MEF-2 function is modified by a novel co-repressor, MITR

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The MEF-2 proteins are a family of transcriptional activators that have been detected in a wide variety of cell types. In skeletal muscle cells, MEF-2 proteins interact with members of the MyoD family of transcriptional activators to synergistically activate gene expression. Similar interactions with tissue or lineage-specific cofactors may also underlie MEF-2 function in other cell types. In order to screen for such cofactors, we have used a transcriptionally inactive mutant of *Xenopus* MEF2D in a yeast two-hybrid screen. This approach has identified a novel protein expressed in the early embryo that binds to XMEF2D and XMEF2A. The MEF-2 interacting transcription repressor (MITR) protein binds to the N-terminal MADS/MEF-2 region of the MEF-2 proteins but does not bind to the related *Xenopus* MADS protein serum response factor. In the early embryo, MITR expression commences at the neurula stage within the mature somites and is subsequently restricted to the myotomal muscle. In functional assays, MITR negatively regulates MEF-2-dependent transcription and we show that this repression is mediated by direct binding of MITR to the histone deacetylase HDAC1. Thus, we propose that MITR acts as a co-repressor, recruiting a specific deacetylase to downregulate MEF-2 activity.

Keywords: co-repressor/HDAC1/histone deacetylase/MEF-2/mHDA1

Introduction

The MEF-2 family of transcription factors are involved in the regulation of many muscle-specific genes (reviewed in Black and Olson, 1998). Initially identified as a DNA-binding activity that recognized an A/T-rich element found in the regulatory regions of skeletal and cardiac muscle structural genes, it is now clear that MEF-2 proteins are essential for differentiation of both striated and smooth muscle cells. Furthermore, in vertebrates MEF-2 proteins have been detected in a variety of non-muscle cell types, suggesting that the functions of these proteins may not be restricted to muscle cell lineages (Dodou *et al*., 1995; Ornatsky and McDermott, 1996).

Diversity of MEF-2 function may in part be explained by the size of the protein family. In vertebrates, four *MEF-2* genes (*MEF-2 A–D*) have been identified, each of which gives rise to several transcripts encoding distinct proteins through alternative splicing. In addition, MEF-2 proteins bind to their target DNA sequence as dimers and can heterodimerize with each other *in vitro*. The DNA binding domain encoded by each *MEF-2* gene is highly conserved and the MEF-2 proteins appear to share a common DNA binding site consensus. However, extensive divergence in the remainder of their protein sequence raises the possibility that individual MEF-2 isoforms, and perhaps individual heterodimer combinations, may possess unique regulatory functions.

In addition to homo- and hetero-dimerization, MEF-2 proteins have also been shown to interact with other tissue or lineage-restricted transcription factors. The best studied examples of such interactions involve the bHLH class of transcription factors. In skeletal muscle cells, MEF-2 proteins can interact either with members of the MyoD family to synergistically activate gene expression (Kaushal *et al*., 1994; Molkentin *et al*., 1995, 1996) or with twist to inhibit myogenesis (Spicer *et al*., 1996). Similarly, during neurogenesis MEF-2 proteins interact with the neurogenic bHLH transcription factor MASH-1 to activate transcription of neural-specific genes (Black *et al*., 1996; Mao and Nadal-Ginard, 1996). In addition to bHLH factors, a number of other transcription factors have been shown to play a role in promoting muscle differentiation by interacting with MEF-2 proteins. These include the ets domain protein PEA3 (Taylor *et al*., 1997), the thyroid hormone receptor TR (Lee *et al*., 1997) and the *Drosophila* PDP1 gene product (Lin *et al*., 1997). In some of these examples, interaction produces a synergistic activation of gene expression by recruitment of the transcriptional adapter proteins p300 and CBP (Eckner *et al*., 1996; Sartorelli *et al*., 1997) to the complex.

In the early *Xenopus* embryo, zygotic expression of XMEF2D (formerly known as SL-1) and XMEF2A (SL-2) is restricted exclusively to muscle lineages (Chambers *et al*., 1992; Wong *et al*., 1994). XMEF2A is expressed in the somitic mesoderm coincident with terminal differentiation markers, and transcripts are subsequently confined to the myotomal muscle of the tailbud embryo. In contrast, XMEF2D expression commences before the onset of terminal differentiation and is also detected later in cardiac precursors (Chambers *et al*., 1992, 1994). Lineage-restricted expression of the *MEF-2* genes is maintained only during early development, and by the swimming tadpole stage both genes are expressed widely throughout the embryo. Similarly, in the adult frog both *MEF-2*

transcripts and DNA binding activity are present in a wide range of tissues. These results indicate first, that each MEF-2 protein may perform different functions during embryonic muscle differentiation and secondly, that these factors have further roles in other cell types as development proceeds.

In order to identify other potential cofactors that may confer either target specificity or unique regulatory properties to individual MEF-2 proteins, we have used a yeast two-hybrid assay with a transcriptionally inactive mutant of *Xenopus* MEF2D as bait. Using this approach, we have isolated a novel protein from an embryonic (neurula stage) cDNA library that binds specifically with MEF-2 factors in a variety of assay systems. Binding requires an intact MADS/MEF-2 domain, but does not require residues previously demonstrated to be required for MEF-2 factor dimerization or MEF-2–myogenic bHLH factor interaction (Molkentin *et al.*, 1995, 1996). This factor, which we have named MEF-2 interacting transcription repressor (MITR) protein, is expressed in the developing embryo in a spatial and temporal pattern that overlaps that of XMEF2D and XMEF2A. Database searches reveal that MITR belongs to a family of highly conserved proteins, some members of which contain a C-terminal histone deacetylase domain implicated in transcription repression. Although MITR itself lacks such a domain, studies *in vivo* suggest that MITR binds to MEF-2 proteins and interferes with their native transcription activation activity. This repression requires a C-terminal domain distinct from the MEF-2 interacting domain, and can be relieved by treatment with trichostatin A (TSA), suggesting that MITR acts through recruitment of a histone deacetylase. We also show that the human homologue of MITR (hMITR) acts as a TSA-sensitive repressor of transcription when fused to a GAL4 DNA binding domain, and that it is able to interact directly with HDAC1. These results suggest that MITR acts as a co-repressor and is likely to alter the function of the MEF-2 proteins as embryonic muscle differentiation proceeds.

Results

Mapping transcription activation domains in a yeast expression system

In our search for proteins able to interact with *Xenopus* MEF-2 proteins, we employed the yeast two-hybrid system (reviewed in Fields and Sternglanz, 1994). Since this requires a 'bait' MEF-2 that lacks transcriptional activation activity, we first sought to map the regions of *Xenopus* MEF2D and XMEF2A that are required for transactivation of target reporter genes.

Introduction of multiple MCK MEF-2 recognition sites upstream of a LacZ reporter gene gave high levels of β-galactosidase activity in yeast, indicating that an endogenous transcriptional activator binds to the MEF-2 site *in vivo* (data not shown). Indeed, two MEF-2-like proteins have been identified in the yeast genome (Watanabe *et al.*, 1995, 1997; Dodou and Treisman, 1997), one of which, Rlm1, is known to have a similar site preference to members of the vertebrate MEF-2 family. For this reason, transcriptional activation assays and the two-hybrid screen in yeast were carried out using fusions of the *Xenopus* MEF-2 proteins with either the LexA or GAL4 DNA binding domains.

A series of N- and C-terminal deletions of XMEF2D fused to the LexA DNA binding domain were used in a yeast system to map the transcriptional activation domain. Full transcriptional activation required amino acids 102–339 of XMEF2D and suggests that only the most C-terminal portion of this domain is essential for transactivation (Figure 1A). This conclusion was confirmed using a series of internal deletions. Removal of a short 23 amino acid region from the C-terminus of the putative transcriptional activation domain (XMEF2DΔ316–339) reduced activity to 27% of the full-length protein, and progressively larger deletions abolished any transcription activation activity above background levels. The transactivation activity of a second member of the *Xenopus* MEF-2 family, XMEF2A, had previously been investigated using transfected COS cells (Wong *et al.*, 1994). Conflicting results were obtained with different reporter genes and, therefore, we re-examined this protein using the yeast assay system (Figure 1A). A series of N- and C-terminal deletions were used to map the transcriptional activation domain to between residues 102 and 383. Thus, XMEF2A contains a transcription activation domain located in a region comparable to that of XMEF2D, C-terminal to the MADS/MEF-2 domain. Furthermore, the potency of both transcription activation domains is similar.

The yeast assay system may not truly represent the *in vivo* activity of these proteins; therefore, we sought to confirm these results by testing a number of the XMEF2D and XMEF2A deletion mutants in an oocyte assay system in which the MEF-2 protein could bind via its MADS/MEF-2 domain to consensus MEF-2 binding sites in a CAT reporter gene construct. In each case, we first established that the truncated proteins retained full DNA binding activity as assessed by gel shift assays using *in vitro* translated protein (data not shown). As shown in Figure 1B, C-terminal deletion of XMEF2D to amino acid 339 had little effect on transcription activation, whereas deletion to amino acid 327 essentially abolished XMEF2D activity. This was identical to the results obtained using the yeast system, mapping the C-terminal extent of the XMEF2D activation domain to residue 339. Similarly, the C-terminal extent of the XMEF2A activation domain was mapped to amino acid 383 (Figure 1C). Finally, internal deletion of either amino acids 278–339, or amino acids 316–339 was sufficient to eliminate XMEF2D activation activity (Figure 1D). In all cases no transcriptional activation was observed using a control reporter in which the MEF-2 sites were mutated to eliminate binding.

These results demonstrate that microinjection of oocytes provides a convenient and homologous assay system to monitor the activity of *Xenopus* transcriptional activators. Furthermore, the agreement in results between the yeast and oocyte systems suggests that both assay systems provide a reasonable reflection of the *in vivo* activities of the *Xenopus* MEF-2 proteins.

Isolation and characterization of the MITR cDNA

In order to retain as much of the XMEF2D protein as possible in the two-hybrid 'bait', we used internal deletion XMEF2DΔ316–378. This protein was inactive in both the yeast and oocyte assay systems, and also lacked a glutamine-rich region (residues 366–377), which was found to cause non-specific interaction with the GAL4

activation domain contained in all library cDNAs. A *Xenopus* neurula (stage 18) cDNA library (Sparrow *et al.*, 1998b) was screened using the optimized procedure previously described (Sparrow *et al.*, 1998a). Transformants (8×10^6) were screened, and 16 independent clones isolated. Sequence analysis showed that these were all derived from the same gene and contained a continuous open reading frame, suggesting that they did not represent the full cDNA. To isolate the remainder of the *Xenopus* transcript, the longest clone (pMITR-

y1) was used as a probe to screen a neurula (stage 17) cDNA library (Kintner and Melton, 1987). Several clones extending to the 3′ end of the cDNA sequence were isolated and sequenced (Figure 2A). No clone was isolated that extended beyond the 5′ end of the original yeast clones or had a termination codon upstream of three in-frame methionine codons.

To eliminate the possibility that the MITR–MEF-2 interaction resulted in some way from the particular juxtaposition of the GAL4 activation domain with the cDNA sequence, a domain swap experiment was performed (Figure 3A). The original MITR fragment was fused in-frame to the GAL4 DNA binding domain and tested for its ability to interact with full-length XMEF2D or XMEF2A fused to the GAL4 activation domain. In each case, transactivation of the reporter was retained, indicating that the interaction between these proteins was not an artefact of the original screen.

MITR is related to a family of histone deacetylases

Database comparisons revealed that MITR is related to a recently reported family of proteins (summarized in Figure 2B). First, the human cDNA KIAA0744 (DDBJ/EMBL/GenBank accession No. AB018287) contained sequences with high homology to the entire MITR coding region, and thus probably represents hMITR. Secondly, two other related proteins contained an N-terminal domain similar to the entire MITR coding region. These are named KIAA0600 (DDBJ/EMBL/GenBank accession No. AB011172) /NY-Co-9 (Scanlan *et al.*, 1998) /mHDA1 (Verdel and Khochbin, 1999) /HDACB (Fischle *et al.*, 1999) /HDAC5 (Grozinger *et al.*, 1999), and KIAA0288 (DDBJ/EMBL/GenBank accession No. AB006626) /HDACA (Fischle *et al.*, 1999) /HDAC4 (Grozinger *et al.*, 1999). Interestingly, both of these proteins also possess a C-terminal domain unrelated to MITR that has been demonstrated to be a functional histone deacetylase (Fischle *et al.*, 1999; Grozinger *et al.*, 1999; Verdel and Khochbin, 1999). This is an intriguing result, since histone deacetylase domains have been demonstrated to be involved in negative regulation of transcription (see Discussion). In summary, MITR belongs to a family of three proteins represented in *Xenopus*, mouse and human. Two family
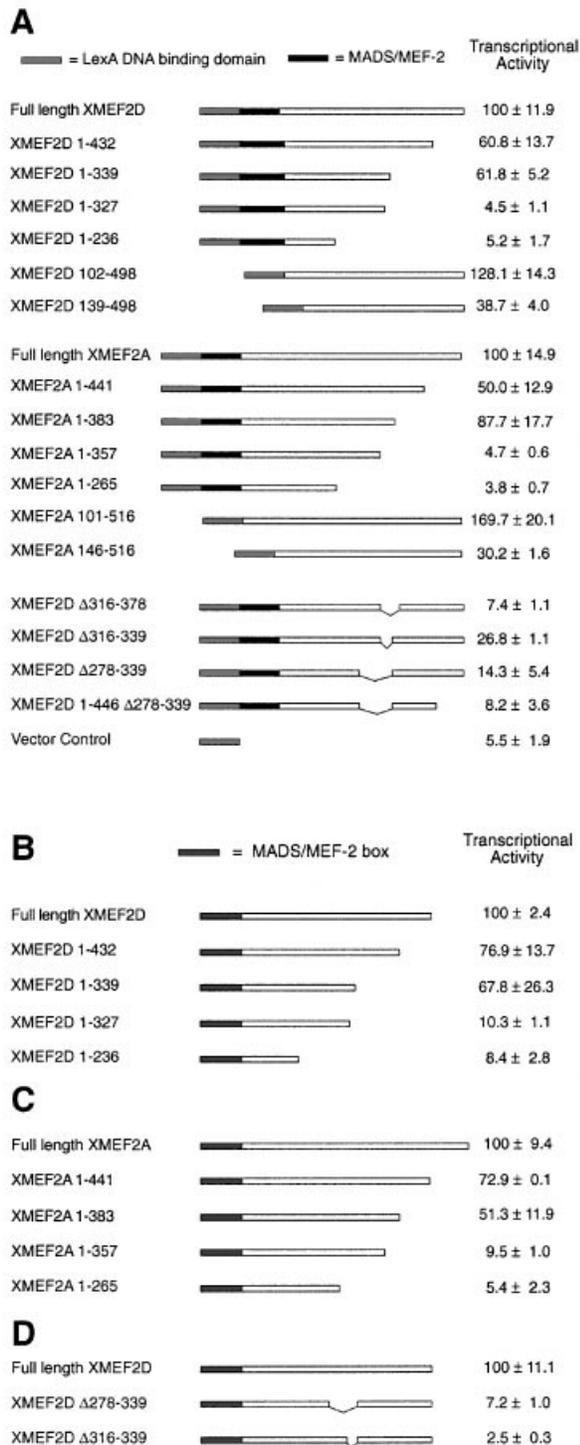

Fig. 1. Transcriptional activity of MEF-2 deletions. (**A**) CTY yeast strain was transformed with a yeast expression vector encoding the indicated LexA–MEF-2 chimeras. Amino acids contained in each mutant are indicated on the left. *LacZ* activity was determined as described in Materials and methods. Values are expressed as the percentage of LexA–MEF2D (or LexA–MEF2A where appropriate) activity observed for each mutant and are averages ± standard errors of at least three experiments. For (B)–(D), synthetic RNAs encoding the indicated deletions of XMEF2D or XMEF2A were injected into the cytoplasm of *Xenopus* oocytes. (**B**) XMEF2D N- and C-terminal deletions. (**C**) XMEF2A N- and C-terminal deletions. (**D**) XMEF2D internal deletions. Amino acids contained in each mutant are indicated on the left. A reporter plasmid containing three MEF-2 binding sites cloned upstream of CAT were injected into the germinal vesicle 18–24 h later. CAT activity was determined as described in Materials and methods. Values are expressed as the percentage of full-length XMEF2D (or XMEF2A where appropriate) activity observed in a representative experiment. Experiments were carried out at least three times and while absolute values varied, the relative values were similar.

members consist of an N-terminal MITR domain and a C-terminal histone deacetylase domain, whereas MITR itself lacks the C-terminal deacetylase domain. A fourth protein, mHDA2 (Verdel and Khochbin, 1999) /HDAC6 (Grozinger *et al.*, 1999), has been placed in this family by virtue of homology within the histone deacetylase domain. Uniquely, it consists of two adjacent histone deacetylase domains. However, it has no region with homology to MITR, and thus may instead represent the founder member of a novel class of histone deacetylase.

Comparison of the *Xenopus* MITR amino acid sequence with that of the MITR domain in other family members reveals a number of blocks of very highly conserved residues (Figure 2C). Searches of the database with either the full amino acid sequence or with any of the conserved domains did not reveal any significant homologies to any other protein.

Genomic organization of hMITR

Searches of sequences from the human genome project revealed that *hMITR* maps to 12 exons on chromosome 7 covering a total of ~173 kb [human BAC clones RG180O01 (DDBJ/EMBL/GenBank accession No. AC002124) and RG317M02 (DDBJ/EMBL/GenBank accession No. AC002433)]. More extensive searches of the chromosome 7 sequence data revealed sequences

A

MITR

potentially coding for a histone deacetylase domain beginning at least 56 kb downstream of the *hMITR* 3′ UTR sequence and extending for a further 228 kb, making the total locus 454 kb in size. Given this genomic organization, it is possible that the two classes of MITR-related clones (those with and without the C-terminal histone deacetylase domain) arise by alternative splicing. Supporting this conclusion, there is evidence for alternative splicing of the family member mHDA1 (Verdel and Khochbin, 1999). Whether such alternative splicing occurs in *Xenopus*, and its significance in the early embryo, is unclear since no evidence for histone deacetylase domain containing forms of MITR was found in preliminary experiments by 3′ RACE using RNA from early embryos or from analysis of the cDNA clones isolated.

Mapping the MITR–MEF-2 interaction domains

The amino acid sequences required for MITR–MEF-2 interaction were investigated using the yeast two-hybrid system. N- and C-terminal deletions of the original fragment (MITR 1–222) were fused in-frame to the GAL4 activation domain and tested with the original XMEF2D 'bait' (Figure 3B). Activation of the LacZ reporter gene was assessed by filter assay (Breeden and Nasmyth, 1985).

N-terminal truncation to amino acid 141 had little

B

C
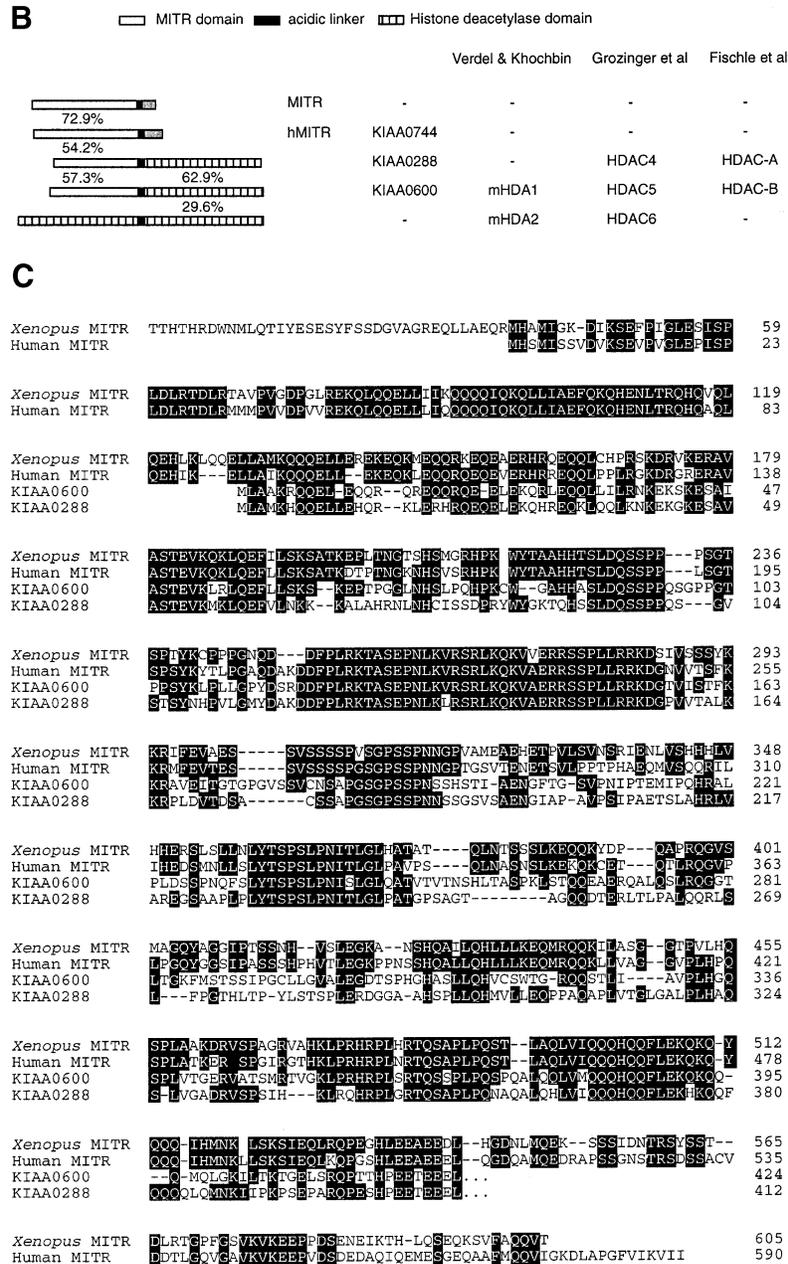
Fig. 2. Sequence of the *Xenopus MITR* gene. (**A**) *Xenopus MITR* cDNA sequence and deduced amino acid sequence. (**B**) A schematic representation of MITR and three related mammalian proteins. The previously assigned names are shown together with the respective citation. The percentage similarity of the N-terminal domains to MITR is indicated, along with the percentage similarity of the histone deacetylase domains to KIAA0288. (**C**) Comparison of MITR with the three related mammalian proteins. Dashes represent gaps introduced to maximize the alignment; conserved amino acids are shaded in black. For clarity, only the N-terminal domains of KIAA0600 and KIAA0288 are shown. The amino acid sequence of KIAA0288 is derived from Grozinger *et al.* (1999) and includes the full N-terminal extension.
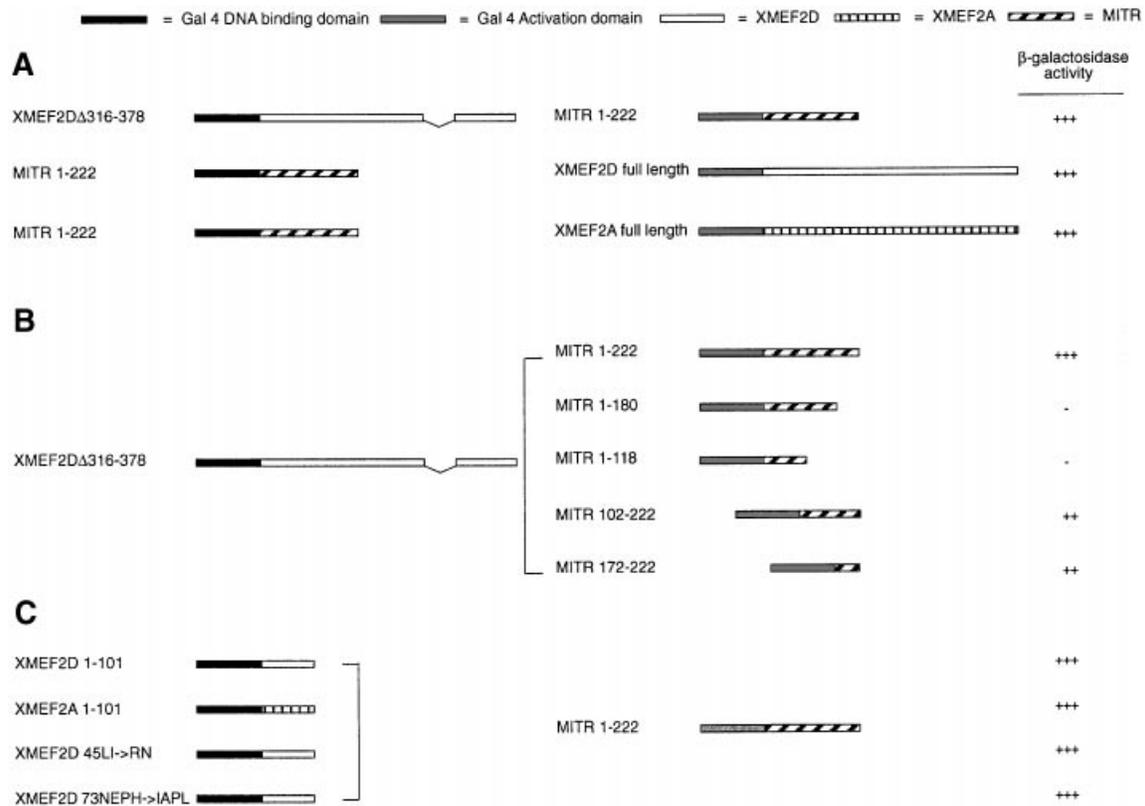
Fig. 3. Mapping regions of MEF-2 and MITR proteins required for protein–protein interaction. The indicated fusions in yeast expression vectors were co-transformed into yeast strain YN166 and grown on SD–Trp–Leu plates at 30°C for 72 h. β-galactosidase activity was measured by filter assay (Breeden and Nasmyth, 1985).

or no effect on MITR–MEF-2 interaction, which was, however, completely abolished by C-terminal truncation to amino acid 180 (Figure 3B). A minimal fragment comprising amino acids 172–222 of MITR was sufficient for MITR–MEF-2 interaction. In reciprocal experiments, the first 100 amino acids of XMEF2D or XMEF2A were both necessary and sufficient for the interaction with the original 222 residue MITR fragment (Figure 3C). This corresponds to the MADS/MEF-2 domain which contains distinct regions for DNA binding (Nurrish and Treisman, 1995; Molkentin *et al.*, 1996), MEF-2 homo- and hetero-dimerization (Molkentin *et al.*, 1996) and interaction with myogenic bHLH factors (Molkentin *et al.*, 1995). To determine whether any of these were also required for binding to MITR, we used mutations of the MADS/MEF-2 domain previously shown to abolish either dimerization of mouse MEF2C (45LI→RN; Molkentin *et al.*, 1996) or its interaction with myogenic bHLH factors (73 NEPH→I-APL; Molkentin *et al.*, 1995). Neither of these mutations had any effect on MITR–MEF-2 interaction (Figure 3C) demonstrating that binding of MITR can be distinguished from other MEF-2 interactions.

Physical interaction between MITR and MEF-2 proteins

Next, we sought to confirm the ability of MITR and MEF-2 proteins to interact, using synthetic protein. ^{35}S-labelled *in vitro* translated full-length MITR and the MITR 1–222 fragment were synthesized using rabbit reticulocyte extract and tested for their ability to bind to glutathione

S-transferase (GST) fusions of XMEF2D, XMEF2A and *Xenopus* serum response factor (XSRF). Both MITR proteins bound specifically to the GST–XMEF2D and GST–XMEF2A proteins (Figure 4A, lane 3; Figure 4B, lanes 3 and 4), whereas their interaction with the related MADS protein, SRF, or GST alone was minimal (Figure 4A, lanes 2 and 4; Figure 4B, lanes 2 and 5). This assay therefore provides independent evidence for the ability of MITR to bind the MEF-2 proteins, and demonstrates that despite their conservation in sequence and structure, the MADS domain of other proteins does not necessarily support this interaction. To test whether the MITR–MEF-2 interaction occurs in an *in vivo* system, we co-expressed XMEF2D and HA-epitope-tagged MITR by injection into *Xenopus* oocytes. Proteins from oocyte extracts were immunoprecipitated using a polyclonal anti-MEF-2 antibody (Dodou *et al.*, 1995) and analysed by SDS–PAGE followed by Western blotting with a rat monoclonal anti-HA antibody (Figure 4C). HA-tagged MITR only immunoprecipitated in the presence of both XMEF2D and the anti-MEF-2 antibody, providing further evidence for specific interaction of these two proteins. As a positive control, a mouse monoclonal anti-HA antibody was used to demonstrate that the HA-tagged MITR could be immunoprecipitated alone (Figure 4C). Thus, we conclude that MITR can interact specifically with MEF-2 proteins *in vivo* as well as *in vitro*. This view is further supported by experiments demonstrating that the hMITR co-immunoprecipitates with hMEF2A in extracts from mammalian cell culture (Miska *et al.*, 1999).
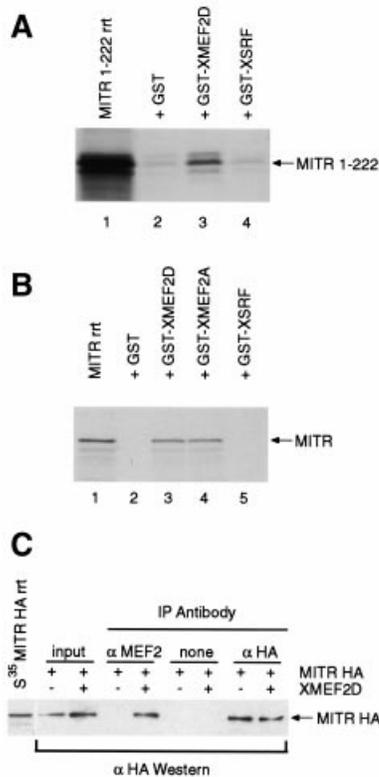
Fig. 4. MITR and XMEF2 interaction assays. ^{35}S-labelled *in vitro* translated MITR 1–222 (A) or full-length MITR (B) were incubated in the presence of the GST-fusion proteins as indicated. The mixture was bound to glutathione–agarose beads and washed extensively. Bound protein was eluted with reduced glutathione and analysed by SDS–PAGE. (**A**) Lane 1, input MITR 1–222; lane 2, GST control; lane 3, GST–XMEF2D; lane 4, GST–XSRF. (**B**) Lane 1, input full-length MITR protein; lane 2, GST control; lane 3, GST–XMEF2D; lane 4, GST–XMEF2A; lane 5, GST–XSRF. Note that in (A) lane 1 is overloaded with input protein. (**C**) Oocytes were injected with RNA encoding either HA-tagged MITR alone or MITR–HA and XMEF2D. After 36–48 h, oocyte extracts were immunoprecipitated with the indicated antibody and analysed by SDS–PAGE followed by Western blotting with an anti-HA antibody. For comparison, levels of MITR–HA in the input extracts and ^{35}S-labelled *in vitro* translated MITR (as a size marker) are shown.

MITR is expressed in an overlapping pattern to Xenopus MEF-2 genes

The physical interaction of MITR and MEF-2 proteins *in vitro* has little biological significance unless the two proteins are present in the same cells *in vivo*. Therefore, we investigated the expression of MITR in the developing embryo and in adult *Xenopus* tissues.

Using an RNase protection assay, we found that levels of *MITR* RNA were extremely low at all stages and in all tissues (Figure 5A and B; compare with the expression of XMax2). Maternally-derived *MITR* transcripts are present in the early embryo and decline during gastrulation (Figure 5A, lanes 3–5). These are replaced by zygotic transcripts from the onset of neurulation, levels remaining approximately constant at least until the swimming tadpole stage (Figure 5A, lanes 7–11). In the adult, low levels of *MITR* RNA were present in a wide range of tissues including intestine, stomach, gall bladder, skeletal and heart muscle, lung and spleen (Figure 5B).

Our protection assay probe always gave a prominent second product (or doublet) resulting from partial protec-
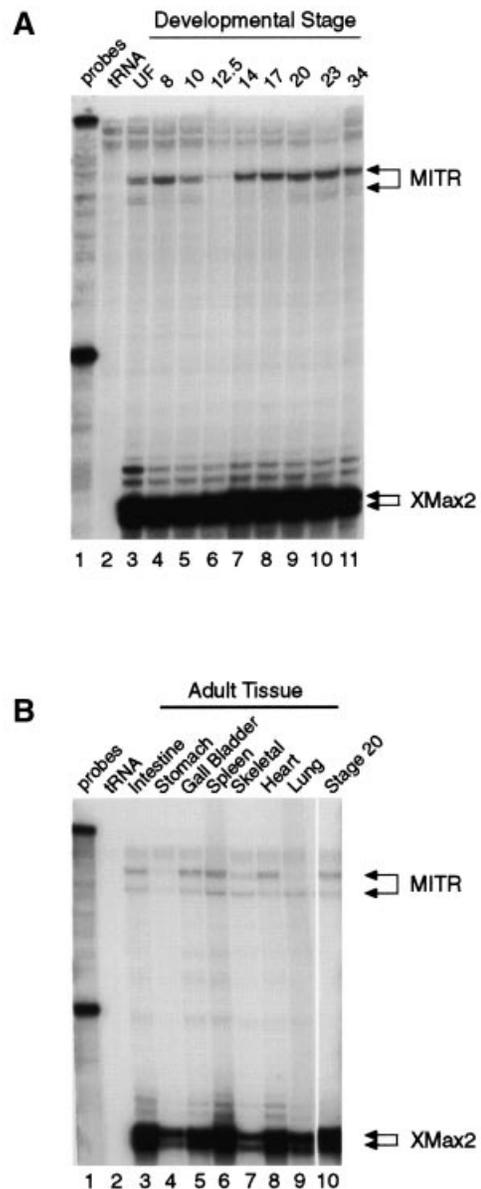
Fig. 5. Expression of *MITR*. (**A**) The distribution of *MITR* transcripts in *Xenopus* embryos was analysed by RNase protection assay. Lane 1, undigested probes; lane 2, tRNA control; lanes 3–11, unfertilized oocyte, stages 8, 10, 12.5, 14, 17, 20, 23 and 34 embryo RNA, respectively. Three embryo equivalents of RNA (15 μg) were used in each case. Full-length protected fragments for each probe are indicated. As an internal control, a probe for *XMax2* (which is expressed at constant levels throughout early development; Tonissen and Krieg, 1994) was included in the *MITR* assay. (**B**) The distribution of *MITR* mRNA in adult frog tissues was analysed by RNase protection assay. Lane 1, undigested probes; lane 2, tRNA control; lanes 3–10, intestine, stomach, gall bladder, spleen, skeletal muscle, heart muscle, lung and tailbud embryo (stage 20) RNA, respectively. Five micrograms of total RNA was used in each assay. Full-length protected fragments for each probe are indicated. As an internal control, a probe for *XMax2* was included in the *MITR* assay.

tion of the probe sequence. There are two possible explanations for this observation. First, this may be due to the presence of transcripts from another *MITR* allele. Co-expression of divergent alleles is not uncommon for *Xenopus laevis*, since it is a pseudotetraploid species (Bisbee *et al.*, 1977). Secondly, as described above, there is evidence that the related gene *mHDA1* is alternatively

spliced (Verdel and Khochbin, 1999). In adult tissues, the relative intensity of the full and partial protection products varies widely between tissues, raising the possibility that this is due to tissue-specific patterns of alternative splicing.

To examine the spatial distribution of transcripts within the embryo, whole-mount *in situ* hybridization was performed with two different probes. The first corresponded to the original *MITR* clone and the second was derived from 1.5 kb of 3′ UTR sequence. Both probes gave identical expression patterns. Beginning from late neurula stages (stage 18), expression was detected as a thin stripe in the older, more anterior somites but was absent from the most recently formed somites at the posterior end of the embryo (Figure 6A). This expression pattern was maintained at least until swimming tadpole stages (Figure 6B). Longitudinal sectioning of stained embryos showed that each stripe of staining corresponded to the aligned nuclei that lie in the middle of each myotome (Figure 6C), suggesting that transcripts may be localized in a perinuclear fashion. However, the significance of this observation is unclear. In *Xenopus* embryos, a somite consists entirely of mononucleated myotomal cells that span each segmental unit (Hamilton, 1969), and thus *MITR* is expressed in the same cells as both *XMEF2D* and *XMEF2A* (Chambers *et al.*, 1992). However, the levels of *MITR* and *XMEF2D* transcripts within these cells are present in a reciprocal pattern since *XMEF2D* is expressed more highly in the younger, more posterior somites as well as the unsegmented paraxial mesoderm (Figure 6D).

MEF-2-specific transcriptional activation is repressed by MITR

Since we had demonstrated that MITR and MEF-2 proteins interact physically, and were co-expressed in the developing embryo, we next investigated what effect ectopic expression of MITR had on normal MEF-2 function during early development. In these experiments, ectopic expression of MITR in one half of the embryo had no discernable effect on the expression of skeletal or cardiac muscle markers as judged by whole-mount *in situ* hybridization for *MLC1/3* and *MLC2A*, despite detection of MITR protein by Western blotting (data not shown). Surprisingly, however, a reproducible percentage of embryos had a partially duplicated or shortened axis (30% of injected embryos) and a phenotype that was both RNA-specific and dose-dependent (data not shown). The explanation for these results is currently unclear; therefore we sought a more direct test of the effects of MITR on MEF-2 function. For this, we used an oocyte expression system in which transcriptional activation of a reporter by MEF-2 proteins could be monitored. Capped RNA encoding the full-length MITR was co-injected into oocytes at increasing amounts relative to *XMEF2D* RNA, and reporter gene activity measured by CAT assay. Even at low doses of MITR, reporter gene expression was significantly reduced and at a 2:1 ratio of MITR:XMEF2D, reporter gene expression was almost abolished (Figure 7A). Since MITR had been shown to interact equally efficiently with XMEF2A, we also tested the effects of MITR on XMEF2A-mediated transcription activation. In these assays, MITR was consistently ~2.3-fold more efficient at repressing XMEF2A activity (Figure 7A).

This observed repression could be due to a number of
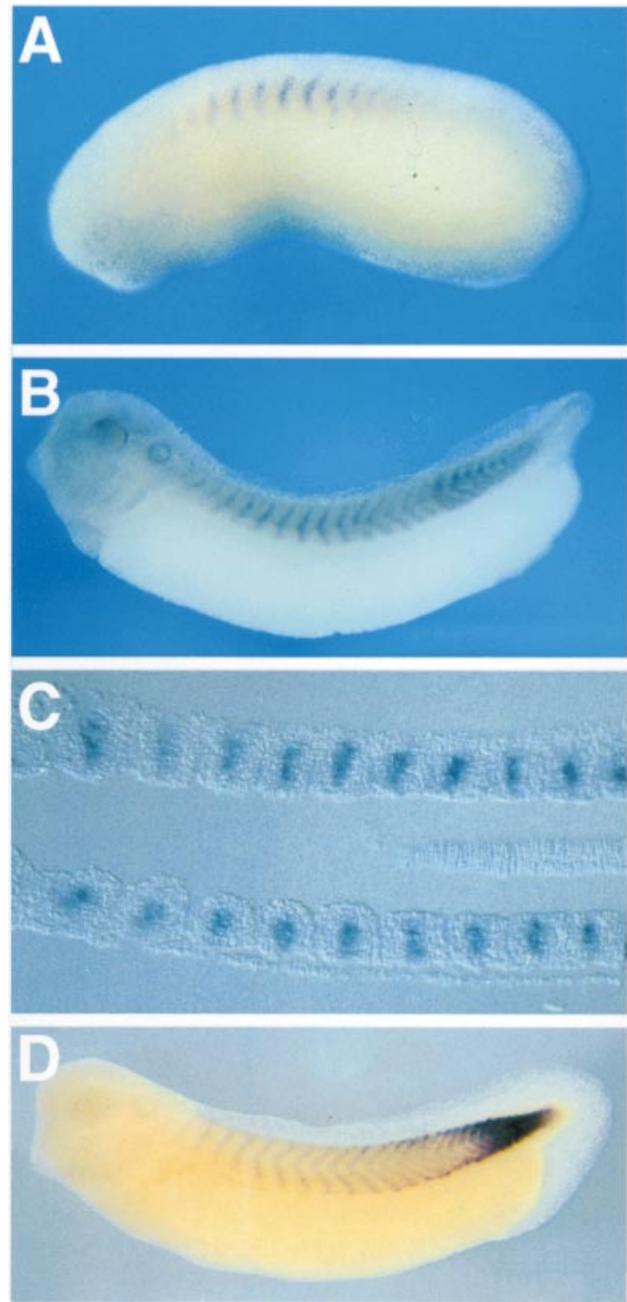
Fig. 6. Whole-mount *in situ* analysis of *MITR* expression. (**A**) Lateral view of a *Xenopus* tailbud embryo (stage 24), with anterior to the left. (**B**) Lateral view of a tailbud stage embryo (stage 32). (**C**) Longitudinal section of a stage 32 embryo processed for RNA whole-mount *in situ* hybridization using a probe specific for *MITR*. (**D**) For comparison, a lateral view of a stage 32 embryo stained for *XMEF2D* expression is shown.

trivial causes. First, co-injection of MITR and XMEF2D RNAs could cause a drop in the amount of XMEF2D protein produced due to translational competition. To test this possibility, an RNA encoding a protein not thought to interact with XMEF2D (XNkx2.5) was co-injected with XMEF2D at a 1:1 ratio. This had no effect on the levels of reporter gene activity (Figure 7A). Secondly, MITR may cause a general repression of all transcription within the oocyte. To eliminate this possibility, MITR was co-expressed with the unrelated transcriptional activator Xbra,

A

MITR	-	+	-	0.5	1	2	-	-	+	-	-
XMEF2D	-	-	+	+	+	+	+	-	-	-	+
XNkx2.5	-	-	-	-	-	-	+	-	-	-	-
XMEF2A	-	-	-	-	-	-	-	+	+	-	-
MITR 1-222	-	-	-	-	-	-	-	-	-	+	+

B

| MITR | - | + | - | + |
| Xbra | - | - | + | + |

C

TSA	-	+	-	+	-	+
XMEF2D	-	-	+	+	+	+
MITR	-	-	-	-	+	+

Fig. 7. MITR represses MEF-2-specific transcriptional activation. Synthetic RNAs encoding the indicated proteins were injected into the cytoplasm of *Xenopus* oocytes. In (**A**) and (**C**) the reporter plasmid contained three copies of the MEF-2 binding site cloned upstream of CAT. In (**B**) the reporter contained a single copy of the *Xenopus* eFGF Brachyury half-site (Casey *et al.*, 1998). CAT activity was determined as described. Values are expressed as the percentage of full-length XMEF2D (A) and (C) or Xbra (B) activity observed.

and the activity of a Brachyury half site CAT reporter gene measured (Casey *et al.*, 1998). In this assay, MITR had no effect on the levels of Xbra-mediated transcription activation (Figure 7B), suggesting that its repressive effect was specific to MEF-2 factors.

To map the region of MITR required for the repression of MEF-2 activity, the original fragment of MITR cloned from the yeast two-hybrid screen was tested in the oocyte transcription assay system. This N-terminal fragment of MITR had no effect on reporter gene activity (Figure 7A), suggesting that sequences C-terminal to the MITR–MEF-2 interaction domain are required for repression.

MITR recruits histone deacetylase activity to repress MEF-2 function

Since MITR belongs to a family of proteins which possess histone deacetylase activity, we used the oocyte transcription system to test the hypothesis that repression 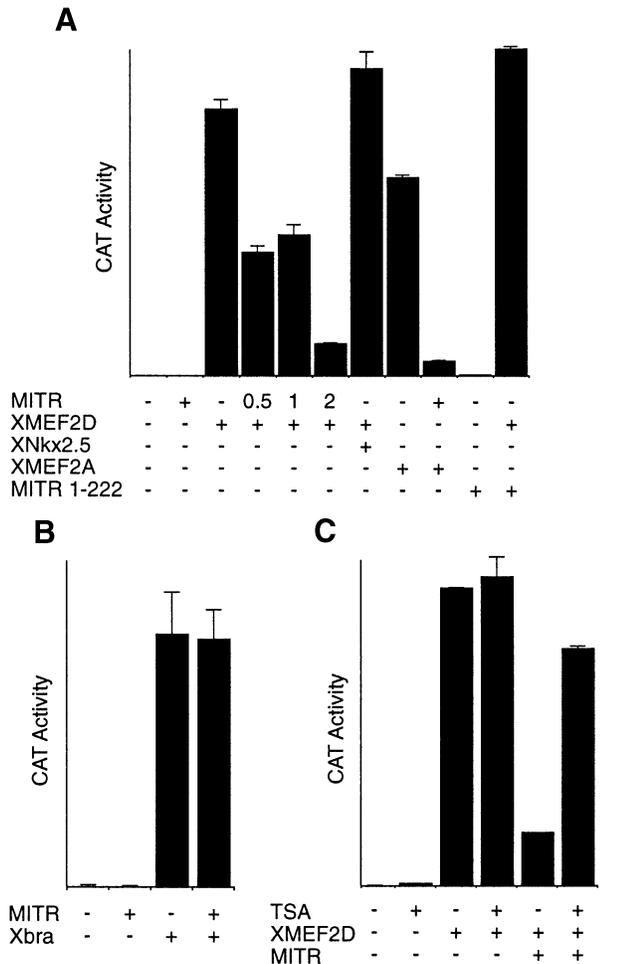of MEF-2 activity was due to recruitment of a histone deacetylase to the synthetic MEF-2 promoter. TSA has been demonstrated to be a potent and specific inhibitor of
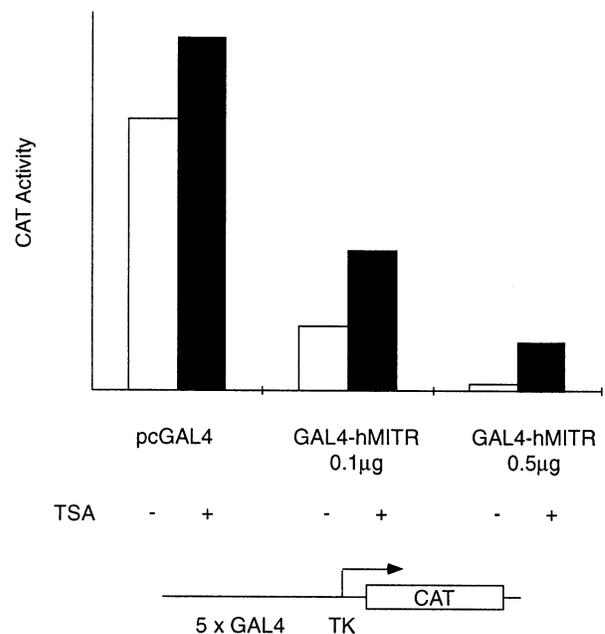
Fig. 8. hMITR represses transcription as a GAL4 fusion. 293T cells were transfected with 0.5 μg of 5× GAL4TK–CAT reporter and either 1 μg of pcGAL4, 0.1 or 0.5 μg of pcGAL4–hMITR. The results are shown as relative CAT activity. TSA was used at 330 nM. All of the GAL4 constructs tested were expressed at equal levels and had no effect on a control promoter lacking GAL4 sites (0× GAL4TK–CAT; data not shown).

deacetylase activity and is functional in *Xenopus* oocytes (Wong *et al.*, 1998). As shown in Figure 7C, TSA treatment had no effect on background reporter gene levels, nor on levels of MEF-2-specific transcription. In contrast, TSA was able to attenuate almost completely the repressive effects of MITR, suggesting that indeed a histone deacetylase activity was involved. This experiment was repeated using HA-epitope-tagged MITR, and protein levels determined by Western blotting using an anti-HA antibody to confirm that TSA presence had no effect on the levels of MITR protein in the oocyte (data not shown).

To investigate further the potential role of histone deacetylase in MITR's function, we switched to using a mammalian cell culture system. To minimize incompatibilities between systems, we used hMITR in all subsequent experiments. First, we fused hMITR to the GAL4 DNA binding domain and tested for its ability to repress transcription from a synthetic promoter consisting of the viral thymidine kinase promoter with added GAL4 binding sites (Morkel *et al.*, 1997). As shown in Figure 8, increasing amounts of GAL4–hMITR were able to repress the high basal level of transcription almost completely, relative to the GAL4 DNA binding domain alone. This repressive effect was substantially relieved by the addition of TSA, providing further evidence for the involvement of a deacetylase activity. The GAL4–hMITR fusion had no effect on the same promoter without GAL4 binding sites (data not shown). The histone deacetylase HDAC1 has recently been shown to interact directly with the retinoblastoma protein (Rb) to mediate transcriptional repression (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). To test whether MITR-specific repression involves a similar mechanism, we co-expressed

A

GAL4-hMITR →

myc-HDAC1   mock

α myc IP
α GAL4 Western

myc-HDAC1 →

input
α myc Western

Controls

GAL4-hMITR →

input
α GAL4 Western

B

GST-E7   GST-hMITR   GST-Rb (379-928)   glutathione sepharose   1% HDAC1-F input

Flag-HDAC1 →

α Flag Western

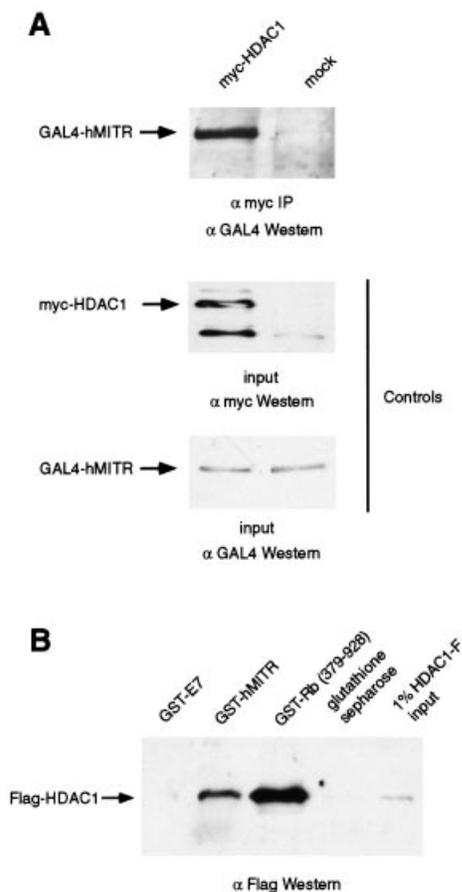**Fig. 9.** hMITR binds the histone deacetylase HDAC1. (**A**) 293T cells were transfected with pcGAL4–hMITR and either pcDNA3.1-A-myc/ his (mock) or pcHDAC1-myc (myc-HDAC1). Cell lysates were immunoprecipitated with an anti-myc antibody and precipitates were subjected to SDS–PAGE followed by Western blotting with an anti-GAL4 antibody. Expression levels of the different proteins in the inputs were verified by Western blotting using the appropriate antibodies (controls). The positions of the proteins are shown by arrows. (**B**) Recombinant purified GST–Rb (379–928), GST–E7 and GST–hMITR bound to glutathione–Sepharose beads were incubated with recombinant purified Flag-tagged HDAC1 (HDAC1-F). Glutathione–Sepharose beads alone were used as a negative control. Bound material was subjected to SDS–PAGE and Western analysis using an anti-Flag antibody. The position of HDAC1 is shown by an arrow.

GAL4–hMITR and myc-tagged HDAC1 in mammalian cell culture. Cell extracts were immunoprecipitated with anti-myc and precipitates analysed by Western blotting using an anti-GAL4 antibody. As shown in Figure 9A, hMITR is specifically precipitated by the anti-myc antibody, suggesting that it interacts with HDAC1 *in vivo*. It is possible that the tissue-culture cells contain essential cofactors for this interaction. To test whether such cofactors were required for interaction, we combined recombinantly produced purified Flag-tagged HDAC1 and either GST–hMITR or GST–Rb *in vitro* and analysed interaction by pGEX pulldown followed by Western analysis with an anti-Flag antibody. Figure 9B shows that in the absence of other proteins, hMITR and HDAC1 are able to bind to each other in the same fashion as previously demonstrated for Rb–HDAC1. As a negative control, we used a protein that is known not to interact directly with HDAC1, namely the human papilloma virus E7 protein (Brehm *et al.*,

1998). Thus, we conclude that hMITR interacts directly with both HDAC1 and MEF-2 proteins, and thus fulfils the definition of a co-repressor.

Discussion

A new class of MEF-2 cofactors
Our attempts to identify cofactors that may confer some specificity to MEF-2 function have yielded a novel protein, MITR, which binds to the MADS/MEF-2 region of both XMEF2D and XMEF2A but not to the DNA binding domain of the related MADS protein XSRF. Database searches identify several related human and mouse clones that probably represent one *MITR* homologue and two *MITR*-related genes. The high level of sequence conservation between clones from different species suggests a common function for this protein in vertebrates. Indeed, we and others have shown that the human MITR-related protein HDAC4/KIAA0288, like *Xenopus* MITR, interacts with MEF-2 proteins *in vivo* (Miska *et al.*, 1999; Wang *et al.*, 1999). Interestingly, the two MITR-related members of this family, but not MITR itself, contain a C-terminal extension with high homology to histone deacetylase domains, and exhibit deacetylase activity (Fischle *et al.*, 1999; Grozinger *et al.*, 1999; Verdel and Khochbin, 1999).

Histone deacetylases mediate the removal of acetyl groups from core histones and basal transcription factors resulting in transcriptional repression. Recently, it has become clear that histone deacetylases can be recruited to specific promoters by interaction with sequence-specific DNA binding proteins forming a three-way complex of DNA-binding protein, co-repressor and histone deacetylase (reviewed in Pazin and Kadonaga, 1997; Struhl, 1998). For example, the transcription factor heterodimers Mad–Max and Mix–Mad interact with the co-repressor Sin3 (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995) as do Ume6 (Kadosh and Struhl, 1997) and YY1 (Yang *et al.*, 1997). Sin3, in turn, binds to the deacetylases HDAC1 and/or HDAC2 (Hassig *et al.*, 1997; Laherty *et al.*, 1997). Similarly, non-liganded nuclear receptors such as TR and RAR interact with the co-repressors SMRT and N-CoR (Chen and Evans, 1995; Horlein *et al.*, 1995; Chen *et al.*, 1996; Sande and Privalsky, 1996), which also bind to HDAC1 and HDAC2 (Nagy *et al.*, 1997). Finally, Rb has been shown to bind directly to HDAC1 (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). This complex is then targeted to DNA by binding to E2F. Strikingly, these DNA binding proteins have all been implicated in control of the processes of cell proliferation and differentiation. *Xenopus* MITR, unlike the MITR-related proteins, does not contain a histone deacetylase domain. Nevertheless, we have found evidence that the inhibitory effect of MITR on transcriptional activation by MEF-2 can be relieved by TSA treatment, suggesting the involvement of a histone deacetylase activity. Furthermore, we have shown that MITR interacts directly with the histone deacetylase HDAC1. Together, these results suggest that MITR performs the function of a co-repressor, serving as a bridge between MEF-2 proteins and HDAC1.

Our two-hybrid screen detected only MITR, but none of the other proteins previously shown to interact with MEF-2 proteins. This may suggest that the MITR–MEF-2 interaction is much stronger than that of MEF-2 with

other proteins. Alternatively, this may reflect the relative abundance of interacting factors at stage 18, or that other interacting factors require MEF-2 to bind to its normal DNA recognition site for high affinity association.

Analysis of human genomic sequences reveals that the *hMITR* locus (including the putative histone deacetylase exons) is located telomeric to the *TWIST* gene at 7p15.3–p21 on chromosome 7. The two genes are convergently transcribed with 100 kb separating the coding regions. *TWIST* has been implicated in the Saethre–Chotzen form of craniosynostosis (el Ghouzzi *et al.*, 1997; Howard *et al.*, 1997), but there is some evidence that other genes in this region of chromosome 7 are also involved. Thus, it is possible that deletion or disruption of the *hMITR* gene contributes to this syndrome. It is interesting in light of this to note that Twist has been shown to inhibit myogenesis, at least in part, by inhibiting transactivation of target genes by MEF-2 proteins (Spicer *et al.*, 1996), and this activity of Twist is similar to that which we have identified for MITR.

MITR function during myogenesis

In *Xenopus*, the onset of myogenesis occurs during gastrulation in the paraxial mesoderm prior to somite formation, and it is in this pattern that XMEF2D transcripts are first detected (Chambers *et al.*, 1992, 1994; Wong *et al.*, 1994). MITR transcripts are not detected until some hours later in the more mature somites of early neurula embryos, coincident with transcripts of XMEF2A and terminal differentiation markers. Similarly, the MITR-related mouse protein mHDA1 has been shown to accumulate in cells only after they have been induced to differentiate (Verdel and Khochbin, 1999). Together, these observations suggest a relatively late function for the MITR–MEF-2 complex during myogenesis. Since we have shown that MITR represses MEF-2 activity, it is possible that MITR acts as part of a molecular switch, repressing the early functions of XMEF2D and XMEF2A in the mature myotome, allowing the onset of the next stage in skeletal muscle differentiation to occur.

A puzzling result is our apparent failure to block the onset of myogenesis in early embryos by ectopic expression of MITR. One explanation may be the absence of HDAC1 or other MITR cofactors in early embryos at gastrulation when myogenesis begins. Alternatively, there may be translational control of the injected *MITR* RNA, preventing accumulation of MITR protein until after myogenesis has been initiated. These possibilities could be addressed by monitoring the temporal and spatial distribution of the endogenous MITR and HDAC1 protein in the developing embryo; however, this will have to await production of suitable antibodies. There are precedents for the unexpected failure to observe phenotypic effects in overexpression studies with *Xenopus* embryos, most notably with the MyoD family of myogenic regulators. Ectopic expression of these factors does not lead to the production of extra muscle tissue, in contrast to their myogenic effect in a variety of cultured cell lines. An alternative method of investigating the potential role of MITR in myogenesis may therefore be to test the effects of overexpression of MITR in myogenic cell lines induced to undergo terminal differentiation.

In the adult, *MITR* is expressed at low levels in a variety of tissues, as is the case for XMEF2A and XMEF2D. In addition, *Xenopus* MEF-2 transcripts are present at increased levels in several tissues such as skeletal muscle and brain, but there does not seem to be a parallel increase in *MITR* levels in these tissues. However, given the widespread distribution of both transcripts it is possible that MITR alters the function of MEF-2 proteins in non-muscle tissues, although we have no evidence for this.

Materials and methods

Construction of deletions and transcription activation domain mapping in yeast

Deletions of the full-length *XMEF2D* and *XMEF2A* genes were made by either exonuclease digestion (Henikoff, 1984) or PCR. 3′ and 5′ deletions of *XMEF2D* and *XMEF2A* were fused in-frame to the LexA DNA binding domain and transformed into yeast strain CTY (Bartel *et al.*, 1993), a gift of Steve Sedgewick (National Institute of Medical Research), by the lithium acetate method (Schiestl and Gietz, 1989). Transformants were plated on SD–Trp and grown for 3 days at 30°C. Levels of LacZ gene expression were determined by liquid culture assay (Johnson *et al.*, 1986). To map the interaction domains of the *Xenopus* MEF-2 factors and MITR, 5′ and 3′ deletions of the original MITR cDNA (pMITR-y1) were made by PCR and fused in-frame to the GAL4 activation domain, before assay in the two-hybrid system using reporter strain YN166 (Sparrow *et al.*, 1998a). All PCR-derived constructs were fully sequenced to ensure that no nucleotide changes were introduced during amplification.

Expression of synthetic RNA in oocytes and embryos

For generating synthetic RNA *in vitro*, the coding region of MITR, as well as the various deletions of XMEF2A and XMEF2D described, were inserted into pT7TS (Cleaver *et al.*, 1996) producing constructs in which both the 5′ and 3′ untranslated regions were replaced by those of *Xenopus* β-globin. RNA was transcribed *in vitro* using T7 RNA polymerase from templates linearized with *Xba*I as described previously (Chambers *et al.*, 1994). Oocyte transcription assays were performed as described (Casey *et al.*, 1998), except that CAT activity was assayed by phase extraction assay (Seed and Sheen, 1988). To create MEF-2 responsive reporters, one, three or five copies of a MEF-2 binding site (CTAGCGCT-CTAAAAATAACCCT) were cloned upstream of a CAT reporter gene in the vector pBLCAT3T (Principaud and Spohr, 1991) linked to a minimal cytoskeletal actin promoter (CSKA) in which the serum response element had been replaced by an *Xba*I site (Mohun *et al.*, 1987). As a negative control, one, three or five copies of a mutated MEF-2 site unable to bind MEF-2 factors were cloned into the reporter (CTAGCGCT-CTAAACATAACCCT, mutation 6; Cserjesi and Olson, 1991). The Xbra-responsive construct driving the CAT reporter gene was as described (Casey *et al.*, 1998). In each case 400–800 pg of reporter was injected. Each deletion construct was assayed a minimum of three times in two independent experiments, and the mean and standard deviation of CAT activity calculated. For experiments involving TSA, after injection of the DNA template, oocytes were transferred to OR2+ containing 20 ng/ml TSA and incubated at room temperature for 16 h prior to assay.

Yeast two-hybrid screen and isolation of the full-length MITR cDNA

A bait for the yeast two-hybrid screen (XMEF2DΔ316–378 pGBT9) was constructed by fusing a transcriptionally inactive internal deletion of XMEF2D in-frame to the GAL4 DNA binding domain. Approximately 8×10^6 independent cDNA clones from a neurula cDNA library fused to the GAL4 activation domain in the Clontech vector pGAD10 (Sparrow *et al.*, 1998b) were screened using this bait as previously described (Sparrow *et al.*, 1998a). Sixteen independent clones encoding the *Xenopus* MITR gene were isolated but none contained a poly A tail. The longest of these was termed pMITR-y1 (nucleotides 1–694 of the full-length cDNA). To isolate the full-length cDNA, a *Xenopus laevis* neurula (stage 17) cDNA library (Kintner and Melton, 1987) was screened with an oligo-labelled probe of the entire pMITR-y1 clone, at a stringency of 0.2× SSC, 60°C. Several positively hybridizing clones were analysed and found to contain sequences overlapping the original clone. The entire *MITR* sequence was derived from one of these clones (pMITR 4.1) that was sequenced using nested deletions (Henikoff, 1984). The

sequence was analysed using the Lasergene suite of programs (DNAS-TAR Inc) and BLAST (at the National Center for Biotechnology Information using the BLAST network server: http://www.ncbi.nlm.nih.gov/). The MITR nucleotide sequence has been submitted to the DDBJ/EMBL/GenBank database under accession No. Z97214.

pGEX pulldown and direct interaction assays
XMEF2D, XMEF2A and XSRF open reading frames were fused in-frame with GST. Expression and purification of GST-fusion proteins were performed as described (Smith and Johnson, 1988). The GST-fusion proteins were analysed by SDS–polyacrylamide gel to ensure integrity and normalize the amount of each protein. ^{35}S-labelled *in vitro* translated full-length MITR was made from a plasmid containing nucleotides 1–3495 subcloned into pBluescript II KS+ (pMITR-ORF), and MITR 1–222 was made from a plasmid containing the original clone (nucleotides 1–694) subcloned into pBluescript II KS+ (pMITR 1–222) using coupled transcription/translation (Promega TNT system) with T7 and T3 RNA polymerase, respectively. Five microlitres of ^{35}S-labelled protein was mixed with ~2–3 µg of GST-fusion protein and rotated at 4°C for 60 min. One hundred microlitres of 10% glutathione-agarose beads was added and the mixture rotated for a further 20 min at 4°C. The supernatant was removed and the beads washed three times with 0.5 ml of wash buffer [10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% NP-40, 2 mM DTT, 2 mM Pefabloc (Pentapharm)]. Bound protein was eluted from the beads by incubation in 20 mM reduced glutathione/50 mM Tris–HCl pH 7.6 at room temperature for 10 min and analysed on a 12.5% SDS–polyacrylamide gel. Gels were fixed for 20 min in 50% methanol/10% acetic acid, washed in distilled water for 20 min, dried and exposed to BioMax X-Ray film (Kodak). In all experiments, two negative controls were used. First, unprogrammed rabbit reticulocyte lysate was used to replace the ^{35}S-labelled MITR or MITR 1–222. Secondly, GST alone was used to replace the GST–XMEF2D/XMEF2A/XSRF protein. For direct interaction assays, the same amount of GST-fusions pre-bound to glutathione–Sepharose beads (Pharmacia), or glutathione–Sepharose beads alone, were incubated in 200 µl of Z' buffer (25 mM HEPES pH 7.5, 12.5 mM MgCl$_2$, 150 mM KCl, 20% glycerol, 0.1% NP-40, 1 mM DTT) with 30 µg of BSA for 10 min at room temperature. One microgram of Flag-tagged HDAC1 was then added to the reactions and incubated at room temperature for a further 1 h. Beads were spun down and after four washes in NETN buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) bound material was resuspended in 1× SDS-loading buffer and subjected to SDS–PAGE followed by Western blotting using an anti-Flag antibody at 4 µg/ml (Martin *et al.*, 1995).

RNA preparation and assay
RNA was prepared from embryos and adult tissue as described previously (Logan and Mohun, 1993). *MITR* transcripts were detected with a probe prepared from nucleotides 643–1002 of pMITR-4.1 and synthesized using *Xba*I linearized template and T3 RNA polymerase. RNase protection assays were performed as described previously (Chambers *et al.*, 1994), with *XMax2* (Tonissen and Krieg, 1994) as a loading control.

Whole-mount RNA in situ hybridization
Albino embryos (stages 14–46) were used for RNA whole-mount *in situ* hybridization as described by Harland (1991) using digoxigenin-labelled probes. For detecting *MITR* transcripts, antisense probes were derived from either *Xho*I linearized pMITR-y1 (nucleotides 1–694) using T7 RNA polymerase, or *Xho*I linearized pMITR-4.1R1 (nucleotides 2882–4368) by T7 RNA polymerase. Sense control probes were synthesized from the same subclones linearized with *Xba*I using T3 RNA polymerase. The probe for *XMEF2D* was as previously described (Chambers *et al.*, 1994). Chromogenic reactions were performed using NBT/BCIP tablets (Boehringer Mannheim). For histological analysis, fixed embryos were embedded in paraffin and 10-µm sections cut.

Cell culture and gene reporter assays
The full-length clone of *hMITR* (KIAA0744) was provided by Osamu Ohara and Takahiro Nagase at the Kazusa DNA Research Institute (Nagase *et al.*, 1998). The hMITR coding region was amplified by PCR and subcloned into pcDNA3, pcDNA3.1-A-myc/his (Invitrogen) and pGEX2TKp to give pchMITR, pchMITR-myc and pGEX-hMITR, respectively. The GAL4 DNA binding domain (amino acids 1–146) was added to the N-terminus of pchMITR and inserted into pcDNA3 to give pcGAL4–hMITR and pcGAL4. All constructs were verified by sequencing. 5× GAL4TK–CAT and 0× GAL4TK–CAT were as described (Morkel *et al.*, 1997). 293T cells were transfected using the

calcium phosphate method as described (Hagemeier *et al.*, 1993). For gene reporter assays, 293T cells were grown in 5-cm diameter dishes and transfected at 40–60% confluency with a total of 10 µg of DNA. Cells were washed 16 h after transfection and incubated for an additional 24 h, either in the presence or absence of 330 nM TSA prior to harvesting. CAT assays were peformed as described (Hagemeier *et al.*, 1993).

Immunoprecipitation
Oocytes injected with synthetic RNA were harvested at 36–48 h by homogenization in IPH buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40). For cell culture experiments, 293T cells in 15-cm diameter culture dishes were transfected with 30 µg of expression vectors. Cells were washed in ice-cold PBS and lysed in IPH buffer at 4°C for 30 min. Lysates were cleared by centrifugation, diluted five times in IPH buffer containing 0.1% NP-40 (input samples taken at this stage) and incubated with 5 µg anti-myc mouse monoclonal antibody (Boehringer Mannheim) for 1 h. Fifty microlitres of a slurry of protein A/G–Sepharose beads (Pharmacia) was added and the incubation continued for 2 h with rotation at 4°C. Precipitates were washed five times in ice-cold IPH and resuspended in loading buffer for analysis by SDS–PAGE followed by Western blotting (Martin *et al.*, 1995) using either an anti-myc antibody at 1 µg/ml, an anti-GAL4 antibody at 0.4 µg/ml or a rat anti-HA monoclonal antibody at 1 µg/ml.

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