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Isolation and Characterization of the *Bacillus subtilis* σ^{28} Factor

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RNA polymerase preparations isolated from vegetatively growing *Bacillus subtilis* cells contain the core subunits β, β′, and α, together with multiple σ factors and other core-associated polypeptides such as δ, ω_1, and ω_2. We have developed an improved, large-scale purification procedure that yields RNA polymerase fractions enriched in both the σ^{28} and δ proteins. These fractions have been used to isolate σ^{28} protein for biochemical characterization and for preparation of highly specific anti-σ^{28} antisera. The amino acid composition of purified σ^{28} protein and the amino acid sequences of tryptic peptide fragments have been determined. Anti-σ^{28} antisera specifically inhibit transcription by the purified σ^{28}-dependent RNA polymerase, yet do not affect transcription by σ^{43}-dependent RNA polymerase. Immunochemical analysis confirms that the σ^{28} protein copurifies with total RNA polymerase activity through the majority of the purification procedure and allows the steps when σ^{28} protein is lost to be identified and optimized. Immunochemical techniques have also been used to monitor the structure and abundance of the σ^{28} protein in vivo. A single form of antibody-reactive protein was detected by two-dimensional gel electrophoresis-isoelectric focusing. Its abundance corresponds to a maximal content of 220 molecules of σ^{28} per *B. subtilis* cell during late-logarithmic-phase growth.

Vegetatively growing *Bacillus subtilis* cells contain multiple forms of RNA polymerase (36). These forms are distinguished by interaction of the catalytically active core RNA polymerase ($\beta\beta'\alpha_2$) with σ factors, regulatory proteins that determine the promoter specificity of the resulting holoenzymes, as well as accessory proteins such as δ, ω_1, and ω_2, whose functions are less well defined. However, the relative yield of these core-associated polypeptides varies greatly with different purification procedures. The major fraction of RNA polymerase in vegetative cells is associated with σ^{43}, the σ subunit that controls the majority of cellular transcription (36). At least six additional σ factors have been identified through biochemical and genetic analysis. These include the σ^{28} (*sigD*) (45), σ^{37} (*sigB*) (4, 11, 18), σ^{32} (29), σ^{30} (*spo0H*) (7), σ^{29} (*spoIIGB*) (16, 31, 43), and *spoIIAC* (46; P. Stragier, FEBS Lett. **195**:9–11, 1986) factors.

Mutations in the genes for three of these factors have been found to impair sporulation (36, 43, 46). Disruption of the σ^{37} and σ^{28} genes, however, does not impair sporulation (11, 20, 25). Disruption of the gene encoding σ^{28} (*sigD*) prevents flagellar synthesis in *B. subtilis*, suggesting that σ^{28} may control genes involved in flagellar morphogenesis and possibly chemotaxis (20).

The σ^{28} factor is a low-abundance protein, and σ^{28} holoenzyme represents no more than 1 to 2% of the RNA polymerase isolated during typical purification procedures (45; data not shown). This has made it difficult to obtain large amounts of the protein for biochemical analysis. Interfering activities in cell extracts also made it necessary to carry out initial purification steps without quantitative measurement of σ^{28} levels, leading to the possibility of substantial losses during these steps. We have recently developed a sensitive and specific in vitro assay for the σ^{28} RNA polymerase that is applicable even to cell extracts. With this assay, we have developed a revised purification of the σ^{28} holoenzyme and the σ^{28} protein that has greatly facilitated study of the structure and properties of this transcriptional factor.

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MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* W168 was used to purify RNA polymerase. Cells were grown on rich media and harvested as described previously (27).

Assays. We have used three different enzymatic assays for the purification and study of the σ^{28} RNA polymerase. The first (assay 1) is a general measure of the total DNA-dependent RNA polymerase activity (primarily σ^{43} holoenzyme). The second (assay 2) is a highly specific and quantitative measure of the amount of active σ^{28} holoenzyme in a fraction. The third (assay 3) is a semiquantitative measure of σ^{28} activity in fractions lacking σ^{43} holoenzyme. All of these assays were performed with reaction mixtures (50 μl) containing 40 mM Tris (pH 8.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 400 μM nucleoside triphosphates. Enzyme and template were added as indicated, and the reaction mixtures were incubated at 37°C for either 6 or 10 min. One unit of enzyme activity was defined as the amount of activity required to give a rate of incorporation of 1 nmol of [α-^{32}P]CMP per h into acid-precipitable product.

(i) Assay 1. To measure the total RNA polymerase activity in a given sample, incorporation of [α-^{32}P]CMP into acid-insoluble material was measured with purified φ29 DNA as template (100 μg/ml). Transcription reaction mixtures were incubated for 6 min at 37°C, the reactions were stopped by addition of 200 μl of YEP (0.5 mg of yeast RNA per ml, 50 mM EDTA, 50 mM sodium PP_i), and the product was precipitated with 2 ml of 4% trichloroacetic acid and collected on GF/C filters (Whatman, Inc., Clifton, N.J.) as described previously (8). Total acid-precipitable radioactivity was determined by liquid scintillation counting. Since DNA isolated from φ29 is a poor template for the σ^{28} holoenzyme and contains strong σ^{43} promoters (12), this assay is biased in favor of the predominant σ^{43} holoenzyme.

(ii) Assay 2. To specifically measure the σ^{28} holoenzyme during early fractionation steps, we have developed a sensitive S1 nuclease protection assay. In vitro transcription reactions involve the use of a supercoiled plasmid template, pJH102T (20 μg/ml). This template contains the strong σ^{28}-dependent promoter $P_{28\text{-}1}$ (12) on a *Hpa*II fragment cloned into the polylinker *Acc*I site of the plasmid pUC8 (37). Transcription initiation within this *B. subtilis* insert is dependent on the σ^{28} holoenzyme, and transcription proceeds toward the *Bam*HI site in the polylinker. Two strong transcription terminators (*E. coli rrnB* T_1 and T_2) have been cloned as a 500-base-pair *Hin*dIII fragment (from plasmid pKK5-1 [5]) upstream of this insert (in the polylinker *Hin*dIII site) to protect this region from readthrough transcription initiated at plasmid promoters (by the σ^{43} holoenzyme, for example). The S1 hybridization probe is end labeled at the *Bam*HI site in the pUC8 polylinker; therefore, this assay is insensitive to both endogenous cellular RNA and chromosomal DNA present in early steps of the fractionation.

To measure σ^{28} RNA polymerase activity, a transcription reaction mixture (50 μl) was incubated at 37°C for 6 min, the reactions were stopped by addition of 50 μl of stop mix (1.5 M ammonium acetate, 40 mM EDTA, 50 μg of tRNA per ml), and nucleic acids were isolated by two or three phenol-SEVAG (1:1, vol/vol) extractions (SEVAG is chloroform-isoamyl alcohol [24:1, vol/vol]). End-labeled single-stranded DNA probe (10,000 to 50,000 cpm) was added, and the recovered RNA was quantitated by S1-nuclease protection and autoradiography as described previously (13).

(iii) Assay 3. To monitor the partitioning of holoenzyme forms during heparin-agarose chromatography, parallel transcription reactions were performed with plasmids pMG201 (20 μg/ml) and pBR322 (20 μg/ml) as templates. RNA was labeled with [α-^{32}P]CMP and quantitated as described for assay 1. Plasmid pMG201 contains the strong σ^{28} promoter $P_{28\text{-}2}$ cloned into pBR322 (12). The σ^{43} holoenzyme uses both templates equally, whereas the σ^{28} holoenzyme is nearly 10 times as active on the template containing the σ^{28} promoter (pMG201).

Purification of *B. subtilis* RNA polymerase. (i) Protein determinations. Protein determinations were performed by using the Bio-Rad protein assay kit (BioRad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard.

(ii) Cell extract. *B. subtilis* W168 cells (500 to 600 g) were homogenized in 2 liters of lysis buffer (50 mM Tris [pH 8.0], 10 mM $MgCl_2$, 2 mM EDTA, 0.1 mM dithiothreitol, 1 mM 2-mercaptoethanol, 233 mM NaCl, 10% [vol/vol] glycerol) and lysed by two passages through a Manton-Gaulin homogenizer at approximately 10,000 lb/in^2. Phenylmethylsulfonyl fluoride (100 mM in absolute ethanol) was added between passages to a final concentration of 1 mM. Following lysis, 2 liters of TGMED (10 mM Tris [pH 8.0], 10% [vol/vol] glycerol, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol)–0.2 M NaCl was added, and the lysate was cleared by centrifugation at 10,000 × *g* for 45 min.

(iii) Polymin P fractionation. The 10,000 × *g* supernatant fraction (ca. 3.5 liters) was mixed with a 10% polyethyleneimine solution (Polymin P; BASF) to a final concentration of 0.5% over a period of 10 to 20 min. The solution was stirred for 30 min at 4°C, and the precipitate was collected by centrifugation for 15 min at 10,000 × *g*. The pellet was washed by resuspension in 1.6 liters of TMED (10 mM Tris [pH 8.0], 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol)–0.4 M ammonium chloride, and the centrifugation was repeated. The supernatant fraction is the 0.4 M wash. The pellet was suspended in 1.0 liter of TMED–1.0 M ammonium chloride, incubated for 5 min at 4°C, and centrifuged again. The supernatant fraction is the 1.0 M eluate.

(iv) Ammonium sulfate fractionation. The 1.0 M eluate was precipitated by addition of solid ammonium sulfate (0.40 g/ml of eluate) while the pH was maintained at 7 to 8 by addition of 5 N ammonium hydroxide. After the mixture had been stirred at 4°C for 30 min, the precipitate was collected by centrifugation at 16,000 × *g* for 1 to 2 h. The ammonium sulfate pellet was suspended in a minimal volume (ca. 150 ml) of TGED (10 mM Tris [pH 8.0], 10% [vol/vol] glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol)–0.05 M NaCl and desalted by passage over a 600-ml P-6 desalting column (48 by 4 cm; Bio-Rad) previously equilibrated in the same buffer.

(v) DNA-cellulose chromatography. Protein-containing fractions from the P-6 column were pooled and applied to a 500-ml DNA-cellulose column (40 by 4 cm) (1) equilibrated in TGED–0.05 M NaCl. After the column had been washed overnight with 1 liter of the same buffer, the polymerase was eluted with a 1.5-liter gradient from 0.05 to 1.0 M NaCl in TGED. $MgCl_2$ was added to a final concentration of 10 mM to all fractions prior to assay. Fractions containing RNA polymerase activity (assay 1) were pooled and precipitated with ammonium sulfate (0.42 g/ml).

(vi) A5M sizing chromatography. The ammonium sulfate pellet was suspended in a minimal volume (ca. 10 ml) of TGMED–0.5 M NaCl. This material was chromatographed on a 500-ml Bio-Gel A5M column (110 by 2.5 cm; Bio-Rad) equilibrated in the same buffer at a flow rate of ca. 25 ml/h. Active fractions (assay 1) were dialyzed into storage buffer (TMED, 50% [vol/vol] glycerol, 100 mM NaCl) and stored at −20°C.

(vii) Heparin-agarose chromatography. To prepare σ^{28}-containing RNA polymerase free from contaminating σ^{43}, A5M purified RNA polymerase was chromatographed on heparin-agarose as described previously (45). The peak eluting at low salt concentrations contained core RNA polymerase and σ^{28} holoenzyme (heparin-agarose peak I). The peak eluting at higher salt concentrations contained the σ^{43} holoenzyme. These fractions were assayed by assay 3, and the presence of σ^{28} in heparin-agarose peak I was confirmed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

Purification and characterization of σ^{28} protein. (i) Isolation of σ^{28}. σ^{28} protein was isolated from A5M- or heparin-agarose-purified RNA polymerase by preparative gel electrophoresis. RNA polymerase was electrophoresed on 1.5-mm-thick 12.5% polyacrylamide gels, and protein bands were visualized by staining with 0.05% Coomassie brilliant blue–7.5% acetic acid–30% methanol and destaining with 45% methanol–7.5% acetic acid. After electrophoretic elution (23), the isolated protein was lyophilized and washed two or three times with acetone-triethylamine-acetic acid (90:5:5, vol/vol) as described previously (33). The acetone pellet was dried under vacuum, suspended in 50 mM ammonium bicarbonate, and stored at −20°C. The isolated protein (gel-purified σ^{28}) was analyzed by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) followed by silver staining (38).

(ii) Amino acid composition analysis. The amino acid composition of gel-purified σ^{28} was determined by complete hydrolysis of 2 μg of gel-purified σ^{28} in 6 N HCl for 24 h at 110°C followed by high-pressure liquid chromatographic analysis (Pico-Tag method; Waters Associates, Inc., Milford, Mass.) of the resulting amino acids after derivatization with phenylisothiocyanate (3). For one sample (trial 2), the protein was reduced and carboxymethylated prior to hydrol-

ysis to recover any cysteine residues as carboxymethylcysteine (19).

(iii) Tryptic peptide sequencing. Gel-purified σ^{28} (20 μg) was digested for 18 h at 37°C with 2 μg of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-trypsin in 0.2 M Tris (pH 8.25) containing 2 M urea and 0.1 mM $CaCl_2$. Tryptic fragments were purified by reverse-phase high-pressure liquid chromatography on a C-18 column (VYDAC, Hesperia, Calif.) with a linear gradient of 0 to 60% 1-propanol in 0.1% trifluoroacetic acid, and the eluate was monitored with a fluorescamine detector (41). The amino acid sequence of selected fractions was determined by microsequencing (21) on a Sequenator (no. 470A; Applied Biosystems, Foster City, Calif.), and the phenylthiohydantoin-amino acids were identified by using a PTH Analyzer (no. 120A; Applied Biosystems).

Immunological techniques. (i) Antibody production. Four female BALB/c mice were immunized by subcutaneous injection with 5 μg each of gel-purified σ^{28} emulsified in Freund complete adjuvant. Mice were boosted at 2- to 3-week intervals with 2 to 3 μg of σ^{28} emulsified in Freund incomplete adjuvant for a total of three to five times. Serum samples were analyzed for anti-σ^{28} antibodies by Western immunoblot analysis against A5M- or heparin-agarose-purified RNA polymerase fractions. Rabbit antisera were prepared by similar techniques, except that rabbits received initial injections of 50 μg and were boosted with 10 μg of σ^{28} at 1-month intervals.

(ii) Preparation of IgG fractions. To partially purify immunoglobulin G (IgG) from rabbit sera, proteins were precipitated with ammonium sulfate added to 50% saturation, dialyzed against 30 mM sodium phosphate (pH 7.2), and passed over a DE-52 column equilibrated in the same buffer (30). The flowthrough fractions were collected, precipitated with ammonium sulfate (50% saturation), and suspended in 80% of the original volume of 10 mM Tris (pH 8.0)–100 mM NaCl.

(iii) Immunochemical analysis. Immunochemical analysis was performed essentially as described previously (20). Anti-σ^{28} antisera were added at a dilution of 1:1,000 in 10 mM Tris (pH 8.0)–100 mM NaCl–1% (wt/vol) bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.). For experiments with mouse antisera, rabbit anti-mouse IgG (final concentration, 1.6 μg/ml) was used as a secondary antibody. Immunoblots were then incubated with horseradish peroxidase-coupled goat anti-rabbit IgG and visualized with 4-chloro-1-naphthol as substrate.

(iv) Two-dimensional immunoblots. Two-dimensional gel electrophoresis was performed as described (40). Immunoblots of two-dimensional gels were performed as described for one-dimensional immunoblots. The pH gradient was measured after isoelectric focusing by using blank tube gels sliced into 0.5-cm slices and equilibrated in boiled, deionized, distilled water.

RESULTS

Purification of *B. subtilis* σ^{28} RNA polymerase. The σ^{28} factor is a low-abundance protein in *B. subtilis*. Therefore, we found it necessary to develop a purification procedure that was both reproducible and applicable to large quantities of bacterial cells. This revised procedure has provided purified σ^{28} RNA polymerase and σ^{28} factor in quantities sufficient for biochemical characterization and antibody production.

To monitor the σ^{28} holoenzyme during purification, we used a transcriptional assay (assay 2) that is highly specific for the σ^{28} RNA polymerase and accurate even for cell extracts. This assay monitors transcripts initiated at a cloned σ^{28} promoter on a supercoiled plasmid template. Because of the sensitivity and specificity of the S1 nuclease protection procedure (13), we can monitor σ^{28} RNA polymerase at very early stages of the fractionation. This has allowed modifications to be introduced into the purification procedure without compromising the recovery of σ^{28} RNA polymerase activity.

The purification procedure we have devised is a modification of our earlier methods (27, 45). Specific modifications include the use of ammonium chloride for elution of RNA polymerase from polymin P, as suggested by Zillig et al. in their original description of polyethyleneimine precipitation (47); the omission of glycerol from the wash and elution buffers during polymin P fractionation; and rapid desalting of the ammonium sulfate pellet by column chromatography prior to DNA-cellulose chromatography. These changes promote efficient and reproducible concentration of the proteins eluted from Polymin P by ammonium sulfate precipitation and improve recoveries of total RNA polymerase activity.

This revised procedure typically yields 80 to 100 mg of total RNA polymerase protein (A5M fraction) from 500 g of cell paste (Table 1): a yield of 40 to 50% of the total RNA polymerase protein in the cell (9). This enzyme contains only 0.01 to 0.02 equivalent of σ^{28} protein. Although this procedure was developed with a σ^{28}-specific assay (assay 2), we routinely use a less specific, but more convenient general RNA polymerase assay (assay 1) to monitor activity during purification. The results of these assays are summarized in Table 1. A Coomassie blue-stained gel of fractions from a typical preparation is shown in Fig. 1A, and the σ^{28} protein is indicated by the arrowhead.

The availability of anti-σ^{28} sera (see below) has allowed us to monitor directly the partitioning of both free σ^{28} polypeptide and σ^{28} holoenzyme during these fractionation steps. An immunoblot analysis of a gel identical to that shown in Fig. 1A is shown in Fig. 1B. The 28-kilodalton band indicated by the arrow is the σ^{28} protein (see below). This protein cofractionates with the pooled RNA polymerase activity through the majority of the steps in the purification. However, significant quantities of σ^{28} protein appear to be lost during Polymin P fractionation (Fig. 1B, lane 2).

Using quantitative immunoblots, we estimated the loss of σ^{28} protein during polymin P fractionation to be 25% of the total σ^{28} protein present in the low-speed supernatant frac-

TABLE 1. Purification of RNA polymerase from *B. subtilis* W168[a]

Fraction	Vol (ml)	Amt of protein (mg/ml)	RNA polymerase activity (U/mg)[b]
10,000 × *g* supernatant	3,820	10.5	57
Polymin P supernatant	3,800	6.9	ND[c]
0.4 M wash	1,600	2.4	5.8
1.0 M eluate	1,040	6.0	56
P-6 pool	190	26.4	644
DNA-cellulose flowthrough	306	15.6	5.4
DNA-cellulose pool	220	1.0	440
A5M pool	21	5.2	788

[a] A total of 586 g (wet weight) of *B. subtilis* cells was used.
[b] Activities were determined by using assay 1 as described in Materials and Methods.
[c] ND, Not determined.

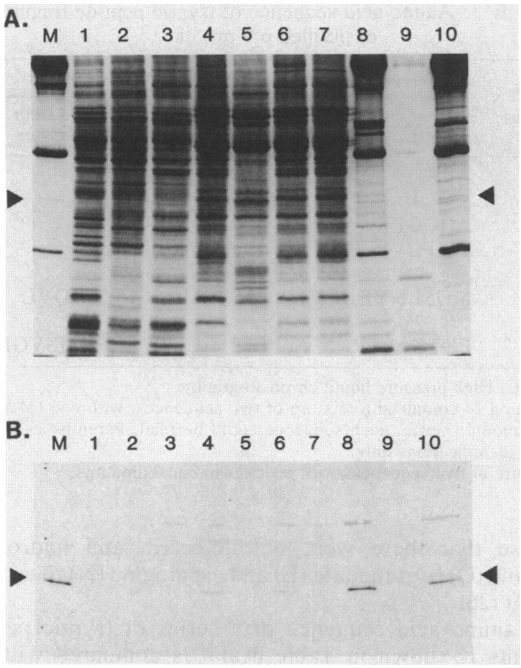

FIG. 1. Purification of *B. subtilis* W168 RNA polymerase. (A) Coomassie blue-stained summary gel. Samples were electrophoresed on an SDS-12.5% polyacrylamide gel. Lanes: M, heparin-agarose peak I RNA polymerase; 1, 2 μl (21 μg) of 10,000 × *g* supernatant fraction; 2, 2 μl (13.8 μg) of Polymin P supernatant fraction; 3, 10 μl (24 μg) of 0.4 M wash; 4, 5 μl (30 μg) of 1.0 M eluate; 5, 20 μl (30 μg) of ammonium sulfate supernatant; 6, 1 μl (26.4 μg) of P-6 pool; 7, 1.5 μl (23.4 μg) of DNA-cellulose flow-through; 8, 20 μl (20 μg) of DNA-cellulose pool; 9, 20 μl (2.8 μg) of ammonium sulfate supernatant; 10, 10 μl (52 μg) of dialyzed A5M fraction. (B) Immunoblot of gel identical to that in panel A with mouse anti-σ^{28} antiserum. The arrowheads indicate the σ^{28} band.

tion (data not shown). In contrast, more than 90% of the β, β′, and σ^{43} subunits were precipitated by Polymin P. We have not been able to modify the Polymin P step to avoid this loss of σ^{28} protein, nor can we explain why this portion of the σ^{28} protein does not remain bound to core RNA polymerase. The levels of Polymin P that remain in the Polymin P supernatant fraction inhibit all transcription assays and preclude direct determination of whether this σ^{28} protein is active.

The σ^{28} holoenzyme elutes near the σ^{43} holoenzyme from both the DNA-cellulose and A5M columns. Figure 2A shows column fractions from a DNA-cellulose column assayed by silver staining, and proteins reactive with mouse anti-σ^{28} antiserum are visualized in the immunoblot shown in Fig. 2B. With this mouse antiserum, some cross-reaction with the σ^{43} factor is observed. Although the σ^{28} holoenzyme eluted slightly ahead of the σ^{43} holoenzyme, all fractions containing σ^{28} protein were included in the activity pool when ϕ29 template DNA (assay 1) was used to monitor RNA polymerase activity. Similarly, the σ^{28} holoenzyme peak is coincident with the σ^{43} peak during A5M chromatography (data not shown). These two holoenzyme forms are resolved during heparin-agarose chromatography as previously described (45).

Isolation and biochemical characterization of σ^{28} protein. Initially, we sought to purify the σ^{28} factor away from other polymerase subunits by using phosphocellulose chromatography (6, 17). However, unlike *Escherichia coli* σ^{70} (6) and *B. subtilis* σ^{43} and δ proteins (17), σ^{28} is not released from core polymerase during chromatography on phosphocellulose under a variety of conditions. Similar results have been reported for σ^{37} (17), σ^{29} (16), and other bacterial σ factors (e.g., *Pseudomonas*, *Micrococcus*, *Calulobacter*, and *Agrobacterium* σ factors [2, 28, 32, 35]). The σ^{28} protein can be eluted from a phosphocellulose column by using buffers containing 4 M urea, but the core subunits also dissociate and α is released from the column along with σ^{28}. Since we were unable to separate σ^{28} from core polymerase under nondenaturing conditions, we chose to use preparative gel electrophoresis after denaturation of RNA polymerase with SDS.

The σ^{28} protein was recovered from preparative SDS-polyacrylamide gels by electrophoretic elution (23). In a typical preparation, 25 mg of heparin-agarose-purified enzyme yielded approximately 40 μg of pure σ^{28} protein. A silver-stained gel of the resulting σ^{28} fraction is shown in Fig. 3. The upward smearing of the purified σ^{28} factor during gel electrophoresis is probably due to modification of the protein during gel purification and electrophoretic elution, since it occurs in σ^{28} fractions isolated from enzyme fractions with no visible contaminants in this region of the gel (Fig. 3, lane 1). We estimate that the overall purification of σ^{28} is about 800-fold with respect to total starting protein for this step.

Using this procedure, we have isolated over 400 μg of σ^{28} protein from nearly 3 kg of cell paste. To confirm the identity of the isolated protein, a sample was renatured from 6 M guanidine hydrochloride (15, 45) and assayed for its ability to form σ^{28} holoenzyme when mixed with purified core RNA polymerase (assay 2). The presence of σ^{28}-specific transcripts in the reconstituted reaction mixture demonstrates

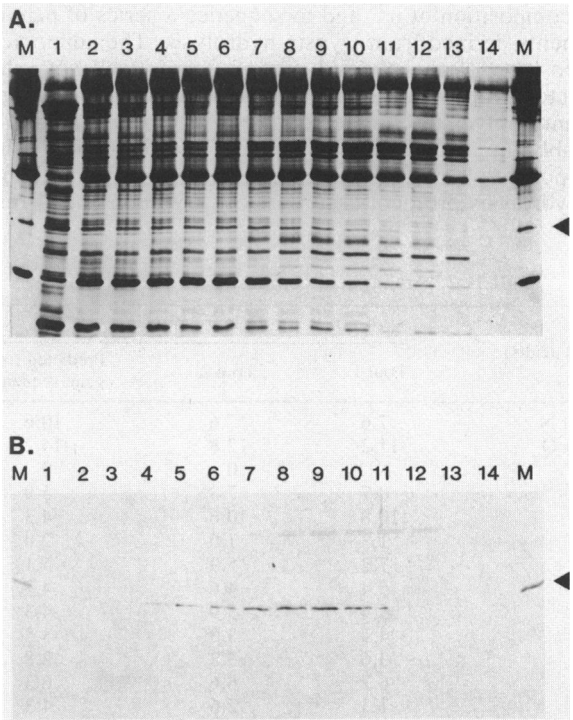

FIG. 2. DNA-cellulose chromatography of RNA polymerase. (A) Silver-stained SDS-polyacrylamide gel electrophoresis analysis of fractions from DNA-cellulose chromatography. The marker lanes (M) contain heparin-agarose peak I RNA polymerase. (B) Immunoblot of gel identical to that in panel A with mouse anti-σ^{28} antiserum. The arrowheads indicate the σ^{28} band.

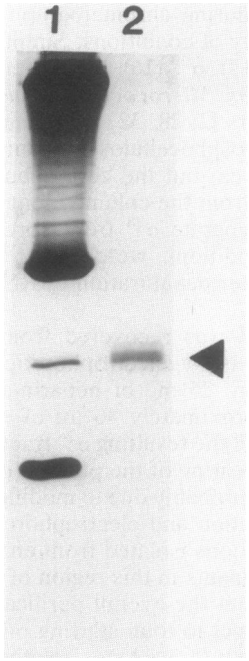

FIG. 3. Gel purification of σ^{28}. Lanes: 1, 15 μg of heparin-agarose peak I (starting material for σ^{28} isolation); 2, Gel-purified σ^{28} protein.

that σ^{28} can be renatured following gel purification and electrophoretic concentration (data not shown).

Purified σ^{28} protein has been used to determine the amino acid composition of σ^{28} and to sequence a series of peptide fragments derived from trypsin hydrolysis. The amino acid composition of the purified protein agrees well with that predicted from the gene sequence (Table 2). The larger amount of glycine (G) detected in the gel-purified protein is probably the result of the use of Tris-glycine buffers during SDS-polyacrylamide gel electrophoresis (23). In addition, the hydrolysis protocol used destroys tryptophan (W) residues, so that these were not detected, and hydrolyzes glutamine (Q) to glutamate (E) and asparagine (N) to aspartic acid (D) (26).

TABLE 2. Amino acid composition of σ^{28} protein

Amino acid(s)	% in σ^{28} protein		
	Trial 1	Trial 2	Predicted from gene sequence
D+N	7.6	7.6	10.6
E+Q	11.2	12.8	14.2
C	ND[a]	0	0
S	8.7	7.3	5.9
G	10.8	10.6	4.3
H	1.3	1.0	2.0
R	7.2	5.9	5.1
T	5.4	4.6	4.3
A	5.4	5.9	4.3
P	4.7	3.3	3.5
Y	3.6	3.3	2.8
V	6.7	6.6	6.3
M	1.3	2.6	4.3
I	5.4	6.6	6.3
L	10.8	11.2	13.0
F	3.4	4.0	2.4
K	6.7	6.6	9.1
W	ND	ND	1.6

[a] ND, Not determined.

TABLE 3. Amino acid sequence of tryptic peptide fragments of purified σ^{28} protein

HPLC[a] fraction no.	Amino acid sequence	
	Found	Predicted from gene sequence
20	ALFK	ALFK
24,1°	AIIDGLR	GAIIDGLR
24,2°	YMPLV(E)(Y)[b]	YMPLVTY
28	MQSLNYEDQVL	MQSLNYEDQVL
22	FDTGAPFR, ISVYLSK[c]	FDTYASFR, ISVGLPK[c]

[a] HPLC, High-pressure liquid chromatography.
[b] Fraction 24 contained a mixture of two sequences, with one (24,1°) in a greater amount; hence, each sequence could be read. Parentheses indicate tentative identifications only.
[c] Mixture of two sequences with residues in equal amounts.

The amino acid sequence of a series of tryptic peptide fragments is shown in Table 3 and is compared with the sequences predicted from the known gene sequence. All of the sequenced tryptic peptides are predicted from the inferred amino acid sequence of the σ^{28} protein (20). One discrepancy is the absence of a glycine in position 1 of the tryptic peptide sequenced in fraction 24. The reason for this discrepancy is not known. The sequence of tryptic peptide 28 has been used to predict an oligonucleotide probe specific for the σ^{28} structural gene (20).

Preparation of anti-σ^{28} antisera. Isolated σ^{28} protein has been used to produce polyclonal antisera in both mice and rabbits. To demonstrate that the antisera are directed against the σ^{28} protein, we determined the effect of purified rabbit IgG fractions on in vitro transcription reactions. The immune IgG fraction, but not the preimmune fraction, specifically inhibits σ^{28}-directed in vitro transcription of the σ^{28} promoter-containing plasmid, pJH102T (Fig. 4). Neither IgG fraction inhibits transcription of the same template by the σ^{43} holoenzyme (data not shown). The lack of effect with σ^{43} RNA polymerase probably reflects the low level of anti-σ^{43}

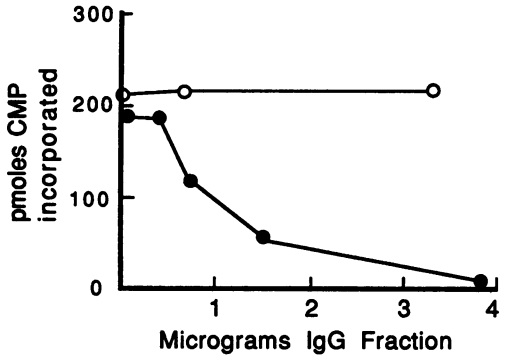

FIG. 4. Inhibition of σ^{28}-directed transcription by anti-σ^{28} antisera. RNA transcription reactions were performed as described for assay 2, except that [α-^{32}P]CTP was included at 150 cpm/pmol and RNA was quantitated by trichloroacetic acid precipitation as described for assay 1. The indicated amounts of immune (●) or preimmune (○) IgG fraction were added 5 min prior to initiation of transcription by shifting the reaction mixtures to a 37°C water bath.

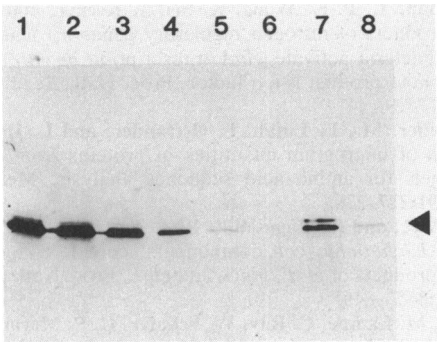

FIG. 5. Quantitative immunoblot analysis of σ^{28} in W168 10,000 × *g* supernatant. Lanes 1 through 6 contain heparin-agarose peak I enzyme (10, 5, 2, 0.5, 0.2, and 0.05 μg, respectively). This enzyme fraction contains at least 0.12% σ^{28} protein by weight as judged by recovery following gel purification. Lanes 7 and 8 contain 39 and 9 μg, respectively, of total protein of 10,000 × *g* supernatant fraction from an RNA polymerase purification similar to that shown in Fig. 1.

antibodies in our purified rabbit IgG fractions (Fig. 5, lanes 7 and 8).

Two-dimensional gel electrophoresis of purified σ^{28} holoenzyme fractions and samples of the low-speed supernatant fraction isolated from *B. subtilis* W168 cells has been used to assess the integrity of the isolated σ^{28} factor (data not shown). In repeated determinations ($n = 5$) with both the low-speed supernatant fraction and the purified σ^{28} RNA polymerase preparation (heparin-agarose peak I), σ^{28} migrates as a single immunoreactive spot with apparent M_r of 28,000 and pI of 5.85 ± 0.10. No other immunoreactive proteins are detected in this size range, indicating that the signal detected in one-dimensional immunoblots corresponds only to the σ^{28} protein. In mixing experiments, the σ^{28} present in purified enzyme preparations superimposes on that present in the initial low-speed supernatant fraction (data not shown).

Quantitative immunoblot analysis has been used to estimate the cellular abundance of σ^{28} in late-logarithmic-phase *B. subtilis* W168 cells (Fig. 5). Since the amount of σ^{28} present in heparin-agarose-purified RNA polymerase fractions is known from both densitometric scanning of Coomassie blue-stained gels and recovery following gel purification, this fraction has been used as a standard. The σ^{28} polypeptide represents between 5×10^{-5} and 10×10^{-5} (by weight) of the soluble protein present in the intial low-speed supernatant fraction extracted from *B. subtilis*. If the protein content of an average *B. subtilis* cell is assumed to be comparable to that of *E. coli* (39), this represents about 220 ± 50 molecules of σ^{28} protein per cell. This is about 10% of the estimated level for σ^{43} and about 40% of that estimated for σ^{37} (29).

DISCUSSION

Bacterial σ factors act as positive regulators of transcription by programming RNA polymerase to recognize specific promoter sites. Alternative σ factors represent one important mechanism by which the expression of unlinked sets of genes can be coordinated. Alternative σ factors in *E. coli* that allow transcription of heat shock (σ^{32})- and nitrogen (σ^{54})-regulated genes have been described (14, 22, 24). In *B. subtilis*, multiple σ factors are implicated in the expression of both vegetative-specific (σ^{28}, σ^{37}, and σ^{32}) and sporulation-specific (σ^{30}, σ^{29}, and *spoIIAC*) functions (36). To better understand the role of the *B. subtilis* σ^{28} factor, we devised an improved, large-scale purification of the σ^{28} protein. This has permitted the isolation, sequencing, and mutational analysis of the σ^{28} structural gene (20).

We have modified our previous procedure (45) for the isolation of σ^{28} RNA polymerase to allow efficient and reproducible purification of large quantities of σ^{28}-containing enzyme. From 500 g of cell paste, we routinely obtain approximately 100 mg of RNA polymerase, which yields 100 μg of σ^{28} after gel purification. This represents a yield of 5 to 10% of the total σ^{28} protein present in the initial low-speed supernatant fraction. This is significantly lower than the overall yield of RNA polymerase (40 to 50%) and probably reflects preferential loss of σ^{28} during some steps. The polymin P fractionation of the initial low-speed supernatant fraction has been identified as one step in which significant amounts of σ^{28} protein, but not of total RNA polymerase, are lost.

The particular purification procedure can greatly influence the transcriptional properties of the final holoenzyme preparation. The current procedure gives relatively high yields of both the σ^{28} and δ proteins. We found that in an alternative procedure involving initial fractionation on heparin-agarose (9), σ^{28} RNA polymerase eluted earlier than the σ^{43} holoenzyme (assay 2). Since nonspecific RNA polymerase assays would not detect σ^{28}-containing enzyme in these crude fractions, the final enzyme fractions are devoid of σ^{28} RNA polymerase activity. Similarly, RNA polymerase purified by the methods of Duffy and Geiduschek (10) and Tjian and Pero (42) fails to transcribe σ^{28}-dependent promoter sites in vitro (27). This underscores the need for an activity assay applicable to early fractionation steps when purifying a particular holoenzyme.

We have used two-dimensional immunoblots to determine whether the isolated σ^{28} protein is representative of that present in cell lysates. The σ^{28} present in purified RNA polymerase fractions comigrates on two-dimensional gels with that present in the initial low-speed supernatant fraction, suggesting that intact σ^{28} is being recovered and that there is no detectable processing of the σ^{28} protein. Consistent with this observation, the amino acid sequence of tryptic peptide 28 is found to correspond to the amino terminus of the predicted protein product of the σ^{28} structural gene (20). This suggests that there is no posttranslational proteolytic processing of the amino terminus of σ^{28} protein. In contrast, the *B. subtilis* σ^{29} protein is synthesized as an inactive precursor of M_r 31,000 and is processed posttranslationally to the active form (44).

The procedure we have developed has allowed the isolation of sufficient quantities of σ^{28} for biochemical characterization and antiserum preparation. Chemical sequencing of purified σ^{28} protein has allowed the cloning of the σ^{28} structural gene (20). This gene can now be used for the overproduction of the σ^{28} protein for further biochemical characterization of this factor and the σ^{28} RNA polymerase.

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LITERATURE CITED

1. **Alberts, B., and G. Herrick.** 1971. DNA-cellulose chromatography. Methods Enzymol. **21**:198–217.
2. **Amemiya, K., C. Wu, and L. Shapiro.** 1977. *Caulobacter crescentus* RNA polymerase: purification and characterization of holoenzyme and core polymerase. J. Biol. Chem. **252**:4157–4165.
3. **Bidlingmeyer, B. A., S. A. Cohen, and T. L. Tarvin.** 1984. Rapid analysis of amino acids using pre-column derivitization. J. Chromatogr. **336**:93–104.
4. **Binnie, C., M. Lampe, and R. Losick.** 1986. Gene encoding the σ^{37} species of RNA polymerase σ factor from *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83**:5943–5947.
5. **Brosius, J.** 1984. Toxicity of an overproduced foreign gene product in *Escherichia coli* and its use in plasmid vectors for the selection of transcription terminators. Gene **27**:161–172.
6. **Burgess, R., A. Travers, J. Dunn, and E. Bautz.** 1969. Factor stimulating transcription by RNA polymerase. Nature (London) **221**:43–46.
7. **Carter, L., and C. P. Moran, Jr.** 1986. New RNA polymerase sigma factor under *spo0* control in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83**:9438–9442.
8. **Chamberlin, M., W. Nierman, J. Wiggs, and N. Neff.** 1979. A quantitative assay for bacterial RNA polymerases. J. Biol. Chem. **254**:10061–10069.
9. **Davison, B., T. Leighton, and J. C. Rabinowitz.** 1979. Purification of *Bacillus subtilis* RNA polymerase with heparin-agarose. J. Biol. Chem. **254**:9220–9226.
10. **Duffy, J., and E. P. Geiduschek.** 1975. RNA polymerase from phage SPO1-infected and uninfected *Bacillus subtilis*. J. Biol. Chem. **250**:4530–4541.
11. **Duncan, M. L., S. S. Kalman, S. M. Thomas, and C. W. Price.** 1987. Gene encoding the 37,000-dalton minor sigma factor of *Bacillus subtilis* RNA polymerase: isolation, nucleotide sequence, chromosomal locus, and cryptic function. J. Bacteriol. **169**:771–778.
12. **Gilman, M., J. Wiggs, and M. J. Chamberlin.** 1981. Nucleotide sequences of two Bacillus subtilis promoters used by *Bacillus subtilis* sigma-28 RNA polymerase. Nucleic Acids Res. **9**:5991–6000.
13. **Gilman, M. Z., and M. J. Chamberlin.** 1983. Developmental and genetic regulation of *Bacillus subtilis* genes transcribed by σ^{28} RNA polymerase. Cell **35**:285–293.
14. **Grossman, A. D., J. W. Erickson, and C. A. Gross.** 1984. The *htpR* gene product of *E. coli* is a sigma factor for heat shock promoters. Cell **38**:383–390.
15. **Hager, D., and R. Burgess.** 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. **109**:76–86.
16. **Haldenwang, W., N. Lang, and R. Losick.** 1981. A sporulation-induced sigma-like regulatory protein from B. subtilis. Cell **23**: 615–624.
17. **Haldenwang, W., and R. Losick.** 1979. A modified RNA polymerase transcribes a cloned gene under sporulation control in *Bacillus subtilis*. Nature (London) **282**:256–260.
18. **Haldenwang, W., and R. Losick.** 1980. Novel RNA polymerase σ factor from *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **77**: 7000–7004.
19. **Heinrikson, R. L., E. T. Krueger, and P. S. Keim.** 1977. Amino-acid sequence of phospholipase A_2-alpha from the venom of *Crotalus adamanteus*: a new classification of phospholipases A_2 based upon structural determinants. J. Biol. Chem. **252**:4913–4921.
20. **Helmann, J. D., L. M. Marquez, and M. J. Chamberlin.** 1988. Cloning, sequencing, and disruption of the *Bacillus subtilis* σ^{28} gene. J. Bacteriol. **170**:1568–1574.
21. **Hewick, R., M. Hunkapiller, L. Hood, and W. Dreyer.** 1981. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256**:7990–7997.
22. **Hirschman, J., P.-K. Wong, K. Sei, J. Keener, and S. Kustu.** 1985. Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription *in vitro*: evidence that the *ntrA* product is a σ factor. Proc. Natl. Acad. Sci. USA **82**:7525–7529.
23. **Hunkapiller, M., E. Lujan, F. Ostrander, and L. Hood.** 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. **91**:227–236.
24. **Hunt, T. P., and B. Magasanik.** 1985. Transcription of *glnA* by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, *glnG*, and *glnL*. Proc. Natl. Acad. Sci. USA **82**:8453–8457.
25. **Igo, M., M. Lampe, C. Ray, W. Schafer, C. P. Moran, Jr., and R. Losick.** 1987. Genetic studies of a secondary RNA polymerase sigma factor in *Bacillus subtilis*. J. Bacteriol. **169**:3464–3469.
26. **Inglis, A. S.** 1983. Single hydrolysis method for all amino acids, including cysteine and tryptophan. Methods Enzymol. **91**:26–36.
27. **Jaehning, J., J. Wiggs, and M. J. Chamberlin.** 1979. Altered promoter selection by a novel form of *Bacillus subtilis* RNA polymerase. Proc. Natl. Acad. Sci. USA **76**:5470–5474.
28. **Johnson, J., M. DeBacker, and J. Boezi.** 1971. Deoxyribonucleic acid-dependent ribonucleic acid polymerase of *Pseudomonas putida*. J. Biol. Chem. **246**:1222–1232.
29. **Johnson, W., 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.
30. **Kane, C. M., and S. Linn.** 1981. Purification and characterization of an apurinic/apyrimidinic endonuclease from HeLa cells. J. Biol. Chem. **256**:3405–3414.
31. **Kenney, T. J., and C. P. Moran, Jr.** 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. J. Bacteriol. **169**:3329–3339.
32. **Knopf, U.** 1974. The nucleoside triphosphate-ribonucleic acid nucleotidyltransferase (E.C. 2.7.7.6) of *Agrobacterium tumefaciens* (Smith and Townsend) Conn. Biochem. J. **143**:511–520.
33. **Konigsberg, W., and L. Henderson.** 1983. Removal of sodium dodecyl sulfate from proteins by ion-pair extraction. Methods Enzymol. **91**:254–259.
34. **LaBell, T. L., J. E. Trempy, and W. G. Haldenwang.** 1987. Sporulation-specific σ factor σ^{29} of *Bacillus subtilis* is synthesized from a precursor protein, P^{31}. Proc. Natl. Acad. Sci. USA **84**:1784–1788.
35. **Lill, U., E. Kniep-Behrendt, L. Bock, and G. Hartman.** 1977. Purification and characterization of the DNA-dependent RNA polymerase and its subunit σ from *Micrococcus luteus*. Hoppe-Seyler's Z. Physiol. Chem. **358**:1591–1603.
36. **Losick, R., P. Youngman, and P. J. Piggot.** 1986. Genetics of endospore formation in *Bacillus subtilis*. Annu. Rev. Genet. **20**: 625–669.
37. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene **19**:269–276.
38. **Morrissey, J.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
39. **Neidhardt, F.** 1987. Chemical composition of *Escherichia coli*, p. 3–6. *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. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
41. **Stein, S., and L. Brink.** 1981. Amino acid analysis of proteins and peptides at the picomole level: the fluorescamine amino acid analyzer. Methods Enzymol. **79**:20–25.
42. **Tjian, R., and J. Pero.** 1976. Bacteriophage SPO1 regulatory proteins directing late gene transcription *in vitro*. Nature (London) **262**:753–757.
43. **Trempy, J., J. Morrison-Plummer, and W. Haldenwang.** 1985. Synthesis of σ^{29}, an RNA polymerase specificity determinant, is a developmentally regulated event in *Bacillus subtilis*. J. Bac-

teriol. **161**:340–346.

44. **Trempy, J. E., C. Bonamy, J. Szulmajster, and W. G. Haldenwang.** 1985. *Bacillus subtilis* σ factor σ^{29} is the product of the sporulation-essential gene *spoIIG*. Proc. Natl. Acad. Sci. USA **82**:4189–4192.
45. **Wiggs, J., M. Gilman, and M. J. Chamberlin.** 1981. Heterogeneity of RNA polymerase in *Bacillus subtilis*: evidence for an additional σ factor in vegetative cells. Proc. Natl. Acad. Sci. USA **78**:2762–2766.
46. **Yudkin, M.** 1987. Structure and function in a *Bacillus subtilis* sporulation-specific sigma factor: molecular nature of mutations in *spoIIAC*. J. Gen. Microbiol. **133**:475–281.
47. **Zillig, W., K. Zechel, and H. Halbwachs.** 1970. A new method of large-scale preparation of highly purified DNA-dependent RNA-polymerase from *E. coli*. Hoppe-Seyler's Z. Physiol. Chem. **351**:221–224.