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ARTICLES

Neural Expression of α-*Internexin* Promoter In Vitro and In Vivo

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Abstract α-Internexin is a 66 kDa neuronal intermediate filament protein found most abundantly in the neurons of the nervous systems during early development. To characterize the function of mouse α-*internexin* promoter, we designed two different expression constructs driven by 0.7 kb or 1.3 kb of mouse α-*internexin* 5′-flanking sequences; one was the enhanced green fluorescent protein (EGFP) reporter for monitoring specific expression in vitro, and the other was the *cre* for studying the functional DNA recombinase in transgenic mice. After introducing DNA constructs into non-neuronal 3T3 fibroblasts and a neuronal Neuro2A cell line by lipofectamine transfection, we observed that the expression of EGFP with 1.3 kb mouse α-*internexin* promoter was in a neuron-dominant manner. To establish a tissue-specific pattern in the nervous system, we generated a transgenic mouse line expressing Cre DNA recombinase under the control of 1.3 kb α-*Internexin* promoter. The activity of the Cre recombinase at postnatal day 1 was examined by mating the *cre* transgenic mice to ROSA26 reporter (R26R) mice with knock-in Cre-mediated recombination. Analyses of postnatal day 1 (P1) newborns showed that β-galactosidase activity was detected in the peripheral nervous system (PNS), such as cranial nerves innervating the tongue and the skin as well as spinal nerves to the body trunk. Furthermore, X-gal-labeled dorsal root ganglionic (DRG) neurons showed positive for α-Internexin in cell bodies but negative in their spinal nerves. The motor neurons in the spinal cord did not exhibit any β-galactosidase activity. Therefore, the *cre* transgene driven by mouse α-*internexin* promoter, described here, provides a useful animal model to specifically manipulate genes in the developing nervous system. J. Cell. Biochem. 97: 275–287, 2006.

Key words: α-Internexin; promoter; intermediate filament; Cre; transgenic mice

Neuronal intermediate filament proteins, including neurofilament triplet proteins, peripherin and α-internexin, have been identified in the developing nervous system [Liem, 1990; Steinert and Liem, 1990; Ho and Liem, 1996; Strelkov et al., 2003]. They are found in differentiated neurons, whereas nestin and vimentin are expressed in neuroepithelial stem cells during early development of the nervous system [Ho and Liem, 1996; Lariviere and Julien, 2004; Wiese et al., 2004]. α-Internexin is an axonal intermediate filament protein found in postmitotic neurons of the central nervous system (CNS) and peripheral nervous system (PNS) [Pachter and Liem, 1985; Kaplan et al., 1990; Vickers et al., 1992; Fliegner et al., 1994; Athlan et al., 1997; Faussone-Pellegrini et al., 1999]. The expression of α-*internexin* is upregulated in the early development of nervous tissues and its mRNA reaches the maximum level on embryonic day 16 and decreases thereafter [Fliegner et al., 1990; Kaplan et al., 1990; Chan and Chiu, 1995]. The spatiotemporal expression pattern of α-internexin implies its function in the neuronal cytoarchitecture during development [Fliegner et al., 1994; Chien et al., 1996, 1998].

Due to their specific patterns of expression, neural intermediate filament proteins have been showed to be regulated by multiple regulatory elements in the 5′-flanking region of their genes, such as nestin [Lothian et al., 1999], vimentin [Sax et al., 1988; Farrell et al., 1990], neurofilament triplet proteins [Julien et al., 1987, 1990], and peripherin [Thompson and Ziff, 1989; Uveges et al., 2002]. Potential regulatory elements in proximal 5′-flanking sequences of rat α-*internexin* have also been

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addressed. By using transient expression systems, Ching and Liem [1991] demonstrated that upto 1.2 kb 5′-flanking sequences of rat *α-internexin* contain the information for basal promoter activity and efficient neuronal transcription. Additionally, Brn-3a, which is closely related to POU (Pit-Oct-Unc) family transcription factors, is expressed predominantly in neuronal cells [reviewed by Latchman, 1999], and is able to modulate the promoter activity of *α-internexin* [Budhram-Mahadeo et al., 1995]. However, other neuron-specific manners of potential regulatory elements have not been clarified in controlling *α-internexin* expression.

On the other hand, gene activation and/or inactivation in spatiotemporally restricted ways are desirable features for the functional analysis of a given gene. The Cre/loxP site-specific recombination system has proved to be a powerful tool to limit gene modifications or deletions in specific cells of interest [Sauer, 1998; Kwan, 2002]. A key element of this strategy is to generate transgenic mice that express the Cre DNA recombinase under the control of a tissue-specific promoter. Subsequent breeding of the *cre* transgenics with mice carrying an allele containing an interesting region flanked by loxP sites results in the excision of the floxed sequence specifically in the tissues expressing the *cre* transgene [Nagy, 2000]. Many studies have demonstrated Cre-mediated recombination in a variety of mammalian tissues, including nervous tissues [Voiculescu et al., 2000; Cinato et al., 2001; Hirasawa et al., 2001; Zhuo et al., 2001, 2002; Tonks et al., 2003].

In the present study, to test the promoter activity of *α-internexin* in vitro and in vivo, we performed a functional analysis of a 1.3 kb 5′-flanking sequence of the mouse *α-internexin* linked with an enhanced green fluorescent protein (EGFP) reporter gene. We monitored the expression levels of the EGFP reporter gene in 3T3 fibroblasts and Neuro2A cells by fluorescence microscopy. Additionally, the activity of functional Cre DNA recombinase driven by *α-internexin* promoter in vivo was also examined via a transgenic approach.

MATERIALS AND METHODS

Construction of Plasmids

A genomic clone corresponding to mouse *α-internexin* has been isolated from a λFIX II genomic library (Stratagene, La Jolla, CA) [Chien and Liem, 1994]. The 1.9 kb 5′ SacI fragment of the 5′-untranslated region was then subcloned into pGEM7Zf(−) vector (Promega, Madison, WI). From analyzing the sequence data and comparing to the rat *α-internexin* promoter [Ching and Liem, 1991], the 1.3 kb 5′ fragment of the mouse *α-internexin* downstream of the start site of the transcription was amplified by PCR using the following primers: T4 primer (forward primer) and 5′TTTGTG-CTCCGCCTCCTCCT3′ (reverse primer, with a modified start codon at the *Hind*III site), which correspond to sequences in the promoter region. The PCR product was cloned into pGEM-T Easy vectors (Promega, Madison, WI). After digestion with restriction enzymes, a 0.7 kb and a 1.3 kb 5′ fragment as promoter candidates were cloned into pEGFP-1 vector (Clontech, Palo Alto, CA) and pBS185 vector (replacing its hCMV promoter) (Gibco/BRL, Grand Island, NY) as the following constructs: p0.7intfs-EGFP, p1.3intfs-EGFP, and p1.3intfs-Cre (Fig. 1).

Cell Transfection

Mouse embryonic 3T3 fibroblast and mouse neuroblastoma Neuro2A cell lines were cultured at cell densities of 1×10^5 cells/ml on coverslips in culture dishes. After changing to serum-free culture medium, a DNA-lipofectamine mixture (3 μg DNA and 10 μl lipofectamine reagent [Life Technologies, Inc., Carlsbad, CA] in 150 μl DMEM) was added to the culture medium and allowed to react with cells for 5 h at 37°C, 5% CO_2. Two DNA constructs p0.7intfs-EGFP and p1.3intfs-EGFP DNA constructs were used to examine expression levels of EGFP, and pDsRed1-N1 vector plasmid (Clontech, Palo Alto, CA) was applied to evaluate the expression of DNA constructs by normalizing the transfection efficiency. Furthermore, p1.3intfs-Cre DNA construct was used to test the efficiency of promoter-driven Cre recombinase expression in the Cre/loxP recombination system. A DNA construct, ploxp*LacZ*, containing the *LacZ* gene flanked by loxP was co-transfected together with p1.3intfs-Cre DNA as a target reporter for Cre-mediated deletion. pBS185 vector was also applied as a positive control. After transfection with lipofectamine for 6–8 h, DMEM containing 20% fetal bovine serum was added back to the cell culture. Three days after transfection, living cells were stained

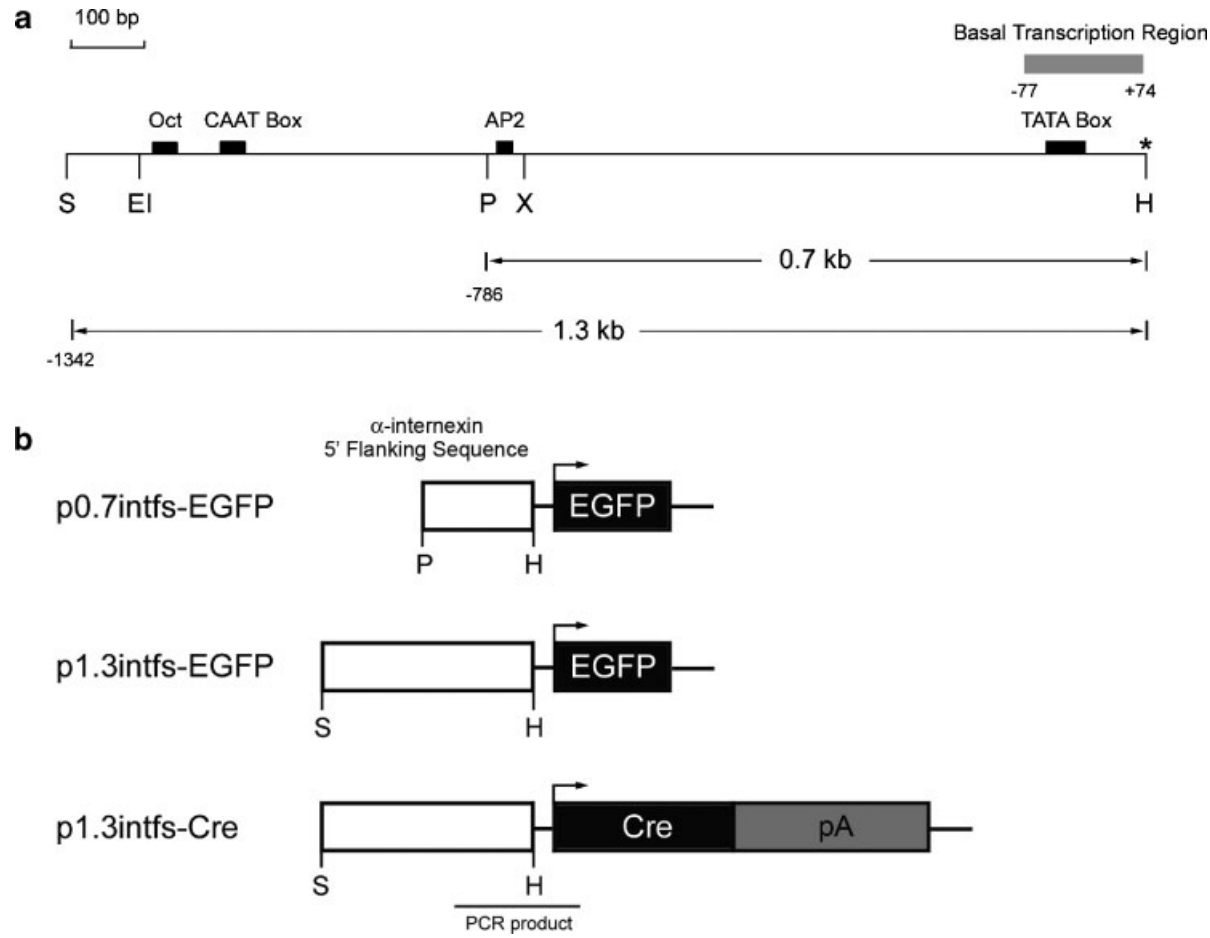


Fig. 1. Structure of the 1.3 kb 5′-flanking sequence of the mouse α-*internexin* and reporter constructs. **a**: The 5′-flanking region of the mouse α-*internexin* upto nucleotide −1342 contains several transcriptional regulatory elements (box), including the TATA box (ATTAAA), potential AP2 binding site, CAAT box, and Oct. The gray bar indicates the basal transcription region of the α-*internexin*, comprising sufficient information for basal promoter activity [Ching and Liem, 1991]. Major restriction enzyme sites relevant to construction are also shown. The asterisk represents the ATG condon being replaced at the *Hind*III restriction site by PCR primer (**ATG**AGTT → **AT**AAGCTT) and destroyed the translation initiation site. The nucleotide +74 to −786 and +74 to −1342, represented as the 0.7 kb and 1.3 kb promoter regions of the mouse α-*internexin*, respectively were used to create DNA constructs. **b**: The 0.7 kb and 1.3 kb promoters of the mouse α-*internexin* are fused to the pEGFP-1 vector, as p0.7intfs-EGFP and p1.3intfs-EGFP, respectively. The p1.3intfs-Cre construct for Cre recombinase expression controlled under the 1.3 kb α-*internexin* promoter was applied to generate the transgenic mice. PCR was used to identify transgenic mice with the primers designed in the promoter and *cre*, and the region of the specific PCR product of 566 bp is indicated. Abbreviations for restriction enzyme sites: EI, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sac*I; X, *Xba*I.

with nuclear dye, H33342 for counting the cell numbers and then observed on a confocal spectral microscope equipped with a water lens (Leica TCS SP2, Heerbrugg, Switzerland) to detect EGFP and DsRed1 expression, or fixed and processed for X-gal histochemical staining. Each experiment was performed in duplicate cover slips and more than 900 cells per experiment were examined. The mean ± SD was calculated from three independent experiments. Means were compared using Student's *t*-test.

Generation of 1.3intfs-Cre Transgenic Mice

The p1.3intfs-Cre construct containing the 1.3 kb α-*internexin* 5′-flanking sequence and Cre recombinase gene was excised by *Bgl*II from vector backbone. The transgenic mouse lines were generated by the Transgenic Core at National Taiwan University. To examine Cre DNA recombinase activity, the transgenic offspring were crossed with ROSA26 reporter (R26R) strain mice (The Jackson Laboratory, Bar Harbor, Maine). The R26R mouse has a

ROSA26 gene that has been modified to harbor a loxP-flanked Neo^R cassette when excised by Cre recombinase allows the *ROSA26* gene promoter to drive expression of the *E. coli LacZ* gene [Soriano, 1999]. All newborn pups were analyzed on postnatal day 1 (P1). Genomic DNA was prepared from fresh limb tissues of the newborns for identification of the genotype. The 566 bp fragment of the integrated 1.3intfs-Cre sequence was PCR amplified, and the forward primer 5′GAAAAACCTCCTCTCCGTGGG3′ and the reverse primer 5′GCGAACCTCATCACT-CGTTG3′ were applied under the following PCR conditions: first at 94°C for 3 min, 35 cycles at 94°C for 30 s, 55.6°C for 30 s, and 72°C for 1 min with a final extension at 72°C for 7 min. PCR analyses for the R26R allele are described in the genotyping protocols of the Jackson Laboratory (http://jaxmice.jax.org/index.html).

X-Gal Histochemistry for Transfected Cells and Whole-Mount Newborn Mice

We followed a well-established protocol for X-gal development by Kawaguchi et al. [2002] to visualize X-gal staining. Transfected cells on coverslips were washed with 0.2 M phosphate-buffered saline (PBS, pH 7.2–7.4) three times, and then fixed in cold methanol at −20°C for 10 min. After washing with PBS at room temperature, cells were rinsed in rinse buffer (2 mM $MgCl_2$, 0.02% NP-40, and 0.01% sodium deoxycholate in PBS). The β-galactosidase activity was detected by reacting in a substrate buffer (5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal] in rinse buffer) at 37°C overnight. Slides were mounted and observed with an Axioskop light microscope (Carl Zeiss, Oberkochen, Germany).

P1 mice were sacrificed by an overdose of choral hydrate (i.p., 400 mg/ kg of body weight), fixed with 4% paraformaldehyde in PBS for 4–6 h, and subsequently washed three times for 20 min in PBS. Fixed pups were embedded with 5% agar in distilled water and cut sagittally from the midline with a vibratome. The β-galactosidase activity was visualized by exposing the subject to the X-gal staining buffer described above at 37°C overnight. Stained subjects were then rinsed three times for 15 min in PBS and were observed and photographed under a dissection microscope (Leica MZ8, Heerbrugg, Switzerland).

Immunohistochemistry

The thoracic walls were dissected from X-gal-stained whole-mount P1 pups and post-fixed for 16 h. After cryoprotection with 30% sucrose in PBS, 16-μm-thick serial coronal sections from tissues were obtained and mounted on silane-coated slides (Muto pure chemicals Co., LTD, Japan). After treating with 0.1% Triton X-100 and 5% normal goat serum in PBS for 1 h, sections were incubated overnight at 4°C with one of the following primary antibodies: 1:200 mouse anti-α-internexin (Chemicon, Temecula, CA), 1: 200 mouse anti-neurofilament light subunit NF-L (Sigma Chemical Co., St. Louis, MO), or 1:200 rabbit anti-peripherin (Chemicon, Temecula, CA). Sections were washed by PBS and then incubated with 1:50 FITC-conjugated goat anti-mouse IgG (Sigma) or FITC-conjugated goat anti-rabbit IgG (Sigma) at 1:50 dilution. After PBS wash, sections were mounted with aqueous mounting medium (Biomeda, Foster City, CA). Images were taken on a Ziess Axiophot epiflurorescence microscope (Carl Zeiss, Oberkochen, Germany), equipped with a Nikon D1X digital camera (Nikon, Tokyo, Japan).

RESULTS

Expression Efficiency of EGFP Reporter and Cre Recombinase Regulated by *α-Internexin* Promoter In Vitro

We first investigated the functional role of 5′-flanking sequences of the mouse *α-internexin* by performing transient expression experiments with EGFP reporter constructs containing two different lengths of the promoter. These constructs were transfected into a non-neuronal (3T3 fibroblasts) and a neuronal (Neuro2A) cell line.

To normalize the transfection efficiency in both cell lines, DsRed1-N1 vector was transfected as a parallel control. The preferential expression of *α-internexin* promoter was revealed by the percentage of EGFP-expressing cells to DsRed1-expressing cells (Fig. 2e). A different expression efficiency of EGFP was observed with the construct containing 0.7 and 1.3 kb of the *α-internexin* 5′-flanking sequences in two cell lines ($P < 0.001$, Fig. 2a–d). In the non-neuronal 3T3 cell line, the expression level was low by the regulation of 1.3 kb 5′-flanking sequence (***, $P < 0.001$, Fig. 2c), whereas in

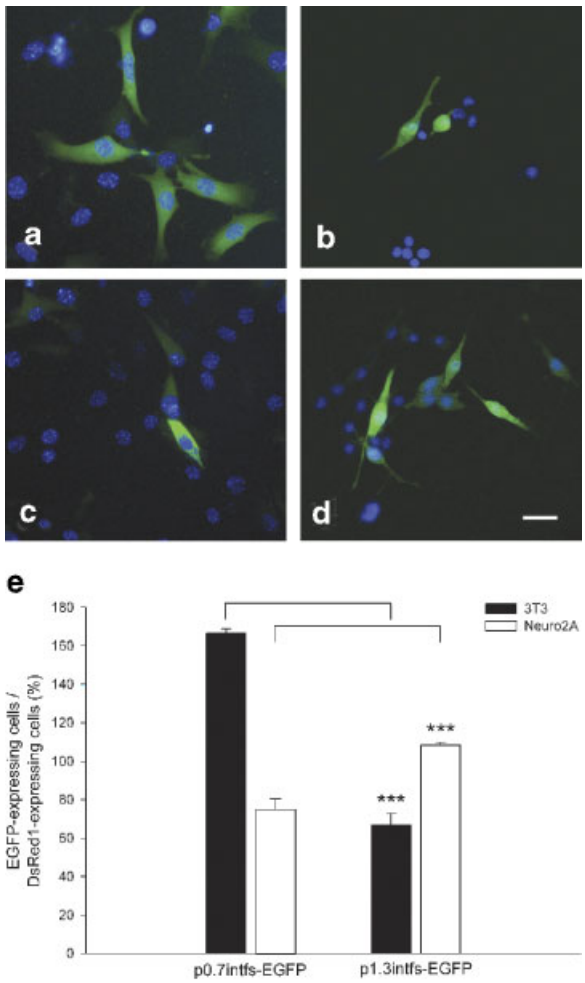


Fig. 2. Expression patterns of the EGFP reporter protein in 3T3 fibroblasts and Neuro2A cells. p0.7intfs-EGFP (**a**, **b**) and p1.3intfs-EGFP (**c**, **d**) were introduced to both 3T3 fibroblasts (**a**, **c**) and Neuro2A cells (**b**, **d**) to express EGFP reporter protein. **e**: Percentages of EGFP-positive cells to DsRed1-expressing cells were calculated in transfected 3T3 fibroblasts and Neuro2A cells. The expression efficiency of EGFP driven by 0.7 kb internexin promoter is higher in 3T3 fibroblasts cells than in Neuro2A cells. However, the above tendency is completely reversed by the activity of 1.3 kb internexin promoter. The statistical data demonstrate that the 1.3 kb promoter exhibits higher (***, $P < 0.001$) and more specific (***, $P < 0.001$) EGFP expression efficiency in Neuro2A cells than the 0.7 kb promoter. Scale bar = 40 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Neuro2A cells, this 1.3 kb α-*internexin* sequence enhanced the EGFP expression (***, $P < 0.001$, Fig. 2d). These results suggest that the mouse α-*internexin* 5′-flanking region upto 1.3 kb confers a preferential expression pattern in neuronal-derived cells. Particularly, α-*internexin* sequences between −1342 bp and −786 bp, contain elements, that is, octamer-like sequences (Oct) that may participate in the cell-type specificity of the gene (Fig. 1a), but these sequences do not seem sufficient to abolish the gene expression completely in non-neuronal cells.

To assess the function of promoter regions in a neuronal expression pattern of α-*internexin*, we constructed 1.3 kb α-*internexin* 5′-flanking regions upstream of the reporter *cre*. The 1.3 kb α-*internexin* 5′-flanking sequence was chosen due to its high selective expression in the neuronal cell line described above. As shown in Figure 3, the β-galactosidase activity detected by X-gal histochemistry was greatly decreased under Cre-mediated deletion in transfected Neuro2A cells. The expression of *cre* under the control of either hCMV promoter (pBS185) or 1.3 kb α-*internexin* 5′-flanking sequences (p1.3intfs-Cre) resulted in a significant decline of β-galactosidase activity (***, $P < 0.001$, Fig. 3d). This reveals the similar expression efficiency controlled by these two different promoters. Our data in vitro showed that 1.3 kb of the mouse α-*internexin* 5′-flanking sequence is sufficient to direct the gene expression in a neuronal manner.

Establishment of 1.3intfs-Cre Transgenic Mice

To study the promoter function of α-*internexin* in vivo, the Cre/loxP site-specific recombination system was employed. It was previously shown that the onset of rat α-*internexin* expression in the developing nervous system occurs on embryonic day 12 [Kaplan et al., 1990]. Accordingly, the 1.2 kb rat α-*internexin* promoter is sufficient to drive the CAT reporter in vitro [Ching and Liem, 1991] and leads to a neuron-specific expression pattern in transgenic mice [Ching et al., 1999]. In the present study, 1.3 kb mouse α-*internexin* promoter was applied to drive the expression of the Cre recombinase. Pronuclear injection of the 1.3intfs-Cre transgene resulted in eight founders and we subsequently obtained two 1.3intfs-Cre transgenic mouse lines.

To test the transgenic lines, 1.3intfs-Cre mice were crossed with R26R mice and the genomic tail DNA of P1 offspring was analyzed by PCR for the presence or absence of Cre and R26R alleles. Data from one litter of 1.3intfs-Cre x R26R mouse offspring showed that one double-transgenic 1.3intfs-Cre/R26R mouse could be identified from the wild type and single transgenic 1.3intfs and R26R mice (Fig. 4).

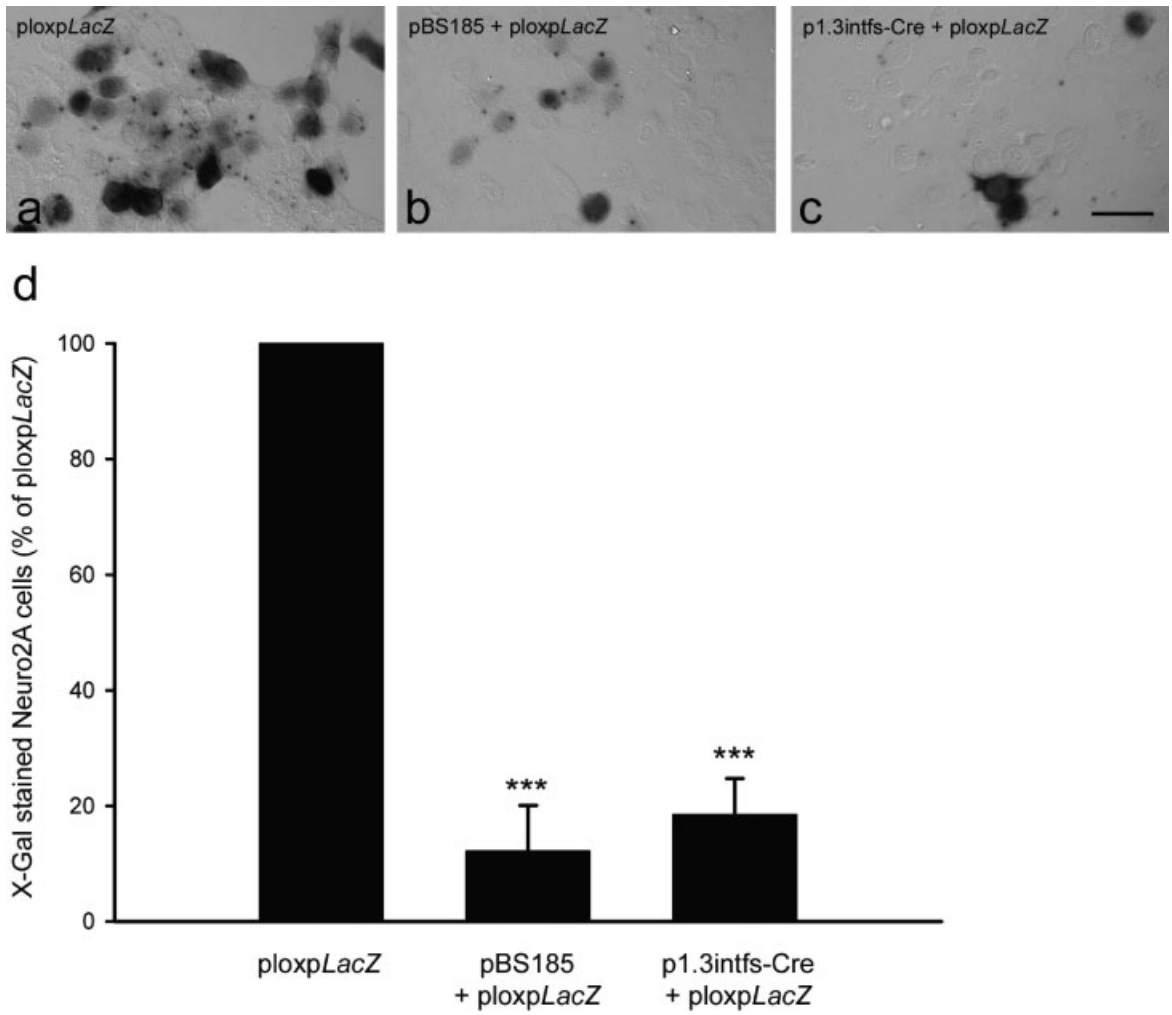


Fig. 3. 1.3 kb mouse α-*internexin* promoter directed Cre DNA recombinase activity in Neuro2A cells. To test Cre recombinase activity in vitro, pBS185 or p1.3itfs-Cre were cotransfected with ploxp*LacZ* into Neuro2A cells. The β-galactosidase activity was produced by ploxp*LacZ* in transfected cells and was monitored by X-gal staining. The X-gal-stained cells resulting from Cre-mediated excision of *LacZ* in pBS185 + ploxp*LacZ* (**b**) or p1.3intfs-Cre + ploxp*LacZ* (**c**) cotransfection are decreased compared with the ploxp*LacZ*-transfected group (**a**). The relative percentage of X-gal-stained cells in the cotransfection group over the ploxp*LacZ* transfected group is shown in (**d**). The statistical data indicate that hCMV promoter-driven (pBS185) and 1.3 kb α-*internexin* promoter-driven (p1.3intfs-Cre) Cre-mediated recombination are equally efficient (***, $P < 0.001$). Scale bar = 40 μm.

Cre-Mediated Expression of *LacZ* in P1 1.3intfs-Cre/R26R Double-Transgenic Mice

Whole-mount X-gal staining of P1 wild type and 1.3intfs-Cre/R26R double-transgenic mice revealed Cre-mediated *LacZ* activation in a peripheral nerve manner (Fig. 5a,b). The Cre-mediated LacZ activity within the 1.3intfs-Cre/R26R double-transgenic mouse was observed to be restricted to the peripheral nervous tissues, and was not seen in the CNS of the whole-mount mouse (Fig. 5b). With the exception of the skeleton, which exhibited non-specific X-gal staining, all other tissues in the body of the mouse did not show any Cre-mediated *LacZ* activation (Fig. 5b). From our comprehensive observations, Cre-mediated *LacZ* expression was specific to spinal nerves, distributing in the thoracic and abdominal walls, pelvis, and limbs (Fig. 5c,d). X-gal staining in the spinal nerves could be seen penetrating the intervertebral foramina at the level of the vertebral column (Fig. 5f). DRG neurons were also X-gal positive. Similarly, *LacZ* expression was limited to the cranial nerves, especially the subcutaneous nerves of the skin and nerves in the tongue (Fig. 5e). In addition, non-specific X-gal staining was also shown in the ribs, vertebrae,

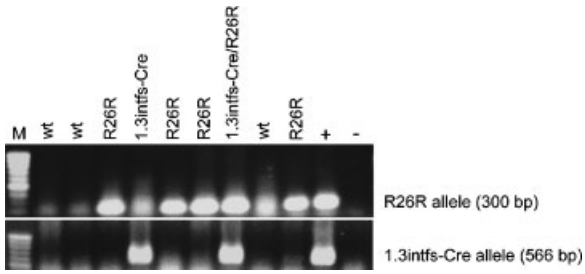


Fig. 4. PCR screening of 1.3intfs-Cre/R26R transgenics. The data represent a typical example (this litter contains nine newborns) from three independent litters. R26R allele-specific PCR and 1.3intfs-Cre allele-specific PCR were performed on mouse tail genomic DNA from the1.3intfs-Cre transgenic line crossed with the R26R strain. One double-transgenic 1.3intfs-Cre/R26R mouse can be identified from the wild type (wt), 1.3intfs, and R26R mice. M: 100 bp DNA ladder marker, +: positive control, −: negative control.

and skeletons of the limbs in both wild-type and 1.3intfs-Cre/R26R newborn mice (Fig. 5a,b).

To clarify the distribution of internexin promoter-driven Cre-mediated *LacZ* activity in the nervous system, more detailed immunohistochemical analysis was applied. All DRG neurons of P1 1.3intfs-Cre/R26R double-transgenic mice were shown to be immunoreactive for α-internexin, NF-L, and peripherin (Fig. 6). Most of them exhibited weak to moderate β-galactosidase activity in cell bodies. At the same section, we observed spinal nerves expending from DRG were NF-L- and peripherin-positive, but α-internexin-negative. All of these nerves showed strong X-gal stain (Fig. 6a–f). However, the β-galactosidase activity was undetectable in α-internexin-positive neurons of spinal cord (Fig. 6g,h).

These results indicated that the mouse α-internexin promoter could be activated in neonatal mice and this specific transgenic activity was dependent upon the presence of 1.3 kb α-internexin promoter.

DISCUSSION

Our in vitro study demonstrated that the 1.3 kb of mouse *α-internexin* 5′-flanking sequence assigned a preferential expression pattern in

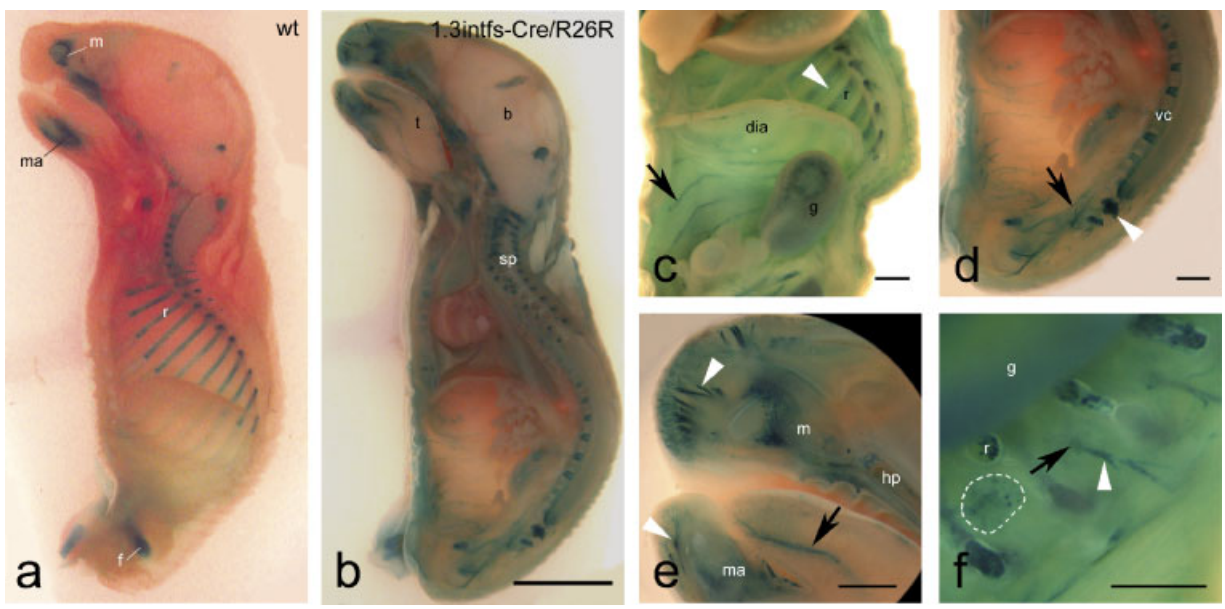
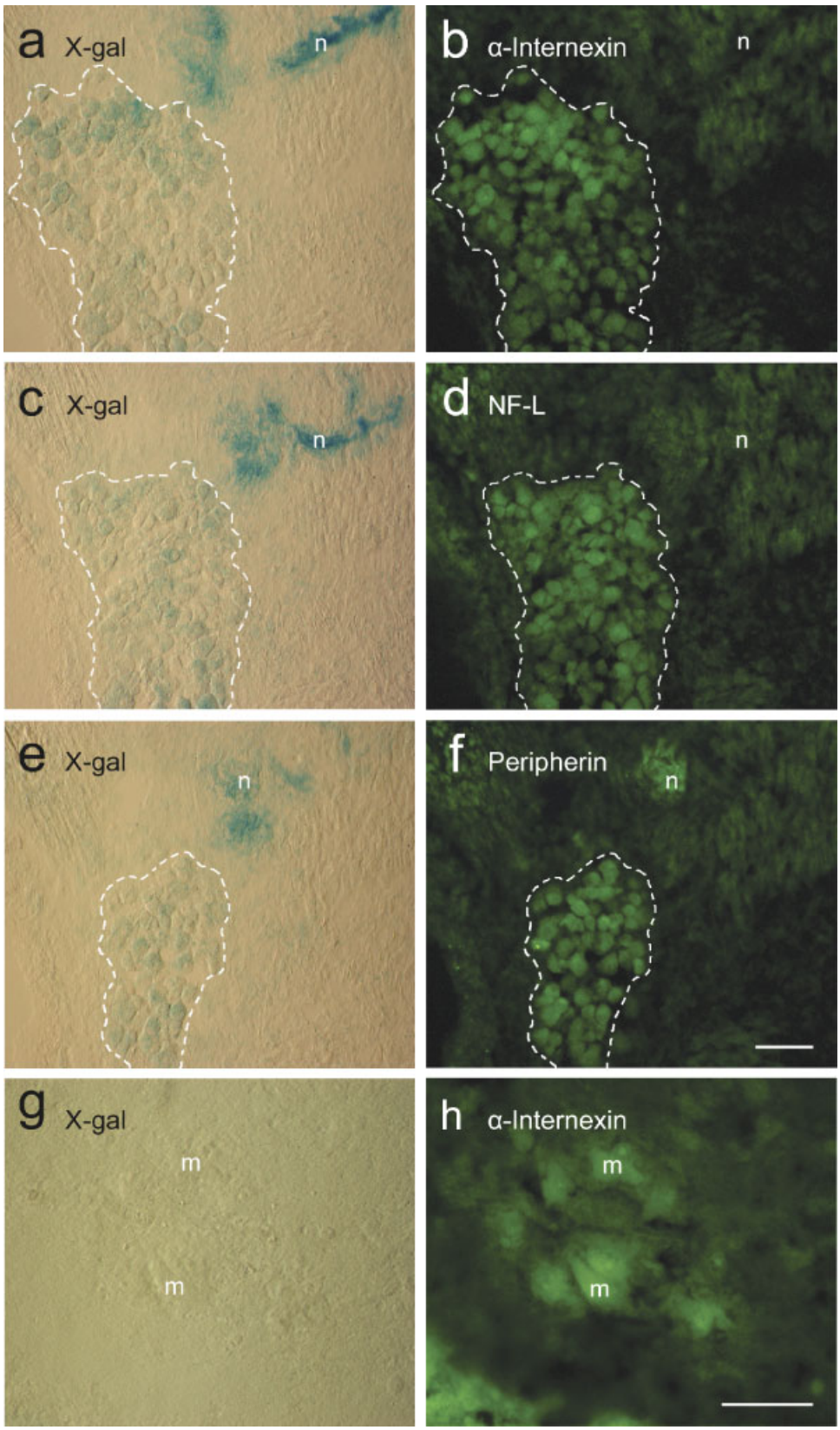


Fig. 5. Histochemical analysis of Cre-mediated β-galactosidase activity in P1 1.3intfs-Cre/R26R double-transgenic mice. Sagittal sections of the wild-type (**a**) and the 1.3intfs-Cre/R26R double-transgenic (**b**) newborns were treated with whole-mount X-gal staining. In addition to the specific expression pattern in nervous tissues, β-galactosidase activity is also detected in the ribs, vertebrae, and bones of the face and limbs of wild-type and 1.3intfs-Cre/R26R mice. β-galactosidase activity can not be detected in the nervous tissues of the brain and spinal cord except for the non-specific ventricular space (**b**). β-galactosidase expression can be found in nerves of the thoracic and abdominal walls (arrowhead and arrow in **c**), roots of the sciatic nerve and nervous plexus in the pelvis (arrowhead and arrow in **d**), and nerves to the skin and the tongue in the mouth region (arrowhead and arrow in **e**). With a high magnification, spinal nerves (arrowhead in **f**) penetrating the intervertebral foramina (arrow in **f**) and DRG neurons with β-galactosidase activity (encircled by a dotted line in **f**) can be observed. The photographs shown here at least from two different litters of 1.3intfs-Cre/R26R mouse. Abbreviations: b, brain; dia, diaphragm; f, femur; g, gut; hp, hard palate; m, maxilla; ma, mandible; r, rib; sp, spinal cord; t, tongue; vc, vertebral column. Scale bar = 5 mm (**a**, **b**) and 1 mm (**c**–**f**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]


a X-gal
n
b α-Internexin
n
c X-gal
n
d NF-L
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e X-gal
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f Peripherin
n
g X-gal
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h α-Internexin
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Fig. 6.

neuronal cells. We also confirmed that the α-*internexin* promoter-driven *cre* expression exhibited efficient transcriptional activity in the Cre/loxP recombination system. Several studies have shown that the promoter regions of several intermediate filament proteins, including nestin [Lendahl et al., 1990; Lothian et al., 1999], peripherin [Karpov et al., 1992], neurofilament triplet proteins [Zopf et al., 1990; Reeben et al., 1993; Schwartz et al., 1994, 1998], and glial fibrillary acidic protein (GFAP) [Brenner et al., 1994; Andræ et al., 2001; Rio et al., 2002], are activated in a spatiotemporal expression pattern. 5′-flanking sequences (1.2 kb) of rat α-*internexin* are shown to contain several potential positive regulatory elements, consisting of SP1, AP2, AP4, and Oct [Ching and Liem, 1991]. Sequence analysis on α-*internexin* promoters of rat and mouse shows that 90% homology and the presence of similar positive transcriptional regulatory elements in the proximal part of the promoters of these two species. Additionally, the CAAT box and octamer-like sequences could only be identified in the mouse α-*internexin* promoter between nucleotide −786 and −1342 (data not showed). The potential regulatory sequences in the promoter may be important in endowing α-*internexin* with neuron-specific properties.

In this study, we observed a low but not completely downregulated EGFP expression in 3T3 fibroblasts transfected by p1.3intfs-EGFP. This suggests that the regulatory elements in the 1.3 kb 5′-flanking sequence are partially but not fully regulated in non-neuronal cells. These results also indicated that a precise expression of the gene may be regulated by other regulatory elements in intragenic regions such as introns, or extragenic part of the 3′-flanking sequence. In previous studies of intermediate filament genes, some regulatory elements were shown to be localized in introns [Charron et al., 1995; Hsu et al., 1995; Lecomte et al., 1999; Lothian et al., 1999]. A study of *nestin* promoter showed that a 120-bp sequence in the second intron of the human *nestin* gene is sufficient to enhance the *LacZ* reporter gene throughout the early developing CNS [Lothian et al., 1999]. Analyses of the intragenic part of the mouse *peripherin* gene have demonstrated that an interplay between intron I and the 5′-flanking sequences of *peripherin* leads to activation and inhibition of gene expression in peripherin-positive and -negative cells, respectively [Lecomte et al., 1999]. Similarly, multiple regulatory elements and enhancers in introns of the *NF-L* gene are contributed to direct both spatial and temporal expression during embryogenesis and in the adult [Beaudet et al., 1992; Leconte et al., 1994–95; Charron et al., 1995; Hsu et al., 1995]. Therefore, other potential regulatory motifs in mouse α-*internexin* may be involved in tuning accurate neuron-specific expression.

The Cre/loxP site-specific recombination system provides a means for restricting somatic mutations to subregions of the nervous system, thus enabling the functional study of neural genes in certain nervous tissues. In order to conduct a site restricted mutation analysis in the nervous system, we established transgenic mice expressing Cre recombinase under the control of the mouse α-*internexin* promoter and utilized R26R mice for cross-breeding. From our phenotyping observations, α-*internexin* promoter can be activated in mouse development, and the activity of this promoter is dependent upon the transcriptional regulatory elements in the promoter. The phenotype we examined was expressed in the PNS during development. Currently, Cre-mediated *LacZ* expression can be observed in branches of cranial and spinal nerves as well as in DRG neurons at P1 indicating an embryonic onset of *cre* expression. We would expect that *cre* expression continues throughout postnatal development.

DRG neurons of double-transgenic P1 mice exhibited immunoreactivities for α-internexin, NF-L, and peripherin. These observations were parallel to the developmental profiles of these neuronal intermediate filament proteins in general. Neurofilaments and peripherin are both expressed in a single neuronal type of

Fig. 6. Presence of Cre-mediated β-galactosidase activity in dorsal root ganglionic neurons and spinal nerves of P1 1.3intfs-Cre/R26R double-transgenic mice. Coronal sections of thoracic wall of double-transgenic mice were stained with X-gal and then immunostained with antibodies for α-internexin (**a**, **b**, **g**, and **h**), NF-L (**c**, **d**), and peripherin (**e**, **f**), respectively. In particular, DRG neurons with weak β-galactosidase activity (**a**, **c**, and **e**) (bounded by dotted lines), were α-internexin-, NF-L- and peripherin-immunoreactive (**b**, **d**, and **f**, respectively). The peripheral spinal nerves (n) with the strong β-galactosidase activity are immunopositive for NF-L and peripherin (**d**, **f**) but not α-internexin (**b**). The β-Galactosidase activity is not detectable in α-internexin-immunoreactive motor neurons (m) of the spinal cord (**g**, **h**). Scale bar = 40 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DRGs at late embryonic period [Golstein et al., 1996], and α-internexin is also found in embryonic DRG neurons although its expression declines postnatally [Kaplan et al., 1990; Fliegner et al., 1994]. However, our recent studies showed that *α-internexin* keeps its expression in the postnatal DRG and its protein mainly found in the central processes (project to the spinal cord) and another peripherin protein is found predominantly in the peripheral processes (spinal nerves) of DRG in the postnatal mice (unpublished data). These data indicates that transgenic manipulations did not change the expression patterns of endogenous α-internexin, NF-L, and peripherin, which normally occur during development. In addition, the observation that peripehrin instead of α-internexin was found in peripheral spinal nerves at P1 may represent the early feature of distribution changes between peripherin and α-internexin.

High levels of Cre-mediated *LacZ* expression were observed in each X-gal-stained peripheral nerve, this indicated that Cre recombinase driven by 1.3 kb of the mouse *α-internexin* promoter is sufficient to result in high *LacZ* expression. However, the level of *LacZ* expression was undetectable in neurons of CNS. Transgenic analyses of NF-L gene promoter also reveal a neuron- and muscle-specific gene expression during embryogenesis [Yaworsky et al., 1997]. The POU-homeodomain transcription factor, Brn3a, which is expressed mainly in PNS neurons and in selected subregions of the CNS, may serve as a candidate for explaining the phenotype in the 1.3intfs-Cre transgenic mice. Studies have shown that Brn-3a plays critical roles in the development and function of the nervous system and stimulates neuronal process outgrowth [Smith et al., 1997a; Latchman, 1999]. Brn-3a is also identified as a neuronal marker for sensory neuron precursors derived from the neural crest [Greenwood et al., 1999]. Brn-3a activates the expression of the *TrkA* gene, which is crucial for differentiation and survival of neural-crest-derived sensory neurons, in a tissue and stage-specific manner [Ma et al., 2003]. In addition, Brn-3a is able to activate rat *α-internexin* promoter, and the responsive elements for Brn-3a may be located close to the transcriptional start site [Budhram-Mahadeo et al., 1996]. Overexpression of Brn-3a results in enhanced neurite outgrowth, the stimulation of neurofilament gene expression and activation of the neurofilament gene promoters [Smith et al., 1997b]. Although the promoters for genes encoding type IV intermediate filament proteins are activated by Brn-3a, the corresponding regions of Brn-3a required for the stimulation of promoters of neurofilament genes and *α-internexin* are different [Budhram-Mahadeo et al., 1996]. It is possible that the alternative activation mechanism of mouse *α-internexin* promoter by Brn-3a may account for the peripheral nerve-specific expression of 1.3intfs-Cre transgenics, and other regulatory elements in introns or the 3′-flanking region may drive specific expression of this *α-internexin* in the CNS.

In several transgenic mice, *cre* expression is driven by intermediate filament gene promoters in certain subregions or specific cell types of the nervous system. Zhuo et al. [2001] reported that widespread gene targeting in multi-potential CNS stem cells is applicable to astrocytes, by using a 2.2 kb 5′-flanking region from the human GFAP gene. Another transgenic mouse, which expressed Cre recombinase under the control of the murine *neurofilament heavy subunit* (*NF-H*) gene promoter was established by Hirasawa et al. [2001]. This transgenic line displayed specific excision of the loxP-flanked gene in neurons of the spinal cord and olfactory bulb area just after embryonic day 18.5 [Hirasawa et al., 2001]. *Peripherin* promoter and its 1.1 kb intragenic sequences were also employed to drive *cre* expression [Zhou et al., 2002]. These *peripherin* transgenic mice exhibited Cre recombinase activity in dorsal root ganglia, trigeminal ganglia, and olfactory epithelium during embryogenesis [Zhou et al., 2002]. This spatiotemporally controlled *cre* expression was similar to the expression of the endogenous mouse *peripherin* gene [Greene, 1989; Belecky-Adams et al., 1993; Chien et al., 1998]. In our analyses of *cre* expression, transient transfection in vitro and *LacZ* expression in vivo allowed us to evaluate the efficiency of promoter-driven Cre DNA recombinase activation. The significant decrease in the X-gal-stained Neuro2A cell population was shown under cotransfection of *α-internexin* promoter-*cre* together with its targeting ploxP*LacZ* vector compared to ploxP*LacZ*-transfection only (Fig. 3). A high level of β-galactosidase activity was seen in the peripheral nerves by mouse *α-internexin* promoter-driven Cre-mediated deletion in the R26R allele, whereas Cre-mediated *LacZ* expression

was not observed in any other region of newborn offspring (Fig. 5). These results provide the methodology for the establishment of a 1.3intfs-*Cre* transgene that preferentially expresses Cre DNA recombinase in peripheral nerves during development.

Additionally, there were quite a bit of non-specific X-gal staining in the control mice, especially in the skeletal system. It may represent the endogenous β-galactosidase activity in these tissues. The presence of β-galactosidase activity in the skeletal system is supported by several studies, which showed β-galactosidase activities in cells of bone and cartilage, including osteoclasts, multinucleate giant cells [Zheng et al., 1991], and chondrocytes [Martin et al., 2004]. This protein was also used as an age-related marker in cartilage and osteoarthritis [Martin et al., 2004], and could be identified by X-gal staining [Dimri et al., 1995]. Therefore, it may be a reasonable explanation for these non-specific X-gal-labeled tissues in control and in transgenic mice.

In summary, we confirmed the preferential neural control of the 1.3 kb 5′-flanking sequence of mouse *α-internexin* and established a *cre* transgenic line that allows conditional expression of genes, particularly in the developing peripheral nerves. Although this phenotype is not completely in line with the endogenous expression pattern of *α-internexin*, the unique features of the 1.3intfs-Cre transgene will provide a valuable tool for functional analysis of genes relevant to the neuronal development and innervation of the PNS.

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