Biochemical and Immunological Heterogeneity of the Ro Ribonucleoprotein Particles

Analysis with Sera Specific for the Ro^{hY5} Particle

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Abstract

Anti-Ro autoantibodies found in sera from patients with systemic lupus erythematosus and related diseases precipitate four RNAs (hY1-hY5) from human cell extracts. We identified two patient sera that selectively immunoprecipitated from such extracts the Ro particle containing the hY5 RNA (Ro^{hY5} particle). Using cell fractions either enriched in or depleted of Ro^{hY5} particles, we have shown that these sera contain autoantibodies that target an antigenic determinant on the 60-kD Ro polypeptide that is expressed only on Ro^{hY5} particles and is absent on the Ro particles containing the hY1-hY4 RNAs ($Ro^{hY1-hY4}$ particles). In a competitive inhibition assay using a cell fraction enriched in $Ro^{hY1-hY4}$ particles but depleted of Ro^{hY5} particles, four of six control anti-Ro sera were also shown to contain antibodies reactive with the epitope specific for the Ro^{hY5} particle. Thus anti-Ro^{hY5} antibodies frequently occur in tandem with anti-Ro antibodies, but are not detected unless inhibition assays are performed. Finally, anti-Ro^{hY5} specific sera do not immunoprecipitate any Ro particles from various nonhuman cell lines. In contrast to other autoantibodies in systemic lupus and related diseases that bind conserved regions on conserved polypeptides, this observation suggests that a portion of the anti-Ro response targets a nonconserved epitope on a conserved autoantigen.

Introduction

The Ro ribonucleoprotein particle consists of the acidic 60-kD Ro polypeptide complexed with two to four small RNAs of 83-112 nucleotides in length (1-3). Recent evidence indicates that a 52-kD polypeptide is also associated with Ro RNAs (4). The number of Ro RNAs varies among mammalian cell types: human HeLa cells contain four, designated hY1-hY5 (hY2 is a slightly degraded form of hY1); murine cells have two, mY1 and mY2 (5); and rat cells have three, rY1a, rY1b, and rY2 (6). The 60-kD Ro polypeptide binds to the Ro RNAs at a specific region within a highly conserved double-stranded stem formed by basepairing of the 5′ and 3′ ends of the RNAs (3); the binding site of the 52-kD polypeptide is not known. The cellular location of the Ro particle is controversial (2, 4, 7, 8) and its biological function remains unknown.

Although the Ro particle is a relatively minor ribonucleoprotein particle, about $1-5 \times 10^5$ copies per cell (3), it is a potent autoimmunogen. Anti-Ro antibodies occur in sera of $\sim$ 30% of patients with systemic lupus erythematosus (SLE)[1] (9, 10) and nearly all patients with Sjogren's syndrome (11). These autoantibodies are also strongly associated with neonatal lupus and complete congenital heart block (12), as well as subacute cutaneous lupus (13). The concentration of anti-Ro in an individual serum may reach 30 mg/dl, constituting a significant proportion of the total antibody concentration (11).

As is the case for nearly all autoantibodies that bind ribonucleoproteins, anti-Ro antibodies bind determinants that reside on the polypeptide components of the Ro particles (3, 14). Indirect evidence suggests that one 60-kD Ro polypeptide associates with only one Ro RNA (3, 15); this implies the existence of distinct populations of Ro ribonucleoprotein particles. Since previously described anti-Ro sera immunoprecipitate all Ro RNAs, however, the antigenic epitopes are thought to be shared among all these different particles.

In the present study, we have identified two sera from patients with SLE that selectively immunoprecipitate the hY5 RNA from HeLa cell extracts. Using biochemically purified Ro particles, we have shown that a subset of anti-Ro sera contain two populations of antibodies, one specific for the Ro particle containing the hY5 RNA (Ro^{hY5} particle) and one directed against all Ro particles. The determinant on the Ro^{hY5} particle recognized by anti-Ro^{hY5} antibodies appears to be conformational, and is not present on Ro particles from cells of nonhuman origin.

Methods

Cells and sera. Human HeLa, mouse Ehrlich ascites, rabbit SIRC, and bovine MDBK cells (the latter three lines initially obtained from American Type Culture Collection, Rockville, MD) were maintained at 37°C under 5% CO_2, in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 60 μg of penicillin per ml, and 100 μg of streptomycin per ml. Sera were obtained from healthy laboratory workers and from American and French Canadian patients with various connective tissue diseases. Control anti-Ro sera were defined as those which immunoprecipitated all four Ro RNAs from HeLa cell extracts labeled in vivo with [^{32}P]-orthophosphate (2).

This study has been presented in part in abstract form (1988. *Arthritis Rheum.* 31:S65).

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1. *Abbreviations used in this paper:* IPP, immunoprecipitation buffer; SLE, systemic lupus erythematosus; TBE, Tris-borate EDTA; TBS, Tris-buffered saline; TSE, Tris-saline EDTA.

Immunofluorescence and immunodiffusion. Indirect immunofluorescence using commercial Hep-2 cell substrates (Immunoconcepts, Sacramento, CA) (16) and Ouchterlony double-immunodiffusion using human spleen extract and affinity-purified bovine Ro polypeptide were performed as previously outlined (17).

Preparation of radiolabeled cell extracts. HeLa cells were radiolabeled for 14 h as previously described (16, 18) with [^{32}P]-orthophosphate (10 μCi/ml cells; Amersham Corp., Arlington Heights, IL) for RNA analysis and with [^{35}S]methionine (10 μCi/ml cells; Amersham Corp.) for analysis of proteins. Cells were collected by centrifugation, washed in Tris-buffered saline (TBS) (10 mM Tris-Cl [7.5], 150 mM NaCl), and sonicated as described (18) in NET-2 buffer (50 mM Tris-Cl [7.5], 150 mM NaCl, 0.05% Nonidet P-40). Other cell lines (murine Ehrlich ascites, rabbit SIRC and bovine MDBK cells) grown to near confluence in 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) were trypsinized, split in two similar flasks in phosphate-free essential medium and labeled with [^{32}P]-orthophosphate (300 μCi per flask) for 14 h. Cells were detached by scraping and processed as described for HeLa cells (16, 18).

Immunoprecipitation procedures. Immunoprecipitation of radiolabeled cell extracts was performed as previously described (19) with modifications (18). 10 μl of patient serum was incubated for 2 h at 4°C with 2 mg of protein A-Sepharose beads (Pharmacia, Inc., Piscataway, NJ) in 500 μl of immunoprecipitation buffer (IPP; 10 mM Tris-Cl [7.5], 500 mM NaCl, 0.1% Nonidet P-40). Beads with bound antibodies were then washed six times in IPP and resuspended in 400 μl of NET-2. For analysis of RNAs, beads were combined with 100 μl of ^{32}P-labeled extract, derived from 2×10^6 cells, and rotated at 4°C for 1 h. After six washes with NET-2, bound ^{32}P-labeled nucleic acids were extracted as previously described (18), fractionated on 10% polyacrylamide-7M urea gels, dried, and detected by autoradiography. For depletion studies, 100 μl of ^{32}P-labeled extracts were sequentially incubated with beads coated with an anti–Ro serum until no Ro RNAs could be immunoprecipitated from these extracts (usually four to five incubations), then with beads coated with an anti–Ro^{hY5} serum. The specificity of the Ro antigen depletion was checked by further incubation of the extracts with an anti–U1 RNP serum. The nucleic acids present in the immunoprecipitates were then extracted and visualized as above. In some experiments, unlabeled HeLa cell extracts (6×10^6 cells per sample) were combined with antibody-coated protein A-sepharose beads and the immunoprecipitated RNAs visualized by silver staining (20).

In experiments using deproteinized nucleic acids, ^{32}P-labeled HeLa cell sonicates were first extracted with phenol/chloroform/isoamyl alcohol (50:50:1). The extracted nucleic acids were precipitated in ethanol, washed in 70% ethanol and resuspended in NET-2 buffer; alternatively, radiolabeled extracts were combined with proteinase K (360 μg/ml; Boehringer Mannheim Biochemicals Indianapolis, IN) at 4°C for 90 min, then made 4.0 mM in phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, MO) (21). Deproteinized extracts were then immunoprecipitated in parallel with untreated extracts as described above.

For protein studies, antibody-coated beads were combined with 400 μl of [^{35}S]methionine-labeled extracts (8×10^6 cells) and rotated at 4°C for 1 h. After six washes with NET-2, the beads were resuspended in SDS-sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris-Cl [6.8], 0.005% bromophenol blue) (22). After heating (90°C for 5 min), the proteins were fractionated on 10% SDS-polyacrylamide gels (acrylamide/bis; 30:1.13), enhanced with 0.5 M sodium salicylate, and dried; labeled proteins were detected by autoradiography.

Two-dimensional RNA fractionation. Two-dimensional RNA fractionation was performed essentially according to Rosa et al. (21) using 10% acrylamide, 0.38% *N,N'*-bisacrylamide, 7 M urea in Tris-borate EDTA (TBE; 0.09 M Tris borate [8.3], 1 mM EDTA) as the first of two dimensions and 18% acrylamide, 0.68% *N,N'*-bisacrylamide in TBE in the second.

RNase digestion experiments. In certain experiments, ^{32}P-labeled HeLa extracts bound to antibody-coated protein A-sepharose beads were washed with NET-2, resuspended in 100 μl of NET-2 containing 5 mM $MgCl_2$ and digested with pancreatic ribonuclease (RNase A; 1 mg/ml; Boehringer Mannheim Biochemicals) as described previously (23), except incubation was at 4°C overnight with rotation. Control samples were handled identically, except for the addition of ribonuclease.

SDS-polyacrylamide gel electrophoresis and immunoblots. Affinity-purified 60-kD Ro polypeptides (4 μg per lane) (kindly provided by Dr. Mark Mamula, Yale University School of Medicine) were fractionated in discontinuous 7.5% polyacrylamide gels (acrylamide/bis; 30:0.8), followed by electrotransfer to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) in 25% methanol, Tris-glycine [8.3] (24). After transfer, the nitrocellulose sheets were blocked with 3% BSA in TBS overnight and subsequently incubated with human antisera diluted 1:50 in TBS containing 0.1% Tween 20 and 1.0% BSA. Bound antibodies were detected with ^{125}I-labeled protein A (1×10^5 cpm/ml; ICN, Irvine, CA) followed by autoradiography on XRP film (Eastman Kodak Co., Rochester, NY).

Biochemical separation of Ro particles. 3 liters of HeLa cells, grown to $6–10 \times 10^5$ cells/ml, was collected by centrifugation, and washed twice in chilled TBS. Subsequently all procedures were performed at 4°C and all buffers were supplemented with 1.0 mM dithiothreitol and 1.0 mM PMSF. A cytoplasmic fraction was prepared without detergent (25). In brief, the cells were allowed to swell in six pellet-volumes of buffer A (10 mM Tris-Cl [7.5], 1.5 mM $MgCl_2$, 10 mM KCl) for 10 min on ice, spun down, resuspended in two pellet-volumes of Buffer A and disrupted by 10 strokes of an all-glass Dounce homogenizer; 100 U of RNasin (Promega Biotec, Madison, WI) were added after disruption of the cells. Extracts were then clarified by centrifugation at 13,000 *g* for 20 min. The salt concentration of the supernatant was increased to 150 mM by adding 0.1 vol of buffer B (0.3 M Tris-Cl [7.5], 1.4 M NaCl, 1.5 mM $MgCl_2$). The extract was then layered over a 7.5-ml sucrose cushion (800 mM sucrose, 10 mM Tris-Cl [7.5], 5 mM $MgCl_2$) in 30-ml tubes and centrifuged at 100,000 *g* for 90 min. The supernatant was collected and diluted 1:1 with Tris-saline EDTA (TSE; 50 mM Tris-Cl [7.5], 150 mM NaCl, 1 mM EDTA) and applied to a 40-ml column of DE 52 (Whatman, Maidstone, Kent, England) preequilibrated in TSE. After extensive washings with TSE, step elutions of the column using TSE containing 175 mM, 210 mM, 250 mM, and 300 mM NaCl were performed. After addition of RNasin (40 U), the eluates were concentrated by vacuum dialysis (Pro-Di-Con, Bio-Molecular Dynamics, Beaverton, OR) against TSE. Elution profiles of the Ro RNAs in each of the eluted fractions were determined by immunoprecipitation with control anti–Ro sera. The cell fraction eluted at 210 mM (enriched in Ro^{hY5} and depleted in Ro particles containing the hY1-hY4 RNAs [Ro$^{hY1-hY4}$ particles]) and the cell fraction eluted at 300 mM (enriched in Ro$^{hY1-hY4}$ and depleted in Ro^{hY5} particles) were used in a competitive inhibition assay (below).

Competitive inhibition of immunoprecipitation. To identify two populations of anti–Ro antibodies in patient sera, a competitive inhibition assay using immunoprecipitation of ^{32}P-labeled HeLa cell extracts was devised. In this assay, the optimal amount of an anti–Ro serum (typically between 0.02 and 0.1 μl) was first defined as the lowest still giving a good visualisation of all the immunoprecipitated Ro RNAs after a 24–48-hour exposure on XRP film (Eastman Kodak Co.). This quantity of serum was then combined with 2 mg of protein A-Sepharose, washed six times in IPP and resuspended in 300 μl of NET-2. Multiple samples were incubated in parallel for 90 min with unlabeled, biochemically purified HeLa cell fractions containing either Ro^{hY5} or Ro$^{hY1-hY4}$ particles, prepared as described above from 1×10^7, 1×10^8, and 4×10^8 cells; control samples were incubated with buffer alone. The cleared sonicate prepared from 1×10^6 ^{32}P-labeled HeLa cells was then added to the mixture and incubated for 1 h. The unlabeled inhibitor was thus used in 0, 10, 100, and 400-fold excess, respectively, relative to the amount of labeled substrate. Subsequently, the beads were washed in NET-2 and the immunoprecipitated ^{32}P-labeled Ro RNAs were extracted and visualized by autoradiography as described. The inhibition of immunoprecipitation was then evaluated with a

scanning laser densitometer (Ultroscan XL, LKB Instruments, Gaithersburg, MD; range absorbance of 0–4 U) (26). Only autoradiographs having net absorbance readings of 0.3–3.0 for the peak intensity of the hY5 RNA were used to calculate the ratio of the immunoprecipitated hY5 RNA to the four immunoprecipitated Ro RNAs. Absorbance readings below 0.3 U were excluded from analysis because below this level the individual RNA peaks could not be accurately identified. Similarly, exposures with a net intensity above 3.0 absorbance units were discarded since the relationship between absorbance and length of autoradiography was nonlinear above this level (26). Evidence of anti-hY5 Ro-specific activity was defined as a specific immunoprecipitation of the hY5 RNA that could not be inhibited by an excess of $Ro^{hY1-hY4}$ particles sufficient to inhibit completely the immunoprecipitation of the hY1–hY4 RNAs.

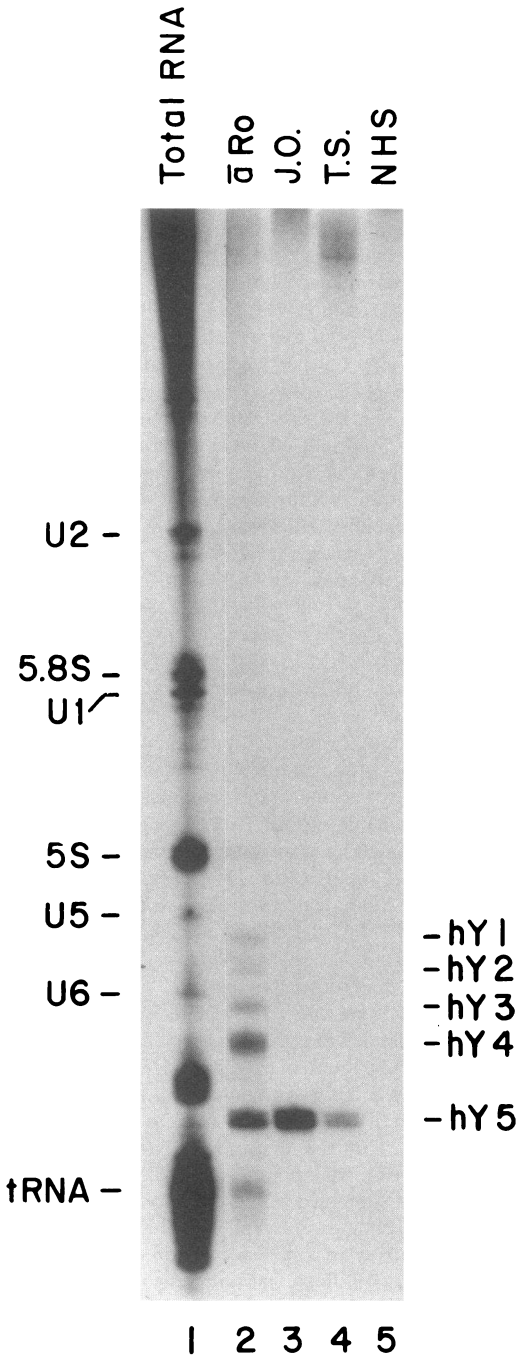

Figure 1. Polyacrylamide gel fractionation of ^{32}P-labeled RNAs in total HeLa cell extracts (lane *1*), and after immunoprecipitation with a control anti-Ro serum (lane *2*), the two prototype sera J.O. and T.S. (lanes *3* and *4*), and a normal human serum (NHS) (lane *5*). Identical amounts of serum (5 μl) and of labeled cells (2×10^6) were used for the immunoprecipitations in lanes *2–5*.

Results

Selective immunoprecipitation of the Ro^{hY5} particle. As originally defined by Lerner et al. (1) and by Hendrick et al. (2), and as shown in Fig. 1 (lane *2*), anti-Ro antibodies immunoprecipitate four small RNAs, hY1–hY5, from HeLa cells labeled in vivo with [^{32}P]orthophosphate. In such experiments, the Ro RNAs are not visible in total RNA extracts (lane *1*), and can only be identified by immunoprecipitation with anti-Ro antibodies. Among 32 sera which contained only anti-Ro antibodies as defined by the ^{32}P immunoprecipitation assay (1–3), we identified two from SLE patients J.O. and T.S., which selec-

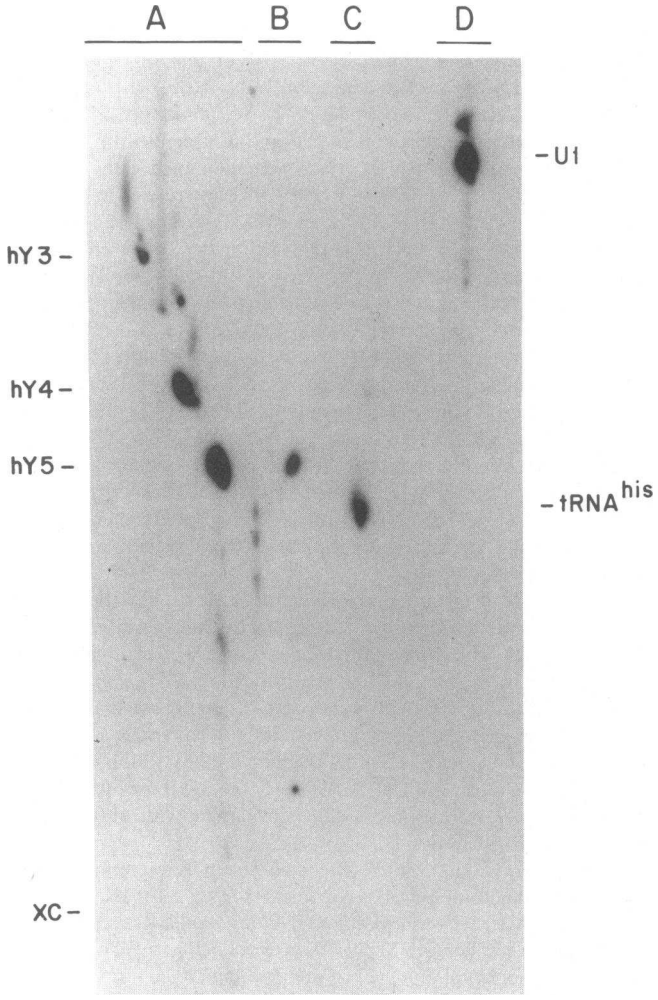

Figure 2. Two-dimensional gel fractionation of immunoprecipitated ^{32}P-labeled HeLa cell RNAs. Immunoprecipitated RNAs were first fractionated in the same 10% polyacrylamide-7 M urea gel, cut out, and then fractionated in a second dimension in an 18% polyacrylamide gel. RNAs were obtained by immunoprecipitation with a control anti-Ro serum (lane *A*), the prototype serum J.O. (lane *B*) and an anti-Jo-1 serum (lane *C*); total U1 RNA is also shown (lane *D*). The hY5 RNA from a control anti-Ro immunoprecipitate and the RNA precipitated by serum J.O. migrate identically in the second dimension. XC, xylene cyanol FF.

tively immunoprecipitated a small RNA that comigrated with the hY5 RNA (lanes *3* and *4*). Like the hY5 RNA, this RNA runs as a tight doublet (lanes *2–4*). The RNA immunoprecipitated by the two prototype sera migrated the same as the hY5 RNA in two-dimensional polyacrylamide gels (Fig. 2; compare the migration in the second dimension of the RNA immunoprecipitated by serum J.O. [lane *B*] with the hY5 RNA immunoprecipitated with a control anti-Ro serum [lane *A*] and with the $tRNA^{his}$ immunoprecipitated by an anti-Jo-1 serum [lane *C*] and with the U1 RNA [lane *D*]). In addition, digestion of the immunoprecipitates from sera J.O. and T.S. with RNase A yielded protected fragments of RNA identical to nuclease-resistant fragments derived from the hY5 RNA when immunoprecipitates from a control anti-Ro serum were similarly digested (data not shown) (3). Moreover, preincubation of HeLa cell sonicates with conventional anti-Ro sera depleted these extracts of the RNA immunoprecipitated by our prototype sera whereas U1 RNPs were not depleted by this preincubation (data not shown). These experiments confirmed that the RNA immunoprecipitated by the two prototype sera was the hY5 RNA.

Since the hY5 RNA constitutes the most intensely radiolabeled of the immunoprecipitated Ro RNAs (compare the intensity of hY5 to hY1–hY4 in Fig. 1, lane *2*), the possibility existed that the prototype sera J.O. and T.S. appeared specific for the Ro^{hY5} particle because they contained low titers of anti-Ro antibodies, and only the hY5 RNA was visualized on autoradiographs. Indeed, both sera gave negative results for anti-Ro antibodies in double immunodiffusion assays and both produced only weak cytoplasmic staining when examined in indirect immunofluorescence using Hep-2 cells. Low antibody titers seemed to be an unlikely explanation for specific immunoprecipitation of the hY5 RNA, however, since this RNA represented > 90% of the radioactivity immunoprecipitated by the two prototype sera over a range of serum dilutions and cell concentrations (Fig. 3 *A*). In contrast, the hY5 RNA constituted only 50–80% of the total radioactivity immunoprecipitated by control anti-Ro sera, with the other Ro RNAs constituting the remainder of the immunoprecipitated radioactivity (Fig. 3 *B*).

Partial biochemical purification of the Ro^{hY5} particle. Since these experiments indicated that the Ro^{hY5} particle was independently targeted by the immune system, we suspected that it might have unique features which would permit its biochemical purification. In agreement with earlier studies we found that cytoplasmic extracts contained most of the Ro particles (27). Accordingly, cytoplasmic extracts of HeLa cells were loaded upon an anion exchange column and eluted stepwise with buffers of increasing ionic strength. As shown in Fig. 4, a control anti-Ro serum immunoprecipitated all four Ro RNAs from HeLa cell extracts before anion exchange chromatography (lane *2*), but only the hY5 RNA from the cell fraction eluted with 210 mM NaCl (lane *3*). All four Ro RNAs were present in the fraction eluted at 250 mM NaCl (lane *4*), although the relative amount of hY5 was diminished in proportion to the other Ro RNAs (compare lanes *2* and *4*). The hY1–hY4 RNAs, with a minimal contamination of hY5, were immunoprecipitated from the fraction eluted at 300 mM NaCl (lane *5*). As expected, the prototype serum T.S. immunoprecipitated the hY5 RNA from the 210 mM NaCl eluate, and a minimal amount of hY5 from the 250 mM eluate, but did not immunoprecipitate any Ro RNAs from the 300 mM eluate (lanes *6–8*).

Selective inhibition of immunoprecipitation of the Ro^{hY5} particle using partially purified Ro^{hY5} particles. To confirm the specificity of the putative anti-Ro^{hY5} antibodies in sera T.S. and J.O., we determined if purified Ro^{hY5} or purified $Ro^{hY1-hY4}$ particles could inhibit the immunoprecipitation of the hY5

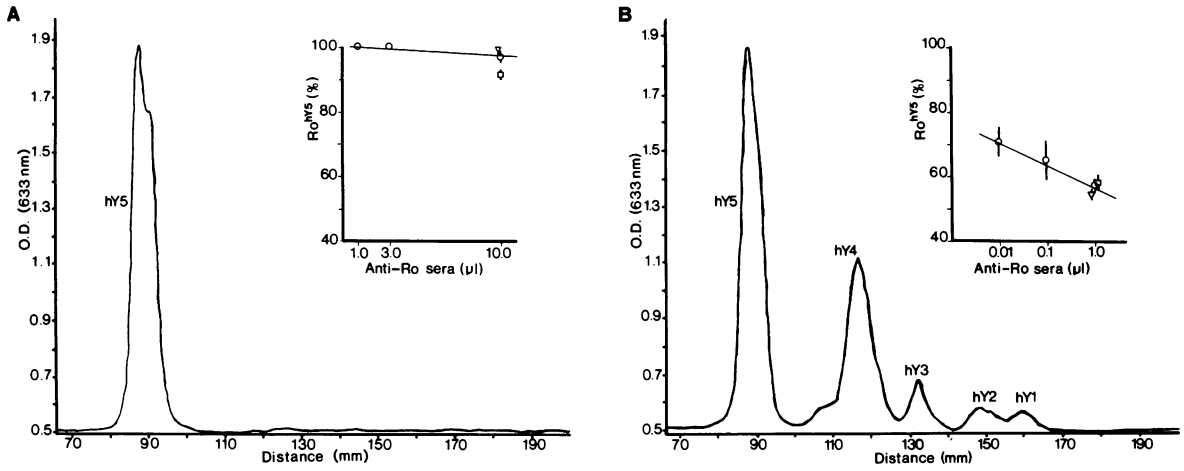

Figure 3. Quantitative determination of the hY5 RNA relative to all the Ro RNAs. The RNAs immunoprecipitated from ^{32}P-labeled HeLa cell extracts were fractionated on a 10% polyacrylamide-7 M urea gel and the autoradiographs scanned with a densitometer (see Methods). (*A*) Densitometry curve corresponding to anti-Ro^{hY5} serum J.O.; ordinate equals optical density at 633 nm, abscissa equals distance in millimeters from the bottom of the gel. (*Inset*) The two anti-Ro^{hY5} sera (J.O. and T.S.) were used to immunoprecipitate HeLa cell extracts; both sera were tested in three amounts from 1.0 to 10.0 µl. Results are expressed as the area under the densitometry curve for the hY5 RNA divided by the sum of the areas of all the Ro RNAs (Ro^{hY5}%; *ordinate*) versus sera amounts (*abscissa*). 10 µl of both sera also were used to immunoprecipitate various amounts of cell extracts: (○) extracts from 2×10^6 cells; (▽) extracts from 2×10^5 cells; and (□) extracts from 4×10^4 cells, respectively. The vertical bars represent the mean±SE. (*B*) Densitometry curve corresponding to a control anti-Ro serum. (*Inset*) Four control anti-Ro sera were used to immunoprecipitate HeLa cell extracts; all four sera were tested in three amounts from 0.01 to 1.0 µl. The vertical bars represent the mean±SE. 1 µl of all four sera also was used to immunoprecipitate various amounts of cell extracts; symbols are as described for the inset in *A*.

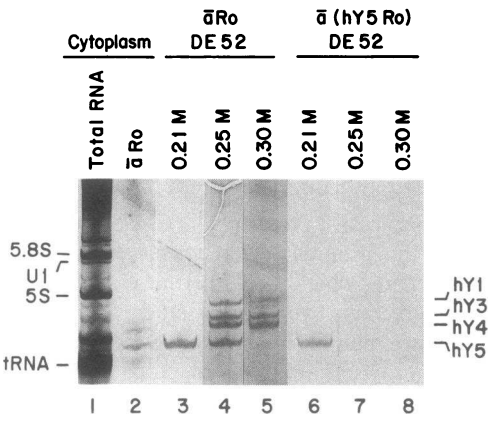

Figure 4. Biochemical fractionation of the Ro^{hY5} and the $Ro^{hY1-hY4}$ particles. Samples from successive steps were either extracted with phenol/chloroform and the total RNAs fractionated on a 10% polyacrylamide-7 M urea gel (lane *1*) or immunoprecipitated with anti-Ro serum W.O. (lanes *2–5*) or with the prototype anti-Ro^{hY5} serum T.S. (lanes *6–8*). Lanes *1* and *2* refer to the total cytoplasmic fraction (note that all four Ro RNAs are present in this fraction; lane *2*); lanes *3* and *6*, *4* and *7*, and *5* and *8* refer to the 210 mM NaCl, the 250 mM, and the 300 mM NaCl eluates, respectively, from a DE 52 cellulose column.

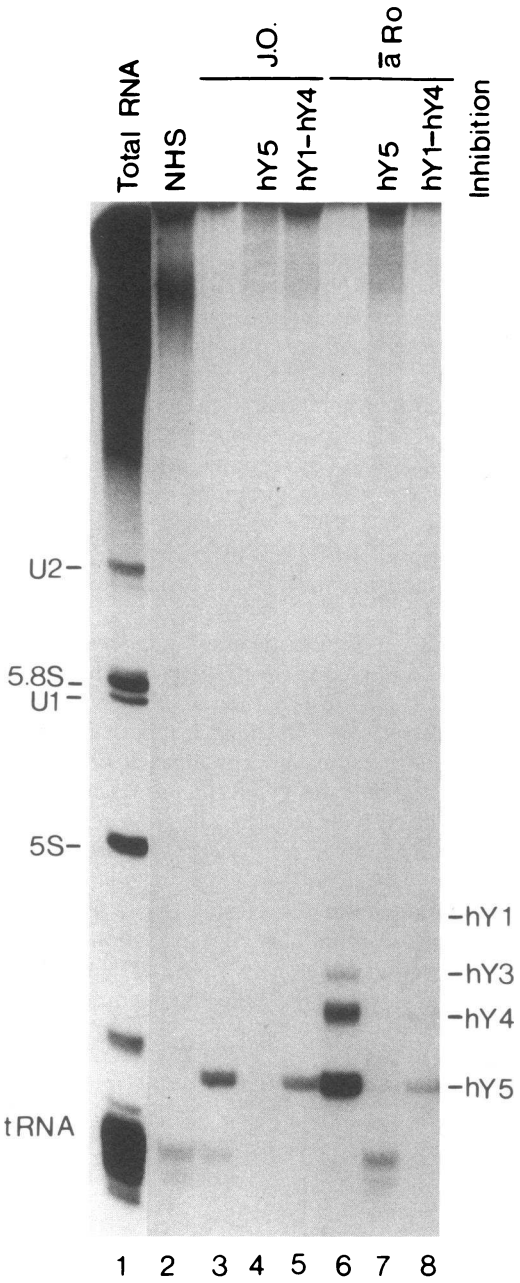

Figure 5. Inhibition of immunoprecipitation of ^{32}P-labeled HeLa Ro RNAs by unlabeled cell fractions enriched in Ro^{hY5} or $Ro^{hY1-hY4}$ particles. RNAs present in total HeLa cell extracts (lane *1*); immunoprecipitate from a normal human serum (NHS; lane *2*); and immunoprecipitates from the prototype serum J.O. (lanes *3–5*) and a control anti-Ro serum W.O. (lanes *6–8*). Before immunoprecipitation of labeled cell extracts, the latter two sera were either incubated with a 200-fold excess of a cell fraction enriched in Ro^{hY5} particles (lanes *4* and *7*) or of a cell fraction enriched in $Ro^{hY1-hY4}$ particles (lanes *5* and *8*). Lanes *3* and *6* show the precipitates from uninhibited sera.

RNA by these sera. In these experiments, sera were used to immunoprecipitate ^{32}P-labeled HeLa cell extracts after being preincubated with an excess of partially purified unlabeled Ro particles. As shown in Fig. 5, the cell fraction enriched in Ro^{hY5} particles, but devoid of $Ro^{hY1-hY4}$ particles, inhibited immunoprecipitation of Ro^{hY5} by the prototype serum J.O. (compare lane *3* with lane *4*) whereas addition of the fraction containing mainly $Ro^{hY1-hY4}$ particles minimally inhibited immunoprecipitation by this serum (lane *5*). This observation confirmed that the prototype serum J.O. contained antibodies specific for an epitope restricted to the Ro^{hY5} particle; this serum was thus designated anti-Ro^{hY5}. In contrast, immunoprecipitation of the Ro RNAs by control anti-Ro serum W.O. was nearly completely inhibited by both cell fractions (compare lanes *7* and *8* with lane *6*), indicating that this serum targeted an epitope common to all the Ro particles, including the Ro^{hY5} particle.

Sera with the anti-Ro specificity contain anti-Ro^{hY5} antibodies. To explore the possibility that autoantibodies specific for the Ro^{hY5} particle are common, we performed immunoprecipitation experiments with a series of patient sera after they had been absorbed with $Ro^{hY1-hY4}$ particles. As shown in Fig. 6 (lanes *2–5*), preincubation with increasing concentrations of $Ro^{hY1-hY4}$ particles preferentially inhibited immunoprecipitation of the $Ro^{hY1-hY4}$ RNAs by serum G.L. Densitometry tracings of autoradiographs confirmed this observation since the percentage of Ro^{hY5} versus the total of the four Ro RNAs increased from 62% in uninhibited immunoprecipitates to 100% in maximally inhibited immunoprecipitates (Table I). Therefore this serum also contains an antibody population specific for the Ro^{hY5} particle. In contrast, preincubation with $Ro^{hY1-hY4}$ particles inhibited immunoprecipitation of all four Ro RNAs by anti-Ro serum W.O. (Fig. 6, lanes *6–9*), indicating that this specimen lacked detectable antibodies specific for the Ro^{hY5} particle. Densitometry tracings confirmed this observation since the hY5 RNA constituted 50% of the total radioactivity immunoprecipitated in the absence of, or in the presence of low concentrations of $Ro^{hY1-hY4}$ particles, with all four Ro RNAs becoming undetectable at high concentrations of inhibitor (Table I). As expected, incubation with excess $Ro^{hY1-hY4}$ particles never completely inhibited immunoprecipitation by the anti-Ro^{hY5} specific serum J.O. (Fig. 6, lanes *10–13;* Table I).

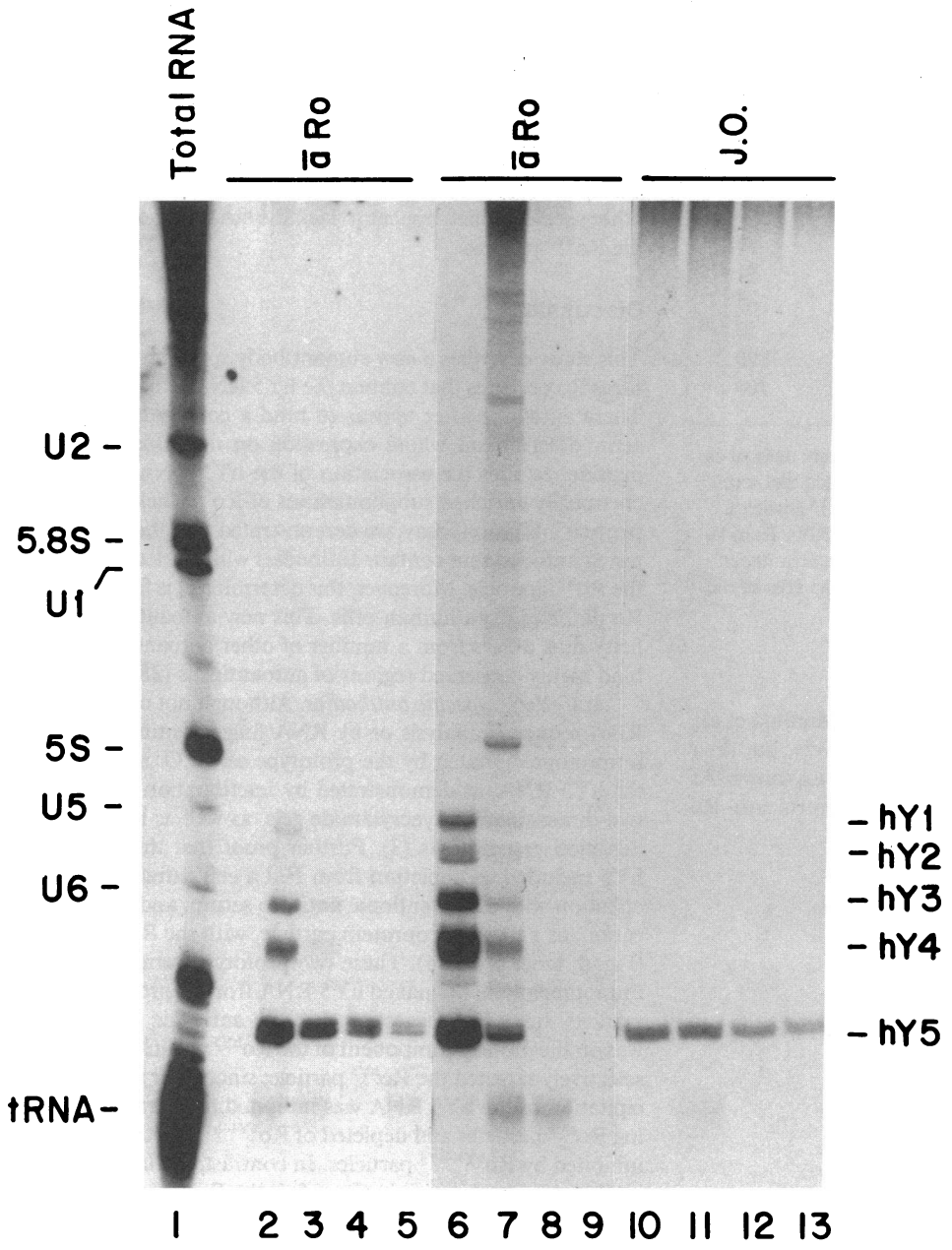

Figure 6. Inhibition of immunoprecipitation of ^{32}P-labeled HeLa Ro RNAs by unlabeled cell fractions enriched in Ro$^{hY1-hY4}$ particles, demonstrating that anti-Ro^{hY5}-specific antibodies are present in anti-Ro sera. Immunoprecipitates from two control anti-Ro sera (serum G.L., lanes *2–5;* and serum W.O., lanes *6–9*) and prototype serum J.O. (lanes *10–13*) are shown. Inhibitor was not added in lanes *2, 6,* and *10.* Cold inhibitor was used at a 10-fold (lanes *3, 7,* and *11*), 100-fold (lanes *4, 8,* and *12*) and 400-fold excess (lanes *5, 9,* and *13*) relative to the amount of ^{32}P-labeled cell extracts. Lane *1* shows the RNAs present in labeled total cell extracts.

Absorption studies using biochemically enriched Ro$^{hY1-hY4}$ particles were carried out on six sera that contained the conventional anti-Ro specificity. Of these, four still immunoprecipitated the hY5 RNA after total inhibition of their capacity to immunoprecipitate Ro$^{hY1-hY4}$ particles (sera G.L., S.A., C.M., and S.C., Table I). Thus these sera also contain antibodies specific for the Ro^{hY5} particle, whereas two sera (sera W.O. and A.S., Table I) appeared to lack such antibodies. Sera containing low titers of anti-Ro^{hY5} might go undetected in these experiments, however, since our Ro$^{hY1-hY4}$ preparation was contaminated with low levels of Ro^{hY5} particles (see Fig. 4, lane *5*).

The Ro^{hY5}-specific autoantigenic epitope requires both RNA and polypeptide components. In anti-Ro sera described to date (3), immunoprecipitation of the Ro particles is dependent upon the Ro polypeptide. Similarly, neither of the anti-Ro^{hY5}-specific sera studied here immunoprecipitated the hY5 RNA after deproteinization of cell extracts by phenol extraction or proteinase K treatment (data not shown), suggesting that the Ro^{hY5} epitope is at least partially constituted by protein. As expected, both sera immunoprecipitated the 60-kD Ro polypeptide from [^{35}S]methionine labeled HeLa cell extracts (Fig. 7, lanes *3* and *4*). Neither of these sera precipitated affinity purified human or bovine Ro polypeptides in double immunodiffusion, however, nor bound these polypeptides in immunoblots (data not shown), suggesting that the Ro^{hY5} epitope requires the presence of hY5 RNA to be expressed.

The Ro^{hY5} antigenic determinant is not evolutionarily conserved. Wolin and Steitz (5) have shown that murine cells contain only two Ro RNAs, mY1 and mY2, which have significant sequence homology with the hY1 and hY3 RNAs from HeLa cells, but lack homology with hY5. Rabbit and

Table I. Screening of Anti-Ro Sera for Anti-Ro^{hY5} Antibodies

Sera	Ro^{hY5}%			
	-fold excess of Ro$^{hY1-hY4}$ inhibitor			
	0	10	100	400
G.L.	62	87	99	100
S.A.	63	ND*	ND	100
C.M.	52	ND	ND	90
S.C.	55	ND	ND	91
W.O.	50	52	50	—‡
A.S.	57	44	47	52
J.O.	100	100	100	100
T.S.	100	100	100	100

Anti-Ro sera G.L., S.A., C.M., S.C., W.O. and A.S. were defined as immunoprecipitating all four Ro RNAs from ^{32}P-labeled HeLa cell extracts in the absence of inhibiting unlabeled Ro$^{hY1-hY4}$ particles. Sera J.O. and T.S. only immunoprecipitated the hY5 RNA from labeled extracts, as described. Abbreviation: Ro^{hY5}%, the area under the densitometry curve for the hY5 RNA divided by the sum of the areas under the curves of all four Ro RNAs × 100%.
* Not done; ‡ not detectable.

bovine cells contain three and four Ro RNAs (Mamula et al., manuscript submitted for publication), respectively, but their structural similarities to the hY1–hY5 RNAs are unknown. As shown by the representative example in Fig. 8, control anti-Ro

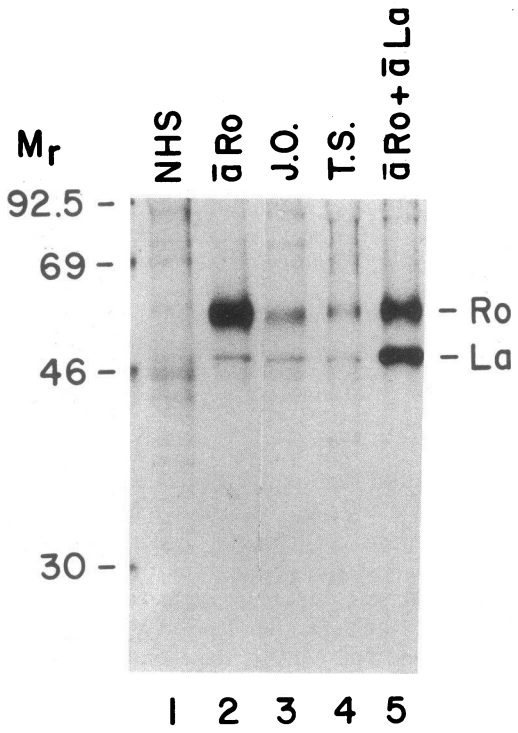

Figure 7. Immunoprecipitation of [^{35}S]methionine labeled HeLa cell extracts. Immunoprecipitates were formed with a normal human serum (NHS, lane *1*), a monospecific control anti-Ro serum W.O. (lane *2*), the prototype anti-Ro^{hY5} sera J.O. and T.S. (lanes *3* and *4*), and a serum with both anti-Ro and anti-La antibodies (lane *5*). The 60-kD Ro and the 50-kD La polypeptides are shown. M_r = molecular mass times 10^{-3}.

sera immunoprecipitated four Ro RNAs from human HeLa cells (panel *A*, lane *2*), three from rabbit cells (panel *A*, lane *5*), two from murine cells (panel *B*, lane *2*), and four from bovine cells (panel *B*, lane *5*). In contrast, as shown by serum J.O., the anti-Ro^{hY5} sera did not immunoprecipitate any RNAs from these three cell lines (panel *A*, lane *6*; panel *B*, lanes *3* and *6*). Thus, the Ro^{hY5} epitope is not present on Ro particles from other species, which appear to lack the structural equivalent of the Ro^{hY5} particle.

Discussion

This study describes a new autoantibody specificity that recognizes Ro particles that contain the hY5 RNA (Ro^{hY5} particles). These autoantibodies appear to bind a conformational antigenic determinant whose expression on the 60-kD Ro polypeptide requires the association of the hY5 RNA. Using biochemically enriched subpopulations of Ro particles in a competitive inhibition assay, we demonstrated that the majority of tested anti-Ro sera contain antibodies which selectively target the Ro^{hY5} particle. Moreover, this determinant is found only in Ro particles from human cells. This new autoantibody specificity thus differs from a number of other autoantibodies that bind highly conserved regions of autoantigens (28–32).

Anti-Ro^{hY5}-specific antibodies. Although not confirmed by RNA sequence analysis or by RNA fingerprinting, the RNA immunoprecipitated by the prototype sera J.O. and T.S. was the hY5 RNA, as demonstrated by fractionation in one- and two-dimensional polyacrylamide gels, as well as by RNAse A digestion experiments (3). Further proof that this RNA was hY5 included its depletion from HeLa cell extracts by preincubation with a conventional anti-Ro serum, and its copurification, as a ribonucleoprotein particle, with the Ro^{hY5} particle (Fig. 4, lanes *3* and *6*). These two prototype sera did not immunoprecipitate the naked hY5 RNA from deproteinized cell extracts, however, suggesting that the antigenic determinant was on the protein component of the Ro^{hY5} particle. These sera selectively targeted the Ro^{hY5} particle, since their immunoprecipitation of the hY5 RNA was inhibited by extracts containing Ro^{hY5} particles and depleted of Ro$^{hY1-hY4}$ particles, but not inhibited by Ro$^{hY1-hY4}$ particles. In contrast, the latter extracts inhibited immunoprecipitation of all the Ro RNAs, including the hY5 RNA, by a control anti-Ro serum. In other words, the Ro^{hY5} particle bears two distinct epitopes: one unique to the Ro^{hY5} particle, and one shared among all Ro particles.

Anti-Ro^{hY5} antibodies are frequent and coexist with anti-Ro antibodies. Although previously unreported, the anti-Ro^{hY5} specificity is not rare. In our laboratory, we routinely screen ANA positive sera by the ^{32}P immunoprecipitation assay which is more sensitive than double immunodiffusion (20, 33); of 32 sera shown to immunoprecipitate the Ro RNAs (but no La-associated RNAs) from HeLa cell extracts, two selectively immunoprecipitated the hY5 RNA. This likely represents a minimum prevalence of sera that only target Ro^{hY5}, however, since the sera in our bank are selected for positive ANAs by indirect immunofluorescence on Hep-2 cells. Both sera containing only anti-Ro^{hY5} antibodies gave faint cytoplasmic immunofluorescence and other sera with the same specificity and weak immunofluorescence could easily go undetected.

Using competitive inhibition experiments, we also uncovered anti-Ro^{hY5} antibodies in four of six control anti-Ro sera

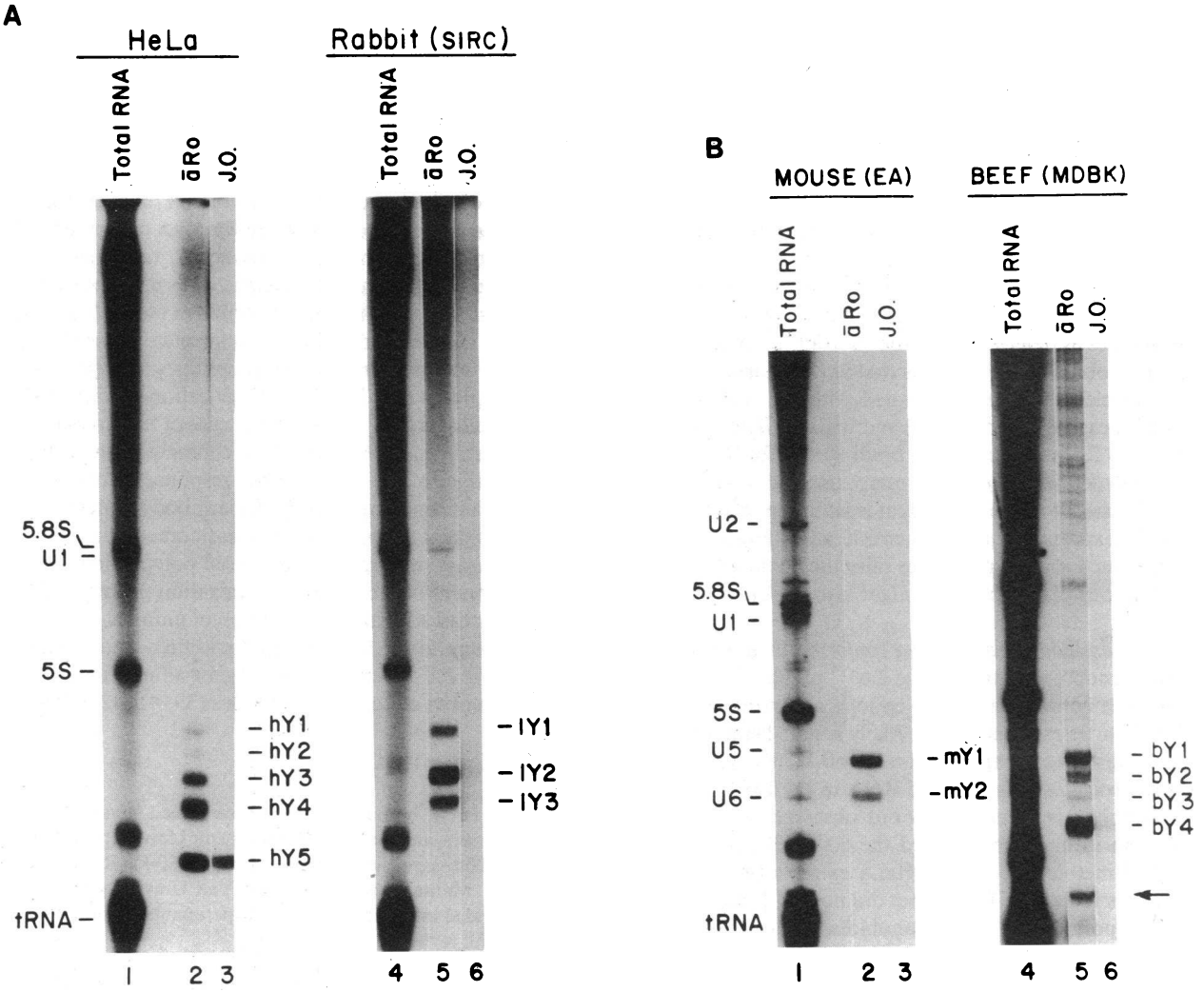

Figure 8. Immunoprecipitation of ^{32}P-labeled Ro RNAs from mammalian cell lines. (*A*)Human HeLa and rabbit SIRC cells. (*B*) Mouse Ehrlich ascites and bovine MDBK cells. The RNAs present in total cell extracts are shown in lanes *1* and *4* (*A* and *B*). Immunoprecipitates from control anti-Ro serum W.O. are shown in lanes *2* and *5* (*A* and *B*); this serum precipitates four RNAs from human cells (hY1–hY5), three from leporine cells (lY1-IY3), two from murine cells (mY1 and mY2), and four from bovine cells (bY1–bY4) (Mamula et al., manuscript submitted for publication). None of the nonhuman cell lines contain a Ro RNA comigrating with the hY5 RNA. RNAs are not immunoprecipitated from the nonhuman cells by the anti-Ro^{hY5}-specific serum J.O. (*A*, lane *6; B*, lanes *3* and *6*), in contrast to immunoprecipitation of the hY5 RNA from HeLa cells (*A*, lane *3*). Similar results were obtained with the anti-Ro^{hY5} serum T.S. *A* and *B* are from different gels. The arrow in *B* points to a probable degradation product of the bovine Ro RNAs, since this RNA was not immunoprecipitated in other experiments.

that had been initially defined by immunoprecipitation of all four Ro RNAs from HeLa cell extracts. These results suggest that anti-Ro^{hY5} antibodies are common and likely occur in tandem with anti-Ro antibodies that immunoprecipitate all the Ro particles. Such a linked occurrence obscures the presence of anti-Ro^{hY5} in the ^{32}P immunoprecipitation assay unless similar inhibition experiments are performed. In the presence of antibodies that immunoprecipitate all four Ro RNAs, however, our method of identification of anti-Ro^{hY5} antibodies would underestimate their frequency because the Ro$^{hY1-hY4}$ substrate we used for inhibition was slightly contaminated by Ro^{hY5} (see Fig. 4, lane *5*). We did not test a larger number of anti-Ro sera because the competitive inhibition assay required large amounts of biochemically enriched Ro$^{hY1-hY4}$ particles, which are in low abundance in cells. Therefore, more precise estimates of the frequency of anti-Ro^{hY5} antibodies must await the development of a less cumbersome and more sensitive assay.

Heterogeneity of the Ro particles. The identification of anti-Ro^{hY5} antibodies indicated that the Ro^{hY5} particle was independently targeted by the immune system, suggesting that at least two populations of Ro particles existed in HeLa cells. Previous investigators, via the identification of a unique polypeptide binding site on all of the Ro RNAs (3), had previously suggested that each Ro RNA was contained in a separate antigenic complex, with a possible stoichiometric relationship of one 60-kD polypeptide bound to one RNA. Analysis of Ro particles by sucrose density centrifugation (3) and gel filtration (15) further supported this hypothesis. The selective immunoprecipitation of the Ro^{hY5} by our prototype anti-Ro^{hY5} sera confirmed that the Ro particles containing the hY5 RNA exist as discrete entities, at least under the conditions of our assays.

We substantiated the heterogeneity of the Ro particles by biochemically separating the native Ro^{hY5} particle away from the other Ro particles. In other experiments, we have further purified the Ro particles and have shown that they segregate into three groups according to their molecular weight, buoyancy in sucrose gradients, and affinity for ligands like hydroxylapatite (34). All three groups of Ro particles contain the 60-kD Ro protein since all are precipitated with control anti-Ro sera specific for this polypeptide. In addition, in the present work, our prototype anti-Ro^{hY5} sera immunoprecipitated the 50-kD La polypeptide from [^{35}S]methionine-labeled HeLa cells (Fig. 6, lanes *3* and *4*), although they did not contain anti-La antibodies as determined by the ^{32}P immunoprecipitation assay. This observation indicates that at least a fraction of the Ro^{hY5} particles are stably associated with La. This is in agreement with earlier reports (3, 35) and with the demonstration that the Ro RNAs have distinct binding sites for both the Ro and La polypeptides (3). Thus, many populations of Ro particles likely coexist in the cell and, at least in the case of the Ro^{hY5} particle, express unique biochemical and immunological properties; such unique properties raise the possibility that the subpopulations of Ro particles might also be functionally distinct.

Anti-Ro^{hY5} antibodies recognize a conformational epitope. Selective immunoprecipitation of the Ro^{hY5} particle indicates that this particle contains a unique epitope not expressed on the $Ro^{hY1-hY4}$ particles. This epitope likely results from a conformational determinant expressed on the 60-kD Ro polypeptide when it associates with the hY5 RNA in the Ro^{hY5} particle. This possibility is supported by our observation that the anti-Ro^{hY5} sera immunoprecipitated the 60-kD Ro polypeptide from [^{35}S]methionine-labeled HeLa extracts (where the Ro^{hY5} particle is intact; see Fig. 2), but did not bind the 60-kD kilodalton Ro polypeptide in immunoblots, where the RNA presumably has been disrupted during SDS-polyacrylamide gel electrophoresis.

An alternative explanation for the selective immunoprecipitation of the Ro^{hY5} particle is that anti-Ro^{hY5} antibodies do not target the 60-kD Ro polypeptide, but another, methionine-deficient protein that selectively associates with the hY5 RNA. The recently reported 52-kD Ro polypeptide is unlikely to be the target of such antibodies, however, since this polypeptide binds all four Ro RNAs and labels well with [^{35}S]-methionine (4). Although the anti-Ro^{hY5} sera also precipitated the 50-kD La protein, this polypeptide probably does not play a major role in the formation of the Ro^{hY5} epitope since it is contained in only a fraction of Ro particles (5). Thus, it appears that the 60-kD Ro polypeptide is the most likely target of anti-Ro^{hY5} antibodies. Studies using the recently cloned cDNA for this polypeptide (36) might definitively identify the peptide sequence(s) involved in the formation of the Ro^{hY5} epitope.

Restriction of the Ro^{hY5} epitope to human cells indicates that autoantibodies may target evolutionary recent epitopes. Anti-Ro^{hY5} sera did not immunoprecipitate any Ro RNAs from animal cell lines, suggesting that cells from lower species lack an equivalent of the Ro^{hY5} particle, or that the targeted epitope is not present on Ro particles from these lines. We suspect the first hypothesis is correct, since no homology was demonstrated between the two murine Ro RNAs, mY1 and mY2, and the hY5 RNA from HeLa cells (5). Furthermore, Ro RNAs from animal cell lines do not correspond in size to the hY5 RNA (Mamula et al., manuscript submitted for publication) (2, 6; Fig. 8). The Ro^{hY5} particle thus appears to be an evolutionary recent addition to cells. The production of autoantibodies that bind epitopes restricted to human Ro particles strongly argues that this particle per se acts as an autoimmunogen (37).

Immunization of animals with autologous (38, 39) or heterologous autoantigens induces the formation of autoantibodies that first target epitopes which are the least conserved (39, 40). The immune response then expands to recognize other portions of the antigen that are selected by properties such as surface accessibility and hydrophilicity index (40, 41). In contrast, human autoantibodies in SLE and related diseases appear to target evolutionary conserved epitopes on highly conserved autoantigens (28–32). The identification of anti-Ro^{hY5} antibodies indicates that, at least in the case of the Ro particle, an evolutionary recent epitope on a conserved autoantigen may be the target of the autoimmune response. If our observations are confirmed with other autoantibody systems, this would indicate that the production of antibodies which recognize nonconserved epitopes on conserved polypeptides represents a component of the initial immunization process in SLE and related diseases, as in immunization of animals. The continuous exposure to the autoantigen could then lead to maturation of the B cell repertoire, with higher-affinity antibodies targeting a number of accessible epitopes (39, 41, 42, 43).

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