JOURNAL OF BACTERIOLOGY, Feb. 1993, p. 661–668
0021-9193/93/030661-08$02.00/0

Vol. 175, No. 3

Transcription of the *Escherichia coli rrnB* P1 Promoter by the Heat Shock RNA Polymerase ($E\sigma^{32}$) In Vitro

JANET T. NEWLANDS,† TAMAS GAAL, JOAN MECSAS, AND RICHARD L. GOURSE*

Department of Bacteriology, University of Wisconsin, 1550 Linden Drive, Madison, Wisconsin 53706

Received 16 September 1992/Accepted 20 November 1992

The P1 promoters of the seven *Escherichia coli* rRNA operons contain recognition sequences for the RNA polymerase (RNAP) holoenzyme containing sigma 70 ($E\sigma^{70}$), which has been shown to interact with and initiate transcription from *rrn* P1 promoters in vivo and in vitro. The *rrn* P1 promoters also contain putative recognition elements for $E\sigma^{32}$, the RNAP holoenzyme responsible for the transcription of heat shock genes. Using in vitro transcription assays with purified RNAP holoenzyme, we show that $E\sigma^{32}$ is able to transcribe from the *rrnB* P1 promoter. Antibodies specific to σ^{70} eliminate transcription of *rrnB* P1 by $E\sigma^{70}$ but have no effect on $E\sigma^{32}$-directed transcription. Physical characterization of the $E\sigma^{32}$-*rrnB* P1 complex shows that there are differences in the interactions made by $E\sigma^{70}$ and $E\sigma^{32}$ with the promoter. $E\sigma^{32}$ responds to both Fis-mediated and factor-independent upstream activation, two systems shown previously to stimulate *rrnB* P1 transcription by $E\sigma^{70}$. We find that $E\sigma^{32}$ is not required for two major control systems known to regulate rRNA transcription initiation at normal temperatures in vivo, stringent control and growth rate-dependent control. On the basis of the well-characterized role of $E\sigma^{32}$ in transcription from heat shock promoters in vivo, we suggest that $E\sigma^{32}$-directed transcription of rRNA promoters might play a role in ribosome synthesis at high temperatures.

In eubacteria, initiation of transcription is mediated by sigma, a subunit of RNA polymerase (RNAP) required to form the RNAP-promoter complex. In *Escherichia coli*, the RNAP holoenzyme containing sigma 70 ($E\sigma^{70}$) is responsible for the initiation of transcription of the majority of genes in the cell under normal growth conditions. Comparison of sequences from 263 *E. coli* promoters known or presumed to be recognized by $E\sigma^{70}$ has revealed a consensus recognition sequence which contains the nucleotide hexamers TTGACA at approximately −35 and TATAAT at approximately −10 relative to the transcriptional start site (18, 19).

Alternative sigma factors were first discovered in *Bacillus subtilis* (29) and later found in *E. coli* and *Streptomyces* spp. (16, 17, 20, 28, 54). These alternative sigma factors bind to core RNAP and reprogram the enzyme to initiate transcription at promoters that have sequences different from those recognized by the primary holoenzyme. The alternative sigma factors direct transcription of subsets of genes that respond to various environmental or nutritional changes, such as heat shock and nitrogen limitation, or they direct cell development (e.g., in *Bacillus* or *Streptomyces* spp.).

The *rrnB* P1 promoter in *E. coli* is similar to the other six *rrn* P1 promoters in that it contains an almost perfect $E\sigma^{70}$ consensus. The interaction of $E\sigma^{70}$ with the *rrnB* P1 promoter has been characterized extensively in vitro (14, 26, 35). *rrnB* P1 also contains sequences that strongly resemble recognition elements for the RNAP holoenzyme containing σ^{32}, $E\sigma^{32}$, which is responsible for the transcription of heat shock genes.

At least 11 promoters that are known to be transcribed by $E\sigma^{32}$ have been found in *E. coli*. The consensus sequence for recognition by $E\sigma^{32}$ slightly resembles the $E\sigma^{70}$ consensus in that there is a conserved TTG motif in the −35 region and an AT-rich element in the −10 region. However, the $E\sigma^{32}$ −10 consensus is characterized by a run of C residues not common to $E\sigma^{70}$ promoters (5, 6).

In this report, we show that $E\sigma^{32}$ recognizes and initiates transcription from *rrnB* P1 in vitro. Antibodies specific to σ^{70} eliminate transcription of *rrnB* P1 by $E\sigma^{70}$ but have no effect on $E\sigma^{32}$-directed transcription. $E\sigma^{70}$ and $E\sigma^{32}$ use the same transcriptional start site. However, footprinting of the $E\sigma^{32}$-*rrnB* P1 complex reveals some differences in the interactions made by $E\sigma^{70}$ and $E\sigma^{32}$ with the promoter. $E\sigma^{32}$ responds to the two modes of upstream activation characterized for $E\sigma^{70}$ at *rrnB* P1: factor-independent activation (26, 38) and Fis-mediated activation (34, 41). These results indicate that upstream activation is not limited to $E\sigma^{70}$, consistent with the idea that activation occurs through another subunit of core RNAP or through a conserved part of sigma.

As a first approach to determining the role of $E\sigma^{32}$ in rRNA transcription in vivo, we tested the effect of deletion of the $E\sigma^{32}$ gene on two major control systems known to regulate rRNA transcription initiation at normal growth temperatures (36). Neither growth rate-dependent regulation nor stringent control requires $E\sigma^{32}$. On the basis of the in vitro transcription of *rrnB* P1 with $E\sigma^{32}$ and the known role of this holoenzyme in transcription from heat shock promoters in vivo, we suggest that $E\sigma^{32}$-directed transcription of rRNA promoters might play a role in ribosome synthesis during conditions of environmental stress, for example at high temperatures.

MATERIALS AND METHODS

Enzymes and proteins. Purified $E\sigma^{70}$ and monoclonal antibody 3D3 (anti-σ^{70}) were provided by Richard Burgess, Dayle Hager, Scott Lesley, and Nancy Thompson (University of Wisconsin). Purified $E\sigma^{32}$ was provided by David Straus and Carol Gross (University of Wisconsin). Restriction enzymes were purchased from New England Biolabs.

* Corresponding author.
† Present address: Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06030.

Moloney murine leukemia virus reverse transcriptase was purchased from Epicentre Technologies.

DNA templates. *rrnB* plasmids used for transcription and footprinting assays contained either *rrnB* P1 or *rrnB* P2 promoter fragments in vector pRLG770 (41). The *rrnB* P1 promoter used contained sequences from −87 to +50 (pRLG1616 [35]). The *rrnB* P2 promoter (about −80 to +250 relative to the P2 start site) was cloned as an *Eco*RI-*Hin*dIII fragment into pRLG770 to make plasmid pRLG965. Plasmid pJET40 was obtained from Jim Erickson and Carol Gross and contains the *dnaK* P1 promoter in a pUC19 derivative (10). The *rrnB* P1 and P2 transcripts terminate at an *rrnB* T1 terminator; the *dnaK* P1 transcript terminates at the *spot42* terminator (23).

In vitro transcription assays. Equimolar amounts of $E\sigma^{32}$ and $E\sigma^{70}$ were used unless otherwise stated. Absolute protein concentrations are reported. The $E\sigma^{70}$ preparation was approximately 40% active in promoter binding (26). While the specific activity of $E\sigma^{32}$ was not determined, and therefore it is not valid to compare transcription by the two preparations quantitatively, the activity of the $E\sigma^{32}$ preparation on the *dnaK* P1 promoter indicates that the $E\sigma^{32}$ preparation's activity is roughly comparable to that of the $E\sigma^{70}$ preparation. Reactions (10 μl) shown in Fig. 2 and 9 were performed at 37°C with DNA concentrations of 0.5 nM and RNAP concentrations of 5.0 or 2.5 nM, in a buffer containing 30 mM KCl, 40 mM Tris-acetate (pH 7.9), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg of bovine serum albumin per ml (KCl buffer). The concentrations of nucleotides were 5 μCi each of [α-^{32}P]UTP and [α-^{32}P]CTP, 500 μM ATP, 50 μM GTP, and 10 μM each CTP and UTP. Transcription was stopped with the addition of 5 μl of 3× transcription stop buffer (30% glycerol, 3× Tris-borate-EDTA buffer [37], 3% sodium dodecyl sulfate, 30 mM Na_2EDTA [pH 8.0], 0.05% xylene cyanol FF, 0.025% bromophenol blue, 7 M urea) (34), and the entire sample was electrophoresed on a 6% acrylamide-bisacrylamide (30:1)–7 M urea denaturing gel and dried on a Savant gel dryer.

For in vitro transcription experiments, anti-σ^{70} 3D3 antibody (47) was purified from contaminating RNase by adsorption to protein A-agarose (Repligen). Fifty nanograms of $E\sigma^{70}$ or $E\sigma^{32}$ was preincubated in KCl buffer (see above) on ice for 60 min with 1.3 μg of antibody in phosphate-buffered saline; 70-ng amounts of plasmid DNA and nucleotides (as specified above) were added to initiate reactions. The final concentrations of reactants were 5.0 nM RNAP, 1.0 nM DNA, and 500 nM antibody. Transcription proceeded for 15 min at 37°C in a total volume of 20 μl and was stopped by the addition of 10 μl of 3× transcription stop buffer. Samples were electrophoresed as described above.

In vitro transcription reactions for testing Fis-dependent activation (Fig. 8) were performed as described previously for $E\sigma^{70}$ (41), with the following two modifications: a lower concentration salt buffer (100 mM NaCl) and a higher concentration of RNAP (1.0 nM) were used in the presence of $E\sigma^{32}$. While these changes allow visualization of transcripts in the lanes without Fis, they reduce the apparent effect of Fis somewhat relative to that observed in our previous studies (34, 41). Likewise, the fold activation of $E\sigma^{32}$ by the upstream activation region (UAR) in the absence of Fis (Fig. 9) should not be compared quantitatively with that reported previously for $E\sigma^{70}$ on *rrnB* P1 (34), since the active concentrations of the two holoenzyme preparations were not identical.

Primer extension of in vitro transcription products for determination of start sites. Transcription with nonradioactive nucleotides (500 μM ATP and CTP; 200 μM UTP and GTP) was performed in KCl buffer (see above) for 15 min at 37°C, using 25 nM RNAP and 2 nM plasmid template. The reaction was initiated by addition of RNAP and stopped by the addition of an equal volume of water-saturated phenol-chloroform-isoamyl alcohol (24:24:1; pH 5.0), after which the mixture was subjected to vigorous vortexing. The aqueous layer was added to 2 volumes of ice-cold 100% ethanol and was precipitated in the presence of 25 μg of glycogen (Boehringer Mannheim). The precipitate was washed with 80% ethanol, dried, and resuspended in 10 μl of distilled water. Two microliters of the sample was combined with 2 μl (2 to 3 pmol) of a γ-^{32}P-labeled sequencing primer (5′-GCTACGGCGTTTCACTTC-3′) which anneals to a region upstream of the *rrnB* T1T2 region of plasmid pRLG770 (41). Annealing was performed in a buffer containing 40 mM Tris-Cl (pH 7.5), 50 mM NaCl, and 14 mM $MgCl_2$ in a final volume of 14 μl by heating to 85°C for 2 min followed by slow cooling to 37°C for 1 h. The mixture was ethanol precipitated at −20°C overnight. The entire sample was used for primer extension in a final volume of 20 μl with Moloney murine leukemia virus reverse transcriptase (Epicentre Technologies) in the buffer supplied by the manufacturer. After 30 to 60 min at 48°C, reactions were stopped by the addition of 15 μl of formamide loading solution (Pharmacia), and products were loaded on a 12% acrylamide–8 M urea sequencing gel. Sequence markers were prepared with the same primer and plasmid used in transcription, as described previously (34).

Footprinting. Reaction mixtures contained 25 nM $E\sigma^{32}$ or $E\sigma^{70}$, 500 μM ATP, and 50 μm CTP (high-pressure liquid chromatography purified; Pharmacia). Heparin (10 μg/ml, final concentration) was added before cleavage or modification except where noted.

(i) DMS footprinting. Complexes containing RNA polymerase and *rrnB* P1 promoter fragments were formed in 30 mM potassium glutamate–40 mM Tris-acetate (pH 7.9)–10 mM $MgCl_2$–0.5 mM dithiothreitol–100 μg of bovine serum albumin per ml (KGlu buffer [27]) and modified with dimethyl sulfate (DMS; 0.4 μl); 9 μl of 5× DMS gel loading buffer (50% glycerol, 2.5× Tris-borate-EDTA buffer, 0.3 mg of yeast RNA per ml, 1.6 M β-mercaptoethanol, 0.05% xylene cyanol FF, 0.025% bromophenol blue) was added, and samples (20 μl) were electrophoresed on a 4% acrylamide gel in 0.5× TBE buffer for 50 min at 200 V to separate the RNAP-DNA complexes from free DNA. After autoradiography, the complexes were excised from the gel, electroeluted, and ethanol precipitated. Samples were resuspended in 100 μl of 1 M piperidine, heated at 90°C for 30 min, dried under vacuum, and resuspended in distilled water, and equivalent amounts of radioactivity were mixed with 5 μl of formamide gel loading solution, heated briefly at 90°C, and electrophoresed on an 9.6% acrylamide–8 M urea–0.5× TBE gel at 2,000 to 2,500 V. Gels were dried and autoradiographed with intensifying screens.

(ii) Hydroxyl radical footprinting. Complexes were formed in KGlu buffer (see above). Hydroxyl radical cleavage was then performed in a reaction volume of 20 μl (35). Reactions were stopped by the addition of 6 μl of 5× OH gel loading buffer (50% glycerol, 2.5× Tris-borate-EDTA buffer, 0.05% xylene cyanol FF, 0.025% bromophenol blue) and immediately electrophoresed as described above. The RNAP-promoter complexes were excised and eluted from the gel by diffusion, phenol extracted, and ethanol precipitated. Samples were resuspended in distilled water, and equivalent amounts of radioactivity were mixed with 3.5 μl of forma-

mide gel loading solution and electrophoresed on denaturing gels as described above.

(iii) $KMnO_4$ footprinting. Potassium permanganate ($KMnO_4$) footprinting was performed in KCl buffer (see above) essentially as described previously (35). In the experiment shown (Fig. 6), heparin was not added to $E\sigma^{32}$ complexes. It was not necessary to separate bound from free DNA to visualize the $KMnO_4$ modifications.

Growth rate dependent regulation and stringent control assays. For measurement of growth rate control, cells were grown at 30°C in defined medium described previously, and RNA/protein ratios were determined (12). For measurement of stringent control, cells were grown in morpholinepropanesulfonic acid (MOPS) medium and labeled as described previously (12) except that starvation was induced by addition of valine (100 μg/ml).

Bacterial strains. RLG1980 is MC4100 [*araD139 ΔargF-lacU169 rpsL150 thiA1 relA1 flbB5301 deoC1 ptsF25 rbsR glnF*/Tn*10* (*glnF208*) *rhoD*]. RLG2800 is a *relA*$^+$ derivative of MC4100 constructed by P1 transduction. RLG2802 (*rpoH*::*kan relA*$^+$ *suhX401*) was constructed from KY1603 (MC4100, *rpoH*::*kan relA1 suhX401*) and P1 transduction. Strains lacking $E\sigma^{32}$ fail to grow at temperatures above 20°C (56) unless the product of the *groE* gene is expressed by virtue of an IS*1* element inserted upstream (25). KY1603 contains an IS*1* element upstream of the *groE* gene, allowing growth at moderate temperatures. RLG1973 is an *rpoH*$^+$ derivative of KY1603 constructed by P1 transduction, generously provided by R. Barber and C. Gross.

RESULTS

Initial observation. Figure 1A illustrates the basic organization of the *rrnB* P1 promoter region. Comparison of the DNA sequence of this region with the $E\sigma^{32}$ promoter consensus sequence shows that the *rrnB* P1 promoter has 5 of 8 matches to the $E\sigma^{32}$ consensus in the −10 region and 6 out of 12 matches at −35 (Fig. 1B). In fact, each of the seven *rrn* P1 promoter regions shows homology to the $E\sigma^{32}$ consensus (Fig. 1C). The $E\sigma^{32}$ consensus overlaps the $E\sigma^{70}$ consensus.

$E\sigma^{32}$ initiates transcription from the *rrnB* P1 promoter in vitro. To determine whether the presence of the $E\sigma^{32}$ consensus contained within the *rrnB* P1 promoter has any functional significance, we performed in vitro transcription assays with $E\sigma^{32}$ (Fig. 2). A specific $E\sigma^{32}$ transcript of approximately the same size as that obtained with $E\sigma^{70}$ was observed from the *rrnB* P1 promoter. On the other hand, only $E\sigma^{32}$ and not $E\sigma^{70}$ made a transcript from the *dnaK* P1 promoter, and only $E\sigma^{70}$ and not $E\sigma^{32}$ produced an RNA I transcript from the plasmid origin of replication.

Because of the relatively low level of *rrnB* P1 transcription by $E\sigma^{32}$, we confirmed that the transcription product did not result from contaminating $E\sigma^{70}$ by transcribing *rrnB* P1 with $E\sigma^{32}$ in the presence of antibody to σ^{70}. The anti-σ^{70} monoclonal antibody 3D3 (47) was previously shown to inhibit transcription of the *lac*UV5 promoter in a coupled transcription-translation system without affecting $E\sigma^{32}$ or $E\sigma^{54}$ transcription (22). Addition of the 3D3 antibody inhibited transcription by $E\sigma^{70}$ from the *rrnB* P1 promoter but had no effect on $E\sigma^{32}$ transcription from *rrnB* P1 or *dnaK* P1 (Fig. 3). Control experiments with the $E\sigma^{70}$-dependent *rrnB* P2 and *lac*UV5 promoters, which do not contain sequences resembling the $E\sigma^{32}$ consensus, indicated that anti-σ^{70} inhibited transcription and that neither *rrnB* P2 (Fig. 3) nor *lac*UV5 (data not shown) was transcribed by $E\sigma^{32}$.

$E\sigma^{32}$ and $E\sigma^{70}$ initiate transcription from the same position.

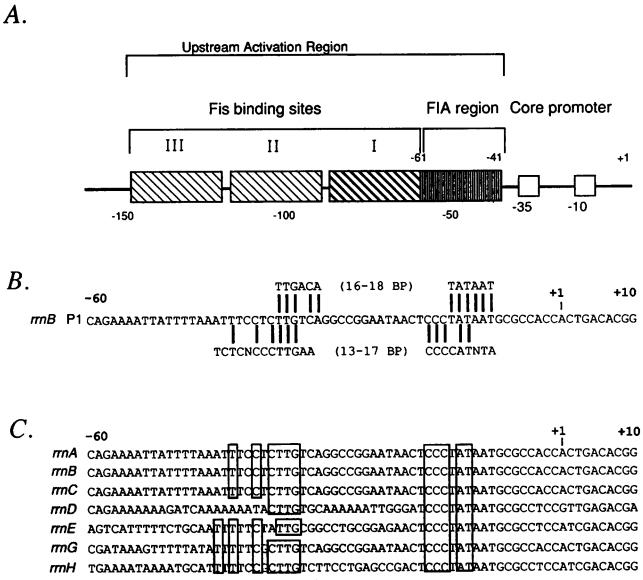

FIG. 1. Structure of the rRNA promoter region. (A) Schematic representation of the *rrnB* P1 promoter region. The region containing the −10 and −35 consensus hexamers recognized by $E\sigma^{70}$ is indicated as the core promoter. The UAR includes Fis sites I, II, and III and the DNA sequences required for factor-independent activation (FIA). (B) Sequence of the *rrnB* P1 promoter from −60 to +10, with consensus sequences for $E\sigma^{70}$ (above *rrnB* P1) and $E\sigma^{32}$ (below *rrnB* P1). Matches to consensus bases are indicated. Consensus sequences are from reference 18 for $E\sigma^{70}$ and from references 5 and 6 for $E\sigma^{32}$. The transcription initiation site (+1) is indicated. (C) Sequences of the seven *E. coli rrn* P1 promoter regions. Matches to $E\sigma^{32}$ consensus bases are boxed.

Although the $E\sigma^{70}$ and $E\sigma^{32}$ products from the in vitro transcription assays appeared to be the same size, slight differences in the start site of transcription would not have been detected. Therefore, we determined the transcript start site by using primer extension from in vitro transcription products generated by $E\sigma^{70}$ and $E\sigma^{32}$. For both holoenzymes, almost all transcription in multiple-round assays starts at the ninth base from the −10 consensus hexamer (Fig. 4), in agreement with the position identified previously in vivo (2, 8), with a minor amount of initiation at the A residue at −3.

$E\sigma^{32}$ and $E\sigma^{70}$ differ in their interactions with *rrnB* P1. We characterized the $E\sigma^{32}$-*rrnB* P1 complex by using base-specific and backbone-specific footprinting reagents. DMS and $KMnO_4$ are footprinting reagents which have been used to characterize protein-DNA complexes in vitro and in vivo (1, 44–46). DMS methylates the N-7 of guanine residues in the major groove and the N-3 of adenines in the minor groove. $KMnO_4$ modifies single-stranded or structurally abnormal pyrimidines. Hydroxyl radicals are used to probe backbone interactions (51).

(i) DMS modification. Positions protected from methylation by $E\sigma^{32}$ overlap but are not identical to the positions protected by $E\sigma^{70}$ (Fig. 5). The guanine at position −5 is very slightly enhanced in the presence of $E\sigma^{32}$ but is strongly enhanced with $E\sigma^{70}$, whereas the G at −7 is methylated more efficiently with $E\sigma^{32}$ than with $E\sigma^{70}$. In addition, the G residues at −15 and −16 are protected in the presence of $E\sigma^{32}$ but not $E\sigma^{70}$, and the G at −32 is protected from methylation by $E\sigma^{70}$ but not by $E\sigma^{32}$. There is a comparable

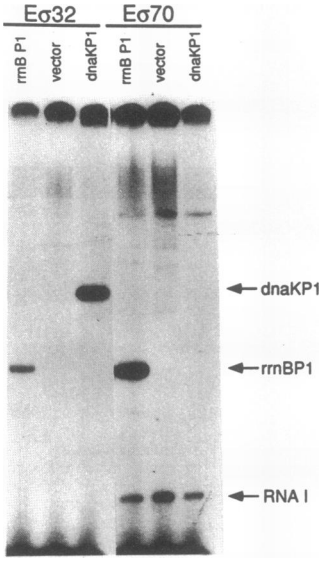

FIG. 2. Multiple-round transcription reactions containing $E\sigma^{32}$ or $E\sigma^{70}$. Reactions were performed in KCl buffer (see Materials and Methods) on supercoiled plasmid DNAs containing the *rrnB* P1 or *dnaK* P1 promoter or lacking test promoters (vector). The approximate transcript sizes are 220 bases for *rrnB* P1 and 350 bases for *dnaK* P1. The holoenzyme used is indicated above each lane. Transcripts are identified with arrows. The transcript labeled RNA I originates from the plasmid vector (33).

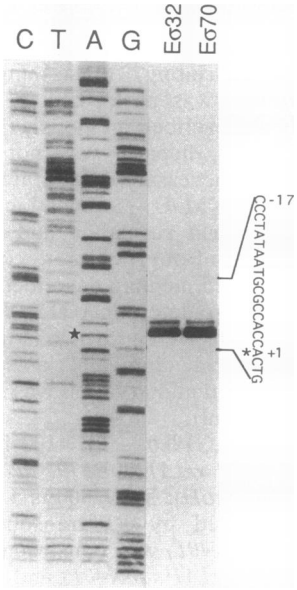

FIG. 4. Determination of transcription start sites. Primer extension was performed on transcription products made from the *rrnB* P1 promoter on supercoiled plasmid pRLG1616 by $E\sigma^{32}$ or $E\sigma^{70}$ (see Materials and Methods). Markers were made by sequencing pRLG 1616 (with T7 DNA polymerase) using the same primer. Volumes of extension products were adjusted to load equal amounts of radioactivity. The holoenzyme used is indicated above the relevant lane.

degree of enhanced methylation in the presence of both holoenzymes at positions +4G and −37G.

(ii) $KMnO_4$ modification. Substantial differences between the two complexes formed with the two holoenzymes are observed on the bottom strand (Fig. 6B). With $E\sigma^{70}$, the T at −3 is very weakly modified relative to the T residues at −10, −11, and −13, while with $E\sigma^{32}$, there is a much greater amount of modification at −3 relative to −10, −11, and −13. In addition, there are minor differences in the modification

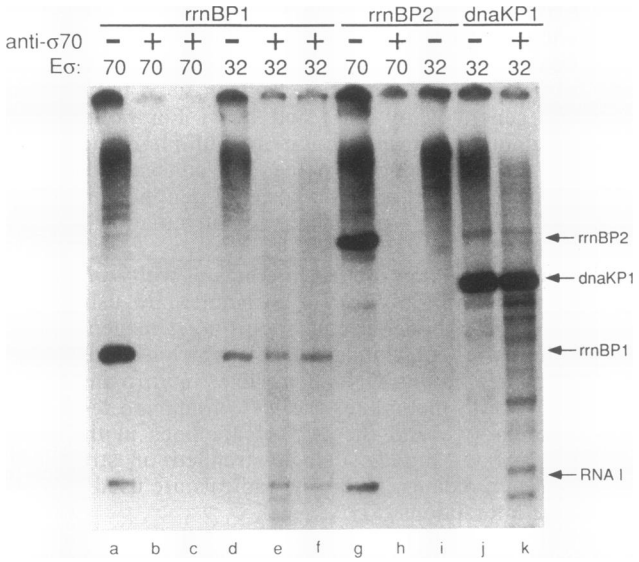

FIG. 3. Transcription reactions containing $E\sigma^{32}$ or $E\sigma^{70}$ performed in the presence of anti-σ^{70} antibody. *rrnB* P1, *rrnB* P2, and *dnaK* P1 promoters were used as templates. The holoenzyme used is indicated above each lane. Transcripts are identified with arrows. Presence (+) or absence (−) of the anti-σ^{70} antibody is indicated. $E\sigma^{32}$ + lanes contain degradation products from minor RNase contamination of the antibody, one of which migrates to a position near the RNA I transcript in the $E\sigma^{70}$ lanes.

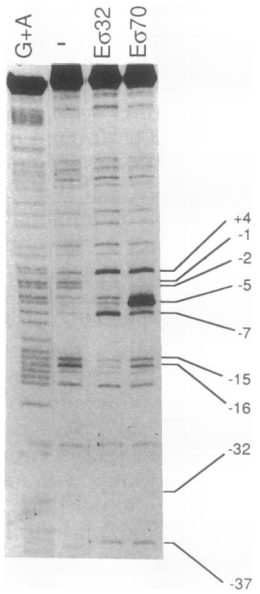

FIG. 5. DMS footprints of $E\sigma^{32}$ or $E\sigma^{70}$ on *rrnB* P1 fragments. The bottom strand (labeled at the *Eco*RI site at −87) is shown. The lines indicate positions discussed in the text. The holoenzyme used is indicated above the relevant lane. Lane G+A, sequencing ladder prepared by the method of Maxam and Gilbert (31); lane −, no RNAP.

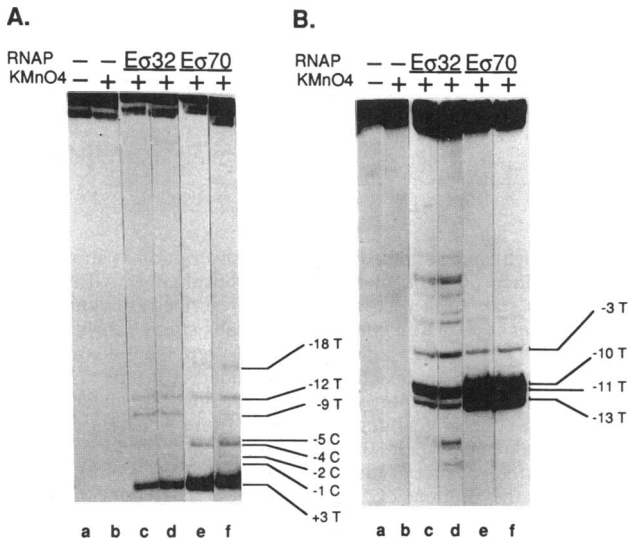

FIG. 6. $KMnO_4$ footprints of Eσ^{32} or Eσ^{70} on *rrnB* P1 fragments. (A) Top strand (labeled at the *Hin*dIII site at +50); (B) bottom strand (labeled at the *Eco*RI site at −87). Lines indicate positions discussed in the text. The holoenzyme used is indicated above the relevant lane. Duplicate samples are shown. Binding and modification for samples in lanes d and f in each panel were performed at 48°C. It is not clear why some C residues within the single-stranded region are not modified in the presence of Eσ^{32}, since single-stranded pyrimidines are usually modified to some extent by $KMnO_4$.

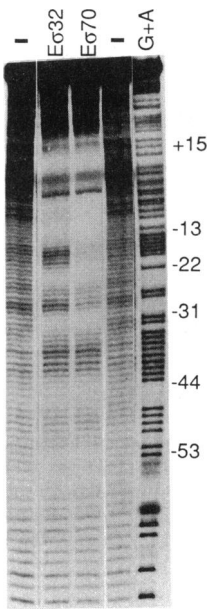

FIG. 7. Hydroxyl radical footprints of Eσ^{32} or Eσ^{70} on *rrnB* P1 fragments. The bottom strand (labeled at the *Eco*RI site at −87) is shown. The holoenzyme used is indicated above the relevant lane. Lane G + A, sequencing ladder prepared by the method of Maxam and Gilbert (31); lane –, no RNAP. The source of the band at +1 in the lanes containing RNAP is unknown.

patterns on the top strand (Fig. 6A). With Eσ^{32} bound, the T at −9 is modified to a greater extent than is the T at −12, while the opposite is true in the presence of Eσ^{70}.

(iii) Hydroxyl radical cleavage. The protection profiles for the two holoenzymes are similar (Fig. 7) except that the backbone positions from about −18 to −14 are not protected by Eσ^{32} but are protected by Eσ^{70}. The −53 region is protected equally by both forms of RNAP. Factor-independent activation of *rrnB* P1 has been correlated with a strong hydroxyl radical protection by Eσ^{70} centered at −53 on the bottom strand (34, 35).

The fact that the complexes formed with the two holoenzyme preparations exhibit differences in their footprints further supports the conclusion that transcription of *rrnB* P1 by Eσ^{32} does not result from contaminating Eσ^{70}.

Eσ^{32} responds to upstream activation. The UAR of the *rrnB* P1 promoter extends from approximately −154 to −40 (15, 26, 38) and has two components. Fis-dependent activation involves the binding of Fis protein to three sites in the UAR between −154 and −60 (41). Factor-independent activation involves the −60 to −40 region, which directly interacts with RNAP (26, 34, 35, 38, 40).

Fis was found to activate Eσ^{32} transcription from *rrnB* P1 (Fig. 8). Eσ^{32} also responds to factor-independent activation. That is, the level of transcription by Eσ^{32} from a template containing *rrnB* P1 sequences from −87 to +50 (+UAR) was significantly greater than that from a template containing sequences only to −50 (−UAR) (Fig. 9).

Eσ^{32} is not required for growth rate-dependent or stringent control of rRNA transcription. The low amounts of Eσ^{32} in cells grown at moderate temperatures make it unlikely that the heat shock RNAP plays a role in regulation of rRNA transcription under these conditions, for example by competing with Eσ^{70} for rRNA promoters. However, to address this question rigorously, we determined whether the two major regulatory mechanisms regulating rRNA synthesis function normally in a strain which makes no Eσ^{32}.

Expression of the *groE* gene product allows strains lacking a functional *rpoH* gene to grow at moderate temperatures (25). Therefore, we were able to examine whether a strain which makes no Eσ^{32} nevertheless regulates rRNA synthesis. Figure 10 shows that growth rate-dependent regulation, as inferred from the increase in RNA/protein ratios in cells grown at different growth rates, is the same within error for a wild-type strain, one expressing the *groE* gene product by virtue of an IS*1* insertion, or one lacking Eσ^{32} (and expressing GroE).

Cells shut off rRNA transcription immediately following

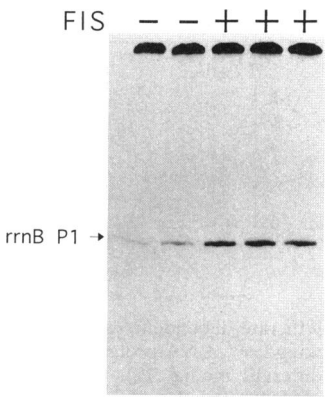

FIG. 8. Fis-dependent transcription activation of Eσ^{32}. Multiple-round transcription reactions containing Eσ^{32} and 0 or 100 nM Fis were performed on supercoiled plasmid DNA containing the *rrnB* P1 promoter as described in Materials and Methods. Replicate lanes are shown.

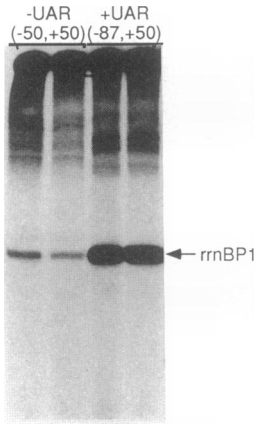

FIG. 9. Factor-independent transcription activation of $E\sigma^{32}$. Multiple-round transcription reactions were performed in low-salt buffer as described in Materials and Methods with 2.5 nM $E\sigma^{32}$ on 0.5 nM supercoiled plasmid DNA containing the *rrnB* P1 promoter with (−87,+50) or without (−50,+50) the UAR. Duplicate samples are shown.

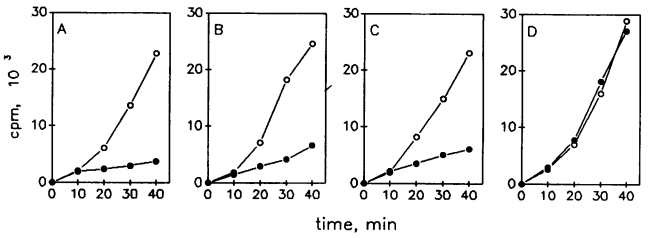

FIG. 11. Stringent control of rRNA transcription in strains lacking $E\sigma^{32}$. Incorporation of radioactive phosphate into RNA after amino acid starvation was measured as described previously (12). Filled circles represent incorporation in cells starved for isoleucine at 5 min by addition of valine; open circles represent incorporation in unstarved cells. Radioactive P_i was added at 0 min. ^{32}P counts incorporated into trichloroacetic acid-precipitable material were corrected for the optical density of the culture at each time point. (A) RLG2800 (wild type); (B) RLG2802 (strain deleted for *rpoH* but expressing the product of the *groE* gene); (C) RLG2804 (*rpoH*$^+$ but expressing the product of the *groE* gene); (D) RLG850 (*relA251* [12]), illustrating that the shutoff of RNA synthesis under these conditions is dependent on the *relA* gene product.

amino acid starvation in a response requiring a functional *relA* gene product (3). Figure 11 shows that *rpoH*::*kan* cells starved for amino acids shut off rRNA transcription (as inferred from reduced incorporation of $^{32}PO_4$ into RNA) in the manner characteristic of stringent control. Therefore, neither growth rate-dependent regulation nor stringent control appears to require $E\sigma^{32}$ function.

DISCUSSION

***rrnB* P1 is transcribed by two holoenzymes.** The similarity of the *rrnB* P1 promoter sequence to that recognized by the heat shock RNAP prompted us to test whether $E\sigma^{32}$ could bind and transcribe this promoter in vitro. We found that although transcription of *rrnB* P1 by $E\sigma^{32}$ is weaker than that by $E\sigma^{70}$ under these solution conditions, $E\sigma^{32}$ binds specifically in footprinting assays and initiates transcription from *rrnB* P1 in vitro. Furthermore, $E\sigma^{32}$ responds to both Fis-mediated and factor-independent activation.

Several genes in *E. coli* are transcribed by more than one RNAP holoenzyme. For example, the operon encoding σ^{70} (*rpoD*) is preceded by tandem $E\sigma^{70}$ promoters and by an $E\sigma^{32}$ promoter directly upstream of the *rpoD* gene itself (49). The gene for σ^{32} (*rpoH*) is transcribed by two holoenzymes, $E\sigma^{70}$ and $E\sigma^{E}$ (11). The *glnALG* operon contains a cyclic AMP (cAMP)-cAMP receptor protein (CRP)-dependent $E\sigma^{70}$ promoter and an NTRC-dependent $E\sigma^{54}$ promoter (39).

The overlapping nature of the $E\sigma^{70}$ and $E\sigma^{32}$ binding regions in the *rrnB* P1 promoter regions is more unusual for *E. coli*. However, several genes with promoter regions containing overlapping holoenzyme specificities have been noted in *B. subtilis*. The *ctc*, *spoVG*, and P43 promoters contain overlapping consensus regions for at least two different RNAP holoenzymes (21, 53). The transcriptional start sites for the two holoenzymes are identical in P43 and *ctc* (21, 48, 53) and are separated by 10 bp in the *spoVG* promoter (21).

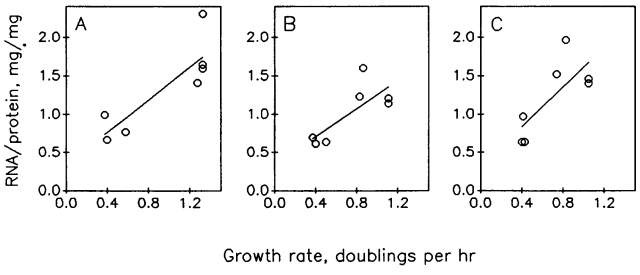

FIG. 10. Growth rate-dependent regulation of rRNA transcription in strains lacking $E\sigma^{32}$. RNA/protein ratios were measured from cells grown in different media (12). Curves represent the linear regressions of measurements made in at least two experiments. Each panel represents a different strain. (A) RLG2800 (wild type), RNA/protein = 1.06 (± 0.29); (B) RLG2802 (strain deleted for *rpoH* but expressing the product of the *groE* gene), RNA/protein = 0.88 (± 0.35); (C) RLG2804 (*rpoH*$^+$ but expressing the product of the *groE* gene), RNA/protein = 1.33 (± 0.48).

Activation of $E\sigma^{32}$ transcription. Both Fis-mediated activation and factor-independent activation are extremely sensitive to the orientation of the activating region with respect to the RNAP binding site (34). The overlap of the $E\sigma^{32}$ and $E\sigma^{70}$ binding sites in the *rrnB* P1 promoter allows both holoenzymes to utilize the upstream activation system. We note that the degree of upstream activation is dependent on reaction conditions and RNAP concentration in vitro. Therefore, we cannot predict the fold activation of $E\sigma^{32}$-directed transcription from the *rrnB* P1 promoter in vivo at this time.

$E\sigma^{32}$ responds to both Fis-mediated and factor-independent activation. This is the first direct demonstration of activation of $E\sigma^{32}$ transcription. This result implies that activation is not mediated by the same elements of RNAP responsible for promoter discrimination. Upstream activation must be mediated by regions conserved between σ^{70} and σ^{32} or by other RNAP subunits, consistent with recent evidence implicating the C terminus of the alpha subunit in factor-independent activation (40).

Similarities between *rrnB* P1-$E\sigma^{32}$ and heat shock promoter-$E\sigma^{32}$ complexes. DMS modification patterns specific to each holoenzyme are observed around the −10 region of *rrnB* P1. For example, a strongly enhanced methylation at −7G and protections at −15G and −16G are observed in the $E\sigma^{32}$-*rrnB* P1 footprint but not in the $E\sigma^{70}$-*rrnB* P1 footprint. We note that this pattern is quite similar to that observed by Cowing and Gross (7) in the $E\sigma^{32}$-*groE* promoter complex.

$E\sigma^{32}$ and $E\sigma^{70}$ protect the −50 region of *rrnB* P1 to the same extent. An interaction of $E\sigma^{70}$ with the −50 region of *rrnB* P1 has been correlated with factor-independent activation (34, 35, 40). The hydroxyl radical footprints of $E\sigma^{32}$ bound to three heat shock promoters (*groE*, *dnaK* P2, and *rpoD* P_{hs}) are similar to that of *rrnB* P1, including the interactions with the −50 regions (32). A relationship between these interactions and promoter activity has not been investigated in these heat shock promoters.

Does $E\sigma^{32}$ play a role in rRNA transcription in vivo? We have shown that transcription of rRNA promoters by $E\sigma^{32}$ does not play a role in two major regulatory mechanisms known to control rRNA synthesis in cells grown at moderate temperatures. However, it seems likely that transcription of rRNA by $E\sigma^{32}$ is not fortuitous and does play some role in cell physiology. For example, it is conceivable that $E\sigma^{32}$-directed rRNA transcription is required for recovery from brief exposure to extremely high temperatures (48 to 55°C) in order to make new ribosomes. At temperatures in this range, $E\sigma^{70}$ is inactivated (11), and rRNA and ribosomes are degraded (50).

Thus far, there is no direct evidence that $E\sigma^{32}$ plays a role in rRNA transcription in vivo. rRNA transcription has been examined during or after heat shock at 40 to 42°C. Transient decreases in rRNA synthesis have been reported following shifts to 40 to 42°C, correlating with a rapid increase in ppGpp levels (30, 42, 43). These changes are obscured by increases in the RNA chain elongation rate due to the higher temperature (43). Zengel and Lindahl (55) and Condon et al. (4) have also reported only very small changes in rRNA transcription after a shift to 42°C. More significantly, the rRNA synthesis rate was not substantially altered in an *rpoH* mutant (55). However, since $E\sigma^{70}$ is not inactivated at 42°C, it is unlikely that a contribution to total rRNA synthesis by $E\sigma^{32}$ would have been detected in any of these experiments. It will be of interest to examine effects of *rpoH* on rRNA transcription at temperatures at which $E\sigma^{70}$ is no longer active.

Factors other than temperature, such as ethanol addition, treatment with puromycin or nalidixic acid, or viral infection, lead to a heat shock response (9, 13, 24, 52). To our knowledge, a role for $E\sigma^{32}$ in rRNA transcription under these conditions has not been investigated.

Determining whether $E\sigma^{32}$ plays a role in rRNA transcription in vivo will depend upon identification of an $E\sigma^{32}$-*rrnB* P1 interaction in the cell. Identification of distinct in vivo transcription products and identification of in vivo footprints are two methods that have been used to examine specific promoter-RNAP interactions in the cell. $E\sigma^{32}$ and $E\sigma^{70}$ initiate transcription from the same position at *rrnB* P1 in vitro, but $E\sigma^{32}$-specific patterns were observed from in vitro footprinting analyses with DMS and $KMnO_4$. Therefore, in vivo footprints in combination with *rrnB* P1 promoter mutations which reduce or eliminate binding by either $E\sigma^{32}$ or $E\sigma^{70}$ might provide direct evidence for *rrnB* P1 promoter-$E\sigma^{32}$ interactions in vivo.

ACKNOWLEDGMENTS

We are grateful to Wilma Ross, Carol Gross, and other members of the Gourse and Gross laboratories for helpful comments. We thank Dayle Hager and Richard Burgess for materials, and Scott Lesley and Nancy Thompson for advice on experiments with anti-σ^{70} antibody. We thank Willy Walter for technical assistance, and Rob Barber, Carol Gross, and Takashi Yura for strains.

This work was supported by NIH grants R01 GM37048 and RCDA AI90035 to R.L.G.

REFERENCES

1. **Borowiec, J. A., and J. Hurwitz.** 1988. Localized melting and structural changes in the SV40 origin of replication induced by T-antigen. EMBO J. **7**:3149–3158.
2. **Borukhov, S., V. Sagitov, C. A. Josaitis, R. L. Gourse, and A. Goldfarb.** Primer-slippage initiation leading to stable early ternary complex formation on the *rrnB* P1 promoter of *Escherichia coli*. Submitted for publication.
3. **Cashel, M., and K. K. Rudd.** 1987. The stringent response, p. 1410–1438. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
4. **Condon, C., J. Phillips, Z. Y. Fu, C. Squires, and C. L. Squires.** 1992. Comparison of the expression of the seven ribosomal RNA operons in *Escherichia coli*. EMBO J. **11**:4175–4185.
5. **Cowing, D. W.** 1988. Ph.D. thesis. University of Wisconsin, Madison.
6. **Cowing, D. W., J. C. A. Bardwell, E. A. Craig, C. Woolford, R. W. Hendrix, and C. A. Gross.** 1985. Consensus sequence for *Escherichia coli* heat shock gene promoters. Proc. Natl. Acad. Sci. USA **82**:2679–2683.
7. **Cowing, D. W., and C. A. Gross.** 1989. Interaction of *Escherichia coli* RNA polymerase holoenzyme containing σ^{32} with heat shock promoters. J. Mol. Biol. **210**:513–520.
8. **deBoer, H. A., and M. Nomura.** 1979. *In vivo* transcription of rRNA operons in *Escherichia coli* initiates with purine nucleotide triphosphates at the first promoter and with CTP at the second promoter. J. Biol. Chem. **254**:5609–5612.
9. **Drahos, D. J., and R. W. Hendrix.** 1982. Effect of bacteriophage lambda infection on the synthesis of *groE* protein and other *Escherichia coli* proteins. J. Bacteriol. **149**:1050–1063.
10. **Erickson, J. W., and C. A. Gross.** 1989. Identification of the σ^{E} subunit of *Escherichia coli* RNA polymerase: a second alternate σ factor involved in high-temperature gene expression. Genes Dev. **3**:1462–1471.
11. **Erickson, J. W., V. Vaughn, W. A. Walter, F. C. Neidhardt, and C. A. Gross.** 1987. Regulation of the promoters and transcripts of *rpoH*, the *Escherichia coli* heat shock regulatory gene. Genes Dev. **1**:419–432.
12. **Gaal, T., and R. L. Gourse.** 1990. Guanosine 3′-diphosphate 5′-diphosphate is not required for growth rate-dependent control of rRNA synthesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87**:5533–5537.
13. **Goff, S. A., and A. L. Goldberg.** 1985. Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. Cell **41**:587–595.
14. **Gourse, R. L.** 1988. Visualization and quantitative analysis of complex formation between *E. coli* RNA polymerase and an rRNA promoter *in vitro*. Nucleic Acids Res. **16**:9789–9809.
15. **Gourse, R. L., H. A. deBoer, and M. Nomura.** 1986. DNA determinants of rRNA synthesis in *E. coli*: growth rate dependent regulation, feedback inhibition, upstream activation and anti-termination. Cell **44**:197–205.
16. **Gross, C. A., M. Lonetto, and R. Losick.** Sigma factors. *In* K. Yamamoto and S. McKnight (ed.), Transcriptional regulation, in press. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Grossman, A., J. Erickson, and C. A. Gross.** 1985. The *htpR* gene product of E. coli is a sigma factor for heat-shock promoters. Cell **38**:383–390.
18. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *E. coli* promoter sequences. Nucleic Acids Res. **15**:2343–2361.
19. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11**:2237–2255.
20. **Helmann, J. D., and M. J. Chamberlin.** 1989. Structure and function of bacterial sigma factors. Annu. Rev. Biochem. **57**: 839–872.
21. **Johnson, W. C., C. P. Moran, Jr., and R. Losick.** 1983. Two RNA polymerase sigma factors from *Bacillus subtilis* discriminate between overlapping promoters for a developmentally regulated gene. Nature (London) **302**:800–804.

22. **Jovanovich, S. B., S. A. Lesley, and R. R. Burgess.** 1989. *In vitro* use of monoclonal antibodies in *Escherichia coli* S-30 extracts to determine the RNA polymerase σ subunit required by a promoter. J. Biol. Chem. **264**:3794–3798.
23. **Joyce, C. M., and N. D. F. Grindley.** 1982. Identification of two genes immediately downstream of the *polA* gene of *Escherichia coli*. J. Bacteriol. **152**:1211–1219.
24. **Krueger, J. H., and G. Walker.** 1984. *groEL* and *dnaK* genes of *Escherichia coli* are induced by UV irradiation and naladixic acid in a *htpR*$^+$-dependent fashion. Proc. Natl. Acad. Sci. USA **81**:1499–1503.
25. **Kusukawa, N., and T. Yura.** 1988. Heat shock protein GroE of *Escherichia coli*: key protective roles against thermal stress. Genes Dev. **2**:874–882.
26. **Leirmo, S., and R. L. Gourse.** 1991. Factor-independent activation of *Escherichia coli* rRNA transcription. I. Kinetic analysis of the roles of the upstream activator region and supercoiling on the *rrnB* P1 promoter *in vitro*. J. Mol. Biol. **220**:555–568.
27. **Leirmo, S., C. Harrison, D. S. Cayley, R. R. Burgess, and M. T. Record, Jr.** 1987. Replacement of potassium chloride by potassium glutamate dramatically enhances protein-DNA interactions *in vitro*. Biochemistry **26**:2095–2101.
28. **Lonetto, M., M. Gribskov, and C. A. Gross.** 1992. The σ^{70} family: sequence conservations and evolutionary relationships. J. Bacteriol. **174**:3843–3849.
29. **Losick, R., and J. Pero.** 1981. Cascades of sigma factors. Cell **25**:582–584.
30. **Mackow, E. R., and F. N. Chang.** 1983. Correlation between RNA synthesis and ppGpp content in *Escherichia coli* during temperature shifts. Mol. Gen. Genet. **192**:5–9.
31. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific cleavages. Methods Enzymol. **65**:499–560.
32. **Mecsas, J., D. W. Cowing, and C. A. Gross.** 1991. Development of RNA polymerase-promoter contacts during open complex formation. J. Mol. Biol. **220**:585–597.
33. **Morita, M., and A. Oka.** 1979. The structure of a transcriptional unit on colicin E1 plasmid. Eur. J. Biochem. **97**:435–443.
34. **Newlands, J. T., C. A. Josaitis, W. Ross, and R. L. Gourse.** 1992. Both fis-dependent and factor-independent upstream activation of the *rrnB* P1 promoter are face of the helix dependent. Nucleic Acids Res. **20**:719–726.
35. **Newlands, J. T., W. Ross, K. K. Gosink, and R. L. Gourse.** 1991. Factor-independent activation of *Escherichia coli* rRNA transcription. II. Characterization of complexes of *rrnB* P1 promoters containing or lacking the upstream activator region with *Escherichia coli* RNA polymerase. J. Mol. Biol. **220**:569–583.
36. **Nomura, M., R. Gourse, and G. Baughman.** 1984. Regulation of the synthesis of ribosomes and ribosomal components. Annu. Rev. Biochem. **53**:75–117.
37. **Peacock, A. C., and C. W. Dingman.** 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide gels. Biochemistry **7**:668–674.
38. **Rao, L., W. Ross, S. Leirmo, P. J. Schlax, and R. L. Gourse.** Unpublished data.
39. **Reitzer, L. J., and B. Magasanik.** 1987. Ammonia assimilation and the biosynthesis of glutamine, glutamate, aspartate, asparagine, L-alanine, and D-alanine, p. 302–320. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
40. **Ross, W., K. K. Gosink, J. A. Appleman, J. Salomon, K. Igarashi, A. Ishihama, and R. L. Gourse.** Unpublished data.
41. **Ross, W., J. F. Thompson, J. T. Newlands, and R. L. Gourse.** 1990. *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. EMBO J. **9**:3733–3742.
42. **Ryals, J., R. Little, and H. Bremer.** 1982. Temperature dependence of RNA synthesis parameters in *Escherichia coli*. J. Bacteriol. **151**:879–887.
43. **Ryals, J., R. Little, and H. Bremer.** 1982. Control of RNA synthesis in *Escherichia coli* after a shift to higher temperature. J. Bacteriol. **151**:1425–1432.
44. **Sasse-Dwight, S., and J. D. Gralla.** 1988. Probing the *Escherichia coli glnALG* upstream activation mechanism *in vivo*. Proc. Natl. Acad. Sci. USA **85**:8934–8938.
45. **Sasse-Dwight, S., and J. D. Gralla.** 1989. $KMnO_4$ as a probe for *lac* promoter DNA melting and mechanism *in vivo*. J. Biol. Chem. **264**:8074–8081.
46. **Siebenlist, U., and W. Gilbert.** 1980. Contacts between *Escherichia coli* RNA polymerase and an early promoter of phage T7. Proc. Natl. Acad. Sci. USA **77**:122–126.
47. **Strickland, M. S., N. E. Thompson, and R. R. Burgess.** 1988. Structure and function of the σ-70 subunit of *Escherichia coli* RNA polymerase. Monoclonal antibodies: localization of epitopes by peptide mapping and effects on transcription. Biochemistry **27**:5755–5762.
48. **Tatti, K. M., and C. P. Moran, Jr.** 1985. Utilization of one promoter by two forms of RNA polymerase from *Bacillus subtilis*. Nature (London) **314**:190–192.
49. **Taylor, W. E., D. B. Straus, A. D. Grossman, Z. F. Burton, C. A. Gross, and R. R. Burgess.** 1984. Transcription from a heat-inducible promoter causes heat shock regulation of the sigma subunit of E. coli RNA polymerase. Cell **38**:371–381.
50. **Tomlins, R. I., and Z. J. Ordal.** 1971. Precursor ribosomal ribonucleic acid and ribosome accumulation in vivo during the recovery of *Salmonella typhimurium* from thermal injury. J. Bacteriol. **107**:134–142.
51. **Tullius, T. D., and B. A. Dombroski.** 1986. Hydroxyl radical "footprinting": high-resolution information about DNA-protein contacts and application to λ repressor and Cro protein. Proc. Natl. Acad. Sci. USA **83**:5469–5473.
52. **VanBogelen, R. A., P. M. Kelley, and F. C. Neidhardt.** 1987. Differential induction of heat shock, SOS, and oxidation-stress regulons and accumulation of nucleotides in *Escherichia coli*. J. Bacteriol. **169**:26–32.
53. **Wang, P. Z., and R. H. Doi.** 1984. Overlapping promoters transcribed by *Bacillus subtilis* σ^{55} and σ^{37} RNA polymerase holoenzymes during growth and stationary phases. J. Biol. Chem. **259**:8619–8625.
54. **Westpheling, J., M. Ranes, and R. Losick.** 1985. RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature (London) **313**:22–27.
55. **Zengel, J. M., and L. Lindahl.** 1985. Transcriptional control of the S10 ribosomal protein operon of *Escherichia coli* after a shift to higher temperature. J. Bacteriol. **163**:140–147.
56. **Zhou, Y.-N., N. Kusukawa, J. W. Erickson, C. A. Gross, and T. Yura.** 1988. Isolation and characterization of *Escherichia coli* mutants that lack the heat shock sigma factor σ^{32}. J. Bacteriol. **170**:3640–3649.