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THE USE OF MONOCLONAL ANTIBODY TO STUDY THE FUNCTIONAL PROPERTIES OF RNA POLYMERASE II SUBUNITS

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SUMMARY: Monoclonal antibodies were raised in mouse against purified calf thymus RNA polymerase II. Three stable hybridoma lines were identified and found to secrete IgM type antibodies. The antibody produced by one clone was found to be directed against the large subunits (II_b and II_c) of the enzyme. In an immuno-blot of electrophoretically separated subunits the monoclonal antibody recognised antigenic determinant on the largest polypeptides of several eukaryotic RNA polymerases II tested. In an *in vitro* transcription system, this antibody is found to inhibit the specific transcription from the adenovirus major late promoter only when the extract is preincubated with the antibody in the absence of DNA.

Three distinct RNA polymerases are present in eukaryotic nuclei (1). These enzymes have been isolated from many eukaryotic tissues (2) and are generally complex multisubunit enzymes. The subunit structure of each class is quite different. Specifically, highly purified RNA polymerase II contains about ten polypeptides in nearly stoichiometric amounts regardless of the organ or animal species from which it is isolated. However, it is not clear whether all of these polypeptides are part of the enzyme or whether some of the smaller polypeptides are proteolytic degradation products of larger subunits which arise during extraction or isolation. Moreover, little is known about the functions of individual subunits. To understand many aspects of gene regulation in eukaryotes, a more detailed knowledge of the function of different subunits of RNA polymerase II is necessary.

It is for this purpose, we, as well as several other investigators,raised

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monoclonal antibodies (3) directed against RNA polymerase II. This report describes the isolation, characterization and properties of one such antibody directed against calf thymus enzyme.

MATERIALS AND METHODS

Preparation of RNA Polymerases: RNA polymerases II from calf thymus (4), mouse plasmacytoma (5), wheat germ (6) and human (7) were purified to near homogeneity as previously described.

Cells: The parental mouse myeloma cell line (nonsecretor) SP 2/0-Ag 14 (8) was provided by the hybridoma center, Washington University, School of Medicine, St. Louis, Missouri. Cells were grown and maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% gama globulin free horse serum, 5% new-born calf serum (Gibco) and 8- azaguanine (10^{-4}M).

Immunoglobulins: Rabbit antimouse IgG_1, IgG_{2a}, IgG_{2b} and IgM were purchased from Gateway, Immunosera, St. Louis, Missouri. Protein A sepharose and protein A were from Sigma Chemical Company.

Immunization: Balb/c mice (Jackson Laboratory) were injected subcutaneously and intraperitoneally with 50 µg of purified calf thymus RNA polymerase II emulsified in complete Freund's adjuvant. Two weeks and two months later booster injection of 50 µg and 25 µg of RNA polymerase II respectively were given in incomplete Freund's adjuvant. Two weeks after the final booster injection, sera of the mice were tested by a solid phase radioimmunoassay as described below. The mouse with the best response was inoculated 4 weeks after the last immunization, intraperitoneally with 25 µg, 25 µg and 30 µg of RNA polymerase II in 0.9% sodium chloride on three successive days.

Cell Fusion: On the following day the mouse was sacrificed by cervical dislocation and a cell suspension was prepared in serum-free DME medium by mincing the spleen with scissors. Erythrocytes were lysed by hypotonic shock in 0.17 M ammonium chloride. Spleen cells were fused to myeloma cells at a 4:1 ratio in the presence of 0.5 ml 50% polyethylene-glycol (PEG 4,000, Fisher) in serum-free DME medium, incubated for 1 min at room temperature and gradually diluted to 50 ml with serum-free DME medium. The cells were pelleted, and resuspended in DME medium containing the standard concentration of hypoxanthine, aminopterin and thymidine (9) and distributed into two 24 well plates (Costar 1524) above a macrophage feeder layer. Hybridoma cells producing antibodies to RNA polymerase II were immediately subcloned by limiting dilution plating in 96-well plates (Costar 3596) in DME medium containg hypoxanthine and thymidine. The positive clones were grown to higher densities in DME medium and further subcloned by growing cells in semi-solid agarose and picking single clone into culture medium (10).

Production of Ascites Fluid and Purification of Antibodies: Balb/c mice were injected intraperitoneally with hybridoma cells ($1X10^6$) three weeks after priming with 0.5 ml pristane (2, 6, 10, 14-tetramethylpentadecane). The antibody, either from culture supernatant or from non-immunized serum was purified by precipitation with ammonium sulphate (40%) followed by chromatography on DEAE cellulose essentially as described before (11). Purified antibodies were dialyzed against 50 mM tris-HCl pH 7.5.

Solid phase radioimmunoassay: Purified calf thymus RNA polymerase II (1 µg) in 50 µl of 25 mM sodium phosphate buffer pH 7.5, 150 mM sodium chloride was absorbed to the wells of polyvinyl microtiter plates (Cooke Laboratories). The subsequent steps were followed as described before (12).

Double Immunodiffusion: The immunoglobulin class of antibodies secreted by the different cell lines was examined by double immunodiffusion (13) using concentrated cell culture supernatants and subclass specific antimouse antibodies.

Immunological Detection of Polymerase II Subunits by Protein Blotting: Protein samples were electrophoresed on 10% Laemmli SDS polyacrylamide gels

(14) and then transferred electrophoretically (15) to diazophenylthioether (DPT) paper (16). Following transfer, the protocol developed by Renart et al. (17) was followed.

Preparation of Soluble Extract and Transcription Assay: Preparation of soluble extract (S100) from HeLa cells and conditions for specific in vitro transcription using the adenovirus major late promoter as a template have been previously described (18). In a typical experiment HeLa cell extract (20 µl, 280 µg protein) was preincubated with different amounts of anti-RNA polymerase antibody at room temperature for 30 min with and without the DNA template. Following incubation, the rest of the components of the transcription reactions were added (includes ribose triphosphates and in some cases templates) and reincubated at 30°C for 1 hr. In a control experiment purified preimmune IgG was used in place of anti-polymerase antibody.

RESULTS AND DISCUSSION

Preparation, purification and characterization of monoclonal antibody: Monoclonal antibodies were prepared against purified RNA polymerase II. When the partially purified antibody was subjected to double immunodiffusion only the IgM type immunoglobulin were found to be secreted by these clones. All experiments described in this report, unless otherwise indicated, were done either using partially purified culture supernatant or ascites fluid of one of the clones.

Conservation of subunits: In order to identify the subunit carrying the antigenic determinant and to compare the conservation of this subunit in a variety of RNA polymerase II enzymes, derived from diverse sources, the purified enzymes were analyzed as described under materials and methods.

One such analysis is presented in Figure 1. Several different polypeptides present in the purified preparation are shown in panel A. The mouse plasmacytoma (lane 1) enzyme has a unique subunit designated as IIo (240 x 10^3 Mr) in addition to the other large subunits IIa (220 x 10^3 Mr) and IIb (180 x 10^3 Mr). However in our wheat germ (lane 2) and calf thymus enzyme (lane 3) this IIo subunit was not detected, but in the case of wheat germ IIa and IIb subunits are present in addition to several smaller subunits. However, calf thymus enzyme in this particular case contains only IIb and IIc (140 x 10^3 Mr) subunits. After electrophoretic transfer (Panel B), when the transferred replica was probed with the monoclonal antibody (Panel C), the largest subunits (IIb and IIc) of the calf thymus

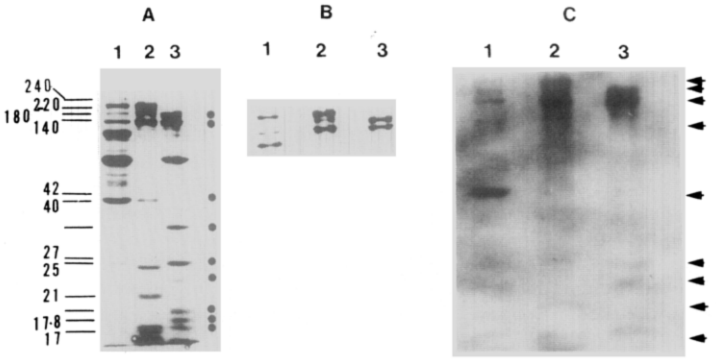

Figure 1: Identification of conserved antigenic subunits by protein blotting: Purified RNA polymerase II (5 μg) from calf thymus, mouse plasmacytoma and wheat germ were subjected to SDS gel electrophoresis and the replica was transferred electrophoretically to diazophenylthioether paper and processed as described under materials and methods. The figure shows (panel A) the subunit pattern of purified RNA polymerase from mouse plasmacytoma cells (lane 1), wheat germ (lane 2) and calf thymus (lane 3) before transfer. Panel B is same as panel A but after electrophoretic transfer to DBM paper. Panel C: autoradiogram of the protein blot after reacting with calf thymus monoclonal antibody followed by ^{125}I second antibody. The largest subunit of enzyme from calf thymus (lane 3), wheat germ (lane 2) and mouse plasmacytoma (lane 1) were strongly reacted. The arrows down the right hand side indicate several smaller subunits reacted with antibody. The numbers on the left side of panel A represent the subunit molecular weights (x 10^3) of the wheat germ enzyme, similar to that from mouse plasmacytoma and calf thymus enzyme. The dots on the right hand side of panel A represent the subunits of calf thymus polymerase II.

enzyme (lane 3) reacted with antibody, although a very minor reaction was also observed with several small subunits. However, in the case of wheat germ (lane 2) and tumor enzyme (lane 1) the IIa and IIb subunits are detected in addition to IIc subunit. Surprisingly, in the case of tumor enzyme another subunit IId (40 x 10^3 Mr) also reacted more strongly than the largest subunit and no other small subunits have been detected. The significance of this interaction is not clear at present.

This result is the most conclusive evidence that the antibody is directed against RNA polymerase II, and that the largest subunits of eukaryotic RNA polymerase II have conserved antigenic determinants. These data are in agreement with those obtained with antibodies to other RNA polymerase II (21-25) and that obtained with calf thymus enzyme by other workers (19,20).

Inhibition of specific transcription: Having determined that the monoclonal

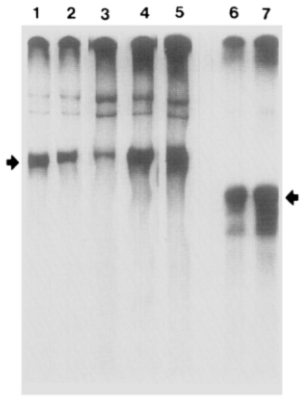

Figure 2: Inhibition of specific transcription by monoclonal antibody directed against RNA polymerase II: An aliquot (20 µl) of HeLa cell extract (S100) was preincubated with 7 µg (lane 2) and 21 µg (lane 3, 4 and 5) of monoclonal antibody or 21 µg of non-immune IgG (lane 7) in absence (lanes 2, 3, 4, and 7) and in presence (lane 5) of template DNA (major late promoter) at room temperature for 30 min. An additional 50 units of exogenous calf thymus RNA polymerase II was added to one of the reactions (lane 4) and transcription was carried out by adding triphosphates (includes α^{32}P GTP) at 30° C for 1 hr. The transcription reaction was described under materials and methods. The autoradiogram shows the gel analysis of *in vitro* RNA synthesized using major late promoter in absence (lane 1 and 6) and in presence (lanes 2-5) of antibody. The scanning of the autoradiogram indicates that there is 30% (lane 2) and 80% (lane 3) inhibition of transcription by anti-RNA polymerase II antibody.

antibody can react with RNA polymerase II, I next examined the functional role of the subunits using an *in vitro* transcription system and the results are presented in Figure 2. As expected, the cell-free extract transcribes the major late promoter in the absence of any other components (lane 1) indicating the presence of saturating amounts of all the transcription factors and RNA polymerase II in the extract. But when this extract is preincubated with a low (lane 2) and a high (lane 3) concentration of IgM antibody in the absence of template, transcription was inhibited to an extent of 30% and 80%, respectively. This inhibition was reversed by adding exogenous RNA polymerase II (50 units) to the reaction (lane 4). Inhibition of transcription was not observed when the extract was treated with the template DNA before antibody treatment (lane 5). Control experiments using preimmune IgG (lane 7) under identical conditions did not inhibit transcription of the major late promoter (lane 6 versus 7). Several points are pertinent from this experiment: (i)

inhibition of transcription from the HeLa cell S100 indicates that the human RNA polymerase II was recognized by the calf thymus RNA polymerase II antibody, (ii) the reversal of inhibition by addition of exogenous calf thymus RNA polymerase II indicates that heterologous enzyme can fulfill the function of the homologous enzyme, (iii) the antibody specifically blocks RNA polymerase II rather than non-specific interaction with other transcription factors, (iv) preincubation with template DNA protected the enzyme from the antibody reaction. Hence, it is likely that the large subunits may participate in the catalytic reaction. However, it is not possible to rule out the possibility that the interaction of other transcription factors with RNA polymerase II was blocked by the antibody. Further studies will undoubtedly help to resolve these possiblities.

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