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***c-Myc* gene single-strand binding protein-1, MSSP-1, suppresses transcription of α -smooth muscle actin gene in chicken visceral smooth muscle cells**

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ABSTRACT

The expression of α -smooth muscle actin is coordinately regulated by positive and negative *cis*-elements in the promoter region. Although *cis*-elements and *trans*-acting factors involved in the positive regulation of the α -smooth muscle (α -SM) actin gene have been well characterized, details of negative regulation remain unclear. In functional analyses using cultured gizzard smooth muscle cells, we identified a sequence ranging from –238 to –219 in the promoter region as a novel negative element. Mutation and deletion analyses further revealed that a sequence, TATCTTA (–228 to –222), is essential for negative regulation. Gel shift assay and Southwestern blotting indicated that a nuclear protein factor specifically interacts with single- or double-strand DNA including this sequence, and the protein factor displays a highly potent binding to the sense strand DNA. cDNA cloning and gel shift analysis using anti-MSSP-1 antibodies revealed that this protein factor is a chicken homolog of human MSSP-1 (*c-myc* gene single-strand binding protein-1). In fact, over-expression of MSSP-1 in cultured smooth muscle cells suppresses the promoter activity. These results suggest a novel function of MSSP-1 regarding the transcriptional regulation of α -sm actin gene.

INTRODUCTION

α -Smooth muscle (α -SM) actin is a well-known molecular marker for a phenotype of vascular smooth muscle cells (SMCs), and its transcriptional regulation has been studied in some cultured cells such as vascular SMCs (1), BC3H1 cells and AKR-2B fibroblasts (2–5). It has been thought that the transcription of the α -SM actin gene is cooperatively regulated by interactions of positive and negative *cis*-elements with their corresponding *trans*-acting factors. The CArG boxes and purine-rich motif are identified as positive *cis*-elements of the promoter in this transcriptional regulation. Serum response factor (SRF) and essential transcriptional enhancer factor-1 (TEF-1), reported as

an M-CAT binding protein (6), are involved in the positive regulation mediated through the CArG box- and purine-rich motif, respectively (1,4,7,8). On the other hand, little is presently known about the negative regulation. It has been solely reported that vascular α -SM actin single-strand binding factors 1 and 2 (VACssBF1 and VACssBF2) extracted from myoblasts or fibroblasts interact with the TEF-1 binding site in a single-strand (ss) DNA-dependent manner (4). Further analyses of site-directed mutagenesis suggest that both factors act as suppressors in the transcription of the α -SM actin gene (9,10).

In this study, we identified a novel negative element (–238 to –219) in the chicken α -SM actin promoter region, which is different from the TEF-1 binding site. Molecular cloning by Southwestern screening revealed that one of the protein factors bound to the novel negative element is a chicken homolog of human *c-myc* gene single-strand binding protein-1 (MSSP-1). We further demonstrated that MSSP-1 functionally suppresses the activity of the α -SM actin promoter in cultured gizzard SMCs. This is the first report regarding MSSP-1-dependent suppression of α -SM actin transcription.

MATERIALS AND METHODS

Chicken MSSP-1 reported in this paper has been submitted to the DDBJ/EMBL/GenBank databank with accession number AB009975.

Cell culture

Gizzard SMCs in primary culture were prepared from 15-day-old chick embryos as described elsewhere (11–13), and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Cloning of the 5'-upstream region of the α -SM actin gene and construction of reporter plasmids

A chicken genomic library was screened using chicken α -SM actin cDNA as a probe. Genomic clones carrying the 5'-upstream region of the α -SM actin gene were characterized by Southern blotting. The 5'-upstream region (–984 to +40) was isolated, and

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then inserted into the *Sma*I site of pUC0CAT, promoter-less chloramphenicol acetyltransferase (CAT) plasmid (14). This plasmid was designated as pActCAT984. Deletions and/or mutations derived from pActCAT984 were constructed.

Southwestern blot analysis

Nuclear extracts from cultured gizzard SMCs were prepared according to a procedure described elsewhere (15). The nuclear proteins were separated in 10% SDS–PAGE and then transferred to a nitrocellulose membrane. After regeneration with guanidine, the membrane was blocked with 5% skimmed milk in binding buffer [5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.8, 5 mM 2-mercaptoethanol, 1 mM EDTA, 60 mM NaCl, 5 mM spermidine, 10% glycerol and 0.1 mg/ml poly(dI–dC)] and hybridized with ³²P-labeled ssDNA probe (CTGCAGTGTGTTTATCTTACAC), in the binding buffer plus 0.2% skimmed milk, at 4°C for 16 h. The membrane was washed three times at 4°C for 2 h with the binding buffer plus 0.2% skimmed milk.

cDNA library construction and screening

A cDNA expression library of cultured gizzard SMCs was constructed using ZAP Express cDNA Synthesis Kit (Stratagene). Recombinant plaques were transferred onto nitrocellulose filters, and the filters were hybridized with ³²P-labeled ssDNA probe (CTGCAGTGTGTTTATCTTACAC) in the binding buffer. Finally, the cloned cDNAs were sequenced.

α-SM actin promoter analysis

Transfection and promoter analysis were carried out as described elsewhere (13,16). In brief, calcium phosphate–DNA precipitates containing 2 µg CAT construct and 1 µg control plasmid carrying the luciferase cDNA under Rous sarcoma virus promoter (pRSV-luciferase) were added to cultured SMCs. In the case of MSSP-1 overexpression, 50 ng of pEF-human MSSP-1 (17,18) were cotransfected with CAT construct and pRSV-luciferase. Standardization of transfection efficiency was carried out using luciferase activity (13,16). We used pUC0CAT and pUC2CAT (14) as negative and positive controls, respectively. The transfection experiments were repeated on multiple sets of cultures with two or three different plasmid preparations. The CAT activities were quantified by Scanning Imager (Molecular Dynamics) and were normalized to the activity of pUC2CAT as 100%.

Purification of recombinant GST fusion protein

We constructed a bacterial expression plasmid (pGEX-6P-1) carrying human or chicken MSSP-1 cDNA. Each fusion protein was produced in *Escherichia coli* BL21(DE3)pTrx transformed by the above plasmids, and was purified by glutathione-coupled Sepharose 4B (Pharmacia) chromatography.

Analysis of DNA–protein interaction by gel shift assay

Double-strand (ds) and ssDNA probes used in gel shift assay are shown in Table 1. To exclude contamination of ssDNAs, the double-strand probe was purified by 20% SDS–PAGE after labelling. For characterization of DNA–protein interaction, nuclear extracts or GST-MSSP-1 fusion protein (4 µg) were mixed with 0.1–0.2 ng of ³²P-labeled probe and 2 µg of

Table 1. Probe used in gelshift assay

	–238	–219		–238	–219
NE20	CTGCAGTGTGTTTATCTTACAC	GACGTCACAAATAGAATGTG	NE20 MUT	CTGCAGTGTGTTTCTCGAGGCAC	GACGTCACAAAGAGCTCCGTG
NE20S	CTGCAGTGTGTTTATCTTACAC		NE20S MUT	CTGCAGTGTGTTTCTCGAGGCAC	
NE20AS	GTGTAAGATAAACACTGCAG		NE20AS MUT	GTGCTTCGAGAACACTGCAG	

Outlined scripts denote mutations.

poly(dI–dC) in the presence or absence of non-radiolabeled competitor in the buffer, using Southwestern blot at room temperature for 20 min. The mixtures were analyzed on 6% polyacrylamide gels in 0.5× TBE buffer. Polyclonal antibodies against human MSSP-1 (19) were used at a final concentration of 3 µg/assay. We confirmed the cross-reactivity of the antibodies to chicken MSSP-1 (data not shown).

Western blot analysis

After washing with PBS, the cells were lysed with 2% SDS sample buffer. The protein samples were separated by SDS–PAGE and transferred to nitrocellulose membranes. Detection of target proteins on the membrane was performed by ECL western blotting detection kit (Amersham) using anti-human MSSP-1 polyclonal antibodies.

RESULTS

Identification of a novel negative element in the α-SM actin promoter

Like vascular SMCs, gizzard SMCs in culture express α-SM actin mRNA and protein. We examined promoter analyses of the α-SM actin gene using cultured gizzard SMCs. Figure 1A shows the schematic diagrams of the 5'-upstream region of the chicken α-SM actin gene from –984 to +40 (20) and a series of CAT constructs used for promoter analyses. The promoter activities of the CAT constructs are shown in Figure 1B. pActCAT193 showed the highest activity compared with the constructs from pActCAT984 to pActCAT238. This result suggests that a sequence from –238 to –194 might be involved in the negative regulation of transcription. Mutation analyses of pActCAT193 revealed that a purine-rich motif (GGAATG) ranging from –181 to –176 and two CArG-box-like elements, CArG B (CCCTATATGG), ranging from –120 to –111, and CArG A (CCTTGTTTGG), ranging from –70 to –61, act as positive elements in cultured gizzard SMCs. These three elements were reported to be positive *cis*-elements in the α-SM actin promoter using cultured rat aortic SMCs (1,5), AKR-2B embryonic fibroblasts and BC3H1 cells (3,4). We also confirmed, using supershift assay, that one of the core factors bound to the CArGs A and B was SRF, and that TEF-1 was bound to the purine-rich motif, as revealed by competition assay using an oligonucleotide including the TEF-1 binding sequence, GAGACACATTCCACACATTCCTG (data not shown).

We further analyzed the negative element in pActCAT238. Although an E box (CAGCTG) is located at –219 to –214, its mutant (pActCAT686) showed a less significant effect on the promoter activity, and pActCAT212, in which the E box was deleted, retained the higher promoter activity (data not shown). These results suggest that the E box is not directly involved in the

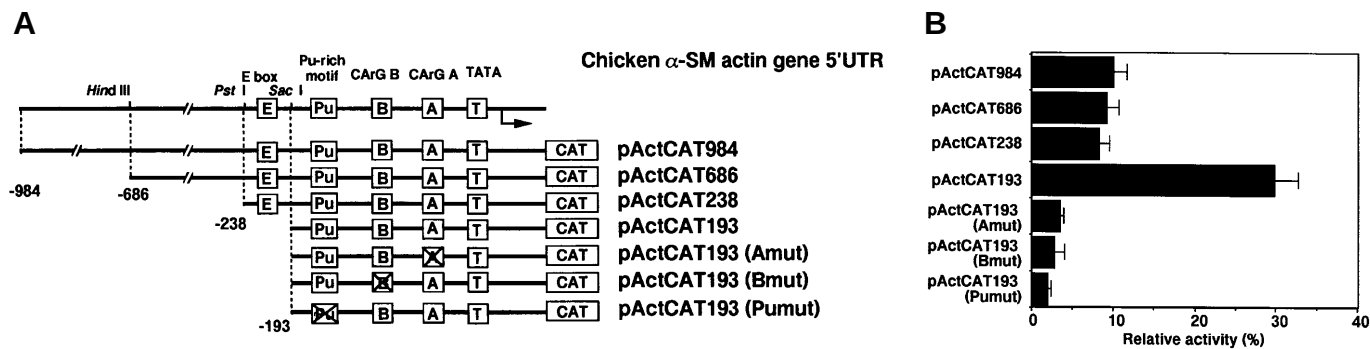


Figure 1. Promoter analysis of α -SM actin gene using a series of deleted and/or mutated CAT constructs in cultured gizzard SMCs. The alignment map of the 5'-upstream region of the chicken α -SM actin gene (–984 to +40) and schematic structures of deleted and/or mutated CAT constructs are shown (A). Locations of canonical *cis*-elements and the transcriptional starting site are indicated in the map; E, E box; Pu, purine-rich motif; B, CArG B; A, CArG A; T, TATA box. The promoter activities of respective constructs are graphically presented (B). The activities were normalized to the activity of pUC2CAT as 100%. To account for differences in transfection efficiencies, the levels of luciferase activity (pRSV-luciferase) were assayed.

negative regulation. To identify the essential sequence from –238 to –219, we constructed site-directed or deleted mutants from pActCAT238 (Fig. 2A), and measured the promoter activities (Fig. 2B). The activities of pActCAT238, pActCAT228 and pActCAT238(NEmut A) showed similarly low levels. In contrast, the activities of pActCAT238(NEmut B) in which a sequence, TATCTTA (–228 to –222), was mutated, as well as pActCAT193, were 3-fold higher than those of the above three constructs. These results indicate that the sequence TATCTTA, ranging from –228 to –222, would be functionally essential for the suppression of α -SM actin promoter activity.

Detection of the negative element binding proteins

We performed gel shift assay to find specific protein factors in SMC nuclear extracts interacting with the negative element. To analyze such protein factors, NE20, NE20S and NE20AS were used as probes, and NE20 MUT, NE20S MUT and NE20AS MUT as specific and non-specific competitors (Table 1). The NE20 is a duplex DNA composed of 20 base pair (bp) nucleotides ranging from –238 to –219, and NE20S and NE20AS are ssDNAs which correspond to the sense and antisense strands of NE20, respectively. Sense and/or antisense strands corresponding to the sequence TATCTTA (–228 to –222) were mutated in NE20 MUT, NE20S MUT and NE20AS MUT. Protein factors in SMC nuclear extracts formed a specific complex with NE20 because this complex formation was completely suppressed by unlabeled NE20, but not by the NE20 MUT (Fig. 3, lanes 1–3). The complex was also efficiently reduced by unlabeled NE20S, NE20 (Fig. 3, lanes 2 and 4) and NE20AS. Non-specific ssDNA, as well as NE20 MUT, were also decreased in the DNA–protein complex, whereas the affinities of the protein factor to non-specific ssDNA and NE20 MUT were lower than that of NE20AS (Fig. 3, lanes 3, 5 and 6). The NE20S formed the complex (Fig. 3, lanes 1 and 7) intensively and this was specifically reduced by unlabeled NE20S, but not by NE20S MUT or non-specific ssDNA (Fig. 3, lanes 8–10). A DNA–protein complex was also formed by NE20AS, while the intensity of this complex was equal to that of the complex formed by NE20 (Fig. 3, lanes 1 and 11). It was slightly reduced by unlabeled NE20AS, but not by NE20AS MUT or non-specific ssDNA (Fig. 3, lanes 12–14). Since the shifted positions of the respective complexes formed with NE20, NE20S or

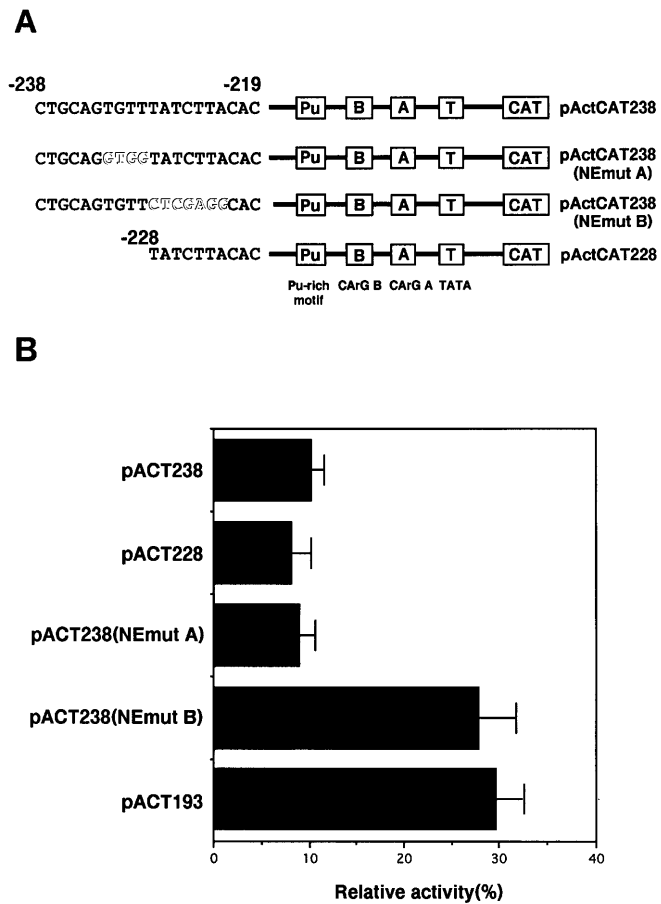


Figure 2. Characterization of the negative element by CAT assay. Schematic structures of CAT constructs including the deleted or mutated negative element are shown (A). Canonical *cis*-elements and the transcriptional starting site are indicated in Figure 1. The nucleotide sequence of the negative element is indicated in respective constructs. Mutations are indicated by outlined scripts. The promoter activities of respective constructs are graphically presented (B). Normalization of the activities was as described for Figure 1.

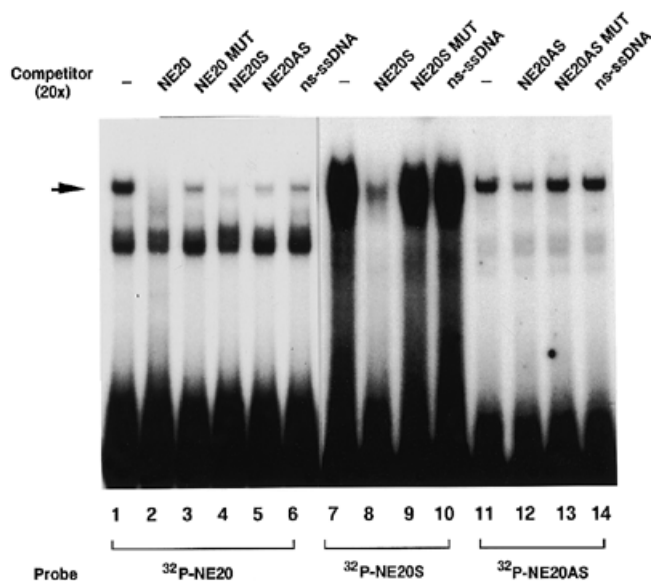


Figure 3. DNA-protein interaction in the negative element was analyzed by gel shift assay. DNA probes are shown in Table 1. Probes and competitors are indicated at the bottom and top of the lanes, respectively. Non-specific ssDNA competitor is an oligonucleotide, CTTACTAGACCACTTGGTTG, in open reading frame of chicken *meta*-vinculin cDNA. The arrowhead indicates a specific DNA-protein complex.

NE20AS were identical, the same protein factor might interact with the probes. There was less significant sequence-specificity of this protein factor to NE20 or NE20AS compared with that to NE20S. We concluded that this protein factor prefers to interact with NE20S, and the target sequence within NE20S is defined as TATCTTA, ranging from -228 to -222. These results also suggest that the sequence TATCTTA is required for DNA-protein interaction and may be involved in the transcriptional suppression (Figs 2 and 3).

Cloning of the negative-element binding protein

To isolate protein factors interacting with the negative element, we performed Southwestern blotting and UV cross-linking using NE20S as a probe. Figure 4 shows the result of Southwestern blotting. The NE20S mainly bound to the two protein factors with M_r of 50 and 100 kDa. Since a 50 kDa protein was most dominant, we aimed to identify this protein (Fig. 4). Under these conditions, a chicken SMC cDNA library was screened by the Southwestern method. Six positive clones were obtained. Sequence analysis revealed that all positive clones coded a chicken homolog of MSSP-1 (submitted to the DDBJ/EMBL/GenBank databank with accession number AB009975); the identity between this protein and human MSSP-1 was 93% (17). Chicken MSSP-1 was composed of 373 amino acids and possessed two conserved RNA binding domains, KGYGFVDF and RGVGFARM, which were responsible for binding to RNA and ssDNA, respectively (17). Northern blotting showed the ubiquitous distribution of MSSP-1 mRNA including gizzard, liver, heart, brain and skeletal muscle (data not shown).

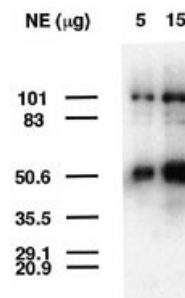


Figure 4. Southwestern blotting. Cultured chicken gizzard SMC nuclear extract (5 and 15 μ g) was separated on 10% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The membrane was hybridized with [32 P]NE20S as a probe. The numbers beside the figure are the molecular weights (kDa).

Involvement of MSSP-1 in the negative regulation of α -SM actin promoter

To characterize the DNA-MSSP-1 interaction, we performed gel shift assay using a GST-fusion MSSP-1 produced in *E.coli*. Fusion protein bound to NE20 or NE20S, and the affinity of this protein for NE20S, was much higher than that for NE20 (data not shown). This result coincided with the binding property of endogenous protein factor to NE20 or NE20S as shown in Figure 3. To confirm whether any protein factor interacting with NE20S is actually MSSP-1, we performed gel shift assay using polyclonal antibodies against human MSSP-1 (Fig. 5). Both NE20S-protein complexes formed with endogenous protein factor and GST-fusion MSSP-1 were reduced by the addition of anti-human MSSP-1 antibodies in a dose-dependent manner, but not by non-immune antibodies. The anti-MSSP-1 antibodies also inhibited the complex formation with NE20 (data not shown). These results suggest that MSSP-1 is involved not only in the NE20S-protein complex formation but also in the NE20-protein complex formation. To investigate the role of MSSP-1 in the transcription of α -SM actin gene, human MSSP-1 (pEF-human MSSP-1) and pActCAT238 were cotransfected in cultured gizzard SMCs. Since the efficiency of human MSSP-1 expression was higher than that of chicken MSSP-1 expression, in this experiment we chose human MSSP-1 for overexpression. The expression of MSSP-1 in the transfected SMCs was confirmed by western blotting (Fig. 6A). Overexpression of MSSP-1 resulted in a 2-fold decrease in the promoter activity of pActCAT238 (Fig. 6B). This result indicates that MSSP-1 acts as a suppressor in the transcriptional regulation of α -SM actin gene in cultured gizzard SMCs.

DISCUSSION

As described in the Introduction, the transcriptional regulation of the α -SM actin gene has been extensively studied using cultured vascular SMCs (1,5), BC3H1 cells and AKR-2B fibroblasts (3,4), and the involvement of *cis*-elements and *trans*-acting factors in cell-type-specific transcription has been reported. Based on these findings, it has been considered that the α -SM actin promoter is controlled by positive and negative regulation. In cultured gizzard SMCs, the purine-rich motif and two CARG boxes (CARGs A and B) also functioned as positive *cis*-elements,

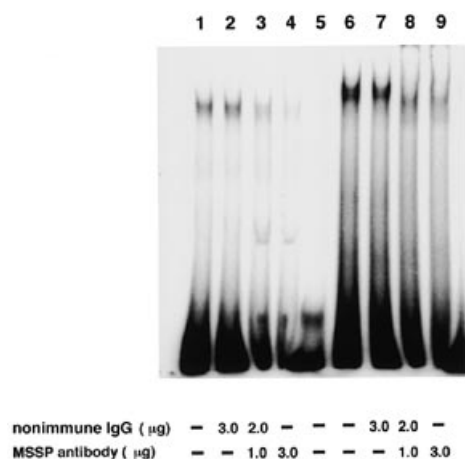


Figure 5. Characterization of MASSP-1 binding to NE20S. Four micrograms of SMC nuclear extracts (lanes 1–4), GST protein (lane 5) and GST-MASSP-1 fusion protein (lanes 6–9) are incubated with [32 P]NE20S. Polyclonal antibodies against human MASSP-1 (lanes 3, 4, 8 and 9) or non-immune antibodies (lanes 2, 3, 7 and 8) are added to the reaction mixtures.

and TEF-1 and SRF bound to them as *trans*-acting factors, respectively (data not shown). With regard to negative elements, Carroll *et al.* have reported a 29 bp sequence ranging from –151 to –123 in chicken α -SM actin promoter as a negative element (21). It has been further demonstrated that the region containing the purine-rich motif acts as a negative element in mouse embryonic fibroblasts and is a target for VACssBF1 and VACssBF2 (4). These *trans*-acting factors specifically bind to ssDNA spanning the purine-rich motif, suggesting candidates for suppressors of mouse α -SM actin promoter (9,10). As demonstrated in this paper, the negative element of chicken α -SM actin promoter is, however, not a target sequence for VACssBF1 and VACssBF2, because pActCAT212 (data not shown) and pActCAT193 (Fig. 1), containing the purine-rich motif, displayed the high promoter activities. This discrepancy may be due to the difference in cell types used for promoter analyses.

Here, we identified a novel sequence, CTGCAGTGTTCATC-TTACAC, ranging from –238 to –219 as a novel negative element in the promoter region of α -SM actin. Further analyses revealed that a sequence, TATCTTA (–228 to –222), was involved in the negative regulation of promoter activity (Figs 1–3). This sequence is partially conserved among the α -SM actin promoter regions from other species such as human, rat and mouse (57% identity). Future studies are required to elucidate whether this element would function as a common negative element of α -SM actin promoters among these species. In contrast with our results, McNamara *et al.* have reported that a partial overlap sequence of our identified negative element, TGTTCATC, is involved in the positive regulation in cultured rat aortic SMCs (22). Their result seems to arise from an analysis using the chicken α -SM actin promoter in cultured rat aortic SMCs.

Molecular cloning using NE20S as a probe revealed that a protein factor bound to the negative element was a chicken homolog of human MASSP-1. Five highly homologous MASSP cDNA clones (MASSP-1, MASSP-2, scr2, scr3 and human YC1) have been reported by two groups (17,19,23). Genomic analysis has revealed that all MASSP cDNAs are encoded by a single gene, MASSP gene 2 (24). The MASSPs are demonstrated to be involved

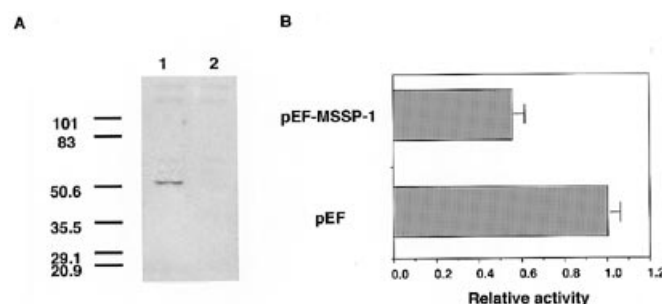


Figure 6. (A) Western blot analysis of pEF-MASSP-1 (lane 1) or pEF control vector (lane 2) transfected gizzard SMCs using MASSP-1 antibodies. (B) The effect of overexpressed MASSP-1 on the promoter activity of pActCAT238. pActCAT238 was cotransfected with pEF-MASSP-1 or pEF control vector in cultured gizzard SMCs. The promoter activities of respective transfectants are graphically represented. Normalization of the activities was as described for Figure 1.

in DNA replication (23) and the regulation of cell cycle (19). The fifth homologous clone, YC-1, is submitted to DDBJ/EMBL/GenBank (accession no. L11289) as a suppressor of HIV-1 and ILR2 α gene transcription; however, the details of this function are unknown. MASSP-1 interacts with the upstream elements of the *c-myc* gene, which are required for DNA replication and active transcription of *c-myc* mRNA. While this factor binds to both ssDNA and dsDNA including the consensus motif (A/TCTA/TA/TT), the binding affinity for ssDNA is stronger than that for dsDNA (17).

The essential sequence in the negative element, TATCTTA, is overlapped by the consensus binding motif of MASSP-1, and is necessary for GST-fusion chicken MASSP-1 binding (data not shown). The endogenous protein factor bound to the negative element also showed a high affinity for ssDNA rather than dsDNA (Fig. 3). The antibodies against MASSP-1 inhibited the binding of endogenous protein factor to the negative element in the same manner as GST-fusion MASSP-1 (Fig. 5). Taken together, we conclude that one of the protein factors in gizzard SMC nuclear extracts interacting with the negative element is MASSP-1. Further, transfection experiments support our present conclusion that overexpressed MASSP-1 in cultured gizzard SMCs suppresses the promoter activity of α -SM actin gene (Fig. 6B).

The molecular mechanism of transcriptional suppression mediated by MASSP-1 remains unclear. We speculate that the interaction of MASSP-1 with the negative element may stabilize the partial ssDNA structure in the α -SM actin promoter and inhibit the interaction of positive regulators, TEF-1 and SRF, with the downstream positive elements, purine-rich motif and CARG boxes. In our preliminary experiment, the negative element had no effect on the caldesmon promoter (data not shown). This finding suggests that the negative element does not suppress the heterologous promoter activities but that the suppression may be closely associated with positive elements in the α -SM actin promoter. With regard to the cell-type-specific expression of α -SM actin, MASSP-1 may inhibit the transcription in other non-muscle cells because MASSP-1 is ubiquitously expressed. In the case of cultured visceral and possibly vascular SMCs, the positive regulatory factors, TEF-1 and SRF, might overcome the negative effect of MASSP-1.

This study is a first report regarding a novel function of MASSP-1 in the transcriptional regulation of α -SM actin gene.

Further studies are necessary to reveal the mechanism of MSSP-1 in suppression of the α -SM actin gene.

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