Msx3 protein recruits histone deacetylase to down-regulate the *Msx1* promoter

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Msx1 promoter is known to be repressed by Msx1 protein [Shetty, Takahashi, Matsui, Iyengar and Raghow (1999) Biochem. J. **339**, 751–758]. We show that in the transiently transfected C_2C_{12} myoblasts, co-expression of Msx3 also causes potent repression of *Msx1* promoter that can be relieved by exogenous expression of cAMP-response-element-binding protein-binding protein (CBP) and p300 in a dose-dependent manner. Co-immunoprecipitation and Western blot analyses revealed that Msx3 interacts with CBP and p300 and this interaction significantly decreases the histone acetyltransferase (HAT) activity of both proteins. We also discovered that Msx3-mediated repression of *Msx1* promoter is synergized by the exogenous co-expression of histone deacetylase 1 (HDAC1). Furthermore, the repression of *Msx1* promoter by Msx3 could be relieved by treating transfected cells with trichostatin A, an inhibitor of HDAC(s). Finally, we show that Msx3 and HDAC1 can be co-immunoprecipitated in a complex that does not contain CBP and that Msx3 and HDAC1 proteins are co-localized in the nucleus. Taken together, our results strongly suggest that two distinct multiprotein complexes are present within the nuclei of C_2C_{12} cells: one containing Msx3 and HDAC(s) and another containing Msx3 and CBP and/or p300. On the basis of these results, we propose a dual mechanism of repression by Msx3 protein that involves the squelching of the HAT activity of co-activators, CBP and p300, and recruitment of HDAC(s).

Key words: chromatin remodelling, CREB-binding protein, histone acetyltransferase, histone deacetylase, protein–protein interaction.

INTRODUCTION

The members of *Msx/msh* family of genes have been identified from sponges to vertebrates and three members, *Msx1*, *Msx2* and *Msx3*, exist in the murine genome [1–3]. The highly conserved structural organization of *Msx*-related genes and their expression at sites of inductive epithelial–mesenchymal interactions in the developing embryo suggest that polypeptides encoded by these genes are crucial to organogenesis. *Msx1* and *Msx2*, which share somewhat overlapping patterns of expression during embryogenesis, are expressed abundantly in the cephalic neural crest, first four branchial arches, mandible, maxilla, eye and ear, as well as in the mesenchyme underlying the apical ectodermal ridge of the developing limbs [1,4]. In contrast, the expression of *Msx3* was observed only in the dorsal neural tube and the adjoining areas of the hindbrain [2].

Spontaneous mutations in *Msx*-related genes as well as their experimental mis-expression are known to cause developmental anomalies. *Msx1*-deficient mice die perinatally owing to craniofacial abnormalities, mainly as a result of incompletely developed mandibles and maxillae and missing teeth [5]. Similarly, disruption of *Msx1* or *Msx2* gene expression in the developing embryos by anti-sense oligonucleotides was shown to cause severe axial and craniofacial dysmorphologies [6]. In humans, chromosomal deletion of MSX1 has been implicated in a craniofacial disorder known as 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 *Msx* genes encode homeodomain-containing DNA-binding transcription factors, their mutated counterparts are likely to perturb the normal expression of their downstream genetic targets. Precise identities of the downstream genetic targets regulated by Msx-related proteins and the mechanistic basis of this regulation are far from clear.

Exogenous overexpression of either *Msx1* or *Msx2* is known to regulate the proliferation and apoptosis of cells while simultaneously inhibiting their ability to differentiate [10,11]. A forced expression of *Msx1* in myoblasts could block their differentiation into myotubes [12]; this inhibitory action of Msx1 on myoblasts was apparently mediated by the repression of Myo D enhancer, which contains a genuine Msx1-binding element [13]. More recent observations have revealed that Msx1 and Msx2 could repress transcription even if their target promoters did not possess cognate Msx1 homeodomain-binding sites [14,15]. This type of transcriptional repression is thought to be mediated through protein–protein interactions between the component(s) of the transcription machinery and Msx1 protein. This mechanistic hypothesis was further supported by the demonstration of binding of Msx1 to the TATA-binding protein TBP [16]. In transient expression assays, Msx1 protein was found to be a potent repressor of its own promoter [17,18]. Furthermore, Msx1-mediated autorepression remained unaffected regardless of the presence or absence of the Msx1 recognition motifs on the promoter [18,19]. It was also demonstrated that exogenous co-expression of TBP, Sp1 or cAMP-response-element-binding

Abbreviations used: CBP, cAMP-response-element-binding protein-binding protein; CMV, cytomegalovirus; HA, haemagglutinin; HAT, histone acetyltransferase; HDAC, histone deacetylase; RSV, Rous sarcoma virus; SV40, simian virus 40; TRITC, tetramethylrhodamine β-isothiocyanate; TSA, trichostatin A.

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protein-binding protein (CBP/p300) effectively counteracted the auto-inhibitory activity of Msx1 in transfected C_2C_{12} cells.

Here we report that Msx3, the most recently discovered member of the Msx family, is also a potent repressor of *Msx1* promoter. We show that the repression of *Msx1* promoter induced by Msx3 could be relieved by the exogenous co-expression of CBP or p300. Msx3 interacts with a multiprotein complex containing CBP and/or p300 and this association decreases the histone acetyltransferase (HAT) activity of both co-activators significantly. Additionally, Msx3-mediated deactivation of *Msx1* promoter can be potently enhanced by the exogenous expression of HDAC1; treatment with trichostatin A (TSA), a known inhibitor of histone deacetylases (HDACs) [20–22], can completely reverse this effect. Co-immunoprecipitation and immunofluorescence microscopy of cells transfected with epitope-tagged HDAC1 and Msx3 suggests that the two proteins are in close physical proximity to each other in the nucleus.

MATERIALS AND METHODS

Cell culture

Murine myoblast cell line C_2C_{12}, bought from the American Type Culture Collection, was cultured and maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) FBS (fetal bovine serum) at 37 °C. Cells were subcultured, before they became confluent, every 48 h.

Plasmid vectors

The *Msx1* promoter–luciferase constructs used in these experiments were made by cloning Msx1 genomic DNA fragment(s) [19] upstream of the luciferase reporter in the pGL2Basic vector, as described previously [17,18]. V5 epitope-tagged Msx3 expression construct, pcDNA3.1-Msx3, was made by cloning PCR-amplified Msx3 cDNA, corresponding to nucleotide residues 96–708 [3]. Msx3 cDNA terminated with *Kpn*I (5′) and *Xba*I (3′) sites were cloned into pcDNA3.1 vector (Invitrogen Corporation, Carlsbad, CA, U.S.A.) linearized with *Kpn*I and *Xba*I enzymes. The CBP expression plasmid, pRSV-CBP (in which RSV stands for Rous sarcoma virus), was provided by Dr Roland Kwok (Vollum Institute, Portland, OR, U.S.A.). The p300 expression vector pCMV β-p300 (in which CMV stands for cytomegalovirus) was a gift from Dr David Livingston (Harvard Medical School, Boston, MA, U.S.A.). HDAC1 expression vector pcDNA 3-HDAC1-F was generously provided by Dr Tony Kouzarides (Wellcome/CRC Institute, Cambridge, U.K.) and pCMV2N3T HDAC1-HA (in which HA stands for haemagglutinin) was a gift from Dr Annick Harel-Bellan (CNRS, Villejuif, France).

Transient transfection and luciferase assay

The methods used for the transfection of C_2C_{12} cells and measurement of transiently expressed luciferase in the extracts of transfected cells have been described previously [17,18]. Briefly, cells (10^5 per well) were seeded in six-well (35 mm) culture dishes 1 day before transfection. Transfections were performed in triplicate, with LIPOFECTAMINE reagent (Gibco–BRL, Life Technologies, Gaithersburg, MD, U.S.A.). Cells were incubated with the DNA–liposome complexes in serum-free Optimem medium (Gibco–BRL) for 5 h, after which the medium was replaced with DMEM supplemented with 10% (v/v) FBS. Transfected C_2C_{12} cells were incubated for 20 h in DMEM containing 10% (v/v) FBS, rinsed in PBS and lysed in 150 μl of cell culture lysis reagent (Promega, Madison, WI, U.S.A.). Lysate (20 μl) was mixed with 100 μl of 470 mM luciferin substrate (Promega) and the light intensity was measured in a TD20e luminometer (Turner Designs, Madison, WI, U.S.A.). The protein content of the lysate was measured by Bio-Rad protein assay system based on the Bradford method [23]. The luciferase activity was expressed as arbitrary light units/μg of total protein. As outlined previously [17,18], the transfection efficiencies were determined by co-transfection of cells with pRSV-lacZ and quantification of β-galactosidase activity in all cell extracts.

To study the effect of exogenous co-expression of Msx3, HDAC1, CBP or p300 on the activity of $-165/+106$ bp *Msx1* promoter–luciferase construct, cells were co-transfected with combinations of vectors as described for individual experiments. The amount of total DNA was kept constant at 1.5 μg per well by adding pGL2 Basic DNA. To determine whether the deacetylation of histones led to altered promoter activity, we treated transfected C_2C_{12} cells with TSA, an inhibitor of HDAC (Sigma, St Louis, MO, U.S.A.), for 6, 12 or 24 h. Stock solution of TSA was prepared in ethanol and added to the culture medium at concentrations of 25, 50 or 100 ng/ml.

Co-immunoprecipitation and Western blotting

Cytoplasmic extracts to be processed for immunoprecipitation and Western blotting were prepared from C_2C_{12} cells as described previously, with minor modification [18]. At 24 h after transfection, C_2C_{12} cells were rinsed twice in ice-cold PBS. Extracts were prepared by suspending the C_2C_{12} cells on ice in lysis buffer [10% (v/v) glycerol/1% (v/v) Triton X-100/1 mM EDTA in Tris/HCl buffer (consisting of 20 mM Tris/HCl, pH 8.0, and 150 mM NaCl)] supplemented with the cocktail of protease inhibitors (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). The lysate was incubated on ice for 20 min with occasional mixing, vortex-mixed for 30 s, incubated for an additional 20 min on ice and then centrifuged in microcentrifuge tubes at 15800 ***g*** (14000 rev./min) at 4 °C for 20 min. The supernatant was transferred to a fresh tube and protein content was estimated with the use of Bio-Rad DC reagent for protein assay. Cell extracts were frozen at −80 °C until needed.

For Western blotting, equal amounts of whole cell extract proteins were boiled for 5 min in Laemmli electrophoresis sample buffer containing 10% (v/v) 2-mercaptoethanol; samples were subjected to SDS/PAGE [4–15% (w/v) gradient mini-gels] (Bio-Rad) at 180 V. To deduce the molecular mass of the polypeptides, known molecular mass markers were also run in a separate lane beside the polypeptides of interest. The size-fractionated polypeptides from total cell extracts were blotted on Immobilon P membranes (Millipore Corporation, Bedford, MA, U.S.A.). The non-specific protein-binding sites on the membranes were blocked by incubation for 1 h in Blotto [5% (w/v) dried milk in TBST, which consisted of TBS containing 0.1% (v/v) Tween 20] at room temperature, with gentle shaking. Finally, the membranes were washed three times (5 min each) in TBST and incubated overnight at 4 °C with gentle mixing in TBST containing appropriately diluted primary antibodies against p300, HDAC1 or V5 epitope as indicated in the individual experiments. The blots were washed three times in TBST and then incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 1 h at room temperature. The immunoblots were then rinsed twice in TBST followed by three more washes of 10 min each in TBST. The polypeptide bands specifically reacting with antibodies were detected by chemiluminescence with Pierce Super Signal Substrate (Pierce, Rockford, IL, U.S.A.) in accordance with the manufacturer's protocol. For re-probing of the membrane with another antibody, the membrane was stripped at 50 °C for

30 min in stripping buffer [62.5 mM Tris/HCl (pH 6.7)/2 % (w/v) SDS, with freshly added 100 mM 2-mercaptoethanol]. The membrane was washed three times in TBST and then blocked and processed in the same way as described above.

For immunoprecipitation, 150 μg of the whole cell extract prepared as described above was incubated with 10 μl of the primary anti-p300 or anti-HDAC1 antibodies for 1 h on ice. Protein A/G (20 μl) plus agarose beads (Santa Cruz) were then added to this suspension and incubated overnight at 4 °C with gentle mixing. Agarose beads were pelleted (15800 ***g*** for 1 min at 4 °C) and washed four times with lysis buffer containing the cocktail of protease inhibitors. The beads were finally suspended in 35 μl of Laemmli sample buffer containing 10 % (v/v) 2-mercaptoethanol, then boiled for 5 min and centrifuged briefly. The solubilized proteins in the supernatant were subjected to SDS/PAGE [4–15 % (w/v) gradient gel], blotted to Immobilon P membranes and immunodetected by Western blotting as described above.

The antibody directed against the V5 epitope tag (catalogue no. R 961-25) was purchased from Invitrogen Corporation. Antibodies recognizing p300 (catalogue no. sc-584), CBP (catalogue no. sc-369) and HDAC1 (catalogue no. sc-6298) were purchased from Santa Cruz Biotechnology. All antibodies were diluted in TBST containing blocking buffer just before use. Optimal concentrations were determined empirically for each antibody.

HAT assay

C_2C_{12} cells were transfected with either expression vector pCDNA-3.1 Msx3 or pGL2 Basic control plasmid. The whole cell extracts were prepared in RIPA buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 % (v/v) Nonidet P40/0.5 % sodium deoxycholate/0.1 % SDS/1 mM EDTA, with a freshly added cocktail of protease inhibitors] from 10^7 cells. Cell extracts were incubated with 20 μl of anti-CBP or anti-p300 antibodies and 40 μl of Protein A/G plus agarose beads and immunoprecipitated as outlined above. Immunoprecipitation was also done with normal rabbit IgG to detect non-specific binding and background HAT activity. The efficiency of immunoprecipitation by antibodies against CBP or p300 was determined by subjecting the immunoprecipitates from extracts from equal numbers of control (pGL2Basic) or Msx3-transfected cells to Western blot analysis; the results from the HAT assays were normalized against the amounts of immunoprecipitated proteins. The HAT assay was performed essentially as described previously [24]. Immunoprecipitated proteins bound to the A/G plus agarose beads were rinsed twice in RIPA buffer followed by three more rinses in HAT buffer [50 mM Tris/HCl (pH 7.5)/1 mM EDTA containing protease inhibitor cocktail]. Finally, the beads were suspended in 21 μl of HAT buffer, 600 nCi of [^{14}C]acetyl-CoA (63 mCi/mmol; ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.); a synthetic biotinylated histone H4 peptide (90 μM final concentration; Chiron Technology, Melbourne, Victoria, Australia) was added to the suspension. The reaction mixture was incubated at 30 °C for 70 min, the reaction was stopped by centrifugation at 15800 ***g*** for 1 min and the supernatant was collected. HAT buffer (500 μl) and 20 μl of prewashed streptavidin–agarose beads (Sigma) were added to the supernatant and the suspension mixture was incubated at 4 °C for 45 min on a rotating wheel. The beads were washed twice with RIPA buffer and suspended in 5 ml of scintillation liquid (Hionic, Packard) and counted using a liquid-scintillation spectrometer (Packard Instruments Co., Downers Grove, IL, U.S.A.).

Immunofluorescence staining

C_2C_{12} cells were cultured on two-well LabTek II chamber slides (Nunc, Naperville, IL, U.S.A.) and co-transfected with V5 epitope-tagged mammalian cell expression vector pCDNA 3.1 Msx3 and HA-tagged pCMV HDAC1. At 20 h after transfection, cells were washed twice in PBS, fixed in 4 % (w/v) paraformaldehyde for 20 min, washed three times with PBS, permeabilized with 0.3 % (v/v) Triton X-100 and quenched in 50 mM NH_4Cl for 30 min. The slides were then incubated in blocking buffer [1 % (w/v) BSA in PBST/0.1 % (v/v) Triton X-100 in PBS, with a freshly added cocktail of protease inhibitors] for 2 h. The cells were then incubated overnight in mouse monoclonal anti-V5 antibodies (catalogue no. R 961-25; Invitrogen) and rabbit polyclonal anti-HA antibodies (catalogue no. sc-805; Santa Cruz Biotechnology). Next morning, the slides were washed five times in PBST, double-labelled with tetramethylrhodamine β-isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (catalogue no. 115-026-072) and FITC-conjugated goat anti-rabbit IgG (catalogue no. 111-096-047) for 1 h. The fluorescence-labelled secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc, West Grove, PA, U.S.A. All the antibodies were diluted in blocking buffer containing a cocktail of protease inhibitors. The slides were washed three times in PBST, incubated in 4,6-diamidino-2-phenylindole (Sigma) at a final concentration of 0.1 μg/ml for 10 min and then washed twice in PBS. Finally, the slides were mounted in Prolong™ antifade kit mounting medium (catalogue no. p-7481; Molecular Probes, Eugene, OR, U.S.A.). The cells were then examined with an Olympus confocal laser-scanning microscope and the images were collected at $\times$ 100 magnification with appropriate filters on a Bio-Rad MRC 1024 scanning system.

RESULTS

Msx3 is a potent repressor of *Msx1* promoter

Msx1, *Msx2* and *Msx3* belong to the *Msx* family of genes that encode homeobox-containing transcription factors. Because

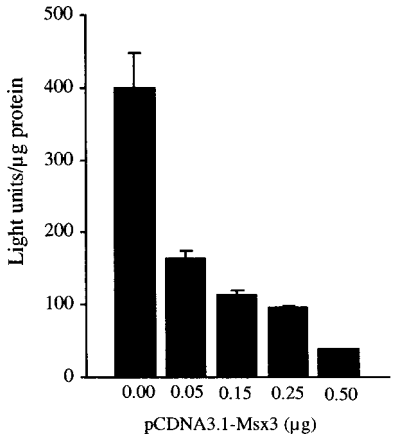

Figure 1 Graded co-expression of Msx3 represses activity of the *Msx1* promoter

C_2C_{12} cells were transfected with fixed amount of *Msx1*-promoter–luciferase DNA (0.5 μg) and various amounts of pcDNA3.1-Msx3. Transfections were performed in triplicate and the final amount of DNA was kept constant at 1.5 μg by the addition of pGL2 Basic DNA. After 24 h the luciferase activity in the cell extract was quantified as light units/μg of total protein; results are shown as means $\pm$ S.E.M.

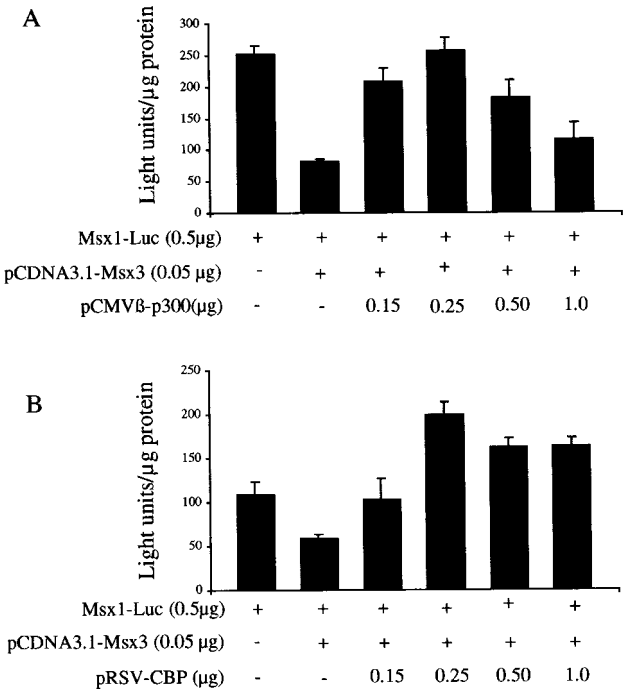

Figure 2 Msx3-induced repression can be relieved by exogenous expression of p300 or CBP

C_2C_{12} cells were transfected in triplicate with 0.5 μg of $-106/+165$ bp *Msx1*-promoter–luciferase plasmid alone or co-transfected with 0.05 μg of pCDNA3.1-Msx3 and an incrementally increasing amount of pCMVβ-p300 plasmid (0–1.0 μg) (**A**), or 0.5 μg of $-106/+165$ bp *Msx1*-promoter–luciferase plasmid alone or 0.05 μg of pCDNA3.1-Msx3 and an incrementally increasing amount of pRSV-CBP plasmid (0–1.0 μg) as indicated (**B**). The final amount of DNA was kept constant at 1.5 μg per well by the addition of pGL2 Basic DNA. Results are shown as light units/μg of total protein and are means $\pm$ S.E.M.

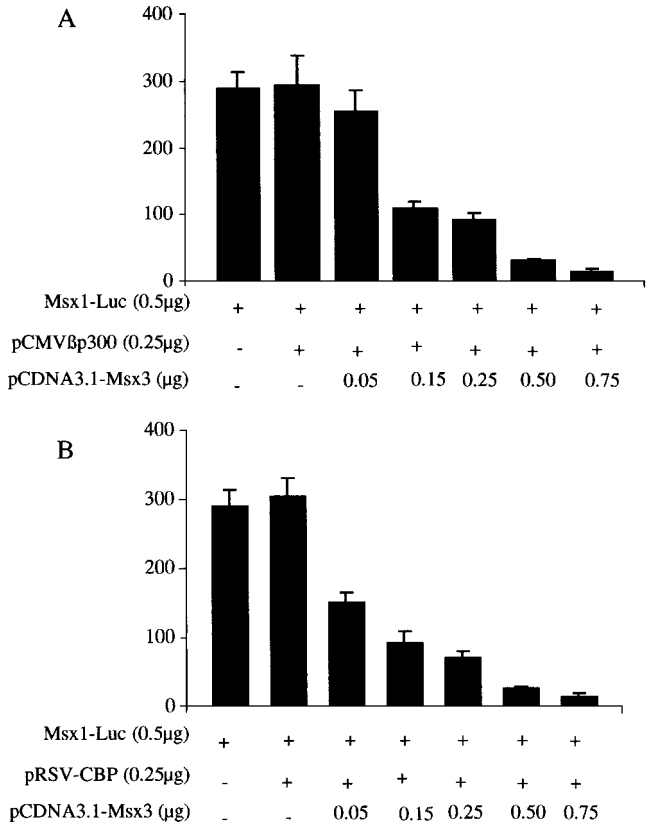

Figure 3 *Msx1* promoter can be repressed by graded co-expression of Msx3 in the presence of either p300 or CBP

C_2C_{12} cells were transfected with 0.5 μg of *Msx1*-promoter–luciferase alone or co-transfected with 0.25 μg of p300 expression vector pCMVβp300 and graded amounts of pCDNA3.1 Msx3 (0–0.75 μg) (**A**), or 0.5 μg of *Msx1*-promoter–luciferase alone or co-transfected with 0.25 μg of CBP expression vector pRSVCBP and graded amounts of pCDNA3.1 Msx3 (0-0.75 μg) as shown (**B**). The final amount of DNA was kept constant at 1.5 μg of DNA per well by the addition of pGL2 Basic DNA. Transfections were performed in triplicate and the luciferase activity is expressed as averaged light units/μg of total protein; results are means $\pm$ S.E.M.

Msx1 has been reported to be a potent repressor of its own promoter activity [18], we were interested in determining whether Msx3 could similarly regulate *Msx1* promoter activity. We therefore co-transfected C_2C_{12} cells with 0.5 μg of the $-165/+106$ bp *Msx1* promoter–luciferase construct and various amounts of Msx3 expression vector, pCDNA3.1-Msx3. We observed that exogenous co-expression of Msx3 repressed the *Msx1* promoter. As shown in Figure 1, as the amount of Msx3 expression vector was increased from 0.05 to 0.5 μg in the transfection mixture, the luciferase activity driven by *Msx1* promoter also decreased 2.5–10-fold. Msx3-mediated repression of *Msx1* promoter was specific because simian virus 40 (SV40) promoter–enhancer was not inhibited under identical conditions (results not shown). The amount of Msx3 expression vector pcDNA3.1-Msx3 was chosen as 0.05 μg for subsequent co-transfection experiments; at this concentration, 2–3-fold repression was observed consistently.

Repression mediated by Msx3 can be relieved by exogenous co-expression of CBP or p300

In the next set of experiments the C_2C_{12} cells were co-transfected with 0.5 μg of $-165/+106$ bp *Msx1* promoter–luciferase construct, 0.05 μg of pcDNA3.1-Msx3 and increasing amounts (0.05–1.0 μg) of either pCMV β-p300 (Figure 2A) or pRSV-CBP (Figure 2B). As expected, exogenous co-expression of Msx3 repressed the *Msx1*-promoter-driven luciferase activity approx. 2-fold and increasing the amounts of pCMVβ-p300 DNA in the transfection mixture relieved the Msx3-mediated repression completely (Figure 2A). Transfection of C_2C_{12} cells with 0.25 μg of pCMVβ-p300 DNA not only reversed Msx3-mediated repression but also enhanced the reporter gene expression above the control (i.e. cells transfected with $-165/+106$ bp *Msx1*–luciferase promoter construct alone). However, there was a decline in the luciferase activity with any further increase in the amount of p300. Similar results were observed when the cells were co-transfected with 0.5 μg of the *Msx1* promoter–luciferase construct, 0.05 μg of Msx3 expression vector pCDNA3.1-Msx3 and incrementally increasing amounts of CBP expression vector pRSV-CBP in the transfection mixture (Figure 2B). We believe that overexpressing p300 and CBP [18] might be toxic to cells.

In the reverse experiment, C_2C_{12} cells were co-transfected with 0.5 μg of *Msx1* promoter–luciferase construct, 0.25 μg of p300 or CBP expression DNA and with increasing amounts (0.05–0.75 μg) of vector pCDNA 3.1-Msx3. A modest enhancement of the promoter activity was observed when p300 expression vector was added alone (Figure 3A). A similar effect was observed with CBP expression vector (Figure 3B). As reported previously [18], we consider that these co-activators might be present in a limiting amount in C_2C_{12} cells. However, the *Msx1*

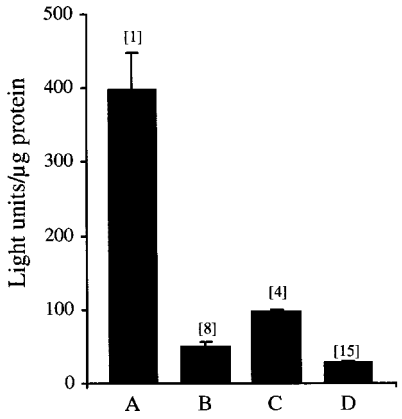

Figure 4 Co-expression of HDAC1 has a potent additive effect on Msx3-induced repression of the *Msx1* promoter

C_2C_{12} cells were transfected with 0.5 μg of *Msx1*-promoter–luciferase alone (column A) or co-transfected with 0.5 μg of *Msx1* promoter and 0.25 μg of pcDNA3-HDAC1 (column B), 0.5 μg of *Msx1* promoter and 0.25 μg of pcDNA3.1-Msx3 (column C), or 0.5 μg of *Msx1* promoter and 0.25 μg each of pcDNA3.1-Msx3 and pcDNA3-HDAC1 (column D) as shown. The final amount of DNA was kept constant at 1.5 μg by the addition of pGL2Basic DNA. Transfections were performed in triplicate and the luciferase activities are expressed as light units/μg of total protein; results are means $\pm$ S.E.M. Fold repressions are shown in square brackets.

promoter–luciferase construct could still be repressed in a dose-dependent manner by Msx3 expression vector pCDNA3.1-Msx3 in cells co-expressing either p300 or CBP.

Repression of $-165/+106$ bp *Msx1* promoter–luciferase by Msx3 can be enhanced by exogenous co-expression of HDAC1

HDACs are known to be associated with transcriptional repression and catalyse chromatin condensation. To test whether Msx3-induced repression was associated with HDAC activity, we co-transfected C_2C_{12} cells with 0.5 μg of $-165/+106$ bp *Msx1* promoter–luciferase, 0.25 μg of Msx3 expression vector pCDNA3.1-Msx3 and 0.25 μg of HDAC1 expression vector pCDNA 3-HDAC1-F. Exogenous expression of Msx3 resulted in approx. 4-fold repression of the *Msx1* promoter–luciferase activity. Exogenous expression of HDAC1 also repressed the minimal *Msx1*-promoter-driven luciferase expression, suggesting that HDACs probably exert a global repressive action. However, such global repression notwithstanding, co-expression of HDAC1 and Msx3 suppressed *Msx1* promoter even more profoundly than either of these effectors alone; a 15–20-fold repression could be seen consistently (Figure 4).

Relief of Msx3-mediated repression by TSA

Because the repression of Msx1–luciferase activity by Msx3 was further enhanced by coexpression of HDAC1, we surmised that histone deacetylation was associated with the transcriptional repression induced by Msx3. To corroborate this hypothesis, we treated transfected C_2C_{12} cells with TSA, to inhibit HDAC. The C_2C_{12} cells were co-transfected with $-165/+106$ bp Msx1–luciferase DNA (0.5 μg) and Msx3 expression vector pcDNA3.1-Msx3 (0.05 μg). At 12 h after transfection, these cells were incubated in culture medium containing various concentrations of TSA (25, 50 or 100 ng/ml) for an additional 6, 12 or 24 h. The cells were then harvested and assayed for luciferase activity. TSA treatment exerted a generalized effect on *Msx1* promoter (Table 1). A similar enhancement of SV40-promoter-driven luciferase activity was also seen (results not shown). As shown in Table 1, the TSA treatment of C_2C_{12} cells abrogated Msx3-mediated transcriptional repression at all TSA concentrations. We did not see any effect of TSA at 6 h of treatment (results not shown). However, when the cells were treated with TSA for 12 h the repressive action of Msx3 was no longer observed. When the cells were treated for 24 h, the relief of repression was more pronounced; however, this was accompanied by greater cell toxicity (results not shown).

Msx3 associates with multiprotein complexes containing either CBP and/or p300, or HDAC1

Because repression induced by Msx3 could be relieved by co-transfection of C_2C_{12} cells with vectors designed to express either CBP or p300, we were interested in studying whether there was a physical interaction between Msx3 and CBP and/or p300. We have demonstrated previously that both Msx1 and Msx3 proteins could be readily detected in the immunoprecipitates obtained with anti-CBP antibody [18]. When C_2C_{12} extracts, prepared from cells co-transfected with pcDNA3.1-Msx3 and pRSV-CBP, were immunoprecipitated with polyclonal antibodies against CBP, a V5 epitope-tagged Msx3 polypeptide could be seen in this complex by Western blot analysis. Here we tested experimentally whether Msx3 was similarly associated with p300 or HDAC1. Whole cell extracts from C_2C_{12} cells transfected with pCMVβ-p300 and pcDNA3.1-Msx3 were immunoprecipitated with polyclonal anti-p300 antibodies and immunoprecipitated polypeptides were subjected to electrophoresis on 4–15% (w/v) gradient gels. As shown in Figure 5 (lane 3), a 26 kDa polypeptide representing V5-tagged Msx3 polypeptide was readily detectable in these immunoprecipitates. A similar but much more intense signal was detected in cell extracts prepared from C_2C_{12} cells transfected with pcDNA3.1-V5 tagged-Msx3 alone and immunoprecipitated with polyclonal anti-p300 antibodies (Figure 5, lane 2). As expected, the V5-tagged Msx3 polypeptide band was not

Table 1 Relief of Msx3-induced repression of Msx1 promoter by TSA

C_2C_{12} cells were transfected with 0.5 μg of $-165/+106$ bp Msx1-promoter–luciferase (Luc) DNA alone or co-transfected with 0.05 μg of pcDNA3.1-Msx3. At 12 h after transfection, cells were treated with different concentrations of TSA as indicated. Each treatment was performed in triplicate. Cells were harvested after 12 h of TSA treatment and luciferase activity was quantified. The luciferase activity is expressed as light units per μg of total protein; results are means $\pm$ S.E.M.

		Luciferase activity (light units/μg)			
Transfection with	TSA (ng/ml)...	0	25	50	100
Msx1-promoter–Luc		197 $\pm$ 27	285 $\pm$ 4	270 $\pm$ 15	225 $\pm$ 20
Msx1-promoter–Luc + pCDNA3.1-Msx3		84 $\pm$ 7	202 $\pm$ 9	204 $\pm$ 21	167 $\pm$ 14

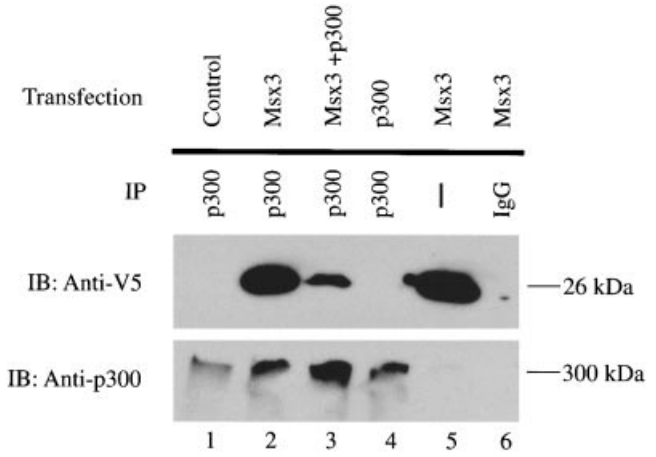

Figure 5 Msx3 is co-localized in the immunoprecipitates obtained with anti-p300 antibodies

C_2C_{12} cells were transfected with pcDNA3.1-Msx3 and/or pCMVβ-p300 as indicated. Whole cell extracts (150 μg) from transfected cells were immunoprecipitated (IP) with polyclonal anti-p300 antibodies (lanes 1–4) or with non-specific IgG (lane 6). Immunoprecipitated polypeptides were analysed sequentially by Western blotting (IB) with anti-V5 (upper panel) or anti-p300 (lower panel) antibodies. Anti-p300 antibody co-immunoprecipitated V5 epitope-tagged Msx3 (lanes 2 and 3), whereas normal IgG did not precipitate either p300 or Msx3 (lane 6). Anti-V5-specific signal was also absent from the immunoprecipitates obtained from untransfected control cells (lane 1). Lane 5 represents the V5-epitope tagged Msx3 polypeptide that could be readily seen in the lysates of C_2C_{12} cells transfected with pcDNA3.1-Msx3, without prior immunoprecipitation.

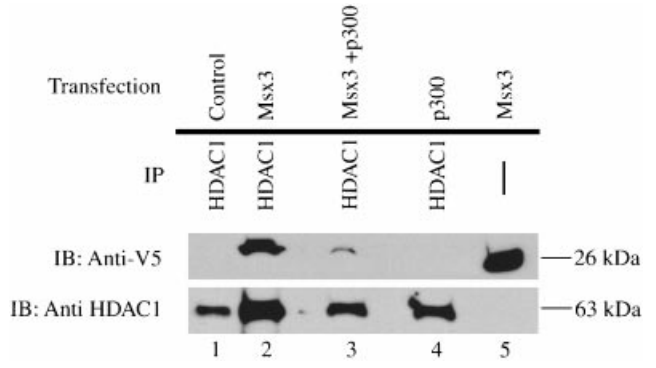

Figure 6 Msx3 interacts physically with HDAC1

C_2C_{12} cells were transiently transfected with pcDNA3.1-Msx3 and/or pCMVβ-p300 as indicated. Whole cell extracts (150 μg) from transfected cells were immunoprecipitated (IP) with polyclonal anti-HDAC1 antibodies and the immunoprecipitates were subjected to Western blot analyses (IB) with anti-V5 (upper panel) and anti-HDAC1 (lower panel) antibodies. Immunoprecipitation with anti-HDAC1 antibody co-immunoprecipitated V5 epitope-tagged Msx3 (lanes 2 and 3). No anti-V5 signal was detected in co-immunoprecipitates from untransfected control cells (lane 1) or cells transfected with only p300 expression vector (lane 4). Lane 5 represents the V5 epitope-tagged Msx3 polypeptide from lysates of Msx3-transfected cells, without prior immunoprecipitation.

observed in the extracts prepared from untransfected control cells (Figure 5, lane 1) or cells transfected with pCMVβ-p300 alone (Figure 5, lane 4). Similarly, no V5-tagged Msx3-specific signal was detected from cell extracts subjected to immunoprecipitation with preimmune rabbit IgG (Figure 5, lane 6). We stripped this blot and re-probed it with anti-p300 antibodies and detected the presence of p300 in both untransfected and transfected C_2C_{12} cells. V5-tagged Msx3 protein was also readily

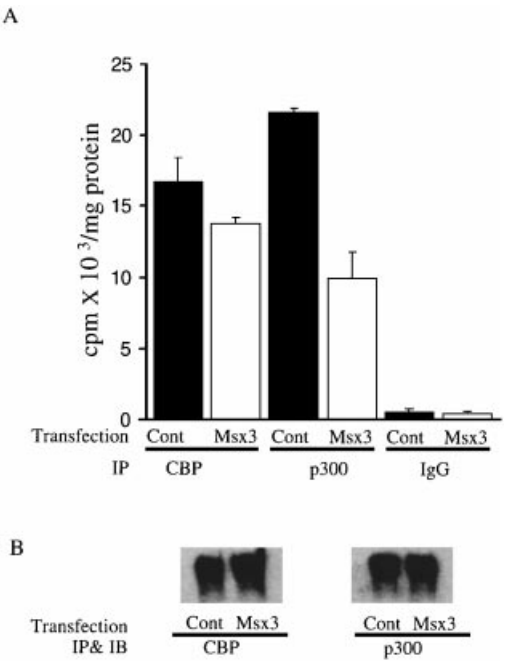

Figure 7 Physical interaction of Msx3 with co-activators CBP and/or p300 reduces their intrinsic HAT activity

(**A**) C_2C_{12} cells were transfected with either unrelated pGL2Basic (control; Cont) or pcDNA3.1-Msx3 plasmids as indicated. The whole cell extracts from transfected cells were immunoprecipitated (IP) with either polyclonal anti-CBP antibodies, anti-p300 antibodies or normal IgG. The beads were washed twice in RIPA buffer and three times with HAT buffer. These immunoprecipitates were then used in HAT assays. The results are expressed as 10^3 c.p.m./mg of total protein. The results are means $\pm$ S.E.M. for six individual HAT assays. (**B**) The amount of protein immunoprecipitated with anti-CBP or anti-p300 antibodies from control and Msx3-transfected cells. Abbreviation: IB, immunoblot.

detected in the whole cell extracts prepared from Msx3-transfected cells without prior immunoprecipitation; such extracts were included as a positive control (Figure 5, lane 5).

The blots containing proteins immunoprecipitated with anti-p300 antibodies were stripped for a second time and reprobed with anti-HDAC1 antibodies. However, we failed to detect any signal. Because repeated stripping and probing of membranes might have caused a loss of signal for HDAC1, we assessed the immunoreactivity of p300 and HDAC1 after two to four strip–probe cycles. Both proteins could be readily detected on control blots after three sequential strippings (results not shown). Therefore a complete lack of HDAC1 signal in the immunoprecipitates of p300 does not reflect an artifact of loss of the protein from immunoblots. These results indicate that Msx3 protein is found in one or more multiprotein complexes that contain p300/CBP and are devoid of HDAC1. This observation is important in light of the results showing that co-transfection of C_2C_{12} cells with V5-tagged Msx3 and pcDNA 3-HDAC1 consistently and significantly enhanced the inhibitory effect of Msx3; furthermore, treatment of transfected C_2C_{12} cells with TSA relieved the Msx3-induced repression. Therefore our results show that Msx3 protein could also associate with and recruit HDAC1.

To test the physical association of Msx3 and HDAC1, whole cell extracts prepared from C_2C_{12} cells transfected with Msx3 and p300 expression vectors were immunoprecipitated with polyclonal anti-HDAC1 antibodies. When the Immobilon P membranes containing immunoprecipitates from C_2C_{12} cells transfected with pcDNA3.1-Msx3 or co-transfected with pcDNA3.1-

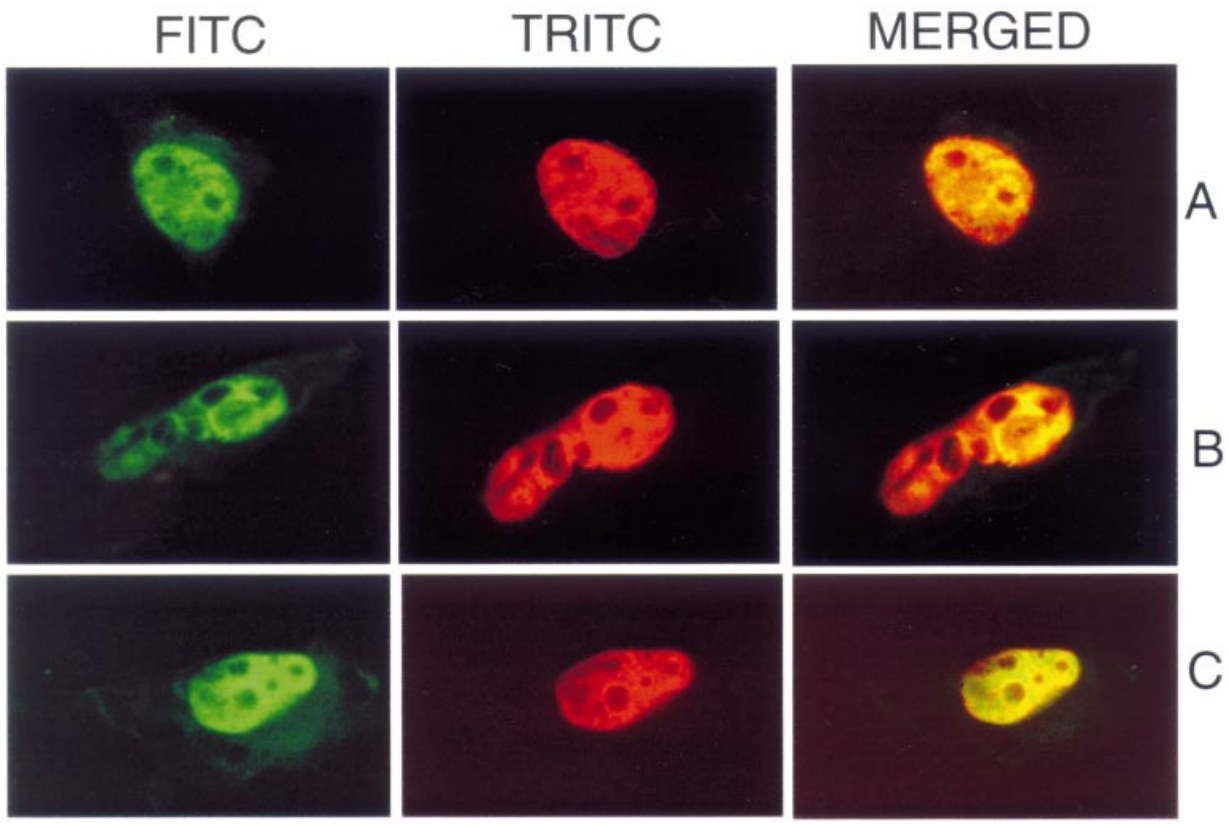

Figure 8 Msx3 co-localizes with HDAC1

C_2C_{12} cells were cultured in slide chambers and co-transfected with V5-tagged Msx3 and HA-tagged HDAC1. Cells were fixed and then reacted with mouse monoclonal anti-V5 and rabbit polyclonal anti-HA primary antibodies. Cells were then incubated with TRITC-conjugated anti-mouse and FITC-conjugated anti-rabbit secondary antibodies and the binding of secondary antibodies was detected by confocal microscopy. The left panels show HDAC1 labelled with green (FITC) and the middle panels Msx3 labelled with red (TRITC). The right panels show merged images to detect co-localization of the two proteins as yellow. (**A**), (**B**) and (**C**) show three representative cells selected from a large population of doubly transfected cells studied for immunofluorescence staining.

Msx3 and pCMVβ-p300 were probed with anti-V5 antibodies, a 26 kDa V5 epitope-tagged Msx3 protein was readily detected (Figure 6). Interestingly, whenever cells were co-transfected with p300 expression vector along with Msx3, the level of immunoprecipitable Msx3 was decreased significantly (Figure 6, lane 3). This difference was seen consistently in several experiments and is unlikely to have been caused by potential variability in the efficiency of immunoprecipitation. We propose that competition between HDAC1 and p300 for Msx3 causes the apparent shift in the amount of immunoprecipitable Msx3 from transfected cells. As expected, no V5 epitope-specific signal was detected when untransfected C_2C_{12} cell extracts were used for immunoprecipitation (Figure 6, lane 1). As another negative control, we used extracts prepared from cells transfected with pCMVβ-p300 alone, for immunoprecipitation; as expected, no signal for V5-epitope was detected (Figure 6, lane 4). We stripped these blots as outlined above and re-probed them with anti-HDAC1 antibodies; a 63 kDa polypeptide band corresponding to HDAC1 was consistently detected in all samples (Figure 6). The whole cell extract prepared from pcDNA 3.1-V5-tagged Msx3-transfected cells, without prior immunoprecipitation, was run as a positive control (Figure 6, lane 5). When these membranes were stripped for a second time and blotted with anti-p300 antibodies, no detectable p300 polypeptide band was seen. These results, along with the experimental analyses outlined above, show that C_2C_{12} cells contain two unique complexes, one enriched in transcriptional co-activators CBP and/or p300 and Msx3 and the other containing HDAC(s) and Msx3.

Association of Msx3 with complex containing CBP and p300 inhibits their intrinsic HAT activity

Because Msx3 interacts with CBP and p300 and these proteins are HATs, we wished to know whether binding of Msx3 affected the HAT activity of CBP and or p300. For this experiment, cell extracts prepared from C_2C_{12} cells transfected with either pGL2Basic (control plasmid) or pcDNA3.1-Msx3 were immunoprecipitated with either polyclonal anti-CBP or anti-p300 antibodies. As an additional control, the same amount of protein (1 mg) was immunoprecipitated with non-specific rabbit IgG and a HAT assay was performed (Figure 7A). The average HAT activity from six independent assays performed on the immunoprecipitated proteins is illustrated in Figure 7(A). As expected, the normal rabbit IgG did not bring down detectable HAT activity from C_2C_{12} cells transfected with either pGL2Basic or pcDNA3.1-Msx3 DNA; untransfected C_2C_{12} cells similarly immunoprecipitated with non-specific IgG were also negative (results not shown). In contrast, HAT activity was readily assayed in immunoprecipitates obtained with anti-CBP or anti-p300 antibodies from C_2C_{12} cells, regardless of whether cells were transfected with pGL2Basic or pcDNA3.1-Msx3 DNA (Figure

7A). HAT activity in the immunoprecipitates obtained from transfected C_2C_{12} cells with epitope-tagged Msx3 was significantly decreased. Complexes containing Msx3 and p300 showed a 50% decrease, and those containing Msx3 and CBP a 20% decrease, in HAT activity. Thus the association of Msx3 differentially affects the HAT activity of CBP and p300. To determine the immunoprecipitation efficiency we performed a Western blot analysis of the immunoprecipitable protein from C_2C_{12} cells transfected with expression vectors, using anti-CBP or anti-p300 antibodies. The amounts of CBP and p300 immunoprecipitated from Msx3 and pGL2 basic transfected cells were similar (Figure 7B).

Immunofluorescence staining of C_2C_{12} cells

To obtain further physical evidence for the association of Msx3 with HDAC(s) we examined C_2C_{12} cells, co-transfected with epitope-tagged Msx3 and HDAC1 expression vectors, by immunofluorescence microscopy. The V5-tagged Msx3 protein was stained red and the HA-tagged HDAC1 protein was stained green; both Msx3 and HDAC1 were confined to the nucleus. The two images were merged to study co-localization of these proteins in the nucleus. Figures 8(A)–8(C) show three different co-transfected cells. These panels show three commonly observed patterns of co-distribution of the two proteins in the co-transfected cells. There were several cells that were transfected only with HA-tagged HDAC1 or V5-tagged Msx3 because they showed either green or red fluorescent labels alone (results not shown). The subpopulation of C_2C_{12} cells that was co-transfected with both vectors showed green and red fluorescence (Figure 8). The merged images clearly show physical co-localization as a yellow colour. In some cells the distribution of both proteins was diffuse (Figures 8A and 8B). In other cells the Msx3 protein was more distinctly associated with the nuclear and the nucleolar membrane, whereas the distribution of HDAC1 was diffuse and these cells showed distinct regions of co-localization (Figure 8C). At present we do not fully understand the reason for these differences. Probably the differences observed in the intensity of the signal could be attributed to the differences in the efficiency of transfection and therefore the amount of the protein present, whereas the differences in the pattern of nuclear distribution could probably be attributed to the stage of the cell cycle.

DISCUSSION

The regulation of eukaryotic gene expression is intimately linked to the packaging of DNA into chromatin, the structural unit of which is a nucleosome. Presumably the condensation and decondensation of chromatin allow DNA to gain reversible access to the regulatory proteins recruited to the promoter and assembled into a 'transcriptosome' [25,26]. This dynamic structural reconfiguration of chromatin is performed by specific chromatin-remodelling proteins. At least two highly conserved and potentially redundant chromatin-remodelling systems have been described. The first of these is represented by NURD, SWI/SNF and RSC complexes in yeast, NURF, CHARF, ACF and Brahma in *Drosophila*, and SWI/SNF complexes in humans [26–29]. The other includes factors that catalyse the acetylation and deacetylation of core histones [30–33]. Several recent studies strongly suggest that HAT and deacetylase activities are required for transcriptional regulation [30,34–36]. It has been documented that transcriptional co-activators have intrinsic HAT activity [37,38] and that targeting HAT complexes to nucleosomes within the vicinity of adenovirus E4 promoter results in transcriptional activation [39]. In contrast, co-repressors of transcription are associated with HDAC activity, and the targeting of Sin3-HDAC/Rpd3 complexes to promoter results in transcriptional repression [29,30,40,41].

We show here that Msx3 protein is a potent repressor of *Msx1* promoter activity and that the Msx3-mediated repression can be reversed completely by the co-expression of CBP or p300 in a dose-dependent manner. We have reported previously that the exogenous expression of CBP also relieves the auto-repressive action of Msx1 and that CBP interacts physically with Msx1 and Msx3 [18]. Here we have extended these results and show that Msx3 protein can also be coimmunoprecipitated with the endogenous p300. At present we do not know whether Msx3 interacts with either CBP and/or p300 proteins directly, or indirectly through some other protein(s). The HAT activity of the immunoprecipitates obtained with anti-CBP or anti-p300 antibodies from C_2C_{12} cells transfected with V5-tagged Msx3 was significantly decreased in comparison with the parallel immunoprecipitates prepared from control cells. The interaction of Msx3 with CBP decreased HAT activity only moderately (approx. 20%), whereas the HAT activity of p300 co-immunoprecipitated with Msx3 was decreased by approx. 50%. Thus we postulate that the interaction of Msx3 with CBP and p300 not only squelches these co-activators but also decreases their HAT activity. Therefore even after these co-activators are recruited to the promoters their functions are significantly compromised.

Because transcriptional repression is often associated with HDACs, we experimentally tested whether these enzymes are also associated with Msx3-induced repression by using transient transfection experiments. Our results show that the exogenous co-expression of HDAC1 strongly enhanced the repressive action of Msx3. A similar increase in the repressive action of retinoblastoma protein by HDAC(s) has been shown previously [42,43]. Our results are consistent with the notion that Msx3 recruits HDAC1 to the target promoters. We interpret our experimental evidence to support the existence of two distinct multiprotein complexes within the C_2C_{12} cells: one complex contains Msx3 and HDAC1; another complex is highly enriched in Msx3 and CBP and/or p300. Our immunofluorescent microscopy results provide further support for a physical association of Msx3 with HDAC1: the two proteins were clearly co-localized in the nucleus. The diffuse patterns of distribution of the two proteins provide further support for our hypothesis that protein–protein interactions are more important than protein–DNA interactions. A similar co-distribution pattern has been observed in mammalian cells overexpressing REST–CoREST and REST–mSin3A [44]. On the basis of these results, we propose that Msx3-mediated repression of its target promoters involves a dual mechanism of squelching HAT activity of the co-activators such as CBP and p300 and the recruitment of HDAC(s). It was recently demonstrated that Cabin 1 represses MEF2-dependent Nur77 expression by controlling the association of HDACs and acetylases with MEF2 [45]. This lends further support for the analogous interaction that might generate two distinct Msx3 co-activator and Msx3 co-repressor complexes. The relief of Msx3-mediated repression by TSA corroborates the involvement of HDAC activity in transcriptional repression by Msx3. A similar relief of repression by TSA treatment was reported for Rb-mediated transcriptional down-regulation [42,43]. The observation that TSA also had a general effect of enhancing the promoter activities of both Msx1 and SV40 promoters is of considerable interest because it is generally accepted that transiently transfected plasmids do not assume a chromatin-like structure. Perhaps we need to re-evaluate this view in view of the reports showing that non-integrated plasmid DNA transfected into mammalian cells can assemble into mini-chromosome-like structure [46]. An alternative suggestion,

that HDAC1 might function by deacetylating one or more non-histone transcription factors [42,43,47], cannot be ruled out by our current results.

In summary, our results show that Msx3 protein can exist in two distinct complexes within the cell, one containing Msx3 associated with co-activators CBP and p300 and the other containing Msx3 associated with HDAC1. Thus we envisage that the binding of Msx3 to CBP and p300 leads to their sequestration and to decreased HAT activity. Concomitantly, Msx3 might specifically recruit HDAC1 to the promoter and suppress its activity by facilitating chromatin remodelling.

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