THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 21, Issue of May 21, pp. 14948–14955, 1999
Printed in U.S.A.

Cloning and Characterization of a Novel Gene, *striamin*, That Interacts with the Tumor Suppressor Protein p53*

(Received for publication, September 10, 1998, and in revised form, January 12, 1999)

Renu Wadhwa‡§, Takashi Sugihara‡, Akiko Yoshida‡, Emma L. Duncan¶, Edna C. Hardeman‖, Hitoshi Nomura‡, Roger R. Reddel‖, and Sunil C. Kaul¶

From the ‡Chugai Research Institute for Molecular Medicine, 153-2 Nagai, Niihari-Mura, Niihari-Gun, Ibaraki 300-41, Japan, the ‖Children's Medical Research Institute, 214 Hawkesbury Road, Westmead, New South Wales 2145, Australia, and the ¶National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, 1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan

Expression analysis of a novel cDNA isolated from immortal murine fibroblasts revealed a single transcript of 3.0 kilobase pairs that was highly expressed in mouse and human striated muscle and in mouse heart. The gene has therefore been named *striamin*. Its expression was confined to skeletal muscle types with a fast glycolytic (2B) contractile phenotype. It was also detected in C2C12 mouse myoblasts and was down-regulated during *in vitro* myogenesis. The cDNA has a single open reading frame encoding a predicted 16.8-kDa protein of 149 amino acids with no homology to known proteins. Microinjection and transfection of green fluorescence protein-tagged *striamin* demonstrated that it localizes to the nucleus. Coimmunoprecipitations revealed that it can interact with p53 (a positive marker for myoblast differentiation) *in vivo* and *in vitro*. Furthermore, it repressed p53 activity in p53-mediated reporter assays. Fluorescence *in situ* hybridization with a mouse P1 genomic clone localized the gene to chromosome 12C3, which is syntenic to human chromosome 14q21–22.

Several genes, such as muscle creatine kinase, troponins, caveolin-3, α-actin, and myosin, have been reported to be predominantly expressed in skeletal muscle. A family of muscle-specific transcription factors such as myoD, myogenin, myf-5, and MRF-4/herculin/myf-6 that regulate muscle-specific gene expression has also been cloned. These are phosphorylated nuclear proteins, containing helix-loop-helix motifs required for dimerization and DNA binding, that can determine a specific cellular differentiation program (1). The myoD family of transcription factors has been shown to direct myogenesis, repress proliferation, activate differentiation, and induce contractile phenotypes. The introduction of any one of these into nonmyogenic cells induces their differentiation into mature muscle cells (2). The MyoD and myf-5 are expressed in proliferating myoblasts whereas myogenin and MRF-4 are not expressed until myoblasts exit the cell cycle in response to mitogen depletion. Therefore, myoD and myf-5 have been implicated as having a role in proliferating myoblasts whereas myogenin and MRF-4 have been shown to activate and maintain muscle gene expression (3). In addition, the cell cycle regulatory proteins such as RB (4, 5), p21 (6), cyclin D, cdk2, cdk4 (7), and p53 (8) have been implicated in the muscle differentiation program. Recently, caveolin-3, α-dystroglycan, and DNA methyltransferase (9–11) have also been assigned a positive role in myogenic differentiation.

While looking for genes involved in senescence and immortalization, we fortuitously cloned a novel gene that is specifically expressed in fast twitch skeletal muscles. The gene is named "*striamin*" because of its specific expression in striated muscle. Cloning of the cDNA, expression analyses, subcellular localization, chromosomal assignment, its interactions with the tumor suppressor p53, and its possible significance during muscle differentiation are reported herein.

EXPERIMENTAL PROCEDURES

Cell Culture—Normal mouse embryonic fibroblasts from the CD1-ICR strain of mouse (CMEF), an immortal clone (RS-4) established from CMEF, and NIH 3T3 cells, initially used for comparison of proteins and cloning studies, were cultured as described (12). C2 cells originally isolated by Yaffe and Saxel (13) and subcloned by Blau *et al*. (14) were grown in DMEM (Life Technologies, Inc., Melbourne, Australia) supplemented with 20% FCS (Commonwealth Serum Labs, Melbourne, Australia) and 0.5% chick embryo extract (Flow Laboratories, North Ryde, Australia). Cells were induced to differentiate by replacing growth medium with mitogen-poor medium, DMEM plus 2% horse serum. COS7 and Rat-1 cells used for transfection and microinjection studies were cultured in DMEM supplemented with 10% FCS.

cDNA Cloning and Sequencing—A cDNA library from RS-4 cells was constructed in the λZAP II vector and was screened with a polyclonal anti-p33 antibody raised against a protein identified from P-100 fractions of NIH 3T3 cells (12). cDNA clones were sequenced by the dideoxy chain termination method, and the reactions were analyzed on an ABI 377 automated sequencing machine. Full sequence of a 2.4-kb cDNA clone designated 336 was derived by generation of nested deletions from the 3′ end by exonuclease III (Deletion kit, Takara, Tokyo, Japan) and primer walking. Subsequently, the 5′ end of the cDNA was obtained by 5′ Marathon™ RACE polymerase chain reaction (PCR) on mouse skeletal muscle cDNA by using three antisense gene-specific primers SP1 (5′-TGT CAC TGC CAC GCC TTC TCG GTG CGC AG -3′), SP2 (5′-TCC CGG CTG CCC TTT GGC CCA TCT TGT CCC -3′) and SP3 (5′-TGA GAA AGC GTT AGA CGC TCT CAG AGC CCT-3′). 5′ Marathon™ RACE PCR was performed as described (CLONTECH).

RNA Isolation—Total RNA was prepared from C2C12 cultures grown in DMEM supplemented with 20% FCS or 2% horse serum (differentiation medium, for 24–96 h) using Trizol (Life Technologies, Inc). Skeletal muscles were excised from B6D2 males (F1 progeny of C57BL/6J female × DBA/2J male matings), frozen in liquid N_2, and homogenized in denaturant. Total cellular RNA was isolated from all muscle samples using the Trizol reagent.

Northern Blot and RT-PCR Analyses—Mouse and human multiple tissue Northern blots containing 2 μg of poly(A)$^+$ RNA per lane were purchased from CLONTECH. Total cellular RNA from C2C12 cultures

* This work was supported by grants from the National Health and Medical Research Council of Australia (to E. C. H.) and a Carcinogenesis Fellowship of the New South Wales Cancer Council (to R. R. R). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF031663.

§ To whom correspondence should be addressed. Tel.: 81-298-30-6211; Fax: 81-298-30-6270. E-mail: renu@cimmed.com.

and B6D2 muscles was denatured and size fractionated on 1% agarose gels containing 2.2 M formaldehyde and transferred to Hybond N membrane (Amersham Pharmacia Biotech). A 1.4-kb 3′-untranslated region fragment obtained by *Bam*HI digestion of pBSSK-336 plasmid was used as a probe. Hybridization was performed at 65 °C in SSC-Denhardt's-SDS buffer. The membrane was washed for 10 min each in 2× SSC, 2× SSC and 0.1% SDS, then washed in 1× SSC and 0.1% SDS twice, and autoradiographed. RNA loading on the blots was determined by hybridization with 18 S ribosomal probe. RT-PCR was performed on total RNA from mouse tissues and C2C12 cells cultured in normal and mitogen poor medium using primers from 5′ and 3′ of *striamin* open reading frame (ORF). Control RT-PCR was performed with glyceraldehyde-3-phosphate dehydrogenase-specific primers.

Cellular Localization of striamin—The *striamin* ORF was ligated in frame C-terminal to the GFP-ORF in pEGFPC1 vector (CLONTECH). The plasmid encoding the GFP-*striamin* fusion protein was transfected into COS7 cells growing on coverslips using LipofectAMINE™ (Life Technologies, Inc.). Coverslips were incubated with nuclear dye, Hoechst 33258 (Sigma) (5–10 μg/ml in culture medium for 10 min before cell fixation), and fixed with methanol:acetone (1:1). After three washings in phosphate-buffered saline, the coverslips were mounted with Fluoromount (Difco). The cells were examined using an Olympus BH-2 microscope with epifluorescence optics or 40× Plan-Neofluar objective on a Zeiss Axiophot microscope (Carl Zeiss, Germany) equipped with a CELLscan system (Scanalytics, Billerica, MA). Microinjections of the pGFPC1/*striamin*, pEGFPC1/N *striamin* (N-terminal 75 amino acids), or pEGFPC1/C *striamin* (C-terminal 74 amino acids) were performed directly into the nuclei of NIH 3T3 cells growing on coverslips using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Cells were fixed and examined for cellular localization of *striamin* as described above.

p53-mediated Reporter Assays—p53−/− mouse embryonic fibroblasts were transfected with a p53-responsive luciferase reporter plasmid, PG-13luc (kindly provided by Dr. Bert Vogelstein). A temperature-sensitive p53 expression plasmid, pMSVp53Val135 (a kind gift from Dr. Paul Jackson) that results in wild-type p53 conformation at 32.5 °C was used for exogenous p53 expression. Control expression plasmid pLK444 (15) or its *striamin*, N *striamin* (N-terminal 75 amino acids), or C *striamin* (C-terminal 74 amino acids) containing derivatives, pLK444/*striamin,* pLK444/N *striamin*, or pLK444/C *striamin*, respectively, were cotransfected with the reporter plasmid. Cotransfections of pRL-CMV were performed as an internal control to determine the efficiency of transfections. Luciferase assays (Dual-Luciferase™ reporter assay system, Promega) were performed 48 h after transfection. Luciferase values were calculated per microgram of protein as determined by Bradford protein assay.

p53−/− mouse embryonic fibroblasts were microinjected with a mixture of plasmids containing 0.1 μg/μl each of the pMSVp53Val135, p53-responsive β-gal reporter pRGCΔfos-lacZ (a kind gift from Dr. David Wynford-Thomas), and pLK444 or pLK444/*striamin*. Control IgG was co-injected for the identification of the injected cells. After overnight incubation at 32.5 °C, the cells were fixed with 4% formaldehyde, permeabilized with phosphate-buffered saline containing 0.1% Triton X-100 for 5 min on ice, washed three times with phosphate-buffered saline, and then stained with fluorescein isothiocyanate-conjugated secondary antibodies to detect injected IgG and β-galactosidase expression using the β-gal staining kit (Roche Molecular Biochemicals). Cells were viewed using a Zeiss microsope. All cells showing any trace of blue staining were scored as positive for expression.

In Vivo Co-immunoprecipitations—COS7 cells were used for high transfection efficiencies. Lysates (400 μg) prepared from pEGFPC1 vector-, pEGFPC1/*striamin*-, pEGFPC1/N *striamin*-, or pEGFPC1/C *striamin*-transfected cells after 48 h of transfection were incubated with anti-p53 antibody (CM-1, Novocastra Laboratories Ltd.) overnight at 4 °C. Immunocomplexes were precipitated by incubating with protein A/G-Sepharose (30 min at 4 °C) and were analyzed for the presence of *striamin* by Western blotting with anti-GFP monoclonal antibody. Precipitation of p53 was detected by Western blotting with anti-p53 monoclonal antibody (Ab-1, Calbiochem).

Preparation of Recombinant striamin Protein—The ORF of *striamin* cDNA was amplified by PCR of pBSSK/*striamin* clone with sense (5′-GGA TCC AAG AAA GGC CTG GCT GGC GAG-3′) and antisense (5′-AAG CTT TCA TGT CAC TGC CAC GCC TTC-3′) primers with *Bam*HI and *Hin*dIII sites, respectively. The PCR-amplified 0.5-kb fragment was first cloned in pGEM-T vector, confirmed to be correct by DNA sequencing, excised with *Bam*HI-*Hin*dIII, and then cloned into pQE30 vector (Qiagen) to yield His-tagged protein. The pQE30/*striamin*, pQE30/N *striamin*, and pQE30/C *striamin* constructs were used to transform M15 bacteria, and cells grown to OD_{580} = 0.6 were induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.2 mM) at 37 °C for 5 h. The bacterial lysates (induced and uninduced with IPTG) were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting with anti-His (Qiagen) and anti-p33 antibodies.

In Vitro Pull-down Assay—His-tagged recombinant *striamin,* N *striamin*, and C *striamin* were purified from *E. coli* transformed with the pQE30/*striamin,* pQE30/N *striamin*, and pQE30/C *striamin* construct, respectively. Cells were centrifuged, and the pellets were resuspended in buffer A (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 20 mM imidazole, 6 M urea, and 5 mM β-mercaptoethanol), sonicated for 2 min on ice, and agitated for 30 min at room temperature. The extract was centrifuged at 15,000 × *g* for 20 min. 0.5 ml of nickel-NTA-agarose affinity resin (Qiagen) was added to the supernatant, and the mixture was agitated at room temperature for 2 h. The mixture was then loaded into a disposable plastic column, followed by washing with 20 ml of 10 mM Tris-Cl, pH 7.5, and 0.5 M NaCl (TBS). The recombinant protein was eluted with 0.5 M imidazole in TBS followed by desalting on a PD-10 column (Amersham Pharmacia Biotech) using TBS as eluent. Aliquots of the purified protein were stored at −20 °C until use. The purity of the preparations was examined by SDS-polyacrylamide gel electrophoresis and Western analysis with anti-His antibody. Purified proteins (2–5 μg) were incubated with GST or GST-p53 (1 μg, Santa Cruz) in an Nonidet P-40-lysis buffer to a final volume of 500 μl. glutathione-Sepharose beads (20 μl) were added after 2 h and rotated at 4 °C for 1 h. Beads were pelleted by centrifugation, washed three times with TBS, boiled in SDS sample buffer, and analyzed by Western blotting with anti-His tag antibody.

Chromosomal Assignment—A mouse P1 genomic clone was obtained by PCR screening of a P1 bacteriophage mouse genomic library with clone 336-specific primers (sense: 5′-TGGTATTCTTATATTGTTTG-CAACTAACTA-3′; antisense, 5′-GGAAGGCCATGTGACCTAATGTT-TCATGTCA-3′). The isolated P1 clone was seen to hybridize with the 3′-untranslated region of the gene, following which it was used for chromosomal localization by fluorescence *in situ* hybridization (FISH). DNA from the mouse P1 clone was labeled with digoxigenin-dUTP by nick translation. Labeled probe was combined with sheared mouse DNA and hybridized to metaphase chromosomes derived from mouse embryonic fibroblasts in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides with fluoresceinated anti-digoxigenin antibodies, followed by counterstaining with 4′,6′-diamidino-2-phenylindole.

RESULTS

Cloning and Characterization of striamin cDNA—A comparison of plasma membrane Triton X-100-insoluble fractions from normal (CMEF) and immortal (NIH 3T3) murine cells revealed a protein of nearly 33 kDa (called p33) present in NIH 3T3 but not in CMEF cells (12). The protein was isolated from SDS-polyacrylamide gels and was used for raising polyclonal antibody. The anti-p33 antibody thus raised was used for cDNA cloning by immunoscreening of a RS-4 cDNA library as described previously (16). Five cDNA clones were obtained and were characterized by partial sequencing. Three clones showed identity to known genes, namely FusCHOP, G-utrophin, and dystrophin, while two clones had no matches in the DNA sequence data bases. The *in vitro* translated products of these two novel clones could not be precipitated with the anti-p33 antibody, indicating that they were not related to the p33. The characterization of one of these clones, 336, is described here.

The complete sequence of 336 (2.4 kb) contained one predicted ORF of 309 base pairs, which was located at the 5′ end of the cloned cDNA. 5′ RACE PCR performed using skeletal muscle cDNA generated an overlapping clone, 5′ 336. The additional 5′ sequence extended the ORF to 447 base pairs with an upstream in-frame stop codon (Fig. 1). The full-length cDNA sequence thus obtained had no homology to any sequence in the DNA sequence data banks. Analysis of the cDNA sequence by BLAST, PROSITE, GCG, and PSORT programs revealed no known motifs that could predict its possible function. The 5′-noncoding sequence of *striamin* contains C/GAAAA repeats and the 3′-noncoding region contains GT repeats; however, the

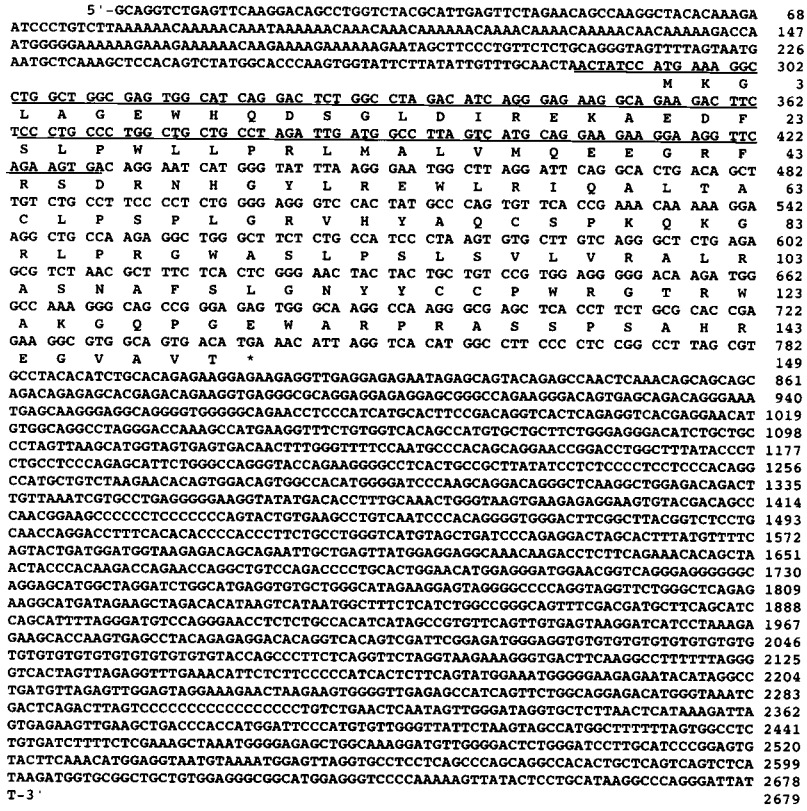


FIG. 1. **Nucleotide and predicted amino acid sequence of *striamin*.** The sequence obtained by 5′ RACE PCR on mouse skeletal muscle cDNA is *underlined* up to the 5′ upstream in-frame stop codon.

functional significance of these repeats is not known. It encodes a 149-amino acid protein (pI 10.2) that also has no significant matches in the protein data base. The ProtPram program predicted a soluble protein with average hydrophobicity of 0.5 and aliphatic index of 0.74. Two protein kinase C phosphorylation sites, SDR at 45–47 amino acid residues and SPK at 78–80 amino acid residues; one casein kinase II phosphorylation site, SGLD at 12–15 amino acid residues; and two myristoylation sites, GNYYCC at 111–116 and GTRWAK at 120–125 amino acid residues, are predicted by the ScanProsite program. Other interesting features of the protein include a high positive charge; a large number of serine, leucine, and proline residues; and the presence of four cysteines. The protein could not be characterized as a member of any known gene family based upon cDNA or protein sequence analyses. In view of its expression in striated muscle (see below), the gene has been named *striamin*.

The predicted ORF was cloned in pBSSK, and *in vitro* translation was performed to confirm the existence of the ORF in the given sequence. A protein of approximately 18 kDa mass was detected (data not shown). The ORF was also cloned into the bacterial expression vector pQE30, and recombinant protein containing a 6×His tag at the amino terminus was obtained. The induction of the promoter by IPTG led to the synthesis of a protein of approximately 18 kDa (data not shown).

Expression Analyses—Northern blotting of mouse and human tissues showed a strong reactivity of the *striamin* probe to a 3.0-kb transcript in mouse and human skeletal muscle and mouse heart (Fig. 2, *A* and *B*). It also reacted very weakly to transcripts of approximately 4.0 and 8.0 kb from human and 4.0 kb from mouse tissues. We therefore performed RT-PCR on mouse tissues and myoblasts with *striamin* primers (Fig. 2*C*). An expected size of DNA fragment was obtained from heart and myoblasts (Fig. 2*C*). This, together with the Northern data, confirmed that the cloned cDNA corresponds to *striamin* that is preferentially expressed in heart and skeletal muscle. We next investigated whether *striamin* exhibits muscle fiber type specificity. Four fiber phenotypes, namely fast twitch fiber types 2A, 2B, and 2X and slow fiber type I, have been defined on the basis of expression of the type of myosin heavy chain isoform (17). *striamin* was predominantly expressed in fast (quadriceps) *versus* slow fibers (soleus) (Fig. 3*A*). Northern analysis using RNA isolated from mouse skeletal muscles of differing fast and slow fiber content (quadriceps: 95% fast 2B, 4% fast 2X (18); extensor digitorum longus: 60% fast 2B, 28% fast 2X, 12% fast 2A (19); superficial gastrocnemius: 100% fast 2B (20); diaphragm: 57% fast 2X, 34% fast 2A, 7% slow (20); and soleus: 45% fast 2A, 55% slow (21)) revealed that *striamin* is expressed preferentially in fast glycolytic (2B) fibers (Fig. 3*B*).

We examined *striamin* expression during *in vitro* myogenesis of C2C12 myoblasts. These cells were cultured in differentiation medium, and RNA was isolated at various time points representing gradual formation of myotubes as observed microscopically. Interestingly, a 4-day culture that showed about 80% myotube formation had negligible expression of *striamin* as compared with the 1- and 2-day cultures, which had about 10–30% myotube formation (Fig. 3*C*). Consistent with this Northern analysis, *striamin* RT-PCR from C2C12 cells cultured in serum-supplemented and -deficient medium for 60–72 h exhibited its down-regulated expression in the latter (differentiated myoblasts) (Fig. 2*C*, *lanes 7* and *8*).

Cellular Localization of striamin—COS7 cells were transfected with a plasmid, pEGFPC1-*striamin*, encoding a GFP-*striamin* fusion protein. Transfected cells had distinct green fluorescence (Fig. 4*A*) in the nucleus that overlapped with the nuclear dye, Hoechst 33258, staining (Fig. 4*B*). pEGFPC1-*striamin*, pEGFPC1/N *striamin* (N-terminal 75 residues), and pEGFPC1/C *striamin* (C-terminal 74 residues) were microinjected into the nucleus of NIH 3T3 cells. Whereas distinct green nuclear fluorescence was detected for *striamin* as in the transfection assays, N *striamin* and C *striamin* were both retained in the cytoplasm (Fig. 4, *a–c*). Three dimensional image scanning of cells revealed that the full *striamin* protein is localized in the nucleus and is clearly excluded from nucleolar structures (Fig. 4*a*). N *striamin* was concentrated around the nuclear mem-

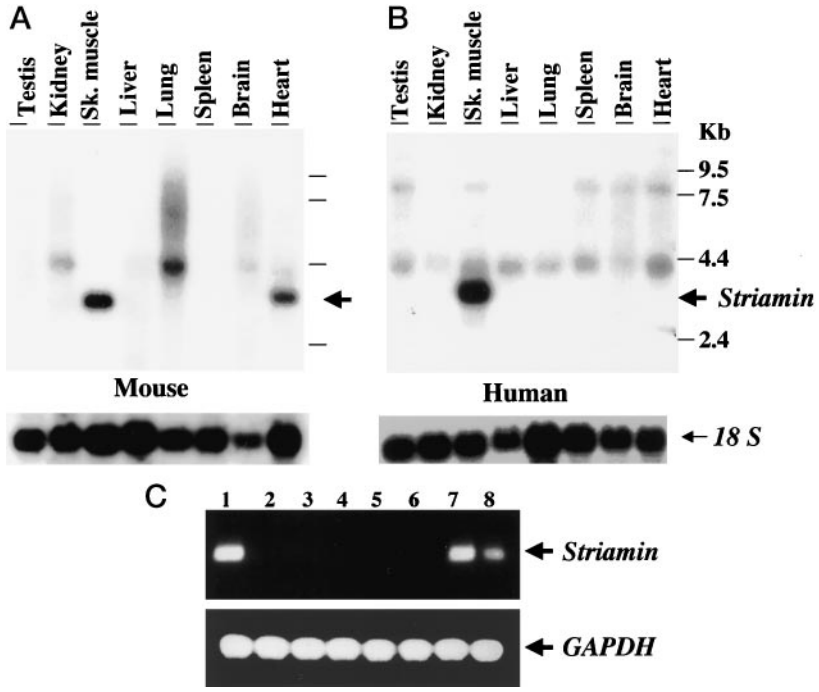


FIG. 2. **Expression of *striamin* in mouse and human tissues.** *A,* mouse multiple tissue Northern blot showing *striamin* expression in skeletal muscle and heart (shown by *arrow).* The probe showed a weak reactivity to a transcript of 4.0 kb in other tissues. *B,* human multiple tissue Northern blot showing *striamin* expression in skeletal muscle (shown by *arrow*). Probe reacted weakly to transcripts of 8.0 and 4.0 kb in some other tissues. RNA loading was assessed by hybridization with an 18 S ribosomal RNA oligonucleotide probe. *C*, *striamin* RT-PCR was performed using total RNA from mouse heart, kidney, testis, liver, brain, spleen, and myoblasts cultured in serum-supplemented and -deficient medium (*lanes 1–8*, respectively). *striamin* expression was detected in heart and myoblasts, and was down-regulated with differentiation (*lanes 7* and *8*).

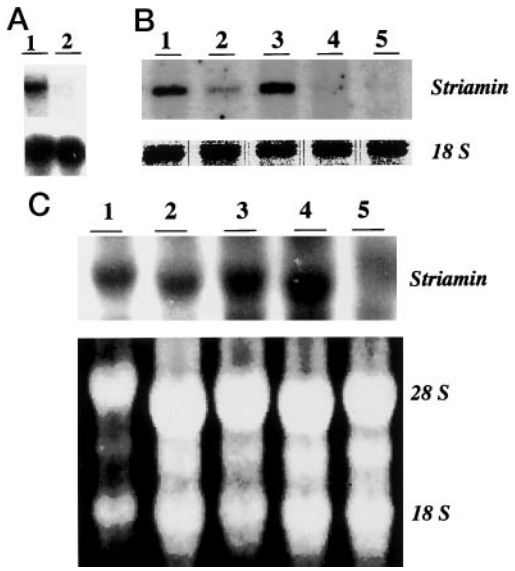


FIG. 3. **Expression in mouse skeletal muscle *in vivo* and *in vitro*.** *A,* Northern analysis for *striamin* expression in different muscle fiber phenotypes. Expression was detected in fast (quadriceps) (*lane 1)* and absent in slow (soleus) twitch fibers (*lane 2). B,* Northern analysis for *striamin* on RNA from muscle fibers with varying composition of fast and slow fibers. *Lanes 1–5* contain RNA from quadriceps (95% fast 2B, 4% fast 2X), extensor digitorum longus (60% fast 2B, 28% fast 2X, 12% fast 2A), superficial gastrocnemius (100% fast 2B), diaphragm (57% fast 2X, 34% fast 2A, 7% slow), and soleus (45% fast 2A, 55% slow). *striamin* is expressed preferentially in fast glycolytic (2B) fibers. The blots were hybridized with 18 S ribosomal RNA oligonucleotide probe for loading controls. *C*, *striamin* expression in C2C12 cells undergoing differentiation *in vitro. Lane 1* has RNA from dividing myoblasts cultured in normal growth medium, and *lanes 2–5* have RNA from cells cultured in differentiation medium for 24, 32, 40, and 88 h and showed approximately 10, 20, 30, and 85% myotube formation, respectively. Absence of *striamin* is seen in the differentiated C2C12 culture (*lane 5*). Ethidium bromide-stained gel with 28 S and 18 S RNA bands is shown as a loading control.

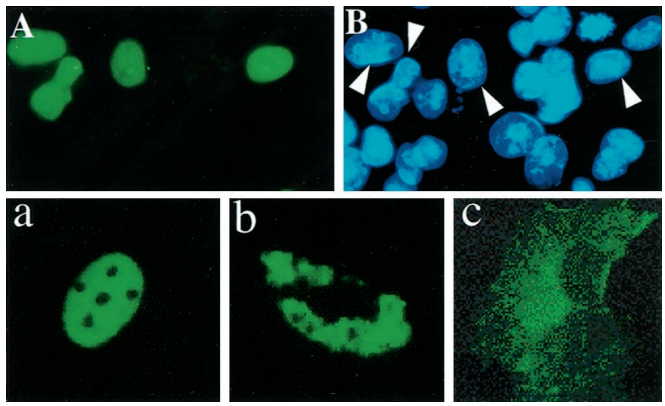
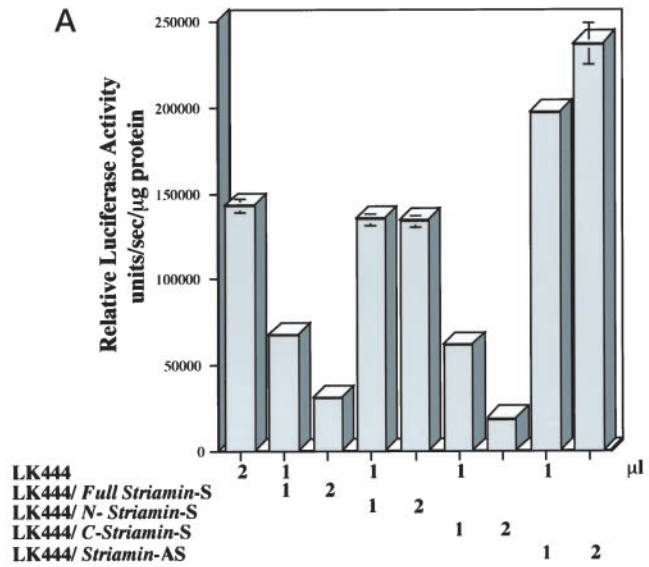
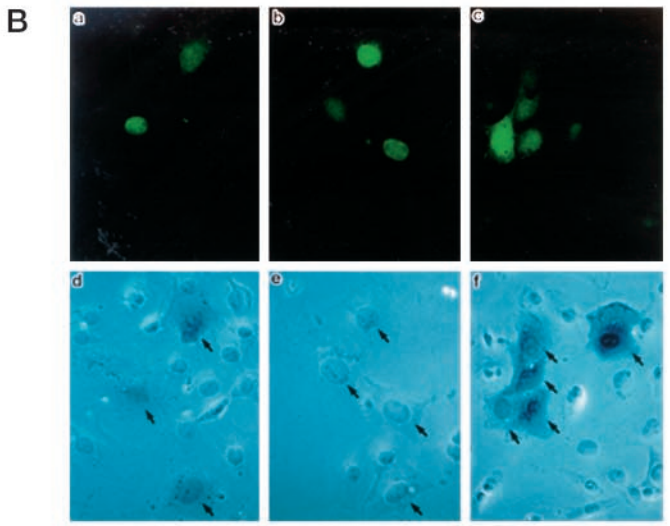


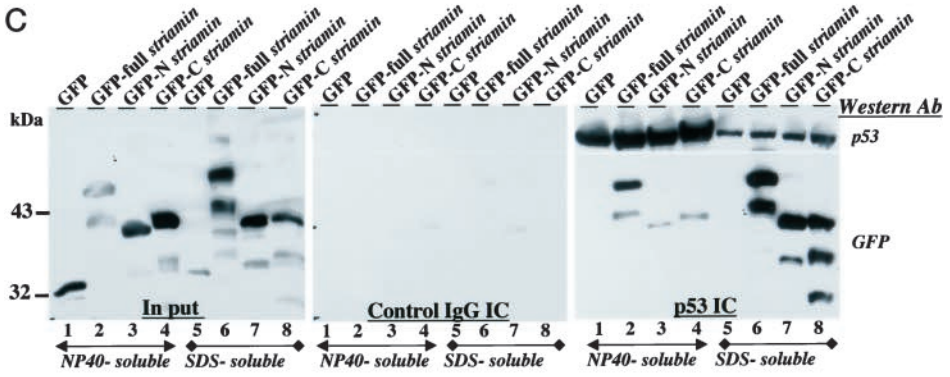
FIG. 4. **Intracellular localization of *striamin*.** Transfection of pEGFPC1-*striamin* resulted in distinct green fluorescence of GFP-*striamin* fusion protein in the nuclei (*A*) of COS7 cells that overlapped with Hoechst dye staining (*B*) shown by *arrowheads*. Microinjection of pEGFPC1/*striamin* (*a*), pEGFPC1/N *striamin* (*b*) and pEGFPC1/C *striamin* (*c*) plasmids into the nucleus of NIH 3T3 cells resulted in nuclear, perinuclear, and cytoplasmic green fluorescence, respectively, as visualized by laser scanning microscopy.

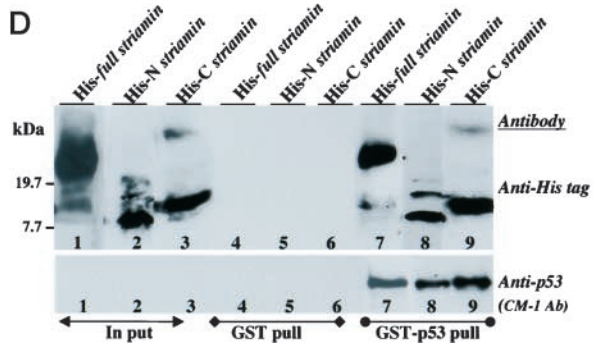
brane (Fig. 4*b*), and C *striamin* in addition to its concentration around the nucleus was also distributed diffusely in the cytoplasm (Fig. 4*c*). The present data and the fact that *striamin* does not contain any known nuclear localization signal could suggest that the predicted high positive charge of the native protein may be responsible for its nuclear localization. Alternatively, *striamin* may translocate to the nucleus by interacting with some nuclear localization signal-containing protein.

striamin Represses p53 Activity—Wild type (wt) p53 has a role during cell differentiation (reviewed in Ref. 22). Evidence in support of this includes the following: (i) overexpression of exogenous wt p53 or endogenous wt p53 following cell irradiation can partially restore differentiation of several tumor cells (23, 24), (ii) up-regulation of p53 mRNA occurs during C2 differentiation (25), and (iii) interference with endogenous wt p53 inhibits hematopoietic and muscle cell differentiation, which is shown to be independent of its cell cycle activity (8). In view of these reports and characteristics of *striamin* such as nuclear localization and down-regulation with myogenic differentiation, we asked whether *striamin* can interfere with p53 activity. p53−/− MEF cells were transfected with the wt p53-responsive luciferase reporter plasmid (PG-13luc), a temperature-sensitive p53 expression plasmid, pMSVp53Val135, and









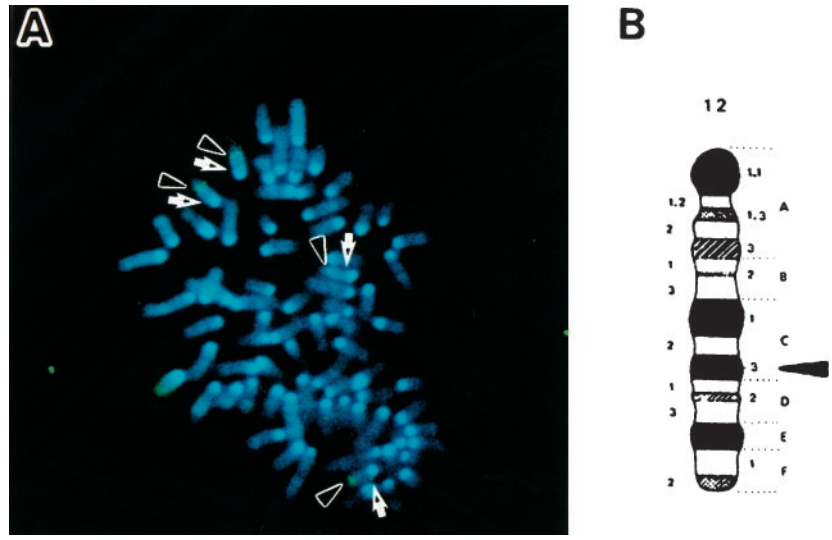


FIG. 6. *A*, chromosomal localization of *striamin* on mouse metaphase chromosomes. *striamin* and chromosome 12-specific probes are seen as green fluorescence, and are also marked by *triangles* and *arrows,* respectively. *B,* diagrammatic representation of *striamin* on mouse chromosome 12C3 region.

either the pLK444 vector or pLK444/*striamin*. The presence of the *striamin* expression construct caused a significant reduction in p53 reporter gene activity in four independent experiments. This result demonstrated that *striamin* can inhibit the transcriptional activity of p53 (Fig. 5*A*). Furthermore, cotransfections of the antisense construct were seen to have a mild positive effect on p53 activity (Fig. 5*A*). Similar results were obtained following microinjection of pMSVp53Val135, the p53-responsive β-gal reporter, pRGCΔfos-lacZ and the various *striamin* expression plasmids. Injected cells were identified by coinjection of rabbit IgG that was visualized by staining with fluorescein isothiocyanate-conjugated secondary antibody. β-Gal staining was observed in 86% and 88% of cells injected with control and antisense *striamin* plasmid, respectively, but only in 5% of cells that were injected with *striamin* sense construct (Fig. 5*B*). These data confirmed the repression of p53 activity by *striamin* and were consistent with its down-regulation observed during C2C12 differentiation. To further characterize the specificity of *striamin*-p53 interactions, we also performed p53 reporter assays in which expression plasmids encoding N-terminal 75 (N *striamin*) or C-terminal 74 (C *striamin*) amino acid residues were transfected. Whereas full and C *striamin* were seen to repress p53 activity, transfections of N *striamin* were neutral (Fig. 5*A*).

In Vivo and in Vitro Interactions of striamin and p53—The effect of *striamin* on the transcriptional activation function of p53 prompted us to investigate a possible interaction between these two proteins. We used COS7 cells for their high amounts of wt p53 and high transfection efficiencies. p53 immunocomplexes from pEGFPC1- and pEGFPC1/*striamin*-transfected cells when analyzed by Western blotting with anti-GFP antibody revealed the presence of GFP-*striamin* (Fig. 5*C*). This demonstrated that the *striamin* interacts with p53 *in vivo*. Similar immunoprecipitations were also performed from cells expressing GFP-N *striamin* and GFP-C *striamin* fusion proteins. The full, N-, and C- *striamin* were found to interact to p53 (Fig. 5*C*); no coimmunoprecipitation of GFP tag was detected in these experiments.

We next performed an *in vitro* pull-down assay for *striamin* and p53. His-tagged recombinant *striamin* was incubated with either GST alone (negative control) or with GST-p53. Western analysis of glutathione-Sepharose-reacting complexes using an anti-His tag antibody revealed the presence of His-tagged *striamin*, demonstrating that *striamin* can physically interact with p53 (Fig. 5*D*). Furthermore, as in the *in vivo* coimmunoprecipitations, both N *striamin* and C *striamin* were found to bind to p53 (Fig. 5*D*).

Chromosomal Localization—A mouse genomic P1 clone containing the *striamin* gene was obtained by PCR screening of a P1 library. In FISH analysis, this clone specifically hybridized to a medium-sized chromosome, which appeared to be chromosome 12 on the basis of 4′,6′-diamidino-2-phenylindole staining. To confirm the localization of *striamin* to mouse chromosome 12, FISH analysis was repeated using the P1 clone and chromosome 12 centromere-specific probe. The *striamin* P1 and chromosome 12 probes localized to the same chromosome (Fig.

Fig. 5. **Interaction of *striamin* and p53.** ***A*,** effect of *striamin* on transcriptional activity of wild type p53. p53-expression (pMSVp53Val135) and p53-responsive luciferase reporter (PG-13luc) plasmids were transfected into p53−/− mouse embryonic fibroblasts (*MEF*) along with antisense (*AS*) or sense (*S*) expression constructs encoding full, N-terminal 75 (N *striamin*), and C-terminal 74 (C *striamin*) amino acid residues. *Numbers* indicate the amount (μl) of each plasmid (0.5 μg/μl) used. *Error bars* represent standard deviation ($n = 3$). A 4.6-fold repression of p53 activity was observed with cotransfection of the full *striamin* sense construct, whereas the antisense construct resulted in 1.6-fold activation. Co-transfections of C *striamin* resulted in repression of p53 activity similar to the full *striamin* protein. N *striamin* was neutral. *B*, repression of p53 activity by microinjected *striamin.* p53−/− cells were microinjected with p53-expression (pMSVp53Val135) and p53-responsive β–gal reporter (pRGCΔfos-lacZ) plasmids with or without *striamin* sense or antisense expression constructs. Injected cells were visualized by staining with fluorescein isothiocyanate-conjugated anti-rabbit IgG (*upper panel)* and are shown by *arrows* in the *lower panel*. Blue staining for β-gal expression was observed in cells coinjected with vector (*a* and *d*) and the antisense construct (c and *f*). Coinjection of *striamin* (*b* and *e*) resulted in repression of p53 activity as demonstrated by the absence of β-gal staining (*e*). Note: dense blue stain in some cells caused interference in visualization of fluorescein isothiocyanate staining (*e* and *f*). *C*, *in vivo* coimmunoprecipitation of *striamin* and p53. p53 immunocomplexes from COS7 cells transfected with GFP vector or the one encoding GFP-*full striamin*, GFP-N *striamin*, or GFP-C *striamin* fusion protein were analyzed by Western blotting with anti-GFP antibody. Protein fractions of the cell lysates that were soluble in Nonidet P-40 lysis buffer and the remaining that were solubilized by boiling in 0.5% SDS were used for immunoprecipitaion. GFP-full *striamin*, -N *striamin*, or -C *striamin* were found to coprecipitate with p53 (*right panel*); no coprecipitation was observed with control antibody (*middle panel*) or of the GFP tag only with anti-p53 antibody (*right panel, lanes 1* and *5*). Input (*left panel*) shows 10% of the protein used for immunoprecipitation. Co-immunoprecipitated p53 was detected by Western blotting with anti-p53 antibody (Ab-1, *upper right panel*). *D*, *in vitro* coimmunoprecipitation of *striamin* and p53. His-tagged recombinant full *striamin,* N-*striamin or* C-*striamin* (5% of the input; *lanes 1–3)* were mixed with GST (*lanes 4–6*) or GST-p53 (*lanes 7–9*) and were precipitated by glutathione-Sepharose beads. GST-Complexes were analyzed by Western blotting with anti-p53 (*CM-1*) and anti-His tag antibody. The full *striamin*, N *striamin*, and C *striamin* were seen to coprecipitate with p53 (*lanes 7–9*).

6). A total of 80 metaphase cells were analyzed, out of which 71 exhibited specific labeling. Measurements of specifically hybridized chromosome 12 in 10 metaphase spreads demonstrated that *striamin* is located at a position that is 57% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome 12, an area that corresponds to band 12C3. This region is syntenic to human chromosome 14q21–22.

DISCUSSION

Here we report cloning and characterization of a novel gene, *striamin*, whose expression is restricted to the striated muscles. The expression pattern of *striamin* shares features in common with a few other genes, but is most similar to that of MyoD. Both are expressed in proliferating myoblasts, decline during differentiation, and yet are present in adult skeletal muscle (26). Furthermore, they appear to be preferentially expressed in fast glycolytic muscle fibers in the adult mouse (27). In adult myofibers myoD is thought to mediate innervation and thyroid hormone effects on fiber type-specific gene expression (26) as well as repress slow isoform gene function (28). Other genes that are specific to fast glycolytic fiber include myosin heavy chain 2B (MyHC 2B) (reviewed in Ref. 29) and a muscle-specific form of the glycolytic enzyme aldolase A (M-aldA) (30). Therefore, *striamin* may function as a mediator of extrinsic factors on gene expression in fast glycolytic fibers, as a determinant of metabolism, or as a determinant of muscle contractile activity.

Adult skeletal muscle can undergo regeneration, repair, and growth in response to injury or various stresses (31, 32). These processes are achieved by the activation of muscle precursor or satellite cells. In normal skeletal muscle, satellite cells are mitotically quiescent, mononucleated cells that are situated between the basement membrane and the myofiber plasma membrane. Injury or stress results in the mitotic activation of the satellite cells, which proliferate and fuse to repair damaged fibers or increase the size of existing fibers. The progression from proliferating to fusion competent satellite cells is marked by a precise order of expression of myogenic regulatory factors and muscle structural proteins. This includes, in order, MyoD, myogenin, α-smooth muscle actin, and sarcomeric myosin (33). Because *striamin* is expressed in myoblasts in culture, it is a candidate marker for activated satellite cells and may play a role in the differentiation process *in vivo*.

striamin is expressed in mouse, but not in human, heart. Differences exist between rodent and human cardiac myofibers in contraction velocities and force production, which in large part reflects the ATPase activity conferred by the MyHC isoform present (34). α-MyHC, the predominant isoform in the rodent heart, confers a faster shortening velocity and low efficiency of force production. In contrast, β-MyHC predominates in the human heart, which has a slower shortening velocity and high efficiency of force production. Rodent and human hearts also differ in the relative amounts of sarcomeric actins present, cardiac and skeletal actin (35, 36), which most likely reflects a difference in force development (37). The combinations of MyHCs and sarcomeric actins in rodent *versus* human heart results in a rodent heart that is more similar in contractile properties to a fast-twitch skeletal muscle fiber, whereas the opposite is true for the human heart. Therefore, the expression of *striamin* in striated muscles and mouse heart is consistent with a role in a fast contractile phenotype.

striamin Was Found to Interact with p53 in Vitro and in Vivo—Repression of p53 activity by *striamin* is consistent with its down-regulation during *in vitro* myogenesis when significant increase in p53 activity has been reported (22). These data suggests that *striamin* may affect myogenesis via a direct interaction with p53. Our data suggested that both the N- and C-terminal halves of *striamin* protein can bind to p53; however, it is the C terminus of *striamin* that represses transcriptional activity of p53. This suggests that there are more than one p53 binding sites in *striamin* protein and *vice versa*. Characterization of these warrant further studies.

The myogenic differentiation program includes activation of myogenic transcription factors, intercellular fusion of myoblasts, their withdrawal from the cell cycle, and terminal differentiation to myotubes. Besides the muscle-specific family of transcription factors, myoD family, several adhesion molecules such as N-CAM, N-cadherin, very late activation antigen 4, vascular cell adhesion molecule 1 (VCAM-1), and meltrin-α have been implicated in this process (38–41). Bone morphogenetic protein-12 and -13, TGF-β, and other members of the TGF-β superfamily (42, 43), ERK-6, a mitogen-activated protein kinase (44), and PAX3 (45) have been shown to interfere with or suppress *in vitro* myogenesis of C2C12 myoblasts. Cyclin D1 is found to be down regulated with myogenesis of C2C12, in contrast to cyclin D2, which showed transient increase, and cyclin D3, which showed 20-fold increase (7, 46). *striamin* does not show any structural homology to any of these proteins that have been implicated in different aspects of muscle differentiation. Of particular interest are its fast fiber specificity, nuclear localization, down-regulation with myogenic differentiation, and functional interactions with the tumor suppressor p53, which may predict it to be an important gene in the regulation of the myogenic differentiation program and warrant further studies to elucidate its role in myogenesis and the fast contractile phenotype.

Acknowledgments—We greatly appreciate the kind assistance of John O'Mahoney, Xavier Badoux, and Vicky Ferguson for Northern analysis

REFERENCES

1. Olson, E. N., and Klein, W. H. (1994) *Genes Dev.* **8,** 1–8
2. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y., and Lassar, A. B. (1991) *Science* **251,** 761–766
3. Emerson, C. P. J.(1993) *Curr. Opin. Cell Biol.* **5,** 1057–1064
4. Shiio, Y., Sawada, J. I., Handa, H., Yamamoto, T., and Inoue, J. I. (1996) *Oncogene* **12,** 1837–1845
5. Wang, J., Guo, K., Wills, K. N., and Walsh, K. (1997) *Cancer Res.* **57,** 351–354
6. Guo, K., Wang, J., Andres, V., Smith, R. C., and Walsh, K. (1995) *Mol. Cell. Biol.* **15,** 3823–3829
7. Kiess, M., Gill, R. M., and Hamel, P. A. (1995) *Oncogene* **10,** 159–166
8. Soddu, S., Blandino, G., Scardigli, R., Coen, S., Marchetti, A., Rizzo, M. G., Bossi, G., Cimino, L., Crescenzi, M., and Sacchi, A. (1996) *J. Cell Biol.* **134,** 193–204
9. Song, K. S., Scherer, P. E, Tang, Z., Okamoto, T., Li, S., Chafel, M., Chu, D., Kohtz, D. S., and Lisanti, M. P. (1996) *J. Cell Biol.* **271,** 15160–15165
10. Kostrominova, T. Y., and Tanzer, M. L. (1995) *J. Cell. Biochem.* **58,** 527–534
11. Takagi, H., Tajima, S., and Asano, A. (1995) *Eur. J. Biochem.* **231,** 282–291
12. Wadhwa, R., Kaul, S. C., Ikawa, Y., and Sugimoto, Y. (1991) *Mutat. Res.* **13. 256,** 243–254
13. Yaffe, D., and Saxel, O. (1977) *Nature* **270,** 725–727
14. Blau, H. M., Chiu, C.-P., and Webster, C. (1983) *Cell* **32,** 1171–1180
15. Gunning, P,, Leavitt, J., Muscat, G., Ng, S. Y., and Kedes, L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4831–4835
16. Wadhwa, R., Kaul, S. C., Ikawa, Y., and Sugimoto, Y. (1993) *J. Biol. Chem.* **268,** 6615–6621
17. Pette, D., and Staron, R. S. (1990) *Rev. Physiol. Biochem. Pharmacol.* **116,** 1- 76
18. Hamalainen, N., and D. Pette (1993) *J. Histochem. Cytochem.* **41,** 733–743
19. Leferovich, J. M., Lana, D. P., Sutrave, P., Hughes, S., and Kelly, A. M (1995) *J. Neurosci.* **15,** 596–603
20. Zardini, D. M., Parry, D. J. (1994) *Muscle & Nerve* **17,** 1308–1316
21. Lewis, D. M., Parry, D. J., and Rowlerson, A. (1982) *J. Physiol.* **325,** 393- 401
22. Prokocimer, M, and Rotter, V. (1994) *Blood* **84,** 2391–2411
23. Soddu, S., Blandino, G., Citro, G., Scardigli, R., Piaggio, G., Ferber, A., Calabretta, B., and Sacchi, A. (1994) *Blood* **83,** 2230–2237
24. Aloni-Grinstein, R., Schwartz, D., and Rotter, V. (1995) *EMBO J.* **14,** 1392-1401
25. Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995) *Science* **267,** 1018–1021
26. Hughes, S. M., Taylor, J. M., Tapscott, S. J., Gurley, C. M., Carter, W. J., and Peterson, C. A. (1993) *Development* **118,** 1137–1147
27. Hughes, S. M., Koishi, K., Rudnicki, M., and Maggs, A. M. (1997) *Mech. Dev.* **61,** 151–163

28. Goblet, C., and Whalen, R. G. (1995) *Dev. Biol.* **170,** 262–273
29. Schiaffino, S., and C. Reggiani (1996) *Physiol. Rev.* **76,** 371–423
30. Colbert, M. C., and Ciejeck-Baez, E. (1992) *Dev. Biol.* **149,** 66–79
31. Antonio, J., and Gonyea, W. J. (1993) *Med. Sci. Sports Exercise* **25,** 1333- 1345
32. Grounds, M. D., and Yablonka-Reuveni, Z. (1993) in *Molecular and Cell Biology of Muscular Dystrophy* (Partridge, T., ed) pp. 210–256. Chapman & Hall, London
33. Yablonka-Reuveni, Z., and Rivera, A. J. (1994) *Dev. Biol.* **164,** 588–603
34. Swynghedauw, B. (1996) *Physiol. Rev.* **66,** 710–771
35. Gunning, P., Ponte, P., Blau, H., and Kedes, L. (1983) *Mol. Cell. Biol.* **3,** 1985–1995
36. Boheler, K. R., Carrier, L., de la Bastie, D., Allen, P. D., Komajda, M., Mercadier, J.-J., and Schwartz, K. (1991) *J. Clin. Invest.* **88,** 323–330
37. Hewett, T. E., Grupp, I. L., Grupp, G., and Robbins, J. (1994) *Circ. Res.* **74,** 740–746
38. Dickson, G., Peck, D., Moore, S. E., Barton, C. H., and Walsh, F. S. (1990) *Nature* **344,** 348–351
39. Knudsen, K. A., McElwee, S. A., and Myers, L. (1990) *Dev. Biol.* **138,** 159- 168
40. Rosen, G. D., Sanes, J. R., LaChance, R., Cunningham, J. M., Roman, J., and Dean, D. C. (1992) *Cell* **69,** 1107–1119
41. Yagami-Hiromasa, T., Sato, T., Kurisaki, T., Kamijo, K. Nabeshima, Y., and Fujisawa-Sehara, A. (1995) *Nature* **377,** 652–656
42. Inada, M., Katagiri, T., Akiyama, S., Namiki, M., Komaki, M., Yamaguchi, A., Kamoi, K., Rosen, V., and Suda, T. (1996) *Biochem. Biophys. Res. Commun.* **222,** 317–322
43. Filvaroff, E. H., Ebner, R., and Derynck, R. (1994) *Development* **120,** 1085- 1095
44. Lechner, C., Zahalka, M. A., Giot, J. F., Moller, N. P. H., and Ullrich, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4355–4359
45. Epstein, J. A., Lam, P., Jepeal, L., Maas, R. L., and Shapiro, D. N. (1995) *J. Biol. Chem.* **270,** 11719–11722
46. Rao, S. S., and Kohtz, D. S. (1995) *J. Biol. Chem.* **270,** 4093–4100

Cloning and Characterization of a Novel Gene,*striamin*, That Interacts with the Tumor Suppressor Protein p53

Renu Wadhwa, Takashi Sugihara, Akiko Yoshida, Emma L. Duncan, Edna C. Hardeman, Hitoshi Nomura, Roger R. Reddel and Sunil C. Kaul

J. Biol. Chem. 1999, 274:14948-14955.
doi: 10.1074/jbc.274.21.14948