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Cloning and characterization of an IGF-1 isoform expressed in skeletal muscle subjected to stretch

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Summary

To ascertain if IGF-1 is a regulator of local muscle growth, total RNA was extracted from rabbit muscle induced to undergo rapid hypertrophy using active stretch and from control muscles. This was analysed by Northern hybridization with a 280 base pair probe containing sequences derived from exons 3 and 4 of the insulin-like growth factor 1 gene. Two types of insulin-like growth factor 1 mRNA were shown to be strong expressed in the stretched muscles. *In situ* hybridization using the same probe (280 base pair) showed that IGF-1 is strongly expressed in muscle that is induced to grow rapidly and is expressed in the muscle fibres themselves. Using RT-PCR a single insulin-like growth factor 1 isoform cDNA (IGF-1Ea) could be cloned from the normal resting muscles. However, an additional isoform of insulin-like growth factor 1 (insulin-like growth factor 1Eb) was found to be expressed in stretched muscle undergoing hypertrophy. The E domain sequence of the additional isoform differs from the liver insulin-like growth factor 1Ea by the presence a 52 base pair insert. This changes the reading frame of the derived carboxyl-terminal resulting in a different precursor insulin-like growth factor 1 isoform. This insulin-like growth factor 1 mRNA probably encodes the precursor insulin-like growth factor 1 isoform that is responsible for local muscle growth regulation in response to mechanical stimulation. To confirm that alternative splicing of the insulin-like growth factor 1 gene occurs in muscle in response to physical activity, oligonucleotide primers were made which specifically amplify the cDNAs of two isoforms (insulin-like growth factors 1Ea and Eb) in the human as well as the rabbit. Following altered physical activity for 2 h to 6 days, appreciable levels of insulin-like growth factor 1Eb (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.

Introduction

Muscle is a tissue which responses to physical activity. Also a large body of literature indicates that insulin-like growth factor 1 (IGF-1) is a major regulator of skeletal muscle growth and maintenance (Loughna *et al.*, 1992). This study was carried out to elucidate the link between the mechanical signal(s) and the regulation of muscle growth. Mature IGF-1 is a 70-residue polypeptide with important functions in the regulation of somatic growth, development and differentiation. The liver is a primary target for pituitary growth hormone (GH) which is stimulated to synthesize IGF-1. The resultant increase in circulating level of IGF-1 promotes cell division and is a major factor in regulating the growth of the body as a whole. In several tissues there is also apparently a local system of growth regulation e.g. skeletal muscle which is able to undergo rapid hypertrophy to adapt to overload. It was, therefore, important to investigate the role of IGF-1 in the locally regulated growth response.

Information on the organization of the IGF-1 gene is now available for several species, among which the human and rat genes are most extensively studied (De Pagter-Holthuizen *et al.*, 1986; Rotwein *et al.*, 1986; Shimatsu & Rotwein, 1987; Tobin *et al.*, 1990; Jansen *et al.*, 1991). The structure of the IGF-1 gene is well conserved among mammals and contains several notable features, including an unexpectedly large size and the presence of alternatively spliced exons. In humans the IGF-1 gene comprises at least six exons (designated exon 1, 2, 3, 4, 5 and 6) spanning a region of over 90 kilobases

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(kb) of genomic DNA. Exons 1 and 2 are alternative leader exons (Tobin *et al.*, 1990; Jansen *et al.*, 1991) with distinct transcription start sites which are differentially spliced to the common exon 3 and produce class 1 and class 2 IGF-1 mRNA transcripts respectively (Adamo *et al.*, 1991; Dickson *et al.*, 1991; Weller *et al.*, 1993). Exons 3 and 4 code for the mature IGF-1 peptide (B, C, A and D domains) as well as the first 16 amino acid of the E domain. Exons 5 and 6 each encodes an alternative part of a distinct extension peptide, named the E domain. This is followed by the termination codons of precursor IGF-1, 3′ untranslated regions and poly(A) addition signal sites (Rotwein *et al.*, 1986). Sequence analyses of IGF-1 peptide purified from human plasma demonstrated that mature IGF-1 contains A, B, C and D domains. The A and B domains are homologous to the A and B chains of insulin (Rinderknecht & Humbel, 1978).

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 (Jansen *et al.*, 1983; Bell *et al.*, 1984; Roberts *et al.*, 1987). 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 known 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 and localized fibre type expression.

Materials and methods

Animals and muscle stretch procedure

New Zealand white rabbits were 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. As was previously reported this results in a 35% increase in muscle mass within a few days (Goldspink *et al.*, 1992). After 6 days the rabbits were killed by intravenous injection of an over dose of sodium pentobarbitone into the marginal ear vein. The EDL was immediately dissected out from both hind legs. The right hind leg served as the control. Each muscle was cut transversely into two parts, one part was fixed in freshly prepared 4% paraformaldehyde fixative at 4° C for 2 h and later processed and embedded in paraffin wax. The second part was packed into a 1.5 ml tube and directly frozen in liquid nitrogen and stored at −70° C to await total RNA isolation.

RNA isolation

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).

Synthesis of probes for Northern blot and in situ hybridization

The oligonucleotide 5′TTGGGCATGTCAGTGTGG 3′ which is complementary to the sequence of exon of the IGF-1 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 oligonucleotide primers (5′GCTTGCTCACCTTTACCAGC3′ and 5′TTGGGCATGTCAGTGTGG3′). A 280 base pair (bp) PCR product covering exon 3 and part of exon 4 of the IGF-1 gene was subcloned into pBS+ phagemid vector (Stratagene) including T_3 and T_7 promoters. Labelled sense

and antisense RNA probes were synthesized by *in vitro* transcription with RNA polymerase using digoxigenin labelled uridine-triphosphate as substrate (Boehringer Mannheim) according to the manufacturers instructions. These probes were used for both Northern blotting and *in situ* hybridization.

Northern blotting

Samples containing the same amount (20 μg) of total RNA were subjected to Northern blotting. The 280 bp antisense probe described above was used. Prehybridization (1 h) and hybridization (15 h) were carried out at 68° C in hybridization buffer (50% formamide; 5 × SSC; 2% blocking reagent; 0.1% N-lauroylsarcosine; 0.02% SDS). Washing was carried out at high stringency 2 × 5 min at room temperature with 2 × SSC and 0.1% SDS, 2 × 15 min at 68° C with 0.1 × SSC and 0.1% SDS. The hybridized probe was detected by chemiluminescence according to the manufacturers instructions (Boehringer Mannheim). The blot filter was exposed to X-ray film for 6 h.

In situ hybridization

The muscle tissues were cut in 10 μm sections. Both transverse and longitudinal sections were taken and mounted onto autoclaved slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma). The sections were dewaxed by washing in xylene three times for 2 min each and rehydrated in a series of methanol solutions. The sections were then denatured by incubating in 0.2 N HCl at room temperature for 20 min, heated in 2 × SSC at 70° C for 20 min and digested for pronase (10 μg ml^{-1}, Boehringer Mannheim) in 50 mM Tris HCl for 15 min and was finally placed in 0.1 M triethanolamine (TEA) buffer, to which acetic anhydride was added to a final concentration of 0.5% and incubated for 10 min in order to block polar and charged groups in the sections. Hybridization was carried out in hybridization buffer (50% deionised formamide; 5 × SSC; 5 × Denhardt's solution; 250 μg ml^{-1} yeast t-RNA; 250 μg ml^{-1} denatured salmon sperm DNA; 4 mM ethylenediaminetetraacetic acid EDTA)) containing the DIG-labelled antisense or sense RNA probe consisting of the 280 bp sequence derived from exons 3 and part of exon 4 of the IGF-1 gene. The final concentration of probe was 1000 ng ml^{-1}. The hybridization was carried out at 68° C for 1 h and then allowed to cool down to 42° C at which it was kept overnight in a human chamber. After hybridization the sections were incubated with RNase A (100 μg ml^{-1}, Sigma) to remove the unbound single strand RNA probe. Washing was carried out at high stringency, 25 min in 2 × SSC at room temperature, 15 min in 1 × SSC at room temperature, 30 min in 0.5 × SSC at 42° C and 30 min in 0.5 × SSC at room temperature. The hybridized probe was detected by anti-digoxigenin-AP antibody conjugate, Fab fragments (1.5 U ml^{-1}, Boehringer Mannheim), according to manufacturers instructions.

Synthesis and molecular cloning of muscle IGF-1 cDNA

The first strand cDNA was synthesized by reverse transcriptase (RAV-2, Amersham) from muscle total RNA with oligo dT primers and then amplified by the 3′ rapid amplification of cDNA ends polymerase chain reaction (3′RACEPCR) with an IGF-1 gene specific primer (5′GCTTGCTCACCTTTACCAGC3′) which is part of the 5′ end sequence of exon 3 of IGF-1. The PCR products were cloned into the pCR™ vector (Invitrogen) for DNA sequencing. Fragments were later sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977). A total of 98% of the DNA sequence was obtained on both strands.

Experiments to confirm the differential splicing of the IGF-1 gene in muscle subjected to altered mechanical activity

The rabbit EDL muscle was subjected to immobilization in the lengthened position for 6 days. Total RNA was extracted and subjected to RT-PCR (Goldspink *et al.*, 1995) using primers which were designed based on the sequence data presented in Fig. 3. These primers enabled the selective amplification of either the IGF-1Ea or the IGF-1Eb (in human, IGF-1Ec; see Chew *et al.*, 1995) cDNA. The 5′ primer sequence (5′GCTTGCTCACCTTTACCAGC3′) which is part of the 5′ end sequence of exon 3 of IGF-1 gene, was common. For the IGF-1Ea the sequence of the 3′ primers was 5′AAATGTACTTCCTTCTGGGTCT3′ which is complementary to the part of exons 4 and 6 of the IGF-1 gene, and for the IGF-1Eb the sequence of the 3′ primer was 5′AAATGTACTTCCTTTCCTTCTC3′ which is complementary to the part of exons 4 and 5 of the IGF-1 gene. The PCR reaction was performed for 35 cycles in thermal reactor (HYBAID) and using the following parameters: 94° C for 1 min; 60° C for 1 min; and 72° C for 1 min. The product of PCR for IGF-1Ea (301 base pairs), and for the IGF-1Eb (353 base pairs) were separated on 1.5% agarose gels. The sequences of these two products were confirmed by Southern bloting using an IGF-1 cDNA which included part of exons 3 and 4 and excluded the primer sequence regions.

These primers were designed so that they would also be appropriate for human exercise experiments. As a initial experiment for forearm flexors muscles of a volunteer were subjected to eccentric exercise using a weighted pully system and a syringe needle aspiration sample (Ennion *et al.*, 1994) was taken from the biceps brachii muscles after 2 h. Total RNA was isolated from the syringe needle aspiration sample and analysed by RT-PCR as for the rabbit muscle.

Results

Northern blotting

The results of Northern blot analysis performed with RNA extracted from normal and stretched EDL are depicted in Fig. 1. 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.

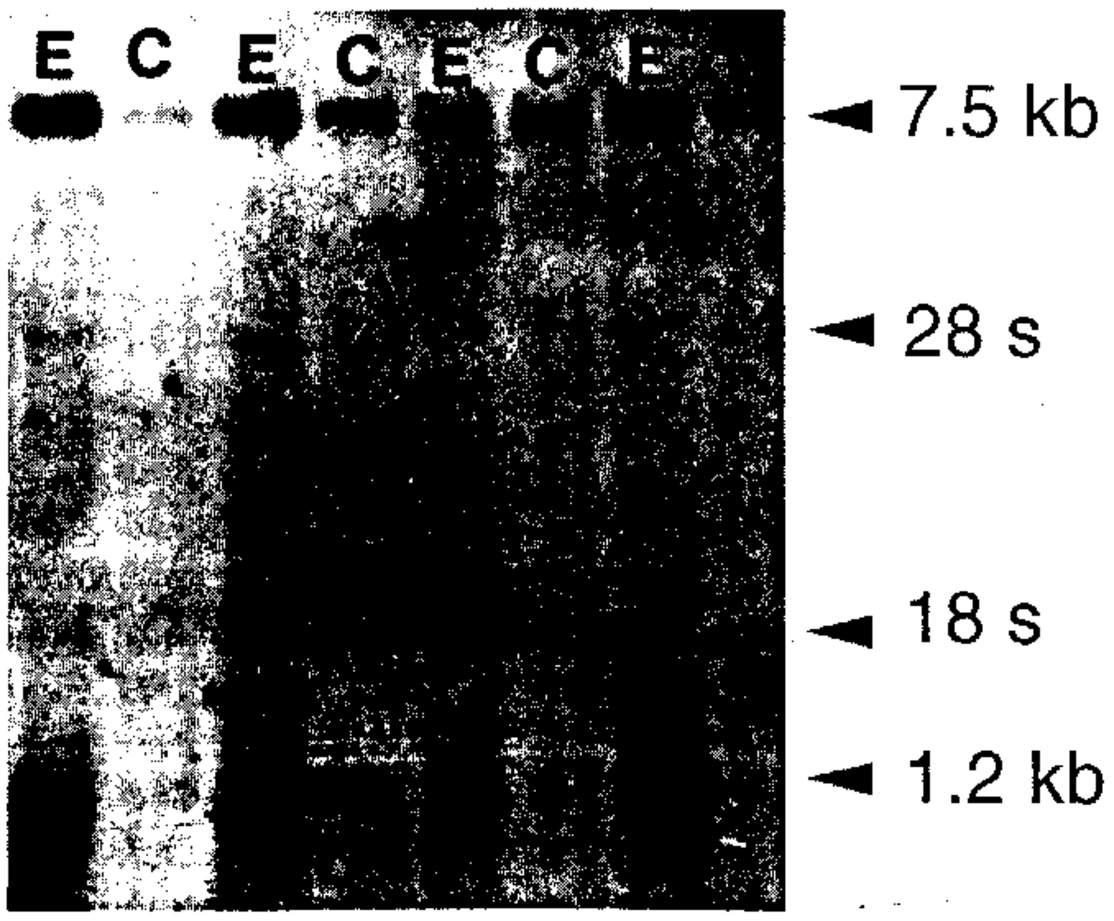

Fig. 1. Expression of IGF-1 mRNA studied by Northern blotting in stretched (E) and control (C) EDL muscle.

Localization of IGF-1 mRNA in normal and stretched 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. 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 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.

Molecular cloning of muscle IGF-1 cDNAs

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 exons 3–6 (which includes the E domain as well as the ligand binding site) 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-1Eb. 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. 3. The sequence may be divided into three 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, 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).

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

Although the RT-PCR method used cannot be regarded as quantitative, it clearly showed that the IGF-1Eb (in human the Ec) isoform was up-regulated following stretch in the case of the rabbit muscles and eccentric exercise in the case of the human muscle. Even with very sensitive PCR methods, only trace of IGF-1Eb was detected in the control muscle (which had still been subjected to mild activity). In contrast although IGF-1Ea was detected at about the same level in all muscles (Fig. 5). These experiments, which will be published in full at a later date, confirmed that the mechanical activity resulted in alterative splicing and that the mRNA for IGF-1Eb (IGF-IEC in the human) was up-regulated.

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

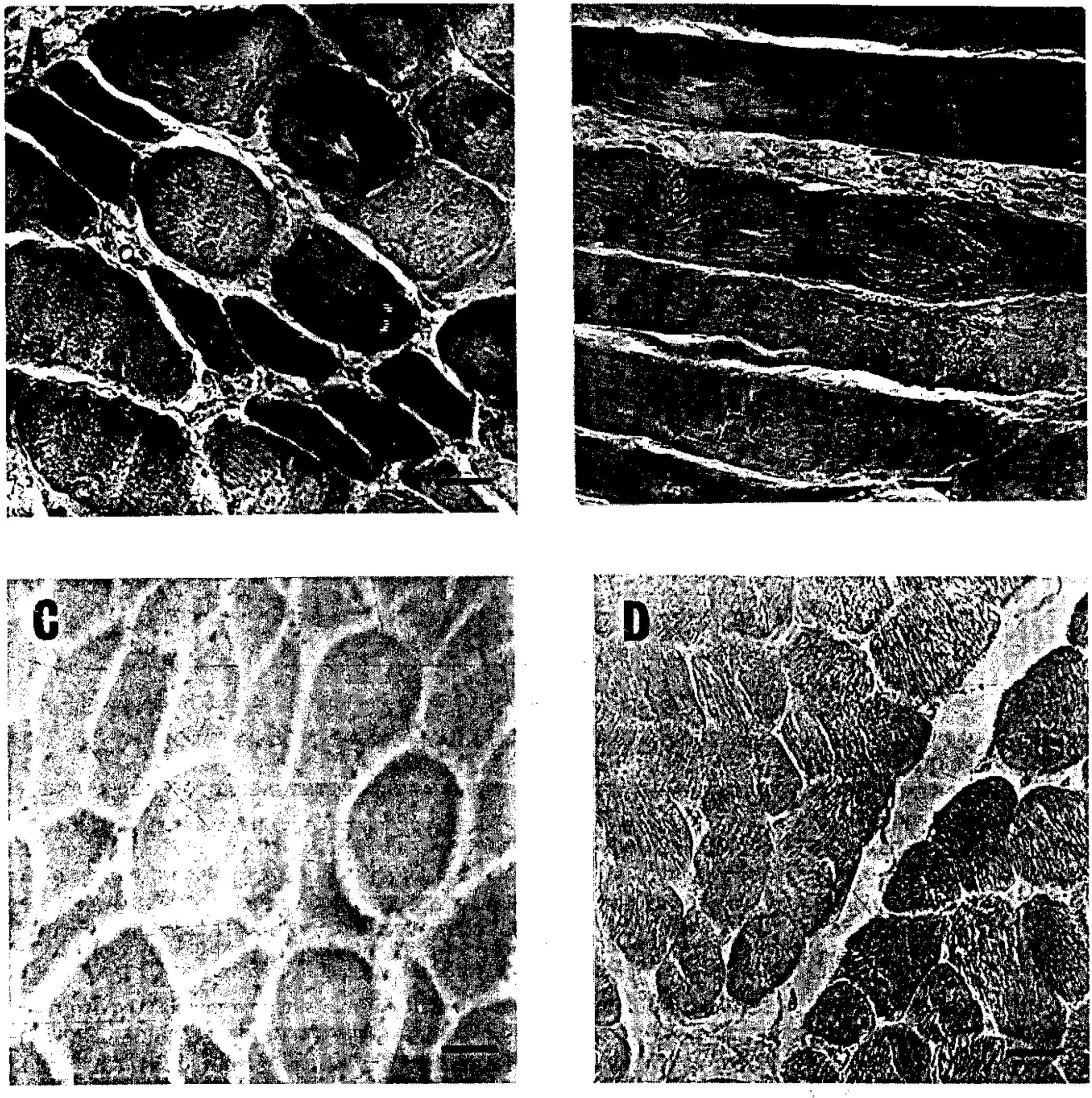

Fig. 2. Localization and distribution of IGF-1 mRNA in stretched (A, transverse section; B, longitudinal section) and control (C) EDL muscle. The sense RNA probe from the same clone was used on the stretched muscle (D) as a negative control. Scale bar = 30 μm.

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. 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 IGF-1Eb (in human the Ec) isoform within a time as short as two hours indicates that its up-regulation is not a consequence of regeneration involving the proliferation of satellite cells, although it may initiate regeneration in case of major injury. 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). Longitudinal growth of skeletal muscle is facilitated by the addition of new sarco-

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TTGCTCACCTTTACCAGCTCGGCCACAGCCGGACCGGAGACGCTCTGCGGTGCTGAGCTG   60
LeuLeuThrPheThrSerSerAlaThrAlaGlyProGluThrLeuCysGlyAlaGluLeu
                                    B-->

GTGGATGCTCTTCAGTTCGTGTGTGGAGACAGGGGCTTTTATTTCAACAAGCCCACAGGA  120
ValAspAlaLeuGlnPheValCysGlyAspArgGlyPheTyrPheAsnLysProThrGly
                                                        C->

TACGGCTCCAGCAGTCGGAGGGCACCTCAGACAGGCATCGTGGATGAGTGCTGCTTCCGG  180
TyrGlySerSerSerArgArgAlaProGlnThrGlyIleValAspGluCysCysPheArg
                                    A-->

AGCTGTGATCTGAGGAGGCTGGAGATGTACTGTGCACCCCTCAAGCCGGCAAAGGCAGCC  240
SerCysAspLeuArgArgLeuGluMETTyrCysAlaProLeuLysProAlaLysAlaAla
                                    D-->

                                                288
CGCTCCGTCCGTGCCCAGCGCCACACCGACATGCCCAAGACTCAGAAGTATCAGCCTCCA  300
ArgSerValArgAlaGlnArgHisThrAspMETProLysThrGlnLysTyrGlnProPro
E-->                                                    Eb-->
                                        340
TCTACCAACAAGAAAATGAAGTCTCAGAGGAGAAGGAAAGGAAGTACATTTGAAGAACAC  360
SerThrAsnLysLysMETLysSerGlnArgArgArgLysGlySerThrPheGluGluHis
                                                GluValHisLeuLysAsnTh
                                        Ea-->                   ◆ ◆

AAGTAGAGGGAGTGCAGGAAACAAGAACTACAGGATGTAGGAAGACCCTTCTGAGGAGTG  420
Lysend
rSerArgGlySerAlaGlyAsnLysAsnTyrArgMETend
  ◆
AAGAAGGACAGGCCACCGCAGGACCCTTTGCTCTGCACAGTTACCTGTAAACATTGGAAT  480
ACCGGCCAAAAAATAAGTTTGATCACATTTCAAAGATGGCATTTCCCCCAATGAAATACA  560
CAAGTAAACATTC
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Fig. 3. 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 bp 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.

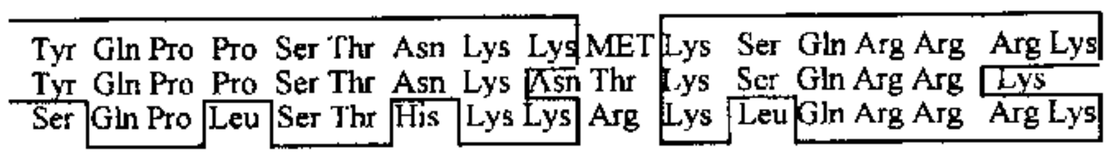

Fig. 4. Alignment of the three derived amino acid sequences of the inserts from rat liver (bottom), human liver (middle) and rabbit stretched muscle (top). Identical amino residues are shown by the boxes.

meres to the ends of the existing myofibrils (Williams & Goldspink, 1971, 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.

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 the 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.

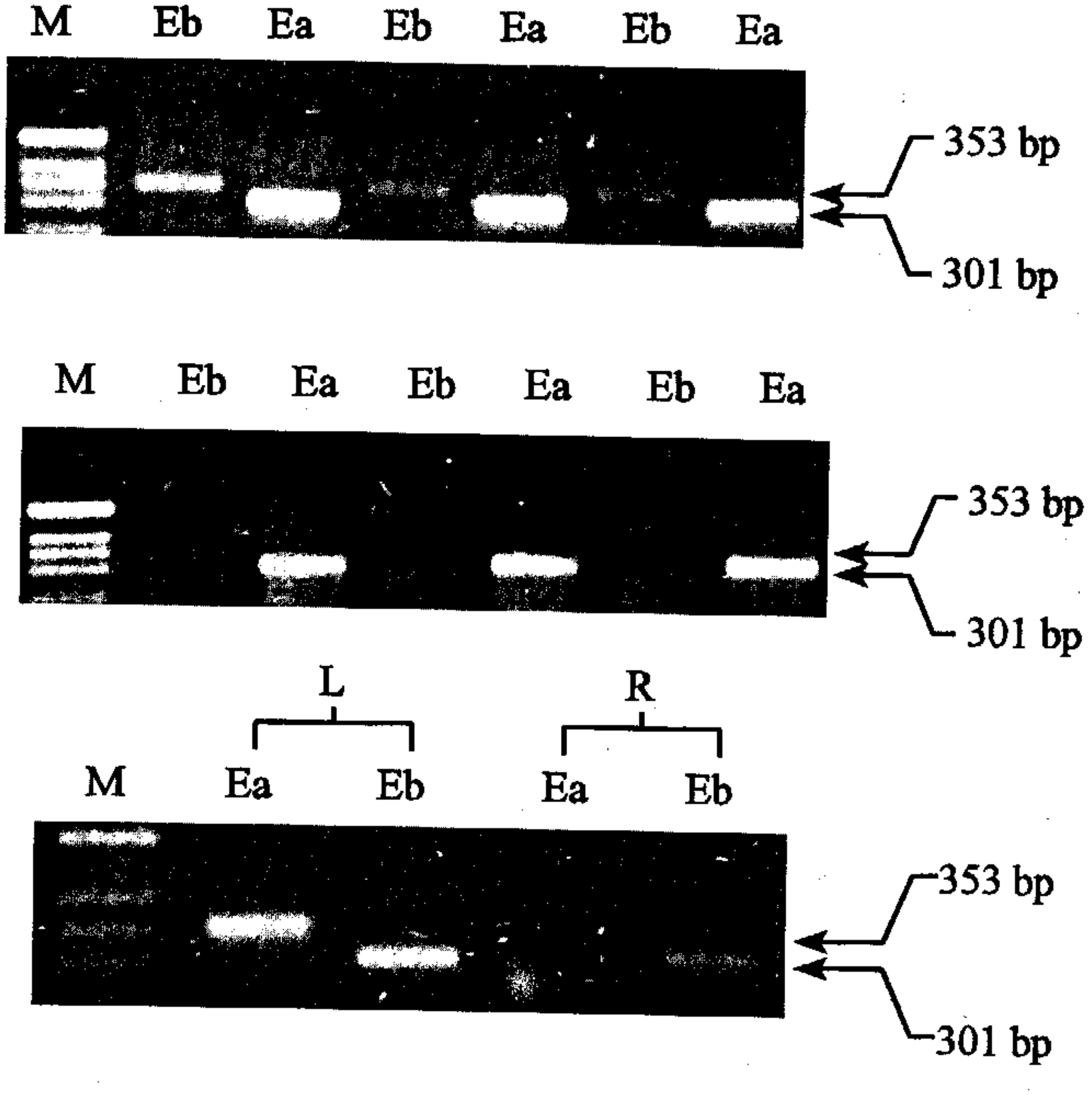

Fig. 5. Using RT-PCR a 353 bp product specific for IGF-1Eb (Eb) was detected in rabbit muscle after it was subjected to stretch for 6 days (top row). No appreciable signal for this isoform could be detected in contralateral muscles (middle row). In contrast the 301 bp product of PCR for IGF-1Ea (Ea) was present in both stretched and control muscles. The PCR product for IGF-1Ec (Eb) was also up-regulated in human muscle when it was subjected to eccentric exercise for 2 h (bottow row, left), but not to be detected in contralateral control muscle (botton row, right). IGF-1Ea (Ea) was again detected in both exercised and control muscles. The sequences of these two products was confirmed by Southern blotting using an IGF-1 cDNA probe excluding the sequence of the primers used in PCR.

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 splicing acceptor site 52-bp from the 3′-end of the pertinent intron. Firstly, 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 and colleagues (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 and colleagues (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-1Eb 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 and colleagues (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 mechanical stimulation, 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.

It is apparent from our study that the different E peptides may play different roles in IGF-1 externalization and 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.

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