

## Identification of Sequences Necessary for Transcription In Vitro from the *Chlamydia trachomatis* rRNA P1 Promoter

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***Chlamydia trachomatis* RNA polymerase was partially purified by heparin-agarose chromatography and used in conjunction with a plasmid-borne G-less cassette template to characterize the *C. trachomatis* rRNA P1 promoter in vitro. Stepwise mutational analysis revealed that sequences in the –10, –25, and –35 regions are necessary for promoter activity, but no sequence upstream of position –40 is required. Partially purified *C. trachomatis* RNA polymerase and purified *Escherichia coli* holoenzyme exhibited some differences in promoter specificity.**

*Chlamydia trachomatis* is a gram-negative obligate intracellular pathogen with a complex life cycle characterized by two serial morphologic forms (reviewed in references 26 and 34). Stage-specific expression has been demonstrated in early (38) and late (1–3, 8, 14, 19, 28) periods of the developmental cycle, but the molecular basis of this gene regulation is not understood. Direct genetic studies of promoter structures have not been feasible; transient transformation of chlamydiae has been described (35), but a practical DNA-mediated transformation system does not exist.

In the absence of a genetic system, several approaches have been taken to identify and characterize chlamydial promoters. The transcription initiation sites of several abundant transcripts have been identified, and their upstream regions have been examined for putative promoter sequences. By inspection, appropriately spaced canonical *Escherichia coli*  $\sigma^{70}$ -type promoter sequences are present in a few of these upstream regions. These include the *C. trachomatis* plasmid countertranscript (PCT) (15), *envA* P1 (13, 22), and the *dnaK* operon of the *C. trachomatis* mouse pneumonitis biovar (11). Others, including the *hctB*, *ltuA*, and *ltuB* genes, have  $\sigma^{70}$  promoter-like sequences at the putative –10 region only. Interestingly, these three genes are all transcribed late in the developmental cycle (14). However, many chlamydial genes are not preceded by  $\sigma^{70}$ -like promoter sequences (i.e., there is no more than a 50% match), and no consensus chlamydial promoter sequence is apparent (8). Explanations for this sequence diversity include the possibilities that (i) some of the presumptive chlamydial transcription initiation sites are instead processing sites, (ii) the putative promoters belong to more than one class of promoters, and (iii) there is latitude in the promoter specificity of *C. trachomatis*  $\sigma^A$  RNA polymerase (RNAP).

The subunits of *C. trachomatis* RNAP have been cloned and sequenced (9, 10, 18, 21).  $\sigma^A$ , a *C. trachomatis*  $\sigma^{70}$  homolog (9, 21), is the only sigma factor identified in chlamydiae to date.  $\sigma^A$  and  $\sigma^{70}$  share striking amino acid sequence conservation in subregions 2.4 and 4.2 (9, 21) of  $\sigma^{70}$ , including the specific amino acid residues which have been shown in *E. coli* to be

involved in promoter recognition at the –10 and –35 positions (reviewed in reference 5). This conservation of amino acid residues involved in promoter recognition contrasts with the observed variability in the DNA sequences of the putative *C. trachomatis* promoters.

Recently, Mathews et al. (23) reported an in vitro transcription system which can be used to functionally characterize chlamydial promoters. Using a high-salt extract from *C. psittaci* and *C. trachomatis* reticulate bodies (RBs), they were able to show  $\sigma^A$ -dependent transcription of several chlamydial genes, including PCT, *hctB*, *ltuA*, and *ltuB* (6, 14, 23). Single mutations in the –10 and –35 regions of the putative PCT promoter region had no effect on promoter activity (24). Multiple mutations were required to reduce or eliminate promoter activity. However, the contribution of sequences outside the –10 and –35 positions was not examined.

We have developed an improved in vitro transcription system to characterize rRNA P1, the highly transcribed promoter common to both *C. trachomatis* rRNA operons. We chose to study this promoter because in *E. coli*, the rRNA promoters are strong promoters that closely resemble the canonical  $\sigma^{70}$  promoter structure. By analogy, the structure of the *C. trachomatis* rRNA P1 promoter may also resemble the canonical *C. trachomatis*  $\sigma^A$  promoter structure. In addition, *E. coli* *rmB* P1 contains a third promoter element, the UP element, which serves as a site of interaction for the  $\alpha$  subunit of RNAP (30). The UP element has been shown to increase the level of transcription of *E. coli* *rmB* P1 up to 30-fold, and it is postulated to be involved in the high-level expression of specific genes required for rapid growth.

We have characterized *C. trachomatis* rRNA P1 by using a stepwise mutational analysis of the putative promoter region. We identified sequences in the approximate –10 and –35 regions and in the –21 to –30 region that are necessary for promoter activity. Interestingly, partially purified *C. trachomatis* RNAP and purified *E. coli* holoenzyme exhibited some differences in promoter specificity.

### MATERIALS AND METHODS

**Reagents.** Products were obtained from the following sources and were used according to the manufacturers' specifications. Restriction enzymes, bacterial alkaline phosphatase, exonuclease III, mung bean nuclease, and T4 DNA ligase were from Gibco BRL (Gaithersburg, Md.); T4 DNA polymerase and T4 polynucleotide kinase were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); RQ1 DNase and RNasin were from Promega Biotech (Madison, Wis.);

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SP6 RNAP was from Ambion (Austin, Tex.); *Thermus aquaticus* DNA polymerase was from Cetus Corp. (Emeryville, Calif.);  $^{32}$ P-containing nucleoside triphosphates were from Amersham Corp. (Arlington Heights, Ill.); SeaPlaque and SeaKem agarose were from FMC Bioproducts (Rockland, Maine); ampicillin, gentamicin sulfate, and rifampin were from Sigma Chemical Co. (St. Louis, Mo.); vancomycin hydrochloride was from Abbott Laboratories (North Chicago, Ill.); and dimethyl sulfoxide was from Fisher Scientific (Pittsburgh, Pa.).

**Nucleic acid preparation and DNA sequencing.** Standard recombinant DNA methods were used for nucleic acid preparation and analysis (31). The dideoxy-chain termination method of DNA sequencing (32) was performed by using a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) on double-stranded plasmid DNA.

**Synthetic oligonucleotides.** The following single-stranded oligonucleotide primers were synthesized by Gibco BRL: M13 forward -40 (5' GTTTT CCCAG TCACG AC), M13 reverse -40 (5' GTTGT GTGGA ATTGT G), pGLS3' (5' ATAGG AGGAA TAATG), rRNA-3 (5' CAGGG TACCA GCCT CCGCG TTCAA GA), rRNA-4 (5' CACGA ATTCC GCGTT CAAGA AAGG), Tx1 (5' AAAGT AACAT CTTAT ATCAA CCTCT), Tx2 (5' AAAGT ATCTT ATATC AACCT CTAT), Tx3 (5' AAAGT ATATA TCAAC CTCTA TTTT), Tx4 (5' CCTGG TACCG CCCGG GAAGA GGGGG TGAGA G), Tx5 (5' ATCAT CCTCA TCCCT ATC), Tx6 (5' AGGGA ATTCA GGGAT ATCTT TAAAC ACACC AAACA TCATA CC), Tx7 (5' AGGAG TGGGT TAGGG CCCTG TGAGA AAAAA TAGAT), Tx8 (5' TGGGT TAGGG AAAGT GTCTC AAAAA TAGAT GCAGA), Tx9 (5' TAGGG AAAGT TGAGA CCCCC TA GAT GCAGA AAAAA), Tx10 (5' AAAGT TGAGA AAAAA GCTCG GCAGA AAAAA TAGAG), Tx11 (5' TGAGA AAAAA TAGAT TACTC AAAAA TAGAG GTTGA), Tx12 (5' AAAAA TAGAT GCAGA CCCCC TAGAG GTTGA TATAA), Tx13 (5' TAGAT GCAGA AAAAA GCTCT GT TGA TATAA GATGT), Tx14 (5' GCAGA AAAAA TAGAG TGGTC TATAA GATGT TACTT), Tx23 (5' AAAGT ATTAT ATCAA CCTCT ATTT), Tx24 (5' AAAGT AATAT CAACC TCTAT TTTT), Tx27 (5' GGGAA GAGGG GGTGA GAG), and Tx28 (5' AAAGT AACAT CCGCG CTCAA CCTCT ATTTT T).

**PCR.** PCR was performed with a Cetus/Perkin-Elmer DNA thermocycler. Reactions were carried out in a volume of 100  $\mu$ l containing 100 pmol each of the 5' and 3' primers, 1 ng of plasmid DNA template, all four deoxyribonucleoside triphosphates (0.2 mM each), 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.1% gelatin, and 2.5 U of *T. aquaticus* DNA polymerase. Amplification was carried out for 40 cycles (denaturation at 94°C for 30 s, annealing at 45°C for 1 min, and polymerization at 72°C for 30 s) unless otherwise stated.

**Purification of *C. trachomatis* RNAP.** The purification method was modified from the heparin-agarose procedure described by Chamberlin et al. (4). *C. trachomatis* L2 was grown in 1 liter of L929 cells (a mouse fibroblast cell line) in spinner flasks in RPMI 1640 tissue culture medium with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 5% fetal bovine serum (Gibco BRL), vancomycin hydrochloride (60  $\mu$ g/ml), and gentamicin sulfate (10  $\mu$ g/ml). RBs were harvested at 18 to 24 h postinfection by centrifuging the infected L cells at 2,500  $\times$  *g* for 10 min in a Beckman JA 10 rotor at 4°C. All subsequent steps were performed at 4°C. The pellet was resuspended in 35 ml of phosphate-buffered saline (pH 7.5), and host L929 cells were lysed in a 40-ml Wheaton homogenizer with an A pestle. The lysate was centrifuged at 3,000 rpm for 10 min (Sorvall RT6000B centrifuge with an H-1000B rotor), and the subsequent supernatant fluid was centrifuged at 17,000  $\times$  *g* for 10 min (Beckman JA 20 rotor). The wet weight of the RB-containing pellet was approximately 400 mg. This RB pellet was resuspended in 2 ml of freshly prepared lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 7.5% [vol/vol] glycerol, 0.3 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride, 150  $\mu$ g of lysozyme per ml, 10  $\mu$ g of pepstatin A per ml) containing 0.6 M NaCl. Twenty microliters of 10% (vol/vol) Nonidet P-40 (Sigma) was added, and the mix was incubated on ice for 5 min. The RBs were lysed by sonication (Branson Sonifier 450 with microtip; two 10-s cycles at high setting). The lysate was centrifuged at 16,000  $\times$  *g* for 30 min (Eppendorf model 5415C centrifuge), and the supernatant fluid was collected and clarified by two to three additional centrifugations of 15 min each. The clarified supernatant fluid was diluted with 1.4 volumes of buffer I (10 mM Tris-HCl [pH 8.0], 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 7.5% [vol/vol] glycerol, 0.3 mM DTT) to give a final concentration of 0.25 M NaCl. This diluted supernatant fluid was mixed for 1 h with 1 ml of heparin-agarose (type II-S; Sigma) previously equilibrated with buffer I containing 0.25 M NaCl. The heparin-agarose column was packed and washed by gravity flow with 20 ml of buffer I containing 0.25 M NaCl. The column was eluted with buffer I containing 0.6 M NaCl, and 0.5-ml fractions were collected. Fractions were dialyzed against storage buffer (10 mM Tris-HCl [pH 8.0], 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.1 mM DTT, 100 mM NaCl, 30% [vol/vol] glycerol) and stored at -70°C. Peak fractions were identified by their *in vitro* transcription activity. Transcription activity was generally recovered in 1 column volume.

**Transcription templates.** The terminator plasmid pMT175 contains *C. trachomatis* rRNA P1 and P2 cloned upstream of a T7 terminator (Fig. 1). The P1 and P2 transcripts are initiated 183 to 185 and 83 to 84 bp, respectively, upstream of the start of the 16S rRNA gene. The two *C. trachomatis* promoters are located within a 305-bp upstream region on a 550-bp *Xba*I-*Sph*I fragment which was derived from plasmid pRC66 (12). This *Xba*I-*Sph*I fragment was subcloned into pMT171 at the *Sma*I polylinker site. pMT171 is a derivative of plasmid pTE103

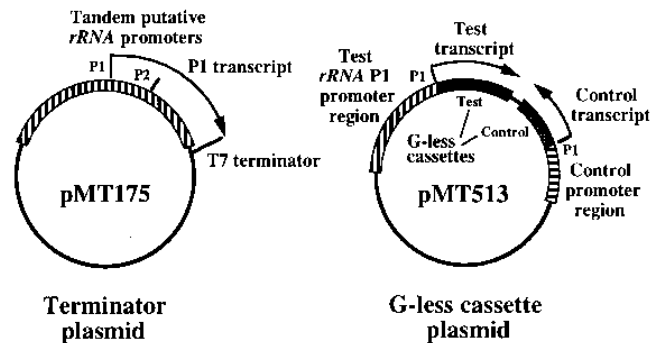


FIG. 1. The T7 terminator and G-less cassette plasmids. The T7 terminator plasmid, pMT175, contains 550 bp of the *C. trachomatis* rRNA gene (hatched box), of which 305 bp are sequences upstream of the P1 transcription initiation site. The two putative promoters, P1 and P2, are shown, together with the P1 transcript which is terminated at a T7 terminator. The G-less cassette plasmid, pMT513, contains two transcription templates cloned in opposite directions, each coding for a transcript of different length. The control promoter region contains sequences of rRNA P1 (+53 to -5) cloned in front of the control G-less cassette. The control transcript is 132 nucleotides long. The test promoter region in pMT513 contains sequences of rRNA P1 (+305 to -5) cloned immediately upstream of the test G-less cassette. The test transcript is 160 nucleotides in length. Other G-less cassette plasmids described in Table 1 contain mutant rRNA P1 promoter region sequences cloned into the test promoter region. Plasmids are not drawn to scale.

(7) in which approximately 230 bp have been deleted, leaving 66 to 67 bp between the multiple cloning site and the T7 terminator. Transcription from P1 and P2, assuming termination at the T7 terminator, is predicted to produce transcripts 303 to 306 and 203 to 205 nucleotides in length, respectively.

The double G-less cassette plasmid contains two transcription templates cloned into the plasmid pGEM-7Zf(+) (Promega Biotech) in opposite directions and coding for transcripts of 160 and 132 nucleotides (Fig. 1). The shorter transcript is encoded by a control transcription template inserted into pGEM-7Zf(+) at the *Sma*I site. This control template consists of *C. trachomatis* rRNA P1 sequences from -53 to +5 (see Fig. 4) cloned immediately upstream of a 127-bp control G-less cassette. The second of the three *in vivo* transcription initiation sites is a guanosine residue, and this was replaced by an adenosine residue (at +2) to allow for transcription in the absence of GTP. The control G-less cassette was derived from plasmid p $\mu$ (-47)-(G-)-I (27) by PCR using primer Tx5 and the M13 reverse -40 primer. This synthetic control template codes for a 132-nucleotide transcript. The test transcription template contains a 155-nucleotide test G-less cassette which was derived from plasmid p $\mu$ (-47)-(G-)-I (27) by PCR using primers Tx6 and Tx4. It was cloned into pGEM-7Zf(+) at the *Eco*RI and *Kpn*I sites, in the opposite direction to the control transcription template. This test G-less cassette contains an *Eco*RV restriction site at its 5' end, providing a convenient site into which a test promoter can be cloned. The plasmid containing the control transcription template and a promoterless test G-less cassette is called pMT504. All the G-less cassette transcription templates described in this report were derived from pMT504 by the cloning of a test promoter region into the *Eco*RI and *Eco*RV sites of pMT504. In each case, the synthetic test template codes for a 160-nucleotide transcript.

Supercoiled DNA plasmids for use in the *in vitro* transcription assays were prepared by using a Qiagen Plasmid Midi kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's directions. The DNA sequences of the promoter templates were determined to ascertain that no mutations had occurred during the PCR and cloning steps.

**Construction of 5'-deletion templates.** pMT513 (Fig. 1) contains 305 bp of rRNA P1 upstream sequences (+305 to -5) which were amplified from plasmid pMT175 (see above) by PCR using primers Tx1 and rRNA-3. The PCR product was digested with *Eco*RI, phosphorylated, and ligated to the double G-less cassette plasmid pMT504 previously digested with *Eco*RI and *Eco*RV. Nested 5' deletions of this 305-bp rRNA P1 upstream region were generated from pMT513 (Table 1) by using exonuclease III and mung bean nuclease as specified by the manufacturer (Gibco BRL).

**Construction of deletion templates.** Mutant templates of rRNA P1 containing deletions near the transcription initiation site (Table 1) were generated by PCR from plasmid pMT514, which contains 53 bp of rRNA P1 upstream sequences as the test promoter. For each PCR, the M13 forward -40 primer was used as the 5' PCR primer. The following 3' PCR primers were used to generate their respective deletion templates: Tx2 ( $\Delta$ 3, deletion of -3 to -1), Tx23 ( $\Delta$ 5, deletion of -5 to -1), Tx3 ( $\Delta$ 6, deletion of -6 to -1), and Tx24 ( $\Delta$ 7, deletion of -7 to -1). Each PCR product was phosphorylated, digested with *Eco*RI, and ligated to plasmid pMT504 previously digested with *Eco*RI and *Eco*RV (Table 1).

TABLE 1. Plasmids used in this study

Plasmid	Description
<b>Starting plasmids</b>	
pMT175	550-bp rRNA promoter region containing P1 and P2 cloned into a T7 terminator plasmid (described in Materials and Methods and shown in Fig. 1)
pMT504	Double G-less cassette plasmid containing (i) a promoterless test G-less cassette and (ii) a shorter G-less cassette under the control of the rRNA P1 -53 deletion template (-53 to +5) (described in Materials and Methods)
<b>5'-deletion templates</b>	
pMT513	-305 deletion template (-305 to +5 of rRNA P1 cloned into pMT504 immediately upstream of the test G-less cassette [Fig. 1])
pMT514	-53 deletion template (-53 to +5)
pMT560	-42 deletion template (-42 to +5)
pMT561	-40 deletion template (-40 to +5)
pMT515	-37 deletion template (-37 to +5)
pMT516	-33 deletion template (-33 to +5)
pMT517	-30 deletion template (-30 to +5)
<b>Deletion templates</b>	
pMT521	$\Delta 3$ deletion template (deletion of -3 to -1 from the -53 deletion template, pMT514)
pMT566	$\Delta 5$ deletion template (deletion of -5 to -1)
pMT522	$\Delta 6$ deletion template (deletion of -6 to -1)
pMT564	$\Delta 7$ deletion template (deletion of -7 to -1)
<b>Base substitution templates</b>	
pMT505	Substitution of -50 to -46 AAAGT by CCCTG in pMT513 (-305 deletion template)
pMT506	Substitution of -45 to -41 TGAGA by GTCTC
pMT507	Substitution of -40 to -36 AAAAA by CCCCC
pMT508	Substitution of -35 to -31 TAGAT by GCTCG
pMT509	Substitution of -30 to -26 GCAGA by TACTC
pMT537	Substitution of -25 to -21 AAAAA by CCCCC
pMT538	Substitution of -20 to -16 TAGAG by GCTCT
pMT539	Substitution of -15 to -11 GTTGA by TGTGC
pMT577	Substitution of -10 to -6 TATAA by GCGCC

**Megaprimer method for generating base substitution mutant templates.** An adaptation of the megaprimer method (37) was used to introduce desired mutations into the rRNA P1 upstream sequence. In the first of two PCRs, an oligonucleotide containing the desired mutation was used to generate a mutant template (the megaprimer). The megaprimer was then extended on a DNA template, so as to incorporate convenient restriction sites into the mutant template. In the second PCR, the extended megaprimer was amplified to produce quantities of the mutant template sufficient for cloning.

For each mutant template, a megaprimer was produced by PCR using plasmid pMT513 as the template, the 3' primer pGLS3', and the respective 5' primer from the following list (with the location of the 5-bp substitution in parentheses): Tx7 (-50 to -46), Tx8 (-45 to -41), Tx9 (-40 to -36), Tx10 (-35 to -31), Tx11 (-30 to -26), Tx12 (-25 to -21), Tx13 (-20 to -16), and Tx14 (-15 to -11) (Table 1). The megaprimer PCR product was electrophoresed on a 3% low-melting-temperature agarose gel (SeaPlaque), and a gel slice containing the 110- to 145-bp product was excised; 1.5 ml of 10 mM Tris-HCl (pH 8.0)-1 mM EDTA was added, and the gel slice was melted at 65°C for 15 min. The melted gel slice was frozen at -70°C for 15 min and thawed at room temperature. Supernatant fluid was collected after centrifugation at 16,000  $\times g$  for 5 min (Eppendorf model 5415C centrifuge), and the megaprimer was recovered by ethanol precipitation. Thirty nanograms of megaprimer was extended with T4 DNA polymerase, using 100 ng of heat-denatured linearized plasmid pMT175 as the template in a 10- $\mu$ l reaction volume. In the second PCR, 2  $\mu$ l of the extended megaprimer mix was used as the template with 100 pmol of primers rRNA-4 and pGLS3'. Each PCR product was digested with *EcoRI* and *DraI* and ligated to plasmid pMT504 previously digested with *EcoRI* and *EcoRV*.

The -10 to -6 base substitution template was generated by PCR using pMT513 as the template and rRNA-4 and Tx28 as the primers. The PCR product was phosphorylated, digested with *EcoRI*, and ligated to plasmid pMT504 previously digested with *EcoRI* and *EcoRV*.

**In vitro transcription.**  $^{32}$ P-labeled RNA was synthesized in a 30- $\mu$ l reaction mixture containing 1 to 3  $\mu$ l of heparin-agarose-purified *C. trachomatis* RNAP,

10 nM DNA template, 1 mM potassium acetate, 8.1 mM magnesium acetate, 50 mM Tris acetate (pH 8.0), 27 mM ammonium acetate, 1 mM DTT, 3.5% (vol/vol) polyethylene glycol (average molecular weight, 8,000; Sigma), 330  $\mu$ M ATP, 330  $\mu$ M UTP, 1  $\mu$ M CTP, 0.21  $\mu$ M [ $\alpha$ - $^{32}$ P]CTP (3,000 Ci/mmol), 100 mM 3'-O-methyl-GTP, Na salt (Pharmacia Biotech, Piscataway, N.J.), and 36 U of RNasin. In some transcription reactions, 0.003 U of *E. coli* RNAP (Epicentre Technologies, Madison, Wis.) was used instead of *C. trachomatis* RNAP and the concentration of potassium acetate used was increased from 1 mM to 100 mM. Other reaction conditions were unchanged. Transcription reaction mixtures were incubated at 37°C for 35 min and terminated by the addition of 70  $\mu$ l of 2.86 M ammonium acetate containing 4  $\mu$ g of glycogen (Boehringer Mannheim Biochemicals).  $^{32}$ P-labeled RNA was recovered by ethanol precipitation of the transcription mix and resuspended in 9  $\mu$ l of sequencing stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Three microliters of the sample was electrophoresed on an 8 M urea-6% polyacrylamide gel. Transcripts were visualized by autoradiography and quantified with a Molecular Dynamics (Sunnyvale, Calif.) PhosphorImager. The size of the transcript was determined by coelectrophoresis with an M13 sequencing ladder.

**Calculation of relative promoter activity.** One consequence of using a plasmid containing two promoters is competition between the promoters for RNAP. These conditions would be expected to underestimate the activities of promoters which are weak relative to a strong control promoter. To minimize this effect, the promoter activity of each test promoter was normalized to that of the control promoter by comparing the PhosphorImager intensities of the test and control transcripts according to the following formula: promoter activity = test transcript intensity/(test transcript intensity + control transcript intensity). The relative promoter activity was calculated as a percentage of the promoter activity of the -305 promoter template; i.e., promoter activity of the -305 promoter template was defined as 100%. Performing these experiments under conditions of RNAP excess or limiting template was not technically feasible. We do not believe that the competitive nature of the transcription reactions alters the conclusions.

**Inhibition of in vitro transcription by rifampin.** Rifampin was dissolved in dimethyl sulfoxide at 25  $\mu$ g/ml and diluted to final concentrations of 16, 160, and 1,600 ng/ml. Components of the transcription reactions, except for the DNA template and the nucleoside triphosphates, were preincubated with rifampin for 15 min on ice. The DNA template, pMT504, and the nucleoside triphosphates were then added to each reaction mix, which was incubated at 37°C for 35 min. All subsequent steps were carried out as described above.

**Primer extension analysis of in vitro transcription products.** Unlabeled in vitro transcripts were synthesized from plasmid pMT513 as described above, with the following changes: each 30- $\mu$ l reaction mixture contained 3  $\mu$ l of heparin-agarose-purified *C. trachomatis* RNAP, 330  $\mu$ M CTP, and no [ $\alpha$ - $^{32}$ P]CTP. RNA from five separate reactions was pooled. In vitro synthesis of RNA by *E. coli* RNAP was performed in a single 30- $\mu$ l reaction containing 0.1 U of *E. coli* RNAP. Prior to primer extension analysis, RNA samples were treated with RQ1 DNase to remove the DNA template. Primer extension analysis was carried out as previously described (12), using  $^{32}$ P-end-labeled primer Tx27, which is complementary to the extreme 3' end of the test G-less cassette template. Primer extension products were electrophoresed next to a Tx27-primed DNA sequence of pMT513.

## RESULTS AND DISCUSSION

**Purification of *C. trachomatis* RNAP by heparin-agarose column chromatography.** *C. trachomatis* RNAP is partially purified by heparin-agarose column chromatography, a procedure that has been used as a single-column purification method for RNAPs from *E. coli*, *Bacillus subtilis*, and several other bacteria (4). Heparin-agarose purification of RNAP is known to remove nucleases and endogenous DNA (4), both of which are present in other *C. trachomatis* transcription extracts (23, 25). The heparin-agarose-purified *C. trachomatis* RNAP prepared in this manner was relatively free of RNase activity (35a). This partially purified RNAP correctly transcribed several *C. trachomatis* promoter templates, including rRNA P1 (described below) and the *groE* and *dnaK* operon promoters (36). Analysis of the partially purified extract by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining revealed many proteins (data not shown), indicating that the extract was still relatively crude.

***C. trachomatis* RNAP is transcriptionally active.** The transcriptional activity of the heparin-purified extract was assayed by using the putative promoter region of the *C. trachomatis* rRNA operons. The *C. trachomatis* genome contains two rRNA operons, each encoding the 16S and 23S rRNA genes (12). The 380-bp upstream regions preceding the start of the

16S rRNA gene in the two rRNA operons are identical and presumably contain the promoter and other regulatory elements. Two *in vivo* 5' RNA ends, P1 and P2, are located 183 to 185 and 84 to 85 bp, respectively, upstream of the 16S rRNA gene (12). However, one or both of these ends could represent RNA processing sites of a longer primary transcript. Examination of the rRNA P1 upstream DNA sequences reveals some similarity to the canonical *E. coli*  $\sigma^{70}$  promoter structure, with three-of-six match at each of the  $-10$  and  $-35$  positions separated by a spacing of 16 nucleotides (8, 12). Alternatively, an extended  $-10$  promoter sequence (TGn-TATAAG) with five-of-six match to the canonical *E. coli*  $\sigma^{70}$  promoter sequence can be found, albeit three nucleotides closer to the apparent *in vivo* transcription initiation site.

To test if these putative promoters are active *in vitro*, the region of the 16S rRNA gene that contains P1 and P2 (extending from positions  $-305$  to  $+239$  relative to P1) was cloned into a T7 terminator plasmid (Fig. 1). *In vitro* transcription of this template using the heparin-agarose-purified *C. trachomatis* RNAP produced a 305-nucleotide transcript (data not shown), corresponding to a transcript initiating at P1 (and extending for 239 nucleotides of rRNA sequence and 66 nucleotides of plasmid sequence that precede the T7 terminator). Initiation at P2 would produce a transcript of 203 to 205 nucleotides in length; no such transcript was observed (data not shown).

For all subsequent analyses, a G-less cassette plasmid was used. The G-less cassette is a synthetic transcription template that does not encode any guanosine residues. Thus, in the absence of added GTP and in the presence of the chain terminator 3'-O-methyl-GTP (33), only transcripts that do not contain guanosine residues can accumulate. The only promoters that can be transcribed are those positioned immediately upstream of or within the G-less cassette; adventitious transcription and read-through transcription originating upstream of the promoter being tested are greatly reduced. RNAP pauses at the end of the G-less cassette where there are three encoded guanosine residues; thus, only single-round transcription occurs. Use of the G-less cassette template improved the transcription signal and virtually eliminated background transcripts, greatly increasing the sensitivity of the *in vitro* transcription system.

The heparin-agarose-purified *C. trachomatis* RNAP was used to transcribe the *C. trachomatis* rRNA P1 region ( $-305$  to  $+5$ ) cloned into the test promoter region of the double G-less cassette plasmid (Fig. 1). An example of this transcription using plasmid pMT513 (Fig. 1 and Materials and Methods) is shown (Fig. 2A, lane 1). Two transcription products, corresponding to the test and control transcripts, were produced. No other plasmid-specific transcripts were observed. No transcription was observed from the rRNA P2 region cloned into a G-less cassette plasmid (data not shown).

The precise start site of the rRNA P1-dependent G-less cassette transcript was verified by primer extension analysis. Figure 3 demonstrates that the *in vitro* transcript mapped to the most 5' of the three adjacent RNA ends mapped *in vivo* (12). It should be noted that in the construction of the G-less cassette plasmid, the second of the three *in vivo* transcription initiation sites was changed from a guanosine residue to an adenosine, so that transcription could be performed in the absence of GTP. The effect of this substitution on the selection of the transcription initiation site is unknown.

**Optimal transcription conditions.** Transcription of linearized or nicked DNA templates was decreased compared to that of supercoiled DNA templates (35a). A 75% decrease in transcription was observed with a linearized DNA template. Transcription was not affected by  $Mg^{2+}$  concentration in the range

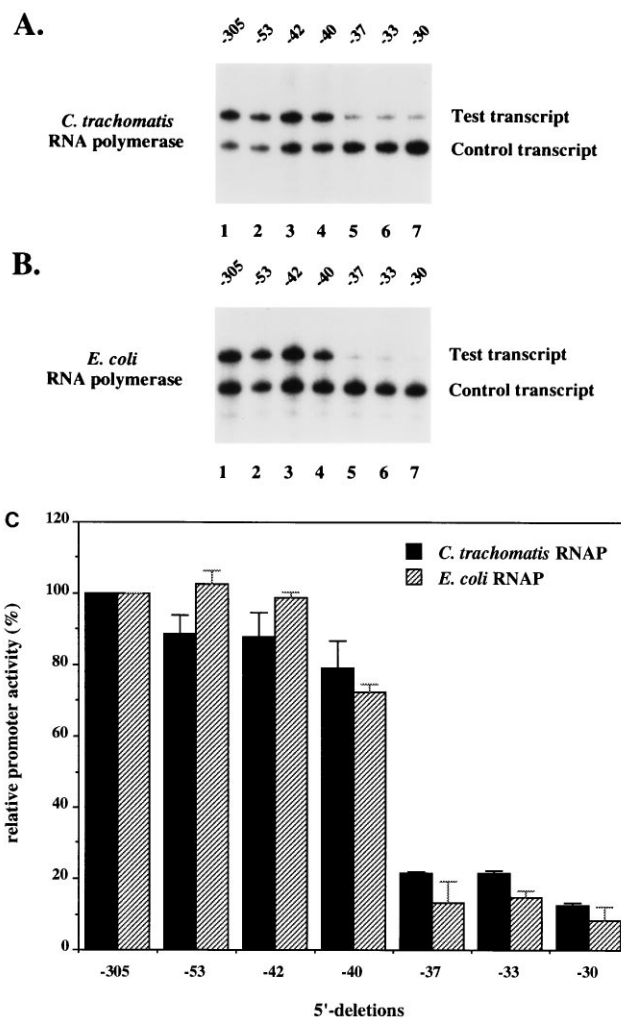


FIG. 2. *In vitro* transcription of 5' nested deletions of rRNA P1. The DNA plasmid for each transcription reaction contains a 5' deletion of rRNA P1 cloned immediately upstream of a test G-less cassette transcription template. The extent of each 5' deletion is indicated. Each plasmid also contains a control promoter template which contains sequences of rRNA P1 ( $+53$  to  $-5$ ) cloned in front of the shorter control G-less cassette. The test and control transcripts are labeled. (A) 5'-deletion analysis with *C. trachomatis* RNAP; (B) 5'-deletion analysis with *E. coli* RNAP; (C) comparison of the relative promoter activities of the 5'-deletion templates as transcribed by *C. trachomatis* RNAP and *E. coli* RNAP. Promoter activity was calculated by comparing the test and control transcript intensities as measured by a PhosphorImager (see Materials and Methods). The relative promoter activity of the  $-305$  deletion template was defined as 100%, and the relative promoter activities of the other 5'-deletions are reported as percentages of this value. The results shown are the averages of three separate experiments.

of 8 to 14 mM. Optimal transcription was observed at a concentration of 1 to 10 mM  $Na^+$  or  $K^+$ . Transcriptional activity of some, but not all, *C. trachomatis* RNAP preparations was inhibited at higher  $Na^+$  or  $K^+$  concentrations. This inhibition appeared to correlate with the presence of an endonuclease activity in the RNAP preparations. An endonuclease activity has been found in RNAP prepared by the high-salt extract method (25). Optimal transcription at lower ionic concentrations is likely explained by the observation that the endonuclease activity is significantly reduced at low ionic strength (35a).

Transcription was equally efficient at 30, 37, and 42°C. Transcription levels at 37°C increased linearly with time up to 30

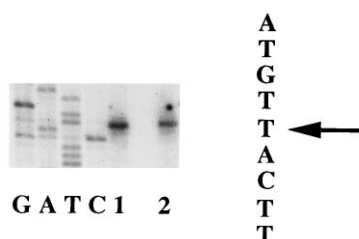


FIG. 3. Primer extension analysis of in vitro transcription products of rRNA P1. Primer Tx27 (complementary to the extreme 3'-end of the test G-less cassette template) was used to map the in vitro transcription initiation site of rRNA P1 cloned upstream of the test G-less cassette template in pMT513. Primer extension products for transcripts produced by *E. coli* RNAP (lane 1) and *C. trachomatis* RNAP (lane 2) were electrophoresed next to a Tx27-primed DNA sequence of pMT513 (marked GATC). The site of in vitro transcription initiation is the same for both *E. coli* and *C. trachomatis* RNAPs and is marked with an arrow.

min, after which they remained at a constant level for up to 60 min. Longer incubation times were not tested.

**The RNAP activity is derived from *C. trachomatis*.** Two criteria were used to demonstrate that the RNAP activity was derived from *C. trachomatis* and not from host eukaryotic cells or from contaminating organisms such as *Mycoplasma* spp. First, a heparin-agarose-purified extract prepared from uninfected HeLa cells showed no transcriptional activity from rRNA P1 (data not shown). Second, the *C. trachomatis* RNAP extract was shown to be inhibited by rifampin at concentrations similar to those which inhibit *E. coli* RNAP (data not shown). This rifampin sensitivity argues against contaminating *Mycoplasma* RNAP activity, as *Mycoplasma* spp. are unique among eubacteria in exhibiting 100-fold greater resistance to rifampin (17). We thus conclude that the transcriptional activity of our RNAP extract is derived from *C. trachomatis*.

**5'-deletional analysis of the *C. trachomatis* rRNA P1 promoter: apparent absence of an UP element.** To further delineate the 5' extent of DNA sequences sufficient for rRNA P1 promoter activity, a series of mutant templates containing nested 5' deletions of the -305-bp rRNA P1 upstream region was constructed. An internal control promoter was included on each plasmid to account for variations in the amounts of supercoiled plasmid and RNAP used. Preliminary experiments using the G-less cassette plasmid demonstrated that sequences from positions -53 to +5 relative to the transcription initiation site (referred to as the -53 deletion template) produced a level of transcription similar to that of sequences from -305 to +5 (the -305 deletion template) as quantified with a PhosphorImager (Fig. 2A; compare lanes 1 and 2). Based on these results, the -53 deletion template was used as the promoter region for the control G-less cassette (illustrated by pMT513 [Fig. 1 and Materials and Methods]). In vitro transcription of a plasmid containing both a test promoter and the

internal control promoter produced two transcripts, a larger test transcript and a shorter control transcript, from which the relative promoter activity of each test promoter template was determined (see Materials and Methods).

Figure 2A shows the results of transcription experiments using 5' deletions of rRNA P1 (described in Table 1). The relative promoter activities of the 5'-deletion templates are shown in Fig. 2C. Compared to the -305 deletion template, the -53 deletion showed a modest decrease in the level of transcription (89%, with the level of -305 deletion normalized to 100%). Levels of transcription of the -53, -42, and -40 deletions were similar to each other, but there was a large decrease in transcription with the -37, -33, and -30 deletion templates. Deletion beyond -27 abolished all transcriptional activity (data not shown). Thus, the 5' extent of the rRNA P1 promoter is likely to be located between positions -37 and -40. The minimal effect on promoter activity seen with deletion of sequences upstream of -40 of *C. trachomatis* rRNA P1 is in marked contrast to the 30-fold decrease in promoter activity seen when the UP element of *E. coli* *rmB* P1 (located within -60 to -40 [Fig. 4]) is deleted (30). These results suggest that *C. trachomatis* rRNA P1 does not contain a stimulatory element upstream of position -40.

***E. coli* RNAP does not require sequences upstream of position -40 for transcription from *C. trachomatis* rRNA P1.** In vitro transcription with *E. coli* RNAP also produced a single prominent transcript from the -305 deletion template (Fig. 2B, lane 1). Primer extension analysis showed that the in vitro transcription initiation sites for *C. trachomatis* and *E. coli* RNAPs are identical (Fig. 3). As seen with *C. trachomatis* RNAP, a similar dramatic decrease in promoter activity between the -40 and -37 promoter templates was also seen for *E. coli* RNAP (Fig. 2B and C). This finding suggests that the two RNAPs recognize a 5' promoter boundary that is in approximately the same location. Of note, the residual promoter activity of the -37, -33, and -30 deletions was greater with *C. trachomatis* RNAP than with *E. coli* RNAP. Since deletion of sequences upstream of -40 had no effect on transcription by *E. coli* RNAP, it is apparent that *E. coli* RNAP does not recognize any UP element upstream of -40 as part of *C. trachomatis* rRNA P1. The UP element is thought to be involved in the elevated expression of particular genes involved in rapid growth. The absence of an UP element in relatively slowly growing chlamydiae would not be inconsistent with this hypothesis. In addition, sequences from -60 to -40 of *C. trachomatis* rRNA P1 do not resemble the sequence of the UP element in *E. coli* (30) or *B. subtilis* (16, 20).

**Deletional analysis of rRNA P1 near the transcription initiation site.** The contribution to promoter activity of DNA sequences immediately upstream of the rRNA P1 initiation site was tested with a series of deletion templates (Table 1). Transcription with *C. trachomatis* RNAP showed significant differences between the wild-type and deletion templates (Fig.

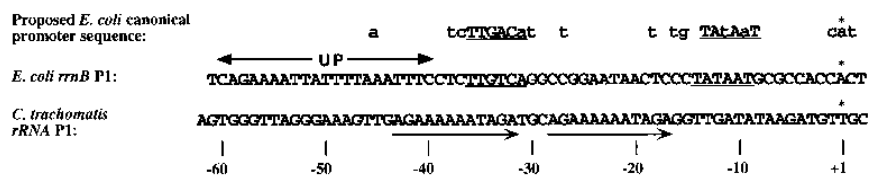


FIG. 4. Comparison of the sequences upstream of selected rRNA operons of *C. trachomatis* and *E. coli*. Sequences of *C. trachomatis* rRNA P1 and *E. coli* *rmB* P1 are aligned at their respective transcription initiation sites (indicated by asterisks). The location of the *E. coli* *rmB* P1 -35 and -10 promoter elements are underlined, and the location of the UP element is indicated. For comparison, the proposed *E. coli* canonical  $\sigma^{70}$  promoter sequence (29) is also shown. The direct repeat sequence in the *C. trachomatis* rRNA P1 region is delineated by two arrows.

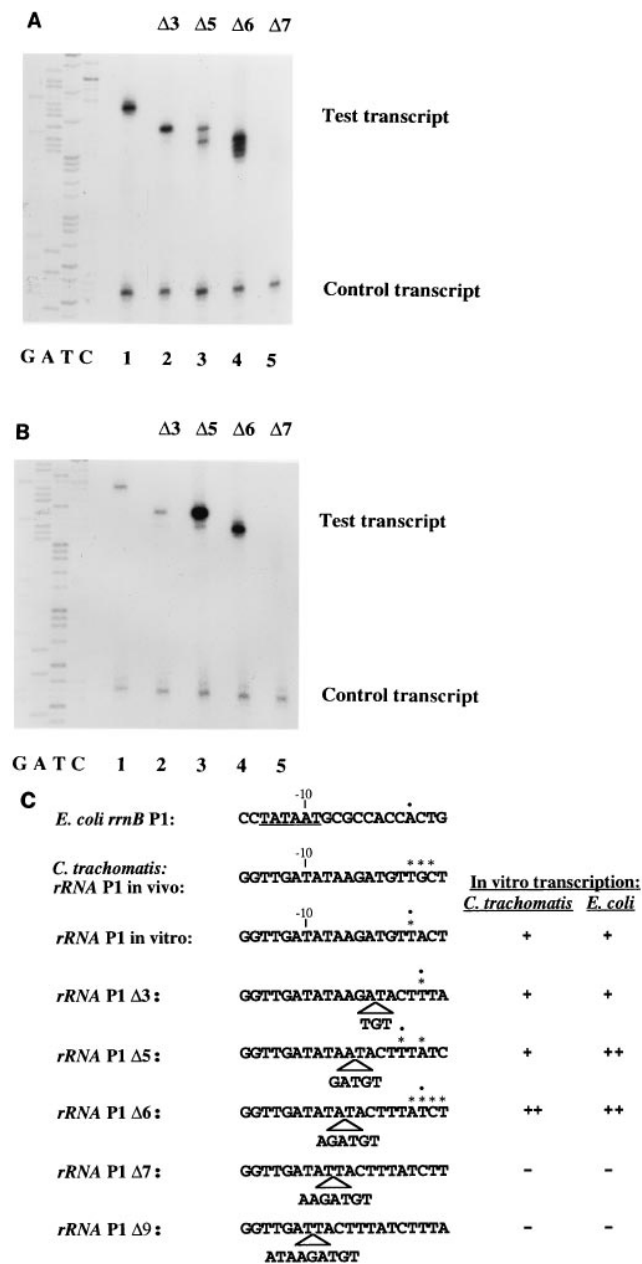


FIG. 5. In vitro transcription of rRNA P1 templates with deletions near the site of transcription initiation. The test template consists of sequences from  $-53$  to  $+5$  (lane 1) or deletions of this region as indicated (lanes 2 to 5) and described in Table 1. The shorter control transcript was transcribed from a control promoter consisting of sequences from  $-53$  to  $+5$  of rRNA P1. (A) Deletion analysis with *C. trachomatis* RNAP; (B) deletion analysis with *E. coli* RNAP; (C) sequences of the deletion templates of rRNA P1. The respective deleted sequences are indicated. The in vitro transcription initiation sites are marked with asterisks for *C. trachomatis* RNAP or dots for *E. coli* RNAP. The levels of in vitro transcription by RNAP from *C. trachomatis* or *E. coli* are also shown for each deletion template.

5A; summarized in Fig. 5C). The  $\Delta 3$  deletion template (deletion of sequences from  $-3$  to  $-1$ ) produced a single test transcript shorter by four nucleotides (lane 2). Transcription of the  $\Delta 5$  and  $\Delta 6$  deletion templates was complex, but in general the approximate transcription initiation position was maintained relative to the upstream promoter sequences. However, the nucleotide sequence in the region of transcription initia-

tion was not preserved in the  $\Delta 5$  and  $\Delta 6$  constructs; we speculate that this accounts for the imprecise initiation sites. The  $\Delta 5$  deletion template (deletion of  $-5$  to  $-1$ ) produced smaller quantities of two transcripts (lane 3) that were four and six nucleotides shorter than that produced from the  $-53$  template. The  $\Delta 6$  deletion template (deletion of  $-6$  to  $-1$ ) produced four prominent test transcripts (lane 4) that were six, seven, eight, and nine nucleotides shorter than the  $-53$  template transcript. The total amount of transcription observed with the  $\Delta 6$  deletion template were consistently higher than for the  $-53$  template.

A sharp decrease in promoter activity was seen for the  $\Delta 7$  (lane 5) and  $\Delta 9$  (data not shown) deletion templates compared to the  $\Delta 6$  template (lane 4). These results suggest that sequences upstream of  $-6$  are required for promoter activity. An alternative explanation that cannot be ruled out is that the failure to see transcription from the  $\Delta 7$  and  $\Delta 9$  deletion templates is due to an unfavorable nucleotide sequence in the region of transcription initiation in these mutant templates.

This deletion analysis was repeated with *E. coli* RNAP (Fig. 5B and C), and a dramatic decrease in promoter activity between the  $\Delta 6$  and  $\Delta 7$  deletion templates was again seen. The  $\Delta 5$  and  $\Delta 6$  deletion templates showed increased transcription levels and a pattern of altered transcript size different from that seen with *C. trachomatis* RNAP. We hypothesize that this result reflects differences in RNAP preference for the initiating nucleotide.

*C. trachomatis* RNAP appears to have a sequence preference for an adenosine or thymidine residue at the site of transcription initiation. The efficiency of transcription initiation from a guanosine residue could not be tested because transcription from the G-less cassette plasmids was carried out in the absence of GTP.

An important caveat to the deletion experiments is that adjacent plasmid sequences may have an effect on promoter activity. That is, it remains possible that one or more of the deletion constructs contain fortuitous promoter sequences comprised of plasmid sequences alone or in combination with native rRNA sequences. Thus, our delineation of the promoter region by deletion analysis to the region  $-37$  to  $-6$  is approximate.

**Multiple base substitution analysis of rRNA P1.** Using the results of the deletion analyses as an approximate guide, we performed a base substitution analysis of the 50-bp region upstream of rRNA P1. This analysis eliminates the potential effect of adjacent plasmid sequences. A series of mutant templates, each containing a substitution of five consecutive base pairs, was constructed in pMT513 (Materials and Methods). A wild-type purine was replaced by the noncomplementary pyrimidine, and a wild-type pyrimidine was replaced by the noncomplementary purine. All other sequences, including flanking sequences from the *C. trachomatis* rRNA P1 upstream region ( $-305$  to  $+5$ ) and plasmid sequences, were identical.

Nine nonoverlapping 5-bp substitution mutants were constructed to scan the region between  $-50$  and  $-6$  relative to rRNA P1 (Table 1). The amounts of test transcript observed for promoter templates containing 5-bp substitutions of  $-40$  to  $-36$ ,  $-35$  to  $-31$ ,  $-30$  to  $-26$ , and  $-25$  to  $-21$  were significantly decreased relative to amounts of the control transcripts (Fig. 6A and C). The amounts of test transcript were also significantly decreased with 5 bp substitutions of  $-15$  to  $-11$  and  $-10$  to  $-6$ . The intervening substitution ( $-20$  to  $-16$ ) showed wild-type promoter activity, suggesting that this 5-bp substitution had no effect on promoter activity. Thus, these mutations define two subregions of the promoter.

This general structure of two separated promoter elements

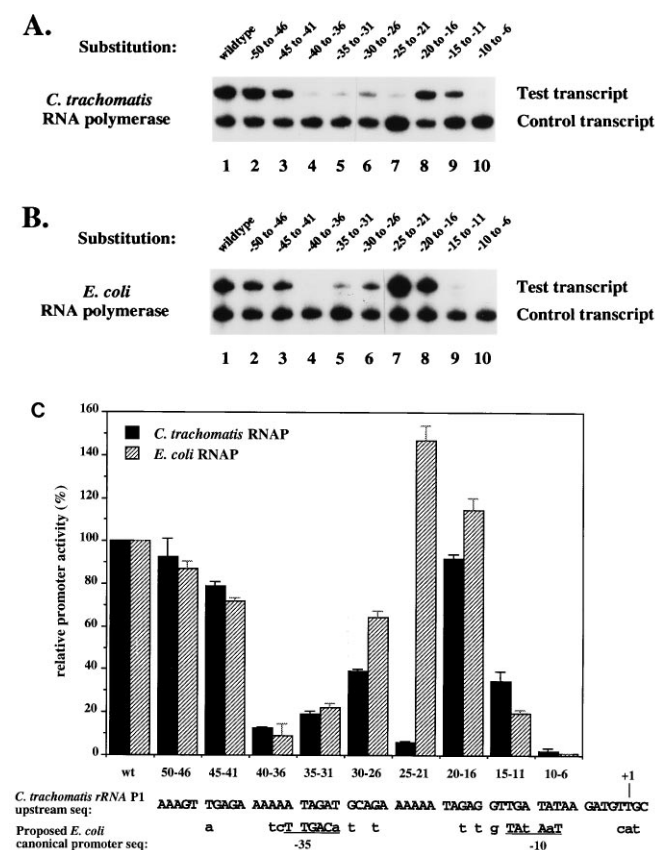


FIG. 6. In vitro transcription of rRNA P1 templates with multiple base substitutions. The test template consists of sequences from  $-305$  to  $+5$  (lane 1) or a 5-bp substitution at  $-50$  to  $-46$  (lane 2),  $-45$  to  $-41$  (lane 3),  $-40$  to  $-36$  (lane 4),  $-35$  to  $-31$  (lane 5),  $-30$  to  $-26$  (lane 6),  $-25$  to  $-21$  (lane 7),  $-20$  to  $-16$  (lane 8),  $-15$  to  $-11$  (lane 9), or  $-10$  to  $-6$  (lane 10) as described in Table 1. The control template contains rRNA P1 sequences from  $-53$  to  $+5$  and produced the shorter control transcript. (A) Multiple base substitution analysis with *C. trachomatis* RNAP; (B) analysis with *E. coli* RNAP; (C) comparison of the relative promoter activity of each base substitution template as transcribed by *C. trachomatis* RNAP and *E. coli* RNAP. The relative promoter activity of the  $-305$  promoter template was defined as 100%, and the relative promoter activities of the base substitution templates are reported as percentages of this value. The sequence of the *C. trachomatis* rRNA P1 region is shown underneath the bar graph, with the wild-type sequence aligned under the bar for each base substitution template. For comparison, the proposed *E. coli* canonical  $\sigma^{70}$  promoter sequence (29) is also shown. The results shown are the averages of three separate experiments.

is analogous to that of many eubacterial promoters, including the *E. coli*  $\sigma^{70}$  promoter. In the latter case, the two promoter elements have been shown to be points of contact with two distinct regions of the  $\sigma$  subunit of RNAP (5). The upstream element of *C. trachomatis* rRNA P1 appears to be more extended than its  $\sigma^{70}$  equivalent. Our results place this upstream element in the approximate region  $-40$  to  $-21$ , but this is an estimate based on the low-level resolution of the 5-bp substitution analysis. Similarly, the downstream element is located within the approximate region  $-15$  to  $-6$ .

***E. coli* RNAP does not recognize the extended portion of the *C. trachomatis* rRNA P1 upstream promoter element.** When the same rRNA P1 base substitution templates were transcribed with *E. coli* RNAP, some similarities as well as an important difference were noted (Fig. 6B and C). Five-base-pair substitutions of  $-40$  to  $-36$ ,  $-35$  to  $-31$ ,  $-15$  to  $-11$ , and  $-10$  to  $-6$  decreased transcription by *E. coli* RNAP. These two regions correspond to the well-described  $-35$  and  $-10$   $\sigma^{70}$

promoter elements, respectively. The promoter activity of substitutions from  $-30$  to  $-26$  and  $-25$  to  $-21$  was not significantly decreased in the reactions using *E. coli* RNAP. This is in sharp distinction to the results seen with *C. trachomatis* RNAP. The contrast is most striking for substitutions of  $-25$  to  $-21$ , which exhibited a modestly increased promoter activity with *E. coli* RNAP but almost no promoter activity with *C. trachomatis* RNAP.

Inspection of the nucleotide sequence of the upstream region reveals the presence of a 12-bp direct repeat spanning the approximate upstream region delineated by the base substitution analysis. The sequence AGAAAAATAGA occurs from  $-43$  to  $-32$  and again from  $-28$  to  $-17$  (Fig. 4 and 6C). Interestingly, the 5-bp CCCCC substitution for the wild-type AAAAA sequences, at  $-40$  to  $-36$  and  $-25$  to  $-21$ , is in the middle of each 12-bp repeat and produced significant decreases in promoter activity at both locations. The effect of the substitution at  $-25$  to  $-21$  is intriguing because the most striking difference in promoter specificity between *C. trachomatis* RNAP and *E. coli* RNAP was seen at this position. To further characterize rRNA P1 and to explore the possible role of the direct repeat sequence, we are currently performing a comprehensive point substitution analysis of the two promoter elements that we have identified. It is possible that the direct repeat represents a DNA binding site for RNAP or other proteins involved in transcription. It is also possible that the high AT content of the direct repeat sequence results in bending of the DNA in this region. The direct repeat sequence bears some resemblance to the *E. coli* UP element (Fig. 4).

These results suggest that the promoter structure of *C. trachomatis* rRNA P1 may differ from the canonical *E. coli*  $\sigma^{70}$  promoter structure. *C. trachomatis* and *E. coli* RNAP also appear to show differences in promoter specificity. However, we note that the *C. trachomatis* RNAP preparation used was not purified to homogeneity. Thus, it is possible that the differences in promoter specificity are due to other *C. trachomatis* proteins present in the extract and thus do not represent an intrinsic feature of the *C. trachomatis* RNAP. It has been reported that no differences can be detected in quantitative transcription assays when highly purified *E. coli* holoenzyme is compared with *E. coli* RNAP purified by the single-step heparin-agarose method (4).

The in vitro transcription system that we have developed to study intrinsic promoter activity is also suitable for studying the regulation of transcription in *C. trachomatis*. *cis*-acting regulatory elements that modify promoter activity can be identified if the necessary transcription factors are also present. Transcription factors may be found in the heparin-agarose-purified RNAP preparation, or they can be added separately in cell extract or purified form. Such an assay system can facilitate the subsequent purification of *C. trachomatis* transcription factors.

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