Evidence for three distinct D proteins, which react differentially with anti-Sm autoantibodies, in the cores of the major snRNPs U1, U2, U4/U6 and U5

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ABSTRACT

Electrophoresis of the mixture of proteins from purified snRNPs U1, U2, U4/U6 and U5 on SDS-polyacrylamide gels that had been allowed to polymerise in the presence of high TEMED concentrations have revealed the presence of proteins in the snRNPs that previously had eluded detection. The most striking case is that of protein D, heretofore generally observed as a single broad band; in high-TEMED gels, this splits into three clearly-separated bands, identified as three distinct proteins. We have denoted these proteins D1 (16 kDa), D2 (16.5 kDa) and D3 (18 kDa). Chemical and immunological studies have shown that D1 is identical with the common snRNP protein D, whose structure was recently resolved by cDNA cloning (Rokeach *et al.* (1988), *Proc. Natl. Acad. Sci. USA*, 85, 4832 – 4836) and that D2 and D3 are clearly distinct from D1 and very probably from each other. In addition to D1, proteins D2 and D3 are present in purified U1, U2, U4/U6 and U5 snRNPs isolated from HeLa cells, so these also belong to the group of common snRNP proteins. They are also found in snRNPs isolated from mouse cells, indicating that the role of these proteins in the structure and/or function of UsnRNPs has been conserved in evolution. Interestingly, patients with systemic lupus erythematosus produce populations of anti-Sm autoantibodies that react differentially with the D proteins; some recognise all of them and others only a subset. The high-TEMED gels allow improved resolution not only of the D proteins, but also of some of the U5-specific proteins contained in 20S U5 snRNPs, in particular the 15-kDa protein. In addition, under these conditions, the common G protein, previously observed as a single band, appears as a doublet. Whether the additional band represents a distinct common snRNP protein or a post-translationally modified version of G is not yet known.

INTRODUCTION

Eucaryotic cells contain a group of small nuclear RNAs, the snRNAs U1, U2, U4, U5 and U6. These are organised as four discrete RNP particles, the snRNPs U1, U2, U4/U6 and U5 (1). All four major snRNPs are essential trans-acting factors in the splicing of pre-mRNA. One of their functions appears to be the recognition of certain signal structures in the pre-mRNA molecule, such as the 5′ and 3′ splicing sites, or the branching point (2–5). The importance of the protein components of the snRNPs in modulating the functions of these particles has been underlined by the finding that snRNP proteins are required for the efficient formation *in vitro* of the complex between U1 RNA and 5′ splicing junctions (6, 7).

The protein constituents of the isolated snRNPs from human cells have until now been analysed mainly by SDS-polyacrylamide gel electrophoresis. There is substantial agreement that at least six proteins, with molecular weights of approximately 29 kDa (B′), 28 kDa (B), 16 kDa (D), 12 kDa (E), 11 kDa (F) and 9 kDa (G) are present in each of the individual snRNPs U1, U2, U4/U6 and U5 (8). A seventh common protein of apparent molecular weight 15.5 kDa has been described by Bringmann and Lührmann (9). This was denoted D′, on account of its similarity in molecular weight with D; however, possible molecular or immunological relationships between D and D′ have not yet been investigated. Recently a protein of size comparable to B′, denoted N, was described; this appears to be expressed in a tissue-specific manner in human and rodent cells, and it also belongs to the group of common proteins (10–12).

In addition to the common proteins, at least U1, U2 and U5 contain particle-specific proteins. U1 snRNPs contain three unique polypeptides of apparent molecular weight 70 kDa (70K), 34 kDa (A) and 22 kDa (C), while U2 snRNPs possess a 31-kDa and a 28.5-kDa protein, respectively denoted A′ and B″. U5 snRNP is the snRNP particle with the greatest proportion of polypeptides; it contains at least seven U5-specific proteins, with molecular weights of 40, 52, 100, 102, 116 and 200 kDa, the latter usually appearing as a double band in electrophoresis (13).

Molecular cloning and sequence analysis of the cDNAs for several snRNP proteins (70K, A, A′, C, B″, B′, B, N, D and E) have yielded information about the primary structure of and molecular relationships among these proteins (for review and references, see van Venrooij and Sillekens (14)).

The association of a set of common proteins (B′ to G) with the various snRNAs suggests the existence of a common RNP structure. The snRNAs appear to provide for this, in that they possess a common structural motif, the domain A or Sm site, which consists of a single-stranded region $PuA(U)_{3-6}GPu$

flanked by double-stranded stems. The Sm site is the only major piece of the snRNAs essential for assembling the common snRNP proteins into the snRNPs (15–17). Association of the common snRNP proteins with the Sm-site of the snRNAs appears to be a prerequisite for the trimethylation of the snRNP cap in the cytoplasm (16).

The snRNP proteins are also interesting from a clinical and immunological point of view. Patients with systemic lupus erythematosus (SLE) or related connective tissue diseases often develop autoantibodies that react with particular snRNP proteins (18, 19). Anti-(U1) RNP autoantibodies react with the U1 polypeptides 70K, A and C, so that they precipitate only U1 snRNPs. In contrast, anti-Sm autoantibodies, which are diagnostic of SLE, precipitate all the nucleoplasmic snRNPs, because the major immunoreactive Sm proteins are the proteins B′/B and D, and these are common to all snRNPs (9, 20). Autoantibodies against the U2-specific polypeptides A′ and B″ and autoantibodies against the common proteins E, F and G have also been found in the sera of some patients, but they occur less frequently (21, 22). Little is known about the aetiology of the production of autoantibodies against nuclear antigens such as the UsnRNPs. A variety of immunological abnormalities, such as B- and T-cell hyper-reactivity, has been observed for human SLE (19). However, experimental evidence is accumulating that the snRNPs play an active role as immunogens in the anti-Sm or anti-RNP autoimmune response, *i.e.*, that this response is antigen-driven (19, 23). Therefore, a detailed knowledge of the number and the chemical nature of the autoimmunising B-cell epitopes on the snRNP proteins can be expected to deepen our understanding of the fundamental interactions involved in the autoimmune response against snRNPs.

In the course of experiments originally intended to give information about the RNA-protein interactions in the snRNP core, we used single snRNP proteins purified by HPLC on reversed-phase columns, and detected two proteins with molecular weights close to that of protein D. Modified SDS-polyacrylamide gel electrophoresis allowed the separation of three distinct proteins, and we have termed these D1, D2 and D3 in order of increasing molecular weight (16, 16.5 and 18 kDa). The clear-cut separation made possible their protein-chemical and immunochemical characterisation. Our results show that the three major snRNPs U1, U2, U4/U6 and U5 all contain the same three D-like proteins, but of these only D1 is identical to the protein D as characterised recently by the cloning and sequencing of its cDNA (24). Immunoblotting studies with anti-Sm sera or with affinity purified anti-Sm antibodies showed that SLE patients produce antibodies that recognise Sm epitopes that are present either on all D proteins or only on a subset of these. The significance of the results will be discussed in relation to the assembly pathway of UsnRNPs in the cell and the role of the snRNPs as immunogens and antigens in the anti-Sm autoimmune response.

MATERIALS AND METHODS

Cells and Antisera

HeLa S3 cells and mouse FM3A cells were raised in suspension culture, as described earlier (9). Sera from SLE patients were kindly given to us by Prof. H. Peter, Universtitätsklinik Freiburg. Monoclonal antibodies (mABs) Y12 (25) and 7.13 (26) were kind gifts from J.A. Steitz and S.O. Hoch, respectively. The other mABs used have also been described in other publications: mABs H20 (27); H57, H304, H111 (28) and C383 (29).

Isolation of snRNP proteins

Nuclear extracts were prepared from HeLa cells or mouse FM3A cells by the method of Dignam et al. (30) as modified by Krainer *et al.* (31). For the isolation of certain proteins by HPLC, we preferred to use snRNP particles that lacked the high-molecular-weight, U5-specific proteins. These partially protein-deficient particles were isolated from nuclear extracts that had been prepared by the 'NX-50' method of Zieve and Penman (32). From these extracts, snRNP particles were purified by immunoaffinity chromatography with the help of anti-m_3G antibodies H20 (27). The proteins were finally separated from the snRNA by addition of SDS to 0.1% w/v and extraction with an equal volume of a phenol/chloroform/isoamyl alcohol mixture (50:48:2 v/v; the phenol had previously been equilibrated with a buffer containing 10 mM Tris/HCl at pH 7.5 and 1 mM EDTA). After extraction the proteins were precipitated by addition of 5 vol. acetone and washed with 80% v/v ethanol/water.

Separation of snRNP proteins and peptide fragments by HPLC

snRNP proteins were separated by reversed-phase HPLC (Separation System A120, Applied Biosystems). The proteins, dissolved in 0.1% TFA, were applied to an Applied Biosystems microbore RP-300 column (C-8) and eluted with a step gradient at a rate of 200 μl min^{-1} (eluent A, 0.1% TFA; eluent B, 80% acetonitrile/0.085% TFA). Separation of the peptide fragments obtained from CNBr cleavage was carried out in the same manner, but with a linear gradient composed of the same two eluents.

Separation of snRNP proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

snRNP proteins were separated according to molecular weight by a method based upon that of Laemmli (33). The stacking gel contained 5% acrylamide in 125 mM Tris/HCl, pH 6.8, and 0.1% (w/v) SDS, and polymerisation was initiated by the addition of 50 μl 10% (w/v) ammonium persulphate (APS) and 25 μl N,N,N′,N′-tetramethylethylenediamine (TEMED) for each 10 ml gel solution. Separation gels contained between 11% and 15% acrylamide in 375 mM Tris/HCl (pH 8.8) with 0.1% w/v SDS. The acrylamide stock solution contained 30% w/v acrylamide and 0.8% w/v 'bis' (N,N′-bis-methylene-acrylamide), giving a 'bis'-to-acrylamide ratio of 2.6% w/w. Higher concentrations of 'bis' were also employed (see Discussion). The solutions were not degassed before polymerisation. Standard gels were polymerised by adding 120 μl 10% w/v APS and 12 μl TEMED to 20 ml gel solution, while high-TEMED gels contained 80 μl APS and 80 μl TEMED in 20 ml gel solution, resulting in polymerisation after about 3 min. Yet greater concentrations of the initiator or catalyst gave such rapid polymerisation that gels could not be cast satisfactorily. The electrode buffer contained 25 mM Tris and 192 mM glycine (pH 8.3) and 0.05% w/v SDS. Proteins were dissolved in sample buffer (50 mM Tris/HCl set to pH 6.8, 10% v/v glycerol, 2% v/v SDS, 5% 2-mercaptoethanol and 0.1% w/v bromphenol blue as tracking dye) and run into the stacking gel at a stabilised current of 15 mA; when they reached the separation gel electrophoresis was continued with a stabilised current of 25 30 mA. These current values apply for gels 0.5 mm in thickness; for thicker gels it was increased proportionately. After electrophoresis (exit of the tracking dye), the proteins were stained with Coomassie Brilliant Blue R250.

Quantitative isolation of proteins from SDS-polyacrylamide gels

Proteins separated by gel electrophoresis were electroeluted from excised fragments of the gels by the method of Hunkapiller *et al.* (34). Polyacrylamide gels were always polymerised on the day before use and kept at 4°C overnight. Oxidation of the proteins was avoided by including 0.1 mM thioglycollic acid in the cathode buffer and 0.1% w/v dithioerythritol in the buffer used for electroelution.

Cleavage of proteins by cyanogen bromide

After removal of residual SDS and Coomassie blue (35), electroeluted proteins were dissolved in 70% v/v formic acid and treated with a tenfold or greater molar excess of CNBr in 70% formic acid. The mixture was shaken for 48 h at room temperature, under nitrogen in the dark. After tenfold dilution with water and freeze-drying, the dried protein fragments were dissolved in 0.1% TFA and separated from one another by HPLC for subsequent amino-acid sequencing. The sizes of the fragments were determined by following the protocol of Schägger and von Jagow (36) with a tricine-SDS gel system.

Amino-acid sequencing

Proteins (electroeluted and freed from residual SDS and Coomassie blue; 35) or HPLC-separated CNBr fragments were dissolved in 0.1% TFA and fixed to glass-fibre filters treated with BioBrene Plus (Applied Biosystems). Sequencing was carried out with a gas-phase sequencer (model 471A, Applied Biosystems). The separation of the phenylthiohydantoin (PTH) derivatives of the amino acids was carried out with the associated isocratic on-line HPLC system employing a C-18 column (Applied Biosystems).

Immunoblotting

For immunological investigation, proteins from the SDS gels were transferred to nitrocellulose by a method similar to that of Towbin et al. (37). Blotting was carried out for about 4h at stabilised voltage (60 V) in an electroblotting apparatus (BioRad) with a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol and 0.1% SDS (resulting pH = 8.3). The protein bands on the blots were made visible with Ponceau S dye. Strips of the nitrocellulose corresponding to the lanes were cut out and saturated by immersion for at least 1 h in solution A (10 mM Tris/HCl set to pH 7.4, 100 mM $MgCl_2$, 0.5% Tween 20, 0.1% Triton X-100, 1% BSA and 5% newborn calf serum) (38, 39). Incubation of the strips with antibodies was performed in dilutions of patient serum (1:100) or supernatants of monoclonal antibodies (1:50) in solution A for 2 h at room temperature or overnight at 4°C. After four washes with solution B (10 mM Tris/HCl set to pH 7.4, 100 mM $MgCl_2$, 150 mM NaCl, 0.5% Tween 20) the strips were incubated with a 1:1000 dilution of the corresponding alkaline-phosphatase-conjugated secondary antibody (goat anti-human IgG, goat anti-mouse IgG, all purchased from Paesel, Frankfurt) in solution A for 90 min at room temperature. After four further washes with solution B, staining was carried out with the reaction mixture (0.1 M diethanolamine, 2 mM $MgCl_2$, 0.01% w/v nitrotetrazolium blue, 1% v/v of a 0.5% w/v solution of 5-bromo-4-chloro-indolyl phosphate in N,N-dimethyl formamide).

Immunoaffinity purification of autoantibodies

Total snRNP protein were separated by SDS electrophoresis on a high-TEMED gel using sample pockets 6.5 cm wide. After transfer to nitrocellulose and staining with Ponceau S, the regions of interest were immediately excised with a scalpel. After saturation with solution A (see above) the strips were incubated overnight with the appropriate patient sera (in 1:50 dilution) as described above. After four washes (as above), the antibodies were eluted by a method similar to that of Smith and Fisher (47), by washing for 3×1 min with the elution buffer (0.1 M glycine/HCl set to pH 2.2, 500 mM NaCl, 0.5% v/v Tween 20 and 1% w/v BSA). The eluates were immediately neutralised with 1 M Tris/HCl at pH 8.0 and were then ready for use in further immune reaction.

RESULTS

Fractionation of three D-sized proteins on reversed-phase columns

During fractionation of the protein mixture from purified snRNPs U1 to U6 on a reversed-phase column under HPLC conditions, we noticed the reproducible occurrence of a closely-spaced triple peak at an eluent ratio of about 55% eluent A to 45% eluent B (see Fig. 1A, fractions 6, 7 and 8 for a typical example). If the proteins from fractions 6, 7 and 8 were run on an 11% SDS-polyacrylamide gel, they all migrated in the region associated with protein D, but, on closer inspection, they revealed small differences in molecular weight (Figure 1B). The similar intensities of the three regions make it improbable that these bands represent three different conformations of one and the same protein, implying rather one of the following possibilities: (i) there are three different post-translational modifications of protein D, (ii) the amonino- or carboxyl-terminal residues of protein D are easily cleaved off, or (iii) three different proteins are present.

Separation of the D-sized proteins by modified SDS-polyacrylamide gel electrophoresis

We first attempted to distinguish experimentally between the three possibilities stated above. This was aided by the serendipitous observation that the D-sized proteins could be separated clearly and reproducibly on SDS-polyacrylamide gels when the polymerisation of the acrylamide was carried out with a concentration of TEMED that was 6 7 times higher than given in generally-used protocols (33, 40, 41). Such gels are referred to in this paper as 'high-TEMED gels'.

Figure 1C shows a separation of the proteins of fractions 6, 7 and 8 of the HPLC chromatogram on a 12.5% polyacrylamide high-TEMED gel. While fractions 6 and 8 each contained mainly a single protein, of molecular weight 18 and 16.5 kDa respectively, fraction 7 contains two proteins, whose molecular weights are 16 and 18 kDa. The greater resolving power of the high-TEMED gel is seen by comparing Figures 1B and 1C. It must be emphasized in this context that the molecular weight assignments are tentative, as the high-TEMED gels showed in this MW range a certain deviation from the usual logarithmic relation between relative mobility and molecular weight (calibration data not shown). Figure 1C shows that the splitting of the D band can also be seen when the total protein mixture from snRNPs U1 U6 is run on a high-TEMED gel (Figure 1C, lane 1). In the following experiments and discussion, the proteins are referred to as D1, D2 and D3 in order of increasing molecular weight (16 , 16.5 and 18 kDa respectively).

In the next experiment, two aliquots of the same preparation of snRNP total proteins were run separately, either in a 15% SDS-polyacrylamide gel with standard TEMED concentration

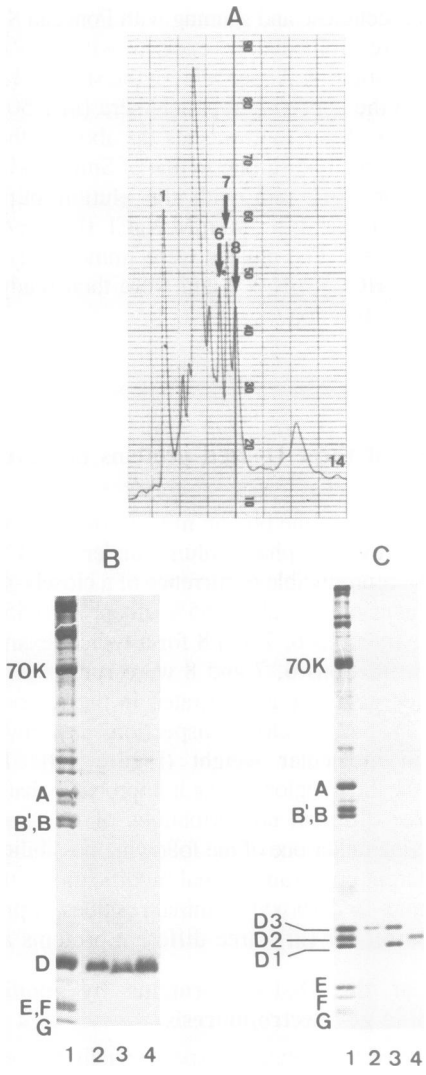

Fig. 1. The separation of D-sized proteins by reversed-phase HPLC. Immunoaffinity-purified snRNPs from NX-50 nuclear extracts were separated into their constituent proteins (see Materials and Methods). (A) shows a part of the elution profile, in which fractions 6, 7 and 8, indicated by arrows, contain D-sized proteins. (B) and (C) show in lanes 2, 3 and 4 the analysis of these three fractions on SDS-polyacrylamide gels, polymerised under standard conditions (B, 11% polyacrylamide) or high-TEMED conditions (C, 12.5% polyacrylamide). Lane 1 in (B) and (C) contained for comparison total UsnRNP proteins from splicing extracts.

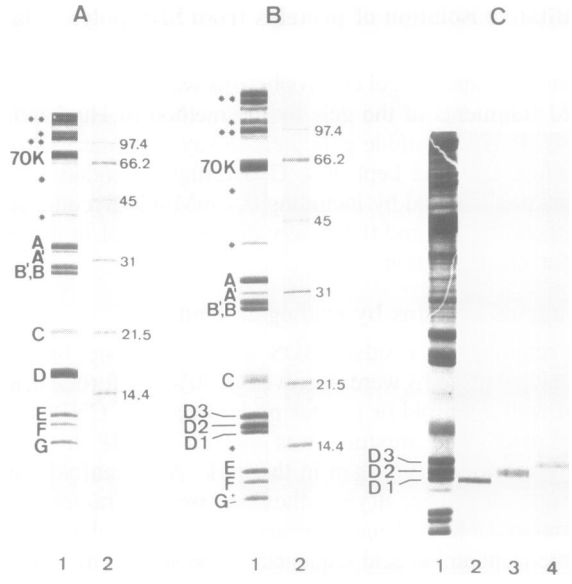

Fig. 2. Separation of total snRNP proteins by high-TEMED SDS-polyacrylamide gel electrophoresis. (A), separation by standard gel (15% polyacrylamide); (B), separation by high-TEMED gel (12.5% polyacrylamide). Identical samples were loaded onto the gels (A and B, lanes 1) and run alongside molecular-weight standards (lanes 2) (BioRad, Low Molecular Weight Standards). Asterisks indicate U5-specific proteins. The symbol + in lane 1 of panel B indicates the presence of a second G-sized protein. The three D-sized proteins run with apparent molecular weights of 16 kDa (D1), 16.5 kDa (D2) and 18 kDa (D3). (C) After electroelution from the gel, the proteins of each resolved D band were subjected to repeated electrophoresis on a high-TEMED gel. They showed exactly the same migration behaviour as in the first electrophoretic run. Lane 1, total snRNP proteins; lane 2, D1; lane 3, D2; lane 4, D3.

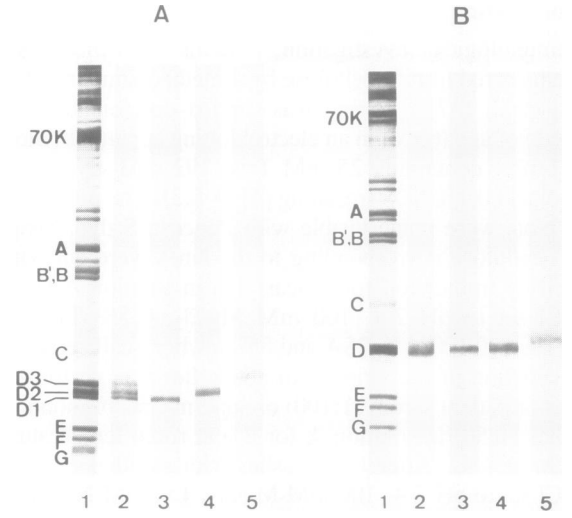

Fig. 3. Evidence that the migratory behaviour of electroeluted D-sized proteins is determined by the gel system only. Separation on a high-TEMED gel (12.5% polyacrylamide) (A) resolved the D bands, while these ran together in the standard gel system (15% polyacrylamide) (B), irrespective of whether they had been prepared by electroelution from a high-TEMED or from a standard gel. Lanes 1, total snRNP proteins; lanes 2, D bands electroeluted from a standard gel; lanes 3, 4 and 5, proteins D1, D2 and D3, respectively isolated from a high TEMED gel.

(Figure 2A) or in a 12.5% SDS-polyacrylamide gel with high TEMED concentration (Figure 2B). Comparison of the two gels showed that, as expected, in the standard gel only a thick D band could be seen, while the high-TEMED gel again revealed three well-resolved bands corresponding to molecular weights 16, 16.5 and 18 kDa. Exact comparison showed that some of the snRNP proteins migrate in the high-TEMED gel at rates slightly different from those in the standard gel: for example, A′ migrates more rapidly in high-TEMED. However, all of the known proteins in the region 8 to 30 kDa (such as B′, B, B″, C, E, F and G) are present, so none of them can account for the extra D-sized bands. This is strong evidence that the three D-sized bands in the high-TEMED gel all originate from the broad D band seen on standard gels. This is also consistent with the fact that the broad band in the standard gel clearly takes up more stain than any of the separate well-resolved bands in the high-TEMED gel stain, while the fact that these stain with roughly equal intensity suggests that they originate from proteins present in roughly equal copy numbers.

The clean separation of the D-sized proteins in the high-

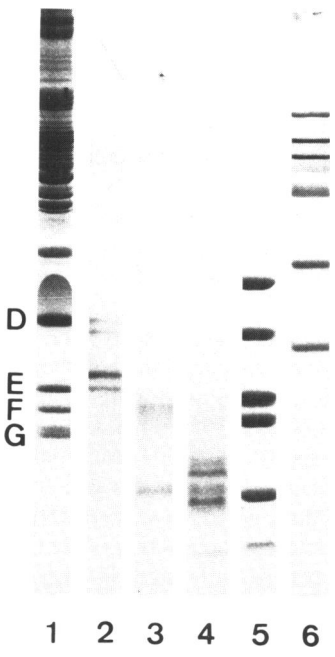

Fig. 4. CNBr cleavage patterns of the proteins D1, D2 and D3. Electroeluted proteins D1, D2 and D3 were cleaved with CNBr and separated on a tricine-SDS gel (see Materials and Methods). The separation gel contained 16.5% polyacrylamide (3% w/w bis:acrylamide), the spacer gel contained 10% polyacrylamide (1.5% bis) and the stacking gel 4% polyacrylamide (1.5% bis). Lane 1 shows total snRNP proteins and lanes 2, 3 and 4 the cleavage products of D1, D2 and D3 respectively. Lane 5 contains molecular weight standards 2.5 to 17 kDa (Protein Standard Mixture I, Merck), and lane 6 molecular weight standards 14.4 to 97.5 kDa (Low Molecular Weight Standard, BioRad).

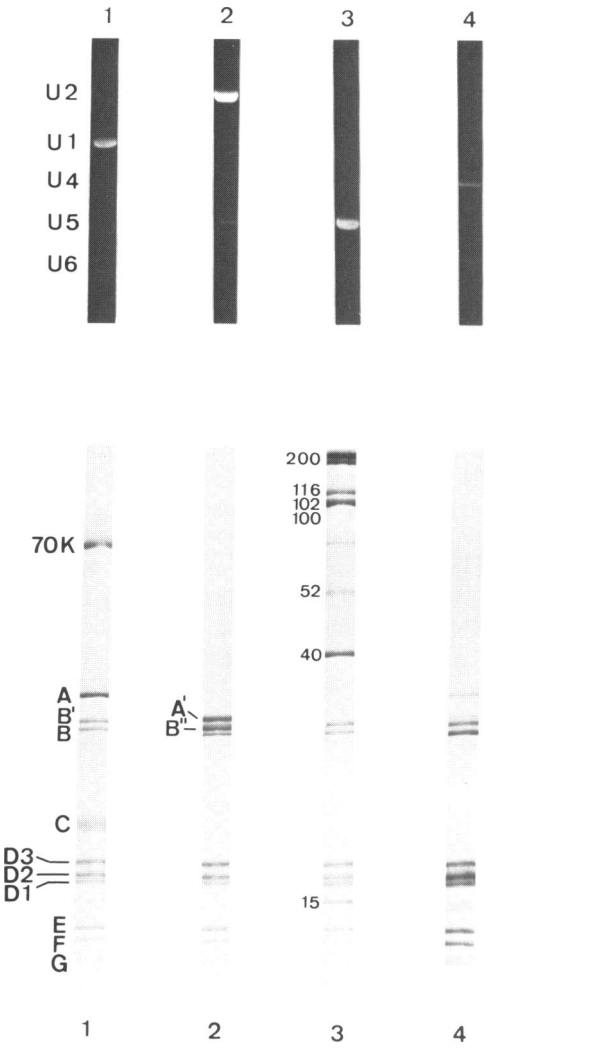

Fig. 5. Presence of proteins D1, D2 and D3 in each of the snRNPs U1, U2, U4/U6 and U5. The individual particles were isolated by the method of Bach *et al.* (13). The upper part of the Figure shows the analysis of the snRNAs, isolated and separated on a 10% urea-TBE-polyacrylamide gel (9). The lower part shows the separation of the protein components on a high-TEMED, 12.5% SDS-polyacrylamide gel. In each gel, lanes 1 4 contain U1, U2, U5 and U4/U6, respectively. The common and the particle-specific proteins are indicated.

TEMED gels allowed a preparative isolation of the three proteins by electroelution from the gel. When the individual proteins D1, D2 and D3 were subjected to repeated gel electrophoresis, they ran exactly as they had done before preparative electroelution (Figure 2C).

A further experiment was performed in order to test our hypothesis that the three proteins separated on the high-TEMED gel correspond to the broad D band on the standard gel (Figure 3). The D band from a standard gel was electroeluted and subjected to repeated electrophoresis, this time in a high-TEMED gel. Proteins with molecular weights 16, 16.5 and 18 kDa were observed (Figure 3A, lane 2). These three proteins electroeluted from high-TEMED gels retained their distinct migratory behaviour (Figure 3A, lanes 3, 4 and 5). Conversely, when these proteins were eluted from high-TEMED gels and re-run on standard gels, they could no longer be distinguished from one another (Figure 3B). This shows that the separation of the proteins in the 16 18-kDa region in high-TEMED gels is not the result of irreversible modification of the proteins caused by the new gel system. We conclude that the polyacrylamide gel matrix as formed under high-TEMED conditions allows better separation of the proteins in this range of molecular weight, and the three bands really correspond to three distinct polypeptide chains that are not resolved under standard conditions.

Biochemical analysis of the proteins D1, D2 and D3

In order to investigate whether the three D proteins result from different post-translational modifications or have fundamentally different primary structures, the individual electroeluted proteins were cleaved with cyanogen bromide and the peptides obtained were fractionated in tricine-containing SDS-polyacrylamide gels. As is seen in Figure 4, the three proteins gave completely different CNBr cleavage patterns, a clear sign that they possess different primary structures.

This was confirmed by partial protein-sequencing. Attempted N-terminal sequencing of these proteins with a gas-phase sequencer showed that only D1 was sequenceable, while the other two had blocked N termini. The first ten N-terminal residues of D1 agreed exactly with the sequence of the protein D as derived from cloned cDNA (24). Furthermore, two CNBr fragments also corresponded to excerpts from the cDNA sequence. However, two internal peptides taken respectively from D2 and D3 showed no overlap with the protein D sequence as deduced from the cDNA (data not shown). We thus conclude that the D1 is identical to the protein D characterised earlier, while the proteins D2 and D3 are different from D1 and, in view of their highly dissimilar CNBr cleavage patterns, very probably from each other too.

Proteins D1, D2 and D3 belong to the group of proteins common to all snRNPs

Preparations of D1, D2 and D3 described so far were made from a total protein mixture from all the major snRNPs U1, U2, U4/6 and U5. In order to find out whether the two new proteins D2 and D3, like D1, are common or particle-specific proteins, we analysed the individual snRNPs by electrophoresis in high-TEMED gels. As Figure 5 shows, the three proteins D1, D2 and D3 occur in each particle in approximately the same amounts. This implies that in addition to D1, D2 and D3 are also common proteins.

Proteins D1, D2 and D3 are also present in mouse snRNPs

Figure 6 shows the separation of a total-protein mixture of snRNPs U1 to U6, isolated from splicing extracts from mouse FM3A cells. On a standard gel (Figure 6A) a broad, intense protein band corresponding to a molecular weight around 16 kDa is seen, while under high-TEMED conditions (Figure 6B) this is resolved into three components of apparent molecular weight 16, 16.5 and 18 kDa. This reproduces exactly the behaviour of human snRNPs, and shows that the occurrence and size of the proteins D2 and D3 have been, at least to some degree, conserved in evolution.

Differential reactivity of proteins D1, D2 and D3 with anti-Sm autoantibodies

To the best of our knowledge, previous work on the reactivity of the protein D with anti-Sm autoantibodies has always been carried out under conditions (immunoblot or ELISA) where, seen retrospectively, the protein D must have been present as a mixture of D1, D2 and D3. With the knowledge that D2 and D3 differ in sequence both from D1 and, in all probability, from each other, it was of interest to see whether D1, D2 and D3 react differentially with anti-Sm autoantibodies.

We first tested the reactivity of various monoclonal antibodies of the Sm type, selected for the property of precipitating all snRNPs. For this purpose, D proteins fractionated on high-TEMED gels were blotted onto nitrocellulose and incubated along with the various antibodies (Figure 7). The monoclonal antibody 7.13, earlier characterised as D-specific (26) reacts exclusively with D1 (Figure 7, lane 1). In contrast, Y12 (25) reacts with proteins D1 and D3 and also with B′/B and E (Figure 7, lane 2). H57, isolated from a mouse that had been immunised against snRNPs U1 U6 (A. Daser, R. Reuter and R. Lührmann, unpublished) reacted only with the common proteins B′/B, but with none of the D proteins (Figure 7, lane 3). As controls, monoclonal antibodies H304, H111 and C383 were used. The first two are U1-specific, as H304 reacts only with the A protein (Figure 7, lane 4) and H111 with the 70K protein (Figure 7, lane 5), while C383 does not react with snRNPs.

Next, we investigated the reaction of the three D proteins with 38 antisera from patients suffering from SLE or related connective tissue disorders. The sera had been pre-selected for the presence of autoantibodies against the Sm-D protein. It was found that the sera could be classified into three major groups on the basis of their pattern of reactivity with proteins D1, D2 and D3 (Figure 8). The largest of these groups (21/38) comprise sera that react with D1 and D3. Sera in the second group (14/38) reacted with all three proteins. Finally, a small number of sera (3/38) reacted strongly with protein D2 and very weakly with the others. In Figure 8, the reactions of representative sera for these groups

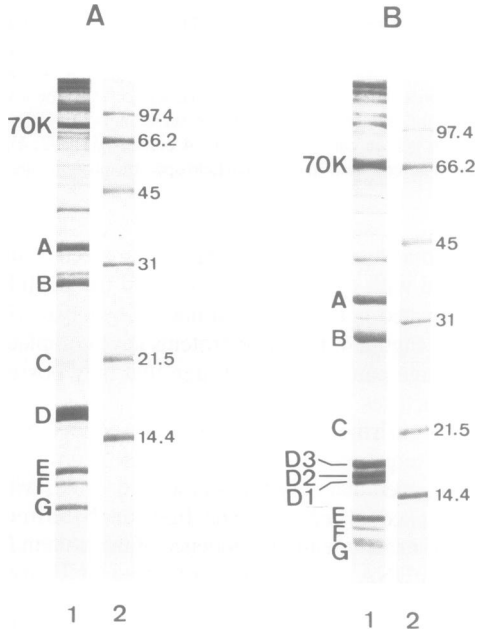

Fig. 6. The separation of snRNP proteins from mouse FM3A cells. Electrophoresis was carried out on a 15% SDS-polyacrylamide gel with standard (A) or on a 12.5% SDS-polyacrylamide gel with high (B) TEMED concentrations. Lanes 1, proteins from affinity-purified snRNPs from mouse cells; lanes 2, molecular weight standards (Low Molecular Weight Markers, BioRad).

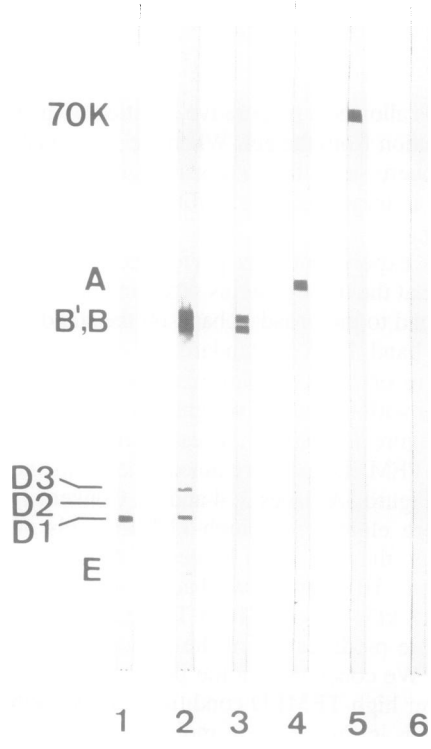

Fig. 7. Reaction of snRNP proteins with various monoclonal antibodies. snRNP proteins were separated electrophoretically on a 12.5% high-TEMED gel and electroblotted onto nitrocellulose. Blot strips were incubated with monoclonal antibodies against snRNP proteins, as follows: lane 1, monoclonal antibodies of the Sm type 7.13 ; lane 2, monoclonal antibody Y12. Other blot strips show for comparison the reactions with other monoclonal antibodies: lane 3, H57 (anti-B′/B); lane 4, H304 (anti-A); lane 5, H111 (anti-70K); lane 6, as a control, anti-ribosomal C383. Some of the snRNP proteins are indicated.

are illustrated. In addition to their reactivity with D proteins, all the sera possessed a high titre of anti-Sm-B/B′ antibodies and many also reacted strongly with the RNP antigens 70K, A and C.

Immunochemical properties of the autoantibodies against snRNP proteins D1, D2 and D3

In order to determine the specificity of autoantibodies against the snRNP proteins D1, D2 and D3, and to detect possible cross-reactivity among these proteins, we affinity purified the respective antibodies. This was carried out with one representative serum from each of the three groups in Figure 8 A C. UsnRNP proteins were purified preparatively on a high-TEMED gel and electroblotted onto nitrocellulose. The nitrocellulose was stained with Poinceau S and the three bands containing D1, D2 and D3 were cut out. As controls, a region of the nitrocellulose with the proteins B′/B and a region bearing no protein were used. After incubation of each of these strips with one of the patient sera, followed by elution of the strip, the affinity-purified antibodies were allowed to react with electroblotted proteins from snRNPs U1-U6.

Figure 9A shows the immunochemical properties of the affinity-purified antibodies from a typical anti-Sm serum that reacts with D1 and D3 but not with D2 (Serum 1 from Figure 8A). Antibodies that were affinity-purified on protein D1 cross-reacted with D3 and B′/B (lane 1). Conversely, anti-D3 antibodies cross-reacted with D1 and B′/B (lane 2). Consistently with this, antibodies against B′/B also cross-reacted with D1 and D3 (lane 3). Control antibodies, eluted from the paper strips to which no protein was bound and thus giving a measure of the background adsorption to the nitrocellulose, showed a light coloration with proteins B′/B (lane 4) that, however, was by no means comparable with the intensity of the reaction of proteins B′/B in lanes 1 3. Furthermore, there is no sign of reaction with proteins D1 D3 in lane 4 (Figure 9A). Analogous cross-reactions of the antibodies against D1, D3 and B′/B as described in Figure 9A were also observed with two other sera from the group represented in Figure 8A (data not shown).

Figure 9B shows the reaction spectrum of the affinity-purified antibodies from an Sm serum that reacts equally strongly with proteins D1, D2 and D3 (Figure 8B). In this case, antibodies that were affinity-purified from D1, D2 or D3 react equally strongly with all D proteins and with B′/B protein (Figure 9B, lanes 1 3). Antibodies against B′/B react equally strongly with D1 and D3, and rather less strongly with D2. This indicates that there may be an epitope common to D1, D2, D3 and B′/B.

Figure 9C shows the investigation of an anti-Sm serum that reacts preferentially with D2 and only very weakly with D1 and D3 (compare Figure 8C). D2-affinity-purified antibodies react strongly with D2 and scarcely react with D1 and D3. In interesting contrast to the situation in Figures 9A and 9B, the reaction of the anti-D2 antibody with B′/B hardly lies above the background level (compare lanes 1 and 3 in Figure 9C). This suggests that this serum contains an antibody specificity that recognises an epitope of D2 only, and fails to cross-react with B′/B. In agreement with this, B′/B-affinity-purified antibodies show hardly any cross-reactivity with D2 and only a little with D1 and D3 (lane 2, Figure 9C). The strong reaction with protein B′/B (lane 2) can thus only be explained by an anti-B′/B antibody population that reacts monospecifically with B′/B. (For this reason, the differential reaction behaviour of the affinity-purified antibodies against proteins D1, D2 and D3 justifies the classification of the anti-Sm antisera into at least three groups.)

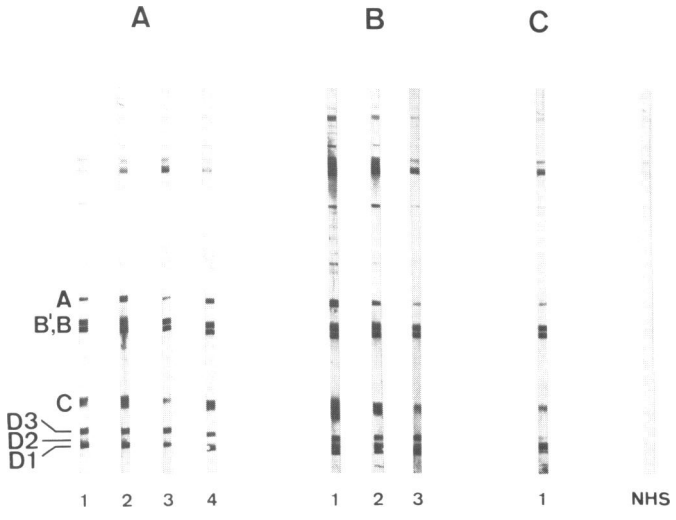

Fig. 8. Reaction of the snRNP proteins with anti-Sm sera from SLE patients. snRNP proteins were separated on high-TEMED gels (11% acrylamide) and blotted onto nitrocellulose strips. The strips were incubated with autoimmune sera from SLE patients. 38 sera were tested, all of which had been shown to react with a D protein. The sera were found to fall into three groups, of each of which a selection is shown here. Group A shows four out of a total of 21 sera that react with D1 and D3 but not with D2. Group B shows three out of a total of 14 sera that react with all three D proteins. C is one of three sera that reacted strongly with D2 and weakly with the others. The final lane (NHS) shows as a control the reaction of a normal human serum. The D proteins and also the proteins 70K, A and B/B′ are indicated.

DISCUSSION

Improvement of the separation performance of SDS-polyacrylamide gels by increased TEMED concentration

In this paper we have described the identification and the protein-chemical and immunological characterisation of two new proteins that are associated with UsnRNP particles and that react with anti-Sm autoantibodies. The critical step in the identification of these proteins was the observation that the broad band of protein D, which migrates at about 16 kDa in gels prepared according to standard protocols, splits into three well-resolved bands when run on gels prepared with a higher concentration of the polymerisation catalyst TEMED (Figure 2B). The apparent molecular weights of the three proteins are 16, 16.5 and 18 kDa, and we have named the proteins D1, D2 and D3 in order of increasing molecular weight.

It may be assumed that the more rapid polymerisation of the 'high-TEMED' gels leads to whatever structural change in the polyacrylamide matrix and is responsible for the improved separation in this region of molecular weight. The improvement appears to be connected with the polymerisation rate rather than with the pore size, as the generation of smaller pores by using a bis-to-acrylamide ratio of 5% (which leads to a minimum pore size irrespective of the absolute acrylamide concentration; 42) did not result in a gel capable of separating the three D proteins unless the concentration of TEMED was also raised (data not shown). A control experiment showed that the pH of the polymerisation mixture was not significantly affected by the presence of the additional TEMED.

The possibility that the high-TEMED gels caused irreversible modification of the proteins, thus leading to an artefactual separation, was excluded by several control experiments. First

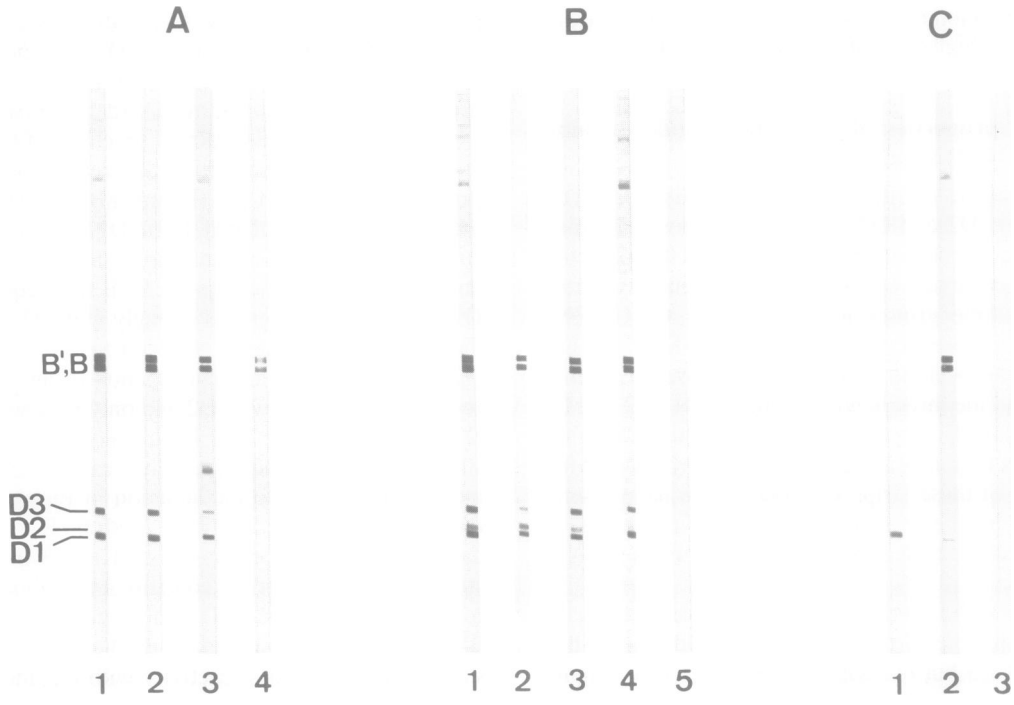

Fig. 9. Reaction of blotted snRNP proteins with affinity-purified Sm-antibodies. snRNP proteins were separated preparatively on a 12.5% high-TEMED gel and blotted onto nitrocellulose. After staining with Ponceau S, the bands of the proteins D1, D2, D3, B'/B and a piece of nitrocellulose bearing no proteins were cut out. The protein strips were incubated with a representative serum from each of the three groups A, B and C in Figure 8 in order to elute the bound antibodies. The affinity-purified antibodies isolated in this way were then allowed to bind to strips onto which the electrophoretically-separated snRNP proteins had been blotted. (A) shows the immune reaction of affinity-purified antibodies from one of the sera in group A of Figure 8. The sera of this group possess antibody specificity against D1 and D3 but not against D2. Lanes 1, 2 and 3, respectively, show the reaction of the autoantibodies that were purified using D1, D3 and B'/B. Lane 4 shows a background control using antibodies eluted from a portion of the nitrocellulose to which no snRNP protein had bound. (B) shows the reaction of autoantibodies affinity-purified from proteins D1, D2, D3 and B'/B (lanes 1 4) after incubation with a representative of sera in group B of Figure 8. Sera in this group recognise all three D proteins. Lane 5 shows the background, as before. (C) shows the immune reaction of autoantibodies that were obtained from the serum in Figure 8C after affinity purification on D2 and B'/B (lanes 1 and 2, with the background control in lane 3).

of all, the proteins separated on high-TEMED gels retained their individual migratory behaviour when re-run on fresh high-TEMED gels (Figures 2C and 3A). When isolated D1, D2 and D3 were re-run on fresh gels with the standard TEMED concentration, they reverted to their usual behaviour and ran as a single, broad band (Figure 3B). Finally, when the broad D band was electroeluted from a standard gel and re-run, it split into the three well-resolved bands when the second electrophoresis was performed under high-TEMED conditions (Figure 3A) but not when a standard gel was used (Figure 3B).

Although the improved resolution of the D proteins is the most striking, improved resolution of the other snRNP proteins is also observed with the high-TEMED gels. We describe here three cases of this.

(1) Electrophoretic fractionation of the proteins of the 20S U5 snRNP gives a clear band corresponding to an apparent molecular weight of 15 kDa (Figure 5, lane 3). In describing the protein composition of the 20S U5 particle, we had earlier noted a band that in standard gels runs somewhat ahead of the broad D band (13), but on account of its poor resolution we did not then attribute it to a U5-specific protein. This 15-kDa protein is found reproducibly in separate U5 snRNP preparations, and we have recently detected it together with other U5-specific proteins in the isolated 25S [U4/U6/U5] tri-snRNP complex (S. Behrens and R. Lührmann, unpublished).

(2) On close examination, it can also be seen that the G protein is split into two bands, although these stain with Coomassie with unequal intensity (Figure 2B). At present it is unclear whether these bands represent two different proteins or two different post-translational modifications of the same protein.

(3) Finally, the C protein is also seen clearly resolved into two bands (Figure 2B). These are probably variants of the same protein with different post-translational modification (43).

It can reasonably be expected that the improved separation by high-TEMED gels may also be applicable to other proteins, at least in some cases.

Three distinct D proteins and the snRNP core structure

Several lines of evidence suggest that the proteins D2 and D3 are structurally distinct from D1 and not post-translational variants of D1. (i) For D1, sequencing of the 10 N-terminal amino acids and of two internal peptides obtained by CNBr cleavage showed complete agreement with the sequence derived from the cDNA sequence obtained by Rokeach *et al.* (24). We can therefore safely take it that D1 is identical to the cloned protein D. (ii) Amino-acid sequencing of one internal, CNBr-cleaved peptide from each of the proteins D2 and D3 (data not shown) reveals no overlap between either of these and the protein sequence of D1 as obtained by cDNA cloning. (iii) Comparison of the CNBr cleavage patterns of the three D proteins shows no similarity of proteins D2 and D3 with D1, or with each other. Furthermore, we have in this work found anti-Sm autoantibodies that react differentially with proteins D2 and D3 (Figures 8 and 9, see also below). There is thus good reason to believe that D1, D2 and D3 have little or no structure in common.

We have previously described a protein denoted D', which is

shared by all snRNPs and migrates slightly faster than the D protein normally observed by conventional SDS-polyacrylamide gel electrophoresis (9). As we do not yet have any protein-chemical data for D', it is not yet possible to say whether D' corresponds to D2, to D3 or to neither of these.

The analysis of snRNP proteins from mouse FM3A cells showed that here, too, the broad 16-kDa band of the D proteins seen in standard gels is resolved into three well-separated bands on high-TEMED gels. These bands correspond to molecular weights similar to those of the human proteins D1, D2 and D3 (Figure 6). This shows that the expression of three distinct D proteins is not a property peculiar to HeLa cells. The presence and sizes of these proteins makes it appear likely that these proteins have been conserved in evolution, at least among the higher vertebrates.

In addition to protein D1, both D2 and D3 occur in all the major snRNPs U1, U2, U4/U6 and U5 (Figure 5), so that these also belong to the common proteins. At present, we cannot completely exclude the possibility that proteins D2 and D3 appear in different forms when they are associated with different snRNP particles. An ultimate answer to this question can only be obtained by the sequencing of D2 and D3 from the various snRNPs.

The occurrence of two further common proteins in the snRNP core raises interesting questions concerning the morphogenesis of the snRNP core structure in the cytoplasm. It has been shown by Fisher *et al.* (43) that proteins D, E, F and G associate spontaneously to give a 6S hetero-oligomer, and it is probably in this form that they bind to the Sm site of a newly-transcribed snRNA. At present, it is an open question whether the three D proteins are all involved in the 6S hetero-oligomer or whether they are incorporated independently of one another into the snRNP core. Findings such as that of Sauterer *et al.* (44), that D-sized proteins towards which mAb 7.13 is unreactive can become integrated into the 6S hetero-oligomer (mAb 7.13 reacts only with D1, Figure 7), may shed light on this.

It is too early to speculate over the function of proteins D2 and D3, since at present none of the tasks of the common snRNP proteins has been assigned. An important condition for answering such questions will be the availability of specific antibodies against D2 and D3.

Three distinct D proteins and anti-Sm autoantibodies

The occurrence of two additional common D proteins in the major UsnRNPs, structurally distinct from the D protein previously presumed to be unique, immediately raised the question of the role of the three D proteins as antigens in the anti-Sm autoimmune response of SLE patients. This was especially important as previous studies aimed at investigating the Sm-antigenic proteins have employed a mixture of the proteins D1, D2 and D3 as antigen.

The results of immunoblot experiments employing anti-Sm sera or monoclonal antibodies have demonstrated a surprising diversity of Sm epitopes, distributed in a complex pattern among the proteins D1, D2, D3 and B'/B, as follows.

1. Epitope I lies on proteins D1, D2 and B'/B. This is the epitope against which patients produce autoantibodies most frequently (55%).
2. Epitope II lies on all D proteins and also on B'/B. 37% of the Sm-D patient sera contained antibodies against this epitope.
3. Epitope III is found on the D2 protein only, and is relatively rarely (8%) recognised by antibodies in patient sera.
4. Epitope IV is defined by the monoclonal antibody Y12 and is found on the proteins D1, D3, B'/B and E. The last of these allows the distinction between epitopes IV and I, as autoantibodies against epitope I do not react with the E protein.
5. Epitope V lies on protein D1 only and is defined by the monoclonal antibody 7.13.

It should be emphasized that none of these autoantibody populations cross-reacts with protein A or protein C. We can thus assume that epitopes I, II and IV, which lie *inter alia* on the B'/B protein, are structurally distinct from the proline-rich B-cell autoimmunising epitope that is common to the proteins B'/B, N, A and C and against which anti-Sm autoantibodies are also produced (45).

How far the differential reactivity with respect to the proteins D1, D2 and D3 of the autoantibody populations observed here can be exploited for diagnostic purposes remains to be seen, and an answer to this can only be awaited as a result of long-term studies.

However, in addition to possible medical application, the observation of diverse Sm epitopes on proteins D1, D2 and D3 is first and foremost of theoretical interest. These results show clearly that D1, D2 and D3 are immunologically distinguishable, which supports our protein-chemical evidence that these proteins are structurally different. Yet the observed cross-reactivity of some antibodies with D1 and D3 or D1, D2 and D3 suggests that they share certain structural features. When the sequences of D2 and D3 are known, it will be possible to look for homologies among the three D proteins.

It is, for example, important to observe that epitopes I, II and IV are shared not only by D1 and D3 or D1, D2 and D3; they are also present on the proteins B'/B, which have no sequence homology with D1 (24, 46). This means either that these epitopes arise from common post-translational modifications, or else that conformation-specific epitopes are present. In the latter case, the cross-reacting conformation-specific epitopes must be produced by particular common constellations of amino-acid residues. The same considerations apply for common epitopes on proteins D2 and D3. It is clear that the molecular characterisation of the cross-reacting epitopes common to the D and B'/B proteins will be a decisive step towards understanding the mechanism by which anti-Sm antibodies appear.

Independently of the chemical nature of the Sm epitope, its simultaneous repetition on several common snRNP proteins is in itself significant for the question of the anti-Sm immune response, in that a high density of repetitive epitopes on the antigen molecule could be important in providing a strong signal to B cells.

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