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Assembly of a Functional cDNA for Human Liver Growth Hormone Receptor: Cloning of Assembled hGHR cDNA

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Human Liver로부터 Cloning한 cDNA성장호르몬 수용체의 기능성 검토

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초 록

사람 성장호르몬 수용체(hGHR) cDNA는 PCR방법에 의하여 fragment로서 보고되어진 바 있으나, liver cDNA library로부터 전장을 cloning한 보고는 없는 실정으로 본 연구에서는 기능을 가진 약 4.6kbp의 cDNA hGHR을 cloning하는데 성공하였다. 먼저 cloning하기 위하여 human liver mRNA와 human breast cancer tissue로부터 회수한 mRNA를 RT-PCR방법에 의하여 human cDNA library와 cloning에 필요한 probe를 제작하였다. human library mRNA는 RT-PCR방법에 의하여 증폭하여 증폭되어진 산물은 λZAP Vector를 이용하여 cDNA library를 구축하였고, screening을 위하여 이미 보고되어진 hGHR fragment native sequence를 기초로 N-terminal부분의 primer를 설계하여 950bp의 probe를 얻는데 성공하였다. 이 probe를 이용하여 준비된 human liver cDNA library로부터 2.5×10^6개의 plaque로부터 6개의 positive clone을 획득하였고, 이들중 poly A signal인 "AATAAA"포함하고 있는 가장 긴 약 3.8kbp의 clone을 sequencing한 결과 open reading frame을 포함하고 있었으나, 5'부분이 결손되어 있었다. 그리하여 이 부분은 human breast cancer tissue로부터 회수한 mRNA를 RT-PCR에 의하여 증폭하였고, sequencing결과 이미 보고되어진 native hGHR와 비교한 결과 하나의 nucleotide가 silent mutation으로 판명되었다. 한편 human liver cDNA library로부터 cloning한 3.8kbp의 positive clone의 5'end의 결손된 부분에 silent mutation된 PCR 산물을 연결함으로써 native hGHR와 유사한 cDNA hGHR subcloning에 성공하였다. 이러한 cDNA hGHR의 clone이 function을 가지고 있는지를 검토하기 위하여 eukaryotic 발현 vector인 pCXN2에 의거 ligation한 후 chinese hamster ovary cell[CHO-KI]에 transfect를 실시하였다. Dexamethasone은 첨가하지 않고 hGH만의 존재하에서 이들 cell을 배양시키고 cell menbrane에서 발현 여부를

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판정키 위하여 hGHR monocloual antibody를 사용하여 flow cytometery해석을 실시하는 한편 ^{125}I-hGH binding assay에 의하여 hGHR binding activity를 측정하였다. 최종적으로 GH signal transduction의 target gene으로 알려져 있는 serine protease inhibitor 2.1(Spi 2.1) gene의 promotor activity를 검토한 결과 hGHR을 transfect한 CHO Cell에 있어서 hGH의 농도에 의존적으로 증가되었다. 따라서 본 실험에서 cloning한 cDNA hGHR는 native hGHR와 같은 기능을 가지는 것으로 판명되었다.

(Key words : human liver mRNA, hGHR, hGH, Spi 2.1, CHO-K1 cell)

INTRODUCTION

The growth hormone (GH) receptor (GHR) is activated on binding of growth hormone to stimulate the growth and metabolism of muscle, bone and cartilage (Nicoll *et al.*, 1986). Molecular analysis of the hGHR and rabbit GHR genes was reported by Leung *et al.* (1987). The receptor contains an extracellular hormone-binding domain (~28kDa), a single transmembrane domain, and intracellular domain (~35kDa). Northern blot hybridization demonstrated the presence of multiple transcripts. The same group of the author proposed that larger mRNA of 3.4 kbp~4.8 kbp codes for the intact receptor, while that the smaller mRNA species of 1.2~1.9 kbp codes for the soluble GH binding protein (Spencer *et al*, 1988). Later, based on the cloning data above, cDNA clones encoding GHR were cloned from a number of species (Barton *et al.*, 1989; Adams *et al.*, 1990; Burnside *et al.*, 1991; Pekhletskii *et al.*, 1991]. GHR action is thus thought to be initiated by hormone-induced dimerization of GHR, leading to activation of the intracellular tyrosine kinase, Janus kinase 2 (JAK 2) and to rapid changes in cytoplasmic and nuclear protein phosphorylation (Hackett *et al.*, 1995). These kinase, in turn, activate of family of STAT (signal transducers and activators of transcription) through phosphorylation of tyrosine residues. The activated STAT proteins are then translocates to the nucleus where they, by themselves or in combination with otherwise weak DNA-biding proteins, bind to specific response elements on responsive genes, and activate transcription (Darnell *et al.*, 1994).

One of the serine, the serine protease inhibitor (Spi 2.1) is to date, the best characterized physiological system for studying GH action. Spi 2.1 expression is greatly reduced by hypophysectomy and can be restored to 60% of its normal level by administration of GH alone. For activating Spi 2.1 transcription, on the other hand, the C-terminal region of the intracellular domain of GHR extending from position 437 amino acid is considered to be necessary (Berry *et al.*, 1986; Thomas *et al*; 1995). Goujon *et al.* (1994) found that Spi 2.1 gene was inducible by GH in CHO cells transfected with the rat GHR cDNA, indicating that CHO cells contained GHINF (or a STAT 5-related factor) which enable to relay the downstream signal transduction (Bergad *et al.*, 1995). Based on the background describe above, the author decided to use this GHR cDNA-transfected-CHO system for analyzing the transducing function of the hGHR cDNA assembled by himself.

We have tried to obtained a whole cDNA sequence with analyzed of the construct could be stably integrated into the cell membrane, the integrated receptor could bind with GH, and the ligand signal could transduce into the cytoplasm.

MATERIALS AND METHODS

1. Phage human liver cDNA library preparation

Human liver mRNA was purchased from Clonetech (Clonetech Lab., Inc., CA, USA, Lot #46018) and 5mg of this preparation was reversely transcribed with Molony Murine Leukemia Virus (MMLV) reverse transcriptase and used to construct a human liver cDNA library. The library was constructed in pBluescript SK+ (Stratgene) after cDNAs were primed with Dynabeads Oligo(dT)25. First and second strand cDNA synthesized by Amersham cDNA synthesis System Plus (Amersham). Double stranded cDNA was ligated to the ENB adaptor (TaKaRa), and then with λZAP II phage DNA arms which had been digested with Eco RI and 59-phosphorylated (T4 polynucleotide kinase, Boehringer Mannheim). Ligated DNA was packaged into phage particle by Gigapack II Gold Packaging Extract (Stratgene). From 100ng of Oligo(dT)25 primed linked cDNA, 2.5×10^7 primary clones were obtained.

2. Preparation of probe for screening form λ phage human liver cDNA library

The primers were designed based on the sequence reported previously (Leung *et al.*, 1987), and to avoid homology to GHRs of other species or the sequences of other GH /prolactin /cytokines superfamily members. The forward primer was; 5'-GATCAGAGGCGAAGCTCGGA-3' (hGHR -32 to -13, 20 bp) and the reverse primer; 5'-TGGGGGCAGAATCAGCATTT 3' (hGHR 943 to 924, 20 bp). Subsequent PCR were carried out with a final volume of 100 μl of 10 mM Tris-HCl buffer (pH 8.3) containing the cDNA template, 50 mM KCl, 25 mM $MgCl_2$, 10 mM dNTP, 0.5 mM of each primer, and 5 unit of amplitaqR DNA polymerase (Sambrook *et al.*, 1989). PCR were performed in a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer Cetus Instrument) for 35 cycle at 94℃ for 1min, 65℃ for 1min and 72℃ for 1.5 min, and the final extension at 72℃ for 10min. Each PCR product forming a band at approximately 950 bp was recovered after electrophoresis. The PCR product was used as the probe for screening hGHR cDNA clones after being labeled with [α-^{32}P] dCTP (3,000 Ci /mmol, Amersham) by random primers. And, approximately 2.5×10^6 plaques of the library were plated and transferred to nylon filters (Hybond-N+, Amersham). This libraries were screened with the probe described above. Transferred colonies were hybridized with the [α-^{32}P]dCTP-labeled probe for 20 h at 42℃ hybridization (Sambrook *et al.*, 1989) mixture (5x SSC, 2.5x Denhardt's reagent, 5mM EDTA, 0.1% SDS, 10% dextran sulfate, 100mg /ml salmon sperm DNA). The membrane was washed once with 0.1 x SSC (containing 0.1% SDS) for 5 min at 55℃ then exposed to X-ray film for 48 h at −70℃, of 2.5×10^6 plaques screened, 6 positive clones were obtained, and inserted cDNAs from these clones were subcloned into Bam HI-digested pUC18 for sequencing.

3. DNA sequencing and sequence analysis

The restriction enzyme map and sequencing strategy is presented in Fig. 1. Sequencing was

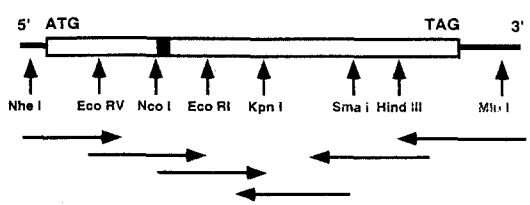

Fig. 1. Schematic structure of and sequencing strategies for the cDNA encoding hGHR. Six restriction enzyme sites are shown, Eco RV (511), Nco I (797), Eco RI (1004), Kpn I (1224), Sma I (1509) and Hind III (1675). the open box indicates the open reading frame (ORF). Sequencing was done according to the direction shown arrows.

performed by the dideoxy chain-termination method using an Auto read sequencing kit and A.L.F. DNA sequences (Pharmacia Biotech). The sequences were obtained for both strands with all restriction sites overlapped in Fig. 2. Sequences analysis was performed with DNSIS-MAC V2.0 computer software (Hitachi).

4. Construction of the hGHR expression vector

The assembled hGHR cDNA described above was integrated into the pUC18 plasmid (pUC18 -hGHR). To expect to enhance the expression efficiency in transfected cells, the hGHR cDNA was integrated into another plasmid pCXN2

```
5'  ATG GAT CTC TGG CAG CTG CTG TTG ACC TTG GCA CTG GCA GGA TCA AGT GAT GCT
    Met Asp Leu Trp Gln Leu Leu Leu Thr Leu Ala Leu Ala Gly Ser Ser Asp Ala
    1                                                                   18
    TTT TCT GGA AGT GAG GCC ACA GCA GCT ATC CTT AGC AGA GCA CCC TGG AGT CTG
    Phe Ser Gly Ser Glu Ala Thr Ala Ala Ile Leu Ser Arg Ala Pro Trp Ser Leu
    19                                                                  36
    CAA AGT GTT AAT CCA GGC CTA AAG ACA AAT TCT TCT AAG GAG CCT AAA TTC ACC
    Gln Ser Val Asn Pro Gly Leu Lys Thr Asn Ser Ser Lys Glu Pro Lys Phe Thr
    37                                                                  54
    AAG TGC CGT TCA CCT GAG CGA GAG ACT TTT TCA TGC CAC TGG ACA GAT GAG GTT
    Lys Cys Arg Ser Pro Glu Arg Glu Thr Phe Ser Cys His Trp Thr Asp Glu Val
    55                                                                  72
    CAT CAT GGT ACA AAG AAC CTA GGA CCC ATA CAG CTG TTC TAT ACC AGA AGG AAC
    His His Gly Thr Lys Asn Leu Gly Pro Ile Gln Leu Phe Tyr Thr Arg Arg Asn
    73                                                                  90
    ACT CAA GAA TGG ACT CAA GAA TGG AAA GAA TGC CCT GAT TAT GTT TCT GCT GGG
    Thr Gln Glu Trp Thr Gln Glu Trp Lys Glu Cys Pro Asp Tyr Val Ser Ala Gly
    91                                                                 108
    GAA AAC AGC TGT TAC TTT AAT TCA TCG TTT ACC TCC ATC TGG ATA CCT TAT TGT
    Glu Asn Ser Cys Tyr Phe Asn Ser Ser Phe Thr Ser Ile Trp Ile Pro Tyr Cys
    109                                                                126
    ATC AAG CTA ACT AGC AAT GGT GGT ACA GTG GAT GAA AAG TGT TTC TCT GTT GAT
    Ile Lys Leu Thr Ser Asn Gly Gly Thr Val Asp Glu Lys Cys Phe Ser Val Asp
    127                                                                144
    GAA ATA GTG CAA CCA GAT CCA CCC ATT GCC CTC AAC TGG ACT TTA CTG AAC GTC
    Glu Ile Val Gln Pro Asp Pro Pro Ile Ala Leu Asn Trp Thr Leu Leu Asn Val
    145                                                                162
    AGT TTA ACT GGG ATT CAT GCA GAT ATC CAA GTG AGA TGG GAA GCA CCA CGC AAT
    Ser Leu Thr Gly Ile His Ala Asp Ile Gln Val Arg Trp Glu Ala Pro Arg Asn
    163                                                                180
    GCA GAT ATT CAG AAA GGA TGG ATG GTT CTG GAG TAT GAA CTT CAA TAC AAA GAA
    Ala Asp Ile Gln Lys Gly Trp Met Val Leu Glu Tyr Glu Leu Gln Tyr Lys Glu
    181                                                                198
    GTA AAT GAA ACT AAA TGG AAA ATG ATG GAC CCT ATA TTG ACA ACA TCA GTT CCA
    Val Asn Glu Thr Lys Trp Lys Met Met Asp Pro Ile Leu Thr Thr Ser Val Pro
    199                                                                216
    GTG TAC TCA TTG AAA GTG GAT AAA GAA TAT GAA GTG CGT GTG AGA TCC AAA CAA
    Val Tyr Ser Leu Lys Val Asp Lys Glu Tyr Glu Val Arg Val Arg Ser Lys Gln
    217                                                                234
    CGA AAC TCT GGA AAT TAT GGC GAG TTC AGT GAG GTG CTC TAT GTA ACA TTT CCT
    Arg Asn Ser Gly Asn Tyr Gly Glu Phe Ser Glu Val Leu Tyr Val Thr Leu Pro
    235                                                                252
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CAG ATG AGC CAA TTT ACA TGT GAA GAA GAT TTC TAC TTT CCA TGG CTC TTA ATT
Gln Met Ser Gln Phe Thr Cys Glu Glu Asp Phe Tyr Phe Pro Trp Leu Leu Ile
253 270
ATT ATC TTT GGA ATA TTT GGG CTA ACA GTG ATG CTA TTT GTA TTC TTA TTT TCT
Ile Ile Phe Gly Ile Phe Gly Leu Thr Val Met Leu Phe Val Phe Leu Phe Ser
271 288
AAA CAG CAA AGG ATT AAA ATG CTG ATT CTG CCC CCA GTT CCA GTT CCA AAG ATT
Lys Gln Gln Arg Ile Lys Met Leu Ile Leu Pro Pro Val Pro Val Pro Lys Ile
289 306
AAA GGA ATC GAT CCA GAT CTC CTC AAG GAA GGA AAA TTA GAG GAG GTG AAC ACA
Lys Gly Ile Asp Pro Asp Leu Leu Lys Glu Gly Lys Leu Glu Glu Val Asn Thr
307 324
ATC TTA GCC ATT CAT GAT AGC TAT AAA CCC GAA TTC CAC AGT GAT GAC TCT TGG
Ile Leu Ala Ile His Asp Ser Tyr Lys Pro Glu Phe His Ser Asp Asp Ser Trp
325 342
GTT GAA TTT ATT GAG CTA GAT ATT GAT GAG CCA GAT GAA AAG ACT GAG GAA TCA
Val Glu Phe Ile Glu Leu Asp Ile Asp Glu Pro Asp Glu Lys Thr Glu Glu Ser
343 360
GAC ACA GAC AGA CTT CTA AGC AGT GAC CAT GAG AAA TCA CAT AGT AAC CTA GGG
Asp Thr Asp Arg Leu Leu Ser Ser Asp His Glu Lys Ser His Ser Asn Leu Gly
361 378
GTG AAG GAT GGC GAC TCT GGA CGT ACC AGC TGT TGT GAA CCT GAC ATT CTG GAG
Val Lys Asp Gly Asp Ser Gly Arg Thr Ser Cys Cys Glu Pro Asp Ile Leu Glu
379 396
ACT GAT TTC AAT GCC AAT GAC ATA CAT GAG GGT ACC TCA GAG GTT GCT CAG CCA
Thr Asp Phe Asn Ala Asn Asp Ile His Glu Gly Thr Ser Glu Val Ala Gln Pro
397 414
CAG AGG TTA AAA GGG GAA GCA GAT CTC TTA TGC CTT GAC CAG AAG AAT CAA AAT
Gln Arg Leu Lys Gly Glu Ala Asp Leu Leu Cys Leu Asp Gln Lys Asn Gln Asn
415 432
AAC TCA CCT TAT CAT GAT GCT TGC CCT GCT ACT CAG CAG CCC AGT GTT ATC CAA
Asn Ser Pro Tyr His Asp Ala Cys Pro Ala Thr Gln Gln Pro Ser Val Ile Gln
433 450
GCA GAG AAA AAC AAA CCA CAA CCA CTT CCT ACT GAA GGA GCT GAG TCA ACT CAC
Ala Glu Lys Asn Lys Pro Gln Pro Leu Pro Thr Glu Gly Ala Glu Ser Thr His
451 468
CAA GCT GCC CAT ATT CAG CTA AGC AAT CCA AGT TCA CTG TCA AAC ATC GAC TTT
Gln Ala Ala His Ile Gln Leu Ser Asn Pro Ser Ser Leu Ser Asn Ile Asp Phe
469 486
TAT GCC CAG GTG AGC GAC ATT ACA CCA GCA GGT AGT GTG GTC CTT TCC CCG GGC
Tyr Ala Gln Val Ser Asp Ile Thr Pro Ala Gly Ser Val Val Leu Ser Pro Gly
487 504
CAA AAG AAT AAG GCA GGG ATG TCC CAA TGT GAC ATG CAC CCG GAA ATG GTC TCA
Gln Lys Asn Lys Ala Gly Met Ser Gln Cys Asp Met His Pro Glu Met Val Ser
505 522
CTC TGC CAA GAA AAC TTC CTT ATG GAC AAT GCC TAC TTC TGT GAG GCA GAT GCC
Leu Cys Gln Glu Asn Phe Leu Met Asp Asn Ala Tyr Phe Cys Glu Ala Asp Ala
523 540
AAA AAG TGC ATC CCT GTG GCT CCT CAC ATC AAG GTT GAA TCA CAC ATA CAG CCA
Lys Lys Cys Ile Pro Val Ala Pro His Ile Lys Val Glu Ser His Ile Gln Pro
541 558

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AGC TTA AAC CAA GAG GAC ATT TAC ATC ACC ACA GAA AGC CTT ACC ACT GCT GCT
Ser Leu Asn Gln Glu Asp Ile Tyr Ile Thr Thr Glu Ser Leu Thr Thr Ala Ala
559                                                                 576
GGG AGG CCT GGG ACA GGA GAA CAT GTT CCA GGT TCT GAG ATG CCT GTC CCA GAC
Gly Arg Pro Gly Thr Gly Glu His Val Pro Gly Ser Glu Met Pro Val Pro Asp
577                                                                 594
TAT ACC TCC ATT CAT ATA GTA CAG TCC CCA CAG GGC CTC ATA CTC AAT GCG ACT
Tyr Thr Ser Ile His Ile Val Gln Ser Pro Gln Gly Leu Ile Leu Asn Ala Thr
595                                                                 612
GCC TTG CCC TTG CCT GAC AAA GAG TTT CTC TCA TCA TGT GGC TAT GTG AGC ACA
Ala Leu Pro Leu Pro Asp Lys Glu Phe Leu Ser Ser Cys Gly Tyr Val Ser The
613                                                                 630
GAC CAA CTG AAC AAA ATC ATG CCT TAG 3'
Asp Gln Leu Asn Lys Ile Met Pro ***
631                         638
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Fig. 2. Nucleotide and deduced amino acid sequences of hGHR. Nucleotide are numbered above and amino acid are numbered below from the first methionine. The cDNA for hGHR contains 1975 nucleotide encoding 638 amino acids. Black box is the silent mutation (G to A), without change of amino acid.

(Miyazaki *et al.*, 1989; Niwa *et al.*, 1991) in Fig. 3. The pUC-hGHR was digested with Nhe I/Mlu I blunted with Xho I and integrated into pCXN2.

5. ^{125}I-hGH binding assay for hGHR expressing CHO cells

The [^{125}I]-hGH binding assay was done for two purpose in this study. One was to select the stable cell line expressing hGHR at a high efficiency, and the other was to character hGHR expressed in that cell line by kinetic analysis. The cells from each clone were seeded in 6 well plate at 1.69×10^6 cells/well until confluence, further incubated for 30 min at 37℃ in a CO_2 incubator (5% CO_2) after the medium was changed to the FCS(−) medium. Then, the medium was again exchanged to 0.1% BSA-PBS containing 3 ng of [^{125}I]-hGH for the selection of the cell line, or 0.1% BSA PBS containing both 3 ng of [^{125}I]-hGH and different concentrations of cold hGH from 10 to 10^5 ng/ml for Scatchard analysis (Scatchard, 1949) in Fig. 4. Next, 200 μl of the cell suspension was dispensed into presence of 10 μg/ml of mouse anti-GHR monoclonal antibody mAb 263 or mAb 5. After being washed two times in PBS containing 0.1% BSA and 0.1% sodium azide, the cells were stained with goat anti-mouse FITC-conjugated F(ab')2 antibodies at a 1:50 dilution and were analyzed on a

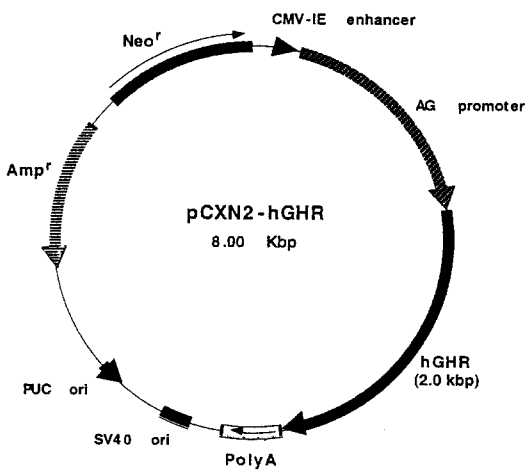

Fig. 3. Construction of hGHR expression vector. A hGHR cDNA was ligated (see materials and methods) into the plasmid pUC18 ori containing element of CMV-IE enhancer with AG promoter, SV40 ori enhancer.

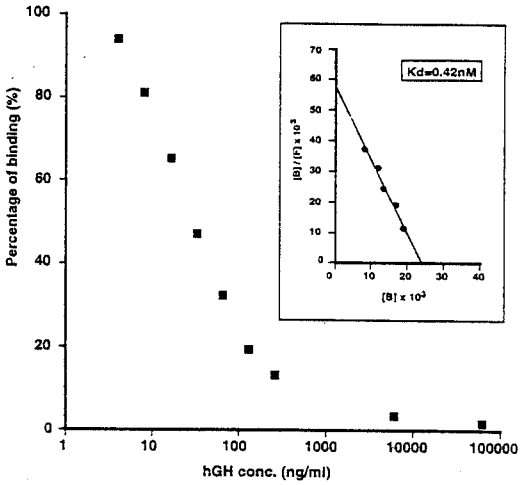

Fig. 4. Binding of [^{125}I]22kDa-hGH to CHO-hGHR (X-A5) cells was measured in the presence of increasing amount of unlabelled 22 kDa-hGH. In the Scatchard analysis, the ratio of bound to free hormones was plotted against the amount of free hormone and the curve of best fit was evaluated by simple regression analysis.

Coulter EPICS (Coulter, Miami, FL).

6. Analysis of signal transduction of CHO-hGHR cells expressing the Spi 2.1 promoter activation assay

Analysis of signal transduction of CHO-hGHR cells was done according to the following strategy. The X-A5 cells, that were proven to express hGHR at high efficiency, was transfected transiently with the construct consisting of the Spi 2.1 promoter (Yoon *et al.*, 1990) and the luciferase reporter genes. Simultaneously with this transfection, the pCH110 reporter plasmid (Promega) expressing the β-galactosidase reporter gene was co transfected to correct the expressing efficiency of individual samples with the amount of the expressed β-galactosidase activities. X-5A cells were seeded in a 24 well plate at 1.2×10^5 cells/well 16 h before the transfection in the medium of OPTI-MEM, and transfected pCH110 (0.75μg/well) and pGL2-RSV (0.25 μg/well) using Lipofectamine kit (GIBCO). After incubation for 5 h, the medium was exchanged with 0.5 ml of the serum free OPTI-MEM medium containing different concentrations of hGH from 0.2 to 2,000 nM and dexamethasone (25 nM). The transfected X-5A cells were further incubated for 48 h lysed with the lysis buffer in the luciferase assay kit, and stored at −80℃. Thawed samples were subjected to the luciferase assay using luciferase assay system with reporter lysis buffer (Promega) and the β-galactosidase assay using β-galactosidase enzyme assay system with reporter lysis buffer (Promega). The luciferase activity of each sample was normalized by the β-galactosidase activity.

RESULTS

The human liver cDNA was constructed in pBluescript SK+ (Stratgene) and 2.5×10^7 primary clones were obtained. To screened positive clones, a probe was synthesized on the human liver cDNA as the template by RT-PCR using the primers designed according to the sequence reported by Leung *et al.* (1987). Sequencing of the probe (PCR product [L]) revealed that it contained a sequence from position −22 to 556 as was expected when cDNA from human liver poly (A) was used as the template. Using this probe, 6 positive clones were obtained. Each insert from the 6 positive clones was subcloned into pUC18 vector. Bam HI-digested of the recombinant plasmid released from approximately 1.5 to 4.0 kbp inserts (the whole hGHR cDNA is 4.6 kbp). Sequencing of these inserts from 5' end demonstrated that all of the 6 inserts were found to be deleted of the initial 5' sequence down to further than the position 328. Two of

these 6 inserts were further deleted of the exon 3 region. The longest inserts of 4.0 kbp was fully sequenced, found to cover from the position 329 to the whole 3' flanking region and was designated as hGHR clone 1. And then, the PCR products originated in human breast cancer tissue mRNA was sequenced and found to cover from position −22 to 955 as expected. But it had a mutation of "G" (the position 703) to "A" at the position of 233 amino acid (Fig. 2), but the mutation is silent and would give the native amino acid residue (Lys).

The assembled hGHR cDNA was first integrated into the pUC18 plasmid, and then into the pCXN2 plasmid to expect enhanced expression efficiency. The later plasmid was transfected into *E. coli* DH5, and 16 clones of 100 transformants were randomly picked up to check the presence and orientation of the insert by Hind Ⅲ digestion. Six clones were found to integrate the insert in right orientation (pCXN2-hGHR). CHO-K1 cells were co-transfected with pCXN2 -hGHR and pRC/RSV, having neomycin resistant gene for selection marker. The ratio of the two plasmid at transfection was set at 20:1 (DNA amount basis) to increase the probability that the neo resistant cells obtained were transfected with hGHR simultaneously. Twenty -four of G418-resistant (neomycin resistant) cell clonies were picked up and the binding activity of each clonal cells to ^{125}I-hGH was assayed after 30 days of culture. By measuring the total radio-activities of the membrane fraction of each clone after incubation with ^{125}I-hGH, the clone showing the highest radioactivity was selected and named as X-A5 cells. The expression of hGHR on the surface of X-A5 cells was confirmed by flow cytometric analysis using the monoclonal anti GHR antibody mAb 263 and mAb 5. When mAb 263 was used, X-A5 cells revealed a positive shift in fluorescence intensity in comparison with non-transfected parental CHO cells (Fig. 5). A similar result was obtained with mAb 5 which seemed to be less reactive to hGHR than mAb 263 (data not shown). When isotope-matched nonspecific mAb was used as a negative control, no shift in fluorescence intensity was detected (data not shown).

To test the signal transducing activity of hGHR cloned by the author, X-5A cells, that were stably transfected with the cDNA encoding hGHR, were transiently co-transfected with the rat Spi 2.1 (−175 to +59) / luciferase construct (Fig. 6), where up-regulation of the Spi 2.1 promoter could be evaluated by the luciferase activity. The Spi 2.1 gene has been known to reside in the downstream of the GH signal transduction (see materials and methods). The −175/+59 fragment of the promoter of the Spi 2.1 gene was reported to show the highest fold induction of CAT activity in the presence of both dexamethasone (Dex) and GH (Allevato *et al.*, 1995), indicating that CHO cells contain some of the appropriate downstream elements,

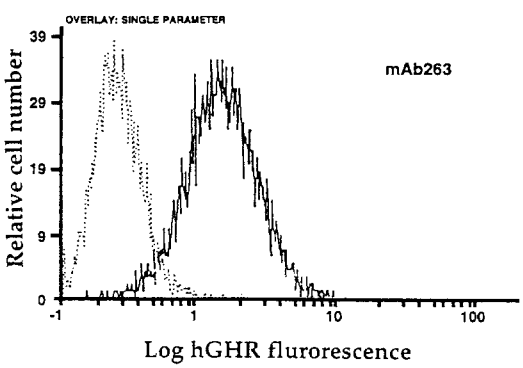

Fig. 5. Comparison of hGHR expression on X-A5 (bold line) and parental CHO cells (dot line). Cells were incubated in the presence of 10 μg/ ml of mouse anti-GHR mAb 263, then stained with goat anti-mouse FITC conjugated F(ab9)2 antibodies. The cells were analyzed on flow cytometry as described in materials and methods.

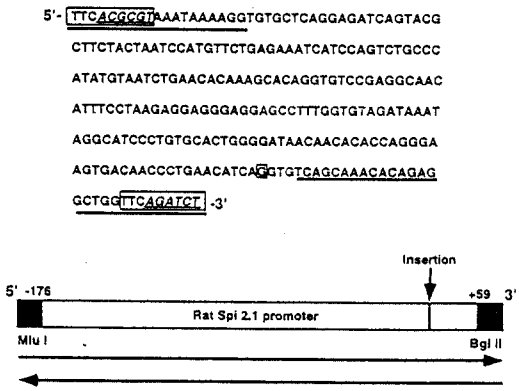

Fig. 6. Upper panel: nucleotide sequence of the PCR product of the rat Spi 2.1 promoter adding with cloning sequences (square boxes) containing Mlu I (forward) and Bgl II (reverse) restriction enzyme sites (indicated with thin underlines). The primers sequences designed are indicated by thick underlines. The "G" in a square box is an insertion of an additional nucleotide based on the original report (Yoon et al., 1990). Lower panel: sequencing strategy. Closed squares at both ends represent additional sequences for cloning.

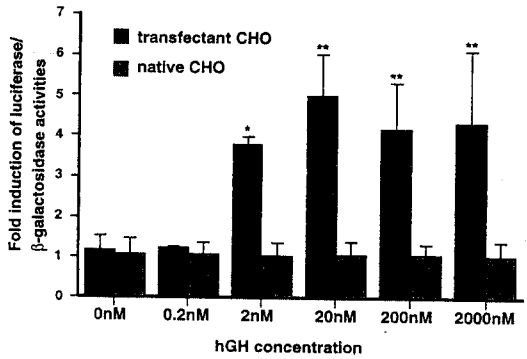

Fig. 7. Effects of increasing construction of hGH on expression of luciferase/ β-gal activity in CHO-K1 cells. CHO cells were transiently co transfected with a plasmid containing the GHR and the fusion gene Spi 2.1/ β-galactosidase. Each luciferase activity is normalized by each β-galactosidase activity. Results are mean + SEM of 3 independent experiment. *: $P<0.05$, **: $P<0.01$ compared with values at 0 nM of the hGH.

including nuclear transcription factors (Yoon *et al.*, 1990). The same sequence of the promoter was used in this study, using pCXN2-CHO (X-A5) cells the author cloned. Neither GH nor Dex per se had significant effects on luciferase activity, but Dex and GH together stimulated luciferase activity (data not presented).

According to this results, increasing hGH concentration on luciferase activity was tested in the presence 250 nM Dex. The effects become significant at 2 nM hGH, with maximal effects at 20 nM hGH. An addition of 20~2,000 nM hGH resulted in approximately five fold significant induction of luciferase activity compared with the control without hGH (Fig. 7).

DISCUSSION

Six positive clones were obtained after screening of approximately 2.5×10^6 plaques of the library with the probe. The rate of the positive clones was extremely very low. The low efficiency suggest that a small number of GHR mRNA molecules were transcribed in the human liver. Because the purification of GHR protein from the human liver was not much suffered from shortage of materials (Leung *et al.*, 1987), the main reason for this inefficiency might be ascribed to the high stability of GHR mRNA, though the true reason for this difficulty is unknown. The 5" ends longer than 340 bp were missing in all the clones screened. One possibility for this deletion may be an inadequate elongation of cDNA sequence during the reverse transcribing procedure. However, Colosi *et al.* (1993) claimed in their report that they also have had difficulties assembling and propagating in *E. coli* cDNA clones coding the full-length human GH

receptor, by referring a report by Leung *et al.* (1987). Another possibility may be related to this difficulties noted by other investigators trying to isolate the whole sequence of hGHR from cDNA libraries. These author wrote the reason for this difficulties was unknown.

Two of the 6 inserts were further deleted of the exon 3 region. A recent report (Wickelgren *et al.*, 1995) suggested that the hGHR existed in 2 isoform; in one form exon 3 was exist (GHR3+), and in the other, exon 3 was absent (GHR3−) which were expressed as tissue specific and/or developmentally regulated manner. Though the author has any direct evidence to discuss the relation between this hypothesis and his result, the existence of 2 isoform is interesting subject to be elucidated in future. Since the longest clone (hGHR clone 1) was found to be lack 5" end down to the position −22 to 955, at the position of 819 (Nco I site). Thus, the full sequence of hGHR cDNA was assembled. On the other hand, the function of this sequence will be analyzed. The functional expression of cloned hGHR is a powerful tool that can help to elucidate the mechanism whereby the ligand binding signal is transduced and interpreted by a responding cell. For the hGHR, ligand binding assays and an analysis of the structure of the ligand receptor complex have provided a wealth of information about the details of the interaction of the ligand with its receptor (Cunningham *et al.*, 1989; Cunningham and Well, 1989; De Vos *et al.*, 1992), including the demonstration that receptor dimerization is the first step in receptor signaling (Fuh *et al.*, 1992). System which show a biological response following transfection of the receptor have been more difficult to establish, although two response systems utilizing the hGH have been described (Colosi *et al.*, 1993; Urbanek *et al.*, 1993; Hackett *et al.*, 1995).

In this study, to have successfully expressed hGHR in CHO-K1 cells, eukaryotic expression plasmid pCXN2, containing chickenβ-promoter was employed. The pCXN2 vector is opposed of a ubiquitously strong promoter, namely modified chicken β-actin promoter with a cytomegalovirus immediate-early enhancer, a rabbit β-globulin 3' flanking sequence and a mutant neomycin phosphor-transferase Ⅱ-encoding gene (Miyazaki *et al.*, 1989; Niwa *et al.*, 1991). Though the author did not conducted strict quantitative comparison with other available plasmid vectors, satisfactory expression of hGHR was achieved in this study, presumably due to the surface expression vector and/or transfection procedure. Emtnter *et al.* (1990) demonstrated GH-stimulated protein synthesis in CHO cells stably expressing the rat GHR. Moller *et al.* (1992) showed that CHO cells stably transfected by the rat GHR cDNA responded to GH by increasing proliferative and MAP kinase activities. It was reported that the C terminal domain of the GHR receptor was not only fully required for required for expressing some biological effects of GH; recent studies (Van Der *et al.*, 1985; Moller *et al.*, 1992) demonstrated that a truncate GHR was still found to be able to transmit the signal for protein biosynthesis, mitogen-activated protein kinase, and JAK-2 kinase activation and mitotic activity in CHO cells, but unable to stimulate Spi 2.1 transcription. Thus, the functional assay of the Spi 2.1 promoter is considered as one of the most useful tool to evaluate the integrity of GHR. The *c-fos* signal pathway is initiated by interaction of GH and GHR, followed by JAK2 phosphorylation and binding of STAT 1-STAT 3 complex to the Sis- inducible element (SIE) in the *c-fos* gene (Allevato *et al.*, 1995). Recently it has been demonstrated that serum does not induce *c-fos* transcription through the SIE, whereas the SIE is essential for full induction by GH, accounting for the different mech-

anism for cell proliferation in GH and in serum (Chen *et al.*, 1995). It may be possible that the proliferative signal induced by serum is partly disturbed by the GH induced signal through a separate signal pathway.

In conclusion, the results presented in this paper illustrate that the approach using co-transfection of the receptor cDNA and a target reporter gene is valid for the hGHR. These suggest that 22kDa-hGH exerts a full agonistic activity for hGHR. In spite of the deleted region involved in the tight binding to hGHR, it is of interest that 22kDa-hGH than 20kDa-hGH is still retains the highly affinity for hGHR. We are now performing an X-ray structural analysis of 20 and 22kDa-hGH, which may explain, at the molecular level, why 22 kDa-hGH has a full effects on the hGHR.

SUMMARY

Though the full sequence of cDNA for human growth hormone receptor(hGHR) was already reported, molecular cloning of the full length of this cDNA from a liver cDNA library was reported to be difficult by some unknown reason. Difficulty to obtain the functional full length of this cDNA made us to try to assemble it. As a result, a funtional cDNA, approximately 4.6 kbp, expressing hGHR by ligating necessary sequences obtained by both cloning from a human liver cDNA library and a RT-PCR amplification using mRNAs originating in a human breast cancer tissue as a template. Human liver mRNAs were amplified by RT-PCR, and the products were integrated into λZAP and a cDNA library was constructed. For screening, a 950 bp probe at N-terminal was amplified by RT-PCR using mixed primers designed according to the reported full sequence of hGHR, and labeled with [α-^{32}P]dCTP.

Using this probe, 6 positive clones were isolated from 2.5×10^6 recombinant. Analysis of the nucleotide sequence revealed that one of the 6 clones of 3.8 kbp cDNA contained a major part of the open reading frame and a poly-adenylation signal preceded by "AATAAA". Because this clone was found to have a deletion at 5' end, this deleted part was amplified by RT-PCR with an appropriate pair of primers using mRNAs extracted from a human breast cancer tissue. Sequencing of this amplified products revealed one nucleotide mutation that still can maintain the native amino acids sequence. This amplified products covering the deletion was inserted into the cloned sequence as it was. Finally, this assembled sequence coding the full length hGHR was integrated in the standard high copy number cloning vector.

The assembled full length construct of hGHR cDNA was integrated into an eukaryotic expression plasmid pCXN2, and transfected into a Chinese hamster ovary cell line (CHO-K1). These cells grew in the presence of GH without dexamethasone. Flow cytometry analysis using specific monoclonal antibody to hGHR indicated expression of GHR on the cell membrane. Binding activity of GHR was confirmed by ^{125}I-hGH binding assay. The promoter activity of serine protease inhibitor 2.1 (Spi 2.1) that was known as one of the target gene of the GH signal transduction, was found to be enhanced by an addition of hGH to the GHR transfected CHO cells. These results demonstrate that the full length hGHR cDNA assembled is functional.

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REFERENCES

Adams TE, Baker L, Fiddes RJ and Brandon MR. 1990. The sheep growth hormone receptor: molecular cloning and ontogeny of mRNA expression in the liver. Molecular Cell Endocrinology, 73:135-145.

Allevato G, Billestrup N, Goujon L, Galsgaard ED, Norstedt G, Postel-Vinay MC, Kelly PA and Nielsen JH. 1995. Identification of phenylalanine 346 in the rat growth hormone receptor as being critical for ligand -mediated internalization and down-regulation. Journal of Biological Chemistry, 270: 7210-17214.

Barton DE, Follmer BE, Wood WI and Francke U. 1989. Chromosome mapping of the growth hormone receptor gene in man and mouse. Cytogenet Cell Genetics, 50:137-141.

Bergad PL, Shih HM, Towle HC, Schwarzenberg SJ and Berry SA. 1995. Growth hormone induction of hepatic serine protease inhibitor 2.1 transcription is mediated by a Stat5-related factor binding synergistically to two gamma-activated sites. Journal of Biological Chemistry, 270:24903-24910.

Berry SA, Manthei RD and Seelig S. 1986. Ontogenesis of growth hormone (GH)-response hepatic gene products: lack of correlation with hepatic GH receptor content. Endocrinology, 119:2290-2296.

Burnside J, Liou SS and Cogburn LA. 1991. Molecular cloning of the chicken growth hormone receptor complementary deoxyribonucleic acid: mutation of the gene in sex-linked dwarf chickens. Endocrinology, 128:3183-3192.

Chen C, Clarkson RWE, Xie Y, Hume DA and Waters MJ. 1995. Growth hormone and colony-stimulating factor 1 share multiple response elements in the *c-fos* promoter. Endocrinology, 136:4505-4516.

Colosi P, Wong K, Leong SR and Wood I. 1993. Mutational analysis of the intracellular domain of the human growth hormone receptor. Journal of Biological Chemistry, 268: 12617-12623.

Cunningham BC and Wells JA. 1989. High-resolution epitope mapping of hGHR-receptor interactions by alanine-scanning mutagenesis. Science, 243:1081-1085.

Cunningham BC, Jhurani P, Ng P and Wells JA. 1989. Receptor and antibody epitopes in human growth hormone identified by homolog scanning mutagenesis. Science, 244:1330-1336.

Darnell JE, Jr, Kerry IM and Stark GR. 1994. Jak-STAT pathway and transcriptional activation in response to IFNs and other extracellular signalling protein. Science, 264:1415-1421.

De Vos AM, Ultsch M and Kossiakoff AA. 1992. Human growth hormone and extracellular domain of its receptor: Crystal structure of the complex. Science, 255:305-312.

Emtner M, Lawrence S, Mathews and Norstedt G. 1990. Growth hormone (GH) stimulates protein synthesis in cells transfected with GH receptor complementary DNA. Molecular Endocrinology, 4:2014-2020.

Fuh G, Cunningham BC, Fukunaga R, Nagata S, Goeddel DV and Wells JA. 1992. Rational design of potent antagonists to the human growth hormone receptor. Science, 256: 1667-1680.

Goujon L, Allevato G, Simonin G, Paquereau L, Le Cam A, Clark J, Nielsen JH, Djiane J, Postel-Vinay MC, Edery M and Kelly PA. 1994. Cytoplasmic sequences of the growth

hormone receptor necessary for signal transduction. Proceeding of the National Academy of Science, USA, 91:957-961.

Hackett RH, Wang YD and Larner AC. 1995. Mapping of the cytoplasmic domain of the human growth hormone receptor required for the activation of JAK2 and STAT proteins. Journal of Biological Chemistry, 270: 21326-21330.

Leung DW, Spencer SA, Cachianes G, Hammonds RG, Collins C, Henzel WJ, Barnard R, Waters MJ and Wood WI. 1987. Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature, 330:537-543.

Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K and Yamamura K. 1989. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. Gene, 79:269-277.

Moller C, Hansson A, Enberg B, Lobie PE and Norstedt G. 1992. Growth hormone (GH) induction of tyrosine phosphorylation and activation of mitogen-activated protein kinase in cells transfected with rat GH receptor cDNA. Journal of Biological Chemistry, 267:23403-23408.

Nicoll CS, Mayer GL and Russell SM. 1986. Structural features of prolactin and growth hormones that can be related to their biological properties. Endocrine Reviews, 7: 169-203.

Niwa H, Yamamura K and Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene, 108:193-199.

Pekhletskii RI, Chernov BK and Rubtsov PM. 1991. Amplification using one-sided polymerase chain reaction, cloning and determination of primary structure of 5'-untranslated cDNA segments coding the somatogenic growth hormone receptor from rat liver. Molecular Biology, 25:1418-1423.

Sambrook J, Fritsch EE and Maniatis T. 1989. Molecular cloning: A laboratory manual, 2nd., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scatchard G. 1949. The attractions of proteins for small molecules and ions. Ann., N.Y. Academy Science, 51:660-672.

Spencer SA, Hammonds RG, Henzel WJ, Rodriguez H, Waters MJ and Wood WI. 1988. Rabbit liver growth hormone receptor and serum binding protein: purification, characterization and sequence. Journal of Biological Chemistry, 263:7864-7867.

Thomas MJ, Gronowski AM, Berry SA, Bergad PL and Rotwein P. 1995. Growth hormone rapidly activities rat serine protease inhibitor 2.1 gene transcription and induces a DNA-binding activity distinct from those of Stat 1, -3, and -4. Molecular Cellular Biology, 15:12-18.

Urbanek M, Russell JE, Cooke NE and Liebhaber SA. 1993. Functional characterization of the alternatively spliced, placental human growth hormone receptor. Journal of Biological Chemistry, 268:19025-19032.

Van Der Putten H, Botteri FM, Miller AD, Rosenfeld MG, Fan H, Evans RM and Verma IM. 1985. Efficient insertion of genes into the mouse germ line via retroviral vectors. Proceeding of the National Academy of Science, USA, 82:6148-6152.

Wickelgren RB, Landin KLL, Ohlsson C and Carlsson LMS. 1995. Expression of exon 3-retaining and exon 3-excluding isoform of the human growth hormone (GH)-receptor is regulated in an individual rather than a tissue-specific manner. Journal of Clinical Endocrinology & Metabolism, 80:2154-2157.

Yoon JB, Berry SA, Seelig S and Towel HC. 1990. An inducible nuclear factor binds to a growth hormone - regulated gene. Journal of Biological Chemistry, 265:19947-19954.

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