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ms1, a novel stress-responsive, muscle-specific gene that is up-regulated in the early stages of pressure overload-induced left ventricular hypertrophy

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Abstract We have identified and characterised a cDNA encoding a novel gene, designated myocyte stress 1 (ms1), that is up-regulated within 1 h in the left ventricle following the application of pressure overload by aortic banding in the rat. The deduced ms1 protein of 317 amino acids contains several putative functional motifs, including a region that is evolutionarily conserved. Distribution analysis indicates that rat ms1 mRNA expression is predominantly expressed in striated muscle and progressively increases in the left ventricle from embryo to adulthood. These findings suggest that ms1 may be important in striated muscle biology and the development of pressure-induced left ventricular hypertrophy.

Key words: Left ventricular hypertrophy; Aortic banding; Gene expression; ms1; Development; Striated muscle

1. Introduction

Left ventricular hypertrophy (LVH) is an adaptation of the heart to chronic pressure/volume overload. Although immediately beneficial in reducing wall stress, in the long term LVH is an important independent risk factor for cardiovascular morbidity and mortality [1]. The cellular hallmark of LVH is hypertrophic growth of the cardiac myocyte. Several genes involved in the pathogenesis of LVH have already been identified, and a characteristic pattern of gene expression has emerged [2]. Shortly after a pressure stimulus, there is an induction of transcription factor-coding early genes in the left ventricle such as the proto-oncogenes c-*myc*, c-*jun* and c-*fos* [3]. This is followed by the re-expression of genes that are only otherwise expressed in the left ventricle during foetal development, such as atrial and brain natriuretic peptides [4] and shifts to foetal β-myosin heavy chain and skeletal α-actin isoforms [5]. An increase in protein content and a re-organisation of contractile elements from a series to a parallel arrangement results in the hypertrophic phenotype.

The molecular mechanisms that govern LVH are unclear. A number of signalling pathways involving downstream molecules such as $G_{\alpha q}$, protein kinase C (PKC), small GTPases, members of the mitogen-activated protein kinase family, calcineurin, and transcription factors such as AP-1, NFAT-3, GATA-4, MEF-2C and NF-κB have all been implicated [6]. However, none of these molecules has been shown to be both necessary and sufficient for hypertrophic growth. Progress in identifying other key regulatory molecules has been hindered, partly due to the candidate gene approach that has been employed. Recent advances in molecular techniques have led to the evolution of expression profiling methods such as differential display [7], serial analysis of gene expression [8], molecular indexing [9] and cDNA array screening [10], enabling a more comprehensive assessment of gene expression in disease states. We have developed an efficient method of expression profiling using the principle of molecular indexing [11]. In the present study, we have applied molecular indexing to study the early stages of pressure-induced LVH in a rat model of aortic banding, in order to identify novel regulatory molecules involved in the initial signalling that leads to the development of LVH. The isolation and characterisation of a novel gene, myocyte stress 1 (ms1; GenBank accession number AF336113), that is markedly up-regulated within 1 h of a pressure stimulus, is described.

2. Materials and methods

2.1. Animals and model

A rat model of pressure overload-induced LVH was generated by supra-renal abdominal aortic banding of 10-week-old male Wistar-Kyoto (WKY) rats, as described previously [12]. Age- and weight-matched banded and sham-operated animals were killed at 30 min, 1 h, 4 h, 1 day, 4 days, 7 days and 28 days post-operation ($n = 10$ per group per time point). Total RNA was prepared from the left ventricles of animals using Trizol (Life Technologies). The development of LVH was assessed by comparing LV mass indices (LV weight/body weight) between groups, and the induction of mRNA expression of the hypertrophic marker atrial natriuretic peptide by Northern blotting [12].

2.2. Molecular indexing

This was performed as described previously [11]. Briefly, double-stranded cDNA produced in parallel from 27 μg of pooled ($n = 10$ animals per group) total LV RNA from 4 h sham and banded groups, was digested with the class IIS enzyme *Bbv*I before ligation to pools of tagged adapters with sequence-specific four-base overhangs of all pos-

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Abbreviations: ms1, myocyte stress 1; EST, expressed sequence tag; LVH, left ventricular hypertrophy; WKY, Wistar-Kyoto; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RACE, rapid amplification of cDNA ends; RH, radiation hybrid; ORF, open reading frame; IRES, internal ribosome entry site; AP, adapter-primer; UTR, untranslated region

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sible sequences. Polymerase chain reaction (PCR) amplification of each adaptered cDNA pool was performed for side-by-side analysis of sham and banded products on a 4.8% polyacrylamide gel and autoradiography. Putative differentially expressed bands were cloned into the TA vector, pGEM-T (Promega), and Northern blotting analysis [11] of pooled 4 h RNA from sham and banded animals was carried out to confirm differential expression. The sequences of cloned differentially expressed fragments were determined by FS dye terminator chemistry (Perkin Elmer), using M13 forward and reverse primers.

2.3. ms1 mRNA analysis

2.3.1. ms1 mRNA expression in left ventricles of individual animals 4 h post-banding. The expression of ms1 in individual sham and banded animals ($n = 5$ per group) was assessed by semi-quantitative reverse transcription-PCR (RT-PCR). Fifty nanograms of cDNA (equivalent from the appropriate RT reaction) were PCR-amplified using forward (5′-ACATTCCCTCTTTAGCCAGTGC-3′) and reverse (5′-TAACATTCCAGAGCAGTTTGCC-3′) primers, of the ms1 full-length cDNA sequence. The thermocycling parameters were 1 cycle of 94°C, 1 min 30 s; 25 cycles of 94°C, 30 s; 60°C, 30 s; 72°C, 1 min 30 s; a final extension step was performed at 72°C for 10 min. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control, and PCR-amplified for 21 cycles under the same thermocycling conditions as for ms1, using forward (5′-GCCAGCCTCGTCTCATAGACAA-3′) and reverse (5′-GGGATGGAATTGTGAGGGAGAT-3′) primers. Relative ms1 expression between groups was assessed by spot densitometry (Alphaimager 2000, Alphaimager Corp.) of bands and corrected for GAPDH.

2.3.2. Temporal expression of ms1 mRNA in left ventricle after banding. Northern blotting was carried out essentially as described previously [12]. A random-prime (Life Technologies) ^{32}P-labelled ms1 indexing-derived cDNA fragment (467 bp) was used as probe and a terminal deoxynucleotide transferase (Amersham-Pharmacia) end-labelled oligonucleotide for GAPDH was used as a loading control.

2.3.3. Tissue distribution. Total RNA was prepared from heart, liver, brain, kidney, testis, adrenals and intercostal muscle from adult WKY rats and also from the A10 rat vascular smooth muscle cell line, using Trizol, and analysed by RT-PCR.

2.3.4. Ontogeny analysis. Total RNA was prepared from the left ventricles of 18-day-old embryos, 2-day-old neonates and 12-week-old adult Wistar rats and analysed using RT-PCR, as described above.

2.4. Full-length sequence of ms1

Rapid amplification of cDNA ends (RACE) was performed from pooled total RNA from the 4 h banded group, using 5′ and 3′ RACE kits (Life Technologies). The kit adapter-primers (AP) used were as follows: AP1, 5′-TAGCACGACTCAGAGCTCAT-3′, AP2, 5′-TAGCACGACTCAGAGCTCATTTTTTTTTTTTTTTTT-3′, AP3, 5′-TAGCACGACTCAGAGCTCATGGGGIIGGGIIGGGIIG-3′. The following ms1-specific primers (designated by the orientation of the cloned ms1 indexing-derived fragment) were used: ms1 sense 1, 5′-CTCAGTCACGACTCGCCAACGC-3′; 2, 5′-ATGGAACAGGAGGAGCCCAAGT-3′; 3, 5′-GGAACAGGAGGAGCCCAAGTGG-3′; ms1 antisense 1, 5′-AGACCTCCGTGGCTTCCTCAGA-3′; 2, 5′-CTCCGTGGCTTCCTCAGAGCCT-3′; 3, 5′-TGGCTTTGGAGACGGAGGTT-3′. Briefly, for 3′ RACE, reverse transcription was performed using primer AP2 before carrying out nested PCR amplifications with primer AP1 in combination with ms1 sense or antisense primers 2 and then 3. In 5′ RACE, the reverse transcription was carried out using ms1 sense or antisense primer 1, before tailing the cDNA and performing nested PCR amplifications using primers AP3 and AP1 in combination with ms1 sense or antisense primers 2 and 3, respectively. The sense and antisense 5′ and 3′ RACE products were gel-purified and cloned into pGEM-T before sequencing five independent clones of each.

2.5. Chromosomal localisation and gene structure of ms1

A subset of 96 hybrids (10 ng DNA each) of the T55 radiation hybrid (RH) rat panel (Research Genetics) was screened by touchdown PCR with 67–61°C annealing temperature, using forward (5′-GGCCGATGAACACATACAGTCA-3′) and reverse (5′-TAACATTCCAGAGCAGTTTGCC-3′) primers located at positions 791–892 and 1267–1246, respectively, of the ms1 cDNA sequence. Samples were electrophoresed on a 3% agarose gel and the data analysed using the MultiMap program [13], before two-point analysis was used to find the nearest RH framework marker. The approximate positions of intron–exon boundaries of the genomic sequence were located by PCR walking using combinations of forward (5′-ACATTCCCTCTTTAGCCAGTGC-3′, 5′-AGGTGGCTGCTGCCAGGATTAA-3′, 5′-TTGCACTCCCAGGCAAACAGAT-3′ and 5′-GGCCGATGAACACATACAGTCA-3′) and reverse (5′-TAACATTCCAGAGCAGTTTGCC-3′, 5′-TCCACTTGGCTGTATTTCCGAT-3′ and 5′-ATGCTCGTACCTGTGGCTCAGG-3′) and primers spanning different regions along the length of the ms1 cDNA sequence and comparing product sizes from RNA and genomic DNA templates. The precise boundaries were then determined by direct sequencing of a PCR product.

2.6. Bioinformatic analysis of rat ms1

A nucleotide database similarity search was performed using the BLAST algorithm [14]. A database search to find functional motifs in untranslated regions (UTRs) was carried out using the UTRScan tool (http://www.bigarea.area.ba.cnr.it:8000/EmbIT/UTRHome/). The ms1 mRNA was translated in all six reading frames by the open reading frame (ORF) finder tool at (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to deduce the putative protein sequence, which was analysed using the integrated PIX program at (http://www.hgmp.mrc.ac.uk).

3. Results

3.1. Molecular indexing

We embarked on a molecular indexing approach [11] to identify genes that are regulated shortly after the imposition of pressure overload on the rat LV. Out of 3181 bands detected in the scan, 124 were identified as potentially differen-

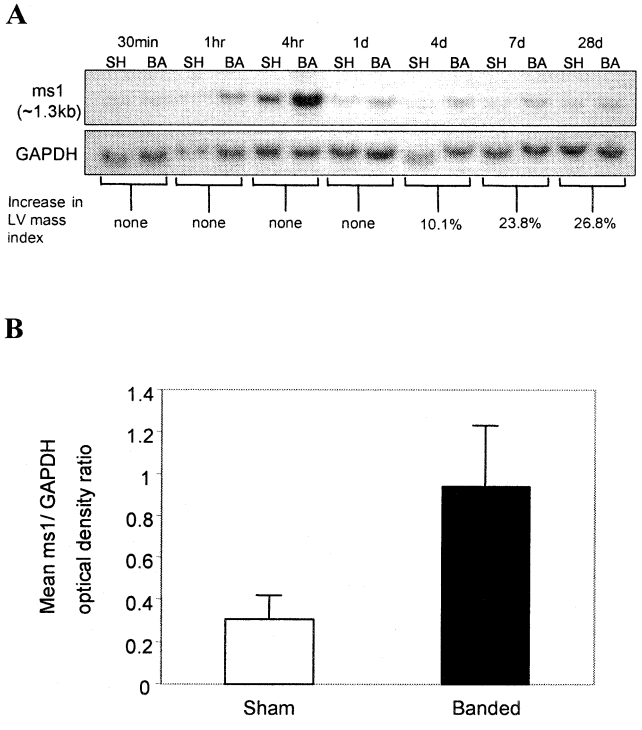

Fig. 1. A: Representative Northern blot of pooled ($n = 6$–10 per group at each time point) LV total RNA showing the time course of ms1 mRNA expression following aortic banding. SH = sham, BA = banded. B: Densitometric analysis of ms1 mRNA expression in 4 h individual animals ($n = 5$ per group). Bar graph shows mean ms1/GAPDH optical density ratios of RT-PCR products from left ventricles of sham and banded animals at 4 h post-operation.

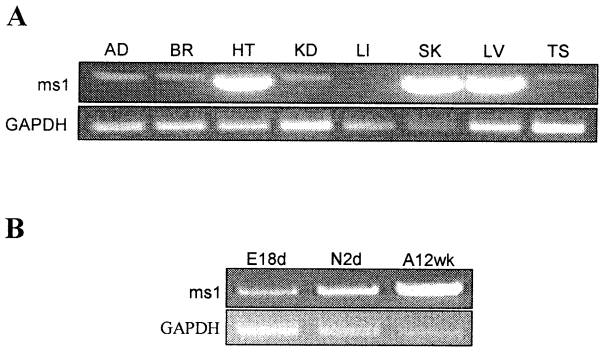

Fig. 2. A: RT-PCR analysis showing the distribution of ms1 mRNA in rat tissues. AD = adrenal gland, BR = brain, HT = whole heart, KD = kidney, LI = liver, SK = skeletal muscle, LV = left ventricle, TS = testis. B: RT-PCR analysis showing the developmental expression of rat ms1 mRNA in the LV. E18 = embryonic day 18, N2d = 2-day-old neonate and A12wk = 12-week-old adult. All experiments were repeated with similar results on independently obtained samples ($n = 3$).

tially expressed. So far differential expression has been confirmed for seven fragments, which include known genes and also a 467-bp fragment with database sequence similarity to expressed sequence tags (ESTs) but not to any known genes. The present report characterises this novel gene that we have named myocyte stress 1 (ms1).

3.2. Up-regulation of ms1 mRNA expression in the left ventricle after aortic banding

The time course of ms1 mRNA expression in the left ventricle after aortic banding is shown in Fig. 1A. The findings indicate a pronounced early up-regulation (within 1 h), with a peak expression before 1 day, well before any detectable increase in LV mass (Fig. 1A). Semi-quantitative RT-PCR analysis confirmed the over-expression of ms1 at 4 h after banding in the left ventricles of individual animals (Fig. 1B). Mean optical density ratios of ms1/GAPDH mRNAs are 0.31 ± 0.105 and 0.94 ± 0.284 for sham and banded groups, respectively, indicating an about three-fold increase in ms1 mRNA expression after banding ($P = 0.002$).

3.3. Tissue distribution and ontogeny of ms1 mRNA expression in myocardium

ms1 mRNA is expressed at high levels in adult rat heart and skeletal muscle, but at very low levels in the adrenals, brain, kidney, liver and testis (Fig. 2A). Fig. 2B shows that ms1 mRNA is expressed in the rat left ventricle during embryogenesis and is post-natally up-regulated through to adulthood.

3.4. Sequence, chromosome localisation and gene structure of ms1

RACE of the ms1 cloned indexing fragment revealed the 5′ and 3′ flanking regions to be 193 bp and 627 bp in length, respectively, indicating that the full-length transcript size for this gene is 1287 bases. The nucleotide sequence is shown in Fig. 3A. Screening of RH panels mapped rat ms1 to chromosome 7 near the microsatellite marker D7Got54 (467cR), with a lod score of 10.9. PCR walking showed that ms1 has a relatively simple genomic organisation of two exons (703 bp and 584 bp) that are located 5′ and 3′, respectively, of an intron of approximately 2.7 kb.

3.5. Bioinformatic analysis of msl cDNA

The largest open reading frame (954 nucleotides) predicts a 317-amino acid protein of approximately 36 kDa (Fig. 3A). The critical nucleotides surrounding the start codon are identical (at the +4 and −3 positions) to the Kozak consensus (**A/GCCAUGG**) ribosome binding sequence [15], suggesting that this is the likely site for translation initiation. This deduction is supported by the immunoprecipitation of an appropriately sized protein produced from in vitro transcribed–translated ms1, by an anti-ms1 antiserum raised to a C-terminal region of this ORF (data not shown). The deduced ms1 protein sequence contains several putative functional domains including two overlapping nuclear localisation sites and protein phosphorylation motifs (e.g. PKA, PKC, casein kinase and tyrosine kinase). In addition, a putative internal ribosome entry site (IRES) is present in the 3′ UTR (Fig. 3A).

BLAST searches revealed that ms1 full-length cDNA and putative protein show no significant homology to any known genes at date of submission. However, there is significant identity of specific regions of ms1 mRNA with cDNA and

Table 1
Database sequence similarity of rat msl to ESTs

Accession number of match	Library source	Area of homology (ms1/db match)	Identity (%) (over length)
BF670302 (896 bp)	*Homo sapiens* skeletal muscle	654–1199/2–548	84.7 (549 bp)
BF549525 (553 bp)	*Rattus norvegicus* embryo, placenta and adult lung, brain, liver, kidney, heart, spleen, ovary, muscle	570–1122/1–550	99.1 (553 bp)
AI172339 (388 bp)	*Rattus norvegicus* normalised adult muscle	1284–938/4–348	99.1 (347 bp)
AW535111 (354 bp)	*Rattus norvegicus* embryo, placenta and adult lung, brain, liver, kidney, heart, spleen, ovary, muscle	1284–966/33–354	93.2 (323 bp)
AI060194 (301bp)	*Rattus norvegicus* placenta, adult lung, brain, liver, kidney, heart, spleen, ovary, muscle	1284–1010/28–301	99.3 (275 bp)
AI716006 (319 bp)	*Rattus norvegicus* normalised eye minus lens	1251–952/9–306	94 (300 bp)
AI510175 (613 bp)	*Mus musculus* embryo, 13.5–14.5 days post-coitum	19–630/1–613	91.4 (613 bp)
AI605651 (576 bp)	*Mus musculus* embryo, 19.5 days post-coitum	441–1017/1–576	92.7 (577 bp)
AI414584 (404 bp)	*Mus musculus* embryo, 19.5 days post-coitum	1221–876/62–404	91 (346 bp)
AV247903 (273 bp)	*Mus musculus* 0-day neonate head	1170–1284/162–268	70.4 (347 bp)
AV599391 (410 bp)	*Bos taurus*, foetal cartilage	1272–899/32–410	75.6 (381 bp)
AI721847 (618 bp)	*Danio rerio* somite, whole embryo and adult liver	760–979/359–587	71.8 (220 bp)
AI533498 (532 bp)	*Drosophila melanogaster*	791–1161/68–434	55.8 (380 bp)
D76164 (360 bp)	*Caenorhabditis elegans*	1072–1033/183–322	65.7 (140 bp)

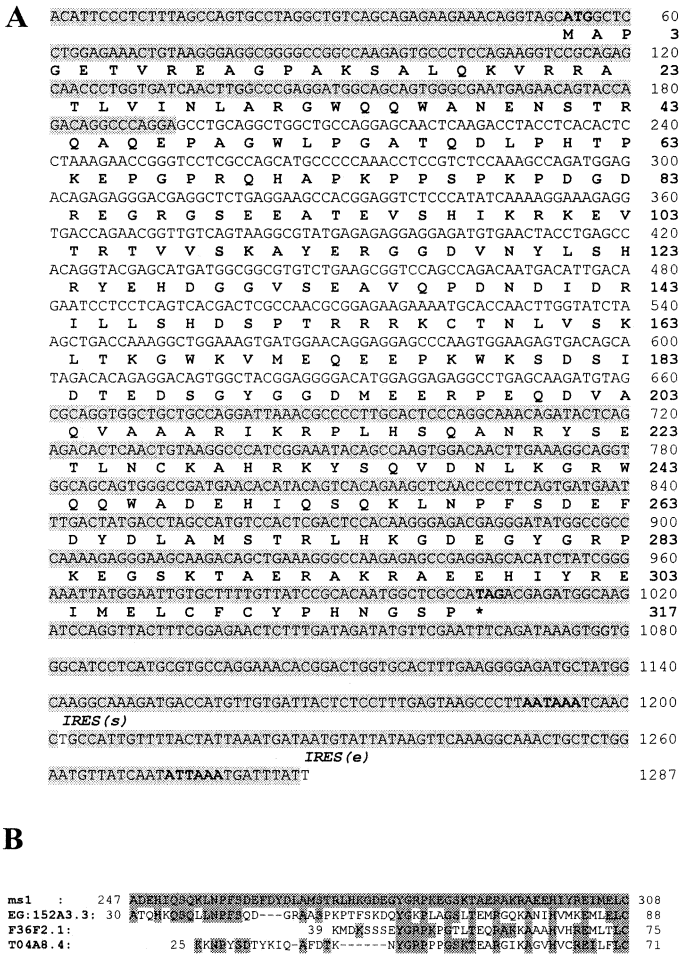

Fig. 3. A: Full-length cDNA (5′ to 3′) and putative protein sequence of ms1. The 5′ and 3′ RACE products are indicated by shaded regions of the nucleotide sequence, and the translation start (ATG) and stop (*) codon encoding the longest open reading frame are shown in bold. The putative polyadenylation signals are in bold and the start (s) and end (e) of a putative IRES element is indicated by unshaded bases. B: Alignment of ms1 putative protein sequence with a protein in *Drosophila melanogaster* (EG:152A3.3) and two in *Caenorhabditis elegans* (F36F2.1 and T04A8.4). Shaded amino acids denote areas of identity with ms1.

genomic sequences from species across the phylogenetic spectrum (Table 1), and significant similarity of the ms1 protein with predicted proteins from invertebrates (Fig. 3B).

4. Discussion

We report here the isolation and characterisation of a novel gene, ms1, which is up-regulated in the very early stages of pressure overload LVH, before any structural changes are apparent. This suggests a possible role for ms1 in the initial signalling of the hypertrophic response. The considerably greater abundance of ms1 mRNA levels in heart and skeletal muscle compared with other tissues suggests that it is highly striated muscle-specific. It is conceivable that ms1 may also have a more fundamental role in the regulation of the 'muscle gene programme' that controls differentiation, growth and sarcomeric organisation of striated muscle [16]. It should also be noted that the normal persistence of ms1 mRNA in the rat left ventricle beyond development and into adulthood indicates that ms1 does not belong to the 'foetal' group of genes that are re-expressed during LVH [4].

The homology of a region of the ms1 mRNA and protein sequences to predicted genes and proteins in *Drosophila melanogaster* and *Caenorhabditis elegans* (Table 1, Fig. 3B) implies evolutionary conservation and thus functional importance. There is a large body of evidence demonstrating the involvement of conserved signalling molecules in growth and stress pathways. For instance, functionally similar sequence homologues of the MAP kinases, p38 and JNK [17], and the cardiac-restricted transcription factors GATA-4 and *Nkx2.5* [18] have been identified in mammals and *D. melanogaster*. The existence of a human homologue of ms1 mRNA is supported by 85% identity of a 3′ region with a 549-base segment of EST BF670302 (from skeletal muscle) and 83–92% identity to different regions of a genomic clone from human Ch8q23 (with segments of this clone almost covering the entire length of ms1 mRNA).

Five rat ESTs exhibit strong identity (93–99%) with different lengths of the 3′ region of ms1 (Table 1). Interestingly, sequence alignments reveal variations in length and sequence amongst the genes represented by ESTs compared to ms1. Although the 3′ terminal 348 bases of EST AI172339 (from a normalised muscle library) is nearly identical to ms1 (99.1% identity), the 40 most 5′ bases of this clone display very weak homology. Three of the ESTs exhibit variation in the length of the 3′-terminus: ESTs AI060194 and AW535111 (from

libraries derived from a mixture of tissues) possess an additional length of approximately 30 non-matching bases, beyond the respective 275 and 323 bases that closely match the 3′-terminus of ms1, whereas AI716006 (from a normalised eye library), is truncated by approximately 40 bases in comparison to ms1. The significance of this potential transcript heterogeneity remains to be determined.

In the 5′ UTR of certain transcripts, an IRES confers the potential for cap-independent translation, and has been identified in various genes involved in cell growth such as c-*myc* [19] and vascular endothelial growth factor [20]. The importance of the IRES is evident when there is a decline in normal cap-dependent protein synthesis during conditions of cellular stress, such as hypoxia [20] and apoptosis [19]. Recently, IRES-like activity has also been demonstrated in the 3′ UTR of certain genes involved in oxidative phosphorylation [21]. Therefore the presence of a putative IRES in the 3′ UTR of ms1 (Fig. 3A) may be relevant to its function.

In spite of intense research on the pathogenesis of LVH, the molecular basis of this disease state remains to be fully understood. Our study has identified a novel gene, ms1, that is upregulated significantly and in a striated muscle-specific manner, during the early stages of LVH development. We propose that ms1 is a potential candidate as an early molecular trigger in the hypertrophic cascade. Future studies will focus on the transcription machinery that governs the regulation and tissue-specific expression of rat ms1 mRNA and specific models of cellular stress will be used to dissect the precise stimulus-specificity of ms1 activation and function.

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