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NOTES

Changes in the Concentrations of Guanosine 5′-Diphosphate 3′-Diphosphate and the Initiating Nucleoside Triphosphate Account for Inhibition of rRNA Transcription in Fructose-1,6-Diphosphate Aldolase (*fda*) Mutants

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Early screens for conditional lethal mutations that affected rRNA expression in *Escherichia coli* identified temperature-sensitive *fda* mutants (*fda* encodes the glycolytic enzyme fructose-1,6-diphosphate aldolase). It was shown that these *fda*(Ts) mutants were severely impaired in rRNA synthesis upon shift to the restrictive temperature, although the mechanism of inhibition was never determined. Here, we bring resolution to this long-standing question by showing that changes in the concentrations of guanosine 5′-diphosphate 3′-diphosphate and initiating nucleoside triphosphates can account for the previously observed effects of *fda* mutations on rRNA transcription.

Alleles of the gene for fructose-1,6-diphosphate (FDP) aldolase (*fda*) were isolated in two independent screens for temperature-sensitive mutants affecting macromolecule biosynthesis in *Escherichia coli* (3, 9). Both *fda* alleles (*h8* [3] and *ts8* [9]) caused severe decreases in the synthesis rate of rRNA at the restrictive temperature in cultures grown in the presence of glucose (or other hexoses). Mutations in *fda* leading to similar phenotypes have also been identified in *Bacillus subtilis* (11, 22). Originally, the *ts8* allele was mapped to another locus (9), but subsequent work definitively mapped the mutation to *fda* and showed that the temperature-sensitive phenotype resulted from a T-to-G mutation at nucleotide position 901 of the *fda* coding sequence, resulting in a Val-to-Gly substitution at amino acid 301 (20). Singer and coworkers showed that both *ts8* and *h8* specifically decreased transcription initiation at *rrn* P1 promoters (21). The concentration of FDP, the substrate for aldolase, increased sharply in the mutant after temperature shift (3), but it was not reported that FDP inhibited rRNA promoter function. Furthermore, in agreement with the original report (9), Singer and coworkers concluded that effects of the *ts8* mutation on guanosine 5′-diphosphate 3′-diphosphate (ppGpp), a direct negative regulator of *rrn* promoters (1), were not sufficient to account for the effects of the *fda* mutations on rRNA transcription (1, 4, 21). Therefore, the mechanism of inhibition has remained unclear.

rRNA promoter strength derives in large part from binding of the transcription factor Fis and the α subunit of RNA polymerase to the region upstream of the −35 element in *rrn* P1 promoters (6). However, neither the binding sites for Fis nor the full α binding site were required for the effects of the *fda* mutation on *rrnB* P1 promoter activity (21), implicating the *rrn* P1 core promoter as the regulatory target of *fda*. Since *rrn* P1 core promoters are regulated directly by the concentration of their initiating nucleoside triphosphate (iNTP) as well as by ppGpp (5, 18), here we tested the hypothesis that changes in the concentrations of ppGpp and the iNTP together account for the effects of *fda* mutations on rRNA transcription.

The *ts8 fda* mutation was moved to an otherwise wild-type background (strain VH1000 [5]) by transduction with phage P1 using a linked Tn*10* (*zdg-210*::Tn*10* [20]) and selecting for tetracycline resistance. Previous reports had suggested that *ts8* strains were not competent hosts for growth of P1*vir* (20); however, we were able to make P1*vir* lysates on a C600 *ts8* strain (a gift from M. Singer, University of California—Davis) using standard protocols (10). We constructed *fda* and wild-type strains carrying a λ prophage containing an *rrn* P1 promoter synthesizing a short-lived *lacZ* mRNA for use as a reporter of rRNA transcription (16). These strains were then transformed with a multicopy plasmid expressing λ cI repressor (pFL122, a gift from M. Filutowicz and J. Wild, University of Wisconsin—Madison) in order to avoid induction of the temperature-sensitive lysogens (*c*I857) following a temperature shift.

We measured RNA synthesis rates from two promoters, *rrnB* P1 and *rrnB* P1(dis), by primer extension (13). *rrnB* P1 is a well-characterized promoter that forms a short-lived open complex and consequently is regulated negatively by increases in the ppGpp concentration and positively by increases in the ATP concentration (1, 5, 18). *rrnB* P1(dis), a variant of *rrnB* P1 with a 3-bp change in the discriminator region (the sequence between the −10 hexamer and the transcription start site), served as a control. *rrnB* P1(dis) makes exactly the same transcript as *rrnB* P1, but it forms a much longer-lived open com-

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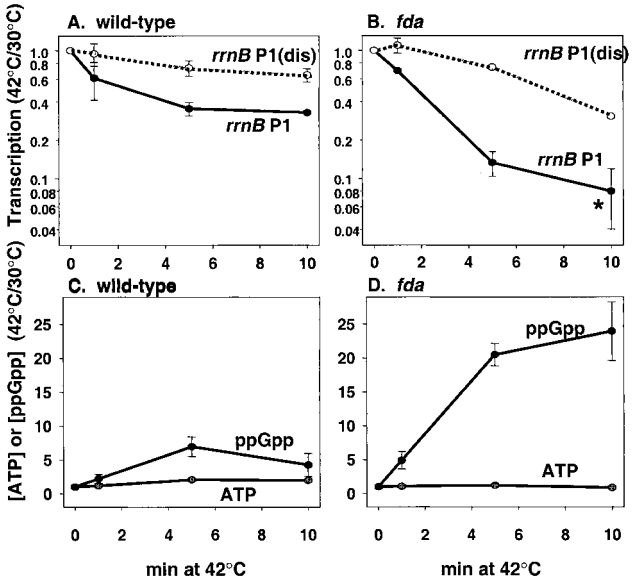

FIG. 1. Effects of the *fda*(Ts) mutation on transcription and on ppGpp and iNTP concentrations. *rrnB* P1 and *rrnB* P1(dis) promoter activities were measured by primer extension (13) in wild-type (A) and *ts8* (B) strains before and as a function of time after a shift from 30 to 42°C. The promoters contained *rrnB* P1 DNA sequences from −66 to + 9 with respect to the transcription start site (+1) and synthesized an unstable RNA (18). Cultures (*rrnB* P1, RLG6256; *rrnB* P1(dis), RLG6257; *rrnB* P1 *fda*, RLG6258; *rrnB* P1(dis) *fda*, RLG6259) were grown in morpholinepropanesulfonic acid (MOPS) minimal medium (15) supplemented with 0.4% glucose, 0.4% Casamino Acids, 40 μg of Trp/ml, 5 μg of thiamine/ml, 1 mM KH_2PO_4, and 100 μg of ampicillin/ml. The asterisk in panel B indicates that promoter activity at this time point was at or below background. ATP and iNTP concentrations were extracted with formic acid and measured by thin-layer chromatography as described previously (18) from wild-type (C) and *ts8* (D) strains grown in parallel with the cultures in panels A and B, except 20 μCi of $KH_2{}^{32}PO_4$/ml was added to the medium. Values were normalized to those at time zero (just before the temperature shift). Each point represents the average of at least two measurements from independent cultures. Error is indicated.

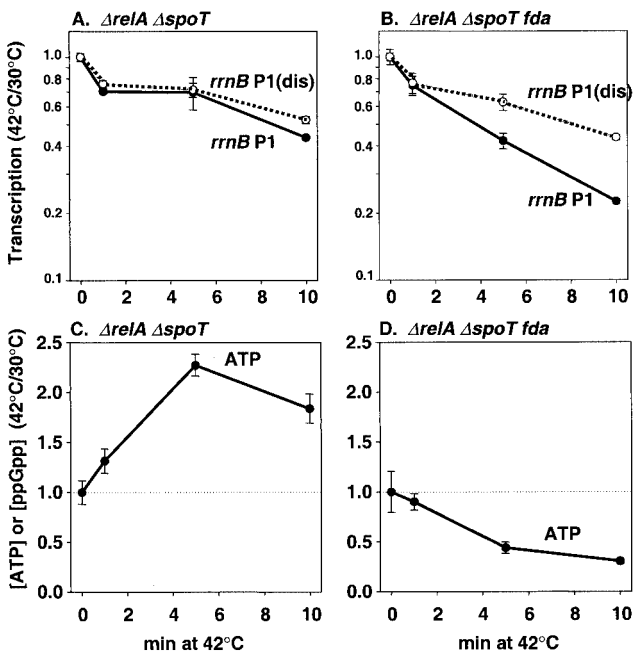

FIG. 2. Effects of *fda*(Ts) on promoter activity and ATP concentrations in Δ*relA* Δ*spoT* strains. *rrnB* P1 and *rrnB* P1(dis) activities were measured by primer extension in Δ*relA* Δ*spoT* (A) and Δ*relA* Δ*spoT* (B) *fda* strains as detailed in the legend to Fig. 1. Strains described in the legend to Fig. 1 were sequentially transduced to Δ*relA* Δ*spoT* as described previously (18) and were grown as indicated in the legend, except that the medium contained 1.0% Casamino Acids. ATP concentrations were measured from Δ*relA* Δ*spoT* (C) and Δ*relA* Δ*spoT fda* (D) strains grown as indicated in the legend for panels A and B except 20 μCi of $KH_2{}^{32}PO_4$/ml was included in the medium. Each point represents the average of at least two measurements from independent cultures. Error is indicated.

plex, resulting in relative insensitivity to changes in both the ppGpp and ATP concentrations (1, 2, 8, 18). We also measured the concentrations of ppGpp and ATP by formic acid extraction and thin-layer chromatography (7).

In the wild-type strain, the activities of both the *rrnB* P1 and *rrnB* P1(dis) promoters decreased slightly after a shift from 30 to 42°C (approximately three- and approximately twofold, respectively) (Fig. 1A), consistent with previous reports describing the effects of high temperature on rRNA transcription (17). In contrast, *rrnB* P1 promoter activity dropped sharply (>12-fold) in the *fda* strain after a shift to the restrictive temperature, much more than the decrease in *rrnB* P1(dis) promoter activity (Fig. 1B). The RNA synthesized by *rrnB* P1 was essentially undetectable by 10 min after the shift (Fig. 1B, asterisk); therefore, the ~12-fold decrease may underestimate the effect of the *fda* mutation on *rrnB* P1 activity.

Concurrent with the drop in *rrnB* P1 promoter activity in the *fda* mutant, there was a large (~25-fold) increase in the concentration of ppGpp at the restrictive temperature, much larger than the increase in ppGpp in the wild-type strain (Fig. 1C and D). We did not detect a significant change in ATP concentration in either the mutant or wild-type strain. Thus, contrary to expectations from previous investigations (9, 21), inactivation of aldolase led to induction of ppGpp, accounting for the large decrease in *rrnB* P1 activity. The mechanisms responsible for induction of ppGpp after inactivation of aldolase, glucose starvation (4), or inhibition of glucose uptake by α-methyl-glucoside (4, 14) remain to be determined.

Previous reports indicated that *rrnB* P1 activity was still inhibited by the *fda* mutation in cells lacking ppGpp (Δ*relA* Δ*spoT*) (21). Since *rrn* P1 promoters can also be regulated by the iNTP concentration (14, 18), we asked whether changes in ATP concentration could account for the ppGpp-independent effect of *fda*. After a shift to 42°C, the activities of the *rrnB* P1 and *rrnB* P1(dis) promoters decreased slightly and to the same extent in a strain that cannot make ppGpp (Fig. 2A). In a Δ*relA* Δ*spoT fda* strain, however, the activity of *rrnB* P1 decreased approximately twofold more than that of the control promoter (Fig. 2B), in agreement with the conclusion reached by Singer and coworkers (21). Notably, the ATP concentration dropped approximately threefold in the Δ*relA* Δ*spoT fda* strain (Fig. 2D), whereas the ATP concentration increased approximately twofold at 42°C in the Δ*relA* Δ*spoT* strain (Fig. 2C). The simplest interpretation of these data is that ppGpp inhibits rRNA synthesis at the restrictive temperature in an *fda* strain, but in a strain without ppGpp (Δ*relA* Δ*spoT fda*), a decrease in the

ATP concentration accounts for the residual inhibition of rRNA transcription. Thus, we suggest that changes in the concentrations of ppGpp and iNTPs are sufficient to account for the observed effects of the *fda* mutation on rRNA transcription (although our data do not rule out the participation of an additional mechanism[s]). These data reinforce the connections between central metabolism and the concentrations of iNTPs and ppGpp (14), although the mechanisms responsible for some of these connections remain to be determined.

A recent report from Morita and coworkers demonstrated that there is an RNase E-dependent decrease in mRNA stability when certain glycolytic enzymes (including fructose-1,6-diphosphate aldolase) (12) are disrupted. This decrease in message stability might contribute to the nonspecific decrease in promoter activity [i.e., that observed on *rrnB* P1(dis)] in the *fda* strain at 42°C.

Although ppGpp and iNTP concentrations have specific, nonredundant roles in the control of rRNA transcription (14), the results reported here illustrate how a decrease in the iNTP concentration can compensate for impaired ability to induce ppGpp. We have observed several other examples where changes in the concentrations of both of these small-molecule regulators compensate for defects in rRNA synthesis: after disruption of the *rrn* antitermination machinery by mutations in *nusB*, after inhibition of UP element function by mutations in *rpoA*, and after deletion of the transcriptional activator *fis* (19). Taken together, our findings support the model that ppGpp and iNTPs form two separate but complementary homeostatic control loops, fine-tuning rRNA synthesis to the growth conditions.

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