The vasorelaxant and anti-mitogenic activities of the atrial and brain natriuretic peptides depend upon their binding to the type A natriuretic peptide receptor (NPR-A) expressed on the surface of vascular cells. Intervention strategies aimed at controlling NPR-A expression are limited by the paucity of studies in this area. Here we identify a sequence CCAAT between −141 and −137 of the NPR-A promoter that, when mutated, reduces promoter activity by 90% in rat aortic smooth muscle (RASM) cells. Protein/DNA cross-linking and immunoprecipitation of electrophoretically shifted complexes formed between RASM nuclear extracts and an oligonucleotide surrounding the CCAAT sequence indicates that the heterotrimeric transcription factor NF-Y binds specifically to the wild-type, but not mutated, CCAAT element. Cotransfection of a dominant negative mutant of the NF-YA subunit results in a concentration-dependent decrease in the activity of the NPR-A promoter in RASM cells confirming that endogenous NF-Y is an activator of the promoter. Mutation of the CCAAT element, in conjunction with mutation of all three Sp1 sites previously shown to be involved in NPR-A promoter regulation, virtually eliminates NPR-A promoter activity in RASM cells. Coexpression of all three NF-Y subunits together with Sp1 in Drosophila cells deficient in these factors indicates that NF-Y and Sp1 act synergistically to reconstitute NPR-A promoter activity. A direct physical association between NF-Y and Sp1 can be demonstrated both in vitro by glutathione S-transferase pull-down assay and in the intact cell by coimmunoprecipitation and functional studies. Together, these studies show that NPR-A promoter activity is dominantly regulated through functional, and possibly physical, interactions of NF-Y and Sp1.

The natriuretic peptides are a family of vasoactive hormones that play an important role in the regulation of blood pressure and cardiovascular homeostasis (1). Atrial natriuretic peptide and brain natriuretic peptide are both produced predominantly in the heart and circulate in plasma. Their natriuretic, diuretic, and vasorelaxant activities are mediated through the type A natriuretic peptide receptor (NPR-A)1 (2) (also known as guanylyl cyclase A) present on the surface of vascular smooth muscle and other cells. Recent studies by two independent groups (3, 4) showed that complete absence of NPR-A in mice leads to hypertension, cardiac hypertrophy, and sudden death, indicating a critical role for NPR-A in the regulation of cardiovascular homeostasis.

The molecular regulation of NPR-A gene transcription is only poorly understood. The rat NPR-A gene has been cloned and sequenced. Sequence analysis identified very few sequence elements for known transcription factors. However, three putative Sp1 consensus binding sites (positioned between −341 and −51) and a CCAAT motif (positioned at −137) were present upstream from the promoter of the NPR-A gene (5). Our earlier studies (6) demonstrated a critical role for the Sp1 family of transcription factors in regulating NPR-A gene transcription in rat aortic smooth muscle (RASM) cells, but the role of the CCAAT sequence remains unknown.

The CCAAT box is present in a number of eukaryotic promoters (7–10) and has been demonstrated to be important for the transcription of many of those genes (7–10). In relatively simple, TATA-less promoters, which, like the NPR-A promoter, contain only one or two additional cis-acting elements, the CCAAT box is absolutely required for regulating gene transcription (10–12). In contrast, the CCAAT box is somewhat less critical for TATA-containing promoters (10–12). Typically, the CCAAT element is found as a single copy in the forward or reverse orientation immediately upstream of the transcription start site. In the TATA-less NPR-A promoter, the CCAAT sequence is positioned in the reverse orientation between −137 and −141 upstream of the transcription start site. In the present study, we show that the CCAAT box is important for NPR-A gene transcription. We have identified the nuclear proteins that associate with the CCAAT box as the heterotrimeric NF-Y complex, and we have demonstrated that functional, and possibly physical, interaction of NF-Y with Sp1 is essential for optimal transcription of the NPR-A promoter in vascular smooth muscle cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit polyclonal antibodies directed against CCAAT/enhancer-binding protein (C/EBPα, C/EBPβ, and C/EBPδ, and goat polyclonal antibody directed against Sp1 were a kind gift from S. McKnight (University of Texas, Southwestern Medical Center, Dallas, TX). Mouse monoclonal antibody directed against nuclear factor-Y (NF-Y) A was purchased from PharMingen (San Diego, CA). Rabbit polyclonal antibody directed against NF-YB was a gift from M. Roberto (University of Milan, Milan, Italy). Poly(dI-dC), glutathione-Sepharose™ 4B, and T7 Sequenase were purchased from Amersham Pharmacia Biotech. Schneider cell medium was obtained from Life Technologies, Inc. All oligonucleotides were synthesized by Cruachem, Inc. Other reagents were obtained through standard commercial suppliers.

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Fig. 1. Mutation of Sp1 and CCAAT sites eliminates rat NPR-A promoter activity in transiently transfected RASM cells. The CCAAT site is on the minus strand in the NPR-A promoter and reads as ATTGG in the figure. Panel A, location and site-directed mutagenesis of the Sp1 and CCAAT sites. WT and MUT represent the wild-type and mutagenized sites. Mutagenized bases are indicated by lowercase letters, and the putative regulatory element is underlined. Panel B, 10 μg of wild-type (WT) −387 NPR-A LUC or the mutant (MUT) indicated were transiently transfected into RASM cells. After 48 h of culture, cells were lysed for luciferase assay. The data represent the mean ± S.D. from four experiments done in triplicate.

Plasmid Construction and Site-directed Mutagenesis—The construction of −387 rat NPR-A luciferase has been described previously (6). The NF-YA expression plasmids pNF-YA (wild-type) and pNF-YA29 (dominant negative form) were provided by R. Mantovani (University of Milan, Milan, Italy), pPacSp1 and Copia β-galactosidase were obtained from R. Tjian (University of California, Berkeley, CA), pPacNF-YA, pPacNF-YB, and pPacNF-YC were provided by T. F. Osborne (University of California, San Francisco, CA). The NF-YA expression plasmids pNF-YA (wild-type) and pNF-YA29 by electroporation (Gene-Pulser, Bio-Rad) at 180 mV and 960 μF. For Drosophila Schneider cells (SI-2) were obtained from the Cell Culture Facility at the University of California (San Francisco, CA). Cells were cultured in Schneider's medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2% (v/v) broth, tryptose phosphate. Drosophila Schneider cells (SI-2) were obtained from the Cell Culture Facility at the University of California (San Francisco, CA). Cells were cultured in Schneider's medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin at 25 °C.

Transfection, Luciferase, and β-Galactosidase Assays—RASM cells were transiently transfected with 10 μg of −387 rat NPR-A luciferase or the relevant promoter mutant and 1–5 μg of pNF-YA or pNF-YA29 by electroporation (Gene-Pulser, Bio-Rad) at 250 mV and 960 μF. For Drosophila Schneider cells, 5 μg of −387 NPR-A luciferase or the relevant promoter mutant and 2 μg of Copia β-galactosidase were cotransfected along with increasing amounts (1–10 μg) of pPacSp1, and/or plasmids encoding NF-Y subunits (pPacNF-YA, pPacNF-YB, and pPacNF-YC), alone or in combination, by electroporation at 180 mV and 960 μF. After transfection, cells were plated on six-well plastic plates and cultured for 48 h. Cells were harvested and lysed in 100 μl of cell culture lysis reagent (Promega, WI). Protein concentration of each cell extract was measured using Coomassie protein reagent (Pierce). Cell lysates were processed (20 μg of protein/sample) and assayed for luciferase as described previously. Measurements of β-galactosidase activity were made using the Galacto-Light Plus® kit from Tropix, Inc. (Bedford, MA).
Preparation of Nuclear Extracts—Cells were harvested and lysed by the addition of 0.5 ml of lysis buffer (containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) on ice for 10 min. Lysates were centrifuged; the pellets were resuspended in buffer B (containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, and the above protease inhibitors) and kept on ice for 30 min. Nuclei were centrifuged at 12,000 rpm for 15 min, and the supernatant extracts were saved. Extracts were stored at −80 °C prior to use.

UV Cross-linking Analysis—Purified [³²P]-end-labeled, double-stranded oligonucleotide containing the CCAAT site in the NPR-A promoter or a control oligonucleotide encoding a C/EBP binding site was incubated with 10 μg of RASM nuclear extract in binding reaction buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 10 μM ZnSO₄, 0.2 mg/ml bovine serum albumin, 10% glycerol, and 0.1% Nonidet P-40) containing 0.5 μg of poly(dI-dC) at room temperature for 30 min. Each reaction mixture was then pipetted onto paraffin and subjected to irradiation for 10–20 min at a distance of 5 cm using an ultraviolet lamp with 254-nm emission. Samples were resolved by electrophoresis on 10% denaturing polyacrylamide gel and exposed to an x-ray film for autoradiography.

Electrophoretic Mobility Shift Assay (EMSA)—The CCAAT oligonucleotides used for EMSAs were as follows: wild-type, 5'-GGTTAAGAGTCAACCCGCGGGGCTCTC-3'; mutant, 5'-GGTTAAGAGTGCGGCCGGCGGCTCTC-3'. Only coding strand sequence is provided; mutagenized bases are identified by lowercase letters, and the CCAAT sequence is underlined. The sequence (coding strand) of the C/EBP binding oligonucleotide is: 5'-TAGCTGAGATCTTGCGTAACC-ATTGCCCAA-3'. Nuclear extracts (10 μg) were incubated in binding reaction buffer containing 0.5 μg of poly(dI-dC) at room temperature for 10 min. Purified [³²P]-end-labeled, double-stranded oligonucleotide was added for an additional 10 min in a total volume of 20 μl. For competition experiments, a 1-100-fold molar excess of unlabeled double-stranded oligonucleotide was added to the binding reaction. For immunoprecipitation experiments, nuclear extracts were incubated on ice for 1 h with 2 μg of polyclonal antibody against C/EBPα, C/EBPβ, C/EBPδ, NF-YA, NF-YB, DBP, TEF, Sp₁, or Sp₃ prior to the addition of labeled probe. Independent studies with the anti-C/EBPα (23) and C/EBPβ (data not shown) antibodies demonstrated that each was capable of disrupting (C/EBPα) or supershifting (C/EBPβ) the relevant DNA-protein complex in the mobility shift assay. All samples were resolved on 5% nondenaturing polyacrylamide gels. Gels were dried and exposed to x-ray film for autoradiography.

GST Pull-down Assay—[³²P]-Labeled Sp₁ protein was synthesized in vitro using the Tn5SP6 quick-coupled transcription/translation system from Promega (Madison, WI) according to the manufacturer's instructions. GST fusion protein expression vectors including pGEX-CBF-A, pGEX-CBF-B, pGEX-CBF-C, and pGEX-Sp₁ were transformed into the BL-21 strain of Escherichia coli (Stratagene, La Jolla, CA), expanded in suspension culture and induced (3 h) with 1 mM isopropyl β-D-thiogalactoside. Cells were pelleted, sonicated in TST buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20, and centrifuged. The resultant supernatant was then added to 300 μl of glutathione-Sepharose beads, mixed on a rotating wheel at 4 °C for 1 h, and centrifuged. The pellet containing the bound GST fusion protein was washed three times with TST buffer, then resuspended in 150 μl of SDS sample buffer, and loaded on a 10% denaturing polyacrylamide gel. The gel was dried and exposed to x-ray film prior to autoradiography.

Coimmunoprecipitation—One mg of RASM nuclear extract was mixed with 2 μg of either anti-Sp₁ antibody or anti-Rel-A (p65) antibody in 200 μl of protein binding buffer. The mixture was incubated at a control, 200 μg of nuclear extract was mixed with either 10 μg of GST alone or GST-Sp₁ bound to glutathione-Sepharose beads. After incubation at 4 °C for 2 h, the reaction mixtures were pelleted, and the precipitates were washed three times with protein binding buffer, resuspended, and boiled with SDS sample buffer. The protein was resolved on 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and immunoblotted with anti-NF-YA antibody. The immunoprecipitated protein signal was detected using the ECL® Western blot detection system (Amersham Pharmacia Biotech).

Statistic Analysis—Data were evaluated by one-way analysis of variance using Newman-Keul's test for significance.

RESULTS

Three Sp₁ consensus sites and one CCAAT site have been identified by DNA sequence analysis in the proximal 5'-flanking sequence of NPR-A gene (5, 6). All of these regulatory elements are present within a segment of the NPR-A gene, extending from 387 base pairs upstream to the transcription start site, which we showed previously to direct the optimal level of NPR-A promoter activity in RASM cells (6). The relative location and specific mutations introduced at each of these sites are presented in Fig. 1A. To determine the relative contribution of each of these putative regulatory elements to NPR-A gene transcription, mutations were introduced into each site within the context of the −387 NPR-A luciferase reporter and transfected into RASM cells. Our previous studies showed that both Sp₁ and Sp₃ bind to each of the three Sp₁ consensus sites in the NPR-A promoter and that mutation of all three Sp₁ sites in concert reduced −387 NPR-A luciferase reporter activity in RASM cells to 10% that of the native promoter. In the present study, we showed that mutation of the CCAAT site resulted in a similar −90% reduction in activity whereas mutation of the CCAAT and Sp₁ sites in combination virtually eliminated NPR-A promoter activity (Fig. 1B). Thus, a CCAAT-binding transcription factor is a dominant activator of the NPR-A promoter.

A number of transcriptional activators that bind to the CCAAT motif have been described. One or more of these may participate in CCAAT-dependent, NPR-A promoter activity. These factors include C/EBP (13–16), NF-Y (17–19), mouse y-box protein 1 (MSY-1) (20), and CCAAT binding transcription factor/nuclear factor-1 (CTF/NF-1) (19, 21). We performed UV cross-linking and electrophoretic mobility shift assays (EMSA) to identify the protein(s) that interact with the CCAAT motif in the proximal NPR-A promoter. UV cross-linking analysis showed at least two protein complexes in RASM nuclear extracts, with molecular mass values of −117 and −78 kDa, that associated with a labeled oligonucleotide spanning the CCAAT motif in the NPR-A promoter, but not with an otherwise identical oligonucleotide containing the mutations that block NPR-A promoter activity (Fig. 2). We also cross-linked the same RASM extracts with an oligonucleotide that binds to
C/EBPs in rat liver tissue and pituitary progenitor GHFT1–5 cells (22, 23). Three nuclear proteins of 42, 48, and 52 kDa were identified. Although a low intensity 42-kDa band was seen in the RASM extracts, the 48- and 52-kDa bands were clearly distinguishable from those interacting with the NPR-A CCAAT sequence (Fig. 2). This suggests that C/EBP proteins are present in the RASM nuclear extract but, for the most part, do not bind to the CCAAT motif in the NPR-A promoter.

EMSA of DNA-binding proteins present in RASM extracts was carried out. A single slowly migrating band was observed which was effectively competed by unlabeled wild-type (WT) oligonucleotide but not by its mutated counterpart (Fig. 3A).

**Fig. 3.** EMSA analysis of the interaction of RASM nuclear extracts with the CCAAT site in the rat NPR-A promoter. Ten µg of RASM nuclear extract was incubated with 32P-labeled, double-stranded NPR-A CCAAT oligonucleotide or 32P-labeled, double-stranded C/EBP-binding oligonucleotide and subjected to EMSA. Panel A, competition of RASM nuclear protein interaction with 32P-labeled CCAAT probe by increasing concentrations (1–100-fold excess) of unlabeled double-stranded NPR-A CCAAT oligonucleotide. Panel B, competition of RASM nuclear protein interaction with 32P-labeled NPR-A CCAAT probe by increasing concentrations (1–100-fold excess) of unlabeled, double-stranded C/EBP-binding oligonucleotide. Panel C, identification of CCAAT binding complexes by EMSA. Ten µg of RASM nuclear extracts was preincubated on ice for 1 h with 2 µg of antibody directed against C/EBPa, C/EBPb, C/EBPd, NF-YA, NF-YB, DBP, TEF, Sp1, Sp3, or with preimmune serum before the addition of labeled NPR-A CCAAT probe. The position of the CCAAT oligonucleotide/NF-Y complex is indicated by the arrow.
NF-Y and Sp1 Co-regulate NPR-A Promoter

EMSA with the C/EBP-specific oligonucleotide demonstrated a faster-migrating protein complex supporting our contention (see above) that C/EBP does not participate in formation of the complex identified on the NPR-A CCAAT element (Fig. 3B). This conclusion drew further support from the failure of the C/EBP oligonucleotide to compete with the native complex on the NPR-A promoter fragment (Fig. 3D). The 117-kDa size of the UV cross-linked protein is similar to the size predicted for the CCAAT-binding, heterotrimeric NF-Y complex, raising the possibility that this transcription factor is involved in regulation of the NPR-A gene. To test this hypothesis, we employed specific antibodies directed against subunits of the NF-Y protein complex. Immunoprecipitation studies showed that the slowly migrating band in the EMSA was supershifted or disrupted by antibody directed against the A or B subunits of NF-Y, but was not recognized by antibody directed against C/EBPα, C/EBPβ, C/EBPδ, DBP, or TEF (Fig. 3C). Collectively, these findings support the hypothesis that the RASM nuclear protein that binds to the CCAAT box of the NPR-A promoter is NF-Y.

NF-Y (also called CBF or CP1) is a ubiquitous transcription factor that binds to CCAAT motifs in the proximal promoters of a large number of mammalian genes (7–12, 17–19). NF-Y/CBF consists of three subunits, A, B, and C, all of which are required for DNA binding. To confirm that the CCAAT box is essential for NPR-A gene expression, we examined the effect of forced overexpression of NF-Y or an NF-Y dominant negative mutant on NPR-A promoter activity. Overexpression of wild-type NF-YA failed to activate the NPR-A luciferase reporter (Fig. 4); however, cotransfection of a dominant negative mutant of NF-YA (pNF-YA29) resulted in a dose-dependent reduction of −387 NPR-A promoter activity in RASM cells (Fig. 4). The mutant NPR-A reporter lacking the CCAAT element was unaffected by pNF-YA29. The failure of NF-YA to activate the NPR-A promoter might reflect the fact that the ubiquitously expressed NF-Y protein, or more specifically NF-YA, is not limiting for NPR-A gene transcription in RASM cells. To confirm that NF-Y is an activator of the NPR-A promoter, we introduced the NPR-A luciferase reporter into Schneider cells, a Drosophila cell line that does not express appreciable levels of a number of mammalian transcription factors, including Sp1 and NF-Y. As shown in Fig. 5A, overexpression of the three NF-Y subunits (A, B, and C) led to a concentration-dependent increase in NPR-A-driven reporter activity in Schneider cells.

 Cooperative interactions among transcription factors have been shown to be important for the regulation of a number of gene promoters. Indeed, simultaneous mutation of all of the Sp1 sites and the CCAAT motif led to a reduction in NPR-A promoter activity to background levels (Fig. 1B). To determine whether Sp1 cooperates with NF-Y at the NPR-A promoter, −387 NPR-A luciferase was cotransfected into Schneider cells along with Drosophila expression vectors encoding Sp1 and the three NF-Y subunits. Whereas expression of either Sp1 or NF-Y activated the NPR-A promoter to a moderate degree, simultaneous expression of both Sp1 and NF-Y together resulted in a dramatic increase in promoter activity (Fig. 5A). This increase exceeded that seen with either transcription factor alone, implying that Sp1 and NF-Y act cooperatively to drive NPR-A gene transcription.

Transcription factors may cooperate at a promoter by separately contacting different rate-limiting targets, by mutually contacting a single rate-limiting target or by interacting with each other to form a novel activity. We performed a GST pull-down assay to probe the potential interaction of Sp1 and NF-Y in vitro. As shown in Fig. 5B, [35S]methionine-labeled Sp1 was able to bind GST-NF-YA and NF-YC, but not GST alone or NF-YB, suggesting that Sp1 interacts physically with the NF-YA and NF-YC subunits. For the converse experiment, nuclear extracts from RASM cells were incubated with GST-Sp1 or GST alone, and the washed beads were electrophoresed, blotted onto filters, and then probed with anti-NF-YA antibody. This demonstrated that endogenous NF-YA in RASM cells also interacted with Sp1 (Fig. 5C). Finally, we conducted coimmunoprecipitation studies to show that Sp1 and NF-YA interact in the context of the intact cell. Immunoprecipitation of Sp1 from the same extracts followed by Western blot analysis for NF-YA revealed a low intensity but specific band, which migrated in a position the size of NF-YA (Fig. 5C). As a control, NF-YA was not coimmunoprecipitated when anti-Rel A antibody was used in place of anti-Sp1 antibody (Fig. 5C). Thus, NF-Y and Sp1 interact both in vitro and in the context of the intact cell. Finally, we asked whether this interaction could be demonstrated to have functional sequelae in the cell. To address this, we transfected NPR-A promoter-driven luciferase constructs, with mutations at either the Sp1 or NF-Y binding sites, into Drosophila Schneider cells, alone or in combination with Sp1 or NF-Y expression vectors. As shown in Fig. 5D, the CCAAT mutant promoter displayed a robust response to co-transfected Sp1 but no response to NF-Y. However, when Sp1 and NF-Y were cotransfected together, there was a small but statistically significant increment in promoter activity. We interpret this increment as reflective of the ability of Sp1 to recruit NF-Y into the regulatory complex through protein-protein interaction. Similar findings were observed with the triple Sp1 mutant promoter. Cotransfection with NF-YA effected a significant increase in promoter activity while Sp1 was completely ineffective. Once again, overexpression of NF-Y and Sp1 together led to an increment in promoter activity that exceeded that seen with either transcription factor alone.

**DISCUSSION**

In this study, we demonstrated that a CCAAT box in the proximal 5' flanking sequence of the NPR-A gene is essential for transcriptional activity. Mutation of the CCAAT motif spanning −142 to −138 relative to the transcriptional start site resulted in 90% reduction in NPR-A promoter activity. A number of nuclear transcription factors have been shown to bind to
the CCAAT box, including C/EBP, NF-Y, MSY-1, and CTF/ NF-1 (13–21). Only NF-Y requires all five base pairs for binding (7). UV cross-linking, EMSA competition, and immunoprecipitation studies demonstrated that the trans-acting factor NF-Y, but not C/EBP, bound to the CCAAT site in the proximal NPR-A promoter. Moreover, forced expression of a dominant negative mutant of NF-YA resulted in a dramatic reduction in NPR-A promoter activity. Together, these data suggest that NF-Y, when bound to the CCAAT element, plays a critical role in the regulation of NPR-A gene transcription.

NF-Y is a ubiquitous heterotrimeric transcription factor, also referred as CP1 or CBF, that consists of NF-YA, NF-YB, and NF-YC subunits with molecular mass values of 42, 36, and 40 kDa, respectively (24–27). NF-YB and NF-YC contain a conserved histone-fold motif, which forms a dimer that interacts with NF-YA (26, 27). All three NF-Y subunits are required for binding to the CCAAT motif (26, 27). Our cross-linking studies demonstrated that at least two complexes with molecular masses of 78 and 117 kDa specifically cross-linked to the CCAAT sequence of the NPR-A promoter. These are very close to the predicted molecular mass of NF-YB/NF-YC dimers and the heterotrimeric complex of all three NF-Y subunits, respec-
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Mantovani, R. Tjian, T. F. Osborne, K.-S. Chang, E. Wintersberger, B. de Crombrugghe, and S. N. Maity, who provided important reagents used in this study.

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Functional Interaction of NF-Y and Sp1 Is Required for Type A Natriuretic Peptide Receptor Gene Transcription
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