A sequence-specific conformational epitope on U1 RNA is recognized by a unique autoantibody
(protein–nucleic acid interactions/autoimmune disease/RNA binding proteins/conformational epitopes)

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Communicated by D. Bernard Amos, January 11, 1988 (received for review September 23, 1987)

ABSTRACT An autoantibody from a patient with lupus-overlap syndrome was found to bind a specific region of U1 RNA. By using RNA sequence analysis, immunoprecipitation, and competition experiments with in vitro synthesized fragments of U1 RNA, a region of 40 nucleotides representing a stem-loop secondary structure was found to be an immunoreactive domain. This antibody recognized a conformational epitope because neither the RNA stem nor the RNA loop alone was immunoprecipitable. Antisense U1 RNA, U1 DNA, and other small RNAs were not reactive with the antibody. While the origins of nucleic acid-binding antibodies are unknown, this RNA-specific autoantibody probably originated by direct presentation to the immune system or as an anti-idiotype against a more common U1 small nuclear ribonucleoprotein-specific autoantibody. Thus, these findings have implications for the mechanisms of autoimmune recognition and provide an immunological approach to probing RNA structure and protein–RNA interactions.

Nucleic acid-reactive antibodies provide models for the study of protein–nucleic acid interactions (1–5). For example, anti-Z-DNA antibodies have been used to probe conformation of DNA and to examine the structure of polytene chromosomes (1, 2). Although nucleic acids are thought to be poor immunogens (6), antibodies to nucleic acids have been experimentally induced (5, 7) and have been obtained from sera of autoimmune patients (2, 7, 8). Most nucleic acid-reactive antibodies recognize DNA (1, 2, 4, 5, 8, 9), although RNA-reactive antibodies that recognize G+C-rich regions (10), tRNA S1a (11), and tRNA Met (12) also have been reported. However, antibody binding sites on RNA have not been defined, and these antibodies appear to lack specificity for defined sequences. The high degree of secondary structure of RNA and the known recognition sites of well-characterized RNA binding proteins, such as ribosomal L18 protein (13) and the phage R17 coat protein (14, 15), suggest that RNA-reactive antibodies may also recognize conformational features of the antigenic molecule. Neither the nature of RNA–antibody recognition nor the mechanism by which nucleic acid-reactive autoantibodies are elicited is understood. We have described RNA-specific autoantibodies (12) that recognize U1 RNA, a small nuclear RNA (snRNA) that is involved in mRNA splicing (16–19). U1 RNA is amenable to the study of nucleic acid–protein interactions because of its small size, the ability to synthesize abundant amounts of the RNA in vitro (20), and the availability of antibodies that react with U1 ribonucleoproteins (RNPs) (16). In this study, a contact site on U1 RNA that is specifically recognized by a U1 RNA-reactive autoantibody was determined. This epitope was synthesized in vitro and retained immunological reactivity as long as the conformation of a specific stem-loop structure of U1 RNA was intact.

MATERIALS AND METHODS

Reagents. Radiolabeled compounds, [32P]UTP, [32P]ATP, and [32P]orthophosphate, were obtained from ICN. Enzymes were obtained from Bethesda Research Laboratories, New England Biolabs, and Pharmacia. Placental ribonuclease inhibitor (RNasin) and phage SP6 polymerase were obtained from Promega Biotec (Madison, WI). Pansorbin was purchased from Calbiochem, RNase T1 and pancreatic nuclease A (RNase A) were obtained from Boehringer Mannheim. Oligonucleotides of >100 nucleotides of poly(C, U), poly(G, U), poly(G), and poly(C) were obtained from Sigma. Autoimmune sera were obtained from Duke patients as described (12, 21). The U1 and U2 cDNAs were gifts of Alan Weiner (Yale University) and Nouria Hernandez (Cold Spring Harbor Lab., Cold Spring Harbor, NY). An SP6 vector expressing adenovirus-associated VA DNA was a gift of Dana Fowkes (University of North Carolina, Chapel Hill).

Cells, Labeling, and RNA Preparation. HEla cells were grown as described (21). In vivo labeled HeLa cell RNA was prepared by incubating cells in the presence of [32P]orthophosphate at 150 μCi/ml (1 Ci = 37 GBq) in phosphate-free medium for 12 hr and processed as described (12).

Immunoprecipitations. Immunoprecipitations were performed essentially as described (12, 21, 22). Immunoprecipitations containing RNA were performed at 4°C in 150 mM NaCl/50 mM Tris, pH 7.4, in the presence of 25 units of RNasin and 20 μg of competitor RNA. Antiserum was added to the reaction for 4 min. Immune complexes were absorbed with Pansorbin for 5 min and washed, and the RNA was extracted (12). RNAs were analyzed on 5% acrylamide/8.3 M urea gels (21).

RNA Labeling. RNA was labeled at its 5′ end with polynucleotide kinase and [32P]ATP as described (23). Uniformly labeled in vitro synthesized RNAs were produced by using the SP6 transcription system in the presence of [32P]UTP. Transcripts of U1 RNA were 300 nucleotides long and included approximately 100 extra bases at the 3′ end (see in Fig. 2 Upper) (20).

Nuclease Digestion of RNA. U1 RNA synthesized in vitro was digested with RNase T1 or RNase A at 37°C for 30 min (nondenaturing conditions) or 68°C for 2 min (denaturing conditions), treated with proteinase K (100 μg/ml), extracted with phenol, and precipitated with ethanol as described (21).

RNA Sequencing. In vitro synthesized U1 RNA was subjected to oligonucleotide fingerprint analysis as follows. RNA was digested with RNase T1 (20 units of T1 per μg of RNA) at 37°C for 30 min, treated with proteinase K (100 μg/ml), extracted with phenol, and precipitated with ethanol before immunoprecipitation in the absence of RNA. Fragments that

Abbreviations: RNP, ribonucleoprotein; snRNA and snRNP, small nuclear RNA and RNP; NHS, normal human serum.
precipitated with antibody were gel-purified and eluted (12), 5' end-labeled (23), and partially digested in formamide at 100°C for 2 hr. The digestion products were separated by electrophoresis on cellulose acetate (pH 3.5) in the first dimension and homochromatography of DEAE-cellulose in the second dimension as described (24). Assignments of the predicted nucleotide sequence were deduced by vectorial shift analysis.

Synthesis of U1 RNA Stem-Loop. U1 RNA corresponding to nucleotides 51–90 (stem–loop) and 66–75 (loop) were generated by synthesis of the corresponding DNA oligonucleotides with 5' HindIII and 3' EcoRI linkers by using an Applied Biosystems (Foster City, CA) synthesizer. Purified oligonucleotides were hybridized (25) and cloned into SP64. Construction of the correct insert was verified by plasmid sequencing (26). The U1 RNA transcripts were synthesized by using the SP6 transcription system after linearization with EcoRI.

RESULTS

Coexistence of Antibodies to RNA and RNP. U1 RNA forms the core of a RNP complex that consists of nine proteins and may never exist as naked RNA in vivo. Three proteins of 70, 30, and 20 kDa recognize specific portions of the RNA (22), while a complex of six proteins (Sm complex) binds a sequence of U1 RNA that is also present in the U2, U4, U5, and U7 snRNAs. Lerner and Steitz (16) first demonstrated that autoantibodies from patients with lupus and related diseases recognize these U1 RNA-binding proteins. The U1 RNP plays an important role in the splicing of precursor mRNA in the nucleus prior to its transport to the cytoplasm (16–19).

We have identified three patients that produce antibodies reactive with U1 RNA (12). In each case, these specificities occurred as a subset of the U1 RNP specificity but appear to be much less common. Serum from a patient with lupus-overlap syndrome and rheumatoid arthritis precipitated U1 and U2 small nuclear RNP (snRNP) complexes by recognition of the associated proteins (Fig. 1 Upper, lanes 2 and 3) as well as deproteinized U1 RNA from HeLa cells (Fig. 1 Upper, lanes 7 and 8). Thus, an antibody in the serum, when presented with total cellular RNA, specifically selected the U1 RNA over all other species. The titer of the anti-U1 RNA activity in the patient's serum remained constant over a 2-year period, and no additional RNA reactivities developed (Fig. 1 Upper, lanes 7 and 8). Further proof that the antibody did not require protein for recognition of U1 RNA was shown by the ability of the antibody to immunoprecipitate U1 RNA synthesized in vitro (Fig. 1 Lower, lanes 1–4). A minor premature termination product of U1 RNA was produced by SP6 transcription. This band of about 220 nucleotides was also precipitable with this antibody and included the entire U1 RNA sequence.

Specificity of the RNA–Antibody Interaction by Competitive Binding. The specificity of antibody recognition of in vitro synthesized U1 RNA was examined by competitive binding assays with various nucleic acids to determine whether the antigen–antibody complex could be dissociated. Fig. 1 Lower shows that unlabeled in vitro U1 RNA effectively competed for binding of in vitro 32P-labeled U1 RNA to the antibody. In addition, various nucleic acid polymers including poly(C), poly(G), or a poly(G)-poly(C) hybrid did not effectively compete for binding to the U1 RNA even at levels of 100-fold molar excess (Fig. 1 Lower). These polymers have been shown to compete for binding of other RNA antibodies to ribosomal RNA (10). Anti-sense U1 RNA also did not compete with U1 RNA. Furthermore, various amounts of tRNA did not compete for binding to the U1 RNA-specific antibody. The failure of these competitors to

block U1 RNA binding by the autoimmune serum indicates that the interaction was highly specific.

To determine whether this antibody recognized only intact U1 RNA or whether a specific region of the molecule was sufficient for binding, 3' portions of the DNA template were deleted. U1-containing SP64 DNA was cleaved at various restriction enzyme sites represented on the diagram in Fig. 2 Upper, and the resultant DNA templates were used to synthesize the corresponding truncated RNAs. Deletion of the 3' end of U1 RNA did not eliminate recognition by the anti-U1 RNA antibody (Fig. 2 Lower, lanes 2, 4, and 5) until
The fragments of U1 RNA immunoprecipitated by the autoantibodies were subjected to RNA sequence analysis. Oligonucleotide fingerprints of the immunoprecipitable T1 fragments labeled at their 5’ ends resulted in three unique ‘wandering spot’ patterns as shown in Fig. 3 Middle. The deduced RNA sequences corresponded to nucleotides 51–85 on the U1 RNA molecule. Some guanosine residues remained RNase T1-resistant, and the 5’ ends of the oligonucleotides in some cases were degraded. This may have resulted from partial digestion conditions, secondary structural features (27, 28), or RNase in the polynucleotide kinase used for end-labeling. A proposed secondary structure of U1 RNA (28) and the U1 RNA fragments that are consistent with the deduced patterns of Fig. 3 Middle are diagrammed in Fig. 3 Bottom. The immunoprecipitable fragments of U1 RNA lie within a predicted middle stem-loop (or B loop) of the U1 RNA molecule.

Conformation of the U1 RNA Epitope. To further define the boundaries of the RNA epitope and to determine the nature of the substructure of U1 RNA that is recognized by the antibody, a DNA corresponding to just the middle stem-loop (Fig. 2 Upper, SLB) was synthesized and cloned into SP64 (Fig. 2 Upper, SLB). The resulting RNA transcript comprising the stem-loop epitope was efficiently immunoprecipitated with anti-U1 RNA antiserum (Fig. 4 Left, lanes 2 and 6). Approximately 45% of the input radioactivity in the stem-loop B transcript was precipitable, while that for the entire U1 molecule was approximately 60%.

Determination of Antibody Binding Site on U1 RNA. Because in vitro synthesized fragments of U1 RNA that lacked portions of the 3’ end were reactive with the antibody, we determined whether fragments generated by direct RNase digestion could be immunoprecipitated. Thus, U1 RNA was synthesized, digested with RNases T1 and A (Fig. 3 Top, lanes 2–5), and immunoprecipitated with anti-U1 RNA antiserum (Fig. 3 Top, lanes 7–11). U1 RNA fragments of approximately 40 and 35 nucleotides generated by digestion with RNase T1 were specifically recognized by the antibody (Fig. 3 Top, lanes 8 and 9). Under denaturing conditions the larger of the two major RNase T1 fragments was less precipitable (Fig. 3 Top, lane 9) suggesting that a folded structure of the 40-nucleotide fragment may be required for antibody recognition. The reduced level of material in lane 9 may also reflect loss of the antibody binding the sequence because of nonspecific RNase digestion of the denatured U1 transcript. However, RNase T1 fragments smaller than 35 nucleotides were not precipitable with the antibody (Fig. 3 Top, lane 9). Small RNA fragments resulting from RNase A digestion shown in Fig. 3 Top, lanes 10 and 11, were poorly recognized by the antibody prior to denaturation. Thus, large nicked fragments of U1 RNA generated by RNase digestion apparently were precipitable with the antibody but separated into multiple small species upon denaturation for PAGE. These findings show that a specific portion of U1 RNA reacts with the autoantibody and suggest that the RNA conformation may affect recognition.

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Fig. 3. (Top) RNase digestion and immunoprecipitation of in vitro SP64-transcribed U1 RNA. $^{32}P$-labeled U1 RNA (lane 1) was digested with RNase T1 (lanes 2, 3, 8, and 9) or RNase A (lanes 4, 5, 10, and 11) and immunoprecipitated with anti-U1 RNA antiserum. Lanes: T, total U1 RNA; 1, immunoprecipitated U1 RNA; 2, 4, 8, and 10, non-denatured prior to nuclease treatment; 3, 5, 9, and 11, denatured prior to nuclease treatment; 6, U1 RNA immunoprecipitated with anti-U1 RNA antiserum; 7, T1-digested U1 RNA immunoprecipitated with NHS. Samples were electrophoresed on 20% polyacrylamide/8.3 M urea gels. (Middle) Oligonucleotide fingerprint analysis of RNase T1 fragments of U1 RNA after immunoprecipitation. Three unique wandering spot patterns (1–3) of $^{32}P$-labeled U1 RNA fragments derived by partial alkali digestion (23). Prior to immunoprecipitation, in vitro synthesized U1 RNA was digested with RNase T1 (20 units of T1 per $\mu$g of U1 RNA) at 37°C for 30 min. Precipitated fragments were gel purified, 5'-end-labeled, and digested in formamide at 100°C for 2 hr. Digestion products were subjected to fingerprint analysis (24). (Bottom) Model of proposed secondary structure of U1 RNA (27) showing the sequences that were consistent with those immunoprecipitated with the anti-U1 RNA antibody. Fragments 1, 2, and 3 correspond to the wandering spot patterns of U1 RNA deduced in Middle. Fragment 3 corresponds to nucleotides 50–60; fragment 2, 62–73; fragment 1, 72–84. The figure also shows restriction enzyme cleavage sites on the corresponding DNA (Fig. 2 Upper).

As expected, when the 3' half of the stem–loop region was deleted by using Msp I-digested template (Fig. 2 Upper, SLB; Fig. 3 Bottom), the antibody no longer recognized the RNA transcript (data not shown). These findings confirmed the RNA sequence analysis (Fig. 3) and suggested that either an intact stem–loop structure or the sequence in the 3' end of the stem was required for antibody recognition.

Based upon predicted models of secondary structure for U1 RNA (26, 27), it is possible that the loop structure

FIG. 4. (Left) In vitro synthesis and immunoprecipitation of the middle stem–loop B "RNA epitope" and loop B region of U1 RNA. DNA oligonucleotides were synthesized corresponding to the middle stem–loop B (nucleotides 51–90) and loop B region (nucleotides 66–75) of U1 RNA together with 5' HindIII and 3' EcoRI linkers. Lanes: T, $^{32}P$-labeled U1 RNA transcripts prior to immunoprecipitation; I, immunoprecipitation of transcripts with U1 RNA antibody unless otherwise stated; 1, 4, and 5, full-length U1 RNA [NHS (lane 4) and anti-U1 RNA antiserum (lane 5)]; 2 and 6, middle stem–loop B of U1 RNA; 3 and 7, loop B region of U1 RNA. (Right) Nuclease digestion and immunoprecipitation of middle stem–loop B "RNA epitope." Lanes: T, $^{32}P$-labeled transcripts prior to immunoprecipitation; I, immunoprecipitation of middle stem–loop B transcripts; 1, $^{32}P$-labeled middle stem–loop B transcript; 2, RNase T1 and A digestion products of middle stem–loop B; 3, transcript immunoprecipitated with NHS; 4, transcript immunoprecipitated with anti-U1 RNA antiserum; 5, immunoprecipitation of RNase T1- and A-digested transcript with NHS; 6, same as lane 5 but with anti-U1 RNA antiserum. The U1 RNA middle stem–loop B transcript together with carrier tRNA (10 $\mu$g) was digested with 1 $\mu$g of RNase T1 and 0.5 $\mu$g of RNase A in 0.01 M Tris-HCl, pH 7.4/0.4 M NaCl at 37°C for 30 min. Samples were processed as described, immunoprecipitated, and electrophoresed on 10% acrylamide/8.3 M urea gels. The stem structure remained intact on 20% acrylamide non-denaturing gels (data not shown) but was fragmented upon denaturation in urea (lane 2).
corresponding to nucleotides 66–75 (Fig. 2 Upper, LB; Fig. 3 Bottom), may protrude from the surface of the molecule and, thus, may be more immunoreactive than the base-paired region of the U1 RNA epitope. To test this, the DNA representing the loop portion of the middle stem–loop (5′ A-U-U-G-C-A-C-U-C-C-3′) was chemically synthesized (Fig. 2 Upper, LB) and cloned into pSP64. However, the RNA transcript was not recognized by the anti-U1 RNA antibody (Fig. 4 Left, lanes 3 and 7). The final possibility, that an intact base-paired stem structure was required for antibody recognition, was examined by digesting the single-stranded loop portion of the in vitro synthesized stem–loop transcript with RNases T1 and A and analyzing the stem under denaturing and nondenaturing conditions. The remaining U1 RNA stem was not precipitable under native conditions (data not shown) or under denaturing conditions (Fig. 4 Right, lanes 2 and 6). Thus, we conclude that the stem and the loop are required for antibody recognition.

DISCUSSION

We have defined an epitope on U1 RNA that is recognized by one or more specific antibodies from an autoimmune patient with lupus overlap syndrome and rheumatoid arthritis. While it is possible that other portions of the RNA are antigenic, we have localized a major immunoreactive domain on the molecule. The epitope on U1 RNA identified in this study appears to encompass nucleotides 51–90, corresponding to a predicted middle stem–loop B region. Deletion of a portion of the 3′ half of this region abolished immunoreactivity. Competition experiments with U1 RNA, antisense U1 RNA, and various oligonucleotides demonstrated specific binding of U1 RNA to the autoantibody. It is unlikely that the antibody is merely recognizing G+C-rich regions (10), because G+C-rich polymers did not effectively compete for binding to the antibody (Fig. 1 Lower). Furthermore, the antibody was specific for U1 RNA, since other RNAs and DNAs synthesized in vitro and in vivo were not recognized (data not shown). This antibody differs from other nucleic acid-reactive antibodies that are reported to recognize broad classes of RNA or DNA molecules (5, 10–12, 29). The specificity of this reaction makes it likely that other in vitro synthesized RNAs, if coupled to this epitope, may be recognized by the antibody. Thus, this short-nucleotide RNA may be useful as an antigenic tag to detect nucleic acid hybridization by RNA or DNA blot analysis.

We also showed that disruption of the conformation of the stem–loop B structure of U1 RNA eliminated recognition by this autoantibody. The involvement of RNA conformation in antibody binding is consistent with properties of other known RNA binding proteins (13–15). In addition, it has recently been shown that a monoclonal antibody that binds DNA may require a cruciform structure for proper binding (5). It is interesting to note that the loop B of U1 RNA, which is part of this epitope, is complementary for 11 nucleotides to a region between bases 112 and 125 of U2 RNA. Whether RNA–RNA interactions play a role in the potential association of U1 and U2 snRNPs in the splicing complex is not known. The U1 RNA-specific antibody described here can be used to probe accessible regions in U1 snRNPs and to determine the binding properties of cell proteins associated with snRNAs.

The mechanism by which anti-RNA antibodies are elicited remains obscure. Other than direct immunological presentation, one likely origin is through an idiotype–anti-idiotype networking mechanism (30–32), which has been implicated in autoimmune diseases such as myasthenia gravis (32), systemic lupus erythematosus (33), myosit (11), and rheumatoid arthritis (34). Presentation of a naked U1 RNA to the immune system seems unlikely because RNA is rarely naked in vivo, ribonucleases are abundant in serum, and the response in this patient was to only a limited portion of the molecule. Although direct evidence is not available, our data are compatible with the suggestion that the anti-U1 RNA antibody is anti-idiotypic in origin and was formed against a separate, but more common, U1 RNP autoantibody (idiotype). The idiotypic autoantibody may have been directed against the RNA binding domain of a protein that recognizes the middle stem–loop of U1 RNA. Consistent with this possibility, the serum containing the U1 RNA-specific antibody also contains antibodies against the proteins bound to U1 RNA (35).

We thank Charles Query for intellectual contributions to this work. This study was supported by grants from the Arthritis Foundation and the National Institutes of Health to J.D.K. S.L.D. is a postdoctoral fellow of the American Cancer Society, and J.D.K. is a Pew Scholar in the Biomedical Sciences.