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Methods to Detect Antifibrillarin Antibodies in Patients With Systemic Sclerosis (SSc): A Comparison

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Autoantibodies against nucleolar antigens are common in systemic sclerosis (SSc). They include autoantibodies against fibrillarin (Fb), which are serological markers for SSc. Fb is associated with the evolutionally-conserved box C/D of small nucleolar RNAs (snoRNAs). We compared indirect immunofluorescence (IIF), Western blot (WB), and immunoprecipitation (IPP) of total small RNAs assays to determine which of these techniques is most specific for the detection of snoRNPs. We also examined the frequency and specificity of autoantibodies from SSc patients to snoRNAs, snRNAs, and scRNAs, and concluded that 1) IIF can not determine autoantibody specificity against Fb, 2) 36% of SSc sera were false-negative by WB, and 3) by IPP, anti-Fb autoantibodies from SSc patients can bind U3, U8, U13, U15, and U22 snoRNAs. J. Clin. Lab. Anal. 18:19–26, 2004. © 2004 Wiley-Liss, Inc.

Key words: immunofluorescence; Western blot; immunoprecipitation snoRNAs; rheumatic diseases

INTRODUCTION

Autoantibodies against nucleolar self-antigens are characteristic of several forms of scleroderma (systemic sclerosis (SSc)), a rheumatic disease with a heterogeneous clinical pattern that includes localized and systemic disease characterized by fibrosis of the skin and internal organs. Autoantibodies against fibrillarin (Fb), DNA topoisomerase I (Scl 70), and RNA polymerases I–III are serological markers for SSc. Anticentromere antibodies and anti-Th antibodies characterize CREST syndrome (calcinosis cutis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasias), and antibodies against U1snRNP, anti Ku, anti PM/Scl, anti SSA/Ro, anti SSB/La, and anti NOR 90 have also been described in SSc and overlapping syndromes. The frequency of detection depends to some extent on the methods used (1–8).

In most cases, antibodies recognize molecular complexes that are formed by one or several small RNAs and proteins. These complexes, called ribonucleoproteins (RNPs), are located in the nuclei, nucleolus, and cytoplasm, and contain different classes of RNA. For instance, snRNPs containing Sm are located in the nucleus, constitute the spliceosomal machinery, and consist of U1, U2, U4/U6, and U5 snRNAs with at least 20 proteins (9,10). Nucleolar RNP (snoRNPs) are formed by several snoRNAs associated with Fb, a 34kD protein. These snoRNAs have a number of important characteristics: they are transcribed by RNA polymerase II (usually in their 5′ caps) to convert hypermethylated (four or more methyl group) to 2,2,7 trimethylguanosine, and are coded in the intronic region of some genes. The snoRNAs can be divided into two major classes. The first class is involved in specific 2′-O-methylation of the methyl group of 2′-O of the ribose

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from rRNA, and in pre-rRNA processing. They possess two conserved sequences called box C/D (these boxes interact with the Fb 34kD protein) (11–13) and include U3, U8, U13, U14, U15, U16, U18, U20, U21, U22, and U24 snoRNAs. The members of the second class (U17, U19, U23, U64, and U72 snoRNAs without box C/D) possess different conserved sequences, called box H/ACA, and are related to pseudourydilation (14–16).

Fb is a 34kD protein that is located in the dense fibrillar component (DFC) of the nucleolus (as determined by immunoelectromicroscopy). Human Fb contains 320 a.a. and consists of three different domains. The glycine-rich amino terminal domain is 80 a.a. in length and contains several residues of dimethylated arginine. The second is a central domain of 90 a.a. residues, in which is located (in position 185-192) the RNA binding site to the box C/D of the snoRNAs. The carboxy terminal domain adopts an α-helical conformation, and has not been shown to possess any defined structural or functional motifs. The snoRNPs play a role in ribosomal RNA transcription, rRNA processing, and ribosomal biogenesis (17–30).

Most of the methods used to search for autoantibodies associated with SSc employ antibodies against protein components of RNP. Therefore, we analyzed sera from 31 patients with SSc using indirect immunofluorescence (IIF), Western blot (WB), and immunoprecipitation of total small RNAs (IPP) assays in order to compare these techniques in terms of their ability to detect anti-Fb antibodies, and to assess their association with snoRNPs.

MATERIALS AND METHODS

Clinical Data and Sera

We studied 31 consecutive patients in whom a diagnosis of SSc had been firmly established. All of the patients fulfilled the classification criteria recommended by the American College of Rheumatology (31). Serum samples were obtained by antecubital vein puncture, stored frozen, and will be studied at once. As positive controls in our assays, we used mouse monoclonal antibodies against human Fb (72B9 and 17C12, from Dr. Eng Tan and Dr. Michael Pollard, respectively), a polyclonal human antibody anti-Fb (from Dr. Marvin Fritzler); a mouse monoclonal antibody against human Sm antigen (Y-12, from Dr. Joan A. Steitz); and a polyclonal antibody anti-Ro 60kD protein (RoM) gentile (donated by Dr. Sandra Wolin). The negative control was pooled normal human sera, which in a regular antinuclear antibody (ANA) immunofluorescence assay did not react against cell structures.

IIF and Slide Preparation

HeLa monolayer cells were grown at an approximately 75–80% confluence on chambered slides. They were maintained at 37°C under 5% CO^2 in DMEM, supplemented with 10% heat-inactivated calf serum, 292 μg/mL L-glutamine, 60 μg/mL penicillin, and 100 μg/mL streptomycin. The cells were prepared by removing the medium from the slide chambers and rinsing the slides in PBS (134.5 mM NaCI, 3.5 mM KCI, 10.5 mM Na_2HPO_4, pH 7.2). After fixation in 4% paraformaldehyde PBS for 10 min at room temperature, the slides were quenched in 0.1 M glycine in PBS for 5 min. They were then permeabilized in either 0.5% Triton X-100 in PBS for 5 min at room temperature, or in –20°C acetone for 10 min each. We found no difference in detection of Fb (33) with either extraction method. In the IIF assay, sera at 1:50 dilution were incubated over the slides for 30 min at room temperature in a humidified chamber and rinsed twice with PBS for 10 min each. This was followed by incubation for 30 min at room temperature in a humidified chamber with antihuman IgG specific antibody conjugated to fluorochrome FITC or Texas Red (Sigma, St. Louis, MO). The slides were rinsed twice with PBS for 10 min each. Then in some cases the slides were stained with DAPI (4,6-diamino-2-phenylindole, 200 ng/mL in PBS; Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 10 min, destained for 5 min with 100% ethanol, and then covered and mounted in 90% glycerol. Images were acquired with an epifluorescence microscope equipped with a photomicroscopy system (Axioskop; Carl Zeiss, Jena, Germany) (32–34).

Cells and Whole-Cell Sonicated RNA Extract

Human HeLa cells were maintained at 37°C under 5% CO^2 in RPMI-1640, supplemented with 10% heat-inactivated calf serum, 292 μg/mL L-glutamine, 60 μg/mL penicillin, and 100 μg/mL streptomycin. HeLa cells were grown to 1×10^5 cells per mL, collected by centrifugation at 2,000 rpm for 5 min at 4°C, and washed three times in chilled TBS (40 mM Tris-HCI, 150 mM sodium chloride, pH 7.4). The harvested cells were disrupted by sonication in NET-2 buffer (150 mM sodium chloride/50 mM Tris-HCI, 0.05% Nonident P-40, pH 7.5), 3 × 30 sec, setting 3, and were incubated on ice for 30 sec between sonications (Sonics & Materials, Inc., Danbury, CT). Finally, the whole-cell sonicate was spun down at 12,000 rpm for 10 min at 4°C (35).

SDS-PAGE and WB

The proteins present in the whole-cell sonicate HeLa cell extract were then separated on 10% SDS-PAGE and transferred to a nitrocellulose sheet. The antigen-antibody reaction was developed with 3,3′-diaminobenzidine. We used a pool of proteins with a molecular range of 12–200 kD (36,37) as molecular markers.

Immunoprecipitation of RNA and RNA Analysis

Protein A-Sheparose-CL-4B, preswollen in NET-2 buffer, was incubated with 10 μL of crude serum overnight at 4°C, and then washed five times with NET-2. The antibody-bound beads were incubated for 2 hr at 4°C with an aliquot of whole-cell sonicate HeLa cell extract (corresponding to 10 mL of cells) and washed five times with NET-2. These samples were immunoprecipitated with phenol-chloroform-isoamyl-alcohol (50:50:1, vol/vol; PCA) and precipitated with 2.5 volume of 100% ethanol absolut and 10% volume of 3M sodium acetate, followed by 3′-end labeling with [5′-^{32}P]pCp (NEN Life Science Products, Boston, MA) and T4 RNA ligase (New England Biolabs, Beverly, MA) (36). The RNAs were then separated on 10% polyacrylamide gels (Sequagel; National Diagnostics, Atlanta, GA). In these assays the positive controls were the same as those used in the IIF assay, with the addition of a total RNA standard. As molecular markers we used a pBR322 DNA-Msp-I- digest (New England Biolabs) and [γ-^{32}P]ATP (NEN Life Science Products), labeled with T4 polynucleotide kinase (New England Biolabs) (38,39).

RESULTS

ANA by IIF

Most patients with SSc had fluorescent ANA when tested on HeLa cells: 84% of the SSc sera were ANA-positive, and only five of 31 sera (16%) had no demonstrable ANA. Eleven sera showed a nucleolar pattern (36%), which was the most common pattern. Homogeneous nuclear and speckled patterns were seen in five sera each, and nucleoplasmic and anticentromere antibodies were detected in two cases each. One serum sample showed an annular nuclear fluorescent pattern. The negative control did not react against any cell structures in any of the experiments (Fig. 1, Table 1).

WB Assay

When antibody specificity was examined using a WB assay, which revealed human IgG antibodies on transferred total HeLa cell extract, only nine sera (29%) from all of the SSc patients studied identified the Fb 34kD protein, and 22 sera (61%) failed to recognize this antigen. However, some sera reacted against proteins that are not related to Fb.

Molecular Specificity and Frequency of Autoantibodies Against snoRNAs in Patients With SSc

Immunoprecipitation detected anti-Fb-associated snoRNAs and other snRNAs and scRNAs more frequently than did autoantibodies in the Wb assay. Fifteen sera recognized Fb-associated snoRNAs, six (20%) reacted exclusively against such protein nucleic acids; nine sera reacted with snoRNAs, snRNAs, and scRNAs (29%); and only one serum was specifically associated with hY1, hY3, hY4, and hY5 scRNAs (which are related to the Ro 60 kD protein antigen. Fifteen (48.5%) additional SSc sera did not recognize RNAs at all (Table 2).

A comparison of IIF, Wb, and IPP in terms of their ability to locate autoantibodies and anti-Fb specific antibodies in SSc sera is displayed at Table 3. SSc sera, which reacted with annular, ACA, or nucleoplasm patterns were always negative for Fb 34kD protein or snoRNA-associated moieties. Nuclear, nucleolar, or speckled IIF patterns were present when anti-Fb or anti-snoRNA antibodies were detected using the Wb or IPP assays. Twelve sera recognized U15, and 11 were associated with U3 snoRNA. Five sera reacted against U13, four sera reacted against U22, and only three sera reacted against U8 (in different combinations). A representative figure showing the IPP patterns observed in SSc sera is displayed in Fig. 2.

DISCUSSION

This study presents a comparative analysis of the IIF, WB, and IPP assays in terms of their ability to detect antibodies against anti-Fb (snoRNPs) in the sera of patients with SSc.

The indirect fluorescence ANA test, which is used as a screening test, is usually positive in SSc patients, and most commonly reveals nucleolar and speckled patterns (40,41). In our study we found ANA in 84% of the patients. A nucleolar pattern was seen in 36%, followed by speckled nuclear and nuclear

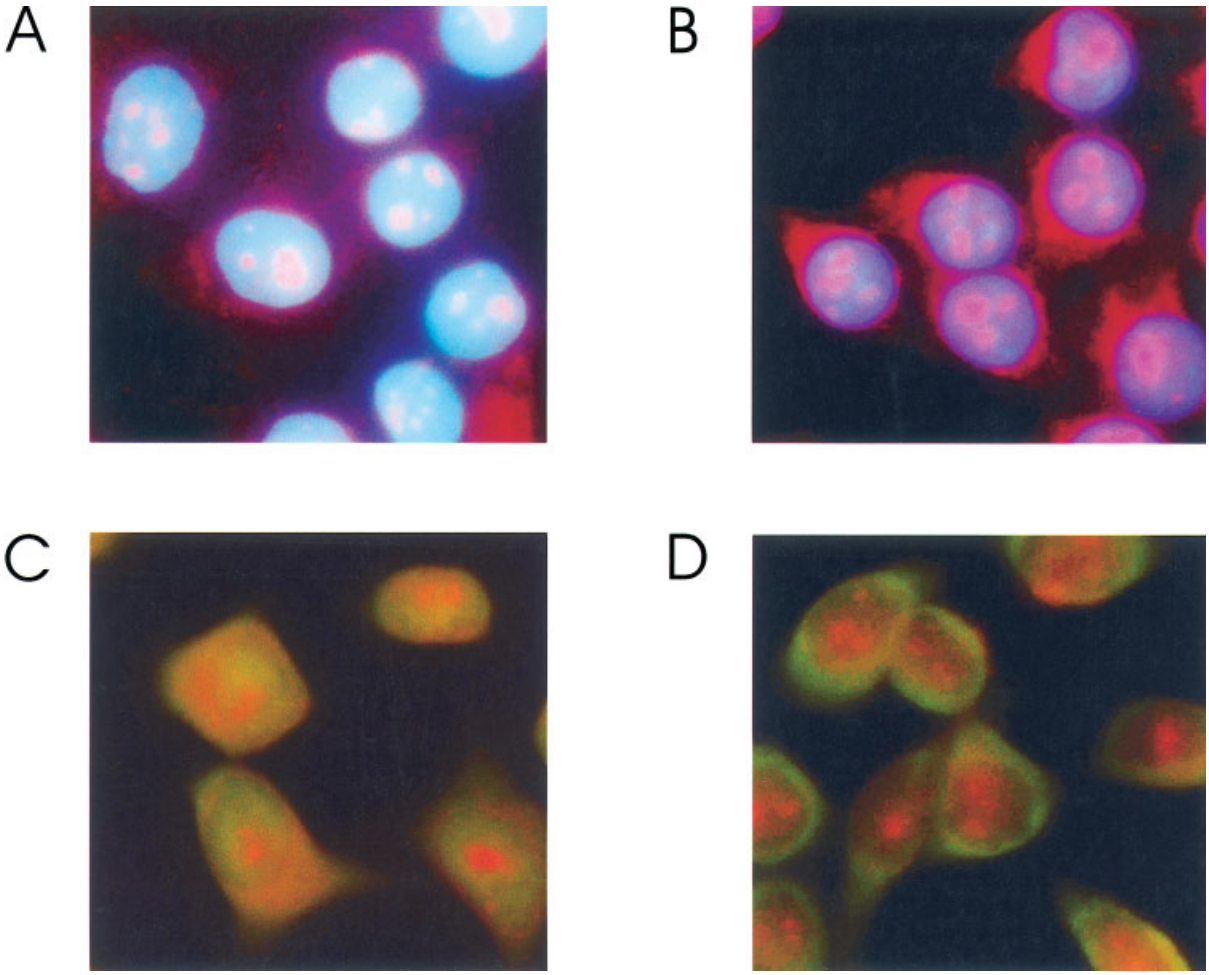

Fig. 1. IIF in HeLa cells using monoclonal or polyclonal antibodies antihuman Fb, Sm, Ro, and U1RNP. **A:** A classic fluorescence pattern by double fluorescence labeling of mouse monoclonal antibody antihuman Fb in red and DAPI in blue. **B:** A classic fluorescence pattern of polyclonal autoantibody antihuman Fb with double labeling (DAPI and Red Texas). **C:** A fluorescence pattern by double labeling, the red in the nucleolus correspond to human antibody anti-Fb and the nuclear spleckleed pattern in green to human anti-Sm antibody. **D:** The detection of Fb in red was observed using a human autoantibody and in green we clearly see a predominant cytoplasm pattern using human autoantibody anti-Ro 60kD.

TABLE 1. Immunofluorescence patterns in sera of patients with SSc

Pattern	Number sera	Frequency
Nucleolar	11	36%
Nuclear	5	16%
Speckled nuclear	5	16%
Nucleoplasmic	2	6.5%
ACA	2	6.5%
Annular	1	3%
Negative	5	16%
Total	31	100%

Western Blot: Only 9 (29%) of the sera patients with SSc identified the Fb protein (34kD protein–antigen). The other 22 (61%) sera patients showed reactivity with other proteins not related with Fb.

patterns in 16%, ACA and nucleoplasmic patterns in 6.5%, and an annular pattern in 3%. Peripheral and annular patterns are only observed when the patients have SSc/SLE overlap syndrome. An anticentromere pattern was present in two sera from patients with clinical features consistent with CREST syndrome.

Several researchers have reported problems with assessing the specificity of autoantibodies using enzyme immunoassay or WB techniques (42–45), particularly in the detection of Fb 34kD protein antigen, because 1) the antibody has low reactivity to the antigen, 2) some anti-Fb antibodies are non-blotter sera, 3) the protein Fb is scarce in the total cell

TABLE 2. Molecular specificity and frequency of snoRNAs, snRNAs And scRNAS detected by autoantibodies from patients with SSc, using IPP

Specificity	Sera number	Frequency
Anti-Fb	6	19.5%
Anti-Fb-Sm	2	7%
Anti-Fb-Ro–Sm	2	7%
Anti-Fb-Ro	1	3%
Anti-Fb-U4-U5-hY5	1	3%
Anti–Fb-Ro-U1	1	3%
Anti-Fb-Ro–U4-U5	1	3%
Anti-Fb-Ro-U2-U1-U4	1	3%
Anti-Ro	1	3%
Associated with RNAs	16	51.5%
Negative	15	48.5%
Total	31	100%

Anti–Fb (U3, U8, U13, U15 or U22 snoRNAs); Anti–Ro (hY1, hY3, hY4 or hY5 scRNAs); Anti–Sm (U1, U2, U4 or U5 snRNAs); Anti–U1nRNP (U1 snRNA).

TABLE 3. Comparative analysis of IIF, WB and IPP techniques in the detection of Fb-snoRNP in patients with SSc

Sera	IIF	WB	IPP de RNAs
1	Annular	–	–
2	ACA	–	–
3	Nucleolar	–	–
4	Speckled Nuclear	–	–
5	Nucleolar	Fb	U13–U15
6	Nuclear	–	U3–U15
7	Speckled Nuclear	Fb	U3
8	Nucleolar	Fb	U3
9	Speckled Nuclear	–	U3–U15
10	Nucleolar	–	U15
11	Nuclear	–	U3–U22–U8
12	Nucleoplasmic	–	–
13	Nuclear	Fb	U8–U15–U22
14	Nucleoplasmic	–	–
15	Speckled Nuclear	–	–
16	Nucleolar	Fb	U3–U15
17	Speckled Nuclear	–	–
18	Nucleolar	Fb	U3–U8–U15
19	Nucleolar	Fb	U3–U13–U15–U22
20	Negative	–	–
21	Nucleolar	–	–
22	ACA	–	–
23	Nuclear	–	U13–U15
24	Nuclear	–	–
25	Negative	–	–
26	Nucleolar	–	U3–U13–U15–U22
27	Nucleolar	Fb	U3–U15
28	Negative	–	–
29	Negative	–	–
30	Nucleolar	Fb	U3–U13–U15
31	Negative	–	–

protein content, and 4) the Fb 34kD protein undergoes conformational modifications during the extraction procedures that alter its affinity for antibody. This may also explain why we detected the Fb 34kD antigen by autoantibodies from patients with SSc in only nine sera (29%) with the WB assay. However, these nine sera were associated with snoRNAs by IPP, which confirms the association of Fb 34kD protein with the snoRNAs.

The frequency of detection reported for Fb in patients with SSc is 4–18% (1–6). In this study, using the IPP of RNAs, we found that 15 SSc sera (48%) were able to bind snoRNAs. Additionally, sera bind to other species of RNAs; therefore, our results show the diversity of RNA-related autoantibodies that are present in patients with SSc. Usually the anti-Fb autoantibodies in patients with SSc are associated with the U3 snoRNA (1,5–7,44,45), but anti-Fb autoantibodies can also bind to U8, U13, U15, and U22 snoRNAs, and may bind to other snoRNAs that possess the two evolutionally-conserved sequences called box C/D (9,12,17,18). It has been reported that anti-U1 (8%), anti-U2 (15%), anti-U4/U6 RNPs (1%), and anti-Sm (3%) antibodies are present in the sera of patients with scleroderma overlap syndrome and SLE-scleroderma overlap syndrome, and the antibodies anti-Ro/anti-La are present in SSc patients (1%); however, no studies have associated these autoantibodies with Fb snoRNPs (1,5–7,46). Here we have shown that the sera of patients with SSc have different specificities and frequencies of autoantibodies (Table 2).

From our comparison of the techniques used to detect molecular RNA-protein nucleolar complexes, we conclude that 1) IIF in patients with SSc cannot determine the specificity of the autoantibody, and a nucleolar pattern did not correspond in all cases to Fb 34kD antigen; 2) with the WB assay, 40% of SSc sera were false-negative; and 3) with IPP of RNAs, we were able to identify with great certainty a variety of nucleolar RNAs, and nuclear and cytoplasmic RNA-protein complexes, that are targeted by SSc sera.

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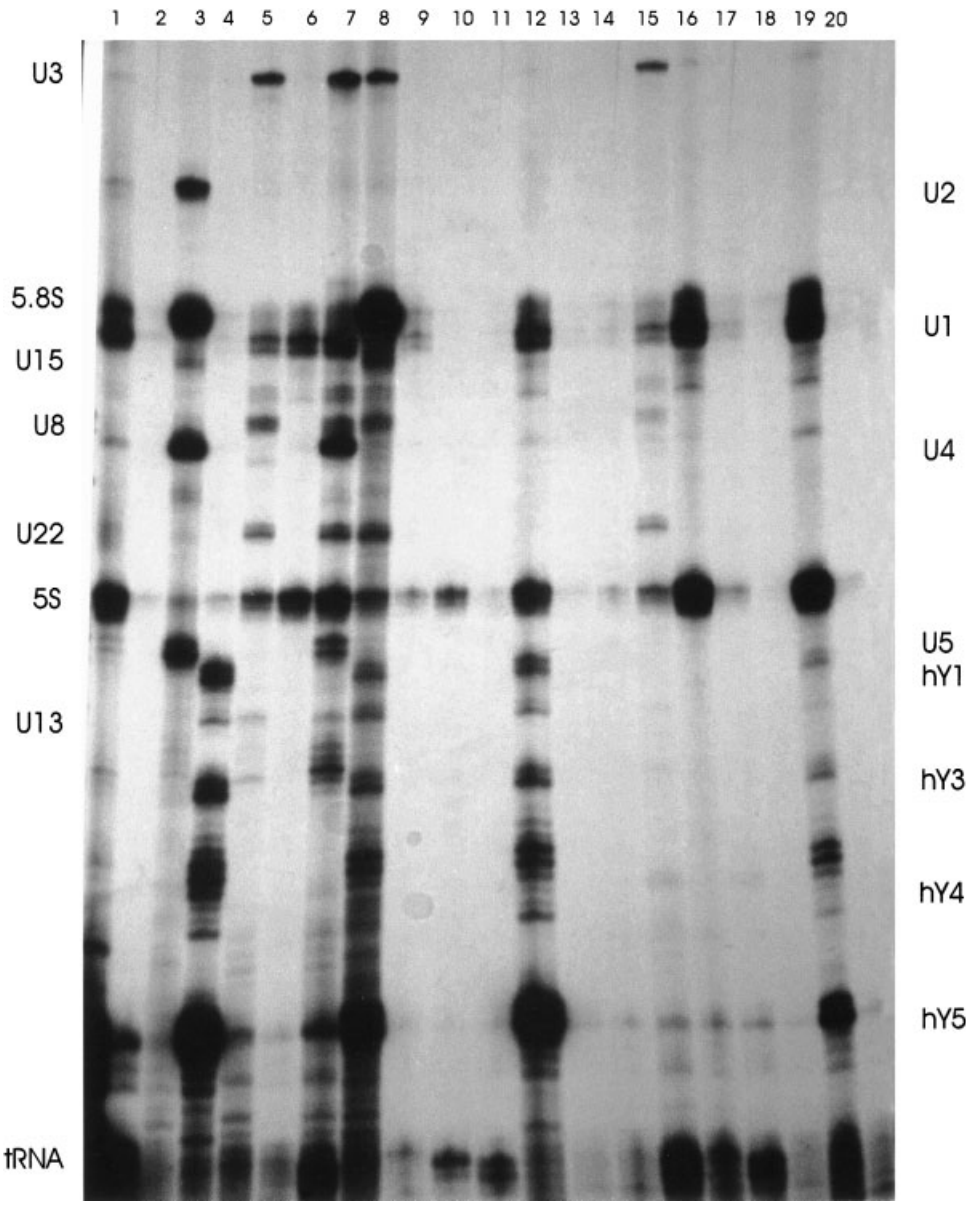

Fig. 2. Polyacrylamide gel fractionation of ^{32}P-labeled RNAs present in total HeLa cell extract using autoantibodies from patients with SSc. Lane 1: Standard RNA from HeLa cells. Lane 2: Negative control (normal human sera). Lane 3: Positive controls (mouse monoclonal antibody antihuman Sm (Y-12) associated with U2, U1, U4, and U5 snRNAs). Lane 4: Human polyclonal autoantibody anti-Ro 60 kD protein (Ro_M) related with hY1, hY3, hY4, and hY5 scRNAs. Lane 5: Mouse monoclonal antibody against human Fb (72B9), in which we can see U3, U15, U8, U22, and U13 snoRNAs. Lanes 6–20 show representative electrophoretic patterns of several snoRNAs, snRNAs, and scRNAs associated with autoantibodies from patients with SSc. Lanes 7, 8, 12, 15, 16, and 19 show anti-snoRNAs particularly associated with U3. Lanes 6–9, 12, 15–17, and 19 also show anti-snoRNAs associated with U15. Lanes 7, 8, and 15 represent anti-snoRNA U8. Lanes 7, 8, and 15 show autoantibodies against snoRNA U22, and lanes 7, 8, 12, and 19 show autoantibodies related with snoRNA U13.

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