Characterization of a cGMP-Response Element in the Guanylyl Cyclase/Natriuretic Peptide Receptor A Gene Promoter

David Hum, Sandrine Besnard, Rocio Sanchez, Dominic Devost, Francis Gossard, Pavel Hamet, Johanne Tremblay

Abstract—Previous studies have shown that atrial natriuretic peptide (ANP) can inhibit transcription of its receptor, guanylyl cyclase A, by a mechanism dependent on cGMP and have suggested the presence of a putative cGMP-response element (cGMP-RE) in the *Npr1* gene promoter. To localize and characterize the putative *cis*-acting element, we have subcloned a 1520-bp fragment of the rat *Npr1* promoter in an expression vector containing the luciferase reporter gene. Several fragments, generated by exonuclease III-directed deletions, were transiently transfected into cells to measure their promoter activity. Deletion from −1520 to −1396 of a 1520-bp-long *Npr1* promoter led to a 5-fold increase in luciferase activity. Subsequent deletion to the position −1307 resulted in a decrease of luciferase activity by 90%. Preincubation of cells with 100 nM of ANP or 100 μM 8-bromo-cGMP inhibited luciferase activity of the 1520-bp and 1396-bp-long fragments, but not the activity of the 1307-bp fragment, suggesting that the cGMP-RE is localized between positions −1396 and −1307. The cGMP regulatory region was narrowed by gel shift assays and footprinting to position −1372 to −1354 from the transcription start site of *Npr1* and indicated its interaction with transcriptional factor(s). Cross-competition experiments with mutated oligonucleotides led to the definition of a consensus sequence (−1372 **A**a**A**tRKaN**TTC**a**A**c**A**K**T**Y −1354) for the novel cGMP-RE, which is conserved in the human (75% identity) and mouse (95% identity) *Npr1* promoters. **(*Hypertension*. 2004;43:1270-1278.)**

Key Words: cyclic GMP ■ natriuretic peptides ■ receptors, atrial natriuretic factor

Atrial natriuretic peptide (ANP) is a cardiac hormone with potent effects in the kidney, vasculature, and nervous system. It has vasorelaxant activity in vascular smooth muscle and promotes urinary sodium and water excretion by the kidney (for review, see Reference[1]). These actions of ANP are brought about via the activation of membrane-bound protein, natriuretic peptide receptor type A (NPR-A), or guanylyl cyclase A.[2–4] NPR-A is a 130-KDa transmembrane protein containing an ANP-binding motif at its extracellular NH_2 terminus and GC activity at its COOH-terminal intracellular domain.[5]

Activation of NPR-A leads to the conversion of GTP to the intracellular second messenger cGMP, which is involved in most of the biological responses associated with natriuretic peptides.[2–5] Increased cGMP yields receptor desensitization, resulting in a decrease of ANP-stimulated cGMP synthesis.[6] Receptor preoccupancy appears to be a mechanism of apparent receptor desensitization[7] but transfection experiments with NPR-A cDNA have revealed that early desensitization by ANP pretreatment can be due to dephosphorylation of NPR-A protein.[8] Besides, a previous study demonstrated that NPR-A activity, as well as its mRNA levels, is diminished in a time- and concentration-dependent manner by ANP or by a cell-permeable cGMP analog, 8-bromo-cGMP.[9] Those results suggest transcriptional regulation via a putative cGMP-response element (cGMP-RE) in the promoter of the *Npr1* gene (gene encoding NPR-A). The sequence of this putative cGMP-RE remains to be identified.

In the present work, we localized and characterized the putative cGMP-RE in the *Npr1* promoter. We found that fragments within the region −1396 to −1290 of the transcription site of *Npr1* are sensitive to ANP and to cGMP analogs. A palindromic sequence was localized by gel shift and DNase I protection assays to the −1387 to −1352 region of the *Npr1* promoter. Cross-competition experiments with mutated oligonucleotides and analysis of the conserved regions between human, rat, and mouse led to the definition of a consensus sequence of this newly-described cGMP-RE.

Materials and Methods

Construction of the *Npr1* Promoter, Directed Enzymatic Deletions, and Reporter Gene Assay

Fragments of the *Npr1* gene 5′ upstream region were generated by polymerase chain reaction (PCR) and then subcloned in the reporter

Received September 30, 2003; first decision November 29, 2003; revision accepted March 22, 2004.

From the Centre de recherche, Centre hospitalier de l'Université de Montréal (CHUM)—Hôtel-Dieu, Montréal, Québec, Canada.

Correspondence to Dr Johanne Tremblay, Centre de recherche, CHUM—Hôtel-Dieu, 3850 St. Urbain St., Montréal, Québec H2W 1T7, Canada. E-mail johanne.tremblay@umontreal.ca

Hypertension **is available at http://www.hypertensionaha.org** **DOI: 10.1161/01.HYP.0000126920.93207.53**

Sequence of Oligonucleotides and EC_{50} in Cross-Competition Experiments

Name			Sequence			EC_{50} (ng)	*P* Value vs P6 (ANOVA)
P6	−1375	5′	AGAAAATAGATTTCAACAGTTTGC	3′	−1352	1.10	-
M9	−1375	5′	AGAAAAT**CTAGTC**CAACAGTTTGC	3′	−1352	>5.81	0.0682
P10	−1375	5′	AGAAAATAGATTTC	3′	−1362	-	-
P11	−1362	5′	CAACAGTTTGC	3′	−1352	-	-
M12	−1375	5′	AGAAAATAGATTTCA**CC**G**GC**TTGC	3′	−1352	>5.81	0.0160
Human P13	−1546	5′	AG**G**AAAT**GTAC**TTCAACA**TTC**TGC	3′	−1523	4.00	0.1322
M14	−1375	5′	AGAAAATAGAT**CTTACCC**GTTTGC	3′	−1352	>5.81	0.0008
M15	−1375	5′	AGA**CAGTCT**ATTTCAACAGTTTGC	3′	−1352	>5.81	0.0087
P16	−1365	5′	TTTCAACAG	3′	−1357	>5.81	0.0006
P20	−1375	5′	AGAAAATAGATTTCAACAG	3′	−1357	0.20	0.0100
P21	−1373	5′	AAAATAGATTTCAACAG	3′	−1357	0.18	0.0016
P22	−1371	5′	AATAGATTTCAACAG	3′	−1357	0.25	0.0239
P23	−1369	5′	TAGATTTCAACAG	3′	−1357	0.50	0.2193
P24	−1367	5′	GATTTCAACAG	3′	−1357	0.65	0.4623
P25	−1365	5′	TTTCAACAGTTTGC	3′	−1352	0.61	0.7467
P26	−1365	5′	TTTCAACAGTTT	3′	−1354	1.00	0.7355
P27	−1373	5′	AAAATAGATTTCAACAGTTTG	3′	−1353	0.50	0.1487
P28	−1373	5′	AAAATAGATTTCAACAGTT	3′	−1355	0.65	0.2714
P29	−1373	5′	AAAATAGATTTCAAC	3′	−1359	4.80	0.0155
P30	−1373	5′	AAAATAGATTTCA	3′	−1361	5.81	0.0059

P values calculated by ANOVA compared the efficiency of nonlabeled probes to displace the P6-labeled probe. Punctual mutations of the P6 segment are indicated in bold. Underlined letters correspond to a palindromic sequence (see first page of this article).

vector pGL3b (Promega, Madison, Wis), or pGL3p. Construct numberings were based on the transcription initiation site of the *Npr1* gene. All fragments were sequenced with the Thermo sequenase kit (Amersham). A 1520-bp-long fragment upstream of the transcription start site of *Npr1* gene was digested with exonuclease III.[10] β-Galactosidase activity was measured in cell extracts with o-nitrophenyl β-D-galactopyranoside as substrate,[10] and luciferase activity of the cell extracts was quantified with the Promega Luciferase Assay System. Sense and antisense oligonucleotides corresponding to the P21 fragment were synthesized, hybridized, and subcloned into the pGL3p vector. A 1396-bp-long partial promoter was modified by deleting the consensus 18 bp (−1373 to −1355) cGMP-RE (KN). The resulting construct preserved 8 bp upstream of the deletion, but the cloning constraint carried away 16 bp (−1396 to −1380) upstream of those 8 bp.

Cell Culture and Expression Studies

Mouse NIH 3T3 cells (ATCC, Rockville, Md) were grown in Dulbecco's modified Eagle's high glucose medium (DMEM) supplemented with 10% calf serum (Life Technologies, Burlington, Ontario, Canada), and 2% penicillin/streptavidin, at 37°C in a 5% CO_2 controlled atmosphere. A10 cells (ATCC) were seeded in DMEM with low glucose, 10% fetal calf serum, and 2% of penicillin/streptavidin. cGMP levels were measured in the culture medium as described.[11]

DNA was transiently transfected into 3T3 and A10 cells according to the calcium-phosphate precipitation procedure or using the Tfx-50 reagent transfection kit of Promega. The cells were plated at a density of 2×10^5 cells/well and transfected with 5 μg/well of plasmid. Transfection efficiency was monitored by cotransfecting 1 μg of a control plasmid, pCMV-β Gal (Clontech, Palo Alto, Calif).

Preparation of Nuclear Extracts, Electrophoretic Mobility Shift Assays, and Footprinting

A10 and NIH 3T3 cells were grown until near confluency, synchronized by 24-hour serum starvation, and then stimulated with ANP or 8-bromo-cGMP for the times indicated. Nuclear extracts were prepared as described,[12] and aliquots were stored at −80°C until used.

Oligonucleotides corresponding to specific regions of the *Npr1* promoter were used as probes (see Table). To each oligonucleotide was added a *BamH*I linker at its 5′ end and a *Bgl*II site at its 3′ end. Punctual mutations of the P6 segment are indicated in bold. Purified ^{32}P end-labeled, double-stranded oligonucleotides were added to 5 μg of nuclear extracts in a total volume of 15 μL. The samples were resolved by 4.5% nondenaturing polyacrylamide gel electrophoresis. Dried gels were exposed to PhosphoImager (Molecular Dynamics, Sunnyvale, Calif).

A 110-bp probe overlapping the gel shift-analyzed region was obtained by digesting 1396 pGL3 plasmid with *Nde*I and *Kpn*I. Only the *Nde*I site was ^{32}P end-labeled. DNase I (50 ng) was added in the reaction for 100 seconds, and the reaction was stopped by 700 μL of dry ice-chilled stop solution (645 μL ethanol 100%, 5 μg tRNA, 50 μL of saturated ammonium acetate) for 15 minutes. The samples were resolved on 6% denaturing polyacrylamide gels that were dried and exposed to film for autoradiography.

Results

Identification of an ANP/cGMP *cis*-Responsive Element

We first confirmed the original findings of Cao et al[9] that NPR-A activity can be downregulated by long-term cell preincubation with its ligand ANP. Thus, preincubation of A10 smooth muscle cells or NIH 3T3 fibroblasts for 18 hours with 10 or 100 nM ANP induced a 45% reduction of ANP-stimulated guanylyl cyclase activity ($P<0.05$).[1] To study *Npr1* gene promoter activity and its regulation by its ligand ANP, we submitted a −1520 to 0 fragment of the *Npr1* promoter to serial, directed deletions with exonuclease III.

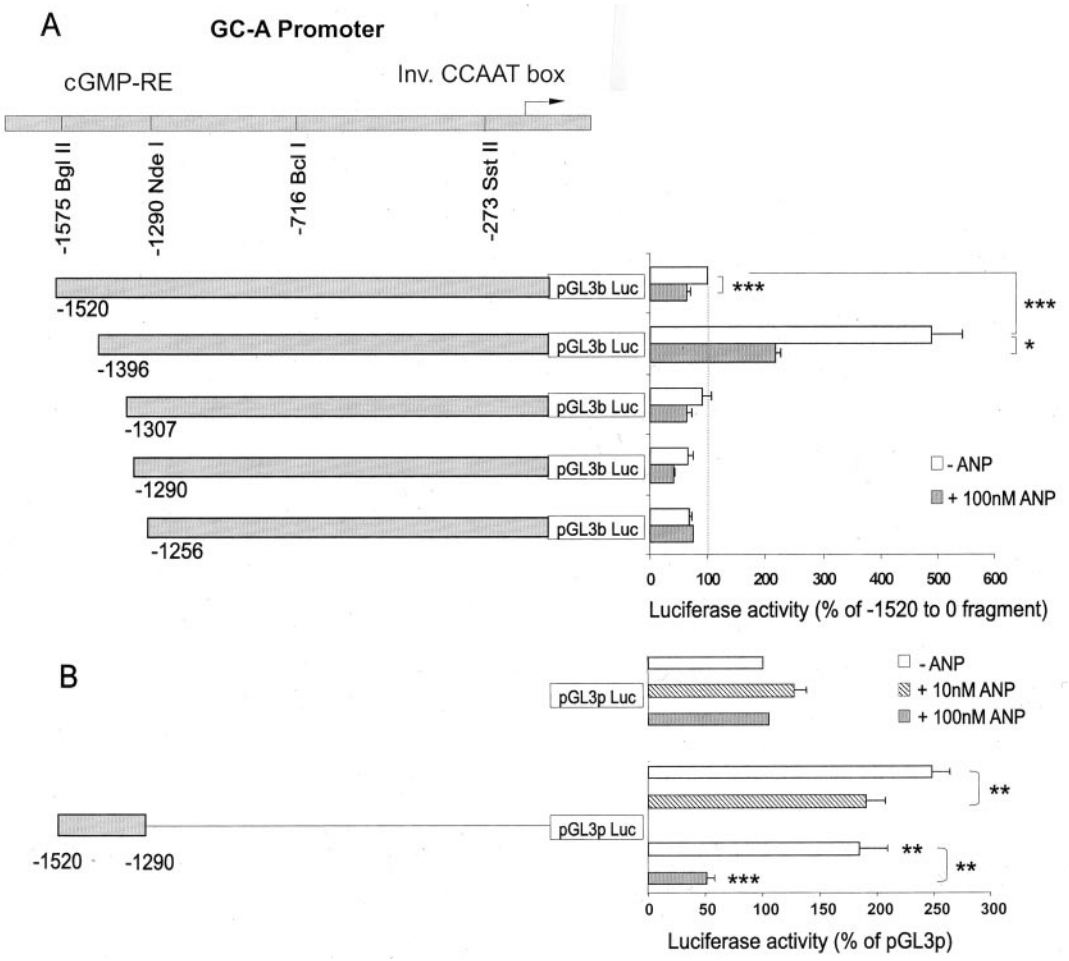

Figure 1. Luciferase activity after transient expression of GC-A promoter fragments in fibroblast cells and effect of ANP preincubation. A, NIH 3T3 cells were cotransfected with 5 μg of the pGC-A-Luc constructs and 1 μg of pCMV-β vector, then exposed or not to 100 nM ANP for 8 hours. All values are expressed as a percentage of luciferase activity from the longest fragment. Values represent means±SEM from 4 different experiments performed in duplicate. *$P<0.04$, **$P<0.006$, ***$P<0.0001$ vs the control group (Student *t* test). B, Luciferase activity of the fragment −1520 to −1290 after exposure to 10 or 100 nM ANP for 8 hours. All values are expressed as a percentage of luciferase activity from pGL3pLuc. Values represent means±SEM from 2 to 4 different experiments performed in triplicate. **$P<0.006$, ***$P<0.0001$ vs control plasmid (Student *t* test).

The resulting fragments were then subcloned in the luciferase reporter plasmid and transfected into 3T3. Deletion of the region −1520 to −1396 of the *Npr1* promoter led to a nearly 5-fold increase in luciferase activity while subsequent deletions from −1396 to −1307 induced a dramatic decrease in the transcriptional activity of the *Npr1* promoter (Figure 1A, open bars). These findings indicate the presence of a negative regulatory element located between −1520 and −1396 and also suggest the presence of a positive regulatory element between −1396 and −1307. When the cells were preincubated with ANP, both the −1520 to 0 and the −1396 to 0 fragments showed a significant decrease in luciferase activity (Figure 1A, gray bars), indicating the presence of a putative ANP-sensitive element located between −1396 and −1307 of the transcriptional start site of the *Npr1* gene.

A −1520 to −1290 subfragment was then placed in the pGL3p plasmid. This 230-bp–long fragment significantly increased the luciferase activity of the SV40-Luc construct (Figure 1B), confirming the presence of a putative activatory element. The effect was inhibited by preincubation of the cells with 10 nM (25%) and 100 nM (50%) ANP, indicating that the ANP regulatory element is located within this region and is functional (Figure 1B, gray bars). To investigate the mechanism of the ANP effect, the plasmid containing the 1396-bp promoter fragment of *Npr1* was transiently transfected into NIH 3T3 or A10 cells. The transfected cells were then preincubated with 100 nM ANP or with increasing concentrations of 8-bromo-cGMP. In Figure 2, we report a similar decrease in luciferase activity with 100 nM ANP and 100 μmol/L cGMP, suggesting that the transcriptional inhibitory effect of ANP depicted in Figure 1 was mediated by cGMP increases and was not restricted to a single cell type. The maximal inhibition obtained with 100 nM ANP or 100 μmol/L 8-bromo cGMP was the same (about 40%). Increasing the concentration of 8-bromo cGMP to 1 mmol/L did not increase the percent of inhibition.

Localization of Putative cGMP-RE

To narrow down the region responsive to cGMP in the *Npr1* promoter and to investigate putative interactions with nuclear

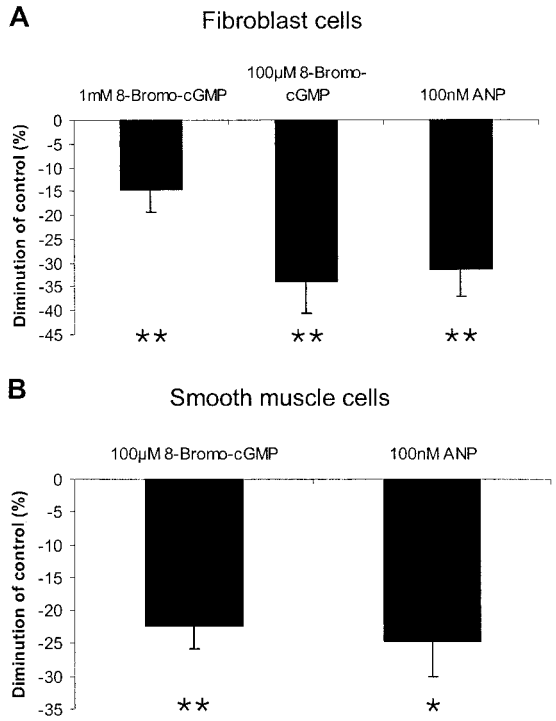

Figure 2. Luciferase activity after transient expression of the −1396 fragment in 2 different cell types: effect of ANP and 8-bromo-cGMP preincubation. A, NIH 3T3 cells were cotransfected with 5 μg of −1396 pGL3 and 1 μg of pCMV-β plasmids and then exposed to 100 nM ANP or 10 or 100 μmol/L 8-bromo-cGMP for 8 hours. B, A10 cells were transfected with 5 μg of −1396 pGL3 and 1 μg of pCMV-β plasmids and then exposed to 100 nM ANP (18 hours) or 100 μmol/L 8-bromo-cGMP (8 hours). Values represent means±SEM of 3 different experiments. *P=0.01, ** P<0.003 vs control group (Student t test).

proteins, we performed electrophoretic mobility shift assays (EMSA) with oligonucleotides spanning the −1396 to −1307 region of the *Npr1* promoter. The region was first divided into 2 fragments of 44 bp each, and corresponding oligonucleotides (Probes P1: −1396 to −1352, and P2: −1352 to −1307) were synthesized and ^{32}P-labeled (Figure 3A). Competition experiments, using excess of unlabeled probes, were performed to determine the specificity of DNA-protein complex formation. Incubation of A10 nuclear proteins with the P1 oligonucleotide resulted in the formation of specific DNA-protein complexes (Figure 3B, lane 1). Specificity of the interaction was shown by its competition with 100-fold excess of the unlabeled P1 probe (Figure 3B, lanes 2 and 4). Incubation of A10 cells with ANP reduced the extent of binding after 6 hours (Figure 3B, lane 3 versus lane 1) of preincubation. This was confirmed by computing data from 3 separate experiments (Figure 3C). The P2 probe did not form any complex with A10 cell nuclear proteins (Figure 3B, lane 6) and did not compete with the P1 probe (Figure 3B, lane 5).

We further divided the P1 probe into 2 fragments of 22 bp each (P3: −1396 to −1374 and P4: −1374 to −1352, Figure 3A). Incubation of nuclear proteins with P3 or P4 oligonucleotide formed 2 DNA-protein complexes (Figure 3D). While absence of competition with up to 250-fold excess of unlabeled P3 probe indicated nonspecific binding (Figure 3D, lane 3), the P4 probe showed specific DNA-protein complex formation, as 30- and 150-fold (Figure 3D, lanes 5 and 6) or 250-fold (Figure 3E, lane 2) excess of P4-specific unlabeled probe competed the interaction. Binding of nuclear proteins to the DNA region corresponding to the P4 sequence was inhibited by preincubation of cells with 8-bromo-cGMP (Figure 3E, lane 4). Thus, the putative cGMP-RE region was narrowed down to a 22-bp-long segment located between positions −1374 to −1352 of the *Npr1* promoter.

Minimal Sequence of cGMP-RE

The sequence of the putative cGMP-RE was confirmed by DNase I protection assay with a 110-bp oligonucleotide corresponding to the gel shift-analyzed region. A protected region was observed and sequenced (Figure 4A). The protected sequence (*AG*AAAATAGATTTC) matched the first 2 bases (AG) of the P3 oligonucleotide and the remaining sequence of the P4 probe (AAAATAGATTTC). It contained a palindromic sequence (underlined).

A 24-bp-long probe (P6) corresponding to the protected sequence plus 10 adjacent nucleotides at its 3′ end (Table) was synthesized and analyzed by EMSA. Again, binding of nuclear proteins to the P6-labeled probe was inhibited by preincubation with ANP (Figure 4B, lanes 1 and 2) or 8-bromo-cGMP (Figure 4B, lanes 3 and 4). When the P6 sequence was further split into halves (P10 and P11 in Table), the binding of nuclear proteins was lost for both segments (Figure 5A, lanes 5 to 6 and 7 to 8). These data indicate that both segments are needed for the binding of nuclear proteins. Finally, we introduced mutations within the palindromic sequence, resulting in the oligonucleotide M9 (Table). This M9 probe formed a specific complex with nuclear proteins (Figure 5A, lanes 3 and 4), suggesting that the mutated nucleotides and the palindromic structure were not essential for binding to nuclear proteins.

Identification of the cGMP-RE Sequence in Human and Mouse *Npr1* Promoters

The definition of a minimal sequence enabled us to search for the existence of putative cGMP-RE in the *Npr1* gene promoter of other species. A site with 75% identity to the rat sequence was found in the human *Npr1* gene at position −1546 to −1523 from the transcription site.[13] In the mouse *Npr1* gene,[14] a region with 95% identity was observed between −1794 to −1771 from the ATG site. To establish the functionality of the identified sequence, an oligonucleotide corresponding to the human sequence (HP13) was synthesized (Table) and analyzed by EMSA. Human P13 was found to bind specifically to nuclear extracts (Figure 5A, lane 9). Furthermore, its binding was partially inhibited by the 100-fold excess unlabeled rat P6 probe (Figure 5A, lane 10).

Cross-Competition Experiments With Mutated Probes

Further mutations were designed near the palindrome of the P6 probe (Table). All mutated sequences were analyzed for homology with known *cis*-acting elements[15] and were used to compete with binding of the P6-labeled probe. As expected, the P6-cold probe was the most potent in competing with its own P6-labeled

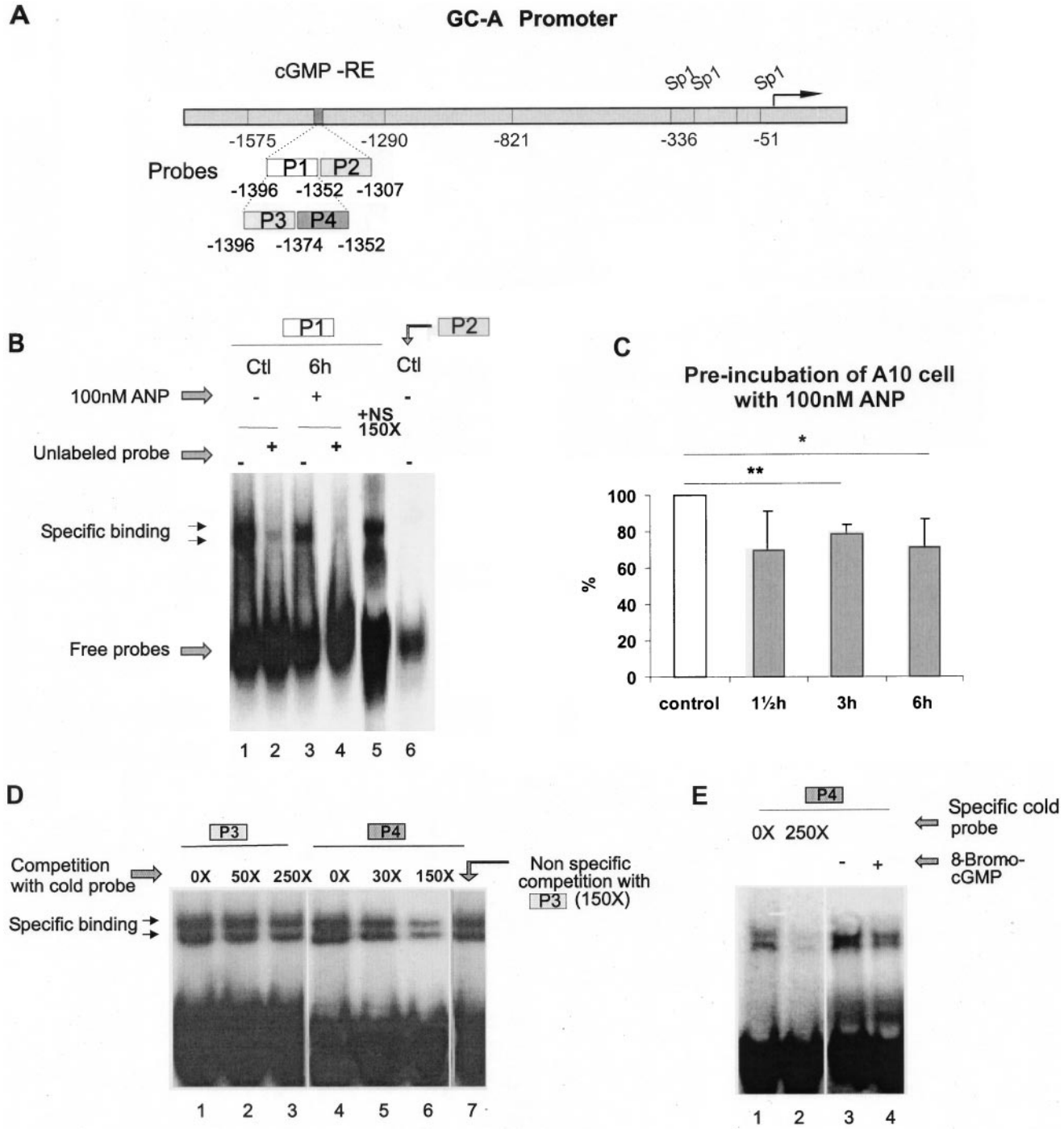

Figure 3. EMSA of the −1396 to −1307 region of the GC-A/*Npr1* promoter. A, The fragment −1396 to −1307 was divided into 2 probes, P1 (−1396 to −1352) and P2 (−1352 to −1307). The P1 probe was further divided into 2 fragments, P3 (−1396 to −1374) and P4 (−1374 to −1352). B, The probes were then ^{32}P-labeled and incubated with 5 μg of A10 nuclear proteins. P1 probe bands correspond to lanes 1 to 5. P2 probe binding corresponds to lane 6. There was no binding of the P2 probe by nuclear protein extracts. Lanes 2 and 4 show specificity of binding of the labeled P1 probe. Lane 3 shows that P1 binding is lower (vs lane 1) when the cells have been preincubated for 6 hours with ANP. C, Densitometric analysis of the time course of the effect of ANP preincubation on P1 binding to nuclear proteins. The intensity of the bands was quantified, and the values correspond to the means±SEM of 3 experiments and are expressed as percentages of the controls. *$P<0.05$, **$P<0.002$ (Student *t* test). D, An excess of specific or unspecific unlabeled probes was added in the reaction mixture for competition of P3 and P4 binding, as indicated. E, Nuclear proteins from control cells (lanes 1, 2, 3) or cells preincubated for 3 hours with 100 μmol/L 8-bromo-cGMP (lane 4).

probe for binding to nuclear proteins, with more than 80% displacement attained at 100-fold excess of unlabeled oligonucleotide (Figure 5B). Although not as efficient as the P6-cold probe in competing for binding, the human P13 oligonucleotide was the second most efficient with 70% displacement of binding of the P6-labeled probe. This indicates that variations in nucleotides between rat and human sequences are less critical for binding to nuclear proteins.

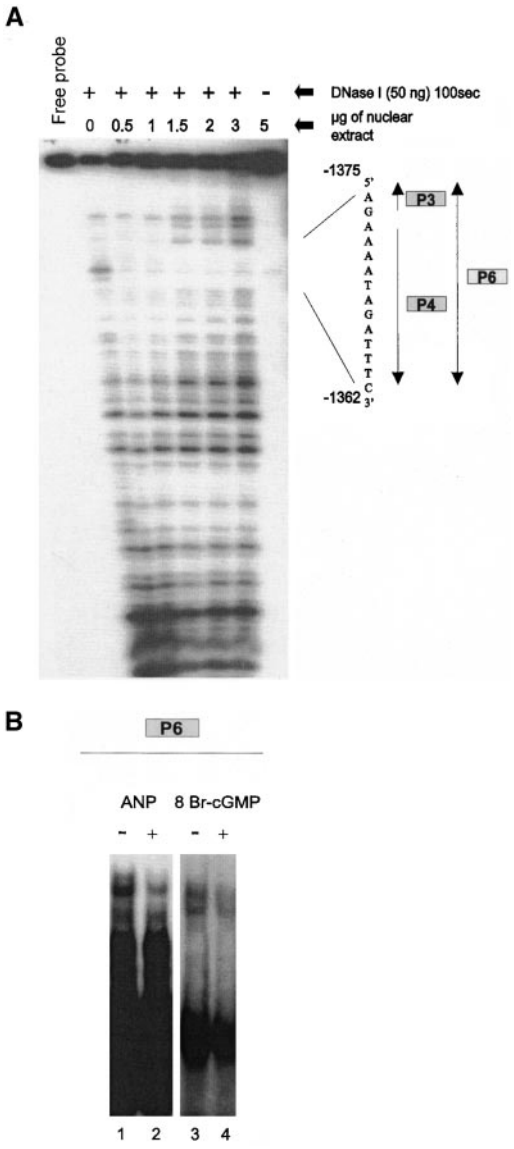

Figure 4. DNase I protection assay. A, The fragment −1396 to −1290 was obtained by digestion of the 1396 pGL3b plasmid vector. One extremity was ^{32}P-labeled and incubated with increasing amounts of A10 nuclear proteins as indicated. B, P6 was synthesized, end labeled, and used in EMSA (Table). Nuclear proteins were extracted from A10 cells preincubated with or without 100 nM ANP (lanes 1, 2) or 100 μmol/L 8-bromo-cGMP for 3 hours (lanes 3, 4).

The mutated probes M12, M14, and M15 were significantly less effective than the P6 probe in cross-competition experiments ($P=0.016$, $P=0.0008$, $P=0.0087$, respectively) (Figure 5B), suggesting that the nucleotides mutated are critical for binding. Based on the results obtained with the M12 and M14 oligonucleotides, a short probe containing 9 nucleotides was synthesized as P16 (Table). This P16 oligonucleotide did not compete with the P6 ^{32}P-labeled probe for its binding to nuclear proteins (Figure 6A and 6B). Adding nucleotides at the 5′ extremity of P16 (Table), as in the P20 to P24 probes, increased the effectiveness of the oligonucleotides to compete with the P6 probe for binding nuclear proteins (Figure 6A). This increase reached a maximum with oligonucleotide P21 ($EC_{50}=0.18$ ng versus 1.1 ng for P6) (Figure 6B and 6C and Table). On the other hand, adding nucleotides at the 3′ end of P16 did not improve competition efficiency, which indicates that nucleotides AA found in P22 but not in P23, GA found in P24 but not in P16, and TTT found in P26 but not in P16 are essential for binding nuclear proteins. To further investigate this possibility, the P21 probe, which was seen to have maximal binding activity, was modified by adding or removing nucleotides at its 3′ end (P27 to P30) (Figure 6). Adding nucleotides at position −1356 to −1353, as in the P27 and P28 cold probes, remained as effective as unlabeled P6 in displacing the P6-labeled probe from nuclear proteins. However, removing 2 or 4 nucleotides of P21 (P29 and P30, respectively) led to a significant loss of binding, suggesting that nucleotides at positions −1360 and −1357 are important for binding.

The P21 fragment was then subcloned in the pGL3 promoter plasmid, transfected in A10 cells, and its luciferase

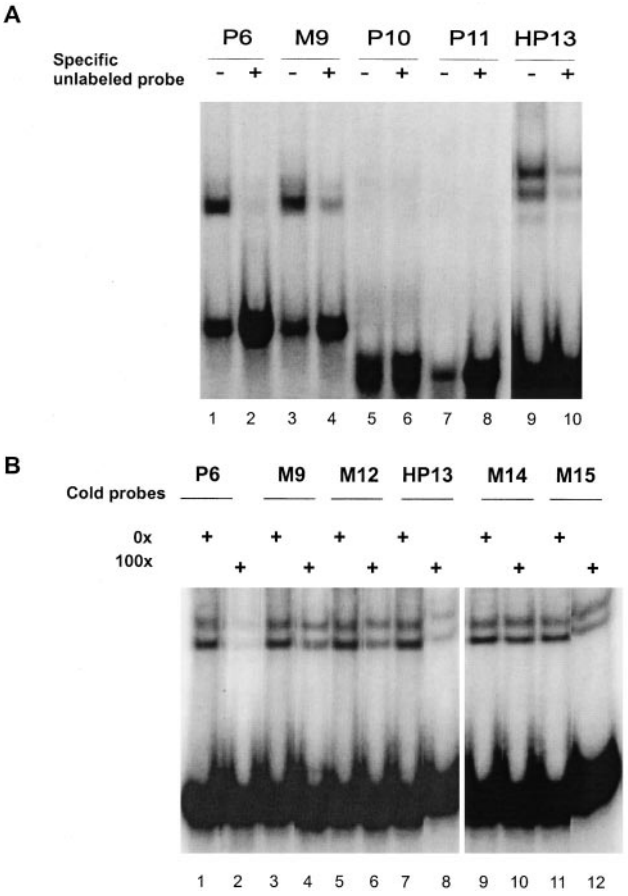

Figure 5. EMSA of the region −1375 to −1352 of the GC-A promoter. A, The sequence of the P6 probe corresponding to the −1375 to −1352 region of the GC-A promoter is shown in the Table. Different mutated probes were designed from the P6 fragment. Mutations were introduced in the palindromic sequence (M9). The P6 probe was separated into 2: P10 and P11 probes. Human putative cGMP-RE was also analyzed by EMSA (HP13). Representative EMSA of the P6 (lanes 1 and 2), M9 (lanes 3 and 4), P10, P11 (lanes 5 to 8), and HP13 (lanes 9 and 10) probes with nuclear proteins in the absence or presence of 200-fold excess cold probe. B, Cross-competition experiments are shown with unlabeled or unlabeled mutated probes added (100-fold excess) together with the P6-labeled probe for competition of nuclear protein binding.

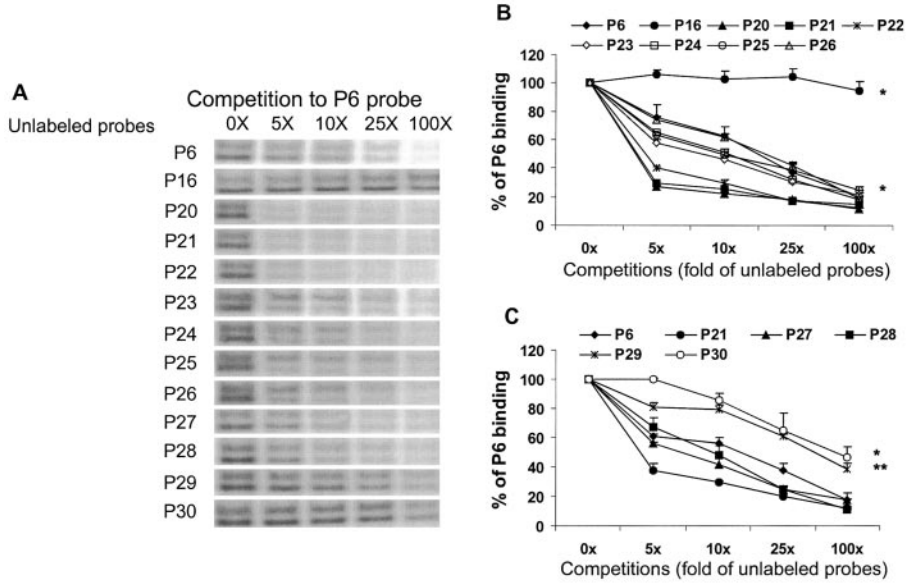

Figure 6. Cross-competiton experiments with truncated probes. A, Increasing concentrations of unlabeled P6 probe or of unlabeled truncated probes were added together with the P6-labeled probe for competition of nuclear protein binding. B and C, Representative competition experiments of P6-labeled probe binding to nuclear proteins. The values corresponding to means±SEM of 3 experiments were expressed as percentages of P6. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (ANOVA).

activity was measured. P21-pGL3p showed slight but significantly higher activity than pGL3p alone. Incubation of cells with 8-bromo-cGMP significantly decreased by 25% the transcriptional activity of the P21 fragment (Figure 7A), indicating that the short P21 fragment site (−1373 to −1357) conferred responsiveness of the *Npr1* promoter to 8-bromo-cGMP.

Finally, we modified the initial −1396 partial promoter by deleting the consensus 18 bp (−1373 to −1355) cGMP-RE (KN). We conducted 3 experiments (n=3 to 5) under previously described conditions. Basal promoter activity was identical between the intact −1396 and the −1380 ΔcGMP-RE clone (KN). Deletion of cGMP-RE almost completely abolished the inhibitory effect (40%±4% versus 5%±9%) of ANP. These results confirm the essential role of the consensus element in the inhibitory effect of ANP on promoter activity. Figure 7 demonstrates reciprocal effects: loss of the ANP effect when the consensus region is deleted from the −1396 partial promoter (Figure 7B) and gain of the cGMP response when the p21 fragment is added to the SV40 promoter (Figure 7A).

Discussion

An ANP regulatory sequence was previously identified in the region between −1575 and −1290 to the transcription start site of the *Npr1* promoter.[9] This region was shown to be also sensitive to 8-bromo-cGMP. In the present study, we identified inside this region 2 regulatory elements, a negative regulatory element located between −1520 and −1397, and a positive regulatory element, between −1396 and −1256. We confirmed the presence of a cGMP-RE in the *Npr1* promoter and delimited its localization to a −1396 to −1307 region.

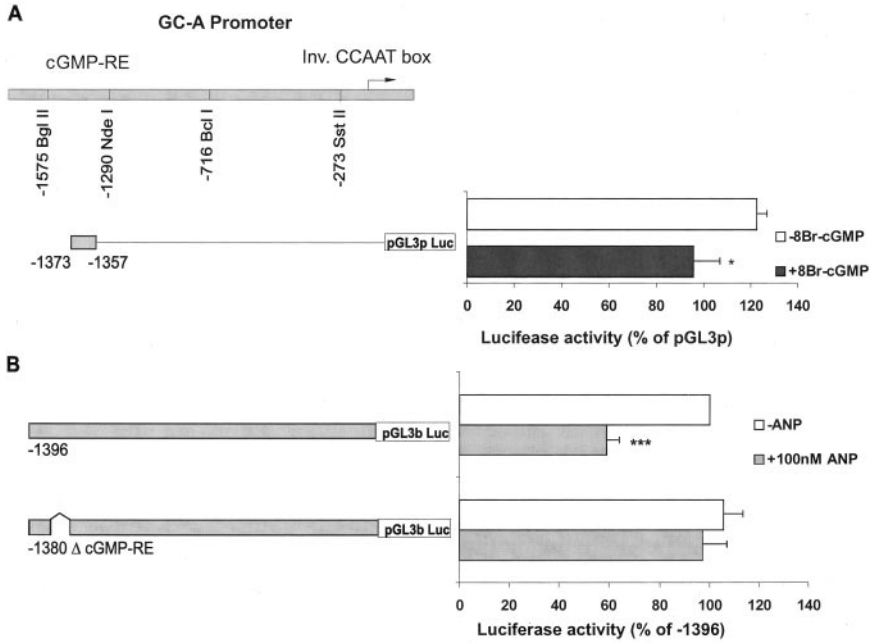

Figure 7. Luciferase activity after transient expression of the P21 fragment and of the deletion construct. A, NIH 3T3 cells were cotransfected with 5 μg of P21-pGL3p-Luc constructs and 1 μg of pCMV-β and exposed to 100 μmol/L of 8-bromo-cGMP for 8 hours. All values are expressed as a percentage of luciferase activity to pGL3p. Values represent the means±SEM of 3 different experiments performed in duplicate. *$P<0.05$ vs control group (Student *t* test). B, The initial −1396 partial promoter was modified by deleting the consensus 18 bp (−1373 to −1355) cGMP-RE (KN). Three experiments (n=3 to 5) were performed under the conditions described in Figure 1A. Basal promoter activity was identical between the intact −1396 and the −ΔcGMP-RE clone (KN). ***$P<0.001$

This region still showed a significant decrease in promoter activity when cells were pretreated with ANP or 8-bromo-cGMP. Furthermore, we narrowed down the cGMP-RE to a 24-bp long fragment located between −1375 and −1352 (P6) of the *Npr1* promoter, which displayed a specific complex formation with A10 cell nuclear proteins. With the DNase I protection assay, we localized a palindromic sequence (5′AAATAGATTT3′) within the P4 probe. However, mutation (M9) within the palindromic sequence did not affect binding, while both adjacent sequences were required for binding nuclear proteins. We then suggested a consensus sequence of **A**a**A**tRKaN**TTC**a**A**c**A**K**T**Y for cGMP-RE. Nucleotides in bold and upper cases are the most important. R (A or G), K (G or T), and Y (T or C) nucleotides were also found in the human sequence. This suggests that these nucleotides are also required for binding. The lower case nucleotides have not been tested.

Our data revealed that binding of the P6 fragment to nuclear proteins was inhibited when the cells were preincubated with ANP or a cGMP analog. This suggests that the binding of unknown proteins or transcription factors is reduced by cGMP elevations, leading to inhibition of *Npr1* transcription. Previous studies have shown that cell pretreatments with ANP or 8-bromo-cGMP led to a decrease in mRNA levels of NPR-A,[6–8] indicating that ANP-induced downregulation of NPR-A is mediated by elevations of intracellular cGMP levels and occurs at the transcriptional level.

To the best of our knowledge, the mechanism by which cGMP influences gene transcription is still unclear. Similarly to cAMP, which acts mainly through cAMP-dependent protein kinases (cAKs), cGMP is able to activate cGMP-dependent protein kinases (cGKs). By phosphorylating nuclear transcription factors, such as members of the CREB/ATF family, cAKs are known to be involved in the regulation of transcription of several genes.[16,17] Since cGKs and cAKs are highly homologous protein kinase families that possess similar substrate specificities, a similar pathway could be proposed for cGMP. Thus, several studies showed that cGMP-dependent protein kinase could interact with transcription factors such as TFII[18] and NFκB.[19] Moreover, cGMP was recently proposed to be involved in other signaling pathways including MAPK1[20] and CaMKinase II cascade.[21] In addition, while recent analysis of the promoter region of murine Npr1 has revealed the presence of several binding sites for a variety of nuclear factors, including HFH-3, CREB, SRY, and USF,[14] no known transcription factors matched the cGMP-RE sequence,[15] suggesting that this is a novel *cis*-acting element. Although the element described here was identified in the promoter regions of mouse, rat, and human *Npr1*, our search did not detect an identical element in the promoters of other cGMP-regulated genes, suggesting a specific mode of regulation for *Npr1*. In order to determine whether G-kinase might alter binding of nuclear proteins to the cGMP-RE, we performed EMSA using specific anti-cGMP–dependent kinase antibody (anti-PKG I). Our preliminary data showed that the protein-DNA complex formed was not supershifted by the anti-PKG I specific antibody (data not shown), suggesting that cGMP-dependent kinase does not affect binding of nuclear proteins to the novel element. Further experiments are needed to identify the mechanism of cGMP regulation of nuclear factor binding to this element.

Perspectives

Since *Npr1* is the receptor of an important regulator of blood pressure and volume homeostasis, the regulation of its gene transcription is of likely physiological importance. Garg and Pandey[22] reported the transcriptional downregulation of *Npr1* by angiotensin (Ang) II, confirming the mutual cross-regulation of the two vasoactive hormones Ang II (vasoconstrictive) and ANP (vasodilatory) in the pathway mediating the pathophysiology of hypertension. Interestingly, we recently showed that the activity of the cGMP-RE was inhibited in spontaneously hypertensive rats. This inhibition was associated with a TA dinucleotide repeat expansion in the *Npr1* promoter with consequence on the expression of the receptor.[23] The present study constitutes the initial characterization of a novel *cis*-acting cGMP-sensitive element in the promoter of *Npr1* of several species, for which nuclear factor(s) binding remain to be identified.

Acknowledgments

This research was supported by a grant from the Canadian Institutes of Health Research (MOP-11463) to JT. S.B. is the recipient of a fellowship from the Canadian Institutes of Health Research (CIHR). R.S. received a fellowship from the MRC/CIHR. We thank Suzanne Cossette for her technical assistance, Ovid Da Silva for his editorial work on this manuscript, and Ginette Dignard for her secretarial skills.

References

1. Tremblay J, Desjardins R, Hum D, Gutkowska J, Hamet P. Biochemistry and physiology of the natriuretic peptide receptor guanylyl cyclases. *Mol Cell Biochem.* 2002;230:41–47.
2. Hamet P, Tremblay J, Pang SC, Garcia R, Thibault G, Gutkowska J, Cantin M, Genest J. Effect of native and synthetic atrial natriuretic factor on cyclic GMP. *Biochem Biophys Res Commun.* 1984;123:515–527.
3. Tremblay J, Gerzer R, Vinay P, Pang SC, Beliveau R, Hamet P. The increase of cGMP by atrial natriuretic factor correlates with the distribution of particulate guanylate cyclase. *FEBS Lett.* 1985;181:17–22.
4. Winquist RJ, Faison EP, Waldman SA, Schwartz K, Murad F, Rapoport RM. Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. *Proc Natl Acad Sci U S A.* 1984;81:7661–7664.
5. Garbers DL. Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. *Cell.* 1992;71:1–4.
6. Cahill PA, Redmond EM, Keenan AK. Vascular atrial natriuretic factor receptor subtypes are not independently regulated by atrial peptides. *J Biol Chem.* 1990;265:21896–21906.
7. Schiffrin EL, Turgeon A, Tremblay J, Deslongchamps M. Effects of ANP, angiotensin, vasopressin, and endothelin on ANP receptors in cultured rat vascular smooth muscle cells. *Am J Physiol.* 1991;260:H58–H65.
8. Potter LR, Garbers DL. Protein kinase C-dependent desensitization of the atrial natriuretic peptide receptor is mediated by dephosphorylation. *J Biol Chem.* 1994;269:14636–14642.
9. Cao L, Wu J, Gardner DG. Atrial natriuretic peptide suppresses the transcription of its guanylyl cyclase-linked receptor. *J Biol Chem.* 1995;270:24891–24897.
10. Sambrook, J, Fritsch, EF, Maniatis, T. *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press; 1989.
11. Hamet P, Pang SC, Tremblay J. Atrial natriuretic factor-induced egression of cyclic guanosine 3′:5′-monophosphate in cultured vascular smooth muscle and endothelial cells. *J Biol Chem.* 1989;264:12364–12369.

12. Dignam JD, Martin PL, Shastry BS, Roeder RG. Eukaryotic gene transcription with purified components. *Methods Enzymol.* 1983;101: 582–598.
13. Nakayama T, Soma M, Takahashi Y, Rehemudula D, Sato M, Uwabo J, Izumi Y, Kanmatsuse K. Nucleotide sequence of the 5′-flanking region of the type A human natriuretic peptide receptor gene and association analysis using a novel microsatellite in essential hypertension. *Am J Hypertens.* 1999;12:1144–1148.
14. Garg R, Oliver PM, Maeda N, Pandey KN. Genomic structure, organization, and promoter region analysis of murine guanylyl cyclase/atrial natriuretic peptide receptor-A gene. *Gene.* 2002;291:123–133.
15. Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL, Kolchanov NA. Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Res.* 1998;26:362–367.
16. Nigg EA, Hilz H, Eppenberger HM, Dutly F. Rapid and reversible translocation of the catalytic subunit of cAMP-dependent protein kinase type II from the Golgi complex to the nucleus. *EMBO J.* 1985;4: 2801–2806.
17. Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W, Tsien R, Montminy MR. Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate-limited by nuclear entry of protein kinase A. *Mol Cell Biol.* 1993;13:4852–4859.
18. Casteel DE, Zhuang S, Gudi T, Tang J, Vuica M, Desiderio S, Pilz RB. CGMP-dependent protein kinase I beta physically and functionally interacts with the transcriptional regulator TFII-I. *J Biol Chem.* 2002;277: 32003–32014.
19. Izumi T, Saito Y, Kishimoto I, Harada M, Kuwahara K, Hamanaka I, Takahashi N, Kawakami R, Li Y, Takemura G, Fujiwara H, Garbers DL, Mochizuki S, Nakao K. Blockade of the natriuretic peptide receptor guanylyl cyclase-A inhibits NF-κB activation and alleviates myocardial ischemia/reperfusion injury. *J Clin Invest.* 2001;108:203–213.
20. Kim E, Park JM. Identification of novel target proteins of cyclic GMP signaling pathways using chemical proteomics. *J Biochem Mol Biol.* 2003;36:299–304.
21. Zhang S, Yang Y, Kone BC, Allen JC, Kahn AM. Insulin-stimulated cyclic guanosine monophosphate inhibits vascular smooth muscle cell migration by inhibiting Ca/calmodulin-dependent protein kinase II. *Circulation.* 2003;107:1539–1544.
22. Garg R, Pandey KN. Angiotensin II-mediated negative regulation of Npr1 promoter activity and gene transcription. *Hypertension.* 2003;41[part 2]:730–736.
23. Tremblay J, Hum D, Sanchez R, Dumas P, Pravenec M, Krenova D, Kren V, Kunes J, Gossard F. TA repeat variation, *Npr1* expression and blood pressure: impact of the ace locus. *Hypertension.* 2003;41:16–24.

Hypertension
Journal of the American Heart Association

Characterization of a cGMP-Response Element in the Guanylyl Cyclase/Natriuretic Peptide Receptor A Gene Promoter

David Hum, Sandrine Besnard, Rocio Sanchez, Dominic Devost, Francis Gossard, Pavel Hamet and Johanne Tremblay

Hypertension. 2004;43:1270-1278; originally published online April 19, 2004;
doi: 10.1161/01.HYP.0000126920.93207.53
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563