Molecular Composition of Ro Small Ribonucleoprotein Complexes in Human Cells

Intracellular Localization of the 60- and 52-kD Proteins

Ameeta Kelekar,* Michael R. Saitta,*‡ and Jack D. Keene*‡

**Department of Microbiology, and ‡Division of Rheumatology and Immunology, Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710*

Abstract

Ro small ribonucleoprotein complexes (RoRNPs) are thought to comprise several proteins, including the 60-kD Ro and the 52-kD Ro proteins, and several small RNAs, designated Y RNAs. Although RoRNPs are fairly ubiquitous in nature, their precise composition remains unknown, their function has been elusive, and their intracellular localization has been controversial. We have analyzed HeLa cell extracts by glycerol density gradient fractionation in order to determine the distribution of the individual protein and RNA components of RoRNPs. We found that 52-kD Ro was not detectable in an RNP complex with the 60-kD protein under a variety of conditions. Pretreatment of cell extracts with ribonuclease affected gradient migration of the 60-kD but not the 52-kD protein, suggesting that the latter is not complexed with RNA. The migration of the hY RNAs in these gradients closely followed that of 60-kD and not 52-kD Ro. Immunofluorescence analysis of two different cell lines with monospecific antibodies against 52- and 60-kD proteins strongly suggests that these two proteins are not present on overlapping sets of structures in vivo. We conclude that the 52-kD Ro protein is not a detectable component of the RoRNP complex under these conditions despite its reactivity with Ro autoimmune antisera. (*J. Clin. Invest.* 1994. 93:1637–1644.)

Key words: autoantibodies • autoantigens • RNA binding proteins • ribonucleoproteins • systemic lupus erythematosus

Introduction

Antibodies to ribonucleoproteins (RNPs)[1] present in patients with various rheumatic diseases have contributed greatly to our understanding of ribonucleoprotein structure and function. Antibodies to RoRNP complexes are found primarily in patients with SLE and Sjögren's syndrome (SS), and appear to be a dominant feature of the autoimmune response (1–3). Although they have been the subject of investigation in a number of laboratories for many years, their precise molecular definition, function, and localization have remained unclear.

RoRNPs are considered to be heterogeneous in terms of their molecular composition. This apparent heterogeneity is manifest at both the RNA and the protein level. RoRNPs, in all mammalian cells examined, comprise one of several different but closely related RNA molecules. In most nucleated human cells there are at least five major RNAs, hY1, hY2, hY3, hY4, and hY5, which range from 84 to 112 nucleotides in length (4). Indirect evidence indicates that each RoRNP probably contains a single hY RNA molecule (5). Physicochemical studies on native RoRNPs indicate that the particles segregate into three discrete subpopulations, one containing hY5, another containing hY4, and a third containing hY1, hY3, and hY4 (6). Initially, it appeared that the 60-kD Ro polypeptide was the sole stable protein component in the complex and that the La protein associated with hY RNAs only in a transient manner just as it associates with all RNA polymerase III transcripts (4, 7). However, Wolin and Steitz (5) showed that hY RNAs in their mature forms were ~ 30% precipitable by anti-La sera and, more recently, Boire and Craft (8) were able to distinguish biochemically certain RoRNPs in which La was a stable component. Additionally, anti-Ro antibodies from SLE and SS sera eluted from Western blots of human cell extracts indicated that a 52-kD protein, in addition to 60-kD Ro, is associated with hY RNA in nucleated cells (9). In red blood cells, however, either a 60- or a 54-kD protein was found complexed with a subset of the hY RNAs, hY1 and hY4 (10, 11). While 60-kD Ro has been shown to be directly associated with the hY RNAs in complexes (12), 52-kD Ro has not been shown to directly contact any of the hY RNAs. However, 52-kD protein–specific antibodies affinity purified from SLE and SS patient sera were able to immunoprecipitate RoRNPs along with the corresponding hY RNAs 1–5 (9). Direct interactions detected between the 52- and 60-kD proteins have led other investigators to conclude that association of 52-kD protein with hY RNA is dependent on the presence of the 60-kD protein (13).

The intracellular localization of the RoRNPs has been a matter of much uncertainty since their original classification as small cytoplasmic RNPs or scRNPs (4) in part because of the cooccurrence of La and Ro antibodies in patient sera. In subsequent studies, anti-60-kD Ro has been detected in the nucleus using autoantibodies affinity purified from Western blots (14). It has also been suggested that RoRNPs reside in intermediate filaments in the cytoplasm (15). More recently, Chan et al. (16) and Slobbe et al. (17), using double immunofluorescence on HEp2 cells, observed identical nuclear and cytoplasmic staining patterns with rabbit anti-60 and human anti-52 sera. They concluded that an intimate physical association existed between the 60- and 52-kD proteins in RoRNPs.

In this paper we have used glycerol gradient fractionation of whole cell extracts to separate RoRNPs and to identify individual subpopulations as a means of understanding their molecular composition. Wherever possible and relevant, we opted to use rabbit antisera raised against recombinant 60- and 52-kD proteins, thus eliminating potential problems of contaminating

Address correspondence to Dr. Jack D. Keene, Department of Microbiology, Duke University Medical Center, Durham, NC 27710.

Received for publication 30 August 1993 and in revised form 21 December 1993.

1. *Abbreviations used in this paper:* RNPs, ribonucleoproteins; SS, Sjögren's syndrome.

J. Clin. Invest.

0021-9738/94/04/1637/08 $2.00
Volume 93, April 1994, 1637–1644

specificities seen in polyclonal patient sera. In addition, we have also used these antisera and two different human cell lines to localize the 60- and 52-kD proteins within cells by immunofluorescence microscopy.

Methods

Glycerol gradient fractionation of HeLa cell extracts. HeLa whole cell extracts were prepared essentially as described by Weil et al. (18), and 5–7 × 10^7 cell equivalents were layered on to a 10–30% preformed glycerol gradient in 12-ml polyallomer tubes (Beckman Instrs., Inc., Fullerton, CA) in buffer containing 10 mM Tris (pH 7.5), 1.5 mM $MgCl_2$, and 10 mM KCl. The concentration of $MgCl_2$ was altered in the buffer from 0 to 10 mM as required during studies to determine the effect of Mg on the integrity of Ro particles. The tubes were centrifuged at 38,000 rpm for 24 h in an SW41 rotor (Beckman Instrs., Inc.) at 4°C, after which 14–15 fractions were collected from the top of the gradient using an Autodensiflow II C fraction collector (Buchler Instruments, Kansas City, MO) and analyzed for their RNA and protein content.

Antisera. Autoimmune patient antisera So and Go were gifts from the Fluorescent Anti-Nuclear Antibody (FANA) Laboratory at the Duke University Medical Center. Go has been characterized by immunodiffusion against standard cell extracts to be a Ro/La antiserum exhibiting strong anti-La activity on Western blots. So has been characterized as an anti-Ro serum by immunodiffusion, however, it recognizes 52-kD Ro almost exclusively on Western blots. The monoclonal antiserum Y12 has been previously described (19). The rabbit anti-60-kD and anti-52-kD sera were produced against partially purified recombinant forms of the corresponding proteins overexpressed in vitro. The characterization of these two antisera will be detailed elsewhere (M. R. Saitta and J. D. Keene, manuscript in preparation).

Immunoblot of gradient fractions. Aliquots of the glycerol gradient fractions were separated on either 10 or 15% polyacrylamide gels and transferred to nitrocellulose using standard methods. The nitrocellulose was then blocked using a solution of 0.5× TBS (20 mM Tris-HCl, pH 7.2, 150 mM NaCl), 0.05% Tween-20, 5% dry milk followed by incubation overnight at 4°C with antiserum diluted 1:1,000 in blocking solution. After washing the nitrocellulose with TBS, 0.05% Tween-20, 2 M urea, 1 μCi [^{125}I]-Staphylococcal protein A (ICN, Costa Mesa, CA) in TBS was added for 1 h at room temperature. After further washing in TBS, 0.05% Tween-20, 2 M urea, the nitrocellulose was air dried and exposed to Kodak XAR autoradiography film.

RNA analyses. A major portion of each gradient fraction was set aside for RNA analyses and the RNA isolated by extensive phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. 1:10 of the total RNA obtained from each fraction was directly analyzed using polyacrylamide gel electrophoresis, followed by silver staining (Bio-Rad Laboratories, Richmond, CA). The remaining RNA was divided into four fractions and each fraction subjected to RNase protection analysis using a different antisense RNA probe. The original clones for the hY1 and hY3 RNAs were a gift from S. Wolin (5), the hY4 clone was a gift from C. A. O'Brien (11), and the hY5 cDNA was isolated in our laboratory using the PCR technique. All four cDNAs were subcloned into pGEM transcription vectors (Promega Biotec, Madison, WI). ^{32}P-labeled antisense transcripts were produced using SP6 or T7 polymerases essentially as described in the Promega Biotec manual. The RNase protection assays were carried out essentially as described in Zinn et al. (20). The labeled and protected RNA-RNA hybrids were analyzed by polyacrylamide gel electrophoresis and visualized by autoradiography using Kodak XAR film.

Immunoprecipitation of labeled cell extracts. 2 × 10^7 HeLa cells were labeled with 10 mCi of [^{32}P]orthophosphate for 12 h in phosphate-free culture medium and extracts were prepared from these cells essentially as described in Weil et al. (18), except that the cells were disrupted by sonication rather than by a Dounce homogenizer. 1:50 volume of the labeled extract was set aside for total RNA analysis and immunoprecipitation controls, and the remainder gradient fractionated as described earlier. Immunoprecipitations of ribonucleoproteins were carried out by incubating the fractions on ice with antibodies immobilized on CNBr-activated protein A–Sepharose, washing several times in NT-2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NP-40), and eluting the antigens with 0.1 M glycine, HCl (pH 3.0). The complexes were deproteinized and the labeled RNAs analyzed by polyacrylamide gel electrophoresis.

Immunofluorescence of tissue culture cells. Cells were fixed by one of the following methods. They were either incubated in fixative containing 50% acetone, 50% methanol (21), or fixed in PLP (10 mM periodate, 75 mM lysine, and 2% paraformaldehyde) for 15 min (22) and then permeabilized in 0.1% Triton X-100 for a further 15 min. After fixation the cells were incubated with the 60- or 52-kD rabbit antiserum or with the corresponding preimmune sera at a dilution of 1:2,000 for 30 min at room temperature. Samples were washed in antibody buffer (1% BSA in PBS, pH 6.5, containing 0.05% Tween-20, 0.005 M EDTA, 1% goat serum) for 30 min, followed by incubation with donkey anti-rabbit IgG conjugated with Texas red (Jackson Immunoresearch) at a dilution of 1:200 for another 30 min at room temperature. The cells were then washed and mounted in 60% glycerol with 2.5% *n*-propyl-gallate (Sigma Chemical Co., St. Louis, MO) and observed under epifluorescence with an ×100 objective (Carl-Zeiss, Inc., Thornwood, NY). Photographs were recorded on Tmax 400 film by Kodak.

Results

The 60- and 52-kD Ro proteins do not cofractionate in glycerol gradients. To distinguish individual components of RoRNPs we separated the particles into subpopulations by gradient fractionation. HeLa cell extracts were prepared and fractionated as described in Methods. Each gradient fraction was analyzed for the presence of 60-kD, 52-kD, and La proteins, and for the distribution of the various hY RNAs. Fig. 1 *A, top,* shows a Western blot of gradient fractions probed with anti-60-kD rabbit serum and with a human autoantiserum, Go, containing strong anti-La reactivity. 60-kD-containing complexes banded between 9S and 12S particle size (lanes *7–9*), averaging a molecular mass of 200 kD. The La protein had a distribution compatible with both its abundance and the fact that it complexes with a wide variety of RNA polymerase III transcripts. Fig. 1 *A, bottom,* shows a Western blot of fractions of the same gradient probed with Ro-specific human antiserum, So, which contains both anti-60 and anti-52 reactivity but reacts only with the 52-kD protein on Western blots (M. R. Saitta and J. D. Keene, manuscript in preparation). It is evident that 52-kD peaked with a sedimentation coefficient of 4S to 5S (lanes *3–5*), suggesting that it is present either as a free protein or in a very small complex. A 52-kD-specific rabbit antiserum made against the partially purified recombinant protein showed a similar distribution of 52-kD (data not shown). It is clear from these data that 60-kD Ro and the 52-kD protein exhibit different distributions by gradient analysis with little overlap, suggesting strongly that the two proteins are not mutually complexed under these conditions.

One cannot eliminate the possibility that a very small proportion of 52-kD protein did cofractionate with 60-kD Ro. There is also the possibility that 52/60-containing RoRNP particles were disrupted during preparation of the cell extract. However, we think this is unlikely. At least three different protocols, previously adopted in the study of Ro ribonucleoprotein particles (9, 6, 13), were used to prepare extracts that were examined on gradients, all of which gave comparable results.

Previous studies of UsnRNPs (23) demonstrated that con-

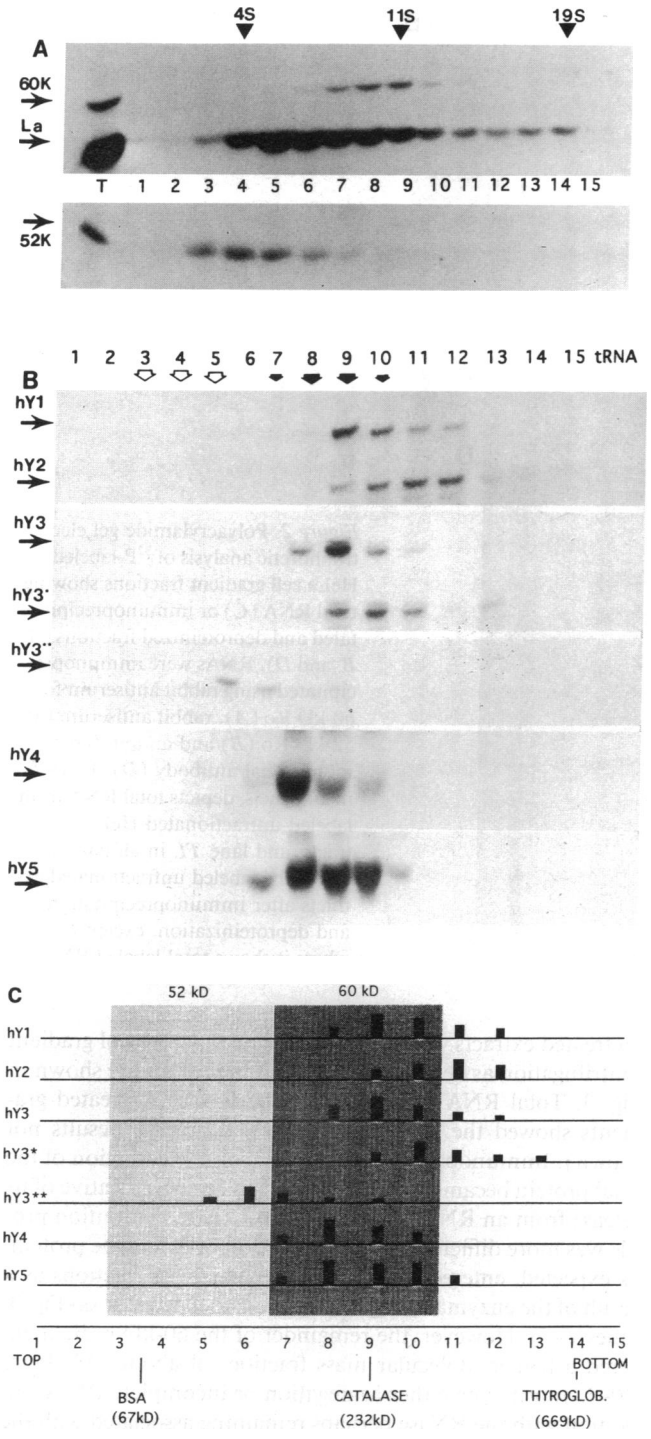

Figure 1. (*A*) Immunoblot of gradient fractions. Equivalent volumes of each gradient fraction were subjected to electrophoresis on 10% (*top*) or 15% (*bottom*) polyacrylamide gels, then immunoblotted with combined rabbit antiserum recognizing the 60-kD protein and a patient antiserum recognizing the La protein (*top*), or a patient antiserum recognizing the 52-kD protein (*bottom*). Lane *T* contains an aliquot of the total HeLa extract that was applied to the gradient. Lanes *1–15* show gradient fractions 1–15, respectively, with fraction 1 representing the top of the gradient and fraction 15 the bottom. The migration position of the bands (*arrows*) was confirmed using recombinant 60-kD, La, or 52-kD protein run in adjacent lanes on the same gel (data not shown). Sedimentation markers are indicated above. (*B*) RNase protection analyses of hY RNAs in gradient fractions. RNA prepared from gradient fractions was hybridized with

centrations of magnesium as high as 10 mM increased the stability of the RNA–protein interactions. In our experiments, however, varying the concentrations of magnesium in extraction and in gradient buffer, from 0 to 10 mM, did not detectably alter the gradient profiles of either the 60- or 52-kD proteins (data not shown).

Distribution of hY RNAs across the gradient. To determine whether hY RNAs comigrated with the 52-kD protein–containing fractions in the gradient, we measured the levels of each of the hY RNAs, both bound and free, across the gradient. Fractions were deproteinized and the RNA subjected to RNase protection analysis using ^{32}P-labeled antisense probes for hY1, hY3, hY4, and hY5 RNAs. Fig. 1 *B* shows that the RNAs sorted into four distinct populations. The hY1 and hY3 RNAs showed similar profiles that peaked in fraction 9, while hY2 and hY3* peaked in fractions 10 and 11. hY4 and hY5 also had similar distribution profiles peaking in fraction 8 while hY3** banded in fraction 5. As depicted in Fig. 1 *C*, when the distribution of the RNAs across the gradient is superimposed upon the protein profiles, it becomes clear that the hY RNAs are located in the fractions that contain 60-kD Ro, except for hY3**, which is known to bind only the La protein (24).

There still remained the possibility that small amounts of hY RNAs or other RNAs, which our assays were unable to detect, were cofractionating with the 52-kD Ro protein. Therefore, it was important to confirm, by other methods, whether the 52-kD protein was detectable in association with an hY RNA–containing particle.

The 52-kD protein was not immunoprecipitable as a complex with hY RNAs. Next we determined the gradient profiles of the subset of total hY RNA that was actually complexed within RoRNPs. Rabbit antisera against recombinant 60- or 52-kD were used to immunoprecipitate the proteins and their putative complexes from ^{32}P-labeled HeLa cell extracts after fractionation over a 10–30% glycerol gradient. The immunoprecipitated extracts were deproteinized and the RNAs analyzed by polyacrylamide gel electrophoresis. The results from a representative gradient are shown in Fig. 2. Fig. 2, *C* and *D*, are controls that show, respectively, the total labeled RNA profile across the gradient and RNAs immunoprecipitated by Y12, a monoclonal Sm antiserum (19). Fig. 2 *A* shows a profile of RNAs found to be immunoprecipitable with 60-kD Ro using rabbit Ro60 antiserum, which does not react with the 52-kD protein (M. R. Saitta and J. D. Keene, manuscript in preparation). Fig. 2 *B* shows immunoprecipitated and deproteinized gradient fractions obtained using 52-kD-specific rabbit antiserum, which immunoprecipitates only the 52-kD protein and not 60-kD from HeLa and HEp-2 cell extracts (M. R. Saitta and J. D. Keene, manuscript in preparation). Longer exposures of the gel in Fig. 2 *B* showed only background traces of 5S and tRNA. In conclusion, the profile of the hY RNAs largely

^{32}P-labeled antisense RNA probes for hY1 (*top*), hY3 (*second* and *third*), hY4 (*fourth*), and hY5 (*fifth*). The hybrids were treated with RNase, deproteinized, and analyzed by gel electrophoresis. Open arrows show fractions in which the 52-kD protein was detected by immunoblotting (as in *A*) and solid arrows point to the 60-kD protein–containing fractions. (*C*) Diagrammatic representation of gradient analyses of RoRNPs by Western blotting and RNase protection analysis (*A* and *B*). The distribution of hY RNAs across the gradients is depicted by solid bars that are superimposed on Ro52 and Ro60 distribution profiles (*shaded*).

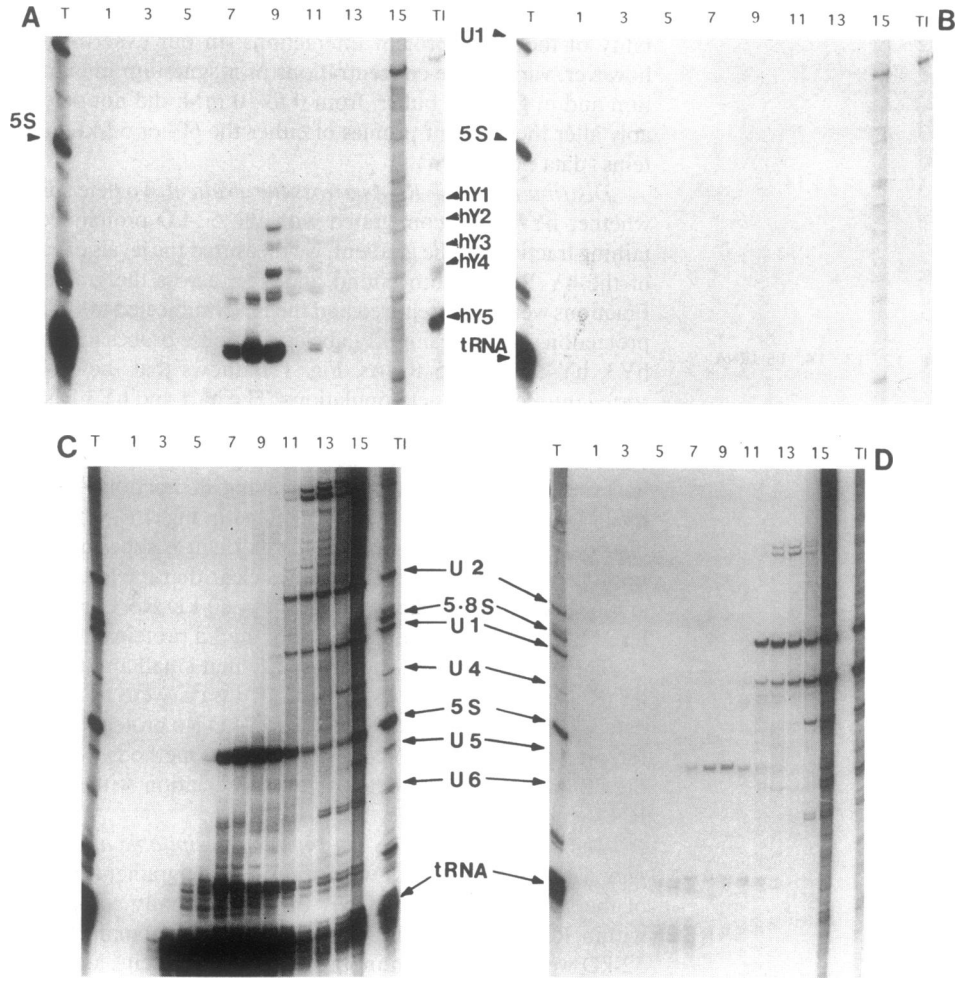

Figure 2. Polyacrylamide gel electrophoretic analysis of ^{32}P-labeled HeLa cell gradient fractions showing total RNA (*C*) or immunoprecipitated and deproteinized fractions (*A*, *B*, and *D*). RNAs were immunoprecipitated using rabbit antiserum for 60-kD Ro (*A*), rabbit antiserum for 52-kD Ro (*B*) and an anti-Sm monoclonal antibody (*D*). Lane *T*, in all panels, depicts total RNA from labeled unfractionated HeLa extracts, and lane *TI*, in all panels, shows the labeled unfractionated extracts after immunoprecipitation and deproteinization, except in *C*, where it shows total labeled RNA.

coincides with that of the 60-kD Ro protein and it is highly unlikely the 52-kD protein is complexed with any hY RNA.

From these data, the 60-kD protein–containing particles (Fig. 2 *A*) can be sorted into three subpopulations, one containing RNAs hY1, hY2, hY3, and hY4 (hY1-4, lanes *9–11*), another containing RNAs hY4 and hY5 (lanes *6–8*), and a third of a higher molecular mass containing only RNA hY5 (lanes *11* and *12*). The differences in detail between the RNA profiles in Figs. 1 *B* and 2 *A* can be attributed to the fact that the experiment shown in Fig. 1 *B* was designed to detect all the hY RNAs in the extract: free forms as well as those complexed with La or any other proteins. The experiment in Fig. 2 *A*, on the other hand, used immunoprecipitation to detect only the hY RNAs that were complexed with 60-kD Ro. In the former (Fig. 1 *B*), the RNAs segregate as subpopulations that include hY1 and hY3, hY2 and hY3* fractionating together, and hY4 and hY5 fractionating together. In the latter (Fig. 2 *A*), however, hY5 particles also fractionate as a separate subpopulation suggesting they may be complexed with additional proteins. No hY3** was observed with anti-Ro immunoprecipitations because this RNA lacks the Ro binding site (24). However, we did detect free hY3** cofractionating with the La peaks as observed in the Western blot (Fig. 1 *A, top*).

The 52-kD protein was not detected in association with any RNA. To consider further the possibility that the 52-kD protein is part of a ribonucleoprotein particle, cell extracts were pretreated with RNase A (100 μg/ml) for 30 min at 37°C. The pretreated extracts were then fractionated by glycerol gradient centrifugation as described earlier and the results are shown in Fig. 3. Total RNA profiles across the RNase A–treated gradients showed the RNA to be largely degraded (results not shown). Immunoblots of La showed that some fraction of the total protein became redistributed in a manner indicative of its release from an RNP complex. The 60-kD redistribution profile was more difficult to interpret. A proportion of the protein, as expected, entered the lower molecular mass fractions as a result of the enzymatic degradation of associated RNAs (Fig. 3, lanes *4–7*). However, the remainder of the 60-kD protein entered a higher molecular mass fraction, of about 14S (lanes *10–12*), implying either aggregation or incomplete RNase digestion with the RNase perhaps remaining associated with the complex. Fig. 3 *B* shows a similar analysis of the 52-kD protein after RNase treatment and gradient fractionation. The 52-kD profile was unaltered by RNase (Fig. 3 *B, bottom*) consistent with its not being physically associated with RNA.

60-kD Ro and 52-kD Ro show dissimilar patterns of intracellular localization. We have used the rabbit sera specific for the 60- and 52-kD proteins to localize the two proteins within human cells. The precise localization of RoRNPs has presented an ongoing dilemma in the field since the first reports that they were exclusively cytoplasmic (4). Many laboratories have observed immunofluorescent staining of nuclei (14, 25) and others report that RoRNPs are cytoplasmic but localized on intermediate filaments (15). Recent studies have used double

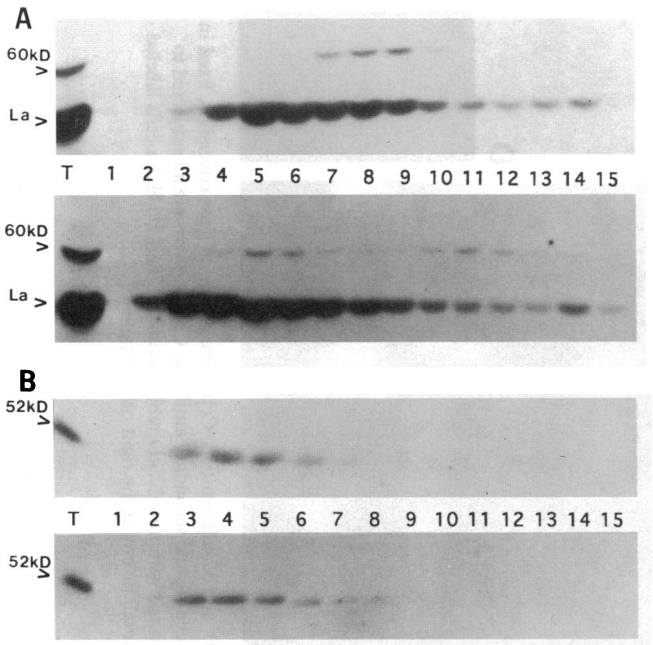

Figure 3. Immunoblot of gradient fractions from HeLa cell extracts pretreated with RNase A. Equivalent volumes of each gradient fraction were subjected to electrophoresis on 10% (*A*) or 15% (*B*) polyacrylamide gels then immunoblotted with a rabbit antiserum recognizing the 60-kD protein and a patient antiserum Go recognizing the La protein (*A*), or a patient antiserum So recognizing the 52-kD protein (*B*). The top of each panel shows the results using non–RNase-treated fractions of HeLa cell extracts and is equivalent to Fig. 1; the bottom of each panel shows the distribution of 60-kD Ro, La, and the 52-kD protein using HeLa cell extract incubated with 100 μg/ml of RNase A before gradient fractionation. Lanes are labeled as indicated in Fig. 1.

immunofluorescence microscopy with rabbit antisera against 60-kD Ro in comparison with patient antisera against the 52-kD protein to localize the particles intracellularly, concluding that the two proteins are present on overlapping subsets of structures (17). Fig. 4 shows immunofluorescence microscopy of two different human cell lines, HEp-2 and HeLa, immunoreacted with rabbit antisera against the two recombinant proteins of interest followed by Texas red–conjugated secondary antibody. A network of fine filaments was observed in the cytoplasm, in addition to bright perinuclear fluorescence and somewhat weaker, but nevertheless detectable, nuclear fluorescence when HEp-2 cells were reacted with the 60-kD rabbit antiserum (Fig. 4 *A*). The 52-kD antiserum, on the other hand, stained the nucleus more prominently than the cytoplasm in these cells, leaving the nucleoli largely unstained, and the overall pattern of fluorescence was more diffuse (Fig. 4 *B*). HeLa cells (Fig. 4, *C* and *D*) exhibited staining pattern differences with the two antisera generally similar to those of the HEp-2 cells. However, the perinuclear nature of the 60-kD immunofluorescence was slightly less pronounced in the case of HeLa cells (Fig. 4 *C*) at the same magnification, and the predominantly nuclear localization of the 52-kD immunofluorescence was less discernible in Fig. 4 *D* due to the high nucleus-to-cytoplasm ratio in HeLa cells. To eliminate the likelihood of misleading observations resulting from cell fixation protocols, two different methods of fixation (21, 22) were used and the results compared. Both acetone/methanol and a modified paraformaldehyde fixation (described in Methods) gave similar results for the two proteins. Our observations suggest very strongly that the 60- and 52-kD Ro proteins localize to different subcellular structures.

Discussion

The RoRNP has been redefined several times with respect to its molecular composition. Initially, only the 60-kD Ro and La were considered to be the protein components of RoRNPs, although it was assumed that La associated with the complex in a transient manner (4). However, hY5 RoRNPs have been isolated that carry La as a stable component (8). La and 60-kD Ro cDNAs were cloned several years ago (26–30) and found to harbor single copies of an RNA recognition motif (RRM), which is characteristic of many known RNA binding proteins (31, reviewed in reference 32). Both La and 60-kD Ro have been shown to interact directly with RNA (12, 28, 30). The 60-kD Ro protein also contains a potential zinc-binding finger motif in its peptide sequence (30). The conformation of 60-kD Ro is vital to its association with hY RNAs since small deletions at either the amino or carboxy terminus of the protein abolished binding (12, S. Deutscher and J. D. Keene, unpublished results, reviewed in reference 32). The 52-kD cDNA has also been cloned (16, 33) and found to share significant homology with *rpt-1*, a mouse T cell downregulatory protein, and with a human transforming protein, *rfp* (9, 16, 34). It harbors in its amino-terminal region a number of potential zinc fingers, within which is located a newly identified cysteine-rich motif found in a number of proteins thought to be DNA binding proteins (35). The 52-kD protein does not contain any RRMs and shares no homology with 60-kD Ro. It has not been shown to contact any of the hY RNAs directly, although anti-52-kD antibodies affinity purified from autoimmune sera were shown to immunoprecipitate RoRNPs along with the corresponding hY RNAs 1–5 (9). Interactions between the 52- and 60-kD proteins have been observed leading to the suggestion that association of 52-kD with hY RNAs is dependent on 60-kD Ro (13). However, a limitation of most of these studies has been the reliance on patient antisera, particularly with respect to the 52-kD protein.

We have used glycerol gradient fractionation of HeLa cell extracts to separate individual RoRNP particles in order to address uncertainties about the RNA and protein components of these particles. In HeLa cell extracts, the 52-kD protein was not detected in association with the 60-kD protein. Based upon its sedimentation coefficient, it appeared to exist as a free protein. Pretreating the extract with RNase A before gradient fractionation altered the behavior of both La and most of 60-kD in a manner that reflected the release of protein from an RNP, but did not affect the distribution of the 52-kD protein. This suggests that very little, if any, of the 52-kD protein is associated with either hY or any other detectable RNA at the time of extract preparation. 60-kD-containing particles appeared to be stable under the experimental conditions used and retained their integrity in the presence of varying concentrations of magnesium. Salt concentrations were maintained < 200 mM because of earlier reports that 60-kD complexes containing hY3 and hY4 RNAs begin to dissociate at salt concentrations of > 200 mM (8). Rabbit anti-52 and anti-60 sera prepared against recombinant proteins were used in the immunoprecipi-

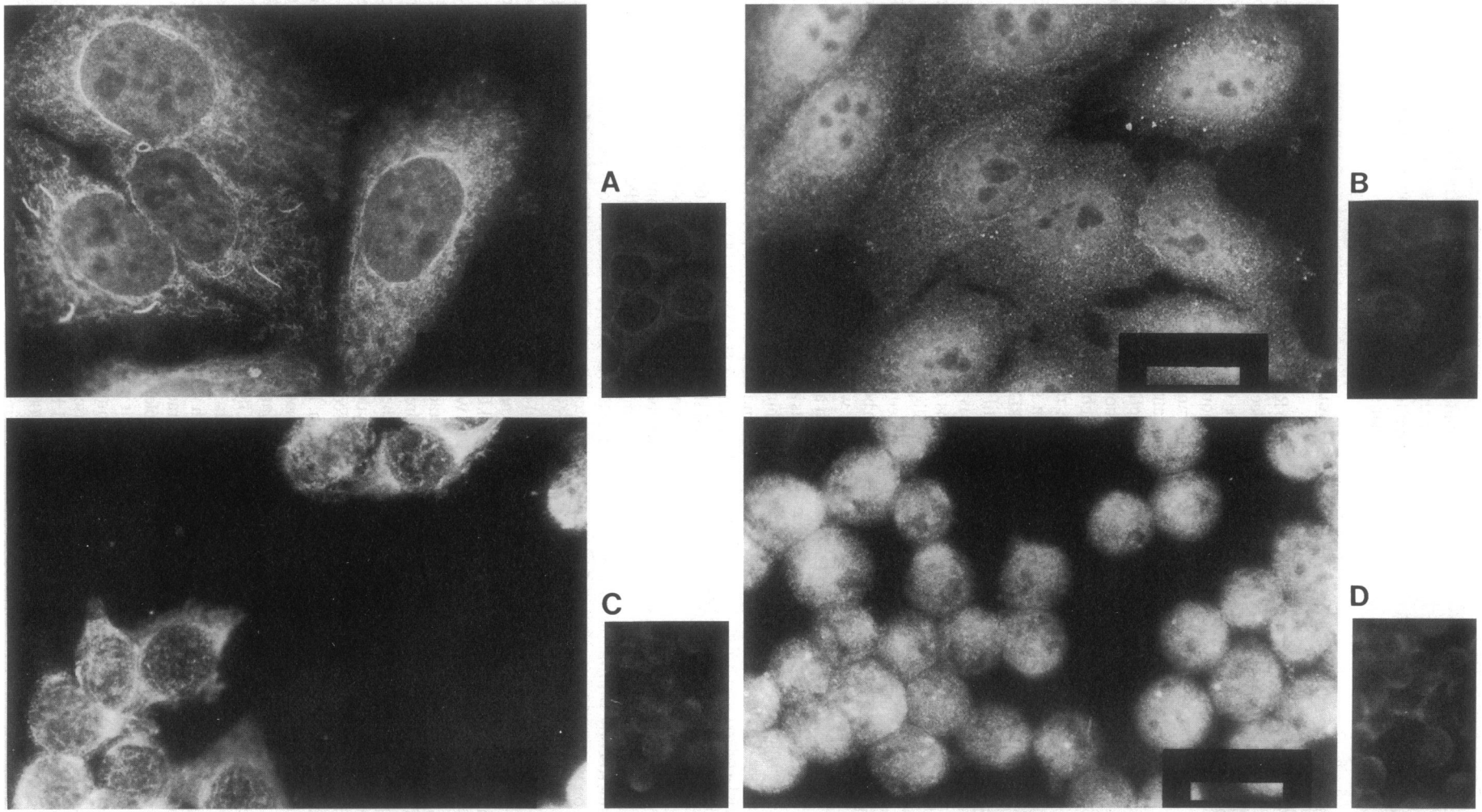

Figure 4. Immunofluorescence microscopy of HEp-2 and HeLa cell lines using rabbit antisera produced against recombinant 60- and 52-kD proteins. Cells were grown on glass coverslips, fixed in paraformaldehyde (see Methods), immunoreacted with either anti-Ro60 or anti-Ro52 serum, and then stained with Texas red–conjugated secondary antibody. Cells shown were all subjected to 15-s exposures and photographed at ×100 under oil. (*A*) HEp-2 cells labeled with anti-60-kD rabbit serum, and (*B*) HEp-2 reacted with anti-52-kD rabbit serum. (*C* and *D*) HeLa cells labeled with anti-60-kD and anti-52-kD sera, respectively. Control cells reacted with the corresponding preimmune sera are shown as small inserts at the right of each panel.

tation and most of the immunoblotting experiments. Of the two rabbit antisera, only the anti-60-kD was able to immunoprecipitate hY RNAs from ^{32}P-labeled HeLa cell (Fig. 3) or HEp2 cell extracts (M. R. Saitta and J. D. Keene, manuscript in preparation). Based upon these data, we conclude that RoRNPs do not contain any detectable 52-kD protein. However, although we consider it very unlikely, we cannot entirely rule out the possibility that a very small proportion of the total 60- and 52-kD Ro proteins interact in vivo.

We have also studied the gradient distribution of the hY RNAs, by RNase protection analyses to detect the total hY RNA, and by direct immunoprecipitation using rabbit antisera to detect the hY RNA complexed with protein in RoRNPs. In general, the hY RNAs could be detected in the same gradient fractions that contained 60-kD Ro. Our observations from both these approaches also confirm earlier reports that there is considerable heterogeneity among RoRNPs at the level of their RNA components (6).

Our explanation for the observations of other researchers who have reported coimmunoprecipitation of 60-kD when using affinity-purified 52-kD antibodies from patient antisera (9, 17) is that certain autoimmune patient sera may not react against a protein immobilized on a membrane while they would recognize it in solution. In other words, patient Ro antisera determined to be monospecific for 52-kD Ro by Western blotting may contain antibodies to discontinuous epitopes on 60-kD Ro that escape detection with this technique. In our hands, several "monospecific" anti-52-kD sera that did not detect 60-kD Ro by Western blotting were still able to immunoprecipitate in vitro translated 60-kD Ro protein (M. R. Saitta and J. D. Keene, manuscript in preparation). Another recent study (36) showed that, in the case of the 52-kD protein, largely sequential or linear epitopes not expressed on the surface of the native protein were recognized by anti-Ro sera. However, most of these antisera recognized only conformational epitopes on the 60-kD protein. Therefore, studies using affinity-purified 52-kD Ro antibodies may have been compromised by the presence of trace amounts of 60-kD antibodies that went undetected by Western blotting.

The results of our immunofluorescence studies using rabbit antisera prepared against recombinant proteins point to two important facts. First, RoRNPs may not be exclusively small cytoplasmic RNPs (scRNPs). Based on reports from several groups, including this study, RoRNP particles have been localized to different subcellular compartments including the cytoplasm, the nucleus, and intermediate filaments (4, 9, 14, 15, 25). In this study, we have provided results of immunofluorescence microscopy using two different cell lines commonly used in the study of RoRNPs, which suggest that 60-kD-containing particles can be localized both to the nucleus and to the cytoplasm. It is possible that individual 60-kD-containing RoRNP subpopulations perform different functions and localize differently within cells. This is an important question and one that may not be resolved until we develop more sophisticated methods to identify and biochemically separate these individual subpopulations.

Our observations also suggest that 52- and 60-kD Ro do not reside on similar or overlapping sets of structures. In both cell lines examined, the 52-kD rabbit antiserum resulted in a punctate nuclear fluorescence that has been reported earlier (9, 17) with some diffuse cytoplasmic staining. On the other hand, the 60-kD rabbit antiserum localized the 60-kD protein quite distinctly on filaments in the cytoplasm, as well as around the nuclear membrane. A similar filamentous pattern of staining has been previously observed and shown, by double immunofluorescence, to be almost identical to the staining pattern exhibited by cytokeratin (15). A nuclear component also reacted with this antiserum in both HEp-2 and HeLa cells, albeit more weakly. We conclude from the above observations, taken together with the results from gradient fractionations, that the 52- and the 60-kD Ro proteins are not present on common particles in cultured human cells under standard growth conditions.

The cooccurrence of 60- and 52-kD antibodies in a large number of SLE autoimmune sera (37) is intriguing. Our results suggest that although cooccurrence of autoimmune reactivities is typically observed for antigenic polypeptides found on the same RNP, this may not necessarily be true in every case. Itoh et al. (38) have provided evidence that suggests that the reactivity of denatured 52-kD Ro represents little more than a cross-reaction with autoantibodies directed against conformational epitopes on the 60-kD Ro antigen. The 52-kD protein does not resemble 60-kD Ro or any known RNA-binding polypeptide component of RNPs in its amino acid sequence. In fact, based upon sequence analysis, it can be predicted that the 52-kD protein is more likely to bind DNA (35). At present very little is known about the function of either the 52-kD protein or 60-kD-containing RoRNPs, but it is tempting to speculate that the 52-kD protein may be involved in regulating RoRNP function, and that the regulation may be manifest at the level of protein–DNA interaction.

Acknowledgment

We thank the members of the Keene laboratory for helpful suggestions, and for critically reading the manuscript.

A. Kelekar was supported by an Arthritis Foundation postdoctoral fellowship and a National Institutes of Health Tumor Immunology training grant (T32 CA-09058). M. R. Saitta was supported by a Physician Scientist Award from the NIH.

References

1. Lerner, M. R., and J. A. Steitz. 1979. Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. *Proc. Natl. Acad. Sci. USA.* 76:5495–5499.

2. Lerner, M. R., N. C. Andrews, G. Miller, and J. A. Steitz. 1981. Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. *Proc. Natl. Acad. Sci. USA.* 78:805–809.

3. Lerner, M. R., J. A. Boyle, J. A. Hardin, and J. A. Steitz. 1981. Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. 1981. *Science (Wash. DC).* 211:400–402.

4. Hendrick, J. P., S. L. Wolin, J. Rinke, M. R. Lerner, and J. A. Steitz. 1981. Ro small cytoplasmic ribonucleoproteins are a subclass of La ribonucleoproteins: further characterization of the Ro and La small ribonucleoproteins from uninfected mammalian cells. *Mol. Cell. Biol.* 1:1138–1149.

5. Wolin, S. L., and J. A. Steitz. 1983. Genes for two small cytoplasmic Ro RNAs are adjacent and appear to be single-copy in the human genome. *Cell.* 32:735–744.

6. Boire, G., and J. Craft. 1989. Biochemical and immunological heterogeneity of the Ro ribonucleoprotein particles. *J. Clin. Invest.* 84:270–279.

7. Rinke, J., and J. A. Steitz. 1982. Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La lupus antibodies. *Cell.* 29:149–159.

8. Boire, G., and J. Craft. 1990. Human Ro ribonucleoprotein particles: characterization of native structure and stable association with the La polypeptide. *J. Clin. Invest.* 85:1182–1190.

9. Ben-Chetrit, E., E. K. L. Chan, K. F. Sullivan, and E. M. Tan. 1988. A

52-kD protein is a novel component of the SS-A/Ro antigenic particle. *J. Exp. Med.* 167:1560–1571.

10. Rader, M. D., C. O'Brien, Y. Liu, J. B. Harley, and M. Reichlin. 1989. Heterogeneity of the Ro/SS-A antigen. Different molecular forms in lymphocytes and red blood cells. *J. Clin. Invest.* 83:1293–1298.

11. O'Brien, C. A., and J. B. Harley. 1990. A subset of hY RNAs is associated with erythrocyte Ro ribonucleoproteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3683–3689.

12. Pruijn, G. J. M., R. L. Slobbe, and W. J. van Venrooij. 1991. Analysis of protein-RNA interactions within Ro ribonucleoprotein complexes. *Nucleic Acids Res.* 19:5173–5180.

13. Slobbe, R. L., W. Pluk, W. J. van Venrooij, and G. J. M. Pruijn. 1992. Ro Ribonucleoprotein assembly *in vitro*. Identification of RNA-protein and protein-protein interactions. *J. Mol. Biol.* 227:361–366.

14. Xia, P., K. A. Fritz, W. D. Geoghegan, and R. E. Jordon. 1987. The particulate (speckled-like thread) nuclear staining pattern: species and cellular distribution of Ro/SSA antigen. *J. Clin. Lab. Immunol.* 22:101–105.

15. Bachmann, M., M. J. Werner, H. C. Schröder, K. Pfeifer, K-H. M. Z. Büschenfelde, and W. E. G. Müller. 1986. Association of La and Ro antigens with intracellular structures in HEp-2 carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 83:7770–7774.

16. Chan, E. K. L., J. C. Hamel, J. P. Buyon, and E. M. Tan. 1991. Molecular definition and sequence motifs of the 52kD component of human SS-A/Ro antigen. *J. Clin. Invest.* 87:68–76.

17. Slobbe, R. L., G. J. M. Pruijn, W. G. M. Damen, J. W. C. M. van der Kemp, and W. J. van Venrooij. 1991. The detection and occurrence of the 60 and 52 kDa Ro (SS-A) antigens and of antibodies against these proteins. *Clin. Exp. Immunol.* 86:99–105.

18. Weil, P. A., J. Segall, B. Harris, S. Ng, and R. G. Roeder. 1979. Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates. *J. Biol. Chem.* 254:6163–6173.

19. Lerner, E. A., M. R. Lerner, C. A. Janeway, and J. A. Steitz. 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: Probes for molecular biology and auto-immune disease. *Proc. Natl. Acad. Sci. USA.* 78:2737–2741.

20. Zinn, K., D. DiMiao, and T. Maniatis. 1983. Identification of two distinct regulatory regions adjacent to the human beta-interferon gene. *Cell.* 34:865–879.

21. Harlow, E., and D. Lane. 1988. Antibodies: A Lab Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

22. MacLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectronmicroscopy. *J. Histochem. Cytochem.* 22:1077–1083.

23. Reveillaud, I., M.-N. Lelay-Taha, J. Sri-Widada, C. Brunel, and P. Jeanteur. 1984. Mg^{2+} induces a sharp and reversible transition in U1 and U2 small nuclear ribonucleoprotein configurations. *Mol. Cell. Biol.* 4:1890–1899.

24. Wolin, S. L., and J. A. Steitz. 1984. The Ro small cytoplasmic ribonucleoproteins: identification of the antigenic protein and its binding site on the Ro RNAs. *Proc. Natl. Acad. Sci. USA.* 81:1996–2000.

25. Alspaugh, M. A., N. Talal, and E. M. Tan. 1976. Differentiation and characterization of autoantibodies and their antigens in Sjögren's syndrome. *Arthritis Rheum.* 19:216–222.

26. Ben-Chetrit, E., B. J. Gandy, E. M. Tan, and K. F. Sullivan. 1989. Isolation and characterization of a cDNA clone encoding the 60kD component of the human SS-A/Ro ribonucleoprotein autoantigen. *J. Clin. Invest.* 83:1284–1292.

27. Chambers, J. C., and J. D. Keene. 1985. Isolation and analysis of cDNA clones expressing human lupus La antigen. *Proc. Natl. Acad. Sci. USA.* 82:2115–2119.

28. Chambers, J. C., D. Kenan, B. J. Martin, and J. D. Keene. 1988. Genomic structure and amino acid sequence domains of the human La auto antigen. *J. Biol. Chem.* 263:18043–18051.

29. Chan, E. K., L. K. F. Sullivan, R. I. Fox, and E. M. Tan. 1989. Sjögren's Syndrome nuclear antigen B (La): cDNA cloning, structural domains and autoepitopes. *J. Autoimmun.* 2:321–327.

30. Deutscher, S. L., J. B. Harley, and J. D. Keene. 1988. Molecular analysis of the 60-kDa human Ro ribonucleoprotein. *Proc. Natl. Acad. Sci. USA.* 85:9479–9483.

31. Chan, E. K., L. K. F. Sullivan, and E. M. Tan. 1989. Ribonucleoprotein SS-B/La belongs to a protein family with consensus sequences for RNA binding. *Nucleic Acids Res.* 17:2233–2244.

32. Kenan, D. J., C. C. Query, and J. D. Keene. 1991. RNA recognition: towards identifying determinants of specificity. *Trends Biochem. Sci.* 16:214–220.

33. Itoh, K., Y. Itoh, and M. B. Frank. 1991. Protein heterogeneity in the human Ro/SSA ribonucleoprotein: the 52- and 60-kD Ro/SSA autoantigens are encoded by separate genes. *J. Clin. Invest.* 87:177–186.

34. Chan, E. K. L., J. C. Hamel, C. L. Peebles, J. P. Buyon, and E. M. Tan. 1989. Protein domain of the 52kD SS-A/Ro ribonucleoprotein particle is homologous to the N-terminal metal-finger of human *ret/rfp* and mouse *rpt-1* proteins. *J. Cell Biol.* 109:14*a* (Abstr.)

35. Freemont, P. S., I. M. Hanson, and J. Trowsdale. 1991. A novel cysteine-rich sequence motif. *Cell.* 64:483–484.

36. Itoh, Y., and M. Reichlin. 1992. Autoantibodies to the Ro/SSA antigen are conformation dependent. I. Anti-60 kD antibodies are mainly directed to the native protein: anti-52 kD antibodies are mainly directed to the denatured protein. *Autoimmunity.* 14:57–65.

37. Ben-Chetrit, E., R. I. Fox, and E. M. Tan. 1990. Dissociation of the immune responses to the SS-A (Ro) 52kD and 60kD polypeptides in systemic lupus erythematosus and Sjögren's syndrome. *Arthritis Rheum.* 33:349–355.

38. Itoh, Y., K. Itoh, M. B. Frank, and M. Reichlin. 1992. Autoantibodies to the Ro/SSA autoantigen are conformation dependent. II. Antibodies to the denatured form of 52 kD Ro/SSA are a cross reacting subset of antibodies to the native 60 kD Ro/SSA molecule. *Autoimmunity.* 14:89–95.