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Distinct Examples of Immunologic Analysis in Molecular and Cellular Biology: Small Nuclear Ribonucleoproteins and the T Cell Receptor

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DISTINCT EXAMPLES OF IMMUNOLOGIC ANALYSIS
IN MOLECULAR AND CELLULAR BIOLOGY :
SMALL NUCLEAR RIBONUCLEOPROTEINS AND THE T CELL RECEPTOR

A thesis submitted to the Faculty of the Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

David E. Fisher

April 1, 1984

The Rockefeller University

New York, New York

Dedicated to Henry G. Kunkel
(1916-1983)

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Part I

Small Nuclear Ribonucleoproteins

Chapter 1

INTRODUCTION

Since the discovery (Tan and Kunkel, 1966; Mattioli and Reichlin, 1971; Sharp, et al., 1972) of human SLE autoantibodies directed against RNA-protein complexes in eucaryotic nuclei, a great deal of interest has been generated regarding both the relationships of antibody specificities to disease and the biological roles of the recognized antigens. These antibodies are some of the many autoantibody species which have been described in systemic lupus erythematosus (SLE) as well as other rheumatic disease conditions. A common feature of many of these reactivities is the apparent nuclear localization of antigen. Study of the antibodies as well as these antigens has led to formation of a particularly unique bridge between clinical medicine and basic science questions in cellular biology and metabolism.

The spectrum of diseases which present with antibodies directed against cellular constituents is vast although certain features of these conditions imply common aspects which may, furthermore, be of some diagnostic use. Familial clustering and HLA associations (Grunet, et al., 1971; Nies, et al., 1974; Kissmeyer-Nielsen, et al., 1975; Arnett and Shulman 1976; Goldberg, et al., 1976; Horn, et al., 1978 Schur and Carpenter 1983) have pointed to genetic factors which may relate to common

etiologies or distinguish among certain of these diseases (Gibofsky, et al., 1973). This group of diseases includes SLE, mixed connective tissue disease (MCTD), scleroderma, Sjogren's syndrome, rheumatoid arthritis, polymyositis, dermatomyositis, and overlap syndromes.

The observation of antinuclear antibodies was probably first reported in the description of the LE cell (Hargraves, et al., 1948). This phenomenon, thought to reflect phagocytosis of antibody coated nuclei by polymorphonuclear leukocytes, has historically provided useful diagnostic information. Three reports in 1957 (Holman and Kunkel, 1957; Friou, et al., 1957; Holborrow, et al., 1957) utilized fluorescent antinuclear antibody binding to demonstrate the presence of antinuclear components in serum of patients with a variety of disorders. The report (Barnett 1977) of low antinuclear antibody titers in a significant proportion of normals has suggested that the simple presence of such immunoglobulins may not be pathological (in low titers). With increasingly sophisticated methods of analysis, more detailed study of these autoantibodies has been carried out resulting in the elucidation of better defined antigen-antibody systems as disease markers and prognostic indicators.

Numerous autoantibody-antigen systems have been described in considerable detail, the majority involving antibodies to nuclear components. The indirect immunofluorescence technique has had very wide use in analysis of such antibodies. Microscopic analysis has revealed several distinguishable patterns of staining which have been shown to correspond to different antigenic specificities (Tan 1982). The diffuse nuclear staining which frequently characterizes anti-DNA antibodies differs from speckled staining (typical of antibodies to certain saline extractable nuclear antigens). Other patterns of staining include "rim," nucleolar,

and centromere/kineticore, each corresponding to distinct antigenic recognition patterns. Two potential hazards in interpretation of the indirect immunofluorescence test are losses of antigen during washing procedures from the fixed cells and possible denaturation effects capable of distorting antigen recognition due to the use of certain fixatives (Tan 1982).

Cytoplasmic antigen recognition has also been seen in a variety of conditions. Antibodies reactive with ribosomes were described in 1967 (Schur, et al., 1967). The two antigen systems Ro and La likely reside, though perhaps not exclusively, in the cytoplasm (Clarke, et al., 1969; Mattioli and Reichlin, 1974).

While antinuclear antibodies are associated with a variety of rheumatic diseases, certain other conditions may also present with this category of antibody reactivity. Nuclear autoantibodies have been described in melanoma, nasopharyngeal carcinoma, and acute and chronic leukemia patients (Morton, et al., 1968; McBride, et al., 1972; Yoshida, et al., 1975; Lamelin, et al., 1978; Klein, et al., 1974; Steiner, et al., 1975). Chronic liver disease (Ziegler, et al., 1976) may also be associated with such autoantibodies including cytoplasmic microsomal reactivity (Davis 1981) and the antimitochondrial antibodies (Davis 1981) seen in primary biliary cirrhosis (Ahrens, et al., 1950).

At approximately the same time as the first reports of nuclear-reactive serum components, came the observations of DNA-reactive antibodies (Robbins, et al., 1957; Cepellini, et al., 1957; Seligmann, 1957). Use of immunodiffusion analysis (Tan, et al., 1966) allowed for the distinction of two differing specificities of DNA reactive antibodies. Sera were described which reacted with both native (double stranded) and heat denatured DNA,

while others reacted with only heat denatured DNA. In addition to these specificities, a third reactivity specific only for native DNA has been described to occur at low frequency (Arana and Seligmann, 1967; Tan and Natali 1970; Koffler, et al., 1971; and Gilliam, et al., 1980). While antibodies reactive with native DNA appear generally to recognize determinants on the sugar-phosphate backbone (Stollar 1970; Picazo and Tan 1975; Samaha and Irwin 1975; Steinman, et al., 1976) single strand DNA specific antibodies appear to recognize the nitrogen bases (Tan 1982). An intriguing distinction between these two anti DNA reactivities is the relative ease with which antibodies to single stranded DNA are induced by immunization in animals as compared with a corresponding difficulty for dsDNA (Tan 1982), although certain animal models of SLE contain such native DNA antibodies.

Clinical correlations with anti-DNA antibodies have been numerous, both in suggesting possible etiology of tissue injury in SLE and as a diagnostic marker. The importance of immune complexes in generation of glomerulonephritis and vasculitis was demonstrated both for exogenous antigen administration in models of serum sickness (Dixon, et al., 1958, 1961) and by direct injection of preformed immune complexes (McCluskey, et al., 1960). Vasculitic and glomerular lesions were shown by immunofluorescence to contain antibody deposits as well as associated C₃, further implicating such immune complexes in a pathogenic role in SLE (Vasquez and Dixon, 1957; Mellors, et al., 1957; Paronetto and Koffler, 1965; Lachmann, et al., 1962). The sequential appearance of DNA reactive antibodies followed by a rise in free serum DNA antigen with loss of serum DNA antibody during a SLE flare was highly suggestive that the DNA antigen antibody system was functionally important in SLE tissue damage (Tan, et

al., 1966). Immunofluorescence and elution techniques demonstrated both DNA antigen and DNA reactive antibodies in affected renal glomeruli. While the involvement of DNA antigen antibody complexes in glomerular and vasculitic lesions appears firmly established, correlations of disease activity with DNA antibody titers cannot exclude consideration of antigen load since these two parameters must likely occur within certain stoichiometric constraints to form pathogenic immune complexes (Tan 1982). Antibody to native DNA is most frequently associated with SLE, although titers (usually low) have been observed in other of the rheumatologic and connective tissue diseases (Tan 1982; Davis 1981). Single stranded DNA reactive antibodies have much wider disease distribution including rheumatoid arthritis, chronic active hepatitis, and infectious mononucleosis (Koffler, et al., 1971).

Histone reactive antibodies (Kunkel et al., 1960; Stollar 1971) have been detected in lupus (both idiopathic and drug induced) and in rheumatoid arthritis (Tan 1982). Lupus induced by pharmacologic agents (e.g. procainamide, hydralazine, and isoniazid) is very frequently associated with histone reactive antibodies (Fritzler and Tan, 1978) while such reactivity is less frequently observed in idiopathic SLE. The possibility that certain purified rheumatoid factors may contain anti DNA-histone reactivity suggests intriguing relationships between these otherwise distinct classes of autoantibodies (Alspaugh, et al., 1978; Agnello, et al., 1978; Hannestad and Stollar 1978).

Other groups of autoantibody-antigen systems include the Ro and La antigens which are RNA containing nucleoprotein complexes. These antibody reactivities are generally thought to be confined to SLE and Sjogren's syndrome (Alspaugh, et al., 1976; Kassan, et al., 1977; Akizuki, et al.,

1977; Provost 1979). Antibodies to proliferating cell nuclear antigen (PCNA) described by Myachi, et al. (1978) are seen in a small proportion of SLE patients. Other autoantibodies include centromere/kinetichore (Moroi, et al., 1980; Brenner, et al., 1981), a 70 kd antigen reactive with certain scleroderma sera (Douvas, et al., 1979b), and a nuclear antigen recognized by antibodies in a significant proportion of rheumatoid arthritis sera (Alspaugh, et al., 1976; Alspaugh and Tan 1976). Several additional systems have been described in dermatomyositis and overlap syndromes. Among these are the PM-1 (Wolfe, et al., 1977), Mi-1 (Nishikai and Reichlin 1980a), Jo-1 (Nishikai and Reichlin 1980b), and Ku (Mimori, et al., 1981) antigen systems.

Many of the nuclear antigens recognized by these numerous categories of autoantibodies are easily removed from nuclei by isotonic extraction. The Sm/RNP antigen antibody system was first described in 1966 (Tan and Kunkel 1966) as a saline extractable nuclear antigen. While other antigens are also saline extractable, the Sm and RNP systems contain several distinguishing features which have facilitated identification of these reactivities. Analysis of precipitin patterns has distinguished these reactivities (Northway and Tan 1972). Use of a hemagglutination assay aided in differentiating between Sm and RNP, and is used as a clinical test for these antigens (Tan and Peebles 1976). Sm and RNP reactive antibodies generally reveal a speckled pattern of nuclear staining by indirect immunofluorescence.

Initial investigations regarding the molecular composition of the Sm/RNP antigens and reactivities revealed RNP sensitivity to RNase digestion while Sm recognition displayed no such sensitivity (Northway and Tan 1972). The anti-Sm antibodies are highly specific for SLE while anti-

RNP antibodies show wider distribution and have helped in defining the mixed connective tissue disease syndrome (Sharp, et al., 1972). This disease was described in rheumatologic patients with features of SLE, scleroderma, and dermatomyositis whose sera contained antibodies directed against an RNase sensitive nuclear component. The frequent occurrence of Raynaud's phenomenon and the absence of renal disease also characterized this disorder. At nearly the same time, Mattioli and Reichlin (1971) reported the occurrence of an RNase sensitive extractable nuclear antigen called Mo. Reactivity with this antigen was seen in 30 to 50% of SLE patients, and it was subsequently shown to be identical to RNP (Tan, et al., 1977). So while Sm reactivity shows high specificity for SLE (although seen in only 30-40% of patients), RNP reactivity can be seen in SLE or MCTD, as well as discoid lupus, scleroderma, rheumatoid arthritis, and Sjogren's syndrome (Tan 1982).

Notman, et al., (1975) tested numerous patients for RNP reactivity and observed uniformly high titers in MCTD patients and lower frequency of high titer in SLE as well as other syndromes. There has been some uncertainty over the classification of MCTD as a distinct disease syndrome. Distinguishing MCTD from dermatomyositis and scleroderma may be of particular importance because of the good response to steroid therapy of the former, but not the latter disease (Tan 1982). MCTD appears to be distinguishable from other related syndromes by the presence of high titers exclusively to RNP- other conditions more likely reveal additional antibody reactivities (Tan and Peebles 1976). Thus, Sm antigen reactivity is seen in 30-40% of SLE patients and represents a specific disease marker; RNP reactivity, while seen in a variety of disorders, may contribute significantly to the diagnosis of MCTD (in the absence of other antibody

reactivities) and is associated with favorable prognosis in those cases.

Since the discovery of Sm antigen reactivity in 1966 (Tan and Kunkel, 1966) and RNP in the early 1970's (Mattioli and Reichlin 1971; Sharp, et al., 1972; Northway and Tan 1972) numerous studies have been carried out, aimed at defining the biochemical composition of these nuclear antigens. Northway and Tan (1972) used DEAE chromatography, gel filtration, and sucrose density centrifugation to show that while some Sm reactivity could be isolated separate from RNP, the rest always copurified with RNP-reactive antigen. This finding was confirmed by Mattioli and Reichlin (1972). Numerous reports have been made regarding the putative protein constituents of snRNPs. While little agreement appeared at first, a consensus is beginning to emerge from reports of recent years. Douvas, et al. (1979a) used anti RNP affinity columns to describe two proteins of 12 and 20 kd. Takano et al. (1981) described RNP antigens of 30 kd, 13 kd, and 55 kd while Sm antigen appeared to be several peptides in the 13 kd weight range. Agelli, et al.. (1980) described a 55 kd protein thought to be Sm. Waelti and Hess (1980) identified putative Sm antigens of 110 and 20 kd. Barque, et al. (1981) described 4 Sm and RNP polypeptides in the 10-20 kd weight range. White and Hoch (1981) described several Sm and RNP peptides using immunoblots. Lerner and Steitz (1979) reported 7 peptides in the weight range of 12-35 kd, immunoprecipitable from ³⁵S-methionine labeled Ehrlich ascites mouse cells. This pattern of peptides ranging from 32,000 d to 9,000 d and named A through G, has been recognized by a number of independant investigators in recent years (Conner, et al., 1982; Matter, et al., 1982; Kinlaw, et al., 1983; Wieben, et al., 1983).

Lerner and Steitz (1979) demonstrated that immunoprecipitates from nuclear extracts using Sm and RNP sera contained U series small nuclear RNA

species. While Sm sera precipitated U¹, U², U⁴, U⁵, and U⁶, RNP sera precipitated U¹ RNA only. These observations both assigned a biochemical niche to these previously described RNA's and provided the suggestion that Sm and RNP antibodies may be of great use in further analysis of structural and functional properties of the U RNA containing complexes.

The U series small nuclear RNAs comprise 0.1-1% of total cellular RNA. They were named (Hodnett and Busch 1969) for their uridylic acid-rich nucleotide composition. Small nuclear RNAs have been identified in normal and malignant cells, vertebrates, invertebrates, dinoflagellates, and plants (Reddy and Busch, 1983). Numerous small RNA species have been identified in addition to these U RNAs. The 7S species (Bishop, et al., 1970) is one which has been demonstrated (Walter and Blobel 1982) to participate in a known biological activity. In association with several peptides, this RNA forms the signal recognition particle shown to be critical in polysome-endoplasmic reticulum interactions during translation of membrane and secretory proteins (Walter and Blobel 1981a,b; Walter, et al., 1981). Another small RNA with known functional importance is the species contained in the e. coli RNase P complex, a particle involved in tRNA maturation (Kole and Altman 1979). These two small RNAs (7S and RNase P) differ from U series RNA in lacking 5' caps. The U RNAs all contain methylated caps at their 5' ends and are RNA polymerase II transcripts, as demonstrated by alpha-amanitin sensitive transcription. Trimethylguanosine (Saponara and Enger 1969) was identified in U¹, U², and U³ RNAs (Reddy, et al., 1972)- present in a 5'-5' triphosphate linkage (Rottman, et al., 1974; Ro-Choi, et al., 1975; Reddy, et al., 1976). Following the discovery of these caps on U RNAs, similar such caps were discovered in viral and eukaryotic mRNAs (Rottman, et al., 1974; Shatkin, et al., 1976) containing

7-methylguanosine.

U1 RNA was identified as a nuclear species by cell fractionation (Weinberg and Penman 1968; Hodnett and Busch 1968). Sequence analysis has led to postulated models which predict a secondary structure involving formation of 5 hydrogen bonded stems (see Figure 1) (Krol, et al., 1981a). Enzymatic digestion followed by immunoprecipitation (Epstein, et al., 1981) suggests 2 regions of susceptibility: the 7 most 5' nucleotides, and the region near nucleotide 107. These regions (particularly the 5' end which undergoes less 2° folding, see Figure 1) are good candidates for participation in the formation of transient base pairs with splice junctions. After U1 RNA the next most abundant small nuclear RNA species is U2. This species contains 2'-O-methylated nucleotides and an unusual number of pseudouridine residues at its 5' end. A U2-like species has been found in wheat and differs only slightly from rat U2. U2 RNA is a nucleolar species, capable of hydrogen bonding to 35S and 28S RNAs (Prestayko, et al., 1971) and is not precipitated by Sm or RNP sera. It resides as a ribonucleoprotein complex in nucleoli (Prestayko, et al., 1971). U4 is another nucleoplasmic species containing the trimethylguanosine cap (Krol, et al., 1981a; Reddy, et al., 1981). It bears extensive homology to U1 and is highly conserved (as is the case with the other U RNAs) (Krol, et al., 1981). U5 RNA is the most uridine rich U RNA (Krol, et al., 1981b). It also contains the trimethylguanosine cap (Krol, et al., 1981a). In contrast to the other U RNAs, the U6 RNA species lacks trimethylguanosine in its cap and contains numerous methylated nucleotides in its central sequences. Other U species are methylated on nucleotides towards the 5' end of the molecule. The suggestion that U6 may associate with newly transcribed RNA (Reddy and Busch, 1983) may implicate

this species in some aspect of RNA processing.

The synthesis and metabolism of U RNAs is of considerable interest in relation to snRNPs. Larger than mature precursors of U1 and U2 have been identified in the cytosol following ^3H -uridine pulse labeling (Elcieri 1974; Fredericksen and Hellung-Larsen 1975; Zieve and Penman 1976). Salditt-Georgieff, et al., (1980) have identified snRNA precursors by virtue of their methylated cap structures. UV irradiation (to which snRNA transcription is sensitive) has suggested transcription units for U1 and U2 as large as 5 kb (Elcieri 1979). Transcription thus appears to be followed by very rapid (<10 minutes) transport of precursor species to the cytoplasm where further methylation (processing) may occur.. Within 15 minutes, mature forms of these U RNAs appear in the nucleus- these nuclear forms being quite stable as would perhaps be expected if the RNAs were associated with proteins. Two additional sets of experiments have been carried out which bear on cellular location of these RNAs. Microinjection of the RNAs in the cytoplasm is followed by transport into the nucleus, where they are associated with snRNP proteins (DeRobertis, et al., 1982). Nuclear transplantation in ameba (Goldstein, et al., 1977) has suggested that U RNAs shuttle between the nucleus and cytoplasm. A considerable proportion of snRNPs have been suggested to associate with the nuclear matrix (Vogelstein and Hunt 1982). Deng, et al. (1981) have shown an intriguing change in location of Sm antigen related to the cell cycle. Sm reactivity can be seen in the nucleus during G1 and S. However, Sm antigen appears to be located outside the nucleus during G2 and away from the chromatin during mitosis. Several unique features of the genes encoding U RNAs have been described. The genes appear to be dispersed throughout the genome although with similar immediate surroundings (Roop, et al, 1981; Manser and

Gesteland 1981; Denison, et al., 1981; Manser and Gesteland 1982). These genes lack intervening sequences and polyadenylation signals (AATAAA near the 3' end of the RNA) (Reddy and Busch 1983; Roop, et al., 1981; Manser and Gesteland 1982). As many as 10 fold more pseudogenes exist for U1-RNAs than true genes (Denison, et al., 1981).

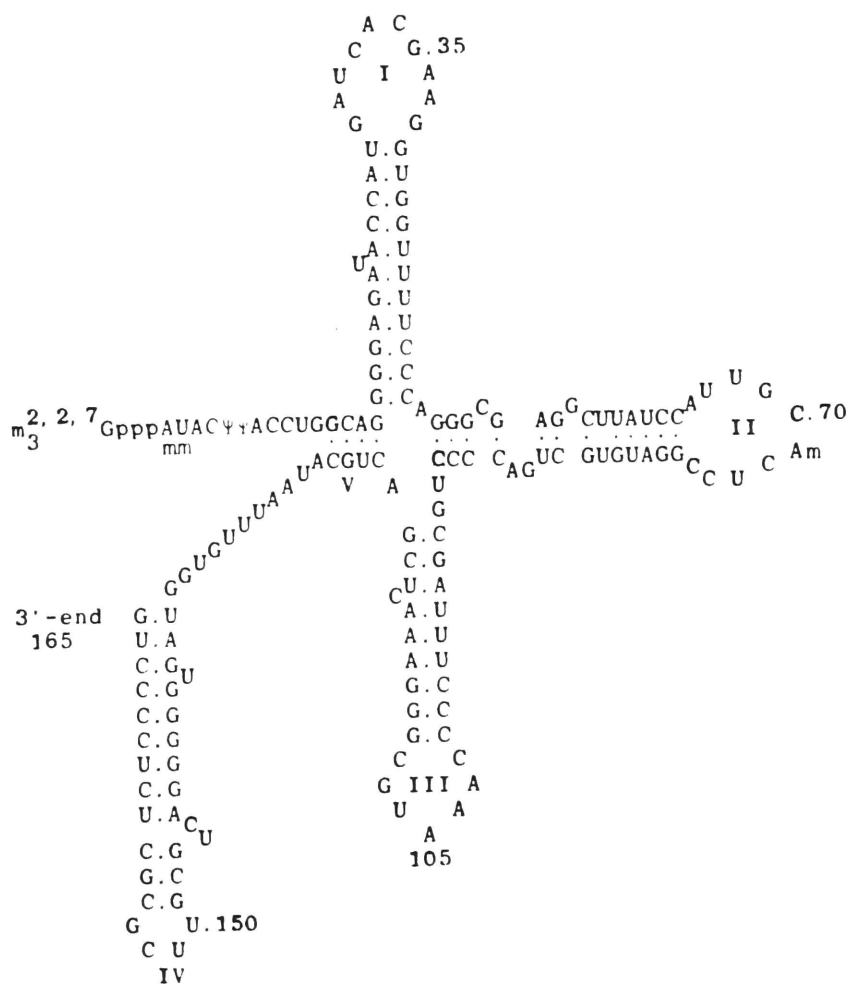
Based on various structural features of the U1 RNA, the suggestions have been made (Lerner, et al., 1980; Rogers and Wall 1980) that this species may participate in RNA:RNA splicing. U RNAs have been seen to associate with hnRNPs (Sekeris and Niessing 1975) and to be capable of hydrogen bonding to hnRNA (Northemann, et al., 1977; Northemann, et al., 1979). Crosslinking studies have also identified U1 and U2 RNAs in association with hnRNA (Calvet and Pederson 1981; Calvet, et al., 1982). A consensus sequence found near the 5' end of U1 RNA bears homology with known splice junctions. This 5' end sequence is identical in all known U1 species. U1 RNA containing snRNPs appear to be capable of binding to 5' splice junctions in vitro (Mount, et al., 1983). Several experiments have demonstrated selective blocking of splicing events when anti-U1 snRNP antibodies were added to isolated nuclear (Yang, et al., 1981) or in vitro (Padgett, et al., 1983) transcription/splicing systems. The splicing activity of another in vitro transcription/splicing system has been shown to reside in a fraction containing snRNPs (Hernandez and Keller 1983). Perhaps arguing against this role for U1 RNA in splicing is the observation of Weissman, et al. (1982). The splicing efficiency of globin precursor RNAs which contained changes in the U1 homologous consensus sequence was high, despite lost complementarity to U1. Other roles for which U RNAs have been postulated include transport (Reddy and Busch, 1983; Hellung-Larsen and Fredericksen 1977), control of transcription (Britten

and Davidson 1969; Prestayko, et al., 1971; Hellung-Larsen and Fredericksen 1977; Light and Molin 1983), and chromatin structure (Pederson and Bhargjee 1979).

Considerably more is known at the structural and biochemical level about the U RNA species than the proteins with which they associate in snRNPs. Since these proteins undoubtedly underly a significant role in the structure and perhaps function of snRNPs, further study of these species may be useful in improving the understanding of snRNP particles. The four following chapters describe experiments carried out with that purpose, making use of the naturally occurring antibodies reactive with snRNPs, those of the Sm and RNP specificities seen in SLE and allied autoimmune diseases.

Figure 1

Proposed secondary structure for rat U-1 RNA.



Chapter 2

Protein Constituents of snRNPs

ABSTRACT

The protein constituents of small nuclear ribonucleoproteins (snRNPs) have been analyzed by ^{35}S -methionine internal labeling followed by immunoprecipitation with SLE sera. The eight peptides termed A-G were identified and further studied by in vitro translation analysis. Each protein was synthesized by the wheat germ cell free extract in the presence of appropriate RNA. Furthermore, protease digestion studies and RNA fractionation coupled to in vitro translation have revealed that these eight snRNP proteins are encoded by separate mRNAs and are not related by post-translational cleavage.

Chapter 2

RESULTS

Endogenous Labeled SnRNP Proteins

Considerable controversy has historically surrounded questions of the identity of protein constituents in small nuclear ribonucleoproteins. Immunoprecipitation studies were carried out to analyze this question, utilizing human autoimmune sera shown to be reactive with these particles. Metabolic labeling was carried out on the human myeloid cell line K562 using ^{35}S -methionine and incubating for 15 hours in RPMI 1640 medium made deficient in methionine. Immunoprecipitation was carried out using serum from a patient with SLE, displaying anti Sm serology as determined by passive hemagglutination. ^{35}S -methionine labeled cells were lysed hypotonically and nuclei extracted using 0.2 M NaCl. Post nuclear supernatant was immunoprecipitated by anti Sm antibodies followed by Protein A Sepharose 4B, washing, elution, and SDS-polyacrylamide gel electrophoretic analysis.

Figure 2 shows the pattern of precipitated ^{35}S -labeled peptides when anti Sm (lane 1) or normal human serum (lane 2) are used. The eight peptides labeled A-G migrate with mobility corresponding approximately to:

- A- 32,000 d
- B- 25,000 d
- B'- 25,000 d
- C- 19,000 d
- D- 16,000 d
- E- 12,000 d
- F- 11,000 d

G- 8,000 d.

Since the snRNP proteins reside in particles in vivo, it was felt to be likely that any nonantigenic snRNP peptides should be visualized following immunoprecipitation by virtue of coprecipitation phenomena (discussed further for the anti RNP serology in Chapter 3). The possibility that other snRNP peptides may show sensitivity, particularly to divalent cation concentrations has been tested under conditions of 5 mM $MgCl_2$ and 25 mM EDTA revealing no apparent sensitivity of snRNP components to these agents (data not shown).

In vitro Translation of SnRNP Proteins

Further analysis of snRNP peptides was carried out by in vitro translation. RNA was extracted from K562 cells and the poly A+ fraction was translated using wheat germ cell-free extract. Immunoprecipitation of translation products is shown in Figure 3. All eight peptides were clearly seen from precipitates of poly A+ RNA translation products. The poly A- fraction (defined only by repeated passage over oligo dT cellulose) appeared to contain mRNAs for most of the peptides, although the possibility certainly exists that residual A+ RNA was present in this fraction. Relative decreases in A, B, and B' are seen as well, in products of total RNA translation. While it is difficult to accurately compare quantitative aspects of these different fractions, the presence of all 8 snRNP peptides in the poly A+ translation is firmly evident. Furthermore, no gross changes in mobility are seen for any of the peptides relative to in vivo labeled snRNPs, an observation which has been made by running in vivo and in vitro generated snRNPs in adjacent lanes of the same gel (data not shown).

Proteolytic Cleavage Patterns

Preliminary experiments were carried out to study relatedness of the various snRNP peptides. Protease cleavage was carried out with chymotrypsin and protease V⁸ to search for possible precursor-product relationships among the proteins. A typical experiment with such analysis is illustrated in Figure 4. In vivo ³⁵S-methionine labeled, immunoprecipitated snRNP peptides A, B, B', and D were cut out of an SDS polyacrylamide gel (as in Figures 2 and 3) and subjected to proteolysis with chymotrypsin according to the method of Cleveland, (1977). Enzyme concentrations of 10, 1, and 0.1 ug per lane were used, as indicated. The untreated peptides are seen to migrate essentially the same as in Figures 1 and 2, with some differences due to the 20% polyacrylamide concentration used in the proteolysis gel (vs. 15% in other experiments). Comparison of the cleavage patterns for these four peptides reveals no striking similarities. While occasional proteolytic fragments comigrate for different peptides, the vast majority of lytic products form migration patterns which appear to be unique for each protein.

Fractionation of RNA Encoding snRNP Proteins

To further study the possibility that precursor-product relationships exist among the Sm-precipitated snRNP proteins, size fractionation of poly A⁺ RNA was carried out prior to in vitro translation. If any two of the snRNP proteins are encoded by the same mRNA, but differ by post-translational cleavage or processing, then their mRNAs should not be separable by size fractionation. K562 poly A⁺ RNA was fractionated on a 10-30% sucrose gradient, each fraction was translated, and the products were immunoprecipitated with Sm serum. Figure 5 shows such a fractionation experiment. In 5a, 3 ul of total translation products were analyzed

(without immunoprecipitation). Clear evidence appears for different sized proteins derived from the RNA gradient fractions. Figure 5b shows translation products immunoprecipitated using an Sm serum. The eight Sm-precipitable proteins are seen to be resolved into seven fractions. The mRNAs for proteins A-G appear to migrate separately in the sucrose gradient. It is noteworthy that the peak fractions of mRNA for proteins B and B' are reversed relative to the sizes of the two proteins. These data suggest that the eight snRNP proteins are translated from separate mRNAs. Furthermore, it was observed that the Sm serum was able to precipitate the eight labeled snRNP proteins in the absence of one another.

DISCUSSION

Immunoprecipitation coupled to cell free translation studies have been carried out to analyze the biochemical identity of snRNP proteins. The results have demonstrated that all 8 snRNP proteins defined by precipitation from ^{35}S -methionine labeled cells, can be translated in vitro and recognized by SLE sera. SDS-polyacrylamide gel electrophoretic migration revealed no apparent differences in mobility of the snRNP proteins generated in vitro and in vivo (Fig. 3) suggesting that major post-translational processing events either do not occur or have occurred to completion in the translation system. The observation that the RNA's encoding the 8 proteins were separable discounts the possibility that these proteins are related as precursor-product. The data suggest that eight separate mRNA's encode snRNP proteins A-G. Furthermore, the relative RNA sizes correspond to the protein molecular weights (as judged by sucrose gradient migration). The only exception is the mRNA for protein B' which

migrates as a larger RNA than that encoding B. In light of the very similar sizes of proteins B and B' as well as the appearance of only one "B" band following immunoprecipitation of rodent snRNP's (Lerner and Steitz 1979), it is of significance that they are translated from separate messages and are not variants of the same primary translation product. Chymotryptic peptides also clearly distinguish the proteins from one another (Figure 4). The reports (Conner, et al., 1982; Petterson, et al., 1984) that a monoclonal antibody is capable of recognizing proteins B and D is of related interest. While the possibility that these proteins are related as precursor-product appears unlikely, the sharing of antigenic determinants suggests the possibility of some relationship among these proteins at either the DNA or mRNA processing levels although no obvious shared proteolytic fragments were observed for these proteins (Figure 4). Shared determinants could suggest that a limited idiotype repertoire may be sufficient for recognition of the many snRNP proteins by autoimmune sera. Several groups have described an antigenic peptide of approximately 60,000 thought to be contained in certain snRNP particles (Hinterberger, et al., 1983; Kinlaw, et al., 1983; White and Hoch 1981). While larger peptides appear in immunoprecipitates and immunoblots with certain sera, screening in this laboratory of numerous MCTD and SLE sera has not revealed correlation of any particular high molecular weight band with either Sm or RNP serology (Conner, et al., 1982). Antibodies against a nuclear matrix protein of approximately the same molecular weight have been described in many MCTD sera Habets, et al. (1983). Further studies regarding the identity and immunogenicity of these larger peptides are currently in progress, utilizing several recently generated monoclonal antibodies (W. Reeves, personal communication). Experiments in this laboratory have not

demonstrated the presence of any additional snRNP peptides in the range of 15,000-33,000d (other than A-G), as reported by several other groups (Hinterberger, et al., 1983; Kinlaw, et al., 1983). While occasional immunoprecipitates have revealed such additional bands, comparison of total lysate proteins has generally revealed those species to correspond to very abundant cellular proteins. Lack of these bands in immunoprecipitates of in vitro translation products from size fractionated poly A+ RNA (Figure 5b) supports this notion.

Figure 2

Small Nuclear ribonucleoproteins recognized by Sm serum. ³⁵S-methionine labeled K562 cells were lysed and immunoprecipitated using normal human serum (NHS) or Sm autoimmune serum (Sm). SnRNP peptides A-G are labeled.

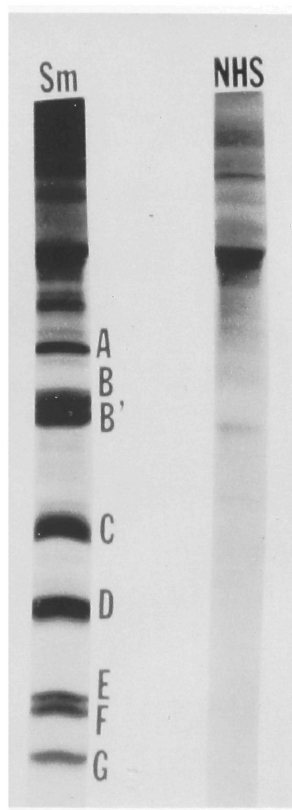


Figure 3

In vitro translation of snRNP proteins. RNA from the K562 cell line was extracted, and either translated directly (tot.) or fractionated into poly A⁺ (A⁺) and poly A⁻ (A⁻) fractions prior to translation using wheat germ cell free extract. Translation products were immuno precipitated with Sm serum.

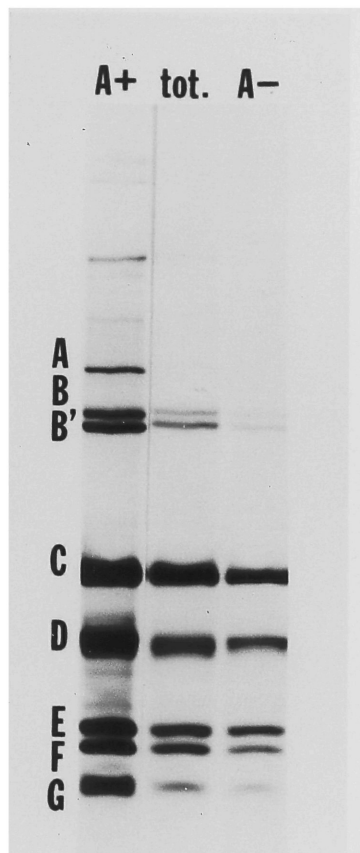


Figure 4

Peptide mapping of 4 snRNP peptides. Peptides A, B, B', and D were excised from a resolving SDS gel as gel slices, which were placed into wells of a 20% SDS-polyacrylamide gel and overlaid with chymotrypsin in loading buffer (either 0.1, 1, or 10 ug enzyme per lane as indicated). Samples receiving no enzyme are labeled "uncut." Peptides were run into the stacking gel, allowed to digest at room temperature, and then resolved as described (Cleveland, et al., 1977). The cleavage patterns do not resemble patterns predicted for post-translationally modified forms of the same peptide.

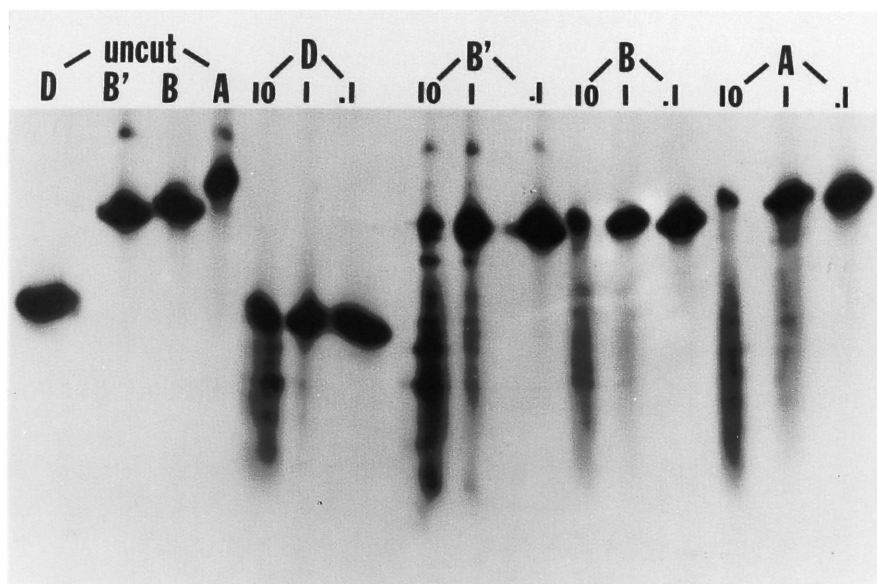
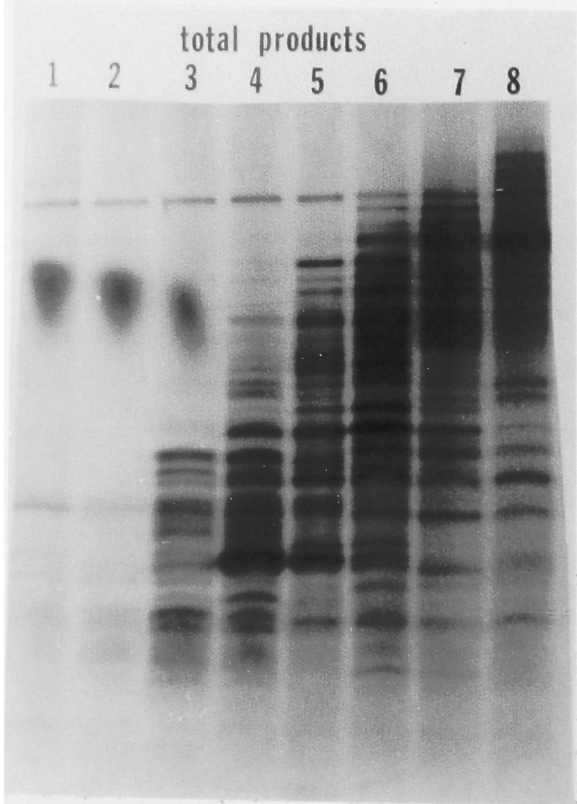


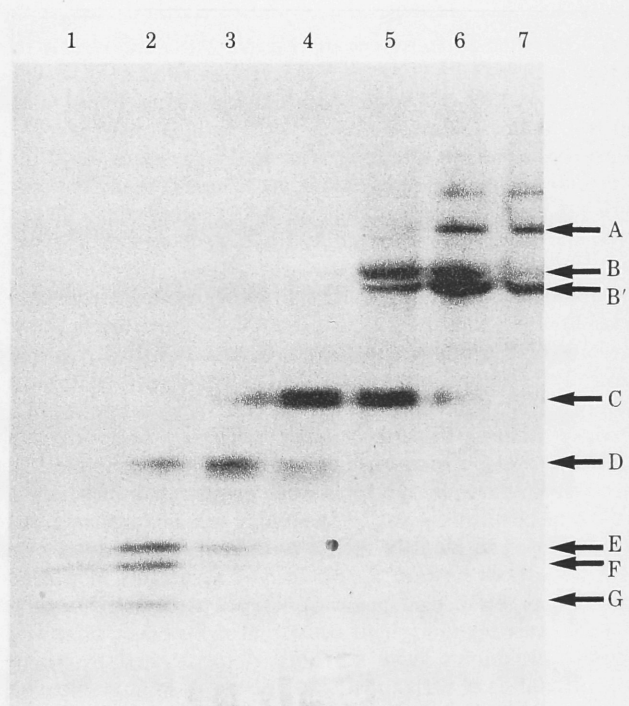
Figure 5

Sucrose gradient fractionation of mRNAs encoding snRNP proteins. K562 poly A+ RNA was fractionated on a 10-30% sucrose gradient and precipitated with ethanol, and each fraction was translated in a cell-free system. Figure 5a shows 3 ul aliquots of total translation products from each fraction. Figure 5b shows Sm immunoprecipitates of translation products from the fractions. Lane 1 represents the top of the gradient.

A



B



Chapter 3

Specificities of the snRNP reactive human autoantibodies, anti-Sm and anti-RNP

ABSTRACT

Antibodies directed against small nuclear ribonucleoprotein (snRNP) particles are found in the Sm and RNP autoimmune sera from numerous patients with systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD). While these autoimmune sera contain snRNP reactive antibodies, distinctions in antigen binding specificity have been difficult to define because of the particulate nature of the snRNP antigen. To overcome this problem, while obtaining the antigen in a native state, cell-free translation of poly A+ RNA was carried out. In addition, cells were pulse labeled with ³⁵S-methionine for 8 minutes to generate radioactive snRNP proteins in forms reflecting incomplete de novo particle assembly. Immunoprecipitation of snRNP antigen prepared in these manners revealed clearly distinct patterns of Sm and RNP immunorecognition. While Sm sera precipitated all 8 labeled snRNP proteins, RNP antibodies precipitated only 2 of the eight. One of the proteins (the A protein) of 32,000d was seen to lose antigenicity upon RNase treatment either when extracted from cells or when generated in vitro. RNase treatment of immunoprecipitated snRNP's released the A protein in an electrophoretically pure form. Analysis of in vitro translated snRNP's has revealed the presence of both unassembled and assembled particles as determined by

sucrose density gradient sedimentation. Post-translational assembly of snRNP's involving both RNA-protein binding (as revealed by A protein antigenicity) and associations of other snRNP proteins occurred in the in vitro system employed here. In addition to antibodies to the other 5 snRNP peptides, all Sm sera tested have been found to contain the RNP-like reactivity with snRNP proteins A and C. Two patients were observed to switch with time from Sm to RNP-like snRNP recognition. Selective reactivity with A and C proteins is of particular interest because these proteins are unique in the metabolism of snRNPs.

Chapter 3

RESULTS

The two serotypes which display reactivity with snRNPs are Sm and RNP. They have been distinguished by virtue of an apparent RNase sensitivity of the antigen recognized by RNP sera (Mattioli and Reichlin 1971; Sharp, et al., 1972; Northway and Tan 1972). Analysis of the protein constituents of snRNPs has resulted in differing, although likely compatible, reports on the precise components of these particles. In vitro translation studies (see Chapter 2) together with the reports (Hinterberger, et al., 1983; Kinlaw, et al., 1983) of nonimmunological snRNP particle purification have allowed at least 8 separately translated proteins to be identified as snRNP constituents.

Identification of which species are specifically recognized by Sm and RNP serum antibodies has been plagued by difficulties related to the noncovalent interactions which hold snRNP particles together. Immunoprecipitation of endogenously labeled snRNPs has therefore been of limited value in delineating distinctions between peptide recognition by Sm and RNP sera. Detergent treatment and immunoblot (Western) analyses have revealed several differences in the serologies. However, identification of all antigenic peptides requires native undenatured proteins since most snRNP peptides are not recognized by SLE antibodies following exposure to denaturing detergents.

Sm and RNP Antigen Recognition of snRNPs subjected to extended labeling

Extended in vivo labeling of snRNPs was carried out by incubation of K562 cells for 15 hours in the presence of ³⁵S-methionine. Lysis and immunoprecipitation results in identification of the eight proteins A-G,

uniquely precipitated by both Sm and RNP sera, but not with normal human serum (Figure 5). Although various higher molecular weight bands have been seen as well, including several uniquely recognized by certain sera, these have not been correlated with either Sm or RNP serology (Conner, et al., 1982).

Sm and RNP recognition of in vitro generated snRNP proteins

Previous work has indicated that bands A-G exist in complex particles which could be dissociated by mild detergent treatment during extraction of antigen from labeled cells (Conner, et al., 1982). Furthermore, unique and distinguishable subsets of these eight proteins (from dissociated particles) are recognized by Sm and RNP sera, although detergent denaturation destroys reactivity with most of the proteins. As an alternative to particle disruption by detergents, incompletely assembled snRNP's might facilitate the analysis of Sm and RNP serological reactivities with native snRNP components. Such reactivities might be identified by immunoprecipitation of products from in vitro translation of snRNP peptides. Wheat germ extract (Erickson and Blobel 1983) was utilized for in vitro translation of K562 poly A+ RNA, followed by immunoprecipitation using Sm and RNP sera. Figure 7 shows typical results of such experiments. As with in vivo generated antigen (lane 1), Sm serum precipitates all eight proteins from translation products (lane 2), while RNP serum (lane 3) precipitates only proteins A and C. Five different Sm and RNP sera gave similar results. Thus markedly different patterns of Sm and RNP antigen recognition have been detected using cell-free translation products. Such unique patterns are not seen when antigen is prepared from cells which have been radioactively labeled for an extended period of time (Fig 5). Precipitation of all snRNP proteins by both sera using snRNP proteins labeled in vivo probably results from associated particle

precipitation by RNP serum, although only two proteins are being immunologically recognized. Analysis of unassociated proteins allows for more accurate determination of individual protein reactivities.

In Vitro Particle Assembly

From the data presented it was impossible to be certain whether the Sm sera used in these studies are recognizing each of the snRNP proteins independantly or precipitating all eight by virtue of narrow specificity and coprecipitation of proteins assembled into particles or particle precursors. Even in the RNA fractionation experiment (Figure 5) the possibility remained that each separate protein was associated with unlabeled proteins or snRNP particles present in the wheat germ extract system. To address this question, products of translation in vitro were subjected to sucrose gradient fractionation followed by immunoprecipitation with Sm serum. As shown in Figure 8, bands B-G are present in the first seven fractions, which represent sedimentation coefficients less than 2S. Band C was seen in the top two fractions upon longer exposure of the gel (data not shown). Mature snRNPs have been reported to sediment at approximately 6-11S (Mattioli and Reichlin 1971; Northway and Tan 1972; Howard 1978; Lerner and Steitz 1979). The presence of these snRNP proteins in fractions of low S value suggests that the proteins are present as separate monomeric species, unassociated into complexes. On the basis of their immunoprecipitation in this form, it is apparent that Sm serum does contain antibodies capable of recognizing proteins B-G individually. The greater mobility of D relative to the other proteins is of some interest. It suggests either that D has associated with other species (e.g. protein or RNA) or that it has an unusual three-dimensional configuration accounting for its larger S value. In this regard it may be of related

interest that D protein appears to split into a doublet upon immunoprecipitation with certain sera (see below). Further examination of Figure 8 also reveals the presence of snRNP proteins in fractions of approximately 7 and 11S. The presence of snRNP peptides in these fractions suggests that limited particle assembly is occurring during or subsequent to translation in vitro. The finding that snRNP proteins are present in approximately 7S and 11S fractions is similar to previous observations (Mattioli and Reichlin 1971; Northway and Tan 1972; Howard 1978; Lerner and Steitz 1979) that there are two size classes of snRNP particles. Although particle assembly can occur in the cell-free translation system, most snRNP proteins exist in unassembled form (Figure 8), a fact that allowed the distinction between Sm and RNP recognition (Figure 7). Nevertheless, the ability to generate assembled snRNPs in vitro may provide a means for analysis of the protein-protein and protein-RNA interactions that undoubtedly underlie the biological functions of these nuclear complexes.

Recognition of pulse labeled snRNPs

A second source of labeled snRNP proteins which may represent incompletely assembled particles may be generated by brief pulse labeling of K562 cells with ^{35}S -methionine, rather than labeling for an extended period of time. A titration experiment was performed in which K562 cells were starved of methionine for various times prior to 8 minute pulse labeling with ^{35}S -methionine, extracting, and anti-Sm immunoprecipitation. As seen in Figure 9, only with some starvation were all eight peptides well labeled. The selective absence of A protein is somewhat striking in the total absence of methionine starvation ("0 starvation") and may relate to the kinetics of A protein metabolism in K562 cells. All pulse-labeling experiments performed subsequently, made use of cells previously starved of

methionine for 0.2 to 2 hours. The results of anti-Sm and anti-RNP immunoprecipitation following an eight minute pulse of ^{35}S -methionine are shown in Figure 10. The three Sm sera shown (lanes 2, 3, and 7) precipitated all eight snRNP proteins, while the three RNP sera (lanes 4, 5, and 6) precipitated proteins A and C only. Normal human serum (lane 1) precipitated none of these proteins. Thus, although all eight particle proteins incorporate ^{35}S -methionine during an eight minute pulse (as illustrated by their precipitation with the Sm sera), only a subset of these proteins, bands A and C, are precipitable by RNP sera. Pulses as short as 2 minutes gave identical results (data not shown). Studies of over 20 Sm and RNP sera, including standards from other institutions demonstrated the same distinct Sm and RNP patterns of reactivity in all cases. Analysis of in vivo and in vitro synthesized snRNP proteins thus demonstrate the same distinguishable patterns of Sm and RNP antigen recognition.

D Band Split

In the process of analyzing snRNP recognition by numerous anti-Sm and anti-RNP sera, a reproducible split in the appearance of the D peptide was seen for certain sera, but not others. No correlation was seen with Sm or RNP serology. Anti-RNP patients which displayed this splitting pattern lacked detectable antibodies to D as demonstrated by in vitro translation and pulse labeling of snRNP proteins. Figure 11 shows an example of such a split D pattern generated by immunoprecipitation of 15 hour labeled snRNPs with a certain RNP serum, Mi, (lane 2). An Sm serum, Tu, which displayed only a single protein is also seen (lane 1). While the possibility exists that one of these bands represents a non-snRNP species such as a histone (against which antibodies are sometimes found in SLE (Kunkel, et al. 1960,

Stollar, 1971), the comigration of both D bands at 10S in sucrose gradients (see Figure 17, chapter 4) suggests that they are true snRNP components. Since antibody specificity is not likely to account for these varying patterns- because the RNP serum has no apparent antibodies to these peptides (Figures 7 and 10), the possibility exists that this splitting pattern represents a serum-specific modification of the antigen which occurs during immunoprecipitation.

To test this possibility, the following experiment was performed. Fifteen hour labeled snRNPs were immunoprecipitated with two sera, one (Tu) which gives a singlet, and the other (Mi) which gives a doublet D. Following adsorption of the immune complexes onto protein A Sepharose, the beads were washed extensively with detergent free buffer. Each set of beads was then incubated with cold (unlabeled) K562 cell lysate plus the other serum for 1 hour at 20°C. Samples were washed, eluted and analyzed electrophoretically. As can be seen in Figure 12a, mixing revealed that treatment of Tu immunoprecipitate with Mi serum plus lysate converted the single D into a doublet. Incubation of Tu serum plus lysate had no apparent effect on Mi immunoprecipitate which still displayed a doublet.

To test whether the activity giving rise to this splitting of D resides in the lysate or is entirely in serum, the above experiment was repeated using only serum in the post-immunoprecipitation mixing steps. Figure 12b shows that the splitting effect was still seen when only Mi serum diluted as before (in lysis buffer) was added to the Tu immunoprecipitate. When the second step was carried out with Mi serum pretreated with 1 ug/ml each of antipain, leupeptin, pepstatin (kindly provided by Dr. Reid Gilmore), 5 mM iodoacetamide, and 0.5 mM phenylmethanesulfonylfluoride, and mixing carried out at 4°C, splitting was

still seen (data not shown) despite the presence of these protease inhibitors. While proteolysis cannot be firmly ruled out by these studies, the possibility remains that the faster migrating form of D protein is modified in a serum specific fashion, and this modification (or removal of a modifying group) may shed some light on the peptide's chemistry and function.

A final possibly related point is the somewhat anomalous migration of unassembled (low S value) D protein, as seen in Figure 8 in products of in vitro translation of snRNPs. Peptides of apparent larger molecular weight migrated slower in the sucrose gradient than the faster SDS-gel migrating D protein. The serum used in the experiment of Figure 8 recognizes a single D band. The possibility therefore exists that the anomalous D peptide mobility in sucrose gradients may relate in some manner to the presence of a modification which is chemically altered specifically by an unidentified serum component in certain individuals.

RNase treatment of snRNPs

To examine the protein and RNA components responsible for immunorecognition by SLE sera, in vivo labeled and in vitro translated snRNP's were treated with RNase prior to immunoprecipitation. Lanes 1 and 3 in Figure 13a demonstrate typical results of immunoprecipitation using 15 hour in vivo labeled antigen, precipitated with RNP and Sm sera respectively. Bands A through G are all present in precipitates using both sera. RNase treatment of extracted cellular antigen prior to immunoprecipitation with RNP and Sm resulted in loss of A band (lanes 2 and 4). Examples of immunoprecipitations of snRNP antigen derived from in vitro translation are shown for Sm and RNP sera in the absence (Figure 13b lanes 1 and 3) or presence (lanes 2 and 4) of RNase treatment. In contrast

to in vivo antigen analysis, precipitation of in vitro translated products gives unique patterns for Sm and RNP sera. In all cases, however, precipitation of the A protein is strikingly affected by RNase treatment. A higher molecular weight protein not known to be associated with snRNP's precipitated preferentially with RNP serum following RNase treatment (Figure 13b lane 4).

In an attempt to recover nonantigenic, RNase treated A protein, in vivo labeled cell extracts were immunoprecipitated onto protein A Sepharose beads, followed by extensive washing of the beads and subsequent RNase treatment. Figure 13c shows RNase treated material which remained bound to the sepharose when precipitated with RNP or Sm (lanes 2 and 4). Loss of A protein again occurred for both sera. When the supernatants of the RNase treatments were run in adjacent lanes (lanes 1 and 3), band A is seen to be essentially the only labeled protein present. In separate experiments it was observed that this band migrated in the exact position of the A protein.

RNase treatment of antigen has thus been shown to abrogate immunoprecipitation of the A protein. While RNP serology has been distinguished from Sm by virtue of an RNase sensitive hemagglutination assay (Tan and Peebles 1976), it is significant that recognition of A protein by Sm serum is equally destroyed by RNase. Similar results have been obtained by using an ELISA for snRNPs (W. Reeves, personal communication). Thus, although RNP antibodies recognize only two of the eight Sm-precipitable proteins,

Sm antibodies display an equal restriction in recognizing the A protein only in the context of an associated RNA. These results are in agreement

with the report (Wieben, et al., 1983) that U1 RNA increases antigenicity of in vitro translated A protein. It is uncertain, however, whether it is specifically U1 RNA that is responsible for the antigenicity of A protein seen in the RNA fractionation experiment (Fig. 5b). Any U1 RNA present in fractions 6 and 7 would exist despite oligo dT affinity chromatography and sucrose gradient fractionation, which was seen to have resolved the RNA's encoding snRNP proteins. While the possibility that trace U1 RNA is present in these fractions cannot be ruled out, alternative explanations exist. An RNA species similar to U1 may be present in wheat germ extract and be responsible for antigenicity of A protein. If so, such a species may be revealing about comparative RNA binding specificity of A protein. The possibility also exists that an entirely different RNA species is binding to A in these fractions. The ability to generate electrophoretically pure native A protein (Fig 13) should facilitate further studies of its biochemical properties.

Shift from Sm towards RNP recognition in two patients In the process of screening numerous patient sera for snRNP reactivity, using pulse labeled cell extract to distinguish Sm and RNP, an unusual observation was made. Two sera from the same patient which differed by several years appeared to generate different patterns of antigen recognition. Further analysis was carried out by immunoprecipitation analysis (of pulse labeled snRNPs) using sera from throughout the patient's disease course at the Rockefeller University Hospital. Results of such analysis are seen in Figure 14. While this patient produced a typical Sm pattern of precipitation in serum from 1978, a shift in reactivity was first detected to have occurred between 1979 and 1981. By June of 1982 the immunoprecipitation pattern is

almost a typical RNP pattern. Some clear evidence exists for B and B' in the immunoprecipitates indicating residual Sm reactivity, however the shift towards RNP-like recognition is quite striking. A similar shift was subsequently detected in a second patient (Figure 15). In this situation first evidence for loss of Sm-specific reactivity began between August and October of 1980. While small quantities of Sm-specific bands appeared for about one and a half years (10/'80-3/'82), further loss of such reactivity continued in 1982. While the possibility is currently being investigated that these observed shifts may correlate with disease-related activities, these examples provide an illustration of the utility of such sensitive analytical methods in the analysis of disease conditions.

DISCUSSION

The previous inability to distinguish the fine nature of antigen recognition of Sm sera from RNP sera is in large part due to the fact that snRNP antigens are complex particles. Antibody recognition of any component in the particle results in immunoprecipitation of the entire particle. Thus when the antigen is radioactively labeled by prolonged incubation with ³⁵S-methionine, little if any difference has been seen in the patterns obtained with Sm and RNP sera (Figure 6). Previous attempts to disrupt the particles using 0.5% SDS treatment have revealed differential recognition of several snRNP peptides by Sm and RNP sera (Conner, et al., 1982). Likewise using SDS gels and immunoblots, several of the snRNP peptides have been observed to be uniquely recognized by Sm and/or RNP sera and monoclonal antibodies (White and Hoch 1981; Conner, et al., 1982; Petterson, et al., 1984). However many of the peptides were not

recognized and the possibility remained that all snRNP peptides are antigenic but SDS treatment seriously alters antigenicity.

For these reasons, the present study was initiated to analyze *in vivo* synthesized, native snRNP peptides. To circumvent the need for particle disruption or detergent treatment, the strategies involved *in vitro* translation and brief pulse labeling with ³⁵S-methionine. By these methods of analysis a clear distinction is seen between Sm and RNP serological immunorecognition (Figures 7 and 10). While Sm sera precipitate all snRNP components, only two of these labeled peptides, A and C, appear in RNP immunoprecipitates.

An understanding of the peptide antigen recognition by Sm and RNP sera is of considerable value in light of the common clinically used assays for these sera. While RNP reactivity has traditionally been distinguished from Sm by RNase sensitivity in hemagglutination and immunodiffusion assays (Northway and Tan 1972; Mattioli and Reichlin 1973), it is of interest that immunoprecipitation of RNase treated snRNP's produces an identical change in antigen recognition by the two sera. Loss of the A protein immunoprecipitation occurs for both Sm and RNP sera. The small nuclear RNA has been shown by Wieben, et. al. (1983) to be critical for A antigenicity. Thus, the finding that RNP sera recognize only proteins A and C may help explain the biochemical basis for the hemagglutination assay since essentially only C protein reactivity remains for RNP sera in RNase treated snRNP's. For reasons of valency, 3-dimensional configuration, or chemical treatments of antigen, the C protein may be poorly reactive in snRNP particles, in agar diffusion plates (Mattioli and Reichlin 1971) or when attached chemically to red blood cells (Tan and Peebles 1975)- particularly since the most common source of antigen is an acetone extract

of rabbit thymus. Antibody to C protein may poorly hemagglutinate or precipitate with antigen under these conditions. If C protein recognition is not predominant in these assays, then A protein antigenicity would be the critical recognition for RNP antibody induced hemagglutination or agar precipitin formation. RNase treatment of snRNP's, then, would be expected to abolish RNP reactivity. Sm serum's additional recognition of proteins B, B', D, E, F, and G, however, would still allow for Sm reactivity in these assays regardless of RNase. This interpretation is further supported by the observation that using an ELISA assay, RNP recognition may be somewhat diminished, but is by no means abolished by pretreatment of snRNP's with RNase (W. Reeves, personal communication). White and Hoch (1981) have also demonstrated RNP antigen recognition in the absence of associated RNA from rabbit thymus acetone extract.

Several groups (Kinlaw, et al., 1983; Hinterberger, et al., 1983) have reported the isolation of two classes of snRNP particles, one of which lacks proteins A and C. This class contains the U2-6 small nuclear RNA species' while the particles containing proteins A and C contain U1 RNA. The other 6 proteins (B, B', D, E, F, and G) are common to both classes of snRNPs.

In light of the finding that RNP sera recognize proteins A and C only, while Sm sera recognize all 8 components, early reports of the relationship between Sm and RNP antigens are better understood. Several groups (Mattioli and Reichlin 1971; Northway and Tan 1972; Sharp, et al., 1972) described RNP (also called "Mo") as an RNA containing antigen. However, fractionation experiments failed to separate it from Sm-reactive material. This was in contrast to the ability to fractionate pure Sm-reactive material away from RNP/Sm reactive antigen. Thus, pure Sm-reactive antigen

likely corresponds to U2-6 containing snRNPs which contain 6 peptides, but lack A and C. Sm/RNP reactive antigen corresponds to U1-containing snRNPs which react with RNP antibodies because of the unique presence in these particles of A protein (with its associated RNA) and C protein. An additional noteworthy observation concerns the finding that all Sm sera studied contain RNP-like reactivity, an observation which has also been made by use of immunoblots by Petterson, et. al. (1984) and an ELISA assay (W. Reeves, personal communication). This clear presence of RNP-like antibodies in Sm sera raises questions regarding similarities and differences in the respective disease states. While the determinants on the A and C proteins recognized by the Sm and RNP antibodies could conceivably differ, it is striking that both sera lose reactivity with A after RNase treatment of antigen. Thus at least one characteristic of the recognition is common to both sera. Relevant to this question is the observation of a temporal decrease in the Sm-specific antibodies in two SLE patients (see Figures 14 and 15).

It is highly interesting that the two peptides (A and C) which distinguish these particle classes contain the precise antigenic determinants for RNP sera. Analysis of in vivo particle assembly has revealed a unique sequence of events which further distinguishes A and C proteins in the kinetics of their incorporation into newly synthesized snRNP particles (chapter 5). Taken together, these considerations would suggest that the immunologic as well as parallel biochemical uniqueness of A and C proteins are not coincidental.

Figure 6

Sm and RNP sera immunoprecipitate the same radiolabeled peptides from 15-
hour, ^{35}S -methionine labeled cells. K562 cells were labeled in vivo with
with ^{35}S -methionine for 15 hours, lysed, and the lysate was precipitated
with normal human serum (NHS), Sm serum (lane 1), and RNP serum (lane 2).

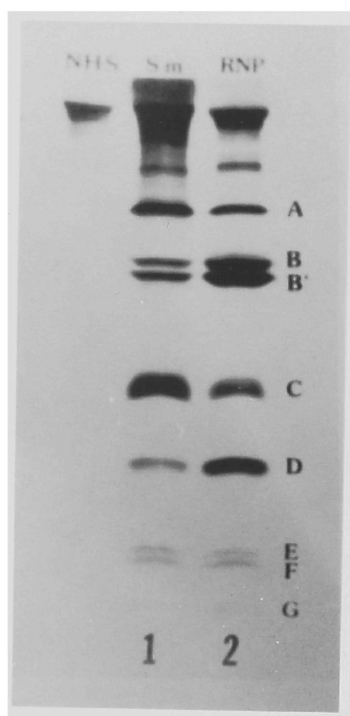


Figure 7

Comparison of Sm and RNP immunoprecipitation of in vitro generated snRNP proteins. 15 hour ^{35}S -methionine labeled K562 cell lysate was immunoprecipitated with Sm serum (lane 1). In vitro translation products of K562 poly A+ RNA were immuno precipitated with Sm (lane 2) and RNP (lane

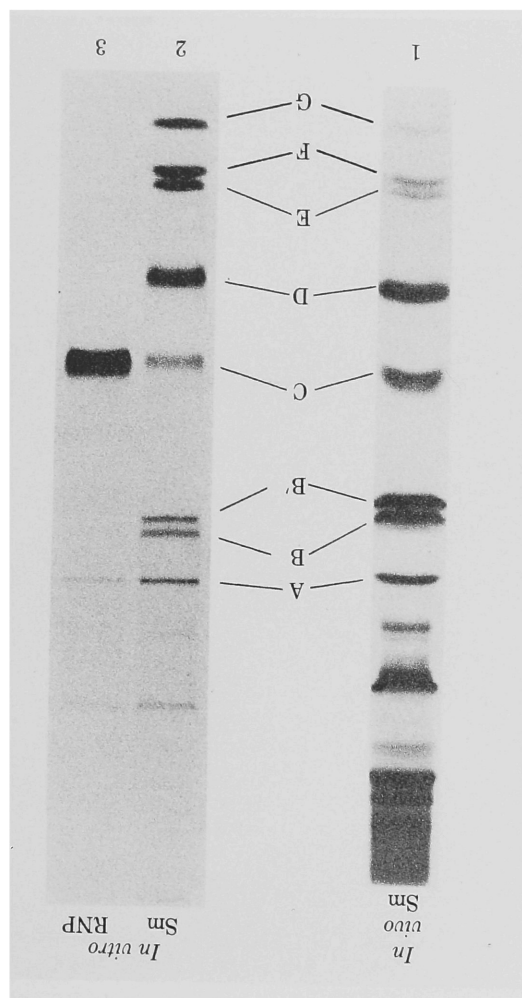


Figure 8

Sucrose density sedimentation of native snRNP proteins translated in vitro.

K562 poly A+ RNA was translated in vitro and total products were fractionated on a 5-20% sucrose gradient. Each fraction was immunoprecipitated with Sm serum and resolved on a slab gel. The pellet from the gradient centrifugation was resuspended and immunoprecipitated as well (lane P). The top of the gradient is indicated on the left. Relative mobilities of marker proteins are indicated with corresponding S values.

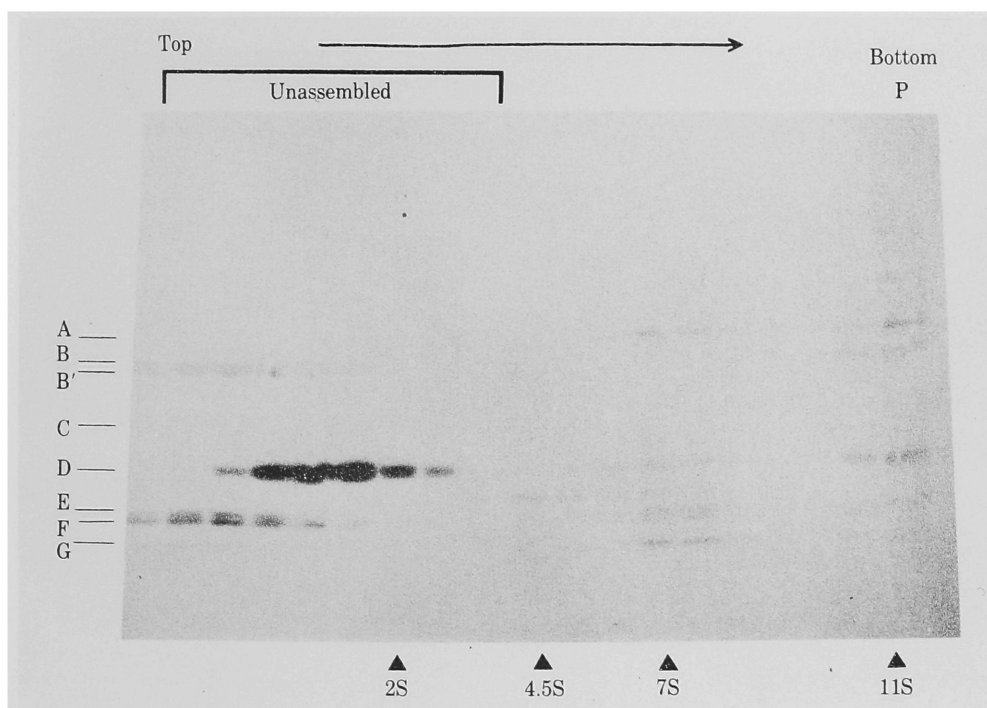


Figure 9

Titration of methionine starvation for pulse labeling of snRNPs. K562 cells were incubated in methionine free medium, and aliquots removed at the time points indicated (0 to 3.25 hours). Such starved cells were then pulsed immediately with 500 uCi of ^{35}S -methionine for 8 minutes. Pulse labeled cells were then lysed and immunoprecipitated with Sm serum.

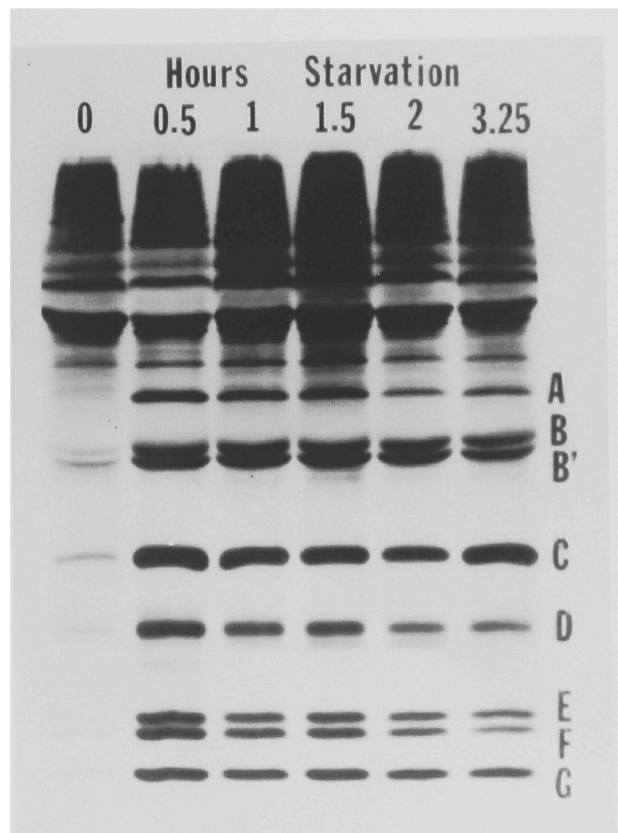


Figure 10

Immunoprecipitation of pulse-labeled snRNP proteins. K562 cells were starved of methionine and then pulse labeled for 8 minutes with ^{35}S -methionine. Following cell lysis, the snRNP proteins were immunoprecipitated with normal human serum (lane 1), 3 different Sm sera (lanes 2, 3, and 7), and 3 different RNP sera (lanes 4-6).

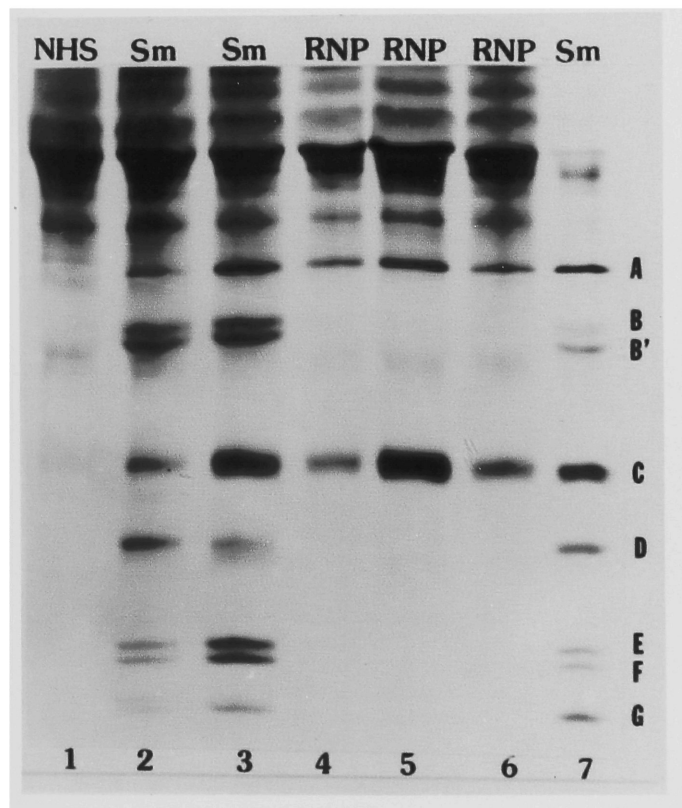


Figure 11

Splitting of D band in immunoprecipitates with certain sera. 15 hour labeled cell lysates were immunoprecipitated with two sera. Serum from patient Tu immunoprecipitates a single D peptide, while the precipitate using "Mi" serum recognizes two bands in the position of D (see arrows).

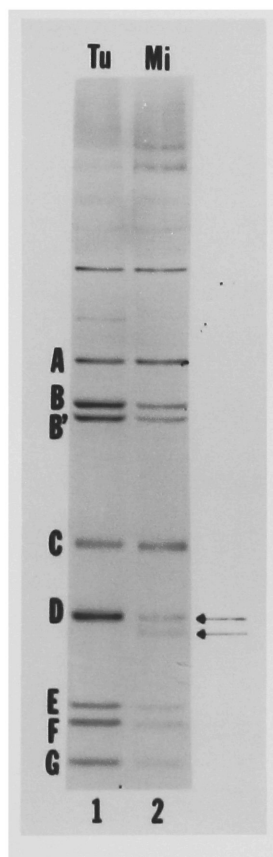


Figure 12

Examination of D band splitting induced by sequential treatments with patient sera. Mixing experiments were carried out on immunoprecipitated snRNP peptides, adsorbed to protein A sepharose beads. A) Lanes 1 and 2 show the doublet and singlet patterns of D protein, respectively. Lane 3 contains products of immunoabsorbed, washed, Mi-serum precipitate further incubated with unlabeled K562 cell lysate plus Tu serum. Lane 4 shows the appearance of a doublet in Tu-immunoprecipitated D protein when further treated with lysate plus Mi serum. B) To test whether the splitting activity is present in the lysate or the serum, an experiment as in "A" was repeated, but with no lysate added in the second incubation. Lanes 1 and 2 show treatment of precipitates with the same serum. Lanes 3 and 4 show incubations with the opposite sera. D splitting is observed by serum incubation alone.

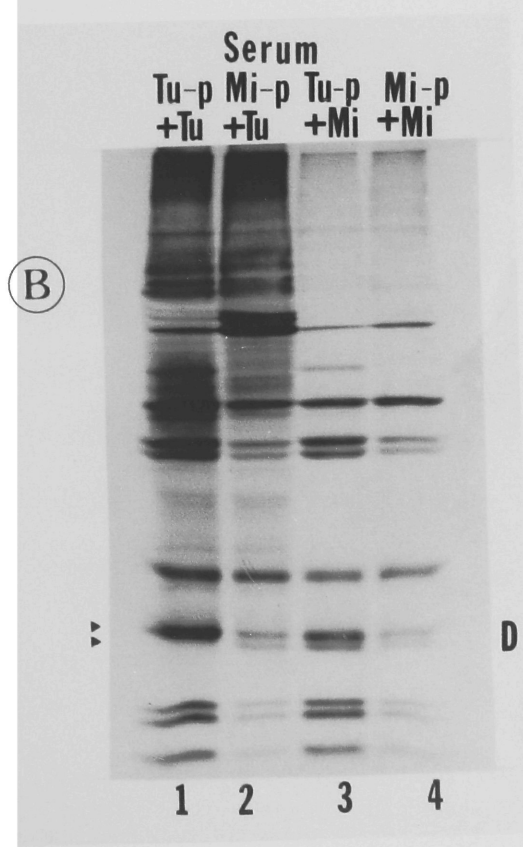
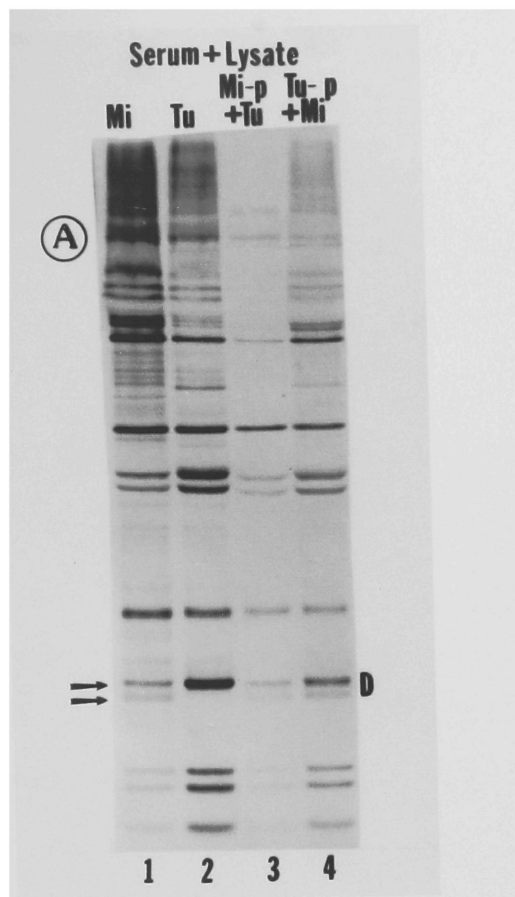


Figure 13

RNase treatment of in vivo and in vitro generated snRNP's. K562 cells (A and C) were grown in ^{35}S -methionine containing medium for 15 hours followed by lysis and extraction. (A) Incubation at 37°C in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 1mg/ml RNase A was followed by immunoprecipitation using RNP (lanes 1 and 2) or Sm (lanes 3 and 4) sera. (B) Translation products of K562 poly A+ RNA were incubated at 25°C for 15 minutes in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 150ug/ml RNase A followed by immunoprecipitation using Sm (lanes 1 and 2) or RNP (lanes 3 and 4) sera. (C) Extract derived from 15 hour labeled K562 cells was immunoprecipitated onto SPA beads using RNP or Sm sera. Following 4 washes, the beads were incubated at 37°C with 1mg/ml RNase A. Supernatants were removed and beads were washed further. Bound proteins were eluted and analyzed on a slab gel. Lanes 2 and 4 show material remaining bound to the SPA beads after RNase incubation for RNP and Sm coated beads, respectively. RNase treatment supernatants showing a single A band appear in lanes 1 and 3 for RNP and Sm immunoprecipitations respectively. The lines at the side of each experiment show the positions of the A-G bands.

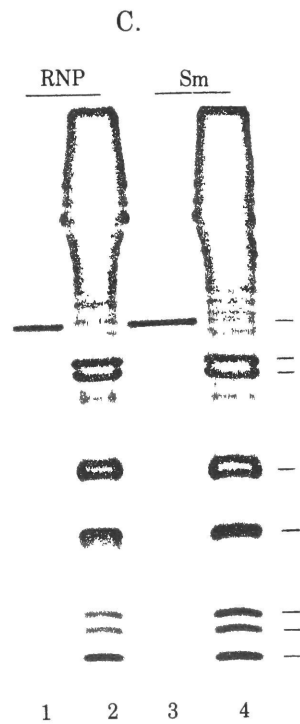
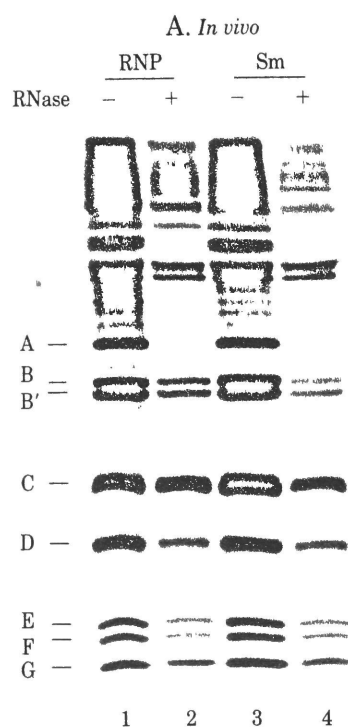


Figure 14

Change with time in Sm vs. RNP pattern of snRNP recognition in serum from an individual patient. Sera from 1978 through June 1982 (6/82) from a patient with SLE were used to immunoprecipitate snRNPs from pulse labeled K562 cell extract. A loss of Sm specific bands appears starting between 1979 and 1981. The RNP-like recognition of A and C proteins remains throughout.

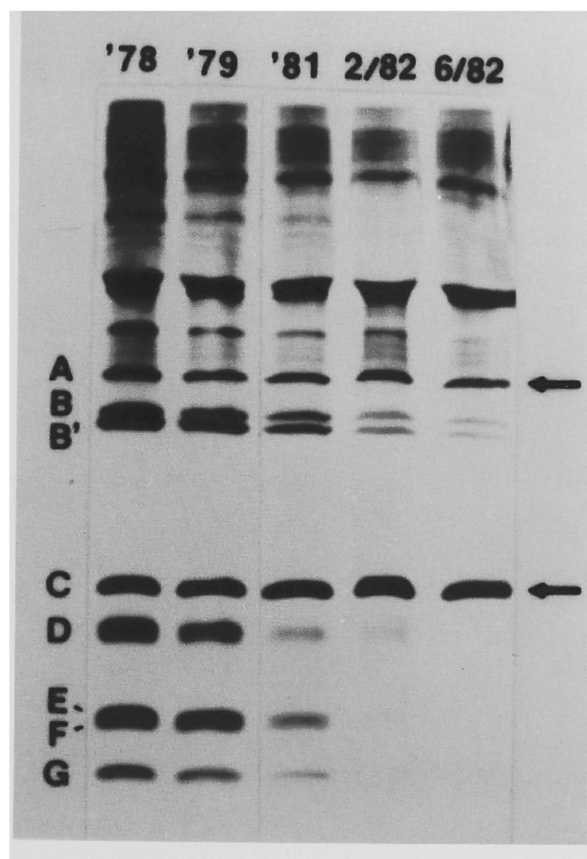
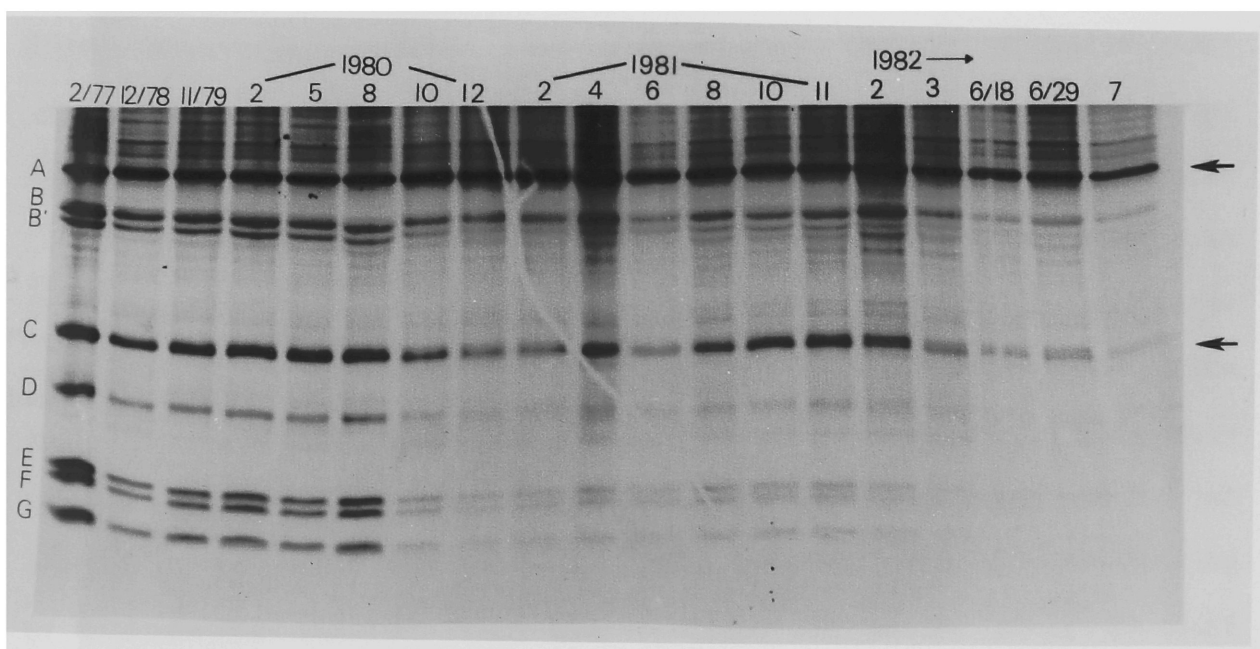


Figure 15

Serial changes in snRNP recognition by sera from a second patient. Sera from February 1977 through July 1982 were tested for snRNP peptide recognition using pulse labeled K562 cell lysate. An abrupt decrease in Sm specific reactivity occurred between 8/80 and 10/80.



Chapter 4

In vivo Synthesis and Assembly of snRNPs

ABSTRACT

The in vivo synthesis and assembly of human small nuclear ribonucleoproteins (snRNPs) have been studied by use of pulse/chase analysis. Antibodies derived from patients with systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) recognize distinguishable subsets of pulse labeled snRNP peptides. These antibodies were used to immunoprecipitate sucrose gradient fractionated pulse-labeled and pulse/chased snRNP proteins. The results indicate that assembly of the anti-RNP reactive (U1) snRNP is a two-step process involving prior assembly of a 6S core particle. Furthermore, a post-translational modification of one of the 10S snRNP specific peptides has been observed, and the kinetics of this process indicate that the modification occurs after particle assembly. Functional and structural implications of a protein core for snRNP particles are discussed.

RESULTS

Immunoprecipitation of Pulse/chased snRNPs.

To explore in vivo events in the assembly of snRNPs, pulse-chase analysis of snRNP biosynthesis was undertaken. K562 cells were pulse labeled for 8 minutes in ^{35}S -methionine and then resuspended in complete medium (containing nonradioactive methionine). Throughout further incubation, aliquots of pulse/chased cells were removed at 0, 0.2, 1, 6, and 15 hours of chase. Cells from each time point were lysed and snRNPs were extracted. Following centrifugation to remove insoluble nuclear material, the extract was subjected to immunoprecipitation by anti-Sm and anti-RNP antibodies, derived from sera of patients with SLE and MCTD, respectively. The results of such pulse-chase analysis are illustrated in Figure 16.

Directly following pulse labeling (0 chase), anti-Sm antibodies precipitate all 8 peptides A-G, while anti-RNP antibodies precipitate labeled snRNP proteins A and C only. Following increased periods of chase the pattern of proteins precipitated by anti-Sm remains essentially the same; all eight peptides are precipitated throughout the chase periods. Anti-RNP antibodies, however, are seen to coprecipitate all eight labeled peptides only after several hours of cold chase. By 6 hours, clear evidence exists of bands B and B', and by 15 hours all 8 peptides are visualized. This eventual coprecipitation of all snRNP particle components by RNP antibodies suggests that de novo snRNP particle assembly occurs in the time-course of such pulse/chase experiments.

Sucrose gradient fractionation of pulse/chased snRNPs.

Since de novo assembly appears to occur within these 15 hours, analysis of this process was undertaken utilizing sucrose gradient centrifugation. K562 cells were pulse labeled or pulse labeled and cold chased for 15 hours. These two cell sources ("pulse" and "chase") were lysed, and their snRNPs extracted as above. The extracts were loaded onto 5-20% sucrose gradients and subjected to centrifugation for 20 hours at 41,000 rpm in an SW50.1 rotor. Gradients were then fractionated, and each fraction was immunoprecipitated using anti-Sm and anti-RNP antibodies. The four immunoprecipitation patterns generated by this analysis are shown in Figure 17.

Anti-RNP immunoprecipitation of "pulse" gradient-fractionated extract is shown in Figure 17- "Pulse-RNP." Of the snRNP proteins, only radiolabeled A and C appear. A small amount of C protein is present near the top of the gradient migrating with sedimentation of less than 4S, and proteins A and C are both present in fractions of approximately 10S. This pattern suggests that immediately following an 9 minute pulse some of the newly synthesized C protein exists in an uncomplexed form (<4S) while some newly synthesized C protein also exists in a complex (~10S) in which ³⁵S-labeled A protein is also present. A complex containing all 8 snRNP proteins with similar mobility has been observed in products of in vitro translated snRNP proteins (see Figure 8, chapter 3). The presence of U₁ RNA in a 10S particle has also been reported (Lerner, et al., 1980).

The pattern obtained from pulse gradient fractionated extract immunoprecipitated with anti-Sm antibodies is shown in Figure 17- "pulse-Sm." As with anti-RNP antibodies, following pulse, C protein is present primarily in two forms, one of <4S, the other of about 10S. Radioactive A protein is again seen in the 10S fractions suggesting that this higher

mobility form of A and C is due to the presence of a complex in those fractions. In contrast to the anti-RNP patterns, however, an additional form of snRNP proteins is present in the anti-Sm immunoprecipitates. In the fractions of approximately 6S, snRNP proteins D, E, F, and G are clearly present. Their coincident sedimentation rate suggests that these peptides also reside in a complex of about 6S. A similar snRNP complex containing these same peptides has also been observed in products of in vitro translated snRNP proteins (Figure 8, chapter 3; the appearance of A protein in 6-7S fractions in Figure 8 probably does not reflect its presence in a snRNP particle because RNP immunoprecipitation of such in vitro translation gradients reveals A protein alone in the same 6-7S fractions). Anti-RNP antibodies appear to lack reactivity with peptides other than A and C, which explains absence of the 6S complex in Figure 14- "pulse-RNP." The bottom fraction in Figure 14- "pulse-Sm" contains a band (see asterisk) of uncertain identity with mobility somewhat greater than the D peptide.

Analysis of fractionated "chase" cell extract immunoprecipitated with anti-RNP is seen in Figure 14- "chase-RNP." The C protein is again seen to be present in the upper two fractions (<4S). Additionally, all eight ³⁵S-labeled snRNP particle proteins (A-G) are seen in fractions (10S) corresponding identically to those in Figure 14- "pulse-RNP" which contain labeled peptides A and C only. The identical migration of these "pulse" and "chase" 10S fractions suggests that the same eight proteins are present in each case, although only A and C are present in labeled form following brief pulse. Of additional interest is the presence of 2 high molecular weight proteins (greater than 50 kd) near the bottom of the gradient (see arrows). While likely antigenic for the serum used here, they appear to be

distinct from snRNPs and migrate broadly as a complex of $>10S$.

Anti-Sm immunoprecipitation of fractionated chase lysate is shown in Figure 14- "chase-Sm." Some C protein is again seen in the top two fractions with a sedimentation rate of $<4S$. While bands D, E, F, and G, are present in the $\sim 6S$ fraction (as in the "pulse" lysate, see Figure 14- "pulse-Sm"), the $10S$ snRNP complex is seen to contain all 8 snRNP proteins in labeled form. No additional labeled peptides appear in the $\sim 10S$ fractions which contain snRNP proteins A-G. The presence of all eight labeled snRNP peptides in the $10S$ fraction suggests that particle assembly has occurred since the pulse analysis, in which peptides A and C were the only labeled species which migrated at $10S$.

Post-translational modification of C protein.

An additional observation relates to the mobility of C protein after pulse and chase. Comparison of the 15 hour chase Sm lane with the 0 chase RNP lane (Figure 16, see arrows) reveals a distinct change in C peptide mobility. Immediately following pulse, the peptide has a greater mobility than after chase with evidence for a shift in mobility present within 1 hour of cold chase. The shift appears to be complete by six hours. When pulsed material is compared to the 15 hour chase, R_f analysis would suggest a shift in mobility corresponding to approximately 1000 d. Preliminary attempts at analysis of this modification did not demonstrate ^{32}P incorporation following ^{32}P -orthophosphate labeling of K562 cells and 0.1 M NaOH incubation of washed immunoprecipitates did not appear to alter the modified C protein (data not shown).

Changes in D protein.

Of further relevance is the appearance in Figure 14- "chase-RNP" of D band as a doublet. Sera from certain patients have consistently given this pattern of two D bands, while others have not. Figure 14 illustrates the observation that both of the split D bands are intrinsic snRNP components. Implications of this split pattern were discussed further in Chapter 3.

Cell fractionation and snRNP assembly.

In an attempt to analyze aspects of the cellular localization of snRNP particle assembly, pulsed and pulse/chased cells were subjected to fractionation. The two types of labeled cells were lysed in hypotonic buffer, nuclei were pelleted by centrifugation, and the cytosol fractions were removed. The nuclear pellet was extracted with 0.2 M NaCl. Labeled snRNP proteins in the cytosol and the nuclear extract were immunoprecipitated using anti-Sm and anti-RNP. Anti-Sm precipitates all 8 labeled peptides from cytoplasmic fractions of both pulsed and pulse/chased cells (Figure 18, lanes 1 and 2). Anti-Sm precipitation of nuclear fractions, however, reveals only labeled A and C peptides following pulse (Figure 18, lane 3), but again all eight peptides following chase (Figure 18, lane 4). Anti-RNP precipitates contain only labeled A and C from pulsed cytoplasmic and nuclear fractions (Figure 18, lanes 5 and 7), while all 8 snRNP peptides are precipitated by anti-RNP following chase- both in cytoplasmic (lane 6) and nuclear (lane 8) fractions. The observations from Figure 17 suggests that pulse labeled A and C proteins reside together in a ~10S particle containing the other unlabeled preexisting snRNP proteins. The results illustrated in Figure 18 demonstrate that pulse labeled A and C can be seen in cytoplasmic fractions where other snRNP peptides reside (presumably as a ~6S form- see Figure 17). However the pulse labeled

nuclear fraction contains A and C as the only radiolabeled snRNP species. Since these peptides reside in a ~10S form (the presence of <4S C peptide is uncertain in the nucleus), these results suggest that after a brief pulse, snRNP proteins A and C have assembled together with the other unlabeled snRNP species into a 10S particle some of which is present in the cytoplasmic fraction, but a good deal of which can be found in the nucleus. Finally, Figure 18 again illustrates the presence of a post-translational modification of C protein. The changing mobility of C peptide with pulse and chase is quite evident. Nuclear versus cytoplasmic origin of the C protein appears not to correlate with the presence or absence of the modification.

DISCUSSION

Three forms of snRNP proteins.

While problems related to coprecipitation have hampered analysis of immunologic specificity using autoimmune sera, this same property has been of great value in studying assembly of the snRNP particles. Sucrose gradient analysis of snRNP's incubated for 15 hours after a brief pulse reveals labeled snRNP proteins in three recoverable forms from within the cell. The three forms are <4S, ~6S, and ~10S (Figure 17). The 10S form appears to represent a mature snRNP particle characterized by others (Lerner, et al., 1980; Hinterberger, et al., 1983; Kinlaw, et al., 1983) and demonstrated to contain U1 RNA. The 6S fractions may include particles which contain the other U series RNA's (U2, U4, U5, and U6), based on purification and characterization of this second class of snRNP particles by Hinterberger, et al. (1983) and Kinlaw, et al. (1983). Proteins D, E,

F, and G are clearly present in the 6S particles. Peptides B and B', while not visualized here, have been suggested to reside in the U2, U4, U5, and U6 snRNP fraction. The third form of snRNP proteins found in vivo sediments at a low S value and likely represents unassembled snRNP protein. Only the C protein is seen in this form. Since A protein antigenicity is lost (Figure 13, Chapter 3) (Wieben, et al., 1983) or greatly reduced (Pettersen, et al., 1984) in the absence of U1 RNA, it is uncertain whether significant quantities of this peptide exist in unassembled form within the cell since they may not be precipitated by these antibodies. However, no significant quantities of unassembled (low S value) B, B', D, E, F, and G proteins were recoverable from K562 cells (Figure 17). Under comparable biochemical conditions, such low S value forms of proteins B-G were all recoverable from in vitro translated snRNP proteins (Figure 2, Chapter 3), suggesting that the peptides other than C protein would not have been missed due to instability or lack of antigenicity in the unassembled state.

Sucrose gradient analysis of in vivo pulse labeled snRNPs (Figure 17) has also revealed three forms of these peptides. The 4S and ~6S forms appear indistinguishable from the snRNP's after a 15 hour pulse/chase. However, the third fraction which has identical mobility to the 10S chase fraction, contains only proteins A and C in labeled form. Most of the other snRNP proteins (D, E, F, and G) all of which are recognized individually by anti-Sm antibodies, reside only in the ~6S fractions immediately following pulse labeling. These labeled proteins appear in the 10S particle, however, following cold chase incubation. On the basis of their identical sedimentation rate, it appears likely that the two 10S snRNP fractions (pulse and chase- derived) are actually the same with regard to protein (and RNA) composition. Only A and C are present in

radiolabeled form following brief pulse. It therefore appears that while the other snRNP peptides are assembled into smaller particles (~6S), proteins A and C have associated with these other components in unlabeled form. Since the only other identified state of these proteins is a ~6S particle (Figure 17), it appears likely that newly translated proteins A and C associate with a preformed ~6S particle containing the other peptides found eventually in the ~10S snRNP.

Two-step model of U1-snRNP assembly. Taken together, these data imply an ordered sequence of events in the biosynthesis and assembly of snRNPs. These events involve the formation of a ~6S particle which is modified further to become a 10S particle (Figure 19). By this model, newly translated snRNP proteins are assembled into a 6S "core" particle prior to the addition of proteins A and C- which is associated with transformation into the 10S particle.

This two step assembly process is based entirely on analysis of protein constituents of snRNPs. However, based on studies by Lerner, et al., 1980, Hinterberger, et al., (1983), and Kinlaw, et al., (1983), certain correlations of these patterns with RNA components can be proposed. Two sets of snRNP fractions with similar protein compositions to the 6S and 10S particles described here have been isolated by these groups and shown to differ in RNA content. The 10S snRNP particle has been demonstrated previously (Lerner, et al., 1980) to contain U1 RNA. Particles similar in protein composition to the 6S particle contain U₂, U₄, U₅, and U₆ small nuclear RNAs. Antigenicity of the A protein appears to require associated U1 RNA (Wieben, et al., 1983), and RNase treatment releases the peptide from snRNP particles (Figure 13, Chapter 3). In light of these results, it

is very likely that the assembly of the 10S snRNP (where A protein is clearly seen) from the 6S core involves U1 RNA addition as well as the presence of A and C proteins. It is unclear whether the 6S core which serves as precursor to the 10S snRNP contains U2, U4, U5, or U6 RNA's or is an RNA-free protein particle. Two proposed schemes for such assembly are shown diagrammatically in Figure 20. While the sucrose gradient analysis employed in this study would not distinguish 6S particles which lack RNA from those which contain RNA (if both populations were present, together), ion exchange chromatography has recently suggested the presence of 2 pI-distinguishable populations of particles with protein composition similar to that of the 6S fraction in Figure 17 (data not shown). Such an RNA-free core might also serve as precursor to the non-U1 snRNPs, by the addition of the respective RNA species'. Kinlaw, et al. (1983) and Mimori, et al. (1984) have described at least one peptide thought to be unique to U2-containing snRNPs. If all snRNP assembly proceeds via a 6S core precursor, then the addition of U2, U4, U5, and U6 RNAs would occur together with the addition of any of these non-U1 snRNP specific proteins. If the core precursors contain any or all of the U₂₋₆ species, transformation to the 10S snRNP would require loss of U₂₋₆ RNA as well as addition of U₁ RNA, and the A and C proteins.

The results of cellular fractionation studies (Figure 16) are consistent with several reports (Elcieri, 1974; Zieve and Penman, 1976; Elcieri, 1980; DeRobertis, et al., 1982; Madore, et al., 1984) which suggest that assembly of the U1-snRNPs occurs in the cytoplasm. By these proposals, precursors to the mature U1 RNA species are transported to the cytoplasm where further processing and presumably ribonucleoprotein assembly occurs. The presence of labeled A and C proteins in the

cytoplasmic fraction of pulsed cells (Figure 18, lane 1) is consistent with the notion that these represent newly assembled 10S particles which have not yet migrated to the nucleus. Some of the newly formed 10S snRNP is also found in the nucleus following pulse (Figure 18, lane 3) consistent with rapid assembly and transport. However, it should be noted that all cellular fractionation experiments may be complicated by leakage between compartments during the fractionation procedures (Scheele, et al., 1978). The above fractionation data are consistent with cytoplasmic assembly, particularly because the ^{35}S -labeled A and C peptides are already seen in the nuclear extract (as well as cytoplasmic) following pulse. Nonetheless, all other possible assembly routes can not be excluded from these data.

Several additional important aspects of this assembly process remain to be understood. A 68,000 d protein reported to be associated with the U1 snRNP (White and Hoch, 1981; Hinterberger, et al., 1983, Kinlaw, et al., 1983) has not been visualized in these experiments. This protein has been inconsistently and nonquantitatively seen by immunoprecipitation (Kinlaw, et al., 1982). An anti-RNP monoclonal antibody (Billings, et al., 1982) recognizes a doublet near 68,000d. A high molecular weight doublet probably distinct from that one is seen by immunoprecipitation using the anti-RNP serum shown in Figure 17- "chase-RNP." These same peptides are also recognized by certain Sm sera while many anti-RNP sera lack the reactivity (Conner, et al. 1982). The monoclonal antibody of Billings, et al. (1982) also recognizes a smaller peptide which may account in part for its snRNP recognition. The 68,000 d peptide may associate transiently into snRNP particles (Kinlaw, et al., 1982), accounting for the difficulty in reproducibly immunoprecipitating it. Further analysis of this and other high molecular weight antigens will be of great interest in further

elucidating structure and function of snRNPs and other antigen-autoantibody systems.

Several implications may follow from the observation of two step snRNP assembly. The possibility exists that the modifications of the core precursor which convert it into the mature 10S snRNP comprise the addition of regulatory and/or specificity elements. One example of such a core-containing multisubunit species is the highly regulated RNA polymerase complex in *e. coli* (Chamberlin, 1974). By analogy, the addition of several peptides and U1 RNA to the snRNP core precursor may confer regulatory or specificity functions resulting in specific enzymatic activities, the best studied being RNA splicing (Lerner, et al., 1980). Based on their protein compositions (Hinterberger, et al., 1983; Kinlaw, et al., 1983) the non-U1 snRNP particles appear likely to contain a similar protein core. If an RNA-free protein core serves as precursor to all classes of U-RNA containing snRNPs, the addition of other proteins and a specific RNA moiety would be of paramount importance in determining any functional properties of the particles. An intrinsic catalytic activity of the RNA such as described in the tRNA processing species RNase P (Guerrier-Takada, et al., 1983) might explain in part the apparent specificity of only the U1 containing snRNPs for mRNA precursor splicing events (Yang, et al., 1981; Padgett, et al., 1983).

In relation to the autoantibody specificity of SLE and MCTD patients it is of interest that a striking feature which distinguishes the Sm and RNP serologies is their nonexclusive versus exclusive recognition and immunoprecipitation of proteins A and C from among all the snRNP proteins. While Sm sera bind components of the core precursor as well as proteins A and C, RNP sera lack any core particle reactivity (see Figure 17). These

differences in core particle recognition may correlate with differences in SLE and MCTD disease manifestations. Perhaps some unique aspect of the state of these snRNP peptides within the cell relates to their unusual recognition by the immune system in disease states.

A final set of observations is the discovery of a post-translational modification of the C protein and an uncharacterized modification in D protein mobility which occurs in a serum specific fashion. The splitting of D protein which is observed following immunoprecipitation with some, but not other anti-snRNP sera, likely relates to an integral snRNP particle component (Figure 17). This shift in mobility may be the result of the addition or removal of some chemical moiety from the D peptide during the immunoprecipitation process. The nature of the moiety remains to be determined.

The other observed snRNP modification involves a shift in mobility of de novo synthesized C protein as revealed by pulse/chase analysis. While R_f analysis would suggest a modification corresponding to ~1000 d, the possibility remains that, based on charge properties, the modification may be much larger or smaller (Weber and Osborn, 1975). An intriguing aspect of this change in C protein is the observation that based on pulse/chase analysis, the modification appears to occur after the C protein (or at least some fraction of it) has been incorporated into the 10S snRNP particle. In this regard, it is of further interest that some labeled C protein is present in unassembled form following 15 hours of cold chase. Since this form is likely to contain the modification (all C appears to be modified in Figure 16), the possibility exists that the unassembled, labeled C protein in chase extracts is material once contained in the 10S particle. As such, this peptide may be capable of particle dissociations

and reassociations, again reminiscent of a class of factors represented by rho and sigma and their regulatory functions for the core enzyme of RNA polymerase (Chamberlin, 1974). Further understanding of the chemical nature of this modification may help elucidate functionally important snRNP properties.

Figure 16

snRNP assembly as revealed by pulse chase time course. K562 cells were pulse labeled with ^{35}S -methionine for 8 minutes. To chase, the pulse labeled cells were resuspended in medium containing unlabeled methionine. Aliquots were removed at 0, 0.2, 1, 6, and 15 hours of cold chase, snRNPs were extracted and immunoprecipitated using anti-Sm and anti-RNP autoimmune antibodies, as indicated. The two arrows point out a post-translational modification of the C peptide.

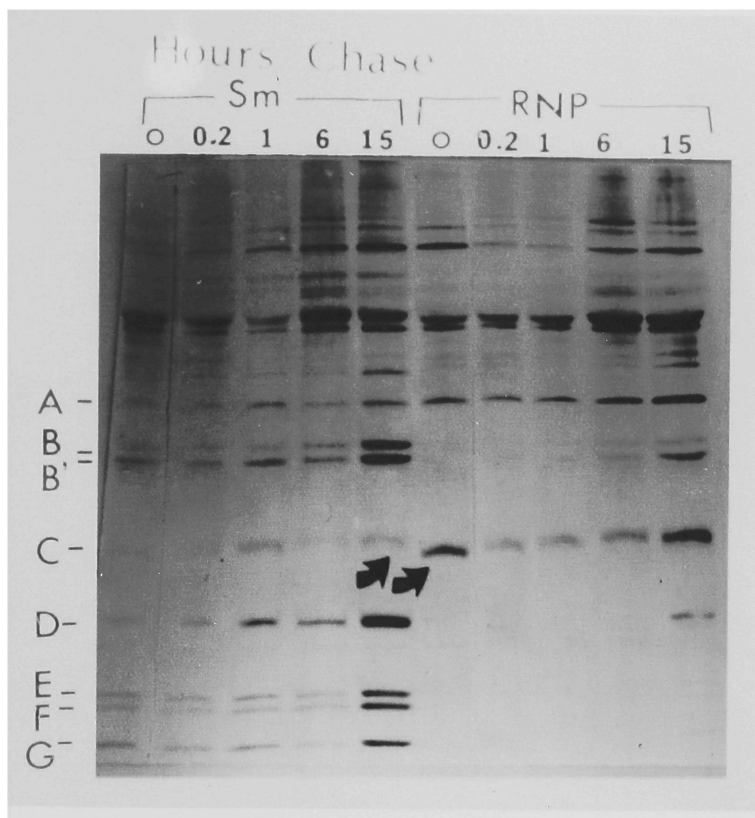
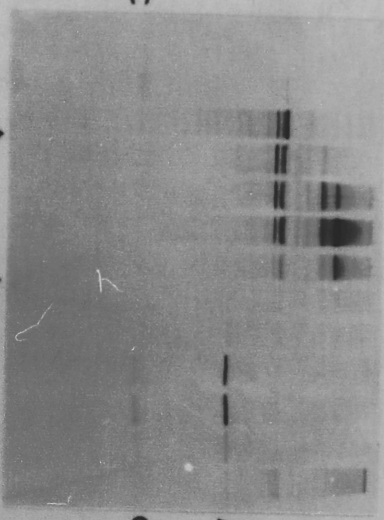


Figure 17

Sucrose gradient analysis of pulse and pulse/chased snRNPs reveals a 2 step assembly process. "Pulse-RNP") 8 minute ^{35}S -methionine pulse labeled K562 cells were extracted and their snRNPs fractionated on a 5-20% sucrose gradient. Fractions were immunoprecipitated using anti-RNP antibodies and protein A sepharose, followed by elution and resolution by SDS-polyacrylamide gel electrophoresis. Standards for the sucrose gradient are human hemoglobin (4.2S), IgG (7S), and catalase (11S). The direction of the gradient (top to bottom) is indicated as left to right. The snRNP peptides A and C are indicated in the margins. "Pulse-Sm") Aliquots of the same fractions used in "Pulse-RNP" were immunoprecipitated using anti-Sm antibodies and analyzed by SDS-polyacrylamide gel electrophoresis. Proteins A-G are identified in the margins. "Chase RNP") 8 minute ^{35}S -methionine pulse labeled K562 cells were subjected to 15 hours cold methionine chase at 37°C . Such cells were extracted and their snRNPs fractionated on a 5-20% sucrose gradient in parallel to the gradients shown in "Pulse-Sm and RNP." Fractions were immunoprecipitated using anti-RNP antibodies and resolved by SDS-polyacrylamide gel electrophoresis. "Chase-Sm") Aliquots of the same fractions used in "Chase-RNP" were immunoprecipitated with anti-Sm antibodies and resolved by SDS-polyacrylamide gel electrophoresis.

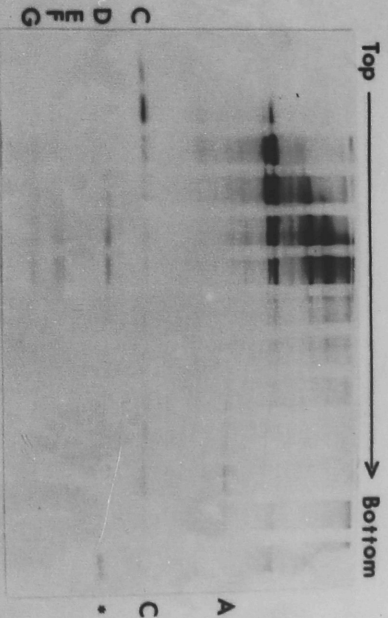
Pulse - RNP → Top Bottom

4.2s 7s 11s



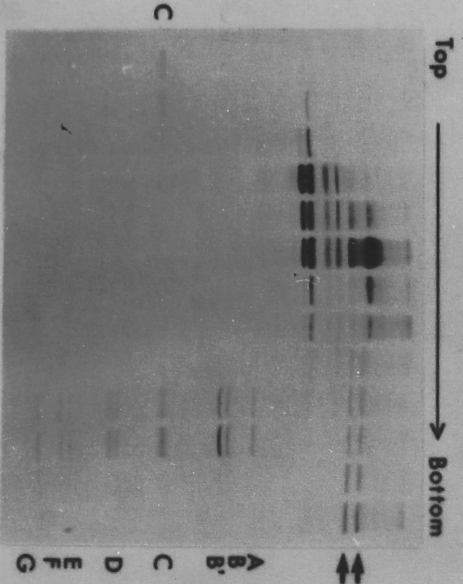
Pulse - Sm → Top Bottom

4.2s 7s 11s



Chase - RNP → Top Bottom

4.2s 7s 11s



Chase - Sm → Top Bottom

4.2s 7s 11s

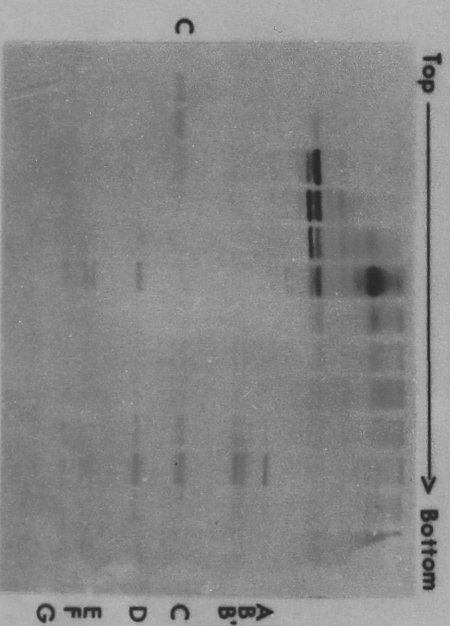


Figure 18

Newly assembled 10S snRNP is localized in the nucleus. Pulsed K562 cells (P) and pulse/chased cells (C) were fractionated by hypotonic lysis (as described in experimental procedures). SnRNPs derived from nuclear (NUC) and cytoplasmic (CYT) fractions were immunoprecipitated with anti-Sm and anti-RNP antibodies as indicated. SnRNP proteins A-G are identified in the left margin.

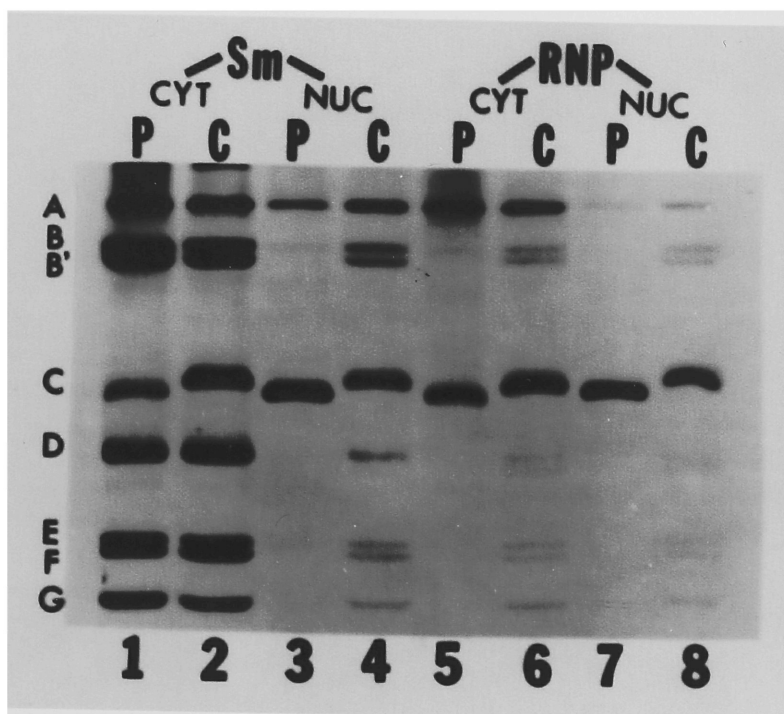


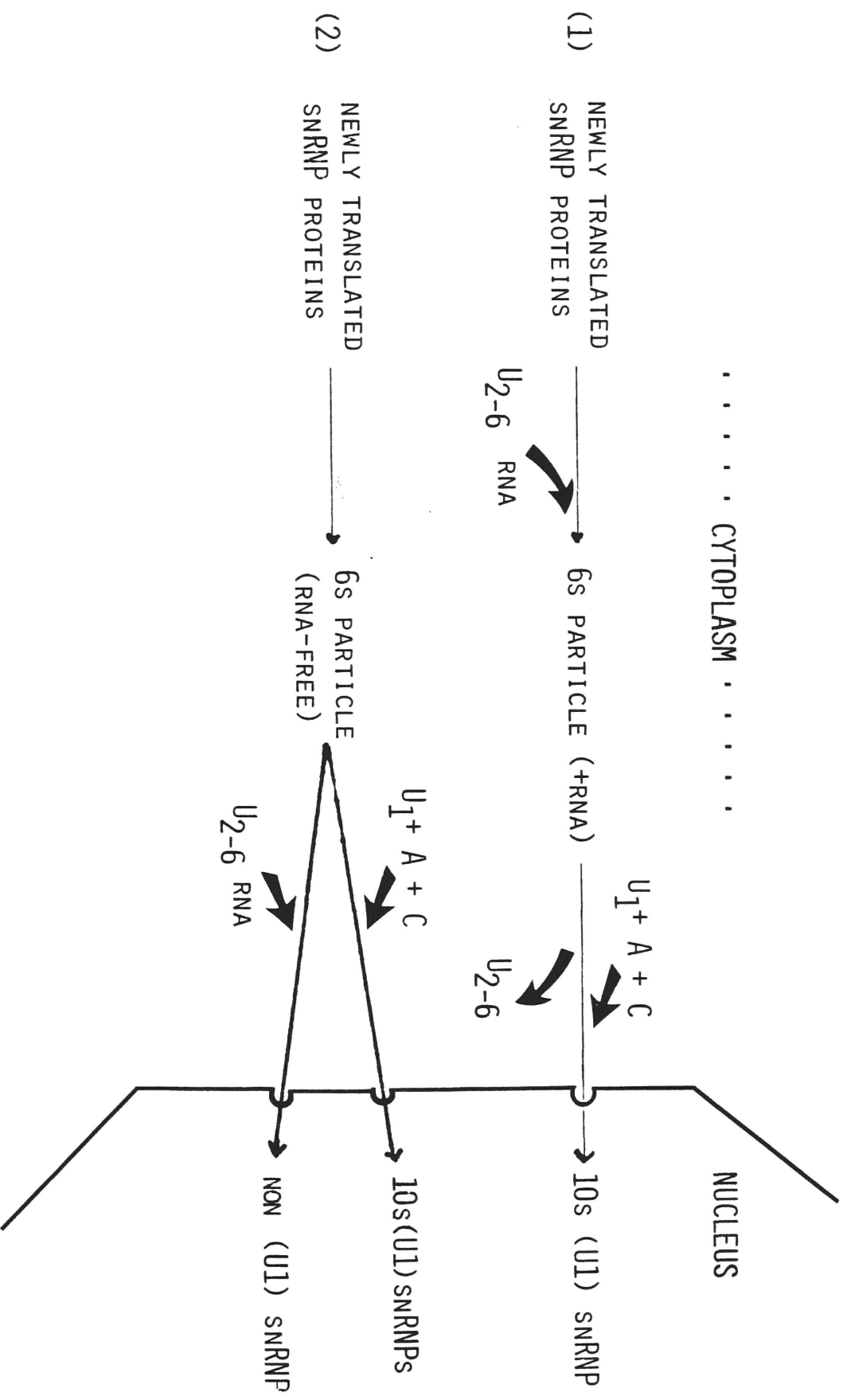
Figure 1^a

Scheme for assembly of the protein constituents of the 10S (U1) snRNP.



Figure 20

Two models for the role of U RNAs in the assembly of small nuclear ribonucleoproteins.



Chapter 5

Towards Isolation of cDNA clones for snRNP Proteins

ABSTRACT

An expression vector library has been constructed in the bacteriophage lambda gt11. RNA was derived from the human cell line 203, which expresses snRNP proteins similarly to other studied cells. The library contains 40% recombinants and contains 1.75×10^6 recombinant members. Preliminary screening using anti-Sm serum of 250,000 such recombinants revealed 44 prospective candidates, nine of which were still positive on secondary screening. Further analysis of these clones may provide isolates of cDNA clones for the snRNP proteins.

RESULTS AND DISCUSSION

A strategy has been devised for isolating cDNA clones encoding the various snRNP proteins. The system of choice was bacteriophage lambda gt11 (Young and Davis 1983). This vector offers the opportunity to screen for the desired clone with antibodies, because the insertion site is within the B-galactosidase gene- an inducible gene in *e. coli*. The method for generating a library and screening is presented in materials and methods, however a brief description will be given here.

Twice poly A⁺ selected RNA from the human tumor cell line 293 (prescreened for snRNP expression by immunoprecipitation), was reverse transcribed followed by second strand synthesis, linker ligation, and ligation into Eco RI cut lambda gt11 DNA. The insert-containing phage DNA was packaged into phage particles followed by infection of *e. coli*. The bacterial strain used for screening contains lac deletions so that there is no background of B-galactosidase synthesis. Plating out a recombinant lambda gt11 library on lac⁻ bacteria in the presence of X-gal generates blue and colorless plaques. The blue plaques retain intact B-galactosidase and likely represent ligation of lambda arms back to each other. The colorless plaques, however, likely represent recombinants in which a cDNA has ligated to the lambda DNA (within the B-galactosidase gene). The cDNA effectively interrupts the B-galactosidase gene preventing its corresponding enzymatic action on the substrate analogue X-gal. The 293 library obtained by these procedures contains 40% colorless plaques (corresponding to 40% recombinants). The library contains a total of 1.75×10^6 such recombinants.

Expression vector screening in lambda gt11 contains several useful

properties relative to other expression vector systems. Being a bacteriophage, one generates plaques by infection rather than transfection (for plasmid vectors). Infection is generally at least an order of magnitude more efficient and thus larger libraries are usually obtained with phage vectors. The lambda gt11 phage in particular has an aspect of its screening designed to circumvent a potential hazard in expression vector cloning. Expression of a hybrid protein (B-galactosidase interrupted by the cDNA) may on occasion be toxic to the infected bacterial cell- preventing the formation of a screenable plaque and effectively leading to loss of that particular clone. To screen lambda gt11, a bacterial strain Y1090 is used, containing a plasmid which expresses the lac Iq gene which acts as repressor for B-galactosidase operon- effectively blocking synthesis of any hybrid (and possibly toxic) proteins. After plaques have grown for several hours, nitrocellulose filters presoaked in IPTG are overlaid onto the plate. IPTG binds the repressor, leading to derepression, or expression of B-galactosidase plus any inserts. After several hours of further incubation, any hybrid protein should have been synthesized and some should be bound in replica form to the nitrocellulose filter. The filter is then screened with antibody and 125 I-protein A, and any positive plaques are further studied.

Screening was carried out for approximately 250,000 colorless plaques. Forty four areas were picked from primary screens and following secondary screening nine possibilities remain. The very low number of prospective positives likely relates to the fact that only one out of six recombinants could be expected to have ligated in both the correct reading frame and orientation for proper expression. An additional concern in relation to the snRNP proteins, is that antigenicity of most of these peptides is quite

sensitive to denaturation (most snRNP peptides are not seen on Western blots- using these same anti-Sm or RNP sera). While not complete, these studies may provide first steps towards isolation of cDNA clones of snRNP proteins.

Chapter 6

Summary and discussion: SLE and autoimmunity- a proposal

Analysis of the antigens recognized by the Sm and RNP sera seen in autoimmune disease have suggested the following. These small nuclear ribonucleoprotein particles consist of two classes- one containing U1 RNA in association with 8 proteins (A-G), the other lacking proteins A and C while presumably containing U2, U4, U5, and U6 RNAs. Only the U1 snRNP is recognized by anti-RNP serum antibodies, because these antibodies recognize snRNP proteins A and C only. Sm sera react with both snRNP particle classes due to the ability of these antibodies to recognize each protein component (A-G). Recognition of the A protein is sensitive to RNase treatment for both Sm and RNP sera. Two patients have been observed to undergo a change in serum antibody from Sm towards RNP-like recognition over the course of time.

Use of the antibody reagents has been of considerable utility in determining aspects of snRNP biochemistry and assembly. Each snRNP protein was observed to be the product of translation from a unique mRNA species. Pulse-chase analysis revealed assembly of U1 (10S) snRNPs to be a 2 step process involving prior formation of a 6S core particle, followed by the addition of A and C proteins. These experiments also revealed the presence

of one and possibly a second post-translational modification of snRNP proteins C and D (respectively). The modification of C protein follows (kinetically) assembly of this species onto the 10S particle. The second modification is observed by the action of a serum component on the C protein and remains undefined at present.

A series of apparent coincidences among these results may bear mention. Specifically, the unique metabolism as well as immunologic recognition of A and C proteins is perhaps of broader significance. These proteins are uniquely added in the second step of U1 snRNP assembly, but are also uniquely (and exclusively) recognized by anti-RNP antibodies as well as antibodies in certain Sm patients who have switched to a RNP-like pattern of recognition. Furthermore one of these two peptides (D protein) is the only snRNP protein recoverable from cells in a detectable unassembled form. The other species (A protein) uniquely requires U1 RNA for antigenicity. As such unassembled A protein is not likely to have been detected even if present. Perhaps metabolic aspects of the handling of these peptides are related to either their presentation or immunologic recognition in autoimmune disease.

* * *

The following is a proposal which relates immunologic recognition to the problem of autoimmunity. While not dealing specifically with any antigen systems in disease, these ideas may be of some interest on a wider scale.

The two observations which serve as the starting point for this proposal are:

1) the vastly disproportionate occurrence of SLE in females over males (~9:1), as well as frequent observations of X-chromosome abnormalities in afflicted males (e.g. Klinefelter's Syndrome, Stern, et al. 1977).

2) several reports of increased HLA-locus compatibility (or identity) between husbands and wives in association with high incidences of spontaneous abortion; that is, sharing of these transplantation antigens is associated with maternal difficulty in successfully bringing a pregnancy to term (Faulk and McIntyre 1981; Komlos, et al., 1977; Beer, et al., 1981; Gerencer, et al., 1979).

While a relationship between these two observations is not overtly apparent, an attempt to draw one will be made here, beginning with the second observation. The maternal-fetal immunologic relationship has long been problematic for immunologists because a fetal "graft," in contrast to other allografts (e.g. skin, kidney) clearly survives despite histoincompatibility. The fact that identical rejection phenomena are not occurring during normal pregnancy is clear, since the vast majority of husband-wife combinations (couples) and maternal-fetal combinations are HLA non-identical. The above observation (#2) would suggest, in fact, that this incompatibility is advantageous vis a vis the maternal-fetal relationship. While seemingly anomalous, analysis of the components of this recognition may perhaps reveal how such a mechanism could have evolved.

Major histocompatibility gene complexes (H-2 in the mouse, HLA in man) were historically appreciated because of properties of allo-recognition.

That is, graft rejection phenomena appeared to involve strong recognition/responses to polymorphisms in proteins encoded at these loci. The striking observation (in the absence of grafts) that polymorphisms of certain regions within these loci correlate with the ability to mount an immune response to a given haptene, gave rise to the notion that "immune response" genes reside as well within these H-2 and HLA loci. Certain allotypes of these immune response gene products (now known as class II histocompatibility antigens or Ia in mouse and Dr in man) were shown to endow an animal with the ability to respond immunologically to certain antigens. But "holes" in immune response potential to certain other defined antigens could be observed if the animal lacked another (different) Ia allotype- one which was associated with ability to respond to certain other antigens. These experiments suggested that diversity of immune response was in some way related to diversity of Ia genes- or Dr in man. Studies of antigen recognition by T lymphocytes revealed the ability (in fact, the necessity) of T cells to simultaneously recognize H-2 (or HLA) in association with antigen on antigen presenting cells (see chapter 8). It would seem reasonable then, to imagine that this Ia or Dr restriction in immune response may be effected biologically at the level of T cell recognition of antigen (+ H-2/Dr). Furthermore, heterozygosity of the two alleles for these immune response genes would be expected to provide a selective advantage relative to homozygosity, because increased diversity of immune recognition correlates with wider spectrum of antigen recognition.

If maternal-fetal recognition were to exist such that diversity of H-2 and HLA loci were selected for, one would predict that a population capable of such "diversity-selection" could have a selective advantage over a

population lacking such recognition. In this regard then, the observation that spontaneous, recurrent abortions are frequently correlated with HLA identity between husband and wife may simply reveal the presence (and effects) of such a "diversity-selection" mechanism.

Further support for the possibility that such recognition may operate in the maternal-fetal relationship comes from reports (though mostly anecdotal) of difficulties encountered during animal inbreeding. These difficulties appear to involve low reproductive efficiency of animals when bred for genetic identity. A further implication of this hypothesis, if correct, would be that females in established inbred strains of animals may have lost or mutated the gene(s) responsible for this "diversity-selection" in the process of inbreeding. Therefore inbred strains of animals (generated frequently to test allorecognition) may contain a (paradoxically, selected for) defect in some aspect of their allorecognition.

This concept of allo vs. self recognition in the maternal fetal relationship carries with it a property uniquely critical to females. Maternal recognition of a genetically identical fetus (appearing as "self"), then, may result in an immunologically mediated reaction to self (or "nonreaction to self") resulting in spontaneous abortion because of failure to support such a fetus or due to specific mechanisms which destroy it. Such properties might well be X-chromosome encoded. What has been described, then, amounts essentially to a female specific recognition of "self" with subsequent destruction of "self." Such a description bears remarkable similarity to the scenario encountered in autoimmune disease--particularly diseases such as SLE which are characterized by female preponderance. Considerable additional evidence has implicated X-

chromosomal genes in immunologically critical activities, as seen, for example, in X-linked immunodeficiency disease of man. In summary, then, this hypothesis would suggest that an immunologically mediated mechanism of "self" recognition normally present in females may have gone awry in certain autoimmune disease.

While little has been done to test this idea, one recent report (Schur and Carpenter, 1983) contributes very useful data (without having addressed this particular issue). In this study of HLA haplotype (A and B loci only) correlations in SLE, the only significant observation related to the HLA types of parents of SLE patients. Of 70 normal parental pairs (controls), only ~4% shared HLA A, B haplotypes. Of 35 SLE parental pairs (35 pairs of SLE patient parents), 7 shared A,B haplotype. Thus 20% sharing was observed in SLE parents as compared to 4% for normals. Given the known genetic influence in acquisition of lupus, this shared haplotype phenomenon may reflect aberrant fetal recognition (at some level) by the lupus mothers. That is normal couples with shared haplotypes may have had less success in producing viable offspring. A second observation which may in some manner relate to the above hypothesis involves the observations (Mund, et al., 1963) of pregnancy's profound influence over the course of disease activity in lupus patients.

Further analysis of genetic loci possibly involved in such a recognition process may in the future be amenable to study by use of restriction fragment length polymorphisms (RFLP) technology (Botstein, et al., 1980).

If "diversity-selection" is, in fact a real biological phenomenon, its biochemical basis would be expected to reside at the level of T lymphocyte recognition of antigen. Delineation of structures possibly involved in

this antigen recognition is the topic of discussion for part two of this thesis.

Part II

Chapter 7

Introduction

Elucidation of structural properties of the T lymphocyte receptor for antigen has represented one of the most profoundly elusive problems in modern immunology. The characterization of numerous functional properties for T cell subpopulations has helped establish the exquisite antigen specificity of these cells. The dual nature of T cell antigen recognition by T lymphocytes was established by the discoveries that antigen recognition occurs specifically in the context of products of the major histocompatibility complex (MHC) (Benacerraf and McDevitt 1972; Zinkernagel and Doherty 1975; Schlossman 1972). This dual specificity of T cells for both antigen and MHC gave rise to two theories which may account for T cell receptor recognition (Zinkernagel 1978). A first model proposes the existence of two distinct receptors: one specific for antigen and another specific for MHC. Presumably appropriate binding to both receptors would serve as antigen recognition. A second model involves the notion of a single receptor which recognizes a single "antigen" which itself contains determinants derived from both MHC and real antigen.

The determination of antigen reactivity by T cells has been studied in vitro by incubation of the T cells with antigen and appropriate accessory

cells. Stimulation by antigen has been measured by ^3H -thymidine incorporation, IL-2 secretion, as well as biological function. The critical role of accessory cells in helping to generate the T cell antigenic response has been established for some time (Hersch and Harris 1968; Cline and Sweet 1968). Numerous studies have argued conclusively for the role of adherent mononuclear cells in this activity (Seeger and Oppenheim 1970; Rosenwasser and Rosenthal 1978). The prevailing notion that the monocyte/macrophage population is primarily responsible for such T cell activation phenomena has recently come into serious question with the reports of highly potent stimulating capacity for dendritic cells (Steinman and Nussenzweig 1980) of mouse (Steinman, et al., 1983; Sunshine, et al., 1983) and man (Crow and Kunkel 1982; Van Voorhis, et al., 1983). The relatively weak stimulating capacity of purified monocytes (Steinman and Nussenzweig 1980) has argued more conclusively for the primary role of dendritic cells in this T cell stimulation.

Use of an in vitro T cell antigen specific system has recently been made to provide strong evidence for the "one receptor" theory of antigen recognition. In these experiments (Kappler, et al., 1981) hybridomas were derived from two T cells each with specificity for a certain antigen and a corresponding MHC type. Resulting hybrids were unable to respond to one antigen plus the other MHC type, but could only respond to the same combinations of antigen plus MHC as the cells used for fusion. This result argued strongly for the likelihood of a single T cell receptor for antigen plus MHC.

Considerable attention has been paid to the possibility that the T cell receptor structure may utilize gene products in common with immunoglobulin. Specifically, the use of anti-immunoglobulin antibodies

appeared to recognize T cell structures which may relate to antigen recognition (Binz, et al., 1976). This and numerous other reports suggesting immunoglobulin characteristics of the T cell receptor were not, however, generalizeable and as such did not lead conclusively to determination of a receptor structure. More recently several laboratories provided evidence for the possibility that idiotypic structures contained in the T cell receptor may be derived from the same gene segments (V_H) as in the case of antibody heavy chain (Binz and Wigzell 1977; Rajewsky and Eichmann 1977; Krammer 1981). Other groups (Hammerling, et al., 1976; Bach, et al., 1979; Mozes and Haimovich 1979; Suzan, et al., 1981; Owen, et al., 1981) described genetic linkage of T cell antigen recognition structures to the immunoglobulin heavy chain locus. Owen, et al. (1979, 1981) furthermore mapped the putative recognition structures to the mouse chromosome 12 between the Igh-1 locus (encoding constant region genes of immunoglobulin heavy chains) and the prealbumin gene. Similar alloantigenic molecules were observed as secreted T cell products (Taniguchi, et al., 1982; Tokuhisa and Taniguchi 1982). A number of studies, however, have demonstrated lack of shared gene products for immunoglobulin and the T cell receptor for antigen. Immunoglobulin joining and constant region gene transcripts could not be detected in T helper or killer cells (Kronenberg, et al., 1980). Furthermore, the use of gene probes derived from V_H gene segments and presumed to be capable of very wide recognition of V_H genes failed to detect homologous transcripts in T cells (Kronenberg, et al., 1983) even when V_H probes were derived from B cells with the same antigen specificity as the T cells (Kraig, et al., 1983). Thus, although the possibility remains that shared determinants may exist for immunoglobulin and the T cell receptor, sharing of actual gene

segments appears to be unlikely.

Considerable advances have recently been made in the understanding of T lymphocyte populations by application of the monoclonal antibody technique (Kohler and Milstein 1975) to the study of T cell surface proteins. Among the more important such markers in humans are the molecules recognized by the antibodies OKT³, T4, and T8 (Reinherz, et al., 1983). T4⁺ T cells appear to recognize class II MHC determinants while T8⁺ cells recognize class I MHC structures (and the cells bearing these markers are mostly helper and cytotoxic cells, respectively). The T³ molecule appears to reside on all mature T cells and has been implicated as important in antigen recognition by virtue of the ability of antibody against this structure to block antigen specific T cell activation. The considerable specificity of monoclonal antibodies directed against T lymphocytes may be of further use in delineating important structures in antigen recognition. It is the use of this methodology which forms the basis of the analyses of the T cell receptor which follow.

Chapter 9

T Cell Receptor Structures on Two T Cell Leukemias

ABSTRACT

Monoclonal antibodies were obtained that displayed unique recognition of a surface protein epitope on each of two T cell leukemias. These reactivities resemble anti-idiotypic antibodies in the specificity of their recognition. For each T cell leukemia, a second monoclonal antibody was obtained which recognized the same molecule as the idiotype-specific antibodies. This second set of monoclonals, however, was capable of staining a certain fraction of normal T cells, suggesting the recognition of a cross-reactive or constant region determinant. The molecules identified in these studies are both disulfide-linked heterodimers. One is 70-75 kd unreduced, and contains a crossreactive determinant with ~2% of normal T cells. The other is 80-90 kd unreduced and contains a determinant present on 30-50% of normal T cells. By virtue of immunochemical properties of these surface proteins, as well as biological effects of the antibodies directed against them, these molecules would appear to be strong candidates for the T lymphocyte antigen receptor.

REAGENTS

The strategy for identification of the T lymphocyte receptor for antigen relies on analogy between this putative structure and the B cell antigen receptor: immunoglobulin. A clone of B cells would likely share antibody idiotypic determinants with no other cells. Antibodies directed against constant region domains of immunoglobulin would, however, be likely to recognize other B lymphocytes as well.

The antibody reagents used in these studies were mouse monoclonal antibodies generated by Dr. Robert Rigler and Dr. David Posnett by immunization of mice with leukemic cells from two T cell leukemia patients.

Patient Su

T lymphocytes from this patient were of the Sezary type. Leukemic cells displayed the phenotype: E rosette +, OKT3+, OKT4+, OKT8-, Ia-, SIg-. Following immunization and fusion, two hybridomas of interest were obtained. Surface staining, as measured by indirect immunofluorescence, revealed one of these antibodies (called SI60) to stain exclusively the immunizing T leukemia cells. Cells failing to show any staining included: autologous and allogeneic normal B and T cells, other T leukemias, myeloid cells, B and T cell lines, activated normal B and T cells. Thus the SI60 antibody appears to be entirely specific for this leukemia cell.

A second monoclonal antibody (called SV11) displayed nearly identical staining patterns, with the important distinction that approximately 2% of normal resting T lymphocytes stained strongly with this antibody. The SV11 antibody therefore recognizes a determinant shared by the leukemic clone of T cells with a small percentage of normal T lymphocytes, but with no other identified cells.

Patient Fi

The immunizing leukemic cells were derived from a T cell chronic lymphocytic leukemia. Phenotypically the cells were E rosette +, OKT3+, OKT4+, OKT8-, Ia-, Tac+, sIg-. From the resulting hybridomas, one (termed FF26) exhibited exclusive staining of the immunizing cells. All other cell populations examined including autologous and allogeneic leukocytes did not stain with FF26. This antibody, therefore, appeared to display complete specificity for the leukemic cell clone. A second monoclonal, termed FFB3, reacted both with the leukemic cells and 30-50% of normal T cells, as well as with several T cell lines. A second recently obtained antibody (FFB3) appears to recognize 30-50% of stimulated normal T cells as well as the immunizing leukemic cells and is discussed below.

RESULTS

Analysis of the structures against which these various hybridomas were directed was carried out by solubilization of the appropriate surface iodinated leukemic cells followed by immunoprecipitation. These results are shown for the SJ60 and SV11 antibodies in Figure 21. Using Su cells, lanes 1 and 5 show unreduced forms of SJ60 and SV11 immunoprecipitates, respectively. A 70,000 d heavily labeled protein appears for both antibodies. Upon reduction with dithiothreitol (lanes 3 and 7), the 70,000 d protein is not seen, but two smaller peptides appear for both antibodies. The smaller component is about 38,000 d and much more heavily labeled than the slightly larger 43,000 d species. Analysis of immunoprecipitates using these same antibodies, but with different cell extracts (T cell line KE37) are shown in odd numbered lanes in Figure 21. In parallel to the staining patterns for these hybridomas, no antigenic peptides are visibly

immunoprecipitated from this T cell source.

Further analysis of the disulfide reduction property of the protein recognized by these two monoclonals was carried out by cutting out the 70,000 d (unreduced) band from a first gel, rehydrating in buffer containing dithiothreitol, and electroeluting into a second SDS-polyacrylamide gel. Figure 22 shows the 70,000 d and two lower bands in lanes 1-3, rehydrated in the absence of reducing agent. However in the presence of reducing agent (lanes 4-6) the 70,000 d band is directly converted into the two lower molecular weight bands. It is of some relevance that the 38,000 d band has uniformly appeared much more heavily labeled than the 43,000 d species. These differences may arise due to differing numbers of iodinated tyrosine residues, or incomplete iodination due to membrane properties of the cell. Analysis by 2-dimensional gel electrophoresis (performed by Dr. C. Y. Wang) has revealed microheterogeneity for both of these peptides, consistent with glycosylation patterns.

Numerous attempts have been made to use these two monoclonal antibodies as aids in obtaining cDNA clones for the molecule under study. RNA extractions were carried out both by NP-40 lysis and phenol extractions (to obtain cytoplasmic RNA) and guanidinium thiocyanate (yielding total cellular RNA). One difficulty in working with these leukemic cells is their extraordinarily small size and their low yield of RNA upon extraction. The similar RNA yields obtained using guanidinium salt extractions argues against degradation as the cause of low RNA yields. Furthermore, oligo dT cellulose chromatography led to the isolation of under 2 ug of poly A+ RNA from 4 mg of total RNA of the Su cells. Therefore, a very powerful cDNA cloning system was needed given such a

small quantity of starting material. Using the lambda gt10 vector a cDNA library was constructed from Su poly A+ RNA which contained approximately 200,000 members.

Parallel experiments were performed to determine a mannerⁱⁿ which one of the monoclonal antibodies might be useful in identifying clones of interest. Attempts at immunoblots (Western blots) failed to display antibody recognition- likely suggesting considerable detergent sensitivity of the antigenic determinants. Immunoprecipitation of Su cell extracts which were previously reduced and alkylated (using iodoacetamide) also failed to reveal antigen recognition by these monoclonals. A realistic conclusion from these studies suggests the likelihood that determinants recognized by both monoclonal antibodies in some way require intact linkage of the two chains together, for successful antibody recognition. Such recognition behavior would be similarly sensitive to reduction and denaturation in the case of antibodies directed against idiotypic determinants on immunoglobulin. Inability to immunoprecipitate Su RNA in vitro translation products using these antibodies further supports this notion (data not shown).

It therefore became evident that more broadly reactive antibodies to the same molecule would be of greater aid for further molecular biological study.

To that end, a strategy was devised to prepare a rabbit antiserum directed against the same molecule. Unlabeled Su leukemic cell lysate was immunoprecipitated onto protein A sepharose beads which were washed and immunized directly in Complete Freund's Adjuvant into two rabbits intraperitoneally. Following 2 months of weekly immunizations, sera were tested for reactivity by immunoprecipitation but failed to reveal any

reactive antibodies. Immunizations were then continued using immune complexes (as above) this time adsorbed onto formalin fixed staph aureus organisms. Following 3 months of further immunization, sera were obtained and used to immunoprecipitate ^{125}I -labeled Su cell extracts, as shown in Figure 23. Patterns are seen both for the SV11 monoclonal antibody (positive control) as well as for the rabbit immunoprecipitates, reduced and unreduced. Clear evidence exists for appearance of the identical protein precipitates, recognized by the rabbit antibodies.

A second monoclonal T cell system was amenable to similar analysis, with the development of monoclonal antibody FF26, reactive with malignant T lymphocytes from patient Fi. Immunoprecipitation of ^{125}I -labeled Fi extracts with FF26 antibody was carried out alongside immunoprecipitates of Su cells using SV11 antibody. As seen in Figure 24 lanes 1 and 2, the FF26 antibody also recognizes a disulfide linked heterodimer. However, several clear properties distinguish this molecule from the pattern obtained by SV11 antibody and Su extract (lanes 3 and 4). The unreduced protein from Fi migrates at about 90,000 d, compared to ~70,000 d for Su. The two reduced peptides migrate at approximately 52 and 43,000 d as compared to 43 and 38,000 d for Su. Both molecules appear to contain a heavily labeled and a lightly labeled component (see reduced forms, lanes 3 and 4). The lightly labeled peptides appear to comigrate indistinguishably for the two cell sources, while the heavily labeled peptides differ considerably in migration. A second monoclonal antibody, FFB3, has recently been obtained which shows reactivity with not only the immunizing T leukemia cells, but also 30-50% of normal T cells and several T cell lines. Sequential immunoprecipitation has indicated (data not shown) that FFB3 recognizes the same molecule as the idiotype-specific antibody, FF26.

Towards eventual isolation of cDNA clones for the proteins studied in Fi cells RNA was extracted and a cDNA library consisting of approximately 100,000 members was constructed in lambda gt10.

Use of T cell-specific cDNA probe

The use of monoclonal antibodies as probes for T cell specific membrane markers has a theoretical parallel utilizing techniques of recombinant DNA. Specifically, the generation of a T-cell "specific" probe would be expected to identify cDNA clones unique to T cells and, perhaps, including the gene(s) encoding the receptor for antigen.

Attempts at such a strategy have been made utilizing the human T cell line HUT-102 from which a 250,000 member cDNA library was generated. The strategy for gene identification involved first generating a T cell specific cDNA probe (by hybrid depletion) and screening of the HUT library for T cell-specific genes. Putative candidates would be screened by Southern blot analysis for the presence of a gene rearrangement by analogy with observations in the immunoglobulin genes of B cells.

Generation of T cell-specific probe employed the use of T cell poly A+ RNA and B cell line (IM-9, kindly provided by Dr. Nicholas Chiorazzi) poly A+ RNA. HUT ³²P-cDNA was hybridized to IM-9 poly A+ RNA in vast excess. Single stranded and double stranded nucleic acids were then separated by use of hydroxyl apatite chromatography. In the presence of 0.1 M phosphate buffer, only double stranded nucleic acids bind to the column. Single stranded nucleic acids (and presumably T cell-specific cDNAs) are thus separable in this step, from double stranded forms, which can be eluted with 0.4 M phosphate. The relative proportions found in single and double-stranded forms are quantitated by counting the various fractions. An index of completion for such nucleic acid hybridization reactions is the

$R_0 t$ value, a number reflecting the influences of both concentration and time on degree of hybridization. In brief, the relationship of $R_0 t$ to completion of the hybridization reaction is given in the following derivation.

For the hybridization reaction: (* connotes ^{32}P -labeled)



If $R = [\text{mRNA}]$ and $C = [\text{cDNA}^*]$ and $R \gg C$ (reaction is run in vast excess), then the rate of this reaction is given by pseudofirst order kinetics and rate of disappearance of cDNA with time is:

$$-(dC/dt) = KRC.$$

K represents a pseudofirst order rate constant which depends on complexity and may (in theory) vary among different cell types. Rearranging the above equation gives:

$$-(dC/C) = KRTdt$$

which may be integrated between times t_0 to t and for cDNA concentrations C_0 to C_t to obtain:

$$\ln(C_0/C_t) = KRT.$$

Rearrangement of this expression gives:

$$C_t/C_0 = e^{-KRT}.$$

Since $R \gg C$, R_0 is approximately equal to R_t . Therefore the equation describing the rate of disappearance of single stranded cDNA probe shows the hybridization to be a function of the two variables R_0 (or B cell A+ RNA concentration) and t (time of hybridization). In the case of HeLa cell poly A+RNA (hybridizing to its own cDNA), hybridization to a $R_0 t$ of 100 ((mol.s)/l) gave approximately 90% S1 nuclease resistant (i.e. double-

stranded) cDNA-RNA hybrids (Bishop, et al. 1974). Hybridization of HUT cDNA with IM-9 A+ RNA was therefore carried out to a R_0t of approximately 2800 (with the purpose of driving the reaction as far as possible). However, Northern blot analysis of this select probe revealed very extensive hybridization to B cell RNA. Further B-T hybridizations were therefore carried out to determine conditions under which a cleaner (more T cell specific) cDNA probe could be generated. Figure 25 shows the proportions of single and double stranded cDNAs following sequential hybridizations to B cell RNA. These results indicated that two rounds of hybridization ($R_0t=500$) left cDNA, only 2% of which was capable of forming hydroxy appetite separable duplexes following a third round of hybridization. Therefore, more cDNA specific probe was generated by 2 rounds of hybridization to IM-9 RNA. Northern blot analysis of this probe appears in Figure 26 and shows considerable enrichment of T cell recognition over B cell. Reactivity with certain specific B cell RNA species remained, and is presumed to represent a population of probe species not amenable to removal by this method.

Such twice-hybridized T cell-select cDNA probe was then used to screen 15,000 clones from the HUT 102 lambda gt11 cDNA library. 245 plaques showed strong binding to this probe representing 1.6% of the clones. An example of one filter showing about 20 positive clones is seen in Figure 27. This same filter was a replica of a plate containing approximately 500 total plaques (thus ~4% of plaques were positive on this plate). Further analysis of these putative T cell specific clones required that they first be plaque-purified (that is, purified by 2^0 screening away from neighboring, contaminating phage). Having purified approximately 50 of these candidates, the first 5 were amplified and tested on Northern blots

for their T cell specificity. These particular Northern blots (Figure 28) contained total RNA from both B (IM-9) and T (HUT 102) cell sources. The unexpected result seen in 3 out of 5 clones tested revealed a heterogeneous smear of hybridization particularly strong in the T cell lane and identification of a small RNA species in both B and T cell RNAs (probes 3, 4, and 5). Intactness of the RNAs in these blots was verified by rehybridization of one of the same blots (after washing) with a separate probe (Figure 29) for a unique sequence gene (provided by Dr. Hung Teh Kao). The suspicion that these unusual hybridization patterns may reflect the presence of repetitive sequences in the probe was tested by hybridization to one of the same blots (after washing and reexposure to ascertain removal of the 1st probe) of ³²P-labeled nick translated human genomic DNA (derived from the Raji cell line). The result, seen in Figure 30, reveals the identical pattern of hybridization, highly suggestive of a pattern for repeat sequences within the probe. The small RNA band likely represents the small RNA 7S which has been shown to contain Alu-type repetitive sequences (Reddy and Busch 1983).

The presence of repetitive sequences among such putative T cell specific clones may be the result of a high level of expression in HUT 102 of mRNAs containing repeat sequences. As such, the increased expression of such repeat-containing genes (relative to IM-9 cells) may perhaps be of relevance to functional properties of this class of sequences. Alternatively this expression pattern may represent a relic of transpositional activity at some stage in the derivation of the HUT 102 cell line and be of little functional significance at present. Methods could be devised (in principle) to overcome this problem. For example, random sequence primers for the ³²P-cDNA probe synthesis could be used in

conjunction with boiling to break the probe into small pieces. Such a mixture should contain representations of all the mRNA sequences, but on numerous small pieces. A hybridization reaction with genomic DNA should remove all fragments containing repetitive sequences, while leaving unique sequence-specific fragments. Such a procedure, however, while theoretically feasible, would require synthesis of an enormous quantity of 32 P-labeled cDNA starting material- an exceedingly difficult task using random cDNA primers. For these reasons, a better approach would likely involve selection of a different T cell source of RNA- particularly if one can be found which lacks the high level of repeat sequence expression as in HUT-102.

DISCUSSION

Several monoclonal antibodies have been described which appear likely to recognize structures with characteristics in common with a putative T cell antigen receptor. Although no direct evidence has been obtained for the notion that the identified structure is an antigen receptor, considerable indirect evidence supports this notion. Antibodies directed against similar disulfide linked heterodimers have been shown to block specific activities in both mouse and cytotoxic human T cell antigen specific systems (Meuer, et al., 1983; Haskins, et al., 1983). A co-modulation phenomenon has also been described (Reinherz, et al., 1982) in which the T cell receptor complex is postulated to contain several elements, one of which is the peptide recognized by the OKT3 monoclonal antibody. In modulation experiments, a given monoclonal antibody is bound to target cells, which are then incubated for 12 hours at 37°C. Upon restaining at that time, the percent positive cells may decrease for a second antibody,

if its target surface antigen has "co-modulated" off the cell surface together with the target for the first antibody. Co-modulation is in some sense considered an index of associations between different proteins in a cell membrane. By these analyses, the SI60 and SV11 antibodies as well as FF26 have all been observed to co-modulate with the OX40 molecule (data not shown), further supporting the possibility that these monoclonal antibodies recognize molecules important in T cell antigen recognition.

Perhaps the most compelling evidence that the targets for these monoclonals are T cell receptor molecules, is the idiotype-like qualities demonstrated by the antibodies. The unique recognition of only a single clone of T cells (as seen with SI60 and FF26) is highly reminiscent of patterns obtained by similar analysis of anti-idiotypic reagents on monoclonal B cell sources. If antigen recognition requires diversity-encoding elements which differ depending on antigen specificity, then the presence of idiotypic determinants would be a prerequisite for the expression of such diversity.

Several other groups have described similar monoclonal antibody systems reactive with T cells in idiotype-like fashion, both in mouse (Allison, et al., 1982; Haskins, et al., 1983) and humans (Meuer, et al., 1983). While the recognized molecules have been identified as disulfide linked heterodimers, the sizes of previously reported molecules have all been similar to the antigen recognized by FF26. The Su molecule appears somewhat smaller than the others, and may correspondingly represent a different isotype for the T cell receptor than has been recognized in the other systems. Particularly striking evidence that the SV11 and FFB2 recognized molecules are antigen receptors relates to the fact that the molecules appear to contain both unique and shared determinants- analogous

to variable and constant domains of immunoglobulin.

The shared SV11 determinant demonstrated by binding to normal T cells of SV11 antibody, is relatively rare among T cells (~2%). It should be noted however, that antibodies directed against constant determinants of (for example) IgA or IgE would similarly recognize only small populations of circulating B lymphocytes. The SV11 antibody, then, may recognize a relatively rare isotype (or "class") of T cell receptor molecule. An alternative type of recognition which may be operating for the SV11 monoclonal is cross-reactive idiotype, wherein a shared determinant is recognized from among variable regions of (isotypically) different molecules. If this were the case, then an antiserum such as that obtained by the rabbit immunizations (see Figure 23) should provide a vehicle for the examination of constant region determinants. Antibodies present in this same serum may as well be of further help in recognition of primary translation products for the various chains, and as such may assist in identifying cDNA clones encoding the proteins of interest. Analysis of structural differences among the molecules on normal T cells reactive with SV11 antibody will be carried out when monoclonal cell lines derived from that population can be expanded appropriately. At that stage, peptide mapping and further serologic studies may reveal more information regarding variable and constant domains of the T cell receptor.

The cross reactive determinant recognized by the FFB3 monoclonal antibody is present on a large population of normal T cells (30-50%). If this recognition involves a distinct isotype, it would be a relatively common isotype of T cells (analogous to IgM or IgG isotypes among B cells). An intriguing recent result is the observation that an 80 kd protein which comigrated with this Fi idiotypic molecule was identified by Western

blotting using a monoclonal anti-HLA antibody. As with the SI60 and SV11 antibodies, FF26 and FFB3 display no recognition on Western blots- presumably due to SDS-denaturation. This HLA monoclonal antibody, however, reproducibly identifies the 45 kd HLA heavy chain by Western blotting (or immunoprecipitation). When this antibody is used to probe a Western blot containing Fi leukemia cell lysate run unreduced, an 80 kd peptide is visualized (as well as the 45 kd HLA). The higher band disappears upon reduction- as predicted for the idiotypic molecule. One possible explanation for these results is that perhaps the smaller peptide in the Fi idiotype molecule is HLA heavy chain. This appears to be unlikely, however, because immunoprecipitation with numerous anti-HLA antibodies including polyclonal rabbit antibodies never resulted in identification of the Fi molecule. A second, more likely, alternative is the possibility that this idiotype molecule contains a determinant in common with HLA, but one which is not normally exposed in the molecule. SDS denaturation may expose the determinant on Western blots. Such homology to HLA would not be unprecedented among surface molecules in the immune system (Williams 1984). Further analysis of these possibilities is underway by use of peptide mapping.

Of particular value is the observation that the FFB3 antibody binds to several immortal T cell lines (Jurkat and Cem-T, data not shown). As such, isolation of large quantities of RNA should be easier than was the case for the 2 leukemia cell sources. Comparison of molecular features at the protein level will be of equal interest, using these reagents.

Finally, several recent reports (Yanagi, et al., 1984; Hedrick, et al., 1984a,b) have described the identification of T cell cDNA clones derived by plus/minus screening or hybrid depletion similar to the

techniques employed in the HUT 102 experiments described here. These gene products appear to contain both constant and variable nucleotide sequences. The molecules encode peptides of approximately 30-35,000d. Considering additional glycosylation, as well as strikingly similar characteristics of specificity, and variable and constant sequences or epitopes, the intriguing possibility exists that these isolated clones may correspond to one of the two chains described in these studies using anti-idiotypic monoclonal antibodies.

Figure 21

Peptide recognition of SI60 and SV11 monoclonal antibodies. ^{125}I labeled Su leukemia cell extract was immunoprecipitated and resolved on a 15% SDS-polyacrylamide gel. Lane 1) before reduction and lane 3) after reduction for antibody SI60; lanes 5 and 7, before and after reduction for antibody SV11. Lanes 2, 4, and 6 represent control precipitations with these antibodies against the KE37 T cell line. Molecular weights are indicated in kilodaltons.

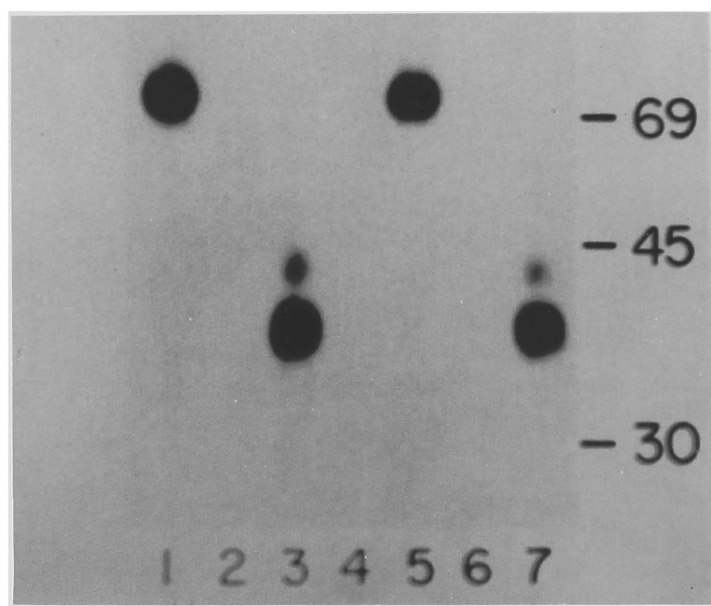


Figure 22

Direct conversion of the 70,000d T cell specific peptide to two smaller chains by disulfide reduction. The upper band, as well as the two reduced bands (as seen in Figure 21) were excised and rehydrated in the absence (lanes 1-3) or presence (lanes 4-5) of 0.1 M DTT. Lane 4 demonstrates the migration of the same protein seen in lane one following disulfide reduction.

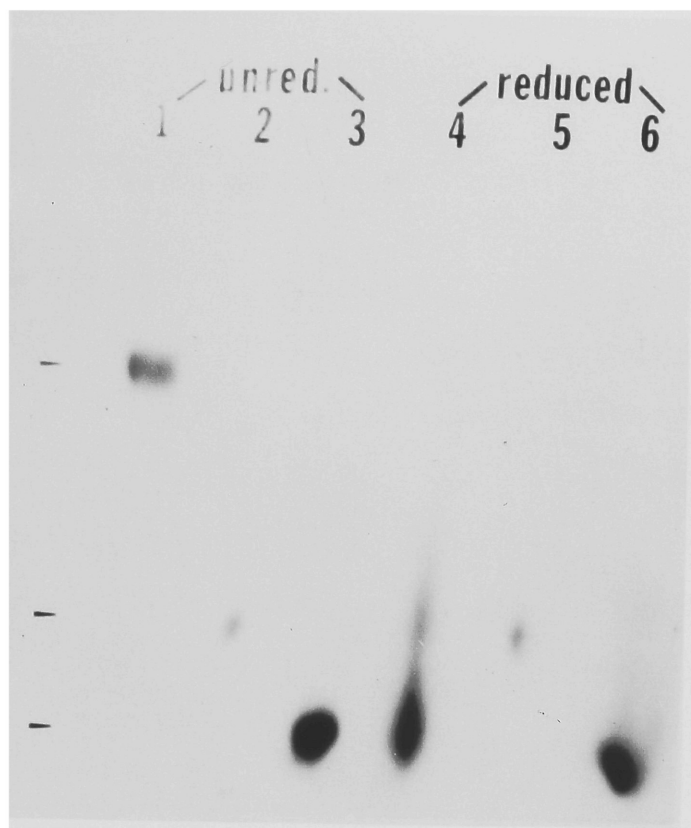


Figure 23

Generation of polyclonal rabbit antiserum to the idiotypic molecule of Su leukemia cells. Immunoprecipitated SV11 antigen, adsorbed to formalin fixed staph. aureus, was injected intraperitoneally into a rabbit for 3 months. Immunoprecipitation with the resulting rabbit serum of ^{125}T -labeled Su cell lysate is shown under reduced (red.) and unreduced (unred) conditions. Identity of the SV11 antigen is seen under the same immunoprecipitation conditions (SV11, red. and unred).

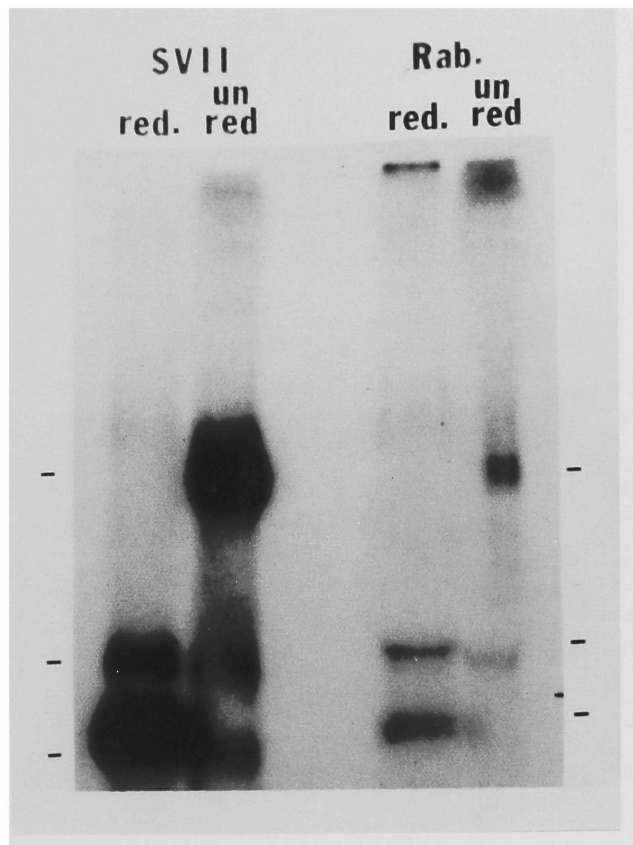


Figure 24

Comparison of immunoprecipitated idiotypic molecules from two different human T cell leukemias. Iodinated extracts of Si cells were immunoprecipitated with SJ50 antibody and run under nonreduced (lane 2) and reduced (lane 4) conditions. Precipitation of labeled Fi cell lysates with FF25 antibody is shown nonreduced (lane 1) and reduced (lane 3).

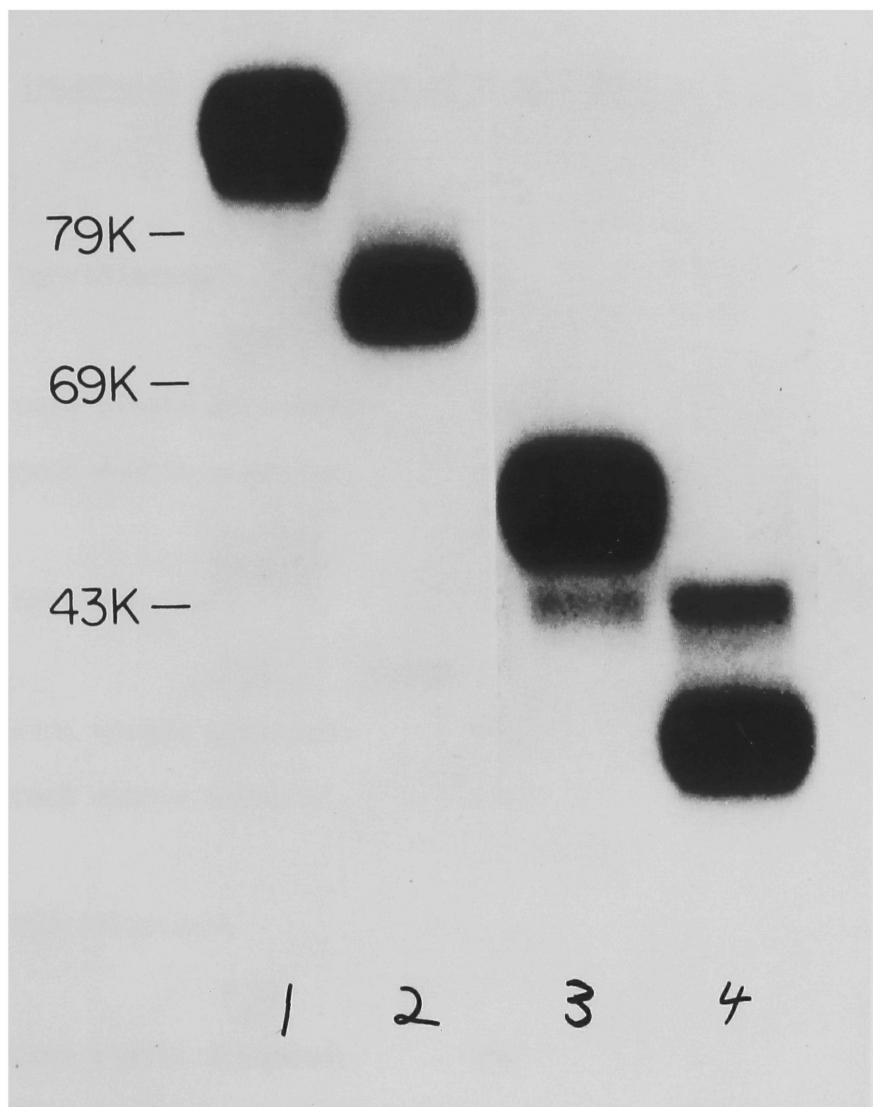


Figure 25Yields from sequential hybridization of T cell cDNA to B cell A+ RNA

1st hybridization:

$R_o t:$	500
percent single stranded:	31%
percent double stranded:	69%

2nd hybridization:

$R_o t:$	500
percent single stranded:	59%
percent double stranded:	42%

3rd hybridization:

$R_o t:$	500
percent single stranded:	98%
percent double stranded:	2%

Figure 25

Northern blot analysis of "specific" probe. 3 ug of T cell and B cell poly A+ RNA were resolved on a formaldehyde gel, followed by blotting to nitrocellulose filter paper. And probing with T cell cDNA probe twice hybridized (selected against) B cell RNA. Markers are indicated in kilobases.

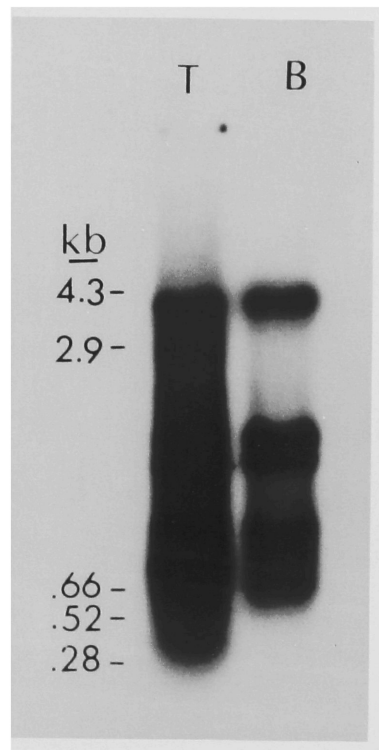


Figure 27

Screening T cell library with T cell cDNA "specific" probe. Twice hybrid depleted T cell cDNA was used to probe nitrocellulose filters containing replicas of a lambda gt10 recombinant phage library from HUT cells. From a total of 15,000 recombinants, 245 were positive with the selected probe. An example is seen here of approximately 20 "positive" plaques on a filter containing approximately 500 total plaques.

Figure 29

Testing intactness of Northern blot RNAs giving rise to heterogeneous patterns. Filter #3 from Figure 28 was washed at high temperature to remove original probe. The same filter was then probed with a known unique sequence gene (generously donated by Dr. Hung Teh Kao). The presence of a clear band in both T and B cell lanes verifies the intactness of both RNA sources.

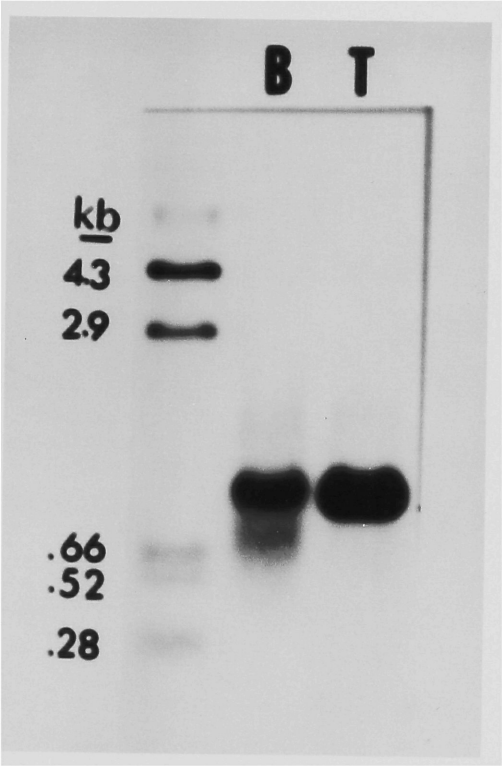
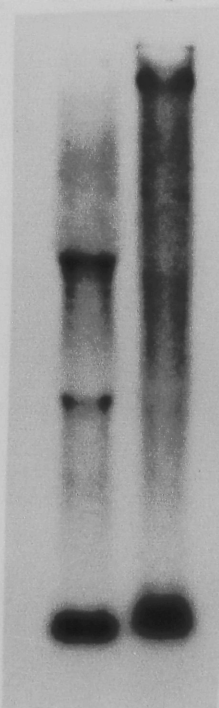


Figure 30

Repetitive sequences in T and B cell RNAs. To test whether the patterns obtained in Figure 28 might be due to repetitive DNA sequences, filter #4 from Figure 28 was washed (reexposed to ascertain removal of the first probe) and probed with nick translated human genomic DNA.

**Genomic
DNA**
B T



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Materials and Methods

Sera, cells, and cell growth. Sm and RNP serological determinations were carried out by the passive hemagglutination assay described by Tan and Peebles (1975). The human myeloid cell line K562 was grown in RPMI 1640 (microbiological Associates, Walkersville, Md.) supplemented with 10% fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N.Y.), 2 mM glutamine, and antibiotics (complete medium).

In vivo labeling. Extended labeling was carried out by resuspending washed K562 cells at 10^6 per ml in methionine-free RPMI 1640 supplemented with 10% FBS, glutamine, and antibiotics as above (met-free medium). To this, 0.5 ug per ml of methionine was added together with 100 uCi per ml of ^{35}S -methionine (800-1000 Ci/mM, New England Nuclear, Boston, Mass.). Following incubation for 15 hours, cells were washed three times in ice cold Dulbecco's phosphate buffered saline (PBS) and lysed. Pulse and pulse-chase procedures were carried out on cells starved (unless otherwise noted) for two hours in met-free medium. Methionine-starved cells (usually 5×10^7) were pelleted and 100 ul met-free medium supplemented with 500 uCi ^{35}S -methionine was added. The cell suspension was incubated for eight minutes at 37°C followed by immediate resuspension in 50 ml of ice cold PBS. In chase experiments, pulsed cells were resuspended in complete medium at a density of 10^6 per ml. During chase, cells were incubated at 37°C and appropriate aliquots were removed, washed in ice cold PBS, and extracted at the time points listed in the legend to Figure 10.

Cell lysis and immunoprecipitation of snRNPs. Following three washes in ice cold PBS, cells were swelled for 10 minutes on ice in 10 mM KCl, 10 mM Tris-Cl (pH 7.6), 5.0 mM iodoacetamide, 0.5 mM phenylmethylsulfonylfluoride, 1.0 mM EDTA. Cells were then lysed by 12 strokes using a Dounce homogenizer. NaCl was added to 0.2 M and the lysate was clarified by centrifugation at 12,000 X g for 5 minutes. Immunoprecipitation of the lysate was carried out by the addition of 5-10 ul of appropriate patient serum per immunoprecipitate. Cells were lysed at 10^6 - 10^7 per ml, and each immunoprecipitate was derived from $1-5 \times 10^6$ cells. After one hour at 4°C, 40 ul Protein A Sepharose 4B (Pharmacia, Piscataway, N. J.) was added and the tubes were rotated for an additional hour at 4°C. Immunoprecipitates were washed four times with 0.5% NaDodSO₄, 2.5% Triton X-100, 50 mM Tris-Cl (pH 7.5), 0.15 M NaCl, 1.0 mM EDTA, and 0.25 M sucrose followed by two washes with 50 mM Tris-Cl (pH 7.5), 0.15 M NaCl, 1.0 mM EDTA, 1 % trasylol.

RNA extraction methods

The perchlorate extraction method for isolating total cellular RNA. This method (Lizardi and Engelberg, 1979) has been used for RNAs prepared for in vitro translation experiments. Washed cells are resuspended in 2.4% SDS, 100 mM NaCl, 7.5 mM EDTA, 20 ug/ml polyvinyl sulfate, 25 mM Tris pH 7.4, and 350 ug/ml proteinase K. Following 15 minute incubation at 37°C, 4 ml of 3.5 M Na Perchlorate was added to 6 ml of cell lysate. The mixture was heated to 50°C until it became clear. Following shearing through a 20 gauge needle, 40 ml of EPR reagent was added. EPR is made by mixing 1

volume of saturated sodium perchlorate (in water) with 4 volumes of EtOH (which is also salt saturated). The EPR reagent is added slowly. The resulting mixture is incubated at 4°C for 30 minutes, and precipitated nucleic acids are collected by centrifugation at 3000xg for 10 minutes (5°C). The pellet is dissolved in 15 ml 25 mM Tris pH 7.4, 0.2% SDS, 1 mM EDTA, and nucleic acids were reprecipitated by addition of NaCl to 0.18 M and 0.6 volumes isopropanol and incubation at -20°C for 4 hours.

Following an EtOH precipitation step (in the presence of 0.2 M NaCl) RNA was reprecipitated in 2.5 M LiCl at 0°C for 10 hours. LiCl precipitation was followed by 3 further EtOH precipitations.

The NP-40 method of preparing cytoplasmic RNA.

To a 1.5-2 ml. prewashed and cooled cell pellet, 9 ml. Isohi pH (10 mM Tris pH 8.4, 140 mM NaCl, 1.5 mM MgCl₂) and 1 ml. 5% NP-40 is Isohi pH are added, on ice. Following handmixing for 5 minutes, nuclei are removed by centrifugation and the supernatant is extracted with phenol (2:1), phenol/chloroform (1:1:1), and chloroform (2:1). RNA is precipitated 3 times in 0.2 M NaCl and 2.5 volumes EtOH.

The Guanidinium/CsCl₂ Method of Preparing Total Cellular RNA. The following solutions are used:

GTC Lysis solution:

47.3 gm Guanidinium Thiocyanate

10 mL 0.5 M EDTA

10 mL 1 M Tris pH 7.4

7 mL straight 2-mercaptoethanol

H₂O to 100 mL

5.7 M CsCl₂/ 50 mM EDTA solution:

96 gm CsCl₂

10 mL EDTA

H₂O to 100 mL

To 1 volume washed packed cells 10 volumes of GTC solution. are added. DNA is sheared by either vortexing, or passage through a gauge 20 needle.

In an SW40 tube, a 2-3 mL 5.7 M CsCl₂/ 50 mM EDTA cushion is first poured. The extract is layered on top of the CsCl₂ cushion. Spin at 25 K at 20° C for 24 hours. The pellet at the end of the spin consists of cellular RNA. Top layers are aspirated off the pellet, which is inverted and drained. The pellet is dissolved in a convenient volume of H₂O and extracted with phenol:chloroform and chloroform. RNA is recovered by ethanol precipitation (twice).

Poly A selection

Total RNA is loaded onto an oligo dT cellulose column, preequilibrated in wash buffer (0.5 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.5% SDS). RNA is reapplied 3-5 times. Column is washed with 20-30 volumes wash buffer and eluted with 10 mM Tris pH 7.5, 1 mM EDTA, 0.5% SDS, in as small a volume as possible. EtOH precipitation is carried out (X3). For more stringent washing, a 10% formamide wash step may be included.

Cell free translation system

Preparation of wheat germ extract

550 ml of CCl_4 and 220 ml of cyclohexane are mixed. 40 gm wheat germ are added (obtained from local health food store). Following brief stirring, remove floating embryos onto filter paper under suction. Dry thoroughly (minimum of one hour). A sephadex G-25 column (2.5x100 cm) should be preequilibrated in a cold room with running buffer (40 mM Hepes pH 7.5, 100 mM K acetate, 4 mM DTT, 5 mM Mg acetate - adjusted to pH 7.6 with KOH). 5 gm of floated embryos are added to a liquid N_2 cooled mortar to which 22 ml of homogenization buffer (40 mM Hepes pH 7.5, 100 mM K acetate, 1 mM MgAcetate, 2 mM CaCl_2 , 4 mM DTT - adjusted to pH 7.6 with KOH) are added, 5 ml at a time. The homogenate (paste) is scraped into a Corex tube and spun at 13,750 rpm using a Sorvall SS34 rotor, for 10 minutes, at 4°C . Remove the yellow lipid (top layer), decant the extract (supernatant), and spin it again as before. The second supernatant is loaded onto the G-25 column and run with the fastest head possible at 4°C . Void volume contains a white slurry which has the most activity. Following one last spin as above, aliquot and quickfreeze (N_2).

Translation

The following conditions were optimized for K562 A+ RNA, extracted by perchlorate method.

Wheat germ 40%, "master mix" 40%, A+ RNA 2-5%. Master mix is composed of: $5\times_{\text{EGS}}$ 50%, $\text{CB}^{10\times}$ 25%, tRNA (2.5 mg/ml) 10%. $5\times_{\text{EGS}}$ consists of 100 mM ATP

6%, 20 mM GTP 2%, 600 mM creatine phosphate 9%, 1 mM 19 amino acids without methionine 10%, ^{35}S -methionine (10 uCi/ul) 5%, 8 mg/ml creatine phosphokinase 4%, 1 M DTT 4%. ^{10}X CB consists of: 1 M Hepes 27%, 2 M K Ac 37.5%, 0.5M MgAc_2 2%, 0.1M spermidine 7.3%, 1M DTT 4%, ddH_2O 22.2%.

Immunoprecipitation of in vitro translation products was carried out by adjusting translation products to 1% Triton X-100, 0.1 M NaCl, 25 mM Tris-Cl (pH 7.4), 5.0 mM EDTA, 2.5% trasylol, 2.5 mg per ml ovalbumin, and 0.1 ug per ml each of antipain, leupeptin, and pepstatin, and addition of 5-10 ul Sm or RNP serum.

Gel analysis. All washed Protein A Sepharose immunoprecipitates were eluted in 0.25 M sucrose, 2.0% NaDodSO₄, 0.1 M Tris-Cl (pH 7.4), 5.0 mM EDTA, 0.1 M dithiothreitol, 0.005% bromphenolblue. Following 5 minutes at 100°C, eluates were removed and applied to 15% NaDodSO₄-polyacrylamide gels (Fisher, et al., 1982) and run at 25 mA for 14 hours. Gels were stained in Coomassie Blue and destained, treated with PPO for fluorography (Bonner and Laskey 1974), and dried prior to autoradiography.

Sucrose gradient analysis

Pulsed and pulse/chased cell lysates were fractionated on 5-20% sucrose gradients in lysis buffer with the addition of NaCl to 0.2 M. Lysates from $1-2 \times 10^7$ K562 cells were loaded onto precooled gradients and subjected to simultaneous centrifugation in an SW 50.1 rotor for 20 hours at 41,000 rpm, and 4°C. Gradients were fractionated with an automated fractionator (model 185, Instrumentation Specialties Co., Lincoln, Nebraska) and fractions were immunoprecipitated with Sm and RNP sera as above. Sedimentation standards

were human hemoglobin (4.2S), IgG (7S), and catalase (11S). Fractionation of in vitro translation products was carried out on 5-20% sucrose gradients in 130 mM K Ac, 3.6 mM DTT, 3 mM MgAc₂, 15 mM Hepes, 80 uM spermine. These gradients were run at 46,000 rpm for 15.5 hours, using RNase A (2S), bovine serum albumin (4.5S), IgG (7S), and catalase (11S) as standards. Poly A⁺ RNA was fractionated on 10-30% gradients in 0.5% SDS, 10 mM NaCl, 5 mM Tris pH 7.4, 0.1 mM EDTA. Sixty micrograms of poly A⁺ RNA in the same buffer was heated to 85°C for 2 minutes, cooled on ice, and centrifuged in a Beckman SW40 rotor for 12 hours at 38,000 rpm, at 20°C. Following automated fractionation, each RNA fraction was twice ethanol precipitated prior to translation.

Cell fractionation

Pulsed or pulse/chased cells were fractionated by lysis in 10 mM KCl, 10 mM Tris pH 7.6, 5 mM iodoacetamide, 0.5 mM PMSF, 1 mM EDTA, for 10 minutes on ice. Nuclei were pelleted by centrifugation at 12,000 x g for 4 minutes. Cytosol fractions (supernate) were made 0.2 M in NaCl and the nuclear pellet was extracted for snRNPs by resuspension in lysis buffer containing 0.2 M NaCl for 5 minutes. The nuclear extract was clarified by centrifugation at 12,000xg for 5 minutes. All procedures were carried out at 0-4°C. Nuclear and cytoplasmic extracts were immunoprecipitated using Sm and RNP sera, as above.

Proteolytic peptide mapping

Radioactive protein bands were cut out from gels which had been only briefly stained and destained (total fixing time: less than 1/2 hour). Following rehydration in 125 mM Tris pH 6.8, 0.1% SDS, 1 mM EDTA, gel

slices were placed into wells of a 20% SDS-polyacrylamide gel (as above with the addition of 1 mM EDTA to gel buffer). Chymotrypsin (various concentrations in loading buffer) was overlaid into the wells, and electrophoresis was started. When the bromphenolblue was near the end of the stacking gel, the power was turned off and digestion allowed to proceed for 1/2 hour, followed by further electrophoresis through the resolving gel (Cleveland, et al., 1977).

Cell surface iodination, extraction, and immunoprecipitation

Cells were iodinated in the presence of 10 ug lactoperoxidase (Sigma) and 0.1 U glucose oxidase (Calbiochem) per 5×10^7 cells and 5 mCi Na^{125}I (New England Nuclear). Cells were extracted in 10 mM Tris pH 8, 0.15 M NaCl, 0.5% NP-40, 1% trasylol for 30 minutes on ice. Where immunoprecipitates were to be analyzed nonreduced, cells were extracted in the presence of 50 mM iodoacetamide. Extracts were cleared by centrifugation at 12,000g for 5 minutes. Protein-A sepharose (PAS) (Pharmacia) beads were coated with Rabbit anti-mouse Ig antibodies (RAM) (kindly provided by Dr. Nicholas Chiorazzi). Extracts were precleared by incubation with RAM-PAS for 2 hours. RAM-PAS beads were incubated with mouse monoclonal antibodies for 2 hours, and added to precleared cell extracts. Immunoprecipitation was carried out for 6-15 hours, and was followed by washing with 10 mM Tris pH 8, 0.5% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mg/ml ovalbumin. Elution and gel analysis were carried out as described, above.

Recombinant DNA methods:

Synthesis of double stranded, linker ligated cDNA. The methods employ

standard technologies (Maniatis) with a few exceptions. First strand synthesis was carried out in the presence of actinomycin D. The second strand reaction was essentially a nick translation utilizing RNase H, *e. coli* DNA polymerase I, and *e. coli* DNA ligase. Following filling ends and methylation, linker ligation was carried out using ^{32}P -labeled Eco RI linkers. Figure 31 shows an autoradiograph of a gel used to monitor linker ligation, before and after Eco RI digestion. Cut linkers were separated from linker-ligated cDNA using a 1 ml sepharose Cl-4B column.

Preparation and ligation of phage DNAs Lambda GT10 and GT11 DNAs are isolated by phenol extraction from CsCl gradient purified phage (Maniatis). Dialysis must be carried out because of a ligation inhibitor in the Cs. Both of these phage systems utilize an Eco RI site for cDNA insertion. Eco RI cleavage is carried out for the purified DNA and monitored by gel analysis in 0.5% agarose gels (in TE), an example of which is shown in Figure 32.

The ratio of lambda arms to ds cDNA should be about 5-15ug arms to 100 ng ds cDNA (2-3x molar excess). Freshly digested arms are mixed with the cDNA, and ligation is carried out as follows:

- 8 ul H₂O with cDNA and lambda arms
- 2 ul 5x ligase buffer
- 1 ul T4 DNA ligase

Incubate at 15⁰ C overnight Success of this ligation reaction may be monitored by testing for the presence of ^{32}P -labeled phage-sized DNA on a 0.5% agarose gel. An example is shown in Figure 33 in which recombinant cDNA (ligated to phage DNA) is visualized near the origin, as well as unligated cDNA of smaller size.

The DNA is now packaged using lysates of mutant phage as described (Maniatis).

Bacterial strains

In the Lambda GT10 cloning system, two bacterial strains are used: C600 and HFL (High Frequency of Lysogeny - a mutant of C600). In the HFL strain, all lambda phage containing an intact repressor gene will lysogenize into the host strain resulting in no lysis (or a minimal background of turbid lysis) during infection. In this cloning system, ds cDNAs are inserted into a unique Eco RI in the lambda repressor. Such recombinant phage, with an interrupted repressor gene can now cause lysis in the HFL strain, and can therefore be propagated using this cell line. This is also the method by which one selects recombinant phage. The C600 strain will lyse upon infection by lambda GT10 regardless of whether the repressor is intact or not. However, recombinant phage will show clear plaques and normal lambda GT10 will show turbid plaques. Using C600, one can determine the proportion of recombinant phage for a given cloning experiment.

In the Lambda GT11 cloning system, ds cDNA is inserted into the B-galactosidase gene via a unique Eco RI site. There are no strains which can select for recombinant phage. Lambda GT11 will NOT form plaques on HFL or C600. Lambda GT11 contains two mutations which are of importance for expression of hybrid galactosidase proteins: a temperature-sensitive repressor (CI857) and an amber mutation in a gene required for lysis (S100). Therefore, at elevated temperatures, plaques will form on C600 (42° C), and turbid plaques will form on Le392 at 37° C. To determine the proportion of recombinant phage in a library for a given cloning experiment

one plates the phage on the bacterial strain Y1090 (which will lyse upon infection by lambda GT11 and does not contain an endogenous galactosidase gene) using the indicators IPTG and XGAL. One then scores for recombinant phage (clear plaques) or intact phage (blue plaques). 2. Plating Procedure

For plating onto an 82mm plate the following procedure is used:

Add 1-3 ul phage

200 ul of overnight bacteria

200 ul of 10 mM MgCl₂

Incubate for 20 minutes at 37° C

Add 4 ml of soft agarose (Kept at 65° C)

= 1 gm NZamine

0.5 gm yeast extract

0.5 gm NaCl

0.2 gm MgCl₂

0.7 gm agarose

100 ml H₂O autoclave mix

Pour onto L broth plates

Screening and Northern blot analysis are carried out as described (Maniatis). For Northern blots, formaldehyde gels are used. For screening, DNA probes are generated by nick translation. For expression vector screening, antibodies are mixed with a solution as follows: 1:100 dilution of serum, 20% supernatant from overnight culture of (lysed) gt11 phage-bacteria, 10 mM Tris pH 7.4, 150 mM NaCl, 3% bovine serum albumin. Filters are incubated with this solution for 1 hour, followed by 5 15 minute washes in 0.5% NP-40, 10 mM Tris pH 7.4, 150 mM NaCl. A last wash

is carried out in the absence of detergent. Following 1 hour incubation in 10 mM Tris pH 7.4, 150 mM NaCl, 3% BSA, containing 10^6 cpm per ml ^{125}I -protein A (New England Nuclear) the filters are washed with detergent, as before.

Figure 31

Linker ligation ladder. Products of the ligation reaction between cDNA and Eco RI linkers are seen in the lane marked "Lig." After digestion with Eco RI, monomeric linkers are seen near the bottom of the gel (7% acrylamide), while linker ligated (32 P- labeled) cDNA is seen at the top of the lane marked "cut RI."

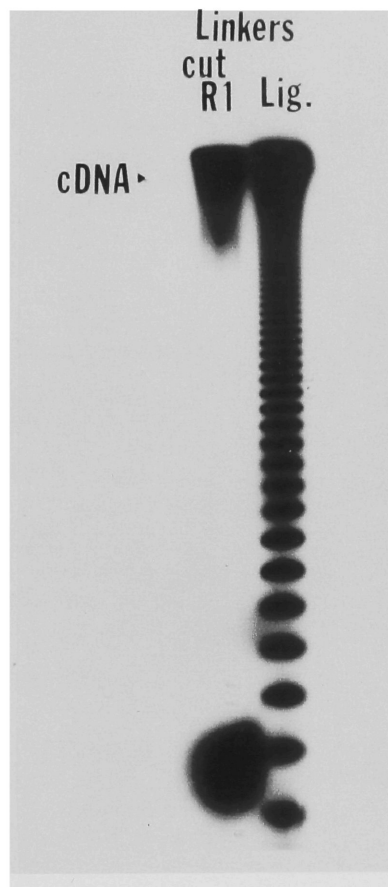


Figure 32

Fragments generated by digestion of DNA from lambda gt11 with Eco R1.
Marker DNA's (M) migrate at 15 and 20 kilobases. Two bands of approximately 18 and 22 kb are the cleavage products of Eco R1 digested (11/R1) phage DNA. A third band migrating at 45-50 kb in the 11/R1 lane represents residual uncut phage DNA.

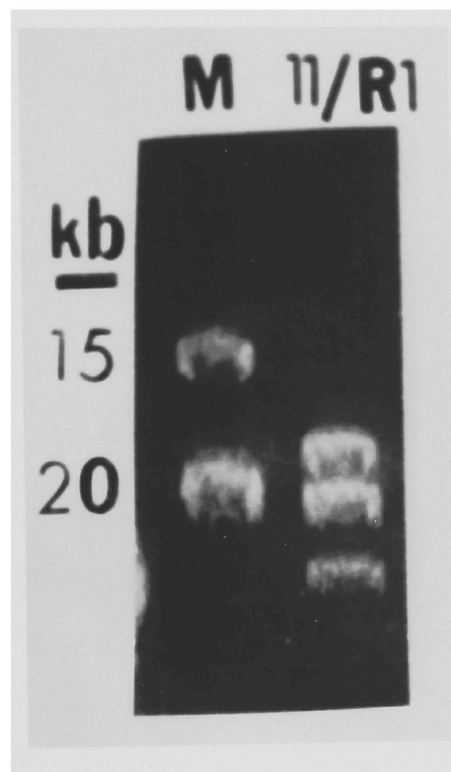
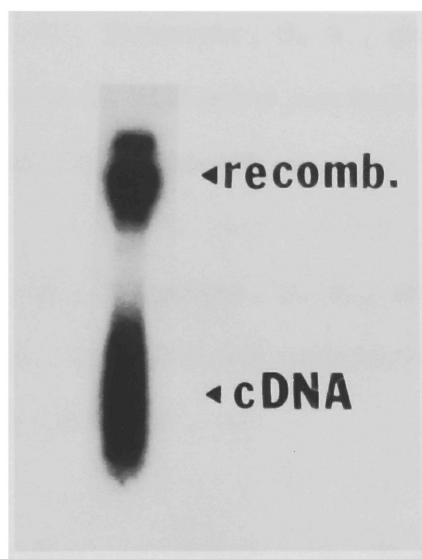


Figure 33

Diagnostic test for the presence of recombinant DNA molecules. Following ligation of cDNA to R1/cut phage, an aliquot is resolved on a 0.5% agarose gel. On such a gel, unligated cDNA runs as a broad fraction (labeled "cDNA") whereas phage DNA, being so much larger remains near the origin. Since the phage DNA itself had not been ^{32}P -labeled prior to ligation, the presence of counts in this slow migrating fraction indicates successful ligation of cDNA to phage DNA.



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