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The rat *Mist1* gene: structure and promoter characterization

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Abstract

Transcription factors of the basic Helix–Loop–Helix (bHLH) protein family play key roles in several developmental processes. *Mist1* belongs to this group of proteins and shares several properties with the other family members. For example, *Mist1* is capable of dimerization with the ubiquitously expressed E2A bHLH proteins and exhibits a strong DNA-binding activity to the core E-box sequence. Using in-situ hybridization and Northern blot hybridization, *Mist1* mRNA has been detected in a variety of embryonic and adult rodent tissues. To understand the molecular mechanisms involved in the expression of the gene, we have cloned the rat *Mist1* gene and analyzed 2.5 kb of its 5' flanking region. The *Mist1* gene spans over 5 kilobases and is composed of two exons separated by a unique intron. The entire coding region is localized in the second exon. Sequence analysis of the promoter region indicated an absence of TATA-box or CAAT-box sequence, but several consensus Sp1-binding sites were present near the transcription start site. Deletion analysis of the promoter region identified a 272 bp proximal fragment to be sufficient to drive expression of a reporter gene in NIH3T3 fibroblasts. Subsequent deletion of potential Sp1 sites results in a marked decrease in promoter activity. Electrophoretic mobility shift assays revealed that Sp1 binds to two different regions in the proximal promoter, a typical Sp1 site located at (–38; –33) and a G/C-rich region between (–67; –62). These data suggest that the basal expression of this TATA-less gene might be driven by general transcription factors, such as Sp1.

Author Keywords: bHLH factor; Promoter sequence; Regulatory elements; Sp1

1. Introduction

The transcription machinery in eukaryotic cells is tightly regulated by a number of key transcription factors that control tissue-specific gene expression patterns. Among the different transcription factor families that have been identified, the basic helix–loop–helix (bHLH) family stands out as an essential regulator of many different developmental pathways. Members of this family exhibit a common structural motif known as the helix–loop–helix (HLH) region, which consists of two α -amphipathic helices separated by a variable loop (Ma and Murre). Immediately adjacent to the HLH is a basic region that is required for DNA-

binding (Blackwell and Weintraub, 1990). The HLH domain is involved in protein dimerization with other bHLH factors, mediating the formation of bHLH dimers that bind to the DNA consensus E-box site (-CANNTG-) found in many structural genes (Murre et al., 1989b). A consequence of the binding of bHLH proteins to DNA targets is the transcriptional activation or repression of specific genes. Based on their expression pattern, bHLH factors are classified in two major categories. The 'tissue-specific' bHLH proteins are only found in a particular tissue or cell-type, i.e. the MyoD family in skeletal muscle (reviewed in Ludolph and Molkentin), the neurogenic Mash1, NeuroD or HES family members in neuronal cells (see Kageyama et al., 1997 for a review) or the Tal-1 protein in hematopoietic cells (Shivadasani and Orkin, 1996). Other factors, such as the E2A gene products, E12 and E47, are called 'ubiquitous' since they are expressed in virtually every cell (Murre et al., 1989a). In general, tissue-specific bHLH factors form heterodimers with a partner of the ubiquitous protein family, i.e. MyoD:E12 in skeletal muscle, and this high-affinity, stable complex binds to an E-box sequence to regulate gene transcription (Blackwell; Lassar and Murre).

We recently identified a novel bHLH factor, Mist1 (Mak et al., 1996), that is capable of forming heterodimers with E12 and E47. In addition, Mist1 forms homodimers that also bind to E-box sequences (Lemerrier et al., 1997). In skeletal muscle, the Mist1 protein accumulates in undifferentiated myoblasts, but rapidly decreases as the cells differentiate into myotubes. Our studies have also shown that Mist1 associates with the myogenic bHLH factor MyoD and represses MyoD from activating gene transcription in myogenic cells (Lemerrier et al., 1998). Thus, Mist1 functions as a bHLH repressor in skeletal muscle. As detected by in-situ hybridization in the mouse embryo, Mist1 mRNA also is found in the primitive gut and organs of the gastro-intestinal tract (pancreas, intestine, submandibular gland), as well as in the olfactory epithelium and lung (Lemerrier et al., 1997). In adult rat tissues, Northern blot analysis indicates that Mist1 mRNA is present in stomach, liver, spleen, skeletal muscle, testis and olfactory epithelium. However, the role(s) of Mist1 in these tissues remains to be elucidated.

The molecular mechanisms that regulate transcription of the Mist1 gene are unknown. Previous studies of eukaryotic class II promoters have shown that they can be dissected into a basal core promoter (frequently containing a TATA box) and distal enhancer elements. The TATA box is typically located at 25–30 bp upstream from the transcription start site and is bound by TFIID, an essential transcription factor composed of the TATA binding protein (TBP) and TBP-associated factors (TAFs) (reviewed in Burley and Hahn). A growing number of genes have been discovered that do not contain a TATA-box element, and it has been found that the transcription initiation complex, including TBP and its associated factors, can still bind to this upstream region if appropriate tethering elements are present. In order to investigate the molecular mechanisms controlling the expression of the *Mist1* gene, we isolated genomic clones and analyzed the 5' flanking region for promoter activity.

2. Materials and methods

2.1. Cell culture

NIH3T3 cells (mouse embryo fibroblasts) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml). C2C7 mouse myogenic cells were maintained in DMEM:Ham F12 (1:1) supplemented as above. Terminal differentiation into myotubes was induced by replacing the culture medium with a high-glucose DMEM containing 2% horse serum for 2 days.

2.2. Northern blots

Total RNA was extracted from mouse cell lines using RNable solution (Eurobio). Fifteen micrograms of total RNA were separated through a denaturing formaldehyde gel, transferred to nitrocellulose (BioBond-NC, Whatman) and hybridized with a ³²P-labeled mouse *Mist* 1 cDNA probe (GenBank Accession No. AF049660). After high-stringency washes, the blots were subjected to autoradiography. To normalize for RNA content, each blot was also probed with a mouse *GAPDH* cDNA.

2.3. Cloning of the rat *Mist1* gene

Two rat *Mist1* genomic clones were obtained by screening an EMBL3 genomic library (Clontech) with a ³²P-labeled *Mist1* cDNA probe. Phage clone I (13 kb insert) was isolated using a rat *Mist1* cDNA probe encoding amino acids 88–197. Subsequently, phage clone II (14 kb insert) was identified using a 5' probe (containing the last 127 bp of intron 1 and part of the coding region) derived from clone I. After restriction digest analysis, a 7 kb *SacI* fragment derived from clone I and a 3.3 kb *SalI* fragment obtained from clone II were subcloned in pBSKS+ plasmid (Stratagene). Inserts were sequenced on both strands, and a DNA sequence analysis was performed using Mac Vector and Blast software programs. Transcription factor consensus binding sites were analyzed with the TFD database. The gene sequence has been deposited in GenBank (AF049874).

2.4. 5' extension analysis

The transcription start site in the rat *Mist* 1 gene was determined by 5' extension analysis according to the manufacturer's instructions (Primer Extension System kit, Promega). The primer designed for this experiment (5'-ACTGGGGATCCGAGGCCGTA-3') is positioned 50 bp upstream of the initiation ATG. The extension reaction was performed from 0.5 µg of rat spleen poly(A)⁺ RNA (Clontech). One tenth of the reaction was loaded on to a 7% sequencing gel, along with ³²P-ΦX174 *HinfI* markers. The gel was exposed to X-ray film with an intensifying screen at –80°C for 15 h.

2.5. Luciferase reporter constructs

A 2588 bp *Mist1* promoter fragment was amplified by PCR from clone II using T3 oligonucleotide and *Mist1* specific primer A (5'-gggtaccGAGGCCGTACGCGCAG-3'). The PCR product was digested with *KpnI* and ligated upstream of the luciferase reporter gene in the pGL2-basic plasmid (Promega). All PCR amplifications were performed using a proofreading polymerase mix of Taq/Pwo (Expand High Fidelity PCR system, Boehringer Mannheim). Deletions in the WT fragment (–2492 to +66) were generated as above by PCR using the following primers (5' to 3'): *Mist1* (–2492 to –198): cgggtaccTTGGGGTCACGAC and T3, *Mist1* (–2492 to –464): cgggtaccTGACAATTGGGAAAAC and T3. For the deletions of the 5' end: primer A (see above) and the following primers (5' to 3'): cgggtaccAATTGTCAGAC (–475 to +66), cgggtaccCCAAGGCCAGGT (–206 to +66), cgggtaccAGGTGCCGCCCTGG (–55 to +66), cgggtaccTGCCGCCCTGGCCCCGC (–51 to +66), cgggtaccAGCGAGCTCATTAC (–34 to +66). The PCR products were cloned into pGL2basic at the *KpnI* site. Mutations of the Sp1 site b in the (–206, +66) construct were achieved by PCR using the overlapping mutated oligonucleotides (5' to 3'): GCCCTGGCCgatCCCAGCTC (+ strand) and CTCGCTGGGatcGGCCAGGCGGCAC (– strand). Mutations were confirmed by DNA sequencing.

2.6. Cell transfections

Transfections in NIH3T3 cells were performed with 2 µg of a calcium phosphate DNA precipitate containing various *Mist1* promoter constructs. After 2 days, cell extracts were

prepared and assayed for luciferase activity and protein content as described previously (Lemerrier et al., 1997). Luciferase values were normalized to protein levels and are expressed as fold over control (pGL2 basic vector). Each value represents the average ($\pm$ SEM) of at least four experiments. Over-expression of Mist1 in NIH3T3 cells was obtained by transfecting the cells with 10 μ g of pcDNA-Mist1 plasmid (Lemerrier et al., 1997).

2.7. Western blot

Western blot analyses were performed as described in detail previously (Lemerrier et al., 1998).

2.8. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from NIH3T3 cells were prepared as described previously (Lassar et al., 1991). Protein concentration was determined using the BioRad protein assay reagent. EMSA was performed as described previously (Lemerrier et al., 1998). Oligonucleotides were end-labeled with γ^{32} P-ATP and T4 polynucleotide kinase. After removal of unincorporated nucleotides, the sense and antisense strands were annealed and used as a probe. The following oligonucleotides were tested (sequence of the + strand, 5' to 3'):

WT -54; -29: AGGTGCCGCCCTGGCCCCGCCAGCG

mut a: AGGTGgatCCCTGGCCCCGCCAGCG

mut b: AGGTGCCGCCCTGGCCgatCCCAGCG

mut a/b: AGGTGgatCCCTGGCCgatCCCAGCG

-63; -38: CGTGGCCGCAGGTGCCGCCCTGGCC

-72; -48: AGCGAGGGGCGTGGCCGCAGGTGCC

mut dist: AGCGAGtaaCGTGGCCGCAGGTGCC

mut prox: AGCGAGGGGCGTGtaaGCAGGTGCC.

For competition experiments a 100-fold molar excess of unlabeled probe was included in the reaction. In supershift experiments, 300 ng of polyclonal antibody directed against Sp1, E47 and AP2 α (Santa Cruz, PEP-2, N-649 and C-18 antiserum, respectively) were preincubated for 15 min at room temperature with the NIH3T3 nuclear extracts prior to the addition of the labeled probe. DNA:protein complexes were resolved on a 4% 0.5 $\times$ TBE acrylamide gel and visualized by autoradiography.

3. Results and discussion

3.1. Isolation and structure of the rat *Mist1* gene

The cloning of the entire Mist1 rat cDNA has been previously reported (Lemerrier et al., 1997). The next step led us to the isolation and the characterization of the *Mist1* gene. A phage EMBL3 rat genomic library was screened using a rat Mist1 cDNA probe encoding for amino acids 88–197. A first positive phage (clone I, 13 kb insert) was isolated (Fig. 1). Subsequently, another screen was performed with the most 5' sequence derived from clone I. A second overlapping phage (clone II) was obtained that contained additional 5' sequence (Fig. 1). Cloning and sequencing of the two clones showed the *Mist1* gene to be composed of two exons separated by a single 656 bp intron (Fig. 2). The intron/exon borders follow the classical GT/AG splice rules. The first exon is 73 bp long and consists entirely of 5' untranslated sequence. The second exon spans over 3.5 kb and contains the entire coding region plus the 3' untranslated sequence. Taken together, the *Mist1* gene is positioned within less than 5 kb of genomic DNA.

3.2. Promoter sequence of the *Mist1* gene

The sequence of approximately 2.5 kb of the 5' region flanking the *Mist1* gene was established from clone II (Fig. 2A). The transcription start site was mapped by 5' primer extension analysis using an oligonucleotide positioned 50 bases upstream of the initiation ATG and poly(A)⁺ RNA obtained from rat spleen. A major product of 75 bases was obtained (Fig. 2B, lane E), which indicates that the 5' untranslated sequence is 124 bp long. These data are in full agreement with our previous report on the isolation of the rat *Mist1* cDNA by 5' RACE strategies (Lemerrier et al., 1997). Therefore, the first nucleotide of the cDNA is referred to as +1, and the first base within the promoter as -1. Sequence analysis and database homology searches revealed that the *Mist1* gene does not exhibit a typical TATA-box motif near the transcription start site but instead has a related TcATTAcAT sequence located at -25 (Fig. 2A). Two consensus Sp1 binding sites (Briggs et al., 1986) are present on the minus strand at positions (-38; -33) and (-49; -44) as well as an AP-2 binding site (GCCGCGGGC, Williams and Tjian, 1991) at -130 and a PU.1 site (GAGGGAAA, Klemsz et al., 1990) at -105. A striking feature in the *Mist1* promoter region is the presence of 21 putative E-box sites (CANNTG), named E1 to E21, raising the possibility that *Mist1* gene expression is regulated through binding of specific bHLH factors.

3.3. The *Mist1* promoter is functional in NIH3T3 and C2C7 cells

In order to confirm the presence of functional promoter regulatory elements, the putative promoter region (about 2.5 kb) plus 66 bp of exon 1 was ligated 5' to a luciferase reporter gene (*Mist* WT-Luc), and the activity of the promoter was tested by transient DNA transfections in NIH3T3 fibroblasts and in C2C7 myogenic cells. Because *Mist1* expression was not detected in a number of tissues (i.e. brain and heart; Lemerrier et al., 1997), we first verified that endogenous *Mist1* was expressed in these two cell lines by testing for the presence of *Mist1* mRNA and protein. *Mist1* mRNA (3.6 kb) was detected in both cell types by Northern blot, indicating that the endogenous *Mist1* gene is active (Fig. 3A). Using a polyclonal rabbit serum raised against a GST-*Mist1* fusion protein, we also confirmed that *Mist1* protein was present in extracts from C2C7 and NIH3T3 cells (Fig. 3B). As reported before (Lemerrier et al., 1998), *Mist1* is expressed in C2C7 myoblasts (MB lane) but not in terminally differentiated myotubes (MT lane). *Mist1* protein was also detected in NIH3T3 control cells (C lane, endogenous protein) and in much larger amounts in cells transfected with a *Mist1* expression plasmid (Tx lane). No signal was obtained with the pre-immune serum or an irrelevant normal rabbit serum (data not shown). Thus, these data indicated that NIH3T3 and C2C7 cells can be used as a model system to study *Mist1* promoter activity.

When the *Mist* WT-Luc plasmid was tested in NIH3T3 cells (Fig. 3C, white bar), 90-fold higher luciferase levels were obtained compared to the promoter-less plasmid (black bar). This result indicates that the 2.5 kb *Mist1* 5'-flanking region contains a functional promoter that allows the transcription of a reporter gene. In C2C7 myogenic cells, we analyzed the expression of *Mist* WT-luc in myoblasts kept in a proliferative state (high serum growth medium) or in cells induced to terminally differentiate into myotubes upon serum deprivation. In this case, a muscle-specific gene such as the troponin I gene (TnI-luc, Johnson et al., 1996) becomes highly expressed in differentiated myotubes (>250-fold over control, gray bar), whereas a low level of TnI-luc activity is detected in myoblasts (Fig. 3C). When tested under similar conditions, a high luciferase expression was obtained with the *Mist* WT-luc reporter in proliferating myoblasts (215-fold above the control), whereas the activity of the promoter decreases as the cells are induced to differentiate into myotubes (77-fold above the control). Thus, the *Mist1* gene behaves in an opposite fashion to the TnI gene in myogenic cells. However, we do not know at present whether the lower activity of *Mist1* promoter in

differentiated cells is due to cell-cycle arrest upon serum deprivation or to a specific inhibition mechanism.

3.4. Deletion analysis of the *Mist1* promoter

To delineate the sequences responsible for the basal promoter activity, several deletion constructs that lacked either the proximal ($\Delta 1$ and $\Delta 2$) or distal promoter sequences ($\Delta 3$ to $\Delta 7$) were generated by PCR. The $\Delta 1$ and $\Delta 2$ constructs, which lack 197 and 463 nucleotides of proximal sequence, respectively, showed a greatly reduced promoter activity when compared to the *Mist1* WT-Luc construct (Fig. 4). In contrast, $\Delta 3$ and $\Delta 4$ constructs, containing only 475 and 206 bp of proximal sequence, exhibited almost full promoter activity in this system. These results indicate that a 272 bp fragment (−206 to +66) contains the regulatory element sufficient to allow the transcription of the luciferase reporter gene. Further deletions of the two E-boxes E3 and E2 and a putative AP-2 site ($\Delta 6$) or the E1 E-box sequence ($\Delta 5$) lead to a marked decrease in luciferase activity. Constructs bearing an additional deletion of the two consensus Sp1 binding sites ($\Delta 7$) did not exhibit promoter activity, suggesting that Sp1 binding at these positions is critical for *Mist1* gene expression. The lack of activity of this latter construct also indicates that the TATA-related sequence at position −25 is not sufficient to confer transcriptional activity on this gene.

3.5. Sp1 has two binding sites in the *Mist1* proximal promoter

To further examine the hypothesis that Sp1 binds to the *Mist1* promoter, EMSA was performed using the two consensus Sp1 binding sites as probes (−54 to −29) and NIH3T3 nuclear extracts. The ³²P-labeled Sp1 probe was incubated with NIH3T3 nuclear extracts and the DNA:protein complexes were resolved on a non-denaturing gel and visualized by autoradiography. As shown in Fig. 5A, three probe-bound complexes were detected. The formation of these complexes was efficiently inhibited by a 100-fold molar excess of the cold probe (100× WT), indicating a specific protein:DNA interaction. Since the oligonucleotide sequence contains two potential Sp1 binding sites, we next looked for the presence of Sp1 protein using an antibody specific for Sp1. When NIH3T3 extracts were incubated with anti-Sp1, complex 1 was greatly reduced, while a supershifted complex (‘*’) appeared. This new complex was not detected when antibodies against unrelated factors (AP2 and E47 proteins) were tested. This result indicates that endogenous Sp1 present in extracts from NIH3T3 cells can bind to the *Mist1* promoter. Further investigations will be required to discover the identity of the proteins contained within complexes 2 and 3. To further characterize the Sp1 binding complexes, EMSA was conducted with mutant oligonucleotide probes. Mutations were introduced in the −54 to −29 DNA probe (see Section 2 and Fig. 6A) to disrupt the putative Sp1 site a (mut a), site b (mut b) or both sites simultaneously (mut a/b). A 100-fold molar excess of cold mut a inhibits Sp1 binding as efficiently as the wild-type (WT) probe (Fig. 5B). Surprisingly, complexes 2 and 3 are also competed by mut a. In contrast, the mut b oligonucleotide, and the double mutant a/b, are unable to compete for Sp1 binding. These data were confirmed by labelling mut a and mut b probes and incubating them with NIH3T3 nuclear extracts. In these conditions, mut a probe, but not mut b, was capable of binding Sp1 (data not shown). Together, these experiments suggest that site b avidly binds Sp1, whereas site a is not functional in this assay.

To confirm the importance of the Sp1 site b in vivo, a mutation was introduced on site b in the $\Delta 4$ *Mist1* promoter fragment (−206; +66) by PCR mutagenesis (CCGCCC altered to CgatCC). The activity of the original and the mutated promoter region was tested in NIH3T3 cells (Fig. 5C). As expected, disruption of the Sp1 site b led to a marked reduction in promoter activity (65% decrease), indicating that Sp1 binding at this position contributes to the activation of the *Mist1* promoter. These data also suggest that additional DNA sequences

in the $\Delta 4$ construct are involved in the activity of the promoter since one-third of the luciferase activity is still detected with the mutated Sp1b construct.

G/C-rich elements are located immediately upstream the E-box E1 (Fig. 6A). Although they do not represent classical Sp1 sites, we performed EMSA with additional oligonucleotides covering the -72 to -48 or the -63 to -38 regions. When increasing amounts of NIH3T3 nuclear extract (0–10 μ g) were incubated with the -72 to -48 probe, a strong Sp1 binding complex could be detected (Fig. 6B, left panel). The signals obtained are comparable to those obtained with the -54 to -29 Sp1 probe (Fig. 6B, right panel). In contrast, only a weak binding activity is detected with the -63 to -38 probe, even in the presence of large amounts of nuclear extract (Fig. 6B, middle panel). Since this latter oligonucleotide contains the Sp1 site a (not functional) and the most proximal G/C element (weak binding), it is likely that the distal G/C site in the -72 to -48 probe represents another strong Sp1 binding site. This issue was addressed in competition experiments with an excess of cold mutant oligonucleotides for the G/C distal or proximal regions (Fig. 6A). As expected, competition for Sp1 binding was obtained with an 100-fold excess of the non-radioactive -72; -48 probe, or with an oligonucleotide bearing a mutated proximal G/C site (Fig. 6C, mut prox), indicating that this proximal G/C element is not necessary for competing Sp1. In contrast, an intact distal G/C element is required, since a mutant distal G/C oligonucleotide (mut dist) has no competitive effect on Sp1 binding to the probe. Finally, we also tested whether cold Sp1 site b could interfere with Sp1 binding to the distal G/C element. Indeed, the incubation of nuclear extracts with an excess of cold -54; -29 DNA inhibited Sp1 binding to the labeled probe, whereas mut b was inefficient (Fig. 6C). This result suggests that the G/C distal element and the Sp1 site b might be functionally equivalent in the *Mist1* promoter.

4. Conclusions

The *Mist1* transcription factor belongs to the basic Helix–Loop–Helix protein family, which is known to play important roles during embryonic development and in cell fate determination. In this work, we have cloned the rat *Mist1* gene, determined its intron/exon structure and analyzed its 5' flanking region for promoter activity. Our analysis showed that the *Mist1* gene consists of two exons separated by a single intron and that the whole gene is contained within less than 5 kb of genomic DNA. Special features in the gene include the presence of an uninterrupted coding region in exon II and a very large 3' untranslated region (2.8 kb).

Sequence analysis of genomic clones containing the promoter region of the rat *Mist1* gene reveals many features that are common to constitutively active eukaryotic promoters, including the absence of classical TATA and CAAT-box sequences and a high GC content. Although no typical initiator element (PyPyCA⁺ NT/APyPy, Smale and Baltimore, 1989) was found, a major product was obtained upon mapping of the transcription start site, indicating that the transcription is not initiated at multiple points but rather at a precise site. Promoter deletion analysis suggests that Sp1 binding sites are necessary for the transcriptional activation of the *Mist1* gene. Pugh and Pugh showed that Sp1 transcription from a TATA-less promoter required a multisubunit TFIID complex that includes TBP, TBP-associated factor (TAFs), coactivators and Sp1. It is thought that Sp1 might be anchoring TBP to the TATA-less promoter via tethering factors such as TAF110 (Hoey et al., 1993). Our study indicates that Sp1 binds to two different regions within the proximal promoter, a typical Sp1 site located at (-38; -33) and a G/C-rich region between (-67; -62). In the context of the TATA-less, Inr-less *Mist1* promoter, it is possible that Sp1 serves as a bridging molecule to allow the assembly of the preinitiation complex. Moreover, two additional factors, that also bind to or near the Sp1 sites, might also contribute to the basal expression of this gene. Our future

studies will aim to identify these two proteins as well as other transcription factors that might regulate the *Mist1* gene during embryonic development and cell differentiation.

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Abbreviations: bHLH, basic Helix–Loop–Helix; EMSA, electrophoretic mobility shift assay

GenBank Accession No. AF049874 (rat *Mist1* gene) and AF049660 (mouse *Mist1* cDNA).

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Fig. 1. Intron/exon structure of the rat *Mist1* gene. The two probes used in the screening procedures are indicated by thick bars. The relative positions of the two isolated genomic clones are given (clone I and clone II).

A -2494 GTCGACAGTATTGCGTCACCCGCTGCTGTCCCGGGCTTGAACAGACGGTGTGTTGGTTTGCCTGCATAGGCAAGCTGGGTCTTGGTACT -2405

-2404 E21 GGACTTGTGCATGTGCCCCTGTTTCGTCCTCCGTCACGGCCAGGGATCTGGGCAGGGCAGTCACACCCATAGCCCGGCACCTACGAGGT -2315

-2314 E20 GTACTTAACACAATCAGATGGGGTTCACGGCAATATAACAGTTAATGAACCTGGAGTTTAAATTGTGTACATTTTAAATTTGAAGATT -2225

T/A rich

-2224 TTACTGTATATTGAAGCTTAGTGTGATTCAATAAATTGCTTGTAAATTTAAAACTATTTAATATTCAAAATAAATATAGTTATATATTTA -2135

-2134 E19 TAATAATCTCCGTTGCTATGTTTATCTGTTTTTCCATGTGTGGGTTTGGCCCACTGATGTAGATCTCTGAGCTCCCCACAGTCCTA -2045

-2044 AGGGCATGGGATGGTTCTCAGGGGACCATAGAGTCTGCCGTGTGGCTGTGGGTTCTGTAATCCAAGCCTTCTACTTACTGAGGAGTTC -1955

-1954 E18 TAGTGAGATCTGGCATGTAAGGATTGGCTTGGCCAGGCCCAATGCTACTCCAGAGATTACGGCTGGCCTTTCCGGCTGGGAAAT -1865

-1864 GGCTTATTGCTGAGACGACCATGTTTCAAGTTTCTAAAACCATGATGTTTGAAAAATGAAAAGGTGAGGTAGAGTGGAGCCTGTGTGCAT -1775

-1774 CGTCCAGTTCTGGGGGAGCAGAGGCGACAGACCCCTTGGGGCTCACTGGCCCGCTAGCAGACTAAGCCAGATCCAGGCCAGTGAGAGATC -1685

-1684 E17 E16 CTGCTCAAAACCAACAAAGTGGTGCACCTGAGAACAACTGATGTTGAAATGAAAAGGTGAGGTAGAGTGGAGCCTGTGTGCAT -1595

-1594 GCACCAAGCCTTCTACTGGGTTTGGTATGTTACATTCCTTTAATACC -1505

-1504 E15 E14 E13 AGCACCTGGGATGCTAAGGCAGGAGATTGCTGTGTGTTCAAAACAGCCCAAGATATGGTACAAATGAGGTCTGTCTCAAAAAACAGT -1415

-1414 E12 TGTATGCAAGTATGGGGCATCTCAGCTCTGACCCCTCTCTCCAGTTCTTTCTGAAAGTCCAAGTGACATGAGGAGGTGACAGCTTTCT -1325

-1324 E11 ACCAGAACCCTAACCACATGCTGTGTCAACAGCCTGAGACCCAAGCCTGCTGAACAGAGAAGTCAACCCAGAGTTATGCAGAGTCCCTGG -1235

-1234 CTGCGTTTTGACTTGTCTTATTAGCCCATGGCGGAAAAAATACTATTCTGTTGAGCAGAGTGACCCCTAATTTCCAGAGTAAGAGGCTGA -1145

-1144 GGCTTGGCTGTGAAGTTTCTGTCTCTTAAAAAACAACAAAGCCAGGGATGTAGTTAGCACACATGAAGCGCTGGGTTTGGTCC -1055

-1054 E9 E8 CTGGACACCACTGCACCACTGTACCGACTGCATCTGTAACCTACAGCACTCACTGGATTAAAGAATGTTTCTACACACGTCACTGTAG -965

-964 E7 E6 TGTGTGCGGTGACCCACTTGACCTTCTAGCCCTTGGCAGGCAGAGGAGGCTGGAGGATCAGGTGTTGCTCAAGGGCAGCCTGCGTGT -875

-874 E5 ACAGCTGGCAAGGTGCTCACCCTTGCCAAACCTAATGACTTGAGTTCAACCCAGCACAGGTGTTGGAAGTAGAGAAGAGACTCCTG -785

-784 CAAGTTGCTCTGACCTATGAGCTTGCACATACACAAAAATAGACAGAAAAGGCTAGCAAGAAAACCTGTTTCTGTGAGCAGCATG -695

-694 ACACGTAGAGAGGTCAGAGGACAACCTGGAGGAGTGGGTTCTCTCTTCCATTATGTGGGCCCCAGACTCATCAGATCTAGTGTCAAGTC -605

-604 CCTCTACCCACGGGCATCTTACTGTCCATAACCTTTTATATTGTAATCCCGGTGAACCTCGGGAAACCGAGGCAGGAGCTAGCAACGG -515

-514 E4 ATAGGCAGCCATGTGGTTGGTGGGTTAGTTGGTTTTCCAAATTGTCAGACTCTTGATAACTGTGACACCCCTGCCACTAGGAGCTCTGGTG -425

-424 ATTGAGTGGCATCCCGATACACCCATTTCAGTGAGGGGAGACAAAAAGGAAGCAGCCGTGTTCTTGAGCCCTTAGGACTCGCAGAGT -335

-334 CCGCTGGAAAAATCAGACGCCCTAGGGCAGGGGAAGAGGCTTGCCAGCTTTGCTCAGGGAGGTGGCTGCGGTTCTGTGAAGACTGCGC -245

-244 E3 E2 AGGGTGGCTTATCTAGACTCTGTTCTGAGTCTGACCCCAAGGCCAGGTGTCCTGGCCAGGTGCGATCCGGAGTTCCCTCCCCCAACC -155

-154 AP2 PU.1 CCGCTCCGGTAAGTCCCCGGGAGGCCGCGGCTCTCAGACCCAGAGGGAAGGGGAGGCGGGTCCGACCCGGTCTCAGAGCGAGGG -65

-64 E1 Sp1 a Sp1 b r GCGTGGCCGAGGTGCGCCCTGGCCCGCCAGCGAGCTATTACATGCTTCCCGACGCCCGCTGTGGTGGCTAAAGCGACGTGTC +26

CTTGTCCCGCTGGGCAGCCTCTGCGCGTACGGCCTCGGATCCCCAGGtaaaccaaggggtttggggaactggatgggactgctgggaag
ggaccagaagcgggaccagaggacctgcagcggtaggggggaaacagatagatggtggtctccactcagactggctgggtgcggtccacg
tggtagatctgggggctgctgcagggcttggccaccgtcagctgacctgtcagctgtccgtgtgtgcatgcactgcggttctggtct
gataatgtcaggactgtgtcatgtccaggagagctgtaaatatagtggtctaaaggggtgctgctgtctcgggtgacagctgtcgggtg
aaccttaagccagggttgggactgtgttctcagtggtttaaacaaattgtcaagtgcctaggctcaggaggttacttgcaacagtaag
gagtaaggggacctgaaagggttcccagatcagtgaggagagagggacggttaggttagagaaaagtggtcagtgtagttctgcccagg
gctttgggaacacaggtgagacaactctgggttcagtgtaattggggagcaccataggtgtccaccgtgcaccagtggtcagctgcactgg
caatgagatcgtgagctgcatgcccactggccacgctgaatgactgcagaggggctgtgttctgaaatttcagTCGGAAGGGCCTCTCC
AGCCACCACCTCTGGGAAAAACAGCCTAGAGCTATG

Fig. 2. (A) Nucleotide sequence of 2.5 kb of the *Mist1* 5'-flanking region, exon I and intron I. The initiation ATG (in bold) is located in exon II. The arrow denotes the transcription start site at +1. Potential transcription factor binding consensus sequences are indicated: E-boxes: E1 to E21, Sp1, PU.1 and AP2 sites, as well as a C/A repeat (boxed) and a T/A-rich region (underlined). (B) Determination of the transcription start site by 5' extension analysis. Rat spleen poly(A)⁺ RNA was used as the starting material. A sequencing ladder and molecular weight markers (lane M, bp) were run on the left- and right-hand sides, respectively. The extension product (lane E) is indicated by an arrow.

Fig. 3. The *Mist1* gene is active in

NIH3T3 and in C2C7 cells. (A) Fifteen micrograms of total RNA were fractionated through a denaturing agarose gel, transferred to nitrocellulose membrane, and subsequently hybridized with a mouse *Mist1* probe and a *GAPDH* cDNA. C2C7: myoblasts, NIH3T3: embryo fibroblasts. The position of the 28S and 18S ribosomal RNA is indicated. (B) Western blot analysis of Mist1 protein expression. Total cell extracts were prepared from C2C7 cells [proliferating myoblasts (MB) or terminally differentiated myotubes (MT)] and from NIH3T3 cells [control (C) or from cells transfected with 10 μ g of pcDNA-Mist1 expression vector (Tx)]. After separation on a 12% SDS-PAGE gel, Mist expression was detected by Western blot using an anti-Mist1 polyclonal serum. Molecular weight markers are given in kDa on the right-hand side. The lower band in the transfected Mist1 lane is due to an internal translation initiation site. (C) A 2.5 kb fragment of *Mist1* 5' flanking sequence allows the expression of a reporter gene in NIH3T3 and in C2C7 cells. NIH3T3 fibroblasts and C2C7 myoblasts (MB) were transfected with 2 μ g of Mist WT-Luc reporter (white bar), with 2 μ g of TnI-luc reporter (troponin I enhancer linked to the luciferase gene, gray bar) or with a promoter-less control plasmid (black bar). Cell extracts were prepared after 48 h and assayed for luciferase activity. In some experiments, C2C7 were induced to differentiate into myotubes (MT) in low serum medium. Data are expressed as fold over the promoter-less control (pGL2 basic).

Fig. 4. Deletion analysis of the *Mist1* 5' flanking region. NIH3T3 cells were transfected with 2 μ g of the various *Mist1* promoter fragments linked to a luciferase reporter gene. Forty-eight hours post-transfection, cell extracts were harvested and assayed for luciferase activity. The data are expressed as fold over the promoter-less control (pGL2 basic).

Fig. 5. Sp1 binds to the Sp1 site b of the -54 to -29 *MistI* DNA fragment. EMSA were performed using NIH3T3 nuclear extracts and 50 fmol of a 32 P-labeled double-stranded oligonucleotide. **(A)** The DNA probe was incubated with NIH3T3 extract (+) alone or with an 100-fold molar excess of the unlabeled oligonucleotide (100× WT) or with 300 ng of polyclonal antibodies raised against Sp1 (α-Sp1), AP2 (α-AP2) or E47 (α-E47). The positions of the three detected complexes are indicated. Sp1 corresponds to complex 1. '*' and F denote the supershifted Sp1 band and the free probe, respectively. **(B)** Mutations disrupting the Sp1 site a (mut a), site b (mut b), or both sites simultaneously (mut a/b), were introduced in the -54 to -29 oligonucleotide. The mutated probes were then tested in competition experiments. NIH3T3 extracts were incubated with 32 P-labelled -54; -29 probe, in the presence of a 100-fold excess of the cold wild type (WT) or the mutated competitors. The data indicate that Sp1 only binds to site b. **(C)** To confirm the importance of the Sp1 site b for *MistI* promoter activity, this region was mutated by PCR to generate the Δ4 mut Sp1b construct linked to the luciferase gene. The activity of this latter construct was then tested by transient DNA transfection in NIH3T3 cells, along with the control counterpart (Δ4). Results are expressed as fold over the promoter-less plasmid (basic).

Fig. 6. (A) Sequence of the -72; -29 promoter region. The mutated nucleotide in mutant distal (mut dist), mutant proximal (mut prox), mutant a (mut a) and mutant b (mut b) are indicated in lower case. (B) Sp1 binds to several sites in the proximal *Mist1* promoter. The presence of additional Sp1 sites was investigated by EMSA. Increasing amounts of NIH3T3 nuclear extract (0–10 μ g) were incubated with 50 fmol of the following oligonucleotides: (-54; -29), (-63; -38) and (-72; -48). Where indicated (+), the identity of the bound proteins was confirmed using the α -Sp1 antibody. '*' and F denote the supershifted Sp1 complex and free probe, respectively. (C) The distal G/C element binds Sp1 in EMSA. 32 P-labelled -72; -48 probe was incubated with NIH3T3 nuclear extracts, in the presence of a 100-fold molar excess of cold competitor. Mutations are present on the G/C distal (mut dist) or proximal (mut prox) region [see (A)]. F: free probe.