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ISOLATION AND CHARACTERIZATION OF TEMPERATURE-SENSITIVE PROTEIN SYNTHESIS MUTANTS OF ESCHERICHIA COLI BY DIRECTED MUTAGENESIS OF THE DEFECTIVE BACTERIOPHAGE LAMBDA FUS2

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ISOLATION AND CHARACTERIZATION OF TEMPERATURE-SENSITIVE PROTEIN SYNTHESIS MUTANTS OF *ESCHERICHIA COLI* BY DIRECTED MUTAGENESIS OF THE DEFECTIVE BACTERIOPHAGE LAMBDA FUS2

A Dissertation

Presented to

the Faculty of the Department of Biochemistry

Quillen-Dishner College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Biomedical Sciences

---

by

Kenton Lee Lohman

December, 1985

APPROVAL

This is to certify that the Graduate Committee of

KENTON L. LOHMAN

met on the

25th day of July, 1985.

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

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Signed on behalf of the Graduate Council

ISOLATION AND CHARATERIZATION OF TEMPERATURE-SENSITIVE PROTEIN SYNTHESIS MUTANTS OF *ESCHERICHIA COLI* BY DIRECTED MUTAGENESIS OF THE DEFECTIVE BACTERIOPHAGE LAMBDA FUS2

by

Kenton Lee Lohman

Mutagenesis of the defective transducing bacteriophage lambda fus2 was used to isolate a collection of temperature-sensitive mutants of *E. coli* in the major ribosomal protein gene cluster. Four mutants were examined in detail. Two of the mutants were resistant to the ribosomal antibiotics neamine and spectinomycin. Another mutant was defective in 50S ribosomal subunit assembly at 42°C. The 30S subunit proteins S17 and S19 were changed in two different mutants. Each protein migrated as a more basic species in two-dimensional gels of ribosomal proteins. Ribosomes from each of the four mutants examined showed a temperature-dependent reduction in translational activity in cell-free assays. The kinetic assays showed declines in both the rate and extent of translation at three temperatures. Ribosomes from three of the four mutants were also found to have an increased rate of heat inactivation at 45°C compared to control particles. Mixed subunit assays identified a t.s. subunit in each mutant. A defect in reassociation at high temperature was found for the subunits from one mutant. Another mutant showed significantly high levels of misreading at 32°C and 42°C. Two mutants showed a decreased ability to bind 14C-phenylalanine tRNA at the two temperatures tested. The increased efficiency and utility of this mutagenesis method for the isolation of protein synthesis mutants is discussed.

ACKNOWLEDGEMENTS

If I had a dime for every time my wife threatened to divorce me during the past three years, I would be wealthy and not have to take a postdoctoral position which will only make me a little less poor and will keep me away from home and in the lab even more than graduate school and all because my committee read this manuscript and said that the only alternative to signing the approval to this dissertation was to give me a job mowing the grass on campus but the Physical Plant would not hire me on account of they said I was over-educated and needed to improve my dexterity skills like picking my nose while driving a tractor-mower over poor defenseless squirrels that were eating the nuts they stole from the medical students' lunches on Tuesday afternoon following the Biochemistry quiz which they all did not pass and blamed on me because they said a tutor was supposed to come with a 30-day money-back guarantee and I am supposed to thank someone for all this?!! Well, if I must then let me thank the members of my committee: Dr. Scott Champney, Dr. Frank Inman, Dr. Ellen Rasch, Dr. Phil Musich, and Dr. Lee Pike for all their help, encouragement, and constant harassment to get me through my degree. Thanks to Ina Pickett for typing my figure legends over three or four thousand times. I would like to acknowledge the Sigma Xi Scientific Society for partially funding my research with a Grant-In-Aid. Once again I would like to thank my wife and son for sticking with me. I would like to thank George for picking with me, Majid for drinking with me, Andrea for laughing with me, Bob for showing me how to shoot off fireworks from the prone position, Craig for fishing the streams and lakes with me, Sharon for being such a "zany", loveable kid, Margie for her ceaseless stories about motherhood, Eric for being hyperspace, Abdol for his volleyball spikes, Joe for being such a Verne, Rhesa for having little Yoda girl, Raymonde for giving us the "Your Daddy Was A Lady and Your Momma Came From Africa Blues", and most of all I would like to thank Scott Champney for taking an old musician under his skinny wing and being a good friend to him.

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Chapter 1

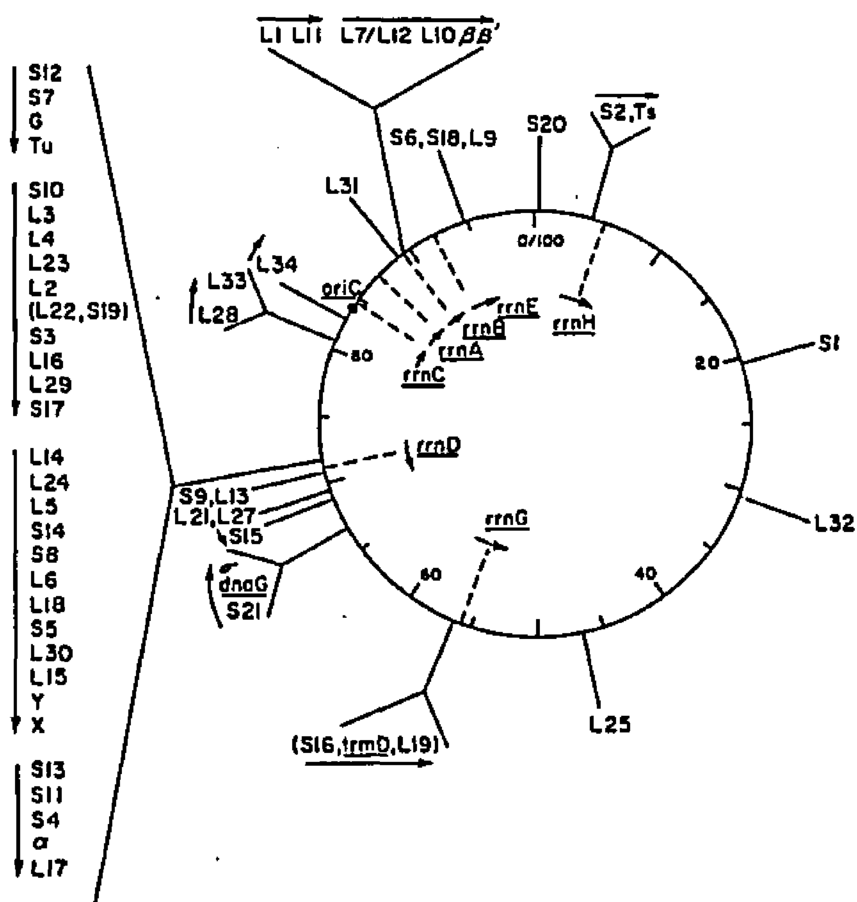
INTRODUCTION

The study of the process of messenger RNA (mRNA) translation in *Escherichia coli* has been extensive. The difficulty of such an investigation has been the large number of cellular components involved in the overall process. In *Escherichia coli* , over 150 individual macromolecules are necessary to translate mRNA into a specific protein. These include 60 tRNA species and about 20 related aminoacyl tRNA synthetases, 3 elongation factors, 3 initiation factors, 3 termination factors, 3 ribosomal RNA's, and 54 ribosomal proteins. A map of the translational and ribosomal component genes of *E. coli* is shown in Figure 1 (Bachmann, 1980; Isono, 1980). The single most complex unit involved in the process is the ribosome. The overwhelming task of studying the structure and function of the bacterial ribosome and its components has involved the use of physical, chemical, biochemical and genetic techniques.

The structures of ribosomal RNAs (rRNA) have been extensively studied. The *E. coli* 5S rRNA was one of the first nucleic acids ever sequenced (Brownlee et al., 1971). Both the 16S (Woese et al., 1983; Brosius et al., 1978; and Carbon et al., 1979) and 23S (Branlant et al., 1975, 1976; Branlant et al., 1981) rRNAs have also been sequenced more recently. Conventional RNA fingerprinting, rapid RNA sequencing methods, and DNA sequencing methods (for cloned

FIGURE 1. THE GENETIC MAP OF TRANSLATIONAL COMPONENTS OF *E. COLI*. THE MAP LOCATIONS IN MINUTES, OF VARIOUS TRANSLATIONAL COMPONENT GENES IS SHOWN OPPOSITE. THE MAJOR RIBOSOMAL PROTEIN GENE CLUSTER WITH 27 GENES IS FOUND AT A POSITION OF APPROXIMATELY 72 MINUTES ON THE *E. COLI* CHROMOSOME. (ISONO, 1980)

GENETIC MAP OF TRANSLATIONAL COMPONENTS OF E. COLI



gene sequences) were used in the sequence determinations. The secondary structures of the rRNAs have been extensively examined. The 5S rRNA secondary structure has been shown to conform to two models. The two models differ in their predicted sensitivity to ribonuclease cleavage, chemical modification, and in the binding of ribosomal proteins to specific domains in vitro (Noller and Garrett, 1979). The two forms seem to have very similar conformations in the ribosomal particle itself (Noller and Herr, 1974) and could be transitional conformers (Wiedner et al., 1977). Due to the complexity and numerous statistical binding possibilities involved, the secondary structures of the two larger rRNAs have been more difficult to characterize. Tinoco et al., (1973) proposed the first 16S rRNA secondary structure based on the most thermodynamically stable base pairing configuration that could be hypothesized from known primary structure. Noller et al., (1980) have been able to ellucidate a stable and statistically accurate model for the 16S rRNA by comparing the sequence data from Escherichia coli with the small subunit RNA's from, Bacillus stearothermophilus , Zea mays and tobacco chloroplasts, and mitochondria from several organism. They also made comparisons between species of 16S rRNA and sequences of 18S rRNA. They then determined which sequences were most conserved (Woese et al., 1983). By examining the consistencies between sequences and the predicted double-stranded regions possible based on these

consistencies, they were able to postulate a general secondary structure model for all 16S-like rRNA's. The model was tested by a number of investigators looking at the susceptibility of certain 16S rRNA bases to T1 and pancreatic RNases, and to chemical modification by bisulfite, kethoxal, and m-chloroperbenzoic acid when the RNA was free, in the ribosome, the 30S subunit, or in various ribonucleoprotein particles.

Possible functional sites on the ribosome involving the rRNA have been examined. Electron microscopy (Sloof et al., 1976) has localized protein binding sites in protein-RNA complexes which stabilize specific folded structures in the rRNA. Vasiliev et al., (1978), have shown that in association with a few of the small subunit ribosomal proteins, the 16S rRNA assumes a folded structure. This structure very closely resembles the intact 30S ribosomal subunit. The effects of buffer conditions on the solution properties of 16S rRNA have been examined by Hochkeppel and Craven, (1977). They showed that acetic acid-urea extracted 16S rRNA had ribosomal protein binding properties not found with 16S rRNA which had been extracted with phenol. Partial nuclease digestion studies by Ehresmann et al., (1977), support the idea of an RNA "core" or nucleus of 500 nucleotides of the 16S rRNA for the 30S particle. Single stranded loops and exposed sites on the rRNA have been postulated as mentioned above, by chemically modifying bases using the single strand, guanine-specific

reagent kethoxal which then renders these sites nuclease resistant (Litt, 1969). Sites involved in important functions or contacts react to a greater or lesser extent with kethoxal under certain conditions. These sites could be potential active sites for ribosomal functions such as tRNA, initiation factor and elongation factor binding to the 16S rRNA. Conformational changes in the 16S rRNA have also been studied in this manner (Ginzberg and Zamir, 1975). Woese et al., (1983) used bisulfite (C-specific), m-chlorobenzoate (A-specific), and glyoxal (G-specific) in similar types of experiments to test for reactive single stranded regions of the secondary structure of 16S rRNA.

Sequence data for double-stranded regions of the RNA have been examined to postulate helical structures and a tertiary structure within the ribosome. Double-strand specific cross-linking reagents, such as psoralen (Karathanasis and Champney, 1979), have been used to probe the double-stranded nature of the secondary structure of the 16S rRNA. Exposure of the RNA or subunit to the reagent and subsequent treatment with a single strand-specific ribonuclease allows double stranded regions with cross-links to be purified. Double-stranded regions which were reactive, were separated by denaturing the RNA and isolating the double-stranded cross-linked RNA which remains. A tertiary structure was postulated by identification of double-stranded regions which existed in the RNA in the particle but were lost when the RNA was free in solution.

The average location of the 16S and 23S rRNAs within their respective subunits was also postulated from neutron and X-ray scattering techniques (Moore et al., 1977; Moore, 1980). It has been through structural studies such as these that information concerning the contribution of the rRNAs to the functional domains of the ribosome have been postulated.

The structures of the ribosomal proteins have also been extensively examined. Kaltschmidt and Wittmann, (1970) electrophoretically separated 21 proteins from the 30S subunit and 34 proteins from the 50S subunit in two dimensional gels. The only protein found in multiple copies on the ribosome is the large subunit protein L12, which consists of four molecules with two modified by acetylation on the NH2 terminus and designated as ribosomal protein L7 (Terhorst et al., 1973). Immunologic studies have shown that S20 and L26 are identical proteins and that their 30S and 50S particle stoichiometry is 0.8 and 0.2 respectively (Weber, 1972). Thus, there is only one copy of this protein per ribosome making the total number of different proteins 52. The difficult task of determining the primary sequences of all of the ribosomal proteins has been completed by Wittmann, (1982). Several proteins are modified posttranslationally. Proteins S5, S18, and L7 have NH2 terminal acetylations (Wittmann-Leibold, 1978; Yaguchi, 1977; Terhorst et al., 1973). Proteins S11, L11, L16, and L33 are methylated, with L11 being the most

highly substiuted (Chen et al., 1977; Dognin and Wittmann-Liebold, 1977).

There are several ways to examine the secondary structure of single ribosomal proteins based on the confirmation suggested by the amino acid sequence (reviewed by Wittmann, 1982; 1983). These conformations include alpha helix, beta helix, random coil and extended structures. Each of the 52 proteins has a distinct amount of each of these conformational forms within their respective structures. Extreme examples are proteins S12 and L29 with 7% and 67% alpha helix respectively. The predictions have also been experimentally tested. ORD and CD have been used to look at protein conformation both on the ribosome and free in solution. The results showed that ribosomal proteins have at least two distinct conformations, in these different states (Allen and Wong, 1978). Nuclear magnetic resonance (NMR) has been used to look at the tertiary structure of ribosomal proteins on the ribosome (Morrison et al., 1977). Such studies have illucidated direct protein-protein interactions. Both low angle X-ray scattering and fluorometric techniques revealed that many of the ribosomal proteins have multiple binding domains on the ribosome and are flexible, making the ribosome a dynamic structure (Osterberg et al., 1978; Labischinski et al., 1979). The technique of neutron scattering has shown that, within the ribosome, some proteins are globular in nature and others are elongated (Moore et al., 1980).

Some of the most valuble information concerning the arrangement and interactions of proteins on the ribosome has been from the cross-linking of neighboring proteins using chemically reactive cross-linkers such as 2-iminothiolane (Traut, et al., 1980). This method has shown which proteins interact structurally but does not necessarily give information about their functional interactions. Ribosomal protein locations on the subunit surfaces have been partially mapped by binding antibody generated against single ribosomal proteins to ribosomal particles and visualizing the binding sites by electron microscopy. The dimeric binding properties of antibodies i.e. a single intact antibody with two Fab regions, allow an antibody to bind two ribosomes or subunits at the same site on the particles and confirm a specific ribosomal protein location on the particle surface. Negative staining and shadow casting techniques have also been used for visualization of intact subunits and 70S ribosomes. In this manner, an overall picture of the shape of the ribosome has been postulated (Boublik et al., 1976; Lake, 1980; Stoffler et al., 1980).

Functional sites on the ribosome have been suggested by a number of structural studies which involve cross-linking ribosomal proteins to each other, to initiaion factors, and to elongation factors. Transfer RNA and mRNA binding studies have given insight into the locations of the functional sites for these translational components on the

ribosome (Lake, 1980; Offengand, 1980). Such studies indicate sites of proximity between ribosomal proteins and other translational components and imply functional sites. Cross-linking occurs at specific sites on individual proteins. These sites are not necessarily involved in a functional site. Therefore, these studies do not prove functional interaction between ribosomal proteins and these translational components. A general sequence for the assembly of ribosomal proteins into each subunit has been constructed from extensive reconstitution experiments (Neirhaus, 1982).

With all the present information available on the structural aspects of ribosomes, there has been relatively little light shed on the contributions of individual ribosomal proteins to the overall structure and function of the 70S bacterial ribosome. Early work in the identification of ribosomal function involved the use of antibiotics. Several translational inhibitors have been found to affect soluble protein factors involved in translation. Fusidic acid sequesters EF-G and GDP on the ribosome and leads to the inhibition of the binding of amino acyl tRNA at the A-site (Bodley et al., 1970). Tetracyline was found to inhibit the binding of formylmethionine-tRNA to the ribosomal A-site (Cundliffe, 1972). Puromycin, an analogue of the 3' end of amino acyl tRNA (amino acyl adenosine), functions at the acceptor site of the peptidyl transferase center on the 50S subunit (Traut and Monro,

1964). It has been used exensively to study the mechanism of peptide bond formation. Once puromycin is in the ribosomal P-site, there is no glycosidic bond available in the antibiotic to facilitate the movement of the amino acid or peptide from the P-site to the amino acyl tRNA in the A-site. The puromycin-peptide is "frozen" in the P-site and translation is halted. Depending on the substrate (tRNA, mRNA, or soluble factors), a number of ribosomal functions can be studied using puromycin.

The use of ribosomal protein mutants to study ribosome function has been an important tool. There have been a number of approaches to mutant isolation. Those which are the most informative have used conditionally lethal mutations such as antibiotic dependence, temperature-sensitivity, cold-sensitivity, and suppressible nonsense mutants (Nomura et al., 1977). Ribosomal antibiotic resistance was used in initial studies of ribosomal protein mutation. Streptomycin has been a well characterized ribosomal antibiotic. The ribosome was first implicated as the site of action of the antibiotic by Gorini, (1969). A spontaneous revertant from streptomycin-dependence was isolated by Brownstein and Lewandowski, (1967). The streptomycin-dependant parent strain could not grow well without streptomycin being present in the growth media. It was shown that revertant growth was inhibited by streptomycin in a manner similar to a wild-type streptomycin-sensitive strain and that the mutations

responsible for both streptomycin dependence and sensitivity in the revertant mapped in the str-spc region of the *E. coli* chromosome. Ozaki et al., (1969), showed that resistance to streptomycin in *E. coli* was due to a mutation in the gene for ribosomal protein S12 (rpsL). Another spontaneous streptomycin-sensitive revertant from a strepomycin-dependant *E. coli* strain was isolated by Birge and Kurland, (1971). Its growth was inhibited by streptomycin in a manner similar to that seen in a wild-type streptomycin-sensitive strain except that the revertant was reversibly inhibited and never died in the presence of streptomycin. The implication was that the revertant was not a true back revertant but that there was a second mutation suppressing the streptomycin-dependent mutation. Additional insight into the nature of streptomycin-dependent mutations was generated by Garvin et al., (1973). It was found that ribosomes synthesized in the presence of streptomycin, showed a high degree of misreading both in vivo and in vitro, even though the concentration of streptomycin in solution was extremely low. Under the identical conditions in the presence of high salt, the misreading was alleviated. That observation lead the investigators to the assumption that streptomycin interferred with the assembly of the ribosome, by inducing a conformational change that caused the misreading. Based on that assumption they felt that streptomycin-dependent mutants required the antibiotic for proper assembly of the

ribosome (Garvin et al., 1973). Thus the conformation-dependent function of the ribosome and its constituents began to be elucidated through the use of ribosomal antibiotics.

The functional interdependence of ribosomal proteins was examined through the use of ribosomal antibiotics. Ribosomal proteins L4 and L22, S5, S12 and the 16S rRNA were studied by taking advantage of each components involvement in resistance to the ribosomal antibiotics erythromycin, spectinomycin, streptomycin, and kasaugamycin respectively (Saltzmann and Apirion, 1976; Cundliffe, 1980). Cabezon et al., (1976) found that mutations in protein S5 (ribosomal ambiguity- ramA) were able to overcome streptomycin dependence in mutants carrying a strA (ribosomal protein S12) mutation. They also isolated revertants from streptomycin-dependent mutants which had altered S17 or S5 ribosomal proteins. One S17 (neaA) mutant was resistant to the related aminoglycoside neamine. The same group found that a mutant resistant to both streptomycin (altered S12) and spectinomycin (altered S5) was also resistant to neamine but had no S17 alteration (Matkovic et al., 1980). Other revertants of strD strains were shown to have many single ribosomal protein alterations (Dabbs and Wittmann, 1976; Dabbs, 1978). Spontaneous revertants from gentamycin-resistant mutants (Buckel et al., 1977), and lincomycin-resistant mutants (Hummel et al., 1979) have defects in large subunit ribosomal proteins L6, and L14 and

L15 respectively. Revertants isolated from erythromycin-dependant mutants are missing one of several ribosomal proteins (Dabbs, 1978). Mutants which were missing ribosomal proteins were unable to bind the antibiotics normally associated with the missing protein.

Genetic approaches to solving the problems of ribosomal fuction have been very informative (Isono, 1980). Mutations in translational and ribosomal components which alter the function of the component have been an important tool in the investigation of ribosomal function. More specifically, point mutations which confer temperature-sensitivity to the host have allowed for the investigation of both the normal and defective functioning of single ribosomal proteins by simply changing the temperature. Temperature-sensitive (t.s.) mutants have been used to study the ability of ribosomes to function both in vivo and in vitro. Wittmann et al., (1974, 1975), isolated temperature-sensitive mutants with defects in the alanyl- and valyl-tRNA synthetases. It was discovered that revertant suppressors of those strains carried defects in ribosomal proteins S5 and S8 respectively. Protein S20 was also found to be altered in the alanyl-tRNA synthetase suppressor mutant (Bock et al., 1974) and completly missing in a revertant of the valyl-tRNA synthetase mutant. Isono et al., (1976, 1978) mutagenized the chromosomal DNA of _E. coli_ with N-methyl-N'-nitro-N-nitrosoguanidine to create temperature-sensitive mutants, many of which contained

altered ribosomal proteins. In general, however, the ribosomal proteins were not the sites of temperature-sensitivity in the majority of those mutants. Isaksson et al., (1978) isolated a t.s. mutant which had multiple defects at high temperature, including fusidic acid resistance, and accumulation of ppGpp and ppGppp. There have also been t.s. mutants isolated in which the post-translational modification of ribosomal proteins has been altered (Isono et al., 1978; Isono, 1980). Mutants affected in acetylation of (proteins S5, S18, and L7), affected in methylation (proteins S11, L3, L11, L7/L12, L16, L33, L1, and L5), and affected in C-terminal modification (protein S6) have been isolated. Many reports of cold-sensitive mutants which are defective in ribosome assembly have been presented (Squires and Ingraham, 1969; Guthrie et al., 1969; Nashimoto et al., 1970, 1971; Bryant and Sypherd, 1974; Geyl et al., 1977). A cold-sensitive pseudorevertant from a temperature-sensitive strain has been isolated in which a spontaneous mutation in a second ribosomal protein overcame the initial t.s. mutation (Mehrpouyan and Champney, manuscript in review). Many such revertants were defective in their assembly of intact functional ribosomes.

An invaluable genetic tool in the isolation of these mutants has been the technique of localized mutagenesis (Hong and Ames, 1971). Point mutations are introduced into genes in any specific portion of the _E. coli_ chromosome

by using a generalized transducing bacteriophage. These phage have the ability to package any piece of bacterial DNA of proper size into the phage capsid following a limited degredation of the host chromosome. Reinfection of a second host population with the phage lysate allows the phage-carried bacterial sequences to recombine with homologous host sequences. This confers a new selectible phenotype on the second host. Mutagenesis of the phage population prior to reinfecting the second host provides a means of recombining mutations into the second host while screening for the new selectable phenotype. Temperature-sensitivity provides a convenient screening procedure for linked mutations in recombinants.

Bacteriophage P22 was first used as a generalized transducing phage in Salmonella typhimurium (Hong and Ames, 1971). The mechanisms of replication and packaging of the P22 phage DNA can be used as a good example of how generalized transducing phage work (Ingraham et al., 1983). Upon infection, the double stranded phage DNA is synthesized as long concatenates made up of about 10 identical phage genomes. The ends of each concatenated genome are terminally redundant which allows for the concatenate formation. A phage coded nuclease cleaves the concatamers as they are individually packaged using the "headful mechanism". Each phage capsid contains at least one whole phage genome but could contain more due to the lack of specificity of the nuclease. The cleavage sites for the

nuclease are determined by the distance from the initial site of cutting until the capsid (phage head) is full of DNA. If the nuclease cleaves the bacterial genome, which it does with a rare frequency, segments of bacterial DNA of the proper size (approximately 1% of the host chromosome) can be packaged by the headful mechanism into phage capsids as if it were phage DNA. A phage particle containing bacterial DNA sequences is termed a "transducing particle". Subsequent infection of a second host with a transducing phage can result in the recombination of the phage-introduced bacterial sequences into the genome of the second host at the site of greatest homology. If this DNA site carries a selectible marker not previously found in the new host (i.e antibiotic resistance versus sensitivity), a transductant can be identified by growing infected cells on plates containing the antibiotic. Only recombinant transductants will survive.

P1, a phage related to P22, has been used with great success for directed localized mutagenesis in our lab and others (Berger et al., 1975; Kushner et al., 1977). Upon infection, the P1 DNA circularizes and replicates as a plasmid. P1 can also lysogenize as a plasmid but more commonly causes a lytic infection which is lethal to the host. Strains of P1 which cannot lysogenize (P1vir) have been isolated and have been especially useful in this method (Berger et al, 1975; Champney, 1979). In a lytic infection, P1 randomly cleaves the host chromosome into several hundred

fragments. Approximately 1 in 10^5 phage from the lytic burst has packaged a piece of bacterial DNA by the headful mechanism and becomes a transducing particle. Another 1 in 10^2 phage carries any given discreet piece of DNA involving approximately 2 minutes (90kb of DNA) on the _E. coli_ map (Birge, 1981). Therefore, 1 out of 100 transducing phage particles or 1 out of 10^7 total phage particles in a single P1 population will contain a specific sequence of interest (i.e. a ribosomal protein gene). Mutagenesis of any specific gene sequence carried by a transducing phage would then require mutagenesis of 10^7 phage particles before "hitting" one transducing phage. The frequency of generating temperature-sensitive mutations with this method is about 1 in 10 affected transducing particles. Thus, the number of mutagenized phage necessary to create a single temperature-sensitive mutation in the desired sequence approaches 10^8. Therefore, about 10^3-10^4 transductants must be screened in order to isolate a single t.s mutation linked to a selective marker gene.

Other bacterial viruses, called specialized transducing phage, have also been used extensively as vectors for recombination in localized mutagenesis (Nomura, 1976; Nomura et al., 1977). Bacteriophage lambda has been used as a specialized transducing phage to characterize _E. coli_ gene organization. Lambda bacteriophage also have a very complex life cycle (Hendrix et al., 1983; Ingraham et al., 1983).

They most efficiently infect E. coli which are metabolizing maltose. The inducible maltose binding protein (MBP) found in the E. coli outer membrane is the site of phage tailpiece attachment and is necessary for efficient infection. After infection, the double stranded linear phage chromosome circularizes by the base pairing of complementary, 12 nucleotide long, single stranded ends termed m and m'. Approximately 180 degrees from the m-m' site on the circular phage genome, is a 15 base pair segment (att lambda P) that is completely homologous with an identical 15 base pair sequence (att lambda B) in the bacterial genome. These sequences are both designated "O". Flanking non-homologous sequences to "O" in the phage DNA are called P and P' and in the bacterial DNA they are called B and B'. Together these sequences form the recombinational sites for the phage called lambda attP or POP' and lambda attB or BOB'. The Campbell Model (Birge, 1981) for recombination leading to lysogeny, places the phage genome into the bacterial genome in the sequence order BOP'-phage genome- POB'. Lysogeny in general, is controlled by a phage coded, DNA binding, repressor protein (cI) which is sequence specific and has a high degree of affinity for its own promotor region on the phage DNA. When the repressor protein is present in sufficient quantity in the cytoplasm of the infected cell, cI binds both its own promotor, P_{RE}, and the main phage promotors P_L and P_R, halting repressor message transcription as well as

transcription from P_L and P_R. This gives cells lysogenized with quiescent lambda prophage. Induction of the prophage leads to cI degredation and both inhibited promotors become functional. The first gene transcribed from the P_L promotor is the N protein gene. N protein binds nusA loci within its own termination site causing antitermination and the continued transcription of the adjacent int and xis genes. Both int and xis gene products are necessary for the excision of lambda from the host chromosome. It is the precision of the excision event that determines if the phage will become defective. Illegitimate excision occurs approximately 10^{-7} times in a lytic burst population (Birge, 1981). The first defective lambda phages isolated and examined were found to have incorporated into their DNA, bacterial DNA normally found adjacent to either side of the lambda attachment site in the *E. coli* chromosome. The gal (galactose metabolism) and bio (biotin utilization) gene loci had been incorporated into the phage DNA. The phage were then able to transduce these alleles into a second host. For the identification of the transductants it was necessary to infect either a gal- or bio- hosts. Deletion of the lambda attachment site from the *E. coli* chromosome was found to cause lambda to insert at random sites around the chromosome at very low frequencies. Many new lambda defective transducing phage were subsequently isolated in the same manner (Hendrix et al., 1983).

The isolation of the transducing phage lambda fus2 used in this study, began with the EC-6, a strain of _E. coli_ which was a lac-/lac+ diploid carrying an F'tslac+ plasmid in the cytosol (Beckwith et al., 1966). F plasmids are small circular double stranded episomal DNAs. F plasmids carry genes specific for the mating transfer of the F plasmid itself. F can also incorporate into the _E. coli_ chromosome at specific sites and the host becomes known as an Hfr strain (high frequency of recombination). If F excises, bringing with it a piece of the _E. coli_ chromosome, it is called an F'plasmid upon recircularization. F'tslac carries a temperature sensitive mutation in the plasmid origin of replication and the _E. coli_ lactose operon (lactose utilization genes). When strain EC-6 was grown on lactose and heated, the t.s. mutation in the plamid prevented plasmid replication and the expression of the phage-carried lactose operon. This resulted in the death of any cell which was unable to incorporate the plasmid into the chromosome where the plamid could be replicated and the lactose utilization genes expressed. One isolate, EC-2, showed that the F'tslac+ had inserted near the aroE locus at 72 minutes. The major cluster of ribosomal protein genes and translational component genes of _E. coli_ also lie in the 72 minute region. Jaskunas et al., (1975d), used EC-2 as a host for infection with lambda lac5, a lambda defective phage which carried the lac operon. EC-2 was deleted for the lambda

attachment site, allowing lambda lac5 to lysogenize at its largest region of homology, the lac operon which was carried by the inserted F'tslac plasmid. They were able to isolate a series of defective phages that contained progressively more bacterial sequences from the 72 min region. Beginning with lambda cI857S7 helper phage, lambda trkA, lambda aroE, lambda spc1, lambda spc2, and finally lambda fus2 and fus3 were isolated. Extensive insight into the organization and the regulation of expression of the ribosomal protein genes in this region has been gained by the use of these phage. It was found that the 27 ribosomal proteins were organized into four different transcriptional units with four promoters (Jaskunas et al., 1976). The direction of transcription of the genes for ribosomal proteins was found to be in the anticlockwise direction on the *E. coli* chromosome (Jaskunas et al., 1975b). A second copy of the gene of EF-Tu was found in this region using these phage (Jaskunas et al., 1975c). The effect of ppGpp and the stringent response on expression of ribosomal proteins from these phage was elucidated (Lindahl et al., 1976). The gene locus for the alpha subunit of RNA polymerase was found in this cluster of ribosomal protein genes (Jaskunas et al., 1975a). They were able to map a large portion of the 72 minute region using these phage (Jaskunas et al., 1977a, 1977b). The regulation of transcription of ribosomal protein genes by ribosomal proteins was also described (Dennis and Nomura, 1975; Nomura and Post, 1980). The large portion of

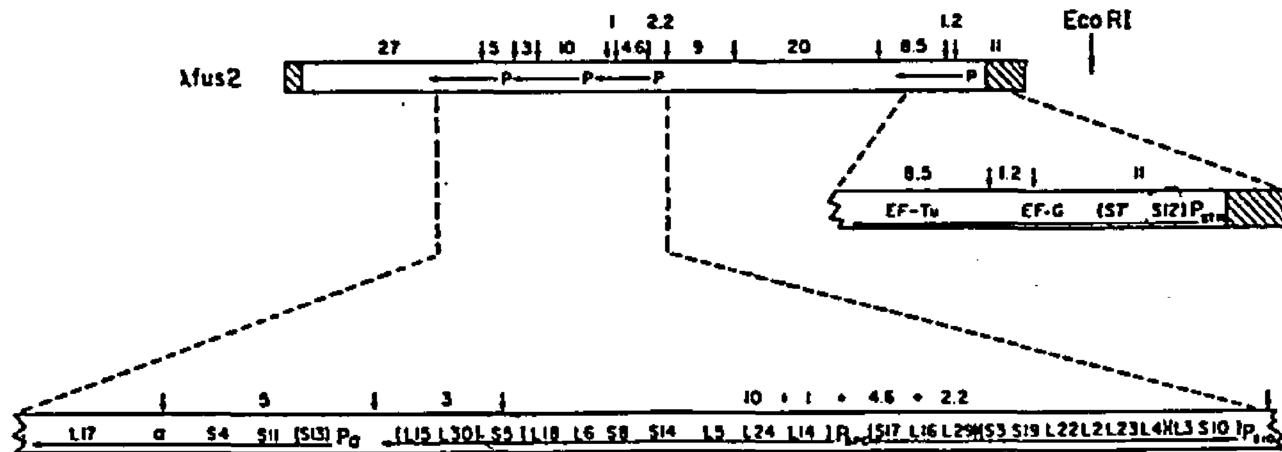
the information concerning regulation and expression of the ribosomal genes in this region was gathered using either phage lambda fus2 or lambda fus3.

We have used the specialized transducing phage lambda fus2 (Jaskunas et al., 1977) in directed mutagenesis of _E. coli_ ribosomal protein genes. Lambda fus2 has 29 X 10^6 daltons of bacterial DNA including 27 _E. coli_ ribosomal protein genes, the alpha subunit of RNA polymerase, and one copy of EF-Tu, and EF-G which is also the fusidic acid locus and the origin of the name of the phage (Fiandt et al., 1976) (Figure 2). This region comprises approximately 1% of the _E. coli_ chromosome. There are a number of advantages in using the specialized transducing phage lambda fus2 to study ribosomal component gene organization and regulation (Nomura, 1976; Nomura et al., 1977).

First, the helper phage, lambda cI857S7, carries a t.s. repressor mutation 857 which allows any lysogenic strain which harbors both phage, to be grown to a high cell density without the cells being lysed. By heating the culture, the t.s. repressor protein breaks down, the helper phage will excise itself and the defective phage from the host chromosome and begin to package both pieces of DNA into phage capsids. The host cells begin to fill up with phage due to the S7 mutation in a phage gene which codes for the lysis protein. The mutation causes the delayed lysis of the host and consequently phage yields approach 1000 phage/cell. The helper phage has a deleted segment of DNA from the

FIGURE 2. GENETIC ORGANIZATION OF LAMBDA FUS2. THE GENE ORGANIZATION OF THE BACTERIAL DNA SEQUENCES CARRIED BY THE DEFECTIVE PHAGE LAMBDA FUS2 AND CHARACTERIZED BY M. NOMURA IS SHOWN OPPOSITE. IT IDENTIFIES 27 RIBOSOMAL PROTEIN GENES, EF-G, EF-TU, AROE, TRKA, AND THE ALPHA-SUBUNIT OF RNA POLYMERASE IN FOUR TRANSCRIPTION UNITS. (JASKUNAS ET AL., 1977A)

FIGURE 2. GENETIC ORGANIZATION OF LAMBDA FUS 2



nonessential region near the middle of the phage genome. The result is that the helper phage particle has less DNA and, therefore, has a lower bouyant density and can be purified away fom the defective phage. This gives almost pure populations of both phage after isopycnic centrifugation. In a P1 phage lysate it is very difficult to separate the transducing particles from those phage particles carrying phage sequences.

A second advantage is the fact that the DNA from the defective phage is approximately 93% bacterial and carries 27 ribosomal protein genes. Mutagenesis of the purified defective phage would be directed primarily against bacterial sequences which are enriched for ribosomal protein genes. Essentially every phage particle is a potential transducing particle. With P1, almost every phage being mutagenized carries phage DNA not bacterial DNA and gives a much reduced efficiency of mutagenesis.

Finally, lambda fus2 carries the strR allele which is recessive to the strS allele. By infecting a host which carries the strS allele, only recombinants incorporating the strR allele from the phage will survive when grown in the presence of streptomycin. This helps to prevent the formation of lysogens because lysogens, being diploid for the streptomycin gene, would express the dominant strS allele and would not survive in the presence of streptomycin. The expression of the streptomycin gene is a particularly effective method of screening for mutant

recombinants because it codes for ribosomal protein S12 which is in the ribosomal gene cluster found on lambda fus2 and any closely linked t.s. mutations have a high probability of involving a ribosomal protein gene. P1 transduction involves the totally random isolation of bacterial genes from around the entire E. coli chromosome making the process comparatively inefficient.

Lambda fus3 is a phage identical to lambda fus2 except that it carries the strS allele instead of the strR allele. Cabezon et al., (1980) have used lambda fus3 to map nonsense mutations within the 72 minute spc-str region. A major difference in the genetic technique used by Cabezon et al. and this work was the selection for monolysogens. They did not use the strR allele as a direct selective marker because lambda fus3 carries the strS allele which is dominant and would have resulted in a negative selection for recombinants (non-survivors). They used instead the aroE locus as their selection and screened aroE+ transductants of an aroE- host for merodiploid monolysogens. They were examining well characterized mutations and not trying to generate new mutations in ribosomal protein genes. Their method is not as generally applicable nor is it as efficient as the method described in this study.

In this work we have exploited the genetic utility of the defective phage lambda fus2 in the localized mutagenesis of ribosomal protein genes. We have taken a purified population of lambda fus2, mutagenized it with

hydroxylamine, infected a strS host, selected for strR recombinants, and screened them for temperature-sensitivity. We used the strR allele carried by lambda fus2 as the direct selection for recombinants because only haploid recombinants of a strS host would survive on media containing streptomycin. The efficiency of our method and the characterization of four of the isolated t.s. mutants will be discussed.

Chapter 2

MATERIALS AND METHODS

Sources of Materials : Acrylamide, methylene bis-acrylamide, hydroxylamine, streptomycin sulfate, neomycin, erythromycin, kanamycin, all 20 L-amino acids, polyuridylic acid, phosphoenolpyruvate (PEP) kinase, tRNA, levigated alumina, ethidium bromide, PPO (2,5-diphenyloxazole), Coomassie Brilliant Blue (R-250 and G-250), ATP (adenosine-5'-triphosphate), GTP (guanosine-5'-triphosphate), PEP, DTT (dithiothreitol), were purchased from Sigma Chemical Corporation.

All culture media used in this study were puchased from Difco. Radio-isotopes (^{3}H-uridine 14 Ci/mmole; ^{14}C-leucine 278, mCi/mmole; ^{14}C-valine, 254 mCi/mmole; ^{14}C-phenylalanine, 525 mCi/mmole) were purchased from New England Nuclear. Toluene, cesium chloride, TCA (trichoroacetic acid), and ETSH (B-mercaptoethanol) were purchased from Fischer Chemicals. Spectinomycin and neamine were gifts from Upjohn Laboratories. Agarose was from E.M. Laboratories. Sodium salicilate was from M.C.B. Laboratories. All buffers are described in the Abbreviations section. Low speed centrifugation (less than 20,000 rpm) was done in a Beckman J2-21 centrifuge. Ultracentrifugation was done in a Beckman L5-55 ultracentrifuge. All rotors used were made by Beckman.

Bacterial Strains : Bacterial strains used in this study are listed in Table I.

TABLE 1. BACTERIAL STRAINS

STRAIN NO.	GENOTYPE	SOURCE
AB2834	F^- aroE353	Pittard and Wallace (1966)
SK901	F^-	aroE$^+$ transductant of AB2834 Kushner et al. (1977)
SK571	recA, Str^S	Kushner et al. (1977)
NO1379	kpdABC5, trkA401 rpsE, rpsL, fusA, recA, (lambda fus2 rpsL) (lambda c1857S7$B_{515}B_{519}$xis6)	Jaskanas, et al. (1976)
KK1901	F^-rpsL	(Str^R) transductant of SK901
KK1902	F^-rpsL	Str^R transductant of SK901
KK2901	F^-rpsE rpsL	Str^R transductant of SK901
KK2902	F^-rpsE rpsL	Str^R transductant of SK901
JC5029	Str^S, Spc^R	Kushner et al. (1977)

Media and Cell Growth : Bacterial strains were stored at $-70^{o}C$ in 1 ml aliquoits of tryptone broth containing 15% glycerol. Cells were grown in Luria Broth (LB), M9 minimal, or A salts minimal solutions. Luria broth consists of 1 gm bacto-tryptone, 1 gm NaCl, 0.5 gm yeast extract, and 0.2 ml 1.0 M NaOH in 100 ml. M9 minimal salts solution contains 7.0 gm Na_2HPO_4, 3.0 gm K_2HPO_4, 0.5 gm NaCl, 1.0 gm NH_4Cl_2, and 0.2 gm $MgSO_4$ per 100 ml of solution. A salts minimal solution per liter contains 10.5 gm K_2HPO_4, 4.5 gm KH_2PO_4, 1.0 gm $(NH_4)_2SO_4$, 0.5 gm sodium citrate, 0.5 ml of 1% thiamine, 10 ml of 20% glucose (Miller, 1972). Plates were made with the same media containing 2% agar. Growth experiments were done in a shaking water bath using 10 ml of cells in 50 ml side-arm flasks. The cell density was determined by using a Klett-Summerson colorimeter with a red (no.66) filter or by reading absorbence at 600nm in a Beckman model 25 spectrophotometer.

Phage Isolation and Mutagenesis : Lambda defective phage were isolated by isopycnic centrifugation in CsCl (Davis et al., 1980) after heat induction of the double lysogen NO1379. NO1379 was grown to a cell density of approximately $2X10^8$ cells/ml at $32^{o}C$, shifted to $42^{o}C$ for 20 minutes, and then returned to $37^{o}C$ for 3 more hours for optimal lysis and phage yield. The defective phage contained approximately 1% contaminating helper phage after CsCl centrifugation as determined by plaque analysis

on strain SK901. Hydroxylamine mutagenesis of lambda fus2 was conducted using $1X10^{11}$ phage in 10 mM Tris-HCl pH 7.6 and a final concentration of 0.5 M hydroxylamine and allowing them to incubate at 37°C for 4, 8, or 12 hours (Miller, 1972). Phage were dialyzed against tryptone broth for 6 hours or overnight and concentrations were estimated by UV absorbence at 260 nm. An A_{260} = 1.0 (absorbence of 1.0 at a wavelength of 260 nm in a 1.0 cm path cell in a volume of 1.0 ml) is equal to 10^{11} phage/ml (Miller, 1972).

Mutant Isolation and Characterization : E. coli strain SK901 was infected with the mutagenized defective phage at a multiplicity of 1 (approximately 1.0 phage/bacterium). On tenth of a milliliter of a phage dilution containing $2X10^{8}$ phage/ml was mixed with 0.1 ml of an SK901 cell culture grown in maltose which contained $2X10^{8}$ cells/ml. The mixture was placed in a water bath at 37°C for 20 minutes (Miller, 1972). The infected cells were centrifuged away from any phage in solution to prevent reinfection and were resuspended in 1.0 ml of LB. Infected cells were allowed to grow overnight for the efficient expression of the recesive streptomycin resistance (strR) allele. Transductants containing lambda fus2 DNA sequences were then selected at 32°C on LB plates containing 100 ug/ml of streptomycin. One hundred strR colonies were picked to master plates and tested for temperature sensitivity by replica plating and incubation at

44^oC. Mutant cells were screened for changes in ribosomal antibiotic sensitivity or resistance by replica plating colonies to LB plates each containing 100 ug/ml of one of the following: streptomycin sulfate, spectinomycin sulfate, erythromycin, neomycin, kanamycin, or neamine. Growth was examined after incubation at 32^oC for 48 hours. Resistance to spectinomycin and neamine were also analyzed in LB containing 0, 50, or 100 ug/ml of the antibiotics. Overnight cultures were each diluted into 1 ml of LB at a density of approximately $2X10^7$ cells /ml (A_{600}= 0.1). Cell densities were measured by absorbence at 600 nm after 48 hours of incubation at 32^oC (Champney, 1979). Mutant reversion frequencies from temperature sensitivity were determined by plating $2x10^8$ cells on LB plates and counting colonies appearing after 48 hours of incubation at 44^oC. The mutants were tested for lambda lysogeny by streaking a line of control cells, a line of each mutant strain, and a line of the lambda lysogen NO1379 on a Luria plate and cross-streaking these lines with a loopful of purified helper phage. The plate was incubated at 32^oC overnight (Miller, 1972). Lambda lysogens would remain resistant to reinfection by helper phage.

Growth Shift : Growth chracteristics of mutant and control strains were tested by diluting overnight cultures into 5 ml of LB to a Klett reading of 10 ($1X10^8$ cells/ml) in 50 ml Ehrlenmeyer sidearm flasks. Flasks were

placed in a shaking water bath at 32°C. When cells reached a density of $2x10^8$ (Klett reading of 30), they were shifted to a temperature of 42°C and shaken for 3 more hours while recording their Klett readings (cell densities).

Ribosomal Subunit Precursor Analysis : The accumulation of ribosomal subunit precursors in mutants at 42°C was tested. Fifteen minutes after a growth shift from 32°C to 42°C, cultures were labeled with 3H-uridine (luCi/ml, 10 ug/ml) for two hours. Uridine (10 ug/ml) was then added for 30 minutes as a chase. Cells were centrifuged in a table top centrifuge and the supernatants were removed. The pelleted cells were washed in 0.5 ml of R-buffer and recentrifuged. Cells were resuspended in 20 ul of lysis buffer containing 25 mM Tris-HCl pH 7.6, 30 mM KCl, 5 mM MgCl, 15% sucrose, and 100 ug/ml of lysozyme. The lysis procedure was completed by freezing and thawing the cells three times by alternately placing the tubes in a Dewar thermos flask containing 100% methanol and solid CO_2 and then into warm water. Sodium deoxycholate and Brig-58 detergents were added to each lysate to a final concentration of 1%. DNAse I was added to 25 ug/ml and the lysates were allowed to stand at room temperature for 15 minutes. Cell debris was removed by a 1.0 minute centrifugation in a microfuge. Ribosomal particles were resolved by electrophoresis of the cell lysates in composite agarose-acrylamide gels.

<u>Composite Gel Electrophoresis</u> : Fifteen milliliter solutions of 5.0% acrylamide and 1.0% agarose were dissolved separately and placed as liquids in 50 ml Ehrlenmeyer flasks at 50°C in a water bath (Stark et al., 1982). The acrylamide solution contained 5.0% (w/v) acrylamide, 0.25% (w/v) N,N'-methylene bisacrylamide, 0.06 ml 1 M $MgOAc_2$, 89 Mm Tris-borate pH 8.3, 10.0 ml dH_2O. The agarose solution contained 15 ml dH_2O and 0.15 gm agarose. Polymerization was catalyzed by the addition of 20 ul N-N'N'N'-TEMED to the acrylamide solution, mixing the acrylamide and agarose solutions together and then adding 10 ul of freshly prepared 10% $NH_4(SO_4)_2$. The solutions were mixed together quickly and poured into a 15cm X 15cm X 1.5mm gel slab mounted on a Hoefer SE-500 gel electrophoresis apparatus. Ten-well combs were placed in the top of the gels to make 30 ul wells. The gels were allowed to stand at room temperature until cool (30 minutes), then were placed at 4°C for several hours to fully gel. Samples with a total volume of 30 ul were loaded onto the gel. The electrophoresis buffer contained 89 mM Tris-borate pH 8.3, and 2 mM $MgCl_2$. Electrophoresis was at 150v for 3 hours at 4°C. Gels were removed from the gel slabs and subunits were visualized by placing the gels in 200 ml of water containing 5 ug/ml of ethidium bromide and allowing them to soak for 15 minutes. The water-EthBr solution was removed and replaced with 200 ml of water. The gel was placed on a UV transilluminator (230-260nm UV light

wavelength range) which illuminated the stained subunits while the gels were photographed with an MP-4 Polaroid Land camera using P/N-55 film with a Wratten 3A UV filter. The gels were soaked in 1.0 M sodium salicylate (pH 6.2) for 30 minutes and dried onto Whatman paper in a Hoefer gel drying apparatus. 3H-uridine labeled subunits and precursors were visualized by fluorography of the dried gel (Chamberlain, 1979). Kodak-XS 5 X-ray films exposed for 24 hours, developed and then scanned with a densitometer to identify and quantitate the separated subunits and precursor particles.

Cell Free Protein Synthesis Component Isolation : 70S ribosomal particles were isolated from 1 liter of cells grown in LB media to mid-log phase ($2X10^8$ cells/ml). Cells were broken by grinding with levigated alumina (Traub et al., 1971). The lysate mixture was diluted with 3 ml of R-buffer and centrifuged at 10,000 rpm (revolutions per minute) for 10 min in a JA-20 rotor. The resulting supernatant (S30) was layered over 3 ml of R-buffer containing 5% sucrose and 1 M NH_4Cl. It was centrifuged in polycarbonate screw top tubes in a Ti-50 rotor for 4 hours at 34,000 rpm (100,000 X g) to pellet ribosomes. The resultant supernatant (S100) containing the soluble protein synthesis factors washed free by the high salt layer, was dialyzed against R-buffer. The ribosomal pellet was resuspended in approximately 0.5 ml of R-buffer to give about 500 A_{260} units of ribosomes/ml (32.5 mg/ml) and

stored at $-70^{o}C$. Ribosomal preparations were reactivated using the procedure of Zamir et al., (1974). Ribosomal stock solutions were brought to a final magnesium concentration of 50 mM with $MgOAc_2$ and to a final ammonium concentration of 200 mM with NH_4Cl. Solutions were then heated at $40^{o}C$ for 30 minutes. The reactivation maximized ribosomal translational activity in polyuridylate dependant assays. S100 protein concentration was determined by the method of Bradford (1976). Bradford reagent was made by mixing 100mg of Coomassie Brilliant Blue G-250, 50 ml 95% methanol, 100 ml of 85% (w/v) phosphoric acid, and dH_2O to give a final volume of 1 liter.

Cell Free Protein Synthesis Assays : Cell free synthesis of polyphenylalanine was based on the method of Traub et.al.(1971), as previously described (Kushner et al., 1977), using in the incubation mixture 3.0 A_{260} units of 70S ribosomes, 65 ug of S100 protein, 3 ug PEP kinase, 40 ug polyuridylic acid, and approximately 200 pmoles 14C-phenylalanine (525 mCi/mmole), 45 ul of MixI, and a final volume of 150 ul was made up with R-buffer. MixI contains 16.7 mM $MgOAc_2$, 66.8 mM NH_4Cl, 16.7 mM Tris-HCl pH 7.6, 3.33 mM ATP, 0.1 mM GTP, 16.6 mM PEP, 3.3 mM DTT, 12 mM ETSH, 0.167 mM each of 19 L-amino acids (no phenylalanine), 3.33 mg/ml *E. coli* total tRNA, with a final volume of 3.0 ml made up with dH_2O.

Assays were incubated for 30 minutes at $32^{o}C$, $37^{o}C$, and $42^{o}C$. Aliquots of 35 ul were removed at

0, 5, 10, and 30 minutes for kinetic assays. Aliquots were placed in 3 ml of 5% TCA and boiled for 15 minutes at 95°C in a heating block then filtered and counted. Incorporation of 14C-phenylalanine was measured by liquid scintillation counting in a Beckman LS-3155T counter with an efficiency of 90%.

Subunit Mixing Assays : Ribosomal subunits were isolated by layering 200 A_{260} units of 70S ribosomes onto 38 ml 5-20% sucrose gradients made with S-buffer. Gradients were spun in cellulose nitrate tubes in an SW27 rotor at 20,000 rpm at 4°C for 13 hours. One milliliter fractions were collected by dripping after bottom puncture of the tubes and the absorbances at 260nm were read and appropriate fractions were pooled. Subunits were pelleted from pooled fractions by ultracentrifugation at 4°C in a Ti50 rotor at 40,000 rpm for 12 hours. Pellets were resuspened in 0.2 ml of R-buffer. Individual 30S or 50S subunits from the mutants were added with the complementary subunits from control ribosomes for the polyuridylic acid directed cell free protein synthesis assays described above except that 0.5 A_{260} unit of 30S subunits and 1.0 A_{260} unit of 50S subunits were used in each assay. Assays were run for 30 minutes at 32°C and 42°C. TCA precipitable material was prepared for counting as described above.

Inactivation Assays : 70S ribosomes from control and mutant strains were inactivated at 45°C by incubating

5.0 A_{260} units of 70S ribosomes in 50 ul of R-buffer. Aliquots of 1.0 A_{260} unit (10 ul) were removed at 0, 60, 120, and 240 minutes. The remainder of the protein synthesis assay components were added to each aliquot, incubated for 30 minutes at 40°C, and the total 14C-phenylalnine incorporation was determined by liquid scintillation counting. Half-times of heat inactivation of ribosomal activity were determined by extrapolation from lines derived by the least squares method (Arkin and Colton, 1970).

Subunit Reassociation Assays : Two A_{260} units of 70S ribosomes from control and mutant strains were dissociated into 30S and 50S subunits by dialysis overnight in S-buffer. The dissociated subunits were placed in 100 ul aliquots and incubated at 42°C for 1 hour. 1 M $MgOAc_2$ was added to give a final concentration of 20 mM and samples were allowed to incubate for 10 more minutes. Ribosomes were then layered onto 5.0 ml 5-20% sucrose gradients made with R-buffer containing 20 mM $MgOAc_2$ and centrifuged in an SW50.1 rotor for 135 minutes at 30,000 rpm. Gradients were fractionated into 30 equal aliquots, diluted in 1.0 ml R-buffer, and the absorbances at 260nm were determined. Areas of 30S, 50S, and 70S peaks were determined by triangulation and percent reassociation was calculated as (Area 70S peak)/(Area 30S + 50S + 70S peaks)x100.

14C-Phenylalanine tRNA Binding Assays : Bulk *E.*

coli tRNA was charged with 14C-phenyalanine using the incubation mixture described for the kinetics assays but were modified to contain 550 ug tRNA. There was no polyuridylate or ribosomes in the mixture. The whole mixture was incubated at 37°C for 1 hour, extracted 2 times with phenol saturated with 10 mM Tris-EDTA buffer. The tRNA was precipitated out of solution by adding 2 volumes of absolute ethanol. The charged bulk tRNA was dried by evaporation under vacuum and resuspended in 100 ul of R-buffer. Specific activity of the 14C-phenyalanine charged bulk tRNA was 2 pmoles/A_{260} unit (1000 cpm/pmole).

The polyuridylic acid directed binding of 14C-phenylalanine tRNA to 70S ribosomes was tested by incubating 1.0 A_{260} unit of 70S ribosomes with 40 ug of polyuridylic acid, 2.5 A_{260} units of tRNA (5000 cpm total input), and R-buffer to a total volume of 50 ul for 30 minutes at 32°C, 37°C and 42°C. Incubated samples were diluted with 1 ml of R-buffer and collected by filtration on 0.45 um nitrocellulose filters. Filters were then washed 2 times with 5 ml of R-buffer, dried and counted. One pmole of 14C-phenylalanine charged tRNA bound to ribosomes equals 1000cpm retained on the filter.

Misreading Assays : The fidelity of the mutant ribosomes in polyuridylic acid directed cell free protein synthesis assays was tested by the misincorporation of 14C-leucine into polyphenyalanine. The standard cell-free system was used as described, with the omission of L-leucine

in the MixI. 200pmoles of L-phenylalanine were added to each incubation mixture to insure polymerization. Two hundred pmoles of ^{14}C-leucine were added to test direct competition between L-phenylalanine and L-leucine for ribosomal binding sites. Assays were run for 30 minutes at $32^{o}C$ and $42^{o}C$. There were approximately 500 cpm/pmole of ^{14}C-leucine.

Isolation of Ribosomal Proteins : Ribosomal proteins were isolated from 20 A_{260} units (20 ug of protein/A_{260} unit) of 70S particles by acetic acid extraction (Hardy et al., 1969). A volume of 1 M $MgOAc_2$ equal to 1/10th the volume of the aliquoted ribosomal stock solution was added. To that, 2 volumes of distilled acetic acid were added and the solution was placed at $4^{o}C$ for 1 hour. The rRNA precipitate was pelleted in a Beckman table top microfuge and the supernatant was placed in a 15 ml graduated conical Corex test tube. The soluble ribosomal proteins were concentrated by dialysis in 5% acetic acid overnight at $4^{o}C$ followed by lyophilization or by precipitation in 5.0 volumes of cold acetone (Barritault et al., 1976) The acetone precipitated protein was pelleted by centrifugation in a JA-20 rotor at $4^{o}C$.

Gel Electrophoresis of Ribosomal Proteins : Two dimensional polyacrylamide gels run as described by Kenny et al., (1979), were used for characterization of the proteins from mutant strains. The gel solution was pH 5.5 and contained 4% acrylamide, 0.066% N-N'

methylenebis-acrylamide, 38 mM bis-Tris, 8.0 M urea, 0.02% (v/v) N-N'N'N'-TEMED, and 3 ul of 10% $NH_4(SO_4)_2$/ml of gel. The upper reservoir buffer contained 10 mM bis-Tris, 3.6 mM acetic acid, and was brought to a pH of 3.8 with 1 M NaOH. The lower reservoir buffer contained 10 mM bis-Tris, 0.4 mM acetic acid, and was brought to a pH of 6.0 with NaOH in 1.0 liter of dH_2O. Gel tubes were 12mmx2mm (interior diameter) and were filled with 0.5 ml of gel up to 10mm. Approximately 10 ul of isobutanol was layered over the gel to allow for anaerobic polymerization and a smooth gel surface. The isobutanol was removed after polymerization (about 20 minutes) and the 25 ul protein sample and 10 ul of 0.5% Basic fuchsin dye in a 5% sucrose dH_2O solution were loaded on top of the gel and any air bubbles were removed. The gels were then loaded into the tube gel apparatus.

The alternative two-dimensional gel electrophoresis method of Howard and Traut (1973) was also used to characterize mutant proteins. The first dimension gel solution for this system was pH 8.6 and contained 4% acrylamide, 0.13% N-N' methylenebis-acrylamide, 20 mM Na_2EDTA , 0.52 M borate, 0.4 M Tris, 6.0 M urea, 1 ul N-N'N'N'-TEMED/ml of gel, and 3 ul of fresh 10% $NH_4(SO_4)_2$/ml of gel. The polymerization and loading procedure were the same as for the above first dimension gel system except that 0.5% pyronine Y was the tracking dye. The 1X upper and lower reservoir running

buffers were made from the same 10X stock which contained 60 mM Tris, 60 mM borate, 3 mM Na_2EDTA, and was brought to a pH of 8.3 with NaOH in a final volume of 1 liter. Pyronine Y was used as the indicator tracking dye. Both first dimension gel systems were run at a constant current of 2.5 mA/gel for 6 hours.

In both cases, the first dimension gels were removed from the glass tubes and soaked for 30 minutes in 10 ml of soaking buffer. Soaking buffer was pH 5.2 and contained 8.0 M urea, 0.75 ml glacial acetic acid, and 0.67 gm KOH dissolved in a final volume of 1.0 liter. Soaked first dimension gels were then loaded onto freshly poured second dimension slab gels and allowed to polymerize in place (approximately 30 minutes). The second dimension gel solution was pH 5.5 and contained 18% acrylamide, 0.25% methylene bis-acrylamide, 0.92 M glacial acetic acid, 0.048 M KOH, 6.0 M urea, 0.058 ml N-N'N'N'-TEMED/10 ml of gel, and 200 ul of fresh 10% $NH_4(SO_4)_2$ (v/v)/10 ml of gel. The gel slab was 15cm X 15cm X 1.5mm and held approximately 30 ml of gel solution. The 10X running buffer was pH 4.0, containing 180 mM glycine, and 6 mM acetic acid. The upper reservoir buffer was 1X and lower reservoir buffer was 0.3X. Pyronine Y was used as the indicator tracking dye. Proteins were visualized by staining with Coomassie Blue G-250 (Reisner, 1984). Photographs of the stained gels were taken with a MP-4 Polaroid Land camera and P/N-55 polaroid film using a yellow filter.

<u>Transductional Mapping of t.s. Mutations</u> : Plvir phage was used to preliminarily map the temperature-sensitive gene locus in each of the four mutants described in this study. Plvir grown on the <u>E. coli</u> strain SK901 was used to reinfect the mutants and make lysates. Overnight 1.0 ml cultures of each mutant were grown at 32°C in LB. The cells were pelleted in a table top centrifuge and the LB was discarded. Cells were resuspended in 1.0 ml of 5.0 mM $CaCl_2$. One tenth of a milliliter of the calcium-cell suspension was mixed with 0.1 ml of the Plvir phage stock ($2X10^{11}$ phage/ml) and placed in a 37°C waterbath for 20 minutes. The infected cells were mixed with 3.0 ml of TP top agar which had been melted and equilibrated to 45°C. The agar-cell-phage mixture was poured and spread onto a LB plates and placed at 32°C overnight. Five milliliters of LB was poured onto the soft agar and the soft agar was scraped off the plate into a 30 ml Corex test tube. The agar, broth, cell debris, and phage were mixed with a glass rod and centifuged in a JA-20 rotor in a at 10,000 rpm for 10 minutes. The 5 ml supernatant of phage was placed in a sterile screw top test tube with 2-3 drops of chloroform, sealed, and stored at 4°C. Dilutions of the phage lysates grown on mutant strains were titered on strain SK901 by the method of Miller, (1972). Both 10^{-3} and 10^{-6} dilutions were made of each lysate in 5.0 mM $CaCl_2$. The 10^{-3} dilution was plated directly to check for possible light lysates as most

titers of such lysates contain approximately 10^{10} phage/ml. The 10^{-6} dilution was subsequently rediluted to 10^{-8} and 10^{-10}. One tenth of a milliliter of each dilution was added to 0.1 ml of an overnight of strain SK901, incubated as before, mixed with soft agar, and plated as described above. Plates were incubated at 32°C overnight. All lysates showed a titer of approximately 10^{-10} phage/ml. E. coli strains used as recipients were strains AB2834, and JC5029 described in Table 1. Overnight 1.0 ml cultures of AB2834 and JC5029 were grown in LB. Strain AB2834 was infected with lysates from KK1901 and KK1902 as described above with the exception that they were plated directly on minimal agar plates to select for aroE+ transductants at 32°C. Strain JC5029 was infected with lysates from KK2901 and KK2902 as described. The infected cells were allowed to grow for 6 hours to express the recombined strR allele before being plated on LB plates containing 100 ug of streptomycin sulfate to select for the streptomycin resistant recombinants at 32°C. One hundred transductants isolated from each of the infections were picked to Luria master plates and then replica plated either to LB plates and placed at 44°C or to LB plates containing 100 ug of spectinomycin and placed at 32°C overnight. Cotransduction was assayed as the inheritance of unselected t.s. or t.r. loci in antibiotic resistant or aroE+ selected transductants.

Chapter 3

RESULTS

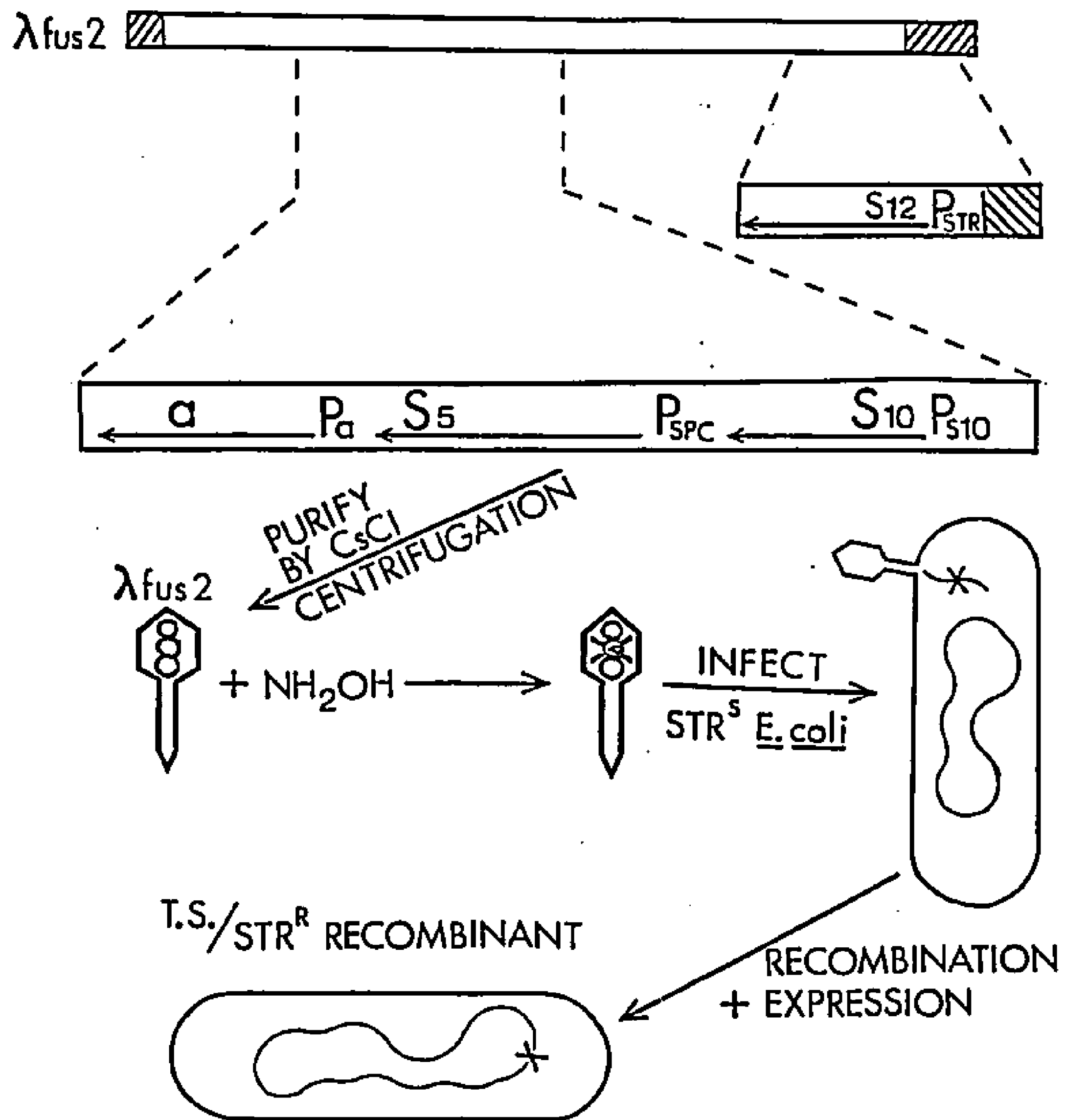
The defective transducing phage lambda fus2 (Figure 2) has been shown to carry genes for 27 ribosomal proteins as well as genes for elongation factors EF-Tu, EF-G, and the alpha subunit of RNA polymerase (Jaskunas et al., 1977a). The purified phage therefore presented an enriched source of genes for E. coli translational components. Mutagenesis of the bacterial DNA carried by the phage would be expected to yield substantial numbers of mutations in the genes for these translational components (Figure 3). The streptomycin-resistance allele (rpsL) carried by the defective phage permitted an efficient selection of generalized recombinants after transduction of an appropriate streptomycin-sensitive recipient cell. Recombinants selected at 32°C were easily tested by replica plating for linked 42°C temperature sensitivity.

Mutant Isolation : The optimum number of strR recombinants was achieved using a cell density of $2X10^8$ cells/ml infected with mutagenized lambda fus2 at an multiplicity of infection of 1. About 10^4 strR recombinants were recovered under these conditions. In two separate experiments, linked temperature sensitive mutants were recovered at a frequency of about 40% (70 out of 170 colonies tested). Cells transduced with phage mutagenized for 4 or 8 hours gave the same total number of transductants but fewer temperature-sensitive colonies. Transduction of

FIGURE 3. LOCALIZED MUTAGENESIS USING LAMBDA FUS2. THE *E. coli* STRAIN NO1379 (FROM M. NOMURA) IS A DOUBLE LYSOGEN HARBORING LAMBDA FUS2 AND THE HELPER PHAGE LAMBDA c1857s7 WHICH CONTAINS A MUTATION CODING FOR A TEMPERATURE-SENSITIVE REPRESSOR PROTEIN. LAMBDA FUS2 CARRIES 27 RIBOSOMAL PROTEIN GENES INCLUDING THE RPSL ALLELE WHICH CODES FOR STREPTOMYCIN RESISTANCE, AS AN ALTERED FORM OF PROTEIN S12.

PHAGE PRODUCTION WAS STIMULATED BY SHIFTING THE GROWING LYSOGEN TO 42°C. THE TWO PHAGE IN THE RESULTING MIXED LYSATE WERE SEPARATED AND PURIFIED BY ISOPYCNIC CENTRIFUGATION IN CsCl. THE DEFECTIVE PHAGE BAND WAS REMOVED FROM THE GRADIENT, DIALYZED, AND TREATED WITH 1.0 M HYDROXYLAMINE FOR 12 HOURS. A STRS STRAIN OF *E. coli* WAS INFECTED WITH THE MUTAGENIZED DEFECTIVE PHAGE, ALLOWED TO EXPRESS OVERNIGHT AT 32°C, AND THEN PLATED ON STREPTOMYCIN. STRR RECOMBINANTS WERE SCREENED FOR TEMPERATURE-SENSITIVITY AT 44°C.

FIGURE 3. LOCALIZED MUTAGENESIS USING LAMBDA FUS 2



an aroE- strain (AB2834) under the same conditions gave similar numbers of aroE+ transductants and linked temperature sensitive mutants when selected on minimal agar plates. The aroE+ locus was also carried by lambda fus2 but was at the opposite end of the phage DNA from the strR locus (Figure 2). The aromatic amino acid requirement allowed another strong positive selection with an efficiency equal to the strR selection, so that strains which were already strR, could also be used as recipients. No transductants were found when the recA strain SK571 was used as the recipient. It was necessary to show that the mechanism described for lambda recombination was in fact the same mechanism by which our strR transductants had been formed. The recA gene product (recA protein) catalyzes several DNA repair processes in E. coli and has an intrinsic role in the molecular mechanism of strand uptake in recombination. Strains of E. coli such as SK571, which have the recA phenotype, are deficient in generalized recombinational processes and do not repair damage to their DNA efficiently. The result is low survival rates after exposure to a DNA damage inducer such as ultraviolet light. Strain SK571 had a 10^6 fold loss of viable cells after a 1.0 minute exposure to U.V. irradiation while the control strain had only about a 10^3 fold loss of viable cells. These results strongly suggest that the transductants we isolated were in fact the result of the recombination of phage-carried bacterial sequences into the chromosome of the

recipient host and that each transductant was selected based on the concomitant expression of those genes.

Initial Characterization of Mutants : The mutants described in this study were not lysogenic for phage lambda as all were sensitive to lambda helper (lambda cI857S7) infection and were strR. Merodiploid lysogens which carried two sets of a number ribosomal protein genes (one set from lambda fus2 and one set from the host) would give experimental results that would be difficult to interpret. For lambda fus2 to be incorporated as a lysogen would require that the helper phage genome be present to provide the necessary phage-coded accessory proteins. Lysogens already harboring the helper phage lambda cI857S7 would be temperature-sensitive and would appear to be t.s. mutants. Such lysogens however, are also resistant to reinfection by helper phage, which provided a convenient method to test for lysogeny in our mutants. The fact that only haploid strR recombinants could survive the selection on streptomycin, gave additional evidence that the mutants were not lysogens.

From these experiments four strR temperature-sensitive mutants were picked for further characterization. Mutants KK2901 and KK2902 showed temperature sensitivity on Luria plates at 37°C as well as at 42°C. An additional screen showed that those two mutants were resistant to spectinomycin. Out of the 170 t.s. strR recombinants which were screened, they were the only two which showed spcR phenotypes. The possibility that they were both double

mutants made them attractive for futher characterization. Mutants KK1901 and KK1902 were temperature-sensitive on Luria plates at 42°C but were chosen to further characterize because they stopped growing, after a growth shift, faster than all of the other mutants which were screened except KK2901 and KK2902. Figure 4 shows the growth characteristics for mutants KK1902 and KK2902 and the control after a temperature shift from 32°C to 42°C. The growth rates of the mutants at 32°C were slightly lower than the control strain and growth was inhibited altogether after 2 hours at 42°C. Mutants KK1901 and KK2901 showed growth shift responses similar to the other two mutants.

Summary of Control and Mutant Phenotypes : A summary of the genetic and phenotypic features of the four mutants selected is presented in Table 2. Temperature resistant revertants were selected from these mutants at frequencies of 10^{-7} to 10^{-8}, suggesting that single point mutations were responsible for the t.s. phenotype (Miller, 1972). Mutants KK2901 and KK2902 were resistant to concentrations of up to 100 ug/ml of spectinomycin. The spcR phenotype was not a property of the defective phage or the host cell, suggesting that these could be double mutants. The same two mutants were found to be resistant to neamine at concentrations up to 100 ug/ml, while the other two mutants showed wild-type sensitivities to the same concentrations of both spectinomycin and neamine. Neamine

FIGURE 4. GROWTH SHIFT EXPERIMENT. LINES REPRESENT SK901 (O) AND MUTANTS KK1902 (□) AND KK2902 (△) IN A GROWTH SHIFT FROM 32°C TO 42°C (ARROW). CELL DENSITY CHANGES ARE MEASURED BY READINGS FROM A KLETT-SUMMERSON COLORIMETER.

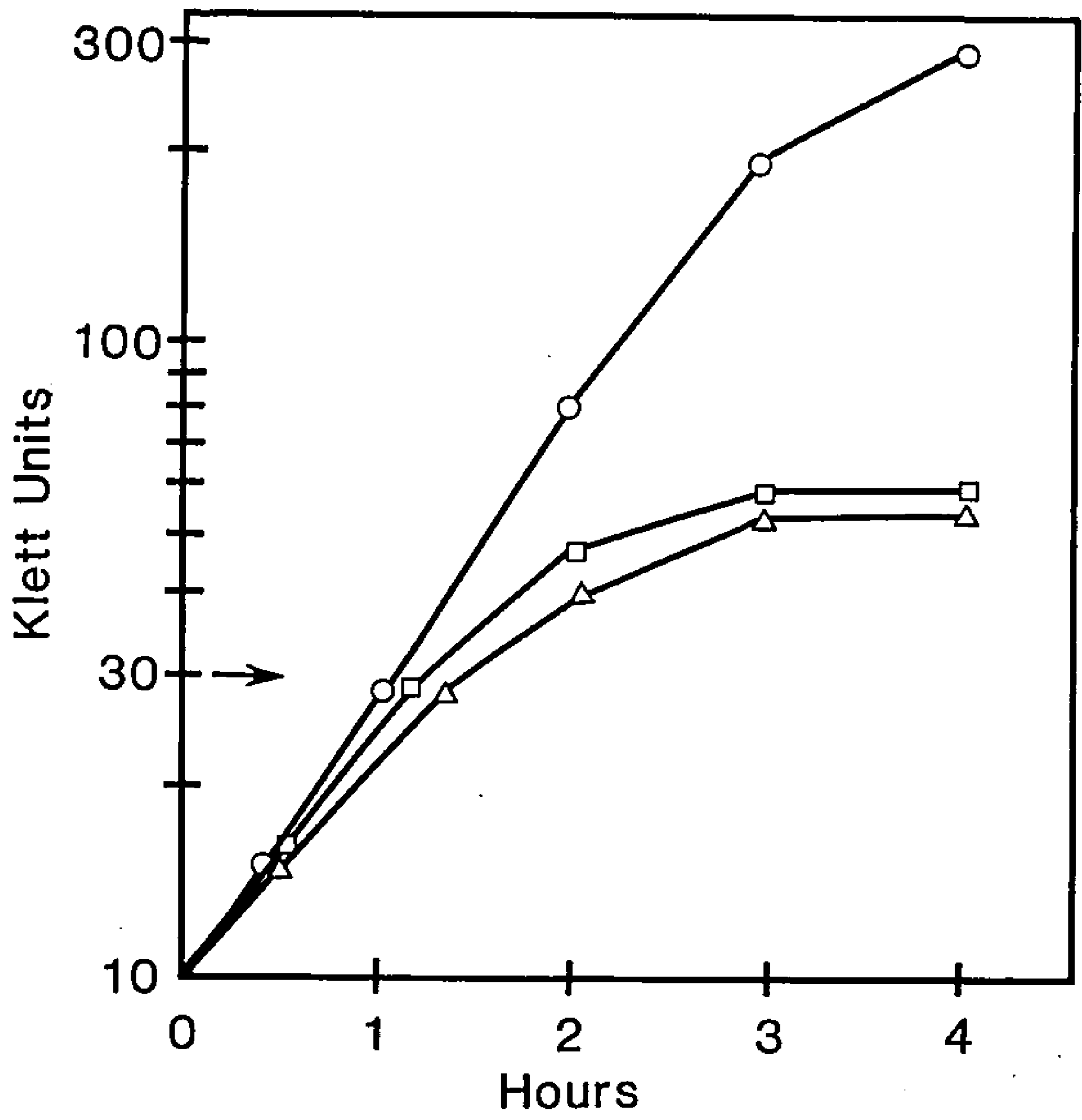

300
100
30
10
Klett Units
0
1
2
3
4
Hours

TABLE 2. SUMMARY OF CONTROL AND MUTANT PHENOTYPES.

STRAIN	GENERATION TIME 32°C	42°C	ANTIBIOTIC S/R*			REVERSION FREQUENCY
SK901	30 MIN	25 MIN	STR^S	SPC^S	NEA^S	-
KK1901	30 MIN	>180 MIN	STR^R	SPC^S	NEA^S	1×10^{-8}
KK1902	45 MIN	>180 MIN	STR^R	SPC^S	NEA^S	$<1 \times 10^{-8}$
KK2901	40 MIN	>180 MIN	STR^R	SPC^R	NEA^R	1×10^{-7}
KK2902	40 MIN	>180 MIN	STR^R	SPC^R	NEA^R	$<1 \times 10^{-8}$

*S = COMPLETE GROWTH INHIBITION AT 37°C ON REPLICA PLATES CONTAINING 100 µG/ML OF STREPTOMYCIN, SPECTINOMYCIN, OR NEAMINE

R = RESISTANCE TO GROWTH INHIBITON BY ANTIBIOTICS INDICATED

STR = STREPTOMYCIN, SPC = SPECTINOMYCIN,

NEA = NEAMINE

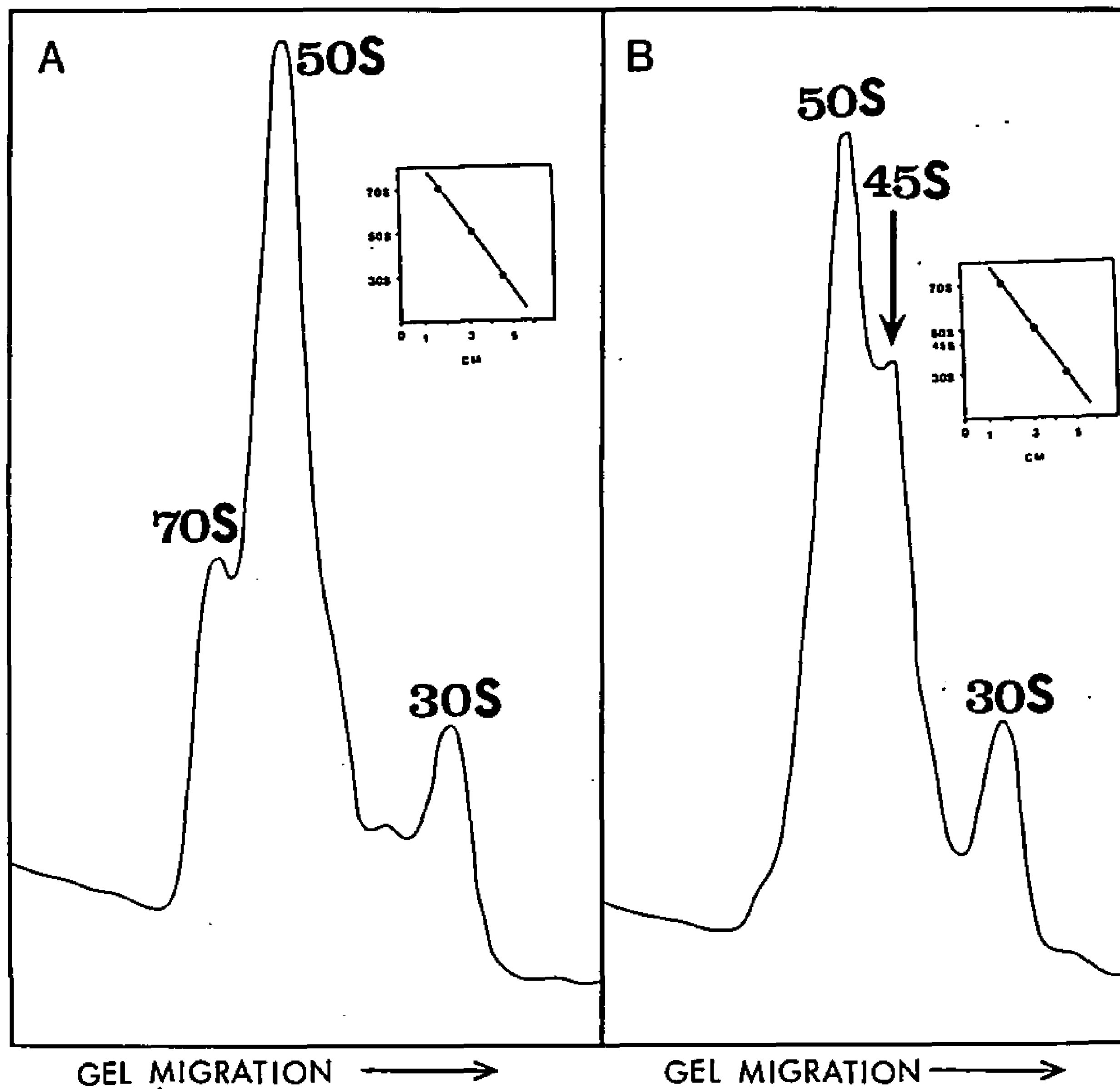
is a hydrolytic product of neomycin (Vasquez, 1979). One molecule of neomycin yields 2 molecules of neamine. All four mutants were similar to the control in their sensitivity to the additional ribosomal antibiotics erythromycin, neomycin, and kanamycin.

Ribosomal Subunit Precursor Analysis : In an attempt to distinguish any ribosomal structural and functional defects which might be associated with the t.s. phenotypes of the four mutants, we first examined ribosomal subunit assembly in vivo. The assembly of normal ribosomal components into functional particles has been broken down into a number of obligate steps for both 30S and 50S subunits (Neirhaus, 1982). Each step in subunit assembly requires specific protein-RNA and protein-protein interactions. The sedimentation coefficient for each precursor particle formed at each step is unique to that particle and can be used as an indication of what constituents of the ribosome are in place on the particle and at what point the assembly process could have been blocked by an abnormal, temperature-induced, conformational change. Such defects have been shown to be characteristics of a number of mutants isolated previously (Held et al., 1974; Neirhaus, 1982). The sedimentation coefficients of the ribosomal particles in growing cells can be determined by extrapolation from a linear correlation generated by plotting known sedimentaion coefficients against migration distance in composite gels.

RNA in control and mutant cells was labeled with 3H-uridine both at 32°C as a control, and after a shift to 42°C. The cells were lysed, and ribosomal particles were separated on composite agarose-acrylamide gels. The initial identification of ribosomal subunit locations in the gels was by EthBr staining. Patterns revealed good separation of the subunits. Densitometer tracings of exposed X-ray films from fluorograms of the dried gels (Figure 5), showed that subunits from mutants labeled at 32°C were not different from control subunits labeled at 32°C. Ribosomal particles from cells labeled at 42°C were also similar to controls labeled at 42°C (Figure 5A), with one exception. Figure 5B shows the densitometric tracing of subunit bands made from the exposed film for mutant KK1901 labeled at 42°C. The tracing shows a 45S, large subunit precursor particle (arrow), which accumulated after the temperature shift. No 70S ribosomal particles were found in that lysate. The sedimentation coefficients of control particles were extrapolated from a linear correlation descibed above (Inset graph Figure 5A). Subunits from mutant KK1901 (Figure 5B) also showed a linear correlation between sedimentation coefficient and particle migration distance (Inset graph Figure 5B) onto which the peak identified by the arrow can be extrapolated as having a sedimentation coefficient of 45S.

Subunit Reassociation Assays : The functional ability of subunits from mutant ribosomes to reassociate into 70S

FIGURE 5. RIBOSOMAL SUBUNIT PRECURSOR ANALYSIS. DENSITOMETER TRACINGS OF A FLUOROGRAM FROM COMPOSITE AGAROSE-ACRYLAMIDE GELS ARE SHOWN. FIGURE 5A SHOWS THE TRACING OF THE RIBOSOMAL PROFILE OF THE CONTROL STRAIN SK901 LABELED WITH ^{3}H-URIDINE AT 42°C. PEAKS FROM LEFT TO RIGHT ARE 70S, 50S AND 30S. FIGURE 5B SHOWS THE TRACING OF THE PROFILE FROM MUTANT KK1901 LABELED AT 42°C. PEAKS FROM LEFT TO RIGHT ARE 50S, 45S (ARROW), AND 30S.

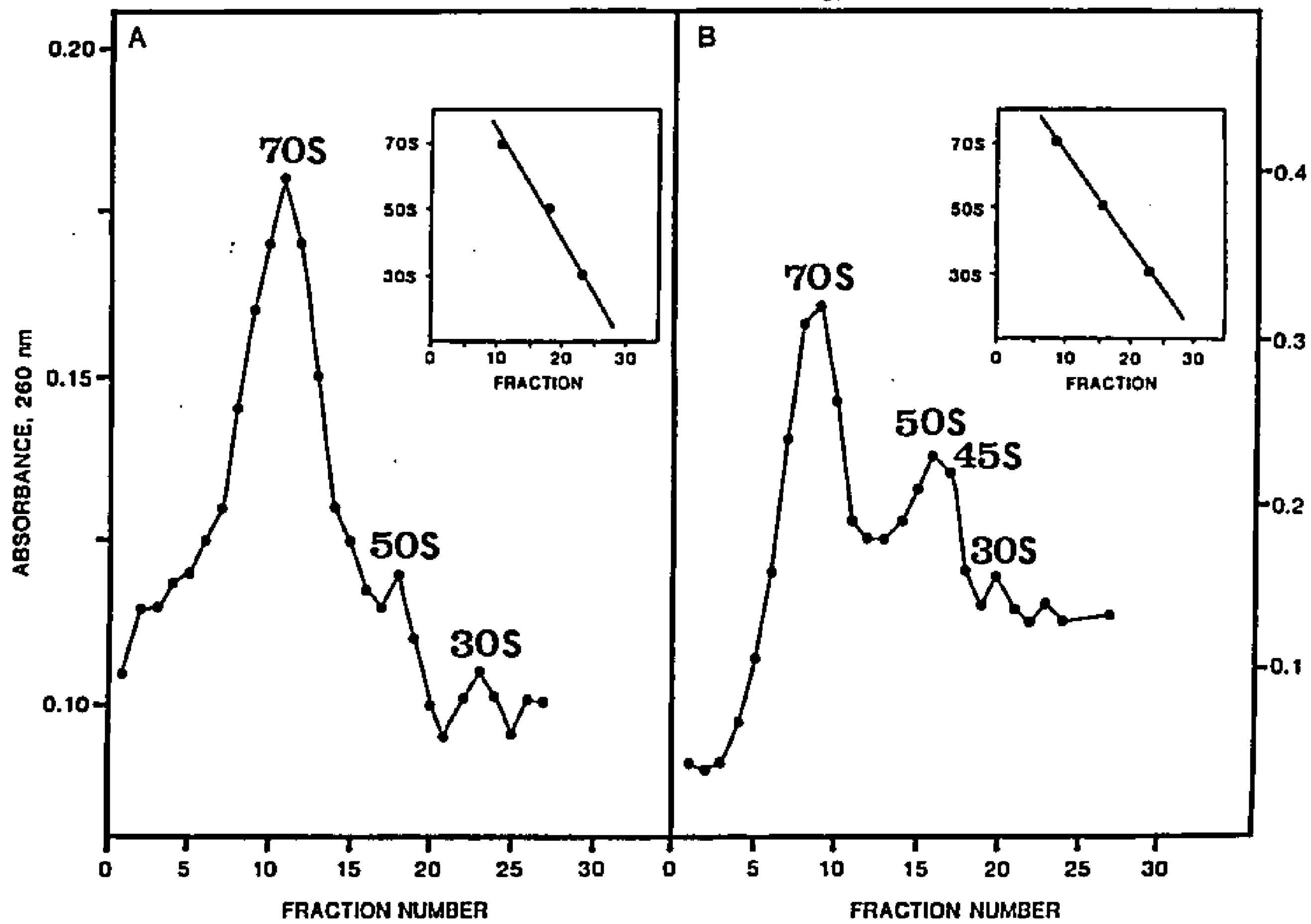


A
50S
70S
30S
GEL MIGRATION
B
50S
45S
30S
GEL MIGRATION

ribosomal particles subsequent to heating at 42°C was assayed by sucrose gradient sedimentation and results are represented in Figure 6. After incubation at 32°C, all 4 mutants and the control had profiles similar to Figure 6A. In addition, at 42°C, the control and three of the mutants also had profiles similar to Figure 6A. By examining peak areas as described in Material and Methods, the profile in Figure 6A represents approximately 89% reassociation of subunits into 70S monomers. The sedimentation coefficient for subunits in representative peaks were determined by extrapolation from a line constructed by plotting particle sedimentation coefficient versus fraction number (inset graphs). Mutant KK1901 had a profile which was similar to the control at 32°C but had only 57% reassociation of its subunits after heating at 42°C. Results in Figure 6B show a reduced 70S peak, a 50S peak with a 45S shoulder, and a 30S peak. Once again the sedimentation coefficients of particles in representative peaks were determined by extrapolation from the line in the inset graph in Figure 6B.

<u>Cell Free Translation Assays</u> : The ribosome was identified as the site of temperature-sensitivity in these mutants by cell-free translation assays. The S100 protein fraction from each mutant supported polyuridylate translation with high efficiency at 42°C using control 70S ribosomes. The S100 supernatant fraction contains the soluble protein factors necessary to support protein

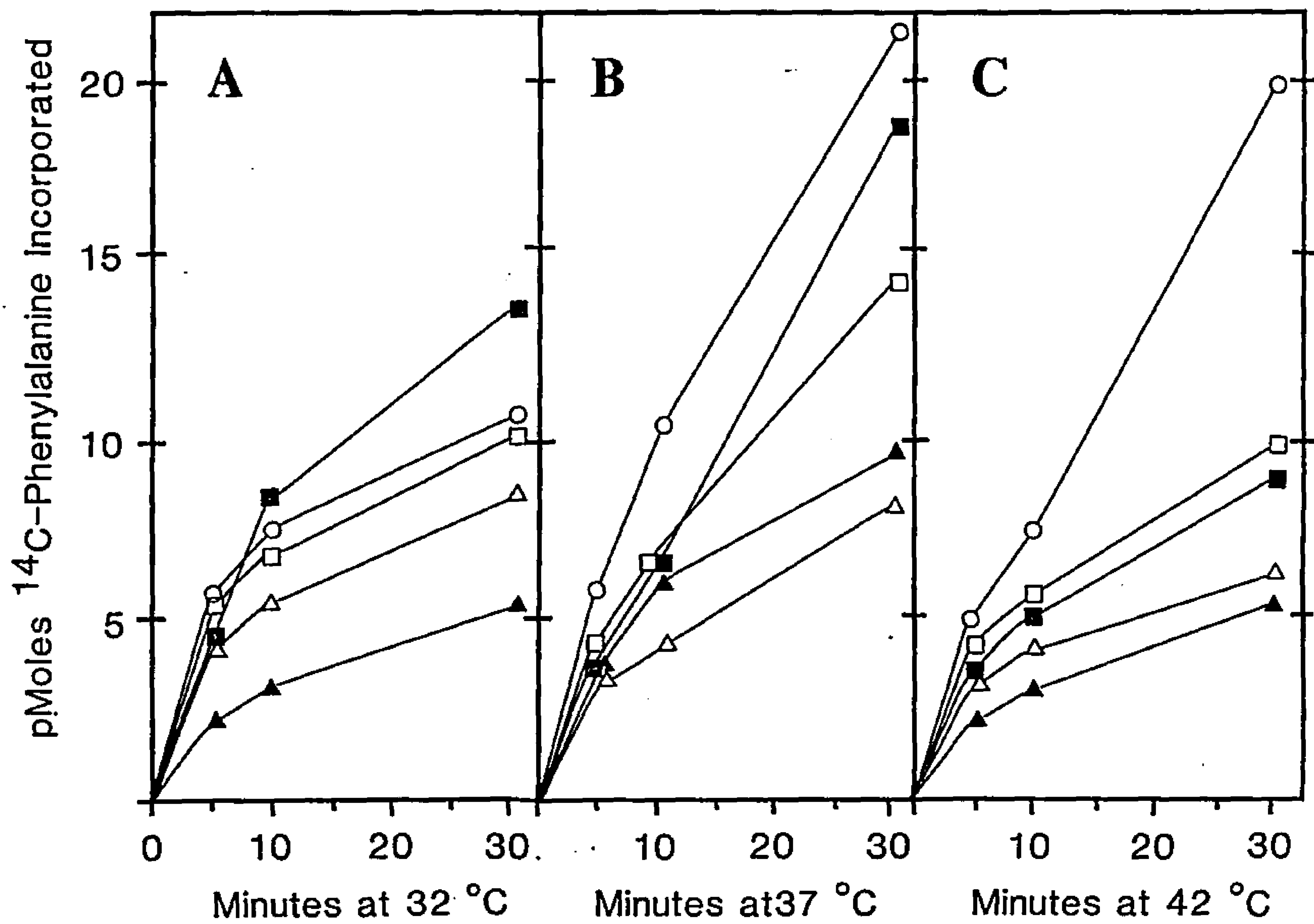
FIGURE 6. SUBUNIT REASSOCIATION PROFILES. THE EXTENT OF REASSOCIATION FOR THE CONTROL SK901 AND THREE MUTANTS WERE SIMILAR AT BOTH 32°C AND 42°C (6A). THEY SHOWED 80-90% REASSOCIATION. MUTANT KK1901 HAD A PROFILE SIMILAR TO THE CONTROL PROFILE AT 32°C BUT HAD ONLY 57% REASSOCIATION AFTER HEATING AT 42°C (6B). INSETS SHOW A LINEAR CORRELATION BETWEEN PARTICLE MIGRATION AND FRACTION NUMBER FROM WHICH THE SEDIMENTATION COEFFICIENTS OF EACH PARTICLE CAN BE EXTRAPOLATED.


A
B
70S
50S
30S
70S
50S
45S
30S
ABSORBANCE, 260 nm
FRACTION NUMBER
FRACTION NUMBER
FRACTION
FRACTION
0.20
0.15
0.10
0.4
0.3
0.2
0.1

synthesis but contains no 70S ribosomes. It was important to test the S100 fraction from each mutant to determine if there was a t.s. defect in any nonribosomal protein synthesis components coded for on lambda fus2 which could have been mutated. The control S100 was also tested without the addition of 70S ribosomes to insure that the supernatant did not show any internal protein synthesis activity which removed the possibility of artificially high or inaccurate incorporations. The 70S ribosomes from all four mutants had reduced levels of protein synthesis at high temperature in single time-point assays.

<u>Kinetics of Protein Synthesis of Mutant Ribosomes</u> : Kinetic assays were performed to examine the relative rates and extents of polyphenylalanine synthesis for the ribosomes from each mutant as a function of temperature. As shown in Figure 7A , at 32°C the initial translation rates with ribosomes from mutants KK1901, KK1902 and KK2902 were comparable to those of the control ribosomes. However, the overall extent of translation was diminished for ribosomes from these three mutants. At 37°C (Figure 7B), the kinetic studies revealed a slightly lower activity for ribosomes from strain KK1901 while the other three mutants showed a substantially reduced activity when compared to the control. The overall extent of translation in the control was maximal at 37°C. Figure 7C shows that at 42°C both the rate and extent of cell-free translation by ribosomes from each mutant were reduced by 50-70% compared

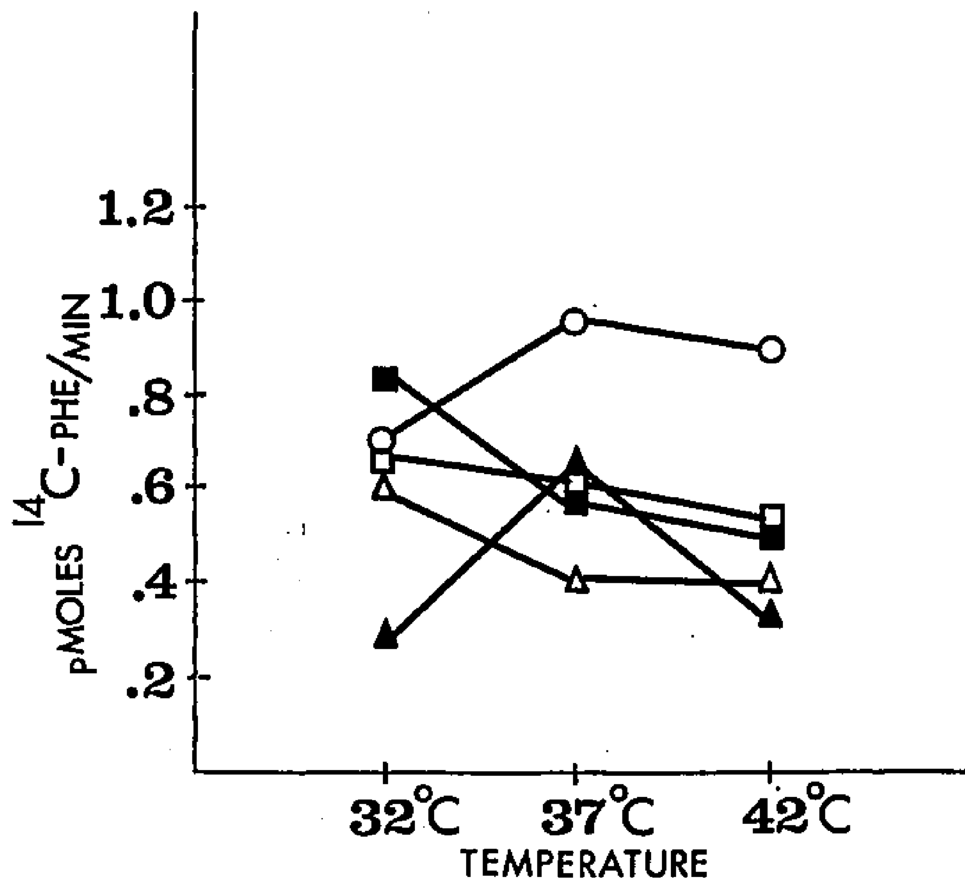
FIGURE 7. KINETIC ANALYSIS OF CELL-FREE PROTEIN SYNTHESIS. THE KINETICS OF PROTEIN SYNTHESIS WITH RIBOSOMES FROM CONTROL STRAIN SK901 (O), AND MUTANT STRAINS KK1901 (■), KK1902 (□), KK2901 (▲), AND KK2902 (△) WERE DETERMINED BY MEASURING POLY-URIDYLATE DIRECTED ^{14}C-PHENYLALANINE INCORPORATION. KINETIC ASSAYS ARE SHOWN AT 32°C (7A), 37°C (7B), AND 42°C (7C). RESULTS ARE THE AVERAGE OF TRIPLICATE ASSAYS. INDIVIDUAL ASSAYS WERE WITHIN $\pm$ 20% OF THE AVERAGE.


A
B
C
pMoles ^{14}C–Phenylalanine Incorporated
20
15
10
5
0
10
20
30
Minutes at 32 °C
Minutes at37 °C
Minutes at 42 °C

with the control activity, consistent with the temperature-sensitive activity seen in vivo. An S100 fraction from wild-type cells was used in each of these assays to compare more directly the effects of temperature on the activity of only the ribosomes from the mutant cells.

Relative Rates of Protein Synthesis : The relative rates of protein synthesis in the mutants were determined by examination of the initial velocities of their individual kinetic profiles. The first three time points from each line were extrapolated to a straight line by the least squares best-fit method (Arkin and Colton, 1970). The slopes of each line from each mutant were plotted as points against each temperature tested and are displayed graphically in Figure 8. It shows the effect of temperature on the relative rates of protein synthesis activity of the ribosomes from the four mutants. At 32°C mutants KK1901, KK1902 and KK2902 showed rates comparable to the control ribosomes. At 37°C they showed a decrease in their rates which remained low at 42°C. These results indicated relatively strong temperature effects in these mutants. Mutant KK2901 showed initially low rates at 32°C. At 37°C, KK2901 showed a marked increase in its relative rate of protein synthesis. It increased in a similar fashion to the control rates which were optimal at that temperature. At 42°C, KK2901 had a marked decrease in its relative rate of protein syntesis. These results might

FIGURE 8. EFFECTS OF TEMPERATURE ON THE RELATIVE RATES OF CELL-FREE PROTEIN SYNTHESIS. SLOPES OF THE INITIAL VELOCITY PORTIONS OF THE KINETIC CURVES FROM FIGURE 7 WERE PLOTTED AGAINST TEMPERATURE FOR THE CONTROL AND THE FOUR MUTANTS. INITIAL VELOCITY LINES WERE REGENERATED BY THE METHOD OF LEAST SQUARES FOR THE FIRST THREE TIME POINTS FROM EACH KINETIC CURVE. RESULTS SHOW THE EFFECT OF TEMPERATURE ON THE RELATIVE RATES OF PROTEIN SYNTHESIS FOR THE CONTROL (○), AND MUTANTS KK1901 (■), KK1902 (□), KK2901 (▲), AND KK2902 (△).



1.2
1.0
.8
.6
.4
.2
pMOLES 14C-PHE/MIN
32°C
37°C
42°C
TEMPERATURE

indicate a more complex defect in this mutant.

Kinetics of Inactivation : The kinetics of heat inactivation of mutant and control ribosomes are shown in Table 3. Inactivation is depicted as the half-time in minutes at 45°C. All mutant strains except KK1902, showed a rate of inactivation at least twice that of the control at 45°C. The 40°C assay temperature was chosen in an attempt to minimize possible reactivation without increasing the inactivation process.

Mixed Subunit Complementation Assays : In an attempt to isolate the subunit location of the t.s. defect in each mutant, mixed subunit complementation assays were performed in four different combinations. Results are shown in Table 4. The values indicate the number of pmoles of 14C-phenylalanine incorporated in 30 minute protein synthesis assays at the temperatures shown using the combinations of subunits indicated. In mutants KK1901, KK2901, and KK2902 the t.s. activity was relieved when control 30S subunits were mixed with the respective mutant 50S subunit, indicating 30S defects in those mutants. Mutant KK1902 showed somewhat ambiguous results which could be interpreted to indicate a t.s. 50S subunit.

14C-Phenyalanine Transfer RNA Binding Assays : Another functional test was performed to determine the efficiency of polyuridylate-programmed mutant ribosomes in binding 14C-phenylalanine tRNA relative to control ribosomes at several temperatures. Results in Table 5 show that mutant

TABLE 3. INACTIVATION RATES FOR RIBOSOMES FROM CONTROL AND MUTANT STRAINS

STRAIN	T 1/2* 45°C
SK901	360
KK1901	175
KK1902	880
KK2901	80
KK2902	140

*T 1/2, THE TIME REQUIRED FOR 50% LOSS OF ACTIVITY WAS CALCULATED FROM THE SLOPES OF INACTIVATION CURVES DERIVED FROM KINETIC EXPERIMENTS WITH 70S RIBOSOMES FROM CONTROL AND MUTANT STRAINS. THE RELATION T 1/2 = -LN2/SLOPE WAS USED IN THE CALCULATION WITH SLOPES COMPUTED BY THE METHOD OF LEAST SQUARES. RESULTS ARE THE AVERAGE OF TRIPLICATE ASSAYS. INDIVIDUAL ASSAYS WERE WITHIN ± 10% OF THE AVERAGE.

TABLE 4. MIXED SUBUNIT COMPLEMENTATION ASSAYS

	32°C			
	30S-C	30S-M	30S-M	30S-C
STRAIN	50S-C	50S-M	50S-C	50S-M
SK901	105.2	-	-	-
KK1901	-	12.3	8.6	103.5
KK1902	-	62.7	111.6	95.5
KK2901	-	10.0	29.3	58.7
KK2902	-	2.3	0.5	16.5

	42°C			
	30S-C	30S-M	30S-M	30S-C
STRAIN	50S-C	50S-M	50S-C	50S-M
SK901	108.2	-	-	-
KK1901	-	39.0	28.9	61.9
KK1902	-	94.4	81.8	66.4
KK2901	-	49.7	40.4	79.8
KK2902	-	4.1	2.9	44.3

THE VALUES INDICATED ARE PMOLES OF ^{14}C-PHENYLALANINE INCORPORATED IN A 30 MINUTE ASSAY AT 32°C AND 42°C WITH MIXED RIBOSOMAL SUBUNITS AS DESCRIBED IN MATERIALS AND METHODS. THE SOURCE OF THE SUBUNIT IS DESIGNATED AS CONTROL (SK901) 30S (30S-C) OR 50S (50S-C) AND MUTANT 30S AS (30S-M) OR 50S (50S-M). RESULTS ARE THE AVERAGE OF TRIPLICATE ASSAYS. INDIVIDUAL ASSAYS WERE WITHIN $\pm$ 20% OF THE AVERAGE.

TABLE 5. ^{14}C-PHENYLALANINE TRANSFER RNA BINDING BY CONTROL AND MUTANT RIBOSOMES

STRAIN	32°C	37°C	42°C
SK·901	2.2	2.0	2.3
KK 1901	2.5	2.3	2.4
KK 1902	2.1	1.7	1.7
KK 2901	0.4	0.6	0.6
KK 2902	0.7	0.5	0.6

Results indicate pmoles ^{14}C-phenylalanine tRNA bound per A_{260} unit of 70S ribosomes in a poly-uridylate dependent assay. Input was 4.5 pmoles ^{14}C-Phe in total tRNA. Results are the average of duplicate assays. Individual assays were within $\pm$ 10% of the average.

KK1901 had activity very similar to control activity at all three temperatures. Mutant KK1902 had control-levels of binding at 32°C but was somewhat reduced at 37°C and remained at this same level at 42°C. Mutants KK2901 and KK2902 both had relatively low levels of activity at all temperatures. KK2901 did, however, show a slight increase in binding activity from 32°C to 37°C where it remained at 42°C. Mutant KK2902 had relatively low activity at all three temperatures. Three of the four mutants were clearly t.s. for ^{14}C-phenylalanine tRNA binding at 37°C and 42°C.

Misreading of Polyuridylate : A third functional assay was used to determine the fidelity with which mutant and control ribosomes translated polyuridylate. Mistranslation was measured by the incorporation of ^{14}C-leucine into polyphenylalanine. Table 6 indicates that control ribosomes and ribosomes from mutants KK1901, KK1902, and KK2902 had between 3-5% misincorporation of ^{14}C-leucine at both 32°C and 42°C. Mutant KK2901 showed a 10% misincorporation of ^{14}C-leucine at 32°C and 28% misincorporation at 42°C.

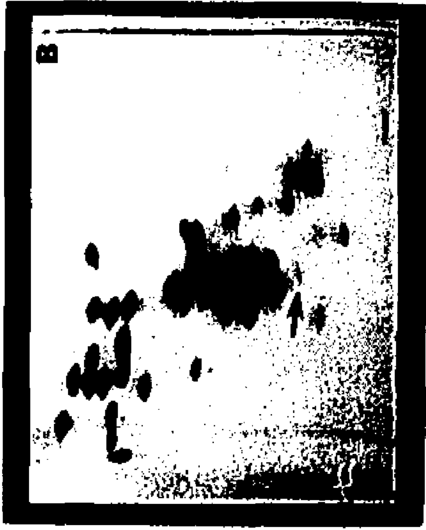
Gel Electrophoresis of Ribosomal Proteins : The functional assays suggested the ribosome as the site of the translational defects. Two dimensional polyacrylamide gels of ribosomal proteins were used to search for possible ribosomal protein changes in these mutants. Figure 9 shows the gel patterns of proteins from SK901, KK1902, and KK2902

TABLE 6. MISREADING OF POLYURIDYLATE BY CONTROL AND MUTANT RIBOSOMES

STRAIN	32°C PMOLES [^{14}C]-PHE	PMOLES [^{14}C]-LEU	% MISREADING	42°C PMOLES [^{14}C]-PHE	PMOLES [^{14}C]-LEU	% MISREADING
SK901	13.8	0.7	5.3	21.2	0.8	3.9
KK1901	35.3	0.9	2.4	25.1	0.9	3.5
KK1902	26.3	1.1	4.2	23.4	1.1	4.9
KK2901	5.3	0.5	10.2	3.3	0.9	28.3
KK2902	21.8	0.7	3.2	18.9	0.8	4.1

THE RESULTS INDICATE PMOLES OF [^{14}C]-PHENYLALANINE OR [^{14}C]-LEUCINE INCORPORATED BY 3.0 A_{260} UNITS OF 70S RIBOSOMES IN 1 HOUR ASSAYS AS DESCRIBED IN MATERIALS AND METHODS. THE PERCENT MISREADING IS BASED ON THE NUMBER PMOLES [^{14}C]-LEUCINE INCORPORATED COMPARED WITH THE NUMBER OF PMOLES [^{14}C]-PHENYLALANINE INCORPORATED BY THE SAME MUTANT AT THE TEMPERATURES INDICATED. RESULTS ARE THE AVERAGE OF DUPLICATE ASSAYS. INDIVIDUAL ASSAYS WERE WITHIN ± 15%.

FIGURE 9. POLYACRYLAMIDE GEL ELECTROPHORESIS OF RIBOSOMAL PROTEINS. TWO DIMENSIONAL POLYACRYLAMIDE GELS OF TOTAL 70S RIBOSOMAL PROTEINS FROM CONTROL AND MUTANTS WERE RUN USING A (pH 5.5) FIRST DIMENSION METHOD. FIGURE 9A SHOWS THE GEL PATTERN OF THE PROTEINS FROM STRAIN SK901. FIGURE 9B SHOWS THE TYPICAL GEL PATTERN OF PROTEINS FROM MUTANT KK2902 WITH PROTEIN S17 IN AN ALTERED LOCATION (ARROW). FIGURE 9C SHOWS A GEL PATTERN OF MUTANT KK1902 WITH PROTEIN S19 MOVED TO A MORE BASIC ELECTROPHORETIC POSITION (ARROW).



using the gel system described by Kenny et al., (1979). This system employs a 4% acrylamide first dimension tube gel at pH 5.5 and an 18% acrylamide second dimension slab gel at pH 4.5. An increased second dimension mobility in the small subunit protein S17 was clearly observed in the gel from strain KK2902 (Figure 9B). The small subunit protein S19 from strain KK1902 migrated as a more basic protein in the second dimension also (Figure 9C). These protein mobility changes were also observed by separating the ribosomal proteins in the alternative first dimension system at pH 8.6 (Howard and Traut, 1973). If a missense mutation in a protein had substituted an amino acid with a different pKa than the original, an extreme change in gel pH in either direction should alter the total charge on the protein sufficiently to result in an electrophoretic mobility shift. The pH 8.6 system was run to try and identify any electrophoretic changes not seen in proteins from mutants KK1901 and KK2901 at pH 5.5 and to attempt to verify the changes indentified in the other two mutants using that same system. There were no new protein changes seen in any of the gels of mutant proteins run at pH 8.6, therefore these gels are not shown.

Transductional Mapping of t.s. Mutations : The exact cotransduction frequencies and map locations for the t.s. mutation in each mutant have not yet been fully determined. However, all the t.s. mutations cotransduce 100% of the time between the aroE and strA genes. That places all the t.s.

mutations within sequences known to be carried by the defective phage lambda fus2.

Chapter 4

DISCUSSION

This study describes a method for the isolation of temperature sensitive mutants in translational components of *E. coli* via mutagenesis of the defective phage lambda fus2. This technique has proven to have several advantages over the previous methods of localized mutagenesis using generalized transducing phage (Hong and Ames, 1971; Kushner et.al. 1977). Isopycnic centrifugal separation of helper phage gives an initial enrichment of the transducing particle population. When using Pl for transduction purposes, the wild-type phage and the tranducing particles cannot be separated due to the infrequency of transducing particles in the population and because there are no differences between particles in bouyant density, molecular weight, or sedimentation coefficient which can be exploited in a separation technique. With lambda fus2, the transducing phage population being mutagenized contains only a particular preselected bacterial gene sequence (i.e. ribosomal protein genes). This leads to a much higher frequency of mutations in the selected gene region. Mutagenesis of a Pl phage population results in the vast majority of damage being inflicted upon phage DNA and not transducing particle bacterial DNA. Even when a transducing particle is mutagenized there is only about 1 chance out of 100 that it carries the bacterial sequence of interest. Finally, in the case of lambda fus2, the mutagenesis affects

basically only bacterial DNA sequences since this phage contains only 7% lambda DNA sequences (Fiandt et al., 1976). These advantages increase the efficiency of the method by a factor of approximately 10^4 compared to mutagenesis of a generalized transducing phage population.

Introduction of homologous bacterial sequences from the defective phage DNA was undoubtedly by generalized recombination since no transductants were found using a recA host. Without a functional recombination system in the host there would be no transduction with lambda fus2 because the antibiotic resistance gene carried by the phage could not be integrated into the host cell DNA and subsequently expressed. In addition, the mutants were not lysogens. Lysogens carrying the helper phage lambda cI857S7 are resistant to reinfection by helper phage. All four mutants described in this study were sensitive to infection and were therefore not lysogens. The allele for sensitivity is dominant. If the transductants were lysogens carrying the defective phage lambda fus2, they would have to also carry the helper phage for proper integration of both phage. Once integrated the cell would be merodiploid for a number of ribosomal genes including the streptomycin allele. They would once again be resistant to reinfection due to the presence of helper phage in the cell. In addition, the strS allele would dominate and lysogens would not survive on streptomycin.

After isolation and purification of the defective

phage, it was necessary to select a mutagen with which to treat the phage. Because of the versatility of the lambda fus2 method, there were a number of different mutagens which could have been employed. Ultraviolet light, transposable genetic elements such as phage Mu and Tn-10, alkylating agents like nitrosoguanidine and ethylmethane sulfonate, and deaminating agents such as nitrous acid were too nonspecific for the generation of t.s. missense mutations.

For this study we chose hydroxylamine (NH_2OH) because it specifically deaminates cytosine residues and forms 6-hydroxymethyl-uracil which has base-pairing properties similar to thymidine (Ingraham et al., 1983). The result is G=C--->A=T transition mutations and because of that specificity, some predictions could be made about the types of mutations that could be expected at the molecular level. By taking into consideration all possible cytosine changes in the genetic code, it was possible to estimate the probability of isolating nonsense mutations, and missense mutations. Only 10% (2 of 21) of all possible cytosine changes would result in nonsense mutations. The mutations of most interest were those missense mutations which would result in amino acids with a different overall charge so that they could be identified by electrophoretic methods. We found that 40% of all cytosine changes possible in either the coding or non-coding DNA strands, would result in amino acid substitutions which would alter the charge of the ribosomal protein.

In this study, four representative temperature-sensitive mutants, isolated as described above, were examined for ribosomal defects. These mutants were selected as strR transductants of the lambda fus2 rpsL gene after infection of a strS host (SK901) with the mutagenized defective phage. They all presumably carry an altered S12 protein as this is the change in rpsL mutants (Ozaki et al., 1969). Two of 100 transductants were picked as additionally spectinomycin resistant (spcR). These most likely are rpsE mutants with an altered protein S5 (Funatsu et al., 1972), and probably represent double mutants (rpsE and t.s.). Hydroxylamine can cause double mutations (Birge, 1981). It is likely that these two mutations were in the same transducing particle because the S12 and S5 genes of _E. coli_ show 50% cotransduction by phage P1. This means that with a streptomycin selection, 50% of the recombinants will have picked up the phage-carried spectinomycin gene (rpsE). The strR-spcR phenotype of these two strains (KK2901 and KK2902) also confered an enhanced resistance to the related aminoglycoside antibiotic neamine, as others have observed (DeWilde et al., 1975). Interestingly, strain KK2902 has an altered S17 protein, like mutants selected directly for neamine resistance. However, the S17 alteration in KK2902 has produced a more basic protein, unlike the _neaA_ mutants, in which S17 has been altered to a more acid protein (Bollen et al., 1975). Mutant KK2901 was also strR, spcR, and neaR, but no ribosomal protein alteration was

apparent on two-dimensional gels. The neamine resistance in both KK2901 and KK2902 was probably due to the cooperative interaction of S12 and S5 and not to the S17 defect. Other cooperative effects in antibiotic resistances of ribosomes involving proteins S17 and S4 have been described (Topisirovic et al, 1977). Since the S17 (rpsQ) gene maps near S5 (rpsE) (Bollen et al., 1975) and since both lie in the gene cluster carried by lambda fus2, it seems reasonable that these mutations were generated by the mutagenesis and transduced into the same host. Protein S17 binds to the 16S rRNA and is part of the core particle in ribosome assembly. It is interesting that this mutant does not show a defect in its ability to assemble its ribosomes. This property will be discussed further with regard to other observations in this mutant.

The gene locus that confers temperature-sensitivity on each of the four mutants has been mapped by transduction with the phage P1vir between aroE at 71 minutes and the strR allele at 72 minutes which would place the mutations within the DNA sequences known to be carried by lambda fus2. This supports the idea that recombinational events between the mutagenized phage and the E. coli chromosome were responsible for the introduction of mutations into bacterial gene sequences.

Spontaneous temperature-resistant revertants were isolated to test the reversion frequency of the mutants from temperature-sensitivity. Miller, (1972) established a range

of frequency within which point mutations fall. That range was set between 10^{-7} to 10^{-8}. All four of the mutants were found to revert at or near these frequencies. Preliminary mapping studies support the reversion frequency data. They also suggest t.s. point mutations in three of the four mutants. The reversion frequencies for frameshift and deletion mutations would be much lower and those types of mutations are not indicated here.

The in vivo assembly of ribosomal subunits proceeds through several discrete precursor particles. Accumulation of ribosomal subunit precursors is a feature of some cold-sensitive and high temperature-sensitive mutants defective in ribosomal assembly at restrictive temperatures (Held et al., 1974; Nomura et al., 1977). Characteristics of precursor accumulation for both subunits in assembly defective mutants have been discussed previously (Nierhaus, 1982). We have observed the accumulation of a 45S precursor particle in strain KK1901 at high temperature. 50S subunit assembly is normal at 32°C. The lack of 70S particle formation at 42°C in vivo may help to explain the substantial reduction in cell-free protein synthesis by ribosomes from this mutant. 30S subunit assembly was apparently not affected at 42°C in this strain. The 45S precursor in this mutant could be related to the 43S precusor seen in normal 50S subunit assembly or to the 47S particle formed with the loss of the 5S rRNA from the 50S subunit. As mentioned earlier, the mutant KK2902 which has

an altered S17 does not have a 30S assembly defect although there are 50S assembly steps which are 30S assembly dependant (Nashimoto et al., 1970). In the processing of rRNA transcripts, the precursor 16S rRNA preceeds the precursor 23S rRNA on the same transcript and must be processed properly before the 23S rRNA can be processed. A defect which hinders 30S subunit assembly or inhibits the processsing of the 16S rRNA, would also affect 50S subunit assembly.

Ribosomes isolated from control and mutant strains grown at 32°C were separated into subunits to see if high temperature affected their normal reassociation activity. Three of the mutants were similar to control at both temperatures in that they reassociated between 80-90% of their subunits into 70S ribosomes. Mutant KK1901 showed only 57% reassociation of its dissociated subunits into 70S ribosomes after heating at 42°C. By extrapolation (insets) the sedimentation coeffient of each particle was deduced. A 45S particle developed in the 50S peak in KK1901 very similar to the 45S peak seen developing in vivo. This suggests that high temperature causes the 50S particle to change conformation after it is fully assembled at low temperature and prevents the particle from reaching total assembly during ribosome synthesis. The inability of mutant KK1901 to reassociate its subunits after heating in vitro may be due to the same 50S defect which affects its normal in vivo ribosome assembly and may also be the source of the

reduced protein synthesis functions at high temperature.

All four mutants were temperature-sensitive for protein synthesis in vitro. These defects in translation of polyuridylate are similar to those which have been previously observed for other protein synthesis mutants derived by P1 localized mutagenesis (Rahi et al., 1979; Champney, 1980) which showed a temperature-dependant decrease in protein synthetic activity. The ribosomes were localized as the site of temperature sensitivity by separation from the cells before use in translational assays.

The kinetic assays for translation are very informative, since both initial velocities and extents of mRNA translation can be measured. The design of this assay, which uses 70S ribosomes as a single variable, provides some of the strongest evidence for a ribosomal source of temperature-sensitive activity (Rahi et al., 1979). Non-ribosomal proteins and factors were tested for temperature-sensitivity by using them in identical assays with control ribosomes. All four mutant S100 fractions were similar in their activity. Since the ribosomes used in these assays were from cells grown at 32°C they should be fully assembled and functional at that temperature.

The kinetics of protein synthesis in the four mutants were examined at 32°C , 37°C, and 42°C. These assays were set up with ribosome concentration as the limiting factor in order to more accurately test the effect

of temperature on the overall efficiency of protein synthesis by control and mutant ribosomes. At 32°C, mutant KK1901 showed 50% more 14C-phenylalanine incorporation than the control, while mutant KK2901 showed only about 50% of control activity. The other two mutants were not remarkably different from the control. Ribosomes from the control and three of the mutants showed optimal activiy at 37°C, although the activities for both mutants KK2901 and KK2902 were reduced by 60-70% compared to the control. The most dramatic effect on the extents of protein synthesis in the four mutants was at 42°C. The fact that ribosomes from all four mutants were at their lowest activity at that temperature while the control ribosomes remained near their optimal activity, gave good evidence that the ribosomes of the four mutants were temperature-sensitive.

Closer examination of the kinetic data revealed information about the direct effect of temperature on the initial rates at which the ribosomes polymerized phenylalanine. The initial rates of protein synthesis for ribosomes from mutants KK1901, KK1902 and KK2902 at 32°C were comparable to that of control ribosomes. Interestingly, these mutants were at maximal activity at 32°C while the control rates were at their minimum level. The initial rates for all three mutants at 37°C were lower than at 32°C while the control ribosomes showed a large increase in their rate and extent of protein

synthesis. Moreover, the overall extent of protein synthesis for these mutants increased between 10-50%. This indicated that the defects in each mutant must have slowed their relative rates of protein synthesis initiation while allowing their efficiency of polymerization to increase toward normal levels as the thermal energy of activation for the ribosomes of each mutant was approached. The relative rates for these three mutants all decreased slightly at 42°C, but the overall extent of incorporation dropped dramatically for all three. This showed that at 42°C the defects in the ribosomes of these mutants must have caused conformational changes in the particles which interfered with both the relative rate and efficiency of polymerization of phenyalanine. The relative rate and efficieny of protein synthesis in the control particles remained high at 42°C. Since the rate limiting factor in protein synthesis is the frequency of initiation (Neirhaus, 1982) the inhibition of the relative initial velocities in these mutants could be attributed to a temperature-sensitive defect in the process of initiation and, presumably, in the 30S subunit.

In contrast to the other three mutants, KK2901 had an initial rate of protein synthesis at 32°C that was almost 60% lower than that of the control ribosomes. This showed that ribosomes from KK2901 were not as efficient at the process of initiation of protein synthesis at this temperature. However, at 37°C there was a comparably

large increase in the relative rate of polymerization in the mutant that was proportional to the increase seen in the control at 37°C. The interpretation is that the ribosomes from this mutant must have been highly inefficient in polymerization at 37°C because, although they were initiating more often (higher relative rate of synthesis), there was a decrease in the overall incorporation. At 42°C, the decline in both the relative rate of protein synthesis and the overall incorporation of ^{14}C-phenylalanine were significant in the mutant while the control levels remained high. Once again the idea is suggested that a temperature-sensitive defect in the ribosome caused a conformational change which greatly inactivated the particle. In the case of KK2901 however, the problem did not seem to lie in initiation as evidenced by the increase seen in the relative rate of protein synthesis at 37°C. The efficiency must have been very poor and the limiting factor in that process is more likely elongation (Neirhaus, 1982). There are a number of functions involved in elongation and most of them are associated with the 50S subunit. The defect in KK2901, therefore, may be in the 50S subunit.

The inactivation of 70S ribosomes from control and mutant strains is an alternative approach to testing the site of temperature-sensitivity (Rahi et al., 1979), since only the ribosomes are heat inactivated. If the t.s. defect is not readily reversible, the heated ribosomes should also

be irreversibly defective in cell-free protein synthesis. First order decay curves indicate single component defects (Rahi et al., 1979). All four mutants showed first order decay rates but KK1902 actually showed a much slower inactivation than control ribosomes. The observed slow inactivation does not correlate with any of the kinetic data for this mutant which showed a relatively rapid loss of activity with time at temperatures lower than that used for the inactivation experiments. The other mutants were heat inactivated from 50-80% faster than the control in this experiment, which was somewhat higher than the inactivation rates seen in the kinetic assays. The kinetic assays however, were conducted at lower temperatures which would account for the slower inactivation.

A set of experiments was performed to test the overall fidelity of protein synthesis in a polyuridylate-directed system. An assay system identical to that used for the kinetic assays was used substituting 14C-leucine for 14C-phenylalnine and providing the ribosomes with 200 pmoles of non-isotopic phenylalanine to facilitate polymerization of both amino acids into polyphenylalanine. Three mutants (KK1901, KK1902, and KK2902) were similar to controls in their incorporation of 14C-leucine. Mutant KK2901 was reduced in its overall activity but also showed a significant degree of misreading at both 32°C and 42°C. It is interesting to note that the level of misreading in the control went down slightly at the higher

temperature while the misreading in all the mutants increased slightly at 42°C. The "wobble" hypothesis was formulated (Crick, 1966) to explain the misreading phenomenon and supports the idea that a single amino acid change in a protein will probably not be lethal to the cell but will allow the ribosome to continue to polymerize amino acids into a generally complete and functional protein.

Transfer RNA binding to active ribosomes is an essential requisite to the fidelity of protein synthesis. Mutant KK1901 was similar to the control in tRNA binding activity. Mutant KK1902 had a slight reduction in binding activity at 37°C which remained lower at 42°C. This is consistent with the kinetics data in that there was reduction in the rate of protein synthesis in this mutant both at 37°C and 42°C. The ability of this mutant to bind phenylalanine-tRNA could be the source of its reduced synthetic rates at high temperature. Mutants KK2901 and KK2902 both had reduced tRNA binding activity at both temperatures tested. Mutant KK2901 showed a 50S assembly defect in vivo and in the reassociation assay, suggesting an elongation defect at high temperature in the kinetic assays. The lack of efficient tRNA binding could be the result of a 50S defect and the cause of the temperature-sensitivity in this mutant. KK2902 showed an initiation problem in the kinetic assays. This could be the cause of the decline in the rate of protein synthesis at high temperature in this cell.

In an attempt to pinpoint the subunit responsible for the mutant t.s. phenotypes and to correlate the functional assay information collected on the four mutants, mixed subunit assays were performed. Mixed subunit complementation assays have been used previously to identify the subunit harboring the t.s. mutation (Rahi et al., 1979). Mutant KK1901 showed a t.s. 30S subunit at both 32°C and 42°C. This correlates well with the the kinetic data which showed the mutant to have an initiation problem which indicated a 30S defect. KK1901 also showed a 45S large subunit precursor which developed in vivo and after heating of subunits in the reassociation assays. A possible explanation is that at high temperature, the defect in KK1901 causes the release of its 5S rRNA. There are 30S subunit proteins which can affect 5S rRNA assembly into and release from the 50S particle (Traut et al., 1980) The release of the 5S rRNA has been shown to reduce the sedimentaion coefficient to the 50S particle to 47S which is very close to the 45S particle we have found in this mutant at high temperature. Loss of the 5S rRNA would also explain the poor ability of the mutant to reassociate both subunits into a functional 70S monomer as this one of the last steps in 50S assembly and has been shown to be involved in the subunit interface (Neirhaus, 1982). Since subunit separation and reassociation is required in the protein synthesis initiation step, the inability of this mutant to put its subunits back together at high temperature would

appear, in the kinetics assay, as an initiation defect.

Mutant KK1902 was ambiguous in the subunit mixing results. It showed an apparent 50S defect at both 32°C and 42°C but a closer examination of the data at 32°C showed that both combinations of subunits in which a control subunit was used showed significantly higher activity than the mutant/mutant combination. This indicated that both subunits could have defects. In this assay both combinations involving control subunits appeared to relieve the t.s. activity. However, at 42°C the correlation between t.s. and the 50S subunit was better. This was not consistent with the data obtained in the kinetic assays which showed an initiation problem and a possible 30S defect. The idea of a double mutation must be entertained since KK1902 had one of the lowest reversion frequencies of the four mutants. The other possibility with KK1902 is that there is a single mutation which affects both subunits. This has been shown to occur before with certain t.s. ribosomal mutants in proteins involved in the binding of the antibiotic erythromycin (Dabbs, 1978).

Mutant KK2901 showed the 30S subunit to be t.s. at both 32°C and 42°C in mixed subunit assays. This did not correlate well with the kinetic data which indicated an elongation problem. This mutant did show a significant amount of misreading at 32°C and even higher levels of misreading at 42°C. If taken as a single piece of information, the misreading data would implicate the 30S

subunit as the site of the t.s. defect. However, KK2901 also showed low levels of tRNA binding a 32°C but showed a slight increase in tRNA binding at the higher temperatures. While the misreading levels tripled, the tRNA binding ability went up by 50%. Consequently as more tRNA was bound the misreading levels increased dramatically. Inefficient tRNA binding was most probably the cause of the extensive misreading which in turn would tend to support the idea that the defect was in the 50S subunit.

Mutant KK2902 showed a 30S defect in the mixed subunit assays which correlated well with the data from the kinetic assay implicating an initiation defect at high temperature. This mutant showed low levels of tRNA binding at all temperatures with a slight drop from 32°C to 42°C. Since the message used in these assays was polyuridylic acid, the specificity of the binding of formylmethionine-tRNA at the P-site was eliminated. The tRNA binding defect seen in this mutant could in fact be associated with tRNA binding at the P-site which would implicate an initiation defect. This would support the idea that the site of the t.s. defect was in the 30S subunit.

In an effort to expose any altered ribosomal proteins from the four mutants which could be implicated as the cause of the t.s. defects in the mutants, two-dimensional polyacrylamide gels were used to separate individual ribosomal proteins. The alterations would be seen as electrophoretic variations caused by missense or nonsense

mutations resulting in a change in the overall charge or size of the protein. As discussed previously, approximately 40% of all cytosine changes in either DNA strand would result in codon changes which would place an amino acid with a different charge into the protein and an additional 10% of cytosine changes would result in new termination codons. A total of 50% of all possible alterations could induce electrophoretic differences in a protein. The other 50% would result in no change but could still induce a temperature-sensitive phenotype and would therefore require different methods to identify.

We were unable to identify specific protein changes by electrophoresis in the proteins from mutants KK1901 and KK2901. Their defects are likely to be missense mutations which result in no charge or molecular weight difference. Beyond the data and explanations given previously for the possible subunit locations of the temperature-sensitive defects in these two mutants, nothing more is known.

Mutants KK1902 and KK2902 however, showed alterations in small subunit proteins S17 and S19 respectively. Both proteins migrated to a more basic position in the second dimension gels suggesting the loss of an acidic or gain of a basic amino acid. There were no detectible electrophoretic variations in those same proteins in either mutant in the first dimension gels at pH 5.5 or pH 8.6. Since the second dimension slab gels were 18% polyacrylamide, they act partially as a molecular sieve. The variation in

electrophoretic movement could have also been due to a size change in the protein caused by a nonsense mutation near the carboxyl terminus or by an altered amino terminal amino acid which would normally have been modified but was no longer capable of being modified. Both situations could have resulted from the point mutations which were indicated in both mutants by reversion frequencies.

Small subunit protein S19 is transcribed from the S10 operon near 72 minutes on the E. coli chromosome (Nomura and Post, 1980) which is a portion of the genetic material carried by the defective phage lambda fus2. It is bound to the 30S particle early in assembly (Neirhaus, 1982) and is essential to the completion of the first precursor or core particle called the RI30 (reconstitution intermediate). S19 must be bound to protein S13 as a complex before both can bind the 16S rRNA in the 3' domain (Dijk, et al., 1977; Zimmermann, 1980). The fact that S19 is bound at the 3' domain of the 16S rRNA, places it near the surface of the particle where it could be involved in binding mRNA, tRNA, or the 50S subunit (Herr et al., 1979). S19 is an elongated protein which has two exposed, antigenically reactive sites and is found in the platform or head of the 30S subunit (Stoffler et al., 1980). It has been crosslinked by the use of 2-iminothiolane to IF-3 which places it in the proximity of the binding site for IF-3 on the 30S subunit (Stoffler et al., 1980). It has been implicated in resistance to the antibiotic tiamulin which is a semisynthetic derivative of

pleuromutilin (Bock et al., 1982). In single component omission studies, S19 was shown to be an important component in the polyuridylic acid directed binding of phenylalanine-tRNA to the ribosome (Nomura et al., 1969). S19 crosslinks to p-azidophenylacyl-phenylalanine-tRNA almost exclusively in the A-site (Offengand, 1980). The involvement of protein S19 in polyuridylate directed phenylalanine-tRNA binding is consistent with our finding that KK1902 loses some phenylalanine-tRNA binding ability at 37°C and 42°C. The fact that it has been crosslinked to IF-3 places it in a position for possible function in the initiation process. The kinetic data suggested that this mutant might have an initiation defect. The further data presented and discussed in this study support the idea that the temperature-sensitive defect in mutant KK1902 is in the 30S subunit and more specifically is due to a single amino acid alteration in protein S19.

Protein S17 (altered in KK2902) is transcribed from the S10 operon at 72 minutes on the E. coli chromosome and is also found on the defective phage lambda fus2. Bollen et al., (1975) described two mutants to neamine resistance which had altered S17 proteins. DeWilde et al., (1975) isolated two spontaneous mutants to neamine resistance which had alterations in both the S5 and S12 small subunit proteins but not in S17. They also found that the combination of strR and spcR phenotypes lead to the neaR phenotype without altering S17. S17 was found to be a 16S

rRNA binding protein and is one of the first proteins bound to the rRNA during assembly (Neirhaus, 1982). S17 binds the 16S rRNA in the same domain as protein S4 (Zimmermann, 1980). Apparent binding constants for S17 to the 16S rRNA in that 5'domain have been calculated (Schwarzbauer and Craven, 1981). This information lead to the conclusion that proteins S4, S5, S12, and S17 all had functional interactions in the fidelity of the translational mechanism (Bollen et al., 1975). This could be the source of the neamine resistance in both mutants KK2901 and KK2902. An S17-S5 (neaR-spcR) double mutant was isolated in which the S17 mutation suppressed the S5 mutation and in the presence of spectinomycin, caused misreading levels to approach those seen in a spcS strain (Matkovic et al., 1980). Herzog et al., (1979) isolated a neamine resistant mutant which had a serine residue replaced by a phenylalanine residue in protein S17 which was responsible for an assembly defect in the 30S subunit. From this information it has been shown that protein S17 plays an important role in the functioning of the E. coli ribosome. As mentioned before, the inefficiency in binding tRNA and the additional data presented and discussed in this study tend to support the hypothesis that the temperature-sensitive defect in mutant KK2902 is localized in the 30S subunit and more specifically is due to a single amino acid alteration in small subunit protein S17.

Overall, the structural and functional studies

TABLE 7. SUMMARY OF RESULTS

Strain	Growth Shift	Kinetics	Rates	Pre-cursor	Reassoc-iation	tRNA Binding	Mistrans-lation	Mixed Subunits	Altered R. Protein	Antibiotic Resistance
SK901	-	-	-	-	-	-	-	-	-	-
KK1901	+	+	IN	50S	+	-	-	30S	-	-
KK1902	+	+	IN	-	-	±	-	±50S	S19	-
KK2901	+	+	EL	-	-	+	+	30S	-	SPC[R] NEA[R]
KK2902	+	+	IN	-	-	+	-	30S	S17	SPC[R] NEA[R]

RESULTS ARE INDICATED AS FOLLOWS:

+ = STRONG T.S. DEFECT ASSOCIATED WITH INDICATED FUNCTION

± = WEAK T.S. DEFECT ASSOCIATED WITH INDICATED FUNCTION

- = NO T.S. DEFECT ASSOCIATED WITH INDICATED FUNCTION

30S = T.S. DEFECT IMPLICATED IN THE SMALL SUBUNIT

50S = T.S. DEFECT IMPLICATED IN THE LARGE SUBUNIT

IN = T.S. DEFECT ASSOCIATED WITH INITIATION PROCESS

EL = T.S. DEFECT ASSOCIATED WITH ELONGATION PROCESS

ALTERED RIBOSOMAL PROTEINS ARE INDICATED.

ANTIBIOTIC[R] NOT SELECTED FOR ARE INDICATED AS: SPC[R] = SPECTINOMYCIN RESISTANCE
NEA[R] = NEAMINE RESISTANCE

performed on these mutants have suggested the subunit location of the temperature-sensitive defect for the four mutants described. A summary of the results and suggested subunit defects in each mutant is described in Table 7.

Strain KK1901 showed the development of a 45S large subunit precursor in vivo. Ribosomes from this mutant showed an inability to reassociate their subunits after heating and again accumulated a 45S particle. Ribosomes from this mutant inactivated twice as fast as the control ribosomes at 45°C. The phenylalanine-tRNA binding ability of these ribosomes was similar to that of control ribosomes at both temperatures tested. There was no significant misreading of polyuridylate. The kinetic assays for cell-free protein synthesis suggested that there was an initiation (30S) defect in the ribosomes. Finally, there were no electrophoretic alterations in any proteins in the gels described.

Mutant KK1902 showed no ribosomal assembly defects in vivo or in vitro. Ribosomes from KK1902 required twice as long to inactivate at 45°C as the control ribosomes. Ribosomes from this mutant showed a slightly decreased ability to bind phenylalanine-tRNA at 42°C. There was no significant level of misreading of polyuridylate by the ribosomes. Cell-free protein synthesis kinetic assays indicated that there was a possible initiation (30S subunit) defect in the ribosomes. Finally, the small subunit protein S19 was found to have moved to a more basic position in the

second dimension of the gels described previously.

Mutant KK2901 showed no ribosomal assembly defects in vivo or in vitro. Ribosomes from this mutant required half the time of control ribosomes to inactivate at 45°C. Phenylalanine-tRNA binding by the ribosomes was significantly lower than control ribosomes at both 32°C, 37°C, and 42°C. Ribosomes from this mutant showed a high degree of misreading of polyuridylate at both 32°C and 42°C. Cell-free protein synthesis kinetic assays implicated an elongation (50S ribosomal subunit) defect. There were no electrophoretic alterations found in any of the ribosomal proteins in two dimensional gels. The fourth mutant, KK2902, showed no ribosomal assembly defects in vivo or in vitro. Ribosomes inactivated 5 times faster than the control ribosomes at 45°C. The ribosomes also showed significantly low levels of phenylalanine-tRNA binding at 32°C, 37°C, and 42°C. Misreading levels however, were similar to control levels at all temperatures. The kinetics of cell-free protein synthesis in this mutant suggested an initiation (30S ribosomal subunit) defect. Two-dimensional polyacrylamide gels showed an S17 protein with a more basic electrophoretic mobility in the second dimension.

In this work we have shown the utility of using a defective lambda phage in the localized mutagenisis of _E. coli_ for the isolation of protein-synthesis mutants. We have shown that the resultant mutants harbor ribosomal

structural and functional defects, altered antibiotic sensitivities, and subunit assembly deficiencies. It is apparent that the ease of isolation and diversity of mutants of *E. coli* derived using this method make it a significant tool in the study of ribosomal component structure and function.

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ABBREVIATIONS

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A260	Absorbance of U.V. light at 260 nm
ATP	Adenosine-5'-triphosphate
aroE	Gene coding for dehydroshikimate reductase
bio	Biotin utilization gene
bis-Tris	Bis-2-(hydroxyethylimino) tris (hydroxymethylaminomethane)
CD	Circular dichroism
CsCl	Cesium chloride
dH20	Distilled water
DTT	Dithiothreitol
EF-G	Elongation factor G
EF-Ts	Elongation factor Ts
EF-Tu	Elongation factor Tu
EthBr	Ethidium Bromide
ETSH	2-mercaptoethanol
g	Gravities
gm	Grams
gal	Galactose utilization
GTP	Guanosine-5'-triphosphate
Hfr	High frequency of recombination
IF-1	Initiation factor 1
IF-2	Initiation factor 2
IF-3	Initiation factor 3
K2HPO4	Potassium phosphate dibasic

KH2PO4	Potassium phosphate monobasic
LB	Luria broth
LB Plates	Luria broth containing 2% Difco agar
M9	Minimal salts media
MBP	Maltose binding protein
MgCl	Magnesium chloride
MgOAc2	Magnesium acetate
MgSO4	Magnesium sulfate
mRNA	Messenger ribonucleic acid
Na2EDTA	DiSodium ethylenediamine tetra acetic acid
Na2HPO4	Sodium phosphate dibasic
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH4Cl	Ammonium chloride
(NH4)2SO4	Ammonium sulfate
(NH4)2(SO4)2	Ammonium persulfate
neaA	Neamine resistance gene locus (rpsQ)
ORD	Optical rotory dispersion
PEP	Phophoenolpyruvate
PEP kinase	Phosphoenolpyruvate kinase
pmole	Picomole
ppGpp	Guanosine tetraphosphate (Magic Spot I)
ppGppp	Guanosine pentaphosphate (Magic Spot II)
PPO	2,5-Diphenyloxazole
R-buffer	10 mM Tris-HCl pH 7.6, 10 mM MgOAc2, 50 mM NH4Cl, 0.2mM ETSH
rRNA	Ribosomal ribonucleic acid

S-buffer	10 mM Tris-HCl pH 7.6, 0.5 mM MgOAc2, 50 mM NH4Cl, 0.2 mM ETSH
spcD	Spectinomycin dependent
spcR	Spectinomycin resistant
spcS	Spectinomycin sensitive
strD	Streptomycin dependent
strR	Streptomycin resistant
strS	Streptomycin sensitive
TP top agar	10 gm tryptone, 8 gm NaCl, 8 gm Difco agar/liter dH2O
TCA	Trichloroacetate
TEMED	N-N'N'N' Tetramethylethylene diamine
Tris-EDTA	10 mM Tris-HCl pH 7.6, 10 mM EDTA
t.s.	Temperature-sensitive
t.r.	Temperature-resistant
tRNA	Transfer ribonucleic acid

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