MOLECULAR AND CELLULAR BIOLOGY, Oct. 1991, p. 5266–5274
0270-7306/91/105266-09$02.00/0

Vol. 11, No. 10

The 40-Kilodalton To Autoantigen Associates with Nucleotides 21 to 64 of Human Mitochondrial RNA Processing/7-2 RNA In Vitro

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A 40-kDa To antigen recognized by sera from some patients with autoimmune diseases is an integral component of both human RNase P and mitochondrial RNA processing (MRP) RNase. Human MRP and RNase P RNAs, synthesized in vitro, readily associate with the To antigen present in the HeLa cell extract. Using this in vitro reconstitution system, the binding site of the To antigen is localized to a 44-nucleotide-long sequence corresponding to nucleotides 21 to 64 of the human MRP RNA. UV cross-linking experiments showed that the To antigen binds directly to MRP RNA and to RNase P (H1) RNA through RNA-protein interactions. Although the MRP RNA and RNase P (H1) RNA show sequence homology in four conserved blocks (H. A. Gold, J. N. Topper, D. A. Clayton, and J. Craft, Science 245:1377–1380, 1989), the To antigen-binding site in MRP RNA does not show any obvious primary sequence homology with H1 RNA. These data suggest that the To antigen binds to a conserved and presumably a common secondary or tertiary structure in human MRP and RNase P RNAs.

Human mitochondrial RNA processing (MRP) RNA (also termed Th or 7-2 RNA) is an integral component of the MRP ribonucleoprotein (RNP). The MRP RNase in mitochondria is responsible for a sequence-specific cleavage of the mitochondrial RNA; this cleavage generates an RNA primer that is used during mitochondrial DNA replication (7, 8). The MRP RNPs are unique in that these particles are present in the mitochondria as well as in the nucleus (8). Most of these RNPs are in the nucleus; less than 1% are in the mitochondria (7, 8). Results obtained in our laboratory showed that rat nucleolar 7-2 RNA is homologous to mouse MRP RNA (31), and recent studies showed that nucleolar 7-2 RNA and MRP RNA are identical (16). These data show that MRP RNase is functional in two different cellular organelles, mitochondria and nucleoli.

MRP RNPs also share several features with RNase P (16); RNase P is an endoribonuclease that processes precursor tRNA transcripts to generate their mature 5′ termini (2). Both RNPs are immunoprecipitable with anti-To/Th antibodies (15, 16, 18, 22), indicating that they share at least one common or antigenically related polypeptide. Both MRP RNA and RNase P RNA are synthesized by RNA polymerase III (3, 18, 30) and show primary sequence homology to each other in four conserved blocks (16). These conserved sequences appear to reside in similar stems in the proposed secondary structures of MRP and RNase P RNAs (4, 28) and are hypothesized to interact with similar or related proteins (16). Since MRP RNP and RNase P are both RNA processing enzymes that make a sequence-specific endonucleolytic cleavage in their RNA substrates, MRP RNase is analogous to RNase P in structure and in function (28).

Some sera from patients with autoimmune diseases contain antibodies, such as anti-Th (18), anti-To (22), and anti-Wa (24), directed against MRP/7-2 RNA-containing RNP particles. Reimer et al. (24) showed that the antigenic protein recognized by anti-To and anti-Wa antibodies was a 40-kDa protein. Using immunoelectron microscopy, they localized the nucleolar 7-2 RNP particles to the granular compartment of the nucleolus. In addition to MRP/7-2 RNPs, anti-To antibodies also precipitate H1 RNA (RNA component of eukaryotic RNase P) from crude cell extracts (15, 16, 18, 22). Gold et al. (16) identified 30 patients with autoimmune diseases whose sera immunoprecipitate the MRP/7-2 RNA; all of these sera also immunoprecipitate H1 RNA of RNase P. These data suggest that MRP RNase, 7-2 RNP, and RNase P share a common autoantigenic polypeptide (16). Interestingly, antibodies against *Escherichia coli* C5 protein (protein component of *E. coli* RNase P) cross-react with a 40-kDa protein integral to both HeLa RNase P and MRP RNPs (21). It is likely that the 40-kDa protein recognized by the anti-C5 protein antibodies and the To antigen are the same protein and the To antigen is the homolog of *E. coli* C5 protein in mammalian cells.

Since MRP RNase and RNase P have features in common, it is very likely that MRP RNA is also a catalytic component in MRP RNase. Although prokaryotic RNase P RNA alone can act as a catalyst in a high (60 mM) concentration of Mg^{2+}, it is apparent that these particular cleavage reactions cannot occur in vivo without the participation of a protein(s) (2). It is hypothesized that the proteins may fine-tune the specificity of RNA enzymes by enhancing the rates of reaction at particular sites and with particular substrates (1). The 40-kDa To antigen is an integral component of both RNase P and MRP RNPs and seems to be the analog of *E. coli* C5 protein, the unique protein component in *E. coli* RNase P. Therefore, studies directed toward understanding the association of the To antigen with MRP/7-2 RNA was undertaken. The results show that the 40-kDa To autoantigen binds between 20 and 65 nucleotides (nt) of MRP RNA in vitro. Surprisingly, this region of MRP RNA is not conserved between RNase P RNA and MRP RNA; instead, it is flanked by two conserved sequence elements.

MATERIALS AND METHODS

Antibodies and DNA templates. Anti-To, anti-Sm, and anti-La antibodies used in this study were characterized

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TABLE 1. Characteristics of human MRP/7-2 plasmid DNA constructs

Construct	Characteristics
MRP/7-2	Human MRP/7-2 RNA gene containing 737 bp upstream, 270 bp corresponding to MRP RNA, and 28 bp downstream inserted into the *Pst*I site of Bluescript vector
MRP(−84/+28)	Human 7-2/MRP gene in Bluescript vector containing 84-bp upstream, 270-bp coding, and 28-bp downstream sequences; produces a full-length MRP RNA
MRP(15-87)	*Ava*I fragment corresponding to nt 15 to 87 of MRP RNA cloned in *Eco*RV site of mU6(−315/1) (11); transcript contains 18 nt from the vector and 73 nt of MRP sequence
MRP(84-270)	Blunted *Ava*I fragment containing sequences between 84 and 270 and 10 bp past the 3′ end (31) cloned in *Eco*RV site of the clone mU6 −315/1 described in 11. The transcript is 204 nt long
MRP(1-64)	84 bp upstream and first 64 bp corresponding to MRP RNA cloned in *Sma*I and *Bam*HI sites of Bluescript vector; transcript is 152 nt long, including 88 nt from the vector
MRP(1-58)	84 bp upstream and first 58 bp corresponding to MRP RNA cloned in *Sma*I and *Bam*HI sites of Bluescript vector; transcript is 146 nt long
MRP(1-51)	84 bp upstream and first 51 bp corresponding to MRP RNA cloned in *Sma*I and *Bam*HI sites of Bluescript vector; transcript is 139 nt long
MRP(1-46)	84 bp upstream and first 46 bp corresponding to MRP RNA cloned in *Sma*I and *Bam*HI sites of Bluescript vector; transcript is 134 nt long
MRP(1-40)	84 bp upstream and first 40 bp corresponding to MRP RNA cloned in *Sma*I and *Bam*HI sites of Bluescript vector; transcript is 128 nt long
MRP(Δ15-21)	7 bp (15 to 21) in MRP RNA gene deleted and replaced by GATCC in clone MRP(−84/+28)
MRP(Δ15-27)	13 bp (15 to 27) in MRP RNA gene deleted and replaced by GATCC in MRP(−84/+28)
MRP(Δ15-34)	20 bp (15 to 34) in MRP RNA gene deleted and replaced by GATCC in MRP(−84/+28)
MRP(Δ15-40)	26 bp (15 to 40) in MRP RNA gene deleted and replaced by GATCC in MRP(−84/+28)
LS(15/21)	7 bp between nt 15 and 21 in MRP RNA gene substituted with GTAGCCTAGGgggaccCCTTCAGCAC
LS(22/27)	6 bp between nt 22 and 27 in MRP RNA gene substituted with CAGTGTGTAGggatccATACAGGCC
LS(28/34)	7 bp between nt 28 and 34 in MRP RNA gene substituted with GAGTCCTCAGggatcccCCTAGGATAC
LS(35/40)	6 bp between nt 35 and 40 in MRP RNA gene substituted with GAACAGAGTggatccTGTGTAGCC
LS(47/51)	5 bp between nt 47 and 51 in MRP RNA gene substituted with GGAAAGGGggatccCAGAGTCCTC
LS(52/58)	7 bp between nt 52 and 58 in MRP RNA gene substituted with CCTAGGCGGgggatccAGGAACAGAG
LS(65/70)	6 bp between nt 65 and 70 in MRP RNA gene substituted with CGGGGACTTggatccAGGCGGAAAG

earlier (22). The human MRP RNA gene with 737-bp upstream sequences and 28-bp downstream sequences was cloned in Bluescript vector and described earlier (30). The 3′ and internal deletion mutants and linker-scan mutants obtained by site-directed mutagenesis are described in Table 1.

In vitro transcriptions. Whole HeLa cell extract was prepared by the method of Weil et al. (29). Three micrograms of supercoiled plasmid DNA was incubated at 30°C for 1 to 3 h in a 50-μl reaction mixture containing 50% (vol/vol) HeLa cell extracts, 0.6 mM each unlabeled CTP, UTP, and ATP, 0.025 mM GTP, 5 to 50 μCi of [α-^{32}P]GTP, 6 mM creatine phosphate, 20 mM KCl, 1 mM dithiothreitol, and 10 mM Tris-HCl (pH 8.1). The transcribed RNAs were subjected to either immunoprecipitation or sodium dodecyl sulfate (SDS)-phenol extraction, precipitation with ethanol, and fractionation by electrophoresis on 10% polyacrylamide–7 M urea gels.

Immunoprecipitations. Immunoprecipitations were carried out as described by Lerner and Steitz (19). The transcription reaction mixture was diluted with 250 μl of phosphate-buffered saline, 100 μg of antibodies in a 10-μl volume was then added, and the mixture was allowed to stand on ice for 15 min. Then 250 μl of Pansorbin (Calbiochem) was added, and the mixture was incubated on ice for an additional 15 min. The immunoprecipitates were washed three times with 10 ml of NET-2 buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 2 mM EDTA, and 0.05% Nonidet P-40). The immunoprecipitated RNAs were extracted with SDS-phenol, ethanol precipitated, and fractionated on 10% polyacrylamide–7 M urea gels.

Analysis of MRP RNA transcripts and RNA fragments. The labeled MRP RNA transcripts or T_1 RNase-resistant fragments were localized on the gel by autoradiography, eluted from the polyacrylamide gel slices, and digested to completion with T_1 RNase. The digests were fingerprinted by electrophoresis on cellulose acetate at pH 3.5 in the first dimension, followed by homochromatography on polyethyleneimine-cellulose sheets with a C15 homomixture in the second dimension. The dried sheets were then subjected to autoradiography as described by Brownlee et al. (6).

UV cross-linking. RNA-protein UV cross-linking was carried out as described by Greenberg (17) and Dreyfuss et al. (12). A 25-μl reaction mixture containing the same buffer used for in vitro transcription, 1 μg of *E. coli* tRNA, 5 μg of poly(dI-dC), and 12.5 μl of HeLa extract was incubated on ice. In competition experiments, 200 to 400 ng of unlabeled HeLa MRP RNA and 5 μg of tRNA or 5S RNA was also included during preincubation. After 10 min of incubation, ^{32}P-labeled MRP(15–87) RNA was added and incubated on ice for 30 min. UV irradiation was done with a G15T8 UV germicidal lamp, kept at a distance of 10 cm for 10 min at 4°C. One unit of T_1 RNase was added to each of the UV-irradiated samples, and digestion was carried out at 30°C for 10 min. An equal volume of 2× Laemmli buffer (1× Laemmli buffer is 60 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 5% glycerol, and 0.001% bromophenol blue) was added to each of the samples. The samples were boiled for 5 min and then immediately loaded and electrophoresed on an SDS–10% polyacrylamide gel (pH 8.1) with a 1-cm-long 5% stacking gel (pH 6.8). The gels were dried and subjected to autoradiography.

RESULTS

MRP RNA is synthesized in vitro and associates with the To antigen. The human MRP RNA gene was transcribed in vitro, using HeLa cell extracts with [α-^{32}P]GTP as the precursor; the transcripts were mainly MRP RNA, 5′-end-

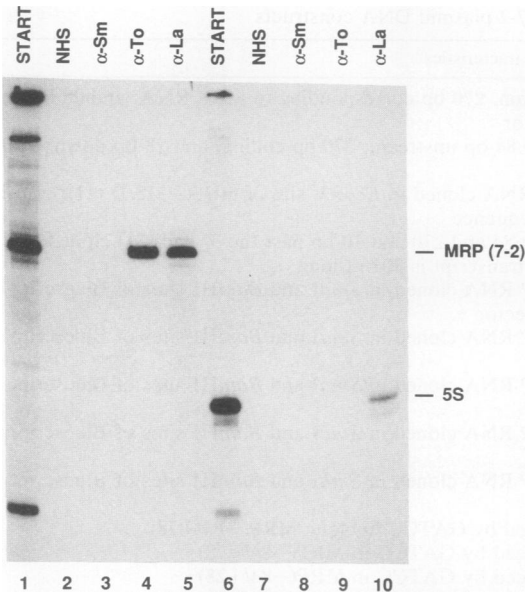

FIG. 1. Immunoprecipitation with different antisera of MRP and 5S RNPs assembled in vitro. Plasmid DNAs containing the human MRP RNA gene (lanes 1 to 5) and the Syrian hamster 5S RNA gene (lanes 6 to 10) were transcribed in vitro with [α-^{32}P]GTP as the precursor. The mixture was precipitated with antibodies indicated above the lanes. START, RNA components in the starting material for immunoprecipitation; NHS, normal human serum. The RNAs in the immunoprecipitates were purified, fractionated on a polyacrylamide gel, and subjected to autoradiography.

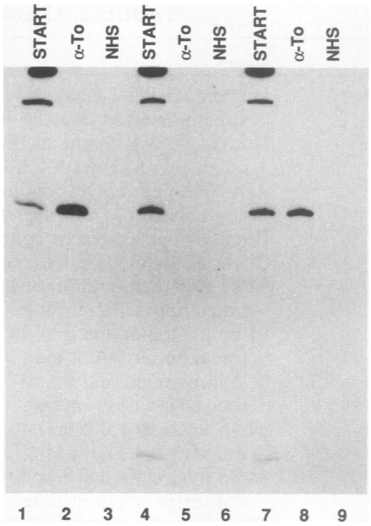

FIG. 2. Reconstitution of isolated MRP RNA with the To antigen. MRP RNA was labeled in vitro, extracted with phenol, and purified on polyacrylamide gels. The RNA was added to the HeLa cell extract, incubated on ice for 30 min, and immunoprecipitated with different antibodies. Lanes: 1 to 3, RNP formed during transcription of MRP DNA in vitro; 4 to 6, MRP RNA isolated from polyacrylamide gels; 7 to 9, RNPs reconstituted by adding protein-free MRP RNA to the extract. The antibodies used to immunoprecipitate are indicated above the lanes.

labeled histidyl-tRNA, and some minor RNA bands (Fig. 1, lane 1). To determine whether MRP RNA synthesized in vitro assembles into an RNP particle, the transcription mixture at the end of the reaction was immunoprecipitated with antibodies from normal human serum and sera with specificities to the Sm, To, and La antigens. Immunoprecipitates obtained with anti-To (lane 4) and anti-La (lane 5) antibodies contained MRP RNA; anti-Sm antibodies (lane 3) or normal human serum (lane 2) did not precipitate MRP RNA. As a control, 5S RNA was synthesized in vitro and immunoprecipitated with different antibodies. Only anti-La antibodies immunoprecipitated 5S RNPs (lane 10). Normal human serum (lane 7), anti-Sm (lane 8), or anti-To (lane 9) antibodies did not react with 5S RNPs. These data show that the association of the To antigen to MRP RNA is specific and that the MRP RNA synthesized in vitro associates with the To and La antigens. In three independent experiments, more than 50% of the MRP RNA synthesized in vitro was precipitable with the anti-To antibodies. The La antigen was found to be associated with many nascent polymerase III transcripts, including newly synthesized 5S RNA and MRP RNA (18, 26). The association of MRP RNA with the La antigen is consistent with all available evidence that the MRP gene is transcribed by polymerase III (18, 30).

Labeled MRP RNA was fractionated on a polyacrylamide gel, extracted by the crush-and-soak method, and purified by precipitation with ethanol. The deproteinized MRP RNA was not recognized by the anti-To antibodies (Fig. 2, lane 5); these data are consistent with those reported by Hashimoto and Steitz (18). To test whether the purified MRP RNA is able to reconstitute into RNP particles by associating with the To antigen, labeled MRP RNA was mixed with the HeLa cell extract under the conditions used for transcription except that the incubation was carried out for 30 min at 0°C. A significant proportion of the MRP RNA was immunoprecipitated with anti-To antibodies (Fig. 2, lane 8). These data show that isolated MRP RNA can form RNP particles by associating with the To antigen in vitro and also suggest that the association of MRP RNA with the To antigen is not coupled to the transcription. A similar reconstitution experiment carried out at 30°C resulted in extensive degradation of the added MRP RNA (data not shown).

In HeLa cells, the To antigen is associated with MRP/7-2 RNA as well as with RNase P (H1) RNA. When we transcribed the human H1 gene in vitro, the H1 RNA also associated with the To antigen and was immunoprecipitated with anti-To antibodies. As controls, human 7SK and U6 small nuclear RNA (snRNA) genes were transcribed; these RNAs did not immunoprecipitate with the anti-To antibodies (data not shown).

To antigen binds between nt 18 and 68 of MRP RNA. To determine the site of To antigen binding to the MRP RNA, the MRP RNA was synthesized in vitro and digested with different concentrations of T_1 RNase. The labeled RNAs were then isolated and fractionated on a polyacrylamide gel (Fig. 3). As expected, the MRP RNA was cleaved by the added T_1 RNase (lanes 2 to 5); the digest obtained with a concentration of 50 U/ml (lane 4) was immunoprecipitated with anti-To antibodies, and one major RNA band (~50 nt long) was observed in the immunoprecipitates (lane 7). Under the same conditions, deproteinized MRP RNA was completely digested by 5 U of T_1 RNase per ml (data not shown). Antibodies with anti-To specificity from six different patients were used, and in all cases only the 50-nt-long RNA fragment was detectable in the immunoprecipitates. This 50-nt-long RNA fragment was not found in the immunoprecipitates obtained with anti-La or anti-Sm antibodies (data not shown). These data suggest that this 50-nt-long

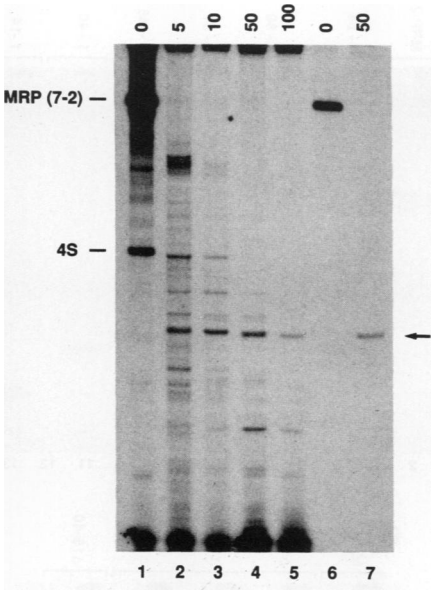

FIG. 3. Partial T_1 RNase digestion of MRP RNA synthesized in vitro and immunoprecipitation with the anti-To antibodies. An in vitro transcription mixture was digested with T_1 RNase at the concentrations indicated above lanes 1 to 5. Aliquots without any added nuclease (lane 6) and with T_1 RNase at 50 U/ml (lane 7) were immunoprecipitated with anti-To antibodies. The purified RNAs were electrophoresed on a 10% polyacrylamide–7 M urea gel and subjected to autoradiography.

RNA fragment is protected from T_1 RNase digestion because of its association with the To antigen.

The 50-nt-long T_1 RNase-resistant MRP RNA fragment was digested to completion with T_1 RNase and fingerprinted (Fig. 4B). Compared with full-length MRP RNA (Fig. 4A), the fingerprint in Fig. 4B contained a limited number of T_1 RNase fragments, and all of these oligonucleotides were from one contiguous region, corresponding to nt 19 to 67 of MRP RNA. The fingerprint of the 50-nt-long RNA fragment found in the immunoprecipitates was found to be identical to the fingerprint shown in Fig. 4B (data not shown). T_1 RNase oligonucleotides 10 (AAAGp) and 11 (CCUGp) were not present in the fingerprint of the 50-nt-long fragment (Fig. 4B). These data show that the G residues corresponding to nt 18 and 69 are not protected by the To antigen. From these data, it is not clear whether the G residues at positions 67 and 68 are protected or not. From the intensity of radioactive Gp and AGp in Fig. 4B, it would appear that the G residue at 68 is not protected. If this G was protected, the radioactivity in Gp would have been equal to or greater than that in AGp; instead, it was found to be less (Fig. 4B). Therefore, the protected region corresponds to nt 19 to 67 of MRP RNA.

Nucleotides 15 to 87 of MRP RNA are sufficient for To antigen binding. Using restriction enzymes *Stu*I and *Ava*I, a DNA fragment corresponding to nt 15 to 87 of MRP RNA was isolated and inserted downstream of a mouse U6 snRNA gene promoter. Similarly, an *Ava*I fragment containing nt 84 to 270 of the MRP RNA gene was cloned into a Bluescript vector downstream of a mouse U6 snRNA gene promoter. With the former construct, a 144-nt RNA transcript containing 73-nt region corresponding to nt 15 to 87 of MRP RNA and 71-nt vector sequences was obtained (Fig. 5, lane 3). This transcript was immunoprecipitated by the anti-To antibodies, showing that nt 15 to 87 of MRP RNA are sufficient for recognition and binding of the To antigen (lane 4). In contrast, the RNA synthesized from the latter construct, MRP(84-270), was not immunoprecipitable with anti-To antibodies (compare lanes 5 and 6). These data show that the region corresponding to nt 15 and 87 of MRP RNA is sufficient for the To antigen recognition and binding and that the rest of the MRP RNA molecule by itself cannot bind the To antigen.

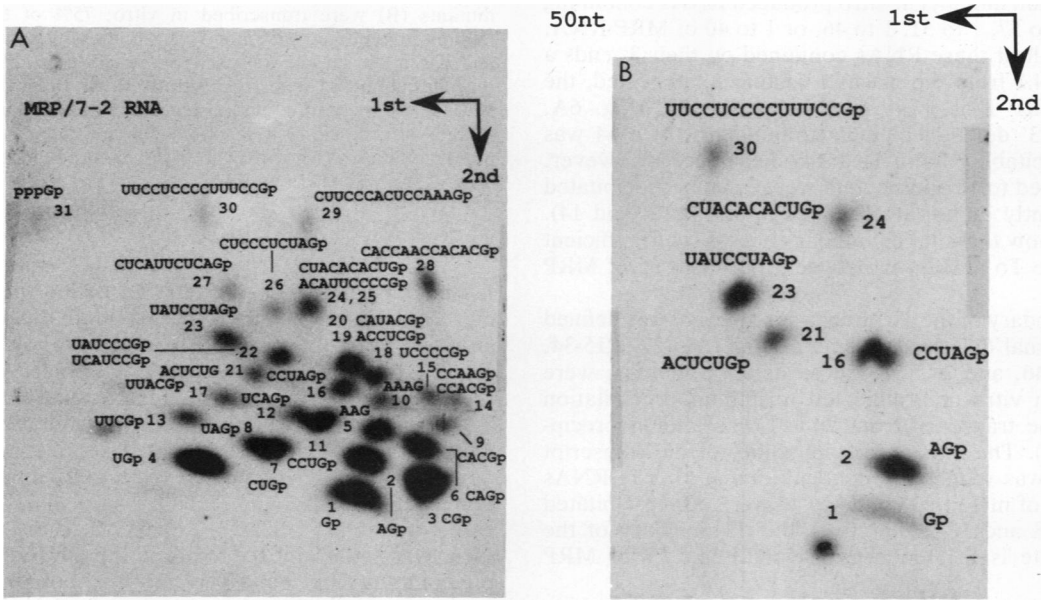

FIG. 4. Analysis of human MRP RNA and T_1 RNase-resistant fragments by fingerprinting. MRP RNA synthesized in vitro and the 50-nt-long T_1 RNase-resistant fragment (see Fig. 3, lane 4) were isolated from a polyacrylamide gel, digested to completion with T_1 RNase, fingerprinted by the procedure of Brownlee et al. (6), and subjected to autoradiography. The first dimension was electrophoresis on cellulose acetate and homochromatography on polyethyleneimine-cellulose 300 plates, with a C15 homomixture in the second dimension.

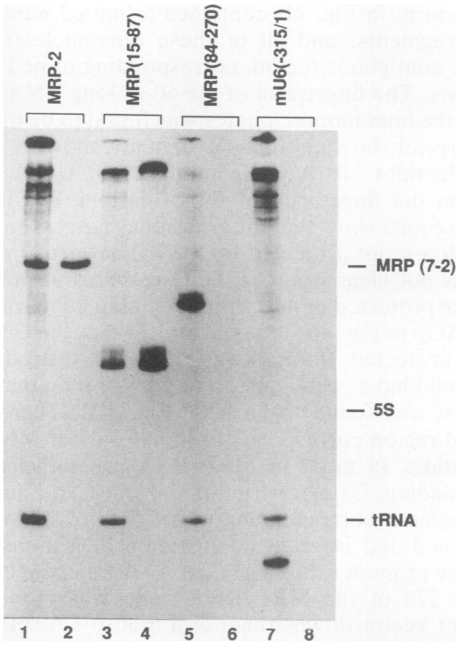

FIG. 5. Immunoprecipitation of MRP(15–87) and MRP(84–270) transcript-containing RNP particles. Samples (3 μg each) of human MRP-2 (lanes 1 and 2), MRP(15–87) (lanes 3 and 4), MRP(84–270) (lanes 5 and 6), and mouse U6(−315/1) (lane 7 and 8) DNAs were transcribed in vitro, using HeLa whole cell extract (lanes 1, 3, 5, and 7). The reaction mixture was immunoprecipitated with the anti-To antibodies (lanes 2, 4, 6, and 8). The RNAs were SDS-phenol extracted, fractionated on a 10% polyacrylamide–7 M urea gel, and subjected to autoradiography.

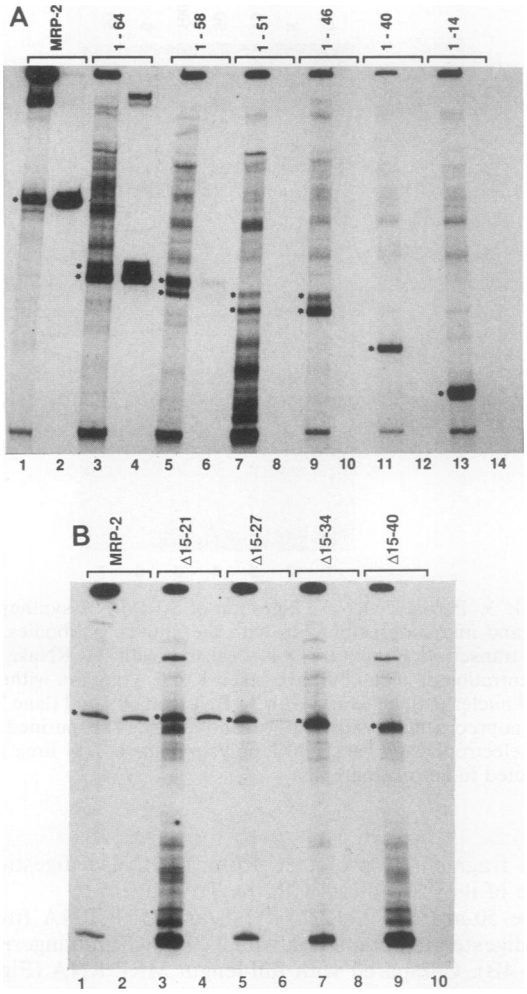

FIG. 6. Determination of the 3′ and 5′ boundaries of the To antigen-binding site. Series of 3′ deletion mutants (A) and internal mutants (B) were transcribed in vitro; 75% of the sample was immunoprecipitated with anti-To antibodies (even-numbered lanes), and 25% of the sample was used to extract RNA (starting material in the odd-numbered lanes). The transcripts containing 3′ truncated MRP RNA are marked with asterisks. Band intensities were quantitated with the aid of a densitometer, and the efficiency of immunoprecipitation is shown in Table 2. The efficiency of full-length MRP RNA was taken as 100%.

The boundaries of the sequences required for To antigen recognition were mapped by using a series of deletion mutants. The 3′ boundary of To antigen binding was determined with a series of 3′ deletion mutants. Transcription of these 3′ deletion mutants in vitro produced RNAs containing nt 1 to 64, 1 to 57, 1 to 51, 1 to 46, or 1 to 40 of MRP RNA. In addition, all of these RNAs contained on their 3′ ends a 75-nt-long RNA from the plasmid vector. As expected, the full-length MRP RNA was immunoprecipitable (Fig. 6A, lane 2). The 3′ deletion mutant containing nt 1 to 64 was immunoprecipitable by anti-To antibodies (lane 4); however, mutants deleted to 58 and beyond were immunoprecipitated very inefficiently or not at all (lanes 6, 8, 10, 12, and 14). These data show that the 3′ boundary necessary for efficient binding of the To antigen is between nt 58 and 65 of MRP RNA.

The 5′ boundary of the To antigen-binding site was defined with the internal deletion mutants Δ15-21, Δ15-27, Δ15-34, Δ15-40, Δ15-46, and Δ15-51. These deletion mutants were transcribed in vitro and subjected to immunoprecipitation (Fig. 6B). The transcript from Δ15-21 was immunoprecipitable (lane 4). The immunoprecipitability of the transcript from Δ15-27 was reduced dramatically (lane 6). The RNAs with deletion of nt 15 to 34 or 15 to 40 were not precipitated at all (lanes 8 and 10). Therefore, the 5′ boundary of the To-binding site is located between nt 21 and 27 of MRP RNA.

Sequences away from the boundaries are also required for binding of the To antigen. The experiments carried out with the 3′ and 5′ deletion mutants indicated that sequences corresponding to nt 21 to 64 of MRP RNA are necessary for the binding of the To antigen. To define this region more clearly, a series of block substitution mutants was constructed. In each mutant, 5 to 7 nt were replaced with the *Bam*HI linker sequence GGATCC. These mutants were transcribed in vitro, and To antigen binding was studied (Fig. 7). As expected, the transcripts from mutants LS15/21 and LS65/70 were immunoprecipitated as efficiently as the unmodified MRP RNA, because these mutations do not reside in the To antigen-binding region (Fig. 7 and Table 2). In the cases of mutants LS22/27, LS28/33, LS34/39, and LS52/58, there was significant reduction in the efficiency of immunoprecipitation (Fig. 7 and Table 2). The mutant in which nt 47 to 51 were substituted was transcribed in vitro and was unable to associate with the To antigen (Fig. 7, lane 12). These data show that sequences away from the 5′ and 3′ boundaries also are important in binding of the To antigen.

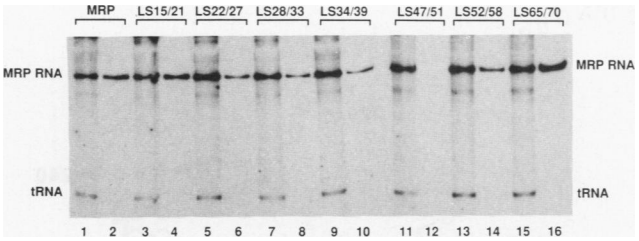

FIG. 7. Transcription and immunoprecipitation of linker-scan mutants. MRP wild-type (lane 1) and LS15/21 (lane 3), LS22/27 (lane 5), LS28/33 (lane 7), LS34/39 (lane 9), LS47/51 (lane 11), LS52/58 (lane 13), and LS65/70 (lane 15) plasmid DNAs were transcribed in vitro in the presence of [α-^{32}P]GTP, 33% of the sample was used for extraction with SDS-phenol, and the RNAs were analyzed; 67% of the sample was precipitated with the anti-To antibodies (lanes 2, 4, 6, 8, 10, 12, 14, and 16). The RNAs in the immunoprecipitates were extracted, loaded next to the corresponding total RNA samples, fractionated on a polyacrylamide gel, and subjected to autoradiography.

The To antigen binds to MRP RNA directly through an RNA-protein interaction. The data presented thus far do not show that the To antigen binds MRP RNA directly. It is conceivable that another protein binds to this region of MRP RNA and that the To antigen in turn binds to this protein. To address this issue, [α-^{32}P]GTP-labeled RNA transcripts corresponding to nt 15 to 87 of MRP RNA were incubated with HeLa cell extract and the complexes were cross-linked by UV irradiation. The results showed that the MRP(15–87) RNA was cross-linked to the 40-kDa protein (Fig. 8, lane 2). Preincubation of the extract with unlabeled HeLa MRP RNA prevented cross-linking to the subsequently added labeled MRP(15–87) RNA (lane 3), while unlabeled tRNA and 5S RNA did not affect the cross-linking (lanes 4 and 5, respectively). When the cross-linked protein shown in lane 6 was electrophoresed next to a nuclear extract and subjected to Western immunoblotting with anti-To antibodies, the immunoreactive To protein and the major cross-linked protein comigrated on the polyacrylamide gel (data not shown). These data show the MRP(15–87) RNA was bound by the 40-kDa To antigen specifically, stably, and directly through an RNA-protein interaction. This conclusion was confirmed by immunoprecipitation of the cross-linked protein with the anti-To antibodies (lane 6). UV cross-linking showed a doublet band, and both bands were immunoprecipitated with the anti-To antibodies (lane 6). One explanation for this doublet band is that there may be size heterogeneity in the human To protein. It is also possible that there are two contact points that can be cross-linked by UV light between the RNA and the To protein.

TABLE 2. Efficiency of the To antigen binding with different structurally altered MRP RNAs

RNA[a]	Efficiency of immunoprecipitation (%)[b]
MRP/7-2 RNA	50 (100)
MRP 1-84	60 (120)
MRP 1-64	45 (90)
MRP 1-58	<5 (<10)
MRP 1-51	<2 (<4)
MRP 1-46	ND[c]
MRP 1-40	ND
MRP Δ15-21	35 (70)
MRP Δ15-27	<5 (<10)
MRP Δ15-34	ND
MRP Δ15-40	ND
MRP LS 15-21	50 (100)
MRP LS 22-27	20 (40)
MRP LS 28-33	10 (20)
MRP LS 34-39	15 (30)
MRP LS 47-51	ND
MRP LS 52-58	15 (30)
MRP LS 65-70	50 (100)

[a] See Table 1 for mutants.
[b] Radioactivity was quantitated by excising bands from acrylamide gels and counting in a scintillation counter. Results are averages of three or more independent experiments.
[c] ND, not detectable.

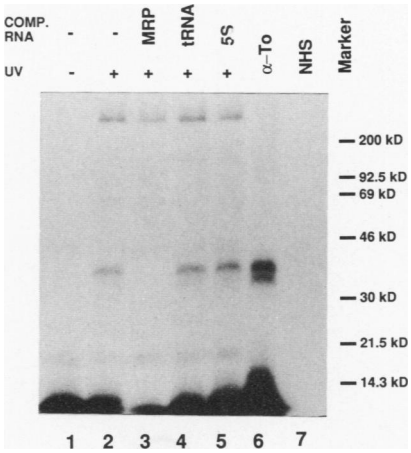

FIG. 8. UV cross-linking of To antigen binding to MRP RNA. [α-^{32}P]GTP-labeled MRP(15–87) RNA was incubated with the HeLa cell extract and then UV irradiated. After T_1 RNase digestion, the samples were loaded on a 10% SDS–polyacrylamide gel. The dried gel was subjected to autoradiography. Lane 1, no UV cross-linking. In lanes 2 to 5, the competitor RNA used is indicated above each lane; in lanes 6 and lane 7, after UV cross-linking and T_1 RNase digestion, the samples were immunoprecipitated with anti-To and normal human serum (NHS), respectively. Molecular mass markers are shown on the right.

The To antigen-binding site on MRP RNA. The binding region of the To antigen on a recently proposed secondary structure model (28) is shown in Fig. 9. In all of the secondary structures proposed for MRP RNA (5, 10, 28, 31), this To antigen-bound region represents a distinct domain. Recently, Forster and Altman (13) proposed a cage structure common to human RNase P and MRP RNAs. Since MRP and RNase P RNAs bind the same To antigen, attempts were made to find common features in the To antigen-binding regions of these two RNAs. The secondary structure proposed for the To antigen-binding region of the MRP/7-2 RNA was a domain containing a stem-loop structure (Fig. 9) proposed by several (9, 13, 28, 31) and is consistent with the proposed cage model (13).

Human RNase P RNA also binds the To antigen in vitro. Since both MRP/7-2 and RNase P RNAs can be immunoprecipitated by anti-To antibodies, we carried out UV cross-linking experiment to determine whether these two RNAs bind the same To protein. When the RNase P RNA was subjected to cross-linking, a 40-kDa protein was efficiently cross-linked to the RNA, though several minor bands were also seen (Fig. 10, lane 1). When the reaction mixture was preincubated with unlabeled MRP/7-2 RNA prior to the

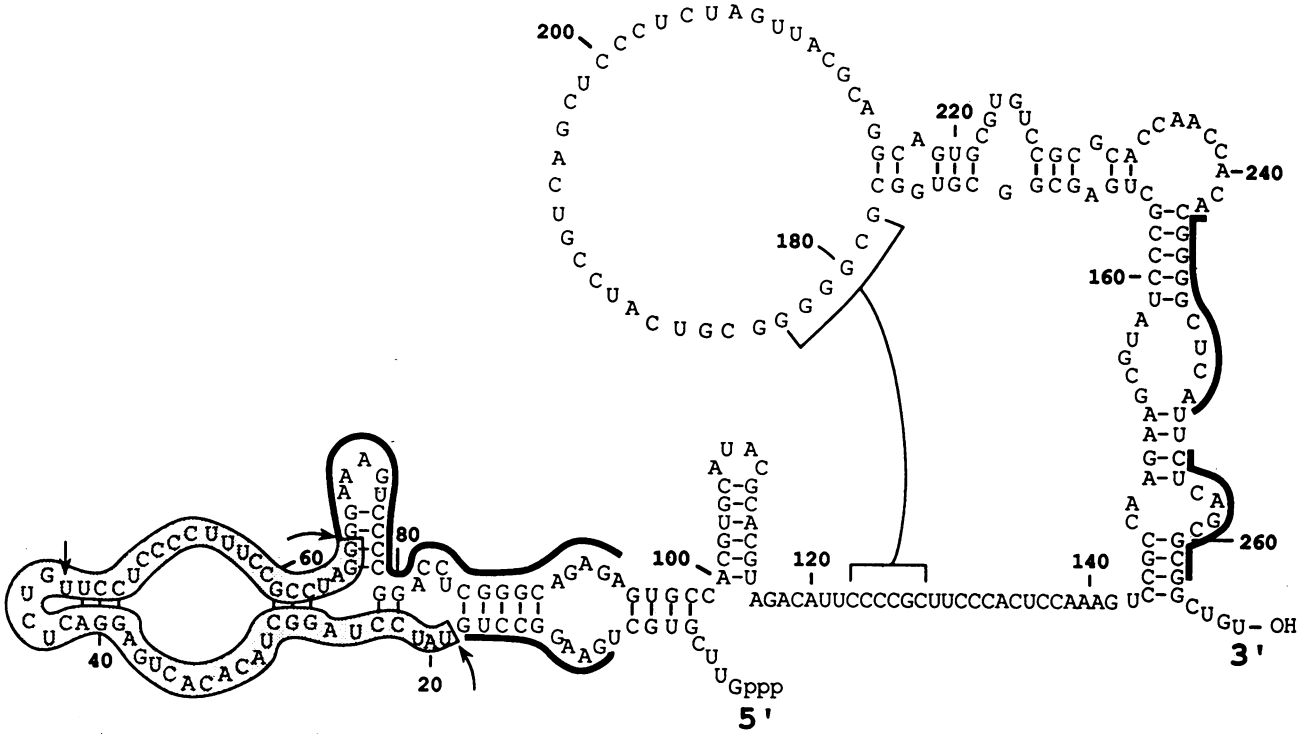

FIG. 9. Secondary structure of the To antigen-binding site in MRP RNA. The region on the MRP RNA secondary structure which is associated with the To antigen is represented by a box. The four regions conserved between MRP RNA and RNase P RNA are indicated by thick lines. Arrows indicate the boundaries of the 50- and 30-nt-long T_1 RNase-resistant RNA fragments in MRP RNA. The secondary structure is essentially as proposed by Topper and Clayton (28).

addition of labeled RNase P RNA, the 40-kDa protein was no longer cross-linked although the minor bands were unaffected (lane 2). The cross-linked 40-kDa protein was immunoprecipitable by anti-To antibodies obtained from two different patients (lanes 3 and 5). These data strongly suggest that the To antigen binds directly to both MRP/7-2 and RNase P RNAs and that the two RNAs compete for binding with each other.

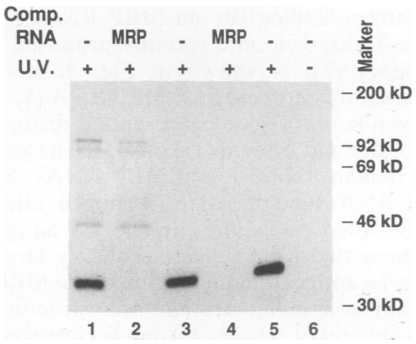

FIG. 10. UV cross-linking of the To antigen bound to RNase P RNA. [α-^{32}P]GTP-labeled RNase P(1–115) RNA was incubated with HeLa cell extract and then UV irradiated. After T_1 RNase digestion, the samples were fractionated on a 10% SDS–polyacrylamide gel, and the dried gel was subjected to autoradiography. The competitor RNA used is indicated above each lane. Lanes: 1 and 2, proteins cross-linked to RNase P(1–115) RNA; 3 and 4, samples immunoprecipitated with anti-To antibodies after UV cross-linking and T_1 RNase digestion; 5, same as lane 3 except that anti-To antibodies from a different patient were used; 6, no UV cross-linking. Molecular mass markers are shown on the right.

DISCUSSION

The antibodies found in patients with autoimmune diseases have proven to be very valuable in understanding the structure and function of many cellular RNPs (20, 27). In our laboratory, one serum, designated anti-To, has been found to contain antibodies against 7-2 and 8-2 RNPs (RNase P) (22). Reimer et al. (24) showed the antigenic protein to be a 40-kDa protein. In addition to the To antiserum, sera from different patients that also precipitate MRP/7-2 RNP have been identified in several laboratories, and the target of these autoimmune sera turned out to be the same antigen, a 40-kDa To protein (16, 18, 22, 23, 24). Besides the To antigen, at least six other polypeptides were reported to be associated in the RNP particle (10). In this study, the region between nt 20 and 65 of MRP RNA was shown to be bound by the To antigen through an RNA-protein interaction. Our results also show that most of this 44-nt-long RNA segment plays a role in binding of the To antigen.

Although we cannot rule out the possibility that more than one polypeptide is involved in protecting the 50-nt region from RNase digestion, it appears from the cross-linking experiment that only the 40-kDa To antigen associates with this region directly. In addition, this whole region is necessary for efficient binding of the To antigen. Since most of the RNA-binding proteins contact a region of less than 10 to 15 nt on RNA, it seems unusual that a relatively small (40-kDa) protein interacts with an RNA region of 45 to 50 nt. This phenomenon could be due to the fact that either the binding

region on RNA is very compact or the protein is very extended.

Since the anti-To antibodies can immunoprecipitate both MRP RNase and RNase P, it was suggested that these two RNA processing enzymes share a common autoantigenic polypeptide or contain the same antigenic epitope. The 5′ end of the human RNase P RNA can be cross-linked to the 40-kDa To antigen, and the MRP/7-2 RNA competes for the same 40-kDa To antigen (Fig. 10). These data suggest and are consistent with the notion that both the MRP/7-2 RNP and RNase P have the 40-kDa To antigen as one of the protein components. Comparison of the primary sequences of MRP RNA and H1 RNA showed four small blocks of conserved sequences, and all of these conserved elements are found in similar stem structures in both H1 RNA and MRP RNA (16) (Fig. 9). It was predicted that these conserved sequences could serve as a common polypeptide-binding site (16). Our data showed the To antigen-binding site in MRP RNA does not reside on any conserved sequence, suggesting that the To antigen recognizes a conserved and presumably common secondary or tertiary structure in human MRP and RNase P RNA. Similarly, RNase P RNA and protein components from human cells and *E. coli* are interchangeable (14), although there is no significant homology in the primary sequences of M1 and H1 RNAs (3). These studies indicate that despite extensive changes in primary sequences, the proteins and RNAs can recognize and bind to appropriate counterparts from distant species.

The antibody against *E. coli* C5 protein cross-reacted with a 40-kDa protein integral in both HeLa RNase P and MRP RNP (21), suggesting that the HeLa 40-kDa protein is the homolog of *E. coli* C5 protein. Isolation of a cDNA clone for the To antigen could be achieved by using an immunoscreening procedure, and comparison of cDNA sequences of the human To antigen and *E. coli* C5 protein may reveal the evolutionary relationship between the To antigen and the C5 protein. Assuming that the human To antigen has homology with its analogs from other sources, it is possible to detect and isolate corresponding RNAs from evolutionarily distant species. Studies from Guthrie's laboratory have shown that the *Xenopus* Sm antigen reconstitutes with yeast U snRNAs (25). It may be possible to immobilize the To antigen on a matrix and allow reconstitution with yeast or plant cell RNAs, wash the column, and elute the bound RNAs.

ACKNOWLEDGMENTS

These studies were carried out by grant CA-10893 from National Cancer Institute.

We thank William Folk for the cloned 5S gene, Sidney Altman for the human H1 RNA gene, Shona Murphy for human 7SK RNA gene, Minyone Finley for HeLa cells, Shashi Gupta for HeLa cell extract, Laura Leo for technical assistance, and Harris Busch for encouragement.

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