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The epitope for the polyol-responsive monoclonal antibody 8RB13 is in the flap-domain of the beta-subunit of bacterial RNA polymerase and can be used as an epitope tag for immunoaffinity chromatography

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Abstract

Polyol-responsive monoclonal antibodies (PR-mAbs) are useful for the purification of proteins in an easy, one step immunoaffinity step. These antibodies allow for gentle purification of proteins and protein complexes using a combination of a low molecular weight polyhydroxylated compound (polyol) and a nonchaotrophic salt in the eluting buffer. mAb 8RB13 has been characterized as one of these PR-mAbs and has been used to purify RNA polymerase from 5 species of bacteria. Here the epitope for 8RB13 has been identified as PEEKLLRAIFGEKAS, a sequence that is highly conserved in the β-subunit of bacterial RNA polymerase. This sequence is located in the "beta-flap" domain of RNA polymerase (and essentially comprises the "flap-tip helix"), an important binding site for sigma70. This location explains why only the core RNAP is purified using this mAb. This amino acid sequence has been developed into an epitope tag that can be used to purify a target protein from either bacterial or eukaryotic cells when genetically fused to a protein of interest.

Keywords

Immunoaffinity chromatography; polyol-responsive; epitope tag; RNA polymerase; beta-flap domain

Introduction

Polyol-responsive monoclonal antibodies (PR-mAbs) are extremely useful in the gentle purification of proteins and protein complexes in their native form while allowing the proteins and protein complexes to retain activity. Many immunoaffinity chromatography procedures use harsh conditions (e.g., low pH or chaotropic reagents) to release the antigen from the antibody. These conditions can result in the protein of interest being denatured. The

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PR-mAb allows for a simple, gentle elution of the protein of interest by using buffers containing low molecular weight polyhydroxylated compounds (polyols) and non-chaotropic salt (1-3). It is currently unknown why some mAbs are polyol-responsive and some are not, but the epitopes for PR-mAbs (designated “softags”) can be used as a way to tag and purify proteins of interest without affecting the protein’s function (4, 5).

In bacterial cells, the RNA polymerase (RNAP) can be in either the core enzyme form or in one of the holoenzyme forms. Core RNAP consists of 5 subunits (β’, β, α_2, and ω). A holoenzyme contains the core subunits plus a sigma factor (β’, β, α_2, ω, and σ); the sigma factor lends specificity to the RNAP to bind to and initiate transcription from different classes of promoters. In *E. coli*, there are 7 sigma factors; thus, there are 7 potential holoenzymes. We have isolated many mAbs that react with the β-subunit, but mAb 8RB13 has proven to be particularly interesting because it is polyol-responsive, and it reacts with the β-subunit of RNAP from many bacterial species (6). Using these properties, we have purified core RNAP from *E. coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Streptomyces coelicolor* (6), *Shewanella oneidensis* (7), and *Clostridium difficile* (our unpublished data). However, immunoaffinity chromatography with mAb 8RB13 isolates only the core form of RNAP from all of these bacteria despite the presence of a holoenzyme.

High-resolution crystal structures of core RNA polymerase, the holoenzyme, and enzyme/DNA complexes have become available (reviewed in 8). This study sought to identify the epitope for mAb 8RB13 to understand why the holoenzyme cannot be purified using this immunoaffinity column. In addition, we were able to demonstrate that fusing this epitope tag to a target protein allows the target protein to be purified from either bacterial extracts or from extracts from mammalian cell culture. By developing a novel epitope tag (designated “softag4”) from the 8RB13 epitope we increase the flexibility of this system to include purification of proteins expressed in eukaryotic cell lines.

MATERIALS AND METHODS

Plasmids and constructs

The plasmids used in this study are listed in Table 1. Plasmids TA501 and TA502, containing the coding regions for the *E. coli* β-subunit in the pET28b vector (Novagen, Madison, WI), have been described previously (9). Plasmid pTA501 contained a N-terminal His_6-tag and pTA502 contained a C-terminal His_6-tag (9). Plasmid pLN06 was constructed by amplifying nucleotides 2364-2870 of the β-subunit by polymerase chain reaction (PCR), using plasmid TA501 as a template. An oligo (5’-cat gcc atg gca tgt gtg tct ctg ggt gaa ccg) containing a *Nco*I site (underlined) on the 5’-end and 15 nucleotides specific to nucleotides 2364-2379 served as the forward primer; an oligo (5’-cgc gga tcc gcc atc gcg agt aaa gac) containing a *Bam*H1 site (underlined) and 15 nucleotides specific to nucleotides 2855-2870 served as the reverse primer. The amplified product was then ligated in frame at the *Nco*1 site contained in the N-terminal domain of human transcription factor IIB (TFIIB), and the *Bam*H1site in contained in vector pET11a. This created a fusion protein, containing amino acids 1-124 of TFIIB and amino acids 770-938 of the β-subunit (Fig. 2A). Plasmid pLH07 was created by subcloning the fusion protein from pLN06 (Nde1/BamH1) into pET33b. Plasmids containing epitope-tagged GFP and YFP for overexpression and purification are described below.

Protein expression

Prokaryotic—Plasmids were transformed into *E. coli* BL21(DE3)pLysS (Novagen). Bacteria were cultured in LB broth containing 30 μg/ml chloramphenicol and either 100 μ g/ml ampicillin (pET11a vector) or 35 μg/ml kanamycin (pET28b and pET33b vectors) at

37°C with shaking. Protein expression was induced when the culture reached an O.D. (600 nm) of 0.6 by the addition of 1 mM isopropylthiogalactoside (IPTG) and the cells were harvested 2.5-3.0 hr later. For epitope-tagged GFP, *E. coli* BL21(DE3) *pLysS* containing over-expressed GroEL and GroES (10) was transformed with the epitope-tagged GFP contained in the pET11a plasmid. The bacteria were cultured in LB broth containing ampicillin at 26°C until the optical density (600 nm) reached 0.3; a 10% solution of arabinose was added to achieve a 0.1% solution to induce the GroEL/GroES chaperones. The bacteria were then cultured until the O.D. reached 0.6, and the epitope-tagged GFP was induced by the addition of 1 mM IPTG. The cells were then cultured at 26°C for 3 hr, harvested by centrifugation and the pellet was frozen at -80°C until use.

Eukaryotic—Plasmids were transfected into HEK293 cells using TransIT-LT1 transfection reagent (MirusBio, Madison, WI) using the manufacturer's recommended protocol, and harvested after 48 hours. HEK293 cells were cultured in DMEM with 10% FBS and 1% Pen/Strep + L-glutamine. Cells were then resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and complete protease inhibitor cocktail from Roche) and placed in an ethanol/dry ice bath for 5 minutes; the cells were returned to the 37°C water bath for 1 min. The freeze-thaw procedure was performed for a total of three cycles.

Antibodies

The preparation of mAb 8RB13 has been described (6). mAb IIB8, that reacts with amino acids 61-68 of human TFIIB, has been described (5).

ELISA-elution assay

The polyol-responsive properties of mAb 8RB13 were observed by using a modified enzyme-linked immunosorbent assay (ELISA), termed ELISA-elution assy as described previously (1, 2). Briefly, antigen was immobilized on the wells of a polystyrene microtiter plate and the plates were blocked. After incubation with the mAb, the wells were treated with 100 μl of TE buffer (50 mM Tris-HCl and 0.1 mM EDTA, pH 7.9) containing varying amounts of ammonium sulfate (0-0.75 M) and propylene glycol (0-40%). After washing, the enzyme-labeled secondary antibody was applied, and then, after incubating and extensive washing, the substrate was applied. A polyol-responsive signal is defined as a reduction in signal of at least 50% in wells treated with buffer containing polyol and salt compared to those treated with TE buffer alone.

Antibody production, purification, and conjugation

mAb 8RB13 was produced in continuous culture, using INTEGRA CELLine CL350 Flasks (distributed by Argo, Elgin, IL) as described (3). Antibody was harvested every 3-4 days by removing the contents of the cell compartment, centrifuging the contents at 1500 rpm, removing the supernatant, and storing it at -20 °C. The cells were then diluted 1:4 with media, and 5 ml of the cells were returned to the growth chamber. The chamber was maintained for about 30-40 days.

Supernatant samples collected from the CELLine flasks were tested for 8RB13 antibody production using an ELISA and *E. coli* core RNAP as an antigen (1). Samples that titered out to at least 1:12,000 were pooled for purification. A typical purification used about 20 ml of the pooled cell culture supernatant. A saturated solution of ammonium sulfate was added to the supernatant to achieve 40% saturation (at 4°C) and stirred for 20 minutes. The precipitate was collected by centrifugation (20 minutes at 7000 rpm at 4 °C). The supernatant was removed, and the pellet was resuspended in 5 ml of antibody buffer (50 mM Tris-HCl, pH 6.9, containing 25 mM NaCl), then dialyzed overnight against 1 liter of

antibody buffer at 4°C overnight. Any precipitate was centrifuged out, and the supernatant was run on a 5-ml DE-52 column (DEAE-cellulose; Whatman) equilibrated with antibody buffer. At this pH and salt concentration, the mAb flows through the column and most contaminating proteins are retained. Fractions (1 ml) were collected and analyzed via SDS-PAGE. Peak fractions were pooled and stored at -20°C.

mAb 8RB13 was conjugated to cyanogen bromide-activated Sepharose 4B (GE Healthcare) as described previously (1, 6).

Mapping the 8RB13 epitope

Rough mapping by the ordered fragment ladder method—The use of the ordered-fragment ladder method for mapping the approximate locations of epitopes on the σ- and β'-subunits of *E. coli* RNAP has been described (4, 11, 12), and a schematic is presented in Fig. 1B. Briefly, the coding region for the *E. coli* β-subunit was analyzed using the MacVector software package (Oxford Molecular Group) to identify sites susceptible to chemical cleavage of the protein. The β-subunit containing either an N-terminal (pTA501) or C-terminal (pTA502) His_6-tag, was expressed in *E. coli*. The inclusion bodies were harvested, washed with TE buffer containing 1% Triton X-100 (Pierce) and solubilized in TE buffer containing 8 M urea. Denatured His_6-tagged β-subunit was then cleaved with hydroxylamine as described previously (9, 12). Denatured and cleaved peptides were then subjected to chromatography on a 1-ml Ni^{+2}-NTA column (Qiagen, Valencia, CA). After extensive washing with TE containing 8 M urea, the His-tagged peptides were eluted with imidazole, separated on a SDS-polyacrylamide gel, and a Western blot was prepared.

Mapping by exonuclease digestion—The Erase-a-Base system (Promega, Madison, WI) that uses Exonuclease III to remove bases at the 3' end of a DNA fragment was used to determine the relative position of the C-terminus of the epitope. Plasmid (pLN07) was digested with *Sac*1 to create a 3'-overhang in the vector to prohibit digestion into the downstream sequencing-primer; the plasmid was then digested with *Bam*H1 to provide a 5'-overhang for digestion into the TFIIB/β-peptide fusion. The plasmid was then digested with Exo III at 22°C to remove about 80 bp/min. Samples were removed at 15 s time-points, and the DNA samples were processed according to the manufacturer's protocol. After transformation into *E. coli* DH5α, clones were chosen, DNA minipreps were prepared (Qiagen), and the DNA was digested with *Nde*1 and *Not*1 in the vector to identify a set of nested deletions. Plasmids were then transformed into *E. coli* BL21(DE3)*pLysS*, induced with IPTG, run on SDS-PAGE, and the expressed proteins detected by Western blotting, using mAbs IIB8 and 8RB13. The exact positions of the truncations were determined by DNA sequencing.

Mapping by oligonucleotide tags—Oligonucleotide tags were used in the fine mapping of the 8RB13 epitope. These were synthesized at the University of Wisconsin Biotechnology Center. The tags were synthesized as complementary oligos with sticky *Nco*1/*Bam*H1ends and two stop codons before the *Bam*H1 site. The oligos were phosphorylated with polynucleotide kinase and ATP, combined in equimolar ratios, heated to 90°C for 20 min, allowed to anneal by slowly cooling to room temperature, and ligated into the *Nco*I site in TFIIB and the *Bam*HI site in the vector, creating a protein with the N-terminal domain of TFIIB and a short C-terminal tag. mAb 8RB13-reactive tags were identified by sequencing the clones, expressing the fusion protein in *E. coli*, and performing Western blots with mAbs IIB8 (in the N-terminal domain of TFIIB) and 8RB13.

Epitope tagging of GFP and YFP

Epitope-tagged GFP for expression in *E. coli* was prepared as follows. Two complementary oligonucleotides were synthesized that contained 5' sticky ends for *Bam*H1, the coding region for amino acids 897-911 of the β-subunit, two stop codons and 3' sticky ends for *Bam*H1. The oligonucleotides annealed as described above. This DNA fragment was ligated into the *Bam*H1 site of GFP contained in pET11a as described (4). DNA sequencing identified a clone that contained only one copy of the epitope in the proper orientation. This plasmid was designated pNT103.

To make the epitope tag for expression in mammalian cell culture, the pcDNA3.1 (+) plasmid (Invitrogen, Carlsbad, CA) with yellow fluorescent protein (YFP) inserted at the *Hind*III site was provided by Jennifer Lamberski (University of Wisconsin-Madison). The mAb 8RB13 epitope was prepared from two complementary synthetic oligonuclotides as above except the 5'- and 3'- ends contained sticky ends for *Kpn*I. Two stop codons were placed 3' to the epitope. The tag was then inserted in frame at *Kpn*I near the C-terminus of YFP. This plasmid was designated pBS1.

Purification of 8RB13-tagged proteins

The bacterial cells (containing pNT103) were lysed by thawing the pellet at room temperature for about 30 min and adding 20 ml (for 1 liter of original culture) of TE buffer containing 0.3 M NaCl. The cell slurry was then sonicated with a Branson digital sonicator equipped with a micro-probe. Five cycles of 15 sec each were applied, using 70% output on ice. The lysate was then centrifuged at 26,000 g for 20 min to remove any inclusion bodies. To the soluble fraction we added a 10% solution of polyethyleneimine (PEI) to achieve a final concentration of 0.3%, and the precipitate was removed by centrifugation (26,000 g for 20 min). The supernatant was applied to the column, washed with TE buffer, and then washed with TE + 0.5 M NaCl. The samples were eluted with TE containing 0.75 M ammonium sulfate and 40% propylene glycol.

A whole cell extract from HEK293 cells expressing epitope-tagged YFP (pBS1) was applied to a 1-ml mAb 8RB13-Sepharose column. The column was then washed with 10 bed volumes of TE + 0.5 M NaCl. Sample was eluted with 40% propylene glycol and 0.75 M ammonium sulfate in TE.

Results/Discussion

Previously, we had roughly determined the locations of epitopes for 7 mAbs on the β-subunit by the use of an "ordered fragment ladder" method (T. Arthur, Ph.D. Dissertation, University of Wisconsin-Madison, 2000); these epitopes are indicated in Fig. 1A. These mAbs were further characterized for the ability to bind to the core RNA polymerase or to the holoenzyme containing sigma70. mAbs 7RB77, 4RB1, 7RB145 and NT63 could all bind to both the core and holoenzyme, however, mAbs 7RB142, 7RB135, and 7RB122 did not bind to either the core or holoenzyme in solution (unpublished data). Curiously, mAb 8RB13, which we isolated at a later date, could bind to the core enzyme but not to the holoenzyme (6, 7). Thus, we sought to map the epitope for this useful mAb.

Epitope-mapping

Numerous methods for mapping epitopes have been described. The β-subunit of *E. coli* RNAP is 1342 residues, one of the largest proteins in *E. coli*. Our previous attempts to map epitopes contained on bacterial antigens by phage-display was not successful, presumably due to interference by the endogenous epitope in the screening process. Thus we chose to

use a combination of peptide generation and isolation, exonuclease digestion, and finally oligonucleotide tagging to identify the epitope for mAb 8RB13.

The ordered-fragment ladder method is shown in the flow chart in Fig. 1B. Using hydroxylamine, we cleaved the denatured β-subunit, separated the fragments containing the terminal His_6-tags by metal affinity chromatography under denaturing conditions, and subjected the fragments to SDS-PAGE and Western blotting. This technique roughly maps the epitopes by comparing the patterns obtained using the N- and C-terminally-tagged fragments. Because preliminary data indicated that the mAb 8RB13 epitope is located toward the C-terminus of the protein, we roughly mapped the epitope for mAb 8RB13 by the ordered-fragment method with respect to the mapped epitopes for mAbs 7RB122 and NT63 (Fig. 1A). The Western blot in Fig. 1B shows that mAb NT63 reacted with the 42 kDa fragment isolated from the C-terminally His_6-tagged protein, while mAbs 7RB122 and 8RB13 did not react with this fragment. mAbs 8RB13 and NT63 reacted with the 53 kDa fragment from the C-terminally tagged protein, but mAb 7RB122 did not react with this fragment. Likewise, mAbs 7RB122 and 8RB13 reacted with the 109 kDa N-terminally His_6-tagged fragment, but mAb NT63 did not. mAb 7RB122 reacted with the 80 kDa N-terminally tagged fragment, but mAb 8RB13 did not. Thus, the epitope for mAb8RB13 lies between the epitopes for mAbs 7RB122 and NT63, a peptide containing amino acids 770 to 938. The roughly mapped epitope is indicated in the map in Fig. 2A.

We amplified the DNA sequence encoding amino acids 770 to 938 of the β-subunit and fused it to the N-terminal domain of TFIIB at the endogenous *Nco*1 site of TFIIB (Fig. 2A). mAb IIB8 reacts with an epitope contained in the N-terminal domain (amino acids 61-70) of human TFIIB (5). The coding region for the fusion protein was sub-cloned into the *Nde*1 and *Bam*H1 site of pET33b (now designated pLN07). This fusion protein (about 28 kDa) could be easily distinguished in an *E. coli* expression system by Western blotting, using mAbs IIB8 and 8RB13. The construct was subjected to a controlled 3' digestion, using Exo III and the Erase-a-Base system (Promega) which resulted in truncations from the C-terminus of the β-subunit peptide. Nested deletions from the 3' end were expressed and assayed by Western blots. The schematic in Fig 2A shows that fusion proteins containing amino acids 770 to 938 and 770 to 921 reacted with both mAbs. However, the peptide containing amino acids 770 to 880 reacted with mAb IIB8, but did not react with mAb 8RB13. Thus the epitope for mAb 8RB13 was localized to residues 881-921 (indicated in Fig. 2B). This region that has been designated the β-flap domain and is important for interaction with region 4 of sigma70 and initiation from promoter sites containing a -35 promoter sequence (13). Also shown in Fig. 2B, is the alignment of the corresponding amino acid sequences from the β-subunits from the other 5 bacterial species from which we have purified RNAP using PR-mAb 8RB13 (6, 7). Not surprisingly, these sequences show a very high degree of similarity.

To identify the 8RB113 epitope, a series of overlapping oligonucleotide tags were synthesized that correspond to the 41-residue region (amino acids 881-921 in *E. coli*) and fused to the N-terminal domain of TFIIB. Of these fusions, the most informative were residues 881 to 911, which reacted with the control mAb IIB8 and mAb 8RB13, and residues 881 to 896, which reacted with the control mAb IIB8, but not with mAb 8RB13 (Fig. 2B). As a result, we have determined the epitope for mAb 8RB13 is contained within amino acids 897-911 (PEEKLLRAIFGEKAS) from the β-subunit of *E. coli* RNAP. This region contains the entire β-flap-tip helix as well as one residue from Flap Arm 1 and five residues the Flap Arm 2 (14). Interestingly, this region is where an important interaction between conserved region 4 of σ70 of *E. coli* and the hydrophobic patch on the flap-tip helix (residues underlined above) seems to be involved in this interaction (14). Thus, the location of the epitope explained the high level of conservation between the listed bacterial species

and why immunoaffinity chromatography using mAb 8RB13 isolates only the core enzyme from all bacteria tested (6, 7).

Epitope tagging GFP and purification from *E. coli*

To establish that this working epitope is sufficient for use in immunoaffinity chromatography, we genetically fused it to the C-terminus of GFP and expressed it in *E. coli* as described in Materials and Methods. Because we wished to purify the epitope-tagged GFP from the soluble fraction, we used an *E. coli* strain that could be induced to produce the GroEL and GroES chaperones as well as the target protein. Induction in the presence of these chaperones helps to increase the solubility of the target protein (10). We cultured the bacteria at 26°C, which also encourages expression of soluble protein. Because the soluble fraction also contained RNAP, which was the origin of the 8RB13 epitope, we removed the endogenous RNAP prior to the purification of the epitope-tagged GFP by treating the soluble fraction with 0.3% PEI in the presence of 0.3 M NaCl as described in Materials and Methods. The PEI-treated material was diluted 4 fold and applied to a 2-ml 8RB13-Sepharose column. After washing with TE buffer, the epitope-tagged GFP was eluted with TE buffer containing 0.75 M ammonium sulfate and 40% propylene glycol. The SDS-PAGE gel in Fig. 3 shows that the epitope-tagged GFP was bound and eluted from the 8RB13-Sepharose.

Examination of the polyol-responsiveness of the epitope-tagged protein

After the 8RB13-tagged GFP was isolated from the *E. coli* extract, we re-examined the ability of ammonium sulfate and propylene glycol to disrupt the antigen-antibody interaction by using the ELISA-elution assay. The purified GFP and the entire core RNA polymerase were immobilized on the microtiter plate. After reaction with mAB 8RB13, the plate was treated with different combinations of ammonium sulfate and propylene glycol. The results are shown in Fig. 4. The mAb eluted from the epitope-tagged GFP in a polyol-responsive manner (Fig. 4B) and actually eluted at lower concentrations of the salt and polyol than it did from the core RNA polymerase (Fig. 4A).

Epitope tagging of YFP for use in mammalian cells

To create a tagged protein for use in a mammalian expression system, the mAb 8RB13 epitope (PEEKLLRAIFGEKAS) was fused to the C-terminus of YFP. We chose YFP for this fusion because it is easy to determine successful transfection rates by simply looking for fluorescence of the cells with UV-microscopy. The fusion was then overproduced in HEK293 cells, a cell extract was prepared, and the soluble material was applied to a mAb 8RB13-conjugated Sepharose column. After washing, the fusion was eluted from the column using polyol and salt and an SDS-PAGE was run. Coomassie blue-staining (Fig. 5A) and western blotting with mAb 8RB13 (Fig. 5B) showed that the epitope-tagged YFP bound to the column and was eluted with salt and polyol. Thus, this epitope tag is useful as a polyol-responsive immunoaffinity tag in mammalian cell culture.

Conclusion

Previously, we identified a PR-mAb 8RB13 that was widely cross-reactive with the β-subunit of RNAP from many bacterial species. We have purified core RNAP from five bacterial species using this mAb (6, 7), and because of its broad cross-reactivity, we suspect core RNAP can be purified from almost any γ-proteobacterium. Using various biochemical methods, we narrowed the epitope to amino acids 897-911 (PEEKLLRAIFGEKAS) of the β-subunit. An alternative method of using peptide arrays was considered, but the expense of this method was beyond the budget for the project. In addition, in retrospect, the surprising length of the epitope (15 amino acids) would have made this approach more difficult. The

epitope sequence is highly conserved among bacterial species (Fig. 2B), explaining the broad cross-reactivity of this mAb.

mAb 8RB13 is unique among the mAbs shown in Fig. 1A, in that it binds to the core enzyme but not to the holoenzyme. The 8RB13 epitope was found to be located in a well-studied region of RNAP called the β-flap, and more specifically, the epitope comprises a specific area called the flap-loop helix. This region is a "stirrup"-shaped protrusion that interacts with region 4 of the σ^{70} subunit. Thus, the epitope is exposed only when the sigma factor is not occupying that binding site, explaining why purification of RNA polymerase on an immunoaffinity column containing mAb 8RB13 isolates only the core polymerase form of the enzyme. We do not know if mAb 8RB13 displaces sigma70 from the holoenzyme or simply binds only to the epitope on the core RNAP. Neither do we know if holoenzymes that contain other sigma factors will bind to 8RB13.

In addition, the sequence PEEKLLRAIFGEKAS can be genetically fused to a protein of interest and the gentle polyol-responsive protocol can be used to purify the protein from both bacterial (Fig. 3) and mammalian cells (Fig. 5), using mAb 8RB13 immunoaffinity chromatography. We have designated this PR-mAb epitope "softag4." When the epitope is fused to the C-terminus of GFP, lower concentration of salt and polyol seems to be effective at disrupting the antigen-antibody interaction than when it is contained in the natural environment of the β-subunit (Fig. 4). We are further investigating the effect of the placement of the epitope on sensitivity to salt/polyol elution. If the apparent increase in poly-responsiveness of the mAb to the epitope tag over that seen with endogenous epitope is established, the usefulness of this epitope tag to recovering tagged proteins, and their protein complexes, from eukaryotic cells will be increased.

Acknowledgments

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References

1. Thompson NE, Hager DA, Burgess RR. Isolation and characterization of a polyol-responsive monoclonal antibody useful for the gentle purification of *Escherichia coli* RNA polymerase. Biochemistry. 1992; 31:7003–7008. [PubMed: 1637835]
2. Thompson NE, Burgess RR. Identification of polyol-responsive monoclonal antibodies for use in immunoaffinity chromatography. Current Protocols in Molecular Biology. 2001:11.18.1–11.18.9.
3. Thompson NE, Foley KM, Stalder ES, Burgess RR. Indentification, production, and use of polyol-responsive monoclonal antibodies for immunoaffinity chromatography. Methods Enzymol. 2009; 463:475–494. [PubMed: 19892188]
4. Thompson NE, Arthur TM, Burgess RR. Development of an epitope tag for the gentle purification of proteins by immunoaffinity chromatography: application to epitope-tagged green fluorescent protein. Anal Biochem. 2003; 323:171–179. [PubMed: 14656522]
5. Duellman SJ, Thompson NE, Burgess RR. An epitope tag derived from human transcription factor IIB that reacts with a polyol-responsive monoclonal antibody. Prot Express Purif. 2004; 35:147–155.
6. Bergendahl V, Thompson NE, Foley KM, Olson BM, Burgess RR. A cross-reactive polyol-responsive monoclonal antibody useful for isolation of core RNA polymerase from many bacterial species. Prot Express Purif. 2003; 31:155–160.
7. Probasco MD, Thompson NE, Burgess RR. Immunoaffinity purification and characterization of RNA polymerase from *Shewanella oneidensis*. Prot Express Purif. 2007; 55:23–30.
8. Lane WJ, Darst SA. Molecular evolution of multisubunit RNA polymerases: Structural analysis. J Mol Biol. 2010; 395:395–704.

9. Arthur TM, Burgess RR. Localization of a σ70 binding site on the N- terminus of the *Escherichia coli* RNA polymerase β' subunit. J Biol Chem. 1998; 273:31381–31387. [PubMed: 9813048]

10. Bu S, Tsang PWK, Fu RZ. GroEL-GroES solubilizes abundantly expressed xylulokinase in *Escherichia coli*. J Appl Microbiol. 2005; 98:210–215. [PubMed: 15610434]

11. Rao L, Jones DP, Nguyen LH, McMahan SA, Burgess RR. Epitope mapping using histidine-tagged protein fragments: Application to *Escherichia coli* RNA polymerase sigma70. Anal Biochem. 1996; 241:141–157.

12. Burgess RR, Arthur TM, Pietz BC. Mapping protein-protein interaction domains using ordered fragment ladder far-western analysis of hexahistidine-tagged fusion proteis. Methods Enzymol. 2000; 328:141–157. [PubMed: 11075344]

13. Kuznedelov K, Minakhin L, Niedziela-Majka A, Dove SL, Rogulja D, Nickels BE, Hochschild A, Heyduk T, Severinov K. A role for the interaction of the RNA polymerase flap domain with the σ subunit in promoter recognition. Science. 2002; 295:855–857. [PubMed: 11823642]

14. Geszvan K, Gruber TM, Mooney RA, Gross CA, Landick R. A hydrophobic patch on the flap-tip helix of *E coli* RNA polymerase mediates σ70 region 4 function. J Mol Biol. 2004; 343:569–587. [PubMed: 15465046]

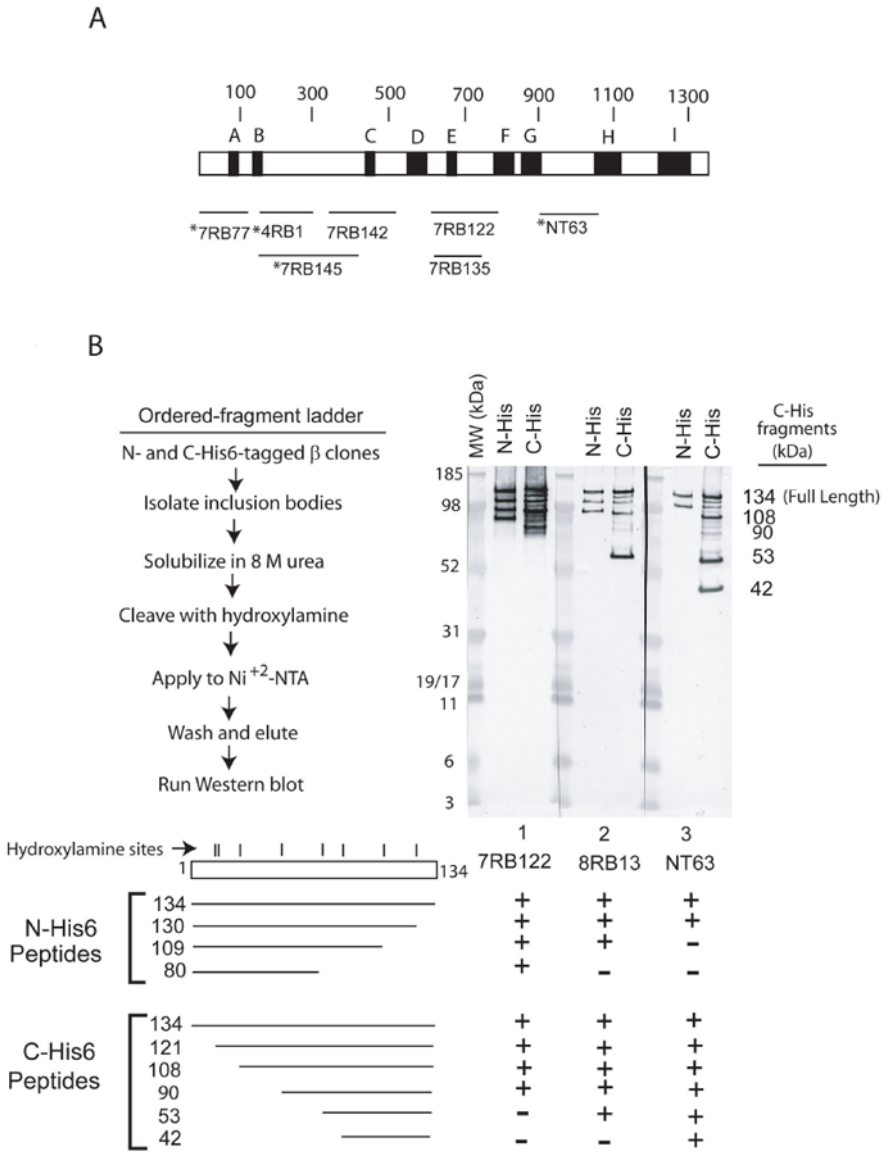

Fig. 1. Epitope mapping of mAbs on the β-subunit of *E. coli* RNA polymerase by the ordered ladder fragment method

(A) Schematic of the β-subunit with the conserved regions (capital letters) and the approximate locations of 7 mAbs that had been previously mapped (Arthur, Ph.D. thesis) using the ordered ladder fragment method. The data were generated using a combination of data obtained using cleavage of the β-subunit with hydroxylamine and with 2-nitro-5-thiocyanobenzoic acid (NTCB) followed by the ordered fragment method for epitope mapping. mAbs 7RB77, 4RB1, 7RB145, and NT63 (designated by asterisk) immunoprecipitate both the core RNA polymerase and the holoenzyme containing sigma70. (B) Ordered fragment ladder, using hydroxylamine cleavage of the β-subunit. On the top left is a flow-chart of the ordered fragment method used to map the location of mAb 8RB13 in relationship to mAbs 7RB122 and NT63. On the top right is a Western blot showing the reactions of the fragments with mAbs 7RB122, 8RB13, and NT63. On the bottom is the summary of the data from the hydroxylamine cleavage and the reactivity of the 3 mAbs with the fragments generated.

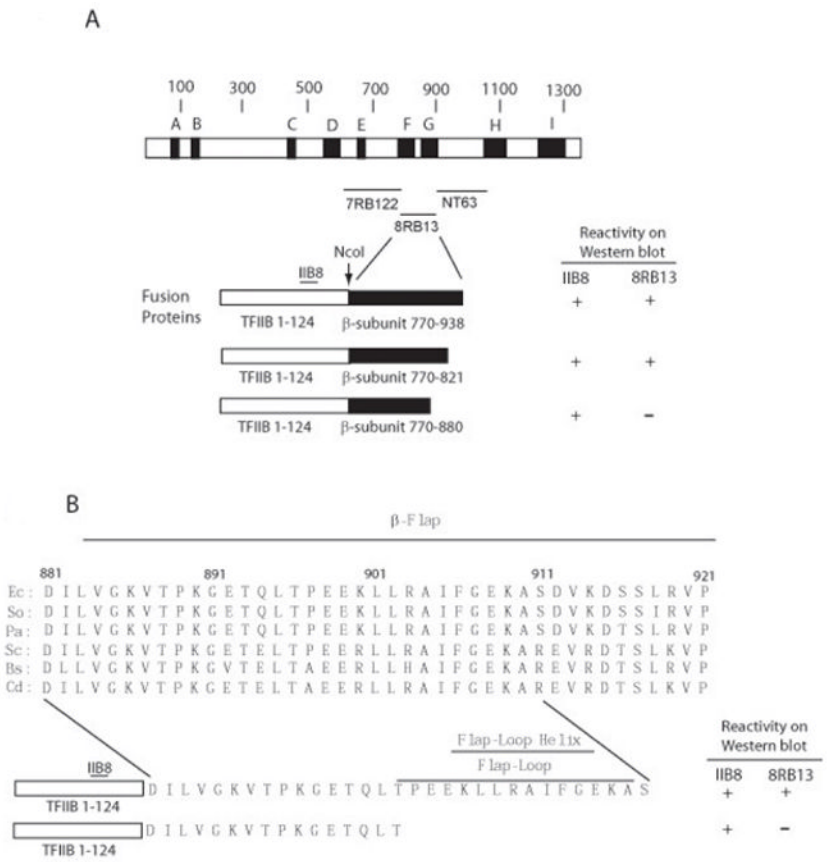

Fig. 2. Fine-structure mapping shows that the epitope for mAb 8RB13 is located in the flap-loop

(A) Location of the 8RB13 epitope between the epitopes for mAbs 7RB122 and NT63 and encompassing conserved regions F and G. This region of the β-subunit (residues 770-938) was amplified by PCR and fused to the N-terminal domain (1-124) of the human transcription factor TFIIB, which contains the epitope for the TFIIB-specific antibody, mAb IIB8 (underlined). Deletions were made from the 3'-end of the chimeric gene using controlled Exo III digestion, the proteins were expressed in *E. coli*, and Western blots were reacted with both mAb IIB8 and 8RB13. Deletion of residues 881-938 resulted in loss of the reactivity with mAb 8RB13, but not mAb IIB8. (B) The sequence of the β-subunit of *E. coli* residues 881 to 921 aligned with the corresponding regions of the β-subunits from the 5 other bacterial species from which the core RNA polymerase has been purified using mAb 8RB13. Abbreviations are: Ec (*Escherichia coli*), So (*Shewanella oneidensis*), Pa (*Pseudomonas aeruginosa*), Sc (*Streptomyces coelicolor*), Bs (*Bacillus subtilis*), and Cd (*Clostridium difficile*). Nested oligonucleoide tags were made from this region, fused to the N-terminal domain of TFIIB, expressed in *E. coli*, and Western blots were probed with mAbs IIB8 and 8RB13. Only tags containing the residues PEEKLLRAIFGEKAS reacted with mAb 8RB13, which is in the flap-loop structure of the β-subunit.

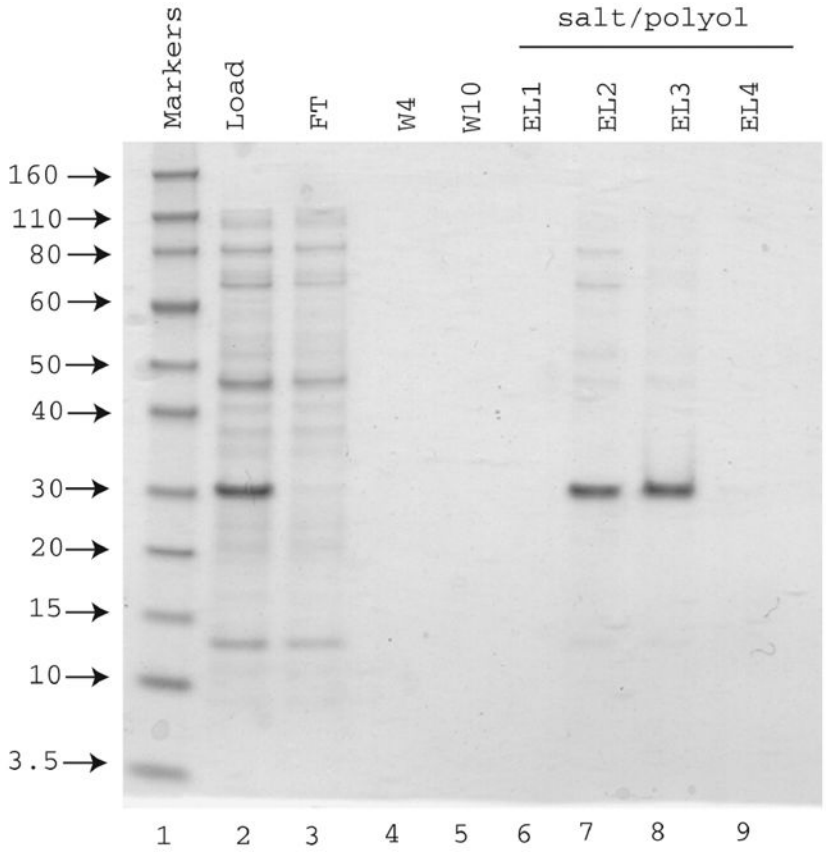

Fig. 3. Purification of epitope-tagged GFP from a bacterial expression system

Plasmid NT103 was transformed into *E. coli* BL21(DE3) *pLysS*, containing overexpressed GroEL and GroES. The soluble fraction was processed as described in the text and loaded (lane 2) onto an 8RB13-Sepharose column. The flow-through faction (Lane 3) was collected, and the column washed with ten 1-ml fractions of buffer (washes 4 and 10 are shown in lanes 4 and 5). The epitope-tagged GFP was eluted with 1-ml fractions of TE containing 0.75 M ammonium sulfate and 40% propylene glycol (lanes 6-9). Lane 1 contained molecular weight markers.

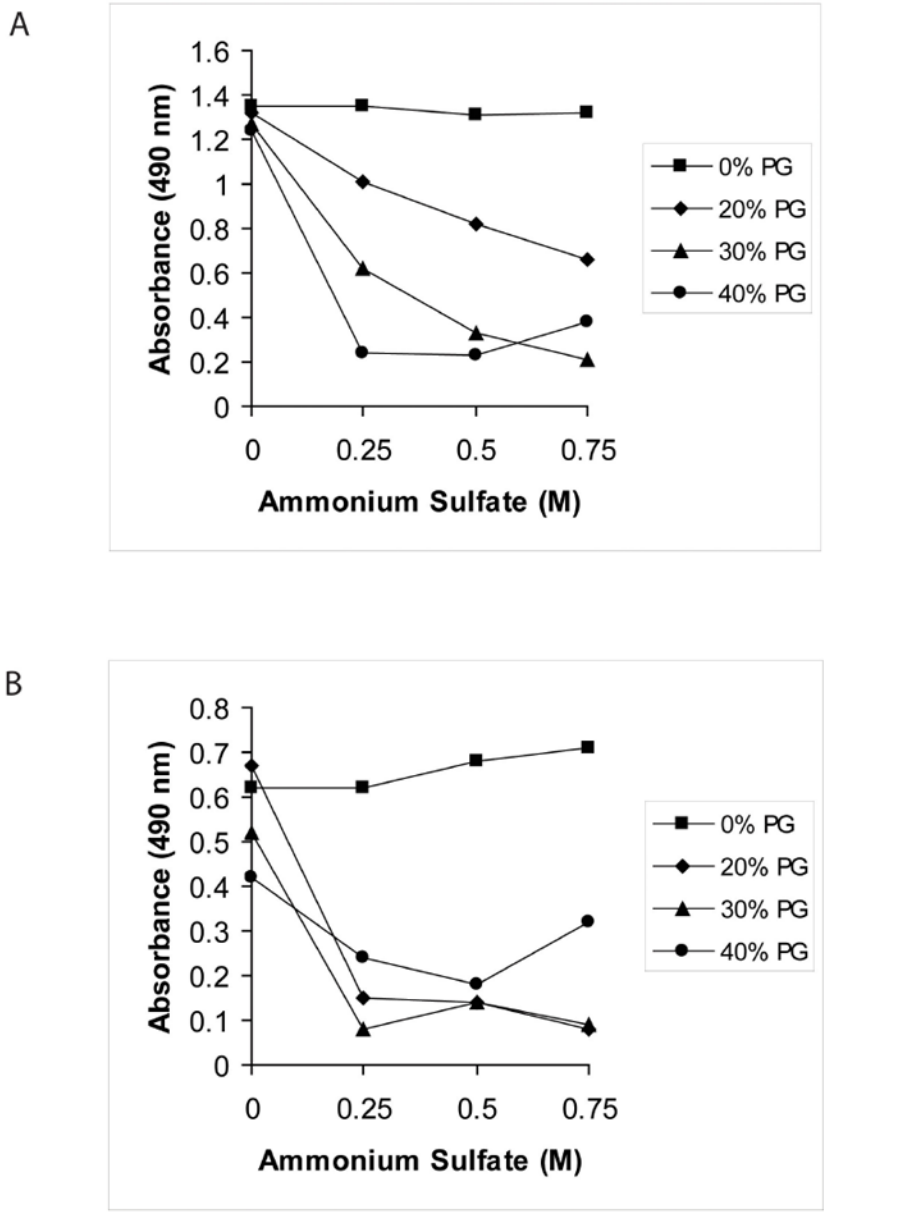

Fig. 4. ELISA-elution assay using mAb 8RB13

(A) The wells of the microtiter plate were coated with 25 ng of *E. coli* core RNA polymerase. The eluting buffer was TE containing 0-0.75 M ammonium sulfate and 0-40% propylene glycol (PG). Elution was measured by the absorbance reading of the substrate used to detect the presence of the secondary antibody bound to mAb 8RB13 after the elution step. (B) Identical to panel A except the 50 ng of epitope-tagged GFP were used to coat each well.

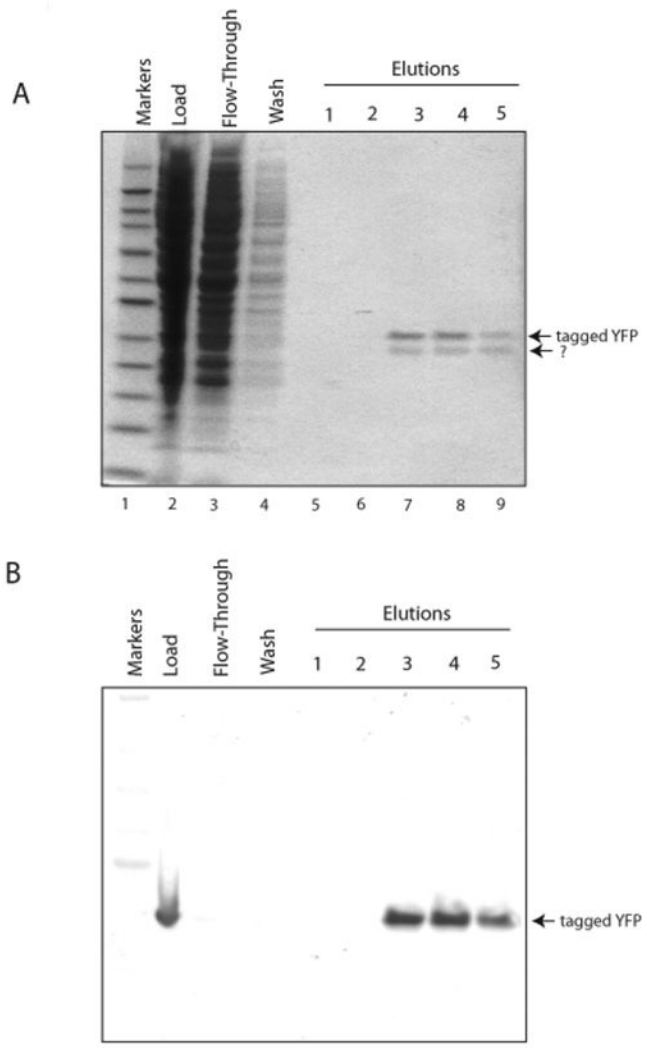

Fig. 5. Purification of epitope-tagged YFP from a mammalian expression system

Plasmid pBS1 containing epitope-tagged YFP was transfected into HEK293 cells. After expression of the YFP (about 48 hrs, confirmed by UV-microscopy) a whole cell extract was applied to a 8RB13-Sepharose column. (A) Coomassie blue–stained gel of the fractions from the chromatography. The identity of the band designated with the question mark is not known. (B) Western blot of an identical gel, using mAb 8RB13. The lanes contained the following: Molecular weight markers (lane 1), 40 μg of whole cell extract that was loaded to the 8RB13 column (lane 2), flow-thrugh fraction (lane 3), buffer wash (lane 4), and the five 1-ml salt/polyol elutions (lanes 5-9).

Table 1

Plasmids used in this study

Plasmid Designation	Construct Description	Description of protein produced	Reference
pTA501	Nucleotides 1-1342 of the β-subunit of *E. coli* RNAP in pET28b	Full-length β-subunit with N-terminal His6-tag	9
pTA502	Nucleotides 1-1342 of the β-subunit of *E. coli* RNAP in pET28b	Full-length β-subunit with C-terminal His6-tag	9
pLN06	Nucleotides 2364-2870 of the β-subunit inserted into the *Nco1* site of human TFIIB and the *Bam* H1 site in pET11a	Fuses aa 770-938 of the β-subunit to aa 1-24 of human TFIIB	This study
pLN07	*Nde1/Bam* H1 (complete fusion construct from pLN06) cloned into pET33b	Fusion protein from pLN06, making it amenable to Exo III digestion	This study
pNT103	Oligo encoding mAb 8RB13 epitope inserted 3' to GFP in pET11a	Fuses aa 897-911 of the β-subunit to C-terminus of GFP	This study
pBS1	Oligo encoding mAb 8RB13 epitope inserted 3' to YFP in pcDNA3.1(+)	Fuses aa 897-911 of the β-subunit to the C-terminus of YFP	This study