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Cloning and Sequencing of cDNA that encodes Ostrich Insulin-like Growth Factor - I

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Summary

The cDNA that encodes ostrich (*Struthio Camelus* (varietas) *domesticus*) insulin-like growth factor - I (oIGF - I) was isolated from an ostrich liver cDNA library. The isolated cDNA is 819 base pairs long, containing an oIGF - I precursor coding region of 459 base pairs, which encodes a polypeptide of 153 amino acid residues. It was predicted that this oIGF - I precursor consists of a 48-amino acid signal peptide, a 70-amino acid mature IGF - I peptide, and a 35-amino acid E domain. Evidence to support this was obtained from amino acid homolgy analyses of IGF-Is hitherto published. The amino acid sequence homology between precursors of oIGF - I and IGF - Is of chicken and turkey was very high (~99%), and that between precursors of oIGF - I and IGF - Is of mice, bovine, goat, and human was also high (84−79%).

Introduction

Insulin-like growth factor - I (IGF - I) is a polypeptide composed of 70 amino acid residues similar to proinsulin (1). Many tissues express IGF - I when stimulated by growth hormone (GH). It has been confirmed that the GH action on tissues is induced by stimulating the local production of IGF - I, which acts in an autocrine/paracrine manner. Transcription of the IGF - I gene mediated many of the biological effects of GH (2 - 6). IGF - I has insulin-like activities, for example, it stimulates glycogen synthesis (7), and functions as a mitogen and as a differentiation factor for various cell lines including preadipocytes (8). The biological actions of IGF - I begin on interaction with its cell surface receptor, which is a ligand-activable tyrosine-specific protein kinase similar to the insulin receptor (9). In the differentiation of cultured cells, transcription of the IGF - I gene is stimulated by GH. IGF - I combined with GH is essential for the differentiation of the cultured cells. This phenomenon is interpreted as follows ; (i) IGF - I secreted from the cultured cells is not active enough to induce the differentiation. (ii) The IGF - I mRNA is translated when cells are cultured in the presence of both GH and IGF - I. (iii) IGF - I is not secreted from the cultured cells without the signal from the IGF - I receptor (10).

The IGF - I DNAs of human (11-12), rat (13-21), bovine (22), sheep (23), goat

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(24, 25), chicken (26, 27), and turkey (28) have been isolated and their nucleotide sequences determined, and the transcriptional regulation of the IGF - I genes of human (19-23), rat (11-16), and goat (25, 26) have been reported. In mouse and rat, the IGF - I genes have two leader exons (exons 1 and 2, resulting in two kinds of mRNAs (classes 1 and 2) (15-18). There is another mRNA species, class 1del., in which a central region of exon 1 is missing (28, 29). Exon 5 is also spliced alternatively, resulting in Ea encoded by exon 4 and 6, and Eb encoded by exons 4, 5, and 6 (15, 21). These diverse IGF - I mRNAs eventually give the same mature protein. The biological significance of the diversity of mRNAs, signal peptides, and E domains is not understood.

The ostrich, *Struthio Camelus* (vairietas) *domesticus,* is an important protein resource, but we have no information about the IGF - I gene structure or amino acid sequence of this animal. We cloned a full length cDNA molecule encoding ostrich IGF - I (oIGF - I), and report here the nucleotide sequence of that cDNA and the deduced primary structure of the precursor protein molecule.

Materials and Methods

Ostrich liver. A 60 day-old ostrich, *Struthio Camelus* (vairietas) *domesticus,* was supplied by Mr. N. Himura, Osaka, Japan. The ostrich was dissected and the liver was isolated by Dr. Y. Karasawa (a professor at the Shinshu University). The isolated liver was stored in a frozen state at -80℃ until used.

Polymerase chain reaction (PCR). For the screening of the 5' terminal region and 3' terminal region of oIGF - I cDNA, PCR was performed at 94℃/30 s, 55 ℃/30 s, and 72℃/60 s for a total of 30 cycles using ExTaq (Takara Shuzo Co.) according to the supplier's manual. For the screening of the full length oIGF - I cDNA, PCR was performed at 94℃/30 s, and 72℃/40 s for a total of 30 cycles using LA Taq (Takara Shuzo Co.) according to the supplier's instructions.

PCR primers. Primer U 1 (22 bp: CCT CAG TGG ATG TTG CCT TTA C) and U 2 (20 bp: GCC TGT ACG GAA GTG TTA CT) correspond to nucleotide sequences 5091-5112 (forward) and 5119-5138 (forward), respectively, both of which are upstream of the *Bgl*II site (5220), of the cloning vector pAP3 sequence (GenBank Accession AB003468).
Primer L 1 (20 bp: CTG GTT CTT TCC GCC TCA GA) and T 3 (20 bp : ATT AAC CCT CAC TAA AGG GC) correlate to nucleotide sequences 8 - 37 (reverse) and 5301-5320 (reverse), respectively, both of which are downstream of the *Not* I site (5295), of the pAP3neo sequence.
Primers F 1, F 2, F 3, R 1, and R 4 are primers corresponding to amino acid sequences of chicken IGF - I precursor which are highly conserved among chicken, human, mouse, and goat IGF-Is. Primers F 1 (20 bp: GGT KGA YGC TCT TCA GTT CG), F 2 (20 bp : GAT GAG TGY TGC TTC CRG AG), and F 3 (20 bp : CTR AGG AGR CTG GAG ATG TA), correspond to amino acids 58-64 (forward), 93-99 (forward), and 102-108 (forward) of the chicken IGF - I precursor, respectively, which are highly conserved between chicken and turkey.

Primers R 1 (20 bp: TCC TGH RYT YCC TCT ACT TG) and R 4 (20 bp: CGA ACT GAA GAG CRT CMA CC) are synthetic nucleotides correspond to amino acids 141-147 (reverse), and 58-65 (reverse) of chicken IGF - I precursor, respectively, which are highly conserved between chicken and turkey.
Forward primer, IGF - I 5' primer (25 bp : ATA GAG CCT GCG CAA TGG AA T AAA G), was synthesized based on the obtained sequence of the 5' terminal region of the oIGF - I cDNA, and reverse primer, IGF - I 3' primer (25 bp : AAA CTC TGG GCT GGT TAA AAC GTT C), was synthesized based on the 3' terminal region of the oIGF - I cDNA.

DNA sequencing. DNA sequencing was accomplished using an ABI PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems) with universal primer U19 (Novagen) and RV-M(Takara Shuzo Co.), and the products were analyzed on a Genetic Analyzer System model 373 (Perkin Elmer).

Results and discussion

Total RNA of ostrich liver was prepared by the acid guanidium-phenol-chloroform (AGPC) method (29). Poly(A)RNA was isolated by passing the total RNA solution through an oligotex(dT) 30-super column (Takara Shuzo Co.). First strand synthesis of cDNA was carried out with 240 μg of the poly(A) mRNA using Superscript Reverse Transcriptase II and RAV-2 reverse transcriptase (Takara Shuzo Co.) with an oligo(dT) 18-linker primer having the *Not*I restriction site. Second strand synthesis was catalyzed using *E. coli* DNA polymerase I in combination with *E. coli* RNase H, *E. coli* DNA ligase, and T 4 phage DNA polymerase. Blunt-end synthesis of the cDNA molecule was done using T 4 phage DNA polymerase. The blunt-end of the cDNA was converted to a terminus that contains a 5' extension by adding a *Bam*HI-*Sma*I adapter (Takara Shuzo Co.). The obtained product was digested with restriction enzyme *Not*I, and ligated with a plasmid vector, pAP3neo, and digested with restriction enzyme *Bgl*II and *Not*I to construct the ostrich liver cDNA library.

To screen the 5' terminal region of oIGF - I cDNA, we subjected the ostrich liver cDNA library to two rounds of nested PCR with U 1, U 2, R 1, and R 4 vector primers, as summarized in Fig. 1. Forward primers U 1 and U 2 are vector primers annealing to upstream of the cloning site of pAP3neo, while reverse primers R 1 and R 4 are primers corresponding to amino acid sequences of chicken IGF - I which are highly conserved between chickens and turkeys, as described in MATERIALS AND METHODS. For primary PCR, the diluted ligation mixture for the construction of the ostrich liver cDNA library was amplified with the outer primer set, U 1 and U 2 at concentrations of 0.2 μM. An aliquot of the primary PCR reaction product was then reamplified with the inner primer set, U 2 and R 4. The expected size PCR products were detected as shown in Fig. 2 (A).

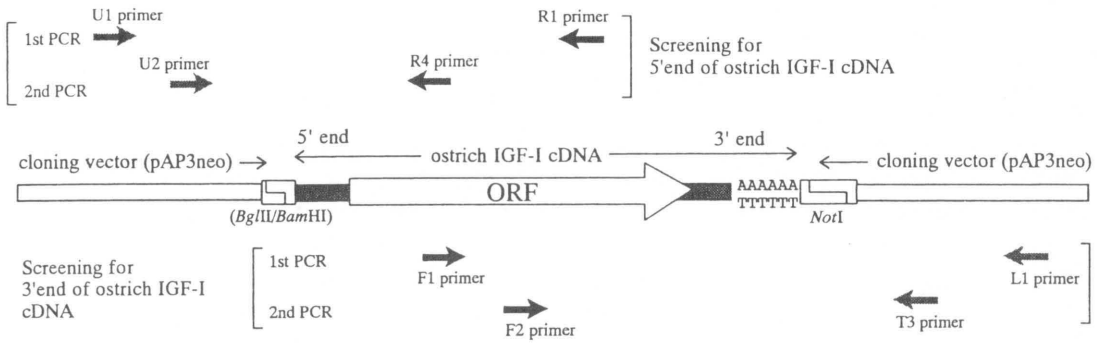

Fig. 1 Schematic representation of primer positions and the nested PCR strategy for screening the 5' terminal and 3' terminal regions of oIGF-I cDNA

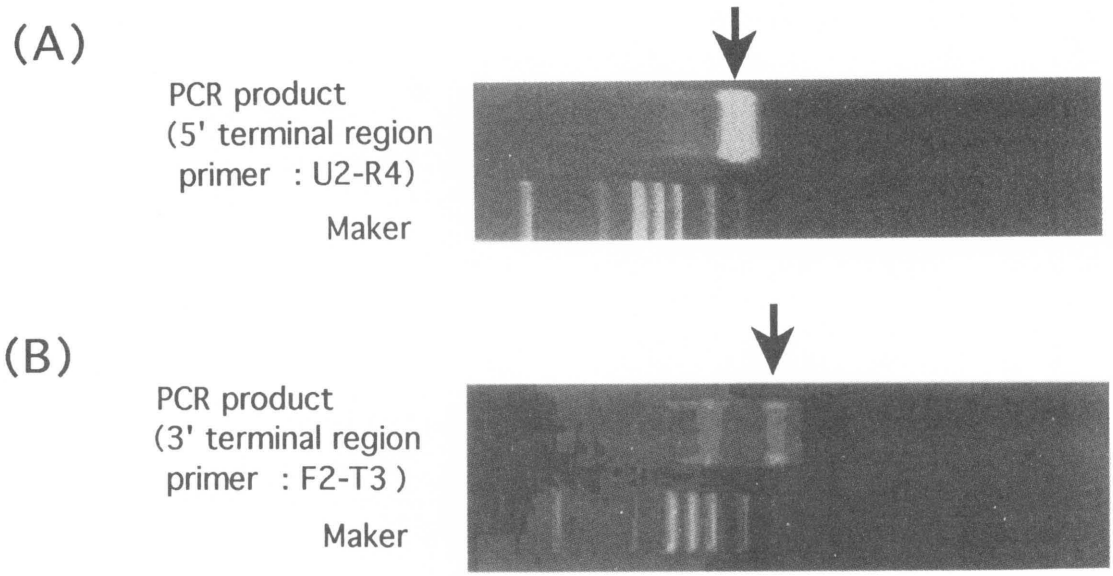

Fig. 2 Gel electrophoresis of nested PCR products. The expected size PCR products from the 5' terminal region of oIGF-I cDNA (A), and those from the 3' terminal region (B) as indicated by the arrows.

To screen the 3' terminal region of oIGF-I cDNA, we subjected the ostrich liver cDNA library to two rounds of nested PCR with L1, T3, F1, and F2 primers as summarized in Fig. 1. Forward primers F1 and F2 are primers corresponding to amino acid sequences of chicken IGF-I which are highly conserved between chickens and turkeys, while reverse primers L1 and T3 are vector primers annealing to down stream of the cloning site of pAP3neo, as described in MATERIALS AND METHODS. For primary PCR, the diluted ligation mixture used for construction of the ostrich liver cDNA library was amplified with the outer primer set, L1 and F1 at concentrations of 0.2 μM. An aliquot of the primary PCR reaction product was then reamplified with the inner primer set, T3 and F2. The expected size PCR products were detected as shown in Fig. 2 (B). The amplified DNA fragments removed from the electrophoresis gels were inserted into a PCR product insertion site of pT7BlueT vector (Novagen), and the sequences of the fragments were determined.

For PCR screening of the full length oIGF-I cDNA, a forward primer, IGF-I 5' primer, and a reverse primer, IGF-I 3' primer, were synthesized. IGF-I 5' primer was synthesized based on the 5'-end 25-bp sequence of the cloned 5' terminal region of IGF-I cDNA, and IGF-I 3' primer was synthesized based on the 3'-end 25-bp sequence of the cloned 3' terminal region of the cDNA. The diluted ligation mixture used for the construction of the ostrich liver cDNA library was amplified with these primers at a concentrations of 0.2 μM. The PCR product was subjected to 1.0% agarose gel electrophoresis, and an amplified band corresponding to about 800 bp was detected. DNA was removed from the gel and was cloned to the PCR product insertion site of pT7BlueT vector. The base sequence of the above obtained DNA fragment corresponding to a full length oIGF-I cDNA was determined by using F1, F2, R1, R4, U19mer primer (Novagen) and RV-M primer (Takara Shuzo Co.), as summarized in Fig. 3.

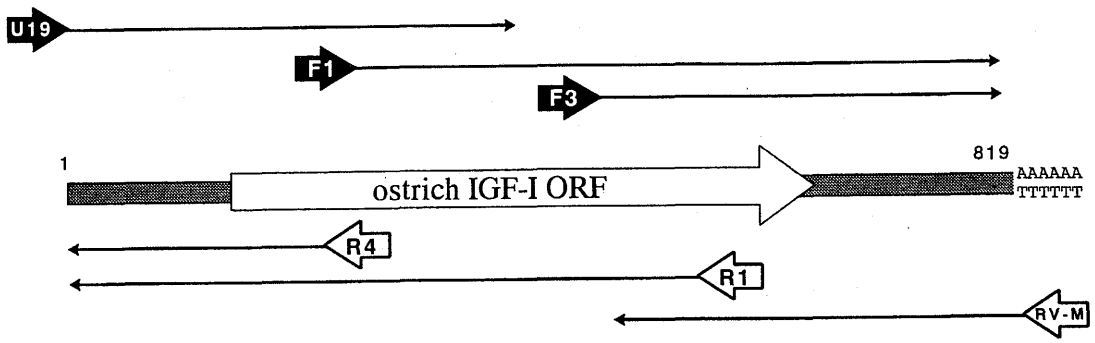

Fig. 3 Schematic representation of primer positions and the sequencing of full length oIGF-I cDNA

It was found that the isolated cDNA fragment contains 819 bp (Fig. 4A). The cDNA has an open reading frame of 459 bp encoding oIGF - I precursor protein, and transcription starts from ATG at position 140-142 and terminates at the termination codon TAA at position 600-602 of the isolated cDNA. It was predicted that the oIGF - I precursor is 153-amino acid residues consisting of a 48-amino acid signal peptide, a 70-amino acid mature IGF - I peptide, and a 35-amino acid E domain which is a COOH-terminal peptide and removed post-translationally, as shown in Fig. 4 B. By sequence analysis, the oIGF - I precursor protein exhibited significant homology with chicken and turkey IGF - I precursors. The homology scores were 100% and 99% at the deduced amino acid level, respectively. The oIGF - I precursor is also highly homologous to the IGF - I precursors of human (84%) (14), bovine (84%) (22), goat (83%) (24), and rat (79%) (15).

(A)

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        10         20         30         40         50         60
ATAGAGCCTG CGCAATGGAA TAAAGTCCTC AATATTGAAA TGTGACATTG CTCTCAACAT
        70         80         90        100        110        120
CTCACATCTC TCTGGATTTC TTTTTTCTCA TCATTACTGC TAACAAATTC ATTTCCAGAC
       130        140        150        160        170        180
TTTGCACTTT TAAGAAGCAA TGGAAAAAAT CAACAGTCTT TCAACACAAT TAGTTAAGTG
       190        200        210        220        230        240
CTGCTTTTGT GATTTCTTGA AGGTGAAGAT GCACACTGTG TCCTACATTC ATTTCTTCTA
       250        260        270        280        290        300
CCTTGGCCTG TGTTTGCTTA CCTTAACCAG TTCTGCTGCT GCCGGCCCAG AGACGCTCTG
       310        320        330        340        350        360
TGGTGCTGAG CTGGTTGATG CTCTTCAGTT CGTCTGTGGA GACAGGGGCT TTTACTTCAG
       370        380        390        400        410        420
TAAGCCTACA GGGTATGGAT CCAGCAGTAG ACGCTTACAC CACAAGGGAA TAGTGGATGA
       430        440        450        460        470        480
ATGCTGCTTC CAAAGCTGTG ACCTGAGGAG GCTGGAGATG TATTGTGCTC CAATCAAGCC
       490        500        510        520        530        540
ACCTAAATCT GCACGCTCTG TACGTGCTCA GCGCCACACT GATATGCCAA AAGCACAAAA
       550        560        570        580        590        600
GGAAGTGCAT TTGAAGAATA CAAGTAGAGG GAACACAGGA AACAGAAACT ACAGAATGTA
       610        620        630        640        650        660
AGAACATGCC ATCCACAAGA GTGAAGAATG AATGTGCCAT CTGCAGGATA CTTTGCTGTA
       670        680        690        700        710        720
AATAAATTAT TTGTTAAACA TTGGAAGACT TAAAAAAAAC TATGGTTTAG TAAGCTTGAT
       730        740        750        760        770        780
GCAATCTCAA CCAATGGGCA TTCTCCCAGT GAACAATGCA ACTAAACATT CCAATATTAA
       790        800        810
GTCTTTAGAG AATAGAACGT TTTAACCAGC CCAGAGTTT
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(B)

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        10         20         30         40         50         60
MEKINSLSTQ LVKCCFCDFL KVKMHTVSYI HFFYLGLCLL TLTSSAAAGP ETLCGAELVD
        70         80         90        100        110        120
ALQFVCGDRG FYFSKPTGYG SSSRRLHHKG IVDECCFQSC DLRRLEMYCA PIKPPKSARS
       130        140        150
VRAQRHTDMP KAQKEVHLKN TSRGNTGNRN YRM
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Fig. 4 (A) cDNA sequence of oIGF - I cDNA.
(B) Deduced amino acid sequence of oIGF - I cDNA

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和文抄録

ダチョウインスリン様成長因子-Iをコードするc DNAのクローン化と塩基配列の決定

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ダチョウ（*Struthio Camelus* (varietas) *domesticus*）のインスリン様成長因子-I（oIGF-I）をコードするcDNAをダチョウ肝臓cDNAより単離した。単離したcDNAは459塩基対のコーデイング領域を含む819塩基対からなっていた。この領域は48アミノ酸残基よりなるシグナルペプチド、70アミノ酸残基よりなる完全長のインスリン様成長因子-I、及び35アミノ酸残基よりなるEドメーンのポリペプチドに相当していた。ダチョウのインスリン様生長因子-Iのアミノ酸配列は、ニワトリおよびシチメンチョウのインスリン様生長因子-Iのアミノ酸配列とは非常に高い相同性（～99%）を示し、またマウス、ウシ、ヤギ、およびヒトのインスリン様生長因子-Iのアミノ酸配列とも比較的高い相同性（84-79%）を示した。