# Probing eukaryotic RNA polymerases B with monoclonal antibodies

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Monoclonal antibodies directed against RNA polymerase B of the fungus *Podospora comata* were selected on the basis of different subunits recognition and inhibitory effect on enzyme activity. A library of 10 antibodies biased toward $B_{180}$, $B_{145}$, $B_{39}$, $B_{23,5}$ and $B_{11}$ subunits was constructed. Most of these anitbodies also recognize yeast, wheat germ and calf thymus RNA polymerase B. Subunits bearing antigenic determinants are not always homologous in *Podospora* and yeast enzyme. As some of these antibodies strongly inhibit enzyme activity they constitute potent probes for functional studies of corresponding subunits.

*Monoclonal antibodies* *RNA polymerase* *Subunits function*

## 1. INTRODUCTION

The transcriptional apparatus of eukaryotes is very complex. It is made up of more than 25 different polypeptides associated in 3 multimeric enzymes called RNA polymerase A or I, B or II and C or III. Each of these enzymes is responsible for the synthesis of a different class of RNA [1]. They are structurally well characterized in a variety of organisms [2]. Due to the great complexity of these enzymes, the function of most of their subunits is still unknown. Few specific inhibitors are available and genetic studies failed to give any information. Some of the subunits may be implicated in the regulation of transcriptional activity during differentiation processes. The lack of specific probes is a limiting factor to progress in investigating the function of the subunits. Immunological studies with polyspecific antibodies directed against isolated subunits have demonstrated the feasibility of using such antibodies as probes [3–5]. However, more specific probes may be obtained by the methodology of monoclonal antibodies [6–8].

Here we report the preparation of a library of monoclonal antibodies directed against RNA polymerase B of the fungus *Podospora comata*. We demonstrate that these monospecific antibodies will be a powerful tool to investigate the functional role of RNA polymerase B subunits in a variety of eukaryotic organisms. These antibodies will also be used to study the role of each enzyme subunit in the regulation of transcriptional activity of RNA polymerase B observed in a temperature-dependent strain of *Podospora* [9,10].

*Abbreviation:* ELISA, enzyme-linked immunosorbant assay

## 2. MATERIALS AND METHODS

Nitrocellulose membranes (HAWP 304FO) were purchased from Millipore. Anti-mouse immunoglobulins antisera were from Nordic Laboratories. $^{125}$I-Labelled protein A was from NEN.

### 2.1. *Purification of* Podospora *RNA polymerase B*

Characterization and purification of RNA

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polymerase B will be described elsewhere. The purification procedure was similar to that described for purification of RNA polymerase C [11] except that heparin Sepharose chromatography was substituted for DNA cellulose chromatography.

### 2.2. *Immunization and cell fusion*

Three month old BALB/c mice were injected intraperitoneally with 50 $\mu$g of RNA polymerase B in 0.25 ml buffer containing 50 mM Tris–HCl, pH 8; 300 mM ammonium sulphate; 0.5 mM EDTA; 10% glycerol emulsified with complete Freund's adjuvant. Booster injections with 25 $\mu$g of RNA polymerase in complete adjuvant were performed 3 and 6 weeks later and then 5 and 7 months later. Four days before cell fusion, the mouse was injected with 50 $\mu$g enzyme. The spleen was removed and spleen cells were fused with SP 2/0 myelomatous cells as in [12]. Hybrid cells were grown in selective medium. Hybridomas-producing specific immunoglobulins were screened by ELISA [13] and cloned twice by limiting dilution. Large amounts of antibodies were obtained from spent supernatants of cultures grown in 50 ml medium. Antibodies were purified by ammonium sulphate precipitation.

### 2.3. *Screening for hybridomas by ELISA and inhibition of activity*

ELISA were done essentially as in [13] using peroxidase-conjugated IgG fraction of rabbit anti-mouse immunoglobulins (Institut Pasteur Production). RNA polymerase B 90% pure in solution at 0.6 $\mu$g/ml in 20 mM carbonate-bicarbonate buffer, pH 9.6, was used as source of antigen for plate coating.

Inhibition of enzyme activity was determined after incubation during 2 h at 4°C of 10 $\mu$l (about 0.5 $\mu$g) of RNA polymerase B with 25 $\mu$l of 20-times concentrated immunoglobulins from spent medium. Residual activity was measured after the addition of nucleotides, DNA and salts to give the final reaction conditions specified in [9].

### 2.4. *Spot-immunodetection and blot-immunodetection*

Spot immunodetection was performed as described in [14] except that an incubation step with rabbit anti-mouse immunoglobulins serum was added before [$^{125}$I]protein-A detection. Subunits of RNA polymerase B were separated by electrophoresis under denaturing conditions on linear gradient gels (7.5–15% acrylamide). Proteins were transferred by diffusion from the gel slab to nitrocellulose as in [6]. Blots were cut in strips and 0.1 $\mu$g of enzymes A, B, C were spotted at the top of the strips. Nitrocellulose membranes were treated as for spot-immunodetection.

## 3. RESULTS

### 3.1. *Screening of hybridomas*

The data presented in table 1 summarize the results of hybridomas selection from cultures producing antibodies directed against RNA polymerase B. They were obtained from one cell fusion realized with spleen cells of a mouse immunized with purified enzyme. Out of 300 wells showing growing hybridomas 16 gave a strong

Table 1

Selection of positive hybridomas

| Culture number | ELISA[a] | Inhibition of enzyme activity (%) | Subunit[b] recognized | Monoclonal[d] hybrid |
|---|---|---|---|---|
| 6 | + + | 7 | 140[c] | N.C. |
| 7 | + + | 2 | $B_{145}$ | C-7 |
| 8 | + + | 9 | $B_{180}$ | N.C. |
| 9 | + + + | 2 | $B_{180}$ | N.C. |
| 10 | + + | 0 | – | N.C. |
| 11 | + + | 8 | $B_{39}$ | C-11 |
| 12 | + + | 0 | $B_{39}$ | C-12 |
| 13 | + + | 4 | $B_{23.5}$ | C-13 |
| 14 | + + | 22 | – | C-14 |
| 15 | + + | 2 | $B_{180}$ | C-15 |
| 16 | + + | 13 | $B_{180}$ | C-16 |
| 17 | + + + | 19 | – | C-17 |
| 18 | + + | 12 | $B_{180}$ | C-18 |
| 19 | + + | 0 | $B_{180}$ | N.C. |
| 20 | + | 16 | $B_{180}$ | C-20 |
| 21 | + + | 28 | $B_{180}$ | † |

[a] The symbols + represent the intensity of the colored reaction

[b] Recognized subunits have been detected by blot-immunodetection. Only subunits giving the strongest reaction with each culture supernatant are indicated

[c] Antibodies from 6 react only with a polypeptide of $M_r$ 140000 which contaminates enzyme preparations

[d] C, cloned; N.C., not cloned; †, lost during cloning

positive response by ELISA. Positive cultures were grown in 1 ml selective medium and the immunoglobulins from culture supernatants were concentrated by ammonium sulphate precipitation and assayed for inhibition of RNA polymerase activity as described in section 2. Six of the cultures contained antibodies which inhibit notably RNA polymerase activity.

Before cloning, primary characterization of antibody specificity towards RNA polymerase subunits was performed by blot-immunodetection. Results are indicated in table 1. Reactions were observed with $B_{180}$, $B_{145}$, $B_{39}$ and $B_{23.5}$ subunits. The largest subunit $B_{180}$ is likely a very immunodominant protein because most of the cultures produce antibodies reacting strongly with this polypeptide. We have cloned the different hybridomas by two successive limit dilutions. Among the hybridomas-producing antibodies against $B_{180}$ subunit, only those producing antibodies which inhibit enzyme activity have been cloned. Antibodies were prepared from the supernatants of large-scale cultures of the different monoclonal hybrids.

### 3.2. *Characterization of monoclonal antibodies reactivity against separated subunits of RNA polymerase B*

Subunits of RNA polymerase B from the *Podospora* bearing the antigenic determinants were identified. The reaction of the different monoclonal antibodies with enzyme components is shown in fig.1. Each of them reacts strongly with one polypeptide except antibodies from clone 7 which react equally with the two large subunits $B_{180}$ and $B_{145}$. Such a result is surprising, as monoclonal antibodies are expected to recognize only one polypeptide site. A similar result has been described in [7]. However, before concluding that the two subunits possess a common antigenic site, we have to check that hybridoma 7 is really monoclonal. Antibody 11-c gives a very strong response with polypeptide C ($M_r$ 39000). This enzyme component is always present in a very low molar ratio in enzyme preparations [10]. This antibody completely inhibits enzyme activity (see table 2) thus providing evidence that this polypeptide is a functional subunit of RNA polymerase B. Antibody from C-13 which reacts with $B_{23.5}$ subunit also recognizes native enzymes A and C.

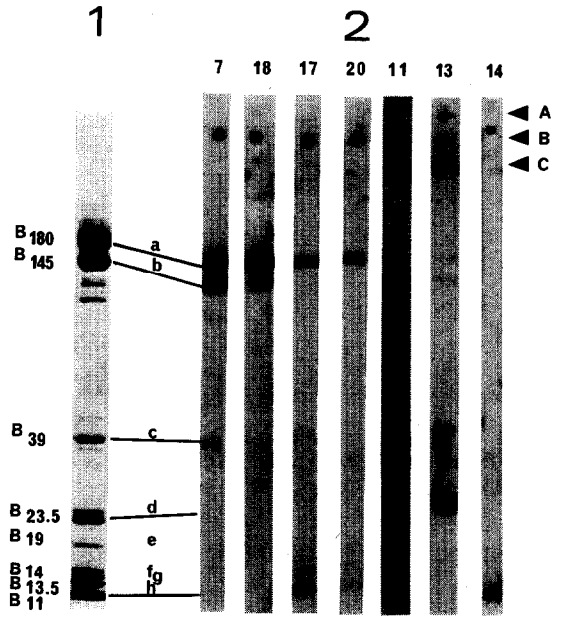


Fig.1. Specificity of monoclonal antibodies towards the subunits of RNA polymerase B. Subunits of RNA polymerase were separated by electrophoresis under denaturing conditions on linear gradient of 7.5 to 15% acrylamide, transferred to nitrocellulose sheets and probed with the different antibodies. (1) SDS–polyacrylamide gel pattern of RNA polymerase B. Subunits are identified on the left by their molecular mass ($\times 10^{-3}$). (2) Autoradiography of the blots treated with the different antibodies. The arrows indicate spots of native RNA polymerase A, B and C. From this experiment antibodies have been named after the number of their culture followed by the subunit(s) they recognize; i.e., 7-a-b, 18-a, 17-a, 20-a, 11-c, 13-d and 14-h.

This confirms previous results that this subunit is common to the 3 enzymes [10]. Clones 17, 18 and 20 produce anti-$B_{180}$ antibodies. The faint reaction observed at the bottom of the strip with antibody 17-a is not reproducibly found and is considered as a non-specific reaction. Antibody 14-h recognizes a small subunit migrating near the electrophoresis front and is identified as $B_{11}$ subunit. No antibodies directed against $B_{19}$, $B_{14}$, $B_{13.5}$ and $B_{10}$ subunits have been obtained neither in this experiment nor in a previous cell fusion. These subunits also react weakly with polyspecific antibodies prepared against RNA polymerase B.

Table 2

Inhibitory effect of monoclonal antibodies on *Podospora* RNA polymerase B activity

| Antibody | % Inhibition | Specificity |
|---|---|---|
| 11-c | 98 | $B_{39}$ |
| 13-d | 34 | $B_{23.5}$ |
| 14-h | 37 | $B_{11}$ |
| 15-a | 16 | $B_{180}$ |
| 17-a | 45 | $B_{180}$ |
| 18-a | 15 | $B_{180}$ |
| 20-a | 48 | $B_{180}$ |

### 3.3. *Specific antigenic determinants are conserved in RNA polymerase B from various organisms*

Using polyspecific antibodies directed against isolated subunits of yeast enzyme B, it has been shown that some antigenic determinants are present in a variety of eukaryotic enzymes B [14]. As polyspecific antibodies recognize many different antigenic sites on the subunits it is not possible to know if the conserved sites are the same on different enzymes. Monoclonal antibodies were used to investigate whether individual antigenic sites are conserved on yeast, plant and mammalian RNA polymerase B. Results of spot-immunodetection (fig.2) show that antibodies 11-c, 14-h, 17-a and 20-a react with a roughly equal intensity with native enzyme B from *Podospora*, yeast, wheat germ and calf thymus. Sites corresponding to 13-d and 15-a are present on wheat germ and calf thymus enzyme. Those corresponding to 16-a and 18-a are borne only by wheat germ RNA polymerase.

### 3.4. *Identification of yeast enzyme subunits bearing the antigenic determinants*

In order to determine if the conserved sites were present on homologous polypeptides in enzyme B from different organisms, we have probed separated subunits of yeast RNA polymerase with the different antibodies. Fig.3 shows that the antibodies which react with yeast enzyme generally recognize subunits which display $M_r$-values related to those of *Podospora* enzyme. However, 17-a and 20-a which react with the $B_{180}$ subunit of *Podospora* recognize $B_{12.6}$ subunit of yeast enzyme. This suggests that conserved sequences are not always present in homologous subunits in enzymes from different sources. Localization of con-

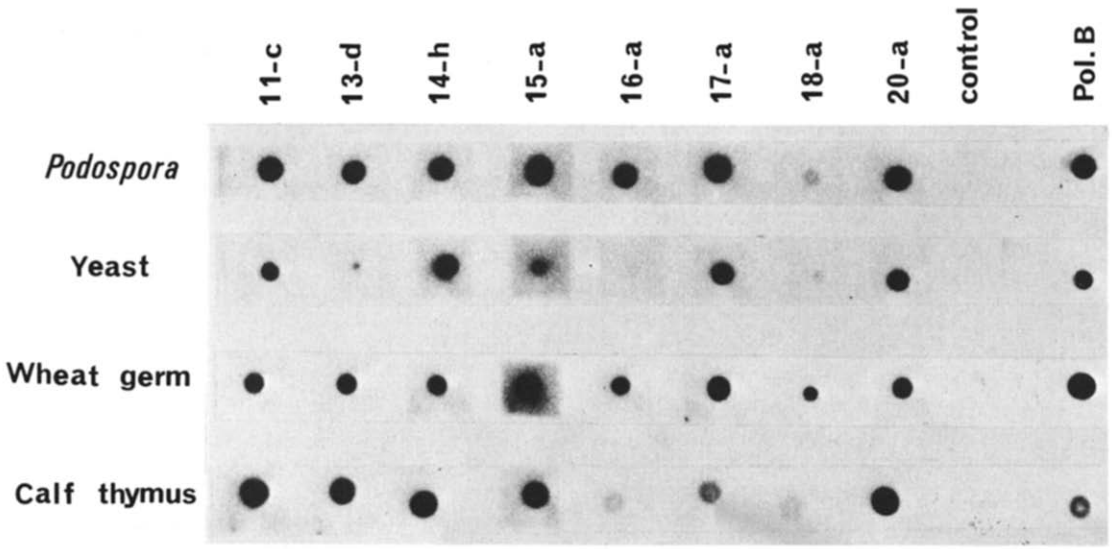


Fig.2. Spot-immunodetection of conserved antigenic determinants in RNA polymerases B from various eukaryotes. About 0.2 $\mu$g enzyme B of *Podospora*, yeast, wheat germ and calf thymus were spotted on nitrocellulose squares and incubated with the different monoclonal antibodies: control IgG, non-specific immunoglobulins; pol. B, rabbit polyspecific immunoglobulins anti-RNA polymerase B.

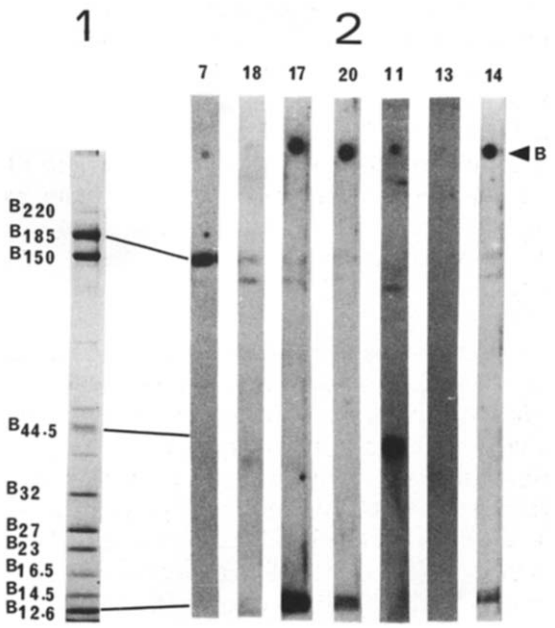


Fig.3. Identification of the subunits of yeast RNA polymerase B recognized by the antibodies: (1) SDS–polyacrylamide gel pattern of yeast RNA polymerase B; (2) immunodetection of blotted subunits with the antibodies. B indicates spots of native yeast enzyme B.

served sequences of wheat germ and calf thymus enzyme is under investigation.

This work confirms that some sequences have been highly conserved in RNA polymerases during evolution. It is likely that they may correspond to regions involved in maintaining the structure and the function of the enzyme. Antigenic determinants may overlap catalytic sites, in such a case, monoclonal antibodies will be useful tools to locate these sites on enzyme and to study their function in transcription. We have assayed the inhibitory effect of antibodies on enzyme activity (table 2). All antibodies inhibit to various extents RNA polymerase B. This demonstrates that subunits $B_{180}$, $B_{39}$, $B_{23.5}$ and $B_{11}$ are functional components of the enzyme. The antibodies which strongly inhibit enzyme activity will thus provide potent probes to study function of the corresponding subunits.

## 4. DISCUSSION

The need for specific probes directed against subunits of *Podospora* RNA polymerase B led us to prepare a library of monoclonal antibodies. The collection of antibodies obtained from one cell fusion is markedly biased towards 5 of the 9 enzyme subunits. These antibodies recognize some immunodominant sites which are also present on yeast, wheat germ and calf thymus RNA polymerase B. The existence of such conserved specific sequences on eukaryotic RNA polymerases has been described, and the hypothesis that structural and functional requirement have forced the conservation of catalytic sequences during evolution has been proposed [14]. Specific probes against these sites will be of great help in studying eukaryotic RNA polymerases. The monoclonal antibodies inhibiting enzyme activity can be used as such probes to investigate the role of the different subunits in the basic steps of enzymatic reaction. They will also be used as specific inhibitors to study the interaction of enzyme subunits with promotors of cloned genes and transcription factors in reconstituted systems.

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