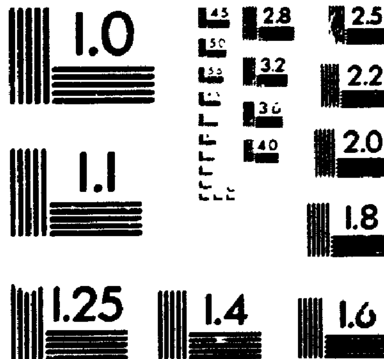


Figure 12

Inhibition of polymerase activity by BuPdGTP

The peak polymerase fraction obtained after ethylene glycol/glycerol gradient centrifugation (fraction 5, see figure 10) was assayed for sensitivity to the inhibitor BuPdGTP. Polymerase assays were performed on activated calf thymus DNA, as described (see 2.11.1), except $8x10^{-3}$ units of polymerase activity, 10μCi[α-^{32}P]dATP, and the indicated amount of inhibitor, were added per reaction. Half maximal inhibition (K_i) by BuPdGTP occurs at $7.9x10^{-8}$M. Inhibition of DNA polymerase δ determined by Lee *et al.*, 1985.

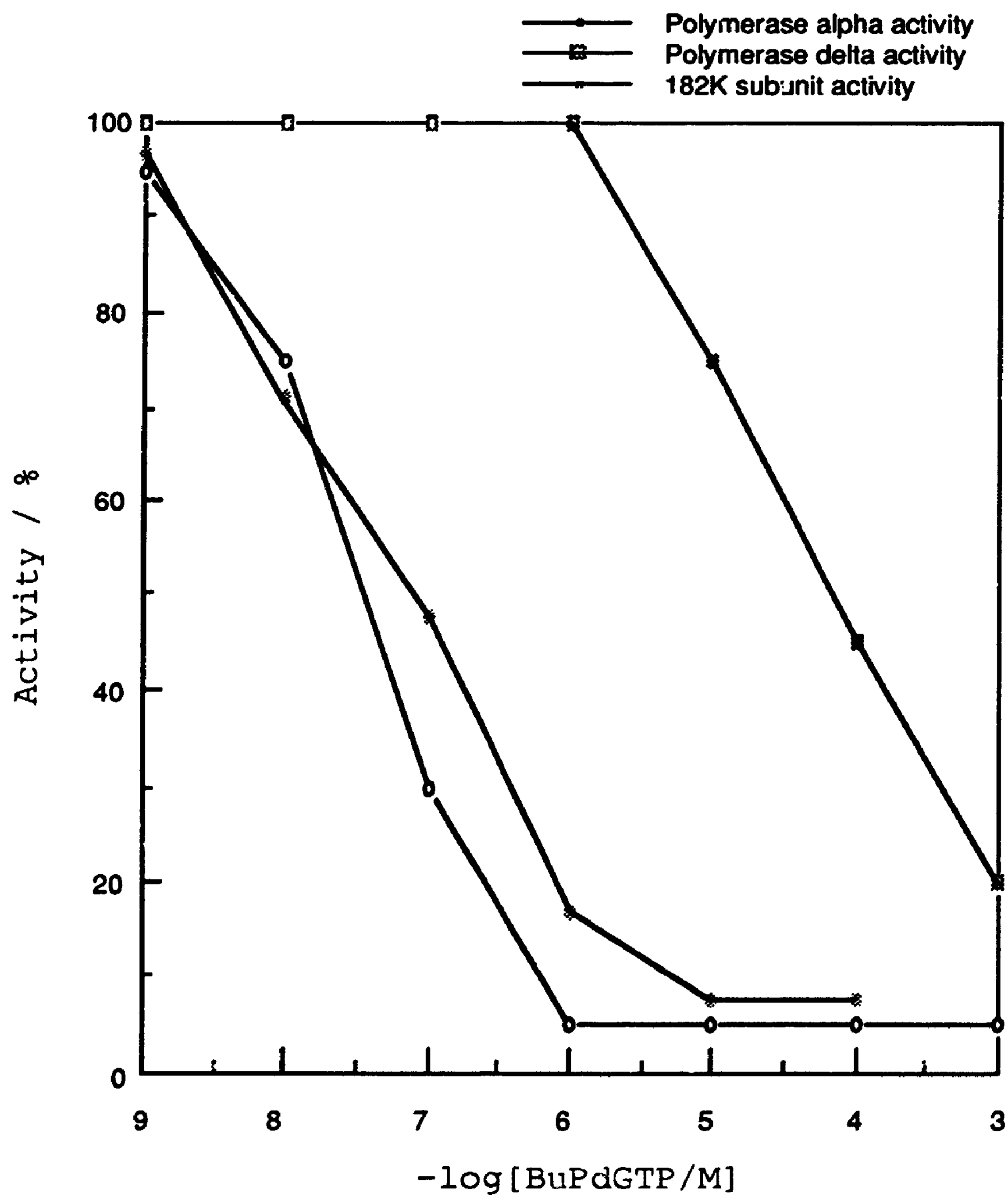
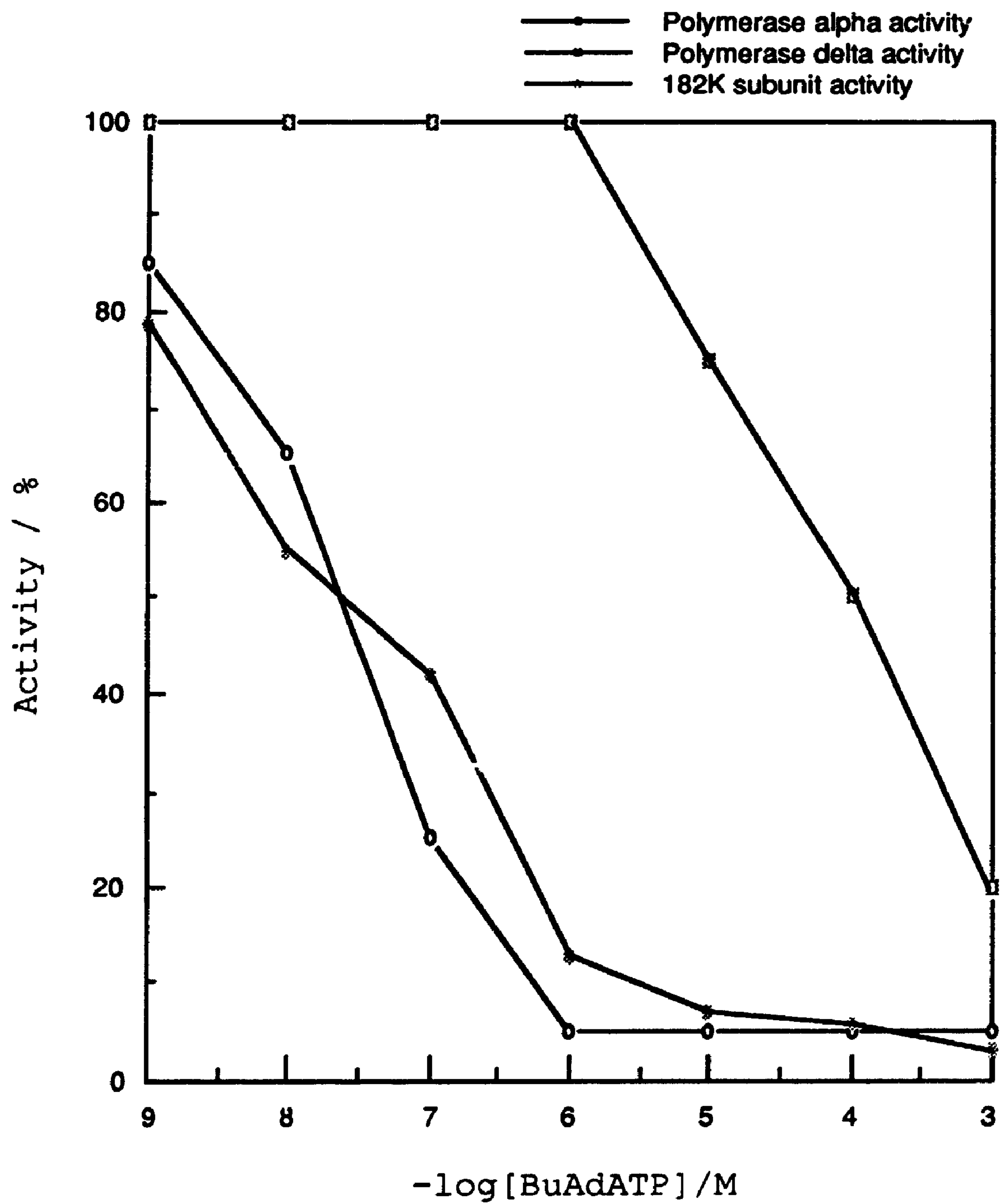

Polymerase alpha activity
Polymerase delta activity
182K subunit activity
100
80
60
40
20
0
Activity / %
9
8
7
6
5
4
3
-log[BuPdGTP/M]

Figure 13

Inhibition of polymerase activity by BuAdATP

The peak polymerase fraction obtained after ethylene glycol/glycerol gradient centrifugation (fraction 5, see figure 3.5) was assayed for sensitivity to the inhibitor BuAdATP. Polymerase assays were performed on activated calf thymus DNA, as described (see 2.11.1), except $8x10^{-3}$ units of polymerase activity, $10\mu Ci[\alpha-^{32}P]dATP$, and the indicated amount of inhibitor, were added per reaction. Half maximal inhibition (K_i) by BuAdATP occurs at $3.2x10^{-8}M$. Inhibition of DNA polymerase δ determined by Lee *et al.*, 1985.


Polymerase alpha activity
Polymerase delta activity
182K subunit activity
100
80
60
40
20
0
Activity / %
9
8
7
6
5
4
3
-log[BuAdATP]/M

3.4 Discussion

We have used a classical purification protocol to purify DNA polymerase α from cultured Ehrlich ascites mouse tumour cells. The use of these cells minimizes degradation, one of the major problems encountered with DNA polymerase α purifications (Holmes et al., 1974; Karkas et al., 1975; Fisher and Korn et al., 1977; Holmes et al., 1976; Matsukage et al., 1976; Brakel and Blumenthal, 1977; Brakel and Blumenthal, 1978; Banks et al., 1979; McKune and Holmes, 1979; Mechali et al., 1980; Villani et al., 1980; Grosse and Krauss, 1980; Hubscher et al., 1981; Filpula et al., 1982; Masakiet al., 1982; Yamaguchi et al., 1982; Wang et al., 1984; Wahl et al., 1984; Chang et al, 1984). We obtain the 182kDa catalytic subunit of DNA polymerase α without the extensive use of protease inhibitors, or the use of antibody affinity purification.

The DNA polymerase complex contains a primase activity, which copurifies with the polymerase, and is associated with the 47 and 55kDa subunits (Kaguni et al., 1983b; Murakami et al., 1986). The DNA polymerase and primase subunits can be dissociated by treatment with ethylene glycol (Suzuki et al., 1985; Yagura et al., 1986).

This work has demonstrated that treatment of a highly purified murine DNA polymerase α with ethylene glycol, followed by centrifugation through a glycerol gradient containing ethylene glycol, can lead to the unmasking of a detectable 3'→5' exonuclease activity in the preparation. This activity is dependent on the ethylene glycol treatment, and is not detectable in purified DNA polymerase α preparation which have not been exposed to ethylene glycol. We have shown by activity analysis (figure 10) that at least the DNA polymerase

and DNA primase activities were resolved by the ethylene glycol treatment. However, we have not been able to demonstrate by polyacrylamide gel electrophoresis and silver staining, that this exonuclease is dependent on the removal of the 70kDa subunit, due to insufficient recovery of protein from the gradient.

Our work contrasts with the report of a cryptic 3'→5' exonuclease in *Drosophila*, in that there is always a low level of exonuclease activity in the untreated *Drosophila* enzyme (Cotterill et al., 1987c). And while we can not rule out that our preparation of DNA polymerase α contains a low level of activity before ethylene glycol treatment, we have been unable to detect any such activity. However, in the case of the *Drosophila* enzyme, the increase in exonuclease activity on treatment with ethylene glycol is about 5 fold. If the same held true in the murine case, detection of the exonuclease activity in native DNA polymerase α would be at the limit of detection of the assay system which we have used.

We have analyzed the purified 182kDa subunit for its response to the DNA polymerase α inhibitors BuPdGTP and BuAdATP. Our results indicate that despite the presence of a 3'→5' exonuclease, inhibition by BuPdGTP and BuAdATP is essentially the same for the purified 182kDa subunit as in the DNA polymerase α complex. This result is in contrast to that reported by Reyland *et al.* (1988), in which the purified 182kDa subunit of *Drosophila melanogaster* DNA polymerase α was shown to be even more resistant to the inhibitor BuPdATP than was *D. melanogaster* DNA polymerase δ

Our goal in determining the relative inhibition of the 182kDa subunit relative to the DNA polymerase α complex was to shed light on the

possible difference between DNA polymerases α and δ. One fundamental difference between these DNA polymerases is the presence of a 3'→5' exonuclease, which may function in proofreading during DNA replication. However, this "difference" has been undermined by the finding that DNA polymerase α can possess a 3'→5' exonuclease, which is masked by its association with the 70kDa subunit (Cotterill et al., 1987c). In addition, DNA polymerase δ may contain an associated primase activity (Crute et al., 1986). The remaining differences between DNA polymerases α and δ are immunological, or involve association with other proteins, notably PCNA (Blow, 1987).

Since mutation of the proofreading ε subunit of *E. coli* DNA polymerase III can lead to an increase in the spontaneous rate of mutation by a factor of 10^5 (Degnen and Cox, 1974; Horiuchi et al., 1978; Echols et al., 1983; DiFrancesco, 1984), and considering the size of eukaryotic genomes, one would expect that a proofreading activity would be present in the eukaryotic DNA replication complex. Thus the association of a 3'→5' exonuclease activity with DNA polymerase α is not surprising.

What is somewhat more surprising is the need for more than one replicative DNA polymerase. Yet, inhibitor studies suggest that both DNA polymerases α and δ are essential for eukaryotic DNA replication *in vivo* (Hammond et al., 1987). *E. coli* DNA replication is catalyzed by DNA polymerase III, a multisubunit enzyme. The enzyme is thought to dimerize through the action of the *dnaZX* gene products (γ and τ subunits), which also creates asymmetry in the two halves of the complex (Johanson and McHenry, 1984; Hawker and McHenry, 1987; Maki and Kornberg, 1988). The use of one catalytic subunit associated with

different subunits, to generate a genuine functionally asymmetric dimer, is thus postulated in *E. coli*. In *S. cerevisiae*, there is evidence for the requirement of two distinct replicative DNA polymerases. DNA polymerase I from *S. cerevisiae* has a subunit structure virtually identical to that found in mammalian DNA polymerase α (Plevani et al., 1985). This enzyme contains a DNA primase activity (Wilson and Sugino, 1985), which has the potential to synthesize mixed oligoribo-deoxyribonucleotides in a similar manner to DNA polymerase α (Wang et al., 1984; Singh et al., 1986; Cotterill et al., 1987a). In *S. cerevisiae* DNA polymerase I is encoded by *POL1*, an essential gene (Johnson et al., 1985). DNA polymerase III from *S. cerevisiae* is immunologically distinct from DNA polymerase I (Burgers and Bauer, 1988). DNA polymerase III copurifies with a 3'→5' exonuclease activity (Bauer et al., 1988), has a subunit structure similar to calf thymus DNA polymerase II (Crute et al., 1986; Bauer et al., 1988), and interacts with PCNA in a similar way to DNA polymerase δII (Bauer and Burgers, 1988). In *S. cerevisiae* DNA polymerase III is encoded by *CDC2*, an essential gene (Sitney et al., 1989). It no longer seems premature to equate the yeast DNA polymerases I and III with higher eukaryotic DNA polymerases α and δ, respectively. It is therefore a reasonable assumption that DNA polymerases α and δ are distinct entities, at least at the DNA polymerase catalytic subunit level. What remains in question is the interaction between the two polymerases, and the possibility of both utilizing the same subunits, at least to some extent. The next piece to this puzzle should be the cloning of the gene for DNA polymerase δ, which should be facilitated by the availability of yeast DNA polymerase III clones.

Chapter 4

DNA polymerase α-primase complex initiates RNA primed DNA synthesis preferentially upstream of 3' CCC and 3' CCA (ψ) sequences

4.1 Abstract

The effect of template DNA sequence on the rate of initiation of RNA primed DNA synthesis by the purified mouse DNA polymerase α-primase complex was examined. We have observed discontinuous DNA synthesis using a minute virus of mice DNA template, which was dependent on ATP or GTP. When DNA polymerization was performed in the presence of GTP, the primers were composed of mixed oligoribo-deoxyribonucleotide chains averaging 5-7 nucleotides long, beginning with pppG residues. Initiation occured within specific priming domains, opposite template C stretches of 3 to 5 nucleotides. However, the presence of stretches of C residues in the template was insufficient to explain the site selection by primase. Two to fourteen nucleotides downstream of the priming sites were hexanucleotides conforming to the formula 3' $C_2A_{1-2}(C_{2-3}/T_2)$, which we have called ψ. To investigate the function of ψ, one ψ containing site was cloned into M13, and subjected to oligonucleotide directed mutagenesis. Mutations in the ψ domain lead to changes in the rate of RNA primed DNA synthesis, and kinetic analysis showed that these changes were due to changes in the K_m with respect to DNA concentration (from an optimal 6pM to 240pM). Only the first three nucleotides of ψ are required for ψ function, and the third nucleotide may be either an A or C residue. Thus, ψ has been redefined as a trinucleotide; 3' CC(C/A). Mutations made in the priming domain, led

to alterations in V_{max} primarily, with template pyrimidines required for priming, and initiation with ATP preferred over initiation with GTP.

4.2 Introduction

The function of a lagging strand DNA polymerase is the initiation and elongation of Okazaki fragments. DNA polymerase α has been shown to catalyze RNA primed DNA synthesis (Spadari and Weissbach, 1975). Further, purified DNA polymerase α is associated with a DNA primase activity (Yagura *et al.*, 1982; Conway and Lehman, 1982). Thus, DNA polymerase α is adapted to both initiate and elongate Okazaki fragments.

Eukaryotic Okazaki fragments are RNA primed DNA fragments of about 200-250 nucleotides (Hand, 1978), and discrete intermediate sizes of 60, 125, and 240 nucleotide Okazaki fragments have been reported (Blumenthal and Clark, 1977a/b). The basic unit of structure in chromatin is the nucleosome, which is a nucleoprotein complex containing about 200 nucleotide pairs of DNA. The similarity in the size of eukaryotic Okazaki fragments, and the length of DNA in nucleosomes has lead to the proposition that chromatin structure determines Okazaki fragment initiation (Hewish, 1976; Rosenberg, 1976). However, experiments conducted *in vitro* have shown synthesis of DNA fragments about 200 nucleotides long when DNA synthesis was performed on naked DNA from the parvovirus minute virus of mice, and the papovavirus SV40 (Faust *et al.*, 1985; Prelich and Stillman, 1988). This suggests that the information dictating Okazaki fragment length is inherent in the DNA polymerase complex and in the DNA sequence, and not directed by chromatin structure.

We have characterized the synthesis of nascent RNA primed DNA chains synthesized *in vitro* on a minute virus of mice DNA template, and have correlated RNA priming with specific priming domains which are

associated with hexanucleotide sequences conforming to the formula $C_2A_{1-2}(C_{2-3}/T_2)$, which we called ψ (Faust *et al.*, 1985). Ψ sequences were located 2-14 nucleotides downstream of AC_4TC, C_5, and several C_3 and C_4 sequences which acted as primase initiation sites, while the same sequences not associated with ψ were not active as the primase initiation sites. We have used oligonucleotide directed mutagenesis to change one priming region. These mutations have been used to analyze the kinetic effects of changes in the priming and ψ domains, as well as to determine the active portion of both the priming and the ψ domains.

4.3 Results

4.3.1 Synthesis of discontinuous MVM dsDNA

When MVM single stranded DNA was incubated with DNA polymerase α in the absence of ribonucleotides, the entire template was copied, resulting in a product which migrated as a 5kbp dsDNA on native agarose gels, and as a 10kb ssDNA on alkaline agarose gels (data not shown). The same result was observed when the pyrimidine nucleotides CTP and UTP were present during complementary strand synthesis. But when either ATP or GTP was present during DNA synthesis, discontinuous DNA synthesis was observed. The products of this reaction migrated as 5kbp dsDNA on native agarose gels, but as less than 10kb ssDNA on alkaline agarose gels (figure 14). The same fragments were observed when the samples were heat denatured, and electrophoresis performed in native agarose gels (data not shown). The major species of DNA generated from DNA synthesis in the presence of GTP migrated on alkaline agarose gels as 0.9, 1.1 (not shown), 2.0, 7.5, 7.7, and 9.0kb ssDNA fragments. Discontinuous DNA synthesis is illustrated schematically in figure 15. Products of >5kb represent the 5kb MVM ssDNA template, elongated at the 3' end, while the smaller (<5kb) species represent nascent RNA primed DNA synthesis, which extended to the 5' end of MVM DNA.

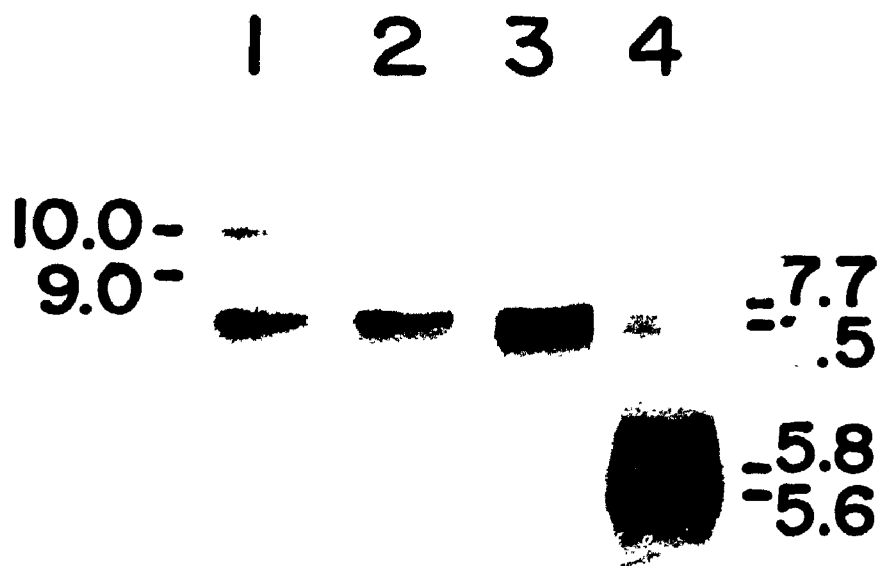
When all 4rNTPs were present during polymerization, the major products generated represented elongation of the 3' hydroxyl by multiples of about 200-300 nucleotides (figure 14, lane 4). Nascent RNA primed DNA fragments were also generated in this case, and these DNA fragments were between 200 and 2000 nucleotides long.

To map the location of the discontinuities, the complementary strand

Figure 14

Alkaline agarose gel electrophoresis of MVM ss-ds assay products

MVM ss→ds DNA assays were performed as described (2.12.1). The reactions contained 0.1, 0.2, or 1.0mM GTP (lanes 1-3), or 1.0mM each ATP/CTP/GTP/UTP (Lane 4). Electrophoresis was performed in 1% alkaline agarose gels (2.4.1). Fragments are described in the text. The 0.9 and 1.1 kb DNA fragments are not shown, but are present primarily in lane 1.

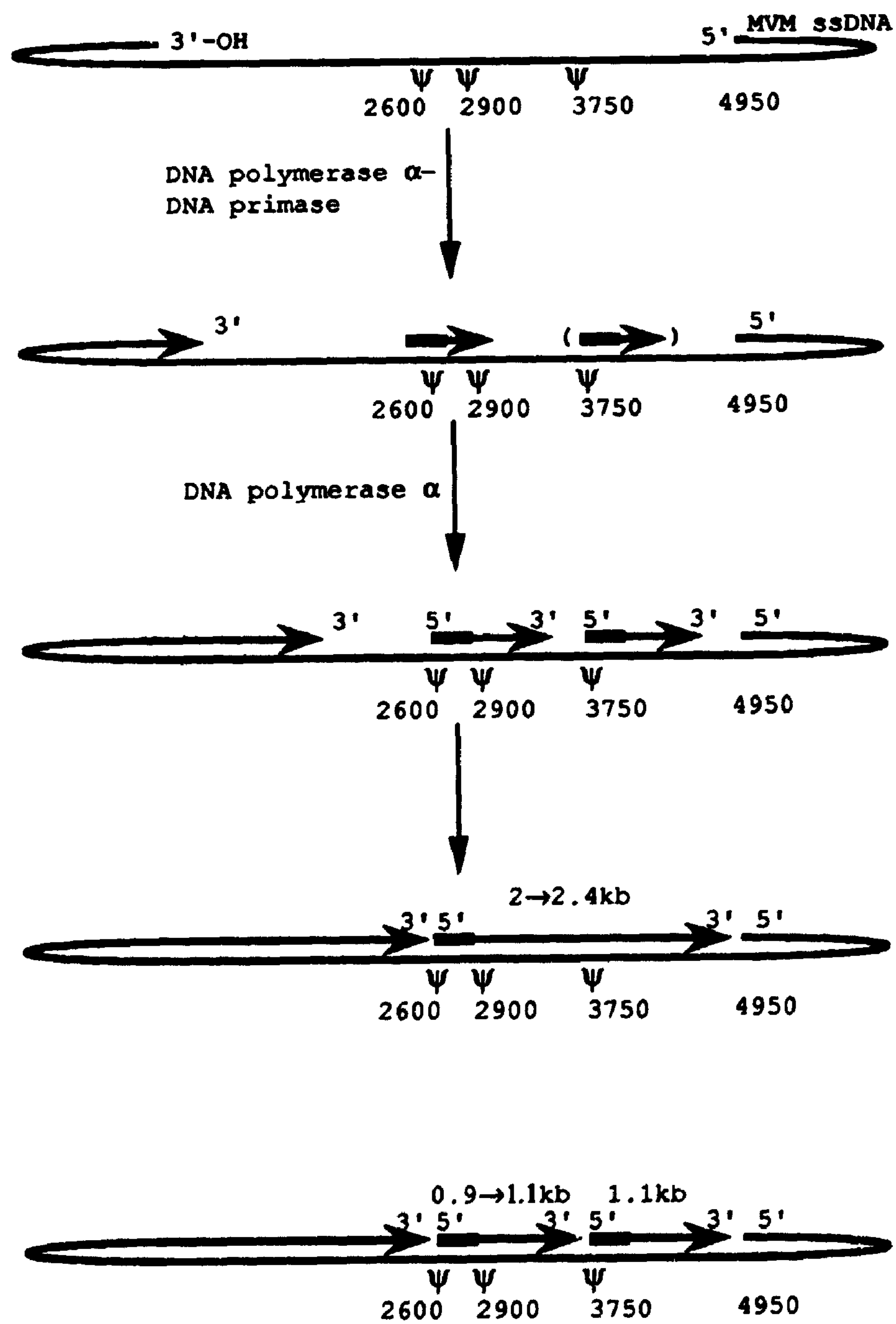


-2.1

Figure 15

Discontinuous MVM ss-dsDNA synthesis schematic

MVM ss→dsDNA synthesis is primed from the 3' hydroxyl. In the presence of GTP, priming may also occur near the major Ψ sites, located near nucleotides 2600, 2900, and 3750 (numbering according to Astell *et al.*, 1986). DNA synthesis continues, generating discontinuous 5kbp dsDNA products. Resolution of the discontinuous products reveals the fragments of 0.9, 1.1, 2.0, 7.5, and 9.0kb, as described in the text.



3'-OH
5'
MVM ssDNA
2600
2900
3750
4950
DNA polymerase α-
DNA primase
3'
5'
2600
2900
3750
4950
DNA polymerase α
3'
5'
3'
5'
3'
5'
2600
2900
3750
4950
2→2.4kb
3'
5'
3'
5'
2600
2900
3750
4950
0.9→1.1kb
1.1kb
3'
5'
3'
5'
3'
5'
2600
2900
3750
4950

was synthesized in the presence of GTP and radiolabeled dNTPs, and the 7.5-9.0kb DNA fragments were purified by alkaline sucrose gradient centrifugation. Under nondenaturing conditions, these MVM DNA species will rapidly reform partial dsDNA regions, due to the continuous nature of the parvovirus primer/template at the 3' end. The renatured species were subjected to restriction endonuclease digestion, and the products analyzed by electrophoresis in polyacrylamide gels. Fragments of labeled DNA present in the purified 7.5-9.0kb DNA, but absent in complete dsDNA, resulted from termination during complementary strand synthesis (figure 16). Single nucleotide resolution was obtained by sizing fragments less than 100 nucleotides in length. Wherever possible, enzymes with a single cleavage site were used to assign termination sites, and in all cases, location of a termination site was confirmed by corresponding fragments generated by digestion with more than one restriction endonuclease. A summary of confirmed termination site locations, and the fragments used in the analysis, are summarized in table 5.

The small nascent DNA fragments were used to map the internal initiation sites. When MVM ss→dsDNA synthesis was performed in the presence of [α-^{32}P]GTP, or [γ-^{32}P]GTP, the 0.9, 1.1, and 2.0kb ssDNA fragments became radiolabeled. MVM dsDNA synthesized in this way was subjected to restriction endonuclease digestion, and analysis by polyacrylamide gel electrophoresis (figure 17). A summary of confirmed RNA priming site locations, and the fragments used in the analysis, are listed in table 5.

The 0.9, 1.1, and 2.0kb ssDNA fragments were synthesized and purified by alkaline sucrose gradient centrifugation, in the same manner as the

7.5-9.0kb DNA fragments. The location of the 5' ends of these fragments represent the location of RNA-DNA junctions, as the RNA primer was degraded during centrifugation through alkaline sucrose gradients. The purified fragments were hybridized to MVM ssDNA, and subjected to restriction endonuclease digestion, and analysis by polyacrylamide gel electrophoresis (figure 18). A summary of confirmed RNA-DNA junction locations, and the fragments used in the analysis, are presented in table 5.

The DNA sequences near the priming regions are presented in figure 19. The sites of termination and reinitiation were found to correlate with two template sequence features. First, initiation occurred opposite a series of template C residues. This was expected, as the only ribonucleotide present during polymerization was GTP; the template must contain enough C residues to form a stable RNA primer. However, not all stretches of C in the template were used as primase initiation sites. Priming was observed at 3 of 4 C_5 stretches, 2 of 8 C_4 stretches, and 2 of 33 C_3 stretches. The second template determinant was designated ψ, for primase initiation site. These corelative studies implicated ψ, a hexanucleotide conforming to $C_2A_{1-2}(C_{2-3}/T_2)$, as the additional determining factor specifying priming. The template C stretches which were located 2-14 nucleotides upstream of ψ were subject to priming, and those that were not located near ψ were not subject to priming. There are two copies of AC_4TC in MVM, one associated with ψ, at which priming occurs, and one not, at which no priming occurs. The corelation of ψ and priming has been further considered by using oligonucleotide mutagenesis in M13.

Figure 16

Acrylamide gel electrophoresis - termination sites

Mapping of the termination sites at single nucleotide resolution. MVM DNA fragments of 7.5-9.0kb DNA were purified by sedimentation through alkaline sucrose greadients at 4°C, for 24 hours, at 36 000 RPM (see 2.2.3). The purified DNA fragments were neutralized, and cleaved with various restriction endonucleases. Electrophoresis performed in 8% denaturing polyacrylamide gels. (a) Lanes 2, 4, 6, 8, and 10; MVM RF DNA synthesized by Klenow polymerase. Lanes 1, 3, 5, 7, and 9; 7.5-9.0kb DNA synthesized in the presence of 0.1mM GTP. Restriction endonuclease digestions were performed with *Hae*III, lanes 1 and 2; *Mbo*I, lanes 3 and 4; *Bgl*I, lanes 5 and 6; *Pvu*II, lanes 7 and 8; *Alu*I, lanes 9 and 10. Even numbered lanes, and the DNA sequencing ladder (lane 11) served as size markers on the gel. (b) Lanes 1, and 3; MVM RF DNA synthesized by Klenow polymerase. Lanes 2, and 4; 7.5-9.0kb DNA synthesized in the presence of 1.0mM GTP. Restriction endonuclease digestions were performed with *Hae*II, lanes 1 and 2; *Alu*I, lanes 3 and 4. Lane 3 and the DNA sequencing ladder (lane 5) served as size markers on the gel.

Arrows indicate the major rGTP dependent termination sites.

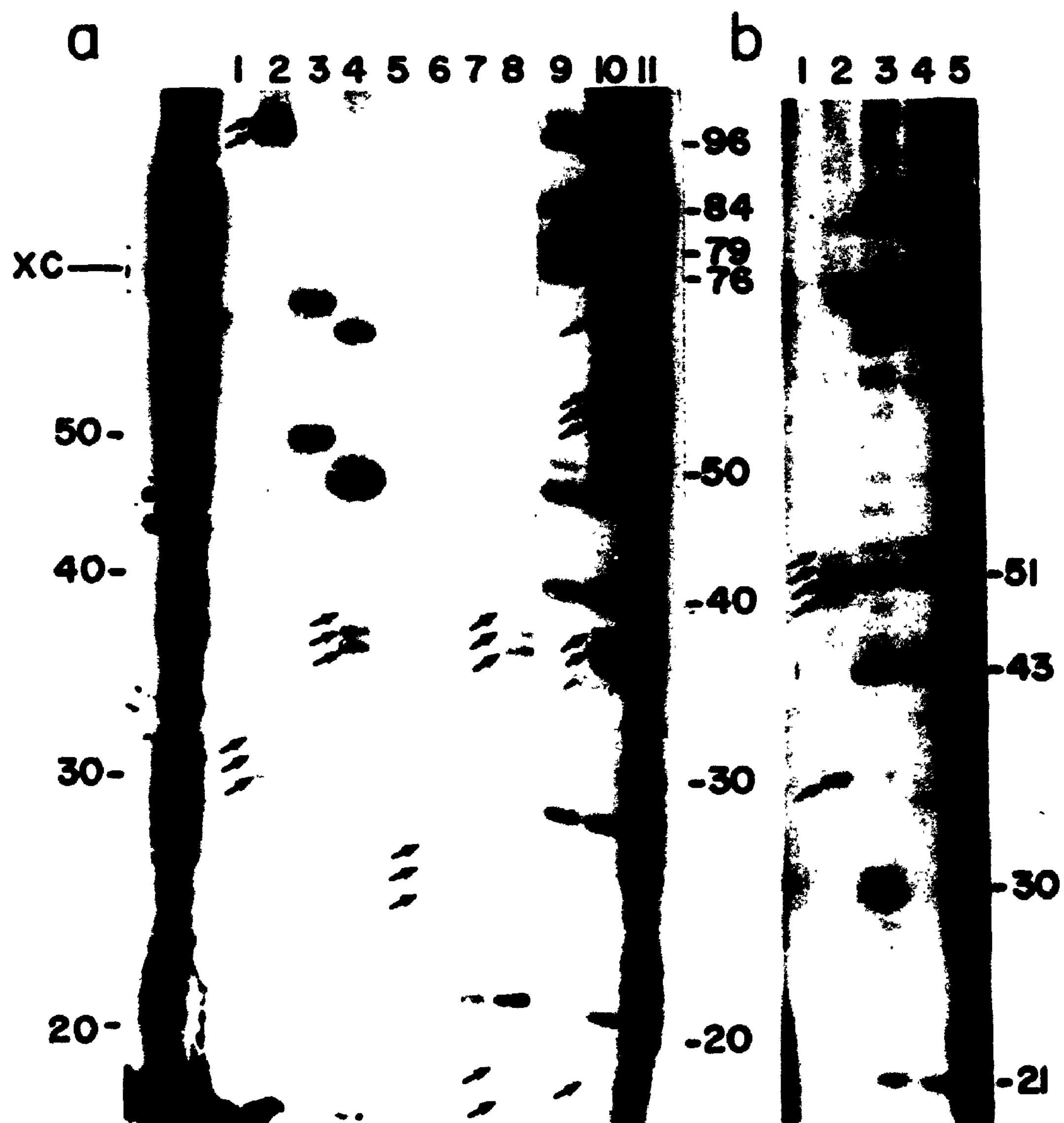
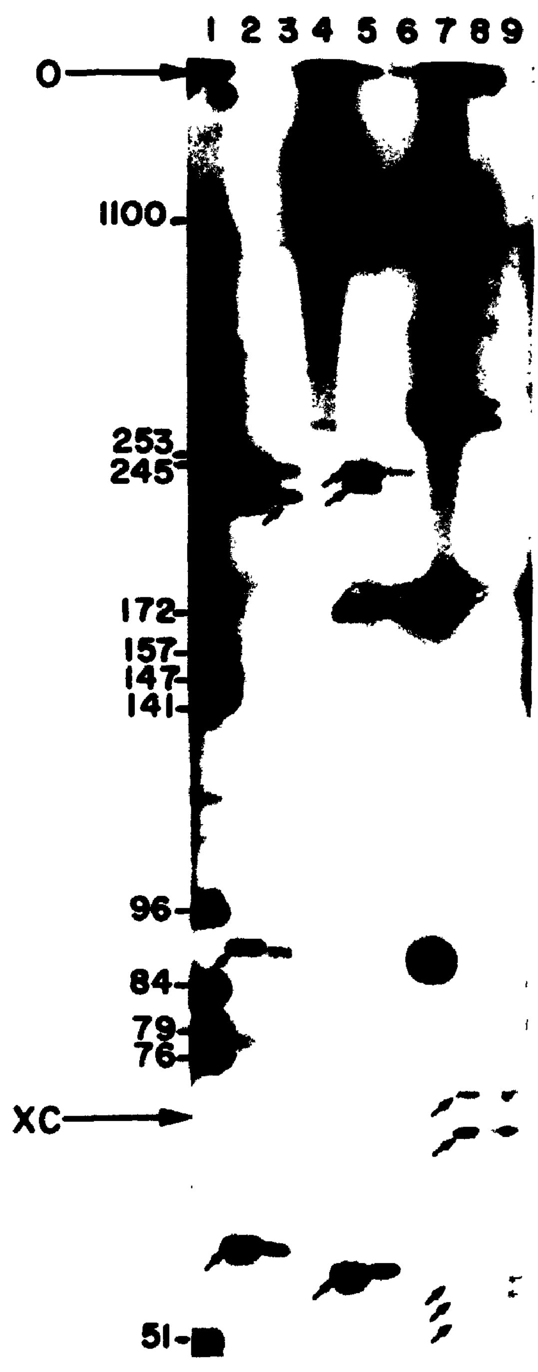

a
1 2 3 4 5 6 7 8 9 10 11
XC
50
40
30
20
96
84
79
76
50
40
30
20
b
1 2 3 4 5
51
43
30
21

Figure 17

Mapping of the primase initiation sites at single nucleotide resolution

DNA was labeled with either [α-^{32}P]GTP or [γ-^{32}P]GTP, and were cleaved with various restriction endonucleases. Electrophoresis was performed in 8% denaturing polyacrylamide gels. (a) Lanes 1, 4, 7; MVM RF DNA synthesized with Klenow polymerase. Lanes 2, 5, 8; MVM ds DNA labeled with [α-^{32}P]GTP. Lanes 3, 6, 9; MVM ds DNA labeled with [γ-^{32}P]GTP. Restriction endonuclease digestions were performed with *Alu*I, lanes 1-3; *Hin*dIII, lanes 4-6; *Hin*fI, lanes 7-9. Lanes 1, 4, and 7 served as size markers. Lanes 1, 4, 7, and a DNA sequencing ladder (not shown) served as size markers. (b) DNA labeled with [α-^{32}P]GTP was digested with various restriction endonucleases, denatured, then treated with tobacco acid pyrophosphatase. Lanes 1, 2, and 4, respectively; *Hin*dIII, *Hin*fI, and *Alu*I digestions of [α-^{32}P]GTP labeled DNA. Lane 3, DNA chain length markers. DNA fragment sized were determined using MVM DNA digested with the restriction endonuclease *Alu*I, and the sequencing ladder in lane 3.

Arrows indicate the major rGTP dependent primase initiation sites.


1 2 3 4 5 6 7 8 9
O
1100
253
245
172
157
147
141
96
84
79
76
XC
51

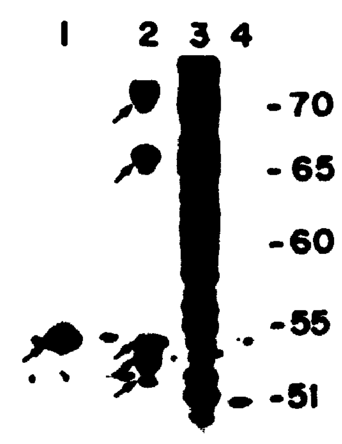

1 2 3 4
-70
-65
-60
-55
-51

Figure 18

Mapping of RNA-DNA junctions at single nucleotide resolution

The 2.1kb labeled DNA fragment was purified by successive rounds of alkaline sucrose gradient centrifugation, at 4°C, for 24 hours, at 36 000 RPM, and for 48 hours at 36 000 RPM (see 2.2.3). The purified DNA was neutralized, and hybridized to excess unlabeled MVM DNA (3.8µg/ml) at 68°C for 5 hours in 100µl of 0.3M NaOAc, 50mM Tris·Cl pH7.5. The hybrid DNA was digested with restriction endonucleases, and electrophoresis was performed in 8% denaturing polyacrylamide gels. Lanes 1,3,5,7, MVM RF DNA synthesized by Klenow polymerase; lanes 2, 4, 6, 8, 2.1kb hybrid DNA. Samples were digested with *Alu*I (lanes 1 and 2), *Hind*III (lanes 3 and 4), *Hinf*I (lanes 5 and 6), and *Mbo*I (lanes 7 and 8). Odd numbered lanes, including the DNA sequencing ladder (Lane 9) were used as size markers. Fragment sizes are given in nucleotides. Arrows indicate the major RNA-DNA junction sites.

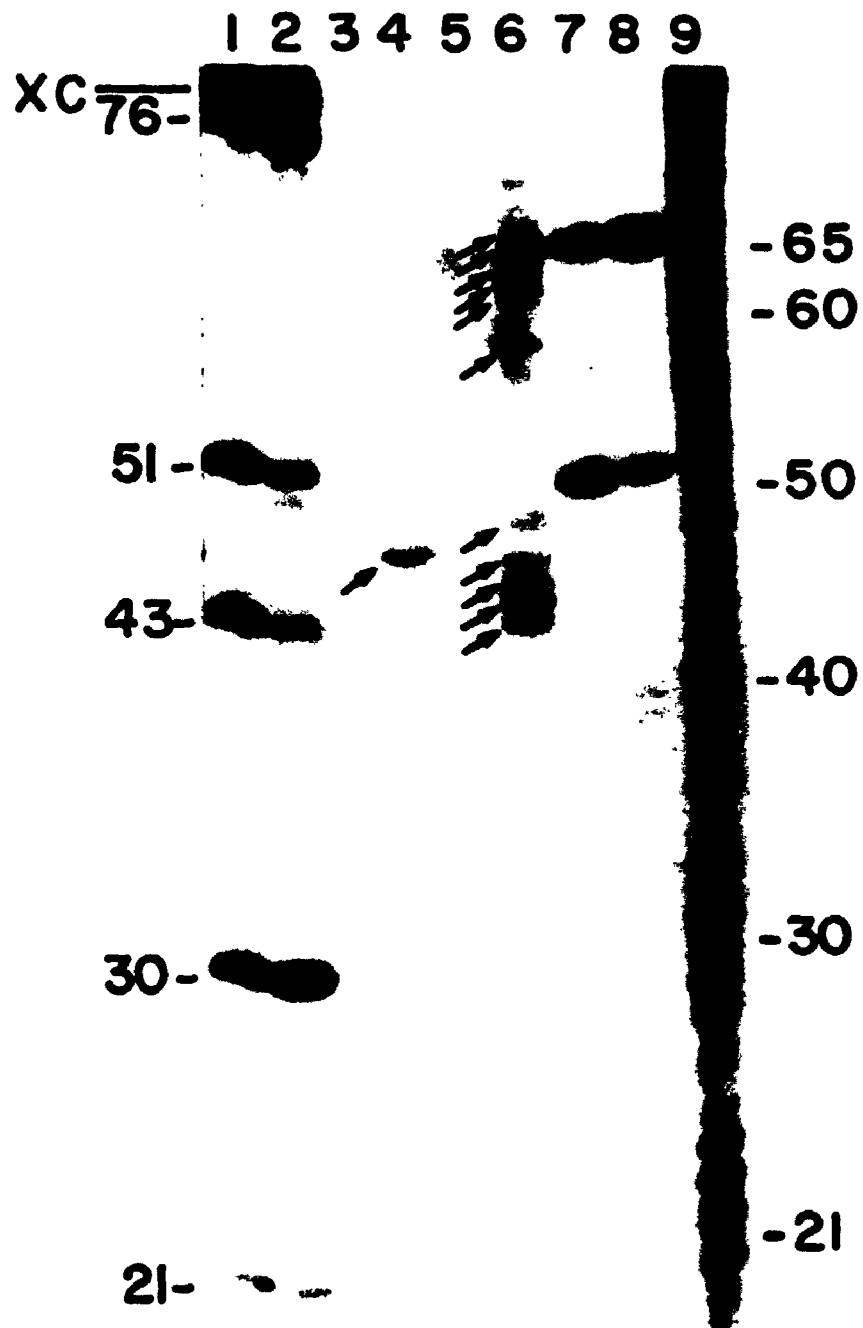

1 2 3 4 5 6 7 8 9
XC
76-
51-
43-
30-
21-
-65
-60
-50
-40
-30
-21

Table 5

Summary of termination, initiation, and RNA-DNA junction sites

Restriction enzyme	Cleavage site*	Fragment size(s)	Site on DNA
		Termination sites	
*Bgl*I	2876	25,26,27	2901,2902,2903
*Pvu*II	2863	17,18,19	2880,2881,2882
		37,38,39	2901,2902,2903
*Hae*III	2870	30,31,32	2901,2902,2903
		94,95	2594,2595
*Mbo*I	2556	37,38,39	2593,2594,2595
*Alu*I	3691	58,59,60	3749,3750,3751
	2863	17,18,19	2880,2881,2882
	2863	37,38,39	2901,2902,2903
*Hae*II	2292	37	2329
	2292	50,51,52,53	2342,2343,2344,2345
		Initiation sites	
*Hin*dIII	2650	54,53	2597,2598
	3993	245†,235†	3751,3761
*Alu*I	2652	56,55	2597,2598
	3838	88,78	3751,3761
	3128	245†,230†	2884,2989
*Hin*fI	2954	54,53,52	2901,2902,2903
	2954	71	2884
	2662	67	2597
		RNA-DNA junctions	
*Hin*dIII	2650	48,47	2603,2604
*Hin*fI	2662	59	2604
	2954	66,64,63,61,60	2888,2890,2891,2893,2894
	2954	48,46,44,43	2906,2908,2910,2911
*Alu*I	2652	50,49	2603,2604

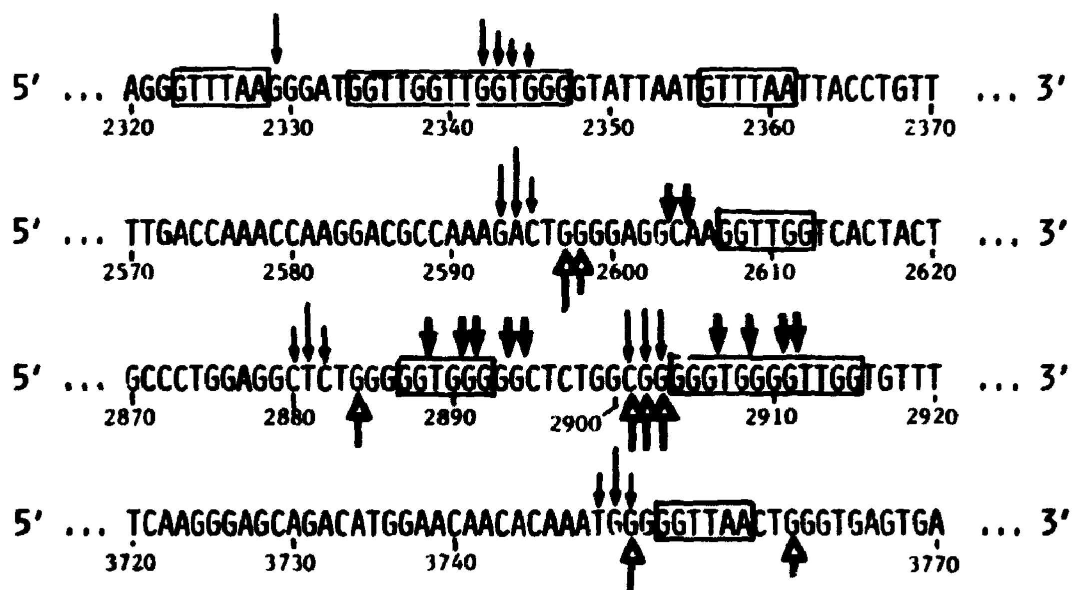
*Cleavage occurs between the nucleotide shown and the one following it.

†Fragment size approximate

Figure 19

Summary of sequence and sites

Summary of RNA priming sites (↑), RNA-DNA junctions (↓), and upstream termination sites (↓). The sequences from which the original definition of ψ was derived are boxed. Nucleotides are identified by the numbering scheme reported by Astell *et al.* (1983).


5' ... AGGGTTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTACCTGTT ... 3'
2320
2330
2340
2350
2360
2370
5' ... TTGACCAAACCAAGGACGCCAAAGACTGGGGAGGCAAGGTTGGTCACTACT ... 3'
2570
2580
2590
2600
2610
2620
5' ... GCCCTGGAGGCTCTGGGGGTGGGGGCTCTGGCGGGGGTGGGGTTGGTGTTT ... 3'
2870
2880
2890
2900
2910
2920
5' ... TCAAGGGAGCAGACATGGAACAACACAAATGGGGGTTAACTGGGTGAGTGA ... 3'
3720
3730
3740
3770

4.3.2 Oligonucleotide directed mutagenesis

The priming site around nucleotide 2600 of MVM was chosen for further study for several reasons. This site could be cloned individually,away from other ψ sites. There is only one copy of ψ at this site, and the ψ sequence at this site is the prototype sequence. Also, the priming and ψ domains are well resolved at this site.

The region of MVM DNA containing ψ 2600 was cloned into M13mp19. The two clones used during mutagenesis were M13mp19·94, and M13mp19·154, the construction of which is described in section 2.5. Mutations have been targeted at several regions of this site, including the nucleotides immediately upstream of the priming domain, the first two nucleotides of the priming domain, within the ψ domain, and into the region between the priming and ψ domains (figure 20). All mutations were introduced by oligonucleotide directed mutagenesis, as described in section 2.6.

The first two mutations to be studied were deletions of ψ, one deleting the six nucleotides of ψ, and one deleting the central two A residues (Δ6 and Δ2, respectively). These mutations were the most direct way to test whether the presence of ψ near primase sites was important for priming, or just coincidental. Mutagenic oligonucleotide 3 changed the two nucleotides immediately upstream of the major priming site to all four possible configurations. This mutation was made to test the influences of nucleotides upstream of the primase domain on priming kinetics. Mutagenic oligonucleotide 4 inserted four nucleotides between the priming domain and ψ, imediately upstream of ψ. The upstream two nucleotides to be inserted were degenerate, allowing incorporation of all four nucleotides at both positions. The

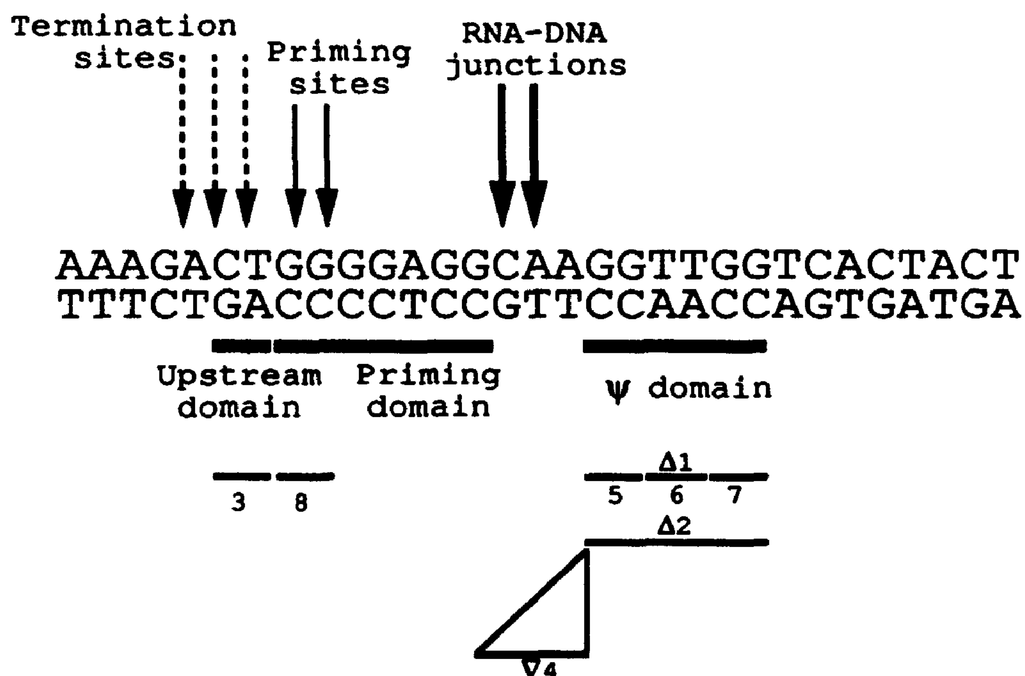
downstream two oligonucleotides inserted two A residues. One of the sixteen possible mutations that this oligonucleotide produced, was a regeneration of ψ, in the exact position which it occupied before the insertion. This internal control was beneficial for testing the spacing requirements between the priming and ψ domains. Mutagenic oligonucleotides 5, 6, and 7, changed the first two, middle two, and final two nucleotides of the ψ sequence, in each case allowing the generation of 16 combinations at the two positions. These mutations were designed to generate a set of point mutations which could be used to define the active sequence within ψ. Mutagenic oligonucleotide 8 changed the first two nucleotides of the priming domain, at which the two main priming events occur, to all 16 possible co[illegible]nations. This oligonucleotide was designed to give information on [illegible]e sequence specificity of the RNA priming event.

Mutations which have been characterized are related to sequence changes by reference to the oligonucleotide used to generate the mutation, and the template sequence of the DNA at the site of mutation. For example, /5.CA would represent a change in the first two nucleotides of ψ from CC to CA. In this case, only the second nucleotide of ψ has actually been altered. In the case of mutations generated by oligonucleotide 4, the template sequence of the two degenerate nucleotides are given. Thus, /4.CC indicates that the insertion has generated a template which regenerates ψ at the same location as prior to the insertion, moving the original ψ sequence four nucleotides downstream, giving CCAACCAACC in the template.

Figure 20

Summary of mutations

DNA sequence and target regions for oligonucleotide mutagenesis at ψ_{2600}. Mutations and rational are described in the text (4.3.2). Also shown are the locations of RNA priming, RNA-DNA junctions, and upstream terminations. The complementary (nascent) strand is written 5'→3', and is positioned above the viral (template) strand.



Summary of mutations:

1. Two nucleotide deletion within ψ.
2. Six nucleotide deletion; all of ψ.
3. Two nucleotide point mutations in the upstream domain.
4. Four nucleotide insertion between the priming and ψ domains. The first two nucleotides generate point mutations.
5. Point mutations in the first and second nuclotides of the ψ domain.
6. Point mutations in the third and fourth nucleotides of the ψ domain.
7. Point mutations in the fifth and sixth nucleotides of the ψ domain.
8. Point mutations in the first and second nucleotides of the priming domain.

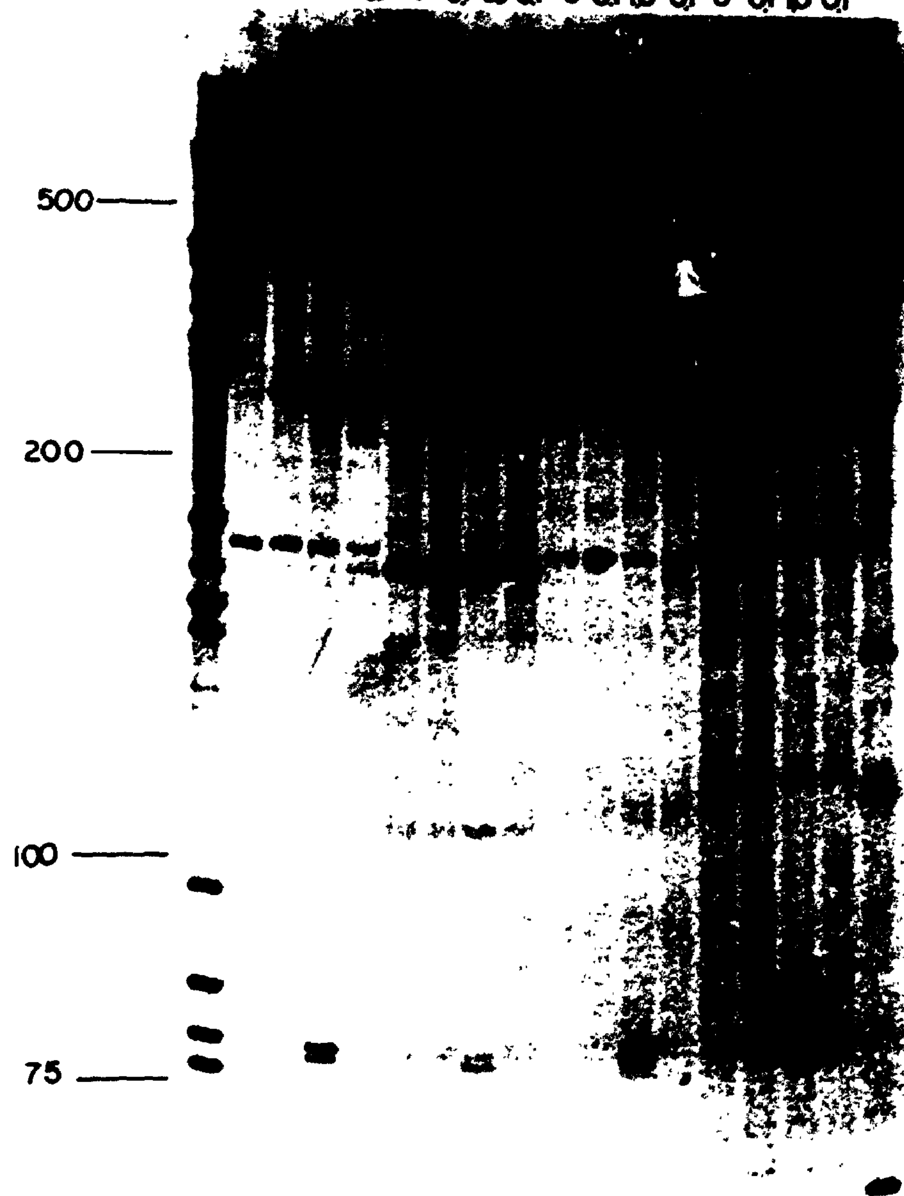
4.3.3 Termination Assay

The first two mutations generated in M13 were derived from M13mp19·94, and were deletions of the internal two A residues of ψ, and of all six nucleotides of ψ. These mutations, as well as a fortuitous mutation generated during oligonucleotide directed mutagenesis, and the wild type, were analyzed for priming by a method similar to that used to detect priming in MVM. Both DNA synthesis and RNA priming were allowed to proceed simultaneously, with DNA synthesis primed upstream of the putative primase site, by the M13 17mer universal primer. If priming occured, DNA synthesis initiated upstream of the priming event would terminate on reaching the downstream double stranded region, since DNA polymerase α catalyzes only limited strand displacement (see chapter 5). If no priming had occurred, DNA synthesis would continue through the priming region. The elongation of the upstream primer was easily followed by radiolabeling the 5' end of the upstream primer, and determination of its length following DNA synthesis. Figure 21 illustrates the results of this assay performed on four DNA substrates. In all cases, the reaction was dependent upon the presence of 1.0mM GTP. Priming occurred at the priming site in 1% of the wild type DNA sequences, as measured by densitometry. The six nucleotide deletion removing ψ lead to a five fold reduction in the amount of priming relative to the wild type sequence. The two nucleotide deletion removing the central two A residues of ψ resulted in a two fold increase in the amount of priming observed. The fourth mutation led to a seven fold increase in the amount of priming observed. This mutation deleted the ψ sequence, but resulted in a duplication of the priming domain. We initially thought that the duplication of the

Figure 21

Termination of DNA primed DNA synthesis in M13

M13 17mer universal primer was 5' end labeled to high specific activity (see 2.3.2), and sequence dependent termination assays were performed as described, using M13mp19·94 w.t., Δ2, and Δ6 (2.12.2). Products were analyzed after electrophoresis in 8% denaturing polyacrylamide gel. Each lane contained 30ng of the specified DNA, which was singly primed with the M13 17mer universal primer. Ribonucleotides (G, GTP; or 4, each of ATP/CTP/GTP/UTP) were present at the concentrations indicated. If RNA priming occured near Ψ, the M13 17mer would terminate synthesis after 78-79 nucleotides. Fragments of this size are present in lanes 4, 8, 12, and 16.


LANE
DNA
rNTP
[rNTP](mM)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
94 W.T.
94Δ6
94Δ2
94Δ6var
- G G 4 - G G 4 - G G 4 - G G 4
0 0.1 1.0 0.1 0 0.1 1.0 0.1 0 0.1 1.0 0.1 0 0.1 1.0 0.1
500
200
100
75

priming domain alone was responsible for the high level of priming observed at this site, but as will become apparent, this duplication also resulted in the regeneration of a ψ sequence.

This method confirmed the general applicability of this method to show RNA primed DNA synthesis using a coupled assay. It also was the first direct implication of ψ as a functional determinant of priming activity, albeit not an absolute requirement. While the coupled assay would allow determination of the sequence requirements for ψ function, a direct method of measuring the rate of RNA primed DNA synthesis was required to determine the kinetic mode of action of ψ.

4.3.4 Sequence dependent priming assay

Single stranded DNA fragments containing mutagenized priming and ψ domains could be purified in large quantities from M13. However, use of the entire M13 genome would inevitably lead to additional interfering primase sites. We adopted the method described in section 2.12.3 to purify ssDNA fragments of about 160 nucleotides containing the primase site near nucleotide 2600 of MVM. Basically, the region of M13mp19·154 containing the cloned ψ sequence was made double stranded by elongation of the M13 17mer universal primer through the cloned DNA region. The DNA was digested with *Hin*dIII and *Kpn*I, which cut on either side of the cloned DNA, generating overhanging DNA on the template DNA strand at both ends. Thus the template DNA strand is 8 nucleotides longer than the nascent DNA strand, and the fragments may be resolved by denaturing polyacrylamide gel electrophoresis. The resolution of these fragments prepared from the wild type and from several mutants is illustrated in figure 22. The purity and integrity

of the purified primase fragments were confirmed by 5' end labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase. No degraded fragments (>20 nucleotides) were detected, and the smaller nascent DNA strand represented less than 0.5% of the total DNA (figure 23; densitometry not shown).

The primase assay is described in detail in section 2.12.3. Priming at ψ in the wild type molecule, followed by elongation to the end of the DNA molecule resulted in a 58 nucleotide RNA primed DNA fragment. This size was somewhat smaller in the deletion mutants, and larger in the insertion mutants, as all deletions and insertions were made between the priming site and the 5' end of the template DNA molecule. Figure 24 illustrates the products of priming reactions performed on the wild type, and the two deletion mutants. A limited amount of products longer than the template DNA molecule were observed. These were the result of transient pairing of the template 3' end with the template molecule, followed by limited polymerization. This was reduced by blocking the 3' ends of the template by the action of terminal deoxynucleotidyl transferase, using dideoxy nucleotides as substrates. However, this reaction was not efficient enough to block all 3' ends, and entirely eliminate a background level of synthesis.

Initial velocities of RNA primed DNA synthesis were determined for the wild type and severa' ısses of mutant priming and ψ sites. Initial velocities were compared to substrate concentration, and least squares analysis was applied to double reciprocal plots of the data. Sequence alteration was correlated with changes in the kinetics of RNA primed DNA synthesis, and the observations grouped into several classes. We will consider the basic classes of mutations separately

Figure 22

Purification of primase fragments

Primase fragments were prepared as described in section 2.12.3, and electrophoresis was performed in 8% denaturing polyacrylamide gels. All DNA samples were derived from M13mp19·154. Lane 1, w.t.; lane 2, Δ2; lane 3, Δ6; lane 4, /4.CA; lane 5, /4.TC; lane 6, /3.GC; lane 7, /7.CG; lane 8, /8.TC.

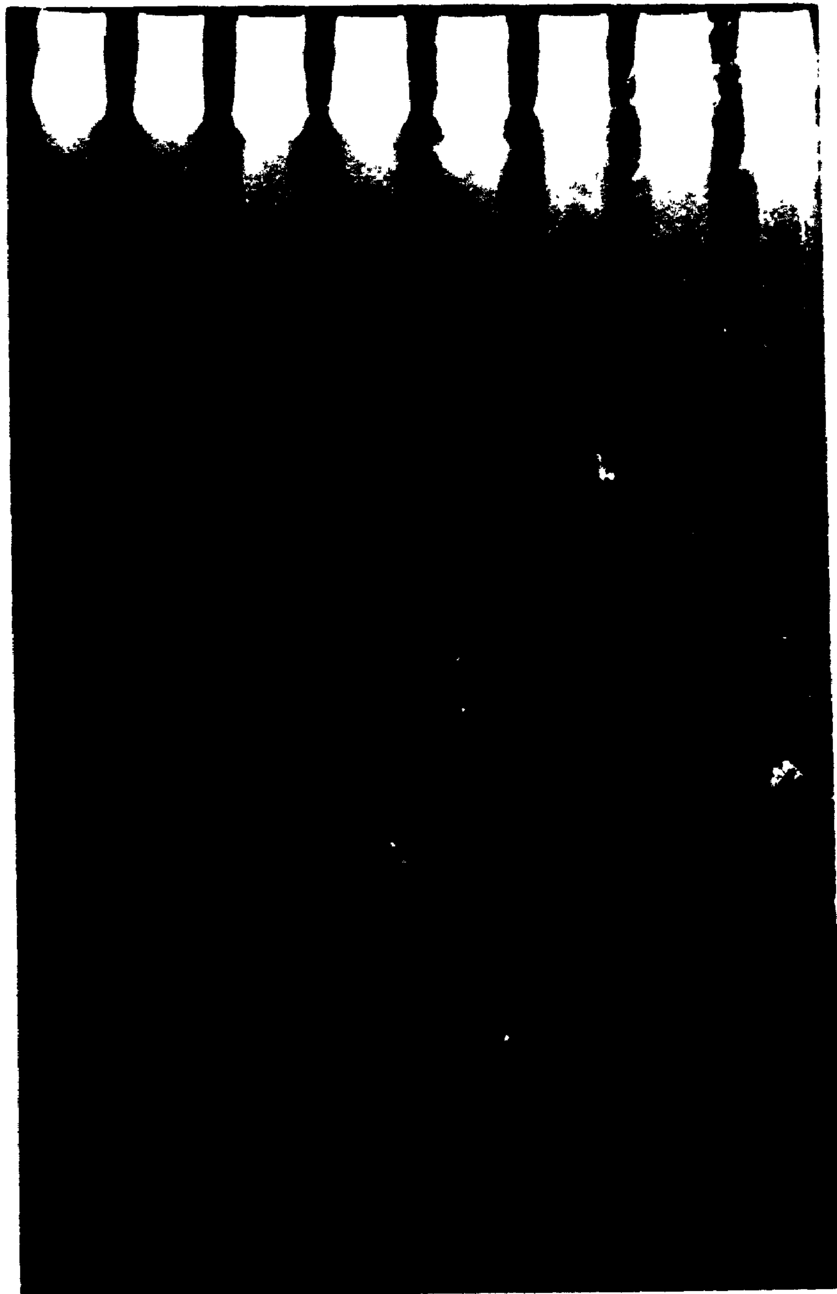

1
2
3
4
5
6
7
8
-161
-153

Figure 23

Purity of primase fragments

Primase fragments were prepared as described in 2.12.3. The purified fragments were treated with calf intestinal phosphatase, and then were 5' end labeled with polynucleotide kinase to a high specific activity. An aliquot of the end labeled material was electrophoresed through an 8% denaturing polyacrylamide gel. The gel was dried and autoradiographed performed at -70°C. Contamination with the smaller DNA fragment generated in primase fragment purification was less that 1% of the total purified DNA. No contaminating fragments of greater than 20 nucleotides were detected. All fragments were derived from M13mp19·154. Lane 1, Δ6; lane 2, Δ2; lane 3, w.t. DNA.

1 2 3
-175
-150
-100
-75

Figure 24

RNA primed DNA synthesis assay products

The products of the RNA primed DNA synthesis assay were resolved on an 8% denaturing polyacrylamide gel. (a) M13mp19·154 w.t. DNA substrate. Primase assays were performed as described in 2.12.3, except the elongation was performed for 1 hour instead of 15 minutes. Lane 1, 8.8pM DNA; lane 2, 10.5pM DNA; lane 3, 19.5pM DNA. (b) Primase assays were performed as described in 2.12.3, with elongation being performed for 15 minutes. Lane 1, 17.2pM M13mp19·154 Δ2; lane 2, 16.7pM M13mp19·154 Δ6.

a.

b.

for clarity. Wild type M13mp19·154 DNA was a substrate for RNA primed DNA synthesis, and exhibited a K_m with respect to DNA concentration of 10pM. The V_{max} for DNA synthesis was 13.3pmol dNTPs incorporated per hour.

Alteration of the first two nucleotides of ψ (mutations /5.CG, /5.CA, and /5.AC) led to large increases in K_m. A mutation in the third nucleotide of ψ, /6.GC, led to a similar change, as did insertions into the region separating the priming and ψ domains. In all six of these cases, where the K_m was increased beyond 100pM, there was an increase in V_{max}. It is not clear whether this is a real change, or simply results from increased error in the measurement when priming is inefficient.

We have detected a class of mutations that increase the rate of RNA primed DNA synthesis by decreasing the K_m. These mutations change the third nucleotide of the ψ domain from A to C. Both the two nucleotide deletion Δ2, and the point mutations /6.CG and /6.CA, change the third nucleotide of ψ from an A to a C, and decrease K_m to 6-7pM (table 6, section d).

Disruption of any of the first three nucleotides of ψ (table 6, sections b and c), with the one exception of changing the third nucleotide from an A to a C (table 6, section d), results in an increased K_m for RNA primed DNA synthesis. The double reciprocal plots of three ψ mutations are presented in figure 25.

Mutations in the priming domain lead to changes which primarily affected the V_{max} of RNA primed DNA synthesis. Table 6 section e illustrates both increased (/8.TC), and decreased (/8.AC, /8.GC) V_{max} values for RNA primed DNA synthesis. The double reciprocal plots of two priming domain mutants are illustrated in figure 26. The V_{max} for

the optimal substrate was 37.7pmol dNTPs per hour, while the worst substrate had a V_{max} of 5.5pmol dNTPs per hour.

Finally, mutations that do not alter the kinetics of the reaction contribute additional information. No change in the kinetics of RNA primed DNA synthesis are observed in the oligonucleotide /7 mutants (table 6, section a). These mutants had altered ψ domains, with changes to the final two nucleotides only. Since changes in the final two nucleotides of ψ do not lead to changes in the kinetics of RNA primed DNA synthesis, the final two nucleotides in ψ as initially proposed, are not required for ψ function. The fourth nucleotide in the original definition of ψ has a small effect on priming. While no change is observed in /6.AG, there is a small increase in K_m for RNA primed DNA synthesis in /6.TT and /6.TC.

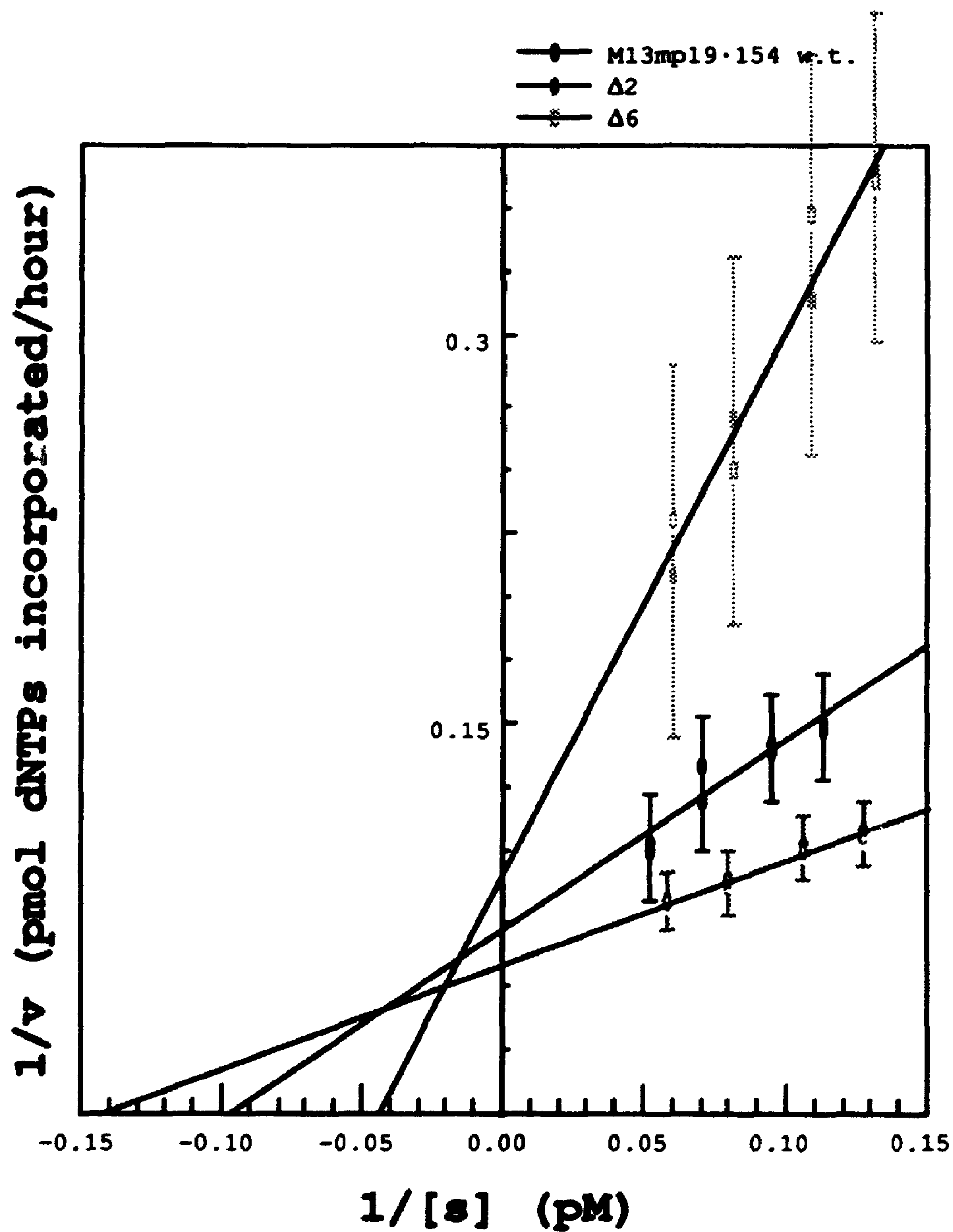
Mutations in the nucleotides immediately preceding the priming domain gave mixed results. While four of the mutations led to wild type kinetics (/3.TA, /3.CA, /3.AA, and /3.GG), an increase in K_m (/3.GT), and an increase in V_{max} (/3.GC), were both observed in single cases.

The general conclusions that we can draw from this work are that there is a sequence dependent aspect to the initiation of RNA primed DNA synthesis catalyzed by DNA polymerase α, through ψ sequences. We have redefined ψ as 3' CC(C/A). Changes in ψ alter the kinetics of RNA primed DNA synthesis primarily through changes in K_m. In addition to ψ, priming is favoured to initiate opposite a template pyrimidine. This result in expected, as DNA polymerase α is known to initiate RNA synthesis with purines only. Changes in the priming domain, in contrast to changes in the ψ domain lead to altered V_{max} values.

Figure 25

Kinetic analysis of RNA primed DNA synthesis: Ψ domain mutations

Sequence dependent RNA primed DNA synthesis reactions were performed as described (2.12.3). DNA substrates were M13mp19·154 w.t. (•), M13mp19·154 Δ2 (o), and M13mp19·154 Δ6 ([]). Substrate concentration is given with respect to the DNA substrate. Error bars give standard deviation of the data.



M13mp19·154 w.t.
Δ2
Δ6
1/v (pmol dNTPs incorporated/hour)
0.3
0.15
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
1/[s] (pM)

Figure 26

Kinetic analysis of RNA primed DNA synthesis: priming mutations

Sequence dependent RNA primed DNA synthesis reactions were performed as described (2.12.3). DNA substrates were M13mp19·154 w.t. (•), M13mp19·154 /8.TC (+), and M13mp19·154 /8.GC (o). Substrate concentration is given with respect to the DNA substrate. Error bars give standard deviation of the data.

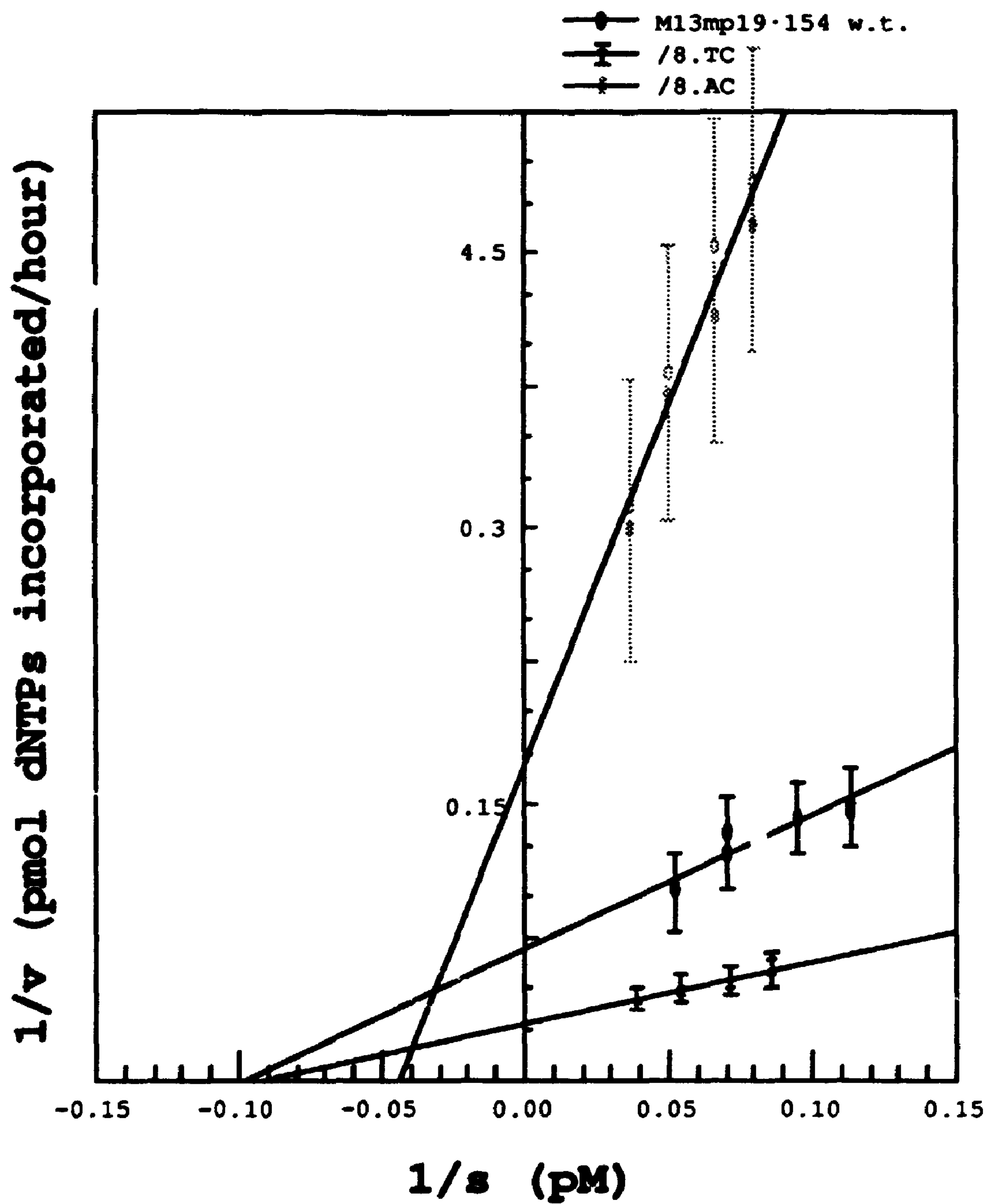

M13mp19·154 w.t.
/8.TC
/8.AC
4.5
0.3
0.15
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
1/v (pmol dNTPs incorporated/hour)
1/s (pM)

Table 6

Summary of sequence dependent changes in RNA primed DNA synthesis

Mutations have been grouped according to the type and extent of kinetic change versus the M13mp19·154 w.t. The K_m and V_{max} values are calculated with respect to the substrate DNA concentration.

Mutant DNA	V_{max} (pmol dNTPs/ hour)	K_m pM
a. Wild type sequences (10pM<Km<22pM)		
w.t.	13	10
/4.CC	15	10
/6.AG	14	10
/7.CG	14	15
/7.TC	15	19
/7.CT	16	19
/7.CA	13	16
/7.AC	15	21
/7.CG	15	20
/3.TA	14	11
/3.CA	16	16
/3.AA	16	19
/3.GG	13	11
/8.CT	12	12
/8.CG	14	15
b. Mutations with very high K_m (Km>100pM)		
/4.AA	28	110
/4.TG	35	150
/4.CA	32	130
/5.CG	37	120
/5.CA	50	180
/5.AC	55	240
/6.GC	25	181
c. Mutations with increased Km (Km>20pM)		
Δ6	10	23
/3.GT	10	32
/4.AA	14	46
/4.TC	16	61
/4.TA	13	37
/4.TA	12	38
/4.GA	16	48
/4.TC	18	59
/6.TT	15	27
/6.TC	13	86
d. Mutations which decrease K_m (Km<10pM)		
Δ2	16	6.9
/6.CG	15	6.7
/6.CA	17	6.2
e. Mutations which alter V_{max}		
/3.GC	38	17
/8.TC	30	11
/8.AC	7.3	38
/8.GC	5.5	22

4.4 Discussion

We have observed nascent RNA primed DNA synthesis during the conversion of MVM single stranded DNA to MVM double stranded DNA. The sites at which RNA priming occurs have been mapped a. high resolution. When GTP is present during polymerization, RNA priming occurs opposite stretches of C residues in the template. This was expected, as the minimum requirement for RNA priming is an area at which a stable primer may be synthesized. However, this was insufficient to explain the specificity of priming, as only 4 of 5 C5 stretches, 2 of 8 C4 stretches, and 2 of 33 C3 stretches were used as primase initiation sites. We looked for an additional determinant, which would correlate primase site selection with the observed data. The result of this search was ψ. Ψ is a hexanucleotide conforming to $C_2A_{1-2}(C_{2-3}/T_2)$. It was found to be located 2-14 nucleotides downstream of the sites of priming. Template C stretches which were not associated with a ψ sequence were not a substrate for RNA priming. The actual sequences from which the above formula for ψ were determined were $C_2A_2T_2$, $C_2A_2T_2$, and C_2AC_3.

We cloned one priming region into M13 to facilitate its study, separate from other priming domains, and to allow for oligonucleotide directed mutagenesis of the sequence. We chose the site depicted in figure 20 for its prototype ψ sequence, and for its well resolved priming and ψ domains. All the kinetic values described in the text are given with respect to the substrate DNA concentration. Thus, alterations in the kinetic parameters which are discussed below, are indicative of changes in the utilization of the DNA substrate by the DNA polymerase α complex.

Mutagenesis of this Ψ sequence has lead to several general conclusions. First, changes in Ψ lead to changes in the K_m for RNA primed DNA synthesis. While changes in V_{max} of the reaction have been observed, the changes in K_m range from a slight decrease, to a 24 fold increase. Changes in V_{max} were all less than two fold, with the exception of one class of mutations, which exhibited the largest change in K_m, and also exhibited three fold increases in V_{max}. Second, the active component of Ψ is a subset of the possible solutions to the above consensus; only the first three nucleotides of Ψ are required for Ψ action. Ψ has been redefined as 3' CC(C/A). Third, initiation of primers may be with a G, or with an A, with the latter favoured. Fourth, changes in the priming domain lead to changes in V_{max} for priming.

While changes in the sequence of the substrate DNA's Ψ domain led to significant changes in the K_m, the V_{max} for the reaction remained constant. This suggests that a change in the sequence of a substrate DNA's Ψ domain does not alter the potential of the DNA to serve as a substrate for RNA primed DNA synthesis, but rather that there is a change in the efficiency of utilization of the DNA substrate. Our data do not address the potential mechanism of this altered efficiency of utilization of the various DNA substrates.

The most studied model for RNA primed DNA synthesis has been SV40. In SV40, replication is semidiscontinuous (Perlman and Huberman, 1977), and RNA primed DNA chains are known to be replication intermediates (Anderson *et al.*, 1977). Preferred sites of Okazaki fragment initiation are known to exist in replicating SV40 (Tapper and DePamphilis, 1980). Both the sites of RNA primer initiation and the

locations of RNA-DNA junctions have been studied *in vitro* (Tseng and Ahlem, 1984; Yamaguchi et *al.*, 1985; Viswanatha et *al.*, 1986), and *in vivo* (Hay and DePamphilis, 1982; Hay *et al*, 1984). *In vitro,* RNA priming initiation has been studied using purified DNA primase (Tseng and Ahlem, 1984), DNA polymerase α-DNA primase (Yamaguchi et *al.*, 1985), and the multienzyme DNA polymerase α_2 (Vishwanatha et *al.*, 1986). These studies have all found two main clusters of RNA primer synthesis near the SV40 origin, one in the direction of early mRNA synthesis, and one in the direction of late mRNA synthesis. Although RNA primer synthesis in the direction of early mRNA synthesis is not associated with ψ as it has been defined here, the large cluster of priming sites in the direction of late mRNA synthesis are associated with many copies of ψ (all 3' CCC).

A distinction must be made between priming of DNA synthesis at an origin of replication, and the priming of Okazaki fragments. Priming at replication origins must be strictly controlled. Any given origin must fire once, and only once, per round of replication. Specific proteins are probably necessary for origin recognition which are not necesary for Okazaki fragment initiation. In SV40, the large tumour antigen, or T antigen, serves as an origin recognition and specificity protein. SV40 T antigen interacts with both the SV40 origin and DNA polymerase α, and presumably localizes the polymerase to a specific location on the SV40 origin. Though DNA polymerase α-primase has been implicated in the initiation of leading strand DNA synthesis in SV40, it is not clear whether DNA polymerase α-primase is responsible for initiation of leading strand replication during cellular DNA replication. This work has not considered the specificity of

initiation of leading strand DNA synthesis at replication origins, a process mediated by origin recognition or specificity proteins. This work has dealt with the selection of Okazaki fragment initiation sites on the lagging strand of DNA synthesis, which we feel is intrinsic to the DNA polymerase, and to the DNA template.

In bacteriophages T4 and T7, site selection by primase is strictly sequence specific. RNA primer synthesis by the T4 gene 61 protein is dependent upon the template sequences 3' TTG and 3' TCG (Cha and Alberts, 1986). In T7, RNA primers are synthesized by the gene 4 product, which recognizes 3' CTGGG/T (Tabor and Richardson, 1981). In both cases, priming begins opposite the second nucleotide in the sequence. Ψ sequences are fundamentally different than these, since the sequences we are studying are not specifically priming sites, rather associated sequences which activate priming upstream. This may explain the lack of consensus when priming has been considered in eukaryotic systems. ψ sequences do not lead to RNA primers with specific sequences. Rather, the consensus is located about ten nucleotides downstream, in a region which would be just within the nascent DNA.

The original definition of ψ was based on correlative data (Faust *et al.*, 1985). The new definition is the result of kinetic studies of ψ and mutant ψ sequences. We have reexamined the data from the initial study, and found the new sequence to be consistent with the previously mapped sites. In one instance, near MVM nucleotide 3750, the ψ sequence which we identified now appears not to be the active site, rather a sequence several nucleotides away is probably the active ψ site.

Finally, we note that the removal of ψ does not completely abolish the initiation of RNA primed DNA synthesis. This suggests that ψ is not an essential template requirement for primase initiation, though our data clearly indicate that it is important. The role of ψ *in vitro*, is in primase site selection. In the DNA polymerase α/δ coupled model (Prelich and Stillman, 1988), DNA polymerase α is the lagging strand polymerase. One problem to be considered in this model is what signals Okazaki fragment switching, that is, the release of one completed Okazaki fragment, and the initiation of a new one. A template sequence specifying primase site selection, such as ψ could function *in vivo* as such a switch.

Chapter 5

Polymerization up to a double stranded region: gap filling

5.1 Abstract

DNA polymerase α was studied in a direct gap-filling assay. Using a defined template, DNA synthesis was primed from the M13 17mer universal primer, and blocked by an oligonucleotide hybridized 56 nucleotides downstream of the primer. DNA polymerase α filled this gap to completion. A time course of the reaction showed that in 50% of the substrate molecules, gaps were filled to completion within 10 minutes. In another 35% of the molecules the final nucleotide was lacking after 10 minutes. This nucleotide was added at a reduced rate, and was not incorporated into all of the molecules even after 6 hours. Limited strand invasion was observed in the remainder of substrate molecules; 42% of substrate molecules had undergone limited strand displacement after 6 hours of incubation. The reduced rate of incorporation of the final nucleotide is reflected in an increased K_m for *de novo* incorporation of one nucleotide at a single nucleotide gap (0.7μM), as opposed to the K_m for incorporation of one nucleotide into singly primed M13 DNA (0.18μM). DNA polymerase α_2, and DNA polymerase purified from cells infected with the parvovirus minute virus of mice, exhibited the same kinetics of gap filling as did DNA polymerase α purified from uninfected Ehrlich ascites mouse tumor cells. T4 DNA polymerase filled gaps to completion in this assay. *E. coli* DNA polymerase I Klenow fragment quantitatively displaced the downstream oligonucleotide, and extended nascent DNA chains for an additional 100 nucleotides.

5.2 Introduction

The ability of DNA polymerase α to fill gaps has implications with respect to its function in DNA replication and repair. DNA polymerase α has been assayed for its ability to fill gaps by several groups (Korn *et al.*, 1978; Bose *et al.*, 1978; Fisher *et al.*, 1979; DePamphilis *et al.*, 1980; Wang and Korn, 1980; Hockensmith and Bambara, 1981; and Mosbaugh and Linn, 1984). However, conflicting data have prevented a consensus with respect to both the optimal gap size and the ability of DNA polymerase α to fill gaps to completion.

Korn *et al.* (1978), and Wang and Korn (1980) studied gap-filling on activated calf thymus DNA which had gaps of defined average size. They showed that human KB cell DNA polymerase α, an enzyme composed of two dissimilar subunits, Mr=76,000, and 66,000 (Fisher and Korn, 1977), left 20-30 nucleotide gaps in activated calf thymus DNA, and was unreactive when the mean gap size was less than 20 nucleotides. Mosbaugh and Linn (1984) studied gap-filling by HeLa cell DNA polymerases α and β, using PM2 DNA that had been digested with HeLa DNase V to generate gaps distributed about a mean size. This study concluded that DNA polymerase α left gaps of 15 nucleotides, which could be extended by DNA polymerase β to generate a substrate for *E. coli* DNA ligase. DePamphilis *et al.* (1980) used gapped SV40 DNA to show that while monkey CV-1 cell DNA polymerase α could not completely fill gaps of 40 nucleotides, DNA polymerase α from calf thymus or HeLa cells apparently could. Furthermore, CV-1 DNA polymerase α could fill gaps in SV40 DNA if the gaps were about 4 nucleotides long. Complete gap-filling in these experiments was judged by the concomitant action of *E. coli* or CV-1 DNA ligase. Bose *et al.* (1978) showed that crude

DNA polymerase α, in the presence of crude apurinic endonuclease and 5'→3' exonuclease, all derived from a human Burkitt's lymphoma line, would perform a nick translation of methyl methanesulfonate treated T7 DNA. Small gaps generated in this manner were filled to an extent that permitted covalent closure by T4 DNA ligase. However, T4 DNA ligase has since been shown to seal single stranded gaps of up to 100 nucleotides, though less efficiently than it seals nicks in DNA (Nilsson and Magnusson, 1982). Hockensmith and Bambara (1981) have described the effect of subunit composition on gap utilization by calf thymus DNA polymerase α. Five forms of DNA polymerase α: A1, A2, B, C, and D, were purified, based on their chromatographic properties. Polymerase α-A1 was more active on a template with an average gap size of 65 nucleotides than on one with an average gap size of 10 nucleotides, while α-C was slightly less active on the template with the larger gap size. This indicates that the extent of gap filling performed by DNA polymerase α is dependent on subunit composition.

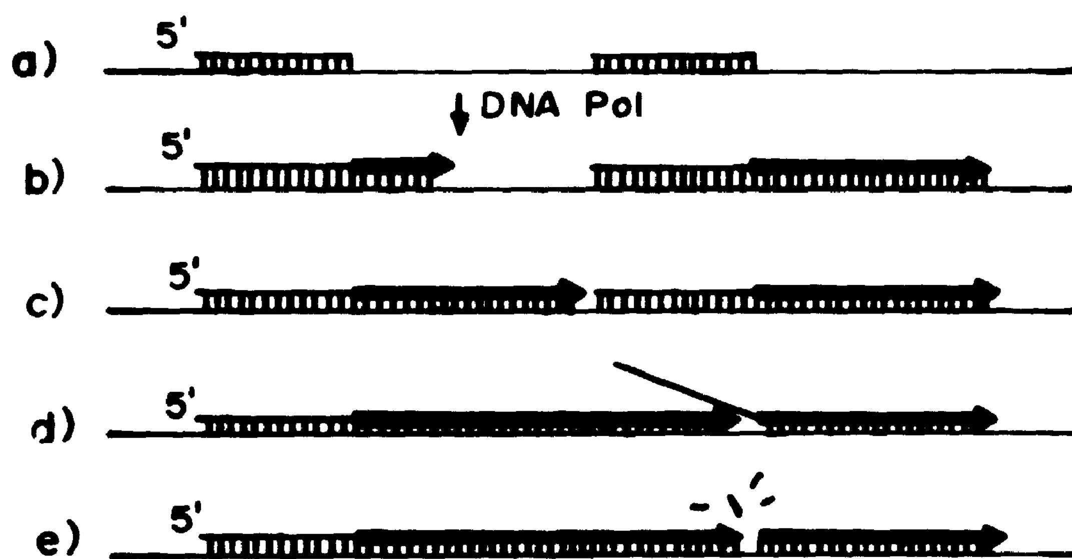
With the availability of highly purified DNA polymerase α, and defined DNA substrates, we have developed a high resolution gap filling assay. The direct gap filling assay utilizes a defined DNA substrate, including template and primer, and a specific oligonucleotide hybridized downstream of the primer which blocks polymerization. DNA polymerase α will utilize M13 DNA as a defined DNA template, with priming occurring at the 3' hydroxyl of the M13 17mer universal primer. A second synthetic oligonucleotide hybridized downstream of the universal primer generates a template with two gaps, one upstream and one downstream of the 17mer. We have chosen an oligonucleotide that hybridizes 56 nucleotides downstream of the M13 17mer universal primer.

In this configuration, both oligonucleotides serve as primers for DNA synthesis, allowing various DNA polymerases to fill the gaps between them (Figure 27). The size of the oligonucleotides after polymerization can be determined by polyacrylamide gel electrophoresis, allowing a precise determination of the extent to which gaps are filled. This approach is capable of revealing whether gaps are filled partially (Figure 27b), completely (Figure 27c), and also whether DNA synthesis proceeds beyond the gap due to strand displacement (Figure 27d) or strand degradation caused by a 5'-3' exonuclease (Figure 27e). This work goes beyond previous gap filling determinations in the use of a fully defined system, and highly purified DNA polymerase α. We have found DNA polymerase α to be capable of filling gaps to completion, in this system, and have characterized a kinetic distinction between DNA polymerization at a single nucleotide gap versus polymerization on singly primed DNA.

Figure 27

Schematic illustration of the gap-filling assay

(a) A gapped DNA substrate is produced by hybridization of two oligonucleotides to a single stranded DNA template. Incubation of this molecule in the presence of DNA polymerase could result in (b) partial gap-filling, (c) complete gap-filling, (d) invasion of the double stranded region, or (e) hydrolysis of the downstream oligonucleotide. The extent to which a gap is filled can be determined by measuring the change in length of the upstream oligonucleotide.


a)
5'
DNA Pol
b)
5'
c)
5'
d)
5'
e)
5'

5.3 Results

5.3.1 Gap-filling by DNA polymerase α

The recombinant M13mp19·529 DNA generated in this study contains a 529 nucleotide DNA fragment, nucleotides 2126 to 2654 of the MVM genome (numbering according to Astell *et al.*, 1986), inserted into M13mp19 DNA, between the *Pst*I and *Hin*dIII restriction sites. The M13 17mer universal primer was hybridized just upstream of the *Pst*I site on M13mp19·529. The second oligonucleotide was hybridized to the MVM portion of M13mp19·529 DNA, downstream of the *Pst*I site (Figure 28). A 56 nucleotide gap is generated between these two oligonucleotides. Both oligonucleotides hybridized to the expected location, since dideoxy sequencing primed from the oligonucleotides yielded the appropriate downstream sequences (data not shown).

DNA polymerase α was incubated in the presence of 5'-^{32}P end labeled M13 17mer universal primer, and unlabeled 20mer downstream oligonucleotide, that had been hybridized to the M13mp19·529 DNA template. The radiolabeled products of this reaction were 72 and 73 nucleotides in length, as assayed by polyacrylamide gel electrophoresis (Figure 29, lanes 1-3). Fragments of 73 nucleotides represent complete gap-filling, indicating that DNA polymerase α has the ability to fill gaps to completion. When the downstream oligonucleotide was omitted from the reaction, DNA synthesis primed at the 17mer generated nascent DNA fragments of 99 and 100 nucleotides in length (Figure 29, lanes 4-6), indicating that production of the 72 and 73 nucleotide fragments is dependent on the presence of the downstream oligonucleotide, and is not due to nonspecific pausing. In these reactions, the radiolabeled

Figure 28

Configuration of oligonucleotides hybridized to M13mp19·529

The template sequence of M13mp19·529 in the region of interest is shown in lower case letters. The sequence is a composite of M13mp19 and MVM sequences described by Norrander (1983), and Astell *et al.* (1986), respectively. The nucleotide sequences and location at which the 17mer universal primer, the gap filling -1 (GF-1) oligonucleotide, and the 20mer downstream (gap filling) oligonucleotide hybridize to the M13 template are shown in upper case letters. The junction between M13 and MVM sequences occurs at the *Pst*I site (nucleotides 59-64). Numbering is arbitrary, starting two nucleotides upstream of the 5'end of the hybridized universal primer. Complete gap-filling will produce the 73 nucleotide fragment indicated.

```
     M13 17mer                                                  -1 Gap Filling       Gap Filling
     Universal                                                  Oligonucleotide      Oligonucleotide
      Primer

 pGTAAAACGACGGCCAGT                                             CGACCTGCAGAAACCCAGAA ACTGGGGAAGCTGGTTCCAA
aacattttgctgccggtcacttaagctcgagccatgggcccctaggagatctcagctggacgtctttgggtcttgtgaccccttcgaccaaggtttcgga
1        10        20        30        40        50        60        70        80        90        100
                                                                        → |  | ← 1 nucleotide
 |←――――――――――――――――――――――――― 73 nucleotides ――――――――――――――――――――――――――→|
```

fragments of 99 and 100 nucleotides were produced by pausing at a region of secondary structure in the DNA template, and could be elongated by DNA polymerase α after further incubation. The products in these reactions were the same when the reaction was performed either without ribonucleotides present (Figure 29, lanes 1 and 4), or with 0.1 or 1.0mM each ATP, CTP, GTP, UTP (Figure 29, lanes 2, 3, 5, and 6). Omitting either the DNA template or DNA polymerase α-primase from the reaction mixtures abolished DNA synthesis (Figure 29, lanes 7-12).

5.3.2 Polymerization at single nucleotide gaps

A time course of DNA synthesis with the gapped M13mp19·529 DNA substrate is shown in figure 30. The results were quantified by densitometry (Table 7). Gaps were filled completely (73 nucleotides) in 52.3% of the molecules 10 minutes after enzyme addition (Table 7). Polymerization at single nucleotide gaps present in the remaining DNA molecules occurred slowly; after 6 hours of incubation 14.2% of the molecules retained a single nucleotide gap (<73 nucleotides). Therefore, the kinetics of addition of the final nucleotide are biphasic.

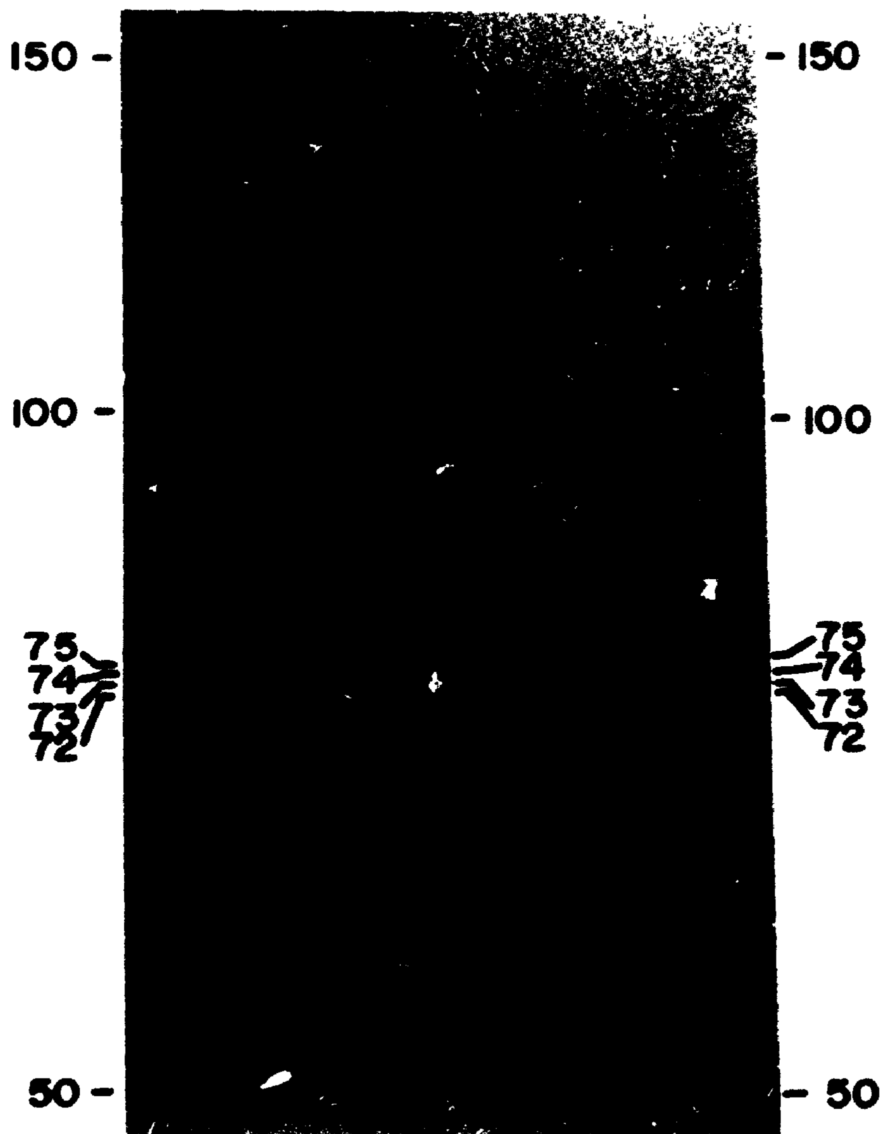
5.3.3 Kinetics of the gap filling reaction

A second priming oligonucleotide (GF-1) was used to study the nature of incorporation of the final nucleotide at a gap. The 3' end of this oligonucleotide hybridized upstream of the gap filling oligonucleotide, leaving a single nucleotide gap when both were hybridized to the M13 template. The single gap could be filled by the incorporaton of a dCTP residue (figure 28), while the downstream oligonucleotide required a

Figure 29

Extent of gap-filling by DNA polymerase α

DNA polymerase α gap filling reactions were performed for 1 hour at 37°C on M13mp19·529, as described (2.13.1), except as noted below. The 20mer downstream oligonucleotide (1.0pmol), and M13mp19·525 ssDNA (0.25pmol) were present (+) or absent (-) during hybridization. DNA polymerase α, "enzyme", (0.1 units) was present (+) or absent (-) during the gap-filling reaction. "rNTPs", ATP, CTP, GTP, and UTP were present during the gap-filling reaction, at 0, 0.1, or 1.0 mM each, as indicated. Products were analyzed by electrophoresis in 8% polyacrylamide/8M urea gels for 1.5 hours at 50mA, and autoradiography was performed for 6 hours at room temperature. Fragment sizes in nucleotides are indicated, based on *Alu*I and *Mbo*I restriction enzyme digests of MVM RF DNA.



LANE 1 2 3 4 5 6 7 8 9 10 11 12
20mer + + + − − − + + + + + +
DNA + + + + + + − − − + + +
ENZYME + + + + + + + + + − − −
[rNTPs]mM 0 0.1 1.0 0 0.1 1.0 0 0.1 1.0 0 0.1 1.0
150
100
75
74
73
72
50

Figure 30

Time course of the gap-filling reaction

DNA polymerase α gap-filling reactions were performed at 37°C for the times indicated. Products were analyzed by electrophoresis in 8% polyacrylamide/8M urea gels for 2.0 hours at 30mA, and autoradiography was performed for 17 hours at -70°C with an intensifying screen. Fragment sizes in nucleotides are indicated, based on Alu I and Mbo I restriction enzyme digests of MVM RF DNA.

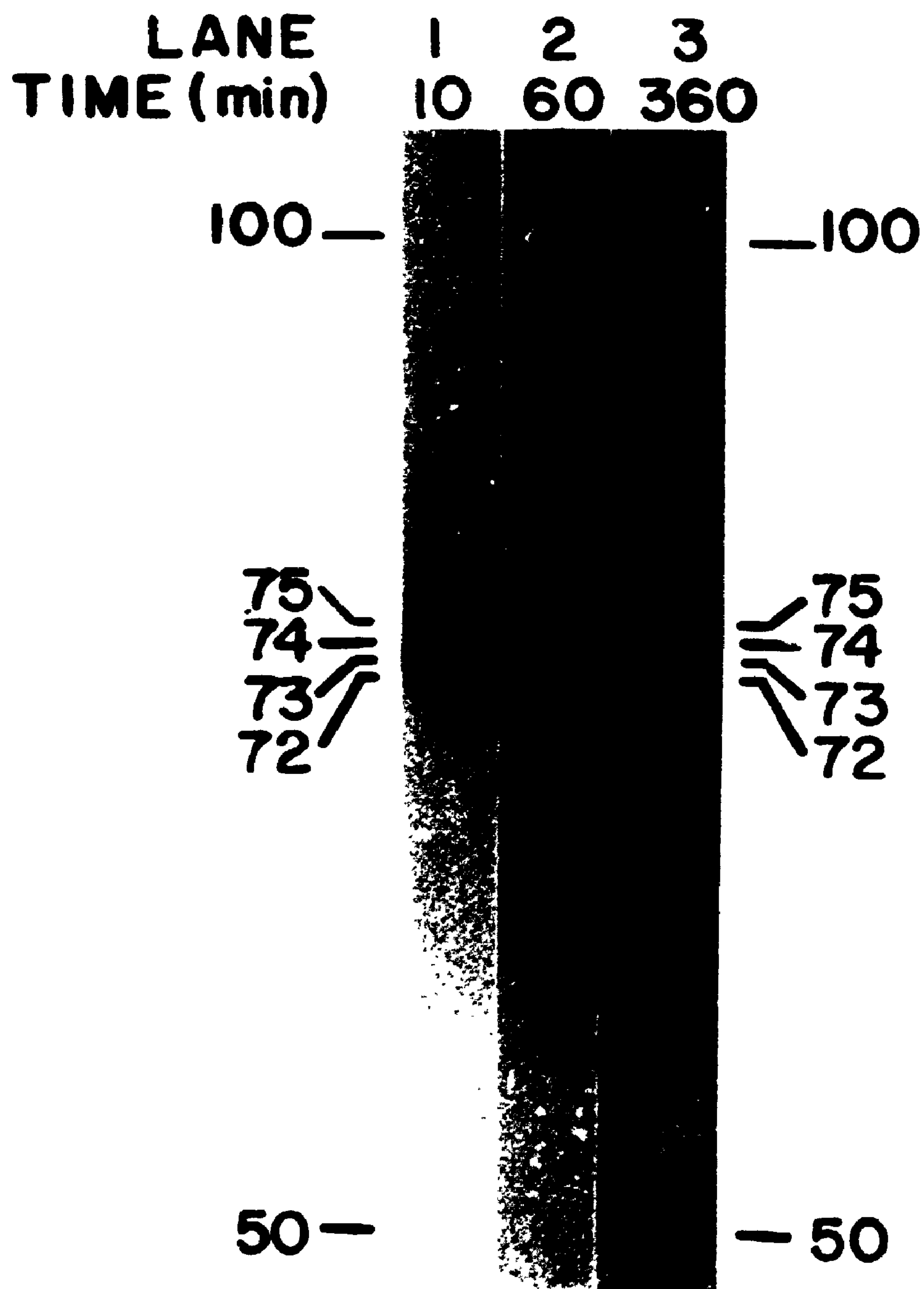

LANE 1 2 3
TIME (min) 10 60 360
100
100
75
74
73
72
75
74
73
72
50
50

Table 7

Distribution of nascent DNA chain lengths among the products of the gap filling reaction

The lanes from figure 30, were analyzed using an LKB UltroScan XL Laser Densitometer. Values were integrated and combined to reflect the percent of the population having chain lengths corresponding to the categories indicated, at various time points.

Time	Percentage of nascent molecules:			
	<73 nucleotides (incomplete gap filling)	≈73 nucleotides (complete gap filling)	>73 nucleotides (invading)	≥73 nucleotides (invading+ complete)
10 min.	47.7	44.2	8.1	52.3
1 hour	28.7	54.9	16.4	71.3
6 hours	14.2	43.0	42.8	85.8

dATP residue for further polymerization. Limited DNA polymerization was performed, with dCTP as the only nucleotide present, so the only primer available for DNA synthesis was the GF-1 oligonucleotide. DNA synthesis was quantitated at various substrate concentrations, both with and without the downstream oligonucleotide hybridized to the substrate DNA molecule. Primer independent DNA synthesis, and limited elongation of the downstream oligonucleotide were both controlled for. In all cases, less than 5% of the substrate was converted to product, in order to ensure initial velocities were obtained. The initial velocities at several substrate concentrations were determined, and presented as double reciprocal plots of initial velocity versus substrate concentration, in the presence or absence of the downstream oligonucleotide (Figure 31).

Least squares analysis was applied to the data in figure 31, to determine the K_m for singly primed M13 DNA 0.18μM with respect to 5' ends, or 1.4mM nucleotides. The K_m for a single nucleotide gap on M13 DNA was determined as 0.7μM with respect to 5' ends. Vmax was not altered by the presence of the downstream oligonucleotide, and was found to be 0.03pmol/hour under the defined gap filling conditions.

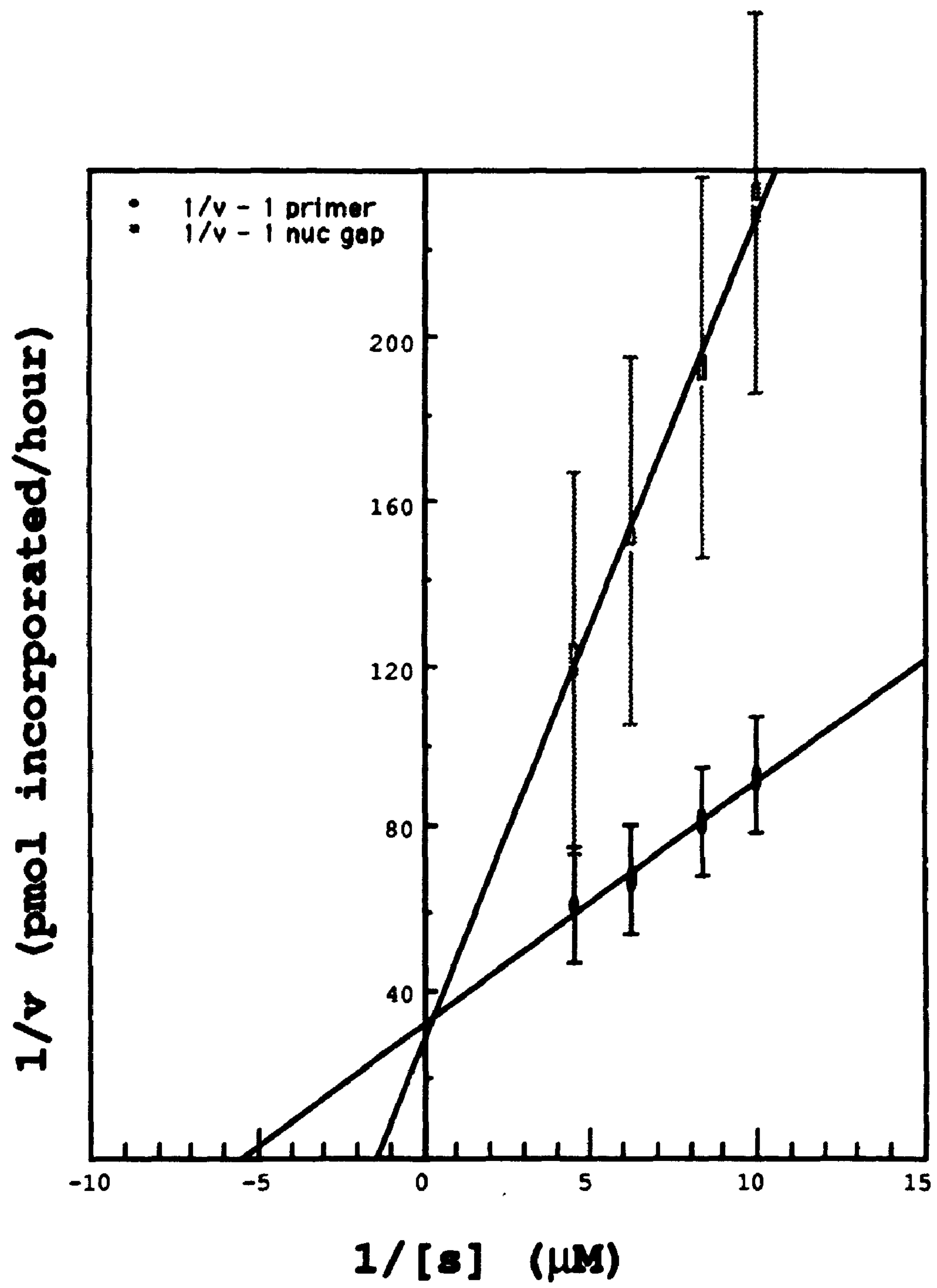
5.3.4 Invasion of double stranded DNA regions

After 6 hours of incubation with DNA polymerase α, radiolabeled DNA molecules up to 85 nucleotides long were produced (figure 30, lane 3). This demonstrates that DNA polymerase α has the ability to extend nascent DNA chains into regions of duplex DNA by up to 12 nucleotides. After 6 hours of incubation, 42% of the nascent DNA chains had been extended into a region of duplex DNA (Table 7).

Figure 31

Determination of the K_m for de novo incorporation of one nucleotide at a single nucleotide gap

Purified 73 nucleotide gap filling (n-1) fragments were annealed to M13mp19·529 in the presence or absence of the downstream gap filling oligonucleotide, and gap filling reactions were performed as indicated, with the following exceptions. Reactions were performed with 5-12pmol 5' ends per reaction, with 0.25μM [α-^{32}P]dCTP (800Ci/mmol) as the only dNTP, in the presence of 0.5 units DNA polymerase α, for 20 minutes, at 37°C. Reactions were assayed for the presence of acid precipitable material, as described in section 2.11.1. The substrate concentration (as 1/[s]), is with respect to the DNA substrate concentration. Error bars are the calculated standard deviation of the data.



1/v - 1 primer
1/v - 1 nuc gap
1/v (pmol incorporated/hour)
200
160
120
80
40
-10
-5
0
5
10
15
1/[s] (μM)

5.3.5 Effect of Inhibitors on the Gap-Filling Reaction

Since the kinetics of addition of the final nucleotide were found to be biphasic, we considered the possibility that our DNA polymerase α preparations were contaminated with low levels of DNA polymerase β, γ, or δ. We examined this possibility initially by using BuPdGTP and BuAdATP, as inhibitors that distinguish between DNA polymerases α and δ (Lee *et al.*, 1985). Inhibition curves were generated by titration of our DNA polymerase α preparations, on an activated calf thymus DNA template, with various concentrations of either BuPdGTP or BuAdATP. Half maximal inhibition of DNA polymerase activity was observed at concentrations of $4.0x10^{-8}$ M and $3.2x10^{-9}$ M, for BuPdGTP and BuAdATP respectively, and the inhibition curves corresponded to those published for DNA polymerase α (Lee *et al.*, 1985). In addition, the gap-filling reactions were performed in the presence of 25μM ddTTP, ddCTP, or a mixture of all four ddNTPs at 0.5mM each (ddNTP/dNTP ratios of 1:1, 1:1 and 20:1 [each] respectively), to test the possibility that DNA polymerase β or γ might be present. Neither the initial rate of DNA synthesis, nor the rate of addition of the final nucleotide, were altered by inclusion of 25μM ddNTPs in the gap-filling reaction mixtures, though DNA synthesis was eliminated by the inclusion of ddNTPs at 0.5mM each (data not shown).

5.3.6 Other Forms of DNA Polymerase α in the Gap-Filling Reaction

DNA polymerase α-primase purified from Ehrlich Ascites mouse tumour cells infected with MVM (Ho *et al.*, 1989), as well as the multiprotein form of HeLa DNA polymerase α2 (Viswanatha *et al.*, 1986), were tested in this assay. A time course of the gap-filling reaction was performed

with each of these forms of DNA polymerase α (figure 32). In all cases, the results were indistinguishable from those exhibited by DNA polymerase α purified from uninfected Ehrlich ascites mouse tumour cells.

5.3.7 Other DNA Polymerases in the Gap-Filling Reaction

Both T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I were tested in this assay (Figure 33). T4 DNA polymerase produced the same radiolabeled DNA products as DNA polymerase α, as well as an additional major DNA species 75 nucleotides long (Figure 33, lanes 5-8). When the downstream oligonucleotide was omitted from the reaction (Figure 33, lane 1), T4 DNA polymerase initially paused at the same site of secondary structure as DNA polymerase α. However, this secondary structure was not sufficient to arrest T4 DNA polymerase at time points longer than 15 minutes (Figure 33, lanes 2-4). Finally, after incubation for four hours, T4 DNA polymerase displaced the downstream oligonucleotide to the same extent as DNA polymerase α, up to 12 nucleotides.

The Klenow fragment of *E. coli* DNA polymerase I extended nascent DNA chains into the double stranded region extensively (Figure 33, lanes 13-16). Under standard reaction conditions, by 30 minutes, the predominant product of elongation was a fragment 200±10 nucleotides long. This DNA fragment likely represents termination of polymerization (or pausing) at a GC rich region in the M13mp19·529 DNA template. Although no stable secondary is predicted there when the DNA is single stranded, polymerization from the 20mer downstream oligonucleotide would render this area double stranded, producing a

strong block to further polymerization.

Figure 32

Gap filling by other forms of DNA polymerase α

DNA polymerase α purified from Ehrlich ascites mouse tumour cells infected with MVM (Lanes 1-8), as well as the multiprotein form of HeLa DNA polymerase α_2 (Lanes 9-12), were tested in this assay. Standard gap filling assays were performed for the lengths of time indicated.

LANE	1	2	3	4	5	6	7	8	9	10	11	12
TIME (min)	15	30	60	240	15	30	60	240	15	30	60	240

100 —

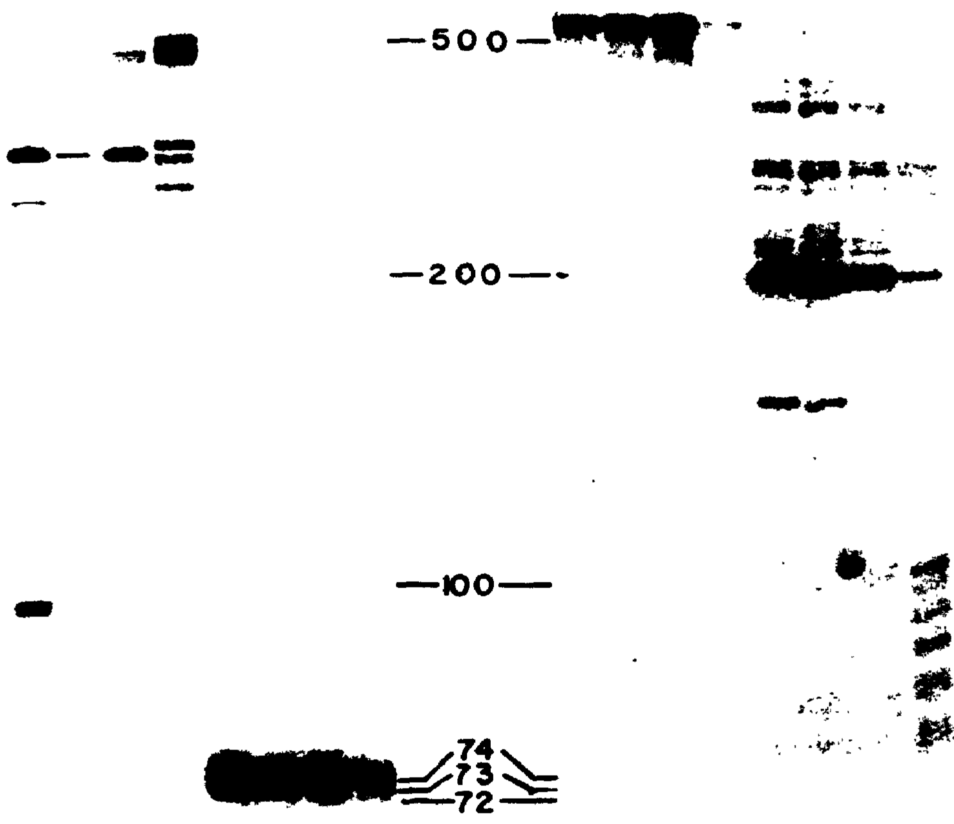
74
73
72
71

50 —

Figure 33

Gap-filling by T4 DNA polymerase and *E. coli* DNA polymerase

T4 c *E. coli* large (Klenow) fragment DNA polymerase gap-filling reactions were performed at 37°C for 15 minutes to 6 hours. Products were analyzed by electrophoresis in 8% polyacrylamide/8M urea gels for 2.0 hours at 50 mA, and autoradiography was performed for 6 hours at room temperature. Fragment sizes in nucleotides are indicated, based on *Alu*I and *Mbo*I restriction enzyme digests of MVM RF DNA.


LANE 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
POLYMERASE T4 KLENOW
20mer – – – – + + + + – – – – + + + +
TIME (min) 15 30 60 240 15 30 60 240 15 30 60 240 15 30 60 240
500
200
100
74
73
72

5.4 Discussion

In this portion of the study we have used a highly purified murine DNA polymerase α to fill a gap of defined size and sequence. By determining the size of a specific primer after elongation, we have shown that the gap can be completely filled. This reaction is dependent on a double stranded DNA region, downstream of the primer, which forms a block to polymerization. In these experiments, we have used a 20mer downstream oligonucleotide, hybridized to the M13 DNA template, to form this double stranded DNA block to polymerization.

While gaps in about half of the molecules were filled to completion within ten minutes of enzyme addition, in the remaining molecules, the final nucleotide was added slowly; gaps were completely filled in only 85% of the molecules even after 6 hours. The fact that the reaction kinetics are biphasic, with different rates for the bulk of elongation as opposed to incorporation at a single nucleotide gap, suggests a fundamental difference in the way DNA polymerase α recognizes single nucleotide gaps versus gaps of 2 or more nucleotides. This idea is supported by the observation that the K_m for incorporation of a single nucleotide at a single nucleotide gap is 4 fold higher than on singly primed M13 DNA. Fisher and Korn (1981) have shown that substrate recognition by DNA polymerase α is ordered, with recognition of the template first, followed by the primer, and then the nucleotide substrate. This suggests that the decreased K_m for *de novo* DNA synthesis at single nucleotide gaps is due to a limiting template length downstream of the primer.

DNA polymerase α has been shown to perform limited strand displacement, probably due to breathing of the 5' end of the downstream

oligonucleotide. So by definition, there is a decrease in processivity of DNA polymerase α at single nucleotide gaps. This may contribute, along with the increased K_m, to the biphasic kinetics that have been observed. The fact that two nucleotide gaps do not accumulate during the course of the gap filling reaction suggests that the altered kinetics observed at single nucleotide gaps do not apply to two nucleotide gaps.

We have eliminated the possibility that a low level contaminant is responsible for the incorporation of the final nucleotide in those molecules where the final nucleotide is added slowly. Titration of the enzyme preparation with BuPdGTP and BuAdATP show the enzyme to be an α as opposed to δ DNA polymerase. Dideoxy nucleotides at concentrations which inhibit β and γ polymerases to greater than 90% do not alter the kinetics of the reaction. These data are in agreement with the premise that DNA polymerase α is responsible for the gap-filling ability demonstrated here. Abbotts *et al.* (1988), reported that DNA polymerase β, purified from chick, calf or HeLa cells, leaves gaps of four to seven nucleotides between oligonucleotides in a direct assay. This further reduces the likelihood that contaminating DNA polymerase β is responsible for the gap-filling observed here.

Other systems used to determine the ability of DNA polymerase α to fill gaps have generated mixed results. A number of explanations for this are possible. Most gap filling determinations were performed before highly purified DNA polymerase α was generally available. Both degradation and contamination could influence an assay for gap filling. Several DNA polymerase α subspecies purified from calf thymus exhibit differential gap filling, suggesting even altered subunit composition

can lead to altered results in a gap filling assay (Hockensmith and Bambara, 1981). Sequence specific contributions to DNA polymerase α activity have the potential to influence results observed in a gap-filling assay. Sequence specificity has been shown to influence DNA polymerase α in RNA priming (Hay *et al.*, 1984; Faust *et al.* 1985), and pausing (Weaver and DePamphilis, 1982; Kaguni and Clayton, 1982; Weaver and DePamphilis, 1984). The use of activated DNA to assess gap-filling is susceptible to sequence effects which are bound to be present due to the random nature of nucleotide sequences in this complex DNA template. Secondary structures likely form in the gapped regions of activated DNA, and if the secondary structures are stable enough to prevent DNA chain elongation, their presence will cause DNA polymerase α to leave spuriously large gaps. The template we have used in this work is free of secondary structure in the 56 nucleotide gapped region. The nearest secondary structure which caused DNA polymerase α to pause is about 80 nucleotides downstream of the 3' end of the 17mer universal primer, and is evident in figure 29, lanes 4-6.

The ability of DNA polymerase α to fill gaps has significance with respect to DNA replication (Edenberg *et al.*, 1978; Depamphilis *et al.*, 1979; Krokan *et al.*, 1979; Anderson and DePamphilis, 1979), and DNA repair (Mosbaugh and Linn, 1983; Mosbaugh and Linn, 1984). Our results do not rule out a role for DNA polymerase α in filling gaps during either of these processes, but the reduced efficiency of incorporation of the final nucleotide suggests that other factors may be required in vivo for efficient gap filling by DNA polymerase α. Accessory proteins may exist which are capable of enhancing the binding of DNA polymerase α-primase at single nucleotide gaps, or alternatively, accessory

proteins may improve processivity as DNA polymerase α approaches a double stranded region. Such factors are also lacking in the multiprotein α_2 form of DNA polymerase α, since this form of DNA polymerase α also fills single nucleotide gaps with reduced efficiency.

The results exhibited by T4 DNA polymerase in this assay are similar to those exhibited by DNA polymerase α. T4 DNA polymerase rapidly fills the 56 nucleotide gap, but displaces one or two nucleotides of the downstream oligonucleotide. The overall extent to which T4 DNA polymerase invades the double stranded region is about 12 nucleotides (Fig. 33, overexposures), which is the same distance as DNA polymerase α. Strand displacement is probably due to slight breathing at the 5' end of the downstream oligonucleotide, since T4 DNA polymerase lacks associated helicase and 5'→3' exonuclease activities (Nossal, 1974).

By contrast to the other DNA polymerases tested, *E. coli* DNA polymerase I Klenow fragment produces nascent DNA chains about 200 nucleotides in length. To distinguish between displacement of the downstream strand by a helicase activity, or the action of a 5'→3' exonuclease to degrade the downstream strand, we radiolabeled the 5' end of the downstream oligonucleotide instead of the upstream primer. After performing the standard gap filling reaction, the 20mer was recovered as a high molecular weight DNA fragment, indicating that strand displacement, and not the action of a 5'→3' exonuclease was responsible for the extension of the upstream oligonucleotide beyond the 5' end of the downstream oligonucleotide. This result was expected, since the Klenow fragment of *E. coli* DNA polymerase I does not contain an associated 5'→3' exonuclease activity.

This approach to gap-filling can be easily adapted to detect

helicase, or 5'→3' exonuclease activity, which would act in association with DNA polymerase α. By using this assay and looking for nascent DNA chains longer than 73 nucleotides, one could assay samples during protein purification. For substantially larger fragments to be generated, a helicase or exonuclease is required, as DNA polymerase α has been shown here to have only a limited ability to extend nascent DNA chains into duplex regions of DNA.

Conclusions

This work is a study of the DNA polymerase α-primase complex. DNA polymerase α is an essential component of eukaryotic replication systems, and is thought to synthesize DNA on the lagging strand at replication forks. We have purified the complex, and studied two aspects of RNA primed DNA synthesis catalyzed by this enzyme complex *in vitro*. These were the sequence specificity of RNA primase initiation, and the ability of DNA polymerase α to fill gaps. These reactions represent two possible control points for DNA synthesis, Okazaki fragment initiation, and Okazaki fragment termination.

DNA polymerase α has been purified from cultured mouse Ehrlich ascites tumour cells, by classical purification methods. The purified DNA polymerase α complex has four subunits, 182, 70, 55, and 47kDa. A cryptic 3'→5' exonuclease activity, revealed by treatment of the complex with ethylene glycol, cosedimented with the DNA polymerase activity, on sedimentation through glycerol gradients. The sensitivity of DNA polymerase α to the butylated nucleotides butylphenyl-dGTP and butylanilino-dATP did not change when the cryptic 3'→5' exonuclease was active. This suggests that the catalytic subunits of DNA polymerases α and δ are distinct. DNA polymerase δ is thought to function on the leading strand of a replication fork, during eukaryotic cellular replication.

The effect of template DNA sequence on the rate of initiation of RNA primed DNA synthesis by the purified mouse DNA polymerase α-primase complex was examined. We have observed discontinuous DNA synthesis, which was dependent on ATP or GTP. RNA primed initiation occured within

specific priming domains, which were located two to fourteen nucleotides downstream of hexanucleotides conforming to the formula 3' $C_2A_{1-2}(C_{2-3}/T_2)$, which we have called ψ. To investigate the function of ψ, one primase initiation site was cloned into M13, and subjected to oligonucleotide directed mutagenesis. Mutations in the ψ domain lead to changes in the rate of RNA primed DNA synthesis, and kinetic analysis showed that these changes were due to changes in the K_m with respect to DNA concentration (from an optimal 6pM to 240pM). Only the first three nucleotides of ψ are required for function, and the third nucleotide may be either an A or C residue. Thus, ψ has been redefined as a trinucleotide; 3' CC(C/A). Mutations in the priming domain lead to alterations in V_{max} primarily, with template pyrimidines required for priming, and initiation with ATP preferred over initiation with GTP. These two kinetically different template determinants may play a role in controlling Okazaki fragment initiation.

DNA polymerase α was also studied in a direct gap-filling assay. Using a defined template, DNA synthesis was primed from the M13 17mer universal primer, and blocked by an oligonucleotide hybridized 56 nucleotides downstream of the primer. DNA polymerase α filled this gap to completion. A time course of the reaction showed that in 50% of the substrate molecules, gaps were filled to completion within 10 minutes. In another 35% of the molecules the final nucleotide was lacking after 10 minutes. This nucleotide was added at a reduced rate, and was not incorporated into all of the molecules even after 6 hours. Limited strand invasion was observed in the remainder of substrate molecules; 42% of substrate molecules had undergone limited strand displacement after 6 hours of incubation. The reduced rate of incorporation of the

final nucleotide is reflected in an increased K_m for *de novo* incorporation of one nucleotide at a single nucleotide gap (0.7μM), as opposed to the K_m for incorporation of one nucleotide into singly primed M13 DNA (0.18μM). DNA polymerase α_2, and DNA polymerase purified from cells infected with the parvovirus minute virus of mice, exhibited the same kinetics of gap filling as did DNA polymerase α purified from uninfected Ehrlich ascites mouse tumor cells.

Taken together, DNA polymerase α is an enzyme complex which is well adapted to synthesis of DNA on the lagging strand, of a replication fork.

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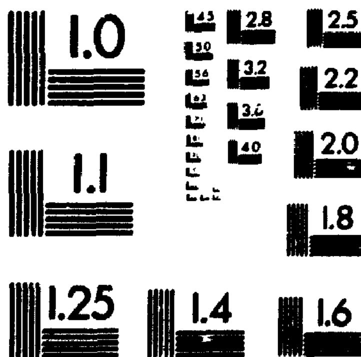
1.0
2.8
2.5
3.2
2.2
3.6
4.0
2.0
1.1
1.8
1.25
1.4
1.6

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