Sequencing of cDNA Clones Expressed in Adipose Tissues of Korean Cattle

J. J. Bong, K. Tong, K. K. Cho[1] and M. G. Baik*

Department of Applied Bioscience and Biotechnology, Biotechnology Research Institute, Inst. of Ag. Sci. and Tech. Chonnam National University, Gwangju 500-757, Korea

ABSTRACT : To understand the molecular mechanisms that regulate intramuscular fat deposition and its release, cDNA clones expressed in adipose tissues of Korean cattle were identified by differential screening from adipose tissue cDNA library. By partial nucleotide sequencing of 486 clones and a search for sequence similarity in NCBI nucleotide databases, 245 clones revealed unique clones. By a functional grouping of the clones, 14% of the clones were categorized to metabolism and enzyme-related group (stearoyl CoA desaturase, lactate dehydrogenase, fatty acid synthase, ATP citrate lyase, lipoprotein lipase, acetyl CoA synthetase, etc), and 6% to signal transduction/cell cycle-related group (C/EBP, cAMP-regulated phosphoprotein, calmodulin, cyclin G1, cyclin H, etc), and 4% to cytoskeleton and extracellular matrix components (vimentin, ankyrin 2, gelosin, syntenin, talin, prefoldin 5). The obtained 245 clones will be useful to study lipid metabolism and signal transduction pathway in adipose tissues and to study obesity in human. Some clones were subjected to full-sequencing containing open reading frame. The cDNA clone of bovine homolog of human prefoldin 5 gene had a total length of 959 nucleotides coding for 139 amino acids. Comparison of the deduced amino acid sequences of bovine prefoldin 5 with those of human and mouse showed over 95% identity. The cDNA clone of bovine homolog of human ubiquitin-like/S30 ribosomal fusion protein gene had a total length of 484 nucleotides coding for 133 amino acids. Comparison of the deduced amino acid sequences of bovine ubiquitin-like/S30 ribosomal fusion protein gene with those of human, rat and mouse showed over 97% identity. The cDNA clone of bovine homolog of human proteolipid protein 2 mRNA had a total length of 928 nucleotides coding for 152 amino acids. Comparison of the deduced amino acid sequences of bovine proteolipid protein 2 with those of human and mouse showed 87.5% similarity. The cDNA clone of bovine homolog of rat thymosin beta 4 had a total length of 602 nucleotides coding for 44 amino acids. Comparison of the deduced amino acid sequences of bovine thymosin beta 4 gene with those of human, mouse and rat showed 93.1% similarity. The cDNA clone of bovine homolog of human myotrophin mRNA had a total length of 790 nucleotides coding for 118 amino acids. Comparison of the deduced amino acid sequences of bovine myotrophin gene with those of human, mouse and rat showed 83.9% similarity. The functional role of these clones in adipose tissues needs to be established. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 4 : 483-489)***

Key Words : Korean Cattle, Bovine cDNA Sequencing, Adipose Tissues

INTRODUCTION

Recent advances regarding the biology of adipose tissue have identified the adipocyte as an important mediator in many physiological and pathological processes regarding energy metabolism (Morrison and Farmer, 2000). Adipocyte is not only metabolically very active, responding quickly to hormonal stimuli in a metabolic interplay with the liver, skeletal muscles, and the heart but it also play major roles in lipid metabolism in the storage of free fatty acid as triacylglycerol and in glucose metabolism. The structural and morphogenesis associated with adipocyte differentiation involves changes in the expression levels of about 300 proteins. Many of these changes occur at the level of gene expression through a series of molecular events involving several transcription factor families that exhibit diverse modes of activation and function. However, the changes have not been understood in bovine adipose tissues. Endocrine function of adipose tissues plays a key role in obesity and associated disorders, and it emphasizes the need for further identification of novel factors produced by adipocyte and characterization of the transcription factors that modulate adipocyte differentiation, function and metabolism (Kim and Moustaid-Moussa, 2000), and several gene function was studied in association with adipose tissue differentiation (Wang et al., 2004).

Quality of beef is dependent on the amount of marbling present in muscle as well as carcass grade. The deposition of intramuscular adipose tissue (marbling) is positively related to beef flavor and palatability (Judge et al., 1989) and it is an important factor for high quality beef, especially in Korean cattle and Japanese Black cattle. However, the molecular mechanism that regulates intramuscular fat deposition and its release has not been well studied. Differences in gene expression have been reported at various adipose tissues. For example, castration in rats resulted in a 50% decrease of the obese gene mRNA level in subcutaneous adipocytes, but resulted in a 92% increase in perirenal adipocytes (Machinal et al., 1999). Our previous results also showed regional differences in obese gene expression in bovine adipose tissues. Moderate levels

* Corresponding Author: M. G. Baik. Tel: +82-62-530-2164, Fax: +82-62-530-2169, E-mail: mgbaik@chonnam.ac.kr

[1] Department of Animal Resources Technology, Jinju National University, Gyeongnam 660-758, Korea.

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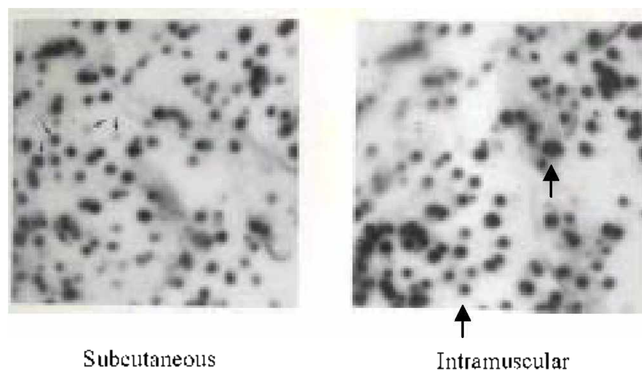

Figure 1. Differential screening of cDNA library of bovine adipose tissues. Approximately 2,000 pfu of phage solution were plated. Duplicate filters were prepared from the same plate and hybridized with ^{32}P-labeled cDNA probes prepared from subcutaneous mRNA or intramuscular mRNA. The arrow heads indicate plaques that showed higher messages in subcutaneous and intramuscular cDNA probe, respectively.

of obese gene mRNA were observed in subcutaneous tissues, but expression levels were very low in intramuscular adipose tissues (Kim et al., 2000). We also produced protein (leptin) of bovine obese gene in *E. coli* (Kim and Baik, 2004).

In Korea, the steer (castrated cattle) is slaughtered at older age and heavier live weight than the normal in order to increase the degree of marbling. But, the increase of slaughter age results in adverse effect in meat yield grade because of increase in back fat thickness (subcutaneous fat depot). Therefore, it is essential to develop method for increasing marbling without adverse effect on yield grade. To produce high marble beef, a study is required to understand the differential mechanism that regulates the deposition and release of fat between intramuscular tissues and external adipose tissues, especially subcutaneous adipose tissues.

The current study was performed to understand molecular events that regulate the deposition of fat and its release in adipose tissues. The primary objective was to identify genes expressed in adipose tissues by differential screening of bovine adipocyte cDNA library. Clones that showed positive signals at either intramuscular tissues or subcutaneous adipose tissues were identified by differential screening, and further characterized by nucleotide sequencing.

MATERIALS AND METHODS

Tissue samples

Nine steer of Korean cattle were used. Immediately after slaughter, pieces of *longissimus dorsi* muscle tissues were obtained. Subcutaneous adipose tissues were collected by dissection. Piece of intramuscular fat tissues were dissected from muscle tissues. Care was taken not to contaminate with muscular tissues and intramuscular fat tissues connected to them. After tissue samples were homogenized in the denaturing solution (guanidinium thiocyanate, Na citrate, 10% sarcosyl, 2-mercaptoethanol), the samples were stored at -70°C.

Differential screening and sequencing

Total RNA was prepared from adipose tissues by acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly (A) RNA was extracted from total RNA using Poly (A) Quit® mRNA Isolation Kit (Stratagene). The 2.5 volume of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) were added to the mRNA solution and the mRNA sample was stored at -70℃ and later used for ^{32}P-labeled cDNA probe.

Subcutaneous and intramuscular cDNA clones were isolated by differential screening (Kim et al., 2001). Bovine adipose tissue cDNA library was previously constructed (Kim et al., 2000, 2001) and used here for the differential screening. Briefly, the library was plated with XL1-Blue cells, and phage DNA was transferred to the membrane in duplicate. Differential hybridization of the membrane was done with ^{32}P-labeled cDNA probe that had been prepared from either mRNAs of subcutaneous or intramuscular adipose tissues. The membranes were washed twice in 2×SSC/0.1% SDS at room temperature for 10 min, twice in 2×SSC/0.1% SDS at 42℃ for 30 min, and twice in 0.1×SSC/0.1% SDS at 65℃ 30 min. After exposure to X-ray film at -70℃ for 48h, the subcutaneous adipose tissue positive-plaques were identified and picked and stored at 4℃ in 200 $\mu\ell$ of phage dilution buffer with 2% chloroform. In the same way, the intramuscular adipose tissue positive-plaques were also picked.

The λDNA containing the cDNA insert was converted into the plasmid by in vivo excision. The conversion of a λZAP Express clone to a pBK-CMV clone was involved in vivo excision and circularization of a complete plasmid from the recombinant phage. The plasmid was isolated using alkaline lysis method. The plasmid DNA was digested with EcoR I and Xho I restriction enzyme, and checked for size of insert.

Partial nucleotide sequences of clones at 5' region of cDNA insert were analyzed by using T3 primer and automatic sequencing protocol (Bioneer, Korea). The selected clones were subjected to nucleotide sequencing using T7 primer in order to obtain full-opening reading region. The sequence of each clone was compared to the sequence of NCBI data.

RESULTS AND DISCUSSION

The cDNA clones expressed in adipose tissues of Korean cattle were identified by differential screening method from adipose tissue cDNA library (Figure 1). The

Table 1. Functional grouping of the cDNA clones expressed in adipose tissues of Korean cattle

Grouping	Percentage of clones (Number)	Gene list
Metabolism and enzyme	14 (34)	Stearoyl-CoA desaturase, lactate dehydrogenase B, fatty acid synthase, ATP citrate lyase, creatine kinase, ADP/ATP translocase, lipoprotein lipase, NADH dehydrogenase, glyceraldehyde phosphate dehydrogenase, acetyl CoA shythetase, fatty acid binding protein, peroxisome biogenesis gene 1, lipoate-activating enzyme, low density lipoprotein-related protein 1, preproadipsin, proteolipid protein 2, adipose tissue-specific protein adipoQ mRNA, etc.
Signal transduction and cell cycle	7 (16)	class I cytokine receptor, protein phosphatase I, insulin-like growth factor binding protein-3, calcium binding protein 1, CCAAT/ enhancer binding protein, retinoic acid receptor responder, cAMP-regulated phosphoprotein, rod photoreceptor transducin alpha1 subunit, stress-induced phosphoprotein 1, thyroid hormone receptor interactor 6, calmodulin, chloride intracellular channel 4, cyclin G1, cyclin H
Cytoskeleton and extracellular matrix components	4 (12)	vimentin, ankyrin 2, gelosin, syntenin, tubulin alpha, type III pro-collagen, collagen type IV alpha1 chain, alpha actin precursor, talin, microtubule-associated protein actin alpha 2, intercellular adhesion molecule precursor, prefoldin 5
Ribosomal proteins	3 (8)	ribosomal protein L17, ribosomal protein L18a, etc.
Mitochondrial genes and others	(76)	Cytochrome oxidase, cytochrome c 1, heat shock protein 27, polyubiquitin, osteonectin, etc.

486 clones were subjected to partial nucleotide sequencing by using T3 primer and automatic sequencing protocol. Sequence similarity was compared with NCBI nucleotide databases, and some genes were abundant: 9 clones were osteonectin cDNA. 6 clones were clone that showed high similarity with human tumor protein translationally controlled 1, 5 clones were elongation factor alpha, 4 clones were clone that showed high similarity with pig mRNA for four and a half LIM domains 1 protein, isoform C, 4 clones were vimentin mRNA, 3 clones were hydroxyacyl glutathione hydrolase, 3 clones were lactate dehydrogenase B, and 3 clones were adipocyte-type fatty acid binding protein. And the 245 clones revealed unique clones. About 17% (42 clones) was identified as known genes in bovine species, over 60% (148 clones) as known genes in human, and 10% (23 clones) of clones revealed the unknown genes. By a functional grouping of the clones (Table 1), 14% (34 clones) were categorized to metabolism and enzyme-related group (stearoyl-CoA desaturase, lactate dehydrogenase B, fatty acid synthase, ATP citrate lyase, creatine kinase, ADP/ATP translocase, lipoprotein lipase, etc), 6% (17 clones) to signal transduction/cell cycle-related group (protein phosphatase I, CCAAT/enhancer binding protein (C/EBP), cAMP-regulated phosphoprotein, cyclin G1, cyclin H, etc), and 4% (11 clones) to cytoskeleton and extracellular matrix components (vimentin, ankyrin 2, gelosin, talin, prefoldin 5, etc). The obtained 245 clones will be useful to study lipid metabolism and signal transduction pathway in adipose tissues and to study obesity in human.

The five clones that showed similarity to other species were subjected to full sequencing in order to obtain nucleotide sequence information of open-reading frame. One clone had a total length of 957 nucleotides coding for 154 amino acids (Figure 2A). It was identified as one encoding bovine prefoldin 5 gene. Comparison of the deduced amino acid sequences of bovine prefoldin 5 with those of human (Vainberg et al., 1998) and mouse (Carninci and Hayashizaki, 1999) showed over 95% identity (Figure 2B).

The cDNA clone of bovine homolog of human ubiquitin-like/S30 ribosomal fusion protein gene had a total length of 482 nucleotides coding for 133 amino acids (Figure 3A). Comparison of the deduced amino acid sequences of bovine ubiquitin-like/S30 ribosomal fusion protein gene with those of human (Kas et al., 1992), pig (Chwetzoff and d'Andrea, 1997), rat (Olvera and Wool, 1993) and mouse (Michiels et al., 1993) showed over 97% identity (Figure 3B).

The cDNA clone of bovine homolog of human proteolipid protein 2 mRNA had a total length of 928 nucleotides coding for 152 amino acids (Figure 4A). Comparison of the deduced amino acid sequences of bovine proteolipid protein 2 gene with those of human (Oliva et al., 1993) and mouse (Carninci et al., 2000) showed 87.5% similarity (Figure 4B). The cDNA clone of bovine homolog of rat thymosin beta 4 had a total length of 602 nucleotides coding for 44 amino acids (Figure 5A). Comparison of the deduced amino acid sequences of bovine thymosin beta 4 gene with those of human (Friedman et al., 1984), mouse (Rudin et al., 1990), and rat (Atkinson et al., 1990) showed 93.1% similarity (Figure 5B). The cDNA clone of bovine homolog of human myotrophin mRNA had a total length of

Figure 2. Nucleotide sequences of bovine prefoldin 5 cDNA and comparison of its amino acid sequences with other species. A. The amino acid was aligned with the second nucleotide of each codon. Asterisk (*) marks stop codon. The start codon, stop codon and polyadenylation signal sequences are underlined. The sequences have been deposited in the NCBI data base (accession no. AF520958). B. Comparison of the amino acid sequences with other species. Dot denotes the amino acid residue identical to that of bovine species.

Figure 3. Nucleotide sequences of bovine ubiquitin-like/S30 ribosomal fusion protein cDNA and comparison of its amino acid sequences with other species. A. The amino acid was aligned with the second nucleotide of each codon. Asterisk (*) marks stop codon. The start codon, stop codon and polyadenylation signal sequences are underlined. The sequences have been deposited in the NCBI data base (accession no. AF520959). B. Comparison of the amino acid sequences with other species. Dot denotes the amino acid residue identical to that of bovine species

790 nucleotides coding for 118 amino acids (Figure 6A). Comparison of the deduced amino acid sequences of bovine myotrophin gene with those of human (Anderson et al., 1999), mouse (Pennica et al., 1995), and rat (Taoka et al., 1992) showed 83.9% similarity (Figure 6B).

Prefoldin (GimC) is a hexameric molecular chaperone complex built from two related classes of subunits and present in all eukaryotes and archaea (Siegert et al., 2000). Function of prefoldin is to deliver nonnative target proteins, principally actins and tubulins, to the eukaryotic cytosolic chaperonin (CCT) for facilitated folding (Hansen et al., 1999). During the course of actin synthesis, several distinct actin-containing species were observed and the composition of each was determined by immunological procedures. After synthesis of the first 145 amino acids, the nascent ribosome-associated actin chain binds to the recently identified heteromeric chaperone protein, prefoldin. Prefoldin remains bound to the relatively unfolded actin polypeptide until its posttranslational delivery to chaperonin containing Tcp1 (CCT). The α- and β-tubulin follow a similar maturation pathway, but to date find no evidence for an interaction between prefoldin and several noncytoskeletal proteins. Prefoldins are members of a recently identified, small-molecular weight protein family

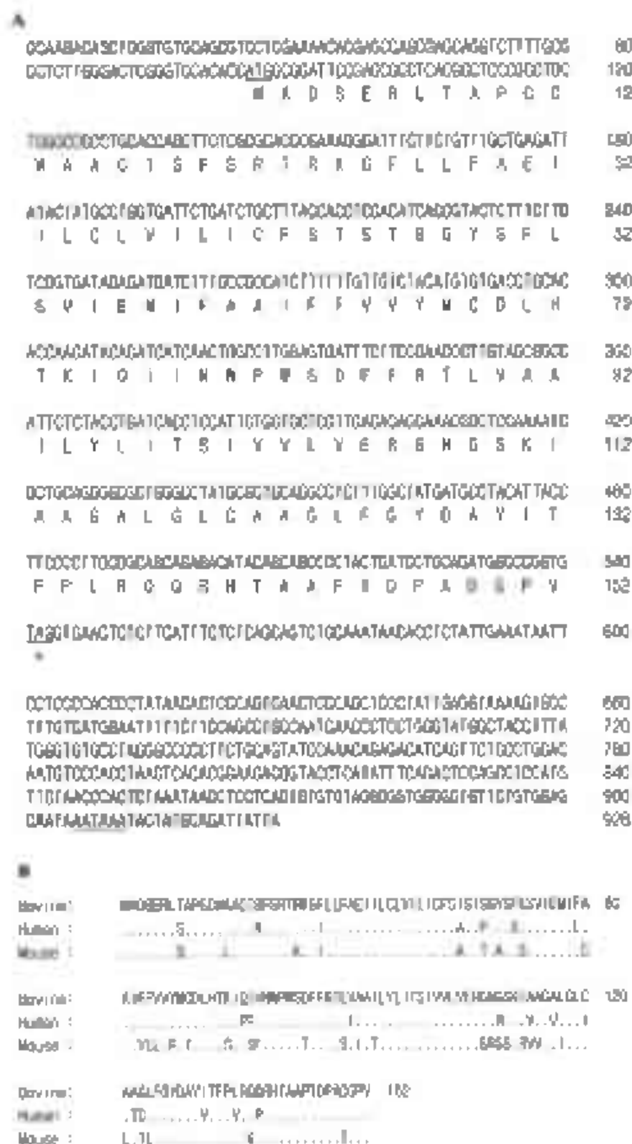

Figure 4. Nucleotide sequences of bovine proteolipid protein 2 and comparison of its amino acid sequences with other species. A. The amino acid was aligned with the second nucleotide of each codon. Asterisk (*) marks stop codon. The start codon, stop codon and polyadenylation signal sequences are underlined. The sequences have been deposited in the NCBI data base (accession no. AY192437). B. Comparison of the amino acid sequences with other species. Dot denotes the amino acid residue identical to that of bovine species.

able to assemble into molecular chaperone complexes. Gstaiger et al. (2003) describe an unusually large member of this family, termed URI, which forms complexes with other small-molecular weight prefoldins and with RPB5, a shared subunit of all three RNA polymerases. Functional analysis of the yeast and human orthologs of URI revealed that both are targets of nutrient signaling and participate in gene expression controlled by the TOR kinase.

The ubiquitin-like/S30 ribosomal fusion protein (fau) has been known to be involved in the ATP-dependent proteolytic activity of ubiquitin (Nakamura and Tanigawa, 1999). The fau gene is the cellular homolog of the fox sequence in the Finkel-Biskis-Reilly Murine Sarcoma Virus (FBR-MuSV). This virus acquired the fau sequence in its reversed transcriptional orientation (Kas et al., 1992). Human and mouse fau cDNAs were identified and both encode a new protein of 133 amino acids. Fau encodes the ribosomal protein S30 fused to an ubiquitin-like protein.

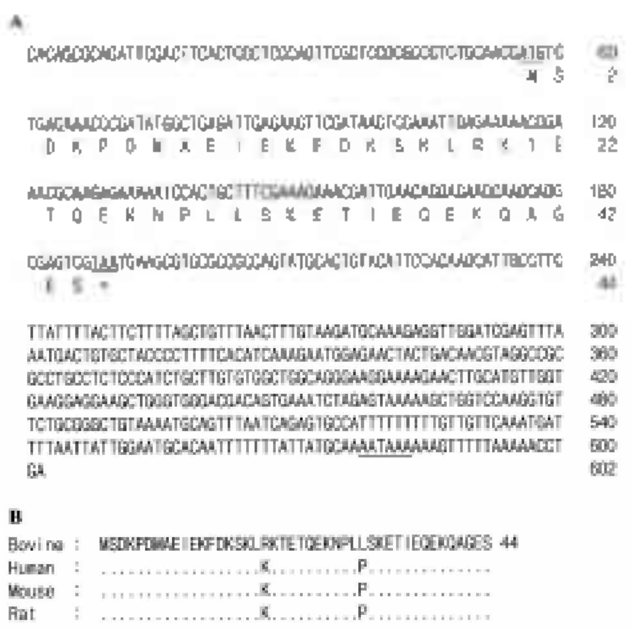

Figure 5. Nucleotide sequences of bovine thymosin beta-4 and its amino acid sequences with other species. A. The amino acid was aligned with the second nucleotide of each codon. Asterisk (*) marks stop codon. The start codon, stop codon and polyadenylation signal sequences are underlined. The sequences have been deposited in the NCBI data base (accession no. AY192438). B. Comparison of the amino acid sequences with other species. Dot denotes the amino acid residue identical to that of bovine species.

Proteolipid protein 2 (PLP2) is maximally expressed at the beginning of gastrulation. Time and site of proteolipid protein 2 gene expression coincide with emerging morphogenetic activities at the posterior pole of the embryo at the beginning of gastrulation (Milde et al., 2001). During development, PLP2 transcript might serve as a molecular marker for the primordium of the primitive streak and the posterior pole of the embryonic disc and might support the concept of the posterior node, with its specific involvement in cell movements at the beginning of gastrulation and in the generation of the extraembryonic mesoderm.

Thymosin beta 4 is a ubiquitous 43 amino acid, 5 kDa polypeptide that is an important mediator of cell proliferation, migration, and differentiation. It is the most abundant member of the beta-thymosin family in mammalian tissue. Thymosin beta 4 is angiogenic and can

Figure 6. Nucleotide sequences of bovine myotrophin and its amino acid sequences with other species. A. The amino acid was aligned with the second nucleotide of each codon. Asterisk (*) marks stop codon. The start codon, stop codon and polyadenylation signal sequences are underlined. The sequences have been deposited in the NCBI data base (accession no. AY192439). B. Comparison of the amino acid sequences with other species. Dot denotes the amino acid residue identical to that of bovine species.

promote endothelial cell migration and adhesion, tubule formation, aortic ring sprouting, and angiogenesis. It also accelerates wound healing and reduces inflammation when applied in dermal wound-healing assays. Thymosin beta 4, a highly conserved peptide with immunomodulatory properties, is a major actin-sequestering peptide in mammalian cells. It is present ubiquitously in most tissues and cell lines and is found in high concentrations in blood platelets and neutrophils (Hannappel et al., 1982). Thymosin beta 4 modulates the formation of F-actin microfilaments by sequestering the monomeric G-actin. Recent studies have shown that overexpression of the thymosin beta 4 gene occurs not only in many human carcinomas but also in the highly metastatic melanomas and fibrosarcomas (Wang et al., 2003). They suggest that the drastic growth property and motility changes of the SW480 cells overexpressing thymosin beta 4 gene are due mainly to a deregulated cell-cell adhesion arisen from the downregulation of E-cadherin, plus uncontrolled cell proliferation owing to the upregulation of beta-catenin, both resulted from a breakdown of actin microfilaments caused by the overexpression of this G-actin sequestering peptide.

Myotrophin is a myocardial hypertrophy-inducing factor initially demonstrated in hypertrophied and cardiomyopathic hearts. Skeletal-myotrophin promotes the hypertrophy of myotube as insulin-like growth factor-I does (Hayashi et al., 2001). Recent evidence suggests an interaction with the transcription factor nuclear factor κB (NFκB), which is activated in heart failure and modulates myocardial protein expression (O'Brien et al., 2003). Gupta and Sen (2002) suggest that probably myotrophin acts as a transcription factor by using κB DNA as a target for its activation and that myotrophin-κB DNA interaction may be an important step in initiating cardiac hypertrophy. The functional role of these clones in adipose tissues needs to be established.

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