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Mehrpouyan, Majid, Ph.D.

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Ann Arbor, MI 48106

A TEMPERATURE-SENSITIVE MUTANT OF *ESCHERICHIA COLI* AFFECTED IN THE ALPHA SUBUNIT OF RNA POLYMERASE

A Dissertation

Presented to

the Faculty of the Department of Biochemistry

James H. Quillen College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Biomedical Sciences

with Emphasis in Biochemistry

---

by

Majid Mehrpouyan

December, 1990

APPROVAL

This is to certify that the Graduate Committee of

MAJID MEHRPOUYAN

met on the

Second day of September, 1989

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

W Scott Champney
Chair, Graduate Committee

David A. Johnson

Arthur E. Hougland

Lee M. Pike

Signed on behalf of
the Graduate Council

Associate Vice-President for Research
and Dean of the Graduate School

ABSTRACT

A TEMPERATURE-SENSITIVE MUTANT OF ESCHERICHIA COLI AFFECTED IN THE ALPHA SUBUNIT OF RNA POLYMERASE

by

Majid Mehrpouyan

A temperature-sensitive mutant of *Escherichia coli* affected in the alpha subunit of RNA polymerase has been investigated. Gene mapping and complementation experiments placed the mutation to temperature-sensitivity within the alpha operon at 72 min on the bacterial chromosome. The rate of RNA synthesis in vivo and the accumulation of ribosomal RNA were significantly reduced in the mutant at 44°C. The thermostability at 44°C of the purified holoenzyme from mutant cells was about 20% of that of the normal enzyme. Assays with T7 DNA as a template showed that the fraction of active enzyme competent for transcription was reduced as a function of assay temperature but that initiation and elongation were not significantly affected by the alpha mutation. A major effect on the fidelity of transcription was observed with the mutant enzyme, with misincorporation on two different templates stimulated about four fold at 37°C. The role of the alpha dimer in the structure and function of RNA polymerase is discussed. In addition during the course of this study a new procedure for the purification of *E. coli* RNA polymerase was developed. This method is rapid, convenient, and useful for the preparation of enzyme from 1-5 grams of cells in two days. The ease and speed of this method allowed the rapid characterization of the mutant enzyme. This system should also find application for the purification of small quantities of other bacterial RNA polymerases that share the general chromatographic properties of *E. coli* RNA polymerase.

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CHAPTER 1

Introduction

Role of RNA Polymerase in Transcription

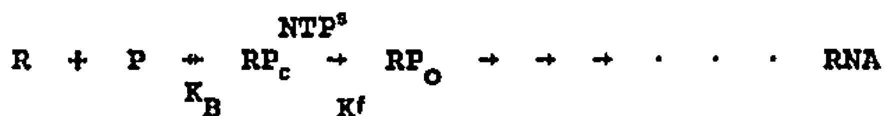
Transcription is the central process in cell growth and development in which genes are selectively located, recognized, and transcribed by DNA-dependent RNA polymerases to produce messenger, structural, and transfer RNA (tRNA) molecules. The DNA-dependent RNA polymerase is the key enzyme responsible for gene transcription in *Escherichia coli* and is thus one of the cell components essential for cell growth.

The basic reaction catalyzed by this polymerase is the transfer of a ribonucleoside monophosphate to the 3'-OH terminus of a growing RNA chain, using ribonucleoside triphosphate as substrate. In order for this polymerization process to occur in a meaningful physiological context, a number of regulatory requirements are imposed on the enzyme. The transcription cycle can be divided into several steps, including initiation steps that yield a tight complex of RNA polymerase with the promoter DNA sequence and allow the formation of the first phosphodiester bond, followed by elongation steps, and finally termination, in which the product (RNA) is liberated and the enzyme becomes available for the next cycle (Von Hipple et al., 1984).

Initiation

Initiation is the best understood of all phases of transcription and is generally described in terms of the following sequence of steps: (i) a recognition sequence (the promoter), usually positioned at the beginning of the sequence to be transcribed, is located and specifically bound by RNA polymerase; (ii) a portion of the promoter is melted to permit appropriate articulation of polymerase with the template DNA strand; and (iii) the first few nucleotides of nascent RNA are polymerized.

Compilations of DNA sequences of promoters have revealed a consensus consisting of the sequence TTGACA-(17 base pairs)-TATAAT-(5 to 9 base pairs)-RNA start (Hawley and McClure, 1983; Siebenlist et al., 1980). The latest compilation by Hawley and McClure (1983) pointed out that both the "-35 region" and the "-10 region" are primarily defined by 3 base pairs, TTG--- for -35 region and TA---T for the -10 region. The effect of DNA sequence on promoter strength has been analyzed quantitatively by studying the effect of base pair substitution on the rate and extent of active open complex formation. Open complex formation is generally described by a two-step interaction of RNA polymerase with the promoter (Chamberlin, 1974):



$$R + P \underset{K_B}{\rightleftharpoons} RP_c \xrightarrow[K^f]{} RP_O \xrightarrow{NTP^s} \rightarrow \rightarrow \cdot \cdot \cdot RNA$$

(i) binding, (ii) isomerization, (iii) promoter clearance.

The first step is described by an equilibrium constant, K_B, and the second step, a slow step termed isomerization, is described by a rate constant, K_f (McClure, 1980). Promoter binding strength assays, utilizing either productive transcription or a steady-state cycling assay have been used to quantitate separately these two constants describing RNA polymerase-promoter interaction. Promoter mutations can affect either K_B, K_f, or both (McClure, 1985). Generally mutations in the -35 region of the promoter can alter either K_B or K_f or both, but mutations in the -10 region of promoter primarily affect the isomerization rate.

Elongation

After the nascent transcript has grown to eight or nine nucleotide residues in length, sigma subunit is ejected from the transcription complex and the tendency of polymerase to "abort" transcription is lost. Also, the "transcription bubble" (the region of melted DNA and the RNA-DNA hybrid lying within it) grows to its mature size. These and other events mark the entry of polymerase into the elongation mode.

Elongation is, in one sense, an enzymatically catalyzed polymerization reaction since it involves the assembly of a high-molecular-weight polymer (RNA) from activated monomers. In this reaction the nucleotide triphosphate (NTP) monomers are selected by the polymerase in an order dictated by their complementary to the base sequence of the template DNA

strand. The details of how this selection works (or fails to work during misincorporation), and the details of the enzymatic chemistry per se, are not fully understood.

In another sense, elongation is a topological reaction. As the polymerase moves down the DNA, a locally melted bubble of DNA moves with it. Within this bubble an RNA-DNA hybrid is transiently formed and pealed apart. The topological requirements of elongation are potentially severe because a helical (rather than just a colinear) relationship exists between the two DNA strands, between the RNA strand and the DNA duplex upstream of the transcription bubble. Elongation thus requires the continuous solution of several unwrapping and rewrapping problems.

The elongation catalyzed by RNA polymerase is totally processive, in that a single polymerase molecule assembles the entire transcript by sequential nucleotide addition steps without dissociating, from either the DNA or the nascent RNA. To fully understand the shift from the initiation mode to the elongation mode, and to understand how transcription suddenly becomes nonprocessive at termination sites, it is necessary to study the interactions that maintain processivity during elongation (Yager and Von Hipple, 1987).

Termination

It is only from within the context of elongation that termination sequences in the DNA are recognized and

transcription is brought to an end. Thus an understanding of elongation is required as the foundation for a reasonable model of termination. Around 1970 it was recognized that, in principle, the cell could regulate gene expression at the level of transcript termination. Two key observations were made: (i) a number of bacterial and phage RNA transcripts were found to have similar 3' ends, suggesting the existence of specific transcription termination sites (Chamberlin, 1974); and (ii) Rho protein was first identified as a factor that induces transcription termination at particular loci (Roberts, 1969). The outline of a mechanism has been established for factor-independent ("simple") termination in a minimal in vitro transcription system containing only template DNA, *E. coli* RNA polymerase, and ribonucleotide triphosphates. However, there is still relatively little known about many aspects of the mechanism of Rho action and the mechanisms (and even the precise roles) of other termination factors (Yager and Von Hipple, 1987).

Structure and Function

E. coli RNA polymerase holoenzyme is a complex enzyme of molecular weight 484,000 and is composed of at least four different polypeptide chains, α, β, β', and δ, the molecular weight of each subunit being 38,500, 155,000, 165,000, and 87,000, respectively (Burgess, 1976). The holoenzyme is believed to have the structure $\alpha_2\beta\beta'\delta$. The core enzyme with the structure $\alpha_2\beta\beta'$, devoid of δ subunit, possesses all the

enzyme activities associated with the polymerase for carrying out RNA synthesis, except that it can scarcely initiate RNA synthesis from a promoter (Chamberlin, 1974).

The structural organization of E. coli RNA polymerase has been studied by electron microscopy (Lubin, 1969), small-angle neutron scattering (Stockel et al., 1980), and X-ray scattering (Meisenberger et al., 1980) as well as by cross-linking with bifunctional reagents (Coggins et al., 1977; Hillel and Wu, 1977) and by partial proteolysis (Lill and Hartmann, 1973; Fisher and Blumenthal, 1980). Subunit-specific polyclonal antibodies have been used to investigate the accessibility and function of the subunits in RNA polymerase (Stender, 1980, 1981) and to study the mode of subunit interaction (Trichelaar et al., 1983). These approaches have provided information regarding the dimensions of RNA polymerase and the gross shape of the individual subunits and the enzyme and have suggested possible modes of subunit interactions. Thus far, the regions involved in subunit-subunit interactions and possible changes in subunit conformation attendant to enzyme assembly remain undefined.

This complexity in structure is paralleled by the complexity of the transcription process catalyzed by RNA polymerase. Evidence has been obtained for the existence of several active sites in the enzyme, for example, two sites for the binding of substrate, one of which has been termed

initiation or product terminus site, and the other, the substrate site (Krakow and Von der Helm, 1971), a product site for the RNA transcript, a DNA-binding site (Jones and Berg, 1966), and different sites for the interaction of RNA polymerase with the specific transcription inhibitor rifampicin (Wehrli and Staehelin, 1971).

It is interesting to note that the bacteriophage-coded RNA polymerase molecules, for example from phage T7 or SP6, are structurally simpler, consisting of single polypeptide chains of molecular weight about 100,000 (Chamberlin et al., 1970). The occurrence of RNA polymerases of relatively smaller size which are able to catalyze the correct and complete transcription of certain templates or reading units shows that the complexity of prokaryotic RNA polymerase is not a mere image of the complexity of the transcription process. The functional difference between large prokaryotic and small "specialized" RNA polymerases is in transcription control. The small enzymes are rigidly programmed for the transcription of only one or a few reading units (Chamberlin and McGrath, 1971). Large RNA polymerases, in contrast, are subject to highly specific regulation of transcription of thousands of different reading units involving factors, effectors, structural modifications of RNA polymerase, and modulation of signals on the template. Thus one is tempted to assume a correlation between structural complexity of RNA polymerases

and multiplicity of control mechanisms. It is therefore important to study the role of each subunit for final understanding of the nature and extent of RNA polymerase participation in transcriptional control.

Roles of RNA Polymerase Subunits

The roles of RNA polymerase in transcription might be studied most directly by analysis of isolated subunits. However, many of the functions associated with each subunit are not manifested in the isolated states; the subunit assembly is required for their intrinsic activities to be manifested. In addition, functions that are dependent on the preceding step or reaction(s) carried out by other subunits cannot be studied in this way. The success of such analysis has been limited, but genetic and other approaches have been employed to elucidate subunit function. Thus the function of each of the subunits is only incompletely understood.

Role of ß Subunit

Various lines of evidence obtained from experiments with *E. coli* RNA polymerase indicate that ß subunits interacts with the antibiotic rifampicin (Krakow et al., 1976; Zilling et al., 1976). All mutations conferring resistance to this antibiotic affect only the ß subunit (Iwakura et al., 1973; Kawai et al., 1976). A derivative of rifampicin has been covalently attached to both ß and δ

subunits (Stender et al., 1975), suggesting that a peptide loop of δ subunit may be located near the rifampicin-binding site of ß on holoenzyme. However, cross-linking studies indicate that the rifampicin binding site is in the vicinity of all of the other subunits of RNA polymerase (Rice and Meares, 1978). Rifampicin bound to RNA polymerase is freely accessible to solvent molecules but not to antibodies to rifampicin, suggesting that it binds in a crevice (Mears and Rice, 1982; Stender and Scheit, 1977). The accessibility of rifampicin to solvent is markedly decreased by the removal of the δ subunit and somewhat decreased by DNA binding, leading Rice and Meares (1982) to suggest that the rifampicin binding site is located in a crevice of the enzyme in the vicinity of the template binding domain held open by the δ subunit. The reason for using rifampicin-resistant (Rif^r) mutations to probe the structure-function relationships of RNA polymerase were two-fold. First, the region of RNA polymerase to which rifampicin binds comprises an important functional domain in the enzyme. Studies on the mechanism of action of rifampicin indicate that rifampicin interrupts transcription by preventing elongation of the nascent RNA chain past a few nucleotides (Johnston and McClure, 1976), probably by binding in the vicinity of the substrate binding site. Either bound rifampicin imposes a steric block to translocation (McClure and Cech, 1978) or promotes dissociation of nascent RNA (Schulz and Zilling,

1981). Rif^r mutations decrease the binding of rifampicin to the enzyme and are likely to affect the function(s) of this region; analysis of such mutations is likely to provide information about structure-function relationship in this domain. Interestingly, other Rif^r mutations have been shown to alter termination at specific terminators. The work of Neff and Chamberlin (1980) was the first to show that in vitro termination is altered by some Rif^r mutations. Recently Junjin et al. (1988) have shown that the Rif^r mutations identify a region of the ß subunit that is involved in termination. Rif^r mutations in the N-terminal and very C-terminal portion of the ß subunit increase termination while those in the central portion of the region decrease termination.

Second, a large number of Rif^r mutations have been used to investigate the involvement of RNA polymerase in a variety of physiological processes. These mutations confer interesting phenotypes which are, in many cases, suggestive of underlying defects in transcription (Yura and Ishihama, 1979).

Role of ß' Subunit

Subunit ß' is able to bind to labeled DNA (Fukuda and Ishihama, 1974). The DNA binding capacity of other subunits is insignificant (Yurbrough and Hurwitz, 1974). Binding of $\beta\alpha_2$ to DNA, however, has been reported on the basis of electrophoretic analysis (Fukuda and Ishihama, 1974).

Subunit ß' binds to the polyanion heparin (Zilling et al., 1971). These findings have been considered as indicating the direct involvement of ß' in the interaction of RNA polymerase with DNA. On the other hand, ß' is the most basic subunit of RNA polymerase. Basic proteins may be expected to bind nonspecifically to polyanions like DNA.

Different types of binding of RNA polymerase to DNA have been described. Nonspecific binding is affected both by core enzyme and, with lower stability, by holoenzyme: site specific, tight binding probably occurs in two different states (Chamberlin, 1974). So far, the binding experiments performed with isolated ß' and other subunits or subassemblies do not allow a specification of the imprecise statement that ß' appears to be involved in template-binding of RNA polymerase.

In all prokaryotic RNA polymerases that have been analyzed by charge density electrophoresis so far, ß' has proved to be strongly basic or at least more basic than ß, suggesting a relation of net basic charge to function (probably DNA-binding) (Zilling et al., 1976; Von Hipple et al., 1984).

Role of δ Subunit

RNA synthesis can be catalyzed by core enzyme alone, but proper initiation from natural promoters on double-stranded DNA requires holoenzyme containing δ subunit (Chamberlin, 1974). Since δ subunit is essential for

selection of promoters by RNA polymerase, the mechanism of δ function has been the subject of intensive study. Although δ is implicated in the primary recognition of promoter DNA sequences, it alone cannot bind to DNA (Zilling et al., 1971). Thus the effect seems indirect, and the DNA-binding specificity of core enzyme may be altered by association with δ subunit. Indeed δ subunit, upon binding to core enzyme, induces a conformational change in the latter (Wu et al., 1976). In concert with this finding, both $\alpha_2\beta$ and ß' subunits interact with δ, and this interaction changes the DNA-binding activity of ß' (Fukuda and Ishihama, 1974). The regulatory function of δ is also in accord with the weak binding of δ to core enzyme (Travers, 1975), the dissociation constant being in the range of 10^{-6}-10^{-8}M (Campbell and Lowe, 1977).

Unlike the native core enzyme, the premature core enzyme is unstable and readily dissociates into $\alpha_1\beta$ and ß' (Ishihama et al., 1979). Premature core enzyme becomes more stable upon incubation at elevated temperatures, and this "maturation" process is promoted markedly by δ subunit (Saitoh and Ishihama, 1976). This δ subunit could work as a protein effector in determining the conformation of core assembly.

Studies of mutants that produce altered δ subunit provide strong evidence for the role of δ in transcription specificity. For instance, mutations in ß subunit often

suppress the phenotype of δ mutations (Yura and Ishihama, 1979). This and other evidence (Stender et al., 1975; Travers, 1975) suggest a close functional interaction between δ and ß subunits during transcription initiation.

Role of α Subunit

That only one type of α subunit is present in *E. coli* RNA polymerase has been shown by analysis of tryptic peptide patterns (Fujiki et al., 1976; Taketo et al., 1976) and by the complete amino acid sequence of purified α polypeptides (Ovichinikov et al., 1977). In contrast to the progress in the structural analysis, no clear function has yet been assigned to α subunit.

Reconstitution of the *E. coli* RNA polymerase core enzyme from its constituent subunits has been extensively studied (Saitoh and Ishihama, 1976; Ishihama, 1981). The first reaction in the assembly sequence is the dimerization of the α subunit and only the dimeric form of the α subunit is able to accept the ß subunit.

RNA polymerase dissociation at low concentration of urea leads to formation of an αß and an αß' complex (Ishihama, 1972). Both combinations of subunits are capable of binding to DNA. Since the isolated ß subunit, but not the ß', lacks the ability to complex with DNA, subunit α seems to be required for the DNA-binding activity to be exposed (Fukuda and Ishihama, 1974). In agreement with this interpretation, Ishihama et al. (1980) found temperature-

sensitive E. coli strains carrying mutations in the α-subunit gene (rpoA). The RNA polymerase from these mutant cells exhibited lower fidelity in transcription than wild-type RNA polymerases, implying that the α subunit is somehow involved in the recognition of the template sequence. Furthermore, one of these mutants showed peculiar properties including thermolability of the purified RNA polymerase and cessation of RNA synthesis after a considerable lag in the temperature rise. These observations suggested a defect in the assembly of RNA polymerase, resulting ultimately in the cessation of RNA synthesis. This indicated that either the dimerization of newly synthesized α subunits or the subsequent association the of altered α dimers with β subunits was blocked in this particular mutant, at non-permissive temperatures (Kawakami and Ishihama, 1980).

There is evidence indicating that the dimeric α subunits in RNA polymerase are not equivalent. Following infection of E. coli with phage T4, initially only one of the α subunits is ADP-ribosylated (Rohrer et al., 1975), indicating that the α subunits are differently arranged in RNA polymerase. Treatment of RNA polymerase with 0.1 mM ρ-(chloromercuri) benzoate results in the release of one of the α subunits (Ishihama, 1981). The unequal exposure of the α subunits in RNA polymerase is also indicated by the data obtained with the monoclonal antibodies (Riftina et al., 1989). It would appear that one of α subunits is

relatively exposed while the other α is positioned with more of its surface shielded by the larger β and β' subunits. This is consistent with the observations of Stender (1980) regarding the low accessibility of core enzyme to polyclonal anti-α antibodies. Trichelaar et al. (1983) showed by immune electron microscopy that almost half of the α surface is covered by the β and β' subunits. On the basis of studies with subunit-specific antibodies, Trichelaar et al. (1983) proposed that δ subunit resides at the concave side of the core where it interacts with the α dimer. Despite all this information on the α subunits of *E. coli* RNA polymerase, the role of the α subunits remains to be defined.

The analysis of functional consequences of mutations within the subunits of RNA polymerase is potentially one of the most powerful tools for investigating the role of subunits in transcription. The following research describes a study of a mutation in the α-subunit gene (rpoA) conferring temperature-sensitivity to the *E. coli* RNA polymerase. The objective is to characterize this temperature-sensitive mutant and to understand how this mutation has affected RNA polymerase function in vitro.

CHAPTER 2

Materials and Methods

Materials

Acrylamide, methylene bis-acrylamide, streptomycin sulfate, all 20 L-amino acids, polyuridylic acid, phosphoenolpyruvate (PEP) kinase, tRNA, levigated alumina, PPO (2,5-diphenyloxazole), Coomassie Brilliant Blue (R-250), dithiothreitol (DTT), calf thymus DNA, double-stranded DNA-cellulose, yeast RNA, Tris base, ethylene diaminetetraacetic acid (EDTA), glycerol, polyethyleneglycol (PEG), and nucleoside triphosphates (ATP, GTP, CTP, and UTP) were purchased from Sigma. [^{3}H]-uridine (14 Ci/mmole), [^{14}C]-leucine (278 mCi/mmole), [^{14}C] phenylalanine (525 mCi/mmole), [^{3}H] UTP, and [^{3}H] CTP (25 Ci/mmol) were purchased from New England Nuclear. Toluene, cesium chloride (CsCl), trichloroacetic acid (TCA), and ß-mercaptoethanol were purchased from Fisher Chemicals. All culture media used in this study were purchased from Difco.

Bacterial Strains

Escherichia coli SK901 (F^-) (Kushner et al., 1977), SK1046 (F^-) (Champney, 1979), and N01379 (rpsL, rpsE, λFusA, RecA) (Jaskunas et al., 1975) were used in this study.

Media and Cell Growth

Bacterial strains were stored at -80°C in 1 ml aliquots of tryptone broth containing 50% glycerol. Cells were grown in Luria broth (LB) or M9 minimal salts solutions. Luria broth consisted of 1 gm bacto-tryptone, 1 gm NaCl, 0.5 gm yeast extract, and 0.2 ml 1.0 M NaOH in 100 ml. M9 minimal salts solution contained 7.0 gm Na_2HPO_4, 3.0 gm K_2HPO_4, 0.5 gm NaCl, 1.0 gm NH_4Cl, and 0.2 gm $MgSO_4$ per 100 ml of solution. Plates were made with the same media containing 2% (w/v) agar. Growth experiments were done in a shaking water bath using 50 ml side-arm flasks. The cell density was determined by using a Klett-Summerson colorimeter with a red (no. 66) filter or by reading absorbance at 600 nm in a Beckman Model 25 spectrophotometer.

Bacteriophage Isolation

Lambda defective phages were isolated by isopycnic centrifugation in CsCl (Davis et al., 1980) after heat induction of the double lysogens. Lysogens were grown to a cell density of approximately 2 x 10^8 cells/ml at 32°C, shifted to 42°C for 20 minutes, and then returned to 37°C for 3 more hours for optimal lysis and phage yield. The defective phage contained approximately 1% contaminating helper phage after CsCl centrifugation as determined by plaque analysis on strain SK901.

Mutational Mapping with Transducing Phages

The mapping of the temperature-sensitive mutation was performed by the following complementation procedure. Cells were grown in tryptone/yeast broth to about 4 x 10^8 cells/ml, centrifuged and suspended in 0.2 volume of λ dilution buffer (10 mM Tris-HCl (pH 7.6), 10 mM $MgSO_4$), and infected with one of the transducing phages (λ Fus 2, λSpc_2, or λ TrkA). After 15 minutes, cells were spread on tryptone plates and were incubated at 44°C for two days. If the transducing phage complemented the temperature-sensitive mutation, abundant cell growth was observed at 44°C. If the phage did not contain the wild-type allele of the temperature-sensitive mutation, the temperature-sensitive cells were not rescued after incubation at 42°C.

Mutational mapping was also carried out by generalized transduction with P_1 phage propagated on the mutant strain, SK1046. Host strain, SC703 (aroE, STR^R), was infected with the P_1(1046) phage at a multiplicity of 0.5 phage particle/cell (Hong and Ames, 1971). After 20 minutes at 32°C cells were centrifuged, washed and resuspended in 0.1 ml of M9/glucose medium, and spread on minimal agar plates. After incubation for 3 to 4 days at 32°C, $aroE^+$ colonies appeared. They were then picked and tested for growth at 42°C and for streptomycin resistance on streptomycin (100 μg/ml) agar plates. Cotransduction was determined as the inheritance of unselected temperature-sensitivity (ts) or

temperature-resistance (tr) in antibiotic-resistant or $aroE^+$ selected transductants.

Cell Free Protein Synthesis Assays

Cell-free synthesis of polyphenylalanine was based on the method of Traub et al. (1971), as previously described (Kushner et al., 1977), using 3.0 A_{260} units of purified 70S ribosomes, 65 µg of S100 protein, 3 µg phosphoenolpyruvate (PEP) kinase, 40 µg polyuridylic acid, approximately 200 pmoles ^{14}C-phenylalanine (525 mCi/mmole), 45 µl of Mix I, a final volume of 150 µl. Mix I contained 16.7 mM $MgOAc_2$, 66.8 mM NH_4Cl, 16.7 mM Tris-HCl pH 7.6, 3.33 mM ATP, 0.1 mM GTP, 16.6 mM PEP, 3.3 mM DTT, 12 mM ETSH, 0.167 mM each of 19 L-amino acids (no phenylalanine), 3.33 mg/ml _E_. _coli_ total tRNA, in a final volume of 3.0 ml.

Assays were carried out at 32°C and 44°C for 30 minutes. Samples of 20 µl were removed at 0, 2.5, 5, 10, 20, and 30 minutes. Samples were mixed with 3 ml of 5% trichloroacetic acid, boiled for 15 minutes at 95°C, filtered on Whatman GF/C filters, and counted. Incorporation of ^{14}C-phenylalanine was measured by liquid scintillation counting in a Beckman LS-3155T counter. Counting was 85% for ^{14}C and 60% for ^{3}H-isotopes.

Kinetics of In Vivo RNA and Protein Synthesis

The effect of culture temperature on in vivo RNA and protein synthesis were carried out following the method of

Ishihama et al. (1980). Cells of E. coli SK901 (wild type) and SK1046 (mutant) strains were grown at 32°C on M9/glucose minimal medium. The cell density of cultures was determined by a Klett-Summerson colorimeter. At a turbidity (Klett reading) of 30, portions of 3 ml were transferred to prewarmed flasks each containing 20 μCi of [^{3}H]-uridine with 20 μg of uridine and 1.0 μCi of [^{14}C] leucine with 14.8 μg leucine. Incubation was continued at either 32°C or 44°C in water baths. Portions of 0.1 ml were taken at 10, 20, 40, and 60 minutes after transfer to determine the incorporation of ^{3}H and ^{14}C radioactivity into acid-insoluble forms. Precipitation was carried out by addition of 2 ml of ice-cold 10% TCA to each 0.1 ml sample, and the mixture was kept on ice prior to filtration on Whatman GF/C filters. Incorporation of radioactive substrates was measured in a liquid scintillation counter as described. Corrections were made for the spillover of ^{14}C-radioactivity into the ^{3}H-channel.

In Vivo Radiolabeling of Ribosomal RNA (rRNA)

For in vivo synthesis of ribosomal RNA, wild type and mutant cells were grown at 32°C as described above. At a turbidity (Klett reading) of 30, portions of 3 ml of each culture were transferred to 32°C and 44°C water baths. After 10, 15, and 20 minutes, samples of 0.5 ml were transferred to tubes containing 1 μCi of [^{3}H]-uridine. Incorporation of labeled substrate was allowed for 3 minutes

followed by addition of 0.1 ml uridine (1 mg/ml) for 1 minute. Samples were then centrifuged and cell pellets were frozen at -20°C until further use.

Lysis of Cells and Extraction of Ribosomal RNA (rRNA)

Cell lysis and rRNA extraction was carried out by the method of Stark et al. (1982). The frozen cell pellets were washed with 0.5 ml lysis buffer (25 mM Tris-HCl pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 20% (w/v) sucrose) and resuspended in 25 µl of the same buffer containing 150 µg lysozyme/ml. After 3 cycles of freezing (in dry ice/ethanol) and thawing (in warm water), the cells were placed on ice and mixed with the following (added as a cocktail): 100 µl TKM buffer (25 mM Tris-HCl (pH 8.0), 60 mM KCl, 5 mM $MgCl_2$), 35 µl of 1% (w/v) sodium deoxycholate, 20 µl of 5% (w/v) Brij 58, 10 µl of DNase I (1 mg/ml), 8.5 µl of 0.1 M $MgSO_4$. The final concentration of Mg^{+2} was 8.0 mM. After 15 minutes at 0°C the cell debris was pelleted in an Eppendorf microfuge (2 minutes at approximately 12,000 revs/minute) and the lysate frozen at -80°C unless required immediately.

Ribosomal RNA was extracted from the lysate by mixing the latter for 30 seconds with an equal volume of water-saturated phenol and 0.1 volume of 10% (w/v) sodium dodecyl sulfate. After a brief centrifugation to separate the phases, ribosomal RNA was recovered from the aqueous phase by precipitation with ethanol (2 volumes) in the presence of

0.1 volume 5 M NH_4 acetate for at least 1 hour at -20°C. Precipitates were collected by centrifugation for 2 minutes in the microfuge, dried in vacuo, and redissolved in 100 µl TKM buffer containing 10 mM $MgCl_2$, 25% (w/v) sucrose, and 0.1% (w/v) xylene cyanol.

Electrophoresis of Ribosomal RNA (rRNA)

Electrophoretic analysis of rRNA was performed in a composite gel containing 2.85% (w/v) acrylamide, 0.15% (w/v) N'-N'-methylene bisacrylamide, and 0.5% (w/v) agarose polymerized at room temperature. Electrophoresis was done at 4°C using a vertical slab gel apparatus. Gels were prepared essentially according to Dahlberg (1982) and were 3 mm thick. Samples of rRNA were electrophoresed for 4 hours at 300 V in gels containing the Tris/borate/EDTA (89 mM Tris, 89 mM borate, 2.5 mM EDTA) buffer of Peacock and Dingman (1967). After electrophoresis, gels containing rRNA were soaked in 1 M Na salisilate for 20 minutes (Chamberlin, 1979), dried down onto Whatman 3 MM paper, and placed against Kodak XR-5 films. Fluorographic exposures were for 1 week at -70°C. The amount of labeled rRNA species was determined by a densitometer tracing of the flurogram.

Buffers and Solutions for Partial Purification of RNA Polymerase

All buffers used in DNA-cellulose column chromatography contained 0.05 M Tris-HCl (pH 7.9, at 25°C), 0.5 mM EDTA, 5%

(vol/vol) glycerol, and 1 mM DTT (TEGD buffer). DTT was added just prior to use from a frozen stock solution (0.1 M). Magnesium has been removed from all buffers, both to prevent DNase action on DNA-affinity columns and also to prevent growth of mold.

The following solutions were used in the preparation of cell extracts: (solution A) 0.01 Tris-HCl (pH 7.9), 25% (w/v) sucrose, and 0.1 M NaCl; (solution B) 0.3 M Tris-HCl (pH 7.9), 0.1 M EDTA, and lysozyme at 4 mg/ml added just before use; (solution C) 1.0 M NaCl, 0.02 M EDTA, pH 7.0, and 0.08% (w/v) deoxycholate; (solution D) 17% (w/v) PEG, 0.157 M NaCl, and 0.01 M DTT added just before use; (solution E) 5% PEG, 2.0 M NaCl, 0.01 M Tris-HCl (pH 7.9), and 0.01 M DTT added just before use. All components except for lysozyme and DTT were sterilized prior to use and stored at 4°C.

Cell Disruption and Preparation of Crude Extract

A crude extract was prepared according to procedure of Gross et al. (1976). One liter of cells grown in rich medium to an A_{450} = 0.6 was centrifuged at 10 K rpm for 10 minutes. The cell pellet was suspended and lysed by addition of 2 ml of solution A. The suspension was kept on ice for 20 mintues followed by 3-4 cycles of freezing and thawing. To this suspension 0.5 ml of solution B was added. After 5 minutes at 0°C, 2.5 ml of solution C was added. The mixture was kept at 10°C for 10 minutes. RNA polymerase was

precipitated with PEG by addition of 7 ml of solution D and the mixture was kept on ice. Thorough mixing was important at this step. After 15 minutes on ice, the precipitated RNA polymerase and DNA (PEG precipitate) was centrifuged at 7 K rpm for 10 minutes, and the supernatant was discarded. At this stage RNA polymerase was extracted from the PEG precipitate by the addition of 2 ml of solution E and dispersion of the pellet. After centrifugation at 10 K rpm for 10 minutes, the supernatant was saved and diluted to 0.15 M NaCl with 26.6 ml of TEGD buffer.

DNA-Cellulose Column Chromatography

A DNA-cellulose column was prepared by suspension of 10 grams of DNA-cellulose in 40 ml TEGD + 0.15 M NaCl at 4°C. After one hour the suspension was poured into a column (1.8 x 10 cm) and packed. The diluted crude extract was passed through the DNA-cellulose column, previously equilibrated with TEGD + 0.15 M NaCl, at flow rate of 50 ml/hour. The column was washed with 40 ml TEGD + 0.15 M NaCl and was developed by with TGED buffer + 0.75 M NaCl. To obtain efficient elution in a small volume, it was necessary to add approximately one half-column volume of TEGD + 0.75 M NaCl, to wait for 15 minutes, and then to add one-column volume of the high salt buffer. The eluted peak of RNA polymerase activity was pooled and dialyzed overnight against storage buffer (TEGD in 50% glycerol) and stored at -20°C until further use.

Assay of RNA Polymerase Activity

RNA polymerase activity was assayed in the following mixture (final volume of 0.1 ml): 25 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 0.2 mM CTP, 0.2 mM GTP, 0.2 mM ATP, 0.05 mM UTP, 1 μCi of [^{3}H] UTP, 6 μg of calf thymus DNA, 1 mM K_2HPO_4 (pH 7.0), 1 mM DTT, and 50 μg of BSA. The final NaCl concentration was adjusted to 0.15 M for each assay. Samples were incubated at 32°C for 10 minutes (unless otherwise indicated) and quenched with 3 ml of cold 10% trichloroacetic acid and kept on ice for at least 10 minutes. Each sample was filtered onto Whatman GF/C filters, washed, dried, and the radioactivity was determined in a scintillation counter.

For RNA polymerase kinetic activity assays, 17 μl samples were taken at 10, 20, 30, 40, and 50 minutes from the assay mixture and the radioactive incorporation for each sample was determined as described above. One unit of RNA polymerase activity was defined as the amount of RNA polymerase that gave a rate of incorporation of one μ mole UMP in 10 minutes at 32°C.

Protein Determination

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Analytical Gel Electrophoresis

Electrophoresis of 5-100 μg samples of protein, reduced with 20 mM mercaptoethanol and denatured with 0.1% SDS, was carried out in 7.5% polyacrylamide gels (1.5 mm thick) as described by Laemmli (1970), using a Bio-Rad Mini-vertical Model 360 slab gel electrophoresis apparatus (8 cm x 8 cm). An KLB 2103 power supplies provided 15 mA during stacking and 20 mA constant current after the tracking dye entered the separating gel. The gels were fixed with 20% trichloroacetic acid for 30 minutes, stained with Coomassie Brilliant Blue dye (1.2 g/liter) in methanol:glacial acetic acid:water (1:1:8) for 2 hours, and destained with ethanol:glacial/acetic acid:water (1:1:8).

Preparation of Single-Stranded DNA-Aragose Column

Single-stranded calf thymus DNA-agarose was prepared as described by Schaller et al. (1972) and Nüsslein and Heyden (1972) with the modifications of Lowe et al. (1979). Calf thymus DNA (600 mg) was thoroughly dissolved in 0.02 M NaOH (40 ml) to a concentration of 15 mg/ml. The homogeneous solution was heated at 55°C and mixed with a half volume of 6% (w/v) agarose which had been boiled and autoclaved to dissolve the agarose and cooled to 55°C. The single-stranded DNA solution was added to the agarose and the mixture was mixed thoroughly for 5-10 minutes at 55°C. The mixture was solifidied rapidly by pouring in a large

shallow, ice-cooled glass dish. The gel was cut into 1-cm cubes and passed twice through a 60-mesh stainless steel sieve. The fragmented DNA-agarose was suspended in a solution of 0.01 M Tris-HCl, pH 7.9, 1 mM EDTA, 0.25 M NaCl, packed into a column, and washed extensively at room temperature with the same buffer until no DNA could be detected in the wash by its absorbance at 260 nm. During the washing procedure about 25% of the input DNA was lost. The final preparation contained 3.5 mg of calf thymus DNA per ml bed volume. The DNA agarose fragments were suspended in 0.01 M Tris (pH 7.5), 1 mM EDTA, 1 M NaCl and stored at 4°C.

Preparation of DNA-Free Cell Extract

DNA-free cell extract was prepared following the method of Humphries et al. (1973). The following operations were carried out at 4°C. A 6 gram sample of frozen cells, which was obtained from 12 liters of rich medium culture (A_{600} = 0.7), was lyzed by grinding with alumina and suspended in 20 ml of TEGD buffer containing 2 M NaCl. The suspension was centrifuged at 10 K rpm for 15 minutes to remove debris. To this suspension 5 ml of TEGD containing 30% (w/v) polyethylene glycol 6000 and 2 M NaCl was slowly added to give a final polyethylene glycol concentration of 6%. After gentle stirring for 15 minutes at 0°C, the cloudy suspension was centrifuged at 10 K rpm for 15 minutes and the clear supernatant was dialyzed for 5 hours against 5 liters of

TEGD buffer containing 50 mM NaCl. After centrifugation at 10 K rpm for 10 minutes to remove a fine precipitate, the supernatant was then ready to be applied to the single-stranded DNA-agarose column.

Affinity Chromatography of Holoenzyme on Single-Stranded DNA-Agarose Column

The DNA-free cell extract was applied at 30 ml/hour to a 50 ml single-stranded DNA-agarose column (2.8 x 17 cm) equilibrated with TEGD buffer + 0.15 M NaCl. The flow was then shut off and the cell extract was allowed to remain in contact with the DNA content of the column for 30 minutes. After washing with TEGD buffer + 0.25 M NaCl until the absorbance at 280 nm was lower than 0.1, core polymerase was eluted with 60 ml of TEGD buffer + 0.4 M NaCl. Holoenzyme was finally eluted with TEGD + 1 M NaCl at 20 ml/hour. Core polymerase and holoenzyme were pooled separately. It was important to decrease the flow rate during the 1 M NaCl elution to allow time for the holoenzyme to dissociate from the column and to increase the concentration of protein in the fractions.

Purification of Holoenzyme by Sephacryl S-200 Gel Filtration

Pooled holoenzyme fractions, from the single-stranded DNA-agarose column, were dialyzed against TEGD buffer + 1 M NaCl containing 20% polyethylene glycol (20,000) for 30 minutes to 1 hour to reduce the volume. The dialyzed

fraction (2 ml) was then applied at 25 ml/hour to a 75 ml Sephacryl S-200 column (2.5 x 17 cm), which was preequilibrated with TEGD buffer + 1 M NaCl. Holoenzyme was eluted in the void volume (33 ml) and was pooled (fractions 10-16) and dialyzed against storage buffer (TEGD + 0.1 M NaCl containing 50% glycerol) overnight and stored at -20°C until further use. Dialysis against storage buffer resulted in an approximately threefold concentration.

RNA polymerase activity, during the purification procedures on single stranded DNA-agarose and Sephacryl gel filtration columns, was monitored by the assay described earlier using calf thymus DNA as a template.

Quantitative Assay for Holoenzyme with Bacteriophage T7 DNA

The quantitative assay for holoenzyme was carried out according to the method of Neff and Chamberlin (1980) and Chamberlin et al. (1979). To carry out the standard assay, a reaction mixture was prepared in a tube at 0°C containing 20-80 μl of AB diluent (10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 10 mM ß-mercaptoethanol, 50 mM NaCl, 10 mM EDTA, 5% glycerol, 100 μg/ml of acetylated bovine serum albumin), 40 μl of solution A (1.4 ml of d.H_2O, 0.4 ml of 1 M Tris-HCl, pH 8.0, 0.1 ml of 1 M $MgCl_2$, 0.1 ml of 1 M ß-mercaptoethanol), 25 μg of T7 DNA, 10 μl of [^3H] UTP (25 Ci/mmol), and a mixture of the three other nucleoside triphosphates (4 mM each). A sample (20 μl) was removed as a zero time control and was mixed with 200 μl of carrier

solution (50 mM sodium pyrophosphate and 50 mM EDTA with 0.5 mg/ml of yeast RNA). Nucleic acids were then precipitated by adding 3 ml of an ice-cold solution of 10% trichloroacetic acid, and the mixture was kept on ice at least for 10 minutes prior to filtration on Whatman GF/C filters.

A sample of RNA polymerase containing 0.2 to 2 milliunit was added to the reaction mixture, and the solution was mixed well. This mixture could be kept on ice up to 10 minutes without affecting the subsequent reaction. RNA synthesis was initiated by transferring the tube to a water bath at 32°C (zero time) with about 10 seconds of gentle shaking to facilitate thermal equilibration. At 1.5 minutes, 4 μl of a solution of rifampicin (1 mg/ml in 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, protected from light, and stored at -20°C) was added to the reaction. Samples of 20 μl were taken at 2, 4, 5, 6, 8, 10, and 12 minutes and were precipitated with TCA like the control. After standing for at least 10 minutes in cold trichloroacetic acid, the samples of labeled RNA were collected by filtration as described earlier. Each filter was washed with 5 ml of 10% TCA and 5 ml of ethanol. After drying, the samples were counted in a toluene-based mixture in a scintillation counter.

One unit of RNA polymerase activity is defined as the amount of RNA polymerase that gives a rate of incorporation

of one μ mole of UMP in 10 minutes at 32°C using T7 bacteriophage DNA as a template.

Kinetics of RNA Polymerase using the Standard T7 Assay

Standard RNA polymerase conditions were employed with rifampicin to inhibit re-initiation of RNA chains as described earlier. The preincubation at various temperatures was for 5 minutes followed by an assay at 32°C or 37°C. Calculations and theory for the kinetic assays are described in Chapter 3.

Electrophoresis of T7 RNA Transcripts

The quantitative assay for holoenzyme using T7 DNA was carried out as described earlier. In some cases the enzymes were preincubated at various temperatures, 37°C or 44°C, for 5 minutes prior to a fixed time point assay (10 minutes) at 32°C. The reaction was terminated by addition of an equal volume of solution containing 0.1 M EDTA, 0.1% SDS, 27 mM Tris, 27 mM boric acid, 0.1% bromophenol blue, and 30% glycerol. The mixture was stored at -20°C until further use.

Electrophoretic separation of T7 RNA transcript was carried out on a vertical slab gel (32 x 10 cm x 10 mm) contianing 2% polyacrylamide, 0.5% agarose, 89 mM Tris, 89 mM borate, and 2.5 mM EDTA (Peacock and Dingman, 1968). Electrode buffer was the same as the gel's except the buffer contained 0.1% SDS. Samples of RNA transcripts were

electrophoresed at 300 V for 4 hours at 4°C. After electrophoresis, gels containing RNA transcripts were dried and exposed for fluorography, as described earlier.

Kinetics of Heat Inactivation

Heat inactivation kinetics were carried out based on the method of Ishihama et al. (1980). A sample of 240 µl of the wild type enzyme (9.12 µg of protein equivalent to 7.38 pmole of active holoenzyme) and 800 µl of mutant enzyme (33.6 µg of protein equivalent to 9.84 pmole of active holoenzyme), in a final concentration of 25% (v/v) glycerol and 0.5 M NaCl, were incubated at 44°C. At 5, 10, 15, 20, and 30 minutes samples of 40 µl of wild type enzyme were removed and assayed for 10 minutes at 32°C in the standard T7 assay in the absence of rifampicin. For the mutant enzyme, samples of 100 µl were removed at 1, 2, 3, 4, 5, 10, and 20 minutes and were assayed at 32°C as for the wild type enzyme. One hundred percent activity corresponded to 1.23 pmole of active holoenzyme, equivalent to 1.52 µg of wild type, and 4.2 µg of mutant RNA polymerase holoenzyme.

Transcriptional Fidelity Assays

Assays for the fidelity of transcription were carried out according to the method of Ishihama et al. (1980). Two different synthetic DNA templates, poly [d(G)]·poly[d(C)] and poly[d(A)]·[d(T)], were used for transcriptional fidelity assays.

Initially, the activity of each enzyme (wild type or mutant) was assayed according to the procedure described earlier (Gross et al., 1976), except that calf thymus DNA was replaced by one of the synthetic DNA templates. Equal activity units of both RNA polymerases (1.5 μg of wild type and 2.1 μg of mutant enzyme) were used for all fidelity assays.

Transcriptional fidelity was assayed in the following mixture (final volume 100 μl): 25 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM K_2HPO_4 (pH 7.0), 1 mM DTT, 50 μg of BSA, and 6 μg of one of the synthetic DNA templates. When poly[d(G)]·[d(C)] was used as a DNA template, 5 μCi of [^{3}H] UTP (25 Ci/mmole) and 0.2 mM GTP were added to the assay mixture. When poly [d(A)]·[d(T)] was used as a template, 5 μCi of [^{3}H] CTP (25 Ci/mmole) and 0.2 mM ATP were used in the assay. The reaction mixture was incubated at 32°C or 37°C and 20 μl samples were taken at intervals. Each sample was quenched with 3 ml of cold 10% (w/v) trichloroacetic acid and kept on ice for at least 10 minutes prior to filtration onto Whatman GF/C filters as described earlier. After drying, the samples were counted in a scintillation counter.

CHAPTER 3

Results

Mutational Mapping

Since the exact location of the structural gene for the α subunit of RNA polymerase (rpoA) on the chromosome of *E. coli* is known (Jaskunas et al., 1975), it is possible to isolate temperature-sensitive (ts) mutations in the rpoA gene by localized mutagenesis of that part of *E. coli* chromosome. Several temperature-sensitive mutations within the strA-aroE region were found. Among those, mutant SK1046 showed the following characteristics. Complementation of the temperature-sensitive mutation by specialized λ transducing phages was carried out as described in Chapter 2. The results are presented in Table 1. Only λ Spc2 and λ Fus2, but not λ TrkA phages, were capable of complementing the mutation in the *E. coli* chromosome, suggesting that the mutation should be located in the Spc or alpha operons. In addition, transductional mapping by P1 transducing phage resulted in 60% co-transduction between the mutational site and the aroE gene, indicating that the temperature-sensitive mutation was in the alpha operon.

In Vitro Protein Synthesis

In order to exclude the possibility of a mutation in the genes coding for ribosomal proteins within the alpha operon, cell-free translation assays were carried out using

Table 1. Complementation of Temperature-Sensitive Mutation with Specialized λ Transducing phages.

λ Phage	Number of Colonies[a]
λ TrkA	5
λ Spc2	540
λ Fus2	560

[a]Colonies appeared on tryptone plates at 44°C after 2 days.

purified ribosomes from the mutant and wild type strains. Synthesis of polyphenylalanine directed by polyuridylate was compared for both sources of ribosomes at a permissive (32°C) and non-permissive temperature (42°C). The results are given in Figure 1. For the mutant the activity was the same as that of the wild type strain at the permissive temperature. The cell-free translational activity of the mutant ribosomes was slightly higher than that of wild type strain at the non-permissive temperature. In addition two dimensional gel electrophoresis of purified ribosomal proteins from both strains showed no altered protein. The results suggest that the ribosomes and translational components were not altered for the mutant strain.

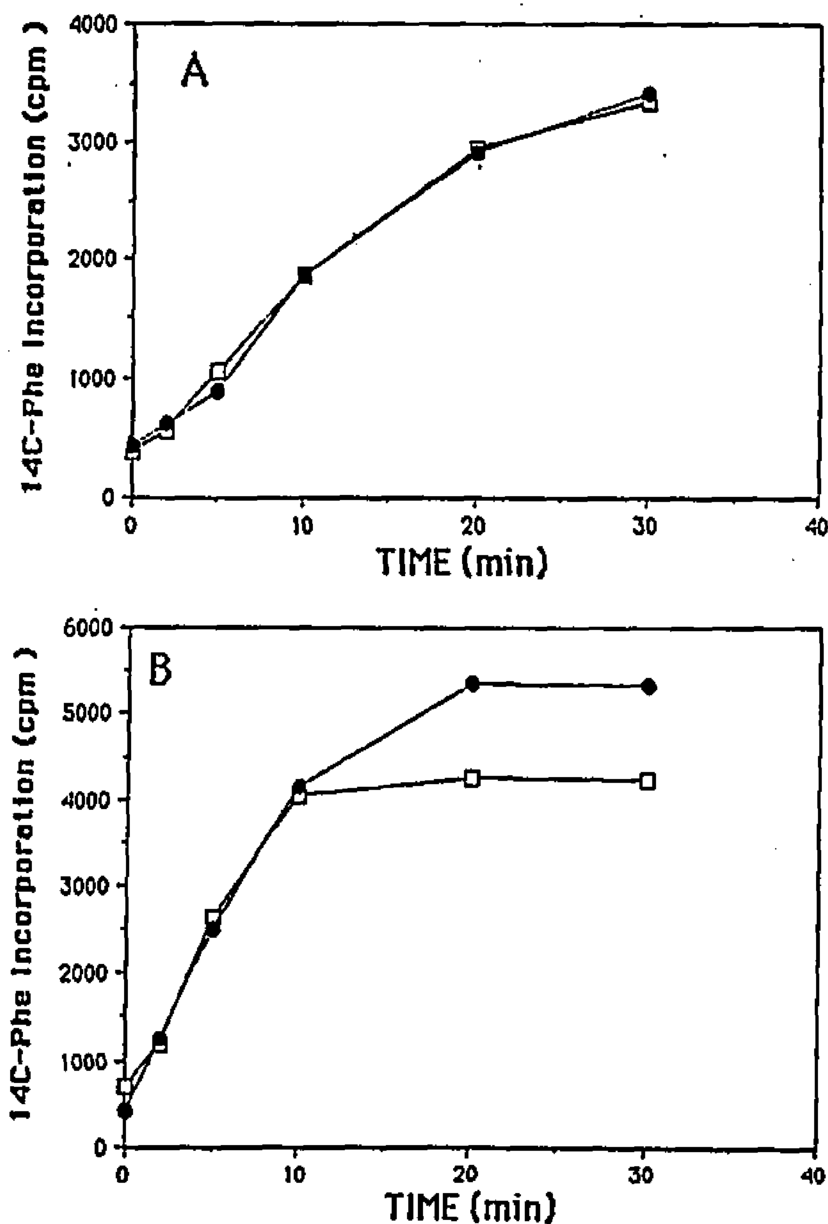
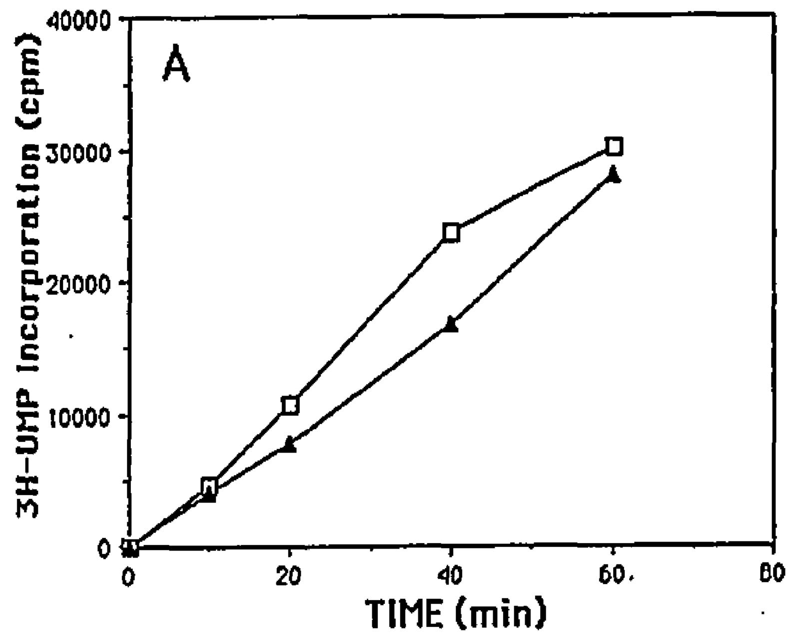


Figure 1. Kinetics of In Vitro Protein Synthesis. Purified ribosomes (3.0 A_{260} units) from wild type and mutant strains as described in Materials and Methods. The assay was carried out at 32°C (A) and 44°C (B). Data are for 20 μl samples taken at intervals during the assay. (□) and (● represent ^{14}C-polyphenylalanine formation for wild type and mutant ribosomes, respectively.

In Vivo RNA and Protein Synthesis

The effect of the cell growth temperature on both protein and RNA synthesis was investigated with the mutant and the wild type strains. Cultures of the mutant and wild type strains, at an equal cell density in early log phase of growth, were transferred from 32°C to 44°C. Samples were taken periodically to monitor the incorporation of labeled substrates. The results are shown in Figures 2 and 3. When a culture of the mutant grown at 32°C was transferred to 44°C, the rate of RNA synthesis constantly decreased after the temperature shift (Figure 2). The average rate of RNA synthesis at 44°C was 3.6 fold lower than that of the wild type strain at the same temperature (Table 2). Protein synthesis also began to decrease in the same manner for the mutant shortly after the temperature shift (Figure 3). The average rate of protein synthesis for the mutant was 2.6 fold lower than that of wild type strain (Table 3), suggesting that the effect of higher temperature on the rate of RNA synthesis was more pronounced than the effect on protein synthesis. Since transcription and translation are closely coupled in procaryotes, a reduction in the rate of RNA synthesis will also result in a slower rate of protein synthesis as observed for the mutant strain at non-permissive temperature. On the other hand, in vitro protein synthesis of the mutant ribosomes showed no temperature-sensitivity at 44°C when it was compared to that of the wild



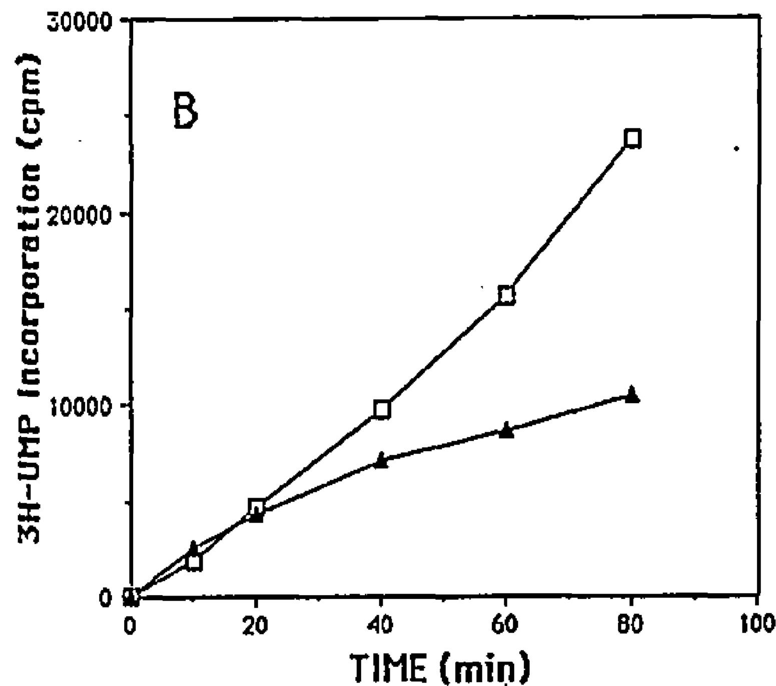
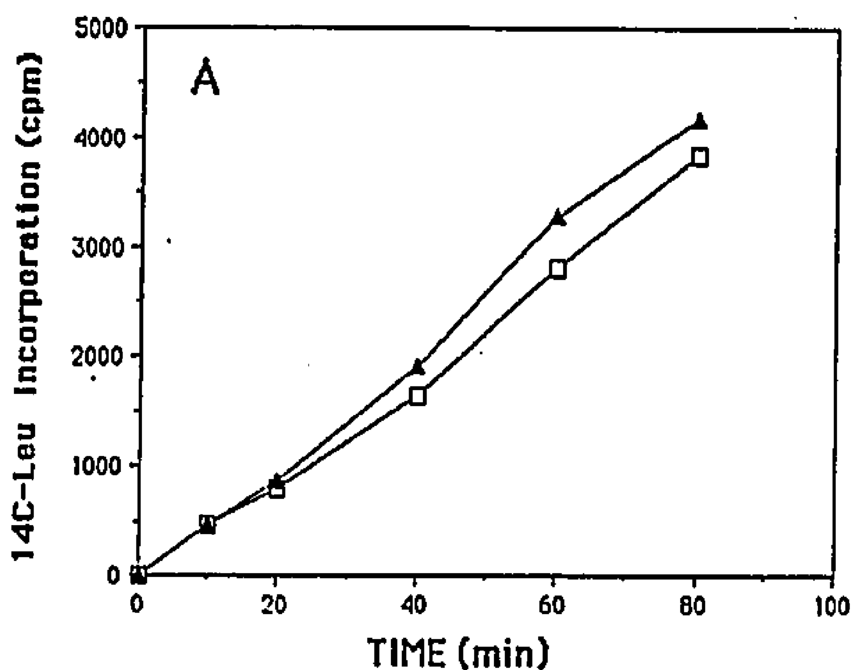


Figure 2. Effect of Culture Temperature on RNA Synthesis. Cells of E. coli wild type (A) and mutant (B) were grown at 32°C on M9/glucose medium. At an equal cell density, portions of cells (3 ml) were transferred to 32°C and 44°C. Samples (0.1 ml) were taken periodically to monitor the incorporation of labeled substrate ([^{3}H]uridine). (□) and (▲) represent incorporation at 32°C and 44°C, respectively.



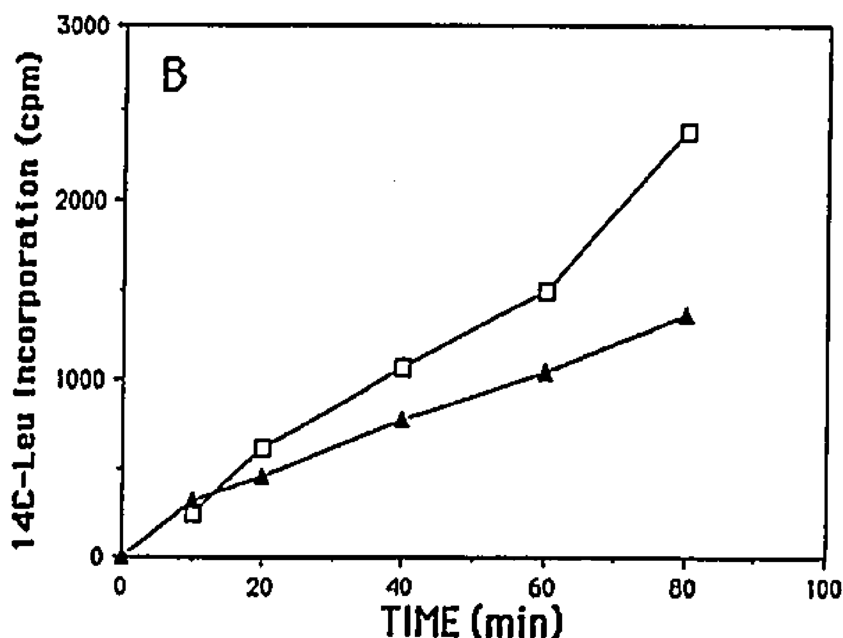


Figure 3. Effect of Culture Temperature on Protein Synthesis. Cells of *E. coli* wild type (A) and mutant (B) were grown at 32°C on Mg/glucose medium. At an equal cell density, portions of cells (3 ml) were transferred to 32°C and 44°C. Samples (0.1 ml) were taken periodically to monitor the incorporation of labeled substrate ([^{14}C]leucine). (□) and (▲) represent incorporation at 32°C and 44°C, respectively.

Table 2. Parameters for In Vivo RNA Synthesis. Parameters were calculated from data of Figure 2.

Strain	Temperature (°C)	Average Rate (cpm/min)	Ratio of Average rate (wild type/mutant)
wild type	32°C	500	at 32°C = 1.6
	44°C	450	
mutant	32°C	300	at 44°C = 3.6
	44°C	125	

type. This clearly implicate a temperature-sensitive function associated with RNA polymerase in the mutant cells. It is noteworthy that even though both the mutant and wild type cultures were shifted at equal cell densities, the ratio of the average rate of RNA or protein synthesis at 32°C remained 1.6 fold lower than that of wild type (Table 2). This also suggests that the rate of in vivo transcription was slower than that of the wild type strain even at the permissive temperature of 32°C.

In vivo synthesis of ribosomal RNA was also monitored in these cells at both permissive and non-permissive temperatures. Cultures of mutant and wild type strains were

Table 3. Summary of RNA Polymerase Purification by DNA-Cellulose Affinity Column.

Step	Volume (ml)	Total protein (mg)	Total activity (milli units)	Specific activity (milli unit/mg)	Purification fold	Yield (%)
wild type crude extract	10	26.5	941	35.6	1	100
DNA-Cellulose	5	3	863	287	8	91
mutant crude extract	10	24	187	7.8	1	100
DNA-Cellulose	5	3	174	58	7.4	93

grown at 32°C to an equal cell density and shifted to 44°C. After 10, 15, or 20 min incubation at 32°C or 44°C, samples were taken and the ribosomal RNA was labeled for 3 minutes with ^{3}H-uridine. The labeled RNA species were separated by gel electrophoresis. Figure 4 shows an autoradiogram of the gel. As is evident from Figure 4, the mutant strain synthesized less ribosomal RNA at each time point after shift to 44°C compared to that of wild type strain. A densitometer tracing of the autoradiogram provided more quantitative results. Figure 5 shows a bar graph representation of labeled ribosomal RNA. It is evident that the amount of ribosomal RNA synthesized at 32°C in the mutant strain was much smaller than that in the wild type. In addition, the amount of ribosomal RNA synthesized in the mutant, after 20 minutes incubation at 44°C, was almost half the amount made at 32°C under the same conditions. This clearly supported the early observation that the rate of total RNA synthesis was severely reduced in the mutant at the elevated temperatures.

Partial Purification of RNA Polymerase

In order to examine the enzymatic characteristics of the mutant RNA polymerase, the enzyme from both strains was partially purified on a double-stranded DNA-cellulose column. Initial purification steps took advantage of the fact that RNA polymerase was bound to DNA at low ionic strength. When the DNA was precipitated by polyethylene

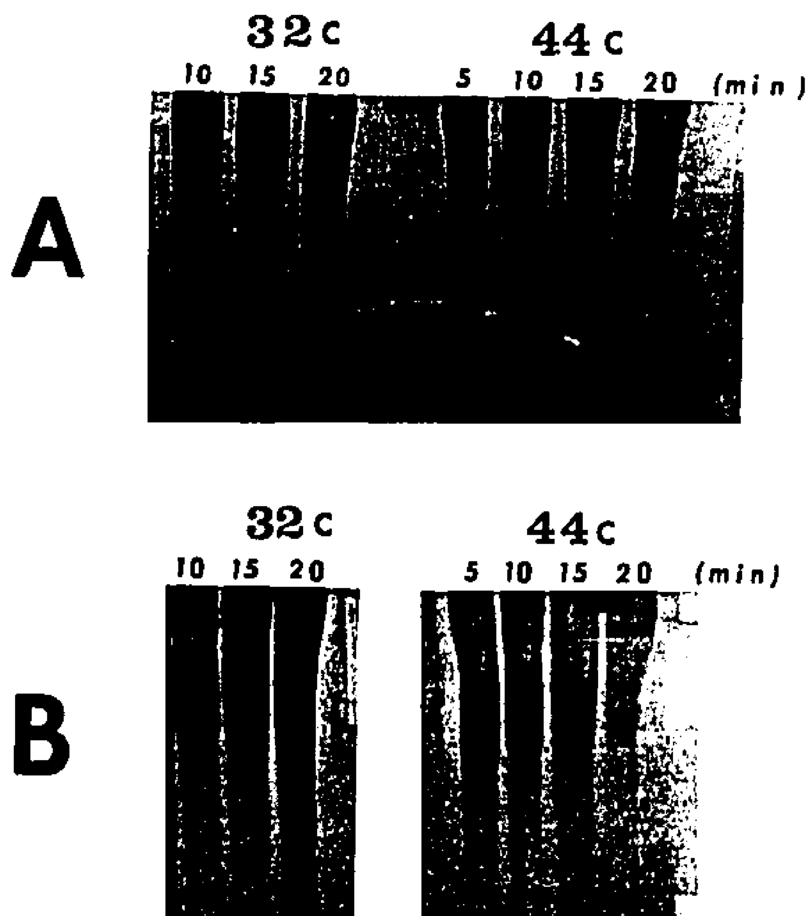


Figure 4. Autoradiogram of Labeled Ribosomal RNA Separated by Gel Electrophoresis. Portions of cultures of wild type and mutant strains growing at 32°C, at an equal cell density, were shifted to 44°C. Samples were taken periodically after shift and were labeled for 3 minutes with [^{3}C]uridine. Labeled ribosomal RNA were separated by composite gel electrophoresis and autoradiographed. (A) wild type and (B) mutant ribosomal RNA.

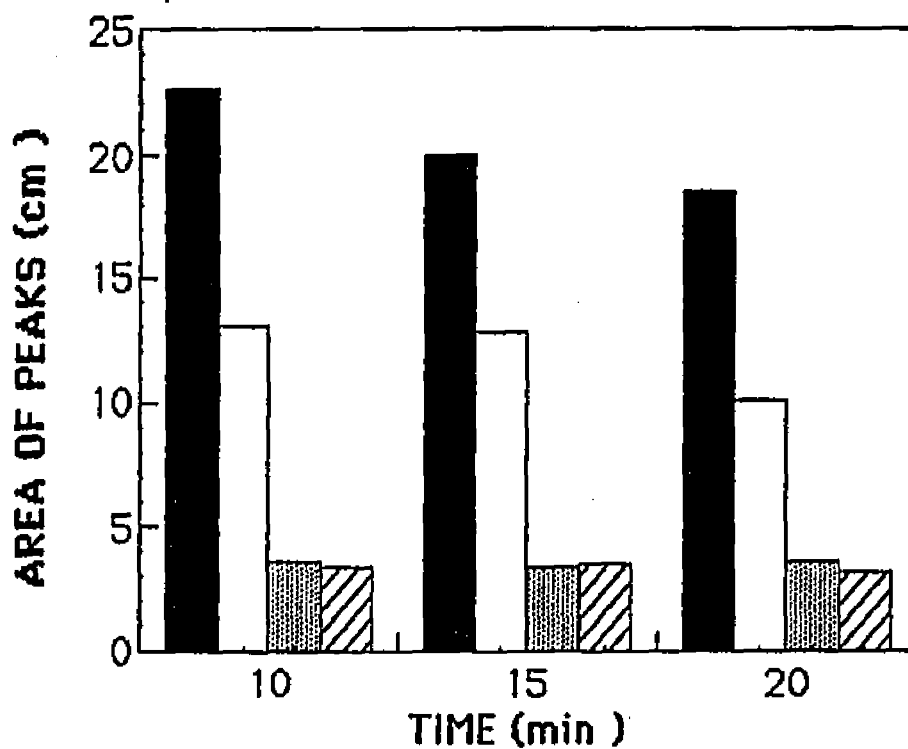


Figure 5. Densitometer tracing of labeled ribosomal RNA. A densitometer tracing was made on the autoradiogram of labeled ribosomal RNA of Figure 4. (■) and (□) represent the area of the peaks (cm^2) from wild type strain at 32°C and 44°C, respectively. (□) and (□) represent the area of the peaks (cm^2) from the mutant strain at 32°C and 44°C, respectively.

glycol 6000 in low salt, RNA polymerase was co-precipitated with the DNA, although most proteins remained in the supernatant. RNA polymerase was then eluted from the PEG precipitate by high salt under conditions where the nucleic acid remained predominantly insoluble. This generated a DNA-free crude extract. Rapid chromatography of the extract with step salt elution from the DNA-cellulose affinity column resulted in the elution of core and holoenzyme as a mixture in a sharp peak of activity. The profile of DNA-cellulose chromatography for both strains is shown in Figure 6 A and B. This procedure can be used to purify polymerase from a number of independent samples. The summary of purification by this method for both strains is given in Table 4. The specific activity of the wild type and mutant enzyme in the pooled fractions was 287 and 58 milliunit/mg, respectively. This procedure resulted in about 90% yield of enzyme. The purity of the pooled fractions was judged by SDS-polyacrylamide gel electrophoresis as shown in Figure 7.

Kinetics of In Vitro RNA Synthesis

In order to assess the effect of temperature on the enzymatic activity of RNA polymerase, in vitro RNA synthesis was conducted at 32°C, 37°C, and 44°C using the partially purified RNA polymerases and calf thymus DNA as a template. Figure 8 shows the results for the control enzyme. It is clear from Figure 8A that the rate of RNA synthesis with the wild type enzyme remained linear for at least 20 minutes.

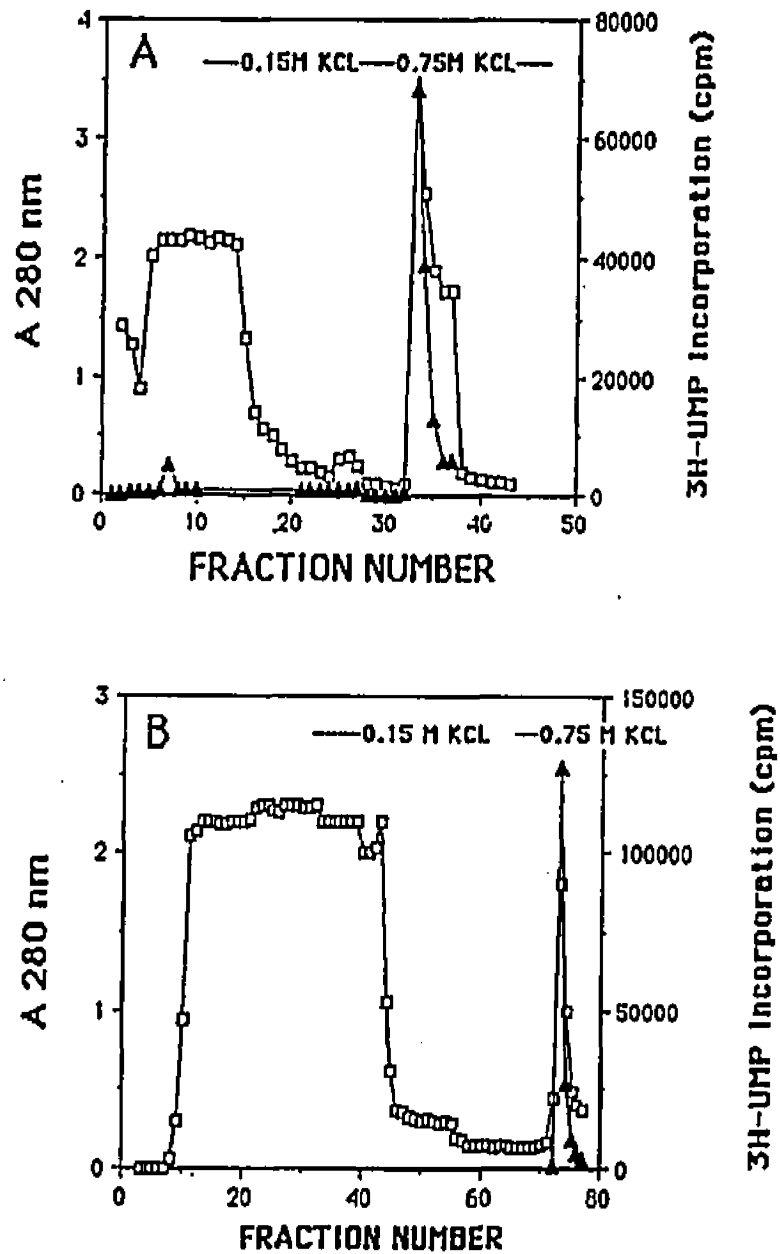


Figure 6. DNA-Cellulose Affinity Chromatography. Cell lysates of Strains wild type (A) and mutant (B) were passed through a DNA-cellulose column. The column was washed with TEGD buffer + 0.15 M NaCl and was then developed with TEGD buffer + 0.75 M NaCl. RNA polymerase activity (-▲-) was eluted in a sharp peak. (-□-) absorbance at A_{280} nm.

Table 4. Quantitative Parameters from Kinetics of In Vitro RNA Synthesis.

Source of enzyme (Strain)	Assay temperature (°C)	Specific Activity (S.A.) (nmole UMP/min/mg)	S.A. at t°C / S.A. at 32°C	Loss of Activity %
	32°C	28.75	1	0
wild type	37°C	25.25	0.91	9
	44°C	25.0	0.87	13
	32°C	5.6	1	0
mutant	37°C	6.25	1.1	0
	44°C	2.3	0.4	60

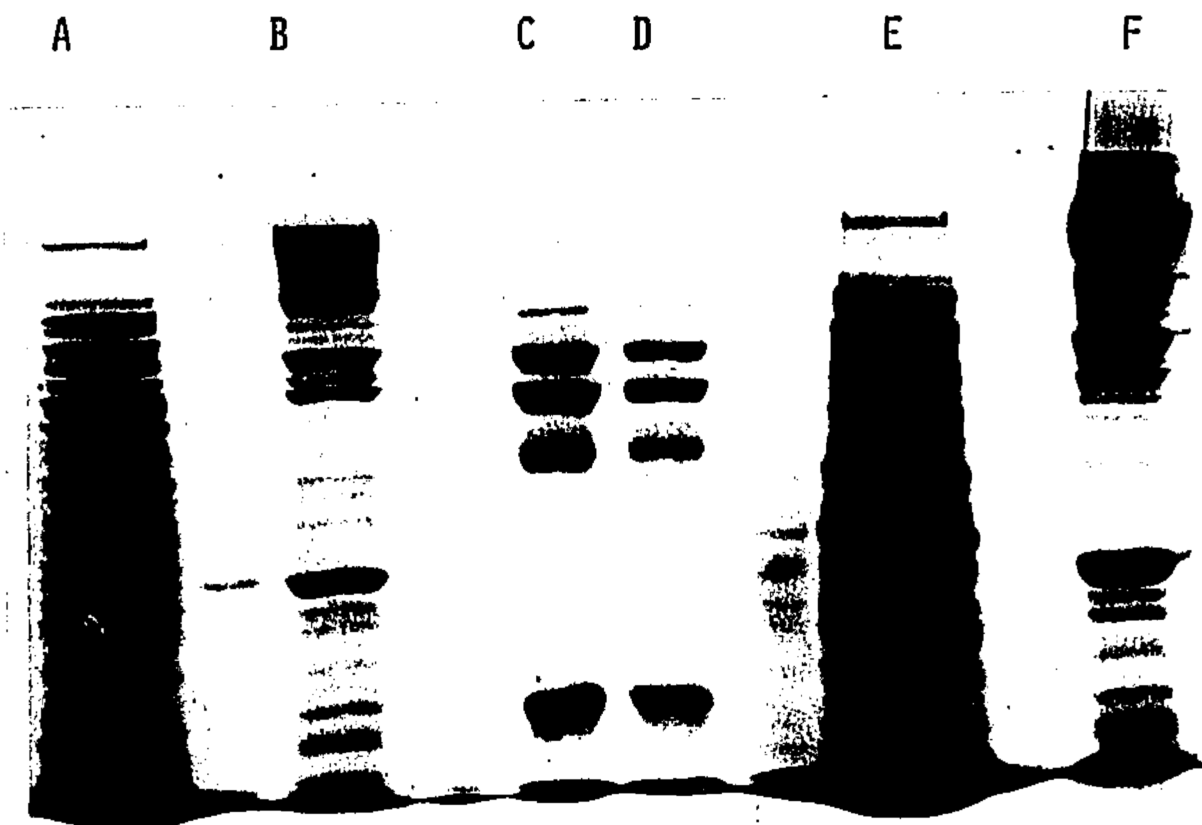


Figure 7. SDS-Polyacrylamide Gel Electrophoresis Analysis of Partially Purified RNA Polymerase. Gel electrophoresis was carried out in a 7.5% polyacrylamide gel with 100 μg of protein in each lane. Lane A, crude extract from wild-type strain; Lane B, DNA-cellulose partially purified enzyme from wild-type strain; Lanes C and D, molecular weight standards; Lane E, mutant crude extract; Lane F, DNA-cellulose partially purified mutant enzyme. RNA polymerase subunits (β, β', δ, and α) are indicated.

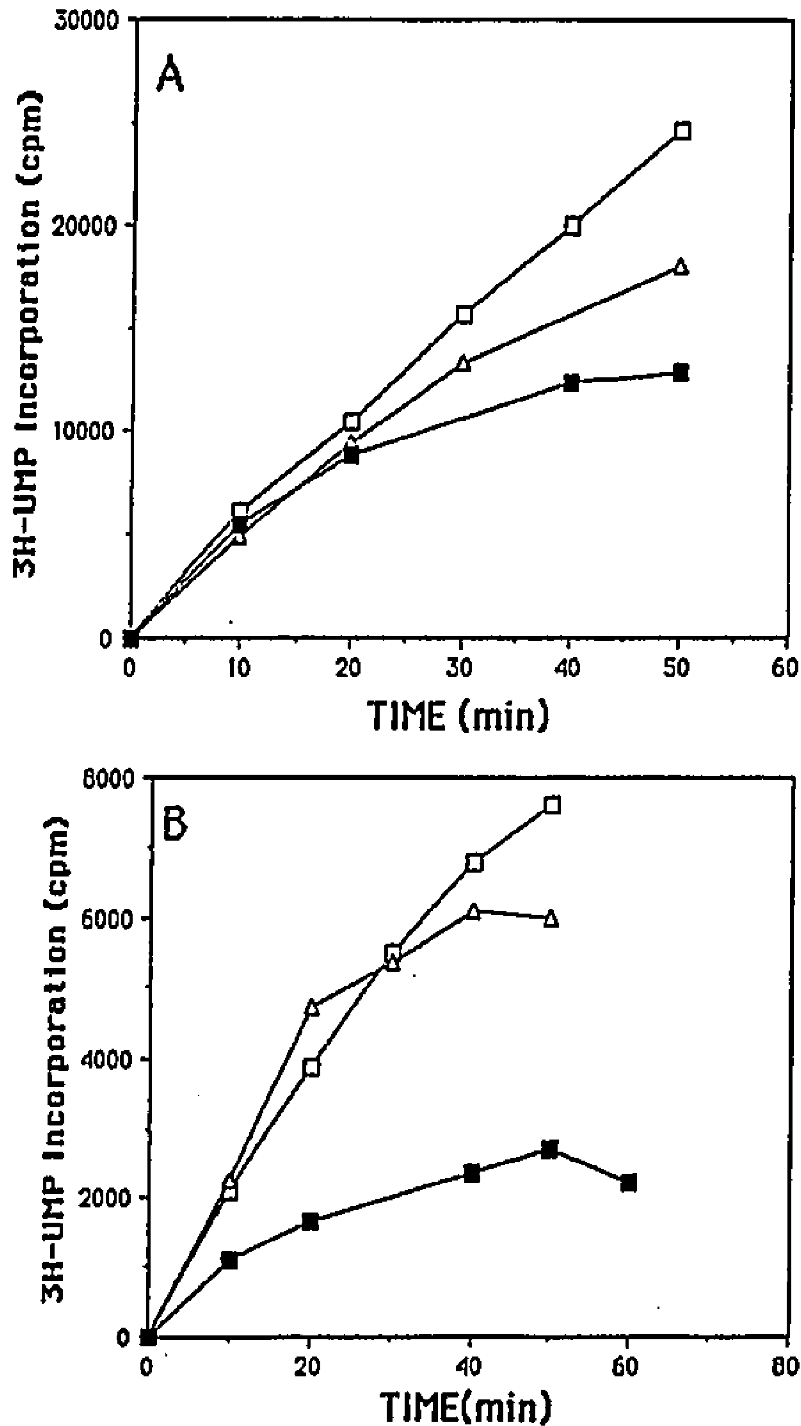


Figure 8. Effect of Temperature on Kinetics of RNA Synthesis Using Partially Purified RNA Polymerase. Samples (17 μl) were removed at intervals from an assay mixture (0.1 ml) containing partially purified RNA polymerase from SK901 (A, 4 μg) and from SK1046 (B, 8 μg), incubating at 32°C (□), 37°C (△), and 44°C (■). The assay mixture contained 6 μg of calf thymus DNA, 0.05 mM UTP, 1 μCi [^{3}H] UTP, and a mixture of all three other nucleoside triphosphates (0.2 mM each).

During this period of time the rate of RNA synthesis was not significantly different at any temperature. The results for the mutant enzyme (Figure 8B) were quite different. The rate of RNA synthesis at 37°C was slightly higher than that at 32°C; however, this rate was considerably reduced at the nonpermissive temperature of 44°C. More quantitative parameters obtained from the kinetic data are shown in Table 4. The specific activity of each enzyme (nmole UMP incorporated/min/mg) was calculated from the nmoles of UMP incorporated at 20 minutes. In addition, the ratio of the specific activity at different temperatures is given for each enzyme. It is evident from Table 4 that the ratio of specific activity of the wild type enzyme at the three different temperatures was only slightly reduced, indicating a small loss of activity at elevated temperatures. Interestingly, the mutant enzyme showed a slight increase in specific activity at 37°C. However, at 44°C the mutant enzyme lost 60% of its activity. This clearly indicates the temperature-sensitive nature of the mutant enzyme. The absolute specific activity of the mutant enzyme was reduced relative to the control activity at any temperature (Table 4). However, from this assay system it is not clear whether the loss of activity was due to the denaturation of the mutant enzyme or to a slower catalytic activity at the nonpermissive temperature. In order to answer this important question, only holoenzyme should be used in a

quantitative assay system, where the different phases of transcription (initiation, elongation, and termination) can be assessed.

Partial Purification of Holoenzyme by Single-Stranded DNA-Aragose Affinity Column

The best methods commonly used for small scale purification of *E. coli* RNA polymerase (Gross et al., 1976; Chamberlin et al., 1983) fail to separate core polymerase from holoenzyme. Since the transcriptional properties, particularly the promoter specificity, of core polymerase and holoenzyme are quite different, it is frequently desirable that holoenzyme be prepared free of core polymerase.

A method was developed for a quick and efficient preparation of RNA polymerase holoenzyme from a crude enzyme fraction. The purification is achieved on a single-stranded DNA-agarose affinity column, to which most contaminating proteins do not bind under the conditions used. By means of their different affinities for single-stranded DNA, the holoenzyme can be separated from core polymerase by stepwise salt elution from the column (Figure 9).

The DNA-free extract from 6 grams of frozen cells was applied at 40 ml/h to a 50 ml (2.8 x 15 cm) single-stranded DNA-agarose column, equilibrated with TEGD buffer + 0.15 M NaCl. Flow was then shut off and the crude extract allowed to remain in contact with the DNA content of the column for

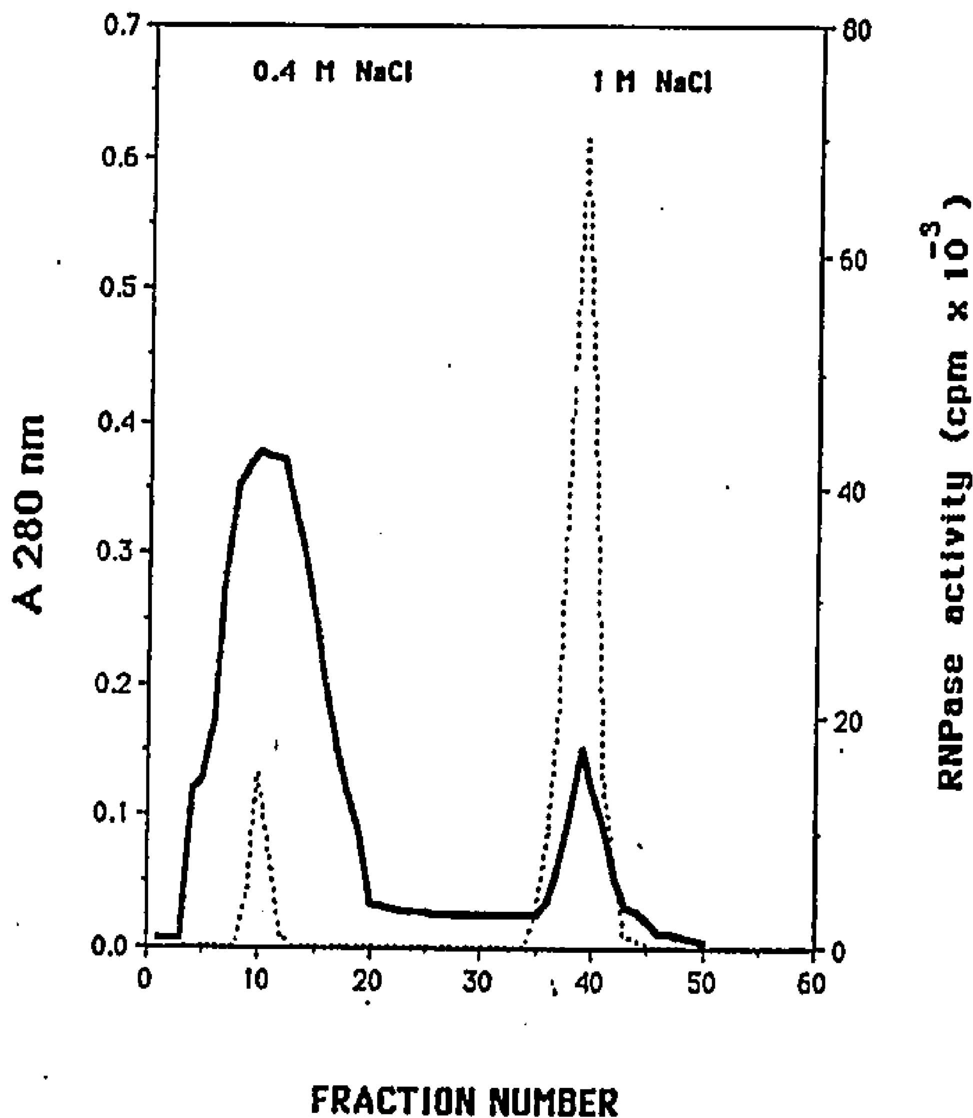


Figure 9. Chromatography of RNA Polymerase Holoenzyme by Single-Stranded DNA-Agarose. DNA-free crude extract was passed through a 50 ml (2.8 x 15 cm) single-stranded DNA-agarose affinity column. After washing with two-bed volumes of TEGD buffer + 0.2 M NaCl, core polymerase was eluted with one-bed volume of TEGD buffer + 0.4 M NaCl. RNA polymerase holoenzyme was finally eluted with TEGD buffer + 1 M NaCl. Core polymerase and holoenzyme were pooled separately. (—) Absorbance at 280 nm. (···) RNA polymerase activity.

30 minutes. After washing with two bed volumes of TEGD buffer containing 0.2 M NaCl, core polymerase was eluted with 60 ml of TEGD buffer + 0.4 M NaCl. Holoenzyme was finally eluted with TEGD buffer containing 1 M NaCl at flow rate of 20 ml/h. The flow rate was decreased during the 1 M NaCl elution to allow more time for the holoenzyme to dissociate from the column and to increase the concentration of protein in the fractions.

Purification of RNA Polymerase by Gel Filtration Chromatography

Since the molecular weight of RNA polymerase holoenzyme is about 480 kilodalton (kd), it is possible to further purify this enzyme by appropriate gel filtration chromatography where the holoenzyme can be eluted in the void volume of the column. The partially purified holoenzyme from a single-stranded DNA-agarose column was dialyzed against TEGD buffer + 1 M NaCl containing 20% polyethylene glycol (20,000) for up to one hour to reduce the volume of the pooled fractions. The dialyzed fraction (2 ml) was applied at 25 ml/h to a 75 ml (2.8 x 17 cm) Sephacryl S-200 column which was preequilibrated with TEGD buffer + 1 M NaCl (Figure 10). Holoenzyme was eluted in the void volume (33 ml) and fractions were pooled and dialyzed against storage buffer (TEGD + 50% glycerol) overnight and stored at -20°C.

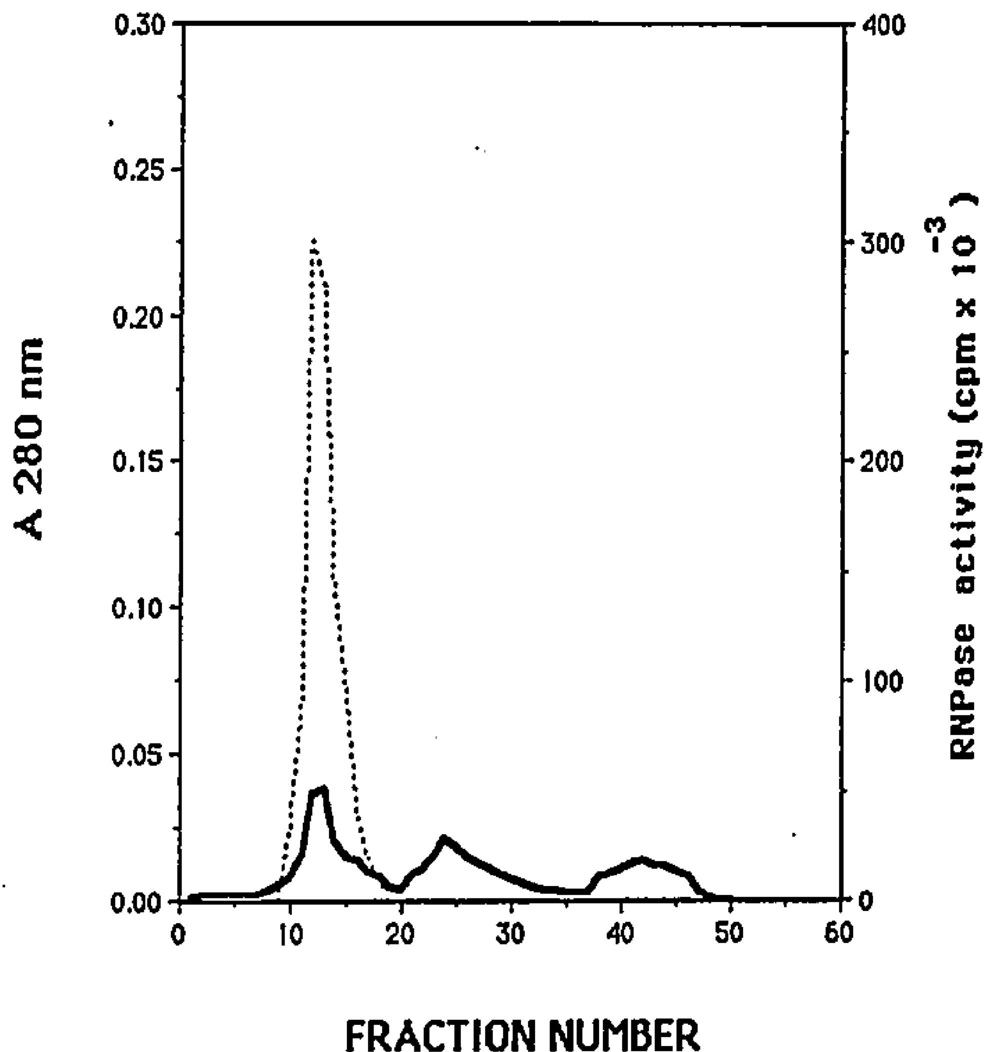


Figure 10. Sephacryl S-200 Gel Filtration Chromatography. Pooled holoenzyme fractions from a single-stranded DNA-agarose column were concentrated and applied to a 75 ml (2.8 x 17 cm) Sephacryl S-200 column, preequilibrated with TEGD buffer + 1 M NaCl. The peak of activity (fractions 10-16) were pooled, dialyzed against storage buffer (TEGD + 0.2 M NaCl and 50% glycerol), and stored at -20°C. (—) Absorbance at 280 nm. (···) RNA polymerase activity.

SDS-Polyacrylamide Gel Electrophoresis

Fractions from the stages of this purification procedure were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Figure 11. Lanes C and D of Figure 11 show the core and holoenzyme proteins from the single-stranded DNA-agarose column. In the core polymerase fraction (Lane C) there is a small amount of δ protein band. This indicates that at 0.4 M NaCl, where core polymerase elutes from single-stranded DNA-agarose column, there was a very slow constant rate of holoenzyme elution. Therefore, the 0.4 M NaCl elution could not be continued much longer than suggested without decreasing the recovery of holoenzyme in the 1 M NaCl step.

The protein content of the holoenzyme after gel filtration on Sephacryl S-200 is shown in Lane E of Figure 11. The two main contaminants of this fraction have molecular weights of 120-130 and 74 kilodaltons. Since these two contaminants coelute with holoenzyme (MW = 480 kd) from the Sephacryl S-200 column, it is possible that they are subunits of an oligomeric protein with a molecular weight close to that of RNA polymerase. Comparison of Lanes D and E of Figure 11 clearly shows that the smaller molecular weight protein contaminants present in the holoenzyme from the single-stranded DNA-agarose (Lane D) have disappeared after gel filtration chromatography on Sephacryl S-200 (Lane E).

Figure 11. SDS-polyacrylamide Gel Electrophoresis of Various Fractions of the Purification. Electrophoresis was carried out in a 7.5% polyacrylamide gel. (A) and (F) protein molecular weight standards, (B) DNA-free crude extract (25 μg of protein), (C) 5 μg of pooled core polymerase from single-stranded DNA-agarose column, (D) 10 μg of pooled holoenzyme from single-stranded DNA-agarose column, and (E) 5 μg of pooled holoenzyme from Sephacryl S-200 column. RNA polymerase subunits (ß, ß', δ, and α) are indicated.

Purity and Yield

The purity of purified holoenzyme was determined by making a densitometer scan of the photographic negative of the SDS-polyacrylamide gel (Lane E, Figure 11). The results are shown in Figure 12. Integration analysis obtained from this densitometer tracing indicated that the contaminants constituted 22.4% of the total proteins. This corresponds to purity of 77.6% of holoenzyme. The sigma subunit of holoenzyme constituted 17.4% of total holoenzyme based on this analysis.

The purification of *E. coli* RNA polymerase from 6 gram of cells (equivalent to 12 liters of culture at OD_{600} = 0.5) is summarized in Table 5. About 0.66 mg of holoenzyme was obtained with an overall yield of 56% in one day. The overall purification steps are presented as a flow chart in Figure 13.

Quantitative Assay for E. Coli RNA Polymerase with Bacteriophage T7 DNA

Rationale for the Assay

When *E. coli* RNA polymerase is added to a solution containing T7 DNA and the four ribonucleoside triphosphates, T7 RNA chains are initiated exclusively from a cluster of three promoter sites at the left end of the genome, designated A1, A2, and A3 (Chamberlin, 1974; Studier, 1975). Each RNA polymerase molecule which initiates an RNA chain

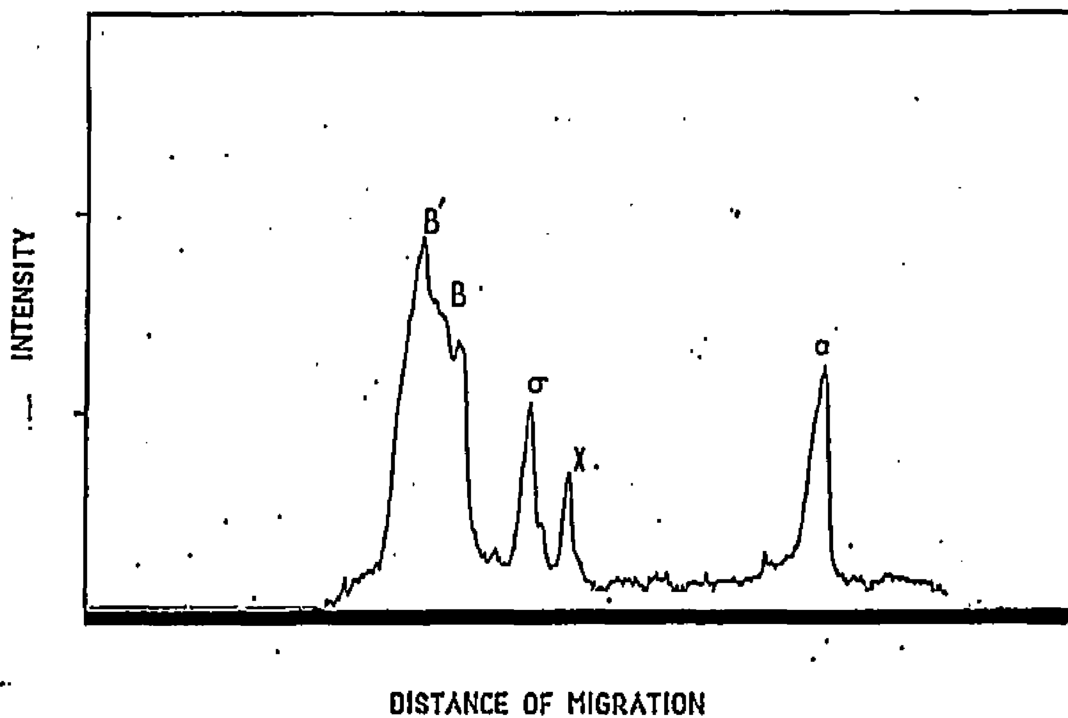


Figure 12. Densitometer Tracing of SDS-Gel Electrophoresis. A densitometer scan was carried out on Lane (E) of the SDS-gel photographic negative (Figure 10). RNA polymerase subunits and contaminants (x) are indicated as in Figure 10.

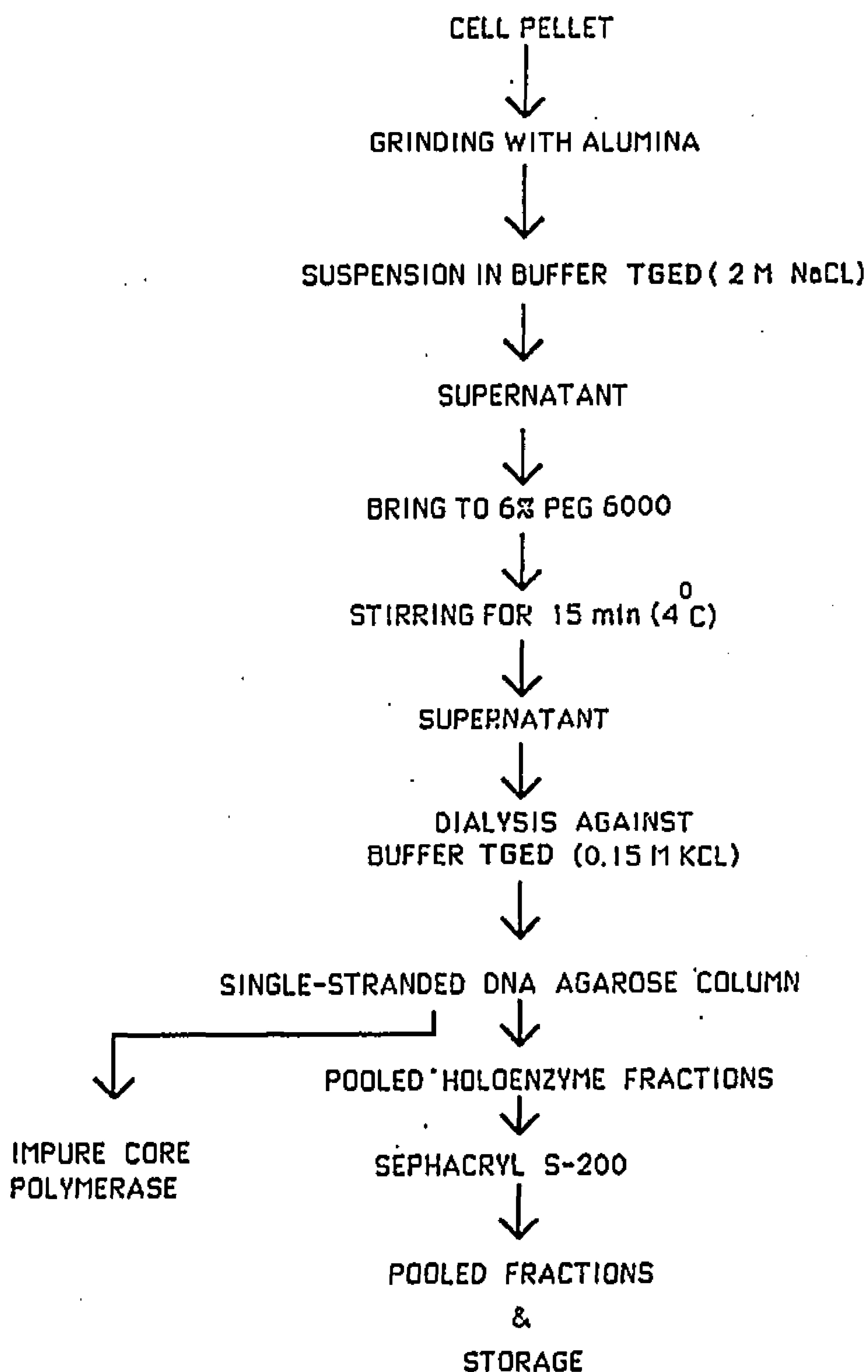


Figure 13. Schematic for the rapid small-scale purification of RNA polymerase.

Table 5. Summary of RNA Polymerase Holoenzyme Purification

Step	Volume (ml)	Total protein (mg)	Total activity[b] (milliunit)	Specific activity (milliunit/mg)	Purification fold	Yield %
DNA-free[a] cell extract	65	41	1430	34.8	1	100
Single-stranded DNA-agarose column:						
Core enzyme	4	0.3	50	166	5	3.5
Holoenzyme	17	1.7	1200	706	20	84
Sephacryl S-200	8	0.3	800	2666	77	56

[a]From 6 gram of cells.
[b]One unit of RNA polymerase is defined as the amount of RNA polymerase that gives the incorporation of one μmole of UMP in ten minutes at 32°C using T7 bacteriophage DNA as a template. For convenience, activities are expressed as milliunit (equal to 10^{-3} unit).

at an A promoter elongates that chain, traversing the early region of the T7 genome until the complex encounters a strong transcriptional termination site at 7720 base pairs (Studier, 1975). At this site about 80% of the RNA polymerase molecules terminate synthesis, generating an RNA chain of average length 7133 base pairs (Chamberlin et al., 1979); the remaining 20% of the RNA polymerase molecules "read through" into the late genetic region and can synthesize RNA chains up to the full length of the T7 genome. The transcriptional program for *E. coli* RNA polymerase acting with T7 DNA is shown in Figure 14. The general form expected for the graph of ribonucleotide incorporation in a standard assay is shown in Figure 15. RNA polymerase locates and forms open promoter complexes at the T7 A promoters. Hence, after a brief initial period of template binding and chain initiation (phase I), up to 30 seconds after addition of enzyme, all RNA polymerase molecules should be growing T7 A RNA chains. In the absence of random chain termination there will be an extended period (phase II) from about 0.5 to 6.9 minutes during which all RNA polymerase molecules are engaged in constant RNA chain elongation. Under conditions where re-initiation of RNA synthesis by these RNA polymerase molecules is blocked (by the addition of the antibiotic rifampicin at an appropriate point during phase II), the rate of ribonucleotide incorporation will diminish

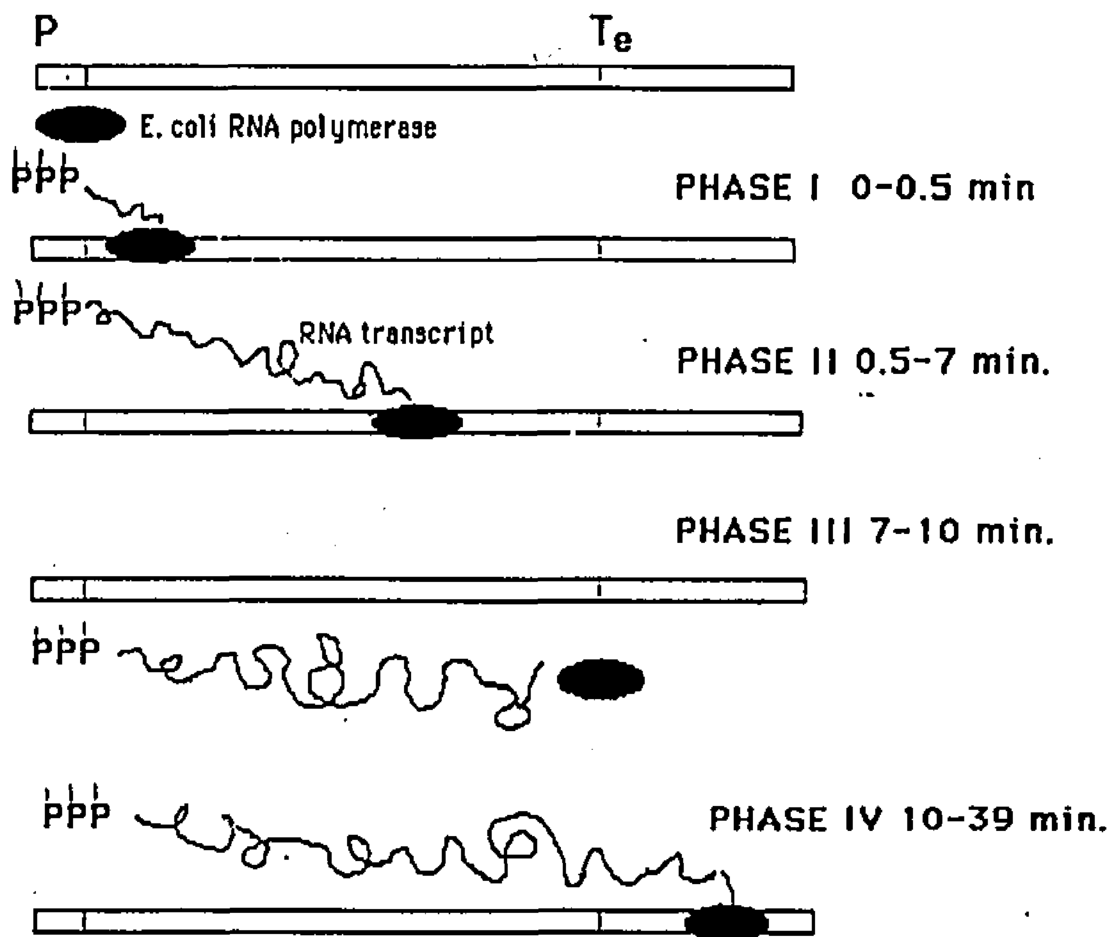


Figure 14. Outline of Transcriptional Program for E. coli RNA Polymerase Acting with T7 DNA. The open bars represent early genetic region of bacteriophage T7 DNA. (P) and Te) represent promoter and terminations equences.

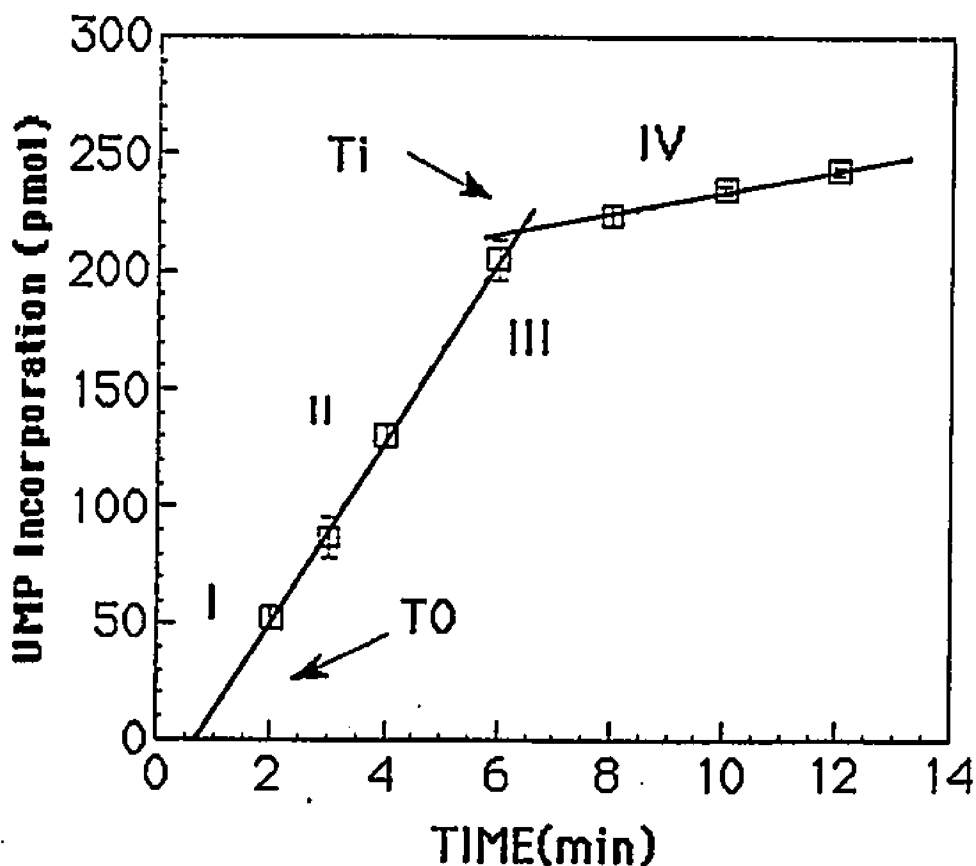


Figure 15. Kinetics of T7 RNA Synthesis With Wild Type RNA Polymerase Holoenzyme. A standard RNA synthesis assay was carried out with purified SK901 RNA polymerase holoenzyme at 32°C. Symbols T_0, T_I are defined in text; I, II, III, and IV refer to phases of transcription as discussed in the text. Data are for 20 µl samples taken during the standard T7 assay in which a total of 1.5 µg (40 µl) of wild type RNA polymerase holoenzyme (specific activity 2.4 unit/mg) was added to the standard assay reaction (200 µl). Each data point represents the average of duplicate determinations from two separate assays. Standard error is indicated by error bars.

substantially as the wave of RNA polymerase molecules elongating chains encounters the T7 terminator at 7720 base pairs on the DNA. This leads to termination and release of the enzyme and RNA chains (phase III). After all elongating RNA polymerase molecules have encountered the terminator, only those molecules which fail to terminate their RNA chains will continue to incorporate ribonucleotides. Hence, phase IV reflects a second linear phase of RNA synthesis carried out by these read-through complexes traversing the remainder of the T7 DNA molecule.

Calculations

Quantitative characterization of bacterial RNA polymerase preparations involves the use of a DNA template bearing a single well-defined transcriptional unit, where the values of different transcriptional parameters can be assayed by following labeled ribonucleotide incorporation during a single cycle of transcription. The procedure measures the amount of active *E. coli* RNA polymerase holoenzyme in the preparation using values measured for the different steps of the transcriptional cycle including (a) the rate of promoter location plus chain initiation, (b) the mean rate of RNA chain elongation, and (c) the efficiency of chain termination at the early T7 termination signal.

The concentration of active RNA polymerase molecules (E_a') is calculated from the amount of ribonucleotide incorporation at the time point T_i, where the phase II and

phase IV curves intercept, if these lines are extrapolated as shown in Figure 14. This is the theoretical point at which each active enzyme molecule would have completed one T7 A RNA chain of average length of 7133 nucleotides, containing equal amounts of the four nucleotide bases (McDonell et al., 1977; Skalka and Hanson, 1972). This is expressed as

$$\text{(total UMP incorporated at time Ti) (Avogadro constant)} = \text{Total number of UMP molecules incorporated at Ti} \quad \text{(I)}$$

If equation (I) is divided by the number of UMP molecules per one transcript (7133/4) equation (II) will result

$$\frac{\text{(moles UMP incorporated at Ti) (Avogadro constant)}}{(7133/4)} = \text{Number of transcripts or number of active RNA polymerase molecules} \quad \text{(II)}$$

It is noteworthy that since reinitiation is inhibited by addition of rifampicin shortly after the onset of transcription each RNA polymerase molecule generates only one transcript. Therefore, in equation (II) the number of transcripts is equal to the number of active RNA polymerase molecules present in the assay. In order to obtain the molarity of active RNA polymerase in the assay, equation (II) should be divided by the Avogadro constant and the result would be the following equation:

$$\frac{\text{Moles UMP incorporated at Ti}}{7133/4} = \text{Moles of active RNA polymerase molecules in the assay}$$

When the data are graphed, as in Figure 14, by the method of least squares, three other useful parameters are easily

calculated. The intercept of the linear phase II curve on the ordinate (T_0) is inversely proportional to the rate at which the enzyme is able to locate the T7 A promoters and initiate an RNA chain. For most polymerases this reflects the rate of promoter site selection and melting since the rate of RNA chain initiation is much more rapid than these earlier steps (Hinkle and Chamberlin, 1972; Mangel and Chamberlin, 1974). It has been shown that for E. coli RNA polymerase T_0 is 30 seconds (Chamberlin et al., 1979).

The time at which the extrapolated phase II and phase IV curves intercept (T_1) is the time at which all of the RNA polymerase molecules in the reaction would complete an RNA chain of average length 7133 nucleotides if they were all growing RNA chains at the mean rate of chain elongation. Hence, 7133 nucleotides divided by (T_1-T_0) in seconds gives the mean rate of chain elongation for the enzyme. Finally, the efficiency of the T7 terminator is calculated from f_{RT}, the ratio of the slopes of phase IV to Phase II. This ratio, f_{RT}, represents the fraction of the active RNA polymerase in the preparation which read through the T7 termination signal, and, hence, is a measure of the efficiency of the signal. Thus the termination efficiency can be written as $1-f_{RT}$ that is expressed as the percentage of RNA polymerase molecules which cannot read through the signal and terminate the transcription.

Standard assay parameters for purified wild type holoenzyme, calculated from Figure 15, are presented in Table 6. The concentration of active enzyme in the assay was 1.23 pmole. The intercept of the linear phase II curve on the ordinate (T_0) was at 30 seconds and the mean rate of chain elongation was 20 nucleotides per second at 32°C. The ratio of the slope of phase IV to phase II (f_{RT}) was 0.15 which corresponded to a termination efficiency ($1-f_{RT}$) of 85%, meaning that 85% of RNA polymerase enzyme terminated the elongation once the enzyme encountered the termination signal. These parameters obtained for the purified enzyme by the small scale purification procedure were very similar to those of highly purified RNA polymerase isolated by a large scale method reported by others (see Table 6) (Chamberlin et al., 1979; Burgess and Jendrisak, 1975).

Transcription of T7 DNA in vitro can be monitored by polyacrylamide gel electrophoresis. Under normal assay conditions, E. coli RNA polymerase holoenzyme has been shown to transcribe primarily a single RNA species of molecular weight of 2.4×10^6 (Millette et al., 1970; Dunn and Studier, 1973) originating from a promoter site at the left end of T7 DNA. Figure 16 shows an autoradiogram resulting from gel electrophoresis of transcription of T7 DNA by wild type holoenzyme under the conditions employed for kinetic analysis described above.

Table 6. Standard T7-Assay Parameters for Wild Type RNA Polymerase Holoenzyme. The assay parameters were calculated from kinetics of T7 RNA synthesis, Figure 14.

Enzyme Fraction	Assay temperature	Specific activity (unit/mg)	Concentration of active enzyme (p mole)	T_o (min)	T_I (min)	Chain growth rate (nucleotides/sec)	Termination efficiency $(1-f_{rt})$
Sephacryl S-200[a]	32°C	2.4	1.23	0.64	6.4	20	85%
ASM 27[b]	30°C	0.55	1.17	0.5	7.2	17	88%

[a]Holoenzyme purified in this study (see Materials and Methods)

[b]Fraction A_{SM27} is the final fraction obtained by the large scale purification method of Burgess and Jendrisak (1975). The assay parameters for this enzyme obtained from Chamberlin et al. (1979).

Quantitative T7 Assay for the Mutant RNA Polymerase

The quantitative assay, using T7 DNA as a template, was carried out at the permissive temperature, 32°C, for the mutant enzyme in order to determine the concentration of active enzyme as well as its assay parameters. The results of this assay are shown in Figure 17. The assay parameters are given in Table 7. The calculated concentration of active enzyme was 0.77 pmole. The standard assay parameters are also given in Table 7. A comparison of T_0, T_1, mean elongation rate and termination efficiency for the mutant enzyme with those of the wild type enzyme (Table 6) showed no significant differences. The specific activity of the mutant enzyme was 46% of that of wild type.

Figure 18 shows all autoradiogram of gel electrophoresis of T7 transcripts made by the mutant enzyme at the permissive temperature. Like with wild type enzyme, the mutant holoenzyme transcribed primarily a single RNA species under the same conditions employed for kinetic analysis described earlier. Kinetic analysis and gel electrophoresis of transcripts of the mutant enzyme collectively suggested that the mutant enzyme behaved the same way as the wild type did at the permissive temperature 32°C.

Effect of Temperature on T7-Assay Parameters

Since the mutant enzyme from strain SK1046 is temperature sensitive, it was of great importance to identify the effect

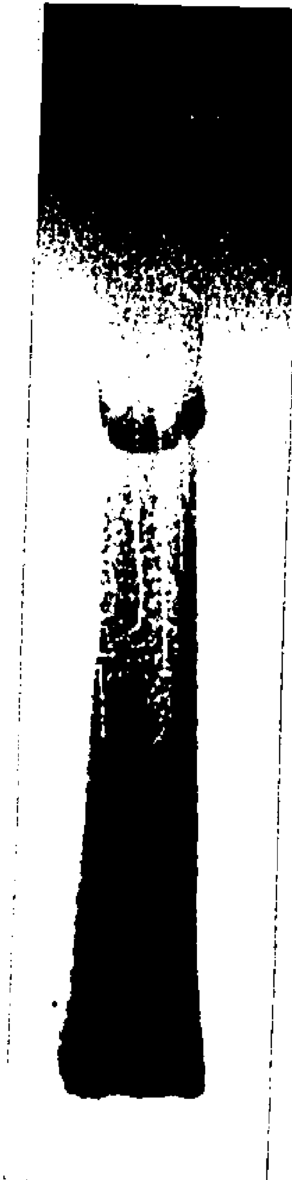


Figure 16. Autoradiogram of Acrylamide-Agarose Gel of T7 RNA Transcripts made by the Wild Type Holoenzyme. T7 RNA synthesis was started with the wild type holoenzyme under standard T7 assay conditions. The reaction was terminated after 10 minutes and the products were analyzed by gel electrophoresis and subsequent autoradiography as described in Materials and Methods.

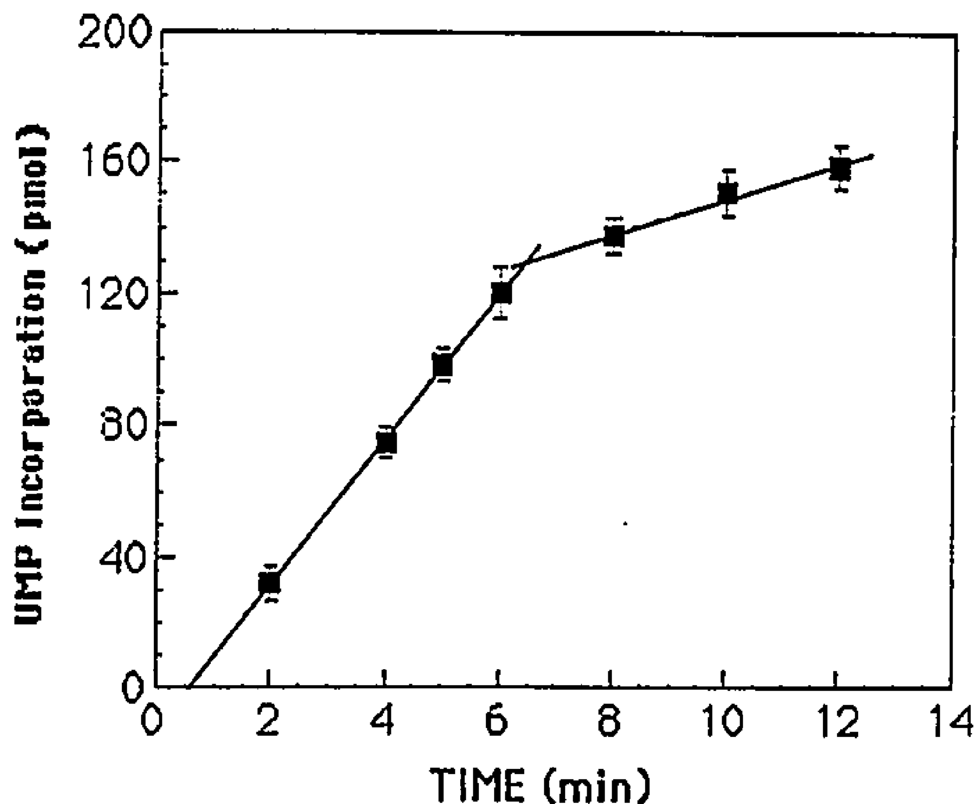


Figure 17. Kinetics of T7 RNA Synthesis by Mutant RNA Polymerase Holoenzyme. Standard T7 RNA synthesis was carried out with purified SK1046 RNA polymerase holoenzyme at 32°C. Data are for 10 µl samples taken during the standard T7 assay in which a total of 2.1 µg (50 µl) of SK1046 RNA polymerase holoenzyme (specific activity 1.1 unit/mg) was added to the standard assay reaction (200 µl). Each data point represents the average of duplicate determinations from two separate assays. Standard error is indicated by error bars.

Table 7. Standard T7 Assay Parameters for Mutant RNA Polymerase Holoenzyme. The assay parameters were calculated from kinetics of T7 RNA synthesis, Figure 15.

Enzyme Fraction	Assay temperature	Specific activity (unit/mg)	Concentration of active enzyme (p mole)	T_o (min)	T_f (min)	Chain growth rate (nucleotides/sec)	Termination efficiency $(1-f_{RT}$
Sephacryl S-200[a]	32°C	1.1	0.77	0.57	6.5	20	80%
A_{5M27}[b]	30°C	0.55	1.17	0.5	7.2	17	88%

[a]Holoenzyme purified in this study (see Materials and Methods)

[b]Fraction A_{5M27} is the final fraction obtained by the large scale purification method of Burgess and Jendrisak (1975). The assay parameters for this enzyme obtained from Chamberlin et al. (1979).

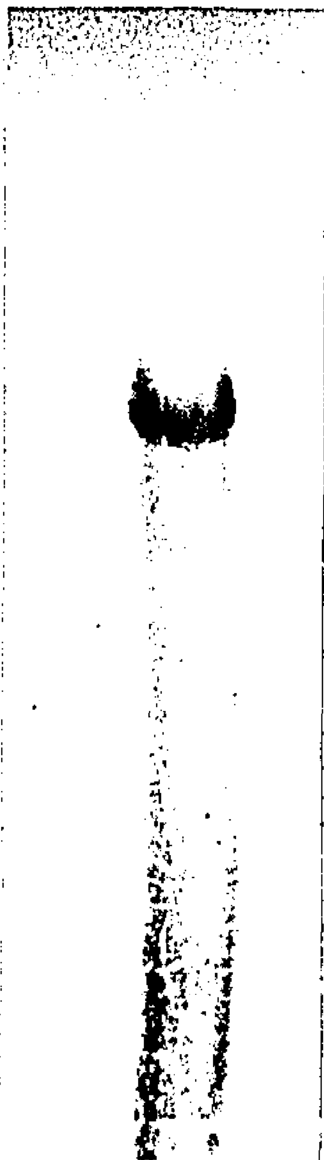


Figure 18. Autoradiogram of Acrylamide-Agarose Gel of T7 RNA Transcripts Made by the Mutant Holoenzyme. T7 RNA synthesis was started with the mutant holoenzyme under standard T7 assay conditions. The reaction was terminated after 10 minutes and the products were analyzed by gel electrohporesis and subsequent autoradiography as described in materials and Methods.

of temperature on the function of this mutant enzyme. The standard T7-assay provide an excellent method for this study. Equal concentration of active enzyme (1.23 pmole, equivalent to 3.6 milliunit), from either the mutant or wild type, was used throughout the subsequent assays. The T7 transcription assay was carried out at the permissive temperature, 32°C. In order to assess the effect of temperature, the enzymes were preincubated for 5 minutes at 37°C or 44°C prior to assay at 32°C. The kinetics results are given in Figure 19 and the data analysis is presented in Table 8. The wild type enzyme lost no activity at 37°C and only 4% of its activity after 5 minutes of preincubation at 44°C. In contrast, the mutant enzyme lost 31% and 61% of its activity after preincubation at 37°C and 44°C, respectively (Table 8). The mutant enzyme preincubated at 44°C ceased transcription, or terminated prematurely, at about 4.5 minutes after the initiation of RNA synthesis on the T7 DNA template (Figure 19). This is due to a dramatic reduction in the concentration of active enzyme after a 5-minute preincubation at 44°C. There was no detectable change in the rate of chain initiation (T_0) or chain elongation (nucleotides/second) for the mutant enzyme (Table 8). The termination efficiency for the mutant enzyme preincubated at 37°C was slightly lower than its efficiency at 32°C (Table 7).

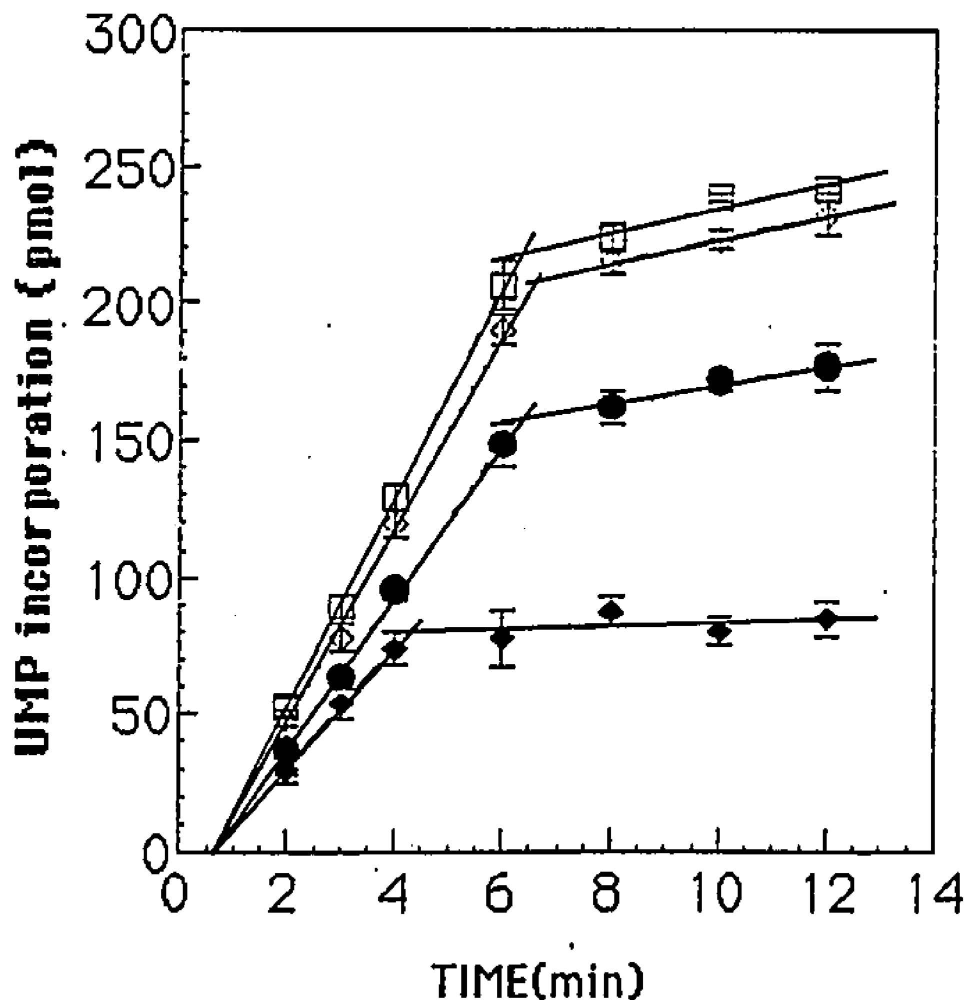


Figure 19. Effect of Preincubation Temperature on the Kinetics of Standard T7 Assay at 32°C. A standard T7 RNA synthesis assay was carried out with purified wild type and mutant RNA polymerase holoenzyme (1.23 pmole, each) as described in the Materials and Methods. Each data point represents the average of duplicate determinations from two separate assays. Standard error is indicated by error bars. (□) and (◇) represent incorporation with wild type holoenzyme after preincubation at 37°C and 44°C, respectively. (●) and (♦) represent incorporation with mutant holoenzyme after preincubation at 37°C and 44°C, respectively.1

Table 8. Effect of Preincubation Temperature on T7 Assay Parameters. The assay parameters were calculated from kinetics of T7 RNA synthesis, Figure 19.

Holoenzyme	Preincubation temperature	Concentration[a] of active enzyme (pmole)	Loss of[b] activity (%)	T_o (min)	T_i (min)	Chain growth rate (nucleotides/sec)	Termination efficiency $(1-f_{RT})$
Wild type	37°C	1.23	0	0.65	6.6	20	85
	44°C	1.18	4	0.72	6.5	20	83
Mutant	37°C	0.84	31	0.69	6.4	20	77
	44°C	0.47	61	0.64	4.5	N.D.	N.D.

[a]Calculated from Figure 16 for each curve.

[b]An equal concentration of active enzyme was used for all assays; hence 1.23 pmole was considered as 100% activity. The specific activities of wild types and mutant holoenzyme were 2.4 and 1.1 unit/mg, respectively.

N.D. not determined due to severe defect of mutant enzyme.

In another set of experiments, the T7 transcription assay temperature was raised to at 37°C for both enzymes after a 5 minute preincubation at 37°C or 44°C. The results are shown in Figure 20. The calculated transcriptional parameters are also given in Table 9. The wild type enzyme lost 26% of its activity after a 5 minute preincubation at 37°C or 44°C followed by an assay at 37°C, compared to a loss of 63% and 81% of activity for the mutant enzyme under the same conditions (Table 9). This suggested that the higher assay temperature (37°C) had enhanced the thermal lability of the mutant enzyme in comparison to an assay at the optimum temperature of 32°C (Table 8). Interestingly, the initiation lag time (T_o) and the rate of chain elongation (nucleotides incorporated/second) were not significantly altered for the mutant enzyme compared to those of wild type (Table 9), suggesting that the mutation has not affected the catalytic activity (i.e., the polymerization rate of nucleosides into RNA products) or promoter binding function of the mutant enzyme. On the other hand the termination efficiency of the mutant enzyme was considerably enhanced (15%) after preincubation and subsequent assay both at 37°C (Table 9), compared to that of wild type enzyme under the same conditions. This increased efficiency of termination indicates that the mutant enzyme had become more sensitive to the termination signal under the conditions employed. It is noteworthy that even though

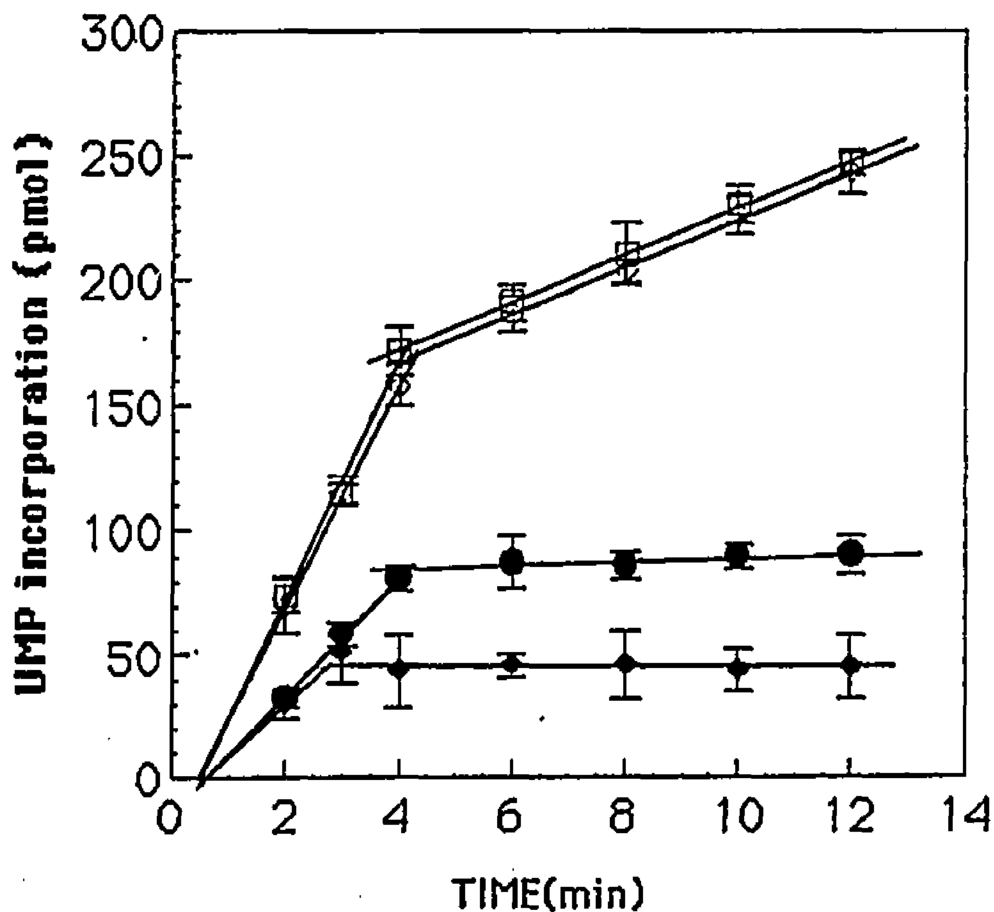


Figure 20. Effect of Preincubation Temperature on the Kinetics of Standard T7 Assay at 37°C. Standard T7 RNA synthesis was carried out with purified wild type and mutant RNA polymerase holoenzymes (1.23 pmole each) as described in Materials and Methods. Each data point represent the average of duplicate determinations from two separate assyas. Standard error is indicated by error bars. (□) and (◇) represent incorporation with wild type holoenzyme after preincubation at 37°C and 44°C, respectively. (●) and (♦) represent incorporation with mutant holoenzyme after preincubation at 37°C and 44°C, respectively.

Table 9. Effect of Preincubation and Higher Assay Temperature on T7 Assay Parameters. The assay parameters were calculated from the kinetics of T7 RNA synthesis, Figure 20.

Holoenzyme	Preincubation temperature	Concentration[a] of active enzyme (pmole)	Loss of[b] activity (%)	T_o (min)	T_i (min)	Chain growth rate (nucleotides/sec)	Termination efficiency $(1-f_{RT})$
Wild type	37°C	0.91	26	0.5	4.2	25	85
	44°C	0.91	26	0.6	4.2	25	78
Mutant	37°C	0.45	63	0.6	4.3	25	95
	44°C	0.45	81	0.52	N.D.	N.D.	N.D.

[a]Calculated from Figure 17 for each curve.

[b]An equal concentration of active enzyme was used for all assays; hence 1.23 pmole was considered as 100% activity. Specific activities of wild type and mutant holoenzyme were 2.4 and 1.1 unit/mg, respectively.

N.D. not determined due to severe defect of mutant enzyme.

the entire transcriptional process (initiation, elongation, and termination) is DNA-dependent, only initiation and termination are DNA sequence specific and of those only the termination step has apparently been affected by this mutation. These observations can be further explained in the context of protein-nucleic acid and protein-protein interactions (see Chapter 4).

Effect of Preincubation Temperatures on T7 Transcription Products

Since the kinetic studies suggested the thermal lability of the mutant enzyme at elevated temperatures, T7 RNA transcripts made by wild type and mutant enzyme were examined by gel electrophoresis (Golomb and Chamberlin, 1974). As with kinetic assays an equal concentration of active enzyme (1.23 pmole) from either the mutant on wild type was preincubated at 32°C, 37°C, or 44°C for 5 minutes prior to the assay. The standard T7 transcripiton assay was started by addition of the preincubated enzymes as described earlier. Synthesis was allowed to continue for 10 minutes at 32°C and the products were analyzed by gel electrophoresis as described in Materials and Methods. A densitometer tracing of fluorogram of these gels is shown in Figure 21. Also Table 10 shows the measured area of the bands obtained from this densitometer tracing. It is evident from Figure 21 and Table 10 that the transcripts formed by the mutant enzyme are considerably less than those

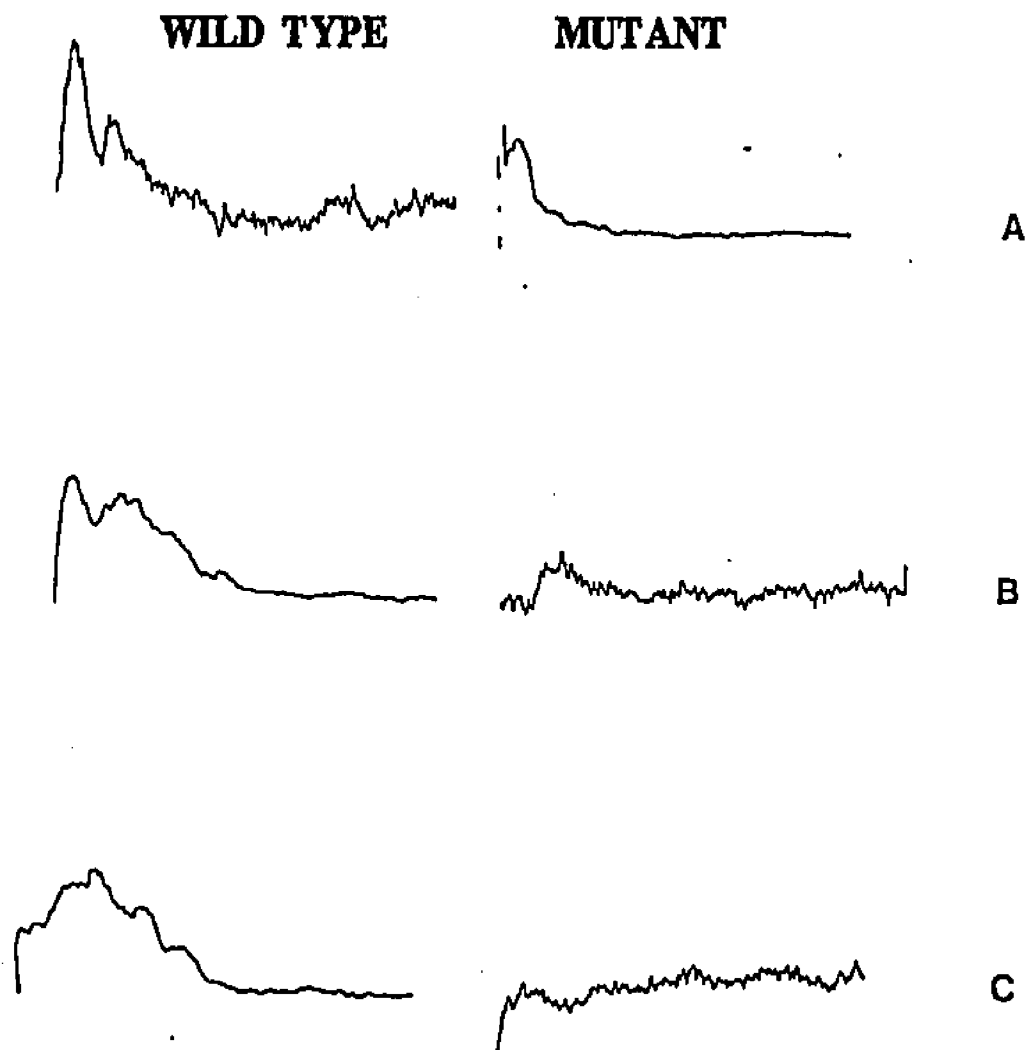


Figure 21. Densitometer Tracing of T7 RNA Transcripts Separated by Gel Electrophoresis. T7 transcripts made by the wild type and the mutant holoenzymes were separated by gel electrophoresis as described in Materials and Methods. The resulting autoradiograph of the gel was scanned. (A), (B), and (C) represent preincubation for 5 mintues at 32°C, 37°C, and 44°C, respectively, prior to the assay.

made by wild type enzyme after 5 minutes preincubation at 37°C or 44°C. The percentage loss of activity calculated from these data for the mutant enzyme after preincubation at 37°C are 33% and 88%, respectively, compared to 31% and 61% loss of activity obtained from kinetic data (Table 8). These results provided further support for kinetic analysis suggesting that thermal denaturation of the mutant enzyme occurred at temperatures above 32°C.

Thermostability of Mutant RNA Polymerase

Since the genetic evidence and the in vitro transcription assays both revealed the temperature-sensitive nature of the mutant enzyme, the holoenzymes were tested for thermostability. Thermostability of the purified enzymes was examined by measuring the rate of heat inactivation. Figure 22 shows the kinetics of heat-inactivation, in which both enzymes were preincubated at 44°C for various periods in the presence of 20% glycerol and 0.5 M NaCl. The activity remaining in each sample was determined at 32°C for 10 minutes using the standard T7 assay, in the absence of rifampicin. The half life ($t_{½}$) for the wild type and the mutant enzyme was calculated from the kinetic data (Figure 22) as the time required to give a 50% inactivation of enzyme. The half life of the wild type and mutant enzymes were 18 and 4 minutes, respectively, showing that the mutant enzyme was 4.5 fold more thermolabile than the wild type enzyme. In addition these results indicate that the mutant

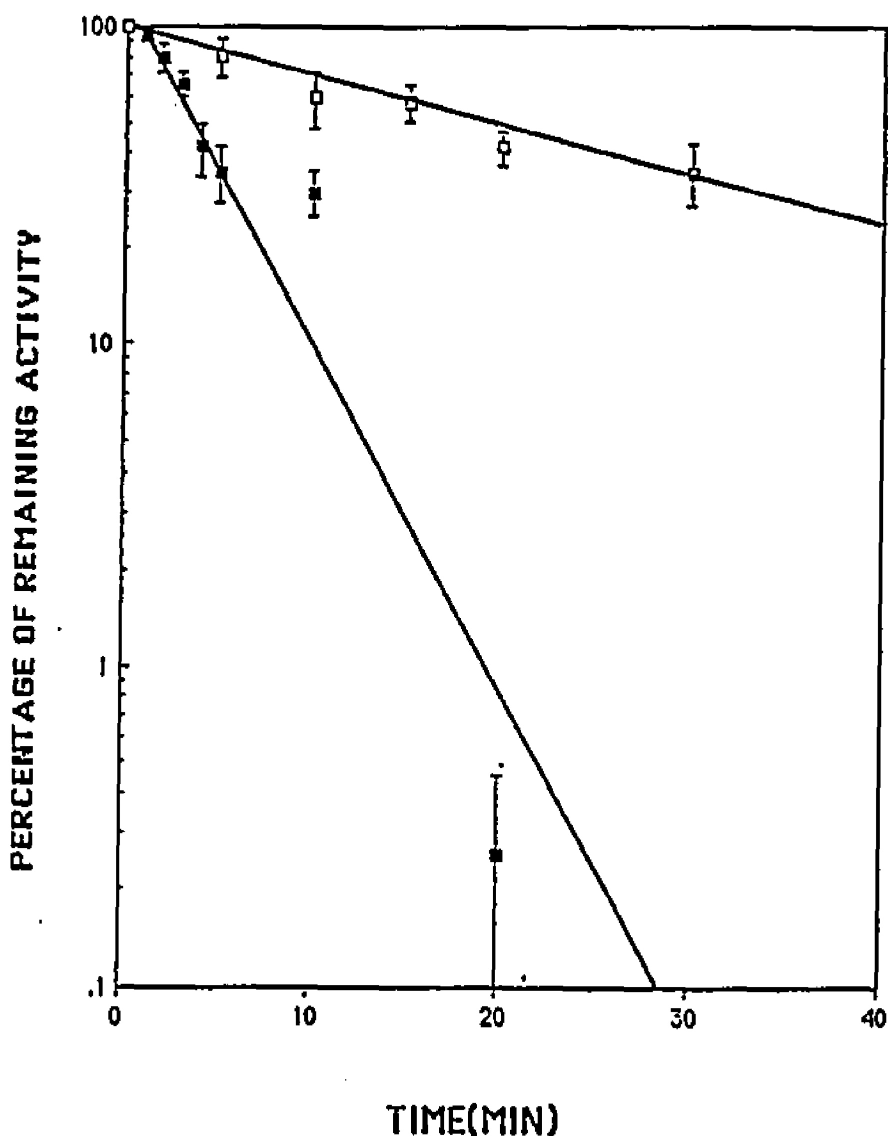


Figure 22. Kinetics of Heat Inactivation. A volume of 240 µl of wild type (9.12 µg of protein equivalent to 7.38 pmole of active holoenzyme) and 800 µl of mutant RNA polymerase (33.6 µg of protein equivalent to 9.85 pmole of active holoenzyme) were incubated at 44°C. At intervals samples of 40 µl of wild type and 100 µl of mutant holoenzyme were removed and assayed at 32°C for 10 minutes, in the standard T7 assay, in the absence of rifampicin. One hundred percent activity corresponds to 1.23 pmole of active holoenzyme, equivalent to 1.52 µg of wild type and 4.2 µg of mutant holoenzyme. Each data point represents the average of duplicate determinations from two separate assays. (□) and (■) represent the percentage of remaining activity for wild type and mutant enzyme, respectively.

Table 10. Relative Area of Scanned T7 Transcripts. The areas were calculated from scanned autoradiogram shown in Figure 18.

Enzyme	Preincubation Temperature (°C)	Scan Area (cm^2)
	32	10.4
Wild Type		
	37	12.4
	44	18.2
	32	7.5
Mutant	37	5
	44	0.9

enzyme became irreversibly inactivated after heat treatment at 44°C.

Transcriptional Fidelity of Mutant RNA Polymerase

The fidelity of transcription by the control and mutant polymerases was examined by measuring the misincorporation of a labeled non-complementary substrate with two different synthetic DNA templates. First, the activity of RNA polymerase from either enzyme was determined in the presence of the complementary substrates at 32°C for 10 minutes, prior to the transcriptional fidelity assay. This gave a

measure of normal incorporation with the correct ribonucleotide substrate. Equal activity units of both enzymes were then used for the fidelity assays at 32°C and 37°C. The results are shown in Figures 23 and 24, and Table 11. When poly[d(G)]·poly[d(C)] was used as a template in the presence of the complementary substrate GTP, and non-complementary substrate [^{3}H]UTP, the mutant enzyme showed 1.6 fold higher misincorporation compared to the wild type enzyme at the permissive temperature, 32°C. However, this value was 3.3 fold higher than that of the wild type enzyme at 37°C (Figure 23 and Table 11). When poly [d(A)]·poly [d(T)] was used as a DNA template, with ATP as a complementary and [^{3}H]CTP as a noncomplementary substrate, the mutant enzyme exhibited 2.7 fold higher misincorporation than the wild type enzyme at 32°C. In contrast, this value was about four-fold higher at 37°C (Figure 24 and Table 11). The significance of these results is two-fold. First, the average amount of misincorporation for the wild type enzyme, either at 32°C or 37°C, was the same. The rate of misincorporation for the wild type enzyme did not change with the temperature, whereas the mutant enzyme showed a higher rate of misincorporation at the elevated temperature, 37°C (Table 11). Secondly, it seems that the mutant enzyme exhibited a higher rate of misincorporation with poly [d(A)]·[d(T)] than with poly [d(G)]·[d(C)] either at 32°C or

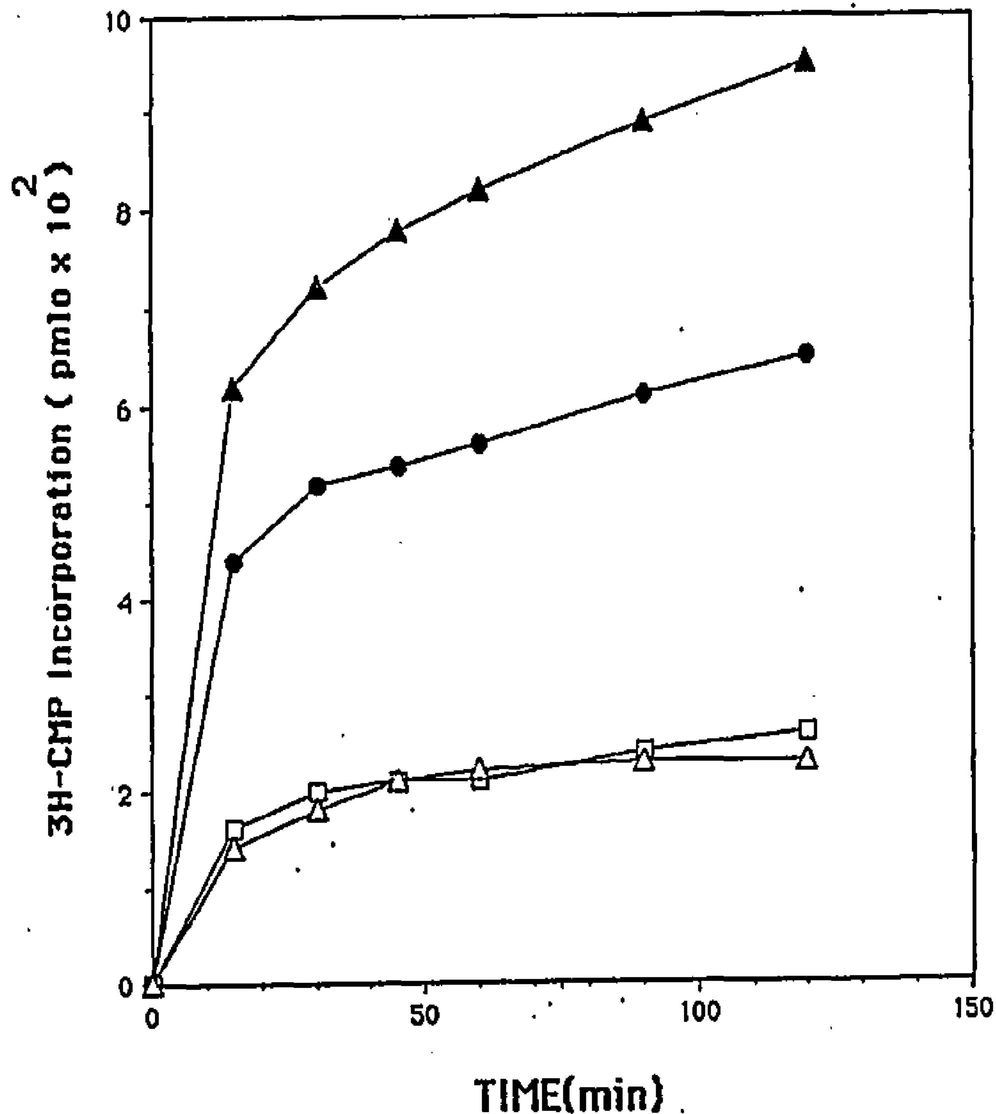


Figure 23. Transcriptional Fidelity Assay With Poly [d(G)]·[d(C)] as a Synthetic DNA Template. Equal activity of holoenzymes (1.52 µg of wild type and 2.1 µg of mutant enzyme), with 6 µg of poly [d(G)]·poly [d(C)] as a template, in the presence of 0.2 mM GTP and 5 µCi of [^{3}H]-UTP (25 Ci/mmole) were mixed in final volume of 100 µl as described in Materials and Methods. The assays were carried out at 32°C and 37°C. Data are for 20 µl samples taken during the assay. (□) and (△) represent misincorporation with wild type enzyme at 32°C and 37°C, respectively. (●) and (♦) represent misincorporation with mutant enzyme at 32°C and 37°C, respectively.

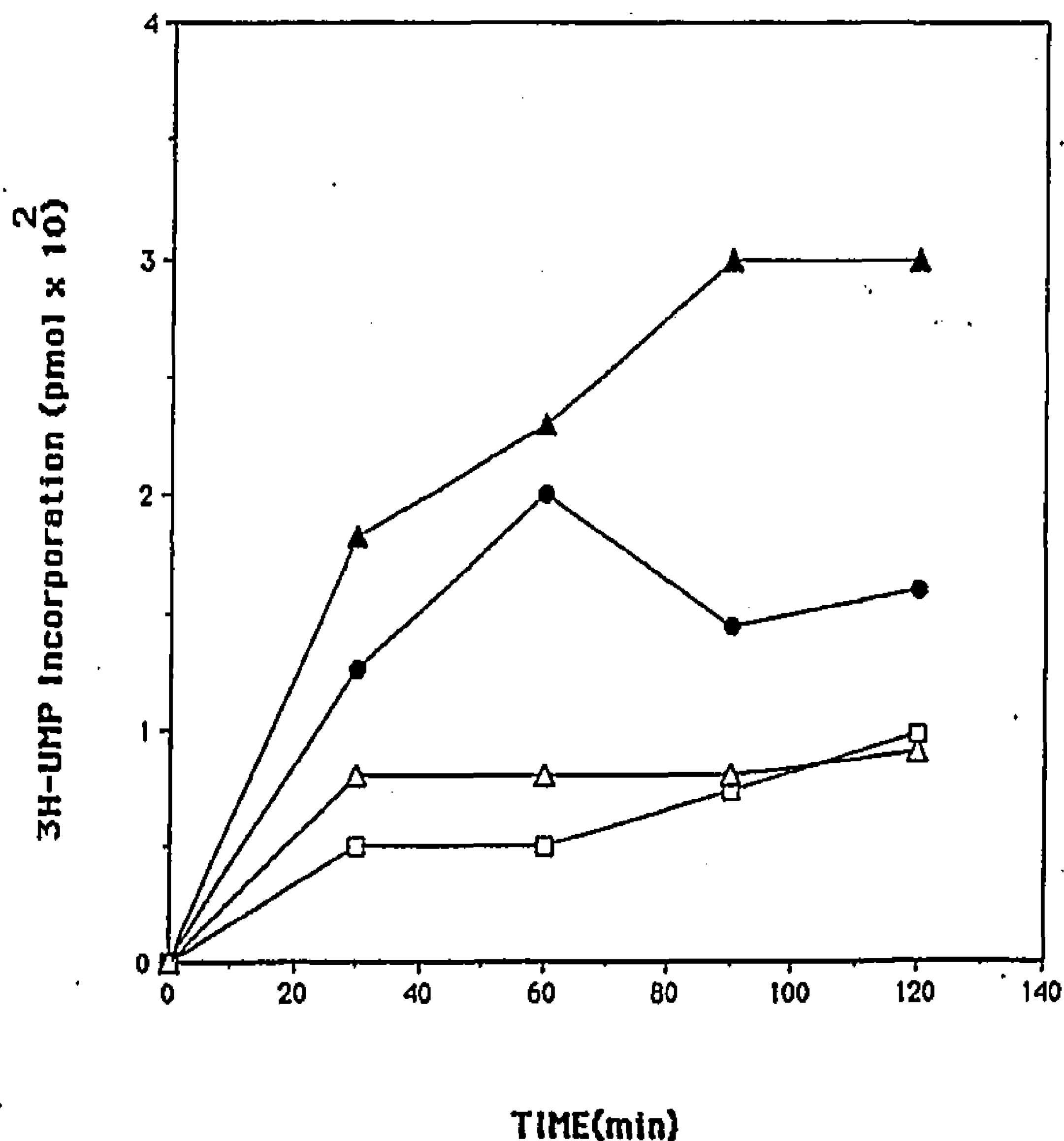


Figure 24. Transcriptional Fidelity Assay With Poly [d(A)]·poly[d(T)] as a synthetic DNA template. Equal activity of holoenzymes (1.52 μg of wild type and 2.1 μg of mutant enzyme), with 6 μg of poly [d(A)]·[d(T)] as a template, in the presence of 0.2 mM ATP and 5 μCi of [^{3}H] CTP (specific activity 25 Ci/mmole) were mixed in final volume of 100 μl as described in Materials and Methods. The assays were carried out at 32°C and 37°C. Data are for 20 μl samples taken during the assay. (□) and (△) represent misincorporation with wild type enzyme at 32°C and 37°C, respectively. (●) and (▲) represent misincorporation with mutant enzyme at 32°C and 37°C, respectively.

Table 11. Summary of Transcriptional Fidelity.

Template	Source of enzyme	Labeled non-complementary substrate	Assay temperature	Average rate of misincorporation (pmole/min)	Ratio of average misincorporation (mutant/wild type)
		32°C	$0.2x10^{-3}$		
	wild type				at 32°C = 2.7
poly[d(A·T)]	[^{3}H]CTP	37°C	$0.2x10^{-3}$		
		32°C	$0.54x10^{-3}$		
	mutant				at 37°C = 4
			37°C	$0.79x10^{-3}$	
		32°C	$0.08x10^{-3}$		at 32°C = 1.6
	wild type				
poly[d(G·C)]	[^{3}H]UTP	37°C	$0.075x10^{-3}$		
		32°C	$0.13X10^{-3}$		
	mutant				at 37° = 3.3
			37°C	$0.25x10^{-3}$	

37°C (Table 11). These results collectively suggest that the mutation in the α subunit of RNA polymerase has somehow affected the recognition of specific nucleotide sequences in DNA molecules.

CHAPTER 4

Discussion

The complex structure of the multi-subunit RNA polymerase is believed to be required to match the complexity of the polymerization process and of transcriptional controls. A number of attempts have been made to reveal the role each subunit plays in transcription and the mechanisms underlying the synthesis and assembly of the subunits of this multimeric enzyme. Detailed analyses of subunit interactions in RNA polymerase are required for a complete understanding of the mutual relationships between RNA polymerase subunit assembly and their significance in the control of transcription.

The role of RNA polymerase subunits in transcription might be studied most directly by analysis of the isolated subunits. However, many of the functions associated with each subunit are not seen in the isolated state. The holoenzyme assembly is required for their intrinsic activities to be exposed. In addition, functions that are dependent on the preceding step reaction(s) carried out by other subunits cannot be studied in this way. Thus, a number of procedures have been developed to elucidate the subunit functions (Zilling et al., 1976; Yura and Ishihama, 1979).

The existence of multiple functional sites required for RNA synthesis was indicated by early studies of chemical modifications (Ishihama and Hurwitz, 1969). Certain modifications with chemical reagents inactivated RNA polymerase, yielding enzyme preparations which were still capable of catalyzing some of the discrete steps of RNA synthesis. A number of reagents which react with sulfhydryl groups rendered the enzyme inactive in binding to DNA, in the initiation of RNA synthesis and in elongation of RNA chains, but had no effect on the binding of ribonucleoside triphosphates to the enzyme. Since initiation and elongation of RNA chains depend on the binding of the enzyme to the DNA, the sulfhydryl groups appear to be essential for this binding reaction. Likewise, amino groups play an essential role in the substrate-binding reaction. Modification of histidine residues of the enzyme results in the formation of an enzyme preparation able to bind DNA and ribonucleoside triphosphates and to catalyze the initiation reaction but unable to catalyze the elongation reaction. These experiments suggested for the first time that multiple functional sites on the enzyme were involved in the enzymatic synthesis of RNA (Ishihama, 1981). The distribution of these sites among different subunits of the polymerase has been investigated by reversible dissociation of the enzyme into partially active subassemblies (Ishihama, 1972). The functional role of each subunit was deduced by

testing whether such subassemblies sufficed for the performance of partial functions. For instance, enzyme dissociation by low concentrations of urea led to formation of $\alpha\beta$ and $\alpha\beta'$ complexes (Ishihama, 1972). Both combinations of subunits are capable of binding to DNA. It thus seems that the DNA binding capacity resides on the β and β' subunits, though the observed DNA binding does not imply the same specific interactions as the holoenzyme. Since the isolated β subunit, but not the β' one, lacks the ability to complex with DNA, subunit α seems to be required for the DNA-binding activity to be exposed (Fukuda and Ishihama, 1974). It has been claimed that isolated β is the only free subunit able to bind labeled rifampicin (Zilling et al., 1976). However, conflicting evidence has been presented (Lill and Hartman, 1973). There is agreement that the complex $\beta\alpha_2$, an intermediate in polymerase assembly, binds rifampicin with high affinity (Lill et al., 1975). The question of whether β can be obtained in the "native" state that allows the binding of the drug or if assembly with α is required for the acquisition of rifampicin binding activity remains to be answered conclusively.

Several specific structural modifications of RNA polymerase are brought about under certain conditions by the appearance of modifying enzymes within the cell, for example, in E. coli after infection with bacteriophage T4 (Rohrer et al., 1975). T4 alteration is effected by an

NAD^+-protein-ADP ribosyl transferase injected by the phage during infection. It results in ADP ribosylation of a particular arginine guanido group on one of the two α chains (Rohrer et al., 1975). T4 modification is effected by a phage gene encoded enzyme synthesized after infection and leads to the same change of α as alteration except that both α chains are modified. The specificity of alteration indicates the non-equivalence of the two α subunits, suggesting that the α subunits are differently arranged in RNA polymerase. Also treatment of RNA polymerase with p-(chloromercuri) benzoate results in the release of one of the α subunits (Ishihama, 1972, 1981). The unequal exposure of the α subunits in RNA polymerase is also indicated by the data obtained with monoclonal antibodies. It would appear that one of the α subunits is relatively exposed while the other is positioned with more of its surface shielded by the larger ß and ß' subunits (Riftina et al., 1989). This is consistent with the observation of Stender (1980) regarding the low accessibility of core enzyme to polyclonal anti-α antibodies. Tichelaar et al. (1983) showed by immunoelectron microscopy that almost half of the α surface is covered by ß and ß' subunits. In addition, chemical cross-linking of the holoenzyme indicated that δ interacts with ß and ß' and with at least one of the α subunits (Hillel and Wu, 1977). This has been supported on the basis of studies with subunit-specific antibodies, since Tichelaar

et al. (1983) proposed that the δ subunit resides at the concave side of the core enzyme where it interacts with the α dimer. The core RNA polymerase modified by T4 phage infection showed a reduced affinity for factor δ, indicating an influence of α on δ-binding (Walter, Seifert and Zilling, 1968; Seifert et al., 1969). In contrast to normal RNA polymerase, the T4-modified enzyme does not support significant expression of a number of E. coli genes in a coupled, cell-free, protein-synthesizing system, although it does promote T4 gene expression normally (Mailhammer et al., 1975). The most probable explanation for this effect is a restriction of promoter specificity, which thus implicates α in promoter interaction. It has also been suggested that the dimeric α subunits may be located near the Pribnow box and/or around the -30 base pair position and it is possible that the subunits bind both to A-T rich regions of the promoter (Scherer et al., 1978; Stender, 1980). In addition, T4-modified RNA polymerase shows a decreased salt sensitivity of termination of transcription by factor ρ (Schäfer and Zilling, 1973). These are the only indications of subunit α having a specific role in RNA polymerase function.

The analysis of functional consequences of mutations within the subunits of RNA polymerase is potentially one of the most powerful tools for investigating the role of subunits in transcription. However, the complexity of

polymerase structure and function and the large size of the subunits require the analysis of large numbers of different mutations covering every subunit structural gene over its entire length. The production of such mutations depends on the knowledge of the positions of structural genes for polymerase subunits within the bacterial chromosome and/or on the elaboration of specific selection procedures (Scaife, 1976).

This study describes the characterization of a temperature-sensitive α-subunit mutant of E. coli RNA polymerase. The temperature-sensitive mutant was isolated by a localized mutagenesis procedure which gave mutants in the 72-minute region of the E. coli chromosome (Kushner et al., 1977; Champney, 1979). Localized mutagenesis was carried out with hydroxylamine as a mutagen which reacts specifically with cytosine and converts it to a modified base that pairs only with adenine, so that a G·C pair ultimately becomes a A·T pair. This results in a point mutation in DNA double helix.

A temperature-sensitive mutation has been located in the α operon in the 72-minute region of the E. coli chromosome. The gene encoding the α subunit of RNA polymerase, rpoA, is located within this operon and shares a bacterial promoter with the genes for ribosomal proteins S13, S11, S4, and S17. The results of genetic analysis have shown that this mutation can be complemented with λ

transducing phages carrying fragments of the bacterial DNA at 72 minutes, including the alpha operon. In addition transductional mapping with bacteriophage P_1 indicated that the mutation is located in the alpha operon. Since the genes encoding other subunits of RNA polymerase, rpoB, rpoC, and rpoD, are located elsewhere in the chromosome, it is clear that the temperature-sensitive mutation is located in structural gene for the alpha subunit of RNA polymerase.

As well as an effect on transcription the mutation affected protein synthesis in vivo but not in vitro. This is due to the fact that in prokaryotes translation is closely coupled to the transcriptional process. Kinetics of both total RNA and ribosomal RNA synthesis in vivo showed that the rate of synthesis in the mutant was severely reduced especially at elevated temperatures. The temperature-sensitive growth of the mutant cell can be explained in terms of the temperature-sensitivity of its RNA polymerase and the reduction of protein synthesis observed is a secondary effect of these coupled processes.

In order to study the consequence of this mutation on the function of the enzyme, RNA polymerase was partially purified on small scale by a commonly used procedure (Gross et al., 1976). The kinetics of in vitro RNA synthesis with calf thymus DNA as a template suggested that the enzyme from the mutant strain became unable to synthesize RNA efficiently at high temperatures. This was primarily due to

a 60% loss of activity or to a two and a half-fold reduction in specific activity of the mutant enzyme at 44°C. These results suggested that the mutation in α subunits had somehow affected the catalytic activity of enzyme. This observation could be due to two different effects. Since there are considerable physical contacts between α and β subunits (Ishihama, 1981), it is possible that small changes in the dimeric α subunits, (due to the mutation) may result in a distortion of catalytic site(s) located in β the subunit. This in turn may affect the rate of elongation or polymerization of nucleoside triphosphates into RNA products. Alternatively, the assembled enzyme may denature or fall apart at higher temperatures. In fact it has been shown that formation of a dimeric α subunit is the first step in the assembly of RNA polymerase (Ishihama, 1981). The temperature-sensitivity of two mutants of the α subunit (Ishihama et al., 1980; Kawakami and Ishihama, 1980) has been attributed to the assembly defect as well as to the thermolability of assembled RNA polymerase. In any case, the calf thymus assay system was incapable of making a clear distinction between these possibilities. In addition the partially purified enzyme employed was a mixture of core polymerase and holoenzyme, which added more to the complexity of this problem.

To solve this second problem, a new small-scale purification method was developed which separated core

polymerase and holoenzyme directly from a crude extract by affinity on a single-stranded DNA-agarose column. Some impurities flow through the column at 0.25 M NaCl. It was found that a step elution was much more satisfactory than the gradient elution of Nüsslen and Heyden (1972) since the enzymes eluted in a much more concentrated form. The optimal salt concentrations used for the elution of core polymerase and holoenzyme were previously determined in the study of de Haseth et al. (1978). At 0.4 M NaCl core polymerase eluted rapidly, but there was a very slow constant rate of holoenzyme elution. Therefore, the 0.4 M NaCl elution could be continued much longer than suggested without decreasing the recovery of holoenzyme in the 1 M NaCl step. The flow rate was decreased during the 1M NaCl elution to allow more time for the holoenzyme to dissociate from the column and to increase the concentration of protein in the fractions. The partially purified holoenzyme was further purified by gel filtration immediately after the affinity chromatography without changing the buffer. The procedure resulted in a 56% yield of enzyme from 6 gram of frozen cells in one day with final purity of 78%.

Using holoenzyme from this new purification procedure it was then important to determine the effect of the mutated α subunit on promoter-binding, elongation, and the efficiency of termination by the enzyme. Bacteriophage T7 DNA is unique as a template in that there is a single major

transcriptional unit for E. coli RNA polymerase which allows the reaction to attain a steady state in which all active RNA polymerase molecules elongate RNA chains at a constant rate. An interesting and profitable feature of the T7 DNA-directed assay is that it reveals several aspects of RNA polymerase activity and function in a single experiment. The assay measures the concentration of active enzyme, the time of promoter site selection, the rate of RNA chain elongation, and the efficiency of termination by the enzyme at the early T7 terminator. Hence, this procedure should be useful in monitoring the potentially subtle changes in the enzymatic properties of the enzyme in vitro, by mutational changes, or by regulatory alternations of the enzyme in vivo, in different growth situations (Chamberlin et al., 1979).

Studies using T7 DNA as a template indicated that the mutant enzyme was severely and irreversibly thermolabile even at 37°C, suggesting thermal denaturation of the assembled enzyme at temperatures above 32°C. As mentioned earlier, formation of the dimeric α subunit is the first step in the assembly of the enzyme, and subsequent addition of other subunits gives rise to a completely assembled RNA polymerase. The mutated α subunit may refold or dimerize differently from the unmodified one. Thus it is likely that this temperature-sensitive mutation weakens protein-protein interactions between RNA polymerase subunits leading to easy

disintegration of the enzyme structure at elevated temperatures. In fact a large number of temperature-sensitive mutants defective in the assembly of RNA polymerase have been well characterized, primarily those of ß and ß' subunits. Those conditionally assembly-defective mutants have shown that the assembly of the enzyme is inefficient at all steps where an altered ß subunit is involved and thus the mutant ß is incapable of associating with the α subunit at non-permissive temperature (Taketo and Ishihama, 1977). On the other hand, it seems that the rate of elongation (the number of nucleotides polymerized per second) has not changed for the mutant enzyme when assayed at 32°C. This indicates that even though there are considerable physical contacts between α and ß subunits (Ishihama, 1981), the catalytic activity of the mutant enzyme has not been affected by this mutation in the dimeric α subunits.

Studies with the present α mutant suggest that the lag time in the T7 assay, which is inversely proportional to the rate at which the enzyme is able to locate the T7 promoters, has not changed for this mutant at 32°C or 37°C. This suggests that the mutation in the dimeric α has not altered the ability of the holoenzyme to locate the T7 promoters and effectively initiate RNA chain synthesis.

Termination sites recognized by E. coli RNA polymerase have been extensively investigated. Terminators are

categorized either as simple (factor-independent) or complex (factor-dependent) (Yager and Von Hippel, 1987). A simple terminator consists of G + C-rich hairpin loop followed by five or more U residues and allows efficient termination in an in vitro transcription system containing only template and RNA polymerase. The hairpin loop signals a transcription pause site while the unstable dA-rU base-pairs promote dissociation of the nascent RNA-DNA hybrid (Farnham and Platt, 1980; Martin and Tinoco, 1980). In contrast, little is known concerning the role of RNA polymerase in termination. One approach to this problem, taken by Glass and his colleagues, has been to make mutations throughout rpoB, the gene encoding the ß subunit of RNA polymerase, and to analyze the mutant phenotypes following suppression with a variety of nonsense suppressors (Nene and Glass, 1984). These researchers identified mutations in several regions of rpoB that may be involved in termination. It has been shown that some rifampicin-resistant (rif^R) mutations alter termination at specific terminators. The work of Neff and Chamberlin (1980) was the first to show that in vitro termination is altered by some rif^R mutations. Further studies by Junjin et al. (1988) indicated that rif^R mutations in the N-terminal and very C-terminal portion of the ß subunit region increased termination while those in the central portion of the region decreased termination. Interestingly, the mutant enzyme exhibited an enhanced

termination at 37°C. It is likely that this mutation in the dimeric α has altered interactions in the N-terminal or C-terminal portion of the β subunit and the result of this altered interaction would be an increase in termination efficiency.

As mentioned earlier, since the isolated β subunit lacks the ability to complex with DNA, subunit α seems to be required for the DNA-binding activity to be exposed (Fukuda and Ishihama, 1974). In agreement with this interpretation, the two temperature-sensitive E. coli strains carrying a mutation in the α-subunit gene (rpoA) of RNA polymerase have exhibited considerably lower fidelity in transcription than wild type RNA polymerase, implying that the α subunit is somehow involved in the recognition of the template sequence (Ishihama et al., 1980). In agreement with this observation, the mutant RNA polymerase showed a lower fidelity in transcription in vitro with two different synthetic DNA templates even at the permissive temperature of 32°C. However, unlike the observations of Ishihama et al. (1980), this mutant enzyme exhibited three-fold lower fidelity with poly [d(A)]·[d(T)] than with [d(G)]·[d(C)] as a template. In addition Springgate and Loeb (1975) reported that the most frequent error catalyzed by the wild type RNA polymerase was the transition d(A)-r(U) to d(A)-r(C). Interestingly, the misincorporation by the mutant enzyme with this transition (when poly[d(A)]·[d(T)] was used) was

much more pronounced than that of the wild type enzyme at all temperatures tested. These observations, along with the higher termination efficiency observed for the mutant enzyme, support the idea that the dimeric α subunit is somehow involved in the recognition of the template sequence (Ishihama et al., 1980).

Studies with a different temperature-sensitive α-subunit mutant have indicated the importance of the dimeric α subunit in the assembly of RNA polymerase enzyme (Kawakami and Ishihama, 1980). Accordingly, it was found that either the dimerization of newly synthesized α subunits or the subsequent association of altered α dimers with β subunits was blocked in this mutant, in particularly at non-permissive temperatures. Heat inactivation studies with the mutant enzyme showed severe and irreversible thermolability at 44°C. The half-life of the mutant enzyme was over four-fold shorter than that of the wild type enzyme, indicating that the mutant was more temperature labile compared to the one studied by others (Ishihama et al., 1980). These results were also supported by the analysis of T7 RNA transcripts and by T7-transcription assays at different temperatures, where the mutant enzyme lost 61% of its activity after 5 minutes of pre-incubation at 44°C. These observations collectively suggested disintegration or disassembly of the mutant enzyme at temperatures higher than 32°C. This severe lack of thermostability could result from

an amino acid substitution in the region of the α subunit which is critical to folding and/or subunit-subunit interactions.

The results presented here support the earlier suggestion (Stender, 1981) that α subunits do not function directly in the catalysis of RNA synthesis or DNA-binding, but serve to maintain an active conformation of the enzyme, presumably by keeping the correct linkage between β and β' subunits. In addition, the results support the notion that the dimeric α subunit is indirectly involved in the recognition of the template sequence (Ishihama, 1981). Further studies including antibody binding, detection of incomplete subassemblies in vivo, proteolytic digestion of the mutant enzyme and determination of the altered amino acid sequence should provide more information concerning the structure-function relationship of this subunit in RNA polymerase.

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CURRICULUM VITA

Majid Mehrpouyan

Address:	Department of Biochemistry, Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614 Business Phone: (615) 929-6211
Personal Data:	Birth: December 6, 1954 Place of Birth: Tehran, Iran Immigration Status: Student Visa (F-1) Marital Status: Married
Education:	East Tennessee State University, Quillen College of Medicine, East Tennessee State University, Johnson City, TN.; Ph.D., Biochemistry, 1990 East Tennessee State University, College of Health Sciences, Johnson City, TN; M.S., Microbiology 1985 University of Maine, Orono, Maine, College of Arts and Sciences,; B.S. Microbiology 1983
Technical Experience:	Extensive training and experience in enzymology, enzyme kinetics, enzyme and protein purification, chromatographic techniques, HPLC, peptide mapping and variety of protein gel electrophoresis. Chemistry and biochemistry of nucleic acids (DNA and RNA), and protein - nucleic acid interactions. Techniques in molecular biology including isolation and preparation of DNA (genomic, plasmid and bacteriophage) and RNA. Cloning techniques. Advanced techniques in molecular genetics of bacteria including lacZ gene and protein fusions, transpositions, targeted mutagenesis, construction of genetic maps.
Professional Experience:	Teaching assistant 1983-1985. College of Health Sciences, East Tennessee State University, Johnson City, TN. Taught undergraduate laboratory courses in general microbiology and microbial pathogens. Research Assistant 1985-1986. College of Medicine, Department of Biochemistry, East Tennessee State University. Dr. David Johnson's laboratory. Enzymology of α_1-PI.
Memberships:	Sigma Xi