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IDENTIFICATION OF THREE DIFFERENT ANTI-4S RNA SERA ASSOCIATED WITH AUTOIMMUNE DISEASE

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Summary: Analysis of antibodies obtained from 10 patients with myositis, 20 with systemic lupus erythematosus and 24 with scleroderma revealed that 2 patients with myositis and one with systemic lupus erythematosus produced antibodies precipitating 4S RNAs. Each of the 3 anti-4S RNA sera precipitated a different subset of the total 4S RNA population. Two sera precipitated deproteinized or gel extracted 4S RNA from Novikoff hepatoma or Dinoflagellate cells. The other serum did not precipitate deproteinized RNA and may precipitate a 4S RNA in a small RNP particle. The reactive 4S RNAs are extensively modified, which suggests that they may be tRNAs, tRNA precursors or previously unknown RNA species.

Recently autoantibodies have become important reagents for immunoprecipitation of small nuclear RNA and RNP species (1,4). Hardin et al. (5) has reported that antibodies of three patients with myositis precipitated tRNAs. In this study, three different antibodies from two patients with myositis and one with systemic lupus erythematosus were identified which precipitate 4S size RNAs.

MATERIALS AND METHODS

[^{32}P] labeled Novikoff hepatoma cells were obtained as described earlier (6). Preparation of Ig fraction by ammonium sulfate precipitation and immunoprecipitation using Pansorbin were carried out as described by Lerner and Steitz (1). The RNAs were identified by electrophoresis on 10%, 12% or 15% acrylamide/7M urea gels. Modified nucleotides were identified by two dimensional chromatography as described by Silberkang et al. (7).

Antigen sources used in different experiments were prepared in several ways. Cell sonicates were prepared by sonication of [^{32}P] labeled Novikoff hepatoma cells in 100 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris pH 8.0 (sonication buffer). The sonicate was centri-

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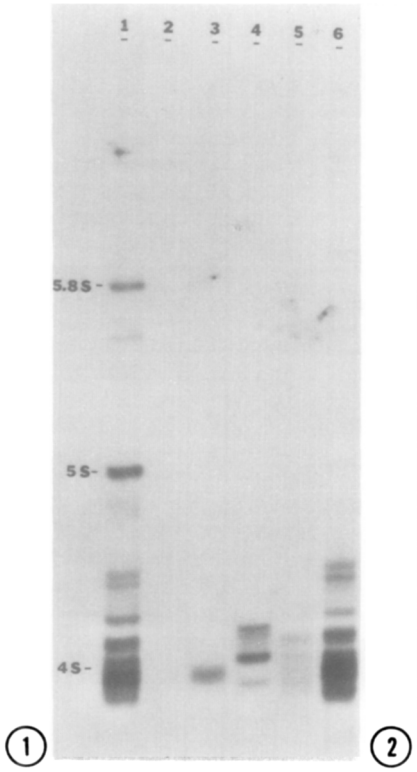
fuged at 12,000 RPM and the supernatant was used as antigen source. High speed supernatants were prepared by centrifugation of cell sonicates at 120,000 g for 2 hr. Deproteinized RNA was prepared by incubation of high speed supernatants in 0.1% SDS with proteinase K by three additions of fresh proteinase K to a concentration of 1 mg/ml at 6 hr. intervals at 22°. Proteinase K was removed by phenol extraction at 60° and ethanol precipitation of the RNA. The RNA was then brought back to the original volume in sonication buffer. Gel extracted RNA was prepared from the 4S region of whole cell RNA, cut from 10% acrylamide/7M urea gels. RNA was extracted by the crush and soak method, ethanol precipitated and dissolved in sonication buffer prior to use. Gel extracted 5S RNA was added as a control for antibody specificity. [^{32}P] end labeled RNA was prepared from 1 M NaCl supernatants of Novikoff hepatoma cell RNA by the method of Donis-Keller et al. (8) for 5'-end labeling or the method of England and Uhlenbeck (9) for 3'-end labeling. Gel purified 5' or 3'-end labeled RNA was dissolved in sonication buffer prior to use. Partial digests of uniformly labeled 4S RNA were made by incubation with T_1-RNase at an enzyme to substrate ratio of 1:1000 for 10 min. at 4° in 200 λ sonication buffer. T_1-RNase was removed by phenol extraction at 60° followed by ether extraction and dilution with 1 ml sonication buffer prior to use. End labeled RNA was digested by heating to 90° for 1 hr. in 200 λ of 0.1 M sodium bicarbonate buffer, pH 8.0 and diluted with 10 volumes of sonication buffer before use as antigen source.

RESULTS

The incidence of anti-4S RNA antibodies in 24 patients with scleroderma, 20 patients with systemic lupus erythematosus and 10 patients with myositis was examined by immunoprecipitation of sonicates of ^{32}P labeled Novikoff hepatoma cells. Of the 54 samples, 2 from myositis patients and 1 from an SLE patient were positive for anti-4S RNA antibodies. The RNAs precipitated by the three sera identified as anti-4S RNA are shown in Figure 1. Antibodies of myositis patient Pa (lane 3), SLE patient Ha (lane 4) and myositis patient Re (lane 5) each precipitated a different subset of RNAs, containing 1, 3 and 6 bands respectively.

Since most small RNAs reactive with antibodies of autoimmune sera are antigenic in association with protein (3,10) it was of interest to determine if the anti-4S antibodies also required protein antigens. Figure 2A shows the RNAs precipitated from Novikoff hepatoma 4S and 5S RNAs which were extracted with SDS/phenol at 60° and isolated on 10% polyacrylamide/7M urea gels prior to use



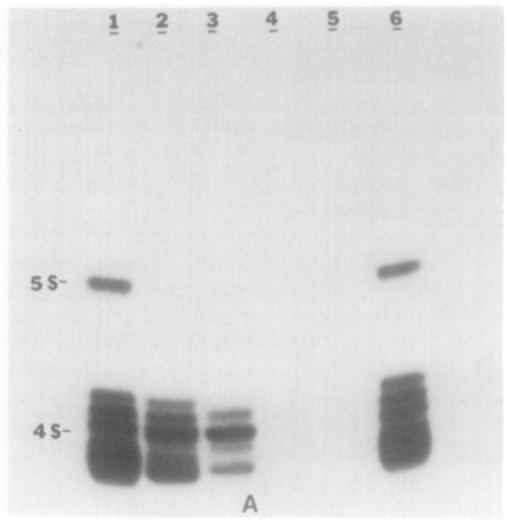


Figure 1. Autoradiogram of a 15% polyacrylamide gel on which immunoprecipitated RNA from a high speed supernatant of sonicated Novikoff hepatoma cells labeled with [^{32}P] was separated: lane 1, 4-8S Novikoff hepatoma RNA; lane 2, 240 µg of normal human Ig; lane 3, 240 ug Ig from patient Pa; lane 4, 20 µg Ig from patient Ha; lane 5, 40 µg Ig from patient Re; lane 6, high speed supernatant.

Figure 2. Immunoprecipitates of gel isolated 4S and 5S RNAs of Novikoff hepatoma (2A) or Dinoflagellate (2B). Figure 2A, lanes 1 and 6, Gel isolated Novikoff hepatoma RNA used as antigen source; lane 2, 40 µg Ig from patient Re; lane 3, 20 µg Ig from patient Ha; lane 4, 100 µg Ig from patient Pa; lane 5, 100 µg of normal human Ig. Figure 2B, lane 1, Gel isolated Dinoflagellate RNA used as antigen source; lane 2, 20 µg Ig from patient Ha; lane 3, 40 µg Ig from patient Re.

as antigen. The isolated 4S RNAs reacted with antibodies from patients Ha and Re but not with antibodies of patient Pa. Experiments using proteinase K digested, high speed supernatants of sonicates of Novikoff cells (data not shown) indicated that this treatment did not inhibit immunoprecipitation with antibodies of patient Re, reduced precipitation with antibodies of patient Ha by 80% and eliminated immunoprecipitation with antibodies of patient Pa.

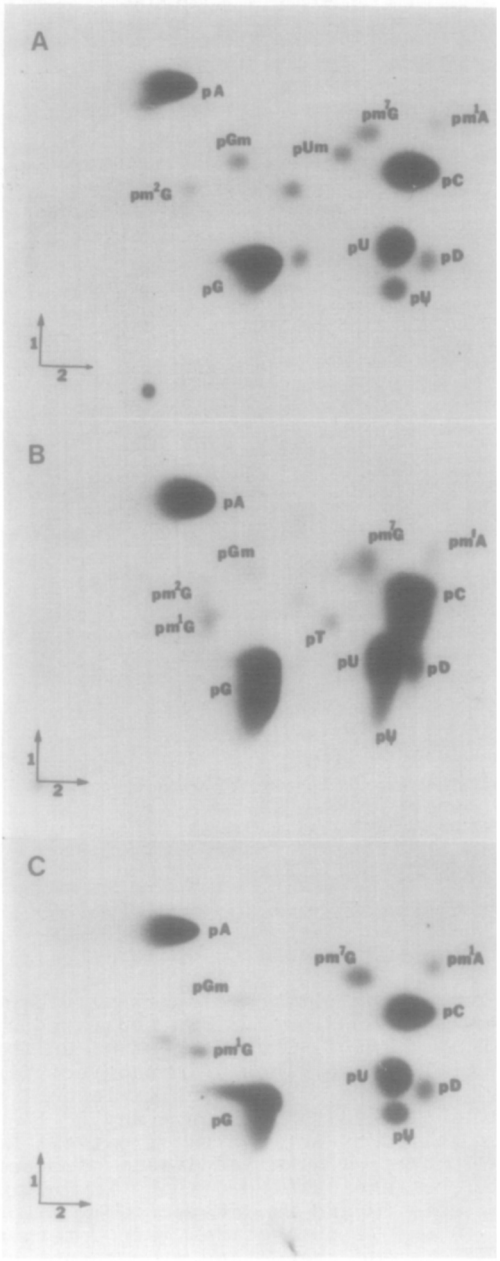


Figure 3. Two dimensional chromatography of P_1-nuclease digests of gel isolated, immunoprecipitated RNAs on cellulose sheets. First dimension, chromatography in isobutyric acid - concentrated NH_4OH-H_2O (66/1/33, V/V/V) and second dimension in 0.1 M sodium phosphate, pH 6.8-ammonium sulfate - n-propanol (100/60/2, V/W/V). A) Uppermost band immunoprecipitated by antibodies of patient Ha; B) Pooled bands immunoprecipitated by antibodies of patient Re; C) 4S RNA immunoprecipitated by antibodies of patient Pa. Nucleotide designations are those which could be determined from the pattern described by Silberkang et al. (7).

Evolutionary conservation of the antigenic determinants in 4S RNAs to antibodies of patients Ha and Re was assessed by pre-

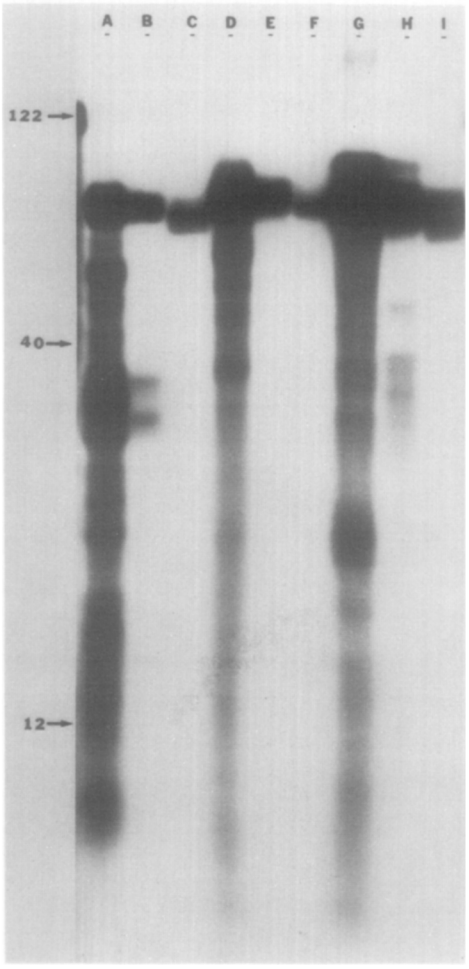


Figure 4. Immunoprecipitates of partially digested, uniformly or end labeled Novikoff hepatoma 4S RNA separated on 12% polyacrylamide gels; lane A, partial T_1-RNase digest of uniformly labeled 4S RNA; lanes B and C, T_1-RNase digests immunoprecipitated by antibodies of patient Re and Ha, respectively; lane D, partial alkaline digest of 5'-end labeled 4S RNA; lanes E and F, 5'-end labeled digests immunoprecipitated by antibodies of patient Re and Ha; respectively; lane G, partial alkaline digest of 3'-end labeled 4S RNA; lanes H and I, 3'-end labeled digests immunoprecipitated by antibodies of patients Re and Ha, respectively. Positions corresponding to 122, 40 and 12 nucleotides were determined by the mobilities of 5S RNA, xylene cyanol and bromophenol blue, respectively (8 and our unpublished results).

cipitation of gel extracted 4S and 5S RNAs from $[^{32}P]$ labeled Dino flagellate cells (Fig. 2B). Conservation of similar epitopes is indicated, since both antibodies precipitated a specific subset of 4S RNAs from Dinoflagellate RNA.

To evaluate the possibility that the precipitated RNAs might be tRNAs, their modified nucleotide content was determined by the

method of Silberkang et al. (7). These results are shown in Figure 3. The precipitated RNAs contain several modified nucleotides including dihydrouridine. The presence of the modified nucleotides and the gel mobility of the precipitated RNAs suggest that some of the reactive RNAs may be tRNAs.

Competition experiments using complete P_1 nuclease digests of unlabeled 4S RNA in 20 fold excess over labeled undigested 4S RNA demonstrated that the digest did not compete for anti-4S RNA antibodies of patients Re and Ha. Since mononucleotides were not antigenic, experiments were performed in an attempt to determine the minimum size and relative position of 4S RNA fragments which could be precipitated by antibodies of patients Re and Ha (Figure 4). Partial T_1-RNase digests of uniformly labeled 4S RNA (Figure 4, lane A) or partial alkaline digests of 5' or 3'-end labeled 4S RNA (Figure 4, lanes D and G) were separated on 12% acrylamide gels to determine approximate fragment length. Immunoprecipitation of the partially digested RNAs with antibodies of patient Ha (Fig. 4, lanes C, F and I) revealed no detectable precipitation of fragments other than full length or nearly full length 4S RNAs. Antibodies of patient Re appeared to precipitate only full length 4S RNA from 5'-end labeled material (Fig. 4, lane E), however, fragments as small as 30 nucleotides were precipitable from uniformly or 3'-end labeled RNA (Fig. 4, lanes B and C). Interestingly, no bands were apparent between approximately 47 and 65 nucleotides in the antibody Re immunoprecipitates of 3' and uniformly labeled RNA. One possible explanation for this is that these intermediate length fragments have an altered secondary or tertiary structure which interferes with antigenicity.

DISCUSSION

Sera from 54 patients with autoimmune disease were analyzed for their reactivity to snRNAs or snRNP particles. Of these three

sera were identified which precipitated RNAs in the 4S region. As indicated by gel mobility and modified nucleotide content, several of the reactive RNAs may be tRNAs.

Interestingly each of the anti-4S RNA sera reacted with a different subset of 4S RNAs. Serum Re precipitated six Novikoff hepatoma 4S RNAs. Serum Ha precipitated three 4S RNAs and serum Pa precipitated one 4S RNA. In contrast to sera Ha and Re, serum Pa was unreactive with deproteinized RNA. Therefore, it is probable that the RNA reactive with serum Pa is antigenic in the form of a small RNP particle.

Reactivity of 4S RNAs to antibodies of patients Re and Ha reside in the polynucleotide structure of these RNAs since they are reactive after deproteinization. Antigenicity does not depend on a particular 5' or 3'-end, nonnucleotide adduct, such as an amino acid, since they are precipitable after 5' or 3'-end labeling. Immunoprecipitations of partially digested 4S RNA suggests that antibodies of patient Ha require an intact 4S RNA structure for antigenicity while antibodies of patient Re react with fragments as small as 30 nucleotides, probably derived from the 3'-end of the RNA. The antigenic determinants for antibodies of patients Ha and Re are conserved in eukaryotic evolution since they are also found in cells as primitive as Dinoflagellates.

In agreement with a previous report of Hardin *et al.* (5) anti-4S RNA antibodies occurred most often in myositis patients; two of the ten myositis sera examined contained anti-4S RNA antibodies. However, one of twenty SLE sera also precipitated 4S size RNAs and therefore the presence of anti-4S RNA antibodies is not a specific indication of myositis. Eilat *et al.* (10) has described spontaneous anti-tRNA antibodies in SLE mice, however, they differed from antibodies of patients Re, Ha and Pa in that they reacted almost equally with all non-tRNA species examined.

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