Expression of the Mature Yellowfin Porgy Growth Hormone in *Spodoptera frugiperda* 21-AE Cells

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A complementary DNA (564 base pairs) encoding for the mature region of the growth hormone (GH) of yellowfin porgy *Acanthopagrus latus* was amplified by polymerase chain reaction. This resultant DNA fragment, ypmGH cDNA, was inserted into baculovirus expression vectors, pAc7, under the control of the polyhedrin promoter. A 22 kDa protein, similar to that of authentic GH, was expressed intracellularly by the insect cell line of *Spodoptera frugiperda* 21-AE infected by recombinant baculovirus. This protein was reactive with rabbit polyclonal antibodies raised against natural chum salmon GH. Furthermore, a protein band with 22 kDa labeled with [³⁵S] methionine was detected in the lysate of cells infected with recombinant virus containing ypmGH cDNA. Results from treatment with tunicamycin, an inhibitor of *N*-link glycosylation, demonstrated that the *N*-glycosylation did not occur in the recombinant GH produced by this system.

The GH cDNA of chum salmon has been cloned and its recombinant GH (rGH) has also been shown to enhance the growth rate of fish.¹² This finding makes GH cDNA potentially useful for the production of rGH in microorganisms for research and other applications. Thus, GH cDNA has been studied extensively in many species of fish, *i.e.* chinook salmon,²² chum salmon,¹² coho salmon,⁴³ eel,⁴³ gilthead seabream,⁵³ rainbow trout,⁶³ red seabream,⁷³ tilapia,⁸³ tuna,⁹³ yellowfin porgy,¹⁰³ and yellowtail.¹¹³ A number of studies of the GH cDNA of these fish focused on expression in either the *Escherichia coli* system^{1,4,6,8-10,11,133} or the *Saccharomyces cerevisiae* system.¹⁴⁻¹⁸³ However, both systems have disadvantages. In the *E. coli* system, the highly expressed products are accumulated within the inclusion bodies in a denatured and unmodified form.¹⁷³ It is a complicated and expensive process to renature the rGH before it is ready to be used. In yeast, the construct plasmids in yeast transformants are generally unstable.¹⁸³

The baculovirus genome offers an alternative expression system for the eucaryotic gene. This interesting gene is ligated in a vector under the control of a polyhedrin promoter, flanked by the viral sequences. The foreign gene with polyhedrin promoter is inserted into the viral genome by

homologous recombination *in vivo*. The resulting recombinant baculovirus is identified by an occlusion negative (Occ⁻) phenotype due to the inactivation of the polyhedrin gene.¹⁹³ Therefore, this baculovirus expression vector system (BEVS) has recently been reported as an effective approach for expressing eucaryotic genes, since BEVS provides correct post-translational processing. However, using BEVS to express fish genes is limited, except in the case of carp gonadotropin.²⁰³

The GH cDNA of yellowfin porgy, *Acanthopagrus latus*, one of the major marine aquacultured fish in Asia, has been cloned from the pituitary gland cDNA library.¹⁰³ The cDNA consists of 915 base pairs (bp) and encodes a protein of 204 amino acids (aa) including a signal peptide with 17 aa. In the present communication, we will describe how yellowfin porgy GH cDNA (ypGH cDNA) is expressed in insect cells under the control of a polyhedrin gene promoter.

Materials and Methods

Virus and Cells

The insect cell line of *Spodoptera frugiperda* 21-AE (Sf21-AE)²¹³ was propagated at 28°C in a spinner culture using a TNM-FH medium²²³ supplemented with 8% fetal bovine serum. The

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wild-type *Autographica californica* nuclear polyhedrosis virus (AcMNPV)¹⁸⁾ and recombinant virus stocks were grown and titrated in a confluent monolayer of Sf21-AE cells.

Polymerase Chain Reaction (PCR)

Two primers, P1 and P2, were used for amplifying GH cDNA without containing signal peptide (ypmGH cDNA). The forward primer (P1), 5'-ATGCAGCCGATCACAGACG-3', was encompassed by the nucleotide region from 104 to 119 of ymGH cDNA but was preceded by ATG. The reverse primer (P2), 5'-ATATAAGCTTTTACTACAGGGTGCAGTTGGC-3', was derived by ATAT, *Hind*III cutting site (AAGCTT) and the antisense strand of one more stop codon (TAA) and the nucleotide region from 667 to 650 of ymGH cDNA. The 915 bp DNA fragment encoding for the full length of ymGH cDNA was used as a template. A reaction in a 100 μ l solution was set up for amplification as described by Innis and Gelfand²³⁾: 2 ng of template DNA, 10 mM of each primer, 10 mM of each dNTP, 10 μ g of bovine serum albumin, 10 μ l of 10 $\times$ reaction buffer and 2 units of Vent DNA polymerase (Biolabs). They were mixed and denatured at 94°C for 5 min before DNA polymerase was added. They were treated in a Thermal Reactor (Hybaid, Hook & Tucker Instruments, U.K.) using the following temperature profile after the first denaturation: annealing at 37°C for 1 min and extension at 72°C for 3 min (1 cycle); denaturation at 94°C for 1 min, annealing at 42°C for 50 s, and extension at 72°C for 80 s (4 cycles); denaturation at 94°C for 50 s, annealing at 60°C for 40 s, and extension at 72°C for 50 s (25 cycles). Cycling was concluded with a final extension at 72°C for 6 min.

Construction of Expression Transfer Vector

The 580 bp DNA fragment obtained from PCR was ligated into the *Sma*I site of pUC19. *E. coli* DH5 α transformants harboring plasmid with the correct polynucleotide sequence of amplified-DNA fragment were chosen. Sequencing was performed by the chain-termination method of Sanger *et al.*²⁴⁾ with a synthesized 13 oligonucleotides primer, 5'-GTCGATGGGGCTG-3', which was complementary to nucleotide position 277 to 289 from the 5' end of GH cDNA.

The 580 bp ypmGH cDNA produced by PCR was recovered from the pUCGH4 by digestion with *Eco*RI and *Bam*HI. The resultant DNA fragment was inserted into the corresponding

sites of pAct7, which is a 5.3 kb of AcMNPV transfer vector.²⁵⁾ The chimeric plasmid construct was designated as pHM4.

Transfection and Isolation of Recombinant Baculovirus

S. frugiperda cells were cotransfected with 1 μ g of wild-type AcMNPV DNA and 20–30 μ g of pHM4 using the calcium-phosphate-DNA precipitation method.²⁶⁾ Plaques produced by each transfection were screened under the microscope for Occ⁻ cytopathic cells.²³⁾ The putative recombinant viruses were confirmed by limiting dilution²⁷⁾ and dot blot hybridization²⁸⁾ with digoxigenin labeled ymGH cDNA. Positive clones of luminescence were identified further by PCR,²³⁾ using one forward primer, 5'-CTATCAATATATAGTTGCTGATATC-3' (primer 3; -115 to -91 of polyhedrin gene) and two backward primers. The latter primers were 5'-GG(T/C)GCGTC(T/G)GG(T/C)GCAAA(T/C)TC(T/C)TT(A/T)AC(T/C)TT-(A/G)AA-3' (primer 4), which is derived from the antisense from 713 to 682 of polyhedrin gene, and 5'-GTCGATGGGGCTG-3' (primer 5), which is derived from the antisense from 289 to 277 of ymGH cDNA. PCR products were analyzed by Southern blotting,²⁹⁾ using ymGH cDNA as a probe.

Analysis of Proteins Synthesized by Insect Cells

Sf21-AE was infected with parental wild-type baculovirus AcMNPV or the recombinant virus containing ypmGH cDNA (AcMNPV-GH4) at a multiplicity of infection (moi) of 10. After 96 h postinfection, cell cultures were harvested at 500 $\times$ g at 4°C and lysed in a sample buffer (10% glycerol, 10% SDS, 5% mercaptoethanol, 0.1% bromophenol blue, and 0.5 M Tris-HCl, pH 6.8). Lysate proteins were analyzed by 12% Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE)³⁰⁾ with silver stain³¹⁾ and by immunochemical reaction after Western blot analysis.³²⁾ Rabbit polyclonal antiserum against natural chum salmon GH, given by Dr. Thomas T. Chen, the Center of Marine Biotechnology, University of Maryland, was used for the latter procedure since there is 62.4% homology of amino acid sequence between chum salmon GH and yellowfin porgy GH.¹⁰⁾ Goat anti-rabbit IgG conjugated with alkaline phosphatase was used for immunoreaction according to previous procedures.¹⁰⁾

Protein Radiolabeling

Cells (6×10^5 per well) in 24-well tissue culture plates were infected by virus of AcMNPV or AcMNPV-GH4 separately at an moi of 10 and incubated in 500 μ l FNM-FH medium at 28°C for 60 h. Then the culture medium was replaced by Grace's methionine-free medium (Gibco). Cultures were labeled with 20 μ Ci of [35 S] methionine (>1000 Ci/mmol, Amershan) for 6 h. At the end of the labeling period, AcMNPV infected cells, AcMNPV-GH4 infected cells and uninfected Sf21-AE cells were separately harvested and lysed. Lysate proteins were resolved on a 12% SDS polyacrylamide gel. After the gel was dried it was exposed to X-ray film (DuPont) at room temperature for 16 h.

Tunicamycin Treatment

Cells of 3×10^5 per well in 24-well tissue culture plates were infected with AcMNPV and AcMNPV-GH4 at an moi of 10, respectively, and incubated at 28°C for 1 h. The culture medium was replaced by 500 μ l serum-free TMN-FH medium³³⁾ containing 5 μ g/ml tunicamycin (Sigma). After the cells had been cultured at 28°C for 4 d, the cells were harvested and their supernatants and lysates were analyzed separately on 12% SDS polyacrylamide gel with silver staining.

Results

Construction of Transfer Vector

A DNA fragment with a molecular weight of 580 bp was obtained from PCR (Fig. 1), which included the 564 bp mature region of GH cDNA. The scheme for plasmid construction is outlined in Fig. 2. The PCR product was ligated in the *Sma*I site of pUC19. Clones harboring the resultant 3.3 kb plasmid construct, pUCGH4, were screened. DNA sequencing showed that some nucleotide sequences of PCR-amplified ypmGH cDNA insert in pUCGH4 were 5'-CCCATGCA-GCCGATCACAGACGGCCAGCGTCTGT-TCTCCATCGCTGTCAGCAGAGTTCAACAC-CT-3', which was the same as that of the sense strand of ypGH cDNA from 104 to 162. In addition, it was preceded by CCC (part of the *Sma*I site) and ATG (an initial codon). The ypmGH cDNA insert was inserted into the pAcT7 and the resultant pHM4 with a molecular size of 5.9 kb was shown on the agarose gel.

Fig. 1. The DNA product (580 bp) of PCR shown on the agarose gel.

The full length of GH cDNA (915 bp, serving as a template DNA) and two primers (see text) were used to amplify cDNA encoding for the mature region of ypGH cDNA. Lane 1, PCR product; lane M, molecular markers, ϕ 174-RF DNA *Hae*III digest in kilobase pairs (kb).

Isolation of Recombinant Baculovirus

After analysis of dot blotting and limiting dilution, one positive cell clone was confirmed to be infected with recombinant virus containing ypmGH cDNA, since its genome generated 0.3 and 1.1 kb DNA fragments when primers 3 and 4 and primers 3 and 5 were used for PCR, respectively. These two resultant DNA fragments also reacted positively with digoxigenin-labeled ypGH cDNA.

SDS-PAGE and Immunochemical Detection

Cell lysates prepared separately from Sf21-AE cells, AcMNPV infected cells and AcMNPV-GH4 infected cells were analyzed by SDS-PAGE. A protein band that migrated with an estimated size of 22 kDa, similar to that of natural GH, was detected in the sample of AcMNPV-GH4 infected cells (lane 1 in Fig. 3A), whereas this band was absent both in the Sf21-AE cells and in the AcMNPV infected cells (lanes C and W in Fig. 3A). Immunoblot analysis showed that the 22 kDa protein of AcMNPV-GH4 reacted with

Fig. 2. Construction of plasmid pHM4.

The PCR product (dark shaded box) was ligated into the *Sma*I site of pUC19. An intermediate plasmid, pUCGH4, was used for partial nucleotide sequence determination. The 5'- and 3'-noncoding region of polyhedrin (PH) gene are represented by light shaded boxes. Arrows indicate the orientation of genes. Amp^r, ampicillin resistance gene; B, *Bam*HI; E, *Eco*RI; S, *Sma*I; MCS, multiple cloning site; P, *Pst*I; P_{PH} and T_{PH}, promoter and transcription terminator of PH gene, respectively.

the chum salmon GH antibody (lane 1 in Fig. 3B). Besides the 22 kDa band, some other bands also reacted with the polyclonal antiserum against the natural chum salmon (Fig. 3B). However, these bands were shared by Sf21-AE cells, AcMNPV infected cells, and AcMNPV-GH4 infected cells.

Radiolabeled Proteins Analysis

Four major and seven minor polypeptides labeled with [³⁵S] methionine were detected in the lysate of AcMNPV-GH4 infected cells (lane 1 in Fig. 4L). Ten out of eleven labeled protein species were shared between the lysates of AcMNPV infected cells and AcMNPV-GH4 infected cells (lanes 1 and W in Fig. 4L). A labeled

Fig. 3. Analysis of cell lysates by SDS-PAGE with silver staining (A) and by immunochemical reaction with antiserum against natural chum salmon GH (B).

Sf21-AE insect cells (lane C), the parental wild-type baculovirus (AcMNPV) infected cells (lane W) and the recombinant virus (AcMNPV-GH4) infected cells (lane 1) are shown. After 96 h postinfection, cultured cells were harvested and their lysates were analyzed on a 12% SDS-PAGE. M, molecular weight markers of proteins in kDa. Arrows indicate the rGH.

band with a molecular weight of 22 kDa was shown in sample of AcMNPV-GH4 infected cells, but was absent both in the Sf21-AE cells and in the wild-type AcMNPV infected cells (lanes 1, W, and C in Fig. 4L).

Proteins in the supernatant of the grown cell culture after 6 h labeling with [35 S] methionine were also analyzed. The result showed that this 22 kDa protein was not detectable in all supernatants of Sf21-AE cells, AcMNPV infected cells, and AcMNPV-GH4 infected cells (lanes C, W, and 1 in Fig. 4S).

Tunicamycin Treatment

Recombinant GH band (22 kDa) was shown in the cell lysate prepared from AcMNPV-GH4 infected cells without treating with tunicamycin (lane 3 in Fig. 5). However, this 22 kDa rGH band did not change its mobility on the gel when AcMNPV-GH4 infected cells were incubated with tunicamycin (lane 4 in Fig. 5).

A structure glycoprotein of baculovirus with a molecular size of 64 kDa was shown on the gel

from the lysates of both AcMNPV and AcMNPV-GH4 infected cells without treating with tunicamycin (lanes 3 and 5 in Fig. 5). This 64 kDa band, however, decreased to 57 kDa after these cells were treated with tunicamycin (lanes 4 and 6 in Fig. 5).

Recombinant GH band did not show on the gel from the samples prepared by the supernatants of AcMNPV-GH4 infected cells either with or without tunicamycin treatment (lanes 1 and 2 in Fig. 5), those prepared by the lysates of AcMNPV infected cells with or without tunicamycin treatment (lanes 5 and 6 in Fig. 5), or those prepared by the cell lysates of Sf21-AE with or without tunicamycin treatment (lanes 7 and 8 in Fig. 5).

Discussion

Results from cell lysate analysis of SDS-PAGE, immunoblotting, and protein labeling showed that rGH with a molecular weight of 22 kDa, similar to that of the authentic fish GH,^{34,35} was

Fig. 4. SDS-PAGE analysis of proteins labeled with [35 S] methionine.

Labeled proteins of cell lysates (L) and of the supernatants (S) from the Sf21-AE insect cells (lane C), the parental wild-type baculovirus AcMNPV infected cells (lane W), and the recombinant virus AcMNPV-GH4 infected cells (lane 1) are shown. After labeling, cells were harvested and proteins from the supernatants (S) and from the cell lysates (L) were resolved on the SDS-polyacrylamide gel before they were exposed to X-ray film. Molecular weight markers of proteins are represented in kDa. Arrow indicates the position of rGH of yellowfin porgy.

expressed by the Sf21-AE insect cells infected with the recombinant virus containing ypmGH cDNA. The maximum expression level of ypGH in this system is 8% of total cellular proteins. Moreover, the rGH was not secreted from cells infected with AcMNPV-GH4, since the insert GH cDNA in the recombinant virus did not include the codons of 17 aa signal peptide. However, we noticed that if cells were not carefully harvested, *i.e.* with a higher centrifugation force ($>500\times g$), a faint protein band with 22 kDa could sometimes be detected in the supernatant of cell culture (data not shown). This band was also positive immunoreaction. We propose that this detectable band is due to the fact that the

rGH is released from the broken cells, not because the rGH is secreted from the intact cells.

The most major protein expressed in the late stage of wild-type AcMNPV infected cells was polyhedrin. The intensity of the polyhedrin band shown on the gel appeared to be the highest of all proteins labeled with [35 S] methionine (Fig. 4L). In the recombinant baculovirus, the coding region of polyhedrin gene was replaced by ypmGH cDNA fragment. After the recombinant virus infection, however, the expression level of ypGH was significantly less than that of polyhedrin, although the ypGH cDNA and polyhedrin gene were driven by the same promoter. Therefore, we suggest that the structures and characteristics

Fig. 5. SDS-PAGE analysis of insect cells cultured with or without tunicamycin treatment.

Samples of the supernatants of AcMNPV-GH4 infected cells without treatment (lane 1) and with treatment (lane 2), the cell lysates of AcMNPV-GH4 infected cells without treatment (lane 3) and with treatment (lane 4), the cell lysates of AcMNPV infected cells without treatment (lane 5) and with treatment (lane 6), and cell lysates of Sf21-AE insect cells without treatment (lane 7) and with treatment (lane 8) were shown on the gel after silver staining. M and M1 are the prestained proteins markers and the stained proteins markers in kDa, respectively. Arrows represent the rGH and stars indicate the protein bands of 64 kDa (upper) and 59 kDa (lower).

of the regulatory region, the immediate upstream of the start codon or even the insert gene *per se* may be involved in the expression for foreign genes under the control of polyhedrin promoter, as was concluded in previous reports.³⁸⁻³⁹ To reach an expression level of fish GH cDNA as high as that of the polyhedrin gene may be an interesting subject for future studies.

A structure glycoprotein of 64 kDa located in the envelope of AcMNPV plays an important role in the ability of the virus to enter insect cells.³⁹ This 64 kDa protein band was reduced to 59 kDa in the presence of tunicamycin, an inhibitor of N-linked glycosylation, since this glycoprotein loses its sugar moiety. Similar to this report, a 64 kDa protein band occurring in the lysates of AcMNPV infected cells decreased to 59 kDa when the cells were treated with tunicamycin (lanes 5 and 6 in Fig. 5). One potential N-glycosylation site located near the C-terminus, Asn-Cys-Thr, of fish GH has been reported by numerous studies.^{1,3,5,9,10} However, we demonstrated that the 22 kDa rGH did not change its molecular size in the presence of tunicamycin (lanes 3 and 4 in Fig. 5). Therefore, we propose that the rGH of

yellowfin porgy expressed intracellularly by the BEVS does not undergo glycosylation at this potential glycosylation site. This suggests that the natural GH of fish may not glycosylated. And this could clarify that the rGH synthesized in *E. coli* is biologically active and has the same potency as natural fish GH, although rGH is not glycosylated.^{1,40}

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