Transcriptional autorepression of Msx1 gene is mediated by interactions of Msx1 protein with a multi-protein transcriptional complex containing TATA-binding protein, Sp1 and cAMP-response-element-binding protein-binding protein (CBP/p300)

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The TATA-less murine Msx1 promoter contains two Msx1-binding motifs, located at −568 to −573 and +25 to +30, and is subject to potent autorepression [Takahashi, Guron, Shetty, Matsui and Raghow (1997) J. Biol. Chem. **272**, 22667–22678]. To investigate the molecular mechanism by which Msx1 represses the activity of its own promoter, we transfected C2C12 myoblasts with Msx1-promoter–luciferase constructs and assessed reporter gene activity, with and without the exogenous expression of Msx1. We demonstrate that Msx1-mediated autorepression remained unaffected, regardless of the presence or absence of the Msx1 recognition motifs on the promoter. Furthermore, graded exogenous expression of TATA-binding protein (TBP), Sp1 or cAMP-response-element-binding protein-binding protein (CBP/p300) could counteract the autoinhibitory activity of Msx1. Finally, we demonstrate that Msx1 protein can be immunoprecipitated in a multiprotein complex containing TBP, Sp1 and CBP/p300. We hypothesize that the interaction of Msx1 protein with one or more ubiquitous or tissue-restricted transcription factors mediates transcriptional autorepression of the Msx1 gene.

Key words: homeobox genes, promoter inactivation, protein–protein interaction, transcriptosome.

INTRODUCTION

The highly conserved structural organization of the members of the Msx family of genes and their abundant expression at sites of inductive cell–cell interactions in the embryo suggest that they have a pivotal role during early development [1]. A somewhat overlapping expression of Msx1 and Msx2 occurs in the mesenchymal cells of the cephalic neural crest, the first four branchial arches, mandible, maxilla, eye and ear, as well as in the mesenchyme underlying the apical ectodermal ridge of the developing limbs [1,2]. In contrast, Msx3 is expressed only in the neural tube and the adjoining areas of the hindbrain [3,4]. Spontaneous or experimental misexpressions of Msx1 or Msx2 are known to be associated with a number of developmental anomalies. Msx1-deficient mice were shown to die perinatally, primarily due to abnormalities in the mandibles and maxillae [5]; similarly, disruption of Msx1 or Msx2 gene expression in the developing embryos by anti-sense oligonucleotides caused severe axial and craniofacial dysmorphologies [6]. In humans, chromosomal deletion of Msx1 has been implicated in the craniofacial abnormalities of Wolf–Hirschhorn syndrome [7]. Selective tooth agenesis and Boston type craniosynostosis have been associated with point mutations in the homeodomains of Msx1 and Msx2 genes respectively [8,9]. Because the protein products of Msx genes are genuine DNA-binding transcription factors, it is believed that the altered transcriptional modulation of the target genes by mutated Msx1 or Msx2 proteins perturbs one or more key steps of cellular differentiation, leading to developmental abnormalities [1].

In vitro, Msx1 and Msx2 genes are capable of regulating proliferation and apoptosis of the target cells while simultaneously inhibiting their differentiation [10]. A forced expression of Msx1 in myoblasts could block their differentiation into myotubes [11]. The transcriptional repression of myoD was shown to result from the binding of Msx1 protein to the two cognate Msx1-binding sites in the myoD enhancer; repression of the myoD enhancer by Msx1 was promoter-specific because Rous sarcoma virus long terminal repeat (RSV-LTR)-driven expression of the reporter gene remained unaffected under identical experimental conditions [12]. The repressive action of Msx1 could be conferred to a heterologous promoter by ligating a multimerized Msx1-binding DNA motif to a basal promoter [13,14]. Subsequently, Abate-Shen and co-workers [15] showed that Msx1 could repress some promoters even if Msx1-binding sites were lacking, presumably through protein–protein interactions between component(s) of transcriptional machinery and Msx1 protein; the demonstration of Msx1 binding to the TATA-binding protein (TBP) is consistent with such a mechanism of transcriptional repression [13–15].

Although the identities of the downstream genetic target(s) of Msx1 *in vivo* remain largely elusive, Msx1 is a potent inactivator of its own transcription *in vitro* [16,17]. Because two *cis*-acting elements capable of binding to Msx1 homeodomain protein are located in the Msx1 promoter [18], it is conceivable that the transcriptional autorepression is mediated through the interaction of the Msx1-binding elements with the Msx1 protein. The TATA-less Msx1 promoter also contains three Sp1-binding sites, the mutation or deletion of which seriously hampered promoter activation, as judged by transient expression experiments; even more importantly, Sp1 was found to be obligatory for the

Abbreviations used: CBP/p300, CREB-binding protein; CMV, cytomegalovirus; LTR, long terminal repeat; MSV, murine sarcoma virus; RSV, Rous sarcoma virus; TBP, TATA-binding protein.

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activation of the −165/+106 bp minimal Msx1 promoter [17]. We therefore envisage that the transcription complex formation on the Msx1 promoter involves a tethering factor [19] between Sp1, bound to the cognate GC boxes in the promoter, and the transcription factor TFIID, which is physically linked to TBP. Such a multisubunit transcriptional complex might be stabilized further through its interactions with TBP-associated factors (TAFs) and other transcriptional co-activators [e.g. cAMP-response-element-binding protein (CREB)-binding protein (CBP)]. Although CBP and its common homologue p300 (CBP/p300) were first identified as transcriptional co-activators of CREB [20], several transcription factors including c-Jun [21], myoD [22], c-Myb [23] and the Drosophila *cubitus interruptus* gene product [24] have subsequently been shown to interact with CBP/p300 [25]. To explore the molecular mechanism of autorepression of the Msx1 gene, we co-transfected C2C12 cells with Msx1-promoter–luciferase and Msx1 expression vector pEMSV-Msx1 (in which MSV stands for murine sarcoma virus) and concomitantly transfected cells with vectors designed to achieve the graded expression of TBP, Sp1 or CBP. We provide evidence that the Msx1-mediated autorepression is most probably mediated by the 'squelching' of component(s) of transcriptional machinery with which Msx1 protein interacts.

MATERIALS AND METHODS

Cell culture

A murine myoblast cell line C2C12, bought from the American Type Culture Collection was cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, at 37 °C in a humidified incubator supplied with air/CO_2 (19:1). Cells were grown to confluence and subcultured every 48 h in Petri dishes 10 cm in diameter [17,18].

Plasmid vectors

Plasmid vectors pEMSV-Msx1(+) and pEMSV-Msx1(−), containing Msx1 cDNA in the sense and anti-sense orientations respectively, driven by an MSV LTR promoter [11], were obtained from Dr. David Sassoon (Brookdale Center of Molecular Biology, Mount Sinai Medical Center, New York, U.S.A.). The Msx1-promoter–luciferase constructs were made by cloning Msx1 genomic DNA fragments upstream of the luciferase reporter into the pGL2-Basic vector, as detailed previously [17,18]. In brief, −3.5 kb/+106 bp and −2.0 kb/+106 bp Msx1 genomic DNA fragments with *Xma*1–*Bam*HI termini were cloned into pGL2-Basic linearized with *Xma*I and *Bgl*II; a −1.4 kb/+106 bp promoter fragment with *Eco*R1–*Bam*HI termini cloned into pBluescript −IISK+ (pBEB) was used as the source of DNA to generate −886/+106 and −165/+106 bp promoter–luciferase constructs. DNA fragments amplified by PCR with the use of forward and reverse oligonucleotides with *Sst*I and *Bam*HI termini respectively were cloned into pGL2-Basic vector linearized with *Sst*I and *Bgl*II. Either one or both of the Msx1 homeodomain-binding sites (+25 to +30 and −568 to −573 bp) were altered from TTAAC to CCGAC by site-directed mutagenesis and mobilized into the −886/+106 bp promoter. Plasmid pAct-TBP [26], in which actin promoter drives the expression of haemagglutinin-tagged TBP, was received from Dr. James L. Manley (Columbia University, New York, U.S.A.). Sp1 expression plasmid pCMV-Sp1 (in which CMV stands for cytomegalovirus) [27] was obtained from Dr. R. Tjian (University of California, Berkeley, CA, U.S.A.). Dr. Roland Kwok (Vollum Institute, Portland, OR, U.S.A.) provided a CBP expression plasmid, pRSV-CBP, driven by RSV-LTR [28]. A full-length Msx1 cDNA fragment with *Xba*I/*Kpn*I termini was generated by PCR and ligated into pcDNA3 (InVitrogen, Carlsbad, CA, U.S.A.) to create a V5 epitope-tagged Msx1 expression vector.

Transient transfections

Cells (10^5 cells per 35 mm diameter well) were seeded in six-well tissue culture dishes 1 day before transfection. Transfections were performed in triplicate by using Lipofectamine (Life Technologies, Gaithersburg, MD, U.S.A.) reagent in accordance with the manufacturer's protocol, as detailed previously [17,18]. Cells were incubated with DNA–lipid complexes for 5 h and then fed with Dulbecco's modified Eagle's medium; 24–48 h after transfection, cells were rinsed and harvested in PBS and lysed in 150 μl of 1×Cell Culture Lysis Reagent (Promega, Madison, WI, U.S.A.). Aliquots (20 μl) of the cell extract were mixed with 100 μl of luciferase assay reagent containing luciferin (Promega) and the light intensity was measured in a TD-20e luminometer (Turner Designs, Madison, WI, U.S.A.). The protein content of cell extracts was quantified by the method of Bradford [29] and luciferase activities were calculated as units of light intensity/μg of protein. Relative expression of the reporter gene, fixed arbitrarily as 100%, was compared with controls. Transfection efficiencies were normalized by co-transfection of cells with pRSV–β-galactosidase and assessing β-galactosidase expression (Promega), as described [17].

Western blotting

Western blotting was done in accordance with the protocol described previously [30], with minor modifications. In brief, cells in 35 mm wells were rinsed twice with ice-cold PBS. Lysis buffer [10% (v/v) glycerol/1% (v/v) Triton X-100/1 mM EDTA in 20 mM Tris/HCl (pH 8.0)/150 mM NaCl (TBS)] (40 μl) with protease inhibitors was added to the cells. Cell lysates were kept on ice for 40 min with occasional mixing and centrifuged (14000 ***g*** for 20 min at 4 °C); supernatants containing equal amounts of protein as determined by the Bradford assay [29] were subjected to PAGE [10% (w/v) gel]. Size-fractionated polypeptides were blotted from gels to Immobilon-P membranes (Millipore Corporation, Bedford, MA, U.S.A.). The blots were soaked for 1 h in Blotto [5% (w/v) dried milk dissolved in 1×TBS containing 0.1% (v/v) Tween 20 (TBS-T)] to block non-specific binding and washed with 1×TBS. The membranes were incubated with anti-V5 antibody (catalogue no. R960-25; InVitrogen) in Blotto overnight at room temperature and then washed with 1×TBS-T. The blots were incubated with horseradish peroxidase-conjugated secondary antibody in Blotto for 1 h at room temperature and the excess antibody was removed by washing in TBS-T. Finally, membranes were treated with enhanced chemiluminescence (ECL®) reagent (Amersham Life Science, Cleveland, OH, U.S.A.) and exposed to X-ray film.

Immunoprecipitation

Cell lysates, prepared as described for Western blotting, were immunoprecipitated in accordance with a procedure described previously [31]. The primary antibodies used for the immunoprecipitation of TBP, Sp1 and CBP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Lysates were incubated with an appropriate amount of primary antibody for 1 h at 4 °C; 20 μl aliquots of Protein A–G PLUS-agarose beads (Santa Cruz Biotechnology) were added to the cell lysate and rocked gently at 4 °C overnight. After being washed four times with lysis buffer, agarose beads were pelleted (1000 ***g*** for 5 min at 4 °C) and taken up in 20 μl of 1×Laemmli sample buffer.

Proteins eluted from beads by being boiled in sample buffer were size-fractionated, electroblotted to membranes and immunodetected by Western blotting.

RESULTS

Msx1 is subject to transcriptional autorepression

With the aim of establishing whether unique *cis*-acting elements mediate the autoinactivation of Msx1 promoter, we transfected C2C12 cells with a number of constructs containing 5′ serially truncated Msx1 promoters driving the expression of luciferase, individually, with or without the Msx1 expression vector pEMSV-Msx1. As judged by the levels of reporter gene expression, activities of all Msx1 promoters, regardless of their lengths, were severely down-regulated in C2C12 cells expressing Msx1 protein (Figure 1A). Although we observed significant variation between experiments, approx. 10-fold repression of promoter activity was generally seen [18]. Exogenous expression of Msx1 suppressed the activities of the −3.5 kb/+106 bp (longest), −886/+106 bp (intermediate) and −165/+106 bp (minimal) Msx1 promoters with equal potency (Figure 1A). This suggested that a large portion of the Msx1 promoter was dispensable for its ability to elicit autorepression. Transcriptional autoinactivation of Msx1 promoter by pEMSV-Msx1(+) was judged to be mediated specifically by the exogenously expressed Msx1 protein, as evident from two types of observation. First, co-transfection of C2C12 cells with pEMSV-Msx1(−) DNA, a plasmid in which Msx1 cDNA is in the antisense orientation, did not adversely affect Msx1 promoter [18]. Secondly, co-transfection of C2C12 cells with Msx1 promoter–reporter constructs with empty vector (pEMSV) was also inconsequential for Msx1 promoter activity [18].

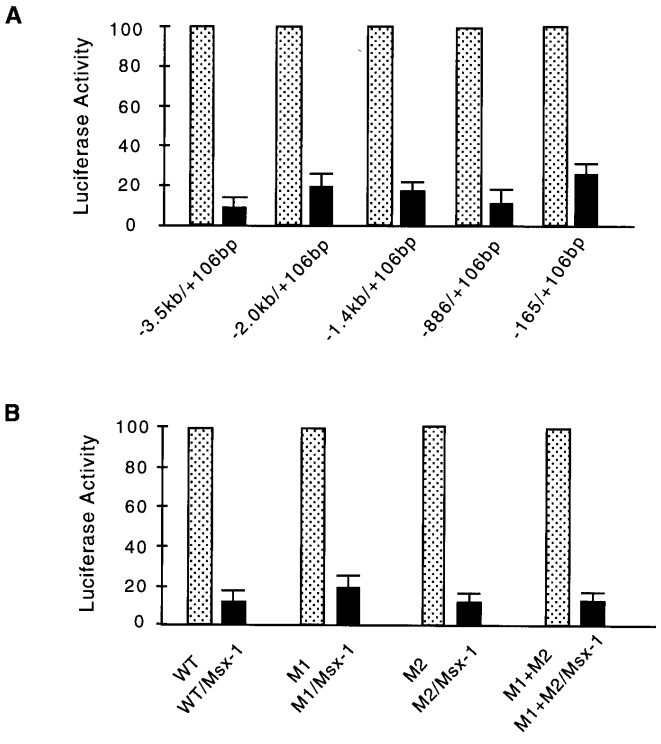

Figure 1 Autorepression of Msx1 promoter is independent of its length or the presence of Msx1-binding motifs

(**A**) Activities of the luciferase from C2C12 cells transfected with Msx1 luciferase alone (stippled bars) or with pEMSV-Msx1 (solid bars) were quantified; activities of the latter are shown as percentages of the former (each set at 100%). The relative activities of the −3.5 kb/+106 bp, −2.0 kb/+106 bp, −1.4 kb/+106 bp, −886/+106 bp and −165/+106 bp Msx1 promoters are shown. (**B**) The luciferase activities in cells transfected with wild-type (WT) or −886/+106 bp Msx1 promoter mutated in the distal (M1), the proximal (M2) or both (M1+M2) Msx1 binding sites, with or without pEMSV-Msx1, are shown; activities in cells co-transfected with pEMSV-Msx1 are shown as percentages of those in cells transfected without pEMSV-Msx1, each set at 100%. Results from three independent transfections were averaged; bars represent S.E.M. values.

Next we experimentally assessed the role of Msx1-binding site(s) on the promoter. The activity of the −886/+106 bp wild-type Msx1 promoter was compared against the activities of the −886/+106 bp Msx1 promoters in which either one or both Msx1 protein-binding elements were mutated [16,18]. As shown in Figure 1(B), luciferase activities driven by both wild-type and mutated Msx1 promoters were decreased to similar extents in cells co-transfected with pEMSV-Msx1. Thus neither of the Msx1 protein-binding elements on the promoter are required for its autoinactivation. We also observed that increasing the ratio of pEMSV-Msx1 DNA in the co-transfection mixture elicited a correspondingly higher degree of repression of Msx1 promoter. Presumably, the graded repression observed in these experiments reflects the dose-dependent effect of Msx1 protein expression on its own promoter. Furthermore, we discovered that the optimal repression of both the longest (−3.5 kb/+106 bp) and the minimal (−165/+106 bp) Msx1 promoters (without poisoning the cells) occurred with 0.05 μg of pEMSV-Msx1 [18]. Therefore, unless otherwise stated, all subsequent co-transfection experiments were done with 0.05 μg of pEMSV-Msx1 DNA.

Finally, because C2C12 myoblasts have a unique phenotype, we were curious to know whether Msx1 promoter autorepression was a special property of these cells or whether similar down-regulation also occurred in other cell types. To address this issue experimentally, we had previously tested and reported that a number of critical parameters of Msx1 promoter activation in C2C12 cells are similar to those in NIH3T3 fibroblasts and a human rhabdomyosarcoma cell line Rh28 [17,18]. Consistent with the previously published results, a graded exogenous co-expression of Msx1 also resulted in the corresponding auto-inhibition of Msx1 promoter-driven luciferase reporter in both NIH3T3 fibroblasts and the rhabdomyosarcoma cell line ([17,18], and T. Takahashi, unpublished work). On the basis of these observations we postulate that the autorepression of Msx1 promoter observed in C2C12 cells is not unique to the myoblast cell line, but occurs in other cells.

Msx1 autorepression can be rescued by graded exogenous expression of TBP, Sp1 and CBP

In the next series of experiments we attempted to compare systematically the repression of the longest and the minimal Msx1 promoters in C2C12 cells transfected to achieve the graded exogenous expression of three key proteins involved in transcriptional regulation. When cells were co-transfected with −3.5 kb/+106 bp Msx1-promoter–luciferase plasmid and 0.05 μg of pEMSV-Msx1, the luciferase activity was repressed to approx. 10%. As shown in Figure 2, co-transfection of C2C12 cells with increasing amounts of the TBP expression plasmid pAct-TBP not only relieved autorepression of the Msx1 promoter in a dose-dependent manner but further enhanced promoter activation above that seen in cells transfected with Msx1-promoter–luciferase alone; thus we observed in cells co-transfected with 0.5 μg of pAct-TBP that luciferase activity was approx. 140% of that in the control transfectants (Figure 2A). A similar enhancement of promoter activity by TBP was seen with the

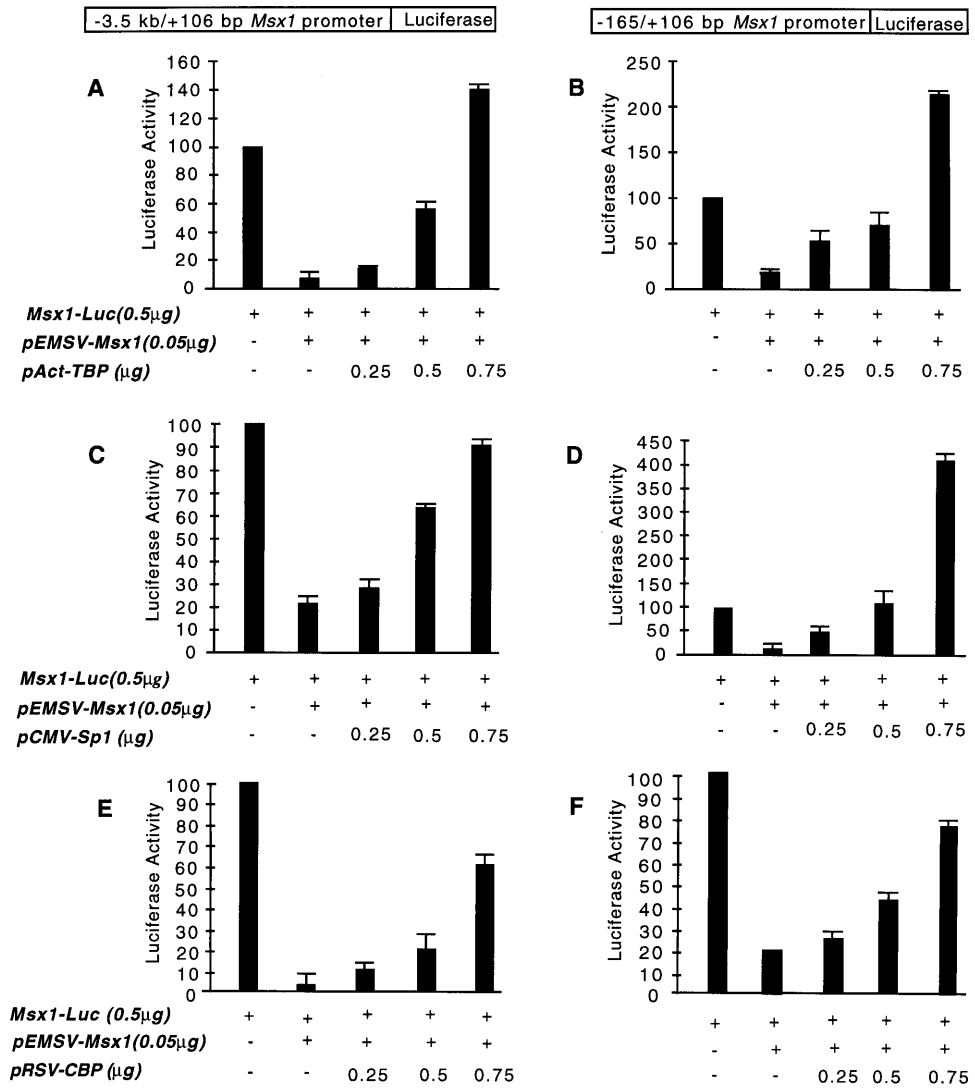

Figure 2 Msx1 autorepression can be rescued by overexpression of TBP, Sp1 or CBP

Graded co-expression of TBP (**A**, **B**), Sp1 (**C**, **D**) or CBP (**E**, **F**) reverses the autoinhibitory action of Msx1 protein on the long (**A**, **C**, **E**) and short (**B**, **D**, **F**) Msx1 promoters. The relative luciferase activities of C2C12 cells transfected with −3.5 kb/+106 bp or −165/+106 bp Msx1-promoter–luciferase reporter plasmids alone or with pEMSV-Msx1, pACT-TBP, pCMV-Sp1 or pRSV-CBP DNA species are shown, as indicated. Results are presented as percentages relative to controls. Experiments were done in triplicate and quantitative results were averaged to calculate S.E.M. values, depicted by bars.

−165/+106 bp Msx1-promoter–luciferase reporter as well. There was a decrease to approx. 20% in the luciferase activity in C2C12 cells co-transfected with −165/+106 bp Msx1-promoter–luciferase and pEMSV-Msx1 (Figure 2B). A graded expression of TBP restored luciferase activity driven by the minimal promoter in a dose-dependent manner, similar to that observed with the longer promoter (Figure 2B). Reversal of repression of both −3.5 kb/+106 bp and the −165/+106 bp Msx1 promoters was also observed if C2C12 cells were co-transfected with the Msx1-promoter–luciferase DNA and the Sp1 expression plasmid CMV-Sp1 (Figures 2C and 2D). A graded expression of Sp1 steadily relieved Msx1 autorepression; co-transfection of cells with 0.75 μg of CMV-Sp1 restored almost completely the activity of the −3.5 kb/+106 bp Msx1 promoter. In contrast, the minimal promoter, which had previously been shown to have an obligatory dependence on Sp1 [17], responded vigorously to Sp1 overexpression; co-transfection of C2C12 cells with 0.75 μg of pCMV-Sp1 resulted in a 2–7-fold enhancement of the minimal Msx1 promoter (Figure 2D).

Co-transfection of Msx1-promoter–luciferase with CBP expression plasmid pRSV-CBP was undertaken to evaluate the role of this transcriptional co-activator in Msx1 autoinactivation. Representative results from these experiment are shown in Figures 2(E) and 2(F). When C2C12 cells were co-transfected with 0.5 μg of Msx1-promoter–luciferase and 0.05 μg of pEMSV, the promoter activity decreased to 20–25%. Increasing amounts of pRSV-CBP DNA included in the co-transfection mixture relieved the autorepression of both the long and short Msx1 promoters in a dose-dependent manner (Figures 2E and 2F). The extent of reversal of the autorepressive action of Msx1 varied significantly from experiment to experiment; nearly complete relief from repression was observed when C2C12 cells were co-transfected with 1.25 μg of pRSV-CBP ([18], and results not shown). Although the exogenous expression of CBP relieved the

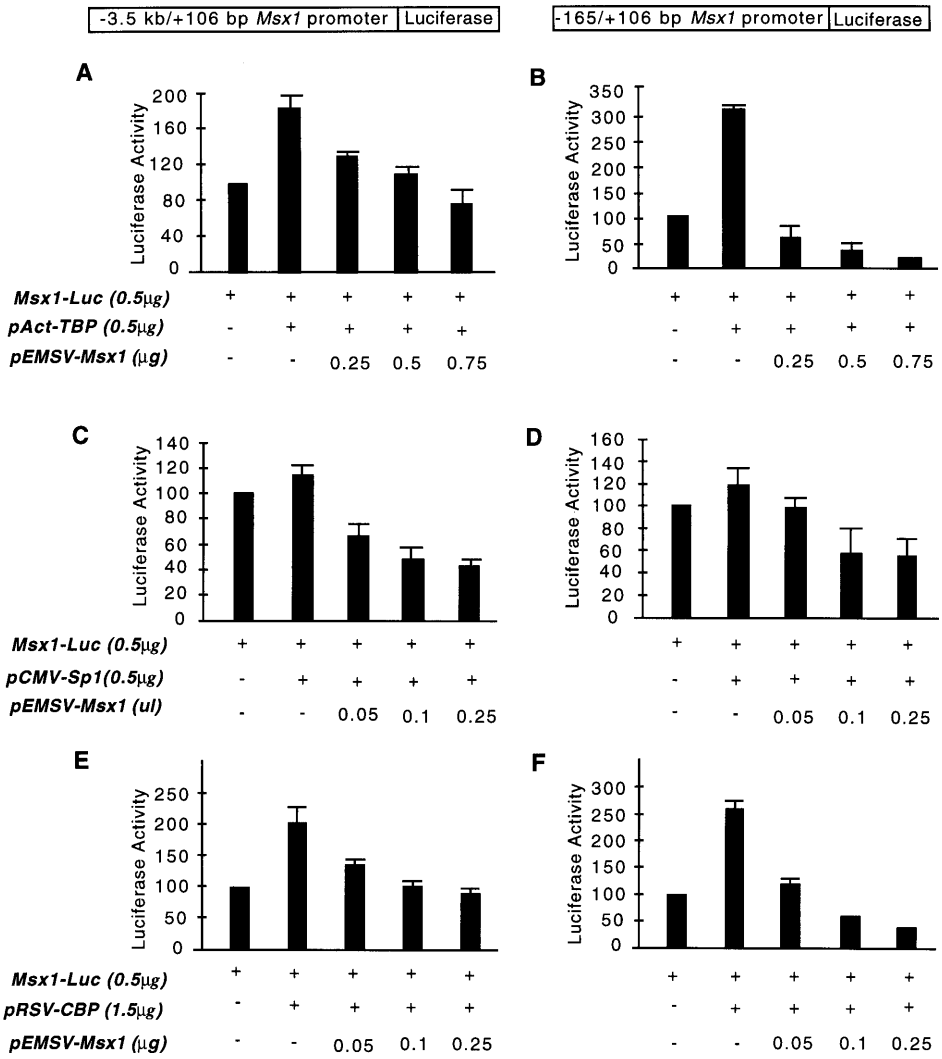

Figure 3 Graded expression of pEMSV-Msx1 can overcome activities of the long and short Msx1 promoters enhanced by co-expression of TBP, Sp1 or CBP

The relative luciferase activities of C2C12 cells transfected with −3.5 kb/+106 bp or −165/+106 bp Msx1-promoter–luciferase plasmid co-transfected with fixed amounts of pAct-TBP, pCMV-Sp1 or pRSV-CBP, and increasing amounts of pEMSV-Msx1, were calculated. Results are presented as percentages relative to controls. Three independent quantifications were averaged; bars represent S.E.M. values.

autorepression of both the long and the minimal Msx1 promoters, the degree of relief elicited by CBP expression, particularly for the minimal promoter, was much less than that seen with equivalent molar co-transfections with TBP or Sp1 expression vectors (compare Figures 2B, 2D and 2F).

In the reverse experiment, the amounts of pAct-TBP, CMV-Sp1 or pRSV-CBP DNA were kept constant and the graded expression of Msx1 protein was attempted by including increasing quantities of pEMSV-Msx1 vector DNA in the transfection mixture. The luciferase activities in extracts from cells transfected with −3.5 kb/+106 bp or −165/+106bp Msx1-promoter–luciferase reporter constructs alone were compared with extracts prepared from C2C12 cells co-transfected with pEMSV-Msx1. Although both the long and the minimal Msx1 promoters showed increased activities when co-transfected with pAct-TBP, the enhanced activation of the −165/+106 bp promoter by TBP was consistently greater than that seen with the −3.5 kb/+106 bp promoter (Figures 3A and 3B). When increasing amounts of pEMSV-Msx1 were co-transfected with the luciferase and pAct-TBP, the luciferase activity steadily decreased in a dose-dependent manner. With the −3.5 kb/+106 bp Msx1 promoter construct, the maximal exogenous expression of TBP resulted in a doubling of the luciferase activity (Figure 3A). Similarly, the greatest expression of TBP resulted in a 3-fold increase in luciferase activity driven by the minimal promoter (Figure 3B). Graded expression of Msx1 in cells co-transfected with pEMSV-Msx1 severely decreased luciferase activity driven by both the long and minimal Msx1 promoters; co-transfection of C2C12 cells with 0.75 μg of pEMSV-Msx1 decreased luciferase activity to 10–20% (Figures 3A and 3B).

Both the long and minimal Msx1 promoters also showed increased activity when cells were co-transfected with CMV-Sp1 or pRSV-CBP. When increasing amounts of pEMSV-Msx1 were co-transfected with constant amounts of Msx1-promoter–luciferase and Sp1 expression plasmids, the reporter activity decreased steadily in a dose-dependent manner. Co-transfection

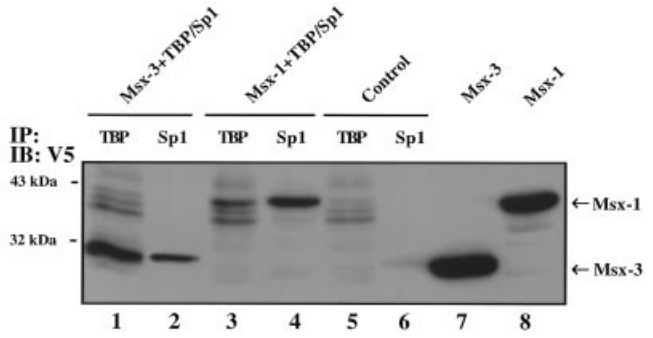

Figure 4 Msx1 interacts with TATA-binding protein and Sp1

C2C12 cells were co-transfected with pcDNA3-Msx3 and pAct-TBP (lane 1), pcDNA3-Msx3 and pCMV-Sp1 (lane 2), pcDNA3-Msx1 and pAct-TBP (lane 3) or pcDNA3-Msx1 and pCMV-Sp1 (lane 4), or transfected individually with pAct-TBP (lane 5), pCMV-Sp1 (lane 6), pcDNA3-Msx3 (lane 7) or pcDNA3-Msx1 (lane 8) DNA vectors. Cell extracts were immunoprecipitated (IP) with polyclonal antibodies against TBP (lanes 1, 3 and 5) or Sp1 (2, 4 and 6), or were subjected to electrophoresis directly without prior immunoprecipitation (lanes 7 and 8), followed by Western blotting (IB) with V5 epitope-specific antibodies, as detailed in the Materials and methods section. Both Msx1 and Msx3 proteins are clearly present in the cell extracts immunoprecipitated with TBP or Sp1-specific antibodies. Polypeptide bands denoting V5 epitope-tagged Msx3 (26 kDa) and Msx1 (39 kDa) are denoted in lanes 7 and 8 respectively. Note that V5 epitope-specific antibody reacted with a number of non-specific polypeptides in the extracts immunoprecipitated with TBP antibodies (compare the V5-tagged Msx1 band in lane 4 with non-specific bands of similar molecular mass seen in lanes 1, 3 and 5).

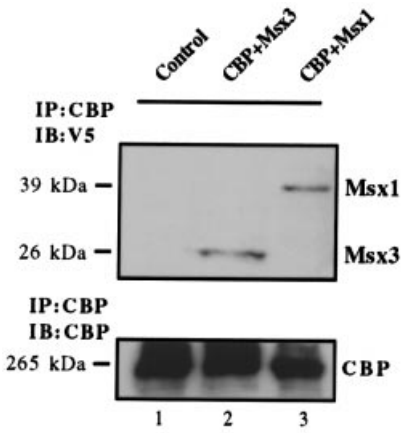

Figure 5 Association of Msx1 and CBP in C2C12 cells

Upper panel: extracts from C2C12 cells transfected with pRSV-CBP alone (lane 1), or co-transfected with pcDNA3-Msx3 and pRSV-CBP (lane 2) or pCDNA3-Msx1 and pRSV-CBP (lane 3). The extracts were immunoprecipitated (IP) with polyclonal antibody against CBP and the blot was probed (IB) with V5 antibody. Lower panel: blots were stripped and reprobed with antibodies against CBP to determine the relative levels of CBP immunoprecipitated from the three cell extracts.

of 0.5 μg of CMV-Sp1 with the -3.5 kb/$+106$ bp and -165/$+106$ bp Msx1 promoters resulted in a modest increase in the luciferase activity (Figures 3C and 3D). Co-transfection of C2C12 cells with increasing amounts of pEMSV-Msx1 caused incremental repression. Although both Msx1 promoters responded positively with pRSV-CBP co-transfection, a 2.5-fold greater amount of pRSV-CBP DNA (1.5 μg compared with 0.5 μg) was required to achieve similar enhancement compared with TBP and Sp1 expression vectors. However, the increasing ratio of pEMSV-Msx1 DNA co-transfected with the Msx1-promoter–luciferase and CBP expression plasmids resulted in a dose-dependent decline in the reporter gene expression, regardless of the length of the Msx1 promoter (Figures 3E and 3F). The ability of TBP, Sp1 or CBP to rescue Msx1-mediated repression was found to be specific for these transcription factors, because C2C12 cells co-transfected with pCMV-Smad1 or pCMV-Smad4 expression vectors failed to reverse Msx1 autoinactivation under identical experimental conditions ([18], and H. Matsui, unpublished work).

Msx1 interacts physically with TBP, Sp1 and CBP *in vivo*

Because the experimental rescue of the Msx1 promoter from autorepression could conceivably be mediated by direct or indirect interactions of Msx1 with TBP, Sp1 or CBP *in vivo*, we experimentally tested the possibility of this interaction. C2C12 cells were co-transfected with V5 epitope-tagged Msx1 expression vector, pcDNA3-Msx1, and with constructs containing TBP, Sp1 or CBP expression cassettes, as outlined in the Materials and methods section. At 24 h after transfection, cell extracts were subjected to immunoprecipitation with antibodies against TBP, Sp1 or CBP, and polypeptides were size-fractionated by SDS/PAGE and probed with V5 epitope-specific antibodies by Western blotting. The blots were incubated with anti-V5 primary antibody (InVitrogen), washed and re-probed with horseradish peroxidase-conjugated mouse secondary antibody (InVitrogen). In Figure 4, lane 8, the extract of C2C12 cells transfected with pcDNA3-Msx1 alone shows a 39 kDa polypeptide representing V5-tagged Msx1. C2C12 cells were also transfected with V5 epitope-tagged Msx3 expression vector, pcDNA3-Msx3, a related member of the Msx gene family but with restricted expression in the embryo. The extract of C2C12 cells transfected with pcDNA3-Msx-3 alone shows a 26 kDa polypeptide band of V5 epitope-tagged Msx3 (Figure 4, lane 7). Lanes 5 and 6 represent extracts from C2C12 cells transfected with either pAct-TBP or CMV-Sp1 plasmids respectively; as expected, V5 epotope-specific antibody failed to react with a specific polypeptide product in these extracts. A number of non-specific polypeptide bands, including a 39 kDa polypeptide, were detected in the extracts precipitated with antibodies against TBP and probed with V5-specific antibodies by Western blotting (Figure 4, lanes 1, 3 and 5). In contrast, the extract of cells co-transfected with pcDNA3-Msx3 or pcDNA3-Msx1 and CMV-Sp1 immunoprecipitated with polyclonal antibodies against Sp1 contained V5-tagged Msx1 protein (Figure 4, lane 2) and V5-tagged Msx3 (lane 4). Similarly, in the extracts of C2C12 cells co-transfected with pAct-TBP and pcDNA3-Msx1 or pAct-TBP and pcDNA3-Msx3, and immunoprecipitated with anti-TBP antibody, V5-tagged Msx3 (Figure 4, lane 1) and V5-tagged Msx1 (lane 3) were readily detectable. The IgG from non-immunized serum failed to bind to a detectable polypeptide band, regardless of whether the specificity of binding was tested by immunoprecipitation or Western blotting ([18], and H. Matsui, S. Shetty and R. Raghow, unpublished work).

Finally, the extract of C2C12 cells co-transfected with 0.5 μg each of pcDNA3-Msx1 and pRSV-CBP was immunoprecipitated with polyclonal antibodies against CBP, and subjected to electrophoresis followed by immunoblotting with antibodies against V5 epitope. As shown in Figure 5 (upper panel, lane 3), a 39 kDa band corresponding to V5 epitope-tagged Msx1 was detected. C2C12 cells were also co-transfected with pRSV-CBP and pcDNA3-Msx3. Cell extracts that were immunoprecipitated with polyclonal antibodies against CBP and probed with V5-specific antibody contained a 26 kDa polypeptide representing Msx3 (Figure 5, upper panel, lane 2). The V5-specific antibody did not react with a specific polypeptide band as seen in the

extract from cells transfected with pRSV-CBP alone (Figure 5, upper panel, lane 1). These blots were stripped and re-probed with antibodies against CBP; it is apparent that similar amounts of CBP could be immunoprecipitated from all three cell extracts (Figure 5, lower panel, lanes 1–3). On the basis of these results we believe that Msx1 protein is capable of interacting with at least three key components of the transcriptional apparatus: TBP, Sp1 and CBP/p300. The binding of Msx-1 to TBP has been documented previously [13–15]. Whether the observed interaction(s) of Msx1 protein with transcription factors Sp1 and CBP/p300 are direct remains to be investigated.

DISCUSSION

The results summarized here show that Msx1 promoter, regardless of its length or the presence or absence of Msx1 homeodomain-binding sites, is subject to transcriptional autorepression. Furthermore, the autorepression of Msx1 could be relieved by the exogenous expression of TBP, Sp1 or CBP. Co-transfection of both the long and the minimal Msx1-promoter–luciferase reporter constructs with pAct-TBP boosted the activity of the luciferase reporter. Therefore either (1) the levels of the endogenous TBP are sub-optimal or (2) the graded overexpression of TBP with Msx1 promoter enhances the rates of formation of the 'transcriptosome'. It remains to be seen whether a similar enhancement of actively transcribing endogenous genetic loci also occurs in the TBP-expressing C2C12 cells. The $-165/+106$ bp minimal Msx1 promoter seems to be more sensitive than the -3.5kb/$+106$bp promoter to activation by pAct-TBP. Nevertheless the promoter activity of Msx1 could be totally repressed by overexpression of Msx1. Because the physical interaction of Msx1 and TBP has been experimentally demonstrated previously [13–15], the overexpression of TBP probably sequesters Msx1 protein, interfering with its repressive action on the transcriptosome and RNA polymerase II. One possible mechanism of the autorepression of Msx1 is the interaction of Msx1 with TBP during the formation of the multisubunit transcription complex, preventing other transcription factors from being recruited to the promoter. Such a hypothesis is consistent with the observation that the shorter promoter, containing only E box and Sp1 motifs [17], was more sensitive to activation by TBP and repression by Msx1. Thus one or more factors located further upstream on the promoter also have a role in the activation of the Msx1 promoter by TBP and its interaction with Msx1 protein.

Promoter-dependent autorepression of Msx1 could also be relieved by the overexpression of Sp1 or CBP. As seen in the experiments with TBP, the $-165/+106$ bp minimal Msx1 promoter was also more responsive than the -3.5 kb/$+106$ bp promoter to the actions of Sp1 and CBP. We hypothesize that this difference in sensitivity to Sp1 might be explained by the fact that the -3.5 kb/$+106$ bp Msx1 promoter has three putative Sp1-binding sites, whereas the $-165/+106$ bp promoter fragment has only one [16,17]. Presumably, the occupancy of all three Sp1-binding sites in the -3.5 kb/$+106$ bp promoter modulates promoter activation and autorepression by slightly different mechanisms. Sp1 is known to recruit TBP to TATA-less promoters via a tethering factor [19]. Site-specific mutation or deletion of the proximal Sp1-binding site can completely abrogate Msx1 promoter activity [17]. The precise role of the distal and the middle Sp1 sequence motifs in the murine Msx1 promoter remains undefined. Most probably Sp1 has an indirect role in the relief of the autorepression of Msx1, by recruiting more TBP to the transcription start site and thus facilitating the interactions between promoter-bound TBP and other transcription factors.

Both the long and truncated Msx1 promoters showed only modest enhancement when co-transfected with pRSV-CBP; this enhancement occurred with much greater amounts of pRSV-CBP. Compared with pAct-TBP and pCMV-Sp1, approx. twice the concentration of pRSV-CBP DNA was required to enhance Msx1 promoter activity. Even more significantly, although pRSV-CBP relieved autorepression by Msx1, it was unable to restore promoter activity completely, even when 40-fold greater concentrations of pRSV-CBP were co-transfected ([18], and results not shown).

Finally, a direct interaction between TBP and specific residues in the N-terminal arm of the Msx1 homeodomain has previously been shown [15]. Therefore it seems that the repressor function of Msx1 involves its interaction with general and/or promoter-specific factor(s) involved in the formation of the 'transcriptosome'. Although overexpression of TBP, Sp1 and CBP could relieve the transcriptional autorepression of Msx1, the exact mechanism of the relief of repression has yet to be elucidated. We hypothesize that transcriptional autorepression is a complex process, involving the interaction of transcription factor(s) and cofactors to form a 'repressor' complex. The results of the Western blotting show that Msx1 can be immunoprecipitated along with TBP, Sp1 and CBP, indicating that Msx1 interacts with TBP, Sp1 and CBP; this strengthens our hypothesis about the existence of a 'repressor' complex as an intermediate in the mechanism of transcriptional repression by Msx1.

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