Acta Biochim Biophys Sin (2008): 526-532 | © 2008 Institute of Biochemistry and Cell Biology, SIBS, CAS | All Rights Reserved 1672-9145
http://www.abbs.info; www.blackwellpublishing.com/abbs | DOI: 10.1111/j.1745-7270.2008.00428.x

# First intron of nestin gene regulates its expression during C2C12 myoblast differentiation

Hua Zhong[1,2], Zhigang Jin[2], Yongfeng Chen[2], Ting Zhang[2], Wei Bian[2], Xing Cui[1]*, and Naihe Jing[2]*

[1] Shan Dong University Medical School, Jinan 250012, China
[2] Laboratory of Molecular Cell Biology, Key Laboratory of Stem Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

**Nestin is an intermediate filament protein expressed in neural progenitor cells and in developing skeletal muscle. Nestin has been widely used as a neural progenitor cell marker. It is well established that the specific expression of the nestin gene in neural progenitor cells is conferred by the neural-specific enhancer located in the second intron of the nestin gene. However, the transcriptional mechanism of nestin expression in developing muscle is still unclear. In this study, we identified a muscle cell-specific enhancer in the first intron of mouse nestin gene in mouse myoblast C2C12 cells. We localized the core enhancer activity to the 291–661 region of the first intron, and showed that the two E-boxes in the core enhancer region were important for enhancer activity in differentiating C2C12 cells. We also showed that MyoD protein was involved in the regulation of nestin expression in the myogenic differentiation of C2C12 cells.**

*Keywords* nestin; C2C12 myoblast; muscle-specific enhancer; MyoD

Skeletal muscle in mammals is a mesodermal derivative and comes from precursor cells present in the somite of embryos [1]. Myogenesis includes generation of the myogenic progenitor cells in the somite, and the differentiation and maturation of these progenitor cells. Under normal growth conditions, newly formed somite rapidly partition into the ventral scelerotome compartment and the dorsal dermomyotome from which muscle cells and dermis are generated. Peripheral muscles, such as those in the limb, are derived from cells that migrate from the lateral part of the somite [1]. The myogenic progenitor cells or myoblasts in the limb bud express the determination-class muscle regulatory factors (MRFs), then exit the cell cycle, and finally differentiate into myocytes. Most myocytes subsequently fuse with each other to form multinucleate myotubes, then mature into myofibers [1]. Myogenesis is regulated by morphogens and myogenic determination factors [2].

Skeletal muscle development is accompanied by changes in the composition of intermediate filaments, where myogenic progenitor cells express nestin and vementin; myocytes express nestin, vementin, and desmin, but mature myofibers only express desmin. Nestin, a class VI intermediate filament protein, is expressed specifically in neuroepithelial stem cells and neural progenitor cells. It has been widely used as a neural progenitor cell marker for the developing central nervous system [3,4]. Nestin expression is also found in myogenic progenitor cells in the dermomyotome of dorsal-lateral somites, and its expression persists in developing thigh muscle until postnatal day 4 of rats [3]. Nestin mRNA is found in developing thigh muscle of rat, from embryonic day 15.5 (E15.5) to postnatal day 21 [5], but not in adult skeletal muscles [3,5,6]. *In situ* hybridization also showed that nestin expression was up-regulated in the developing mouse limb bud during myogenesis, and down-regulated during chondrogenesis [7].

The nestin gene has been cloned from human, rat, and mouse. It shares considerable similarity in gene structure between different species, and contains three introns and four extrons [6,8–10]. Studies in transgenic mice showed

Received: April 2, 2008 Accepted: April 30, 2008
This work was supported by the grants from the National Nature Science of China (Nos. 30623003 and 30721065), the National Key Basic Research and Development Program of China (Nos. 2005CB522704, 2006CB943902, and 2007CB947101 to N.J.), and the National High Technology Research and Development Program of China (No. 2006AA02Z186 to N.J.)
*Corresponding authors:
Naihe Jing: Tel, 86-21-54921381; Fax, 86-21-54921011; E-mail, njing@sibs.ac.cn
Xing Cui: Tel, 86-536-88382459; E-mail, cuixing77@sdu.edu.cn

that the second intron of the nestin gene can drive reporter gene expression in developing neural precursor cells, and the first intron can induce reporter gene expression in both neural and muscle precursor cells [8]. Further studies revealed that the second intron of the nestin gene contained a central nervous system tissue-specific enhancer [8], and the *cis*-elements of POU and Sox binding sites in the 3′ half of the second intron were important for this neural enhancer activity [11,12]. However, the molecular mechanism that regulates nestin expression in developing muscles and in myogenesis is still unclear. In this study, we showed that a muscle-specific enhancer, localized in the first intron of mouse nestin gene, drove reporter gene expression during C2C12 myoblast differentiation.

## Materials and Methods

### Cell culture

C2C12 mouse cells were obtained from American Type Culture Collection (No. CRL 1772; Rockville, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, New York, USA) supplemented with 20% fetal bovine serum (HyClone, Logan, USA) at 37 ºC with 5% $CO_2$. The medium was changed into Dulbecco's modified Eagle's medium supplemented with 2% horse serum (Invitrogen, Carlsbad, USA) as C2C12 cells were induced to form myotubes. The cells were routinely passaged every 2 d, with passage density 60%, and induce density 80%.

### Generation of reporter gene constructs

The first intron was cloned into pGL3-px' and pGL3-TK vectors to generate pNH200 and pNH201, respectively. A series of luciferase reporter gene constructs was prepared by amplifying the first intron of mouse nestin gene with different sets of primers and inserting these polymerase chain reaction (PCR) fragments into pGL3-px' vectors. The promoter of mouse nestin gene (pNesP-3809/+183) was cloned as previously described [8]. The generation of the site-mutated construct was carried out by PCR using two sets of primers, as described previously [13]. In these constructs (p476/492-MT and p539/555-MT), the sequence of one E-box, 5′-CAGATG-3′, was mutated to 5′-CTCGAG-3′, and the other, 5′-ACCTGC-3′, was mutated to 5′-GAATTC-3′. The expression constructs of MyoD, myf5, and myogenin were obtained from Prof. Lin Li (Institute of Biochemistry and Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, Shanghai, China).

### Reverse transcription (RT)-PCR

Total RNA was extracted at different time points using Trizol reagent (Invitrogen). The RT-PCR was carried out in a 20 μl final volume containing 5 μg total RNA, 1× RT buffer, 1 μl random primer, 1 μl dNTP (10 mM), 2 μl dithiothreitol (0.1 M), and 1 μl SSRT at 42 ºC for 1 h. All RT reagents were purchased from Invitrogen. Aliquots of cDNA synthesized above were used as a template for semiquantitative PCR analysis. Nestin primers [5′-gaatcagatcgctcagatcc-3′ (forward) and 5′-gcacgacaccagtagaactgg-3′ (reverse)] were used, and a cDNA fragment of nestin was ampilified ($T_m$, 56 ºC, 30 cycles, PCR products 487 bp). As a loading control, the housekeeping gene *β-actin* was amplified using a primer set [5′-tcgtcgacaacggctccggcatgt-3′ (forward) and 5′-ccagccaggtccagacgcaggat-3′ (reverse); $T_m$, 56 ºC, 21 cycles, PCR products 520 bp].

### Luciferase activity

For transfection of plasmids, $3\times10^4$ C2C12 cells mixed with 1 ml growth medium were seeded into each well of 12-well plates and cultured at 37 ºC. When cells reached 70%–80% confluence, 0.4 μg luciferase reporter plasmid with different DNA fragments of the first intron and 0.2 μg control pRL-TK plasmid (Promega, Madison, USA) were co-transfected into cells using FuGENE HD reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. After 7 h, the culture medium was changed into differentiated medium containing 2% horse serum to induce C2C12 cell differentiation. After 3 d of culture, the cells were harvested as the differentiation 3 d (D3) group. The control cells were continually cultured in growth medium containing 20% fetal bovine serum for 3 d, and harvested as the undifferentiated group (D0). Cells were washed with phosphate-buffered saline and lysed with passive lysis buffer (Promega), and the supernatant was analyzed for luciferase reporter activity on a 20/20n luminometer (Turner BioSystems, Sunnyvale, USA) by the dual luciferase reporter system (Promega). The transfection efficiency was normalized by *Renilla* luciferase. In MRF co-expressing experiments, 0.4 μg MRF-expressing plasmids plus 0.4 μg luciferase reporter construct and 0.2 μg pRL-TK plasmid were co-transfected into C2C12 cells. After 2 d, C2C12 cells were harvested and the supernatant was analyzed with luciferase assay.

### Statistical analysis

The data were expressed in the form mean±SD. Student's *t*-test was applied to study the relationship between the different variables. All experiments were repeated at least three times and similar results were obtained.

## Results

### First intron of mouse nestin gene regulates its expression during C2C12 differentiation

C2C12 myoblasts were derived from adult mouse muscle, and could be induced by 2% horse serum to differentiate into multinucleated myotubes *in vitro* [14]. RT-PCR was used to detect nestin gene expression during C2C12 cell differentiation. The results showed that nestin mRNA could be detected in un-induced C2C12 cells (D0) at a low level, and its expression gradually increased after induction with a peak at D3 [**Fig. 1(A)**].

To identify the regulator sequence of the nestin gene during C2C12 cell differentiation, luciferase reporter constructs with the nestin gene promoter (pNesP-3809/+183), the second intron (pNes2In32/1628) and the first intron of mouse nestin gene (pNes1In1/1003) were transfected into C2C12 cells. After inducing C2C12 cell differentiation for 3 d (D3), the luciferase activities were detected and compared with that of the uninduced C2C12 cells (D0). The first intron-containing construct pNes1In1/1003 in the D3 group showed much higher luceferase activity than that of the D0 group, whereas neither of the constructs containing the promoter (pNesP-3809/+183) nor the second intron (pNes2In32/1628) displayed any activity difference between the D3 and D0 groups [**Fig. 1(B)**].

To eliminate promoter interference with reporter gene expression, we inserted the first intron of mouse nestin gene behind the nestin promoter (pNH200), TK promoter (pNH201), or SV40 promoter [**Fig. 1(C)**, upper panel] and introduced these constructs into C2C12 cells. All constructs showed a significant luceferase activity increase between the D3 and D0 groups [**Fig. 1(C)**, lower panel]. To test the cell-type specificity of this enhancer, we introduced the pNes1In1/1003 construct into different cell lines, such as P19EC, F9EC, NIH3T3, CHO, and SH-SY5Y cells, and found that the first intron did not display any enhancer activity in these non-muscle cells (data not shown). Together, these results suggest that the first intron of mouse nestin gene possesses enhancer activity during C2C12 myoblast differentiation.

### Core enhancer sequence in first intron of nestin gene

To identify the core enhancer sequence in the first intron of the nestin gene, we generated a series of deletion and truncation reporter constructs and transfected them into C2C12 cells (**Fig. 2**). After 3 d of induced differentiation, luciferase assays were carried out. We found that the full-length first intron (pNes1In1/1003) and its 5′ deletion

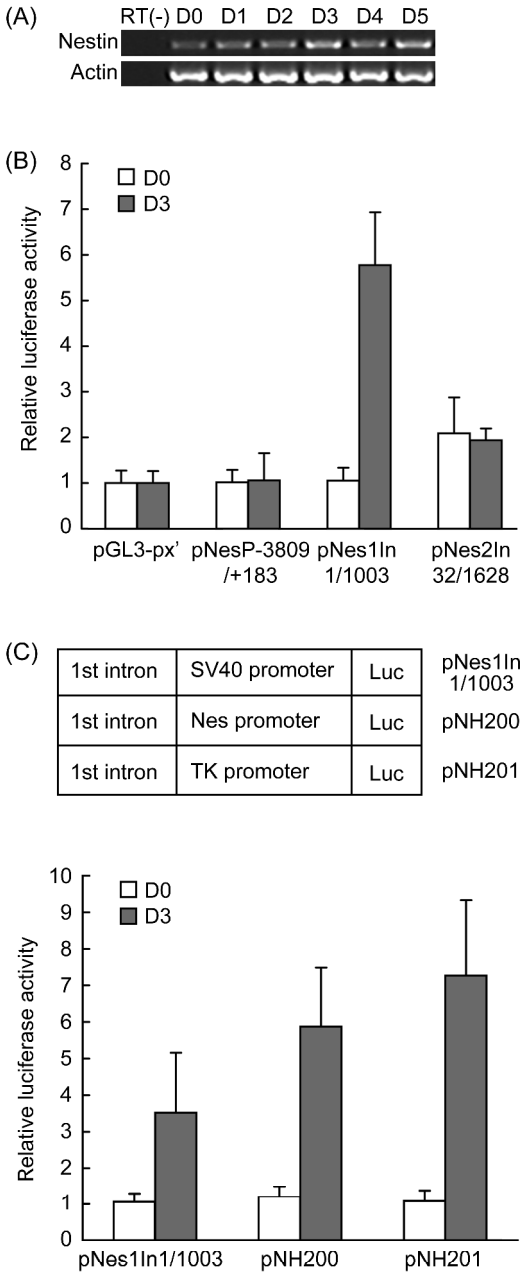


**Fig. 1 Expression of nestin gene is controlled by its first intron during C2C12 myoblast differentiation** (A) C2C12 myoblasts were induced to differentiate into myotubes. Nestin mRNA was determined by reverse transcription-polymerase chain reaction (RT-PCR). (B) Reporter gene expression in undifferentiated (D0) C2C12 cells and after 3 d of induced differentiation (D3) under different sequences of the nestin gene, including 4 kb 5′ flanking promoter (pNesP-3809/+183), and first (pNes1In1/1003) and second (pNes2In32/1628) introns. (C) Nestin first intron was cloned into reporter gene expression vectors with different promoters [SV40 promoter, nestin (Nes) promoter, and TK promoter]. All three reporter genes were transfected into C2C12 cells and reporter activities were determined. The results are presented as the mean±SD. Each experiment was repeated at least three times, and similar results were obtained.

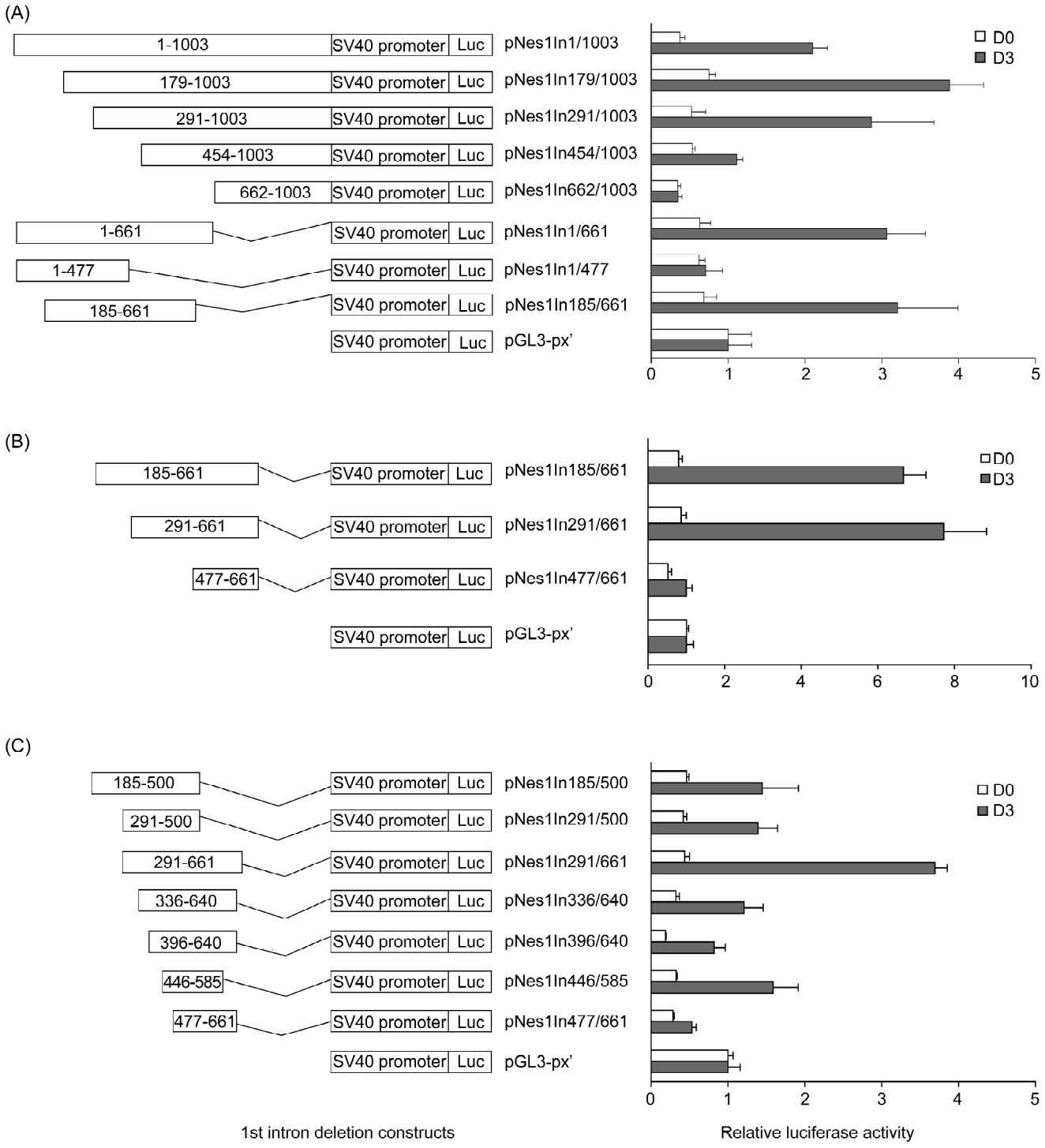


**Fig. 2 Analysis of core enhancer sequence in first intron of mouse nestin gene during C2C12 cell differentiation** Left panel, schematic representation of the enhancer deletion and truncation reporter constructs used for transfection. Right panel, deletion and truncation reporter constructs transfected into C2C12 cells. Cells were either undifferentiated (D0) or induced to differentiate for 3 d (D3) and reporter activities were determined. (A) Enhancer activity in the first intron was analyzed by 5′ serial deletion and reporter activities were determined by luciferase (Luc) assays. (B) Detailed analysis of enhancer activity in the region 185–661 in the first intron. The sequence 185–661 was further truncated from the 5′ end. The sequence required for specific enhancer activity was pinned down to 291–661. (C) Serial deletion and truncation of constructs from sequence of 291–661 in the first intron, and reporter activities were determined by luciferase assays. The results are presented as the mean±SD. Each experiment was repeated at least three times, and similar results were obtained.

constructs, pNes1In179/1003 and pNes1In291/1003, displayed higher luciferase activity in the D3 group than in the D0 group. For further 5′ deletion, including constructs pNes1In454/1003 and pNes1In662/1003, much reduced enhancer activity was found when compared with the full-length first intron. The 3′ truncation experiments showed that both constructs pNes1In1/661 and pNes1In185/661 possessed high luciferase activity, whereas 3′ truncation to 476 (pNes1In1/477) abolished the enhancer activity [**Fig. 2(A)**]. These results suggest that the core enhancer sequence is within the 185–661 region of the first intron of the nestin gene.

To narrow down the enhancer sequence even further, we carried out 5′ deletion to positions 290 and 476 and found that the pNes1In291/661 construct still displayed strong enhancer activity, whereas deletion to 476 (pNes1In477/661) totally eliminated enhancer activity [**Fig. 2(B)**]. Consistently, further deletion and truncation experiments within the 185–661 region showed that only construct pNes1In291/661 had full enhancer activity, and other constructs displayed reduced activities [**Fig. 2(C)**].

In summary, these data suggest that the minimal sequence for the muscle-specific enhancer of the nestin gene is located in the region 291–661 in the first intron of mouse nestin gene.

## Two E-boxes involved in enhancer activity of first intron of nestin gene

To search for the *cis*-elements responsible for the enhancer activity if the first intron of the nestin gene, we analyzed the 291–661 region with Genomatix software (http://www.genomatix.de) and found several putative E-boxes (CANNTG) in the promoters or enhancers of muscle-specific genes. Among these putative E-boxes, two at the positions 476–492 and 539–555 attracted our attention because deletion [**Fig. 2(A)**, pNes1In1/477] or truncation [**Fig. 2(B)**, pNes1In477/661] in the 476–492 region always caused abolishment of enhancer activity of the nestin gene, and the E-box at position 539–555 was very similar to the MyoD binding site. To validate the importance of these two sites, we constructed plasmids p476/492-MT and p539/555-MT with mutations at the 476–492 and 539–555 binding sites, respectively. After transfecting them into C2C12 cells, we found that both mutations had reduced enhancer activity. The enhancer activity of p476/492-MT decreased, and the significant drop of enhancer activity was observed in p539/555-MT ($P<0.05$, p476/492-MT vs. pNes1In291/661; $P<0.01$, p539/555-MT vs. pNes1In291/661) (**Fig. 3**), suggesting that these two E-boxes are involved in muscle-specific enhancer activity of mouse nestin gene.

## MyoD protein involved in regulating nestin gene expression in C2C12 cells

The MRFs, MyoD, myf5 and myogenin, play very important roles during determination and terminal differentiation of skeletal muscle [15], and they also have important functions in the differentiation of C2C12

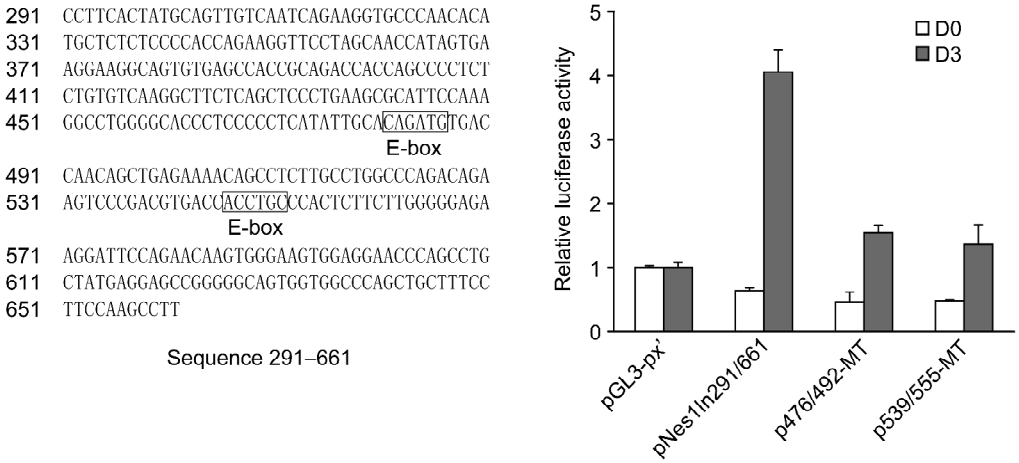


**Fig. 3 *cis*-Elements for enhancer activity of mouse nestin gene** Left panel, sequence analysis showing the putative *cis*-elements (boxed) in the region 291–661. Two E-boxes were mutated: 5′-CAGATG-3′ mutated to 5′-CTCGAG-3′; and 5′-ACCTGC-3′ mutated to 5′-GAATTC-3′. Right panel, luciferase activity of the intact (pNes1In291/661) and mutated (p476/492-MT, p539/555-MT) reporter constructs. The cells were induced to differentiate for 3 d and reporter activities were determined. The results are presented as the mean±SD. Each experiment was repeated at least three times, and similar results were obtained.

myoblasts [16]. To determine which MRF is involved in regulating muscle-specific enhancer of the nestin gene, we co-transfected the full-length first intron with expression vectors encoding MyoD, myf5, and myogenin into C2C12 cells. Luciferase assays showed that overexpression of MyoD (pCMV-MyoD) was able to significantly increase enhancer activity ($P$<0.01, pCMV-MyoD vs. pCMV-HA), modest effects could be detected with myf5 (pCMV-myf5), and myogenin (pCMV-myogenin) produced very little increase [**Fig. 4(A)**]. Consistently, co-transfection of shorter fragments of the first intron, pNes1In185/661 and pNes1In291/661, with MyoD expression plasmid could also increase luciferase activity significantly ($P$<0.01, pCMV-MyoD vs. pCMV-HA) [**Fig. 4(B)**], suggesting that MyoD protein was involved in regulating the muscle-specific enhancer activity of the nestin gene.

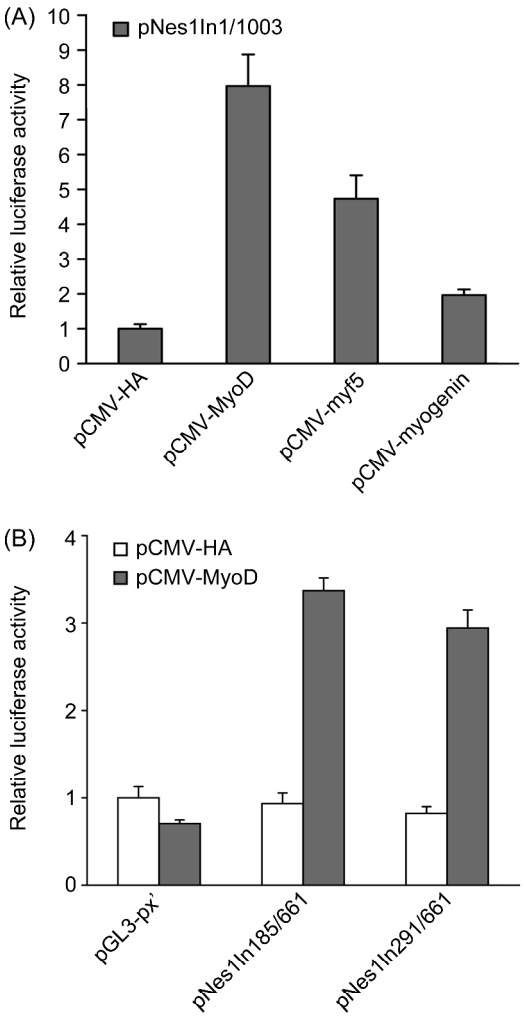


**Fig. 4 MyoD protein regulates expression of mouse nestin during C2C12 differentiation** (A) Expression vectors of MyoD, myf5, and myogenin were co-transfected with reporter gene driven by the full length of the first intron of the nestin gene (pNes1In1/1003) into C2C12 cells, and reporter activities were determined. (B) Co-transfection of MyoD expression plasmid with deletion constructs of the first intron (pNes1In185/661 and pNes1In291/661) into C2C12 cells, and reporter activities were determined. The results are presented as the mean±SD. Each experiment was repeated at least three times, and similar results were obtained.

## Discussion

Intermediate filament protein, nestin, is expressed specifically in neuroepithelial stem cells and muscle progenitor cells in the myotome of rat and mouse embryos [3]. It is reported that the second intron of the nestin gene contains the neural-specific enhancer [8]. However, the detailed relationship between the first intron of the nestin gene and its expression in developing muscles has not been explained. In this study, we identified a muscle cell-specific enhancer in the first intron of mouse nestin gene, and characterized the core enhancer activity in the region 291–661 of the first intron. We also showed that two E-boxes were important for the expression of the nestin gene during induced C2C12 cell differentiation. Finally, we found that MyoD protein was involved in nestin gene expression in C2C12 cells.

It has been reported that MyoD binds to its consensus sequence CANNTG (E-box) in the regulatory region of muscle-specific genes and regulates their expression [17, 18]. In this study, we showed that the first intron of mouse nestin gene possessed the muscle cell-specific enhancer activity (**Fig. 1**), and there were two E-boxes within this region (**Fig. 3**). Further studies showed that the two E-boxes and MyoD protein were involved in nestin gene expression in C2C12 cells (**Figs. 3** and **4**). Interestingly, we found that there was no enhancer activity when we co-transfected MyoD expression plasmid with reporter gene construct pNes1In477/661, in which one E-box (539–555) was intact but the other (476–492) was disrupted (data not shown). This result suggests that both E-boxes are important for the expression of the nestin gene in C2C12 cells. It is also possible that MyoD protein needs to bind cooperatively to two sites at a distance, as is the case with muscle-specific enhancer of creatine kinase [19].

## Acknowledgements

We thank Prof. Lin Li (Institute of Biochemistry and Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, Shanghai, China) for kindly pro-

viding the MRF expression constructs.

## References


1 Buckingham M. Molecular biology of muscle development. Cell 1994, 78: 15–21
2 Olson EC, Brennan TJ, Chakraborty T, Cheng TC, Cserjesi P, Edmondson D, James G *et al*. Molecular control of myogenesis: antagonism between growth and differentiation. Mol Cell Biochem 1991, 104: 7–13
3 Lendahl U, Zimmerman LB, Mckay RD. CNS stem cell expresses a new class of intermediate filament protein. Cell 1990, 60: 585–595
4 Steinert PM, Leim RK. Intermediate filament dynamics. Cell 1990, 60: 521–523
5 Sejersen T, Lendahl U. Transient expression of the intermediate filament nestin during skeletal muscle development. J Cell Sci 1993, 106: 1291–1400
6 Dahlstrand J, Zimmerman LB, Macky RD, Lendahl U. Chracterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments. J Cell Sci 1992, 103: 589–597
7 Wroblewski J, Engström M, Edwall-Arvidsson C, Sjöberg G, Sejersen T, Lendahl U. Distribution of nestin in the developing mouse limb bud *in vivo* and in micro-mass culture of cells isolated from limb buds. Differentiation 1997, 61: 151–159
8 Zimmerman L, Parr B, Lendahl U, Cunningham M, McKay R, Gavin B, Mann J *et al*. Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 1994, 12: 11–24
9 Jin Z, Liu L, Zhong H, Zhang K, Chen Y, Bian W, Cheng L, Jing N. Second intron of mouse nestin gene directs its expression in pluripotent embryonic carcinoma cells through POU factor binding site. Acta Biochim Biophys Sin 2006, 38: 207–212
10 Cheng L, Yang J, Jing N. Cloning and preliminary characterization of regulatory domain of mouse nestin gene. Acta Biochim Biophys Sin 2000, 32: 649–652
11 Josephson R, Müller T, Pickel J, Okabe S, Reynolds K, Turner PA, Zimmer A *et al*. POU transcription factors control expression of CNS stem cell-specific genes. Development 1998, 125: 3087–3100
12 Tanaka S, Kamachi Y, Tanouchi A, Hamada H, Jing N, Kondoh H. Interplay of SOX and POU factors in regulation of the nestin gene in neural primordial cells. Mol Cell Biol 2004, 24: 8834–8846
13 Cheng L, Jin Z, Liu L, Yan Y, Li T, Zhu X, Jing N. Characterization and promoter analysis of the mouse nestin gene. FEBS Lett 2004, 565: 195–202
14 Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 1977, 270: 725–727
15 Berkes CA, Tapscott SJ. MyoD and the transcriptional control of myogenesis. Semin Dev Biol 2005, 16: 555–595
16 Dedieu S, Mazeres G, Cottin P, Brustis JJ. Involvement of myogenic regulator factors during fusion in cell line C2C12. Int J Dev Biol 2002, 46: 235–241
17 Blackwell TK, Weintraub H. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 1990, 250: 1104–1110
18 Lassar AB, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschka SD, Weintraub H. MyoD is a sequence-specific DNA-binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. Cell 1989, 58: 823–831
19 Weintraub H, Davis R, Lockshon D, Lassar A. MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. Proc Natl Acad Sci USA 1990, 87: 5623–5627