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Autoantibodies in human anti-Ro sera specifically recognize deproteinized hY5 Ro RNA

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SUMMARY

We report the existence of a novel autoantibody specificity linked to anti-Ro antibodies. Sera from two patients with anti-Ro ribonucleoprotein (RNP) antibodies also contained antibodies that immunoprecipitated specifically either the deproteinized RNA component of the Ro^{hY5} RNP particle, or intact *in vitro* transcribed hY5 RNA. No serum recognized specifically the other hY RNAs. A mutant hY5 RNA with additional nucleotides (nt) at both extremities was not immunoprecipitated, possibly because of altered secondary structure. Following digestion of hY5 RNA with ribonuclease T1, the smallest immunoprecipitable RNA fragments were 27 and 31 nt long, and respectively mapped to the 5′ and 3′ ends of hY5 RNA, excluding the La-binding region. Base pairing between the 27 and 31 nt long fragments was required for recognition by antibodies. Our data indicate that the epitope bound by anti-hY5 RNA antibodies is conformational. We have previously reported that most anti-Ro sera contain a population of antibodies specific for the Ro^{hY5} RNP. Since antibodies to the deproteinized hY RNAs within anti-Ro sera are also restricted to anti-hY5 RNA, a direct role for the human-specific Ro^{hY5} particles in the immunization process leading to the production of anti-Ro antibodies is suggested.

Keywords anti-RNA antibodies anti-Ro antibodies RNA binding ribonucleoprotein particles systemic lupus erythematosus

INTRODUCTION

Sera from patients with connective tissue diseases frequently contain autoantibodies to small ribonucleoproteins (RNPs) [1]. Among the most frequent targets of these autoantibodies are the Ro RNPs, a minor cellular component (about 10^5 copies/cell) of unknown function [2]. Human Ro RNPs consist of at least one of two polypeptides (60-kD Ro (Ro60), and La) associated with one of four small RNAs of 83–112 nucleotides (nt) in length called hY1, hY3, hY4, and hY5 [2,3]. A 52-kD protein (Ro52) recognized by most anti-Ro sera was initially reported to be part of the Ro RNPs [4,5], but this association has been recently challenged ([6]; Boire *et al.*, manuscript submitted). Little or no naked Ro RNAs is thought to be present *in vivo* [2]. The Ro60 protein associates directly with the Ro RNAs through a highly conserved double stem formed by base pairing of their 5′ and 3′ ends [2]. The La protein interacts with the 3′ poly(U) end of the Ro RNAs, as it does with all other (mostly immature) RNA polymerase III transcripts [7].

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La has been implicated in the termination of transcription by RNA polymerase III [8] and in the modulation of poliovirus RNA translation [9]. Additional sites of interaction of the La protein with Ro RNAs have been proposed, but have not been characterized [5].

Antibodies to Ro RNPs present in patient sera usually target protein antigens [10]. Antibodies to Ro52 tend to recognize continuous epitopes on the denatured protein [11], while antibodies to Ro60 tend to target conformational epitopes [12,13]. Anti-La antibodies are rarely, if ever, found in a serum without anti-Ro antibodies [10]. Since the Ro60 and La polypeptides do not share sequence or epitope similarities [4,14–16], this antibody linkage suggests that relatively intact Ro complexes act as direct immunogens [17]. The role of native Ro complexes in autoimmunization is also supported by our observation that most anti-Ro sera contain antibodies specifically recognizing the hY5-containing Ro (Ro^{hY5}) RNPs, presumably through recognition of a human specific conformational epitope formed on Ro60 protein when associated with the hY5 RNA [13].

Anti-RNA antibodies are commonly found in sera from patients with connective tissue diseases, but they are rarely specific for any particular RNA [18–20]. Notable exceptions

are the recently described autoantibodies recognizing U RNAs [21–24], tRNAs [24,25] and a ribosomal RNA [26]. Specific anti-RNA antibodies often target regions of the RNAs that are functionally important (e.g. the anticodon loop of $tRNA^{Ala}$ [27], the GTPase centre of 28S rRNA [26], and the 2,2,7-trimethylguanosine cap of U RNAs [23]). Thus antibodies against the Ro RNAs, which have not yet been described, could provide useful information about functional domains of these molecules as well as the process of autoantibody production.

For these reasons, we screened 50 anti-Ro RNP-positive sera for the presence of immunoprecipitating anti-hY RNA antibodies. Two patients with severe systemic lupus erythematosus (SLE) produced high titres of antibodies specifically immunoprecipitating the hY5 RNA from a mixture of deproteinized Ro and U1 RNAs. No serum specifically recognized the other hY RNAs. Using mutant RNAs containing extra 5′ and 3′ nt and RNase T1 degradation of *in vitro* transcribed hY5 RNA, we showed that the epitope recognized by anti-hY5 RNA antibodies in sera of these two patients is conformational, and maps primarily to the central portion of the molecule that includes the Ro60 binding region.

PATIENTS AND METHODS

Patient sera

Sera used in this study were sent to the Laboratoire d'Immunologie-Rhumatologie of the University of Sherbrooke for characterization of antinuclear antibodies. These autoantibodies were characterized by double immunodiffusion, immunoblotting, and immunoprecipitation of HeLa cell extracts, as previously described [12,13]. Monospecific anti-Ro sera did not recognize another antigen in any of these three assays. Charts of selected patients were reviewed retrospectively to establish the clinical diagnosis, using criteria published by the American College of Rheumatology.

Immunoprecipitation of Ro RNPs and RNAs

HeLa cells were maintained at 37°C under 5% CO_2, in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 60 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were radiolabelled for 14 h with ^{32}P-orthophosphate (10 μCi/10^5 cells/ml; Amersham Corp., Arlington Heights, IL), collected by centrifugation, washed three times in Tris-buffered saline (10 mM Tris–Cl pH 7·5, 150 mM NaCl), and sonicated in NET-2 buffer (50 mM Tris–Cl pH 7·5, 150 mM NaCl, 0·05% Nonidet P-40). Sonicates were clarified at 14000 *g* for 15 min. The supernatant was filtered on 0·2-μm filters and used as a source of antigens. Immunoprecipitation of radiolabelled and unlabelled cell extracts was performed as previously described [3,12,13]. Nucleic acids present in the immunoprecipitates were extracted with phenol/chloroform/isoamyl alcohol (50 : 50 : 1) containing 0·1% 8-hydroxyquinoline, followed by fractionation on 10% polyacrylamide–7 M urea gels and detection by autoradiography.

In some experiments, deproteinized RNAs were prepared by phenol extraction of anti-Ro and anti-U1 RNP immunoprecipitates from cell extracts. These deproteinized RNAs or *in vitro* transcribed RNAs were used as antigens in immunoprecipitation assays of naked RNAs. The naked RNAs were resuspended in NET-2 containing 1 mg/ml heparin, 200 μg/ml tRNA, 40 U/ml RNas in (Promega, Madison, WI), and 2 mM dithiothreitol. Immunoprecipitation was performed as described for immunoprecipitation of HeLa cell extracts.

Cloning of the hY5 RNA and of hY5 RNA mutants

Ro RNAs immunoprecipitated from HeLa cell extracts by an anti-Ro serum were used in hY5 cDNA production. Using the following primers (5′-AATCTAGAGTTGGTCCGAGTG-3′ and 5′-TTAAGCTTAAAACAGCAAGCTAGTCAAGCG-3′), first strand cDNA synthesis was obtained by reverse transcription followed by second strand synthesis and amplification using Taq DNA polymerase [28]. After digestion with XbaI and HindIII, the cDNA was inserted into pGEM 3Zf(+) (Promega) previously digested with the same enzymes. Sequencing of insert-containing plasmids confirmed the presence of a cloned hY5 cDNA sequence (pY5–125). To produce hY5 RNA with authentic 3′ and 5′ ends (Y5–84 RNA), our first hY5 cDNA (pY5–125) was amplified by polymerase chain reaction (PCR) using the primers 5′-AAATCTAGATAATACGACTCACTATAAGTTGGTCCGA-3′ (containing the T7 RNA polymerase promotor sequence) and 5′-TTAAGCTTTAAAACAGCAAG-3′. After digestion with XbaI and HindIII, the PCR product was subcloned into pUC 19 to yield plasmid pY5–84.

In vitro *transcription of hY5 RNA and hY5 RNA mutants*

In vitro transcriptions by T7 and SP6 RNA polymerases were performed according to the manufacturers' recommendations using α-^{32}P-UTP or α-^{32}P-CTP (Amersham; 800 Ci/ml). All RNA transcripts were gel purified using the crush and soak technique [29]. Plasmid pY5–84 was linearized with DraI and transcribed by T7 RNA polymerase to yield authentic hY5 RNA (Y5–84; 84 nt long) [29]. Plasmid pY5–125 was digested with HindIII to generate a 125 nt long RNA (Y5–125) when transcribed by the T7 RNA polymerase. This longer transcript corresponded to hY5 RNA with 36 extra 5′ nt and five extra 3′ nt. hY5 antisense RNA (102 nt) was also produced by XbaI cleavage and SP6 transcription of pY5–125.

T1 RNase digestions

Y5–84 RNA synthesized *in vitro* using plasmid pY5–84 linearized by DraI was partially digested on ice with 0·05 U/μl of T1 RNase (Calbiochem, La Jolla, CA) in an OPA+ restriction buffer (Pharmacia Biotech Inc, Baie d'Urfe, Québec, Canada) for 20 min (T1 LOW conditions) or with 0·25 U/μl of T1 RNase at 30°C for 20 min (T1 HIGH conditions). The resulting RNA fragments were then fractionated on denaturing gels or used directly in immunoprecipitation experiments. To identify fragments produced by RNase digestion and recognized by anti-hY5 RNA antibodies, immunoprecipitated fragments were gel-purified, precipitated in ethanol, and resuspended in OPA+ restriction buffer containing 2 U/μl T1 RNase at 30°C for 30 min. The digestion products were separated electrophoretically on a 20% polyacrylamide–7 M urea gel and detected by autoradiography. In control experiments, RNase T1 fragments were boiled for 2 min and cooled on ice before addition to IgG-coated protein A Sepharose beads.

PAGE and Western blotting

PAGE under denaturing and reducing conditions was performed in 15% discontinuous polyacrylamide gel slabs

(acrylamide: bisacrylamide 172: 1) [30]. Proteins from these gels were transferred to nitrocellulose in 20% methanol, Tris/glycine pH 8·3 [31]. Incubation of the blots with patient sera was performed in a Miniblotter II apparatus (Immunetics, Cambridge, MA). Bound immunoglobulins were detected using alkaline phosphatase-labelled goat anti-human IgG (BioCan Scientific, Mississauga, Ontario, Canada).

Computer-predicted secondary structures of RNAs

Predicted RNA secondary structures were deduced using the FOLD program of Zuker & Stiegler [32] and energies defined by Freier *et al.* [33]. We used the previously reported primary sequences of hY5 RNA [34], hY4 RNA [35], hY1 and hY3 RNAs [36].

RESULTS

Anti-hY5 RNA antibodies in human anti-Ro sera

To detect antibodies to the Ro RNAs, we screened 50 sera that contained immunoprecipitating anti-Ro RNP antibodies. These sera were from patients with various clinical diagnoses, predominantly SLE and rheumatoid arthritis (RA). As controls, we tested anti-Ro-negative sera from 20 autoimmune and five normal individuals. In this assay, we used as antigen deproteinized Ro and U1 RNAs prepared from immunoprecipitates of ^{32}P-labelled HeLa cell extracts (Fig. 1, lane 3). We identified two young SLE patients with severe disease who produced antibodies that specifically immunoprecipitated hY5 RNA from this mixture of RNAs (Fig. 1, lanes 6 and 9 (patient 1) and 8 (patient 2)). Patient 2 serum also precipitated some deproteinized U1 RNA. The apparently specific immunoprecipitation of hY5 RNA did not result from degradation of the other hY RNAs, since the latter RNAs were present in mostly intact form in the supernatants (data not shown). Both patient 1 and patient 2 sera immunoprecipitated all four Ro RNPs from HeLa cell extracts (Fig. 1, lane 4 (patient 1) and data not shown (patient 2)) and contained antibodies that recognized Ro60 and Ro52 proteins in immunoblot assays (data not shown). As shown in Fig. 1, neither normal human sera (lane 5) nor a number of anti-Ro sera (e.g. serum TC, lane 7) recognized deproteinized RNAs. Although closely related to hY5 RNA, Ro RNAs hY1, hY3 and hY4 were not immunoprecipitated by any of the 75 sera tested.

To exclude the possibility that a fragment of Ro60 protein still attached to *in vivo* labelled hY5 RNA was responsible for the immunoprecipitation of hY5 RNA by patient 1 and patient 2 sera, we performed immunoprecipitation using RNA synthesized *in vitro*. Two cDNAs encoding the hY5 RNA were thus

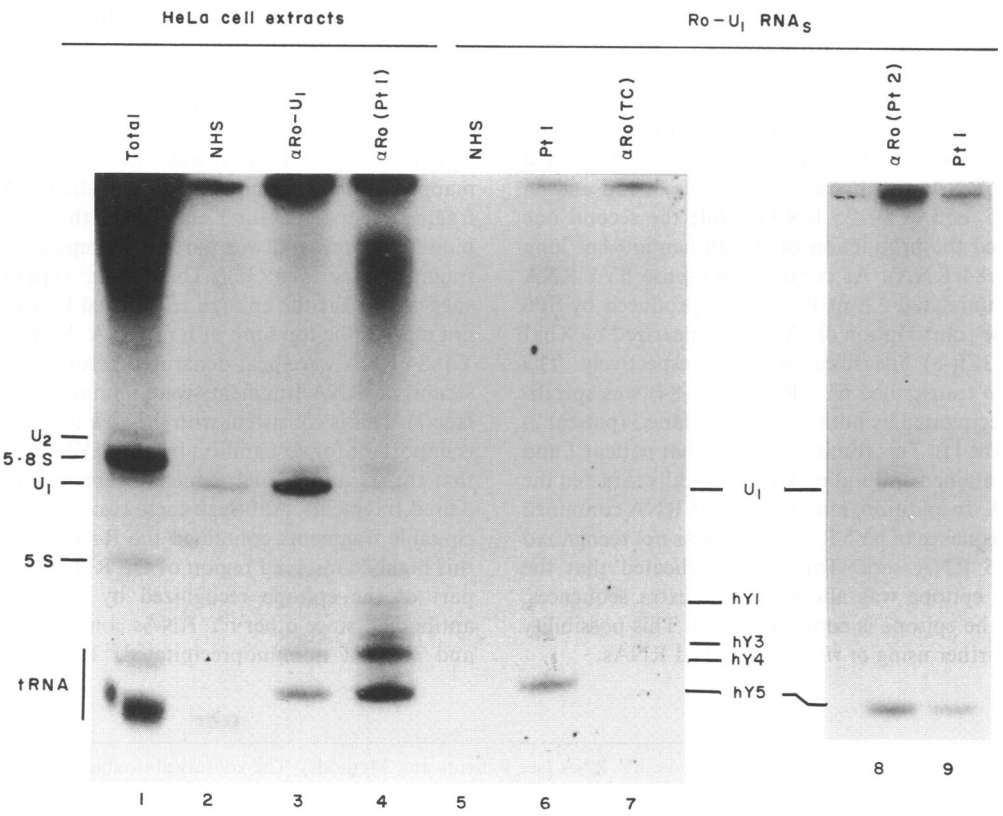


Fig. 1. Immunoprecipitation of HeLa cell extracts (lanes 1–4) and of cellular RNAs prepared by deproteinization of anti-Ro and anti-U1 ribonucleoprotein (RNP) immunoprecipitates (lanes 5–9). Patient 1, 2 and TC sera (only patient 1 serum is illustrated, lane 4) specifically immunoprecipitated all four Ro RNAs from HeLa cell extracts and are thus anti-Ro sera. The ^{32}P-labelled RNAs were separated on a 10% denaturing polyacrylamide–7 M urea gel before autoradiography. Lane 1 contains the total RNAs present in HeLa cell extracts. Lane 3 contains the cellular Ro and U1 RNAs used for immunoprecipitation of naked RNAs; these were obtained by immunoprecipitation of HeLa cell extracts using an anti-Ro and anti-U1 RNP serum. Lanes 2 and 5, normal human serum (NHS); lanes 4, 6 and 9, anti-hY5 RNA patient 1 serum; lane 7, anti-Ro serum TC; lane 8, anti-hY5 RNA patient 2 serum.

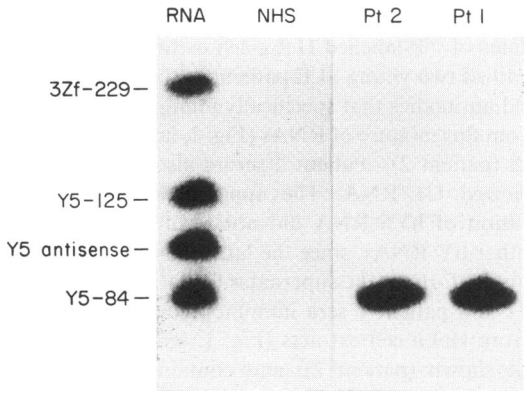


Fig. 2. Immunoprecipitation of *in vitro* transcribed RNAs. The RNAs were separated on a 10% denaturing polyacrylamide–7 M urea gel before autoradiography. The 229 nucleotide (nt) long RNA (3Zf-229) represents the product of SP6 RNA polymerase transcription of wild type pGEM 3Zf(+) linearized with BglI. Y5–125 RNA (125 nt) was transcribed using T7 RNA polymerase from recombinant plasmid pY5–125 linearized with HindIII. Antisense hY5 RNA (102 nt) was transcribed by SP6 RNA polymerase from plasmid pY5–125 linearized with XbaI. Y5–84 RNA (84 nt) was transcribed using T7 RNA polymerase from plasmid pY5–84 linearized with DraI and represented the authentic 84 nt long hY5 RNA. All four RNAs were mixed together before immunoprecipitation (lane 1). Normal human serum (NHS) recognized no RNA (lane 2). Patient 2 and patient 1 sera specifically recognized Y5–84 RNA (lanes 3 and 4).

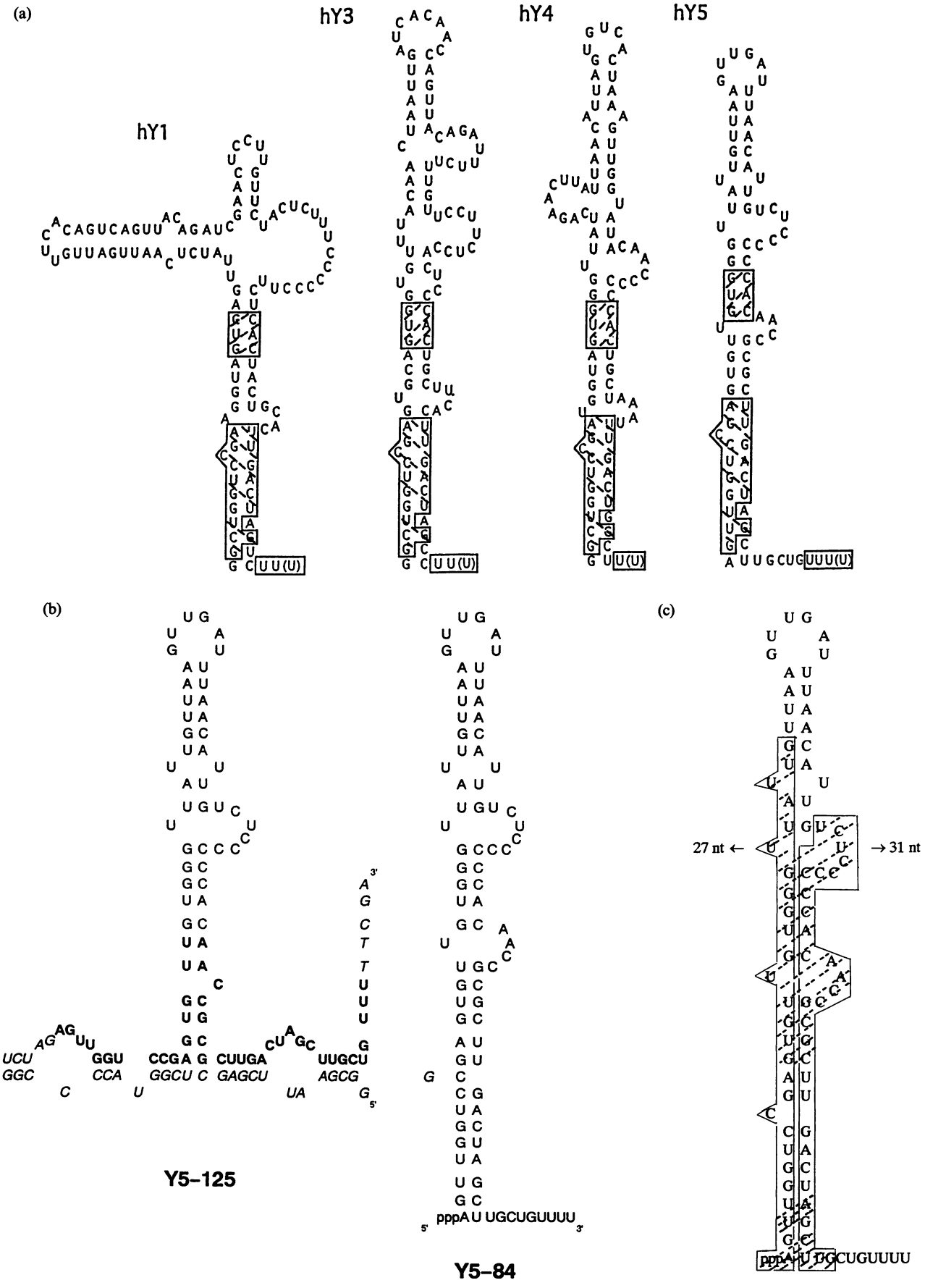
cloned into plasmid vectors and RNAs were transcribed using T7 RNA polymerase. The first cDNA (pY5–125) yielded a 125 nt long hY5 RNA with five extra nt at the 3′ end and 36 extra nt at the 5′ end (Y5–125 RNA), while the second one (pY5–84) allowed the production of the authentic 84 nt long hY5 RNA (Y5–84 RNA). As controls, antisense hY5 RNA (102 nt) and an unrelated 229 nt RNA were produced by SP6 RNA polymerase transcription of pY5–125 linearized by XbaI and of pGEM-3Zf(+) linearized by BglI, respectively. The authentic *in vitro* transcribed hY5 RNA (Y5–84) was specifically immunoprecipitated by both sera (Fig. 2, lane 3 (patient 2) and lane 4 (patient 1)). This result confirmed that patient 1 and patient 2 sera contained antibodies that specifically targeted the naked hY5 RNA. In addition, mutant Y5–125 RNA contained all the primary sequence of hY5 RNA, but it was not recognized by the anti-hY5 RNA sera. This result indicated that the structure of the epitope was altered by the extra sequences, suggesting that the epitope is conformational. This possibility was examined further using *in vitro* transcribed RNAs.

Characterization of the epitope bound by anti-hY5 RNA antibodies

Ro RNAs are small and relatively well conserved in structure [37]. In particular, they share a Ro60 binding site consisting of a stem formed by base-pairing their 5′ and 3′ ends (Fig. 3a). Human Ro RNAs also have a conserved poly(U) tail where La protein binds. Since anti-hY5 RNA antibodies exclusively recognized hY5 RNA and not the other hY RNAs, the epitope was expected to reside in less conserved regions of the RNA. In Fig. 2, authentic Y5–84 was immunoprecipitated while Y5–125 was not. Interestingly, computer predictions indicated that addition to hY5 RNA of the extra sequences present in Y5–125 RNA did not affect the predicted secondary structure of its middle portion, but that the predicted structure of its 3′ and 5′ ends was profoundly altered (Fig. 3b). Based on those considerations, the middle part of hY5 RNA (still present in Y5–125) would not contain the epitope recognized by patient 1 serum, or a novel structure might interfere with epitope recognition. Thus, the epitope could be located in areas adjacent to the Ro60 and/or La binding sites (Fig. 3c, hatched areas in boxes).

To define the epitope in more detail, we performed T1 ribonuclease digestions of Y5–84 RNA followed by immunoprecipitation of the resulting fragments using patient 1 serum. As shown in Fig. 4a, the shortest fragments recognized by patient 1 antibodies were 31 and 27 nt long. Patient 1 serum was used initially because it was more readily available, and because of a higher titre of anti-hY5 RNA antibodies. However, patient 2 serum yielded identical results (data not shown). The identity of these immunoprecipitated fragments recovered from denaturing gels was addressed by digestion to completion with RNase T1. As illustrated in Fig. 4b, the 27 nt fragment mapped at the 5′ terminal portion of the RNA, while the 31 nt fragment mapped at the 3′ end, lacked the single-stranded 3′ La binding region, and was partially complementary to the 27 nt fragment (see Fig. 3c). These data support our previous suggestion that the epitope recognized by patient 1 serum did not map to the top loop of hY5 RNA. When the T1 digests of Y5–84 RNA were heat-denatured before addition to patient 1 serum, no RNA fragments were immunoprecipitated (Fig. 4a, lane 7). This is consistent with the idea that secondary structure is important for recognition by anti-hY5 RNA antibodies, and that the 31 and 27 nt fragments were immunoprecipitated as paired fragments. Although these two base-paired immunoprecipitable fragments contained the Ro60-binding double stem, this highly conserved region of the RNA is not expected to be part of the epitope recognized by specific anti-hY5 RNA antibodies, since other hY RNAs contain an identical domain and are not immunoprecipitated. The minimal hY5 RNA

Fig. 3. (a) Predicted secondary structures of the hY RNA (see Patients and Methods). The conserved double-stranded 60-kD Ro binding region is hatched ▨ and the 3′ poly(U) La binding region is boxed. An additional conserved sequence is also hatched ▨. (b) Predicted secondary structures of Y5–84 and Y5–125 RNAs. Sequence of hY5 RNA with altered structure (in Y5–125 RNA) is printed in bold. Sequence unrelated to hY5 RNA in Y5–125 RNA (originating from the multiple cloning site of the plasmid vector) is shown in italics. Sequence of hY5 RNA with conserved structure (in both RNAs) is in regular print. (c) The 27 and the 31 nucleotide (nt) long fragments of hY5 RNA (mapping at the 5′ and at the 3′ end of hY5 RNA, respectively) that are immunoprecipitated by anti-hY5 RNA sera after RNase T1 digestion of Y5–84 RNA (see Fig. 4) are shown in boxes. The 27 nt long and the 31 nt long fragments map to nt 1 to 27 and nt 47 to 77 of the hY5 RNA, respectively. Putative areas contributing to epitope formation on Y5–84 RNA are hatched ▨. These areas are not conserved among hY RNAs, and are present in the base-paired 27 and 31 nt long fragments that are immunoprecipitated.


(a)
hY1
hY3
hY4
hY5
(b)
Y5-125
Y5-84
pppA U UGCUGUUUU
(c)
27 nt ←
→ 31 nt
pppA U UGCUGUUUU

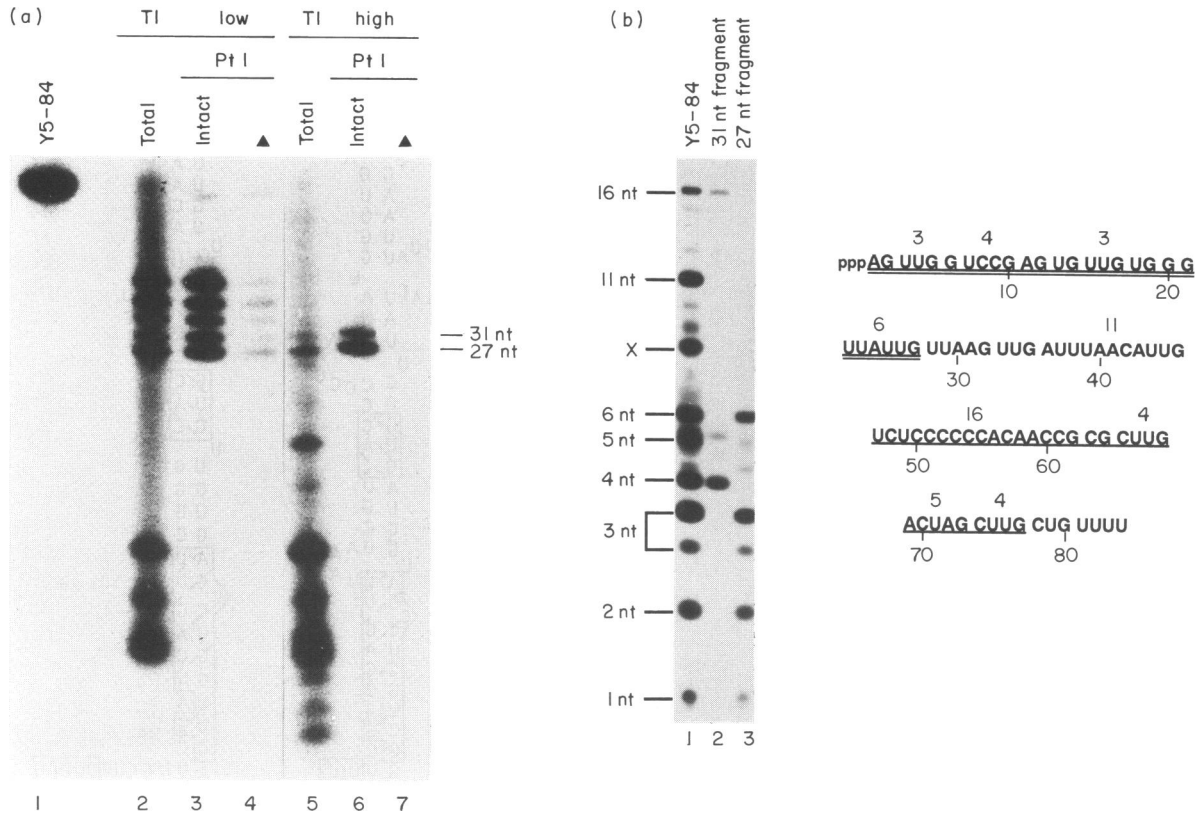


Fig. 4. (a) Immunoprecipitation of T1 RNase digests of Y5–84 RNA. RNAs were run on a denaturing 18% polyacrylamide–7 M urea gel before autoradiography. Y5–84 RNA is shown in lane 1. Lane 2, incomplete digestion of Y5–84 RNA by T1 RNase ('T1 low'); lanes 3 and 4, fragments of RNA immunoprecipitated by patient 1 serum from 'T1 low' digestion before (lane 3) and after (lane 4) heat denaturation of the RNA fragments; lane 5, almost complete digestion of Y5–84 RNA by T1 RNase ('T1 high'); lanes 6 and 7, fragments of RNA immunoprecipitated by patient 1 serum from 'T1 high' digestion before (lane 6) and after (lane 7) heat denaturation (▲) of the RNA fragments. The larger fragments immunoprecipitated by patient 1 serum in lane 3 (T1 low) were shown to contain either the 31 or the 27 nucleotide (nt) RNase T1-resistant fragments, and thus represent incomplete digestion products (see below). (b) Mapping of the immunoprecipitated 31 and 27 nt long fragments immunoprecipitated by patient 1 serum using complete T1 RNase digestion. RNAs were run on a denaturing 20% polyacrylamide–7 M urea gel before autoradiography. Complete T1 RNase digestion of full length Y5–84 RNA (lane 1), of the 31 nt immunoprecipitable fragment (lane 2), and of the 27 nt immunoprecipitable fragment (lane 3). Note that the RNA was labelled using α-^{32}P-UTP, and that T1 RNase leaves a 3′ phosphate after cutting at G. Thus the nucleotide directly upstream of a U will be labelled due to nearest neighbour transfer of label. Fragments originating from the 31 nt fragment (mapping at the 3′ end of hY5 RNA) are underlined, and those originating from the 27 nt fragment (mapping at the 5′ end of hY5 RNA) are double underlined. X, Fragment presumably resulting from incomplete digestion.

epitope would therefore consist of area(s) adjacent to the Ro60 binding site (Fig. 3c, hatched areas). These results are now being confirmed by more precise epitope mapping.

DISCUSSION

We have defined a novel autoantibody specificity targeting the deproteinized hY5 RNA. These autoantibodies were found only in sera from patients with anti-Ro antibodies. The two prototype anti-hY5 RNA sera were from young patients with severe SLE. Few specific anti-RNA antibodies have been described [21–26], and their clinical significance remains poorly defined. Hoet *et al.* have suggested that changes in serum levels of anti-U1 RNA antibodies may correlate with changes in disease activity in mixed connective tissue disease (MCTD) patients [38]. By analogy, an eventual prognostic or diagnostic potential of anti-hY5 RNA antibodies in anti-Ro-positive patients is currently being investigated.

Patient 1 and 2 sera recognized a conformational epitope on the hY5 RNA. Two sets of evidence support this notion. First, disruption of the structure of the RNA by addition of extra 5′ and 3′ nt to the full hY5 RNA sequence abolished antibody binding. Second, base pairing of two RNase T1-resistant fragments of hY5 RNA was required for recognition by patient antibodies. These results also suggest that region(s) of RNA adjacent to the Ro60-binding double stem (shown in hatched areas in Fig. 3c) are part of the epitope recognized by anti-hY5 RNA antibodies. No protein or nucleic acid has yet been shown to interact with the central portion of hY5 RNA. The production of antibodies directed against this part of hY5 RNA may indicate that this region is exposed on the Ro^{hY5} RNP, and thus, by analogy with other RNA epitopes, is available to interact with other molecules. Anti-hY5 RNA

antibodies might thus be used to probe the binding properties of these RNA sequences with proteins or other RNA molecules. This information may prove useful in our continuing search for the elusive function of Ro RNPs.

The detection of anti-hY5 RNA antibodies adds to the enlarging family of autoantibodies targeting components of the Ro RNPs that frequently coexist in the same anti-Ro sera (e.g. anti-La, anti-Ro60, anti-Ro RNP, and anti-Ro^{hY5} RNP antibodies). The multiplicity of antibody specificities within a given serum directed against different components of a macromolecular assembly is highly suggestive of a situation where the immune response is antigen-driven [1,17]. The humoral response to hY5 RNA also presents other characteristics of an antigen-driven maturation [39]. First, the recognition of hY5 RNA is mediated by IgG antibodies. This is shown by the fact that IgG are the major immunoglobulin binding on the protein A Sepharose beads during immunoprecipitation assays. In addition, purified IgG from patient 1 serum also recognized hY5 RNA (data not shown). Second, the recognition of hY5 RNA is highly specific, since patient 1 and 2 sera failed to recognize the closely related hY1, hY3 and hY4 RNAs as well as other unrelated RNAs. Third, anti-hY5 RNA antibodies have a high affinity for their antigen. In our assay, immunoprecipitation was performed under very stringent conditions (1 mg/ml heparin and 200 μg/ml tRNA). This represents more than a 10^6-fold molar excess of cold tRNA relative to labelled hY5 RNA. Thus, our data add to the evidence suggesting a role for the autoantigens themselves in initiation and/or perpetuation of the B cell response [1,17,39].

The mechanisms leading to the production of anti-RNA (and anti-DNA) antibodies are being unravelled [40]. Naked nucleic acids are not immunogenic [41]. Little or no Ro RNAs is thought to exist in the cell unassociated with Ro60 or La proteins [2]. Since the Ro60 binding site itself is conserved on all hY RNAs, an idiotype–anti-idiotype mechanism originating from antibodies that recognize the RNA binding area of the Ro60 protein is unlikely to lead specifically to anti-hY5 RNA antibodies. Alternatively, direct immunological presentation of intact Ro^{hY5} RNP complexes (with or without putative Ro^{hY5} RNP-specific proteins) might represent the immunogenic stimulus leading to the production of anti-hY5 RNA antibodies [39,40]. Definition of the implicated mechanism must await the results of ongoing studies aimed at precise epitope mapping of anti-hY5 RNA antibodies and at more complete characterization of the structure of the Ro^{hY5} RNPs.

The reasons why hY5 RNA (and not the other hY RNAs) is preferentially targeted by autoantibodies are intriguing. hY5 RNA-containing (Ro^{hY5}) RNPs are themselves specific targets of antibodies present in most anti-Ro sera [13]. Interestingly, no equivalent of Ro^{hY5} RNP was found in non-human cell lines [13]. Inhibition of immunoprecipitation experiments has shown that Ro^{hY5} RNPs contain all the antigenic determinants present on Ro RNPs, while the other human Ro RNPs do not. The highly specific production of antibodies against hY5 RNA and against Ro^{hY5} RNP (as opposed to the other Ro RNAs and Ro RNPs) in a significant proportion of anti-Ro sera suggests that Ro^{hY5} RNPs are particularly immunogenic. Because autoantibodies frequently recognize functional areas on autoantigens [1,39], this enhanced immunogenicity might be a clue to special or unusual cellular interactions or functions. Preliminary data suggest that the proteins associated with purified Ro^{hY5} RNPs, but not those associated with purified $Ro^{hY1-hY4}$, are able to reconstitute *in vitro* all the RNP complexes formed between hY5 RNA and cellular proteins [42]. It is thus possible that Ro^{hY5} RNPs constitute the functional and immunological quintessence of human Ro RNPs.

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