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Autoimmune epitopes in messenger RNA

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ABSTRACT

Patients with systemic autoimmune disorders produce autoantibodies against sequence-specific conformational RNA epitopes on U1 snRNA, 28S rRNA, and transfer RNAs. The molecular basis for immunological reactivity with these highly abundant and stable RNAs is not understood. Here, we report the existence of discrete RNA epitopes in messenger RNAs that are generally less abundant and less stable than snRNAs and tRNAs. An iterative selection and amplification procedure using pooled autoimmune patient sera identified immunoreactive mRNA species. Following deconvolution of the pools to identify the reactive sera, several mRNAs recognized by these autoantibodies were cloned and sequenced. Detailed analysis using one particular serum indicated reactivity against the messages encoding alternative splicing factor (ASF/SF2) and calmodulin. Deletion and site-directed mutagenesis determined that an epitope recognized by this serum is located in a 17-base stem-loop structure common to both messages. This serum was then used to immunoprecipitate native mRNAs encoding ASF/SF2 and calmodulin from total HeLa cell RNA. Our results demonstrate that despite its low abundance and instability, messenger RNA is capable of reacting with autoantibodies generated during an autoimmune response. These data are consistent with direct presentation as a model to explain the generation of RNA conformation-specific autoantibodies.

Keywords: autoantibodies; autoimmunity; molecular mimicry; RNA antibodies; RNA–protein interactions

INTRODUCTION

The reactivity of autoantibodies with specific conformational epitopes in RNA provides an intriguing model of molecular recognition. Autoantibodies are often reporters of underlying molecular events and, as such, have helped elucidate the mechanisms of splicing, translation, and other cellular processes (reviewed in Tan, 1991; Saitta & Keene, 1992).

Work to date has identified autoepitopes in a limited number of cellular RNAs. One of the best characterized set of RNA autoepitopes are in the U1 small nuclear RNA, which contains conformational epitopes on stem-loops II and IV (Wilusz & Keene, 1986; Deutscher & Keene, 1988; van Venrooij et al., 1990). Certain autoimmune patient sera react with initiator methionine tRNA (Sri-Widada et al., 1986; Wilusz & Keene, 1986) or alanine tRNA (Bunn & Mathews, 1987). Sjogren's syndrome patients with specificity for the Ro protein antigen also produce antibodies against hY5 RNA (Boulanger et al., 1995). Another conformational epitope recognized by systemic lupus erythematosus (SLE) patient sera resides in a unique region of the 28S rRNA molecule involved in GTP hydrolysis (Chu et al., 1991; Uchiumi et al., 1991).

The appearance of autoantibodies against both DNA and RNA is enigmatic, as nucleic acids are poor immunogens. RNA-specific autoantibodies differ from anti-DNA autoantibodies in that they may recognize specific sequences and structures in a restricted set of RNA molecules, although autoantibodies with generic reactivity to all RNA molecules have also been reported (Stollar, 1994). DNA-reactive autoantibodies show little specificity, although they may preferentially react with G/C rich sequences (Stollar, 1994; Pisetsky, 1999). Understanding the mechanism by which anti-nucleic acid autoantibodies arise may provide valuable information concerning the etiology of autoimmune disorders.

In this article, we extend the search for autoimmune epitopes in RNA to include mRNA. Messenger RNA is less abundant and less stable than other forms of cellular RNA, suggesting that the methodologies employed to discover autoimmune epitopes in small cellular RNAs or ribosomal RNA may not have been sensitive enough to reveal epitopes in mRNA. We used a natural sequence library for iterative selection with pools of autoimmune patient sera to find antibodies with anti-mRNA reactivity and to identify the mRNAs with which they react. We focused on serum from patient PL that recognized a common epitope in the mRNAs encoding

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ASF/SF2 and calmodulin. The minimal sequence in these messages required for recognition by serum PL forms a predicted stem-loop secondary structure containing identical bases at several positions. Site-directed mutagenesis determined that the epitope in ASF/SF2 is located in this 17-base stem-loop structure. Serum PL also selectively immunoprecipitated the full-length mRNAs for ASF/SF2 and calmodulin from total HeLa cellular RNA, indicating the epitope is formed and recognized in the cellular mRNAs for ASF/SF2 and calmodulin. The elucidation of autoimmune epitopes in mRNA may advance understanding of fundamental principles of molecular recognition, as well as help elucidate underlying mechanisms of autoimmunity.

RESULTS

In vitro selection of mRNA epitopes from a natural sequence library

Given the existence of autoantibodies recognizing specific epitopes in small nuclear, transfer, and ribosomal RNA molecules, we undertook a search for such epitopes in messenger RNA. The relative low abundance of mRNA would have precluded observing autoimmune epitopes in mRNA by the simple immunoprecipitation methodology employed in previous studies. To detect reactivity against mRNA, we used a novel approach using pooled autoimmune sera in an iterative selection and amplification method (Fig. 1A). This in vitro selection from a natural sequence library is based upon previous selection methods developed in our lab (Gao et al., 1994; Keene, 1996b). It allows very rare as well as abundant mRNA sequences to be selected. An amplified full-length cDNA library was prepared using total HeLa cell RNA. Transcripts were generated from the amplified cDNA library using a T7 promoter sequence included in the 5′ PCR primer and subjected to immunoprecipitation using four pools each containing four autoimmune patient sera. Pooling of patient sera expanded the number of sera that could be tested. The mRNA molecules bound by the four pools of sera were iteratively reverse transcribed, amplified, and immunoprecipitated by the pools of sera.

Six rounds of selection and amplification generated four mRNA populations enriched for binding by autoimmune antibodies (Fig. 1B). A control selection using no autoimmune sera (protein A beads alone) did not yield any products. The autoimmune sera pools formed two categories differing in the apparent type of mRNA populations they selected and the number of rounds necessary to visualize these mRNA populations as

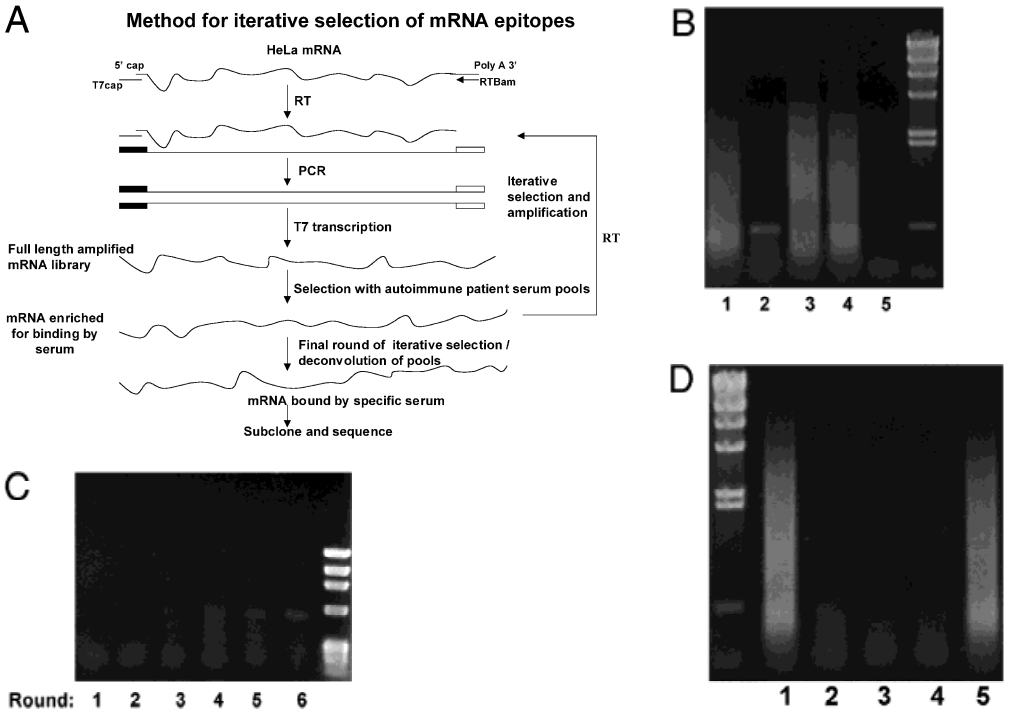


FIGURE 1. Selection of HeLa mRNA recognized by pooled autoimmune patient sera. Iterative in vitro selection (**A**) of HeLa RNA was done using four pools containing sera from four patients with systemic autoimmune disorders using primers designed to produce an amplified full-length mRNA library. **B:** The RT-PCR products after six rounds of selection and amplification. Lanes 1–4: RT-PCR products for autoimmune sera pools 1–4, respectively; lane 5: protein A beads alone (no serum). **C:** The emergence of enriched pool 2 product over the six rounds of selection (lanes 1–6). **D:** Deconvolution of pool 1 to determine which serum in the pool was reactive. RT-PCR products from precipitation of round 6 transcripts using the entire pool 1 (lane 1) and individual sera 1–4 from pool 1 (lanes 2–5) are shown.

RT-PCR products. Pools 1, 3, and 4 generated smears of RT-PCR products ranging in size from 250 bp to 4,000 bp. Pool 2 was unique in that it selected a predominant product of approximately 550 bp (Fig. 1B). Selection products were discernible after one round of selection for pools 1, 3, and 4, but pool 2 did not exhibit any discernible RT-PCR products until three rounds of selection were completed (Fig. 1C). The degree of enrichment for products interacting with antibodies in the sera pools over the course of the selections was determined by comparing the proportion of starting material versus products of the final round of selection bound by a given pool, a standard measure of enrichment (Conrad et al., 1995). Radioactively labeled transcripts from the unselected HeLa cell cDNA library and from the round 6 products of pool 1 and pool 2 were compared for binding by pool 1 and pool 2, respectively. Binding of pool 1 and pool 2 products from round 6 were 12-fold and 6-fold enriched, respectively, over the binding levels of these pools for the HeLa cell mRNA starting material (data not shown). We conclude that the selected RNA was enriched substantially over the course of these iterative selection experiments to yield products preferentially bound by antibodies in these sera.

Deconvolution of patient serum pools

The serum pools were then deconvoluted to determine which of the sera in each pool possessed anti-mRNA reactivity. Each serum from the pools was tested for the ability to immunoprecipitate the round 6 mRNA selected by that serum's pool. The results for pool 1 were typical (Fig. 1D); within pools 1, 2, and 4 there was one serum responsible for that pool's immunoreactivity against the mRNA population selected. In pool 3, however, three of the four sera showed reactivity against the mRNA selected by that pool. These deconvolution experiments demonstrate the validity of pooling patient sera in order to investigate a larger number of sera for mRNA reactivity. Under the conditions employed here, no dilution effects masked detection of reactivity among the pooled sera. These results also provide a preliminary indication of the prevalence of anti-mRNA reactivity among autoimmune patient sera. Six of the 15 autoimmune sera assayed, or 40%, had reactivity against mRNA, whereas the other 9 did not immunoprecipitate mRNA to measurable levels after six rounds of selection. The sample size is reduced from 16 to 15 because one of the patient sera included in the pools was found to be from a normal patient rather than a patient with autoimmune disease as originally thought when it was randomly chosen for pooling from a collection of patient sera. This normal human serum, as well as nine of the autoimmune patient sera, did not select any mRNA species during the six rounds of selection performed here. This finding suggests that the mRNA fragments that arose during selections with the other mRNA-reactive autoimmune sera represented genuine mRNA autoantigens rather than aptamer RNA sequences that evolved during selection to fit the binding requirements of some member of the constellation of antibodies found in human serum.

The sera with anti-mRNA reactivity were used to probe western blots to determine what major protein autoantigens they also recognize. Three of the six sera were reactive against the RNA binding protein La. Reactivity against this autoantigen is correlated with Sjogren's syndrome and SLE (Lerner & Steitz, 1979; Rinke & Steitz, 1982; Saitta & Keene, 1992). Two of the sera were reactive against the SmB/B′ component of the U1 snRNP complex. One of these sera also had reactivity for the U1 70K component of the UL snRNP. Patients with SLE often produce antibodies against these antigens (Hardin, 1986; van Venrooij & Sillekens, 1989). The other serum with anti-mRNA reactivity had western blot reactivity for numerous proteins, with no predominant specificity for a known autoantigen.

Identity of mRNAs selected in vitro

The RT-PCR products for pools 1 and 2 were subcloned and sequenced to identify the messages selected by these two pools. Most of the subclones contained imperfect repeats of short stretches of an mRNA sequence due to internal priming by the T7cap primer in conjunction with some unknown concatemerization process. Nonetheless, pool 1 and pool 2 selected portions of several known mRNAs (Table 1). Pool 1 selected two independent clones of over 400 bp of the arginosuccinate synthetase message. Another pool 1 clone contained repeats from the ferritin heavy chain message. Three additional clones having no matches in the NCBI BLASTN database were selected by pool 1. Pool 2 selected clones containing repeats from the messages of alternative splicing factor/SF2 (ASF/SF2), calmodulin, and complement C3.

In vitro binding studies were performed to confirm that the serum used to select them recognized the subcloned message fragments. Labeled transcripts were generated for the arginosuccinate synthetase message fragment selected by pool 1 and the ASF/SF2 message fragment selected by pool 2. As expected, pool 1 immunoprecipitated the in vitro transcript of the arginosuccinate synthetase message and pool 2 immunoprecipitated the ASF/SF2 transcript (Fig. 2). We also tested the specificity of interaction for each pool; pool 1 bound the pool 2-selected ASF/SF2 transcript at much lower levels than the arginosuccinate synthetase transcript. Pool 2 also bound the pool 1-selected transcript arginosuccinate synthetase at lower levels than the ASF/SF2 transcript. These results illustrate the specificity of interaction, as each pool bound the transcript it selected much more strongly than the transcript se-

TABLE 1. Messenger RNA fragments subcloned after six rounds of selection.

mRNA	Portion subcloned	Accession number
Pool 1		
Arginosuccinate synthetase	Nt 892 to 1322 of coding	X01630
Ferritin heavy chain	Nt 699 to 790 of coding	M11146
Pool 2		
Alternative splicing	Nt 214 to 301 of coding	M72709
Calmodulin	Nt 1204 to 1321 of 3′ untranslated region	J04046
Complement C3	Nt 429 to 612 of coding region	K02765

lected by the other serum pool. The reactive serum from pool 1, serum from patient MJ, and the reactive serum from pool 2, serum from patient PL, were tested individually and found to bind these products in the same selective manner (data not shown).

Competition and deletion studies to define a common autoepitope in ASF/SF2 and calmodulin mRNAs

Competition experiments were performed to determine whether two transcripts selected by reactive serum PL from pool 2 were bound by the same antibody within that serum. Labeled ASF/SF2 transcript was immunoprecipitated by serum PL in the presence of increasing amounts of unlabeled calmodulin transcript. Addition of as little as 0.01 μg of cold competitor calmodulin transcript significantly abrogated ASF/SF2 binding (64% of control with no competitor), and use of 5 μg of competitor reduced binding to 13% (Fig. 3). By contrast, a control transcript from the 3′ untranslated region of neurofilament M mRNA did not interfere with binding of ASF/SF2 at levels up to 10 μg. The converse experiment using labeled calmodulin transcript and ASF/SF2 transcript as competitor produced similar results (data not shown). Two transcripts selected by serum PL thus compete for binding to an antibody in this serum, implying that they contain the same epitope.

Transcripts consisting of single repeats of either the ASF/SF2 or calmodulin message fragments were used in *in vitro* binding assays with serum PL to determine whether one repeat was sufficient for recognition by the antibody (Fig. 4). The *Bsa*JI transcript from the ASF/SF2 subclone was bound at significant levels by serum PL, as was the *Xho*I transcript from the calmodulin subclone. We conclude that the epitope recognized by serum PL is present in a single copy of the repeated ASF/SF2 and calmodulin sequences, and is not an artifact of the repeat sequences in the selected clones.

PCR clones of ASF/SF2 and calmodulin were generated that represented more of the full-length message than contained in the short repeats of the original subclones (Fig. 5A). Serum PL immunoprecipitated an ASF/SF2 transcript consisting of nt 67 to 565 of the ASF/SF2 mRNA, as well as a calmodulin transcript consisting of nt 1103 to 1504 from the 3′ untranslated

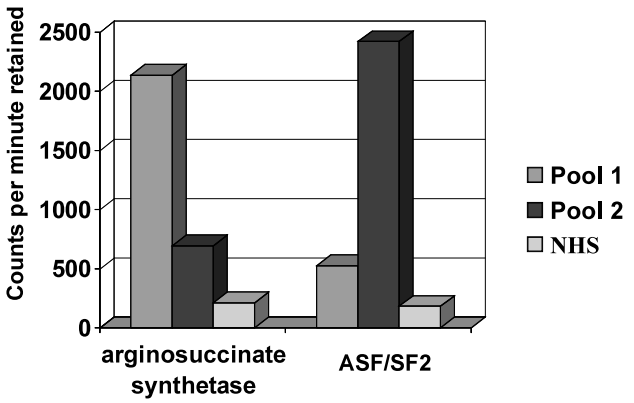


FIGURE 2. Pools 1 and 2 selectively bind in vitro transcripts of their selected products. Radiolabeled transcripts of the arginosuccinate synthetase and ASF/SF2 subclones selected by pools 1 and 2, respectively, were immunoprecipitated using protein A beads coated with pool 1, pool 2, or normal human serum (NHS). After washing, aliquots of the beads were analyzed by Cerenkov counting.

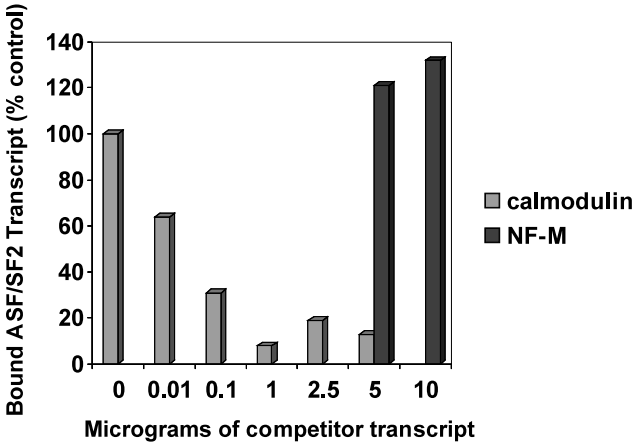
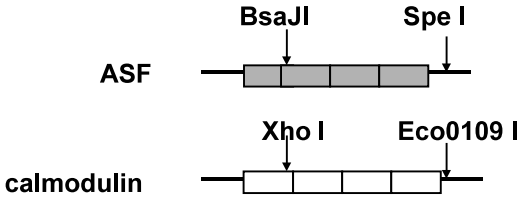


FIGURE 3. ASF/SF2 and calmodulin compete for binding by an autoantibody in serum PL. Radioactively labeled ASF/SF2 transcript was immunoprecipitated by protein A beads coated with serum PL in the presence of increasing amounts of unlabeled calmodulin transcript or unlabeled NF-M transcript. After washing, aliquots of the beads were analyzed by Cerenkov counting.

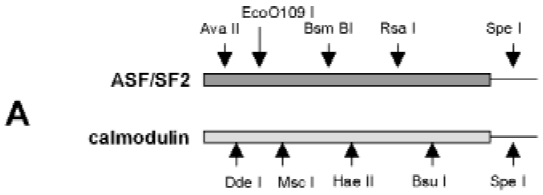


Transcript	cpm retained	
ASF	**serum PL**	**NHS**
Bsa JI	5402	59
Spe I	4237	68
calmodulin	**serum PL**	**NHS**
Xho I	10082	213
Eco0109 I	9673	102

FIGURE 4. A single short sequence from the ASF/SF2 or calmodulin subclones is sufficient for binding to serum PL. Subclones containing four imperfect repeats of either ASF/SF2 or calmodulin were digested at the indicated restriction sites and labeled transcripts containing either one or four repeats were immunoprecipitated with either serum PL or normal human serum (NHS). After washing, aliquots of the beads were analyzed by Cerenkov counting.

region of the calmodulin message (Fig. 5B, *Spe*I digests of ASF/SF2 and of calmodulin, respectively). Shorter transcripts produced by restriction digests of the templates were also recognized by serum PL. We conclude that recognition of the ASF/SF2 and calmodulin subclones was not an artifact due to the tandem repeats of message fragments contained in the subclones obtained from the selection experiments.

Further restriction digestion of the ASF/SF2 and calmodulin templates produced truncated transcripts that we used to determine the minimal sequence element in these messages recognized by serum PL. Progressive truncation revealed the minimal sequence required for binding of ASF/SF2 to reside between the *Eco*O109I site and *Ava*II sites (Fig. 5C). Similar experiments with calmodulin ascertained that the minimal binding sequence is between the *Dde*I and *Msc*I sites (Fig. 5C). Because these mRNA fragments can compete with each other for binding by serum PL antibodies, some common epitope must be present in both these minimal binding sequences. Computer generated secondary structures for these messages revealed that both are capable of forming a secondary structure with a stem surrounding a loop of five bases (Fig. 5D). Both the ASF/SF2 and calmodulin stem-loop structures share a number of bases in the same relative positions (indicated in Fig. 5D). Although all predictions of RNA secondary structure are fraught with potential uncertainty, predicted structures for the two mRNAs that bind this antibody are strikingly similar. The secondary structures taken together with the competition data for these



B

ASF/SF2	cpm retained	calmodulin	cpm retained
Bsm BI	441	Hae II	4890
Rsa I	563	Bsu I	3340
Spe I	1448	Spe I	3359

C

Transcript	Counts per minute retained	
ASF/SF2	**serum PL**	**NHS**
Ava II	90	51
EcoO109 I	3411	74
Spe I	5044	89
Calmodulin	**serum PL**	**NHS**
Dde I	63	33
Msc I	603	199
Hae II	3283	195

D

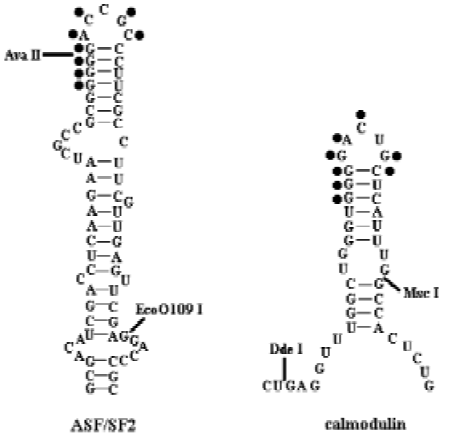


FIGURE 5. Serum PL recognizes a minimal sequence from ASF/SF2 and calmodulin mRNAs that both form a similar predicted stem-loop secondary structure. Portions of the ASF/SF2 and calmodulin mRNAs were obtained by PCR cloning, and digested at the indicated restriction sites to produce labeled transcripts (**A**). The transcripts were immunoprecipitated with protein A beads coated with serum PL (**B**), or with serum PL and normal human serum (NHS) (**C**). Transcripts corresponding to various lengths of the ASF/SF2 and calmodulin mRNAs were precipitated by serum PL in **A**. The results of experiments with progressive truncations to define the minimal sequences from these mRNAs required for recognition by serum PL are shown in **B**. RNA folding of these portions of the ASF/SF2 and calmodulin mRNAs was generated using the Wisconsin GCG Squiggles program (**D**). The locations of the restriction sites marking the boundaries of the minimal binding sequence are indicated, along with the nucleotides common to both sequences.

two mRNA fragments suggests the two mRNA fragments share a common conformational epitope.

To define the bases comprising the epitope in the ASF/SF2 message recognized by serum PL, several

mutants were constructed with altered bases in the region of sequence similarity between ASF/SF2 and calmodulin. Radiolabeled transcripts of these mutants were used along with the unaltered ASF/SF2 mRNA in immunoprecipitation experiments with serum PL and NHS to determine whether the mutations affected the ability of serum PL to recognize the transcripts (Fig. 6). Altering the five bases comprising the loop in a conservative fashion (purine bases switched for purine bases and pyrimidines switched for pyrimidines) reduced binding serum by PL to 55% of wild-type ASF/SF2 levels. Nonconservative switches of the bases in the loop (purines switched for pyrimidines and pyrimidines switched for purines) reduced binding by serum PL to 40% of the wild-type ASF/SF2 levels. Flipping the three bases in the stem of the ASF/SF2 structure that are closest to the loop from one side of the stem to the other reduced binding to 49% of wild-type levels. Flipping every other base in the stem region closest to the loop had no effect on binding. A mutant with nonconservative switches of the loop and the three bases closest to the loop flipped had negligible binding by serum PL. Taken together, these results determine that the epitope in ASF/SF2 recognized by serum PL is indeed located in the 17-base stem-loop region sharing common sequence with the calmodulin message also recognized by this serum.

Immunoprecipitation of ASF/SF2 and calmodulin mRNAs from total cellular RNA

To further establish that serum PL was able to recognize native mRNAs for ASF/SF2 and calmodulin, we used this serum to immunoprecipitate total HeLa RNA that had been deproteinized by purification in guanidinium and phenol followed by proteinase K treatment and phenol extraction. The precipitated RNA was reverse transcribed using random hexamer primers. Reverse transcriptions were also performed using the supernatant as well as total HeLa cell RNA as templates. Primers specific for ASF/SF2, calmodulin, and various control genes such as actin, GAPDH, and glucose transporter 1 were used to amplify the reverse transcription products. Serum PL immunoprecipitated the ASF/SF2 and calmodulin messages but did not immunoprecipitate any of the negative control messages (Fig. 7). Control immunoprecipitation with normal human serum did not precipitate any mRNA as determined by the same RT-PCR measurements (data not shown). We conclude that the antibodies contained

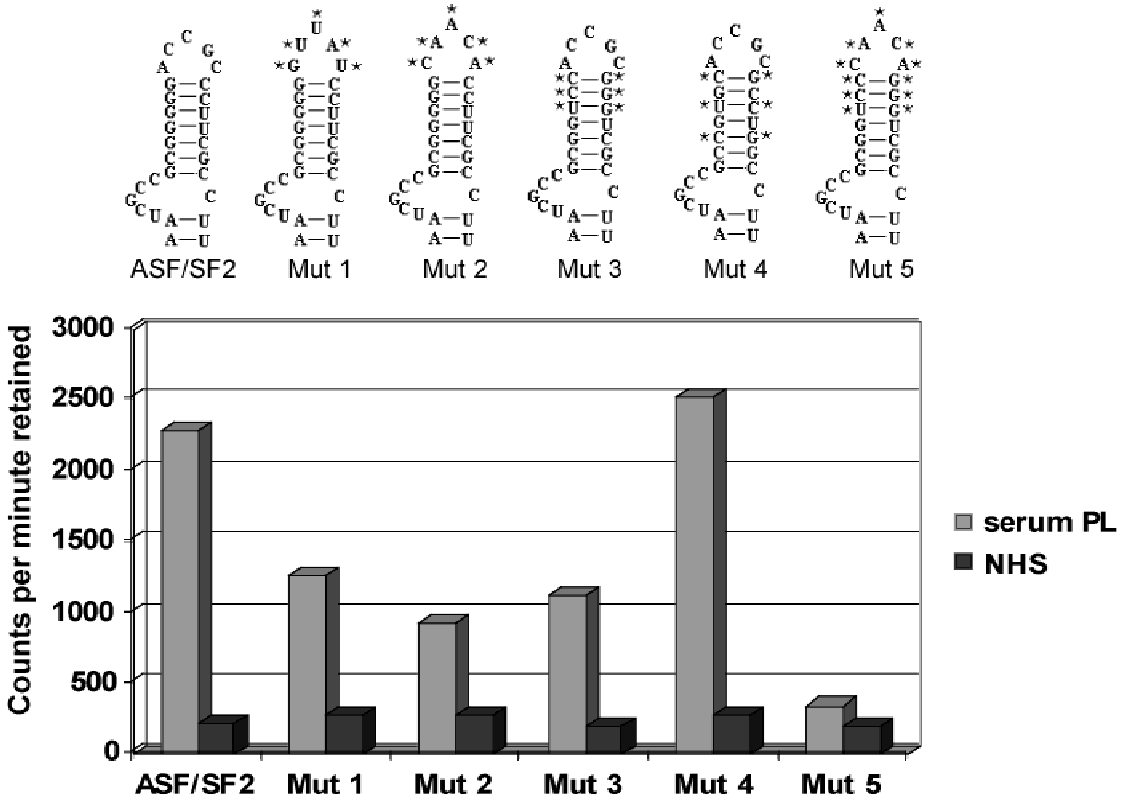


FIGURE 6. The epitope in ASF/SF2 recognized by serum PL resides in a stem-loop structure. Site-directed mutagenesis was used to produce four templates encoding transcripts with altered bases, indicated by stars. Immunoprecipitations of these mutant transcripts and wild-type ASF/SF2 were performed using protein A beads coated with serum PL or normal human serum (NHS). After washing, aliquots of the beads were analyzed by Cerenkov counting.

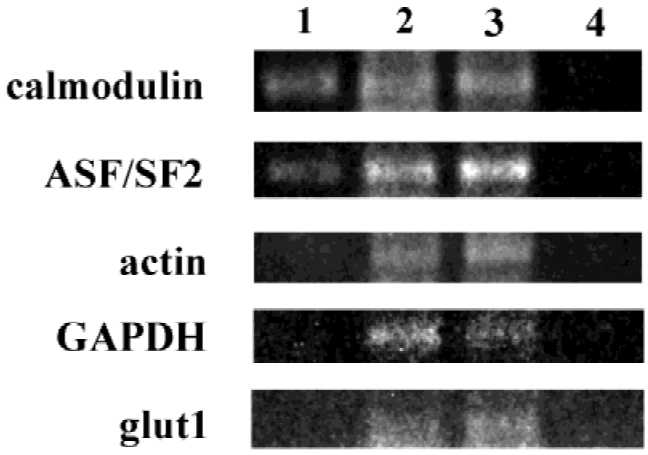


FIGURE 7. Serum PL selectively immunoprecipitates full-length ASF/SF2 and calmodulin mRNAs from HeLa total RNA. Total RNA from HeLa cells was immunoprecipitated with serum PL. The bound RNA was eluted and then reverse transcribed using random hexamers. Reverse transcription was performed using the RNA remaining in the supernatant, as well as total HeLa cell RNA. Primers specific for ASF/SF2, calmodulin, glyceraldehyde 3-phosphate dehydrogenase, and glucose transporter 1 were used. In each panel, lanes 1–4 show precipitated RNA, supernatant RNA, total HeLa cell RNA, and no template PCR control, respectively.

in serum PL are capable of specifically recognizing an epitope in the cellular mRNAs for ASF/SF2 and calmodulin.

DISCUSSION

We have identified anti-mRNA reactivity in sera from patients with systemic autoimmune diseases using a strategy of pooling patient sera for iterative in vitro selection of mRNA from a natural sequence library. Under proper conditions, in vitro selection should enable cloning and identification of any message recognized by an autoantibody. The selection scheme employed in these studies did not produce full-length clones of these messages. However, in vitro selection from this natural sequence library allowed the identification of autoimmune epitopes in mRNAs. Several mRNAs containing autoepitopes were identified: arginosuccinate synthetase, ferritin heavy chain, alternative splicing factor/SF2, and calmodulin. The patient sera recognizing these mRNAs also had immune specificity for the La autoantigen.

One explanation for the origin of anti-RNA antibodies involves the broadening of an original immune response toward a protein antigen that spreads to the RNA molecules when complexed with the autoantigenic protein. Numerous patient sera with anti-RNA autoantibodies have been shown to also contain autoantibodies against RNA-binding proteins that are complexed with the RNA antigens in various RNP particles (reviewed in Keene, 1996c). For example, certain patients with SLE or Sjogren's syndrome have antibodies against the hY5 RNA as well as the Ro RNA-binding protein (Boulanger et al., 1995). The most thoroughly characterized example of this phenomenon is the U1snRNP, in which the U1 snRNA as well as the various RNP proteins are autoreactive.

Emerging data in our lab suggest the mRNA epitopes described here could possibly have generated immune responses as a result of being complexed to autoantigenic RNA binding proteins. Recently, we reported the development of methods for identification of clustered mRNAs using antibodies against RNA-binding proteins to immunoprecipitate RNP complexes containing RNA-binding proteins (Tenenbaum et al., 2000). The mRNAs contained in the immunoprecipitated RNP are identified and quantitated using cDNA microarray technology. Employing this method, patient sera with reactivity against the RNA-binding protein La were used to immunoprecipitate and identify the mRNAs present in La-containing RNP complexes. It is intriguing to note that the four known mRNAs shown here to be reactive against La sera were strongly represented in these La RNP complexes: the calmodulin, arginosuccinate synthestase, ferritin heavy chain messages, and ASF/SF2 were all immunoprecipitated by this anti-La serum (S.A. Tenenbaum & J.D. Keene, unpubl. data). These findings are consistent with direct immunological presentation of the La protein, followed by spreading of the immune response to messages found in the La RNPs, resulting in autoantibodies against these mRNAs.

The apparent paucity of epitopes found in RNA molecules argues against mere chance presentation of randomly exposed portions of the RNA molecules complexed to protein antigens as the exclusive mechanism giving rise to anti-RNA autoantibodies. Epitopes have only been found in a small number of cellular RNAs: U1 snRNA alone among the snRNAs, hY5 Ro alone among the five hY Ro RNAs, and only one small portion of the 28S rRNA molecule (reviewed in Keene, 1996a). If anti-RNA reactivity resulted merely from chance presentation of RNA molecules complexed to protein autoantigens, one would expect a broader response against these RNA molecules. A set of criteria for molecular recognition must govern which RNA molecules and which portions of those molecules generate an autoimmune response.

We have previously hypothesized that structural mimicry between a protein autoantigen and an RNA molecule is involved in the generation of RNA autoepitopes (Keene, 1996a). According to this hypothesis, an antigenic protein is the original immunogen and anti-RNA reactivity results from cross-reactivity due to structural mimicry. Examples have been found using in vitro selection experiments with combinatorial RNA libraries of RNA molecules that compete with peptides or proteins for binding to an antibody generated against that peptide or protein (Tsai et al., 1992; Hamm, 1996). RNAs

selected by a monoclonal antibody against the insulin receptor cross-react with autoantibodies from insulin-resistant patients (Doudna et al., 1995), indicating RNA can mimic the structure of an autoantigenic epitope. Certain RNA molecules are clearly capable of mimicking the protein structures of antigens. Perhaps the mRNA epitopes described here resemble a proteinaceous epitope during presentation to the immune system, causing the formation of antibodies to those messages. Thus, molecular mimicry could form the basis for determining which RNA molecules are immunogenic and which are not.

Structural mimicry between RNA and protein surfaces, as indicated by recognition by a common antibody, may also reflect functional mimicry. The mimetic RNA and protein could act in a *trans* fashion at the same site within the cell (Keene, 1996a). Accumulating data show that protein factors involved in protein biosynthesis are structural and possibly functional mimics of tRNA (reviewed in Nissen et al., 2000). For example, crystallography data indicate strikingly similar shapes for the bacterial elongation factor EF-G and the ternary complex of phenylalanine tRNA with elongation factor Tu, providing the basis for interaction of the elongation factor with the A site of the ribosome (Nissen et al., 1995).

Consistent with the findings of this study, mRNA molecules represent a library of sequences capable of forming secondary structures that may mimic the shapes of proteins and have regulatory functions. Although no examples of mimicry between a message and a protein have been elucidated to date, there is evidence for sequences in mRNA having the ability to act in *trans* to regulate cellular processes. For example, the 3′ UTRs of troponin I, α-tropomyosin, and α-cardiac actin function as *trans*-acting regulators to cause tumor suppression (Rastinejad & Blau, 1993; Rastinejad et al., 1993). Tropomyosin is believed to exert this action by activating the cellular kinase PKR (Davis & Watson, 1996), and this may account for its *trans* effects in growth suppression.

Direct presentation of mRNA resulting from a broadened immune response and the possible contribution of molecular mimicry of proteins by RNA are both fascinating explanations for the mRNA epitopes reported here. Further work will be necessary to determine if either one or both of these mechanisms are involved in the generation of anti-RNA reactivity. Regardless of how they arise, it is certain that autoantibodies against mRNA epitopes will be invaluable tools to permit dissection of mRNP particles. Such autoantibodies will aid in the study of the posttranscriptional processing of messages and perhaps shed light on how messages are grouped and moved throughout the cell. Autoantibodies are also potentially useful tools for identifying regions of mRNA molecules involved in *trans* active functions. The findings presented here thus reveal the existence of anti-mRNA antibodies as exciting new tools for the study of messenger RNA biology.

MATERIALS AND METHODS

Immunoprecipitation of RNA

Immunoprecipitations were performed as previously described (Tsai et al., 1992). Briefly, protein A-Sepharose beads (Sigma, 8 μg/100 mL of reaction volume) were washed in NT2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Nonidet P-40) and mixed with either 10 μL of pooled patient sera or 4 μL of individual patient serum overnight at 4 °C with rotation. The beads were washed four times in 1 mL NT2 buffer and resuspended in 100 μL KNET buffer (50 mM Tris, pH 7.4, 80 mM NaCl, 20 mM KCl, 2 mM EGTA, 0.05% Nonidet P-40, 1 mM $MgCl_2$, 2.5% polyvinyl alcohol, RNasin at 40 U/mL, yeast tRNA at 100 μg/mL, bovine serum albumin at 50 μg/mL). RNA was heat denatured for 3 min at 85 °C, allowed to refold for 10 min at room temperature, and added to the protein A bead/KNET mixture. After incubation with mixing at room temperature for 7 min, the beads were washed five times in 1 mL of NT2 buffer. The immunoprecipitated mRNA was recovered by proteinase K treatment, phenol/chloroform/isoamyl alcohol extraction, and ethanol precipitation.

Iterative selection

An adaptation of the method of Gao et al. (1994) was used to select mRNA sequences from a natural sequence library. An amplified cDNA library was made from 1.5 mg total HeLa RNA by the cap-switch reverse transcription method (Clontech). Full-length cDNAs were produced by including an oligonucleotide (T7CAP; 5′-ACCTGGATCCTAATACGACTCACT ATAGACGCGGG-3′) complementary to the mRNA cap structure. The Superscript II reverse transcriptase (Life Technologies) was primed by a modified oligo (dT) primer (RTBAM; 5′-CATGGAATTCGGATCCT$_{(25)}$N$_1$N-3′, where N = A, C, G, or T and N$_1$ = A, C, or G), and then transcribed to the 5′ end, where it switched strands and transcribed the T7CAP sequence. Oligonucleotides T7CAP and RTBAM were used as primers for subsequent amplification of the cDNAs using the polymerase chain reaction. Reactions were performed using TaqPlusLong (Stratagene) in the manufacturer's buffer. An initial cycle of 95 °C for 2 min was followed by 20 cycles of 95 °C for 1 min with a combined annealing and extension at 68 °C for 5 min. The library was checked for representation of various rare messages through PCR with primers specific for those gene products.

Oligonucleotide T7CAP contains a T7 RNA polymerase promoter sequence, allowing PCR amplification products to be in vitro transcribed into full-length messages. One-fourth of each PCR reaction's products were used as template in a transcription reaction containing polymerase buffer (40 mM Tris, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 0.4 mM each ATP, CTP, GTP, and UTP, 1.5 mM dithiothreitol, 20 U RNasin (Promega), and 20 U T7 RNA polymerase. After 1 h at 37 °C, RNase-free DNase was added to a concentration of 1 U/μg template for 30 min at 37 °C to destroy the template. The transcripts were purified through G-50 columns, then phenol extracted and ethanol precipitated.

Autoimmune sera pools were created by mixing equal volumes of four patient sera. The pooled sera were adsorbed to protein A-Sepharose beads and used to immunoprecipitate mRNA as described above. The eluted RNA was reverse transcribed and amplified as above, then T7 transcribed for another round of immunoprecipitation. Following the final round of iteration, PCR products were subcloned using the TA cloning vector system (Invitrogen).

Immunoprecipitation of radiolabeled RNA

Radioactively labeled in vitro transcripts were generated from linearized plasmids using the Ribopobe in vitro transcription system (Promega). ^{32}P-labeled transcripts were purified through G-50 spin columns (5 Prime→3 Prime). For experiments comparing binding of multiple labeled transcripts, the number of radioactive counts used for each transcript were normalized for content of the labeled residue. Immunoprecipitation was carried out as above, followed by five 1-mL washes in NT2 buffer. The protein A-Sepharose beads were resuspended in 150 μL of NT2. A 50-μL aliquot was spotted on a GF/C filter (Whatman) for Cerenkov counting to determine the number of counts immunoprecipitated.

Site-directed mutagenesis

A cDNA clone containing bases 67–984 of the ASF/SF2 mRNA was used to generate site-directed mutants. The QuikChange site-directed mutagenesis system (Stratagene) was used. Oligonucleotides from each strand containing the desired base changes were used to amplify the wild-type template plasmid with the non-strand-displacing PfuTurbo DNA polymerase. The parental DNA was digested with *Dpn*I and the remaining mutated circular DNA was transformed into XL-Blue cells. Mutations were confirmed by DNA sequencing. The wild-type and mutant plasmids were digested with *Rsa*I to produce templates for radiolabeled in vitro transcripts.

Immunoprecipitation and RT-PCR of full-length mRNA

Total HeLa RNA was prepared by Trizol (Life Technologies) extraction of HeLa S3 cells followed by Proteinase K digestion and phenol/chloroform/isoamyl alcohol extraction. Fifteen micrograms of RNA was gently agitated with protein A-Sepharose beads coated with either autoimmune serum PL or normal human serum for 7 min at room temperature in KNET buffer. After removing the supernatant, the beads were washed six times in 1-mL NT2 buffer. The precipitated RNA was eluted by proteinase K digestion followed by phenol/chloroform/isoamyl alcohol extraction. RNA was ethanol precipitated from the supernatant and precipitate fractions and reverse transcribed at 50 °C using random hexamer primers with Thermoscript reverse transcriptase (Life Technologies) in manufacturer's supplied buffer and reagents. Aliquots of the reverse transcription were used in PCR reactions with primers specific for ASF/SF2 (ASFfwd99: GTTCTCGAGTC CGCACTTTT; ASFrev99: TGCCATCTCGGTAAACATCA, annealing temperature of 50 °C), calmodulin (calmfwd99: GAGGAATTGTGGCGTTGACT; calmrev99: TTAGCTAGC CCAGCATGGTT, annealing temperature of 50 °C), glucose transporter (glut1fwd: CCTAATACGACTCACTATAGGGCAA GTGTGAGGAGCCCCA; glut1rev: CTAAAAACCAGCCAT TTA, annealing temperature of 50 °C), m-actin (m-actin1: TTGCTGGCGACGACGCCCCC; m-actin2: GTCTGTCAG GTCCCGGCCAG, annealing temperature of 55 °C), and glyceraldehyde dehydrogenase (GAPDHfwd: TCCTGCACCACCA ACTGC; GAPDHrev: TCATACCAGGAAATGAGC, annealing temperature of 55 °C). PCR conditions were 94 °C for 2 min, followed by 25 cycles of 94 °C for 1 min, annealing at the appropriate primer temperature for 1 min, and extension at 72 °C for 2 min, followed by a final extension step of 7 min of 72 °C. Products were visualized on an agarose gel.

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