Research article

TRANSCRIPTIONAL REGULATION OF MOUSE MESENCEPHALIC ASTROCYTE-DERIVED NEUROTROPHIC FACTOR IN Neuro2a CELLS

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Abstract: Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a novel type of trophic factor. Recent studies indicate that the MANF gene is induced in response to endoplasmic reticulum (ER) stress through ER stress response element II (ERSE-II) in its 5'-flanking region. In this study, we evaluated the roles of six ER stress response transcription factors in the regulation of the promoter activities of the mouse MANF gene via ERSE-II using various types of mutant MANF luciferase reporter constructs. Treatment with thapsigargin (Tg) induced MANF mRNA generation in parallel with the elevation of ATF6α, sXBP and Luman mRNA levels in Neuro2a cells. Of the six transcription factors, ATF6α most strongly increased the MANF promoter activity via ERSE-II, while the effects of ATF6β and sXBP1 were moderate. However, overexpression of Luman or OASIS did not enhance ERSE-II-dependent MANF promoter activity in Neuro2a cells. To evaluate the relationships between transcription factors in the regulation of ERSE-II-

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dependent MANF promoter activity, we transfected two effective transcription factor constructs chosen from ATF6α, ATF6β, uXBP1 and sXBP1 into Neuro2a cells with the MANF reporter construct. The MANF promoter activity induced by co-transfection of ATF6α with ATF6β was significantly lower than that induced by ATF6α alone, while other combinations did not show any effect on the ERSE-II-dependent MANF promoter activity in Neuro2a cells. Our study is the first to show the efficiency of ER stress-related transcription factors for ERSE-II in activating the transcription of the mouse MANF gene in Neuro2a cells.

Key words: ATF6α, ATF6β, ERSE-II, ER stress, Luman, MANF, OASIS, sXBP1, uXBP1

INTRODUCTION

Mesencephalic astrocyte-derived neurotrophic factor (MANF) was first identified as arginine-rich, mutated in early stage of tumors (Armet) and recognized as a protein with a high mutation rate in various tumors and an unknown precise function [1, 2]. Petrova *et al.* reported that MANF is secreted from a rat mesencephalic type 1 astrocyte cell line and that it acts as a selective neurotrophic factor for dopaminergic neurons [3]. Its neurotrophic potency is comparable to that of glial cell line-derived neurotrophic factor rather than brain-derived neurotrophic factor. Accordingly, Armet is referred to as MANF, even though its precise mechanisms for preventing neuronal cell death remain unknown [3-6].

The endoplasmic reticulum (ER) is responsible for folding and modifying newly synthesized transmembrane and secretory proteins [7, 8]. Some pathophysiological conditions disrupt ER functions and cause the accumulation of unfolded and/or misfolded proteins in the ER [9, 10]. These occurrences, known as ER stress, activate various stress responses that are mediated by three ER-resident stress sensors: PERK [11], IRE1 [12] and ATF6α [13, 14]. Various genes have been identified as downstream targets of these sensors. ATF6α recognizes the ER stress response element, ERSE (CCAAT-N(9)-CCACG), by forming complexes with the NF-Y family to induce the expression of several ER stress-related genes, including ER resident chaperones, which control the quality of newly synthesized proteins in the ER [13-17].

Mizobuchi *et al.* were the first to demonstrate that MANF is one of the genes that is induced via ERSE-II (CGTGG-N-CCAAT) in response to ER stress [18]. ERSE-II is another type of ATF6α-recognizing sequence in which the binding sites for ATF6α and NF-Y are separated by only 1 bp [17, 19]. sXBP1, an IRE1-spliced transcription factor, is also reported to recognize ERSE-II in the absence of NF-Y [17].

In addition to the above-mentioned canonical ER stress sensors, others that are structurally similar to ATF6α have been identified, and their targets have been explicitly characterized [20-26]. They are localized in the ER lumen in a quiescent state, but their N-terminal halves translocate into the nucleus and

induce the transcription of specific genes following their cleavage by regulated intramembrane proteolysis (RIP) in response to ER stress. Among these novel sensors, ATF6β [20] and Luman [21] are reported to recognize ERSE and ERSE-II, respectively. However, the roles of these transcription factors in regulating the promoter activity of the MANF gene are not fully understood. In this study, we evaluated the effects of ER stress-related transcription factors on potentiating mouse MANF promoter activities in Neuro2a cells using various types of mutant MANF luciferase reporter constructs.

MATERIALS AND METHODS

Construction of plasmids

To prepare reporter constructs for the mouse MANF promoter, genomic DNA from Neuro2a cells was extracted, and the mouse MANF promoter (-129/+34) was amplified using PCR and then cloned into the pGL3-Basic (pGL3b) vector (Promega). Other constructs containing deleted and mutated mouse MANF promoters were also prepared using PCR. The promoter region was defined using the database and RIKEN functional annotation of a full-length mouse cDNA collection (FANTOM; NM_029103.3 and AK131997).

To prepare mouse ATF6α and Luman (CREB3), they were amplified from Neuro2a cell cDNA using PCR. For the preparation of the expression constructs of mouse ATF6β (G13 protein) and human OASIS (CREB3L1), cDNA was purchased from DNAFORM and the RIKEN BRC. The active forms of the N-terminal fragment of the transcription factors N-ATF6α (1-366 aa), N-ATF6β (1-392 aa), N-Luman (1-225 aa) and N-OASIS (2-374 aa) were amplified using PCR and then cloned into the pFLAG-CMV vector.

Cell culture and treatment

Neuro2a cells were maintained in Dulbecco's modified Eagle's minimum essential medium containing 8% fetal bovine serum (FBS). C6 glioma cells were cultured in HAM F10 medium containing 3% horse serum and 7% FBS. Vector transfection was performed using Lipofectamine Plus Reagent (Life Technologies) according to the manufacturer's instructions. For cell treatment, Neuro2a cells were treated with thapsigargin (Tg; 0.1 μM; Sigma-Aldrich) or MG132 (20 μM; Peptide Institute) for the indicated time.

Reverse transcription polymerase chain reaction

To estimate the expression level of each gene via RT-PCR, total RNA was extracted from cells lysed with TRIzol reagent (Life Technologies) and converted to cDNA via reverse transcription using random nine-mers as primers for superscript III RNase⁻ reverse transcriptase (RT; Life Technologies), as previously described [27]. Specific cDNAs were mixed and amplified with a PCR mixture (Taq PCR Kit, Takara). The RT-PCR primers used in this study were:

MANF sense primer, 5'-GTTTGTATTTCTTATCTGGG-3'
MANF antisense primer, 5'-TCTTCTTCAGCTTCTCACAG-3'
ATF6α sense primer, 5'-GTTCTGTCGTCTGCTCAGC-3'
ATF6α antisense primer, 5'-ACTTGGGACTTTGAGCCTCT-3'
ATF6β sense primer, 5'-CTCCTGCCCGCCTGAAGT-3'
ATF6β antisense primer, 5'-CTGATTGGCAGGGCTCACACT-3'
XBP1 sense primer, 5'-ACGCTTGGGAATGGACACG-3'
XBP1 antisense primer, 5'-ACTTGTCCAGAATGCCCAAAAG-3'
Luman (CREB3) sense primer, 5'-AGAAGGTGTATGTCGTGGGGCT-3'
Luman (CREB3) antisense 1 primer, 5'-GGAGAACACGAGGACCAGAACA-3'
Luman (CREB3) antisense 2 primer, 5'-AAGTGCTGCCGCTGCTGGTTTT-3'
OASIS (CREB3L1) sense primer, 5'-TACAGATGCCTCCAACACCC-3'
OASIS (CREB3L1) antisense primer, 5'-CACCTTCTTCCACAGTTC-3'
GAPDH sense primer, 5'-ACCACAGTCCATGCCATCAC-3'
GAPDH antisense primer, 5'-TCCACCACCCTGTTGCTGTA-3'

The typical reaction cycling conditions were 30 sec at 96ºC, 30 sec at 58ºC, and 30 sec at 72ºC. The results represent 20 to 34 cycles of amplification. Then, cDNAs were separated via electrophoresis on 2% agarose gels and visualized using ethidium bromide.

Reporter assay
Each reporter construct (0.05 μg/well) and the internal control (0.02 μg/well pGL4.70 vector; Renilla luciferase; Promega) were transfected into Neuro2a cells in a 48-well plate as previously described [27]. After 24 h, the cells were treated with Tg (0.1 μM) or vehicle for 10 h. To determine the effects of each transcription factor on reporter activities, the expression vectors of the indicated transcription factors or an empty vector (mock; 0.005 μg/well) were co-transfected with reporter constructs into the cells and cultured for 36 h. In some experiments, a different amount of the indicated expression vector was transfected together with each luciferase reporter, as described in the figure legends. After incubation, the cells were lysed and luciferase activities in each lysate were measured using a Dual-Luciferase assay system (Promega). Reporter activity in each lysate was normalized to the co-transfected Renilla luciferase activity. The results are shown as relative luciferase activity. Transfection of some transcription factors increased the luciferase activities, even in cells with the empty pGL3b vector. Therefore, the relative MANF promoter activities related to the transfection of each transcription factor were normalized to the values in cells into which pGL3b was transfected with an individual transcription factor. Experiments were repeated more than twice and reproducibility was confirmed.

Western blot analysis
To determine the expression level of each Flag-tagged transcription factor in the Neuro2a cells, the cells were lysed with homogenate buffer consisting of 20 mM Tris-HCl (pH 8.0) containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% TritonX-100, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A. To evaluate the amount of the indicated transcription factor in the nucleus, the nuclear fraction was prepared as described previously [28]. Cells were lysed with 10 mM HEPES buffer (pH 7.5) containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.2% (v/v) Nonidet-P40, 0.2 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A. After centrifugation at 4,000 rpm, the cell pellet was resuspended with 20 mM HEPES buffer (pH 7.5) containing 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A, and incubated on ice for 30 min. After centrifugation at 15,000 rpm, the soluble fraction was collected and used as the nuclear fraction. After the protein concentration was determined, each cell lysate was dissolved in SDS-Laemmli sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS and 10% glycerol. Equal amounts of cell lysate were separated on 10% SDS-polyacrylamide electrophoresis gels, immunoblotted onto polyvinylidene difluoride membrane (GE Healthcare) and identified through enhanced chemiluminescence (GE Healthcare) using an antibody against the Flag-epitope (Sigma-Aldrich). Signals detected using the anti-Flag antibody were properly validated using a control cell lysate that did not express the Flag-tagged protein.

Statistical analysis
The results are expressed as means ± SD of the indicated number. Statistical analyses were carried out using one-way ANOVA followed by Scheffé's method. $p < 0.05$ was considered to be statistically significant.

RESULTS

First, we evaluated the expression levels of MANF and several ER stress-related transcription factors in Neuro2a cells. The expression levels of MANF, ATF6α, Luman and sXBP1 mRNA were upregulated in Neuro2a cells 6 and 12 h after Tg treatment (Fig. 1A). In addition, the level of sXBP1, which is a spliced form of uXBP1 through IRE1, was remarkably increased, while that of uXBP1 was proportionally decreased. Consistent with the Tg-induced expression of MANF mRNA, the luciferase activity of the mouse MANF reporter gene, including that of ERSE-II (-129/+34), was dramatically elevated by Tg treatment (Fig. 1B). Transfecting N-ATF6α, the N-terminal fragment of ATF6α (active form), with the mouse MANF reporter construct (-129/+34) also increased the luciferase activity in Neuro2a cells. By contrast, mutations of the ATF6α- or NF-Y-binding sites (-129/+34 m1 or m2, respectively) or deletion of the ERSE-II motif
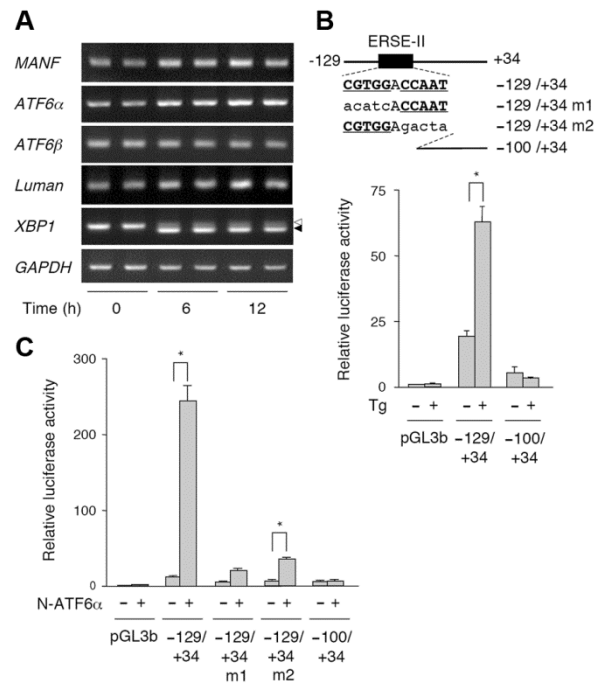
Fig. 1. Thapsigargin induced the generation of MANF mRNA in parallel with activation of its promoter activity via ERSE-II in Neuro2a cells. A – Neuro2a cells were treated with Tg (0.1 µM) for the indicated time. The expression of each mRNA was evaluated using RT-PCR as described in the Materials and Methods section. Open and closed arrow-heads respectively indicate uXBP1 and sXBP1. B – 24 h after transfection of each MANF reporter construct, Neuro2a cells were treated with Tg (0.1 µM) or vehicle for an additional 12 h. ATF6- and NF-Y-binding sequences in the ERSE-II motif and their mutated sequences (-129/+34 m1 and m2) are respectively shown with bold type and small letters. C – 36 h after transfection of a Flag-tagged N-ATF6α construct or an empty vector with each of the reporter constructs and pGL4.70, Neuro2a cells were lysed, and each construct's luciferase activity was measured as described in the Materials and Methods section. Values represent the means ± SD from 3 independent cultures and are expressed relative to the basal activity of the cells transfected with the pGL3-Basic vector. Data were analyzed using one-way ANOVA followed by Scheffé's method to evaluate the effects of Tg treatment (B) or N-ATF6α overexpression (C) on the activity of each MANF promoter. Values marked with an asterisk are significantly different from the MANF promoter activity of the untreated cells ($p < 0.05$).

(-100/+34) almost abolished the ATF6α response (Fig. 1C). These results indicate that the ERSE-II motif in the mouse MANF reporter is indispensable for both basal and ER stress-induced promoter activities in Neuro2a cells.

To evaluate the ERSE-II regulation of mouse MANF promoter activities in addition to those of ATF6α, we compared the functional abilities of ATF6β, uXBP1 and sXBP1 to potentiate the promoter activity. Fig. 2A shows the expression of each transcription factor in the total lysate of Neuro2a cells 32 h
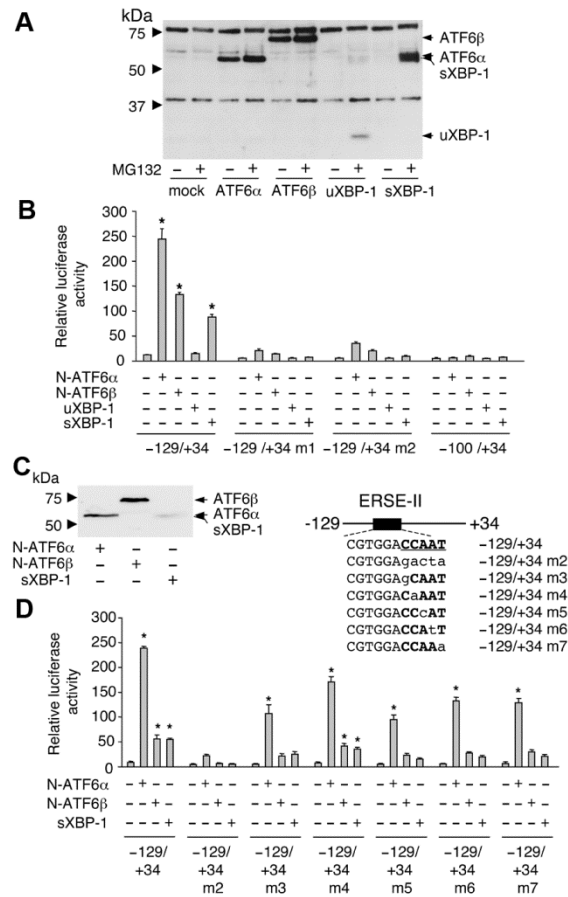
Fig. 2. Characterization of the role of ATF6α, ATF6β and XBP1 in potentiating the luciferase activity of mouse MANF promoter constructs in Neuro2a cells. The expression levels of each transcription factor in the total cell lysate (A) and nuclear fraction (C) are shown. A – 24 h after transfecting the indicated expression constructs, Neuro2a cells were treated with MG132 (20 μM) or vehicle for an additional 8 h. C – 36 h after transfection of the indicated expression constructs, the cells were harvested for preparation of the nuclear fraction. The procedure for the preparation of the total cell lysate (A) and nuclear fraction (C) and the detection of each transcription factor are described in the Materials and Methods section. B and D – 36 h after transfecting the indicated transcription factors with each of the reporter constructs and pGL4.70, Neuro2a cells were lysed, and the luciferase activity of each sample was measured. ATF6- and NF-Y-binding sequences in the ERSE-II motif and their mutated sequences (-129/+34 m2-m7) are respectively shown with bold type and small letters (D). Values represent the means ± SD from 3 independent cultures and are expressed relative to the luciferase activity of the cells co-transfected with the pGL3-Basic vector and the indicated transcription factors. Data were analyzed using one-way ANOVA followed by Scheffé's method to evaluate the effects of co-expression of the indicated transcription factors on each of the MANF promoter activities. Values marked with an asterisk are significantly different from the MANF promoter activity (-129/+34) without co-transfection of each transcription factor ($p < 0.05$).

after transfection. The expression of uXBP1 and sXBP1 proteins in Neuro2a cells was negligible in the quiescent state, but treatment with MG132, a proteasome inhibitor, increased the expression levels of both. Likewise, the expressions of N-ATF6α and the N-terminal fragment of ATF6β (N-ATF6β) were also upregulated by MG132. Transfection of each transcription factor construct with the mouse MANF promoter construct showed different effects on the reporter activity (Fig. 2B). N-ATF6α showed the strongest ability to increase the mouse MANF promoter activity, while the abilities of N-ATF6β and sXBP1 were moderate: less than half that of N-ATF6α. By contrast, overexpression of uXBP1 barely increased the reporter activity. The effects of N-ATF6α, N-ATF6β and sXBP1 on the reporter activity were similarly abolished by mutations of the N-ATF6α- or NF-Y-binding sites or deletion of the ERSE-II motif in the mouse MANF reporter construct.

We further evaluated the amount of three potent activators for the mouse MANF promoter, N-ATF6α, N-ATF6β and sXBP1, in the nuclear fraction (Fig. 2C). Consistently with the amount of each activator in the total lysate (Fig. 2A), the amount of Flag-tagged sXBP1 in the nuclear fraction was apparently lower than that of the other activators.

We then investigated whether N-ATF6α, N-ATF6β and sXBP1 recognize the NF-Y-binding site in the mouse MANF promoter using 5 additional reporter constructs in which 1 nucleotide within the site is substituted with another nucleotide. Each of the point mutations (-129/+34 m3 through m7) in the NF-Y-binding site slightly but non-significantly decreased the basal promoter activity. The responsiveness to N-ATF6α, N-ATF6β or sXBP1 of each reporter with a point mutation partially decreased compared with that of the MANF promoter without such mutation (-129/+34), but was in each case higher than that of the promoter construct with the NF-Y-binding site fully mutated (-129/+34 m2; Fig. 2D).

We also investigated whether other ER stress-related transcription factors participate in the regulation of mouse MANF promoter activity. As shown in Figs 1A and 3A, Luman mRNA was slightly induced 12 h after Tg treatment. The OASIS gene, the product of which is structurally similar to ATF6 and Luman, was expressed in C6 glioma cells without stimulation, whereas OASIS mRNA was not detected in Neuro2a cells even after Tg stimulation. On the other hand, we found that adequate amounts of N-Luman and N-OASIS were accumulated after transfection of their expression vectors into Neuro2a cells (Fig. 3B). However, the co-transfection of the N-Luman or N-OASIS expression vectors at a low concentration (0.0005 μg/well), i.e. a hundredth of the amount of the MANF reporter construct, did not enhance the promoter activities. In the case of high expression vector concentration (0.005 μg/well), i.e. one-tenth of that of the reporter construct, the co-transfection rather attenuated each of the luciferase activities in the Neuro2a cells without significant difference (Fig. 3C).
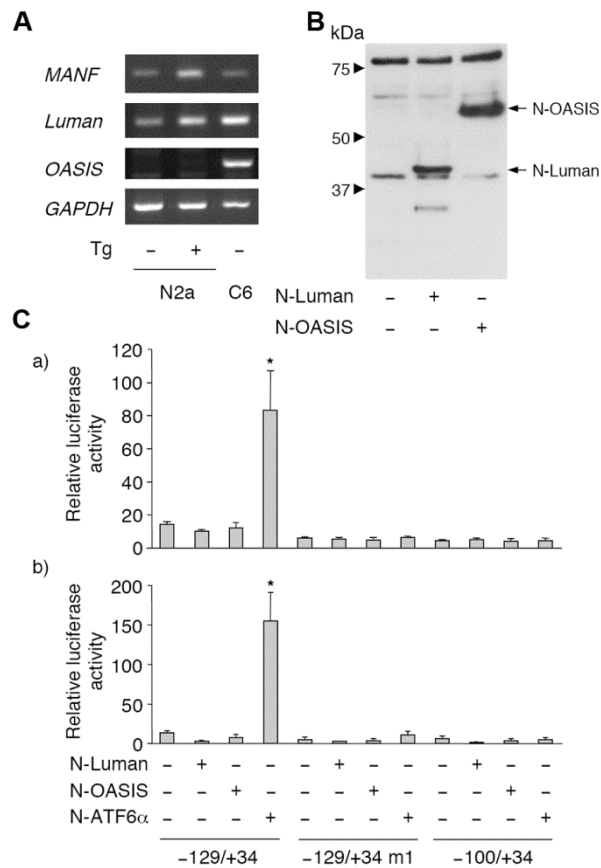
Fig. 3. Overexpression of Luman and OASIS did not activate the MANF promoter activities in Neuro2a cells. A – The expression of the indicated mRNA in Neuro2a and C6 glioma cells treated with Tg (0.1 μM) or vehicle for 12 h was evaluated via RT-PCR. B – 24 h after transfection of the indicated expression vectors into Neuro2a cells, the expression of each transcription factor was detected using western blot analysis as described in the Materials and Methods section. C – The indicated transcription factors at low (a, 0.0005 μg/well) or high (b, 0.005 μg/well) concentrations, together with each of the MANF reporter constructs and pGL4.70, were transfected into Neuro2a cells. 36 h after transfection, the cells were lysed, and the activity of each luciferase was measured. Values represent the means ± SD from 5-6 independent cultures and are expressed relative to the luciferase activity of the cells co-transfected with the pGL3-Basic vector and the indicated transcription factors, respectively. Data were analyzed using one-way ANOVA followed by Scheffé's method to evaluate the effects of co-expression of the indicated transcription factors on the activities of each of the MANF promoters. Values marked with an asterisk are significantly different from the MANF promoter activity (-129/+34) without co-transfection of each transcription factor ($p < 0.05$).

Fig. 4. Synergetic effect of N-ATF6α, N-ATF6β and XBP1 on the mouse MANF promoter activities in Neuro2a cells. A, B − The indicated transcription factors with the MANF reporter construct (-129/+34) and pGL4.70 were transfected into Neuro2a cells. 36 h after transfection, the cells were lysed and luciferase activity was measured as described in the Materials and Methods section. Values represent the means ± SD from 3 independent cultures and are expressed relative to the luciferase activity of the cells co-transfected with the pGL3-Basic vector and the construct of indicated transcription factors. Data were analyzed using one-way ANOVA followed by Scheffé's method to evaluate the effects of co-expression of the indicated transcription factors on the MANF promoter activity. Values marked with an asterisk are significantly different from the MANF promoter activity (-129/+34) withou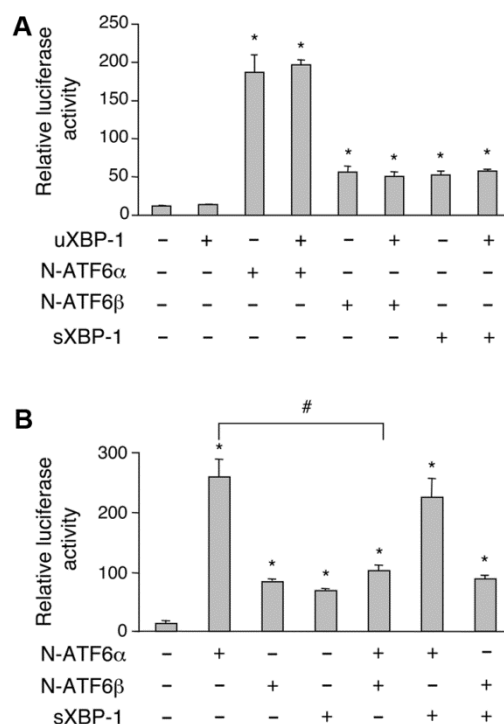t co-transfection of each transcription factor ($p < 0.05$). Luciferase activity values marked with a hash (#) are significantly different ($p < 0.05$).

Finally, we characterized the relationships between N-ATF6α, N-ATF6β, uXBP1 and sXBP1 in regulating the mouse MANF promoter activity through co-transfection of two transcription factors with the reporter construct (-129/+34). Transfection of either XBP1 construct with another transcription factor construct did not affect the luciferase activities (Fig. 4A). Interestingly, the luciferase activity due to the co-transfection of N-ATF6α and N-ATF6β was markedly lower than that due to N-ATF6α overexpression alone (Fig. 4B). However, N-ATF6β co-transfection did not attenuate sXBP1-induced reporter activity in Neuro2a cells.

DISCUSSION

It was recently suggested that MANF and cerebral dopamine neurotrophic factor (CDNF) should be classified as a novel family of trophic factors for dopaminergic neurons and several other types of cell, even though their precise cytoprotective mechanisms are unknown [3-6, 29, 30].

Using DNA array analysis of cells from XBP1-knockout mice, Lee *et al.* first suggested that MANF is relevant to ER stress [31]. As ER stress has been reported to be associated with the onset and progression of various types of disease, including neurodegenerative conditions [9, 10], MANF is one of many promising agents for disease management as an ER stress-inducible gene. In addition, the ERSE-II motif in the 5'-flanking promoter region of the MANF gene was reported to play a pivotal role in inducing gene expression [18]. In contrast to the canonical ERSE, the recognition of ERSE-II is not restricted by complexes such as ATF6 and NF-Ys [17-19].

In this study, we characterized the promoter activities of the mouse MANF gene via the ERSE-II motif at the 5'-flanking region through co-transfection with canonical and novel ER stress-related transcription factors, which are suggested to recognize ERSE and/or ERSE-II, excluding OASIS. We showed that Neuro2a cells intrinsically expressed each of the genes studied here, and Tg-treatment increased MANF mRNA levels in parallel with ATF6α, Luman and sXBP1 transcripts. The absence of OASIS mRNA in Neuro2a cells, even after Tg-treatment, is consistent with the reports that OASIS is only expressed in specific types of cells, such as osteoblasts [32] and astrocytic cells, including C6 glioma cells [22]. Among the ubiquitously expressed transcription factors, ATF6α, Luman and sXBP1 seem to be the ER stress-inducible factors in Neuro2a cells. Namba *et al.* demonstrated that the transcriptional activation of the ATF6α gene is mediated via putative ATF6-binding sites in its promoter region [33], but the mechanisms for the moderate induction of Luman mRNA by Tg-treatment in Neuro2a cells are still unclear.

An evaluation of the potency of transcription factors in activating the ERSE-II motif of the mouse MANF gene revealed that N-ATF6α has the strongest effect on MANF promoter activity. sXBP1 significantly induced the MANF gene in this experiment although sXBP1 was known to be relatively unstable and its amount in the nuclear fraction was much lower than that of N-ATF6α (Fig. 2C). sXBP1 is probably one of the important factors that induces the transcription of the MANF gene in Neuro2a cells. These results are consistent with those published in an earlier report, which stated that the individual effects of XBP1-knockout or ATF6α-knockdown on MANF induction were weak, but the combination of the two dramatically downregulated MANF expression [31]. The overexpression of N-ATF6β actually upregulated the ERSE-II-mediated MANF promoter activity (Fig. 2B and D). We also observed that N-ATF6α, N-ATF6β and sXBP1 had similar potencies for activating the mouse MANF promoter when the untagged transcription factors were individually transfected into

Neuro2a cells (Suppl. Fig. 1 at http://dx.doi.org/10.2478/s11658-013-0096-x). Thuerauf *et al.* reported that ATF6β overexpression marginally induced luciferase activities via ERSE in the human GRP78 promoter [34]. In our experiment, N-ATF6β did not elevate the promoter activity of the human GRP78 gene (Suppl. Fig. 2A), although the overexpression of N-ATF6β induced luciferase activity in the case of 1× UPRE-containing pGL3-promoter [17] (Suppl. Fig. 2B) or ERSE-containing CRELD2 promoter [27] (data not shown). Therefore, the differences between the ERSE, ERSE-II and UPRE motifs should not be attributed to the fact that ATF6β is inactive against the ERSE of the human GRP78 promoter. Nevertheless, our study shows that ATF6β could participate in the transcription of the MANF gene in Neuro2a cells.

We also measured the recognition of the NF-Y-binding site (CCAAT) of the mouse MANF promoter by N-ATF6α, N-ATF6β and sXBP1, although their capabilities to enhance promoter activity were quite different. Each of the point mutations in the motif partly decreased the basal promoter activity and responsiveness to N-ATF6α, N-ATF6β and sXBP1. N-ATF6α and N-ATF6β, but not sXBP1, are reported to recognize the ERSE/ERSE-II motifs in cooperation with NF-Ys. Further studies are required to characterize the differences in the cofactors that are recruited into ERSE-II in the MANF promoter together with ATF6s and/or sXBP1 under pathophysiological conditions.

Recently, novel types of ER stress transducers have been identified, such as Luman, OASIS, BBF2H7, CREBH and CREB4, all of which have analogous molecular features to the canonical ATF6 [21-26]. These transducers predominantly localize in the ER through a corresponding transmembrane region without stimulation, but they are processed by RIP, and their N-terminal halves are translocated into the nucleus once they perceive the ER stress. These ATF6-related transmembrane transcription factors are considered to regulate the expression of specific genes, but not all of their targets have been identified.

Luman is suggested to activate the promoter activity of the homocysteine-induced endoplasmic reticulum protein (Herp), specifically recognizing ERSE-II but not ERSE [19, 21]. Furthermore, it has been suggested that the CCACG sequence of the ATF6-binding site in the ERSE-II of the Herp promoter is enough for recognition by Luman. In this study, transfection of N-Luman into Neuro2a cells was found to accumulate an adequate amount of this protein, but its overexpression did not activate the promoter activity of the mouse MANF gene. Instead, Neueo2a cells co-transfected with pGL3b and a high amount of the N-Luman construct showed a higher relative luciferase activity compared to that of the control (data not shown). These results were even observed in cells with the reporter construct containing the mutated ATF6-binding site (-129/+34 m1). After the normalization of the value in cells expressing the reporter (-129/+34) together with N-Luman to that in cells expressing pGL3b with N-Luman, it was found that N-Luman overexpression did not induce but rather decreased the mouse MANF promoter activities in Neuro2a cells.

Among the nucleotide sequences flanking the ERSE-II motif of the mouse MANF promoter, there is a unique sequence (GCACGTGG), which is analogous to the OASIS-recognizing sequence in type I collagen promoter (CGACGTGG), i.e. it is a CRE-like sequence [32]. Although the Neuro2a cells did not intrinsically express OASIS mRNA, in contrast to C6 glioma cells [22], we evaluated the possibility that OASIS enhanced the mouse MANF promoter activities by transfecting N-OASIS into Neuro2a cells. Similarly to N-Luman, the N-OASIS overexpression also increased the luciferase activity, even in the empty pGL3b-transfected cells, and the normalized values indicate that N-OASIS seems not to recognize the putative CRE-like sequence or ERSE-II. Therefore, this CRE-like sequence flanking the ERSE-II motif of the mouse MANF promoter might not be the target of OASIS.

There are two possible reasons for the unresponsiveness of N-Luman to the mouse MANF promoter. First, to recognize this ERSE-II motif and/or have N-Luman-induced activation of the promoter, other factors that are not expressed in Neuro2a cells might be required. Another reason for the unresponsiveness is that Luman could specifically participate in the activation of the human Herp promoter via ERSE-II [21]. Therefore, we examined the effect of N-Luman on the promoter activity of the mouse Herp gene containing ERSE and ERSE-II (Suppl. Fig. 3). However, the overexpression of N-Luman barely enhanced the mouse Herp promoter activity while N-ATF6α increased it by about 3-fold. The reasons why N-Luman did not upregulate the Herp promoter activity are unclear. Neuro2a cells might not have indispensable co-factors regulating the Luman activity appropriately. Further characterization of Luman is required to resolve these inconsistencies because the targets of Luman require further identification.

In the human genome, more than 50 transcription factors containing a basic leucine zipper (bZIP) domain have been identified [35]. Some of these factors are known to interact with one another and modify their stability and transcriptional activity. uXBP1 is reported to interact with sXBP1, ATF6α and ATF6β, while uXBP1 overexpression facilitates the degradation of sXBP1 and ATF6α in a proteasome-dependent pathway [36, 37]. ATF6β is suggested to have the opposite effect to ATF6α, downregulating the promoter activity mediated by ERSE [34, 38]. However, there have been no studies on the synergetic effects of these transcription factors on ERSE-II-mediated promoter activities. Overexpression of sXBP-1 or uXBP1 together with either N-ATF6α or N-ATF6β minimally affected the promoter activities compared with each factor alone. Because of the instability of the two types of XBP1 in Neuro2a cells, these factors could not influence the promoter activities when transfected alone. However, N-ATF6β overexpression downregulated the N-ATF6α-mediated promoter activity but not that of sXBP1. These results indicate that ATF6β might bind to ERSE-II in the same manner as ATF6α and compete with ATF6α to attenuate ERSE-II-dependent promoter activity.

Under ER stress conditions, each of these factors forms complexes more elaborately on the ERSE-II motif to regulate the expression of MANF genes. Very recently, Wang *et al.* reported that sXBP1 activity is regulated by acetylation and deacetylation, which are respectively mediated by p300 and Sirt1 [39]. However, nicotinamide (NA), an inhibitor of Sirt1, did not upregulate the expression of MANF mRNA in the presence or absence of Tg, while the level of sXBP1 mRNA was remarkably elevated by Tg treatment (Suppl. Fig. 4). It is possible that the protein acetylation and deacetylation requirements for regulating the cellular events differ between cell lines. Differences in the cell types and the reporter constructs might give rise to these inconsistencies.

In this study, we characterized the roles and the relationships of the transcription factors responsible for MANF induction in the regulation of ERSE-II-mediated promoter activity using several types of mutant reporter constructs. We demonstrated that ATF6α potently activates the MANF promoter and that the efficacy is different between the sXBP1 and ATF6 families. In addition to the modification of transcription factors, for example through phosphorylation and acetylation [39-41], other factors, such as C/EBPβ [42, 43] and YY1 [44], are reported to participate in activating the ERSE-containing promoter.

It is suggested that MANF might be a promising factor in the treatment of neurodegenerative diseases, such as Parkinson's disease and ischemic dysfunction [3-6, 29, 30], because the neurotrophic potency of MANF is comparable to that of glial cell line-derived neurotrophic factor rather than that of brain-derived neurotrophic factor [3]. Further characterization of the mechanism for regulating the expression of the MANF gene might provide new insight into the prevention of the onset and progression of these neurodegenerative diseases.

REFERENCES

1. Shridhar, R., Shridhar, V., Rivard, S., Siegfried, J.M., Pietraszkiewicz, H., Ensley, J., Pauley, R., Grignon, D., Sakr, W., Miller, O.J. and Smith, D.I. Mutations in the arginine-rich protein gene, in lung, breast, and prostate cancers, and in squamous cell carcinoma of the head and neck. **Cancer Res.** 56 (1996) 5576-5578.

2. Evron, E., Cairns, P., Halachmi, N., Ahrendt, S.A., Reed, A.L. and Sidransky, D. Normal polymorphism in the incomplete trinucleotide repeat of the arginine-rich protein gene. **Cancer Res.** 57 (1997) 2888-2889.

3. Petrova, P., Raibekas, A., Pevsner, J., Vigo, N., Anafi, M., Moore, M.K., Peaire, A.E., Shridhar, V., Smith, D.I., Kelly, J., Durocher, Y. and

Commissiong. J.W. MANF: a new mesencephalic, astrocyte-derived neurotrophic factor with selectivity for dopaminergic neurons. **J. Mol. Neurosci.** <u>20</u> (2003) 173-188.

4. Voutilainen, M.H., Bäck, S., Pörsti, E., Toppinen, L., Lindgren, L., Lindholm, P., Peränen, J., Saarma, M. and Tuominen, R.K. Mesencephalic astrocyte-derived neurotrophic factor is neurorestorative in rat model of Parkinson's disease. **J. Neurosci.** <u>29</u> (2009) 9651-9659.

5. Airavaara, M., Shen, H., Kuo, C.C., Peränen, J., Saarma, M., Hoffer, B. and Wang, Y. Mesencephalic astrocyte-derived neurotrophic factor reduces ischemic brain injury and promotes behavioral recovery in rats. **J. Comp. Neurol.** <u>515</u> (2009) 116-124.

6. Yu, Y.Q., Liu, L.C., Wang, F.C., Liang, Y., Cha, D.Q., Zhang, J.J., Shen, Y.J., Wang, H.P., Fang, S. and Shen, Y.X. Induction profile of MANF/ARMET by cerebral ischemia and its implication for neuron protection. **J. Cereb. Blood Flow Metab.** <u>30</u> (2010) 79-91.

7. Gething, M.J. and Sambrook, J. Protein folding in the cell. **Nature** <u>355</u> (1992) 33-45.

8. Helenius, A., Marquardt, T. and Braakman, I. The endoplasmic reticulum as a protein-folding compartment. **Trends Cell Biol.** <u>2</u> (1992) 227-231.

9. Kim, I., Xu, W. and Ree, J.C. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. **Nat. Rev. Drug Discov.** <u>7</u> (2008) 1013-1030.

10. Lindholm, D., Wootz. H. and Korhonen, L. ER stress and neurodegenerative diseases. **Cell Death Differ.** <u>13</u> (2006) 385-392.

11. Harding, H.P., Zhang, Y. and Ron, D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. **Nature** <u>397</u> (1999) 271-274.

12. Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G. and Ron, D. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. **Nature** <u>415</u> (2002) 92-96.

13. Zhu, C., Johansen, F.E. and Prywes, R. Interaction of ATF6 and serum response factor. **Mol. Cell. Biol.** <u>17</u> (1997) 4957-4966.

14. Haze, K., Yoshida, H., Yanagi, H., Yura, T. and Mori, K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. **Mol. Biol. Cell** <u>10</u> (1999) 3787-3799.

15. Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M. and Mori, K. Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6α and 6β that activates the mammalian unfolded protein response. **Mol. Cell. Biol.** <u>21</u> (2001) 1239-1248.

16. Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A. and Mori, K. Transcriptional induction of mammalian ER quality

control proteins is mediated by single or combined action of ATF6α and XBP1. **Dev. Cell** <u>13</u> (2007) 365-376.

17. Yamamoto, K., Yoshida, H., Kokame, K., Kaufman, R.J. and Mori, K. Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II. **J. Biochem.** <u>136</u> (2004) 343-350.

18. Mizobuchi, N., Hoseki, J., Kubota, H., Toyokuni, S., Nozaki, J., Naitoh, M., Koizumi, A. and Nagata, K. ARMET is a soluble ER protein induced by the unfolded protein response via ERSE-II element**. Cell Struct. Funct.** <u>32</u> (2007) 41-50.

19. Kokame, K., Kato, H. and Miyata, T. Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. **J. Biol. Chem.** <u>276</u> (2001) 9199-9205.

20. Haze, K., Okada, T., Yoshida, H., Yanagi, H., Yura, T., Negishi, M. and Mori, K. Identification of the G13 (cAMP-response-element-binding protein-related protein) gene product related to activating transcription factor 6 as a transcriptional activator of the mammalian unfolded protein response. **Biochem. J.** <u>355</u> (2001) 19-28.

21. Liang, G., Audas, T.E., Li, Y., Cockram, G.P., Dean, J.D., Martyn, A.C., Kokame, K. and Lu, R. Luman/CREB3 induces transcription of the endoplasmic reticulum (ER) stress response protein Herp through an ER stress response element. **Mol. Cell Biol.** <u>26</u> (2004) 7999-8010.

22. Kondo, S., Murakami, T., Tatsumi, K., Ogata, M., Kanemoto, S., Otori, K., Iseki, K., Wanaka, A. and Imaizumi, K. OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes. **Nat. Cell Biol.** <u>7</u> (2005) 186-194.

23. Kondo, S., Saito, A., Hino, S., Murakami, T., Ogata, M., Kanemoto, S., Nara, S., Yamashita, A., Yoshinaga, K., Hara, H. and Imaizumi, K. BBF2H7, a novel transmembrane bZIP transcription factor, is a new type of endoplasmic reticulum stress transducer. **Mol. Cell Biol.** <u>27</u> (2007) 1716-1729.

24. Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D.T., Back, S.H. and Kaufman, R.J. Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. **Cell** <u>124</u> (2006) 587-599.

25. Stirling, J. and O'hare, P. CREB4, a transmembrane bZip transcription factor and potential new substrate for regulation and cleavage by S1P. **Mol. Biol. Cell** <u>17</u> (2006) 413-426.

26. Asada, R., Kanemoto, S., Kondo, S., Saito, A. and Imaizumi, K. The signalling from endoplasmic reticulum-resident bZIP transcription factors involved in diverse cellular physiology. **J. Biochem.** <u>149</u> (2011) 507-518.

27. Oh-hashi, K., Koga, H., Ikeda, S., Shimada, K., Hirata, Y. and Kiuchi, K. CRELD2 is a novel endoplasmic reticulum stress-inducible gene. **Biochem. Biophys. Res. Commun.** <u>387</u> (2009) 504-510.

28. Oh-hashi, K., Maehara, K. and Isobe, K. Hydrogen peroxide induces GADD153 in Jurkat cells through the protein kinase C-dependent pathway. **Redox Rep.** 9 (2004) 173-178.
29. Lindholm, P. and Saarma, M. Novel CDNF/MANF family of neurotrophic factors. **Dev. Neurobiol.** 70 (2010) 360-371.
30. Glembotski, C.C., Thuerauf, D.J., Huang, C., Vekich, J.A., Gottlieb, R.A. and Doroudgar, S. Mesencephalic astrocyte-derived neurotrophic factor (MANF) protects the heart from ischemic damage and is selectively secreted upon ER calcium depletion. **J. Biol. Chem.** 287 (2012) 25893-25904.
31. Lee, A.H., Iwakoshi, N.N. and Glimcher, L.H. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. **Mol. Cell Biol.** 23 (2003) 7448-7459.
32. Murakami, T., Saito, A., Hino, S., Kondo, S., Kanemoto, S., Chihara, K., Sekiya, H., Tsumagari, K., Ochiai, K., Yoshinaga, K., Saitoh, M., Nishimura, R., Yoneda, T., Kou, I., Furuichi, T., Ikegawa, S., Ikawa, M., Okabe, M., Wanaka, A. and Imaizumi, K. Signalling mediated by the endoplasmic reticulum stress transducer OASIS is involved in bone formation. **Nat. Cell Biol.** 11 (2009) 1205-1211.
33. Namba, T., Ishihara, T., Tanaka, K., Hoshino, T. and Mizushima, T. Transcriptional activation of ATF6 by endoplasmic reticulum stressors. **Biochem. Biophys. Res. Commun.** 355 (2007) 543-548.
34. Thuerauf, D.J., Morrison, L. and Glembotski, C.C. Opposing roles for ATF6α and ATF6β in endoplasmic reticulum stress response gene induction. **J. Biol. Chem.** 279 (2004) 21078-21084.
35. Newman, J.R. and Keating, A.E. Comprehensive identification of human bZIP interactions with coiled-coil arrays. **Science** 300 (2003) 2097-2101.
36. Yoshida, H., Oku, M., Suzuki, M. and Mori, K. pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response. **J. Cell. Biol.** 172 (2006) 565-575.
37. Yoshida, H., Uemura, A. and Mori, K. pXBP1(U), a negative regulator of the unfolded protein response activator pXBP1(S), targets ATF6 but not ATF4 in proteasome-mediated degradation. **Cell Struct. Funct.** 34 (2000) 1-10.
38. Thuerauf, D.J., Marcinko, M., Belmont, P.J. and Glembotski, C.C. Effects of the isoform-specific characteristics of ATF6α and ATF6β on endoplasmic reticulum stress response gene expression and cell viability. **J. Biol. Chem.** 282 (2007) 22865-22878.
39. Wang, F.M., Chen, Y.J. and Ouyang, H.J. Regulation of unfolded protein response modulator XBP1s by acetylation and deacetylation. **Biochem. J.** 433 (2011) 245-252.
40. Luo, S. and Lee, A.S. Requirement of the p38 mitogen-activated protein kinase signalling pathway for the induction of the 78 kDa glucose-regulated protein/immunoglobulin heavy-chain binding protein by azetidine stress:

activating transcription factor 6 as a target for stress-induced phosphorylation. **Biochem. J.** <u>366</u> (2002) 787-795.

41. Donati, G., Imbriano, C. and Mantovani, R. Dynamic recruitment of transcription factors and epigenetic changes on the ER stress response gene promoters. **Nucleic Acids Res.** <u>34</u> 3116-3127.

42. Matsuda, T., Kido, Y., Asahara, S., Kaisho, T., Tanaka, T., Hashimoto, N., Shigeyama, Y., Takeda, A., Inoue, T., Shibutani, Y., Koyanagi, M., Hosooka, T., Matsumoto, M., Inoue, H., Uchida, T., Koike, M., Uchiyama, Y., Akira, S. and Kasuga, M. Ablation of C/EBPβ alleviates ER stress and pancreatic β cell failure through the GRP78 chaperone in mice. **J. Clin. Invest.** <u>120</u> (2010) 115-126.

43. Gade, P., Ramachandran, G., Maachani, U.B., Rizzo, M.A., Okada, T., Prywes, R., Cross, A.S., Mori, K. and Kalvakolanu, D.V. An IFN-γ-stimulated ATF6-C/EBP-β-signaling pathway critical for the expression of Death Associated Protein Kinase 1 and induction of autophagy. **Proc. Natl. Acad. Sci. USA** <u>109</u> (2012) 10316-10321.

44. Li, M., Baumeister, P., Roy, B., Phan, T., Foti, D., Luo, S. and Lee, A.S. ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. **Mol. Cell. Biol.** <u>20</u> (2000) 5096-5106.