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Identification of the cis-element and bZIP DNA binding motifs for the autogenous negative control of mouse NOSTRIN

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ABSTRACT

mNOSTRIN is the mouse ortholog of hNOSTRIN. Unlike hNOSTRIN, which is alternatively spliced to produce two isoforms (α and β), only a single isoform of mNOSTRIN has been detected in either the nucleus or cytoplasm/membrane. Because mNOSTRIN represses its own transcription through direct binding onto its own promoter, this protein is constantly expressed in a temporally regulated pattern during differentiation of F9 embryonic carcinoma cells. In this study, we identified the specific cis-element in the mNOSTRIN regulatory region that is responsible for negative autogenous control. This element exhibits inverted dyad symmetry. Furthermore, we identified a putative bZIP motif in the middle region of mNOSTRIN, which is responsible for DNA binding, and showed that disruption of the leucine zippers abolished the DNA-binding activity of mNOSTRIN. Here, we report that a single form of mNOSTRIN functions in both the nucleus and cytoplasm/membrane. In the nucleus, mNOSTRIN acts as a transcriptional repressor by binding to the cis-element through its bZIP motif.

1. Introduction

mNOSTRIN is the mouse ortholog of human NOSTRIN (hNOSTRIN) [1], which is involved in secretion and trafficking of eNOS (nitric oxide synthase) [2]. Both hNOSTRIN and mNOSTRIN share an FCH (Fes/Cip4 homology) domain at the N-terminus, two coiled-coil domains in the middle region, and an SH3 domain at the C-terminus. hNOSTRIN functions in a multi-step process, acting as an adaptor for eNOS trafficking while simultaneously associating with multiple binding partners via its SH3 domain [3,4], with which eNOS also interacts. Homo-oligomerization of the protein is required for this process, and the second coiled-coil region is responsible for this activity [4]. The FCH domain governs the subcellular localization of hNOSTRIN. hNOSTRIN is usually localized at the plasma membrane and on intracellular vesicles, and it is partially associated with filamentous structures. Deletion of the FCH domain causes the protein to be localized in the nucleus, and further deletion of the coiled-coil region causes the protein to be diffusely distributed throughout the cytoplasm and nucleus [5]. mNOSTRIN is expressed in a retinoic acid (RA)-responsive manner during RA-induced differentiation of F9 cells into visceral endoderm-like cells [1]. However, we could detect neither expression nor activity of eNOS in RA-treated F9 cells. Endocytosis is one of the main processes observed in visceral endoderm [6,7]; hNOSTRIN, which belongs to the BAR protein family, is likely to be involved in membrane dynamics associated with endocytosis. We suggested previously that mNOSTRIN induced in RA-treated F9 embryonic carcinoma cells might play a role in a process other than eNOS trafficking [8]. In the meantime, another study showed that hNOSTRIN and mNOSTRIN control their own gene expression in an autoregulatory negative-feedback loop by binding to their own 5′ regulatory regions [8,9]. Via alternative splicing, the hNOSTRIN gene produces another isoform (NOSTRIN β; amino acids (a.a.) 79–506 of NOSTRIN α) that carries out its role in the nucleus [9]. Deletion of the N-terminus (a.a. 1–164) abolishes the DNA-binding activity of NOSTRIN β; a putative bZIP motif present in the deleted region has been postulated to be involved in the DNA binding [9]. In a yeast two-hybrid assay of hNOSTRIN, however, a deletion mutant containing only the first coiled-coil region (a.a. 1–288 of hNOSTRIN) did not dimerize, whereas a deletion mutant containing the second coiled-coil region (a.a. 323–470) did [4].

We have not observed multiple isoforms of mNOSTRIN; the protein has the same size in both the nucleus and the cytoplasm/membrane. This observation implies that a single form of the protein plays dual roles in two different cellular compartments.

Here, we present novel data regarding the DNA cis-element of mNOSTRIN, the putative DNA binding domain of mNOSTRIN, and how mNOSTRIN functions as a trans-element through its putative bZIP motif.

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2. Materials and methods

2.1. Plasmid construction

The 5′ flanking DNA fragment of the mNOSTRIN gene (−200, −400) from pGL3::−200/0, pGL3::−400/0 [8] and the amplification product genomic DNA using specific primers (5′-ttggtacctccca ctggctcttttgacccag-3′ and 5′-aaagatctgttgaaatgctgtctgctttttc-3′) were cloned the pGL4.10 plasmid (Promega). pEF-flag::mNOSTRIN was generated by ligation of the DNA fragment to the pEF-mDaIP2, described previously [8]. To obtain C-terminally tagged mNOSTRIN, cDNAs were amplified with specific primer sets not containing the stop codon. Amplified DNA was ligated into the pEF1/Myc-His vector (Invitrogen). Complement 2× repeated inverted repeat (2×IR) synthesized oligonucleotides were ligated into the pGL4.23, which includes a minimal promoter (Promega). pEF-HA-mNOSTRIN, pET14b-mNOSTRIN, and pGEX-4T3-mNOSTRIN were described previously [1,8]. To generate deletion constructs of mNOSTRIN, each fragment was amplified by PCR using site-specific primers flanked with restriction enzyme sites (*Mlu*I and *Bgl*II). Each PCR product was cloned into pET14b (Novagen). Each plasmid was verified by automated sequencing.

2.2. Luciferase assay and mammalian one-hybrid assay

F9 cells were seeded in 24-well culture plates on the day before the transfection, and then the cells were incubated with a mixture of DNA and Lipofectamine 2000 reagent, according to the manufacturer's instructions. pGL4.10-promoter construct (0.3 μg) and pEF1/Myc-His expression vectors were co-transfected. The bait plasmid was pCMV-AD harboring mNOSTRIN fused with the NF-kB activation domain, and pGL4.23::2×IR was used as the prey. Each plasmid was co-transfected into Cos-1 cells with linear polyethyleneimine (L-PEI). Passively lysed cell extracts were measured using a Luminoskan Ascent Microplate luminometer (Thermo scientific). Plasmids pGL4.10 and pRL-TK were used as negative and internal controls, respectively.

2.3. Recombinant protein purification

GST-mNOSTRIN was purified from *Escherichia coli* DH5α using batch Glutathione Sepharose™ 4B (Amersham Pharmacia Biotech), according to the manufacturer's instructions. His-mNOSTRIN, His-FCH, and His-ΔSH3 were purified from *E. coli* Rosetta2 (DE3) pLysS using a HisTrap HP column (GE Healthcare) according to the manufacturer's instructions. The purity of His_6-tagged recombinant proteins in each fraction was analyzed on SDS–PAGE following by staining with Coomassie blue R-250. Pools of eluted fractions were concentrated using an Amicon Ultra centrifugal filter unit (Millipore). Chromatography was performed at 4 °C.

2.4. Electrophoretic mobility shift assay

Fully annealed electrophoretic mobility shift assay (EMSA) probes were labeled with biotin (Cosmogenetech). Binding reactions were conducted using LightShift® Chemiluminescent EMSA Kit (ThermoScientific) with 50 fmol of purified His-mNOSTRIN, according to the manufacturer's instruction. The reaction mixtures were then loaded onto 5% polyacrylamide gels in TBE. The competition assay, a 50-fold or 100-fold molar excess of unlabeled probe was mixed with purified proteins and then the labeled probe was added to the mixture. The reaction mixtures for the super-shift experiment were incubated for 20 min with 1 μl of anti-His antibody (Amersham Pharmacia Biotech) at room temperature, after the binding reactions.

2.5. Cell culture and retinoic acid treatment

F9 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum on culture plates coated with 0.1% gelatin at 37 °C in a 5% CO_2 incubator. To elicit differentiation, cells were treated with 1×10^{-6} M all-*trans*-Retinoic acid for 2, 4, or 6 days. Cos-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS).

2.6. Subcellular fractionation

RA-treated F9 cells were collected by centrifugation. Cytoplasmic and nuclear fractions were prepared in buffers A and C, respectively [10]. The protein from each fraction was separated by SDS–PAGE (8% gel) and analyzed by immunoblotting, using mNOSTRIN antisera. Anti-c-fos and anti-ERK2 (Santa Cruz Biotechnology) were used as nuclear and cytoplasmic controls, respectively.

2.7. Immunofluorescence

pEF-HA-mNOSTRIN Transfected F9 cells were washed in PBS and fixed in 3.7% formaldehyde/PBS. After being washed twice in PBS-Blotto (1% skim milk in PBS), cells were permeabilized with methanol for 10 min at −20 °C and 0.5% Triton X-100 in PBS for 10 min, washed again in PBS, incubated in PBS-Blotto with anti-HA mouse monoclonal antibody (Santa Cruz Biotechnology) for 1 h at 37 °C, washed again, and incubated with FITC-conjugated goat anti-mouse IgG (Sigma) for 1 h at 37 °C. Cells were also stained with DAPI/PBS (1 μg/ml) for 1 min to identify the nucleus. Cells were visualized using an inverted microscope (Zeiss) equipped with fluorescence illumination.

2.8. Co-immunoprecipitation and Western blotting

Transfected Cos-1 cells were washed in cold PBS and lysed with 1% NP-40 in PBS. One mg of each protein sample was incubated with 1 μg of the indicated antibodies for 16 h at 4 °C. Then, A/G agarose slurry (Santa Cruz) was added to the mixture and incubated for 4 h. After five washes, half of the boiled samples were immunoblotted with anti-FLAG antibody, and the other half with anti-Myc antibody.

2.9. Site-directed mutagenesis

pGL4.10::−223 plasmid was amplified using mutation primer (5′-GCCGGTACCTCCCACT**TT**C**GG**TTTTGACCCAGAGC-3′) and whole mNOSTRIN expression plasmids were amplified using mutation primers (L359F: 5′-GAAACTAGACCTTTT**C**CAAGCGAACTCCTAC-3′; L366P: 5′-GAACTCCTACAAAC**C**GTCGTCAGTGTTG-3′; L373P: GTGTTGGCAGACC**C**GGAGCAACGGCC). Amplified DNAs were digested with *Dp*nI, ligated for 4 h, and then transformed to competent cells. Sequence-verified coding sequences were subcloned into each new vector.

3. Results

3.1. Identification of the specific binding site for mNOSTRIN

As we reported previously, mNOSTRIN transcriptionally represses its own promoter. Prior to this study, however, the cis-element responsible for this negative regulation had not been identified. To find this element, we analyzed the region of the 5′ upstream region from −400 to −200 bp upstream of the start codon (Fig. 1A). Our analysis revealed a distinctive inverted repeat

sequence between −218 and −201 bp (CTGGCTCTTTTGACCCAG). In preliminary DNase I footprinting assays, we observed weak protection around this region (data not shown).

To determine the role of this sequence element in mNOSTRIN self-inhibition, we used a luciferase assay system with pGL4.10 and pRL-TK as a normalization control (Fig. 1B). Exogenous mNOSTRIN expressed in wild-type F9 cells caused slightly different expression patterns in pGL4.10::−223 and pGL4.10::−200. There were slight inhibitions in −400 to −223 regions with mNOSTRIN expression, while the inhibition was not detected in +1 to −200 region. To confirm the effect of mNOSTRIN on inhibition, we used stepwise increments of ectopic mNOSTRIN expression. Luciferase activities of pGL4.10::−223 decreased sequentially when mNOSTRIN expression was increased in RA-treated or -untreated F9 cells, while pGL4.10::−223 mutant which CTGGCTC was substituted to CTTTCGG (Fig. 1A) didnot (Fig. 1C). These results suggest that the inhibitory effect of mNOSTRIN on its own promoter is related to the region between −223 and −200 bp. Therefore, we investigated whether the inverted repeat region is a cis-element for mNOSTRIN, using a mammalian one-hybrid assay (Fig. 1D).

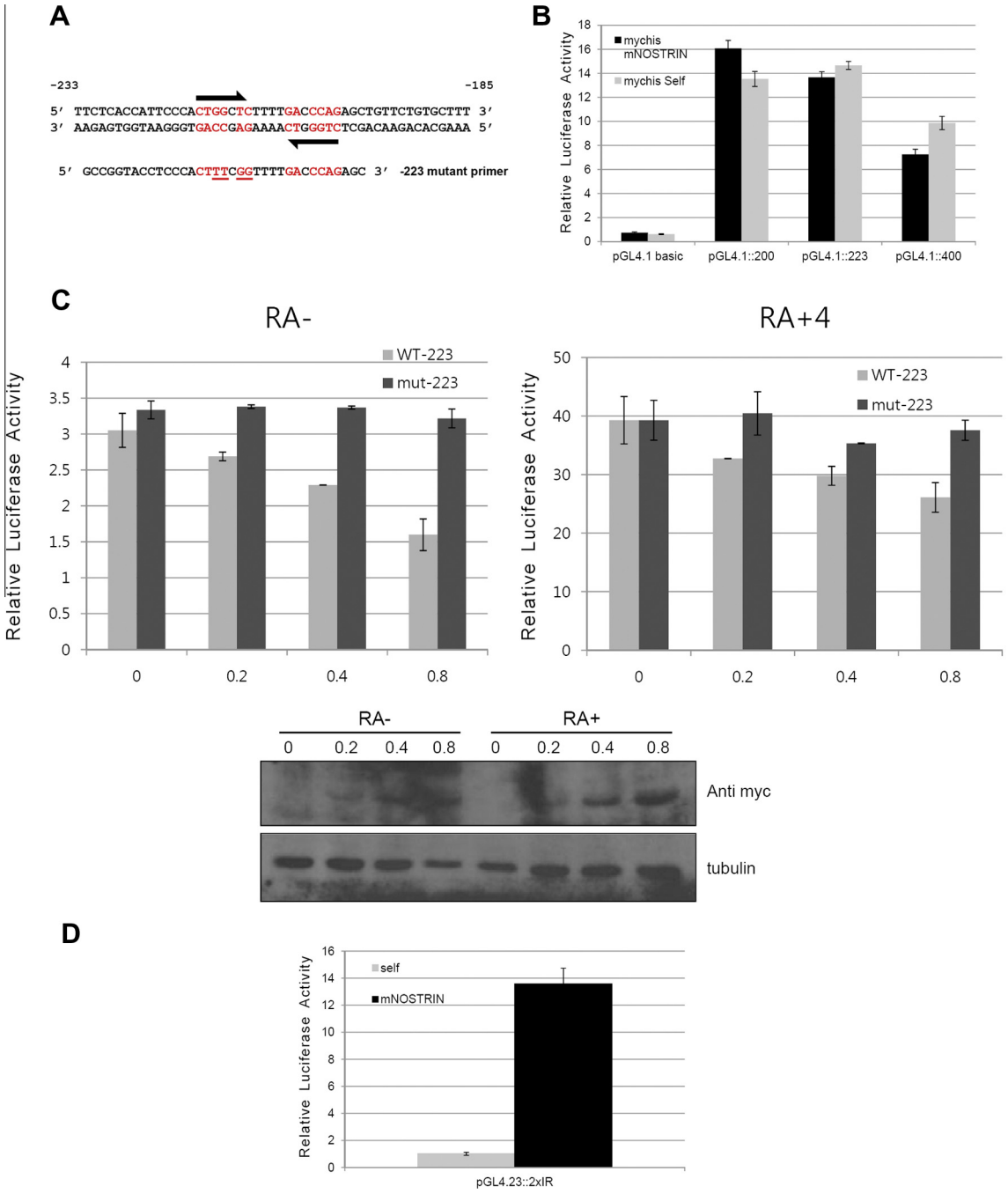

Fig. 1. Identification of the mNOSTRIN relative regulatory region. (A) The inverted repeat in the 5′ regulatory region of the mNOSTRIN gene. A pair of arrows indicates the inverted repeat sequence, (B) Sequential deletion-mutant of the mNOSTRIN promoter region. pGL4.10::−200, −223, and −400 plasmids were analyzed for their effect on mNOSTRIN expression. (C) Stepwise expression of mNOSTRIN effect on −223 promoter region. RA-treated or -untreated F9 cells were co-transfected with pGL4.10::−223 or pGL4.10::−223 mutant and the indicated amount (μg) of pEF1/Myc-His::mNOSTRIN expression vector. Extracts from each cell type were measured using a luminometer. (D) The interaction of mNOSTRIN protein and the putative inverted repeat sequence was further analyzed with mammalian one-hybrid experiments in Cos-1 cells. pCMV-AD and pCMV-AD-mNOSTRIN were used as baits. Cells were co-transfected with pGL4.23::2×IR, as well as each bait construct.

Plasmid pCMV-AD contains the transcriptional activation domain (AD) of NF-kB, and was used here for expression of AD-mNOSTRIN fusion protein. The prey vector contained two copies of the inverted repeat region (2×IR) in the pGL4.23 reporter plasmid, which includes minimal promoter and firefly luciferase genes. We detected a 13-fold increase of luciferase activity in cells expressing the AD-mNOSTRIN fusion protein, relative to cells transfected with pCMV-AD alone.

We designed two DNA oligomers as probes for an EMSA. Each oligomer contained a half site of the inverted repeat (−207 to −183 and −231 to −212) (Fig. 2A). One of them (probe#1) alone exhibited a weak mobility shift, but the other one (probe#2) did not (data not shown). We designed another probe containing the full inverted repeat (−223 to −196) (Fig. 2A). Mobility shift was exhibited in both left half and inverted repeat site. Stronger binding was detected in the inverted repeat than in the half site (Fig. 2B, lanes 2 and 9). In cross competition assay between the half and inverted repeat sites (Fig. 2B, lanes 5, 6, and 10–13), complete competition with the inverted repeat appeared against both half and inverted repeat sites, compared with the half site competition. This specific binding was supported by a super-shift assay carried out using an antibody against mNOSTRIN (Fig. 2B, lanes 7 and 14). These data indicate that mNOSTRIN specifically binds to the inverted repeat region of the mNOSTRIN promoter, and that this region is a core cis-element for the binding. Our in vitro data showed that mNOSTRIN asymmetrically binds to the left half site only. We do not know why such an asymmetric binding occurs. We surmise that the asymmetric binding may be due to the different flanking sequences of the inverted repeat, suggesting that these sequences may be involved in the binding. And also it is weird that the half site did show an incomplete competition with 50-fold half site cold probe (Fig. 2B, lane 3), while the competition was complete when 100-fold cold probe was added (lane 4). Probably we thought that it was a kind of experimental error.

3.2. Characterization of mNOSTRIN expression during F9 differentiation and domain for binding

The expression of the mNOSTRIN gene increases as F9 cells differentiate into visceral endoderm under RA treatment [1]. We carried out subcellular fractionation of mNOSTRIN to analyze the changes in its localization that accompany F9 cell differentiation. Nuclear localization of the protein increased as cell differentiation proceeded (Fig. 3A). Immunofluorescence assays revealed that the protein resided either in the nucleus or the cytoplasm over the course of differentiation (Fig. 3B). Some cells exhibited a distinct localization of mNOSTRIN in the nucleus. In subcellular fractionation assays, the concentration of the protein in the nucleus increased gradually as the cells differentiated into visceral endoderm. We believe that the mNOSTRIN protein is present in both nucleus and cytoplasm during the process of differentiation, and that the biased localization of the protein into the nucleus may reflect further differentiation into visceral endoderm.

In the case of hNOSTRIN, the NOSTRIN β isoform is localized in the nucleus in cirrhotic cells. However, mNOSTRIN is unlikely to produce another isoform during the F9 cell differentiation process, as reflected by the observation that the protein was the same size in both nucleus and cytoplasm in this experiment (Fig. 3A).

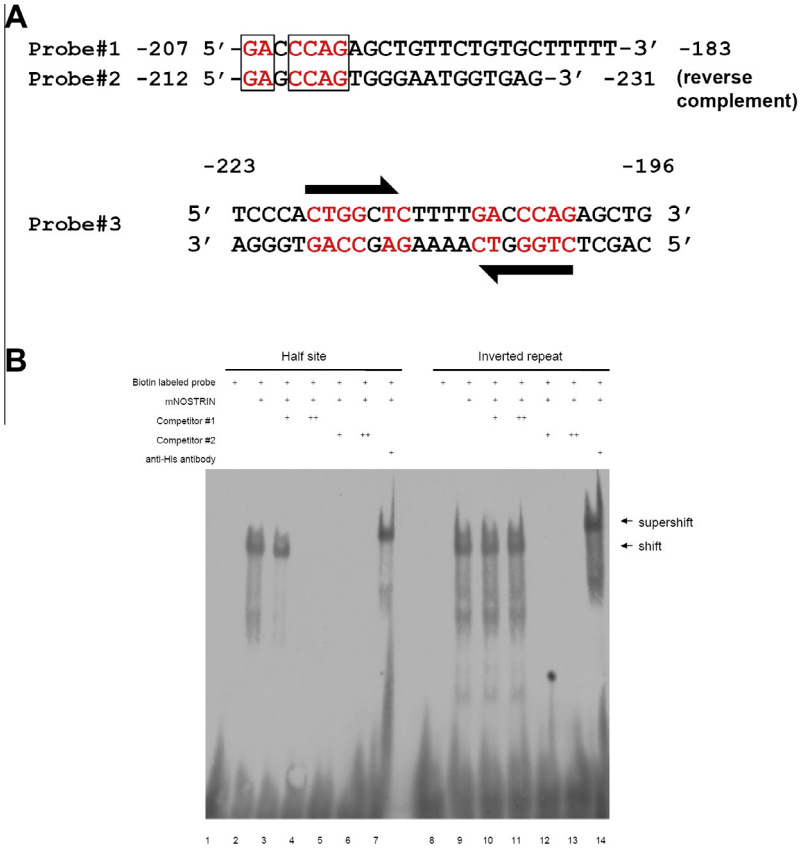

Fig. 2. mNOSTRIN binding to the inverted repeat in the 5′ regulatory region of the mNOSTRIN gene. (A) DNA probes used in the electrophoretic mobility shift assay (EMSA). (B) Purified His-mNOSTRIN was used for EMSA. The half of inverted repeat (5′-GACCCAGAGCTGTTCTGTGCTTTTT-3′, corresponding to −207 to −183) and inverted repeat (5′-TCCCACTGGCTCTTTTGACCCAGAGCTG-3′, corresponding to −223 to −196) were labeled with Biotin and used as probes. Competitor #1 (unlabeled half site) was used in 50-fold (lanes 3 and 10) or 100-fold molar excess (lanes 4 and 11). Competitor #2 (unlabeled inverted repeat) was also used in 50-fold (lanes 5 and 12) or 100-fold molar excess (lanes 6 and 13). Anti-His antibody was used for the super-shift (lanes 7 and 14).

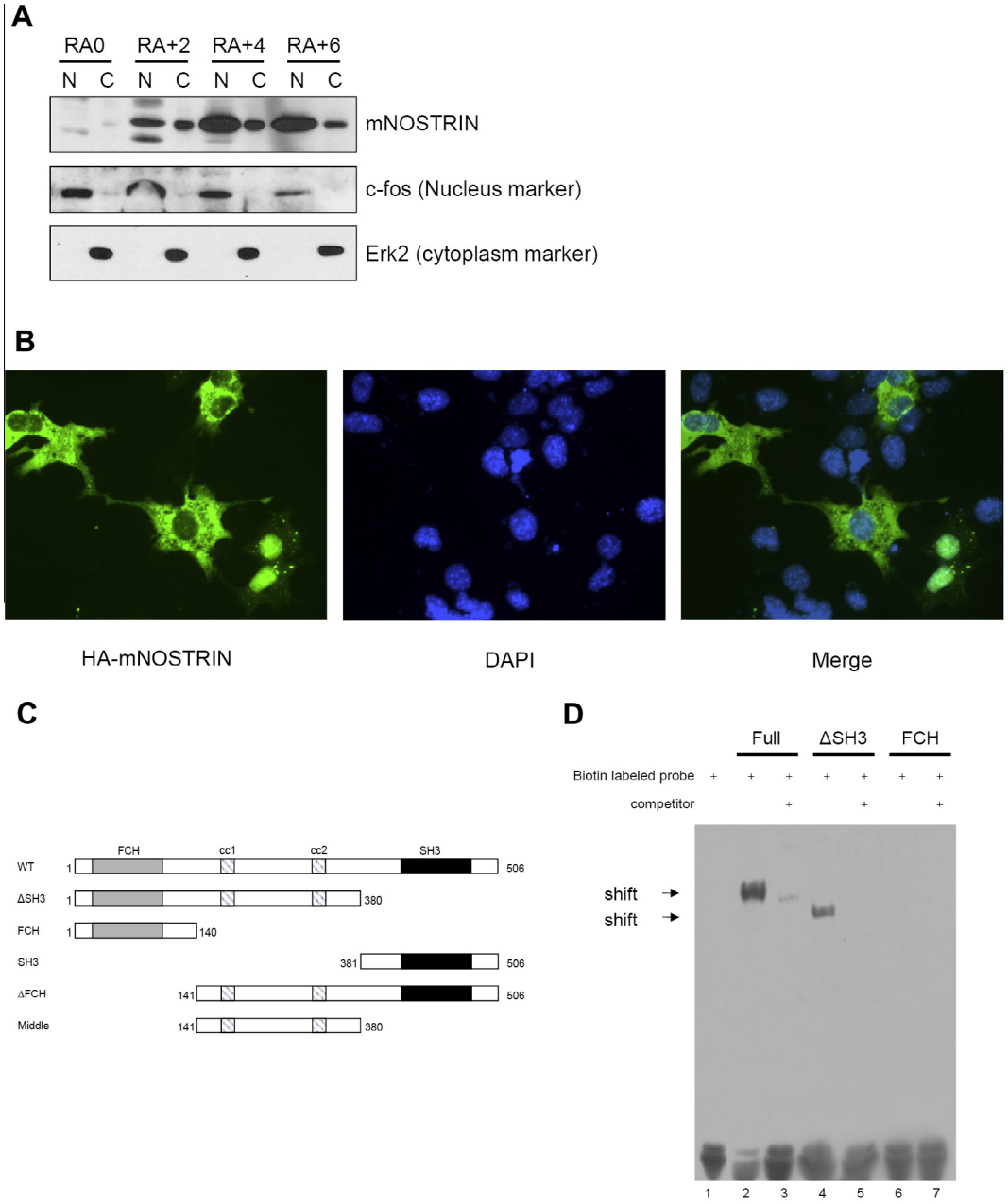

Fig. 3. Characterization of mNOSTRIN expression during F9 differentiation and binding assays using deletion mutants of mNOSTRIN. (A) Subcellular localization of mNOSTRIN. RA-treated F9 cells were separated into nuclear and cytoplasmic fractions. Equal amounts of nuclear and cytoplasmic proteins were analyzed by SDS–PAGE and immunoblotted with mNOSTRIN antisera. The same blots were stripped and reblotted for Erk2 (as a cytoplasmic marker) or c-fos (as a nuclear marker). N, nuclear fraction; C, cytoplasmic fraction. (B) Indirect immunofluorescence to determine localization of mNOSTRIN. RA-treated F9 cells were transfected with pEF-HA-mNOSTRIN. The HA-mNOSTRIN protein was observed under a fluorescence microscope. (C) Schematic diagram of mNOSTRIN deletion mutants used in EMSA. (D) Full-length mNOSTRIN (WT) and deletion mutants (ΔSH3, FCH) were used in EMSA. The inverted repeat was labeled with Biotin and used as a probe. For the competition assay, a 100-fold molar excess of unlabeled inverted repeat was added (lanes 3, 5, and 7). Shift was observed in the presence of WT and ΔSH3 mNOSTRIN (lanes 2, 3, and 4).

Because the inverted repeat region is quite likely to be the cis-element for mNOSTRIN binding, we investigated which part of the mNOSTRIN protein is critical for promoter binding. Several mNOSTRIN deletion-mutant plasmids were constructed using the pET14b vector, which includes a His-tag, and the resultant proteins were expressed and purified. The deletion constructs were FCH (a.a. 1–140, including the FCH region), ΔSH3 (a.a. 1–380, excluding the SH3 region), WT (a.a. 1–506, including the whole protein), SH3 (a.a. 38–506, including the SH3 region), ΔFCH (a.a. 141–506, excluding the FCH region), and Middle (a.a. 141–380, excluding the FCH and SH3 regions) (Fig. 3C). Middle, SH3, and ΔFCH could not be obtained in practical quantities, because they were so low in solubility and were unable to renature and refold efficiently. Therefore, we conducted the binding assay using only three forms of the protein: WT, FCH, and ΔSH3. Two of them, WT and ΔSH3, bound to the inverted repeat, whereas FCH did not (Fig. 3D).

3.3. The DNA-binding domain is a putative leucine zipper homodimer

The middle region of mNOSTRIN is responsible for DNA binding. A previous report [4] showed using GST-pull down and yeast two-hybrid assays that hNOSTRIN functions as a homotrimeric adaptor when eNOS is internalized, and that the a.a. 324–434 region is critical for self-binding. According to our results, this region

overlaps with the middle region containing the second coiled-coil. We used two different tags (Myc and FLAG) to make fusion proteins with mNOSTRIN, and performed co-immunoprecipitation to observe self-binding (Fig. 4A). Cell extract from Cos-1 cells transfected with Myc- and FLAG-tagged mNostrin expression cassettes were precipitated with anti-Myc antibody and immunoblotted

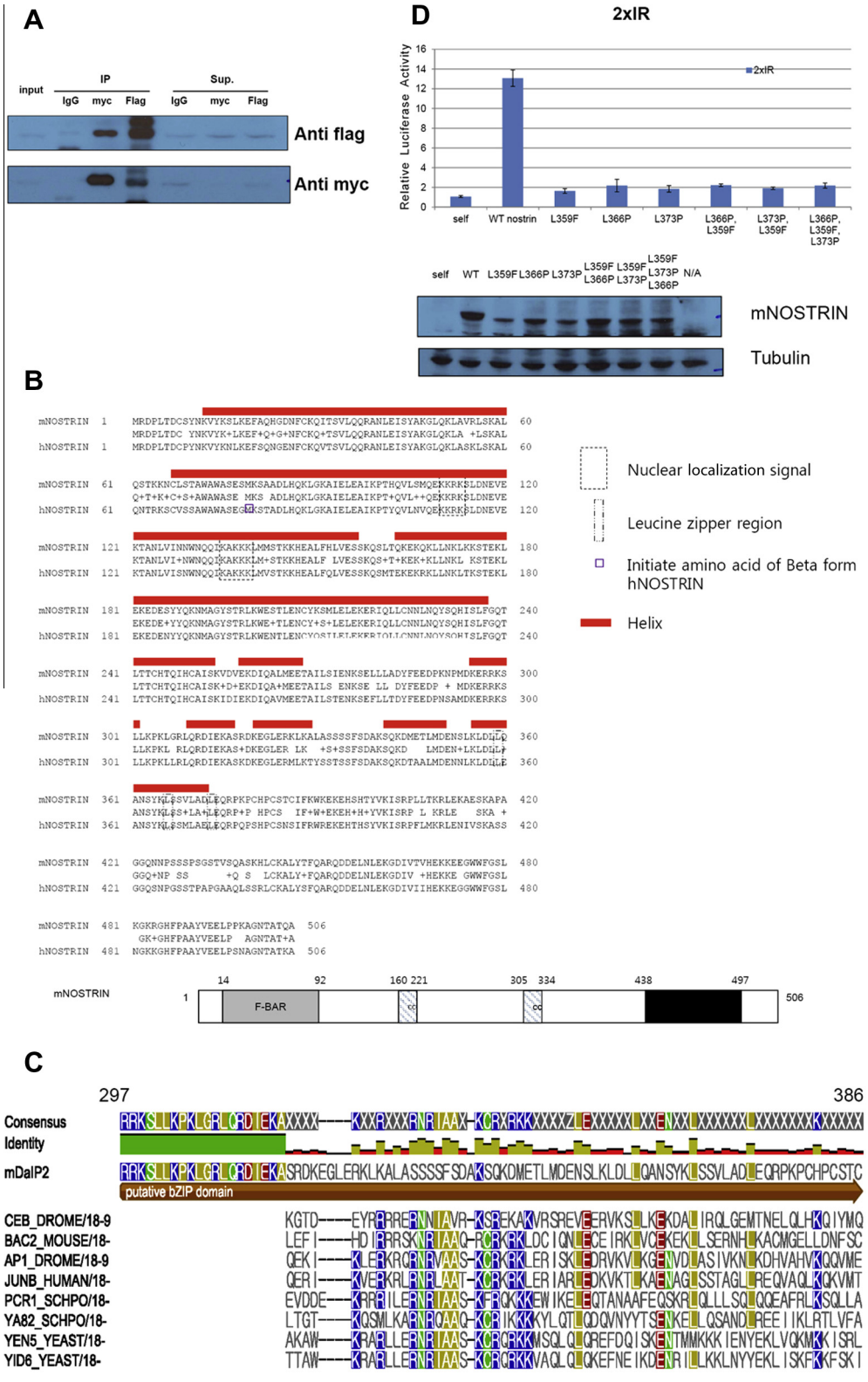

Fig. 4. DNA-binding domain as a putative leucine zipper homodimer. (A) Interaction between mNOSTRIN proteins with different tags. Cos-1 cells were co-transfected with pEF1/Myc-His::mNOSTRIN and pEF-FLAG::mNOSTRIN. Total extracts were prepared and immunoprecipitated with anti-FLAG and anti-Myc antibodies. Each precipitate was immunoblotted using both antibodies. (B) Amino-acid sequences of mNOSTRIN analyzed using the PSIPRED web software. (C) Comparative analysis of representative bZIP superfamily proteins and mNOSTRIN. (D) Interaction between WT or mutant mNOSTRIN and the 2× inverted repeat region, as determined by mammalian one-hybrid assay. pCMV-AD::mNOSTRIN WT and the indicated substitution mutants were used as baits. pGL4.23::2×IR and each bait were co-transfected, and whole-cell extracts were assayed using a luminometer.

with anti-FLAG antibody. A large amount of the FLAG-mNOSTRIN was co-immunoprecipitated with Myc-mNOSTRIN, but neither was detected in precipitations with pre-immune serum. This finding implies that the mNOSTRIN undergoes oligomerization and binds to the inverted repeat.

One of the most well-known three-dimensional structural motifs involved in DNA binding by transcription factors is the basic leucine zipper (bZIP). This motif exhibits common patterns: dimerization, formation of parallel alpha helices, and leucines at 7-residue intervals. We analyzed the mNOSTRIN amino-acid sequences using the PSIPRED computational prediction software to identify the location of alpha helices. The results revealed that the a.a. 295–372 region has a tendency to form alpha helices (Fig. 4B). A comparison of the amino-acid sequence of mNOSTRIN with the conserved amino acids of eight representative bZIP superfamily proteins revealed the presence of basic amino acids and multiple leucines at 7-residue intervals (Fig. 4C). We replaced these leucine residues with phenylalanine and proline (L359F, L366P, and L373P), and subcloned the resultant mutants into the pCMV-AD mammalian one-hybrid expression vector. DNA-binding activities significantly decreased, not only in the triple mutant (L359F, L366P, and L373P), but also in the single mutants (Fig. 4D). These observations suggest that the putative bZIP located in the a.a. 297–386 region of mNOSTRIN is critical for DNA binding.

4. Discussion

Two isoforms of hNOSTRIN have been observed in cirrhotic cells. One isoform (α) is full-length hNOSTRIN, and the other (β) is a shortened variant. The α isoform is involved in endocytosis, whereas the β isoform is involved in negative autoregulation [9]. At present, no other spliced form of mNOSTRIN has been observed; the protein is the same size in both nucleus and cytoplasm (membrane fraction included) in RA-treated F9 cells differentiating into visceral endoderm. Maternal nutrients are actively transported into the embryo through endocytosis in visceral endoderm [6,7]. mDab2, along with cublin and amnionless, is one of the major proteins involved in this process [11–13]. mNOSTRIN and mDab2 are expressed in similar temporally regulated patterns, and are associated with each other during F9 cell differentiation into visceral endoderm [1]. This observation implies that mNOSTRIN may be involved in endocytosis along with mDab2. Based on our observations, it is likely that a single spliced form of mNOSTRIN plays dual roles in two or more different compartments, as in the cases of Epsin and APC [14,15]. At present we do not understand why hNOSTRIN and mNOSTRIN, which share 83% amino-acid identity, use different strategies to carry out the same mode of regulation. Moreover, we do not understand the function of negative autogenous control of *Nostrin* gene expression. Is the maintenance of NOSTRIN protein *in vivo* critical for a certain function, such as endocytosis or cell differentiation? Further research will be required to understand these mechanisms.

The responsive element for the negative autogenous control of mNOSTRIN lies from −218 to −201 bp and exhibits typical inverted dyad symmetry. We believe that oligomerized mNOSTRIN (dimer or tetramer) binds to the cis-element and acts as a transcriptional repressor. hNOSTRIN forms a homo-dimer or -trimer via the second coiled-coil region, which simultaneously associates with N-WASP, dynamin, and eNOS itself during eNOS trafficking [4,16]. In computational analysis (using PrebZIP), both hNOSTRIN and mNOSTRIN were determined to contain two putative bZIP motifs, one in each coiled-coil region. In EMSA, a deletion mutant of NOSTRIN β lacking the first coiled-coil region, including the putative bZIP, failed to bind to the upstream region of the hNOSTRIN promoter [9]. Furthermore, in a yeast two-hybrid assay, the first coiled-coil region did not dimerize, whereas the second coiled-coil region did [4]. In our mammalian one-hybrid assay, disruption of the bZIP motif in the second coiled-coil almost abolished reporter enzyme activity. This observation implied that the second bZIP motif is responsible for mNOSTRIN binding to its responsive element. These results can be summarized as follows: (i) The first coiled-coil containing a putative bZIP motif does not oligomerize on its own, but is required for DNA binding. (ii) The second bZIP motif is critical for oligomerization and DNA binding, but alone it cannot bind to the specific cis-element. (iii) Disruption of either of these motifs abolishes the DNA-binding activity of mNOSTRIN, i.e., the two motifs cooperate in binding to the cis-element.

This cooperative binding is a distinctive feature of mNOSTRIN protein. Transcriptional regulators such as Jun and Fos, which have a single bZIP motif per molecule, must oligomerize to bind to their dyad symmetrical cis-elements. By contrast, mNOSTRIN has two putative bZIP motifs. To achieve cooperative binding, the bZIP motifs might associate in at least two ways: intramolecular association between two bZIPs within a single mNOSTRIN protein, or intermolecular association via two bZIPs in different mNOSTRIN proteins. Whatever the association mode, the findings described here suggest that mNOSTRIN binding to its cis-element is cooperative. It is evident that the protein must be oligomerized to maintain a multivalent state during eNOS trafficking in the cytoplasm; consequently, the intermolecular association seems more plausible. However, the intramolecular association cannot be excluded, because the association can be able to project two basic regions toward the responsive element in the nucleus. The crystallization of the protein–DNA complex is currently underway, with the goal of precisely determining the details of this association.

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References

[1] Y.J. Choi, S.Y. Cho, H.W. Kim, J.A. Kim, S.H. Bae, S.S. Park, Cloning and characterization of mouse disabled 2 interacting protein 2, a mouse orthologue of human NOSTRIN, Biochem. Biophys. Res. Commun. 326 (Jan 21) (2005) 594–599.
[2] K. Zimmermann, N. Opitz, J. Dedio, C. Renne, W. Muller-Esterl, S. Oess, NOSTRIN: a protein modulating nitric oxide release and subcellular distribution of endothelial nitric oxide synthase, Proc. Natl. Acad. Sci. USA 99 (Dec 24) (2002) 17167–17172.
[3] B.R. Carlson, K.E. Lloyd, A. Kruszewski, I.H. Kim, R.M. Rodriguiz, C. Heindel, et al., WRP/srGAP3 facilitates the initiation of spine development by an inverse F-BAR domain, and its loss impairs long-term memory, J. Neurosci. 31 (Feb 16) (2011) 2447–2460.
[4] A. Icking, S. Matt, N. Opitz, A. Wiesenthal, W. Muller-Esterl, K. Schilling, NOSTRIN functions as a homotrimeric adaptor protein facilitating internalization of eNOS, J. Cell Sci. 118 (Nov 1) (2005) 5059–5069.
[5] A. Icking, K. Schilling, A. Wiesenthal, N. Opitz, W. Muller-Esterl, FCH/Cdc15 domain determines distinct subcellular localization of NOSTRIN, FEBS Lett. 580 (Jan 9) (2006) 223–228.
[6] R. Sharma, S. Peel, Uptake of marker proteins by glycoprotein-containing cells of the pregnant rat uterus and placenta, J. Anat. 129 (Dec) (1979) 707–718.
[7] B.L. Hogan, R. Tilly, Cell interactions and endoderm differentiation in cultured mouse embryos, J. Embryol. Exp. Morphol. 62 (Apr) (1981) 379–394.
[8] H.W. Kim, Y.J. Choi, J.A. Kim, S.H. Bae, K.R. Kim, S.S. Park, Mouse disabled 2 interacting protein 2 functions as a transcriptional repressor through direct binding onto its own promoter, Biochem. Biophys. Res. Commun. 337 (Nov 11) (2005) 75–81.
[9] A. Wiesenthal, M. Hoffmeister, M. Siddique, I. Kovacevic, S. Oess, W. Muller-Esterl, et al., NOSTRINbeta – a shortened NOSTRIN variant with a role in transcriptional regulation, Traffic 10 (Jan) (2009) 26–34.
[10] N.C. Andrews, D.V. Faller, A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells, Nucleic Acids Res. 1 (May 11) (1991) 2499.
[11] M.E. Maurer, J.A. Cooper, Endocytosis of megalin by visceral endoderm cells requires the Dab2 adaptor protein, J. Cell Sci. 118 (Nov 15) (2005) 5345–5355.

[12] S. Strope, R. Rivi, T. Metzger, K. Manova, E. Lacy, Mouse amnionless, which is required for primitive streak assembly, mediates cell-surface localization and endocytic function of cubilin on visceral endoderm and kidney proximal tubules, Development 131 (Oct) (2004) 4787–4795.
[13] B.T. Smith, J.C. Mussell, P.A. Fleming, J.L. Barth, D.D. Spyropoulos, M.A. Cooley, et al., Targeted disruption of cubilin reveals essential developmental roles in the structure and function of endoderm and in somite formation, BMC Dev. Biol. 6 (2006) 30.
[14] J. Hyman, H. Chen, P.P. Di Fiore, P. De Camilli, A.T. Brunger, Epsin 1 undergoes nucleocytosolic shuttling and its eps15 interactor NH(2)-terminal homology (ENTH) domain, structurally similar to Armadillo and HEAT repeats, interacts with the transcription factor promyelocytic leukemia Zn(2)+ finger protein (PLZF), J. Cell Biol. 149 (May 1) (2000) 537–546.
[15] M. Brocardo, B.R. Henderson, APC shuttling to the membrane, nucleus and beyond, Trends Cell Biol. 18 (Dec) (2008) 587–596.
[16] K. Schilling, N. Opitz, A. Wiesenthal, S. Oess, R. Tikkanen, W. Muller-Esterl, et al., Translocation of endothelial nitric-oxide synthase involves a ternary complex with caveolin-1 and NOSTRIN, Mol. Biol. Cell 17 (Sep) (2006) 3870–3880.