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Expression of Rainbow Trout Growth Hormone cDNA in Yeast

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Huai-Jen Tsai, Chun-Feng Tseng and Tsong-Teh Kuo (1993) Expression of rainbow trout growth hormone cDNA in yeast. *Bull. Inst. Zool., Academia Sinica* **32**(3): 162-170. An episomal expression plasmid with an insert of rainbow trout growth hormone (rtGH) cDNA under the control of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter was constructed. The resultant plasmid was harbored by *Saccharomyces cerevisiae* 20B12 (designated as strain Y-105) and maintained in amounts of around 20-25 copies per cell. This yeast-produced recombinant GH is a fusion protein in which the mature rtGH (188 amino acids) was preceded by six deduced amino acids: Met-Gly-Gln-Gly-Ala-Ala. Northern blotting showed that the rtGH cDNA was transcribed into mRNA in yeast cells. A 22 kilo-daltons (kDa) protein band was detected in the soluble fraction of cellular proteins extracted from the Y-105 strain. This band was found to be immunoreactive to an antiserum raised against natural chum salmon growth hormone.

Key words: Glyceraldehyde-3-phosphate dehydrogenase promoter, Expression plasmid, Immunoreaction, Recombinant growth hormone.

Growth hormone (GH) is a polypeptide hormone produced by proximal pars distalis cells of the anterior pituitary gland which regulates growth and metabolism in vertebrates (Ganong 1983). GH enhances appetite, feed efficiency, and growth rate in fish (Donaldson 1979); thus, GH may be the most promising agent for growth promotion in aquaculture (Zohar 1989). However, the availability of natural GH is extremely limited, since its preparation from fish pituitary glands is cost-prohibitive.

Most fish GH cDNA have been expressed in *Escherichia coli* (Sekine *et al.* 1985, Agellon and Chen 1986, Saito *et al.* 1988, Rentier-Delrue *et al.* 1989, Sato *et al.* 1989, Tsai *et al.* 1993). However, the greatest disadvantage of this expression system is that the biosynthesized GH accumulates within the inclusion body in a denatured and unmodified form. As a result, this recombinant GH (rGH) has to be processed in a complicated and expensive way in order to renature it to its native form before it can be used.

Saccharomyces cerevisiae is considered a better host cell for expressing GH cDNA because: (a) it is capable of processing post-translational modification; (b) the gene product exists in a natural form; (c) it costs less for culturing than tissue culture; and, (d) it

is a safe organism which has been used as food additive, including an additive to fish meal. However, little information is available regarding the expression of fish GH cDNA in yeast (Hayami *et al.* 1989) as compared to an *E. coli* expression system.

Recombinant GH has been shown to be potent in accelerating the growth rate of fish by injection (Sekine *et al.* 1985, Agellon *et al.* 1988, Sato *et al.* 1988), immersion (Agellon *et al.* 1988, Schulte *et al.* 1989), implantation (Down *et al.* 1988) and oral-intubation (Moriyama *et al.* 1989, McLean *et al.* 1990, Hertz *et al.* 1991). However, the administration of exogenous rGH to fish *via* these methods is not very practical for aquaculture. The most desirable approach would be oral administration with yeast containing rGH. With this goal in mind, we will describe how rtGH cDNA is expressed in yeast cells under the control of the GAPDH promoter, which is a highly efficient promoter, since GAPDH gene encodes an active glycolytic enzyme (Bitter and Egan 1984).

MATERIALS AND METHODS

Strains, transformation, and screening

The plasmid was introduced into *S. cerevisiae* 20B12 [MAT-α, *pep*4-3, *trp*1 (Jones 1976)] *via* the lithium acetate method (Ausubel *et al.* 1989). Transformants were selected from a YM (0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, and 2% dextrose) plate at 30°C for 3 to 5 days. Colonies were cultured in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) and confirmed by restricting their containing plasmid after the plasmids were isolated and back-transformed (Ausubel *et al.* 1989) into *E. coli* JM109 (Yanisch-Perron *et al.* 1985) which was grown at 37°C in LB (1% Bacto-peptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose; pH 7.2) plates containing 50 μg/ml ampicillin.

Plasmid construction

The rtGH cDNA from pAF51 (Agellon *et al.* 1986) ligated into the *Eco*RI and *Hin*dIII sites of pGEM7Zf (Promega) was pGEMRT1. After *Nde*I linker (5′-CCATATGG-3′) was added to the *Sma*I site of pGEMRT1, a pGEMRT2 was constructed. An rtGH cDNA was obtained by cutting pGEMRT2 with *Sph*I and *Bam*HI, and then ligating it into the *Sph*I and *Bam*HI sites of pUC18. The resultant plasmid was pUCRT. The rtGH cDNA resulting from the digestion of pUCRT with *Eco*RI was inserted into the *Eco*RI site of pYE8 (Schaber *et al.* 1986)— an expression vector controlled by a GAPDH promoter.

Nucleotide (nt) sequence determination

The nt sequence from the TATA box (at nt position −145 to −138) through the 15th codon (at nt position 45) was determined by dideoxynucleotide chain termination (Sanger *et al.* 1977) using a synthetic oligomer (5′-AAAGACGGTAGG-3′) as a sequencing primer. This 12 oligomer corresponded to nt −140 to −129 of the GAPDH gene (Dobson *et al.* 1980).

Northern blot analysis

Yeast transformants were cultured in 10 ml YM medium at 30°C overnight. Total RNA was extracted by the SDS-phenol method (Schmitt *et al.* 1990) and separated on a denaturing gel (Ausubel *et al.* 1989). After being transferred to a Hybond N-membrane (Amersham, UK), the filter was hybridized with rtGH cDNA labeled with ^{32}P *via* random priming (Feinberg and Volgestein 1984). The hybridized filter was washed three times in 2 x SSC (0.3 M NaCl, 30 mM sodium citrate; pH 7.0) and 0.1% SDS at 68°C for 15 min each time before being

exposed to X-ray film at −70°C.

Copy number

Transformant was grown in 10 ml YM medium at 30°C with aeration until it reached middle-log phase (3.2×10^6 cells/ml), late-log phase (2×10^7 cells/ml), stationary phase (2×10^8 cells/ml) and stationary phase after 10 generations. Total DNA was extracted (Mann and Jeffrey 1989) and cut with *Eco*RI. After Southern blotting (Southern 1975), the DNA was hybridized with probes—including rtGH cDNA and the *leu2* gene. The *leu2* gene, a single copy gene in haploid yeast (Lopes *et al.* 1989), was prepared by electroeluting the 4.5 kb DNA fragment from the digesting pSZ20 (Szostak and Wu 1979) with *Sph*I and *Pst*I. This fragment was further cut by *Eco*RI and *Sal*I, and the resultant 1 kb fragment contained a *leu2* gene. To determine the relative copy number of plasmid containing the rtGH cDNA, the *leu2* signal was used to normalize the DNA for loading. A densitometer was used to compare the intensity of the hybridized bands of both *leu2* and rtGH cDNA.

Analysis of biosynthesized rGH

Yeast strains C and Y-105 grown separately in YM were harvested, resuspended in TE buffer (50 mM Tris-HCl, and 10 mM EDTA; pH 8.0), and disrupted by vortexing with acid-wash beads. Supernatants (S) were obtained after the homogenates were centrifuged. Debris was washed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4; pH 7.3), centrifuged, resuspended in TE containing 1% Triton, then vortexed for 5 min. The Triton-treated samples (T) were obtained from the supernatant fluid after centrifugation. Additionally, this debris was rewashed with PBS, centrifuged, resuspended in TE containing 8 M urea, vortexed, and recentrifuged. Our urea-treated samples (U) were obtained from the supernatants. S, T, and U samples were separately dissolved in Laemmli buffer (Laemmli 1970) and analyzed *via* sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) in a 13.5% gel followed by Coomassie blue staining (Hames 1990) and immunoblotting with an antibody raised against natural chum salmon GH (Towbin *et al.* 1979).

Zymolase treatment

Yeast transformants [one (strain C) harboring pYE8 and two (strains Y-105 and Y-122) harboring pYERT] were cultured in YPD medium at 30°C until they reached mid-log phase. Cells were harvested and resuspended in 100 μl sorbitol solution (1 M sorbitol, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 μg/ml phenylmethylsulfonylfluoride). About one tenth of the final volume of 3 mg/ml zymolase (ICN Biomedicals, Inc., USA) was added and incubated at 30°C for 30 min. Non-salt lysis buffer (1% Nonidet P-40, 50 mM HEPES, 1 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonylfluoride) was then added, and cells were incubated at 4°C for 10 min (Mann and Jeffrey 1989). The supernatants obtained after culturing were centrifuged at 13500 x g for 10 min, then used for protein analysis with SDS-PAGE and Western blotting.

RESULTS AND DISCUSSION

Plasmid construct, sequences, and yeast transformants

The construction scheme for the episomal and shuttle plasmid pYERT (7.2 kb) is shown in Fig. 1. We determined the DNA sequence from nt at −128 through the 15th codon (Fig. 2). A conserved sequence (5′-CACACA-3′) which was found upstream of

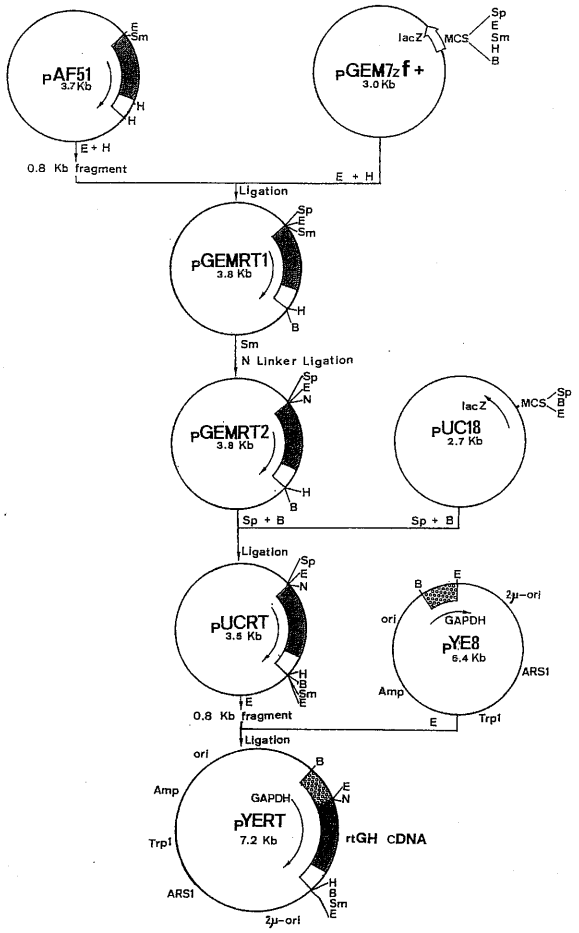

Fig. 1. Scheme for pYERT construction. The GAPDH promoter (stipple box), rtGH cDNA (hatch box) and 3′-noncoding region of GH (empty box) are shown. ori, origin of replicon; Amp, ampicillin resistance gene; Trp1, N-(5′-phosphoribosyl)-anthranilate isomerase; ARS, autonomous replicating sequences; 2μ-ori, *ori* site of 2μm-circle plasmid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; lacZ, encodes β-galactosidase; MCS, multiple cloning site. Arrow shows transcription direction. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N, *Nde*I; Sm, *Sma*I; Sp, *Sph*I.

ATG in several yeast genes (Dobson *et al.* 1982) was included at the 5′-region of pYERT. Six deduced amino acids (Met-Gly-Gln-Gly-Ala-Ala) preceded isoleucine— the first amino acid of mature rtGH (Agellon and Chen 1986).

Strain Y-105 (the transformant of *S. cerevisiae* 20B12 harbored pYERT) contained plasmid in which having both 6 and 1.2 kb *Bam*HI fragments; strain C (the transformant harbored expression vector pYE8 with no insert) contained plasmid in which having a 6.4 kb *Bam*HI fragment. pYERT maintained a minimum of 50 generations in YM medium.

Northern blot analysis

About 60 μg of total RNA was extracted separately from 10 ml culture of both strain C and strain Y-105. When 15 μg of RNA from each strain was loaded onto a gel, the resultant electrophoretic patterns were identical (Fig. 3A). After Northern blotting, the RNA of strain Y-105 showed a positive reaction band with the rtGH cDNA probe, which was located between 1200 nt and 1600 nt. However, there was no positive hybridization band for RNA obtained from strain C (Fig. 3B).

At the 3′-untranslated region of rtGH cDNA, only 200 bp after the stop codon was used to construct in pYERT. Thus, the transcription terminator had to be dependent on the FLP termination which was located in the expression vector pYE8. This construction resulted in the transcribing of rtGH cDNA into a longer length than was expected.

Copy number of pYERT

A smeared pattern appeared on the gel if the total DNA extracted from either strain C or Y-105 was digested with *Eco*RI (Fig. 4A). Southern blotting showed that two bands were positively hybridized with probes (Fig. 4B); one band (0.8 kb) was the rtGH cDNA, and the other (6 kb) was the *leu2* gene. When comparing the relative intensity of these two bands, the average copy

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-147  5'---AGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAATCT

-104  ATTTCTTAAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTT

 -52  TAAAACACCAAGAACTTAGTTTCGAATAAACACACATAAAGAATTCCCCCAT

                                        *
   1  ATG  GGA  CAA  GGG  GCA  GCG  ATA  GAA  AAC  CAA
   1  met  gly  gln  gly  ala  ala  ile  glu  asn  gln

  31  CGG CTC TTC AAC ATC ---3'
  11  arg leu phe asn ile  .
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Fig. 2. Nucleotide sequence between the TATA box of the GAPDH promoter and the 15th codon of rtGH cDNA in pYERT. The number above each sequence is the nucleotide order, which is numbered beginning with the first codon. The numbers below each sequence indicate the order of amino acid position. Underlines represent the synthetic oligomer as a sequencing primer. Ile-7 (marked with an asterisk) was the first amino acid of the mature rtGH.

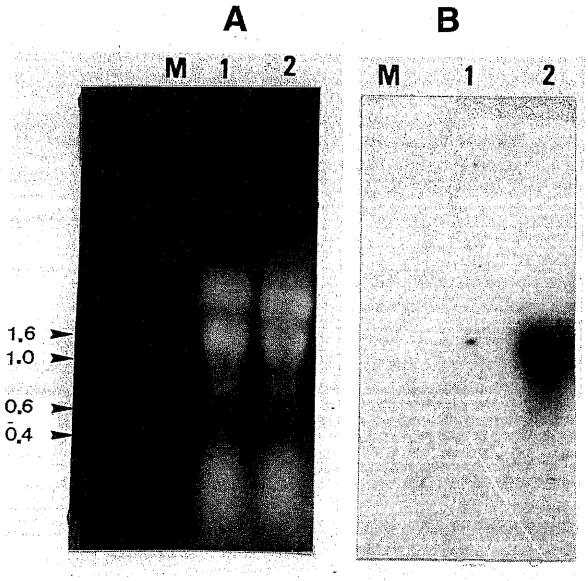

Fig. 3. Analysis of RNA obtained from the recombinant strains of *S. cerevisiae* 20B12 by hybridization with rtGH cDNA. (A): electrophoresis pattern shown on the 1.3% denaturing gel; 15 μg of RNA were loaded per lane. (B): autoradiogram of Northern blot hybridization corresponding to (A). Lane 1, strain C, harboring pYE8— a vector without insertion of foreign DNA; lane 2, strain Y-105, harboring pYERT— a vector with insertion of rtGH cDNA; lane M— molecular weight markers of RNA in kilo-nucleotides.

number of pYERT was 20-25 per cell. Furthermore, this plasmid number did not vary considerably for either the physical stages examined or after 10 generations in a selective medium (lane 4 of Fig. 4B). This data is consistent with results reported by Jayaram *et al.* (1983) and Rose and Broach (1990), who found that the average number of a 2μm-based vector was 10 to 40.

Analysis of PAGE and Western blot

A 22 kDa protein band— the same size as that of natural fish GH— was detected in the supernatant sample obtained from strain Y-105 (lane 2 of S in Fig. 5A). As expected, this band was absent in the supernatant prepared from strain C (lane 1 of S in Fig. 5A). Immunoblot analysis (lane 2 of S in Fig. 5B) showed that this protein band specifically reacted with antisera against chum salmon GH (which shares identical amino acid sequences with rtGH) (Gonzalez-Villasnor *et al.* 1988). This band was not detected in the soluble fraction from strain C (lane 1 of S in Fig. 5B). The amount of

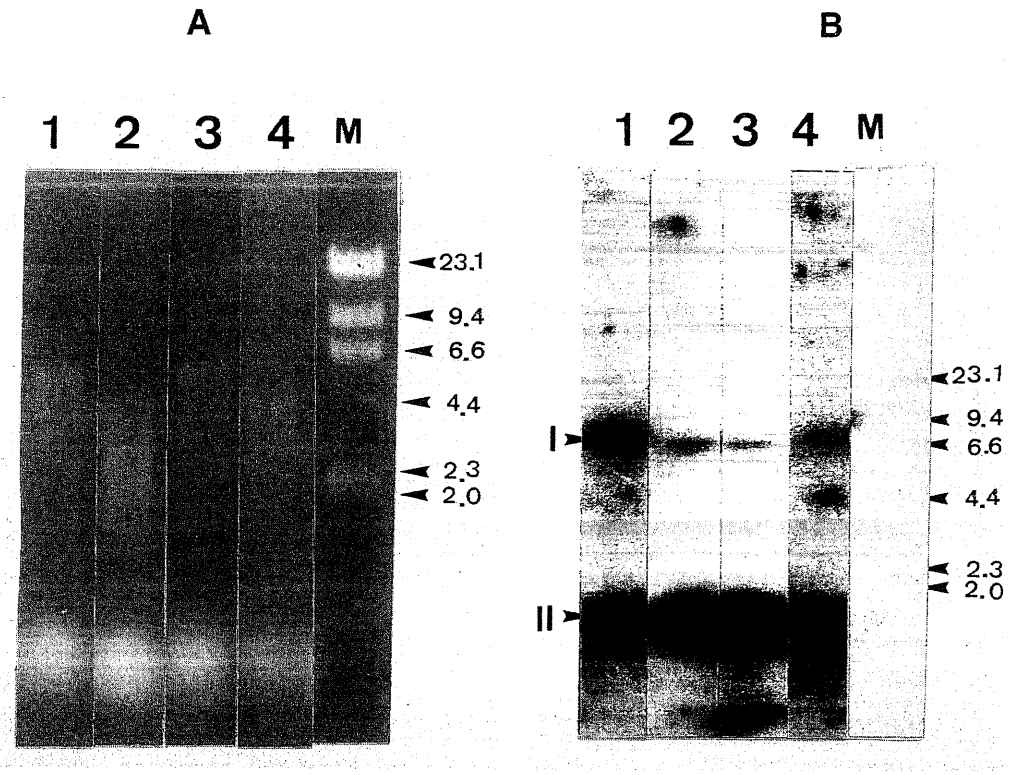

Fig. 4. Estimation of the copy number of pYERT harbored in *S. cerevisiae* Y-105. (A), the *Eco*RI-digestion pattern of total DNA extracted from Y-105 if cultured in YM medium at a density of: 3.2×10^6 cells/ml (middle-log, lane 1); 2×10^7 cells/ml (late-log, lane 2); 2×10^8 cells/ml (stationary, lane 3); and at 2×10^8 cells/ml after 10 generations (stationary, lane 4). (B), autoradiogram of Southern hybridization corresponding to (A). M, molecular marker of linear DNA fragments in kilo-base pairs. Positive band I and II represent the *leu2* gene and the GH cDNA, respectively. The copy number was determined by using a densitometer to compare the relative intensity of band I to band II.

rtGH synthesized in the yeast cell was about 0.5% of total cytosolic proteins.

Electrophoretic patterns of supernatant fractions (T) after Triton treatment showed no difference between strain C and Y-105; furthermore, an immunoreactive band was not detected (data not shown). This result disagrees with Hayami *et al.* (1989), who reported that part of recombinant tuna GH expressed in yeast could be detected in the supernatant fraction after treatment with Triton. However, when the Y-105 cell pellet obtained from a Triton treatment was broken further by glass beads in the presence of urea, an 22 kDa immunoreactive band was detected (U of Fig. 5B); however, the SDS-PAGE patterns showed a slight difference from the strain C band (U of Fig. 5A).

After the cell walls of the yeast transformants were removed with zymolase, yeast cells were lyzed and their soluble proteins extracted. A clear immunoreactive band with M. W. of 22 kDa appeared on the membrane for strains Y-105 (lane 2 of Fig. 5C) and Y-122, with another yeast transformant containing pYERT (lane 3 of Fig. 5C). However, this band was absent from the strain C extract (lane 1 of Fig. 5C). The intensity of the positive band was much stronger than that in samples without zymolase treatment (Fig. 5B). This evidence indicates that glass bead disruption of yeast is more effective

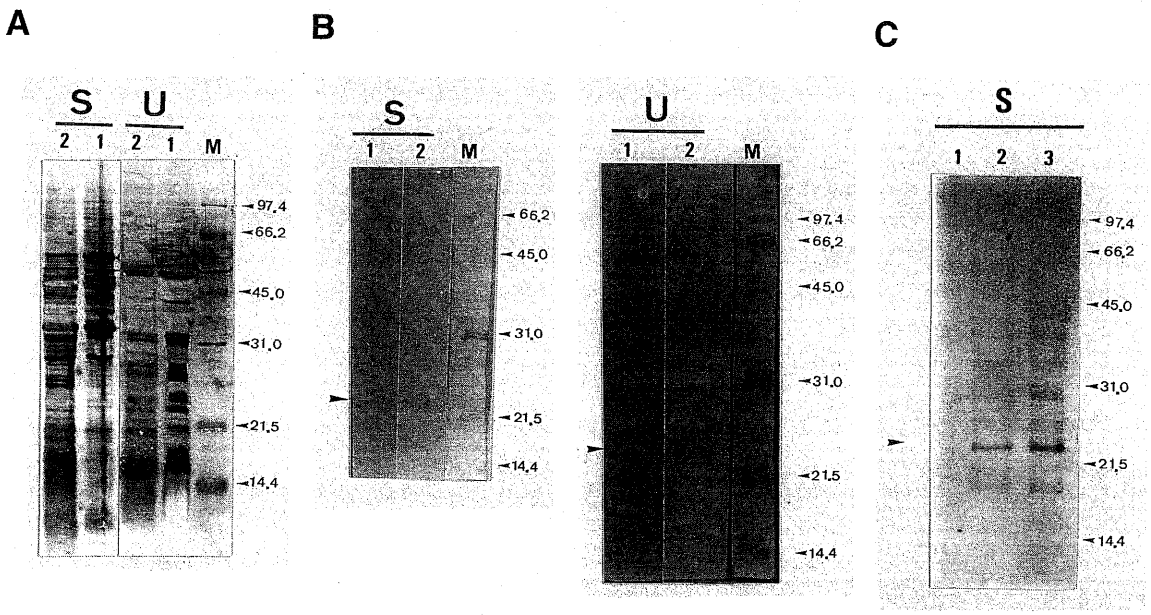

Fig. 5. Protein analysis. (A), about 40 μg of proteins extracted from *S. cerevisiae* 20B12 transformants were analyzed by SDS-PAGE and Coomassie blue staining in a 13.5% acrylamide gel. (B), Western blotting analysis corresponding to (A). (C), Western blotting analysis for yeast cells treated with zymolase. Lane 1, strain C (harboring expression vector pYE8 without DNA insertion); lane 2, strain Y-105 (harboring pYERT, a pYE8 with insertion of rtGH cDNA); lane 3, strain Y-122 (another transformant harboring pYERT); lane M, protein markers in kilo-daltons. The proteins in the supernatant fraction were obtained after yeast cells were lysed and centrifuged (S), or after the Triton-treated pellets were vortexed in the presence of 8 M urea, then centrifuged (U).

for cells treated with zymolase than for untreated cells.

Future perspectives

We have demonstrated that rtGH cDNA is expressed in *S. cerivisiae* cells under the control of a GAPDH promoter. The rGH which was biosynthesized by yeast was detectable in the cell lysate. It is likely that fish growth would be enhanced by direct feeding of recombinant yeasts containing rGH, since the intact form of GH or active fragment thereof may be absorbed by fish intestines following oral administration. This approach does not require procedures for cell lysis, isolating inclusion bodies, or re-naturing rGH. Moreover, it does not cause any stress to fish during treatment.

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虹鱒生長激素cDNA在酵母菌體的表現

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利用遺傳工程的技術將虹鱒生長激素cDNA剪接到酵母甘油醛磷酸去氫酶基因的起動子後，再接到含有2μm ori的episomal plasmid上。這個質體爲7.2kb的pYERT。經核苷酸定序後推演這個基因組合生長激素爲Met-Gly-Gln-Gly-Ala-Ala再接上虹鱒生長激素(GH)不含分泌性訊號的188個胺基酸。當pYERT轉形到酵母菌（*Saccharomyces cerevisiae* 20B12）時，篩選到含虹鱒生長激素cDNA的酵母菌轉形株（strain Y-105），並用南氏浸漬法，北方浸漬法和免疫沉澱法，分別檢測strain Y-105的DNA, RNA及蛋白質。結果證實該轉形株每個細胞含有20-25個pYERT；該段cDNA能被轉錄成GH mRNA；並且在酵母菌萃取液中，有一條外來22kDa的蛋白質；此蛋白質與生長激素抗體有免疫反應。