# Regulation of muscle fibre phenotype, muscle mass and IGF-1 gene expression in skeletal muscle in response to mechanical activity

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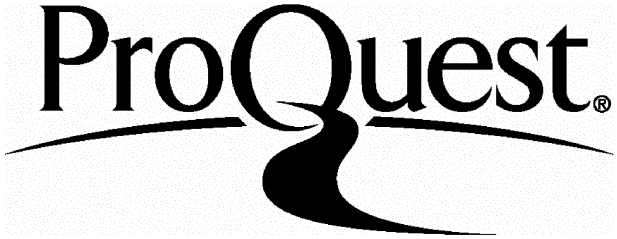

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# Abstract

This thesis is concerned with the regulation of muscle mass , muscle fibre phenotype and the role of IGF-1 expression in skeletal muscle in response to mechanical activity. Initially this work was done by Northern hybridization and then by *in situ* hybridization. The latter indicated that the end of normal adult fibres is the region of the longitudinal growth and that IGF-1 is involved in this process. By combining *in situ* hybridization and immunohistochemistry procedures, the effects of passive stretch and disuse of muscle on the expression of IGF-1 mRNA at the individual muscle fibre level and the fibre type composition of the muscles were studied. The result indicated that stretch also induced an increase in the percentage of fibres expressing neonatal and slow myosin and that IGF-1 is involved not only in muscle hypertrophy, but also in muscle fibre conversion.

Using RT-PCR a single IGF-1 isoform cDNA (IGF-1Ea) could be cloned from the normal resting muscles. However, an additional isoform of IGF-1 (IGF-1Eb) was found to be expressed in stretched muscle undergoing hypertrophy. The latter IGF-1 mRNA probably encodes the precursor IGF-1 isoform that is responsible for local muscle growth regulation in response to mechanical signals. To confirm that alternative splicing of the IGF-1 gene occurs in muscle in response to physical activity, oligonucleotide primers were made which specially amplify the cDNAs of two isoforms (IGF-1 Ea and Eb) in the human as well as the rabbit. Following altered physical activity for 2 hours to 6 days, appreciable levels of IGF-1 Eb (in human the Ec) isoform were detected in skeletal muscle by using RT-PCR and RNA protection. These data suggest that the IGF-1 Eb be a link of mechanical activity and the expression of muscle genes in adaptive hypertrophy and repair processes.

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# Abbreviations

| | |
|---|---|
| A | adenine |
| APTES | 3'-aminopropyltriethoxysilane |
| bp | base pairs |
| C | cytidine |
| cDNA | complementary DNA |
| dATP | deoxyadenosine 5' triphosphate |
| dCTP | deoxycytidine 5' triphosphate |
| dNTP | deoxyribonucleoside 5' triphosphate |
| dUTP | deoxyuridine 5' triphosphate |
| DEPC | diethylpyrocarbonate |
| DDT | dithiothreitol |
| DIG | digoxigenin |
| E | E-domain |
| EDL | extensor digitorum longus muscle |
| EDTA | ethlylenediaminetetracetic acid |
| FGF | fibroblast growth factor |
| G | guanine |
| GH | growth hormone |
| HEPES | 2-Hydroxyethyl-piperazine-N-2'-ethaneslfonic acid |
| IGF | insulin-like growth factor |
| IGFBP | insulin-like growth factor binding protein |
| IgG | immunoglobulin G |
| IPTG | isopropyl-β-D-galactoside |
| Kb | Kilobasepairs |
| min(s) | minute(s) |
| MOPS | 3-N-(Morpholino) propanesulfonic acid |
| MyHC | myosin heavy chain |
| nt | nucleotide |
| OD | optical density |

| | |
|---|---|
| PBS | phosphate buffered saline |
| PFA | paraformaldehyde |
| PCR | polymerase chain reaction |
| RACE | rapid amplification of cDNA ends |
| RT | room temperature |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SDS | sodium dodecyl sulphate |
| SOL | soleus muscle |
| SSC | saline sodium citrate |
| T | thymidine |
| TA | tibialis anterior muscle |
| TBE | Tris-borate-EDTA buffer |
| TE | Tris EDTA buffer |
| TEMED | N,N.N',N'-tetramethylethylenediamine |
| TGF | transforming growth factor |
| $T_m$ | melting temperature |
| Tris | Tris[hydroxymethyl]aminoethane |
| Tris HCl | Tris[hydroxymethyl]aminoethane hydrochloride |
| tRNA | transfer RNA |
| UTP | uridine 5'triphosphate |
| UTR | untranslated region |
| UV | ultra violet |
| Xgal | 5-bromo-4-chloro-3indolyl-β-D-galactoside |

# Chapter One
# General introduction

## 1.1. Introduction

Three entirely different observations have led to the discovery of the insulin-like growth factors (IGFs).

An experiment carried out in 1957 by Salmon and Daughaday showed that growth hormone (GH) itself exercises no direct metabolic action on skeletal tissue, but rather acts through a second substance, which is detectable in normal plasma. The term "sulphation factor" was applied to this plasma activity because the assay used for its detection depended upon the *in vitro* incorporation of radioactive sulphate ($^{35}$S) into proteoglycans of cartilage. This factor appears to be growth hormone-induced, since it appears in the plasma of hypophysectomized rats after the administration of growth hormone (Salmon & Daughaday, 1957).

In 1972 Pierson and Temin extracted factors from calf serum which stimulate the multiplication of fibroblast in tissue culture and termed as multiplication-stimulating activity (MSA ). These factor had molecular weights of about 6000 Dalton (Pierson & Temin, 1972).

The third observation was based on the fact that serum exerts insulin-like growth activity on insulin target tissues such as muscle and adipose tissue. These effects are much greater than would be expected on the basis of the insulin content of serum. Furthermore these effects were not suppressed by the addition of anti-insulin serum, contrary to the effects of insulin and termed as non-suppressible insulin-like activity (NSILA). It was suggested that these substances may be closely related to the insulin molecule (Froesch E. *et al.,* 1963).

Although the mechanism of production and ultimate physiological role of these substances remained to be defined at that time, their importance in the growth and anabolic responses of both skeletal and certain non-skeletal tissues can be in no doubt. It was, therefore, later decided that these three activities represented similar

responses and were termed "somatomedins" (Daughaday *et al.*, 1972).

Purification of somatomedin from human serum led to the identification of a neutral peptide of about 7000 Dalton termed somatomedin-A (Hall, 1972; Uthne, 1973) and a basic peptide termed somatomedin-C (Van Wyk *et al.*, 1974). Both somatomedins were found to be under growth hormone control.

Analysis of NSILAs from human serum resulted in the first elucidation of the primary structures of two peptides that were termed human IGF-1 (Rinderknecht & Humbel, 1978b) and human IGF-II (Rinderknecht & Humbel, 1978a). Purification and analysis of MSAs in the serum free medium conditioned by the Buffalo rat liver cell line led to structural characterization as the rat counterpart of human IGF-II (Marquardt *et al.*, 1981). Sequence analysis of somatomedin-C confirmed that somatomedin-C is identical in structure with IGF-1 (Klapper *et al.*, 1983). Somatomedin-A was shown to represent a mixture of IGF-1 and IGF-II (Spencer *et al.*, 1983).

In 1987, to prevent confusing nomenclature in the literature, workers in this field proposed that IGF-1 and IGF-II terminology be adopted, and described IGF-1 as a basic 70 amino acid residue single chain peptide structurally related to human proinsulin, IGF-II as a 67 residue neutral peptide that is structurally similar to IGF-1, but much less GH-dependent (Daughaday *et al.*, 1987a; Daughaday *et al.*, 1987b; Daughaday *et al.*, 1987c).

## 1.2. Source of production

The IGF-1 gene was traditionally considered to be expressed in the liver. There is now evidence that IGF-1 is synthesized at multiple sites (D'Ercole *et al.*, 1984).

### 1.2.1. Liver

Schwander et al (1983) used isolated livers of normal and hypophysectomized rat with or without GH replacement therapy to study synthesis and secretion of insulin-like growth factor in liver. They showed that the liver is the main source of circulating IGF-1 and that rates of hepatic secretion of IGF-1 are sufficient to account for level of IGF-1 found in plasma, which would be involved in growth regulation in an endocrine manner. IGF is continuously synthesized and released by liver depending on GH status and it is synthesized rapidly before secretion (Schalch *et al.,* 1979; Schwander *et al.,* 1983).

### 1.2.2. Skeletal muscle

Skeletal muscle, the major component of lean body tissue and the largest protein store in the body, is capable of undergoing rapid and extensive alterations in mass. It is also believed to be one of main targets of IGF-1 and muscle growth in response to GH administration was not under the control of circulating IGF-1 acting in an endocrine manner (Loughna *et al.,* 1992). Low expression of IGF-1 in skeletal muscle has been reported. The IGF-1 content in skeletal muscle extracts decreases after hypophysectomy and is restored after treatment *in vivo* with growth hormone (D'Ercole *et al.,* 1984; Murphy *et al.,* 1987; Isgaard *et al.,* 1988; Isgaard *et al.,* 1989). Interestingly in hypophysectomized animals, high level of IGF-1 mRNA is expressed after injury (Edwall *et al.,* 1989), indicating that IGF-1 expression after skeletal muscle injury may be independent of GH.

### 1.2.3. Other tissues

In addition to muscle tissue, IGF-1 is also present in numerous organs and tissues. These organs and tissues are lung; kidney; heart; brain; testes; prostate; thymus; lymph nodes; cartilage (sternum); fat pad (perirenal), submaxillary gland, spleen, brain and epididymal (white) adipose tissue, but was barely detectable in kidney,

brain and thymus (D'Ercole *et al.*, 1984; Han *et al.*, 1987). It is possible that in these tissues IGF-1 acts in a paracrine or autocrine fashion and is likely to have a role in regulating organ-specific growth. An exception is IGF-1 message in brain which was not influenced by hypophysectomy (Gosteli-Peter *et al.*, 1994).

## 1.3. Biological activity of IGF-1

The two main biological actions of the IGFs may be summarized as an insulin-like metabolic action and a growth-promoting action. The end result of IGF action will depend on the responsiveness of their target cells. That is to say, if the cells are in the hyperplastic phase, then IGF acts as a mitogen. However, if the cell are undergoing hypertrophic growth, or are mature nondividing cells such as neurons, then other anabolic processes, such as protein synthesis, are stimulated.

### 1.3.1. *In vitro*

The *in vitro* action of IGF-1 has been well established in a wide variety of cells. These studies have been reviewed by Sara and Hall (1990). Effects of IGF-1 *in vitro* are now commonly classified into short term insulin-like effects such as stimulation of glucose uptake, glycogen and lipid synthesis in adipose tissue and long term effects such as stimulation of RNA, DNA and protein synthesis and involvement in cellular proliferation and differentiation.

The IGFs have been demonstrated to stimulate the differentiation of myoblast (Schmid *et al.*, 1983; Florini *et al.*, 1986), osteoblast (Schmid *et al.*, 1984), adipocyte (Smith *et al.*, 1988) and oligodendrocytes (McMorris *et al.*, 1986). IGF-1 also induces erythropoiesis (Claustres *et al.*, 1987), granulopoiesis (Merchav *et al.*, 1988) and chemotaxis in endothelial and melanoma cells (Grant *et al.*, 1987; Stracke *et al.*, 1988). In rat skeletal muscle cells , IGF-1 has been shown to stimulate expression of oncogene c-fos (Ong *et al.*, 1987). It also affects proliferation, differentiation and protein turnover in skeletal muscle cells. IGF-1 stimulates proliferation (Ballard *et al.*,

1986; Hill *et al.,* 1986; Ewton *et al.,* 1987) and differentiation (Ewton & Florini, 1981; Schmid *et al.,* 1983; Ewton *et al.,* 1987) in cultured myogenic cells of both mammalian and avian origin. The IGF-1 also enhances protein synthesis in L6 and L8 myoblast (Ballard *et al.,* 1986; Gulve & Dice, 1989) and ovine myotubes (Roe *et al.,* 1989) and decreases protein degradation in L6 myotubes (Ewton *et al.,* 1987), L8 myotubes (Gulve & Dice, 1989), and ovine myotubes (Roe *et al.,* 1989).

**1.3.2. *In vivo*:**

Studies of the biological actions of the IGFs *in vivo* were pioneered by the groups in Amsterdam and Zurich using crude preparations. The availability of recombinant IGF-1 in recent years has opened the way for numerous investigations and has confirmed these early findings. The effects of the IGFs *in vivo* depend on their mode of administration (Zapf *et al.,* 1984). With intravenous bolus injections, IGFs cause acute insulin-like effects. With long-term subcutaneous administration they have anabolic and growth-promoting actions (Sara & Hall, 1990). This is clearly because of a difference in the availability of the IGFs to the target cells.

In terms of insulin-like effects of IGF-1, using both normal and hypophysectomized rats, the intravenous bolus injection of both IGF-I and IGF-II induced hypoglycaemia and enhanced glucose uptake from serum and incorporation into glycogen. The hypoglycaemic effect of IGF-1 tended to be more potent than IGF-II (Zapf *et al.,* 1986). In healthy man, IGF-1 given intravenously in dose of 100 $\mu$g per kg of body weight decreases blood glucose equipotent to the effect of 0.15 iu insulin per kg body weight (Guler *et al.,* 1987). The hypoglycaemic effect of IGF-1 is due to the concentration of free IGF-1, that is the molecule not bound to IGF binding protein (IGFBP), whereas bound IGF-1 to IGFBP has no insulin-like action. The insulin-like effect of IGF-1 on glucose homeostasis was confirmed in studies on depancreatized dogs (Giacca *et al.,* 1990). During a 90-minute infusion of IGF-1 or insulin in doses that induced an identical decline in blood sugar, the decrease in glucose production was significantly less with IGF-1 ( 25%) than with insulin (42%). Glucose utilization

increased more with IGF-1 (68%) than with insulin (38%), resulting in a higher metabolic clearance with IGF-1. It was thought the main site of action of IGF-1 on glucose metabolism is not likely to be liver or adipose tissue, the target tissue of insulin. The rise in plasma lactate is in keeping with an IGF-1 effect on the muscle and is consistent with a higher IGF-1: insulin potency ratio in muscle than in adipose tissue. In summary, IGF-1 *in vivo* has pronounced metabolic effects on glucose and lipid metabolism in man and animals. IGF-1 is proposed to be of therapeutic value during conditions involving insulin resistance such as type 2 diabetes, obesity and hyperlipidaemia in which the pattern of effects of IGF-1 may be preferable to that of insulin.

Long-term subcutaneous infusions of recombinant IGF-1 stimulates the growth of hypophysectomized rats (Guler *et al.,* 1988; Skottner *et al.,* 1987), rapidly growing post-weaning normal rats (Hizuka *et al.,* 1986), neonatal and pre-weaning rats (Philipps *et al.,* 1988) and insulin-dependent diabetes mellitus (IDDM) rats (Scheiwiller *et al.,* 1986). The original studies of the *in vivo* actions of the IGFs used preparations that were purified from human serum. These studies demonstrated that IGF-1 was able to stimulate the growth of hypophysectomized rats (Schoenle *et al.,* 1985) as well as Snell dwarf mice (van Buul-Offers *et al.,* 1986).

During tissue repair and periods of localized growth the paracrine/autocrine IGF-1 is of greater importance. Induction agents other than growth hormone may have a primary role in its secretion (Clemmons & Shaw, 1983; Chatelain *et al.,* 1987; Canalis *et al.,* 1989; Norstedt *et al.,* 1989).

## 1.4. Mechanism of IGF-1 activity

In recent years it has become apparent that the IGF-1 has widespread actions on multiple cell types and can act in an endocrine, paracrine, or autocrine manner. The widespread actions of IGFs raise the question of what dictates the specificity of IGF action in different tissues at appropriate stages in development and in response to

tissue- and cell- specific stimuli. Some evidence suggests that specific IGF binding proteins (IGFBP) can dictate transcapillary transport of IGFs and regional distribution of the IGFs within tissues (Bar *et al.,* 1990a; Bar *et al.,* 1990b). Furthermore, the biological actions of the IGFs are mediated by cell surface receptors called type I and type II IGF receptor.

### 1.4.1. IGF-binding proteins

IGF-1 exists in two forms, a free and a bound form with high affinity to and specificity for various soluble IGF-binding proteins. Traditionally, the IGFBP were thought to be liver-derived proteins that bound circulating IGF-1, limited the availability of free IGF-1, and prolonged the plasma half-life of IGF-1. It is now clear that there is a family of six distinct IGFBPs, numbered 1-6, that share similar structure features and properties (Shimasaki *et al.,* 1991; Shimasaki & Ling, 1991; Drop *et al.,* 1992). Each of these IGFBPs appears to show distinct developmental and tissue-specific pattern of expression. The different IGFBPs also are regulated in a distinct fashion by hormones and other physiological stimuli, such as nutrient status (Martin & Baxter, 1990; Clemmons *et al.,* 1991; Shimasaki & Ling, 1991) . Together they bind most of the IGF-1 in the circulation, leaving less than 10% of the total serum concentration of IGF-1 in the free form (Zenobi *et al.,* 1992). Studies in many cells in culture indicate that IGFBPs can either inhibit or potentiate IGF action, possibly depending on whether they are secreted or membrane associated (Martin & Baxter, 1990; Rosenfeld *et al.,* 1990; Clemmons *et al.,* 1991). Thus, the IGFBPs may be integral to IGF actions.

IGFBPs specifically bind IGF-1 and IGF-II but not insulin. IGFBPs 1, 3 and 4 bind IGF-1 and IGF-II with similar affinity, IGFBPs 2, 5 and 6 have higher affinity for IGF-II than IGF-1 (Rechler & Brown, 1992; Roghani *et al.,* 1991; Bautista *et al.,* 1991)

The purification and structural characterization of distinct forms of IGFBP and the

development of specific antisera and cDNA probes has allowed the identification of several forms of IGFBP in blood and tissues. Characteristics of IGFBPs are depicted in Table 1.1.

**Table 1.1**

| Characteristic | IGFBP-1 | IGFBP-2 | IGFBP-3 | IGFBP-4 | IGFBP-5 | IGFBP-6 |
|---|---|---|---|---|---|---|
| Sequence (KDa) | | | | | | |
| Human | 25.2 | 31.3 | 28.7 | 26 | 31-32 | ? |
| Rat | 29.6 | 29.5 | 28.8 | 25 | 28-29 | 33 |
| Electrophoresis (kMr) | | | | | | |
| Human | 29 | 34 | 39-43 | 24 | | |
| Rat | 31 | 32 | 39-43 | 24 | | |
| Cysteine residues | 18 | 18 | 18 | 18 | | |
| N-glycosylation | Absent | Absent | Present | Present | Present | Present |
| RGD sequence | Present | Present | Absent | Absent | | |
| Affinity (nM) | | | | | | |
| IGF-1 | 6.5 | | 2.1 | | | |
| IGF-II | 3.2 | | 3.3 | | | |
| m RNA size (bp) | | | | | | |
| Human | 1600 | 1800 | 2500 | 2600 | 6000 | ? |
| Rat | 1600 | 1800 | 2400 | 2600 | 6000 | 1400 |

An important biological property of the IGFBPs is their ability to increase the circulating half-life of the IGFs (Davis *et al.*, 1989; Guler *et al.*, 1989; Hodgkinson *et al.*, 1989). Free IGF-1 has a half-life in blood of less than 10 minutes, whereas when bound to the 50000-Mr complex (primarily unsaturated IGFBP-1 and 2), it has a half-life time of approximately 30 minutes. The half-life time of IGF bound to

IGFBP-3 is even longer ( more than 6 hours). The IGFBPs also have the potential to prolong the half-life of IGFs in extracellular fluids and in the interstitium.

Both [$^{125}$I] IGFBP-1 and 2 are transported across intact endothelium (Bar *et al.*, 1990; Bar *et al.*, 1990), and this may be an important biological property of the two proteins. IGFBP-1 apparently serves as a shuttle transporter of IGF-1 from the serum to the interstitial fluid, and it also controls the concentration of free IGF-1 at its site of action (Holly *et al.*, 1991). The physiological role of IGFBP-2 is poorly understood, but it may serve as a shuttle transporter of IGF-1 between intravascular and interstitial spaces of target organs. It is the predominant form of IGFBP in cerebrospinal fluid. As the levels of both IGFBP-1 and 2 in blood are regulated, this could provide a mechanism for controlling the rate of transport of IGFs out of the intravascular compartment.

Because of its size , the IGFBP-3 complex is probably not transported across the endothelial blood barrier and simply acts as a reservoir of IGF. This storage is likely to account for the relatively prolonged half-life of IGF in blood.

IGFBPs also modulate the biological activity of IGFs. Addition of IGFBP-1, for example, depresses IGF-1 stimulated amino acid transport of human choriocarcinoma JEG-3 cells (Ritvos *et al.*, 1988), DNA synthesis of FRTL5 rat thyroid follicular cells (Frauman & Moses, 1989) and blocks the growth of chick embryo pelvic cells (Draznin *et al*, 1979). Similarly , addition of IGFBP-2 blocks the metabolic and mitogenic activity of IGF-1 on chick embryo fibroblasts (Knauer & Smith, 1980). IGFBP-3 also blocks IGF-1-stimulated, bone-cell proliferation (Mohan *et al.*, 1989). The proposed, simple model of these actions of IGFBPs is that the IGFBPs simply act as blocking agents preventing IGFs association with cell-surface binding sites.

In summarily, the various forms of IGFBP play distinct roles in controlling the availability of IGF to tissues, regulating the half-life of circulating IGF and modulating the biological activity of IGF. There are no definitive data to indicate a

biological activity attributing to the IGFBPs independent of the IGFs. Thus it is important not only to know the level of a particular form of binding protein in a biological fluids but to know its degree of saturation with one of its two ligands. The application of IGFBPs in both human medicine and agriculture is an impetus to further investigation into the role of these proteins in inhibiting or stimulating the activity of IGFs.

### 1.4.2. IGF receptors and intracellular signal transmission

The biological activity of any hormone depends on the ability of the target cell to respond to the signal in the extracellular milieu. This is a function of cell receptors as well as post-receptor mechanisms. Two distinct IGF receptors have been characterized. The type I receptor preferentially binds IGF-1 and is commonly called the IGF-1 receptor. Similarly the type II receptor preferentially binds IGF-II and is commonly referred to as the IGF-II receptor.

The subunit structure of the IGF-1 receptor were first revealed by [$^{125}$I]-IGF-1 affinity cross-linking (Chernausek *et al.*, 1981; Kasuga *et al.*, 1983; Massague & Czech, 1982) and photoaffinity labelling techniques (Bhaumick *et al.*, 1981). The IGF-1 receptor is a glycoprotein with a molecular weight of between 300KDa and 350KDa, which consists of two extracellular α-subunits (about 130KDa) and two transmembrane β-subunits (about 95KDa). The human IGF-1 receptor gene has been mapped to chromosome 15q25-26 (Ullrich *et al.*, 1986). This receptor preferentially binds IGF-1, but the homologous IGF-II also cross-reacts to a lesser degree. At high concentrations insulin cross-reacts weakly in the IGF-1 receptor. The hormone binding site is located in the extracellular α-subunit and possibly also the extracellular domain of the β-subunit. The carboxyl-terminal region of α–subunit and as well as the amino-terminal region of the β-subunit of the IGF-1 receptor are hydrophobic region that are likely to be exposed extracellularly to provide a site for ligand binding.

The IGF-1 receptor is an IGF-1 stimulated, tyrosine-specific, protein kinase. In intact

cells, IGF-1 stimulates the tyrosine phosphorylation of its receptor and of other cellular proteins as well (Jacobs *et al.,* 1983; Jacobs & Cuatrecasas, 1986; Kadowaki *et al.,* 1987; Shemer *et al.,* 1987). Similarly, in solubilized preparations of receptor, IGF-1 stimulates tyrosine phosphorylation of the receptor and of added exogenous proteins and synthetic peptide substrates (Rubin *et al.,* 1983; Zick *et al.,* 1984). This tyrosine kinase activity is intrinsic to the receptor as it co-purified with the receptor (LeBon *et al.,* 1986), and as the IGF-1 receptor cDNA contains a clear, tyrosine-kinase domain sequence.

There is a strong general consensus that the tyrosine kinase activity of IGF-1 receptor is involved in the process by which it generates a cellular signal. In support of a role for the tyrosine kinase activity of IGF-1 receptor, Izumi et al (1988) found that anti-peptide antibodies directed against the tyrosine kinase domain of the receptor inhibited IGF-1 stimulation of membrane ruffling in KB cells.

The structure of IGF-1 receptor has been reviewed in detail by Yarden and Ullrich (1988). It consist of a large glycosylated, extracellular ligand binding domain; a single, hydrophobic transmembrane region and a cytoplasmic domain containing a tyrosine kinase catalytic region. As a consequence of this structure, the ligand binding domain and the tyrosine kinase activity are separated by the plasma membrane. Therefore, receptor activation by extracellular ligand binding must be translated across the membrane to result in activation of the cytoplasmic domain. In order to produce such an effect, contemporary studies suggest that ligand binding to IGF-1 receptor results in conformational alterations of the extracellular domain. These conformational changes result in the interactions of both $\alpha\beta$ dimers so that $\alpha-\alpha$ and $\beta-\beta$ are associated (Boni-Schnetzler *et al.,* 1988; O'Hare & Pilch, 1988) and this interaction is necessary for transmission of the signal and activation of the tyrosine kinase. The IGF-1 receptor , therefore, is capable of transmitting a signal initiated by a conformational change in the extracellular domain to the cytoplasmic domain, without altering the monomeric structures of the transmembrane domain ( Fig. 1.1 and 1.2).

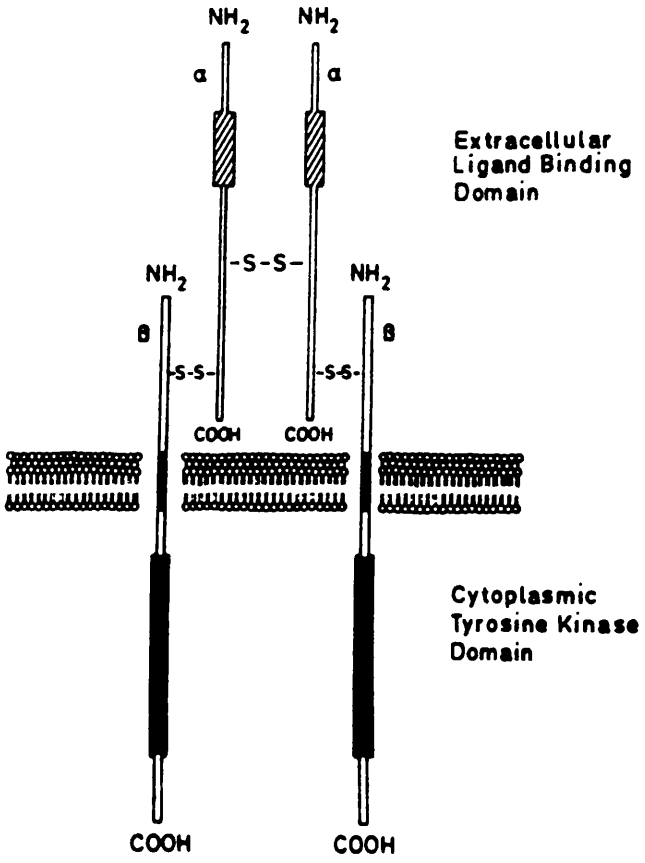



**Fig. 1.1.** Schematic representation of the transmembrane IGF-1 receptor. The IGF-1 receptor is glycoprotein with an alpha, extracellular ligand binding subunit, and a beta, cytoplasmic subunit that contains a tyrosine kinase domain. (re-drawn from Ward *et al*, 1994). As a consequence of this structure, the ligand binding domain and the tyrosine kinase activity are separated by the plasma membrane. Therefore, receptor activation by extracellular ligand binding must be translated across the membrane to result in activation of the cytoplasmic domain.

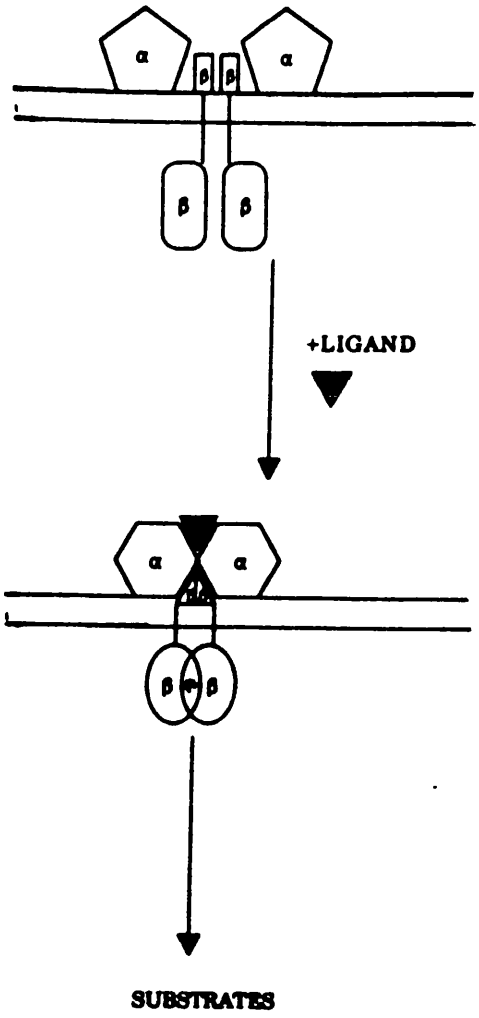



**Fig. 1.2.** Schematic representation of the major signal transduction pathway mediated by activation of IGF-1 (re-drawn from Ward *et al*, 1994). The IGF-1 receptor exist as heterotetrameric protein. Ligand binding to the receptor results in conformational alterations of the extracellular domain. This conformational changes results in the interactions of both α and β dimers so that α-α and β-β are associated. The latter then activate the intrinsic tyrosine kinase. This receptor is autophosphorylated and the tyrosine kinase also phosphorylates various cellular substrates. * indicates activated tyrosine kinase.

Ultimately, the importance of the tyrosine kinase activity of the IGF-1 receptor in producing a biological response will require identification of its cellular protein substrates. Several, possible, physiologically relevant substrates have been identified which are a series of proteins (Kasuga *et al.,* 1990). Their physiological function is not known yet.

## 1.5. Structure of IGF-1: In relationship to function

### 1.5.1. Primary structure of IGF-1

The accuracy of the primary structure of IGF-1, which was isolated from adult human plasma by Rinderknecht and Humbel (1978b), was confirmed by the analysis of cDNA encoding it (Jansen *et al.,* 1983; Bell *et al.,* 1986; Murphy *et al.,* 1987).

IGF-1 is a single-chain polypeptide consisting of 70 amino acid residues with a predicted molecular weight of 7648.7. Similar to proinsulin, IGF-1 contains an amino-terminal B region and an A region that are separated by a short connecting C region. The A and B region of IGF-1 are similar to those of proinsulin, giving a sequence homology to proinsulin of 43%. Unlike proinsulin, however, IGF-1 also contains a D-region extension peptide at the carboxyl-terminus. (Fig. 1.3). Especially important is the conservation of the cysteine and glycine residues between IGF-1 and proinsulin, which allows the prediction that the tertiary structure of the IGF-1 will be rather similar to that of insulin. The hydrophobic core of insulin: A2 Ile, A16 Leu, B12 Val, B15 Leu and B24 Phe (insulin notation) is conserved as well. The most obvious differences between IGF-1 and insulin are in the C-domain.

IGF-1 contains three motif-specific disulphide bonds. Narhi et al (1993) demonstrated the presence of all three disulphide bonds [18-61, 6-48, 47-52] is required for the native function, structure and stability of IGF-1. (Fig. 1.4).

## B domain

```
Human pro-insulin     FVNQHLCGSHLVEALYLVCGERGFFYTPKT
Human IGF-1           GPET---AE--D--QF---D---YFNKP-
                          *** ***** ******* *
```

## C domain

```
Human pro-insulin     RREAEDLQVGQVELGGGPGAGSLQPKAKEGSLQKR
Human IGF-1           GYGSSSRRAP-T
                                *
```

## A domain

```
Human pro-insulin     GIVEQCCTSICSLYQLGNYCD
Human IGF-1           ---DE--FRS-D-RR-EM--A
                      *** **   * *  *  **
```

## D domain

```
Human pro-insulin     .....
Human IGF-1           PLKPAKSA
```

**Fig. 1.3.** Comparation between the primary sequences of human pro-insulin and IGF-1. The A and B region of IGF-1 are similar to those of proinsulin, giving a sequence homology to proinsulin of 46-47%. Unlike proinsulin, IGF-1 also contains a D-region extension peptide at the carboxyl-terminus. The most obvious differences between IGF-1 and proinsulin are in the C-domain. The homologous amino residues are marked by underneath *.

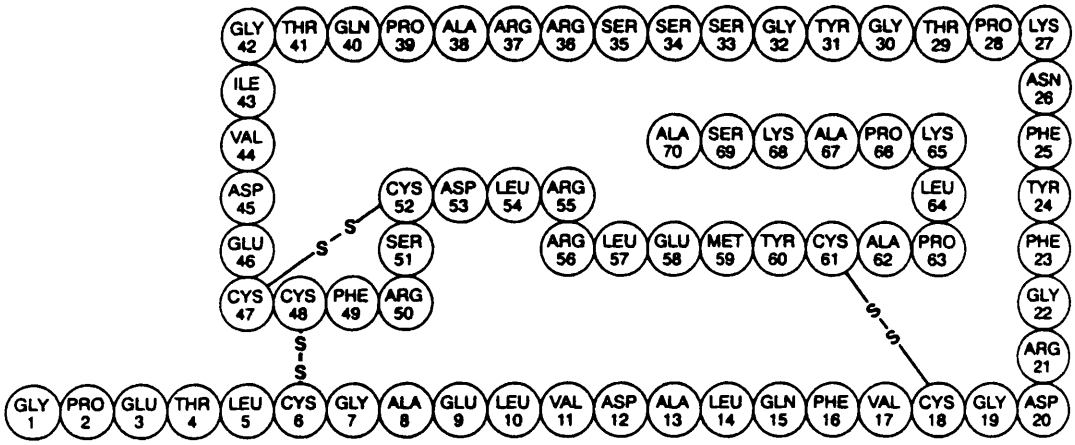



**Fig. 1.4.** Sequence of mature human IGF-1 (re-drawn from Narhi et al, 1993). IGF-1 contains three native (insulin-like) disulphide bonds, [6-48; 18-61; 47-52], indicated by -S-S-. The presence of all these three disulphide bonds is required for the native function, structure and stability of IGF-1.

The similarity of the IGFs and insulin has allowed prediction of the regions involved in their interactions. Experimental evidence obtained with natural IGF-1 variants, products of post-translational processing, and synthetic hybrid or mutated IGFs has led to elucidation of physiological roles of different region of IGF-1. The B domain (specially B23-24) is believed to have important role on the interactions of IGF-1 with insulin receptor. The A chain is involved in the interactions of IGF-1 with IGF-1 receptor and the effectiveness of this interaction depends on positioning the molecule correctly, possibly influenced by its D-domain. The C-domain supplies the specific residues for IGF-1 interacting with IGF-1 receptor (Hampton *et al.,* 1989). Blundell et al (1983) have pointed to the hydrophobic surface patch involving B13, 16, 17 and A13 as a possible binding site for IGFBPs. A more detail picture was obtained from the studies of Bagley et al (1989), which showed that residue B3 in IGF-1 is critical for binding. Fine mapping of the site of interaction with the different BPs is not yet possible. Clearly more studies with pure substances are required for further refinement of the interfaces of interaction with IGFBPs.

### 1.5.2. The secondary structure of IGF-1

The secondary structure of the IGFs has not yet been determined by X-ray or nuclear magnetic resonance. However, by analogy with the known structure of insulin, and based on first principles, Blundell et al (1978) has proposed three-dimensional model of IGF-1. The model is depicted in Fig 1.5.

Two of the disulphide bridges are in the core of the molecule [47-52, and 61-18], whereas the third S-S bridge is on the surface [6-48]. The C and D domain are two prominent regions of the IGF molecules. The C-domain is rather hydrophilic. The distance between carboxyl-terminus of the B-domain and amino-terminus of A-domain is small. It is proposed that the D-domain has a hairpin configuration: the two branches are held together by hydrophobic interaction and allow hydrophilic side chain to be exposed to the solvent.

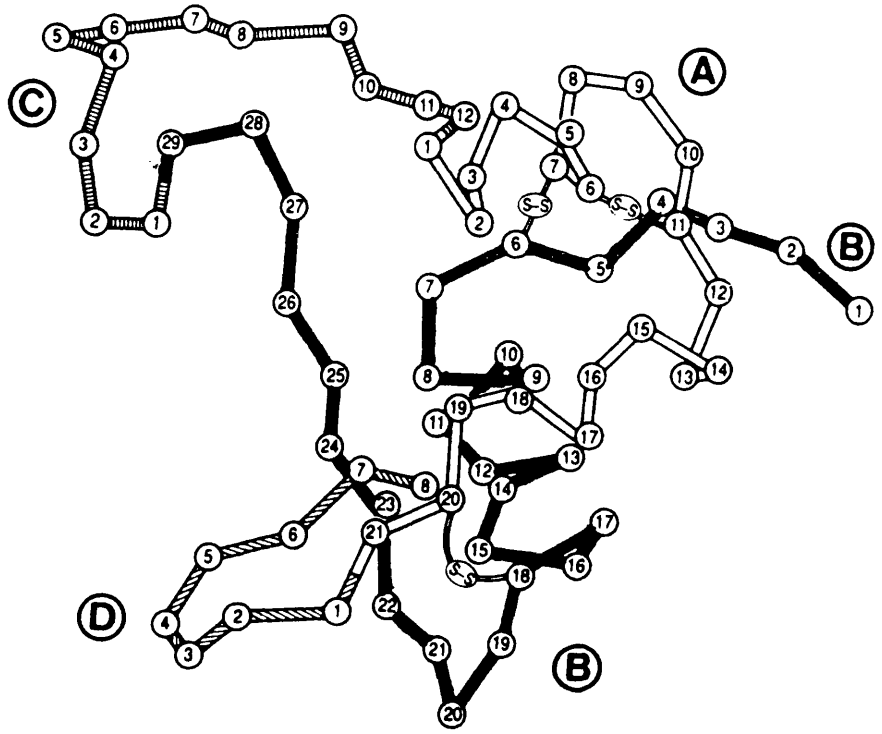



**Fig. 1.5.** Tertiary structure of IGF-1 (re-drawn from Blundell et al, 1983), residues are numbered by domain. Two of the disulphate bridges are in the core of the molecule [B18-A20; A6-A11], whereas the third S-S bridge [B6-A7] is on the surface. The C and D domain are two prominent regions of the IGF-1 molecule. The C-domain is rather hydrophilic. The distance between carboxyl-terminus of the B-domain and amino-terminus of A-domain is small. It is proposed that the D-domain has a hairpin configuration, i.e. the two branches are held together by hydrophobic interaction and allow hydrophilic side chain to be exposed to the solvent. This is incomplete as it does not show the E domain, the presence of which was not known at the time.

### 1.5.3. IGF-1 variants

Two IGF-1 variants have been found so far. The first is a processed IGF-1 lacking the first three N-terminal residues, termed as des(1-3)-IGF-1, as discovered by Sara et al (1986) in human fetal and adult brain extracts. This variant was also in other tissues, for example, porcine uterus (Ogasawara *et al.,* 1989), human platelet lysates (Karey *et al.,* 1989). But it has never been identified in adult plasma or its derivatives. Interestingly, this truncated IGF-1 has a 100-fold reduction in affinity for BP1 (Carlsson-Skwirut *et al.,* 1989) and 2-3 times lower for BP3 (Forbes *et al.,* 1988), whereas it has strongly increased biological activity, varying between 4 and more than 10 times the potency of intact IGF-1 (Ballard *et al.,* 1987; Ballard *et al.,* 1988; Szabo *et al.,* 1988; Carlsson-Skwirut *et al.,* 1989; Ogasawara *et al.,* 1989). It is also interesting to note that the tripeptide Gly-Pro-Glu, liberated on cleavage, appears to have intrinsic neurotransmitter activity (Sara *et al.,* 1989).

Another IGF-1 variant is a IGF-1 like peptide cleaved between two arginines at C7-C8 (termed as IGF-1, c7-8 cleaved). This cleavage could be an artefact of the isolation procedure or occur naturally. In either case it supplies an interesting model for studying the relationship between structure and function (Van den Brande *et al.,* 1990).

### 1.5.4. IGF-1 in different species

The primary sequences of IGF-1 in a number of mammalian species have now been disclosed (Marquardt *et al.,* 1981; Bell *et al.,* 1986; Honegger & Humbel, 1986; Shimatsu & Rotwein, 1987a; Francis *et al.,* 1988; Francis *et al.,* 1989a; Francis *et al.,* 1989b; Tavakkol *et al.,* 1988; Tamura *et al.,* 1989; Weller *et al.,* 1993; Dickson *et al.,* 1991). The homology between the IGF-1 of mammals is remarkable (table 1.2). IGF-1 is 100% identical in man, cow, pig and sheep. In rat three and in mouse four residues are different in the B, C and D domains. The A domain is 100% identical.

**Table 1.2**

| Domains | B domain | C domain | D domain | D domain |
|---|---|---|---|---|
| Species | B 21 | C 6 | D 2 | D 4 |
| Human | Arg | Ser | Leu | Pro |
| Porcine | | | | |
| Bovine | | | | |
| Ovine | | | | |
| Rat | Pro | Ile | Thr | |
| Mouse | Pro | Ile | Thr | Ala |

## 1.6 IGF-1 gene

The first IGF-1 cDNA to be characterized was isolated from a human liver cDNA library by Jansen et al (1983). From then a number of groups isolated similar rat IGF-1 cDNAs from liver cDNA libraries or from cDNA libraries derived from other nonhepatic tissues (Casella *et al.*, 1987; Roberts *et al.*, 1987; Shimatsu & Rotwein, 1987a; Murphy *et al.*, 1987). The exact length of the IGF-1 gene is unknown because of an undetermined gap within the largest intron between exons 3 and 4 and the uncertainly about the definition of the 5' end of the gene.

### 1.6.1. IGF-1 gene structure

Among the mammals, the human and rat genes have been the most extensively studied, and in these species IGF-1 is encoded by a single-copy gene.

**A. Human IGF-1 gene:** The human IGF-1 gene has been mapped to the long arm of chromosome 12 (Brissenden *et al.*, 1984; Tricoli *et al.*, 1984; Hoppener *et al.*, 1985). It consists of at least six exons spanning a region of more that 95 kb of chromosome DNA (De Pagter-Holthuizen *et al.*, 1986; Rotwein *et al.*, 1986;

Gilmour, 1994). The structure of the human IGF-1 gene is shown in fig. 1.6. Exons 1 and 2 are alternative leader exons (Tobin *et al.,* 1990; Gilmour, 1994) with distinct transcription start sites which are differentially spliced to the common exon 3 and produce class 1 and 2 IGF-1 mRNA transcripts respectively (Weller *et al.,* 1993). Exons 3 and 4 code for the mature IGF-1 (B, C, A and D domains) as well as the first 16 amino acid of the E domain. Exons 5 and 6 each contains the sequences encoding the alternative parts of the E domain together with 3' untranslated regions, which is followed by alternative polyadenylation signals.

Alternate splicing or inclusion of exons leads to a family of IGF-1 mRNAs that all encode the same mature IGF-1 but differ in sequences 5' or 3' to the IGF-1 coding sequence (Daughaday & Rotwein, 1989; Hepler & Lund, 1990). In term of 5' alternative splicing, Exons 1 and 2 are each termed 5' leader exons. 3' alternative splicing of the primary IGF-1 gene results in six different IGF-1 mRNA, i.e. class 1 IGF-1Ea (exons 1, 3, 4 and 6) (Sussenbach *et al.,* 1992; Gilmour, 1994),class 1 IGF-1Eb (exons 1, 3, 4 and 5) (Rotwein *et al.,* 1986; Gilmour, 1994) class 1 IGF-1Ec (Exons 1, 3, 4, part of 5 and 6)(Chew *et al.,* 1995), class 2 IGF-1Ea, class 2 IGF-1Eb and class 2 IGF-1Ec mRNAs. Although the biological activities generated from these alternative precursors have yet to be determined, alternative RNA splicing may provide a mechanism for regulating IGF-1 biosynthesis and subsequent function.

**B. Rat IGF-1 gene:** Studies from a number of laboratories elucidated the complete structure of the rat IGF-1 gene (Shimatsu & Rotwein, 1987a; Bucci *et al.,* 1989; Hoyt *et al.,* 1992; Simmons *et al.,* 1993). The gene is large, spanning more than 80 kb of genomic DNA, and contains six exons (Fig.1.7). Exons 3 and 4 encode the identical sequence found in all rat IGF-1 cDNA, including the IGF-1 coding sequence. Class 1 and 2 IGF-1 mRNA derive from the use of different transcription start sites in exon 1 and 2 (Adamo *et al.,* 1991; Hall *et al.,* 1992; Simmons *et al.,* 1993). Exons 1 and 2 are each, therefore termed 5' leader exons.

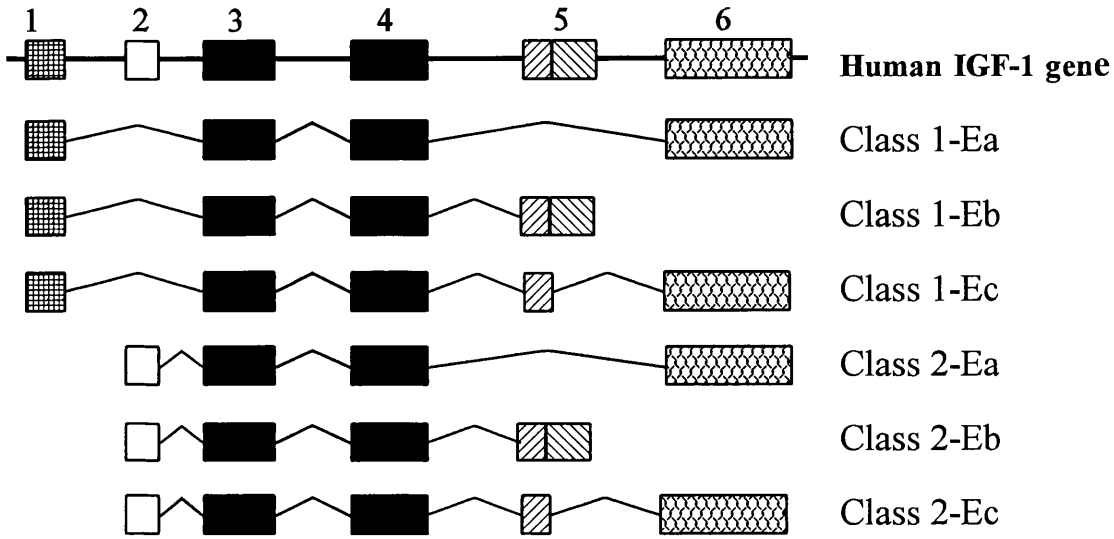



**Fig. 1.6.** Schematic of human IGF-1 gene (top row) which consists at least 6 exons spanning a region of more than 95 kb of genomic DNA. Exons 1 and 2 are alternative leader exons with distinct transcription start sites which are differentially spliced to the common exon 3 and produce class 1 and 2 IGF-1 mRNA transcripts respectively. Exons 3 and 4 code for the mature IGF-1 peptide as well as the first 16 amino acid of the E domain. Exons 5 and 6 each contains sequences encoding the alternative parts of the E domain with 3' untranslated regions. 3' alternative splicing leads to Ea, Eb and Ec distinct E domains. Together of 5' and 3' alternative splicing or inclusion exons result in a family of IGF-1 mRNAs (2-7 rows).

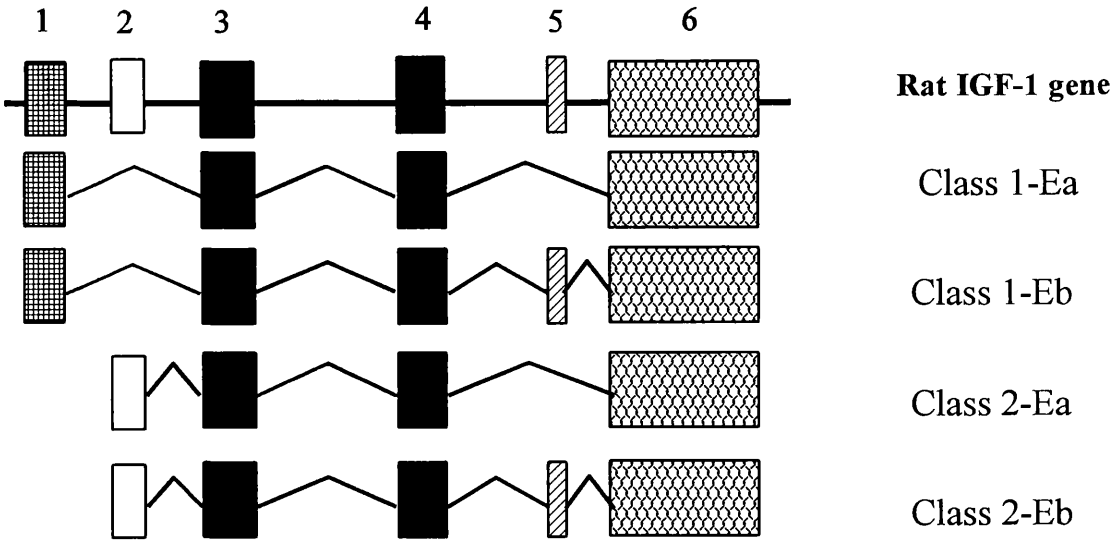
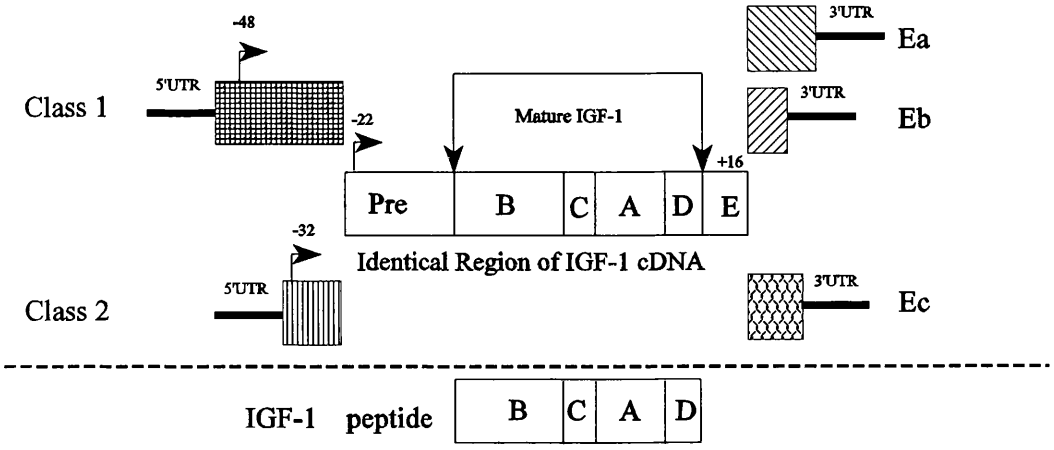



**Fig. 1.7.** Schematic of rat IGF-1 gene (top row) and derived IGF-1 mRNAs (bottom rows). Similar to its human counterpart, the rat IGF-1 gene is large, spanning more than 80 kb of genomic DNA, and contains 6 exons. Class 1 and 2 IGF-1 mRNA derive from the use of different transcription start sites in exons 1 and 2. Exons 3 and 4 (black boxes) encode the mature IGF-1 peptide and the first 16 amino acid of the E domain. Unlike its human counterpart, exon 5 of rat IGF-1 gene is a cassette exon of 52 base-pairs (bp), rather than a terminal exon. 3' alternative splicing by inclusion of exon 5 (i.e. splicing of exons 4-5-6), or exclusion of exon 5 (i.e. splicing of exons 4-6) results in IGF-1 Ea and Eb mRNAs. Class 1 and 2 sequences is each associated with Ea or Eb 3' coding sequences, therefore, rat IGF-1 gene encodes class 1 Ea, class 1 Eb, class 2 Ea and class 2 Eb mRNAs (bottom rows).




Structure of IGF-1 gene

Mature IGF-1 peptide

**Fig. 1.8.** Schematic of IGF-1 cDNA and encoded precursors. The bottom row shows a schematic of mature IGF-1 which contains B, C, A and D domains. The upper panels show a schematic of an IGF-1 cDNA encoding IGF-1 in the midregion and flanking sequences for amino-terminal (Pre) and carboxy-terminal (E) precursor peptides. Arrowed numbers -48 in exon 1, -32 in exon 2 and -22 in the identical region indicate the location of different in-frame AUG codons. Codons -48, -32 and -22 is relative to the first codon for mature IGF-1 peptide.

Rat IGF-1 mRNAs encoding the 35-amino-acid Ea domain derive from the splicing together of exons 4 and 6 and exclusion of exon 5. Rat IGF-1 mRNAs encoding the 41-amino-acid Eb domain derive from splicing together exons 4, 5 and 6 ( Fig. 1.7). Actually, exon 5 is a cassette exon of 52 base pairs (bp), rather than a terminal exon (Roberts *et al.*, 1987).

Based on available rat IGF-1 cDNA sequences, it appears that in the liver, class 1 or class 2 5' sequences can each be associated with Ea- or Eb- type 3' coding sequences. Rat IGF-1 gene, therefore, encodes class 1-Ea, class 1-Eb, class 2-Ea and class 2-Eb mRNA (Fig. 1.7). Here it should be noted that the rat Eb peptide is a rat counterpart of human Ec peptide instead of human Eb peptide.

The precise structure of amino-terminal precursor was, and remains, uncertain. This is because the 5' ends of characterized rat IGF-1 cDNAs contain more than one in-frame methionine codon, each of which could serve as translation initiation codons. In rat there are two in-frame AUG codons that could specify amino-terminal precursors of ether 48 or 22 amino acids, depending on which codon is used for translation initiation. Human IGF-1 cDNAs similarly contain AUGs at codons -48 and -22, relative to the start of the IGF-1 coding sequence (fig. 1.8)

### 1.6.2. IGF-1 expression

IGF-1 expression has been demonstrated in various tissues from several mammalian species, including humans (Jansen *et al.*, 1983; Le Bouc *et al.*, 1986; Rotwein *et al.*, 1986; De Pagter-Holthuizen *et al.*, 1989), rat (Lund *et al.*, 1986; Beck *et al.*, 1987; Roberts *et al.*, 1987; Shimatsu & Rotwein, 1987a), mice (Beck *et al.*, 1987), pig (Tavakkol *et al.*, 1988; Weller *et al.*, 1994; Weller *et al.*, 1993), sheep (Le Bouc *et al.*, 1986; Dickson *et al.*, 1991; Saunders *et al.*, 1991; Pell *et al.*, 1993) and guinea pig (Bell *et al.*, 1990). The expression of IGF-1 in various tissues has led to the suggestion that IGF-1 was not simply endocrine hormone but that it also was

autocrine or paracrine hormone.

In human, multiple IGF-1 mRNA species that range from 7.6 to 1.0 bk have been identified in both fetal and adult tissues. The wide variety of size classes of transcripts may reflect the use of alternative 5' untranslated regions as well as variable polyadenylation sites at the 3' untranslated region. In adult liver, the major IGF-1 mRNA is 7.6 and 1.1 kb, although hybridizing bands at about 6.3, 3.7 and 1.7 kb have been observed (Rotwein, 1986; Rotwein *et al.*, 1987). In comparison to the adult, a lower level of IGF-1 expression is found in the fetal liver. The developmental pattern of IGF-1 gene expression in human liver follows the IGF-1 level in the circulation, thus supporting the concept that the liver is the major source of circulating IGF-1 and that the greater part of endocrine IGF-1 production begins after birth in humans.

In rats, multiple IGF-1 mRNA species of 7.5, 4.7, 1.7 and 1.2 kb have been identified in fetal and adult tissues (Lund *et al.*, 1986; Murphy *et al.*, 1987; Shimatsu & Rotwein, 1987a). The IGF-1 mRNA was more abundant in foetal than in adult tissues, with the exception of the liver where a dramatic increase in the adult was observed (Lund *et al.*, 1986; Hoyt *et al.*, 1988). Similarly, the liver is implicated as the major source of IGF-1 production and circulating plasma component in the adult rat. Both IGF-1 Ea and Eb mRNA are present in all species of transcripts (Lowe *et al.*, 1988; Shimatsu & Rotwein, 1987a). Both IGF-1 Eb and IGF-1Ea mRNA are regulated by GH, although a greater proportional increase in IGF-1 Eb mRNA was reported in the liver (Lowe *et al.*, 1988; Murphy *et al.*, 1987). Tissue-specific expression of the alternative 5' untranslated regions of the IGF-1 mRNA has also been observed in rats (Lowe *et al.*, 1988; Roberts *et al.*, 1987). Thus IGF-1 gene expression, involving the use of alternative 5' untranslated regions and the predominance of IGF-1 Ea or IGF-1 Eb mRNA, is tissue and developmentally specific.

It is very interesting to note that IGF-1Ea , but not IGF-1 Eb, was glycosylated after

*in vitro* translation in the presence of microsomal preparation (Bach *et al.,* 1990). This was consistent with E peptide region of precursor IGF-1 Ea, although the glycosylation at these sites remains to be demonstrated *in vivo.* It seems possible that this post-translational mechanism might effect either, or both, intracellular transferring and biological activity of IGF-1 Ea.

### 1.7. Regulation of the production of IGF-1

IGF-1 gene expression is influenced by hormonal, nutritional, tissue-specific and developmental factors.

#### 1.7.1. Hormone regulation

Among the hormones, the growth hormone was shown to be the most important factor controlling IGF-1 secretion although other hormones were also shown to be important.

**A. Growth hormone:** Growth hormone is a positive regulator of IGF-1 synthesis (Daughaday & Rotwein, 1989; Cohick & Clemmons, 1993). The induction of IGF-1 synthesis by GH is associated with an increase in IGF-1 mRNA level and IGF-1 gene transcription (Bichell *et al.,* 1992). The question of whether the two IGF-1 promoter elements are equally responsive to transcriptional activation by GH has not yet been resolved. Some experiments performed with rats and sheep (Pell *et al.,* 1993) have suggested that GH differentially stimulates transcription initiated at the P2 promoter. However, in hypophysectomized rats receiving a single injection of GH, the P1 and P2 promoters are activated equally (Bichell *et al.,* 1992). The inducement of IGF-1 mRNA by GH is very rapidly. For example, in the hypophysectomized rat there is virtually no detectable IGF-1 mRNA, as measured by Northern blot. Within 2 hours of GH injection, IGF-1 mRNA is easy to be seen, with peak levels being reached by about 4 hours and then declining (Rotwein *et al.,*1993).

**B. insulin:** IGF-1 gene transcription is low in streptozotocin diabetic rats and is restored to normal by treatment with insulin, suggesting that insulin is also a positive regulator of IGF-1 gene transcription (Pao *et al.*, 1993). Insulin also increases IGF-1 mRNA level in cultured hepatocytes, consistent with a direct positive effect of insulin on IGF-1 gene expression (Johnson *et al.*, 1989; Phillips *et al.*, 1991). Whether or not similar sites mediate the effects of GH and insulin on IGF-1 transcription remains to be determined.

In addition to the above growth hormone and insulin, there are other hormones which effect the expression of IGF-1 gene (Table 1.3).

**Table 1.3.**

| Hormones | Effects |
|---|---|
| Thyroxine | Decrease in hypothyroidism in proportion to the decrease in GH secretion |
| Prolactin | In the absence of GH, prolactin is a weak stimulus |
| Oestrogen | Physiologic oestrogen replacement causes an increase |

Recently the relationship between GH-IGF axis and cortisol was investigated in sheep fetuses (Li *et al.*, 1993; Li *et al.*, 1996). The results showed that cortisol suppressed IGF-II gene expression in the liver of the sheep fetus (Li *et al.*, 1993), but it is a physiological regulator of hepatic GH receptor and IGF-1 gene expression in fetal sheep during late gestation (Li *et al.*, 1996). When the effects of thyroid hormone and glucocorticoids (dexamethasone) upon the expression of the GH receptor and IGF-1 genes was studied (Brameld *et al.*, 1995), it was demonstrated that there was one possible mechanism for interactions of glucocorticoids and thyroid hormones with the GH-IGF axis. Clearly more work is needed to elucidate the relationship between GH-IGF axis and various hormones.

### 1.7.2. Nutrition regulation

Nutrition is an very important regulator of IGF-1 synthesis, and this regulation of IGF-1 may be a key control point for nutritional regulation of growth. In human children that are growth-arrested because of protein-energy malnutrition, IGF-1 levels are decreased, and the decreased IGF-1 may be causally related to the growth arrest (Soliman *et al.,* 1986). Similarly, in experimental models in which animals are fed diets containing inadequate amounts of protein or energy, reduced growth velocity is consistently associated with reduced level of plasma IGF-1 (Cohick & Clemmons, 1993; Soliman *et al.,* 1986; Straus & Takemoto, 1991). The lowering of IGF-1 caused by nutritional insufficiency is dominant to the positive effects of pituitary GH. For example, in humans with protein-energy malnutrition, level of IGF-1 are decreased even though the circulating level of GH is generally elevated (Soliman *et al.,* 1986). Furthermore, in protein-restricted experimental animals, injection of additional GH fails to restore circulating IGF-1 level to normal (Thissen *et al.,* 1991). This dominant role of nutrition in regulating IGF-1 level is consistent with the dominant role of nutrition in regulation of animal growth.

In the fasting rat, the decrease in somatic level of IGF-1 is accompanied by a decrease in the abundance of IGF-1 mRNA in liver and other tissues (Straus & Takemoto, 1990; Thissen *et al.,* 1991). The pattern of decrease of IGF-1 mRNA differs in different models of nutritional restriction. In energy-restricted animals, the three major size species of IGF-1 mRNA decrease in a coordinated manner (Straus & Takemoto, 1991). In contrast, in the protein-restricted animals there is a pronounced decrease in the large IGF-1 mRNA species and a lesser decrease in the smaller IGF-1 mRNA species (Soliman *et al.,* 1986; VandeHaar *et al.,* 1991; Miura *et al.,* 1992).

IGF-1 gene transcription exhibits a downward trend in fasting animals, suggesting that IGF-1 mRNA abundance may be regulated at least partly at the transcriptional level (Straus & Takemoto, 1990). Evidence also points to post-transcriptional regulation of IGF-1 level, for example, in protein-deprived rat GH normalizes the

abundance of IGF-1 mRNA but not plasma IGF-1 concentration (Thissen *et al.*, 1991).

### 1.7.3. Local regulation

A wide variety of cells and organs produce IGFs as local paracrine hormones. This was first suggested by Hall and Bozovic (1969). It was later demonstrated that multiple tissues and organs of adult (Hizuka *et al.*, 1986; Lund *et al.*, 1986) and fetal (D'Ercole *et al.*, 1980) released IGF-1 into the medium. Further studies have shown the expression of the IGF-1 gene as well as the presence of the peptides in most fetal and adult tissues, thus establishing the IGF-1 local biosynthesis and paracrine role. It now seems clear that trophic hormones, such as adrenocorticotropic hormone (ACTH), thyrotropin (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), can, in addition to their classic action on secondary hormone production, stimulate the paracrine biosynthesis of IGF-1 from their target organs. For example, in the gonads IGF-1 production from granulosa and Sertoli cells is stimulated by FSH and LH (Chatelain *et al.*, 1987; Hammond *et al.*, 1985; Maruo *et al.*, 1988; Tres *et al.*, 1986). The enhancement of local IGF-1 biosynthesis also occurs in response to local injury. For example, an elevation in IGF-1 immunoreactivity during regeneration after injury of peripheral nerves, skeletal muscle and endothelial cells of arteries (Hansson *et al.*, 1986; Hansson *et al.*, 1987; Jennische *et al.*, 1987).

### 1.7.4. Developmental regulation

IGF-1 expression in the rat begins well before the developmental onset of GH action. GH receptors do not appear in the liver until about 2 weeks after birth (Mathews *et al.*, 1986; Tiong & Herington, 1992). By contrast serum IGF-1 levels increase by nearly 20-fold during the first 14 days of life (Donovan *et al.*, 1991; Kikuchi *et al.*, 1992). It is thus likely that the early postnatal expression of IGF-1 is GH independent. The mechanism of the neonatal rise in serum IGF-1 was investigated by focusing on

IGF-1 gene expression in the liver. During the first 2 postnatal weeks of life, IGF-1 mRNA level rise progressively to reach values that were nearly 50% those seen in the adult (Daughaday & Rotwein, 1989). This increase contrasted with the disappearance of IGF-II mRNA during the same interval (Norstedt *et al.,* 1988; Kikuchi *et al.,* 1992). The mechanism of induction of IGF-1 mRNA was primarily transcriptional. Similarly, human IGF-1 expression pattern is well established (Hall *et al.,* 1988). The IGF-1 level are low prenatally and at birth but rise during childhood to high levels during puberty , after which they decline with increasing age (Hall *et al.,* 1980).

## 1.8. Aspect of muscle growth

Muscle, the major component of lean body tissue and the largest protein store in the body, is capable of undergoing rapid and extensive alterations in mass. Such alteration in the growth of this tissue can be produced in response to a variety of different stimuli, including altered nutritional and endocrine status as well as neural and mechanical influences.

Growth of muscle can occur in two ways: (1) by an increase in muscle cell number, (2) by an increase in muscle cell size. Both of two mechanisms are involved in muscle growth. Growth in cell number, however, is limited to the prenatal and immediately period, with the animals and human being born with or soon reaching their full complement of muscle cells. Postnatal muscle growth is frequently considered to be due primarily to muscle cell hypertrophy, as contrasted to prenatal muscle fibre hyperplasia (Allen *et al.,* 1979)

### 1.8.1. Muscle Developmental growth

With one or two exceptions, skeletal muscle is derived from lateral plate and paraxial somitic mesoderm. The main mass of muscle arises from the inner part of each myotome. As soon as the myoblast occurs it will differentiate into myotube, the latter develops to the small phase fibre and fibre number becomes fixed before birth or

shortly after birth. During the post-natal growth of the animal, the growth of muscle occurs only due to muscle fibre increase in size. Muscle fibre hypertrophy happens in two ways: (1) by increase in length and (2) by increase in circumference or girth.

**A. Growth in the length of myofibrils:** The limbs of most species of animals increase in length by several times during post-natal growth. The increase in limb length is accompanied by an increase in the fibres of individual muscles of the limb. It has been shown that increase in muscle fibre length is a consequence of an increase in the number of sarcomeres. Williams and Goldspink (1971) have shown that longitudinal growth in vertebrate muscle takes place by adding to the number of sarcomeres in series. Griffin et al (1971) demonstrated that the end of the muscle fibres are growth regions. Using such data, Goldspink (1983) demonstrated that the number of sarcomeres in the postnatal soleus muscle of the mouse increases from about 700 to 2200 in 0 to 20 weeks of age. Most of the increase actually occurred during the first 7 weeks of age, which coincides with the rapid growth phase of the mouse. Goldspink (1983) has also indicated that extra long sarcomeres are added to the ends of the myofibrils. Adaptation in sarcomere numbers for the growing animals is of significance physiologically since the degree of force that a muscle can generate is dependent on the degree of overlap by the thick and thin filaments. Thus, the optimum sarcomere length is that which allows the maximum amount of interaction between the myosin cross bridges and the actin filaments. Since the length of both the thick and thin filaments are fixed, the only mechanism by which the muscle fibre can adjust its sarcomere length is to regulate the number of end-to-end sarcomeres in the myofibrils. It seems probable then that each muscle can sense when its mechanical output decreases and add or remove sarcomeres to maintain the maximum functional overlap of the thick and thin filaments (Goldspink, 1983). Thus, the amount of tension generated appears to be responsible for controlling the number of in-series sarcomeres. But the signals including any putative growth factor which links the force or tension generated and addition of sarcomeres to the myofibrils remains to be elucidated.

**B. Growth in fibre diameter:** The physiological reason for the conversion of the small phase fibres into large phase fibres becomes apparent when the ultrastructure of fibres in these two phases of development is examined. Indeed the increase in girth of the fibre can be explained almost entirely by the increase in the number and size of the myofibrils, the number of the myofibrils within an individual muscle fibre of the mouse may increase during growth by more than 15 times (Goldspink, 1970). Evidence obtained by examining muscle fibres at different stages of growth strongly suggests that this proliferation of myofibrils is the result of longitudinal splitting of the existing myofibrils once they attain a certain size. It has been shown that muscle fibres increase in girth is in a discontinuous way (Alnaqeeb & Goldspink, 1987). In large muscles the fibres probably undergo several increase in size over a relatively long period of time before they attain their ultimate size. The signal transduction involved in this process is not known yet.

### 1.8.2. Muscle compensatory growth

Like most organs of the body, muscle changes in mass with changes in physiological demand; increased work leads to rapid hypertrophy, while decreased work causes atrophy. These adaptive responses are probably of important selective advantage to the organism and enable it to acquire new skill and to compensate for disease or injury (for example, cardiac hypertrophy). This hypertrophy is called compensatory growth.

In order to investigate the muscle compensatory growth, a simple and highly reproducible experimental system for inducing hypertrophy of skeletal muscle was required. Goldberg (1967) used a method in which the gastrocnemius portion of the achilles tendon was sectioned on one limb of rat, leaving the remaining plantaris and soleus muscle to support the body weight on that side and which undergoes rapid in compensatory growth. Using this method, twenty-four hours after operation increase in mass was already apparent and by the end of five days, it was essentially complete (as this is now believed to be induced by stretch). Another simple procedure inducing

rapid muscle hypertrophy was designed by Goldspink et al (1992) in which the tibialis anterior (TA) muscle of rabbit was stretched by immobilization of one limb in the extended position. As the result the TA muscle is induced to synthesize much new protein and to grow by as much as 30% within a period as short as 4 days. This very rapid hypertrophy was found to be associated with an increase of up to 250% in the RNA content of the muscle.

A number of studies were undertaken to clarify the relationship between muscle postnatal growth and muscle compensatory growth. It has long been known that postnatal growth requires the presence of pituitary growth hormone (GH). But the pituitary growth hormone is not essential for muscle compensatory growth, as the same results of muscle compensatory growth were obtained in the hypophysectomized rats (Goldberg, 1967). Also muscle compensatory growth process can occur in animals deprived of food and thus in negative nitrogen balance in a manner similar to that seen with normal animals (Goldberg & Goodman, 1969a). Together these findings suggest that compensatory hypertrophy clearly takes precedence over hormonal growth. The differences of requirements for different muscle growth are summarized in Table 1.4.

**Table 1.4**

| Muscle Developmental Growth | Muscle Compensatory Growth |
|---|---|
| Pituitary hormones (growth hormone, TSH etc.) | Occurs in hypophysectomized (non-growing) animals |
| Adequate diet | Occurs in food-deprived animals (despite general muscle wasting) |

Although both of these different processes affect the composition of the tissue in distinct ways (Goldberg & Goodman, 1969b), both involve increased protein and DNA synthesis in muscle.

In order to look at fibre changes in the muscle which undergoing rapid hypertrophy by increased work load, Rowe and Goldspink (1968) using essentially the same procedure found that the mouse soleus muscle which normally has a unimodal distribution of fibre sizes become bimodal. In this case the increased work load had not caused all the fibre to increase in size but caused a certain percentage of them to increase in cross-sectional area by 3 or 4 times. The response of the muscle to different work load poses the question as to what is the nature of the link or feedback mechanism between the mechanical event and the biochemical processes involved in the synthesis of more muscle proteins.

## 1.9. Skeletal muscle genes and their expression

Muscle is made up of cellular units called muscle fibres, which are 20-100 μm in diameter. The muscle fibre contain rod-like contractile structure, myofibrils, which are about 1 μm in diameter. These are made up of protein filaments arranged in units called sarcomeres. Each sarcomere consists of one set of thick (myosin) filaments and two sets of thin (actin) filaments.

### 1.9.1 Myosin and myosin heavy chain

The thick filament, myosin, is the most abundant protein within muscle (Goldspink, 1996), and the individual myosin molecule consists of two heavy chains, which are wound around each other except for their globular heads or S1 regions, and four light chains (Weeds & Lowey, 1971). There are different myosin heavy chain isoforms with different functional properties, some of which are preferentially present in fast skeletal muscle, some in slow skeletal muscle. The different myosin heavy chain isoforms are coded for by separate gene, termed as myosin heavy chain (MyHC) genes. In human skeletal muscle there are at least seven separate skeletal myosin heavy chain genes, including the embryonic, neonatal and adult fast and slow myosin genes, which are arranged in series on chromosome 17 (Leinwand *et al.*, 1983a; Leinwand *et al.*, 1983b). In addition to being expressed in different tissues and in

different cell types, different myosin heavy chain genes, including embryonic and neonatal isoforms are expressed at various stages during development (Butler-Browne & Whalen, 1984; Swynghedauw, 1986). To date a total of 14 distinct myosin heavy chain isoforms have been identified in mammalian skeletal muscle (Goldspink, 1996).

### 1.9.2. The muscle fibre phenotype

Skeletal muscle fibres can be divided into three main types. One is adapted for a high power output over a short period (fast, glycolytic or type IIb), the second one is adapted for a high power output over a longer period (fast, oxidative, glycolytic or type IIa) and the last one is the slow oxidative type I fibre that has a form of myosin which hydrolyses ATP only slowly, produces economically slow repetitive movements and sustains isometric force which is needed during standing, walking and running.

Muscle fibre types differ phenotypically in that they express different subsets of myofibrillar isoform genes with different specific ATPase activities as well as different types and level of metabolic enzymes (Goldspink, 1996). The inherent ability of skeletal muscle to adapt to mechanical signals is related to its ability to induce or repress the transcription of different isoform genes and to alter the general level of expression of different subsets of genes. The fact that there are several myosin heavy chain isoforms means that muscle fibres can change their contractile properties either during development or in response to mechanical activity by rebuilding their myofibrils by means of myosin heavy chains with the necessary slow or fast cross-bridge cycling rate (Goldspink, 1996).

### 1.9.3. Determination of muscle fibre phenotype

The expression of MyHC genes is tightly regulated in a developmental stage-specific and tissue-specific manner (Butler-Browne & Whalen, 1984; Mahdavi *et al.*, 1986;

Nguyen *et al.*, 1982; Weydert *et al.*, 1987; Weydert *et al.*, 1985; Whalen, 1985). Phenotypic expression of these genes is also influenced by thyroid hormone (Izumo *et al.*, 1986; Lompre *et al.*, 1984), altered patterns of innervation and physical activity including passive stretch (Russell & Dix, 1992; Dix & Eisenberg, 1990; Goldspink *et al.*, 1992). In the rabbit, chronic low-frequency stimulation induces pronounced fast-to-slow transitions in both myosin isoforms and fibre types (for reviews see Pette and Vrbova 1992; Pette 1992). Both stretch alone (Gupta and Zak 1992) and electrical stimulation alone (Pette and Dusterhoft 1992) caused some activation of the slow type and repression of the fast-type genes. However, a more complete switch in myosin heavy chain gene expression was achieved when these mechanical stimuli were combined (Goldspink *et al.*, 1991). This leads to the conclusion that muscle fibre adult phenotype is determined by stretch and force generation and that is controlled at the level of gene transcription (Goldspink, 1992). However, very little is known about the mechanotransduction mechanism involved and what growth factors or muscle regulatory factors may be involved that induce changes in transcription of specific genes in skeletal muscle.

## 1.10. Muscle growth, muscle fibre phenotype and IGF-1

Although IGF-1 was initially considered to be derived from the liver, both their expression and local production have since been demonstrated in numerous organs and tissues of many animal species. The functions of locally produced IGFs are largely unknown but are beginning to be clarified.

### 1.10.1. IGF-1 and post-natal muscle growth

IGF-1 mRNA is comparatively high in neonatal rat muscle (Adamo et al 1989), although this level decreases gradually during postnatal development. IGF-1 can be demonstrated by immunostaining in developing rat muscle during the first days post partum (Jennische and Olivecrona 1987). However, this expression decreases with increasing maturation of the muscle fibres. In human fetal muscle, IGF-1 mRNA is

considerably less abundant than IGF-II mRNA (Han *et al* 1988) and it can be demonstrated in the muscle fibres of human fetuses by immunostaining (Han *et al* 1988; Hill *et al* 1989). In adult rats there is low expression of IGFs in skeletal muscle. The IGF-1 content decreases after hypophysectomy and is restored after treatment with growth hormone (D'Ercole *et al* 1984; Murphy *et al* 1987 and Isgaard *et al* 1988 and 1989). However, in adult muscle fibres no immunostaining for IGF-1 was found (Jennische *et al* 1987).

### 1.10.2 IGF-1 and compensatory muscle hypertrophy

The compensatory hypertrophy of plantaris and soleus muscle of hypophysectomized and normal rat was induced by tenotomy of the synergistic gastrocnemius. The skeletal muscle hypertrophy was accompanied by marked increase in IGF-1 mRNA in skeletal muscle of both of normal and hypophysectomized rat. IGF-1 mRNA, quantified by a solution-hybridization, nuclease-protection assay, rose by nearly threefold on day 2 and remained elevated throughout the experimental period. A more dramatic response was seen in hypophysectomized rats, where IGF-1 mRNA levels rose by 8- to 13-fold. These results indicate that modulation of IGF-1 mRNA level can occur in the absence of growth hormone (Devol *et al* 1990).

Clearly a deal of further work will be required to determine exactly how IGF-1 influences accretion of muscle protein and whether different transcripts are produced in response to different stimuli thus channelling responses to production of the same IGF-1 protein. Other possible common mediators of muscle growth promoting stimuli may also be identified but at least in some cases IGF-1 would appear to play an important role.

### 1.10.3. IGF-1 and muscle fibre phenotype

By using one animal model overloading the rat soleus and plantaris muscles by tenotomy of the synergistic gastrocnemius, the increased growth in these muscles has

been associated with increased protein synthesis rates and alterations in contractile protein isoforms at both mRNA and protein level. Although the hypertrophy of these muscles has been shown associated with elevated IGF-1 mRNA levels in these muscles (D'Ercole *et al.,* 1984; Czerwinski *et al.,* 1994), very little is known about the relationship between the alteration of fibre phenotype and the expression of IGF-1 mRNA in skeletal muscle.

## 1.11. Aims of these studies

There is currently widespread interest in the IGFs and their roles in the regulation of growth and differentiation of an ever increasing number of tissues. It is also apparent that the regulation, biological actions and potential clinic use of IGF-1 in muscle is under active investigation. This selective review focused on the current state of our knowledge about the gene structure and action mechanism of mammalian IGF-1 and its roles on developmental, compensatory muscle growth as well as on expression of muscle genes. The overall purpose of the experiments presented in this dissertation was to determine exactly how IGF-1 influences accretion of muscle protein, whether different transcripts are produced in response to different stimuli and IGF-1 is involved in muscle fibre conversion in response to passive or negative stimuli.

# Chapter Two

# Regulation of muscle mass and IGF-1 mRNA by mechanical activity

## 2.1 Introduction

Muscle increases in size is response to increased physiological demand. Unlike developmental growth which needs pituitary growth hormone, this work-induced muscle hypertrophy, sometimes referred to as compensatory growth, is a type of growth hormone-independent hypertrophy (Goldberg, 1967). It has been shown that the stretch results in a rapid increase in the number of sarcomere in series (Williams & Goldspink, 1973) and increase in mass that is associated with an increase in the rate of protein synthesis (Goldspink *et al.*, 1986; Loughna *et al.*, 1986). An essential step for investigation of the biochemical events responsible for compensatory hypertrophy was the development of a simple and highly reproducible experimental system for inducing hypertrophy of skeletal muscles. A simple procedure inducing rapid muscle hypertrophy was designed by Goldspink and Williams (1971) in which the tibialis anterior (TA) muscle of rabbit was stretched by immobilization of one limb at extended position. As the result the TA muscle is induced to synthesize much new protein and to grow by as much as 30% within a period as short as 4 days.

The purpose of the first study was to investigate the regulation of muscle mass and expression of IGF-1 mRNA in response to immobilization of one limb at extended and short position.

## 2.2 Materials and methods

### 2.2.1 Animals and muscle stretch procedure

6 New Zealand white adult rabbits were used. The extensor digitorum longus (EDL), tibialis anterior (TA) muscles were subjected to acute stretch by immobilizing the left hind limb in the appropriate position using a plaster cast for 6 days. In this case the soleus muscle (SOL) was in the shortened position. The right hind leg was left in its rest position and served as the control.

### 2.2.2 Muscle sampling and preparation

**A. Extraction of tissues:** After 6 days casting, rabbits were sacrificed by intravenous injection of a large dose of pentobarbitone sodium into the ear vein. The EDL, TA and soleus muscles were dissected immediately from both hind legs. After dissection, each muscle was quickly weighed in cold conditions and cut transversely into three parts. One part was packed into 1.5 ml tube and directly frozen in liquid nitrogen and stored at -70 °C to await total RNA isolation. The second part was trimmed to a suitable size (about 1 $cm^3$) and mounted transversely on a piece of cork with embedding medium (Bright). The cork with the sample on it was then immediately frozen in isopentane suspended in liquid nitrogen to almost its frozen point. This allows the tissues to cool and pass through $0^0$ C faster than if dropped directly into liquid nitrogen and prevents the formation of water crystals which leads to cracking and fracturing of the subsequent sections. The tissues were wrapped with foil and stored in liquid nitrogen and later at -70$^0$C until cryosectioning. The last part of the muscle was fixed in freshly prepared 4% paraformaldehyde (PFA) fixative as described below in section B.

**B. Paraformaldehyde fixation and paraffin wax embedding of tissues:** The tissues were fixed in freshly prepared 4% PFA at 4$^0$C for 2 hours. After fixation was completed, the fixative was poured off and replaced with 50% ethanol. The 50% ethanol was immediately changed with fresh 50% ethanol and incubated for 20 minutes. The 50% ethanol was changed in same way for 3 times, each time the samples were incubated at room temperature for 20 minutes. The samples were dehydrated continuously by incubating three times in 70% ethanol, 20 minutes for each time, at room temperature. At this stage samples can be stored in 70% ethanol for a few days, if desired. The samples were continuously dehydrated through a graded series of ethanol dilutions by immersion for 2 hours in 80%, 90%, 90%, 100%, 100% ethanol, xylene, xylene, wax, wax in automaton. After dehydration was completed, the embedding mould were prepared and filled with molten paraffin wax using a hot glass pipette. The samples were immediately transferred to the wax-filled

mould using hot forceps. An embedding ring was placed on the mould and filled with paraffin wax and labelled to facilitate future identification of samples. The cast blocks were left at room temperature to allow the wax to become hard. After the wax hardened, the cast blocks were removed from embedding mould and stored in a dry place at room temperature until required.

### 2.2.3 Slide preparation and muscle sectioning

**A. Slide preparation** Glass slides were washed with 1% Decon for 1 hour and rinsed in distilled water for twice, 5 minutes for each before it was baked at 180 $^0$C overnight. The slides were then coated through the following solutions:

2% 3-aminopropyltriethoxy-silane (APTES) in acetone for 5 minutes;

Acetone for 2x5 minutes;

Diethyl pyrocarbonate (DEPC) treated water for 5 minutes;

The slides were then dried at 37-42 $^0$C for 2- 3 days and stored at room temperature until required.

**B. Sectioning of wax blocks:** The wax blocks were sectioned (10 μm) using a microtome ( American optical) at room temperature. The sections were floated on warmed DEPC-treated water (45 °C) to prevent creasing, mounted onto autoclaved slides coated with 2% 3-aminopropyltriethoxy-silane, and dried at 42 °C for 30 minutes. The slides containing sections were incubated at 37 °C for 2 days to firmly attach sections to subbed slides and stored in a slide box with desiccate at -20 °C until required (the sections can be stored for up to several weeks in this way).

**C. Cryosectioning:** The specimen stored in - 70 °C were transferred to the prechilled microtome ( Bright), mounted onto a chuck and orientated so as to give transverse sections. 10 μm sections were cut at -20 °C. The sections were thaw-mounted onto autoclaved slides coated with 2% 3-aminopropyltriethoxy-silane (SIGMA). Samples were allowed to dry for 30 minutes at room temperature before being fixed in 4% paraformaldehyde at 4 °C for 5 minutes , washed twice in 1 x PBS, and dehydrated

in an ascending ethanol series. The slides were dried and stored with desiccant at -70 °C in an airtight box until required (the slides can be stored in this way for up to several weeks). Before opening the box to use the slides, the box was brought to room temperature.

### 2.2.4 Measurement of total RNA in muscle tissues

Total cellular RNA was isolated from stretched and normal muscle using the single-step method with acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987). The concentration, and to some degree the quality, of RNA dissolved in water could be assessed by scanning spectrophotometry in a 1 cm path length Quartz cuvette. 50 µl of total RNA stock solution was taken from -70$^0$C and spun down in 14000 rpm at 4$^0$C for 30 minutes. The RNA pellets were washed in 50 µl cold (-20$^0$C) 70% ethanol for 3 times. Finally the RNA was recovered by resuspension in 500 µl diethyldicarbonate (DEPC) treated water and scanned at 260 nm using an UV/VIS kinetics spectrometer (UNICAM). The RNA concentration in solution was determined by its absorbency at 260 nm on the theory of an absorbency of 1 in a 1 cm path length cuvette at 260 nm is equivalent to 40 µg/ml RNA. The RNA concentration in tissues was calculated according to the RNA concentration in solution and the amount of tissues used for preparation of total RNA.

### 2.2.5 Expression of IGF-1 mRNA in hypertrophic muscle studied by Northern Blotting

**A. Synthesis of probes for Northern blot and *in situ* Hybridization:** The oligonucleotide 5' TTGGGCATGTCAGTGTGG 3' which complements to the sequences of exon 4 of IGF-I gene were used as the reverse primer to synthesize IGF-I cDNA by reverse transcriptase(RAV-2, Amersham). The cDNA was then amplified by PCR using two oligonucleotide primers (5' GCTTGCTCACCTTTACCAGC 3' and 5' TTGGGCATGTCAGTGTGG 3') . The

280 base pair PCR product covering exon 3 and part of exon 4 was subcloned into pBS+ phagemid vector (Stratagene) including $T_3$ and $T_7$ promoters from which the labelled sense and antisense cRNA probe was synthesized by *in vitro* transcription with RNA polymerase using digoxigenin labelled uridine-triphosphate as substrate (Boehringer Mannheim ). These probes were used for both Northern blotting and *in situ* hybridization.

**B. Northern Blotting:** Formaldehyde was used to achieve complete denaturation of RNA for electrophoretic separation and subsequent Northern transfer onto a membrane.

250 ml of diethylpyrocarbonate (DEPC)-treated autoclaved water and 30 ml of 10 x MOPS buffer were added to an RNase-free flask containing 3 g agarose. The agarose was dissolved in microwave oven. 1 µl ethidium bromide solution was introduced into agarose solution. In a fume hood, 15 ml 37% formaldehyde was introduced into the agarose solution after it cooling to 50 °C. The agarose solution was made to a final 300 ml volume with DEPC-treated water, mixed well and then poured into the an RNase-free 20 x 18 cm gel tray. The gel was allowed to sit for 1 hour before use. Prior to loading the gel, the sample wells of the gel were flushed by pipetting electrophoresis buffer in and out of the wells.

20 µg of total RNA stock solutions (the volume of solution depends on the concentration of total RNA in stock solutions) was added to an empty 1.5 ml tube and then spun down in 14000 rpm at 4 °C for 20 minutes. The RNA pellets were washed in 50 µl of cold 70% ethanol for 3 times and then dried under vacuum for 5 minutes, finally resuspended in 5 µl of diethylpyrocarbonate (DEPC)-autoclaved water.

The 25 µl of electrophoresis sample buffer (deionized formamid 50%; 1 x MOPS; formaldehyde 0.66 M; glycerol 6.7%; bromophenol blue 0.08%) was introduced into each sample. The samples were heated to 65 °C for 15 minutes and then put onto the

ice for denaturing the RNA before they were loaded. Finally the sample were loaded into the wells and then separated by electrophoresis at 30 volts (constant) at room temperature for 18 hours.

Following electrophoresis the gel was photographed on UV transilluminator. The gel was soaked for 2 x 20 minutes in 10 x SSC (made in DEPC-water) at room temperature with gentle shaking. The RNA was transferred to the membranes (Boehinger Mannheim) in 10 x SSC by capillary action using soft tissues to enhance capillary action. After the RNA was transferred completely, the membranes were baked for 2 hours at 80 $^0$C for fixing the RNA to the membranes and used directly for hybridization or stored dry between two filter paper in plastic at room temperature until required

Prehybridization (1 hour ) and hybridization ( 15 hours ) were carried out at 68 $^0$C in hybridization buffer [50% formamide; 5xSSC; 2% blocking reagent; 0.1% N-lauroylsarcosine; 0.02% SDS ]. Washing was carried out at high stringency 2x5 minutes at room temperature with 2xSSC and 0.1% SDS, 2x15 minutes at 68 $^0$C with 0.1xSSC and 0.1% SDS. The hybridized probe was detected by chemiluminescence according to the manufacturers instruction. The blotted filter was exposed to X-ray film for 6 hours.

### 2.2.6 Time course of IGF-1 mRNA changes within muscle following stretch

10 New Zealand white adult rabbits were used to investigate the time course of IGF-1 mRNA changes within muscle following stretch. The extensor digitorum longus (EDL) muscle was subjected to acute stretch in the same way as described on 2.2.1 (page 54). The animals were randomly assigned to 4 groups and examined at 8 hours, 3 to 4 days and 6 days after immobilization(two animals in each of 8 hours, 6 days and control groups; 4 animals in 3 to 4 days group), the control group was not immobilized. Muscle sample and total RNA was prepared as described on 2.2.2 (page 55) and 2.2.3 (page 57). The abundance of IGF-1 mRNA was determined through

the use of specific RNase protection assays. Briefly, 10 μg of total RNA was hybridized in solution (50 °C for 18 hours) using a $^{32}$P-labelled antisense probe containing part of exon 3 and part of exon 4 of the rabbit IGF-1 gene. The probe is 300 bases long and the protected fragments was 280 bases long. The hybrids were then treated with RNase ONE ™ (Promega) to remove single stranded ends resulting in 280 base double-stranded fragments. Samples were then denatured and electrophoresed on an acrylamide gel, dried and exposed to X-ray film (Kodak) for 24 hours (for detail see 5.2.5 on page 131). The intensity of each mRNA signal was quantitated using an LKB Ultroscan Scanning Densitometer.

### 2.2.7 Statistical analysis

Statistical significance of the differences between the passive stretched and normal muscle, as well as between the disused and normal muscle were analysed by the paired *t* test using a statistical software program (UNISTAT, Unistat Ltd.).

## 2.3 Optimization of *in situ* hybridization

The study of gene products using biochemical and molecular techniques often requires tissue samples containing a considerable amount of the target molecule. This presents a difficulty when the gene of interest is expressed in a minority of cells within a complex tissues. For this purpose *in situ* hybridization can be used so that the presence of a specific mRNA can be related to the cytology and the cell type in what it is expressed. This part was carried to optimize the condition for *in situ* hybridization.

The *in situ* hybridization technique was originally developed by Pardue and Gall (1969). At that time radioisotopes were the only labels available for nucleic acid, and autoradiography was the only means of detecting hybridized sequences. Furthermore, as molecular cloning was not possible in those days, *in situ* hybridization was restricted to those sequences that could be purified and isolated by conventional

biochemical methods. Molecular cloning of nucleic acid sequences and improved radio-labelling techniques have changed this picture dramatically. Some years ago, the application of chemically synthesized, radioactively labelled oligonucleotides, especially used for *in situ* mRNA detection, was reviewed (Coghlan *et al.,* 1985). In spite of the sensitivity and wide applicability of *in situ* hybridization techniques particularly for diagnostic procedures, its use has been limited. This is probably attributable to the problems associated with radioactive probes, such as the safety measures required, limited shelf life, extensive time required for autoradiography and cost. Clearly, the production of nucleic acid probes with a stable nonradioactive label removed some of the major obstacles. Digoxigenin labelling was developed (Heiles *et al.,* 1988) and expanded (Martin *et al.,* 1990; Muhlegger *et al.,* 1990; Holtke *et al.,* 1990; Kessler *et al.,* 1990; Holtke & Kessler, 1990) recently. Digoxigenin labelled cRNA probes were used in the following studies because of their stability and convenience of use.

Three areas of technical expertise are required for performing nucleic acid hybridization. First, preparation of a suitable nucleic acid probe demands an understanding of the principles of molecular biology (e.g. subcloning, plasmid preparation, *in vitro* transcription etc.). Successful tissue preparation requires practical experience in the art of histology. Finally, as with all morphological techniques, the correct interpretation of the experiment results requires familiarity with cell biology, anatomy, or embryology. In order to optimize the conditions of *in situ* hybridization on muscle tissue, a few critical parameters were established in the following manner.

### 2.3.1 Preparation of probe

The oligonucleotide 5' TTGGGCATGTCAGTGTGG 3' which complements to the sequences of exon 4 of IGF-I gene were used as the reverse primer to synthesize IGF-I cDNA by reverse transcriptase(RAV-2, Amersham). The cDNA was then amplified by PCR using two oligonucleotide primers (5'

GCTTGCTCACCTTTACCAGC 3' and 5' TTGGGCATGTCAGTGTGG 3'). The 280 base pair PCR product was subcloned into pBS+ phagemid vector (Stratagene) including $T_3$ and $T_7$ promoters and confirmed by DNA sequencing that it is covering part of exon 3 and part of exon 4 of the IGF-1 gene.

After linearization of the template DNA at a suitable site, the RNA polymerases were used to generate "run off" transcripts. Digoxigenin labelled uridine-triphosphate (Boehringer Mannheim) was used as substrate and incorporated into the transcript. The probe was then analyzed by 1.2% agarose-gel electrophoresis and ethidium bromide staining (see fig. 2.1). The amount of newly synthesized labelled RNA was approximated by comparison with the standard RNA and the labelling was checked by direct detection. When the cRNA probes were analyzed on agarose-gel a denaturing loading buffer (0.75 ml deionized formamide, 0.15 ml 10XMOPs, 0.24 ml formaldehyde, 0.1 ml deionized RNase-free water, 0.1 ml glycerol, 0.08 ml 10% bromophenol blue; freshly prepared prior to loading or store at -20 $^0$C in small aliquot) was used instead of a general DNA loading buffer.

### 2.3.2 Optimization of permeability

A challenge for successful *in situ* hybridization to mRNA is to preserve the morphology, and at the same time permeabilize the sample so that the target mRNA is accessible to the probe. The optimal fixative for *in situ* hybridization have been shown to be paraformaldehyde (Zeller *et al.*, 1987), although other fixatives have been used successfully. However, the permeabilization conditions need be determined for every cell and tissue and for muscle tissue these had not been optimized.

**A. Determination of the degree of proteolysis:** Unlike the hybridization of nucleic acid in solution, the RNAs in tissue is generally bound to proteins within the tissues. These proteins must be denatured and removed in order to make RNA more accessible to probe. The degree of proteolysis must be carefully balanced to permit adequate morphology while increasing signal. Four different concentration (5, 7, 10

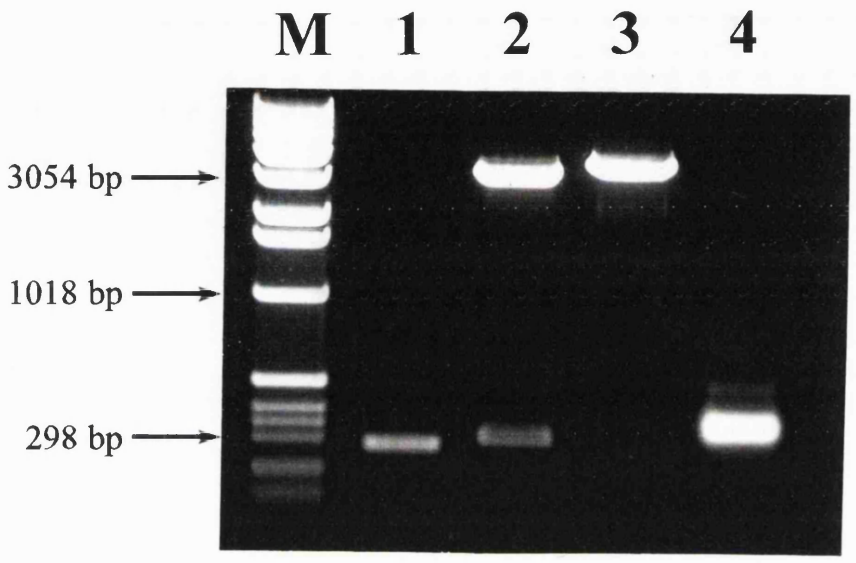



**Fig. 2.1.** Preparation of IGF-1 cRNA probe for Northern blotting and *in situ* hybridization. A oligonucleotide which is complementary to the sequence of exon 4 of the IGF-I gene was used as primer to synthesise cDNA of the IGF-1 mRNA by reverse transcriptase (RAV-2, Amersham). The cDNA was then amplified by PCR using two specific oligonucleotide primers for IGF-1 (lane 1, 280 bp PCR product). The PCR product was then subcloned into pBS+ phagemid vector (Stratagene) (lane 2), This PCR product was confirmed by DNA sequencing that it is covering part of exon 3 and part of exon 4 of the IGF-1 gene. After linearization of the template DNA at a suitable site (lane 3), the cRNA probe (lane 4) was synthesized by *in vitro* transcription of DNA with $T_3$ RNA polymerase using digoxigenin-labelled uridine-triphosphate as substrate. The yield of labelled RNA can be estimated from the ratio of DNA to RNA band. The lane M is a DNA marker lane (1 Kb ladder, Gibco BRL).

and 15 μg/ml in 50 mM Tris.HCl pH 7.5) of self-digested pronase combining three different length (10, 15, and 20 minutes) of digestion were tested. The 15 minutes digestion with 10 μg/ml of self-digested pronase at 37 °C gave the best morphology with the best signal.

**B. Ascertainment of necessity of the pre-hybridization step:** Pre-hybridization incubation in hybridization solution lacking probe is included by some workers in order to prevent background staining. This step, however, can substantially dilute the small volume of probe, leading to decreased signal. Some workers have stated the pre-hybridization step is not necessary (Wilkinson 1992). Comparison between including and excluding the pre-hybridization step was carried out and indeed the results showed that the pre-hybridization is not necessary for muscle sections.

### 2.3.3 Optimal conditions for hybridization:

**A. The melting temperature ($T_m$):** The melting temperature of hybrids is influenced by a number of factors. For RNA-RNA hybrids in standard hybridization buffer (containing 5XSSC and 50% formamide), the following formula was used to predict the melting temperature:

$T_m = 79.8+18.5 \log$ (molarity of monovalent cations) + 0.58 (%GC content of probe) +0.12 (%GC content)$^2$ - 820 / (length of probe in bases) - 0.35 (% formamide).

For RNA-RNA hybrids, the *in situ* hybridization reaction is usually completed at a temperature 30 ° C below the $T_m$, so the temperature for *in situ* hybridization should be $T_m - 30^0$ C. 45.3 $^0$ C was chosen as *in situ* hybridization based on the calculation of $T_m$ using 280 base IGF-1 cRNA probe.

**B. Pre-heating step:** Initially the lower signal is the main problem in our *in situ* hybridization result. This was mainly dues to the probe binding to tissues non-specifically within the sections during the procedure at room temperature. The non-

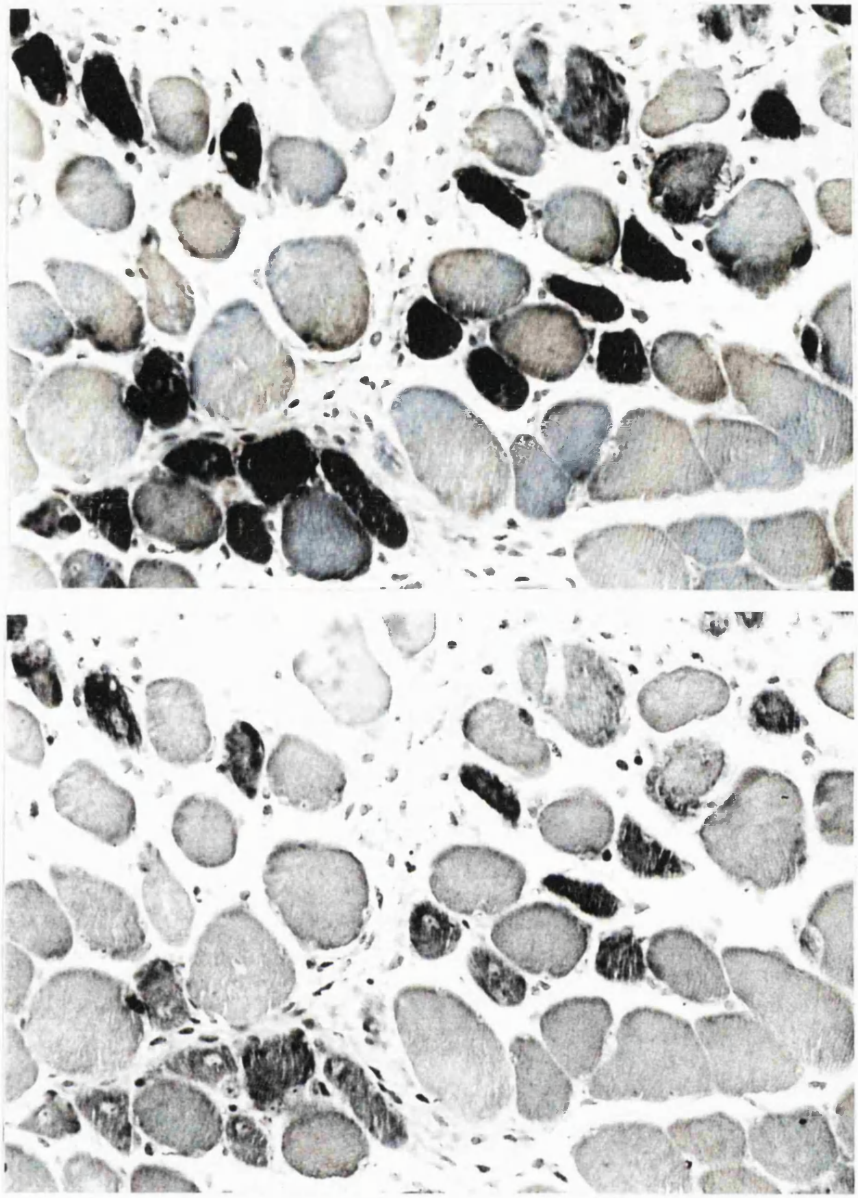


**Fig. 2.2.** The pre-heating step included (top row) in *in situ* hybridization give a much higher ratio of message signal to background than exclusion of the pre-heating step (bottom row). This step (the sections with probe in hybridization buffer were incubated at 68 °C for 1 hour before the sections were incubated at annealed temperature) increases the ratio of signal to background probably because it could help to unbind the non-specifically bonded probes, to denature the mRNA in sections and to make mRNA in muscle sections more accessible to hybridization and allowed the higher concentration of probes to hybridize to target mRNA specifically.

specifically bound probe was later digested by RNase resulting in lower signals. In order to resolve this problem, to denature the mRNA in sections and to make mRNA in muscle sections more accessible to hybridization and allowed the higher concentration of probes to hybridize to target mRNA specifically, a pre-heating (68 $^0$C) step ( the sections with probes in hybridization buffer were incubated at 68 °C for 1 hour between applying the probes on the sections and hybridization at annealed temperature) was tested. The result showed that preheating step can increase signal (fig. 2.2).

### 2.3.4 Standard procedure for *in situ* hybridization:

After empirical optimization of several critical parameters, the following standard *in situ* hybridization protocol was chosen for the following studies in this thesis.

**A. Standard protocol:**

| Step | Procedures and Commentary | Freq x Times |
|---|---|---|
| 1 | Move slides from -20 ° C to RT | |
| 2 | If **WAX** sections, preheat slides to 60 °C<br>This step is to soften wax. | 5 mins |
| | Dewax in Xylene at RT for 3 times | 3x2 mins |
| | Rehydrate in methanol 100% at RT<br>95%<br>70%<br>50% | 2x2 mins<br>2 mins<br>2 mins<br>2 mins |
| 2 | If **FROZEN** sections, fix in 4%PFA at 4 ° C | 20 mins |
| 3 | Denature in 0.2 N HCl at RT<br>This step denatured proteins and nick DNA , but also partially reversed the paraformaldehyde fixation step. | 20 mins |

| | | |
|---|---|---|
| 4 | Denature in 2 x SSC at 70 °C<br>This step denatured RNA and probably also remove some of the protein to make RNA in sections more accessible to hybridization. | 15 mins |
| 5 | Rinse in 1 x PBS at RT | 2 mins |
| 6 | Digest in 10 µg/ml pronase in 50 mM Tris-HCl at 37 °C<br>Optimized step, see 2.3.2. A | 15 mins |
| 7 | Stop digestion with 2 mg/ml glycine at RT | 30 sec. |
| 8 | Post-fix in fresh 4% PFA at 4 °C | 5 mins |
| 9 | Block fixation in 3 x PBS at RT | 5 mins |
| 10 | Rinse in 1 x PBS at RT | 2 x 30 sec. |
| 11 | Equilibrate in fresh 100 mM TEA buffer | 2 mins |
| 12 | Transfer to new TEA buffer, add acetic anhydride to a concentration of 0.25% while stirring and incubate<br>Add additional acetic anhydride to a final concentration of 0.5% while stirring and incubate<br>This step blocked polar and charged groups on sections, which will cause nonspecific sticking of probes.<br>NB: The acetic anhydride was added while solution was stirred and the sections were incubated because of the short half-time of acetic anhydride in solution. | 5 mins<br><br>5 mins |
| 13 | Block in 2 x SSC | 5 mins |
| 14 | Applied 50 µl of hybridization solution containing probes onto each section, covered with coverslip, placed in a humidified box and incubated at 68 °C. | 1 hour |
| 15 | Gradually cool to $T_m$ 45.3 °C and continue to incubate | overnight |
| 16 | Remove coverslip by shaking in 2 x SSC at RT | |

| | | |
|---|---|---|
| 17 | Wash in 2 x SSC at RT | 15 mins |
| 18 | Incubated with 100 µg/ml RNase in 2xSSC at RT<br>This step was only included while the cRNA probe was used as it removed the nonspecific bound cRNA probe | 30 mins |
| 19 | Wash in 2 x SSC at RT<br>Wash in 0.5 x SSC at 45 °C<br>Wash in 0.5 x SSC at RT | 15 mins<br>30 mins<br>30 mins |
| 20 | Equilibrate in wash buffer at RT | 1 mins |
| 21 | Incubate in buffer 2 at RT<br>This step block sections for sequential detection | 30 mins |
| 22 | Applied 100 µl diluted (1:500 in bluffer 2) anti-DIG antibody solution onto each slide and incubated in a humid chamber at RT | 1 hour |
| 23 | Wash slides in buffer 1 at RT<br>This step removed unbound antibody | 2 x 15 mins |
| 24 | Equilibrate in buffer 3 at RT | 2 mins |
| 25 | Apply 500 µl of colour solution on each slide, incubated in the absence of light, in a humid chamber at RT<br>NB. This step must be in the absence of light in order to avoid of high background | overnight |
| 26 | Wash in buffer 4 at RT | 30 mins |
| 27 | Remove excess fluid and mount in glycerin jelly | |

**B. Solutions**

RNA degradation by RNase within the sections is a major concern during the *in situ* hybridization procedure. This omnipresent enzyme (fingertips, dust etc.) is heat stable

and does not require co-factors for its enzymatic activity. This makes it difficulty to handle the samples. Thus, it is very important to make the following solutions which would be used in the per-treatment and hybridization steps RNase-free. Making solutions with diethylpolycarbonate (DEPC) treated distilled water or treating with the solutions (except of Tris solutions) by DEPC can avoid the problems of RNase contaminations.

**DEPC treated water:** Mix DEPC with redistilled water in a concentration of 0.1% and incubate at room temperature overnight, and then autoclaved.

**DEPC treated solutions:** Mix DEPC with the solutions in a concentration of 0.1% and incubate at room temperature overnight, and then autoclaved.

**Hybridization buffer:** 50% deionised formamide; 5xCCS; 5xdenhardt's solution; 250 μg/ml yeast t-RNA (sterile-filtered); 250 μg/ml denatured salmon sperm DNA; 4 mM ethylenediaminetetraacetic acid (EDTA). Hybridization buffer should be prepared freshly or per-prepared and stored -70 °C in aliquot. The hybridization solution included probe in a concentration of 500 ng/ml in hybridization buffer.

**Buffer 1:** 100 mM maleic acid; 150 mM sodium chloride; pH 7.5 (20 °C); Adjusted with concentrated NaOH and autoclaved.

**Buffer 2:** 1% Blocking reagent (Boehringer Mannhein) in buffer 1, autoclaved. Buffer 2 should be prepared freshly or per-prepared and stored at -20 °C in aliquot.

**Buffer 3:** 100 mM Tris-HCl; 100 mM NaCl; 50 mM $Mg_2Cl$; pH 9.5 (20 °C).

**Buffer 4:** 10 mM Tris-HCl; 1 mM EDTA; pH 8.0 (20 °C).

**Washing buffer:** 0.3% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20) in buffer 1.

**Colour solution:** 45 μl nitroblue tetrazolium salt (NBT) and 35 μl X-phosphate solution in 10 ml of buffer 3. The colour solution must be prepared immediately before use.

**Anti-DIG antibody:** The anti-DIG antibody (Boehringer Mannhein) is polyclonal sheep anti-digoxigenin Feb-fragments, conjugated with alkaline phosphatase, (750 U/ml)

## 2.4 Results

### 2.4.1 Changes of muscle weight

Changes in muscle wet weight in the fast-twitch extensor digitorum longus (EDL) and tibialis anterior (TA) muscle induced by passive stretch and in the slow-twitch soleus (SOL) muscle induced by immobilization in the shortened position (disuse) are shown in fig. 2.3. Six days of positively stretch resulted in an 19.3% increase in EDL wet mass (2.50±0.04 vs.2.99±0.13) and an 33% increase in TA wet mass ( 2.39± 0.11 vs. 3.19±0.20). These differences were statistically significant ($P<0.01$). However, Six days of disuse resulted in a significant ($P<0.001$) decrease in soleus wet mass (2.15±0.10 vs 1.44±0.11).

### 2.4.2 Concentration of tissue total RNA

The concentration of total RNA in muscle tissue was significantly elevated by passive stretch (757.2±43.8 vs. 1381.7±162.9, $P < 0.02$, EDL; 994.7±112.3 vs. 1698.9±150.6, $P < 0.01$, TA), (see fig. 2.4). Certainly, the increase of total RNA content in stretched muscle means that extra ribosomes are available to translate the message, whatever it may be, therefore, the rapid synthesis of more ribosomes seems to be the first step in producing muscle fibre hypertrophy. In contrast, the concentration of total RNA in muscle tissue was not significantly different between

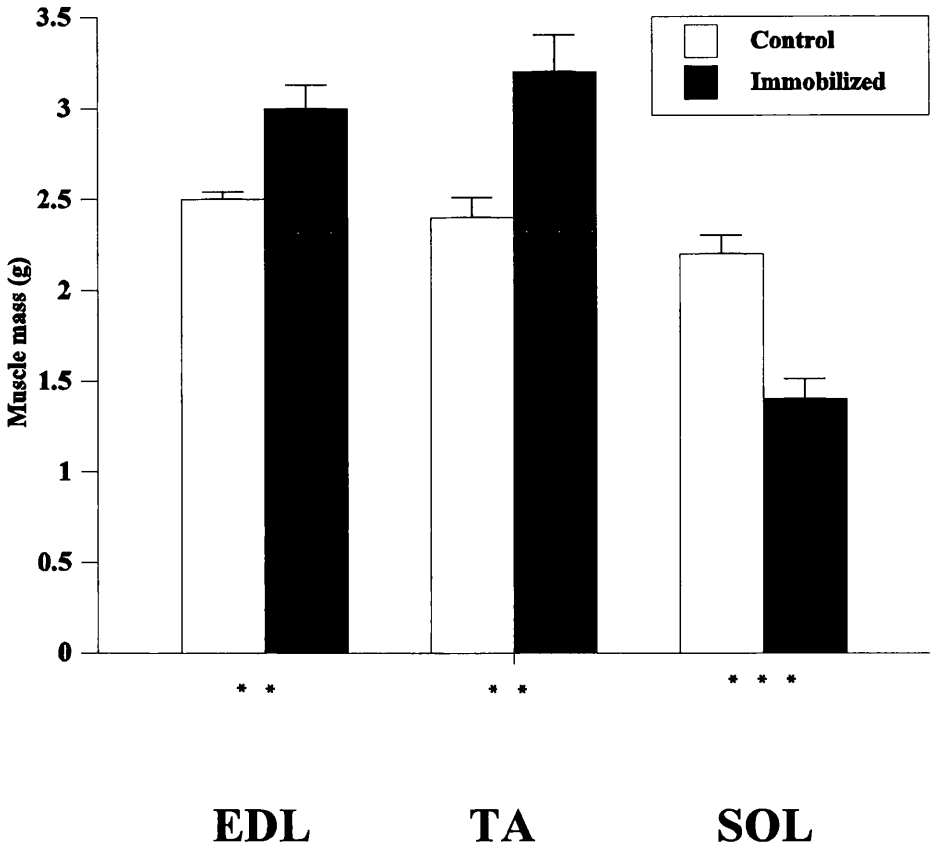



**Fig. 2.3.** Changes in wet weight of the extensor digitorum longus (EDL), tibialis anterior (TA) muscles in response to immobilization in their lengthened position and of the soleus (SOL) muscle in its shortened position for 6 days. The standard error is given for each mean and the level of the significance difference between the means is denoted below the experimental and control columns as ***P<0.001, **P<0.01; (n=6).

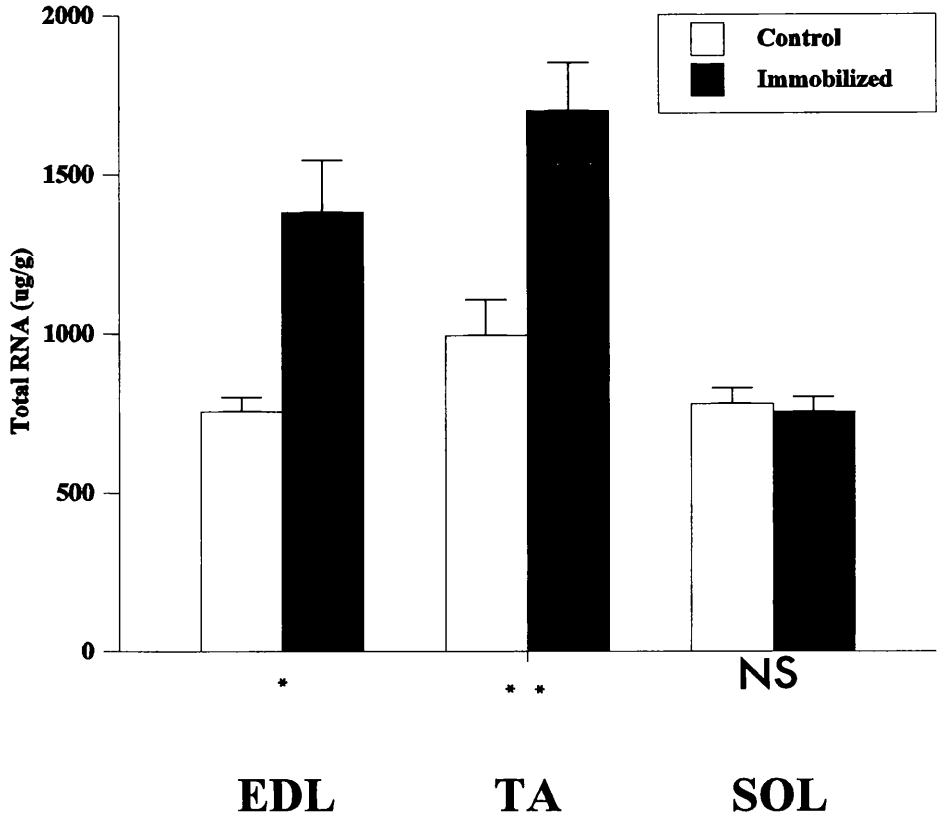



**Fig. 2.4.** Changes in concentration of total RNA in the extensor digitorum longus (EDL), tibialis anterior (TA) in response to immobilization in their lengthened position and of the soleus (SOL) muscle in its shortened position for 6 days. The standard error is given for each mean and the level of the significance difference between the means is denoted below the experimental and control columns as **P<0.01, * P <0.05, NS P >0.05; (n=6).

disused and normal soleus muscles (754.4±45.04 vs.779.8±48.9, P > 0.7), (see fig. 2.4).

### 2.4.3 Myotube formation in myotendinous region

After 6 days of stretch, many small myotube expressing embryonic and neonatal myosin heavy chain were present among mature fibres in the transverse sections from the end of stretched muscle ( see fig. 2.5). These myotubes usually have central nuclei and also react weakly with anti slow myosin heavy chain antibody. However, these myotubes were not present in the same region of the control muscle. It is also very interesting to note that high expression of the mRNA for IGF-1 within these myotubes with central nuclei ( see fig. 2.5).

### 2.4.4 Expression of IGF-1 mRNA studied by Northern blotting

The results of Northern blot analysis performed with RNA extracted from normal and stretched extensor digitorum longus (EDL) are depicted in fig. 2.6. The 280 bp IGF-1 antisense probe containing sequences derived from exon 3, and 4 of the IGF-1 gene hybridized with the two prominent IGF-1 mRNA species, 1.2 kb and 7.5 kb long. The expression of both types of mRNA species was greater in stretched muscle, although in some muscles the control muscle expressed more 7.5 kb mRNA than the stretched muscle.

### 2.4.5 Localization of IGF-1 mRNA in growing muscle

Expression of IGF-1 mRNA within normal and stretched muscle studied by *in situ* hybridization using antisense and sense RNA probe is shown in fig. 2.7. The *in situ* hybridization data demonstrates that the mRNA of IGF-1 is produced in response to stretch at the muscle fibre level as result of mechanical stimulation. This work showed that IGF-1 gene expression is not confined to the satellite cells but is up-regulated in the muscle fibres themselves. In transverse sections the IGF-1 message was localized

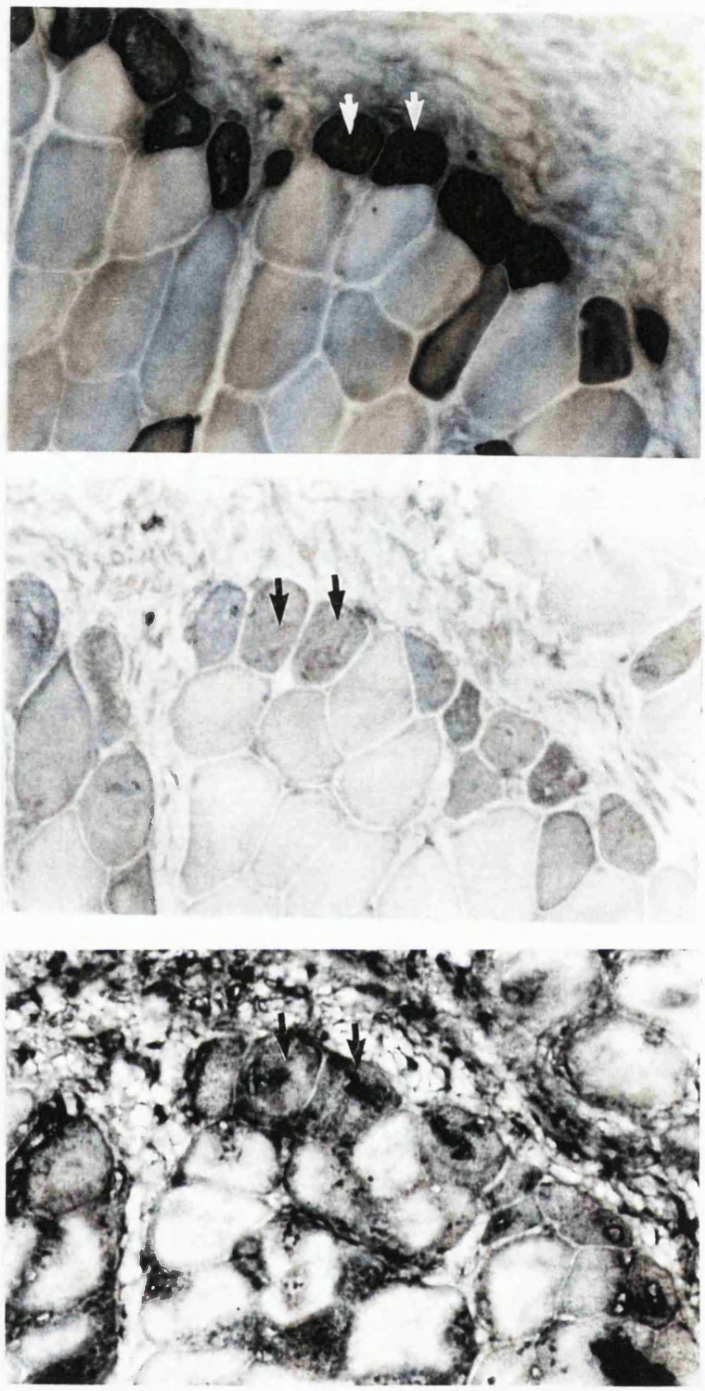


**Fig. 2.5.** Myotube formation in myotendinous region in response to stretch. After 6 days of stretch, many small myotubes (marked by arrow) expressing embryonic and neonatal myosin heavy chain (top row) were present in the transverse sections from the end of stretched muscle. These myotubes also reacted weakly with anti-slow myosin heavy chain antibody (middle row). It is also interesting to note that high expression of IGF-1 mRNA within these myotubes with central nuclei (bottom row).

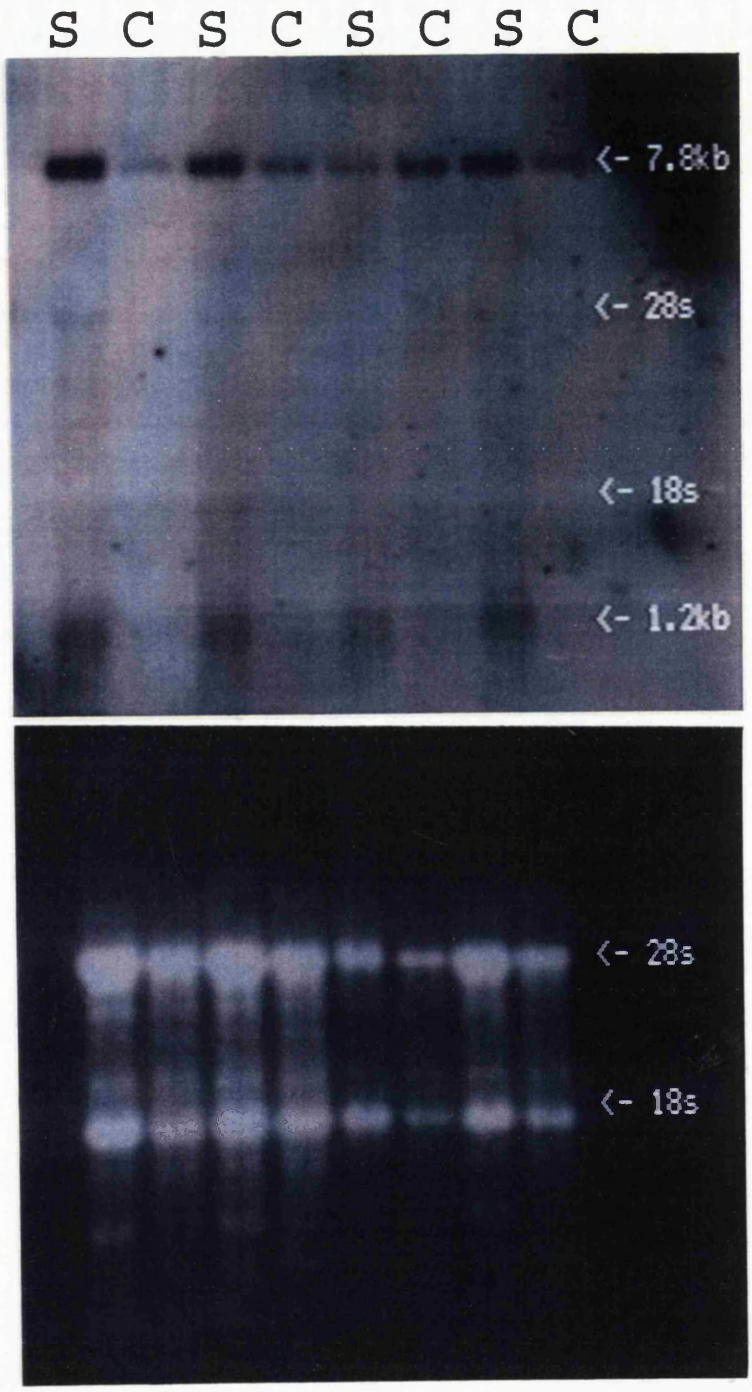



**Fig.2.6.** Expression of IGF-1 mRNA studied by Northern blotting (top row) in stretched (S) and control (C) extensor digitorum longus (EDL) muscle. 20 μg of total RNA was separated by electrophoresis on 1% agarose gel and then photographed on UV transilluminator with ethidium bromide staining (bottom row). After RNA was transferred to the membrane and probed by a DIG-labelled cRNA probe containing sequences derived from exons 3 and 4 of IGF-1 gene, two prominent IGF-1 mRNAs, 1.2 kb and 7.5 kb, were showed to be expressed in muscle and both of them were up-regulated by stretch.

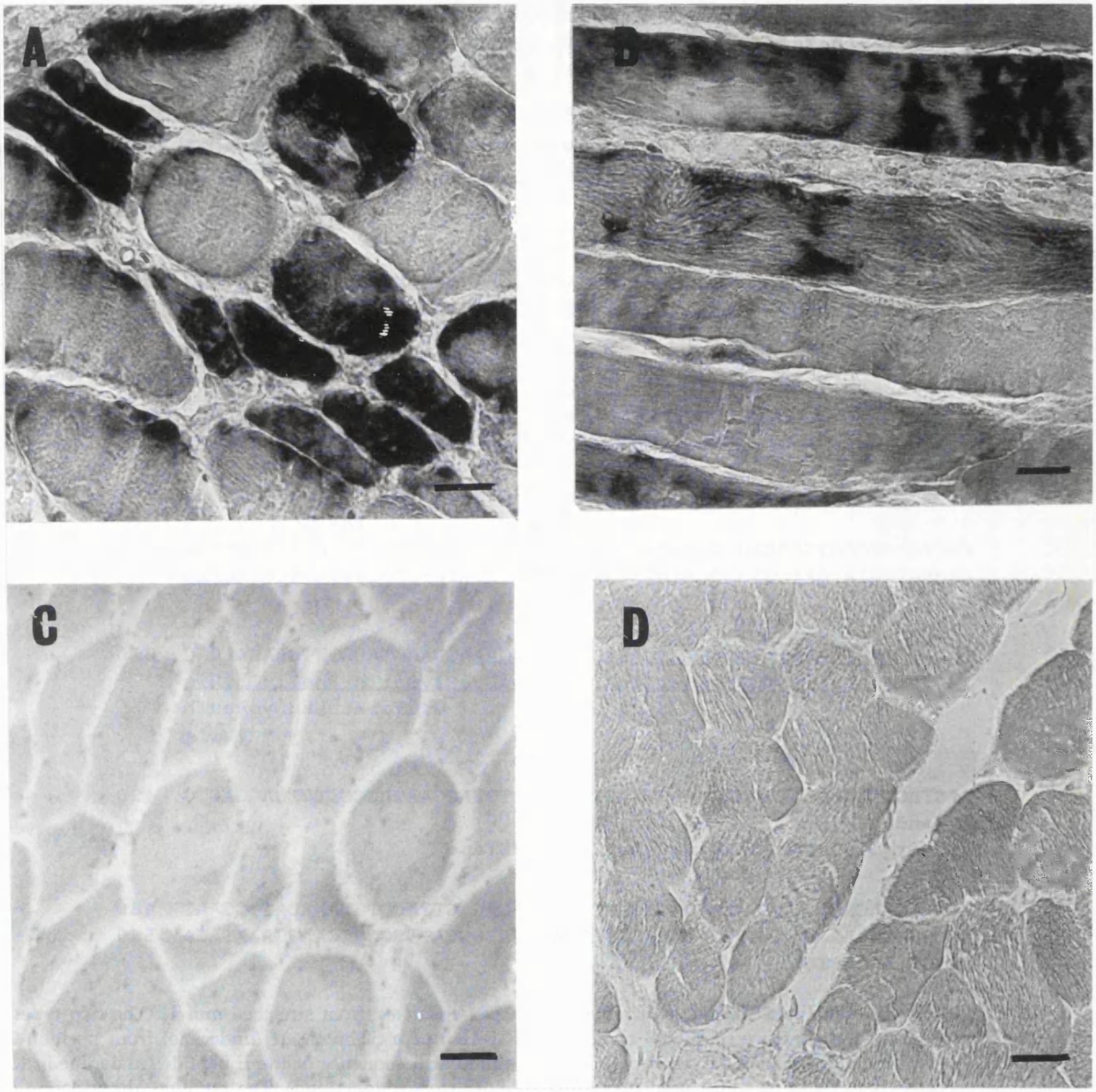



**Fig. 2.7.** Localization and distribution of IGF-1 mRNA in stretched (A, transverse section; B, longitudinal section) and control (C) extensor digitorum longus (EDL) muscle. The sense RNA probe from the same clone was used on the stretched muscle (D) as a negative control. Scale bar, 30 μm.

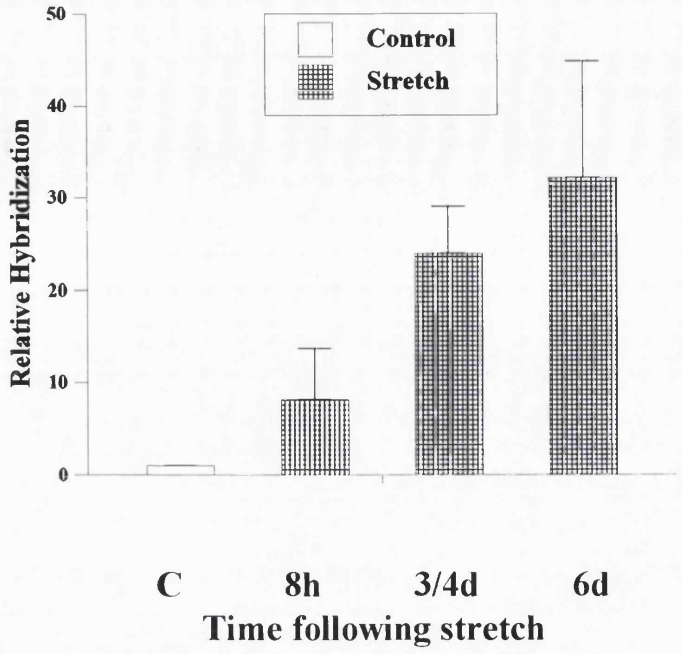



**Fig. 2.8.** Relative abundance of IGF-1 mRNA within hypertrophic EDL muscle after different times of stretch. Bar graphs depict the relative level of IGF-1 mRNA which is expressed relative to the control values for each muscle. Values are means ± SE. Time points are indicated on the abscissa. For control (C) n=2; 8 hours (8h) n=2; 3/4 days (3/4d) n=4; 6 days (6d) n=2.

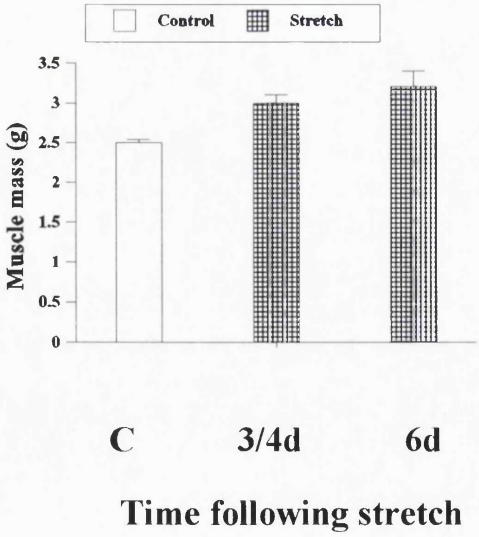



**Fig. 2.9.** Changes in wet weight of EDL muscle after different time course of stretch. Values are means ± SE. Time points are indicated on the abscissa. For control (C) n=12; 3/4 days (3/4d) n=6; 6 days (6d) n=6.

to large muscle fibre but tended to be expressed strongly in the small fibres which represent the tapered ends of fibres terminating in the muscle belly (Rosser *et al.*, 1995). In a few muscles some evidence of degeneration and regeneration was noted with high IGF-1 mRNA levels. These regions were superficial and indicated that in these cases the plaster cast was too tight. The *in situ* hybridization study however showed that with the use of the simple stretch model, the up-regulation of IGF-1 occurred in undamaged fibres.

### 2.4.6 Time course of IGF-1 expression within muscle after stretch

Relative abundance of IGF-1 mRNA within hypertrophic EDL muscle after different time course of stretch was shown in Fig. 2.8. After stretch for 8 hours IGF-1 mRNA levels were significantly higher than the unstretched control muscle. This increase of IGF-1 mRNA level caused by stretch was continued up to 6 days. The increase of IGF-1 mRNA was accompanies by increase of muscle mass shown in Fig. 2.9.

## 2.5 Discussion

### 2.5.1 Regulation of muscle mass in response to stretch

A work-induced hypertrophy model (Williams & Goldspink, 1971), in which EDL and TA muscle was immobilized in its lengthened position, was used to investigate IGF-1 gene expression during local growth and inactivity of skeletal muscle. Apparently significant increases in both muscle wet weight and RNA content were observed in EDL and TA muscles which underwent hypertrophy by passive stretch. In contrast, soleus muscle which was immobilized in the shortened position showed a significant decrease in wet mass, but not in RNA contents. This is due to the fact that immobilization alters the functional length of muscle and that adult muscle can adapt to an increase or decrease in the functional length by gaining or losing sarcomeres (Williams & Goldspink, 1973).

There are two main ways in which protein accumulates during muscle growth. One way is to increase the rate at which proteins are synthesized. The other is to decrease the rate at which they are degraded. It has been reported that passive stretch has little or no effect on protein degradation (Goldspink, 1976; Loughna *et al.*, 1986; Goldspink *et al.*, 1986). It is, therefore, clear that in this study the increase of muscle wet mass of both EDL and TA is due to increasing the rate of protein synthesis within muscle induced by passively stretch. Since over 80% of the total RNA in muscle is ribosomal, changes in protein synthesis per unit RNA are believed to imply a change in the ribosomal activity in the synthesis of new protein (Waterlow *et al.*, 1978). The increase of total RNA content in these two muscles passive stretched for six days, therefore, is consistent with the synthesis of new proteins.

The total RNA content is not significantly changed in inactive muscle and this would support the hypothesis that the decrease of soleus wet mass induced by immobilization in the shortened position is mainly due to increase of the rate at which the protein in muscle are broken down. This result is consistent with the report in which the atrophy of the soleus muscle induced by disuse using a suspension model was brought about by a small fall in the fractional rate of protein synthesis and a large increase in rate of protein degradation (Loughna *et al.*, 1986). Levels of IGF-1 mRNA, studied by *in situ* hybridization, (described in chapter 3) is not significantly difference between atrophic and normal soleus muscle indicating that IGF-1 seems not involved in muscle atrophy induced by inactivity. This means IGF-1 seems not to play role on protein degradation in skeletal muscle.

### 2.5.2 Fibre lengthening in response to stretch during longitudinal muscle growth

It has been demonstrated that during stretch-induced muscle growth sarcomeres are added in series at the ends of fibres (Williams & Goldspink, 1973; Goldspink, 1985). Protein synthesis is high at the ends of normal fibres, and this accelerates when fibres are lengthening in response to stretch (Goldspink *et al.*, 1986; Goldspink *et al.*, 1974;

Williams *et al.,* 1986). Myotube formation at the ends of muscles stretched for 6 days is another evidence of stretch activation at the myotendinous region. The explanation for this observation is that these myotubes are nascent fibres that will fuse with and become extensions of existing fibres. It has been demonstrated that chronically stretched muscles lengthen by the addition of nascent fibres derived from satellite cells to the ends of existing fibres, and these nascent fibres repeat the MyHC transitions observed during development (Williams & Goldspink, 1971; Moss & Leblond, 1971; Dix & Eisenberg, 1990).

The forces generated by muscle contraction are transmitted from the ends of muscle fibres to the collagenous tissue of tendon, periosteum and/or other muscle fibres (Trotter, 1993). The observation of the normally complex morphology of the myotendinous junction can be greatly altered by myopathies (Desaki, 1993; Law & Tidball, 1993; Law *et al.,* 1994) and ageing (Trotter *et al.,* 1987) suggested that the myotendinous region is a important site for force generation and muscle adaption in response to mechanical activity. The fact of up-regulation of IGF-1 mRNA within these myotubes at this region suggested that IGF-1 may play the role in this muscle adaption process.

### 2.5.3 Up-regulation of IGF-1 mRNA in stretched muscles

The estimation of the expression of the IGF-1 mRNA by Northern blotting suggests that both the 7.5 kb and 1.2 kb IGF-1 mRNA species are specifically induced by mechanical stimulation, but their increase seems to be independent of each other. The 1.2 kb mRNA was increased in all stretched muscle which was not always the case for the 7.5 kb mRNA. At this stage we do not know which is transcript for the IGF-1Eb. Further work is needed to characterise these mRNA and to determine what coding signal sequence and other elements they include. Time course of IGF-1 mRNA changes within muscle following stretch was investigated by use of specific RNase protection assays. Results showed that coincident with rapid increase of muscle mass, there is an increase in steady-state level of IGF-1 mRNA. This

suggested that IGF-1 gene was activated by stretch. The results presented here agree with those of Czerwinski et al (1994) who used a chicken model to stretch wing muscle and found IGF-1 mRNA level peaked at 11 days during stretch. In our study time course of expression of IGF-1 mRNA was examined only up to 6 days, clearly further studies are needed to investigate the effect of longer period of stretch on expression of IGF-1.

### 2.5.4 Localization of IGF-1 mRNA in hypertrophic muscle

The results presented here agree with published work (Beck *et al.*, 1987; Han *et al.*, 1987; Caroni & Schneider, 1994) that IGF-1 mRNA is expressed in muscle tissues. However, they also show that the IGF-1 gene is expressed in the muscle fibres themselves and not solely in satellite and connective tissue cells. The expression of the IGF-1 transcripts was not uniform and it is usually the smaller fibres that show high levels of IGF-1 mRNA. A study of transverse and longitudinal sections showed that the small fibre which expressed IGF-1 mRNA also express the neonatal myosin heavy chain (MyHC). It has also been shown that small diameter fibres containing neonatal MyHC are the tapered ends of the larger fibres terminating within the belly of the muscle (Rosser *et al.*, 1995). As mentioned previously, longitudinal growth of skeletal muscle is facilitated by the addition of new sarcomeres to the ends of the existing myofibrils (Williams & Goldspink, 1971; Williams & Goldspink, 1973) and the initial stage involves the laying down of neonatal myosin (Rosser *et al.*, 1995). These data support the hypothesis that the ends of normal adult fibres are the region for longitudinal growth and that IGF-1 is involved in this process.

### 2.5.5 Methodological aspects

The nonradioactive detection method of hybridized nucleic acid probes used in this study allows a resolution of the signal at the cellular level. It is, however, important to point out that more prolonged protease pretreatment of muscle sections or several denature steps are required to make mRNA in muscle sections more accessible to

hybridization. On the other hand, prolonged protease treatment reduced signal intensities.

Firstly we prolonged protease pretreatment of muscle sections and combined this protease treatment with HCl and heating denaturing steps. This give us much better signals than any single step own. This was probably due to the presence of myofibrils inside skeletal muscle fibres which need strong methods and longer to denature. Secondly, between application the probes on the sections and hybridization at annealed temperature we heated the sections with probes in hybridization buffer up to 68 ℃ for 1 hour. By using this step, the ratio of message signals in background is much higher than the no heating step. This probably because the heating step could help to unbind the non-specifically bonded probes, to denature the mRNA in sections and to make mRNA in muscle sections more accessible to hybridization and allowed the higher concentration of probes to hybridize to target mRNA specially. Thirdly, in the protease treatment step, pronase was used instead of protease K. This also give us a better result then protease K digestion. This probably because of pronase is less special than protease K which lyses more specific proteins.

When the paraffin and cryosection were compared, the paraffin sections have several advantages. Firstly, paraffin wax block is easier to get satisfactory sections with good morphology. Secondly, routine histology and *in situ* hybridization can be performed on serial sections. Thirdly, paraffin wax blocks can be stored indefinitely at room temperature.

## 2.6 Summary

In this study a stretch, hypertrophy model in which the EDL and TA muscle was immobilized in their lengthened position and the soleus in its shortened position, was used to investigate IGF-1 gene expression. The estimation of the expression of the IGF-1 mRNA by Northern blotting suggests that both the 7.5 kb and 1.2 kb IGF-1 mRNA species are specifically induced by mechanical stimulation, but their increase

seems to be independent of each other. The 1.2 kb mRNA was increased in all stretched muscle which was not always the case for the 7.5 kb mRNA. Time course of IGF-1 mRNA changes within muscle following stretch showed that coincident with rapid increase of muscle mass, there is an increase in steady-state level of IGF-1 mRNA. The expression of the IGF-1 transcripts was not uniform and it is usually the smaller fibres that show high levels of IGF-1 mRNA. It has also been shown that small diameter fibres containing neonatal MyHC are the tapered ends of the larger fibres terminating within the belly of the muscle. Longitudinal growth of skeletal muscle is facilitated by the addition of new sarcomeres to the ends of the existing myofibrils and the initial stage involves the laying down of neonatal myosin. These data support the hypothesis that the ends of normal adult fibres are the region for longitudinal growth and that IGF-1 is involved in this process.

# Chapter Three

# The expression of IGF-1 and conversion of fibre type in growth muscle in response to mechanical activity

## 3.1 Introduction

Skeletal muscles consist of populations of slow-contracting oxidative fibres that are adapted for slow repetitive or postural-type contractile activity and fast-contracting fibres that are recruited for fast phasic movements. These muscle fibre types differ phenotypically in that they express different subsets of myosin heavy chain (MyHC) genes. The isoforms of myosin heavy chains have been shown to be products of a multigene family, and their expression is tightly regulated in a developmental stage-specific and tissue-specific manner (Butler-Browne & Whalen, 1984; Mahdavi *et al.*, 1986; Nguyen *et al.*, 1982; Weydert *et al.*, 1987; Weydert *et al.*, 1985; Whalen, 1985). Phenotypic expression of these genes is also influenced by thyroid hormone (Izumo *et al.*, 1986; Lompre *et al.*, 1984), altered patterns of innervation, alterations in both energy intake and environmental temperature and physical activity including passive stretch (Russell & Dix, 1992; Dix & Eisenberg, 1990; Goldspink *et al.*, 1992).

A unique characteristic of skeletal muscle is its diversity. Different anatomical muscle and different fibres within those muscles express different subsets of genes and in this way become adapted to the kind of contractile activity they are required to perform. The extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of mammals, for example, are fast-twitch muscles which consist of predominant fast-contracting fibres that are recruited for fast, phasic movements. In contrast, the soleus muscle of mammals is slow-twitch muscle which consists of predominant slow-contracting oxidative fibres and is adapted for slow repetitive or postural-type contractile activity. The latter type of fibres are able to carry out these functions economically because of their predominant myosin crossbridges have a slow rate of ATP hydrolysis; the latter being replenished by oxidative pathways (Goldspink & R.Mc.Alexander, 1992)

.

Both of these two distinct muscle types are able to respond to altered demand by undergoing a series of physical and biochemical adaptation (Loughna *et al.*, 1986). For example, when subjected to stretch by plaster cast limb immobilization, the fast

contracting tibialis anterior muscle in the rabbit is induced to synthesize much new protein and to grow by as much as 30% within a period as short as 4 days (Goldspink *et al.*, 1992). Stretch has been shown to result in a rapid increase in the number of sarcomere in series (Williams & Goldspink, 1973) and increase in mass that is associated with an increase in the rate of protein synthesis (Goldspink *et al.*, 1986; Loughna *et al.*, 1986). It has been suggested that stretch or increased tension on a muscle is a major component contributing to muscle mass increase (Goldberg *et al.*, 1975; Goldspink, 1977). Recently it has been shown that stretch is more important than contractile force produced by electrical stimulation in inducing increased protein synthesis in muscle (Goldspink *et al.*, 1995).

Skeletal muscles also undergo rapid atrophy under conditions in which active stretch is reduced and contractile activity is significantly less than normal. Such atrophy occurs in humans during prolonged bed rest (Greenleaf & Kozlowski, 1982), plaster cast immobilization (MacDougall *et al.*, 1977) and space-flight (Waterlow *et al.*, 1978). The effect of inactivity on protein turnover in phasic and postural muscles has been investigated (Loughna *et al.*, 1986) and indicated that the muscle atrophy due to inactivity is mainly a result of elevated protein degradation. Initially this is preceded by down regulation of gene expression first at the level of translation and then transcription (Booth & Seider 1980).

As well as undergoing changes in mass, skeletal muscle is also a tissue that possesses an intrinsic ability to phenotypically adapt to the type of physical activity it is required to perform, i.e. remodelling by physical activity. For example, when subjected to stretch, skeletal muscle apparently adapts to a more postural type of role by expressing the slow and repressing the fast myosin genes as well as producing higher levels of mitochondrial genes (Goldspink *et al.*, 1992). However, very little is known about the mechanotransduction mechanism involved and what growth factors or muscle regulatory factors may be involved that induce changes in transcription of specific genes.

Increases of IGF-1 mRNA of muscle have been observed during work-induced compensatory hypertrophy (DeVol *et al.*, 1990) and passive stretch-induced muscle growth (Goldspink *et al.*, 1995). It has been suggested that increased secretion of IGF-1 during work-induced hypertrophy (DeVol *et al.*, 1990) may promote the accumulation of proteins in skeletal muscle cells by an autocrine mechanism. The level of IGF-1 release from skeletal muscle cells undergoing hypertrophy has been measured *in vitro* (Perrone *et al.*, 1995) but it was not known whether the muscle fibres themselves express the IGF-1 and in what form and whether IGF-1 is involved in muscle fibre conversion in response to passive stretch or disuse.

By combining *in situ* hybridization and immunohistochemistry procedures, the effects of passive stretch and disuse of muscle on the expression of IGF-1 mRNA at the individual muscle fibre level and the composition of fibre types in the muscle were studied. This approach has been used to identify in which fibre type the IGF-1 is expressed when muscle fibre undergoing phenotypic conversion and/ or hypertrophy in response to passive stretch. The data presented here indicates that IGF-1 is not only associated with muscle hypertrophy, but also with muscle fibre conversion as the individual fibres undergoing the conversion are the ones which express IGF-1.

## 3.2 Materials and Methods

### 3.2.1 Animals and stretch procedure

The rabbit was chosen for this study as fibre type conversion by acute stretch (Goldspink *et al.*, 1992) or chronic stimulation result in conversion of fast type II fibres into slow type I fibres. The IIX ( IID) fibres which are the predominant fast fibres in human muscle (Ennion *et al.*, 1995; Smerdu *et al.*, 1994) are also relatively abundant in the rabbit (Aigner *et al.*, 1993). The extensor digitorum longus (EDL), tibialis anterior (TA) muscles in 6 New Zealand white adult rabbits were subjected to stretch by immobilizing the left hind limb in the appropriate position using a plaster cast. In this case the soleus muscle (SOL) was in a disused condition. After 6 days

post casting, euthanasia was induced by intravenous injection of an overdose of sodium pentobarbitone into the marginal ear vein. The EDL, TA and SOL were immediately dissected out from both hind legs. The right hind leg served as the control. The validity of using the contralateral muscle was checked by taking the same anatomical muscles from non-experimental animals of about the same at size. After dissection each muscle was quickly weighed, put on ice and cut transversely into two parts. One part was packed into 1.5 ml tube and directly frozen in liquid nitrogen and stored at -70 $^0$C to await total RNA isolation. Another part was trimmed to a suitable size (about 1 $cm^3$ ) and mounted in transverse on a piece of cork with embedding media (Bright). The cork with the sample was then immediately plunged into isopentane prechilled to almost its freezing point by liquid nitrogen. The samples were then stored in the liquid nitrogen until they were cut on a cryostat. The samples were sectioned transversely in series at 10 micrometers (μm) on a cryostat at -20 $^0$C to -25 $^0$C and mounted onto autoclave slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma). The sections from stretched and control muscles of the same rabbit were mounted side by side on the same slide and were allowed to air dry in cryostat chamber for 30 minutes (to prevent endogenous RNase activity) before being stored at -70 $^0$C until further processing. Four adjacent serial sections were used for identifying the different muscle fibre types and testing the composition of fibre types using specific antibodies directed to different isoforms of myosin heavy chain by immunohistochemistry. One was used for testing IGF-1 mRNA expression using IGF-1 anti-sense RNA probe by *in situ* hybridization procedure.

### 3.2.2 Synthesis of probe for *in situ* hybridization

The IGF-1 cRNA probe was synthesised using the same method as the chapter 2 (2.3.1). The slow myosin heavy chain (MyHC) gene cDNA clone including the regions of ATP binding site was subcloned into the polylinker site of an appropriate transcription vector (Bluescript II KS, Stratagene) which contains a promoter for $T_7$ and $T_3$ RNA polymerase. The slow MyHC gene cRNA probe was synthesised *in vitro* by using digoxigenin labelled uridine-triphosphate (Boehringer Mannheim) as

substrate.

### 3.2.3 *In situ* hybridization

The standard protocol (2.3.4) was used in *in situ* hybridization. For detection of hybridized slow MyCH gene probe, anti-digoxigenin-fluorescein,(Fab fragments) was used instead of the anti-digoxigenin (Feb-fragments), conjugated with alkaline phosphatase (Boehringer Mannhein). The final signal of slow MyHC labelled with fluorescein was visualized under fluorescence microscopy.

### 3.2.4 Measurement of IGF-1 mRNA using *in situ* hybridization sections

A comparator projecting microscope was used to view stained muscle cross-sections. Intensity of staining (grey value) within *in situ* hybridization sections was measured using a Kontron KS 400 image analyzer with a Photometer Science cooled CCD camera. All measurements were made under a preset lamp setting.

### 3.2.5 Immunohistochemical procedure

The serial slices were used to demonstrate the presence of the distinct myosin heavy chain (MyHC) isoforms. The anti-slow MyHC monoclonal antibody were kindly donated and characterized by Dr. Sant'Ana Pereirra (1995). The anti-neonatal MyHC monoclonal antibody was purchased (Novocastra Laboratories Ltd) and is known to stain embryonic as well as the neonatal MyHC isoforms. The muscle sections were incubated with the primary antibody for 3 hours at room temperature and then incubated with horse anti-mouse IgG biotinylated antibody (Vector Laboratories) in HEPES buffer with 10 % horse serum for 2 hours at room temperature. The last incubation was followed by the incubation with Avidin-horseradish peroxidase (Avidin-HRP) for 1 hour at room temperature. The immunocomplex was visualized by incubating with 3.3'-diaminobenzidine (0.2 mg/ml), $H_2O_2$ (0.02%) in 50 mM Tris/HCl (pH 7.4).

### 3.2.6 Fibre counting and fibre composition analyses

A comparator projecting microscope was used to view stained muscle cross-sections. For the calculation of the number of type 1 and neonatal fibre type per $mm^2$ area in the sections, the cross-sections were projected using a projection microscope (Olympus Vanox-T) on a white board. The number of type I and neonatal fibres in a certain area of cross-sections of muscle appropriately stained was counted. A Kontron KS400 image analyzer with a photometer science cooled CCD camera was used to make positive fibre counts.

### 3.2.7 Identification of the fibres which produce IGF-1 mRNA

Identification of the fibres which produce IGF-1 mRNA was obtained by comparing the *in situ* hybridization stained sections with the different fibre type stained sections.

### 3.2.8 Statistical analysis

Statistical significance of the differences between the passive stretched and normal muscle, as well as between the disused and normal muscle were analysed by the paired *t* test using a statistical software program (UNISTAT, Unistat Ltd.).

## 3.3 Results

### 3.3.1 Expression of MyHC gene in muscle related to mechanical activity

*In situ* hybridization using an anti-sense cRNA probe for the slow (type 1) MyHC mRNA (fig. 3.1) on the stretched and control muscles showed that the message of this gene is localised around the periphery where the myonuclei are located while the sense cRNA probe was only bound to the myonuclei. It is apparent that in stretched muscle there are many more fibres expressing the slow MyHC gene and they appeared as a bundle while in the control muscle the fibre expressing the slow MyHC

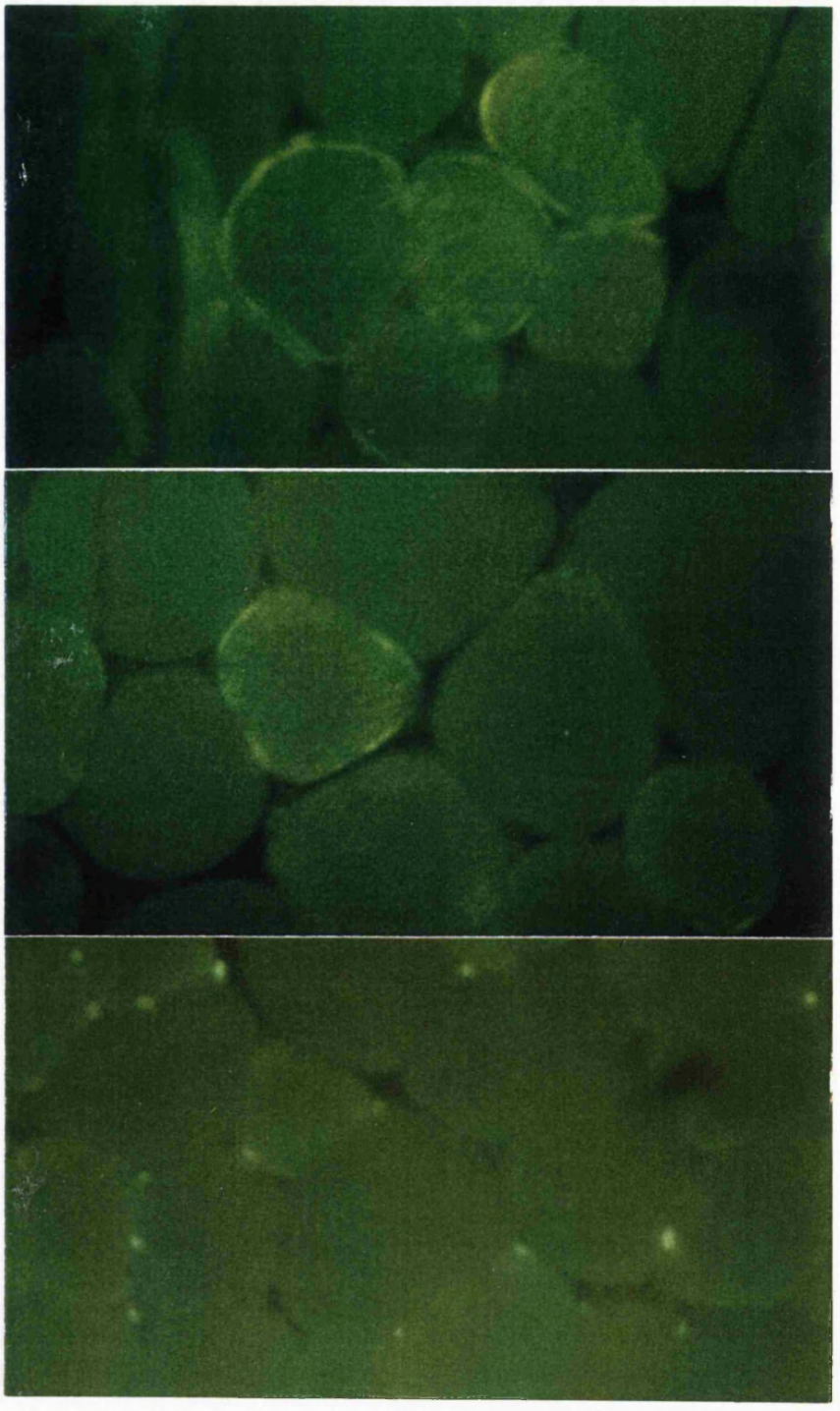


**Fig. 3.1.** *In situ* hybridization of anti-sense cRNA probe for the slow (type 1) myosin heavy chain (MyHC) mRNA in the stretched (top) and control extensor digitorum longus (EDL) muscle (middle) showed that the message of this gene is localised around the periphery where the myonuclei are located while the sense cRNA probe was only bound to the myonuclei (bottom). It is apparent that in stretched muscle there are many more fibres expressing the slow MyHC gene and they appeared as a bundle while in the control muscle the fibre expressing the slow MyHC gene appeared as a single individual fibre.

gene appeared as a single individual fibre. This result indicated that the slow myosin heavy chain gene was activated by passive stretch.

The expression of a gene can be controlled at multiple levels. These are usually categorized as pre-translational (including transcription, mRNA processing, and mRNA stability), translational and post-translational (protein processing, transport, assembly and degradation). The results present here suggest that an adaptive change of specific skeletal muscle proteins in response to an inherent change in contractile activity involves pre-translational control of gene expression, although it has also been shown that control of growth can take place at multiple levels (Booth & Kirby, 1991).

### 3.3.2 Fibre composition within both passively stretched and normal muscles

Myosin heavy chain (MyHC) isoforms in skeletal muscle follow a sequential transition from embryonic and neonatal to the adult isoforms, which in the rat is normally completed in the first few weeks of postnatal life (Whalen *et al.*, 1981; Mahdavi *et al.*, 1986). Figures 3.2 to 3.5 show the results of stretch on the number of fibres expressing the neonatal and the type 1 MyHC fibre type per unit area of cross-section. In the EDL muscle these were increased significantly ($45 \pm 6$/ mm$^2$ vs $65 \pm 5$ / mm$^2$, $P < 0.02$ in the case of neonatal MyHC; $23 \pm 6$ / mm$^2$ vs $37 \pm 6$ /mm$^2$, $P < 0.02$ in the case of type 1 MyHC). In the TA muscle the significant increase only occurred in neonatal MyHC fibre type ($40 \pm 26$ / mm$^2$ vs $87 \pm 7$ / mm$^2$, $P < 0.05$ ), whilst the increase in the number of type 1 fibres per cross-sectional area was not significant ($41 \pm 15$ / mm$^2$ vs $24 \pm 8$ / mm$^2$, $P > 0.05$). The sum of the above results indicates that stretch can increase translation of neonatal and embryonic myosin heavy chains in both EDL and TA muscles. However, stretch only increases translation of slow (type 1) myosin heavy chains in EDL muscle, but not in TA muscle. This may be due to the anatomical difference of these two muscles, and one muscle (e.g. EDL) was more activated then the another muscle (e.g. TA) by stretch. Nevertheless, these results indicated that the control of skeletal muscle gene

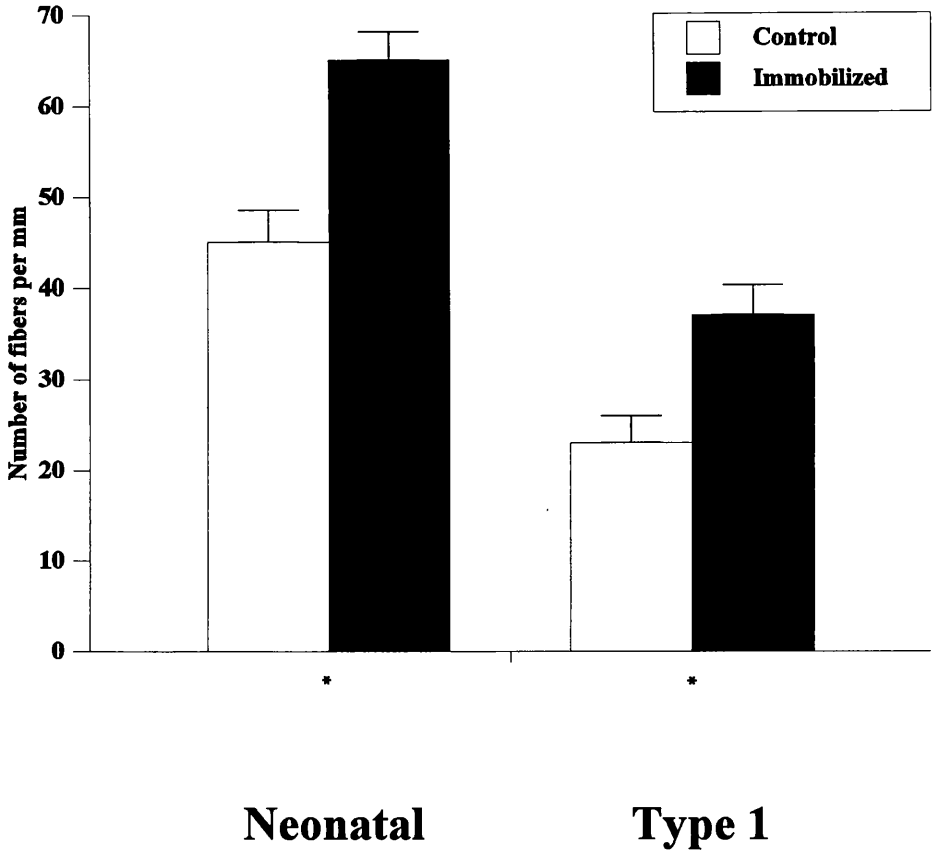



**Fig. 3.2.** Data showing the increase in the number of fibres expressing neonatal and type 1 MyHC per unit area in cross-section of the extensor digitorum longus (EDL) following 6 days of stretch. The standard error is given for each mean and the level of the significance difference between the means is denoted below the experimental and control columns as * $P < 0.05$; (n=6).

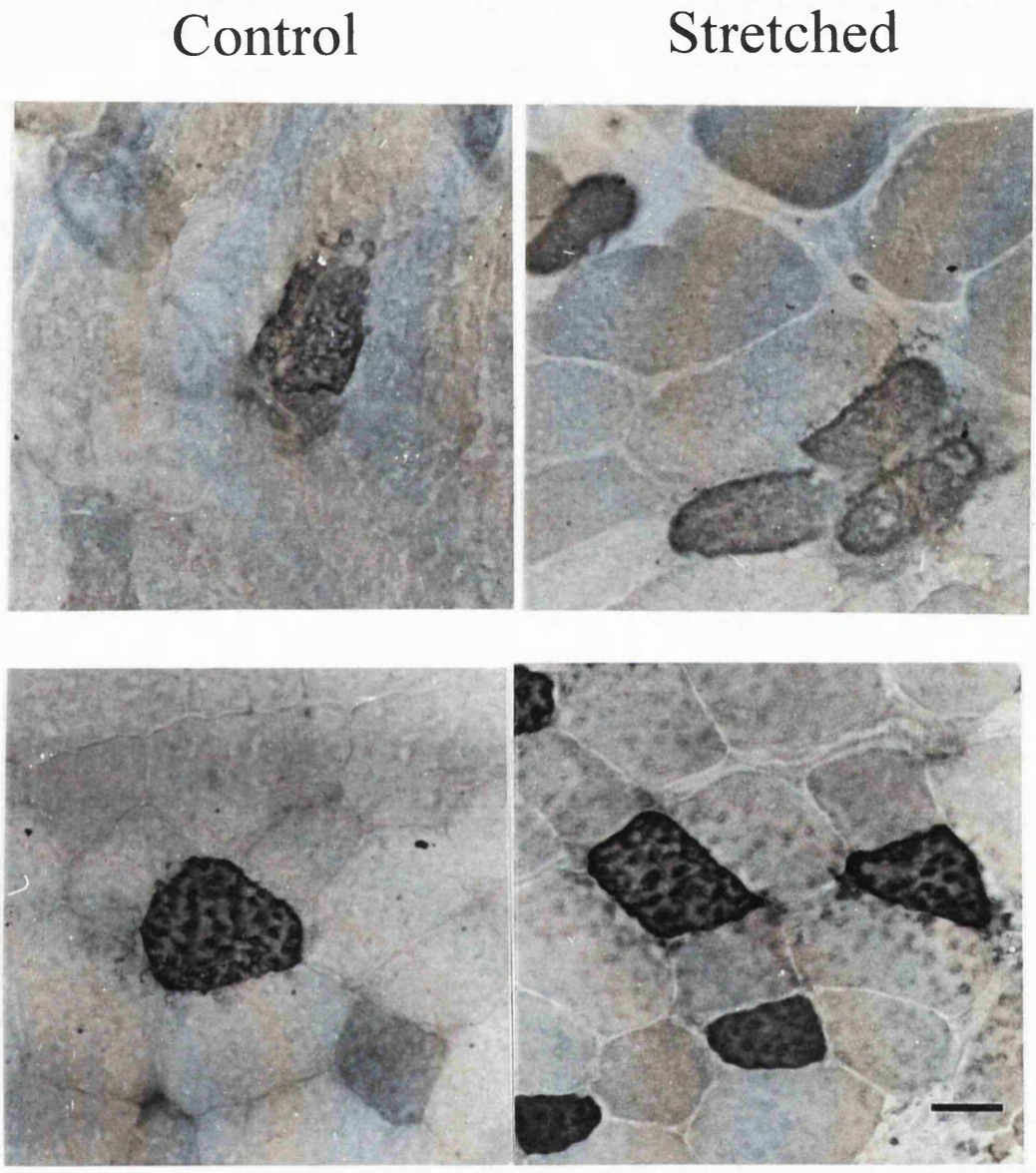



**Fig. 3.3.** Staining of transverse sections of stretched (right) and control (left) extensor digitorum longus (EDL) using monoclonal antibody to neonatal skeletal myosin heavy chains (top) and slow skeletal myosin heavy chains (bottom). After 6 days of stretch many more fibres are seen to be expressing neonatal and slow type myosin (dark staining). Scale bar, 30 μm.

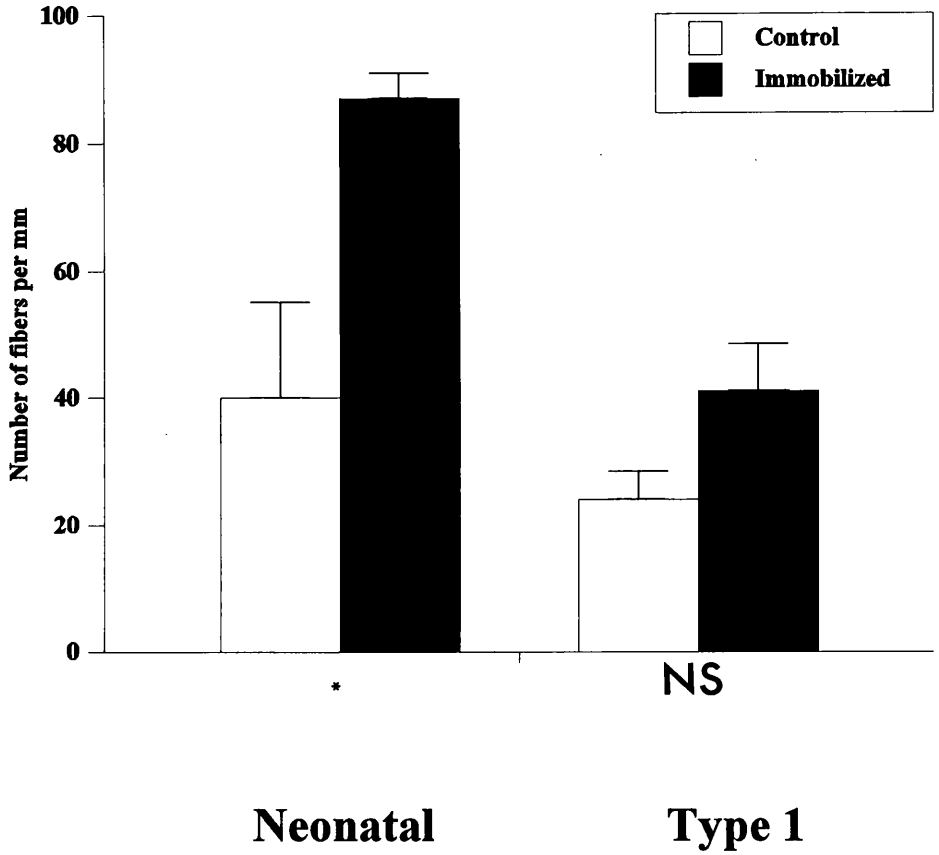



**Fig. 3.4.** Data which shows that after six days of stretch, the number of fibres expressing the neonatal MyHC per unit area in cross-section of tibialis anterior (TA) muscle increased significantly. The standard error is given for each mean and the level of the significance between the means is denoted below the experimental and control columns as * $P < 0.05$, NS $P > 0.05$; (n=6).

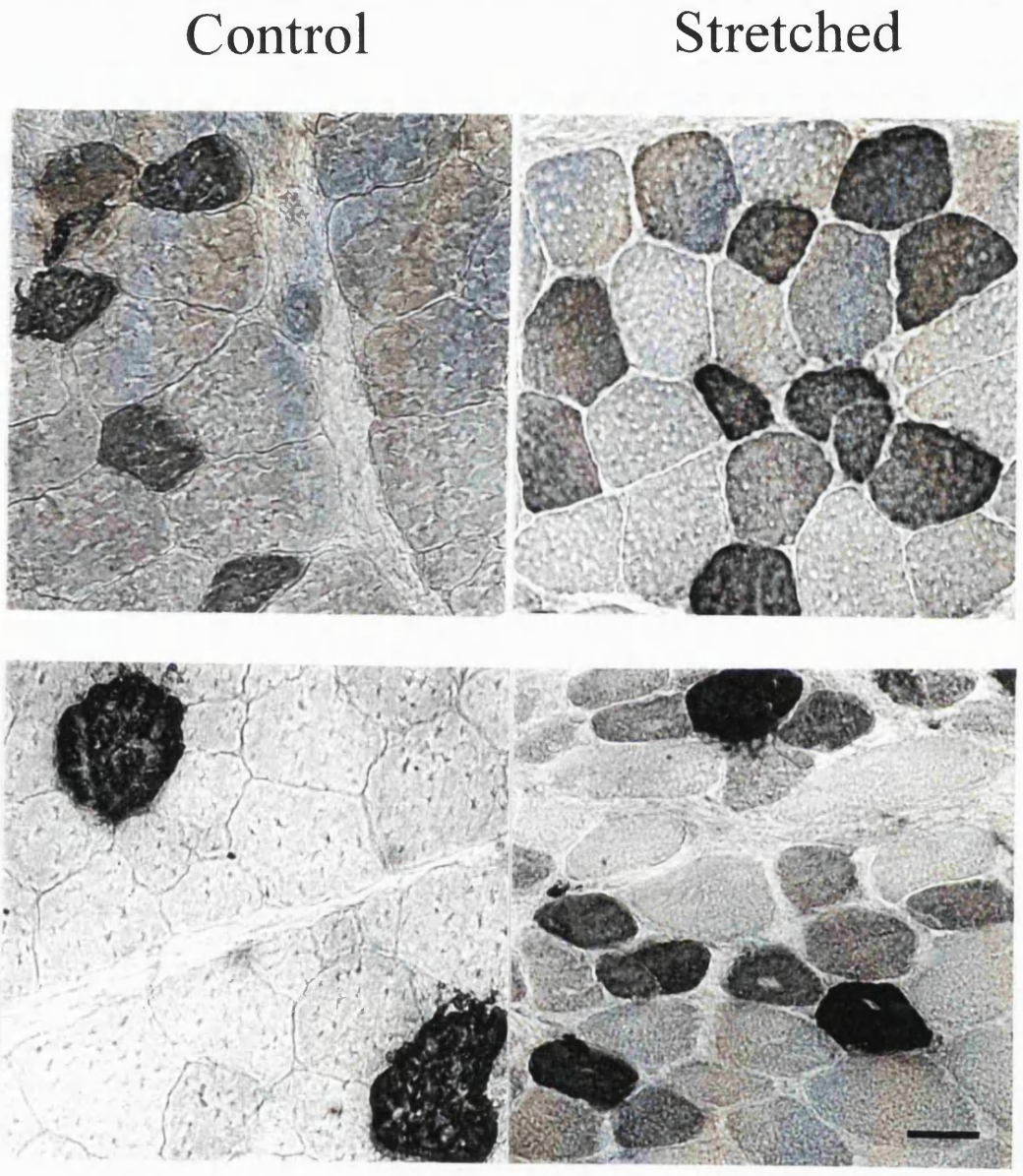



**Fig. 3.5.** Staining of transverse sections of stretched (right) and control (left) tibialis anterior (TA) using monoclonal antibody to neonatal skeletal myosin heavy chains (top row) and slow skeletal myosin heavy chains (bottom row). After 6 days of stretch many more fibres are seen to be expressing neonatal and slow type myosin (dark staining). Scale bar, 30 μm.

expression in response to alteration of mechanical activity also involves the translation level.

### 3.3.3 Level of IGF-1 mRNA in both stretched, disused and normal muscle

The level of IGF-1 mRNA in passive stretched EDL and TA muscles studied by *in situ* hybridization was up to 22 times higher than that in the unstretched muscle. However, the level of IGF-1 mRNA was not changed in the soleus muscle immobilized in the shortened position ($P > 0.8$), (fig. 3.6 and 3.7). This means that although increased muscle activity results in a change of IGF-1 mRNA expression, decreased muscle activity has no effect on IGF-1 mRNA expression. The distribution of IGF-1 mRNA in different regions of stretched EDL was investigated. The results (Fig. 3.8) showed that there was a significantly higher percentage of fibres that express IGF-1 mRNA in myotendinous region than in the middle region of muscle. This is another evidence of stretch activation at the myotendinous region and confirmed the finding in chapter 2 which showed additional sarcomere formation at the myotendinous region.

### 3.3.4 Identification of the fibres which produce IGF-1 mRNA

Serial sections of the muscles were used for *in situ* hybridization and immunohistochemical staining in order to correlate IGF-1 positive fibres to MyHC phenotype. It was seen that by six days of stretch, the fibres producing the IGF-1 mRNA were those that stained strongly for neonatal myosin or those that were hybrid fibres expressing neonatal and type 1 myosin. It is interesting to note that in these hybrid fibres, the greater intensity of type 1 staining was found within the larger diameter fibres (Fig. 3.9). This may indicate that both adaptive responses, i.e. changes in fibre phenotype and hypertrophy, were more complete in these particular fibres.

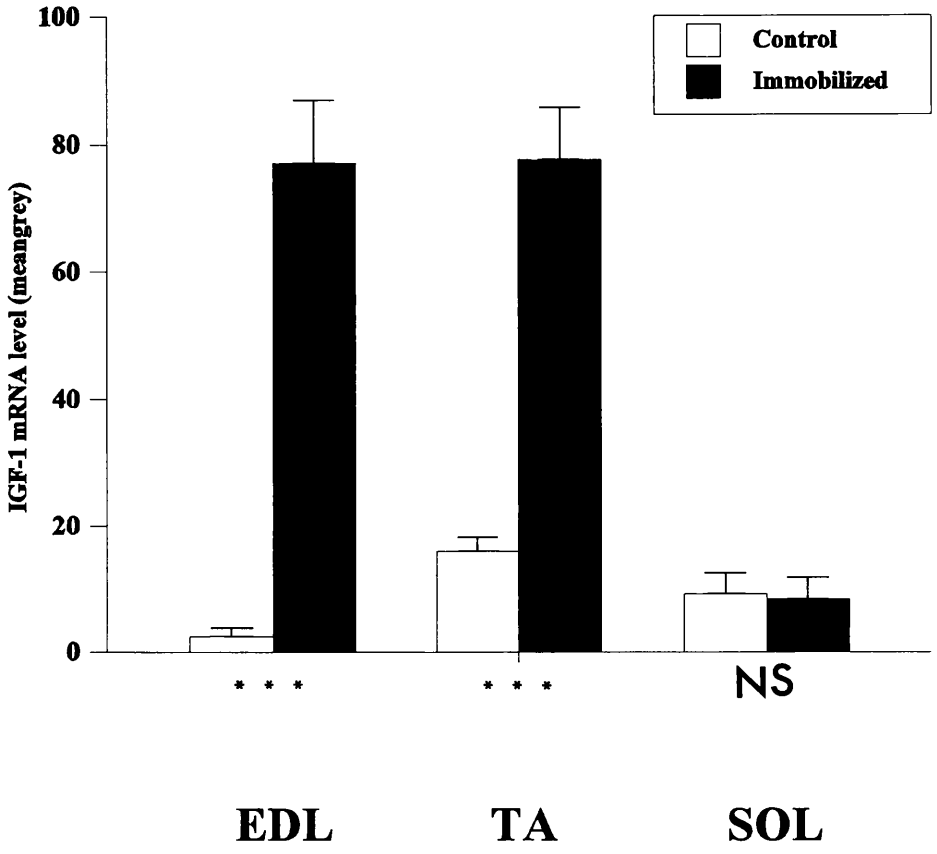



**Fig. 3.6.** Changes in level of IGF-1 mRNA, studied by *in situ* hybridization, in the extensor digitorum longus (EDL), tibialis anterior (TA) muscles after six days of stretch, in the soleus (SOL) after six days of disuse. The standard error is given for each mean and the level of the significance difference between the means is denoted below the experimental and control columns as *** $P < 0.001$, NS $P > 0.05$; (n=6).

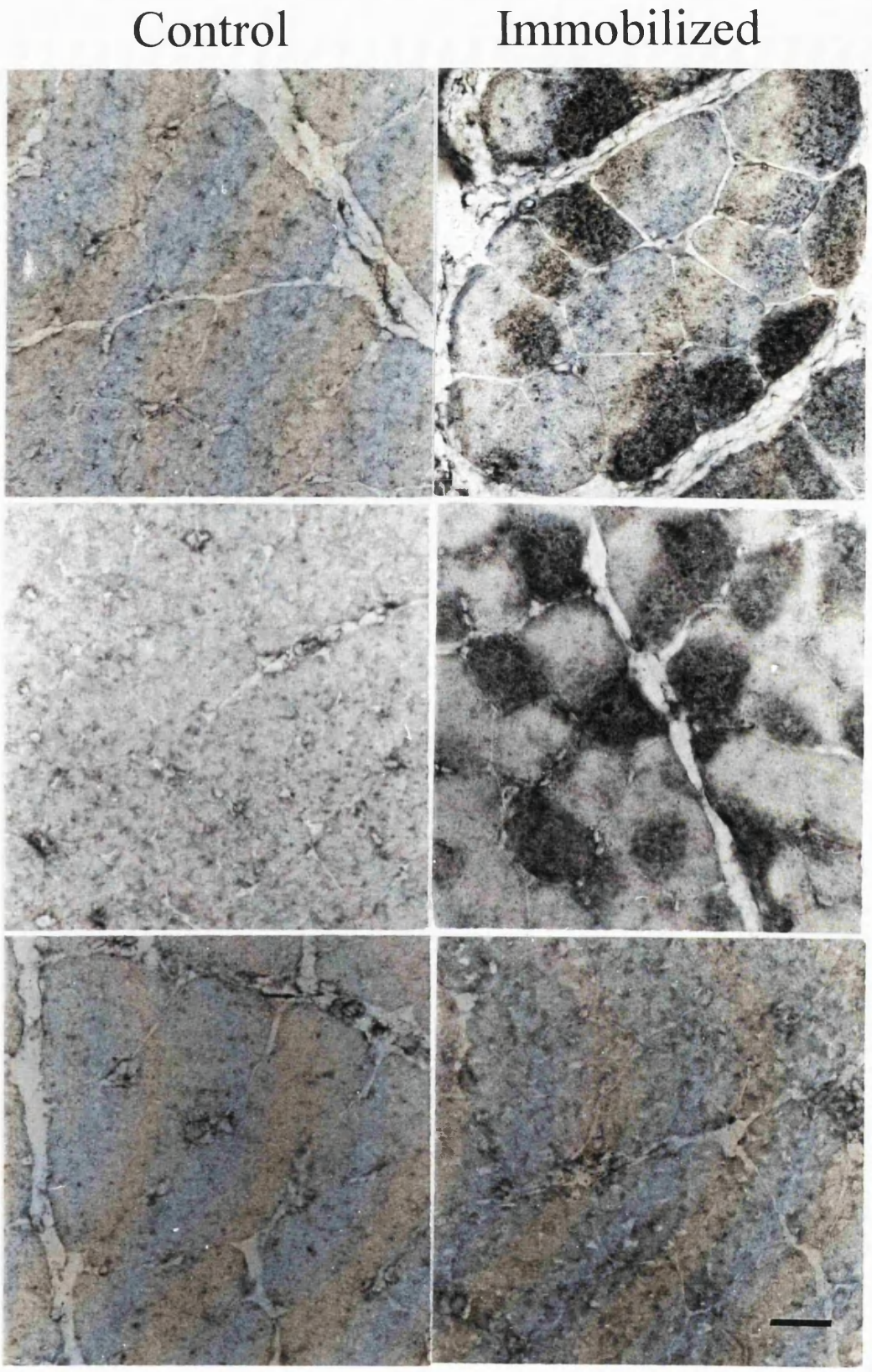



**Fig. 3.7.** A photomicrograph of a transverse section showing expression and localization of IGF-1 mRNA by *in situ* hybridization in the immobilized (right) and control (left) extensor digitorum longus (top row), tibialis anterior (middle row) and soleus muscles (bottom row). EDL and T.A muscles were immobilized in their lengthened position while soleus muscle in its shortened position. Scale bar, 30 μm.

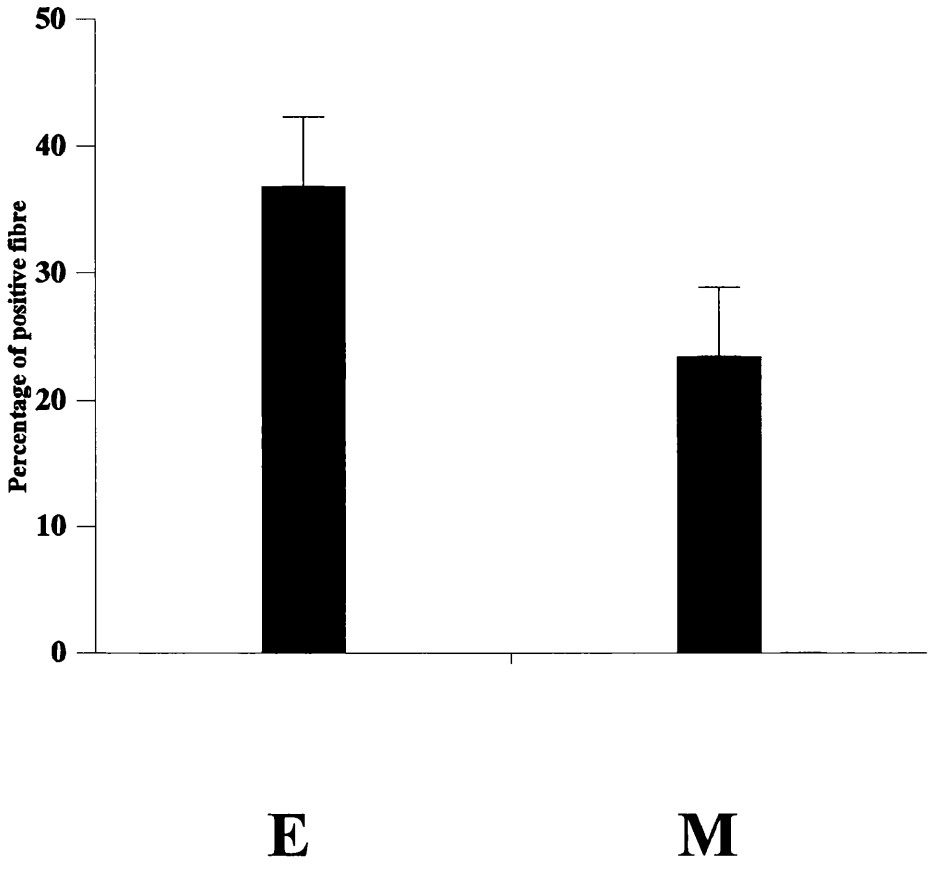



**Fig. 3.8.** Percentage of positive fibres staining for IGF-1 mRNA in the myotendinous region (E) and the middle region (M) of stretched EDL. It is apparent that after stretch for 6 days there is significant ($P > 0.01$) higher percentage of fibres which express IGF-1 mRNA in myotendinous region than in the middle region of muscle. The standard error is given for each mean; (n=5).

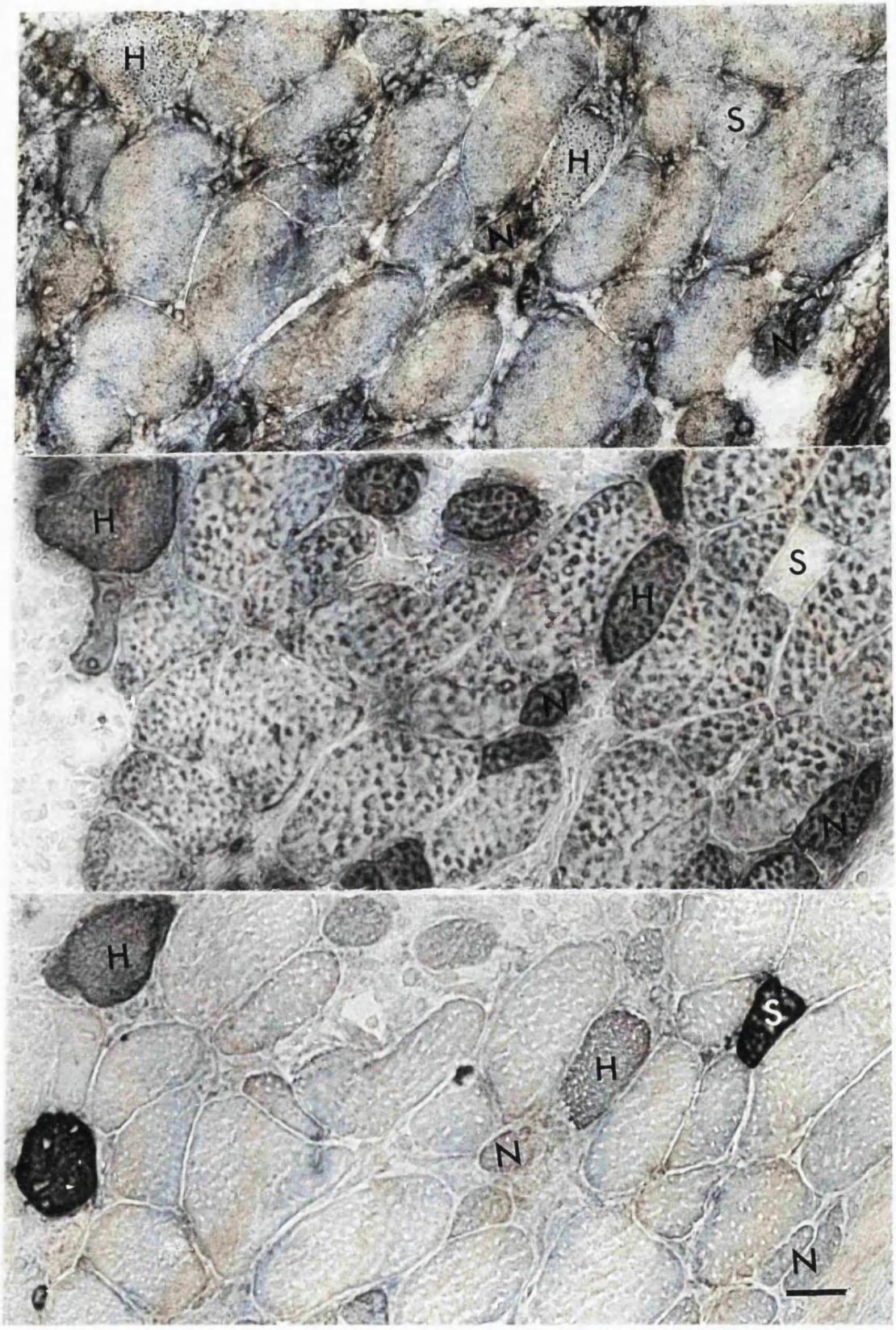



**Fig. 3.9.** Staining for IGF-1 mRNA (top), neonatal myosin (middle) and slow myosin (bottom) on serial transverse sections of the EDL subjected to stretch for 6 days. It was seen that by six days of stretch the fibres producing the IGF-1 mRNA were those that stained strongly for neonatal myosin (N) and those that were hybrid fibres (H) expressing neonatal and slow myosin, but not those that stained for slow myosin (S) only. These hybrid fibres are believed to be undergoing the conversion. This suggests that IGF-1 may be associated with muscle phenotypic conversion. Scale bar, 30 μm.

## 3.4 Discussion

### 3.4.1 Activation of slow myosin heavy chain gene by passive stretch

Muscle is a tissue in which gene expression is regulated to a large extent by mechanical signals (Goldspink, 1992). The inherent ability of skeletal muscle to adapt to mechanical signals is related to its ability to induce or repress the transcription of different isoform genes and to alter the general levels of expression of different subsets of genes (Goldspink, 1996). The fact that there are several myosin heavy chain isoforms means that muscle fibres can change their contractile properties either during development or in response to exercise by rebuilding their myofibrils by means of myosin heavy chains with the necessary slow or fast cross-bridge cycling. Analysis of expression of MyHC gene by Northern blotting showed that there was re-programming of the muscle in response to stretch. In a situation in which the muscle is synthesizing much new myosin, the fast myosin heavy chain gene is apparently repressed as its message level is decreased (Goldspink *et al.*, 1992). In this study the expression of slow MyHC gene was studied by *in situ* hybridization. The results showed that the slow MyHC gene was rapidly activated by passive stretch. The message for this gene is localised around the periphery where the myonuclei are located. Also the results suggested that an adaptive change of specific skeletal muscle proteins in response to a change in contractile activity involves pre-translational as well as translational control of gene expression.

### 3.4.2 Changes of muscle fibre phenotype in muscles subjected to simple stretch

There was also a rapid and marked change in the fibre type composition in muscles subjected to simple stretch. By 6 days slow myosin expression in stretched fibres increased such that a significant proportion of fibres had shifted from the fast type towards an intermediate type or hybrid type (Russell & Dix, 1992). The elevated total RNA content in stretched EDL and TA muscle presumably facilitates both rapid growth and the remodelling of the muscle. The switch to the "slow" (type 1) MyHC

fibre type in stretched muscle is believed to be a mechanism for adapting to a postural-type contractile activity by expressing a more economical "slow" MyHC protein instead of "fast" MyHC (Goldspink *et al.*, 1992). According to the data presented here, this switch is not the case of the "fast" MyHC directly replaced by the "slow" (type 1) MyHC. It is initially replaced by the developmental MyHC and then the slow MyHC. The degree of tension may contribute to this switch process. For example, in EDL muscle, in which there may be more tension by stretch, there was the increase of translation of both neonatal/embryonic MyHC and slow MyHC. In TA muscle, in which there may be less tension by stretch, there was only increase of translation in neonatal/embryonic MyHC, but not yet in slow MyHC.

Furthermore, single fibre dissection has demonstrated that certain fibres can co-express two or more MyHC genes (Biral *et al.*, 1988; Staron & Pette, 1987). Observations on chronically stimulated rat (Termin *et al.*, 1989) and rabbit (Maier *et al.*, 1988) muscle indicated that transforming fibres may contain more than two myosin heavy chains and also the transforming fibres temporarily expressed developmental myosin heavy chain isoforms, i.e. embryonic and neonatal myosins. The results here showed that there are many of hybrid fibres co-expressing slow and developmental myosins. It is probable that these hybrid fibres were in the process of undergoing phenotypic conversion in response to stretch and that the switching of the myosin isoform genes reiterates the developmental pattern but is still incomplete at 6 days.

Interestingly, at six days the most striking observation was that the fibres expressing IGF-1 at high level are those which expressed neonatal myosin and/or type 1 myosin and some of these fibres have central nuclei. This suggested that IGF-1 plays a role in the remodelling of the muscle including muscle phenotype as well as increasing tissue mass.

### 3.4.3 Muscle gene expression is activity dependent

It is well established that adult skeletal muscle is not an inert organ. Instead, the plasticity of muscle is demonstrated by its adaption to alterations in its inherent type of contractile activity (Booth & Thomason, 1991). Such adaption of adult skeletal muscle is characterized by differential gene expression. For example, in an animal or human undergoing a programme of physical training which consists of 30 minutes of running, skeletal muscle recruited during running exhibits an adaptive increase in mitochondrial density without a change in muscle mass (Holloszy & Booth, 1976). In contrast, skeletal muscle trained by weight lifting displays increase muscle mass and unchanged or slightly decreased mitochondrial density (Tesch *et al.*, 1989). Such observations imply differential skeletal muscle gene expression in response to alterations in the inherent type of contractile activity. The lack of adaptation in muscle not recruited during running or weight lifting training supports the idea that differential gene expression is a consequence of the specific pattern of contractile activity.

However, very little is known about the mechanism of transduction of the mechanical signals that induce changes in gene expression in muscle tissue. It has been well established that expression of muscle gene is subject to control by hormones and growth factors. So far, the most potent and most extensively studied agents are fibroblast growth factor (FGF), the insulin-like growth factors (IGFs) and transforming growth factor-β (TGF-β). The results present here indicated that at least in some cases IGF-1 would appear to play a very important role in this mechanism. A great deal of further work will be required to determine exactly how IGF-1 influences accretion of muscle protein and whether different IGF-1 transcripts are produced in response to different stimuli.

### 3.4.4 Relationship between the expression of IGF-1 and MyHC genes

The relationship between the up-regulation of the IGF-1 gene and the increased

expression of the developmental MyHC genes is of considerable interest. There are two explanations for this observation, one is that passive stretch causes damage to muscle fibres leading to their degeneration and subsequent regeneration. The latter then induces a re-expression of the developmental program of MyHC isoforms including neonatal MyHC. Several groups have demonstrated a re-expression of the developmental program of MyHC isoforms during injury-induced regeneration (Cerny & Bandman, 1987; Matsuda *et al.*, 1983; Saad *et al.*, 1987).The embryonic and/or neonatal MyHC are probably more suitable for assembling the initial sarcomere ultrastructure. This would then be replaced by the adult "slow" type 1 isoform by law of mass action exchange (Russell & Dix, 1992). Studies of distribution of myosin mRNA in regenerating adult muscle showed that accumulation of myosin mRNA could have important effects on myofibrillogenesis in regions of focal damage or rapid growth (Dix & Eisenberg, 1991). It may be therefore that some local damage is a prerequisite for both hypertrophy and muscle phenotype change and that IGF-1 is only produced as a result of damage. Another explanation is that there is a mechanotransduction system in the muscle fibres which results in the production of IGF-1 even before damage occurs and that couples reprogramming and increased gene expression. Our present result can not distinguish between these two processes. Indeed these are not mutually exclusive as there is evidence (Petrof *et al.*, 1993a; Petrof *et al.*, 1993b) that the membranes of muscle fibres are occasionally ruptured by stretch and/or stimulation. It is likely therefore that IGF-1 is expressed in response to this microdamage in order to initiate the repair mechanisms. This would involved an increase in protein synthesis and then the prevention of apoptosis. The finding that muscle regulatory factors are also expressed by muscle subjected to overload (Jacobs-El *et al.*, 1995) suggested that change in fibre type from fast to slow does involve re-inducing the developmental program. However, IGF-1 is also known to have effects on the expression of muscle-specific genes in transfected L6E9 myoblasts (personal communication from Dr. Nadia Rosenthal).

The level of IGF-1 mRNA, studied by Northern blots or RT-PCR was demonstrated to increase in muscle tissue after passive stretch or overload (DeVol *et al.*, 1990;

Goldspink *et al.*, 1995). But it was not known whether this change occurs in muscle fibres, satellite cells, fibroblasts or other all type cells within the muscle. The results presented here showed that muscle fibres themselves express IGF-1 and this occurs in those fibres that are undergoing a phenotype change as well as those that are apparently undergoing hypertrophy. Previously, it has been reported that muscle can be induced to undergo hypertrophy in hypothesectomized animals (Goldberg, 1967) indicating that unlike the stimulation in the liver, the expression of IGF-1 gene in muscle is not totally dependent on growth hormone (GH). Indeed, the present work shows that it is induced by mechanical signals.

Transgenic animal experiments in which the IGF-1 gene was expressed in skeletal muscle using an avian actin promoter showed that the transgenic mice had larger muscle fibres although the IGF-1 serum level was not elevated. This suggests an autocrine/paracrine action (Coleman *et al.*, 1995). Further studies are required to elucidate the mechanism of IGF-1 paracrine/autocrine action *in vivo* and the mechanism via which specific genes are regulated during postnatal growth and fibre type determination.

### 3.4.5 Regulation of mass, expression of MyHC gene and IGF-1 mRNA in muscle atrophy by decreased activity

As mentioned in chapter 2 the concentration of total RNA in muscle tissue was not significantly different between disused and normal soleus muscles. This suggests that the control of gene expression in muscle atrophy seems not to occur at pre-translational level. The results in this study showed that the level of the mRNA for IGF-1 in atrophy muscle, studied by *in situ* hybridization, was not changed. Regulation of IGF-1 could also occur at either the point of gene transcription to mRNA or at translation of message to protein. Therefore, it can not be clarified whether IGF-1 plays a role in regulation of muscle mass and muscle gene expression in the atrophied muscle. The conclusion from these data agrees with the observation on the denervated atrophy skeletal muscle (Czerwinski *et al.*, 1993) and the

regression of hypertrophy that occurs after removal of the stretch stimuli (Czerwinski *et al.*, 1994).

Observation from both animal and human studies suggest that under conditions of hypokinesia and hypodynamia, muscle loses proteins differentially, depending on muscle function and fibre type composition. Atrophy is more extensive and rapid in slow-twitch postural muscles than fast-twitch phasic muscles (Fell *et al.*, 1985; Musacchia *et al.*, 1983). Further study of the effect of inactivity on protein turnover in postural rat muscles (Goldspink *et al.*, 1986; Loughna *et al.*, 1986) showed that the atrophy of soleus muscle was explained by a decrease in the fractional rate of protein synthesis and increase in the rate of protein breakdown. When this inactive soleus muscle was permanently stretched, the rate of protein synthesis increase markedly while the rate of protein breakdown was not affected. Taken together these results suggest that the mechanisms controlling the processes of protein synthesis and protein breakdown during muscle disuse atrophy may be independent of each other. Compared with hypertrophy induced by stretch which is mainly due to the increase of the rate of protein synthesis, the atrophy by disuse which mainly was caused by the increase of the rate of protein breakdown and slight decrease of the rate of protein synthesis (Loughna *et al.*, 1986) may be controlled by a different mechanism.

With respect to expression of MyHC within atrophied muscle, the expression of MyHC in response to immobilisation in shortened positions were also observed (Loughna *et al.*, 1990). The adult soleus muscle if deprived of stretch and contractile activity, the MyHC gene begin to switch to the fast myosin heavy chain from slow myosin heavy chain. In the young animal when the soleus was immobilized in the shortened position, it did not fully differentiate into a slow postural muscle. Therefore, the fast adult myosin heavy chain genes are the default genes. When the muscle is subjected to stretch or to repeated stimulation it will express the slow adult genes.

In summary, skeletal muscle is a tissue that possesses an intrinsic ability to adapt to

the type of physical activity it is required to perform. Adaptation takes place during normal growth and as a response to alteration of mechanical activity. With the emergence of methods that enable us to study changes in gene expression, we can now begin to understand adaptation in terms of control of gene expression of individual gene and subsets of genes. This will enable us to obtain an understanding that will range from the whole tissue to the gene level, to open the way to manipulating muscle development in a scientific manner and to present the possibility of using a gene therapy approach to cure some myopathies and inherent diseases.

## 3.5 Summary

In this study, three questions were addressed by combining *in situ* hybridization and immunhistochemistry procedures. The effects of six days of passive stretch and disuse of muscle on the expression of IGF-1 mRNA on the muscle fibre level and the composition of fibres in the muscle were tested. This also involved identifying the fibre type in which the IGF-1 was expressed when the muscle undergoing to growth and muscle fibre undergoing to be converted resulted from passive stretch. The data presented suggested that IGF-1 is involved not only in muscle hypertrophy, but also in muscle fibre phenotype determination. However, it is not involved in muscle atrophy induced by disuse. As the changes could be detected at the individual fibre level this indicated that there is local (autocrine/paracrine) control of muscle growth.

# Chapter Four

# Isolation and molecular cloning of different IGF-1 cDNA from skeletal muscle related to mechanical activity

## 4.1 Introduction

In recent years it has become apparent that the IGF-1 has widespread action on multiple cell types and can act in an endocrine, paracrine, or autocrine manner. The widespread action of the IGF-1 and the widespread distribution of the IGF receptors that mediate their action raise the question of what dictates the specificity of IGF-1 action in different tissues at appropriate stages in response to tissue- and cell-specific stimuli? It has been suggested that the specificity of IGF-1 action is mediated by a number of factors including multiple precursors that may target sites of IGF-1 action.

Analysis of liver IGF-1 cDNA sequences also demonstrated the presence of an E peptide domain which was an extension of the D peptide domain (Roberts *et al.*, 1987; Jansen *et al.*, 1983; Bell *et al.*, 1984). A later study using antibodies directed against the E peptide of human IGF-1 confirmed that the mRNA sequence encoding the E peptide is actively translated and suggested that the E peptide circulates as part of the IGF-1 prohormone (Powell *et al.*, 1987). In rat liver, IGF-1 mRNAs code for a 35-amino-acid E peptide sequence (IGF-1Ea). However an isoform (IGF-1Eb) with a different 41-amino-acid Eb domain has been detected at very low levels (Lowe *et al.*, 1988). These two mRNAs encode alternative E peptide due to the presence (IGF-1Eb) or absence (IGF-1Ea) of a 52 base insert in the region coding the E domain (Roberts *et al.*, 1987; Lowe *et al.*, 1988). In human there are also IGF-1 cDNAs encoding three different Ea, Eb and Ec domains. The Ea and Eb-type cDNAs contain entirely different 3' sequences which specify different 3' untranslated sequences as well as different E domain coding sequences (Rotwein *et al.*, 1986). This is due to splicing in 3' exons (Rotwein *et al.*, 1986). The Ec is a exon 4-5-6 spliced cDNA which predicts a precursor IGF-1 of 158 amino-acid residues and is the human counterpart of the rat Eb (Chew *et al.*, 1995). The physiological role of the alternative E peptide generated from IGF-Ea, IGF-1Eb and IGF-1Ec remains unknown.

Skeletal muscle has been shown to increase in mass very rapidly in response to

passive stretch. The mature rabbit anterior tibialis is able to increase in mass by 35% in 4 days in this way (Goldspink *et al.*, 1992). From previous work (Tabary *et al.*, 1972; Williams & Goldspink, 1973; Goldspink *et al.*, 1986) this was known to be associated with the rapid production of new sarcomeres which are added serially at the ends of the fibre to existing myofibrils. Muscle stretch has been shown to result in a marked increase in muscle protein synthesis (Goldspink & Goldspink, 1986) and stretch rather than electrical stimulation is associated with an increase of IGF-1 mRNA as measured by RT-PCR (Goldspink *et al.*, 1995). However, it is not know whether the IGF-1 gene is expressed by the muscle fibres themselves or by satellite cells or what isoforms of IGF-1 are involved. The regulation of muscle growth *in vivo* remains poorly understood, although the original observations on compensatory muscle hypertrophy implied that a component of muscle growth regulation is a localized, self-contained, and self-limiting process.

Against this background, the current study was designed to determine whether local induction of muscle growth *in vivo* may involve alternative IGF-1 gene expression with different mRNA splicing for IGF-1 specially in 3' area.

## 4.2 Materials and methods

### 4.2.1 Animals and muscle stretch procedure

The same animal model as in the chapter 2 was used. The extensor digitorum longus (EDL) muscle was subjected to acute stretch by immobilizing the left hind limb in the extended position using a plaster cast for 6 days. The right hind leg served as the control.

### 4.2.2 RNA isolation

**A. Preparation of tissues for extraction of total RNA:** Euthanasia of all rabbits was induced by intravenous injection of a large dose of pentobarbitone sodium into the

ear vein. The extensor digitorum longus (EDL) were dissected immediately and then placed into 1.5 ml microtubes which were cold quickly by dropped into liquid nitrogen and later stored at -70$^0$C until required.

**B. Extraction of total RNA from muscle tissue:** The extraction of undegraded template RNA from tissues is particularly difficult because of its labile nature and the presence of active ribonuclease in the tissue itself. Digestive enzymes including RNase are synthesized on ribosomes on the cytoplasmic surface of the endoplasmic reticulum, then pass through into the internal compartment before being packaged into secretory granules. This process effectively separates the synthetic pathways of the cytoplasm from the degradative function of these enzymes. However, disruption of the cell during an extraction procedure would allow RNase to act on nuclear and cytoplasmic RNA. RNase are also very active enzymes that do not require co-factors to function (Lynas *et al.*, 1989). The single-step method of RNA extraction with acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987) overcomes this problem by lysing tissue in denaturing solution (including guanidium thiocyanate). The denaturing solution supplies a chemical environment that results in the denaturation of RNase at the same time as extracting RNA from tissue.

The cellular total RNA was isolated from both stretched and control EDL muscles of rabbit. Total RNA extractions were performed as follows:-

**Step 1.** All solutions and instruments to be used were placed on ice.

**Step 2.** The tissues to be extracted were taken from -70$^0$C, weighed accurately and placed in 5 ml of ice cold denaturing solution (D.S: 4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 m 2-mercaptoethanol) as quickly as possible. The samples were then homogenized twice, each time for 30 seconds using Ultra-turrax T25 homogenizer (IKA-Labortechnik). During homogenizing the tubes were kept on ice at all times.

**Step 3.** After the tissue was homogenized the tubes were still kept on ice and 0.3 ml of 3 M sodium acetate (pH 4), 5 ml of phenol and 1 ml of chloroform : isoamyl alcohol mixture (24:1) were added to each tube . The samples were mixed well by inversion after each addition.

**Step 4.** Each tube was vortexed for 2x5 seconds, care was taken to keep tubes off ice for shortest possible time.

**Step 5.** Whole solutions were transferred to ice cold 15 ml tubes ( this can reduce the area of the interface between two phases after centrifugation ) and then incubated on ice for 15 minutes.

**Step 6.** The samples were centrifuged at 6000 rotor per minute (rpm) at $4^0$C for 20 minutes. The upper layer ( aqueous phase ) of the solutions was then transferred to the new empty 15 ml tubes. 10 ml of absolute ethanol was added to each tube for precipitating RNA.

**Step 7.** The RNA was precipitated at -20 $^0$C for at least 1 hour preferably overnight. And then was spun down in 6000 rpm at $4^0$C for 20 minutes.

**Step 8.** The RNA pellet was resuspended in 0.5 ml of D.S. completely, and 1 ml of cold absolute ethanol was added to each tube. The samples were stored at -$70^0$C until required.

### 4.2.3 Determination of total RNA concentration in solution

The concentration, and to some degree the quality, of RNA dissolved in water was assessed by scanning spectrophotometry in a 1 cm path length Quartz cuvette. 50 µl of total RNA stock solution was taken from -$70^0$C and spun down in 14000 rpm at $4^0$C for 30 minutes. The RNA pellets were washed in 50 µl cold (-$20^0$C) 70% ethanol for 3 times. Finally the RNA was recovered by resuspension in 500 µl DEPC-treated

water and scanned at 260 nm using an UV/VIS kinetics spectrometer (UNICAM). The RNA concentration in solution was determined by its absorbency at 260 nm on the theory of an absorbency of 1 in a 1 cm path length cuvette at 260 nm is approximately equivalent to 40 μg/ml RNA.

### 4.2.4 cDNA synthesis

RoRidT17 oligonucleotide primer (5' ATCGATGGTCGACGCATGCGGATCCAAAGCTTGAATTCGAGCTCTTTT TTTTTTTTTTTT 3') was designed to synthesize cDNA from mRNA. Because mRNA contains poly (A) tails, we have included a complementary dT sequence in the primer to aid annealing of the primer at the 3' end of the mRNA. The primer also contains known restriction enzyme sites, it is therefore easier to manipulate cDNA by restriction enzymes.

20 μg of total RNA ( the volume depends on the concentration of total RNA in the stock solution) was taken from stock solution, and then spun down in 14000 rpm at $-4^0$ C for 30 minutes. The pellets were washed for 3 times with 50 μl of cold 70% ethanol and dried under volume for 5 minutes. The pellets were resuspended in 12.5 μl of diethylpyrocarbonate (DEPC)-treated water. The RNA was denatured by heating in 65 °C for 5 minutes and then rapidly cooled on ice. To this RNA solution 40 units of RNase inhibitor (Boehringer Mannheim) was added to inhibit possible contaminating RNases. To this solution 5 μl of 5 x reverse transcriptase buffer [500 mM Tris-HCl (pH 8.15 at 42 °C); 600 mM KCl; 100 mM $MgCl_2$]; 5 μl of dNTP stock solution (5 mM for each nucleotide); 500 ng of the RoRidT17 oligonucleotide primer; 0.5 μl of 1 M DTT; 7.5 units of Rous associated virus reverse transcriptase (Amersham) were added. The reactions were then incubated at 42 °C for 2 hours and then stored at -20 °C until required.

### 4.2.5 Amplification of IGF-I cDNA by PCR

The polymerase chain reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA and permits the easy analysis of DNA fragments by molecular cloning.

**A. Primer designation:** The 3' end untranslated region of a gene usually contains specific sequences and could be isolated as specific probes to investigate the expression of different mRNA. The 3' end untranslated region of IGF-I mRNA could be reversed transcribed to cDNA with oligonucleotide dT as a primer by reverse transcriptase, the cDNA was then amplified by PCR. Ro primer (5' ATCGATGGTCGACGCATGCGGATCC 3') which is part sequence of RoRidT17 primer used to reverse transcribing the IGF-I mRNA (4.2.4) and the oligonucleotide 5' GCTTGCTCACCTTTACCAGC 3', which is part of 5' end sequence of exon 3 of IGF-I, were designed to amplified IGF-I cDNA sequence from exon 3 to 3' end untranslated region. This oligonucleotide, which show good homology between species, was chosen so that the PCR product to bridge several introns and would not amplify from traces of genomic DNA in RNA samples.

**B. The PCR reaction:** 1 µl of first strand cDNA solution from 4.2.4 step was mixed with 5 µl of 10 x TAQ DNA polymerase buffer, 1 µl of $MgCl_2$ (50 mM), Ro and exon 2 primer (125 pM for each ), and 2 µl of 4 dNTP stock solution (5 mM for each nucleotide). The volume was brought to 49.5 µl with distilled water. 50 µl of mineral oil was placed on the surface of the reaction solution to prevent any evaporation. The reaction solution was heated to $95^0$ C for 5 minutes to denature first strand of cDNA and then applied 0.5 µl (2.5 U) of TAQ DNA polymerase (Boehringer Mannheim). The DNA was amplified using an intelligent heating block (Techne) with sequential denaturation ($94^0$ C, 1 minute), annealing ($65^0$ C, 1 minute) and elongation ($72^0$ C, 1 minute) for up to 30 cycles before a final elongation step for 7 minutes at $72^0$ C.

The PCR products were then sized on a 1.5% agarose gel by electrophoresis at 70

voltage at room temperature for 1-2 hours and either purified from the gel using pre-a-gene DNA purification matrix (BIO-RAD) for ligation into a plasmid vector later.

### 4.2.6 Molecular cloning of different IGF-1 cDNA

*Taq* DNA polymerase normally adds a single non-template nucleotide (nearly always A) to the 3 ' end of all duplex DNA strands. This extra A can be used as a single-base overhang to ligate the PCR products into either a vector which had been linearized with a blunt-end cutting enzyme to which a single T was added, or a vector which had a single T in its blunt-end supplied by Invitrogen. The cloned PCR products could then be further characterised by DNA sequencing and subsequently used to investigate the expression of the gene in tissue as the probes.

**A. Purification of PCR products:** Before being ligated into the vector, the PCR products should be purified. The PCR products were separated by electrophoresis on 1.5% ethidium bromide stained agarose gel. The desired DNA band was excised from the gel, and then purified using pre-a-gene DNA purification matrix according to manufacturers instructions (BIO-RAD).

**B. Ligation of PCR products into pCR$^{TM}$II vector:** The pCR$^{TM}$II vector was purchased from Invitrogen which had been linearized with a blunt-end added a single T base which supplies a complementary base to the PCR product and made it more efficient to ligate the PCR product into the vector. 6 µl of purified PCR products from step A were incubated with 1 µl of 10 x $T_4$ ligase buffer, 2 µl of resuspended pCR$^{TM}$ vector (500 ng) and 1 µl of $T_4$ DNA ligase (Invitrogen) at $12^0$ C overnight.

**C. Introduction of plasmid vector containing PCR products into competent cells:** 5 µl of ligation reaction solution from step B was mixed with 100 µl of XL-1 Blue competent cells and incubated on ice for 10 minutes. The cells were shocked by incubating at $42^0$ C for 2 minutes to make cells more accessible to pick up the plasmid DNA. To the cell and vector mixture 1 ml of LB (appendix) medium without

antibiotics was added and incubated at $37^0$ C for 30 minutes to allow the cells to recover before selecting on agar plates containing ampicillin (50 μg/ml), X-gal (20 μg/ml) and IPTG (0.1 mM, appendix). The plates were incubated overnight at $37^0$ C.

**D. Identification of positive clones:** White colonies need to be identified if they contain the plasmid vector which had been ligated into the expected DNA before the cloned DNA being sequenced. 1.5 ml of overnight cultured LB containing white colonies was applied to a 1.5 ml tube. The cells were recovered by spinning down at 13000 rpm for 10 seconds, decanting supernatant LB and remaining 50-100 μl of LB with cell pellet. The cells were resuspended in remained LB with vortexing, mixed with 300 μl of TENS buffer [10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0; 0.1 M NaOH and 0.5% SDS ] and incubated on ice for 15 minutes. 150 μl of 3 M sodium acetate (pH 5.2) was added to the solution to precipitate the proteins. The solution was spun down at 13000 rpm for 2 minutes. The supernatant was transferred to a new 1.5 tube and introduced 0.9 ml of cold 100% ethanol to precipitate the plasmid DNA which was then recovered by spinning down at 13000 rpm, washing in 70% ethanol and resuspending in 100 μl of TE buffer (appendix).

The plasmid DNA was digested with appropriate restriction enzymes (for example, EcoRI), and separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide. With positive colonies the expected size DNA band can be seen on UV transilluminator.

### 4.2.7 DNA sequencing

**A. Preparation of plasmid DNA for sequencing:** Double strand plasmid DNA was isolated for sequencing using the magic™ minipreps DNA purification system (Promega), suspended in 20 μl of TE buffer (appendix) and stored at -20 $^0$C until use. 1 μl of all minipreps was checked by digesting with restriction endonuclease and running on 1.5% agarose gel stained with ethidium bromide.

**B. DNA sequencing reaction:** DNA sequencing by dideoxy chain termination method ( Sanger et al 1977) was performed using sequenase (United States Biochemical). For each sequencing reaction 5 μg of DNA was used. This was made up to a volume of 20 μl with distilled water and 2 μl of freshly prepared 2 M NaOH. After 5 minutes at room temperature 8 μl of 5 M ammonium acetate (pH 7.5) and 100 μl of 95% ethanol were added. The mixture was left at -20 $^0$C for 30 minutes before being centrifuged for 30 minutes at 14000 rpm (4$^0$C). The precipitate was washed with 500 μl of cold 70% ethanol, dried and then resuspended in 7 μl of distilled water in preparation for sequenase reaction.

To the resuspended, denatured (single strand) DNA solution, 2 μl of 5 x sequenase reaction buffer and 1 μl of the chosen primer (0.5 pmol/ul) were added. The tubes were placed in a beaker filled with 65 $^0$C water and cooed at room temperature slowly to < 37 $^0$C to allow annealing to occur. While cooling 2.5 μl of each termination mixture were placed in separate labelled eppendorf tubes (ddA, ddT, ddG, ddC) and prewarmed at 37 $^0$C .

Once the annealing mixture had cooled to below 37 $^0$C 1 μl of 1 M DDT (appendix), 2 μl of sequenase labelling mix, 0.5 μl a [$^{35}$S] dATP and 2 μl of diluted sequenase (1:8) were added. After 5 minutes at room temperature , 3.5 μl of the labelling mixture was transferred to each of the four termination tubes and incubated for a further 5 minutes at 37 $^0$C. Finally 4 μl of the stop solution was added to each tube to stop reaction.

**C. Preparation of sequencing gels:** Sequencing gels containing 7.2% polyacrylamide were prepared by mixing 33 g urea (molecular biology grade, Sigma), 13 ml of 40% acrylamide with 2% bis-acrylamide (both Sigma), 0.7 ml of 10% ammonium persulphate (BDH)< 7.7 ml of 10 x TBE (appendix), 70 μl of N,N.N',N'-tetramethylethylenediamin (TEMED, Sigma) and 33 ml of distilled water and polymerized between glass plates 0.4 mm apart.

**D. Electrophoresis:** The sequencing reaction samples were heated to above 70 $^0$C for 2 minutes and then put on ice immediately prior to loading on a sequencing gel. The gel was run at 30 watts for approximately 2 hours, fixed in 10% acetic acid for 15 minutes and dried for 1 hour in a gel drier (BIO-RAD) before exposure to an X-ray film (Kodak) overnight at room temperature.

**E. Sequence read and analysis:** All sequencing gels were read manually and the data compiled and collated using a DNA analysis computer software. A total of 98% of the DNA sequence was obtained on both strands.

## 4.3 Results

### 4.3.1 Preliminary PCR amplifications

The most prominent amplified band in each sample was between 500 and 550 base pair (bp) . In the no template cDNA reaction no identical cDNA band was isolated, which excludes the possibility of contamination in the PCR. The 75 bp band observed in both sample and no template control is due to primer dimerisation (fig. 4.1).

### 4.3.2 cDNA sequences

This study was designed to investigate if different isoforms of IGF-1 are expressed in muscle when it is subjected to mechanical activity. Ten clones covering the E domain ( exons three to six) were isolated and sequenced from stretched and from contralateral control muscle. Two classes of cDNA clone were obtained using RNA isolated from stretched muscle. Among these clones, 30 % contain the sequences coding for IGF-1Ea and 70% for IGF-1 Eb. However, even after repeated attempts, only IGF-1Ea type clones could be isolated from unstretched rabbit muscle. The cloned cDNA sequence starts from exon 3 which codes for mature IGF-1. The sequences of the two classes of IGF-1 cDNA isolated from total RNA of the stretched EDL muscle are shown in fig. 4.2. The sequence may be divided into three

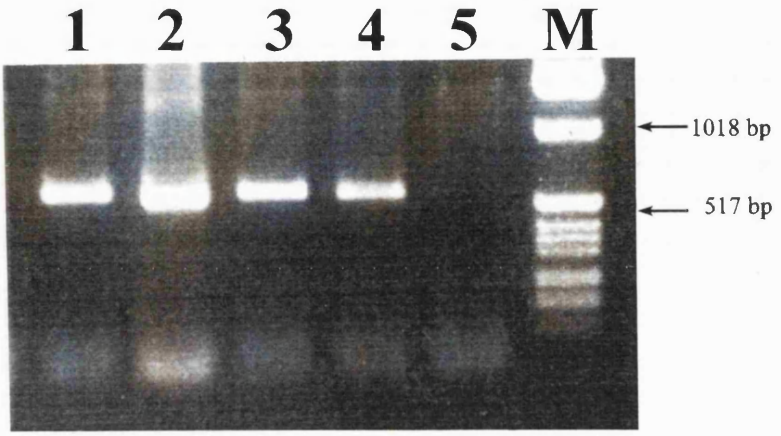



**Fig. 4.1.** PCR products. The prominent amplified band in each sample was between 550 and 600 base pair (lanes 1 to 4). In the no template cDNA reaction no identical cDNA band was isolated (Lane 5), which excludes the possibility of contamination in the PCR. The 75 bp band observed in both sample and no template control is due to primer dimerisation. The lane M is a DNA marker lane (1Kb ladder, Gibco BRL)

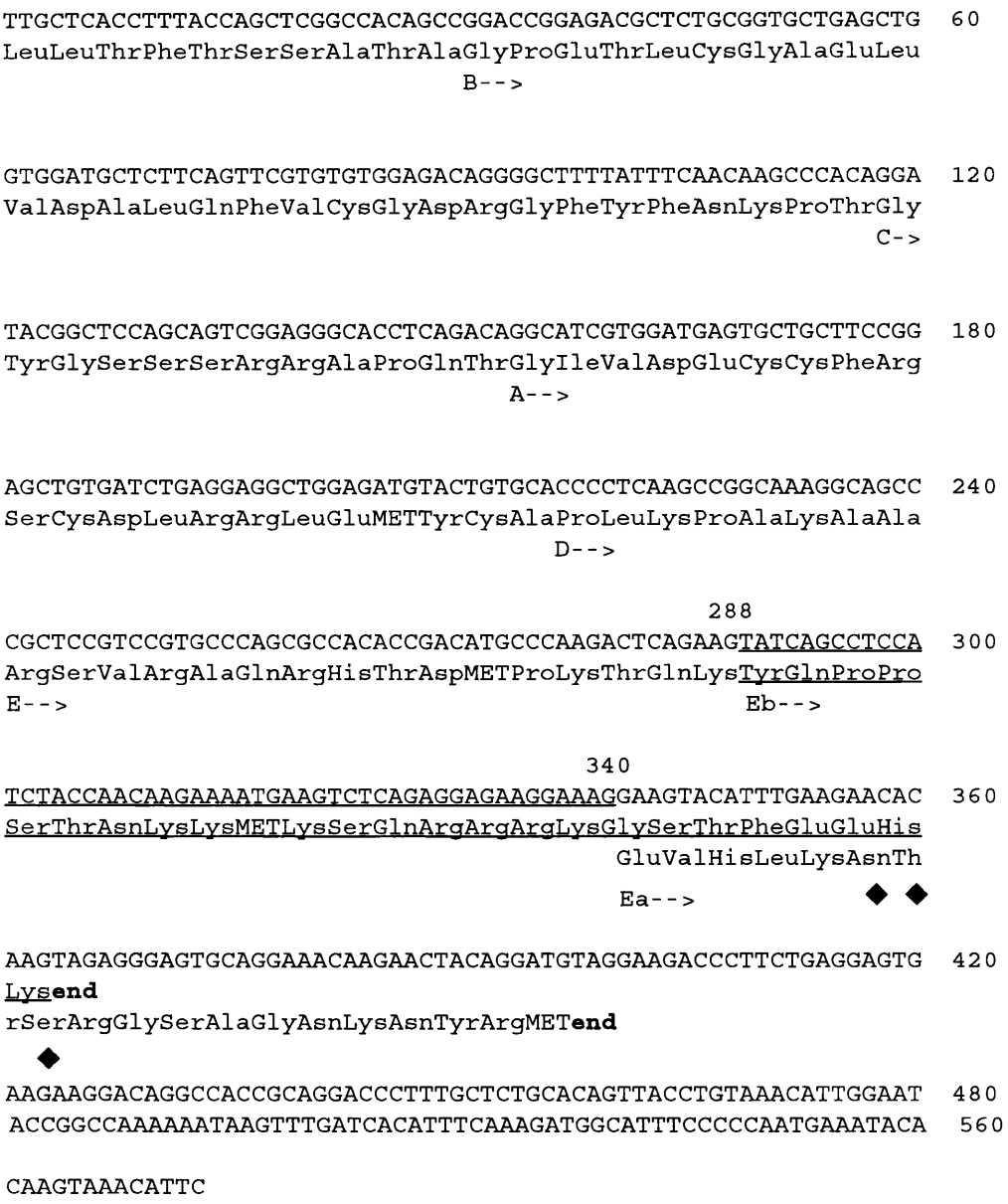



**Fig. 4.2.** DNA and derived amino acid sequences of rabbit IGF-1 cDNA isolated from stretched muscle: The two types of cDNA sequence differ by the presence (IGF-1Eb) or absence (IGF-1Ea) of a 52 base pair insert (underline)from position 288 through position 340. The insert altered the derived C-terminal amino acid sequence of the E peptide (underline in IGF-1Eb case), changed the reading frames and used two different TAG stop codons (end). The putative glycosylation site (Asn-Thr-Ser) (marked by ◆◆◆) is present in the Ea but not in the Eb peptide.

sections. A region which encodes mature IGF-1 (peptides B, C, A and D), an extension E peptide which in IGF-1Eb has a 52 base insert which is lacking in IGF-1Ea, (fig. 4.2) and a common 3' untranslated region. In terms of the carboxyl-terminal extension (E) peptide, the rabbit amino acid sequence is identical to the human sequence up to residue E 16. At the first base of the codon for residue E 17, the amino acid sequences of the two cDNA clones diverge due to the 52 -bp insert in the IGF-1Eb clone. The insert changes the derived amino acid sequence as well as the reading frame, resulting in two possible carboxyl-terminal E peptide sequences and the presence of two different UAG stop codons in end variants.

Comparing the 52-bp insert from rabbit muscle with the 52-bp insert in the IGF-1Eb expressed in rat liver in very low amounts (Lowe *et al.*, 1988) and IGF-1Ec which has recently been detected in human liver (Chew *et al.*, 1995), the positions where the insert occurs is the same. The rabbit cDNA sequence shows 77% homology with rat IGF-1Eb, with 12 of the 17 expected amino acid sequences being identical and 94% with human IGF-1Ec, with 13 of 16 expected amino acid sequence being identical (fig. 4.3).

### 4.3.3 Evolution of IGF-1 gene

Predicted amino acid sequences of vertebrate mature IGF-1 peptide and the E peptide were aligned (fig. 4.4).This accumulated evidence suggests that the IGF-1 gene emerged at a very early stage in vertebrate evolution and has been highly conserved through out vertebrate evolution. In addition to the highly conserved mature peptide, the E peptide (Ea and Eb, in human Ec) has been conserved highly as well. This suggests evolutionary pressure to conserve the heterogeneity, and therefore provide indirect evidence for its functional significance. If the evolutionary conservation among the human, rabbit, rat and mouse between the Ea and Eb peptide was compared, the Eb peptide has thus far only been found in mammals, however, it appears to be less conserved than the Ea peptide.

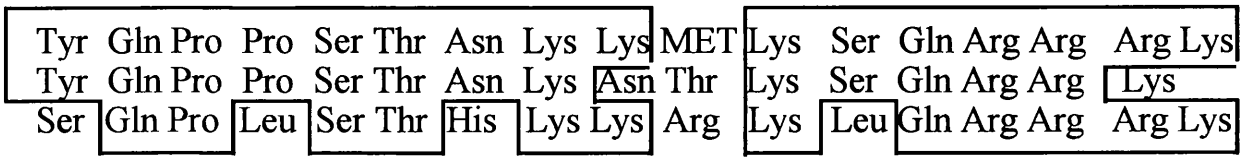



**Fig. 4.3.** Alignment of the three derived amino acid sequences of the inserts from rat liver (bottom), human liver (middle) and rabbit stretched muscle (top). Identical amino residues are shown by the boxes.

Mature peptide

| | B domain | C domain | A domain | D domain |
|---|---|---|---|---|
| Human: | GPETLCGAELVDALQFVCGDRGFYFNKPT | GYGSSSRRAPQT | GIVDECCFRSCDLRRLEMYCA | PLKPAKSA |
| Pig: | ---------- | ---------- | ---------- | -------- |
| Cow: | ---------- | ---------- | ---------- | -------- |
| Guineapig: | ---------- | ---------- | ---------- | -------- |
| Sheep: | ---------- | ---------- | ---------- | ---A---- |
| Rabbit: | ---------- | ---------- | ---------- | ------A- |
| Rat: | ----------P--------- | -----I------ | ---------- | ----T--- |
| Mouse: | ----------P--------- | -----I------ | ---------- | ----T-A- |
| Chicken: | ----------S--- | --------LHHK | --------Q---------- | -I--P--- |
| Frog: | ----------T----------S--- | ----NN--SHHR | --------Q---F-------- | -A------ |
| Salmon: | ----------T------E-----S--- | ---P----SHNR | --------Q--E--------- | -V-SG-A- |

E peptide

| | Common region | Ea | Eb(in human Ec) |
|---|---|---|---|
| Human: | RSVRAQRHTDMPKTQK | EVHLKNASRGSAGNKNYRM | YQPPSTNKNTKSQRR KGSTFEERK |
| Rabbit: | ---------- | ------T---------- | --------KM-----R-------H- |
| Rat: | --I---------- | ------T--------T--- | S--L--N-KR-L---R----L--H- |
| Mouse: | --I---------- | ------T--------T--- | SPSL----K--L---R-------H- |
| Pig: | ----------A-- | ----------S------- | |
| Cow: | ----------A-- | ------T---------- | |
| Sheep: | ----------A-- | ------T---------- | |
| Guinea pig: | ---------- TQK | ---------- | |
| Chicken: | ----------A-- | ------T---NT--R---- | |
| Frog: | ----------A-- | ---P--T---NT-SRGF-- | |
| Salmon: | ----------R-P-(27mino) | ---Q--S---NT-GR---- | |

**Fig. 4.4.** Comparison of the amino acid sequences of IGF-1 mature peptide and E peptide from several vertebrate species. Dashes indicate amino acid identity with the human sequence. Domains are indicated above the sequence. This accumulated evidence indicate that the IGF-1 gene emerged at a very early stage in vertebrate evolution. In addition to the highly conserved mature peptide, the E peptide has been conserved highly as well. This provide indirect evidence for its functional significance.

## 4.4 Discussion

Experimental models of muscle regeneration indicated that IGF-1 may act as a trophic factor in muscle regeneration (Jennische *et al.*, 1987) and it is expressed in proliferating myoblast and satellite cells (Edwall *et al.*, 1989). In this study we have analysed IGF-1 mRNA in skeletal muscle induced to undergo rapid longitudinal growth. This model was chosen as there is very little injury to the muscle fibres. The results presented here agree with published work (Beck *et al.*, 1987; Han *et al.*, 1987; Caroni & Schneider, 1994) that IGF-1 mRNA is expressed in muscle tissues.

The isolation of two classes of cDNA clones (IGF-1Ea and IGF-1Eb) from stretched muscle indicates that both forms of IGF-1 mRNA are present in the stretched muscle. The IGF-1Ea and IGF-1Eb cDNA 3' sequences differ by the presence of a 52-bp insert which in the latter alters the derived carboxyl-terminal amino acid sequence. Three mechanisms may account for the 52-bp insert. Firstly, the insert could be generated by an alternate splice donor site 52-bp into the 5' -end of an intron present at this position in the IGF-1 genomic sequence. Alternatively, it may be generated by the use of an alternate splice acceptor site 52 bp from the 3' -end of the pertinent intron. Finally, the 52-bp insert could arise from a completely separate exon (Roberts *et al.*, 1987).

A comparison of the sequence of the rabbit IGF-1Eb with sequences of the rat IGF-1Eb (Roberts *et al.*, 1987) and human IGF-1Ec (Chew *et al.*, 1995) showed that the IGF-1Eb, which is markedly up-regulated in stretched muscle, is apparently the rabbit counterpart to the rat IGF-1Eb and the human IGF-1Ec. Our results showed rabbit IGF-1Eb (the equivalent of human IGF-1Ec) was only detectable in stretched muscle. The fact that this is inducible isoform is consistent with the results of Chew et al (Chew *et al.*, 1995) who demonstrated that after stimulation with physiological levels of GH, human IGF-1Ec transcript was increased in human hepatoma HepG2 cells (a hepatoma line), relative to human IGF-1Ea. The site for IGF-1 binding proteins (BP) is believed to be in the B domain (De Vroede *et al.*, 1985). Also C and D-domains

are thought to be "active regions" (Pietrzkowski *et al.*, 1992) . However, the physiological role of the alternative E peptide generated from IGF-1Ea and IGF-1Eb mRNA remains unknown (Lowe *et al.*, 1988). It has been suggested that it could affect the interaction of IGF-1 with its receptor or its binding proteins. It has been also suggested that the E-peptides themselves may also have distinct biological roles after being cleaved from the pro-protein (Lowe *et al.*, 1988). Recently, part of the E-peptide has been shown to contain an amidated growth-promoting peptide with specific binding sites in human tissues (Siegfried *et al.*, 1992). According to our findings, the Eb-peptide appears to be induced only in the stretched muscle. This suggests the Eb-peptide may play a role in local growth control as exemplified by skeletal muscle fibre increasing in length and mass in response to mechanical stretch. The Eb peptide may be involved in the externalization of IGF-1 and also the binding of IGF-1 to muscle receptors.

There is evidence which suggests that the Ea peptide may be glycosylated *in vivo.* Bach et al (Bach *et al.*, 1990) found that the Ea peptide can be glycosylated following *in vitro* translation in the presence of microsome. No putative glycosylation sites were noted from the muscle IGF-1 Eb sequence data. Possible functions for the differences in glycosylation of Ea and Eb include the reduction of the half life of IGF-1Eb, differential localization of the two forms and differential affinities for binding proteins. Therefore, the stretched muscle type IGF-1Eb may be much smaller but with a shorter half-life than the isoforms produced by normal muscle and the liver.

Devol et al (1990) reported that IGF-1 mRNA in skeletal muscle is independent of GH and other pituitary hormones and demonstrated a link between local stimulation of skeletal muscle growth and IGF-1 gene expression. The Eb expressed only in stretched muscle indicates that the expression of IGF-1Eb mRNA might be switched on by stretch, which is known to induce rapid muscle growth (Goldspink *et al.*, 1992). The Eb peptide may then be a specific factor distinguishing mechanical stimulation and associated mechanisms of muscle growth.

In summary, Only one isoform of IGF-1 cDNA could be cloned from the normal resting muscles. However, two isoforms of IGF-1 cDNA were cloned from stretched muscle undergoing hypertrophy. The E domain sequence of the additional isoform differs from the liver IGF-1Ea by the presence a 52 base pair insert. This changes the reading frame of the derived carboxyl-terminal resulting in a different precursor IGF-1 isoform (IGF-1Eb). The muscle IGF-1 mRNA probably encodes the precursor IGF-1 isoform that is responsible for local muscle growth regulation in response to mechanical stimulation. There are differences in the potential glycosylation of Ea and Eb peptide and this may contribute to reduced half-time which may favour autocrine / paracrine action over systemic effects.

It is apparent from our study that the different E peptides may play different roles in IGF-1 activity. Further studies are required to elucidate whether the E peptide of these alternative IGF-1 mRNA interact differently with the IGF-1 receptor or with IGF-1 binding proteins, or whether the alternative E peptide alone enables the growth factor to act in an autocrine fashion.

## 4.5 Summary

Using RT-PCR a single IGF-1 isoform cDNA (IGF-1Ea) could be cloned from the normal resting muscles. However, an additional isoform of IGF-1 (IGF-1Eb) was found to be expressed in stretched muscle undergoing hypertrophy. The E domain sequence of the additional isoform differs from the liver IGF-1Ea by the presence a 52 base pair insert. This changes the reading frame of the derived carboxyl-terminal resulting in a different precursor IGF-1 isoform. This IGF-1 mRNA probably encodes the precursor IGF-1 isoform that is responsible for local muscle growth regulation in response to mechanical stimulation.

# Chapter Five

# Differential splicing of IGF-1 gene in skeletal muscle is response to mechanical activity

## 5.1 Introduction

The primary transcript of the IGF-1 gene can be alternatively spliced to result in at least four mRNAs encoding precursor proteins differing in their amino-terminal and carboxyl-terminal amino acid extensions (Gilmour, 1994). Exons 1 and 2 contain discrete sites for the initiation of transcripts which are alternatively spliced (i.e. either exon 1 → 3 or exon 2 → 3) to the primary mRNA (Gilmour, 1994). Hall and his co-workers (1992) reported the complete organization of rat IGF-1 gene and identified several transcription start sites within exons 1 and 2 of IGF-1 gene which are adjacent to presumptive promoters 1 and 2 respectively. Both exons 1 and 2 have potential coding regions and result in distinct amino-terminal extensions to the signal peptide as a consequence of 5' alternative splicing events.

In the human alternative polyadenylation signals in the 3' untranslated region of the gene are also detected (Rotwein, 1986). This differential splicing results in either IGF-1 Ea or IGF-1 Eb mRNA. Recently, another alternatively spliced human IGF-1 transcript (the exon 4-5-6-transcript) was identified, termed as IGF-1 Ec (Chew *et al.*, 1995). Thus in the human the IGF-1 gene encodes three precursor proteins, i.e. a precursor protein of 153 amino acid, including an E peptide of 35 amino acids, a precursor protein of 195 amino acids, including an E peptide of 77 amino acids and a precursor protein of 158 amino acids with an E peptide of 24 residues. Rat, mouse and rabbit IGF-1 genes, similar to the human IGF-1 gene, also encode two IGF-1 precursors that differ in their carboxyl-terminal peptides (Bell *et al.*, 1986; Shimatsu & Rotwein, 1987b; Shimatsu & Rotwein, 1987b; Tobin *et al.*, 1990).

Some investigation carried out on the rat showed that a pro-IGF-1 with an intact Ea domain is secreted from cultured fibroblasts (Conover *et al.*, 1989) and macrophages (Kirstein *et al.*, 1992). These observations also suggested that the E peptide region of IGF-1 Ea is released as part of the prohormone form in cultured human fibroblasts, and that the levels of this prohormone are regulated by growth hormone (Conover *et al.*, 1989). After *in vitro* stimulation with advanced glycosylation end (AGE)

proteins, the normal human blood monocytes also expressed IGF-1 Ea mRNA leading to the secretion of IGF-1 Ea prohormone (Kirstein *et al.*, 1992).

Although additional studies are clearly required to defined the functional role of IGF-1 Eb precursors, there is some evidence indicating that Eb peptides may have a biological role distinct from that of mature IGF-1 (Siegfried *et al.*, 1992). In chapter 4 evidence was presented that IGF-1 Eb (in the human IGF-1Ec) expressed in muscle may be responsible for local muscle growth regulation in response to mechanical stimulation. On the base of the data in chapter 4, this chapter was designed to investigate the expression of IGF-1 Eb mRNA in response to mechanical stimulation by using RT-PCR and RNA protection. The purpose of this aspect of the research was to confirm that alternative splicing of the IGF-1 gene occurs in muscle in response to physical activity.

## 5.2 Materials and methods

### 5.2.1 Muscle stretch procedure

New Zealand white rabbits were used. The extensor digitorum longus (EDL) muscle was subjected to acute stretch by using the same procedure as mentioned in chapter 2. The EDL muscle was collected as same as mentioned in chapter 4. In order to verify that alternative splicing of the IGF-1 gene also occurs in human muscle in response to physical activity, human exercise experiments was included. As a initial experiment the forearm flexors muscles of a volunteer were subjected to eccentric exercise using a weighted pulley system and a syringe needle aspiration sample (Ennion *et al.*, 1994) was taken from the biceps brachii muscles after 2 hours. Total RNA was isolated from the syringe needle aspiration sample and analysed by RT-PCR as for the rabbit muscle. The human needle aspiration samples were taken after ethical permission had been given by the Royal Free Hospital to Professor Archie Young.

### 5.2.2 RNA preparation and cDNA synthesis

The total cellular RNA was prepared from muscle using the same methods as the chapter 4 (4.2.2 on page 111). And the cDNA was synthesized from the total RNA using the same methods as the chapter 4 (4.2.4 on page 114).

### 5.2.3. RT-PCR reaction

The polymerase chain reaction (PCR) is an invaluable technique for the analysis of gene expression since mRNA reverse transcribed into cDNA may be subjected to conventional PCR amplification. This is particularly useful when analysing low abundance mRNAs.

**A. Primer design:** Primers which were designed based on the sequence data presented in chapter 3. The 5 ' primer sequence (5' GCTTGCTCACCTTTACCAGC 3') which is part of the 5' end sequence of exon 3 of IGF-I gene, was common. For the IGF-1 Ea the sequence of the 3' primers was 5'AAATGTACTTCCTTCTGAGTCT 3' which is complementary to the part of exons 4 and 6 of the IGF-I gene, and for the IGF-1 Eb the sequence of the 3' primer was 5'AAATGTACTTCCTTTCCTTCTC 3' which is complementary to the part of exons 5 and 6 of the IGF-I gene. These primers were designed so that they enable the selective amplification of either the IGF-1Ea or the IGF-1Eb ( in human, IGF-1Ec) cDNA. The product of PCR for IGF-1 Ea should be 301 base pairs long, and for the IGF-1 Eb 353 base pairs long.

**B. PCR conditions:** The PCR reaction was performed for 35 cycles in thermal reactor (HYBAID) and using the following parameters: 94 °C for 1 minute; 60 °C for 1 minute; and 72 °C for 1 minute followed by an extension of 72 °C for 10 minutes which ensures that all PCR products are complete and blunt-ended.

**C. Sequencing of PCR products:** The product of PCR for IGF-1 Ea (301 base

pairs), and for the IGF-1 Eb (353 base pairs) were then separated on 1.5% agarose gels. The desired DNA band was excised from the gel, and then purified using "prep-a-gene" DNA purification matrix according to manufacturers instructions (BIO-RAD). The purified PCR products were then subcloned into pCR™II vector and sequenced as mentioned in chapter 4.

### 5.2.4 Confirmation of PCR products by Southern blotting

**A. Separation of PCR products by agarose gel electrophoresis:** After PCR is completed, 12 µl of PCR reaction was loaded with 1 µl of DNA loading buffer on 1.5% agarose gel. The fragments of DNA was separated by electrophoresis. When electrophoresis is finished, DNA was then photographed in agarose gels stained with ethidium bromide by illumination with UV light.

**B. Transfer of DNA onto nitrocellulose filters:** After electrophoresis, the gel was transferred to a glass baking dish. The DNA on the gel was denatured by soaking the gel for 45 minutes in several volumes of 1.5 M NaCl, 0.5 M NaOH with constant, gently agitation. The gel was then rinsed briefly in deionized water and neutralized by soaking gel for 30 minutes in several volumes of a solution of 1 M Tris (pH 7.4), 1.5 M NaCl at room temperature with constant, gentle agitation.

The gel was removed from neutralization solution and placed inverted on the support. The DNA in the gel was transferred to the membranes (Boehinger Mannheim) in 10 x SSC by capillary action using soft tissues to enhance capillary action. After the DNA was transferred, the membranes were baked for 2 hours at 80 °C for fixing the DNA to the membranes and used in hybridization directly or stored dry between two filter paper in plastic at room temperature.

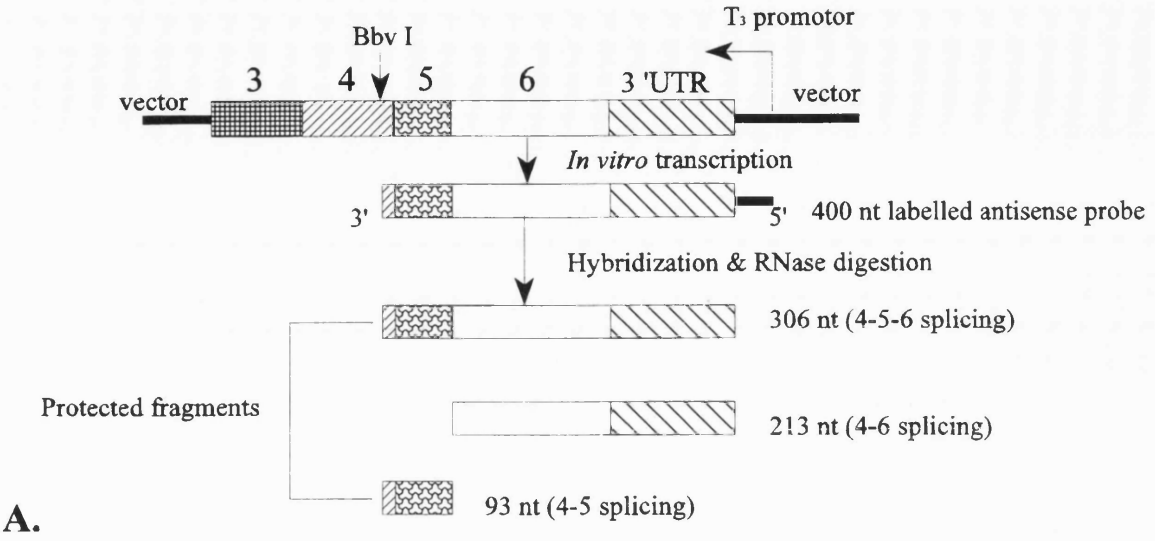
**C. Preparation of cRNA probes for Southern blotting:** The template DNA was linearized by EcoNI, extracted by acid phenol:chloroform and purified using wizard™ DNA clean-up system kit (Promega) according to the manufacturers instructions. The

α [$^{32}$P] cRNA probe was then synthesised *in vitro* from the template DNA by $T_3$ RNA polymerase as following. 1 μg linearized template DNA was mixed with 3 μl 10 x transcription buffer, 4 μl dNTP (10 mM of each ATP, GTP, UTP) mixture, 1 μl CTP (100 μM), 1.5 μl dithiothreitol (DTT), 2 μl RNase inhibitor (Promega), 7 μl [$^{32}$P] α CTP (800Ci/mM) and 2 μl $T_3$ RNA polymerase (Ambion). The final volume of reaction was made to 30 μl by DEPC treated distilled water and the reaction was carried at 37 ° C for 2 hours. The labelled probe was identified extracted by acid phenol:chloroform, identified and purified by electrophoresis on a 10 % polyacrylamide gel.

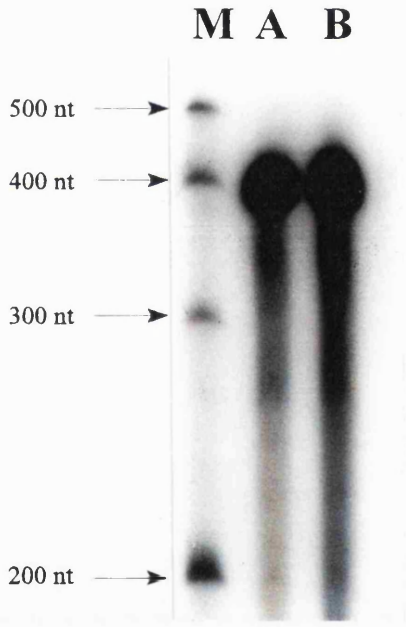
**D. Southern hybridization:** Prehybridization (4 hours) and hybridization (overnight) were performed at 42 °C in hybridization buffer [ 50% formamide; 5 x SSC; 50 mM sodium phosphate (pH 6.8); 5 x Denhardt's solution and 0.05% SDS]. Post-hybridization wash was carried out at high stringency 2x15 minutes at room temperature with 2xSSC and 0.1% SDS, 2x15 minutes at 50 $^0$C with 0.1xSSC and 0.1% SDS. The membranes were expose to Kodak film for 30 minutes.

### 5.2.5 RNA protection

**A. Probe preparation for RNA protection:** The IGF-1 Eb cDNA (see chapter 4) was subcloned into pBS+ phagemid vector (Stratagene) including $T_3$ and $T_7$ promoters so as the sequence of IGF-1 Eb cDNA can be labelled by *in vitro* transcription. The template DNA was linearized by Bbv I, extracted by acid phenol:chloroform and purified using wizard™ DNA clean-up system kit (Promega) according to the manufacturers instructions. The sequences of this template DNA include part of exon 4 and whole of exons 5, 6 and 3' UTR region (fig 5.1). The α [$^{32}$P] cRNA probe was then synthesised *in vitro* from the template DNA by $T_3$ RNA polymerase as following. 1 μg linearized template DNA was mixed with 3 μl 10 x transcription buffer, 4 μl dNTP (10 mM of each ATP, GTP, UTP) mixture, 1 μl CTP (100 μM), 1.5 μl dithiothreitol (DTT), 2 μl RNase inhibitor (Promega), 7 μl [$^{32}$P] α CTP (800Ci/mM) and 2 μl $T_3$ RNA polymerase (Ambion). The final volume of




**A.**




**B.**

**Fig. 5.1.** Preparation and protected fragments of the probe for RNase protection assay. The IGF-1 Eb clone was linearized at Bbv I site shown in panel A. A 400 nucleotides (nt) [$^{32}$P] cRNA probe ( A and B in panel B, M lane in panel B is a RNA marker lane) was synthesised by *in vitro* transcription with $T_3$ RNA polymerase. By using this probe, it was possible to identify different 3' splicing isoforms of IGF-1, i.e. 4-5-6, 4-6 and 4-5 splicing (panel A).

reaction was made to 30 µl by DEPC treated distilled water and the reaction was carried at 37 ° C for 2 hours. The labelled probe was extracted by acid phenol : chloroform, identified and purified by electrophoresis on a 10 % polyacrylamide gel.

**B. Co-precipitation of target and probe RNA:** 100 µl (10 µg) total RNA was mixed with 2.59 µl [$^{32}$P] RNA probe (6 x $10^4$ cpm), 10.3 µl 3 M sodium acetate and 300 µl absolute ethanol (cold at -20 °C) and precipitated at -20 °C overnight.

**C. Hybridization of radiolabelled RNA probe:** The precipitated total RNA and probe RNA was spun down at 4 °C with 14000 rpm for 20 minutes. The pellet was washed with 1 ml 75% ethanol (cold at -20 °C), dried at room temperature for 15 minutes and resuspended in 30 µl hybridization buffer (80% deionized formamide; 1 mM EDTA; 40 mM PIPES pH 6.4 and 200 mM sodium acetate). The sample was then heated to 95 °C for 5 minutes and vortexed briefly to help dissolving the RNA. The total RNA and probe RNA was finally denatured at 95 - 100 °C for 5 minutes. The hybridisation was carried out at 68 °C for 2 hours and then at 50 ° C overnight.

**D. Digested with RNase ONE ™ and electrophoresis:** After hybridization, the samples were briefly spun down to collect the condensate, diluted with 270 µl RNase ONE ™ digestion buffer (10 mM Tris-HCl, pH 7.5; 5 mM EDTA; 200 mM sodium acetate) and digested with 7 units of RNase ONE ™ at 30 °C for 1 hour. The digestion was stopped by adding 30 µl stop solution (10% SDS; 1.0 mg/ml tRNA). The hybridized RNA was precipitated by adding 825 µl cold absolute ethanol and incubating at -70 ° C for 15-30 minutes. The pellet was washed with 300 µl of cold 70% ethanol and resuspended in 10 µl of gel loading buffer (80% deionized formamide; 1 mM EDTA; 0.1% bromophenol blue; 0.1% xylene cyanol; 0.1% SDS). The hybridized RNA fragments was then denatured by heating at 95 °C for 5 minutes and separated by electrophoresis through a polyacrylamide/8 M urea gel under standard conditions using 1 x TBE buffer. The labelled fragments were detected by autoradiography of the dried gel using 1 week exposure.

## 5.3 Results

### 5.3.1 PCR products

A 353 bp PCR product specific for IGF-1 Eb was detected in EDL muscle after it was subjected to stretch for 6 days. No appreciable signal for this isoform could be detected in the control contralateral muscle. In contrast the 301 bp product of PCR for IGF-1 Ea isoform was present in both stretched and control muscles. The PCR product for IGF-1 Ec was also up-regulated in human muscle when it was subjected to eccentric exercise for 2 hours, but not to be detected in contralateral control muscle. IGF-1 Ea (Ea) was again detected in both exercised and control muscles (fig. 5.2). Although the RT-PCR method used can not be regarded as quantitative , it clearly showed that the IGF-1 Eb (IGF-1 Ec in the human) was up-regulated following stretch or eccentric exercise. Even with very sensitive PCR methods, only trace of IGF-1 Eb was detected in the control muscle (which had still been subjected to mild activity). In contrast although IGF-1 Ea was detected at about the same level in all muscles. These experiments confirmed that the mechanical activity resulted in alterative splicing and that the mRNA for IGF-1 Eb (IGF-1 Ec in the human) was up-regulated .

### 5.3.2 Southern blotting

Southern blotting analyses confirmed that these two different size PCR products included the IGF-1 sequences (Fig. 5.3). By using an internal common cDNA probe including the sequence of exon 4 which presents in both IGF-1 Ea and IGF-1 Eb isoforms, two different PCR products could be detected. However, when an internal oligo probe including the sequence of exon 5 which only presents in IGF-1 Eb isoform, only 353 bp PCR product could be detected. This means that the 353 bp product is specific for IGF-1 Eb. Also it was showed that IGF-1 Eb is up-regulated by stretch, although a trace of IGF-1 Eb was detected in the control muscle (which had still been subjected to mild mechanical activity).

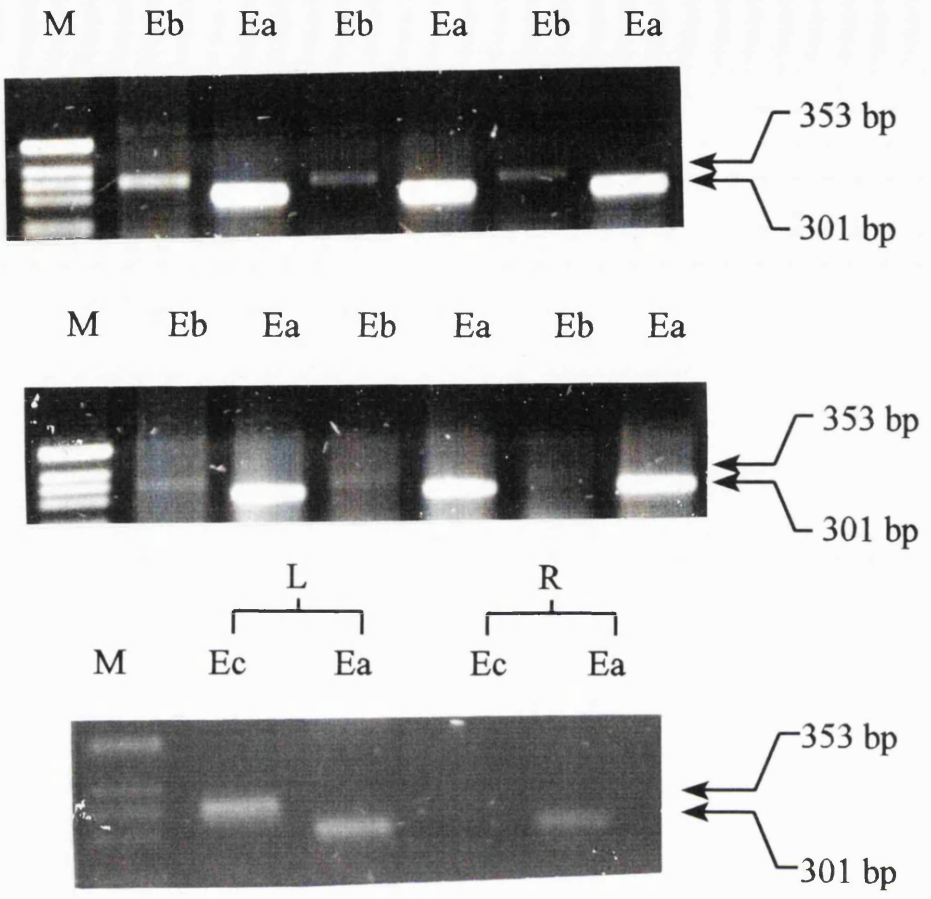



**Fig. 5.2.** Using RT-PCR a 353 bp product specific for IGF-1 Eb (Eb) was detected in rabbit muscle after it was subjected to stretch for 6 days (top row). Only trace of the messenger for this isoform could case just be detected in contralateral control muscle (middle row). In contrast the 301bp product of PCR for IGF-1 Ea (Ea) was present in both stretched and control muscles. The PCR product for IGF-1 Ec (Ec) was also up-regulated in human muscle when it was subjected to eccentric exercise for 2 hours ( bottom row, L), but not to be detected in contralateral control muscle (bottom row, R). IGF-1 Ea (Ea) was again detected in both exercised and control muscles. The lane M is a marker lane (1Kb ladder, Gibco BRL). The human needle aspiration samples were taken after ethical permission had been given by the Royal Free Hospital to professor Archie Young.

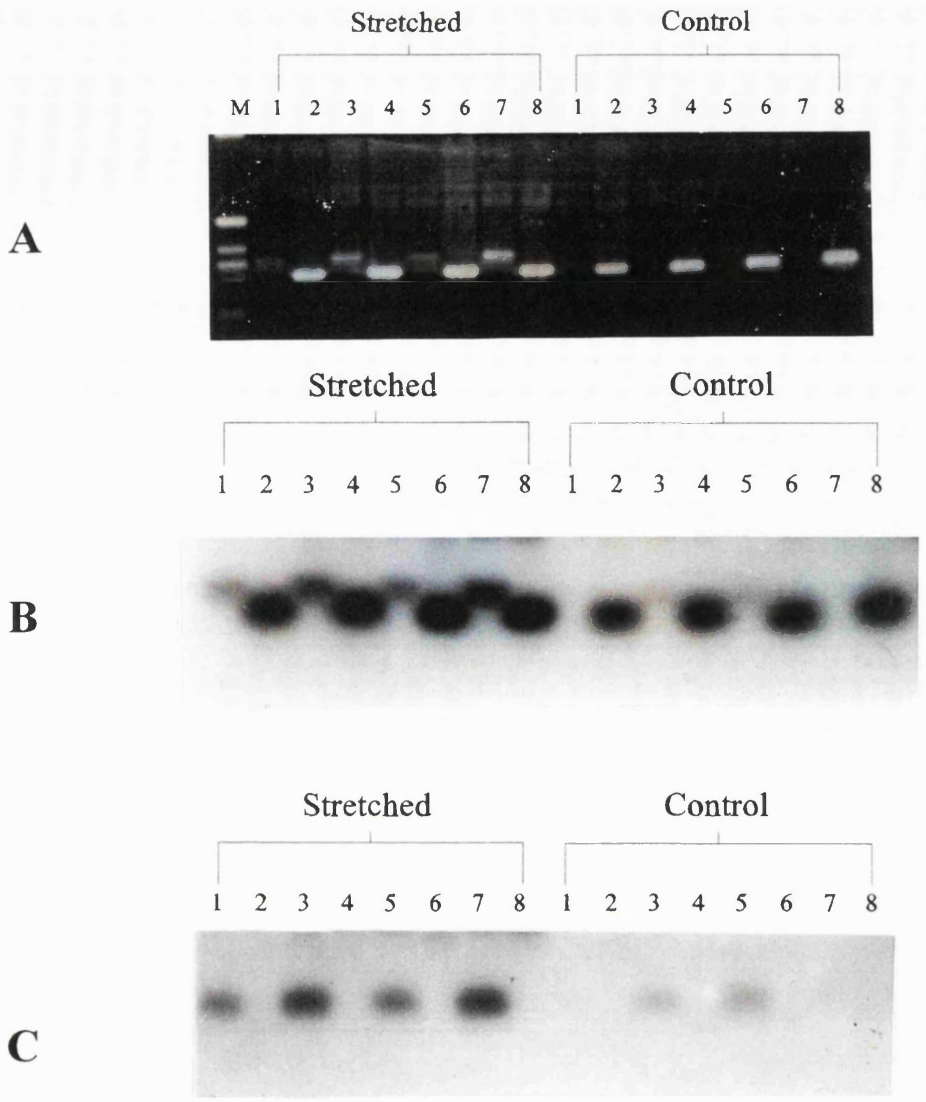



**Fig. 5.3.** Southern hybridization of IGF-1 Ea and Eb PCR products. 12 µl of PCR reaction (described as in 5.2.3.) was separated by electrophoresis on 1.5% agarose gel and then photographed on UV transilluminator with ethidium bromide staining (panel A). The DNA was then transferred and fixed onto nitrocellulose filters. The latter was probed by an IGF-1 cRNA probe excluding the sequence of the primers used in PCR reaction (panel B). Two isoforms of IGF-1 (Ea and Eb) were identified, Because the sequence of this probe covers the exons 3 and 4 of IGF-1 gene which is common to IGF-1 Ea and Eb (Ec in human). When the DNA was hybridized with a oligonucleotide probe which is specific for IGF-1 Eb (Ec in human) but not includes the sequences of the primer used in PCR reactions, only 353 bp PCR product was detected (panel C). This indicates that the 353 bp product is specific for IGF-1 Eb (Ec in human). Also it was showed that IGF-1 Eb ( lane 1, 3, 5 and 7 ) is up-regulated by stretch, although a trace of IGF-1 Eb was detected in the control muscles which had still been subjected to mild mechanical activity. IGF-1 Ea (lane 2, 4, 6 and 8) was detected in both stretched and control muscle.

### 5.3.3 RNase protection

The expression of different IGF-1 transcripts was also studied by RNase protection as this is very sequence specific and does not have the same selective amplification problems of PCR. By using a probe (fig. 5.1) including the sequences of exons 4, 5 and 6, it was possible to identify different 3' splicing isoforms of IGF-1, i.e. 4-5, 4-6 and 4-5-6 splicing isoforms. The results (fig. 5.4) showed that the 4-6 splicing isoform represented by exon 6 and 3' untranslated region (UTR) (213 bp) is the main isoform in both muscle and liver. Also the 4-5-6 splicing (306 bp) was detected in live as well as stretched muscle but not in the control muscle. The signals for the 4-5-6 splicing isoform were weaker than would be expected on the basis of data from RT-PCR. The probe used here was relatively long which might result in secondary structure formation and decreased hybridization to target mRNA. This explanation was confirmed by Dr. McKoy who used the same cDNA clone but reduced its size. By using the shorter probe she obtained strong signals for the 4-5-6 splicing isoform within stretch muscle, but again not in the control muscle. The shorter probe has been used for further studies and these data will be published later. It was noted that there were another additional two bands (250 bp and 150 bp) in liver to those in muscle. This maybe due to the different sizes of the 3' UTR of liver IGF-1 mRNA transcripts. The RNase protection analyses again showed that mechanical activity (stretch) can induce 4-5-6 splicing expression to produce a different isoform of pre-IGF-1.

## 5.4. Discussion

Examination of responses of different 3' spliced IGF-1 mRNAs in skeletal muscle to mechanical stimuli demonstrated that IGF-1 Eb was up-regulated by stretch. In a survey of IGF-1 expression in different tissues, it has been reported that skeletal muscle only expresses the class 1 5' untranslated region (Lowe *et al.*, 1987). If this is the case there should be two transcripts which were expressed in the muscle tissue, i.e. class 1 IGF-1 Ea and Eb (in human Ec). The results of chapter 4 indicated that

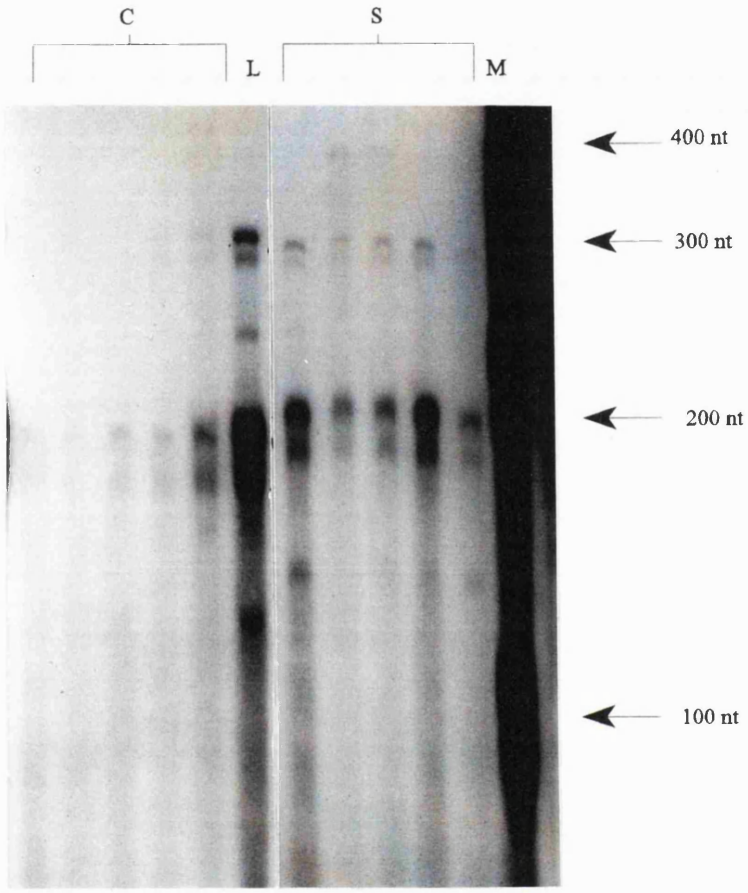



**Fig. 5.4** Autoradiograph of RNase protection assay of tissue RNA from stretched (S), control (C) muscles and Liver (L) with a rabbit IGF-1 Eb riboprobe described as in fig. 5.1. Lane M is a RNA marker. It is apparent that the exon 6 including 3' untranslated region (UTR) (213 bp) which represents the 4-6 splicing is the main isoform in both muscle and liver, and the 4-5-6 splicing (306 bp) is present in some stretched muscle as well as liver but not in the control muscle. The 4-5 splicing (93 bp) is not present in both muscle and liver. There are another additional two bands (250 bp and 150 bp) in liver to those in muscle. This maybe dues to different size of 3' UTR of IGF-1 mRNA in liver. The RNase protection analyses showed that mechanical activity (stretch) can induce 4-5-6 splicing expression to produce a different isoform of pre-IGF-1.

only IGF-1 Eb (in human is IGF-1 Ec) is responsible for local muscle growth regulation in response to mechanical stimulation. This means that only class 1 IGF-1 Eb (in human Ec) is responsible to mechanical activity in muscle tissue. Furthermore the results also indicated that the 3' splicing of IGF-1 gene which involves differential exon usage may determine the mode of action of the pro IGF-1 peptide.

In recent years it has become apparent that the insulin-like factor system involves a network of molecules that includes the IGFs themselves, IGF receptors, IGF-binding proteins and IGFBP proteases. The widespread action of the IGF-1 and the widespread distribution of the receptors that mediate its action raises the question as to what dictates the specificity of IGF action in different tissues at appropriate stages in development and in response to tissue- and cell- specific stimuli? Lund (1994) have suggested that the specificity of IGF action is mediated by a number of factors, including (1) multiple transcriptional and post-transcriptional mechanisms that control levels and sites of IGF synthesis and levels of circulating IGFs. (2) multiple precursors that may target sites of IGF action, and (3) multiple IGFBPs that may target sites of IGF action and modulate this action. This chapter was designed to investigate the expression of IGF-1 Eb mRNA in response to two forms of mechanical stimulation namely stretch (rabbit) and repeated contraction (human) by using RT-PCR and RNA protection. The results suggested that different usage of 3' region of IGF-1 gene is the regulation of IGF-1 synthesis and action in skeletal muscle related to mechanical activity.

More detailed analysis has increased our understanding of the regulation of IGF-1 gene expression and suggested differential splicing of IGF-1 may have a role in the regulation of IGF-1 expression. Two major classes of mRNA transcripts have been identified in hepatic and non-hepatic tissues, each coding for the same mature peptide but containing different 5'-untranslated regions, derived from transcription initiation at different transcription start sites in exons 1 and 2 (Lowe *et al.*, 1987; Pell & Gilmour, 1993; Pell *et al.*, 1993; Weller *et al.*, 1993; Foyt *et al.*, 1992). The effect of GH on choice of transcription initiation sites was studied in hypophysectomized rats;

a marked decrease in exon 2-derived transcripts, termed as class 2, was seen in liver following hypophysectomy, with a 10-fold increase after repeated GH injections (Lowe *et al.,* 1987). In contrast, the steady-state levels of exon 1 mRNA transcripts in all tissues studied were significantly less affected by these manipulation. The sum of these observation suggested that exon 2 transcripts regulated primarily by GH perhaps encode the endocrine form of IGF-1. Similarly, exon 1 transcripts may be considered to encode the paracrine and/or autocrine form of IGF-1, as mentioned above, exon 1 mRNA transcripts are expressed in all tissues studied. The observation by Yang and his colleagues (Yang *et al.,* 1995) demonstrated that IGF-1 mRNAs potentially encode multiple forms of pre-pro IGF and that specific differences in their 5'- untranslated regions provide a molecular basis for translational control of IGF-1 biosynthesis.

The 3 ' splicing of IGF-1 gene results in differential exon usage inducing three distinct E peptides which are extensions of carboxyl-terminal D domain. The role of the E peptides, either as a part of the IGF-1 prohormone or possible as a free peptide, remains unknown. Further observation of the tissue distribution and regulation by GH of the IGF-1 Ea and Eb (in human is Ec) mRNA demonstrated that these two transcripts are present in low abundance in all tissues studied (Lowe *et al.,* 1988). Although multiple forms of pre-pro IGF-1 have been described, this appears to be the first time that mechanical activity of muscle induces the 3' alternative splicing of IGF-1 gene *in vivo.*

The relationship between the different 5'-untranslated regions and 3' alternative splicing has been studied. It has been shown that the 4-5-6 transcript was associated with the one of alternative 5' untranslated regions (Lowe *et al.,* 1988). Studies demonstrating the association of the 4-5-6 transcripts with another class of 5'-untranslated region are lacking, but 4-5-6 transcripts presumably may contain any of the alternative 5'- untranslated regions. In some other genes splicing events at the 5' -end determine exon use at the 3' -end (Breitbart *et al.,* 1987). However, in IGF-1 mRNA processing the relationship between 5' and 3' splicing events is not clear yet.

If the choice of 5' -untranslated region and usage of exon at 3'-end are independent events, there should be at least 6 types of IGF-1 mRNA are able to be transcribed, so far of which 4 IGF-1 mRNAs have been identified (Gilmour, 1994; Shimatsu & Rotwein, 1987a). It is apparent, then, that multiple mRNAs encoding IGF-1 are capable of being transcribed. Further studies are clearly required to explore the physiological role of the peptide products of these alternative IGF-1 mRNAs, whether these peptide products interact differently with the IGF-1 receptor or with the IGF-1 binding proteins, or whether the alternative E peptides alone have a biological role.

## 5.5. Summary

In this study experiments were designed to investigate the expression of IGF-1 Eb mRNA in response mechanical stimulation by using RT-PCR and RNA protection in stretched rabbit muscle and human biceps subjected to repeated contraction. In order to verify that alternative splicing of the IGF-1 gene also occurs in human muscle in response to physical activity, a human exercise experiments was also included. Following physical activity for 2 hours to 6 days, appreciable levels of IGF-1 Eb (in human the Ec) isoform were detected in skeletal muscle by using RT-PCR. In contrast, very little if any of this splice variant could be detected in control muscle not subjected to stretch or extra physical activity. The results confirmed that IGF-1 Eb mRNA probably encodes the precursor IGF-1 isoform that is responsible for local muscle growth regulation in response to mechanical stimulation.

# Chapter Six

# General Discussion

## 6.1 Muscle mass and mechanical activity

Hypertrophy of skeletal muscle fibres as a result of certain forms of exercise has been an accepted fact for many years. It has been appreciated that there is local control growth of muscle because if a muscle is exercised it is only that muscle which undergoes hypertrophy and not all the muscles of the limb. Indeed, skeletal muscle size and shape is not apparently strictly pre-programmed but regulated to a large extent by mechanical factors. The number of muscle fibres apparently does not increase during postnatal growth or as a result of exercise training at reasonable intensity level. However, the mean cross-sectional area of the fibres does increase considerably. This increase in fibre cross-sectional area is associated with a large increase in the myofibrillar content of the fibres.

It has been shown that stretch is the major mechanical signal for the addition of sarcomeres (Williams & Goldspink, 1973; Goldspink, 1976; Williams & Goldspink, 1978). Sarcomere number of muscle fibre is not fixed and even in adult muscle it is capable of either increasing or decreasing (Tabary *et al.*, 1972; Williams & Goldspink, 1973). This occurs during muscle immobilization. In muscle immobilized in the shortened position, sarcomeres are lost and the remaining sarcomeres are altered to a length that enables the muscle to develop its maximum tension at the length which corresponds to the immobilized position (Williams & Goldspink, 1978). In muscle immobilized in the lengthened position, sarcomeres are added on and this results in sarcomere length being reduced as compared with non-adapted muscle fixed in a similar position. Maximum tension again is found to be developed at the new functional length. It has been noted that during stretch-induced muscle growth sarcomeres are added in series at existent fibre ends (Williams & Goldspink, 1973; Goldspink, 1985); in a similar way to that which occurs during normal post-natal growth (Griffin *et at.*, 1971). Protein synthesis is high at the ends of normal fibres (Griffin *et al* 1971), and this accelerates when fibres are lengthening in response to stretch (Goldspink *et al.*, 1986; Goldspink *et al.*, 1974; Williams *et al.*, 1986). When the new sarcomeres were added to this region, the satellite cells play a very important

role. Satellite cells defined as the cells that sit dormant under the basement membrane of multinucleated muscle fibre have little cytoplasm and express no muscle proteins. During muscle hypertrophy satellite cells divide repeatedly and function as a source of new nuclei (Moss & Leblond, 1971). After overload the satellite cells take several hours to be mobilized (Kennedy *et al.,* 1988). After 6 days of stretch there were many myotubes with central nuclei at the end of the muscle, and these myotubes were believed to be differentiated from satellite cells and to fuse with existing fibres (Dix & Eisenberg, 1990; Russell *et al.,* 1992).

The results of the first study (chapter 2) showed that high expression of the mRNA for IGF-1 within the fibres with central nuclei and which express developmental myosin isoforms, i.e. embryonic and neonatal MyHC. Furthermore, the data of the second study (chapter 3) showed that high expression of the IGF-1 mRNA also occurs in hybrid fibres expressing the neonatal and slow MyHC which are apparently undergoing a phenotype change. This suggests that IGF-1 also plays the role on muscle cell transformation, i.e. fast to slow in response to overload. A study carried out on myoblasts *in vitro* has also demonstrated that IGF-1 stimulates proliferation and differentiation of skeletal muscle satellite cells (Allen & Boxhorn, 1989) also supported our conclusions. The fact of maximum stimulation of proliferation was observed in the presence of both fibroblast growth factor (FGF) and IGF-1 indicated that this regulatory process is complex.

Muscle undergoes rapid atrophy under conditions in which force production is reduced and contraction is significantly less than normal. Such atrophy occurs in humans during prolonged bed rest (Greenleaf & Kozlowski, 1982), plaster cast immobilization (MacDougall *et al.,* 1977) and space-flight (Waterlow *et al.,* 1978). In fact, changes in protein mass of skeletal muscle are attributable to alterations in the relative rates of protein synthesis and protein breakdown. The effect of inactivity on protein turnover in phasic and postural muscles has been investigated (Loughna *et al.,* 1986) and indicated that the muscle atrophy due to inactivity is caused mainly by elevation of protein degradation and only partly by reduction of protein synthesis.

The fact of the level of total RNA content and IGF-1 mRNA was not affected by disuse (chapter 2 and 3) indicates that IGF-1 appears not to play a role in skeletal muscle atrophy, but is only involved in the positive growth mechanism.

## 6.2 Relationship between muscle fibre phenotype and mechanical activity

It is well established that muscle gene expression is activity dependent (see chapter 3). However, the nature of the link between the mechanical signal and the regulation of structure gene expression needed to be elucidated. Skeletal muscle fibre types differ phenotypically in that they express different subsets of myofibrillar isoform genes with different specific ATPase activities as well as different types and level of metabolic enzymes.

The inherent ability of skeletal muscle to adapt to mechanical signals is related to its ability to induce or repress the transcription of different isoform genes and to alter the general level of expression of different subsets of genes. The fact that there are several different myosin heavy chain isoforms means that muscle fibres can change their contractile properties either during development or in response to exercise by rebuilding their myofibrils. This includes changing the myosin cross bridges with their characteristic slow or fast cross-bridge cycling rates (Goldspink, 1996). The isoforms of myosin heavy chain have been shown to be encoded by individual genes which are members of a multigene family. Of which the expression is tightly regulated in a stage-specific and tissue-specific manner (Butler-Browne & Whalen, 1984; Mahdavi *et al.*, 1986; Weydert *et al.*, 1987). It is also influenced by hormones (Lompre *et al.*, 1984; Gustafson *et al.*, 1986) the type of innervation (Pette & Vrbova, 1985) and mechanical activity (stretch and electrical stimulation)(Goldspink *et al.*, 1992). With regard to the latter it was shown that both stretch alone and stimulation alone caused repression of fast type and activation of the slow myosin genes. When subjected to mechanical overload skeletal muscle apparently adapts to a more postural type of role

by expressing the slow isoform gene as well as higher level of mitochondrial genes (Goldspink *et al.*, 1992).

The result presented in the second study (chapter 3) showed that the slow MyHC gene, studied by *in situ* hybridization and using a slow MyHC cRNA probe, was rapidly activated by passive stretch. As documented by mATPase histochemistry, normal rabbit EDL and TA muscle contain only a minor percentage (3-5%) of slow type 1 fibres. In agreement with this, the majority of the fibres in the normal muscles did not bind the slow MyHC probe following *in situ* hybridization. However, in stretched muscle there are many more fibres were expressing the slow MyHC gene and they appeared as bundles. While in the same normal muscles (EDL and TA) the fibre expressing the slow MyHC gene appeared as single widely spaced individual fibres. When fibre composition within both stretched and normal muscles was studied by immunohistochemical procedure, stretch also resulted in a increase translation of neonatal, embryonic and slow (type 1) myosin heavy chains. These data indicate that the enhanced transcriptional activity of the slow(type 1) MyHC gene is followed by an increased translation of the transcript.

When the relationship between expression of IGF-1 mRNA and changes in muscle fibre phenotype in response to stretch was studied by combining *in situ* hybridization and immunohistochemistry procedures, it is apparent that it was these fibres undergoing change in fibre phenotype and hypertrophy that express higher levels of IGF-1 mRNA. This indicates that when subjected to mechanical overload skeletal muscle apparently adapts to a more postural type of role by increasing transcriptional and translational level of slow (type 1) MyHC. IGF-1 appears to be involved in this adaption and because of the appearance of the fibres and clustering effect it suggests an autocrine/paracrine mechanism.

## 6.3 Regulation of IGF-1 mRNA in skeletal muscle in response to stretch

IGF-1 was originally considered to be produced solely by liver and to act via an endocrine mechanism. However, it was found that this growth factor can be synthesized at multiple sites (D'Ercole *et al.*, 1984). It has been suggested that some forms of IGF-1 may act through autocrine or paracrine mechanisms, although there was no firm evidence for this mode of action. IGF-1 mRNA accumulation in skeletal muscle during growth hormone stimulated muscle hypertrophy seemed to support this suggestion (Turner *et al.*, 1988). However, hypertrophy of skeletal muscle in response to overload in hypophysectomized rats demonstrated that signal was independent of growth hormone (DeVol *et al.*, 1990). Hence the finding presented here are in accord with the concept that modulation of IGF-1 mRNA level can occur in the absence of growth hormone and that produced IGF-1 might play a role in local control of skeletal muscle growth.

The estimation of the expression of the IGF-1 mRNA in stretched skeletal muscle by Northern blotting (chapter 2) suggested that two species of IGF-1 mRNA (7.5 kb and 1.2 kb) are specifically induced by mechanical stimulation, although there is a family of IGF-1 mRNAs that range in size from 7-8 kb to 0.8-1.2 kb (Rotwein, 1986; Lund *et al.*, 1986; Han *et al.*, 1988; Hoyt *et al.*, 1988), of which 4 IGF-1 mRNAs have been identified in rate liver (Shimatsu & Rotwein, 1987). The results presented in chapter 2 also indicated the 1.2 kb IGF-1 mRNA was increased in all stretched muscle which was not always the case for the 7.5 kb IGF-1 mRNA. By using oligomer-directed ribonuclease H mapping, the 7.5- to 7.0-kb rat mRNAs were demonstrated differing in size from the smaller-molecular-weight IGF-1 mRNAs due to differences in the length of 3' untranslated (3' UTR) region and that the 7.5- to 7.0 kb rat IGF-1 mRNAs have unusually long 3' UTR (Lund *et al.*, 1989). Studies on different half-lives of IGF-1 mRNAs that differ in length of 3' UTR sequence demonstrated that the 7.5- to 7.0-kb rat liver IGF-1 mRNAs are less stable *in vitro* and *in vivo* than the smaller IGF-1 mRNAs (Hepler *et al.*, 1990). Whether the

different responses of 1.2 kb and 7.5 kb IGF-1 mRNAs in skeletal muscle to stretch is due to this different stability remains to be investigated further.

The expression of IGF-1 mRNA in hypertrophic muscle, studied (chapter 2) by *in situ* hybridization, demonstrated that IGF-1 mRNA is expressed in the muscle fibres themselves and not solely in satellite and connective tissue cells. The level of IGF-1 mRNA is elevated up to 22 times in muscle fibres in response to stretch for 6 days compared with unstretched muscle. Its expression was not uniform and it is usually the smaller fibres that showed higher levels. A study of transverse and longitudinal sections showed that the small fibre which expressed IGF-1 mRNA also express the neonatal myosin. It has also been shown that small diameter fibres containing neonatal MyHC are the tapered ends of the larger fibres terminating within the belly of the muscle (Rosser *et al.*, 1995). As mentioned previously, longitudinal growth of skeletal muscle involves the addition of new sarcomeres to the ends of the existing myofibrils (Williams & Goldspink, 1971; Williams & Goldspink, 1973) and the initial stage involves the laying down of neonatal myosin (Rosser *et al.*, 1995). These data support the hypothesis that the ends of normal adult fibres are the region for longitudinal growth and that IGF-1 is involved in this process.

Studies (chapter 3) on serial sections of the muscles by *in situ* hybridization and immunohistochemical staining showed that by six days of stretch, the fibres producing the IGF-1 mRNA were those that stained strongly for neonatal myosin or those that were hybrid fibres expressing neonatal and type 1 myosin. It is interesting to note that in these hybrid fibres, the greater intensity of type 1 staining was found within the larger diameter fibres. This may indicate that both adaptive responses, i.e. changes in fibre phenotype and hypertrophy, were more complete in these particular fibres. Also that IGF-1 plays a role in the remodelling of the muscle including muscle phenotype as well as increasing tissue mass.

In the third study (chapter 4), using RT-PCR only a single IGF-1 isoform cDNA (IGF-1Ea) could be cloned from the normal resting muscles. However, an additional

isoform of IGF-1 (IGF-1Eb) cDNA was cloned from stretched muscle undergoing hypertrophy. The E domain sequence of the additional isoform differs from the liver IGF-1Ea by the presence a 52 base pair insert. This changes the reading frame of the derived carboxyl-terminal resulting in a different precursor IGF-1 isoform. This IGF-1 mRNA probably encodes the precursor IGF-1 isoform that is responsible for local muscle growth regulation in response to mechanical stimulation. Bach et al (1990) found that the Ea peptide was glycosylated following *in vitro* translation in the presence of microsome preparation. However, no putative glycosylation sites are noted from the muscle IGF-1 Eb sequence data (chapter 4). Possible functions for the differences in glycosylation of Ea and Eb include the reduction of the half life of IGF-1Eb, differential localization of the two forms and differential affinities for binding proteins. Therefore, IGF-1Eb produced in response to stretch and overload may be much smaller but with a shorter half-life than the isoforms produced by normal resting muscle and by the liver.

To confirm that alternative splicing of the IGF-1 gene occurs in muscle in response to physical activity, oligonucleotide primers were made which specially amplify the cDNAs of two isoforms (IGF-1 Ea and Eb) in the human as well as the rabbit. Following altered physical activity for 2 hours to 6 days, appreciable levels of IGF-1 Eb (in human the Ec) isoform were detected in skeletal muscle by using RT-PCR. In contrast very little if any of this splice variant could be detected in control muscle not subjected to stretch or extra physical activity.

The expression of different IGF-1 transcripts in response to stretch, studied by RNase protection, indicated that mechanical activity (stretch) can induce to expression of 4-5-6 splicing isoforms of IGF-1. The data presented in chapters 4 and 5 indicated that IGF-1 Eb (in human Ec) is the isoform that is responsible for local muscle growth regulation in response to mechanical stimulation.

## 6.4 IGF-1 and the general anabolic response

As mentioned previously local up-regulation of IGF-1 expression has been implicated as a mediator of not only stretch-induced muscle hypertrophy, but also regeneration (DeVol *et al.*, 1990; Edwall *et al.*, 1989). It is plausible that stretch induced expression of IGF-1 in muscle fibres results in hypertrophy through one or a combination of several mechanisms. First, IGF-1 is known to elicit numerous effects on the metabolism of skeletal muscle that are anabolic in nature such as the stimulation of amino acid and glucose uptake and enhancement of net myofibrillar protein accretion. Therefore, up-regulation of muscle mass by stretch observed in the second study (chapter 3) could be primarily due to the cumulative anabolic effects of IGF-1. Another possibility is that stretch naturally results in a combination of several mechanisms, and IGF-1 is one system of these mechanisms. This explanation is also supported by a finding from a recent study carried by Hantai et al (1995). The result of this study showed that long term (6 weeks) subcutaneous administration of recombinant human IGF-1 (rhIGF-1) (1 mg/kg, each day) to wobbler mice, which has been characterized as a model of lower motoneuron disorder with associated muscle atrophy, results in a increase in body weight and in mean skeletal muscle fibre diameter.

When an *in vitro* model of stretch-induced skeletal muscle growth was used to investigate expression of IGF-1 gene in skeletal muscle cell (Perrone *et al.*, 1995). It was found that the acute release of endogenous IGF-1 with stretch was 20-40-fold more effective than the equivalent amount of recombinant IGF-1 required to stimulate skeletal muscle cell growth. This suggested that the stretch-induced autocrine production of IGF-1 is more biologically active as far as muscle cells than exogenously added recombinant IGF-1. This observation indirectly supported the results presented in chapter 4 and 5 which proposed that E peptide, either as a part of the pre- IGF-1 or as a free peptide may be responsible for local muscle growth regulation in response to mechanical stimulation.

## 6.5 IGF-1 and expression of muscle gene

Although it has been established that muscle gene expression is activity dependent and high expression of IGF-1 mRNA in skeletal muscle is related to overload or stretch, very little is known about the relationship between IGF-1 and skeletal muscle gene expression. An observation about effects of growth hormone treatments on the proportion of the type 1 and type 2 fibre in skeletal muscle of hypophysectomized rats demonstrated that growth hormone treatment, presumably acting through increased expression of IGF-1, elicited an increase in the relative proportion of type 1 fibres (Ayling *et al.*, 1989). A collaborative study was formed with a group (Wahib et al) using the IGF-1 probes designed as described in chapter 2. The subsequent results which involved *in situ* hybridization studies upon muscles sections from growth hormone deficient human patients demonstrated that 70% IGF-1 mRNA is distributed in type 1 fibres. Together these observations indicate that IGF-1 may be related to the expression of specific myosin genes. These also indirectly supported the observation as described in chapter 3 that suggested IGF-1 may play a role in fibre type in transition. Further investigation is needed to address the exact relationship between IGF-1 and muscle gene expression and to determine if there are IGF-1 response elements in the 5' or 3' flanking sequences of different MyHC isoform genes.

## 6.6 A proposal mechanisms of the regulation of endocrine and autocrine/paracrine forms of IGF-1

Since IGF-1 gene was to be found activated in skeletal muscle by overload, it was suggested that modulation of IGF-1 gene expression in skeletal muscle is controlled by (at least ) two mechanisms, one that is dependent on systemic growth hormone and possibly other hormones, whereas the other is a consequence of unknown local factors (DeVol *et al.*, 1990). Both mechanisms lead to the enhancement of IGF mRNAs within muscle, and both play a role in regulation of skeletal muscle growth. The fact of exon 2 of IGF-1 gene is more responsive to growth hormone treatment than exon 1 (Lowe *et al.*, 1987) indicated that exon 2 transcripts encode the

"endocrine" form of IGF-1 which was regulated primarily by growth hormone. The results of third (chapter 4) and fourth studies (chapter 5) indicated that IGF-1Eb (in human Ec) is probably responsible for local muscle growth regulation in response to mechanical stimulation.

## 6.7 Future work

Inappropriate growth and maintenance of groups of muscle mass usually results in postural abnormalities and remain major medical problems. A long term (6 weeks) subcutaneous administration of recombinant human IGF-1 (rhIGF-1) (1 mg/kg, each day) to wobbler mice has been shown to result in a increase in body weight and in mean skeletal muscle fibre diameters (Hantai *et al.*, 1995). The increase in body weight, likely reflects the increase in skeletal muscle mass since the latter represents more than 40% of total body weight. It has also been found that the acute release of endogenous IGF-1 with stretch was 20-40-fold more effective than the equivalent amount of recombinant IGF-1 required to stimulate skeletal muscle cell growth (Perrone *et al.*, 1995). This suggested that the stretch-induced autocrine production of IGF-1 is more biologically active as far as muscle cells than exogenously added recombinant IGF-1. In chapter 4 and 5, IGF-1 Eb (Ec in human) mRNA has been found probably encodes the precursor IGF-1 isoform that is responsible for local muscle growth regulation in response to mechanical stimulation and cDNA for this isoform has been cloned. Recently a new method of gene transference by simple intramuscular injection has been investigated (Wolff *et al.*, 1990; Hansen *et al.*, 1991; Wells *et al.*, 1992). Professor Goldspink's group has shown that the foreign engineered gene under the control of a muscle specific promoter result in good uptake and expression (Hansen *et al.*, 1991; Wells *et al.*, 1992). We can now use this approach to introduce this IGF-1 Eb cDNA into muscle. This gene transfer study will not only enable us to study local action of the muscle growth factor on the motor nerves as well as the muscle fibres but also to eventually develop a gene therapy approach for enhancing muscle growth and possibly maintaining neuronal and bone tissue.

A synthesis of peptides for IGF-1 Eb (Ec in human) supplies anther way for researching the physiological function of this isoform. The PinPoint Xa protein purification system can be used to synthesize and purify the peptide by IGF-1 Eb cDNA sequences. The cDNA will be cloned into downstream of the coding region for the biotinylated segment of the PinPoint Xa vector which encode the preferred cleavage sequence for endoproteinase factor Xa at the carboxyl terminus of the biotinylated region which will be used subsequently to cleave and purify the peptide. As soon as the peptide is synthesized and purified, the physiological function of IGF-1 Eb (Ec in human) can be investigated by intramuscular injection directly or tested on C2C12 myoblasts and other cells (e.g. MT 4Hl cell) in culture. The synthesized and purified peptide also can be used to generate the antibodies for IGF-1 Eb isoform which can be used in the binding studies, physiological studies etc.

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# Appendices

# Appendix 1

## Commonly used buffers and solutions

**20 x SSC**

| | | |
|---|---|---|
| 3.0 M | sodium chloride | |
| 0.3 M | sodium citrate | pH 7 |

**10 x TBE**

| | | |
|---|---|---|
| 0.89 M | boric acid | |
| 0.2 M | Tris | |
| 0.025 M | EDTA | pH 8 |

**10 x MOPS**

| | | |
|---|---|---|
| 0.2 M | 3-[N-morpholino]-propane-sulphonic acid (MOPS) | |
| 0.05 M | sodium acetate | |
| 0.01 M | EDTA | pH 8.3 |

**TE**

| | | |
|---|---|---|
| 0.01 M | Tris HCl | |
| 0.001 M | EDTA | pH 8.0, pH 7.6, pH 7.4 |

**1 x TAE**

| | | |
|---|---|---|
| 0.04 M | Tris acetate | |
| 0.001 M | EDTA | pH 8.5 |

**Denature solution** (used in RNA preparation)

| | | |
|---|---|---|
| 4 M | guanidium thiocyanate | |
| 0.025 M | sodium citrate | pH 7.0 |
| 0.5% | N-lauroyl sarcosin (sodium salt) | |
| 0.1 M | mercaptoethanol (to be added just before use) | |

***In situ* hybridization buffer**

| | | |
|---|---|---|
| 50% | formamide (deionized) | |
| 5x | SSC | |
| 5x | Denhardt's solution | |
| 250 µg/ml | yeast tRNA (sterile-filtered) | |
| 250 µg/ml | salmon sperm DNA (denatured) | |
| 0.004 M | EDTA | |

**TENS**

| | | |
|---|---|---|
| 0.01 M | Tris | pH8.0 |
| 0.001 M | EDTA | |
| 0.1 M | sodium hydroxide | |
| 0.5% | SDS | |

# Appendex 2

# Publications and Abstrcts

**Publications**

YANG, SY., ALNAQEEB, M., SIMPSON, H. & GOLDSPINK, G. (1996) Cloning and characterization of an IGF-I isoform expressed in skeletal muscle subjected to stretch. *Journal of Muscle Research and Cell Motility* **17**, 487-495.

GOLDSPINK, G., YANG, SY., SKARLI, M. & VRBOVA, G. (1996) Local growth regulation is associated with an isoform of IGF-I that is expressed in normal muscle but not in dystrophic mdx or dydy mouse muscle when subjected to stretch. *Journal of Physiology* **495.P**, 162p-163p.

YANG, SY., ALNAQEEB, M., SIMPSON, H. & GOLDSPINK, G. (1996) Changes in muscle fibre type, muscle mass and IGF-I gene expression in rabbit skeletal muscle subjected to stretch. *Journal of Anatomy* (in press).

**Abstracts**

Shiyu Yang and Geoffrey Goldspink **Expression of IGF-1 mRNA in stretched skeletal muscle** 14th alternative Muscle Club, University of Newcastle-upon-tyne, 19-21 December 1993.

Shiyu Yang and Geoffrey Goldspink **Localization of IGF-1 mRNA in skeletal muscle during muscle growth in response to stretch** 15th Alternative Muscle Club, University of Leeds 18-21 December 1994.