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Association of La and Ro antigens with intracellular structures in HEp-2 carcinoma cells

(small nuclear ribonucleoproteins/small cytoplasmic ribonucleoproteins/monoclonal antibodies/cytoskeleton)

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ABSTRACT **Monoclonal antibodies were raised against homogeneous Ro and La antigens, two proteins associated with Ro and La ribonucleoproteins (RNPs). The specificity of the monoclonal antibodies was proven by immunoblot analysis and by immunoprecipitation. The anti-Ro antibody reacted with a M_r 95,000 protein in a mouse lymphoma cell extract and with a M_r 60,000 polypeptide in extracts from human spleen. The anti-La antibody recognized a M_r 50,000 polypeptide in the mouse L5178y cell extract. The two monoclonal antibodies precipitated RNPs that contained the typical RNA species of Ro or La RNPs. The localization of Ro and La antigen was performed by direct immunofluorescence microscopy. It was found that the anti-Ro antibody reacted with a fibrous network that behaves like cytokeratin, one of the intermediate filament systems. The anti-La antibody reacted with nuclear structures that gave a speckled-type pattern.**

Four different major types of autoantibodies to ribonucleoproteins (RNPs) have been identified in patients with various rheumatic diseases: anti-U1 RNP, anti-Sm, anti-Ro, and anti-La antibodies (1). Several studies indicate that these patients often produce a set of antibodies recognizing different epitopes on RNPs (reviewed in ref. 2)—e.g., anti-Ro antibodies are mostly accompanied with anti-La antibodies (3, 4). RNA analysis revealed that Ro small cytoplasmic RNPs (scRNPs) carry the La as well as the Ro determinants and, consequently, were a subclass of La RNPs (5). Based on immunoprecipitation studies, the molecular weight of the Ro antigen(s) was determined to be 94,000 (90,000) (6), while analysis by immunoblotting revealed a M_r of 57,000 (7). Comparative experiments using the techniques of counterimmunoelectrophoresis and immunoblotting led to the assumption that the Ro antigenic determinant is labile when exposed to $NaDodSO_4$ (8). Recently we succeeded in the purification of the Ro antigen, characterized by a M_r of 94,000. Moreover, this protein was identified in the RNP particle exhibiting endoribonuclease VII activity (ref. 9; unpublished data). It is generally agreed that the molecular weight of the La protein is around 50,000 (2, 7). Anti-Ro antibodies were determined to immunoprecipitate five small RNAs from human cells termed hY1-5 (hY2 is processed from hY1) (10), while anti-La antibodies immunoprecipitate a highly banded spectrum of small RNAs (11).

Here we report a more extensive characterization of the Ro and La antigens by means of monoclonal antibodies (mAbs) raised against them. We show that the Ro antigen is associated with cytokeratin, while the La antigen primarily resides in the nucleus.

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MATERIALS AND METHODS

Materials. Materials were obtained as follows: CH-activated Sepharose 4B and protein A-Sepharose from Deutsche Pharmacia (Freiburg, F.R.G.); anti-mouse IgG (whole molecule from rabbit, peroxidase-conjugated; catalogue no. A 2028), deoxyribonuclease I (D 4763), ribonuclease A (insoluble enzyme, 120 units per g of solid; R 1626), anti-mouse IgG (I 5381), fluorescein isothiocyanate (FITC; F 7250), and rhodamine B isothiocyanate (RITC; R 1755) from Sigma; anti-cytokeratin mAb no. 18 (mouse) from Boehringer Mannheim; anti-α tubulin mAb (mouse) from Amersham Buchler International (Buckinghamshire, England); and rhodamine phalloidin from Molecular Probes (Junction City, OR).

Preparation of the Anti-Ro and Anti-La mAbs. Homogeneous Ro and La antigens were prepared from L5178y mouse lymphoma cells (38). Briefly, crude cell extracts were enriched for Ro and La antigens by ammonium sulfate fractionation (45–80% saturation), ribonuclease A treatment, and Sephadex G-150 gel filtration. This extract was further purified by immunoaffinity column chromatography with monospecific antibodies against the Ro and La antigens according to the procedure described below for the application of mAbs. The antisera were obtained from patients with autoimmune disorders. The homogeneity of the antigen preparations was proven by $NaDodSO_4$/gel electrophoresis, and their specificities, by immunoblotting (12) with standardized reference antisera (anti-Ro and anti-La; Centers for Disease Control, Atlanta). General procedures for preparation of mAbs (12, 13) were used. Female BALB/c AnHan mice were injected intraperitoneally with 100 μg of La or Ro antigen. After two further booster injections, spleen cells were prepared and fused with P3X63-Ag 8.653 myeloma cells. Culture supernatants were tested by an enzyme-linked immunosorbent assay (12) using a crude extract from L5178y mouse lymphoma cells as antigen at 1 mg/ml. Positive cultures were recloned (12), and the antibodies in the culture supernatants were isolated and purified by $(NH_4)_2SO_4$ precipitation [45% (wt/vol) final concentration] and by affinity chromatography on protein A-Sepharose (12). In a further step, the antibodies were purified by immunoaffinity column chromatography using normal anti-mouse IgG coupled to Sepharose 4B (14). The purification procedure was given earlier (14). The final preparations were supplemented with Merthiolate (0.02% final concentration) and stored at 4°C.

For the documented studies, one stable clone producing antibodies against the Ro antigen (Ro2F4) and a second clone producing anti-La antibodies (La1B5) were selected. Both antibodies belong to the IgG class, as proven by Ouchterlony

Abbreviations: FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate; RNP, ribonucleoprotein; scRNP, small cytoplasmic RNP; mAb, monoclonal antibody.

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test procedure using class-specific goat anti-mouse antibodies (Medac, Hamburg, F.R.G.).

Isolation of the La and Ro Antigen by Immunoaffinity Chromatography. L5178y mouse lymphoma cells (5×10^9) were harvested from the ascites of 12 mice and collected by centrifugation ($1000 \times g$ for 10 min at 4°C). The cells were washed with buffer A (10 mM Tris·HCl, pH 7.4/150 mM NaCl) and resuspended in 60 ml of buffer A and homogenized for 5 min by an Ultra Turrax (Janke-Kunkel, Staufen, F.R.G.). Subsequently, the homogenate was sonicated and centrifuged (15). The supernatant (crude extract) was brought to 45% saturation with $(NH_4)_2SO_4$. After centrifugation ($12,000 \times g$ for 15 min at 4°C), $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 80%. The precipitate formed was collected by centrifugation, dissolved in 20 ml of buffer A, and dialyzed against the same buffer. During dialysis, the fraction was treated with insoluble ribonuclease A (10 units) and deoxyribonuclease I (250 μg/ml) in the presence of protease inhibitors (leupeptin, chymostatin, and pepstatin, each at 5 μg/ml, and 0.5 mM phenylmethylsulfonyl chloride). Then the fraction was passed through a Sephadex G-150 column (4 × 30 cm); the antigen-containing fractions, which were eluted in a V_e/V_o range (16) between 1.3 and 1.9, were collected (260 ml) and concentrated by $(NH_4)_2SO_4$ precipitation (80% saturation).

The pellet (enriched antigen fraction) was dissolved in 40 ml of buffer A and subjected to immunoaffinity chromatography on mAb-conjugated Sepharose. The mAbs were coupled to CNBr-activated Sepharose 4B (10 mg of the respective mAbs per 2 ml of Sepharose 4B; ref. 17). The antigen-containing fraction was passed through the anti-La mAb-Sepharose column. The flow-through fraction was loaded again on this column. The washing procedure with buffer A was continued until the fractions were negative for La antigen in the enzyme-linked immunosorbent assay (12). For this procedure, 200 μl of the respective fraction was added to a well. After washing with phosphate-buffered saline (39), 1 μg of purified anti-La mAb was added per well, and the reaction was monitored with anti-mouse IgG peroxidase conjugate. The flow-through fraction was applied to the 2-ml anti-Ro mAb-Sepharose column and washed with 4 column volumes of buffer A. The La or Ro antigens were eluted from the corresponding mAb-Sepharose column with buffer B (200 mM glycine·HCl, pH 3.0/500 mM NaCl). The purified fractions were dialyzed against buffer A, lyophilized, and redissolved in buffer A at 1 mg/ml. To remove stable La/Ro complexes from free La antigen, the La eluate was passed again through the anti-Ro mAb-Sepharose column. The flow-through fractions were found to be negative for Ro antigen in the enzyme-linked immunosorbent assay. The obtained Ro and La antigens were assayed by immunoblotting (18) with standardized antisera from the Centers for Disease Control. The antigens were determined to be positive with the respective antisera.

$NaDodSO_4$/Polyacrylamide Gel–Nitrocellulose Sheet-Blotting Technique. Polyacrylamide (6%) gel electrophoresis in the presence of 0.1% $NaDodSO_4$ was performed by the system of Laemmli (19); the gels were stained with silver (20). Immunoblotting was performed as described (21). Briefly, after the electrophoretic transfer of the proteins, the nitrocellulose sheet (BA 85; Schleicher & Schüll, no. 401180) was incubated first for 4 hr in blocking solution (buffer A containing 1% bovine serum albumin) and second for 2 hr in buffer A containing 100 μg of a serum from a nonimmunized animal per ml. After the sheets were washed with buffer A, they were incubated (30 min) with the respective mAbs at 15 μg/ml in buffer A (supplemented with 0.1% bovine serum albumin) and subsequently washed with buffer A (supplemented with 0.5% Tween 20). The bound antibodies were detected by reaction with peroxidase-labeled rabbit anti-mouse IgG, followed by incubation with 4-chloro-1-naphthol (12).

For these experiments, crude extracts from L5178y cells (see above) or human spleen were used; they were prepared as described (22).

Immunoprecipitation and RNA Analysis. Ten milliliters of a human spleen extract, isolated as described by Mattioli and Reichlin (22) (protein concentration, 10 mg/ml), was incubated (15 min at 37°C) with 500 μg of anti-Ro or anti-La mAb. The formed immunocomplexes were isolated by affinity chromatography on protein A-Sepharose (23). RNA was isolated from these complexes and fractionated on one-dimensional polyacrylamide gels as described (15).

Labeling of Antibodies. mAbs against the Ro and La antigen were conjugated with fluorescein isothiocyanate (FITC), whereas mAbs to cytokeratin and mAbs to α tubulin were labeled with rhodamine isothiocyanate (RITC) as described (24).

Immunofluorescence Microscopy. Human epithelioma cells (HEp-2) were obtained from Antibodies Inc. This cell line was originally propagated from a human laryngeal carcinoma. They were grown on coverslips, washed with phosphate-buffered saline, and fixed in acetone at −20°C for 5 min. For double-staining, the general procedures described by Vandesande (25) were followed. Directly FITC-labeled anti-Ro mAb was applied first, followed by RITC-labeled anti-cytokeratin or anti-α tubulin antibody or RITC-phalloidin. Stained cells were analyzed with a Leitz Orthoplan microscope equipped with epifluorescence optics. A super-pressure mercury lamp served as light source. An excitation filter BP 450–490 (for fluorescence of FITC) or BP 530–560 (for RITC) was used; a cut-off filter LP 515 (FITC) or LP 580 (RITC) was inserted into the microscope tube. Photographs were taken with oil-immersion ×100 objectives and Kodak 400 Tri-X Pan film. Control experiments revealed that these filter systems were specific for the fluorochrome dye used (results not shown).

Analytical Methods. Protein concentration was determined spectrophotometrically at 230 and 260 nm (26).

RESULTS

Characterization of the mAbs Against the Ro and La Antigen. BALB/c mice were immunized with homogeneous Ro or La antigen to obtain mAbs directed against these proteins. Two hybridoma clones, one producing anti-Ro antibodies (Ro2F4) and a second one secreting anti-La antibodies (La1B5), were selected. Both types of mAbs belong to the IgG class.

Fig. 1*A* shows that, by application of an immunoaffinity chromatographical procedure, the anti-La mAb reacted with a M_r 50,000 polypeptide (lane a), and the anti-Ro mAb reacted with two proteins of M_r 95,000 and M_r 90,000 (lane b) in the enriched antigen fraction from L5178y cells. Moreover, immunoblot analysis (Fig. 1*B*) confirmed the reliability of the antibody–antigen reactions both for La (lane b) and Ro (lane c), determined in the immunoaffinity experiments.

Immunoprecipitation experiments were performed to define the mAbs as anti-Ro or anti-La (Fig. 2*A*). The anti-Ro mAb was shown to react with RNPs containing the four major RNA species, designated hY1 and hY3–hY5 (lane a). This RNA pattern has been shown to be typical for human Ro scRNPs (2, 5). In contrast, anti-La mAb reacted with RNPs containing a more complex RNA pattern (lane b). A similar RNA composition has been found to be characteristic for La RNPs (2, 5).

In accordance with the literature (reviewed in ref. 2), we found that a M_r 50,000 polypeptide carries the La antigenic determinant (Fig. 1). The anti-Ro mAb studied in the present investigation bound to M_r 95,000 and M_r 90,000 polypeptides

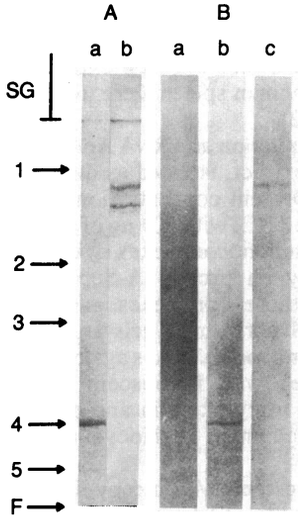

FIG. 1. Characterization of anti-Ro and anti-La mAbs. (*A*) $NaDodSO_4$/polyacrylamide gel analysis of La antigen (lane a) and Ro antigen (lane b) isolated from L5178y mouse cells by immunoaffinity chromatography with anti-La and anti-Ro mAb-Sepharose columns. (*B*) $NaDodSO_4$/polyacrylamide gel–nitrocellulose sheet blot. A freshly prepared antigen-enriched extract from L5178y mouse cells was fractioned by $NaDodSO_4$/polyacrylamide gel electrophoresis; the proteins were electrophoretically transferred onto nitrocellulose sheets and treated with control mouse IgG (lane a), mouse anti-La mAb (lane b), or mouse anti-Ro mAb (lane c). The proteins were identified by reaction with anti-mouse IgG. Molecular weight standards (arrows): 1, phosphorylase a (M_r 98,000); 2, transferrin (M_r 77,000); 3, bovine serum albumin (M_r 66,000); 4, gamma globulin (heavy chain) (M_r 50,000); 5, ovalbumin (M_r 45,000). SG, stacking gel; F, front. Further details are given in text.

present in the crude extract from L5178y cells (Fig. 1). Usually, anti-Ro mAbs have been determined to bind to a M_r 60,000 protein (reviewed in ref. 2). Therefore, we performed comparative studies (Fig. 2*B*) and could demonstrate that

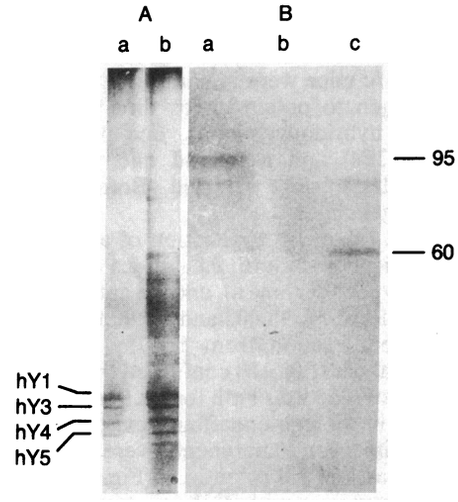

FIG. 2. (*A*) Immunoprecipitation of RNPs from crude human spleen extract *in vitro*. The extract was treated with anti-Ro or anti-La mAb. The formed immunocomplexes were isolated, and their RNA constituents were extracted and analyzed by gel electrophoresis. RNA patterns of RNPs precipitated by anti-Ro mAb (lane a) or anti-La mAb (lane b) are shown. Lines mark the position of the small RNAs hY1 and hY3–hY5. (*B*) Immunoblot experiments with a crude extract from mouse L5178y cells (lane a) and from human spleen (lane c). Lane b is a control with anti-mouse IgG (peroxidase-conjugated). The proteins were treated with anti-Ro mAb as described.

indeed our mAb against the Ro antigen recognized predominantly a M_r 60,000 polypeptide in the crude extract from human spleen (Fig. 2*B*, lane c), whereas it reacted with a M_r 95,000 (50,000) protein in the mouse L5178y cell extract (Fig. 2*B*, lane a).

Intracellular Localization of Ro and La Antigens in HEp-2 Cells. Immunofluorescence staining of HEp-2 cells with mAbs to Ro antigen revealed that this antigen is organized in the cytoplasm in a filamentous network (Fig. 3). To determine whether the fine network of fibers, visualized by

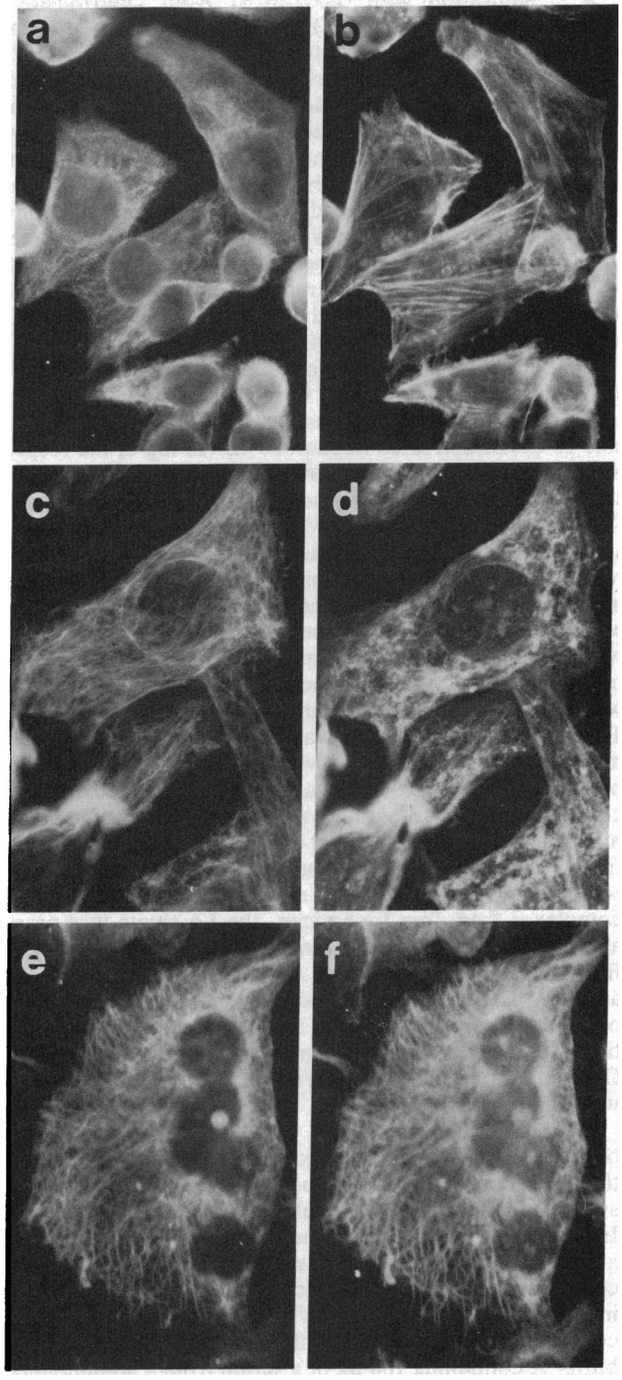

FIG. 3. Double direct immunofluorescence microscopy of HEp-2 cells. Cells were treated with FITC-labeled anti-Ro mAb (*a–f*), followed by RITC-labeled phalloidin (*a* and *b*) or RITC-labeled mAbs to α tubulin (*c* and *d*) or RITC-labeled mAbs to cytokeratin (*e* and *f*). (*a*, *c*, and *e*) Fluorescein patterns. (*b*, *d*, and *f*) Rhodamine pattern of the same cell. (×1100.)

FITC-labeled anti-Ro mAb, has similarities to one of the three major fibrous structures in animal cells, we performed double direct immunofluorescence studies with RITC-labeled anti-α tubulin (staining of microtubules), RITC-labeled phalloidin (staining of microfilaments), or RITC-labeled anti-cytokeratin (staining of intermediate filaments). Observations by this technique revealed that the pattern obtained with anti-Ro mAb is not identical with that network reacting with phalloidin (Fig. 3 *a* and *b*) or with anti-α tubulin antibodies (Fig. 3 *c* and *d*). However, an obviously complete superposition of the labeling pattern is obtained with anti-cytokeratin and anti-Ro antibodies (Fig. 3 *e* and *f*).

In contrast to the Ro antigen, the La antigen was localized predominantly in the nucleus (Fig. 4 *a* and *b*). The pattern is of the speckled type.

DISCUSSION

Ro and La proteins are known to be prone to partial proteolysis in crude tissue extracts (7). In the present study we used mAbs directed against the Ro and La proteins to determine the molecular weight of these antigens in different tissues by immunoblotting. The anti-La mAb identified a single M_r 50,000 immunoreactive polypeptide in a crude extract from L5178y mouse cells; this finding is in agreement with other studies (2). The anti-Ro antibody recognized two proteins of M_r 95,000 and M_r 90,000 in this extract. Comparative studies revealed that this anti-Ro antibody reacted predominantly with a M_r 60,000 polypeptide in a human spleen extract. The latter finding is in accordance with other reports (2). The reason for the discrepancy in the molecular weight of the Ro antigen in the different tissues is unknown. One explanation may be that the M_r 60,000 polypeptide results from proteolytic degradation or is a processed form of the M_r 95,000 protein (that is also visible in the blot from human spleen extract; see Fig. 2*B*, lane c) as suggested earlier (7). The specificity of the anti-Ro and anti-La mAbs was additionally established by immunoprecipitation experiments. It was found that anti-La and anti-Ro mAbs reacted with distinct RNP particles comprising the respective typical RNA species (2, 5, 10, 11).

We used the defined mAbs for an analysis of the intracellular distribution of La and Ro antigen in the human epithelioma cell line HEp-2. By immunofluorescence it was shown that the anti-La mAb gave a characteristic speckled nuclear fluorescence (27) and little cytoplasmic staining. At present it is unknown with which supramolecular structures the La antigen, characteristically described as nuclear antigen (5, 28, 29), is associated. It might be interesting that a similar speckled intranuclear pattern has been obtained by Chaly *et al.* (30), using a mAb produced against isolated nuclear matrices, and by van Venrooij and his colleagues (31), using sera from patients with mixed connective tissue disease.

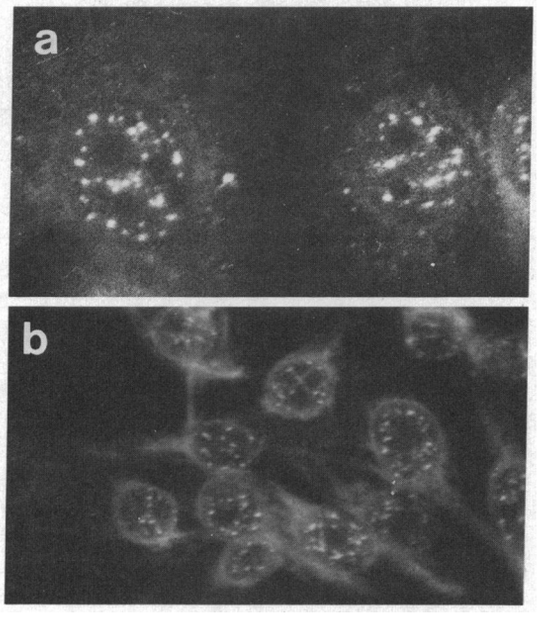

FIG. 4. Single direct immunofluorescence microscopy of HEp-2 cells with FITC-labeled anti-La mAb. (*a*, ×1600; *b*, ×600.)

The Ro antigen is localized in the cytoplasm and found to be associated with one of the three major cytoskeletal elements, the intermediate filaments. By using the technique of double immunofluorescence staining, mAbs to the Ro antigen and to cytokeratin stained the same filamentous system in the cytoplasm of HEp-2 cells. In preliminary experiments, it was found that in other cell types not containing cytokeratin, other intermediate filament systems are stained with mAbs to the Ro antigen (unpublished data). No significant superposition of the patterns obtained with anti-Ro and anti-tubulin or rhodamine-phalloidin (which stains actin; ref. 32) was obtained.

The biological function of the Ro scRNPs remains to be discovered. It had been speculated that they participate in translation-related events (33)—perhaps in selection of distinct subsets of mRNAs abundant in brain and heart (4). Thus, the presented finding that the Ro antigen is associated with cytokeratin filaments corroborates the intriguing possibility that binding of mRNA to this cytoskeletal element might be required for translation (34–36). This view is also consistent with the finding of Zumbé *et al.* (37), indicating cap-binding proteins to be involved in linking host mRNAs to intermediate filaments.

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