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A functional site of the GTPase-associated center within 28S ribosomal RNA probed with an anti-RNA autoantibody

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An anti-RNA autoantibody (anti-28S) was employed to identify structural and functional elements characteristic of a domain termed the 'GTPase center' in eukaryotic 28S ribosomal RNA. This antibody, an inhibitor of ribosome-associated GTP hydrolysis, has a unique property: it binds to the RNA domain of eukaryotes but not to that of prokaryotes. The antibody binding occurred in the presence of Mg^{2+} and protected from chemical modification three conserved bases (U1958, G1960 and A1990) and the base G1959 which is replaced by A in prokaryotic 23S rRNA (A1067 in *Escherichia coli*). *In vitro* substitution of G1959 to A drastically weakened the antibody binding, and the reciprocal substitution, A1067→G of the *E.coli* domain conferred the binding ability. This suggests that the G base determines the specificity of antibody binding. The G1959 was also protected by the association of ribosomes with elongation factor EF-2. The result, together with protection of *E.coli* base A1067 by EF-G [D.Moazed, I.M.Robertson and H.F.Noller (1988) *Nature*, 334, 362–364], suggests that the position of G1959 in eukaryotes and A1067 in prokaryotes constitutes at least part of the factor binding site irrespective of the base replacement during evolution.

Key words: anti-RNA/autoantibody/EF-2/ribosome/ribosomal RNA

Introduction

Antibiotics have been powerful tools for studying functional RNA elements in prokaryotic ribosomal RNA (Moazed and Noller, 1987; Egebjerg *et al.*, 1989; Woodcock *et al.*, 1991). Thiostrepton, one such compound, inhibits elongation factor-dependent processes in protein synthesis (reviewed by Cundliffe, 1980; Gale, 1981) through binding to a specific RNA region within domain II of *Escherichia coli* 23S rRNA (Thompson *et al.*, 1982; Egebjerg *et al.*, 1989). This RNA region is termed the 'GTPase center' or the L11 binding domain and is believed to participate in triggering the GTPase reaction on elongation factors EF-Tu and EF-G, members of the G protein superfamily (Bourne *et al.*, 1991). A residue A1067 within this RNA domain plays a pivotal role in thiostrepton binding (Thompson *et al.*, 1982, 1988; Thompson and Cundliffe, 1991). The A base at this position is well preserved in prokaryotes (Gutell and Fox, 1988). Studies by cross-linking (Sköld, 1983) and chemical footprinting (Moazed *et al.*, 1988) have shown that A1067 directly interacts with elongation factor EF-G.

The GTPase center is evolutionarily conserved to a considerable extent in primary and secondary structures even between prokaryotes and eukaryotes. The conservation of the GTPase center is also shown by heterologous interaction of an *E.coli* protein, L11; this protein directly binds to the RNA domain from archaebacteria (Beauclerk *et al.*, 1985), yeast and mouse (El-Baradi *et al.*, 1987). In spite of such conservation of the RNA domain, eukaryotic ribosomes are insensitive to thiostrepton. The base at the position equivalent to *E.coli* A1067 is changed to G in eukaryotes (Gutell and Fox, 1988), suggesting that this base replacement is at least partly responsible for the thiostrepton insensitivity. Thompson *et al.*, however, suggested that this insensitivity cannot be attributed solely to the A→G base substitution (Thompson *et al.*, 1988) and is possibly due to the exchange of L11 protein with the eukaryotic homolog (Thompson *et al.*, 1993). Thus, thiostrepton has served as an informative probe for analyzing structural differences in the functional domain between prokaryotes and eukaryotes.

We recently found a new ligand to the eukaryotic GTPase center, anti-28S autoantibody, in sera of patients with systemic lupus erythematosus (Uchiumi *et al.*, 1991). This antibody binds to the eukaryotic rRNA with much higher affinity than to the prokaryotic rRNA. This binding specificity is in contrast to that of thiostrepton. Anti-28S strongly inhibits interaction of eukaryotic ribosomes with elongation factors and the associated GTP hydrolysis, but not their peptidyltransferase activity. In this study, we use this antibody as a probe to identify structural and functional elements characteristic of the eukaryotic RNA domain. Our results strongly suggest that the guanine base at the position equivalent to *E.coli* A1067 determines an identity of the eukaryotic GTPase center.

Results

Anti-28S antibody obtained from patients with autoimmune disease strongly inhibits the elongation factor-dependent GTPase activity of eukaryotic ribosomes. This antibody specifically protects a fragment containing residues 1944–2002 of human 28S rRNA against RNase T1 digestion (Uchiumi *et al.*, 1991). This region corresponds to residues 1052–1110 covering the GTPase center of *E.coli* 23S rRNA. To determine sites involved in interaction with the antibody at the nucleotide level, we used chemical probing methods using dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-*p*-toluene sulfonate (CMCT) and kethoxal (KE) (Moazed and Noller, 1986). The position and extent of chemical modification were monitored by

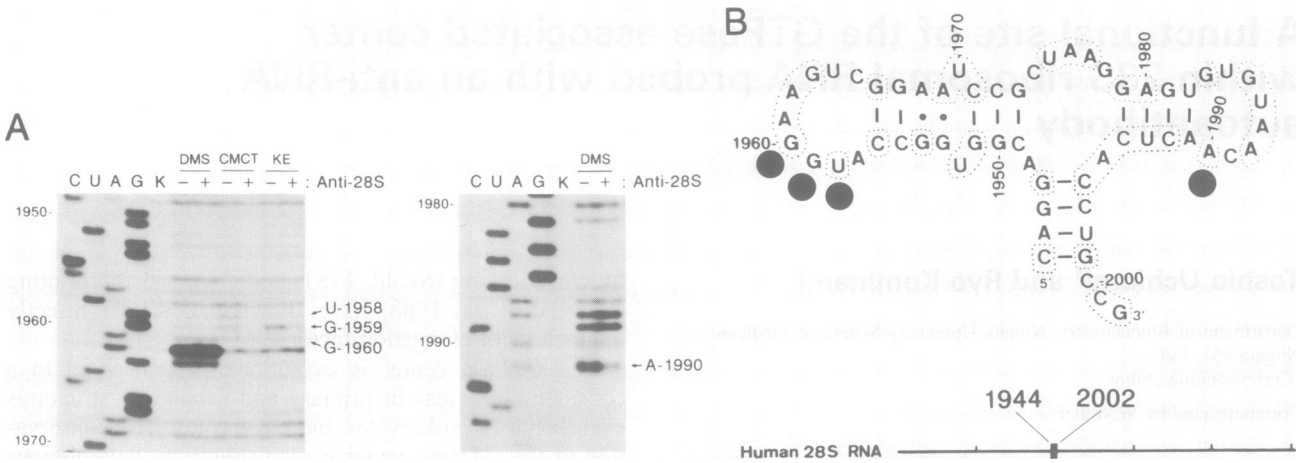

Fig. 1. Protections of bases in the GTPase center by the binding of anti-28S antibody. (**A**) Autoradiographs of gels containing primer extension products and sequencing ladders. Protected sites of U1958, G1959 and G1960 (left) and A1990 (right) are shown. The 28S rRNA was incubated in the presence (+) or absence (–) of anti-28S IgG as described in Materials and methods. The samples were modified with dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-*p*-toluene sulfonate (CMCT) and kethoxal (KE). Lane K, a control reverse transcription sample using unmodified template; lanes C, U, A and G, samples terminated with respective dideoxynucleotides. The nucleotide numbering of human 28S rRNA (Gonzalez *et al.*, 1985) is utilized here. Protections of the four bases were reproducible in three experiments, although the G1960 protection was not striking compared with the others in all these experiments. (**B**) Positions of the bases protected by anti-28S antibody (●) on a possible secondary structure of the GTPase center (Gorski *et al.*, 1987). Nucleotides identical to those of the equivalent positions in *E.coli* 23S rRNA are enclosed with dotted lines.

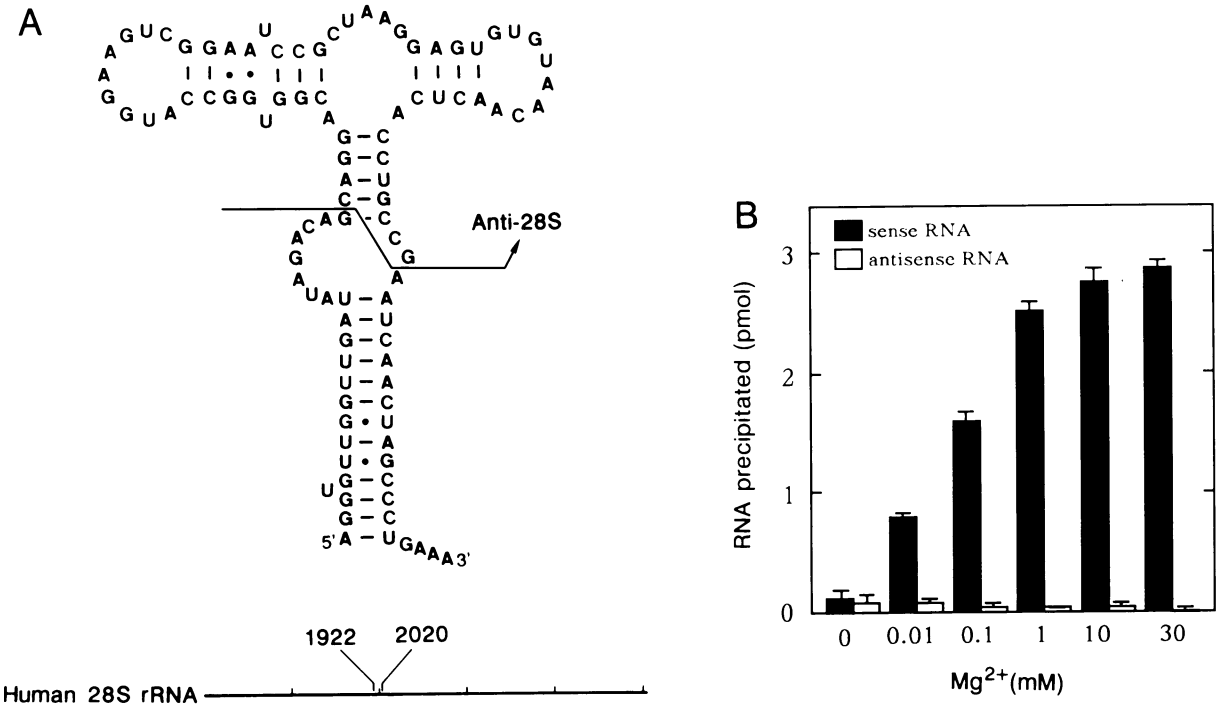

Fig. 2. Effect of Mg^{2+} on binding of anti-28S to the immunoreactive RNA domain. (**A**) A possible secondary structure (Gorski *et al.*, 1987) of the synthetic RNA fragment covering the GTPase center of human 28S rRNA. The upper half marked with an arrow is the region protected by anti-28S antibody against RNase T1 digestion (Uchiumi *et al.*, 1991). (**B**) Immunoprecipitation of the transcript containing residues 1922–2020 of human 28S rRNA and its antisense RNA in indicated concentrations of $MgCl_2$. Each RNA sample (10 pmol) was incubated with 5 μg of anti-28S IgG and the antibody-bound RNA was then recovered with protein A–Sepharose.

primer extension with reverse transcriptase, which paused at the modified bases. Binding of anti-28S to 28S rRNA resulted in protection of four bases, U1958, G1959, G1960 and A1990; the reactivity of CMCT was reduced in the former three bases, and that of KE and DMS was reduced in G1959 and A1990, respectively (Figure 1A). The three bases U1958, G1959 and G1960 are located in the loop containing residues 1957–1965 and A1990 is located in the loop of residues 1983–1990 (the left and right loops of the secondary structure in Figure 1B, respectively). These protections were also observed in intact 60S ribosomal subunits (not shown). On the other hand, we did not observe any antibody-dependent protection of phosphates in the RNA backbone against modification

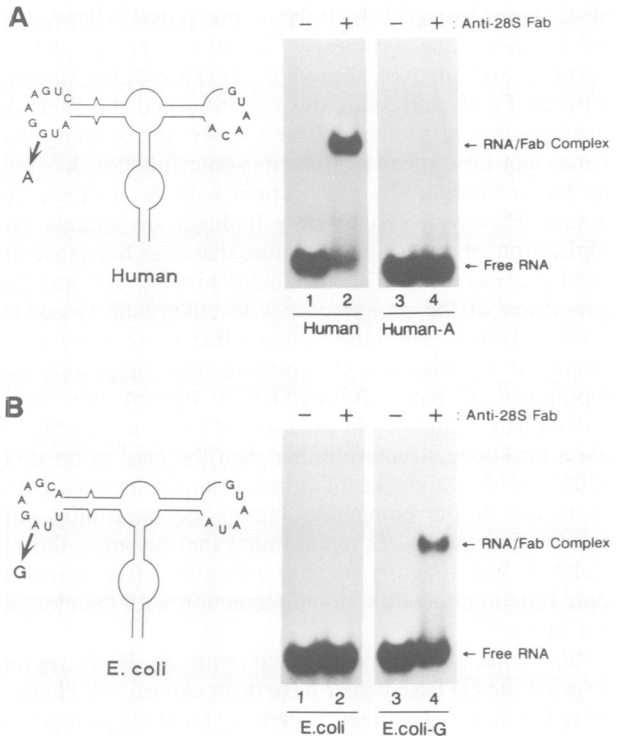

Fig. 3. Effect of base substitution on anti-28S binding: G1959 to A of the human GTPase center (**A**) and A1067 to G of the equivalent *E.coli* domain (**B**). Diagrams illustrate the position of these base substitutions. The right panels show gel retardation assays using the wild-type RNAs (lanes 1 and 2 in both A and B) and their mutants (lanes 3 and 4) as probes: lanes 1 and 3, anti-28S Fab not added; lanes 2 and 4, anti-28S Fab added.

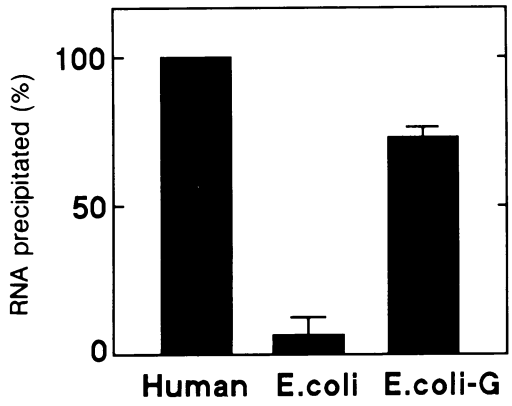

Fig. 4. Antibody binding capacity of the *E.coli* G1067 mutant RNA. The transcripts (10 pmol) containing residues 1922–2020 of human 28S rRNA (Human), residues 1029–1127 of *E.coli* 23S rRNA (*E.coli*) and its G1067 mutant (*E.coli*-G) were each incubated with 5 μg of anti-28S IgG in 5 mM $MgCl_2$, 300 mM KCl and 20 mM Tris–HCl (pH 7.5), and then immunoprecipitation was performed with protein A–Sepharose as described in Materials and methods. Data represent the means of three assays.

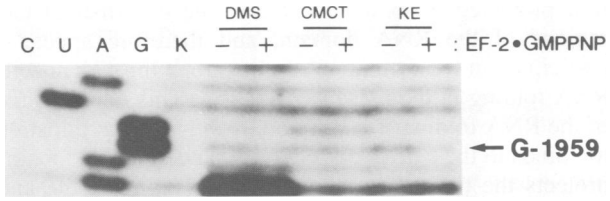

Fig. 5. Protection of the base G1959 of 28S rRNA by the binding of EF-2 to ribosomes. The 80S ribosomes were incubated in the presence (+) or absence (–) of EF-2, and chemical modification and primer extension were performed as described in Materials and methods. Lane notations are as described in the legend to Figure 1A. The G1959 protection was reproducible in three experiments.

with ethylnitrosourea (our unpublished observation). These results suggest that the anti-28S antibody binds to 28S rRNA on four bases within two separate loop regions of the GTPase center.

We synthesized a 99 nucleotide RNA fragment covering the GTPase center by *in vitro* transcription (Figure 2A) and used it for further analysis. This fragment retains the ability to bind to mammalian ribosomal protein L12 and a P protein complex (Uchiumi and Kominami, 1992) as well as anti-28S antibody (Chu *et al.*, 1991; Uchiumi *et al.*, 1991). The binding of anti-28S to this transcript required Mg^{2+} (Figure 2B). At Mg^{2+} concentrations higher than 1 mM, 5 μg of anti-28S IgG immunoprecipitated ~3 pmol of the RNA fragment, but none of its antisense RNA. Binding of L12 protein to the RNA also required Mg^{2+} (not shown). These results suggest that Mg^{2+} induces a unique RNA conformation of the GTPase center, resulting in the protected four bases being brought close together, and that the structure forms an autoimmune epitope (discussed later). A similar folding model was also proposed for the equivalent domain in *E.coli* 23S rRNA (Egebjerg *et al.*, 1990).

The three bases U1958, G1960 and A1990 protected by anti-28S (Figure 1B) are well conserved from prokaryotes to eukaryotes. In contrast, the remaining base (G1959) is unique to eukaryotes; the equivalent position in prokaryotic 23S rRNA has A (A1067 in *E.coli*) which is required for efficient binding of thiostrepton (Thompson *et al.*, 1988; Thompson and Cundliffe, 1991). We therefore speculated that the base G1959 participates in anti-28S binding specific to eukaryotic 28S rRNA. To test this possibility, we examined the effect of the G→A base substitution on anti-28S binding. The substitution drastically weakened the binding to anti-28S Fab fragments, as revealed by gel retardation assay (Figure 3A). The reciprocal substitution, A1067→G, in the *E.coli* RNA domain greatly increased the Fab binding (Figure 3B). The binding capacity of the G1067 mutant RNA was estimated by immunoprecipitation using anti-28S IgG, which showed 75% of that for the human wild-type RNA (Figure 4). These results strongly suggest that G1959 is responsible for the specificity of anti-28S binding to the eukaryotic GTPase center.

G1959 was also protected by eukaryotic elongation factor EF-2 against modification with KE, when complexes between 80S ribosomes and EF-2 were stabilized with the non-hydrolyzable GTP analog GMPPNP (Figure 5). No such protection was observed in the absence of GMPPNP (not shown). The finding that EF-2 and anti-28S both protect G1959 can explain the inhibition by anti-28S of the interaction between ribosomes and EF-2. The EF-2-dependent protection is consistent with the previous report that prokaryotic EF-G protects A1067 of *E.coli* 23S rRNA (Moazed *et al.*, 1988). These data suggest that the positions of G1959 in eukaryotes and A1067 in prokaryotes are required for action of the translocation factors EF-2 and

EF-G, respectively, and therefore the two positions are functionally equivalent.

Discussion

The present study demonstrates that the anti-28S autoantibody recognizes base G1959 of 28S rRNA, a site for interaction with EF-2. The G base at this position within the large subunit rRNA is conserved throughout all determined eukaryotic rRNA sequences except those of *Giardia*, a unicellular intestinal parasite lacking mitochondria (van Keulen *et al.*, 1992). The base at the equivalent position in prokaryotic 23S rRNA is changed to A which is not recognized by the antibody. This binding specificity of anti-28S exhibits a striking contrast with that of thiostrepton which binds to the prokaryotic GTPase center. Thiostrepton shows a 10-fold higher binding affinity to *E.coli* 23S rRNA than to a mutant with the G1067 substitution (Thompson and Cundliffe, 1991) and to yeast 26S rRNA (Thompson *et al.*, 1993).

In addition to the base G1959, anti-28S also protected three conserved bases, U1958, G1960 and A1990. These four protected bases are located in the two distinct loop regions of the RNA domain, and therefore appear to participate in the formation of an epitope through specific RNA folding in the presence of Mg^{2+}. This interpretation of the RNA folding is supported by the fact that thiostrepton binds to the equivalent prokaryotic domain and mainly protects the two loop regions corresponding to the anti-28S-protected regions (Egebjerg *et al.*, 1989); the three sites protected by thiostrepton coincide with the positions of G1959, G1960 and A1990 in the eukaryotic domain. Furthermore, our finding that the substitution A1067→G in the *E.coli* GTPase center conferred the anti-28S binding ability, strengthens the view of a highly conserved structural feature of the GTPase center between eukaryotes and prokaryotes except the difference of the bases G1959 and A1067, respectively.

The two ligands thiostrepton and anti-28S inhibit elongation factor-dependent GTP hydrolysis associated with prokaryotic and eukaryotic ribosomes, respectively (Cundliffe, 1980; Uchiumi *et al.*, 1991), which provides evidence for functional equivalence of the target RNA sites in the two kingdoms. The functional conservation of the GTPase center was also shown by exchanging the domain between *E.coli* and yeast that did not show gross loss of ribosomal function (Musters *et al.*, 1991; Thompson *et al.*, 1993), although the activities of these hybrid ribosomes were examined in the presence of inseparable wild-type ribosomes. Footprinting studies by Moazed *et al.* (1988) and by us (present study) have shown that elongation factors, EF-G in prokaryotes and its eukaryotic homolog EF-2, protect an equivalent position within the GTPase center, although the base in this position is changed from A1067 in the prokaryotic sequence to G1959 in the eukaryotic one. This suggests that the A1067/G1959 position plays the equivalent role in the elongation factor-dependent ribosomal function.

The functional significance of the A→G base replacement during evolution is not clear at present. *Escherichia coli* ribosomes containing an A1067→G mutation and mutations to C and to U showed no detectable loss of EF-G-dependent GTP hydrolysis, nor poly(U)-dependent polyphenylalanine synthesis (Thompson *et al.*, 1988). Besides, exchange of the whole GTPase RNA domain between *E.coli* and yeast did not influence the function much, as described above. These observations imply that it may not be critical for the ribosome function whether the factor binding site is occupied with A or G (or the others). However, evolutionary findings are against this implication: all ribosomes in nature that have been investigated possess A or G at the factor binding site and the conversion of the prokaryotic A to eukaryotic G occurs at this position. The facts suggest that these bases have functional significance. Our present data suggesting the importance of bases A1067/G1959 support this view. Although the paradox may be resolved by the interpretation that a backbone structure rather than the base at position A1067/G1959 might be of primary importance as a site for action of the elongation factor, the possibility still remains that the A→G replacement that occurred during evolution had a significant, but unknown, effect on ribosome function, most likely on interaction with the elongation factor.

Ribosomal proteins associated with an RNA region covering the GTPase center have been extensively characterized in *E.coli* ribosomes. Protein L11 and the pentameric complex $L10(L7/L12)_4$ cooperatively bind to the RNA domain (Beauclerk *et al.*, 1984; Egebjerg *et al.*, 1990). A number of functional studies have shown that L7/L12 protein plays important roles in the interaction of ribosomes with EF-G and EF-Tu and in the associated GTPase activity (reviewed by Möller and Maassen, 1986). L11 is also involved in interaction with EF-G (Maassen and Möller, 1974, 1978; Stark *et al.*, 1980). On the other hand, eukaryotic homologs of the L7/L12 protein (Möller *et al.*, 1975; Sanchez-Madrid *et al.*, 1981; MacConnell and Kaplan, 1982; Uchiumi *et al.*, 1990) and of L11 (El-Baradi *et al.*, 1987; Uchiumi and Kominami, 1992) have been demonstrated to play equivalent functional roles. It is therefore suggested that the A1067/G1959 RNA region, together with these ribosomal proteins, constitutes a functional site involved in the elongation factor-dependent process of translation within ribosomes.

Anti-28S activity has been detected in sera from 17% of patients with systemic lupus erythematosus (T.Sato, T.Uchiumi and R.Kominami, manuscript in preparation), although the etiology of the production of this antibody remains obscure. Anti-28S antibodies from two patients examined so far show similar RNA binding properties: requirement of Mg^{2+} and recognition of the base G1959. Anti-28S used here is polyclonal, but it seems likely that a single species of antibody recognizes the GTPase center because of the following observations. First, a clear single band of the RNA–Fab complex was observed on the gel (Figure 3A), regardless of the amount of Fab added. Second, a mutation in one loop lost the antibody binding ability (Figure 3A and unpublished experiments).

Human autoantibodies have contributed to elucidation of cellular processes such as RNA splicing and DNA replication (reviewed by Tan, 1989, 1991). The present study shows that the autoantibody serves as a probe of functional structure in eukaryotic rRNA as antibiotics have done in prokaryotic rRNA. The data on anti-28S

therefore support the hypothesis that human autoantibodies are regarded as 'reporter molecules' for the functional site of subcellular macromolecules (Tan, 1989).

Materials and methods

Ribosomes, ribosomal RNA and EF-2

High salt-washed 80S ribosomes and 28S rRNA were prepared from rat liver (Uchiumi *et al.*, 1991). EF-2 was purified from pig liver (Iwasaki and Kaziro, 1979; Uchiumi *et al.*, 1986).

Anti-28S autoantibody

Patients' sera containing anti-28S antibody were obtained from K.Elkon (Cornell University Medical College) and T.Sato (Niigata University School of Medicine). The anti-28S IgG and its Fab fragments were prepared as described previously (Uchiumi and Kominami, 1992).

Chemical modification and primer extension

The 28S rRNA–antibody complex was formed by incubation at 30°C, 10 min in 50 μl of solution containing 10 pmol of 28S rRNA, 40 μg of anti-28S IgG, 50 mM potassium cacodylate (pH 7.2), 5 mM $MgCl_2$ and 100 mM KCl. The ribosome–EF-2 complex was formed by incubation at 37°C for 5 min in 50 μl of a buffer containing 10 pmol of 80S ribosomes, 20 pmol of EF-2, 50 mM potassium cacodylate (pH 7.2), 0.1 mM guanyl-5′-yl imidophosphate (GMPPNP), 5 mM $MgCl_2$, 50 mM KCl and 0.2 mM dithiothreitol. For CMCT modification, the incubation volume was 30 μl and 50 mM potassium borate (pH 8.0) was used instead of potassium cacodylate. Chemical modification was started by addition of DMS (1 μl, 1:4 dilution in ethanol), CMCT (20 μl, 42 mg/ml in modification buffer), or KE (2.5 μl, 37 mg/ml in water), followed by incubation at 30°C for 20 min. RNA extraction, primer extension and gel electrophoresis were performed as described by Moazed and Noller (1986). The primer used was 5′-GTATGGGCCCGACGCTCCAG-3′, which is complementary to residues 1949–1968 of rat 28S rRNA (Chan *et al.*, 1983) and corresponds to residues 2031–2050 of human 28S rRNA (Gonzalez *et al.*, 1985). The nucleotide numbering of human 28S rRNA is utilized instead of that of rat 28S rRNA used here, since sequence in this region is identical in all known mammalian 28S rRNA.

In vitro RNA synthesis

The DNA fragments corresponding to residues 1922–2020 of human 28S rRNA and to residues 1029–1127 of *E.coli* 23S rRNA were amplified using the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) and inserted into *Hin*dIII and *Xba*I sites of an expression vector, pSPT18 (Uchiumi *et al.*, 1991). Base substitution was performed by oligonucleotide-directed mutagenesis using PCR (Higuchi *et al.*, 1988). The plasmid DNAs were linearized with *Xba*I and transcribed with SP-6 polymerase in the presence of [^{32}P]UTP (Uchiumi *et al.*, 1991). The antisense version of the human wild-type RNA was synthesized with T7 polymerase after linearization of the plasmid with *Hin*dIII. Specific activity of the transcripts used was 960 c.p.m./pmol for the sense RNA and 1480 c.p.m./pmol for the antisense RNA.

Immunoprecipitation

Each labeled RNA fragment (10 pmol) was incubated with 5 μg of anti-28S IgG in 100 μl of a buffer containing 300 mM KCl, 20 mM Tris–HCl (pH 7.5), 5 μg of *E.coli* tRNA with various concentrations of $MgCl_2$ at 30°C for 10 min. Each sample was mixed at 4°C for 1 h with 1 mg of protein A–Sepharose (Pharmacia LKB Biotechnology) which is sufficient for complete adsorption of input IgG. The antibody-bound beads were then washed four times with buffer containing the same concentration of $MgCl_2$, and then the radioactivity was counted.

Gel retardation assay

Each RNA fragment (10 pmol) was incubated in 10 μl of solution containing 10 mM $MgCl_2$, 300 mM KCl, 20 mM Tris–HCl (pH 7.5) in the presence or absence of 15 μg of anti-28S Fab fragments at 30°C for 10 min. Samples were electrophoresed through 6% polyacrylamide gel in the presence of 5 mM $MgCl_2$ as described previously (Uchiumi and Kominami, 1991).

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