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Secondary σ factor controls transcription of flagellar and chemotaxis genes in *Escherichia coli*

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ABSTRACT **The genes specifying chemotaxis, motility, and flagellar function in *Escherichia coli* are coordinately regulated and form a large and complex regulon. Despite the importance of these genes in controlling bacterial behavior, little is known of the molecular mechanisms that regulate their expression. We have identified a minor form of *E. coli* RNA polymerase that specifically transcribes several *E. coli* chemotaxis/flagellar genes *in vitro* and is likely to carry out transcription of these genes *in vivo*. The enzyme was purified to near homogeneity based on its ability to initiate transcription of the *E. coli tar* chemotaxis gene at start sites that are used *in vivo*. Specific *tar* transcription activity is associated with a polypeptide of apparent 28-kDa molecular mass that remains bound to the *E. coli* RNA polymerase throughout purification. This peptide behaves as a secondary σ factor—designated σ^F—because it restores specific *tar* transcription activity when added to core RNA polymerase. The σ^F holoenzyme also transcribes the *E. coli tsr* and *flaAI* genes *in vitro* as well as several *Bacillus subtilis* genes that are transcribed specifically by the σ^{28} form of *B. subtilis* RNA polymerase. The latter holoenzyme is implicated in transcription of flagellar and chemotaxis genes in *B. subtilis*. Hence *E. coli* σ^F holoenzyme appears to be analogous to the *B. subtilis* σ^{28} RNA polymerase, both in its promoter specificity and in the nature of the regulon it controls.**

Flagellar, chemotaxis, and motility genes in *Escherichia coli* and *Salmonella typhimurium* are coordinately controlled in a large and complex unit often called the flagellar, chemotaxis, and motility regulon (1–3). Genetic analysis suggests that in *E. coli* these genes fall into five transcriptional subclasses, all dependent on the *flbB/flaI* operon (1, 2, 4). However, little is known about the mechanisms by which gene expression in the regulon is controlled.

A number of flagellar and chemotaxis genes have been sequenced. While the transcriptional start sites of most of such genes have not been characterized, regions just upstream of the coding sequences contain conserved sequences (5–7) that strongly resemble promoters utilized by the σ^{28} holoenzyme from *Bacillus subtilis* (8). This vegetatively active RNA polymerase is implicated in the transcription of chemotaxis and flagellar genes in *B. subtilis* (ref. 9; D. Mirel and M.J.C., unpublished data). In addition, the gene encoding σ^{28} is itself homologous to *flbB* and *flaI* (9). These findings suggested to us that the products of one or both of these genes might be an alternative σ factor responsible for the transcription of *fla/che/mot* genes in *E. coli* (7). This hypothesis led us to search in *E. coli* for an RNA polymerase activity that could efficiently initiate transcription of an *E. coli* chemotaxis gene at transcription start sites used *in vivo*.

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MATERIALS AND METHODS

Strains and Plasmids. *E. coli* strains RP437 (10) and MS4136 (11) and plasmids pWK56 (*tar*; ref. 12), pCK210 (*flaAI*; ref. 13), pMG201 (8), and pJH102T (14) are described in the cited references. Plasmid pPA63, bearing the *E. coli tsr* gene, was provided by Peter Frederikse and Lucille Shapiro. Plasmid pDNA1 bearing the transcriptional start site of the *tar* gene was constructed by insertion of a 305-base-pair (bp) *Hpa* II fragment (from position −230 to +75 with respect to the translational start site of the *E. coli tar* gene) from pWK56 into the *Acc* I site of pUC18 (15), followed by insertion of a 508-bp *Hin*dIII fragment of pKK5-1 (16) containing the T_1 and T_2 ribosomal terminators into the *Hin*dIII site of the pUC18 polylinker 5′ to the *tar* gene fragment.

Assays. S1 nuclease mapping was carried out as described (17). A 348-nucleotide S1 probe was generated by digesting pDNA1 with *Pst* I and *Eco*RI, treating the digest with calf intestinal alkaline phosphatase, and 5′-end-labeling the products with phage T4 polynucleotide kinase and [γ-^{32}P]ATP (18). The single-stranded probe was isolated on a 5% polyacrylamide sequencing gel. *E. coli* RP437 and MS4136 containing plasmid pDNA1 were grown at 30°C in LB medium (18) containing 50 μg of ampicillin per ml. RNA was isolated from logarithmically growing cultures (OD_{600} = 0.7) and purified as described (17), except that subsequent to sonication the cell lysates were extracted once with 50°C phenol and four times with phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol). Maxam–Gilbert sequencing of the S1 nuclease probe was carried out as described (18).

Total RNA polymerase activity was determined by using a phage T7 DNA template as described by Chamberlin *et al.* (19). σ^F RNA polymerase activity was followed by primer extension analysis of *in vitro* products transcribed from pDNA1 DNA. A sample (2 μl) of the enzyme fraction was mixed with 48 μl of transcription buffer (40 mM Tris chloride, pH 8/10 mM 2-mercaptoethanol/10 mM $MgCl_2$/0.1 mg of acetylated bovine serum albumin per ml/40 μg of pDNA1 per ml/1 mM NTP (ATP, UTP, CTP, and GTP) and was incubated at 30°C for 1 min. The reaction was stopped by addition of 1:1 (vol/vol) phenol/chloroform, reextracted with 24:1 (vol/vol) chloroform/isoamyl alcohol, precipitated with ethanol, and dried. A 5′ ^{32}P-labeled DNA primer (5′-GCGAATACCCCCAGGACCATTACC-3′; 100–500 fmol per reaction) complementary to the *tar* gene mRNA was used to detect the presence of unlabeled *tar* mRNA by primer-extension analysis as described (20). Reactions were extracted with phenol/chloroform prior to electrophoresis on 6% polyacrylamide sequencing gels. DNA oligonucleotides were synthesized on a Biosearch 8750 DNA synthesizer and were purified by polyacrylamide gel electrophoresis.

Enzyme Purification. σ^F RNA polymerase was prepared from *E. coli* RP437 by the method of Burgess and Jendrisak (21) from cells grown in LB medium (18) at 30°C to an OD_{600} of 0.8. The peak of RNA polymerase activity eluted from the DNA cellulose column, as determined with phage T7 DNA

template, was pooled, diluted with TGED [10 mM Tris chloride, pH 8/0.1 mM EDTA/0.1 mM dithiothreitol/5% (vol/vol) glycerol] to a conductivity equal to TGED containing 200 mM NaCl, loaded on a phosphocellulose column, and eluted with a linear gradient from 200 mM NaCl to 800 mM NaCl. Fractions were dialyzed into RNA polymerase storage buffer (50 mM Tris chloride, pH 8/50 mM NaCl/1 mM dithiothreitol/10 mM $MgCl_2$/0.1 mM EDTA/50% glycerol) and stored at −20°C.

Core RNA polymerase lacking any σ^{70} was obtained by poly(rC) chromatography (22), and the absence of σ^{70} protein was confirmed by immunoblot analysis using σ^{70} polyclonal antibody.

Enzyme Reconstitution. The reconstitution procedure followed the method of Hager and Burgess (23). σ^F holoenzyme from phosphocellulose fraction 56 (30 μg) was electrophoresed on a 12.5% polyacrylamide/$NaDodSO_4$ gel. A portion of the gel containing size markers and the edge of the preparative track were silver-stained and used as a guide to slice the gel. Each slice was crushed, and proteins were eluted in elution buffer (23) (300 μl) for 1 hr at room temperature. The gel fragments were removed by centrifugation, the proteins in the supernatants were precipitated (30 min at −70°C) with 1.2 ml of acetone, and pellets were washed with 500 μl of 80% acetone/20% 10 mM Tris chloride, pH 8 (vol/vol), and dried. The eluted proteins were denatured by resuspending them in 5 μl of dilution buffer containing 6 M guanidine hydrochloride (23), incubated 15 min at room temperature, diluted 1:50 with dilution buffer (final volume, 250 μl), and allowed to renature at room temperature for 1 hr. Renatured proteins (15 μl or 6% of the total) were added to 0.53 μg of *E. coli* RNA core polymerase, incubated 15 min at 0°C, and assayed by primer-extension analysis for transcriptional activity on the *tar* promoter. The remaining portion of the $NaDodSO_4$/acrylamide gel (shown in Fig. 4*A*) was stained with Coomassie blue.

Transcription and Primer-Extension Reactions. Transcription reactions contained 1.9 μg of *E. coli* σ^{70} holoenzyme (24) and 10 μg of heparin-agarose-purified σ^{28} holoenzyme (14) or 2 μg of phosphocellulose-purified σ^F holoenzyme (fraction 56). Primer-extension reactions were carried out by using the following DNA primers: 5′-GCCAAAAACGGCCAAAACCAGCAG-3′ (*tsr*), 5′-CTGGCACAGGCCGCGAGGGTAATG-3′ (*flaAI*), 5′-CGGACAATTAGCCAGTTCTCCCAT-3′ ($P_{28\text{-}1}$), and 5′-CCATCCCGACGCTCAATGCCACAA-3′ ($P_{28\text{-}2}$). Primer-extension products were electrophoresed on 6% polyacrylamide sequencing gels adjacent to sequencing ladders. Sequencing of the transcription templates was performed by using the primers and a Sequenase dideoxynucleotide sequencing kit (United States Biochemical) according to the manufacturer's instructions for supercoiled template sequencing.

RESULTS

Identification of Transcription Start Sites for the *E. coli tar* Gene *in Vitro* and *in Vivo*. To identify the promoter sequences responsible for transcription of *E. coli* chemotaxis genes, we mapped the transcription start sites for the *tar* gene *in vivo*. Total RNA was isolated from wild-type *E. coli* RP437 and a nonmotile strain, MS4136 (*flaI*$^-$), each of which had been transformed with pDNA1, a plasmid containing the *E. coli tar* gene. RNA was also prepared *in vitro* by transcribing the *tar* gene with the *B. subtilis* σ^{28} polymerase, since we had found that this enzyme can specifically transcribe *E. coli* chemotaxis and flagellar genes (7).

The 5′ ends of the different RNA preparations were analyzed by S1 nuclease mapping with a DNA probe prepared from the plasmid pDNA1. Because the labeled end of the probe originates from vector DNA, only transcripts from the plasmid itself were detected, not transcripts from the chromosomal *tar* gene. *Tar* transcripts were found in RNA from the wild-type *E. coli* strain and from *in vitro* transcription of pDNA1 by σ^{28} RNA polymerase. No *tar* transcripts were detected in the *flaI*$^-$ strain (Fig. 1). Hence, the use of these start sites appears to be physiologically correct, judged by the requirement for the *flaI* gene product (1, 2). The transcripts begin at adenine residues eight and nine nucleotides from the end of the characteristic conserved sequence, a position expected if the conserved 5′ region is a promoter recognized by a σ^{28}-like RNA polymerase.

Identification of an Enzymatic Activity for *tar* Transcription in *E. coli* Extracts. We exploited our identification of the start region for *tar* transcription to detect and measure the corresponding RNA polymerase activity *in vitro*. The method of primer extension was used to identify transcripts starting at the *tar* promoter. This technique can also quantitatively measure the amount of a particular transcript under appropriate conditions (D. Mirel and M.J.C., unpublished data). Extracts of wild-type *E. coli* cells, but not those from a *flaI*$^-$ mutant, contained an activity that initiates transcription of the *tar* gene at the sites used *in vivo* (data not shown). We show below that this activity is due to programming of the core RNA polymerase by a secondary σ factor, which we designate σ^F.

Purification of the σ^F Holoenzyme from *E. coli*. A variety of bacterial RNA polymerases have been purified previously (19, 21, 22) by fractionation with polymin P (BASF) followed by chromatography on DNA-cellulose. Cell extracts from

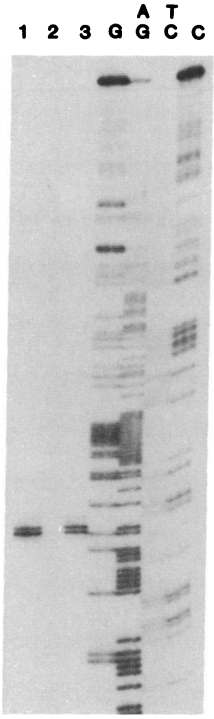

FIG. 1. S1 nuclease mapping of the *tar* gene transcriptional start sites. Lanes: 1, RNA from *E. coli* RP437 (wild type) transformed with pDNA1; 2, RNA isolated from *E. coli* MS4136 (*flaI*$^-$) transformed with pDNA1; 3, RNA produced by *in vitro* transcription of pDNA1 with the *B. subtilis* σ^{28} RNA polymerase. Maxam–Gilbert ladders of the S1 nuclease probe were used to map the start sites; DNA fragments produced by this sequencing technique migrate faster (equivalent to a difference in size of 1–2 nucleotides) than fragments from S1 nuclease digestion.

wild-type *E. coli* were fractionated by this procedure; total cellular RNA polymerase was detected by transcription of phage T7 DNA template (19). The presence of *E. coli* RNA polymerase core subunits was followed by NaDodSO$_4$/polyacrylamide gel electrophoresis. σ^F holoenzyme activity was followed by using the *tar*-specific template pDNA1.

σ^F activity was eluted with a linear NaCl gradient from DNA-cellulose columns in a broad peak that began at about 350 mM NaCl, nearly coincident with total RNA polymerase activity. Chromatography of these fractions on phosphocellulose led to further purification of the σ^F holoenzyme and release of most of the σ^{70} in the preparation. Several other secondary σ factors are known to remain with the holoenzyme during phosphocellulose chromatography (25, 26); σ^F holoenzyme activity was eluted just prior to the peak of core RNA polymerase; the bulk of σ^F activity appeared in fractions 56 and 57 (Fig. 2). The initial σ^F fraction (56) contained proportionately less core RNA polymerase than did later fractions and was used for reconstitution and template-specificity assays. Usually, σ^F activity was eluted as a single peak from phosphocellulose. In this experiment, a smaller "second peak" in fractions 60–62 is probably the tail of the first peak; an irregularity in the salt gradient occurred that is believed to have caused the separation.

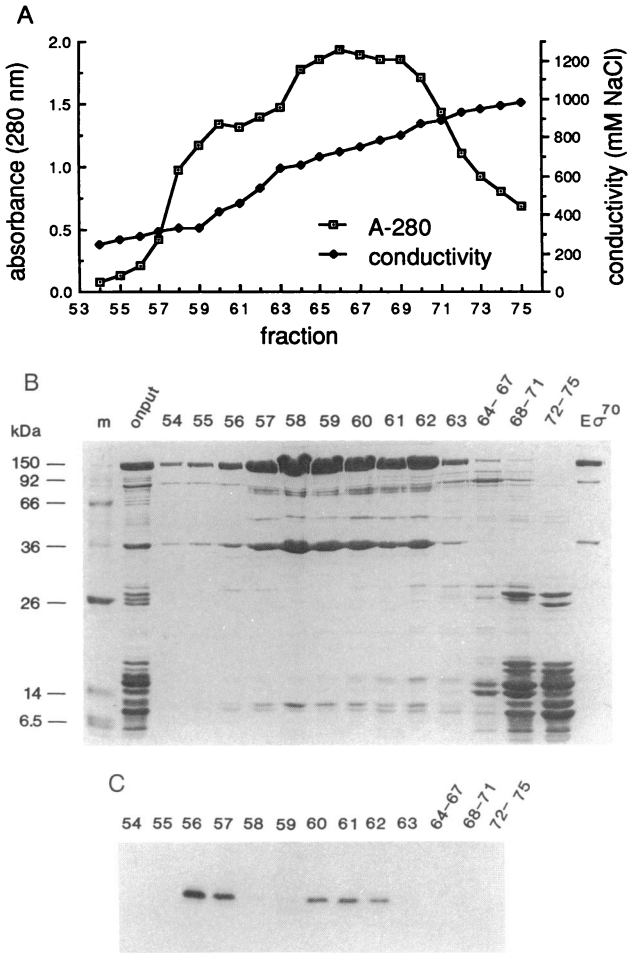

FIG. 2. Phosphocellulose chromatography of the σ^F holoenzyme. (*A*) Proteins eluted from the column in a 200–800 mM NaCl linear gradient. ⊡, A_{280}; ●, conductivity. The column (8.5 × 2.2 cm) was eluted with a 200-ml linear gradient. The preparation began with 155 g of frozen cells. (*B*) NaDodSO$_4$/polyacrylamide gel analysis of column fractions. Lanes: m, markers; onput, 14 μg of pooled fractions from DNA-cellulose column; 54–75, 8 μl of fractions 54–75; Eσ^{70}, 3 μg of purified σ^{70} RNA polymerase. (*C*) σ^F activity present in fractions from phosphocellulose chromatography. Samples (2 μl) were assayed by using pDNA1 as template. mRNA was detected by primer-extension analysis as described.

In the course of several different purifications, a 28-kDa protein was fractionated together with σ^F activity (Fig. 3). This band is clearly seen in fractions 56 and 57 in Fig. 2. The σ^F peak fractions were not free of σ^{70} holoenzyme as judged by protein gels (Fig. 3) and activity assays (not shown); however, the σ^{70} polymerase itself did not use σ^F promoters, and the σ^{70} protein was not required for reconstituting σ^F activity (see below). In addition, σ^F polymerase was not inhibited by σ^{70} antiserum (data not shown).

Reconstitution of σ^F Activity by a 28-kDa Protein. A number of σ factors have been identified by elution and renaturation of specific peptide bands from NaDodSO$_4$/polyacrylamide gels (23, 27–29). Phosphocellulose-purified σ^F holoenzyme was subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis, and peptides eluted from the gel were precipitated with acetone and renatured from guanidine hydrochloride solutions. σ^F activity was detected when proteins from a gel slice containing the 28-kDa protein were added to core RNA polymerase (Fig. 4). The core polymerase used here was prepared by a procedure that eliminates all σ^{70} (22); hence, that factor is not involved in σ^F activity. The σ^F activity was not enhanced or reduced when proteins present in other gel slices were added. However, we estimate that less than 5% of the activity was recovered after renaturation.

Template Specificity of the σ^F RNA Polymerase. The σ^F holoenzyme was purified by its ability to initiate transcription accurately solely at the *tar* promoter. To characterize the enzyme's promoter specificity, we have tested its ability to transcribe other *E. coli* and *B. subtilis* flagellar and chemotaxis genes. The holoenzyme was markedly less active on linear templates than on supercoiled plasmids (data not shown), so runoff transcription reactions could not be used to characterize initiation sites. Instead, primer-extension analysis was used to detect and map 5′ ends of transcripts generated *in vitro* from supercoiled DNA templates containing the *E. coli tsr* and *flaAI* genes and the *B. subtilis* $P_{28\text{-}1}$ and $P_{28\text{-}2}$ promoters. This method also gives a relative measure of the amount of transcription from each template. In each case, the σ^F holoenzyme used the *E. coli* promoters, initiating transcription 7–10 nucleotides 3′ to the −10 region of promoter homologies (Figs. 5 and 6). The σ^F holoenzyme also initiated accurately from the *B. subtilis* $P_{28\text{-}1}$ and $P_{28\text{-}2}$ promoters (Figs. 5 and 6) as well as from the *B. subtilis* flagellin gene promoter (not shown), which is transcribed by σ^{28} polymerase *in vivo* and *in vitro* (D. Mirel and M.J.C., unpublished data). None of the promoters was utilized by the σ^{70} holoenzyme (Fig. 5). These promoters were also transcribed by the *B. subtilis* σ^{28} RNA polymerase; for each promoter, the start site is the same for the σ^{28} and the σ^F holoenzymes. However, the *tsr* promoter, which lacks the first "T" in the −10 region, was transcribed only very weakly by the σ^{28} holoenzyme.

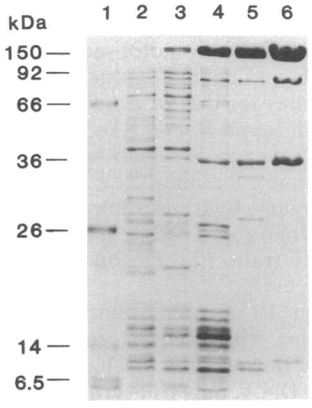

FIG. 3. Purification summary of the σ^F polymerase. Samples were electrophoresed on a NaDodSO$_4$/12.5% polyacrylamide gel and stained with Coomassie blue. Lanes: 1, markers; 2, cell extract; 3, DNA-cellulose onput; 4, pooled fractions from DNA-cellulose column; 5, fraction 56 from phosphocellulose chromatography (10 μg); 6, purified σ^{70} RNA polymerase. The 28-kDa peptide (σ^F) is visible in lane 5.

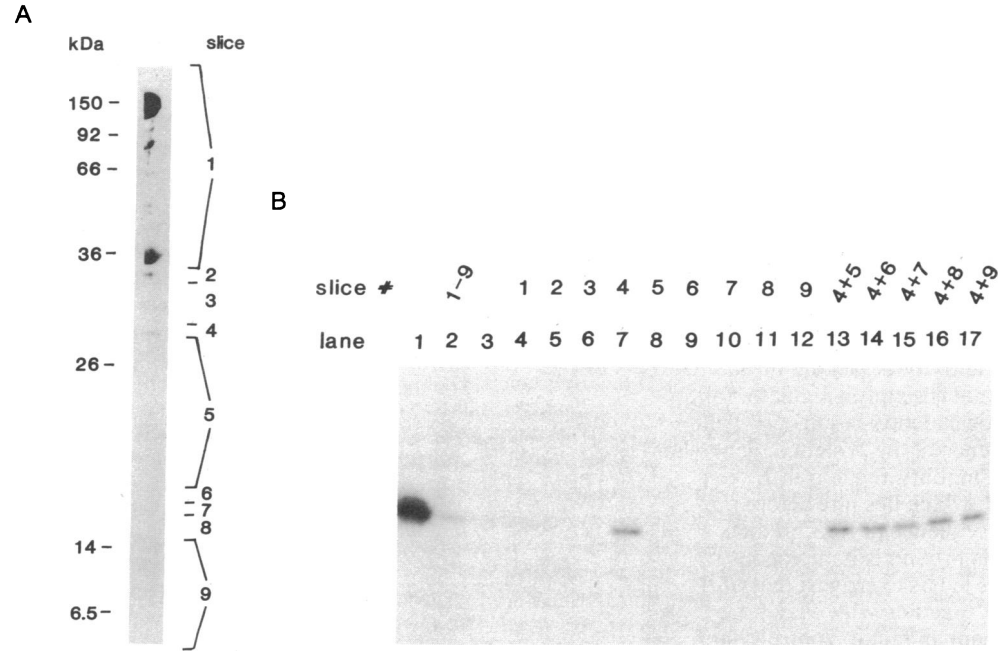

FIG. 4. Reconstitution of the σ^F holoenzyme. (*A*) Preparative $NaDodSO_4$/polyacrylamide gel stained with Coomassie blue showing the edge of the lane from which gel slices were cut. Slice 4 contains the 28-kDa peptide. (*B*) Primer-extension analysis of transcription products. pDNA1 was transcribed by using 1.25 μg σ^F holoenzyme (fraction 56) (lane 1) or 0.53 μg of *E. coli* core RNA polymerase plus proteins renatured from the gel slices indicated.

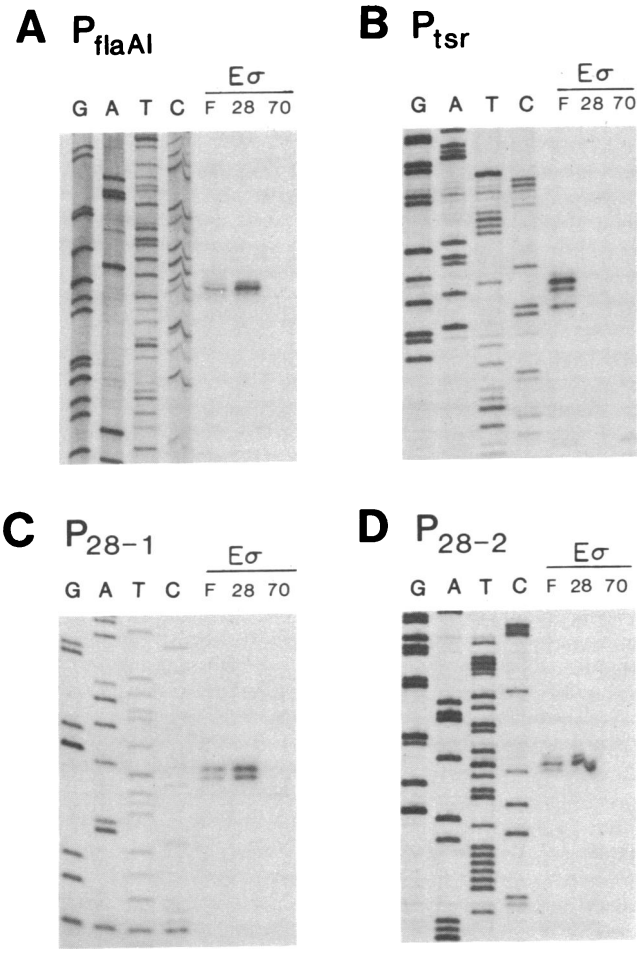

FIG. 5. Transcriptional specificity of the σ^F and σ^{28} RNA polymerases. Supercoiled templates containing the promoters were transcribed by purified σ^F, σ^{28}, or σ^{70} polymerases, and RNA was characterized by primer-extension analysis. (*A*) *E. coli flaAI* promoter. (*B*) *E. coli tsr* promoter. The σ^{28} polymerase lane contains barely visible bands that comigrate with the σ^F products. (*C*) *B. subtilis* $P_{28\text{-}1}$ promoter. (*D*) *B. subtilis* $P_{28\text{-}2}$ promoter.

DISCUSSION

We have purified a form of RNA polymerase from *E. coli* that selectively transcribes several *E. coli* and *B. subtilis* genes involved in flagellar structure and chemotaxis. The most highly purified fractions contain predominantly core RNA polymerase subunits, together with a peptide of 28 kDa. This latter peptide is sufficient to reconstitute transcriptional activity for the *E. coli tar* gene when added to highly purified core RNA polymerase, which lacks the *E. coli* σ^{70} subunit. This 28-kDa peptide behaves as a σ factor that confers a unique transcriptional specificity on the core RNA polymerase; we have designated it σ^F.

The promoters recognized by the σ^F holoenzyme have conserved sequences in the -35 and -10 regions that are distinct from those used by the *E. coli* σ^{70} holoenzyme (30). These sequences are virtually identical to those found for the *B. subtilis* σ^{28} RNA polymerase (8) and to those of the conserved regions identified for *E. coli* flagellar and chemotaxis genes (5–7). The σ^{28} and σ^F holoenzymes each transcribe several *E. coli* and *B. subtilis* promoters having this consensus. In each instance, transcription initiates at sites 7–10 bp from the end of the -10 consensus region; for the one promoter investigated *in vivo*, the start site is the same *in vivo* and *in vitro*. In a prior report, it was suggested that the start site of transcription *in vivo* of the *flaAI* gene lies 8 nucleotides 5′ to the site that we have mapped (31). We do not know the reason for this discrepancy.

The results confirm our earlier suggestion that *E. coli* possesses a secondary σ factor that shares a common pro-

E. coli		
tar	cagcaa TAAA	gtttccccctcctt GCCGATAA cgagatcaactt
flaAI	ttgcgc TCAA	gacgcaggataatta GCCGATAA gcagtagcgaca
tsr	tattca TAAA	gtttttcctttccag GCCGAAAA tcttgcatcggt
B. subtilis		
$P_{28\text{-}1}$	agaagc TAAA	tgattctgtttttat GCCGATAT aatcactagaaa
$P_{28\text{-}2}$	tgtccc TAAA	gttccgggcaccaaa ACCGATAT taaccatagaca

FIG. 6. Transcriptional initiation regions for promoters recognized by σ^F and σ^{28} RNA polymerases. Data are from Figs. 1 and 5.

moter selectivity with the *B. subtilis* σ^{28} factor and controls a homologous regulon in *E. coli* (7). The reconstitution studies and the analysis of promoter sequence specificity of the σ^F holoenzyme establish beyond doubt that this activity is not due to the σ^{70} holoenzyme interacting with some unknown regulatory protein.

The most plausible candidate for the σ^F gene is the *E. coli flaI* gene. This locus has been shown to control synthesis of all motility and flagellar genes in *E. coli* (1, 2), and a related gene (*flaE*; ref. 32) is implicated in regulation in *S. typhimurium*. DNA sequence analysis predicts a size of 22 kDa for FlaI (3, 6); however, some σ factors have $NaDodSO_4$ gel mobilities that are significantly lower than expected from the true molecular mass (33–35). σ^F activity is lacking in *flaI*$^-$ strains, but it is still possible that FlaI is simply a regulator for σ^F activity or synthesis. Unequivocal identification of σ^F will require determination of the sequence of the protein or gene.

Regulation of the flagellar and motility regulon in *E. coli* also involves a second gene, *flbB*, which lies just before *flaI* (6, 11). The expected amino acid sequence of *flbB* predicts a protein of 13.5 kDa (3, 6), and this protein is homologous to the *B. subtilis* σ^{28} subunit (9). This led us to suggest that *flbB* might specify a peptide required together with FlaI for σ^F function. A similar two-component σ factor controls late transcription in *B. subtilis* cells infected with phage SPO1 (36). Our studies to date provide no evidence supporting the idea that σ^F activity depends on the *flbB* gene. Peptides of this size are not required for reconstitution of σ^F activity. However, it cannot be ruled out that the core RNA polymerase used for reconstitution normally contains the FlbB protein as a contaminant. Resolution of the role of the *flbB* gene will depend on studies of σ^F activity in *flbB* mutants and on isolation and study of the FlbB peptide.

The flagellar and motility regulon of *E. coli* is organized in a complex hierarchy (1–3); it appears from our studies that one alternative σ factor, σ^F, is responsible for the transcription of many or all of these genes, including the sensory transducers *tar* and *tsr*. Genes higher up in the hierarchy such as *flaU* control expression of genes near the bottom (1–3), perhaps by acting as transcriptional activators or repressors in concert with σ^F holoenzyme. The molecular mechanisms by which flagellar and motility genes are coordinately regulated can now be studied by using an *in vitro* transcription system with purified σ^F polymerase.

It is interesting that transcriptional regulatory signals for flagellar and chemotaxis genes are so highly conserved between bacteria separated by a large evolutionary distance, such as *E. coli* and *B. subtilis*. One possibility is that these particular functions are so essential to primordial bacterial growth that they were established early in evolution. Since chemotaxis and motility require a large number of different genes, mutations that change all of the promoters of this class of genes and the specificity of the alternative σ factor would be rare.

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