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Characterization and tRNA Recognition of Mammalian Mitochondrial Seryl-tRNA Synthetase*

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Animal mitochondrial protein synthesis systems contain two serine tRNAs (tRNAs^{Ser}) corresponding to the codons AGY and UCN, each possessing an unusual secondary structure; the former lacks the entire D arm, and the latter has a slightly different cloverleaf structure. To elucidate whether these two tRNAs^{Ser} can be recognized by the single animal mitochondrial seryl-tRNA synthetase (mt SerRS), we purified mt SerRS from bovine liver 2400-fold and showed that it can aminoacylate both of them. Specific interaction between mt SerRS and either of the tRNAs^{Ser} was also observed in a gel retardation assay. cDNA cloning of bovine mt SerRS revealed that the deduced amino acid sequence of the enzyme contains 518 amino acid residues. The cDNAs of human and mouse mt SerRS were obtained by reverse transcription-polymerase chain reaction and expressed sequence tag data base searches. Elaborate inspection of primary sequences of mammalian mt SerRSs revealed diversity in the N-terminal domain responsible for tRNA recognition, indicating that the recognition mechanism of mammalian mt SerRS differs considerably from that of its prokaryotic counterpart. In addition, the human mt SerRS gene was found to be located on chromosome 19q13.1, to which the autosomal deafness locus DFNA4 is mapped.

The fidelity of protein synthesis relies on the specific attachment of amino acids to their cognate tRNA species. This process is catalyzed by aminoacyl-tRNA synthetase (ARS),[1] which discriminates with high selectivity among many structurally similar tRNAs and amino acids (1, 2). To avoid misacylation of tRNAs from any of the 19 noncognate groups within each tRNA sequence, tRNAs possess identity elements that are unambiguously recognized only by the cognate synthetase. These recognition elements are most commonly located in the tRNA anticodon, the acceptor stem and the "discriminator" base at position 73 (2–5). However, in the *Escherichia coli* system, several biochemical approaches have revealed that identity elements of the tRNA^{Ala} and tRNA^{Ser} isoacceptors are not located in the anticodon and discriminator (4, 6–9). In the case of tRNA^{Ala}, the G3-U70 base pair in the acceptor stem is a major determinant of tRNA^{Ala} identity (8, 9).

tRNAs can be divided into two groups according to the length of the extra arm: those with a short extra arm of 4–5 nucleotides (type 1) and those with a long extra arm of at least 11 nucleotides (type 2) (10). tRNAs that belong to the latter type are restricted to only three species in prokaryotes: tRNAs^{Tyr}, tRNAs^{Leu}, and tRNAs^{Ser}, and two species in eukaryotes: tRNAs^{Leu} and tRNAs^{Ser} (Fig. 1). Biological experiments have shown that the long extra arm of *E. coli* tRNA^{Ser} contributes the most to the specificity of serylation (6, 7, 11–13). Moreover, Himeno *et al*. (6) reported that the different orientations of the long extra arms in these three species are a key element for discrimination by *E. coli* seryl-tRNA synthetase (SerRS), which is a plausible reason why neither the length nor the sequence of the extra arm is conserved among tRNA^{Ser} isoacceptors (14).

These results are consistent with the crystallographic structures of SerRS-tRNA^{Ser} complexes from *E. coli* and *Thermus thermophilus* (15–17). tRNA^{Ser} binds across both subunits of the dimer. The terminal part of the acceptor end contacts the active site of one subunit, whereas the rest of the tRNA^{Ser} is bound to the other subunit, in which is located the N-terminal long helical arm-like domain that is important for recognition of the long extra arm and TΨC loop of tRNA^{Ser}. In eukaryotic systems, cytoplasmic tRNA^{Ser} also has a long extra arm (Fig. 1), and several biochemical studies on *Saccharomyces cerevisiae* and human tRNAs^{Ser} have indicated that the major identity element of tRNA^{Ser} is located in this arm (18–21). Thus, it can be concluded that the major identity element of both prokaryotic and eukaryotic cytoplasmic tRNAs^{Ser} for specific recognition by SerRS is located in the characteristic long extra arm. The recognition mechanism using the long extra arm

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB029947 (bovine mt SerRS), AB029948 (human mt SerRS), and AB029949 (mouse mt SerRS).

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[1] The abbreviations used are: ARS, aminoacyl-tRNA synthetase; SerRS, seryl-tRNA synthetase; mt, mitochondrial; tRNA$^{Ser}_{GCU}$, serine-specific tRNA corresponding to the anticodon GCU; tRNA$^{Ser}_{UGA}$, serine-specific tRNA corresponding to the anticodon UGA; tRNA$^{Phe}_{GAA}$, phenylalanine-specific tRNA corresponding to the anticodon GAA; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; np, nucleotide position(s); bp, base pair(s); UTR, untranslated region; RT, reverse transcription; PCR, polymerase chain reaction; EST, expressed sequence tag; FPLC, fast protein liquid chromatography; NRF, nuclear respiratory factor.

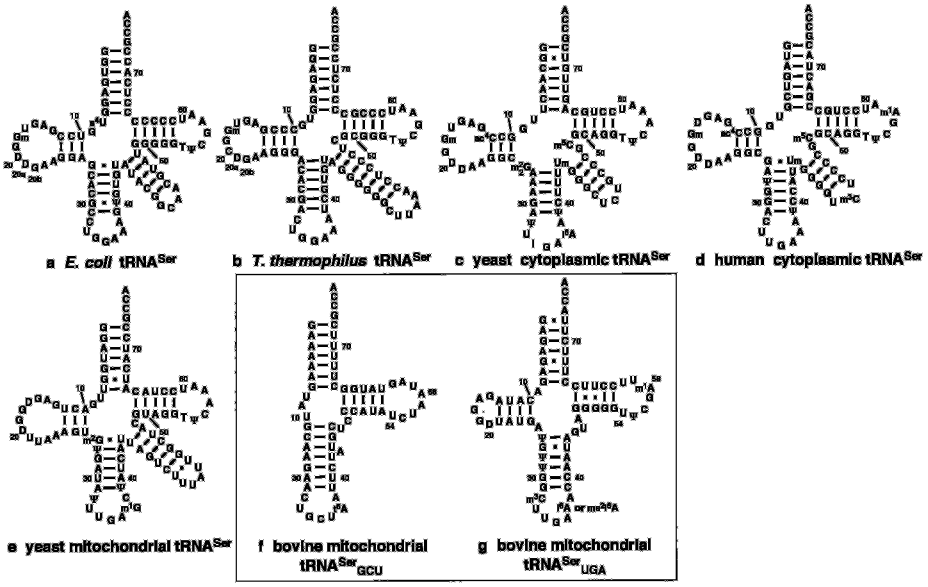

FIG. 1. **Secondary structures of tRNAs^{Ser} from several organisms.** The base and tRNA numberings conform to the rule proposed by Sprinzl *et al*. (14). *a*, *E. coli* tRNA^{Ser} RS1663. *b*, *T. thermophilus* tRNA^{Ser} determined by Biou *et al.* (16). *c*, *S. cerevisiae* cytoplasmic tRNA^{Ser} RS6281. *d*, human cytoplasmic tRNA^{Ser} RS4001. *e*, *S. cerevisiae mt tRNA^{Ser} RS9991*. *f*, bovine mt tRNA$^{Ser}_{GCU}$ RS5360. *g*, bovine mt tRNA$^{Ser}_{UGA}$ determined by Yokogawa *et al.* (23).

appears evolutionarily conserved in the tRNA^{Ser}-SerRS system.

On the other hand, because all animal mitochondrial (mt) tRNAs^{Ser} possess a short extra arm (10), the recognition mechanism described above would not be applicable in the mt system. Also, animal mt tRNA^{Ser} isoacceptors differ structurally from those of other mt tRNAs; the tRNA^{Ser} specific for codons AG*Y* (*Y* = C or U; tRNA$^{Ser}_{GCU}$) lacks the entire D arm (22), whereas the isoacceptor for codons UC*N* (*N* = A, G, C, or U; tRNA$^{Ser}_{UGA}$) lacks the invariant U8 between the acceptor and D stems and has a small D loop and an extended anticodon stem consisting of 6 base pairs (23) (Fig. 1). The primary and secondary structures of these two tRNAs^{Ser} are too different for a common region in these tRNAs to be identified. To date, it remains unclear whether the single ARS recognizes two cognate tRNAs with apparently different structures, like animal mt tRNAs^{Ser}. It thus is of interest to ascertain whether the single mitochondrial seryl-tRNA synthetase (mt SerRS) recognizes the two distinct tRNA^{Ser} isoacceptors and, if so, what kind of tRNA recognition mechanism is needed for the system.

To obtain information on the recognition mechanism of animal mt SerRS, we previously studied the recognition sites of bovine mt tRNA$^{Ser}_{GCU}$ (24). We have recently undertaken further biochemical investigations to elucidate the recognition mechanism of animal mt SerRS by purifying bovine mt SerRS from bovine liver, cloning its gene, and characterizing the native bovine mt SerRS. The results are presented here.

EXPERIMENTAL PROCEDURES

Materials—Phenylmethylsulfonyl fluoride (PMSF) and DEAE-Sepharose were purchased from Sigma; hydroxyapatite and a protein assay kit were from Bio-Rad; Centriprep-10, Centricon-10, and Microcon-10 were from Amicon; [^{14}C]L-serine (4.4 GBq/mmol) was from NEN Life Science Products; and Superdex 200 prep grade, HiTrap heparin (1 ml), Mono S (HR5/5), and Mono Q (HR5/5) were from Amersham Pharmacia Biotech. Other chemicals were from Wako Pure Chemicals. *E. coli* total tRNAs were from Roche Molecular Biochemicals. Native mt tRNAs^{Ser} and mt tRNA$^{Phe}_{GAA}$ were purified from bovine mitochondria by the selective hybridization method using a solid phase DNA probe as described by Wakita *et al*. (25).

Purification of SerRS from Bovine Liver Mitochondria—Procedures were generally performed at 4 °C; only the FPLC system (Amersham Pharmacia Biotech) was operated at room temperature. For For step 1, digitonin-treated bovine liver mitochondria, isolated mt pellets, and the mt S-30 fraction were prepared as described previously (26, 27). For step 2, fresh S-30 (2800 mg) was applied onto a DEAE-Sepharose column (2.7 × 17.5 cm) equilibrated and washed with Buffer A (20 mM Tris-HCl (pH 7.6), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 6 mM β-mercaptoethanol, 10% glycerol, and 100 μM PMSF), and developed with a linear gradient (1000 ml) from 40 to 500 mM KCl in Buffer A. Fractions (10 ml) were collected at a flow rate of 1.0 ml/min. Active fractions were precipitated with ammonium sulfate (60% saturation). For step 3, the above precipitate was dissolved and dialyzed extensively against Buffer B (10 mM potassium phosphate (pH 7.4), 6 mM β-mercaptoethanol, 10% glycerol, and 100 μM PMSF). The dialyzed sample (360 mg of proteins) was applied onto a hydroxyapatite column (1.5 × 11 cm) equilibrated with Buffer B and developed with a linear gradient (200 ml) from 10 to 200 mM potassium phosphate in Buffer B. Fractions (5 ml) were collected. Aliquots (200 μl) were taken from every second fraction and dialyzed against Buffer C (20 mM Hepes-KOH (pH 7.0), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 6 mM β-mercaptoethanol, 10% glycerol, and 100 μM PMSF) with Microcon 10 to remove phosphate. These were used for the aminoacylation assays. The concentrated sample (5 ml, 55 mg of proteins) collected by Centriprep 10 from active fractions was immediately applied onto a Superdex 200 column (2.5 × 60 cm) equilibrated with Buffer C. For step 4, the column was developed with Buffer C. Fractions (5 ml) were collected at a flow rate of 0.5 ml/min. Active fractions were concentrated with Centriprep 10. This procedure was used in the subsequent steps. For step 5, the concentrated sample (5.6 mg of proteins) was diluted with Buffer D (20 mM Hepes-KOH (pH 7.0), 1 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, and 10% glycerol) 4-fold and immediately applied onto a HiTrap heparin column (1 ml), which was developed with a 25-ml linear gradient from 0 to 500 mM KCl in Buffer D at a flow rate of 0.5 ml/min using a FPLC system. Fractions of 1 ml were collected. For step 6, the sample (0.36 mg) dialyzed against Buffer D with Centricon 10 was immediately applied onto a Mono S column (0.5 × 5 cm) and developed with a 20-ml linear gradient from 0 to 400 mM KCl in Buffer D at a flow rate of 0.5 ml/min by FPLC. Fractions of 1 ml were collected. For step 7, the sample (0.14 mg) dialyzed against Buffer D with Centricon 10 was immediately applied onto a Mono Q column (0.5 × 5 cm) and developed with a 25-ml linear gradient from 0 to 300 mM KCl in Buffer D at a flow rate of 0.5 ml/min by FPLC. Fractions of 1 ml were collected. To check their purity, active fractions were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli (28). The mt SerRS fraction was frozen quickly and stored at −70 °C.

Native PAGE—The tRNA^{Ser}-SerRS complex was formed by incubation at 37 °C for 10 min in a 10-μl aliquot containing 50 mM Tris-HCl (pH 8.5), 15 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermine, about 0.02 A_{260} unit of mt tRNA, and about 0.5 μg of mt SerRS fraction. Native PAGE was done as described by Hornung *et al.* (29), and the gels were stained with both Coomassie Brilliant Blue and toluidine blue to analyze the components of the tRNA^{Ser}-SerRS complex. The band of the complex was cut out and subjected to SDS-PAGE, and the gel was silver-stained.

Determination of mt SerRS Amino Acid Sequence—About 15 μg of the purified mt SerRS was digested with 1 μg of lysyl endopeptidase at 37 °C overnight in a 50-μl aliquot containing 100 mM Tris-HCl (pH 9)

TABLE I
Purification of mitochondrial seryl-tRNA synthetase from bovine liver

Purification step	Protein	Total units	Specific activity	Total recovery	Purification
	mg	*units*	*units/mg*	*%*	*fold*
Mitochondrial extract (S-30)	2800	8000	2.8	100	1
DEAE-Sepharose	460	6800	15	85	5.3
60% Ammonium sulfate precipitation	360	5600	16	70	5.6
Hydroxyapatite	55	3200	64	40	23
Superdex 200	5.6	3200	580	40	210
Heparin	0.36	780	2200	9.8	790
Mono S	0.14	420	3000	5.3	1100
Mono Q	0.032	220	6800	2.8	2400

and 20 mM EDTA. The resultant product was loaded onto a C8 column (2.1 × 30 mm) in a high performance liquid chromatography system and separated at a flow rate of 0.2 ml/min with a 6-ml linear gradient from 0 to 35% acetonitrile containing 0.1% trifluoroacetate and then with a 3-ml linear gradient from 35 to 70% acetonitrile containing 0.1% trifluoroacetate. The amino acid sequence of each separated peptide was determined with an Applied Biosystems 477A/120A protein sequencer. In parallel, the sequences of peptides digested with endoproteinase V8 were obtained according to the method of Cleveland *et al.* (30) with the modifications indicated in Ref. 31.

Assays of Bovine mt SerRS Activity—The assays were carried out at 37 °C for 5 min with reaction mixtures (15 μl) containing 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 60 mM KCl, 2 mM ATP, 10 mM dithiothreitol, 42 μM [^{14}C]L-serine, 0.5 A_{260} unit of *E. coli* total tRNAs, and an appropriate amount of the enzyme fraction (32). One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 pmol of seryl-tRNA^{Ser} for 1 min. The protein concentration was determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

Aminoacylation reactions to determine the kinetic parameters of bovine mt SerRS were carried out at 37 °C in a buffer containing 50 mM Tris-HCl (pH 8.5), 15 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermine, 2 mM ATP, 60 mM KCl, 33 μM [^{14}C]L-serine (5.59 GBq/mmol) purchased from Amersham Pharmacia Biotech, and 5.7 nM purified bovine mt SerRS. Although L-serine was used at the subsaturating concentration, it was slightly above K_m (23 μM) of bovine mt SerRS according to Kumazawa *et al.* (33), and we made compromise between unreliable results because of the concentration around K_m and low counting efficiency because of low specific activity of the labeled L-serine caused by dilution with nonlabeled L-serine (18). The initial rates of aminoacylation were determined by using six different concentrations of native tRNAs^{Ser} ranging from 0.04 to 1.5 μM (0.04, 0.10, 0.30, 0.70, 1.0, and 1.5 μM) for tRNA$^{Ser}_{GCU}$ or from 0.03 to 1.3 μM (0.03, 0.10, 0.25, 0.60, 0.90, and 1.3 μM) for tRNA$^{Ser}_{UGA}$ at a fixed concentration of mt SerRS, which gave reasonable kinetics plots for determining the apparent K_m and k_{cat} values.

cDNA Cloning of Bovine mt SerRS—Partial peptide sequences of bovine mt SerRS were subjected to a BLAST search of the DDBJ/EBI/GenBank™ nucleotide sequence data bases and a human EST clone (accession number T78174) was obtained. A sense primer (np 1207–1227; see Fig. 4) and an antisense primer (np 1453–1472) were designed from the partial region in the clone that was highly identical to the partial peptide sequences of bovine mt SerRS. To obtain the bovine cDNA clone, RT-PCR was performed using these two primers, 2 μg of laboratory stock bovine poly(A)-tailed mRNA, and a TaKaRa RNA PCR kit (AMV) version 2.1. cDNA screening, cloning, and sequencing of the plasmid DNA obtained were done according to Takeuchi *et al.* (34). The 5′-region of the cDNA corresponding to the N-terminal region of the mature mt SerRS was obtained by RT-PCR. First strand cDNA synthesis and first and nested PCR were carried out according to Nakayama (35) with some modifications. A degenerate sense primer (np 103–122) was designed from the N-terminal peptide sequence. Antisense primers (np 1099–1118 and 1123–1143) were designed from the cDNA sequence obtained by cDNA screening and respectively used for first and nested PCR. The predominant PCR product was purified by agarose gel electrophoresis and cloned into a pCR®2.1-TOPO vector (Invitrogen). A Marathon™ cDNA amplification kit (CLONTECH) was used to further determine the 5′-region of the bovine mt SerRS cDNA. Antisense primers (np 151–168 and 126–144) were designed from the 5′-region sequence of the mature mt SerRS and used, respectively, for first and nested PCR. Sequencing was done using a Dye Terminator Cycle sequencing kit (Perkin-Elmer) and an ABI PRISM™310 genetic analyzer.

Determination of Human mt SerRS cDNA Sequence—The major part of the putative human mt SerRS cDNA sequence and the whole putative cDNA sequence of mouse mt SerRS were obtained by connecting several EST clones whose peptide sequences are very homologous to that of bovine mt SerRS. The few unknown regions in the human mt SerRS cDNA were determined by RT-PCR using RT-PCR high (Toyobo). Primers were designed from the determined sequences on both sides.

RESULTS

Purification of mt SerRS and Its Recognition of tRNAs^{Ser}—To elucidate whether the single animal mt SerRS recognizes the two tRNA^{Ser} isoacceptors, which differ considerably in their secondary structures, we purified mt SerRS to homogeneity from bovine liver mitochondria by successive column chromatographies as described under "Experimental Procedures." Only one peak fraction exhibiting serylation activity was observed in each step. The purification scheme resulted in 2400-fold purification of mt SerRS with 2.8% recovery (Table I). In the final step, the serylation activity completely coincided with the Mono Q column absorbance profile (Fig. 2*A*). The molecular mass of mt SerRS was estimated to be about 53,000 Da by SDS-PAGE (Fig. 2*B*). On the other hand, mt SerRS was eluted in the region of a molecular mass exceeding 100,000 Da on Superdex 200 column chromatography (data not shown). Because all the SerRSs known so far have an α_2 subunit structure, bovine mt SerRS is thought to be a dimer.

To ascertain whether the single bovine mt SerRS recognizes the two mt tRNAs^{Ser}, we carried out gel retardation assays and aminoacylation reaction experiments. Fig. 3*A* shows that the main protein band was shifted as a consequence of adding mt tRNA$^{Ser}_{GCU}$ and mt tRNA$^{Ser}_{UGA}$ to mt SerRS, whereas no such shift was observed when mt tRNA$^{Phe}_{GAA}$ was used. Furthermore, the shifted band was found to contain both a 53,000-Da protein and the mt tRNA^{Ser} on the SDS-containing gel (Fig. 3*B*). It was thus demonstrated that the single mt SerRS recognizes and binds to the two tRNA^{Ser} isoacceptors with different structures.

The kinetic parameters of aminoacylation by the purified bovine mt SerRS are shown in Table II. The bovine mt SerRS is seen to aminoacylate the two tRNAs^{Ser} almost equally. The K_m values determined in the present study are rather different from those reported previously using partially purified bovine mt SerRS (36). The present data appear more reasonable because the K_m values for each cognate tRNA^{Ser} in the previous data differ considerably.

Determining the Peptide Sequence of mt SerRS and Its cDNA Cloning—To obtain cDNA clones of mt SerRS, partial peptide sequences were determined. N-terminal sequencing revealed that mt SerRS has two heterologous termini: NH_2-ATERQDRNLLYEHAR and NH_2-ERQDRNLLYEHAR (Fig. 4). Subsequently, five internal peptides were sequenced (Fig. 4) that were subjected to a BLAST search through the human EST data base. The search revealed one EST clone (accession number T78174) containing portions of the two peptide sequences at the C-terminal region of bovine mt SerRS (Fig. 4). For cDNA screening, a cDNA clone was obtained by RT-PCR using bovine mRNA with primers designed from the sequences of this clone

(Fig. 4). The cDNA screening gave one cDNA clone 998 base pairs (bp) in length that corresponded to the C-terminal region (np 892–1889) of mt SerRS (Fig. 4). Subsequently, the N-terminal region was amplified by RT-PCR using a degenerate primer based on the N-terminal peptide sequence, and one cDNA clone 1118 bp in length (np 103–1220) was obtained.

Through 5′-rapid amplifiation of cDNA ends, four clones with identical sequences but different lengths were obtained. The longest cDNA fragment, 210 bp in length, contained one ATG codon. Assuming this to be the initiation codon, the 5′-untranslated region (UTR) consists of only 12 bases. It is possible that another ATG codon further upstream in the 5′-region functions as the initiation codon. However, human mt SerRS has a TAA codon at position −48 in frame that strongly suggests that the relevant ATG codon functions as the initiation codon. Additionally, the initiation context found in both sequences, possessing A at position −3 and G at position 4, conforms to the consensus feature for eukaryotic genes (37) (Fig. 4). These facts strongly suggest that the sole ATG codon found in the cDNA sequence of bovine mt SerRS is the actual initiation codon. It is now clear that the bovine mt SerRS cDNA is composed of at least 12 bp of 5′-UTR, a 1557-bp coding sequence, and 331 bp of 3′-UTR. All the sequences of the five peptide fragments derived from bovine mt SerRS were identified within its complete amino acid sequence (Fig. 4).

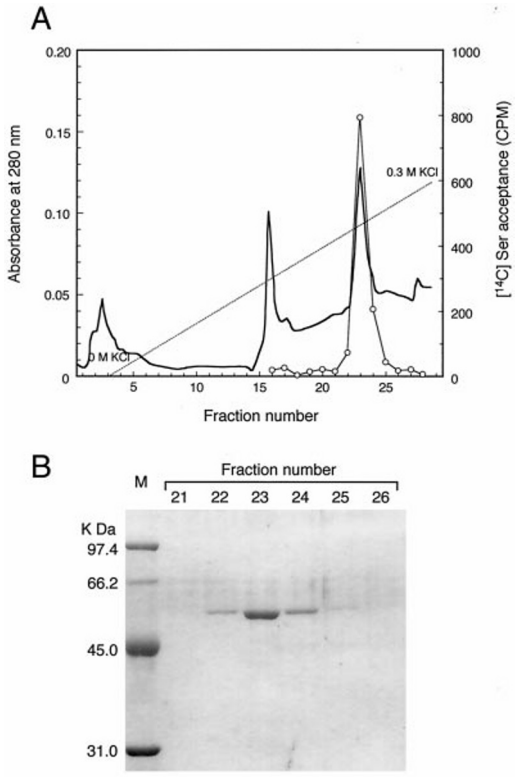

FIG. 2. **Purification of bovine mt SerRS.** *A*, elution profile of mt SerRS in MonoQ column chromatography. The *circles*, the *solid line*, and the *dotted line* show the serylation activity, absorbance at 280 nm, and KCl concentration, respectively. *B*, SDS-PAGE analysis of fractions obtained by MonoQ column chromatography (10-μl samples from fraction numbers 21–26). *Lane M*, molecular mass markers with their sizes indicated in kDa. The gel was stained with Coomassie Brilliant Blue.

Based on the amino acid sequence of the 1557-bp coding sequence, analysis of the mature bovine mt SerRS revealed that the N-terminal 34-amino acid sequence of the precursor protein functions as the targeting peptide (Fig. 4). However, as noted above, two different N-terminal peptide fragments (*i.e.* two different precursor cleavage sites) were observed. This leaves the possibility of alternative cleavage of the mt SerRS precursor by the matrix processing protease (38).

We next confirmed the C-terminal peptide of bovine mt SerRS. After digesting the mt SerRS with trypsin, peptide fragments were analyzed by liquid chromatography/mass spectrometry using electrospray ionization/iontrap mass spectrometry. The C-terminal peptide, LPGQPASS, was identified as a slightly charged ion with an *m/z* of 756.4 Da (data not shown). Peptide fragments generated from digestion by trypsin in $H_2{}^{18}O$ were similarly analyzed. No change in the molecular mass of the relevant fragment was observed, showing that the actual termination is executed at the putative termination codon expected from the bovine mt SerRS cDNA sequence. Thus, it was concluded that the cDNA sequence determined in this work is actually derived from the mature mt SerRS.

cDNA of Mammalian mt SerRS—The putative human mt SerRS cDNA was obtained by connecting human EST clones and RT-PCR (Fig. 5*A*); it is composed of at least 160 bp of 5′-UTR, a 1557-bp coding sequence, and 337 bp of 3′-UTR. The putative mouse mt SerRS cDNA was acquired only by connecting mouse EST clones (Fig. 5*A*); it consists of at least 20 bp of 5′-UTR, a 1557-bp coding sequence, and 281 bp of 3′-UTR.

Information on the position of the human mt SerRS gene on the genome was obtained by subjecting its cDNA sequence to a BLAST search. One of the acquired clones contained the complete human mt ribosomal protein S12 (*MRPS12*) gene (acces-

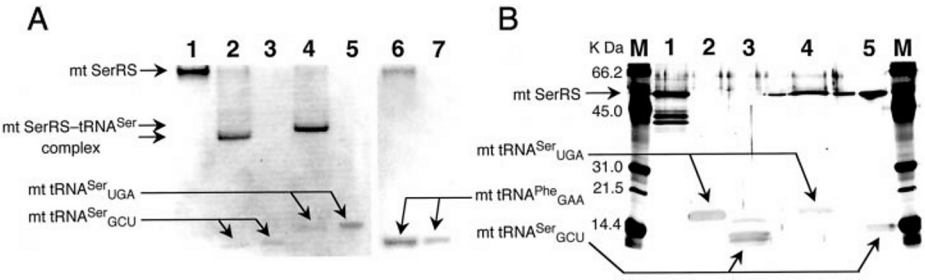

FIG. 3. **PAGE analyses to confirm complex formation between mt tRNAs and mt SerRS.** *A*, native PAGE analysis showing that mt SerRS formed a stable complex with mt $tRNA^{Ser}_{GCU}$ and mt $tRNA^{Ser}_{UGA}$. *Lane 1*, mt SerRS (0.5 μg) alone. *Lane 2*, mt SerRS and 0.02 A_{260} unit of mt $tRNA^{Ser}_{GCU}$. *Lane 3*, mt $tRNA^{Ser}_{GCU}$ (0.01 A_{260} unit) alone. *Lane 4*, mt SerRS and 0.02 A_{260} unit of mt $tRNA^{Ser}_{UGA}$. *Lane 5*, mt $tRNA^{Ser}_{UGA}$ (0.01 A_{260} unit) alone. *Lane 6*, mt SerRS and 0.02 A_{260} unit of mt $tRNA^{Phe}_{GAA}$. *Lane 7*, mt $tRNA^{Phe}_{GAA}$ (0.01 A_{260} unit) alone. *Lanes 6* and *7* were derived from another gel. The gels were stained with Coomassie Brilliant Blue and toluidine blue. *B*, SDS-PAGE analysis of the complex band in *A* with blank lanes between each sample lane. *Lane 1*, mt SerRS (0.5 μg) alone. *Lane 2*, mt $tRNA^{Ser}_{UGA}$ (0.01 A_{260} unit) alone. *Lane 3*, mt $tRNA^{Ser}_{GCU}$ (0.01 A_{260} unit) alone (see below). *Lane 4*, the band of the mt $tRNA^{Ser}_{UGA}$-mt SerRS complex. *Lane 5*, the band of the mt $tRNA^{Ser}_{GCU}$-mt SerRS complex. The gel was run with blank lanes between each $tRNA^{Ser}$-mt SerRS complex lane to prevent carryover from the adjacent lanes, and then it was silver-stained. Because completely purified mt $tRNA^{Ser}_{GCU}$ was used in this work, the *shadows* around the main band and an unknown band appearing above the main band in *lane 3* in *B* are thought to be the artifacts arising from the silver staining because of its high sensitivity. However, further efforts to clarify these phenomena are indispensable.

sion number AF058761). Only the first exon of the human mt SerRS gene was found in the sequence of the above-mentioned EST clone (Fig. 5*B*). It is of interest that one of the putative binding sites of nuclear respiratory factor-1 (NRF-1), one of the transcription factors, is located in the coding sequence of human mt SerRS in the opposite direction (39).

Comparison of Amino Acid Sequences of Mammalian mt SerRSs with Those of Other SerRSs—According to the coding sequence of bovine mt SerRS, the predicted translation product has 518 amino acids. Mammalian mt SerRS has a long C-terminal sequence, but it is different from a basic C-terminal lysine-rich extension found in all eukaryotic cytoplasmic SerRSs that may be important for both stability and optimal substrate recognition (40). Though it displays only 28–34% homology with both prokaryotic and eukaryotic cytoplasmic counterparts and even with yeast mt SerRS, relatively high homology is observed in the C-terminal region among all the sequences (Fig. 6).

Analyses of the crystal structures of *E. coli* and *T. thermophilus* SerRSs revealed that prokaryotic SerRS discriminates $tRNA^{Ser}$ from other noncognate tRNAs by means of the long helical arm located in the N-terminal region and interacts with serine and ATP by the residues mainly located in the C-terminal region, in particular in motifs 2 and 3, which are highly conserved active sites among class II ARSs (Fig. 6). The high homology in the C-terminal region between prokaryotic SerRS and mammalian mt SerRS indicates that the C-terminal region also functions as the catalytic core in the latter, whereas the low homology in the N-terminal region accords well with the lack of the long extra arm in most animal mt $tRNAs^{Ser}$ from the perspective of the co-evolution of ARS and its cognate tRNA.

TABLE II
Kinetic parameters in aminoacylation of bovine mitochondrial serine tRNAs

Experimental conditions for aminoacylation are described under "Experimental Procedures."

Substrate	K_m	k_{cat}	k_{cat}/K_m
	μM	$1/s$	$1/(\mu M \times s)$
$tRNA^{Ser}_{GCU}$	0.37 ± 0.10	0.35 ± 0.10	0.95
$tRNA^{Ser}_{UGA}$	0.22 ± 0.03	0.63 ± 0.06	2.86

DISCUSSION

Our work has shown that the two distinct mitochondrial $tRNA^{Ser}$ isoforms are recognized by a single bovine mt SerRS, a 54,635-Da polypeptide. The low homology in the N-terminal region between mammalian mt SerRS and other SerRSs is consistent with the recognition mechanism of mammalian mt SerRS differing from that of prokaryotic SerRSs so far elucidated. On the other hand, the high homology in the C-terminal region is indicative of the conservation of the catalytic core in mammalian mt SerRSs, except for some residues involved in the interaction with the acceptor stem of tRNA. This local difference seems to be in agreement with the unique recognition mechanism of mammalian mt SerRS. Relevant details of our inspection of the C-terminal region of bovine mt SerRS are as follows.

In the crystal structure of *T. thermophilus* SerRS, ATP is bound to the active site through interactions with Arg^{256},

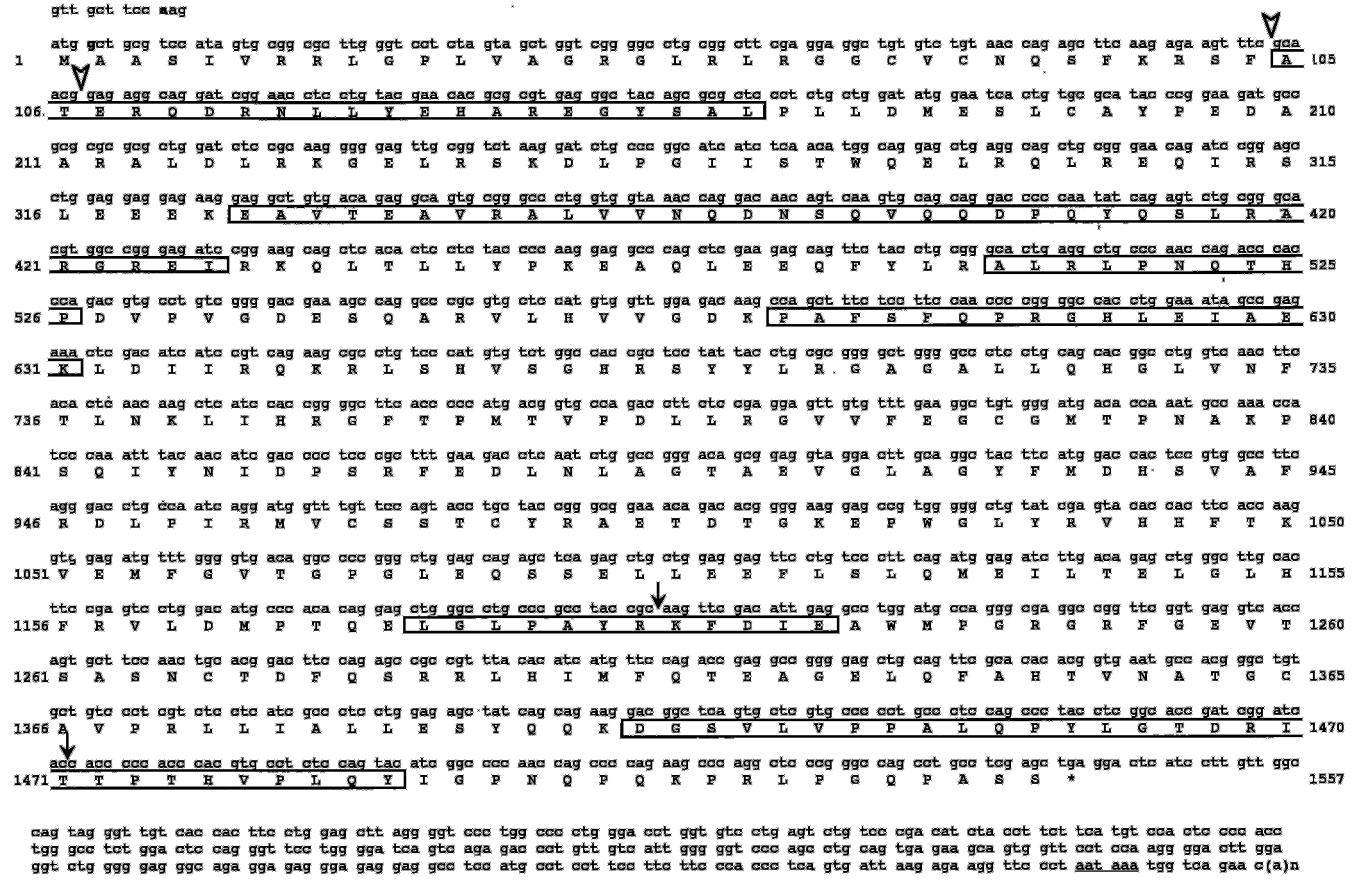

FIG. 4. **Nucleotide sequence of cDNA and the deduced amino acid sequence for bovine mt SerRS.** The sequence of the cDNA probe used for cDNA screening is located between the two *downward-pointing arrows*. The base numbered +1 corresponds to the first base of the open reading frame of mt SerRS. The putative polyadenylation signal (aataaa) is *underlined*, and *(a)n* denotes the poly(A) tail. An *asterisk* marks the stop codon. Sequences of the peptide fragments derived from mt SerRS are *boxed*. The two *downward-pointing wedges* indicate the possible cleavage sites observed in the purified bovine mt SerRS. A at position −3 and G at position 4 are emphasized by *bold letters* conform to the consensus for eukaryotic genes (36).

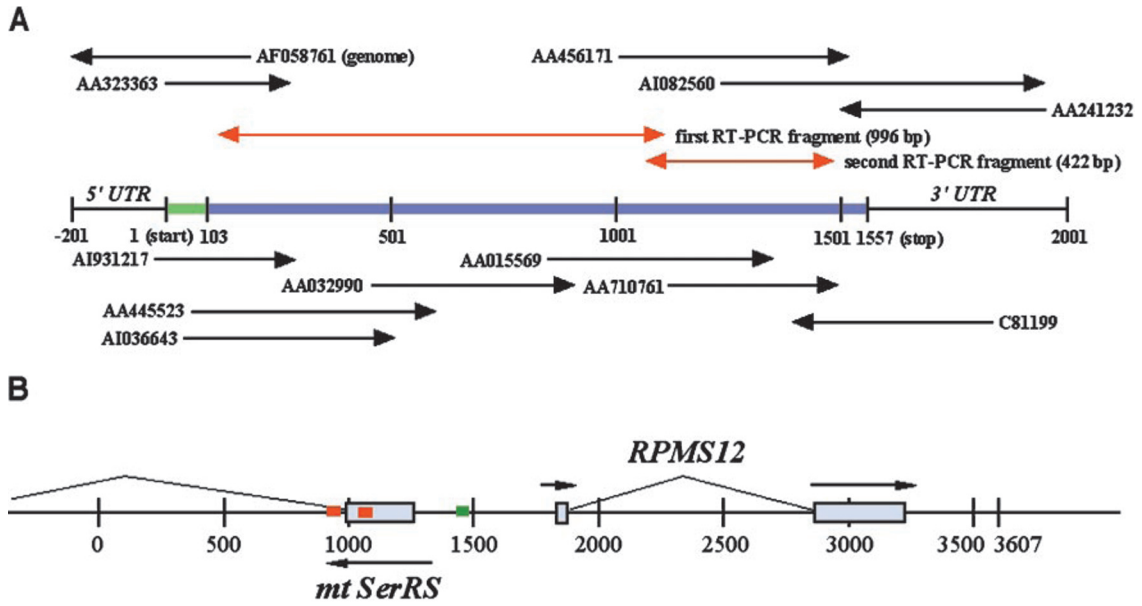

FIG. 5. **cDNA structures of mammalian mt SerRS.** *A*, schematic alignment of EST sequences with cDNAs of human and mouse mt SerRS. The representative EST clones used to obtain putative human mt SerRS cDNA (*upper part*) and mouse mt SerRS cDNA (*lower part*) are aligned with the corresponding bovine mt SerRS gene (*control part*). The protein-coding regions of bovine mt SerRS are indicated by a *green rectangle* for the targeting peptide and a *purple rectangle* for the mature form of bovine mt SerRS. Accession numbers are shown to the *right* or *left* of the *black arrows* representing the EST fragments. RT-PCR was performed twice to compensate for the blank and unknown nucleotides in human mt SerRS cDNA. The amplified regions obtained by the first and second RT-PCR are also indicated by *orange arrows*. *B*, gene organization at human chromosome 19q13.1. The numbering conforms to the sequence of a human EST clone (accession number AF058761) that contains the full *RPMS12* gene. *Light purple rectangles* indicate coding sequences. The putative binding sites of NRF-1 and NRF-2 are shown by *orange rectangles* and a *green rectangle*, respectively. One of the NRF-1 binding sites is located in the coding sequence of mt SerRS.

Glu^{258}, Arg^{271}, Phe^{275}, Glu^{345}, Glu^{348}, and Arg^{386} (16, 17, 41). (Fig. 6) Furthermore, serine specificity is ensured by the interaction of the hydroxyl group in the side chain of serine with Tyr^{380} in motif 3 (41). In particular, Glu^{281} in yeast cytoplasmic SerRS, equivalent to Glu^{258} in *T. thermophilus* SerRS, is reported to be important for the binding of ATP and to contribute to the stabilization of the motif 2 loop (42). All of these residues are also conserved in mammalian mt SerRS (Fig. 6). As reported by Cusack *et al*. (17), the motif 2 loop of *T. thermophilus* SerRS can take either of two quite different conformations: one in the presence of tRNA (the T-conformation) and the other in the absence of tRNA but in the presence of ATP (the A-conformation). These two ordered conformations are each stabilized by different sets of interactions, often involving the same residues. The side chains of Glu^{258} and Arg^{271}, key residues in the conformation switch, alter the conformation and bind to either ATP or tRNA in each conformation. These two residues are conserved in the mammalian mt SerRSs. On the other hand, Ser^{261}, Phe^{262}, and Arg^{267}, which are involved in interactions with several bases in the acceptor stem in the T-conformation (17), are scarcely conserved in mammalian mt SerRS. Cusack *et al*. (17) speculate that the occurrence of two glycines in the motif 2 loop (Gly^{260} and Gly^{263}) surrounded by small residues (Ala, Thr, or Val) in positions 259 and 266 may provide the flexibility necessary to facilitate the conformational switch. However, Gly^{260} and Val^{266} in *T. thermophilus* SerRS are not conserved in mammalian mt SerRSs.

The conservation of Glu^{258} and Arg^{271} (according to the *T. thermophilus* numbering) in the motif 2 loop of mammalian mt SerRSs also suggests the existence of the conformational switch from the serine activation step to the aminoacylation step in these enzymes. However, the lack of two out of the several residues necessary for providing flexibility to the motif 2 loop may reduce the flexibility of mammalian mt SerRS. Because the motif 2 loop of SerRS is the longest among other class II synthetases (17), residues of the long motif 2 loop are able to extend down to the fifth base pair of the acceptor stem of *T. thermophilus* $tRNA^{Ser}$. The apparently lower flexibility of the motif 2 loop and the low level of conservation of Ser^{261} and Arg^{267} (*T. thermophilus* SerRS numbering) in mammalian mt SerRS (Fig. 6), raise the possibility that mammalian mt SerRS does not interact with the bases of the acceptor stem. This is fully consistent with our previous finding that substitution of A-U base pairs in the acceptor stem of bovine mt $tRNA^{Ser}_{GCU}$ with C-G pairs did not severely impair the charging activity of $tRNA^{Ser}_{GCU}$ by bovine mt SerRS (24).

We previously demonstrated the significance of U^{54} and A^{58} of the T-loop in the recognition of bovine mt $tRNA^{Ser}_{GCU}$ by bovine mt SerRS (24). The corresponding residues are also found in another isoacceptor, $tRNA^{Ser}_{UGA}$, as U^{54} and m^1A, respectively. Because the present work has shown that the single mt SerRS can aminoacylate the two structurally distinct $tRNAs^{Ser}$, it is reasonable to assume that both $tRNA^{Ser}_{GCU}$ and $tRNA^{Ser}_{UGA}$ have the same recognition elements. Because tertiary U^{54}-A^{58} pairing is widely conserved among nonmitochondrial tRNAs and is considered to play a general role in maintaining the L-shape of the tRNA molecule (43), it seems unlikely that this pairing is critical for enzyme recognition. Further study is necessary to determine the recognition elements common to both bovine mt $tRNAs^{Ser}$. Kumazawa *et al*. (44) showed that bovine mt SerRS not only charges cognate *E. coli* $tRNA^{Ser}$ species but also extensively misacylates several noncognate *E. coli* tRNA species, whereas *E. coli* SerRS is unable to aminoacylate bovine mt $tRNAs^{Ser}$. This unilateral aminoacylation mechanism between bovine mitochondria and *E. coli* will be also elucidated through further research.

A human EST data base search revealed that the human mt SerRS gene is located at a position 5′ adjacent to the *RPMS12* gene on chromosome 19q13.1 (Fig. 5*B*) (39, 45). Recently, the autosomal dominant deafness locus DFNA4 was also mapped to 19q13.1 (46). Because the ribosomal protein S12 is known to act as a core component of the highly conserved accuracy center

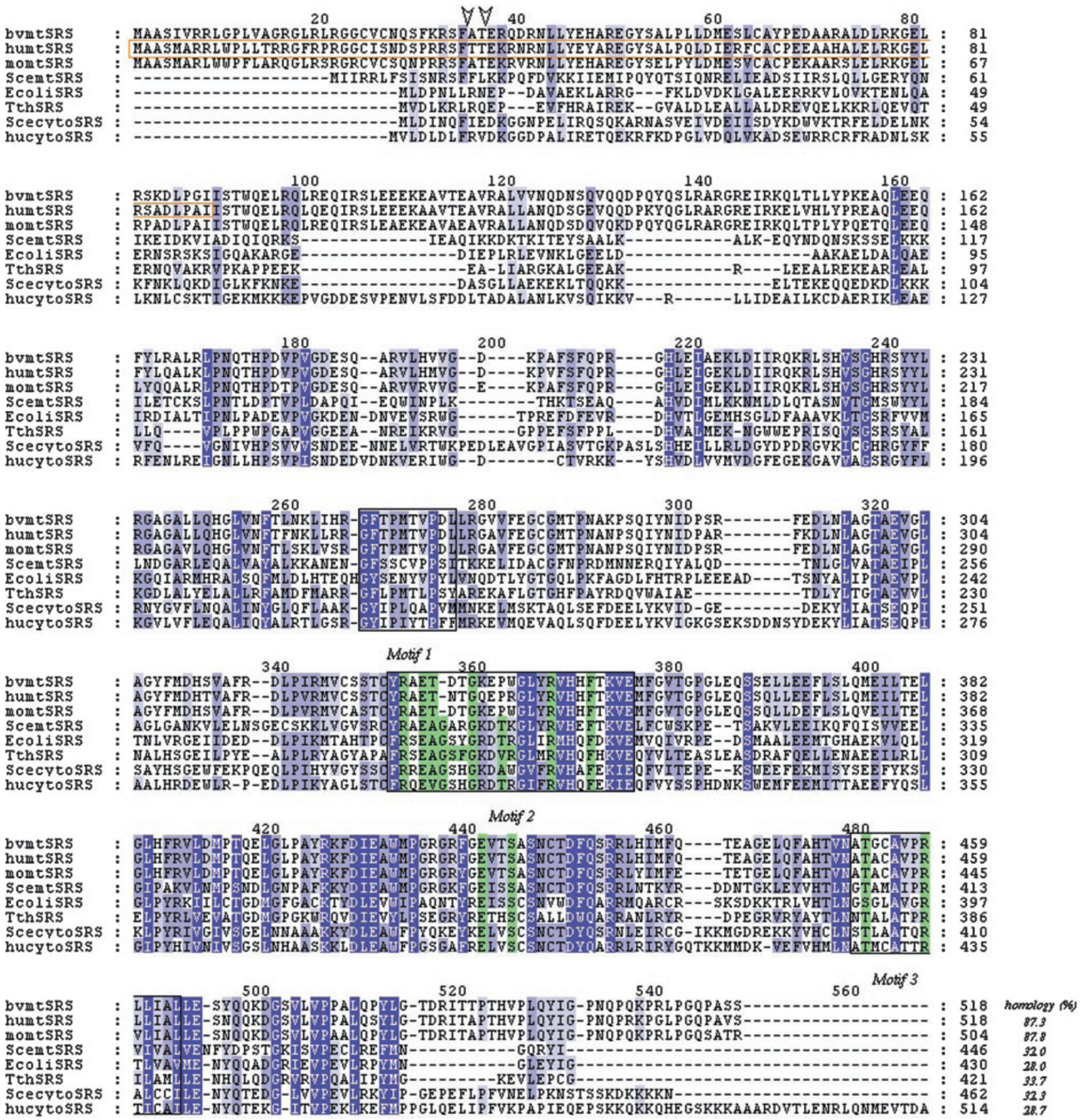

FIG. 6. **Sequence alignment of SerRS polypeptides from various sources.** The organisms used for the sequence alignment and corresponding accession numbers of the Swiss Protein Data Base are as follows: bovine liver mitochondria (*bvmtSRS*), human mitochondria (*humtSRS*), mouse mitochondria (*momtSRS*), *S. cerevisiae*, mitochondria (putative) (*ScemtSRS*; P38705), *E. coli* (*EcoliSRS*; P09156), *T. thermophilus* (*TthSRS*; P34945), *S. cerevisiae* cytoplasm (*ScecytoSRS*; P07284), and human cytoplasm (*hucytoSRS*; P49591). Multiple sequence alignment of SerRS polypeptides was done with the CLUSTAL X program. Three motifs highly conserved among the prokaryotic class II ARSs are *boxed* in *black*. When more than five residues in the compared eight sequences are identical or very similar, they are indicated by *normal* or *outlined letters* with colored backgrounds as follows: all residues, *dark purple*; six or seven residues, *purple*; and five residues, *light purple*. The two *wedges* indicate two possible cleavage sites for producing mature mammalian mt SerRS. Residues in the catalytic domain discussed in the text are indicated by *green backgrounds*. In addition, the N-terminal domain of human mt SerRS (residues 1–89, *boxed* in *orange*) is encoded in its first exon located adjacent to the human *RPMS12* gene (see Fig. 7). The homology values between the amino acid sequence of bovine mt SerRS and the sequences of other the SerRSs are shown at the *side* of the alignment. These values were calculated by using GENETYX-MAC version 7.3.

in the ribosome, it is supposed that mutations in the S12 gene result in inaccurate mt translation (47). A genetic study of the fruit fly indicates that a single point mutation in the mt ribosomal protein S12 causes a bang-senseless mutant called tko (48), the phenotype of which resembles a sensorineural hearing loss related to mt dysfunction (49). Although human *RPMS12* has been suggested to be responsible for DFNA4 hearing loss (39, 45), the human mt SerRS gene may also be a possible candidate, because mt SerRS contributes to the maintenance of translational fidelity in the mt protein synthesis reaction.

Although many biochemical experiments on recognition elements in tRNAs, especially those of prokaryotes, have been reported, there has been no study in which the recognition mechanism of structurally different tRNAs by a single synthetase was elucidated. We have discussed the recognition mechanism of bovine mt SerRS in the light of the information re-

vealed in the present study. Further experimental investigation will certainly reveal the essential recognition mechanism between SerRS and $tRNAs^{Ser}$ and thereby deepen our understanding of the animal mitochondrial translation system.

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REFERENCES

1. Normanly, J., and Abelson, J. (1989) *Annu. Rev. Biochem.* **58,** 1029–1049
2. Schulman, L. H. (1991) *Prog. Nucleic Acids Res. Mol. Biol.* **41,** 23–87
3. Kisselev, L. L. (1985) *Prog. Nucleic Acids Res. Mol. Biol.* **32,** 237–266
4. Shimizu, M., Asahara, H., Tamura, K., Hasegawa, T., and Himeno, H. (1992) *J. Mol. Evol.* **35,** 436–443
5. Saks, M. E., Sampson, J. R., and Abelson, J. N. (1994) *Science* **263,** 191–197
6. Himeno, H., Hasegawa, T., Ueda, T., Watanabe, K., and Shimizu, M. (1990) *Nucleic Acids Res.* **18,** 6815–6819
7. Sampson, J. R., and Saks, M. M. (1993) *Nucleic Acids Res.* **21,** 4467–4475
8. Hou, Y. M., and Schimmel, P. (1988) *Nature* **333,** 140–145
9. McClain, W. H., and Foss, K. (1988) *Science* **240,** 793–796
10. Lenhard, B., Orellana, O., Ibba, M., and Weygand-Durasevic, I. (1999) *Nucleic Acids Res.* **27,** 721–729
11. Asahara, H., Himeno, H., and Shimizu, M. (1991) *Chem. Lett.* 363–366
12. Schatz, D., Leberman, R., and Eckstein, F. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6132–6136
13. Asahara, H., Himeno, H., Tamura, K., Nameki, N., Hasegawa, T., and Shimizu, M. (1994) *J. Mol. Biol.* **236,** 738–748
14. Sprinzl, M., Hartmann, T., Weber, J., Blank, J., and Zeidler, R. (1989) *Nucleic Acids Res.* **17,** 1–172
15. Price, S., Cusack, S., Borel, F., Berthet-Colominas, C., and Leberman, R. (1993) *FEBS Lett.* **324,** 167–170
16. Biou, V., Yaremchuk, A., Tukalo, M., and Cusack, S. (1994) *Science* **263,** 1404–1410
17. Cusack, S., Yaremchuk, A., and Tukalo, M. (1996) *EMBO J.* **15,** 2834–2842
18. Achsel, T., and Gross, H. J. (1993) *EMBO J.* **12,** 3333–3338
19. Dock-Bregeon, A. C., Garcia, A., Giege, R., and Moras, D. (1990) *Eur. J. Biochem.* **188,** 283–290
20. Wu, X. -Q., and Gross, H. J. (1993) *Nucleic Acids Res.* **21,** 5589–5594
21. Himeno, H., Yoshida, S., Soma, A., and Nishikawa, K. (1997) *J. Mol. Biol.* **268,** 704–711
22. Steinberg, S., Gautheret, D., and Cedergren, R. (1994) *J. Mol. Biol.* **236,** 982–989
23. Yokogawa, T., Watanabe, Y., Kumazawa, Y., Ueda, T., Hirao, I., Miura, K., and Watanabe, K. (1991) *Nucleic Acids Res.* **19,** 6101–6105
24. Ueda, T., Yotsumoto, Y., Ikeda, K., and Watanabe, K. (1992) *Nucleic Acids Res.* **20,** 2217–2222
25. Wakita, K., Watanabe, Y., Yokogawa, T., Kumazawa, Y., Nakamura, S., Ueda, T., Watanabe, K., and Nishikawa, K. (1994) *Nucleic Acids Res.* **22,** 347–353
26. Schwartzbach, C. J., Farwel, M., Liao, H. X., and Spremulli, L. L. (1996) *Methods Enzymol.* **264,** 248–261
27. Schwartzbach, C. J., and Spremulli, L. L. (1989) *J. Biol. Chem.* **264,** 19125–19131
28. Laemmli, U. K. (1970) *Nature* **227,** 680–685
29. Hornung, V., Hofmann, H., and Sprinzl, M. (1998) *Biochemistry* **37,** 7260–7267
30. Cleveland, D. W., Fisher, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102- 1106
31. Omori, A., and Yoshida, S. (1994) *J. Protein Chem.* **13,** 471
32. Nishimura, S., and Novelli, G. D. (1964) *Biochim. Biophys. Acta* **80,** 574–586
33. Kumazawa, Y. (1988) *Studies on the Mitochondrial Protein Synthesis*. Ph.D. Thesis, The University of Tokyo, Tokyo, Japan
34. Takeuchi, N., Kawakami, M., Omori, A., Ueda, T., Spremulli, L. L., and Watanabe, K. (1998) *J. Biol. Chem.* **273,** 15085–15090
35. Nakayama, H. (1996) *Baio Jikken Irasutoreiteddo* **3,** 85–100
36. Yokogawa, T., Kumazawa, Y., Miura, K., and Watanabe, K. (1989) *Nucleic Acids Res.* **17,** 2623–2638
37. Kozak, M. (1997) *EMBO J.* **16,** 2482–2492
38. Gavel, Y., and von Heijne, G. (1990) *Protein Eng.* **4,** 33–37
39. Johnson, D. F., Hamon, M., and Fischel-Ghodsian, N. (1998) *Genomics* **52,** 363–368
40. Weygand-Durasevic, I., Lenhard, B., Filipic, S., and Söll, D. (1996) *J. Biol. Chem.* **271,** 2455–2461
41. Härtlein, M., and Cusack, S. (1995) *J. Mol. Evol.* **40,** 519–530
42. Lenhard, B., Filipic, S., Landeka, I., Skrtic, I., Söll, D., and Weygand-Durasevic, I. (1997) *J. Biol. Chem.* **272,** 1136–1141
43. Rich, A., and Kim, S. H. (1978) *Sci. Am.* **238,** 52–62
44. Kumazawa, Y., Himeno, H., Miura, K., and Watanabe, K. (1991) *J. Biochem.* (*Tokyo*) **109,** 421–427
45. Shah, Z. H., Migliosi, V., Miller, S. C. M., Wang, A., Friedman, T. B., and Jacobs, H. T. (1998) *Genomics* **48,** 384–388
46. Chen, A. H., Ni, L., Fukushima, K., Marietta, J., O'Neill, M., Coucke, P., Wiliems, P., and Smith, R. J. H. (1995) *Hum. Mol. Genet.* **4,** 1073–1076
47. Alskne, L. E., Anthony, R. A., Liebman, S. W., and Warner, J. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9538–9541
48. Shah, Z. H., O'Dell, K. M. C., Miller, S. C. M., An, X., Jacobs, H. T. (1997) *Gene* (*Amst.*) **204,** 55–62
49. Prezant, T. R., Agapian, J. V., Bohlman, M. C., Bu, X., Ötzas, S., Qiu, W. -Q., Arnos, K. S., Cortopassi, G. A., Jaber, L., Rotter, J. I., Shohat, M., and Fischel-Ghodsian, N. (1993) *Nat. Genet.* **4,** 289–294