STRUCTURAL ANALYSIS AND EXPRESSION OF THE GENES FOR GOAT GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I

Author(s)
Mikawa, Satoshi

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Kyoto University (京都大学)

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GROWTH FACTOR-I

SATOSHI MIKAWA
1995
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>dCTP</td>
<td>deoxycytidine 5'-triphosphate</td>
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<td>DNA</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>nucleotide</td>
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<td>ORF</td>
<td>open reading frame</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PIPES</td>
<td>piperazine-$N,N'$-bis-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEA</td>
<td>triethanolamine</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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INTRODUCTION

An essential element in the development and functional integrity of all organism is intercellular communication. This is achieved by the secretion of soluble messenger molecules which interact with its receptor on the target cell surface. Hormones are traditionally defined as the messengers synthesized by endocrine glands. Unlike hormones produced by endocrine glands, growth factors are hormone-related substances produced by many tissues and play an important role in controlling growth and development. While the exact physiological roles of growth factors have not yet to be elucidated, they play important roles in the regulation of cellular proliferation and/or differentiation. During recent years there has been a substantial increase in research related to peptide hormones, growth factors, and their receptors. With the discovery and characterization of numerous growth factors, it became clear that these growth factors had multiple features in common with classic hormones.

Growth hormone (GH)

Growth hormone is a peptide hormone, which consists of 191 amino acid residues. GH is produced in anterior pituitary and secreted into blood. The secretion of GH is controlled by two neuropeptides produced in hypothalamus. One is growth hormone releasing factor, which stimulates the secretion of GH from pituitary, the other is somatostatin, which suppresses the secretion of GH. Physiological activities of GH are stimulation of growth, protein assimilation, carbohydrate metabolism, lipid metabolism, electrolyte metabolism, and so on. The most important function of GH is stimulation of growth. Animals, which could not synthesize enough GH, did not grow up normally and intravenously injection of GH could make up for its growth. Over production of GH caused gigantism and acromegaly. Growth is, however, very complicated phenomena induced by the combination of many factors and hormones, and detailed function of GH in growth have not been understood.
The gene and cDNA of the GH receptor were isolated and characterized. But it have been not clear how GH binding to its receptor causes changes in cell. Recently, it was reported that GH receptor binding its ligand associated Jak2 protein. Jak2 is a member of JAK family of PTKs (protein tyrosine kinases), which were discovered by polymerase chain reaction (PCR) using degenerate oligonucleotide primers based on highly conserved catalytic domain motifs of PTKs. JAK family of PTKs have two kinase domains, so JAK is an acronym of both Just Another Kinase and JAnus Kinase (Janus is two faced Roman god). cDNA encoding Jak1, Jak2, and Jak3 were cloned and characterized and were demonstrated to share structural homology with Tyk2. To date, these four proteins belong to JAK family of PTKs.

The receptors of Interferons also associate JAK family of PTKs. The Interferons (IFNs) are responsible for a diverse range of biological actions, such as inhibition of virus proliferation, inhibition of tumor proliferation, strengthen of natural killer cell activity. Stimulation of cells by IFNs leads the activation of latent cytoplasmic transcription factors that translocate to the nuclei and bind specific sequences located in the promoters of various IFN-inducible genes. For example IFN-α binding to the IFN-α receptor leads to activation by tyrosine phosphorylation and formation of the latent cytoplasmic transcription factor Interferon Stimulated Gene Factor 3α (ISGF3α). ISGF3α then complexes with a second component, a myb-related DNA-binding protein ISGF3γ (p48). This activated transcription complex then translocates to the nuclei where it binds to an Interferon Stimulated Response Element (ISRE). ISGF3α complex consists of three distinct polypeptides-p113, p91 and p84. These polypeptide have recently become the subject of an attempt to rationalize their nomenclature and are now known respectively as STAT2, STAT1α, and STAT1β (Signal Transducer and Activators of Transcription). Interestingly, STAT proteins contain both SH2 and SH3 domains (Src Homology domain). STAT1α and STAT1β are splicing variants of the same gene product, with STAT1α having a 38-amino acid C-terminal extension. It now appears that this 38-amino acid tail is essential for DNA binding. While STAT1β is capable of replacing STAT1α in a mutant cell line deficient in STAT1α, it is able to do so only up to the point of DNA transcription. STAT1β is incapable of binding DNA to activate transcription.

GH binding to its receptor leads to activation of tyrosine kinase of Jak2 and phosphorylation of cellular proteins with molecular masses of 93 kDa, 91 kDa, and 84 kDa. These phosphorylated proteins assembled and translocated from cytoplasm to nucleus. These proteins activated by GH are antigenically similar STAT1α, and GH signaling and IFNs signaling are thought to be very similar and it is possible that they share the common proteins for signaling.

Insulin-like growth factor-1 (IGF-I)

IGFs were originally recognized as GH dependent serum factors termed somatomedins, which mediated GH action on cartilage in vitro. While the source of somatomedins was at first unknown, liver was widely suspected. In 1970, it was reported that activities of somatomedins released from hypophysectomized rat liver were increased by injection of GH. This finding has been confirmed by studies with isolated hepatocyte. It was also reported that human serum contains an insulin-like activity not suppressible by insulin antibodies (NSILA). Purified preparation of NSILA mimics most effects of insulin on adipose tissue and muscle in vitro and in vivo. In addition, NSILA had growth-promoting properties for cartilage and fibroblasts in vitro. In 1976, two forms of NSILA (NSILA-I and II) were isolated from human serum and characterized as single-chain polypeptide with an approximate molecular weight of 6,000. Amino acid sequences of NSILA were analyzed and found that their structures were similar to insulin. The term NSILA was replaced by the more general term insulin-like growth factor, IGF.
stimulated by GH. This has led to the view that GH action on tissues is exerted by stimulating local production of IGF-I which acts in an autocrine/paracrine mode.

Biological action of IGF-I is initiated by its binding to the receptors on the cell surface. The IGF-I receptor shares a large degree of structure as well as functional properties with the insulin receptor. Both receptors are composed of two α subunits (Mr ~135,000) and two β subunits (Mr ~95,000) disulfide-linked to an α2β2 hetero tetrameric complex. The IGF-I and insulin receptor α subunits reside exclusively on the extracellular face of the plasma membrane and anchored to the plasma membrane by disulfide linkages to the extracellular domain of the transmembrane β subunits. In analogy with the low density lipoprotein receptor, the α subunits of the IGF-I and insulin receptors contain a highly related N-terminal cysteine rich domain thought to be the high affinity ligand binding domain. The intracellular region of the β subunits contains the ATP binding domain, the intrinsic tyrosine-specific protein kinase domain, tyrosine-specific autophosphorylation acceptor sites, and serine and threonine phosphorylation acceptor sites for other endogenous protein kinases.

Binding of insulin and IGF-I to their receptors stimulates β-subunit tyrosine kinase activity, leading to receptor autophosphorylation and tyrosine phosphorylation of several cellular substrates. Insulin receptor substrate-1 (IRS-1) is the major target for the insulin and IGF-I receptor tyrosine kinases and is tyrosine-phosphorylated on multiple sites by both receptors. It has been proposed that the tyrosine-phosphorylated form of IRS-1 acts as a docking protein by associating certain proteins containing SH2 domains. Indeed, after insulin and IGF-I receptor activation, the tyrosine-phosphorylated form of IRS-1 binds the 85 kDa regulatory subunit of the phosphatidylinositol 3'-kinase and this interaction results in the activation of the enzyme. More recently, it has been shown that IRS-1 can also interact with other signaling proteins via their SH2 domains such as the GRB2-SOS complex and the SH2 containing tyrosine phosphatase (Syp). SOS is a guanine nucleotide exchange factor of low molecular G proteins and activated by binding of the GRB2-SOS complex to IRS-1. SOS activates Ras by converting Ras from GDP-binding form to GTP-binding form. GTP-binding Ras triggers the activation of MAP kinase cascade (Raf-1, MAP kinase kinase, MAP kinase, and RSK), leading to phosphorylation and activation of nuclear transcription factors. Thus, insulin and IGF-I progress the cell cycle.

References
Chapter I. Structure of the Locus for Goat Growth Hormone Genes

Growth hormone (GH) is a peptide hormone and is required for the growth of animals. GH is produced in pituitary by the stimulation of growth hormone releasing factor from hypothalamus. GH stimulates insulin-like growth factor-I (IGF-I) transcription in many tissues and IGF-I mediates the functions of GH, such as growth of bone and proliferation of cells. GH belongs to a family of hormones with chorionic-somatotropin (CS) and prolactin (PrL), and they evolved from a common ancestral gene. In the case of human growth hormone gene family, the GH and CS genes are more closely related to each other than to the PrL gene. The GH and CS genes are located on the chromosome 17 and the PrL gene is located on the chromosome 6. From these data, it is postulated that the primordial gene was duplicated giving rise to two separate precursors, one for the GH and CS genes and the other for the PrL gene. The GH and CS genes are located on the chromosome 17 and the PrL gene is located on the chromosome 6. From these data, it is postulated that the primordial gene was duplicated giving rise to two separate precursors, one for the GH and CS genes and the other for the PrL gene. The chromosomal locus of the GH and CS genes is 5'--(GH-1)-(CS-5)-(CS-1)-(GH-2)-(CS-2)-3' in the region spanning about 48 kbp of DNA. It is thought that the locus of GH and CS genes was formed by the gene duplications. Though the instability of GH and CS genes is not known, high number of Alu family and several large direct repeats are thought to participate in the gene duplications. In the case of various mammalian species, such as rat, bovine, and porcine, except for human only a single copy of the GH gene, and no other closely related sequences were reported. In this chapter, the author shows the mapping and nucleotide sequences of the goat GH genes and repetitive sequences flanking of them. The author also shows the polymorphism of the GH genes arrangement in goat.

Materials and Methods

Materials

[a-32P]dCTP was purchased from Amersham. Restriction endonucleases were from Takara. Cellulose nitrate membrane (BA85) was from Schleicher & Schuell.

Construction of the goat genomic DNA library

High molecular weight DNA was extracted from the spleen of the goat (Capra hircus) by the method of Blin and Stafford. This DNA was completely digested with the restriction endonuclease Bgl II, and DNA fragments hybridizable with the goat GH cDNA were recovered from the gel. These fragments were ligated into BamHI "arm" of the cloning vectors Charon28 and EMBL3. The recombinant DNA was packaged in vitro into particles.

Plaque hybridization

Phages of the library were cultured at 10⁵/400 cm² and transferred to cellulose nitrate membrane. The membrane was immersed in denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 1 min, transferred into neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.4]) for 5 min, and rinsed in 2x SSC. The membrane was incubated with the 32P-labeled goat GH cDNA in 5x SSC, 50% formamide, 5x Denhart’s solution, 10% dextran sulfate, and 0.5% SDS for 12 hr at 42°C. The membrane was washed in 2x SSC and 1% SDS for 20 min and exposed X-ray film for 12 hr.

Southern hybridization

DNA fragments separated by agarose gel electrophoresis were transferred to cellulose nitrate membrane by a capillary transfer method and hybridized with the 32P-labeled goat GH cDNA described as plaque hybridization.
DNA sequence analysis

Overlapping DNA fragments containing a part of the goat GH genes and the repetitive sequences were subcloned to the M13 vectors. Sequencing was carried out by the dideoxy chain termination method using 7-DEAZA sequencing kit (Takara).

Results

Cloning of the goat GH genes

To find the GH genes in the goat chromosome, genomic Southern hybridization was done (Fig. 1). The results showed that the goat GH genes was included in around the 20 kbp Bgl II segment, and in the 12 kbp and 5 kbp BamHI segments. The genomic library, Bgl II-cleaved goat genomic DNA cloned into the vector EMBL3, was screened for positive clones by plaque hybridization using 32P-labeled goat GH cDNA as a probe. Six positive clones were isolated out of 6 x 10^5 independent plaques. Based on the restriction enzyme mapping, two kinds of clones were obtained. One (CgGH) was the same clone as one isolated previously from the library using Charon 28 as a vector. The other (EgGH) 21 kbp in length, was about 5 kbp longer than CgGH (Fig. 2). The results of Southern hybridization and nucleotide sequence analysis showed that CgGH contained the gGH1 gene, and EgGH contained the gGH2 and gGH3 genes. EgGH contained 491 bp inserted sequence, just upstream of the gGH3 gene, which was not present in the clone CgGH.

Fig. 1. Detection of GH Sequences in Total Goat DNA. High molecular weight DNA was digested with restriction endonucleases: (A) Bgl II; (B) BamHI. GH specific fragments were detected by Southern hybridization with the goat GH cDNA as a probe.

Fig. 2. Restriction Map of CgGH and EgGH Clones that Contain the goat GH Genes. Filled boxes indicate the goat GH genes (gGH1, gGH2, and gGH3). Arrows indicate the repetitive sequences. Restriction endonuclease cleavage sites are indicated as follows. B, BamHI; E, EcoRI; H, HindIII; K, Kpn I; X, Xho I.
Characterization of repetitive sequences around the Goat GH genes

The author found the repetitive sequences (named as la, lb, lc, and ld) in the flanking regions of the gGH1 gene and defined the consensus sequence (Fig. 3). Based on the consensus sequence, oligonucleotides containing a part of it were synthesized, and were used as the hybridization probes for detecting the repetitive sequences in the restriction fragments of the EgGH DNA. The hybridization and nucleotide sequencing data indicated that there were eight repetitive sequences in the EgGH DNA, and they were named as 2a, 2b, 2c, 2d, x, 3b, 3c, and 3d, as aligned in the order from 5' to 3'. Repetitive sequence x was found in the 491 bp insert (Fig. 2).

Fig. 3. Comparison of the Repetitive Sequences (la, lb, lc, and ld). The nucleotide sequences are aligned by the introduction of gaps to maximize the homology. The consensus sequence of the repetitive sequences are shown in the bottom line. R indicates the purin nucleotide, Y indicates the pyrimidine nucleotide and N indicates the nucleotide R or Y. The tandem repeats of AACTG or A rich sequences at 3' end are indicated by double underlines. Terminal direct repeats at 3' and 5' end are indicated by italic. Nucleotide sequences of the synthetic probes for hybridization with the subfragments of EgGH are indicated by underlines in the lc and x sequence.

Analysis of the 491 bp inserted fragment

Comparison of the restriction maps of CgGH and EgGH showed that EgGH has duplicated GH genes (gGH2 and gGH3) and has the 491 bp fragment, which was not present in CgGH (Fig. 2). The author found repetitive sequence x and TATA box, CAAT box, and small ORF in this insert (Fig. 4).

Fig. 4. The Nucleotide Sequence between the BamHI Site and the HindIII Site of EgGH. The 491 bp inserted sequence is indicated by italic. The repetitive sequences x and 2d are underlined. One of the candidates for the transcription start site of the gGH3 is indicated by +1. The 491 inserted sequence is located at 24 bp upstream of the gGH3 gene. The consensus sequence of TATA box and CAAT box is found in the inserted sequence, but it is uncertain if these sequences are function for the transcription of any gene, or not.
Detection of the Polymorphism in Arrangement of the Goat GH Genes. High molecular weight DNA was prepared from white blood cells of a pair of goat and their three children, and the DNA was digested with the restriction endonuclease BamHI. The intensity of the bands corresponding to the 12 and 5 kbp fragments is almost the same in goat number 5. The 5 kbp fragment is stronger than the 12 kbp band in the others.

Arrangement of the GH genes among individual goats

To examine the differences in the arrangements of the GH genes among individual goats, genomic Southern hybridization was done using goat GH cDNA as a probe. The genomic DNA was prepared from white blood cells of five goats, a couple of goats and their children, and the DNA was digested with the restriction endonuclease BamHI. The author could detect the 5 kbp fragment originating from the EgGH fragment and 12 kbp fragment from the CgGH fragment in every goats. Intensity of the bands corresponding to these fragments differed among the individual goats (Fig. 5).

Discussion

The copy number of the GH gene or other closely related sequences have been discussed on the basis of the genomic Southern analysis for many animals. In the case of the rat genome, the GH gene was found in the 11 kbp EcoRI fragment. It was reported that porcine, bovine, and rat genomes contained a single copy of the GH sequence, but human genome contained two copies of the GH gene. In bovine, Gordon et al. reported there were two EcoRI fragments, 8.4 kbp and 4.3 kbp, hybridizable with the GH cDNA. Woychik et al. reported there were two EcoRI fragments, 11 kbp and 4.3 kbp, hybridizable with the GH cDNA. They found the GH gene in the 4.3 kbp fragment, and the nucleotide sequence of the GH gene was determined.

The nucleotide sequence of the gGH1 gene is homologous with that of the bovine GH gene. In goat, two BamHI fragment of 12 kbp and 5 kbp contained the goat GH genes, while in bovine only a single BamHI fragment of 12 kbp contained the GH gene. To identify the arrangements of the GH genes in the goat genome, the author cloned the Bgl II fragments, which contained GH genes, in λ cloning vectors and determined the restriction maps of these fragments. The author got two clones that contained the 21 kbp and 16 kbp inserts. The 16 kbp fragment contained the gGH1 gene and 21 kbp fragment contained the gGH2 and gGH3 genes. These genes were hybridizable with the goat GH cDNA, and the nucleotides of them were sequenced.
The nucleotide sequences of the promoter regions of these genes were compared with one another. Although the gGH1 and gGH2 had very similar nucleotide sequences including the TATA sequence, the gGH3 had very different nucleotide sequence (Fig. 4). This result indicates that the gGH1 and gGH2 may be functional genes. Judging from the data of primer extension experiment, the mRNAs of the goat GH genes were transcribed from three sites, cytidine, adenine and guanine (data not shown). The adenine residue was the same as the transcription start site of bovine GH.\(^7\)

As the author got two types of clones, one containing the gGH1 and the other containing the gGH2 and gGH3, the author investigated the presence of these GH genes of individual goats. When the Southern hybridization analysis was done using BamHI digested goat chromosomal DNA, differences in the intensity were found between the bands corresponding to the 12 kbp and 5 kbp fragments. These results suggest that the individual goats have the gGH1, gGH2, and gGH3 with different copy numbers, and CgGH and EgGH DNA segments may be allelic on the goat chromosome.

The author did a Southern hybridization experiment, using the synthetic oligodeoxynucleotides as probes, and 12 repetitive sequences were found in the flanking regions of the GH genes. The consensus sequence of the repetitive sequences was compared with that of the bovine proopiomelanocortin (POMC) and goat \(\beta\)-globin genes (Fig. 6). These sequences may belong to the same family of the repetitive sequences. Watanabe et al. found out about \(10^5\) copies of the repetitive sequences in the bovine genome.\(^15\) These repetitive sequences may be widely distributed in the genomes of these animals. The repetitive sequences found in the present study had the tandem repeats of AACTG sequences of A rich sequences at the 3' end and some repetitive sequences were flanked by direct terminal repeat at the 5' and 3' ends. This is the common feature of the Alu family sequences. In human, two GH genes and three CS genes existed in the segment spanning about 48 kbp of DNA, and there were many Alu family sequences around these genes. On the basis of the restriction endonuclease mapping, Barsh et al. proposed a hypothesis for the evolution of the human GH and CS locus, involving the gene duplication mediated by the recombination between the Alu sequences. Recently, Hirt et al. also proposed the mechanism of the evolution of the human GH and CS locus via repetitive sequences by Southern hybridization using human Alu sequence as a probe.

The nucleotide sequence between the HindIII site and the BamHI site upstream of the gGH3 was determined. Judging from the sequence, it was evident that 491-nt long DNA segment was inserted just 24 bp upstream of the putative transcription start site of the gGH3 gene. This DNA segment contained a repetitive sequence x. Though the author could not have the evidence for the gene duplication mechanism for the GH genes, it is conceivable that the gene duplication occurred first, followed by insertion or deletion of DNA segments. The repetitive sequences found in the present work might be involved in these process in some manner.

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![Fig. 6. Comparison of the Consensus Repetitive Sequences in the Regions Adjacent to the Goat GH, Bovine POMC, and Goat \(\beta\)-Globin Genes. The nucleotide sequences were aligned by the introduction of gaps to maximize the homology. Asterisks indicate the nucleotides identical to those of the goat GH genes.](image-url)
Chapter II.
Structure of Goat Insulin-like Growth Factor-I Gene and its Multiple Transcripts

Insulin-like growth factors IGF-I and II are polypeptide hormones structurally homologous to proinsulin. Their precursors consist of a signal peptide, a mature protein, and an E domain, which is removed post-translationally. The two growth factors share the functions of regulation of development and somatic growth, but IGF-I acts postnatally and IGF-II acts prenatally.1-10) IGF-I is synthesized in a variety of tissues and the transcription of its gene is regulated by growth hormone (GH). The biological actions of IGF-I begin by interaction with its cell-surface receptor, which is a ligand-activated tyrosine-specific protein kinase with structural and functional similarities to the insulin receptor.11) IGF-I can bind the insulin receptor also, but the affinity between them is about one-hundredth that between IGF-I and the IGF-I receptor.

The genes and cDNA coding for IGF-I of some animals have been isolated (Fig. 1). The rat IGF-1 gene consists of six exons and five introns, and is more than 80 kbp long.12) Many kinds of mature mRNA are synthesized from the IGF-I gene through multiple polyadenylation sites and alternative splicing. In rat13-15) and human,16-18) because of two leader exons (1 and 2), there are two kinds of IGF-I mRNAs: classes 1 and 2. There is the third kind of mRNA in rat: class 1del., which lacks the central region of exon 1 from class 1 mRNA.19,20) There are two kinds of E domains. In rat, the Ea is encoded by exons 4 and 6, and the Eb is encoded by exons 4, 5, and 6.21) In human, the Ea is encoded by exons 3 and 4, and the Eb is encoded by exons 3 and 5.18,22) In ovine, another leader exon (exon 1W) was found, resulting in class 1W mRNA, but exon 5 was not found.23) The IGF-I precursors encoded by the multiple mRNAs differ in their signal peptide and E domain but are processed to become the same mature IGF-I. The biological significance of the diversity in the signal peptides and E domains is unknown, but perhaps the
secretion of IGF-I is regulated in different ways specified by the different signal peptides or by differences in the processing of the IGF-I precursor. In this chapter, the author shows the structure of the goat IGF-I gene and its multiple transcripts.

![Illustration of gene structure](image)

**Fig. 1. Structure of the Human, Rat, and Ovine IGF-I Genes.** Introns are represented by lines and exons by boxes. Solid boxes correspond to the open reading frames encoding the IGF-I precursors. The hatched area of the rat IGF-I gene indicates the sequence that is spliced out, giving the class 1del. mRNA.

**Materials and Methods**

**Materials**

Reverse transcriptase from moloney murine leukemia virus (MMLV-RT) was purchased from Gibco BRL. Ribonuclease inhibitor RNasin was from Promega. Taq DNA polymerase was from Takara. Restriction endonucleases were from Takara and New England Biolabs. [\(\gamma\)-\(^{32}\)P]ATP was from Amersham.

**Animals**

The liver and spleen were isolated from a female Shiba goat (*Capra hircus*), 3 years old.

**Isolation of the goat IGF-I cDNA by RT-PCR**

For isolation of the goat IGF-I cDNA, the author prepared primer 5' (ATGTGACATGGCTCTCAGCAT), which was in the 5'-flanking region conserved among the human\(^{22}\) and ovine\(^{23}\) IGF-I cDNAs, and primer 3' (GCAGTGAAGAAGTCAAAAT), which was complementary to the 3'-terminal part of the goat IGF-I cDNA cloned from a goat liver cDNA library (Fig. 2). Total RNA was prepared from the goat liver by the acid-guanidine thiocyanate-phenol-chloroform (AGPC) method.\(^{24}\) One microgram of the total RNA was transcribed into cDNA with 200 units of MMLV-RT in 20 \(\mu\)l of a reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl\(_2\), 0.1 mg/ml BSA, 1 mM each dNTP, 0.5 mM random hexamer, and 20 units of ribonuclease inhibitor RNasin. The cDNA synthesized during 1 hr of incubation at 42°C was used as a template for PCR in a reaction mixture containing 5 units of Taq DNA polymerase, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl\(_2\), 0.1 mg/ml BSA, 0.2 mM each dNTP, and 0.2 mM each of the two primers. After 25 cycles (1 min at 95°C, 1 min at 55°C, and 2 min at 72°C) of the PCR, the mixture was electrophoresed through a 1% agarose gel, and the DNA fragments were isolated and subcloned into pBluescriptII. Sequence analysis was done for twelve clones from three independent RT-PCR.

**Cloning of the goat IGF-I gene**

A genome DNA library was constructed from the goat spleen DNA that had been partially digested by Sau3AI, and the phage vector EMBL3. Clones having segments of the goat IGF-I gene were isolated by plaque hybridization with the goat IGF-I cDNA as a probe. The DNA of the positive clones was digested with restriction endonucleases, and Southern hybridization analysis was done using the goat IGF-I cDNA as the probe.
Detection of additional leader exons

For detection of exons, the author prepared primer 1W (TTTACCCCGT-CGTTTGAGG), primer 1 (AGACTTTGCACTCAGAAGCA), and primer 2 (TGACCTGCTGTAAAGATCT) with sequences found in the regions of the goat IGF-I gene corresponding to exons 1W, 1, and 2 of the ovine IGF-I gene, respectively, and prepared primer 6 (GTGCAGAGCGAAGGATTG), which was complementary to the 20-nt sequence in exon 6 of the goat IGF-I gene. RT-PCR was done using goat liver RNA as described above.

Primer extension

The author prepared primer p1W (AAGACGGCGCTGGGATGACCTCCGTCCTGGTTGCC) complementary to a 36-nt sequence in exon 1W, primer p1 (CGCAGGCTCTATCTGCTCTG) complementary to a 20-nt sequence in exon 1, and primer p2 (TCACCTGTGTAGGTGTAAACC) complementary to a 20-nt sequence in exon 2. Ten picomoles of each synthetic primer were 5' -end labeled with [γ-³²P]ATP. One picomole of a labeled primer and 6 ~g of polyadenylated RNA from goat liver were hybridized to each other for 12 hr at 30°C in 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, and 40% formamide. After precipitation of the nucleic acids with ethanol, chain extension from the 3'-ends of the primers was done with 200 units of MMLV-RT for 2 hr at 37°C in a mixture of 50 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM MgCl₂, 1 mM each dNTP, 1 mM DTT, and 50 mg/ml actinomycin D. After treatment with ribonuclease A, and then with phenol/chloroform (1:1, v/v), the samples were analyzed on a denaturing polyacrylamide gel.

Results

Cloning of the goat IGF-I cDNA

The author isolated the goat IGF-I cDNA from goat liver RNA by RT-PCR. Sequence analysis showed that this cDNA was homologous to the rat class 1 IGF-I cDNA. This cDNA was 969 nucleotides long, and from the open reading frame found in it, the author predicted a 154-amino acid protein consisting of a 49-amino acid signal peptide, a 70-amino acid mature IGF-I peptide, and a 35-amino acid E domain (Fig. 2). Another kind of cDNA was isolated. It was 966 nucleotides long and lacked the trinucleotide

Fig. 2. Nucleotide Sequence of the Goat IGF-I cDNA and the Deduced Amino Acid Sequence. The goat IGF-I cDNA was isolated by RT-PCR as described in Materials and Methods, and its nucleotides were sequenced with an ABI 373A DNA sequencer (Applied Biosystems). The underlined amino acid sequence is the mature IGF-I protein, which consists of the B, C, A, and E domains. The E domain corresponds to a COOH-terminal peptide, which is removed post-translationally. The nucleotide sequences with double underlining are the locations of the primers for RT-PCR. The shorter goat IGF-I cDNA, 966 bp long, lacks the italic trinucleotide (A₁₄₁A₁₄₂G₁₄₃).
C141A142G143, so that it encoded an IGF-I precursor that lacked one amino acid, Gln 22, in its signal peptide.

In the nucleotide sequence of the coding region, the goat IGF-I cDNA was highly homologous (93%) to the human IGF-I cDNA.16-18) Only one amino acid (Thr 67) of the mature IGF-I protein was different from the residues in the same position in human and bovine25) IGF-I (Ala 67).

Cloning of the goat IGF-I gene

Upon screening of 2 x 10^5 clones by plaque hybridization, four positive clones (A.ggiGF 1, 3, 5, and 6) were isolated. Southern hybridization and sequence analysis showed that A.ggiGF 3 contained exons 1 and 3 and regions corresponding to exons 1W and 2 of the ovine IGF-I gene, that A.ggiGF 5 contained exon 4, and that A.ggiGF 1 and 6 contained exon 6 (Fig. 3). Exons 1 and 3 encoded the signal peptide, and exons 3 and 4 encoded the mature IGF-I (B, C, A, and D domains). The E domain was encoded by exons 4 and 6. The trinucleotide C141A142G143 missing in the shorter cDNA was found to be at the 5' splicing site of exon 3. Each exon was numbered as in the human IGF-I gene.

Detection of exons 1W and 2

It has been reported that some mammalian IGF-I genes have multiple leader exons. For example, the human IGF-I gene has two leader exons (1 and 2),22) and the ovine IGF-I gene has three leader exons (1W, 1, and 2).23) The nucleotide sequence of exon 1 was mapped in the goat IGF-I gene, and was homologous to the sequences of the human and ovine IGF-I genes. The author searched for additional leader exons in the goat IGF-I gene by RT-PCR using goat liver RNA as described in Materials and Methods (Fig. 4). When primers 1W and 6 were used for detecting exon 1W, two DNA fragments were amplified. One was 600 bp long and its nucleotide sequence showed that it contained exons 1W, 3, 4, and 6. The other was longer than expected, 680 bp. Sequence analysis showed that this DNA fragment contained the 3'-portion of exon 1 inserted between exons 1W and 3. With primers 1 and 6 designed to detect exon 1, a 545 bp DNA fragment was amplified that contained exons 1, 3, 4, and 6. When primers 2 and 6 were used, a 510 bp DNA fragment was amplified that contained exons 2, 3, 4, and 6. The results showed that the IGF-I gene had three leader exons (1W, 1, and 2, from upstream), and that the gene was transcribed and processed into three kinds of mature mRNAs for the leader exon (classes 1W, 1, and 2). The goat IGF-I gene was transcribed into a fourth kind of mRNA, which had both exon 1W and the 3'-portion of exon 1 (class 1W-1del.; Fig. 4).

Exon 1 of the rat IGF-I gene and exon 1 of the goat IGF-I gene were homologous to each other. Exon 1 of the rat IGF-I gene is transcribed into two kinds of mature mRNAs, one of which contains intact exon 1 (class 1), and the other of which lacks the central region of exon 1 (class 1del.).20) Sequence analysis around the splicing recipient sites in exon 1 of the goat class 1W-1del. cDNA and the rat class 1del. cDNA showed that these splicing recipient sites were in identical regions and were conserved between rat and goat (Fig. 5).
Goat IGF-1 gene

Fig. 4. Detection of Leader Exons. a, RT-PCR was done with total RNA from goat liver for detecting exon 1W (lane 1), exon 1 (lane 2), and exon 2 (lane 3) as described in Materials and Methods. b, Sequence analysis showed that the goat IGF-1 gene had three leader exons (1W, 1, and 2), which were transcribed to three kinds of mRNAs (classes 1W, 1, and 2). The forth kinds of mRNA contained the 3'-portion of exon 1 inserted between exons 1W and 3 (class 1W-1del.). Mature mRNAs are represented by bold lines connected by thin lines. Arrows above exons 1W, 1, 2, and 6 show the position of primers 1W, 1, 2, and 6, respectively.

Fig. 5. Comparison of the Splice Recipient Sites in Exon 1 of the Rat and Goat IGF-1 Genes. Exons are shown by boxes; solid areas are translational regions and hatched areas show the regions spliced out, giving the rat class 1del. and the goat 1W-1del. mRNAs. The nucleotides in the rat class 1del. mRNA and the goat class 1W-1del. mRNA are shown in uppercase letters. The nucleotides that are removed upon splicing are shown in lower-case letters. The nucleotide sequences of these two splice recipient sites indicated by bold letters are conserved.

Mapping of transcriptional initiation sites

To map transcriptional initiation sites for each leader exon, primer extension analysis was done (Fig. 6). With primer p1W, a transcriptional initiation site was mapped 274 bp upstream from the 3'-end of exon 1W (panel a; lane W). With primer p1, major initiation sites were mapped 247, 251, and 253 bp upstream from the 3'-end of exon 1 and there were some minor sites around there (panel b; lane 1). The author could not exclude the possibility that there are other signals because of smearing. With primer p2, initiation sites were mapped 68 and 69 bp upstream from the 3'-end of exon 2 (panel c; lane 2). Upstream from exons 1W, 1, and 2 of the goat IGF-1 gene, CAAT and TATA boxes were not found. Upstream from exon 2, two cAMP response element-like sequences were found. In exons 1W and 1, multiple translation initiation codons (ATG) were found, but were followed immediately by stop codons. The author found that each of exons 1, 2, and 3...
had an ATG codon organizing an open reading frame encoding the IGF-I precursor, but exon 1W did not have it. (Fig. 7).

Discussion

The author isolated two kinds of class 1 IGF-I cDNAs from goat liver RNA, which differ from each other only in the trinucleotide C141A142G143. This trinucleotide is not present in the human,16-18 rat,14,21 mouse,26 and swine27) IGF-I cDNAs. Sequence analysis of the goat IGF-I gene showed that the trinucleotide was at the 5'-splicing site of exon 3. The nucleotide

Fig. 6. Mapping of Transcriptional Initiation Sites of Exons 1W, 1, and 2. Primer extension analysis was done with 6 μg of poly(A) RNA from goat liver and with primers p1W (panel a), p1 (panel b), and p2 (panel c). The products of primer extension were separated by electrophoresis through a 6% polyacrylamide gel containing 50% urea, and used to expose X-ray film for 2 days.

Fig. 7. Nucleotide Sequence of the Goat IGF-I Gene. Nucleotide sequences of the exons are shown in upper-case letters and those of the introns in lower-case letters. Underlining indicates the location of the primers for RT-PCR and primer extension analysis. Dots above the nucleotide sequences of exons 1W, 1, and 2 indicate the transcriptional initiation sites found by primer extension analysis. Asterisks show the nucleotides corresponding to transcriptional initiation sites of exons 1 and 2 of the ovine IGF-I gene reported by Ohlsen et al. Wavy lines show the regions corresponding to those in which transcriptions start in human and rat. The italic trinucleotide (CAG) at the beginning of exon 3 is missing from the goat IGF-I cDNA that is 966 bp long. Doubly underlined sequences are mentioned in the text.
sequence including the splicing site was CAGCAGGTGAAG, and the two 5'-splicing sites of exon 3 were cagCAGGTGAAG and cagcagGTGAAG. In ovine, the nucleotide sequence of this region is conserved as reported by Wong et al.\textsuperscript{28} and Ohlsen et al.\textsuperscript{29} The ovine class 1 and 2 cDNAs have this trinucleotide, but the ovine class 1W cDNA does not. The author also detected two kinds of goat class 2 mRNAs, in which one had this trinucleotide, but the other did not, in goat liver RNA (data not shown). However it is not known whether splicing of exon 3 is regulated differently for tissues or for each class of cDNA.

Results of RT-PCR using goat liver RNA showed that the goat IGF-I gene had three leader exons (1W, 1, and 2), and that they resulted in four kinds of mature mRNAs (classes 1W, 1W-1del., 1, and 2) in liver. A preliminary study with RT-PCR showed that class 1 mRNA was expressed in various tissues, and that class 1W, 1W-1del., and 2 mRNAs could be detected only in liver, uterus, and ovary (data not shown). In exons 1W and 1, the author found multiple translation initiation codons (ATG) followed immediately by stop codons. These regions may help to regulate translation. The translational initiation sites of the four kinds of goat mature IGF-I mRNAs were not located, but the author found that each of exons 1, 2, and 3 had an ATG codon organizing an open reading frame encoding the IGF-I precursor (Fig. 7). A translational initiation codon was not found in exon 1W, so translation of the class 1W mRNA may start at an ATG in exon 3. In this case, the putative signal peptide consists of 25 amino acid residues. It is possible that the class 1W mRNA is not translated at all. In the class 1W-1del. and 1 cDNAs, the putative translational initiation codons, found in exon 1, were identical. The signal peptides of the IGF-I precursors encoded by the class 1W-1del. and 1 mRNAs were 49 amino acid residues long. In exon 2 of the class 2 cDNA, the author found an ATG leading to a signal peptide 33 amino acid residues long. If all the four kinds of mRNAs are translated, there are three kinds of IGF-I precursors, which differ from one another only in their signal peptides. Activity of promoters upstream of each leader exon may determine the abundance of each type of the signal peptide in cell. Moreover, for transcripts containing exon 1W, post-transcriptional processing may participate in selection of types of the signal peptides.

The amino acid sequences were highly homologous among human, bovine, and goat mature IGF-I. Only the 67th amino acid in the mature IGF-I was different (threonine in goat IGF-I and alanine in human and cattle). The conserved structure of the mature IGF-I may be functionally advantageous for interaction with various factors (IGF binding proteins and receptors). The signal peptide of each class of IGF-I precursor was also conserved among human, bovine, and goat. It is not known whether the difference in signal peptides has any special biological significance, but it is possible that the IGF-I is secreted in a different way under the direction of the different signal peptides, resulting in paracrine, autocrine, and endocrine actions.

Four kinds of goat IGF-I mRNAs encoded the same mature protein and the E domain. For rat, two kinds of E domains have been identified, one of which is encoded by the exons 4 and 6 (Ea), and the other by exons 4, 5, and 6 (Eb). None of the DNA products of the RT-PCR contained a sequence inserted between exons 4 and 6, so the goat IGF-I gene may have no exon corresponding to exon 5 of the rat IGF-I gene. In the human IGF-I precursor, two kinds of E domains have been found, encoded by exons 4 and 6 (Ea) or by exons 4 and 5 (Eb). The author prepared mix primers with sequences encoding the part of human Eb domain, and RT-PCR was done using goat liver RNA. But no DNA fragments was amplified (data not shown) To find whether the goat IGF-I gene has exon 5, the author have to isolate the region of the goat IGF-I gene corresponding to exon 5 of the human IGF-I gene and analyze the transcription of this region.

Transcriptional initiation sites of exon 1 reported in rat and human were mapped at two conserved regions,\textsuperscript{30-32} which were about 250 and 350 bp upstream from the 3'-end of exon 1. In ovine, initiation sites were mapped about 180 and 350 bp upstream from the 3'-end of exon 1.\textsuperscript{29} In goat, initiation sites were mapped at 247, 251, and 253 bp upstream from the 3'-end.
of exon 1 and conserved like that of rat and human. Around 180 and 350 bp upstream of the 3'-end of exon 1, no initiation site was detected. Transcriptional initiation sites of exon 2 were reported in rat, human, and ovine. Major initiation sites were mapped about 50 bp upstream from the 3'-end of exon 2 in rat and ovine,23) at 70 bp upstream from the 3'-end of exon 2 in human,21) and about 90 bp upstream from 3'-end of exon 2 in ovine.29) In goat, initiation sites were mapped at about 70 bp upstream from the 3'-end of exon 2, and was conformable to those of human. Additional initiation sites which were mapped 700-850 bp further upstream in rat, human, and ovine, were not detected in goat. Because the transcripts from this region were small in rat, human, and ovine, the author cannot rule out the possibility that initiation sites were not detected in this region because of the low sensitivity. Upstream of exons IW, 1, and 2, CAAT and TATA boxes were not found. This may be a reason why transcription initiates at multiple sites, and why initiation sites were not defined unequivocally. Upstream of exon 2, two cAMP response element-like sequences were also found, suggesting that this element may be involved in cAMP-dependent stimulation of the transcription of the IGF-I gene as reported previously.33,34)

References
Chapter III. 
Expression of Goat Insulin-like Growth Factor-I mRNAs in Tissues of each Developmental Stages

The author cloned the gene and cDNAs for the goat IGF-I.\(^1\) Nucleotide sequence analysis of the gene and cDNAs showed that the gene had at least six exons (1W, 1, 2, 3, 4, and 6) containing three leader exons (1W, 1, and 2). The gene was transcribed into three kinds of mRNAs for the leader exon (classes 1W, 1, and 2). The 4th kind of mRNA was found and it had the 3'-portion of exon 1 inserted between exons 1W and 3 (class 1W-1 del.; Fig. 1).

The goat IGF-I precursors encoded by these cDNAs were different from each other only in their signal peptides. The signal peptide encoded by the class 1W cDNA was 25 amino acids long, the signal peptides encoded by class 1W-1 del. and class 1 were identical and 49 amino acids long, and the signal peptide encoded by class 2 was 35 amino acids long. It is not known whether these different signal peptides have any special biological significance, but it is possible that the goat IGF-I is secreted in a different way under the direction of the different signal peptides, resulting in a paracrine, an autocrine, and an endocrine action.

To study the role of each signal peptide of the goat IGF-I precursors, the author analyzed expression of the four kinds of goat IGF-I mRNAs in various tissues of each developmental stages by a reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

Materials and Methods

Animals and tissues

Brain, lung, liver, spleen, kidney, uterus, ovary, testis, heart, and skeletal muscle were isolated from fetal, neonatal, and adult (3 years old) Shiba goat (Capra hircus).
Fig. 1. Structure of the Goat IGF-I Gene. Exons are represented by boxes and introns by lines. Solid areas show coding regions and a hatched area shows the region of exon 1 spliced out, giving the class 1W-1del. mRNA. Mature mRNAs are represented by bold lines connected by thin lines.

A RT-PCR assay

Total RNA was isolated from tissues by the acid-guanidine thiocyanate-phenol-chloroform (AGPC) method. RT-PCR was done described as in chapter 2. For detecting four types of mRNAs, the author prepared primer 1W (TTACCCAGTCTTGAAGG), primer 1 (CCTGCTAACATTTCT), and primer 2 (TGACCTGCTATAAGATCT) with sequences in exons 1W, 1, and 2 of the goat IGF-I gene, respectively, and the author prepared primer 6 (GTGCAGAGCGAAGGTGCT), which was complementary to a 20-nt sequence in exon 6 of the goat IGF-I gene. For detecting β-actin mRNA, the author prepared primer β-5 (GCCATCCCCAGGCTGTGCT) and β-3 (CCACTGCTGCTGAGT). After 25 cycles (1 min at 95°C, 1 min at 55°C, and 2 min at 72°C) of the PCR, the reaction mixtures detecting four classes of IGF-I mRNA and β-actin were mixed and electrophoresed through a 1% agarose gel. The gel was stained by ethidium bromide and a photograph of it was taken using incident ultraviolet. The intensity of each signal on the negative was measured by densitometry using an LKB-XL laser photodensitometer.

Results

The author analyzed three adult females, two adult males, two neonatal females, one neonatal male, and three fetuses. Although there are some problems for measuring the amount of mRNAs by RT-PCR, the conditions for PCRs were adjusted as possible so that the amount of amplified DNA fragments represented the amount of target mRNA. Under these conditions, the intensity of signal for β-actin mRNA was almost equal among tissues. Then the author used the intensity of signal for β-actin mRNA as a control of cDNA synthesis and DNA amplification of PCR. The results for a neonatal female are shown in the Figure 2.

Table showed the relative amount of each class of the goat IGF-I mRNA to β-actin mRNA. In adults, class 1 mRNA was found to be expressed in all tissues examined. Among them, it was expressed at a high level in liver,
uterus, and ovary. Class 1W and 1W-1del. mRNAs were expressed in only liver, uterus, and ovary. Class 2 mRNA was expressed in liver, uterus, and ovary and its amount was large in female liver and uterus. In contrast to ovary, class 1W, 1W-1del., and 2 mRNAs were not detected in testis. In young, class 1 mRNA was expressed in all tissues examined. Class 1W and 1W-1del. mRNAs were expressed only in liver. Class 2 mRNA was expressed in liver, uterus/ovary, heart, and skeletal muscle. In fetus, class 1 mRNA was expressed at a low level in tissues except for heart.

Table. Expression of the Four Kinds of the Goat IGF-1 mRNA in Tissues of each Developmental Stage.

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Intensity of signal for detecting the four kinds of the goat IGF-1 mRNAs was measured by densitometry. Relative amounts of them to β-actin mRNA are shown. 0 < < 50, 50 < ++ < 100, 100 < +++ (amount of β-actin mRNA = 100), - means "not detected", nd means "not done"

Discussion

The data are based on the use of RT-PCR. Although there are reservations on the quantitative aspects of RT-PCR, the conclusions based on the present experiments are supported by a number of controls. 1) only one band was visible on the gel by each PCR and that band hybridized to a specific IGF-1 probe. 2) Amplification efficiency of the PCR detecting each of the four kinds of mRNAs was almost equal judging from the results of control PCRs using goat IGF-1 cDNAs as templates. 3) Using 0.5 μg, 1 μg, and 2 μg of total RNA from tissues, the conditions for PCRs detecting four kinds of IGF-1 and β-actin mRNAs were adjusted so that the amount of amplified DNA fragments was proportional to the amount of the total RNA. 4) Contamination of DNA is irrelevant since the author prepared primers located on different exons.

In rat, tissue specific expression of IGF-1 mRNA was reported by Lowe et al. In class 1 mRNA was expressed in all tissues examined containing heart, lung, brain, testis, liver, muscle, stomach, and kidney. Class 2 mRNA was expressed in only liver at a low level. In goat, class 1 mRNA was expressed in almost all tissues and class 2 mRNA was expressed in liver as in rat. In goat, class 2 mRNA was detected also in uterus and ovary. In hypophysectomized rat, class 1 mRNA increased 2- to 3-fold and class 2 mRNA increased more than 6-fold in response to growth hormone treatment. It is unknown in goat whether class 2 mRNA is more sensitive to growth hormone than class 1 mRNA. In rat, expression of IGF-1 mRNA during development was analyzed by Adamo et al. In liver, heart, and kidney, IGF-I mRNA levels were low at birth and increased during the 50-day postnatal period. In contrast, stomach and muscle IGF-I mRNA levels were highest at the earliest stage and declined to the levels observed in 50-day-old rats. In goats, liver, uterus, and ovary IGF-1 mRNA level was raised during development. In heart, IGF-I mRNA could not detected in fetus but it was expressed in young. In kidney, IGF-I mRNA was expressed at the same level during development.

It is noted that a large amount of class 1 mRNA was expressed in adult uterus and ovary, but in testis it expressed at the same level as other tissues. This suggests that IGF-1 may be important in ovary and uterus but not testis. In rat, human, and porcine, IGF-I acts with follicle-stimulating hormone (FSH) to increase steroidogenesis (progesterone production) in ovary
granulosa cells. In goat, class 2 mRNA was expressed in young heart and skeletal muscle. This result suggests that IGF-I may participate in the formation of muscle in goat. IGF-I was reported to increase glucose transport and utilization in rat muscle cells\(^8\),\(^9\) and stimulate protein synthesis in freshly isolated cardiac myocytes.\(^{10}\) Class 2 mRNA was expressed at a high level only in female liver and uterus, but its role was not known. Recently it was reported that IGF-I and its receptor are necessary for fetal development as IGF-II in rat.\(^{11,12}\) In goats, only class 1 mRNA was expressed in fetal tissues and thought to play a role in fetal development.

References

Chapter IV.
Regulation of Insulin-like Growth Factor-I Expression in Mouse Preadipocyte Ob1771 Cells

Mouse preadipocyte Ob1771 cells\(^1\) which cease proliferating at confluence, can differentiate to adipocyte. Growth hormone (GH) has a strong adipogenic activity in Ob1771 as well as in 3T3-F442A\(^2,4\) and 3T3-L1\(^5\) preadipocyte. In Ob1771 cells, GH stimulates the formation of diacylglycerol without accumulation of inositol phosphate,\(^6\) modulates the transcription of the lipoprotein lipase gene,\(^7\) and transiently increases the transcription of the c-fos gene.\(^8\) GH also stimulates transcription of the insulin-like growth factor-I (IGF-I) gene,\(^8\) which is thought to participate in induction of the differentiation to adipocyte. In differentiated Ob1771 cells, enzymes for lipid synthesis including glycerophosphate dehydrogenase (GPDH), are activated\(^9\) and oil droplets were accumulated.

Transcription of the IGF-I gene was stimulated by GH, but IGF-I and GH were essential in medium for differentiation of Ob1771 cell. To study the role of exogenous IGF-I protein, the author analyzed expression of IGF-I mRNA and protein in Ob1771 during induction of differentiation. In this chapter, the author shows that IGF-I in the medium caused the change of IGF-1 mRNA species and stimulated the production of IGF-I protein.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium was purchased from Nissui. ASF104 medium was from Ajinomoto. Biotin, pantothenate, calf serum, triiodothyronine (T\(_3\)), 3-isobutyl-1-methylxanthine (Mix), dihydronicotinamide adenine dinucleotide (NADH), and dihydroxyacetone phosphate were from Sigma. Recombinant human insulin-like growth factor-I (IGF-I) was from Bachem. Cellulose nitrate membranes (BA85) was from Schleicher & Schuell. Nylon membrane, Gene Screen Plus was from DuPon. Monoclonal anti-human insulin-like growth factor-I antibody was from Upstate Biotechnology. Goat anti-mouse IgG hors eradish perox dyase conjugated was from Bio Rad.

Cell culture

Ob1771 cells were plated at 10\(^3\)/cm\(^2\) in Dulbecco's modified Eagle's medium supplemented with 200 units of penicillin/ml, 50 \(\mu\)g of streptomycin/ml, 33 \(\mu\)M biotin, 17 \(\mu\)M pantothenate, and 10% (v/v) calf serum. This medium was termed standard medium. At confluence (day 0, ~5 days after seeding), cells were exposed to standard medium supplemented with 2 nM T\(_3\), 100 \(\mu\)M Mix, which was termed differentiation medium. When indicated, differentiation medium was supplemented with 10 nM recombinant goat GH purified from E. coli and with 10 nM recombinant human IGF-I from day 0 to day 4. Cells were cultured in standard medium for more four days (~day 8) and used for GPDH assay. Both standard and differentiation medium were changed every 2 days.

GPDH assay\(^{10,13}\)

After induction of differentiation (day 8), cells were washed with PBS and harvested in 0.5 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM \(\beta\)-mercaptoethanol. Cells were sonicated for 5 seconds at 0°C with the microtip of a sonifier (Handy sonic, model UR-20P TOMY). The extracts were centrifuged for 10 minutes at 10,000 g at 4°C and the supernatants were stored at -20°C. GPDH activity was analyzed by measurement of oxidation of NADH at 340 nm in 100 mM TEA-HCl (pH 7.5), 2.5 mM EDTA, 0.2 mM dihydroxyacetone phosphate, and 0.12 mM NADH. The results were shown with relative increase of GPDH activities to that in full-differentiated cells cultured with 10 nM GH and 10 nM IGF-I.
Western blotting

Cell extracts, which were prepared as described above, and media were diluted in sample buffer containing 50 mM Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.001% bromophenol blue. Equivalent amount of proteins was separated by SDS-PAGE, transferred to cellulose nitrate membranes with a Trans-Blot SD Semi-dry Transfer Cell (Bio-Rad) in 48 mM Tris base, 39 mM glycine, and 0.004% SDS. Membranes were blocked overnight in PBS containing 0.1% Tween 20 (PBS-T) with 5% skim milk, and incubated with monoclonal anti-human insulin-like growth factor-I antibody in PBS-T for one hour. Membranes were washed twice for 15 minutes with PBS-T and incubated with goat anti-mouse IgG horseradish peroxidase conjugated in PBS-T for one hour. After washing, antibody binding was visualized using an ECL detection system (Amersham).

RT-PCR and Southern hybridization

For detection of both class 1 and 1del., the author prepared primer 1-1del. (ATGGGAAAAATCAGCAGTC) in the sequence of exon 1. For detection of only class 1, the author prepared primer 1 (TCAAAAITGAAATGTGAC) in the sequence of exon 1 spliced out, resulting in class 1del. The author also prepared primer 6 (AGGTCITGTITCCTGCAC), which is complementary to the sequence in exon 6. Total RNA was prepared from cells by the acid-guanidine thiocyanate-phenol-chloroform (AGPC) method. RT-PCR was done described as in chapter 1. After 25 cycles (1 min at 95°C, 1 min at 55°C, and 2 min at 72°C) of the PCR, DNA fragments were separated by a 1% agarose gel and transferred to nylon membrane (Gene Screen Plus) by capillary transfer method. Hybridization and detection using mouse IGF-I cDNA as a probe were done with a ECL direct nucleic acid labeling and detection system (Amersham).

Results

Transcription of IGF-I stimulated by GH in Ob1771 cells

Northern blot analysis of the total RNA from Ob1771 cells is shown in Figure 1. When post-confluent Ob1771 cells were cultured in the differentiation medium with neither GH nor IGF-I, IGF-I mRNA was not detected. When post-confluent Ob1771 cells were cultured with GH or with GH and IGF-I, IGF-I mRNA was accumulated. Multiple mRNA species of 15, 7.5, 1.5, and 0.8 kbp were seen. It was also reported by A. Doglio et al. and reported to be due to multiple polyadenylation sites. The amount of mRNA was larger in cells cultured with only GH than in cells with GH and IGF-I.

Requirement of GH and IGF-I for differentiation of Ob1771 cells

Post-confluent Ob1771 cells were cultured in differentiation medium supplemented with 10 nM GH or with 10 nM GH and 10 nM IGF-I, and glycerophosphate dehydrogenase (GPDH) activities were analyzed (Fig. 2). Ob1771 cells cultured with neither GH nor IGF-I did not differentiate and GPDH was not activated. Using differentiation medium with GH and IGF-I, the cells differentiated to adipocyte and GPDH was activated and many oil droplets were observed. Using differentiation medium with GH, GPDH activities increased only 5.8% of that in full-differentiated cells cultured with GH and IGF-I. This result showed that exogenous IGF-I combined with GH was needed for differentiation of Ob1771 cells although IGF-I mRNA was accumulated by only GH.

Expression of each class of IGF-I mRNA during induction of differentiation

The rat IGF-I gene has two leader exon (exons 1 and 2) and each of them was spliced to exon 3, resulting in two kinds of mRNAs (classes 1 and 2). Exon 1 was spliced to another mRNA (class 1del.), which was missing central region of exon 1. The author analyzed IGF-I mRNA species during induction of the differentiation by RT-PCR (Fig. 3). Using differentiation medium with
GH, transcription from exon 1 was stimulated during induction (day 2-4) and major splicing pattern was class 1del. Using the differentiation medium with GH and IGF-1, transcription from exon 1 was stimulated as well, and major splicing pattern was changed to class 1. In both case, class 2 mRNA was expressed transiently at day 4 in induction. This result showed that the splicing pattern of IGF-I mRNA was regulated by IGF-I itself.

Fig. 1. Northern Hybridization of RNA Prepared from Ob1771 Cells. Post-confluent Ob1771 cells were cultured in differentiation medium supplemented with non (control), with 10 nM GH and 10 nM IGF-I (GH + IGF-I), or with 10 nM GH (GH). At day 4, total RNA was prepared from cells by AGPC method and 10 μg of RNA was separated through the 1.2% agarose gel containing 6% of formaldehyde. RNA was transferred to the nylon membrane, Gene Screen Plus (DuPon). Hybridization and detection using mouse IGF-I cDNA as a probe were done in ECL system (Amersham).

Fig. 2. Differentiation of Ob1771 Cells by GH and IGF-I. Ob1771 cells were cultured in standard medium and at confluence (day 0) medium was changed to differentiation medium supplemented with 10 nM GH and 10 nM IGF-I (GH + IGF-I), or with 10 nM GH (GH). At day 4, medium was changed to standard medium and cells were cultured for more four days. At day 8, cells were harvested and cell extracts of them were used for measurement of GPDH activities.

Requirement of both GH and IGF-I for synthesis and secretion of IGF-I protein

Post-confluent Ob1771 cells were induced differentiation by GH or by GH and IGF-I. At indicated day, medium was changed to serum free medium ASF104 (ajinomoto) and 24 hr later, cells and media were collected and IGF-I protein was analyzed with anti-IGF-1 antibody by ECL system (Fig. 4). Using differentiation medium with only GH, IGF-I protein could be detected neither in cells nor in medium. Using differentiation medium with GH and IGF-I, IGF-I protein could be detected in both cells and medium from day 2 to day 4. The result showed that translation of IGF-I required IGF-I with GH in medium.
Fig. 3. Expression of IGF-I mRNAs during Induction of Differentiation. RNA was prepared from the Ob1771 cells cultured in standard medium (day -2 and 0), during induction of differentiation by 10 nM GH or by 10 nM GH and 10 nM IGF-I (day 2-4), and after induction (day 6). Using 1 μg of RNA, RT-PCR was done with primers 1 and 6 (class 1), with primers 1-1del. and 6 (class 1 + class 1del.), and with primers 2 and 6 (class 2) and amplified DNA fragments were analyzed by Southern hybridization as described in Materials and Methods.

Fig. 4. Synthesis and Secretion of IGF-I Protein in Ob1771 Cells. Post-confluent Ob1771 cells were cultured in differentiation medium supplemented with 10 nM GH (GH) or with 10 nM GH and 10 nM IGF-I (GH and IGF-I) from day 0 to day 4. At indicated day, medium was changed to serum free medium ASF104 and 24 hr later, cells and media were collected and IGF-I protein was analyzed by western blot using mouse anti-human IGF-I monoclonal antibody.

Biological activity of IGF-I secreted from Ob1771 cells
Post-confluent Ob1771 cells were cultured for two days (day 0 ~ day 2) in differentiation medium with GH or with GH and IGF-I. After washing, cells were maintained differentiation medium supplemented with nothing for one more day. These medium were termed as GH conditioned medium and GH-IGF-I conditioned medium, respectively. In GH-IGF-I conditioned medium, IGF-I protein was detected and though to be secreted from Ob1771 cells (data not shown). In GH conditioned medium, IGF-I protein could not be detected. Differentiation of another post-confluent Ob1771 cells was induced using these conditioned medium and GPDH activities were analyzed.
increased 17% of that of the full-differentiated cells. This result showed that IGF-I protein secreted from Ob1771 had activity to induce differentiation.

**Fig. 5. Biological Activity of IGF-I Secreted from Ob1771 Cells.** From day 0 to day 2, Ob1771 cells were cultured in differentiation medium with 10 nM GH or with 10 nM GH and 10 nM IGF-I. After washing, cells were maintained differentiation medium supplemented with nothing for one more day. These media were termed as GH conditioned medium and GH-IGF-I conditioned medium, respectively. Other Ob1771 cells were induced differentiation using these conditioned medium supplemented with GH. At day 8, cells were harvested and analyzed GPDH activities in cells cultured in differentiation medium with 10 nM GH (lane 1), in differentiation medium with 10 nM GH and 10 nM IGF-I (lane 2), in GH-IGF-I conditioned medium with 10 nM GH (lane 3), and in GH conditioned medium with 10 nM GH (lane 4).

**Requirement of IGF-I in early period of induction of differentiation**

The author analyzed when IGF-I was required for differentiation. During four days of induction, IGF-I was added to the differentiation medium with GH only in the early two days or in the late two days (Fig. 6). When IGF-I was added in the medium for the early 2 days, GPDH in the cells was activated to 96% of that of the full-differentiated cells with GH and IGF-I for all four days. When IGF-I was added for the late 2 days, GPDH activity of the cells was 40% of that of the full-differentiated cells with GH and IGF-I for all the four days. These results showed that IGF-I was required in early period of the induction of differentiation of Ob1771 cells.

**Fig. 6. Analysis of the Period in which IGF-I is Required for the Differentiation of Ob1771 Cells.** During four days of induction, different medium was used in early two days and in late two days. Ob1771 cells were cultured in differentiation medium, when indicated, supplemented with 10 nM GH and 10 nM IGF-I. At day 8, cells were harvested and analyzed their GPDH activities.
Discussion

Ob1771 cells is preadipocyte established from mouse adipose tissues and its differentiation to adipocyte is dependent on both GH and IGF-1.\(^1,16,17\) Transcription of the IGF-1 gene was stimulated by GH in Ob1771 cells, but IGF-1 protein was needed in medium for differentiation to adipocyte. The author thought some possibilities for requirement of exogenous IGF-1 for differentiation. 1) IGF-I mRNA is not translated unless signal from IGF-I receptor. 2) IGF-I protein is not secreted from the cell unless signal from IGF-I receptor. 3) IGF-I secreted from Ob1771 cells does not have biological activity. In this case, activity of IGF-I may be inhibited by IGF binding proteins or the amount of secreted IGF-I may not be sufficient to induce the differentiation. To study the reason for requirement of exogenous IGF-I, the author analyzed mRNA species, production and secretion of IGF-I protein, and activity of IGF-I secreted from Ob1771 cells.

When Ob1771 cells were cultured with GH and IGF-I, IGF-I protein was produced and secreted to the medium and class 1 mRNA was major species in cells. When cells were cultured with only GH, IGF-I protein was not produced and class 1del. mRNA was major species. These results showed that exogenous IGF-I caused change of IGF-I mRNA species. The unknown protein, which production is induced by IGF-I, may bind primary transcripts of the IGF-I gene and inhibit the splicing within exon 1. The results also showed that exogenous IGF-I was required for the production of endogenous IGF-I. Two reason were possible for it. 1) Class 1 mRNA can be translated but class 1del. mRNA cannot be translated for its higher structure. 2) Both class 1 and 1del. mRNAs can be translated and translation machinery for them is not activated unless signal from IGF-I receptor. To certify these two possibility, the author have to analyzed the efficiency of the translation of class 1 and 1del. mRNAs and activities of translation machinery for IGF-I in nuclei of the cells incubated with GH, and with GH and IGF-I.

![Fig. 7. Model of Initiation of IGF-I Production.](image)

The author found that GH-IGF-I conditioned medium, which contained IGF-I secreted from Ob1771, induced differentiation of other Ob1771 cells. The author thinks that it was due to IGF-I in medium. However, it cannot rule out the possibility that another adipogenic factor is secreted from Ob1771 cells incubated with GH and IGF-I, and induced the differentiation. To certify it, the author have to analyze the significance of IGF-I in medium using antibody which inhibits the binding of IGF-I to its receptor. Moreover, exogenous IGF-I was required only in early period of induction. Exogenous IGF-I may stimulate the production and secretion of endogenous IGF-I, which could act in an autocrine/paracrine fashion and stimulate the subsequent production and secretion of endogenous IGF-I. Therefore once endogenous IGF-I was produced and secreted, exogenous IGF-I may not be needed in medium. From these results, the author suggests the following model for differentiation to adipocyte (Fig. 7). GH stimulates the transcription of the genes needed for differentiation in preadipocyte. GH also stimulates the transcription of IGF-I but its mRNA cannot be translated to protein. IGF-I produced in other tissues...
such as liver acts in an endocrine fashion and activates the translation of IGF-I, probably by changing the splicing pattern of IGF-I mRNA from class Idel. to class I. IGF-I secreted from preadipocyte can act in an autocrine/paracrine fashion and stimulates the more production of endogenous IGF-I, which stimulates the subsequent changes in cells and induced differentiation to adipocyte. So, IGF-I acting preadipocyte in an endocrine fashion triggers the differentiation by causing initiation of endogenous IGF-I production.

References
CONCLUSION

Chapter I

The author isolated two clones which contained the goat GH genes. One clone CgGH contained the gGH1 gene and the other clone EgGH contained the gGH2 and gGH3 genes. The gGH1 and gGH2 genes had very similar sequences and thought to be functional genes but the gGH3 had different sequence in 5'-flanking region and thought to be pseudogene. The author found that the individual goats have the gGH1, gGH2, and gGH3 genes with different copy numbers, and CgGH and EgGH DNA segments may be allelic on the goat chromosome. Repetitive sequences were found flanking the gGH1, gGH2, and gGH3 genes. The consensus sequence of them was homologous to that found in the flanking of the bovine proopiomelanocortin (POMC) gene and the goat β-globin gene.

Chapter II

The author isolated the gene and cDNAs for the goat IGF-I. The gene had six exons (1W, 1, 2, 3, 4, and 6) containing three leader exons (1W, 1, and 2), resulting three kinds of mRNAs (classes 1W, 1, and 2). Another RNA species was detected by RT-PCR analysis of exon 1W and found to have both exon 1W and the 3'-portion of exon 1. These four kinds of mRNAs encoded the same mature IGF-I and E domain, but encoded different signal peptide. It is not known whether the difference in signal peptides has any biological significance, but it is possible that the IGF-I is secreted in a different way under the direction of the different signal peptides, resulting a paracrine, an autocrine, and an endocrine action.

Chapter III

The author analyzed the expression of the goat IGF-I mRNAs in various tissues of each developmental stage. Class 1 mRNA was expressed in all tissues examined. In liver, uterus, and ovary, class 1 mRNA was expressed at a high level. Class 1W and 1W-1del. mRNAs were expressed in only liver, uterus, and ovary. Class 2 mRNA was expressed in liver, adult uterus and ovary, and young heart and skeletal muscle. IGF-I produced from class 2 mRNA may have a function in the formation of muscle in young as well as a function in the adult uterus and ovary.

Chapter IV

The author analyzed the regulation of the IGF-I expression in preadipocyte Ob1771 cells. When cells were cultured with GH, the IGF-I gene was transcribed but IGF-I protein was not produced. In this case, the major species of mRNA was class 1del. When cells were cultured with GH and IGF-I, the major species of mRNA was changed to class 1 and IGF-I was produced and secreted from cells. IGF-I secreted from cells could act in an autocrine fashion and stimulate the subsequent production and secretion of endogenous IGF-I. IGF-I acting preadipocyte in an endocrine fashion triggers the adipose conversion by causing the initiation of endogenous IGF-I.
PUBLICATIONS


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Satoshi Mikawa 1995