Mechanism of Stimulation of Ribosomal Promoters by Binding of the +1 and +2 Nucleotides*

Chih M. Lew and Jay D. Gralla‡

From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, California 90095-1569

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The rate of transcription of Escherichia coli ribosomal RNA promoters is central to adjusting the cellular growth rate to nutritional conditions. The +1 initiating nucleotide and ppGpp are regulatory effectors of these promoters. The data herein show that in vitro transcription is also regulated by the +2 nucleotide. Both the +1 and +2 nucleotides act by driving polymerase into an altered conformation rather than by increasing the lifetime of transcription complexes. The unique design of the ribosomal promoters may stabilize a distorted state of polymerase that is relieved by the binding of the two nucleotides required for transcription initiation.

Transcription of ribosomal RNA is the limiting step in ribosome production (1). The ribosomal promoters sense the overall availability of nutrients and respond by producing amounts of rRNA that can support the maximal growth possible given the nutritional environment. Nucleotides have been shown to be critical effectors of this promoter response. The signal nucleotide ppGpp is produced in inverse concentration to growth rate, and this may help keep the rate of rRNA transcription at an appropriate level (2, 3). The ribosomal promoters are also unusually sensitive to the concentration of the initiating nucleotide (4, 5). During transitions to slow growth, the nucleotide concentration can be depressed, and this prevents unnecessary transcription of ribosomal components (3).

The mechanism by which nucleotides affect transcription from ribosomal promoters has received a great deal of attention. In the case of the initiating nucleotide, it was proposed that it works by stabilizing polymerase-promoter DNA complexes (4). This effect is thought to be specific to the ribosomal promoters because their complexes have short half-lives and are therefore uniquely unstable. ppGpp also reduces the complex half-life (6) and competes with the initiating nucleotide (5).

There are uncertainties associated with this mechanism of nucleotide control of ribosomal transcription. In order for the short lifetime of ribosomal complexes to reduce transcription, dissociation would need to occur before the first RNA bond is formed, but this is typically quite rapid (7–10). How these unstable complexes are uniquely stabilized by the +1 nucleotide is not known. In addition, recent experiments in other transcription systems have shown that the +2 nucleotide can also have stimulatory effects. This occurs during initiation by the viral T7 RNA polymerase (11) and during elongation by the Escherichia coli RNA polymerase (12). Nucleotides that do not match the +1 position on the template can stimulate the isomerization of E. coli RNA polymerase in complexes with fork-junction DNA. The structure of such complexes is known (13) and gives no obvious clue as to how this stabilization by nucleotides could occur.

For these reasons we have re-evaluated the role of nucleotides as effectors of ribosomal transcription. The new data show that both the +1 and +2 nucleotides can act as effectors, unifying the properties of the bacterial and viral RNA polymerases.

EXPERIMENTAL PROCEDURES

Plasmids and Proteins—E. coli holoenzyme was purchased from Epicentre. Plasmid rrnB P1-pTH8 is a derivative of pTH8 (14) with the rrnB P1 promoter replacing the glnA promoter. rrnB P1-pTH8 was constructed as follows: complementary rrnB P1 promoter oligonucleotides from −61 to +15 with 5′-BamHI and 3′-HindIII sites (Qiagen) were phosphorylated, annealed, and inserted into digested pTH8. Plasmid rrnB P1(+2G)-pTH8 was created by site-directed mutagenesis (Stratagene). The pTH8-UV5 plasmid was as described previously (15).

Transcription—This was conducted as described in Refs. 4, 16, and 17, with minor changes. 5 nM rrnB P1-pTH8 or rrnB P1(+2G)-pTH8 was incubated in Buffer A (4) with 40 mM Tris acetate, pH 7.9, 10 mM MgCl2, 100 μM acetylated bovine serum albumin, 170 mM KCl, 1 mM β-mercaptoethanol, and 50 nM E. coli RNA polymerase at 37 °C for 5 min. For nucleotide titration experiments, the nonradioactive NTPs were at 200 μM with 25 μM [α-32P]UTP at 0.1 μCi/μl. Transcription was for 10 min at 37 °C.

In NTP stabilization experiments, 2 μM specified NTP was preincubated with the transcription complex for 5 min at 37 °C. For hepatitis challenge experiments, 100 μg/ml heparin was added to the transcription complex for 3 min at room temperature prior to the addition of the transcription nucleotide mix. A nucleotide mix of 200 μM ATP, 200 μM CTP, 200 μM GTP, and 25 μM [α-32P]UTP at 0.1 μCi/μl final concentration was then added for 10 min at 37 °C in a final volume of 10 μl. In the half-life experiment, a scaled-up reaction with 2 μl holoenzyme was incubated with 1 μM pTH8-rrnB P1 in Buffer A for 5 min at 37 °C. 20 μM BamHIII-cut pTH8-rrnB P1 competitor was then added. An aliquot was removed and added to a nucleotide mix as indicated above at various time intervals. Transcription was allowed to proceed for 10 min at 37 °C. A control was run to obtain the background transcription with BamHIII-cutt pTH8-rrnB P1, and this background was subtracted from the signal. In the control, 2 μl holoenzyme was preincubated with 20 μM BamHIII-cut pTH8-rrnB P1 for 5 min at 37 °C, and then 1 μl uncut pTH8-rrnB P1 was added and transcription proceeded for 10 min at 37 °C. The controls for reactions with the nucleotide used for the preincubation contained 2 μM of the indicated nucleotide in the incubation with holoenzyme-BamHIII-cut pTH8-rrnB P1. A formamide-urea dye mix was added to stop the

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‡ To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry and the Molecular Biology Inst., UCLA, P. O. Box 951569, Los Angeles, CA 90095-1569. Tel.: 310-825-1620; Fax: 310-267-2302; E-mail: gralla@chem.ucla.edu.

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RESULTS

High Concentration of Both the +1 and +2 Nucleotides Are Required at the rrnB P1 Promoter—A key property of the rrnB P1 promoter and its variants is that it transcribes poorly when the concentration of the +1 initiating nucleoside triphosphate is low. Experiments indicated that nucleotide positions downstream from +1 do not induce this property (4). We repeated these experiments under different conditions, namely substituting 37 °C for lower temperature and using a template that contained the natural upstream sequence that enhances transcription (the “UP” element) (18, 19). The experiment involved titrating the concentration of a single NTP, leaving the concentrations of the other 3 NTPs constant and fixed. The ribosomal promoter (rrnB P1) RNA sequence has an A at position +1 and a C at position +2 (Fig. 1, top). The transcripts obtained in the experiments, where +1 ATP or +2 CTP were titrated up to 200 μM, are shown in the middle panel of Fig. 1. The data were normalized to the amount of transcription at 200 μM CTP value. Transcription continues to rise at higher concentrations of ATP and CTP.

Because the +2 effect had not been seen previously, we repeated the type of experiment that previously supported the role of the +1 nucleotide (4). The +2 nucleotide was changed from C to G, and the experiment was repeated. The RNA sequence then had a +2 G, and the +1 A and all other nucleotides were left unchanged. The data (Fig. 2) show that the mutant promoter has a high concentration requirement for ATP and GTP, corresponding to its +1 and +2 positions. CTP, which behaved in this manner on the wild type promoter with a +2 C, no longer did so when +2 was changed to G. The magnitude of the effect was slightly reduced in the mutant, but the switch in specificity was clear. We concluded that both the +1 and +2 nucleotides have a higher concentration requirement at the rrnB P1 promoter.

The +1 or +2 NTP Can Activate Heparin-resistant Complexes—Previous experiments under lower ionic strength conditions have shown that, in the absence of initiating nucleotides, RNA polymerase binds the rrnB P1 promoter in a stable closed complex (20, 21). Under similar conditions, preincubation with the +1 (but not the +2) nucleotide induce more complexes to survive dissociation in a heparin challenge protocol (4). In view of the additional effect of +2 CTP in the above experiments, we reassessed the role of the +1 and +2 NTP under these experimental conditions.

Fig. 3A shows the results of an experiment evaluating the effects of the +1 or +2 NTP in forming heparin-resistant complexes. In these experiments, each NTP was preincubated with the transcription complex, and then heparin was added for 3 min. At that point, transcription was initiated by adding the remaining NTPs. The data show that preincubation with either +1 ATP (Fig. 3A, lane 2 versus lane 1) or +2 CTP (lane 3 versus lane 1) increased transcription but that +4 GTP had no effect (lane 4 versus lane 1). This is consistent with the specific need for high concentrations of the +1 ATP and +2 CTP in the titration experiments (Fig. 1). The effect of +1 (reported previously in Ref. 4) is greater than that of +2, which is clear but smaller.

Other experiments indicated that preincubation is not required to achieve the stimulatory effect of the nucleotide. Fig. 3B shows that, if either the +1 or +2 NTP is added after heparin, the results are unchanged from those described above. That is, there was still a specific stimulation (Fig. 3B, panel B...
is similar to panel A). In control experiments, the heparin was found to inactivate ∼85% of the complexes if added directly to the polymerase prior to DNA (at the lac UV5 promoter, the effectiveness was greater than 95%, Fig. 3C). The results suggest that an initiating NTP can convert a poorly transcribing transcription complex to a better transcribing one, even in the presence of heparin.

It was reported previously that preincubation with the +1 NTP increases transcription by stabilizing an intrinsically unstable transcription complex as assessed in a heparin challenge assay (4). The incomplete effect of heparin on inactivation at high ionic strength made this assay somewhat problematic. The prior heparin challenge assay used to determine complex lifetime was done using a lower KCl concentration (30 mM instead of 170 mM) (4), and we were able to reproduce the sensitivity to heparin under these low salt conditions (data not shown). We also found that the specificity of initiation was weak under these conditions, consistent with prior reports on the same promoter DNA (22). To bypass these potentially complicating factors, we developed a modified assay to assess the lifetime of rrnB P1 promoter complexes at higher ionic strength, where NTP effects on transcription have been demonstrated (see above and Ref. 4).

High Concentrations of the +1 and +2 NTPs Do Not Significantly Stabilize Polymerase at the rrnB P1 Promoter—To measure the lifetime of RNA polymerase bound to the rrnB P1 promoter, we established a system wherein RNA polymerase does not efficiently reinitiate transcription from the template to which it is initially bound. In this system, RNA polymerase is prebound to the usual supercoiled template. A large quantity of competitor DNA was used, which was of the same template, except that it was cleaved prior to the usual transcription termination site so that its transcripts would be shorter and more distinguishable. Titration determined the amount of competitor that bound nearly all of the polymerase released after transcribing the original template. The small amount of reinitiated transcripts constituted a small background, which was corrected in the analysis. Under these conditions, polymerase initiated from the original template but could not rebind because of the large quantity of nearly identical promoter DNA, which acts as the competitor.

The response to the nucleotide was not changed under these conditions (data not shown). The effect of the nucleotide on inhibition by ppGpp was also tested using this competitor (Fig. 3D). ppGpp inhibited transcription 3-fold in the absence of nucleotide and only slightly less in the presence of the +1 or +2 NTP. The use of a DNA competitor did not appear to alter the regulatory properties of the promoter.

To measure the half-life of polymerase bound to the rrnB P1 promoter, complexes were assembled in the absence of NTPs, and competitor DNA was then added. At various subsequent times, aliquots were removed and NTPs were added to allow those complexes that had not dissociated to initiate transcripts. The amount of RNA at each time, corresponding to the number of functional complexes, was measured. The dissociation curve, which is plotted as the appropriate semilog plot, is shown in Fig. 4A. The half-life of the complexes under the experimental
conditions used here is 3–4 min, only slightly longer than that seen using the lower KCl conditions and heparin as a competitor (4).

When the experiment was repeated using transcription complexes, which had been preincubated with either the +1 ATP (Fig. 4B) or the +2 CTP (Fig. 4C), no significant change in the lifetime was seen. These lifetimes were much shorter than those obtained after the first bond formation (Ref. 6 and data not shown), ruling out the effects of NTP contamination in these experiments. In all three cases, the lifetime is 3–4 min (see legend to Fig. 4). It is important to note that these results were obtained under the same experimental conditions as described above, where preincubation with these nucleotides stimulated transcription. We infer that the cause of nucleotide-dependent stimulation of transcription is not stabilization of polymerase at the promoter. Instead, the nucleotide must change some other property of the polymerase.

**DISCUSSION**

These experiments have addressed how the *rrnB* P1 ribosomal promoter is stimulated by the nucleotide, an effect thought to be central to regulation during growth phase transitions, where NTP concentrations change (3). The main new result was that this stimulation is associated with both the +1 and +2 initiating nucleotides. The effect was previously thought to be specific for +1 NTP (4). Although the stimulation by +2 was not anticipated, it helps unify our understanding of nucleotide effects; the +2 NTP was previously shown to be an effector of elongation by *E. coli* RNA polymerase (12) and of initiation by T7 RNA polymerase (11). Under “Discussion,” we place these results in context and suggest why ribosomal promoters are uniquely built to require higher concentrations of initiating nucleotides.

**How Do the +1 and +2 NTPs Assist Ribosomal Transcription?**—The data show that *rrnB* P1 transcription is dependent on high concentrations of the +1 and +2 NTPs, using two different protocols. First, low concentrations of an NTP corresponding to either the +1 and +2 RNA position led to low transcription (Fig. 1); low concentration of an NTP incorporated at a downstream position did not lower transcription (Fig. 1). This experiment differed from a prior one, which showed only a +1 dependence (4) using a higher temperature (37 °C) and a promoter that contained the natural upstream sequence containing the UP element described previously under “Results.” Second, when *rrnB* P1-polymerase complexes were preincubated with either the +1 or +2 NTPs, transcription was increased in a heparin challenge protocol. Taken together, the data show that transcription depends on the independent binding of the two nucleotides needed to form the first RNA bond.

The stimulation by the +1 NTP has been suggested to be related to stabilizing the short-lived heparin-resistant complexes that form at ribosomal promoters (4). When we measured stability under conditions used here for transcription, the half-life was indeed short (3–4 min), but neither the +1 nor the +2 NTP lengthened it substantially. It is possible that the short half-lives are a consequence of unusual features of the transcription complex but are not the basis for stimulation by the +1 and +2 NTPs.

Instead, the +1 and +2 nucleotides appeared to increase the ability of bound RNA polymerase to transcribe. Fig. 3B shows that the +1 or +2 NTP can increase transcription even after heparin was added to the transcription complexes under these conditions (4). This indicates that the NTPs can convert inactive complexes to active ones. The conformation of polymerase at the *rrnB* P1 promoter appears to be distorted from that of a normal preinitiation complex, because NTPs have an abnormally high *Km* (Fig. 1 and reported previously for the +1 NTP) (4), indicating that their binding sites are hindered or malformed. Thus, high concentrations of initiating NTPs are needed to fill these sites, but once bound, should induce restoration of the site configuration and activate the polymerase. The specific requirement for the +1 or +2 NTP is likely a consequence of the unique existence of these two sites in the preinitiation complex. *rrnB* P1 transcription needs high concentrations of these NTPs because its complexes, which contain these sites, may be distorted compared with the usual state at nonribosomal promoters.

**A Model for the Unique Role of Nucleotides at Ribosomal Promoters**—Even nonribosomal promoters have a slightly increased *Km* for binding the +1 and +2 NTPs compared with those used in elongation (9). Somehow this effect is exaggerated at ribosomal promoters to the extent that it allows the promoters to be regulated when NTP concentrations drop. Several features of the promoter are known to contribute to this effect (23). One prominent feature is the uniquely short spacer length of ribosomal promoters, which is central to control by both the initiating nucleotide (23) and growth rate regulation (24). The short spacer is interesting in several regards. It should distort the transcription complex, because a suboptimal-length spacer DNA cannot be accommodated without either the DNA changing its twist or the polymerase distorting to match the improperly presented –10 and –35 regions (25). Region 3 of ω senses the spacer length (26) and would likely be distorted at the ribosomal promoters. Two lines of evidence indicate that a region 3.2 distortion could be associated with creating a requirement for high concentrations of initiating NTPs. First, mutations and a truncation that includes region 3.2 lead to a decrease in abortive transcription (27, 28), which can be compensated for by higher concentrations of initiating NTPs. First, mutations and a truncation that includes region 3.2 lead to a decrease in abortive transcription (27, 28), which can be compensated for by higher concentrations of initiating NTPs (28). Second, region 3.2 is close to an NTP binding site, as demonstrated by its ability to cross-link to a bound ATP (29). Thus, the unique short spacer of the ribosomal promoters may cause region 3.2 to occlude the nearby NTP binding sites. This can be corrected by driving the binding with high concentrations of the initiating NTP.

Although the short spacer is a hallmark of ribosomal promoters and contributes to its regulation, another unusual feature is a long stretch of DNA between the downstream promoter element and the start site. The discriminator for ppGpp control is found here, and mutations (23) in this region result in a general loss of regulation. This long distance may increase the distortion of the complex. The manner of accommodation of the unusually large DNA segment could be affected by the sequence elements in the single-stranded DNA. Taken together, these features could be associated with distortions in the +1 and +2 NTP binding sites, which create the need for high concentrations to fill them. Once they are filled, the complex likely becomes undistorted, as a cross-linking study shows that NTP binding can alter the polymerase conformation to induce engagement of the +1 position on the template strand.1

These same features may also contribute to the down-regulation of ribosomal transcription by the nucleotide effector ppGpp. It was recently suggested that ppGpp competes with ATP for its binding site at *rrnB* P1 (5). ppGpp may be a particularly effective competitor at ribosomal promoters because the NTP binding sites are distorted and have a high *Km* for the +1 and +2 NTPs. Under certain conditions of nutritional deprivation, there may be synergistic down-regulation via nucleotides because the low ATP concentration leads to less transcription (3) and also allows more competition by the inhibitor ppGpp (5). Although the loss of NTPs may be accompanied by an increase in nucleoside diphosphate amounts, nucleo-
side diphosphates have a significantly higher $K_m$ (not shown) and would be ineffective stimulators. When these nucleotide effects are coupled with repression caused by the reduction in DNA supercoiling (30), very substantial down-regulation of ribosomal transcription occurs.

REFERENCES
1. Nomura, M., Gourse, R., and Baughman, G. (1984) Annu. Rev. Biochem. 53, 75–117
2. Reiness, G., Yang, H. L., Zabay, G., and Cashel, M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2881–2885
3. Murray, H. D., Schneider, D. A., and Gourse, R. L. (2003) Mol. Cell 12, 125–134
4. Balke, V. L., and Gralla, J. D. (1987) J. Bacteriol. 169, 4499–4506
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