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Second Intron of Mouse Nestin Gene Directs its Expression in Pluripotent Embryonic Carcinoma Cells through POU Factor Binding Site

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Abstract Nestin, an intermediate filament protein, is expressed in the neural stem cells of the developing central nervous system. This tissue-specific expression is driven by the neural stem cell-specific enhancer in the second intron of the nestin gene. In this study, we showed that the mouse nestin gene was expressed in pluripotent embryonic carcinoma (EC) P19 and F9 cells, not in the differentiated cell types. This cell type-specific expression was conferred by the enhancer in the second intron. Mutation of the conserved POU factor-binding site in the enhancer abolished the reporter gene expression in EC cells. Oct4, a Class V POU factor, was found to be coexpressed with nestin in EC cells. Electrophoretic mobility-shift assays and supershift assays showed that a unique protein-DNA complex was formed specifically with nuclear extracts of EC cells, and Oct4 protein was included. Together, these results suggest the functional relevance between the conserved POU factor-binding site and the expression of the nestin gene in pluripotent EC cells.

Key words nestin; embryonic carcinoma cells; enhancer; POU factor

Nestin, an intermediate filament protein, is expressed in the neural stem cells (NSCs) of the developing central nervous system (CNS), and is used as an NSC marker [1–4]. *In situ* hybridization results showed that nestin mRNA was found predominantly in the neuroepithelial and radial glia cells of the neural tube of mouse embryos at embryonic day 10.5 [4]. During CNS development, NSCs differentiate into mature neurons and glial cells, concomitantly nestin expression is downregulated and replaced by neurofilament and glial fibrillary acidic protein [1,4]. Nestin expression shows a sharp decline in the motor neurons of the spinal cord and marginal layer neurons of the telencephalon when NSCs become post-mitotic young neurons [4].

The nestin gene is well conserved in its genomic structure in human, rat and mouse, and it contains four exons and three introns [2,3,5]. Transgenic mice showed that the rat nestin gene had two tissue-specific enhancers in the first and second introns, which could drive nestin gene expression in muscle progenitor cells and NSCs, respectively [6]. The NSC-specific enhancer resides in the 3′ region of the second intron, and a *cis*-element for the POU transcription factors is responsible for nestin expression in the developing CNS [7–9]. It has been shown that group B1/C Sox and Class III POU factors might interact synergistically to determine nestin expression in neural primordial cells [10]. We previously described the cloning of the mouse nestin gene, and found that nestin protein existed in the developing eye, the growth cones of P19 neurons and the mouse cerebellar granule cells [11–13]. We further characterized the promoter of the mouse nestin gene, and showed that transcription factors Sp1 and Sp3 were involved in the regulation of nestin expression [14].

The onset of nestin expression occurred in the neural plate of mouse embryos at approximately embryonic day 7.5 [4,7]. Using more sensitive methods, the expression

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of nestin was detected in embryonic day 7.0 mouse embryos [15,16]. Recently, however, weak expression of nestin was found in the inner cell mass of mouse blastocysts as well as in human and mouse embryonic stem (ES) cells, and the expression was upregulated upon differentiation from ES cells into NSCs [17,18]. We previously reported nestin expression in undifferentiated mouse embryonic carcinoma (EC) P19 cells and retinoic acid-induced P19 NSCs at both mRNA and protein levels [13,19]. In contrast to the well-known mechanisms of expression regulation of the nestin gene in NSCs, little is known of the molecular basis of earlier nestin expression in pluripotent stem cells. In this study, we showed the nestin gene expression in pluripotent EC cells, and found that the POU factor binding site in the second intron of the mouse nestin gene was probably involved in this cell type-specific gene expression.

Materials and Methods

The luciferase reporter constructs pNH92 and pNH94 were generated in our laboratory previously using the vector pGL3-Px′ which contained the SV40 promoter [5]. We also prepared the luciferase construct, in which the mini-enhancer was placed in front of the nestin endogenous promoter (pNH142). To prepare enhanced green fluorescent protein (EGFP) reporter constructs, the vector ptkEGFP (kind gift from Dr. H. KONDOH, Osaka University, Osaka, Japan) was modified in multiple cloning sites (named pxtkEGFP) and the fragments of nestin enhancer were inserted into pxtkEGFP at the proper cloning sites. The generation of POU site-mutated constructs was performed by polymerase chain reaction (PCR) using two sets of primers as described previously [14], and in these constructs (pNH166 and pNH135), the sequence of the POU site, 5′-attagcat-3′, was mutated to 5′-aactagtt-3′. All of these constructs were confirmed by restriction enzyme digestion and DNA sequencing.

Cell lines, including mouse embryonic carcinoma P19, F9, mouse fibroblast NIH3T3, Chinese hamster ovary CHO, African green monkey kidney Cos7 and human neuroblastoma SH-SY5Y, were maintained in DMEM/F12 (1:1) medium (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, USA) at 37 ºC in 5% CO_2. Cells were seeded in 24-well plates for 24 h, then 0.4 μg of firefly luciferase reporter constructs and 0.1 μg of pRL-TK plasmid (Promega, Madison, USA) were cotransfected into cells with FuGENE6 reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. Forty hours later, cells were washed with phosphate-buffered saline and lysed with Passive lysis buffer (Promega). The supernatant was analyzed for luciferase reporter activity on a 20/20^n Luminometer (Turner Biosystems, Sunnyvale, USA) by the dual luciferase reporter system (Promega). The transfection efficacy was normalized by *Renilla* luciferase. All experiments were carried out at least three times and similar results were obtained.

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, USA). Reverse transcription (RT) was performed with 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). PCR reaction consisted of denaturation at 94 ºC for 45 s, annealing for 45 s, and extension at 72 ºC for 45 s. PCR primers and reaction parameters for each gene are shown in **Table 1**. The sequences of primers are perfectly conserved in different species and the primers were designed accordingly to decipher the genuineness of amplified products, based on their size from cDNA, but not from genomic DNA. PCR products were separated on agarose gel. Reverse transcription-minus RNA samples were used as negative controls, and no positive band was detected. PCR analyses were repeated three times for each gene, and similar results were obtained.

Nuclear extracts were prepared from cells as described previously [14]. Nuclear proteins (3–6 μg) were preincubated with 2 μg poly(dG-dC)·poly(dG-dC) (Amersham, Uppsala, Sweden) on ice for 30 min in 5×binding buffer containing 50% glycerol, 60 mM HEPES (pH 7.9), 20 mM

Table 1 Polymerase chain reaction primers and reaction parameters for different genes

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Annealing temperature (ºC)	Cycles	Size (bp)
Nestin	gaatcagatcgctcagatcc	gcacgacaccagtagaactgg	56	30	487
Oct1	tgaccttgaggagcttgagcag	ctctgcatcatttagccacttctc	56	28	214
Oct4	cagaagaggatcaccttggg	gtgagtgatctgctgtaggg	60	28	324
β-actin	tcgtcgacaacggctccggcatgt	ccagccaggtccagacgcaggat	56	21	520

Tris-HCl, 300 mM KCl, 5 mM EDTA and 5 mM dithiothreitol. ^{32}P-labeled DNA probe was added to the binding reaction mixture and incubated for an additional 30 min at 25 ºC. Competition experiments were performed with preincubation of a 100-fold molar excess of unlabelled probe together with nuclear extracts. In the case of the supershift assay, 1 μg of antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) against Oct1 and Oct4 were added to the reaction mixture before the addition of labeled probes. Protein-DNA complexes were fractioned on a pre-run non-denaturing 6% polyacrylamide gel and subjected to electrophoresis in 0.5×TBE buffer. Gels were dried and exposed to X-ray film with an intensifying screen at −80 ºC. The oligonucleotide probe was designed with complementary single-stranded oligonucleotides containing 4 bp overhangs at the 5′ end (in lowercase). The sequence of probe POU is: 5′-tagtGTGGACAAAAGGCAATAATT-AGCATGAGAATC-3′. The POU factor binding site is underlined. The sequence of probe SIE as irrelevant competitor is: 5′-GATCCAGTTCCCGTCAATCGATC-3′.

Results and Discussion

To survey nestin gene expression in different cell lines, we analyzed nestin transcript with RT-PCR. As shown in **Fig. 1(A)**, nestin mRNA could be detected in mouse EC P19 and F9 cells, though the expression level is relatively low in F9 cells. However, the expression of the nestin gene was even low or undetectable in differentiated cell lines, such as mouse fibroblast NIH3T3 cells, Chinese hamster ovary CHO cells, human neuroblastoma SH-SY5Y cells and Africa green monkey kidney Cos7 cells. To confirm the pluripotent EC cell-specific expression of the nestin gene, and to identify the regulatory elements driving nestin gene expression in EC cells, we scanned the genomic sequence of the mouse nestin gene with luciferase reporter gene assay. Our previous study, which showed no cell type specificity in the promoter region up to the 4 kb 5′ flanking sequence [14], prompted us to survey the first and second introns of the mouse nestin gene. The second intron, in both the full length of 1.6 kb (pNH92) and the 3′ 320 bp region (pNH94, mini-enhancer), did show higher luciferase activities in pluripotent P19 and F9 cells than in other cell lines [**Fig. 1(B)**]. In contrast, the first intron could not drive reporter gene expression in any cell line. The second intron also enhanced the expression of the reporter gene in P19 EC cells when the SV40 promoter was replaced by the endogenous promoter of the mouse nestin gene (data not shown), suggesting that the regulatory elements were indeed located in the second intron. Thus, these results show a cell type-specific expression of the nestin gene in EC cells, and this cell type specificity is probably conferred by the enhancer in the second intron of the mouse nestin gene.

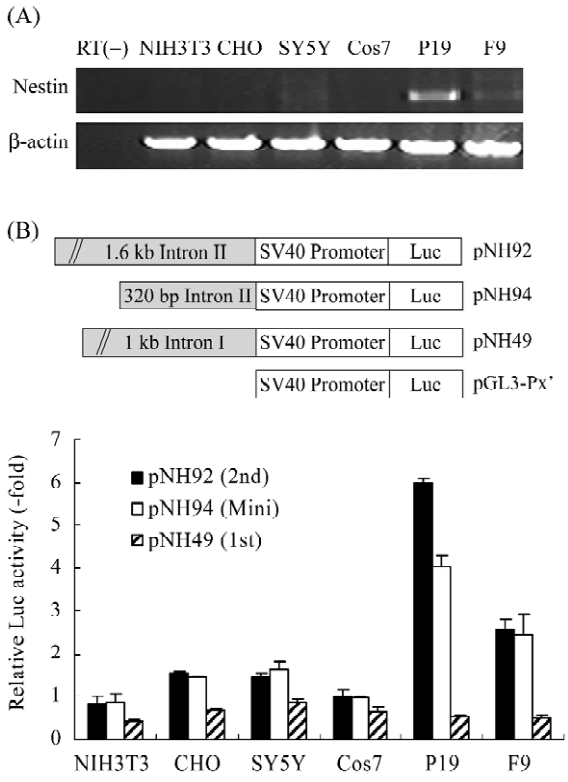

Fig. 1 Second intron of mouse nestin gene directs nestin expression in embryonic carcinoma (EC) cells

(A) Nestin expression in different cell lines. Total RNA from different cell lines was reverse transcribed and nestin gene expression was detected by reverse transcription-polymerase chain reaction. Reverse transcription-minus RNA samples RT(−) were used as negative controls. (B) The second intron of the mouse nestin gene drives reporter gene expression in P19 and F9 EC cells. Luciferase activities in different cell lines are expressed as times more than that of the pGL3-Px′ control vector. The results are presented as mean±standard deviation. Each experiment was repeated at least three times, and similar results were obtained.

The POU factor binding site in the second intron of the nestin gene has been shown to be critical for its expression in NSCs of developing CNS [8]. To examine whether this binding site is also essential to nestin expression in EC cells, we transfected the EGFP reporter gene under the control of the mini-enhancer of the second intron (pNH161) and its POU site-mutated counterpart (pNH166) into P19 cells. As shown in **Fig. 2(A)**, this mini-enhancer could induce reporter gene expression in P19 cells, but the expression of EGFP was severely reduced in the cells transfected with the mutant construct. To further validate the

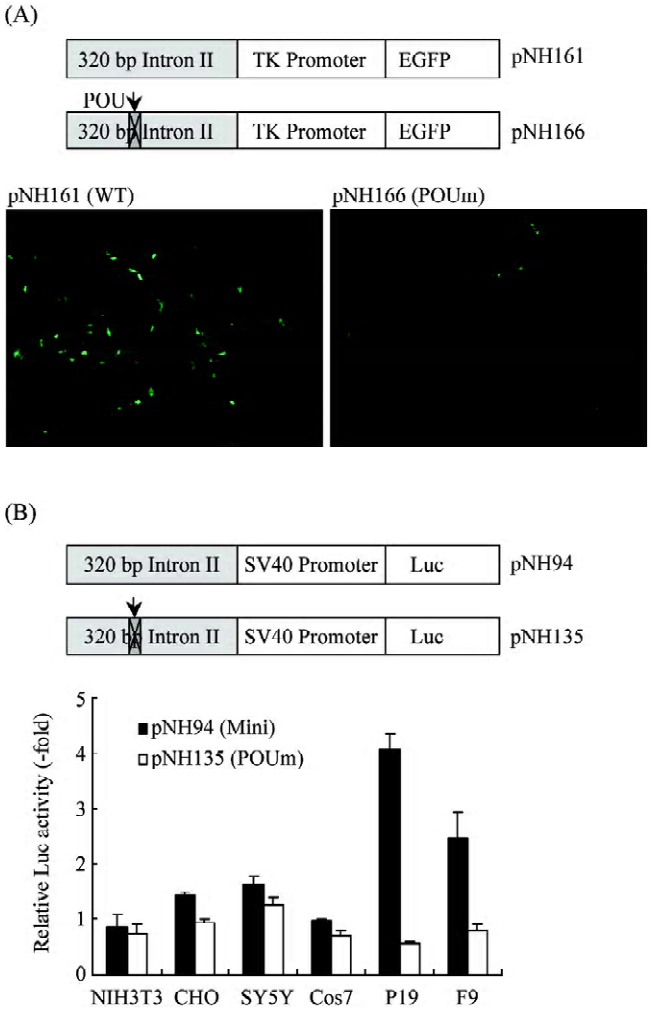

Fig. 2 Mutation of POU factor binding site abolishes the enhancer activity in embryonic carcinoma cells

(A) Transfection of enhanced green fluorescent protein (EGFP) reporter constructs containing wild-type (pNH161) and POU site-mutated mini-enhancer (pNH166) in P19 cells. EGFP expression was severely reduced when the POU site was mutated. Transfection efficacy was corrected by co-transfection of pCMV-SV2-mRFP and no obvious difference of mRFP expression was observed. (B) Transfection of luciferase constructs containing wild-type (pNH94) and POU site-mutated mini-enhancer (pNH135) in different cell lines. Luciferase activities in different cell lines are expressed as times more than that of the pGL3-Px′ control vector. The results are presented as mean±standard deviation. Each experiment was repeated at least three times, and similar results were obtained.

importance of this POU factor binding site in EC cells, we transfected, in different cell lines, the mini-enhancer construct (pNH94) and POU site-mutated construct (pNH135) linked with the luciferase reporter gene. It was found that the mutation of this binding site caused a sharp reduction of luciferase activity in P19 and F9 EC cells, but it did not induce significant changes in other cell lines [**Fig. 2(B)**]. Taken together, these results show that the POU factor binding site is required for the enhancer activity in pluripotent EC cells.

To search for the possible *trans*-factors binding to the POU site, we examined the expression patterns of members of the POU transcription factor family by RT-PCR (**Fig. 3**). Oct1, the Class II POU factor, was expressed in

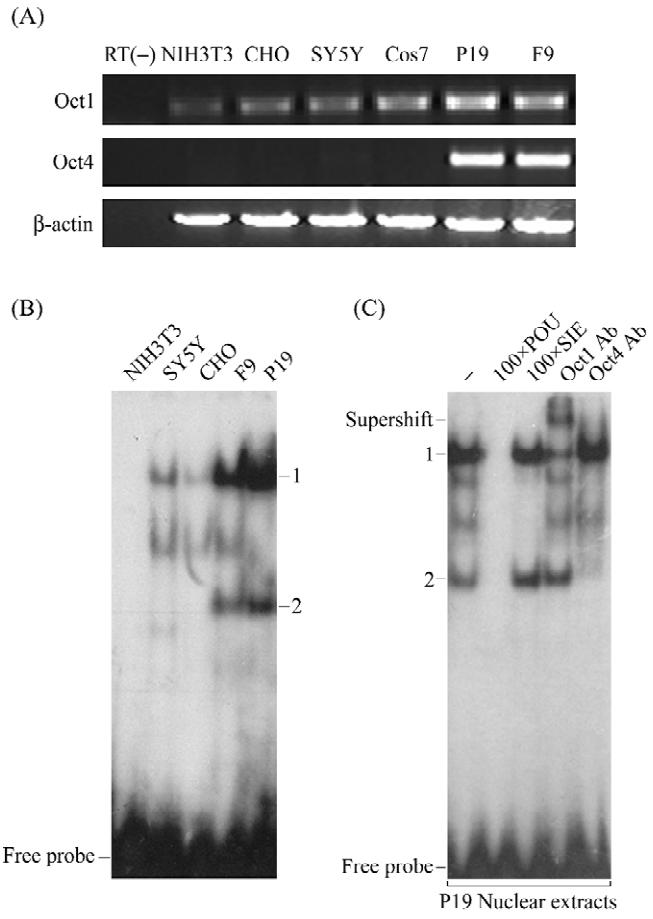

Fig. 3 Expression of POU factors and DNA-protein complex formation in embryonic carcinoma (EC) cells

(A) The expression of POU transcription factor family members in different cell lines. Total RNA from different cell lines was reverse transcribed, Oct1 and Oct4 expression were detected by reverse transcription-polymerase chain reaction. Oct1 is expressed in all cell lines, but Oct4 is expressed specifically in EC cells. Reverse transcription-minus RNA samples RT(–) were used as negative controls. (B) Electrophoretic mobility-shift assay using ^{32}P-labeled probe containing the POU factor binding site with nuclear extracts from different cell lines. A low mobility band (band 1) could be detected in SY5Y, CHO, F9 and P19 cells, and a fast migrating band (band 2) existed only in F9 and P19 cells. (C) Competition experiment and supershift assay using nuclear extracts from P19 cells. The formation of band 1 and band 2 resulted from specific binding of nuclear proteins to the POU site, whereas the other bands were non-specific binding. Nuclear proteins in band 1 and band 2 were recognized by antibodies against Oct1 and Oct4, respectively. Ab, antibody; –, non-labeled probe.

each cell line, and showed no cell type specificity. In contrast, Oct4, the Class V POU factor, was only expressed in the EC cells [**Fig. 3(A)**]. This is consistent with the notion that Oct4 is a very important transcription factor for the self-renewal and pluripotency of ES cells [20–22]. To test whether there are nuclear proteins actually binding to the POU factor binding site, we used nuclear extracts from different cells and performed electrophoretic mobility-shift assay with a ^{32}P-labeled oligonucleotide probe containing the POU site. As shown in **Fig. 3(B)**, a low mobility band (band 1) could be detected in SY5Y and CHO cells as well as F9 and P19 cells, and a fast migrating band (band 2) existed only in F9 and P19 cells. The protein-binding specificity of the POU site was confirmed because these two bands (band 1 and band 2) were effectively competed by a non-radiolabeled probe, but not by an irrelevant competitor, probe SIE [**Fig. 3(C)**].

To identify the proteins binding to the POU site, supershift assays were performed. Results showed that band 1 was supershifted by antibody against Oct1, and band 2 was specifically diminished by antibody against Oct4 [**Fig. 3(C)**]. Thus, POU factors Oct1 and Oct4 did bind to the POU site in P19 EC cells. Interestingly, the *Sox2* gene was expressed in pluripotent ES cells and a core enhancer in the 5′ flanking region activated transcription in ES cells depending on POU factor binding sites [23,24]. In the Sox2 5′ enhancer, two bands of protein-DNA complexes were formed between the POU binding sites and nuclear extracts from ES cells, in which the fast migrating band was the complex with Oct4 protein and the slow band was with Oct1 [23]. The binding of Oct4 to POU sites in the Sox2 5′ enhancer might impart its specific expression in ES cells. Based on these observations, we speculate that Oct4 and Oct1 proteins might directly bind to the POU factor binding site and regulate nestin expression in EC cells.

In summary, we showed that the nestin gene was expressed specifically in pluripotent EC cells, and that this cell type-specific expression was regulated by the NSC-specific enhancer in the second intron of the mouse nestin gene. The conserved POU factor binding site in this enhancer mediated nestin gene expression in EC cells. These results shed light on the molecular mechanisms of regulation of nestin gene expression in pluripotent stem cells.

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