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Regulation of RNA Polymerase Synthesis in *Escherichia coli*: A Mutant Unable to Synthesize the Enzyme at 43°

(localized mutagenesis/rifampicin resistance/radiochemical labeling/ sodium dodecyl sulfate gel electrophoresis/regulation of cell growth)

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ABSTRACT · **We report the isolation of a mutant of *E. coli* in which the capacity to synthesize RNA polymerase (EC 2.7.7.6) (the β and β' subunits) is rapidly lost at 43°. The mutation has no effect on the stability or activity of the polymerase itself. The mutation is recessive and is closely linked to the *rif* locus (the structural gene for the β subunit). Using strains carrying the mutation, we have shown that polymerase is present in excess in rapidly growing *E. coli* cells.**

In the *Escherichia coli* cell one enzyme, RNA polymerase (EC 2.7.7.6; nucleosidetriphosphate:RNA nucleotidyltransferase), carries out all of the synthesis of RNA from DNA templates (1–4). The intracellular concentration of active RNA polymerase molecules directly affects the rate of cell RNA and protein synthesis (5). The concentration of active enzyme is therefore potentially a critical element in determining the rate of cell growth and replication. The enzyme is composed of four subunits ($\alpha_2\beta\beta'$) (6). The synthesis of the β and β' subunits has been observed to be coordinately regulated (5, 7–11), which is consistent with the conclusion that these two subunits are encoded in an operon (12, 13). Nothing is known at present about the regulation or genetics of the α subunits. Gene dosage appears to have little effect on the rate of synthesis or final concentration of the enzyme (9, 13–16), suggesting a transcriptional mechanism of regulation. On the other hand, Hayward, Austin, and Scaife (16) have suggested that the regulation of the concentration of RNA polymerase may be post-translational and carried out by the degradation of uncomplexed subunits. The minimum concentration of active RNA polymerase also appears to be regulated, suggesting a mechanism that maintains the intracellular concentration of polymerase at a constant concentration (5). The intracellular concentration of active RNA polymerase molecules is also relatively unaffected by the cell growth rate (9–11), with the enzyme apparently in excess under all growth conditions (9–11).

We have begun to investigate the cellular mechanism(s) that regulate the synthesis and concentration of RNA polymerase in *E. coli*. We have isolated mutants that conditionally alter the intracellular concentration of the RNA polymerase complex ($\alpha_2\beta\beta'$). In this report we describe a mutant that cannot synthesize new RNA polymerase molecules at 42°. The stability and activity of intact polymerase molecules appear to be unaffected by the mutation even at the nonpermissive temperatures. The growth pattern of strains carrying the mutation after shift to nonpermissive temperature (where no new polymerase molecules are synthesized) supports the conclusion that the concentration of RNA polymerase is not the rate-limiting factor in cell growth, since the enzyme is apparently present in excess even in rapidly growing cells.

MATERIALS AND METHODS

Bacterial Strains. All bacteria were derivatives of *E. coli* K-12. They were MX245 (F$^-$, *trp, galK, galE, ara, lac, tsx, str, sup*), MX390 (F$^-$, *leu, argH, trp, galK, galE, lacZ, tsx, str, sup*), MX386 (F$^-$, *leu, metB, trp, galK, galE, lacZ, tsx, str, sup*), MX387 (F$^-$, *leu, metB, trp, galK, galE, lacZ, tsx, str*), and MX504 (F$^-$, *ppc, pur, trp, galK, galE, lacZ, tsx, str, sup*). These five strains were constructed in our laboratory and were derivatives of M238, which was kindly provided by John Abelson. F110/KL131 (F'*metB*$^+$/*metB*$^-$, *aroB, argG, leu, recA*) and MX255 (*metB, aroB, argG, leu, recA, val*^{r}) were constructed from strains obtained from K. B. Low. The Hfr strains used in the mapping were those described by K. B. Low (17) and were kindly provided by him.

Media, Chemicals, and Isotopes. R broth (18) was used as the standard medium. Medium E (19) was used to score recombinants in the P1 crosses. When required, the medium was supplemented with amino acids to a final concentration of 0.6 mM. Tyrosine decarboxylase-digested Y broth (M. P. Oeschger and J. E. Cronan, Jr., manuscript in preparation) was used for the labeling experiments, and consists of 0.5% yeast extract (Difco), 1.0% casamino acids (Difco) and 1/10× medium E. Amino acids and streptomycin were obtained from Sigma. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) was obtained from Aldrich and rifampicin (rifamycin) was the kind gift of Dr. Hans Heymann of Ciba/Geigy. [^{14}C]-Tyrosine (specific activity about 500 mCi/mmol) was obtained from Amersham-Searle.

Mutagenesis and Selection of Mutants. Derivatives of MX245 carrying nonsupplementable (R broth) temperature-sensitive mutations located in the region of the genes for RNA polymerase β and β' subunits were isolated with nitrosoguanidine,

Abbreviations: Nitrosoguanidine, *N*-methyl-*N'*-nitro-nitrosoguanidine; ts, temperature-sensitive; *rif*^{s}, rifamycin sensitivity; *rif*^{r}, rifamycin resistance; other gene symbols as in Taylor and Trotter (31).

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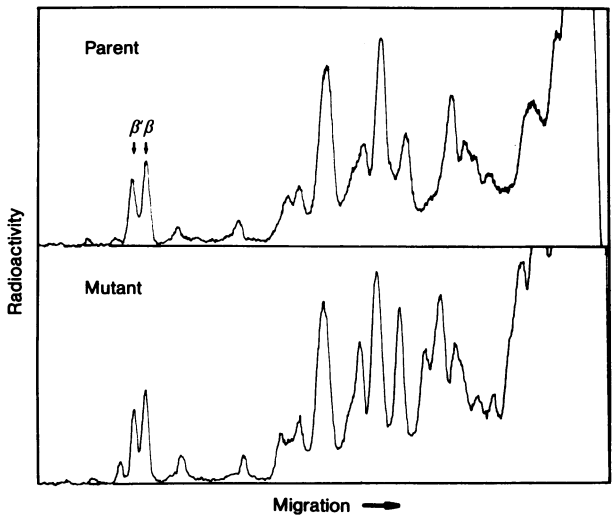

FIG. 1. Rate of β and β' subunit synthesis at 25°. Logarithmic phase cultures of PM142 and MX245, growing at 25° in digested Y broth, were labeled with [^{14}C]tyrosine for 40 min (20 min, MX245) and chased with unlabeled tyrosine for 20 min (10 min, MX245) according to the procedures described in *Materials and Methods*. Polyacrylamide gels (5%) were used, fractionating proteins of greater than 60,000 molecular weight (30). Radioactivity is expressed in arbitrary units.

as described (20). The selection took advantage of the potential of nitrosoguanidine to cause clustered multiple mutations (21), and all the isolates were primarily selected for rifampicin-resistance. Strains with temperature-sensitive mutations in the *rif* region [as determined by conjugational analysis (20)] were selected for further investigation.

Rate of Polymerase Synthesis. The rate of RNA polymerase (β and β' subunit) synthesis was determined in the following way: 1 ml portions of logarithmic phase cultures growing in digested Y broth at 25° were incubated with 1 μCi of [^{14}C]-

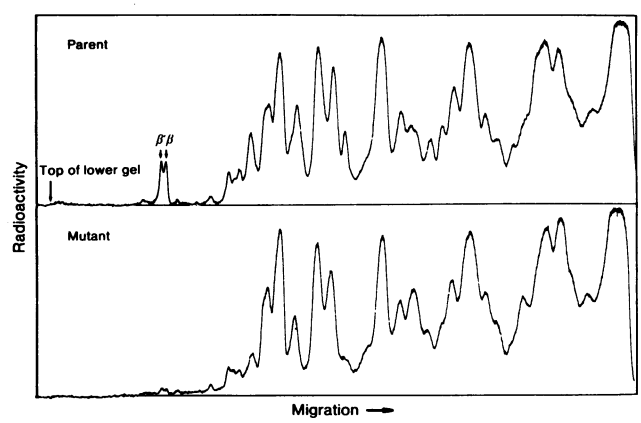

FIG. 2. Rate of β and β' subunit synthesis at 43°. Logarithmic phase cultures of PM142 and MX245, growing in digested Y broth at 25°, were shifted to 43°. Five minutes after the shift [^{14}C]tyrosine was added to the cultures and incubation was continued for 15 min (9 min, MX245), when unlabeled tyrosine was added. Incubation was continued for another 20 min (10 min, MX245), when the cells were harvested. Cells were washed and extracts prepared as described in *Materials and Methods*. Polyacrylamide gels (7-1/2%) were used, fractionating proteins of greater than 30,000 molecular weight (30). Radioactivity is expressed in arbitrary units.

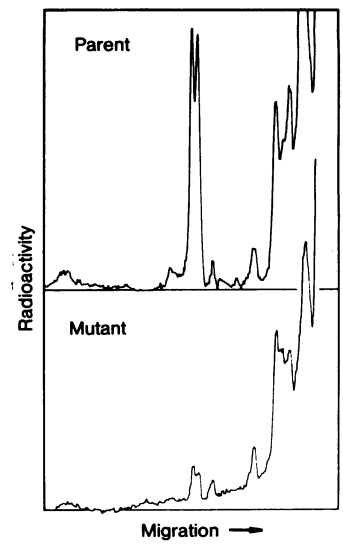

FIG. 3. Rate of β and β' subunit synthesis at 43°. The region of the gel depicted in Fig. 2, fractionating proteins between 100,000 and 180,000 molecular weight, is presented in an expanded tracing. See legend of Fig. 2 for experimental details.

tyrosine. Labeling was stopped by the addition of 0.4 μmol of tyrosine followed by 5-fold dilution into ice-cold saline. The cells were harvested by centrifugation, washed with ice-cold saline, and stored at −20°. The frozen cell pellets were resuspended in 0.1–0.2 ml of sample buffer (22) and immediately incubated at 90° for 3 min. Portions (25–50 μl) of the cell lysates were used for polyacrylamide gel fractionation and analysis according to the method of Maizel (22). The radioactivity of the fractionated proteins was determined by quantitative microdensitometry of autoradiograms of the dried gels with a Joyce-Loebl recording microdensitometer.

P1 Phage Crosses. Stocks of P1 vir, hereafter called P1, for transduction were obtained by growth in soft agar overlays containing R broth. The phage lysates were extracted from the agar by centrifugation and stored in R broth containing 10 mM $MgCl_2$ and 5 mM EDTA over $CHCl_3$.

Transductions were carried out according to the method of Cronan *et al.* (23). Recombinants were obtained with selective medium E plates incubated at 25°. Unselected markers were scored by replica plating isolates after recycling on selective medium E against R broth plates at 25 and 42° and R broth plates containing 40 μg/ml of rifampicin at 25°.

Dominance. Logarithmic phase cultures of F110/KL131 and the mutant strains grown in R broth at 37 and 27°, respectively, were mixed (five donors per recipient) to a total density of 5×10^8 cells per ml and incubated for 30 min with gentle shaking at 30°. Recombinants were selected by plating the mating mixtures on R broth plates containing 100 μg/ml of streptomycin and incubating at 42°. The formation of F′ heterodiploids, as opposed to chromosomal recombinants, was verified by transfer of F110 from the recombinants in a second mating. MX255 (F^-, *metB*) was used as the recipient, and the matings were carried out on selective medium E plates containing tyrosine, tryptophan, phenylalanine, arginine, leucine, and valine at 37°.

Physiological Studies. Logarithmic phase cultures of strains growing in R broth at 27° were transferred to 43°. Their

growth was monitored at 600 nm in a Coleman Jr. II spectrophotometer and by serial dilution and plating on R broth plates for colony-forming units at 25°. The morphology of the cells was monitored by phase contrast light microscopy.

RESULTS

Isolation and Characterization of Mutants. Two assumptions were used in designing the scheme for the isolation of RNA polymerase mutants: first, that the mutants would be lethal; second, that such mutations would be located in or near the structural genes for the enzyme. Conditional lethal temperature-sensitive mutants that mapped in the region of the structural genes for RNA polymerase were selected and tested for their capacity to synthesize RNA polymerase at 42°. Comutagenesis with nitrosoguanidine was used to enrich the yield of mutants located in the *rif* region of the chromosome (20). Resistance to the antibiotic rifampicin was used as the comutagenic selective marker. This marker was chosen because it was amenable to positive selection and because of its location in the desired region of the chromosome; it is actually a part of the structural gene for the β subunit of RNA polymerase (24).

Mutants that could not form colonies at 42° on R broth plates and that mapped in the *rif* region of the chromosome were tested for their ability to synthesize the RNA polymerase β and β′ subunits at 42°. Polymerase subunit synthesis was measured by determining the radioactivity of the protein fractionated from extracts of cultures pulse-labeled with [^{14}C]tyrosine (see *Materials and Methods*). Many of the mutants could not be grown in defined media, and so the labeling experiments were carried out in broth. Tyrosine was chosen as the amino acid for labeling because it is efficiently taken up by prototrophic *E. coli* strains (M. P. Oeschger, unpublished) and because nonradioactive tyrosine can be readily removed from broth medium by treatment with tyrosine decarboxylase (M. P. Oeschger and J. E. Cronan, Jr., manuscript in preparation).

The results of such labeling experiments are shown in Figs. 1, 2, and 3. Mutant isolate PM142 shows a normal (control) rate of β and β′ subunit synthesis at 25° but a markedly inhibited rate of synthesis of these two proteins at 42°. In experiments where no chase was used, similar results were obtained (data not shown).

Genetic Analysis. P1 phage transduction was used to further localize the mutation. P1 phage were grown on PM142, and *arg*$^+$ recombinants were selected with MX390 and *met*$^+$ recombinants with MX386 and MX387. The recombinants were scored for rifampicin-resistance and temperature-sensitive growth on R broth plates. As shown in Table 1, 85–90% of the *rif*^{r} transductants were temperature-sensitive; 100% of the *ts* transductants, however, were *rif*^{r}. Thus, mutant PM142 carries at least two mutations: a *rif*^{r} mutation which does not confer temperature-sensitivity; and a *ts* mutation which may also be in the *rif* locus or may be in a separate, closely linked locus. The fact that 68 out of 68 *ts* transductants were also rifampicin-resistant suggests that the former possibility is correct. Spot checks of a number of the recombinants confirmed that the temperature-sensitive phenotype correlated with defective RNA polymerase synthesis at 42°.

The mutation was observed to be recessive by heterodiploid analysis. The incorporation of F110, an episome that carries the region of the *E. coli* chromosome between 77 and 81 minutes (25), into PM142 cured the temperature-sensitivity and restored the capacity to synthesize the β and β′ subunits at 43° (data not shown). The formation of heterodiploids, as opposed to recombinants, was verified by the continued ability of the F110/PM142 isolates to transfer F110 in a second mating.

Revertants, isolated as colony formers at 42° on R broth plates, regained normal levels of β and β′ subunit synthesis at all temperatures.

TABLE 1. *Cotransduction of the rif*^{r} *and ts mutations*

Distribution of *rif* and ts markers in the *arg*$^+$ recombinants					Frequency (%)	
rif^{r}ts	*rif*^{r}ts$^+$	*rif*^{s}ts	*rif*^{s}ts$^+$	Total	*rif*^{r}ts/*rif*^{r}	*arg*$^+$*rif*^{r}/*arg*$^+$
68	9	0	123	200	88	39

Transductions and selection of recombinants were carried out as described in *Materials and Methods*. MX390 was used as the recipient, and *arg*$^+$ colonies were selected. ts and *rif*^{r} cotransductants were scored by replica plating. To establish the coincidence of the ts mutations and the regulation of RNA polymerase synthesis, random ts transductants were tested for their ability to synthesize RNA polymerase at 43° as described in the legend of Fig. 2.

Stability of the Enzyme. No difference in the rates of turnover of the β and β′ subunits was detected in a comparison of the mutant and parent strains at 25 or 43°. Cultures were labeled with [^{14}C]tyrosine at 25°, chased (as described in *Materials and Methods*), and maintained at 25° or transferred to 43°. Samples of the cultures were analyzed by sodium dodecyl sulfate gel electrophoresis. The amount of radioactivity in the β and β′ subunits was compared to that of the bulk cellular protein. The RNA polymerase subunits were observed to be stable (turn over at the rate of the cellular protein) in both the parent and mutant strains at both 25° and 43° (Table 2).

Physiological Studies. The growth and viability of transductants derived from PM142 were monitored after shift to 43°. The transductants were able to increase their mass 7- to 8-fold before the cessation of growth. All through the period

TABLE 2. *Ratio of pulse-labeled β and β′ RNA polymerase subunits to total protein in whole cells*

	Minutes of chase				
	25°			43°	
Strain	10	25	60	25	60
MX245	1.02	0.95	0.75	0.60	0.57
PM142	0.59	0.70	0.70	0.36	0.45

Logarithmic phase cultures of PM142 and MX245, growing in digested Y broth, were pulse-labeled with [^{14}C]tyrosine for 10 min. Labeling was terminated by the addition of unlabeled tyrosine, and a portion of each culture was immediately transferred to 43°. Samples were taken at the times indicated and prepared for electrophoresis. The details of the procedure were as described in *Materials and Methods*. Planimeter tracings of expanded densitometer tracings were used to determine the amount of ^{14}C-labeled β and β′ subunits and whole cellular protein. The ratios given are arbitrary units.

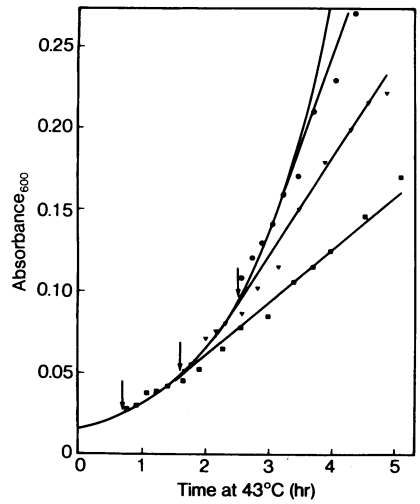

FIG. 4. Cell growth at 43°. The growth curves of a representative mutant (a transductant of PM87 into MX387*) and MX504 are compared at 43°. Portions of a logarithmic phase R broth culture of the mutant were transferred from 27 to 43° at the times indicated by the arrows. MX504 was maintained at 43° throughout the experiment and is represented in the figure by the exponential curve without points. ■, ▼, ●, growth of the mutant strain from three initial cell densities.

* A transductant was used to eliminate any possible extraneous effects arising from other mutations present in the original mutant strain.

of growth at 43° the cells remained small and were comparable microscopically to logarithmic phase cells taken from control cultures. The viability of mutant cultures held at the nonpermissive temperature for periods of up to 8 hr showed, at most, a 2-fold drop when compared to the colony-forming units of control cultures at the same turbidity (data not shown). The cessation of polymerase synthesis did have a definite effect on the rate of growth of the cultures. Upon shift from 25 to 43° the exponential increase in cell number continues for one doubling and then slows, possibly becoming arithmetic (Fig. 4), indicating that the concentration of RNA polymerase has become the rate factor in cell growth.

DISCUSSION

We have isolated a mutant of *E. coli* that is unable to synthesize RNA polymerase at 43°. The mutation affects the synthesis, not the degradation, of the enzyme by the following criteria: (*i*) β and β′ subunits made before the temperature shift are stable; (*ii*) the cells continue to grow for three generations after temperature shift to 43°; (*iii*) colony-forming units can be recovered after prolonged incubation at 43°; (*iv*) pulse-labeling experiments at 43° without a chase show no incorporation of radioisotope into the β and β′ polymerase subunits; and (*v*) the mutation is recessive in a heterodiploid analysis, ruling out a selective protease, mRNA degrading activity, or selective inability to translate polymerase mRNA.

The ts mutation can be transferred from one bacterial strain to another by P1 phage transduction. The distribution of recombinant genes among the transductants reveals a close linkage of the ts mutation to a second, *rif*^{r}, mutation (85–90% cotransduction). In recent studies by Stadler and Kariya correlating cotransduction frequency with physical distance between closely linked markers, a 90% cotransduction frequency was observed for *trp* mutations separated by less than 6700 base pairs (26, 27). The cistrons for the β and β′ subunits of RNA polymerase are part of an operon (12, 13) and account for approximately 9000 DNA base pairs (6). Therefore, it is apparent that the mutations could fall within the β′ subunit or just beyond it, depending on the spacing between β and β′ and on where in β *rif* is located. The successful transfer of the mutation to both su$^+$ and su$^-$ strains indicates that the mutation does not involve an amber nonsense suppressor. The fact that no *rif*^{s}*ts* recombinants were recovered indicates that either the *rif*^{r} locus is required for phenotypic expression or that there are two *rif*^{r} mutations, one of which conveys temperature-sensitivity. The *rif* region is subject to gene duplications which can be induced by nitrosoguanidine mutagenesis (28). Determination of the exact nature of the alteration in the DNA resulting in ts RNA polymerase synthesis will require further investigation.

The mutation causes a rapid and coordinate loss of the capacity to synthesize the β and β′ subunits of RNA polymerase after a shift in the culture temperature to 43°. This selective loss of protein-synthesizing ability could be accounted for by several mechanisms affecting the transcription and translation of polymerase mRNA. At this time we have no evidence in support of any one mechanism, but our data make the following translational or post-translational possibilities unlikely:

(*i*) the selective proteolytic breakdown of the polymerase subunits at 43° is not supported by the labeling experiments, even without a chase (Fig. 2);

(*ii*) there is no evidence for the accumulation of a precursor or higher-molecular-weight protein as a result of the mutation (Fig. 2);

(*iii*) a selective inhibition of translation due to collapse of nascent polymerase peptides on the ribosome appears to be unlikely by the following reasoning: a lowering of the concentration of active polymerase molecules results in a marked stimulation of polymerase synthesis (ref. 5 and M. P. Oeschger, unpublished results). The projected numbers of ribosomes taken from the protein-synthesizing pool by the enhanced rate of polymerase synthesis and ribosome inactivation would result in a continued slowing of the growth rate. Our results indicate that such is not the case, for the cells continue to grow without a decrease in rate for an extended period (Fig. 4).

The decision between transcription and translation will best be made by mRNA hybridization experiments with phage carrying the polymerase genes (13, 14). The effect of the mutation on the synthesis of the α and σ proteins has not been determined.

The inhibition of polymerase synthesis is reversible, for cells kept at 43° for up to 8 hr retain colony-forming ability. This result speaks for the prolonged stability of intracellular polymerase, in accord with the conclusions of Iwakura *et al.* (11), for the presence of active RNA polymerase is essential to cell recovery. In contrast to Iwakura *et al.* (11), we found no evidence for a nonpolymerase protein which migrates along with the β′ subunit (Figs. 2 and 3). The mutant did show some changes in the rates of synthesis of proteins other than the β and β′ subunits synthesized after shift to 43° (Fig. 2). This result may be due in part to a general phenomenon of reprogramming of gene expression which follows temperature shift (M. P. Oeschger, unpublished). The reprogramming encompasses the labeling period, making it very difficult to ob-

tain identical rates of synthesis for all the proteins from two separate cultures. The differences in labeling between the mutant and parent cultures seen in Fig. 2, other than that of the β and β' subunits, show no consistent pattern in duplicate experiments and, therefore, may be due to uncontrolled variations in experimental conditions. An alternate explanation is that the mutation has a pleiotropic effect on the rate of synthesis of a number of proteins in the cell. We cannot make a definite decision between the two possibilities at the present time.

The cellular factors that regulate the growth rate of cells have not been established. RNA polymerase has the potential to be a growth-limiting factor by determining the number of ribosomes, tRNAs and mRNAs available for protein synthesis. The number of RNA polymerase molecules involved in RNA synthesis has been calculated from the rates of RNA chain growth *in vivo* and the amounts of total RNA synthesis in cells growing at different rates (29). A comparison of the values with the total number of polymerase molecules per cell, as determined by sodium dodecyl sulfate gel electrophoresis of whole cell extracts, indicates that there is an excess of polymerase molecules at all rates of cell growth (9–11). The intracellular concentration of RNA polymerase does not increase in proportion to the growth rate, the calculated excess being at a minimum under optimum growth conditions (9–11). While the majority of promotors are, therefore, saturated by the concentration of RNA polymerase (apparent polymerase excess), there may exist a class of low affinity promotors that are not saturated and are thus directly affected by the polymerase concentration. If the gene products regulated by such promotors are critical for cell growth, the concentration of RNA polymerase which appears to be in excess may in reality be the rate-determining factor in cell growth. The experiments that determine RNA polymerase to be present in excess (9–11) are therefore open to the criticism that they ignore the possible existence of such a class of promotors.

Our mutant provides an alternate method to determine whether polymerase is present in excess or is a rate-determining factor in cell growth. Experimentally, the rate of growth of a culture is monitored after a shift in the growth temperature from 25 to 43°. At 43° no new polymerase molecules are made, although the molecules already present are stable and continue to function. The period of maintenance of exponential growth measures the extent of polymerase excess. The advantage of this method is that it specifically measures the excess of polymerase with respect to the transcription of essential gene products.

Since the apparent excess of polymerase is at a minimum in optimally growing cells (9–11), we carried out the temperature shift experiments in broth. We observed mutant cultures to double their mass before the onset of linear growth (Fig. 4). From these data we can estimate that RNA polymerase is present in a 2-fold excess even in rapidly growing cells, and that the concentration of growth-determining factors is probably not controlled by low efficiency promotors. Therefore, RNA polymerase, at the concentrations normally maintained within the cell, is not a rate-limiting factor in the growth of *E. coli*.

Note Added in Proof. We have recently investigated another ts RNA polymerase synthesis mutant which at 25° contains twice the parental amount of polymerase. The excess polymerase appears to be functional since the mutant continues to grow exponentially for two generations upon shift to 43°. The mutant grows at the parental rate at permissive temperatures, which supports the conclusion that RNA polymerase is in functional excess in normal cells.

We thank Ted Reid for his encouragement and help in initiating these experiments, John Cronan and Brooks Low for numerous invaluable discussions and free access to their strain collections, Winona Wagner for excellent technical assistance, Dick Burgess for pointing out to us that the β and β' subunits of RNA polymerase can be quantitated from sodium dodecyl sulfate gels of whole cell extracts, John Wohlheiter for the use of his microdensitometer, and Ellen Baron for help in preparation of the manuscript. This work was supported by Grant DRG-1109 from The Damon Runyon Memorial Fund and Grant VC-122 from The American Cancer Society.

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