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Visualization and quantitative analysis of complex formation between *E.coli* RNA polymerase and an rRNA promoter *in vitro*

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ABSTRACT

We have established conditions that stabilize the interaction between RNA polymerase and the rrnB P1 promoter in vitro. The requirements for quantitative complex formation are unusual for E. coli promoters: (1) The inclusion of a competitor is required to allow visualization of a specific footprint. (2) Low salt concentrations are necessary since complex formation is salt sensitive. (3) The addition of the initiating nucleotides ATP and CTP, resulting in a low rate of dinucleotide production, is required in order to prevent dissociation of the complexes. The complex has been examined using DNAase I footprinting and filter binding assays. It is characterized by a region protected from DNAase I cleavage that extends slightly upstream of the region protected by RNA polymerase in most E. coli promoters. We find that only one mole of active RNA polymerase is required per mole of promoter DNA in order to detect filter-bound complexes. Under the conditions measured, the rate of association of RNA polymerase with rrnB P1 is as rapid as, or more rapid than, that reported for any other E. coli or bacteriophage promoter.

INTRODUCTION

In E. coli, ribosome synthesis rates are regulated to meet the cell's requirements for protein synthesis. At high growth rates, as much as 50% of the cell's instantaneous RNA synthesis is rRNA (1). To meet this need, rRNA promoters have the capacity to be the strongest known in E. coli, approaching the maximum theoretical rate of transcription initiation (2). At the same time, rRNA synthesis rates are subject to a delicate system of controls (3-7). However, the molecular effectors that interact with rRNA and tRNA promoters, RNA polymerase, or both have not been identified conclusively.

Only sequences between -50 and -4 with respect to the transcription start site are required for growth rate regulation of the rrnB P1 promoter (7) or for stringent control of a tRNA promoter (8,9). However, sequences outside this region are important to the absolute rRNA synthesis rates. The major determinant of the absolute level of rRNA synthesis is a short sequence (the Upstream Activator Sequence, or UAS) between -51 and -88 of the P1 promoter start site, which has a 10-15 fold stimulatory effect in vivo (7). Regions analogous to the rrnB P1 UAS have been identified upstream of the E. coli tyrosine tRNA (tyrT) promoter (10) and the Salmonella histidine tRNA promoter (11). Restriction fragments carrying the UAS region migrate abnormally slowly

on acrylamide gels (7,11). This implies that this region of DNA has an aberrant conformation (a "static bend"; 12,13) which could affect the way RNA polymerase or other factors interact with the promoter.

Previous attempts to understand the mechanisms responsible for activation and regulation have been hampered by the limited amount of information available about the conditions required for optimum RNA polymerase binding and rRNA transcription initiation *in vitro* at rRNA promoters. Therefore, we have determined solution conditions required for complex formation between RNAP and *rrnB* P1, and we have characterized the products of the reaction under these conditions. We have found evidence by DNAase I footprinting (14) of differences in the character and limits of protection by RNAP compared to the situation with most other *E. coli* promoters. Finally, we have quantified the association of RNAP with *rrnB* P1 using filter binding assays (15) to measure the stoichiometry, overall kinetics, and unique nucleotide requirements of the binding reaction.

MATERIALS AND METHODS

DNA fragments

The DNA fragments used for footprinting and filter binding assays were isolated from a series of plasmids described earlier (7) derived from Bal 31 treatment of longer fragments containing the *rrnB* P1 promoter. Specifically, fragments were used with upstream endpoints at -152 or -115, and downstream endpoints at +28 or +50. DNA containing the *lac*UV5 promoter (-140 to +63) was generously provided by G. Bellomy. The wild type *lac* promoter fragment (-82 to +59) was from pUC19 (17). After digestion with restriction endonucleases, separation on acrylamide gels, and electroelution into dialysis membranes, fragments were concentrated and purified by BND cellulose (Boehringer-Mannheim) chromatography and ethanol precipitation. Fragments were dephosphorylated with calf intestinal alkaline phosphatase and radioactively labeled with polynucleotide kinase (Boehringer-Mannheim) and γ^{32}P ATP (New England Nuclear). The DNA concentrations of *rrnB* P1 fragments used in filter binding and footprinting experiments were measured spectrophotometrically.

RNA Polymerase

RNA polymerase was a generous gift from D. Hager, S. Leirmo, and R.R. Burgess. The activity of the enzyme was determined by S. Leirmo using lambda P_R templates for filter binding and run-off transcription assays, as described previously (15). The preparations used here were 5.9 x 10^3nM in protein concentration and 40% active ±10%. When necessary, the enzyme was diluted on ice and used within a few minutes. As judged from the formation of filter bound complexes, the RNAP was active in promoter binding at the lowest concentrations tested (0.05nM).

Filter binding

Filter binding assays used BA85 nitrocellulose filters (Schleicher and Schull) in manifolds purchased from Hoefer. The standard conditions for the filter binding and footprinting reactions (50 μl) were 40mM Tris-Acetate (pH

7.9), 30mM KCl, 10mM $MgCl_2$, 100 μg/ml BSA, 1mM DTT, 0.01-1.0nM DNA, and, when indicated, 0.05-50nM RNAP, 500μM ATP, 50μM CTP, 50μM UTP. All reactions were performed at 37°C. For experiments done at maximum RNAP-promoter occupancy (i.e. after the binding reaction had gone to completion), heparin (Sigma) was added to a final concentration of 10μg/ml and vortexed gently. The sample was filtered under vacuum, and the filter was washed with 1 ml 10mM Tris-Acetate pH8, 30mM KCl, 0.1mM EDTA. For measuring kinetics, 50 μl aliquots were removed from larger reaction volumes at the indicated times to tubes containing 2 μl 250 μg/ml heparin, vortexed gently, and filtered as above. Nucleotides were purchased from Pharmacia (FPLC Ultrapure), and dinucleotides were purchased from Sigma. A linear least squares analysis was performed in order to determine slopes for TAU plots of the filter binding data, and computer software from Jandel Scientific (SigmaPlot) was used for graphical representations.

DNAase I Footprinting

Footprinting experiments (14) were performed under the conditions described above after RNAP binding had reached a maximum level at 37°C as determined from the filter binding kinetics. Heparin was added to 10μg/ml for 10 seconds, followed by DNAase I (Worthington) digestion at 4μg/ml for 15 seconds, and phenol extraction. NaCl was added to 0.1M, and tRNA (10-20μg) was added as carrier for ethanol precipitation. The samples were dried, resuspended in 7M urea, 0.05M NaOH, bromphenol blue, and xylene cyanol, boiled for 30 seconds, and electrophoresed on 40cm long, 0.4mm thick 10% acrylamide gels (30:1 acrylamide:bisacrylamide), containing 0.5X TBE (18) and 7M urea. Gels were dried and autoradiographed with Kodak XAR-5 film, sometimes using Dupont Cronex Lightning Plus intensifying screens at -70°C. Sequence markers were produced with the chemical method (19).

Analysis of nucleotide substrates and products

30nmoles each of ATP, CTP, UTP, ApC, and CpA were analyzed for purity by ascending thin layer chromatography on PEI cellulose in 1.0 M LiCl (20) and by paper chromatography on Whatman 3MM in water: saturated ammonium sulfate: 2-propanol (18:80:2; ref. 21). No contaminants were detected. 10ul binding reactions contained rrnB P1 DNA fragments (-152, +28) at a concentration of 5-10nM, 25-50nM RNAP (active concentration), 500 μM ATP, and 0.3 μM α^{32}P CTP (3000 Ci/mmol; NEN), with the same temperature, buffer, and competitor conditions as described above. The reactions were electrophoresed on 25% acrylamide-0.8% bisacrylamide-7M urea gels (22). After autoradiography, the bands were excised, soaked in water, and the eluates were concentrated and chromatographed on paper in the system described above. Reactions were also spotted directly onto chromatograms. Where indicated, they were first treated with 1 unit of calf intestinal alkaline phosphatase (Boehringer-Mannheim) for 5 minutes at 37°C. or filtered onto nitrocellulose and washed as above. In the latter case, the radioactivity was eluted from the filter in 0.5 ml of water for 1 hour at room temperature, then concentrated and spotted on Whatman 3MM for chromatography. After autoradiography, the spots were quantitated by scintillation counting.

RESULTS

Requirements for complex formation

In order to optimize reaction conditions to achieve a specific, quantitative interaction of RNA polymerase (RNAP) with the rrnB P1 promoter, we systematically examined salt, competitor, and nucleotide requirements. Since previous experiments had shown that sequences just upstream of the normal RNA polymerase binding site (the UAS) are required for maximal rRNA synthesis in vivo (7), fragments used for footprinting and filter binding studies retained this region as well as the normal RNA polymerase binding site identified in other E. coli promoters. The promoter regions contained in these fragments are sufficient to give maximal rRNA synthesis rates in vivo (7).

Preliminary experiments, in which salt concentration (KCl or KGlutamate), RNA polymerase concentration, RNAP:DNA ratio, or DNAase I concentration or incubation time were varied in the presence or absence of a competitor (the polyanion heparin), failed to yield specific footprints. Specific protection could be visualized only when the nucleotides ATP and CTP (the initiating nucleotides for rrnB P1) were added. In Figures 1-3, the salt, competitor, and nucleotide requirements are examined using the footprinting assay. In each case, a comparison is made with a lacUV5 promoter fragment, showing that the unusual requirements for binding to the rrnB P1 promoter fragment are a property of the promoter itself and do not result from some other variable that we have unknowingly introduced into the experiment.

Figure 1, lanes a-d, illustrates the requirement for a competitor in the binding reaction. In the absence of competitor (in this case heparin), there is protection from DNAase digestion that extends well upstream of the normal RNAP binding site, virtually to the end of the fragment. This protection pattern in the absence of heparin (lane b) is complicated most likely by end-binding (23) so that it is difficult to distinguish where end-binding stops and heparin-sensitive protection begins. The heparin-sensitive protection occurs in the presence (not shown) or absence of nucleotides, and is especially obvious with higher RNAP concentrations (e.g. 10-100nM), preventing identification and visualization of specific interactions at the promoter. With the lacUV5 promoter fragment, a specific footprint is visible in the presence or absence of heparin (lanes e-h). Protection outside the normal RNA polymerase binding site is not seen. While the nature of RNAP binding to rrnB P1 in the absence of competitors deserves further study, for the purposes of investigating interactions at the normal RNAP binding site, we limit our analysis in this paper to those complexes stable to a short heparin challenge.

Figure 2 illustrates the salt dependence of the binding of RNAP to the rrnB P1 promoter fragment in the presence of the initiating nucleotides. As the KCl concentration increases, the extent of protection decreases (lanes b, d, f, and h). (The cleavage by DNAse I becomes slightly less efficient at higher salt concentrations, but the reduction in binding can be observed when compared to the appropriate control digest in lanes a, c, e, and g). The inclusion of K-glutamate instead of K-chloride allows protection to be seen at slightly higher salt concentrations, but the optimum footprint is still seen

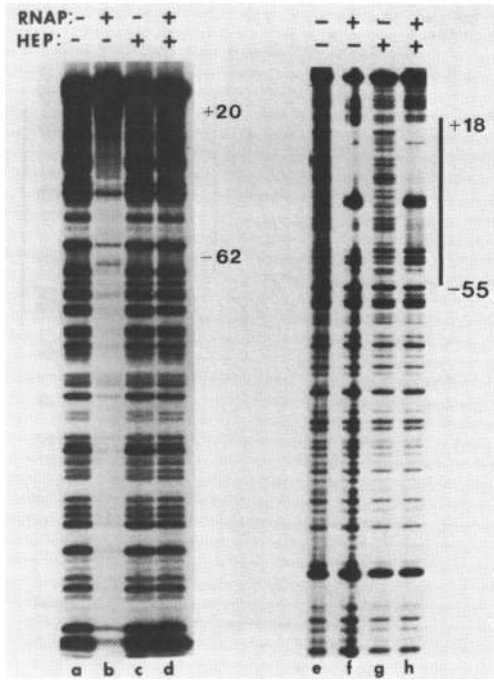

Figure 1. Competitor requirement for defined rRNA promoter - RNA polymerase complexes. Footprints were performed as described in Materials and Methods. The RNAP:DNA ratio was 5:1, the RNAP concentration was 10nM, and the heparin (HEP) concentration was 10μg/ml. Lanes a-d: rrnB P1 promoter fragment (-152 to +50, labeled at the upstream site on the top strand). Lanes e-h: lacUV5 promoter fragment (-140 to +63, labeled at the upstream site on the top strand). Numbers refer to positions relative to the transcription start site and are shown only to orient the reader. No nucleotides were present in the reactions, so no specific footprint is seen in lane d. The line adjacent to lane h shows the approximate position of the footprints in lanes f and h.

at 30mM (data not shown). No requirement for low salt concentrations is seen in the lacUV5 footprint with KCl (lanes i-m) or with KGlutamate (data not shown).

Transcription experiments from several laboratories (24-27) have shown that RNAP does in fact recognize rRNA promoters in vitro. Therefore, we reasoned that the inclusion of the initiating nucleotides might stabilize the initiating complex making it possible to visualize a footprint on the rrnB P1 promoter. Since the rrnB P1 transcript starts with pppApCpUpG..., various combinations of nucleotides (either ATP alone, ATP and CTP, or ATP, CTP, and UTP) were added to the binding reaction (Figure 3). At 30mM KCl with a brief heparin challenge, addition of no NTPs (lane b), ATP alone (lane d), or CTP alone (not shown) is not sufficient to allow visualization of a footprint. Including ATP and CTP (lane f) or ATP, CTP, and UTP (lane h) results in good

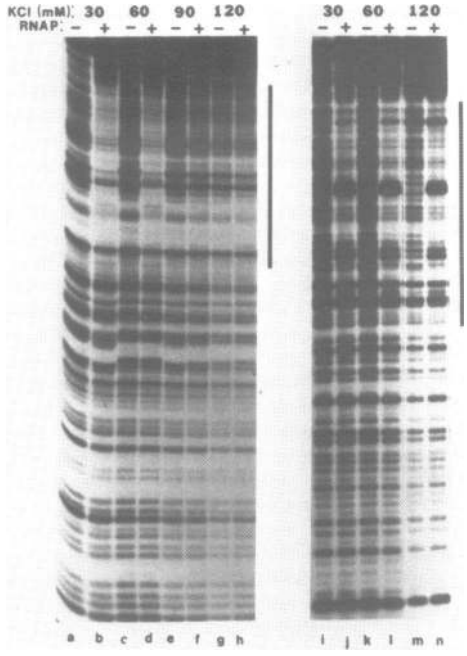

Figure 2. Salt sensitivity of rrnB P1-RNAP complex formation. Footprints were performed as described in Materials and Methods except the KCl concentration was varied from 30-120mM as indicated. The RNAP concentration was 10nM, and ATP and CTP were included in the reaction. DNA fragments were the same used in Figure 1. Lanes a-h: rrnB P1. Lanes i-n: lacUV5.

protection. With the lacUV5 promoter (lanes i-k), which begins with pppApApU..., the inclusion of ATP is not required for footprints to be seen, and has only a very slight effect on the region protected (28). The addition of the starting nucleotides is not sufficient to stabilize the interaction of RNAP with the wild type lac promoter (lanes l-n) in the absence of the accessory protein, cAMP-CAP, known to be required for activation of this promoter. While this result does not exclude the possibility that an accessory protein is required in rrnB P1, it does show that inclusion of initiating nucleotides cannot always drive promoter complexes into a stable conformation.

Nucleotide requirement

The requirement for the initiating nucleotides has also been examined by the filter binding assay as adapted by Record and collaborators (15). It was found that the conditions required for quantitative complex retention on filters were the same as those required for specific footprints, i.e. low KCl concentrations, the initiating NTPs, and a brief challenge with heparin (or poly dA-dT).

The nucleotide sequence in the vicinity of the transcription start site

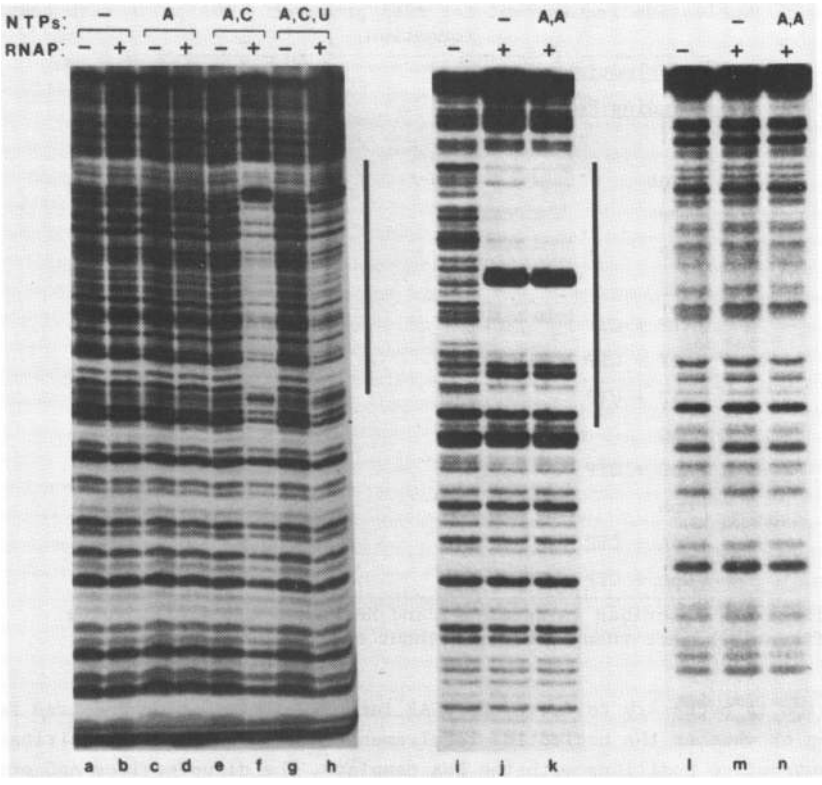

Figure 3. Nucleotide requirement for the rrnB P1 promoter footprint. Samples were prepared as described in Materials and Methods except nucleotides (500μM ATP, 50μM CTP and UTP) were added as indicated. The sequence at the 5' end of rrnB P1 is pppApCpU. That for lac is pppApApU. Lanes a-h: the rrnB P1 promoter fragment (-152 to +50, labeled at the downstream site on the bottom strand). Lanes i-k: the lacUV5 promoter fragment (-140 to +63, labeled at the upstream site on the top strand). Lanes l-n: the wild type lac promoter fragment (-82 to +59, labeled at the downstream site on the bottom strand).

is C (-1), A (+1), C (+2), (U +3), G (+4). Various combinations of nucleotides were added to the binding reaction, and the fraction of rrnB P1 fragments retained was measured at a time sufficient for maximal promoter occupancy (Table 1). As expected from the footprinting experiments, without the addition of nucleotides, there was only minimal filter retention. Increasing the RNAP concentration to 50nM and the RNAP:DNA ratio to 100:1 did not significantly increase formation of stable complexes (unpublished results). In the presence of ATP alone, CTP alone, or UTP alone, there were still only minimal levels of binding, while the addition of ATP and CTP or ATP, CTP, and UTP gave almost quantitative retention. In an attempt to determine if phosphodiester bond

Table 1. Nucleotide requirement for rRNA promoter - RNA polymerase complex formation.

Nucleotides in Binding Reaction	Fraction DNA Bound to Filter
none	0.11
ATP	0.12
CTP	0.12
UTP	0.14
ATP + CTP	0.79 *
ATP + CTP + UTP	0.84 *
ATP + UTP	0.14
ApC	0.14
ApC + UTP	0.40 *
CpA	0.18
CpA + CTP	0.89 *
CpA + CTP + UTP	0.99 *

Conditions are described in Materials and Methods. * indicates value significantly higher than obtained without nucleotide addition.

formation is necessary to drive the RNAP into the conformation required for binding or whether the nucleotide requirement reflects a need for pairing of two consecutive positions with the DNA template, the dinucleotides ApC or CpA were included in the binding reaction. Neither alone was sufficient to give significant levels of binding. If the addition of nucleotides provides energy derived from phosphodiester bond formation to stabilize the complex, then we reasoned that the addition of single nucleotides to the dinucleotides might restore bound complexes. As shown in Table 1, the addition of CTP to the CpA dinucleotide did, in fact, result in filter binding. Likewise, the addition of UTP to the ApC dinucleotide increased binding significantly, but not to the same extent as the (A,C), (A,C,U), and the (CpA + C) combinations.

Products of the binding reaction in the presence of the initiating nucleotides

Several laboratories have attempted to use the abortive initiation assay (2) to measure the kinetics of rRNA transcription initiation in vitro, but have found that the level of abortive product formed was too low to allow accurate measurement of the reaction rate (B. Hoopes and W. McClure, unpublished; S. Leirmo, R.L. Gourse, and M.T. Record, unpublished), although longer abortive products of reactions primed with ApCpU have recently been reported (29). We were able to visualize the products of binding reactions containing only the starting two nucleotides by increasing the DNA and RNAP concentrations and the specific activity of the α^{32}P CTP (Figure 4). In panel A, the products are displayed on a 25% acrylamide denaturing gel. Two major bands are specific to the lane containing RNA polymerase, ATP, and α^{32}P CTP.

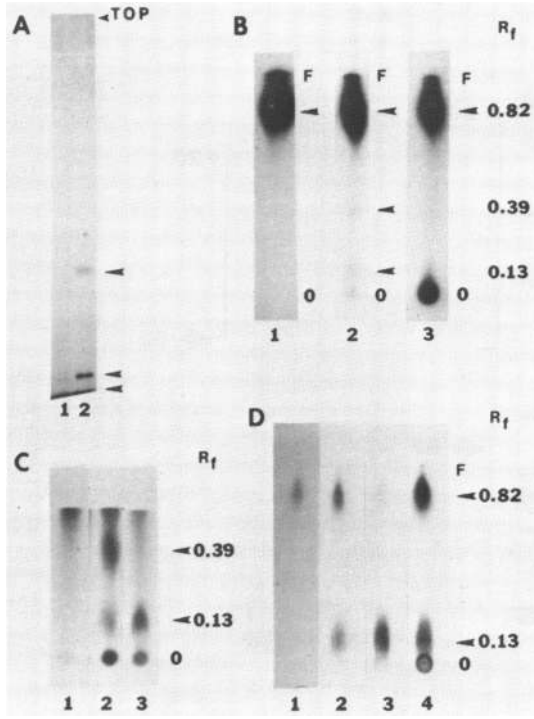

Figure 4. Products of the RNAP-rrnB P1 promoter binding reaction. Panel A: Samples were prepared and electrophoresed on 25% acrylamide gels as described in Materials and Methods. Lane 1 is a 10 min. binding reaction with DNA (-152, +28), ATP, α^{32}P CTP, and buffer components but without RNAP. Lane 2 is the same reaction with RNAP. Panel B: paper chromatogram on which lanes 1 and 2 are the same reactions as in panel A, and lane 3 is a reaction with ATP, α^{32}P CTP, UTP, and GTP. Relative mobilities (R_f) are indicated for the radioactive spots. O = origin; F = solvent front. Panel C, lanes 1 and 2: reactions with ATP and α^{32}P CTP as in lanes 1 and 2 of Panel B. Lane 3: same as lane 2 except the sample was treated with alkaline phosphatase. The top of the chromatogram was cut off before autoradiography to facilitate visualization of the reaction products. Panel D: Samples were incubated for various lengths of time, treated with heparin, filtered onto nitrocellulose, and the eluate was chromatographed. Lane 1: no RNAP, 10 min. incubation. Lane 2: 1 min. incubation with RNAP. Lane 3: 3 min. incubation with RNAP. Lane 4: 10 min. incubation with RNAP.

One band, just above the α^{32}P CTP, is at the approximate position expected for the 2-mer product pppApC. Another product was found in significant amounts, migrating more slowly than the expected 2-mer. Longer products, presumably resulting from trace contamination with other nucleotides, could be seen in 30-fold longer x-ray exposures, but were in very low yield compared to the two seen in Figure 4A.

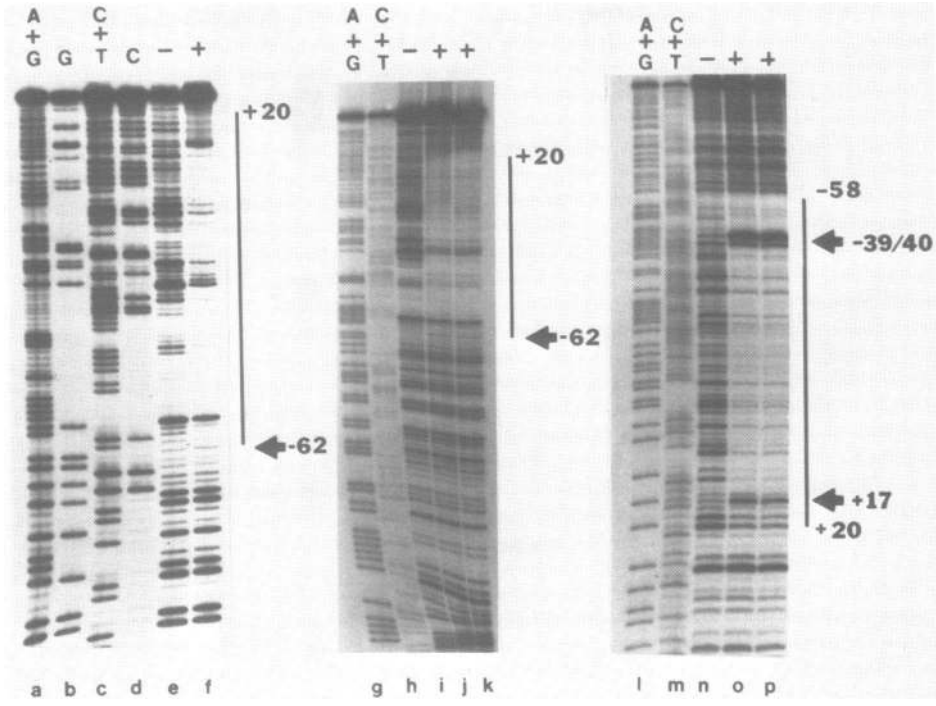

Figure 5. Footprints of rrnB P1 on the top and bottom strands. Lanes a-k: rrnB P1, top strand. Lanes l-p: rrnB P1, bottom strand. The DNA fragment used in lanes a-f extends from -115 to +28 with respect to the transcription start site; that used in lanes g-p extends from -152 to +50. The radioactive label is at the upstream end of the top strand and at the downstream end of the bottom strand. Lanes a-d, g-h, and l-m are Maxam-Gilbert sequence markers as indicated. Vertical lines mark the limits of the protected regions. Solid arrows indicate hypersensitive positions. The DNA concentration used in lane e-f was 0.25 nM, and the RNAP concentration used in lane f was 2.5 nM. In lanes i-k and n-p, the DNA concentration was 0.2 nM. The RNAP concentration in lanes j and o was 1.0 nM and in lanes k and p was 10nM.

The reactions were also examined by paper chromatography. As expected from the gel results and the abortive initiation experiments cited above, the products were in very low yield (on the order of no more than a few moles per mole of template per minute; Figure 4B, lane 2 and S. Leirmo and R. Gourse, unpublished results). On the other hand, if all four nucleotides were included in the reaction, 35-fold more product at the origin could be seen (Figure 4B, lane 3).

The mobilities of the products (0.82, 0.39, 0.13) correlate well with the mobilities reported by McClure et al. (21) for CTP (0.81), pppApC (0.38), and ApU (0.12; ApC was not reported). The identification of the slower migrating spot as the dephosphorylated form, ApC, of the 2-mer, pppApC, is

supported by the experiment illustrated in Figure 4C: treatment of the binding reaction with alkaline phosphatase results in the disappearance of the spot at 0.39 and the appearance of more radioactivity at 0.13. Commercial ApC comigrated with the 0.13 spot (not shown). In order to correlate the gel bands with the chromatogram spots, the gel bands indicated with arrows in Figure 4A were excised, and the radioactivity was eluted and chromatographed (not shown). The fastest migrating gel band corresponded to the spot migrating fastest on the chromatogram (CTP); the middle gel band corresponded to the pppApC spot; and the slowest gel band migrated like ApC. We have not as yet determined the cause of the dephosphorylation of the 2-mer reaction product.

In the 10 minute reactions containing ATP and CTP (Figure 4B and C), a small amount of material remained at the origin, presumably a mixture of longer oligomers deriving from trace amounts of other nucleotides present in the commercial preparations. The potential contribution of these longer products to filter retention was evaluated by filtering reactions done in the presence of non-radioactive DNA, ATP, and α^{32}P CTP at various times after RNAP addition and then examining the retained radioactivity by chromatography. [Control experiments under these same conditions but with radioactive DNA and non-radioactive ATP and CTP (not shown) indicated that the low CTP concentrations used above were sufficient for maximal filter retention of complexes at the earliest time point taken, 30 seconds after addition of RNAP.] Figure 4D shows that the filter bound material derived from a short incubation with RNAP contains almost exclusively 2-mers. We think it unlikely that longer products contribute significantly to filter retention at the 500-fold lower RNAP concentrations used in the kinetic assays (see below). That is, complex formation is virtually instantaneous at the high RNAP concentrations (necessitated by the high DNA concentrations) used in the chromatography reactions, yet the accumulation of significant amounts of the material at the origin (longer oligomers) was seen only in the longer incubations.

Limits of protection from DNAase I

We have examined the footprint of RNAP on each strand of rrnB P1 for both the limits of the DNA protected from DNAase I and the pattern of hypersensitive sites within the protected region (Figure 5) and compared the footprint with that of the lacUV5 promoter. The rrnB P1 footprints extend further upstream than in lacUV5, from -61 to +20 on the top strand (left panel) and from about -58 to +20 on the bottom strand (right panel). The middle panel in Figure 5 displays a larger region of the rrnB P1 DNA fragment in order to show that the protection of rrnB P1 does not extend past -61. (The bands at the bottom of this gel cover sequences to -117 with respect to the transcription start site.) On the bottom strand, inefficient DNAase cleavage in the -55 to -60 region prevents definitive identification of the exact protection endpoint. There are strong hypersensitive sites at -40 and -39 on the bottom strand and much weaker ones at -62 (top) and +17 (bottom).

Other laboratories have shown that RNAP protects about 70 bp from DNAase I cleavage in the lacUV5 promoter, from -56 to +15 on the top strand and from

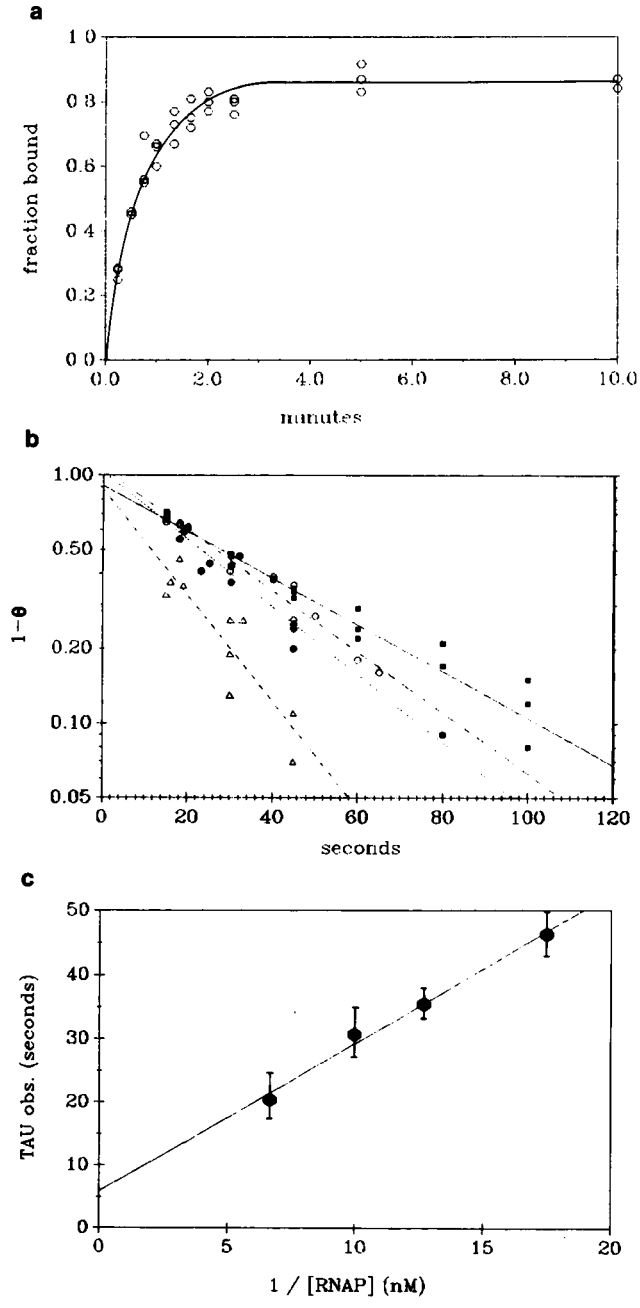

Figure 6. Association kinetics for RNA polymerase with rrnB P1. Kinetics were measured in the presence of ATP and CTP using the filter binding assay as described in Materials and Methods. Points from at least three separate

experiments are plotted together. Panel (a) shows the time dependence of formation of filter-retainable complexes stable to a heparin challenge at one RNA polymerase concentration (0.057nM). The DNA concentration was 0.01nM. Radioactivity retained in the absence of RNAP, usually about 2% of the input, has been subtracted. Panel (b) shows semilog plots of $1-\theta$ versus time for the data of (a) and for three other RNAP concentrations. θ is the ratio of the fraction bound to the plateau value. The slopes are used to derive estimates of the pseudo first-order rate constant, k_{obs}, for each RNAP concentration (2,15). Filled squares = 0.057nM, $k_{obs} = 2.2 \times 10^{-2}$; open circles = 0.079nM, $k_{obs} = 2.8 \times 10^{-2}$; filled circles = 0.1nM, $k_{obs} = 3.3 \times 10^{-2}$; open triangles = 0.15nM, $k_{obs} = 4.9 \times 10^{-2}$. The quantity "TAU observed" is the reciprocal of k_{obs}. The kinetic data obtained for different RNAP concentrations are displayed as a TAU plot in panel (c). The second-order association constant ($k_a = K_B k_f$) is the reciprocal of the slope, $4.3 \pm 0.35 \times 10^8$ $M^{-1}s^{-1}$, and the Y-intercept gives a value for $1/k_f$ of 6 ± 2 sec. (which is not significantly different from 0 by the Student's T test).

about -46 to +22 on the bottom strand (30) or -51 to +20 and -52 to +17, top and bottom respectively (31) and that there are up to 13 sites that are hypersensitive to DNAase cleavage in the presence of RNAP. Spassky (28) has shown that the addition of the initiating nucleotides to footprinting reactions of the lacUV5 promoter has no effect on the positions of the DNAase-hypersensitive sites or the upstream limit but extends the downstream protection by two bp on the top strand. Our lacUV5 footprints are in good agreement with those reported by previous investigators, i.e. -54 to +18 on the top strand, -48 to +16 (bottom strand) with very hypersensitive sites at -46, -24, and -23 (top strand) and -49, -39, -26 (bottom strand) and somewhat enhanced sites at other positions. Two additional positions on the top strand (-60, -61) showed minor protection from DNAase cleavage in some experiments, but never as much as that observed in the -58 through -61 region of rrnB P1. The protection in rrnB P1 immediately upstream of the region protected in lacUV5, and the absence of almost all hypersensitive positions within the protected region represent differences between most other E. coli promoter footprints and that on rrnB P1 (See Discussion).

Kinetics of formation of complexes

The kinetics of formation of initiating complexes prior to the catalytic steps of RNA chain elongation play the major role in controlling the rate of rRNA synthesis in vivo, since DNA sequences downstream of the promoter region are not required for activation, growth rate regulation, or stringent control (7,32). Having obtained the above information about the requirements for filter retention of rrnB P1-RNAP complexes, we quantitated the kinetics of the reaction in vitro. Because the reaction between RNAP and rrnB P1 was so rapid, it was necessary to use low RNAP concentrations [0.05-0.2 nM active concentration of RNAP (as determined by filter binding and transcription assays on the lambda P_R promoter with the same RNAP preparation; ref. 15; S. Leirmo and M.T. Record, unpublished)] and low DNA concentrations (0.01 - 0.02 nM DNA fragment) in order to keep the RNAP:DNA ratio at least 5:1. At low salt and RNAP concentrations, with initiating nucleotides, and under pseudo first-order conditions of excess RNAP, the reaction is essentially

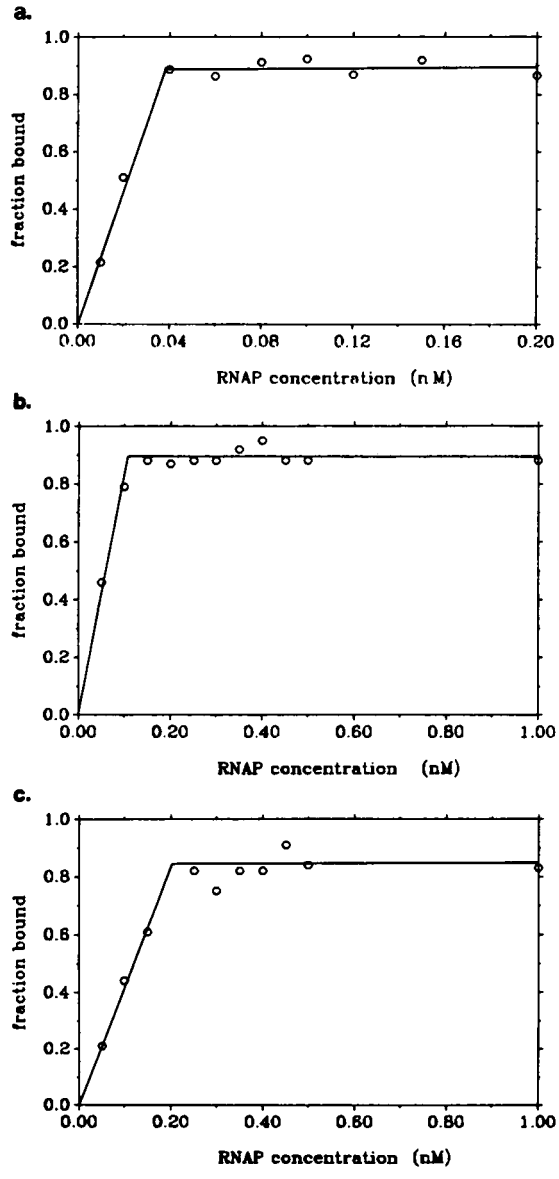

Figure 7. Stoichiometry of the binding reaction between RNAP and rrnB P1. The fraction of radioactive DNA retained in the filter binding assay is plotted versus the active RNAP concentration. The RNAP concentration was determined as described in Materials and Methods, and the DNA concentration was determined spectrophotometrically. The three panels are experiments using different DNA concentrations: (a) 0.04nM, (b) 0.1nM, (c) 0.2nM. In all three cases, the saturating level of RNAP occurs at 1 mole RNAP per mole DNA.

irreversible during the time course of the assay (data not shown). As expected from the rate of rRNA synthesis *in vivo*, the rate of complex formation was found to be extremely rapid, with an overall second-order association constant ($k_a = K_B k_f$) of 4.3 ± 0.35 x 10^8 $M^{-1}s^{-1}$ at 37°C. (Figure 6a-c). Variation of the RNAP concentration allows dissection of the association reaction into two kinetic steps (2). K_B defines the formation of the closed complex, while k_f defines the isomerization steps leading to the complex capable of initiating transcription. The results are illustrated as a TAU plot (2) in Figure 6c. The y-intercept gives a k_f of approximately 1.7 x 10^{-1} sec^{-1}, and the binding constant ($K_B = k_a/k_f$) therefore is estimated at about 2.5 x 10^9 M^{-1}.

Stoichiometry

The stoichiometry of the binding reaction is of interest, because it has been proposed (33,34) that some of the regulatory properties specific to stable RNA promoters might be explained if RNAP binds to stable RNA promoters as a dimer rather than as a monomer. We have measured directly the RNAP:DNA ratio required for maximum retention of *rrnB* P1 fragments on filters (Figure 7). The *rrnB* P1 fragment concentration was measured spectrophotometrically, and increasing amounts of RNAP were allowed to bind. For the RNAP-promoter binding reaction to go to completion (i.e. to form a complex), only 1 active RNAP molecule is required per molecule of DNA.

DISCUSSION

Requirements for complex formation.

Initiating nucleotides. The pathway leading to prokaryotic transcription initiation can be thought of in terms of the kinetic steps indicated below, where R = RNA polymerase, P = promoter, RP_c = the "closed" complex, RP_o = the "open" complex, and RP_{init} = the complex formed after addition of the first few nucleotides (reviewed in ref. 35; intermediate steps in the formation of the closed and open complexes have been omitted for simplicity):

$$R + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o \xrightarrow{k_3} RP_{init} \xrightarrow{k_4} RNA$$

We have manipulated solution conditions in order to stabilize an RNAP - rRNA promoter interaction. At the same time, the complex formed must be specific enough so that surrounding DNA sequences are not protected from the cleaving agent. Our footprinting and filter binding experiments indicate that the *rrnB* P1 promoter is not populated by RNAP to any great extent unless low salt concentrations and the initiating nucleotides are used to drive the reaction into a complex which is essentially irreversible. The relative occupancy of RP_c and RP_o in the absence of the starting NTPs is not known. However, increasing the RNAP concentration was not sufficient to eliminate the NTP requirement, even at low salt concentrations, implying that the rate k_{-2} is substantial *in vitro*. For most promoters, k_{-2} is apparently slow enough that it does not contribute to the overall rate *in vitro*. Although the contribution of k_{-2} to instability of the *rrnB* P1 open complex is unlikely to

be significant under normal *in vivo* conditions where NTPs are likely to be present, a substantial k_{-2} may account for some of the unusual characteristics of rRNA promoter behavior *in vitro*. It is also possible, of course, that nucleotide availability in some physiological circumstances serves to regulate rRNA transcription initiation.

Recently, it was reported that footprints were achieved using a phage T7 promoter and T7 RNA polymerase only after addition of the initiating nucleotide GTP (36, 37). In this case, the transcript begins with pppGpGpGpG..., so it was not as convenient to investigate the nature of the nucleotide requirement as it is in the *rrnB* P1 case. Furthermore, it has been found that the nucleotide requirement in the T7 system was eliminated by changing the solution conditions, i.e. by lowering the magnesium and salt concentrations and by omitting tRNA from the binding buffer (38). Lowering the salt and magnesium concentrations and substituting glutamate for chloride as the anion does not stabilize the *rrnB* P1 - promoter complex sufficiently to alleviate the nucleotide requirement, even at high RNAP concentrations (unpublished results).

The results listed in Table 1 suggest that phosphodiester bond formation may be required to stabilize the complex, and Figure 4 shows that a phosphodiester bond is in fact being made under our conditions. A working hypothesis is that the energy derived from bond formation drives a conformational change in the RNAP that allows for altered, more stable DNA contacts. It seems plausible that the stability afforded by nucleotide addition and the low rate of formation of abortive initiation products may be related.

RNAP is thought to undergo major conformational changes in moving from an "open" to "elongating" mode. Recent work suggests that a stable ternary complex is formed only after about 10 bases have been polymerized (29). The complex described here may be an intermediate in that pathway, stable enough to be retained on filters or footprinted, but not a true elongation complex. Evidence that the complex formed in the presence of the initiating nucleotides is not an elongation complex is (a) the DNAase footprints obtained do not extend downstream of those seen in open or initiating complexes at other *E. coli* promoters; (b) the upstream boundary of the footprint implies that the contacts in the -35 region are still present; and (c) the predominant products of the reaction are 2-mers, and not longer products. Although we do not understand fully the chain of events involved in formation of the observed complex, operationally the addition of the initiating nucleotides allows measurements of the rRNA promoter - RNAP binding process that are removed by as few steps as possible from the original interaction. Since kinetic step(s) beyond formation of open complexes are included in this rate measurement, the rates are all the more extraordinary (see below).

Ionic conditions. Salt sensitivity of promoter-RNAP complexes has been noticed previously for stable RNA promoters (rRNA, 26, 39, 40; tyrosine tRNA, 41). The effects of ions on transcription initiation of other promoters have been

studied in detail by Record and collaborators (42-44). One possibility with rrnB P1 is that the low salt concentration reduces the rate of dissociation from the closed complex (i.e. slowing down k_{-1}) so that formation of the complex proceeds by a "sequential" rather than "rapid equilibrium" mechanism. It should be possible to distinguish between different possible kinetic mechanisms based on the effects of temperature and salt on the association and dissociation rates.

Competitor. We have utilized heparin to prevent binding that occurs outside of the normal promoter region in order to facilitate footprint analysis of the complex and to prevent non-specific binding. However, the heparin-sensitive binding component of the rRNA promoter-RNAP interaction might conceivably play a role in the mechanism of the upstream activation phenomenon and in the formation of the closed complex and certainly deserves further investigation. It was reported previously that an rRNA promoter was more sensitive than non-rRNA promoters to the addition of a competitor in an in vitro transcription assay (27).

The requirements for the initiating nucleotides to drive the initiation reaction to the right, for low salt to prevent dissociation to the reactants, and for a competitor to prevent binding outside the normal RNAP binding region explain why we had been unable previously to utilize footprinting to determine the DNA sequences within an rRNA promoter that interact with RNAP. The occupancy time of RNA polymerase in the open complex (the form normally capable of giving footprints for other promoters) must be short compared to the dissociation rate.

The ribosomal RNA promoter has an unusual interaction with RNA polymerase. Upstream limits of protection. The footprints described here define the rrnB P1 promoter region that interacts with RNA polymerase in a complex that presumably is competent for initiation. The limits of protection extend somewhat further upstream than is normally found with other E. coli promoters, e.g. lac (28,30,31,45-47), gal (48), ara (49), bla (50), lambda P_R (51), tetR (52). However, there have been other reports of RNAP ($E\sigma^{70}$) DNAase footprints with protection extending somewhat upstream of the normal promoter region [tyrT (41); pap (53); fd PVIII (54)]. In addition, DNAase footprints of RNAP holoenzyme containing the heat shock sigma factor ($E\sigma^{32}$) on the heat shock promoters dnaK P2, groE, and rpoD P_{hs} have upstream limits at about -62 (D. Cowing and C. Gross, personal communication), and a B. subtilis promoter has been reported to have an extended footprint (55).

The more conservative estimates of protection reported for tyrT (41), from +19 to -59 on the bottom strand and to -63 on the top strand, closely match those found here for rrnB P1, as expected for promoters with the same regulatory properties. However, end binding of RNAP to the tyrT promoter DNA fragments makes it difficult to evaluate protected regions further upstream in those experiments. The heparin-sensitive upstream protection found in rrnB P1 could correlate with the additional upstream protection noted in tyrT. Of course, the tyrT and rrnB P1 promoter interactions with RNAP need not be identical.

The interesting possibility that a second RNA polymerase molecule might interact with the region upstream of the tyrT promoter has been suggested by Travers and collaborators (41). To further assess this possibility for rrnB P1, we have examined the stoichiometry of RNAP - rrnB P1 complex formation using the filter-binding assay. Our experiments indicate that 1 active RNAP per promoter is sufficient to achieve maximal filter binding under conditions where specific footprints are obtained. However, we cannot rule out the possiblity that the actual complex includes a dimer of one active RNAP and one inactive RNAP per rrnB P1 promoter. Finally, our results do not exclude a role for RNAP dimers (perhaps related to the heparin-sensitive upstream binding component) in kinetically facilitating closed or open complex formation.

Effects of conformation. The DNA bend immediately upstream of the rrnB P1 promoter (7) conceivably could contribute to the upstream protection we see (i.e. to -61) by preventing DNAase from interacting with the DNA in that region. For example, if the upstream DNA bends around RNA polymerase, it may not be accessible to cleavage even though no extra contacts to RNAP are made. Alternatively, this upstream protection may derive from actual additional DNA contacts with RNAP upstream of the contacts normally found with other promoters. Footprints with small chemical probes are underway to distinguish between these possibilities.

We see very few hypersensitive sites within the protected region in rrnB P1, even though some positions which are enhanced in other promoter footprints are clearly suitable substrates for DNAase cleavage in the control lacking RNAP. This result also suggests that the interaction of RNAP with the rRNA promoter is in some way different than it is with most other promoters. A difference in the conformation of the complex could result from the DNA sequence within the normal RNAP-binding region itself or from the influence of the upstream region or from a combination of both.

The footprints and binding kinetics reported here were done on linear templates. The association rate constant (k_a) we have measured here of about 4 x 10^8 $M^{-1}s^{-1}$ is approximately equal to that measured for the strongest phage promoters (56) and close to the diffusion limit. It seems unlikely that supercoiled templates would increase the association rate greatly under these solution conditions. Furthermore, supercoiled templates do not alleviate the requirement for the initiating nucleotides (S. Leirmo and R.L. Gourse, unpublished experiments). However, it is conceivable that supercoiled templates might facilitate steps after complex formation or that higher molecular weight templates might increase the association rate by providing interactions at upstream or downstream sites that could be followed by facilitated diffusion to the promoter.

rRNA transcription initiation.

Kinetics. It is tempting to compare the rapid association kinetics described here in vitro with the kinetics described for other promoters and with the approximate rate of rRNA synthesis measured in vivo. The rapid binding kinetics we observe in vitro, i.e. a bimolecular rate constant at or near the diffusion limit, meet the criteria expected for the strongest promoters (2)

and at least are consistent with the idea that the complexes studied here are physiologically significant. There is often a good correlation between *in vitro* and *in vivo* transcription initiation rates (57, 58). However, it must be taken into consideration that the *in vitro* solution conditions used here differ from those used in most studies of other promoters and may be quite different from the cellular milieu. For example, the ionic environment can have a far greater effect than even RNAP concentration on the kinetics of transcription initiation (43,44).

Control. Although guanosine tetraphosphate (ppGpp) has long been implicated in the control of rRNA transcription, its role remains controversial (see refs. 1, 59-61 for discussions). We have not yet undertaken a study of the effects of ppGpp on the steps involved in complex formation between RNAP and *rrnB* P1. It is our hope that identification of a kinetic step affected by ppGpp and quantitation of that effect (if observed) under various solution conditions will aid in the interpretation of the relevance of ppGpp to regulation *in vivo*.

In summary, we have determined assay systems that allow the visualization and quantitation of RNAP binding to rRNA promoters *in vitro*. The requirements for complex formation are clearly unusual, but the observed kinetics suggest they are physiologically significant. The position of the enzyme in footprints and the analyses of the reaction products indicate that RNAP has not moved from the promoter in this complex. The systems reported here should prove useful in the characterization of both wild type and mutant rRNA promoters in order to define specific base sequences and protein contacts that correlate with kinetic steps in rRNA transcription initiation. It is our hope that a better understanding of the RNAP - rRNA promoter interaction will help elucidate the role proposed for ppGpp and facilitate the identification of factors and sequences that specifically influence rRNA transcription initiation and its regulation.

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