Identification of the Epitope for a Highly Cross-Reactive Monoclonal Antibody on the Major Sigma Factor of Bacterial RNA Polymerase

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A highly cross-reactive monoclonal antibody (MAB), 2G10, was found to react in a conserved region of *Escherichia coli* RNA polymerase σ^{70} . The epitope was localized to amino acids 470 to 486, which included part of conserved region 3.1. The epitope for MAB 3D3, a MAB which maps close to the 2G10 epitope, was also determined.

Escherichia coli core RNA polymerase, which consists of four subunits (α , β , β' , and ω), is capable of RNA synthesis but lacks promoter binding specificity. The addition of a sigma subunit creates a holoenzyme form of RNA polymerase, capable of promoter selection depending upon the specificity determining sigma factor (for reviews, see references 15, 16, 18, and 31). The major sigma factor (σ^{70} in *E. coli*) is responsible for directing RNA polymerase to the majority of promoters and making RNA chain initiation efficient.

Monoclonal antibodies (MABs) have been important tools in studying the structure and function of sigma factors (1, 2, 5, 7, 11–13, 20, 24). Strickland et al. (29) mapped seven MABs to various regions along the σ^{70} polypeptide. One of these MABs, 2G10, cross-reacts with the major σ factor from a wide range of bacterial species, including such diverse bacteria as *Chlamydia trachomatis* (8), *Mycobacterium smegmatis* (21), *Staphylococcus aureus* (23), *Bordetella pertussis* (28), and *Rhodobacter sphaeroides* (6), as well as *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, *Streptococcus faecalis*, *Arthrobacter globiformis*, and an *Anabaena* sp. (our unpublished data). Clearly, MAB 2G10 is an important tool for identifying the major sigma factor in bacteria. In addition, MAB 2G10 also inhibits transcription (29). Thus, the epitope for MAB 2G10 could represent an important feature of sigma function, vital not only to *E. coli* but to eubacteria in general. In other words, as this epitope is strikingly conserved among many species, it may correspond to a sequence essential for proper structure and function of sigma. Furthermore, even though many regions of *E. coli* σ^{70} are highly conserved with major sigmas from other organisms, most anti- σ^{70} MABs that we have isolated are not widely cross-reactive. Because MAB 2G10 had been previously mapped to amino acid residues 456 to 496 (29), it was believed that a more precise mapping of the epitope for this MAB would be useful in helping to identify the function of this important region of σ^{70} .

In addition, Strickland et al. (29) mapped the epitope for MAB 3D3 to amino acid residues 449 to 456 of σ^{70} . However, we found that *Bacillus subtilis* RNA polymerase σ^A , which is 100% conserved with *E. coli* σ^{70} between residues 449 and 456,

failed to react with MAB 3D3 (Fig. 1, lane 2). However, a protein fragment containing amino acids 456 to 613 did not react with MAB 3D3 (Fig. 1, lane 3), indicating that at least part of the epitope is contained within amino acids 1 to 456.

Therefore, to identify the N-terminal boundaries of the epitopes for MABs 2G10 and 3D3, a series of truncated proteins were synthesized from PCR-generated DNA templates (Fig. 2). All primers were made by the University of Wisconsin Biotechnology Center. Forward primers carried a ribosome binding site, an *Xba*I site, a methionine codon, and the codons for the first five amino acids to be synthesized. These primers were used with an oligonucleotide containing an *Eco*RI site, two translational termination codons, and five codons complementary to the last five codons of σ^{70} . Templates were amplified from the pMRG8 plasmid (3), a construct containing the coding region for σ^{70} under the control of the bacteriophage λ P_L promoter.

A similar method was employed to elucidate the C-terminal end of the epitope for MABs 2G10 and 3D3. The forward primer used to amplify codons starting at amino acid 1 of σ^{70} contained a *Bgl*II site followed by the T7 promoter. Reverse primers, which were complementary to the last five codons of interest, included two translational termination codons followed by an *Eco*RI site. Templates were amplified from pLN12, a construct containing the coding region of σ^{70} in the pET11T vector (22). The subsequent linear double-stranded DNA fragment contained a promoter, a ribosome binding site, an *Xba*I site, and the desired coding region.

These PCR-generated templates were then gel purified by running the PCR products in agarose gels onto DEAE-cellulose paper discs (26). DNA was eluted with NaCl, ethanol precipitated, washed with 70% ethanol, resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.9), and used in the *E. coli* S-30 extract system for linear DNA (Promega Corp., Madison, Wis.). This in vitro transcription-translation system has been used previously for epitope mapping (10). Reaction mixtures were incubated at 37°C for 1 h and then suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. It was found, however, that while the S-30 system worked well for DNA truncations of the C terminus of the protein, results with DNA fragments corresponding to N-terminal truncations were mixed and unpredictable, possibly due to the shorter length of the N-terminally truncated polypeptides.

Two PCR fragments, F470-613 and F474-613 (Fig. 2), could

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FIG. 1. Western blot analysis of *B. subtilis* RNA polymerase σ^A and *E. coli* σ^{70} protein fragment F456-613. Purified *E. coli* σ^{70} (lanes 1 and 4), purified *B. subtilis* RNA polymerase (lanes 2 and 5), and partially purified σ^{70} fragment F456-613 (lanes 3 and 6) were reacted with MAbs 3D3 (lanes 1 to 3) and 2G10 (lanes 4 to 6).

not be expressed in the S-30 system and were subsequently cloned into pET11a. A third fragment, F456-613, which was generated by taking advantage of a convenient *Nde*I site at amino acid 456, was also cloned into pET11a. These three constructs were transformed into *E. coli* BL21(DE3)(pLysS) and induced with 1 mM isopropylthiogalactoside (30).

Whether generated by PCR/S-30 or through overexpression of cloned gene fragments, protein fragments were separated

from endogenous σ^{70} by SDS-PAGE. Samples were subjected to SDS-PAGE (9) and were run on a Hoefer Mighty Tall apparatus with 10 or 12% gels for C-terminally truncated fragments and 15% gels for N-terminally truncated fragments. Western blots were performed according to the method of Strickland et al. (29), except that the secondary antibody was peroxidase-labeled goat anti-mouse immunoglobulin G (American Qualex, La Mirada, Calif.) (1:1,000 in 1% nonfat dry milk), and the blots were developed with enhanced chemiluminescence Western blotting detection reagents (Amersham Life Sciences, Arlington Heights, Ill.).

Primary antibody dilutions in 1% nonfat dry milk varied based on the antibody titer (1H6, 1:5,000; 3D3, 1:250; 2G10, 1:1,000; 1S4, 1:250). MAb 1H6, an N-terminal antibody which maps to amino acids 167 to 215 on σ^{70} (22), was used as a positive antibody control for the C-terminal truncations. The control antibody for N-terminal truncations was 1S4, which maps between amino acids 500 and 541 (29).

It was found that MAb 2G10 reacted with F1-486 (Fig. 3a) and failed to react with F1-481 (Fig. 3b). On the N-terminal end of the epitope, F470-613 reacted with MAb 2G10 (Fig. 3c), while F474-613 produced no signal with MAb 2G10 (Fig. 3d). Therefore, the epitope for MAb 2G10 includes at least part of the region between amino acids 470 and 486, a 23-amino-acid reduction from the previously determined epitope for MAb 2G10.

Truncations were also used to map the epitope for MAb 3D3. These results are summarized in Fig. 2. MAb 3D3 reacted with F1-471 but failed to react with F1-466. The N-terminal end of the epitope for MAb 3D3 was found with a positive reaction with F449-613 and no reaction with F456-613. Therefore, it can be determined that the epitope for MAb 3D3

FIG. 2. Schematic representation of all fragments synthesized through PCR/S-30 or gene cloning. +, a positive reaction between the antibody and fragment tested; -, no observable reaction between the antibody and fragment tested. Proposed boundaries of the epitopes for MAb 3D3 and MAb 2G10 are noted underneath *E. coli* σ^{70} residues 449 to 496. Secondary structure was predicted by the method of Rost and Sander (25).

FIG. 3. Analysis of the C-terminal and N-terminal boundaries of the epitope for MAb 2G10 by Western blot assays. (a and b) Reactions with F1-486 and F1-481, respectively. Protein fragments were synthesized in the S-30 system from PCR-generated templates (lanes 3, 6, and 9). Purified σ^{70} (lanes 1, 4, and 7) and an S-30 reaction mixture containing no added DNA (lanes 2, 5, and 8) served as controls. Blotted proteins were reacted with MAbs 1H6, 3D3, and 2G10 as indicated. The C-terminally truncated fragments are located under the breakdown product of endogenous sigma. (c and d) Cloned gene fragments corresponding to *E. coli* amino acids 470 to 613 and 474 to 613, respectively. The protein fragments were expressed by induction of the cloned gene fragment by the addition of isopropylthiogalactoside to logarithmically growing cells. Purified σ^{70} and uninduced culture served as controls. Induced (ind) samples were taken at 1.5 and 3 h. Blotted proteins were reacted with MAbs 3D3, 2G10, and 1S4 as indicated.

required at least part of the region between amino acids 449 and 471, a broader region than was originally proposed.

This paper defines the N-terminal and C-terminal boundaries of the epitope for one MAb that cross-reacts with many diverse bacteria and documents the epitope for another MAb which maps nearby. These epitopes can now be narrowed to a 17-amino-acid region for MAb 2G10 and 23-amino-acid region for MAb 3D3. However, as most contiguous epitopes contain 6 to 8 amino acids, it may be hypothesized that the epitopes for MAb 2G10 and MAb 3D3 are noncontiguous. Nevertheless, both MAbs react well with σ^{70} blotted out of SDS-PAGE gels onto nitrocellulose membranes. If the epitope consisted of two sites separated by approximately 10 amino acids, it is likely that this epitope would reform efficiently on the membrane. Of course, with the experimental design employed it is not possible to state which specific amino acids of that 17- or 23-amino-acid region are necessary to bind MAb 2G10 and MAb 3D3. In addition, because 4 to 5 amino acids were removed with each truncation, the exact end of each epitope is not known. It should be noted that Rao et al. (22) proposed that the epitope for MAb 2G10 is located between amino acids 434 and 475 $\pm$ 10. This method, which uses a His-tagged protein, is dependent upon estimations of molecular weights of

protein fragments on SDS-PAGE. The epitope map reported here (470 to 486) is more precise but consistent with the location proposed by Rao et al. (22) within the error of the His-tagged method.

The proposed epitope for MAb 3D3 (449 to 471) is 77% conserved in *E. coli* σ^{70} relative to *B. subtilis* σ^A and 63% conserved between *E. coli* σ^{70} and *C. trachomatis* σ^{66} . One part of that epitope (449 to 456) is highly conserved, while the region from 457 to 471 is weakly conserved. This may explain why MAb 3D3, unlike MAb 2G10, exhibits little cross-reactivity with the major sigma factors from other bacteria.

In addition, we have found that σ^F reacts with MAb 2G10 but σ^S does not (Fig. 4). Both of these minor sigma factors show a high degree of conservation within this region. A comparison of the region corresponding to the 2G10 epitope in the major sigma factors that cross-react with MAb 2G10 (for which the amino acid sequence is known) is shown in Table 1. Also shown in Table 1 is the corresponding region of σ^F and σ^S . While σ^F is highly conserved on both the N and C termini of the sequence, σ^S shows a high degree of conservation only in the C-terminal part of the sequence. A synthetic peptide containing the sequence EMGREPTPEELAE, which corresponds to amino acids 473 to 485 of *E. coli* σ^{70} , did not react with MAb

FIG. 4. Reactivity of MAb 2G10 with *E. coli* σ^F . Purified sigma factors (100 ng per lane) were analyzed by Western blot. Lanes 1 to 3 were reacted with MAb 2G10; lanes 5 to 7 were reacted with MAb 1RS1, a MAb specific to σ^S (20). Lane 4 contained prestained molecular weight markers that allowed the blot to be cut apart for the antibody reactions and then realigned precisely.

2G10 (data not shown). Because F470-613 did react with MAb 2G10 and F474-613 did not react with MAb 2G10, it is likely that this synthetic peptide did not contain the N-terminal portion of the epitope.

TABLE 1. Comparison of the 2G10 epitopes of different species^a

SIGMA	SEQUENCE	Reacts with 2G10	Match to underlined consensus
<i>Ec</i> <i>rpoD</i>	⁴⁷⁰ MLQEMGREPTPEELAE ⁴⁸⁶	Yes	15/15
<i>Ct</i> <i>rpoD</i>	LMETCYKEPTPEELGEE x x	Yes	13/15
<i>Bs</i> <i>rpoD</i>	LLQDLGREPTPEELAE	Yes	15/15
<i>Pa</i> <i>rpoD</i>	MLQEMGREPTPEELGER	Yes	15/15
<i>An</i> <i>rpoD</i>	LSQEMGRKPTPEELIATR x x x x	Yes	11/15
<i>Sa</i> <i>rpoD</i>	LLQDLGRDPAPEETIGEE x	Yes	14/15
<i>Ec</i> <i>fliA</i>	LEQELGRNATEVEAER x x x x x	Yes	10/15
Consensus	<u>MLQEMGREPTPEELAE</u>		
<i>Ec</i> <i>rpoS</i>	LSKLDHRPSAVEIAEQ x x x x x	No	8/15

^a Sequences are aligned according to the method of Lonetto et al. (16). The sequence of *E. coli* FliA (14, 19) is identical to that of *S. typhimurium* FliA. A consensus sequence, corresponding to the most highly conserved amino acids among the major sigma factors (RpoD) and *E. coli* FliA (σ^F) that react with MAb 2G10, is shown. Underlined amino acids in the consensus sequence include those residues which represent complete identity or a highly conservative change in five or more of the seven sequences that react with MAb 2G10. An amino acid in a given sequence is considered to match the underlined consensus if it is identical or a highly conservative change. Amino acid residues of the eight sigma sequences (including the sequence of *E. coli* σ^S [RpoS] which does not react with MAb 2G10) that are not identical to or a highly conservative change from the underlined amino acids of the consensus are indicated by an "x" underneath the residue. Note that *E. coli* RpoS shows the least match to the underlined consensus and that the mismatches are clustered in the region corresponding to *E. coli* σ^70 positions 471 to 481. Abbreviations: *Ec*, *E. coli*; *Ct*, *C. trachomatis*; *Bs*, *B. subtilis*; *Pa*, *P. aeruginosa*; *An*, *Anabaena* sp.; *Sa*, *S. aureus*.

Recently, a crystal structure for a protease-resistant fragment of *E. coli* σ^70 has been solved (17). This fragment contains residues 114 to 448 but does not include the region presented in Table 1. However, a secondary-structure prediction for the region containing amino acids 449 to 496, using the program PHDsec (25), suggests α -helices for amino acids 454 to 474, 480 to 487, and 491 to 501 and a loop at 475 to 479. This secondary-structure prediction is shown in Fig. 2. A helix-loop-helix motif for the epitope would explain why the epitope requires about 17 amino acids and why sequence conservation on the ends of the epitope in σ^F is important. Finally, MAb 2G10 binds to holoenzyme and inhibits transcription (29). We have also found that 2G10 binds native σ^70 in solution (our unpublished data), indicating that this epitope is exposed in free σ^70 as well as in the holoenzyme. Thus, MAb 2G10 probably binds to an exposed helix-loop-helix.

The role of region 3 is not yet understood. Mutations in σ^70 at positions 504 and 506 result in ppGpp-independent transcription of several amino acid synthesis operons (4), but these amino acids are almost 20 amino acids C terminal to the 2G10 epitope. Likewise, Severinov et al. (27) have found that nucleotides bound in the initiating site in open complexes can cross-link to σ^70 in the C-terminal portion of region 3 (amino acids 508 to 561). These results, along with our data, suggest that region 3 contains several structural and functional features.

Now that the 2G10 epitope has been localized to 17 amino acids, site-directed mutagenesis on the 2G10 epitope may be conducted in an effort to learn more about the function of this region of the major sigma factor of bacteria.

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