

A monoclonal antibody specific for snRNPs U1 and U2

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A monoclonal antibody (D-5) is described which selectively precipitates snRNPs U1 and U2. The antibody was derived from a mouse immunized with extracts from chick embryonic nuclei. By immunoblotting on either total proteins from purified snRNPs U1–U6, U2–U6 or U1 only, we could demonstrate that the monoclonal antibody cross-reacts with the U1 RNP specific polypeptide A and the U2 RNP specific polypeptide B^{*}, thereby establishing that the two snRNP proteins share at least one epitope. D-5 precipitates snRNPs U1 and U2 from a variety of species, including man, chicken, mouse, rat kangaroo and *Xenopus laevis*. It will thus be a useful tool for studying structure function relationships of the two snRNP species in different cell systems.

U snRNP RNA processing Splicing Monoclonal antibody Lupus erythematosus Antigenic structure

1. INTRODUCTION

All eucaryotic cells contain a group of small nuclear RNAs, the snRNAs U1, U2, U4, U5 and U6, which are organized in the nucleoplasm in four discrete RNP particles [1–4]. These are the snRNPs U1, U2, U5 and U4/U6. Biochemical fractionation, immunoprecipitation, and protein blotting techniques have identified 9 polypeptides as components of U1 RNPs, the proteins 70 kDa, A, B', B, C, D, E, F and G [5–8,12]. Six of these (B', B, D, E, F, G) are also found in the U2 RNP [5,6] as well as in the snRNPs U5 and U4/U6 (Bringmann, P. and Lührmann, R., in preparation). The U2 RNP further possesses at least two unique polypeptides of *M*_r 32000 (A') and 28500 (B'') [6,9,10]. Patients with connective tissue diseases often develop autoantibodies reacting with some of the snRNA associated proteins [2]. Anti-RNP antibodies selectively precipitate U1 RNPs while anti-Sm antibodies react with all snRNPs U1–U6 [2]. More recently, two further types of autoantibodies have been detected which

are less common among patients and which react with U2 RNPs [9,10].

In the nucleus the U snRNPs are associated with hnRNPs [13–15], consistent with the current thinking that all snRNPs may contribute to pre-mRNA processing [16]. So far it has been experimentally verified that the snRNPs U1 and U2 are implicated in pre-mRNA splicing [17–20]. Their exact function in this process is not yet understood, however. A role for U4 RNA in 3'-end processing of mRNA precursors has been proposed [21], but experimental proof for this notion is still lacking. One approach to a better understanding of the functions of individual U snRNPs is to use snRNP class-specific antibodies for neutralizing the function of the snRNP species in question, either in vivo or in appropriate in vitro systems. Using anti-RNP and anti-Sm autoantibodies [17,22] as well as snRNA cap specific antibodies [18], this approach has already been successfully applied for demonstrating the involvement of U1 RNP in splicing reactions. The usage of naturally occurring snRNP-reactive autoantibodies for the unravelling of the functions of other snRNPs is hampered, however, for several

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reasons. Either snRNP class-specific autoantibodies have not yet been detected (which is true of snRNPs U5 and U4/U6), or they often are contaminated by other autoantibodies, a general drawback of using autoantisera. One approach which bypasses these problems is the use of monoclonal antibodies; these reagents may be expected to react specifically with only one or at most a defined subset of the snRNPs. We here describe a monoclonal antibody (D-5) that reacts selectively with snRNPs U1 and U2 due to cross-reactivity with the U1 specific polypeptide A and the U2 specific polypeptide B".

2. MATERIALS AND METHODS

2.1. Antibodies

The immunization of Balb/c mice with extracts from chick embryonic nuclei, fusion of spleen cells to myeloma cells, and the establishment of permanent hybridoma cell lines have been described [23]. Monoclonal antibody D-5 (an IgG₁) was harvested from hybridoma supernatants, precipitated by 45% saturated ammonium sulfate and dialysed against PBS, or purified by immuno-affinity chromatography with rabbit anti-mouse IgG coupled to CNBr-Sepharose. Antibodies specific for 2,2,7-trimethylguanosine (m₃G) were obtained as in [24] by immunizing rabbits with a conjugate of human serum albumin and m₃G.

2.2. Cell culture

PtK₂ rat kangaroo cells and XLKE *Xenopus laevis* cells were grown as monolayers in the presence of 10% fetal calf serum (Boehringer, Mannheim). PtK₂ cells were kept in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories) at 37°C, while *Xenopus* cells were cultured at 25°C in DMEM diluted 6:7 with sterile water. Cells were labelled for 16 h with [³²P]orthophosphate (10 µCi/ml) in phosphate-free Eagle's minimal essential medium (MEM, Flow Laboratories) supplemented with non-essential amino acids (Flow Laboratories) and 10% dialyzed fetal calf serum. HeLa and Ehrlich ascites cells (EATC) were grown in suspension culture at 37°C in a medium consisting of Eagle's MEM, 5% newborn calf serum (Biochrome, Berlin), 50 µg/ml penicillin and 100 µg/ml streptomycin. They were labelled with [³²P]orthophosphate (10 µCi/ml) in

phosphate-free MEM with 5% dialyzed fetal calf serum. D-5 hybridoma cells were grown in RPMI 1640 (Flow Laboratories) supplemented with 10% fetal calf serum.

2.3. Radioimmunoprecipitation and immunoblotting

Cellular and nuclear extracts were prepared as in [25]; precipitation of antigen-antibody-complexes by Protein A-Sepharose was carried out as described by Matter et al. [26]. In the case where monoclonal antibody D-5 was used, rabbit anti-mouse IgG were prebound to protein A-Sepharose beads. Immunoblotting was performed according to Towbin et al. [27], and visualization of antigenic proteins on nitrocellulose strips was achieved essentially as described by Habets et al. [28].

2.4. Isolation of snRNPs

Total nucleoplasmic snRNPs U1–U6 from nuclear extracts of HeLa cells were purified in a one-step procedure by affinity chromatography using antibodies specific for m₃G as described [29]. Bound snRNPs were desorbed specifically from the column with an excess of nucleoside m₃G. U1 snRNPs were obtained from anti-m₃G affinity-purified snRNPs U1–U6 by chromatography on DEAE-Sepharose [5]. To obtain an snRNP mixture containing only snRNPs U2, U5 and U4/U6 (U2–U6), anti-m₃G affinity-purified snRNPs U1–U6 (see above) were depleted of U1 snRNPs by passing the total snRNP mixture twice over an anti-RNP immune affinity column according to Billings and Hoch [7], except that anti-(U1)RNP IgG from a MCTD patient serum was used instead of monoclonal anti-(U1)RNP antibody.

3. RESULTS AND DISCUSSION

The D-5 monoclonal antibody, identified here as a reagent specific for snRNPs U1 and U2, was originally raised against chicken nuclear antigens [20]; its production and a preliminary account of its reactivity toward chick embryo fibroblasts have been presented [23]. Because of a conspicuous immunoblotting reactivity toward a protein doublet migrating in the region of snRNP core proteins, D-5 hybridoma supernatant was tested for anti-snRNP reactivity. In a sensitive ELISA assay, D-5

was found to react strongly with purified snRNPs U1–U6 from HeLa cells (not shown).

Reactivity of D-5 with snRNPs was further verified by immunoprecipitation assays using total nuclear extracts of 32 P-labelled HeLa cells as a source of snRNPs. Antibody-RNP complexes were precipitated with *Staphylococcus aureus* cells and RNAs contained in the immune precipitates were identified by polyacrylamide gel electrophoresis. Most interestingly, D-5 selectively precipitated the snRNPs U1 and U2, while absolutely no reaction was observed with any of the other snRNPs (fig.1). As D-5 did not react with deproteinized snRNAs (not shown) these data indicated that precipitation of snRNPs U1 and U2 was due either to reaction with a yet undiscovered protein shared only by the two snRNP species, or to cross-reaction with different but structurally related proteins residing on snRNPs U1 and U2.

The second alternative turned out to be true as demonstrated by protein blotting. When total protein from purified snRNPs U1–U6 was used for immunoblots, monoclonal antibody D-5 reacted with two proteins of about 34 and 28.5 kDa (fig.2B), suggesting that D-5 cross-reacts with the U1 specific polypeptide A and the U2 specific polypeptide B" (for the protein composition of the snRNPs U1 and U2, see section 1). This supposition could be confirmed by the following experiments. With total protein from purified U1 RNPs, D-5 decorated solely the 34 kDa protein on immunoblots (fig.2C). This clearly demonstrates that D-5 does not react with the proteins B and B' of 28 and 29 kDa, respectively, which are present in all snRNP particles U1–U6. When, on the other hand, proteins from purified snRNPs U2–U6, which completely lacked U1 RNPs, were immunoblotted, D-5 recognized only the 28.5 kDa protein (fig.2D). We therefore conclude that precipitation of snRNPs U1 and U2 is due to reaction of the monoclonal antibody D-5 with the polypeptides A and B", which reside on the snRNPs U1 and U2, respectively.

Though the epitope recognized by D-5 is shared by the two snRNP proteins it does not appear to be distributed among other cellular proteins. If total protein from HeLa cell nuclei was used for immunoblotting D-5 recognized only the 34 and 28.5 kDa proteins (fig.2A). As shown previously, D-5 also reacted with only two proteins when

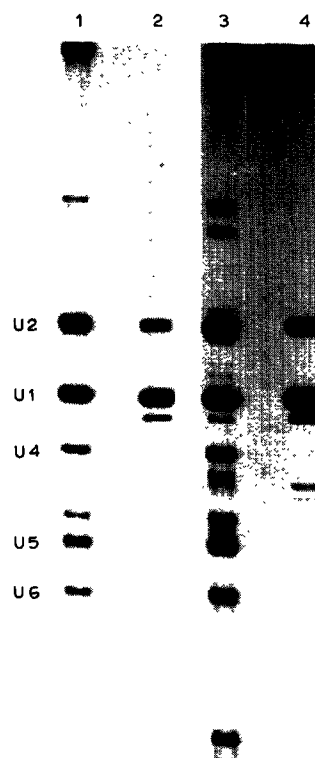


Fig.1. Specificity of monoclonal antibody (mAb) D-5 for snRNPs U1 and U2 as demonstrated by radioimmunoprecipitation. Radioimmunoprecipitation assays were performed as described in section 2. RNAs were recovered from the immunoprecipitates by phenol extraction, fractionated by electrophoresis in a 10% polyacrylamide gel containing 7 M urea, and autoradiographed. Lanes: 1, RNA content of the nuclear extract from HeLa cells; 2, RNAs immunoprecipitated from the extract by mAb D-5. In lanes 3 and 4 the X-ray film has been exposed 3-times as long as in lanes 1 and 2. (The RNA band moving just ahead of U1 RNA represents a fragment of U1 RNA, the faster moving double band probably represents degradation products of U2 RNA.)

assayed by immunoblotting on total chicken nuclear proteins. Moreover, as determined by indirect immunofluorescence microscopy, the subcellular distribution of the D-5 antigens was confined to the extra-nucleolar regions of the nucleus [23].

The antigenic determinant shared by the proteins A and B" appears to be highly conserved in evolution, as monoclonal antibody D-5 reacts with snRNPs U1 and U2 not only from human and

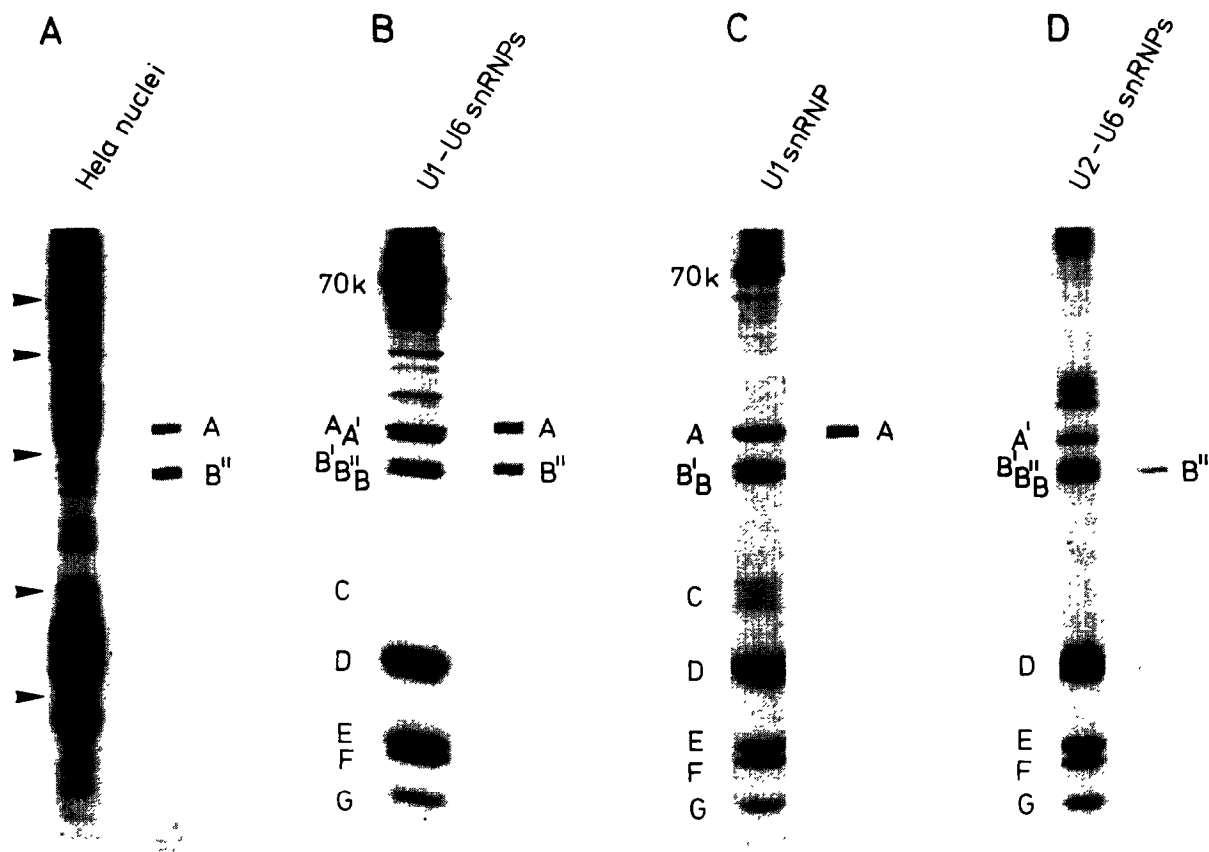


Fig.2. As revealed by immunoblotting, mAb D-5 reacts with the A protein of U1 snRNP and the B'' protein of U2 snRNP. Proteins from whole nuclei of HeLa cells (A), from anti-m₃G affinity purified snRNPs U1-U6 (B), from U1 snRNP (C) and snRNPs U2-U6 (D) were fractionated by electrophoresis in a 15% SDS-polyacrylamide gel [32] and were either stained with Coomassie brilliant blue R250 (all to the left) or electroblotted to nitrocellulose sheets and immunostained with mAb D-5 (all to the right) as described in section 2. Arrows indicate positions of marker proteins of 66, 45, 31, 21.5 and 14.4 kDa (from top to bottom).

chicken cells but also from mouse, rat kangaroo and amphibian cells (fig.3). This will allow the use of monoclonal antibody D-5 for the analysis of structure-function relationships of snRNPs U1 and U2 in different cell systems; most importantly also for microinjection in *X. laevis* oocytes.

The cross-reactivity of polypeptides A and B'' with monoclonal antibody D-5 is the second incidence of a shared epitope among distinct proteins residing on different snRNP particles. Previously it had been shown that a monoclonal autoantibody reacted simultaneously with the polypeptides B, B' and D and, to some extent, with E [12,30]. It is possible, therefore, that snRNA-associated polypeptides may comprise a family of structurally

related proteins, as observed in the case of hnRNP core proteins [31].

The distinctive reactivity of the D-5 monoclonal antibody is highly reminiscent of the properties of a novel autoimmune serum which has recently been detected in rare cases of patients with connective tissue disorders [10]. These antisera were noticeable for their selective precipitation of snRNPs U1 and U2. By immunoblotting techniques, and using purified antibody fractions eluted from individual antigen bands on nitrocellulose blots, it could be shown that the anti-(U1,U2) sera shared autoantibodies cross-reacting with the snRNP polypeptides A and B'' [10]; in addition, however, they contained further

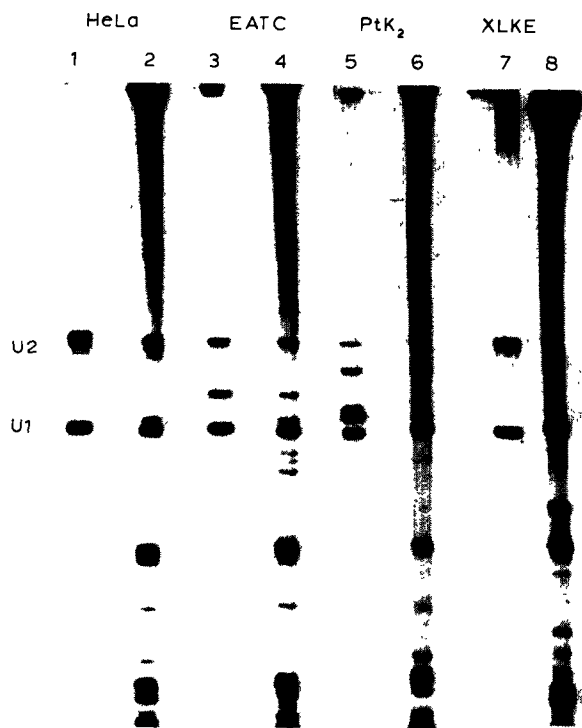


Fig.3. The antigenic determinant recognized by mAb D-5 is highly conserved during evolution. Radio-immunoprecipitation assays with mAb D-5 using cellular extracts from different species as antigens were performed as described in section 2. Immuno-precipitates were processed as described in the legend to fig.1. The autoradiograph shows the RNAs in the cellular extract of HeLa cells (lane 2), mouse Ehrlich ascites tumor cells (lane 4), rat kangaroo PtK₂ cells (lane 6) and *X. laevis* kidney epithelial cells (lane 8). Lanes 1, 3, 5 and 7 display RNAs present in immuno-precipitates prepared with mAb D-5 from the corresponding cellular extracts (lane 3: mouse cells contain two species of U1 snRNA, U1b and U1a (2); lane 5: the RNA band migrating ahead of U2 most probably represents a 5'-terminal fragment of native U2 RNA, as indicated by the finding that this RNA also reacts with anti-m₃G IgG (not shown). The lower U1 snRNA band lacks a m₃G cap and is most likely degraded from the native U1 snRNA.

autoantibodies against other U1 and U2 specific proteins [10]. Competitive binding studies showed that binding of the monoclonal antibody D-5 and the anti-(U1,U2) autoantibodies to isolated snRNPs is mutually exclusive (unpublished), indicating that these antibodies recognize the same

region of the snRNP polypeptides A and B". Thus, the D-5 monoclonal antibody may not only be valuable in further elucidating the biological role of the snRNPs U1 and U2, but may also be used to measure the occurrence of anti-(U1,U2) autoantibodies in sera from patients with connective tissue disorders by a simple competition assay.

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