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Autoantigens Interact with *cis*-Acting Elements of Rubella Virus RNA

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Rubella virus (RV) infections in adult women can be associated with acute and chronic arthritic symptoms. In many autoimmune individuals, antibodies are found targeting endogenous proteins, called autoantigens, contained in ribonucleoprotein complexes (RNPs). In order to understand the molecular mechanisms involved in the RV-associated pathology, we investigated the nature of cellular factors binding RV RNA and whether such RNPs were recognized by antibodies in infected individuals. Previously, we noted that cellular proteins associated with the RV 5′(+) stem-loop (SL) RNA are recognized by serum with Ro reactivity. To better understand the nature of the autoantigens binding RV *cis*-acting elements, serum samples from individuals with various autoimmune diseases were tested for their ability to immunoprecipitate RNPs containing labeled RV RNAs. A subset of serum samples recognizing autoantigen La, or Ro and La, immunoprecipitated both the RV 5′(+)SL and 3′(+)SL RNA-protein complexes. Autoantigens binding the RV 5′(+)SL and 3′(+)SL RNAs differed in molecular mass, specificities for respective RNA binding substrates, and sensitivity to alkaline phosphatase treatment. The La autoantigen was found to interact with the RV 5′(+)SL RNA as determined by immunological techniques and binding reactions with mixtures containing recombinant La protein. To test whether there is a correlation between La binding to an RV RNA element and the appearance of an anti-La response, we measured anti-La titers in RV-infected individuals. Significant anti-La activity was detected in approximately one-third of RV-infected individuals 2 years postinfection.

Rubella virus (RV) is the etiological agent of German measles, a common, benign exanthematous childhood disease characterized by a rash and mild fever (1). However, considerable clinical concern arises with regard to RV infections in women during the first trimester of pregnancy. Transmission of RV transplacentally to a fetus during early stages of development often leads to severe congenital malformations, including cataracts, deafness, and cardiac problems, collectively referred to as congenital rubella syndrome (48).

RV infection or vaccination in adult women may lead to other complications, including transient arthritis and other debilitating symptoms (6, 7, 18, 37, 45, 46). The occurrence of acute arthralgia and arthritis following RV infection or vaccination of adult women was noted in the earliest vaccine trials (8a, 9, 27, 38, 44), and several retrospective studies have reported that as many as 50% of adult female vaccinees may be affected (3, 16, 31). In contrast, studies have identified only ~7% of vaccinated adult men experiencing similar symptoms (16, 46). Among seronegative vaccinees who experience arthritic symptoms following RV exposure, up to 50% of adult females may develop persistent or recurrent arthritis (7, 13, 16). RV was recovered from synovial fluids, breast milk, and peripheral leukocytes of patients, some suffering from chronic arthritis, several years following natural infection or vaccination (6–8, 22, 45, 46).

Many vaccinees experience joint symptoms, which are also a feature of certain autoimmune disorders (16, 18). With many autoimmune connective tissue disorders, patients generate antibodies against certain self proteins termed autoantigens (42), and the association of cellular proteins with viral proteins has been shown to stimulate an autoimmune reaction (12). Interestingly, host proteins that are recognized autoantigens have been implicated in RV RNA replication (1, 24, 29). The participation of host proteins in the replication of RV RNA was first hypothesized when it was noted that actinomycin D has profound inhibitory effects on RNA replication and accumulation of the RV capsid and E1 and E2 glycoproteins (25). Subsequently, *cis*-acting sequences within the virus genome have been determined (11, 24, 29) and shown to be necessary for both in vivo translation and RNA replication of chimeric RV-reporter gene RNAs (24, 29). The active sequence elements mapped to stem-loop (SL) RNA structures present at the 5′ and 3′ termini of the genomic plus-strand RNA. We have demonstrated that host-encoded proteins specifically bind each of these terminal RV SL structures (23, 29). Proteins binding the 5′(+)SL of RV RNA were shown to be potential autoantigens because an autoimmune serum immunoprecipitated complexes containing this RV RNA sequence (29). Calreticulin, a putative autoantigen (20), has been conclusively shown to specifically bind the 3′(+)SL RNA of RV both in vitro and in RV-infected cells (1, 2, 36). The binding of calreticulin to the RV RNA is dependent on its phosphorylation status, which is in turn modulated by RV infection (1, 2). These

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host-virus interactions suggest a role for autoantigen-RNA complexes in the replication and pathogenesis of RV.

As a first step towards further understanding the association of RV with autoimmune disease and RV-associated arthritis, we have characterized the nature of host proteins interacting with the RV 5′ and 3′ terminal sequences. We have also investigated the distribution and prevalence of autoantibodies recognizing ribonucleoprotein complexes (RNPs) containing both RV 5′(+)SL and 3′(+)SL RNAs in groups of patients with a variety of autoimmune disorders, RV vaccinees, and individuals with natural RV infections. These studies provide important insight into the nature of proteins binding essential RV *cis*-acting sequences and provide a basis to design future studies to determine the role of these interactions in virus pathogenesis.

MATERIALS AND METHODS

Virus infection of cells. Vero 76 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone Laboratories, Inc.). Cells were infected with the plaque-purified M33 strain of RV (5 PFU per cell) as previously described (23).

Preparation of cell lysates. Cell lysates from both mock- and RV-infected cells were prepared as described previously (23) in a cytolysis buffer (25 mM Tris-HCl buffer [pH 7.5] containing 40 mM KCl, 1% Triton X-100, 50 μM *p*-nitrophenyl-*p*′-guanidinobenzoate, and 20 μg of leupeptin per ml).

In vitro synthesis of RNA transcripts. The synthesis and purification of oligonucleotide templates that include a 17-base T7 promoter were performed as previously described (23). Transcription reactions with T7 polymerase and [α-^{32}P]CTP were performed by using the T7 Megascript kit (Ambion, Inc.) according to the manufacturer's protocol. The reverse-complement sequences of the putative structures presented in Fig. 1C and D constitute the oligonucleotide sequences used for the synthesis of the wild-type RV 5′(+)SL and 3′(+)SL RNAs. The oligonucleotide sequence for the wild-type 5′(+)SL (complementary sequence of the T7 promoter is underlined) is 5′ACCTCATCTAGGAGTTTC TCCATGGGAATGGGAGTCCTAAGCGAGGTCCTATAGTGAGTCGTAT TA. The oligonucleotide sequence for the wild-type 3′(+)SL is 5′CTAGCGCG CTATAGCGCCGCGCAAGCCTATAGTGAGTCGTATTA.

RNA gel retardation assays. RNA gel retardation assays were conducted as previously described (23). For antibody inhibition-supershift studies, following incubation of the probe with the cytolysate for 30 min, 2 μl of antibodies was added to a reaction mixture and incubated for an additional 30 min at room temperature. RNA-protein complexes were resolved for 1.5 h in a 4% polyacrylamide gel (Protogel; National Diagnostics) buffered in TBE (0.089 M Tris-HCl [pH 8.3] containing 0.089 M boric acid and 0.002 M EDTA). Following electrophoresis, the gels were dried, and RNA-protein complexes were visualized by subsequent autoradiography.

In vitro UV-induced cross-linking and immunoprecipitation of RNA-protein complexes. UV cross-linking and subsequent immunoprecipitation of RNA-protein complexes were performed as described previously (29). All immunoprecipitates were subjected to extensive washings with NET (50 mM Tris-HCl [pH 8.0] containing 400 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.57 μM *p*-methylbenzone sulfonylfluoride, and 0.27 μM *N*-tosyl-L-phenylalanine chloromethyl ketone) buffer. Samples were boiled in Laemmli sample buffer and analyzed by separation in sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and subsequent autoradiography. A Molecular Dynamics Personal Laser Densitometer was used to quantify the autoradiographic results. Competition studies and alkaline phosphatase treatment of cytolysates were performed as described previously (23).

Serum samples. Serum samples from normal individuals and patients with a spectrum of autoimmune disorders were either obtained by us or generously provided by E. K. L. Chan (Scripps Institute, La Jolla, Calif.), J. Keene (Duke University), D. Kenan (Duke University), and R. Miller (Center for Biologics Evaluation and Research, Food and Drug Administration). Diseases represented among patients donating serum samples include Sjogren's syndrome, systemic and cutaneous lupus, rheumatoid arthritis, juvenile arthritis, and myositis. Protein concentrations were determined by the bicinchoninic acid assay. RV immunoglobulin G (IgG) was measured (in international units per milliliter) with the Enzygnost Anti-Rubella-Virus/IgG kit (Behringwerke AG, Marburg, Germany), while RV IgM was measured with the Eti-Rubek-M reverse kit (Sorin Biomedica, Duesseldorf, Germany). The autoimmune disease diagnosis was provided with serum samples by the source and in many cases was confirmed by the ANA/AMA Immunoblot (Biermann, Bad Nauheim, Germany) and ANA-Profil (ELIAS, Frieburg, Germany) tests. The anti-La activity measurements were performed by using the Anti-La enzyme-linked immunosorbent assay (ELISA) system (ELIAS).

Production of rLa protein. *Escherichia coli* BL21(DE3)(pLysS) bearing the pET-8c vector containing the human La-coding region was obtained as a kind gift of J. Keene and D. Kenan (Duke University). The production and purification of recombinant La (rLa) protein were performed according to published procedures (39). The final protein was purified to near homogeneity as judged by Coomassie blue staining of SDS-polyacrylamide gels. The identity of the protein as La was confirmed by Western blotting (immunoblotting) with 6181 serum (a kind gift of J. Keene and D. Kenan), which was initially raised against rLa protein.

Immune selection of anti-La antibodies. rLa protein was loaded at 1 μg per well, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto a nitrocellulose membrane. The location of the La protein on the membrane was confirmed by Ponceau S protein staining, and this region was excised and incubated with 200 μl of MA (Ro+/La+) serum diluted with 200 μl of TBST (20 mM Tris-HCl [pH 7.4] containing 150 mM NaCl and 0.05% Tween 20) at 4°C with rocking. Following approximately 72 h of incubation, the nitrocellulose membrane fragments were removed from the serum mixture and washed twice with 1 ml each of TBST buffer and then twice with 1 ml each of 10 mM Tris (pH 7.4) containing 500 mM NaCl. Pure antibodies were eluted from the membranes in 0.5 ml of 3.8 M $MgCl_2$ and dialyzed against phosphate-buffered saline (1.7 mM KH_2PO_4 and 5.0 mM Na_2HPO_4 containing 150 mM NaCl and 0.005 M phenylmethylsulfonyl fluoride). The presence of anti-La antibodies in the immune-selected solution, the depleted MA serum, and the whole MA serum was confirmed by Western analysis with rLa as the antigen. These results confirmed that the immune-selected solution contained approximately one-half of the anti-La activity in the nonselected whole MA serum and that the selection process had not completely depleted the aliquot of MA serum of its anti-La antibodies.

RESULTS

Immunoprecipitation of proteins binding the RV 5′(+)SL RNA correlates with Ro/La positivity of autoimmune serum. Previously, we have observed that incubation of radiolabeled RV 5′(+)SL RNA (Fig. 1C) with cytolysates derived from mock- or RV-infected Vero 76 cells followed by exposure to UV light resulted in specific labeling of 59- and 52-kDa RNPs (29) (Fig. 1A, lanes 1 and 4). Further, a serum, from a patient suffering from an autoimmune disorder, recognizing the Ro antigen was capable of immunoprecipitating both the 59- and 52-kDa RNPs containing the RV 5′(+)SL RNA (29). In order to gain a better understanding of the autoantigenic nature of proteins binding the 5′(+)SL RNA and of the distribution of autoantibodies, we screened serum samples from patients with various autoimmune diseases for their abilities to immunoprecipitate in vitro-reconstituted RNPs containing the RV 5′(+)SL RNA.

Sera with autoimmune specificities for only the U1-RNP, Sm, Scl, CENP, and Jo autoantigens and sera from rheumatoid and juvenile arthritis patients failed to immunoprecipitate the RNPs containing the RV 5′(+)SL RNA (30). In contrast, a subset of sera recognizing the Ro (Ro+) and/or La (La+) antigens (for example, MA [Ro+/La+]) immunoprecipitated 59- and 52-kDa RNPs containing the labeled 5′(+)SL RNA (Fig. 1A, lanes 2 and 5). The immunoprecipitated 52-kDa RNP migrates slightly lower on this gel (Fig. 1A, lanes 2 and 5), probably because of SDS-PAGE conditions. Migration in the correct position was normally observed (see Fig. 2, 3, and 5). Essentially identical patterns of labeled complexes were immunoprecipitated by each serum testing positive (30). A similar pattern of RNPs was also immunoprecipitated by the Ro+/La+ human serum from cytolysates prepared from either mock- or RV-infected Vero 76 cells (Fig. 1A, lanes 2 and 5). Other autoimmune serum samples, including BL (Ro+) as a representative (Fig. 1, lanes 3 and 6), and 22 normal human serum samples (30) did not immunoprecipitate the RNPs containing the 5′(+)SL RNA, even upon very extended exposures. The proportion of sera, with reactivity to a known autoantigen, capable of immunoprecipitating RNPs containing the 5′(+)SL RNA and the approximate exposure times necessary to visualize the products upon autoradiography are presented in Fig. 1B. In general, sera recognizing only the Ro antigen were severalfold less reactive with proteins associated with the

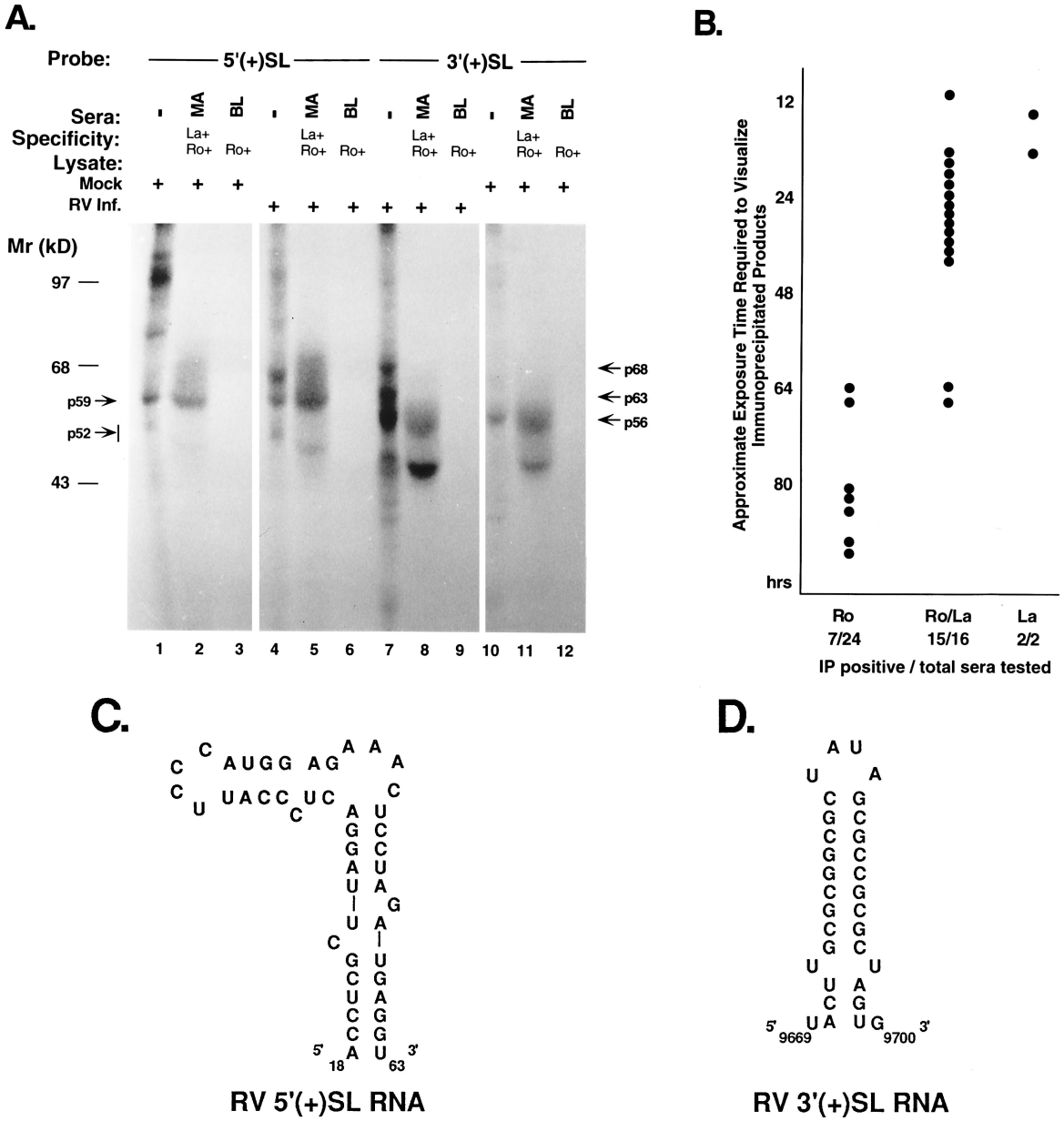

FIG. 1. Immunoprecipitation of RNPs containing radiolabeled RV 5′(+)SL and 3′(+)SL RNAs by human autoimmune sera. (A) Radiolabeled RNA (5×10^4 cpm) corresponding to the RV 5′(+)SL (lanes 1 and 4) or RV 3′(+)SL (lanes 7 and 10) was incubated with 28 μg of cytosolic lysates derived from mock-infected (lanes 1 and 10) or RV-infected (Inf.) (lanes 4 and 7) Vero 76 cells and subjected to UV cross-linking procedures. The mobilities of the 59- and 52-kDa proteins binding the 5′(+)SL RNA and of the 68-, 63-, and 56-kDa proteins binding the 3′(+)SL RNA are indicated by arrows at left and right, respectively. The mobilities of these proteins provided a point of reference for proteins identified in immunoprecipitation reactions. For immunoprecipitation of RNPs containing the RV RNAs, mock- and RV-infected cell lysates (60 μg) were incubated with radiolabeled RV 5′(+)SL (1×10^6 cpm) or 3′(+)SL (0.5×10^6 cpm) and subjected to UV cross-linking procedures followed by immunoprecipitation with human autoimmune sera with either Ro+ specificity (BL) (lanes 3, 6, 9, and 12) or Ro+/La+ specificity (MA) (lanes 2, 5, 8, and 11). Proteins bearing transferred label were resolved by SDS-PAGE and visualized by subsequent autoradiography. (B) Each autoimmune serum capable of immunoprecipitating (IP) RV 5′(+)SL RNPs (●) is plotted above the serum specificity type, with increased height indicating stronger reactivity (exposure time required to visualize immunoprecipitated products). Below each serum specificity type, the number of samples immunoprecipitating products and total number of samples tested are given. (C and D) Predicted secondary structures of the 5′(+)SL RNA (C) and 3′(+)SL RNA (D) structures of RV RNA used as probes in this study.

5′(+)SL RNA than sera recognizing both Ro and La antigens (Fig. 1B).

Immunoprecipitation of RNPs containing the RV 3′(+)SL RNA also correlates with Ro/La positivity of autoimmune serum. Incubation of the radiolabeled RV 3′(+)SL RNA (Fig. 1D) with cytolysates from mock-infected cells followed by exposure to UV light results in the labeling of an ~56-kDa RNP (Fig. 1A, lane 10), whereas four complexes of ~45, ~56, 63, and 68 kDa are labeled in UV cross-linking assays with cytosolic extracts from RV-infected cells (Fig. 1A, lane 7) (23). The ~56-kDa protein binding the RV 3′(+)SL RNA has been conclusively demonstrated to be the simian homolog of human calreticulin (36). To further characterize the nature of the proteins binding the 3′(+)SL RNA in RV-infected extracts, we

screened the panel of human autoimmune sera for their abilities to immunoprecipitate in vitro-reconstituted RNPs. As observed for the 5′(+)SL RNA, sera with autoimmune specificities for only U1-RNP, Sm, Scl, and CENP; sera from patients with diseases not inducing an anti-La or anti-Ro response; and all normal sera failed to immunoprecipitate complexes containing the 3′(+)SL RNA (30). In contrast, autoimmune sera with Ro+/La+ specificities (for example, MA), which were also capable of immunoprecipitating RV 5′(+)SL RNPs, precipitated RNPs containing the 3′(+)SL RNA (Fig. 1A, lanes 8 and 11). Essentially identical patterns of labeled complexes containing the 3′(+)SL RNA were immunoprecipitated by MA (Fig. 1A, compare lanes 8 and 11) and by all positive sera from either mock- or RV-infected cytolysates. The predominant RNP containing the 3′(+)SL RNA, formed by proteins in cytolysates from mock- or RV-infected cells and precipitated by the human sera, was ~56 kDa (Fig. 1A, lanes 8 and 11) and corresponded in size to the previously characterized RV RNA-binding protein simian calreticulin (35). A second, smaller (~45-kDa) complex observed with the immunoprecipitation of the 3′(+)SL RNA (Fig. 1A, lanes 8 and 11) did not appear consistently. Other autoimmune sera not recognizing the La antigen (for example, BL [Fig. 1A, lanes 9 and 12]) failed to immunoprecipitate RNPs containing the 3′(+)SL RNA.

Proteins immunoprecipitated in the RV 5′(+)SL and 3′(+)SL RNPs can be distinguished from one another. The observation that particular human serum samples immunoprecipitated proteins associated with RV 5′(+)SL and 3′(+)SL RNPs prompted experiments to test whether the binding proteins were the same. Comparisons of immunoprecipitated proteins show that the major complexes containing the 5′(+)SL are 59 and 52 kDa, whereas the RNPs containing the 3′(+)SL had different molecular masses (56 and 45 kDa) (Fig. 1A, compare lanes 5 and 8).

To further investigate the relationship among the proteins binding the 5′(+)SL and 3′(+)SL RNAs, we compared the specificities of cellular proteins binding to their cognate RV RNA. RNA binding reactions were performed in either the presence or absence of nonlabeled homologous or nonhomologous RNAs as competitors, and this was followed by UV cross-linking and immunoprecipitation with a human serum recognizing both RNPs. Coincubation of up to a 200-fold excess of nonlabeled 3′(+)SL RNA with the radiolabeled 5′(+)SL RNA had a minimal effect on the recovery of the 59- and 52-kDa RNPs containing the 5′(+)SL probe (Fig. 2A, compare lanes 1 and 2). Conversely, a >90% decrease in the amount of labeled protein associated with radiolabeled RNA of the same sequence and polarity was observed upon coincubation of a 100- or 200-fold excess of nonlabeled 5′(+)SL RNA (Fig. 2A, compare lane 1 with lanes 3 and 4). Similarly, addition of a 25-fold excess of nonlabeled 3′(+)SL RNA to binding reaction mixtures containing the homologous radiolabeled RNA reduced the amount of labeled 56-kDa complex immunoprecipitated by a Ro+/La+ autoimmune serum by 80% (Fig. 2A, lane 6). However, 3′(+)SL binding reaction mixtures coincubated with similar excesses of nonlabeled 5′(+)SL RNA displaced the labeled 3′(+)SL RNA probe by only 40% (Fig. 2A, lane 7).

Detailed studies have demonstrated that calreticulin of simian or human origin specifically binds the RV 3′(+)SL RNA sequence in vitro and in RV-infected cells (2, 36). The binding of cellular proteins derived from RV-infected cells (23) and of calreticulin (36) to the RV 3′(+)SL RNA is highly dependent on their phosphorylation state. Pretreatment of cytolysates derived from mock- or RV-infected cells (23) or of recombinant calreticulin (2, 36) with alkaline phosphatase eliminates binding to the RV 3′(+)SL RNA probe. We therefore compared the effects of alkaline phosphatase treatment of cytolysates on the binding of proteins to the 5′(+)SL and 3′(+)SL RNAs which were immunoprecipitated by autoimmune sera (Fig. 2B).

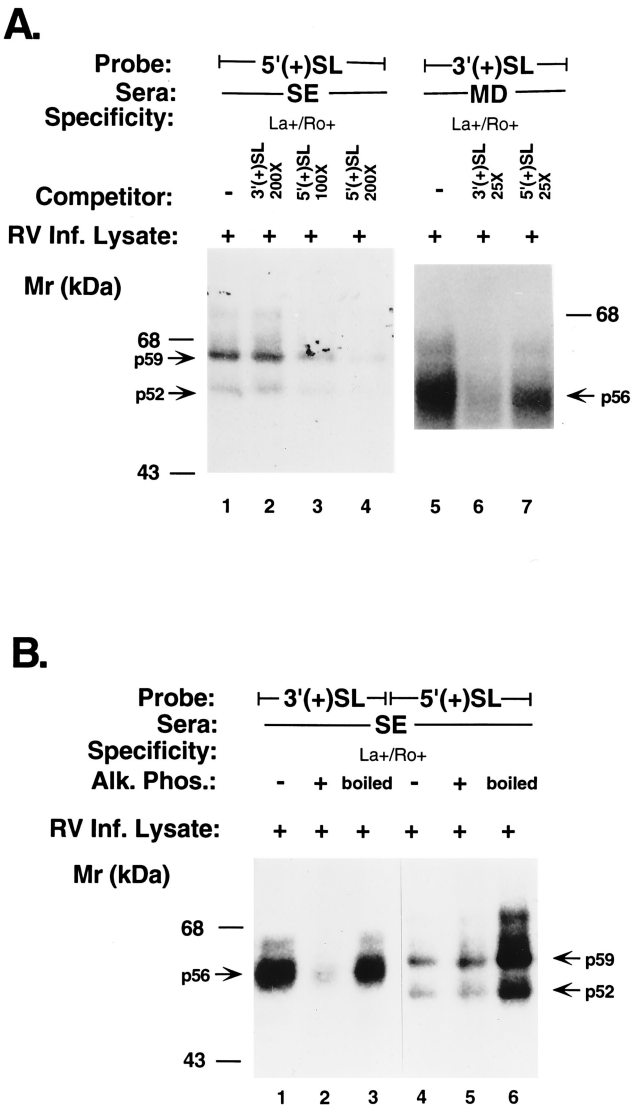

FIG. 2. Differentiation of proteins interacting with the 5′(+)SL and 3′(+)SL RNAs by binding specificity and sensitivity to alkaline phosphatase treatment. (A) RNA corresponding to the RV 5′(+)SL was incubated with cytosolic lysates derived from RV-infected (Inf.) cells without (lanes 1 to 4) or with the addition of nonlabeled 3′(+)SL RNA (lane 2; 200-fold molar excess) or 5′(+)SL RNA (lanes 3 and 4; 100- and 200-fold molar excesses, respectively). Reaction mixtures were subjected to UV cross-linking procedures, subjected to immunoprecipitations with a human autoimmune serum (SE) with Ro+/La+ specificity, and resolved by SDS-PAGE. Similar reaction mixtures containing the 3′(+)SL RNA alone (lane 5) or with the addition of a 25-fold molar excess of either nonlabeled 3′(+)SL RNA (lane 6) or 5′(+)SL RNA (lane 7) were subjected to immunoprecipitation with a human autoimmune serum (MD) with Ro+/La+ specificity and analyzed similarly. (B) Cytolysates derived from RV-infected cells were incubated with alkaline phosphatase (Alk. Phos.) (20 U) for 30 min at 37°C (lanes 2 and 5), with boiled inactivated alkaline phosphatase (lanes 3 and 6), or with no enzyme (lanes 1 and 4). Radiolabeled RNA probes corresponding to the 3′(+)SL (lanes 1 to 3) or 5′(+)SL (lanes 4 to 6) RNA were added to each lysate-enzyme mixture, and resulting RNPs were immunoprecipitated with a human autoimmune serum (SE) with Ro+/La+ specificity. RNPs were analyzed as described in the legend to Fig. 1. The mobilities of the 59- and 52-kDa proteins associated with the 5′(+)SL RNA and of the 56-kDa protein binding the 3′(+)SL RNA are indicated by arrows.

Pretreatment of cytolysates with alkaline phosphatase reduced recovery of RV 3′(+)SL RNPs by >90% when a Ro+/La+ autoimmune serum was used (Fig. 2B, compare lanes 1 and 2). In contrast, treatment of cytolysates with the previously boiled alkaline phosphatase did not alter the recovery of RV 3′(+)SL RNPs (Fig. 2B, lane 3). Alkaline phosphatase treatment of cytolysates prior to the formation of the RV 5′(+)SL RNP had a minimal effect on the immunoprecipitation of labeled protein from the UV cross-linking reaction (Fig. 2B, lanes 4 and 5). The enhanced recovery of proteins binding 5′(+)SL RNA when the cytolysates were incubated with boiled alkaline phosphatase (Fig. 2B, lane 6) was not a consistent observation. Nevertheless, these data show that the proteins associated with the 5′(+)SL and 3′(+)SL RNAs and recognized by human autoimmune sera can be distinguished from one another on the basis of the sizes of the RNPs they form, specificities of binding to their respective RNA substrates, and sensitivities to phosphatase treatment.

La sera immunoprecipitate proteins bound to the 5′(+)SL RNA but not the 3′(+)SL RNA. We have previously shown (29) that antibodies raised against recombinant 60-kDa Ro protein did not precipitate RNPs containing the 5′(+)SL RNA. Additionally, this serum did not react with proteins bound to the 3′(+)SL RNA (30). The results in Fig. 1B suggest that there is a strong correlation between the ability of an autoimmune serum to recognize the La antigen and its ability to immunoprecipitate proteins bound to RV RNA sequences. This raised the possibility that La, or antigens associated with La, comprised the major binding factors. Therefore, we obtained a human serum which contained autoimmune specificity restricted to the La antigen (Gore) and a rabbit serum raised against recombinant La protein (serum 6181) (kind gifts of J. Keene and D. Kenan, Duke University) (4, 5). These sera were used to immunoprecipitate reconstituted RNPs containing either the radiolabeled 5′(+)SL or 3′(+)SL RNAs and associated RNA-binding proteins from cytolysates of RV-infected Vero 76 cells. An autoimmune serum, TH, with Ro+/La+ specificity precipitated the characteristic RNPs associated with either RV RNA (Fig. 3, lanes 2 and 6). In contrast, immunoprecipitations with the Gore and 6181 sera precipitated complexes containing only the 5′(+)SL and not the 3′(+)SL RNA (Fig. 3, compare lanes 7 and 8 with lanes 3 and 4). In particular, only the 59-kDa RNP containing the 5′(+)SL RNA was precipitated by either La-specific serum (Fig. 3, compare lane 6 with lanes 7 and 8). These results strongly suggest that the protein constituent of the 59-kDa RNP, which specifically binds the 5′(+)SL RNA, is the La antigen or an antigenically related protein. The identity of the protein within the 52-kDa RNP which also specifically binds the 5′(+)SL RNA in crude Vero 76 cytolysates (Fig. 3, lane 5) and is immunoprecipitated by autoimmune serum samples (Fig. 3, lane 6) remains unclear.

To provide further evidence that the 59-kDa protein binding the 5′(+)SL RNA was the La antigen, we tested various human sera for their abilities to affect the formation of RNPs in RNA mobility shift assays. Two specific complexes (a and b) were formed upon incubation of the 5′(+)SL RNA with cytolysates derived from RV-infected Vero 76 cells (Fig. 4A, lanes 2 and 6). More slowly migrating complexes associated with the 5′(+)SL RNA were shown to be nonspecific by previously published competition and mutant RNA comparison studies (30). Coincubation of the cytolysates with radiolabeled 5′(+)SL RNA and human serum with Ro+/La+ specificity (MA) (Fig. 4A, lane 4) or only La+ specificity (Gore) (Fig. 4A, lane 7) resulted in selective inhibition of the formation of the two specific complexes (a and b) without affecting the nonspecific RNPs. In contrast, addition of serum with only Ro+ specificity (BL) to binding reactions did not specifically block the formation of any RNPs (Fig. 4A, lane 3).

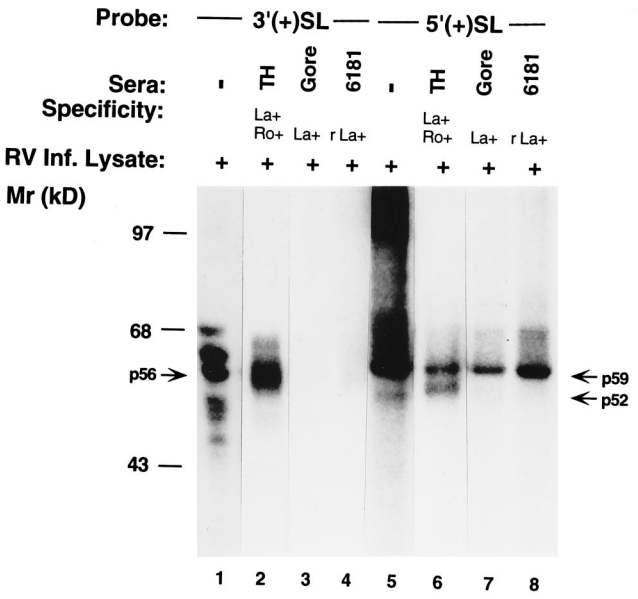

FIG. 3. The 59-kDa protein specifically binding the RV 5′(+)SL RNA is immunoprecipitated by anti-La sera. Cytolysates from RV-infected (Inf.) cells were incubated with radiolabeled RV 5′(+)SL or 3′(+)SL RNA and subjected to UV cross-linking and immunoprecipitation procedures followed by SDS-PAGE analysis. The human autoimmune sera used in immunoprecipitation reactions included TH (Ro+/La+ specificity) (lanes 2 and 6), Gore (La+ specificity, diluted 1/100) (lanes 3 and 7), and 6181 (rabbit serum raised against recombinant La) (lanes 4 and 8). UV cross-linking reaction mixtures, without immunoprecipitation, contained cytolysates from RV-infected cells and either the 3′(+)SL (lane 1) or 5′(+)SL (lane 5) RNA. The mobilities of the 59- and 52-kDa proteins associated with the 5′(+)SL RNA and of the 56-kDa protein binding the 3′(+)SL RNA are indicated by arrows.

To test the specificity of the interaction of the La antigen with the 5′(+)SL RNA, gel mobility shift assays were also conducted with the 3′(+)SL RNA coincubated with human autoimmune serum (Fig. 4B). Without the addition of serum, we observed two RNPs with the 3′(+)SL RNA, designated I and II (Fig. 4B, lane 2). Addition of sera with specificities for both Ro+/La+ (MA), La+ alone (Gore), or Ro+ alone (BL) did not eliminate the formation of the two specific complexes with shifted mobilities (I and II) or induce the formation of novel, supershifted RNPs (Fig. 4B, lanes 3 to 5). However, the addition of a human serum to RNA mobility shift assays resulted in a general reduction of the intensities of RNPs in these experiments. The data shown in Fig. 4 strongly suggest that La+ sera specifically inhibit the binding of cellular proteins to the 5′(+)SL RNA.

Recombinant La binds the RV 5′(+)SL RNA. In order to confirm that the La antigen was indeed binding the 5′(+)SL RNA, we compared the 5′(+)SL RNA binding activities and immunoprecipitation patterns of proteins present in Vero cytolysates with those of recombinant La protein by using Ro+/La+ human sera and immune-selected anti-La antibodies. rLa was expressed in *E. coli* and purified (39) to near homogeneity, as judged by Coomassie blue staining of SDS-PAGE gels (30). Anti-La antibodies were selected from MA serum (Ro+/La+) (Fig. 1A) as indicated in Materials and Methods. Binding reactions were performed by mixing of radiolabeled 5′(+)SL RNA with either Vero cytolysates (Fig. 5, lanes 1 and 2) or rLa protein (Fig. 5, lanes 3 to 5) followed by exposure to UV light and immunoprecipitation of RNPs by either whole MA serum

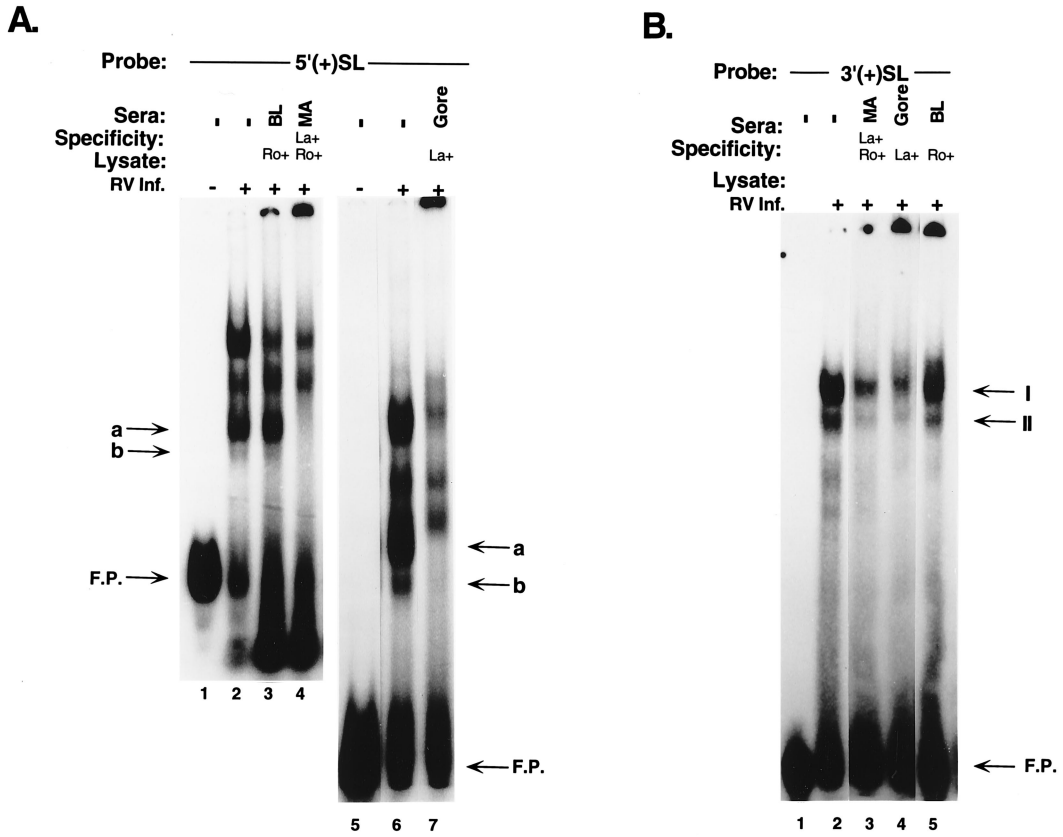

FIG. 4. Autoimmune serum that is reactive to the La antigen will specifically inhibit the formation of RNPs containing the 5′(+)SL RNA and cellular proteins. (A) A radiolabeled 5′(+)SL RNA probe was incubated with cytolysates from RV-infected (Inf.) cells (lanes 2 to 4, 6, and 7) for 30 min, and then 2 μl of human autoimmune serum with Ro+ (BL) (lane 3), Ro+/La+ (MA) (lane 4), or La+ (Gore) (lane 7) specificity was added. Lanes 2 and 6, RNA mobility shift assays without the addition of serum. RNPs were separated in a nondenaturing gel and visualized by autoradiography. RNPs specific for the 5′(+)SL RNA are indicated by a and b. (B) Radiolabeled 3′(+)SL RNA was incubated with cytolysates from RV-infected cells for 30 min, and then 2 μl of human autoimmune serum with Ro+/La+ (MA) (lane 3), La+ (Gore) (lane 4), or Ro+ (BL) (lane 5) specificity was added. Lane 2, RNA mobility shift assay without the addition of antibody. Specific complexes containing the 3′(+)SL RNA are indicated by I and II. F.P., migration of the free RNA probe.

(Fig. 5, lanes 1 and 4) or immune-selected anti-La antibodies (Fig. 5, lanes 2 and 5). Both 59- and 52-kDa RNPs containing the 5′(+)SL RNA were immunoprecipitated from Vero cytolysates with either the MA serum or selected anti-La antibodies (Fig. 5, lanes 1 and 2). The incubation of the 5′(+)SL RNA and the rLa protein, followed by UV exposure, resulted in the labeling of a 59-kDa RNP (Fig. 5, lane 3). A complex with a similar molecular mass was immunoprecipitated from reaction mixtures containing rLa by whole MA serum or immune-selected anti-La antibodies (Fig. 5, lanes 4 and 5). The correspondence in size of the RNP identified in Vero cytolysates with that formed between rLa protein and the 5′(+)SL RNA, as well as the ability of immune-selected anti-La antibodies to immunoprecipitate both RNPs, confirms that the RNA-binding protein in the 59-kDa RNP is the La autoantigen. In similar experiments, rLa was not immunoprecipitated in association with the 3′(+)SL RNA (30).

Appearance of anti-La antibodies in patient sera following RV infection. As previously indicated, RV infection or vaccination can be associated with symptoms resembling autoimmune disease (16, 18). Since a known autoantigen, La, binds an important *cis*-regulatory element of RV RNA, we were interested in determining whether autoantibodies against La could be detected in individuals following RV vaccination or infection. We monitored four separate groups, two groups of RV vaccinees and two groups with primary RV infections, for the presence of anti-RV titers and autoantibodies specific for the La antigen. Eight vaccinees who were initially seronegative for anti-RV antibodies and seven vaccinees whose low RV titers were boosted by vaccination were monitored for anti-La activity, as measured by ELISA, for 26 weeks following exposure to RV. No anti-La activity was measured at any of the time points in these individuals; however, these individuals continue to be monitored. Similarly, 40 individuals with primary RV infections were monitored at the onset of the rash and 2 weeks postdiagnosis for anti-La activity. In each of these samples, no anti-La activity was detected 2 weeks after RV infection.

In contrast, a group of 16 individuals, with a similar age profile, contracting primary RV infections were monitored from the onset of the rash through 2 years postinfection. All individuals in this group were negative for anti-La activity at the onset of the rash; however, significantly elevated anti-La titers (between 5 and 15 ELISA units) were measured in 5 of the 16 individuals 2 years postinfection. The presence of anti-La antibodies in these sera was confirmed by subsequent Western blot analysis (30). All individuals in this group exhibited comparable anti-RV IgM and IgG levels, and no elevated titers for human immunodeficiency virus type 1, poliovirus, or Epstein-Barr virus were observed in comparisons of samples taken at the onset of the rash through 2 years postinfection (30). The modest levels of anti-La titers observed in these RV-infected individuals are significant because anti-La activity

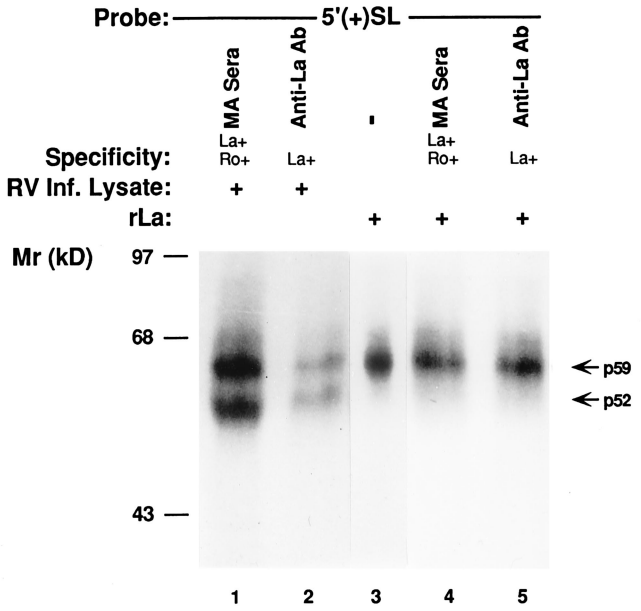

FIG. 5. The 59-kDa protein specifically binding the RV 5′(+)SL RNA is the La antigen. Cytolysates from RV-infected (Inf.) cells (lanes 1 and 2) or rLa protein (50 ng) (lanes 4 and 5) were incubated with radiolabeled RV 5(+)SL RNA and subjected to UV cross-linking and immunoprecipitation procedures followed by SDS-PAGE analysis. Human autoimmune serum MA (Ro+/La+ specificity) (lanes 1 and 4) or immune-selected anti-La antibodies (Anti-La Ab) (lanes 2 and 5) were used in immunoprecipitation reactions. Lane 3, UV cross-linking reaction, without immunoprecipitation, containing rLa protein and the 5′(+)SL RNA. The mobilities of the 59- and 52-kDa proteins associated with the 5′(+)SL RNA are indicated by arrows at the right, while mobilities of molecular mass markers are indicated at the left. Lane 3 represents an approximately threefold shorter exposure of the SDS-PAGE gel than used for the other lanes.

was absent in over 300 control samples (the majority seropositive for RV) from individuals with similar geographic origin, age, and gender profiles assayed by this anti-La ELISA test (30).

DISCUSSION

RV infection in women following natural infection or immunization can result in chronic arthritis and other symptoms resembling autoimmune disease. In autoimmune diseases, endogenous components, identified as autoantigens, are targeted by the immune system (42, 43). The contribution of autoantibodies to RV-associated disease is presently unclear. Previous studies suggested that cellular proteins specifically binding RV RNA sequences, having *cis*-acting functions in viral replication, were autoantigens (29, 36). In this study, we confirmed this observation and have defined the reactivity within human sera responsible for the recognition of RNPs containing both RV 5′(+)SL and 3′(+)SL RNAs. Recognition of these RNPs was dependent on serum reactivity to the Ro and La autoantigens (Fig. 1B). A human serum (Gore) known to contain a high antibody titer to the La antigen, rabbit antiserum raised against rLa, and immune-selected anti-La antibodies specifically precipitated the 59-kDa complex in association with the 5′(+)SL RNA. Indeed, rLa binds the 5′(+)SL and is immunoprecipitated as a 59-kDa RNP by La+ human sera and immune-selected anti-La antibodies. These results coupled with the ability of anti-La sera to selectively inhibit the formation of 5′(+)SL-containing RNPs (Fig. 4) conclusively demonstrate that the 59-kDa RNP contains the La antigen.

It is interesting that the rLa protein, which migrates as an ~47-kDa monomer upon SDS-PAGE analysis (data not shown), behaves like a 59-kDa RNP when associated with the 5′(+)SL RNA (Fig. 5). This RNP is identical in size to the RNP formed by Vero cytosolic proteins and the 5′(+)SL RNA (Fig. 5). The increased molecular mass associated with the La-5′(+)SL RNP most probably results from residual RNA remaining in the RNP, which is protected from degradation.

The identity of the protein constituent of the 52-kDa RNP containing the 5′(+)SL RNA has not been conclusively demonstrated. Autoantibodies in various human autoimmune sera precipitate this RNP, but antibodies raised against recombinant 59- or 52-kDa Ro proteins (29) or La proteins (Fig. 3) do not. However, immune-selected anti-La antibodies do precipitate the 52-kDa RNP from Vero cytolysates (Fig. 5), and extended exposures of immunoprecipitations with the Gore serum reveal a complex of this size but in low abundance (30). The intensity of this immunoprecipitated complex also varied depending on the time of cytolysate preparation (compare Fig. 1 and 5), and it appears to be least abundant in freshly prepared lysates (Fig. 1 and 3). These observations suggest that the protein contained within the 52-kDa RNP is at least antigenically related to La or possibly in physical association with the La-RNPs. Further, this complex may represent a lower-molecular-mass La-RNP (compared with the well-characterized 59-kDa RNP) resulting from limited degradation of its protein or RNA constituents.

Not all sera capable of precipitating RV 5′(+)SL RNA-associated binding proteins were found to be La positive by immunodiffusion assays. However, these Ro+ sera, including the previously reported Ge serum (29), were inefficient at immunoprecipitating the RNPs compared with sera also testing positive for La. The ability of apparent Ro+ sera to immunoprecipitate the La protein associated with the 5′(+)SL RNA may speak to the sensitivity of our assay system, allowing the detection of anti-La antibodies present at an extremely low abundance in a given serum.

The La antigen is a known RNA-binding protein (17) which has been shown to interact with nascent Pol III transcripts (32, 40) and numerous viral RNAs, including Epstein-Barr virus-encoded RNAs (34), the VA RNAs of adenovirus (14), the leader sequences of vesicular stomatitis virus (47), poliovirus (21), and the human immunodeficiency virus type 1 TAR element (5). Several putative functions have been ascribed to the La antigen, ranging from transcriptional termination (15, 19) to translation-enhancing activities (5, 21, 41). Although La is known to interact with a variety of RNA substrates, previous studies (29) and those whose results are presented in Fig. 2A demonstrate that the La protein binds the RV 5′(+)SL RNA with high specificity, as shown in RNA binding assays conducted with specific and nonspecific RNA SL structures, including the 3′(+)SL RNA, as unlabeled competitors. The physiologic relevance of both the binding of the La antigen to the 5′(+)SL RNA and its effect on the translation and replication of RV RNA are presently under investigation.

We found that autoimmune sera reacting with 5′(+)SL RNA-binding proteins also recognized the 3′(+)SL RNA-binding proteins. However, the RNPs containing the RV 3′(+)SL RNA are distinct, as judged by molecular mass, the divergent specificities of the cellular proteins for each RV SL RNA, and the sensitivity of the RNA binding activities to phosphatase treatment. The characteristics of 3′(+)SL RNA-binding proteins were consistent with those of cellular and recombinant calreticulin (1, 2, 36). However, the mechanism by which the calreticulin-3′(+)SL RNP is immunoprecipitated by autoimmune sera is unclear. Using antibodies raised against recombinant calreticulin or peptide constituents, we have not

been able to immunoprecipitate the 3′(+)SL RNA in association with recombinant human calreticulin or calreticulin present in cytolysates with the same efficiency as with the human autoimmune sera (30). This inconsistency is in line with previous observations detailing limitations associated with antibodies raised against calreticulin (26, 33, 36). However, systemic lupus erythematosus sera have been shown to recognize recombinant calreticulin in Western blot assays (33), although the sera used in this study did not show similar reactivity (30). These observations imply that intracellular calreticulin has peculiar immunogenic properties, especially when complexed with RNA. Further, the binding of RNA by phosphorylated calreticulin may reveal cryptic epitopes specifically recognized by antibodies in human sera (10). It is known that autoantibodies often recognize conformational epitopes composed of discontinuous amino acids or resulting from multimeric complexes (43), accounting for the lack of reactivity with denatured protein upon Western blotting.

The difficulty in confirming the presence of antibodies specific for calreticulin in these human autoimmune sera could also be explained by the existence of a multimeric complex containing RV 3′(+)SL RNA bound by calreticulin, which is in turn associated with another protein. It is interesting that calreticulin has recently been identified in Ro RNPs and can physically associate with the 52-kDa Ro protein (8a). Autoantibodies in sera reactive with the associated protein could immunoprecipitate this complex. Since the 3′(+)SL is cross-linked only to calreticulin, calreticulin would be the only protein visualized upon SDS-PAGE analysis and autoradiography. An assessment of this type of association and of the ability of autoimmune sera to immunoprecipitate multiprotein complexes will require rigorous investigation, which is currently under way in our laboratories.

Many studies have indicated that a relationship between viral infection and certain autoimmune disorders may exist (18, 26, 28, 42, 43). Recently, several investigators have provided molecular evidence suggesting that complexes of viral proteins and cellular proteins can challenge self-tolerance. Such an association is seen in apoptotic cells infected with Sindbis virus, which produce cell surface blebs containing the Ro autoantigen colocalizing with viral glycoproteins (35). In addition, inoculation of animals with p53 complexed with the simian virus 40 T antigen can lead to an autoimmune response driven by p53 alone (12). In this context, the identification of cellular proteins binding RV RNA regulatory sequences as autoantigens raises the possibility that RV infection may promote the development of autoantibodies and may contribute to further disease progression.

The appearance of anti-La antibodies in patients after RV infection or vaccination could imply a connection between these two events. Sera from all patients in this study with natural infection assayed at the onset of the RV-induced rash and from all individuals at the time of vaccination showed no reactivity to La. Further, no reactivity was observed in these individuals up to 26 weeks later. However, at 2 years postinfection, 5 of 16 RV-infected individuals had significant levels of anti-La activity. This observed frequency of La seropositivity is not expected in the general population, as evidenced from a sample of 300 normal individuals of similar age and geographic origin who were negative for this activity. Even though both normal and RV-infected individuals showed RV seropositivity, the appearance of anti-La antibody in RV-infected individuals may be transient and dependent on the time postinfection. Further, the 16 RV-infected individuals showed no serological evidence of other infection, such as with human immunodeficiency virus type 1, poliovirus, or Epstein-Barr virus, agents known to be associated with the La protein, which excludes these agents as the source of the observed antibody response.

Since it is not known at present whether anti-La activity contributes directly to arthritic conditions, further studies will be required to establish that the observed association between RV and La contributes to postrubella arthritis.

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