Structure and Function of the σ-70 Subunit of *Escherichia coli* RNA Polymerase. Monoclonal Antibodies: Localization of Epitopes by Peptide Mapping and Effects on Transcription†

Marie S. Strickland,‡ Nancy E. Thompson, and Richard R. Burgess*
McArdle Laboratory for Cancer Research, University of Wisconsin—Madison, 450 North Randall Avenue, Madison, Wisconsin 53706
Received January 21, 1988

ABSTRACT: Murine monoclonal antibodies reactive with the major σ subunit (σ-70) of *Escherichia coli* RNA polymerase were obtained by standard hybridoma techniques. Western blot analyses established that seven antibodies had unique specificities after various chemical and enzymatic methods were used to fragment σ. Peptides were purified by HPLC using size-exclusion, reverse-phase, or ion-exchange chromatography. The epitopes for six of these antibodies have been localized to specific peptides. These peptides were further characterized by amino acid composition and N-terminal sequencing. σ, which has a molecular weight of 70.2K, runs as 83K on SDS gels in this study. This anomalous behavior has been localized to the very acidic N-terminal half of the molecule. One antibody is unable to bind to native σ. Two others do not bind well to σ when it is contained in holoenzyme, indicating that their epitopes are in regions of σ which are inaccessible in the holoenzyme complex. All three of these antibodies fail to inhibit in vitro transcription by holoenzyme. The other four antibodies all can inhibit in vitro transcription.

Escherichia coli DNA-dependent RNA polymerase consists of a core polymerase ($\alpha_2\beta\beta'\omega$) and a σ subunit which determines the specificity for promoter sites. The major σ factor (σ-70) was originally isolated as a factor that stimulated in vitro transcription (Burgess et al., 1969). Many properties of the protein have been reported previously (Lowe et al., 1979), but the relatively small amount of protein available has slowed the study of the structural and functional properties. The DNA sequence of *rpo*D, the gene encoding σ-70, has been determined (Burton et al., 1981) and a plasmid constructed that overexpresses the protein (Gribskov & Burgess, 1983). Milligram quantities can now easily be purified, making possible the present study. As part of a detailed study of the structure and function of σ-70, we report the isolation of seven anti-σ-70 monoclonal antibodies and localization of their epitopes. Studies of the effects of monoclonal antibodies to *E. coli* RNA polymerase β and β′ subunits have been reported (Rockwell et al., 1985). We hope to use the antibodies reported here to help elucidate the function of σ-70.

Materials and Methods

All reagents were from Sigma Chemical unless otherwise specified. Milli-Q water (Millipore) was used throughout.

Purification of σ-70. σ was purified from an overproducing strain as described previously (Gribskov & Burgess, 1983) with the exception that a Mono Q column (5 mm × 5 cm, Pharmacia) was used instead of the Sephacryl S-200 column in the final purification step. The 0.3 M NaCl elution peak from the DE-52 column was loaded onto the Mono Q column which had been equilibrated with 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl),[1] pH 8.0, 0.1 mM EDTA, 0.1 mM DTT, and 0.3 M NaCl, and the σ was eluted with a 0.3–0.6 M NaCl, 30-mL gradient. Two peaks containing σ elute from this column at about 0.42 and 0.54 mM NaCl, respectively. The earlier peak (which is the major fraction) was used for all studies in this paper. The concentration of σ was determined using $E^{1\%}_{280nm} = 8.4$ (Lowe et al., 1979).

Preparation of Hybridomas. Adult female Balb/c ByJ mice (Jackson Laboratories, Bar Harbor, ME) were injected with 20 μg of purified σ contained in Freund's complete adjuvant (Difco), administered subcutaneously; 1 month later, the injection was repeated with Freund's incomplete adjuvant. Approximately 1 month later and 3 days prior to the fusion, mice with titers greater that 1:2400, as determined by ELISA (Voller et al., 1978), were injected intraperitoneally with 20 μg of σ contained in PBS. Spleen cells were fused with either NS1 or SP2/0 myeloma cells, using 40% poly(ethylene glycol) 1000 (Baker) according to standard methods (Fazekas de St. Goth & Scheidegger, 1980). Fusions were screened for specific antibody-producing cells by ELISA, using 96-well polystyrene plates coated with 0.8 μg of σ/mL and blocked with either 3% BSA or 1% BLOTTO (Carnation non-fat dry milk; Johnson et al., 1984). Culture fluid from each well was reacted with the immobilized σ, and the presence of σ-specific antibody was detected by using a horseradish peroxidase labeled goat antibody prepared against the mouse γ heavy chain (Zymed, South San Francisco, CA). Cells were cloned twice by limiting dilution, using spleen cells from nonimmunized Balb/c ByJ mice as helper cells. Stable cell lines were injected into Pristane-primed Balb/c ByJ mice for production of ascites fluid.

Purification of Antibodies. Each antibody was precipitated from about 10 mL of cell-free ascites fluid by the addition of

† Supported by NIH Research Grants GM 28575, CA 23076, and CA 09230.
* Address correspondence to this author.
‡ Present address: Biotechnology Center, University of Wisconsin—Madison, 1710 University Ave., Madison, WI 53705.

[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EIA, enzyme immune assay; ELISA, enzyme-linked immunosorbent assay; Gdn-HCl (GuHCl in figures), guanidine hydrochloride; HFBA, heptafluorobutyric acid; kDa, kilodalton(s); MAb, monoclonal antibody; NTCB, 2-nitro-5-thiocyanobenzoate; PBS, 10 mM phosphate (pH 7.4) buffered saline (150 mM NaCl); TBS, 10 mM Tris (pH 7.4) buffered saline (150 mM NaCl); TBST, TBS + 0.1% Tween-20 [poly(oxyethylene) sorbitan monolaurate]; TGED, 10 mM Tris (pH 7.9), 5% glycerol, 0.1 mM EDTA, and 0.1 mM DTT; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-5755$01.50/0

$(NH_4)_2SO_4$ to 45% saturation. Precipitated material was dissolved in 50 mM Tris-HCl, pH 6.9, and 25 mM NaCl and dialyzed against the same buffer. The material was passed through a column (approximately 1.0 × 10 cm) of DEAE (Whatman DE-52) equilibrated in the Tris buffer. MAbs 1 H6, 3D3, 2G10, 2D1, and 1S4 flowed through the column under these conditions and were judged to be greater than 90% pure as determined by SDS–PAGE. MAbs 2F8 and 2D4 adsorbed to the DE-52 under these conditions and when removed by gradient elution (25–500 mM NaCl) did not yield an antibody preparation of equivalent purity to the other five MAbs. Antibody 2F8 was further purified on a Mono Q column using the same buffer and gradient elution as described for the DE-52 column. Antibody 2D4, an IgA, proved more difficult to purify. The 45% $(NH_4)_2SO_4$ pellet from the 2D4 ascites fluid, after solubilization and dialysis into Tris-HCl, pH 7.0, and 200 mM NaCl, gave almost complete inhibition of activity when about 1 μg of protein was used. This extract was the most impure and had some RNase activity. After further purification on DE-52, 3 μg of protein inhibited activity by about 40%. The fraction from DE-52 was used in all the studies reported in this paper. Concentration estimates of MAb were based on an assumed $E^{1\%}_{280nm} = 13.8$.

Sepharose-Conjugated Antibodies. Each purified antibody (from DE-52) was dialyzed against 100 mM $NaHCO_3$, pH 8.5, and 500 mM NaCl. Approximately 8.75 mg of antibody in 10 mL of buffer was conjugated to 1 g of CNBr-activated Sepharose 4B (Pharmacia) by mixing for 2 h at room temperature. Unreacted sites were blocked by reacting the Sepharose with 1 M ethanolamine for 2 h at room temperature. In all cases, greater than 95% conjugation of the antibody to the Sepharose was achieved, as determined by optical density readings at 280 nm. Control Sepharose was subjected to the same procedure except that the coupling buffer contained no protein.

Characterization of Antibodies. Antibodies that reacted with purified σ in the ELISA screening system were tested for the ability to react with σ in Western blot assays (described below). The unique specificity of each antibody was determined by reactions obtained in Western blot assays using limited tryptic digests of σ. The class, subclass, and light-chain type of each antibody were determined by reactions obtained by using an isotyping kit (Hyclone Laboratories, Logan, UT) in an ELISA system.

The ability of each antibody to react with σ contained in holoenzyme was determined by using the antibody-conjugated Sepharose preparations. A slurry was prepared by making 1 g (3.5 mL) of antibody-conjugated Sepharose up to 10 mL with bicarbonate buffer. Samples (100 μL containing about 0.875 μg of MAb) of the slurry were washed twice in 1 mL of PBS and reacted with 450 μL containing either 9 μg of σ or 27 μg of holoenzyme in PBS for 2 h at room temperature. The slurry was centrifuged, the supernatant fluids were concentrated by evaporation in a Speed-vac concentrator (Savant) and resuspended in an appropriate amount (45–55 μL, depending upon the residual glycerol) of SDS sample buffer. Samples were then run on 15% gels to determine if the conjugated antibody was able to remove the holoenzyme from solution as effectively as it removes the purified σ. Control Sepharose, containing no antibody, was tested concomitantly to determine the degree of nonspecific binding of σ and holoenzyme to Sepharose.

Transcription Assays. The assay used was based on the σ-70 assay described previously by Lowe et al. (1979) which measures promoter-directed RNA synthesis. Holoenzyme was purified from the holo overproducing strain (NO3273) and core from the core overproducing strain (NO3176) described by Bedwell and Nomura (1986). Purification of proteins was essentially as described previously (Burgess & Jendrisak, 1975; Lowe et al., 1979) except that the single-stranded DNA–agarose column was replaced with a Q Sepharose (Pharmacia) column; the elution buffer was TGED with a 15–500 mM NaCl gradient. Each 100 μL of transcription assay mixture contained 40 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.05% BSA (acetylated Sigma B2518), 10 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM DTT, 1 mM EDTA, ATP/GTP/CTP each at 200 μM, 50 μM UTP (containing 1 μCi of [^{3}H]UTP), and 1 μg of pBR322 DNA. The mixture was stored on ice, and the DNA was added just before use. In this assay, incorporation of ^{3}H into RNA was stimulated at least 35-fold when 1 μg of core polymerase was supplemented with 0.3 μg of σ-70. The effect of the MAb on holoenzyme was measured in assay mixtures which contained 0.7 μg of holo (1.5 pmol if fully saturated with σ-70) treated with 0.2–5.0 μg of MAb. Assuming that the MAb react monovalently, 1 μg corresponds to a 4.4 molar excess of an IgG or 2.2 for an IgA. The MAbs were diluted in 40 mM Tris-HCl, pH 7.0, 200 mM NaCl, and 0.05% BSA, and 10 μL was added to the holoenzyme diluted in 10 μL of pH 8.0 buffer (40 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.05% BSA). The 20-μL mixture was stored on ice for at least 1 h before 100 μL of assay mix was added, followed by incubation at 37 °C for 10 min. The assay was terminated by the addition of 25 μL of 0.2 M EDTA; then 100 μL of the mixture was spotted onto DEAE-derivatized filter paper disks and allowed to air-dry. Filters were washed with two changes each of 5% Na_2HPO_4, water, and 95% ethanol (10 mL per filter for each 15-min wash). After air-drying, the filters were counted in 5 mL of nonaqueous scintillation fluid. Assays were done in duplicate. Controls were done with boiled MAb and were compared to the minus MAb controls to measure any decrease in the amount of [^{3}H]RNA binding to the filters due to the presence of heat-stable RNase in the MAb preparations. Such RNase activity was detected only in the solubilized 45% $(NH_4)_2SO_4$ pellet from the 2D4 ascites fluid.

Polyacrylamide Gel Electrophoresis, Western Blotting, and EIA. Samples were subjected to SDS–PAGE on 15% gels essentially as described by Laemmli (1970) and modified by Thomas and Kornberg (1975). Samples were heated at 37 °C for 1 h in sample buffer before electrophoresis, since σ is partially cleaved by the usual 2–3-min heat treatment at 100 °C. The acrylamide to bis(acrylamide) ratio is 200:1, and the separating gel and running buffer are used at twice the buffer concentration of that used by Laemmli. SDS gels with a 10–20% gradient of polyacrylamide [acrylamide to bis(acrylamide) ratio of 37.5:1] and 0–75% gradient of glycerol were also used. A 5% gel of the same buffer composition as the lower gel was added to the top of the gradient gel just before used as a mold for the comb. This was more convenient than adding combs directly to the multiple gradient gels when they were poured; fewer combs are needed, all wells can be visualized, and the shape of the comb can be chosen as desired. The gels were 8 × 10 cm and 0.75 mm thick. The combs used most frequently were one with 15 wells (3 mm) and one with a large 65-mm well and two 3-mm wells. A Hoefer Mighty Small apparatus was used. Sigma Chemical molecular weight markers ($\times 10^{-3}$) were the following: SDS-7, containing bovine serum albumin (66), ovalbumin (45), glyceraldehyde-3-phosphate dehydrogenase (36), carbonic anhydrase (29), trypsinogen (24), soybean trypsin inhibitor (20.1), and α-

lactalbumin (14.2); SDS-17, containing myoglobin (17) and myoglobin fragments (14.4, 8.2, 6.2, and 2.5). Prestained low molecular weight markers (BRL), which were used to orient positions on Western blots, contained ovalbumin (43), α-chymotrypsinogen (25.7), β-lactoglobulin (18.4), lysozyme (14.3), bovine trypsin inhibitor (6.2), and the α and β chains of insulin (3). Linear calibration curves were obtained on gradient gels with SDS-17. The Sigma company suggests that only the Swank and Munkres (1971) method would yield such a calibration curve for the small peptides in the MW-SDS-17 mixture. We obtained good results with the method as outlined here and find it convenient since the same buffer system can be used for all gels. Gels were stained with Coomassie brilliant blue R250. Silver staining (Wray et al., 1981) is more sensitive but was not used since it was noted that a large CNBr peptide (CNBr-9) did not stain with silver. All chemicals for electrophoresis were obtained from Bio-Rad except SDS which was from BDH Chemicals.

Proteins were blotted onto nitrocellulose (0.45 μm, Schleicher & Schuell) as in Towbin et al. (1979) except that SDS was added to 0.05% in the transfer buffer. The blots or 0.5-mm strips of the blots were subjected to EIA essentially as described by Blake et al. (1984). The nitrocellulose blots were blocked in TBST for 1–3 h. The blots were incubated with 0.3–0.5 μg of anti-σ antibodies/mL of 1% BSA in TBS for 1 h. Blots of whole gels (8 × 9 cm) were incubated in 10 mL of diluted MAb in 150-mm-diameter petri dishes which were mixed on a rotary shaker. Strip blots (0.5 cm × 8 cm) were incubated in 1 mL of diluted MAb in 13 × 100 mm screw-cap tubes (Falcon 2027) which were mixed on a rocking platform. The blots were then washed 3 times for 10 min with TBST. Goat anti-mouse IgG coupled to alkaline phosphatase (Boehringer Mannheim) was diluted 1:2000 in BSA and incubated with the blots for 1 h. The blots were then washed 3 times for 10 min with TBST. Strip blots were processed individually to this point. The blots were rinsed with 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$ and then incubated with 75 mM BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 20 mM NBT (nitro blue tetrazolium), which were dissolved in the pH 9.5 buffer above, until sufficient color developed.

Immunodot Assay. A Bio-Rad Bio-Dot microfiltration apparatus was used to immobilize protein or peptide samples on nitrocellulose. This apparatus has a 12 × 8 matrix like that of 96-well microtiter plates. Fractions from reverse-phase HPLC columns were reduced in volume in a Speed Vac; an aliquot was diluted into 100 μL of TBS (containing 500 mM NaCl) and applied to the well. The sample was pulled through by vacuum, and the wells were washed with 200 μL of TBS (500 mM NaCl) by vacuum. The higher concentration of NaCl in the TBS was necessary to prevent protein binding to the plastic of the apparatus. At this point, the filter was removed from the apparatus, blocked with TBST, and cut up, if necessary, before incubation with MAb as described above for EIA of Western blots.

Cleavage of σ-70. (A) Tryptic Digestion at Lysines and Arginines. Protease-resistant regions in σ have been noted previously (Lowe et al., 1979; Gribskov, 1985). Partial tryptic digestions were done after urea denaturation to obtain a more random yield of partial fragments for initial screening of MAb on Western blots. A titration of the amount of trypsin necessary was performed by keeping the time constant and varying the amount of trypsin added. In a typical titration experiment, 25 μg of protein was denatured in 25 μL of 8 M urea, 100 mM Tris-HCl (final pH 8.0), 0.1 mM EDTA, and 1 mM DTT for 30 min at 37 °C. The mixture was diluted to 2 M urea by the addition of 3 volumes of Tris buffer. Trypsin (Cooper) in 1 mM HCl was added to 20-μL aliquots of protein in substrate:trypsin ratios of 50:1, 100:1, 200:1, 400:1, and 800:1 (w/w), and the mixture was incubated 1 h at 37 °C before addition of a 2-fold excess of soybean trypsin inhibitor (dissolved in Tris buffer). The amounts of trypsin and trypsin inhibitor were estimated by assuming an $E^{1\%}_{280nm}$ of 10. The degree of digestion was monitored by mixing an appropriate volume of 4× SDS sample buffer with 2–15 μL (0.5–3.75 μg of σ), loading into a 3-mm well, and running on a gradient gel. Typically, a 100:1 substrate:trypsin ratio for 1 h gave good visualization when 3–5 μg was loaded and the gel stained with Coomassie blue. If a Western blot was to be done, 30 μg of digest was loaded in a 65-mm well. Tryptic digests were also done on native protein at a protein concentration of 0.3 mg/mL in 25 mM NH_4HCO_3, and titration experiments were done as above. Less trypsin was required than in the urea experiments. In a typical preparative limited digestion, 1 mg of native σ was digested at a 1000:1 ratio for 30 min at 37 °C. Usually about 500 μg was loaded onto 4.6-mm-diameter HPLC columns to separate the peptide fragments.

(B) NTCB Cleavage at Cysteines (Jacobson et al., 1973; Stark, 1977). Protein was denatured at 1 mg/mL in 8 M urea and 200 mM Tris (final pH 9.0) with a 5-fold molar excess of DTT over the cysteines in the protein. After incubation for 1 h at 37 °C, a 5-fold excess of NTCB (over the total sulfhydryls in the mixture) was added, and the mixture was incubated 20 h at room temperature. The digest was loaded directly onto SDS gels or subjected to column chromatography. Typical amounts used were similar to those described for trypsin digestion.

(C) Carboxymethylation of Protein. In a typical experiment, 1–2 mg of σ was dissolved in 1 mL of 6 M Gdn-HCl and 100 mM Tris-HCl (final pH 8.0) with a 5-fold molar excess of DTT. After incubation at 37 °C for 1 h, a 5-fold excess of sodium iodoacetate was added. After 15 min, the reaction was terminated by addition of excess DTT. The excess reagents were removed by dialysis against 10 mM NH_4HCO_3. All operations after the addition of sodium iodoacetate were performed in the dark.

(D) CNBr Cleavage at Methionines (Gross, 1967). The carboxymethylated protein, usually about 500 μg (1 mg/mL in 10 mM NH_4HCO_3), was made up to 70% formic acid by adding 2.8 volumes of 95% formic acid. A 100-fold molar excess of CNBr over the methionines was added in 70% formic acid, and cleavage was allowed to occur for 24 h at room temperature. The excess reagents and formic acid were removed by drying in a Speed Vac. The peptides were taken up in a small volume (200 μL) of 8 M urea. If SDS gels were run, about 5 μg of total digest was loaded per 3-mm well.

Purification of Peptides. Separation of peptides was performed on a Beckman Model 344 gradient HPLC system which has a Model 165 dual-channel variable-wavelength detector. Fractions were collected in 6-mL polypropylene test tubes. Excess reagents were removed by dialysis against 10 mM NH_4HCO_3 or, if volatile, by evaporation in a Speed Vac. Samples were not dried completely on the Speed Vac (some peptides were difficult to redissolve) but merely reduced in volume and then made up to a known volume with H_2O.

Peptide NTCB-1 was purified on a Mono Q column. The eluant was 6 M urea and 50 mM Tris-HCl (pH 8.0) with a 0.1–0.5 M linear gradient of NaCl. The CNBr peptides were first fractionated on a Protein-Pak 125 column (7.8 mm × 30 cm, Waters). The eluant was 10 mM HEPES, pH 7.0, and

Table I: Monoclonal Antibodies to *E. coli* σ-70

antibody designation	isotype	MAb conjugate binds holoenzyme[a]	inhibits transcription by holoenzyme
2D1	IgG2b	–	–
1H6	IgG2a	–[b]	–
3D3	IgG2a	+	++
2G10	IgG2b	+	++
2F8	IgG2b	+	++
1S4	IgG1	–[c]	–
2D4	IgA	+	+

[a] Determined by using antibody-conjugated Sepharose as described under Materials and Methods. [b] Does not bind all free σ in solution. [c] Does not bind to some preparations of σ.

100 mM NaCl. Peptide CNBr-9 was further purified on a C3 column (Altex Ultrapore, 4.6 mm × 7.5 cm) using acetonitrile (Burdick and Jackson) gradients containing 0.1% TFA (Pierce). The other CNBr peptides were further purified on a Vydac C4 reverse-phase column (4.6 mm × 25 cm). Tryptic peptides were purified on Vydac C4 and C18 columns. Some peptides were further purified by rerunning on the reverse-phase column with acetonitrile gradients containing 0.05% HFBA (Pierce) instead of TFA to take advantage of the different selectivity imparted by another ion-pairing reagent (Guo et al., 1987).

Amino Acid Compositions, Amino Acid Analysis, and N-Terminal Sequencing. The amino acid compositions of the protein and peptides were calculated from the gene sequence (Burton et al., 1981) with the Peptide Sort program from the University of Wisconsin Genetics Computer Group. PTC-amino acid analysis (which involves the formation of a phenylthiocarbamyl derivative of the amino acids; Bidlingmeyer et al., 1984) and amino acid sequencing were performed at the University of Wisconsin Biotechnology Center by the Protein/DNA Sequence/Synthesis Facility which is supported in part by NIH Grant DMB 8514305.

Results

Characterization of Antibodies. The antibodies, isolated from two fusions, used in this study are listed in Table I along with some pertinent characteristics. All of the antibodies are of the IgG class except for antibody 2D4 which is an IgA antibody. However, this antibody can be detected with commercially available enzyme-linked secondary antibodies prepared against the mouse γ heavy chain. Evidently, there is enough cross-reactivity in these secondary antibody preparations to effectively detected the IgA class. All of these antibodies contained the κ light chain.

All of the MAbs react with σ-70, and its peptide fragments blotted out of SDS gels onto nitrocellulose. Western blots and EIA of limited tryptic digests were used to screen the MAbs to select the ones with different epitopes (Figure 1). The MAbs which exhibited distinct reaction patterns to the complex peptide mixture generated by limited tryptic digestion were considered to bind to different sites. Two MAbs (1H5 and 2B8) were not studied further since they had similar cleavage patterns to two of the others and could not be demonstrated to be different by any other criterion. The remaining seven antibodies had distinct reaction patterns (Figure 1). Two pairs of antibodies (3D3/2G10 and 2F8/1S4) have similar reaction patterns, but Western blots of other types of digests demonstrated that they were different (see below).

Each of these MAbs was conjugated to Sepharose 4B and tested for its ability to bind σ-70 and holoenzyme. Antibodies 3D3, 2G10, 2F8, and 2D4 effectively remove both σ and the holoenzyme from solution. Antibody 2D1 removes σ from solution but does not remove holoenzyme efficiently. The

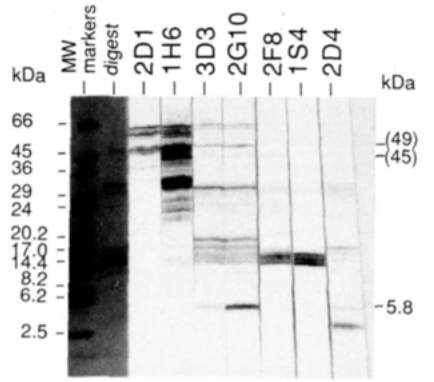

FIGURE 1: Western blot and EIA of fragments produced by limited tryptic digestion (1.5 h at 500:1) of urea-denatured σ-70. 30 μg of cleaved protein was run on a 10–15% gradient gel. The left two lanes were cut from the gel and stained prior to electroblotting of the remainder of the gel. They show molecular weight markers and the digest, respectively. The other lanes show strips of the blot after incubation with the various antibodies indicated. On the right are indicated the positions and probable identities (by size) of three partial fragments (see also Figures 5 and 6).

amount of free σ removed from solution by the 1S4 Sepharose conjugate varied between σ preparations. Two preparations of σ reacted well while a third showed almost no reaction. Some difference in the preparation or storage method may account for this unexplained observation. All preparations of σ had one major band at 83K on SDS gels and stimulated core enzyme to a similar extent. Antibody 1H6 does not remove either σ or the holoenzyme from solution, although it reacts well, as do the other six MAbs, with σ immobilized on polystyrene (in the ELISA) or on nitrocellulose (in the immunodot assay or Western blots). These results are summarized in Table I.

Each of these MAbs was tested for its ability to inhibit in vitro transcription by RNA polymerase. Antibodies 3D3, 2G10, and 2F8 have the most dramatic effect on transcription by holoenzyme, giving at least 90% inhibition with 0.5 μg of MAb. Antibody 2D4 seems to have a more moderate action, but this is difficult to quantitate since this antibody has not been purified to the same extent as the others. Antibodies 2D1, 1H6, and 1S4 at 5 μg do not inhibit transcription activity. This is expected since, when conjugated to Sepharose, none of them reacts with holoenzyme in solution and 1H6 does not even react well with free σ in solution. These results are summarized in Table I.

Purification of Immunoreactive Peptides. We purified and characterized immunoreactive peptides from various cleavage mixtures, including NTCB, CNBr, and limited or complete tryptic digestion. Peptide numbers are those given by the Peptide Sort program beginning at the N-terminus. These results are summarized in Figure 2.

Immunoblotting experiments on NTCB digests of σ revealed the expected three large peptides and two partial cleavage products (Figures 2 and 3). A very small peptide (NTCB-3) would not be detected on these blots. The smallest detectable NTCB peptide, which reacts with 2D1, was purified and identified as NTCB-1. This peptide, which spans positions 1–131 of σ (Figure 2, Table II), was identified by amino acid analysis (Table III). Antibody 1H6 reacts with the next biggest NTCB fragment (NTCB-2), which is predicted to span positions 132–290. MAbs 3D3, 2G10, 2F8, 1S4, and 2D4 all react with NTCB-4 (Figure 3).

The CNBr immunoblot (Figure 4) shows that 1H6 reacts with the largest CNBr fragment. The identity of this peptide (CNBr-9, positions 106–273, Figure 2) was confirmed after

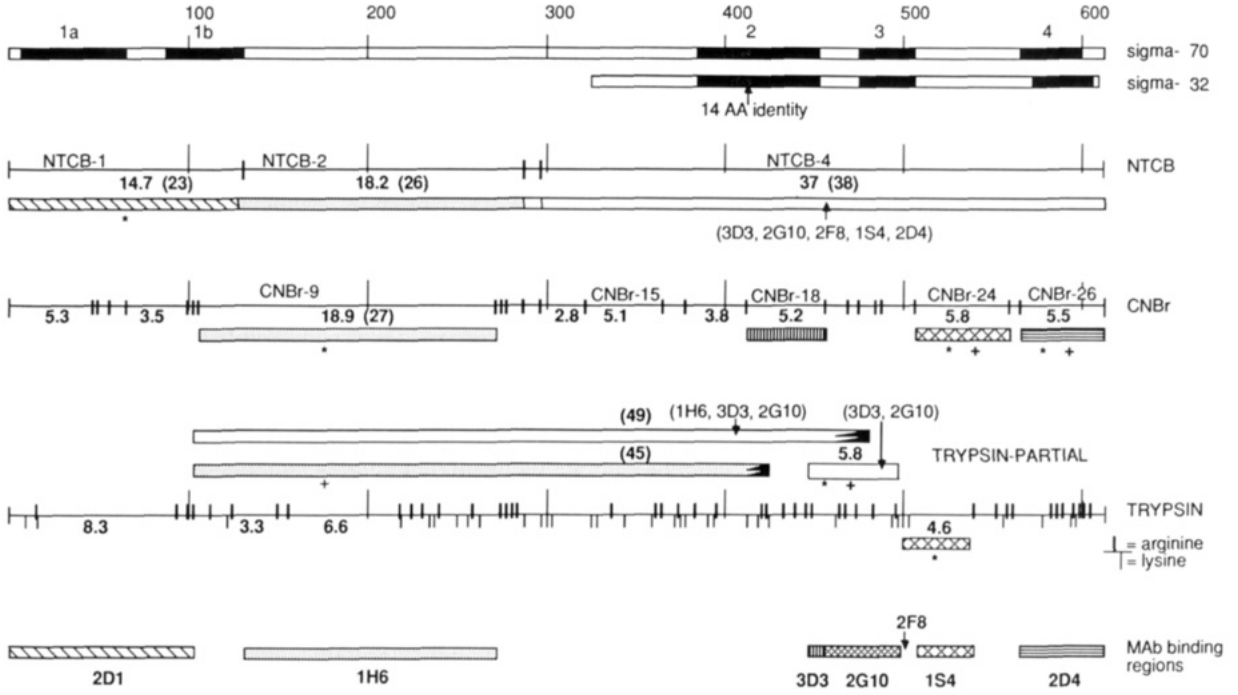

FIGURE 2: Diagrammatic representation of the predicted cleavage points of σ-70 after the treatments indicated. The peptides which were purified and/or visualized on immunoblots are shown. The numbers under the peptides are the sizes in kilodaltons as deduced from the gene sequence or, if in parentheses, the observed molecular weight on SDS gels. The top two lines showing homology between σ-70 and σ-32 (black bars) are from Gribskov and Burgess (1986). A summary of the maximum extent of the monoclonal antibody binding regions is given at the bottom of the figure. Peptides that were identified by amino acid analysis (asterisks) and/or by N-terminal sequencing (+) are indicated.

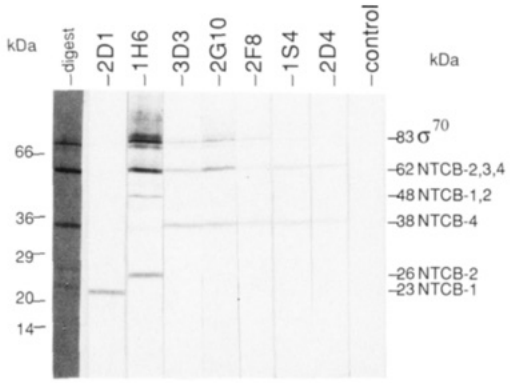

FIGURE 3: Western blot and EIA of fragments produced by cleavage of σ-70 with NTCB. 40 μg of cleaved protein was run on a 15% gel. The left lane was cut from the gel and stained prior to electroblotting of the remainder of the gel and shows the total digest. The positions of molecular weight markers are shown at the left. The apparent molecular weights of the fragments are given on the right. The other lanes show strips of the blot after incubation with the antibodies indicated. The control strip was processed without anti-σ antibody.

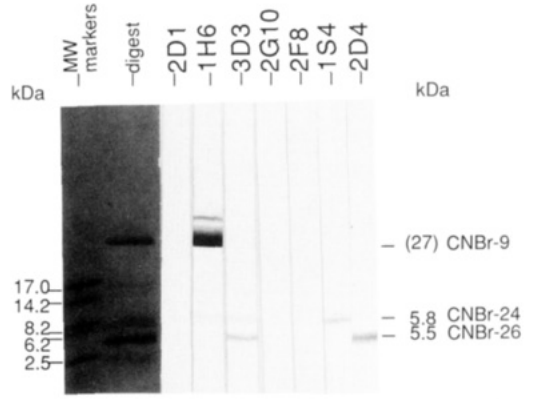

FIGURE 4: Western blot and EIA of fragments generated from σ-70 by treatment with CNBr. 100 μg of cleaved protein was run on a 10–20% gradient gel. The left two lanes were cut from the gel and stained prior to electroblotting of the remainder of the gel. They show molecular weight markers and the digest, respectively. The molecular weight for the large CNBr fragment was calculated on a 15% gel. The other lanes show strips of the blot after incubation with the various antibodies indicated. The 3D3 peptide does not separate well from the 2D4 peptide on this gel, but on other gels, it can be seen to run slightly slower than the 2D4 peptide.

Table II: σ-70 and Certain of Its Peptides: Comparison of Apparent Size on SDS Gels with That Predicted from the Gene Sequence

	inclusive amino acids	predicted size ($\times 10^{-3}$)	obsd size ($\times 10^{-3}$)	charge
NTCB-1	1–131	14.7	23	−22
NTCB-2	132–290	18.2	26	−23
NTCB-4	295–613	37.0	38	+2
NTCB(-1-2)	1–290	32.9	48	−45
NTCB(-2-3-4)	132–613	55.2	62	−22
CNBr-9	106–273	18.9	27	−29
σ-70	1–613	70.2	83	−44

it was purified and amino acid analysis was performed (Table III). Smaller fragments react with 3D3, 1S4, and 2D4. Two of these peptides were purified and subjected to amino acid analysis (Table III) and N-terminal sequencing (data not shown). They were found to contain the expected amino acid compositions and sequences for CNBr-24 (positions 508–561) and CNBr-26 (positions 568–613). CNBr-24 reacts with 1S4, and CNBr-26 reacts with 2D4 (Figure 2). The fragment which reacts with 3D3 could not be purified by the methods used for the other peptides as multiple immunoreactive peaks were always recovered after the C4 column. This peptide could be partially purified (separated from all the other immunoreactive peptides) on the Protein Pak 125 column. A major reactive peak and a minor reactive peak were also noted for antibodies 1S4 and 2D4 on the C4 column. The 3D3-reactive peptide is presumed to span amino acids 414–456 (CNBr-18). Several other acids were tried as solvents for CNBr cleavage, including 5 N acetic acid, 0.2 N HCl, and 50% TFA, but partial cleavage was obtained in all cases.

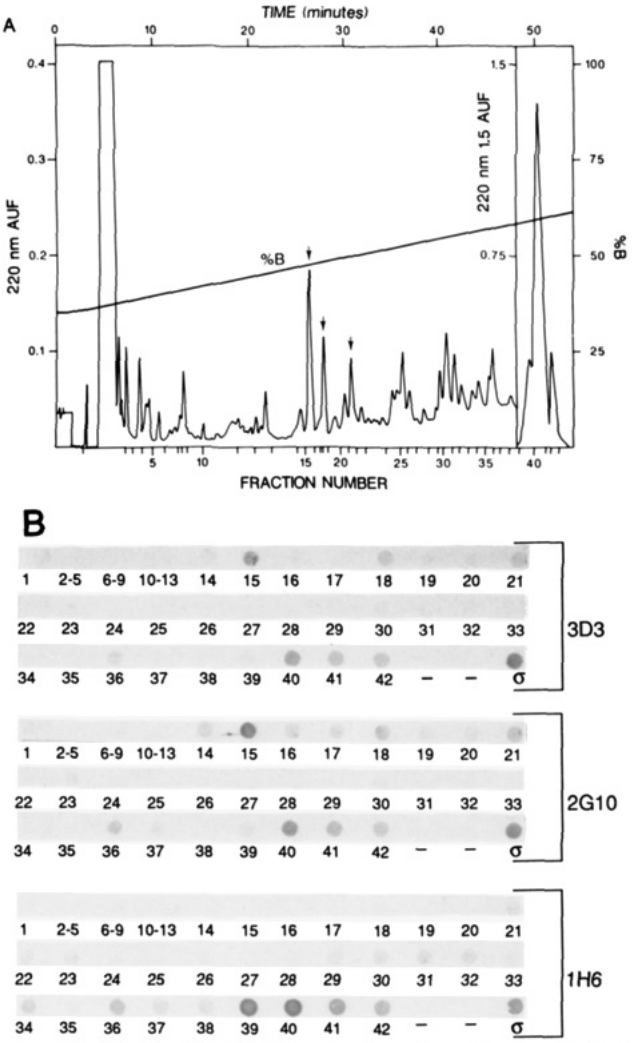

FIGURE 5: (A) Gradient HPLC separation of peptides from limited tryptic digest of σ-70. Protein was digested at 800:1 for 30 min at 37 °C. The column load was 300 μg (4.3 nmol). Column, Vydac C4, 4.6 mm × 25 cm, 5 μm; eluent A, 0.1% TFA; eluent B, 0.075% TFA in 80% acetonitrile; gradient, 35–65% in 60 min; flow rate, 1 mL/min. Fractions were reduced in volume by vacuum and then made up to 300 μL. Panel B shows immunodot assays of fractions for three antibodies (5 μL was used for all fractions except fraction 40, 2 μL). The arrows in panel A indicate fractions in the early part of the gradient which along with fractions 40, 41, and 42 were analyzed by gels and Western blots (Figure 6). Amino acid analysis was performed on 100 μL of fraction 15, and the yield was about 180 pmol (540 pmol total for the fraction).

NTCB-1, NTCB-2, and CNBr-9 all run much slower on SDS gels than expected. Table II lists the expected and observed molecular weights calculated for certain NTCB and CNBr peptides. The sum of the observed sizes for NTCB-1 and -2 (49K) is very close to that of a partial cleavage fragment (48K). The sum of NTCB-1, -2, and -4 (87K) is close to the observed molecular weight of intact σ-70 (83K). The slow migration of intact σ, which has a calculated molecular weight of 70.2K but runs as 83K on 15% SDS gels, seems to be due to the acidic N-terminal 290 amino acids since the 37K C-terminal NTCB-4 fragment runs close to its expected size.

Several tryptic peptides have been purified from complete and partial digests. Figure 5A illustrates the purification of various fragments from a limited digest of native σ. Dot blot immunoassays were performed on all column fractions for the seven MAbs, and representative blots are shown in Figure 5B. Aliquots of the main immunoreactive peaks from the column run illustrated in Figure 5A were run on three identical gels. One gel was stained, and the other two were subjected to

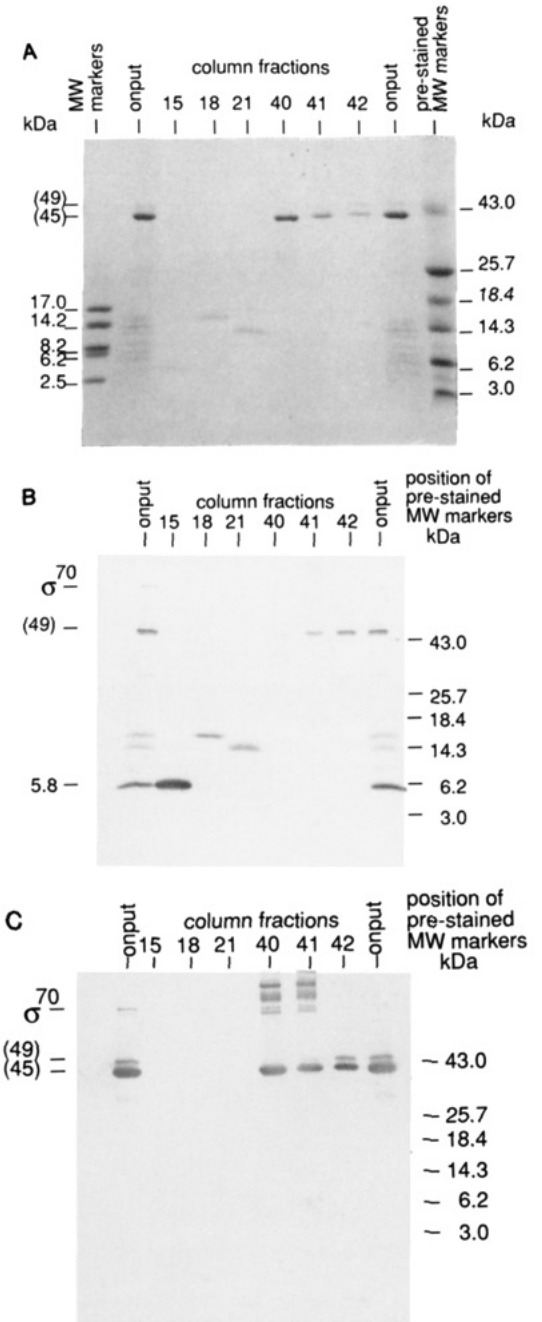

FIGURE 6: Gradient SDS–PAGE, Western blot, and EIA of selected fractions from the C4 column run (separation of peptides from a limited tryptic digest of σ-70) illustrated in Figure 5. (A) Coomassie-stained gradient gel; 2.5 μg of total digest was loaded in the onput lanes; 20 μL of fractions 15, 18, 21, 41, and 42 and 5 μL of fraction 40 were loaded. The markers shown in the left lane are SDS-17. The apparent molecular weights, in parentheses, for the 45- and 49-kDa fragments were calculated on 15% gels with SDS-7 markers. (B) Western blot and EIA of a gel identical with the ones shown in panels A and C reacted with antibody 2G10. (C) Western blot and EIA of a gel identical with the ones shown in panels A and B reacted with antibody 1H6. Prestained molecular weight markers were used to orient the positions on the filter.

Western blot and EIA with 1H6 and 2G10, respectively (Figure 6A–C). The large molecular weight 1H6-reactive bands seen in fractions 40 and 41 (Figure 6C) are apparently aggregates induced by the fractionation procedure since they do not appear in the material applied to the column.

A large partial tryptic fragment of about 45K, which is presumably the same as the one seen in the present experiment (Figures 2, 5, and 6), has been purified previously and found

Table III: Amino Acid Composition and Some Characteristics of σ-70 Peptides Predicted from the Gene Sequence Compared with Purified Peptides

	NTCB-1	2D1 + peptide	CNBr-9	1H6 + peptide	CNBr-24	1S4 + peptide	CNBr-26	2D4 + peptide	Trp 55–63	3D3/2G10 + peptide	Trp 65–66	1S4 + peptide
mol wt (×10^{-3})	14.7	(23)[b]	18.9	(27)	5.8	(5.5)	5.5	(5.2)	5.8	(4.0)	4.6	(5.4)
position	1–131		106–273		508–561		568–613		449–496		500–541	
charge	−22		−29		−9		+1		+1		−10	
amino acid												
Asx	19	18.2	26	20.2	7	6.3	5	3.5	3	2.7	6	5.3
Glx	27	21.2	32	27.4	6	6.5	7	6.9	9	7.0	6	6.0
Ser	5	5.0	9	8.2	3	2.2	3	2.4	1	0.8	4	3.3
Gly	7	7.6	7	7.3	3	4.3	1	1.0	1	1.0	2	1.8
His	1	0.9	2	0.4	2	1.4	1	0.9	1	0.8	1	0.9
Arg	6	6.1	9	9.6	3	2.8	7	6.6	6	5.7	1	1.0
Thr	7	6.9	9	8.0	7	5.1	3	2.5	3	2.2	5	4.1
Ala	11	10.9	15	14.9	6	5.6	2	2.1	1	1.1	2	2.0
Pro	4	5.3	5	6.2	2	2.0	1	1.2	4	3.0	3	2.7
Tyr	3	3.2	5	5.6	0	0	1	0.6	0	0	0	0
Val	9	7.8	11	9.7	2	2.7	3	2.9	1	1.2	0	0.2
Met	8	6.4	1	nd	1	nd	0	0	5	3.8	1	1.2
Cys(CMC)[a]	0	nd[c]	1	0.7	0	0	0	0	0	nd	0	nd
Ile	11	8.6	9	8.1	2	2.2	2	1.8	6	7.0	4	3.2
Leu	10	9.7	15	14.8	8	6.3	5	4.5	4	5.0	5	4.8
Phe	0	0	5	4.2	1	0.7	2	1.8	0	0.2	1	1.2
Lys	3	2.8	7	6.7	1	1.0	3	2.6	3	3.0	1	1.2

[a] CMC = *S*-(carboxymethyl)cysteine [b] Apparent molecular weight on SDS gels. [c] Not determined.

by N-terminal sequencing to begin at position 104 (Gribskov, 1985). This fragment reacts only with 1H6. Amino acid analysis gave inconclusive results and could not be used to calculate the C-terminus of the peptide. By analogy to CNBr-9, which spans positions 106–273, it was assumed that part of this large tryptic peptide from positions 104–274 (tryptic peptides 7–23) could be assigned an apparent molecular weight on SDS gels of 27K. Since we have shown previously that the deduced molecular weights of certain σ peptides on SDS gels are additive, and that the peptide derived from the C-terminal half of σ behaves normally on SDS gels (Table II), we can simply add the calculated molecular weights of the expected tryptic peptides from peptide 24 onward until a molecular weight near 45K is obtained. If this is done, the C-terminus of the 45K peptide is predicted to be about position 426 (after peptide 51, 44.8K). Another slightly longer fragment (49K) which reacts with 1H6 and also with 3D3 and 2G10 is presumed also to start at position 104 and extend further toward the C-terminus than the 45K fragment (Figure 2).

Fractions 18 and 21 (Figure 5) showed positive reactions on dot blots to all of the MAbs except 2D4 and 2D1. The reaction with 1H6 was weak but indicated that the fractions contain more than one peptide, since the size of the peptides on gels (Figure 6A) indicates that they are too small to include the epitopes from 1S4/1H6 (Figure 2).

A small fragment of 5.8K (positions 449–496) was isolated (fraction 15, Figure 5) and identified by amino acid analysis (Table III) and N-terminal sequencing (data not shown). This fragment reacts with both 3D3 and 2G10. The epitopes for these two antibodies are apparently closely linked but separated by a methionine (position 456), as a CNBr fragment (CNBr-18) has been observed which is presumed to end at that position and reacts only with 3D3 (Figure 2).

The position of the epitope for antibody 2F8 can be deduced to be near position 500 (Figure 2). MAb 2F8 reacts with the C-terminal NTCB fragment (positions 295–613) but not with the 45K partial tryptic fragment which extends to about position 426 or with a larger partial tryptic fragment which includes the epitopes of 3D3 and 2G10. Tryptic peptide (positions 449–496) reacts only with 3D3 and 2G10 and is nearly contiguous with another partial tryptic peptide (positions 500–541) which is positive only for 1S4. During the purification of σ, a protein of slightly smaller size was observed which reacted on Western blots with all the antibodies except 1S4 and 2D4, indicating that it likely was a proteolytic breakdown product of σ lacking a C-terminal region. Since 2F8 reacts with this fragment, its epitope must be N-terminal to those of 1S4 and 2D4 (Figure 2) and to lie between 2G10 and 1S4. The epitope for 2F8 may be destroyed by tryptic cleavage at position 496.

Discussion

This paper presents a detailed structural and immunological study of the σ-70 subunit of *E. coli* RNA polymerase. Seven monoclonal antibodies have been characterized and their epitopes mapped. These results are summarized in Figure 2.

Information on the conformation of σ in holoenzyme is obtained from the studies with antibody conjugates. Antibodies 2D1 and 1S4 when immobilized on Sepharose 4B do not remove holoenzyme from solution. This would indicate that regions near their epitopes are involved in σ–core interaction or that core binding induces conformational changes which make the epitopes inaccessible. The observation that preparations of σ vary in reactivity with the 1S4–Sepharose conjugate indicates the existence of conformational variation between different preparations of σ.

The observation that 1H6 does not react well with σ in solution indicates that the region containing the epitope for 1H6 is tightly organized. Further evidence is the observation of a protease-resistant region that includes the 1H6 epitope, extending from about positions 104–426 (Figure 2). Protein folding evidently excludes the antibody and also protease molecules. This region of σ-70 apparently resists denaturation with 8 M urea, or refolds rapidly in 2 M urea, as a peptide of about 45K (reactive with 1H6) can be seen in the partial tryptic digest of urea-denatured σ illustrated in Figure 1. The 45K peptide includes a sequence unique to σ-70 (positions 130–374) and extends into a proposed core interaction site between positions 403 and 431. Several groups have reported comparisons of various σ-like factors including σ-43, σ-32, σ-29, SPO1 gp28, and SPO1 gp34 (Landick et al., 1984; Yura et al., 1984; Stragier et al., 1985; Gribskov & Burgess, 1986). Despite the extensive homology between σ-32 and σ-70 (Figure 2), none of the MAbs in this study cross-reacts with σ-32 in Western blot analysis (Lesley et al., 1987). σ-70 is released

from core during chromatography on BioRex-70, but σ-32 is not (Grossman et al., 1984). Also, σ-32 is able to bind to core and function despite the presence of large amounts of σ-70, implying a difference in their affinities for core. Perhaps changes in the conformation of the unique highly acidic σ-70 sequence may be involved in modulation of core binding.

Antibody 2D4 is reactive with region 4 (Figure 2) of σ-70 which contains a conserved helix–turn–helix (HTH) unit proposed to be involved in promoter recognition (Stragier et al., 1985; Gribskov & Burgess, 1986). Antibodies 3D3, 2G10, and 2F8 react near conserved region 3 (Figure 2) which has been noted to contain a possible HTH region (Stragier et al., 1985; Gribskov & Burgess, 1986). Antibodies 2D4, 3D3, 2G10, and 2F8 all inhibit in vitro transcription by holoenzyme. The effect of 3D3, 2G10, and 2F8 may be similar since their epitopes are closely linked (Figure 2). We are in the process of a more detailed study of these four MAbs to determine precisely at which step they inhibit (e.g., at the binding of holoenzyme to promoter DNA, at the melting of DNA to form an open promoter complex, or at a subsequent step involved in actual initiation of an RNA chain). This may involve experiments with Fab or Fab′ fragments and kinetic studies with various promoters.

Antibodies 2D1, 1H6, and 1S4 do not affect in vitro transcription activity. Apparently, these antibodies cannot react with holoenzyme because the epitopes for antibodies 2D1 and 1S4 are buried in holoenzyme while the epitope for 1H6 is buried in σ even before it combines with core. It is interesting to note that while the 2F8 and 1S4 epitopes are located near each other (Figure 2) and these two MAbs show almost identical patterns of reactivity to partial tryptic fragments (Figure 1), they nevertheless differ dramatically in their ability to bind to holoenzyme and inhibit transcription.

Some observations of interest to protein chemists are presented here. The SDS gel migration of many peptides of known composition has been noted. NTCB-1, NTCB-2, and CNBr-9 all run slower on SDS gels than expected, and this seems to be correlated to their acidic nature. Possibly they assume a more extended conformation or bind SDS differently than the C-terminal half of σ. The SDS migration behavior of several smaller peptides has been observed on gradient gels (Table III). The molecular weight standards used on these gels gave linear calibration plots. Some peptides in this study have predictable mobilities while those of others are very unpredictable. For instance, a tryptic fragment (peptides 55–63) has a deduced size of 5.8K and runs as if it were smaller (4K) while CNBr-24 runs bigger that its predicted size. The peptides with very negative charges migrate slower, while those with positive charges migrate faster. It is evident that the SDS–PAGE system used in this study cannot be used to calculate precise molecular weights of peptides. It is, however, useful to indicate approximate size and to monitor purity. Peptides generated by CNBr were difficult to purify due to the presence of multiple immunoreactive peaks after reverse-phase HPLC. These multiple peaks may be due to modifications to the peptides during the CNBr cleavage in formic acid. CNBr-18 contains two serines, four threonines, three tyrosines, two glutamines, three lysines, and two tryptophans which are likely candidates for formyl esterification, deamidation, amino group reactions, or oxidation (Tarr & Crabb, 1983).

ACKNOWLEDGMENTS

The initial isolation of some of the MAbs was carried out during a sabbatical leave by R.R.B. at Genetic Systems Corp., Seattle, WA, and was supported in part by the Guggenheim Foundation. The help of the staff at Genetic Systems is gratefully acknowledged. Studies by M. R. Gribskov were an important foundation for the work presented here. We thank Gayle Hagar for preparing the holoenzyme and core polymerase.

REFERENCES

Bedwell, D. M., & Nomura, M. (1986) *Mol. Gen. Genet. 204*, 17–23.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984) *J. Chromatogr. 336*, 93–104.

Blake, M. S., Johnston, K. H., Russell-Jones, G. J., & Gotschlich, E. C. (1984) *Anal. Biochem. 136*, 175–179.

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4638.

Burgess, R. R., Travers, A. A., Dunn, J. J., & Bautz, E. K. F. (1969) *Nature (London) 221*, 43–46.

Burton, Z., Burgess, R. R., Lin, J., Moore, D., Holder, S., & Gross, C. A. (1981) *Nucleic Acids Res. 9*, 2889–2903.

Fazekas de St. Goth, S., & Scheidegger, D. (1980) *J. Immunol. Methods 35*, 1–21.

Gitt, M. A., Wang, L., & Doi, R. H. (1985) *J. Biol. Chem. 260*, 7178–7185.

Gribskov, M. R. (1985) Ph.D Thesis, University of Wisconsin—Madison.

Gribskov, M. R., & Burgess, R. R. (1983) *Gene 26*, 109–118.

Gribskov, M. R., & Burgess, R. R. (1986) *Nucleic Acids Res. 14*, 6745–6763.

Gross, E. (1967) *Methods Enzymol. 11*, 238–255.

Grossman, A. D., Erickson, J. W., & Gross, C. A. (1984) *Cell (Cambridge, Mass.) 38*, 383–390.

Guo, D., Mant, C. T., & Hodges, R. S. (1987) *J. Chromatogr. 386*, 205–222.

Jacobson, G. R., Schaffer, M. H., Stark, G. R., & Vanaman, T. C. (1973) *J. Biol. Chem. 248*, 6583–6591.

Johnson, D. A., Gautsch, J. W., Sportsman, J. R., & Elder, J. H. (1984) *Gene Anal. Tech. 1*, 3–8.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Landick, R., Vaughn, V., Lau, E. T., VanBogelen, R. A., Erickson, J. W., & Neidhardt, F. C. (1984) *Cell (Cambridge, Mass.) 38*, 175–182.

Lesley, S. A., Thompson, N. E., & Burgess, R. R. (1987) *J. Biol. Chem. 262*, 5404–5407.

Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344–1352.

Rockwell, P., Beasley, E., & Krakow, J. S. (1985) *Biochemistry 24*, 3240–3245.

Stark, G. R. (1977) *Methods Enzymol. 47*, 129–132.

Stragier, P., Parsot, C., & Bouvier, J. (1985) *FEBS Lett. 187*, 11–15.

Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462–477.

Tarr, G. E., & Crabb, J. W. (1983) *Anal. Biochem. 131*, 99–107.

Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626–2630.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Voller, A., Bartlett, A., & Bidwell, D. E. (1978) *J. Clin. Pathol. 31*, 507–520.

Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.

Yura, T., Tobe, T., Ito, K., & Osawa, T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6803–6807.