A New Mutation Affecting Ribosomal RNA Synthesis in *Escherichia coli*†

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ABSTRACT: A temperature-sensitive mutant of *Escherichia coli* is described. At the nonpermissive temperature there is a 12-fold reduction in the rate of rRNA synthesis, while tRNA and mRNA syntheses are affected to only a slight extent. Both protein and DNA syntheses also continue at nearly the normal rate. The mutation appears to affect the synthesis of 16S and 23S rRNA equally and has no detectable affect on rRNA maturation. The temperature-sensitive lesion appears to be caused by a single point mutation lying between minutes 21 and 27. It is suggested that this mutation seems to define a new genetic locus involved in the regulation of rRNA synthesis.

The synthesis of rRNA in *Escherichia coli* is closely regulated in response to the availability of amino acids, carbon source, and overall growth rate of the bacteria. Many features of the regulation of rRNA synthesis in response to the amino acid supply are fairly well understood at the present time. Certainly the steps required for the synthesis of guanosine 5′-diphosphate 3′-diphosphate (ppGpp)[1] in response to amino acid starvation in a stringent (*rel*+) strain have been well characterized (Haseltine et al., 1972; Haseltine and Block, 1973; Ramagopal and Davis, 1974). Furthermore, there is ample evidence that the accumulation of ppGpp does, in fact, correlate with the shut-off of rRNA synthesis (Cashel, 1969; Khan and Yamasaki, 1974).

The situation with respect to the regulation of rRNA in response to carbon source deprivation and growth rate is somewhat more confused. Relaxed mutants (*rel*$^-$) still regulate their rRNA content normally during carbon source deprivation and in response to changes in growth rate (Neidhardt, 1968). Moreover, although ppGpp does accumulate during carbon source downshift (Harschman and Yamasaki, 1971), the increase is slow and variable compared to the effect of the downshift on RNA synthesis (Lazzarini et al., 1971; Gallant et al., 1972). Thus, it appears possible that an independent genetic locus other than *rel* may also be involved in regulation of rRNA synthesis.

In this paper we report the characterization of a temperature-sensitive mutant of *E. coli* which has a defect in the synthesis of 16S and 23S rRNA at 42 °C.

Materials and Methods

Strains and Media. D10 (*met*$^-$, *rns*$^-$, *rel*$^-$) has been described by Gesteland (1966). 2S142 (*met*$^-$, *rns*$^-$, *rel*$^-$, *ts*$^-$, *ilv*$^-$) was isolated as a temperature-sensitive survivor of a nitrosoguanidine treated culture of D10 (Chaney and Schlessinger, 1975). It retains all of the other characteristics of the parental strain except that it is also *ilv*$^-$. The strain is stable with a reversion frequency of 10^{-7} and is only weakly temperature sensitive. That is, single colonies will grow on agar plates at 42 °C, although more slowly than at 30 °C (in contrast, D10 grows much more rapidly at 42 °C). CP76 (*met*$^-$, *rel*$^+$), a methionine requiring strain used in the determination of ppGpp levels, is a spontaneous revertant of the Borek relaxed strain CP71. The HFr's (high frequency of recombination) and F-primes (F′) used in mapping the temperature-sensitive lesion in 2S142 are shown in Table I. All of these strains were obtained from the *E. coli* Genetic Stock Center (Department of Human Genetics, Yale University School of Medicine, New Haven, Conn. 06510). Strains 5153 (*thi*$^-$, *pyrC*$^-$, *relA*$^-$, *lac*$^-$) and 4211 (*thi*$^-$, *pyrD*$^-$, *his*$^-$, *trp*$^-$, *thyA*$^-$, *mtl*$^-$, *xyl*$^-$, *mal*$^-$, *gal*$^-$, *strA*) were also obtained from the *E. coli* Genetic Stock Center. S52 (*thi*$^-$, *man*$^-$, *mal*$^-$, *str*^{r}, *his*$^-$, *try*$^-$, *gal*$^-$, *lac*$^-$, *pro*$^-$, *thr*$^-$, *leu*$^-$) is the Adelberg strain AB2103 obtained from Dr. David Schlessinger.

The cells were usually grown on M9 medium (Adams, 1966) supplemented with 0.2% Difco casamino acids and either 0.2% glucose or glycerol. In some experiments they were grown on LB medium (Miller, 1972). D10 grows at 30 °C with a doubling time of 55 min on the M9 + glycerol, 46 min on the M9 + glucose, and 35 min on LB medium. 2142 has a doubling time approximately 1.8 as great on all 3 media. It is distinctly temperature sensitive on all three media.

Experimental. For determination of RNA, DNA, and protein, aliquots of exponentially growing cells were precipitated with 5% trichloroacetic acid and fractionated as described previously (Jackson and Chaney, 1976). Cells were permeabilized with EDTA by a modification (Jackson and Chaney, 1976) of the method described by Rose et al. (1970). The preparation and electrophoretic analysis of the rRNA and tRNA were carried out essentially as described previously (Jackson and Chaney, 1976). The β-galactosidase assay was carried out essentially as described by Miller (1972).

The initial mapping of the temperature-sensitive locus in 2S142 was carried out by the rapid mapping method (method A) of Low (1973) using the Hfr's listed in Table I. A *str*^{r}, *rif*^{r} derivative of 2S142 (obtained by P1 transduction) was used as the recipient in these experiments. Episome transfers were carried out as described by Miller (1972) using the F′ strains

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; ATP, GTP, CTP, and UTP, adenosine, guanosine, cytosine, and uridine triphosphate; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; rRNA, tRNA, and mRNA, ribosomal, transfer, and messenger ribonucleic acid, respectively.

TABLE I: Strains Used to Map the Genetic Lesion in 2S142.

CGSC designation[a]	Other designation	Hfr's Point of origin[c] (min)	Direction of transfer[b]
5051	P801	2	c
4312	BW113	7	c
4314	KL208	33	c
4240	KL983	50	c
4245	KL16	61	c
4318	KL228	83	c
4315	KL209	90	c
259	HfrH	97	cc
4241	Ra-2	90	cc
4244	KL25	83	cc
4294	KL14	66	cc
312	AB312	63	cc
4316	PK191	44	cc
5015	Hfr Broda 8	8	cc

CGSC designation[a]	F's Other designations	Area covered by episome[c] (min)
4287	F152/KL253	14-17.5
5104	F1-gal/PA2005^R	14-18
2605	F8/W3104	16-17.5
4323	KLF47/KL262	16-21
4309	KLF6/KL181	19-21
4253	KLF26/KL181	16.5-33
4320	KLF25/KL181	21-33
4256	KLF23/KL181	27-33

[a] *E. coli* Genetic Stock Center designation. [b] c = clockwise, cc = counterclockwise. [c] Positions based on the revised linkage map of *E. coli* (Bachman et al., 1976).

listed in Table I and the *rif*^{r}, *str*^{r} derivative of 2S142. P1 transductions were carried out essentially as described by Lennox (1955).

Results

The Synthesis of RNA, DNA, and Protein at 42 °C. The accumulation of RNA, DNA, and protein during growth at 42 °C was measured chemically using orcinol, diphenylamine, and biuret determinations (data not shown). 2S142 does show a short (10 or 15 min) burst in RNA accumulation immediately after the shift to 42 °C. Following this burst, however, the accumulation of RNA virtually ceases, while protein synthesis continues linearly at approximately 70–80% of the pre-shift rate. During a 2-h incubation at 42 °C there is a 2.2× increase in protein and DNA content but only a 40% increase in RNA, with essentially all of that occurring in the first 15 min at 42 °C. Clearly then, 2S142 continues to accumulate significant levels of both DNA and protein at 42 °C, even though net accumulation of RNA has ceased.

The Effect of Temperature on Total RNA Synthesis. Initially we wished to measure the effect of growth at 42 °C on the rate of total RNA synthesis (stable + unstable RNA) carried out by 2S142. A reasonable estimate of the total rate of RNA synthesis can be obtained from short pulse labels if one takes into account both permeability affects and RNA turnover. Initially, estimates of the rate of RNA synthesis were obtained by simply pulse labeling aliquots of a growing culture for 60 s with 5 μCi/mL of [^{3}H]uridine. When 2S142 is shifted to 42 °C, the incorporation of [^{3}H]uridine in a pulse label rapidly falls to about 15% of the observed incorporation at 30 °C (data not shown).

To evaluate this information, it was first necessary to estimate the extent to which permeability changes were affecting

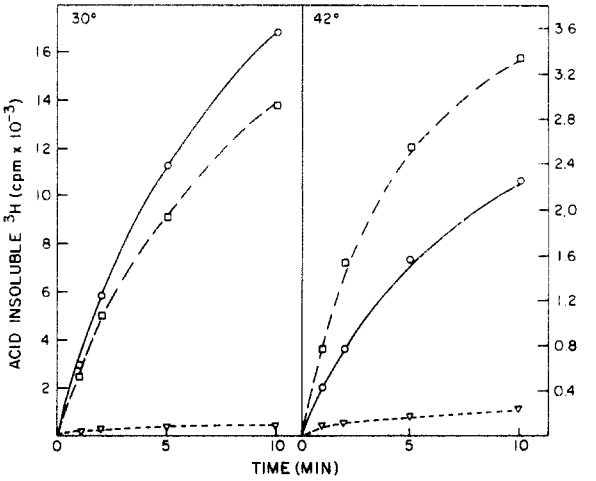

FIGURE 1: Effect of EDTA treatment on [^{3}H]uridine incorporation by 2S142 at 30 and 42 °C. Cells were grown at 30 °C in M-9 medium + glucose and casamino acids to a cell density of 1 × 10^8 cells/mL. At this point the culture was divided into 6 aliquots, 3 of which were incubated at 30 °C and 3 at 42 °C. After 45 min two of the 30 °C aliquots and two of the 42 °C aliquots were carried through the permeabilization procedure described under Materials and Methods (EDTA-treated cells). As controls, another pair of aliquots was carried through the same procedure except that M-9 salt medium was used instead of Tris and no EDTA was added (control or untreated cells). In each case, 0.1 μCi/mL of [^{3}H]uridine was added to the treated cultures and incorporation of the ^{3}H into acid-insoluble material was measured by standard techniques. In the case of the cells grown at 42 °C, both the incorporation study and each step of the permeabilization were also carried out at 42 °C: (○—○) control cells; (□ - - - □) EDTA-treated cells; and (▽ - - - ▽) EDTA-treated cells assayed in the presence of 20 μg/mL rifampin.

the uptake of label. This was done by treating cells grown at 30 or 42 °C with EDTA. These permeabilized cells were then assayed for their ability to incorporate [^{3}H]uridine (Figure 1). The EDTA treatment has little effect on the uptake of [^{3}H]-uridine at 30 °C, but does substantially increase the rate of incorporation at 42 °C, indicating that at least part of the observed decrease in ^{3}H uptake at 42 °C is due to permeability effects. However, even in the permeabilized cultures, the rate of total RNA synthesis appears to be three- to fourfold reduced in 2S142 at 42 °C.

Since previous data have shown that the extent and rate of degradation of unstable RNA are significantly greater in these mutant strains at 42 °C than at 30 °C (Chaney and Schlessinger, 1975), one is likely to obtain an underestimate of the relative rate of RNA synthesis at the high temperature by merely comparing the incorporation of [^{3}H]uridine during a fixed time period at the two temperatures, especially if the time period involved is a relatively long one. This effect can, however, be minimized by extrapolating to the shortest possible labeling time. This was done by calculating the ratios of ^{3}H uptake at 42 and 30 °C after various times of incubation from the data in Figure 1 and extrapolating to an infinitely short pulse time (Figure 2). In this manner, we estimate that 2S142 shows about a threefold decrease in the rate of total RNA synthesis at 42 °C.

The Effect of Temperature on β-Galactosidase Synthesis. The mutant strain continues to synthesize protein at 42 °C although net accumulation of RNA has ceased. Since there is no evidence for increased stability of mRNA at 42 °C (Chaney and Schlessinger, 1975) it seems likely that mRNA synthesis must be continuing at 42 °C. Accordingly, we tested the ability of this strain to synthesize new β-galactosidase at 42 °C (Table II). Clearly, 2S142 is capable of synthesizing β-galactosidase de novo at 42 °C. While the rate of β-galactosidase production is lower at 42 °C than it is at 30 °C, this

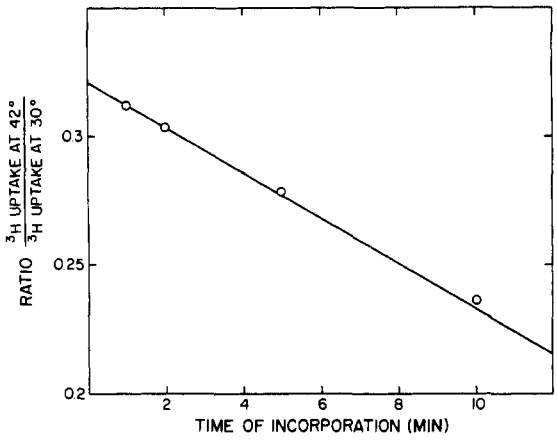

FIGURE 2: The ratio of [^{3}H]uridine uptake at 42 and 30 °C after various labeling times. The ratio of [^{3}H]uridine uptake at 42 °C/[^{3}H]uridine uptake at 30 °C after 1, 2, 5, and 10 min of labeling was calculated from the data presented in Figure 1 for the EDTA-treated cells.

TABLE II: Rate of β-Galactosidase Production at 30 and 42 °C.[a]

Strain	Condition	Rate of β-gal production[b] (units min^{-1} 10^{-8} cells)	Ratio Rate at 42 °C/ rate at 30 °C
D10	30 °C only	11.9	
D10	42 °C for 45 min	16.0	1.34
2S142	30 °C only	10.8	
2S142	42 °C for 45 min	2.81	0.26

[a] Cells were grown on M-9 medium + glycerol and casamino acids at 30 °C to a cell density of 1 × 10^8 cells/mL. Each culture was then split into two aliquots, one of which was incubated at 30 °C for 45 min and the other at 42 °C. At this point 5 × 10^{-3} M isopropyl β-D-thiogalactoside was added to each culture. Samples were removed at 5, 10, 15, and 20 min after addition of inducer and assayed for β-galactosidase activity as described by Miller (1972) (one unit of β-galactosidase hydrolyzes 1 nmol of *O*-nitrophenyl β-D-galactoside/min at 30 °C and pH 7.0). [b] The rate of β-galactosidase production was obtained from the slope of the line obtained by plotting β-galactosidase activity vs. time after isopropyl β-D-thiogalactoside addition.

observed decrease correlates fairly well with the previously observed decrease in total RNA synthesis. Thus, we assume that the synthesis of at least one class of mRNA can occur at 42 °C and that mRNA can be translated into functional protein.

The Effect of Temperature on the Synthesis of rRNA and tRNA. Since net accumulation of RNA ceases at 42 °C while the synthesis of at least some mRNA continues, one would expect to observe a preferential decrease in the synthesis of stable RNA at 42 °C. In fact, previous studies (Chaney and Schlessinger, 1975) using competition hybridization to measure the rRNA have shown that pulse-labeled RNA produced by 2S142 at 42 °C contains only about one-fourth as much rRNA as pulse-labeled RNA produced at 30 °C. To confirm this observation and to extend it to synthesis of tRNA, the RNA produced after 5, 10, and 15 min of labeling at 30 and 42 °C was examined by gel electrophoresis as described under Materials and Methods. Typical results obtained with the RNA extracted from the parental strain, D10, after 15 min of labeling at 30 or 42 °C are shown in Figure 3. Comparable data obtained with 2S142 are shown in Figure 4. As expected, the 2S142 incorporates less [^{3}H]uridine at 42 °C, reflecting both the previously observed decrease in permeability and the decrease in net RNA synthesis. However, when one compares

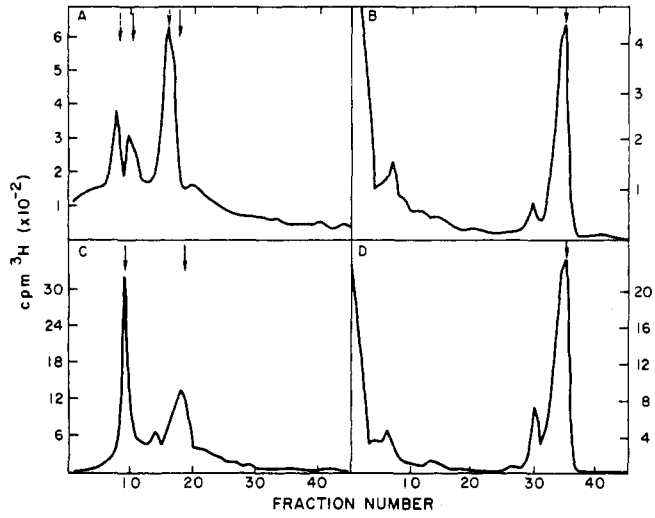

FIGURE 3: Gel electrophoresis of the RNA produced by D10 after 15 min of labeling at 30 and 42 °C. Cells were grown at 30 °C in M-9 medium + glucose, casamino acids, and 1.5 × 10^{-5} M carrier guanosine to a cell density of 1.2 × 10^8 cells/mL. At this point the cells were divided into two equal aliquots, one of which was incubated at 30 °C for 45 min and the other at 42 °C for 45 min. [^{3}H]Guanosine (10 μCi/mL) was then added to each culture. After 5, 10, and 15 min of labeling samples were removed and the RNA extracted as described under Materials and Methods. Electrophoresis was carried out on 3.6% polyacrylamide gels for 6.5 h (rRNA) and on 6.0% polyacrylamide gels for 2 h (tRNA) at 5 mA per tube. The gels were then fractionated and counted as described under Materials and Methods. Only the data obtained with the RNA extracted after 15 min of labeling are shown above. The position of the mature 4S, 16S, and 23S RNAs was determined by coelectrophoresis of each sample with ^{14}C-labeled stable RNA prepared as described under Materials and Methods. The solid arrows indicate the positions of the mature 4S, 16S, and 23S peaks and the dotted arrows indicate the positions of the p16S and p23S peaks on these gels: (A) D10 at 30 °C, 3.6% gel; (B) D10 at 30 °C, 6.0% gel; (C) D10 at 42 °C, 3.6% gel; (D) D10 at 42 °C, 6.0% gel.

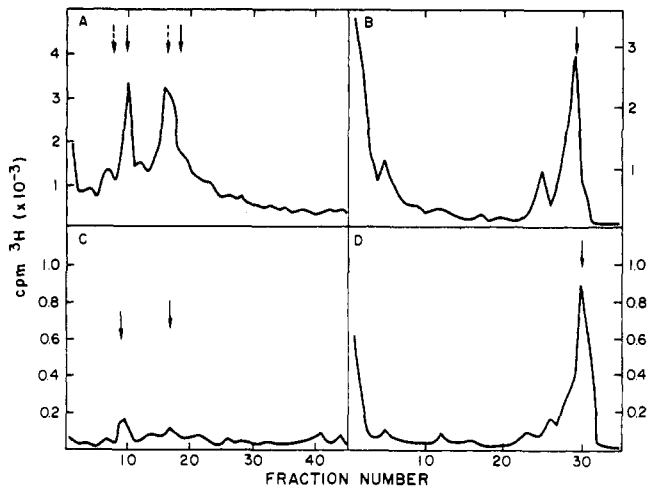

FIGURE 4: Gel electrophoresis of the RNA produced by 2S142 after 15 min of labeling at 30 and 42 °C. The conditions used were the same as described in the legend to Figure 3 except that the labeling at 42 °C was done with 20 μCi/mL [^{3}H]guanosine in the absence of any added carrier guanosine (final concentration = 1 × 10^{-6} M): (A) 2S142 at 30 °C, 3.6% gel; (B) 2S142 at 30 °C, 6.0% gel; (C) 2S142 at 42 °C, 3.6% gel; (D) 2S142 at 42 °C, 6.0% gel.

the relative size of the rRNA and tRNA peaks after various times of incubation at 30 or 42 °C, it is also apparent that the appearance of label into the rRNA peak is selectively decreased at 42 °C relative to the tRNA peak. This can best be seen by calculating the area under the rRNA and tRNA peaks

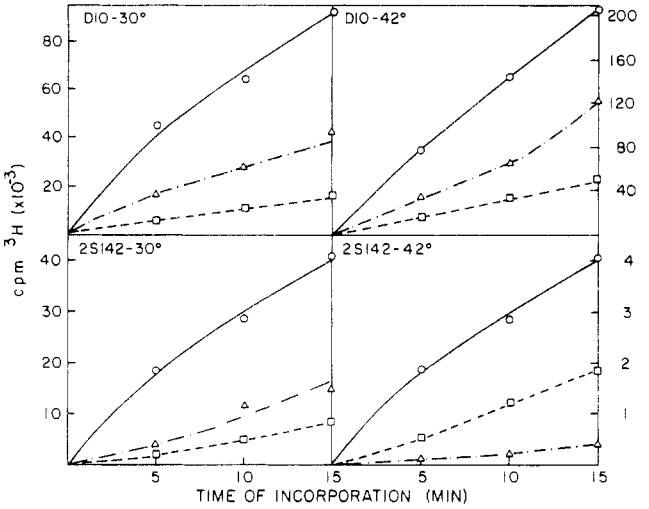

FIGURE 5: Composition of the RNA synthesized by D10 and 2S142 at 30 and 42 °C. The incorporation of [^{3}H]uridine into the total acid-insoluble fraction and into rRNA and tRNA was measured in the experiments described in Figures 3 and 4: (○—○) total acid-insoluble radioactivity; (△- · -△) 16S + 23S RNA; (□ - - - □) 4S RNA.

and comparing these values to the total Cl_3AcOH precipitable ^3H at the corresponding times. Figure 5 summarizes these calculations for D10 and 2S142.

Genetics. 2S142 has a reversion frequency of 10^{-7}, indicating a likelihood that the temperature sensitivity is due to a single point mutation. Preliminary experiments involving generalized P1 transductions indicated that the temperature-sensitive lesion was not co-transducible with the *ilv*, *rif*, *relC*, or *spoT* loci (Chaney, 1975). Thus, to more clearly define the map position of this lesion, 2S142 was mated with the Hfr's listed in Table I. Method A as described by Low (1973) was used. This method involves the replication of the entire set of Hfr cells, all on one plate, onto a lawn of 2S142. For these experiments a *rif^r*, *str^r* derivative of 2S142 (prepared by P1 transduction) was used and either rifampin or streptomycin used to counterselect against the Hfr's. The clockwise Hfr's 4314, 4240, and 4245 and the counterclockwise Hfr's 5015 and 259 all gave large numbers of recombinants, indicating a map position of between 8 and 33 min.

The map position was further delineated in a series of matings between the F''s listed in Table I and the *rif^r*, *str^r* derivative of 2S142. Only the F''s 4253 and 4320 gave rise to temperature-resistant colonies when mated with 2S142 (*str^r*, *rif^r*). Since the effectiveness of all of the F''s used in this study was verified independently (Harris, 1976), these results indicate a map position of between 21 and 27 on the *E. coli* chromosome.

The *pyrD* (min 21), *pyrC* (min 23), and *trp* (min 27) loci are all within this region of the chromosome. Linkage of the *ts* lesion to these loci was tested by P1 transduction. A P1 lysate of 2S142 was used to infect strains 5153 (*pyrC$^-$*), 4211 (*pyrD$^-$*), and S52 (*trp$^-$*). In each case over 200 transductants were selected which no longer showed the designated nutrient requirement. None of these transductants were temperature sensitive. In parallel experiments, P1 lysates of 5153, 4211, and S52 were used to produce *ts$^+$* transductants of 2S142. None of these transductants had an additional nutrient requirement. Thus, no linkage was observed between any of these gene loci and the temperature-sensitive lesion in 2S142.

Discussion

This mutant, 2S142, was selected from a class of mutants which all cease net RNA accumulation at 42 °C while continuing to synthesize protein. Previous studies have suggested that this mutant was defective in the synthesis of rRNA at the restrictive temperature (Chaney and Schlessinger, 1975). The reversion frequency suggested that this lesion was probably due to a single point mutation. Thus, it became of interest to investigate the effects of temperature on all aspects of RNA metabolism.

The effects of temperature on total RNA synthesis (stable and unstable RNA) were studied using EDTA treated cells with the effect of RNA turnover minimized by extrapolating to the shortest possible labeling time. From these data, we estimate that total RNA synthesis is reduced threefold when cells are shifted to 42 °C.

The effect of high temperature on the accumulation of stable RNA was measured directly by gel electrophoresis of the RNA produced at 30 and 42 °C. The results clearly indicated a preferential effect on rRNA accumulation at 42 °C, with the percentage of rRNA which accumulated during the first 15 min of labeling decreasing fourfold while the percent tRNA actually increased by just over twofold. Given a fourfold reduction in the percentage of rRNA synthesized and a threefold reduction in the rate of RNA synthesis, the rate of rRNA synthesis must be reduced by about 12-fold at 42 °C. While these experiments themselves do not differentiate between an effect of the restrictive temperature on rRNA synthesis or rRNA maturation, previous hybridization experiments (Chaney and Schlessinger, 1975) have also shown a fourfold reduction in the percent rRNA in pulse-labeled RNA. Thus, it is likely that the synthesis of rRNA is primarily affected at 42 °C. If one looks at individual species of rRNA, both 16S and 23S rRNA seem to be affected equally at the restrictive temperature (Figure 4). Nor is there any evidence for a significant delay in processing either species of rRNA (data not shown). The 5S rRNA is not sufficiently resolved from tRNA on our gels for us to unambiguously determine the effects of high temperature on its synthesis.

Based on the assumption that the percent tRNA which accumulates in the same experiment is also proportional to the rate of tRNA synthesis at the two temperatures, one can also estimate the effect of temperature on the rate of tRNA synthesis. In this case there is around a twofold increase in the percent tRNA synthesized. Coupled with the observed threefold reduction in total RNA synthesis, this would indicate that the rate of tRNA synthesis at 42 °C is probably about two-thirds of the rate at 30 °C.

The best direct evidence that mRNA synthesis continues at 42 °C is the fact that these cells are inducible for β-galactosidase at the high temperature. This clearly indicates that mRNA synthesis can occur at 42 °C and that the mRNA can be translated into functional protein. While the quantitative effect of high temperature on mRNA synthesis is somewhat more difficult to estimate, it appears likely that the reduction in mRNA synthesis is comparable to the observed reduction in tRNA synthesis and far less than the reduction in rRNA synthesis.

Clearly then, the experiments reported in this paper confirm that 2S142 is specifically defective in the synthesis of rRNA at 42 °C. This biochemical defect appears to result from a genetic lesion located between min 21 and 27 on the *E. coli* chromosome. Only two other ribosomal mutations, *rimC* (Bryant and Sypherd, 1974) and *mac* (Sparling and Blackman, 1973), have been reported in this area. It is unlikely, however, that either of these mutations is identical with the lesion in 2S142. Mutants of the *rimC* class appear to be specifically defective in ribosome maturation, accumulating a 32S precursor particle. The *mac* mutation results in erythromycin

dependence and is very closely linked with the *trp* locus. As discussed previously, the lesion in 2S142 shows no detectable linkage with the *trp* locus. Thus, the mutation in 2S142 appears to define a new gene locus required for rRNA synthesis in *E. coli*.

The biochemical nature of that lesion has not been determined at present. Recent experiments in our laboratory suggest that cells grown at 42 °C have a diminished capacity for RNA synthesis in a complex in vitro system, but contain identical levels of fully active RNA polymerase (Williams et al., manuscript in preparation). Thus, we currently favor the hypothesis that 2S142 may be temperature sensitive for an auxiliary protein other than RNA polymerase which is required for rRNA synthesis.

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