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Accelerated Publications

Chemical Cross-Linking of Sm and RNP Antigenic Proteins†

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ABSTRACT: Nuclear extracts, competent for in vitro premessenger RNA splicing, were chemically cross-linked with thiol-reversible reagents in order to study the organization of proteins within ribonucleoprotein particles (RNPs) containing uridine-rich small nuclear RNAs (UsnRNPs). The distribution of select UsnRNP antigens within cross-linked complexes was determined by Western blotting of diagonal two-dimensional gels. On the basis of calculations from the molecular weights of cross-linked complexes containing UsnRNP common proteins B′, B, and D, it is proposed that each of these proteins was associated with UsnRNP common proteins E and G. In addition, D′ is proposed to be positioned close to D. The spatial distribution of UsnRNP common proteins was such that B′ and B could not be cross-linked to D. The data also suggested that the 63-kDa U1 snRNP specific protein was cross-linked to other U1-specific proteins, particularly C, but not to the UsnRNP common proteins. We propose that part of the UsnRNP core of common proteins contains at least two asymmetrical copies of B′:B:D:D′:E:G with stoichiometries of 2:1:1:1:1:1 and 1:2:1:1:1:1.

Chemical cross-linking has been widely used for mapping extra- and intracellular protein interactions. The potential power of this technique in revealing the protein organization within ribonucleoprotein particles such as those that package premessenger RNA (hnRNPs)[1] or uridine-rich small nuclear RNAs (UsnRNPs) has not been exploited. Biochemical

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[1] Abbreviations: RNP, ribonucleoprotein particle; UsnRNP, RNP containing uridine-rich small nuclear RNA; hnRNP, RNP containing heterogeneous nuclear RNA; PMSF, phenylmethanesulfonyl fluoride; TEO, triethanolamine; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DTBP, 3,3′-dimethyldithiobis(propionimidate); PAGE, polyacrylamide gel electrophoresis; 2D, two dimensional; Tris, tris(hydroxymethyl)aminomethane; NEM, *N*-ethylmaleimide; NaDodSO$_4$, sodium dodecyl sulfate.

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analyses have characterized hnRNPs in terms of their size and properties and, importantly, have shown that a sextet of 30–45-kDa proteins, designated A–C core proteins, is the major structural component of hnRNPs (Pederson, 1974; Beyer et al., 1977; Stevenin et al., 1978; Choi & Dreyfuss, 1984; Lahiri & Thomas, 1985; Lothstein et al., 1985; Wilk et al., 1985). Chemical cross-linking coupled with biochemical analyses has suggested that monoparticles consist of three to four repeating units, each having a stoichiometry for A1, A2, B1, B2, and C1, and C2 of 3:3:1:1:3:1 (Lothstein et al., 1985).

As with hnRNPs, the major UsnRNP proteins represent a subset of the total nuclear proteins and have been given alphabetical designations. B′ (29 kDa), B (28 kDa), D (16 kDa), D′ (15.5 kDa), E (13 kDa), F (11 kDa), and G (9 kDa) are components of U1, U2, U5, and U4/U6 snRNPs (Lerner & Steitz, 1979; Hinterberger et al., 1983; Kinlaw et al., 1983; Billings & Hoch, 1984; Mimori et al., 1984; Bringmann & Lührmann, 1986). In addition to these UsnRNP common proteins, U1 snRNPs uniquely contain A (34 kDa), C (22 kDa), and a 70-kDa protein, whereas A′ (33 kDa) and B″ (28.5 kDa) proteins are unique components of U2 snRNPs. Ribonuclease digestion studies have suggested that E and G proteins may form a core that binds to a conserved RNA sequence motif (Liautard et al., 1982; Riedel et al., 1987). Analyses of UsnRNP assembly using mutant snRNAs have suggested that B′, B, D, E, F, and G bind within a common 3′ or central domain of U1 and U2 snRNAs, respectively, which includes the conserved sequence motif (Mattaj & DeRobertis, 1985; Mattaj, 1986; Hamm et al., 1987; Patton & Pederson, 1988). The UsnRNP unique proteins A, A′, B″, C, and 70 kDa were bound to different domains of snRNA sequence or secondary structure.

In this paper, we demonstrate an application of protein–protein cross-linking technology for analysis of UsnRNP protein organization.

Experimental Procedures

Nuclear extracts were prepared from spinner culture HeLa cells grown to mid log phase in Joklik's media containing 10% fetal calf serum essentially as described by Dignam et al. (1983). Modifications included TEO, pH 7.9, as a buffer, DTT was omitted, and 1 mM PMSF, 0.05 μg/mL leupeptin and aprotinin, and 20 units/mL soybean trypsin inhibitors were included in all buffers. Protein concentrations of the extracts averaged 6 mg/mL.

In vitro RNA splicing reactions were carried out under optimized conditions (Grabowski et al., 1985) with SP-6 transcripts (Padgett et al., 1983; Konarska et al., 1984). Spliceosome assembly was determined by electrophoresing one-fifth of each in vitro splicing reaction into native, low-porosity agarose–acrylamide composite gels (Zillmann et al., 1987).

12-Å Cross-Linking with 3,3′-Dimethyldithiobis(propionimidate) (DTBP). The conditions were modifications of those described originally by Wang and Richards (1974, 1975). Extracts were diluted to 2 mg/mL protein in in vitro splicing reaction buffer minus DTT and cross-linked with 6 mM DTBP on ice for 1 h. The reactions were then quenched with 100 mM ammonium acetate and brought to 10 mM *N*-ethylmaleimide to prevent free sulfhydryl exchange (on ice for 30 min). Proteins were acetone precipitated and dissolved in 62.5 mM Tris, pH 6.8, 2% $NaDodSO_4$, 9 M urea, and 10 mM NEM at 65 °C before electrophoresis.

Cross-linked proteins were resolved on 5–18% gradient gels [30:0.8 acylamide:bis(acrylamide)] with the Tris, glycine, and $NaDodSO_4$ system described by Laemmli (1970). Following

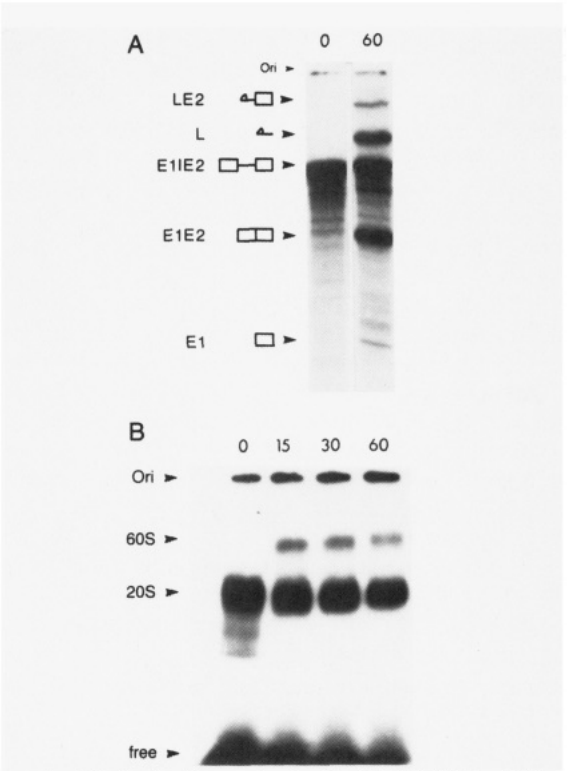

FIGURE 1: (A) Autoradiograph of radiolabeled RNA splicing precursors and products resolved by PAGE following 0- and 60-min (left and right lanes, respectively) in vitro reaction as described under Experimental Procedures. The precursor RNA (E1IE2), splicing intermediates (LE2 and E1), and products (L and E1E2) are indicated at the left of the figure. E, exon; I, intron; L, lariat intron. (B) Aliquots from the reaction in (A) were removed at the times (minutes) indicated above the lanes resolved on native RNP gels and autoradiographed. The positions of the prespliceosome complex (20S) and the in vitro assembled spliceosome (60S) containing the radiolabeled RNA splicing precursor are shown to the left of the figure.

electrophoresis (20 mA for 3 h), individual lanes were incubated at 65 °C for 30 min in cross-link cleavage buffer (2% $NaDodSO_4$, 62.5 mM Tris, pH 8.8, 10% glycerol, 20 mM DTT), followed by incubation at 24 °C in the same buffer at pH 6.8. For the second-dimensional electrophoresis, thiol-treated first-dimensional gels were placed onto the stacking gel of the second-dimensional 10.5% acrylamide Laemmli gel [30:0.8 acrylamide:bis(acrylamide)] and electrophoresed for 3 h at 35 mA.

One-dimensional or diagonal two-dimensional (2D) gels were electrophoretically transferred to nitrocellulose (0.45-μm pore size) for 12 h at 75–100 mA at 7 °C in the Tris, glycine, and methanol buffer of Towbin et al. (1979). These conditions left no silver-stainable material in the gels. Following transfer, the nitrocellulose blots were incubated for 2–12 h in blocking buffer containing 5% horse serum and processed as described by Smith et al. (1986) using peroxidase-conjugated secondary antibodies and the 3,3′-diaminobenzidine substrate system.

Results and Discussion

Nuclear extracts converted approximately 50% of the radiolabeled precursor RNA to products (ligated exon 1-2 and free lariat) during 60 min of in vitro incubation (Figure 1A). Native PAGE revealed that 60S splicing complexes (spliceosomes) were assembled within 15 min of incubation (Figure 1B). Extract competence for in vitro spliceosome assembly and RNA splicing demonstrated the presence of functional, and therefore presumably structurally intact, UsnRNPs.

One-dimensional gel Western blots for the four antibodies used in this study are shown in Figure 2. Autoimmune patient

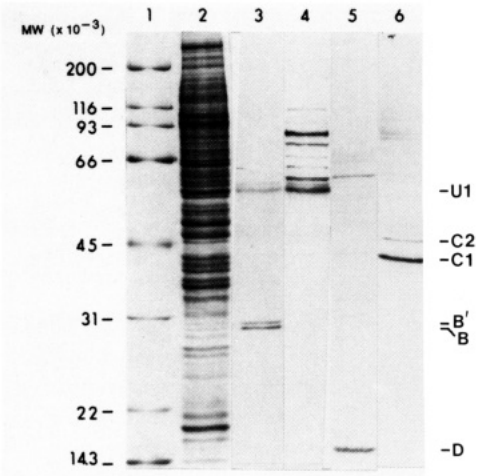

FIGURE 2: Nuclear extract proteins were resolved by one-dimensional 10.5% PAGE, Western transferred, and probed with UsnRNP- and hnRNP-specific antibodies as described under Experimental Procedures. Lanes 1 and 2 show the electrophoretic distribution of molecular weight markers (masses indicated to the left) and total nuclear extract proteins stained with Coomassie blue. Lanes 3–6 are Western blots of parallel lanes containing extract proteins probed with autoimmune patient serum [reactive with the U1 snRNP specific 57-kDa protein and UsnRNP common proteins B′ (29 kDa) and B (28 kDa)], monoclonal antibodies reactive with the U1-specific 57-kDa protein, monoclonal antibodies reactive with UsnRNP D protein (16 kDa), and monoclonal antibodies reactive with hnRNP C1 (41 kDa) and C2 (43 kDa) proteins, respectively.

serum (categorized as mixed Sm/RNP) reacted with nuclear extract UsnRNP antigens having M_r of 28 000 (B) and 29 000 (B′) and the U1 snRNP specific 57-kDa protein. U1 snRNP specific monoclonal antibodies (Billings et al., 1982) reacted with the 57-kDa protein as well as with several other proteins with higher molecular weights. Monoclonal Sm antibodies (Billings et al., 1985) primarily reacted with the 16-kDa UsnRNP D protein. Finally, monoclonal antibodies were used that reacted with hnRNP proteins C1 (41 kDa) and C2 (43 kDa) (Dreyfuss et al., 1984).

The system for analyzing macromolecular assemblies involved chemically cross-linking extract proteins through their free amine residues separated by 12-Å distances. Cross-linking of proteins therefore can suggest certain spatial relationships between components within a macromolecular assembly but does not prove that there are structural or functional interactions. Prior to the first-dimensional PAGE, native macromolecular assemblies were dissociated with 9 M urea and $NaDodSO_4$, leaving only cross-linked complexes intact. First-dimensional gels were subsequently treated with DTT to cleave the chemical cross-links, and the proteins were resolved by second-dimensional PAGE. Non-cross-linked monomers appeared along a diagonal extending from high to low molecular weight (upper right to lower left in Figure 3A). Log molecular weight versus relative mobility plots were generated for the diagonal lines by using the molecular weights of the antigens as internal standards as well as the migration of molecular weight standards on parallel amido black stained blots.

Had thiol-sensitive, cross-linked complexes of proteins been present in the first-dimensional gel, monomers from these complexes would have appeared to the right of the diagonal line (off-diagonal spots) following thiol treatment and electrophoresis into the second-dimensional gel. A few such off-diagonal spots became apparent following silver staining of non-cross-linked protein (Figure 3B) and indicated that there were some endogenous interprotein disulfide bonds. Blots of similar gels as in Figure 3, simultaneously reacted with

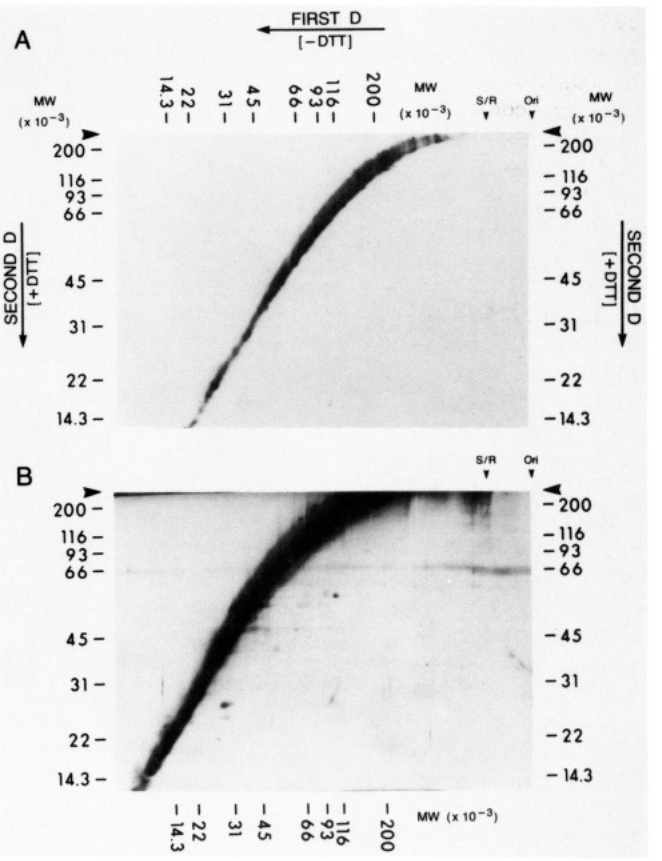

FIGURE 3: Non-cross-linked nuclear extract proteins resolved on diagonal 2D gels and stained with (A) Coomassie blue or (B) silver. Molecular weight calibrations (based on the distribution of amido black stained molecular weight standards blotted from gels run in parallel) are shown at the top or bottom (first dimension) and along the sides (second dimension) of (A) and (B). The direction of electrophoresis in the first- and second-dimensional (D) gels is indicated with long arrows. Small arrowheads [top right of (A) and (B)] indicate where the first-dimensional gel's stacking gel origin (ori) and stacking gel/running gel interface (S/R) were. The large arrowheads [upper right and left of (A) and (B)] indicate where the second-dimensional gel's S/R was.

monoclonal antibodies for hnRNP C proteins and the autoimmune patient serum, failed to show any of the antigens in off-diagonal positions (Figure 4A). Similarly, control blots reacted with C protein antibodies and monoclonal antibodies for the UsnRNP common protein D also showed no off-diagonal monomers (Figure 4B).

Blots from gels upon which cross-linked nuclear extract had been resolved were also treated with both hnRNP C protein reactive monoclonal antibodies and the autoimmune patient sera and developed. Subsequently, these blots were reincubated with monoclonal antibodies against the UsnRNP D protein and developed. An example of the result from these sequential reactions is shown in Figure 5.

The cross-linking pattern for hnRNP C proteins served as an internal standard for assessing intra-RNP cross-linking conditions and both confirmed and extended prior cross-linking data for these proteins (Lothstein et al., 1985). The major hnRNP C1 and C2 off-diagonal monomers, C1a, C1b, C2a, and C2b, corresponded to cross-linked complexes with composite molecular weights of 123 000, 246 000, 129 000, and 258 000, respectively (Table I). These values were consistent with the data of Lothstein et al. (1985), who suggested that C1 exists as a trimer in hnRNP monoparticles, and in addition, our data suggested cross-linked C1 hexamers (and possibly nanomers, C1c) and C2 trimers and hexamers were also present. Further, off-diagonal C1 monomers immediately

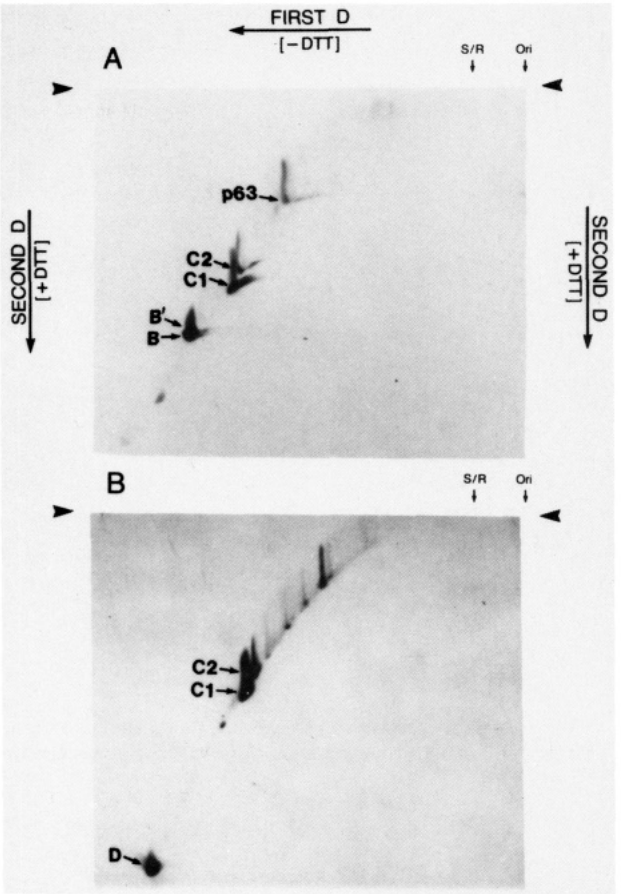

FIGURE 4: Non-cross-linked nuclear extract proteins were resolved on diagonal 2D gels, Western transferred, and probed with (A) both autoimmune patient serum reactive with the 63-kDa U1 snRNP specific protein and UsnRNP common proteins B′ (29 kDa) and B (28 kDa) and monoclonal antibodies reactive with hnRNP C1 (41 kDa) and C2 (43 kDa) proteins or (B) both monoclonal antibodies reactive with the UsnRNP common D protein (16 kDa) and monoclonal antibodies reactive with the hnRNP C proteins as described under Results and Discussion. Gel coordinates were the same as described for Figure 3 and are indicated along the sides of the figure.

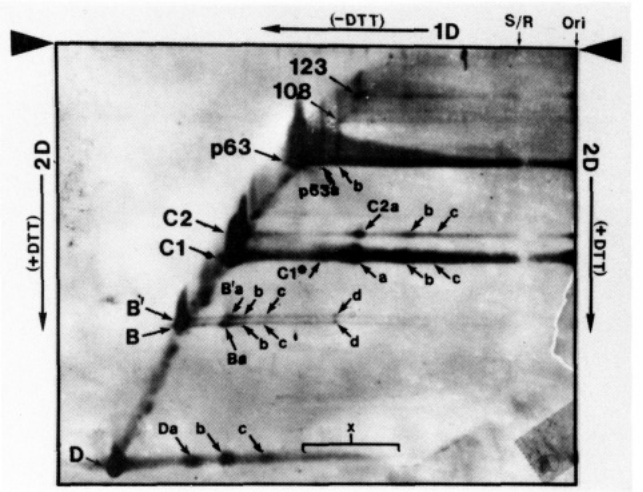

FIGURE 5: Cross-linked nuclear extract proteins were resolved on a diagonal 2D gel, Western transferred, and probed with antibodies reactive with U1 snRNP specific 63-kDa protein, UsnRNP common proteins B′ (29 kDa), B (28 kDa), and D (16 kDa), and hnRNP C1 (41 kDa) and C2 (43 kDa) proteins as described under Results and Discussion. Arrows within the figure indicate the position of off-diagonal monomers for each antigen and are labeled alphabetically according to increasing molecular weight of their corresponding cross-linked complexes. Gel coordinates are indicated at the top and to the sides of the figure and are the same as described for Figure 3. The apparent high background on this blot resulted from trans-illumination and photography while the blot was still wet. This was done because the off-diagonal Dc and Dx monomers faded rapidly upon drying.

preceding C1a (C1*) corresponded to 82-kDa cross-linked complexes that might have been C1 dimers. Off-diagonal, horizontal streaks, spots at 108 and 123 kDa, and spurious spots along the diagonal were primarily due to cross-linked complexes that had escaped DTT cleavage. The streaking between monomer spots was most likely due to C protein overloading in the gel, which was necessary in order to visualize the UsnRNP monomers (see below).

The additional homotypic cross-linked complexes for C1 and the novel C2 cross-linked complexes were presumably detected in this study but not by Lothstein et al. (1985) because Western blotting is more sensitive than Coomassie blue staining. We have now observed identical cross-linked complexes of C proteins in sucrose gradient purified hnRNP monoparticles (manuscript in preparation), and therefore the differences do not simply reflect cross-linking of nuclear extracts versus isolated hnRNP monoparticles. An interesting possibility suggested by these data was that the folding and conformational changes observed for nascent hnRNPs in situ (Skoglund et al., 1983; Oshein et al., 1985) might be brought about, in part, by C protein oligomerization from their peripheral positions (Holuobek, 1984) in hnRNP monoparticles.

B′ and B were observed as a parallel series of off-diagonal spots with major monomers B′a, B′d, Ba, and Bd corresponding to 38-, 108-, 37-, and 107-kDa cross-linked complexes, respectively (Table I). Less abundant and less well-defined cross-linked complexes were suggested by off-diagonal B′ and B monomers at positions "b" and "c", which corresponded to cross-linked complexes of 41–42 and 52–58 kDa. The parallel nature of the B′ and B off-diagonal complexes suggested that they had similar macromolecular organization. Unlike the hnRNP C proteins, which also showed a parallel pattern of off-diagonal monomers, the abundance of B′ and B complexes was comparable. We have observed the same cross-linked complexes when cross-linking was performed on sucrose gradient partially purified UsnRNPs using both autoimmune patient antibodies and monoclonal antibodies reactive with B′ and B (manuscript in preparation).

On the basis of the difference in molecular weight between the cross-linked complexes and the antigen monomers, we speculate that the off-diagonal B′ and B "a" monomers were derived from 38- and 37-kDa cross-linked complexes (respectively), which contained one copy each of B′, G or B, G (Table I). By similar reasoning, it is proposed that the less abundant B′b and Bb off-diagonals were derived from B′E and BE cross-linked complexes. A combination of B′ a and b complexes would have a composite molecular weight of 80 000 (of 78 000 in the case of the B complexes), which differs from the molecular weight of the "d" complexes (108 000) by 28 000, or the molecular weight of one B monomer (or B′ monomer, in the case of the Bd complex). We speculate therefore that the cross-linked complexes responsible for the off-diagonal B′d and Bd monomers contained B′:B:E:G in 2:1:1:1 and 1:2:1:1 stoichiometry, respectively (Table I). This proposed stoichiometry is supported by the presence of the off-diagonal B′c and Bc monomers, which correspond in position to cross-linked complexes having molecular weights equal to homotypic and heterotypic dimers of B′ and B (Table I). Moreover, well-resolved B′ and B monomers corresponding to cross-linked complexes of 80 kDa have been detected with the 16-Å cross-linker 2-iminothiolane (manuscript in preparation).

As with B′ and B, the D protein of UsnRNPs has been proposed to be a component of a common protein core (Mattaj

Table I: Theoretical Protein Composition of Cross-Linked Complexes Based on Molecular Weight Determinations[a]

antigen	monomer M_r ($\times 10^{-3}$)	off-diagonal position	cross-linked complex M_r ($\times 10^{-3}$)	possible components	possible stoichiometry
HnRNP					
C1	41	*	82	C1	dimer
		a	123	C1	trimer
		b	246	C1	hexamer
C2	43	a	129	C2	trimer
		b	258	C2	hexamer
U1 snRNP					
p63	63	a	85	63:C	1:1
		b	108	63:C	1:2
UsnRNP					
B′	29	a	38	B′:G	1:1
		b	42	B′:E	1:1
		c	52–58	B′	dimer
				B′:B	1:1
		d	108	B′:B:E:G	2:1:1:1
B	28	a	37	B:G	1:1
		b	41	B:E	1:1
		c	52–58	B	dimer
				B:B′	1:1
		d	107	B:B′:E:G	2:1:1:1
D	16	a	31.5	D	dimer
				D:D′	1:1
		b	37	D:E:G	1:1:1
		c	54	D:D′:E:G	1:1:1:1

[a] The data in this table were calculated from the blots in Figures 5 and 6 on the basis of migration of molecular weight standards and the molecular weights of the antigens and previously identified UsnRNP proteins (Bringmann & Lührmann, 1986) as described under Experimental Procedures and Results and Discussion.

& DeRobertis, 1985; Mattaj, 1986; Hamm et al., 1987). Molecular weight based calculations suggested that the three well-resolved off-diagonal D monomers were derived from cross-linked complexes of D:D′ (31.5 kDa), D:E:G (37 kDa), and D:D′:E:G (54 kDa) (Table I). Although the D monomer for the 37-kDa cross-linked complex (Db) was positioned immediately below the off-diagonal Ba and B′a monomers, the combined molecular weight of D with B or B′ is inconsistent with these proteins having been components of the same cross-linked complexes. It is conceivable that the E and G proteins cross-linked to D are different than those cross-linked to B′ and B. In other analyses (manuscript in preparation), we have observed faint 140-kDa off-diagonal B′ and B monomers, which suggests the possibility that D, D′, B′, B, E, and G were components of these complexes. Off-diagonal D monomers in the less well-resolved "x" position are certainly consistent with this interpretation (Figure 5).

We interpret these data to indicate that at least part of the Sm UsnRNP protein core consists of two asymmetrical halves, differing in the B′:B stoichiometry (B′:B:D:D′:E:G = 2:1:1:1:1:1 and 1:2:1:1:1:1). The overall UsnRNP stoichiometry for these six proteins would be B′:B:D:D′:E:G = 3:3:2:2:2:2. Our analyses have not permitted us to assign the F protein within this core stoichiometry. One possibility is that F is more closely associated with D′ whereas E and G are associated with D. Naturally one must be aware that, because the molecular weights of these proteins are so close, accurate assignment of the components within any cross-linked complex must await development of additional antibodies and/or characterization of the individual components following purification of the cross-linked complexes. These efforts are now in progress.

Western blots probed with either the autoimmune patient serum (Figure 5) or the RNP monoclonal antibody (Figure 6) revealed that, under the conditions of the diagonal two-dimensional gel system, the U1 snRNP specific protein migrated as a 63-kDa protein. Off-diagonal monomers p63a and p63b corresponded to 85- and 108-kDa cross-linked complexes

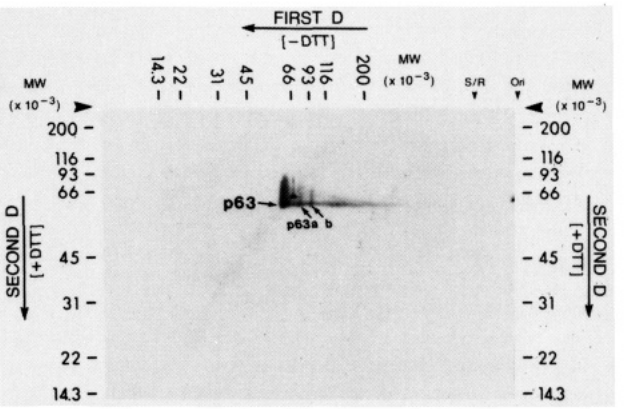

FIGURE 6: Western transfer of the diagonal 2D gel pattern from cross-linked nuclear extract proteins probed with monoclonal antibodies reactive with the 63-kDa U1 snRNP specific protein. Arrows indicate the positions of off-diagonal p63 monomers a and b. Gel coordinates are the same as described for Figure 3 and are indicated along the sides of the figure.

(Table I). We still do not understand why the U1-specific antigen migrates as either a 57- or 63-kDa protein in different gel systems; however, one possibility is that the urea in the diagonal two-dimensional gel system removes some bound RNA and thereby reduces the protein's net charge, and hence migration. The possibility that factors other than protein size influence the relative electrophoretic migration of the 70-kDa protein is supported by recent cDNA sequence analyses suggesting that the size of the protein is actually 52 kDa (Spritz et al., 1987).

As both polyclonal and monoclonal antibody blots show, in addition to well-defined off-diagonal monomers, the bulk of p63 off-diagonal monomers was poorly resolved as a streak extending to the gel origin. Whether the streak resulted from numerous heterogeneously sized cross-linked complexes or simply poor solubility in the first-dimensional gel is not clear. Conditions for analyzing this protein are being reevaluated by using affinity-purified U1 snRNPs.

Given that the RNP antigen is 63 kDa, we propose that the off-diagonal monomer position p63a corresponded to cross-linked complexes that also contained the U1-specific C protein (22 kDa) (Table I). It is presently not clear which components are cross-linked to p63 to give rise to complexes corresponding to the off-diagonal p63b. Cross-linked complexes of the three U1-specific proteins, p63, C, and A (34 kDa), would have a composite molecular weight (119 000) greater than that determined for p63b (108 000). Certainly, given the tendency of this protein to migrate differently depending on the gel system, it might be predicted that p63 cross-linked to A, or A and C, might migrate as a 108-kDa complex. At this point in our analysis, however, an equally likely possibility is that A is not proximal to p63 within U1 snRNPs and that the p63b monomer corresponds to p63:C complexes with 1:2 stoichiometry. Even though p63b and B′ and B d off-diagonal monomer positions indicated similarly sized cross-linked complexes, molecular weight based calculations did not suggest that there was an association. This would be consistent with data from quite different analyses suggesting that the RNA and Sm antigens occupy distinct RNA binding domains (Mattaj & DeRobertis, 1985; Mattaj, 1986; Hamm et al., 1987; Patton & Pederson, 1988).

In conclusion, Western blotting combined with reversible chemical cross-linking of proteins and diagonal two-dimensional gels has been used successfully on nuclear extracts to begin to map the organization of proteins within UsnRNPs. The extracts used in developing the cross-linking system were chosen as a starting material because they contain RNP assemblies that we have demonstrated to be functionally intact (as evident from in vitro RNA splicing analyses) and therefore presumably structurally similar to their in situ state. Efforts are now under way to determine the cross-linking pattern of proteins in highly purified preparations of UsnRNPs. With this information, specific cross-linked complexes can be purified and their components characterized by direct biochemical means.

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