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MULTIPLE mRNA SPECIES TRANSCRIBED FROM MULTIPLE GENES CONTAIN DIVERGED NUMBERS OF EXACT 39-BASE (13-AMINO ACID) REPEATS*

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Polysialoglycoprotein (PSGP) of unfertilized eggs of rainbow trout (Salmo gairdneri) consists of tandem repeats (about 25) of a glycotridecapeptide, Asp-Asp-Ala-Thr*-Ser*-Glu-Ala-Ala-Thr*-Gly-Pro-Ser-Gly (* denotes the attachment site of a polysialylglycan chain) (Kitajima, K., Inoue, Y., and Inoue, S. (1986) J. Biol. Chem. 261, 5262-5269). By using oligodeoxynucleotide probes based on the above sequence, we isolated a genomic clone for apoPSGP which contains 39-base pair repeats (5'-GACGAGCCACCTCTGAAGCT-GCGACCGCCCGTCTGGCC-3') encoding the tridecapeptide. Using a fragment of this genomic DNA as a probe, we next screened a cDNA library constructed with mRNA from immature ovaries of rainbow trout. Nucleotide sequencing analyses of cDNA clones thus obtained revealed that apoPSGP is encoded by multiple mRNA species consisting of diverged numbers (6-32) of the 39-base repeat encoding the tridecapeptide unit and homologous 5'- and 3'-bordering regions. The encoded protein consists of three distinct regions: the N-region consisting of a putative signal peptide and a pro-peptide, the R-region containing diverged numbers of the tandem repeat of 13-amino acid residues, and the C-region with six amino acid residues. Southern blot analysis showed that multiple mRNAs are transcribed from multiple genes for apoPSGP containing diverged numbers of the 39-base pair repeat. Thus, the genes for apoPSGP constitute a multigene family. Expression of the mRNAs is stage and organ specific; i.e. they are expressed only in immature ovaries and not in mature ovaries or in any other organ.

A highly sialylated glycoprotein was first isolated from the unfertilized eggs of rainbow trout (1). This sialylated glycoprotein is unique because most of the sialic acid residues exist as polysialyl groups (2). Thus, it was designated as polysialoglycoprotein (PSGP)* (1, 2). The presence of PSGP in unfertilized eggs has been demonstrated for eight different species from three genera of Salmonidae fishes so far examined (3, 4). The existence of similar polysialylated molecules has been reported in tissues and cultured cells of higher animals (5). Neural cell adhesion molecules (N-CAM) of humans, mice and chickens (6-8), and glycoproteins of rat-developing brain (9) are examples of glycoproteins that have polysialylglycans and play roles as ligands in cell-cell interaction and binding.

The PSGPs of Salmonidae fishes have the following distinct features. 1) Most sialic acid residues exist in the form of polysialyl groups (-8NeuGca2), (1, 2). The polysialyl groups are partly capped with deaminoneuraminic acid (KDN, 3-deoxy-D-glycero-D-galacto-nonulosonic acid or 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid), and consequently, they are resistant to various sialidases (10). 3) The core protein contains exact tandem repeats of the same peptide. In the case of rainbow trout, there are an average of 25 repeats of a glycotridecapeptide (11), Asp-Asp-Ala-Thr*-Ser*-Glu-Ala-Ala-Thr*-Gly-Pro-Ser-Gly, where * denotes the attachment site of a polysialylglycan chain. 4) PSGP is a major component of the cortical alveoli (vesicles) of unfertilized eggs (12). In response to egg activation, PSGP is discharged by exocytosis into the space between the vitelline envelope and the plasma membrane, i.e. the perivitelline space, where the 200-kDa PSGP molecules undergo rapid and dramatic depolymerization by proteolysis into glycotridecapeptides with the least repeating structural units (9 kDa) (13). Thus, it is now evident that PSGP molecules have unique structural features which are reminiscent of their multiple physiological functions (10-13). Since the presence of cortical vesicles is ubiquitous among animal eggs of divergent sources from sea urchin to mammals (14, 15), elucidation of fertilization-related events concerning PSGP at the molecular level is essential for a better understanding of fertilization and subsequent early development. We, therefore, started the molecular cloning and characterization of apoPSGP of rainbow trout eggs. In this paper, we report the isolation and characterization of cDNA clones encoding apoPSGP and show the existence of multiple mRNA species that contain diverged numbers of a structural unit and are transcribed from multiple genes only in immature oocytes of rainbow trout.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli DNA ligase and avian myeloblastosis virus reverse transcriptase were obtained from P-L Biochemicals Inc. and Bio-Rad, respectively. DNA Polymerase I from E. coli, its Klenow fragment, bacterial alkaline phosphatase, T4 polynucleotide kinase, and Bal31 nuclease were purchased from Takara Shuzo Co. Restriction enzymes used were products of Takara Shuzo Co., Toyobo Co., and P-L Biochemicals Inc. The oligo(dt)12-18 cellulose was a product of P-L Biochemicals Inc. A nick translation kit and [γ-32P]ATP (-3000 Ci/mnmol) were obtained from Amersham Corp. [α-32P]dCTP
were obtained through the courtesy of the Gunma Prefectural Fishery Experimental Station at Kayaba, Japan. They were immediately frozen at -80 °C after dissection and kept frozen until use.

Sequence-specific oligodeoxynucleotide probes for apoPSGP, the following synthetic oligodeoxynucleotide probes based on the amino acid sequence of the tridecapeptide of apoPSGP (11) were used. Probes A and B, corresponding to the sequence, had the nucleotide sequence (A) 5'-d[GC(T or C)TC(G or A)TC(G or A)TC(G or A)TC(G or A)TC(G or A)TC]-3' (20 mer, 512 mix), (B) 5'-d[GC(T or C)TC(G or A)TC(G or A)TC(G or A)TC(G or A)TC]-3' (20 mer, 516 mix), and probe C, corresponding to the sequence, Glu-Ala-Thr-Gly-Pro, had the sequence (C) 5'-d[GGCCNGTNGC(G or A)TC(G or A)TC]-3' (17 mer, 515 mix) which N means all of four deoxynucleotides. These were synthesized with a DNA synthesizer (Applied Biosystem Inc. model 380 B) and labeled at their 5'-ends with [γ-32P]ATP and T4 polynucleotid kinase in a buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol, and 0.1 mM spermidine.

Construction of Genomic Library—Genomic DNA was extracted with the method described by Maniatis et al. (16) with slight modifications. The gut, liver, and testes of rainbow trout liver frozen at -80 °C were crushed into powder in a mortar and incubated at 50 °C in a solution containing 0.5 M EDTA, 0.5% Sarcosyl, 50 mM Tris-HCl, pH 7.4, and 0.2 mg/mI proteinase K for 5 h with gentle mixing. Then, the solution was extracted three times with phenol, and dialyzed against 10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 10% sucrose, and dialyzed again against the same buffer. The DNA was partially digested with Sau3A-I and fractionated by low-melting-temperature agarose gels. The DNA was then ligated into bacteriophage particles using the packaging kit GigaPack gold (Stratagene), and grown on E. coli NM555.

Isolation of Genomic Clones for ApoPSGP—Recombinant plaques were transferred onto nylon filters (Hybond® N, Amersham Corp.) and fixed with ultraviolet light. The filters were prehybridized for 10 h in 50 mM Tris-HCl, pH 8.0, containing 1 M NaCl, 10 mM EDTA, 10% SDS, 0.2 mg/ml heat-denatured E. coli DNA, and 50% (v/v) formamide, and hybridized using a cDNA fragment as probe under the same conditions. The filter was finally washed with 0.1 × SSC containing 0.1% SDS at 55 °C and then exposed to films.

Results

Isolation and Identification of Genomic Clones—From 3 × 10⁹ independent plaques of a λEMBL3 genomic library of rainbow trout, three clones were identified that hybridized with both probes A and C. One of them, designated λRTg-10, was subjected to further analyses and its restriction map is shown in Fig. 1. Since Southern blot analysis revealed that a 2.6-kb HindIII fragment was hybridized with the probes, the DNA fragment was excised from a gel, partially digested with Bal31 nuclease, and inserted into pUC8. Sequence analysis revealed that one of the truncated subclones, designated pRTg-10HB21, contained on one end of its insert more than 11 repeats of a 39-base sequence (5'-GAC GAC GCC ACC TCT GAA GCT GCG ACC GGC CCG TCT GGC-3') encoding the tridecapeptide sequence of apoPSGP (Asp-Ala-Thr-Ser-Glu-Ala-Ala-Thr-Gly-Pro-Ser-Gly) (11). Thus, the 1.6-kb insert of pRTg-10HB21, which began in the middle of the open box in Fig. 1 and ended between the first EcoRV site and third HindIII site from the left end, was isolated and used as a probe to identify mRNA and cDNA clones for apoPSGP.

cDNA inserts prepared by EcoRI digestion of recombinant phage DNA were subcloned into pUC18. Recombinant plasmids were introduced into E. coli HB101 as described above, and colonies containing the cDNA insert were selected by hybridization with the probe used for isolation of cDNA clones. The cDNA inserts and those of their subclones were sequenced by the dyelexie method (22) and by the chemical modification method of Maxam and Gilbert (23). The precise number of repeating units was determined as follows. 5'-End-labeled HindIII-EcoRV fragments of repeating regions were chemically modified and degraded by the method of Maxam and Gilbert (23) and electrophoresed with size markers in 6% acrylamide gels containing 8.3 M urea for 3, 8, and 15 h at 30 V/cm and for 25 h at 40 V/cm. The gels were then exposed to Fuji RX films and repeating patterns were counted.

RNA Blot Hybridization—Total RNA was extracted from various organs of rainbow trout as described for the construction of the cDNA library. RNA samples (40 µg) were denatured and electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and 40 mM MOPS buffer, pH 7.0 (16). After electrophoresis, the RNA was transferred onto a nitrocellulose membrane (Schleicher & Schuell Inc.), fixed by baking at 80 °C, prehybridized at 42 °C in a solution containing 3× SSC, 8.3 × Denhardt's solution, 40 µg sodium phosphate buffer, pH 7.0, 0.2% SDS, 8.3% Dextran sulfate, 0.2 mg/ml heat-denatured E. coli DNA, and 50% (v/v) formamide, and hybridized using a cDNA fragment as probe under the same conditions. The filter was finally washed with 0.1 × SSC containing 0.1% SDS at 55 °C and then exposed to films.

Genomic DNA Blot Hybridization—Four µg of the genomic DNA prepared as described above were digested with various restriction enzymes, fractionated on agarose gels, electrophoresed with size markers in 6% acrylamide gels containing 8.3 M urea for 3, 8, and 15 h at 30 V/cm and for 25 h at 40 V/cm. The gels were then exposed to Fuji RX films and repeating patterns were counted.

Restriction map of a genomic clone for apoPSGP, λRTg-10. The open box denotes the region that has the 99-nucleotide repeats. The bar represents 1 kb. Letters represent the sites of restriction endonucleases as follows: H, HindIII; P, PstI; V, EcoRV; B, BamHI; E, EcoRI. A 0.8-kb HindIII fragment of this clone was subcloned and sequenced as described under "Results." Thus, the orientation of the gene for apoPSGP was determined as indicated by the arrow.
Isolation and Identification of cDNA Clones—Since RNA from immature ovaries (6 months before ovulation) contained a significant amount of mRNA which hybridized with the above genomic DNA probe (see Fig. 3), we screened an λgt10 cDNA library of an immature ovary of rainbow trout. Most of the cDNA clones started with the repetitive sequence of 39 bp at the 5' termini which was located at opposite ends of the poly(A) sequence, and only one clone, designated λRTc-5-1, contained a nonrepetitive sequence at the 5' terminus of the cDNA insert, followed by the repetitive sequence (see Fig. 2). Then we again screened the same cDNA library using the nonrepetitive cDNA fragment as a probe to obtain full-length cDNA clones. Four clones (λRTc-5-2–5) thus isolated were subjected to further analyses together with the original clone, λRTc-5-1.

Sequence Analysis of cDNA Clones for ApoPSGP—From sequence analysis of cDNA clones for apoPSGP described below, it is concluded that the consensus structure of mRNAs of rainbow trout apoPSGP could be drawn as shown in Fig. 2A and that the mRNAs could be divided into three domains, N-, R-, and C-domains.

The cDNA inserts of λRTc-5-1-5 were subcloned into pUC18 and sequenced by the dideoxy method (22) from both ends as shown in Fig. 2B. The inserts of λRTc-5-1, 3, 4, and 5 contained poly(A) sequences at one corresponding to the 3' termini of the mRNAs. The repeating sequence ends about 100 bp upstream from the poly(A) sequence. The nucleotide sequences of these 3'-nonrepetitive regions were the same in all four clones.

The nucleotide sequences of the cDNA inserts corresponding to the 5'-terminal region of the mRNA preceding the repetitive sequence of 39 bp were also identical in the five clones, except that the 5'-terminal positions were different as shown in Fig. 2. From these results, it was concluded that the cDNA inserts of all five clones had the same structure, although their repetitive regions contained different numbers of the 39-bp repeating unit. Thus, the nucleotide sequences of the five clones were completely determined as shown in Fig. 2C.

These nucleotide sequencing results can be summarized as follows: 1) In the R-domains, the number of repetitions varies, 6, 16, 32, and 26 units, for λTRc-5-1, 3, 4, and 5, respectively. The G/C content of the R-domain is very high (72%) and is ascribed to the high frequency of G or C residues in the third letters of several amino acid residues; 2) 2 for Asp, 2 of 2 for Thr, 2 of 2 for Gly, 1 of 1 for Pro and 2 of 3 for Ala. 2) As for the N-domain, the five clones had essentially the same nucleotide sequence with only four nucleotides changed among the five different clones (Fig. 2). The last 14-amino acid residues encoded by the N-domain were homologous to the repeating unit of the R-domain: an extra alanine residue is inserted between the 3rd and 4th residues of the tripeptide. 3) The C-domains of the four clones had the same nucleotide sequences, including the 3'-noncoding region. The encoding region contains only 18 nucleotides, and the deduced amino acid sequence is partially homologous to the repetitive peptide sequence. A sequence, ATTAAA, was found at the upstream of the poly(A) sequence, which is similar to the universal poly(A) addition signal sequence, AATAAA (41, 42), but is different at the second residue. However, all cDNA clones contained poly(A) sequence of 17–57 bp, and the sequence, ATTAAA, should function as a poly(A) addition signal.

To confirm the initiation Met and transcription initiation site, a 0.8-kb HindIII fragment of the genomic clone pRTg-10 (see HindIII sites in Fig. 1) was sequenced because it should include a region corresponding to the N-domain of the cDNA clone. The result is included in Fig. 2C (nucleotide residues numbers –27 to –7). Further, using the genomic DNA fragment from –80 to 32 as a probe (see Fig. 2C, but complete

![Fig. 2](image-url)
sequence data was not shown) an S1-mapping experiment revealed the transcription initiation sites at residues −27 and −10 (overlined A residues in Fig. 2C). From the result that the upstream region contains an in-frame termination codon (residue numbers −18 to −16), together with the fact that the nucleotide sequence around Met-1 in Fig. 2C matched Kozak’s (24) “first Met rule,” we concluded that this Met residue is the first candidate for the initiation site, although the second ATG may function as an alternative initiation codon.

The primary structure of pre-pro-apoPSGP, thus definitely determined, can be divided schematically into three regions (N-, R-, and C-regions) in the same manner as the nucleotide sequence. Approximately the first 25 residues of the N-region are hydrophobic and are considered to form a signal peptide sequence. According to von Heijne’s (25) method, the cleavage site is postulated as the COOH terminus of the twenty-first valine, although its S-value (25) is rather low (S = 1.63). The existence of a signal peptide region is reasonable because apoPSGP is transferred into the Golgi apparatus and excreted from the cell. The remaining portion of the N-region is a pro-peptide region which is not contained in the mature PSPG molecule. A sequence homologous to the pre-pro-peptide or tricapeptide sequence of apoPSGP was not found in the protein sequence database (National Biomedical Research Foundation Data Bank). The R-region is an apoPSGP region, and the C-region, comprising 6-amino acid residues, is not contained in the mature PSPGP whose COOH terminus is Gly (11).

Distribution of mRNA for ApoPSGP—As noted in the section Isolation and Identification of cDNA Clones, immature ovaries contain a significant amount of mRNA for apoPSGP. The expression of apoPSGP mRNA in various organs of rainbow trout was examined using R-probe (indicated in Fig. 2B). As shown in Fig. 3, apoPSGP mRNA was detected only in the immature ovary (at 6 months before ovulation) (lane 9), and not in the mature ovary, i.e. ovulated eggs, nor in any other organs examined (lanes 8 and 1-7, respectively) even when exposed for longer periods or large amounts of total RNA (1-10 μg) were used (data not shown). This indicates that the expression of the apoPSGP mRNA is organ- and stage-specific. The apoPSGP mRNA detected in the immature ovary showed a smeared pattern expanding from 0.8 to 6 kb in length (lane 9). This shows that the mRNA for apoPSGP is heterogeneous in length due to diverged numbers of repeating units (39 base) as revealed by the analyses of cDNA clones (Fig. 2).

Identification of the Genes for ApoPSGP—To identify the genes for apoPSGP, chromosomal DNA of rainbow trout was digested with various restriction enzymes and subjected to a total genomic Southern experiment using three cDNA fragments corresponding to the three domains (Fig. 2) as probes. When the R-probe corresponding to the R-domain was used, multiple bands were detected in all cases (Fig. 4, lanes 1-5). Since, judging from the restriction map shown in Fig. 1, HindIII digestion of the apoPSGP gene should generate a single band hybridized with the R-probe, the multiple bands in the HindIII digestion (lane 3) indicate the presence of multiple genes for apoPSGP.

Hybridized bands gave various staining intensities. This is not due to heterogeneity in the sequence because essentially the same pattern was obtained when synthetic probes (probes A and C, see “Experimental Procedures”) were used under stringent conditions (data not shown). Therefore, the staining intensity corresponds to the number of genes with similar lengths. The number of genes for apoPSGP was calculated to be more than 100 on the assumption that the faintest band...
represents a single gene for apoPSGP. Since there is no intervening sequence between the HindIII and EcoRV sites spanning the coding region of λRTg-10 (Fig. 1), the length of the HindIII-EcoRV fragments hybridized with R-probe represent the lengths of the repeating regions (R-domain) from 0.5 to 7.5 kb (lane 5). This implies that the apoPSGP gene is heterogeneous with respect to the numbers of the repeating unit in the R-domain and that the numbers of repetition range from 10 to 200.

When the N-probe was used, almost the same profiles were obtained for EcoRI, BamHI, and EcoRV digestions (lanes 6, 7, and 9, respectively) as were obtained with the R-probe, confirming the multiple nature of the apoPSGP gene. In the case of HindIII or HindIII/EcoRV digestion (lane 8 or 10) which separates the N-domain from the R-domain (see HindIII sites of the cDNA clones in Fig. 2B), however, the patterns were totally different from those of the R-probe; the N-probe revealed a major band of 0.80 kb and a minor band of 0.56 kb, indicating that the apoPSGP genes contain highly homologous 5' regions (N-domain). As proof, we isolated genomic clones for apoPSGP that had a 0.80-kb HindIII fragment in the N-domain (data not shown). Though a 0.56-kb HindIII fragment has not yet been identified, it might represent minor mutants that have deletions in the N-domain or point mutations generating another HindIII site. These facts are consistent with the analyses of the cDNA clones as shown in Fig. 2.

Similar results were obtained when the C-probe was used (data not shown). The patterns of the EcoRI, BamHI, and HindIII digestions were similar to those of the R-probe. In the case of the HindIII/EcoRV digestion, which separates the R- and C-domains (see EcoRV sites of the cDNA clones in Fig. 2B), however, the C-probe revealed only one band 1.5 kb long. Therefore, C-domains of the multigenes for apoPSGP are also highly homologous.

Thus, the genes for apoPSGP constitute a gene family that has conserved N- and C-domains and an R-domain of diverged length. Most of the multiple genes are transcribed into multiple mRNAs containing diverged numbers of the repeating unit as revealed by sequence analyses of cDNA clones (Fig. 2) and Northern blot analysis (Fig. 3).

**DISCUSSION**

In this study, we isolated and sequenced several cDNA clones for apoPSGP. Nucleotide-sequencing analyses revealed that mRNA for apoPSGP contains tandem repeats, each composed of 39 bases encoding a tridecapeptide and that the sequences of the repeating units are completely conserved at the nucleotide level as well as at the amino acid level. Moreover, multiple mRNA species are transcribed from multiple genes for apoPSGP having diverged numbers of the repetitive sequence.

The nucleotide sequence of the R-domain is quite interesting in the following points. 1) The number of repeating units of 39 bp, which encode a tridecapeptide (Asp-Asp-Ala-Thr-Ser-Glu-Ala-Ala-Thr-Gly-Pro-Ser-Gly), is diverged among the cDNA clones. 2) The nucleotide sequences of the 39-bp repeats within a single cDNA clone and in different cDNA clones are completely identical. 3) A single codon is used for Asp, Thr, Ser, and Gly residues which appear twice in the repeating unit. The 3 Ala residues in the repeating unit, on the other hand, are encoded by three different codons. As discussed above, the codon usage of the 3 Ala residues is conserved among all repeating units. Therefore, the strict conservation of the nucleotide sequence in the repeating units is not due to a preferential codon usage in rainbow trout but may have some biological importance in itself, e.g. in transcriptional regulation, gene duplication, etc.

Several cases of tandem repeating structures in proteins and genes are known, for example, malaria circumsporozoite antigen (26), human involucrin (27), winter flounder antifreeze proteins (28), the proline-rich proteins from the salivary glands of various animals (29-32), epidermal filaggrin (a histidine-rich basic protein) present in cornified cells (33), human liver histidine-rich glycoprotein (34), silkmoth chorion protein (35), collagens (36-38), ubiquitin (39), and apolipoprotein(a) (40). Among these proteins, the genes for winter flounder antifreeze proteins, rat and mouse proline-rich proteins, silkmoth chorion proteins, collagens, and ubiquitin constitute multigene families like apoPSGP genes. However, in these cases, members of the multigene families are much fewer than those of apoPSGP, and significant heterogeneities are observed among members and only consensus sequences are described. In this respect, apoPSGP is unique because its heterogeneity is found only in the number of repeating units and the total sequence, including the 5' and 3' noncoding sequences, is well conserved. In some cases function of repeating structures is known: to be concerned with their specific three-dimensional structures. For example, it is well known that collagen has a very distinctive structure called triple helix or collagen helix (36-38), and the antifreeze properties of antifreeze protein are due to its structure containing tandem repeats (28). The high antigenicity of malaria circumsporozoite antigen is ascribed to its repeating structure (26), which contains a β-sheet structure. On the other hand, in the case of ubiquitin precursor (39) and apolipoprotein(a) (40), their repeating units function independently after processing to their unit peptides. PSGP belongs to the latter group.
because PSGP is cleaved into its units at fertilization or at time of activation of eggs (13).

Fig. 5 shows a schematic pathway for the biosynthesis and modifications of PSGP based on present results. In immature ovaries of rainbow trout, multiple apoPSGP genes are transcribed into multiple mRNAs having varying numbers of the 39-base repeat unit bordered by highly homologous sequences. The multiple mRNAs are translated into pre-pro-apoPSGP with varying numbers of the tridecapeptide repeat. Following transport to the Golgi apparatus, proteolytic cleavage and extensive glycosylation occur to produce mature PSGP comprised solely of tandem repeats of glycotridecaglycopeptides (11). This mature PSGP is localized in the cortical alveoli of eggs. Upon activation of the egg (or fertilization), the cortical alveoli undergo exocytosis, and PSGP is discharged into the perivitelline space and cleaved by the action of a specific protease, designated as PSGPase, into a low molecular weight PSGP consisting of the tridecapeptide with three polysialylglycan chains (11, 12). During fertilization and/or early development these tridecaglycopeptides play important roles, for example, they prevent polyspermy or are involved in the formation of a fertilization membrane. Then, why are they synthesized as a large precursor? This may be because the precursor form (mature PSGP) is inactive and a large amount of active peptides are needed immediately at fertilization and/or at a very early developmental stage.

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