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Temperature-Sensitive RNA Polymerase Mutants with Altered Subunit Synthesis and Degradation

(*Escherichia coli*/complementation groups/gel electrophoresis)

JOEL B. KIRSCHBAUM, IVAN V. CLAEYS, SERGIO NASI, BRUCE MOLHOLT*, AND JEFFREY H. MILLER

Département de Biologie moléculaire, Université de Genève, 30, quai Ernest Ansermet, 1211 Genève 4, Switzerland

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ABSTRACT **A temperature-sensitive mutant having a lethal mutation in the gene for the β subunit of RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) exhibits an apparent 2- to 3-fold decrease in the rates of both β and β′ subunit synthesis at the non-permissive temperature, relative to total protein. In contrast, a temperature-sensitive mutant with a lethal mutation in the gene encoding β′ has a 5- to 6-fold increase in the rates of β and β′ synthesis at 42°. These β and β′ mutants also exhibit rapid degradation of both subunits at the high temperature.**

RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) is responsible for the synthesis of most, if not all, of the RNA molecules coded for by the bacterial chromosome. The isolation of mutants with altered RNA polymerases is, therefore, essential to the study of transcription and its control. Compared to most other enzymes, RNA polymerase of *Escherichia coli* is extremely complex, consisting of at least four different subunits in the arrangement $\alpha_2\beta\beta'\sigma$ (1–3). The possible existence of a new subunit, σ', has recently been reported (4). Mutations in the individual subunits would not only shed light on their roles in the complex process of RNA synthesis, but might also provide information on the regulation of subunit synthesis itself.

To date, only the location of the β and β′ genes is known. The genes for these subunits form a single operon, located at about 79 min, which is transcribed in a clockwise fashion on the standard genetic map (5). The gene for the β subunit was originally identified because mutants resistant to the antibiotic rifampicin synthesized an RNA polymerase altered in this polypeptide (6). We recently isolated a large number of temperature-sensitive mutants having lethal mutations in the *rif* region of the chromosome, with the hope that some of these mutations would be in the structural genes for β (*rif*), β′, and whichever other subunit genes of RNA polymerase might be present in the same immediate region†.

This collection of 68 mutations has been divided into five complementation groups (Fig. 1) by complementation tests performed in *recA* strains. (For a complete description of the isolation, map position, and complementation properties of these mutations, see footnote.†) In order to determine which complementation groups corresponded to cistrons coding for the various RNA polymerase subunits, we employed two amber mutations in the gene for the β subunit. One of these mutations, *rif*°III/8, has been reported to be polar on the synthesis of the β′ subunit (7), while the other, *rif*°D12, has been reported to have no effect on β′ synthesis (7). We have independently verified the polar and nonpolar nature of these two amber mutations†. In addition, we found that all members of complementation group E (Fig. 1) complement the *rif*°D12 mutation, whereas none of the mutations in this group complement the polar mutation *rif*°III/8. Members of group D failed to complement either amber mutation. All mutations in groups A, B, and C complemented both amber mutations. These combined results† allowed us to assign group D to the cistron encoding the β subunit of RNA polymerase, and group E to a cistron corresponding to another gene in the same operon. Group E mutations also failed to complement *Ts*X, a temperature-sensitive RNA polymerase mutation isolated by Khesin *et al.* (8) which is known to be a β′ mutation as shown by the *in vitro* reconstitution of the RNA polymerase from purified subunits (9). We, therefore, have assigned complementation group E to the structural gene for β′†.

Here we report biochemical experiments performed on representative β and β′ mutants, and show that these mutations affect the rate of synthesis and breakdown of RNA polymerase subunits *in vivo*. These observations raise the possibility that the β and β′ subunits might act in some way to regulate the synthesis of the RNA polymerase.

MATERIAL AND METHODS

Bacterial Strains. For purposes of biochemical analysis, the temperature-sensitive mutations were transferred by

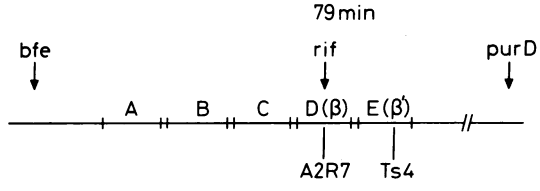

FIG. 1. Genetic map of the *rif* region. A, B, C, D, and E correspond to five complementation groups in the *rif* region of the chromosome in which temperature-sensitive lethal mutations have been found. Group D has been identified as the structural gene for the β subunit of RNA polymerase and group E as the cistron encoding the β′ subunit†.

* Present address: Institut für Molekulare Genetik, der Universität Heidelberg, Berlinerstr. 15, 69 Heidelberg 1, West Germany.

† I. Claeys, S. Van den Elsacker, B. Molholt, S. Nasi, J. Kirschbaum, and J. Miller, manuscript submitted.

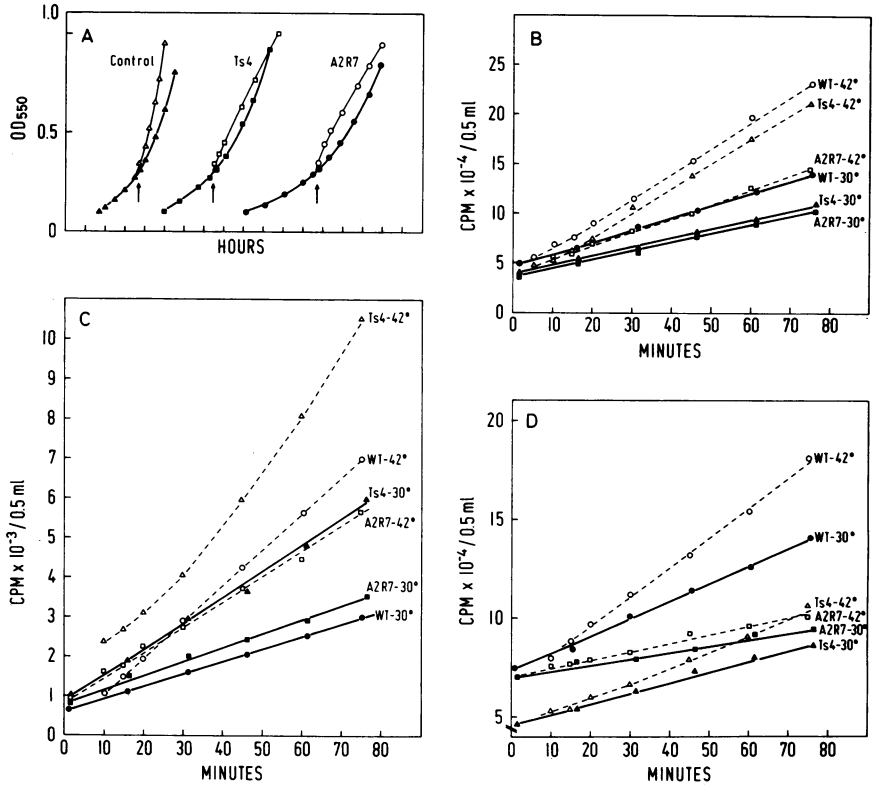

FIG. 2. (A) Growth curves of wild-type (WT) and mutant cultures at 30° and 42°. A sample of each culture growing exponentially at 30° (in M9 glucose minimal medium containing 1 μg/ml of vitamin B1 and 40 μg/ml of methionine and histidine) was shifted to 42° at the time indicated by the arrow. Turbidity of the cultures (OD_{550}) is plotted on a linear scale as a function of time (hours). Closed symbols represent growth at 30°, and the open symbols depict growth at 42°. (B) Leucine incorporation by mutant and wild-type strains at 30° and 42°. (C) Thymidine incorporation by mutant and wild-type strains at 30° and 42°. (D) Uridine incorporation by mutant and wild-type strains at 30° and 42°. Wild type: 30° (●——●), 42° (○- - -○); *A2R7:* 30° (■——■), 42° (□- - -□); *Ts4:* 30° (▲——▲), 42° (△- - -△).

Incorporation is expressed as cpm of trichloroacetic-acid-insoluble material per 0.5 ml of cell culture. All data have been corrected for nonspecific background incorporation. Cultures were grown in labeling medium (M9 glucose minimal medium containing 40 μg/ml of methionine and histidine, 20 μg/ml of uridine, 10 μg/ml of leucine and thymidine and 1 μg/ml of vitamin B1) at 30°. Each culture was divided into three aliquots and the various aliquots were labeled with radioactive leucine, uridine, or thymidine, at a final concentration of 1 μCi/ml. (For thymidine labeling, the corresponding aliquot was made 200 μg/ml in deoxyadenosine 15 min prior to the addition of label.) Following addition of label, the cultures were incubated for 1 hr at 30° to ensure complete equilibration of the label with the internal pools. At time zero, when the cultures were at density of 1×10^8 cells per ml, each labeled culture was then divided into two equal portions which were incubated at 30° and 42°. Samples of 0.5 ml, withdrawn at various times after the temperature shift, were added to tubes containing 5 ml of ice-cold trichloroacetic acid. The samples were chilled for 30 min in an ice-water bath and filtered on Whatman GF/C filters. The filters were extensively washed, dried, and analyzed in 5 ml of toluene-based scintillation fluid using a liquid scintillation spectrometer.

bacteriophage P1 transduction into *E. coli* strain X239 (F^- *his thi metB bfe purD argH-2 strA lac*) by selecting Arg^+Pur^+ transductants at 30°. Purified transductants were scored for inheritance of the temperature-sensitive mutation, and for loss of resistance to bacteriophage BF23 (*bfe*). The isogenic control strain for these experiments (X240) was prepared in an identical manner, using a P1 lysate grown on a wild-type donor.

Radioisotopes. [*5*-^{3}H]Uridine, 1 mCi/ml, 29 Ci/mmol, (Amersham: TRK 178): L-[*4,5*-^{3}H]leucine, 1 mCi/ml, 54 Ci/mmol, (Amersham: TRK 170): [*methyl*-^{3}H]thymidine, 1 mCi/ml, 20 Ci/mmol (New England Nuclear: NET 027X).

Gel Electrophoresis. Preparation of samples: Incorporation of L-[*4,5*-^{3}H]leucine by growing cultures was halted by the addition of NaN_3 to a 5 ml sample of the culture (final concentration of 0.05 M). The samples were chilled in an ice/water bath, centrifuged at 4°, and the cell pellets (containing about 10^9 cells) were washed once with ice-cold 0.01 M $MgSO_4$. The final cell pellets were drained and suspended in 0.2 ml of gel sample buffer (containing no Na dodecyl sulfate). The suspensions were made 3% in Na dodecyl sulfate and were heated for 3 min at 100°. The radioactivity in 10 μl was measured in 5 ml of toluene-based scintillation fluid containing 0.01% Na dodecyl sulfate and 10% NCS tissue solubilizer in order to determine total incorporation. Values obtained this way are in good agreement with values based on the measurements of trichloroacetic-acid-insoluble material.

Electrophoresis. Gel sample buffer and Na dodecyl sulfate/polyacrylamide gels were prepared according to Laemmli (10). The gels consisted of a 5% lower slab gel (9 cm long) and a 3% upper stacking slab gel. Samples (50 μl) of the labeled extracts were applied to the sample wells together with 0.2 μg of purified RNA polymerase holoenzyme, which serves as

an internal marker for the β and β' subunits. Samples were stacked for 3 hr at 10 mA, and electrophoresis was performed for 5.5 hr at 30 mA. The slab gel was stained for 1 hr at room temperature in a solution of methanol/glacial acetic acid/H_2O 5:1:5 containing 0.05% Coomassie brilliant blue. Destaining was performed with gentle agitation overnight in a solution of 7.5% glacial acetic acid/5% methanol. For each sample applied, a 2 cm long section of the slab gel containing the stained β and β' subunits was dissected from the main slab, and each such gel strip was further sliced into 1 mm sections with a razor blade slicing device. The individual 1 mm slices were transferred to counting vials containing 5 ml of a mixture of toluene-based scintillation fluid, 0.01% Na dodecyl sulfate, and 10% NCS tissue solubilizer. The capped vials were incubated for 6 hr at 37° with occasional agitation (the time determined to be necessary to completely solubilize the labeled protein), and the samples were analyzed in a liquid scintillation spectrometer. About 90% of the radioactive material applied to the gel is recovered by this method.

RESULTS

The temperature-sensitive β and β' mutations we have studied are designated *A2R7* and *Ts4*, respectively. The *A2R7* β mutation is actually a double mutation consisting of the temperature-sensitive mutation *A2*, known to map in the β subunit gene†, and the spontaneous rifampicin resistance mutation *R7*. Strains carrying this double mutation are more temperature sensitive in single colony formation at 42° than strains carrying *A2* alone†. Isogenic derivatives containing the wild-type, *Ts4*, and *A2R7* alleles were constructed as described in *Material and Methods*.

These strains grow exponentially in glucose minimal medium at 30° with doubling times of about 80, 100, and 140 min, respectively. When log phase cultures are shifted to 42°, turbidity continues to increase in all cases for several hours after the shift. However, unlike the wild-type control culture, which exhibits an exponential increase in turbidity at 42°, both mutant cultures display linear growth kinetics (Fig. 2A). The cells in these cultures do continue to divide during this linear growth phase, since only unit length and double unit length cells can be seen in the 42° population (i.e., the cells do not form snakelike elongated structures). Protein, DNA, and RNA synthesis in the mutants are also not significantly affected by the shift to high temperature. Figs. 2B, C, and D show that there is no rapid shutoff of incorporation of leucine, thymidine, or uridine into acid-insoluble material after the shift to 42°.

The mutants do, however, show a striking deviation from the wild type at the level of RNA polymerase subunit synthesis and breakdown. Because of their extremely large sizes, the β and β' subunits can be separated from all other bacterial proteins in crude cell extracts by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (ref. 11; see legend to Fig. 3A). Thus, the *in vivo* levels of these two subunits can be measured by expressing the amount of a labeled amino acid incorporated into these purified proteins as the percent of incorporation into total protein.

We have measured the levels of β and β' in the mutants, using a variety of pulse-chase labeling experiments. In the initial experiment, cultures of wild-type *A2R7*, and *Ts4* were shifted to 42° for 15 min, pulse-labeled for 2 min with [^{3}H]-leucine, and were then chased for 30 min at 42° in the presence

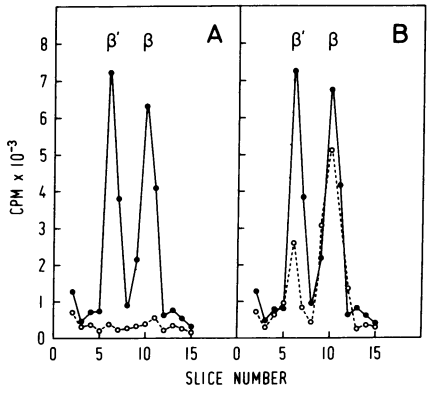

FIG. 3. (A) $\beta\beta'$ gel profile of *A2R7* and wild-type; (B) $\beta\beta'$ gel profile of *Ts4* and wild type. In both figures, the data for the mutants (○) have been normalized relative to wild type (●) with respect to total cpm applied to the gels. (The curves depicting *A2R7* and *Ts4* were generated using normalization factors of 1.3 and 1.7, respectively.) Recently, Iwakura *et al.* (4) observed an additional polypeptide in crude extracts of *E. coli* K12 W3350 unrelated to the RNA polymerase subunits which migrated in the immediate vicinity of the β' subunit on Na dodecyl sulfate/polyacrylamide gels. This polypeptide was present in about twice as many copies as β' itself and interfered with measurements of β' levels by this method. However, this protein is apparently not present in our test strain background, as can be seen in (A).

of a large excess of unlabeled leucine. Extracts were prepared and fractionated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Fig. 3A and B, which depicts the $\beta\beta'$ profiles from the gels, show the amounts of pulse-labeled β and β' subunits remaining in the mutants after the 30 min chase, relative to wild type. Under these conditions, no detectable labeled β or β' can be found in extracts of *A2R7* (Fig. 3A). *Ts4* also shows a reduction of both subunits, with the β' subunit being preferentially affected (Fig. 3B).

This behavior of both mutants at 42° can be shown to be due to a combination of two different effects: (*a*) a change in the instantaneous rate of subunit synthesis, together with (*b*) the rapid degradation of newly synthesized subunits. Fig. 4A and B present the results of more extensive pulse-chase analyses performed with the mutant and wild-type strains at both 30° and 42°. In these experiments, cultures grown at 30° were divided in half and incubated at 30° and 42°. After 15 min at 42°, both sets of cultures were pulse-labeled for 2 min with [^{3}H]leucine, a large excess of unlabeled leucine was added, and samples were withdrawn at the times indicated for the measurement of subunit levels. The values corresponding to the time zero points in these figures, which are taken as estimates of the instantaneous rates of subunit synthesis, were actually determined on samples withdrawn after a 3 min chase. This brief chase is necessary to allow for completion of those nascent β and β' subunit chains (4) that would otherwise have gone undetected in the gel assay because of their smaller sizes.

Fig. 4A shows that at 30°, *A2R7* synthesizes both β and β' at a slightly higher rate than wild-type, and that these subunits are degraded with half-lives of about 63 and 35 min, respectively. Fifteen minutes after the culture is shifted to 42°, not only does this mutant appear to synthesize both subunits at a 2- to 3-fold lower rate than at 30°, but also it degrades both subunits with a half-life of about 8 min. Conse-

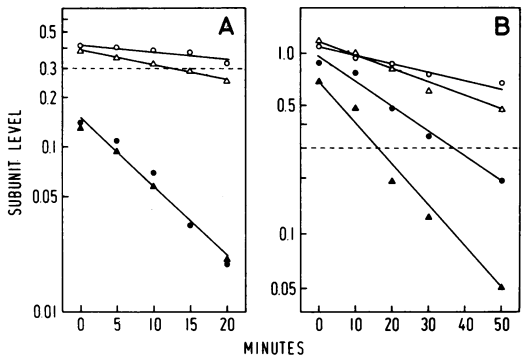

FIG. 4. Pulse-chase analysis of the β and β' subunits synthesized in *A2R7* (A) and *Ts4* (B). Subunit level is given as the percent of the total incorporation in the individual subunits, as determined in *Material and Methods*. β subunit levels are indicated by circles (open, 30°; closed, 42°); β' levels are represented by triangles (open, 30°; closed, 42°). Both the individual β and β' subunit levels for the wild-type control are identical at 30° and 42°, and are represented simply by the broken line for clarity. Cultures of wild-type and mutant strains *A2R7* and *Ts4* were grown at 30° in M9 glucose minimal medium containing 40 μg/ml of methionine and histidine, and 1 μg/ml of vitamin B1. At a cell density of 1×10^8 cells per ml, a sample of each culture was shifted to 42° for 15 min. At this time, both the 30° and 42° cultures were pulse-labeled for 2 min with [^{3}H]leucine at a final concentration of 10 μCi/ml; unlabeled leucine was then added to a final concentration of 200 μg/ml and incubation was continued at the respective temperatures. Samples (5.0 ml) of the labeled cultures were withdrawn at various times, and were processed as described in *Material and Methods*.

quently, by 20–30 min after pulse-labeling at 42°, there are no significant amounts of either labeled β or β' present in the cell (see also Fig. 3A). To determine whether subunits synthesized at 30° are also rapidly degraded at 42°, a culture of *A2R7* was pulse-labeled for 2 min and chased for 10 min at 30°, and was then shifted to 42° and chased for an additional

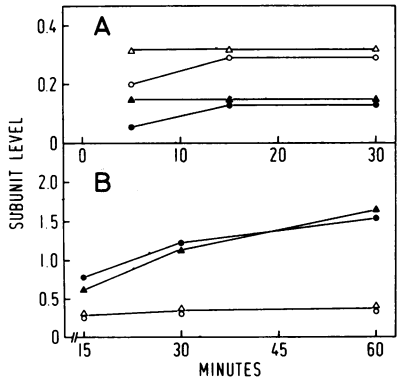

FIG. 5. Instantaneous rate of β and β' synthesis in *A2R7* (A) and *Ts4* (B) at various times after the shift to 42°. Subunit levels in the wild type and mutants are represented by open and closed symbols, respectively: β, circles; β', triangles. Cultures of the wild-type and mutant strains *Ts4* and *A2R7* were grown at 30°, as described above, to a density of 1×10^8 cells per ml. At this time, each culture was divided into three aliquots, and all aliquots were simultaneously transferred to 42°. At the indicated times following the temperature shift, cultures were pulse-labeled for 2 min and chased for 3 min. Labeled samples were processed as described in *Material and Methods*.

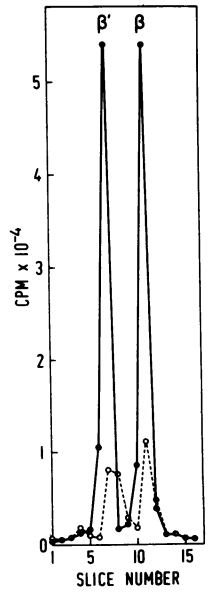

FIG. 6. Overproduction of $\beta\beta'$ in *Ts4* relative to wild type as seen by pulse-labeling 60 min after the shift to 42°. The $\beta\beta'$ gel profiles used in the analysis described in Fig. 5B are shown here for the wild type (○) and mutant (●). The data for the mutant have been normalized relative to wild type with respect to total cpm applied to the gels by using a normalization factor of 1.7.

30 min. About 50% of the newly synthesized β and β' was degraded under these conditions (data not shown). Thus, in *A2R7*, subunits synthesized at 30° are degraded less rapidly at 42° than subunits synthesized at the high temperature. Fig. 5A, which presents the instantaneous rates of β and β' synthesis in *A2R7* as a function of time after the shift to 42°, shows that the apparent 2-fold reduction in synthesis rate observed earlier occurs within 5 min after the shift, and is not further affected by longer preincubations at the high temperature.

Mutant *Ts4* displays a somewhat different behavior in an analogous set of experiments. This mutant overproduces both subunits about 3-fold, relative to wild type, at 30°, and both subunits are subsequently degraded with estimated half-lives of 52 min for β and 36 min for β' (Fig. 4B). Fifteen minutes after the shift to 42°, the mutant still exhibits significant subunit overproduction, relative to wild type, and degrades β and β' with half-lives of 23 and 12 min, respectively. When a culture of *Ts4* was pulse-labeled for 2 min and chased for 10 min at 30°, and was then shifted to 42° and chased for an additional 30 min, the decay of the labeled subunits followed the kinetics observed for subunits synthesized at 42° (data not shown). Consequently, subunits synthesized at 30° are no more stable at 42° than subunits newly synthesized at the high temperature. Also the $\beta\beta'$ overproduction initially observed at 42° in this mutant becomes even more pronounced with longer preincubation times at the high temperature. The instantaneous rate of synthesis of both subunits after 1 hr at 42° is 5- to 6-fold greater than wild type (Figs. 5B and 6).

Preliminary screening of four other mutants belonging to the β cistron, including the single mutant *A2*, shows that these members are similar to the *A2R7* mutant in their patterns of $\beta\beta'$ synthesis at 42°. Likewise, five other β' mutants behave in an analogous fashion to *Ts4*. However,

analysis of one representative member of each of the other three complementation groups indicates that these mutants do not differ from wild type in their patterns of $\beta\beta'$ synthesis (Kirschbaum, unpublished results).

DISCUSSION

We have investigated certain *in vivo* properties of temperature-sensitive lethal mutations mapping in the genes encoding the β and β' subunits of RNA polymerase†. Neither the β mutant (*A2R7*) nor the β' mutant (*Ts4*) exhibits rapid cessation of RNA synthesis within 1 hr after the shift to the nonpermissive temperature. However, during this time interval, both mutants show striking deviations from the wild type with regard to the synthesis and stability of the $\beta\beta'$ subunits.

In particular, within 5 min after the shift to 42°, *A2R7* (the β mutant) synthesizes both subunits at an apparent 2- to 3-fold lower rate than wild type and both subunits are subsequently degraded at the same rapid rate such that after 20 min chase there is no detectable pulse-labeled β or β' in the cell. Although *Ts4* (the β' mutant) actually overproduces these subunits at 42°, both β and β' are also degraded rapidly at exponential rates. A possible explanation for both the linear kinetics of growth exhibited by both mutants at 42°, and the inability of these strains to form single colonies at 42° would be that the overall levels of RNA polymerase in the cell rapidly become limiting at the high temperature due to subunit degradation. Additional rounds of cell division only further dilute the amounts of enzyme present until the cells can no longer grow and divide.

Although the β and β' subunits synthesized in wild-type cells are completely stable at 42°, conceivably these subunits normally resist degradation only by virtue of being assembled into the core or holoenzyme complex. No significant amounts of free β or β' have been detected in wild-type crude extracts fractionated by glycerol gradient centrifugation (4). Also, under certain conditions, cells that synthesize β' in excess to β have been reported to rapidly degrade these additional β' subunits (7). The *A2R7* and *Ts4* mutations could, therefore, possibly interfere with the normal assembly of β and β' into a stable complex, since a mutation in one subunit apparently affects the stability of both proteins. Alternatively, it is possible that, once assembled, the RNA polymerase itself is unstable in these mutants.

These mutations also affect the instantaneous rates of β and β' subunit synthesis. The *A2R7* mutation, which we have assigned to the gene coding for the β subunit, results in an apparent 2- to 3-fold reduced rate of $\beta\beta'$ synthesis at 42°. (It is unlikely that this effect could be caused by degradation of the labeled subunits during the 2 min pulse and 3 min chase period used for this measurement, because the subunits would have to have half-lives of less than 5 min.) Recently, Oeschger and Berlyn have reported the isolation of a temperature-sensitive mutant which is likewise defective in the synthesis of β and β' at 42° (12).

In contrast, the *Ts4* mutation, a mutation in the gene for β', causes significant overproduction of both subunits. These observations raise the interesting possibility that the individual RNA polymerase subunits might play important roles in regulating the overall rate of enzyme synthesis. Such autogenous mechanisms of regulation have been hypothesized for RNA polymerase (13, 14), and are well documented for a variety of other systems (15).

It would be valuable to extend the subunit overproduction studies on *Ts4* to include measurements of α and σ levels, to determine whether synthesis of these subunits is affected coordinately with β and β'. In addition, quantitative hybridization experiments using the DNA of the specialized transducing phage λ*frif*^{d}18 (16), recently shown to carry the genes for at least β and β' (17), could reveal whether the subunit overproduction results from an increase in specific mRNA synthesis. The existence of mutants, such as *A2R7*, which synthesize subnormal amounts of β and β', creates the possibility of generating mutations in the genetic controlling elements for RNA polymerase by screening for pseudorevertants able to form colonies at 42° which synthesize higher levels of these subunits.

In addition to the analyses of the *A2R7* β mutant, and the *Ts4* β' mutant reported here, we have also examined four additional β and 5 additional β' mutants, all isolated independently. All of these mutants resembled *A2R7* and *Ts4* in that they exhibited abnormal pattens of subunit synthesis. However, amongst the additional β' mutants studied, we have identified a class of mutants which, unlike *Ts4*, immediately stop incorporating uridine into acid-insoluble material after the shift to 42°†.

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