Transcriptional Regulation of Type B Human Natriuretic Peptide Receptor Gene Promoter

Dependence on Sp1

Dolkun Rahmutula, Junfeng Cui, Songcang Chen, David G. Gardner

Abstract—The type B natriuretic peptide receptor (NPR-B) is the cognate receptor for the C-type natriuretic peptide and, as such, is responsible for signaling growth-suppressant activity in vascular smooth muscle cells. Here we report the isolation and characterization of the human (h) NPR-B gene promoter. Using 5′ rapid amplification of cDNA ends analysis, we have identified the 5′ terminus of the hNPR-B gene transcript ~732 base pairs upstream from the presumed translation start site of the protein. We generated a series of 5′ deletion mutants linked to a luciferase reporter and introduced these constructs into rat aortic smooth muscle cells or neonatal rat cardiac fibroblasts. Maximal expression was seen with a construct harboring 441 base pairs of 5′ flanking sequence. Site-directed mutagenesis of the proximal promoter revealed a series of GC-rich sequences, 5 of which contributed modestly (∼25%) to basal hNPR-B promoter activity. Mutation of a sixth GC-rich sequence led to a >90% reduction in promoter activity. This sequence was shown to associate with Sp1 and Sp3 in vitro. The same mutation that resulted in loss of functional activity also resulted in loss of binding activity in vitro. Overexpression of Sp1 or Sp3 in Drosophila Schneider cells resulted in an increase in hNPR-B promoter activity that was completely nullified with the Sp1 binding site mutation described above. These studies provide the first description and characterization of the NPR-B gene promoter and suggest that this promoter’s activity is dominated by a single cluster of Sp1-binding elements in the proximal 5′ flanking sequence of the gene.

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Key Words: natriuretic peptide receptor ■ transcription ■ muscle, smooth, vascular

The natriuretic peptides constitute a family of vasoactive peptide hormones with profound vasorelaxant, hypotensive, natriuretic, and antiproliferative activity in target tissues.1 Atrial natriuretic peptide and brain natriuretic peptide are produced primarily in the atria and ventricles of the heart, respectively, whereas C-type natriuretic peptide (CNP) is produced in the central nervous system, reproductive tract, and endothelium of the vasculature, but only to a limited degree in the heart. Atrial natriuretic peptide and brain natriuretic peptide share a common receptor in target tissues, the type A natriuretic peptide receptor (NPR-A), and it is believed that most of their natriuretic activity is mediated through this receptor.2 CNP operates largely through the type B receptor (NPR-B). Each of these receptors links a large extracellular ligand-binding domain through a single transmembrane-spanning segment to a catalytic effector domain harboring particulate guanylyl cyclase activity.3 The ligand-dependent generation of cyclic GMP is thought to represent the primary signaling mechanism for these hormones. A third type of receptor (NPR-C), appears to function predominantly in a clearance mode.

Although the role of NPR-A in signaling the cardiovascular and renal effects of the natriuretic peptides has been examined in detail, the physiological function of NPR-B is less well understood. Roles for CNP and NPR-B in the regulation of renal function, skeletal growth, central nervous system homeostasis, and reproductive function have been suggested.4–6 Interestingly, the role of CNP/NPR-B in the vasculature appears to involve growth control in the vascular wall. Treatment of rats with CNP following balloon injury to the carotid artery results in a significant reduction in smooth muscle cell proliferation during the recovery phase.7–9

Whereas several reports have helped to define the regulatory factors governing NPR-A10–14 and NPR-C15 gene promoter activity, relatively little is known about the regulation of the NPR-B gene, particularly at a transcriptional level. This article describes the cloning and sequence analysis of the human (h) NPR-B gene, identification of the 5′ transcription start site, and promoter regulation in transiently transfected rat aortic smooth muscle (RASM) and rat cardiac fibroblast cells. Our findings demonstrate that the NPR-B promoter is avidly expressed in these cells, largely under the control of a
small cluster of Sp1 binding sites in the proximal 5′ flanking sequence (5′ FS) of the gene.

**Methods and Materials**

Sources of materials and additional methodological details are available in an online supplement at http://www.hypertensionaha.org.

**Cell Culture**

Neonatal RASM cells were obtained from H. Ives at University of California at San Francisco and cultured as described previously. Fibroblasts were isolated at the preplating step from the hearts of 1-day-old neonatal Sprague-Dawley rats and expanded in culture as described. Drosophila Schneider cells (SL-2) were cultured at 25°C in Schneider medium (GIBCO) using a conventional protocol.

**Isolation and DNA Sequencing of hNPR-B**

**Genomic Clone and Determination of Transcription Start Site**

We used a pair of oligomers (sense: 5′-CAGAACACACAA-TGAGCTATGGC3′; antisense: 5′CGAACAGGGTAGATAATGG-3′) containing sequence from the hNPR-B coding sequence to screen a human genomic DNA P1 library (Genome Systems Inc; St. Louis, Mo). A single P1 clone was identified and restriction mapped using conventional endonuclease digestion and Southern blot hybridization. A 9 kb restriction fragment harboring the 5′ FS of the hNPR-B gene was subcloned into the polylinker of the pBluescript KS vector (Stratagene) and sequenced using chain termination methodology in the Biomolecular Resource Center at the University of California at San Francisco. Total RNA was prepared from human ocular trabecular meshwork cells and the start site was determined using 5′ rapid amplification of cDNA ends (RACE; Gene Racer Kit, Invitrogen, Carlsbad, Calif).

**Construction of hNPR-B Luciferase Expression Vectors**

To obtain deletion constructs, polymerase chain reaction was performed using LA-Taq DNA polymerase (Takara La Taq), a subcloned 9 kb human genomic clone as a template, and a series of sense primers (NPR-B A1 to 10) together with antisense primer (NPR-B ΔR1 or NPR-B ΔR2) positioned between +148 to +166 and +223 to +245, respectively, relative to the transcription start site. This chimeric sequence was inserted between the KpnI/BglII sites of pGL3 (Promega). The structure of all constructs was confirmed by DNA sequencing.

**Site-Directed Mutagenesis**

Site-directed mutagenesis was carried out with the QuikChange kit (Stratagene) using conditions recommended by the manufacturer.

**Transfection, Luciferase, and β-Galactosidase Assays**

RASM cells were transiently transfected with the relevant plasmids by electroporation (Gene-Pulser, Bio-Rad Laboratories Inc) at 250 mV and 960 microfarads, SL-2 at 180 mV and 960 microfarads, and rat cardiac fibroblasts at 250 mV and 960 microfarads. Cell lysates were processed (30 μg protein per sample) and assayed for luciferase activity using a commercial kit (Promega Corp). Measurements of β-galactosidase activity were made using the Galacto-Light Plus kit (Tropix Inc). Luciferase measurements were normalized for β-galactosidase activity individual samples.

**Preparation of Nuclear Extracts**

Nuclear extracts were prepared from RASM cells as described previously and stored at −80°C until use.

**Electrophoretic Mobility-Shift Assay**

The oligonucleotides used for electrophoretic mobility-shift assays (EMSAs) are presented in Table II available online. EMSAs were carried out as described previously.

**Statistical Analysis**

Data were evaluated by 1-way ANOVA and the Newman-Keuls test for significance.

**Results**

We used a pair of oligonucleotides derived from the 5′ untranslated region of the hNPR-B cDNA to screen a human genomic DNA P1 library (Genomic Systems Inc) and identified a single ~160 kb P1 clone containing NPR-B genomic sequence. The clone was amplified, and DNA was isolated and subjected to restriction mapping and Southern blot analysis. A 9 kb SpeI restriction fragment harboring the 5′ FS was identified, subcloned, and subjected to DNA sequence analysis. Approximately 6 kb of 5′ FS was contained in this clone. Sequence of the 893 bp most proximal to the 5′ border of the published cDNA sequence is shown in Figure I available online; remainder of 5′ FS is available at GenBank accession number AY528561. Comparison with the human genome sequence (www.ncbi.nlm.nih.gov/NT_008413), which was completed while this study was in progress, identified relatively few areas of disagreement (Table I, available online), and none of these disagreements appeared to fall within or near potential regulatory elements (see below).

We used 5′ RACE to locate the 5′ terminus of the hNPR-B mRNA transcript and inferentially identify the transcription start site. 5′ RACE analysis identified a 5′ terminus (as indicated by arrow in Figure I) approximately 452 bp upstream from the 5′ terminus of the previously published cDNA sequence and 735 bp upstream from the presumed translation start site (boxed ATG). This site is surrounded by GC-rich sequence with a number of putative transcription regulatory elements in the region immediately upstream (Figure I). We generated a −2129 hNPR-B luciferase construct (NPR-B sequence extending from −2129 to +165 relative to the transcription start site identified above) and introduced it into cultured RASM cells by transient transfection. Expression of the reporter was robust in these cells. 5′ Deletion analysis demonstrated that the majority of promoter activity was retained as the deletions were pushed to −441 relative to the transcription start site (Figure II available online). Activity of this deletion, in fact, was 4-fold higher than that seen with −2129 hNPR-B luciferase. There was a progressive reduction in reporter activity as the deletions were pushed from −441 to −84, and activity was reduced to background levels when the deletion was positioned at −34 relative to the transcription start site.

With this information in hand, we focused our efforts on potential transcriptional regulatory elements positioned between −441 and the start site. Conventional sequence analysis identified a number of potential regulatory elements in this region dominated by GC-rich sequences like those known to bind to the transcription factors Sp1, Sp3, and AP2 (Figure I). We used the −441 hNPR-B luciferase construct to introduce site-directed mutations into a number of these sites...
before introducing the modified reporters into RASM cells. As shown in Figure 1A, 10 independent mutations were introduced into the wild-type (WT) sequence. Of the group, the only mutations that resulted in a significant reduction in promoter activity were M3, M4, M5, M6, and M7. With the exception of M7, each of these mutations resulted in \( \sim 25\% \) reduction in NPR-B promoter activity. M7, which represents a mutation of 2 overlapping Sp1 binding sites extending between \( 115 \) and \( 103 \), resulted in a 94% reduction in activity relative to the WT promoter (shown in Figure 1B).

The \( -441 \) hNPR-B luciferase reporter was also highly expressed in neonatal rat cardiac fibroblasts (Figure III, available online), demonstrating that expression of this reporter is not idiosyncratic to cultured RASM cells. Again, the M7 mutation resulted in a pronounced reduction in basal promoter activity, implying that the targeted Sp1 binding element is of critical importance in at least 2 different cell types expressing this gene.

As shown in Figure 2, radiolabeled WT sequence positioned between \( -118 \) and \( -83 \) bound to several proteins in the extract resulting in a shift in migration of the oligonucleotide to a higher position in the gel. Immunoperturbation studies, carried out with antibody directed against Sp1, eliminated the most slowly migrating of these bands, supershifting it to a higher position in the gel (not well seen in this autoradiograph), and thereby identifying it as Sp1-bound oligonucleotide. Addition of antibody directed against Sp3 eliminated the 3 lower bands in the pattern, identifying them as Sp3-bound oligonucleotide, whereas addition of antibodies against both Sp1 and Sp3 eliminated the binding pattern completely. These interactions were specific. Antibodies directed against peroxisome proliferator-activated receptor \( \gamma \) or early growth response-1 factor, another transcription factor known to associate with GC-rich sequence,\(^1\) failed to alter the binding pattern.

In an effort to better localize the site of Sp1 binding, we used a series of 5′ double-stranded oligonucleotides with mutations designed to interfere with Sp1 binding positioned at different locations in the WT sequence (Table II). As shown in Figure 3A, each of the mutant oligonucleotides, with the exception of M7, competed effectively for Sp1 and Sp3 binding to the WT sequence extending between \( -115 \) and \( -103 \), implying that M7 harbors the mutation that
selectively interferes with binding of these transcription factors. This was confirmed in direct binding studies shown in Figure 3B. Radiolabeled M3, M4, M5, M6, and M8 associated with Sp1 and Sp3 in the RASM cell extracts; however, M7 was devoid of binding activity.

Finally, we attempted to assess the ability of Sp1 overexpression to drive functional activity of the NPR-B promoter in transient transfection analyses. Because concentrations of Sp1 and Sp3 are typically not limiting in most mammalian cells in culture, we chose to carry out these studies in Drosophila Schneider cells, which are known to be relatively deficient in mammalian transcription factor equivalents. These studies were performed by cotransfecting Sp1, Sp2, or Sp3 expression vectors driven by the Drosophila-specific actin (Pac) gene promoter together with the −441 hNPR-B luciferase reporter. As shown in Figure 4A, Sp1 and Sp3, but not Sp2, each led to a dose-dependent increase in NPR-B promoter activity. Noteworthy, the combination of Sp1 and Sp3 was not synergistic in driving this increase, suggesting that they operate over a shared pathway. This pathway appears to require the Sp1 binding element described above because the M7 mutation eliminated the ability to respond to either Sp1 or Sp3 (Figure 4B). These functional data support the in vitro mobility shift analyses presented above, which demonstrate that each of these 2 transcription factors is capable of independently binding to this element.

Discussion

The article describes, for the first time to our knowledge, the cloning of the NPR-B gene and functional analysis of its promoter in RASM cells. The promoter is highly active in these cells, relying heavily on a cluster of Sp1 binding sites for maintenance of this activity.

cDNA sequence for the hNPR-B was reported initially in 1989.17 NPR-B coding sequence was assembled using a combination of conventional cDNA and genomic DNA sequencing. At the time only limited 5′ untranslated sequence was reported (∼283 nucleotides upstream from the ATG thought to initiate translation). Genomic sequencing of the putative 5′ FS of the hNPR-B gene was subsequently carried out by Rehemedula et al using a PCR-based technique; however, recombinant clones were not obtained for subsequent analysis.20 This sequence extended approximately 750 bp upstream from the ATG codon. 5′ RACE analysis carried out in the same study placed the 5′ border of the transcribed mRNA at a position that was only 14 bp upstream from the ATG codon. Our data using cloned NPR-B genomic sequence indicate that the bulk of the sequence reported in the work of Rehemedula et al20 is correct; however, 5′ RACE analysis conducted in this study places the transcription start site more than 721 bp farther upstream. This discrepancy could reflect an error in the previous start site assignment or differences in the source of RNA for the 2 studies (human trabecular meshwork cells in our study and human pituitary gland cells in that of Rehemedula et al). This would imply that different
transcription start sites are used in a tissue-specific fashion. If this is the case, it could lead to differences in the array of regulatory elements governing tissue-specific expression of this gene. Noteworthy, whereas functional promoter activity is clearly present in sequence positioned upstream from the start site that we have identified, similar constructs generated using the candidate start site of Rehemedula et al20 as a reference point were uniformly inactive in RASM cells (D.R., J.C., D.G.G., unpublished observations, 2001).

Expression of the NPR-B promoter is heavily regulated by the transcription factor Sp1. Sp1 is a transcription factor that has been shown to govern expression of a number of genes expressed in the heart21 and vessel wall.22,23 Of note, the NPR-A gene is also under Sp1 control.12 A series of 3 Sp1 binding sites and a single NF-Y binding site account for almost 100% of basal NPR-A gene promoter activity.13 Given the reliance of both receptor subtypes on Sp1, it will be of interest to determine whether and how other transcription factors interact with Sp1 to establish tissue specific differences and regulatory differences in the expression of these 2 receptor genes. Sp1 has been invoked as a protein that increases in response to promitogenic stimuli in vascular smooth muscle cells (VSMCs) and may play a role in controlling the growth response that is activated by these stimuli.22,23 Our data would suggest that Sp1 may accomplish this, at least in part, through activation of counter-regulatory pathways (ie, NPR-A and NPR-B) with antimitogenic properties that would serve to mitigate the growth response. Ultimately, this would provide the target cell with considerable flexibility in fine tuning the response to hormonal and mechanical stimuli that promote growth.5 A recent study suggests that CNP may, in fact, represent effective therapy for achondroplastic dwarfism, a defect in long bone growth.27 NPR-B is also expressed in VSMCs, and in 1 study expression increased in replicating VSMCs in culture relative to nondividing cells in the vessel wall.28 The NPR-B ligand CNP is expressed in vascular endothelial cells where it increases following treatment with a variety of cytokines and trophic factors, including transforming growth factor β,29 interleukin 1α, interleukin 1β, tumor necrosis factor α, and lipopolysaccharide,30 and is suppressed by insulin and vascular endothelial growth factor.31,32 The proximity of ligand- and receptor-expressing cells has led to the hypothesis that the CNP–NPR-B system acts as a local paracrine mechanism to control VSMC proliferation, particularly following endothelial damage. A number of studies have reported that CNP inhibits VSMC proliferation in vitro33,34 and in the wall of the carotid artery following balloon injury in vivo,7–9 a finding which lends support to the model described.

Perspectives

In summary, we have isolated and characterized the promoter region of the hNPR-B gene. We have mapped the transcription start site to a position more than 721 bp upstream from that previously identified.20 The hNPR-B promoter is highly active in cultured RASM cells and neonatal rat cardiac fibroblasts, and it relies on a series of proximally positioned Sp1 binding sites to attain maximal activity. One pair of these sites in particular (ie, that targeted by the M7 mutation) plays a dominant role in the control of promoter activity. This information should prove useful in dissecting out the mechanisms governing transcriptional regulation of this gene in a variety of systems.

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References


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