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Relationship between Intron 4b Splicing of the Rat Geranylgeranyl Diphosphate Synthase Gene and the Active Enzyme Expression Level

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Geranylgeranyl diphosphate synthase (GGPS) is a branch point enzyme in the mevalonate pathway that catalyzes the synthesis of geranylgeranyl diphosphate used for the geranylgeranylation of Rho, Rac and Rab proteins. The current study showed the production of multiple forms of GGPS mRNA from a single GGPS gene in rat. The mRNAs resulted from combinations of multiple alternative introns and two poly(A) sites in the 3′-translated and 3′-untranslated regions. These are classified into 1a-type and 1b-type mRNAs, based on the splicing of intron 4b resulting in the difference in deduced amino acid sequence between the C-terminal regions. The 1a-type and 1b-type proteins expressed in both *Escherichia coli* and HeLa cells were active and inactive, respectively. In the case of HeLa cells, the latter protein expression level was about 10% relative to the former one. This was also observed for Cos-7 and 293 cells. When fusions of β-galactosidase with C-terminal regions differing between the 1a-type and 1b-type proteins were expressed in HeLa cells, the expressed fusion proteins were both found to be active but the latter fusion protein expression level was considerably low compared with the former one. The expression level of 1a-type mRNA was higher than that of 1b-type mRNA in brain, liver, heart, and thymus, but the two expression levels were the same in testis and ovary. During testis development the total GGPS mRNA expression level increased, accompanied by an increase in 1b-type mRNA, the expression level of 1a-type mRNA encoding active GGPS remaining kept unchanged. These results indicate that the expression level of rat active GGPS is at least regulated through the splicing of intron 4b of its gene.

Key words: alternative splicing, geranylgeranyl diphosphate synthase, post-transcriptional regulation, protein prenylation, testis development.

Abbreviations: BGH pA, bovine growth hormone receptor polyadenylation signal; CMV, cytomegalovirus; DMAPP, dimethylallyl diphosphate; EST, expression sequence tag; FPP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; HRP, horseradish peroxidase; *lacZ*, β-galactosidase gene; GAPDH, glyceroaldehyde-3-phosphate dehydrogenase; GGPP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; IPTG, isopropyl 1-thio-β-D-galactopyranoside; UTR, untranslated region.

Protein prenylation is one of the post-translational modifications that occur in mammalian cells (*1*). Ras proteins involved in cell growth, Rho and Rac proteins involved in cytoskeletal organization, and Rab proteins involved in vesicular trafficking are prenylated, and this lipid modification is essential for the association of proteins with cellular membranes and the function of proteins (*2–7*). The lipid precursors for protein prenylation are 15-carbon farnesyl diphosphate (FPP) and 20-carbon geranylgeranyl diphosphate (GGPP) (*8*, *9*), and they are synthesized as intermediates in the mevalonate pathway. It is very important to clarify how the syntheses of these FPP and GGPP differ from each other in order to understand the biosynthesis of prenylated proteins. However, the intermediate FPP has also been shown to be converted into divergent metabolites other than prenylated proteins, such as cholesterol, ubiquinone, dolichol, and heme a (*10*). The intermediate GGPP has also been shown to act as a negative regulator of the DNA binding of liver X receptor (LXR) (*11*, *12*). Therefore, study of FPP synthase (FPS) and GGPP synthase (GGPS) responsible for the syntheses of intermediates is not limited to the understanding of prenylated protein biosynthesis but also covers that of divergent metabolites including prenylated proteins in the mevalonate pathway.

FPS, which catalyzes the synthesis of FPP from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), has been extensively studied: it behaves as a dimer (*13*) and its expression is transcriptionally regulated by the cellular cholesterol level (*14–16*). On the other hand, GGPS, which catalyzes the synthesis of GGPP from IPP and FPP, was recently cloned and characterized: it behaves as an oligomer (*17*, *18*) and its expression is not transcriptionally regulated by cholesterol (*19*). Vicent *et al.* have reported that three sizes of GGPS mRNAs are overexpressed in *ob*/*ob* mouse (*20*). We demonstrated in a previous study (*18*) that two sizes of mRNA were detected in various human tissues, implying the presence of multiple GGPS genes or splice variants.

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Table 1. **Primers used in this study.**

Primer	Use	Position	Sequence (5′–3′)
rgg-s1	RT-PCR	–30 to –6[a,b]	atgaggcagaggggaggtggcatac
rgg-s2	RT-PCR	1 to 22[a,b]	ctgcgcagtgtgatagggatgg
rgg-a1	RT-PCR	3150 to 3122[a]	ggcaatacaactgacctttattcttgcag
rgg-a2	RT-PCR	2474 to 2541[b]	gagttagcaccaggccaaaattaatttaattagg
rGGPS-gw3	Genome walking	Exon 5b	cttgaagaggcagaagccaggctaatag
rGGPS-gw4	Genome walking	Exon 5b	gagttctaggccagctagaattgcatag
rGGPS-gw7	Genome walking	Intron 4b–Exon 4b	cctgtagcttgtcttctggaactttcagc
rGGPS-gw8	Genome walking	Exon 4b	gtgattaaatgcctgtgaaagtttggttc
rGGPS-gw9	3′-RACE and Genome walking	Exon 5	gcaattctagctggcctagaactccagg
rGGPS-gw10	3′-RACE and Genome walking	Exon 5	ctattagcctggcttctgcctcttcaag
rGGPS-gw11	Genome walking	Exon 2	ggctctagaagaattctttcagctttctctttag
rGGPS-gw12	Genome walking	Exon 2–Intron 1	ctccatgttttattaatttcaaagttgacctg
rGGPS-gw21	Genome walking and Southern blotting	Exon 1	tagggatggcgtattttcttacacc
rGGPS-gw23	Genome walking	Upstream of Exon 1	gtccaatcttccaggtcaatcggtaaac
rGGPS-gw27	Genome walking	Intron 1	acttctctggcctcaagagcaatattc
rGGPS-gw28	Genome walking	Intron 1	ttatccattaaaaaaatcagcaactcatg
rGGPS-gw33	Southern blotting	Intron 1	ccttaaccatccttcctcacctctc
rGAPDH-qs1	Quantitative RT-PCR	404 to 428[c]	tgaaccacgagaaatatgacaactc
rGAPDH-qa1	Quantitative RT-PCR	967 to 943[c]	tgttgctgtagccatattcattgtc
rGGPS-qs3	Quantitative RT-PCR	483 to 507[d,e]	cctcatgcagttgttctctgactac
rGGPS1a-qa1	Quantitative RT-PCR	826 to 802[d]	cgatttgtttgtaggctttagcttc
rGGPS1b-qa1	Quantitative RT-PCR	785 to 761[e]	tcatggtaggacacacatttgattc

[a]rGGPS1a1 cDNA and [b]rGGPS1a3 cDNA relative to the putative transcription start site. [c]Rat GAPDH cDNA, [d]rGGPS1a cDNA, and [e]rGGPS1b cDNA relative to the translation start site. Underlines indicate polyadenylation signals (complementary).

In the current study on rat GGPS, we isolated two cDNAs differing in the 3′-sequence, suggesting the presence of splice variants. Further studies showed that multiple mRNAs for GGPS were derived from a single GGPS gene in rat. Also, these multiple mRNAs were classified into two types, 1a-type mRNAs encoding active GGPS and 1b-type mRNAs encoding inactive enzymes. The expression levels of 1a-type and 1b-type mRNAs were different in rat tissues and also in testes during development. In this paper, we describe the results.

MATERIALS AND METHODS

cDNA Cloning—A λTriplEx rat testis cDNA library (Clontech) was screened by plaque hybridization using the coding region of human GGPS cDNA (hGGPS1a) as a probe (*18*). Two positive clones were isolated, which were sequenced in both directions using a LI-COR 4200 sequencer (Li-COR). A search for DNA homology was performed using the NCBI BLAST network service. The partial cDNAs were amplified with a SMART RACE cDNA amplification kit (Clontech) using primer rGGPS-gw9 and rGGPS-gw10 (Table 1), and rat testis mRNA. The full-length cDNAs were amplified from rat testis mRNA by RT-PCR under the following conditions: 30 s at 95°C and 1 min at 63°C for 35 cycles, with the following primers: rgg-s2 and rgg-a1 or rgg-s2 and rgg-a2 (Table 1). The amplified cDNAs were cloned into pCR-2.1 (Invitrogen) and their sequences were determined.

Genome Walking on the Rat GGPS Gene—Genome walking on the rat GGPS gene was performed with a genome walker kit (Clontech). The primers used are shown in Table 1. Primary PCR was performed under the following conditions: 2 s at 94°C and 6 min at 68°C for 7 cycles, and 2 s at 94°C and 6 min at 63°C for 42 cycles. Secondary PCR was performed using a 50-fold diluted primary PCR product as a template under the following conditions: 2 s at 94°C and 6 min at 68°C for 5 cycles, and 2 s at 94°C and 6 min at 63°C for 30 cycles. The five PCR products were about 3.2 kbp (with primer pairs of rGGPS-gw3 and 4), 3.1 kbp (rGGPS-gw7 and 8), 2.5 kbp (rGGPS-gw9 and 10), 4.8 kbp (rGGPS-gw11 and 12), and 2.5 kbp (rGGPS-gw21, 23, 27, and 28) in length, and each was cloned into pT7Blue (Novagen) or pCR-2.1. The products were subcloned into pUC18 and then sequencing was performed as described above. For sequence alignment, GENETYX-Win 5.1 and GENETYX-WIN/ATSQ 1.03 software was used.

Southern Blot Analysis—Five μg of rat genomic DNA was completely digested with *Eco*RV, *Dra*I, *Pvu*II, or *Sca*I, followed by separation by 1× TAE–0.8% agarose gel electrophoresis and transfer to a nylon membrane. A DIG-labeled PCR probe was synthesized with a set of primers, rGGPS-gw21 and 33 (Table 1), and a PCR DIG probe synthesis kit (Roche). Hybridization was performed with 15 ng/ml of DIG-labeled probe at 42°C for 16 h in DIG Easy Hyb (Roche), and the hybridized membrane was washed twice with 2× SSC–0.1% SDS at room temperature for 5 min and then twice with 0.1× SSC–0.1% SDS at 68°C for 15 min. Detection was performed with anti-DIG alkaline phosphatase and CSPD reagent (Roche).

Expression of Rat GGPS1a, 1b, and 1bΔ in Escherichia coli cells—The coding regions of the rGGPS1a, 1b, and 1bΔ cDNAs were ligated into the *Nde*I and *Bam*HI sites of pET-15b (Novagen) to generate pET-rGG1a, pET-rGG1b, and pET-rGG1bΔ, respectively. The sequences of the plasmids were confirmed before transformation. *E.*

coli BL21 (DE3) cells transformed with each pET plasmid were grown to an A_{600} of 0.6. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.3 mM, and then the cells were cultured at 18°C for 6 h. Whole cell extracts were separated by SDS-polyacrylamide (12.5%) gel electrophoresis and then stained with Coomassie brilliant blue. For detection of GGPS, the separated proteins were transferred to a PVDF membrane and visualized with guinea pig anti-bovine GGPS polyclonal antibodies (*18*), goat anti-guinea pig IgG alkaline phosphatase (BETHYL), and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate. For *in vitro* GGPS assaying, crude soluble proteins were prepared, and enzymatic activity was measured with [1-^{14}C]IPP (Amersham Pharmacia Biotech) and FPP as described previously (*18*). For *in vivo* GGPS assaying, *E. coli* BL21 (DE3) cells harboring pACYC-crtIB (a kind gift from Dr. Hemmi, Tohoku University) (*21*), which contains *crtI* and *crtB* encoding carotenoid biosynthetic enzymes, were transformed with each pET plasmid and then plated on LB plates containing 100 μg/ml ampicillin and 12 μg/ml tetracycline. Resistant colonies were replated on LB plates containing the same antibiotics and cultured at 20°C for 3 d, the change in color of colonies due to the accumulation of lycopene being examined. The His-tagged rGGPS1a and 1b proteins were purified by nickel affinity column chromatography, and the enzymatic products were analyzed by normal-phase thin-layer chromatography using a 2-propanol/ammonia/H_2O 6:3:1 (v/v) solvent system as described previously (*22*). For autoradiography, the thin-layer plates were exposed on a Fuji imaging plate and then examined with a Fuji BAS 1000 bioimage analyzer.

Expression of Rat GGPS1a, 1b, and 1bΔ in HeLa Cells—The coding regions of rGGPS1a, 1b, and 1bΔ were ligated into the *Bam*HI site of pcDNA3.1-HisC (Invitrogen) to generate pcDNA-rGG1a, pcDNA-rGG1b, and pcDNA-rGG1bΔ, respectively. The plasmid sequences were confirmed before transfection. HeLa cells (a kind gift from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University) were maintained in Minimum Essential Medium containing 10% fetal bovine serum (Gibco) and kanamycin sulfate (100 μg/ml) at 37°C in a 5% CO_2 incubator. HeLa cells (8×10^5) were seeded into 60 mm dishes the day before transfection. Expression plasmids (3.0 μg) were transfected into HeLa cells using PolyFect transfection reagent (Qiagen) according to the manufacturer's protocol. The transfected cells were cultured for 24 h.

Total RNA was extracted with an RNeasy Mini Kit (Qiagen). The RNA obtained was treated with DNase I for 30 min at 37°C and then with DNase inactivation reagent (Ambion, Texas USA). Twenty μg of each total RNA was separated on a 1× MOPS–1.5% agarose gel containing 0.66 M formaldehyde, followed by transfer to a nylon membrane. A DIG-labeled antisense RNA probe was synthesized with the pBSK-Com600 plasmid, which contains a partial rGGPS1a cDNA (1–600; relative to the translation start site) in the antisense direction under the T7 promoter, T7 RNA polymerase, and DIG RNA Labeling Mix (Roche). Hybridization was performed with 100 ng/ml DIG–labeled RNA probe at 68°C for 16 h in DIG Easy Hyb, the hybridized membranes were washed, and then the RNA was detected as described above.

Crude soluble proteins were prepared as described previously (*17*). Twenty μg of crude soluble proteins was separated by SDS–polyacrylamide (12.5%) gel electrophoresis, and followed by staining with anti His-HRP antibodies (Qiagen) and ECL advance reagent (Amersham Pharmacia Biotech). Five μg of crude soluble proteins was incubated with 100 μM DMAPP, GPP, or FPP and 100 μM [1-^{14}C]IPP in 100 μl of 50 mM potassium phosphate buffer (pH 7) containing 50 mM L-phenylalanine, 5 mM $MgCl_2$, 2 mM DTT, and 1 mg/ml BSA at 37°C for 2 h. The enzymatic products were extracted with 1-butanol saturated with H_2O and then treated with acid phosphatase at 37°C for 12 h (*18*). The liberated products were extracted with hexane and then the hexane soluble products were analyzed by reverse-phase LKC-18 thin-layer chromatography in acetone:water (7:1). For autoradiography, the thin-layer plates were analyzed as described above. GGPS and FPS activities were calculated from the amount of geranylgeraniol produced from FPP and [1-^{14}C]IPP, and on that of farnesol produced from GPP and [1-^{14}C]IPP, respectively.

Expression of the β-Galactosidase Gene with or without Its Stop Codon Fused with the 3′-Coding Regions of Rat 1a-Type and 1b-Type GGPS mRNAs in HeLa Cells—Plasmid pcDNA3.1-HislacZ, which encodes *E. coli* β-galactosidase under the control of the cytomegalovirus (CMV) promoter, was purchased from Invitrogen. The 3′-regions of rGGPS1a (691–903, relative to the translation start site), 1b (691–828), and 1bΔ (691–804) were ligated into the *Xho*I site (41 nucleotides downstream from the *lacZ* stop codon) of pcDNA3.1-HislacZ to generate pLacZ-rGG1a691–903UTR, pLacZ-rGG1b691–828UTR, and pLacZ-rGG1bΔ691–804UTR, respectively. These plasmids express β-galactosidase mRNAs containing the 3′-coding regions of GGPS mRNAs within the 3′-UTR. The coding region of β-galactosidase without its stop codon was amplified by PCR and then ligated into the *Bam*HI site of pcDNA3.1HisC to generate pLacZTAA(–). The 3′-regions of rGGPS1a (691–903, relative to the translation start site), 1b (691–828), and 1bΔ (691–804) were ligated into the *Xho*I site (49 nucleotides downstream of the AAA codon encoding the most C-terminal lysine residue of β-galactosidase) of pLacZTAA(–) to generate pLacZ-rGG1aCterm, pLacZ-rGG1bCterm, and pLacZ-rGG1bΔCterm, respectively. Transfection into HeLa cells was performed as described above. Twenty μg of crude soluble proteins was separated by SDS-polyacrylamide (7%) gel electrophoresis, followed staining with anti His-HRP antibodies and ECL reagent (Amersham Pharmacia Biotech). For β-galactosidase assaying, 1 μg of crude soluble proteins was incubated with 1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside in 67 μl of 75 mM sodium phosphate buffer (pH 7) containing 7.5 mM KCl, 0.75 mM $MgSO_4$, 29 mM β-mercaptoethanol at 37°C for 1 h. The reaction was terminated by the addition of 125 μl of 1 M sodium carbonate and then the absorbance at 420 nm was measured to determine the relative β-galactosidase activity.

Analysis of mRNA Expression by Real-Time PCR—Three Sprague-Dawley rats (male or female, age 3–10 wk) were killed by decapitation, and then tissues (brain,

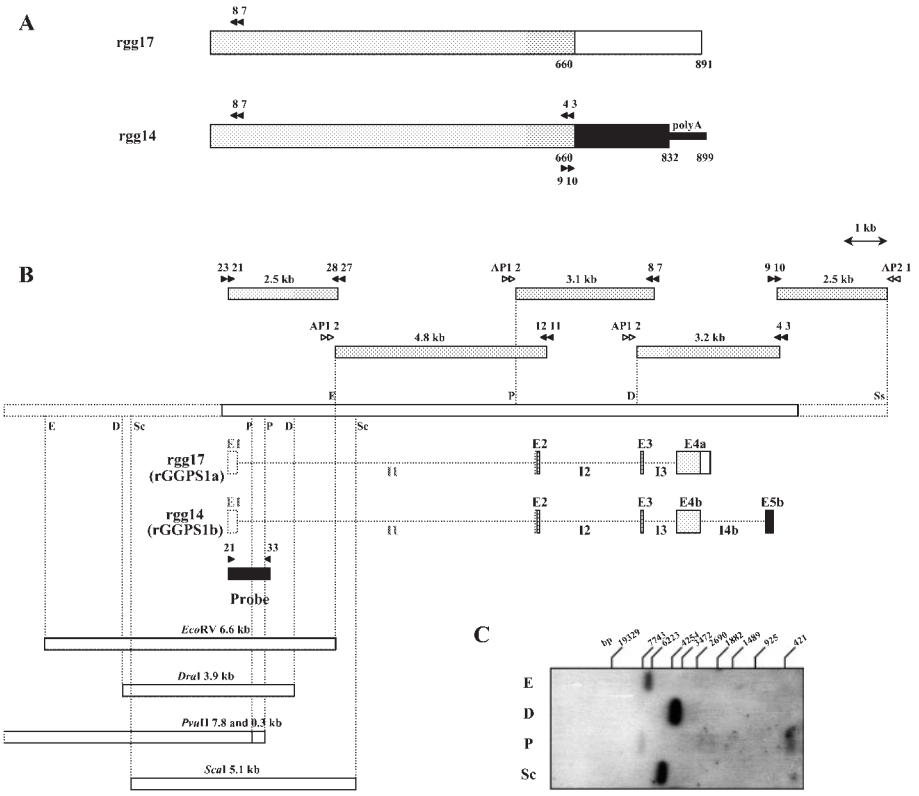

Fig. 1. **Two partial cDNAs of rat GGPS and genomic organization of the rat GGPS gene.** (A) The two cDNAs (rgg17 and rgg14) screened from the rat testis cDNA library are illustrated. rgg17 (891 bp) and rgg14 (899 bp) have the same 660 bp sequence (gray box) in the 5′-region but their 3′-regions (open and black boxes) are different. The entire rgg17 sequence is similar to that of human GGPS1, and rgg14 has a poly(A) tail. Arrowheads 8, 7, 4, 3, 9, and 10 indicate the primers used for the following genomic walking experiments (see Table 1). (B) Analysis of the genomic structure of the rat GGPS gene. The longest open bar in the center shows the genome (E, *Eco*RV; D, *Dra*I; P, *Pvu*II; Sc, *Sca*I; and Ss, *Ssp*I). Using primers (arrowheads, see Table 1), and adaptor primers 1 and 2 (AP1 and 2), five PCR products (upper gray boxes, 2.5, 4.8, 3.1, 3.2, and 2.5 kbp) were obtained and analyzed. The exon-intron organization of rgg17 and rgg14 corresponding to parts of rGGPS1a and rGGPS1b, respectively, is also shown under the genome open bar. E and I indicate exon and intron, respectively. (C) Southern blot analysis of rat genomic DNA. The genomic DNA (5 μg) digested with E (*Eco*RV), D (*Dra*I), P (*Pvu*II), or Sc (*Sca*I) was separated by agarose gel electrophoresis and then transferred to a nylon membrane. Hybridization was performed with a DIG-labeled PCR probe (position +14 to +955, relative to the putative transcription start site) as described under "MATERIALS AND METHODS." The DIG-labeled PCR probe (black box) is shown in B. The five genomic bands detected were found to correspond to *Eco*RV 6.6, *Dra*I 3.9, *Pvu*II 7.8, *Pvu*II 0.3, and *Sca*I 5.1 kbp shown in B (open bars).

liver, thymus, heart, testis, and ovary) were isolated and crushed in liquid nitrogen. Total RNA was extracted with an RNeasy Midi Kit (Qiagen) and mRNA was purified with an Oligotex-dT column (Takara). Each mRNA was treated with DNase I before reverse transcription. Reverse transcription was performed with 1 μg of each mRNA for 1 h at 42°C using a Ready-To-Go T-Primed First-Strand Kit (Amersham Pharmacia Biotech), and reverse transcriptase was inactivated for 5 min at 90°C. All primers used for quantitative RT-PCR were 25 nucleotides in length with a T_m value of 60–63°C (Table 1). First, PCR was performed to confirm the primer specificity (agarose gel electrophoresis and DNA sequencing) and to confirm there was no genomic DNA contamination (PCR using mRNA without reverse transcription). Second, quantitative PCR was performed with a Light Cycler apparatus (Roche) with the Fast start DNA SYBR Green Master Mix (Roche) according to the manufacturer's instructions using appropriate dilutions of reverse transcription products, specific primer pairs (500 nM), and 3 mM $MgCl_2$. The PCR conditions were as follows: rat GAPDH cDNA (1 s at 95°C, 10 s at 55°C, and 20 s at 72°C, generating a 564 bp product), rGGPS1a (1 s at 95°C, 10 s at 55°C, and 14 s at 72°C, generating a 344 bp product), and rGGPS1b (same conditions as for rGGPS1a, generating a 303 bp product). Sequence-specific standard curves were generated using 5-fold serial dilutions of a plasmid containing each cDNA. A relative value for the initial target concentration in each reaction was determined from the standard curve. The relative target/GAPDH mRNA level is presented as the mean ± S.D. of triplicate determinations.

RESULTS

Screening of GGPS cDNA, Genomic Organization of the GGPS Gene, and Southern Blot Analysis—When we screened a human testis cDNA library with a human GGPS cDNA (hGGPS1a) probe, several clones were obtained. However, sequence analysis revealed no clone different from hGGPS1a. Similarly, we screened a rat testis cDNA library with the same human probe and obtained several clones. Sequence analysis revealed that two clones (rgg17 and rgg14) are the same in the 5′-region (Fig. 1A, gray boxes) but differ in the 3′-region (open and black boxes). The identity between rgg17 and hGGPS1a was 89% at the cDNA level and 95% at the amino acids level, suggesting that rgg17 and rgg14 are a counterpart (rGGPS1a) of hGGPS1a and a splice variant of rGGPS1a, respectively. To determine the genomic organization of the rat GGPS gene, we first focused on the differences between rgg17 and rgg14 in the 3′-region and started genome walking. Five PCR products (Fig. 1B, upper gray boxes) were cloned and sequenced, giving a 13 kbp genomic sequence for the GGPS gene containing 4 or 5 exons and 3 or 4 introns. rgg17 and rgg14 correspond to parts of rGGPS1a and a variant (rGGPS1b) on intron 4b splicing, respectively.

To determine whether the rat GGPS gene is single, we performed Southern blot analysis, using a DIG-labeled

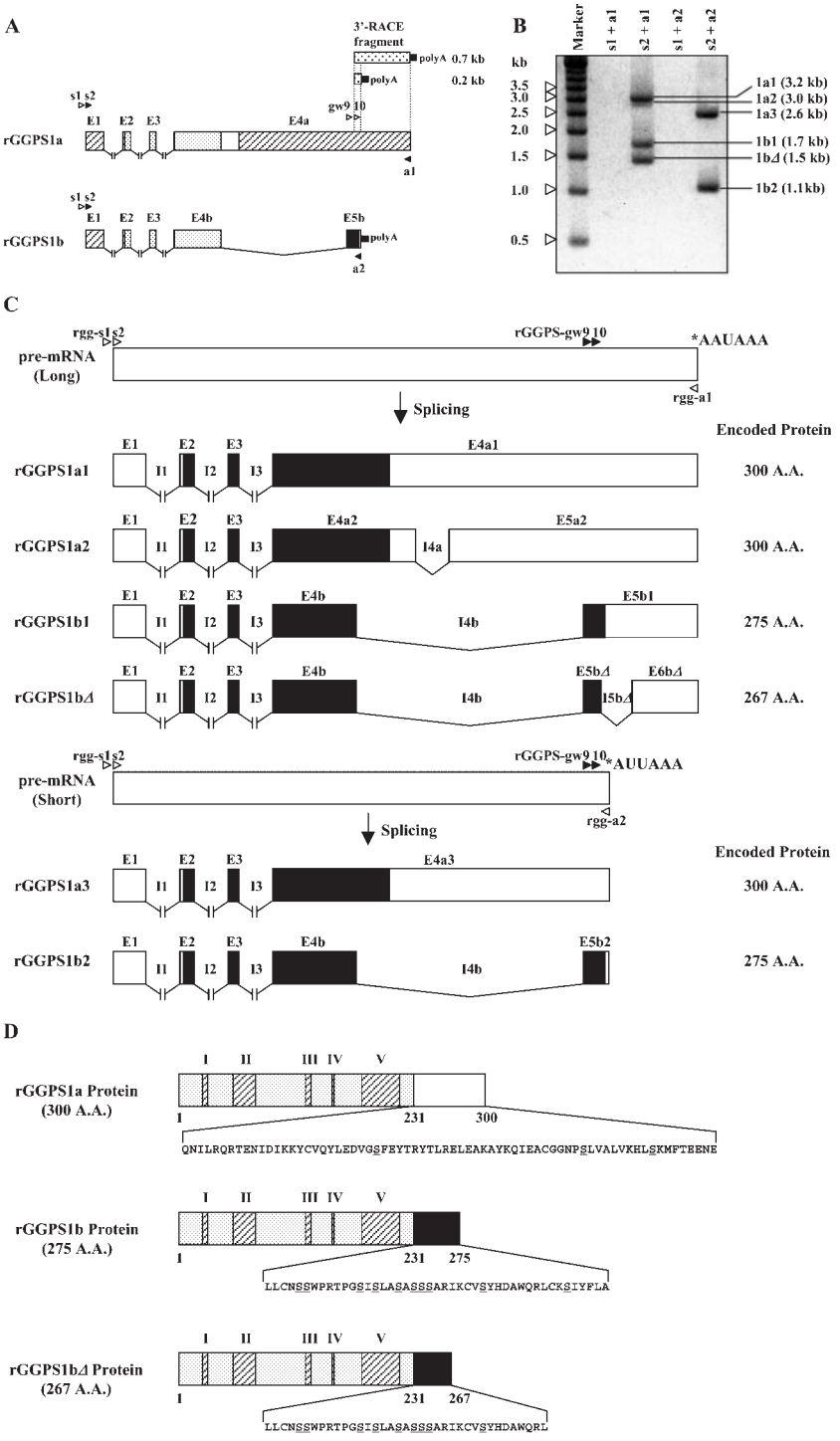

Fig. 2. **Analysis of six cDNAs of rat GGPS.** (A) Confirmation of two poly(A) sites in the 3′-UTR of rGGPS1a and 1b. The two cDNA fragments (0.7 and 0.2 kbp) obtained by the 3′-RACE method are shown above rGGPS1a (upper two dotted boxes). Primers (arrowheads, gw9 and 10, see Table 1) were used for PCR reactions to detect poly (A) tail (black bar)-containing products. The common 690 bp coding sequence (gray boxes) in the 5′-region, the 210 bp coding sequence (open) of rGGPS1a in the 3′-region, the 135 bp coding sequence (black) of rGGPS1b in the 3′-region, and UTR (hatched) are shown. (B) Agarose electrophoresis of RT-PCR products. The sense primers (arrowheads, s1 and s2) and antisense primers (arrowheads, a1 and a2) shown in A were used for full-length RT-PCR. The four products (3.2, 3.0, 1.7, and 1.5 kbp) amplified using primer sets s2 and a1 were found to be rGGPS1a1, 1a2, 1b1, and 1bΔ, respectively, and the two products (2.6 and 1.1 kbp) amplified using primer sets s2 and a2 were found to be rGGPS1a3 and 1b2, respectively. (C) Analysis of the exon-intron junctions of six mRNAs. Two pre-mRNAs (long and short open bars) are shown at the top and center, respectively. The asterisks on the long and short pre-mRNAs show poly(A) sites following AAUAAA and AUUAAA poly(A) signal sequences, respectively. Four mRNAs (rGGPS1a1, 1a2, 1b1, and 1bΔ) with the AAUAAA poly(A) signal sequence are shown under the long open bar. Two mRNAs (rGGPS1a3 and 1b2) with the AUUAAA poly(A) signal sequence at the 3′-terminus are shown under the short open bar. Numbers of amino acids of proteins encoded by these mRNAs is shown in left. E and I indicate exon and intron, respectively and the black boxes show coding regions. A.A. indicates amino acid. (D) The primary proteins structures deduced from rGGPS1a, 1b, and 1bΔ mRNAs. Each C-terminal region is illustrated with the amino acid sequence. rGGPS1a, 1b, and 1bΔ proteins share the same N-terminal region (230 amino acids) including five conserved regions (I, II, II, IV, and V) common to all-*trans* prenyltransferases shown as hatched boxes, and the C-terminal regions of the GGPS1b and 1bΔ proteins are shown as black boxes to emphasize the difference from that of the GGPS1a protein shown as an open box. Serine residues found in the C-terminal regions of the rGGPS1a, 1b and 1bΔ proteins are underlined.

cDNA probe (genomic position +14 to +955, relative to the putative transcription start site). The results are shown in Fig. 1C. Digestion with *Eco*RV, *Dra*I, or *Sca*I gave a single band corresponding to the size of 6.6, 3.9, or 5.1 kbp, respectively. On the other hand, digestion with *Pvu*II gave two bands corresponding to sizes of about 7.8 and 0.3 kbp. These bands matched the fragments expected from the cleavage sites for four restriction enzymes on the genome sequence. These results indicate that the rat GGPS gene is single like the human one (*19*, *23*).

Cloning of Six Rat GGPS cDNAs—The two cDNAs (rgg17 and rgg14) obtained on rat testis cDNA library screening were parts of rGGPS1a and 1b, respectively, so we next analyzed the full length form (1a and 1b). First, we obtained two partial cDNAs that have two distinct poly(A) signals by 3′-RACE using primer sets rGGPS-gw9 and rGGPS-gw10 (Fig. 2A). The longer cDNA contained the sequence AATAAA and the shorter one ATTAAA as a poly(A) signal. Next, we designed two sense primers (rgg-s1 and rgg-s2) and two antisense

Table 2. **Exon-intron boundaries of the rGGPS gene.**

	Exon	Intron
rGGPS1a1 (3,150nt)		
1st	*CTGCGC-(211nt)-GGAACC	gtgagc-(6,751nt)-taacag
2nd	GTCAAC-(93nt)-TACCAG	gtaaca-(2,301nt)-ttgcag
3rd	AAACAG-(71nt)-CTACAG	gtatct-(741nt)-ctctag
4th	ATTATC-(2,775nt)-ATTGCC	
rGGPS1a2 (2,928nt)		
1st	*CTGCGC-(211nt)-GGAACC	gtgagc-(6,751nt)-taacag
2nd	GTCAAC-(93nt)-TACCAG	gtaaca-(2,301nt)-ttgcag
3rd	AAACAG-(71nt)-CTACAG	gtatct-(741nt)-ctctag
4th	ATTATC-(932nt)-CAGTCA	gtgctc-(222nt)-gctcag
5th	TGGTTA-(1,621nt)-ATTGCC	
rGGPS1a3 (2,574nt)		
1st	*CTGCGC-(211nt)-GGAACC	gtgagc-(6,751nt)-taacag
2nd	GTCAAC-(93nt)-TACCAG	gtaaca-(2,301nt)-ttgcag
3rd	AAACAG-(71nt)-CTACAG	gtatct-(741nt)-ctctag
4th	ATTATC-(2,199nt)-TAACTC	
rGGPS1b1 (1,667nt)		
1st	*CTGCGC-(211nt)-GGAACC	gtgagc-(6,751nt)-taacag
2nd	GTCAAC-(93nt)-TACCAG	gtaaca-(2,301nt)-ttgcag
3rd	AAACAG-(71nt)-CTACAG	gtatct-(741nt)-ctctag
4th	ATTATC-(546nt)-ACCCAG	gtacag-(1,483nt)-agacag
5th	GTTTTA-(746nt)-ATTGCC	
rGGPS1b2 (1,091nt)		
1st	*CTGCGC-(211nt)-GGAACC	gtgagc-(6,751nt)-taacag
2nd	GTCAAC-(93nt)-TACCAG	gtaaca-(2,301nt)-ttgcag
3rd	AAACAG-(71nt)-CTACAG	gtatct-(741nt)-ctctag
4th	ATTATC-(546nt)-ACCCAG	gtacag-(1,483nt)-agacag
5th	GTTTTA-(170nt)-TAACTC	
rGGPS1bΔ (1,468nt)		
1st	*CTGCGC-(211nt)-GGAACC	gtgagc-(6,751nt)-taacag
2nd	GTCAAC-(93nt)-TACCAG	gtaaca-(2,301nt)-ttgcag
3rd	AAACAG-(71nt)-CTACAG	gtatct-(741nt)-ctctag
4th	ATTATC-(546nt)-ACCCAG	gtacag-(1,483nt)-agacag
5th	GTTTTA-(115nt)-GACTGT	gtaagt-(199nt)-attcag
6th	AAAAGA-(432nt)-ATTGCC	

*Putative transcription start site deduced on RT-PCR.

primers (rgg-a1 and rgg-a2) containing anti poly(A) signals (TTTATT and TTTAAT, respectively). Surprisingly, we obtained six cDNAs differing in length by RT-PCR from testis mRNAs (Fig. 2B). 1a1 (3.2 kbp), 1a2 (3.0 kbp), 1b1 (1.7 kbp), and 1bΔ (1.5 kbp) were amplified using primer sets rgg-s2 and rgg-a1. 1a3 (2.6 kbp) and 1b2 (1.1 kbp) were amplified using primer sets rgg-s2 and rgg-a2. Since no amplified cDNA was detected using rgg-s1 (position –30 to –6, relative to the putative transcription start site) as a sense primer, it is expected that the transcriptional start sites of these mRNAs are located in the region between rgg-s1 and rgg-s2. As shown in Fig. 2C, 1a1, 1a2, and 1a3 had the same coding region corresponding to a GGPS of 300 amino acids but had different 3′-untranslated regions (UTRs) due to the splicing of intron 4a or the presence of two poly(A) signals. Both 1b1 and 1b2 mRNAs had the same coding region corresponding to a truncated protein of 275 amino acids but had different 3′-UTRs due to the presence of two poly(A) signals. 1bΔ had a coding region corresponding to a more truncated protein of 267 amino acids. All of the introns found in these cDNAs contained the consensus sequence GT at their 5′-ends and AG at their 3′-ends (Table 2).

Comparison of the 1a, 1b and 1bΔ proteins is shown in Fig. 2D. All of these putative proteins have five conserved regions (I, II, III, IV, and V, hatched box) common to members of the all-*trans* prenyltransferase family (*24*) within their N-terminal 230 amino acids (gray box). The 1b and 1bΔ proteins have a unique serine-rich sequence at the C-terminus (black boxes, serine residues are underlined).

Expression of Rat GGPS1a, 1b, and 1bΔ in E. coli Cells—PSORT analysis (*25*) of these three putative proteins revealed that they have no transmembrane region and are localized in the cytoplasm. So, we first tried to express them in *E. coli* cells. As Fig. 3A shows, the His-tagged 1a, 1b, and 1bΔ proteins (36.9, 33.7, and 32.8 kDa, respectively) were overexpressed by the addition of IPTG. These recombinant proteins were also detected using anti-bovine GGPS polyclonal antibodies (Fig. 3B). The 1a and 1bΔ proteins were also weakly detected without IPTG induction. The weak signal corresponding to about 30 kDa detected for cells transformed with pET-rGG1a

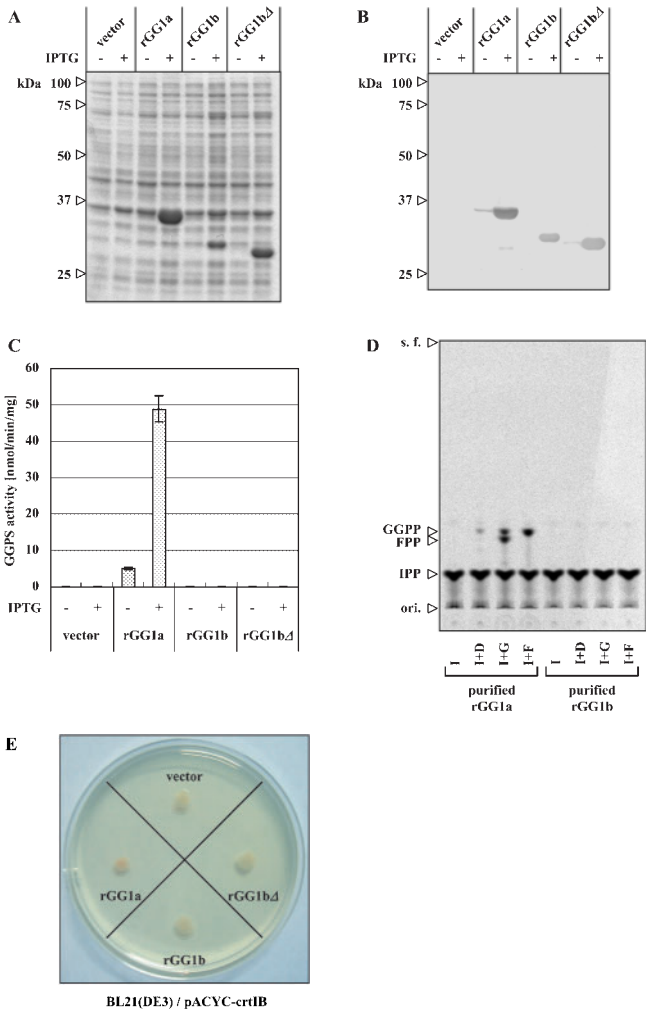

Fig. 3. **Expression of rat GGPS1a, 1b, and 1bΔ in *E. coli* cells.** (A) SDS-polyacrylamide gel electrophoresis of the His-tagged rGGPS1a, 1b, and 1bΔ proteins expressed in *E. coli* BL21 (DE3) with or without IPTG induction. (B) Western blot analysis of (A) with anti-bovine GGPS polyclonal antibodies. (C) GGPS activity of crude soluble fractions obtained from *E. coli* cells shown in A. Activity was measured with the combination of [1-^{14}C]IPP and FPP. (D) Silica-gel TLC of products of the prenyltransferase reaction with the purified His-tagged rGGPS1a and 1b proteins with [1-^{14}C]IPP and allylic substrates. Large capitals I, D, G, and F indicate isopentenyl, dimethylallyl, geranyl, and farnesyl diphosphates, respectively. s.f. and ori. indicate the solvent front and origin, respectively. (E) Carotenoid–color production testing of *E. coli* BL21 (DE3) cells harboring pACYC-crtIB and the pET plasmid expressing rGGPS1a, 1b, or 1bΔ gene. Resistant colonies were replated on an LB plate containing appropriate antibiotics and then cultured at 20°C for 3 d, as described under "MATERIALS AND METHODS."

might represent a partially degraded 1a protein. On *in vitro* GGPS assaying with crude soluble proteins and the combination of [1-^{14}C]IPP and FPP, enzymatic activity was observed in the case of 1a but not in the case of 1b or 1bΔ (Fig. 3C). We purified the His-tagged 1a and 1b proteins by nickel affinity chromatography and analyzed their enzymatic products (Fig. 3D). The 1a protein showed the ability to produce GGPP with the combination of [1-^{14}C]IPP and DMAPP, GPP, or FPP. However, the purified 1b protein did not show any ability to produce GGPP with the same substrate combination. We also performed a carotenoid-color production test to detect GGPS activity, especially 1b and 1bΔ-derived activity. The *in vivo* GGPS assay system has been established to be sensitive enough to detect the activity of a mutated 1a protein, which is 0.15% active relative to the 1a protein in an *in vitro* assay (unpublished data). As shown in Fig. 3E, *E. coli* cells harboring pET-rGGPS1a turned red, but ones harboring pET-rGGPS1b or pET-rGGPS1bΔ remained pale yellow similar to the control cells. These results indicate that the 1a protein is active, while the 1b and 1bΔ proteins are inactive or, if active, less than 0.15% activity relative to the 1a protein level.

Expression of Rat GGPS1a, 1b, and 1bΔ in HeLa Cells—We next tried to express the His-tagged 1a, 1b, and 1bΔ proteins in HeLa cells. On Northern blot analysis, their mRNAs were significantly detected using a common DIG-labeled antisense RNA (600 nucleotides) probe corresponding to the N-terminal 200 amino acids (Fig. 4A). On Western blot analysis of crude soluble fractions, the proteins derived from these mRNAs were detected, but the expression levels of the 1b and 1bΔ proteins were about 10% relative to that of the 1a protein (Fig. 4B). We assayed prenyltransferase activity using crude soluble fractions and analyzed the products (Fig. 4C). Radioactive farnesol (C_{15}OH) was commonly detected when four fractions including the control were assayed with the combination of [^{14}C]IPP and DMAPP or GPP. On the other hand, radioactive geranylgeraniol (C_{20}OH) was significantly detected only with the soluble fraction derived on 1a transfection, as determined with the combination of [^{14}C]IPP and DMAPP, GPP or FPP. Fig. 4D and E show the quantitative analyses of radioactive farnesol with the combination of [^{14}C]IPP and GPP, and of radioactive geranylgeraniol with the combination of [^{14}C]IPP and FPP, as shown in Fig. 4C, respectively. The FPP-synthesizing activity (Fig. 4D) was a little high for the soluble fraction derived on 1a transfection. The GGPP-synthesizing activity of the same fraction (Fig. 4E) was about 20-fold as high as that of the control fraction, and neither an increase nor decrease in GGPP-synthesizing activity was observed in the cases of 1b and 1bΔ transfection compared with the control. We also tried to express the 1a and 1b proteins in Cos-7 cells or 293 cells, however, the low expression levels of the 1b proteins compared with that of the 1a protein was similar to in the case of HeLa cells (data not shown). These results suggest that in mammalian cells 1a-type (1a) mRNAs are translated to give active GGPS and that 1b-type (1b and 1bΔ) mRNAs are translationally repressed and/or inactive 1b-type proteins are unstable.

Expression of the β-Galactosidase Gene with or without Its Stop Codon Fused with the 3′-Coding Regions of Rat 1a-Type and 1b-Type GGPS mRNAs in HeLa Cells—Concerning the 3′-coding regions of 1a-type and 1b-type GGPS mRNAs, we performed the following two experiments. In one experiment, each 3′-coding region was inserted at 41 nucleotides downstream (derived from the multi-cloning site of the pcDNA vector) from the *lacZ* stop codon within the 3′-UTR (Fig. 5A; 2, 3, and 4), and these plasmids were transfected into HeLa cells. As shown in Fig. 5B and C (1, 2, 3, and 4), there was no change in the expression levels of β-galactosidase pro-

teins or in their activities. This means that the different 3′-coding regions are not necessarily related to translational control. In the other experiment, the same 3′-coding regions were similarly inserted at 49 nucleotides downstream of the β-galactosidase gene without its stop codon (Fig. 5A; 5, 6, and 7). These plasmids express fusions of β-galactosidase with the C-terminal regions of the 1a, 1b, and 1bΔ proteins containing an 18 amino acid spacer sequence derived from the multi-cloning sites of the pcDNA vector. In this case, fusion proteins differing in the C-terminal region were detected, and their expression levels were different (Fig. 5B; lanes 5, 6, and 7). In particular, the expression levels of fusions of β-galactosidase with the C-terminal regions of the 1b (lane 6) and 1bΔ (lane 7) proteins were much lower than that with the C-terminal region of the 1a protein (lane 5). The β-galactosidase activities of these fusion proteins were almost the same as those of the expressed proteins (Fig. 5C; lanes 5, 6, and 7). This means that β-galactosidase mRNAs without the stop codon fused with different 3′-coding regions of 1a and 1b or 1bΔ mRNAs are expressed to produce active fusion proteins, but the presence of 1b and 1bΔ-derived C-terminal peptides in fusion proteins is related to the lower detection levels of the fusion proteins. These results support that the low expression levels of 1b-type GGPS proteins observed in HeLa, Cos-7 and 293 cells are not due to the translational repression attributed to the 3′-coding region of 1b-type mRNAs but to the instability of 1b-type proteins attributed to their C-terminal regions.

Tissue Distribution of Rat 1a-Type and 1b-Type GGPS mRNAs—To better understand the two types of mRNAs, we analyzed the expression levels of 1a and 1b mRNAs in various tissues. Since the difference between 1a-type and 1b-type mRNAs is due to alternative splicing of intron 4b (Figs. 2C and 6A), assaying of 344 and 303 bp PCR product was performed to detect each mRNA. Another long product (1,789 bp) was not observed under the assay conditions used. The results of quantitative RT-PCR are shown in Fig. 6B. The numbers under the graph are the ratio of 1a-type and 1b-type mRNAs. In most tissues of a male rat (age 3 wk), 1a-type mRNAs were expressed more than 1b-type mRNAs, but in testis 1a-type and 1b-type mRNAs were expressed at almost the same levels. The comparative occurrence of both mRNAs was also observed in ovary (age 3 wk), though the total level was low compared with in the case of testes. These results suggest that the splicing of intron 4b is differently regulated in various tissues.

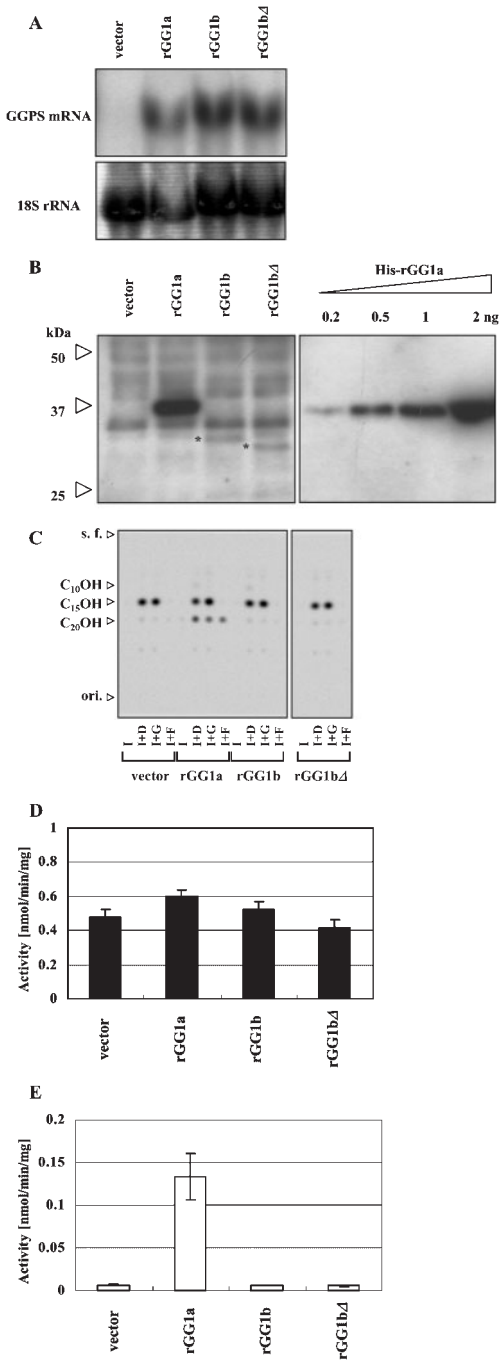

Fig. 4. **Expression of rat GGPS1a, 1b, and 1bΔ mRNAs in HeLa cells.** (A) Northern blot analysis of rGGPS1a, 1b, and 1bΔ mRNAs expressed in HeLa cells. Total RNAs (20 μg) were analyzed with a common 600 nucleotide DIG-labeled antisense RNA probe (upper), and 18S rRNAs were detected by ethidium bromide staining (lower), as described under "MATERIALS AND METHODS." (B) Western blot analysis of crude soluble fractions (20 μg) of transfected HeLa cells. Detection was performed with anti-His-HRP antibodies. Purified His-tagged 1a proteins (0.2, 0.5, 1, and 2 ng) were also loaded as standards. Asterisks indicate the bands corresponding to the 1b and 1bΔ proteins. (C) Analysis of enzymatic products with crude soluble fractions of transfected HeLa cells with [^{14}C]IPP and allylic substrates. Prenyl alcohols obtained on the treatment of enzymatic product with acid phosphatase were analyzed by reverse-phase LKC-18 thin layer chromatography in acetone:water (7:1). The abbreviations, I, D, G and F, are the same as in Fig. 3D. C_{10}OH, C_{15}OH, and C_{20}OH are geraniol, farnesol, and geranylgeraniol, respectively. s. f. and ori. indicate the solvent front and origin, respectively. Each datum is one of three independent similar results. (D) Quantitative analysis of FPS activities calculated from radioactive farnesol with the combination of [1-^{14}C]IPP and GPP shown in C. Activities are presented as means ± SD of triplicate determinations. (E) Quantitative analysis of GGPS activities calculated from radioactive geranylgeraniol with the combination of [1-^{14}C]IPP and FPP shown in (C). Activities are presented as means ± SD of triplicate determinations.

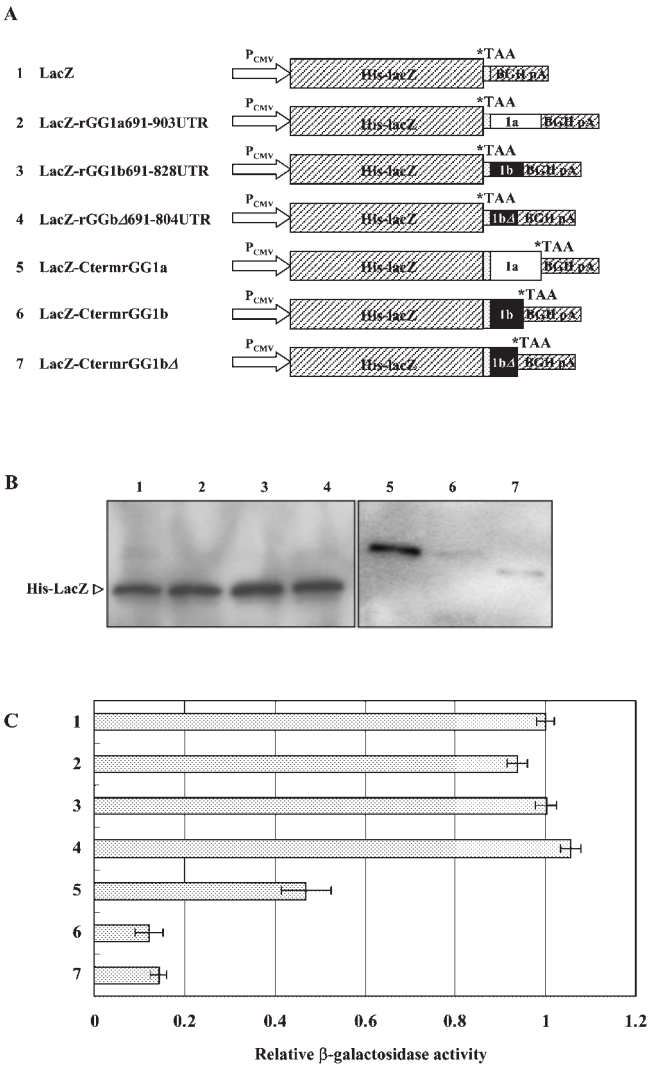

Fig. 5. **Expression of the β-galactosidase gene with or without its stop codon fused with the 3′-coding regions of rat 1a-type and 1b-type GGPS mRNAs in HeLa cells.** (A) Construction of fusion β-galactosidase expression plasmids. Construct 1 is a control plasmid encoding His-tagged *E. coli* β-galactosidase (hatched box) between the cytomegalovirus promoter (P_{CMV}, shown as an arrow) and the bovine growth hormone receptor polyadenylation signal (BGH pA, hatched bar). Plasmids 2, 3, and 4 contain the 3′-coding regions of rat GGPS1a (691–903, relative to the translation start site, open bar), 1b (691–828, black bar), and 1bΔ (691–804, black bar), respectively, at 41 nucleotides downstream (derived from the vector multi-cloning sites, shown as a gray bar) from the *lacZ* stop codon within the 3′-UTR. Plasmids 5, 6, and 7 encode fusions of β-galactosidase with the C-terminal regions of rat GGPS1a (amino acids, 231–300, shown as an open box), 1b (231–275, black box), and 1bΔ (231–267, black box) containing an 18 amino acid sequence (GSSVVEFCRYPAQWRPLE, shown as a gray box) derived from the vector multi-cloning sites as a spacer. The positions of stop codons (TAA) are shown by asterisks. (B) Western blot analysis of crude soluble fractions of transfected HeLa cells expressing β-galactosidase (lanes 1, 2, 3, and 4) and β-galactosidase fusions (lane 5, 6, and 7). Twenty μg of soluble proteins was separated by SDS-polyacrylamide (7%) gel electrophoresis, followed by staining with anti-His-HRP antibodies. (C) Analyses of β-galactosidase activities of crude soluble fractions shown in (B). One μg of crude soluble proteins was used for the enzyme assay, as described under "MATERIALS AND METHODS." Relative β-galactosidase activities are presented as means ± SD of triplicate determinations.

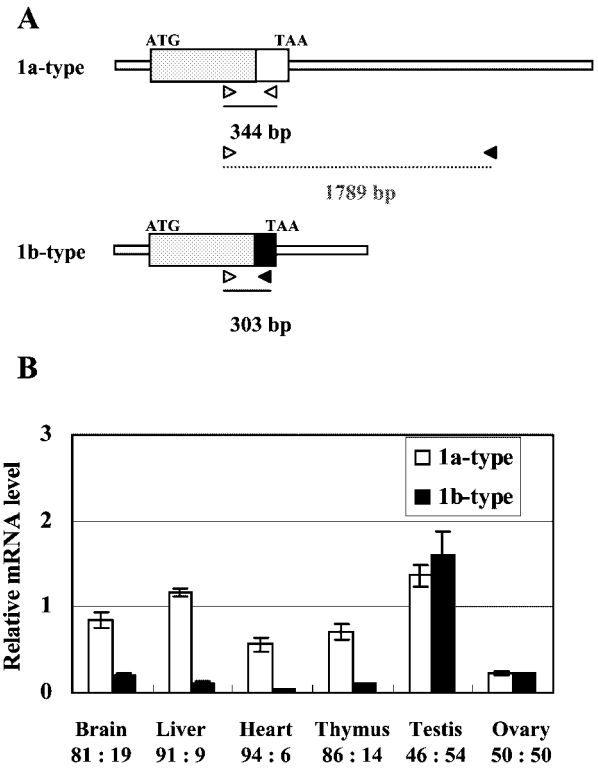

Fig. 6. **Tissue distribution of 1a-type and 1b-type GGPS mRNAs in rat.** (A) Location of the two cDNA probes (344 and 303 bp) used to determine the 1a-type and 1b-type mRNAs expression levels. The 344 and 303 bps probes were obtained by RT-PCR with the combinations of rGGPS-qs3 (gray arrowhead) and rGGPS1a-qa1 (open arrowhead), and rGGPS-qs3 and rGGPS1b-qa1 (black arrowhead), respectively. (B) Relative mRNA levels (1a-type/GAPDH and 1b-type/GAPDH, %). Total mRNAs were prepared from various tissues of 3-wk-old male or female rat, and then analyzed by quantitative RT-PCR with a Light Cycler as described under "MATERIALS AND METHODS." The values presented are means ± SD of triplicate determinations. The numbers under the graph are the ratios of 1a-type and 1b-type mRNAs.

Expression Level of Rat 1a-Type and 1b-Type GGPS mRNAs, and Enzymatic Activity during Testis Development—We also examined the expression levels of 1a-type and 1b-type mRNAs in rat testis during testis development. As shown in Fig. 7A, the total expression level of GGPS mRNAs (1a plus 1b mRNAs) increased. Interestingly, the level of 1a-type mRNA was almost the same in testes at 3 to 10 wk, whereas that of 1b-type mRNA increased in testes of 3 to 4 wk. We also examined GGPS activities during testis development. The GGPS activity level remained almost unchanged from 3 wk to 10 wk (Fig. 7B). These results suggest that GGPS activity during testis development is regulated at the splicing step to a certain extent.

DISCUSSION

The original aim of our study was to learn how GGPS and its gene are regulated, because this enzyme is responsible for the synthesis of GGPP leading to the formation of important geranylgeranylated proteins in the mevalonate pathway. In the present study, we have identified

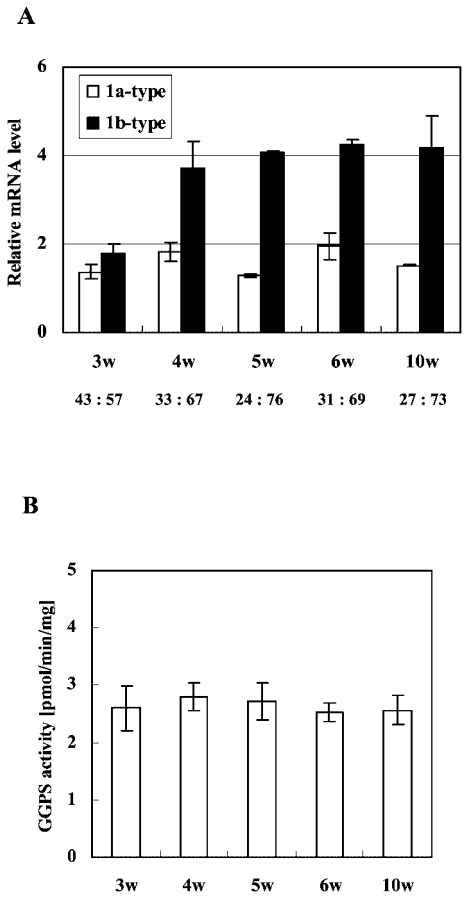

Fig. 7. **Expression levels of 1a-type and 1b-type GGPS mRNAs, and enzymatic activity during rat testis development.** (A) Relative mRNA level (1a-type/GAPDH and 1b-type/GAPDH, %). Total mRNA was prepared from rat testes (age 3, 4, 5, 6, and 10 wk) and analyzed by quantitative RT-PCR with a Light Cycler as described under "MATERIALS AND METHODS." The values presented are means ± SD of triplicate determinations. The numbers under the graph are the ratios of 1a-type and 1b-type mRNAs. (B) GGPS activities of crude soluble proteins prepared from rat testes (age 3–10 wk). The assay was performed with the combination of [1-^{14}C]IPP and FPP as described under "MATERIALS AND METHODS." The values presented are means ± SD of triplicate determinations.

multiple GGPS mRNAs produced from a single GGPS gene in rat. These multiple mRNAs can be grouped into two types, 1a-type mRNAs encoding GGPS of 300 amino acids and 1b-type mRNAs encoding truncated GGPS of 275 or 267 amino acids. The 1a-type and 1b-type proteins were expressed in *E. coli* and HeLa cells, and determined to be active and inactive, respectively. But, in the case of HeLa cells, the expression level of 1b-type proteins is quite lower than that of the 1a-type protein, though the amounts of their mRNAs expressed are similar (Fig. 4A). This was also the case for Cos-7 and 293 cells. These findings raised the possibility that 1b-type mRNAs are translationally repressed and/or 1b-type proteins are unstable in mammalian cells. The difference between 1a-type and 1b-type mRNAs is in their 3′-regions. Some of the mRNAs containing stem-loop structures within 5′-UTR and 3′-UTR are recognized by RNA-binding proteins and translationally repressed (*26*, *27*). We predicted the RNA secondary structure of each 3′-coding region of 1a-type and 1b-type mRNAs using GENETYX-Win 5.1 software. Several different stem-loop structures were predicted between their 3′-regions corresponding to 70, 45, or 37 amino acid sequences. We inserted each 3′-coding region within the 3′-UTR of the β-galactosidase gene with or without its stop codon, and then analyzed the protein expression levels. The insertion did not result in reduction of the non-fusion β-galactosidase expression. In the case of fusion β-galactosidase expression, 45 and 37 amino acids corresponding to the C-terminal region of 1b-type proteins caused a dramatic reduction compared with 70 amino acids corresponding to those of 1a-type proteins. These results support that the difference in protein expression between rat GGPS1a and 1b or 1bΔ in mammalian cells is due to the instability of the expressed proteins due to the C-terminal peptides of 1b-type proteins. Coupled with these results, we at present conclude that the expression level of active GGPS is at least regulated by the splicing of intron 4b of its gene in rat.

For mouse, a similar GGPS1b cDNA sequence is registered in the GenBank database. The coding region is shorter than that of rat due to a frame shift of one nucleotide, so the mouse 1b protein has a short polypeptide of SLTMQF at the C-terminus compared with the polypeptides of 45 or 37 amino acid residues of the corresponding rat 1b-type proteins. In other words, the 3′-regions of 1b-type mRNAs are almost completely conserved between rat and mouse, while the encoded C-terminal amino acids sequences are not conserved between them. In an expression experiment involving mouse 1b mRNA, low level protein expression was also observed in HeLa cells (unpublished data). We cannot at present conclude that the reduction of the mouse 1b protein expression level is due to the instability of the expressed proteins, because we have not yet performed β-galactosidase fusion experiments as to the mouse 3′-region. However, we expect that the mouse 1b protein is also inactive and unstable, similar to rat 1b-type proteins.

In man, we demonstrated in a previous study the occurrence of two sizes of mRNA (1.5 and 3.1 kb) for GGPS (*18*) on Northern blot analysis. Comparison of the shorter cDNA obtained by our group, a longer cDNA reported by Ericsson *et al.* (*19*), and the available human genome DNA (*28*) revealed that the two sizes of mRNA are due to different poly(A) signals in exon 4 of the GGPS gene (NT004836) located on 1q43. We could not isolate any human 1b-type cDNA on library screening. We also could not find any 1b-type cDNA in a database search on the NCBI BLAST network, but could find another type of splice variant in man. The sequences, BE780949, CD103564, and BI963107, lack exon 2 containing a translation initiation codon. We grouped them as 1c-type mRNAs. The human 1c-type mRNAs encode a protein of 154 amino acids without three (I, II, and III) of five conserved regions commonly observed in all-*trans* prenyltransferases, suggesting that the protein is inactive. Human splice variants (1c-type) are different from rodent variants (1b-type), but these splice variant-derived GGPS proteins other than rat 1b-type proteins might be inactive. If this is the case, we can conclude that mammalian active GGPS expression is regulated commonly through alternative splicing of their mRNAs.

The occurrence of multiple mRNAs has also been found in studies on other mevalonate pathway enzymes. Multiple mRNAs for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase are produced due to multiple transcription initiation sites and alternative introns in the 5′-region (*29*). Multiple mRNAs for FPS are produced due to multiple transcription initiation sites (*30*). Some of these mRNAs contain several out-of-frame upstream ATGs in their 5′-UTR and regulate the translation level. These multiple mRNAs may play a role in regulating gene expression of these enzymes at the translational step in addition to finely tuned transcriptional control regulated by the cellular cholesterol level (*31*). Since the occurrence of multiple mRNAs for GGPS is due to alternative splicing of exon 2 (human) or intron 4b (rodent), it is very interesting that the splicing type of GGPS is different from that of HMG-CoA reductase or FPS. This difference seems to reflect the difference in the regulation of each biosynthetic step corresponding to mevalonate, FPP, and GGPP syntheses.

Ericsson *et al.* reported that GGPS transcription is not regulated by the cellular cholesterol level (*19*), unlike in the cases of HMG-CoA reductase (*10*) and FPS (*14–16*). This seems to be reasonable because the product GGPP is a nonsterol compound and not an intermediate for cholesterol synthesis. However, GGPP acts as a negative regulator for the DNA binding of LXR (*11*, *12*). LXR is an orphan member of the nuclear hormone receptor superfamily and plays a central role in cholesterol metabolism (*32*). Study on regulation of GGPS is very important not only for understanding protein prenylation but also for understanding LXR regulation. In the current study we focused on the splicing of intron 4b. The difference in expression level between 1a-type and 1b-type mRNAs or between their proteins during rat testis development suggests that transcription and splicing are closely related with each other in GGPS expression. Vicent *et al.* also reported differences between three sizes of mRNA and protein expression levels among different tissues of *ob/ob* mice (*20*). To understand the coupling of the transcription and splicing mechanisms for mammalian GGPS genes, further experiments are in progress.

The nucleotide sequences reported in this paper have been submitted to the GenBank with accession numbers AB118237 (rat geranylgeranyl diphosphate synthase gene), AB118238 (rGGPS1a1 cDNA), AB118239 (rGGPS1a2 cDNA), AB118240 (rGGPS1a3 cDNA), AB118241 (rGGPS1b1 cDNA), AB118242 (rGGPS1b2 cDNA), and AB118243 (rGGPS1bΔ cDNA). We would like to thank Professor Tanetoshi Koyama and Dr. Seiji Takahashi for the helpful discussions, and also Professor Tokuzo Nishino and Dr. Toru Nakayama for the use of the Light-Cycler. We also thank Dr. Hisashi Hemmi for providing us with the pACYC-crtIB plasmids. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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