

# Anti-TS1-RNA: Characterization of Novel Antibodies Against Sequence-Specific RNA by Random RNA Selection in Patients with Sjögren's Syndrome

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**ABSTRACT. Objective.** To define a novel RNA epitope recognized by serum from a patient with Sjögren's syndrome (SS) from a randomized RNA epitope library and investigate the epitope reactivity of the anti-RNA antibodies in patients with various connective tissue diseases.

**Methods.** Serum from a patient with SS was used to select ligands from a library of RNA oligomers with a central region of 25 degenerate nucleotides. Bound RNA was recovered by reverse transcription, PCR amplification, and subcloning. The relationship between the antibodies to the selected RNA and disease specificity was studied using immunoprecipitation.

**Results.** From the random RNA library, several unique RNA sequences were obtained. Sera from 32 of 61 patients with SS (52.5%) precipitated with one of the selected RNA (TS1-RNA), whereas sera from 8 of 41 patients with systemic lupus erythematosus (19.5%) and 3 of 25 patients with rheumatoid arthritis (12.0%) precipitated. Although the frequency of reactivity to the TS1-RNA was higher in anti-SSA/Ro positive sera, the presence of either native or recombinant SSA/Ro antigen showed no detectable competition, and no apparent sequence homology was found between the TS1-RNA and hY RNA.

**Conclusion.** These data suggest that anti-TS1-RNA is a novel antibody against sequence-specific RNA in many patients with SS. (J Rheumatol 2002;29:931-7)

## Key Indexing Terms:

ANTI-RNA

SJÖGREN'S SYNDROME

ANTIBODIES

ANTI-SSA/RO

Sera from patients with various connective tissue diseases often contain autoantibodies against cellular components such as DNA, RNA, or protein. Recent studies have shown that RNA-protein complexes are the common targets for such immune responses<sup>1</sup>. In addition to antibodies directed to the protein component, antibodies to the RNA moiety have recently been found. Some patients produce autoantibodies directed against naked RNA that include U1 RNA<sup>2-4</sup>, hY5 SSA/Ro RNA<sup>5</sup>, alanine transfer RNA<sup>6,7</sup>, initiator methionine transfer RNA<sup>2,8</sup>, histidine transfer RNA<sup>9</sup>, and 28S ribosomal RNA<sup>10,11</sup>. Whereas anti-DNA antibodies tend to have only general sequence preference for double or single stranded DNA, antibodies against these RNA have been shown to be highly sequence-specific<sup>2-4,10</sup>. It has been found that the antibody levels to certain RNA epitopes of U1

RNA appear to correlate with disease activity in patients with mixed connective tissue disease and thus might play a role in the pathogenesis of the disease<sup>12</sup>. However, the antigenic determinants of the RNA and the clinical presentation of patients possessing such antibodies have not been investigated extensively.

In this study, a random RNA library was used in a direct immunoprecipitation assay to select RNA species capable of binding to antibodies present in sera obtained from patients with various connective tissue diseases. Bound RNA was recovered by standard reverse transcription, polymerase chain reaction (PCR) amplification, and subcloning. This approach allows the determination of RNA epitopes using autoimmune sera in cases where the ligand is not known. We defined a novel RNA epitope recognized by serum from a patient with Sjögren's syndrome (SS) from a randomized RNA epitope library and investigated the epitope reactivity of the anti-RNA antibodies in patients with various connective tissue diseases.

## MATERIALS AND METHODS

**Patients.** Sera obtained from 2 patients with SS and 2 patients with systemic lupus erythematosus (SLE) were used in the random RNA selection procedure. Of these, serum from a 59-year-old Japanese woman (Patient TS) who had biopsy proven SS was shown to bind sufficiently labeled transcript after multiple rounds of RNA selection. Sera from 60

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other patients with SS and 41 patients with SLE, 25 patients with rheumatoid arthritis (RA), and 14 healthy control subjects were used to test reactivity to this RNA ligand selected from the random RNA library. All patients with SS were diagnosed according to the criteria proposed by the European Community<sup>13</sup>. All patients with SLE and RA met the relevant American College of Rheumatology classification criteria<sup>14,15</sup>, and did not have SS.

**Preparation of RNA.** Double stranded transcription template was prepared by subjecting 10 ng of linear oligonucleotide (Figure 1) to 25 cycles in a Techne Thermal Cycler (1 min at 94°C, 1 min at 50°C, and 2 min at 72°C) in the following buffer: 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1 μg T7Univ primer, 0.1 μg RevUniv primer<sup>16</sup>, 200 μM dNTP, and 2.5 units Taq DNA polymerase (Takara, Tokyo, Japan). Any tandem products were reduced to monomers by cutting with BamHI. The DNA template was then transcribed using T7 RNA polymerase (Life Technologies Inc., Rockville, MD, USA).

**Selection of specifically bound RNA by immunoprecipitation.** The transcripts were immunoprecipitated with patients' sera as follows: protein A-Sepharose CL-4B beads (Sigma, St. Louis, MO, USA; 4 mg per 100 μl of reaction mixture) were washed in NT2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.05% Nonidet P-40)<sup>17</sup>, mixed with 2 μl of the patient serum, incubated on ice for 10 min, and washed in NT2 buffer. The beads were then resuspended in 100 μl of RNA binding buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM KCl, 1 mM EGTA, 0.05% Nonidet P-40, 1 mM MgCl<sub>2</sub>, 2.5% polyvinyl alcohol, RNasin at 40 units/ml, poly(A) RNA at 50 μg/ml, *Escherichia coli* transfer RNA at 0.5 mg/ml, 1 mM DTT, bovine serum albumin at 100 μg/ml, 0.4 mM vanadyl ribonucleoside complex)<sup>17</sup>. Roughly 200 to 500 ng of RNA was added, and the reactions were incubated at room temperature for 10 min. The pellets were washed 5 times with NT2 buffer including 0.5 M urea. RNA was recovered by phenol extraction and ethanol precipitation. RNA was then reverse transcribed using 0.1 μg RevUniv primer with AMV reverse transcriptase (Life Technologies) for 1 h at 42°C using conditions recommended by the supplier, and the resultant cDNA was subjected to 25 cycles of PCR under the conditions already noted. The amplified templates were used to repeat the above cycle for 6 additional rounds. The final PCR product was digested with BamHI, subcloned into pGEM-7Zf(+) (Promega, Madison, WI, USA), and sequenced using Sequenase Version 2.0 (United States Biochemical, Cleveland, OH, USA).

**Reactivity to the selected RNA by immunoprecipitation.** cDNA encoding the selected RNA was transcribed *in vitro* with T7 RNA polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]UTP. The transcripts were purified by gel filtration on a Sephadex G-50 column (Pharmacia LKB Biotechnology Inc., Upsala, Sweden). The <sup>32</sup>P labeled transcripts (approximately 400,000 cpm) were immunoprecipitated with 2 μl of patient sera as described above. The

bound RNA was analyzed on an 8% polyacrylamide-8 M urea gel followed by autoradiography or quantitated by scintillation counting, and the binding was calculated as follows: percentage binding = 100 × (cpm of <sup>32</sup>P labeled RNA precipitated with patient serum)/(cpm of <sup>32</sup>P labeled RNA added in the binding reaction).

**Preparation of SSA and U1 RNP antigen.** Recombinant 60 kDa and 52 kDa SSA/Ro proteins derived from cDNA clones as described<sup>18,19</sup> were gifts from Dr. E.K.L. Chan (W.M. Keck Autoimmune Center, Scripps Research Institute, La Jolla, CA, USA). Native SSA/Ro and U1 RNP antigens were purified from calf thymus extract by a combination of ammonium sulfate fractionation, anti-SSA and anti-U1 RNP Sepharose 4B affinity chromatography, and high pressure liquid chromatography gel filtration, as described<sup>20</sup>.

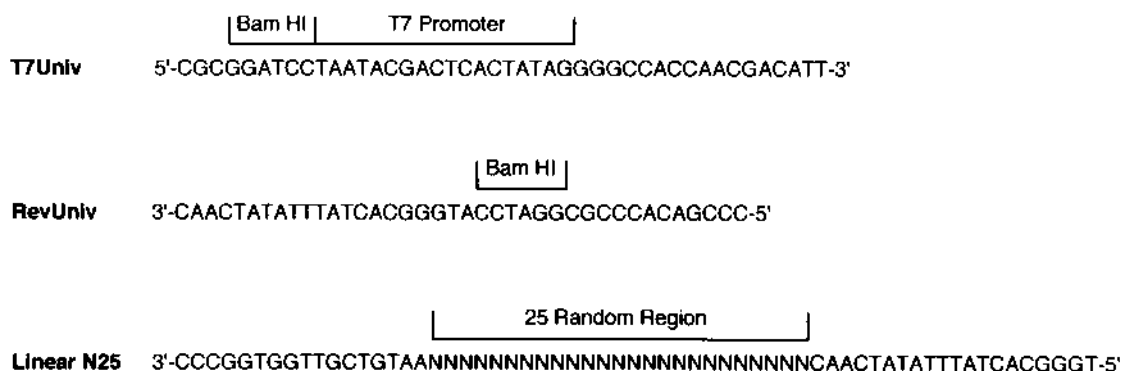
**Inhibition tests by immunoprecipitation.** Immunoprecipitation was used in inhibition tests to analyze the cross-reactivity between TS1-RNA and SSA and U1 RNP antigens. SSA and U1 RNP antigens, serially diluted from 100 μg/ml to 0.3 μg/ml, were preincubated with serum from Patient TS. Unlabeled TS1-RNA and random RNA from the original pool, serially diluted from 7.5 μg/ml to 0.3 μg/ml, were also prepared and used as a positive and negative control. After incubating for 1 h at room temperature, a fixed amount of <sup>32</sup>P labeled TS1-RNA was added and immunoprecipitation was done as described above.

**Other methods.** Anti-SSA and anti-SSB antibodies were detected by double immunodiffusion<sup>21</sup>. Nucleotide sequence analysis was performed using the Human Genome Center databases (Institute of Medical Science, The University of Tokyo). Statistical significance was assessed by chi-square test with Yates' correction (2 tailed test).

## RESULTS

### *Serum from a patient with SS binds specific RNA sequences.*

A library of 3 × 10<sup>11</sup> RNA molecules was generated, with each molecule containing a 25-nucleotide-long region of random sequence flanked by defined sequence<sup>16</sup>. An RNA selection procedure was performed consisting of 7 cycles of successive transcription, direct RNA immunoprecipitation by patients' sera, reverse transcription, and PCR before vector cloning. Antibodies to RNA were detected in serum of Patient TS by immunoprecipitation using <sup>32</sup>P labeled RNA, and then final PCR products were subcloned into pGEM-7Zf(+) for sequence analysis. The sequences of 10 individual clones, designated TS1-RNA to TS10-RNA, each representing a selected RNA species, are shown in Figure 2.



**Figure 1.** Oligonucleotides used in the random RNA selection. The DNA oligonucleotides containing internal randomized region (Linear N25) are synthesized by programming all 4 nucleotide substitutions at each of the randomized positions labeled N.

Some of the RNA sequences were found to be present multiple times (TS1-RNA to TS5-RNA, and TS6-RNA to TS7-RNA).

To rule out the possibility of nonspecific RNA binding to either normal antibodies or to the protein A sepharose beads, <sup>32</sup>P labeled TS1-RNA and TS6-RNA were precipitated under identical conditions with either normal human serum or Patient TS serum. TS1-RNA and TS6-RNA were found to bind only Patient TS serum (Figure 3, lanes 1 and 3) and not normal serum (Figure 3, lanes 2 and 4). Other RNA was tested and found not to be precipitable with Patient TS serum. For example, a negative control consisting of linear RNA of the same length and containing identical flanking PCR primer regions was not precipitated with either serum (Figure 3, lanes 5 and 6). These results confirmed that these RNA epitopes are specific for the patient's serum and that

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TS1  CGAAAGUCCGAUCGGCGUAAUGUCA
TS2  CGAAAGUCCGAUCGGCGUAAUGUCA
TS3  CGAAAGUCCGAUCGGCGUAAUGUCA
TS4  CGAAAGUCCGAUCGGCGUAAUGUCA
TS5  CGAAAGUCCGAUCGGCGUAAUGUCA
TS6  GUUUGGCGACGUCCACAUGAGCC
TS7  GUUUGGCGACGUCCACAUGAGCC
TS8  GUUUGAUGGAUACCUAUCCUUGGCC
TS9  GUUUGGUACGACCUGAUCAUGC GCC
TS10 AUGUCACGUAUUCUUCAAGAUCGAAG
  
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Figure 2. Selected RNA sequences to bind Patient TS serum. The selected sequences corresponding to the degenerate regions are shown for each isolate.

the sites of interaction are not in the flanking PCR primer regions, but rather at the specific sequences present in each selected isolate.

A search of the nucleic acids data bank (Human Genome Center) with TS1- and TS6-RNA revealed no extensive homology.

*Disease specificity of the reaction of antibodies to the selected RNA.* The relationship between the antibodies to the selected RNA and disease specificity was studied using immunoprecipitation. TS1-RNA was chosen for further characterization because it appeared most frequently (5 times) between TS1- and TS10-RNA. For the quantitative measurement of antibody levels, the supernatants and bound <sup>32</sup>P labeled TS1-RNA were measured by scintillation counting and the binding was calculated as described. The results of immunoprecipitation for anti-TS1-RNA in various connective tissue disease groups and controls are shown in Figure 4. Immunoprecipitation results were positive in 32 of 61 patients with SS (52.5%), 8 of 41 patients with SLE (19.5%), and 3 of 25 patients with RA (12.0%) when values greater than 2 SD above the mean of 14 healthy controls were considered to be positive. No healthy control had anti-TS1-RNA. Although 7 of 8 patients with anti-TS1-RNA positive SLE had anti-SSA antibody, neither 8 patients with SLE nor 3 patients with RA who had anti-TS1-RNA had any clinical symptoms associated with SS. Three patients with RA who had anti-TS1-RNA did not have anti-SSA.

Since anti-TS1-RNA antibodies were detected more frequently in the SS patients than in the other disease groups investigated, anti-TS1-RNA levels in patients with connective tissue disease were grouped according to their positivity for anti-SSA and anti-SSB antibodies (Figure 5).

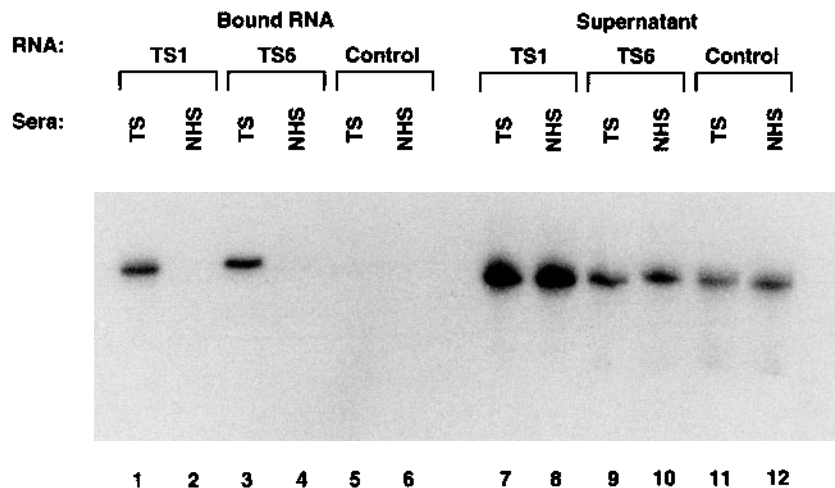


Figure 3. Selected RNA bind specifically to Patient TS serum. *In vitro* transcribed <sup>32</sup>P labeled RNA were immunoprecipitated by either Patient TS serum (TS) or normal human serum (NHS). RNA was analyzed on a urea-8% acrylamide gel and visualized by autoradiography. Lanes 1 to 6, RNA extracted from the Staph A pellets after 5 washes; 7 to 12, transcripts remaining in the supernatants after the first step of binding; 1 and 7, TS1-RNA with TS serum; 2 and 8, TS1-RNA with normal serum; 3 and 9, TS6-RNA with TS serum; 4 and 10, TS6-RNA with normal serum; 5 and 11, control RNA with TS serum; 6 and 12, control RNA with normal serum.

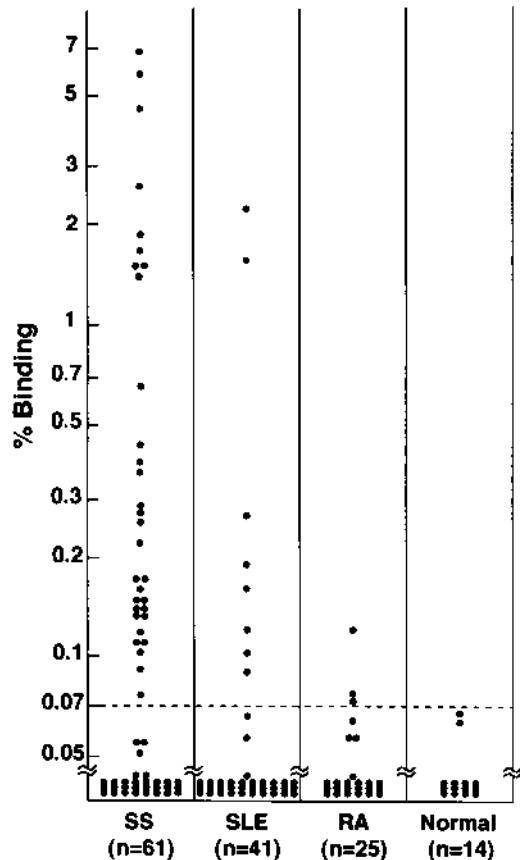


Figure 4. Reactivity to TS1-RNA by sera from patients with SS, SLE, and RA. Broken line represents the upper limit of the normal range: 2 SD above the mean value in 14 healthy controls.

The frequency of positivity for TS1-RNA was significantly higher among sera positive for anti-SSA (52.0%; 39 of 75) than among sera negative for anti-SSA (16.6%; 7 of 42) ( $p < 0.0005$ ). The frequency of positivity for TS1-RNA also tended to be higher among sera positive for anti-SSB (62.5%; 15 of 24, vs 33.3%; 31 of 93).

#### Inhibition tests with SSA and U1 RNP against TS1-RNA.

The results shown above suggested the possibility of cross-reactivity between TS1-RNA and SSA antigen. Therefore, the inhibition test with SSA against TS1-RNA was performed. When serial dilutions of purified SSA antigen, recombinant 60 kDa SSA antigen, recombinant 52 kDa SSA antigen, purified U1 RNP antigen (Figure 6A), original pool RNA, and TS1-RNA (Figure 6B) were added as inhibitors, the binding of Patient TS serum to  $^{32}\text{P}$  labeled TS1-RNA was not blocked by purified SSA, recombinant 60 kDa SSA, recombinant 52 kDa SSA, purified U1 RNP, or original pool RNA. In contrast, labeled TS1-RNA was only blocked by unlabeled TS1-RNA. These results suggest that the antibodies directed against TS1-RNA are not cross-reactive with either SSA or U1 RNP.

Since Patient TS serum also bound TS6-RNA, TS6-RNA was also tested as a competitor for antibody binding to

TS1-RNA. The sequences of TS1-RNA and TS6-RNA are remarkably dissimilar. Figure 6B indicates TS6-RNA did not significantly inhibit Patient TS serum binding to TS1-RNA. This result would provide even more support for sequence-specific binding. The result would also indicate that there are multiple, independent RNA binding antibodies produced in SS.

## DISCUSSION

Autoantibodies are known to have nucleic acid binding properties, but only a few examples of sequence specificity have been described. Attempts to use synthetic DNA followed by iterative selection and amplification for determining DNA binding sites of anti-DNA autoantibodies have not revealed strong definitive consensus sequences, although recent studies have shown that DNA size influences the binding of anti-DNA in sera of patients with SLE<sup>22</sup>. This suggests that either anti-DNA antibodies do not have strong sequence specificity or the preferred binding sequences are so diverse that they are unrecognized among the selected population. On the other hand, certain anti-RNA antibodies have been shown to be highly sequence-specific. For example, sera reactive against U1 RNA and certain transfer RNA were shown to selectively bind these species from total HeLa cell RNA<sup>2,23</sup>. In the case of U1 RNA, autoantibody epitopes had been defined by the selection of discrete RNA fragments from a pool of partially digested *in vitro* transcripts<sup>3</sup> or by binding to truncated *in vitro* transcripts that were amplified by subcloning<sup>3,4</sup>. Although these approaches have been useful in identifying general RNA epitopes, the precise nucleotides that interact with the antibodies were not determined and the methods are limited because they require the previous identification of potential RNA ligands. Ultimately, Tsai, *et al* pinpointed the numbers and locations of RNA epitopes on U1 RNA using a random RNA selection protocol<sup>24</sup>. This epitope selection through the use of degenerate nucleic acid pools, followed by multiple rounds of amplification and selection, allows the screening of billions of sequences at one time. We applied this method to characterize RNA epitopes capable of binding to antibody present in a patient with SS.

To identify the RNA epitopes in SS serum, the pool of degenerate RNA was subjected to direct immunoprecipitation. After 7 rounds of high stringency selection, the cDNA were cloned and individual isolates sequenced. Examination of the sequences revealed some consensus sequences. The consensus TS1-RNA and TS6-RNA were specific for the Patient TS serum. Immunoprecipitation experiments with the labeled TS1-RNA suggest that the antibodies to TS1-RNA appeared to be present in SS patients at the highest frequency, although they were also detected at substantial frequencies in other connective tissue diseases.

The random RNA selection process may be biased by several factors. First, some species may not be selected

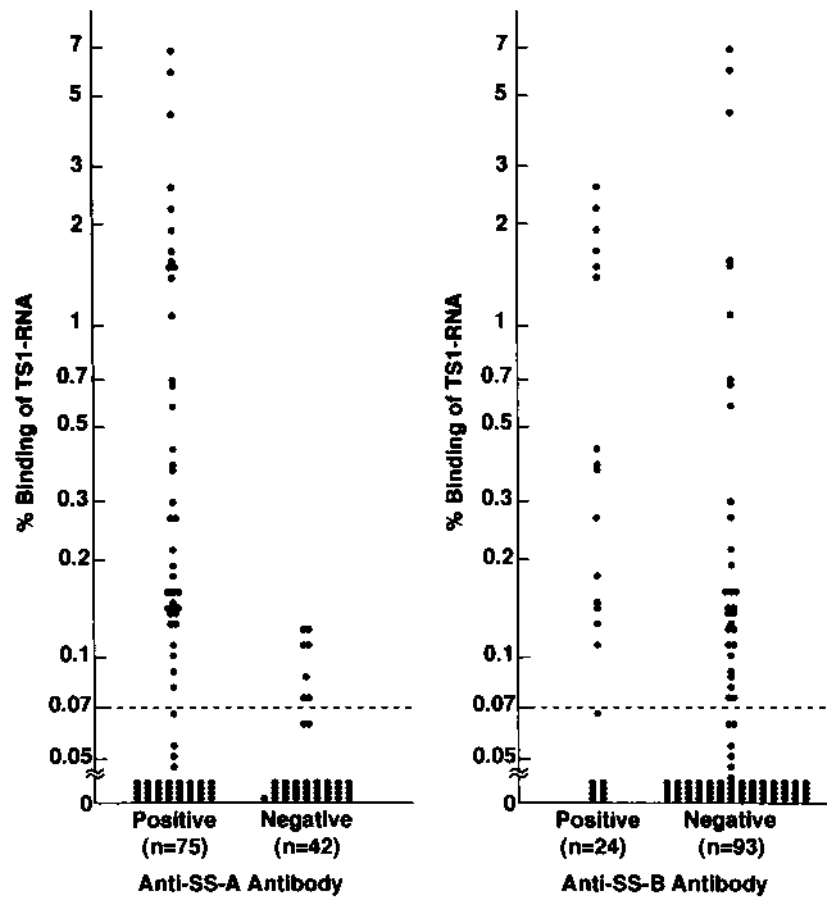


Figure 5. Comparison of reactivity to TS1-RNA in anti-SSA or anti-SSB antibody positive vs negative patients.

because they are not represented in the degenerate pool. Assuming a degeneracy of 25,  $1.1 \times 10^{15}$  are possible, however, only  $3 \times 10^{11}$  RNA molecules were present in the 10 ng of linear oligonucleotide that was used to prepare the RNA. The odds of finding the right RNA sequence by anti-RNA antibodies in Patient TS serum were thus less than 1:1000. This may explain why TS1-RNA has no sequence similarity with a known cellular RNA, as it may represent the best fit available in a small sample of the total possible RNA. Second, the selection will be influenced by the stringency of the washing conditions used in the binding reactions. Finally, the conformation of the RNA may be important for sequence-specific recognition. The binding affinity of relevant sequences may be strongly influenced by their presentation in a higher order structure that is not represented in our RNA pool. However, in the case of the U1-A protein, the same sequence was selected in both the stem-loop or linear context<sup>16</sup>. TS1-RNA does not have the stem-loop structure although the RNA epitopes that were discovered over the past decade occur in regions of the RNA that involved higher order structures and that overlap both paired and unpaired RNA bases. Future work will address

the structure more directly through chemical probing of the RNA and RNA-antibody, and by means of nuclear magnetic resonance and x-ray crystallography.

In the inhibition test, cross-reactivity with recombinant SSA and purified SSA and U1 RNP against TS1-RNA was not found. However, the competing antigens were either made from recombinant proteins without RNA or of affinity purified protein from a tissue extract where RNA is likely to be degraded, thus the possibility that TS1-RNA is cross-reactive with hY RNA or U1 RNA could not be excluded. Further studies will be needed to identify anti-TS1-RNA and their associations with human cellular RNA.

It remains unknown why autoantibodies can bind RNA when both RNA and DNA are considered to be poorly immunogenic. The reactivity of RNA with antibodies has been viewed as a result of accidental cross-reactivity of RNA with other cellular immunogens, or from the presentation of RNA fragments to the immune system following a breakdown in discrimination of self from non-self. But presentation of naked RNA to the immune system seems unlikely because RNA is rarely naked *in vivo* and ribonucleases are abundant in serum. If the origin of the anti-TS1-



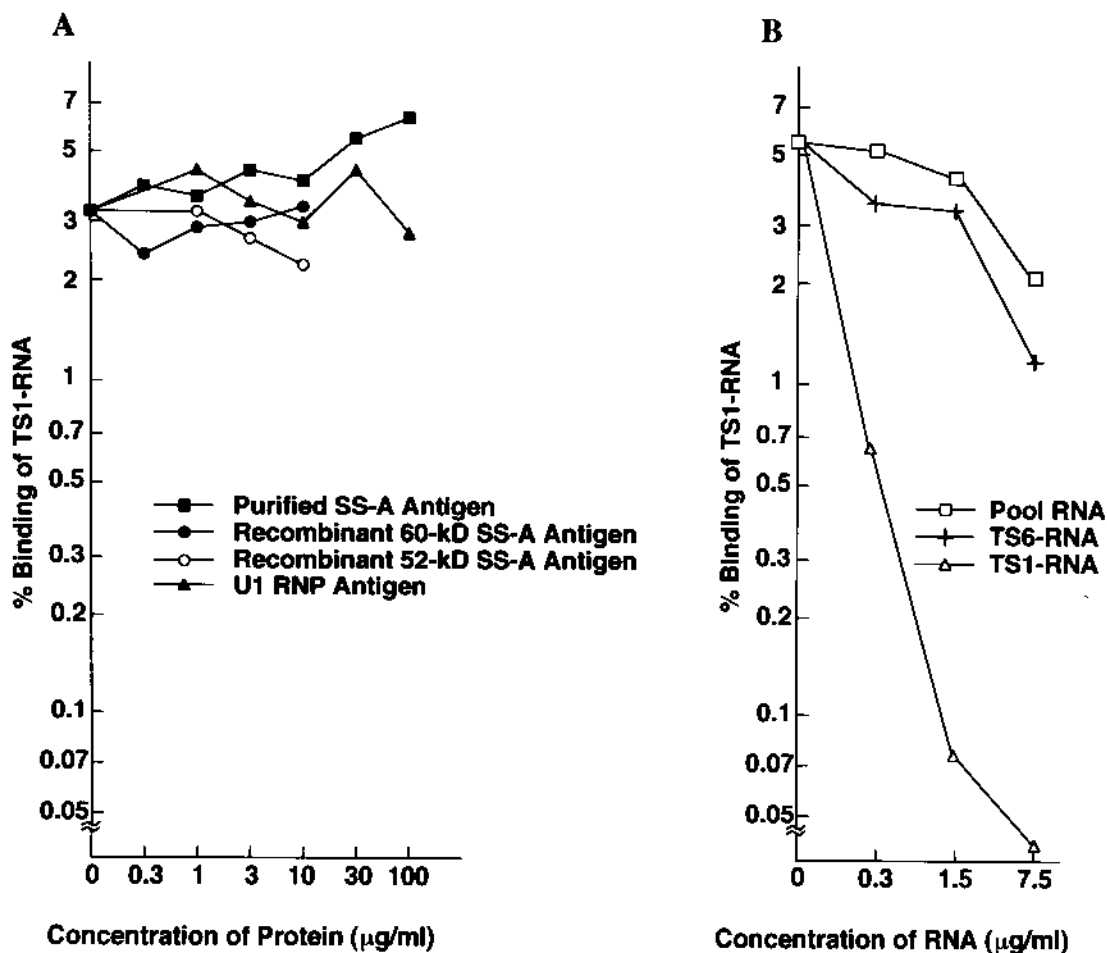


Figure 6. Inhibition test with SSA, U1 RNP antigens (A), pool RNA, TS1-RNA, and TS6-RNA (B), against labeled TS1-RNA binding to Patient TS serum. Serial dilutions of purified SSA antigen, recombinant 60 kDa SSA protein, recombinant 52 kDa SSA protein, and purified U1 RNP antigen, and each unlabeled RNA were preincubated with Patient TS serum, then  $^{32}\text{P}$  labeled TS1-RNA was immunoprecipitated. The amount of bound RNA was quantitated as described.

RNA response in SS is due to cross-reactivity with a protein epitope, then the RNA selection procedure might yield a novel RNA epitope that is optimal for that particular antibody; thus, the epitope may not necessarily exist in nature. Another potential application of RNA selection against antibodies is the ability to block the native autoantibody. Doudna, *et al* isolated RNA ligands against a monoclonal antibody to the insulin receptor. These selected RNA reacted with autoantibodies in the serum of patients with severe insulin resistance and could function as RNA decoys to block interaction between the antibodies and the insulin receptor<sup>25</sup>. These findings further suggest that RNA and proteins can recognize identical or overlapping topological surfaces on proteins despite the fact that RNA and protein are chemically very different and are not expected to form the same shapes or to present the same chemical moieties on

their surfaces. Recent data indicate that conformational RNA epitopes may result from cross-reactivity between RNA and proteins that is not merely due to coincidence<sup>24-26</sup>. On the contrary, such RNA epitopes may indicate that functional mimicry between proteins and RNA is an important and general biological phenomenon<sup>27</sup>.

In a preliminary study, the presence of anti-TS1-RNA in SS patients did not appear to correlate with activity or severity of the disease (data not shown). However, the clinical significance of antibodies directed against such RNA has not been investigated sufficiently and more intensive clinical surveys may be necessary to elucidate the pathogenic roles of anti-RNA antibodies.

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