Dorsal Root Ganglia Neuron-specific Promoter Activity of the Rabbit β-Galactoside α1,2-Fucosyltransferase Gene*

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The rabbit H-blood type α1,2-fucosyltransferase (RFT-I), gene and its biosynthetic products, H antigens (Fucα1,2Galβ), are abundantly expressed in a subset of dorsal root ganglia (DRG) neurons. To investigate the regulatory mechanisms for the RFT-I gene expression, we determined the genomic structure and promoter activity of this gene. PCR amplification of the 5′ cDNA end analysis revealed two transcriptional start sites, 498 and 82 nucleotides upstream of the translational initiation codon, the latter site yielding a major 3.1-kb transcript specifically expressed in DRG, as revealed by Northern blotting. Promoter analysis of the 5′-flanking region of the RFT-I gene using a luciferase gene reporter system demonstrated strong promoter activity in PC12 cells, which express the rat H-type α1,2-fucosyltransferase gene, and Neuro2a mouse neuroblastoma cells. Deletion analysis revealed the 704-base pair minimal promoter region flanking the translational initiation codon, for which two distinct promoter activities were detected and differentially used in PC12 and Neuro2a cells. The minimal promoter region contained a GC-rich domain (GC content 80%), in which a Sp1 binding sequence and a GSG-like nerve growth factor-responsive element were found, but lacked TATA- and CAAT-boxes. Promoter analysis with a primary culture of DRG neurons demonstrated that the minimal promoter region of the RFT-I gene was sufficient for the expression of a reporter gene in DRG neurons. We conclude that the TATA-less GC-rich minimal promoter region of the RFT-I gene controls DRG small neuron-specific expression of the RFT-I gene.

The H-blood type antigens (Fucα1,2Galβ) are synthesized by GDP-L-fucose:β-D-galactoside 2-α-L-fucosyltransferase (α1,2-FT)1 (for a review, see Ref. 1). The expression of H determinants is strictly regulated temporally and spatially during vertebrate development (2–4). The H antigens are rarely detected in adult nervous tissues of human and other mammals but are present on a subset of neurons. Analyses with Ulex europaeus agglutinin 1 lectin, which binds to type 2 H (Fucα1,2Galβ1,4GlcNAc) determinants, and anti-H antibodies revealed that the expression of H antigens was restricted to olfactory bulb and cochlear hair cells in rats (5, 6) and to primary sensory neurons and their axons in human and other primates (7–9). Most of the H-positive axons of primary sensory neurons were unmyelinated and thought to be C-fibers that mediate nociceptive or thermoreceptive inputs or both.

We have extended the analysis of the expression of H determinants in the mammalian nervous system using anti-fucosyl GM1 antibodies and U. europaeus agglutinin 1 lectin (10–13). In the human and rabbit nervous systems, H antigens are abundantly expressed in dorsal root ganglia (DRG), which consist of several types of primary sensory neurons. The anti-fucosyl GM1 antibodies and U. europaeus agglutinin 1 lectin recognized a subpopulation of neurons in DRG and the dorsal horn of the spinal cord. The anti-fucosyl GM1 antibodies also bound to the satellite cells surrounding the fucosyl GM1-positive neurons (10, 12). In addition, in rabbits, the anti-fucosyl GM1 antibodies bound to the axons and the myelin of the small myelinated fibers in the dorsal root and the large neurons in the ventral horn (13). In rabbit DRG, fucosyl GM1 is readily detectable immunohistochemically on embryonic day 25, followed by the appearance of U. europaeus agglutinin 1 lectin-reactive antigens postnatally (12, 13).

The expression of H antigens in human and rabbit DRG neurons seems to be under similar control, although little is known about the molecular basis of their regulated expression. To investigate the mechanisms underlying the regulation of the biosynthesis of H antigens in DRG neurons, we recently cloned three types of rabbit α1,2-FT gene, i.e., one H-type and two Se-type genes, as judged from the results of kinetic studies (14, 15). Analysis of the expression of these genes revealed that all three α1,2-FT genes were expressed in DRG of late embryonic rabbits but that only the H-type α1,2-FT gene, the RFT-I gene, was expressed postnatally (16). In situ hybridization demonstrated that abundant RFT-I mRNA was present in adult rabbit DRG neurons of small diameter. We have shown that the RFT-I gene specifies the mRNAs of a major 3.1-kb transcript in DRG and a minor 4.2-kb transcript broadly expressed in rabbit nervous tissues. These results indicate that the 3.1-kb transcript of the RFT-I gene is under the control of DRG small chain reaction; CHO, Chinese hamster ovary; NGF, nerve growth factor; bp, base pair(s); kb, kilobase pair(s); CMV, cytomegalovirus; NGFI, nerve growth factor-induced gene. The nomenclature for gangliosides and glycolipids follows the system of Svennerholm (38).
neuron-specific promoter activity. In this study, we determine the genomic structure of the RFP-I gene and the promoter activity of the 5′-flanking region using a primary culture of DRG neurons.

**EXPERIMENTAL PROCEDURES**

**PCR Rapid Amplification of 5′ and 3′ cDNA Ends (RACE)—**Poly(A)-rich RNAs were extracted from adult rabbit DRG by the guanidinium isothiocyanate method and purified with Oleosogen-dT30 (Takara-Shuzo, Japan). Amplification of the 5′-end of the RFP-I cDNA was performed essentially according to the procedure of Frohman et al. (17). cDNA was synthesized by reverse transcription (Superscript II; Life Technologies, Inc.) of 5 μg of rabbit DRG poly(A)-rich RNA using primer H4B3, 5′-AAACCAAGAGGCCAGACAGGCTG-3′, which is complementary to nucleotides +45 to +22 (taking the translational initiation site as +1) of the RFP-I gene. The excess primer and deoxynucleotide were removed by passage of the cDNA through a MicroSpin S-400 column (Amersham Pharmacia Biotech). The cDNA was A-tailed with 0.6 units of terminal deoxynucleotidyltransferase (Boehringer Mannheim), using (dT)18 (Amersham Pharmacia Biotech). The cDNA was end-labeled with [32P]dCTP using Klenow polymerase.

**Plasmid Construction—**To obtain various lengths of the 5′-flanking region relative to the translation initiation site, we performed Northern blot analysis using probes A, B, or C. We previously reported two RFP-I mRNA isoforms, pH4-SK3.6 and pH4-SK3.6-I, which is complementary to nucleotides 1-903 of the RFT-I gene. The excess primer and deoxynucleotide were removed by passage of the cDNA through a MicroSpin S-400 column (Amersham Pharmacia Biotech). Two consecutive PCRs were performed with two nested sets of primers; for the first PCR, the forward primer was NotI-(dT)18 (Amersham Pharmacia Biotech), and the reverse primer was H4B3; for the second PCR, the forward primer was as above but without the T-stretch, and the reverse primer was HinClI (Invitrogen). The PCR products were then digested with NotI and HinClI and ligated into NotI-HinClI-digested pPGBII, respectively. As negative and positive controls, the pPGBII and pBII-SV40 plasmids, respectively, were also transfected.

**Plasmid pH4-SK3.6, which contains 5′-untranslated region, the entire coding region, and 3′-untranslated region with a poly(A) signal, was linearized with HindIII, combined with pTK-Hyg (CLONTECH), and then transfected into Neuro2a and PC12 cells. After culturing for 72 h, the cells were selected with 0.2 μg/ml of hygromycin (Life Technologies, Inc.) and subsequently subcloned.

**Cultures of DRG neurons and cerebellar granule cells were obtained as described (18).** Briefly, 5 × 10^5 cells were collected by centrifugation and then resuspended in 0.4 M sucrose and centrifuged. The cell pellet was then resuspended with buffer D (50 mM HEPES, pH 7.5, 2 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/mL of leupeptin), buffer A containing 0.2% Nonidet P-40, and buffer A containing 0.2 M sucrose and centrifugation at 800 g. The cell pellet was then resuspended with buffer D (50 mM HEPES, pH 7.9, 400 mM KCl, 10 μg/mL of leupeptin, 0.1 mM dithiothreitol, and 10 μg/mL leupeptin) and centrifuged. Protein concentration of supernatant was determined by a Bio-Rad protein assay.

**Drugs**

- **Narcotic administered in vivo**: buprenorphine (Buprenex; Abbot, Japan; 0.1 mg/kg i.m.) or saline (0.1 ml/kg i.m.) was administered once daily for 4 days and was continued at the same dose during the experiment. Drug-injected and saline-injected groups were analyzed separately.
- **Narcotic administered in vitro**: 10-20 μM of agonists was added into the medium and incubated for 1 h prior to the addition of the agonist. The response was determined as the maximum response reached within 1 h of agonist application. The maximally achievable response was determined using the same agonist in the absence of any other agonist. The EC50 values were determined from the dose-response curves with the aid of GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA)

**Data Analysis**

- **EC50 values** were determined using Graphpad Prism 3.0 (Graphpad Software Inc., San Diego, CA, USA). The EC50 values were calculated as the concentration of agonist that resulted in 50% of the maximal response.
- **Statistical Analysis**
  - **Data Analysis:** All data are expressed as the mean ± SEM. The Student's t-test for unpaired observations was used to determine the statistical significance of differences between treatment groups. Differences were considered statistically significant at p < 0.05.
Egr-1 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added where indicated. After incubation, the samples were loaded onto a 4% polyacrylamide gel in 0.5 × TBE. The gel was run in the cold at 200 V and dried, and then the radioactivity was detected with a BAS 2000 image analyzer (Fuji Film, Japan).

RESULTS

Determination of the Transcriptional Start Site of the RFT-I Gene—We previously showed that two RFT-I mRNA transcripts, 4.2 and 3.1 kb, were expressed in rabbit nervous tissues (16). The 4.2-kb transcript was widely found in the central and peripheral nervous tissues examined but in low amounts, whereas the 3.1-kb transcript was abundantly and specifically observed in DRG neurons of small diameter. To isolate the 5'-end of the RFT-I cDNA, we performed RACE-PCR using poly(A) RNA from adult rabbit DRG, and found two transcriptional start sites, at positions −498 and −82 (Figs. 1 and 2). The RACE-PCR results revealed that there are three types of mRNA transcripts; one transcript (3.1-kb mRNA in Fig. 1) started at position −82 and contained no intron, the second one (3.3-kb mRNA in Fig. 1) started at position −98 and skipped the intron of nucleotides −265 to −3, yielding an additional 3.3-kb transcript, and the last one (4.2-kb mRNA in Fig. 1) also started at position −498 but without splicing out the 3.3-kb mRNA intron sequence. We also performed 3' RACE-PCR and found a poly(A) signal at position +2579 without an intron.

To analyze the differential use of transcriptional start sites in rabbit nervous tissues, we next performed Northern analysis using three kinds of probes (probes A, B, and C in Fig. 1). Probe A, recognizing 3.3- and 4.2-kb mRNAs, gave weak signals at the corresponding bands in both DRG and brain RNA (Fig. 3). Probe B gave weak signals at 4.2-kb in DRG and brain RNA. Probe C hybridized to a 3.1-kb transcript in DRG RNA strongly enough to be detected on short exposure. These results showed that the 3.1-kb mRNA of the RFT-I gene was abundantly and specifically expressed in DRG and that the 3.3- and 4.2-kb mRNAs were broadly found in DRG and other nervous tissues but in low amounts.

Promoter Activity Analysis—To determine the RFT-I gene promoter activity, we used three types of cells: PC12 rat pheochromocytoma cells expressing rat H-blood type α1,2-FT, a counterpart of RFT-I; Neuro2a mouse neuroblastoma cells originating from the neural crest but not expressing α1,2-FT; and nonneuronal CHO cells. A series of reporter plasmids containing progressive deletions of the 5'-flanking region of the RFT-I gene fused to the promoterless luciferase gene were mixed with an internal control plasmid, pSR-β-Gal, carrying a β-galactosidase gene under the control of the SRα promoter, and then transfected into cells. The luciferase activity due to each luciferase reporter plasmid was normalized as to the β-galactosidase activity. The promoter activity was calculated relative to the SV40 promoter activity taken as 100%.

pBH4-BP3.0, containing the 3.0-kb 5'-flanking sequence of the RFT-I gene (−2970 to −4), showed high levels of promoter activity when expressed in PC12 and Neuro2a cells but not in CHO cells (Fig. 4). The pH4-SP0.7 reporter plasmid also showed high promoter activity in PC12 and Neuro2a cells, although the activity was lower than that of pBH4-BP3.0. The 0.7-kb 5'-flanking region was divided into three portions, and each fragment was ligated into a pPGBII plasmid. The pH4-SSm0.2 plasmid, containing the 0.2-kb sequence (−707 to −539), showed a substantial level of promoter activity when expressed in PC12 but not in Neuro2a cells, whereas the pH4-NP0.3 plasmid, containing the 0.3-kb sequence (−294 to −4), showed a level of promoter activity comparable with pH4-SP0.7 in Neuro2a cells but not in PC12 cells. pH4-Sma0.3 showed little promoter activity in both types of cells. These results suggested that the promoter activity was differentially regulated in PC12 and Neuro2a cells.

To determine the effect of the differential promoter activity on the transcriptional pattern, plasmid pH4-SK3.6, containing the 5'- and 3'-flanking regions and the entire coding region of the RFT-I gene, was stably transfected into Neuro2a and PC12 cells. The transcriptional pattern was analyzed by reverse transcription-PCR using three pairs of primers; the H4A5 and H4B4 primers could discriminate the presence of a 3.3-kb mRNA, the H4A5 and H4B5 (5'-GGAGGAGGTCTGGGAAAA-GAGGCC-3') primers could detect a 4.2-kb mRNA, and the H4A4 and H4B4 primers could detect 3.1- and 4.2-kb mRNAs but could not discriminate them. All pairs of primers gave positive bands when RNA from Neuro2a cell-derived stable transfecants with pH4-SK3.6 was analyzed (data not shown), indicating the presence of 3.3- and 4.2-kb transcripts of the RFT-I gene in these cells, although this result did not exclude the possibility of the presence of a 3.1-kb transcript. On the contrary, a positive band was amplified only by the H4A4 and H4B4 primers in PC12 cell-derived stable transfecants with pH4-SK3.6 (data not shown), showing the presence of a 3.1-kb transcript of the RFT-I gene.

Effects of NGF and Mutations on Promoter Activity—A data base search for possible binding of transcription factors revealed several Sp1 binding sites and a N-Myc binding site in the region of nucleotides −707 to −4. Among possible Sp1
binding sites, one (nucleotides -650 to -638) showed the highest homology to the consensus sequence and overlapped a GSG (GCGGGGCG)-like motif (nucleotides -645 to -637). To determine whether or not these elements were functional, we constructed plasmids pBH4-SacB8 (containing nucleotides -707 to -626) and pBH4-SacB8m (the same region with mutations) and transfected them into PC12 cells. PC12 cells transfected with pBH4-SacB8 and treated with 100 ng/ml of NGF for 48 h showed 2-fold higher luciferase activity than those without NGF (Fig. 5A). The mutations in the Sp1 and GSG overlapping domain decreased the luciferase activity and abolished the effect of NGF treatment.

We then examined whether or not a possible N-Myc binding site (nucleotides -244 to -233) found within the pBH4-NP0.3 construct could be demonstrated functionally. We constructed plasmids pBH4-A6Pst (containing nucleotides -264 to -4) and pBH4-A6mPst (the same region with mutations) and transfected them into Neuro2a cells. The pBH4-A6Pst and pBH4-A6mPst constructs showed comparable promoter activities when transfected into Neuro2a cells (Fig. 5B), suggesting that the N-Myc was not a major factor for the activity.

Electrophoretic Mobility Shift Assay—To confirm the actual binding of Sp1 to the putative Sp1 binding site between nucleotides -650 to -638, we performed an electrophoretic mobility shift assay. In the mobility shift experiments involving the DNA fragments of nucleotides -707 to -626, recombinant Sp1 bound to the DNA fragments (Fig. 6A, lane 2). The shifted band completely disappeared in the presence of the nonlabeled spe-
NGF treatment. When nuclear protein extracts of Neuro2a and PC12 cells with and without Neuro2a cells, we next performed the assay using nuclear mutations.

specific competitor (DNA fragments from −707 to −626, the same as the labeled probe; Fig. 6A, lane 3) and partially disappeared in the presence of the same region of the DNA fragments with mutations (lane 4). Recombinant Sp1 did not bind to the labeled probe of the DNA fragments from −707 to −626 with mutations.

To determine the transcriptional factors that regulate the specific promoter activity of this region in PC12 but not in Neuro2a cells, we next performed the assay using nuclear protein extracts of Neuro2a and PC12 cells with and without NGF treatment. When nuclear protein extracts of Neuro2a and PC12 cells were used, the labeled DNA fragment of nucleotides −655 to −626 (A8B8) appeared as two shifted bands with apparently the same electromobility (Fig. 6B, lanes 2–4, bands a and c). An additional band (band b) was observed when using a nuclear extract of PC12 cells treated with NGF for 90 min, and this shifted band disappeared upon the addition of the anti-Egr-1 polyclonal antibodies (Fig. 6B, lane 7). All shifted bands completely disappeared in the presence of the nonlabeled specific competitor (A8B8, lane 5) but were not abolished by the mutant competitor (A8mB8m, the same region of the DNA fragments with mutations; lane 6). No shifted band was found when using the labeled DNA fragment of nucleotide −655 to −626 with mutations (A8mB8m) and nuclear protein extracts of Neuro2a and PC12 cells.

Promoter Activity Analysis of Cultures DRG Neurons—

DRG neurons were cultured and transfected with reporter plasmids containing several lengths of the 5′-flanking region of the RFT-1 gene. The pBH4-SP0.7 construct showed the highest level of promoter activity when expressed in DRG neurons (Fig. 7), suggesting that the high promoter activity of the pBH4-SP0.7 construct could be due to the expression of the reporter gene in DRG neurons. Next, we determined whether or not the promoter activity of the pBH4-SP0.7 construct was specific to DRG neurons. The pBH4-SP0.7 and other constructs showed lower promoter activity when expressed in cerebellar granule cells, another type of neuron, than in DRG neurons (Fig. 7).

DISCUSSION

We previously showed that the expression of RFT-1 is strictly regulated spatially and temporally in the rabbit nervous system and abundant in adult DRG neurons of small diameter (16). In this study, we determined the genomic organization and promoter activity that regulates the DRG neuron-specific expression of the RFT-1 gene. Our results demonstrated that the RFT-1 gene used two transcriptional start sites yielding three types of mRNA and that the minimal promoter region flanking the translational initiation codon of the RFT-1 gene was sufficient for DRG neuron-specific expression of the gene. DRG neuron-specific promoter activity has not been detected so far, and analysis of the proteins binding to the minimal promoter region will facilitate understanding of the differentiation of DRG neurons.

The RFT-1 gene specified three types of mRNA; a 3.1-kb transcript starting at position −82 was abundantly and exclusively expressed in DRG neurons, and 3.3- and 4.2-kb transcripts both starting at position −498 were broadly found in the rabbit nervous system but in low amounts. Recently, evidence of multiple transcriptional start sites in such glycosyltransferase genes as the rat and human a2,6-sialyltransferase genes (19–21), murine β1,4-galactosyltransferase gene (22), human β1,6-N-acetylgalcosaminyltransferase V gene (23), human β1,4-N-acetylgalcosaminyltransferase gene (24), and human α1,3- and α1,2-fucosyltransferase genes (25, 26) has been accumulated. Similar to our results for the RFT-I gene, the murine β1,4-galactosyltransferase gene uses at least two transcriptional start sites yielding 4.1- and 3.9-kb mRNAs; the former is ubiquitously expressed in all tissues, and the latter is abundantly expressed in lactating mammary glands (22). The differential use of multiple transcriptional start sites and associated promoters of the glycosyltransferase gene could regulate the tissue- and stage-specific expression of glycosyltransferase genes and subsequent glycosylation patterns.

The distribution of H antigens in the nervous system is quite similar in human and rabbit; in both species, small neurons of DRG are positive for both U. europaeus agglutinin 1 lectin staining and anti-fucosyl GM1 antibody immunostaining (8, 11, 12). The human H-blood type α1,2-FT FUT1 gene is known to control the H antigens on erythrocytes, whereas the expression of the FUT1 gene in human nervous tissues remains undetermined. The H antigens on DRG small neurons in humans seem to be synthesized by FUT1, because FUT1 preferentially uses type 2 precursor glycochain as an acceptor to yield type 2 H, which the U. europaeus agglutinin 1 lectin recognizes, rather than FUT2 (27–29). The exon-intron organization and splicing patterns of FUT1, which are different from those of the RFT-I gene, have been determined in human bone marrow cells, HEL human erythroleukemic cells, and MCAS human ovarian cancer cells (26). It would be interesting to analyze the transcriptional pattern of FUT1 in human DRG neurons and to compare it with that of the RFT-I gene.

In the present study, we detected the promoter activity in the 5′-flanking region (nucleotides −707 to −4) of the RFT-I gene when reporter plasmids were transfected into PC12 and Neuro2a cells. PC12 (rat pheochromocytoma) and Neuro2a (mouse neuroblastoma) cells share a common developmental origin, the neural crest, with DRG neurons. Although the species of PC12 and Neuro2a cells and rabbit DRG neurons are all different, common mechanisms and highly conserved factors might function to control the expression of H-type α1,2-FT or other neural crest-originating neuron-specific genes. Indeed,
the pBH4-SP0.7 construct containing the region of nucleotides −707 to −4 showed high promoter activity in PC12 and Neuro2a cells. In this region, at least two promoter domains are thought to exist; the pBH4-SSm0.2 (nucleotides −707 to −539) construct showed the promoter activity when expressed in PC12 cells, and the pBH4-NP0.3 (nucleotides −294 to −4) construct showed the promoter activity when expressed in Neuro2a cells.

The region of nucleotides −707 to −539 included a GC-rich domain of 80% GC content, in which several Sp1 binding sites were found in a database search for transcription factors. One of the Sp1 binding sites showing the highest homology with the consensus sequence overlapped a GSG-like (8/9 consensus) NGF-responsive element (30, 31). Promoter analysis involving pBH4-SSm0.2 with and without mutations demonstrated the reduction of the promoter activity and the abolition of NGF responsiveness upon the insertion of mutations, suggesting that these elements are functional. An electrophoretic mobility shift assay involving recombinant Sp1 and the labeled DNA fragment (nucleotides −707 to −626) with and without muta-

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**FIG. 4.** RFT-I gene promoter activity analysis. Shown is a schematic representation of DNA constructs containing various lengths of the 5′-flanking region of the RFT-I gene. Each construct and the pSR-β-gal plasmid were transfected into PC12, Neuro2a, or CHO cells. Luciferase activity was normalized as to β-galactosidase activity, and the relative promoter activity was calculated relative to SV40 early promoter activity taken as 100%. Each experiment was performed in duplicate, and the results are the averages of four experiments.

|                | PC12 | Neuro2a | CHO |
|----------------|------|---------|-----|
| pBH4-SP0.7     | 322  | 258     | 50  |
| pBH4-SP0.7     | 300  | 140     | 48  |
| pBH4-SSm0.2    | 163  | 35      | 47  |
| pBH4-SacB8m    | 110  | 24      | 47  |
| pBH4-NP3.0     | 57   | 151     | 40  |

**FIG. 5.** Effects of NGF and mutations on the promoter activity. A, PC12 cells were transfected with the pBH4-SSm0.2, pBH4-SacB8, and pBH4-SacB8m constructs and then cultured with and without 100 ng/ml of NGF for 48 h. The wild type (pBH4-SSm0.2 and pBH4-SacB8) and mutational (pBH4-SacB8m) sequences of the Sp1 binding site and the overlapping GSG-like (8/9 consensus) elements are shown. B, Neuro2a cells were transfected with the pBH4-A6Pst and pBH4-A6mPst constructs, and then cultured for 48 h. The wild type (pBH4-A6Pst) and mutational (pBH4-A6mPst) sequences of the inverted N-Myc binding site are shown. Luciferase activity was normalized relative to β-galactosidase activity, and the relative promoter activities were calculated with SV40 early promoter activity taken as 100%. Each experiment was performed in duplicate, and the data are the means ± S.E. for three experiments.
tions confirmed the actual binding of Sp1 to the putative Sp1 binding site of nucleotides −650 to −638. The partial disappearance of signals upon the addition of an excess amount of unlabeled DNA fragments with mutations could be due to the weak binding of Sp1 to another Sp1 binding site (nucleotides −658 to −646) exhibiting lower homology with the consensus sequence.

The GSG element is a consensus binding motif recognized by members of the early response gene family, such as NGFI-A (32)/Egr-1 (33), Krox-20 (34), Wilms’ tumor gene product (30), and NGFI-C (31). Among them, NGFI-A/Egr-1 and NGFI-C are rapidly and temporally induced in PC12 cells by NGF stimu-

![Fig. 6. Electrophoretic mobility shift assay. A, the labeled probes of the DNA fragments from −707 to −626 without (SacB8, lanes 1–4) or with (SacB8m, lanes 5–8) mutations in the Sp1 binding site were incubated with 0.4 footprinting units of recombinant human Sp1 either alone (lanes 2 and 6) or with the nonlabeled wild-type (lanes 3 and 7) and mutant (lanes 4 and 8) competitors in a 20-fold molar excess. The arrow indicates the binding of recombinant Sp1 to the labeled SacB8 probe, which completely disappeared in the presence of the nonlabeled SacB8 DNA fragment and partially disappeared in the presence of the nonlabeled SacB8m DNA fragment. B, the labeled probes of the DNA fragments from −655 to −626 without (A8B8, lanes 1–7) or with (A8mB8m, lanes 8–11) mutations in the Sp1 and GSG overlapping domain were incubated with 0.5 µg of nuclear extract from Neuro2a (lanes 2 and 9) and PC12 cells with (lanes 4–7 and 11) and without (lanes 3 and 10) NGF treatment. The nonlabeled wild-type (lane 5) and mutant (lane 6) competitors in a 20-fold molar excess or anti-Egr-1 polyclonal antibodies (lane 7) were also added.](image)

![Fig. 7. RFT-I gene promoter activity in DRG neurons. Primary cultures of DRG, DR, and cerebellar tissue were transfected with reporter plasmids, pBH4-SSP1.3, pBH4-SP0.7, pBH4-SSm0.2, and pBH4-NP0.3 and then cultured for 48 h. The viability of cells and actual transfection of the reporter plasmids were verified by transfection of the pCMV-EGFP construct to another primary culture preparation and observation by fluorescence microscopy. Luciferase activity was normalized relative to β-galactosidase activity, and the relative promoter activity was calculated with SV40 early promoter activity taken as 100%. Each experiment was performed in duplicate, and the results are the averages of three experiments.](image)
moter sequences sufficient for the DRG neuron-specific gene expression.

The pBH4-SSm0.2 and pBH4-NP0.3 constructs showed relatively low promoter activity as compared with the pBH4-SP0.7 construct, when transfected into a primary culture of DRG neurons. DRG neurons are known to express trk (neurotrophin receptor family genes) and to be dependent on neurotrophin-mediated signaling (36, 37). Most of the small neurons of DRG bearing H antigens on their surface express TrkA, which binds to NGF, as in the case of PC12 cells. Despite the fact that the DRG neurons were cultured with 100 ng/ml NGF, the pBH4-SSm0.2 construct showed a low level of promoter activity in DRG neurons comparable with that in dorsal roots and cerebellar granule cells. Some other factors binding to the Sp1 binding site and the overlapping GSG-like element could act in a stage-specific manner during the late embryonic period when DRG neurons are critically dependent on NGF for their survival.

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