Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8717–8721, November 1989
Biochemistry

An immunological determinant of RNase P protein is conserved between *Escherichia coli* and humans

(epitope/ribonucleoprotein/tRNA processing)

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Communicated by Edward A. Adelberg, August 28, 1989 (received for review July 18, 1989)

ABSTRACT **RNase P, an enzyme with RNA and protein subunits, cleaves tRNA precursor molecules to form the 5′ termini of mature tRNAs in both prokaryotes and eukaryotes. Rabbit antibodies made against the protein subunit, C5 protein, of *Escherichia coli* RNase P bound RNase P protein from *E. coli* and *Bacillus subtilis* in immunoblots and solid-phase immunoassays. These rabbit anti-C5 antibodies also bound a protein ($M_r \approx 40{,}000$) in preparations of RNase P from human (HeLa) cells and depleted the enzymatic activity from preparations of RNase P from both human and *E. coli* cells. Finally, rabbit anti-C5 antibodies immunoprecipitated from crude extracts of human cells a ribonucleoprotein complex containing H1 RNA, the putative RNA component of human RNase P. These results show that an antigenic determinant is shared by C5 protein from *E. coli* RNase P and a protein component of RNase P from human cells.**

RNase P, a ribonucleoprotein, is an endoribonuclease that has been found in the extracts of many organisms (1). This enzyme carries out the biosynthesis of the 5′ termini of mature tRNA in both prokaryotes and eukaryotes. Under appropriate conditions, *in vitro*, the catalytic activity of the enzyme from several eubacterial sources resides in the RNA subunit alone (2, 3). The RNA subunit from eukaryotes has not yet been shown to have catalytic activity by itself *in vitro*. The protein subunit of eubacterial RNase P has a considerable stimulatory effect on the k_{cat} of the reaction and is also capable of altering the rate of the reaction with specific tRNA precursor molecules (2).

RNase P from any one species can cleave tRNA precursors from any other species (1). Furthermore, active hybrid enzymes can be reconstituted from subunits isolated from very distantly related species (2, 4). Thus, even though the sequences of the genes coding for the RNA and protein subunits of RNase P have drifted rapidly (4–6), important functional features of the enzyme must have been conserved during evolution and may be reflected in structural features of the ribonucleoprotein complexes. The conservation of structural features may also extend to the nature of the surface epitopes of the protein subunit(s) of the enzyme from different sources if their surface features have an important biological function. Accordingly, we have investigated the antigenic properties of the protein subunit of RNase P from various sources and, indeed, we have found that an immunologic determinant is shared between RNase P proteins from *Escherichia coli* and human cells.

MATERIALS AND METHODS

Preparation of RNase P Enzymes. RNase P from HeLa cells and C5 protein from *E. coli* were prepared as described (7, 8). *Bacillus subtilis* RNase P protein was a gift of N. Pace (Indiana University, Bloomington, IN). M1 RNA was generated by transcription *in vitro* using T7 RNA polymerase (8). *E. coli* RNase P holoenzyme was prepared by mixing pure C5 protein with M1 RNA transcribed *in vitro* in PA buffer (50 mM Tris/100 mM NH_4Cl/10 mM $MgCl_2$, pH 7.5) and incubating the mixture at 37°C for 5 min.

Immunization of a Rabbit with Recombinant C5 Protein. Antibodies to pure C5 protein were raised in one New Zealand White rabbit. The rabbit was immunized at three subcutaneous sites and one intramuscular site with a total of 100 μg of C5 protein in 500 μl of complete Freund's adjuvant. At 3 weeks and 5 weeks after the initial immunization, the rabbit was given subcutaneous booster injections of 100 μg of C5 protein in incomplete Freund's adjuvant. The rabbit was bled 10 days after the last boost and at weekly intervals thereafter. The IgG fraction of the rabbit serum was purified by passage through DE-52 anion-exchange resin (Whatman) with 10 mM sodium phosphate buffer (pH 7.4).

Western Immunoblotting of RNase P Protein. Western immunoblots were performed by a procedure modified from that of Towbin *et al.* (9). Briefly, ≈1 μg of *E. coli* or *B. subtilis* RNase P protein or 10 μg of partially purified RNase P from HeLa cell extracts (see ref. 7) were electrophoresed in SDS/10% polyacrylamide minigels (Bio-Rad) and transferred to nitrocellulose. The nitrocellulose was blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 10 mM sodium phosphate/150 mM NaCl, pH 7.4), incubated with rabbit anti-C5 serum or human anti-RNase P serum (10^{-2} dilution in PBS), and visualized by incubation with ^{125}I-labeled protein A (Amersham) and autoradiography (10).

Detection of Anti-RNase P by Enzyme-Linked Immunosorbent Assay (ELISA). ELISAs were performed with modifications of an established procedure (11). Purified *E. coli* C5 protein (10 μg/ml) or HeLa cell RNase P (30 μg/ml; see ref. 7) was adsorbed to the solid phase of microtiter plates. Plates were blocked with 1% BSA in PBS followed by the addition of rabbit anti-C5 serum or preimmune serum (10^{-3} dilution in PBS). Binding was detected with anti-rabbit IgG-alkaline phosphatase (Sigma) and *p*-nitrophenyl phosphate as substrate. In some assays, serum dilutions were preincubated for 4 hr at 25°C with purified C5 protein (20 μg/ml) or HeLa RNase P (60 μg/ml) prior to serum application to antigen-coated plates. Percent binding inhibition was calculated as [1 − (inhibited OD_{405}/untreated OD_{405})] × 100.

Immunoprecipitation of RNase P from Human HeLa Cell Extract. Ribonucleoproteins were immunoprecipitated from extracts of HeLa cells by a procedure modified from Forman *et al.* (12). HeLa cells were sonicated in NET-2 buffer (50 mM Tris/150 mM NaCl/0.05% Nonidet P-40, pH 7.4) and centrifuged at 10,000 × *g* for 30 min to remove cell debris. Preimmune rabbit serum (80 μl), anti-C5 rabbit serum (80 μl),

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Abbreviation: BSA, bovine serum albumin.

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or human anti-RNase P serum (10 μl) were incubated with protein A-Sepharose 4B and then mixed with HeLa cell extract. After 2 hr incubation at 4°C, the samples were washed five times with NET-2 and extracted with phenol/chloroform/isoamyl alcohol (50:50:1, vol/vol). The samples were then precipitated with cold ethanol, electrophoresed in 7 M urea/5% polyacrylamide gels, and stained with silver (12).

Nucleotide Sequence Analysis of RNAs Immunoprecipitated from HeLa Cells. RNA from HeLa cell extract was immunoprecipitated as described above with rabbit anti-C5 serum or with serum from a patient with connective-tissue disease previously determined to possess antibodies to human RNase P (10). The RNAs were phenol-extracted, ethanol-precipitated, and sequenced as described by Tabler *et al.* (13). A deoxynucleotide primer complementary to the middle of the H1 RNA sequence was radiolabeled by using [γ-^{32}P]ATP and polynucleotide kinase (Promega) as described (14). Aliquots of the primer (1 pmol/μl) were annealed to the immunoprecipitated RNAs in hybridization buffer [100 mM Tris (pH 8.3)]. Extension reactions were carried out as described (12) using reverse transcriptase (Amersham) in the presence of 2′-deoxy- and 2′,3′-dideoxynucleoside triphosphates (dNTPs and ddNTPs). After phenol extraction and ethanol precipitation the reaction products were analyzed in a 7 M urea/8% polyacrylamide gel.

Depletion of RNase P Activity by Immunoprecipitation with Anti-RNase P Antibodies. Preimmune rabbit serum and anti-C5 serum were incubated with protein A-Sepharose 4B in NET-2 buffer. After 2 hr, the beads were washed three times with NET-2 and once with either PA buffer, if *E. coli* RNase P was to be immunoprecipitated, or RM buffer (30 mM Tris/100 mM NH_4Cl/5 mM $MgCl_2$/0.1 mM EDTA/0.1 mM 2-mercaptoethanol, pH 8.0), if HeLa RNase P was to be immunoprecipitated. Identical amounts of either *E. coli* or HeLa cell RNase P were then incubated with antibody–protein A-Sepharose samples for 2 hr at 4°C with gentle mixing. The samples were centrifuged and the supernatants were assayed for RNase P catalytic activity.

Assays for RNase P Catalytic Activity. Assays of *E. coli* RNase P holoenzyme were carried out as described (15), using PA buffer and ^{32}P-labeled precursor to *E. coli* tRNA^{Tyr} (pre-tRNA^{Tyr}) transcribed *in vitro* as substrate. Assays of HeLa cell RNase P activity were performed with RM buffer and ^{32}P-labeled precursor to yeast tRNA^{Ser} (pre-tRNA^{Ser}) transcribed *in vitro* as substrate. The original clone was a gift of D. Soll (Yale University, New Haven, CT). Cleavage assays were performed in the linear range of kinetics. Products of tRNA processing were subjected to electrophoresis in denaturing polyacrylamide gels. The products of the reaction were analyzed by densitometry and the percent depletion of RNase P activity (as described above) was quantified. Percent depletion of RNase P enzymatic activity was calculated as [1 − (% cleavage of rabbit anti-C5-adsorbed samples per minute/% cleavage of preimmune rabbit serum-adsorbed samples per minute)] × 100.

RESULTS

Immunoblot Analysis of Rabbit Anti-C5 Antibodies. Serum from a rabbit immunized with *E. coli* C5 protein was analyzed in immunoblots for its ability to bind to various RNase P proteins including C5 protein, *B. subtilis* P protein, and a partially purified RNase P preparation from HeLa cells (Fig. 1, lanes 2–4). The rabbit serum bound the *E. coli* C5 protein, ($M_r \approx 13{,}800$; lane 5) and the *B. subtilis* P protein (lane 6). Although the exact identity of the protein component(s) of the human RNase P ribonucleoprotein are presently unknown, the rabbit anti-C5 antibodies bound a single protein ($M_r \approx 40{,}000$) from partially purified HeLa RNase P preparations (lane 7). Preimmune rabbit serum failed to bind any RNase P proteins (lanes 8–10).

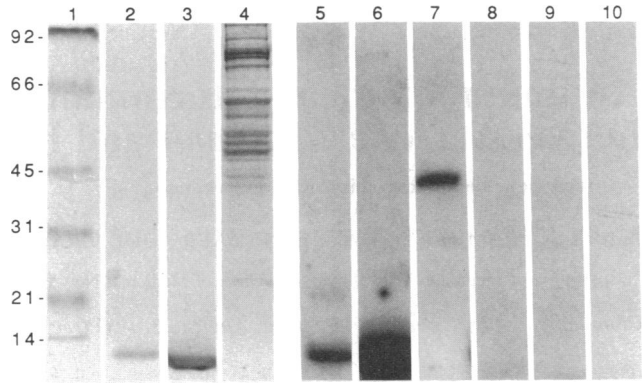

FIG. 1. Western immunoblots of rabbit anti-C5 antibodies to RNase P. *E. coli* C5 protein, *B. subtilis* RNase P, and HeLa RNase P were electrophoresed in SDS/10% polyacrylamide gels and transferred to nitrocellulose. Preimmune or immune rabbit serum at a dilution of 10^{-2} was incubated with RNase P proteins and binding was detected with ^{125}I-labeled protein A. Lanes 1–4: nitrocellulose stained with amido black. Lane 1, molecular weight standards ($M_r \times 10^{-3}$ at left); lane 2, *E. coli* C5 protein; lane 3, *B. subtilis* RNase P protein; lane 4, HeLa cell extract enriched in RNase P catalytic activity lanes 5–10: nitrocellulose membranes probed with rabbit anti-C5 serum (lanes 5–7) or preimmune rabbit serum (lanes 8–10). Lanes 5 and 8, *E. coli* C5 protein; lanes 6 and 9, *B. subtilis* RNase P; lanes 7 and 10, HeLa RNase P.

ELISAs. The binding specificity of rabbit anti-C5 antibodies was investigated in solid-phase immunoassays using C5 protein and HeLa RNase P protein antigens (Fig. 2). With *E. coli* C5 protein adsorbed to microtiter plates, binding of rabbit anti-C5 antibodies was detectable at a serum dilution of 10^{-5} relative to preimmune serum controls (a positive signal is defined as the OD_{405} for a given dilution of rabbit anti-C5 that is >2 standard deviations above the OD_{405} for the same dilution of preimmune serum; see *Materials and Methods*). When rabbit anti-C5 serum at a dilution of 10^{-3} was first preincubated with C5 protein or HeLa RNase P, binding to C5 protein was inhibited 72% and 56%, respectively (Fig. 2*A*). Binding to HeLa RNase P antigen was detected by rabbit antiserum at a dilution of 10^{-4} and was inhibited 47% by preincubation with C5 protein and 60% with HeLa RNase P protein (Fig. 2*B*).

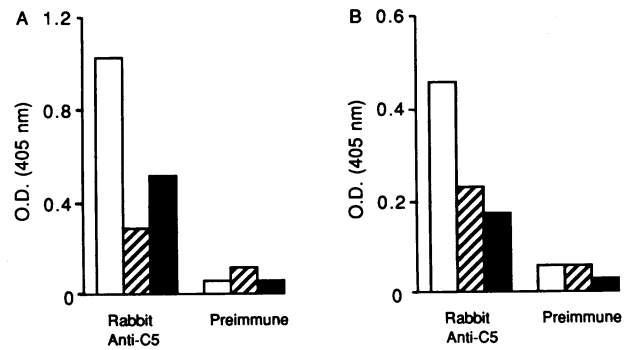

FIG. 2. Solid-phase immunoassay of rabbit anti-C5 antiserum binding to C5 and HeLa RNase P proteins. *E. coli* C5 antigen (*A*) or partially purified HeLa cell RNase P (*B*) was adsorbed to microtiter plates and incubated with rabbit anti-C5 antiserum or preimmune rabbit serum (open bars) at a dilution of 10^{-3}. As a measure of the specificity of antibody binding, serum dilutions were preincubated with purified C5 protein (hatched bars) or HeLa RNase P (solid bars) and the residual antigen binding activity was then measured on antigen-coated microtiter plates.

Depletion of RNase P Enzymatic Activity with Anti-C5 Antibodies. Preimmune rabbit antibodies or rabbit anti-C5 antibodies attached to protein A-Sepharose 4B were incubated with *E. coli* or HeLa RNase P to determine the effectiveness of the antibodies in depleting RNase P enzymatic activity in solution. Residual catalytic activity of *E. coli* or HeLa cell RNase P was measured in the supernatants of adsorbed enzyme preparations by the ability to process substrate precursor tRNA. Assays of *E. coli* RNase P activity with pre-tRNA^{Tyr} substrate are shown in Fig. 3*A*. *E. coli* RNase P adsorbed with preimmune rabbit antibodies demonstrated no detectable difference in cleavage activity compared to controls adsorbed with protein A-Sepharose 4B alone (Fig. 3*A*, lanes 1–4). Adsorption of *E. coli* RNase P with rabbit anti-C5 antibodies depleted 76% of cleavage activity as compared to cleavage activity of *E. coli* RNase P adsorbed with preimmune serum (Fig. 3*A*, lanes 3 and 4 compared to lanes 5 and 6). In similar assays using human RNase P, rabbit anti-C5 antibodies depleted 56% of precursor tRNA cleavage as compared to preimmune serum controls (Fig. 3*B*, lanes 3 and 4 compared to lanes 5 and 6). However, a minimal amount of inhibition of HeLa cell RNase P activity was observed with preimmune rabbit serum adsorption as compared to protein A-Sepharose 4B adsorption controls (Fig. 3*B*, lanes 1 and 2 compared to lanes 3 and 4).

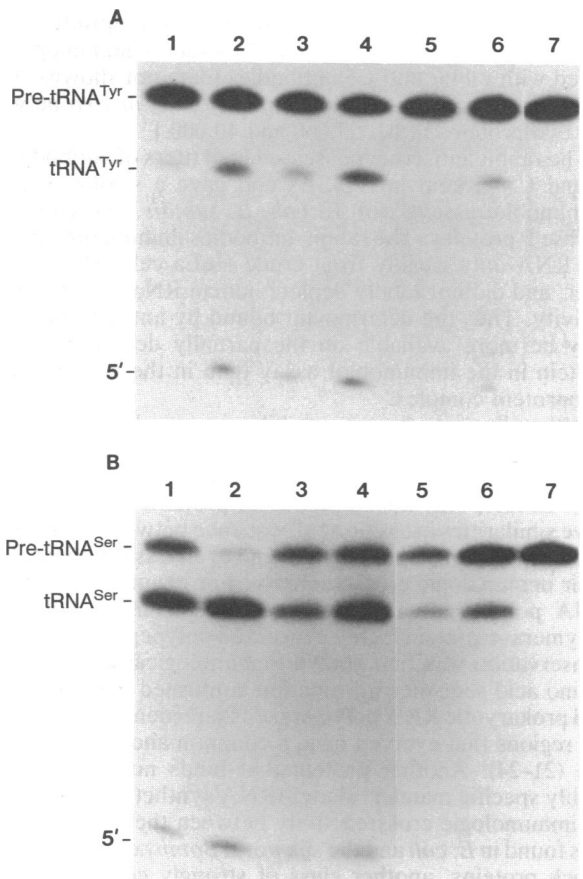

FIG. 3. Depletion of RNase P enzymatic activity with rabbit anti-C5 antibodies. (*A*) *E. coli* RNase P was incubated with substrate pre-tRNA^{Tyr} for 3 min (lanes 1, 3, and 5) or 6 min (lanes 2, 4, and 6). Lanes 1 and 2, cleavage of pre-tRNA^{Tyr} by *E. coli* RNase P after mock adsorption with only protein A-Sepharose 4B. Lanes 3 and 4, cleavage after adsorption of *E. coli* RNase P with preimmune rabbit serum antibodies attached to protein A-Sepharose 4B. Lanes 5 and 6, cleavage after adsorption of *E. coli* RNase P with rabbit anti-C5 antibodies attached to protein A-Sepharose 4B. Lane 7, pre-tRNA^{Tyr} substrate control with no RNase P. (*B*) HeLa RNase P incubated with pre-tRNA^{Ser} for 3 min (lanes 1, 3, and 5) or 6 min (lanes 2, 4, and 6). Lanes 1 and 2, cleavage of pre-tRNA^{Ser} by HeLa RNase P after mock adsorption with protein A-Sepharose 4B. Lanes 3 and 4, cleavage after adsorption of HeLa RNase P with preimmune rabbit antibodies attached to protein A-Sepharose 4B. Lanes 5 and 6, cleavage after adsorption of HeLa RNAse P with rabbit anti-C5 bound to protein A-Sepharose 4B. Lane 7, pre-tRNA^{Ser} substrate control with no RNase P.

Immunoprecipitation of Human Ribonucleoprotein Particles. Sera from certain patients with connective-tissue diseases possess autoantibodies that immunoprecipitate human RNase P ribonucleoprotein particles and are capable of depleting RNase P enzymatic activity (10). Rabbit anti-C5 antibodies were similarly assessed for their ability to immunoprecipitate human ribonucleoprotein particles. As demonstrated in Fig. 4, lane 2, a prototype human serum from a patient with connective-tissue disease immunoprecipitated the presumed human RNase P RNA, designated H1, and Th RNA, an unrelated RNA moiety known to coimmunoprecipitate with the H1 RNA (10). Rabbit anti-C5 serum immunoprecipitated RNA in a band comigrating with the H1 RNA and another minor species in a band comigrating with Th RNA (Fig. 4, lane 4). The rabbit serum did not immunoprecipitate RNAs from phenol-extracted HeLa cell lysate (data not shown), suggesting that the RNAs were immunoprecipitated by virtue of their associated protein(s). The purified IgG fraction of rabbit anti-C5 serum also immunoprecipitated RNA in a band comigrating with the H1 RNA (lane 6), while normal human serum and preimmune rabbit serum did not immunoprecipitate either H1 or Th RNAs (lanes 3 and 5, respectively). Other species migrating above or below the H1 and Th RNAs were observed when immunoprecipitations were performed with both preimmune and immune rabbit

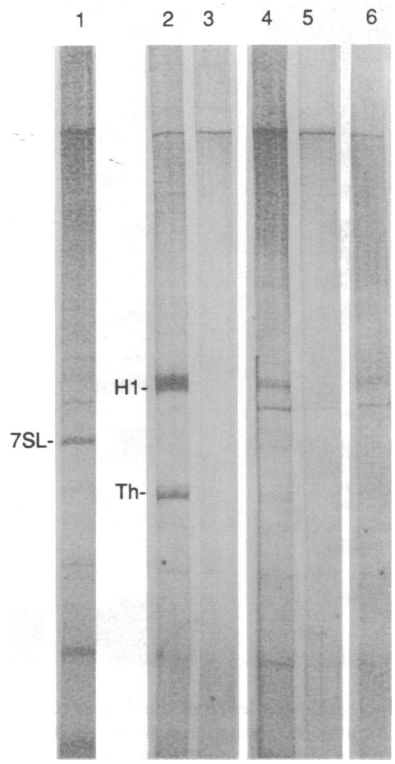

FIG. 4. Immunoprecipitation of RNAs from HeLa cell extract. Lane 1, 7SL RNA size marker. Lane 2, immunoprecipitation using serum from a patient with connective-tissue disease which possesses antibodies to human RNase P and Th ribonucleoproteins. Lane 3, normal human serum immunoprecipitation. Lane 4, rabbit anti-C5 immunoprecipitation. Lane 5, precipitation with preimmune rabbit serum. Lane 6, precipitation with the purified IgG fraction of rabbit anti-C5 serum.

sera, and were thus nonspecifically immunoprecipitated (lanes 4 and 5).

Nucleotide sequence comparisons were performed to determine whether the RNA immunoprecipitated with rabbit anti-C5 antibodies from human cells was identical to the previously identified H1 RNA (7). RNAs immunoprecipitated by the prototype autoimmune patient serum, rabbit anti-C5 serum, and preimmune rabbit serum were sequenced by using reverse transcriptase and a primer specific to a known internal sequence of the H1 RNA (Fig. 5). Nucleotide sequences were identical between RNAs immunoprecipitated with human anti-RNase P antibodies (Fig. 5 *Left*) and rabbit anti-C5 antibodies (*Center*) and were identical to the known sequence of H1 RNA (7). RNAs immunoprecipitated with preimmune rabbit serum failed to be recognized by the H1-specific primer and thus did not yield any nucleotide sequence data (Fig. 5 *Right*).

DISCUSSION

Immunization of a rabbit with the protein component of *E. coli* RNase P, C5, produced antibodies that bound C5 protein in immunoblots and in solid-phase immunoassays and that depleted catalytic activity from preparations of *E. coli* RNase P. The rabbit anti-C5 antibodies also depleted enzymatic activity from preparations of HeLa cell RNase P and immunoprecipitated the presumed RNA component of human RNase P, H1 RNA, from HeLa cell extract. In solid-phase immunoassays, both HeLa cell RNase P preparations and purified C5 protein effectively inhibited binding of the rabbit anti-C5 antibodies to either the C5 protein or HeLa cell

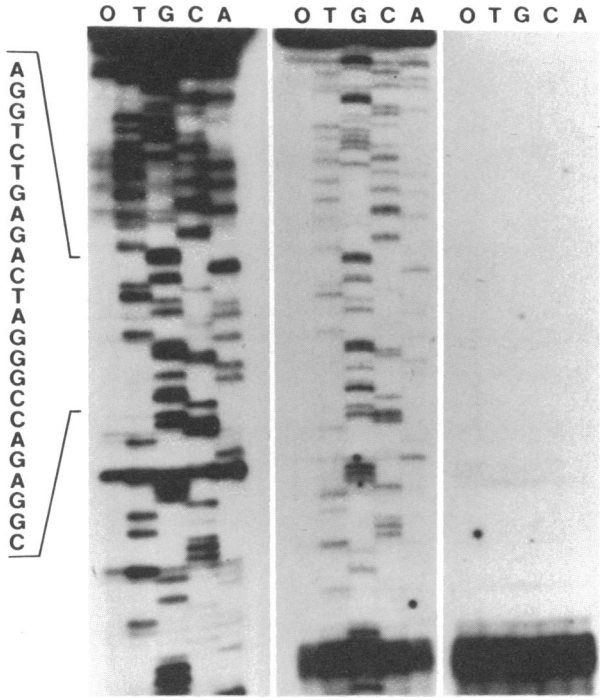

FIG. 5. Nucleotide sequence derived from reverse transcription of HeLa RNAs immunoprecipitated with rabbit and prototype human sera. Reverse transcription was performed using a primer that hybridized to the middle of the H1 RNA. The reaction products were analyzed by electrophoresis in a 7 M urea/8% polyacrylamide gel and autoradiography. (*Left*) Reverse transcripts of RNA immunoprecipitated with serum from a systemic lupus erythematosus patient known to possess anti-RNase P and anti-Th antibodies. (*Center*) Reverse transcripts of RNA immunoprecipitated with rabbit anti-C5 antibodies. (*Right*) Reverse transcripts of RNA immunoprecipitated with preimmune rabbit antibodies. Lanes O, no ddNTPs; lanes T, ddTTP; lanes G, ddGTP; lanes C, ddCTP; lanes A, ddATP.

RNase P. These results demonstrate that an immunologically defined determinant is shared by *E. coli* and human RNase P proteins.

An indication of this relationship also appeared in studies of the interaction of autoantibodies from patients with autoimmune disease with RNase P from humans and other sources (10, 16). While the protein components of human RNase P are unknown, rabbit anti-C5 antibodies identified a single polypeptide (M_r 40,000) in immunoblots of partially purified HeLa cell RNase P. Sera from patients with connective-tissue disease containing anti-RNase P antibodies identify a polypeptide of the same electrophoretic mobility (ref. 16; M.J.M., unpublished data) implicating the M_r 40,000 polypeptide as a component of human RNase P. A recent study (17) demonstrated that the human RNase P and Th particles may be structurally related via a common polypeptide. Since the Th RNA is weakly immunoprecipitated by rabbit anti-C5 antibodies from crude HeLa cell extracts, the M_r 40,000 polypeptide may be present on a complex of both RNase P and Th RNAs. [We have also found that a protein of $M_r \approx 55{,}000$ is recovered from precipitates with [^{35}S]methionine-labeled HeLa cell extract is immunoprecipitated with rabbit anti-C5 antibodies (data not shown). Thus a precursor–product relationship may exist *in vivo* between the two proteins of M_r 55,000 and 40,000.]

The rabbit antiserum possessed high titers of antibody that bound C5 protein in ELISAs and gave a strong signal in immunoblot assays with *E. coli*, *B. subtilis*, and HeLa cell RNase P proteins. The rabbit antibodies immunoprecipitated H1 RNA only weakly from crude HeLa cell extract, however, and did not totally deplete human RNase P enzymatic activity. Thus the determinant bound by anti-C5 antibodies may be more available on the partially denatured human protein in the immunoblot assay than in the native ribonucleoprotein complex.

Although some proteins exhibit immunologic crossreactivity across several species within a particular kingdom, very few span the range between bacteria and human cells (18). In fact, only a small group of proteins are known that have similarities in amino acid sequence between prokaryotic and eukaryotic species (19, 20), and even less is known about their immunologic crossreactivity. For example, eukaryotic RNA polymerase II and the β subunit of *E. coli* RNA polymerase contain highly conserved polypeptides (21). This conservation was first shown immunologically; subsequent amino acid sequence information confirmed that eukaryotic and prokaryotic RNA polymerases share identical peptides at six regions that evolved from a common ancestral polymerase (21–24). Another protein that binds nucleic acid in a highly specific manner, alanyl-tRNA synthetase, also exhibits immunologic crossreactivity between the molecular species found in *E. coli* and the silkworm *Bombyx mori* (25). Heat shock proteins, another class of strongly conserved molecules (26) from diverse eukaryotes, demonstrate immunologic crossreactivity, too (27).

Regions of ribonucleoproteins other than RNase P, such as the Sm polypeptides of the U-series RNAs, involved in splicing of pre-mRNA, and La(SSB), a transcription termination factor, are highly conserved among lower and higher eukaryotes (28, 29). The latter ribonucleoproteins, like RNase P, are important targets of autoantibodies in patients with connective-tissue diseases (10, 30). The evolutionarily conserved properties of these ribonucleoprotein particles may be important in their role as autoantigens (30).

While the *B. subtilis* and *E. coli* RNase P proteins appear to share an antigenic determinant, their overall amino acid sequence homology is only 25% (31, 32). However, selected regions between these two species of RNase P proteins share over 60% homology, and one sequence of 7 amino acids is identical. The region of identical amino acid sequence may be

the immunologic determinant shared between these and other RNase P proteins.

We have demonstrated that an immunologically recognized determinant is shared between prokaryotic and eukaryotic RNase P. Perhaps domains essential for the maintenance of essential biologic activity of these ribonucleoproteins have been preserved across species lines by selective pressures while regions of the proteins not critical in their biological function have been allowed to diverge. These conserved features may exist only for very ancient and essential enzymatic functions such as RNase P, RNA polymerase, and some aminoacyl-tRNA synthetases. That RNA and protein from RNase P of different species can reassociate into a catalytically active complex (2, 4) also shows that functional domains are conserved despite a lack of significant homology in the coding sequences for either RNase P proteins or RNAs between individual species.

We are grateful to Marcjanna Bartkiewicz for HeLa cell extracts enriched in RNase P activity, to John Arnez and Augustine Vioque for purified C5 protein, to Heidi Gold for helpful discussion and valuable advice in immunoprecipitation experiments, and to Tony Chou for help in preparation of antibodies. We thank Dr. John A. Hardin for advice and support during this project. This work was supported by grants from the National Institutes of Health (GM19422 to S.A.; AI26853 to J.C.; AR32549-02 and AM07109-11 to John A. Hardin); the Connecticut chapter of the Lupus Foundation, the Arthritis Foundation and its Connecticut chapter, and the Scleroderma Society of New York (to John A. Hardin); the United Scleroderma Foundation (to J.C.); and the National Science Foundation (DMB-8722644 to S.A.). J.C. is a Pew Scholar in the Biomedical Sciences. M.J.M. is the recipient of a postdoctoral fellowship from the Arthritis Foundation.

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