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Genetic requirements for growth of *Salmonella typhimurium* lacking the proofreading subunit of DNA polymerase III

Lancy, Edward Donald, Jr., Ph.D.

Case Western Reserve University (Health Sciences), 1990
GENETIC REQUIREMENTS FOR GROWTH OF SALMONELLA TYPHIMURIUM

LACKING THE PROOFREADING SUBUNIT OF DNA POLYMERASE III

by

Edward Donald Lancy, Jr.

Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

Thesis Advisor: Russell A. Maurer, Ph.D.

Department of Molecular Biology and Microbiology
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May 20, 1990
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GRADUATE STUDIES

We hereby approve the thesis of

Edward Donald Lancy, Jr.

candidate for the Ph.D.
degree.*

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GENETIC REQUIREMENTS FOR GROWTH OF SALMONELLA TYPHIMURIUM LACKING THE PROOFREADING SUBUNIT OF DNA POLYMERASE III

Abstract

by

Edward Donald Lancy, Jr.

DnaQ (mutD) encodes the editing exonuclease subunit (epsilon) of DNA polymerase III. Deletion-substitution alleles of the dnaQ gene were constructed in vitro and then introduced into the Salmonella chromosome to yield dnaQ null mutants. These null mutants exhibited a growth defect and as a consequence of the poor growth of dnaQ mutants, these strains were replaced within single colonies by derivatives carrying an extragenic suppressor mutation that compensated the growth defect.

Sixteen independently derived suppressors mapped in the vicinity of dnaE, the gene for the polymerization subunit (alpha) of DNA polymerase III. Using a combination of nucleotide sequencing and marker rescue experiments, the alteration in one such suppressor was localized to the dnaE gene and results in a valine to glycine substitution at amino acid 832 of the 1,160 amino acid alpha polypeptide. Partially purified DNA polymerase III containing this altered alpha subunit was active in polymerization assays and lacked any detectable editing activity.

A two hundred nucleotide region encompassing the site of the valine to glycine substitution mutation was analyzed in fourteen
other suppressor mutants using a combination of the polymerase chain reaction and DNA sequencing. Three of the fourteen suppressor mutants contained the valine to glycine substitution at position 832. The other eleven contained valine (wild type) at position 832 and did not contain any other changes from the wild type sequence.

In addition to their dependence on a suppressor mutation affecting alpha for healthy growth, the dnaQ null mutants strictly required DNA polymerase I for viability. All three activities of DNA polymerase I were required in the dnaQ null mutants when a suppressor mutation was not present. In the presence of a suppressor mutation, the dnaQ null mutants no longer required the 3'→5' exonuclease of DNA polymerase I.

These studies indicate that in the absence of epsilon, DNA replication falters unless secondary mechanisms come into play, including a genetically-coded alteration in the intrinsic replication capacity of alpha and an increased use of DNA polymerase I. Thus, epsilon plays a role in DNA replication distinct from its known role in controlling spontaneous mutation frequency.
DEDICATION

I dedicate this thesis to my parents and to my fiancée, JingJing, for their constant love and support.
ACKNOWLEDGEMENTS

I express sincere thanks to Dr. Russell A. Maurer for his support and guidance throughout the course of this work.

I thank the members of my thesis committee, Drs. T. Nilsen, C. Miller, P. DeHaseth, and C. Cooper, for their guidance and input. Specifically, Dr. Nilsen for his persistent advice to "just sequence the gene"; and Dr. Miller for his comments and suggestions.

I also thank the members of Dr. Maurer's laboratory, past and present, for providing a stimulating and amicable work environment. Specifically, Annette Wong and Joyce Engstrom for their friendship, encouragement and help in getting me started in the laboratory; Miriam Lifsics for her many helpful suggestions, her assistance in proofreading this thesis, and her biochemical contribution to this work which helps to create a more complete picture; and to Paresh Shrimankar for his friendship, encouragement, discussions, and company in the lab during the late night/early morning hours.

Finally, I thank my family - my parents and my sisters, Sherri and Janet, for their constant support and encouragement. Special thanks are extended to Sherri, Tom, and Shane for welcoming me into their home for the past two years.
Chapters II and III of this thesis have previously appeared in
the literature and are presented in their entirety (see Literature
Cited, Lancy et al., 1989a and 1989b). Furthermore, Chapters II and
III contain appendices which include additional experiments that were
not included in the published reports.

The contribution by Dr. Miriam Lifsics to portions of the work
presented in Chapters II and III is hereby acknowledged. Dr. Lifsics
is responsible for the biochemical analysis of the alpha subunit and
of DNA polymerase III from Salmonella typhimurium. These experiments
culminated in the data presented in Table 2 and Figures 5 and 6 of
Chapter II. In Chapter III, Dr. Lifsics carried out the identifica-
tion of the gene products expressed in the vicinity of the dnaE gene,
the results of which are summarized in Figure 9.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Tn10dTc</td>
<td>derivative of Tn10 conferring resistance to tetracycline</td>
</tr>
<tr>
<td>Tn10dKm</td>
<td>derivative of Tn10 conferring resistance to kanamycin</td>
</tr>
<tr>
<td>Tn10dCm</td>
<td>derivative of Tn10 conferring resistance to chloramphenicol</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N' tetraacetic acid.</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>Tc^R</td>
<td>tetracycline resistance</td>
</tr>
<tr>
<td>Tc^S</td>
<td>tetracycline sensitivity</td>
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<tr>
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<td>kanamycin resistance</td>
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<tr>
<td>Cm^R</td>
<td>chloramphenicol resistance</td>
</tr>
<tr>
<td>Sm^R</td>
<td>streptomycin resistance</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

In the enteric bacterial species *Escherichia coli* and *Salmonella typhimurium*, DNA polymerase III is the enzyme responsible for the majority of chromosomal replication, including synthesis of the entire leading strand (continuous synthesis) and the Okazaki fragments of the lagging strand (discontinuous synthesis). DNA polymerase I, an enzyme distinct from DNA polymerase III, matures the DNA by filling any short gaps between Okazaki fragments and replacing RNA primers used to initiate Okazaki fragment synthesis. A third polymerase, DNA polymerase II, has no known role in the replication of the chromosome and is more likely involved in repair of DNA. This is based on the inducibility of this enzyme by DNA damage and its ability to copy damaged DNA (Bonner et al., 1988).

The work presented in this thesis involves the characterization of *Salmonella typhimurium* mutants which contain an altered form of the multisubunit DNA polymerase III. This altered polymerase lacks the epsilon subunit which, through the action of its 3'→5' exonuclease activity, is normally responsible for removing replication errors at the replication fork. The absence of the epsilon subunit imparts a severe growth defect on the cells. This growth phenotype is susceptible to modulation by mutations at two other loci: *dnaE*, encoding the polymerization subunit of DNA polymerase III, and *polA*, encoding the single subunit DNA polymerase.
I. In this introduction, I discuss studies which address the structure and function of DNA polymerase III. Also, a comparison is made between DNA polymerase III and other DNA polymerases, both procaryotic and eucaryotic, in order to gain some insight as to what common mechanisms or activities occur in different polymerases. Finally, since the epsilon subunit is so important for fidelity, the role of DNA polymerases in fidelity is also discussed.

A. STRUCTURE AND FUNCTION OF DNA POLYMERASE III.

1. DNA polymerase III holoenzyme and its subassemblies.

DNA polymerase III holoenzyme is a multisubunit enzyme consisting of ten distinctive polypeptides: alpha, epsilon, theta, tau, gamma, delta, delta prime, chi, psi, and beta (Kornberg, 1988; Makishima et al., 1988). Three of the subunits, alpha (α), epsilon (ε), and theta (θ), together form DNA polymerase III core, defined as the smallest active subassembly prepared from DNA polymerase III (McHenry and Crow, 1979). The remaining seven subunits are designated as auxiliary subunits as defined by Kornberg (reviewed in Kornberg, 1988). This definition contrasts with that of Wickner (1976) who believes that the auxiliary subunits are not part of the holoenzyme but are instead, separate proteins which act as replication factors. These replication factors would modulate replication but would not necessarily remain attached to the polymerase throughout replication. These different views are discussed below in the context of the current models of DNA replication by DNA polymerase III.

The DNA polymerase III core is active only on double-stranded
DNA substrates containing short single-stranded gaps. The core polymerase has a low processivity (defined as the number of nucleotides incorporated per binding event of the polymerase) of 10 to 20 nucleotides and a low catalytic activity of 20 nucleotides inserted per second per molecule (Fay et al., 1981; Maki et al., 1986). The processivity and catalytic activity of the purified holoenzyme are >5000 nucleotides and 500 nucleotides per second per molecule, respectively (Fay et al., 1982; O'Donnell and Kornberg, 1985) and holoenzyme is active on long single-stranded DNA such as M13 or ϕX174. Intermediate forms of DNA polymerase III have been isolated and found to possess processivities in between those of the core and holoenzyme (see Table; reviewed in McHenry, 1988). The

<table>
<thead>
<tr>
<th>Polymerase form</th>
<th>Subunit composition</th>
<th>Ratea</th>
<th>Processivityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holoenzyme</td>
<td>α,ɛ,θ,τ,γ</td>
<td>500</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Pol IIIα</td>
<td>α,ɛ,θ,τ</td>
<td>ndd</td>
<td>50</td>
</tr>
<tr>
<td>Pol IIIβ</td>
<td>α,ɛ,θ</td>
<td>nd</td>
<td>30</td>
</tr>
<tr>
<td>Pol III core</td>
<td>α,ɛ,θ</td>
<td>20</td>
<td>10-20</td>
</tr>
</tbody>
</table>

a nucleotides incorporated per second per molecule  
\(b\) nucleotides incorporated per binding event of polymerase  
\(c\) γ refers to the gamma complex which consists of gamma, delta, delta prime, chi, and psi  
\(d\) not determined

Analysis of these intermediates and the availability of cloned subunit genes have yielded an increased understanding of the roles of the individual subunits in DNA replication.
2. Functions of the DNA polymerase III core subunits.

The α subunit contains the polymerase activity of the enzyme and is encoded by the dnaE gene (Welch and McHenry, 1982; Maki and Kornberg, 1985). The ε subunit, encoded by the dnaQ gene, contains the 3'→5' editing exonuclease activity (Scheuermann et al., 1983; Scheuermann and Echols, 1984). The function of the θ subunit and the identity of its gene are not known.

Recently, Studwell and O'Donnell (1990) have carried out reconstitution studies using purified DNA polymerase III holoenzyme subunits to investigate the separate contributions of the core subunits to processivity of the polymerase. The reconstitutions were carried out in the presence of all the auxiliary subunits except tau, which had previously been shown to increase the processivity of the core only 2- to 4-fold (Fay et al., 1982). When DNA polymerase III was reconstituted with only the α subunit of DNA polymerase III (along with the auxiliary subunits, minus tau), the processivity was reduced approximately 2-fold compared with the polymerases reconstituted with either the core or the α·ε dimer (Studwell and O'Donnell, 1990). This 2-fold reduction in processivity is likely an underestimate since it is imagined that the processivity of DNA polymerase III holoenzyme in vivo is virtually unlimited (Kornberg, 1988) and the substrate for measuring processivity in the reconstitution experiments was only 5.4 kilobases in length. Nevertheless, it is clear that the lack of the ε subunit has a detrimental effect on the processivity of DNA polymerase III in vitro. Whether this reduction in processivity results primarily from
the absence of the exonuclease activity or from the absence of the ε subunit itself remains to be determined.

The decrease in processivity of DNA polymerase III reconstituted in the absence of epsilon may be due to the disruption of a functional interaction between the alpha and epsilon subunits. Evidence for a functional interaction between these two subunits was obtained by Maki and Kornberg (1987) who demonstrated that, in an in vitro polymerase reaction using purified α subunit, the activity of the subunit was enhanced 2-fold by the addition of the ε subunit to the reaction. Likewise, the addition of the polymerase subunit to the ε subunit increased the exonuclease activity of the subunit by 10- to 80-fold. Although this enhancement of the exonuclease activity was attributed to a greatly increased affinity of the ε subunit to the 3'-hydroxyl terminus, it nevertheless suggests a functional interaction between the two proteins.

In addition to the demonstration of a functional interaction between the two subunits, genetic evidence also points to an interaction between the epsilon and alpha subunits. Maurer et al. (1984a) isolated extragenic suppressor mutations of a temperature-sensitive allele of dnaE, the gene encoding the α subunit of DNA polymerase III which were shown to be located in dnaQ, the gene encoding the ε subunit. Also, Horiuchi et al. (1981) have shown that a double mutant, containing temperature-sensitive mutations in dnaE and dnaQ, is much more temperature-sensitive than either of the single temperature-sensitive mutations alone. While these studies do not address the specific functions of the epsilon and alpha subunits
that are affected by the mutations, they nonetheless demonstrate an interaction between these two subunits.

The role of the editing exonuclease in fidelity will be considered in a later section.

3. Functions of the auxiliary subunits (except beta).

The addition of the tau subunit to the DNA polymerase III core forms the subassembly DNA polymerase III' (McHenry, 1988) which has an increased processivity of 30 nucleotides compared to that of DNA polymerase III core (10-20 nucleotides) (Fay et al., 1982). In addition, tau endows the DNA polymerase III' subassembly the ability to dimerize (McHenry, 1982). This property of tau initially led to the possibility that DNA polymerase III existed as a dimer in vivo. However, while further evidence does in fact support the existence of a dimeric DNA polymerase III as will be discussed below, it is not known which subunit (or combination of subunits) is responsible for the dimerization of DNA polymerase III.

In reconstitution studies of DNA polymerase III, Maki and Kornberg (1988c) found that when only the tau subunit was lacking, the reconstituted polymerase was more prone to pause upon encountering secondary structures in the template (such as a hairpin region in the single strand or a duplex region formed by a strand annealed to the template). On the other hand, the polymerase reconstituted with tau was able to replicate through the secondary structures. Maki and Kornberg (1988c) interpreted this to mean that tau functions by causing the polymerase to be bound more tightly to
the template.

The tau subunit, a 71 kilodalton polypeptide, and the 52 kilodalton gamma subunit, are encoded by the dnaZX gene (Mullin et al., 1983; Kodaira et al., 1983; Hawker and McHenry, 1987; Maki and Kornberg, 1988a). The dnaZX gene contains a single open reading frame (Yin et al., 1986; Flower and McHenry, 1986) from which the tau and gamma subunits are generated. The tau subunit is proteolytically cleaved at its carboxyl end to produce the gamma subunit (Maki and Kornberg, 1988a). The protease responsible for the cleavage and the regulation of this cleavage event are not known.

Prior to the demonstration that the dnaZX gene was responsible for the synthesis of both subunits, dnaX and dnaZ were thought to represent separate, but closely linked, genetic loci since plasmids from the Clarke-Carbon collection could be isolated which complemented mutations in only dnaX, dnaZ, or both (Henson et al., 1979). It has subsequently been shown that the tau subunit, the full length gene product, is able to complement both dnaX(Ts) and dnaZ(Ts) mutant cell extracts whereas the gamma subunit, the proteolytically-cleaved product, is capable of complementing only cell extracts from dnaZ(Ts) mutants (Maki and Kornberg, 1988a). Thus, the tau subunit possesses two functions, one which is identical to that of the gamma subunit (gamma is derived from tau by the removal of tau's C-terminus), and the other which is specific to the tau subunit. This tau-specific activity is attributable to the carboxyl domain of tau, which is lacking in the gamma subunit.

The gamma subunit was initially isolated in a complex, called
gamma-delta, which reconstituted holoenzyme activity when added to DNA polymerase III core and the beta subunit (Hurwitz and Wickner, 1974; McHenry and Kornberg, 1977). Recently, the gamma-delta complex has been isolated by an improved purification procedure and was shown to contain five different polypeptides (Maki and Kornberg, 1988b). This assembly of subunits, now termed the gamma complex, contains the gamma (52 kilodaltons), delta (35 kilodaltons), delta prime (33 kilodaltons), chi (15 kilodaltons) and psi (12 kilodaltons) subunits of DNA polymerase III. With the exception of the dnaZK gene for the gamma subunit, none of the genes for these subunits has been identified. The addititon of the gamma complex to DNA polymerase III' forms the subassembly DNA polymerase III* (McHenry, 1988). DNA polymerase III* has a slightly greater processivity than does DNA polymerase III' (50 versus 30 nucleotides) (Fay et al., 1982).

O'Donnell and Studwell (1990) have recently reported the purification of the delta subunit, the delta prime subunit, and a gamma-chi-psi complex from the five protein gamma complex which had been initially isolated and described by Maki and Kornberg (1988b) (see above). Using these preparations, O'Donnell and Studwell (1990) were able to reconstitute ATP-activated initiation complexes on primed templates with the beta subunit and either gamma-delta or tau-delta prime heterodimers. When assembled in the presence of the alpha-epsilon dimer, both reconstituted polymerases, as well as polymerase reconstituted with the five protein gamma complex, yielded highly processive synthesis. Thus, while the ability to reconstitute different functional polymerases from different subsets of subunits
supports the presence of a dimeric polymerase, these experiments did not, however, show any functional differences between the different reconstituted polymerases. These experiments did demonstrate that the chi and psi subunits were not necessary for in vitro processive synthesis by the reconstituted polymerases. The functions of these two subunits remain unknown.

4. The beta subunit, an initiation factor.

Addition of the beta subunit to DNA polymerase III* results in the formation of holoenzyme (McHenry, 1988). The presence of beta results in a 25-fold increase in the processivity of the holoenzyme over that of DNA polymerase III' (Fay et al., 1981). The beta subunit is one of the first subunits to bind to the primed template in the formation of the initiation complex (see below). The beta subunit, a 37 kilodalton polypeptide, is encoded by the dnaN gene (Burgers et al., 1981).

Maki and Kornberg (1988c) have demonstrated in reconstitution studies that the beta subunit binds to the primed template in an ATP-dependent manner, through the catalytic action of the gamma complex, and forms an initiation complex, consisting of DNA polymerase III core, beta, and the gamma complex. Initiation complex formation is dependent upon beta as an anti-beta antibody has been shown to inhibit initiation (Johanson and McHenry, 1982).

Once beta is in place, the addition of DNA polymerase III core creates a processive polymerase. The basic unit of the processive polymerase consists of only DNA polymerase III core and the beta
subunit since in reconstitution assays, the gamma complex does not remain attached to the polymerase but rather recycles, supporting the formation of multiple initiation complexes (Maki and Kornberg, 1988c). The beta subunit remains attached to the DNA polymerase during replication (Maki and Kornberg, 1988c) and is embedded in the complex as elongation is insensitive to the action of an anti-beta antibody (Johanson and McHenry, 1982).

The binding of DNA polymerase III core to the beta subunit may be facilitated through an interaction between the alpha and beta subunits. That these two subunits interact was suggested by the isolation of a mutation in the dnaN gene that was able to suppress a temperature-sensitive dnaE allele (Kuwabara and Uchida, 1981).

In summary, the following model can be envisioned for DNA replication by DNA polymerase III from the current knowledge of the activities of the various subunits. In an ATP-dependent reaction, the beta subunit forms the initiation complex with DNA polymerase III core and the gamma complex (gamma, delta, delta prime, chi, and psi) which acts catalytically. From the work of O'Donnell and Studwell (1990), it is known that the requirement of the gamma complex can be substituted for by heterodimers of either gamma·delta, tau·delta prime, or tau·delta. The gamma·delta prime heterodimer does not support initiation complex formation. This implies that the carboxyl end of the tau subunit, lacking in gamma, is essential for interacting with delta prime to produce an initiation-competent heterodimer (tau·delta prime). The tau subunit, in addition to supporting initiation complex formation (in a dimer with either delta
or delta prime), causes the polymerase to bind to the template more tightly (Maki and Kornberg, 1988c). These two activities may represent the two roles of the tau subunit.

Once an initiation complex has been formed, polymerization can begin. During polymerization, the gamma complex or presumably, the initiation-competent heterodimers defined by O'Donnell and Studwell (1990), would recycle to form new initiation complexes. This recycling would be especially useful during lagging strand synthesis where many initiation events occur. This notion of polymerase subunits recycling during polymerization supports the original hypothesis of Wickner (1976) who proposed that auxiliary polymerase subunits are not part of a polymerase holoenzyme, but rather, are separate replication factors that need not remain associated with the polymerase throughout replication.

5. The asymmetric dimeric DNA polymerase III hypothesis.

The initial observation that led to the proposal that DNA polymerase III holoenzyme was dimeric was the demonstration that the various intermediates of DNA polymerase III, with the exception of DNA polymerase III core, were able to form dimers in solution (reviewed in McHenry, 1988). The current proposal that DNA polymerase III is an asymmetric dimer with distinguishable leading and lagging strand polymerases was initially proposed by Johanson and McHenry (1984). Their proposal was based on experimental evidence which suggested two slightly different holoenzyme populations in solution, one that could form initiation complexes in the presence of
adenosine 5'-O-(3-thiotriphosphate) (ATP-gamma-S) and one that can form complexes only with ATP and could be dissociated by ATP-gamma-S (Johanson and McHenry, 1984). In addition, a strong positive cooperativity for the binding of both ATP and ATP-gamma-S to DNA polymerase III was demonstrated and suggests the presence of a strong interaction between the ATP binding sites of the proposed dimeric enzyme (McHenry et al., 1987). This interaction would not be expected for an enzyme that functions as a monomer.

Additional evidence relating to the asymmetric dimeric holoenzyme DNA polymerase enzyme has been obtained in the Kornberg lab (Maki and Kornberg, 1988c; Maki et al., 1988). Maki and Kornberg (1988c) demonstrated that a more processive polymerase resulted when the holoenzyme was reconstituted in the presence of tau than in its absence. The reconstituted tau-less polymerase was more prone to dissociation from the template and thus was less processive. In analyzing the stoichiometric content of a dimeric form of DNA polymerase III*, Maki et al. (1988) obtained suggestive, but not conclusive, evidence that the tau subunit was present in only one half of the enzyme. From these results, a model in which an asymmetric polymerase is able to concurrently replicate both the leading and lagging strands is feasible. The holoenzyme half containing tau, the processive polymerase, would be responsible for the continuous synthesis of the leading strand whereas the less processive tau-less polymerase would be well suited for the discontinuous synthesis of the lagging strand.

The functional redundancy of the heterodimers (gamma-delta and
tau-delta prime) in the formation of initiation complexes observed by O'Donnell and Studwell (1990) is also indicative of a dimeric polymerase. Each heterodimer could reside in separate halves of the polymerase yielding an asymmetric enzyme with the tau-containing half being the processive polymerase and the gamma-containing half being the less processive polymerase.

B. COMPARISON OF DNA POLYMERASE III WITH OTHER DNA POLYMERASES.

In the following section, a variety of procaryotic and eucaryotic DNA polymerases are considered. The DNA polymerases all carry out the same basic reaction, polymerization – the addition of dNMP onto the 3' terminus of the primer strand. In addition, some of the polymerases considered contain up to two additional enzymatic activities, a 3'-5' exonuclease and a 5'-3' exonuclease. The focus of the following discussion is a comparison of the various DNA polymerases on a structural basis in order to examine if, in addition to possessing similar enzymatic activities, the various DNA polymerases are also structurally related to one another. The mechanism of polymerization as it relates to replication fidelity, is discussed in a later section.

1. DNA polymerase I of *Escherichia coli*.

The first DNA polymerase to be studied in great detail, as well as the first DNA polymerase to be crystallized, is DNA polymerase I of *Escherichia coli*. For this reason, DNA polymerase I has served as the prototype DNA polymerase for the comparison and analysis of other
DNA polymerases. As mentioned above, during DNA replication DNA polymerase I fills any gaps remaining between adjacent Okazaki fragments and also replaces the RNA primers that are used in initiating Okazaki fragment synthesis (Kornberg, 1980). Both of these activities occur during the synthesis of the lagging strand. DNA polymerase I has three enzymatic activities, (1) 5'→3' polymerase, (2) 3'→5' exonuclease, and (3) 5'→3' exonuclease (Kornberg, 1980). The 5'→3' exonuclease is used in the removal of the RNA primers from the Okazaki fragments. This activity is not present in DNA polymerase III.

DNA polymerase I, unlike the multisubunit DNA polymerase III, is a single polypeptide. DNA polymerase I can be proteolytically cleaved into two active fragments, a C-terminal two thirds of the polypeptide, the Klenow fragment, which contains the polymerase and the 3'→5' exonuclease activities and a small N-terminal fragment containing the 5'→3' exonuclease (Brutlag et al., 1969; Klenow and Henningsen, 1970).

The Klenow fragment of DNA polymerase I has been crystallized and was shown to consist of two domains (Ollis et al., 1985). The smaller domain was demonstrated to bind dTMP which suggested that this domain comprised the 3'→5' exonuclease active site since dNMP is the product of the 3'→5' exonuclease activity (Ollis et al., 1985). That the smaller domain of the Klenow fragment does contain the 3'→5' exonuclease active site was borne out by the characterization of mutant proteins that contained amino acid changes in the dNMP binding sites (Derbyshire et al., 1988). These mutant proteins, while
retaining full polymerase activity, were deficient in the exonuclease activity. Additional evidence was obtained from studies which demonstrated a single-stranded DNA binding region in the smaller domain of the Klenow fragment (Freemont et al., 1988).

The larger domain of the Klenow fragment was shown to possess a cleft which was the appropriate size and shape for binding double-stranded DNA (Ollis et al., 1985). This cleft was located approximately 30 Å away from the dNMP binding site. The polymerase active site was shown to be present on this domain by the cloning and expression of a DNA fragment encoding only this domain (Freemont et al., 1986). The resulting protein was shown to possess significant DNA polymerase activity with no measurable 3'→5' exonuclease activity. An attempt to demonstrate binding of double-strand DNA to the cleft in crystals of Klenow fragment failed because the high ionic strength conditions of the crystallization caused the DNA to bind to the 3'→5' exonuclease active site (Freemont et al., 1988).

Once the crystal structure of the Klenow fragment was known, the amino acid sequences of other DNA polymerases were compared with the sequence of the Klenow fragment. One of the earlier comparisons made was with the bacteriophage T7 DNA polymerase (Ollis et al., 1985). This comparison yielded nine polypeptide regions, ranging in length from 9 to 45 residues, that showed significant (33 to 58%) amino acid sequence identity between the two proteins. Eight of these regions were located in the larger Klenow fragment domain containing the polymerase active site.

Recently, the sequence of the *Escherichia coli* dnaE gene, which
encodes the alpha subunit of DNA polymerase III, was determined (Tomasiewicz and McHenry, 1987). Tomasiewicz and McHenry (1987) compared the amino acid sequence of the alpha subunit with that of DNA polymerase I, concentrating on the nine regions of homology previously found between the Klenow fragment and T7 DNA polymerase (Ollis et al., 1985). Five regions of homology between the alpha subunit and DNA polymerase I were found which ranged between 16 to 32% identical residues. This was less than the homology between the same regions of DNA polymerase I and T7 DNA polymerase which ranged between 33 to 58% identical residues. Surprisingly, one of the regions of homology was located in the small domain of the Klenow fragment which contains the 3'→5' exonuclease active site, an activity which is not present in the alpha subunit of DNA polymerase III but rather, resides on the epsilon subunit. The significance of this homology was not addressed by Tomasiewicz and McHenry (1987).

In a study to examine if the alpha subunit of DNA polymerase III was homologous to other DNA polymerases, Franden and McHenry (1988) isolated three anti-alpha monoclonal antibodies and used these to test for cross reactivity with other DNA polymerases. One of the monoclonal antibodies was shown to bind strongly to DNA polymerase I and less strongly to bacteriophage T4 DNA polymerase. T7 DNA polymerase did not bind to the antibody. The region of DNA polymerase I that was binding to the antibody was localized to the N-terminal domain of DNA polymerase I, the domain containing the 5'→3' exonuclease which is separated from the Klenow fragment upon proteolytic digestion. This result is surprising since DNA
polymerase III lacks this enzymatic activity. Antibody binding to
the alpha subunit of DNA polymerase III could be competed only with
either heat denatured alpha subunit or DNA polymerase I suggesting
that the conserved domain was embedded in the protein. The exact
nature of this conserved domain is not known.

2. Polymerase domain of DNA polymerases.

On the basis of amino acid sequence homology, DNA polymerases
have been organized into two groups, the DNA polymerase I-like
polymerases and the human alpha-like polymerases (reviewed in Hall,
1988). The DNA polymerase I group includes T7 DNA polymerase (Ollis
et al., 1985) and also the bacteriophage T5 DNA polymerase (Leavitt
and Ito, 1989). Both of these polymerases lack 5'→3' exonuclease
activities and thus are similar to the Klenow fragment of DNA
polymerase I. The alpha subunit of DNA polymerase III would appear
to belong to this group based on its limited homology with DNA
polymerase I and T7 DNA polymerase (Tomasiewicz and McHenry, 1987) as
well as the demonstration of strong cross reactivity between an anti-
alpha antibody and DNA polymerase I (Franden and McHenry, 1988).

The human alpha group contains DNA polymerases from a diverse
group of organisms (Hall, 1988). These include human alpha,
adenovirus type 2, herpes simplex types 1 and 2, vaccinia, and
bacteriophages T4 and phi29 polymerases. These polymerases contain
homologous sequences which are widely dispersed throughout the
proteins. However, none of these polymerases have been crystallized
and thus it is not known whether any structural similarities exist
between the DNA polymerase I and human alpha polymerase groups.

The presence of the widely dispersed homologous regions in the human alpha-like proteins has led to the thought that the polymerase active site in these polymerases, unlike DNA polymerase I, is not organized into a discrete domain. In support of this theory, a linker-insertion mutagenesis analysis of adenovirus type 2 DNA polymerase demonstrated essential regions for polymerase activity scattered throughout the entire molecule (Chen and Horwitz, 1989). However, these essential regions were not limited to the regions of homology shared with the other human alpha-like polymerases. Thus, it appears that, for at least adenovirus type 2 DNA polymerase, the functions of the polymerase are not organized into discrete domains. This also suggests that while the homologous regions are conserved between the different polymerases and are useful in comparing DNA polymerases, they do not necessarily comprise the polymerase active site.

In contrast to the adenovirus type 2 DNA polymerase study, a recent study utilizing intragenic complementation of T4 DNA polymerase mutants demonstrated that the T4 DNA polymerase, a human alpha-like polymerase, contains two domains, a C-terminal polymerase domain and an N-terminal domain (Reha-Krantz, 1990). The N-terminal domain was found to be similar to sequences found in the 5'-3' exonuclease domain of E. coli DNA polymerase I and in E. coli ribonuclease H suggesting that this domain may contain a nuclease activity. These results suggest that although T4 DNA polymerase and DNA polymerase I are not homologous in the polymerase domain, these
polymerases may nonetheless be structurally similar. Additional evidence that structural similarities may exist between the DNA polymerase I and the human alpha groups was demonstrated by the binding of an antibody, directed against the alpha subunit of *E. coli* DNA polymerase III, a DNA polymerase I-like polymerase, to T4 DNA polymerase, a human alpha-like polymerase (Fraden and McHenry, 1988).

3. 3′→5′ exonuclease domain of DNA polymerases.

Unlike the DNA polymerization domain, the 3′→5′ exonuclease domain of DNA polymerase I of *Escherichia coli* is conserved among most of the DNA polymerases examined, including DNA polymerase I-like and human alpha-like polymerases (Bernad *et al.*, 1989). This domain is also conserved in the epsilon subunit of DNA polymerase III (Bernad *et al.*, 1989). The 3′→5′ exonuclease active site is comprised of three highly conserved regions which, in *E. coli* DNA polymerase I, are involved in metal binding and catalysis (Derbyshire *et al.*, 1988).

The conserved 3′→5′ exonuclease active site is also present in DNA polymerase III of *Bacillus subtilis*, a polymerase which shows no homology, except for the 3′→5′ exonuclease site, to either DNA polymerase I or the human alpha-like polymerases and may represent a third group of polymerases (Sanjanwala and Ganesan, 1989). Like DNA polymerase III of *E. coli*, the *B. subtilis* DNA polymerase III is responsible for chromosomal replication but unlike DNA polymerase III of *E. coli*, the *B. subtilis* DNA polymerase is a single polypeptide
which contains a 3'-5' exonuclease activity (Sanjanwala and Ganesan, 1989). The 3'-5' exonuclease domain of *B. subtilis* DNA polymerase III, present in the N-terminal portion of the protein, has 26% homology to the epsilon subunit of DNA polymerase III (Sanjanwala and Ganesan, 1989).

The occurrence of the highly conserved 3'-5' exonuclease domain in a diverse group of procaryotic and eucaryotic DNA polymerases demonstrates the importance of this catalytic domain.

4. **Asymmetric dimeric DNA polymerases: Application in eucaryotes?**

Recent evidence has been obtained which suggests that eucaryotic DNA polymerases may also be functioning in a dimeric state at the replication fork. Of the four eucaryotic DNA polymerases identified, the alpha and delta polymerases are thought to be involved in chromosomal DNA replication (reviewed in Lehman and Kaguni, 1989). The alpha polymerase contains an intrinsic DNA primase activity and is thus believed to be responsible for lagging strand synthesis (Lehman and Kaguni, 1989). The delta polymerase, on the other hand, is stimulated by proliferating-cell nuclear antigen (PCNA) but, when PCNA is omitted from the SV40 replication system, only short nascent strands are synthesized that are derived from the lagging strand (Frelich and Stillman, 1988; Wold et al., 1989). This suggested that the delta polymerase performs leading strand synthesis whereas alpha is responsible for lagging strand synthesis.

Weinberg and Kelly (1989) recently demonstrated that both the alpha and delta polymerases are required for the replication of SV40
DNA in vitro supporting the requirement of both polymerases. Tsurimoto and Stillman (1989), utilizing primed single-strand M13 DNA, examined the effects of various replication factors on DNA synthesis carried out by either alpha or delta polymerase. While the alpha polymerase was independently stimulated by either replication factor A (RF-A) or replication factor C (RF-C), the delta polymerase required both replication factors as well as PCNA for significant DNA synthesis. These results are consistent with the model that the two polymerases function at the replication fork, the alpha polymerase synthesizing the lagging strand whereas the delta polymerase synthesizes the leading strand. These results led Tsurimoto and Stillman (1989) to propose that these two polymerases, through protein-protein interactions, coordinate the synthesis of the leading and lagging strands at the replication fork. This model is similar to that of the current model for the dimeric DNA polymerase III holoenzyme in which the asymmetric distribution of auxiliary subunits supposedly allows concurrent synthesis of the leading and lagging strands at the replication fork.

C. FIDELITY OF DNA REPLICATION.

The replication of the bacterial chromosome is carried out with great accuracy. In *Escherichia coli*, error frequencies are $10^{-9}$ to $10^{-10}$ per base pair replicated (Drake, 1969). This exceptional fidelity arises from the involvement of DNA polymerase III and of post-replicative mismatch repair systems. Mismatch repair accounts for a factor of approximately $10^2$ to $10^3$ fold to the overall fidelity
of DNA replication (Claverys and Lacks, 1986). The contribution by DNA polymerase III occurs in two steps: the initial base selection during polymerization and a 3'-5' exonucleolytic editing of incorrectly inserted deoxynucleotides at the 3' end of the growing chain (proofreading).

Fersht et al. (1982) have defined the overall specificity of a DNA polymerase as being dependent upon three discrimination factors. The first discrimination factor involves the selection of the nucleotide to be inserted and is defined as the ratio of the rate of insertion of a correctly matched nucleotide to the rate of insertion of an incorrectly matched nucleotide. The two additional discrimination factors determine the proofreading specificity of the polymerase. These factors are defined as the ratio of the rates of elongation from a correct match versus elongation from a mismatch and the ratio of the rates of excision of a mismatch versus excision of a correct match. The cost of editing is defined as the proportion of correctly matched nucleotides that are removed and is determined by the discrimination factors of proofreading.

1. Nucleotide selection by DNA polymerases.

That DNA polymerase III of Escherichia coli plays an active role in the selection of correct nucleotides during polymerization was suggested by the isolation of mutations, located in the polymerase subunit gene (dnaE), which resulted in increased mutation rates (Hall and Brammer, 1973; Sevastopoulos and Glaser, 1977; Konrad, 1978). However, the mechanism by which DNA polymerases participate in
discriminating between correct and incorrect nucleotides, aside from proofreading, has remained unknown. Two mechanisms which have been proposed for how a polymerase might distinguish between correct and incorrect base pairs are (1), differential binding in the active site ($K_m$ discrimination) and (2), differential catalysis of phosphodiester bonds, dependent upon prior binding of the correct pair ($V_{\text{max}}$ discrimination) (reviewed by Loeb and Kunkel, 1982).

A mechanism of nucleotide discrimination involving differential binding of correct and incorrect nucleotides would be reflected in different $K_m$'s for the correct and incorrect nucleotides. In this model, $K_m$ discrimination, the rates of binding at the active site would be similar for correct and incorrect nucleotides. Discrimination would occur due to free energy differences between correctly versus incorrectly inserted nucleotides. The free energy differences would arise from differences in the hydrogen bonding and stacking interactions at the active site of the polymerase for the correct versus incorrect nucleotides. This would result in mismatched nucleotides dissociating faster than correctly inserted nucleotides.

In studies examining nucleotide discrimination by Klenow fragment, a small contribution from selective dNTP binding for enhanced discrimination was observed. Fersht et al. (1983) found that the $K_m$ for incorrect nucleotides increased $>100$-fold with a poly(dA)-oligo(dT) substrate. In addition, El-Deiry et al. (1984, 1988) found that the $K_m$ for the insertion of the non-complementary nucleotide was 6- to 17-fold higher than that for the complementary
nucleotide.

Also in support of the $K_m$ discrimination model, Boosalis et al. (1987), using purified Drosophila DNA polymerase α holoenzyme which is devoid of 3' exonuclease proofreading activity, found that the nucleotide insertion fidelity was attributed to a much higher dissociation constant for wrong versus right nucleotide substrates. The ratios of the $K_m$ values (incorrect/correct) for G-T and C-T misincorporations were 1100 and 2600, respectively. In a similar study using the purified α subunit of DNA polymerase III of E. coli, it was shown that base selection by the polymerase was primarily governed by a differential $K_m$ of the enzyme for the correct versus incorrect nucleotides (Sloane et al., 1988).

The $K_m$ discrimination model was refined by Petruska et al. (1986) to account for the discrepancy observed between the measured fidelity of DNA polymerases in vitro ($10^{-3}$ to $10^{-5}$) and the free energy difference between base mispairs observed in aqueous solution (between 1 and 3 kcal·mol$^{-1}$). This range of free energy differences would account for only a 5- to 150-fold discrimination against insertion of an incorrect nucleotide which is several orders of magnitude lower than the DNA polymerase insertion accuracies measured in vitro.

In their attempt to resolve this discrepancy, Petruska et al. (1986) compared the base-pair dissociation energies measured in water, measured in the presence of polymerase, and the theoretical values calculated from base-stacking and hydrogen bonding interactions in vacuum. They concluded that the free energy
differences between correct and incorrect nucleotide insertions are
enhanced in the active site of the polymerase by the exclusion of
water. This enhancement of discrimination, in the absence of water,
would thus be adequate for the polymerase to discriminate between
correct and incorrect nucleotides to achieve the insertion accuracies
obtained in vitro. In an in vitro test of this model, using purified
Drosophila DNA polymerase α holoenzyme which lacks a 3' exo-
nuclisolytic proofreading activity, Petruska et al. (1988) found
that the free energy differences between correct and incorrect base
pairs in the active site cleft of polymerase appeared to be ten-fold
greater than the difference observed in aqueous medium. Thus, for
Drosophila DNA α polymerase, it seems that the free energy
differences between correct and incorrect nucleotides are enhanced in
the presence of the polymerase and play a major role in the
polymerase's ability to discriminate between correct and incorrect
nucleotides.

An alternative mechanism for base discrimination by the
polymerase is based on the differential catalysis of the
phosphodiester bond, \( V_{\text{max}} \) discrimination. \( V_{\text{max}} \) discrimination has
been shown to be operative during nucleotide selection by Klenow
fragment. El-Deiry et al. (1984, 1988) found that the \( V_{\text{max}} \) for the
insertion of the correct nucleotide was 1600-fold higher than that
for the incorrect nucleotide. Kuchta et al. (1988) also observed a
greatly increased rate of phosphodiester bond formation for correct
nucleotides. In addition, in a study examining the initial rates of
dTTP incorporation into poly(dA)-oligo(dT) during template-directed
synthesis by the Klenow fragment of DNA polymerase I, Mizrahi et al. (1985) found evidence for a rate-determining conformational change prior to phosphodiester bond formation. In this study, the incorporation of incorrect nucleotides was not examined and thus it could not be determined if this conformational change influenced nucleotide selection by the polymerase.

In a more recent study, Kuchta et al. (1988) measured the kinetic parameters of the Klenow fragment involved in the polymerization of correct and incorrect nucleotides. For several of the misincorporations examined, no $K_m$ discrimination was observed. Instead, however, there was a marked decrease in the rate of phosphodiester bond formation for incorrect nucleotides which resulted in a $1.1 \times 10^4$- to $>1.2 \times 10^6$-fold increase in fidelity (Kuchta et al., 1988).

In summary, nucleotide discrimination can occur either by differential binding ($K_m$ discrimination) or differential catalysis ($V_{\text{max}}$ discrimination) of the correct and incorrect nucleotides. These two mechanisms are not mutually exclusive as demonstrated by Klenow fragment which utilizes both mechanisms in the selection of nucleotides. On the other hand, for the Drosophila alpha polymerase, $K_m$ discrimination appears to be the major determinant in base selection by the polymerase. $K_m$ discrimination also appears to be the major mechanism during nucleotide selection by the alpha subunit of DNA polymerase III, although a small contribution of $V_{\text{max}}$ discrimination has been observed (Sloane et al., 1988). Thus it appears that for the majority of polymerases examined, a combination
of the two mechanisms is utilized to effect nucleotide discrimination. The extent to which each mechanism contributes depends on the individual polymerase as well as the individual misincorporation event being considered (G:T versus C:T, for example).

2. Contribution of proofreading to the fidelity of DNA replication.

Proofreading is the 3'→5' exonucleolytic removal of nucleotides from the 3' terminus during polymerization. An exonuclease is considered to have proofreading activity if it prefers a single-stranded to double-stranded DNA substrate, preferentially excises a mispaired rather than correctly paired primer terminus, is physically associated with the polymerase, either as part of the same polypeptide or as an associated subunit, and acts coordinately with the polymerase to enhance the fidelity of DNA synthesis (Kunkel, 1988). The contribution of proofreading to the fidelity of DNA replication is estimated by comparing the accuracy of synthesis under conditions that either allow or inhibit proofreading.

As stated above, the proofreading specificity of DNA polymerases comprises two discrimination steps: the relative rates of, 1) elongation from either a correct or incorrect nucleotide and 2) excision of either a mismatch or match (Fersht et al., 1982). When an incorrect nucleotide is inserted by the Klenow fragment of DNA polymerase I, a conformational change occurs after the phosphodiester bond is formed (Kuchta et al., 1988). The authors suggested that this conformational change results in a slower dissociation of the incorrect DNA product from the Klenow fragment and thus allowing the
exonuclease more opportunity to excise the error. This step
increases fidelity 4- to >61-fold (Kuchta et al., 1988). Presumably,
when a correct nucleotide is inserted and the phosphodiester bond is
formed, this conformational change does not occur and would allow the
rapid addition of the next nucleotide. In addition, the Klenow
fragment was shown to polymerize the next correct dNTP onto a
mismatch very slowly and this step adds an additional 6- to >340-fold
increase to fidelity (Kuchta et al., 1988).

The cost of editing, the proportion of correctly matched
nucleotides that are removed, is determined by the proofreading
discrimination steps. The cost of editing has been measured using
purified DNA polymerase III holoenzyme in an in vitro λX174 DNA
replication system (Fersht et al., 1982). The costs of incorporating
dATP, dTTP, dGTP, and dCTP, were found to be 13%, 10%, 5%, and 6%,
respectively. Thus, between 5 to 13% of the nucleotides incorporated
were eventually excised. The addition of dGMP, an inhibitor of the
3'-5' exonuclease of DNA polymerase III, resulted in a decrease in
the cost of editing but an increase in the frequency of errors
(Fersht et al., 1982). This suggests that the editing exonuclease
activity is acting at a near optimum level since any increase in the
specificity of the exonuclease would increase the cost. With an
increased cost there would inevitably be a decrease in replication as
the rate of the exonuclease would eventually approach that of
polymerization.

In a kinetic analysis of polymerization by the Klenow fragment
of DNA polymerase I, it was observed that the 3'-5' exonuclease did
not differentiate between correctly and incorrectly base-paired nucleotides (Kuchta et al., 1988). The discrimination arose due to an increased lifetime of the enzyme-DNA complex containing the misincorporated base. Therefore, these results, obtained with the Klenow fragment, suggest that the proofreading specificity depends not on discrimination by the exonuclease, but instead, on the mechanisms inherent in the polymerization reaction.

The demonstration that the exonuclease active site is located 30 Å away from the polymerization site in DNA polymerase I (Ollis et al., 1985) was intriguing since it was difficult to imagine how the exonuclease was able to coordinate its activity with polymerization. Studies with the Klenow fragment have demonstrated that the switch from excision by the exonuclease to incorporation by the polymerase, as it occurs in an idling-turnover reaction (alternating polymerization and editing at the 3' terminus without any net synthesis), occurs without the polymerase dissociating from the DNA substrate (Mizrahi et al., 1986). This suggests that the enzyme and the DNA move relative to each other in order to position the necessary active site at the primer terminus.

Joyce and Steitz (1987) have proposed that in order for the exonuclease to be active at the primer terminus, the DNA would have to slide about eight base pairs while three to four bases at the primer terminus would have to be single-stranded. In defining the DNA substrate structural requirements for the exonuclease and polymerase activities of DNA polymerase I, Cowart et al. (1989) have recently obtained compatible results which demonstrated that in order
for the exonuclease to be able to remove nucleotides from the primer terminus, at least four base pairs of the primer strand must melt out. This requirement for strand separation for exonuclease activity was also seen for T4 and T7 DNA polymerases where two and three nucleotides, respectively, melt out (Cowart et al., 1989). To determine the necessary DNA binding region required for the exonuclease activity of Klenow to function, Cowart et al. (1989) prepared a series of DNA template primers which contained a covalently-linked biotin avidin complex located at various distances upstream of the primer terminus. It was shown that if a biotin-avidin complex was located closer than fifteen nucleotides upstream of the primer terminus, the exonuclease was inactive (Cowart et al., 1989).

As analyzed by similar techniques, the structural requirements of the DNA polymerase I polymerase activity differ from those of the editing activity (Cowart et al., 1989). For polymerase activity, the DNA duplex was not required to undergo strand separation. Furthermore, the biotin-avidin complex was effective in inhibiting polymerase activity only when it was located six nucleotides, or closer, upstream of the primer terminus. These results further support the notion that in order for the exonuclease to act at the primer terminus, the DNA moves, relative to the enzyme, to position the primer terminus at the exonuclease active site.

3. Proofreading and the epsilon subunit of DNA polymerase III.

In DNA polymerase III of E. coli and S. typhimurium, the epsilon
subunit, encoded by the *dnaQ* gene, contains the 3'-5' exonuclease responsible for proofreading (Scheuermann et al., 1983; Scheuermann and Echols, 1984). The contribution of the epsilon subunit to the overall fidelity of the polymerase has been estimated to be 200 fold (Fersht and Knill-Jones, 1983). The *dnaQ* gene was first identified in *E. coli* as a mutator allele and was initially designated as mutD (Degnen and Cox, 1974). *MutD5* mutants possessed very high mutation rates when grown in rich media (10^4 fold above wild type) but low mutation rates when grown in minimal media (10 to 50 fold above wild type). This medium effect has been attributed to the presence of thymidine in the rich media (Erlich and Cox, 1980). However, thymidine is not the only effector responsible for increased mutation rates in rich media as mutants defective in thymidine metabolism still retain high mutation rates in rich media (Erlich and Cox, 1980).

Recently, it was shown that the mutational spectrum of the *mutD5* strain grown in rich media was different than that when the strain was grown in minimal media (Schaaper, 1988). In addition to having this medium effect on mutational spectra, the *mutD5* strain, when grown in rich media, was also defective in methyl-directed mismatch repair, the final step in determining the overall fidelity of DNA replication. It was further shown that the extreme mutator phenotype of *mutD5* strain was due to saturation of the mismatch repair system by an excess level of DNA replication errors (Schaaper, 1989; Schaaper and Radman, 1989; Damagnez et al., 1989). The defect in the *mutHLS* mismatch repair system in the *mutD5* strain was only transient
and was able to be rescued by either inhibiting chromosomal DNA replication or by supplying the cells with a multicopy plasmid expressing the mutH or mutL gene (Schaaper and Radman, 1989; Damagnez et al., 1989).

The dnaQ gene was located on the E. coli genetic linkage map at about 5 minutes whereas dnaE, the gene encoding the α subunit of DNA polymerase III, was placed at approximately 4 minutes (Horiuchi et al., 1978). The dnaQ gene from E. coli was cloned via complementation using a dnaQ conditional lethal mutation (Horiuchi et al., 1981). The dnaQ gene product, a 25 kilodalton protein, was identified on the basis of the correlation of the insertion of a gamma-delta insertion sequence with the loss of dnaQ complementing activity (Horiuchi et al., 1981). The mutD5 and dnaQ49 mutants were found to be defective in the 3'→5' exonucleolytic editing activity of DNA polymerase III holoenzyme (Echols et al., 1983). It was subsequently shown that the dnaQ gene product, identified by Horiuchi et al. (1981), was in fact, the epsilon subunit of DNA polymerase III holoenzyme (Scheuermann et al., 1983). Scheuermann and Echols (1984) further showed that the epsilon subunit possessed the 3'→5' exonuclease activity of DNA polymerase III. Thus, for DNA polymerase III, in contrast to E. coli DNA polymerase I and phage T4 DNA polymerase, the polymerase and editing exonuclease activities reside on separate subunits.

The wild type dnaQ gene and several of the mutant alleles, which lead to increased mutation rates, have been sequenced (Maki et al., 1983; Takano et al., 1986; Cox and Horner, 1986). Compared to the
wild type gene, the mutD5 allele, which is dominant over the wild-
type allele (Maruyama et al., 1983), contained two amino acid
substitutions (Takano et al., 1986). Both of these substitutions, by
themselves, were able to confer the dominant mutator phenotype when
present on a high copy number plasmid (Takano et al., 1986).
However, only one of the substitutions, Ala164 to Val, was able to
effect the mutator phenotype when present on a low copy number
plasmid. The dnaQ49 allele is temperature sensitive for DNA
replication at 44.5°C, exhibits high mutator activity at permissive
temperatures, and is recessive to wild type (Horiuchi et al., 1978;
Maruyama et al., 1983). The dnaQ49 allele contains one base change
from the wild type allele which results in a Val96 to Gly amino acid
substitution (Takano et al., 1986).

It is interesting to note that both mutant alleles, mutD5 and
dnaQ49, encode the full length protein, i.e. neither mutation results
in a truncation of the protein coding sequences by the introduction
of a frame shift or stop codon in the coding sequence. Another
mutant allele of dnaQ which results in high mutator activity, dnaQ61,
contains an amino amino acid substitution at position 193 and an
amber mutation at position 195, which causes a carboxyl truncation of
the 243 amino acid subunit (Maki et al., 1983). This result suggests
that the carboxyl terminal portion of dnaQ is necessary for the
exonuclease activity of the enzyme.

D. RATIONALE AND RESEARCH AIM.

Genetic and biochemical studies have demonstrated that the
epsilon subunit of DNA polymerase III, encoded by the dnaQ gene, possesses a 3'→5' exonuclease activity which is involved in proofreading during replication. The isolation of dnaQ mutants which are defective in the 3'→5' exonuclease and exhibit a mutator phenotype, implies that the exonuclease activity of the epsilon subunit is not required for cell viability. However, since none of the dnaQ mutations reported in the literature is an unambiguous null mutation, the requirement for the epsilon subunit for viability remained an open question.

The initial objective of the work presented in this thesis was to examine the cellular requirement for the epsilon subunit of DNA polymerase III in Salmonella typhimurium. This was addressed by determining the consequences of removing the epsilon subunit from the cell by deletion of its gene. It was found that deletion of dnaQ conferred a marked growth defect. In subsequent experiments, this growth phenotype was characterized and determined to be modulated by the genotype at two other loci intimately involved in DNA replication: dnaE, encoding the polymerase subunit of DNA polymerase III, and polA, encoding DNA polymerase I.
CHAPTER II

ISOLATION AND CHARACTERIZATION OF MUTANTS WITH DELETIONS IN dnaQ, THE GENE FOR THE EDITING SUBUNIT OF DNA POLYMERASE III IN Salmonella typhimurium

A. INTRODUCTION

The ε subunit of DNA polymerase III is a fidelity factor for DNA replication by virtue of its ability to catalyze excision of erroneously inserted nucleotides at the growing point of a new chain (Scheuermann et al., 1983; Scheuermann and Echols, 1984). The antimutagenic effect of ε is not essential for cell viability, since E. coli readily tolerates a wide range of spontaneous mutation rates. Indeed, the mutD5 mutation of dnaQ increases the spontaneous mutation frequency at some loci as much as 10^5-fold without causing general lethality (Degner and Cox, 1974). None of the dnaQ mutations described in the literature is an unambiguous null, however, and for this reason the cellular requirement for ε for viability has remained an open question.

In this paper, we report the construction and properties of null mutations in dnaQ. We first constructed defined mutations (deletion-substitutions) in cloned dnaQ, then used these constructs to replace chromosomal dnaQ. Our main finding was that loss of dnaQ caused a severe growth defect, but this phenotype was well-suppressed by a class of mutations in dnaE, the gene for the polymerization subunit (α) of DNA polymerase III (Welch and McHenry, 1982). Survival in the
absence of c required DNA polymerase I in addition to the genetically altered DNA polymerase III. Our evidence also suggested that the suppressor mutations were specific for the growth phenotype of dnaQ and not for its mutator phenotype. On the basis of these and other observations, we argue that dnaQ normally plays an important, if not essential, role in promoting successful DNA replication, and that this role is formally separable from the nonessential role of dnaQ in promoting faithful DNA replication.

B. MATERIALS AND METHODS

1. Bacterial strains and media. Bacterial strains used are described in Table 1. Generalized transduction was carried out using phage P22 int HT12/4 (Schmieder, 1972). General bacteriological media and procedures were as described (Maurer et al., 1984b). Drugs at the following concentrations (micrograms per milliliter) were added to plates as needed: tetracycline HCl, 25; kanamycin, 50; chloramphenicol, 20; rifampicin, 100; and nalidixate, 50. Tetracycline plates contained, in addition, 10 mM EGTA to prevent reinfection of transductants by P22.

2. dnaQ constructs. Plasmids and λ phages carrying mutated dnaQ genes of S. typhimurium may be considered derivatives of pFF16 (Figure 1) and λRM354 (Maurer et al., 1984b), respectively. Insertions of Tn10dtc in λRM354 were isolated as described (Maurer et al., 1984b) and among these, insertions specifically in dnaQ were identified by red-plaque test (Maurer et al., 1984b). As judged by
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU10</td>
<td>thp dae</td>
<td>Secor et al., 1946</td>
</tr>
<tr>
<td>HU87</td>
<td>[HU31] nagA256-µth10dm6</td>
<td>This laboratory</td>
</tr>
<tr>
<td>HU38</td>
<td>[HU10] hisG9+ / hisG10</td>
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<tr>
<td>HU11</td>
<td>3-aminosensitive strain</td>
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<tr>
<td>HU709, 709, 710, 711, 712, 713, 714, 715, 716, 717, 718, 812, 813, 814, 815, and 819</td>
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</tr>
<tr>
<td>HU1655, 821, 822, 823, 830, 832, 834, 835, 836, 843, 845, 847, 848, 849, 850, 851, and 852</td>
<td>Transformation of HU10 to $T^8$ using $T^8$ grown on HU709, etc.</td>
<td></td>
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<tr>
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<td>[HU11] nagA256-µth10dm6</td>
<td>HU31 + HU(21000)</td>
</tr>
<tr>
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<td>HU31 + HU(21000)</td>
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<td>HU31 + HU(21000)</td>
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<td>HU748</td>
<td>[HU10] nagA256-µth10dm6</td>
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<td>Transformation of HU10 to $T^8$ with LBN600</td>
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<td>HU768</td>
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<td>low-652 polA2</td>
<td>C. Miller (TH247), Yeh et al., 1967</td>
</tr>
<tr>
<td>HU1748</td>
<td>low-652 polA2</td>
<td>Transformation of HU14 to $T^8$ from a pool of common $T^8$/Dm6 insertions$^a$</td>
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<td>HU1848</td>
<td>[HU8] polA2</td>
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</table>

$^a$All strains are $T^8$ (see reference Secor et al., 1946 for comments on the use of HU11 and its derivatives). A strain number in brackets indicates that all the mutants of the depicted strain are included in the genotype.

$^b$Unusualized transitions are depicted by "wildtype = $T^8$/(domain)". Selections were always for a drug markers $T^8$ for $dcm$ alleles, $T^8$ for nerve alleles, and $T^8$ for polA. Unselected mutants were scored as indicated in Materials and Methods for $T^8$, and by sensitivity to methyl-methane-sulfonate for polA.

$^c$RpsL was obtained from J. Roth in strain TH201.

$^d$The $T^8$ strain of this series was not kept.

$^e$hisG9 is approximately 4% extrodinarily compatible with polA.
Figure 1. DnaQ constructs (see text).
pFF16 \[\xrightarrow{\text{HindIII site modification}}\] pFF73

\[x\beta\text{I cleavage}\]

\[\xrightarrow{\text{BslI digestion}}\]

\[\xrightarrow{\text{add HindIII linkers and digest with HindIII}}\]

\[\xrightarrow{\text{circularize}}\]

\[\xrightarrow{\text{open at HindIII site and add rest of Tn10}}\]

\[\xrightarrow{\text{sequence deletion endpoints}}\]

\[\text{dnaQ::Tn10 (e.g., dnaQ201)}\]
restriction enzyme analysis (not shown), the most widely separated Tn10dTc insertions in dnaQ having the same orientation differed by about 300 base pairs in their sites of insertion; these insertions were found in λRM81 and λRM164. To create the dnaQ200 allele in which Tn10dTc exactly replaced these ~300 base pairs, a fragment from λRM81 containing one end of dnaQ and the adjacent portion of Tn10dTc was joined to a fragment from λRM164 containing the rest of Tn10dTc and the other end of dnaQ, all in plasmid pUC8 (Vieira and Messing, 1982). Neither fragment contained the ~300 base pair region between the two sites of insertion. In effect, an unequal crossover between the two transposons was produced; this was plasmid pFF16.

Further manipulations of pFF16 enlarging the extent of DNA replaced by Tn10dTc are illustrated in Figure 1. Plasmid pFF73 was derived from pFF16 by filling in the HindIII site near the 5' end of dnaQ with DNA polymerase I Klenow fragment. Plasmid pFF73 was linearized by cutting within Tn10dTc with XbaI. The linearized DNA was digested to various extents with Bal-31 to produce a series of deletion endpoints within dnaQ from digestion at one end, and deletion endpoints within Tn10dTc from digestion at the other end. The ends were ligated to HindIII linkers (duplex DNA having the sequence 5'GCAAGCTTGGC on both strands; Bethesda Research Laboratories, Inc.), and the DNA was then digested with HindIII. This procedure had the effect of reducing all the deletion endpoints on one side to the HindIII site near the end of Tn10dTc. The digested DNA was circularized to yield plasmids pFF113 to pFF124, which conferred resistance to ampicillin but not to tetracycline. In
the final step, the intact Tn10dTc was restored by inserting, in the 
HindIII site of these plasmids, a HindIII fragment obtained from 
λRM637, a dnaC clone containing an insertion of Tn10dTc.

In plasmids pFF113 to pFF124 (Fig. 1), a unique portion of Tn10 
abutted different parts of dnaQ. Using an oligonucleotide primer 
corresponding to the Tn10 sequence (5' GATCCAGAGAACCAC; nucleotides 
1550 to 1534 as numbered in Schollmeier and Hillen, 1984), we 
sequenced the adjoining dnaQ segments. The information obtained from 
this sequence, shown in Fig. 2, was sufficient to establish several 
points. First, the target of the transposon insertions was the 
authentic dnaQ gene as judged by its extensive homology to E. coli 
dnaQ. Second, the ~300 base pair deletion in dnaQ200 affected the 3' 
portion of dnaQ. Third, the more extensive deletions (dnaQ201 to 
dnaQ206) removed additional portions of dnaQ towards the 5' end of 
the gene. The sequence information shown in Fig. 2 will appear in 
the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the 
accession number M26045.

Each mutant dnaQ allele formed in a plasmid was transferred to 
λRM354 by homologous recombination. This was accomplished by 
propagating λRM354 through an E. coli strain harboring the particular 
plasmid and selecting a TcR λ recombinant (Maurer et al., 1984b). 
The expected physical map of each recombinant was verified by 
restriction enzyme analysis.

3. Formation of chromosomal dnaQ::Tn10 strains. The first 
chromosomal dnaQ::Tn10 strains were made by infecting a λ-sensitive
Figure 2. Partial $dnaQ$ sequence and deletion endpoints. The main sequence was obtained from a series of Bal-31 deletions derived from plasmid pFF16 (see MATERIALS AND METHODS and Fig. 1). Except for a one-base deletion in 5' flanking region (hyphen at -24), the sequence aligns precisely with $dnaQ$ from $E. coli$ (Cox and Horner, 1986); differences at the nucleic acid and protein levels are shown. Numbering is with reference to the beginning of the protein-coding sequence. A few bases that could not be read because of a compression artifact are shown as N; a region corresponding to 53 bases in the $E. coli$ sequence that was not read because of its distance from the nearest deletion endpoint is given by (...). The base shown as each Bal-31 deletion endpoint is the terminal deleted base. The plasmids and $\lambda RM354$ derivatives in which each deletion can be found, as well as the $dnaQ$ allele number, are given (boxed). Additional deletions not shown ended in Tn10dTc itself. The largest such deletion removed all but 218 bp from the end of the transposon. Sequencing on this template allowed unambiguous determination of the position of transposon interruption of $dnaQ$ in pFF16 ($dnaQ200$). Since Salmonella $c$ is similar in size to $E. coli$ $c$ on Western blots (not shown), and the latter is 243 amino acid residues in length, it follows that $dnaQ200$ eliminates the C-terminal -half of $c$. 
S. typhimurium sequence

E. coli differences

Protein changes
Deletion endpoints

-11 AAATGACCGAT ATG AGC ACT GCA ATT ACA CGA CAG ATC GTC CTC GAT ACC GAA ACC
   C T
   pF123/125, λRM781 (dnaQ202) pF124/127, λRM780 (dnaQ201)

46 ACC GGT ATG AAT CAG ATA GGC GCG CAC TAT GAA GGT CAC AAG ATT ATT GAG ATC
   C T T C C T

100 GGT GCG GTT GAG GTG ATA AAC CGT CTG ACC GGC AAC AAT TTT CAT GTT TAC
   C A G G G T C C T T
   I-V

154 CTG AAG CCC GAT CGC CTT GTC GAT CCA GAG GCT TTT GGC GTA CAC GGT ATT GCC
   C A G G G A C T
   pF120/220, λRM787 (dnaQ208)

208 GAT GAG TTT CTG CTG GAT AAG CCG GTT TTT GCT GAT GTG GTC GAT GAG TTT CTT
   A T C C A A C C A G
   V+T D+E V+A L+M
   pF119/219, λRM786 (dnaQ205)

262 GAT TAT ATN NNN GGC GCG GAG CTG GTC ATC CAT AAC GCA T..................
   C T CGC T G
   pF118/218, λRM785 (dnaQ204)

355 CCT AAA ACC AAT ACT TTC TGC AAA GTT ACC GAC AGC CTG GCG
   G G T G C T T
   pF16, λRM612 (dnaQ200)
Salmonella strain with λdnaQ::Tn10dTc and selecting for TcR survivors (Maurer et al., 1984b). For the experiments reported in this paper, we used λdnaQ derivatives in which Tn10dTc replaced part of dnaQ, in contrast to the simple Tn10dTc insertions used earlier. We shall use the generic designation dnaQ::Tn10 to refer to both insertion and substitution alleles. Regardless of the exact dnaQ allele used, introduction of dnaQ::Tn10 into the bacterial chromosome always resulted in the isolation of survivors bearing a suppressor mutation linked to dnaE (see next paragraph and RESULTS). In subsequent steps, the dnaQ::Tn10 allele was moved to other strains by P22-mediated transduction, again with selection for TcR. dnaQ::Tn10 strains with a known suppressor mutation were constructed in two steps. First, the suppressor mutation was moved into a dnaQ+ strain by P22 transduction using selection for a linked kanamycin-resistance (KmR) marker (see below for scoring the suppressor genotype). In the second step, a dnaQ::Tn10 allele was introduced by an additional round of transduction.

4. Mapping of suppressor genes. Mapping of suppressor genes was facilitated by a correct hunch that dnaE was the suppressor locus. The suppressors characterized in this study were isolated in strain RM97, in which the vicinity of dnaE was marked by a transposon insertion conferring KmR. Each of the immortal survivors of dnaQ200::Tn10dTc insertion in RM97, carrying a presumptive suppressor, was used to propagate phage P22. These lysates were used to transduce strain RM10 to KmR. Ten colonies from each transduction, in most cases, were scored for the suppressor genotype
by judging colony appearance following transduction to TcR using the same lysate a second time. Typically, cotransduction of suppressor and Kmr was about 50%, but this number should be considered a rough estimate in view of the small sample size and occasional difficulty in judging the suppressor phenotype. In a few cases, more than ten transductants had to be tested to find one that clearly carried the suppressor mutation. The cotransduction frequency of Kmr and dnaE698(Ts) (Maurer et al., 1984b), measured in a separate experiment, was 68%; dnaE698 maps to the same half of dnaE as does spq-2 in marker rescue experiments (chapter III, Lancy et al., 1989b).

5. Molecular cloning of suppressor genes. Genomic libraries of strains RM746, RM812, RM813, and RM815 were prepared in the vector λ1059 as described (Maurer et al., 1984b). DnaE clones from these libraries were identified by red-plaque complementation of dnaE(Ts) strains (Maurer et al., 1984b). Presence of a functional suppressor gene in these clones was assayed by a red-plaque suppression test (Maurer et al., 1984a). This suppression test determined the ability of a λ clone to restore growth at 40.5°C of Salmonella strain RM858, a temperature-sensitive survivor of TnlOdTc insertion in dnaQ. Control experiments showed that dnaE+ clones from wild type did not restore growth to RM858, whereas dnaQ+ clones did. Thus, the failure of RM858 to grow at high temperature resulted from failure to suppress the growth defect conferred by dnaQ::TnlOdTc, rather than from an ordinary temperature-sensitive mutation in dnaE.

6. Nomenclature. Although all suppressors mapped to the vicinity of dnaE, only one suppressor mutation has been explicitly
identified as a dnaE allele. For this reason, and for clarity, in this paper we have designated all of the suppressors by spq (suppressor of dnaQ).

7. Determination of mutation rates. On several widely-separated occasions, one or more cultures of each strain to be examined were grown to saturation in 5 ml of LB broth plus thymine (10 μg/ml). A mutant-free inoculum (typically a few thousand cells) from each culture was used to seed a subculture, which was also allowed to grow to saturation. The total titer and the titer of drug-resistant mutants in each subculture were determined by plating samples on plates with no drug, nalidixate, or rifampin. Colonies were counted after one day except for the rifampin-resistant colonies, which were counted after two days. The raw data from all the subcultures were pooled and used to estimate mutation rate using the method of maximal likelihood (Lea and Coulson, 1949).

8. Purification of DNA polymerase III. DNA polymerase III activity from strain RM822 was prepared by modification of procedures of Maki and Kornberg (1985) and McHenry and Crow (1979; Table 2). A cell lysate was prepared by incubating 200 g of cell paste at 0° for 60 min in 200 ml of 50 mM Tris-HCl, pH 7.5, 10% (w/v) sucrose, 5 mM dithiothreitol, 10 mM spermidine HCl, and 0.05% (w/v) lysozyme. The lysate was cleared of cell debris by centrifugation at 31,700 x g for 60 min to give Fraction I. Fraction I was treated with ammonium sulfate (0.25 g per ml of Fraction I) for 30 min with stirring at 0°. The precipitate was collected by centrifugation at 31,700 x g for 45 min, dissolved in 20 ml of buffer A (50 mM Tris-HCl, pH 7.5, 20%
Table 2. DNA Polymerase III activity from RM822

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Activity (10^3) U</th>
<th>Sp. Act. (10^3) U/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>I  Lysate supernatant</td>
<td>3600</td>
<td>840</td>
<td>0.23</td>
</tr>
<tr>
<td>II Ammonium sulfate</td>
<td>390</td>
<td>200</td>
<td>0.51</td>
</tr>
<tr>
<td>III Heparin-agarose</td>
<td>46</td>
<td>210</td>
<td>4.6</td>
</tr>
<tr>
<td>IV Sephacryl S-300</td>
<td>2.3</td>
<td>81</td>
<td>35</td>
</tr>
<tr>
<td>V' Mono Q, peak I</td>
<td>0.015</td>
<td>2.2</td>
<td>150</td>
</tr>
<tr>
<td>V'' Mono Q, peak II</td>
<td>0.007</td>
<td>1.8</td>
<td>260</td>
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</table>
(v/v) glycerol, 1 mM EDTA and 10 mM β-mercaptoethanol), and dialyzed overnight against 2 liter of buffer A plus 20 mM NaCl to yield Fraction II. Fraction II was diluted with buffer A plus 20 mM NaCl until its conductivity matched that of the diluent, and was then applied to a 100 ml column (5 by 5 cm) of heparin-agarose equilibrated with diluent. The column was washed with 5 column volumes of diluent, and activity was eluted with a 20 column volume linear salt gradient (20 to 400 mM NaCl in buffer A) at a flow rate of 70 ml/hr. Peak fractions were pooled to yield Fraction III.

Protein was precipitated from Fraction III by addition of 0.4 g ammonium sulfate per ml of fraction. The suspension was stirred for 30 min and held at 0°C for an additional 12 h. The precipitate was collected by centrifugation at 12,250 x g for 30 min at 0°C, dissolved in 1 ml buffer A, and gel-filtered on a 350 ml column (2.5 x 72 cm) of Sephacryl S-300 equilibrated in buffer A plus 40 mM NaCl. Fractions were collected at a flow rate of 20 ml/hr, and peak fractions were pooled to yield Fraction IV. Fraction IV was applied to a 1 ml Mono Q HR 5/5 anion-exchange column equilibrated in buffer A plus 40 mM NaCl. The column was washed with 2 ml of equilibration buffer and activity was eluted by a 20 column volume linear salt gradient (40 to 400 mM NaCl in buffer A) at a flow rate of 12 ml/hr. Activity eluted in two peaks, Fractions V and V'.

9. Enzyme assays. DNA polymerase activity was followed by incorporation of [³²P]dTTP into gapped salmon sperm DNA (McHenry and Crow, 1979). One unit of activity is the amount catalyzing incorporation of 1 pmol of dNTP per min at 30°C. Exonuclease
activity (Echols et al., 1983) was determined using a 3' mismatched substrate, \((dT)_{17}^{-}(^{3}H)dC)_{1.6}/(dA)_{1500}\), prepared by the terminal transferase method (Brutlag and Kornberg, 1972).

10. Other biochemical methods. Standard procedures were followed for SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), silver staining (Wray et al., 1981), and determination of protein concentration (using bovine serum albumin as standard) by the method of Bradford (1976). Western blots were prepared by electrophoretic transfer of proteins from 6% SDS-polyacrylamide gels (Towbin et al., 1979) and stained with a Vecta-stain ABC kit (Vector Laboratories).

C. RESULTS

1. Null phenotype of dnaQ. The reconstruction experiment of Fig. 3 illustrates the null phenotype of dnaQ. When wild-type cells were transduced to tetracycline-resistance (Tc\(^R\)) using a donor strain with Tn10 in dnaQ or a control donor with Tn10 in the nonessential bio genes, the dnaQ::Tn10 transductant colonies differed from the controls in three visible features: they were smaller, they had an irregular border, and they had a mottled surface appearance (compare Fig. 3A and B). The control colony morphology was stable, as shown by replating (Fig. 3D). The "sick" dnaQ::Tn10 colony morphology, in contrast, was unstable, since dnaQ::Tn10 colonies gave rise to some colonies of wild-type appearance upon replating (Fig. 3E). We deduced that the sick cells gave rise to the healthy ones, and not vice versa, because primary transductants replated at earlier times had few, if any, healthy cells in them, and because primary
Figure 3. *DnaQ* null phenotype. Freshly growing cells were transduced at a multiplicity of 0.1 (PFU per cell) with P22 lysates propagated on strain RM238 (*bio*:Tn10) or RM978 (*dnaQ201*:Tn10). After 15 min of incubation at 37°C, the transduction mixes were diluted with an equal volume of LB broth containing 20 mM EGTA to prevent reinfection of transductants. The mixes were then incubated with aeration at 37°C for 2 h to allow expression of tetracycline resistance, and plated on LB plates containing tetracycline and EGTA. After 24 h of incubation at 37°C the plates were photographed, and then incubation was continued for an additional 24 h. After the additional incubation, single colonies from each plate were resuspended, diluted, and replated on the identical medium. These plates were also photographed after 24 h of incubation at 37°C. A, RM820 (dnaE<sup>+</sup>) x *bio*:Tn10; B, RM820 x *dnaQ201*:Tn10; C, RM821 (spq-2) x *dnaQ201*:Tn10; D, E, F, replated transductants from A, B, and C respectively. Bar, 0.5 mm.
Figure 4. Southern analysis of dnaQ mutants. EcoRI-digested genomic DNA was probed with a plasmid containing the indicated 3.1-kilobase (kb) \textit{dnaQ}^+ restriction fragment. Lane 1, \textit{dnaQ}^+ (RM749); lane 2, \textit{dnaQ201::Tn10} (RM976); lane 3, \textit{dnaQ201::Tn10} (RM978). The latter two strains differ in that RM976 presumably contains an uncharacterized suppressor arising subsequent to introduction of the \textit{dnaQ} mutation, whereas RM978 was constructed by introducing the \textit{dnaQ} mutation into an \textit{spq-2} strain. The blot shows that the \textit{dnaQ} gene in the wild type strain resides on a single EcoRI fragment whereas in the \textit{dnaQ201::Tn10} strains, two bands are detected as expected since the deletion-substitution in \textit{dnaQ} introduces two new EcoRI sites. \textit{H}, HindIII; \textit{R}, EcoRI.
transductants allowed to grow on plates for 72 h formed sick colonies in which several papillae or outgrowths with wild type morphology could be seen in each colony. The healthy cells exhibited the expected mutant structure of the dnaQ region (Fig. 4), and we will show below that these cells also contained a suppressor mutation. Therefore, the sick cells represented the unaltered original transductant genotype, and they define the dnaQ null phenotype as "poor growth" under the conditions illustrated. There was some variability in the severity of the growth defect among many repetitions of this experiment, the precise cause of which we do not understand.

Although the reconstruction experiment of Fig. 3 used a single dnaQ allele, a single host strain, and a single method of strain construction, the growth defect was not specific to these conditions. We observed similar behavior in λ-sensitive Salmonella transduced to dnaQ::Tn10 via λ infection. Moreover, the same phenotype was produced by every insertion or substitution allele of dnaQ in our collection. This included simple insertions of Tn10Δ16Δ17 near the middle of dnaQ or near its 3' end, and internal substitutions encompassing the 3' half of dnaQ and extending to varying degrees towards the 5' end (Fig. 1 and 2). All of these dnaQ alleles shared the feature that expression of the carboxyl portion of c was disrupted. Thus, the carboxyl portion of c appears to be necessary for c to carry out its growth-promoting function.

2. Suppressors of dnaQ::Tn10. The observations on the appearance and behavior of freshly made dnaQ null mutants suggested
the presence of a suppressor mutation in their healthy progeny. This suggestion was confirmed by showing that the factor responsible for these cells' improved growth was a genetic element that could be transduced from one cell to another by phage P22. When a cell harbored such a suppressor, it could tolerate the subsequent introduction of a dnaQ null allele as evidenced by the formation of transductant colonies of normal and stable morphology (Fig. 3C and F). In the best-studied case, three lines of evidence agree that one of these suppressors, called spq-2, is a mutation of dnaE, the gene for the polymerization subunit (α) of DNA polymerase III. These lines of evidence include transductional linkage of spq-2 to a KmR marker near dnaE, molecular cloning of spq-2 on a fragment of DNA that also includes dnaE, and marker rescue of the spq-2 phenotype by a fragment of DNA whose only difference from wild-type (determined by complete DNA sequence of the fragment) encodes a valine → glycine change in α (chapter III; Lancy et al., 1989b).

The presence of two cell types, one sick and without a suppressor, the other healthy and having a suppressor, in each dnaQ::Tn10 transductant colony exemplified in of Fig. 3B implied that the suppressors arose subsequent to the time of plating and therefore must have arisen independently in each colony. In addition to spq-2 described above, we partially characterized fifteen other suppressors obtained from different primary transductant colonies. All of these suppressors were linked to dnaE by P22 cotransduction with a KmR marker near dnaE (see RM1655 et seq. in Table 1), and spq-17, -14, and -16 were, in addition, molecularly cloned in phage λ on a
fragment of DNA that also included dnaE. These data are not sufficient to prove that the additional suppressors are in fact alleles of dnaE, but the example of spq-2 makes this likely. In a few cases (spq-11, -13, -14, and -16) the cotransduction frequency of suppressor with KmR was suspiciously low (<10% cotransduction versus 68% cotransduction of KmR with dnaE698), perhaps indicating a second locus in the dnaE vicinity that can give rise to suppressor mutations. However, other possible explanations for the low cotransduction frequency, such as error or ambiguity in scoring the suppressor phenotype, have not been ruled out, and the cloning results mentioned above imply that spq-13, -14, and -16 are closer to dnaE than indicated by their cotransduction frequency.

All of the suppressors were generated with the dnaQ200 allele, in which the 3' portion of dnaQ was substituted whereas the 5' portion of dnaQ encoding the N-terminal 132 amino acid residues of c remained intact (Fig. 2). When tested, however, all sixteen suppressors were able to suppress the more inclusive dnaQ201 substitution allele encoding only 13 N-terminal residues (Fig. 2). This observation was consistent with the apparent equivalence of the various dnaQ alleles in their growth phenotype.

3. Additional properties of spq-2 and dnaQ200, spq-2 strains.

We examined several aspects of the physiology of strains RM821 (spq-2) and RM822 (spq-2 dnaQ200) as exemplars of the consequences of loss of dnaQ and its suppression. These two strains grew in LB broth with a doubling time of 35 to 40 minutes at 37°C, the same as their wild-type ancestor, RM10 (data not shown). Strain RM822 was a mutator by
a factor of about $10^3$ at two loci tested, but strain RM821 exhibited nearly wild-type spontaneous mutation rate (Table 3; other suppressors tested behaved similarly). Strains RM821 and RM822 were not more sensitive to ultraviolet light than RM10, and in fact, dnaQ200 afforded some degree of protection from UV killing (S. Slater and R. Maurer, manuscript in preparation).

4. Active DNA polymerase III in a dnaQ null strain. A biochemical analysis of DNA polymerase III was undertaken to test two opposing hypotheses about the nature of the $spq-2$ suppressor mutation. According to one hypothesis, in the absence of $c$ the remaining DNA polymerase III components interfere with some alternative mechanism of DNA replication. The $spq-2$ mutation in $c$ inactivates DNA polymerase III, potentiating the alternative mechanism. According to the opposing hypothesis, the $spq-2$ mutation restores some critical property of DNA polymerase III that is lost when $c$ is missing. An active DNA polymerase III species was isolated from strain RM822 ($dnaQ200 spq-2$). The polymerase activity of Fraction IV was decreased 5-fold in 150 mM KCl and 10-fold in N-ethylmaleimide. This pattern of sensitivity is characteristic of DNA polymerase III and not of DNA polymerase I (Kornberg, 1980). Fraction V' consisted of four major polypeptides, one of which was identified as the $a$ subunit of DNA polymerase III based on its apparent molecular weight, 128 kDa, and its cross-reactivity on Western blots to a polyclonal antibody specific to purified E. coli $a$ (Fig. 5). No 3'-5' exonuclease activity was detectable in Fraction V'', although such activity was readily detected in a comparable
Table 3. Mutation rates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of cultures</th>
<th>Mutations/10^8 cells per generation ± variance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rifampin resistance</td>
</tr>
<tr>
<td>RM820</td>
<td>dnaE&lt;sup&gt;+&lt;/sup&gt; (spq&lt;sup&gt;+&lt;/sup&gt;), dnaQ&lt;sup&gt;+&lt;/sup&gt;</td>
<td>15</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>RM821</td>
<td>spq-2, dnaQ&lt;sup&gt;+&lt;/sup&gt;</td>
<td>15</td>
<td>0.58 ± 0.13</td>
</tr>
<tr>
<td>RM822</td>
<td>spq-2, dnaQ200::Tn10</td>
<td>19</td>
<td>375 ± 22</td>
</tr>
</tbody>
</table>

<sup>a</sup> The strains are otherwise isogenic.
Figure 5. DNA polymerase III from strain RM822. A. Fraction V'' (1 μg) was electrophoresed through a 10% to 15% SDS-polyacrylamide gradient gel and visualized by silver staining. Marker proteins (Bio-Rad Laboratories) were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa). B. Fraction V'' (1 μg) was electrophoresed through a 6% SDS-polyacrylamide gel and visualized by staining with polyclonal antibody to E. coli σ subunit. Pre-dyed protein standards (Bio-Rad) and their approximate molecular weights were myosin (200 kDa), phosphorylase B (97.4 kDa), and bovine serum albumin (68 kDa). Additional marker proteins were not resolved in the 6% gel.
preparation of wild-type enzyme (Fig. 6). Use of up to five times more units of mutant enzyme than used for Fig. 6 failed to reveal any evidence of exonuclease (not shown). The absence of exonuclease suggests that no active c fragment purified with the DNA polymerase III activity and provides further evidence for the absence of DNA polymerase I from this preparation. All of these observations argue that spq-2 encodes an active rather than an inactive DNA polymerase III α subunit.

5. Requirement for DNA polymerase I in dnaQ null strains. Even though strain RM822 contained an active DNA polymerase III, it was still possible that cell survival required assistance from additional DNA polymerase(s). To test whether DNA polymerase I was required for the survival of dnaQ null mutants, we repeated the transduction test illustrated in Figure 3 using recipient strains with a mutant DNA polymerase I gene, polA2 (Table 4). The polA2-encoded enzyme exhibits reduced, but detectable, polymerase and editing activities (Whitfield and Levine, 1973; Engler and Bessman, 1979). All of the polA2 strains were transduced to TcR by P22 with near-normal efficiency (50-100%) compared to their polA+ parents when the donor strain contained Tn10 located in a nonessential site (data not shown). When the donor strain contained Tn10 in dnaQ (dnaQ200), the frequency of TcR transductants was decreased more than two orders of magnitude in both the dnaE+ recipient (experiment analogous to those shown in Fig. 3B and E) and in spq-2, -3, and -6 recipients (analogous to data in Fig. 3C and F). From these results, it is clear that dnaQ200 cells exhibited a more stringent requirement for
Figure 6. 3'-5' exonuclease activity in DNA polymerase III preparations. A. \textit{spq}-2 polymerase (fraction V'; 6.5 U). B. Wild-type polymerase (fraction V; 6.5 U). This enzyme was prepared from RM10 by the same procedure used for \textit{spq}-2 enzyme except that fraction V was prepared by DEAE chromatography. dGMP (5 mM) is a specific inhibitor of c exonuclease (Scheuermann and Echols, 1984).
Table 4. Requirement for DNA polymerase I for dnaQ::Tn10 viability

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>DnaQ::Tn10 transductants (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM2685</td>
<td>dnaE+ (spq+), polA+</td>
<td>665</td>
</tr>
<tr>
<td>RM1835</td>
<td>dnaE+ (spq+), polA2</td>
<td>0</td>
</tr>
<tr>
<td>RM2686</td>
<td>spq-2, polA+</td>
<td>630</td>
</tr>
<tr>
<td>RM1836</td>
<td>spq-2, polA2</td>
<td>0</td>
</tr>
<tr>
<td>RM2687</td>
<td>spq-3, polA+</td>
<td>597</td>
</tr>
<tr>
<td>RM1837</td>
<td>spq-3, polA2</td>
<td>0</td>
</tr>
<tr>
<td>RM2688</td>
<td>spq-6, polA+</td>
<td>409</td>
</tr>
<tr>
<td>RM1838</td>
<td>spq-6, polA2</td>
<td>1</td>
</tr>
</tbody>
</table>

*Each entry shows the number of TcR transductants obtained from 5 x 10^6 P.F.U. of P22 lysate propagated on strain RM711 (dnaQ200::Tn10 spq-5). Colonies were counted after 2 days.*
DNA polymerase I for viability than did dnaQ+ cells, and this requirement was not relieved by spq suppressor mutations.

D. DISCUSSION

Loss of dnaQ leads to a severe growth defect illustrated by the formation of small colonies of abnormal morphology. We have not been able to study dnaQ::Tn10 strains directly because of their genetic instability. Instead, we have studied derivatives of these strains that carry a mutation that suppresses the growth defect.

Elevated mutation rate is a familiar feature of previously described mutant alleles of dnaQ and is accounted for, at least in part, by their exonuclease deficiency (Degnen and Cox, 1974; Horiuchi et al., 1978; Cox and Horner, 1982; Echols et al., 1983, Schaspar, 1989). We observed a thousand-fold elevation of mutation rate in dnaQ::Tn10 strains carrying spq-2 (Table 3) or other spq mutations (data not shown). Since the suppressor mutations by themselves had little effect on mutation rate, this thousand fold elevation of mutation rate is a reasonable approximation of the effect to which the dnaQ::Tn10 cells are subjected as they await development of a suppressor mutation. Such an increase in mutation rate presumably increases the likelihood that a suitable suppressor mutation will occur within each single colony.

Several lines of evidence indicate that the spq-2 dnaE gene encodes an active, rather than an inactive, a subunit of DNA polymerase III. Most significantly, an active DNA polymerase III species has been isolated from strain RM822 (Fig. 5 and 6; Table 2).
Moreover, α subunit purified from an E. coli strain that overproduces dnaE (spq-2) is as active as wild-type α in gap-filling activity (data not shown). The interpretation of this biochemical evidence is consistent with two in vivo results as well. In spq-2 strains, the spontaneous mutation rate varies with the dnaQ genotype (Table 3). Since ε acts as an intrinsic subunit of DNA polymerase III, this observation implies that DNA polymerase III, and specifically its spq-2-encoded α subunit, is functional. It has also been observed that dnaQ::Tn10 transductants do not appear if the recipient strain carries a temperature-sensitive mutation in dnaE. This observation implies that the suppressor mechanism, whatever it is, can work only in the context of a functional α protein (A. Wong and R. Maurer, unpublished data).

A plausible view, then, is that ε normally provides a function critical to the operation of DNA polymerase III. When ε is lacking, a suppressor mutation operates at the level of the polymerase α subunit to restore or bypass the critical function. It appears that the critical aspect of ε is not its fidelity function, for spq-2 by itself has little effect on the fidelity of DNA replication as judged from mutation rates (Table 3) nor does it unveil or recruit any detectable novel exonuclease activity that purifies with the polymerase (Fig. 6).

If fidelity is not the growth-promoting function of ε, then what is? There is biochemical evidence that α is more active and more stable when bound to ε than it is alone (Maki and Kornberg, 1987) and genetic and biochemical evidence that α and ε interact (Horiuchi et
al., 1981; Maurer et al., 1984a; Takano et al., 1986; Maki and Kornberg, 1987). Also, the full replication competence of DNA polymerase III requires several other subunits in addition to α and ε (McHenry, 1988); conceivably, one or more of these subunits could attach to the polymerase complex through ε and would be lost or loosely bound in the absence of ε (Shwartz et al., 1988). (The major peptides, other than α, that purify with our ε-free gap-filling activity have not been identified explicitly, but as judged by apparent molecular weight, they could be the β, γ, and δ subunits that have been described previously.) These considerations lead to several possible hypotheses connecting ε to a property of DNA polymerase III such as its rate, processivity, intrinsic stability, ability to dimerize, ability to extend a mismatched primer terminus, or ability to participate in methyl-directed mismatch repair (Lahue and Modrich, 1988). Deterioration of any of these characteristics of the polymerase might impair a cell's ability to complete DNA replication, yet could be remedied, in principle, by a modification of α. Analysis of possible alteration in such properties of α by spq mutations will require the application of more specific tests of polymerase activity than the general gap-filling assay we have used. This work is currently in progress.

**Role of DNA polymerase I.** *DnaQ* null mutants, even after acquisition of a suppressor mutation, exhibit a more stringent requirement for DNA polymerase I than do *dnaQ*+ strains. Elucidation of the physiological basis of this requirement will require further investigation because pol I possesses three distinct activities
(Joyce and Steitz, 1987), any of which (or some combination thereof) could be required, depending on the actual nature of the growth function of c. The pol I mutant used in observing this requirement has reduced polymerization and editing capacities, whereas the effect of the mutation on its 5'-3' exonuclease activity is unknown (Whitfield and Levine, 1973; Engler and Bessman, 1979). If the loss of c makes DNA polymerase III a less processive enzyme, this could lead to a higher demand for DNA polymerase I polymerase activity to fill additional gaps in the replicated DNA. If such gaps are characterized by RNA primers on one side (as in normal Okazaki fragment synthesis), the additional demand would include the 5'-3' exonuclease activity. Note that the dnaQ200::Tn10 allele used in the DNA polymerase I experiments leaves enough of dnaQ intact to insure normal promoter activity of the divergently oriented RNaseH (rmh) gene (Nomura et al., 1985; Cox and Horner, 1986). This may be important if RNaseH contributes to the removal of RNA primers of DNA synthesis. The editing exonuclease activity of DNA polymerase I could be used to erase a fraction of nucleotides misinserted by DNA polymerase III and resistant to further extension. Note, however, that the elevated mutation frequency of dnaQ::Tn10, spq strains indicates that many misinsertions are not corrected. Finally, another possible source of increased demand for DNA polymerase I would be in post-replication repair of mismatches which will be more frequent in the absence of editing at the replication fork. In the specific case of the mutHLS methyl-directed mismatch repair system, which normally utilizes DNA polymerase III (Lahue and Modrich, 1988),
involvement of DNA polymerase I might arise if the absence of α or the suppressor-encoded alteration in α interfered with participation by DNA polymerase III.

The requirement for DNA polymerase I in dnaQ::Tn10 strains differs in two respects from another report of a special situation when DNA polymerase I becomes essential for replication. Niwa et al. (1981) found that DNA replication strictly depends on DNA polymerase I in dnaH(Ts) mutants at high temperature, and in unsuppressed dnaH(αm) mutants (Bryan et al., 1988), provided that a mutant allele of a poorly characterized gene, pchA, is present. In the dnaQ::Tn10 strains, in contrast, the requirement for dnaE is not bypassed, and indeed, survival appears to depend on an alteration in some property of the dnaE product. Moreover, wild-type Salmonella clearly exhibits the PchA+ phenotype since dnaH(Ts) mutations are temperature-sensitive lethals (Maurer et al., 1984b). Salmonella that were once dnaQ::Tn10 and then later transduced to dnaQ+ are also PchA+ (data not shown), showing that survival following introduction of dnaQ::Tn10 does not require induction of a mutation in pchA such as described by Bryan and Moses (in E. coli, pchA is not linked to dnaE or dnaQ; Bryan and Moses, 1984).

No requirement for RecA. When normal DNA replication in E. coli is blocked or slowed, either by treatment with DNA-damaging agents or by raising the temperature in certain temperature-sensitive replication mutants, a complex response (SOS) involving induction of a set of genes ensues. In a variety of ways, the induced genes aid the cell in recovering from the inducing condition. A key positive
regulatory element in the response is the RecA protein, whose activation leads to proteolysis of LexA protein, the common repressor of the induced SOS genes (Walker, 1984). Salmonella clearly possesses a similar system under analogous control (Orrego and Eisenstadt, 1987). The fact that we (S. Slater and R. Maurer, unpublished data) have been able to make a RecA derivative of an spq-2 dnaQ200::Tn10 strain argues that survival of this strain does not require chronic induction of any SOS proteins. However, this result does not address whether transient induction of SOS might play a role in promoting the persistence of freshly made dnaQ::Tn10 transductants until the development of a suppressor mutation.

Other roles for c? The isolation of strains devoid of c subunit opens up the opportunity to investigate the role of c, if any, in several aspects of cell physiology. For example, the role of c in inhibiting or possibly promoting DNA synthesis on UV-damaged DNA is controversial (Villani et al., 1978; Woodgate et al., 1987; Jonczyk et al., 1988; Shwartz et al., 1988; Quinones et al., 1989). Whether any SOS-induced proteins needed for UV-mutagenesis have c as their primary target is also unknown. It will also be of interest to study the structure and genetic requirements of DNA polymerase III holoenzyme in dnaQ::Tn10 strains to learn whether any holoenzyme subunits use c as their site of attachment to the polymerase.

E. APPENDIX

1. Growth rates of spq-2 strains. In the initial characterization of the strains which had survived the deletion of
the dnaQ gene, the growth rates of the wild type strain (RM10, dnaE<sup>+</sup> dnaQ<sup>+</sup>) and the spq-2 strains (RM821, spq-2 dnaQ<sup>+</sup> and RM822, spq-2 dnaQ<sub>2</sub>:Tn10) were measured to determine if the presence of the suppressor mutation or the dnaQ mutation affected the growth of the cells. Duplicate cultures of each strain were grown in LB broth plus thymine at 37°C with constant aeration. The growth of the cultures was monitored by turbidity at various time points using a Klett-Summerson colorimeter. The growth curves of these strains are shown in Figure 7. The three strains grew at similar rates with a doubling time of 35 to 40 minutes. Therefore, we conclude that the spq-2 suppressor mutation, either in the presence of a functional c subunit, or in its absence, does not have an effect on the overall growth rate of the cell compared to the dnaE<sup>+</sup> strain.

2. Mutation rates of spq-2 strains containing various dnaQ alleles. The spq-2 mutant was isolated using the dnaQ200::Tn10 allele which encodes a truncated N-terminal 132 amino acid fragment of c (see Fig. 2). To examine the possibility that this N-terminal fragment possessed any residual activity, further deletions of the 5' portion of dnaQ were been made. This resulted in a set of deletion alleles, dnaQ201-dnaQ206, which are shown in Fig. 2. These additional dnaQ alleles, when introduced into the spq-2 strain, were all suppressed by the spq2 mutation. The mutation rates of the spq-2 strains containing the various dnaQ alleles were determined to test if the dnaQ alleles differentially affected the mutation rates. The mutation rates of these strains, determined as above (see Materials...
Figure 7. Growth curves of strains RM10, RM821 and RM822 (see text for details).
and methods), are listed in Table 5. All six dnaQ deletion alleles gave mutation rates similar to those of the dnaQ200 allele (Table 3). Based on this result, it appears that the N-terminal 132 amino acid fragment of dnaQ200 does not contain any residual activity that influences the mutation rate of the spq-2 strain. Furthermore, since all the dnaQ alleles behave similarly, including the ones in which the majority of dnaQ has been removed (e.g. dnaQ201 and dnaQ202), these dnaQ alleles can thus be considered as dnaQ null mutants.

3. Effect of spq allele on mutation rate. In an attempt to categorize the different spq mutations, the mutation rates of dnaQ200::Tn10 and dnaQ201::Tn10, in combination with a variety of independently obtained spq alleles, were determined. As shown in Table 6, the mutation rates of all the spq dnaQ::Tn10 strains were elevated over those of the dnaQ+ dnaQ+ and the spq dnaQ+ strains as was observed with the spq-2 strains (Table 3). No systematic differences between dnaQ200 and dnaQ201 were observed, supporting the conclusion of the previous paragraph. Nor could we discern any systematic distinctions among the spq alleles. Even the information from DNA sequencing that spq-2, spq-7, and spq-9 form a distinct class of mutations (Table 2) did not allow a classification based on mutation rate to be devised. Therefore, it is apparent that the suppressor mutations cannot be differentiated based on their mutation rates. A similar increase in the mutation rate is characteristic of all spq, dnaQ::Tn10 strains.
Table 5. Mutation rates of different dnaQ strains

<table>
<thead>
<tr>
<th>spq allele</th>
<th>dnaQ allele</th>
<th>No. of cultures</th>
<th>Mutations/10^8 cells per generation ± variance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rifampin resistance</td>
</tr>
<tr>
<td>spq^+</td>
<td>dnaQ^+</td>
<td>21</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>dnaQ^+</td>
<td>15</td>
<td>0.58 ± 0.13</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>19</td>
<td>375 ± 22</td>
</tr>
<tr>
<td>2</td>
<td>203</td>
<td>3</td>
<td>289 ± 40</td>
</tr>
<tr>
<td>2</td>
<td>204</td>
<td>3</td>
<td>569 ± 69</td>
</tr>
<tr>
<td>2</td>
<td>205</td>
<td>3</td>
<td>196 ± 31</td>
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<tr>
<td>2</td>
<td>206</td>
<td>3</td>
<td>182 ± 28</td>
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<tr>
<td>2</td>
<td>201</td>
<td>16</td>
<td>247 ± 17</td>
</tr>
<tr>
<td>2</td>
<td>202</td>
<td>3</td>
<td>514 ± 65</td>
</tr>
</tbody>
</table>

^a spq^+ is the same as dnaQ^+.

^b The dnaQ alleles are listed in the order of increasing deletion size. The deletion endpoints of the dnaQ alleles, with the exception of dnaQ203, are shown in Figure 2. dnaQ203 is identical to dnaQ200, i.e., both contain the same portion of the dnaQ sequence.
Table 6. Mutation rates of various \textit{spq} strains.

<table>
<thead>
<tr>
<th>\textit{spq} allele</th>
<th>\textit{dnaQ} allele</th>
<th>No. of cultures</th>
<th>Mutations/10^8 cells per generation ± variance</th>
</tr>
</thead>
</table>
| 3 \( spq^+ \)       | 200 \( dnaQ^+ \)    | 21             | \begin{tabular}{c|c|c} Rifampin resistance & 0.15 ± 0.05 & 0.35 ± 0.08 \\
| 3 201               | 17                   | 93 ± 7         | 2.3 ± 0.5 \\
| 4 200               | 4                    | 497 ± 55       | 76 ± 12 \\
| 4 201               | 3                    | 217 ± 35       | 46 ± 10 \\
| 5 200               | 4                    | 289 ± 35       | 170 ± 23 \\
| 5 201               | 3                    | 224 ± 35       | 85 ± 16 \\
| 6 200               | 4                    | 96 ± 15        | 64 ± 11 \\
| 6 201               | 3                    | 103 ± 18       | 83 ± 16 \\
| 7 200               | 4                    | 315 ± 24       | 99 ± 10 \\
| 7 201               | 3                    | 286 ± 28       | 244 ± 27 \\
| 8 200               | 4                    | 810 ± 85       | 71 ± 12 \\
| 8 201               | 3                    | 119 ± 21       | 44 ± 10 \\
| 9 200               | 4                    | 598 ± 63       | 162 ± 22 \\
| 9 201               | 3                    | 233 ± 36       | 44 ± 10 \\
| 10 200              | 4                    | 301 ± 36       | 147 ± 23 \\
| 10 201              | 3                    | 895 ± 108      | 662 ± 98 \\
| 12 200              | 4                    | 562 ± 59       | 189 ± 26 \\
| 12 201              | 3                    | 932 ± 113      | 308 ± 43 |
4. Examination of Tc\textsuperscript{R} dnaQ::Tn10 polA2 transductants. Although polA2 strains (either dnaE\textsuperscript{+} or spq) were transduced to dnaQ::Tn10 with a much lower efficiency than polA\textsuperscript{+} strains, a few Tc\textsuperscript{R} transductants were nonetheless obtained. We were intrigued by the formation of these transductants and decided to investigate them further. Each polA2 strain in Table 4 (RM1835, RM1836, RM1837, and RM1838) was retransduced with dnaQ200::Tn10 to generate additional Tc\textsuperscript{R} transductants for this analysis.

A trivial possibility was that the Tc\textsuperscript{R} colonies were not transductants derived from the polA2 mutants but instead were contaminants in the experiment. To test this, several isolates from each of the four transductions were streaked onto LB plus thymine plates containing either kanamycin or chloramphenicol to check, respectively, for the drug markers near the spq mutation (but not dnaE\textsuperscript{+}) and the polA2 allele. The Tc\textsuperscript{R} transductants of the dnaE\textsuperscript{+} strain (RM1835) were Km\textsuperscript{S} and Cm\textsuperscript{R}, as expected, and the Tc\textsuperscript{R} transductants of the spq strains (RM1836, RM1837, and RM1838) were Km\textsuperscript{R} and Cm\textsuperscript{R}, as expected (data not shown). Thus, the possibility that the Tc\textsuperscript{R} transductants were contaminants was ruled out.

The second possibility was that the transductants were revertants or pseudorevertants of the polA2 allele. This was tested by plating the Tc\textsuperscript{R} transductants on LB plus thymine plates containing 3 mM methyl methanesulfonate (MMS) which inhibits the growth of polA mutants. All of the Tc\textsuperscript{R} transductants tested (either dnaE\textsuperscript{+} or spq) failed to grow on the MMS plates and were therefore still phenotypically PolA\textsuperscript{−}, ruling out the possibility that the
transductants were true revertants of polA2. However, pseudorevertants (including extragenic suppressors) of polA2 cannot be ruled out as their behavior in the EMS test is unpredictable (see below).

A third possibility was that the TcR transductants contained a chromosomal duplication in the region of the dnaQ gene and thus contained both dnaQ+ and dnaQ200::Tn10 alleles. To test this possibility, the segregation of the TcR marker in the RM1835, RM1836, RM1837, and RM1838 dnaQ200::Tn10 transductants was examined. Four isolates from each transduction were purified on LB plus thymine plates in the absence of tetracycline. The purified isolates were then tested for their tetracycline phenotype and all were found to be TcR. The TcR strains were then grown to saturation in liquid culture under nonselective conditions (no drug) and plated onto LB plus thymine plates for single colonies. The colonies were then tested for their tetracycline phenotype. None of the colonies, derived from the dnaQ::Tn10 transductants of RM1835 (82 colonies), RM1836 (124 colonies), RM1837 (87 colonies), and RM1838 (88 colonies) was TcS, indicating that the TcR marker was stable in these strains. Thus, chromosomal duplications that could account for the viability of the TcR dnaQ::Tn10 polA2 transductants were not detected in these strains.

It has been reported that haploid segregants have been found at frequencies of 1 to 30% in overnight cultures of duplication-containing Salmonella strains when grown under nonselective conditions (Sonti and Roth, 1989). In a similar study, Ames et al.
(1978) determined that duplications in the histidine transport operon in Salmonella were resolved at a frequency of 5%. Consequently, it is possible that dnaQ duplications were present in the dnaQ::Tn10 polA2 transductants and were not detected in the above analysis. This would be applicable if the dnaQ duplications were resolved at low frequencies since only a small number of isolates from each strain was examined. Therefore, without the analysis of a larger number of isolates, we cannot absolutely rule out the possibility that dnaQ duplications were present in the dnaQ::Tn10 polA2 transductants.

The exact identity of the TcR polA2 dnaQ::Tn10 transductants remains unknown. We have argued that the transductants are not polA revertants and possibly do not contain dnaQ duplications. We cannot even be sure that the transductants are genetically distinct from their non-growing siblings. It may be that ~1% of polA2 cells have enough DNA polymerase I to allow colony formation by dnaQ::Tn10 derivatives. Alternatively, it is possible that additional suppressors are generated in the polA strains (dnaE+ or spq) when dnaQ is deleted. Such suppressors would have to be distinct from spq because rare TcR polA2 dnaQ::Tn10 transductants are obtained from both dnaE+ and spq strains. Therefore, the hypothesized suppressors generated in these strains would most likely be compensating for the polA2 defect without relieving the cells of their MMS sensitivity. It would be interesting to learn whether these strains carry such a suppressor and what its mechanism is, as this information would clarify the role of DNA polymerase I in promoting the growth of
DNAQ::Tn10 strains.
CHAPTER III

NUCLEOTIDE SEQUENCES OF dnaE, THE GENE FOR THE POLYMERASE SUBUNIT
OF DNA POLYMERASE III IN Salmonella typhimurium, AND A VARIANT THAT
FACILITATES GROWTH IN THE ABSENCE OF ANOTHER POLYMERASE SUBUNIT

A. INTRODUCTION

In Escherichia coli and Salmonella typhimurium, DNA replication
is carried out by the multisubunit DNA polymerase III. The intrinsic
polymerization activity of this enzyme complex lies in the α subunit,
encoded by dnaE (Welch and McHenry, 1982; Maki and Kornberg, 1985).
Other subunits, by associating with α, refine many of its properties,
including its rate, processivity, thermostability, and tendency to
dimerize (Maki and Kornberg, 1987; McHenry, 1988). In addition, the
α subunit encoded by dnaQ endows the polymerase complex with an
editing capacity (Scheuermann et al., 1983; Scheuermann and Echols,
1984). These biochemical interactions between α and other polymerase
subunits may underlie several genetic interactions that have been
described between dnaE and genes for other polymerase subunits
(Horiuchi et al., 1981; Kuwabara and Uchida, 1981; Maurer et al.,
1984a).

In the accompanying paper (chapter II, Lancy et al., 1989a), we
have shown that Salmonella null mutants of dnaQ, although viable,
exhibit a severe growth defect that can be remedied by suppressor
mutations mapping in or near dnaE. Here we present nucleotide
sequence data for wild-type dnaE and for one of the suppressor variants, spq-2. We also present evidence from marker rescue experiments that the single sequence difference in dnaE between these two alleles accounts for the suppressor phenotype of spq-2. These results provide further evidence for the functional significance of an α-ε interaction by showing that the growth-defective phenotype of DnaQ− can be remedied by an alteration of α.

B. MATERIALS AND METHODS

1. Strains. In addition to strains listed in Table 1 (chapter II, Lancy et al., 1989a), we used strains RM1757 and RM1759, derivatives of strain RM10 carrying dnaE698(Ts) and dnaE693(Ts), respectively (Maurer et al., 1984b).

2. Clones and subclones. Wild-type dnaE was subcloned from λRM310 (Maurer et al., 1984b) on a 7.0-kilobase (kb) SmaI fragment in the vector pUC8 (Vieira and Messing, 1982) to give plasmid pFF325. A Southern blot confirmed that dnaE is found on a 7.0-kb SmaI fragment in genomic DNA of both wild-type and spq-2 strains (data not shown). A subclone of the spq-2 7.0-kb fragment was made in several steps beginning with preparation of a library in the vector bacteriophage λ1059 by using genomic DNA of strain RM746 (chapter II; Maurer et al., 1984b; Lancy et al., 1989a). A dnaE-complementing clone, λRM740, isolated from this library, was the source of the 7.0-kb fragment which was subcloned into pUC8 to give pFF221. Four additional pUC8 plasmid constructs containing incomplete dnaE genes were derived from pFF221 and pFF325 by subcloning fragments having
one end at a unique PstI site corresponding to codons 600 to 601 in dnaE and the other end at either a BamHI site or an EcoRI site derived from the pUC8 multiple cloning site. The plasmids thus formed were pFF377, carrying the 3' portion of spq-2; pFF378, carrying the 3' portion of dnaE+; pFF381, carrying the 5' portion of spq-2; and pFF382, carrying the 5' portion of dnaE+.

3. Marker rescue experiments. dnaE(Ts) strains RM1757 and RM1759, and derivatives carrying plasmids pFF377, -378, -381, and -382, were grown to saturation at 32°C in LB plus thymine. A 0.1-ml sample of the culture was plated on LB plus thymine agar. After the plate was incubated at 42°C overnight, colonies were counted. In cases where the presence of a plasmid yielded a marked excess of temperature resistant revertants compared with the number obtained in the control (no plasmid) experiment, individual revertants were purified by restreaking at 32°C. In most cases, these purified revertants were found to have lost their respective plasmids, but the precise point at which plasmid loss occurred was not determined. These revertants were then tested for suppressor phenotype by scoring colony morphology after transduction to dnaQ::Tn10 (chapter II; Lancy et al., 1989a).

4. DNA sequence methodology. DNA sequence was determined by using double-stranded plasmid DNAs (pFF221 and pFF325) as the template. Sequencing was by the dideoxy method, using modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp.). Initial sequence was obtained by using a primer based on the published dnaE sequence of E. coli (Tomasiewicz and McHenry, 1987). As the S. typhimurium
sequence was revealed, additional primers were designed to allow the sequence to be extended and confirmed on both strands. In addition to the sequence presented in Fig. 8, sequence to the 3' side was determined to the end of the 7.0-kb fragment for both wild type and spq-2. The nucleotide sequence data reported in this paper, as well as flanking sequences not shown, will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number M26046.

5. Expression and labeling of proteins expressed under T7 RNA polymerase control. Suitable restriction fragments containing wild-type dnaE or spq-2 were inserted into pT7 plasmids containing a T7 promoter (Tabor and Richardson, 1985). Proteins were expressed under T7 control after thermal induction of a strain in which the gene for T7 RNA polymerase was controlled by a temperature-sensitive λ repressor (Tabor and Richardson, 1985). After the thermal induction, further host RNA synthesis was shut off by the addition of rifampin, to which T7 RNA polymerase is insensitive. The protocols were slightly different in detail in isotopic labeling experiments and overproduction experiments. For labeling, cells were grown at 30°C in LB, washed three times, and resuspended in minimal (M9) medium supplemented with thiamine and 18 amino acids (minus cysteine and methionine). After additional growth at 30°C for 1 hr, cells were shifted to 42°C for 15 min. Next, rifampin was added at 400 μg/ml, and incubation was continued at 42°C for 20 min. Cells were then shifted to 37°C for 20 min, and labeling with [35S]methionine took place for 5 min at 30°C. For DNA polymerase overproduction, thermal
induction was in LB for 20 min at 42°C; rifampin was added to a final concentration of 100 μg/ml, and incubation was continued at 42°C for 20 min; the culture was then shifted to 30°C for 1 h before harvesting. DNA polymerase activity in fraction I was determined as described in the accompanying report (chapter II; Lancy et al., 1989a).

C. RESULTS

1. DNA sequences of the wild type and spg2 dnaE genes. The S. typhimurium dnaE (wild type) open reading frame (ORF) and some of its flanking sequence are shown in Fig. 8. The 3,480-base ORF encodes an 1,160-residue protein of calculated molecular weight 130,133. That this ORF is in fact dnaE was supported by several pieces of evidence, including (i) the striking similarity at the amino acid level between this sequence and the E. coli α peptide sequence deduced from the E. coli dnaE gene sequence (Tomasiewicz and McHenry, 1987); (ii) the ability of this sequence, when present on a λ phage or a plasmid, to complement authentic dnaE(Ts) mutations of E. coli or S. typhimurium (Maurer et al., 1984b; unpublished data); (iii) synthesis of a protein of the predicted size when a fragment containing this region in the appropriate orientation was transcribed from a phage T7 promoter (Fig. 9, lanes 1, 3, and 4); (iv) overproduction of DNA polymerase activity dependent on T7 RNA polymerase in vivo from such constructs (Fig. 9); and (v) loss of genetic complementation of mutants, overproduction of polymerase activity, and synthesis of the presumptive α protein, when the reading frame was truncated (Fig. 9, lane 2).
Figure 8. *DnaE* (wild-type) sequence. The strand with the same sense as mRNA is shown. Sequence gaps needed to maintain the alignment of the *E. coli* and *S. typhimurium* sequences are indicated by hyphens.

In *spq-2* DNA, codon 832 (nucleotides 2494-2496, boxed) is changed to GGC (see Fig. 10).
Figure 9. Expression of gene products under T7 RNA polymerase control from spq-2 and wild-type DNA fragments, and overproduction of DNA polymerase activity. The plasmids used in lanes 1 and 4 were identical except that the entire T7 transcription unit was in the same orientation as the plasmid β-lactamase gene in lane 1 and in the opposite orientation in lane 4. The plasmid for lane 2 differed from that for lane 1 by a deletion of 2.3 kb from the 3' end of the transcription unit. The plasmid for lane 3 differed from that for lane 4 by a deletion of 1.2 kb from the 5' end of the transcription unit.
<table>
<thead>
<tr>
<th>Insert</th>
<th>(aq 0.7) zths</th>
<th>(aq 0.7) zths</th>
<th>(aq 0.7) zths</th>
<th>(aq 0.7) zths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>truncated α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25 kd</td>
<td>22 kd</td>
<td>18 kd</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43 kd</td>
<td>68 kd</td>
<td>97 kd</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 kd</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

β-lactamase

Polymerase Sp. Act. (U/mg) | 498 | 95 | 589 | nd | (no insert=106)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
The same region was sequenced by using \textit{spq-2} DNA as the template. The only change observed in the entire \textit{dnaE} reading frame was found at codon 832. In the \textit{spq-2} gene, the sequence encoded glycine in place of the wild-type valine (Fig. 10). There were no differences found in any flanking sequences.

2. \textbf{Marker rescue of spq-2 and localization of dnaE(Ts) mutations.} Without sequencing much more flanking DNA on the 5' side of \textit{dnaE}, we could not be sure that the valine 832-to-glycine change found in \textit{spq-2} \textit{dnaE} was the only difference between the \textit{spq-2} and wild type clones. Instead, we carried out a marker rescue experiment to show that the identified change was sufficient to produce the suppressor phenotype. We prepared four plasmids, which we shall refer to as 5' wild type, 5' \textit{spq}, 3' wild type, and 3' \textit{spq}. Each plasmid contained approximately half of \textit{dnaE} (the dividing point being at a PstI site corresponding to codons 600 to 601), as well as flanking sequences on the same side, amounting to 506 bases on the 3' side and about 3,000 bases on the 5' side. These plasmids were introduced into two different \textit{dnaE(Ts)} mutants of \textit{S. typhimurium}, where, as expected, they did not complement the temperature sensitivity. Plasmid-containing cells, propagated at 32°C, were plated at 42°C to isolate temperature-resistant revertants, the number of which would be expected to be increased if the plasmid, by recombination with the host chromosome, could replace (rescue) the mutation causing the temperature sensitivity. Rescue occurring with the 3' \textit{spq} plasmid might result in placement of the valine 832-to-glycine mutation into the chromosome, where, in the context of a
Figure 10. Portion of a DNA sequence gel showing the difference between wild-type and spq-2 (arrow).
complete dnaE gene, its ability to confer the suppressor phenotype on the cells could be assessed as described in the accompanying report (chapter II; Lancy et al., 1989a).

dnaE698 was efficiently rescued by the 3' wild type and by the 3' spq plasmids, but not by the 5' plasmids (Table 7). Moreover, all of 16 temperature-resistant revertants recovered from the experiment with the 3' spq plasmid and none of 16 from the experiment with the 3' wild type exhibited the suppressor phenotype. It is important to note that complete sequence of the 3' plasmids was known, including the flanking regions for both wild type and spq-2; since these plasmids differed only by the valine-to-glycine change, the recovery of suppressor cells must be attributed to this difference. This experiment incidentally localized dnaE698 to the 3' portion of dnaE, and most probably to a region close to the spq-2 mutation, since the sites of dnaE698 and spq-2 were not separated in any of 16 recombinants. Another dnaE allele, dnaE693, was not efficiently rescued by either 3' or 5' plasmids. This result may be explained if the mutation in dnaE693 was located close to the PstI site in dnaE, as this positioning would tend to diminish the frequency of recombination with the plasmid that ought to give marker rescue in principle.

D. DISCUSSION

The main result presented here is the determination of the S. typhimurium dnaE sequence for both the wild-type gene and a mutant allele that promotes growth of DnaQ- strains. We argue elsewhere
Table 7. Marker rescue

<table>
<thead>
<tr>
<th>DnaB(Ts) Allele</th>
<th>Colonies at 42° per 0.1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Plasmid</td>
</tr>
<tr>
<td>DnaE693</td>
<td>7</td>
</tr>
<tr>
<td>DnaE698</td>
<td>47</td>
</tr>
</tbody>
</table>
(chapter II; Lancy et al., 1989a) that the spq-2 allele of dnaE encodes an active α subunit of DNA polymerase III. This argument is reinforced by the data presented here showing overproduction of DNA polymerase activity in vivo from an spq-2 expression plasmid (Fig. 9). We infer, therefore, that the change of valine 832 to glycine alters some property or properties of α in a way that mimics the effect of the normal α-ε interaction. Although valine 832 could be a residue that normally interacts with ε, our data do not require this to be so. Studies of the wild type and spq-2 α proteins, now in progress, have so far failed to identify any property of α that is altered in the mutant.

The sequence change in spq-2 dnaE and its location within dnaE offer little help in understanding the mode of action of the spq-2 suppressor. In general, dnaE shows little similarity to other proteins of related function (Tomasiewicz and McHenry, 1987), and codon 832 does not lie within any regions that have previously been suggested as significantly similar to other polymerases. A data base search for protein sequences similar to the 201 amino-acid dnaE subsequence surrounding codon 832 failed to reveal any instructive similarities. Moreover, codon 832 is not the only site that can give rise to suppressor mutations. The vicinity of codon 832 has been sequenced in seven additional independently obtained spq mutants. Two of the mutants were identical to spq-2, whereas the wild-type sequence was found at codon 832 in the other five mutants (data not shown). The actual mutational change has not been identified for any of the other five spq mutants.
spq-2 not an intragenic suppressor of dnaE693(Ts). For any
dnaE(Ts) mutation that cannot be rescued by the 3' wild type test
plasmid, the result of attempted marker rescue by the 3' spq-2
plasmid, pPF377, reveals whether spq-2 can suppress the temperature
sensitive mutation intragenically. For example, from the dnaE693(Ts)
strain carrying pPF377, there should have developed a number of cells
carrying the doubly mutated dnaE693(Ts) spq-2 gene in the chromosome.
However, the number of temperature-resistant revertants of the
dnaE693 strain carrying pPF377 was not elevated in comparison with
its control. We infer that the doubly mutated gene (dnaE693(Ts) spq-
2) encodes a temperature-sensitive product and that spq-2 is not an
intragenic suppressor of dnaE693. That spq-2 fails to stabilize this
explicitly temperature-sensitive α protein does not preclude the
possibility that it could help to stabilize wild-type α. This is one
of several hypotheses for the mode of action of spq-2 discussed
elsewhere (Lancy et al., 1989a) and is a particularly attractive one
because it would form a coherent picture with data showing that α
(whose function we suppose spq-2 mimics) stabilizes wild-type α (Naki
and Kornberg, 1985; Takano et al., 1986).

General comparison of dnaE between E. coli and S. typhimurium.

Like other DNA replication genes whose sequences are known from both
E. coli and S. typhimurium (dnaA, dnaB, dnaG, and fragments of dnaN
and dnaQ [Erickson et al., 1985; Lancy et al., 1989a; Skovgaard and
Hansen, 1987; Wong et al., 1988]), the dnaE genes and even more so
their encoded protein sequences are extensively similar in the two
species. The predicted proteins are identical in length, nearly
identical in charge, and identical in 97% of amino acid residues. Among the 36 amino acid differences, many are conservative substitutions, including glutamate for aspartate, lysine for arginine, isoleucine for valine or leucine, and threonine for serine. The distribution of substitutions is not random, since changes are concentrated towards the amino-terminal portion of the protein and are correspondingly scarce in the middle of the protein. For example, there are 23 substitutions within the first 350 residues but only 3 substitutions within the next 350 residues. On average, given the total of 36 differences between the two proteins, about 11 changes would be expected over an interval of 350 residues. The uneven distribution of substitutions may indicate the presence of important functional domains in the most strongly conserved regions, but the small total number of substitutions and their predominantly conservative character make it difficult to put much confidence in this interpretation.

Although the two DnaE proteins are identical in length, they are not perfectly aligned. To maintain the alignment shown in Fig. 8, it was necessary to place a single codon gap in the E. coli sequence following codon 159 and to match this with a single codon gap in the S. typhimurium sequence following codon 180. Given this adjustment, the intervening sequences encode identical amino acids in 18 of 20 positions. Apparently, α can tolerate such variation in the precise disposition of these amino acid residues, whose role in polymerase activity is unknown.

In E. coli, dnaE is embedded in a proposed operon containing at
least seven genes including lpxA and lpxB (Crowell et al., 1986; Crowell et al., 1987; Tomaszewicz and McHenry, 1987; Coleman and Raetz, 1988). The order of the genes and the size of their protein products (subscripts, expressed in kilodaltons) are 5'-ORF7-ORF17-lpxA28-lpxB42-ORF23-dnaE130-ORF37. The 7.0-kb fragment of Salmonella DNA used in our studies gave rise to four labeled translation products (including the presumptive dnaE product) when it was transcribed in vivo by T7 RNA polymerase in one direction (Fig. 9, lane 4) and no products when transcribed in the other direction (data not shown). By similar analysis of smaller fragments related to the 7.0-kb fragment by deletion at one end or the other (Fig. 9, lanes 2 and 3), it was possible to arrive at an unambiguous order for the ORFs in S. typhimurium: 5'-ORF25-ORF22.5-dnaE130-ORF18.5. We know from the sequence determination that the 18.5-kilodalton translation product resulted from truncation of a longer ORF at the SmaI site.

We did not detect any labeled product similar in size to the E. coli lpxB protein, but even in the experiments of Crowell et al. (1986), this protein was poorly labeled and ran as a diffuse band. The 7.0-kb fragment is sufficiently large to encode a protein of 42-kilodaltons in addition to the proteins we detected. If such an ORF were to be located between ORF25 and ORF22.5, then the organization of genes and the sizes of gene products would be similar in E. coli and S. typhimurium. This is our working model for this portion of the chromosome.

Another feature of dnaE in E. coli is an overlap of five bases between the end of the upstream reading frame (ORF23) and the dnaE
reading frame. In *S. typhimurium*, this overlap is disrupted by two deletions. These deletions are highlighted in Fig. 11, which shows the sequence through this region and the translation products for *E. coli* and *S. typhimurium*. The more important deletion is that of a single nucleotide 40 bases upstream of the *dnaE* initiation codon. This deletion brings a termination codon into frame after five additional amino acid residues. The other deletion, of 13 nucleotides, does not affect the reading frame since it is in the newly created intergenic region. The net result is that *dnaE* is separated from the upstream ORF by 26 nucleotides in *S. typhimurium*. This difference may be significant for the expression of *dnaE*, since it has been hypothesized that the overlapping reading frames of the genes of this operon in *E. coli* lead to translational coupling of their expression (Crowell et al., 1987; Tomaszewicz and McHenry, 1987; Coleman and Raetz, 1988). In *S. typhimurium*, such coupling could not occur. Instead, the level of translational activity for *dnaE* message would depend on the exposure and affinity of a ribosome-binding site and might be greater or diminished compared with that of the translationally coupled *E. coli* gene.

E. APPENDIX

1. **Sequence analysis of the region encompassing codon 832, the site of the *spq-2* mutation, in other *spq* mutants.** Having identified the mutation responsible for suppression in the *spq-2 dnaQ* null mutant, we set out to examine the region encompassing the site of the *spq-2* mutation in other *spq* mutants. This was done to determine if
Figure 11. Nucleotide sequence and translation termination of the open reading frame upstream of dnaE in E. coli and S. typhimurium. The correct reading frame for the S. typhimurium upstream sequence was established by additional sequence data (not shown), which encoded a translation product highly homologous to ORF23 of E. coli. The portion of the sequence shown in the figure (as well as the next 115 nucleotides on the 5' side) was determined on both strands, and the two deletions were visualized clearly on both strands.
<table>
<thead>
<tr>
<th></th>
<th>AlaLeu GlyLeuValSerTER</th>
<th>(dnaE) MetSerGlu</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.t.</td>
<td>CTCT-GGGACCTTGTGTCCTGATTCTTTGATCGAGA-------------AATCTGAAGATGTCTGAA</td>
<td></td>
</tr>
<tr>
<td>E.c.</td>
<td>CACTTGGGACTTGCGTCCTGATTCTTTGTCGAGATTAAGTAAACCAGAATCTGAAGATGTCTGAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AlaLeuGlyThrCysValLeuIleLeuValSerArgLeuSerLysProGluSerGluAspValTER</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(dnaE) MetSerGlu</td>
<td></td>
</tr>
</tbody>
</table>
other *spq* mutations are identical to *spq-2*, and if not identical, if any of the other mutations reside in the same region of the *dnaE* gene. In this way, we began to address the question of how many sites in the *dnaE* gene can give rise to suppression. To analyze the other *spq* mutants, a 710 base pair fragment, containing the site of the *spq-2* mutation, was amplified for each mutant using the polymerase chain reaction (PCR). The resulting products were then subjected to DNA sequence analysis to determine the nucleotide sequence of a 202 base pair region surrounding the site of the *spq-2* mutation.

PCR amplification involves the use of two oligonucleotide primers which bracket the target DNA. The primers hybridize to opposite strands of the DNA and are oriented in such a manner that DNA synthesis directed by each primer, occurs in the region between the primers. The amplification process involves repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with Taq polymerase, a thermostable DNA polymerase. The primers are used in equimolar amounts to ensure synthesis of double-stranded products. Since the resulting products can also bind the primers, each cycle results in an exponential accumulation of the target DNA. Consequently, it is important that the primers and deoxynucleotides are present in excess to facilitate complete synthesis of the target DNA. In this way, PCR amplification can result in a selective enrichment of the target fragment by a factor of 10⁶ (Saiki et al., 1988).
To make PCR more amenable to direct DNA sequencing, a modification of the above method has been devised. This modification, termed asymmetric PCR, biases the reaction to the production of single-stranded DNA which can then be used directly for DNA sequencing (Gyllensten and Erlich, 1988; Shyamala and Ames, 1989). To enhance the production of single-stranded DNA, one of the primers is present in a 50-100 fold molar excess over the other, limiting, primer. The amplification is performed as with symmetric PCR with repeated cycles of denaturation, annealing, and polymerization. The accumulation of the double-stranded DNA product, resulting from the utilization of both primers, occurs exponentially as with symmetric PCR. However, when the limiting primer is exhausted, the synthesis of single-stranded DNA begins as directed by the excess primer. The accumulation of single-stranded DNA is linear since the number of available template molecules remains constant. The end result of asymmetric PCR is the presence of an excess amount of single-stranded DNA over that of double-stranded DNA. The samples are purified to remove the remaining primer molecules and deoxynucleotides and the single-stranded DNA is then used for sequencing.

The strategy for analyzing other spq mutants in the vicinity surrounding the spq-2 mutation site is shown in Figure 12. Two primers, 2803(+) (nucleotides 2199 to 2215 as numbered in Fig. 8) and 3496(-) (nucleotides 2876 to 2892), were used to amplify a 710 base pair fragment encompassing the spq-2 site. PCR amplification was performed using Taq DNA polymerase and a GeneAmp kit (Cetus Corp.).
Figure 12. Strategy utilized in analyzing the other *spq* mutations in the vicinity of the site of the *spq-2* mutation.
2803(+) PCR primer

2992(+) sequencing primer

3130

3210(–) sequencing primer

3495(–) PCR primer
The amplifications were done asymmetrically using a modified procedure of Shyamala and Ames (1988). Both combinations of excess and limiting primers were used in order to determine the sequence on both strands of each mutant, with one exception (see Table 8). DNA that was amplified was either genomic DNA, for those spq mutations which had not been cloned, or λ DNA, for the spq-13, spq-14, and spq-16 mutations which had been previously isolated from genomic libraries. For the genomic samples, 1 μg of DNA was used whereas 10 ng of λ DNA was used. 60 pmol of the excess primer (60 pmol) was used with either 0.6 pmol or 1.2 pmol of the limiting primer. The amplification consisted of 40 cycles which were composed of 1 minute at 94°C, 2 minutes at 55°C and 3 minutes at 72°C. After the last cycle, there was an additional 7 minute incubation at 72°C to allow for the final extension of incomplete substrates. The amplified single-stranded DNA was purified by passage through a Sephacryl S-300 column to remove excess primers and deoxynucleotides. The DNA was concentrated by ethanol precipitation and was sequenced using modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp.) according to the manufacturer’s specifications. The primers used for sequencing, 2992(+) (nucleotides 2388 to 2404 as numbered in Fig. 8) and 3210(-) (nucleotides 2590 to 2606), are located internally on the amplified 710 base pair fragment (see Figure 12). The sequences obtained from the use of these two primers overlap by 202 base pairs and include the site of the spq-2 mutation.

The results of the analysis of the other spq mutants in the vicinity of the spq-2 mutation are summarized in Table 8. spq-2,
Table 8. Analysis of other *spq* mutants

<table>
<thead>
<tr>
<th><em>spq</em></th>
<th>Genomic/λ DNA amplified</th>
<th>Codon 832</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RM723</td>
<td>GGC (spq-2)</td>
</tr>
<tr>
<td>2</td>
<td>λ740</td>
<td>GGC (spq-2)</td>
</tr>
<tr>
<td>3</td>
<td>RM824</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>4</td>
<td>RM827</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>5</td>
<td>RM830</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>6</td>
<td>RM833</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>7</td>
<td>RM836</td>
<td>GGC (spq-2)</td>
</tr>
<tr>
<td>8</td>
<td>RM839</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>9a</td>
<td>RM842</td>
<td>GGC (spq-2)</td>
</tr>
<tr>
<td>10</td>
<td>RM845</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>11</td>
<td>RM773</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>13</td>
<td>λ832</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>14</td>
<td>λ836</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>15</td>
<td>RM814</td>
<td>GTC (wt)</td>
</tr>
<tr>
<td>16</td>
<td>λ857</td>
<td>GTC (wt)</td>
</tr>
</tbody>
</table>

*Only one strand was sequenced.*
present on a λ clone, was also subjected to PCR and sequence analysis and was shown to contain the same mutation identified using the plasmid pFF221 (Figure 8). Of the other fourteen *spq* mutants analyzed, three of the *spq* mutations, *spq*-1, *spq*-7, and *spq*-9, are identical to *spq*-2. The rest of the *spq* mutants analyzed did not contain any differences from the wild type sequence and thus must be located outside the 202 base pair region analyzed.

The fact that four of the fifteen *spq* mutations analyzed contain the valine to glycine change at codon 832 suggests that the number of sites in the *dnaE* gene that can give rise to suppression is limited. However, without the identification of the others, we do not know how many other sites can give rise to suppression. The fact that only glycine is found at position 832, instead of valine, suggests that this may be the only substitution at codon 832 that, in addition to retaining activity, results in suppression. Experiments that would address this include site directed mutagenesis at position 832 to introduce other amino acid substitutions to determine if other functional substitutions can also suppress the *dnaQ* deletion. A similar analysis could be performed with the other *spq* mutation sites, once they are identified.

A logical approach to identify the other *spq* mutations would be to target the location of each mutation to a small region of the gene and sequence that region to determine the mutational change. This would eliminate the laborious task of sequencing the entire 3480 base pair mutant genes. To localize the other *spq* mutations to a particular region of the *dnaE* gene, a number of methods could be
utilized. All of these methods rely on the formation of heteroduplex molecules consisting of the wild type gene and its mutant allele which contains a single base mutation. The heteroduplexes can be either DNA:DNA hybrids (Novack et al., 1986; Cotton et al., 1988), DNA:RNA hybrids (Myers et al., 1985), or RNA:RNA hybrids (Winter et al., 1985). Once formed, the heteroduplexes are treated with either chemical reagents or enzymes which effect cleavage at the site of the single base pair mismatch. The cleavage products are then analyzed on a gel to determine their size which is used to localize the mutation on the fragment. The mutational change can then be identified by sequencing that region of the gene.

Alternatively, if the above methods fail to localize the other spq mutations, the approach then would be to choose an unidentified spq mutation and sequence the entire mutant gene to identify the mutation. PCR and DNA sequencing would then be used to examine the site of this mutant in the other as yet unidentified spq mutants as was done above for the spq-2 mutation site.
CHAPTER IV

THE ROLE OF DNA POLYMERASE I IN Salmonella typhimurium STRAINS

LACKING THE EPSILON (EDITING) SUBUNIT OF DNA POLYMERASE III

A. INTRODUCTION

In Escherichia coli, DNA polymerase I has been shown to function both in excision repair and in the processing of Okazaki fragments during chromosomal replication (Kornberg, 1980). DNA polymerase I is a single polypeptide which possesses three enzymatic activities: polymerase, 3'→5' exonuclease, and 5'→3' exonuclease. The enzyme can be separated by proteolysis into two domains - a large C-terminal fragment which contains the polymerase and 3'→5' exonuclease activities (Klenow fragment) and a smaller N-terminal fragment having the 5'→3' exonuclease activity (Brutlag et al., 1969; Klenow and Henningsen, 1970). DNA polymerase I is required for growth on rich medium but not on minimal medium (Joyce and Grindley, 1984). The growth defect on rich medium was eliminated when either Klenow fragment or the 5'→3' exonuclease was present on a plasmid in the polA deletion strain.

DNA polymerase I in Salmonella typhimurium is analogous to the E. coli enzyme and mutants of the S. typhimurium polA gene have been isolated and characterized (Whitfield and Levine, 1973; Engler and Bessman, 1979). However, the growth requirement for DNA polymerase I in S. typhimurium has not been addressed and the only mutant that has been characterized in any detail, polA2, has reduced, but detectable
polymerase and 3'-5' exonuclease activities (Whitfield and Levine, 1973; Engler and Bessman, 1979). The effect of the polA2 mutations on the 5'-3' exonuclease is unknown.

In chapter II, we have demonstrated an increased requirement for DNA polymerase I in Salmonella typhimurium dnaQ null strains. In strains containing the polA2 mutation, neither wild type (dnaE+) nor suppressor-containing (e.g. spq2) strains were able to be transduced to tetracycline resistance with high efficiency using a dnaQ::Tn10 lysate. The conclusion from these studies is that DNA polymerase I is required for growth of dnaQ null mutants, including both defective growth exhibited by dnaE+ strains and proficient growth exhibited by spq mutants. Although we have demonstrated that DNA polymerase I is required for viability of these cells, we do not know which of its activities (or combination of activities) is essential. In the experiments presented in this chapter, the role of the three activities of DNA polymerase I in the dnaQ null mutants was investigated.

B. EXPERIMENTAL PROCEDURES

1. Bacterial strains and media. Bacterial strains used in these experiments, in addition to strains already listed in Table 1, are listed in Table 9. General bacteriological media and procedures were as described (Maurer et al., 1984a). Generalized transductions were carried out using phage P22 int HT12/4 (Schmeiger, 1972). Bacterial matings were performed as outlined in Miller (1972). Drugs at the following concentrations were added to plates as needed:
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2207</td>
<td>LE215/gC100 (F' piliD')</td>
<td>Joyce and Grindley</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1984)</td>
</tr>
<tr>
<td>C2102</td>
<td>LE215/gC102 (F' B6 400')</td>
<td>Joyce and Grindley</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1984)</td>
</tr>
<tr>
<td>C332</td>
<td>LE215/gC103 (F' Elamow 400')</td>
<td>Joyce and Grindley</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1984)</td>
</tr>
<tr>
<td>C351</td>
<td>LE215/gC103 (F')</td>
<td>C. Joyce</td>
</tr>
<tr>
<td>C356</td>
<td>C35129 pspID 400/gC102</td>
<td>C. Joyce</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. typhimurium Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB526</td>
<td>LE215/42A(00') M1356A(00')</td>
<td>Bulles and Eps</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1983)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB4456, MB447, MB448, MB449 and MB450</td>
<td>LE215 containing pC100,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB446 x C2207,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C2101, C2103, C2105 and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C2105, respectively</td>
</tr>
<tr>
<td>MB4470</td>
<td>[MB4456] pspID 400 (00')</td>
<td>MB4455 +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P22(MB4475)</td>
</tr>
<tr>
<td>MB4457</td>
<td>[MB447] pspID 400 (00')</td>
<td>MB4475 +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P22(MB4474)</td>
</tr>
<tr>
<td>MB4470</td>
<td>[MB447] pspID 400 (00')</td>
<td>MB4475 +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P22(MB4474)</td>
</tr>
<tr>
<td>MB4479</td>
<td>[MB447] pspID 400 (00')</td>
<td>MB4475 +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P22(MB4474)</td>
</tr>
<tr>
<td>MB551</td>
<td>T° MB2476</td>
<td>Selection on</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fumaric acid</td>
</tr>
<tr>
<td>MB552</td>
<td>MB551 eng/1256::thai404</td>
<td>MB550 + P22(MB550)</td>
</tr>
<tr>
<td>MB553</td>
<td>T° MB2477</td>
<td>Selection on</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fumaric acid</td>
</tr>
<tr>
<td>MB554</td>
<td>T° MB2478</td>
<td>Selection on</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fumaric acid</td>
</tr>
<tr>
<td>MB555</td>
<td>T° MB2479</td>
<td>Selection on</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fumaric acid</td>
</tr>
<tr>
<td>MB556, MB557, MB558, MB559, MB560, MB561, MB562, MB563 and MB564</td>
<td>MB555 containing pC100,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB555 x MB544,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB557, MB558, MB560,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB559 and MB564</td>
</tr>
<tr>
<td>MB569, MB569, MB561, MB562, MB563, MB564, MB565 and MB566</td>
<td>MB565 containing pC100,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB565 x MB564,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB567, MB568, MB569,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB570 and MB571</td>
</tr>
<tr>
<td>MB569, MB569, MB561, MB562, MB563, MB564, MB565 and MB566</td>
<td>MB565 containing pC100,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB565 x MB564,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB567, MB568, MB569,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB570 and MB571</td>
</tr>
<tr>
<td>MB569, MB569, MB561, MB562, MB563, MB564, MB565 and MB566</td>
<td>MB565 containing pC100,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB565 x MB564,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB567, MB568, MB569,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MB570 and MB571</td>
</tr>
</tbody>
</table>

* A strain number in [brackets] indicates that all the members of the bracketed strain are included in the genotype. For the genotype of these strains, see Table 1.

+ Transconjugations are denoted by "recipient + F decac.". Selection was always for drug marker T° for placing the marker near pspID and T° for the mother near F°. Bacterial conjugations to introduce the T° plasmids are denoted by "recipient + F decac.". Selected strains containing the F° plasmids were selected for by T° in conjunction with either T° (MB562 recipient) or T° (MB563 recipient).

+ eng/1256 is an insertion in a nonessential site near fpr.
tetracycline HCl, 25 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 20 µg/ml; streptomycin, 25 µg/ml; fusaric acid, 12 µg/ml. Tetracycline plates contained, in addition, 10 mM EGTA to prevent reinfection of transductants by F22.

In the initial experiments in which DNA polymerase I was shown to be required for the viability of the dnaQ null mutants (chapter II, Lancy et al., 1989a), the polA2 mutants contained a chloramphenicol resistance (CmR) marker which is linked to the polA gene. These strains were not suitable for the present experiments because the E. coli polA alleles to be tested are also linked to a CmR marker (see below) which is needed for their introduction into the various strains to be tested. To alleviate this problem, the CmR marker near the polA2 allele was replaced with a nearby tetracycline resistant (TcR) marker (47 linked). CmR polA2 strains RM1835, RM1836, RM1837 and RM1838 (Table 1) were transduced to TcR using F22(RM1674). The TcR transductants were tested for their Cm phenotype to obtain strains RM2476, RM2477, RM2478, and RM2479 which are chloramphenicol sensitive (CmS).

Since the ultimate test of the polA2 strains containing the various E. coli polA alleles involved the transduction of the strains to dnaQ::Tn10 (selecting for TcR), the tetracycline marker near the polA2 allele had to be removed. To do this, the TcR strains RM2476, RM2477, RM2478, and RM2479, were plated onto fusaric acid plates (Bochner et al., 1980) and the respective TcS strains, RM2851, RM2853, RM2854, and RM2855, were isolated. Fusaric acid inhibits the growth of TcR cells and has no effect on TcS cells. Thus,
spontaneous Tc$^S$ mutants of Tc$^R$ strains can be readily obtained by simply plating a Tc$^R$ strain on a fusaric acid plate. To ensure the stability of the mutations to Tc$^S$ in RM2851, RM2853, RM2854, and RM2855, these strains were grown non-selectively in liquid culture (LB plus thymine) to saturation and then plated on tetracycline plates to measure the reversion frequency to Tc$^R$. None of the four strains (RM2851, RM2853, RM2854, and RM2855) yielded any Tc$^R$ revertants when $>10^8$ cells were plated. It is important to note that strains RM2851, RM2853, RM2854, and RM2855 most likely do not contain the same Tc$^S$ mutation and therefore are no longer isogenic in that region of the chromosome.

2. *Escherichia coli* DNA polymerase I F' plasmids. F' plasmids containing various alleles of *E. coli* DNA polymerase I were obtained from Dr. Catherine Joyce. These plasmids are listed in Table 10. pCJ100 contains the entire polA gene and thus encodes all three activities of DNA polymerase I: polymerase, 3'-5' exonuclease, and 5'-3' exonuclease. pCJ103 contains the 5'-truncated polA gene which encodes the Klenow fragment (polymerase and 3'-5' exonuclease), amino acids 324 to 928 of the 928 amino acid polypeptide. pCJ102, which contains the polA1 amber allele and produces a 341 amino acid fragment, encodes for only the 5'-3' exonuclease. The possibility that a readthrough product would be formed in this construct is eliminated by the presence of a deletion which removes the coding sequence for the carboxyl terminal 413 amino acids of the polypeptide. The entire polA gene resides on pCJ145 and contains two point mutations, D355A and E357A, which eliminate the 3'-5' exonuclease of
Table 10. *E. coli* DNA polymerase I alleles.

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>DESCRIPTION</th>
<th>DNA POLYMERASE I ACTIVITY PRESENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCJ100</td>
<td>F&lt;sup&gt;'&lt;/sup&gt; polA&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>polymerase, 3′→5′ exo, 5′→3′ exo</td>
</tr>
<tr>
<td>pCJ102</td>
<td>F&lt;sup&gt;'&lt;/sup&gt; 5′ Exo Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>5′→3′ exo</td>
</tr>
<tr>
<td>pCJ103</td>
<td>F&lt;sup&gt;'&lt;/sup&gt; Klenow Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>polymerase, 3′→5′ exo</td>
</tr>
<tr>
<td>pCJ105</td>
<td>F&lt;sup&gt;'&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>NONE</td>
</tr>
<tr>
<td>pCJ145</td>
<td>F&lt;sup&gt;'&lt;/sup&gt; polA D355A, E357A Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>polymerase, 5′→3′ exo</td>
</tr>
</tbody>
</table>
DNA polymerase I (Derbyshire et al., 1988). The polA alleles in pCJ100, pCJ102, and pCJ145 are expressed from the natural polA promoter whereas in pCJ103, the Klenow fragment is expressed from the lacUV5 promoter. pCJ105 serves as a control and does not contain any polA gene.

C. RESULTS

We have shown previously (chapter II; Lancy et al., 1989a) that there is an increased requirement for DNA polymerase I in both wild type and spq-containing strains upon deletion of the dnaQ gene, which encodes the epsilon (editing) subunit of DNA polymerase III (Table 4). This was observed as an ~100 fold reduction in transducing efficiency of dnaQ::Tn10 when the recipient strain was polA2 versus polA+. In the experiments presented here, the requirements for the individual activities of DNA polymerase I were examined. This was accomplished by introducing various F' plasmids containing the E. coli polA alleles into S. typhimurium polA2 strains (dnaE+, spq-2, spq-3, and spq-6) and then transducing the strains to TcR using a dnaQ::Tn10 P22 lysate. The results of these transductions are listed in Table 11. All of the polA2 strains were transduced to TcR by P22 with near normal efficiency (50-100%) compared to their polA+ parents when the donor strain contained Tn10 in a nonessential site (data not shown).

1. Requirement for DNA polymerase I in dnaE+ (spq+) strains.

When dnaE+ strains (RM2894, RM2895, RM2896, RM2897, and RM2898) were transduced with the dnaQ::Tn10 lysate, only RM2894, containing
Table 11. Requirement of DNA polymerase I activities in dnaQ null strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>F' Plasmid</th>
<th>Transductants (no.)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM2852</td>
<td>dnaR+ (spq+) polA2</td>
<td>NONE</td>
<td>37b</td>
</tr>
<tr>
<td>RM2894</td>
<td>dnaR+ (spq+) polA2</td>
<td>pCJ100</td>
<td>3040c</td>
</tr>
<tr>
<td>RM2895</td>
<td>dnaR+ (spq+) polA2</td>
<td>pCJ102</td>
<td>22</td>
</tr>
<tr>
<td>RM2896</td>
<td>dnaR+ (spq+) polA2</td>
<td>pCJ103</td>
<td>28</td>
</tr>
<tr>
<td>RM2897</td>
<td>dnaR+ (spq+) polA2</td>
<td>pCJ105</td>
<td>38</td>
</tr>
<tr>
<td>RM2898</td>
<td>dnaR+ (spq+) polA2</td>
<td>pCJ145</td>
<td>38</td>
</tr>
<tr>
<td>RM2853</td>
<td>spq-2 polA2</td>
<td>NONE</td>
<td>32b</td>
</tr>
<tr>
<td>RM2899</td>
<td>spq-2 polA2</td>
<td>pCJ100</td>
<td>3940</td>
</tr>
<tr>
<td>RM2900</td>
<td>spq-2 polA2</td>
<td>pCJ102</td>
<td>28</td>
</tr>
<tr>
<td>RM2901</td>
<td>spq-2 polA2</td>
<td>pCJ103</td>
<td>3860</td>
</tr>
<tr>
<td>RM2902</td>
<td>spq-2 polA2</td>
<td>pCJ105</td>
<td>33</td>
</tr>
<tr>
<td>RM2903</td>
<td>spq-2 polA2</td>
<td>pCJ145</td>
<td>5600</td>
</tr>
<tr>
<td>RM2854</td>
<td>spq-3 polA2</td>
<td>NONE</td>
<td>48b</td>
</tr>
<tr>
<td>RM2904</td>
<td>spq-3 polA2</td>
<td>pCJ100</td>
<td>6460</td>
</tr>
<tr>
<td>RM2905</td>
<td>spq-3 polA2</td>
<td>pCJ102</td>
<td>25</td>
</tr>
<tr>
<td>RM2906</td>
<td>spq-3 polA2</td>
<td>pCJ103</td>
<td>4960</td>
</tr>
<tr>
<td>RM2907</td>
<td>spq-3 polA2</td>
<td>pCJ105</td>
<td>29</td>
</tr>
<tr>
<td>RM2908</td>
<td>spq-3 polA2</td>
<td>pCJ145</td>
<td>5360</td>
</tr>
<tr>
<td>RM2855</td>
<td>spq-6 polA2</td>
<td>NONE</td>
<td>35b</td>
</tr>
<tr>
<td>RM2909</td>
<td>spq-6 polA2</td>
<td>pCJ100</td>
<td>5860</td>
</tr>
<tr>
<td>RM2910</td>
<td>spq-6 polA2</td>
<td>pCJ102</td>
<td>39</td>
</tr>
<tr>
<td>RM2911</td>
<td>spq-6 polA2</td>
<td>pCJ103</td>
<td>3320c</td>
</tr>
<tr>
<td>RM2912</td>
<td>spq-6 polA2</td>
<td>pCJ105</td>
<td>32</td>
</tr>
<tr>
<td>RM2913</td>
<td>spq-6 polA2</td>
<td>pCJ145</td>
<td>4920</td>
</tr>
</tbody>
</table>

---

a Each entry shows the number of TcR transductants obtained from 1 x 107 P.F.U. of P22 lysate propagated on strain RM978 (dnaQ201::Tn10 spq-2). Colonies were counted after 2 days.

b The basis for the formation of these transductants was further investigated (see chapter II appendix).

c These transductants were much smaller than the other transductants which were similar in size to the colonies obtained with the control transductions.
pCJ100, gave an increased frequency of Tc\textsuperscript{R} transductants compared to the parent strain without any plasmid (RM2852). The RM2894 transductants obtained were much smaller than the control transductants of Tn10 in a nonessential site. The appearance of the small colonies was not surprising since RM2894 does not contain a suppressor mutation and thus a slow growth phenotype is expected when dnaQ is deleted. The effect observed for pCJ100 was specific to the polA allele present on the plasmid and was not due to the presence of the F' plasmid itself, as pCJ105 (no polA gene) gave a similar number of transductants as did the parent strain without any plasmid (RM2852).

2. Requirement for DNA polymerase I in spq-2, spq-3, and spq-6 strains. When spq strains containing the various E. coli polA alleles were transduced to Tc\textsuperscript{R} using a dnaQ::Tn10 P22 lysate, a much different result was obtained compared to the transduction of the dnaA\textsuperscript{+} strain (Table 11). In the presence of a preexisting suppressor mutation, plasmids pCJ100, pCJ103, and pCJ145 were all able to rescue the spq polA2 strains as evidenced by an increased frequency of Tc\textsuperscript{R} transductants. Thus, the requirement for DNA polymerase I in dnaA\textsuperscript{+} strains was partially suppressed in spq mutants. The specificity of this suppression provides a potential clue about the nature of the spq alteration (see DISCUSSION).

These experiments also revealed a difference between spq-6 on one hand and spq-2 and spq-3 on the other. Whereas Klenow fragment sufficed to allow formation of normal-looking dnaQ::Tn10 transductants in spq-2 and spq-3, the transductants of spq-6/pCJ103
(Klenow fragment) were small (sick). Thus, \textit{spq-6} exhibits a more stringent requirement for the 5'-3' exonuclease domain or activity than does \textit{spq-2} and \textit{spq-3}.

D. DISCUSSION

Utilizing the \textit{polA2} allele of DNA polymerase I, we have demonstrated that DNA polymerase I is necessary for viability upon deletion of \textit{dnaQ} in both wild type (\textit{dnaE}+) and \textit{spq} mutant \textit{S. typhimurium}. The \textit{polA2} mutation results in a polymerase with reduced polymerase and editing activities whereas the effect of the mutation on the 5'-3' exonuclease is unknown (Whitfield and Levine, 1973; Engler and Bessman, 1979). In the present study, we have examined the role of the individual activities of DNA polymerase I in complementing the lethality of \textit{polA2}, \textit{dnaQ} null mutants.

The interpretation of these experiments is not entirely straightforward because the various \textit{polA} constructs are not isomorphic. The most closely related constructs are pCJ100 (\textit{polA}+) and pCJ145 (deficient in the 3'-5' exonuclease). These differ only by two point mutations affecting the 3'-5' exonuclease active site. Crystallographic studies of Klenow fragment containing the same two substitutions show that its structure is nearly superimposable on that of the wild type Klenow fragment (Derbyshire et al., 1988). Therefore it is reasonable to expect that the complete DNA polymerase I proteins encoded by pCJ100 and pCJ145 will behave identically in every respect except for the 3'-5' exonuclease activity. Plasmid pCJ103 encodes the Klenow fragment, a truncated form of DNA
polymerase I lacking the N-terminal (5'→3' exonuclease) domain.

Transcription of the Klenow fragment gene is controlled by a lac promoter in pCJ103; therefore the level of expression of the Klenow fragment is likely to be different from that of polA+ (pCJ100), the 3'→5' exonuclease mutant of polA (pCJ145), or the isolated 5'→3' exonuclease domain (pCJ102), all of which are controlled by the natural polA promoter. In fact, since S. typhimurium does not contain a lac region, the Klenow fragment gene would be constitutively expressed due to the absence of lac repressor.

Whether the absence of the 5'→3' exonuclease domain of DNA polymerase I affects in any way the ability of Klenow fragment to provide polymerase and 3'→5' exonuclease activities in vivo is uncertain. Finally, pCJ102 encodes the 5'→3' exonuclease domain. As with Klenow, it is not known whether the absence of the other domain affects the ability of this protein fragment to provide its activity in vivo, but in vitro, the 5'→3' exonuclease activity is reduced in the absence of concurrent polymerization (Kelly et al., 1970). There is evidence that the 5'→3' exonuclease domain and Klenow fragment have at least some activity in vivo since each is able to support growth of E. coli in rich medium (Joyce and Grindley, 1984).

In view of these considerations, the strongest inference can be drawn from comparison of the effect of pCJ100 and pCJ145. These comparisons reveal the role of the 3'→5' exonuclease activity. In dnaE+ cells, we found that pCJ100 was sufficient for dnaQ::TnlO viability (giving sick colonies) whereas pCJ145 was not; therefore the 3'→5' exonuclease is required. In contrast, pCJ145 worked as
well as pCJ100 in \( spq^-2 \), \( spq^-3 \), or \( spq^-6 \) cells. Thus, the \( spq \) mutations relieve the requirement for the \( 3'\rightarrow 5' \) exonuclease.

Comparison of the effect of pCJ100 (\( polA^+ \)) and pCJ103 (Klenow fragment) reveals the role of the \( 5'\rightarrow 3' \) exonuclease domain. As with the \( 3'\rightarrow 5' \) exonuclease, the \( 5'\rightarrow 3' \) exonuclease domain appears to be required for viability of \( dnaQ:\text{Tn}10 \) in \( dnaE^+ \) cells but not in \( spq \) mutants. However, because the \( 5'\rightarrow 3' \) domain itself is absent from Klenow fragment, not just the exonuclease activity, it is possible that the requirement in \( dnaE^+ \) cells is not for the exonuclease per se, but for some structural feature embedded in the domain.

Determination of the requirement for the polymerase activity of DNA polymerase I depends on an indirect argument since no version of DNA polymerase I deficient only in polymerase is available. As stated above, \( spq \) mutations (especially \( spq^-2 \) and \( spq^-3 \)) relieve the requirements for the \( 5'\rightarrow 3' \) exonuclease domain and the \( 3'\rightarrow 5' \) exonuclease activity when these requirements are tested separately. A simple extrapolation of these data would be that the \( spq \) mutants retain a requirement for the polymerase activity. However, we cannot rule out the formal possibility that in \( spq \) mutants there is a requirement for an exonuclease activity, which may be satisfied by either the \( 3'\rightarrow 5' \) exonuclease or \( 5'\rightarrow 3' \) exonuclease activity of DNA polymerase I. In biochemical terms this hypothesis makes little sense since the specificity of the two exonuclease activities is opposite, making it hard to imagine any \textit{in vivo} DNA substrate that could be processed alternatively by either exonuclease. Notwithstanding this biochemical argument, however, Joyce and
Grindley (1984) discovered that either the 5'→3' exonuclease domain, or Klenow fragment, was adequate to support growth of *E. coli* in rich medium. This observation may have an explanation other than equivalency of the two exonucleases (see below). If there is a requirement for either exonuclease, there is probably an associated requirement for the polymerase activity, too, since the isolated 5'→3' domain (pCJ102) is not sufficient to support growth. Thus we conclude that in *spq* mutants and presumably in *dnaE*+, there is a requirement for polymerase activity (and possibly for either exonuclease activity).

This interpretation of the DNA polymerase I complementation experiments may be summarized as follows. In *dnaE*+ cells, growth of *dnaQ* deletion strains requires the 3'→5' exonuclease activity, the polymerase activity, and the 5'→3' exonuclease domain or activity. In *spq* mutants, growth of *dnaQ* deletion strains require the polymerase activity, and possibly one or the other exonuclease, but more likely neither of them. Thus, the *spq* mutations alleviate the requirement for one or both exonucleases. An exception to this generalization is *spq*-6, which fully relieves the requirement for the 3'→5' exonuclease but only partially relieves the requirement for the 5'→3' exonuclease activity (or domain) leading to small colonies with pCJ103.

In developing a model for the role of DNA polymerase I in epsilon-deficient replication, it is useful to begin with a description of the role proposed for DNA polymerase I in normal replication. DNA polymerase I is required for the processing of
Okazaki fragments during lagging strand synthesis. Okazaki fragment synthesis, initiated from RNA primers, is carried out by DNA polymerase III. Synthesis of each Okazaki fragment proceeds until DNA polymerase III runs into the next Okazaki fragment and falls off, presumably to be recycled to the next Okazaki fragment. The resulting structure on the lagging strand consists of Okazaki fragments containing RNA at their 5' end, and nicks, possibly sometimes gaps, between adjacent Okazaki fragments. Maturation of these structures is accomplished by DNA polymerase I which replaces the RNA with DNA through the coupling of its polymerase and 5'→3' exonuclease activities (nick translation). This process leaves a nick in the DNA which is then sealed by DNA ligase to complete lagging strand synthesis.

In *E. coli*, DNA polymerase I is entirely dispensable during slow growth (Joyce and Grindley, 1984). This suggests that there are alternative mechanisms, less efficient than DNA polymerase I, for the maturation of Okazaki fragments. It is not known with certainty how the RNA is removed, but an appealing candidate for this role is RNaseH. For this mechanism to work, it might be necessary for RNaseH to act before completion of the next Okazaki fragment. In this way, the replacement DNA could be synthesized readily by DNA polymerase III. Even at rapid growth rates, intact DNA polymerase I is not required: either the 5'→3' exonuclease alone, or Klenow fragment, suffices for viability. The 5'→3' exonuclease could work in analogous fashion to the proposed role of RNaseH. Klenow fragment could function by a strand displacement mechanism, with an
unspecified nuclease degrading the displaced segment. According to this hypothesis, the functional equivalence of 5'→3' exonuclease and Klenow fragment is based on the capabilities of the polymerase domain of Klenow, not the exonuclease domain.

The requirement for DNA polymerase I in epsilon-deficient cells differs in several characteristics from the requirement observed in normal cells. First, the 3'→5' exonuclease of DNA polymerase I is not required in normal cells but is definitely required for "sick" growth of epsilon-deficient cells (dnaE+). Secondly, the Klenow enzyme is sufficient to satisfy the requirement of normal cells but not the requirement of epsilon-deficient cells (dnaE+). Lastly, the 5'→3' exonuclease domain is sufficient to satisfy the requirement of normal cells but not the requirement of epsilon-deficient cells, whether dnaE+ or spq.

These differences suggest that in epsilon-deficient cells, additional demands are placed upon DNA polymerase I. Most of the observed requirements of DNA polymerase I in epsilon-deficient cells can be accounted for by postulating the occurrence of many additional gaps in the replicated DNA resulting from premature termination by DNA polymerase III either at random or following a misinsertion. The former event would occur whenever a DNA polymerase III of diminished processivity dissociated from the template and would be expected in both dnaE+ and spq strains. Once this occurred, DNA polymerase I would then be able to complete the fragment. These events would account for the requirement for polymerase activity. The second type of event would occur when DNA polymerase III dissociated from the
template after making a misinsertion. On the evidence of Sloane et al. (1988) we can estimate that misinsertions would occur on the order of hundreds of times per round of replication. DNA polymerase I would come in, excise the mismatch using the 3'-5' exonuclease, and then complete filling the gap. This type of event would account for the 3'-5' exonuclease requirement and by hypothesis either would not occur in spq mutants, or would be processed differently (see below).

In proposing the additional demands placed upon DNA polymerase I in epsilon-deficient cells, we have not specified the strand on which the additional gaps appear since presumably, they would appear on both strands. Although DNA polymerase I is normally active on only the lagging strand, this restriction is almost certainly the result of the continuity of synthesis on the leading strand. There is no theoretical barrier to DNA polymerase I, in the epsilon-deficient cells, participating in the rectification of incomplete substrates on both strands.

This model, however, does not readily account for the requirement for the 5'-3' exonuclease seen in dnaE+, but not in spq, strains. One trivial possibility that cannot be excluded by available data is that the requirement is not for the activity per se, but for its domain, in order to obtain maximal activity of the other two functions. The extra gaps needing filling in the dnaE+ case (i.e., gaps provoked by misinsertion) would demand maximum DNA polymerase I activity. The difficulty with any hypothesis requiring the 5'-3' exonuclease activity is that it is hard to imagine any substrate susceptible to 5'-3' exonuclease yet refractory to strand
displacement by Klenow.

Recently, Studwell and O'Donnell (1990) reported the first successful reconstitution of a holoenzyme-like activity lacking epsilon. The reduced processivity of this reconstituted enzyme (averaging a few kilobases) lends support to our hypothesis that DNA replication in the absence of epsilon leaves behind many gaps, explaining the stringent requirement for DNA polymerase I. We would also predict that a fraction of the terminations seen by Studwell and O'Donnell are characterized by 3' mismatches, but whether this was the case was, unfortunately, not determined.

A striking and unexpected result of these experiments was the clear demonstration that the details of the DNA polymerase I requirement were different in dnaE+ and spq mutant strains. Specifically, spq-2, spq-3, and spq-6, the only alleles tested, completely abolished the requirement for the 3'→5' exonuclease activity of DNA polymerase I. This observation strongly implicates 3' terminal mismatches as critical substrates governing the success of DNA replication. We could find no evidence that spq-2 provides an alternative editing capacity (chapter II, Lancy et al., 1989a). The elevated mutation frequency in dnaQ-, spq strains makes it unlikely that spq allows DNA polymerase III to avoid making mis insertions. One is left with the hypothesis that spq diminishes the tendency of DNA polymerase III to convert mismatches into structures needing maturation by DNA polymerase I. One simple way (simple in principle) this could be done would be if spq mutations enhanced the capacity of DNA polymerase III to extend a mismatched primer terminus.
At least two types of alterations to the polymerase endowing it with a mismatch-extension capacity can be imagined. The first would be a change leading to higher affinity of the polymerase for DNA. This would lengthen the retention time of the polymerase at the primer terminus and give the polymerase a chance to extend the mismatch (a reaction of which it may be intrinsically capable; Sloane et al., 1988). This kind of alteration would also be expected to increase the overall processivity of the enzyme. The second type of alteration would somehow promote localized template-primer realignment to give a base-paired terminus that can easily be extended. Realignment following misinsertion has been proposed for frameshift mutagenesis (Kunkel and Soni, 1988). According to this idea, an spq mutant would be a frameshift-specific mutator. Our analysis of mutation rates in spq strains would not have detected frameshifs because the mutations we were detecting occur exclusively by base substitutions.

The results of this chapter also compel reconsideration of the issue of the rate of spontaneous mutagenesis in unsuppressed dnaQ null mutants. We have previously supposed that this rate, which could not be measured directly because of genetic instability of the strains, was the same as the rates in suppressed (spq) strains. This supposition was based on the failure to elicit any evidence that the spq mutations affect fidelity (chapter II, Lancy et al., 1989a). But the evidence of this chapter shows that DNA polymerase I editing is essential to the feeble growth of unsuppressed strains. Thus, at least some polymerase errors are being corrected by editing.
We envision that after each misinsertion, further replication can take place according to one of two schemes (in the absence of epsilon). If DNA polymerase III dissociates from the template, then the mispaired primer end becomes accessible to DNA polymerase I, which edits the error before resuming synthesis. Alternatively, if DNA polymerase III succeeds in extending the mispair, then the error is retained and DNA polymerase I does not enter the picture. Clearly, one would like to know the relative propensity of the DNA polymerase III to dissociate or extend from a mispair, but for the moment this information is not available. If dissociation is favored, then most misinsertions would end up being edited, and the mutation rate will be close to that of wild type. If extension is favored, then most misinsertions will end up being extended, and the mutation rate will be elevated. To the extent that editing by DNA polymerase I is reducing the mutation rate in unsuppressed strains, it is even more surprising that each "sick" colony gives rise to one or more suppressor clones as described in chapter II, since we know that only a few sites can mutate to the suppressor genotype and therefore suppressor mutations should be rare unless the cells are suffering a high rate of mutagenesis.
CHAPTER V

SUMMARY

The epsilon subunit of DNA polymerase III, encoded by the dnaQ gene, plays an intricate role, through its 3'→5' exonuclease activity, in the maintenance of fidelity during DNA replication. While this enzymatic activity is essential for the removal of replication errors by the subunit, the 3'→5' exonuclease is not required for cell viability since dnaQ mutants, defective in the 3'→5' exonuclease activity, are viable. Prior to the work presented in this thesis, the possibility that epsilon possesses an additional function, aside from its exonuclease capacity, had not been addressed.

The studies presented here demonstrate that epsilon is required for normal growth. Upon the removal of epsilon from Salmonella typhimurium, by the replacement of the wild type dnaQ gene with a null mutant allele, a severe growth defect was observed. The growth defect was readily relieved by the presence of extragenic suppressor mutations. The suppressor-containing strains were shown to possess a mutator phenotype which is indicative of the loss of epsilon.

Sixteen independently derived suppressor mutations mapped in the vicinity of dnaE, the gene encoding the alpha subunit of DNA polymerase III. One of these mutations, spq-2, was localized to the dnaE gene and results in a valine to glycine amino acid substitution at position 832 of the alpha polypeptide. This mutation, first
identified by DNA sequence analysis, was subsequently shown to be responsible for the suppressor phenotype. In addition, three other suppressor mutants contained the identical mutation found in the \textit{spq-2} mutant. None of the other twelve suppressor mutations have been identified.

In addition to their dependence on a suppressor mutation, the \textit{dnaQ} null mutants required DNA polymerase I for continued viability. All three activities of DNA polymerase I, polymerase, 3'→5' exonuclease and 5'→3' exonuclease, were required in the absence of a suppressor mutation. In the presence of a suppressor mutation, the DNA polymerase I requirements were slightly altered. The 3'→5' exonuclease activity of DNA polymerase I was no longer required in the presence of the three suppressor mutations tested, \textit{spq-2}, \textit{spq-3}, and \textit{spq-6}. This suggests that a major problem in epsilon-deficient cells (without a suppressor) is the inability to process misinsertion events, which are more easily dealt with in suppressor-containing cells.

Partially purified DNA polymerase III from the \textit{spq-2} mutant was shown to be active in polymerization assays implicating an active role of DNA polymerase III in promoting suppression. In addition, DNA polymerase III from the \textit{spq-2} mutant did not contain any detectable 3'→5' exonuclease activity, confirming the absence of the epsilon subunit. This also rules out the possibility that suppression is being achieved by a restoration of the editing capacity of the polymerase.

The results from these studies imply that epsilon, the editing
subunit of DNA polymerase III, possesses a second function, apart from its exonuclease activity, which is required for the normal growth of the cell. The loss of this additional function of epsilon, which is currently unidentified, can be replaced by the combined action of a genetically-altered alpha subunit, the polymerization subunit of DNA polymerase III, and DNA polymerase I.

The characterization of the epsilon-deficient cells and the identification of one of the mutations responsible for the suppressor phenotype in the present studies have initiated the investigation of the growth-promoting function of the epsilon subunit. An extension of the above studies would be the identification of the other sites in the dnaE gene that give rise to the suppressor phenotype. Furthermore, additional amino acid substitutions could be engineered at those sites which give rise to suppression and tested for their ability to suppress.

The growth-promoting function of epsilon may be deduced by the determination of the suppression mechanism in the epsilon-deficient cells. The approach that is currently being used to determine the mechanism of suppression is the biochemical analysis of the purified spq-2 alpha subunit. The properties of the purified subunit that will be analyzed and compared to those of the wild type alpha subunit include the rate of polymerization, processivity, thermostability, and the ability to extend 3' mismatches. Thus, the identification of the altered property of the spq-2 alpha subunit should result in the determination of the suppressor mechanism and hopefully, lead to the identity of the growth-promoting function of the epsilon subunit.
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