Characterization of monoclonal antibodies against *Escherichia coli* core RNA polymerase

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Multiple interactions with DNA, RNA and transcription factors occur in a transcription cycle. To survey the proximity of some of these factors to the *Escherichia coli* RNA polymerase surface, we produced a set of nine monoclonal antibodies (mAbs) against the enzyme. These mAbs, located at different places on the surface of the enzyme, were used in a co-immunopurification assay to investigate interference with the binding of NusA, σ70, GreB and HepA to core RNA polymerase. One of these mAbs turned out to be the first antibody inhibitor of the binding of NusA and σ70; it did not affect GreB and HepA interactions. Its epitope was located on the β' subunit at the C-terminus of region G. The properties of this mAb reinforce the idea that the mutually exclusive binding of NusA and σ70 to core RNA polymerase is due to, at least partially, overlapping binding sites, rather than allosteric interaction between two distant binding sites. This mAb is also useful to understand the occupancy of σ70, NusA and Gre proteins on core RNA polymerase.

Key words: inhibitor, protein–protein interactions, σ70, transcription, SPR.

INTRODUCTION

The DNA-dependent core RNA polymerase of *Escherichia coli* is composed of three subunits (α_2, β and β') and is able to processively synthesize an RNA chain from a DNA template. Additional factors are needed, however, to transcribe biologically relevant DNA [1], to increase elongation efficiency and to recognize transcription terminators [2–4]. Over the years, biochemical, biophysical and genetic data have accumulated on this enzyme. The basic mechanistic aspects of the transcription cycle are now more clearly understood, and most of the factors interacting with core RNA polymerase have been identified. A necessary step to further address the functions of these transcription factors would be to locate their binding site(s) on the primary sequence of core RNA polymerase, their occupancy at the surface of the enzyme and the conformational changes they induce at each transcription step. Unfortunately, structure–function relationship studies are impaired by the lack of a high resolution three-dimensional structure of core RNA polymerase, either free or in complex with RNA, DNA or transcription factors. Recently the high-resolution structure of *Thermus aquaticus* RNA polymerase was published [5]. Its high degree of sequence similarity with the *E. coli* enzyme allows the superposition of structural data from *T. aquaticus* and functional data from *E. coli*. This strengthens the interest of new structure–function studies with anti-(RNA polymerase) monoclonal antibodies (mAbs).

mAbs have already proved their usefulness to the study of the *E. coli* RNA polymerase [6–10]. Although some of the mAbs directed against *E. coli* core subunits give hints about the regions involved in core assembly [11], only one mAb directed against the α subunit exhibits slightly different binding properties against core RNA polymerase and holoenzyme [11]. To our knowledge there is still no report describing an anti-(RNA polymerase core) mAb whose binding is affected by NusA, GreA, GreB or HepA.

Antibodies against *E. coli* RNA polymerase have often been obtained by immunization with individual subunits [9,12]. In order to probe the location of the transcrption factors, and the conformational changes induced by the transcription factors at the surface of the RNA polymerase, we developed a set of mAbs directed against the entire core enzyme, hoping that these antibodies would be more representative of the whole enzyme structure. We show in the present study that one of these mAbs, directed against a highly antigenic and divergent region of β', behaves very differently from the previously described mAb [7,9]. This mAb turned out to be strongly affected by σ70 and NusA binding and to be a very potent transcription inhibitor, but did not affect GreB or HepA binding.

MATERIALS AND METHODS

Materials

E. coli core RNA polymerase was purchased from Epicentre Technologies (Madison, WI, U.S.A.). σ70, σ70 Δ1.1 (σ70 deleted from amino acids 1 to 102), σ70 Δ3–4 (σ70 deleted from amino acids 449 to 614), β'1 (amino acids 1–600 of β'), β'2 (amino acids 300–1000 of β'), β'3 (amino acids 821–1407 of β'), NusA, GreB and HepA were cloned in frame in the *Nde*1–*Not*1 sites of a pET21a vector. A C-terminal histidine tag was introduced at the C-termini of β'1, β'2 and β'3. σ70, β'1, β'2 and β'3 were expressed in *E. coli* BL21λDE3 cells (Novagen). Cells were harvested 4 h after induction with 1 mM isopropyl β-D-thiogalactoside, lysed and the proteins were extracted from inclusion bodies with 40 mM Tris/HCl (pH 8), 5 M urea, 10 mM 2-mercaptoethanol and 10 % (v/v) glycerol. Proteins β'1, β'2 and β'3 were then submitted to Ni^{2+}-nitrilotriacetate (Ni-NTA)–agarose (Qiagen) chromatography in the same buffer, eluted with this buffer supplemented with 200 mM imidazole, and stored in 40 mM Tris/HCl (pH 8), 5 M guanidinium chloride, 10 mM 2-

Abbreviations used: mAb, monoclonal antibody; Ni-NTA, Ni^{2+}-nitrilotriacetate; SPR, surface plasmon resonance.

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mercaptoethanol and 50 % (v/v) glycerol at −80 °C. σ70 was further purified according to Igarashi and Ishihama [13].

Standard protocols were used to fuse myeloma cells with immune spleen cells and to produce mAbs from ascites. The mAbs were purified by affinity chromatography on Protein A. All these mAbs are IgG1.

Epitope mapping of the polymerase by surface plasmon resonance (SPR)

A rabbit anti-mouse IgG1 Fc (RAM-G1; Biacore AB, Uppsala, Sweden) (60 μl; 0.33 μM) in 10 mM sodium acetate buffer (pH 5.0), was immobilized on a sensor chip surface, previously activated with 100 μM *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodi-imide hydrochloride/400 μM hydroxysuccinimide. The excess of activated carboxy groups was blocked with 1 M ethanolamine hydrochloride (pH 8.5). Each anti-core mAb was injected at a concentration of 0.33 μM in HBS [Biacore AB; 10 mM Hepes (pH 7.4), 150 mM NaCl, 3.4 mM EDTA and 0.005 % (v/v) Biacore™ surfactant]. The injection was performed at a flow rate of 20 μl/min, and 10 μl of 0.1 M HCl acid was used to regenerate the sensor chip. Complementarities of the mAbs were determined by a sandwich method: each mAb (mAb1) was captured on the sensor chip by a RAM-G1 antibody directed against the IgG Fc portion. After adding core RNA polymerase (30 nM; 60 μl) a second mAb (mAb2) was added. A volume of 60 μl of mAb1 in HBS buffer was injected at a flow rate of 20 μl/min and bound to the immobilized RAM-G1 antibody. The unoccupied sites of RAM-G1 were blocked with 3×60 μl of an unrelated mAb to avoid binding of mAb2 to unoccupied ligand sites. Core RNA polymerase (60 μl) was injected followed by 60 μl of mAb2. The surface was regenerated with 0.1 M HCl. Background levels of mAb2 binding were measured by replacing the core solution with buffer.

Co-immunopurification assay

The plasmids used in the *in vitro* translation experiments were purified with a Qiagen Midiprep kit according to the manufacturer's instructions. To avoid RNase contamination, an additional phenol extraction followed by an ethanol precipitation was performed. The ^{35}S-labelled proteins (σ70, σ70 deletions, NusA, GreB and HepA) were produced by *in vitro* transcription and translation in a reticulocyte lysate (STP3 T7 kit; Novagen) in the presence of 1 μg of plasmid and 40 μCi of [^{35}S]methionine, and stored at −80 °C. For immunoprecipitation, 2 μl of crude *in vitro* translated protein was incubated for 30 min at 22 °C, with 10 pmol of core polymerase in IP buffer [20 mM Tris/HCl (pH 8.4), 10 mM $MgCl_2$, 100 mM NaCl and 0.2 % (w/v) BSA], in the presence of 4 units of RNase A (Pharmacia). Protein A–Sepharose (50 μl; Pharmacia) was loaded in a minispin column and washed with 100 μl of IP buffer. Purified mAb (20 pmol) in 100 μl of IP buffer was added, and the column was washed three times with the same buffer to remove unbound mAbs. This amount of mAb was more than necessary to saturate the column. The core–^{35}S-labelled transcription factor complex was then loaded on to the column. Following brief centrifugation (3000 ***g*** for 30 s) the flow-through was collected, and after three successive re-loadings and centifugations, the column was washed three times with 100 μl of IP buffer. The bound complexes were eluted with 50 μl of sample buffer and loaded on to an SDS/15 % (w/v) polyacrylamide gel [14]. The gel was dried and exposed under autoradiography to Kodak Biomax-ms films and intensifying screens.

In vitro transcription assay

The well-characterized rrnB P1 promoter was PCR-amplified from *E. coli* K12 genomic DNA using the following primers: 5′-ATTATTGCCCGTTTTACAGCG-3′ and 5′-TCCGTATC-TTCGAGTGCCCA-3′. The transcription buffer consisted of 40 mM Tris/HCl (pH 8), 50 mM NaCl, 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The mAb (5 pmol) was incubated with RNA polymerase (2 pmol) in 10 μl of transcription buffer for 10 min at 37 °C. σ70 (1 pmol in 1 μl of transcription buffer) and the DNA template (1 pmol in 1 μl of transcription buffer) were then added, and the mixture was incubated again for 10 min at 37 °C. Transcription was initiated by adding 5 μl of NTP mixture {0.5 mM GTP, 0.5 mM ATP, 0.5 mM [^{32}P]UTP (4000 c.p.m./pmol), 0.5 mM CTP and 125 μg/ml rifampicin} in transcription buffer at 37 °C. Transcription was stopped at the indicated time with 150 μl of stopping buffer [40 mM Tris/HCl (pH 8), 20 mM Na_3EDTA, 250 mM NaCl, 0.4 % (w/v) SDS and 250 μg/ml yeast RNA]. Transcripts were purified by phenol extraction and ethanol precipitation, and were resolved on 6 % (w/v) polyacrylamide gels containing 7 M urea. The gels were dried and submitted to autoradiography.

RESULTS

Characterization of a set of mAbs raised against the core RNA polymerase

Mice were immunized with *E. coli* core RNA polymerase and after fusion with lymphoma cells a set of nine mAbs were selected by ELISA (11D11, 3C2, 2E11, 12D8, 3E10, 3E11, 8F4, 16F8 and 12D9). In a first approach, to make sure that the mAbs were located on different sites at the surface of the polymerase, we checked the independence of their binding sites using SPR. SPR allows real-time analysis of biospecific interactions without the need for labelling. Purified mAbs were non-covalently immobilized, using rabbit anti-mouse IgG1 covalently bound to the chip. A blocking, unrelated antibody was then injected three times to saturate the rabbit anti-mouse antibody. It was verified that no more antibody binding occurred after the third pulse.

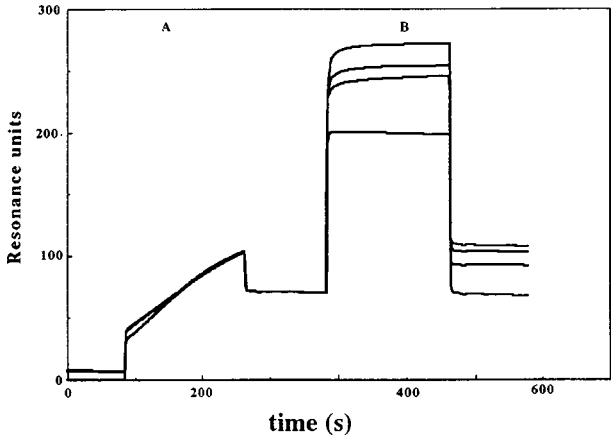

Figure 1 Evaluation of the complementarities between mAbs using SPR

A typical sensorgram of the mapping, performed as described in Table 1 and the Materials and methods section, is presented. mAb1 (12D8) was injected first (not shown), followed by the saturating unrelated mAb (not shown), core RNA polymerase (**A**) and mAb2 (**B**); top trace to bottom trace: 11D11, 3C2, 3E10 and 12D8.

Table 1 Evaluation of the complementarities between mAbs using SPR: summary of the results

The complementarities between mAbs were determined by a sandwich method as described in the Materials and methods section. Briefly, a rabbit anti-mouse IgG1 Fc was covalently immobilized on to a sensor chip. Each mAb (mAb1) was captured on the sensor chip by the rabbit anti-mouse antibody, and the unoccupied sites were blocked with an unrelated mAb to avoid binding of the second mAb. After adding core RNA polymerase, a second mAb (mAb2) was added. Results are presented as the percentage of inhibition of mAb2 binding to the polymerase. It was assumed that the molecular mass of an antibody is 42% of the molecular mass of core RNA polymerase, and that consequently saturating mAb2 concentrations should induce a 42% increase in resonance units due to core RNA polymerase.

mAb1	mAb2 ...	11D11	3C2	2E11	12D8	3E10	3E11	8F4	16F8	12D9
11D11		92	30	17	32	85	26	0	0	0
3C2		10	88	0	23	54	49	0	0	68
2E11		15	37	79	3	50	49	0	36	75
12D8		0	37	8	97	57	80	0	41	100
3E10		0	12	6	0	78	0	0	0	23
3E11		27	0	9	0	0	100	0	12	64
8F4		0	0	5	0	0	58	100	90	55
16F8		83	50	100	0	65	76	13	100	73
12D9		0	0	0	21	0	11	0	8	100

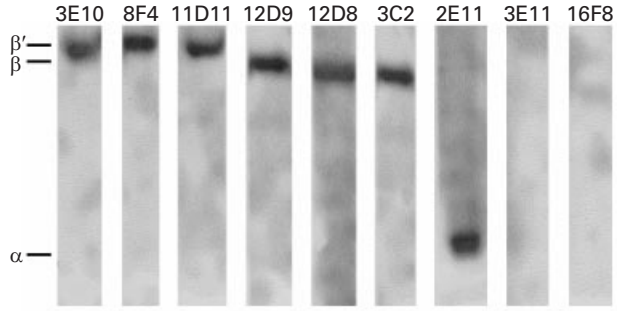

Figure 2 Determination of mAb specificity

Core RNA polymerase (10 pmol) in sample buffer was loaded on to an SDS/polyacrylamide gel [12% (for α) or 6% (for β and β') (w/v) polyacrylamide]. The proteins were transferred on to a nitrocellulose membrane, and the subunits were revealed by ECL® after incubation with each mAb, followed by incubation with an Fc-specific anti-mouse peroxidase conjugate.

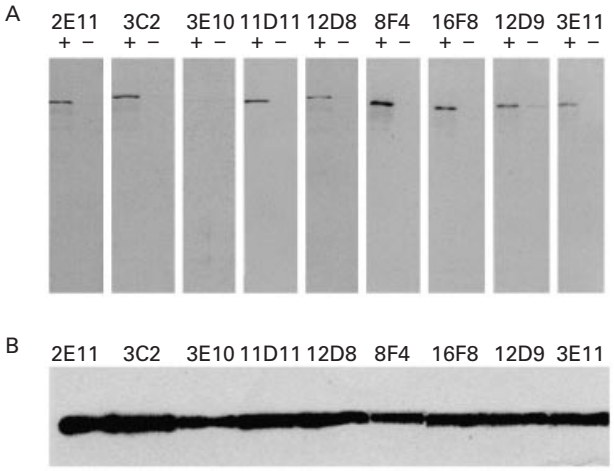

Figure 3 Co-immunopurification of σ70 and core RNA polymerase with the anti-(RNA polymerase) mAb

(**A**) The *in vitro* translated σ70 was incubated for 30 min at 22 °C with 10 pmol of core RNA polymerase to form the holoenzyme. Purified mAb (20 pmol) in 100 μl of IP buffer (+) or 100 μl of IP buffer only (−) was loaded on to a Protein A–Sepharose minispin column, and the column was washed three times with the same buffer to remove unbound mAbs. The core–^{35}S-labelled σ70 complex was then loaded on to the column. Following brief centrifugation, the column was washed with IP buffer, and the complexes were eluted with 50 μl of sample buffer and loaded on to an SDS/15% (w/v) polyacrylamide gel. The gel was dried and autoradiographed. Fractions corresponding to the loading, flow-through, washes and elution were loaded on to the gel; however, only the elution is presented to simplify the Figure. (**B**) The binding properties of each mAb for core RNA polymerase was evaluated by the same immunopurification method. Purified mAb (20 pmol) in 100 μl of IP buffer was loaded on to a Protein A–Sepharose minispin column, and the column was washed three times with the same buffer to remove unbound mAbs. Core RNA polymerase (20 pmol) was loaded on to the column. Following brief centrifugation, the column was washed with IP buffer, and the complexes were eluted with 50 μl of sample buffer and loaded on to an SDS/15% (w/v) polyacrylamide gel. The proteins were transferred on to a nitrocellulose membrane and the β' subunit was revealed by ECL® (Amersham Pharmacia Biotech) with the 11D11 peroxidase conjugate.

The core polymerase was then loaded, followed by one of the nine mAbs. All of the selected mAbs bound efficiently to the chip and interacted with the core polymerase. When the core polymerase immobilized by a first antibody was further incubated with this same antibody, no binding or little non-specific binding was detected. In most cases (61 experiments out of 81) the binding of an antibody to the core RNA polymerase did not significantly inhibit the interaction with any other antibody: Figure 1 shows a typical sensorgram obtained in the cross-competition assay. The results of the complete study are summarized in Table 1. In a few cases, complete asymmetric blocking was observed (16F8/11D11, 16F8/2E11 and 12D8/12D9); the binding was strong in one orientation and less efficient (40% or less) in the other orientation. This is frequently observed and can be explained by partial overlapping of the binding sites or by distal conformational changes induced by the first antibody. These results were confirmed by immunoprecipitation of core RNA polymerase by an antibody-saturated Protein A support in the presence of an excess of a second antibody (results not shown), but the extent of the asymmetric blocking was in all cases less marked compared with previous experiments. Therefore none of these antibodies bind to exactly the same site at the surface of the enzyme. This also suggests that these nine mAbs are not representative of all the epitopes at the surface of core RNA polymerase. More than 40 antigenic determinants can be recognized by a polyclonal rabbit antiserum against thyroglobulin, a 600000 kDa globular protein, and more than ten determinants were reported on this same protein using mAbs [15]. We also verified by Western-blot analysis (Figure 2) that

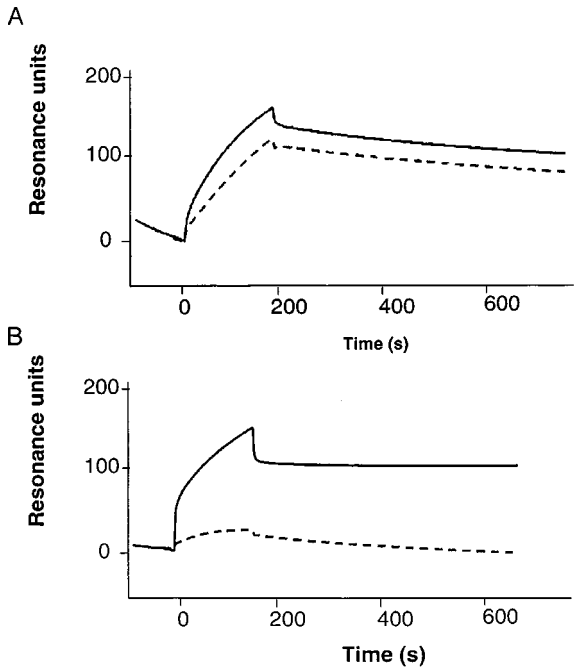

Figure 4 SPR evaluation of the affinity of 11D11 and 3E10 for core RNA polymerase and holoenzyme

The affinities of 3E10 and 11D11 were measured by SPR as described in the Materials and methods section, except that no saturating unrelated mAb and no mAb2 were injected. Briefly, a rabbit anti-mouse IgG1 Fc was covalently immobilized on to the surface of a sensor chip. The 11D11 (**A**) and 3E10 (**B**) mAbs were captured on the sensor chip by the rabbit anti-mouse antibody. Core RNA polymerase (continuous line) or holoenzyme (dashed lane) was then injected. Background levels of polymerase binding were measured by replacing the mAb solution with buffer.

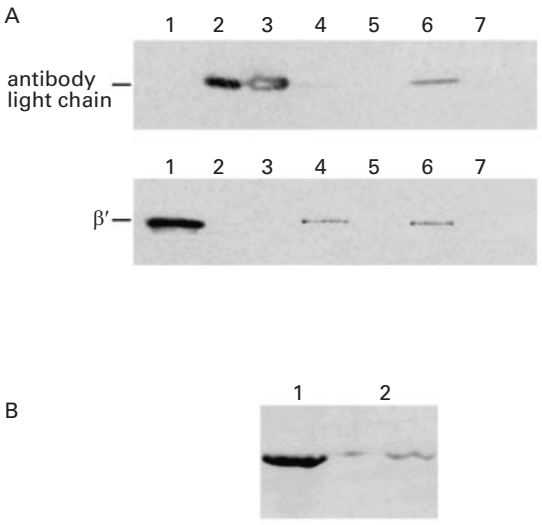

Figure 5 Co-immunopurification of 3E10 and core RNA polymerase with σ70 immobilized on a solid support

(**A**) σ70 was immobilized on *N*-hydroxysuccinimido-activated Sepharose 4B according to the manufacturer's instructions. As a control the same support was processed without σ70. Solid support (50 μl) with (lanes 4 and 6) or without (lanes 5 and 7) σ70 was loaded on to a minispin column, followed by 20 pmol of core RNA polymerase in 100 μl of IP buffer. The column was washed three times with the same buffer to remove unbound core polymerase. 3E10 (lanes 4 and 5) or 11D11 (lanes 6 and 7) mAbs (20 pmol) in 100 μl of IP buffer was loaded, and after three washes with the IP buffer, the complexes were eluted with 50 μl of sample buffer and loaded on to an SDS/15% (w/v) polyacrylamide gel. The proteins were transfered on to a nitrocellulose membrane, and the β′ subunit was revealed by ECL® with an 11D11 mAb covalently linked to horseradish peroxidase and an anti-mouse Fc-specific antibody covalently linked to horseradish peroxidase. Lane 1 corresponds to polymerase loaded on to the column, and lanes 2 and 3 correspond to 11D11 (lane 2) and 3E10 (lane 3) mAbs loaded on to the column repectively. (**B**) Solid support (50 μl) with σ70 was loaded on to a minispin column, followed by 20 pmol of core RNA polymerase without (lane 1) or with (lane 2) 100 pmol of σ70 in 100 μl of IP buffer. The column was processed as previously described and the proteins were transfered on to a nitrocellulose membrane. The β′ subunit was revealed by ECL® with a 11D11 peroxidase conjugate.

antibodies specific for all of the polymerase subunits were selected. Only one of these antibodies (2E11) recognizes α, within amino acids 94–171. β′ is recognized by 3E10, 8F4 and 11D11, and β is recognized by 12D9, 12D8 and 3C2. No signal was observed with 3E11 and 16F8 in this assay. The epitopes of these two antibodies are more dependent on the assembly of the enzyme.

Competition between mAbs and transcription factors

Having nine mAbs directed against nine completely, or at least partially, independent sites on core RNA polymerase, we set up a binding assay that could be used to rapidly characterize competition between antibodies and transcription factors. This assay is based on the affinity co-purification of a ^{35}S-labelled transcription factor with an immobilized anti-core mAb in the presence of core. We first focused on σ70, and a plasmid coding for this protein, under the control of a T7 promoter, was *in vitro* transcribed and translated in the presence of [^{35}S]methionine. The crude radioactive protein was immunopurified with each of the nine mAbs immobilized on Protein A–Sepharose, in the presence or absence of core polymerase. The *in vitro* translated σ70 efficiently bound to the enzyme; depending on the mAb up to 90% of the radioactive protein was found in the fraction bound to core RNA polymerase. This binding was highly specific; almost no ^{35}S-labelled σ70 bound to the mAb in the absence of core (Figure 3A) or in the presence of unlabelled σ70 (results not shown). Among these nine mAbs, only 3E10 was affected by the binding of σ70 (Figure 3A), whether or not σ70 was pre-complexed with core (results not shown). Although this inhibition was severe, we compared the immunoprecipitation efficiency of RNA polymerase core with each mAb. The amount of core polymerase immunoprecipitated by the mAbs, including 3E10, was comparable (Figure 3B). Therefore we were able to exclude that the lack of σ70 signal was due to the poor binding properties of 3E10, in line with the SPR data showing that 3E10 binds core with a K_d of 2.6×10^{-10} M. SPR competition experiments were attempted in order to confirm the inhibition of σ70 binding by 3E10 observed in our immunoprecipitation assay. The binding of holoenzyme and RNA polymerase core to 3E10 and 11D11 mAbs immobilized on the surface of a chip was compared. Both mAbs bound core RNA polymerase efficiently (Figures 4A and 4B); but even though the binding of 11D11 to the holoenzyme remained unaffected (Figure 4A), the binding of 3E10 was inhibited by more than 90% in the presence of σ70 complexed to the polymerase (Figure 4B). The affinity of 3E10 for the holoenzyme ($K_d = 2.6 \times 10^{-10}$ M) is approx. 40-fold lower compared with the affinity for core RNA polymerase. Calculation of the association and dissociation constant suggests that the association rate constant k_a was almost unchanged with or without σ (4.7×10^5 $M^{-1} \cdot s^{-1}$ and 3.02×10^5 $M^{-1} \cdot s^{-1}$ respectively), whereas the dissociation rate constant k_d was strongly affected (3.99×10^{-3} s^{-1} and 7.88×10^{-5} s^{-1} respectively). This could imply that 3E10 binding is affected by conformational changes of the

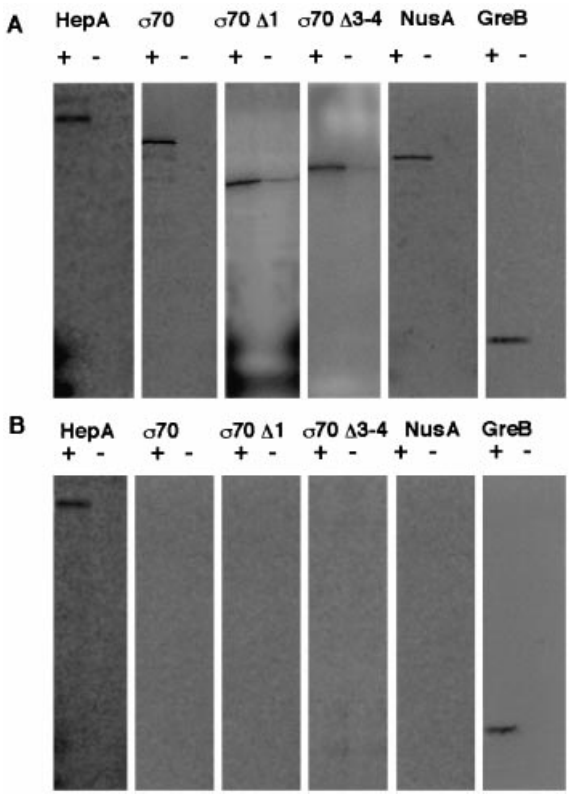

Figure 6 Co-immunopurification of NusA, GreB, HepA, σ70, σ70 deletions and core RNA polymerase with the anti-(RNA polymerase) mAb

In vitro translated protein (2 μl) was incubated for 30 min at 22 °C with 20 pmol of core RNA polymerase to form a complex. Purified mAb [20 pmol; 11D11 (see Figure 5A) or 3E10 (see Figure 5B)] in 100 μl of IP buffer (+) or IP buffer only (−) was loaded on to a Protein A–Sepharose minispin column. The column was washed three times with the same buffer to remove unbound mAbs. The core–^{35}S-labelled protein complex was then loaded on to the column. Following brief centrifugation the column was washed with IP buffer, and the complexes were eluted with 50 μl of sample buffer and loaded on to an SDS/15% (w/v) polyacrylamide gel. The gels were dried and autoradiographed.

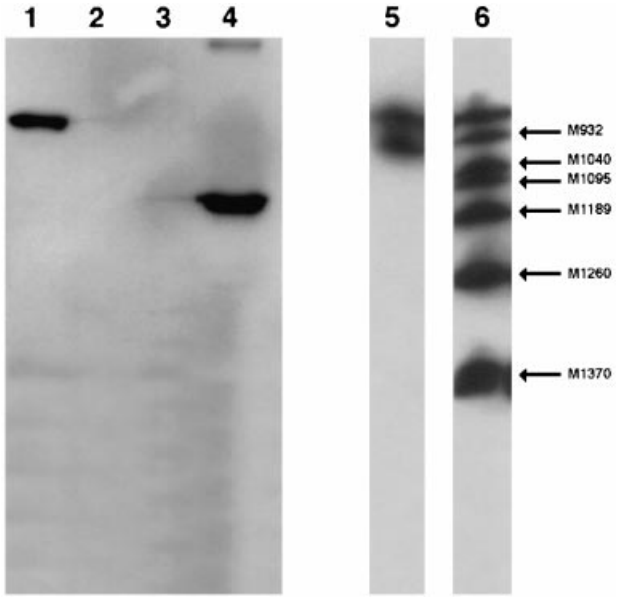

Figure 7 Epitope mapping of 3E10

Core RNA polymerase (4 μg; lane 1) or 1 μg of one of the three overlapping β′ fragments, β′1 (1–600; lane 2), β′2 (300–1000; lane 3) or β′3 (821–1407; lane 4), was loaded on to an SDS/12% (w/v) polyacrylamide gel. The β′3 fragment (20 μg) was subjected to limited cleavage with CNBr (lanes 5 and 6). After freeze-drying and solubilization, the fragments containing the C-terminal histidine tag were selected by precipitation with 20 μl of Ni-NTA agarose. The support was resuspended in 100 μl of loading buffer and the fragments were separated on an SDS/17% (w/v) polyacrylamide gel. The proteins on both gels were transferred on to nitrocellulose membrane and detected with an anti-histidine tag peroxidase conjugate (lane 6) or the 3E10 peroxidase conjugate (lanes 1–5).

holoenzyme rather than by competition with σ70. However, the signal obtained with 3E10 on the holoenzyme was weak and could be impaired by the presence of core dissociated from σ70. It is therefore difficult to take these values into account. We noticed a strong non-specific binding to the chip when RNA polymerase core was injected in the presence of a 5-fold excess of σ70, thus preventing us from using saturating σ70 concentrations (results not shown). These two results suggest that σ70 prevents the binding of 3E10, either through direct competition for the same region at the surface of core RNA polymerase or through allosteric interactions between the σ70 binding site and the 3E10 binding site. Such a hypothesis is not unlikely; the low resolution structure of core polymerase in the presence or absence of σ70 revealed conformational changes [16], and recent observations demonstrated that the interactions of σ70 with core are allosterically regulated by the nascent RNA [17]. In order to discriminate between these two mechanisms, we covalently immobilized σ70 on to a solid support, and we tried to affinity-purify a core–mAb complex. σ70 immobilized on *N*-hydroxysuccinimido-activated Sepharose binds to core polymerase (Figure 5), and this binding can be inhibited by free σ70. When mAbs 11D11 or 3E10 were loaded on to the immobilized holoenzyme, 11D11 was retained on the support with greater efficiency than 3E10 (Figure 5). Therefore the binding of σ70 and 3E10 to core RNA polymerase was almost completely mutually exclusive, and it is likely that both proteins have the same binding site or at least occupy in part the same place at the surface of the core. Recent studies of σ70 domain organization revealed that a central domain (amino acids 114–448) was sufficient to bind core RNA polymerase [18]. To determine more precisely the regions of σ70 involved in the competition with 3E10, we deleted the N-terminal portion (region 1.1; σ70 Δ1.1) or the C-terminal part (region 3 and 4; σ70 Δ3–4) of the protein. As expected from a previous report [18], these two deletions did not strongly affect binding to core RNA polymerase, and 11D11 co-purified core and both σ70 Δ 1.1 and σ70 Δ3-4 (Figure 6A). However, 3E10 did not bind wild-type core–σ70 complexes, and behaved in the same way with core–σ70 Δ1.1 and core–σ70 Δ3–4 (Figure 6B). Therefore the central domain of σ70 regions is probably involved in the competition with 3E10. In order to confirm the specificity of the inhibition observed with 3E10, we also compared the effects of this mAb with the binding of other transcription factors that interact with core RNA polymerase. 11D11 specifically co-purified the transcription factors NusA, GreB, HepA and σ70 in the presence of the polymerase (Figure 6A). The intensity of the signal obtained with GreB and HepA was somewhat lower than that obtained with σ70. This could be explained by the low affinity of GreB [19], and possibly HepA, for the polymerase. In comparison, 3E10 co-purified GreB and HepA as efficiently as 11D11, but not σ70 and NusA (Figure 6B). Using C-terminal histidine-tagged NusA immobilized on to Ni-NTA agarose, we verified that core RNA polymerase bound to NusA was also unable to bind 3E10 (results not shown). Therefore the characterization of a common

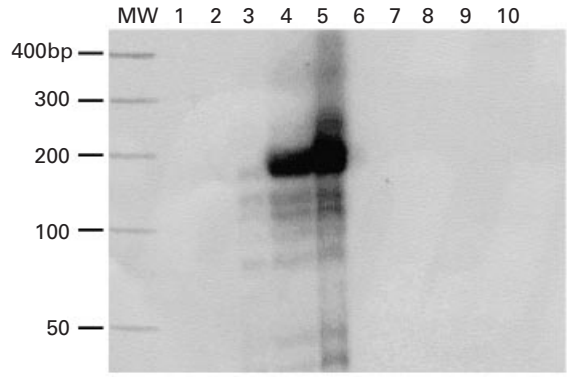

Figure 8 Inhibition of transcription by 3E10

Transcription of the rrnB P1 promoter template was performed as described in the Materials and methods section. mAbs 11D11 (lanes 1–5) or 3E10 (lanes 6–10) were preincubated with RNA polymerase in 10 μl of transcription buffer for 10 min at 37 °C. σ70 (1 pmol in 1 μl of transcription buffer) and the DNA template (1 pmol in 1 μl of transcription buffer) were added, and the mixture was incubated again for 10 min at 37 °C. Transcription was initiated by addition of the NTP mixture, and was stopped after 0 (lanes 1 and 6), 1 (lanes 2 and 7), 3 (lanes 3 and 8), 7 (lanes 4 and 9) or 15 min (lanes 5 and 10). A ^{32}P-labelled DNA fragment ladder (geneRuler 100 bp ladder; MBI Fermentas, Hanover, MD, U.S.A.) was loaded in the left-hand lane (MW).

competitor for NusA and σ70 tends to demonstrate that both proteins share, at least in part, the same place on core RNA polymerase. However, the possibility that σ70 and NusA have overlapping, but slightly different, binding sites cannot be ruled out.

Epitope mapping and transcription inhibitory effects of 3E10

Western-blot analysis identified β' as the subunit interacting with 3E10 (Figure 6); this binding site was specified by immunoprecipitation using three overlapping fragments of β', $\beta'1$, $\beta'2$ and $\beta'3$ (see the Materials and methods section), and by limited cleavage of $\beta'3$ with CNBr according to Rao et al. [20]. The results of these experiments indicate that fragments deleted from their N-terminus by CNBr cleavage were recognized up to Met^{309}; when cleavage was made beyond this, 3E10 no longer recognized the protein (Figure 7). Therefore the epitope is located at the C-terminus of the $\beta'3$ conserved region G, or in the divergent domain next to it. We did not map the epitope using fragments deleted from the C-terminus, and therefore we do not know where precisely the C-terminal limit of the epitope is. However, the *E. coli* RNA polymerase is organized in well-defined domains and the epitope is likely to be between Met^{932} and Met^{1040}. We failed to map it more precisely using the spot multiple peptide sythesis technique [21], suggesting that the epitope is not linear (results not shown). Two classes of antibodies have already been identified in this highly immunogenic region. The first class binds within amino acids 1145–1198 and does not significantly affect transcription. The second class binds a discontinuous epitope that is affected by two deletions 90 amino acids apart (amino acids 941–1000 and 1091–1130), and inhibits transcription and transcript cleavage induced by GreB [7]. Interestingly, the affinity of this second class of antibodies was 5-fold less for core RNA polymerase compared with free β', but not much difference was observed between core polymerase and the holoenzyme [9]. Finding two or more different epitopes in a 200-amino-acid long sequence is common. For example, the 49-amino-acid long peptide insulin accommodates four epitopes [22]. Furthermore, immunization with proteins in complexes is frequently used to isolate conformation-dependent antibodies [23]; this could explain why the previously characterized antibodies obtained after immunization with free β' had an affinity 10–70-fold lower for the holoenzyme compared with free β' [9], and did not compete for σ70. The affinity of 3E10 for core RNA polymerase was high ($K_D = 3 \times 10^{-10}$ M), but decreased for denatured core ($K_D = 2 \times 10^{-8}$ M). It was recently reported that β' subunits deleted in this region fail to assemble with α and β [7]. To further compare 3E10 with the other antibodies described in this region, we tested its effects in an *in vitro* transcription assay on the rrnB P1 promoter. No transcription was detected when the antibody was preincubated with core RNA polymerase prior to σ addition (Figure 8). No inhibition of transcription was detected under the same experimental conditions with an irrelevant antibody (results not shown).

DISCUSSION

In the present study we report the isolation of the first anti-(core RNA polymerase) mAb that competes with σ70 and NusA binding. This mAb was found in a set of nine mAbs interacting at different locations on the enzyme surface. No competitor was found for GreB and HepA, supporting the view that these mAbs are not representative of the epitopes of the entire polymerase surface, and that despite their bulkiness, antibodies can be very selective competitors of proteins interacting with core RNA polymerase. Local conformational changes between core polymerase and holoenzyme have been reported [16]. Data obtained with these nine mAbs scattered on the surface of the polymerase imply that σ70, NusA, GreB and HepA occupy discrete places on core RNA polymerase as suggested by targetted footprinting experiments [24], and do not induce extensive conformational changes of the whole enzyme surface.

The epitope bound by 3E10 was located at the C-terminus of the β' region G, a sequence conserved between *E. coli* and *T. aquaticus*, or in the divergent 200-amino-acid sequence located next to it and absent from *T. aquaticus*. This epitope corresponds exactly to the domain cross-linked by the 3′-end of a 27mer transcript carried by a non-productive ternary elongation complex [25]; a different site located between β' regions F and G was cross-linked by Koulich et al. [26] with the 3′-end of a 6mer transcript.

The high resolution structure of the *T. aquaticus* RNA polymerase [5] was used to superpose structural data with functional data from *E. coli*. *T. aquaticus* RNA polymerase has the same size and shape as the *E. coli* RNA polymerase and shares extensive sequence homology. The conserved N-terminal region of the 3E10 epitope is located at the surface of the enzyme in the proximity of the catalytic site (the distance between the Cα of β' Met^{1238} and magnesium is 20 Å; where 1 Å = 0.1 nm) at the boundary between the primary and the secondary channel. Depending on its precise orientation on the epitope, 3E10 can protrude either into the primary or the secondary channel. The location of the epitope at the surface of the enzyme could account for the striking differences observed between 3E10 and previously described antibodies which bind to the same region but do not compete with σ70 [7,9]. The average surface occupied by an antibody is in the range of 400–600 Å^2 [27]. If we assume, according to biochemical data, that regions of σ70 are in close proximity to the catalytic site [28], the distance between the catalytic site and the epitope is compatible with direct competition with σ70. However, this conformational epitope probably could involve a divergent region at the C-terminus of region G, present

in *E. coli* and absent from *T. aquaticus*, and consequently its exact position is impossible to determine.

σ70 binds core RNA polymerase and allows promoter-specific recognition and transcription initiation. An initial deletion analysis identified σ70 region 2.1 as being necessary for the binding of σ70 to core RNA polymerase [29]. This finding was extended to other regions using a variety of techniques (for a review see [1]). Recent data [30] demonstrated that a region located in the N-terminal part of β' could be the other side (or part of it) of the interface with region 2 of σ70, but additional interactions are also likely. Targetted protein footprints [24] with σ70 and NusA, have suggested that contacts of these proteins with core RNA polymerase involve several identical regions of the enzyme. When positioned on the structure of *T. aquaticus* polymerase, most of the cutting site appears to be close to the high affinity σ70 binding site, or the catalytic site. One of the major cutting sites on β' is located in-between amino acids 410–450, at less then 8 Å from magnesium, and at approx. 20 Å from the 3E10 epitope on the same face of the enzyme; a minor cutting site is located in the region recognized by 3E10. These results are in good agreement with the properties of 3E10, which competes with NusA and σ70, and with the mutually exclusive binding of NusA and σ70 to core RNA polymerase [31]. The finding of a common competitor to NusA and σ70 supports the idea that both proteins occupy, at least in part, the same place at the surface of core polymerase, and that the competition is probably not the result of allosteric interaction between NusA and σ70 binding sites.

We evaluated the distance between the epitope of 3E10 and the putative σ70 major binding site, located in the template channel in the upstream portion of the lower jaw. The closer amino acids are separated by more then 46 Å (distance between the Cα of β' Met^{1238} and the Cα of Pro^{522}). However, σ70 region 2.1, involved in the binding to β', was also shown to be close to positions -4 to $+3$ of the lacUV5 promoter, together with region 3.1 [32]. Mutations that destabilize σ–core interaction have been identified in region 3 [33], and region 3.1 was mapped in the catalytic site [28]. σ70 region 1 is also a candidate to interact with core polymerase [1]. In order to discriminate between the σ70 regions involved in competition with 3E10, we performed immunoprecipitations of core RNA polymerase with 3E10 in the presence of σ70 lacking regions 1, or 3 and 4. A strong inhibition of the binding of σ70 to core polymerase was observed in the presence of 3E10. Therefore we concluded that the central domain of σ70 is sufficient to inhibit the binding of this mAb. The central domain of σ70 could be closer from the 3E10 epitope than expected from the recent interpretation of a low resolution structure of a σ70–polymerase complex [34].

Gre factors induce the cleavage of the nascent transcript in ternary elongation and trigger the endonucleolytic activity of the catalytic site located 40 Å away from its binding site [35]. Unlike NusA, Gre factors do not compete with σ70 for core RNA polymerase binding [19], despite that Gre A, NusA, and σ70 apparently cleave core polymerase at very similar locations in targetted chemical protease experiments [24]. How proteins such as σ70 and Gre factors, with apparently similar occupancy, can bind to core simultaneously [19] is still not understood and needs to be confirmed using other techniques. 3E10 recognizes a domain already involved in Gre protein function [7], but it is able to bind core–Gre B complex but not core–σ70 or NusA complexes. Therefore the occupancy of these two groups of proteins, in this region of core polymerase, is at least in part different and can be discriminated with 3E10.

Our results, together with previous data, illustrate the use of combined approaches to understand where transcription factors bind on core, what conformational changes they induce, and how intricate the contacts in this limited area of the core RNA polymerase can be. Genome-wide two-hybrid screening already provides new candidates for interaction with core polymerase. This set of mAbs will certainly be useful to investigate the relationships between the RNA polymerase and these putative new partners.

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