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FAST TRACK

Identification of the Increased Expression of Monocyte Chemoattractant Protein-1, Cathepsin S, UPIX-1, and Other Genes in Dystrophin-Deficient Mouse Muscles by Suppression Subtractive Hybridization

Jin Fang,[1] Guo-Ping Shi,[2] and Pal L. Vaghy[1]*

[1]Department of Molecular and Cellular Biochemistry, College of Medicine and Public Health, The Ohio State University, Columbus, Ohio 43210
[2]Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Abstract The lack of dystrophin results in muscular dystrophy characterized by degeneration, inflammation, and partial regeneration of skeletal muscles. The fate of these muscles may be determined by the extent of adaptation to the defect and the efficiency of regeneration that is affected by inflammatory cells. We have used suppression subtractive hybridization and quantitative Northern blot analysis to identify differentially expressed genes. Increased expression of murine monocyte chemoattractant protein-1 (JE/MCP-1), cathepsin S, UPIX-1, nmb, cathepsin B, and lysozyme M mRNAs were identified in 2-month-old *mdx* mouse leg muscles. UPIX-1 is a novel gene. Although it was not expressed in control muscles, it was expressed in control brain, heart, and spleen. JE/MCP-1 and cathepsin S proteins in *mdx* muscles, as well as JE/MCP-1 protein in the serum of *mdx* mice were also detected. JE/MCP-1 may be responsible for attraction of inflammatory cells, and cathepsin S, a potent elastolytic protease, may contribute to the remodeling of the extracellular matrix that is required for the migration of these cells to the injured muscles. J. Cell. Biochem. 79: 164–172, 2000.

Key words: Duchenne muscular dystrophy; *mdx* mouse; gene expression; MCP-1; macrophage; extracellular matrix

Duchenne muscular dystrophy (DMD) is a childhood disease characterized by the lack of dystrophin, progressive loss of muscle function, and severe clinical disability. The rapid progression of the disease is caused by myofiber necrosis, inefficient regeneration and replacement of necrotic muscle fibers with fibrotic and adipose tissue [Dubowitz, 1995]. The *mdx* mouse is a genetic homologue of DMD [Partridge, 1991]. In contrast to DMD, a remarkable regeneration occurs in *mdx* mouse muscles after an early degenerative period that starts at 2 to 3 weeks of age. The more efficient regeneration occurring in the mouse than in human muscles could be responsible for the milder phenotype [Partridge, 1991].

Previous studies have demonstrated an increased presence of several proteins in dystrophin-deficient muscles. These included cytoskeletal proteins [Law et al., 1994], cytokines and growth factors [Tidball et al., 1992], desmin [Niiyama et al., 1999], caveolin-3 [Vaghy et al., 1998], and matrix metalloproteinases [Kherif et al., 1999]. A recent study using suppressive subtraction hybridization (SSH) has revealed that the mRNAs of many more known and unknown genes may be differentially expressed in *mdx* mouse muscles [Tkatchenko et al., 2000]. SSH is a powerful new procedure for identification of differentially expressed genes in tissues [Diatchenko et al., 1996]. Theoretically, SSH is appropriate for

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Guo-Ping Shi's present address: Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of California San Francisco, 505 Parnassus Avenue, L-1312, Box 0130, San Francisco, CA 94143-0130.

*Correspondence to: Pal L. Vaghy, Department of Molecular and Cellular Biochemistry, The Ohio State University, 466 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210-1218. E-mail: vaghy.1@osu.edu

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identification of all differentially expressed genes between two comparable tissues. However, there are practical limitations of this method. The subtracted cDNA libraries generated by elimination of most mutually expressed genes consist of thousands of bacterial colonies containing putative differentially expressed cDNA fragments. Only rigorous screenings and cDNA sequencing can positively identify the truly differentially expressed mRNAs. Furthermore, the identification of the differentially expressed mRNAs does not necessarily indicate altered expression of the biologically active proteins, just as an altered presence of a protein is not always the result of altered gene expression.

We performed SSH and Northern blot analysis to identify differentially expressed mRNAs, and verified previous reports demonstrating the upregulation of cathepsin B, lysozyme M, and nmb in *mdx* mouse muscles [Sano et al., 1988; Tkatchenko et al., 2000]. More importantly, we show here, for the first time, that mRNAs for murine monocyte chemoattractant protein-1 (JE/MCP-1), cathepsin S, and UPIX-1, a novel gene, are differentially expressed in the *mdx* mouse muscles. Finally, we provided evidence for the expression of JE/MCP-1 and cathepsin S in *mdx* mice by identification of the protein products of these genes.

MATERIALS AND METHODS

RNA Isolation

Control (C57BL/10SnJ) and *mdx* (C57BL/10ScSn Dmd^{mdx}) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Total RNAs were isolated from the hindlimb muscles of 2-month-old animals according to Chomczynski and Sacchi [1987]. Poly(A)$^+$RNAs were purified from total RNAs using PolyATract mRNA Isolation System III Kit (Promega, Madison, WI) according to the manufacturer's protocol.

Suppressive Subtraction Hybridization

Reverse transcription of poly(A)$^+$ RNAs and generation of subtracted libraries were performed using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol with modifications. The conditions of primary polymerase chain reaction (PCR) were as follows: 75°C for 5 min; 94°C for 25 s followed by 27 amplification cycles (94°C for 20 s, 66°C for 30 s, 72°C for 1.5 min), and a final extension at 68°C for 5 min. The conditions of secondary PCR were as follows: 94°C for 10 s followed by 12 amplification cycles (94°C for 10 s, 68°C for 15 s, 72°C for 1.5 min) and a final extension at 72°C for 7 min. All PCR amplifications were carried out using the AdvanTAge cDNA polymerase mix (Clontech) and an Eppendorf Mastercycler. The PCR products were resolved by electrophoresis using a 2% agarose gel.

cDNA Cloning and Screening

The secondary PCR products were subcloned into pT-Adv plasmid vector using the AdvanTAge PCR Cloning Kit (Clontech) according to the manufacturer's protocol. For colony dot blot screening, identical blots were prepared by arraying 2 μl of bacterial overnight cultures onto Nytran nylon membrane filters (Schleicher & Schuell, Keene, NH) placed on the top of LB/ampicillin agar plates. After growing overnight at 37°C, the bacteria were lysed, the DNA was denatured and neutralized, and the filters were baked for 2 h at 80°C. Forward- and reverse-subtracted cDNAs were used as hybridization probes after the following treatments. The adapters were removed by digestion with *Rsa*I and *Sma*I restriction enzymes, and the cDNA was resolved by electrophoresis on a 2% agarose gel. The adaptor-free cDNA was removed from gels by Concert Gel Extraction Systems (GIBCO BRL, Rockville, MD). The cDNA was labeled with [P^{32}]-dCTP (ICN Biochemicals, Costa Mesa, CA) by random labeling method. Prehybridization was carried out at 65°C for 1 h with 10 ml Rapid-hyb buffer (Amersham, Piscataway, NJ). The hybridization was performed at 65°C for 4 h with 0.5×10^6 cpm/ml in the Rapid-hyb buffer. The membranes were washed once for 15 min with 1 × SSC and 0.5% sodium dodecyl sulfate (SDS) at room temperature, and twice, 20 min each, with 0.2 × SSC and 0.5% SDS at 65°C. The membranes were exposed with X-ray films at −80°C for 4–6 h.

For cDNA dot blot screening, the inserts were amplified using 1 μl bacterial cultures as templates and primers flanking the cloning site. The PCR was performed at 94°C for 30 s followed by 23 cycles of 95°C for 30 s and 68°C for 3 min. The PCR products were analyzed by electrophoresis of a 5-μl aliquot from each reaction on a 2% agarose gel. Identical cDNA dot blots were prepared by arraying 5-μl aliquots from each reaction onto Nytran nylon mem-

brane filters. Prehybridization and hybridization were performed as described for colony dot blots.

Northern Blot Analysis

Poly(A)$^+$RNA from 2-month-old control and *mdx* muscles were resolved on 1.2% agarose formaldehyde gels and transferred to Nytran membranes. Normal adult mouse multiple tissue Northern blot was purchased from Clontech. PCR products from inserts of selected clones were random labeled with [P^{32}]-dCTP and used as probes. Hybridization and washing conditions were as described above. Autoradiography was performed at −80°C for 52–112 h. Quantitation of hybridization signals was performed by Kodak BioMax 1D image analysis software (Kodak, Rochester, NY). The hybridization signals were normalized to a housekeeping gene, GAPDH, to compensate for unequal loading.

cDNA Purification and Sequencing

cDNA was prepared from overnight bacterial cultures by using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). DNA sequencing was performed at the DNA Sequencing Facility of the Ohio State University Neurobiotechnology tk;4Center using an ABI PRISM Big Dye Terminator Cycle Sequencing Reaction Kit (PE Applied Biosystems, Foster City, CA) and an ABI373XL Stretch DNA sequencer. The cDNA sequences were subjected to BLAST homology search to compare them with sequences present in the GenBank [Altschul et al., 1997].

Western Blot Analysis

For detection of JE/MCP-1 protein, control and *mdx* mouse tibialis anterior muscle homogenates and sera were separated by SDS–polyacrylamide gel electrophoresis (PAGE) using 15% polyacrylamide mini gels and transferred to 0.45 μm nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were first reacted with goat anti-JE/MCP-1 polyclonal antibody (R & D Systems, Minneapolis, MN) diluted 1:500 in 10 mM Tris, pH 8.0, 140 mM NaCl, and 0.05% (vol/vol) Tween 20 (TBST) for 1 h. The membranes were washed six times, 5 min each, in TBST and then reacted with peroxidase-conjugated affinity purified donkey anti-goat IgG secondary antibody (Jackson Immunoresearch Laboratory, West Grove, PA) diluted 1:100,000 in TBST for 1 h. The antigen–antibody complexes were detected by enhanced chemiluminescence (Amersham). C-terminal truncated 8,525-Da recombinant mouse JE/MCP-1 (R & D Systems) was used for positive control.

For detection of cathepsin S protein, control and *mdx* mouse tibialis anterior muscle homogenates were separated by SDS-PAGE using 12% acrylamide gels. The proteins were transferred to 0.45-μm nitrocellulose membranes (Schleicher & Schnell), and blocking was performed overnight with 5% nonfat dry milk in TBST. The proteins were then reacted with anti-cathepsin S primary rabbit or mouse polyclonal antibodies diluted 1:1,000 in TBST with 0.3% bovine serum albumin (BSA). Membranes were washed four times, 5 min each, in TBST and then reacted with peroxidase-conjugated affinity purified goat anti-mouse IgG secondary antibody (Jackson Laboratory) diluted 1:50,000 in TBST with 1% BSA. The antigen–antibody complexes were detected by enhanced chemiluminescence. Mouse cathepsin S transfected 293 cells expressing both the biologically active mature 28-kDa enzyme and the 37-kDa pro-cathepsin S were used for positive controls.

RESULTS

Colony Dot Blot Screening of an Equalized and Subtracted cDNA Library Revealed the Presence of Putative Differentially Expressed Genes in *mdx* Muscles

SSH and T/A cloning techniques were used to generate an equalized cDNA library enriched in clones of genes that are expressed in 2-month-old *mdx* mouse limb muscles but not in controls. The screening of 576 randomly selected white bacterial colonies by dot blot analysis identified 108 clones that hybridized with the *mdx*-specific probes but not with control-specific probes (Fig. 1). These clones were subjected to PCR amplification to determine the cDNA insert sizes. The first 11 clones that contained PCR-amplifiable cDNA inserts were used for Northern blot analysis to positively identify mRNAs that are differentially expressed in *mdx* mouse muscles.

Identification of Differentially Expressed mRNAs by Northern Blot Analysis, cDNA Sequencing, and BLAST Search

Seven of the 11 cDNA inserts hybridized with mRNAs expressed at much higher levels

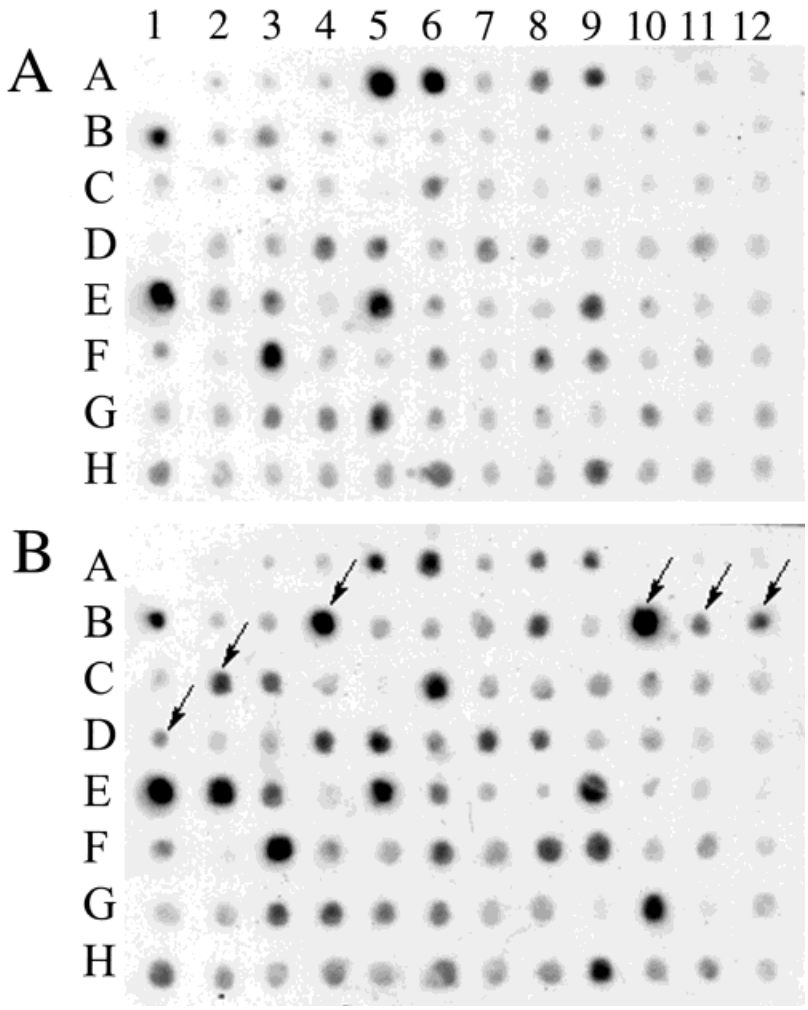

Fig. 1. Representative colony dot blot analysis of differentially expressed genes. Two identical dot blots were prepared using overnight cultures of white bacterial colonies randomly picked from the normalized and subtracted cDNA library enriched in clones of genes differentially expressed in the limb muscles of *mdx* mice. **Blot A** was hybridized with the control-specific cDNA probe, and **blot B** was hybridized with the *mdx*-specific cDNA probe. Six bacterial colonies that did not hybridize with the control-specific cDNA probe but did hybridize with the *mdx*-specific cDNA probe are indicated by arrows.

in *mdx* muscles than in controls. cDNA sequencing followed by BLAST search of GenBank determined that two of the seven cDNAs, both reacting with 1.8-kb mRNAs, derived from the same gene, mouse lysozyme M. The remaining five cDNAs, however, all had different length and cDNA sequences and hybridized with different RNAs (Fig. 2). One cDNA insert hybridized with a 3-kb mRNA specifically present in *mdx* muscles. The sequence of this 583-bp-long cDNA was 99.5% identical with a segment of nmb, a recently identified but uncharacterized putative transmembrane glycoprotein (gb AJ251685). Another cDNA insert hybridized with 2.2-kb mRNAs present in both control and *mdx* muscles. The sequence of this 458-bp-long cDNA was 100% identical with a segment of mouse cathepsin B (gb X54966). Approximately twice as much cathepsin B mRNA was detected in *mdx* than in control muscles (Fig. 2). The sequence of a 434-bp-long cDNA that hybridized with a 1.7-kb mRNA selectively present in *mdx* muscles was 99.8% identical with cathepsin S (gb AJ002386). The sequence of an 804-bp-long cDNA that hybridized with a 0.8-kb mRNA present primarily in *mdx* mouse muscles was 99% identical with JE/MCP-1 (gb J04467). Quantitative analysis indicated the presence of 22-times more JE/MCP-1 mRNA in *mdx* than in control muscles.

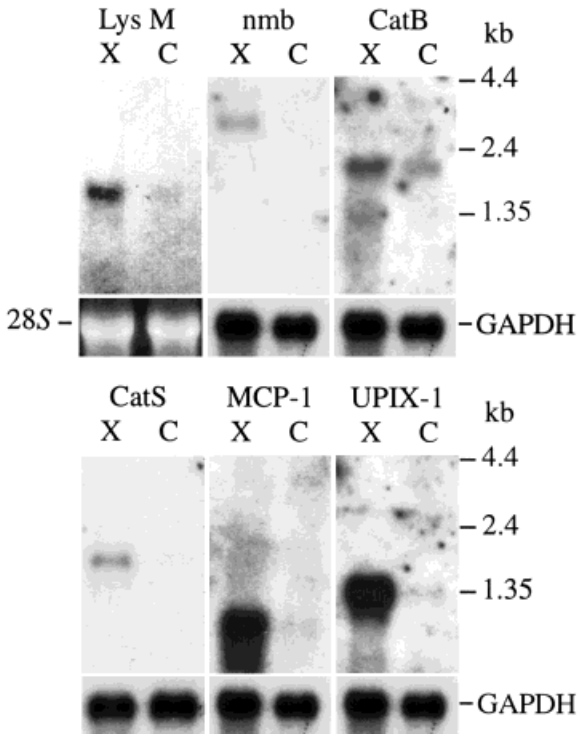

Fig. 2. Northern blot analysis of differentially expressed mRNAs. Total and poly(A)$^+$RNAs from 2-month-old *mdx* (X) and control (C) mouse leg muscles were separated on 1.2% agarose gels and transferred to Nytran membranes. One blot containing 20 μg total RNAs/lane was hybridized with lysozyme M (Lys M) cDNA. The 28*S* rRNA on this blot is shown for reference. A blot containing 4 μg poly(A) $^+$RNAs/lane was hybridized with nmb, cathepsin B (CatB), JE/MCP-1 (MCP-1), UPIX-1, and GAPDH cDNA probes. The blot containing 2 μg poly(A) $^+$RNAs/lane was hybridized with cathepsin S (CatS) and GAPDH cDNA probes. The sizes of mRNAs are indicated on the right in kilobases.

Currently, there is no full-length nucleotide sequence in the GenBank corresponding to the cDNA sequence of the insert purified from one clone. Therefore, this 362-bp-long cDNA, which hybridized with a 1.4-kb mRNA selectively expressed in *mdx* mouse muscles, is a fragment of a novel gene that we named UPIX-1 after UP-regulated In *mdX*. However, the UPIX-1 sequence is 95% identical with the 5′ 116-bp segment of a 203-bp-long mouse EST 3′ sequence (RIKEN clone 6430533L01, gb AV343083). The 95% identity in the overlapping region suggests that the two cDNA sequences may have derived from the same gene. Sequence alignment and analysis allowed us to determine the orientation of the partial UPIX-1 sequence (Fig. 3A). The UPIX-1 fragment was generated by digestion with *Rsa*I, which has a cutting site at GT/AC. Indeed, the sequence of UPIX-1 fragment ends with GC. More importantly, a GTAC sequence can be identified in the clone 6430533L01 sequence at the position where the UPIX-1 sequence ends. Furthermore, the EST-3′ end sequence of clone 6430533L01 contains two putative polyadenylation sites, one of which is 18 bases upstream from the 3′ end. All these data together suggested that the combined sequences may represent the 3′-end of a novel gene. The 362-bp partial sequence of UPIX-1 has been deposited in the GenBank (gb AF230110).

According to the information available in the GenBank (gb AV343083), the RIKEN clone 6430533L01 was derived from a mouse brain cDNA library. If the partial UPIX-1 and RIKEN clone 6430533L01 3′ sequences are in fact different parts of the same gene, as the identity of the overlapping regions suggested, then UPIX-1 mRNA must be expressed in the normal mouse brain. This possibility was tested by multiple-tissue Northern blot analysis. The data shown in Figure 3B revealed that the 1.4-kb UPIX-1 mRNA is indeed expressed in the normal mouse brain. Moreover, we also show that normal heart and spleen and, to a much smaller extent, lung and liver also express the UPIX-1 mRNA. At the same time, UPIX-1 mRNA was not detected in normal skeletal muscle.

Identification of Differentially Expressed JE/MCP-1 and Cathepsin S Proteins in *mdx* Mouse Muscles

Because altered expression of mRNAs may or may not be followed by changes in the protein expression, it was important to determine whether the protein products of these mRNAs are also differentially expressed in *mdx* mouse muscles. We performed Western blot analysis to examine expression of JE/MCP- 1 and cathepsin S proteins. The data shown in Figure 4 indicate that the 16.5-kDa JE/MCP-1 and the mature, 28-kDa cathepsin S are present in at least 10-fold higher levels in *mdx* mouse muscles than in age-matched control muscles.

Identification of JE/MCP-1 Protein in the Serum of *mdx* Mice

It has been well established that MCP-1 produced by the injured tissue is secreted into the

A

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UPIX-1:      actctgcctcctcaagctggaattattggtgtgctgttgtgttacctggaatttacatga   60
UPIX-1:      gttctggattccaaattgtagatttcttgcttcgggaacaagagctttaattgctgagcc   120
UPIX-1:      atctcttcagcccaagaaagaaatcagatgtggtagttatgaaaatagcttttcggattt   180
UPIX-1:      cctgttaagatgatgagaaataccaacattggaaaacggcaaactcccatcataccagca   240
UPIX-1:      caccaggggctgtcaaggctgctgacaaatactgacttgacaacaaattttaagaatgac   300
                  |||||||||||||||||||||||||||||| ||||||||| ||||||||||||||
6430533L01:       gggctgtcaaggctgctgacaaatactgatttgacaactaattttaagaatgac    54
UPIX-1:      ttctgtgcaagaaaatgcagagtgtcttaaaatcttgcaccttataataaagcaaatatg   360
             |||||||||||||||||||||||| ||||| | |||||||||||||||||||||||||
6430533L01:  ttctgtgcaagaaaatgcagagtgttttaaatttttgcaccttataatAAAgcaaatatg   114
UPIX-1:      gt                                                            362
             ||
6430533L01:  gtaccagtttttccccaaatttgacatgacactgctgatgaagaattatgactgaagttt   174
6430533L01:  actgaattattAATAAAgaacccaagcaa                                 203
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B

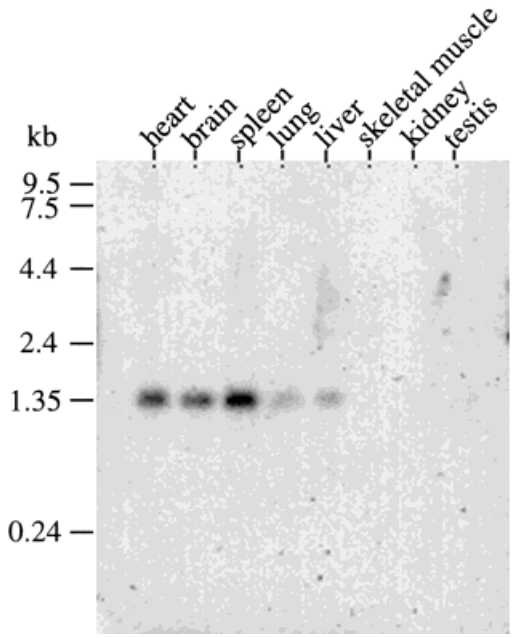

Fig. 3. Nucleotide sequence and tissue-specific expression of UPIX-1. **A:** Alignment of the 362-bp UPIX-1 (gb AF230110) and the 203-bp RIKEN clone 6430533L01 3′ end (gb AV343083) sequences. Bold letters show the *Rsa*I cutting sites. The putative polyadenylation sites are underlined. **B:** Tissue-specific expression of UPIX-1 mRNA. An adult mouse multiple-tissue Northern blot containing 2 μg Poly(A) $^{+}$RNA on each lane was hybridized with the 362-bp UPIX-1 cDNA probe. The sizes of mRNAs are indicated on the left in kilobases.

blood to attract various inflammatory cells to the site of injury [Inadera et al., 1999]. Therefore, we examined whether JE/MCP-1 protein was present in the serum of *mdx* mice. We determined that the heavily glycosylated, 25-kDa JE/MCP-1 protein is present in the serum of *mdx* mice but not in the serum of control mice (Fig. 4).

DISCUSSION

Recent advances in biotechnology have made the efficient identification of differentially expressed genes in diseased cells and tissues possible [Carulli et al., 1998]. Of the various procedures currently in use, SSH is particularly useful for identification of known as well as

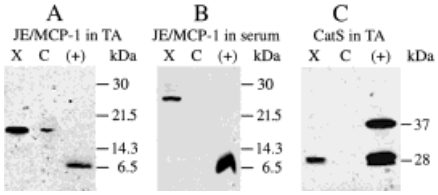

Fig. 4. Western blot analysis of JE/MCP-1 and cathepsin S proteins. **A:** Tibialis anterior (TA) muscle homogenates from 2-month-old *mdx* (X) and control (C) mice (35 μg protein/lane) were separated on a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel, and after blotting to nitrocellulose membranes, JE/MCP-1 was detected by a goat anti-JE/MCP-1 polyclonal antibody. Recombinant, C-terminal truncated JE/MCP-1 (8,525 Da) was the positive control (+). **B:** Four-microliter serum samples from 2-month-old *mdx* (X) and control (C) mice were separated on a 15% SDS polyacrylamide gel and the JE/MCP-1 was detected as described above. **C:** TA muscle homogenates from 2-month-old *mdx* (X) and control (C) mice (60 μg protein/lane) were separated on a 12% SDS polyacrylamide gel, and cathepsin S was detected by an anti-cathepsin S polyclonal antibody. Mouse cathepsin S transfected 293 cell lysate was the positive control (+).

unknown rare genes that often escape detection because of the low abundance of their mRNAs. This is achieved by equalization of high- and low-abundance sequences [Diatchenko et al., 1996]. We have used SSH in combination with Northern blot analysis, cDNA sequencing, and BLAST search of nucleotide data banks to identify mRNAs that are expressed in 2-month-old *mdx* mouse leg muscles but not in the age-matched controls of the same mouse strain. We have determined that JE/MCP-1, cathepsin S, UPIX-1, nmb, and lysozyme M mRNAs are upregulated at least 10-fold and that the cathepsin B mRNA is doubled in *mdx* muscles relative to controls. The identification of upregulated JE/MCP-1, cathepsin S, and UPIX-1 mRNAs is a novel finding of this study. We determined that UPIX-1, a novel gene that is upregulated in *mdx* muscles, is also expressed in normal mouse brain, heart, spleen, lung, and liver but not in normal skeletal muscle. We also provided evidence for the translation of JE/MCP-1 and cathepsin S mRNAs by demonstrating their protein products in the muscles of *mdx* mice. Finally, by showing the presence of JE/MCP-1 protein in the serum of *mdx* mice, we provided evidence for secretion of this C-C chemokine to the blood.

JE was first identified in mouse fibroblasts as a platelet-derived growth factor–inducible early-response gene [Cochran et al., 1983; Rollins et al., 1988; Kawahara and Deuel, 1989]. After determination that JE is the murine homologue of the human MCP-1, its name was changed to JE/MCP-1. JE/MCP-1 is a 16.3-kDa C-C chemokine. However, the secreted JE/MCP-1 is heavily glycosylated, and its size varies between 25 and 30 kDa [Ernst et al., 1994; Luo et al., 1994]. The role of JE/MCP-1 in attraction of monocytes, T lymphocytes, basophils, and mast cells to the site of injury has been well established [Gu et al., 1999; Van Coillie et al., 1999]. Attraction of circulating monocytes to the injured muscles has been attributed to "wound hormones" such as platelet-derived growth factor, basic fibroblast growth factor, interleukin-1, and products of complement activation [Tidball, 1995]. However, a delay exists between the peak release of "wound hormones" from the damaged myofibers and the infiltration of circulation macrophages. This suggested that "wound hormones" first activate fibroblasts and/or resident macrophages, which then produce the substance that directly attracts macrophages to the injured myofibers [Tidball, 1995]. However, such a substance has not been identified. JE/MCP-1 is a candidate both because of its presence in muscles and serum of *mdx* mice and because of its well-established role in the attraction of monocytes and other inflammatory cells to the site of injury.

Recent evidence suggests that cathepsin S, a member of the lysosomal cysteine protease family, has specific intracellular and extracellular effects. Inside the cells, cathepsin S regulates major histocompatibility complex class II–mediated antigen presentation [Pierre and Mellman, 1998; Driessen et al., 1999]. When secreted, cathepsin S plays an important role in the remodeling of the extracellular matrix [Reddy et al., 1995; Sukhova et al., 1998; Watari et al., 1999; Shi et al., 1999]. In the extracellular matrix, cathepsin S specifically affects the storage and release of growth factors necessary for proliferation and differentiation [Liuzzo et al., 1999]. Thus, the expression of the mature cathepsin S protein in *mdx* mouse muscles suggests that this protease may affect the growth factors that are modifying the function of myofibers, fibroblasts, and satellite cells. Cathepsin S is also one of the most potent elastolytic enzymes [Shi et al., 1994; Chapman et al., 1994; Petanceska et al., 1996]. By its elastolytic activity, cathepsin S could also have a role

in creation of a path for the inflammatory cells migrating to the site of injury.

The upregulation of cathepsin B, nmb, and lysozyme M in *mdx* mouse muscles has been described before. Specifically, Sano et al. demonstrated by immunohistochemical methods a two- to threefold increase in cathepsin B protein in 2-month-old *mdx* mouse muscles [Sano et al., 1988]. Our finding of an approximately twofold upregulation of cathepsin B mRNA is in agreement with these data and indicates that SSH is a useful procedure for identification of differentially expressed genes in dystrophic muscles. The differential expression of nmb and lysozyme M mRNAs in 3-month-old *mdx* mouse muscles was recently described by Tkatchenko et al., who used SSH as we did [Tkatchenko et al., 2000]. Our identification of the increased expression of nmb and lysozyme M mRNAs in the 2-month-old *mdx* mouse muscles shows not only that SSH is a reproducible procedure, but also that the expression of these genes is initiated at a much earlier age of *mdx* mice.

Remarkably, skeletal muscle can survive the lack of important proteins such as creatine kinase by inducing the expression of other proteins that have complementary roles [Steeghs et al., 1998]. Such response is similar to the adaptation of the muscle to changes in functional demands, which is also characterized by altered expression of numerous genes simultaneously [Pette and Vrbova, 1999]. A point mutation in the dystrophin gene results in the lack of dystrophin protein but only a mild dystrophic phenotype in mice. The structural and functional changes occurring in the *mdx* mouse muscles include an elevated intracellular calcium concentration, signs of oxidative stress, increased cell membrane fragility, myofiber apoptosis and necrosis, accumulation of various inflammatory cells, activation of satellite cells resulting in regeneration, and a complex remodeling of the extracellular matrix [Gillis, 1999; De La Porte et al., 1999]. Such changes together suggest that the entire tissue with all of its components works together to adapt to the damage and maintain function. The altered expression of several genes simultaneously is an indication of such an effort.

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