Cloning and Characterization of Ovine Insulin, Insulin-Like Growth Factor-I and -II Genes

by

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Abstract

Genes encoding ovine insulin like growth factor-I (oIGF-I), -II (oIGF-II) and insulin were cloned, sequenced and characterized. The oIGF-I gene contains six exons spanning greater than 30 kilobases. Class 1 and class 2 oIGF-I transcripts contained exons 1 and 2 alternatively-spliced to exon 3, respectively. A novel oIGF-I exon (W) was located upstream of exon 1 and was found alternatively spliced to exon 3. No in-frame methionine codon was found in exon W and therefore translation is proposed to initiate at the methionine codon present in exon 3. Using primer extension, the ovine transcription initiation sites were mapped and found to be well conserved among mammalian and avian IGF-I genes. Expression of exon 1-, 2- and W-specific transcripts was examined in seven tissues from adult or fetal sheep using a reverse transcription-polymerase chain reaction (RT-PCR) assay. Exon 1 transcripts were the most abundant and found in all fetal and adult tissues. Exon 2 transcripts were found in all tissues and in general showed the highest expression in adult liver. Exon W transcripts were expressed at low levels in all tissues examined.

To confirm that exon W mRNA produced biologically active IGF-I, an exon W containing cDNA was cloned under the control of a glucocorticoid-inducible MMTV promoter (pMMTV-IGF-IW) and transfected into a bovine mammary epithelial cell line (MAC-T). Stable transfectants were induced with a synthetic glucocorticoid to produce secretable IGF-I. Transcript expression of pMMTV-IGF-IW was confirmed by Northern blot analysis and IGF-I was quantified in the medium of growing cells with RIA. Biological activity of the secreted IGF-I was assayed by measuring the incorporation of [^{3}H]-thymidine into DNA of test MAC-T cells. Media harvested from the pMMTV-IGF-IW transfected clones stimulated labeling of MAC-T cells greater than that of conditioned media from MAC-T cells. Thus, biologically active IGF-I was secreted from pMMTV-IGF-IW cells.

The oIGF-II gene is composed of 9 exons that span approximately 25 kilobases. Approximately 750 nucleotides upstream of oIGF-II exon 1 are the three exons of the ovine insulin gene which are transcribed in the same direction as oIGF-II. Four putative promoters direct transcription of six 5' non-coding exons (1, 3, 4, 5, 6, and 7), which are alternatively spliced to exons 8, 9, and 10. An ovine exon comparable to human exon 2 has not been identified. Multiple transcription initiation sites were identified for exons 1 and 6 by primer extension analysis. Using a RT-PCR assay, exon 1 and 3 transcripts were shown to be expressed in adult but not fetal liver. In addition, a novel transcript that contained exon 1 spliced directly to exon 8 was detected in adult liver. Exon 4 transcripts were not detected, whereas exons 5, 6 and 7 transcripts were detected in both fetal and adult liver. Like the human and rodent genes, the regulation of expression of the oIGF-I and oIGF-II genes are under complex control.

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CHAPTER 1
Literature Review

Introduction

The aim of this Literature Review is to summarize current information pertaining to the complex structure and expression of the insulin-like growth factor I and II genes (IGF-I and -II). This review will begin with a brief history and characterization of the IGF, continue with a description of the physiological regulators of the IGF during growth and development and conclude with detailed structures of the IGF-I and -II genes and their putative promoters.

History

Over thirty five years ago, Salmon and Daughaday (1957) were the first to isolate a "sulfation factor", which was hypothesized to function as a growth hormone (GH)-intermediate. The sulfation factor soon became known and accepted as a somatomedin, a word derived from the combination of soma (referring to the somatotropin hormonal relationship) and medin (denoting its intermediary action). Experiments indicated that circulating GH was unable to directly induce mitotic growth in target tissues of hypophysectomized animals. Therefore, it was

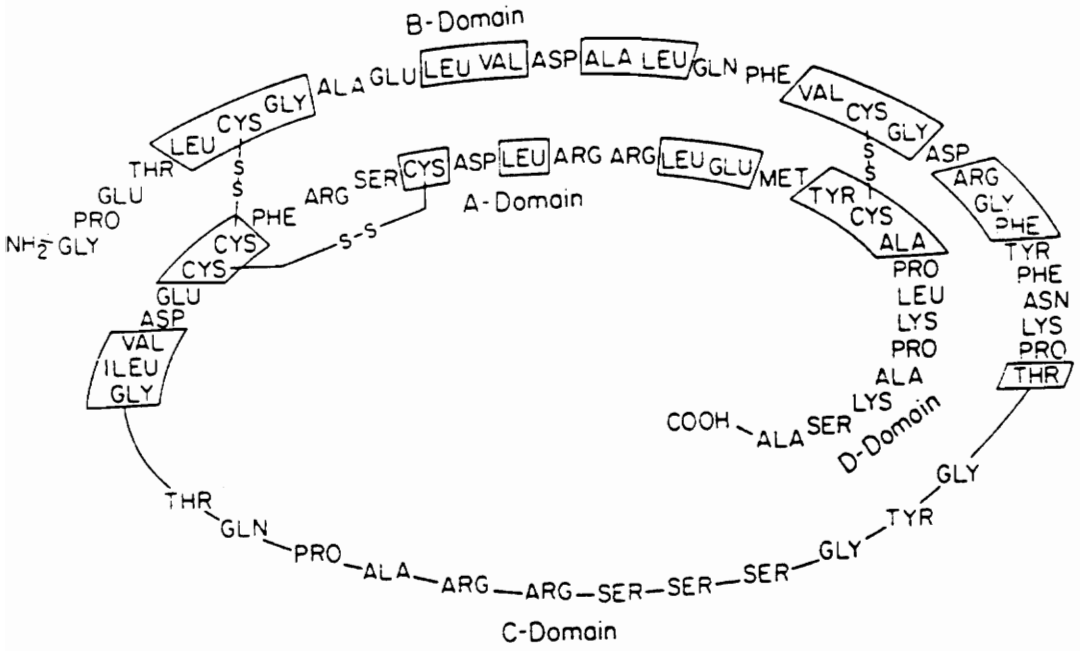
hypothesized that an intermediary factor found in the serum "mediated" the actions of GH (Salmon and Daughaday, 1957). This hypothesis is more formally referred to as the somatomedin hypothesis and the somatomedins were proposed to function as these GH-intermediates.

Somatomedins (C and A) and IGF (I and II) were isolated from human serum in independent investigations (Van Wyk *et al.*, 1975; Hall, 1972; Rinderknecht and Humbel, 1978). The somatomedins were characterized by their ability to incorporate sulfate and thymidine into cartilage. The IGF were characterized by their similarity to the structure of insulin, as well as their similarities in metabolic and mitogenic effects. In conjunction with the isolation and characterization of the somatomedins and the IGF, other research groups reported the identification of similar growth factors such as non-suppressible insulin-like activity (NSILA) and multiplication stimulation activity (MSA) peptides (Froesch *et al.*, 1963; Moses *et al.*, 1980; Yang *et al.*, 1985). Amino acid sequencing of these peptide growth factors demonstrated that somatomedin-C and -A were identical to IGF-I (Klapper *et al.*, 1983), and that MSA was the rat homologue to human IGF-II (Marquardt *et al.*, 1981). To avoid further confusion in the literature, leading researchers agreed to refer to these factors as IGF-I and IGF-II (Daughaday *et al.*, 1987).

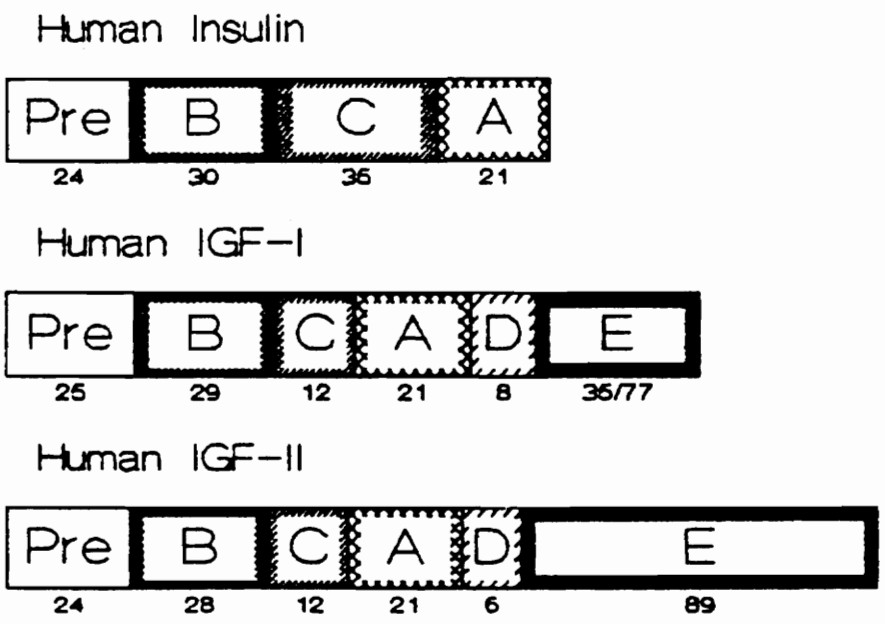
Characterization of IGF-I and IGF-II

Extensive characterization of human IGF-I and -II confirmed their similarity to the insulin peptide. Both IGF-I and -II polypeptides are single chained peptides of 70 and 67 amino acid residues, respectively. While human IGF-I is a basic peptide (pI 8.5 ± 0.5) with a molecular weight of 7649 g/mol, human IGF-II is slightly more acidic (pI 6.2 ± 0.5) with a molecular weight of 7471 g/mol. The three-dimensional structure of both IGF-I and -II is governed by cross linkage in the form of three disulfide bridges (Figure 1-1). Like proinsulin, both peptides consist of

Figure 1-1. Amino acid structure of IGF-I.
A.) Amino acid structure of mature IGF-I (Adapted from Clemmons, 1989).
B.) Comparison of peptide domains of preproinsulin, preproIGF-I and preproIGF-II. Individual domains are represented by boxes which indicate the number of amino acids contained in each.



The structure of insulin-like growth factor I.



B, C, and A domains. However, unlike insulin the mature IGFs retain the C peptide and contain an additional D domain at the carboxyterminus (Figure 1-1). Analysis of IGF-I and -II cDNA sequences of human, rat, mouse, pig and sheep predicted the presence of additional pre- and E-peptide domains (Jansen *et al.*, 1983; Shimatsu and Rotwein, 1987; Bell *et al.*, 1986; Honeggar and Humbel, 1986; Tavakkol *et al.*, 1988; Wong *et al.*, 1989). Both of the pre- and E-peptide domains are removed during proteolytic processing of the IGFs, resulting in mature IGF-I and -II polypeptides of 70 and 67 amino acids, respectively.

Both IGF-I and -II have variant forms of amino acid sequences. A truncated IGF-I (des-IGF-I) missing the first three amino acids at the aminoterminus was isolated from fetal and adult brain tissue as well as bovine colostrum (Sara and Hall, 1990; Sara *et al.*, 1986; Carlsson-Skwirt *et al.*, 1986; Francis *et al.*, 1986). This peptide modification is presumed to result from post-translational modification of the proIGF-I since the site of truncation was not at an exon/intron junction and no cDNA sequence has been identified which encodes the des-IGF-I. IGF-II variants also have been found in human serum. A 10 kDa peptide containing a tripeptide insertion and a carboxy-terminal extension of 21 amino acid residues has been reported by Zumstein *et al.* (1985). Again, the insertion does not occur at an exon/intron junction and raises the possibility of a second IGF-II gene. A second variant form of IGF-II has been reported by Jansen *et al.* (1985) in which a tetrapeptide insertion at the human exon 8/9 junction occurs. This insertion was confirmed by cDNA sequencing.

Serum concentrations of IGF-I and IGF-II appear to fluctuate during development (Zapf *et al.*, 1981; Bala *et al.*, 1981). In fetal serum, circulating IGF-II is significantly greater than IGF-I. Throughout fetal development, IGF-II concentrations decrease until parturition. Differences between human and rat IGF-II concentrations were observed after parturition (Moses *et al.*, 1980). During the first year, human IGF-II concentrations increase to adult levels of 600-800 ng/ml

(Daughaday and Rotwein, 1989) whereas rat IGF-II serum concentrations remain low throughout adulthood. In contrast to IGF-II concentrations, human IGF-I in the serum rises during postnatal development, peaks at puberty and levels off to approximately 180 ng/ml in adults 20-30 years of age (Underwood *et al.*, 1986). Consistent with the somatomedin hypothesis, the increase in serum concentrations of IGF-I during postnatal development are paralleled by increases in circulating GH and cell surface GH-receptors (Mathews *et al.*, 1989; Tiong and Herington, 1992).

Endocrinological and Developmental Regulation of IGF-I

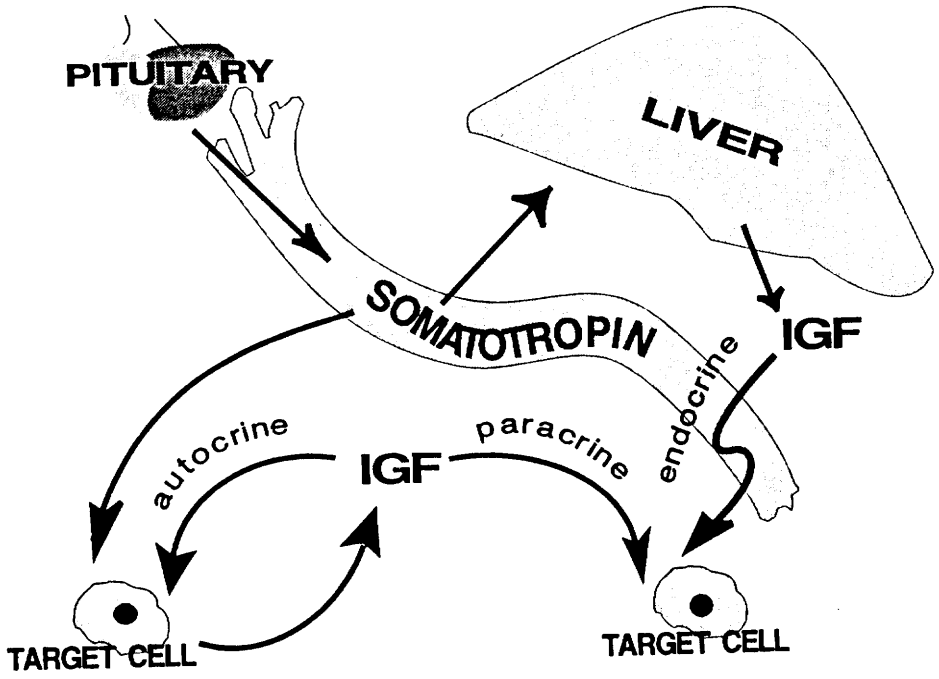
Growth Hormone Regulation

Physiological studies have shown that GH treatment coordinately increased IGF-I mRNA transcripts in the liver (Mathews *et al.*, 1986; Murphy *et al.*, 1987a), but had no effect on IGF-II transcripts (Turner *et al.*, 1988). Thus, secreted IGF-I was shown to be GH dependent and acted as an endocrine growth factor. The liver was once considered to be the only organ involved in IGF-I and -II synthesis and secretion, but it is now known that most if not all tissues produce IGF-I and -II (D'Ercole *et al.*, 1984). The detection of IGF-I and -II mRNA in a number of extra-hepatic tissues led to the hypothesis that the liver produces a GH responsive endocrine form of IGF-I while the extra-hepatic sources of IGF-I and -II function as autocrine/paracrine growth factors (Figure 1-2).

Other Endocrine Regulators of IGF-I

In addition to GH, many other endocrine factors appear to be involved in the regulation of IGF-I gene expression. It is proposed that the coupling of steroid and/or protein hormones with tissue-specific nuclear proteins may potentially

Figure 1-2. Possible endocrine, autocrine and paracrine actions of the insulin-like growth factors (McGuire et al., 1992).


PITUITARY
LIVER
SOMATOTROPIN
IGF
endocrine
autocrine
IGF
paracrine
TARGET CELL
TARGET CELL

regulate IGF-I synthesis at the transcriptional level. Addition of these factors to *in vivo* and cell culture systems has resulted in either increases or decreases in production of IGF-I, depending on the tissue-type. For example, addition of dexamethasone (DEX), a synthetic glucocorticoid, to rat neuronal and glial cells either in culture or by injection decreased all of the IGF-I mRNAs in primary cell culture and tissue samples (Adamo *et al.*, 1988; Luo and Murphy, 1989). This contrasts with experiments that showed the addition of DEX had no effect on IGF-I production in the rat ovary, and that in combination, DEX and insulin prevented the normal reduction of IGF-I gene expression (Botero *et al.*, 1993). Additionally, GH and estrogen were shown to independently affect IGF-I synthesis in the rat uterus, while only GH stimulated the same response in the liver (Murphy *et al.*, 1987b; Murphy and Freisen, 1988). When GH and estrogen were added together, IGF-I mitogenic response in both the liver and uterus was different from that when added individually (Murphy and Freisen, 1988). How these extrinsic and tissue-specific factors interact to regulate IGF-I synthesis is still being investigated.

Nutritional Regulation of IGF-I

Serum and tissue concentrations of IGF-I in animal models are dependent on nutritional status. IGF-I has little to no diurnal variation in response to feed intake in well nourished animals, and is predicted not to play a role in the regulation of daily nutrient absorption (Franklin and Cameron, 1978; Van Wyk and Underwood, 1978). However, changes in IGF-I status were observed during fasting and protein-deprivation studies. Thus the functional role of GH and IGF-I was theorized to adapt protein synthesis and growth rates to acceptable levels of nutrition (Riis, 1983).

Fasting and malnutrition result in a state of low serum IGF-I which is associated with GH resistance. The number of GH receptors in the liver decreased during fasting (Maiter *et al.*, 1988; Thissen *et al.*, 1990), reducing the response to

circulating GH at the liver. The reduction in GH-receptors led to decreased serum IGF-I and treatment with GH after fasting did not increase serum IGF-I concentrations (Clemmons and Underwood, 1991). The number of IGF-I transcripts was reduced during fasting, but no detectable changes have been found in the rate of IGF-I transcription (Adamo *et al.*, 1991a; Emler and Schalch, 1987; Straus and Takemoto, 1990b). It has been proposed that the decrease in IGF-I transcripts was due to a post-transcriptional block. Increasing serum concentration of IGF-I to normal levels in fasted rats restored near normal growth rates (Schalch *et al.*, 1989).

IGF-I concentrations are reduced in growing rats that are fed a low protein diet. Unlike fasting, the number of liver GH receptors remained normal during protein deprivation studies (Ketelslegers *et al.*, 1993). Therefore, the reduced serum IGF-I during protein depletion was due to a reduction in IGF-I gene expression. Analysis of rat liver and muscle mRNAs during protein-deprivation feeding trials showed a consistent decrease in the number of IGF-I transcripts (Straus and Takemoto, 1990a; VandeHarr *et al.*, 1991). Increasing the serum IGF-I concentration to normal physiological concentrations did not result in an increase of whole body growth, but instead resulted in specific tissue/organ effects similar to that reported in transgenic IGF-I mice (Thissen *et al.*, 1991).

Animals Divergently Selected for IGF-I

Because IGF-I was originally thought to act as the intermediate mitogenic factor of GH (Somatomedin hypothesis), it was predicted that increases in serum IGF-I concentrations would result in increased whole body growth as seen in the transgenic mice overproducing GH (Palmiter *et al.*, 1982, 1983). However, increases in IGF-I serum concentration had different physiological effects. Mice divergently selected for high plasma concentrations of IGF-I (200-2000 ng/ml) showed an increase in whole body growth. However, an additional 30% increase in splenic

growth was not simply due to an increase in liveweight (Siddiqui *et al.*, 1992). Similar results were found when lambs were treated for 8 weeks with IGF-I (Gallaher *et al.*, 1993). Lamb spleen weights corrected for body weight increased by 40%. Since the selectively bred mice were never exposed to an alternative source of IGF-I (i.e., human recombinant IGF-I), accelerated splenic growth was not thought to be a result of a secondary immune response but instead due to the direct consequence of elevated circulating IGF-I. Current research is addressing the differences in growth response induced by GH and IGF-I.

Developmental and Endocrinological Regulation of IGF-II

Developmental regulation of IGF-II gene expression has been characterized in a number of species. Similar studies showed that IGF-II gene expression during fetal development could be detected in a number of tissue types up to birth, after which dramatic decreases in IGF-II mRNA were seen in all tissue types with the exception of the central nervous system and the liver of sheep and human (Hynes *et al.*, 1988; de Pagter-Holthuizen *et al.*, 1988; O'Mahoney *et al.*, 1991; Delhanty and Han, 1993).

Unlike IGF-I, IGF-II does not respond to GH stimulation or other trophic hormones which are known to either increase or decrease the circulating IGF-I concentration (Hynes *et al.*, 1987; Mesiano *et al.*, 1989; Owens *et al.*, 1990). Also, significant changes in serum IGF-II concentration did not correlate with changes in nutritional status. Experiments have shown decreases in fetal IGF-II as a result of maternal fasting, but also have shown that upon fasting, changes in serum IGF-II either decrease, remain unchanged, or elevate in lambs of 2 and 14 days, 4-6 months and 1-2 years respectively (Oldham *et al.*, 1993). No consistent decreases in IGF-II expression have been be correlated with poor nutritional status as seen for IGF-I gene expression.

One physiological regulator, cortisol, has been shown to direct hepatic IGF-II gene expression. *In vivo* studies showed that in both sheep and rat, IGF-II gene expression decreased either a few days before parturition or 18 to 20 days after parturition, respectively. This decrease correlated with escalating levels of fetal cortisol (Silver, 1990; Beck *et al.*, 1988). Li *et al.* (1993) showed that adrenalectomy of fetal sheep prevented the normal decreases in IGF-II mRNA expression. Follow-up treatment of the adrenalectomized fetal sheep with cortisol stimulated the IGF-II decrease in liver mRNAs but had no effect on extra-hepatic IGF-II expression. Similar results were reported in the treatment of primary fetal hepatocytes in cell cultures with dexamethasone (Townsend *et al.*, 1991).

Although glucocorticoids are well established as inducers of gene expression, gene repression also has been reported in the regulation of opiomelanocortin, prolactin, and nerve growth factor gene expression (Charron and Drouin, 1986; Camper *et al.*, 1985; Siminoski *et al.*, 1987). While the mechanism by which IGF-II expression is regulated by cortisol remains unclear, further analysis of transcript-specific expression may identify the IGF-II promoter involved.

IGF Binding Proteins

Unlike other endocrine factors, the IGFs are not stored in secretory vesicles of an endocrine organ, but are found in circulation, bound to insulin-like growth factor binding proteins (IGFBPs). Six different IGFBPs, designated IGFBP-1 through -6, have been characterized and were found to consist of 200-300 amino acids with 20-40 amino acid signal sequences (Brewer *et al.*, 1988; Lee *et al.*, 1988; Brown *et al.*, 1989; Wood *et al.*, 1988; Binkert *et al.*, 1989; Shimasaki *et al.*, 1991). Eighteen cysteines were highly conserved in the IGFBPs and are thought to play a role in determining the three-dimensional structure of the IGFBPs. In circulation the major form of the IGFBPs is the 150 kDa complex which is comprised of three

subunits, an acid-labile component (80 kDa), an acid stable component (IGFBP-3 42-45 kDa) and IGF-I or -II (7 kDa). In the complex, IGF-I or -II is biologically inactive. Thus, the binding protein complex acts as an inhibitor of mitogenic stimulation and also prolongs the half-lives of IGF-I and -II in circulation (Adamo *et al.*, 1992). Specific IGFBPs such as IGFBP-1 and -2 also may be involved in the transportation of IGFs from circulation to potential target tissues (Bar *et al.*, 1990).

The IGFBP affect IGF-I and -II bioactivity and receptor interaction (reviewed in Adamo *et al.*, 1992; Cohick and Clemmons, 1993). Changes in the binding affinity of the IGFBPs for both IGF-I and -II are thought to regulate IGF-receptor interactions. When IGFBP-3, the major IGFBP found in serum, was either pre- or co- incubated with IGF-I in fibroblast cell cultures, the activity of IGF-I was either potentiated or inhibited (Conover *et al.*, 1990; Baxter and Martin, 1989). The mechanism by which IGFBP enhances IGF-I-receptor interaction is unknown but is thought to require both IGFBP and receptor binding to IGF-I (Clemmons *et al.*, 1990). Amino acid residues of IGF-I and -II which are critical for binding IGFBP-3 are also important for binding to IGFBP-1, -2 and -4 (Oh *et al.*, 1993), but are different from those thought to be important for receptor interaction (Cascieri *et al.*, 1988a; Bayne *et al.*, 1989). Differences in IGFBP phosphorylation also have been shown to change the affinity of IGFBPs for IGF-I (Jones *et al.*, 1991; Frost and Linda, 1991). Phosphorylated IGFBP-1 has a six-fold greater affinity for IGF-I than dephosphorylated IGFBP-1. Thus the increase in IGF-I stimulated mitosis was a result of reduced affinity between IGF-I and dephosphorylated IGFBP-1 and an increased interaction between IGF-I and its cell surface receptor. The mechanism regulating the phosphorylation state of IGFBP-1 has yet to be determined.

IGF Cell Surface Receptors

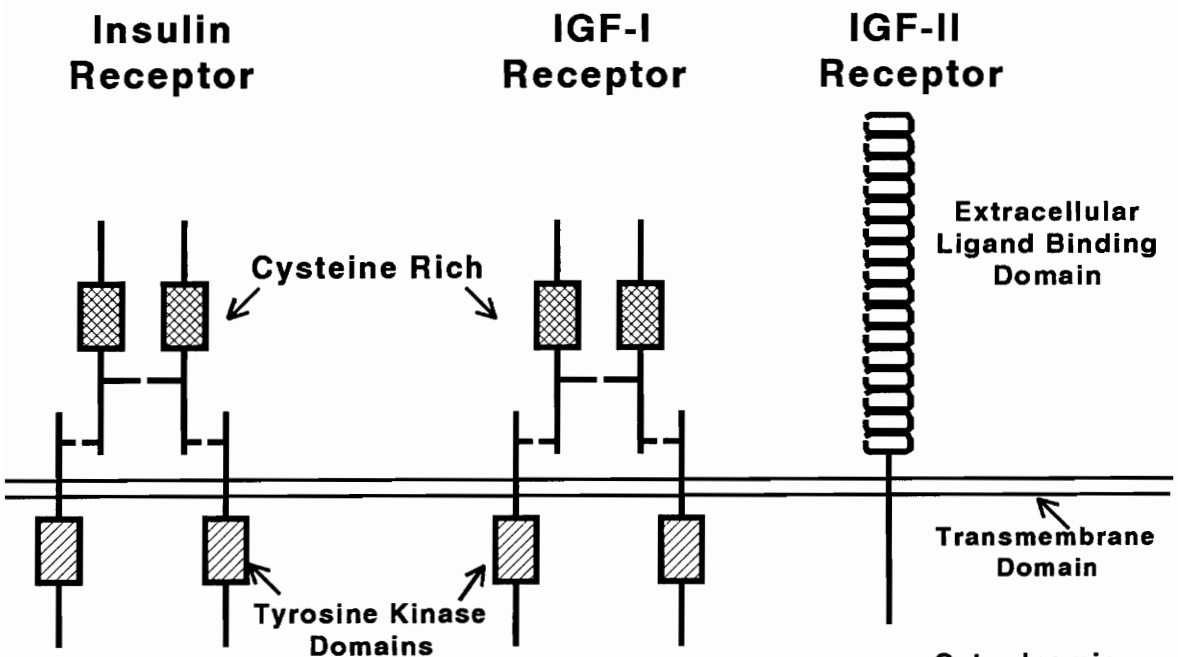
The biological actions of IGF-I and -II are mediated through cell surface receptors. IGF-I "type one" receptors (IGF-IR) have the greatest affinity for binding IGF-I, ten-fold less affinity for IGF-II, and 100-500 fold less for insulin (Cohick and Clemmons, 1993). The IGF-IR is a tetramer (Figure 1-3) consisting of two α-subunits which contain hormone binding domains and two ß-subunits which have a tyrosine kinase activity (Ullrich *et al.*, 1986). This heterodimeric structure is also found in the insulin receptor. Although the IGF-IR and the insulin receptor have similar organizations, the IGF-IR has been shown to mediate mitogenic effects while the insulin receptor regulates metabolic activity (reviewed by Adamo *et al.*, 1992). These actions are thought to be regulated by the distribution and abundance of the receptor on the cell surface.

Upon binding IGF-I, II or insulin, the tyrosine kinase domain in the beta subunit of the IGF-IR receptor phosphorylates endogenous cellular substrates (Sun *et al.*, 1991; Rothenberg *et al.*, 1991). Receptor substrates such as phosphoproteins 185 and 175, have been found associated with other cellular proteins, such as phosphatidylinositol 3' kinase, a well known signal transduction molecule (Yamamoto *et al.*, 1992; Myers *et al.*, 1993). Defects in the IGF-IR tyrosine kinase activity reduced IGF-I stimulated DNA synthesis which was thought to be activated through this signaling pathway (McClain *et al.*, 1990).

Recently, a hybrid receptor which can bind both IGF-I and insulin was identified (Soos *et al.*, 1990). This receptor consisted of an α- and ß-subunit from both the IGF-IR and the insulin receptor. Differential tissue distributions of the hybrid receptors may account for unique mitogenic and metabolic responses upon stimulation (Soos and Siddle, 1989).

A specific IGF-II receptor was also isolated and characterized (Roth *et al.*, 1990). This "type two" receptor is identical to the mannose-6-phosphate (M6P)

Figure 1-3. Comparison of the insulin, IGF-I and IGF-II receptors. The insulin and IGF-I receptors consist of two α domains and two β domains held together by disulfide bonds depicted with dashed lines. The IGF-II receptor consists of a large single-chained polypeptide domain. The small stacked boxes represent the numerous disulfide bonds in the type two receptor.



(adapted from Adamo et al., 1992)

receptor which targets proteins to the lysosome through the M6P modification (Kiess *et al.*, 1988). Unlike the IGF-IR, the type two IGF-II receptor consists of a large single-chain polypeptide domain, a single transmembrane domain and a short cytoplasmic region which lacks protein kinase activity (Figure 1-3). Separate binding sites for IGF-II and M6P have been mapped to the extracellular domain of the receptor (Braulke *et al.*, 1988; Tong *et al.*, 1988). Although IGF-I cross reacts at the IGF-II receptor, insulin does not (Massaque and Czech, 1982). Only a limited number of studies have been conducted to investigate the mechanism of IGF-II signal transduction through the IGF-II/M6P receptor. Recently, G proteins (GTP-binding proteins) were implicated as a part of the IGF-II stimulatory response (Nishimoto *et al.*, 1989). This activation was inhibited by M6P and required a 14 amino acid segment of the cytoplasmic domain (Murayama *et al.*, 1990; Okamoto *et al.*, 1990; Okamoto and Nishimoto, 1991). Further investigations of the interactive role between G proteins and IGF-II receptor signal transduction are ongoing.

Characterization of the IGF-I Gene

IGF-I gene sequence and gene structure have been found to be well conserved in human, rat, chicken and sheep (de Pagter-Holthuizen *et al.*, 1986; Rotwein *et al.*, 1986; Shimatsu and Rotwein, 1987; Kajimoto and Rotwein, 1991; Ohlsen *et al.*, 1993). Confirmation of a single gene responsible for encoding the IGF-I peptide was demonstrated by chromosomal mapping (Brissenden *et al.*, 1984; Tricoli *et al.*, 1984). The human and mouse IGF-I genes have been mapped to chromosomes 12 and 10, respectively, and found to span greater than 80 kb of DNA.

The current number of IGF-I exons has been determined by cDNA sequence analysis and identification of corresponding exon/intron splice site junctions. IGF-I exon/intron junctions have been identified by the application of the "GT-AG rule" in which the 5' and 3' ends of introns share a conserved GT and AG sequence,

respectively. In most mammalian species, the IGF-I gene consists of 6 exons (Figure 1-4). However, only 5 exons have been identified in the chicken IGF-I gene; exon 2 has not been detected (Kajimoto and Rotwein, 1991). Alternative splicing of exon 1 or 2 to exon 3 results in production of a heterogenous population of IGF-I mRNAs (Figure 1-4). Transcripts containing either exon 1 or 2 have been isolated and characterized as cDNAs from human, rat, and sheep (Jansen *et al.*, 1983; Le Bouc *et al.*, 1986; Rotwein, 1986; Casella *et al.*, 1987; Murphy *et al.*, 1987a; Roberts *et al.*, 1987; Wong *et al.*, 1989) and have been classified as "class 1" and "class 2" IGF-I transcripts, respectively. A third class of IGF-I transcripts appears to be specific to rat mRNAs (Shimatsu and Rotwein, 1987). A post-transcriptional deletion of a 186 nucleotide sequence within rat exon 1 resulted in the "class 1 del" transcript. All three classes of transcripts were associated with polysomal RNA of which the number of class 1 del transcripts appeared to be enhanced, thus indicating that all transcript variants were translated into IGF-I precursors (Foyt *et al.*, 1991). Additional alternative splicing also was found near the 3' end of the gene and will be discussed later.

PreproIGFs with different signal peptides are encoded in class 1 and 2 transcripts. Class 1 mRNA transcripts encode a 48 amino acid IGF-I signal peptide and class 2 mRNAs encode a 32 amino acid signal peptide. Similar signal peptide sequences of 49 and 33 amino acid residues were deduced from ruminant cDNA sequences (Figure 1-5). The extra amino acid was derived from an insertion of a glutamine codon (Wong *et al.*, 1989; Fotsis *et al.*, 1989).

Additional 5'-untranslated regions (5'-UTR) in both exons 1 and 2 were found to precede the signal peptide sequence. The lengths of the 5'-UTR of exon 1 and 2 were determined by methods used to identify the 5' end of an exon such as RNase protection and primer extension. Although the location of a single start site has not been found for either class 1 or 2 transcript populations, clusters of start sites have been mapped in human, rat, and chicken (Adamo *et al.*, 1991b; Jansen *et al.*, 1991;

Figure 1-4. Genomic organization of rat IGF-I. Exons are numbered 1-6. Alternative splicing creates three different classes of IGF-I mRNAs. Open and filled boxes represent untranslated and translated regions, respectively.

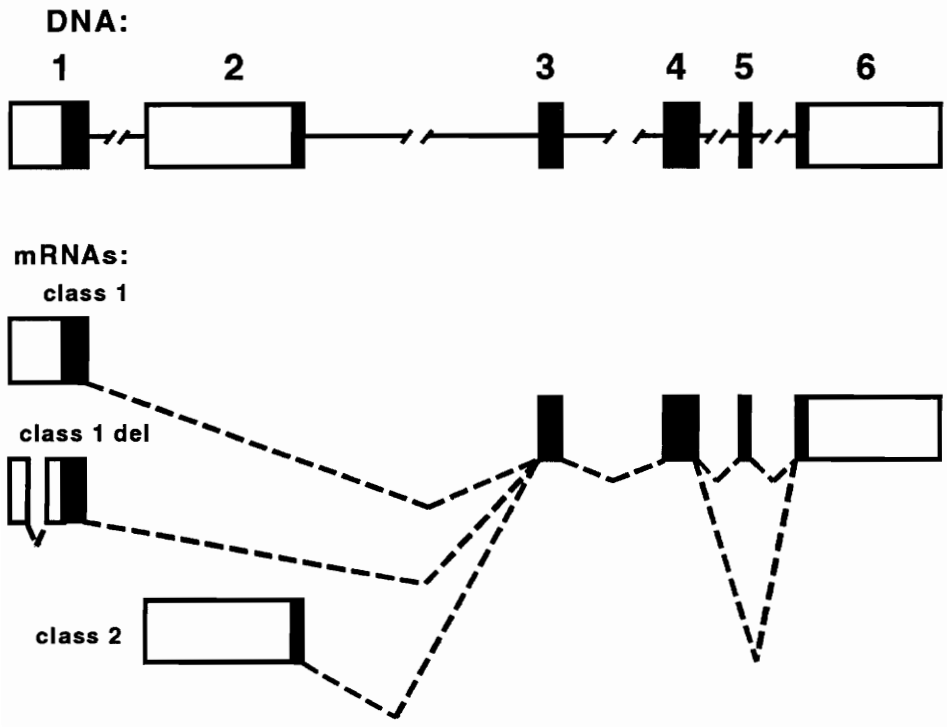

DNA:
1
2
3
4
5
6
mRNAs:
class 1
class 1 del
class 2

Figure 1-5. Ovine class 1 and 2 IGF-I leader peptides.
Class 1 mRNA transcripts encode a 49 amino acid IGF-I signal peptide. The first 21 amino acids (indicated in italics) are encoded within exon 1. The additional 27 amino acids are encoded in exon 3. Class 2 mRNAs encode a 33 amino acid signal peptide, of which 5 and 28 amino acids are encoded in exons 2 and 3, respectively.

Class 1 IGF-I Transcripts

exon 1
GAATAAAGTCCTCAAAATTGAAATGTGACATTGCTCTCAACATCTCCCATCTCCCTGGAT

TTCTTTTTGCCTCATTATTTCTGCTAACCAATTCATTTCCAGACTTTGCACTTCAGAAGC

MetGlyLysIleSerSerLeuProThrGlnLeuPheLysCysCysPheCysAspPheLe
AATGGGAAAAATCAGCAGTCTTCCAACCCAATTATTTAAGTGCTGCTTTTGTGATTTCTT

exon 3
uLys *GlnValLysMET...*
GAAG CAGGTGAAGATG...

Class 2 IGF-I Transcripts

exon 2 exon 3
METValThrProThr *GlnValLysMET...*
CTGTAAAAGATCTGGAACAAACAAAAATGGTTACACCTACA CAGGTGAAGATG...

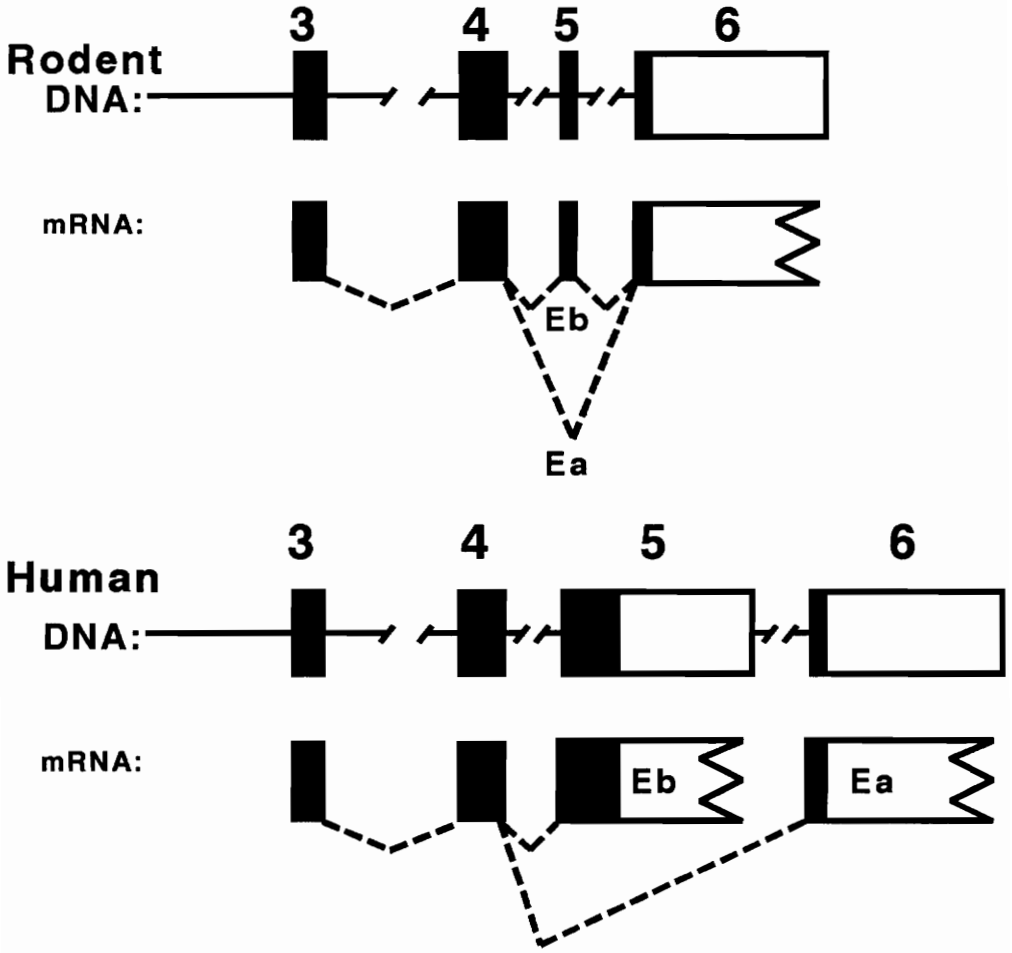
Kajimoto and Rotwein, 1991; Kim *et al.*, 1991; Simmons *et al.*, 1991). A more detailed description of these 5'-UTRs and transcription initiation sites will be discussed in chapter two.

Alternative splicing at the 3' end of the IGF-I gene generates two different COOH-terminal E-peptide domains in human, rat and mouse cDNA sequences (Jansen *et al.*, 1983; Bell *et al.*, 1986; Lowe *et al.*, 1988; Roberts *et al.*, 1987; Shimatsu and Rotwein, 1987). Human peptide sequences of 19 and 61 amino acid residues were found encoded in the cDNAs and were termed Ea and Eb IGF-I domains (Rotwein, 1986). Ea-peptides were generated by the alternative splicing of exon 4 directly to exon 6 in both human and rodent systems, whereas divergent splicing of either exon 4 to exon 5 in humans or exon 4 to 5 to 6 in rodents produced Eb-encoded peptides (Figure 1-6). Lowe et al. (1988) showed IGF-I Ea and Eb transcripts were differentially regulated in hypophysectomized, GH treated rats. In kidney, lung, and heart, both Ea and Eb transcript expression was coordinately regulated by GH while in liver tissue IGF-I-Eb transcripts were preferentially expressed (3-fold). Thus, processing of liver Ea and Eb transcript was shown to be different from non-hepatic tissues.

Two putative glycosylation sites in the Ea-peptide domain have been identified and are active *in vitro*, while no sites have been found in peptides with the Eb-domain (Bach *et al.*, 1990). Differential glycosylation of peptides with either Ea or Eb-peptide domains by *in vitro* translation is thought to reflect *in vivo* post-translational processing. Thus, differential processing and glycosylation may be a mechanism for intracellular identification of the different proIGF-I peptides.

In IGF-I cDNAs, additional 3'-untranslated regions extend beyond the encoded E-peptide domain. Mapping of the 3' ends of IGF-I mRNAs revealed a number of polyadenylation signal sequences. Termination of transcription at these various sites resulted in the production of mRNAs with different 3' termini. Although the biological significance of IGF-I mRNAs of different size has not yet

Figure 1-6. Comparison of alternative splicing at the 3' end of the rodent and human IGF-I genes. Alternative splicing of IGF-I exon 4 to exons 5 and 6 is depicted by the dashed lines. Splicing results in different mRNAs which code for distinct E-peptides. Open and filled boxes represent untranslated and translated regions, respectively.


Rodent
DNA:
3
4
5
6
mRNA:
Eb
Ea
Human
DNA:
3
4
5
6
mRNA:
Eb
Ea

been determined, variation in the length of the 3'-untranslated regions might influence the half-life of the IGF-I mRNA (Hepler *et al.*, 1990). It has been shown in both *in vitro* and *in vivo* studies that rat IGF-I transcripts 0.9-1.2 or 7.0-7.5 kb, which result from the alternative use of two different polyadenylation sites, have different rates of degradation. The half-life of the 7.0-7.5 kb transcripts were much shorter compared with the 0.9-1.2 kb mRNAs. The mechanism which regulates RNA stability is still to be determined.

Regulation of IGF-I Gene Expression

Differential IGF-I gene expression results in the production of multiple RNA transcripts in a variety of tissues at different stages of development. Alternative splicing, multiple transcription initiation sites and polyadenylation sites all play significant roles in determining the length of the expressed transcript. Sizes of IGF-I transcripts have been determined by Northern blotting of polyadenylated RNA from human, rat and mouse tissues (Shimatsu and Rotwein, 1987; Bell *et al.*, 1986; Rotwein, 1986; Roberts *et al.*, 1987; Murphy *et al.*, 1987a; Lund *et al.*, 1986; Han *et al.*, 1988). Analysis of IGF-I mRNA transcript expression has shown that distinctive transcripts are expressed in various tissue types during different stages of development.

During the first week of postnatal development in rats, serum IGF-I levels increase. Increases in transcriptional activation lead to a rise in serum IGF-I (Kikuchi *et al.*, 1992). Quantitation of rat IGF-I transcripts indicated that over 90% of the transcripts found in the liver at early development contain exon 1. Detection of increased DNase I hypersensitivity in the rat IGF-I gene correlated with the increased expression of class 1 transcripts and gene activation (Kikuchi *et al.*, 1992). The increasing number of DNase I hypersensitive sites at the 5' end of the rat IGF-I

gene upstream of exon 1 indicated that throughout development, transcriptional activation of class 1 transcripts were preferentially increased over class 2 transcripts.

Unlike class 1 transcripts, exon 2 containing transcripts were not detected during early postnatal development and appeared 15-22 days after partuition (Kikuchi *et al.*, 1992). Interestingly, these data correlated with reported developmental expression of rat GH receptor mRNA which were not expressed until at least 14-20 days of age (Mathews *et al.*, 1989; Tiong and Herrington, 1992).

Growth Hormone Regulation of Class 1 and 2 IGF-I Transcripts

The regulation of class 1 and 2 transcript expression at the molecular level by GH has been investigated recently. Class 2 IGF-I transcript expression has been shown more responsive to changes in GH status (Lowe *et al.*, 1987). Stimulation of transcription in rats with GH treatment increased both exon 1 and 2 transcripts, however the largest increases were seen in mrnas containing exon 2. GH treatment in sheep also resulted in up-regulation of ovine class 2 transcripts (Saunders *et al.*, 1991; Pell *et al.*, 1993). In addition, further analysis of transcriptional start site usage in exon 1 and 2 transcripts in hypophysectomized and GH treated rats supports the hypothesis that class 2 IGF-I mRNAs levels are more responsive to GH status of the animal (Foyt *et al.*, 1992). Although the length of class 1 and 2 transcripts appeared to have an effect on its translatability, this might reflect putative tissue-specific factors involved in the cellular regulation of IGF-I production (Foyt *et al.*, 1992). Currently, studies are being conducted to elucidate the tissue- and development-specific mechanisms of IGF-I gene regulation.

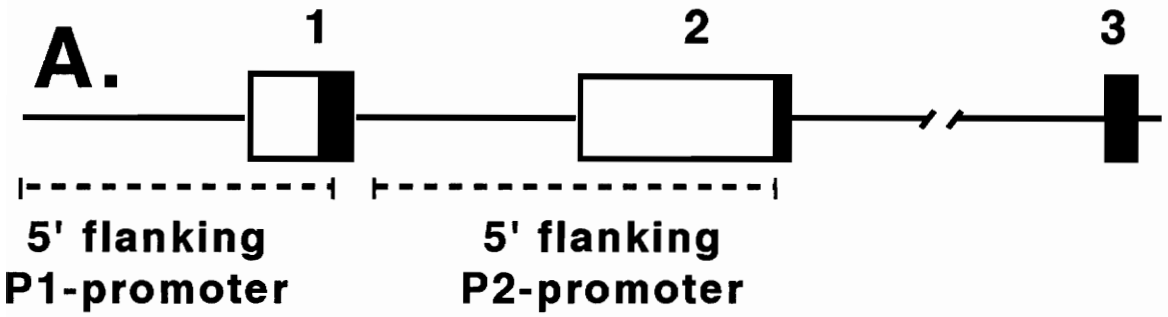
IGF-I Promoters

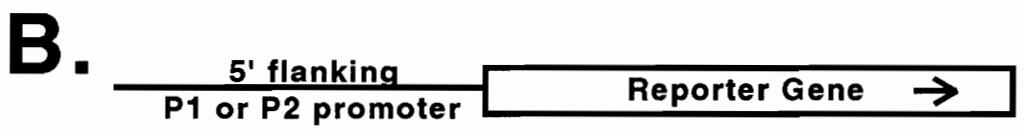
Initial analysis of exon 1 and 2 promoter regions indicated that two independent promoters were regulating class 1 and class 2 transcript synthesis. To test this hypothesis, transgenes were constructed in which putative 5' IGF-I regulatory regions of both exons 1 and 2 were cloned upstream of a reporter gene such as the firefly luciferase gene. Promoter regions for exons 1 and 2 were designated P1 and P2, respectively. Expression of the reporter genes demonstrated that the promoter regions were able to regulate gene expression (Figure 1-7).

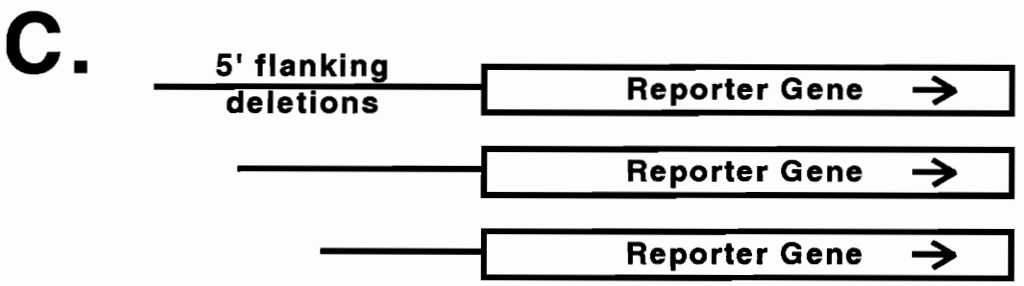
Transgenic constructs which contained deletions of exon 1 (P1-promoter) 5'-flanking regions were searched for potential promoter elements. Analysis of greater than 1600 nucleotides of the rat, human and chicken 5' flanking regions indicated a number of regulatory sequences (Adamo *et al.*, 1993; Lowe *et al.*, 1992; Hall *et al.*, 1992; Kim *et al.*, 1991; Jansen *et al.*, 1992; Kajimoto and Rotwein, 1991; Kajimoto *et al.*, 1993). These regions function in either a positive or negative manner to regulate transcription of exon 1. Deletion constructs were created and transfected into different cell lines by independent laboratories to test the activity of these putative promoter elements. As described below, conflicting results from the expression of these constructs assayed in different cell lines may indicate possible cell-specific regulatory differences.

Comparable results were reported when rat P1-promoter constructs were transfected into Chinese hamster ovary (CHO) and rat dermal fibroblast cells (Adamo *et al.*, 1993; Lowe *et al.*, 1992). Fragments of 5'-flanking regions containing nucleotides -133/+362 were found to maximally stimulate reporter gene activity in an orientation-independent manner, such that cloning of the identical fragment in a forward or backward direction resulted in similar stimulation of reporter gene expression. The localization of a putative negative regulatory element

Figure 1-7. Strategy for analysis of IGF-I promoter regions. A.) The IGF-I promoters are located upstream of exons 1 and 2 and are marked by a dashed underline. B.) Promoter function was tested by cloning the promoters upstream of a reporter gene. C.) Deletion analysis of the promoter regions was used to identify functional promoter elements.


A.
1
2
3
5' flanking
P1-promoter
5' flanking
P2-promoter


B.
5' flanking
P1 or P2 promoter
Reporter Gene


C.
5' flanking
deletions
Reporter Gene
Reporter Gene
Reporter Gene

was confirmed by both transfection studies and was localized to a region ranging from 787 nucleotides upstream of the 3' splice site of exon 1 to 74 nucleotides downstream (-787/+74). Constructs containing this region were found to have reduced reporter gene expression, while constructs lacking this region from -787/+74 showed increased expression. These results contrast with those reported by Hall *et al.* (1992), Jansen *et al.* (1992) and Kajimoto *et al.* (1993) in which rat, human, and chicken 5'-IGF-I fragments extending beyond the putative negative element (-787/+74) were transfected into human neuroepithelioma (SK-N-MC), human ovarian carcinoma (OVCAR-3) or human HepG2 cell lines. These constructs containing the large P1-promoter fragments enhanced reporter gene expression in comparison to the shorter P1-promoter fragments of -400 to -120 nucleotides which exhibited little or no promoter activity. Host cell-specific factors may be responsible for differential expression of these reporter gene constructs. Subsequent studies showed that mutations in the putative promoter element of the chicken IGF-I exon 1 flanking region may inhibit activation of an IGF-I promoter (Kajimoto *et al.*, 1993). This putative promoter element was found within 600 nucleotides of the exon 1 5'-flanking sequence and was thought to be regulated by a member of the protein kinase C pathway. Comparable evidence for this putative promoter element has yet to be discovered in mammalian systems.

The putative promoter elements of exon 2 (P2) have been initially characterized. Unlike P1-promoter regions, characteristic eukaryotic elements such as CCAAT and TATA boxes were identified in the 5'-flanking sequence of rat exon 2 and are thought to direct binding of RNA polymerase II (Adamo *et al.*, 1991b). Difficulties in the early attempts to express P2-promoter/reporter gene constructs in SK-N-MC cell lines were reported (Kim *et al.*, 1991; Hall *et al.*, 1992). More recently, expression constructs containing P2-promoter elements were successfully expressed in SK-N-MC, OVCAR-3, HepG2 and CHO cells (Jansen *et al.*, 1992; Adamo *et al.*, 1993) due to changes in the transfection procedure and/or changes in

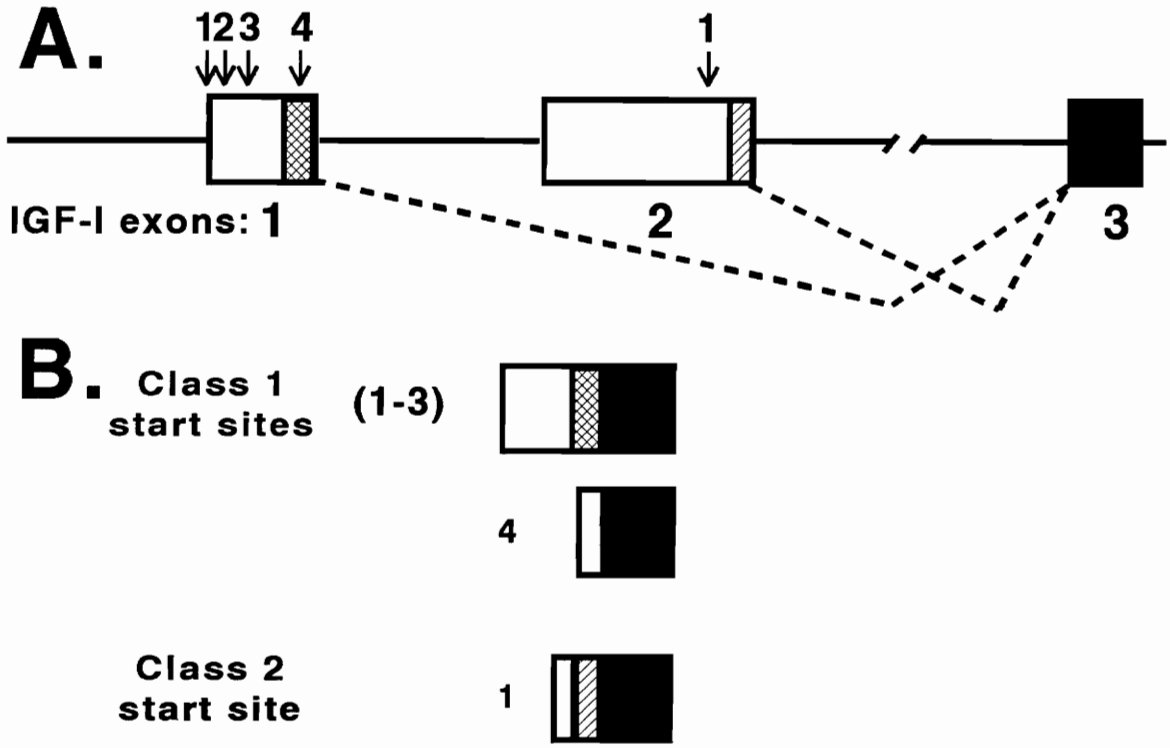
cell type. In general, P2 promoter activity was found to be less than P1 activity, except in OVCAR-3 cells. Stepwise deletions from the 5' end of a greater than 1.2 kb P2 fragment in both human and rat systems resulted in small increases in promoter activity (Adamo *et al.*, 1993). Identification of tissue- and development-specific P2 promoter elements has not yet been accomplished.

Transcription Initiation Sites in Exons 1 and 2

Differential usage of exon 1 and 2 transcription initiation sites demonstrate potential development- and tissue-specific regulation of IGF-I gene transcription. Four major start sites have been characterized for transcription initiation of rat exon 1 (S1-4) (Adamo *et al.*, 1991b; Simmons *et al.*, 1991). All were located approximately 250-350 nucleotides upstream of the 3' splice site of exon 1 (Figure 1-8). Transcription initiated at start sites 1, 2, or 3 generated mRNAs that encoded the 48 amino acid signal peptide. However, start site S4 was located 3' of the methionine predicted to initiate translation of the 48 amino acid signal sequence. It is proposed that transcription initiation at S4 leads to translation initiation at a methionine codon in exon 3 which generates a signal peptide of 22 amino acids.

Tissue specific expression of transcripts initiating at specific start sites has been described by Shemer *et al.* (1992). Extra-hepatic rat tissues such as testis, lung, kidney, heart, brain, muscle, and stomach were analyzed for specific transcript expression. Start sites S1 and S4 were utilized in all tissues at extremely low levels, while transcripts initiating at S2 and S3 were the most abundant. In liver tissue, the quantity of transcripts initiating at S2 and S3 were predicted to be identical while in testes and lung tissue, the S3 initiation site was selectively used. Stomach, heart and muscle tissue were also found to express the majority of class 1 transcripts by using start site S3. No developmental changes were seen in start site usage for lung and stomach tissue. However, start sites used in the developing kidney shifted

Figure 1-8. Rat IGF-I transcription initiation sites in exons 1 and 2. A.) The location of the rat initiation sites are indicated by numbered arrows and alternative splicing is depicted by the dashed lines. Open boxes represent untranslated regions while hatched and cross-hatched boxes depict translated sequences. B.) Transcripts which initiate upstream of the exon 1 and 2 coding regions (class 1 sites 1-3 and class 2 site 1) encode 48 and 32 amino acid signal peptides illustrated by the cross-hatched and hatched boxes inculudes the amino acid residues encoded within exon 3. Class 1 transcripts which initiate at start site 4 within the exon 1 signal peptide sequence have a 5'-untranslated region and encode only the 22 amino acid signal peptide originating within exon 3 (Adamo et al., 1991b).


A.
123 4
1
IGF-I exons: 1
2
3
B.
Class 1
start sites
(1-3)
4
Class 2
start site
1

from transcripts starting at only start site S3 to those starting at both S2 and S3 during late fetal/early postnatal development.

Only one major transcription initiation site located approximately 70 nucleotides upstream of the 3' end of exon 2 was analyzed for tissue-specific expression (Shemer *et al.*, 1992). While exon 2 transcripts were highly expressed in liver tissue, analysis of the extra-hepatic tissues revealed that exon 2 was also expressed in testes, lung, kidney, and stomach. The relative amount of the exon 2 transcripts in these tissues was considerably less than that detected in liver, and no class 2 transcripts were found in heart, brain, and muscle. The developmental profile exhibited in extra-hepatic tissues resembled expression in the kidney of exon 1 transcripts initiated at start site S2. This suggests that regulation of transcript initiation at start site S2 in exon 1 and the exon 2 site may be controlled during development through a similar mechanism.

Characterization of the IGF-II and Insulin Genes

IGF-II and insulin (II) genes are found linked on chromosomes 11, 1 and 7 in human, rat, and mouse, respectively (Tricoli *et al.*, 1984; Brissenden *et al.*, 1984; Soares *et al.*, 1985, 1986; Lalley and Chirgwin, 1984; Brilliant *et al.*, 1987). Two insulin genes (I and II) were identified in rat and mouse genomes. The following cross-species comparisons will refer only to the insulin (II) gene. Like IGF-I, the genes encoding the IGF-II and insulin (II) proteins consist of multiple exon and intron regions. The insulin (II) gene contains three exons separated by two small introns (Bell *et al.*, 1985; Soares *et al.*, 1985; O'Malley and Rotwein, 1988) and spans approximately 2 kb. The insulin (II) gene immediately precedes the IGF-II gene and is transcribed in the same direction. The distance between the insulin (II) and IGF-II gene varies between species ranging from 1.4 to 18 kb in humans and rodents,

respectively (de Pagter-Holthuizen *et al.*, 1987; Ikejiri *et al.*, 1990; Rotwein and Hall, 1990).

The 2 kb insulin (II) gene is much smaller than the IGF-II gene, which spans 25-30 kb (Bell *et al.*, 1985). Human, rat, and mouse IGF-II genes each contain 10, 6, and 6 exons, respectively (Figure 1-9). Homologues to human exons 1, 2, 3, and 5 have not been identified in either rodent genes. The preproIGF-II peptide was entirely encoded within exons 8, 9, and 10 in humans and exons 4, 5, and 6 in rodents. Alternative splicing of the 5'-untranslated exons (1-7) to human exon 8 or 5'-untranslated exons (1-3) to rodent exon 4 generate a heterogenous population of IGF-II mRNAs. The alternative splicing pattern of human and rodent IGF-II genes are shown in Figure 1-10. Of the complex population of human IGF-II gene transcripts, five variant forms were identified. Exons 1, 2, and 3, exons 4 and 5, exon 4, exon 6, or exon 7 were alternatively spliced to human exon 8. The transcripts of all these alternatively spliced forms were isolated as cDNAs and their corresponding transcript sizes are indicated in figure 1-10 (Bell *et al.*, 1985; Dull *et al.*, 1984; Frunzio *et al.*, 1986; Soares *et al.*, 1986; de Pagter-Holthuizen *et al.*, 1987; Chiariotti *et al.*, 1988; Ikejiri *et al.*, 1991).

In contrast to IGF-I, alternative splicing of the IGF-II gene does not affect the amino acid sequence of preproIGF-II because exons 1-7 (human) and 1-3 (rodents) are untranslated. The IGF-II signal peptide of 24 amino acid residues is encoded in human exon 8 and rodent exon 4. The mature IGF-II peptide of 67 amino acid residues is encoded in human exons 8 and 9 or rodent exons 4 and 5. An additional E-carboxyl terminal domain of approximately 89 amino acids is encoded primarily within human exon 10 and rodent exon 6. The fate of this large E-peptide domain is unknown, but is thought to be proteolytically removed during post-translational processing.

Human exon 10 spans approximately 4.1 kb, of which only the first 234 nucleotides encode the carboxylterminus of the IGF-II E-peptide. Two

Figure 1-9. Genomic organization of human, rat and mouse insulin and IGF-II genes. Exons are numbered 1-10 and open and filled boxes represent untranslated and translated regions respectively. Ins indicates the insulin gene and $\Psi_1$and $\Psi_2$are the pseudo exons identified in the mouse IGF-II gene (Rotwein and Hall, 1990).

Insulin and IGF-II Genes

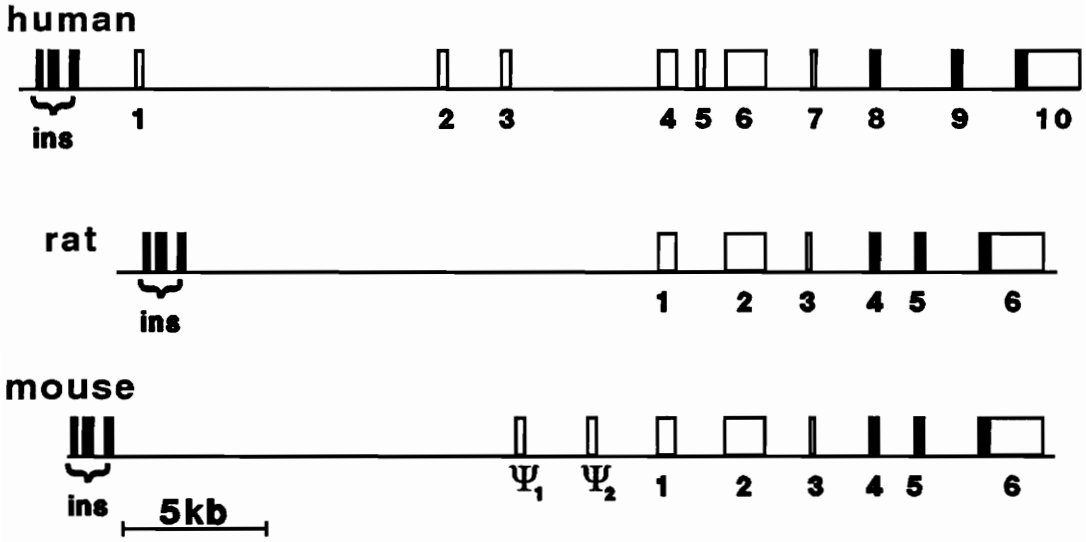
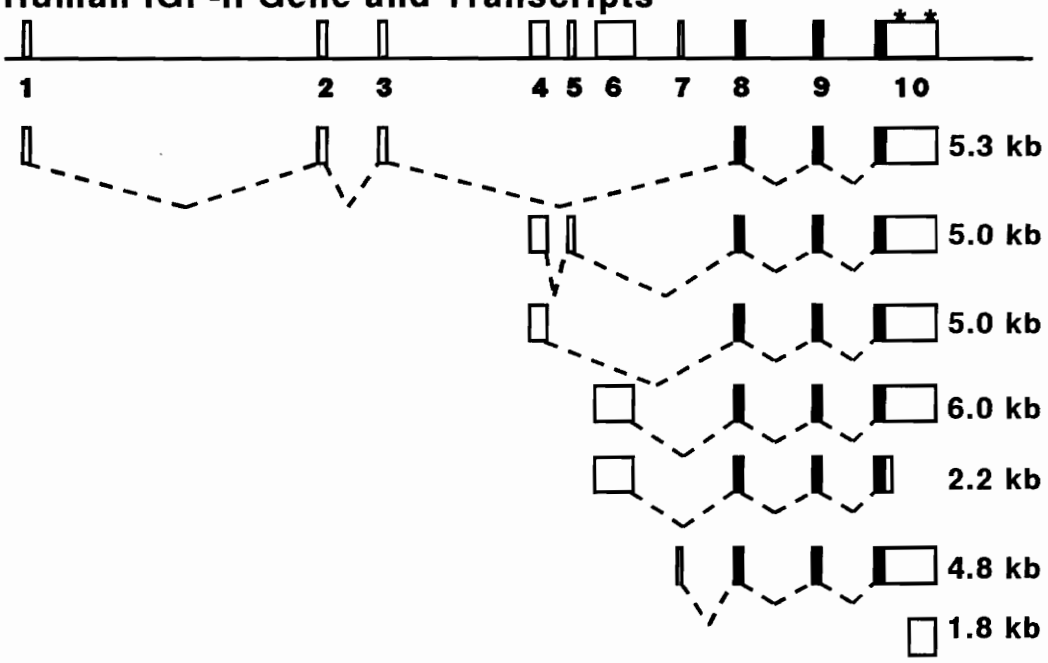
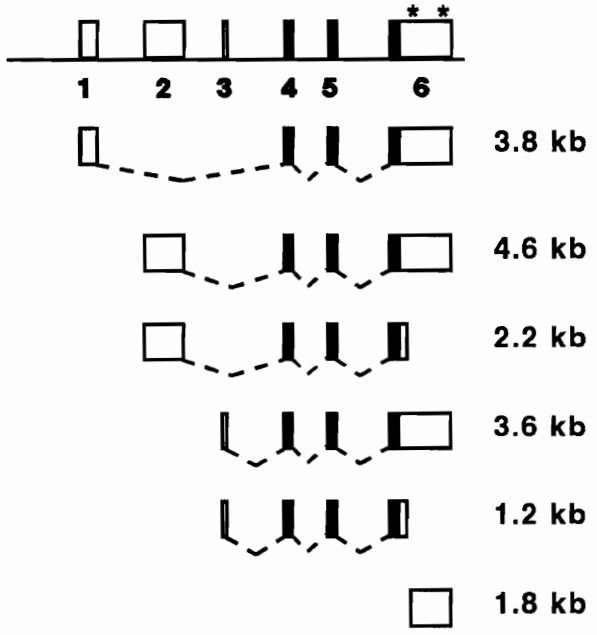


Figure 1-10. Human and rat IGF-II genes and mRNA transcripts. Human and rat IGF-II exons are numbered 1-10 and the asterisks indicate polyadenylation signals in human exon 10 and rat exon 6. Sizes of IGF-II mRNA transcripts are reported in the right hand column in kilobases (kb). Untranslated regions are depicted by open boxes and translated regions by filled boxes (Jansen *et al.*, 1990).

Human IGF-II Gene and Transcripts



Rat IGF-II Gene and Transcripts



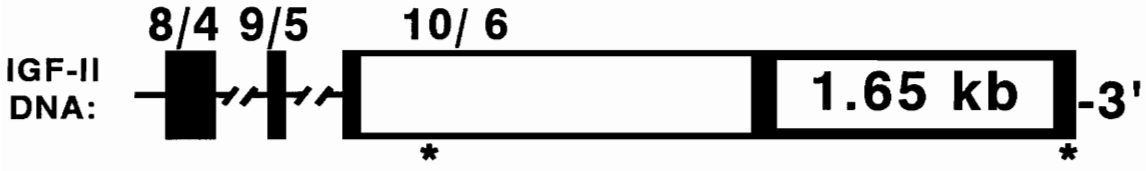
major polyadenylation sites were mapped within the 3'-UTR and are found to be approximately 2.9 kb apart. The alternative use of polyadenylation sites resulted in further heterogeneity in the sizes of IGF-II mRNAs as indicated by the 1.2 and 2.2 kb transcripts in Figure 1-10. Also transcribed within human exon 10 is a novel 1.8 kb transcript (de Pagter-Holthuizen *et al.*, 1988). This 1.8 kb RNA is polyadenylated at the corresponding distal IGF-II site and has a 5' end 1.65 kb upstream from its polyadenylation site with a 252 nucleotide open reading frame (Figure 1-11). Meinsma *et al.* (1991) showed that this 1.8 kb transcript has no independent promoter and arose from endonucleolytic cleavage of IGF-II mRNAs. Expression of similar RNA transcripts were detected in rat and mouse as well (Chiariotti *et al.*, 1988; Meinsma *et al.*, 1992). More recently, two cleavage sites were mapped in the 3'-UT region and were shown to be essential for the production of the 1.8 kb RNA. Although the physiological function of the 1.8 kb transcript is still unknown, synthesis and cleavage of this transcript are thought to be associated with IGF-II gene regulation (Meinsma *et al.*, 1992).

Regulation of IGF-II Gene Expression

Although multiple promoters regulate the expression of the IGF-II gene, little is known about the molecular mechanisms that control the activity of these promoters. Because human promoters P2, P3, and P4 are analogous to rat promoters P1, P2, and P3, respectively, they will be analyzed in pairs. No counterpart to human P1 is present in rodents. In Figure 1-12, four human IGF-II promoters and three rat promoters are shown in relation to the IGF-II gene structure. Analysis of the promoters in human and rat systems has confirmed some similarities and differences between species.

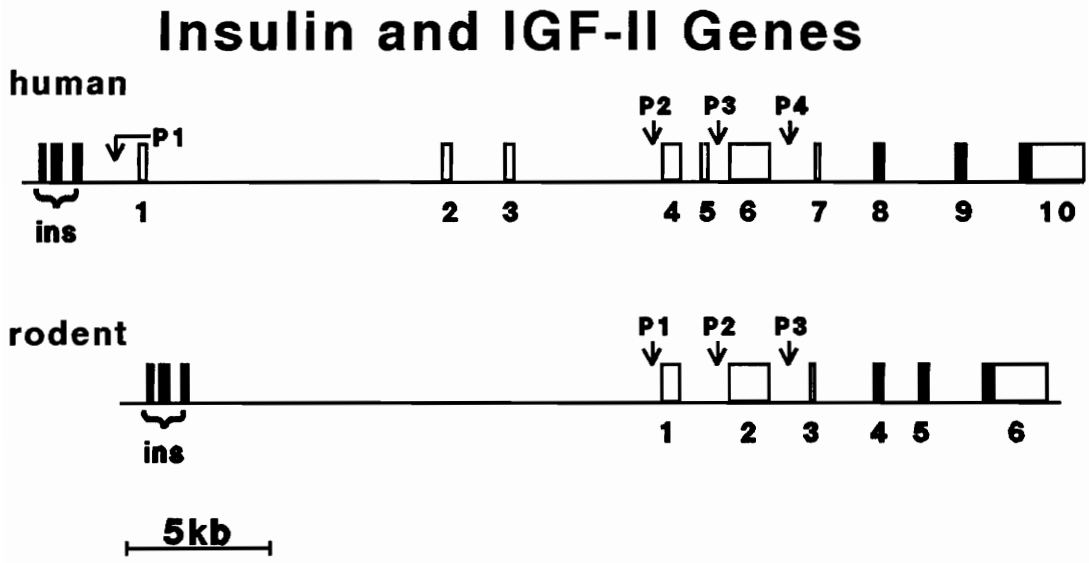
Figure 1-11. Location of the human exon 10 and rodent exon 6 transcriptional unit. The box at the 3' end of human exon 10 and rodent exon 6 (1.65 kb) depicts the coding region of a unique 1.8 kilobase (kb) mRNA transcript. Polyadenylation of the 1.65 transcript results in a mRNA of 1.8 kb.The two asterisks indicate the position of IGF-II polyadenylation signals. Open and filled boxes denote untranslated and translated regions, respectively.

Human/Rodent exons



*= poly (A+) sites

Figure 1-12. Human and rodent (rat and mouse) IGF-II promoter regions. Boxes denote exons 1-10 of the IGF-II gene. Translated regions are depicted by filled boxes and untranslated regions by open boxes. Promoters P1-P4 are marked with vertical arrows.


Insulin and IGF-II Genes
human
P1
P2
P3
P4
ins
1
2
3
4
5
6
7
8
9
10
rodent
P1
P2
P3
ins
1
2
3
4
5
6
5kb

IGF-II Human P1-Promoter

Upstream of human IGF-II exon 1 is a promoter region that regulates the transcription of an RNA containing exons 1, 2, 3, 8, 9, and 10. No homologous exons to human exons 1, 2, and 3 have been found in the rodent genes (Ikejiri *et al.*, 1990; Rotwein and Hall, 1990). Hybridization of Northern blots demonstrated that exon 1 transcripts were the only RNAs expressed in human adult liver (de Pagter-Holthuizen *et al.*, 1987). Thus, the hP1-promoter showed tissue- and development-specific regulation since exon 1 containing transcripts were not expressed during fetal development or in non-hepatic tissues. No common eukaryotic promoter elements (i.e., TATA and CCAAT boxes) were identified in the hP1-promoter region, with the exception of a couple of GC-rich regions thought to play a role as potential binding sites for the transcription factor, Sp1 (Dynan, 1986). Transfection of hP1-promoter/reporter gene constructs showed little reporter gene activity (van Dijk *et al.*, 1991). The exon 1 containing RNA transcripts were not characteristically found in the transfected cell types, suggesting that necessary nuclear factors required for native expression may not be present. Recently, a CCAAT/enhancer binding protein (C/EBP) site has been mapped approximately 100 nucleotides upstream of the transcription initiation site (van Dijk *et al.*, 1992). The C/EBP protein has been shown to regulate liver-specific gene expression in other model systems (Friedman *et al.*, 1989) and was predicted to regulate liver-specific expression of IGF-II exon 1 transcripts. Although footprinting analysis shows that C/EBP protein can bind in the hP1-promoter region, *in vivo* studies have yet to show C/EBP as a functional binding protein of the IGF-II gene.

IGF-II Human P2-Promoter and Rat P1-Promoters

Analysis of the human P2 (hP2) and rat P1 (rP1) promoter regions has been difficult. Similar to hP1-promoter studies, hP2-promoter/reporter gene constructs were transfected into hepatic Hep3B and ovarian HeLa cells (van Dijk *et al.*, 1991). Since Hep3B cells synthesize low levels of endogenous IGF-II transcripts from promoter hP2, this cell line was thought to be an appropriate cell for analysis. Unfortunately, detection of reporter gene expression was not found in either cell line. Use of histiocytoma and leiomyosarcoma tumor cells, where hP2-promoters have enhanced expression, may prove to be a better model system for future promoter analysis (Holthuizen *et al.*, 1990; Ikejiri *et al.*, 1991). Difficulty in the analysis of the rP1-promoter also was reported when rP1-promoter/reporter gene constructs were transfected into three different cell lines (Matsuguchi *et al.*, 1990). Detection of reporter gene expression was also very low in comparison to rP2 and rP3-promoters.

IGF-II Human P3-Promoter and Rat P2-Promoters

IGF-II human P3- (hP3) and rat P2-promoters (rP2) regulate the majority of fetal transcript expression. The hP3- and rP2-promoters contain TATA and CCAAT boxes which are similar to other RNA polymerase II-dependent promoters. Deletion of these elements greatly reduced reporter gene expression (van Dijk *et al.*, 1991; Evans *et al.*, 1988). In addition, transfection studies of hP3-promoter constructs, which have been truncated at the 5' end, confirmed the location of upstream elements which had strong stimulatory effects on reporter gene expression (van Dijk *et al.*, 1992; Raizis *et al.*, 1993). Raizis *et al.* (1993) reported the identification of an additional 5' promoter element which may correspond to the hP2-promoter. A 2.3 kb fragment which contained the complete hP3-promoter sequence, exon 4 and 5

sequences, and part of the hP2-promoter region showed greater expression in human hepatoma HepG2 cells than ovarian HeLa cells. This tissue-specific expression may indicate that HepG2 cells contain essential hP2 transcription factors not found in hepatic Hep3B or ovarian HeLa cells. Analysis of hP2-promoter/reporter gene constructs transfected into HepG2 has not been reported. A total of nine hP3-promoter binding sites have been reported, most of which have weak binding affinity for putative regulator molecules. Thus, strong hP3-promoter activity appears to result from many weak protein/DNA interactions (Raizis *et al.*, 1993).

In contrast, analysis of the rP2-promoter showed that the homologous promoter elements located in the 5' region of the hP3-promoter were not necessary for a functional rP2-promoter (Evans *et al.*, 1988; Matsuguchi *et al.*, 1990). Thus, the rP2-promoter was a simple promoter consisting of no more than 141 nucleotides upstream of the rat exon 2 transcription initiation site.

IGF-II Human P4-Promoter and Rat P3-Promoters

A comparison of the human P4- (hP4) and hP3-promoters demonstrates tissue-specific expression. In three cell lines tested for both hP3- and hP4-promoter expression, the hP4-promoter region was less active than the hP3-promoter, except in a human colon carcinoma cell line (SW613) in which hP4-promoter expression was greater than hP3 (Schneid *et al.*, 1993). Truncation of the 5' end of the hP4-promoter/reporter gene construct decreased reporter gene expression in both Hep3B and HeLa cells (van Dijk *et al.*, 1991). It was concluded that for maximal hP4-promoter activity, additional upstream sequences and factors present in the SW613 cells were required.

The rP3-promoter is different from the hP4-promoter in that only 128 nucleotides were sufficient for transcription. Expression of the rP3-promoter constructs containing additional 5' sequence did not increase promoter activity like

the human constructs and deletions of the 128 bp region greatly reduced promoter function (Evans *et al.*, 1988; Matsuguchi *et al.*, 1990) . Nucleotide sequence analysis of the 128 bp region revealed a TATA box with four upstream GC-boxes which bind the general transcription factor Sp1. These sequence elements also were found in the hP4-promoter region and are thought to play a similar role in transcriptional regulation of human IGF-II gene expression.

Recombinant IGF Cell Lines and Transgenic Animals

Both mammalian and bacterial transgenic expression systems have been used to synthesize biologically active IGF-I (Buell *et al.*, 1985; Peters *et al.*, 1985; Nilsson *et al.*, 1985; Bayne *et al.*, 1987; Cascieri *et al.*, 1988b; Dai *et al.*, 1992; Romagnolo *et al.*, 1992). Initially, the expression of human IGF-I cDNAs in *Escherichia coli* resulted in production of a secreted IGF-I peptide which was denatured and inactive (Peters *et al.*, 1985) Although the peptide could be renatured under *in vitro* conditions, these manipulations complicated interpretation of biological activity of recombinant IGF-I. IGF-I also was expressed as a fusion protein in *Staphylococcus aureus* (Nilsson *et al.*, 1985). This resulted in secretion of a non-denatured IGF-I protein, but still required chemical treatment to remove additional staphylococcal protein A associated with the fusion protein. Expression of the first biologically active synthetic IGF-I gene was reported by Bayne *et al.* (1987). A synthetic IGF-I gene was constructed and cloned with a bovine growth hormone (bGH) signal peptide sequence previously shown to function in an expression vector system. Transient transfection of mouse fibroblasts (L cells) with the IGF-I expression vector resulted in synthesis of a fusion IGF-I protein. The bGH signal peptide was removed during processing, and secreted IGF-I was biologically active.

Similar studies reporting the synthesis and secretion of IGF-I have appeared in the literature since 1987. Overexpression of IGF-I in rat thyroid follicular cells

(FRTL-5 cells), mouse fibroblast cells (L cells), Chinese hamster ovary cells (CHO cells) and bovine mammary epithelial cells (MAC-T cells) resulted in various cellular responses to the IGF-I secreted into the medium of the growing cell cultures (Cascieri *et al.*, 1988b; Bovenberg *et al.*, 1990; Dai *et al.*, 1992; Romagnolo *et al.*, 1992). While FRTL-5, CHO, and MAC-T cells demonstrated a mitogenic response to the IGF-I in the medium, prolonged exposure to high concentrations of IGF-I in transfected mouse L cells inhibited IGF-I stimulated DNA synthesis. The loss of responsiveness was not due to the loss of IGF-I cell surface receptors, but was attributed to postreceptor desensitization, which eliminated cellular responsiveness to IGF-I (Cascieri *et al.*, 1988b).

Transgenic model systems also have been created to test overexpression of IGF-I *in vivo*. The growth promoting potential of IGF-I-transgenic mice was different from previously reported GH-transgenics (Mathews *et al.*, 1988; Palmiter *et al.*, 1982). The weight/growth ratio and skeletal growth were reduced in the IGF-I-transgenic mice while reproductive maturation was slowed as compared to the GH-transgenics. Increases in splenic, pancreas and kidney organ growth were observed also. These results were consistent with previously mentioned accelerated spleen growth identified in IGF-I treated mice and sheep (Siddiqui *et al.*, 1992; Gallaher *et al.*, 1993). Cross breeding between the IGF-I-transgenics and GH-deficient mice generated progeny in which weight and linear growth were indistinguishable from nontransgenic siblings (Behringer *et al.*, 1990). The overproduction of IGF-I in the cross bred mice acted as a physiological substitute for GH. While IGF-I was important in mediating GH stimulation, these experiments also suggested that both GH and IGF-I have independent functions, since unique growth promoting effects were observed in liver and brain.

Gene targeting experiments which disrupt normally functioning genes were used to study the role of IGF-II during growth and development. DeChiara *et al.* (1990) showed that mutations in the IGF-II gene result in reduced birth weights up

to 40%, indicating the necessity of IGF-II production for normal fetal growth. In addition, IGF-II gene targeting experiments showed the mouse IGF-II gene undergoes tissue-specific parental imprinting (DeChiara *et al.*, 1990). This imprinting results in expression of IGF-II from the paternally inherited chromosome and repression of the maternally inherited allele in most tissues of the developing embryo. Little is known about the molecular basis of imprinting, but the IGF-II gene is currently being used as a model system in which to study the chromatin and methylation state of the gene during transcription (Sasaki *et al.*, 1992).

Conclusion

In conclusion, the role of the IGFs during differentiation, growth and development is very complex. The structures of both IGF-I and IGF-II genes are remarkably similar. Both genes consist of small exons dispersed among large regions of chromosomal DNA. Alternative splicing of either untranslated or translated first exons results in the production of a heterogenous population of IGF mRNAs. IGF-I and IGF-II gene expression appears to be controlled by multiple promoters which are regulated in a development- and tissue-specific manner. It has been proposed that this "insulin gene family" is derived from one common ancestral gene and that duplication of this ancestral gene during evolution led to the unique IGF genes. To better understand the role of IGFs in physiology, additional molecular analysis of these genes and their expression is necessary.

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CHAPTER II
Characterization of Multiple Transcription Initiation Sites of the Ovine Insulin-Like Growth Factor-I Gene and Expression Profiles of Three Alternatively Spliced Transcripts.

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Ohlsen, S. M., D. M. Dean, and E. A. Wong. 1993. Characterization of multiple transcription initiation sites of the ovine insulin-like growth factor-I gene and expression profiles of three alternatively spliced transcripts. DNA Cell. Biol. 12:243.

Abstract

Alternative splicing of ovine insulin like growth factor-I transcripts generates three different mRNAs. Class 1 and class 2 transcripts contain exons 1 and 2 spliced to exon 3, respectively. A novel IGF-I mRNA containing exon W is spliced to exon 3 and has been located upstream of exon 1. No in-frame methionine codon was present in exon W and therefore translation is proposed to initiate at the methionine codon present in exon 3. Using primer extension, transcription initiation sites were found 179, 336, and 368 nucleotides upstream of exon 1 and 86, 96, 131, and approximately 850 nucleotides upstream of exon 2. The location of these transcription initiation sites are well conserved among mammalian and avian IGF-I genes. Expression of exon 1, 2 and W specific transcripts was examined in brain, heart, kidney, liver, lung, skeletal muscle, and spleen from adult ewes or 75 day fetal lambs using a reverse transcriptase-PCR assay. Exon 1 transcripts were the most abundant and found in all fetal and adult tissues. Exon 2 transcripts were found in all tissues and were generally expressed the highest in adult liver. Exon W transcripts also were found expressed in all tissues examined. Thus, the three alternatively spliced ovine IGF-I transcripts were expressed in a variety of fetal and adult tissues.

Introduction

We have previously cloned and sequenced three different cDNAs (Wong *et al.*, 1989) encoding ovine IGF-I (oIGF-I). Two of these oIGF-I cDNAs represent class 1 and class 2 IGF-I transcripts. The third oIGF-I cDNA contained a unique 5' sequence designated exon W which has not been reported previously in other species. In order to study the role of these multiple IGF-I mRNAs in the regulation of ruminant growth, we have cloned and sequenced the oIGF-I gene and mapped putative transcription initiation sites for both class 1 and 2 transcripts as well as exon W transcripts. Class 1, 2 and W specific transcripts were found to be expressed in all fetal and adult tissues examined.

Materials and Methods

Animals and tissue collection:

Pregnant and non-pregnant crossbred ewes were used for fetal and adult tissue collection. Ewes were slaughtered when fetuses were 75 days of gestation. Brain, heart, kidney, liver, lung, spleen and skeletal muscle samples were collected, minced, immediately frozen in liquid nitrogen, and stored at -80°C until analyzed. Three fetal and three adult animals were used for analysis.

Cloning and sequencing:

Two genomic sheep libraries were constructed using adult liver and lambda phage EMBL3 or purchased pre-made from Clontech Laboratories (Palo Alto, CA). The libraries were screened either with an ovine IGF-I cDNA (Wong *et al.*, 1989) or exon-specific oligonucleotide probes. Positive lambda clones were mapped with restriction enzymes and exon containing fragments were subcloned into phagemid

vector pTZ18R (Pharmacia, Piscataway, NJ). Nested sets of deletions were constructed using an exoIII/mung bean nuclease kit (Stratagene, LaJolla, CA) and sequenced using the Sequenase version 2.0 kit from United States Biochemicals (USB, Cleveland, Ohio). Sequencing compressions were resolved by the substitution of deaza-GTP or dITP for dGTP in the sequencing reactions. Much of the introns represent sequencing on one strand only, with the exception of most of the region between exons 1 and 2 which was sequenced on both strands.

Sequence analysis software from the Genetics Computer Group (Madison, WI) was used to align overlapping clones and process sequence data. Comparison of IGF-I sequences across species was accomplished by pairwise alignments of different IGF-I sequences. Percent identity was calculated by dividing the number of mismatches by the total number of nucleotides aligned.

RNA isolation and primer extension:

Putative oIGF-I mRNA initiation sites were identified in fetal and adult ovine tissues by primer extension. Oligonucleotide primers specific for exon 1 (oligo 1a: 5' TGCTTCTGAAGTGCAAAGTCTG 3'; nt 1481-1502), exon 2 (oligo 2a: 5' TGTTTGTTCCAGATCTTTTACA 3'; nt 3341-3362), and exon W (oligo Wb: 5'GGAGGATGGGCGAGCAAT 3'; nt 975-992) were end-labeled with [γ-^{32}P] ATP (DuPont/NEN, Boston, MA) and T4 polynucleotide kinase (USB). The labeled oligonucleotides were separated from unincorporated [γ-^{32}P] ATP using Sephadex G-25 spin columns. Total RNA was isolated from frozen tissue samples by acid-phenol-guanidinium thiocyanate extraction (Puissant and Houdebine, 1990) and hybridized to labeled oligonucleotides by boiling for 2 min followed by incubation on ice for 15 min. Primer extension reactions were carried out in 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, 0.5 mM dNTPs, 20 units rRNasin (Promega, Madison, WI) and 500 units Moloney murine leukemia virus (M-MLV) reverse transcriptase (USB) for 60 min at 37°C. The cDNA products were purified

by the method of Livak (1990) and analyzed on 7% denaturing polyacrylamide gels.

Reverse transcription and PCR amplification:

Total RNA was used as a template for the synthesis of partial cDNAs. Hybridization of 100 ng oligo 3a (5' GTCTCCGCACACGAACTGGA 3'; exon 3, nt 306-325) to 6 μg total RNA was performed by heating at 95°C for 5 min followed by slow cooling to 37°C. Partial oIGF-I cDNAs were synthesized in 1 X PCR buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% Triton X-100), 100 units M-MLV reverse transcriptase (Promega), 1.5 mM $MgCl_2$, 2.5 mM dNTPs, and 20 units rRNasin at 37°C for 1 hr. Samples were fractionated on Sephadex G-50 spin columns to remove excess primers and then incubated at 37°C in 0.1 N NaOH and 0.03 M EDTA to remove RNA. Reactions were neutralized with 0.3 M Tris-HCl (pH 7.4) and ethanol precipitated in the presence of 2.5 M ammonium acetate. The purified cDNAs were resuspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and three equal aliquots were used for PCR amplification.

Oligonucleotide primers complementary to the three oIGF-I 5'-untranslated regions were used to amplify the cDNAs. These primers included oligo Wa (5' ATTGCTCGCCCATCCTCC 3'; nt 975-992) oligo 1b (5' ACATCTCCCATCTCCCTG 3'; nt 1421-1438) and oligo 2b (5' TACCCACCCTGACCTGCT 3'; nt 3324-3341). In pairwise combinations with oligo 3b (5' GAGCATCCACCAACTCAGCC 3'; exon 3, nt 286-305), oligo Wa, 1b, and 2b generated PCR products of 160, 269, and 181 bp, respectively. For PCR amplification, 150 ng of oligo Wa, 1b, or 2b, and 150 ng of oligo 3b were added to the tubes containing cDNA and Taq DNA polymerase (Promega). The reaction was brought to a final volume of 100 μl with 1 X PCR buffer and topped with a thin layer of mineral oil. Each reaction was subjected to approximately 45 PCR cycles (94°C for 60s, 45°C for 135s, 72°C for 60s) using a Precision GTC-2 thermocycler (Chicago, IL). Mineral oil was subsequently removed by extraction with chloroform.

Amplified DNA fragments were electrophoresed on 2% agarose gels and transferred to Magna nylon membranes (MSI, Westboro, MA) by Southern blotting. Hybridization with a labeled probe, oligo 3c (5' GATGCCAGTCACATCCTCCTCGC 3'; exon 3, nt 190-212) was carried out according to the protocol recommended by MSI. Blots were exposed to Kodak XAR-5 film with intensifying screens at -80°C.

Results

Cloning and sequencing of the ovine IGF-I gene

Screening of two ovine genomic libraries with an oIGF-I cDNA and exon-specific oligonucleotide probes resulted in the isolation of three non-overlapping lambda phage containing oIGF-I exons. Lambda 21 contained exons W, 1, 2, and 3, lambda 187 contained exon 4, and lambda 71 contained exon 6. The arrangement of the six oIGF-I exons are shown in Fig. 2-1. We and others (Dickson *et al.*, 1991) have found no evidence for exon 5 in oIGF-I cDNAs. Because recombinant lambda clones containing the complete introns between exons 4 and 6 have not been isolated, it is estimated that the oIGF-I gene is greater than 30 kilobases.

The DNA sequence encoding these six exons and part of the flanking introns are shown in Fig. 2-2. With the exception of a few nucleotide changes, this sequence is essentially the same as that reported by Dickson *et al*, (1991). Transcribed regions are shown in upper case type and introns are shown in lower case type. All of exon 6 is presumed to be transcribed because we have previously observed a 7.5 kb oIGF-I transcript (Wong *et al.*, 1989), and Steenbergh *et al.* (1991) have shown that the high molecular weight, 7.6 kb human IGF-I transcript contains a large 3' untranslated sequence which is derived from a single exon.

Previously, we have reported that different exons can be alternatively spliced

Figure 2-1. Organization of the ovine IGF-I gene. Exons W, 1 and 2 are alternatively spliced to exon 3. Exon 5 has not been identified in the oIGF-I gene.

Ovine IGF-I Gene

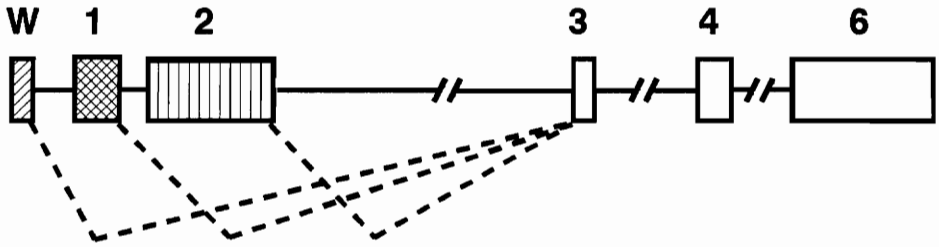

Figure 2-2. Nucleotide and predicted amino acid sequence of the ovine IGF-I gene. Transcribed regions are shown in upper case type and introns are shown in lower case type. Exon W is also underlined. Transcription initiation sites for exons 1 and 2 are in bold type. For exon 2, the transcribed region for only the proximal transcription initiation sites are in upper case type, because the precise start of transcription has not been determined for the distal site. The oIGF-I nucleotide sequence data reported will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession numbers X69472 (exons W, 1 and 2), X69473 (exon 3), X69474 (exon 4), and X69475 (exon 6).

EXONS W, 1 and 2

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1     caacccctttcaaaaccaatcaggttttcctttgagggcacccacctctgcaacagcttcctggccatggcataaagacc
81    tagacaagagtttcaagcaaaccttgtttttagtcgaagcatcaggatcactgtgtcccctgagaataagagggagaaa
161   tacaaggtccaggctactcccaccattccagaaaaccatgccccaatagaccccagggaaagtgggatgtctaacctggg
241   cttttgcaatcttatttcataatccactttcttatcgcctccttcacaaaactgatgagaaattggtacaaactctatcg
321   acaaaagatcacaacttgatcctcaatggcaaaggcaagtatacattataaatagcaaaacagctggcttggaccatgtt
401   gctggccactcatccagctgagagatttgaatgacatcataacccttgagagggtattgctagccagctggtgttattta
481   gaatacacaaaaaggggggaaagaaaatgcactcacatgcacacacacacaaatacacacacacacacaggttcaagt
561   tatgcagaaaaatatgaacagtgggaaaatcatttgcctctcagatgcccttcccctggtgtggggtgggggtcggggtg
641   ggggccaagcagcagaatagaggaaggaagaaagagattcgatttttatttttcagttggctttacagctcagcaaaatc
721   tttgccctgtcgtgggcaaaaagcatgagacagtgtcctgaggggagccaattacaaagctgcctgccccttccaggtt
801   ctaggaaatgagatcattcccctcacttggcaaccaggacgaggggtcatcccagcgccgtcttccagtctagtttaccc
881   caGTCGTTTGAGGGTTAAAATCATAGAGTATGCTTGAGATGGTCTTTTTTTCATTTCTTGTTTTTTAAATTTTGTGTTGG
961   CTCTGGAATATAAAATTGCTCGCCCATCCTCCACGAATATTCCTTTCATACGGgtaaggtgtattagcagatgtgtgtgt
1041  cttcatgcccggtagaaagttaatcagaggacagcatcaggattttaatgtctgctcctcttgtcactaacacacattct
1121  tttaagggaaaaaaatgcttctgtgctctagttttaaaatgcaaaggtatgatgttatttgtcaccatgcccaaaaaAGT
1201  CCTTACTCGATAACTTTGCCAGAAGAGGGAGAGAGAGAGAAGGCAAGCGTTCCCCCAGCTGTTTCCTGTCTACAGTGTCT
1281  GTGTTTTGTAGATAAATGTGAGGATTTTCTCTAAATCCCTCTTCTGTTTGCTAAATCTCACTGTCACTGCTAAATTCAGA
1361  GCAGATAGAGCCTGCGCAATGGAATAAAGTCCTCAAAATTGAAATGTGACATTGCTCTCAACATCTCCCATCTCCCTGGA
1441  TTTCTTTTTGCCTCATTATTTCTGCTAACCAATTCATTTCCAGACTTTGCACTTCAGAAGCAATGGGAAAAATCAGCAGT
                                                                    METGlyLysIleSerSer
1521  CTTCCAACCCAATTATTTAAGTGCTGCTTTTGTGATTTCTTGAAGgtaaatatttcttactctttgaagtcattggggaa
      LeuProThrGlnLeuPheLysCysCysPheCysAspPheLeuLys
1601  ttttctttaaattgtgtactgcttgcttctgcttagaaatgttcttcactttagaattttcattgtttcggcactgggag
1681  ttatttataaattgctgaatatgcaattctgtgggatctgaaaaaatagctccgggagataaatgcctttgcacagatat
1761  ctgtatgagtaaaaactattgcaaggtacttatgctaaatcctccacttcttcagggcttgagtggtgtcattatagaag
1841  attcctttaaatcctgtctatggttaagggctatagggcatggatatgaacttttggatttttttgcaggtgcagatgt
1921  tttatttttaagaccatgttcatgtgtatgtagggctgtgtggtgtgtgtgtgtgcgtgtgtgtaactggccaggacttt
2001  tgattacaagacatgccacatacaaagagttacgttttaaatgatattaaagctttaaatatgatctttggagctaagg
2081  tcccggaactctctgcacttatgcccagagagtcaaagttagagtgaagtttcatttgctcttctgaaaaagaactcctt
2161  aagaactcctgctgaccttgcatattcggataatttaaacaaatgcacactgtatatggaaagcggaaactttctagcaa
2241  ctgctattccaagtttttttcttttgaagaggactctcaggggcgaagtttggacttggggtcttgtgttgtaaaacacgg
2321  gttttgtatttgggattgtaaagtctctttaggtgaatttggctagcgctgtcaatgcactgacttcgcctttccaataa
2401  ctggccctagttcaagtttccattctcagcaaaattatatctttcaagacttgtatttttccaatttgcaagcattttta
2481  agctgctgtcactggctccccgatgcaattgcctgtgggctcaattcatgatccgtccctaccgcttagtccaacactta
2561  tttgccggctttccagcactccaccactaggacgtttcttagtcaagtcagtggcttaggagttaagaatacatccttgt
2641  cgggcacctgatcctgccgcccttgagaagccaagatgcacactgcctaccttgcttttaaaagaaatgaagtcagtata
2721  cacatatgctatgtggtctgacagctggggattttgtatccctacttagaggatcctaaaaggggctgtgtagtgttatc
2801  tctgcattaattacaagtttggaaaactccaaatgaactttccatgctgtgtatgctgaacttttcagaagtagagctag
2881  ctagccataagttgttgctttttcctgtacttgaagcaggaagtggtttcagggagctacgtggttctttcaaatgtaat
2961  tcaatgagtaaaggtgtctgccaggcagagctcacaagctgattgtactgtgagtctcaagatatttccaagtgtttgag
3041  tcagaggaaagagggcacaggggaggactggagcttcggtccctgtccaggacggctacaataggcacacgatggaaatc
3121  agtggcttgattgggaggaaaagattgactcagatcccagccgtgcaatttgtttattgtctgaatggacaaaaggcagt
3201  ttacccaggctcgtagcatacctgcctgggtgtccaaatgtaactaGATGCTTTCACAAACCCCACCCACAAAGCAGCAC
3281  ATGTTTTTAAGTCCTCAGTTTTCTATTCACATCAGTCTCATAATACCCACCCTGACCTGCTGTAAAAGATCTGGAACAAA
3361  CAAAAATGGTTACACCTACAgtgagtattttcttatgactattgccctcaaattttgctgggcatttttattataaccca
           METValThrProThr
3461  gacatctggaaccaattgatattccatttatttaagataaaaagaaaggtttttaaaattttggatttgtgaatgatttt
3521  tgagaaagagtgctctgaaatttttttttctcgtctctttctagttcttctttcatttctttctttcagatctctccttt
3601  ctctctcttcatttctttttgtgtttggaaaaataaaagaccaaggaaatgatgaacatatggggccactcatttcaaact
3681  tttaactcctaagcaagttcttcattgttttctttttgggggaacataatgtgataaattctctggttctctgtgggtgtg
3761  atggactg                    ~4 kb
```

EXON 3

```
1     aaagtcagatagcatgatgccaagacctgctcagaggtcactcacacaccttgttgcactcctgaggggagagttttgat
81    taataacaggaatgcagagatggggtaaaggcacccttgccttgcccccactcccctggcaaggacccaggaggaagatg
161   accctccttctgctctttcagCAGGTGAAGATGCCAGTCACATCCTCCTCGCATCTCTTCTATCTGGCCCTGTGCTTGCT
                           GlnValLysMetProValThrSerSerSerHisLeuPheTyrLeuAlaLeuCysLeuLe
241   CGCCTTCAGCAGCTCTGCCACGGCGGGACCCGAGACCCTCTGCGGGGCTGAGTTGGTGGATGCTCTCCAGTTCGTGTGCG
      uAlaPheSerSerSerAlaThrAlaGlyProGluThrLeuCysGlyAlaGluLeuValAspAlaLeuGlnPheValCysG
321   GAGACAGGGGCTTTTATTTCAgtaagtagcctccctctcgctgctctgtggatttacaactgcggggagtgtgtgaagag
      lyAspArgGlyPheTyrPheA
401   ctgaatgaccgcctgtggctggcagccatcctcagcctccgagattcctcaccctaatgcgagcctagtgtttcagcggg
481   gccgtggcacgttttgcagattttggatggagttttcctccttagcagtttgtcttttaactactcccactaggctttcc
561   cgcaactgttctg
```

EXON 4

```
1     gtagcagtgaacaccgcctattatcccactctaaaactaggcctctctctgatctgcacagACAAGCCCACGGGGTACGG
                                                                   snLysProThrGlyTyrGl
81    CTCGAGCAGTCGGAGGGCGCCCCAGACAGGAATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTGAGGAGGCTGGAGA
      ySerSerSerArgArgAlaProGlnThrGlyIleValAspGluCysCysPheArgSerCysAspLeuArgArgLeuGluM
161   TGTACTGTGCGCCTCTCAAGGCCGCCAAGTCGGCCCGCTCAGTCCGTGCCCAGCGCCACACCGACATGCCCAAGGCTCAG
      etTyrCysAlaProLeuLysAlaAlaLysSerAlaArgSerValArgAlaGlnArgHisThrAspMetProLysAlaGln
241   AAGgtaagcccaccaggggcggcggtgagggtcggccatcttcgcgagatctgagttatgtggttgtagcaacttagcag
      Lys
321   cagcagcatctgaatagtaattcataccctaatagacttctggctcatagggtcaaaagagctccattctcccctgagag
401   gttcatcttttctgtcactccacattataaattctcccttccaccgctgtggcccatctgatcaccagaagatggtggag
481   aagagtgactaaacctgggctttggcatcagacaaaactgggatcc
```

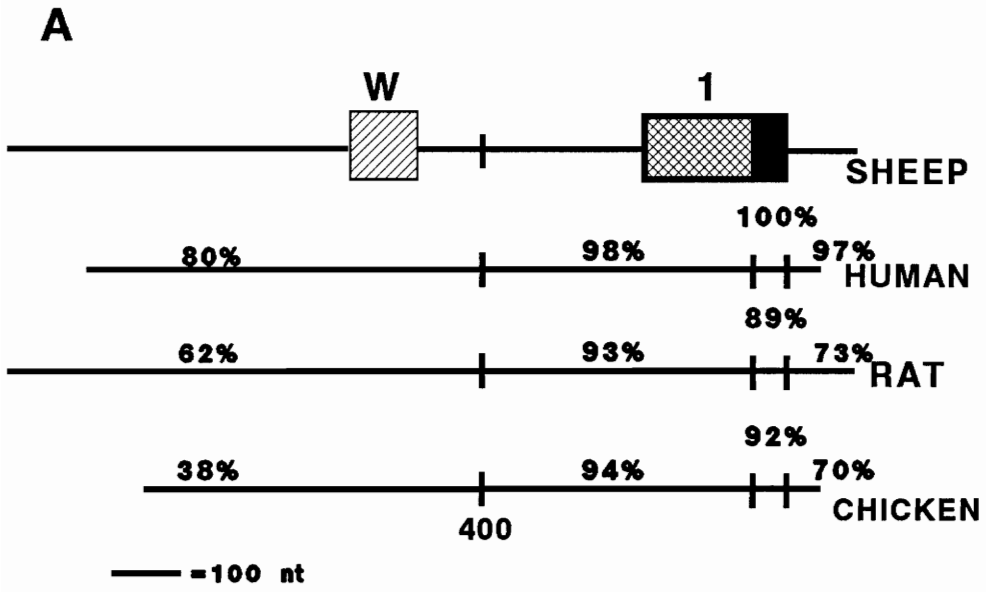
EXON 6

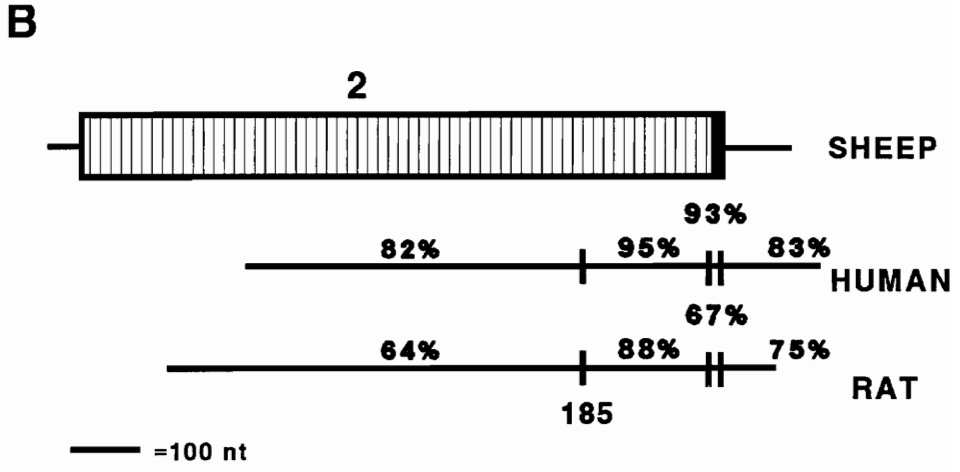
```
1     gtgagacataaggtatgacggttgagatccagtgttagaaatagataggttggatacataaacattcatcaaattaatgt
81    cacttttctcccttatttttagGAAGTACATTTGAAGAACACAAGCAGAGGGAGTGCAGGAAACAAGAACTACAGAATG
                           GluValHisLeuLysAsnThrSerArgGlySerAlaGlyAsnLysAsnTyrArgMet
161   TAGGAAGCCCTTCCTAAAGAGCGAAGAATGACATGCCACCGGCAGGATCCTTCGCTCTGCACGAGTTACCTGTTCAACAC
241   CAGAAGACCTACCAAAAATAAGTTTGATAACATTTCAAAAGATGGGCATTTCCCCCAATGAAATAAGTAAACATTCCAAC
321   ATTGTCTTTAGGAGTGATTGTTCAAAGCTTTGCACCTTGCAAAAATGACCCTGGAGTTGGTAGATTGCTGTTGATCCTTT
401   ATCAATAATGTTCTATAGAGAAAATATATATATAGATCTTAGTCCCTGCCTCTCAAGAGCCACAAATGCATGGGTGTTGT
481   ATAGATCCAGTTGCACTAAATTTATCTCTGAATCTTGGCTGCTAGTGACATTCATTCAGCAGGCTTGTCTAAGTGGTTTA
561   TTAATTTTTTATTTGCACTTCTTTCTACGCAACACAGGCTGTTTGTTTTACAGTGTCTGATAATCTTGTTCCTCTATCCC
641   GCTCCCTTCTCCATCCCCTTTATATTTGCTGAATTTGGCCTCCTAAACAGCAGCAGCAAGCAGTTAAGTAGCATACAGTT
721   TTAACCCACAAGATTCATCTGTGGCATGTGTACAAATATAGTTGGATGCATTTATTTAGACACAAGCTTTATTTCTTCCA
801   CATCTTGCTAAAAGATGCATGTAGTTACAACTTTGAGGCCAATCATTTTAGGTGTATGTTTTAAGCATAGAAAGTCTCAA
881   ACTCTGAATGGTTCCTTCAAGAGATGAGTTAGTGTGTGACCTGATCAGTACTTCTCTCTTTTTATTTCTTCCATATAGAG
961   CACTATGTAAATTTAGCATATCAATAATACAGGATATATCAAACAGTATGTAAAACTCTGTTTTTTAGTACAATGGTGCT
1041  ATTTTGTAGTTTGTTATATGAAAGAGTCTGGCCAAAACGGTAAAAGGTGAAAGCAAAACTAAAGAGGAAGCCTGGAGCCA
1121  AAGGTGACACAGAGGGGAAGGGTACTCAAAACTCAAACATTTGGGAAAGAAAAGCTCCCCAATTATGCCTTCCAAGAAAA
1201  ATAAGACACAAAGTCCACTGATAACAAATTTGATTGGCGAGTCCAGAGAGGAGTAGAAAAAGCAGAAGGCTAGGAATTTA
1281  ATAATCCTGGTTTCTTATTCTCACTGAAGAAACAAACATCTGACGACTCTGCCACGGACTCACCACTGTGTGACCTTGGG
1361  CAAGTCACTTCACCTCTCTGTGCCTCAGTTTC
```

to oIGF-I exon 3. Class 1 and class 2 transcripts, comprised of exon 1 spliced to exon 3 and exon 2 spliced to exon 3, respectively, have been identified in sheep, rat, and human IGF-I cDNAs. The extra glutamine predicted from cDNA analysis to be present in the leader peptides of oIGF-I class 1 or 2 transcripts was clearly due to a glutamine codon at the 5' end of exon 3 and not part of exons 1 and 2 as previously reported (Wong *et al.*, 1989). In sheep, a variant cDNA was identified and found to contain an unknown sequence, designated exon W. Exon W was found upstream of exon 1 at nt 883-1013. Splicing of exon W to exon 3 occurred at an alternative splice site resulting in the deletion of a CAG triplet at the 5' end of exon 3.

Sequence alignment of oIGF-I gene regions spanning exons W, 1, and 2 with corresponding gene sequences reported for human, rat, and chicken IGF-I, revealed that the 5' regions of the IGF-I gene were well conserved (de Pagter-Holthuizen *et al.*, 1986; Rotwein *et al.*, 1986; Shimatsu and Rotwein, 1987; Kajimoto and Rotwein, 1991). Human, rat, and chicken gene sequences from the MET codon in exon 1 to a point 400 nt upstream were 98, 93, and 94% identical with the oIGF-I sequence, respectively (Fig. 2-3A). In the case of rat (89%) and chicken (92%), this 5'-untranslated (5'-UTR) sequence was more conserved than the protein coding sequence of exon 1. Further upstream of this arbitrary 400 nt position, the human, rat, and ovine sequences were still relatively well conserved while the chicken sequence showed only 38% identity. Similar sequence alignment analysis was performed for regions flanking exon 2 (Fig. 2-3B). Human and rat gene sequences from the MET codon in exon 2 to a point 185 nt upstream showed 95 and 88% identity, respectively, with the oIGF-I sequence. Exon 2 has not been identified in the chicken IGF-I gene. Again, this 5'-UTR region was more conserved than the coding region of exon 2. Upstream of this arbitrary 185 nt position human IGF-I was 82% identical and rat was 64% identical to the oIGF-I sequence. In addition, the intron sequences 3' of exons 1 and 2 were well conserved among rat, human, sheep and chicken IGF-I.

Figure 2-3. Comparison of nucleotide sequence of sheep, human, rat, and chicken IGF-I exons 1 and 2. Regions compared include available sequence A) 400 nt upstream of the protein coding region, from -400 to the coding region, coding region, and intron 3' of exon 1; B) 185 nt upstream of the protein coding region, from -185 to the coding region, coding region, and intron 3' of exon 2. The -400 and -185 nt positions were chosen to encompass the mapped transcription initiation sites.


A
W
1
SHEEP
100%
80%
98%
97%
HUMAN
89%
62%
93%
73%
RAT
92%
38%
94%
70%
CHICKEN
400
=100 nt


B
2
SHEEP
93%
82%
95%
83%
HUMAN
67%
64%
88%
75%
RAT
185
=100 nt

Ovine IGF-I transcription initiation sites

Transcription initiation sites were identified for oIGF-I exons 1, 2 and W by primer extension analysis (Fig. 2-4A, exon W results not shown). Major initiation sites for class 1 transcripts were identified using oligo 1a which was specific for exon 1. Extension products (Fig. 2-4B) synthesized from this primer were 305, 273, and 116 nt (bands a, b, and c, respectively) and corresponded to transcription initiation sites located 368, 336, and 179 nt upstream of the 3' end of exon 1. Multiple initiation sites were also identified for class 2 transcripts. Major extension products of approximately 840, 116, 81, and 71 nt (bands d, e, f, and g, respectively) were observed utilizing oligo 2a. These products corresponded to the initiation of transcription ~850, 131, 96, and 86 nt upstream of the 3' end of exon 2. Band f was readily visible in other gels with adult liver RNA (lane 3). Numerous extention products ranging from 50-400 nt in length were generated with oligo Wb. No major exon W transcription initiation sites were identified by primer extension. Additional studies are necessary to confirm the putative use of these multiple start sites. Other minor bands were detected for all primer extension reactions upon longer exposure. No difference in the pattern of primer extension products was observed when total RNA was isolated from adult or fetal liver.

A comparison of reported IGF-I transcription initiation sites, determined by multiple methods, are shown in Fig. 2-5, A and B. The overall pattern of sites are conserved in the different species examined. This conservation of transcription initiation sites correlates well with the conservation of nucleotide sequence among different species within these regions shown in Fig. 2-3.

Expression of exon 1, 2 or W-specific oIGF-I transcripts

The three different oIGF-I mRNA transcripts were detected by a reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The combination of oligonucleotide Wa, 1b, or 2b with an exon 3-specific primer (oligonucleotide 3b)

Figure 2-4. Identification of transcription initiation sites for oIGF-I gene. A). Major transcription initiation sites for oIGF-I exons 1 and 2 were determined by primer extension and are positioned relative to the 3' end of the exons. B). Primer extension products for ovine exon 1 using oligonucleotide 1a (lanes 1 and 2) and exon 2 using oligonucleotide 2a (lanes 3 and 4). RNA was extracted from adult liver (lanes 1 and 3) and fetal liver (lanes 2 and 4). Identification of the transcription initiation sites were determined by genomic sequencing of exon 1 primed with oligonucleotide 1a (left panel) or exon 2 with oligonucleotide 2a (right panel). The approximate size of band d was estimated by comparison to a 123 bp ladder (GIBCO-BRL).

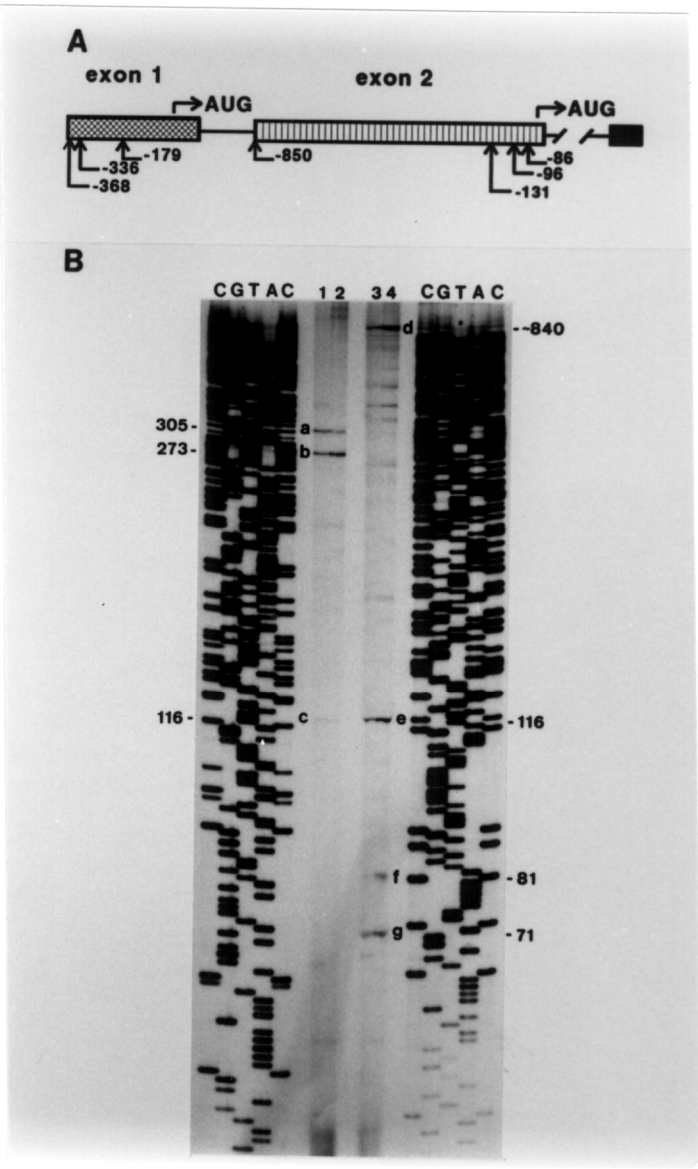
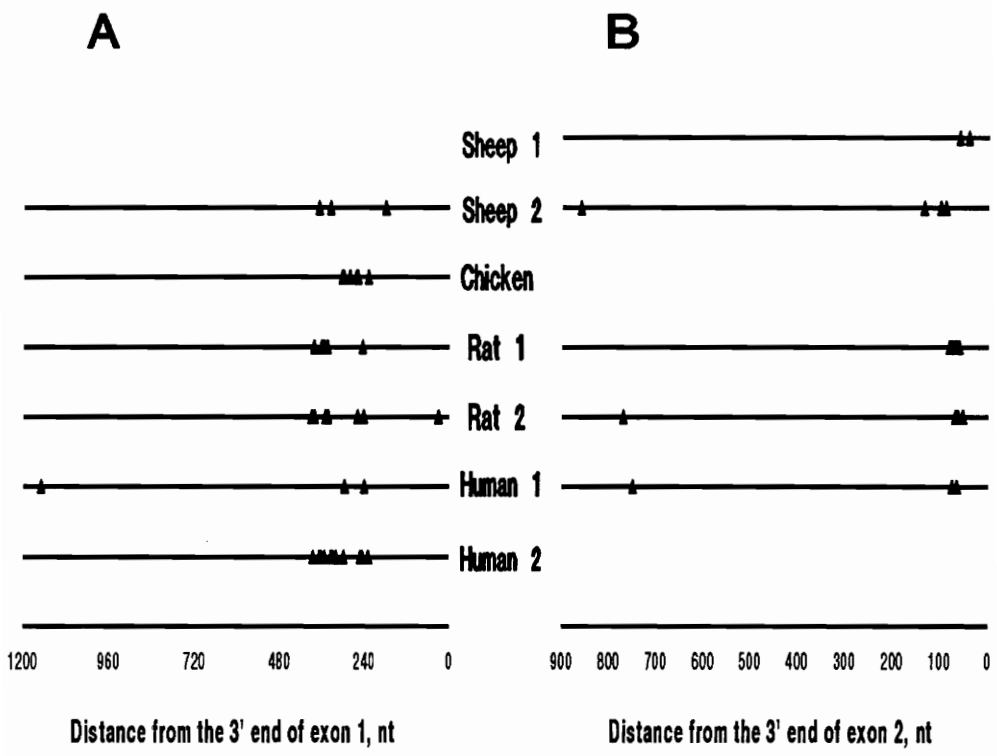

A
exon 1
exon 2
AUG
AUG
-179
-336
-368
-850
-86
-96
-131
B
CGTAC
1 2
3 4
CGTAC
-840
305-
273-
a
b
116-
c
e
-116
f
-81
g
-71

Figure 2-5. Comparison of the transcription initiation sites for sheep, rat, human, and chicken IGF-I genes. References include: sheep 1 (Dickson *et al.*, 1991); sheep 2 (present study); chicken (Kajimoto and Rotwein, 1991); rat 1 (Simmons *et al.*, 1991); rat 2 (Adamo *et al.*, 1991); human 1 (Jansen *et al.*, 1991); human 2 (Kim *et al.*, 1991).


A
B
Sheep 1
Sheep 2
Chicken
Rat 1
Rat 2
Human 1
Human 2
1200
960
720
480
240
0
900
800
700
600
500
400
300
200
100
0
Distance from the 3' end of exon 1, nt
Distance from the 3' end of exon 2, nt

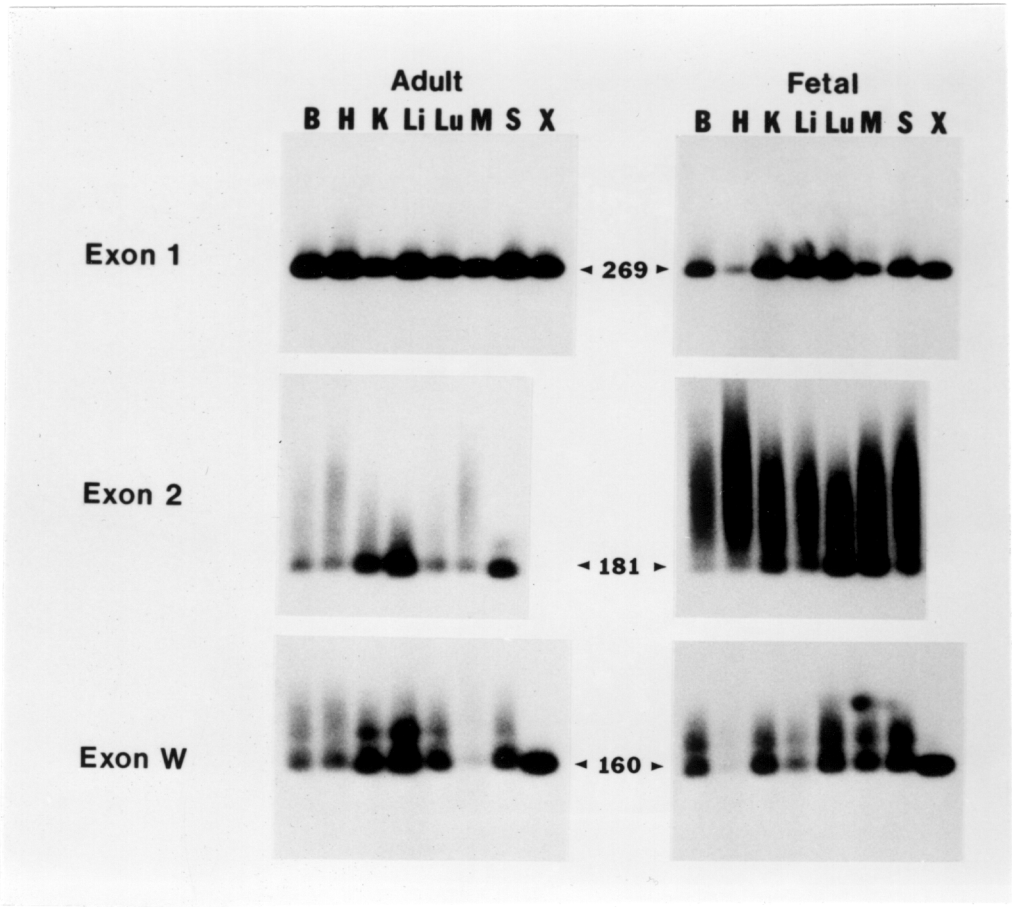
generated PCR products of 160, 269, or 181 nt, respectively. In Fig. 2-6, expression profiles of three alternatively spliced oIGF-I transcripts in fetal and adult brain, heart, kidney, liver, lung, skeletal muscle, and spleen are shown. Exon 1 transcripts were the predominant species detected in all fetal and adult tissues examined. Exon 2 transcripts were also detected in all fetal and adult tissues. We consistently observed more intense hybridization to adult liver mRNA, suggesting that exon 2 transcripts were more abundant in liver tissue. Exon W transcripts also appeared to be expressed in all fetal and adult tissues. The origin of the higher molecular weight hybridizing bands for exon 2 and exon W specific transcripts is unknown.

The exposure time for the fetal RNA blots was approximately four times that of the adult RNA blots, indicating that more oIGF-I mRNA was present in adult versus fetal tissues. However, the relative intensities of hybridization for different tissues were not consistently observed when comparing results obtained from other comparable animals. For this reason and the fact that the RT-PCR assay is not strictly quantitative, the percentage of total oIGF-I mRNAs for each exon-specific transcript could not be reliably determined.

Discussion

Comparison of our oIGF-I gene sequence with an oIGF-I gene previously reported by Dickson *et al.* (1991) showed a few differences consisting of single nucleotide substitutions or small insertions/deletions, occurring mainly in introns or non-coding regions. These discrepancies may reflect individual or breed differences or technical problems, e.g., in exon 3 (nt 248-253), we found AGC triplets while Dickson *et al.* (1991) reported ACG triplets (nt 5053-5058). These substitutions would result in a change in the amino acid sequence of the oIGF-I prepropeptide from serine to threonine at these positions. When comparing our cDNA and genomic sequences, a silent substitution was detected in the oIGF-I coding region.

Figure 2-6. Expression of oIGF-I exon-specific transcripts.
Ovine IGF-I transcripts containing exons W, 1 or 2 were identified by the presence of 269, 181, or 160 nucleotide bands, respectively on Southern blots probed with oligonucleotide 3c. Tissues were from an adult ewe or a 75d fetus: B (brain), H (heart), K (kidney), Li (liver), Lu (lung), M (skeletal muscle), and S (spleen). Lane X shows the position of the expected PCR product after amplification with the respective cloned cDNAs. A standard for exon 2 transcripts was not available because the cloned cDNA containing exon 2 did not include the 5' oligonucleotide primer site. Exposure time for the adult samples was 4 hours and for the fetal samples was 16 hours.


Adult
B H K Li Lu M S X
Fetal
B H K Li Lu M S X
Exon 1
269
Exon 2
181
Exon W
160

At nt 271 in exon 3, the substitution of a cytosine in the gene sequence for a thymidine in the cDNA sequence still encoded a proline at this position. This change probably reflects individual or species differences since the cDNA and genomic libraries were generated from different sheep.

In exon W, the sequence of Dickson *et al.* (1991) predicted an in-frame methionine codon (nt 1574-1576); whereas our sequence data did not. The difference between the two oIGF-I sequences was the insertion of a guanine nucleotide (nt 1577, Dickson *et al.*, 1991), which resulted in a shift of an adjacent ATG codon into an open reading frame. Our genomic and cDNA sequences for exon W were identical and both lacked this additional guanine nucleotide and thus the in-frame methionine. In fact, just upstream of this ATG sequence is a stop codon (TGA, nt 915-917) in our reading frame. Therefore, we conclude that exon W transcripts do not initiate translation in exon W but probably utilize the ATG codon in exon 3.

A transcription initiation site has been reported for rat IGF-I which is located only 30 bases upstream of the 3' end of exon 1 (Adamo *et al.*, 1991b). RNA transcription initiating at this site (start site 4) would preclude the upstream methionine codon at -48, and result in translation initiation at the methionine codon at -22 in exon 3. The oligonucleotide used for primer extension analysis of oIGF-I exon 1 is complementary to a sequence upstream of this proximal start site and therefore the existence of a comparable site for oIGF-I has yet to be determined. Therefore, it appears that two potential mechanisms for the production of a IGF-I prepropeptide with the 22 amino acid leader peptide are alternative transcription initiation (start site 4) or alternative splicing to an exon (W) lacking an in-frame methionine codon.

The tissue distribution of oIGF-I mRNA spliced variants show similarities and some differences to that found for rat IGF-I (Lowe *et al.*, 1987; Adamo *et al.*, 1989). Like rat IGF-I, ovine exon 1 transcripts were the predominate mRNA and were

present in a wide variety of both fetal and adult tissues. Ovine exon 2 transcripts were detected in all of the adult and fetal tissues examined and generally the most intense hybridization was found in RNA from adult liver. This latter observation is consistent with the demonstration that exon 2 transcripts are highly inducible in the liver by growth hormone treatment in rats (Lowe *et al.*, 1987) and sheep (Saunders *et al.*, 1991). The detection of exon 2 transcripts in all tissues examined is in contrast to the results reported in rats where expression of exon 2 (class A/B) transcripts were undetectable in muscle, heart, and brain utilizing a solution hybridization/RNase protection assay (Lowe *et al.*, 1987; Adamo *et al.*, 1989) or undetectable in a variety of fetal and adult non-hepatic tissues on Northern blots (Hoyt *et al.*, 1988). This variability may be due to the difference between sheep and rats and/or the method of detection. We measured mRNA utilizing the very sensitive reverse transcriptase/PCR assay while the work on rats utilized Northern blots and solution hybridization/RNase protection assays.

Exon W transcripts also were detected in all fetal and adult tissues, indicating that the isolated exon W cDNA (clone A46, Wong *et al.*, 1989) was not the result of a cloning artifact. Analysis of the rat and human IGF-I gene sequences, corresponding to the 3' end of ovine exon W, revealed similar potential splice sites. The 3' splice site of ovine exon W contained the sequence ATACGG/GT, whereas rat and human contained the sequence AT($^{C}/_{A}$)CTG/GT. This high sequence identity suggests that exon W containing transcripts may exist for rat and human IGF-I.

The precise role for IGF-I mRNAs with alternatively spliced first exons is unknown. However, one potential role for these mRNA variants is translational discrimination. Foyt *et al.* (1991) have shown that rat IGF-I mRNAs with different 5'-UTRs are differentially associated with polysomes in vivo, although all spliced variants are found associated with polysomes. Likewise, human IGF-II mRNAs with different 5'-UTRs also show translational discrimination (Nielsen *et al.*, 1990).

The significance of the alternative splicing of exon W to a different acceptor site at the 5' end of exon 3 is unknown (Wong *et al.*, 1989). The sequence surrounding this splice site in exon 3 is 5' CAGCAGGTG 3' (nt 179-189). Exons 1 and 2 are spliced to exon 3 following the first AG pair and thus include the CAG triplet encoding glutamine; while exon W is spliced to exon 3 following the second AG pair. Because our sequence data indicates that exon W does not contain an in-frame methionine codon, this alternative splicing event does not affect the length or composition of a leader peptide because translation presumably initiates from the methionine codon in exon 3. This repeating CAG triplet only has been found in the oIGF-I gene. Rat and human IGF-I have distinctive consensus sequences at this splice site (5' NGNCAG/NTN 3') which would not favor this alternative splicing event. Alternative splice site selection to adjacent CAG triplets also has been reported for the human IGF-I receptor gene (Yee *et al.*, 1989). In this case alternative splicing results in a change in the IGF-I receptor from Thr-Gly in the CAG+ receptor to Arg in the CAG- receptor.

Multiple transcription initiation sites for both exons 1 and 2 appear conserved across species. RNase protection, 5'-RACE, and primer extension assays have been used to map putative transcription start sites in human, rat, chicken, and sheep. Initiation sites for class 1 transcripts appear to have two conserved sites at approximately 200 and 350 nt from the 3' end of exon 1. One long transcript has been identified by Jansen *et al.* (1991) in human but similar size transcripts have not been reported for other species. Transcription initiation sites for class 2 mRNAs are almost identical in human, rat, and sheep; no exon 2 has been identified in chicken (Kajimoto and Rotwein, 1991). The majority of the sites have been mapped 60-80 nt upstream of the 3' end of exon 2, while one site has been found 700-850 nt further upstream in human, rat, and sheep. Class 2 transcription start sites have been reported previously for oIGF-I 46 nt from the 3' end of exon 2 with some minor sites at -64 nt (Dickson *et al.*, 1991). We have detected sites further upstream at 89, 99,

134, and ~850 nt from the 3' end of exon 2, but did not detect the more proximal transcription initiation sites of Dickson *et al.* (1991). These differences may be due to the location of the hybridizing oligonucleotides used in our primer extension experiments.

Although sequence homology is well conserved across species in regions containing these initiation start sites, no predominant well defined site of initiation has been found for either exon 1 or 2. This may be due to the lack of CCAAT and TATA sequences upstream of the start of transcription initiation. The IGF-I gene promoter appears to be a member of the class of eukaryotic promoter elements which lack a TATA and CCAAT sequences and are not GC-rich. Instead, the chicken (Kajimoto and Rotwein, 1991), rat (Hall *et al.*, 1992), and human (Kim *et al.*, 1991) IGF-I genes contain a sequence resembling the "initiator sequence" originally defined by Smale and Baltimore (1989) for the mouse terminal deoxynucleotidyl transferase gene. The initiator sequence defines the start of transcription in the absence of a CCAAT and TATA box. The sheep IGF-I gene (nt 1199-1214) contains a sequence identical to that of the rat IGF-I initiator sequence. The rat IGF-I receptor gene also contains a sequence surrounding a transcription start site which is similar to the initiator sequence (Mamula and Goldfine, 1992). In contrast to its role in the mouse TdT or rat IGF-I receptor gene, the initiator sequence does not appear to define a unique transcriptional initiation site as indicated by the multiple sites reported. Clearly more studies need to be conducted to identify the cis-acting elements and trans-acting factors that regulate expression of the IGF-I gene.

Acknowledgments

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CHAPTER III
Expression of a Variant Ovine Insulin-like Growth Factor I (IGF-I) cDNA in Bovine Mammary Epithelial Cells

Abstract

Alternative splicing of ovine insulin like growth factor-I (oIGF-I) transcripts generates three different mRNAs. A novel oIGF-I mRNA containing exon W is spliced to exon 3 and has been located upstream of oIGF-I exon 1. No in-frame methionine codon was found in exon W and therefore translation is proposed to initiate at the methionine codon present in exon 3. To determine the translatability of the ovine-specific exon W mRNA a plasmid which overexpresses an exon W cDNA was constructed. The cDNA was cloned under the control of a glucocorticoid-inducible MMTV promoter (pMMTV-IGF-IW) and transfected into a bovine mammary epithelial cell line (MAC-T). Selected stable transfectants were induced with dexamethasone (DEX) and transcription of pMMTV-IGF-IW was confirmed by Northern blot analysis. Expression of the pMMTV-IGF-IW transcripts was either induced or constitutive, producing transcripts of 1.6 and 1.4 kilobases, respectively. Concentrations of secreted IGF-I, detected by RIA, were associated with expression of IGF-I mRNA. Ligand blot analysis of conditioned medium detected changes in the profiles of insulin-like growth factor-binding proteins 2 and 3 (IGFBP-2 and -3). Parental MAC-T cells only secrete IGFBP-2 while transfectants produced either IGFBP-3 or both IGFBP-2 and IGFBP-3. Biological activity of secreted IGF-I was assayed by measuring the incorporation of [^{3}H]-thymidine into DNA of test MAC-T cells. Media harvested from the pMMTV-IGF-IW clones stimulated labeling of MAC-T cells greater than that of conditioned medium from MAC-T cells. Thus, expression of pMMTV-IGF-IW mRNA was associated with altered IGFBP profiles and the production of biologically active IGF-I.

Introduction

Three different cDNAs encoding ovine IGF-I (oIGF-I) were previously cloned and sequenced (Wong *et al.*, 1989). Two of these oIGF-I cDNAs represent class 1 and class 2 IGF-I transcripts. The third oIGF-I cDNA contained a unique 5' sequence, designated exon W, which has not been reported previously in other species. Exon W was initially described as an unique 5' end of an oIGF-I cDNA (clone A46) and mapped 553 nucleotides upstream of the 3' end of oIGF-I exon 1 (refer to Chapter 1, Figure 1-1). No open reading frame was found in exon W. Thus, transcripts containing exon W were predicted to initiate translation using the methionine codon present in IGF-I exon 3, resulting in a shortened signal peptide of 25 amino acids (Ohlsen *et al.*, 1993). Exon W transcripts were of low abundance in a variety of tissues and detectable only by PCR (Ohlsen *et al.*, 1993; Pell *et al.*, 1993).

Romagnolo *et al.* (1992) previously reported the construction of an oIGF-I class 2 cDNA under the control of the glucocorticoid-inducible MMTV promoter. This construct was transfected into MAC-T cells, a bovine mammary epithelial cell line. Stable transfectants that overexpressed oIGF-I showed enhanced growth. To investigate the translatibility of exon W transcripts, an exon W containing cDNA was cloned under the control of the MMTV promoter and transfected into MAC-T cells. RIA analysis of medium collected from transfected cells indicated that IGF-I was synthesized and secreted from oIGF-IW transcripts.

Materials and Methods

Plasmid construction

An expression plasmid containing exon W IGF-I cDNA under the control of a glucocorticoid-inducible MMTV promoter was constructed. A 0.6-kb oIGF-I cDNA

fragment was isolated from clone A46 by a *Hin*dIII/*Bam*HI restriction enzyme digest (Wong *et al.*, 1989), purified by Geneclean (BIO 101, Inc. La Jolla, CA) and blunt-ended with T4 DNA polymerase. This fragment was inserted into the *SmaI* site of the 7.6-kb expression vector pMSG (Pharmacia, LKB Biotechnology, Inc., Piscataway, NJ). The resultant pMMTV-IGF-IW construct was verified by DNA sequencing.

Isolation of stable transfectants

Bovine mammary epithelial cells (MAC-T) (Huynh *et al.*, 1991) were cotransfected by calcium phosphate precipitation with 10 μg of pMMTV-IGF-IW DNA and a plasmid containing a gene encoding resistance to hygromycin-B (HYG-B) (Blochlinger and Diggelmann, 1984). Stable transfectants were selected in media containing 200 μg/ml HYG-B (Sigma, St. Louis, MO). Clonal lines were established by limiting dilution in 96-well plates. Genomic DNA from individual clones was analyzed by Southern blots and probed with a nick-translated oIGF-I cDNA fragment to verify that stable transfectants also incorporated the non-selected pMMTV-IGF-IW plasmid.

Northern blot analysis

pMMTV-IGF-IW clones were cultured in three 35-mm tissue culture wells with Dulbecco's Modified Eagles's Media (DMEM)-10% FCS and 200 μg/ml HYG-B to 85% confluency. Media were removed and cells were washed with Dulbecco's PBS (DPBS). The clones were induced for 48 hr with 1 μM dexamethasone (DEX) in DMEM media. An aliquot of media was saved for RIA analysis. Total RNA was extracted directly from individual wells by scraping the plates and extracting with acid-phenol-guanidinium thiocyanate (Puissant and Houdebine, 1990). RNA samples were size fractionated on 1% agarose gels and blotted onto Magna membrane (MSI

Inc., Westborough, MA), hybridized with a nick-translated oIGF-I cDNA fragment and washed according to the manufacturer's directions. Blots were exposed to Kodak XAR-5 film with intensifying screens at -80°C.

RIA for IGF-I and ligand blots for IGFBPs

Media samples were assayed by RIA for IGF-I following methanol-formic acid extraction to dissociate IGF from IGF binding proteins (IGFBP). The RIA was performed as described previously (Herring and McFadden, 1990). Ligand blotting was performed to determine the profile of secreted IGFBPs. Conditioned media was concentrated by lyophilization and an aliquot was applied to a 12.5% SDS polyacrylamide gel. Proteins were transferred from the gel to nitrocellulose (45 μm pore size). The blot was incubated with [^{125}I] IGF-I as described by Hossenlopp *et al.* (1986). Identification and molecular weight estimation of the IGFBPs were determined by previous Western blot analysis and the migration of protein standards (Diversified Biotech, Newton Center, MA).

Assay for biological activity of secreted oIGF-I

Conditioned media were collected from the pMMTV-IGF-IW clones as well as MAC-T controls. Cells were plated at a density of 1 X 10^6 in 100-mm tissue culture plates for 72 h in the presence or absence of 1 μM DEX. At the end of the induction period, conditioned media were collected and used to culture MAC-T cells seeded at a density of 5 X 10^4 cells per well. After 16 h, cells were pulse-labeled for 2 h with [^{3}H]-thymidine (68 Ci/mM, ICN Biomedicals, Inc., Costa Mesa, CA) and incorporation was measured as described by Zhao *et al.* (1992).

Results

Construction of pMMTV-IGF-IW expression vector

An oIGF-I cDNA (A46) containing a variant 5' exon referred to as exon W, was cloned under the control of a mouse mammary tumor virus (MMTV) promoter (Fig. 3-1). The MMTV promoter is inducible by the glucocorticoid, dexamethasone (DEX). Transcription of the pMMTV-IGF-IW construct is predicted to initiate 134 nucleotides 5' to the polycloning site *SmaI* and terminate 822 nucleotides 3' of the cloned fragment at a simian virus-40 (SV40) polyadenylation signal sequence. The predicted size of the transcription product from pMMTV-IGF-IW is 1.6 kilobases (kb). Exon W contains no in frame methionine codon, therefore translation initiation of the oIGF-I prepropeptide is predicted to occur within oIGF-I exon 3. This would result in a oIGF-I prepropeptide of 130 amino acids including a 25 amino acid leader peptide.

Bovine MAC-T cells were co-transfected with plasmid pMMTV-IGF-IW and a plasmid conferring resistance to HYG-B and selected in the presence of HYG-B. A number of stable HYG-B-resistant MAC-T cell lines were isolated and shown by Southern blotting to contain plasmid pMMTV-IGF-IW. A unique banding pattern was identified for each individual clone, thereby indicating independently isolated transfectants. The majority of the clones isolated contained between 2-5 integrated copies of the pMMTV-IGF-IW construct (Fig. 3-2).

Inducible and constitutive expression of oIGF-I RNA

Stable transfectants grown in the presence or absence of the MMTV inducer, DEX, were categorized by Northern blot analysis as having two types of oIGF-I transgene expression (a subset of clones are shown in Fig. 3-3). Group one clones (2, 6 and 11) expressed the predicted pMMTV-IGF-IW transcript of 1.6 kb which was inducible with DEX, while group two clones (9 and 12) constitutively expressed

Figure3-1. Structure of the pMMTV-IGF-IW expression vector. The ovine exon W containing cDNA (A46) was cloned 3' to the pMSG mouse mammary tumor virus long terminal repeat (MMTV-LTR) depicted by the left arrow, and 5' of a SV40 polyadenylation signal depicted by the right arrow. Open and filled boxes of the cDNA represent untranslated and translated regions, respectively. Gray and black boxes depict signal and mature IGF-I peptide coding regions, respectively.

pMMTV-IGF-IW Expression Vector

Figure 3-2. Southern blot analysis of pMMTV-IGF-IW DNA. Total DNA was isolated from individual clones and digested with *Eco RI*. Numbers above the lanes indicate clone number. The MAC-T cells (M) were used to identify the parental IGF-I bands indicated by the three arrows on the left of each panel. The λ */Hind III* molecular weight marker is shown by dashed lines to the right of each panel in kilobases (kb).

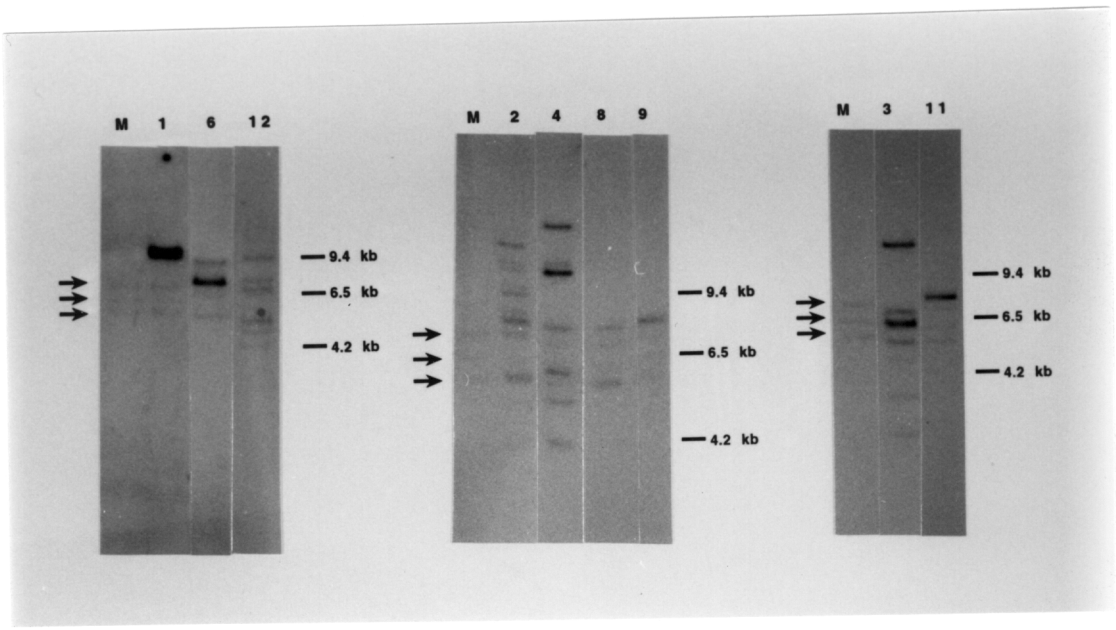
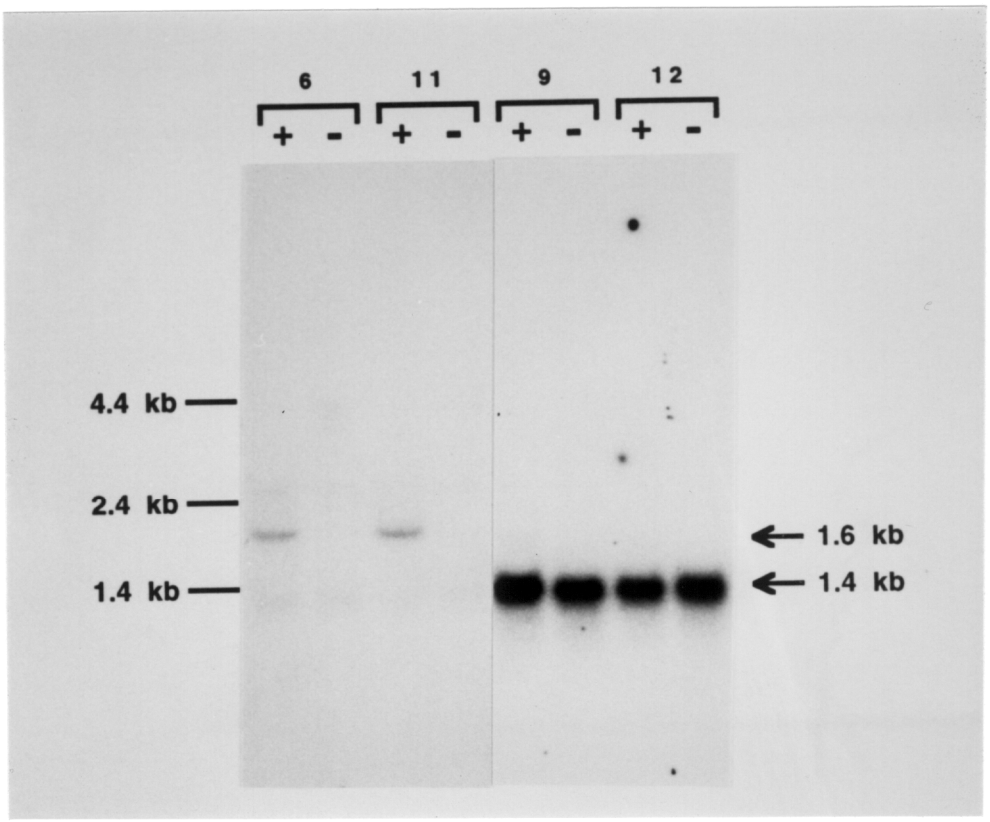

M 1 6 12
9.4 kb
6.5 kb
4.2 kb
M 2 4 8 9
9.4 kb
6.5 kb
4.2 kb
M 3 11
9.4 kb
6.5 kb
4.2 kb

Figure 3-3. Northern blot analysis of pMMTV-IGF-IW RNA. Total RNA (10 μg/ml) isolated from stably transformed pMMTV-IGF-IW clones 6, 11, 9, and 12 was hybridized with a radiolabeled oIGF-I cDNA probe. Odd lanes represent clones which were grown in the presence of 1 μM DEX (+) and even lanes were grown without DEX (-). RNA transcript sizes were determined by an RNA molecular weight marker indicated at the left and band sizes are indicated by arrows on the right.


6
11
9
12
+ - + - + - + -
4.4 kb
2.4 kb
1.4 kb
1.6 kb
1.4 kb

a novel 1.4 kb transcript in theabsence or presence of DEX. Two clones had no detectable transcripts while one clone had both the 1.4 and 1.6 kb transcripts (clone 8). Of the nine pMMTV-IGF-IW cell lines examined, four were randomly chosen to represent the two types of RNA expression, inducible (clones 6 and 11) and constitutive (clones 9 and 12). Endogenous expression of IGF-I in MAC-T cells was not detectable. The mechanism by which the smaller transcript is generated is unknown, but may be due to alternative initiation sites or polyadenylation signal sequences in the pMMTV-IGF-IW construct.

RIA of the medium collected from the four cell lines (clones 6, 11, 9 and 12) during the DEX induction experiment corresponded to the two modes of pMMTV-IGF-IW expression (Fig. 3-4). Clones 6 and 11 showed inducible IGF-I secretion. In contrast, clones 9 and 12 showed constitutive secretion of IGF-I, which was at a concentration greater than that of clones 6 and 11, even after induction.

Biological activity of oIGF-I

The biological activity of the oIGF-I secreted into conditioned media collected from clones 6, 11, 9, and 12 was assayed by measuring the incorporation of [^{3}H]-thymidine into DNA of test MAC-T cells. Fig. 3-5 demonstrates that media harvested from the transgenic clones stimulated labeling of MAC-T cells greater than that of conditioned medium from MAC-T cells. The presence of DEX also appeared to increase [^{3}H]-thymidine incorporation for all conditioned media tested. Differences in the ability to stimulate [^{3}H]-thymidine incorporation were detected between the clones, however there were no consistent correlations with the concentration of IGF-I in the conditioned medium of the growing cells and ability to stimulate [^{3}H]-thymidine incorporation. For example, conditioned medium from clone 12 which contained the highest concentration of IGF-I had limited ability to stimulate [^{3}H]-thymidine incorporation.

Figure 3-4. IGF-I in medium of transfected cells. Media were collected from clones 6, 11, 9 and 12 treated with (filled bars) or without (hatched bars) dexamethasone (DEX). IGF-I was assayed by radioimmunoassay (RIA) from growth medium collected at 48 hours as described in material and methods.

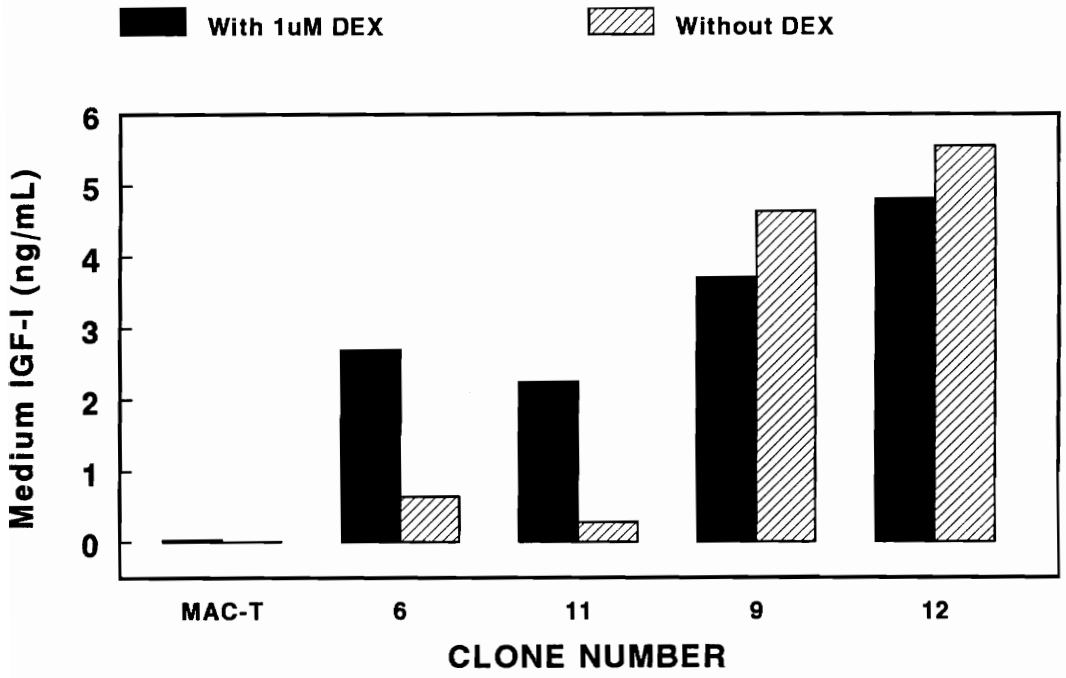
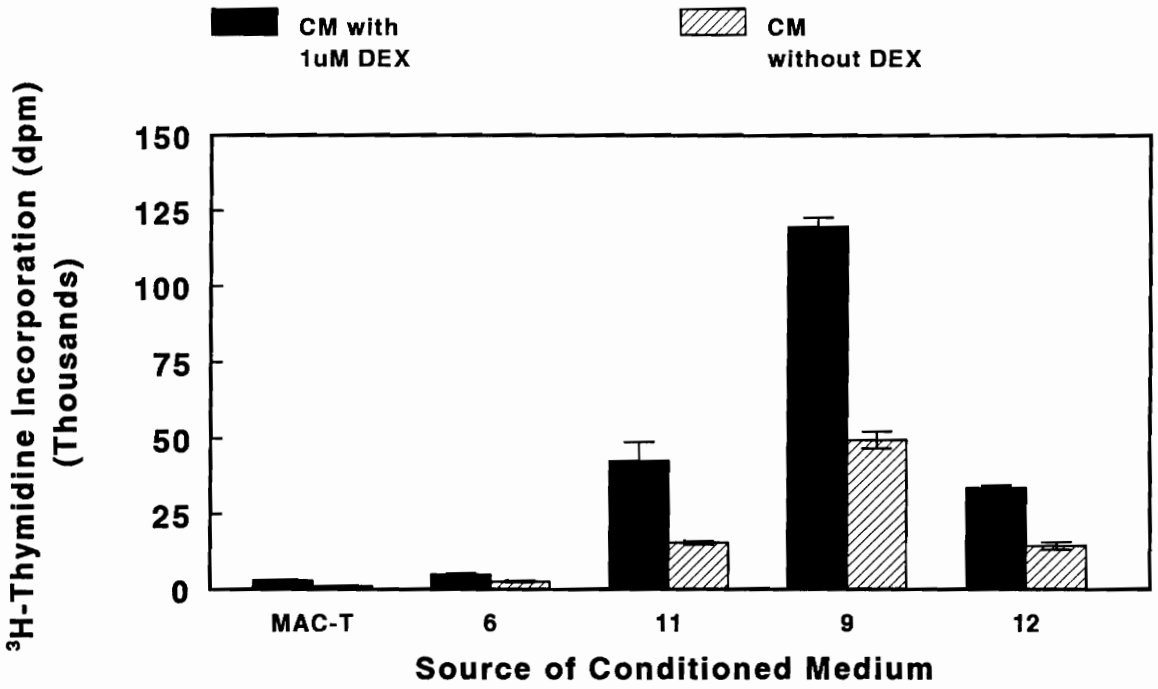

With 1uM DEX
Without DEX
Medium IGF-I (ng/mL)
6
5
4
3
2
1
0
MAC-T
6
11
9
12
CLONE NUMBER

Figure 3-5. Stimulation of MAC-T DNA synthesis by conditioned medium from pMMTV-IGF-IW clones. Conditioned media were collected from cells cultured in serum free media with (filled bars) or without 1 μM dexamethasone (hatched bars) for 72 hours. [^{3}H]-Thymidine incorporation was carried out as described in material and methods. The data shown are averages of four replicates.


CM with
1uM DEX
CM
without DEX
^{3}H-Thymidine Incorporation (dpm)
(Thousands)
150
125
100
75
50
25
0
MAC-T
6
11
9
12
Source of Conditioned Medium

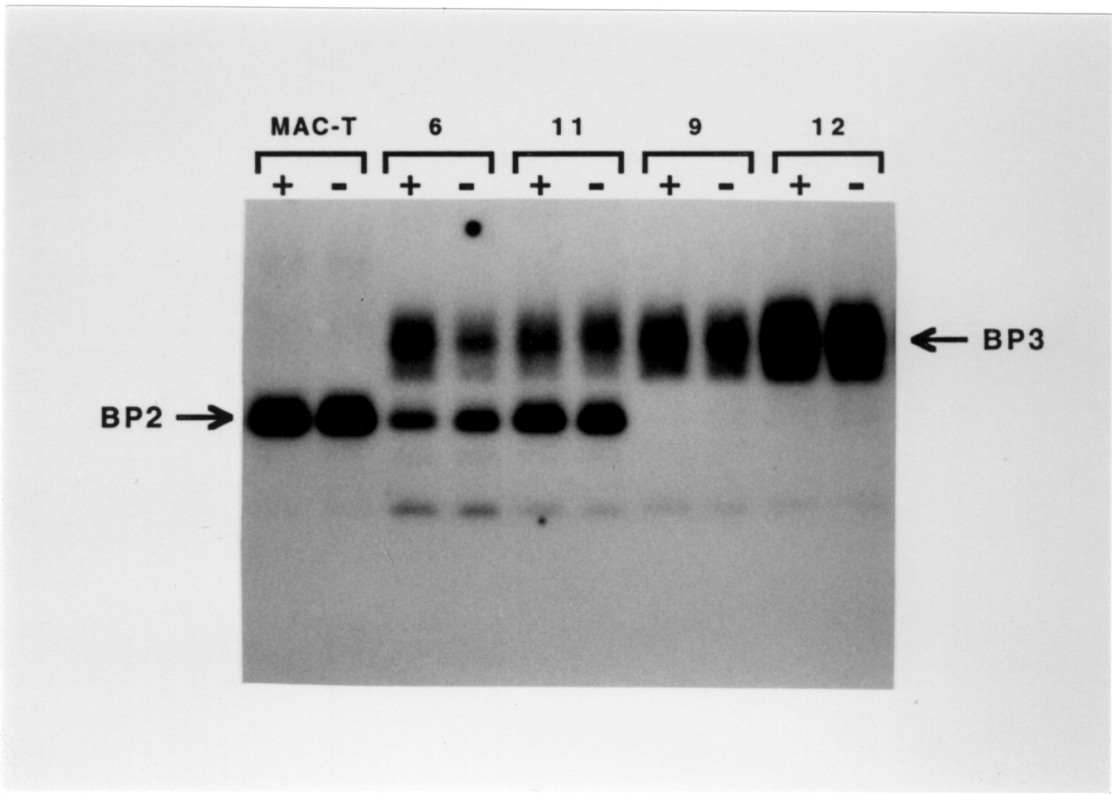
Changes in the IGFBP profiles

Because IGF-I binding proteins (IGFBP) have been shown to modulate the activity of IGF-I, conditioned media harvested from clones 6, 11, 9, and 12 were also analyzed for differences in the IGFBP profiles. Fig. 3-6 shows a ligand blot in which the profiles from clones 6 and 11 (lanes 3-6) differed markedly from that of clones 9 and 12 (lanes 7-10). Two IGFBPs of approximately 30 kDa (IGFBP-2) and 42-43 kDa (IGFBP-3) were present in the media from clones 6 and 11. In contrast, the media from clones 9 and 12 appeared to only contain the 42-43 kDa IGFBP-3. Parental MAC-T cells only secreted IGFBP-2. In addition, the IGFBP profiles of group 1 or 2 clones did not appear to be influenced by the induction of the MMTV promoter with DEX. Thus, for each clone the DEX and non-DEX treated cells have identical IGFBP profiles.

Discussion

Exon W containing transcripts only have been reported in sheep and are normally expressed in a variety of ovine tissues but at levels only detectable by a reverse transcriptase-PCR assay (Ohlsen *et al.*, 1993). Because exon W lacks an in-frame methionine codon, translation is predicted to initiate at the methionine codon in exon 3, generating an IGF-I prepropeptide with a 25 amino acid leader sequence. In rats, putative translation initiation from the methionine codon in IGF-I exon 3 is achieved through an alternative mechanism. A transcription initiation site (start site 4) has been reported which is located only 30 bases upstream of the 3' end of exon 1 (Adamo *et al.*, 1991). RNA transcripts initiated at this site would preclude the upstream methionine codon in exon 1 leaving the methionine codon in exon 3 as the only available translational start site. Transcripts initiated at start site 4 were also found to be in low abundance in a number of tissues (Adamo *et al.*, 1991; Shemer *et al.*, 1992).

Figure 3-6. Identification of IGFBP-2 and -3 by ligand blot. Conditioned media were collected from MAC-T and transfected clones 6, 11, 9, and 12 after 48 hours in serum free media with (+) or without (-) 1 μM DEX.


MAC-T
6
11
9
12
+ - + - + - + - + -
BP2
BP3

An exon W containing oIGF-I transgene (pMMTV-IGF-IW) under the control of the MMTV promoter directed the synthesis of IGF-I in stably transfected bovine mammary epithelial cells (MAC-T). A preproIGF-I containing a 25 amino acid leader peptide is sufficient for correct processing and secretion of biologically active IGF-I into the medium. Bovenberg *et al.* (1990) also have reported that a transgene containing a truncated human IGF-I cDNA, which encoded a preproIGF-I with a 25 amino acid leader sequence, directed the secretion of IGF-I into the medium of a mouse Ltk cell line.

Expression from the pMMTV-IGF-IW construct in MAC-T cells did not always follow the predicted pattern. The MMTV promoter is inducible with DEX and should direct the synthesis of a 1.6 kb transcript. We identified two classes of transformants. Group 1 transfectants showed the expected DEX-induced expression of a 1.6 kb transcript. In contrast, group 2 transformants constitutively expressed a 1.4 kb transcript. It is not known why one class of transfectants not inducible by DEX generated a shortened transcript. One possibility is that a cryptic transcription initiation site which is unresponsive to DEX (glucocorticoids) was utilized.

The concentration of IGF-I present in conditioned media did not reflect the ability to stimulate [^{3}H]-thymidine incorporation into MAC-T cells. For example, conditioned media from clone 12 had the highest IGF-I levels yet showed only moderate ability to induce thymidine incorporation. The high level of IGFBP-3 secreted into the medium by clone 12 presumably complexed with the secreted IGF-I rendering it biologically inactive. Although conditioned medium from transformed cells was able to stimulate thymidine incorporation into MAC-T cells, transformed cells were unable to grow autonomously in the absence of serum (data not shown). Only control MAC-T and clone 6 showed minimal growth in the absence of serum while clones 9, 11, and 12 showed cell death after 6 days in culture. This contrasts with transformed MAC-T cell line, MD-IGF-I, which contains an ovine exon 2 cDNA under the control of the same MMTV promoter (Romagnolo *et al.*, 1992). This cell

line secretes IGF-I and shows autonomous growth in the absence of serum. The lack of autonomous growth stimulation by the secreted IGF-I in the exon W transformants may result from an overexpression of IGFBP-3 to bind up all free IGF-I or a downregulation of the IGF-I receptor. In other transformants (Romagnolo *et al.*, submitted), overexpression of IGF-I leads to a decrease in the abundance of IGF-I cell surface receptors. The number of IGF-I receptors in the exon W transfectants has not yet been examined.

Overexpression of IGF-I transgenes changed the profile of secreted IGFBPs. Following transfection with the pMMTV-IGF-IW construct, stable transfected cells shifted from IGFBP-2 to IGFBP-3 secretion. In two clones (6 and 11) there was secretion of both IGFBP-2 and IGFBP-3; whereas in clones 9 and 12, the shift was complete with only IGFBP3 secreted. Romagnolo *et al.* (1992) previously have shown that in MD-IGF-I cells, transfected with an ovine exon 2 containing transgene, secretion of IGFBP-2 was inhibited and secretion of IGFBP-3 was induced. Clones 6 and 11 appear to be in the transition between downregulating IGFBP-2 synthesis and upregulating IGFBP-3 synthesis. The putative threshold at which the IGFBP-2 to IGFBP-3 shift in both pMMTV-IGF-IW and MD-IGF-I cells yet has to be determined.

The mechanism by which secreted IGF-I alters the binding protein profile is unknown. The addition of exogenous IGF-I to the medium of MAC-T cells fails to alter the binding protein profiles of MAC-T cells (Akers, unpublished observation). Thus, the IGFBP shift does not appear to be a mechanism conveyed through an IGF-I cell-surface receptor but appears to be associated with enhanced endogenous IGF-I synthesis. This shift from IGFBP-2 to IGFBP-3 has been reported in other cell systems and appears to be cell-specific. An IGFBP-2 to IGFBP-3 shift also can be induced in MCF-7 cells by treatment with retinoic acid (Adamo *et al.*, 1992). However, rat thyroid follicular cells (FRTL-5) respond to overexpression of endogenous IGF-I by increasing the synthesis and secretion of IGFBP-5 (Dai *et al.*,

1992) while MCF-7 breast carcinoma cells respond to exogenous IGF-I by increasing the synthesis and secretion of IGFBP-2 (Adamo *et al.*, 1992).

This IGFBP shift also may be due to additional factors such as a putative IGFBP-2 protease. Recently, a potential IGFBP-2 protease (rat urokinase-type plasminogen activator molecule) has been reported and is proposed to function in decreasing IGFBP-2 (Koutsilieris *et al.*, 1993). This reduction in IGFBP-2 may result in a coordinated increase of IGFBP-3, similar to what has been reported for IGFBP-3 protease activity in prostate cancer patients (Karasik *et al.*, 1993)

In summary, the transfection of an MMTV-IGF-IW-expression construct into MAC-T cells resulted in the isolation of unique clones which were able to secrete immuno-reactive IGF-I. This study demonstrates that a shorten signal peptide of 25 amino acids is sufficient for initiation of translation. Multiple clones with induced or constitutive IGF-IW expression produced different sized transcripts. The study of this expression system may help elucidate the mechanism of expression (induced or constitutive) during transcription. In addition, detection of clonal variation of IGFBP production also may provide clarification to the interactive role between IGF-I secretion and IGFBP action. Further research to identify putative factors that regulate these distinct types of expression will lead to a better understanding of the role of this ovine specific mRNA.

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CHAPTER IV
Characterization of the Linked Ovine Insulin and Insulin-like Growth Factor-II Genes

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Abstract

The ovine insulin-like growth factor-II (oIGF-II) gene is composed of 9 exons that span approximately 25 kilobases. Approximately 750 nucleotides upstream of oIGF-II exon 1 are the three exons of the ovine insulin gene which are transcribed in the same direction as oIGF-II. The genomic organization and expression of the oIGF-II gene is similar to the human IGF-II gene. Four putative promoters direct the transcription of six 5' non-coding exons (1, 3, 4, 5, 6, and 7) which are alternatively spliced to the shared exons 8, 9, and 10. An ovine exon comparable to human exon 2 has not been identified. Multiple transcription initiation sites were identified for exons 1 and 6 by primer extension analysis. Using a reverse transcription-polymerase chain reaction (RT-PCR) assay, exon 1 and 3 transcripts were expressed in adult but not fetal liver. In addition, a novel transcript which contained exon 1 spliced directly to exon 8 was detected in adult liver. Exon 4 transcripts were not detected in either fetal or adult liver, whereas exon 6 and 7 transcripts were detected in both fetal and adult liver. Exon 5 transcripts also were expressed in both fetal and adult liver which is in contrast to the tumor cell-specific expression of human exon 5. Like the human and rodent genes, the regulation of expression of the oIGF-II gene is under complex control.

Introduction

The role of IGF-II gene expression in fetal growth and development has been well established (Gray *et al.*, 1987; de Pagter-Holthuizen *et al.*, 1987; Bell *et al.*, 1980). As described in the literature review, the human, rat, and mouse IGF-II genes have been cloned and all show complex patterns of gene expression. In ruminants, cDNAs encoding bovine and ovine IGF-II and bovine insulin have been cloned (D'Agostino *et al.*, 1987; Brown *et al.*, 1990; Ohlsen, 1990; O'Mahoney *et al.*, 1991; Demmer *et al.*, 1993); however, cloning of the ruminant IGF-II and insulin genes have not been reported. In order to study the role of IGF-II in ruminant growth and development, we have cloned the contiguous ovine insulin and IGF-II genes and have found that the exon arrangements and pattern of expression are similar to those of the human genes.

Materials and Methods

Similar techniques were previously described in the materials and methods section of chapter 2.

Animals and tissue collection:

Crossbred ewes were used for fetal and adult tissue collection. Ewes were slaughtered when fetuses were 75 days of gestation. Livers were collected, minced, immediately frozen in liquid nitrogen, and stored at -80°C until analyzed. Two fetal and two adult animals were used for analysis.

Cloning and sequencing:

A sheep liver cDNA library and genomic library were purchased from Clontech Laboratories (Palo Alto, CA). The cDNA library was screened with a human IGF-II cDNA kindly provided by Dr. Susan Berry (Univ. of Minnesota). The genomic library was screened with either the cloned ovine IGF-II cDNA or exon-

specific oligonucleotide probes. Positive bacterial or lambda clones were mapped with restriction enzymes and exon containing fragments were subcloned into phagemid vector pTZ18R (Pharmacia, Piscataway, NJ). Nested sets of deletions were constructed using an exoIII/mung bean nuclease kit (Stratagene, LaJolla, CA) and sequenced using the Sequenase version 2.0 kit from United States Biochemicals (USB, Cleveland, OH). Sequencing compressions were resolved by the substitution of deaza-GTP or dITP for dGTP in the sequencing reactions. The oIGF-II cDNA and insulin genomic exons were sequenced on both strands whereas the oIGF-II exons and introns represent sequencing on one strand only. Sequence analysis software from the Genetics Computer Group (Madison, WI) was used to align overlapping clones and process sequence data.

RNA isolation and primer extension:

Putative oIGF-II transcription initiation sites were identified in fetal and adult liver tissues by primer extension. Two oligonucleotide primers specific for exon 1 (1b, 5'-CCAATCTGCGCGCACAGG-3'; nucleotides 2062-2079) and exon 6 (6a, 5' CCTTCCTGCTGCGTATTGC-3'; nucleotides 3956-3974) were end-labeled with [γ-^{32}P]-ATP (DuPont/NEN, Boston, MA) and T4 polynucleotide kinase (USB). The labeled oligonucleotides were separated from unincorporated [γ-^{32}P]-ATP using Sephadex G-25 spin columns. Total RNA was isolated from frozen tissue samples by acid-phenol-guanidinium thiocyanate extraction (Puissant and Houdebine, 1990) and hybridized to labeled oligonucleotides by boiling for 2 min followed by incubation on ice for 15 min. Primer extension reactions were carried out in 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM dNTPs, 20 units RNasin (Promega, Madison, WI) and 500 units Moloney murine leukemia virus (M-MLV) reverse transcriptase (USB) for 60 min at 37°C. The cDNA products were purified by the method of Livak (1990) and analyzed on 7% denaturing polyacrylamide gels.

Reverse transcription and PCR amplification:

Total RNA was used as a template for the synthesis of partial cDNAs. Hybridization of 150 ng oligonucleotide 8c (5'-TGAAGTAGAAGCCGCGGTCC-3'; exon 8, nucleotides 608-627) to 40 μg total RNA was performed by heating at 95°C for 5 min followed by slow cooling to 37°C. Partial oIGF-II cDNAs were synthesized in 1 X HRT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$), 200 units M-MLV reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), 2.5 mM dNTPs, and 40 units rRNAsin (Promega) at 37°C for 1 hr. Samples were treated initially with 10 μg RNase A for 15 min at 37°C and then incubated in 0.1 N NaOH and 0.03 M EDTA to remove RNA. Reactions were neutralized with 0.3 M Tris-HCl (pH 7.4) and then fractionated on Sephadex G-50 spin columns to remove excess primers. Samples were ethanol precipitated in the presence of 2.5 M ammonium acetate. The purified cDNAs were resuspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and equal sized aliquots were used for PCR amplification.

Oligonucleotide primers complementary to oIGF-II 5'-untranslated regions (UTRs) were used to amplify the cDNAs. Primers for exon 1 (1a: 5'-CCTGTGCGCGCAGATTGG-3'; nucleotides 2062-2079), exon 3 (3b: 5'-CACCTTGAGGACGAGGAG-3'; nucleotides 261-278), exon 4 (4c: 5'-CTTCCAACGGACTGGGCGTTG-3'; nucleotides 1289-1309), exon 5 (5a: 5'-AGGCGAGCTGCTTAGACGCAC-3'; nucleotides 2107-2127), exon 6 (6b: 5'-TACGCAGCAGGAAGGTGG-3'; nucleotides 3960-3977), and exon 7 (7a: 5'-AGCTTCTCCTCGGAGGCAGCCT-3'; nucleotides 187-208) were paired with an oligonucleotide specific to exon 8 (8a: 5'-CAGCATAGCAGCACGAGG-3'; nucleotides, 526-543) for PCR amplification. The predicted sizes of the PCR products for exon arrangements 1-8, 1-3-8, 3-8, 4-8, 4-5-8, 5-8, 6-8, and 7-8 are 123, 342, 279, 228, 393, 122, 120, and 171 nucleotides, respectively.

The PCR reaction contained the cDNA, 150 ng of the 5'-UTR oligonucleotide, 150 ng of oligonucleotide 8a, and 1 unit of Taq DNA polymerase (Promega). The

reaction was brought to a final volume of 100 μl with 1 X PCR buffer and topped with a thin layer of mineral oil. Each reaction was subjected to approximately 40 PCR cycles (94°C for 90s, 50°C for 135s, 72°C for 45s) using a Precision GTC-2 thermocycler (Chicago, IL). Mineral oil was subsequently removed by extraction with chloroform. Amplified DNA fragments were separated on 2% agarose gels and sized using a 123 bp DNA ladder (GIBCO-BRL).

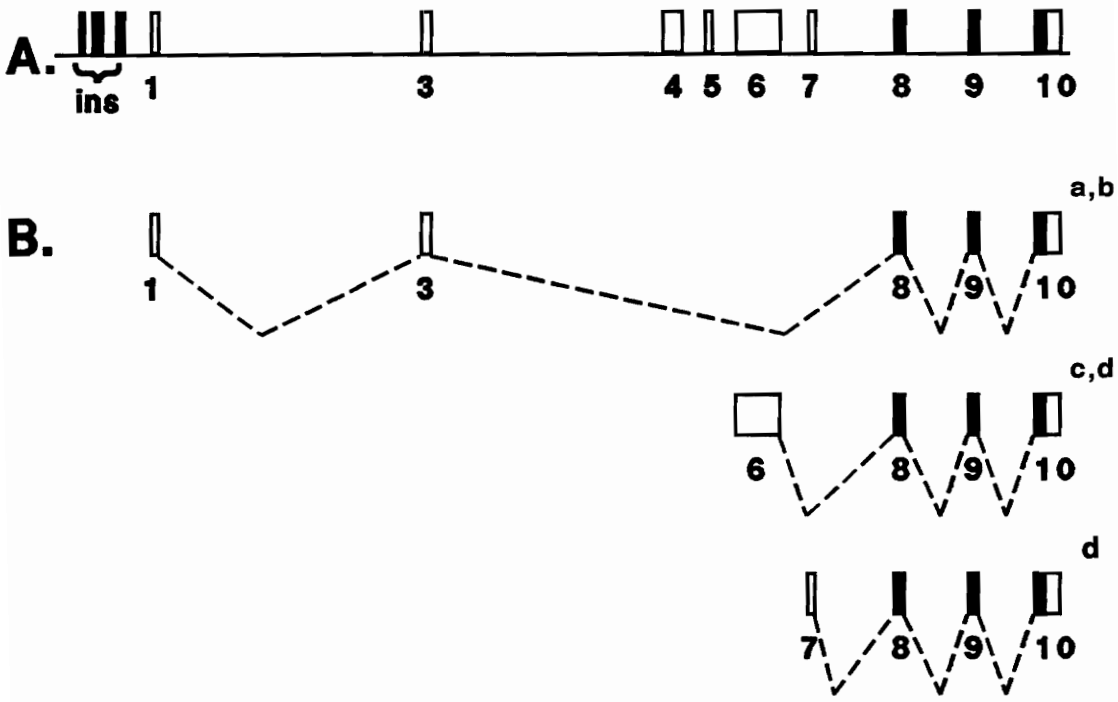
To verify the identity of the PCR products, the DNA fragments were transferred to Magna nylon membranes (MSI, Westborough, MA) by Southern blotting. Blots were hybridized with a [^{32}P]-labeled oligonucleotide specific to exon 8 (8d, 5'-AAGCGCCAGCATCGACTTTCCT-3'; nucleotides 485-506) and washed according to the protocol recommended by MSI and then exposed to Kodak XAR-5 film with intensifying screens at -80°C. In addition, PCR products were cloned into phagemid pTZ18R by dT/dA tailing using terminal deoxynucleotidyl transferase (Promega) and sequenced as described above (data not shown).

Results

Organization and sequence of the ovine insulin and IGF-II genes

Based on the sequence of the cloned oIGF-II cDNAs and sequence identity to human IGF-II exons, nine putative oIGF-II exons have been identified and found to span a region of approximately 25 kilobases (Fig. 4-1A). Because we found an ovine exon homologous to the novel human exon 5 identified by Ikejiri *et al.* (1991), the ovine IGF-II exons are numbered based on the 10 exon arrangement proposed by Ikejiri *et al.* (1991) rather than the 9 exon arrangement originally proposed by Holthuizen *et al.* (1991). An ovine exon homologous to human IGF-II exon 2 has not yet been identified. The three exons of the ovine insulin gene were located upstream of oIGF-II exon 1. As found for human, rat and mouse insulin and IGF-II genes, the ovine genes were transcribed from the same strand.

Figure 4-1. Organization and expression of the ovine insulin and IGF-II genes. A) The three exons for ovine insulin (ins) are shown. Numbered boxes indicate the nine identified oIGF-II exons. Numbering of oIGF-II exons is based on the nomenclature of Ikejiri *et al.* (1991). Open boxes indicate 5' and 3' untranslated regions and solid boxes indicate translated regions.
B) Arrangement of alternatively-spliced oIGF-II transcripts from cloned cDNAs. References are: a, Ohlsen (1990); b, O'Mahoney *et al.* (1991); c, Brown *et al.* (1990); and d, Demmer *et al.* (1993).


A.
ins
1
3
4
5
6
7
8
9
10
B.
a,b
1
3
8
9
10
c,d
6
8
9
10
d
7
8
9
10

The sequence of the nine oIGF-II exons, three insulin exons, part of the flanking introns, and the complete intergenic region between insulin and IGF-II are shown in Fig. 4-2. The entire intron sequences between oIGF-II exons 4, 5, and 6 have been determined and the divisions between these exons have been arbitrarily set. The proposed transcribed region shown for ovine insulin exon 1 is based on the human insulin gene (Bell *et al.*, 1980). Transcribed regions shown for oIGF-II exons 1, 6, and 7 are based on the 5' most sequence of cloned oIGF-II cDNAs. Because an exon 4 containing oIGF-II cDNA has not yet been isolated, the proposed transcribed region for oIGF-II exon 4 is based on transcription of hIGF-II exon 4 (Holthuizen *et al.*, 1990).

The nucleotide sequence of the ovine insulin gene predicts a prepropeptide of 101 amino acids including a 24 amino acid leader peptide. The predicted amino acid sequence for ovine proinsulin is identical to that determined previously by amino acid sequencing (Brown *et al.*, 1955; Peterson *et al.*, 1972). A putative TATA box (nucleotides 80-85) and polyadenylation signal (AATAAA, nucleotides 1357-1362) were identified flanking the coding region. The intergenic region between insulin and IGF-II had a high G+C content, in particular the region between nucleotides 1392 and 1476 which was 96% G+C. The distance between the stop codon of the ovine insulin gene and the 3' splice site of oIGF-II exon 1 was 762 nucleotides. This was approximately 750 nucleotides shorter than the comparable distance between human insulin and IGF-II exon 1.

In Fig. 4-3, a comparison of the genomic organization of the ovine, human, rat, and mouse IGF-II and insulin genes is shown. The organization of the oIGF-II gene very closely resembles that of the hIGF-II gene. The rodent IGF-II genes differ in that they lack promoter P1, and functional exons 1, 2, 3, and 5. However, the intergenic distance between the insulin gene and the coding exons of IGF-II (human exons 8, 9, and 10 or rodent exons 4, 5, and 6) is conserved. Based on conservation of exons, it is likely that ovine promoters, analogous to human promoters P1-P4, are

Figure 4-2. Partial nucleotide sequence for ovine insulin and IGF-II genes. Uppercase letters represent transcribed regions and lowercase letters represent introns or intergenic regions. Bold letters highlight potential TATA- and CCAAT-boxes, a Sp1 recognition site, and a polyadenylation signal sequence. The nucleotide sequence data reported will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession numbers U00659 (insulin) and U00662 to U00668 (IGF-II).

oInsulin Exons 1, 2, and 3

```
1     ctgcagcctcagccccgggccatctgccgcactcctggaggccctgatgagaggccaggtggtggcgggctcaggggct
81    ataaagccggcaggccgtggcagcccccTGCCCTCAGGACCGGCTGCATTCGAGGCTGTCAGCAAACAGgtccgtccttg
161   ggctctgcgtccgcctgggtccggggctgctctgctggggctcagggcatcagggcccaggtccaccgagcagcaggagg
241   atgtgcgctctctcagagcaccctggtgggggcaccctgactgggggcgctgggttagaggccctgactgggggacgct
321   gggttaggggccctgactgggggcgctgggttgggggccctggttgaggggggctgggtttgaggggcaggcactagttga
401   aggggctgggctggggccctggttgaggtgttgggtttgggggggcaggcgctggttgagggcgctgggttgggggcgagc
481   cctgactgagggtgctgagcgccctgccccagcctcagccccgctgctgcccagGTCCTCGCAAGCCCGCCATGGCCCT
                                                                          METAlaLe
561   GTGGACACGCCTGGTGCCCCTGCTGGCCCTGCTGGCACTCTGGGCCCCCGCCCCGGCCCACGCCTTCGTCAACCAGCACC
      uTrpThrArgLeuValProLeuLeuAlaLeuLeuAlaLeuTrpAlaProAlaProAlaHisAlaPheValAsnGlnHisL
641   TGTGCGGCTCCCACCTGGTGGAGGCGCTGTACCTGGTGTGCGGAGAGCGCGGCTTCTTCTACACGCCCAAGGCCCGCCGG
      euCysGlySerHisLeuValGluAlaLeuTyrLeuValCysGlyGluArgGlyPhePheTyrThrProLysAlaArgArg
721   GAGGTGGAGGGCCCCCAGGgtgagcccccgctccccccggtccccggtcctcccggcccctaccttggcctcccgctg
      GluValGluGlyProGlnV
801   gcgcccagcgggaaatcaagagagatttttaaaaaggaaaaccatgtcctgtccacgtcctggaagtgaccagctcccta
881   ggggctaagccatgacgacccccaaggggcctcaccccatctatcctgccttcttacccgggccccctcgcctcctcac
961   acgagggcagctctaggggggatctgacgatgcgaggccaatccaggggccgagggtctcggtgggcagcccttggtggc
1041  gatggagtggtgcccatgggagacgcccgtcacccggagagagggccccgtcccgggtgggctggcgggggtggaggg
1121  gaggggaaaagggcgcctgggcagggccccgctccaaggccaggtagggcgggcaggggggcgtccgctacctgaccgt
1201  ctccccgcagTGGGGGCGCTGGAGCTGGCCGGAGGCCCCGGCGCGGGTGGCCTGGAGGGGCCCCCGCAGAAGCGTGGCAT
                  alGlyAlaLeuGluLeuAlaGlyGlyProGlyAlaGlyGlyLeuGluGlyProProGlnLysArgGlyIl
1281  CGTGGAGCAGTGCTGCGCCGGCGTCTGCTCTCTCTACCAGCTGGAGAACTACTGTAACTAGacctggcccgccgccaata
      eValGluGlnCysCysAlaGlyValCysSerLeuTyrGlnLeuGluAsnTyrCysAsn***
1361  aagccctgacgagcccgctgcgtctctgtgtggcctggggtcccggcgcccgtgggaggggggcgggggggggggaggggggc
1441  gggggggggcgggggggcggcccccggcccgagacccctttctggtctcactgcgtcggcttttctgagctgcctccacacg
```

oIGF-II Exon 1

```
1521  tgctgggtgcacgaggcggggccctccaaggtggcgcgctcccgtcttcaggggtcctgctcacaaggtgggctccacag
1601  gcacctgcccaggccaccacccgtcacacccaagagcccggggctgccggggttggccaaggctccggaggtcagcgggt
1681  gacctggctgtaccaaggtctggccagcctgccagccggccaggccaaaccaatctcctcctctcctgaaggtgccaccg
1761  ggccggggcttggggtggctgggcctggggctgggcacctgggctcccgtggctgcagaccactcagatgccagcctggt
1841  gcccggctggcggggcagtcctctgtgtaccccaaaggctctcaccctgtcacccctcctcggggctacatcaacaccc
1921  cccaggcctccagaaggccccgcccctggggatggccagggttgtgcaggcaggtggccggccggacactggacccgga
2001  agaggagggagtgggggggctgggatgagcagtgatctgcccgtagcgTCACGCAGGTGGCCCTGTGCGCGCAGATTGGG
2081  GCAGGGGTGCCCCGCAGGAGCTGAGgtatgtgggtgcctggagcggcctggcaggggggcaactggggctgggaagtgggc
2161  tggtcatcaccaggctgccctcaaggccctgccagctggtgagaccctggggaccacctggacactcccccctcccctct
2241  ggtgccaacagactctgtagagagggcaaggaggcctttagggtcccggggtgcatggggccgtcccagtatgccctggt
2321  gcaggatgccctgggtgacatgggctgggggctcgggggaggggtgcacgggggcaaggctgtgggtcacggt
```

oIGF-II Exon 3

```
1     atggggaagttcagctatggggaactgtggacacttctcttcctcccttcttcagaaaaggcagagctgggcggccagag
81    ggacaggcaggggagccgtcacccaaacctgacaaggtgacccgagggcctctccacctgacagccagtggaggggcagg
161   ggccagggcctggctgggtgtgtgcacatgtgtggatttgttttaagacaccatcttaagaacacccccctctctccccca
241   gGGGACGAAGAGTCACTGTTCACCTTGAGGACGAGGAGGCGGCCTTCAGCTCCCAGCCCCAGGGCCCCACACCCAGGCCA
321   GGTCAGCCCTTTGCCCAGGCTGCCCCCCGGCCAGAGCCTGCAGCAGGCCAGAAACCCCAGCCCGGGAAGTCAGCTGCTGT
401   GACCCGTGGCCCTCAATCCCCACCTGCTCAGAACTGAGGCCCACGGCCCAGCCCGCAGAGgtgagtgctcctgacgctcc
481   caggcaggaccgcgtgcctagtcagcgccacaggctctggacaggggcctgccatcctccagggctcagagcccctcggc
561   agctcctgtggccatcacccctttcccaaggagtggggccaccaggggctagggtcatatcagggtgacacagcaggaa
641   tctgggttccagtggtcagctcctgggggctcagctgcaggcagggcaggcccc
```

oIGF-II Exon 4

1 aaaattattttctggggcctccggattgcgatgctgcctgctagccctccaaaataaactgagacttttgccccttctc
81 cacaaaacaactgaacttttttgagcactgatttatgggtcccctgattttatgatccttaccctgagtcctaaatttag
161 gcaatttttccacaggcctcccaaaatgaaattgcccagataccgaaaaatacccccaggtcctggaaatctcaggtatcc
241 taggcctgcgtcctataagactccttgctattaaccaggttccctgaaatttagataccagtctctactgaacatttccc
321 atggatcccccacactttcgccagtgcttcctaggggtccccaaatgcagacagtgcccaggaaaatatttttttttcct
401 gttcaccaaaaataaactaatcgaaacccatttaaaaattaaaatattaaaaagacattgattttctgccttctccaaat
481 gtactgatcacaccctaggttgccccaaatttagactgtgtttcttgagtctctcaaggacattgagaatttttccagga
561 gccacaattatggaagtgattttcctcgggcgctgcaacggtccgctaacctgaatttctaaatcttggaaatgattctc
641 cactcctctccaccgcacctggatccccaaggcccacccactgtttttcttccccgaattttagacccttgccctttg
721 gaacctcccaagttccacactgaggattttgctccggtcatggacatccgcagacctactgccctacaaatggggctgcc
801 ccacgagcctgctgacctacatgcctgggtcctcacagggtagcctcggctccccgtgccccccggctgcacggagcgcct
881 ccgcggcccccagactcgggttctttttctggagtctcgaacttagtcattaatcacagctcctcaccccgacgcaccct
961 cctttgggtccccacttcaccaccttcgttctctgccttgcaaaccaggcggctccgcactgcgttcccggcagttgggc
1041 ATTGCTCCCGGCCCCAGCGCGGTCTCTGCAGCTTCCGCGGCCCGCACCCCGCGGGCACCGGTTCTGGGCCTCTCCGCGTC
1121 TGCTGCGAAGTCCCCGGAGCTCCTTGGATGCGGGAAGTTTCTCTCGTCCTCCTCACACACATTCGGTCGGGCAGCTTGTC
1201 CGCCAGGCAGTAGGCAGGGCTTCTCCCGCGTCCAGAACGACTGGGCATTGCCCCCAGTTTCCCCCAAATTTGGGCATTGT
1281 CCCCGGGTCTTCCAACGGACTGGGCGTTGCCCCGGACGCTGGGGACTGCCCCGGGGTCTCGCTCACCTTCAGCGCGCCTA
1361 CCGCCCGCCGCAGATCGCTCGCTCGCTGCCTCGACTCCTCCGCGCCCGCGACGCAGCCTTCGGCCTCCAGCCTCGCGgtg
1441 agctccccgccgcctcgcgtccgggcctggctcccaaacccacccgccgccgtcccgccgtcccgagcggctgctgggga
1521 caccggcgtggggcgcgggagccgtgcggcgggccagcccctcggccgccccgcgcgctgggtcgagccccggagaccgc
1601 gccgctttcctttgctctgtgcaaagatcacaacgagggttaaggccctcggcgacctccaagcggggccggcagggcgc
1681 agggcaggagggacgcggaggagaggcgcaccccgctcaggcggcgcggcgcctccagccgccgggcgcctccagcgccc

oIGF-II Exon 5

1761 ggcggaggactcgggtaggcggccccgagcgcgggagacgtgattgatggcggagcgaggggcgacggcctcggcttcc
1841 gcgccggcagccccttcccccttggagaggcgcgcgtggtcggggctcctggggcgggcggggcgcggggactcgtgcgtg
1921 gttttgacttggtaaaaagaaaaaaaaaatcacaccgacttcttacatcgtccaaaccctctagGAGATGGTTTCCCCAG
2001 ACCCTCAAATGACCGTGGTGCGCCCCGGGGCTGAACCCGAATCTACGCAAGTCCAACGCATAGAGGACGGGGGAACCATT
2081 ATCCGGATATTTTGGGTGGGCCCCAAAGGCGAGCTGCTTAGACGCACCCCGGTGAGCTCGGCCATTCAGgtaggatttga
2161 acgacgtttcccgccctgctcgtctcgcccctggcacacaggcgcagcccgggccggccccatgcctggcccagattcgg
2241 gcctggcccggctggccctctccacgttccgcacctggcgtccgccgaggctcctccccggttcccgtgcggcaccgggg
2321 ggcgctcgggctccggctgcagctcgaggtgctgcgcctgctcgggcaggtggaggctgcgcgcctcgcccgcgcccaga
2401 gactccccacttccccagcaggtccccggcgggactcggggacccgccacctgggcccgcacacacagggcaccgtcgcc
2481 gcctagccccggtggggcgcggggatccggccgtcgctagggcatgtgacgccgcccaccctggtcgtcccgggtccccc

oIGF-II Exon 6

2561 gtgtgctgtgtcggactctggcaccccggaacctcagacagggcccccaaacactttagaatagtgattctagatgtctt
2641 tcgaactctgaggatccctgccccactccaatctcgtccccgcgcaccccacagccccccccctcaccgctgccccccg
2721 ggaccccaagcctggcccccccttggctcgagttgcagaggcggttcgggggcggggcgaggaccccctcgacgcccattg
2801 gcgcgggcactcggccagcggggcccgagcgggcgccgagccgcgggtggctcgactataagagccgcgttgcgcccaga
2881 gttcgcctgctctccggcggagctgcgtgagccaggcagccccggccccccttccggccgccctcgcctcctggccctc
2961 gcccgccagcgcctccacccggctggcggccccgcgtcgacgccgtccgctacgctgctgacccccatcgcgggcgccgt
3041 cggcgggccgcgctccgccggcctgcggattcCCCGCCGCCTCCTCTTCGTCTACCTCAACGCCCGTCCCGCTTCGCCCG
3121 AGGAGGCGGTCCCCCCCGCAGGCAGTCCGGCTCGCAGGCCGCCGGCGTTGTCATCCCCCGCGCTCCCCCCCGGCCCTCCC
3201 CCGGCGCGCAGCCCGCCGCTCCCCCTCCTCGCTGCGTCCCGAGCGGCCCGGGCGCCCGCACCGCCACCCCCCCCCCGAGG
3281 CCCGGGCTCGCGACGGCCGAGGGCTCCGTCGGCCCAAGCCGAGCTGGGCGCCCGCGGCCCGGGTGACGCCTCCGCTCCGC
3361 CCCCCTCAGCACCTTCCCCGGCCCCCGACGTTGCGCCCTTTCCTCGGCTTCTCTGCGCTCCCCGGCCCCTCTCCGGCCTC
3441 TGGTCCCGGCCCCCAGTCTTCTTCCGCAGCCTTCCCTTCGCTCCCTCCCGTCCCCCCCAGCTCCTTGCCTCCAACTCCCT
3521 CCCCCTCCACGCCCGCCCTCTCGCCTTCGCCGTCCCAAAGTGGATTAATTATACGCTTTCTGTTTCTCTCTCCGCTGCAC
3601 TCTCCCGCTGTGAGCCTGCCCGCCTCTCGCTGTCCTCTCTCCCTCTCGCCCTCTCTTCGGCCCCCCCTTTTCACGTTCAC
3681 CCTGTCTCTCTCACTATCTCTGCCCCTCTCTGTCCTTGATACAACAGCTGACCTCATTTCCCGATACCCTTTCCCCCCCT
3761 AAAAGTACAACATCTGGCCCGCCCCGGCCCGCAGACAGCCCGTCCTCCCTAAAGAATCAGACGAAAATTTCCCCCCCCCC
3841 CAAAAACAAGCCATCCCCCCGTTCTGCCCCGTCGCACATTCGGCCCCCGCGACTCGGTCAGAGCGGCGCTGGCAGAGGAG
3921 TGCCCGGCAGGAGGGCCTTCGCCGCTGTTCGGTTTGCAATACGCAGCAGGAAGGTGGGCGGCTCCGGTGCCGGCTTCCAG
4001 gtaagcggcgtgcgggccgggccgcgcggggcggcggggccgcggcagacggggctggggtgctggcggtggcgtccccc

oIGF-II Exon 7

```
1    cgctagggctagcggttgggggtgcaggagaggaggggctggctgggaggacggaggggggcgggacggaaggggggcgggg
81   ggagtggtcagctgggagcggggtgggggcagggtggagcccgggctgggaggagccggcccagacataaaagctgaggc
161  actgaccagcctgcaaactggacatTAGCTTCTCCTCGGAGGCAGCCTTCCAGACTCCTCCTCCTCCTCCTCCTCCTCAT
241  CCTCCTTCAGCCCCAGCGAGCCTCCTGTCCAGCTGCAGgtaaccgcggctctggagcaggaccccgctgcgctgcggctc
321  ccgcccgccgcagccgcttggctcctgcccgccgcccctcactcagccccccaccccctcctctctgcctctgccccccac
401  ccctcctctgcccactggctcacctggcctgcgtggctgcctgcctcctgtccagtctcggctctccgctggtgtcctgg
481  actggaccccacaccaagtggctgggcttgcagaaagatggagttgccgctcggacagcaggaattc
```

oIGF-II Exon 8

```
1    ggctcagggaggctcaagaagtggccactgccttgtgctggccccgccccccatgacttcataggatggaggccaggtcca
81   gtgtccagcactggcccttgccgctctgggtgtggccaagcctcggcaataatacgaggggacagtgatccccacgtga
161  aggtactgaccagcacttggggcccatcagagaggctccgccccagcccttccctcctcgtggggtgatttctgcatccc
241  accagcctcaacagagaaagctcttgttgttcctgtaattaaagcaattaagattaaggagaaagccaggctttaagtct
321  gcaacggggcttggacttagaaggtggctgggcttagggtccaaaggagcagccggcacgtcctggctgagccatccggg
401  ggcctgccggagggcgggccggccctgggcggggtggtggctcacgcctctgtctctcccgcagACATCAATGGGGATCA
                                                                    METGlyIleT
481  CAGCAGGAAAGTCGATGCTGGCGCTTCTTGCCTTCTTGGCCTTCGCCTCGTGCTGCTATGCTGCTTACCGCCCCAGCGAG
     hrAlaGlyLysSerMetLeuAlaLeuLeuAlaPheLeuAlaPheAlaSerCysCysTyrAlaAlaTyrArgProSerGlu
561  ACTCTGTGCGGCGGGGAGCTGGTGGACACCCTCCAGTTTGTCTGTGGGGACCGCGGCTTCTACTTCAgtgagtagctccc
     ThrLeuCysGlyGlyGluLeuValAspThrLeuGlnPheValCysGlyAspArgGlyPheTyrPheS
641  tggggccaggggaggggtgggcgcacgggccctgcgtagatgctggggtcctcctcacagtcctcagtcgcctctgcagg
721  ccctccagcctccctggctcgggggggctggacccaggcttctagccacacgacttacagtcaagggagaatggacaggtg
801  tgtccacaagtggccagacctggagccgcaagtgcgggggggggggcgggggtggaggcggctgaaggccagttccggtgt
881  ggcgggggg
```

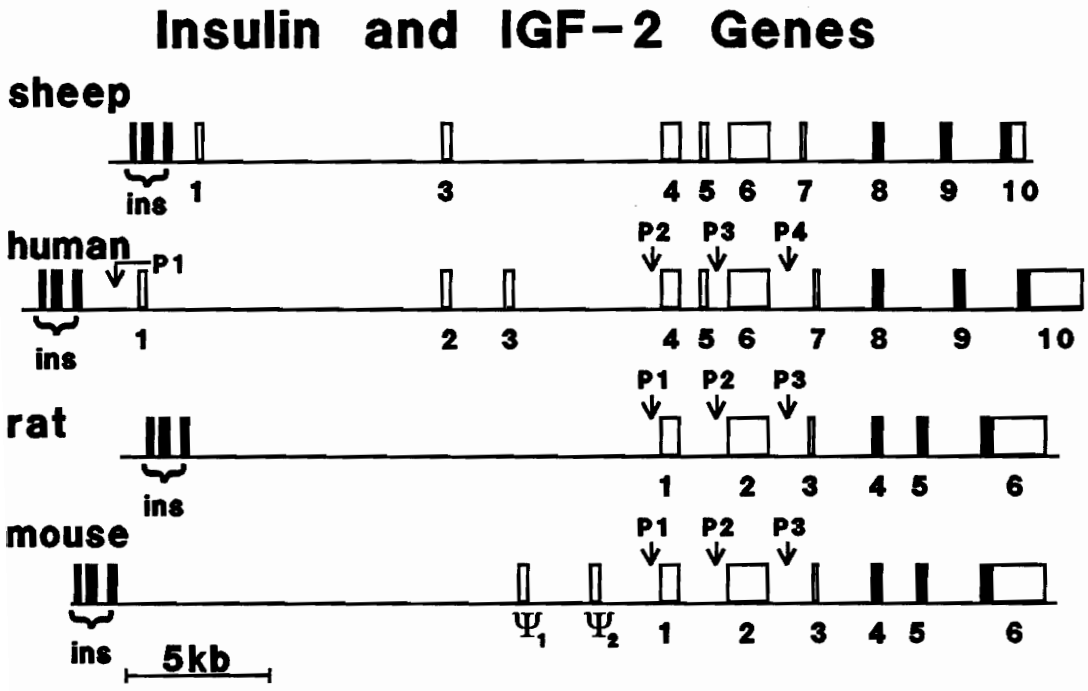
oIGF-II Exon 9

```
1    tgatgttcgaggaggaggcgcaggacgcaggggctggctctggtcactcacgctcactgtcccctctgtccctttctgggc
81   ccgcctctcgcttccttctccactccaggctgcggctggctgtgctgcagggccaccgggtgtcccgtcccaagggtggg
161  ggcgggggggctggccgaggacccccgctctgccaggtgcgggcactgacctgtcctgcccttcctccctgggggaccacc
241  agGCCGACCATCCAGCCGCATAAACCGACGCAGCCGTGGCATCGTGGAAGAGTGTTGCTTCCGAAGCTGCGACCTGGCCC
       erArgProSerSerArgIleAsnArgArgSerArgGlyIleValGluGluCysCysPheArgSerCysAspLeuAlaL
321  TGCTGGAGACTTACTGTGCCGCCCCCGCCAAGTCCGAGAGGGATGTGTCTGCCTCTACGACCGTGCTTCCGgtaagacag
     euLeuGluThrTyrCysAlaAlaProAlaLysSerGluArgAspValSerAlaSerThrThrValLeuPro
401  ccccccagctccagcccagccccgggggggaaccctcccctctctccctctgcagccccgcggggctgtccccttggagcc
481  aggggaccagggcacagcagctgctgagctgagagagcagccagggagtcctttacactctgcccactgggggcgctggc
561  cagcagcgggcgggcactggctgtcacacctgccatcacacctggggcacc
```

oIGF-II Exon 10

```
1    cagctcaggcgttgccccgccgtgttccctggggcctgggcagcccctgactcactcttcctctcccagGACGACTTCAC
                                                                    AspAspPheTh
81   AGCATACCCCGTGGGCAAGTTCTTCCAATCTGACACCTGGAAGCAGTCCACCCAGCGCCTGCGCAGGGGCCTGCCCGCTT
     rAlaTyrProValGlyLysPhePheGlnSerAspThrTrpLysGlnSerThrGlnArgLeuArgArgGlyLeuProAlaP
161  TCCTGCGAGCACGCCGGGGTCGCACGCTCGCCAAGGAGCTGGAGGCGCTCAGAGAGGCCAAGAGTCACCGTCCGCTGATC
     heLeuArgAlaArgArgGlyArgThrLeuAlaLysGluLeuGluAlaLeuArgGluAlaLysSerHisArgProLeuIle
241  GCCCTGCCTACCCAGGACCCTGCCACCCACGGGGGCGCCTCTTCCGAGGCATCCAGCGATTAGAAGTGAGCCAAAGTGTC
     AlaLeuProThrGlnAspProAlaThrHisGlyGlyAlaSerSerGluAlaSerSerAsp***
321  GTAATTCTGCCAAGTGACACCATCTACCTCGCGCCGTCCTCCTGACCGGGACTGCCCCACTAGGTCTCTCTCTGAAATCC
401  CTGTACCGTCCTGTCTGCGGGCTCCCCTGACCCAGCCTCTGTGCCCCAACCTCCCCACGTCAGGCAAGTCCCCCTCGGCC
481  CCCTCCATCTGGCCGAGGGGATCAGAACAACATCTCTAAAAATGTACAAAACCAATTGGCTTTAAATATCCCCCCAAATT
561  ATCACCCCCCAAATTACCCCCAAATTATACAACCAAAATTGCAATCATAAACCCCTCAATCAGCCCCCTTGAAATGAATT
641  GGCTTTTTAGCAACACCAGAAAAGCAAACTAGCTTTCCAAAAACTTCTTAAAAAAAAAAAAAAAATCAATTGGCTGAAAAA
```

Figure 4-3. Comparison of the genomic organization of the ovine, human, rat, and mouse IGF-II and insulin genes. Ins represents the insulin gene and the numbered boxes represent the IGF-II exons. Open and solid boxes indicate untranslated and translated regions, respectively. $\Psi_1$and $\Psi_2$are the two pseudo exons identified in the mIGF-II gene by computer sequence anlysis (Rotwein and Hall, 1990). Promoters are indicated by P1-P4.


Insulin and IGF-2 Genes
sheep
ins
1
3
4 5 6 7 8 9 10
human
P1
P2 P3 P4
ins
1
2 3
4 5 6 7 8 9 10
rat
P1 P2 P3
ins
1 2 3 4 5 6
mouse
P1 P2 P3
ins
Ψ1 Ψ2
1 2 3 4 5 6
5kb

located at comparable positions preceding ovine exons 1, 4, 6, and 7.

Identification of alternatively spliced oIGF-II cDNAs

Screening of a sheep liver cDNA library with a human IGF-II cDNA probe resulted in the isolation of an oIGF-II cDNA of 1225 nucleotides in length (Ohlsen, 1990; accession no. X55638). This cDNA is almost identical to the cDNA reported by O'Mahoney *et al.* (1991) with the exception of 49 additional nucleotides at the 5' end but differs at the 5' end from the cDNAs reported by Brown *et al.* (1990) and Demmer *et al.* (1993). The oIGF-II cDNAs with different 5' termini are shown in Fig. 4-1B and are generated by alternatively splicing exons 1 and 3, exon 6, or exon 7 to exons 8, 9, and 10. Ovine IGF-II cDNAs containing exons 4 and 5 have not yet been isolated.

The 3' termini of all the identified oIGF-II cDNAs end with a stretch of 9-17 adenine nucleotides. This poly A tract was proposed to represent a site of polyadenylation; however from the genomic sequence this stretch of adenine residues is clearly encoded in the gene (exon 10, nucleotides 690-704). Thus, the cDNAs were most likely generated by reverse transcription following hybridization of the oligo dT primer to this poly A tract. Similar poly A tracts are not found in the human, mouse or rat IGF-II genes.

Comparison of different mammalian IGF-II gene sequences

A comparison of the nucleotide sequences between the homologous sheep, human, rat, and mouse IGF-II exons are shown in Table 4-1. Between 72 and 91% sequence identity was observed for sheep exons and those of the other mammalian species, including the 5'-non-coding exons 1, 3, 4, 5, 6, and 7.

In Fig. 4-4, a comparison of sequences upstream of exon 1 revealed the lack of sequence elements in the ovine gene that were present in the human gene. The deletion of DNA sequences in the ovine gene relative to the human gene (between

Table 4-1

Percent Sequence Identity to Sheep IGF-II Exons

Sheep exons	1	3	4	5	6	7	8	9	10
human	75%	72%	83%	90%	91%	83%	91%	83%	83%
rat			79%		86%	77%	86%	86%	80%
mouse			77%		84%	76%	84%	87%	80%

Figure 4-4. Comparison of the nucleotide sequence upstream of ovine and human exon 1. Uppercase letters indicate identical nucleotides in the ovine and human sequences and lowercase letters indicate differences. Periods indicate spaces inserted to maximize sequence alignment. Numbering of the human IGF-II sequence is from van Dijk *et al.* (1991). The positions in the human IGF-II gene of the PE1-1 and PE1-2 binding sites are underlined and the C/EBP binding site is double underlined. The location of the proximal 67 bp repeat is in bold and a putative CCAAT site is in bold italics.


1623
Ovine GtcacacCcA agAgCccGGG GCTGCCgGGG ttggCcaaGG CtccGGAGGT CAGCGGGTGA CCtggctgta CC.aaGGtct GGCCAGcCTG CCaGCCggCC
Human GggcagaCgA gaAcCtaGGG GCTGCCtGGG cctaCtgtGG CctgGGAGGT CAGCGGGTGA CCctagctac CCtgtGGctg GGCCAGtCTG CCtGCCacCC
1722
Ovine AGGCCAAACC AATCTcCtCC TcTCCTGA.A GgTgCcaCCg GGcCgGGGCT tGGGgTGGCT GGGCCTGGGG CTGGgcaCcT GGGCTcccGT GGCTGCAGAC
Human AGGCCAAACC AATCTgCaCC TtTCCTGAgA GcTcCacCCa GGgCtGGGCT gGGGaTGGCT GGGCCTGGGG CTGG...CaT GGGCT...GT GGCTGCAGAC
67 base pair repeat
1821
Ovine CACTcagA.. .TG..... ....CC AGCctGGtgC CcGGCtggcG GGGCAGtCCT cT.....G tgtacccCaa AggCtcTCaC
Human CACTgccAgc tTGggcctcg aggccaggag ctcaccctCC AGCtaGGgaC CtGGCcactG GGGCAGcCCT gTtcctgaaG ctctgagCtc AccCctTCcC
-188 PE1-2 -150
1886
Ovine CcTGtCacCc cCtcCtCggg gCtACatcaA cAcccCcCAG gCCtCcagaA GgCCCcgCcC CttgGgGatG GCCAgGGtTG tGCAGGCaGG TGGcCGGCCG
Human CaTGaCcaCa tCagCcCccc tCcACccagA gAtgtCaCAG cCCcCagctA GcCCCacCtC CagaGtGggG GCCAaGGcTG gGCAGGCgGG TGGaCGGCCG
C/EBP -70 PE1-1 -43
1986
Ovine GACACTGGaC CCGGAAGAGG AGGGAGtGgG GgGGCTGGGA TgaGCAGtgA tCtGcCCgTa GcgtCACgCA GgtGGCCCtg tgCGCgCaGa TtGgGgCAGG
Human GACACTGGcC CCGGAAGAGG AGGGAG.GcG GtGGCTGGGA TcgGCAG.cA gCcGtCCaTg GgaaCACcCA GccGGCCCca ctCGCaCgGg TaGaGaCAGG
2086
Ovine GGtGCCCcGC aGGAGCTGAG GTATGTGgGt gC
Human GGcGccCtGC tGGAGCTGAG GTATGTGaGc tC
exon 1 ↑ intron

nucleotides 1830-1831) resulted in the partial loss of elements corresponding to the human nuclear factor binding site, PE1-2, (van Dijk *et al.*, 1991) and the proximal 67 base pair inverted repeat (de Pagter-Holthuizen *et al.*, 1987) present in the P1 promoter region. No intergenic ovine sequence was found comparable to the distal 67 base pair inverted repeat. The loss of these elements was probably due to the shortening of the ovine intergenic region by approximately 750 nucleotides relative to the human intergenic region. In addition, a proposed CCAAT/enhancer binding protein (C/EBP, nucleotides -99/ -91, van Dijk *et al.*, 1992) site located upstream of human exon 1 was not conserved in the ovine gene. However, further upstream of the proposed C/EBP site is a conserved region between sheep (nucleotides 1720-1736) and human which may represent a potential CCAAT binding site.

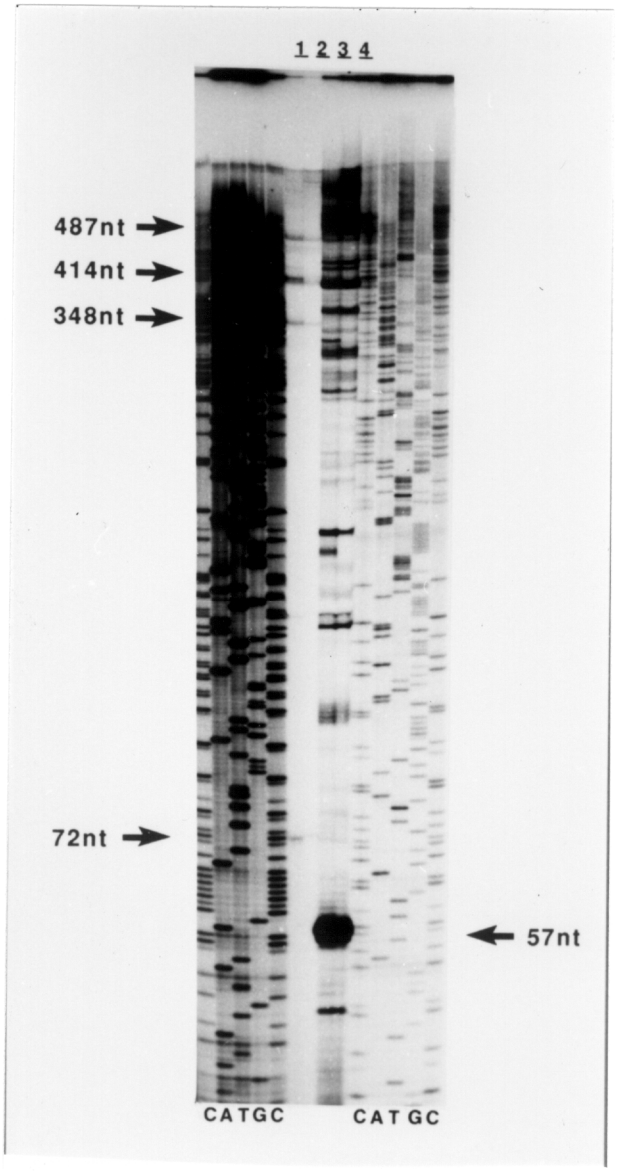
Identification of transcription initiation sites

Using primer extension analysis, transcription initiation sites were identified for exons 1 and 6 using total RNA extracted from liver tissue of 75-day fetuses and adult ewes. In Fig. 4-5, extension products of 72, 348, 414, and 487 nucleotides were detected with an oligonucleotide specific for exon 1. These products correspond to transcription initiation sites located 98, 374, 440, and 513 nucleotides from the 3' end of exon 1. For exon 6, a major extension product of 57 nucleotides was seen in addition to multiple fragments of larger size. The major site was located 83 nucleotides from the 3' end of exon 6. The transcription initiation sites for exons 1 and 6 were similar for fetal and adult hepatic RNAs with only a few minor differences. The assignment of these sites is tentative until confirmed by an alternative method such as RNase protection.

Expression of oIGF-II transcripts

To analyze expression of the alternatively spliced mRNAs, exon-specific transcripts were detected by a reverse transcriptase-polymerase chain reaction

Figure 4-5. Mapping of transcription initiation sites for oIGF-II exons 1 and 6. Major transcription initiation sites were determined by primer extension using oligonucleotides specific for exons 1 (lanes 1 and 2) or 6 (lanes 3 and 4). Total RNA from fetal (lanes 1 and 3) or adult (lanes 2 and 4) liver were used as templates for the reverse transcription reaction. Fragment sizes are determined based on the sequence ladder generated by sequencing of exon 1 or 6 containing fragments with the same oligonucleotides used for primer extension.

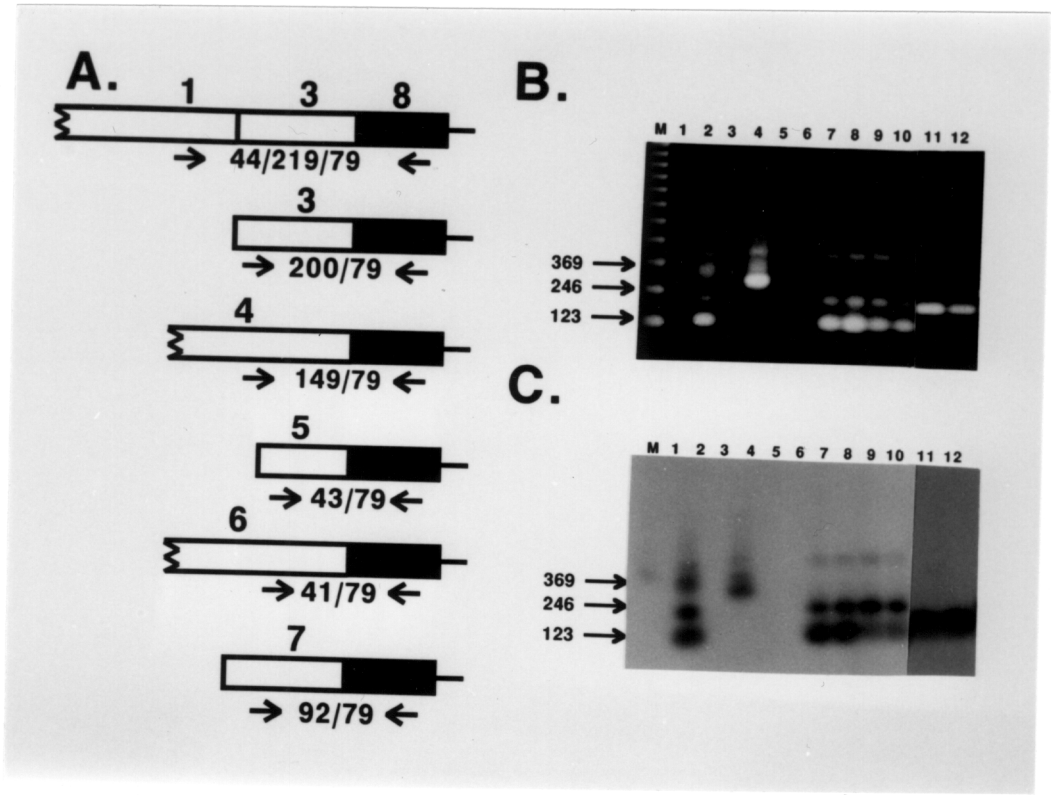

1 2 3 4
487nt
414nt
348nt
72nt
57nt
CATGC
CATGC

(RT-PCR) assay. Oligonucleotides specific for exons 1, 3, 4, 5, 6, and 7 were paired with an oligonucleotide complementary to exon 8. The predicted sizes of the PCR products for exon arrangements 1-8, 1-3-8, 3-8, 4-8, 4-5-8, 5-8, 6-8, and 7-8 were 123, 342, 279, 228, 393, 122, 120, and 171 nucleotides, respectively (Fig. 4-6A). Total RNA from fetal and adult liver were analyzed by the RT-PCR assay (Fig 4-6B). To confirm that these PCR products were specific for oIGF-II transcripts, the gels were Southern blotted to nylon membranes and probed with a nested oligonucleotide specific to oIGF-II exon 8 (Fig. 4-6C). Results from two different fetal and adult liver RNA samples were identical (data not shown).

For exon 1 a number of bands appeared in the adult sample (lane 2), of which one PCR product approximated the predicted size of 342 nucleotides for the exon 1-3-8 arrangement and a second of 123 nucleotides which could represent an alternatively spliced message containing exons 1 and 8. The 1-8 exon arrangement was verified by cloning and sequencing the PCR product corresponding to these exons. The origin of the other bands present in the adult sample has not yet been determined. When the exon 3-specific oligonucleotide was used (lane 4), there appeared to be only an adult liver-specific product corresponding to exon arrangement 3-8, which was verified by cloning and sequencing of the PCR product. Expression of exons 1 and 3 in adult and not fetal liver is consistent with the adult liver-specific expression of hIGF-II exons 1, 2, and 3 (de Pagter-Holthuizen *et al.*, 1987).

Transcripts containing exon 4 were not detected in either fetal or adult liver RNA (lanes 5 and 6). A second oligonucleotide specific to oIGF-II exon 4 was also used for PCR amplification, again with negative results. However, an oligonucleotide specific to ovine exon 5 generated PCR products of approximately 125 and 210 nucleotides in both fetal and adult liver RNA (lanes 7 and 8). The two products were generated by amplification from oligonucleotide 8a and residual oligonucleotide 8c, which had been utilized for reverse transcription. This exon 5-8 arrangement was

Figure 4-6. PCR amplification for the detection of alternatively spliced oIGF-II mRNAs. Total RNA from fetal or adult liver was reverse-transcribed with an oligonucleotide primer specific to the shared exon 8. PCR reactions were carried out with a nested primer in exon 8 and one additional primer specific to exon 1, 3, 4, 5, 6, or 7. A. Schematic representation of the predicted sizes of PCR products for different exon combinations. B. Ethidium bromide stained agarose gel containing PCR products for exon combinations 1-8 (lanes 1 and 2), 3-8 (lanes 3 and 4), 4-8 (lanes 5 and 6), 5-8 (lanes 7 and 8), 6-8 (lanes 9 and 10), and 7-8 (lanes 11 and 12). Odd numbered lanes show PCR products from fetal liver and even numbered lanes are from adult liver. Fragment sizes were estimated from a 123 bp ladder (M). C. Southern blot of the agarose gel shown in part B, probed with an exon 8-specific oligonucleotide.


A.
1
3
8
44/219/79
3
200/79
4
149/79
5
43/79
6
41/79
7
92/79
B.
M 1 2 3 4 5 6 7 8 9 10 11 12
369
246
123
C.
M 1 2 3 4 5 6 7 8 9 10 11 12
369
246
123

verified by cloning and sequencing of both PCR products. Human exon 5 is a novel alternatively spliced exon that was detected only in human histiocytoma tissue (Ikejiri *et al.*, 1991b). However, it appears that the putative ovine exon 5 is a normally utilized exon in adult and fetal sheep liver; but exon 4 is not expressed in liver.

The promoters for human exon 6 (P3) and exon 7 (P4) are predominantly active in fetal tissues (de Pagter-Holthuizen *et al.*, 1987). Utilizing an oligonucleotide to ovine exon 6 (lanes 9 and 10) or exon 7 (lanes 11 and 12), the predicted size PCR products for exon arrangements 6-8 or 7-8 were detected in both fetal and adult liver. As for exon 5, the two PCR products were the result of amplification with oligonucleotides 8a and 8c. Both 6-8 and 7-8 exon arrangements were verified by cloning and sequencing of the adult and fetal liver PCR products. This result demonstrates that the promoters for ovine exon 6 and 7 are different from that of human in that they appear to be active in both fetal and adult liver.

Discussion

The organization of the ovine IGF-II and insulin genes is very similar to the human genes. Presently, neither exon 2-containing oIGF-II cDNAs nor the genomic region corresponding to human exon 2 have been cloned. In the rat genome, regions corresponding to human exon 1 have been deleted completely while regions corresponding to exons 2 and 3 have been conserved nearly completely or in a highly deviated form, respectively (Ikejiri *et al.*, 1990). In sheep, the intergenic region between insulin and IGF-II is approximately 750 nucleotides shorter than the human intergenic region and contains a stretch of 85 nucleotides that is 96% G+C. This shorter intergenic region deletes the 67 base pair inverted repeats found in the human intergenic region (de Pagter-Holthuizen *et al.*, 1987), although the functional significance of these inverted repeats is not known. Because the insulin and IGF-II

genes are located in close proximity and transcribed in the same direction, some mechanism must be active to prevent transcription run through. Perhaps the inverted repeats for the human genes or the high G+C sequence act as barriers to the cotranscription of the two genes.

Transcription initiation sites tentatively mapped for ovine exon 1 were different from those mapped for hIGF-II exon 1. Four sites were mapped 98, 374, 440, and 513 nucleotides from the 3' end of ovine exon 1. This contrasts to the single cluster of sites that was mapped 115-118 nucleotides from the 3' end of hIGF-II exon 1 (dePagter-Holthuizen *et al.*, 1987). The deletion of a region upstream of oIGF-II exon 1 (between nucleotides 1830-1831) eliminates the nuclear factor binding site PE-1 (van Dijk *et al.*, 1991). In addition, a putative C/EBP site identified in the human sequence is not conserved in the sheep sequence. Because C/EBP is expressed predominantly in fat, liver, and lung tissue (Birkenmeier *et al.*; 1989) and is a potent activator of the liver-specific, serum albumin promoter (Friedman *et al.*, 1989), the C/EBP binding site was hypothesized to be important for mediating adult liver specific expression of promoter, P1 (van Dijk *et al.*, 1992). Due to the poor conservation of this element in the ovine gene, another mechanism may promote adult-specific expression in sheep liver. Further upstream of the proposed C/EBP site is a conserved region between sheep and human which may represent a functional CCAAT binding site.

A major transcription initiation site was present 83 nucleotides upstream of the 3' end of exon 6. In addition, other initiation sites were mapped further upstream which were different than those identified for the hIGF-II gene (de Pagter-Holthuizen *et al.*, 1987). Transcription initiation sites further upstream of the major initiation site were consistent with the cDNA (accession no. M89789) reported by Demmer *et al.* (1993) which contained 931 nucleotides of exon 6. The 5' end of this cDNA terminated approximately 200 nucleotides short of the corresponding position for the proposed transcription initiation site (oIGF-II exon 6, nucleotide 2881) for

human exon 6 (de Pagter-Holthuizen *et al.*, 1987). Further upstream of this corresponding position were a potential CCAAT box (CCATT; nucleotides 2795-2799), TATA box (TATAA; nucleotides 2857-2861), and Sp1 recognition site (nucleotides 2767-2776). These putative regulatory elements also were found conserved not only in hIGF-II exon 6 but also in the homologous rat and mouse exon 2 gene sequences (Rotwein and Hall, 1990; Soares *et al.*, 1986; Evans *et al.*, 1988).

The tissue- and development-specific regulation of the putative ovine IGF-II promoters is similar to human IGF-II promoters. Based on the sequence of oIGF-II cDNAs previously reported and the results of our RT-PCR assays, the oIGF-II gene also is likely to contain four promoters preceding exons 1, 4, 6, and 7. Ovine IGF-II cDNAs corresponding to exons 1 and 3, 6, and 7 spliced to the shared exons 8, 9, and 10 have already been cloned. Ovine IGF-II cDNAs containing exons 4 or 5 have not been reported.

Similar to the hIGF-II gene, the ovine promoter for exon 1 is expressed in adult and not fetal liver. Human promoter P1 (exon 1) has been shown to be expressed specifically in adult liver. Mouse and rat IGF-II genes lack a promoter homologous to human P1 and therefore do not express IGF-II in adult liver (Frunzio *et al.*, 1986; Soares *et al.*, 1986). Consistent with the adult liver-specific expression of hIGF-II exons 1, 2, and 3, sheep exon 1 or exon 3 containing transcripts were detected only in adult liver and oIGF-II cDNAs containing exons 1 and 3 were isolated from adult sheep liver cDNA libraries (Ohlsen, 1990; O'Mahoney *et al.*, 1991). Using the RT-PCR assay, we have identified also a novel exon arrangement containing exon 1 spliced directly to exon 8. In adult sheep liver, oIGF-II transcripts of 5.3 kb (Demmer *et al.*, 1993), 5.1 kb (O'Mahoney *et al.*, 1991; Delhanty and Han, 1993), or a doublet of 5.0 and 4.7 kb (Wong and Ohlsen, unpublished) were detected. These transcripts were likely initiated from promoter P1 and contain exon 1 and/or 3. The two oIGF-II transcripts that we detect may represent mRNAs with either a 1-3-8 or 1-8 exon arrangement.

We have further shown by RT-PCR that oIGF-II mRNAs containing exon 5 spliced to exon 8 are present also in fetal and adult liver tissue. However, exon 4-8 or 4-5-8 arrangements were not detected by RT-PCR. This is consistent with the absence of exon 4 containing transcripts in adult human liver and barely detectable presence in fetal liver; but enhanced expression in histiocytoma and leiomyosarcoma tumor cells (Holthuizen *et al.*, 1990; Ikejiri *et al.*, 1991). In human tumor cells, exon 5 is sometimes present in an exon 4-5-8 arrangement or spliced out. This situation is analogous to our finding that oIGF-II exon 3 is sometimes present in an exon 1-3-8 arrangement or spliced out. Since we have not detected an oIGF-II exon 4-5 arrangement, it is not known what leader exon, if any, is associated with oIGF-II exon 5 transcripts. However, because the 5' splice site of ovine exon 5 is almost 100% identical to that of human exon 5, this would suggest that ovine exon 5 is spliced to an upstream exon rather than transcribed from a unique promoter. Nevertheless, it is clear that both fetal and adult liver express oIGF-II transcripts containing exon 5.

Human promoters P3 and P4 which transcribe exon 6 and exon 7 have been shown to be active in fetal liver and turned off in adult liver (de Pagter-Holthuizen *et al.*, 1988). However, our RT-PCR results using oligonucleotides specific to ovine exons 6 and 7 show that exon 6 and exon 7 containing transcripts were present in both fetal and adult liver tissue. Demmer *et al.* (1993) have cloned oIGF-II exon 6 and exon 7 containing transcripts from a lamb liver library and Brown *et al.* (1990) have cloned exon 6 containing transcripts from a fetal sheep library. These results support the conclusion that ovine promoters P3 and P4 are active both fetally and postnatally.

The overall organization and expression of the oIGF-II gene is similar to that of hIGF-II, with only minor species differences. Four putative promoters for oIGF-II have been identified which need to be confirmed by transfection studies with promoter-reporter gene constructs. In addition, trans-acting factors which

differentially activate these promoters need to be identified to further understand the mechanisms which control the activation of the ovine IGF-II gene.

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Summary and Implications

The cloning and characterization of oIGF-I and -II genes provide fundamental information required to investigate the molecular regulation of these growth promoting peptides. Both oIGF-I and -II genes have been shown to be well conserved in sequence, structure and organization with IGF-I and IGF-II genes of other species. These genes have been resistant to change throughout evolution thus indicating their importance in regulating growth and development. Identification of factors involved in initiating gene transcription and generating multiple mRNAs by alternative splicing to produce heterogenous populations of mRNAs will help determine how oIGF-I and -II gene expression is regulated.

Three different oIGF-I transcripts were found to be expressed in fetal and adult tissues. Two of the three mRNAs are similar to transcripts identified in other species, thus suggesting the mechanism used to regulate oIGF-I gene transcription is similar to other non-ruminant species. Class 1 transcripts appear to be expressed in many fetal and adult tissues and the growth hormone inducible class 2 transcripts are expressed predominantly in liver. The third oIGF-I transcript contained a novel exon W sequence that was expressed at low levels in both fetal and adult tissues. Homologous transcripts to the exon W mRNA have not been described in other species indicating possible ovine-specific gene regulation. All three transcripts result in encoding distinct proIGF-I signal peptide sequences. These leader peptide sequences may alter the post-translational processing of the different pro-oIGF-I peptides. IGFBP changes in bovine mammary epithelial cells that express exon W and class 2 transcripts indicate that IGF-I production may also influence the regulation of IGFBP expression and/or secretion. Understanding the interactive role of between IGF-I and IGFBPs at the cellular level will be necessary to determine the growth promoting effects of oIGF-I.

Organization and expression of the oIGF-II gene are comparable to the human IGF-II gene. While the majority of oIGF-II transcripts are similar to those expressed in human and rodents, two oIGF-II transcripts were found to be unique to sheep. Ovine mRNAs that contain exon 1 or 5 spliced directly to exon 8 were expressed in sheep liver and corresponding transcripts have not been characterized in human or rodent IGF-II gene expression. These transcripts appear to be expressed in a tissue- and development-specific manner such that exon 1 transcripts were specifically expressed in adult liver tissue while exon 5 transcripts were detected in both fetal and adult samples. Identification of putative regulatory binding factors which function at oIGF-II promoter regions may help determine how tissue- and development-specific expression is controlled during oIGF-II gene expression. The selection for multiple promoters may have been necessary to insure the continuous production of oIGF-II throughout development because the putative oIGF-II binding factors may themselves be regulated in a tissue- or development-specific manner.

The cloning and characterization of the oIGF-I and -II genes are the initial steps in understanding the role these peptides play in controlling ruminant growth. Discerning how ruminant-specific growth control mechanisms are regulated at the molecular level may eventually lead to new production practices in which tissue- and/or development-specific growth can be achieved. Changes in animal selection, nutrition, or treatment practices based on these molecular findings will provide for improved growth efficiencies and product quality.

Vita

Susan Marie Ohlsen was born on April 8, 1965 in Marshall, Minnesota, the daughter of Dr. Jay and Joyce Ohlsen. She graduated from Marshall Senior High School in 1983 and continued her education at Concordia College in Moorhead, Minnesota, where she received a Bachelor of Arts degree in Biology and Chemistry in May of 1987. During June, 1987 through August 1988, she was employed by Business Credit Leasing (BCL) as a medical marketing manager. In September, 1988 she began her graduate studies at the University of Minnesota where she was awarded a Masters of Science degree in Animal Physiology in October, 1990. She pursued her graduate studies at Virginia Polytechnic Institute and State University and completed her doctoral studies in Genetics in October, 1993. While at Virginia Tech she was a member of the Graduate Student Assembly, the American Association for the Advancement of Science, and the Endocrine Society.

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