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Biochemistry

Selection of an RNA molecule that mimics a major autoantigenic epitope of human insulin receptor

(antibody–RNA interaction/autoimmunity/*in vitro* selection/RNA structure)

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Contributed by Thomas R. Cech, December 15, 1994

ABSTRACT **Autoimmunity often involves the abnormal targeting of self-antigens by antibodies, leading to tissue destruction and other pathologies. This process could potentially be disrupted by small ligands that bind specifically to autoantibodies and inhibit their interaction with the target antigen. Here we report the identification of an RNA sequence that binds a mouse monoclonal antibody specific for an autoantigenic epitope of human insulin receptor. The RNA ligand binds specifically and with high affinity (apparent $K_d \simeq 2$ nM) to the anti-insulin receptor antibody and not to other mouse IgGs. The RNA can also act as a decoy, blocking the antibody from binding the insulin receptor. Thus, it probably binds near the combining site on the antibody. Strikingly, the RNA cross-reacts with autoantibodies from patients with extreme insulin resistance. One simple explanation is that the selected RNA may structurally mimic the antigenic epitope on the insulin receptor protein. These results suggest that decoy RNAs may be useful in the treatment of autoimmune diseases.**

Expression of TAR and RRE sequences has been shown to render $CD4^+$ T cells resistant to human immunodeficiency virus (HIV) replication by inhibiting the functions of the RNA binding proteins tat and rev (1–3). Because they both bind and block their cognate proteins, these transcripts have been termed decoy RNAs. In this study, we tested whether *in vitro* selection could be used to isolate a "designer" decoy RNA for a large, clinically relevant, non-RNA binding protein. Our system involves human insulin receptor, a tetrameric protein consisting of two extracellular insulin-binding α subunits and two transmembrane β subunits (4–10). Patients with extreme insulin resistance type B often carry autoantibodies that bind this receptor (11–13). As a model for such autoantibodies, we used a mouse monoclonal antibody (MA20), specific for an antigenic epitope on the α subunit of human insulin receptor (14). The same epitope is commonly recognized by autoantibodies generated against insulin receptor (11).

To identify RNA ligands for the monoclonal antibody, we used an approach developed recently for the *in vitro* selection from random sequence libraries of RNA molecules that bind with high affinity and specificity to proteins or small biomolecules (15–17). These methods have previously been used to isolate RNA molecules that bind to T4 DNA polymerase (15), bacteriophage R17 coat protein (18), HIV Rev protein (19), HIV reverse transcriptase (20), basic fibroblast growth factor (21), organic dyes (16), ATP (22), theophylline (23), and several amino acids (24–26).

Using similar methodology, Keene and coworkers (27) isolated an RNA that bound to antibodies in a polyclonal mixture raised in a rabbit immunized with a 13-amino acid peptide. This suggested that antibodies that do not naturally recognize nucleic acids might be reasonable targets for *in vitro* selection. The antigen-combining site of an immunoglobulin is a complex surface and it might bind a specific RNA in any of a variety of modes. Surprisingly, however, we present evidence below that an RNA that binds to an antibody may in fact structurally resemble a complex protein antigen.

MATERIALS AND METHODS

Antibodies and Insulin Receptor. Monoclonal antibody MA20 was purchased from Amersham. Purified soluble ectodomain of the human insulin receptor protein (IR921) was a gift from Erik Schaefer and Leland Ellis (Texas A & M University, Houston). Monoclonal antibody 83-7 was a gift from Kenneth Siddle (Addenbrooke's Hospital, University of Cambridge, Cambridge, U.K.). Serum samples from three patients with extreme insulin resistance type B, referenced as B10, B7, and Bd, were a gift from Domenico Accili and Simeon Taylor (National Institutes of Health, Bethesda).

Selection Procedure. A random pool of RNA oligonucleotides of sequence 5′-GGGAGAGCGGAAGCCGUGCUG-GGGCCN_{40}CAUAACCCAGAGGUCGAUGGAUC-3′ (where N_{40} represents 40 nucleotides with equimolar A, G, C, and U at each position) was generated by *in vitro* transcription of a synthetic DNA template. For the initial round of selection, 30 μg of RNA ($\approx$1 nmol) was incubated with 5 μl of naive mouse IgGs (1 mg/ml) in a binding buffer containing 30 mM Tris·HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1% bovine serum albumin, and 5 units of RNasin (Promega) in a total vol of 100 μl. After a 30-min incubation at 25°C with gentle shaking, 20 μl of goat anti-mouse IgG-coated magnetic beads (Dynal, Oslo) was added and allowed to incubate an additional 30 min at 25°C. The beads were then pelleted with a magnet, and the supernatant was moved to a new tube and incubated with 5 μl of mouse monoclonal antibody MA20 (0.5 mg/ml) for 30 min at 25°C. The antibody was then precipitated using the magnetic beads as before, and the pellets were washed twice with 0.5 ml of the binding buffer. RNA was eluted from the pellets in 100 μl of 0.1 M EDTA; the EDTA chelates magnesium ions, which might be required for RNA structure, thus effectively denaturing the RNA. The eluate was then applied to a Sephadex G-25 spin column to remove EDTA and salts. A 20-nt DNA primer (1 μM), complementary to the 3′ sequence of the original pool RNA, was added to the eluted RNA, and cDNAs were generated by using avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). After a 30-min incubation at 37°C, a second DNA primer (1 μM) was added, which was identical to the 5′ end of the original RNA pool and contained the 17-nt promoter sequence for T7 RNA polymerase. Thirty cycles of PCR were carried out using *Taq* DNA polymerase (Perkin–Elmer). Amplified DNA was phe-

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nol extracted, precipitated, and resuspended in 20 μl of 10 mM Tris·HCl, pH 8/1 mM EDTA. Half of this solution was used as the template for an *in vitro* transcription reaction with T7 RNA polymerase in 100-μl total vol. Subsequent cycles of selection were performed in the same way using either 50% (rounds 1–8) or 20% (rounds 9–11) of the transcribed RNA. In rounds 9–11, MA20 concentration was reduced by 80%. Prebinding to naive mouse IgGs was performed only on alternate cycles.

Analysis of Selected RNAs. Plasmid DNA encoding selected RNA 1 or 9 was transcribed *in vitro* with T7 RNA polymerase in the presence of [α-^{32}P]ATP. Resulting transcripts contain both the selected insert sequences and the fixed flanking sequences listed above. RNA was typically incubated in 100 μl of binding buffer with antibody for 30 min at 25°C with shaking. Goat anti-mouse IgG magnetic beads or protein G-Sepharose beads (Pharmacia) were added, incubated for 15 min at 25°C with shaking, and recovered with a magnet or by pelleting in a microcentrifuge. Pellets were washed twice with 500 μl of binding buffer, and the immunoprecipitated RNA was either quantitated in a liquid scintillation counter or eluted by the addition of 10 μl of 500 mM EDTA and analyzed by gel electrophoresis.

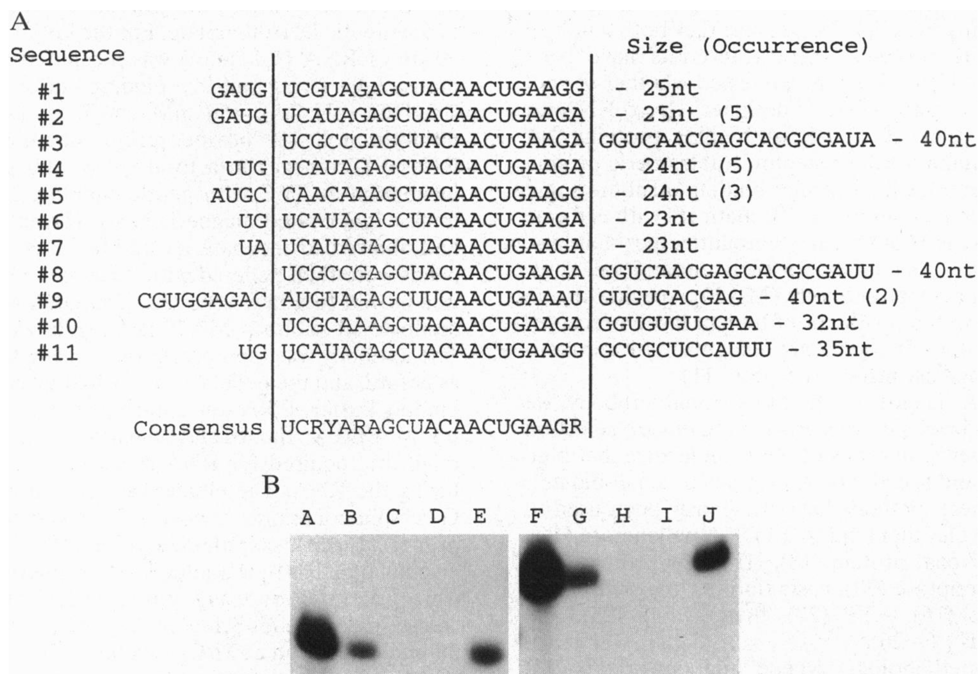
RESULTS

A library of ≈10^{14} RNA molecules was generated, with each molecule containing a 40-nt-long region of random sequence flanked by defined sequences. The RNA library was first incubated with normal mouse IgGs, and antibody–RNA complexes were immunoprecipitated and discarded. This step served to remove RNAs that bound to the constant region of the antibodies or that adhered nonspecifically to the magnetic beads used in the immunoprecipitation. The supernatant from this step (containing the bulk of the RNA pool) was then incubated with the mouse MA20 monoclonal antibody, and MA20–RNA complexes were immunoprecipitated in the same way. RNA was eluted from the precipitated complexes with EDTA, reverse transcribed, and amplified by PCR. The amplified DNA was then transcribed to generate RNA for the next cycle of selection. After eight selection cycles, the stringency of the selection was increased by reducing the concentration of RNA and MA20 protein during the binding step and increasing the number of washes of the precipitated complexes. After three further cycles of selection, the amplified DNA was cloned, and 22 individual clones were sequenced.

Eleven different RNA sequences were found within the section of the oligonucleotide originally randomized, and all contained the 21-nt consensus sequence shown (Fig. 1*A*). Many of the selected RNAs were shorter than the original 89-nt pool RNAs because of deletions within the randomized region that presumably arose during reverse transcription and PCR amplification.

Two of the RNAs, numbers 1 and 9 in Fig. 1*A*, were chosen for further characterization. Radiolabeled RNA was used in an immunoprecipitation experiment to test the specificity of RNA binding to the MA20 antibody. The RNA from both of these clones was found to bind to MA20 regardless of the type of beads used to immunoprecipitate the antibody complexes (Fig. 1*B*). Neither RNA bound to IgGs from nonimmunized mice or to magnetic beads alone. The RNA also did not cross-react with other mouse monoclonal antibodies with different specificities (data not shown). Thus, the RNA ligand apparently binds to the variable region of the MA20 antibody.

To estimate the equilibrium dissociation constant (K_d) for this binding, RNA 9 was immunoprecipitated with increasing concentrations of antibody. The same apparent K_d of ≈2 nM was obtained at three concentrations of RNA 9 (Fig. 2). This



A

Sequence			Size (Occurrence)
#1	GAUG	UCGUAGAGCUACAACUGAAGG	- 25nt
#2	GAUG	UCAUAGAGCUACAACUGAAGA	- 25nt (5)
#3		UCGCCGAGCUACAACUGAAGA	GGUCAACGAGCACGCGAUA - 40nt
#4	UUG	UCAUAGAGCUACAACUGAAGA	- 24nt (5)
#5	AUGC	CAACAAAGCUACAACUGAAGG	- 24nt (3)
#6	UG	UCAUAGAGCUACAACUGAAGA	- 23nt
#7	UA	UCAUAGAGCUACAACUGAAGA	- 23nt
#8		UCGCCGAGCUACAACUGAAGA	GGUCAACGAGCACGCGAUU - 40nt
#9	CGUGGAGAC	AUGUAGAGCUUCAACUGAAAU	GUGUCACGAG - 40nt (2)
#10		UCGCAAAGCUACAACUGAAGA	GGUGUGUCGAA - 32nt
#11	UG	UCAUAGAGCUACAACUGAAGG	GCCGCUCCAUUU - 35nt
	Consensus	UCRYARAGCUACAACUGAAGR	

FIG. 1. Selected RNA sequences and their specific, high-affinity binding to the MA20 antibody. (*A*) After 11 rounds of *in vitro* selection, selected RNAs were reverse transcribed, and the resulting cDNAs were PCR amplified and subcloned. Twenty-two clones were sequenced and were found to encode 11 different RNA insert sequences. These RNAs all contain a highly conserved 21-nt sequence (R, purine; Y, pyrimidine). Several of the RNA sequences were found to be present multiple times (numbers in parentheses), and most had inserts shorter than 40 nt. (*B*) Internally labeled RNA 1 (lanes A–E) or 9 (lanes F–J) (1 nM) was incubated with the MA20 antibody (10 mg/ml) (lanes B, E, G, and J), with normal mouse IgGs (20 mg/ml) (lanes C and H), or without any antibody (lanes D and I). The antibody–RNA complexes were immunoprecipitated with goat anti-mouse IgG magnetic beads (lanes B–D and G–I) or with protein G-Sepharose beads (lanes E and J). Immunoprecipitated RNAs were separated by gel electrophoresis on a 10% polyacrylamide gel containing 7 M urea. Control lanes A and F contain one-half the amount of the labeled RNAs 1 (lane A) and 9 (lane F) that was added to the immunoprecipitations.

is likely to be an upper estimate, since the immunoprecipitation procedure involves several steps including extensive washing. Hence, the RNA binds extremely tightly to the antibody, consistent with the stringency of the conditions under which the selection was performed.

MA20 has been shown to bind the insulin receptor near the site of insulin binding. However, the antibody does not directly compete with insulin for binding to the receptor, and, in fact, antibody binding stabilizes preformed insulin–insulin receptor complexes (14, 28). We took advantage of this feature of MA20 to determine whether the selected RNA bound at or near the antibody combining site.

Purified ectodomain of the human insulin receptor (10) was prebound to ^{125}I-labeled insulin and then incubated with the MA20 antibody in the presence (Fig. 3, samples B–E) or absence (sample A) of competitor RNA. Complexes were immunoprecipitated and assayed for ^{125}I-insulin content. In the presence of a 100-fold excess of RNA 9 over receptor, the amount of ^{125}I precipitated was not detectable above background counts (sample B). With only a 10-fold excess of this competitor RNA, we observed a nearly 90% decrease in the amount of ^{125}I-labeled complexes precipitated (sample C). In contrast, no significant reduction in immunoprecipitation was observed when 100-fold excess of the original pool RNA was added as a nonspecific competitor (sample D). In addition, no inhibitory effect of RNA 9 was observed (sample E) when immunoprecipitating ^{125}I-labeled receptor complexes with a different monoclonal antibody, 83-7, which recognizes a different epitope on the human insulin receptor (8, 11, 29). This last sample not only illustrates the specificity of the RNA–antibody interaction but also demonstrates that the selected RNA does not displace ^{125}I-insulin from the receptor. Thus, RNA 9 is an effective decoy RNA, which suggests that it interacts with MA20 at or near the site on the antibody used for insulin receptor binding.

These results raised the intriguing possibility that the selected RNA might be a structural mimic of the antigenic epitope of the insulin receptor that is recognized by the MA20 antibody. To explore this possibility, we obtained samples of serum from three patients diagnosed with extreme insulin resistance (type B). This autoimmune disorder is often characterized by the presence of autoantibodies against the same epitope of human insulin receptor that is recognized by the MA20 mouse antibody (11).

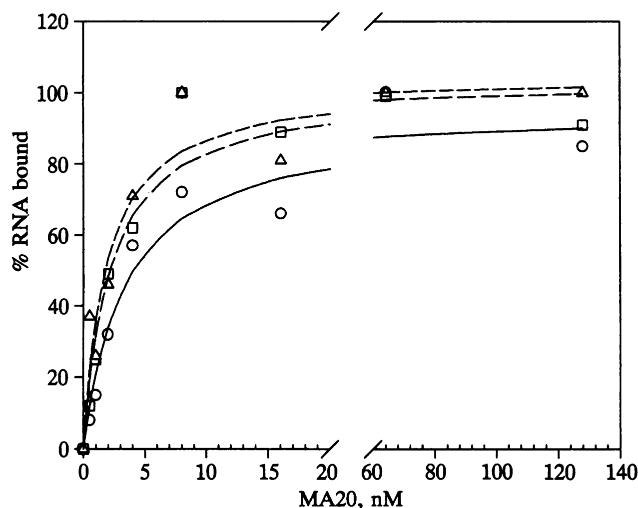


FIG. 2. A selected RNA binds to the MA20 antibody with high affinity. ^{32}P-labeled RNA 9 (10, 20, and 40 pM) was incubated with increasing amounts of MA20 antibody and the RNA–antibody complexes were immunoprecipitated with protein G-Sepharose beads. A maximum of 40–60% of the total counts were immunoprecipitated even when incubated with extremely high antibody concentrations, so plotted numbers have been normalized to that amount. Data were fit with a hyperbolic function for saturation binding. ○, 10 pM RNA; □, 20 pM RNA; △, 40 pM RNA. K_d values determined at these RNA concentrations were 3.4, 2.2, and 1.9 nM, respectively.

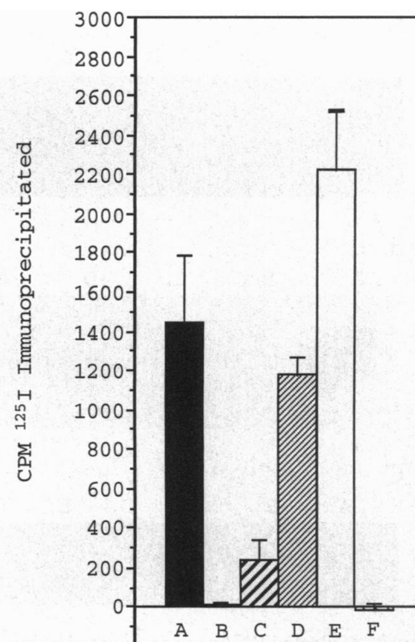


FIG. 3. A selected RNA can block the binding of the MA20 antibody to the human insulin receptor. Purified ectodomain of the α subunit of the human insulin receptor (10 nM) was preincubated with ^{125}I-insulin (1 nM) in 100 µl of binding buffer at 4°C for 20 min with shaking. Then either the MA20 (20 nM) or the 83-7 (1 µl of ascites fluid) anti-insulin receptor antibody was added in the presence or absence of competitor RNAs along with protein G-Sepharose beads, and immunoprecipitation was carried out as described. A, no RNA added; B, 100-fold excess of RNA 9 (1 µM) over insulin receptor; C, 10-fold excess of RNA 9 (0.1 µM) over insulin receptor; D, 100-fold excess of nonselected pool RNA (1 µM) over receptor; E, 100-fold excess of RNA 9 (1 µM) over receptor, 83-7 monoclonal antibody instead of MA20 antibody (see text); F, no insulin receptor in reaction. Values shown are averages of measurements performed in triplicate. Error bars represent SD. A sample containing no antibody was used to determine the background level of ^{125}I-insulin found in the pellets. Numbers shown have been corrected by subtracting this value (180 cpm).

Since the serum antibodies are of indeterminate concentration and also contain an abundance of contaminating nucleases, the antibodies were prebound to protein G-Sepharose beads and washed. Then, radiolabeled RNAs 1 and 9 were immunoprecipitated with the antibody-coated beads. All three of the autoimmune sera were found to contain antibodies that can precipitate the selected RNAs (Fig. 4 *A* and *B*). Examination of the supernatants revealed considerable nuclease degradation of the unbound RNA (data not shown), so the signal in lanes D–F represents almost complete binding of the intact RNA. Neither RNA was precipitated by antibodies in normal human serum (lanes C).

To determine whether the RNA binds specifically to these autoantibodies, a competition assay was performed. Radiolabeled RNA 9 was incubated and immunoprecipitated with the antibodies from one of the patients in the presence or absence of a 10,000-fold excess of nonlabeled competitor RNA 9 or

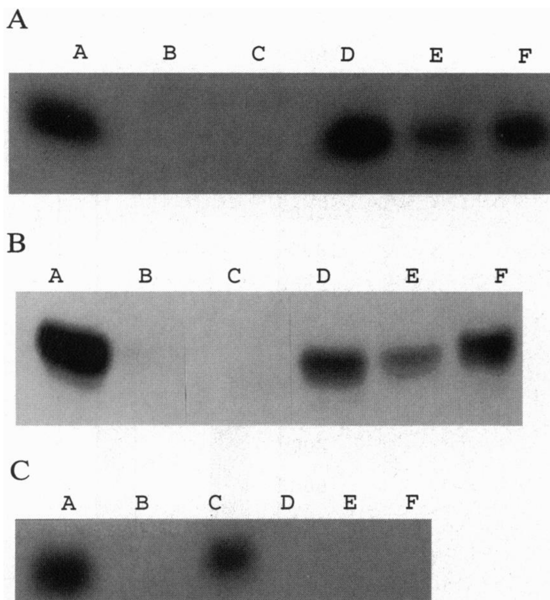


FIG. 4. Selected RNAs are recognized by human autoimmune sera from patients with severe insulin resistance type B. Selected RNA 1 (*A*) or 9 (*B*) was immunoprecipitated with the MA20 antibody (lane A), normal mouse IgGs (lane B), antibodies from normal human serum (lane C), or antibodies from three autoimmune patient sera (lane D, serum B10; lane E, serum B7; lane F, serum Bd). MA20 antibody (1 μl; final concentration, 2.5 μM), normal mouse IgGs (1 μl; final concentration, 5 μM), normal human serum (30 μl), or an autoimmune serum (30 μl) was prebound to 20 μl of the protein G-Sepharose beads in 250 μl of binding buffer. The beads were pelleted in a microcentrifuge, washed with 500 μl of binding buffer, and repelleted. Next they were resuspended in 100 μl of binding buffer containing 100 pM selected RNA end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The binding reaction mixtures were incubated and assayed by gel electrophoresis as described. (*C*) Binding of selected RNA 9 can be blocked by competition with specific but not nonspecific RNA. Protein G-Sepharose beads were prebound either to antibodies from B10 patient serum (lanes A–C) or to normal human serum (lanes D–F) as described above. End-labeled RNA 9 (100 pM) was added with no competitor RNA (lanes A and D), with unlabeled RNA 9 (1 μM) (lanes B and E), or with unlabeled original pool RNA (1 μM) (lanes C and F), and binding and analysis of bound RNAs were performed as described above.

original pool RNA. As shown in Fig. 4*C*, only RNA 9 can compete for antibody binding. Thus, the cross-reactivity is specific to the selected RNA, and it cannot be ascribed to anti-RNA antibodies that might be present in autoimmune sera.

As a starting point for testing the structural features of RNA 1 and 9, their most stable secondary structures were calculated (refs. 30–32; Fig. 5*A*). The predicted structures consist of three stems separated by unpaired nucleotides and are capped at one end by an 8-nt loop consisting of some of the most highly conserved nucleotides in the sequence.

To test the validity of this predicted secondary structure, several variants of RNA 1 were synthesized and tested for binding to the MA20 antibody (Fig. 5*B*). The full-length RNA 1 was again bound (lane 1). A shorter version of the RNA, lacking unpaired flanking sequences, bound to the antibody (lane 3), as did a version lacking stem I (lane 2). Disruption of stem II resulted in loss of binding (lane 6), which could be regained with compensatory changes to reform the stem (lane 7). From these results, we conclude that stem I is dispensable, and stem II is required for RNA folding but not for sequence-specific recognition by the antibody. When the sequence UAG was mutated to disrupt stem III (Fig. 5*A*), the RNA no longer bound to the antibody (lane 4). Restoration of base comple-

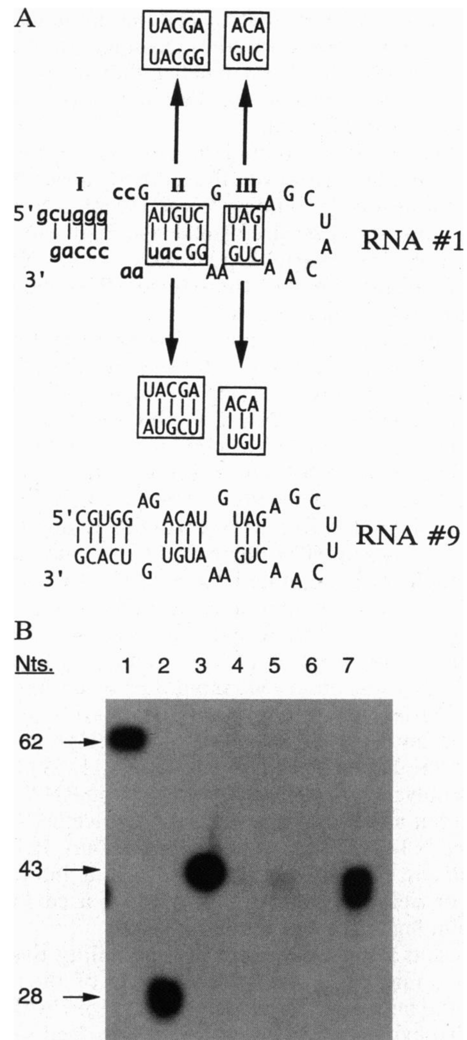


FIG. 5. (*A*) Predicted secondary structure of selected RNAs 1 and 9. Nucleotides in lowercase letters originate from the fixed flanking sequence surrounding the selected variable sequence of RNA 1. Additional flanking sequences are not shown. Proposed stems are labeled with roman numerals. (*B*) Autoradiogram of 10% polyacrylamide gel containing radiolabeled RNAs immunoprecipitated with the MA20 antibody. Lanes: 1, RNA 1 full-length sequence (62 nt); 2, RNA 1 lacking stem I (28 nt); 3, RNA 1 lacking most of flanking primer binding sequences (43 nt); 4, RNA 1 with stem III UAG changed to ACA (43 nt); 5, RNA 1 with stem III UAG changed to ACA and stem III CUG changed to UGU (43 nt); 6, RNA 1 with stem II AUGUC changed to UACGA (43 nt); 7, RNA 1 with stem II AUGUC changed to UACGA and stem II GGCAU changed to UCGUA (43 nt).

mentarity in stem III did not restore binding (lane 5). Thus, the sequence UAG must be involved in an interaction different from or in addition to stem III. Future work will address the structure more directly through chemical probing of the RNA and RNA–antibody complex and by means of NMR and x-ray crystallography.

DISCUSSION

We have used *in vitro* selection and amplification (15, 17, 33) to identify a small RNA molecule that binds to a mouse monoclonal antibody, MA20, which recognizes the human insulin receptor. The selected RNA ligand binds to the antibody specifically and with high affinity (apparent $K_d \approx 2$ nM). Furthermore, the RNA can serve as a decoy RNA, blocking the antibody from binding the insulin receptor. Patients with

extreme insulin resistance (type B) often carry autoantibodies to the same epitope of the insulin receptor recognized by MA20. The selected RNA cross-reacts with these autoantibodies in sera from three patients.

The three sets of human autoantibodies recognize an epitope contained within amino acids 450–601 of the α subunit of the insulin receptor (11). However, there is evidence that these autoantibodies are not identical: B7 bound efficiently to a recombinant chimeric receptor that contained only residues 450–601 of the insulin receptor, whereas B10 and Bd bound very weakly to the chimeric protein (11). Thus, although the sequences of the three sets of human autoantibodies are unknown, it is unlikely that they are identical to each other or to the MA20 mouse antibody.

Why, then, do all these antibodies cross-react with the RNA selected for binding to MA20? A simple and very exciting possibility is that the selected RNA folds into a three-dimensional structure resembling that of the major antigenic epitope of the insulin receptor, which would explain their common recognition by the various antibodies. Although RNA and protein are constructed from chemically different building blocks, it seems possible that they could present a similar array of functional groups such as hydrogen bond donors and acceptors, anionic moieties, and planar aromatic groups (see also refs. 34 and 35). An alternative possibility is that the selected RNA does not resemble a portion of the insulin receptor but binds in a very different mode to some structural feature shared by the various anti-insulin receptor antibodies but absent in control antibodies.

An immune response occurs by *in vivo* selection from a large pool of protein sequences of an antibody that binds specifically to a target macromolecule. In autoimmune diseases the normal controls have gone awry, resulting in antibodies that recognize self-antigens. By undertaking a second selection process, this time *in vitro* using a monoclonal antibody as the target, we have identified RNA decoys that appear to structurally mimic the insulin receptor epitope that elicited the antibody. Such molecular mimics could potentially be used to inhibit autoantibodies in patients with extreme insulin resistance without interfering with their normal immune system function. In a broader sense, RNAs that mimic protein structures may prove to be generally useful for experimental and therapeutic purposes.

Note Added in Proof. Comparison of the structures determined by x-ray crystallography of a portion of elongation factor G and transfer RNA bound to EF-Tu provides a striking example of structural similarity between protein and RNA (P. Nissen, M. Kjeldgaard, S. Thirup, G. Polekhina, L. Reshetnikova, B.F.C. Clark, and J. Nyborg, personal communication).

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