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REGULATION OF INSULIN-LIKE GROWTH FACTOR II GENE TRANSCRIPTION DURING MUSCLE DIFFERENTIATION

by

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A THESIS

Presented to the Department of Cell and Developmental Biology

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Master of Science

September 2006

TABLE OF CONTENTS

ACKNOWLEDGMENTS

To my mother who has always supported and believed in me, my brother for keeping me laughing, my daughter for giving life light and reason and all of the friends whose personal sacrifices and support I could never do without.

I would also like to thank my mentor, Peter Rotwein, and Elizabeth Wilson, senior scientist, for their guidance and support in my graduate work.

This study was supported by grants from the American Heart Association, Tartar Trust of Oregon Health Science University and a scholarship from MENSA

ABSTRACT

Muscle cell differentiation, maturation and regeneration depend on ongoing interactions between signaling pathways activated by growth factors, hormones, and myogenic transcription factors including MyoD and related proteins. The insulin-like growth factors (IGFs) play vital roles in prenatal muscle development and are important for coordinating muscle regeneration and re-innervation following neuro-muscular injury in the adult. IGF action also is critical for maintaining skeletal and cardiac muscle mass during aging.

IGF-II is important during prenatal development to promote growth, survival and differentiation of many cell types and tissues. The focus of my study is the regulation of IGF-II gene expression in muscle. Previous studies have shown that IGF-II gene transcription and mRNA expression are induced during the differentiation of skeletal myoblast cell lines. However, little has been done to unravel the steps that control cell-type specific IGF-II gene expression. The IGF-II gene is reciprocally imprinted with the adjacent H19 gene, leading to expression of IGF-II from only the paternally derived chromosome in most tissues, and H19 expression from the maternal chromosome. While it is postulated that DNA elements within the IGF-II-H19 locus may play a role in imprinting and thus in overall control of IGF-II gene expression, the specific biochemical mechanisms of gene regulation have not been elucidated.

To address this, experiments were done to determine whether IGF-II gene expression is induced during myogenic differentiation of mouse mesenchymal stem cells. Reverse transcriptase polymerase chain reaction (RT-PCR) of whole cell RNA isolated

during muscle cell differentiation, demonstrated that indeed IGF-II gene expression is induced and furthermore transcripts are produced only from IGF-II promoter 3 during muscle differentiation. Further studies showed that the increase in IGF-II gene expression during muscle cell differentiation correlates to an increase in IGF-II transcription and that the transcription of myogenin, H19 and IGF-II is impaired in myoblasts lacking normal IGF-II expression.

Previous attempts to identify the regulatory regions within the IGF-II/H19 gene locus have yielded inconclusive results. Transgenic experiments have shown that mesoderm-specific enhancers are not located within the area 14.5 kb upstream to 6.5 kb downstream of IGF-II [1, 2], or within 11 kb upstream to 12 kb downstream of H19. However, the H19 and IGF-II genes share a complex pattern of gene expression, suggesting their transcriptional activation is linked mechanistically. This led me to search for more distal regulatory regions surrounding the two genes on mouse chromosome 7. Four putative mesodermal and/or muscle-specific DNA control regions were tested for their ability to act as enhancers of the IGF-II promoter 3, driving luciferase expression, during muscle cell differentiation. An area shared by two of the DNA control elements, overlapping by approximately 300 base pairs, was found to be a potent enhancer of IGF-II P3 during differentiation of both a mesenchymal stem cell line and a myoblast cell line.

This myogenic enhancer region is well conserved among mammalian species and contains several potential binding sites for muscle-specific transcription factors. These results demonstrate an important contribution to understanding the complex regulation of IGF-II gene expression during muscle differentiation and pave the way to further studies

of this important enhancer region and its role in regulating the complex process of muscle formation.

INTRODUCTION

Muscle cell differentiation, maturation and regeneration depend on ongoing interactions between signaling pathways activated by growth factors, hormones, and myogenic transcription factors including MyoD and related proteins. The insulin-like growth factors (IGFs) play vital roles in prenatal muscle development and in promoting growth, survival and differentiation of many cell types and tissues. They are also important for coordinating muscle regeneration and re-innervation following neuro-muscular injury in the adult. The focus of my work is the regulation of IGF-II gene expression in muscle.

Discovery of IGFs

Nearly 50 years ago Salmon and Daughaday attempted to develop an assay to measure growth hormone (GH) activity in human plasma [3]. These studies demonstrated that sulphate uptake in cartilage of hypophysectomized rats is mediated through an intermediate serum factor which they termed sulfation factor (SF). It was later determined that serum stimulated protein and DNA synthesis in cartilage in a GH-dependent fashion [4, 5] and that SF was active in muscle as well [6]. These discoveries led to the renaming of SF to the more general term of somatomedin(s) (SM) and the development of the somatomedin hypothesis. This hypothesis states that circulating somatomedin(s) is/are synthesized by a GH stimulus to indirectly mediate the effects of GH activity on body tissues [7]. Somatomedins A, B and C were eventually termed to describe the neutral, acidic and basic fractions, respectively, that were subsequently purified and characterized [8-10].

During this time, another line of research was being conducted, on a different biological system, which would eventually lead to the discovery of the same family of proteins. Following the development of a radioimmunoassay for insulin, it was noted that most of the insulin-like activity measured by this bioassay in serum could not be suppressed by the addition of anti-insulin serum [11, 12]. This was referred to as nonsuppressible insulin-like activity (NSILA) and in human serum was found to be composed of two components- a large molecular-weight acid-insoluble fraction, NSILA-P, and a smaller acid-soluble fraction, NSILA-S [11, 13]. Nearly a decade later, Rinderknecht and Humbel isolated two forms of insulin-like factors from NSILA-S and determined the amino acid sequence of these peptides, which were subsequently named Insulin-like Growth Factor-I and –II (IGF-I and -II), for their close similarity with proinsulin [14-16].

In 1983 the growth field (SM) and metabolic field (NSILA) merged when Klapper et al. demonstrated that IGF-I was identical to SM-C and later when Enberg et al. showed that SM-A was also IGF-I and had been characterized as a separate fraction due to differing isolation techniques [17, 18]. IGF-II was then isolated from rat liver cell conditioned medium by a group that had initially set out to isolate a family of small polypeptides termed Multiplication Stimulating Activity for their ability to stimulate DNA synthesis in chick embryo fibroblasts [19-21]. In 1987, Daughaday et al. suggested that IGF-I and -II become the common terms for all fields, gradually replacing 'Somatomedins' [22].

Molecular Biology of IGF-II – Peptides, Genes and mRNAs

In 1978, Rinderknecht et al published the amino acid sequence of IGF-II [16]. Later, cDNA cloning allowed the full code of human IGF-II to be unraveled confirming the correctness of the peptide sequence and its similarity with proinsulin [23, 24]. By the end of the 1980's the structure of the IGF-II gene and its mRNAs had been determined for humans and many other species [25-32].

Human IGF-II is a small 7.5kDA, single-chain peptide of 67 amino acids with similarity to both IGF-I and insulin [16]. The 180 amino acid (20.1 kDa) IGF-II preprohormone contains a carboxy-terminal peptide of 89 amino acids and a signal peptide of 24 amino acids, both of which are cleaved to produce the 67 amino acid monomeric plasma protein. The sequence is well-conserved among mammals with 61 out of 67 amino acids identical among species with published amino acid sequences [33]. The mammalian IGF-II peptide consists of 4 domains: B (N-terminal), C, A and D (C-terminal) generally containing 28, 12, 21 and 6 residues respectively (Figure 1). Mature IGF-II displays 47% amino acid sequence identity with insulin, and owing to strict conservation of the 3 interchain disulphide bridges and residues in the hydrophobic core, has similar 3D structure [34].

Since the elucidation of the primary form of human IGF-II, several protein variants have also been described. These include a larger, 70 amino acid, form which arises from an alternative splice site in exon 9 and a 69 amino acid form, both with additional amino acids falling within the C domain [35-37]. These variants have a reduced affinity for the IGF-I receptor however, their biological significance is unknown [33, 36].

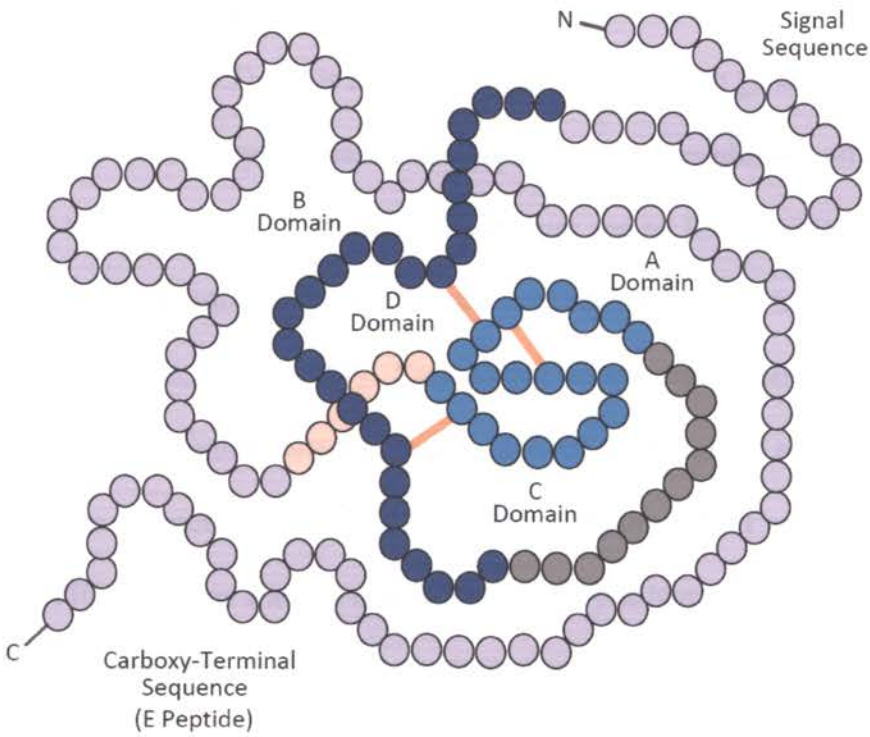


Figure 1: Structure of IGF-II preprohormone. Prepro IGF-II consists of a signal peptide of 24 residues, 67 amino acids of mature peptide and 89 amino acids in a carboxy-terminal extension termed the E-domain. Similar to proinsulin, IGF-II is divided into A, B and C domains. A and B domains are bridged by two inter-domain disulphide bonds (orange bars), with one internal disulphide bond in the A domain. The A and B domains are connected by a C-domain, which unlike the insulin C-domain is not proteolytically cleaved during structural maturation. The D-domain is not present in insulin. The carboxy-terminal sequence (E domain) of the preprohormone is removed during processing (based on a review by Nielsen [38]).

The mouse IGF-II gene extends over ~12 kilobases of distal chromosome 7 (human chromosome 11p15.5) and is located 18 kb 3' to the insulin gene and 80 kb 5' to the H19 gene. The tyrosine hydroxylase gene resides just 5' to insulin and all four genes are in the same transcriptional polarity [23]. The mouse and rat IGF-II genes contains six exons with exons 1-3 encoding distinct 5' untranslated regions that are transcribed by three different promoters, P_1, P_2, and P_3. Each unique leader exon is spliced into a cassette of coding and 3' untranslated sequences, derived from exons 4 to 6 [39]. The mouse IGF-II gene also contains two 5' pseudo exons, which share 81 and 53% identity with the human IGF-II exons 2 and 3, respectively [39]. The human IGF-II gene contains 10 exons and four promoters, the most 5' of which does not appear to have a structural or functional homologue in the mouse IGF-II gene. In all species there are three coding exons; in humans, exons 8-10, and in rodents, exons 4-6. Multiple mRNA transcripts are produced for the IGF-II gene in mammalian species through a variety of mechanisms including alternative transcription initiation sites, use of different promoters, variable RNA polyadenylation and cleavage of mature mRNAs [33]. Figure 2 details these mRNAs for the human gene.

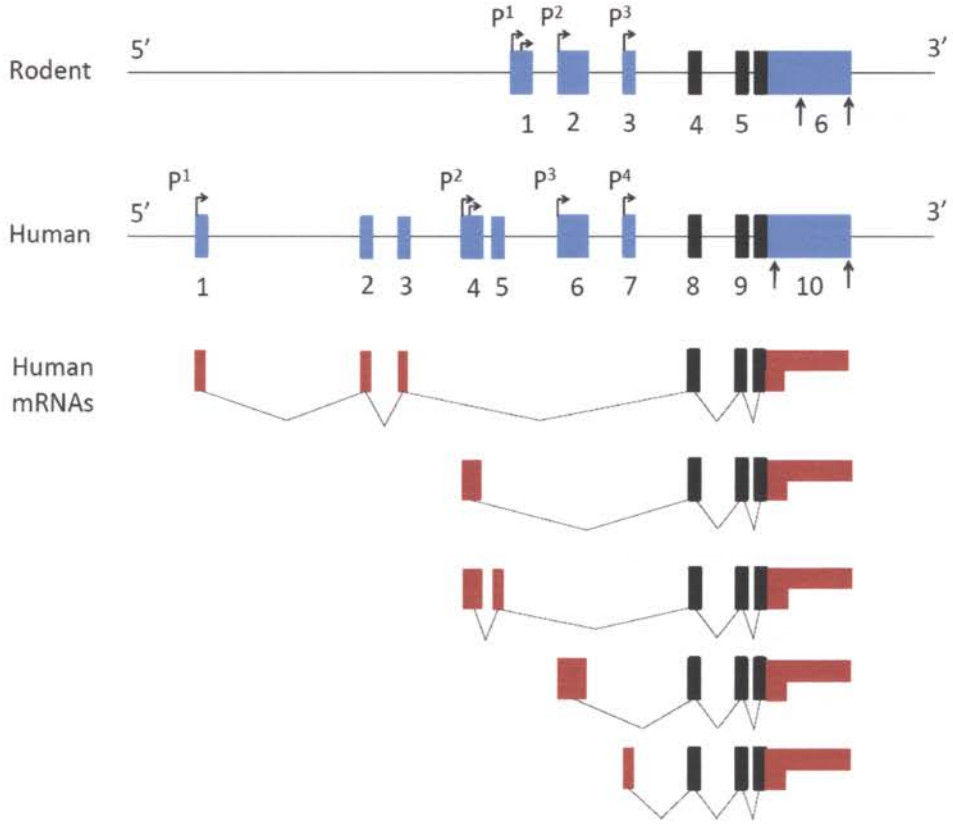


Figure 2: Structure of human and rodent IGF-II genes. Exons are numbered with coding regions in black. Promoters are labeled "P", transcription start sites with bent arrows and polyadenylation sites by vertical arrows. Structures of human IGF-II transcripts are below in red. Sites of pre-mRNA splicing are show with thin lines and locations of differential polyadenylation by boxes of different lengths.

Regulation of IGF-II Expression

Regulation of the IGF-II gene is complex. Expression is controlled at many levels including tissue-specific and developmental stage-dependent transcription initiation, imprinting mechanisms, alternative splicing, multiple polyadenylation sites, endonucleolytic cleavage of IGF-II mRNAs and translational control [40].

Developmental and tissue-specific expression

IGF-II gene expression has been detected in mouse embryos starting at the two-cell stage [41]. The important role of IGF-II in promoting growth of the developing embryo has been clearly seen in IGF-II null mice which show a 40% reduction in body weight compared to their wild type littermates [42]. IGF-II mRNA and protein levels are high in most fetal tissues, especially in the liver [43]. After birth, IGF-II expression remains high in humans, but decreases in all rodent tissues except the choroid plexus and leptomeninges [44]. This difference may be accounted for by the presence of an adult stage-specific promoter, P_1, found in humans but not in rodents ([45] and Figure 2).

Imprinting

Previous studies have shown that the IGF-II gene is regulated at the level of transcription in muscle cell lines [1]. However, little is known about how this regulation occurs. Studies involving IGF-II gene regulation have largely been focused on the reciprocal imprinting of the IGF-II and H19 genes. Genomic imprinting is the process by which gamete-specific epigenetic modifications control the differential expression of the two alleles of a gene. The tightly linked H19 and IGF-II genes are expressed in tissues of both endodermal and mesodermal origin, with H19 being expressed from the maternal chromosome and IGF-II from the paternal chromosome in most tissues [46].

Several models have been proposed to explain the reciprocal imprinting of these genes, most of which share the following common elements. Chromosome-specific expression is thought to be governed by competition between promoters for a common set of enhancers that are activated during various stages of development. A differentially methylated domain (DMD) found 5' of the H19 gene functions as a methylation-sensitive insulator. On the maternal allele, where H19 is transcribed, the zinc finger protein, CCCTC-binding factor (CTCF) binds to the unmethylated DMD to insulate the IGF-II promoter from enhancers located 3' to H19. This allows H19 exclusive access to these elements and associated transcription factors. On the paternal allele, the methylated DMD silences H19, with methylation subsequently spreading to the H19 promoter region to lock it in a transcriptionally inactive state. The hypermethylated DMD cannot be bound by CTCF, thereby preventing the formation of an insulator and allowing IGF-II access to downstream enhancers ([46] & Figure 3).

An additional DMD is present near exons 5 and 6 of the IGF-II gene but has not been found to be crucial for imprinting [47]. To date, tissue specific enhancers directing expression of both genes in liver, gut endoderm, and sclerotome have been characterized and are located approximately 10 kb downstream of H19 [48, 49]. In addition to shared regulatory elements, IGF-II may also possess independent enhancers since it has been shown to escape imprinting in some tissues and be expressed from both chromosomes [50, 51].

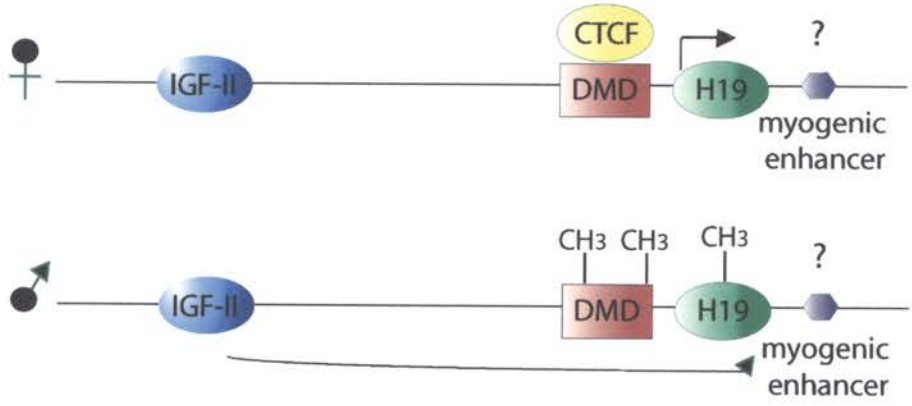


Figure 3: Imprinting model of the H19 and IGF-II genes. Chromosome-specific expression of the H19 and IGF-II genes is established through an insulator element that blocks access of the genes to shared enhancers downstream of the H19 gene. CTCF binds the differentially methylated domain on the maternal chromosome allowing the H19 gene exclusive access to the shared enhancers. Methylation of the DMD and the H19 gene prevents binding of CTCF on the paternal chromosome and prevents activation of the H19 gene allowing IGF-II exclusive access to downstream enhancers.

mRNA Processing

Regulation of the IGF-II gene also occurs at the level of posttranscriptional mRNA processing. Expression of IGF-II in human adult liver tissue is derived from promoter P_1. This promoter gives rise to a 5.3 kb mRNA and is the main activator of IGF-II transcription in the adult liver, which functions as the source of circulating endocrine IGF-II. Since this promoter is absent in rodents, expression of IGF-II is largely shut-off after birth [52]. Before birth, IGF-II transcription is directed by promoters P2, P3 and P4 with P3 yielding a 6.0 kb mRNA transcript which is the most abundant in prenatal development. In rodents and humans, a non-IGF-II-encoding RNA of 1.8 kb has also been detected by Northern blots using a probe specific to the 3'-UTR of IGF-II [52]. This non-IGF-II encoding RNA does not correspond to any of the promoters and has been identified as a product created by endonucleolytic cleavage of IGF-II mRNAs in the 3' UTR [53]. This mRNA is polyadenylated and quite stable although its biological relevance has yet to be elucidated.

IGF-II Signaling Pathways and Muscle Differentiation

Skeletal muscle development in vertebrates proceeds when progenitor cells within the somites receive cues to migrate and differentiate into muscle precursor cells. This process begins with the induction of two muscle-specific transcription factors, Myf-5 and MyoD, members of the basic helix-loop-helix (bHLH) transcription factors of the MyoD family, which also includes myogenin and Muscle Regulatory Factor 4 (MRF4). These proteins are critical for muscle development in the embryo and are capable of inducing a variety of cell types to be converted into muscle when overexpressed in cultured cells [54]. These proteins dimerize with E2A gene products to bind E-boxes in the regulatory

regions of skeletal muscle-specific genes for transcriptional activation [55]. Double knockout mice of Myf-5 and MyoD die at birth from a lack of any skeletal muscle or precursor myoblasts suggesting an essential role of these proteins in myogenesis and myoblast specification [56]. Similar studies of myogenin knockout mice have demonstrated a role for this protein in differentiation of myoblasts into myocytes [57].

IGF-II has also been shown to have important roles in normal muscle growth and development and can promote proliferation or enhance differentiation depending on the signaling cues and cellular environment [58]. IGF-II expression in skeletal muscle cell lines has been shown to be linked to the differentiation state of the cells. In mouse C2 myoblast cells serum withdrawal leads to terminal differentiation causing a marked increase in IGF-II mRNA and autocrine peptide production [59, 60]. This increase in IGF-II expression during C2 cell differentiation was shown to be due to transcriptional activation of the gene through promoter 3 [1].

During muscle cell differentiation newly produced IGF-II will bind to and activate the IGF-I receptor. This leads to receptor tyrosine phosphorylation and the subsequent tyrosine phosphorylation of adaptor molecules, IRS1 and IRS2, followed by the recruitment and activation of Phosphoinositide-3 kinase (PI3-kinase), and production of the signaling lipid, PIP3 (Figure 4). This triggers a signaling cascade that leads to the phosphorylation and activation of Akt. Unknown downstream signaling steps then stimulate the activity of MyoD, which is bound in conjunction with other basic-helix-loop-helix transcription factors to a DNA element termed the E box in chromatin at the promoters of muscle-specific genes, leading to their transcriptional activation (Figure 4).

Some of the signaling pathways involved in the switch of IGF action from a mitogenic to a differentiative response have begun to be determined through the use of pharmacological blocking agents. Inhibiting MAP Kinase Kinase (MEK) 1 and 2 blocks IGF-mediated replication by preventing activation of the Mitogen Activated Protein (MAP) kinases, Erks 1 and 2, while inhibiting the PI3-kinase pathway does not significantly interfere with IGF-mediated muscle cell proliferation [61, 62]. Conversely, the role of IGFs in maintaining muscle cell viability was shown to proceed through the signaling intermediates PI3-kinase and Akt (Figure 4) by the induction of the cyclin-dependent kinase inhibitor, p21 [63]. Further work is needed to identify the specific co-factors and signaling pathways of IGF-II as many additional proteins are suggested to be involved in the complex process of myogenesis.

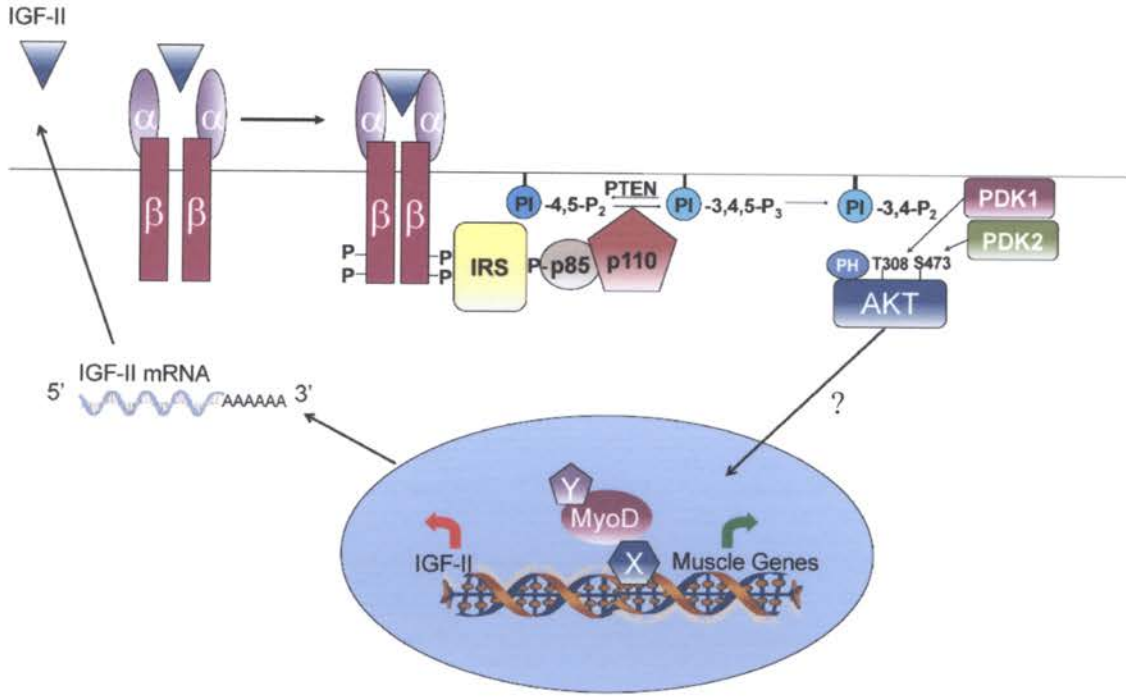


Figure 4: Autocrine signaling by IGF-II during muscle differentiation. Binding of IGF-II to the IGF-1 receptor leads to autophosphorylation of the receptor. Docking proteins (IRS) bind to the Tyrosine phosphorylation sites and recruit the regulatory subunit of PI3 kinase, p85. P85 then brings the catalytic subunit, p110, in proximity of the cell membrane where it can now convert Phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphate idylinositol (3,4,5)-trisphosphate (PIP3). This phosphorylation event leads to activation of phosphoinositide-dependent protein kinase 1 (PDK 1) which phosphorylates Akt on T308, next either PDK 1 or an unidentified kinase, PDK 2, phosphorylates Akt on S473 leading to complete activation of the protein. Unknown signaling events then lead to binding of MyoD family members with other yet to be identified bHLH transcription factors, "X", to DNA control regions termed E boxes in the promoters of muscle-specific genes. Transcriptional coactivators, "Y", are recruited leading to the transcription of muscle-specific genes.

The IGF System

IGF-II is a member of the IGF family that contains two growth factors, IGF-I and –II, two receptors, IGF-IR and IGF-IIR, and six high-affinity binding proteins, IGFBP-1 to –6 [64, 65]. IGF-I, a 70 amino acid protein also structurally similar to insulin (50% homology), promotes cell proliferation and differentiation in multiple tissues. The IGFs exert their effects on target tissues through at least three different receptor types, IGF-IR and –IIR as well as the insulin receptor. The biological effects of both IGFs are largely mediated by the IGF-IR, a ligand activated tyrosine protein kinase related to the insulin receptor [64, 65]. The IGF-IR consists of a $\alpha_2\beta_2$ heterotetramer. The α subunits are located extracellularly and mediate ligand binding, whereas the two β subunits are intracellular and possess intrinsic tyrosine kinase activity. Ligand binding to the extracellular part of the receptor triggers autophosphorylation of the β subunit and stimulates tyrosine kinase activity. By contrast, the IGF-IIR, which is identical to the mannose-6-phosphate receptor, promotes sequestration and degradation of IGF-II, and also functions in targeting lysosomal enzymes [65]. The homology in both ligand and receptor structures allows insulin and the IGFs to cross-bind each other's receptors, however, the affinities with which they bind are 10- to 100-fold reduced compared with binding to their own receptor.

The primary regulators of IGF expression are nutritional factors and growth hormone (GH); however, the developmental expression of IGF in various tissues precedes that of GH, supporting an independent role of IGFs in embryonic and fetal life. IGFBPs bind IGFs with affinities higher than those of the receptors and function primarily to modulate growth factor action, although other, IGF-independent, effects

have been described [66]. Most of the IGF molecules in serum are found in a 150 kDa ternary complex formed by an IGF, IGFBP-3 or 5 and a glycoprotein known as acid labile subunit (ALS) [67, 68]. Affinities of the IGFBPs for either of the IGFs can vary widely and their expression is developmentally regulated, tissue-specific and under hormonal control (reviewed in [33]). Less than 1% of IGFs circulate freely. The IGF/IGFBP complex is acted upon by proteases at target organs/tissues where IGF is released and is available for biological actions. The GH/IGF-I axis is the primary regulator of postnatal growth while IGF-II, which is relatively independent of GH, has an important role during fetal development [69].

IGF-II in Disease

IGF-II action plays an important role in normal growth and development as well as in a variety of disease pathologies. IGF-II is an imprinted gene which gives it a pivotal role in cancer and tumorigenesis when a loss of imprinting (LOI) of this gene occurs. One of the more widely described syndromes resulting from highly elevated IGF-II transcripts and protein levels is Beckwith-Wiedemann syndrome, characterized by neonatal overgrowth, exomphalos, macroglossia and an increased risk of developing Wilms kidney tumors. LOI or a relaxation of imprinting often accompanies this syndrome and sporadic Wilms tumors, resulting in expression of IGF-II from both parental alleles [70].

Altered IGF-II expression has also been shown in a number of other cancers including but not limited to: breast, prostate, cervical and lung cancer. Prostatic stromal cells and epithelial cells in primary culture secrete IGFBPs and express the IGF-IR and

stromal cells produce IGF-II [71]. Changes in the expression levels of these components have been associated with prostate cancer risk [72-74]. Similarly, the IGF-II systems appear to be interrelated in cervical cancer, contributing to early malignant cell proliferation and lympho-vascular metastasis [75]. In addition, an IGF-II induced phosphorylation of cAMP response element binding protein (CREB) has been shown to play a significant role in lung adenocarcinoma formation [76]. Several studies over the last couple of years have also demonstrated that in women with breast lesions, free IGF-II levels are clearly correlated to the size of a breast cancer, indicating an involvement in tumor growth in this tissue as well [77-79]. Recent evidence has also pointed to IGF-II playing an important role in muscle regeneration [80-82] although a clear understanding of the signaling pathways and mechanisms involved in this process has only begun to be elucidated.

Hypothesis

IGF-II is an important growth factor in prenatal muscle development, adult muscle regeneration, and repair after injury. The regulation of IGF-II occurs at many levels and is quite complex. It is likely, however, that the regulation of IGF-II during muscle cell differentiation occurs at the level of transcription since this has been seen in some myoblast cell lines. It is my hypothesis that IGF-II gene expression and transcription is upregulated during muscle cell differentiation and that a conserved DNA regulatory region within the IGF-II/H19 gene locus functions as an enhancer during muscle cell differentiation. The specific aims of my studies were:

I. Determine if IGF-II gene expression is upregulated during MyoD-induced conversion and differentiation of C3H 10T1/2 mesenchymal stem cells.

II. Determine if the increase in IGF-II expression seen during differentiation of C3H 10T1/2 cells occurs through an upregulation of IGF-II gene transcription and determine which of the three IGF-II promoters are activated.

III. To identify and characterize the critical control region for muscle differentiation-dependent activation of IGF-II gene transcription.

MATERIAL AND METHODS

Reagents

Fetal calf serum, newborn calf serum, horse serum, Dulbecco's modified Eagle's medium (DMEM), trypsin and phosphate-buffered saline (PBS) were purchased from Mediatech-Cellgrow (Herndon, VA). Trypsin/EDTA was from Invitrogen Inc. (Carlsbad, CA). Protease inhibitor tablets were purchased from Roche Applied Sciences (Indianapolis, IN), okadaic acid from Alexis Biochemicals (San Diego, CA), and sodium orthovanadate from Sigma. *Trans*IT-LT-1 was from Mirus Corp. (Madison, WI). The BCA protein assay kit was from Pierce (Rockford, IL). Immobilon-FL was from Millipore Corporation (Billerico, MA), AquaBlock tm/EIA/WIB solution was from East Coast Biologicals (North Berwick, ME). Restriction enzymes, buffers, ligases and polymerase were purchased from Roche Applied Sciences, BD Biosciences (Clontech), and Fermentas (Hanover, MD). The AdEasy adenoviral recombinant kit was from Q-BIO Gene (Carlsbad, CA). Cell culture lysis reagent and luciferase reporter assay kit were from Promega (Madison, WI).

Antibodies

Monoclonal antibodies	Company	Western Blot Dilution
Myogenin* (F5D cell line)	Dev. Studies Hybridoma Bank, Iowa City, IA	1:100
MHC* (MF20 cell line)	Dev. Studies Hybridoma Bank, Iowa City, IA	1:100
Troponin T (CT3)	Dev. Studies Hybridoma Bank, Iowa City, IA	1:1000
MyoD	BD Biosciences/Pharmingen, San Diego, CA	1:3000
α-tubulin	Sigma Aldrich, St. Louis, MO	1:2000

Polyclonal antibodies	Company	Western Blot Dilution
Akt	Cell Signaling Technology, Beverly, MA	1:1000
phosphor-Akt (Ser^{473})	Cell Signaling Technology, Beverly, MA	1:1000
Sp-1 (PEP2)	Santa Cruz Biotechnology, Santa Cruz, CA	1:2000

Antibody Conjugates	Company	Western Blot Dilution
Goat α-mouse IgG_1-Alexa 488	Molecular Probes, Eugene, OR	1:5000
Goat α-mouse IgG_{2b}-Alexa 594	Molecular Probes, Eugene, OR	1:5000
Goat α-mouse IgG-Alexa 680	Molecular Probes, Eugene, OR	1:5000
Goat α-rabbit IgG-IR800	Rockland Immunochem., Gilbertsville, PA	1:5000

*Antibodies prepared from hybridoma cell lines by Elizabeth Wilson in our lab as described [83].

Cell Cultures

C3H 10T1/2 mouse embryonic fibroblasts (ATCC catalog number CCL226) were incubated on gelatin-coated tissue culture dishes in growth medium (DMEM with 10% heat-inactivated fetal bovine serum) at 37°C in humidified air with 5% CO_2, until they reached 50% of confluent density for acute infection with recombinant adenovirues. Cells were grown on non-gelatin-coated plates for general use. Differentiation was initiated one day later when cells reached ~95% of confluent density after washing twice with PBS and adding differentiation medium (DM), consisting of DMEM plus 2% horse serum.

Murine C2 myoblast cells were plated 100,000 cells per ml on gelatin-coated plates in growth medium (DMEM with 10% heat-inactivated fetal bovine serum and 10% newborn calf serum) at 37°C in humidified air with 5% CO_2. Differentiation was initiated one day later after washing twice with PBS and changing to DM.

Construction of Mouse IGF-II P3 Expression Plasmids

Mouse IGF-II promoter 3 (P3) (see appendix A for sequence) was cut out of an exhisting Bluescript/IGF-II P3 plasmid in the lab with BstX1 filled in as a blunt end and a

Xho I sticky end. This was cloned into pGL3 using Hind III filled-in and the Xho I site. Putative enhancer regions A, B and C (see Figure 10) were generated by PCR from mouse genomic DNA using the following primers. *Sal I* or *Xba I* restriction sites were added to the ends of each primer (underlined):

A:
Top Strand: 5'-GCCGTCTAGAAGCCCAGCTGGGACAGCAAAC-3'
Bottom Strand: 5'-GCGCGTCGACCCTTCTGACCAGCTCCAAGCT-3'

B:
Top Strand: 5'-GCGCTCTAGACAGCTGCTGGCCTTCAAAGAG-3'
Bottom Strand: 5'-GCGCGTCGACCCTGGTAATCGGGGCTCTTCT-3'

C:
Top Strand: 5'-GCGCTCTAGACCCTGAAACCTTGCAAAGTCT-3'
Bottom Strand: 5'-GCGCGTCGACCCCTGTGTCCCTGCGATGAGG-3'

Fragments A, B and C were then cloned into IGF-II P3/pGL3 through the *Sma I* and *Xho I* sites. A plasmid containing putative enhancer D was obtained from Dr. Jie Chen at the University of Illinois, Urbana and the region of interest excised using *Spe I* and *Eag I*, filled-in and subsequently inserted into the IGF-II P3/pGL3 plasmid through the *Sma I* site. This 4.1 kb putative enhancer was also used to generate three smaller, overlapping fragments (D1-D3) which were cloned into the IGF-II P3/pGL3 plasmid. Additional IGF-II P3/pGL3 plasmids were produced containing the 291 bp overlapping region of C and D3 and two smaller fragments of this region. These were generated by PCR using the following primers which added *Asp718* and *Sac I* to the ends of the fragments for ease of cloning back into IGF-II P3/pGL3 through the *Kpn I* and *Sac I* sites:

Overlap of C/D3:
Top Strand: 5'-CGCGGGTACCCCTGAAACCTTGCAAAGTCT-3'
Bottom Strand: 5'-CGCGGAGCTCGGCCGGTACATAACGGGAAAA-3'

Overlap of C/D3 + 25 bp of C:
Top Strand: 5'-CGCGGGTACCCCCTGAAACCTTGCAAAACTCTA-3'
Bottom Strand: 5'-CGCGGAGCTCAGGAGCAGCTGTTCTCCTCATC-3'

Overlap of C/D3 + 100 bp of C:
Top Strand: 5'-CGCGGGTACCCCCTGAAACCTTGCAAAACTCTA-3'
Bottom Strand: 5'-CGCGGAGCTCAGCTAAATCCTCCTTGGCCCCT-3'

Recombinant Adenoviruses

All recombinant adenovirues were generated previously in our lab and isolated via a protocol supplied by Q-BIO Gene [58]. A recombinant adenovirues encoding β-galactosidase (Ad-β-Gal) was a gift from Dr. J. Molkentin, University of Cincinnati School of Medicine. All viruses were purified on discontinuous cesium chloride gradients and titered by optical density. For infections, recombinant adenoviruses were used at a multiplicity of infection of (MOI) of 250. They were diluted in half of the usual well per dish volume of DMEM plus 2% fetal bovine serum, filtered through a Gelman syringe filter (0.45μM), and added to C3H 10T1/2 mouse embryonic fibroblasts cells at 37°C for 120 minutes. Then an equal volume of DMEM with 20% fetal bovine serum was added to produce an ~11% serum medium, cells were incubated for a further 24 h, then washed twice with PBS and put in DM. This leads to 90% or more of the cells infected as verified by immunocytochemical staining with appropriate antibodies.

Immunocytochemical Stainings

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized with a 50:50 mixture of methanol and acetone for 2 min before blocking in 0.25% normal goat serum for 1 h at room temperature. Primary antibodies diluted in blocking buffer were added for 16 h at 4°C (anti-MHC 1:50 dilution, anti-myogenin,

1:250 dilution). After washing, cells were incubated for 2 h at 20°C in goat anti-mouse IgG_{2b}-Alexa 594 (red), goat anti-mouse IgG_1-Alexa 488 (green), and Hoescht, each diluted 1:1000 in blocking buffer. Images were captured with a Roper Scientific Cool Snap FX CCD camera attached to a Nikon Eclipse T300 fluorescent microscope using IP Labs 3.5 software.

SDS-PAGE and Western Blotting

Whole cell protein lysates were prepared after washing cells with PBS and incubating on ice for 15-20 min in RIPA Buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% IGEPAL CA-630) with 1X Protease Inhibitor Cocktail and 1μM okadaic acid, and 0.5mM NaVanadate . Lysates were scraped and passed through a 21-gauge needle and centrifuged at 14,000 rpm at 4°C, for 10 min to remove insoluble material. Protein concentrations were determined using the BCA protein assay kit and protein extracts were aliquoted and stored at -80°C until they were assayed. Protein samples were separated by SDS-polacrylamide gel electrophoresis, with a 4% stacking gel and 10% separating gel. Molecular weight markers were run simultaneously (Sigma Broad Range Marker). Following SDS-PAGE, the proteins were transferred to Immobilon-FL, blocked in Aquablock, and incubated with the appropriate primary antibodies (diluted in 50% Aquablock, 50% PBS and 0.1% Tween 20) 24-48 h at 4°C or 2 hours at room temperature. Membranes were washed four times in TBS-T for 5 min each and incubated with secondary antibodies for 1 hour (diluted in 50% Aquablock, 50% PBS, 0.1% Tween 20, and 0.01% SDS). Detection was

made using an Odyssey Infrared Imaging System by LiCoR Biosciences (Lincoln, NB) and v1.2 analysis software.

Isolating Nuclei

Cells were lysed by resuspending in Buffer A (10 mM HEPES pH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 1X Protease inhibitor cocktail, 500 μM sodium orthovanadate, 1μM okadaic acid, 1 mM DTT, 0.5% Triton), incubating on ice for 15 min and passing through a 23 gauge needle 4-8 times. Nuclei were then pelleted by centrifuging at 14,000 rpm for 2 min at 4°C. The supernatant (cytosolic fraction) was removed and snap-frozen in a dry ice ethanol bath and stored at -80°C in aliquots. The nuclei were then washed in Buffer A (without Triton) and repelleted at 14,000 rpm for 2 min. Nuclei were then used to isolate RNA or protein.

Nuclear Protein Extracts

Nuclei were isolated as described above and the nuclei were resuspended in Buffer C (20 mM HEPES pH7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1X Protease inhibitor cocktail, 500 μM sodium orthovanadate, 1μM okadaic acid, 1 mM DTT) and vortexed at 4°C for 15 min. Samples were then centrifuged for 10 min at 4°C and the supernatant snap-frozen in a dry ice ethanol bath and stored at -80°C in aliquots.

RNA Isolation and Analysis

Whole cell RNA was isolated as described previously [65]. RNA concentration was determined spectrophotometrically at 260 nm, and its quality assessed by agarose gel electrophoresis. RNA (2.5 μg) was reverse-transcribed in a final volume of 20 μl using a

RT-PCR kit (Invitrogen) with oligo(dT) primers. Each PCR reaction contained 1.0 µl of cDNA. The linear range of product amplification was established for each primer pair, and the cycle number representing the approximate midpoint (20-30 cycles) was used in final experiments. Results were quantified by densitometry after electrophoresis through 1% agarose: formaldehyde:MOPS/EDTA gels after staining with ethidium bromide.

Reporter Assays

Cells were seeded at 1 x 10^5 cells per well on gelatin-coated 12-well tissue culture dishes. 24 h later cells were transfected in duplicate (2 wells per plasmid) with 250 ng DNA and 1.25 µl of *Trans*IT LT-1 per well. The next day C3H 10T1/2 cells were infected with Ad-MyoD or Ad-β-Gal as described above, 24 h later cells were washed twice with PBS and put into DM or harvested (Time 0) by scraping into 200 µl of lysis buffer and freezing at -80°C. Additional plates were allowed to incubate in DM for 24 or 48 hours and then harvested. Once all the samples were collected they were thawed, vortexed and centrifuged at 4°C for 5 min at max speed. Samples were assayed for luciferase enzymatic activity using a Promega Vertitas microplate luminometer and readings were normalized to protein concentration as determined by BCA protein assay kit. C2 myoblast cells were assayed the same way except without recombinant adenovirus transduction.

Statistics

Student's t-test was used for comparisons between promoter-only reporter vector and putative enhancer vectors. $P<0.05$ was considered significant.

RESULTS

C3H 10T1/2 cells can be acutely converted to myoblasts by quantitative infection with a recombinant adenovirus, encoding mouse MyoD: C3H 10T1/2 cells are a mouse mesenchymal stem cell line that proliferates in growth medium [GM - Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum]. These cells are particularly useful as they contain no endogenous MyoD and can therefore provide a tightly controlled model of muscle cell differentiation. Previous studies have shown that MyoD is a vital transcription factor in muscle development [84, 85]. C3H 10T1/2 cells have been used extensively in our lab to characterize signaling mechanisms activated during muscle differentiation [86, 87]. This cell line can be converted to myoblasts after transduction with a recombinant adenovirus encoding MyoD (Ad-MyoD). A major advantage of recombinant adenoviruses is their ability to infect both dividing and non-dividing cells at high efficiency, ensuring nearly all cells will express the protein of interest. In order to determine if expression of MyoD is able to convert C3H 10T1/2 cells to myoblasts, in my hands, C3H 10T1/2 cells were incubated on gelatin-coated tissue culture dishes until they reached 50% confluency. Recombinant adenoviruses were infected at a multiplicity of infection of 250 and incubated for 24 hours, to reach >90% confluency, before being placed in differentiation medium for up to 48 hours. As seen in Figure 5, Ad-MyoD infected cells demonstrate progressive formation of multi-nucleated myotubes, indicative of muscle differentiation.

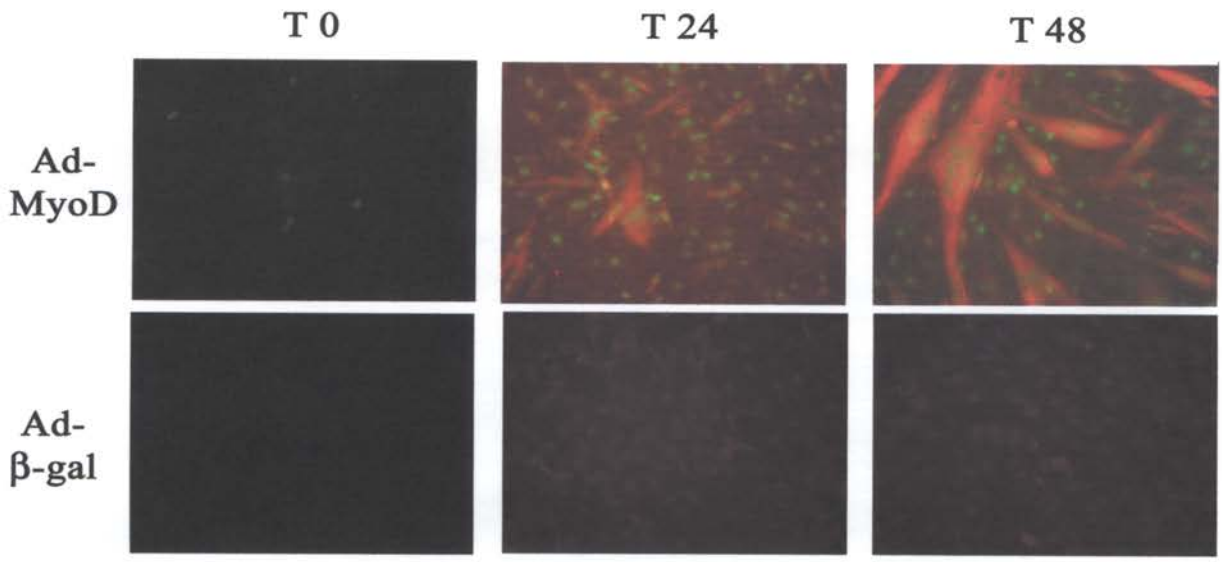


Figure 5: Ad-MyoD promotes myoblast conversion and differentiation of C3H 10T1/2 cells. Results of Immunocytochemistry as shown in cells infected with Ad-MyoD or Ad-β-Gal and induced to differentiate for 24 or 48 hours. Myosin heavy chain is shown in red and myogenin in green.

IGF-II gene expression is induced in Ad-MyoD converted C3H 10 T1/2 fibroblasts: Previous studies have shown that IGF-II gene expression is activated during differentiation of established muscle cell lines [1]. To investigate whether stimulation of IGF-II expression is a general component of myoblast differentiation, Ad-MyoD converted C3 10T1/2 fibroblasts were studied. RNA was isolated from cells during incubation in DM for up to 48 hr, and IGF-II, myogenin, and myosin heavy chain (MHC) mRNAs were measured by semi-quantitative RT-PCR. As seen in Figure 6, myogenin and IGF-II transcripts were induced in abundance during early differentiation. MHC was also increased. In contrast, no IGF-II, myogenin or MHC transcripts were detected in C3H 10T1/2 cells infected with Ad-β-galactosidase.

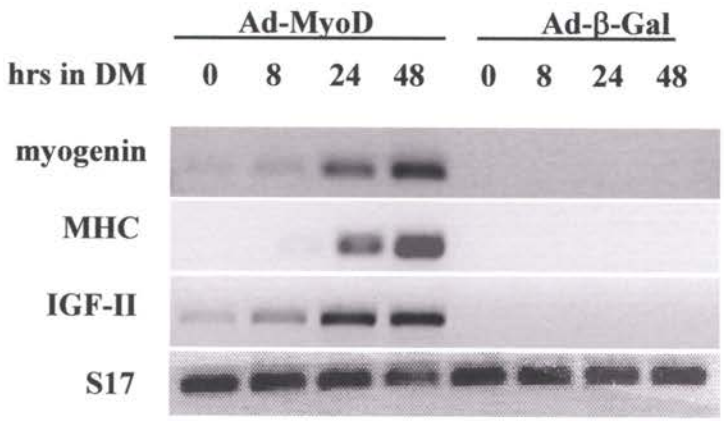


Figure 6: Induction of IGF-II gene expression during MyoD-mediated muscle differentiation. Results are shown of RT-PCR experiments in C3H 10T1/2 fibroblasts infected with either Ad-MyoD or Ad-β-galactosidase and incubated in differentiation medium up to 48 hours. S17 served as an unchanging loading control.

Only IGF-II promoter 3 is active during Ad-MyoD induced muscle cell differentiation of C3H 10T 1/2 cells: The mouse IGF-II gene contains three tandem promoters, each containing a unique 5' untranslated exon [39]. Previous studies have shown that promoter 3, and to some extent promoter 2, are activated during myoblast differentiation of established muscle cell lines [1]. To determine which promoters are active during differentiation of C3H 10T 1/2 cells, time course experiments were performed to measure the accumulation of IGF-II transcripts by reverse transcription polymerase chain reaction (RT-PCR). 10T1/2 fibroblasts infected with Ad-MyoD or Ad-β-Gal, were incubated in DM for 0, 8, 24 and 48 hours. I employed unique 5' PCR primers to each of the exons corresponding to the three promoters (E1-E3, Figure 7) along with a common 3' PCR primer (E4 Rev). PCR primers from the coding exons 5 and 6 were used as positive PCR controls (E5 and E6 Rev). In all experiments a negative control, lacking the reverse transcription enzyme, was also included to ensure lack of contamination by chromosomal DNA. Additionally, mouse fetal liver RNA was used as a positive control since transcripts from all three promoters are produced in this tissue prenataly [88]. Results show increasing levels of transcripts from all three promoters in mouse fetal RNA but transcripts are only produced from promoter three in Ad-MyoD transduced C3H 10T 1/2 cells (Figure 7).

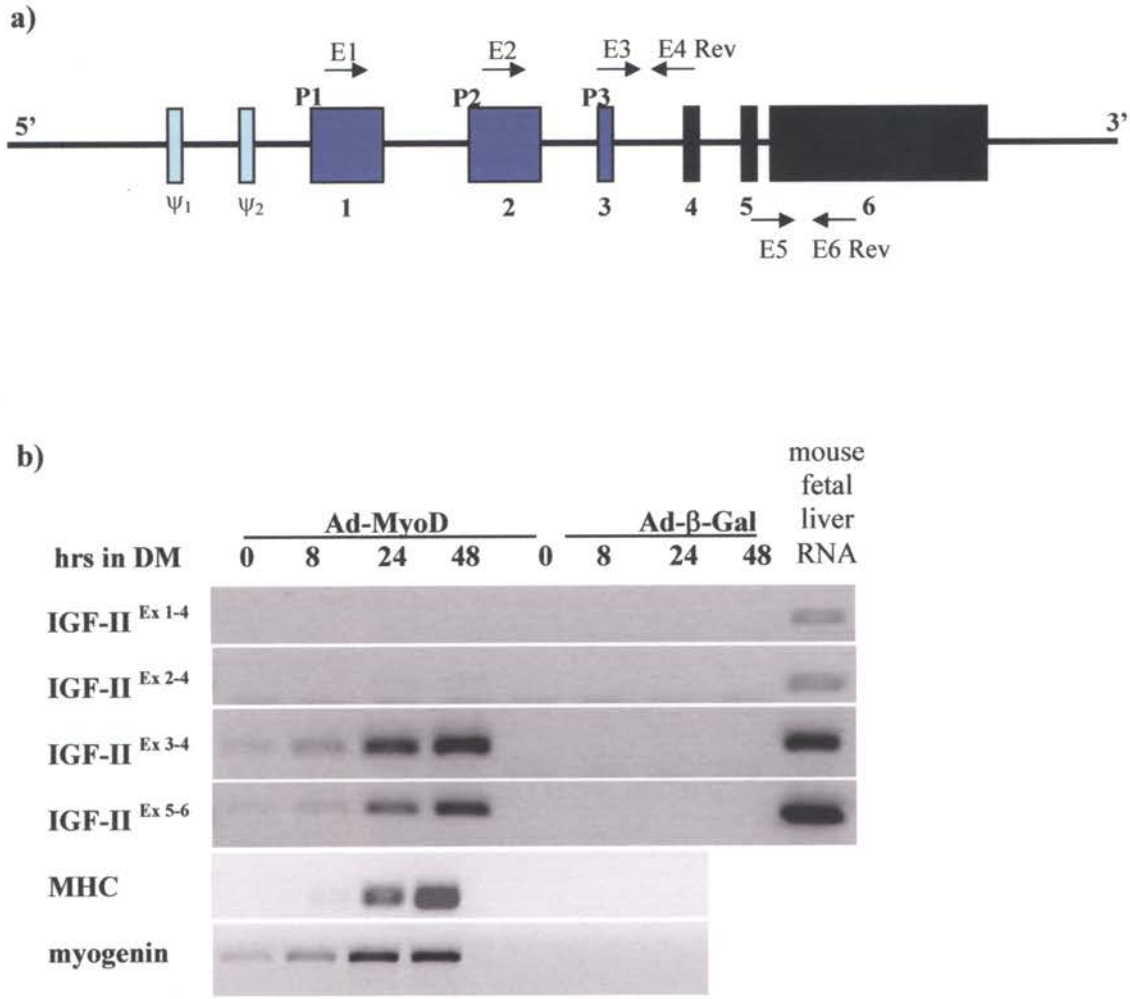
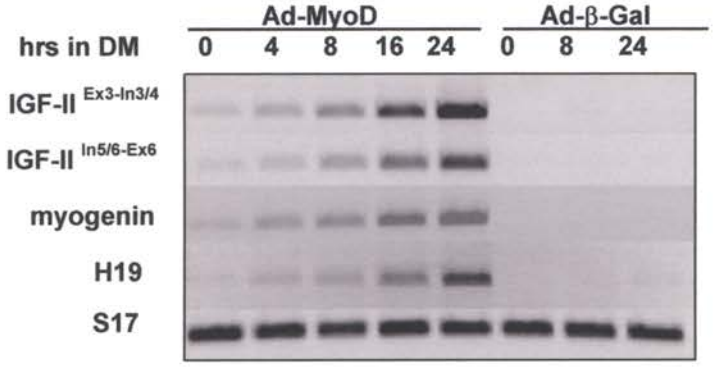


Figure 7: a) Locations of primers along the IGF-II gene. b) Time course experiments by RT-PCR using C3H 10T1/2 cells infected with either Ad-MyoD or Ad-β-Gal and incubated in DM up to 48 hours. Results show the progressive production of transcripts from only IGF-II promoter 3, and the myogenin gene starting early in differentiation, with a later accumulation of MHC transcripts from Ad-MyoD transduced cells.

Transcription of the IGF-II gene is rapidly induced in Ad-MyoD converted C3H 10 T1/2 fibroblasts. To determine if the increase in IGF-II expression is due to an increase in transcription, nascent nuclear RNA was isolated from cells during incubation in DM for up to 24 hours and IGF-II, myogenin, H19, and S17 (loading control) mRNAs were measured by semi-quantitative RT-PCR. As seen in Figure 8, myogenin, H19 and IGF-II transcripts were progressively induced during early differentiation of Ad-MyoD treated cells but not in Ad-β-Galactosidase (β-Gal) transduced cells.



Gene	Location	DNA sequence	Product Size (bp)
IGF-II	exon 3 intron 3-4	5'-GCAAACTGGACATTAGCTTCT 5'-CCCTTGGGTAACTAAAATCATCTT	597
IGF-II	intron 5-6 exon 6	5'-CAAAGAGACCACTCACTTCTTGATT 5'-GGTCACAGATTGATGGTACTA	492
myogenin	intron 2-3 exon 3	5'-GGGATCACTCAGTCAGTGTTGTAA 5'-TCTCTGCTTTAAGGAGTCAGCTAAA	537
H19	exon 1 intron 1-2	5'-TAGAGAGAAGAAAGAAGAGGTGCAG 5'-GAAGACATGAGTTAATTGAACTTGC	943
S17	exon 2 intron 2-3	5'-ATCCCCAGCAAGAAGCTTCGGAACA 5'-GAACCGACTTTGTCTCTACATCAAG	439

Figure 8: Induction of IGF-II gene transcription during MyoD-mediated muscle differentiation. Results are shown of time course experiments by RT-PCR using C3H 10T1/2 fibroblasts infected with either Ad-MyoD or Ad-β-Gal and incubated in differentiation medium up to 24 hours. Myogenin, IGF-II and H19 transcripts are progressively induced during differentiation of the Ad-MyoD transduced cells but not the Ad-β-Gal cells.

Transcriptional activation of myogenin, IGF-II and H19 is impaired in myoblasts lacking normal IGF-II expression: Previous studies in our lab have shown a requirement for autocrine activation by IGF-II of the IGF-I receptor and the signaling molecules PI3-kinae and Akt in myoblast differentiation induced by MyoD in C3H 10T1/2 mesenchymal stem cells. These studies found that inhibition of IGF-II expression by a doxycycline-suppressible adenovirus encoding an IGF-II cDNA in the anti-sense orientation (Ad-IGF-II^{AS}) reversibly blocked Ad-MyoD-mediated differentiation by preventing expression of muscle-specific proteins and formation of multinucleated myotubes [88]. In this study, I analyzed nascent nuclear RNA of myogenin, IGF-II and H19 by RT-PCR under these same conditions and found that inhibiting IGF-II expression blocks transcription of myogenin, IGF-II and H19 (Figure 9).

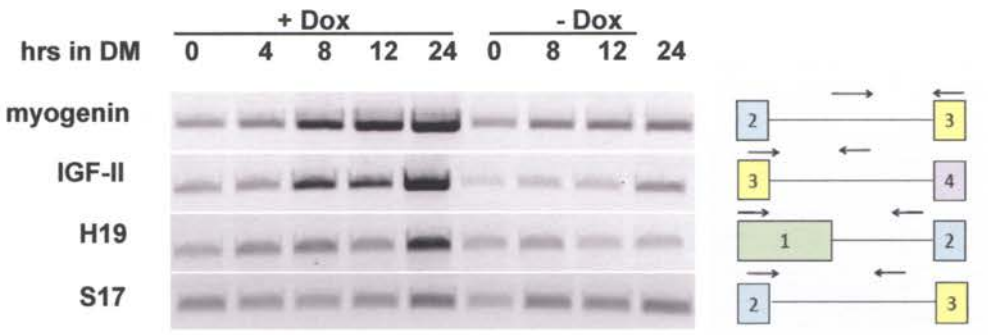


Figure 9: Transcriptional activation of myogenin, IGF-II and H19 is impaired in myoblasts lacking normal IGF-II expression. +Dox: normal IGF-II expression/anti-sense IGF-II is suppressed. S17 serves as an unchanging loading control. (H19 28 cycles, all others 30 cycles)

Identifying DNA control elements that mediate stimulation of IGF-II gene transcription during muscle cell differentiation: Previous attempts to identify the regulatory regions within the IGF-II gene have yielded inconclusive results. Transgenic experiments have shown that mesoderm-specific enhancers are not located within the area 14.5 kb upstream to 6.5 kb downstream of IGF-II [1, 2], or within 11 kb upstream to 12 kb downstream of H19 [89, 90]. However, the H19 and IGF-II genes share a complex pattern of gene expression, suggesting that their transcriptional activation is linked mechanistically. This led me to search for more distal regulatory regions surrounding the two genes on mouse chromosome 7. Through a review of literature mostly focused on identifying mechanisms of imprinting between these two genes, I identified four putative mesodermal and/or muscle-specific DNA control regions that I tested [91-93]. The locations of each region are outlined in Figure 10.

The first putative enhancer (A) was chosen from a paper by Ainscough et al which claimed to identify a repressor for the IGF-II gene within a 3 kb region containing intergenic DNase I hypersensitive sites [94]. This area is approximately 40 kb downstream of the gene but also contained a second evolutionarily conserved region (Region 2). I hypothesized this second region may function as an enhancer since the transgenes they used contained a deletion of Region 1 but left the second conserved region intact. These studies showed increasing IGF-II expression in muscle with increasing copy number of the transgene. An alternative explanation for Region 1 being a repressor is that the second conserved region functions as an enhancer since additional copies of this regio to a parallel increase in IGF-II gene expression.

The next two enhancers, B and C, were chosen from a study by Ishihara et al which identified 10 chromosomal regions located downstream of the H19 gene that were conserved between mouse and human genomes. This study utilized comparative genomic sequencing to identify methylation-sensitive factors involved in imprinting. Two of the evolutionarily conserved fragments, CS6 or "B" in my studies and CS9 or "C", were shown to drive expression of a lacZ reporter in the myotome (rib primordial and intercostal muscles) of transgenic mice at embryonic day 12.5 [92]. These two conserved regions exhibit 68% and 71% sequence homology between mouse and human. A third conserved segment, CS7, drives expression in the mesenchymal condensation of the limb buds but did not have wide-spread skeletal muscle staining and therefore was excluded from my initial studies. A final putative enhancer chosen from a paper by Kaffer et al. is ~4 kb in length and is located from +22.9 to +27.5 kb downstream of H19. This segment was found to confer muscle specific expression from a SV40 promoter-lacZ reporter when stably transfected into differentiating Sol8 muscle cells, enhancing expression 400 to 500-fold [93]. This region is labeled "D" in Figure 10 and through sequence analysis of the C and D regions I found that these two areas overlap by 291 bp on mouse chromosome 7.

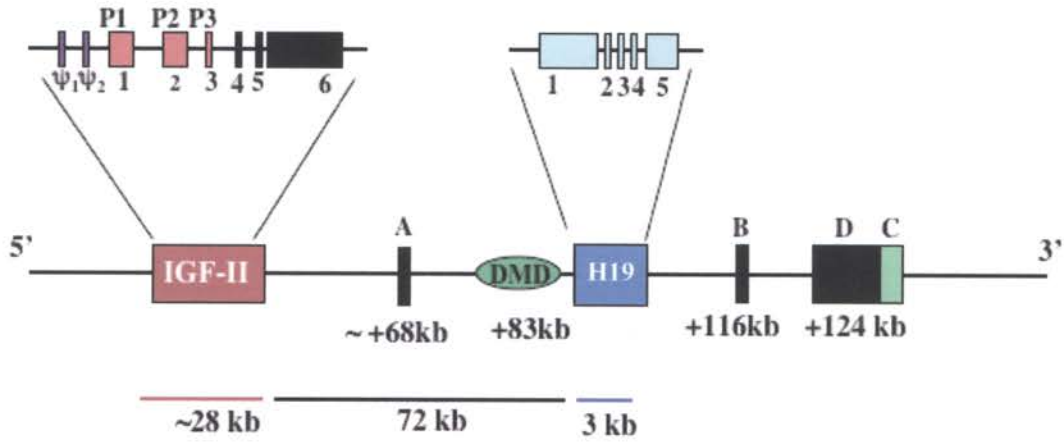
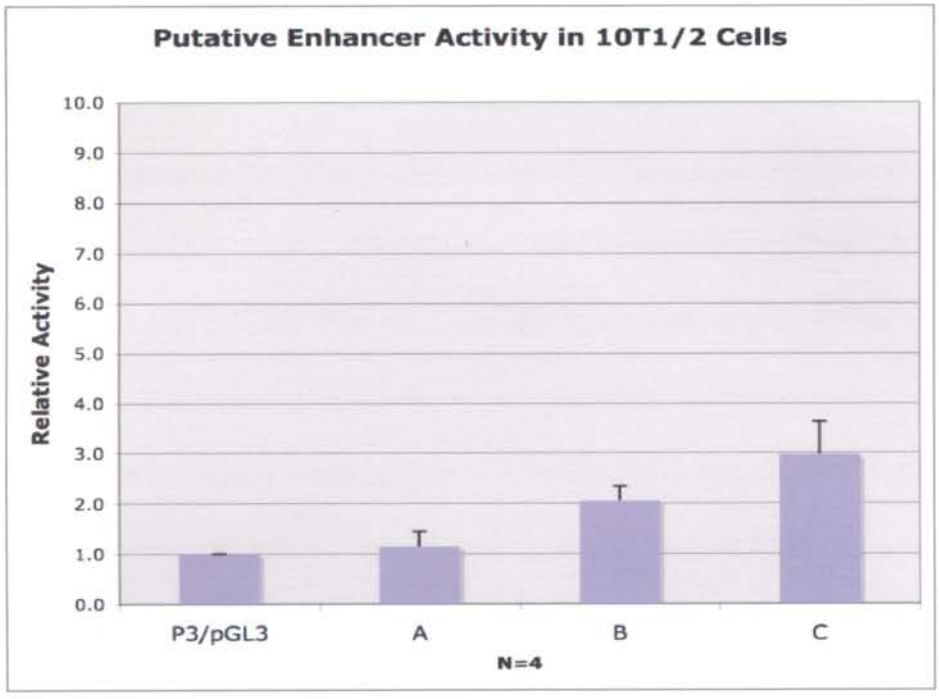


Figure 10: Location of the IGF-II and H19 genes on mouse chromosome 7, including DNA regions (A-D) studied and the differentially methylated domain (DMD) described in the text. Positions are marked relative to the start of the IGF-II gene.

The identified regions exhibit functional activity in C3H 10T1/2 cells: To determine if any of these regions function to stimulate IGF-II gene transcription, two series of experiments were performed using reporter plasmids containing IGF-II P_3. In the first group of experiments, each of the DNA fragments outlined in the map in Figure 10 was cloned 5' to the IGF-II promoter 3 in a luciferase reporter plasmid. These promoter - reporter plasmids were transiently transfected into C3H 10T1/2 mesenchymal stem cells, followed by measurement of luciferase activity 24 or 48 hours later, to determine if any of the fragments encode transcriptional enhancers. The largest fragment, D, was dissected into smaller overlapping pieces (D1-D3) and tested in the same functional assay. All of the plasmids exhibit only limited enhancer activity when transfected into C3H 10T1/2 cells with enhancer plasmid expression levels ranging from approximately two to three-fold higher than the promoter only vector (Figure 11).



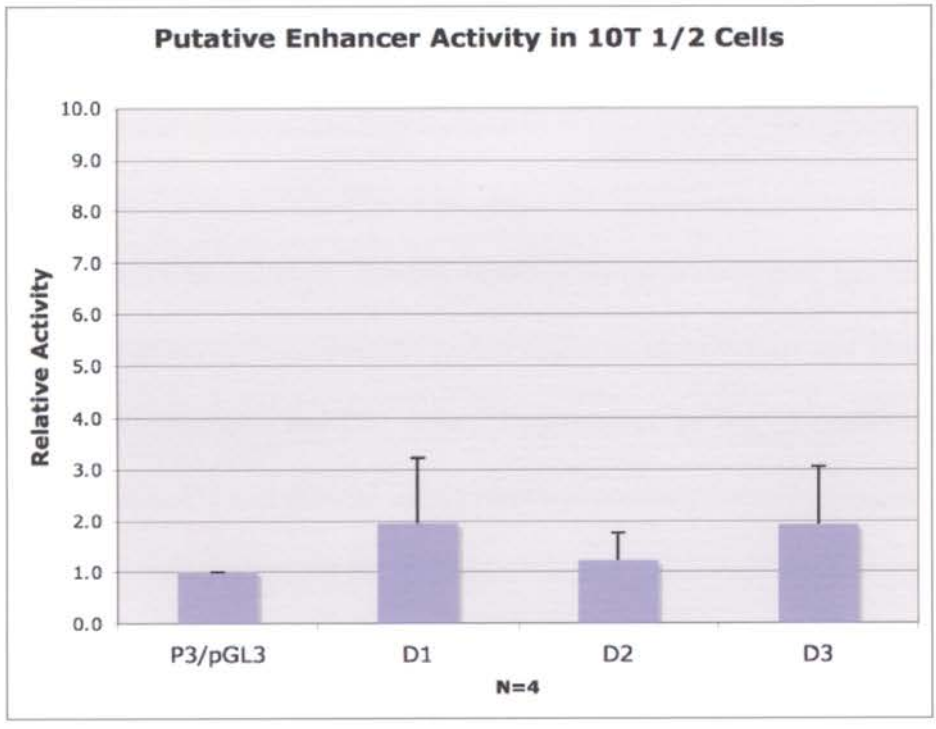


Figure 11: C3H 10T1/2 cells were transiently transfected with the reporter plasmids. Limited to no enha tivity is seen for all of the plasmids compared to promoter only vector.

Regions D1, D3 and C greatly enhance IGF-II promoter activity during muscle differentiation: To determine if any of these regions functions to increase IGF-II P_3 expression during muscle cell differentiation, each of the promoter – reporter fusion genes was transfected into C3H 10T1/2 cells followed by infection with Ad-MyoD or Ad-β-gal, incubation in DM for 24 or 48 hours, and measurement of luciferase enzymatic activity. The IGF-II promoter 3-pGL3 vector, pGL3 and the original C and D1 enhancer plasmids were also included for comparison. To verify that differentiation is proceeding equally in all samples, Western blots were done for myogenin and MHC, with tubulin as an unchanging loading control. Differentiation was also assessed morphologically by immunocytochemistry as described previously [58] using antibodies to MHC and myogenin as well as Hoechst staining to visualize nuclei. These experiments were done in duplicate wells and confirmed in four independent experiments. As a positive control for gene activation during muscle differentiation, a reporter plasmid containing the mouse myogenin promoter was included. Parallel experiments were performed in differentiating C2 murine myoblast cells, to validate the results found in MyoD-converted fibroblasts, using DNA control regions C and D3. Area D3 was chosen for the C2 studies since D1 entirely encompasses D3 and showed nearly identical luciferase activity (Figure 12). Results show that fusion genes for D1, D3 and C show a significant increase in luciferase activity during muscle differentiation (Figures 12 and 13). Biochemical markers of differentiation and myogenin promoter activity also show appropriate increases during differentiation of b[illegible] l types (data not shown).

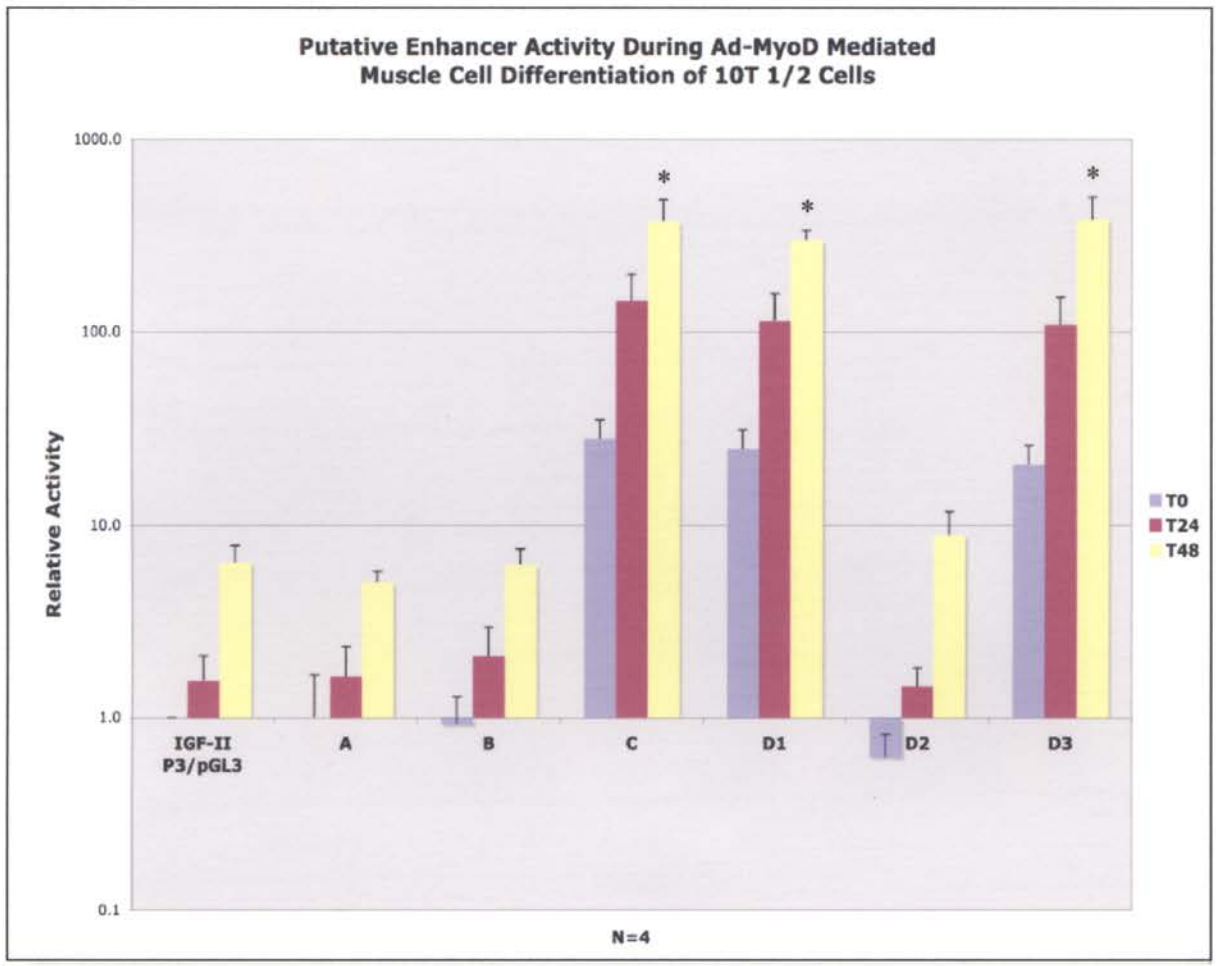


Figure 12: C3H 10T1/2 cells were transiently transfected and infected with Ad-MyoD 24 hours later. Cells were induced to differentiate by changing media to DM ~24 hours after infection and cells were harvested at 0, 24 and 48 hours in DM. Results are shown on a log scale with DNA control regions D1, D3 and C significantly increasing the expression of the IGF-II P_3 reporter plasmid. Relative activity = luciferase units/protein concentration with promoter only vector, at 0 hrs in DM, set to 1, *P<0.05 compared to promoter-only vector, ANOVA.

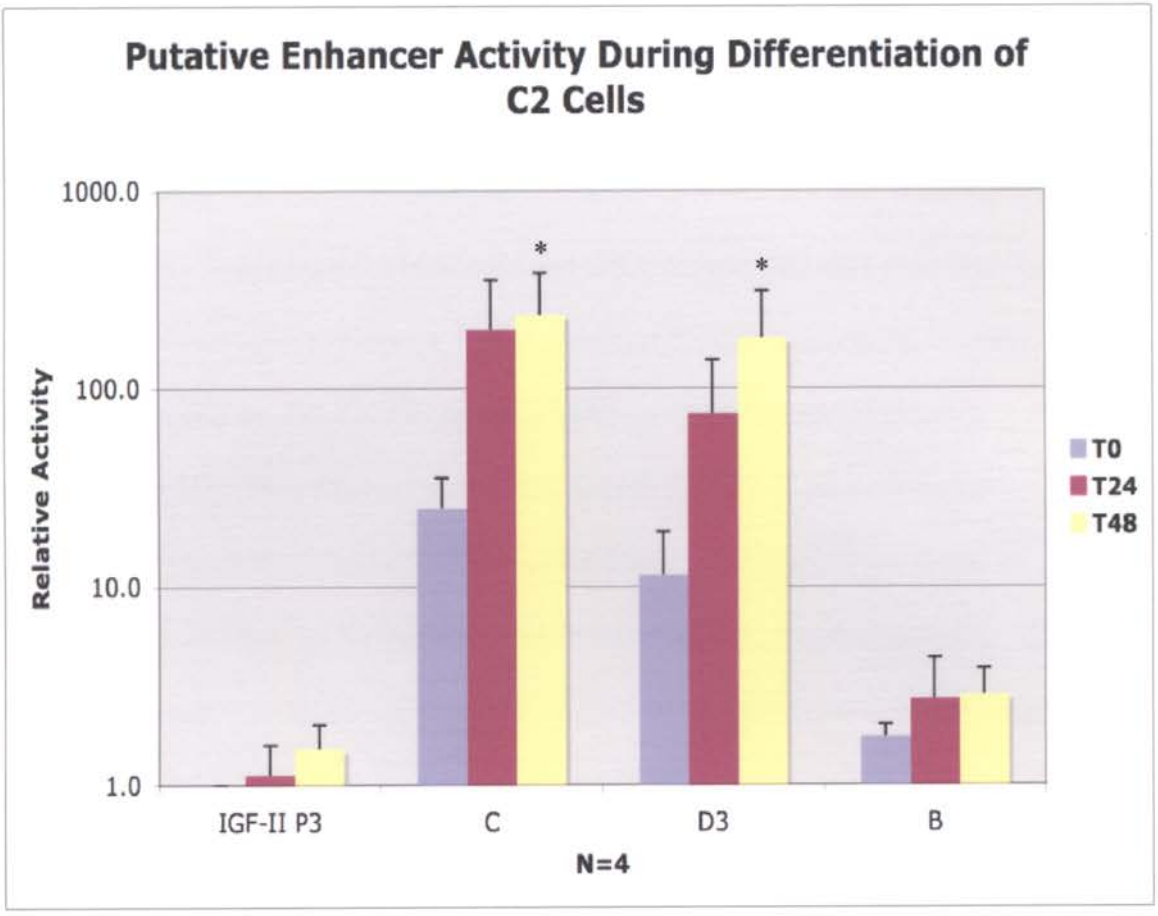
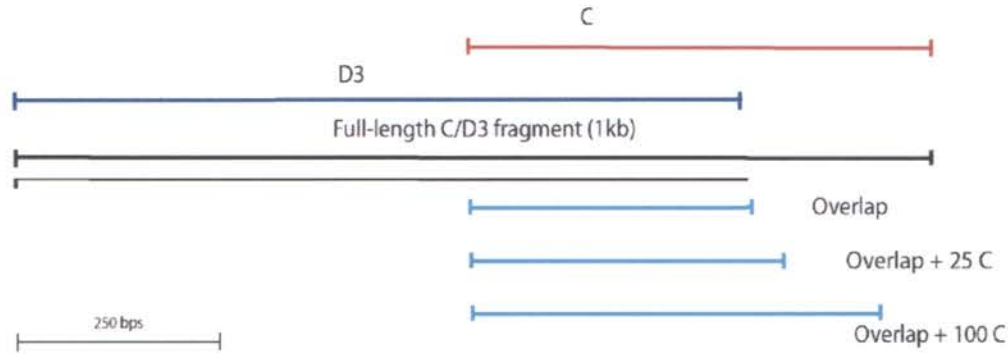


Figure 13: C2 myoblast cells were transiently transfected with the reporter plasmids and induced to differentiate by changing media to DM 24 hours later. Cells were harvested at 0, 24 and 48 hours in DM. Results are shown on a log scale with DNA control regions D3 and C significantly increasing the expression of the IGF-II P_3 reporter plasmid. Region B now serves as a negative control since it does not show an increase in reporter gene activity in C3H 10 T ½ cells (Figure 12). Relative activity= luciferase units/protein concentration with ter only vector, at 0 hrs in DM, set to 1. *P<0.05 compared to promoter-only vector, ANOVA.

A well-conserved, 316 bp region lies approximately 127 kb downstream of the IGF-II gene and is able to significantly enhance luciferase enzymatic activity through the IGF-II promoter 3 during muscle cell differentiation. As seen in Figures 12 and 13, DNA segments C, D1 and D3 exhibit a significant enhancer effect on the IGF-II promoter 3 during myogenic differentiation of both C3H 10T 1/2 cells transduced with Ad-MyoD and C2 myoblasts. These potential DNA control elements represent the smallest functional units to increase IGF-II promoter 3 expression during muscle differentiation and are 470 bp, 750 bp and 1.1kb respectively with D1 entirely encompassing D3. They lie ~127 kb downstream of the IGF-II gene on mouse chromosome 7 with the 5' end of enhancers D1 and D3 overlapping the 3' end of enhancer C by 291 bp. To further refine the location of the myogenic enhancer within the ~1.1 kb region encompassing C and D3, experiments were done in which overlapping fragments of the C/D3 region were generated by PCR according to Figure 14. Each of these fragments were cloned into the luciferase reporter vector (pGL3) containing the IGF-II promoter 3. These plasmids were then transfected into C2 myoblasts which were then induced to differentiate in DM. Cells were harvested at 0, 24 and 48 hours in DM and luciferase enzymatic assays were performed as before and normalized for protein concentration. Differentiation was again confirmed by Western blot and immunocytochemistry. As shown in Figure 14, a 316 bp fragment encompassing the overlap between D and C as well as 25 bp of C represents the minimal area of peak functional activity during muscle cell differentiation. Sequence analysis of this region using Mac Vector 9.0 and ECR Browser (http://ecrbrowser.dcode.org) showed that this area is well conserved among mammals, having 79% sequence homology with human and chimp, 75% with dog and 92% with rat. Additionally, transcription factor database

screens located two completely conserved putative E boxes within this region providing a possible mechanism for regulation of muscle cell differentiation through transcription factor binding. Future studies identifying the precise mechanisms by which this area functions to enhance IGF-II activity during muscle cell differentiation will be vital to understanding this key regulatory region.



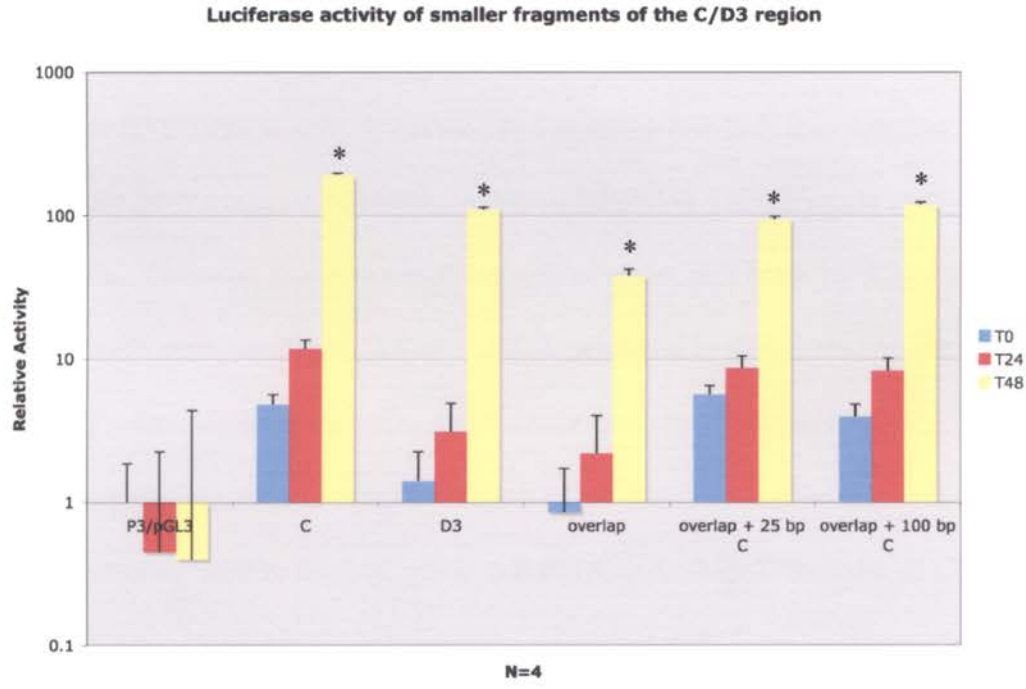


Figure 14: A 316 bp fragment encompassing the overlap between D and C as well as 25 bp of C represents the minimal area of peak functional activity during C2 myoblast differentiation. C2 myoblast cells were transiently transfected with the reporter plasmids and induced to differentiate by changing media to DM 24 hours later. Cells were harvested at 0, 24 a hours in DM. Results are shown on a log scale. Relative activity= luciferase units/protein concentration with promoter only vector, at 0 hrs in DM, set to 1. *P<0.05 compared to promoter-only vector, ANOVA.

SUMMARY AND CONCLUSIONS

In my studies, I have attempted to define how the IGF-II gene is regulated during muscle cell differentiation. Studies were done in two cell lines, C3H 10T1/2 cells and C2 myoblast cells. This allowed me to study muscle differentiation under tightly controlled conditions using a recombinant adenovirus encoding MyoD to infect the C3H 10T1/2 cells and then induce them to differentiate through low serum media. The C2 myoblast cells provided confirmation of my results in a muscle cell line.

In C3H 10T1/2 cells, I was able to determine that IGF-II expression is induced quite early during muscle cell differentiation, following a similar time course as myogenin. This was shown through analysis of whole cell RNA by RT-PCR of C3H 10T1/2 cells transduced with Ad-MyoD and incubated in differentiation medium up to 48 hours. To determine which of the three mouse promoters accounted for the increase in IGF-II expression, primers specific to each of the exons corresponding to the three promoters were used to analyze whole cell RNA in Ad-MyoD transduced C3H 10T1/2 cells. The results of this experiment were consistent with those seen previously in C2 myoblast cells with transcripts only produced from mouse promoter 3 of the IGF-II gene.

Since IGF-II transcripts could clearly be seen between zero and eight hours in my studies on expression levels, I adopted a more refined time course analyzing nascent nuclear RNA at five time points, over a 24 hour period, to determine if the IGF-II gene is regulated at the level of transcription during Ad-MyoD induced differentiation of C3H 10T1/2 cells. In addition, I analyzed the H19 gene in this experiment since the two genes are reciprocally imprinted. RT-PCR using intron/exon primer pairs clearly demonstrated that the increase in IGF-II gene expression in this model system results from a

corresponding increase in IGF-II gene transcription. The next study done looked at the effect of blocking IGF-II expression on the transcriptional activation of myogenin and H19 and demonstrated that IGF-II expression levels are directly linked to the level of transcription of these genes as well.

The most novel aspect of my studies was the work done identifying a distal DNA control element within the IGF-II/H19 gene locus which is able to enhance luciferase enzymatic activity through the IGF-II promoter 3 in reporter assays during both C2 myoblast and C3H 10T1/2 cell differentiation. This well conserved enhancer is quite strong under these experimental conditions and could account for the large increase in IGF-II expression seen during muscle cell differentiation. Further analysis of transcription factor binding sites within this region will clearly be of great interest for future studies of this region. In addition, unraveling the mechanisms behind which this DNA control element is able to act on a gene approximately 127 kb upstream of it warrants attention. Chromosome conformation capture (3C) assays would be a good tool to determine if there is a direct interaction between the IGF-II gene and the enhancer region as has been shown for other regulatory regions of this locus as well as other gene loci containing distal DNA control regions [95-97].

In summary, my results clearly demonstrate that IGF-II gene expression is upregulated at a transcriptional level during differentiation of C3H 10T/2 cells into muscle cells and that this increase occurs through IGF-II promoter 3. In addition, a DNA regulatory region within the IGF-II/H19 locus functions as a well-conserved myogenic enhancer of the IGI ne.

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APPENDIX A

Sequence of the IGF-II promoter 3 used for reporter assays:

CTCGAGGTCGACTTTGTGCCAATGAGACAGGAAGGAAAAGTGGGTGGGCG
GGGCCAAGAGCCCCCCAAAATTGGTAAAAGAGTCAAGCCAGACCCCACAG
TTAGCTTTTCCCCCCACCCACACACCCCAACTCTGTCCCCAACATTCCCA
ACCTGGAATGTTGGGGGGAGAGGGGACGGTTTCGGGCTCAGAAGGGCAGA
AATGTTACAGTTCAAAGAGAAATAACTGCCCCTGCCTTTTACTGGGTGTA
ACTTTCCATAGGAGGATGTGGGAAGGAGCCCCCTCCCTCCCCCCATTGGT
GTGTGCAGAAAGGGCTGGGGGTCGCACGATGAGGCCCCCCTCCCACTGGT
GGTGCTTTGCTAAGGAATGGTCCAAGGCTAGCTCTTGGGGGTGCAGGAGA
AAAGGGACTGGCTGGAAGGAGGGAGGGGGCGGGTGCAAAGGGGGCGGGGG
GAGTGGTCAGCAGGGAGGGGGTGGGGGGTAGGGTGGAGCCGGGACTGGGA
GGAGCCACTCAGACATAAAAAGCGGAGGCACTGACCAGTTCGCAAACTGG
ACATTAGCTTCTCCTGTGAGAACCTTCCAGCCTTTTCCTGTCTTCATCCT
CTTCCAGCCCCAGCGGCCTCCTTACCCAACTTCAGGTAACCAGAGCT