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Cloning of cDNA and estrogen-induced hepatic gene expression for choriogenin H, a precursor protein of the fish egg envelope (chorion)

(choriogenin/egg envelope/fish)

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ABSTRACT **A cDNA for choriogenin H (Chg H; formerly high-molecular weight spawning female-specific substances, or H-SF), a precursor protein of the inner layer subunits of egg envelope (chorion) of the teleost fish, *Oryzias latipes*, was cloned and analyzed. The clone consisted of 1913 bp and contained an open reading frame encoding a signal peptide of 22 aa and Chg H protein of 569 aa. The Chg protein possessed three potential N-glycosylation sites and Pro-X-Y repeat sequences in the first two-fifths of the N terminus. There were amino acid sequence similarities between Chg H and a gene product expressed in the liver of female winter flounder during vitellogenesis. Moreover, the amino acid sequence of Chg H is similar to that of ZP2 rather than ZP3 of zona pellucida of some mammals. Northern blot analysis indicated that gene expression for Chg H occurred only in the livers of spawning female fish and 17β-estradiol-treated male fish, but not in the ovary of the spawning female fish. Gene expression for Chg H and Chg L (formerly low-molecular weight spawning female-specific substance, or L-SF) was induced and increased in parallel in the male fish liver after 17β-estradiol treatment.**

The egg envelope encasing an animal egg plays significant roles in reproductive and developmental processes as an interface between the egg and sperm and/or as an interface between the embryo and its environment. The fish egg envelope (chorion), especially its major portion, the inner layer, has been conventionally regarded as a so-called primary egg envelope that is of oocyte origin (1–3). However, recent studies have accumulated the data that strongly suggest hepatic synthesis of precursor proteins of the inner layer subunits under the influence of estrogen (4–11).

In freshwater teleost fish, the Japanese medaka (*Oryzias latipes*), the chorion inner layer comprises two groups of subunits, ZI-1,2 and ZI-3. The former group was presumed at first to be composed of two proteins, ZI-1 and ZI-2 (12), but is now considered to consist of three similar proteins with relative molecular masses from 74,000 to 76,000 (ref. 13; H.S., K.M., I.I., K. Nomura, and K.Y., unpublished work). The latter group consists of a single protein, ZI-3, with an M_r of 49,000 (ref. 12; H.S., K.M., I.I., K. Nomura, and K.Y., unpublished work). ZI-1,2 proteins together and ZI-3 protein have been isolated by SDS/PAGE and by column chromatography and partly characterized (12–14). Through biochemical and immunochemical analyses using specific antibodies against each subunit group, we found precursor proteins of both subunit groups were synthesized in the livers of spawning female fish and estrogen [17β-estradiol (E_2)]-treated male fish under natural and experimental conditions, respectively (5, 6, 9, 10, 13). Thus, the precursors of ZI-1,2 and ZI-3 have been called high- and low-molecular weight spawning female-specific substances (H- and L-SF), respectively (9). We purified L-SF and H-SF from ascites fluid that was accumulated in E_2-treated male fish and found the former was a single protein similar to ZI-3 and the latter comprised three proteins corresponding to ZI-1,2 proteins (12, 13). In a previous paper (15), we reported isolation and analysis of a cDNA clone for L-SF, describing a predicted primary structure that was coincident in some portions with purified ZI-3 and L-SF, and an exclusive expression of the gene in the liver but not in the ovary as detected by Northern blot analysis using the cDNA as a probe.

In the present study, we performed cloning and analysis of a cDNA for a protein of H-SF and confirmed that there was also no dual expression of the gene; the gene expression occurred only in the liver but not in the ovary. According to the results, precursor proteins of both the two major subunit groups of fish egg envelope are now purified and characterized and their hepatic synthesis is confirmed. Thus, a general name of choriogenin (Chg) should be proposed for such liver-derived precursor proteins of the inner layer subunits of fish egg envelope (chorion)—i.e., medaka Chg L for L-SF and Chg H for H-SF—since this new name would better represent the biological nature of the precursor proteins. Recently, we were aware that a similar naming had been done for cDNAs for *Fundulus heteroclitus* chorion proteins (16). Although our proposal was done independently of it, an identical term is now applied to the liver-derived precursors of fish chorion subunits (see also ref. 17). Thus, the present report describes the cloning and analysis of a cDNA and the gene expression for medaka Chg H.

MATERIALS AND METHODS

Fish. Adult fish of commercial orange-red variety of the medaka, *O. latipes*, were fed on Tetrafin flakes (TetraWerke, Melle, Germany) in outdoor tanks in summer. To construct a cDNA library, the livers of spawning female fish were used. Some fish were kept in indoor tanks at about 27°C on 14 hr light/10 hr dark regime to keep them in breeding condition. For estrogen administration to the fish, E_2 (Sigma) was given

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Abbreviations: E_2, 17β-estradiol; H- and L-SF, high- and low-molecular weight spawning female-specific substances, respectively; Chg H and L, choriogenin H and L, respectively; SLIC, single-strand ligation to single-stranded cDNA.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. D89609).

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to the experimental male fish either *per os* by the use of E_2-dusted diet (1 mg of E_2 per g of Tetrafin flakes) for 7–14 days (6, 13) or by rearing the fish in water containing E_2 (100 ng/ml). In the latter case, E_2 dissolved previously in 0.1 ml of ethanol was added to 600 ml of water as described previously (10). Fish were treated with E_2 for the periods ranging from 2 to 72 hr. As controls, fish were reared in water of the same concentration of ethanol containing no E_2. The experimental and control fish were kept under nonbreeding conditions—i.e., on 10 hr light/14 hr dark regime at 15°C to minimize any stimulatory influence on their reproductive activity and to keep the E_2-treated fish in healthy condition.

cDNA Cloning and Sequencing. For construction of a cDNA library, poly(A)$^+$ RNA was purified by two cycles of chromatography through an oligo(dT)-cellulose column from the bulk RNA of the liver of spawning female or E_2-treated male fish.

cDNA synthesis was performed with a kit (ZAP-cDNA Synthesis Kit, Stratagene) using an oligo(dT) primer with *Xho*I linker. The cDNA was size-fractionated on a Sephacryl S-400 column with a lower cut-off value of 0.5 kb and inserted into λ ZAP arms following addition of *Eco*RI adaptor.

Anti-Chg H IgG as a probe was obtained from the mouse immunized with purified Chg H. This IgG was absorbed with a lysate of *Escherichia coli* XL1-Blue cells. The absorbed antibody that was not reactive with Chg L but only with Chg H was used for screening the recombinants that were expressing Chg H by the plaque method. The screening of the λ ZAP library was carried out according to the immunological method by the use of avidin:biotinylated enzyme complex technique with Vectastain ABC kit (Vector Laboratories; ref. 18).

The sequence of the biggest-sized positive clone (H-SF11) obtained by immunological screening was determined. According to the stepwise deletion method (19), the overlapping deletion mutant subclones of H-SF11 were constructed and generated. The inserts of these subclones and the PCR products (see below) were subjected to sequence analysis by using *Taq* Dye Primer Cycle Sequence Kit and Dye Terminator Cycle Sequence Kit (Applied Biosystems and Amersham) with a DNA thermal cycler (Perkin–Elmer/Cetus), and automatic sequencing was performed on a 373A DNA sequencer (Applied Biosystems).

Single-Strand Ligation to Single-Stranded cDNA (SLIC)-PCR. SLIC-PCR was performed according to the method of Edwards *et al.* (20). The single-stranded cDNA was synthesized with Takara PCR kit (Takara Shuzo, Otsu, Japan). Total RNA obtained from the spawning female liver in RNAase-free distilled H_2O was boiled at 65°C for 10 min and immediately stored in ice. To perform reverse transcription (RT) reaction, the total RNA (5 μg in 6 μl of distilled water) was mixed with 1 μl (6 pmol) of H-SF11-specific primer 1 (P1, 5′-GTTCTTGCCTGGATCTTGAG-3′), 2 μl of 10× RT buffer (100 mM Tris·HCl/900 mM KCl, pH 8.3), 2 μl of 0.1 mM DTT, 4 μl of 2.5 mM dNTPs, 4 μl of 25 mM $MgCl_2$, and 1 μl of murine leukemia virus reverse transcriptase (5 units) and incubated at 37°C for 1 hr and then at 90°C for 5 min. To remove the excess primer, the RT products were washed three times with TES (20 mM Tris·HCl, pH 8.0/1 mM EDTA/0.15 M NaCl) by filtration through a SprecII centrifuge tube (Takara Shuzo). Thus obtained sample (250 μl) was mixed with 37.5 μl of 2 M NaOH and incubated at 50°C for 30 min. After neutralization with 37.5 μl of 2.2 M acetic acid, the sample was precipitated by the addition of 15 μl of glycogen (0.5 μg/μl), 35 μl of 10 M LiCl and 700 μl of ethanol, and the precipitate was resuspended in 5 μl of water. Thus obtained single-stranded cDNA (1 μl) and the anchor oligomer (5′GCACTTGACTATGACTGACTGACTGAATTCTTTAGTGAGGGTTAATTGCC-3′) were incubated in RNA ligase buffer (50 mM Tris·HCl/10 mM $MgCl_2$/10 mM DTT, pH 7.5) containing 1 mM ATP, 0.1 mg/ml BSA, and 24% polyethylene glycol with T4 RNA ligase (20 units; Takara Shuzo) at 22°C for 48 hr. The product of single-strand ligation was used for amplification by PCR. The reaction mixture (100 μl) consisted of 1 μl of the product and 99 μl of PCR cocktail (Gene *Taq*; Nippon Gene, Toyama, Japan), containing 20 pmol each of the primers that are complementary to the ligated anchor oligomer (5′CAATTAACCCTCACTAAACA-3′) and the original H-SF11-specific primer 2 (P2, 5′-GATTGCTAGGCTTCCCATCT-3′), 0.2 mM dNTPs, and 2.5 units of recombinant *Taq* DNA polymerase. Thirty-five cycles of the first-round PCR amplification were performed (temperature profile, 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C) in a DNA thermal cycler (Perkin–Elmer/Cetus). The PCR amplification product was cloned into pCRTM2 vector (Invitrogen) using a DNA ligation kit (Takara Shuzo).

Protein Sequencing. Chg H and ZI-1,2 were purified according to the methods of Murata *et al.* (13) and Sugiyama *et al.* (14), respectively. However, as the sequencing of the purified Chg H and ZI-1,2 was unsuccessful, the N-terminal amino acids of both Chg H and ZI-1,2 were presumed to be blocked. Therefore, analysis of the sequences of Chg H and ZI-1,2 were performed after partial digestion of them with endopeptidase Lys-C (sequencing grade, Promega) as described before (13, 14). The sequence analysis of the purified Chg H was also done after a direct digestion with the medaka hatching enzyme (step 1, purified sample, ref. 21). The digests were fractionated by reverse phase HPLC with a Shodex C18–5A column (Showa Denko, Tokyo). The amino acid sequences of polypeptide fragments in some peaks of the elution pattern were determined with an automatic amino acid sequencer (Shimadzu model PSQ-1).

Amino Acid Composition. The data of amino acid compositions of the purified Chg H and ZI-1,2, cited from Murata *et al.* (13), were used to compare with that of the deduced amino acid sequence of the cloned Chg H cDNA (H-SF11 connected with H-SF38).

Northern Blot Analysis. For Northern blot analysis of various organs, the experimental fish were given E_2 *per os*. The liver, gut, muscle, and the whole head of spawning female fish, E_2-treated male fish, and control (nontreated) male fish, and the ovary of the spawning female fish, were used as the sources of total RNAs, which were extracted according to the guanidinium/hot phenol method (22). Total RNA (10 μg) obtained from these organs was electrophoresed on a formaldehyde/1% agarose gel, transblotted onto a nylon filter (Hybond-N, Amersham) according to the method of Maniatis *et al.* (23), and hybridized with the random-primed ^{32}P-labeled probes (Amersham) of the *Xho*I–*Eco*RI fragments of the cDNA clone, H-SF11.

For analysis of the time course of expression of the genes for both Chg H and Chg L, E_2 treatment was performed by rearing the experimental male fish in water containing E_2, since this method permitted quantitative administration of E_2 to all experimental fish uniformly (10). For every sample, 5 μg of total RNA was extracted from the livers of five fish, electrophoresed, and transblotted as described above. Gene transcripts for Chg H and Chg L were visualized by the use of ^{32}P-labeled probes of H-SF11 and L-SF41 (15), respectively.

***In Situ* Hybridization.** The livers of spawning female fish were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 30 min. After trimming, they were fixed again with the same concentration of paraformaldehyde buffer at 4°C overnight, dehydrated through a graded alcohol series (70, 80, 90, and 100% ethanol for 30 min once each, absolute ethanol for 30 min twice, xylene for 15 min at 4°C twice, and finally xylene for 15 min at room temperature once) and embedded in paraffin at 52°C. The sections cut at 6-μm thickness were deparaffinized (xylene for 10 min three times, 100, 90, and 80% ethanol for 15 sec each once, and 0.1 M phosphate buffer (pH 7.4) for 15 sec twice). The sections were treated with proteinase K (15 μg/ml in 10 mM Tris·HCl, pH

8.0/1 mM EDTA) for 15 min, 4% paraformaldehyde in phosphate buffer for 10 min, phosphate buffer for 1 min, 0.2 M HCl for 10 min, phosphate buffer for 1 min, 0.1 M triethanolamine-HCl (TEA; pH 8.0) for 1 min, 0.1 M TEA-0.25% acetic anhydride for 10 min, phosphate buffer for 1 min, 70, 80, and 90% ethanol for 15 sec each, and 100% ethanol for 15 sec twice and then dried. The gene transcripts for Chg H and Chg L were visualized by hybridization with digoxigenin-labeled probes of the whole H-SF38 and the *Xho*I–*Spe*I fragments of the cDNA clone, L-SF41 (15), respectively, according to the method of Hirota *et al.* (24) using a commercial kit (DIG DNA Labeling and Detection kit, Boehringer Mannheim).

Computer-Aided Analyses of Sequence and Hydropathy. The similarity search of the predicted amino acid sequence of the obtained cDNA for Chg H was practiced using Swiss Protein Sequence Data Bank and EMBL and GenBank databases. Hydropathy of the Chg H predicted from the cDNA was performed according to the method of Kyte and Doolittle (25).

RESULTS

Cloning and Analysis of a cDNA for Chg H. By immunological screening of 1×10^5 recombinant phage, we isolated ≈20 positive cDNA clones. They were ≈2 kb or shorter. These clones were presumed not to include cDNA clones for Chg L, since we used anti-Chg H IgG, which was not reactive with Chg L as a probe. In fact, partial sequencing revealed that most of them were derived from the same cDNA. As apparent relative molecular mass of Chg H was found to range from 74,000 to 76,000 on SDS/PAGE, the largest clone, named H-SF11, was presumed to be most probably corresponding to Chg H. The H-SF11 was 1667 bp and contained a long open reading frame but was incomplete, as it did not possess an initiator Met. Thus, we cloned its missing 5′ end portion by SLIC-PCR from mRNA of the spawning female liver. The obtained clone, H-SF38, was 460 bp. It comprised a 3′ side portion of 214 bp that was overlapping and coincident with the 5′ side portion of H-SF11, and a 5′ side extension of 246 bp (Fig. 1*A*).

Fig. 1*B* shows the nucleotide sequence and the predicted amino acid sequence of a Chg H cDNA that was made by combination of those of H-SF11 and H-SF38. The clone consisted of 1913 bp and contained an open reading frame encoding 591 aa. A partial sequence from Gly-29 to Pro-62 was coincident with that determined for three proteins together of purified Chg H. Moreover, another sequence from Trp-462 to His-507 was also identical with that determined for three proteins together of purified ZI-1,2. The sequence of the N terminus was rich in hydrophobic amino acid residues, characteristic of signal peptide. The cleavage site by signal peptidase was deduced to be between Thr-22 and Glu-23 according to the (−3,−1) rule and signal peptidase cleavage pattern (26, 27).

To determine whether the obtained cDNA was a full-length sample or not, primer extension analysis was performed using mRNA from the spawning female fish liver and a synthetic oligonucleotide primer (5′-GGAAAGAACATAGCAGAGCTAGTGC-3′), which was end-labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (28). We obtained an extended fragment of 94 bp, and the result showed that the 5′ end of the Chg H mRNA was 3 bp longer than the 5′ end of the Chg H cDNA (H-SF11 combined with H-SF38).

Thus, this sequence was regarded as the full-length cDNA for a precursor protein of the Z-I,2 subunits. There is a poly(A)$^+$ addition signal at the position of 17 bp upstream of poly(A)$^+$ tail of the nucleotide sequence. Comparison of amino acid compositions between purified Chg H, ZI-1,2, and the predicted amino acid sequence of the Chg H cDNA indicated that they were very similar (data not shown). Characteristics of their amino acid compositions were high contents

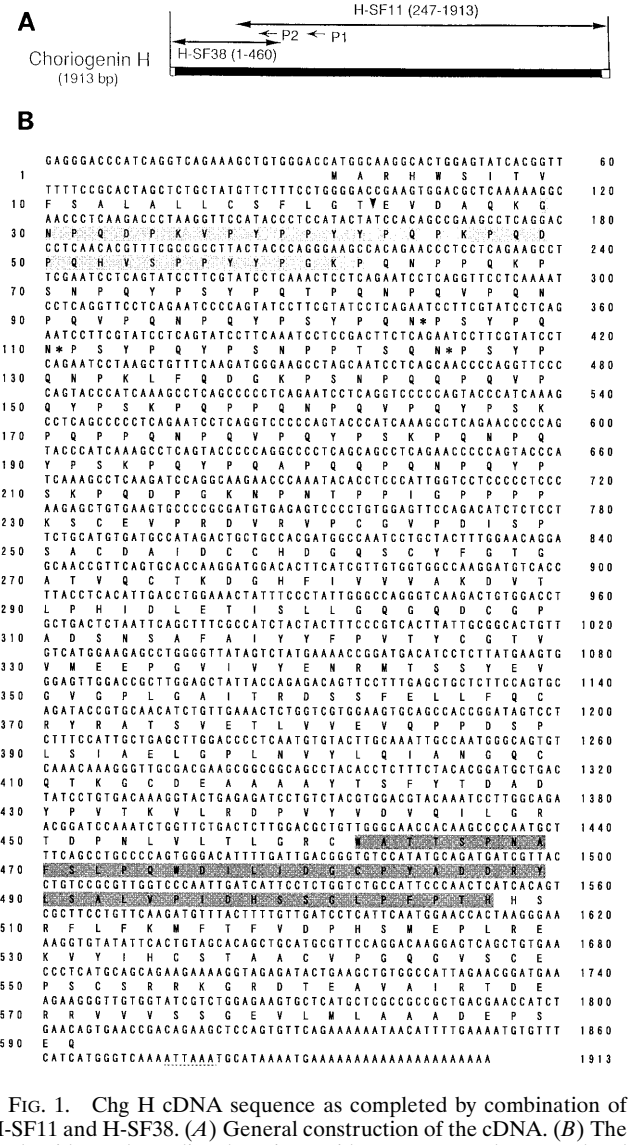

FIG. 1. Chg H cDNA sequence as completed by combination of H-SF11 and H-SF38. (*A*) General construction of the cDNA. (*B*) The nucleotide and predicted amino acid sequences. The arrowhead indicates the signal sequence cleavage site. Asterisks show possible N-glycosylation sites. The lightly and darkly shaded portions refer to the amino acid sequences that coincided with those of purified Chg H (three proteins together) and purified ZI-1,2 (three proteins together), respectively. The underdotted part is the poly(A)$^+$ addition signal.

of Pro (17.58% for Chg H, 19.99% for ZI-1,2, and 17.18% for the Chg H cDNA) and Glu/Gln (13.34%, 15.46%, and 13.18%, respectively). In the first two-fifths of the N terminus, there are Pro-X-Y repeat sequences, where most Xs are Gln and some are Ser. Relative molecular mass of the predicted amino acid sequence was 62629.75, while those of purified Chg H and ZI-1,2 were both apparently 74,000 to 76,000 on SDS-PAGE. The discrepancy in molecular size may be attributable to carbohydrate moieties, since three potential N-linked glycosylation sites (Asx-X-Ser) were observed in the predicted amino acid sequence.

Northern Blot Analysis of Gene Expression in Various Organs. Gene expression for Chg H was found to occur only in the livers of spawning female fish and E_2-treated male fish (Fig. 2). There were no positive signals with RNAs derived from the other organs, including the ovary of spawning female fish. This fact clearly indicated that gene expression for Chg H did not occur in the oocyte, like that for Chg L (15); there were no dual syntheses of Chgs in the liver and the ovary of spawning female fish. In the livers of both spawning female and E_2-

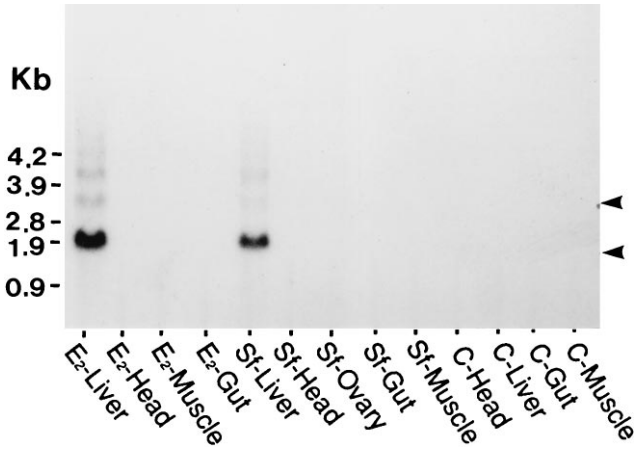

FIG. 2. Northern blot analysis of gene expression for Chg H. Total RNAs (10 μg each) obtained from various organs of the male control (C), E_2-treated male (E_2, administered *per os* for 10 days), and spawning female (Sf) fish were electrophoresed, transblotted onto a filter, and hybridized with ^{32}P-labeled probes of *Xho*I–*Eco*RI fragments of H-SF11. Upper and lower arrowheads refer to the positions of 28S and 18S ribosomal RNAs, respectively.

treated male fish, at least two weak signals >1.9 kb were detected, this fact suggesting that some higher-molecular weight gene transcripts for Chg H were synthesized.

Sequence Similarity Between Chg H and Some Other Egg Envelope Proteins. Fig. 3 illustrates the results of computer-aided search for protein sequences homologous to Chg H. Amino acid sequence of the Chg H cDNA showed a high degree of identity (54.5%) to that of the wf♀ gene product that was expressed in the liver of the female winter flounder, *Pseudopleuronectes americanus*, during oocyte growth phase and was significantly similar to zona pellucida proteins of a rabbit gene (*rc55*) and a mouse gene (*zp2*; ref. 29). Hydropathy profiles of Chg H and the winter flounder protein were markedly similar (data not shown). Different from cDNA for Chg L (L-SF41), which resembled ZP3 rather than ZP2 of some mammalian zona pellucida (15), the obtained Chg H cDNA (H-SF11 combined with H-SF38) was more closely similar to ZP2 than ZP3 of mouse and human zona pellucida. The Chg H cDNA contained a domain of Pro-X-Y repeat (where X was mostly Gln) in its N terminus. The Pro-X-Y repeat sequence is absent from mammalian zona pellucida but present in the gene product of winter flounder. Thus, the Chg H cDNA includes a ZP-domain of ZP2, which is widely distributed in several mammalian egg envelope (zona pellucida) and a domain of Pro-X-Y repeat sequence that seems to be characteristic of fish egg envelope.

Concurrent Gene Expression for Chg H and Chg L in the Livers of E_2-Treated Male Fish and Spawning Female Fish. Time course of intrahepatic gene expression for Chg H, and Chg L for comparison, in male fish following E_2-treatment is shown in Fig. 4 *A* and *B*. Under the experimental conditions, Chg H mRNA could be detected first in the liver 4 hr after the start of E_2-treatment. The time of the first appearance of Chg H mRNA was apparently coincident with that of Chg L mRNA. The concentration of mRNAs for both Chgs increased in parallel with the time length of E_2-treatment up to 48 hr, when the concentration reached maximum. As described above, some Chg H-positive signals for larger-sized molecules than Chg H mRNA were found. Such molecules were not found for Chg L-positive signals. It was reported that both Chg proteins became first detectable coincidentally 16 hr after the E_2 treatment under the same experimental conditions (10). Therefore, it probably takes ≈12 hr to translate mRNAs and synthesize significant amounts of Chg H as well as Chg L.

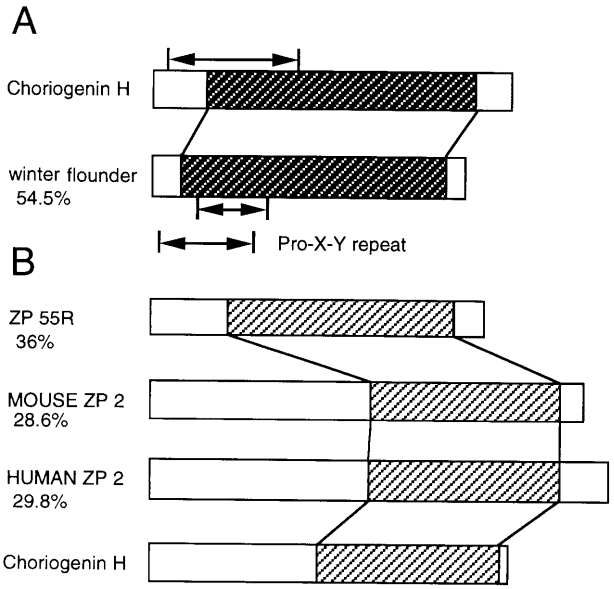

FIG. 3. Similarities in amino acid sequence between Chg H and egg envelope proteins of some other animals. (*A*) Similarity between Chg H and the wf♀ gene product of female liver of winter flounder. Hatched portions refer to their homologous domains. (*B*) Similarities between Chg H and zona pellucida proteins (ZP2) of some mammalian species. ZP 55R refers to a rabbit zona pellucida protein encoded by rc55 and reportedly homologous to winter flounder protein (29). Hatched portions refer to homologous domains. The percentage refers to the similarity between Chg H and respective proteins.

Under natural conditions, Chg H seems to be synthesized concurrently with Chg L in the hepatocytes of spawning female fish. Fig. 4 *C–F* show the gene transcripts for Chg H and Chg L in the hepatocytes of spawning female fish as visualized by *in situ* hybridization. mRNAs for both Chgs were detected in every parenchymal cell. Also in E_2-treated male fish, gene expression for both Chgs occurred similarly in every parenchymal cell (data not shown). In a previous paper (10), we reported that both Chg proteins (H-SF and L-SF) were immunologically detected to appear concurrently in every parenchymal cell after E_2 administration. The present results coincided with the previous results.

DISCUSSION

In the present study, a cDNA of a precursor of one of three proteins constituting ZI-1,2 subunit group of the inner layer of medaka egg envelope (chorion) was cloned and analyzed. According to our partial characterization of the isolated ZI-1,2 subunits, the peptide portions of the three proteins were very similar, since their peptide maps were almost the same and amino acid sequencing of the three proteins together produced one and the same sequence. Therefore, the three proteins seem to be isomers with different carbohydrate moieties. As mentioned before, Chg H (or H-SF), the precursor of ZI-1,2, also comprises three proteins, which are also presumed to be similar. In fact, partial sequencing of some other cDNA clones indicated identity of their sequences with that of the present Chg H cDNA (data not shown). Thus, the primary structures of three proteins of Chg H may be represented by the cloned Chg H cDNA.

It is of great interest to note that there is a considerable similarity in amino acid sequence between Chg H and a gene product expressed in the liver of winter flounder (29). In some fishes other than medaka, extraovarian and/or hepatic syntheses of egg envelope (chorion)-like proteins have been strongly suggested (11, 16, 30–33). The winter flounder gene product (29) should also be one of the egg envelope subunits.

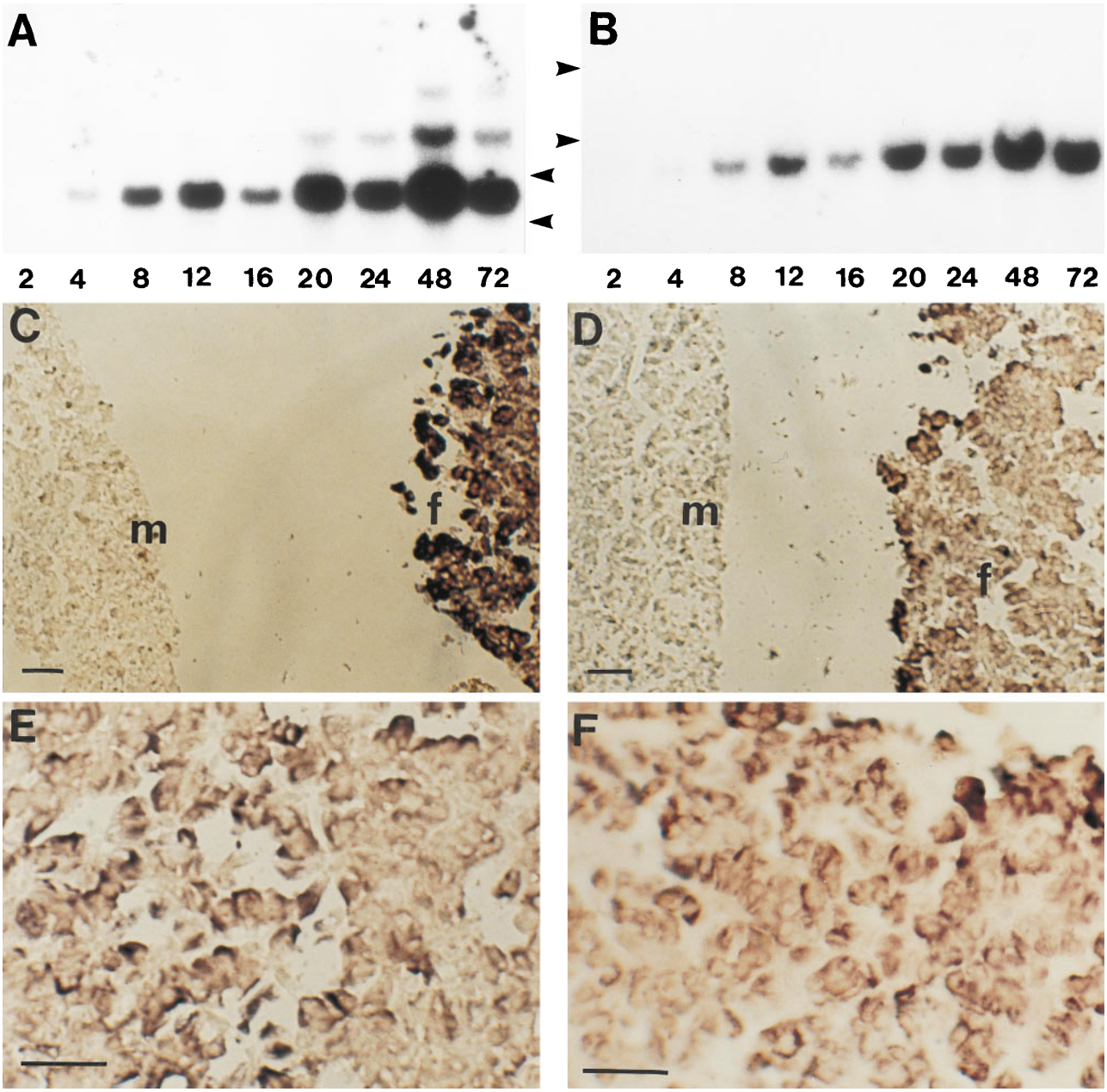

FIG. 4. Expression of the gene transcripts for Chg H and Chg L for comparison in the livers of E_2-treated male fish and spawning female fish. (*A* and *B*) Time courses of expression of the gene transcripts for Chg H and Chg L, respectively, in the male livers following E_2 treatment. E_2 was given to the experimental fish by rearing them in water containing E_2. Total RNA (5 μg) extracted from the liver of the E_2-treated male fish was electrophoresed in each lane and visualized as described. The numbers along the bottom indicate the time (in hr) of E_2-treatment. The upper and lower arrowheads refer to the positions of 28S and 18S ribosomal RNAs, respectively. (*C* and *D*) Visualization of the gene transcripts for Chg H and Chg L, respectively, in the livers of spawning female fish by *in situ* hybridization following the procedures described. m, Male fish; f, spawning female fish. (Bar = 40 μm.) (*E* and *F*) Magnification of a part of the spawning female livers in *C* and *D*, respectively. (Bar = 40 μm.)

Presence of Pro-X-Y repeat sequence in it strongly suggests that it is a winter flounder homolog of medaka Chg H but not of Chg L, since Chg L does not contain Pro-X-Y repeat (15). As reported previously (34), a Pro-X-Y-rich domain of the hardened chorion subunits of medaka was abundant also in γ-Glu–ε-Lys crosslink and presumably plays some roles in chorion hardening on fertilization or activation. Like the winter flounder protein, Chg H cDNA also showed a similarity in amino acid sequence to ZP2 rather than ZP3 of some mammalian zona pellucida. As reported previously (15), Chg L cDNA showed a sequence similarity to ZP3 more than ZP2. Recently, it was reported that mouse *Zp1* encoded a protein homologous to the winter flounder protein and mammalian ZP2 (35). This protein seems to be more similar to Chg H than to Chg L. Thus, two major subunits of the fish chorion inner layer seem to be homologous to two major subunits of mammalian zona pellucida, although the sites of synthesis of them are different—i.e., oocytes in mammals, while liver in fish. It remains obscure at present whether hepatic synthesis of egg envelope subunits is restricted to some fish species or can be generalized to any fish species. As in the case of gene expression for Chg L (15), gene transcripts for Chg H were found only in the liver, these facts excluding a possibility of dual syntheses of all medaka Chgs in both the liver and ovary.

In Northern blots, there were found some positive signals of larger-sized molecules than Chg H (H-SF) mRNA (Fig. 2). Although their nature is not clear at present, they might be related to some Chg H-like immunoreactive proteins with

larger molecular size than Chg H, which were found to be synthesized concurrently with Chg H in the male fish liver on treatment with E_2. Further analysis of their nature should be of great interest in connection with vitellogenins as well as Chg L, which are also synthesized in the liver under the influence of E_2.

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