Cooperative Activation by GATA-4 and YY1 of the Cardiac B-type Natriuretic Peptide Promoter*

Suparna S. Bhalla‡§, Lynda Robitaille‡, and Mona Nemer‡§§

From the ‡Laboratoire de Développement et Differenciation Cardiaques, Institut de Recherches Cliniques de Montréal and §Département de Pharmacologie, Université de Montréal, 110 des Pins Ouest, Montréal QC, H2W 1R7, Canada and ¶Department of Medicine, Division of Experimental Medicine, McGill University, Montréal QC, H3A 1A3, Canada

YY1, a multifunctional protein essential for embryonic development, is a known repressor or activator of transcription. In cardiac and skeletal myocytes, YY1 has been described essentially as a negative regulator of muscle-specific genes. In this study, we report that YY1 is a transcriptional activator of the B-type natriuretic peptide (BNP) gene, which encodes one of the heart major secretory products. YY1 binds an element within the proximal cardiac BNP promoter, in close proximity to the high affinity binding sites for the zinc finger GATA proteins. We show that YY1 cooperates with GATA-4 to synergistically activate BNP transcription. Structure-function analysis revealed that the DNA binding domain of YY1 is sufficient for cooperative interaction with GATA-4, likely through corecruitment of the CREB-binding protein coactivator. The results suggest that YY1 and GATA factors are components of transcriptionally active complexes present in cardiac and other GATA-containing cells.

The B-type natriuretic peptide (BNP)1 is a peptide hormone that is synthesized and secreted from the heart and plays an important role in cardiovascular homeostasis (reviewed in Ref. 1). We have previously demonstrated that the proximal 114 bp of the rat BNP promoter are sufficient for maximal BNP expression in cardiomyocytes (1). Deletion analysis established three regulatory regions within this proximal promoter that are required for maximal transcriptional activity. Further analysis of one of these elements, which contains a binding site for the GATA family of zinc finger proteins, led to the isolation and characterization of transcription factor GATA-4 (1). A cardiac-restricted member of the GATA family of proteins, GATA-4, was since shown to be a key regulator of several cardiac genes (reviewed in Ref. 2) and an essential factor for cardiac gene expression (3–5). A 5′ deletion that removed the GATA motifs resulted in a 3- to 4-fold decrease in promoter activity; additionally, removal of a 20-bp element (−80 to −60 bp), which contains putative binding sites for YY1 and CACC box-binding proteins, led to a further 3-fold loss of BNP promoter activity in cardiomyocytes (1). We therefore undertook to examine the potential role of the YY1 protein in the regulation of BNP transcription.

YY1 is a 65-kDa multifunctional zinc finger DNA binding transcription factor, belonging to the human GLI-Krüppel family of nuclear proteins (6) and essential for mammalian embryonic development (7). Identified as an initiator binding protein (6, 8), it can either activate or inhibit transcription depending on the promoter context (9–12). A potent transcriptional repressor, YY1 is hypothesized to be associated with the repression of a variety of cellular and viral genes including c-fos (13), β-casein (14), α-globin (15), serum amyloid A1 (16), cytomegalovirus (17), and the mouse mammary leukemia virus (18). In addition to its role as a repressor, YY1 was shown to act as an activator of some promoters like the c-Myc (9, 10), the dihydrofolate reductase (11), the IgH intronic enhancer (19), the ribosomal gene promoters (20), and more recently, the myeloid-specific gp91 (21). YY1 binding sites have been identified within the regulatory regions of several cardiac and skeletal muscle genes, including muscle creatine kinase (22), α-skeletal actin, and cardiac actin (10, 23, 24). The finding that deletion of YY1 sites within these promoters results in a small, albeit reproducible gain in promoter activity, together with studies showing that YY1 competed with the action of serum response factor led to the suggestion that YY1 behaves as a repressor of cardiac genes (22, 25).

The mechanisms by which YY1 mediates its pleiotropic responses remain undefined although it is clear that the promoter context and the cellular environment, including coactivators and corepressors, influence YY1 action. The CAMP-response element-binding protein (CBP) belongs to a class of transcriptional cofactors that link upstream transactivators and the basal transcription machinery (26–28). In skeletal muscle, the CBP/p300 proteins act as transcriptional adapters for MyoD and MEF-2 in the regulation of myogenesis (27, 29–31). In the heart, CBP/p300 plays an important role in maintaining the terminally differentiated state of cardiomyocytes by both preventing reinitiation of DNA synthesis and activating subsets of cardiac-specific genes (32). Although the exact transcription factors with which CBP/p300 interact to regulate cardiac gene expression remain unclear, activity of the GATA proteins is likely to be modulated by these coactivators given that CBP cooperates with GATA-1 (33) and p300 was recently shown to act as a coactivator for GATA-5 in the regulation of the cardiac atrial natriuretic factor gene (34).

In the present work, we demonstrate that YY1 functions as a transcriptional activator of the cardiac BNP promoter and we provide evidence that YY1 cooperates with GATA-4 to enhance BNP transcription. This synergy requires GATA but not YY1 DNA binding sites and is mediated via CBP that likely serves as a bridge between the two factors allowing the formation of a
Fig. 1. A, structural organization of the proximal BNP promoter. Regulatory elements in the proximal BNP promoter are highlighted, and their position relative to the start site is indicated. rBNP, rat BNP. B, mapping regulatory domains on the BNP proximal promoter. Primary atrial and ventricular cardiomyocyte cultures obtained from 4-day-old rats were transiently transfected with BNP-luciferase constructs containing the indicated BNP promoter. The results are expressed relative to BNP-114 and represent the mean of three independent experiments carried out in duplicate. The Rous sarcoma virus-human growth hormone plasmid was used as an internal control.

Fig. 2. Identification of sequence-specific DNA-binding proteins that interact with the proximal BNP element. A, three different radiolabeled 20-bp oligonucleotides corresponding to the sequence between −80 and −60 bp (WT BNP) and others that contain mutations within the putative YY1 site or the CACC site were incubated with HeLa cell nuclear extracts as described under “Experimental Procedures.” The sequence-specific binding species obtained were competed with a 100-fold molar excess of cold competitor oligonucleotide that corresponds to a consensus YY1 site found in the adenovirus associated P+5 (adeno-YY1) promoter. B, supershift analysis using anti-YY1 or anti-SP1 on the wild type BNP probe revealed that the complex with the slowest mobility contained an SP1-like species. The two complexes with faster mobility correspond to YY1 as they are completely abrogated in the presence of YY1 antibody. C, the oligonucleotide containing the adenovirus consensus YY1 site was used as probe. Incubation with HeLa nuclear extracts revealed the presence of two sequence-specific complexes that were similar in mobility to those obtained on the BNP wild type probe and correspond to YY1 as confirmed by incubation with the YY1 antibody. Note that the SP1 antibody had no effect on YY1 binding. D, predominance of the fastest mobility YY1 complex in cardiac extracts prepared from neonate ventricular cardiomyocyte cultures. A similar pattern is observed in neonate atrial cardiomyocytes and in adult heart extracts. Also note the absence of SP1 binding in cardiac extracts.
transcriptionally competent complex. These findings suggest that cardiac-specific genes are controlled by complex transcriptional pathways involving combinatorial interactions between cell-restricted (GATA-4) and ubiquitous factors (YY1 and CBP); such multicomponent complexes likely provide additional levels of gene expression control.

EXPERIMENTAL PROCEDURES

Plasmids—Rat BNP luciferase constructs were obtained by cloning appropriate promoter fragments generated using restriction enzymes or PCR into PXP2, as previously described (1). Point mutations in the proximal BNP promoter were generated by PCR, and constructs were confirmed by sequencing. The expression vectors for the intact and mutated YY1 were generous gifts of Dr. Kenneth Walsh and Dr. Robert Schwartz and have been previously described (10, 13). The GATA expression vectors used have been described previously (35, 36). The expression vector for the human CBP was a gift from Dr. Robert Rehfeld (McGill University, Montréal, CA).

Cell Cultures and Transfections—HeLa or CV1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Primary cardiocyte cultures were prepared from 14-day-old Sprague-Dawley rats and kept in synthetic medium as previously described (37). 24 h after plating, cells were transfected using the calcium phosphate technique. Rous sarcoma virus-humanized growth hormone was added (2 μg per dish) to normalize for transfection efficiencies. The total amount of DNA was kept constant (maximum 10 μg per 35-mm dish) by the addition of PC86 or pBluescript DNA. Cells were harvested, and the medium was collected 36 h after transfection. Luciferase activity was measured with an Amersham Pharmacia Biotech luminometer, and the human growth hormone was measured in the cell media by radioimmunoassay as previously described (38). The results reported were obtained from at least four independent experiments, each carried out in duplicate using two different DNA preparations.

Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from cells according to Schreiber et al. (39). Extracts from HeLa cells overexpressing YY1 were prepared from 200 mm dishes transfected with 10 μg of expression vectors. The probe for YY1 binding, GGTCTCCATTTTGAAGCG, corresponds to the adenovirus-associated virus 5 +1 site and has been previously described (6). The probes used from the BNP promoter correspond to sequences from −84 to −60 bp as follows: wild type, TCAGAGATCTCCACCCACTTTAGAAGC; YY1m, TCAGAGATCTCCACCCACTTTAGAAGC; CACCm, TCAGAGATCTCCACCCACTTTAGAAGC.

Binding reactions were performed at room temperature for 20 min in a 20-μl reaction mixture containing 3–5 μg of nuclear extracts, 10 mM Tris-HCl, pH 7.9, 60 mM KCl, 5 mM MgCl2, 1 mM poly dI-dC oligonucleotides, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. Unlabeled double-stranded oligonucleotides were added as competitors at 100-fold molar excess when required. Binding reactions were analyzed on a 5% Tris borate-EDTA gel. The DNA-protein complexes were resolved on a 5% polyacrylamide nondenaturing gel in 0.5 M glycerol. Gel migration properties, this complex corresponds to the slower migrating YY1 complex observed in HeLa cells. Based on YY1 antibody recognition and gel migration properties, this complex was identified as YY1; both complexes are specifically competed with a consensus YY1 site from the adenovirus-associated P5 promoter and blocked by a YY1 antibody (Fig. 2A). Binding of YY1 to its cognate juxtaposed sites occurs independently, because mutation of the YY1 site (YY1m) does not affect YY1 binding. Similarly, mutation of the SP1 site, which abrogates SP1 binding, does not qualitatively or quantitatively alter the YY1 complexes (Fig. 2A).

RESULTS

YY1 Binds to and Transactivates the BNP Promoter—Deletion analysis of the proximal rat BNP promoter indicated that sequences between −80 and −60 bp contribute significantly to BNP promoter activity. Their deletion decreases promoter activity by 3- to 4-fold in atrial primary cardiocyte cultures (Fig. 1A). The −80- to −60-bp region harbors two potential transcription factor binding sites, a CCACC box, which can interact with two classes of zinc finger proteins, SP1, and members of the EKLF-Krüppel family (40–42), and adjacent to it, a CCAT core, which is a putative binding site for the YY1 zinc finger protein (43, 44). Gel shift analyses showed that both SP1 and YY1 interact with the BNP sequences (Fig. 2) as evidenced by competition analysis and antibody recognition. Incubation of a 20-bp oligonucleotide corresponding to the −80- to −60-bp region with HeLa cell nuclear extracts resulted in the formation of three major sequence-specific DNA binding complexes (Fig. 2A). The complex with the slowest mobility was identified as an SP1-like protein based on competition with an unlabeled high affinity SP1 site and its interaction with the SP1 antibody (Fig. 2 and data not shown). The two faster migrating complexes were identified as YY1; both complexes are specifically competed with a consensus YY1 site from the adenovirus-associated P5 promoter and blocked by a YY1 antibody (Fig. 2B). In addition, they show similar mobility as complexes obtained on the well studied adenovirus YY1 binding site (Fig. 2C). Binding of SP1 and YY1 to their cognate juxtaposed sites occurs independently, because mutation of the YY1 site (YY1m) does not affect SP1 binding. Similarly, mutation of the SP1 site, which abrogates SP1 binding, does not qualitatively or quantitatively alter the YY1 complexes (Fig. 2A). YY1 is expressed in cardiomyocytes (22, 24, 45), and it has been shown to negatively regulate several cardiac promoters including α-skeletal actin (10, 24), cardiac actin (23), and muscle creatine kinase (22). SP1 levels are very low in postnatal cardiomyocytes but appear to increase following trophic growth stimulation (46). When gel shift analyses were carried out using cardiacomyocyte nuclear extracts, the predominant complex observed over the BNP probe corresponded to YY1 (Fig. 2D). Moreover, the majority of YY1 binding in cardiomyocytes corresponded to the faster migrating YY1 complex observed in HeLa cells. Based on YY1 antibody recognition and gel migration properties, this complex likely represents an alternatively spliced YY1 form containing essentially the DNA binding zinc finger region.

The functional consequences of YY1 binding to the BNP promoter were tested. Transient cotransfection analysis revealed that full-length YY1 is a potent transactivator of the BNP promoter (Fig. 3). Other related zinc fingers such as Egr-1.
and the KRAB domain containing protein ZNF74 (47) had no effect on BNP promoter activity (data not shown). YY1 transactivation of the BNP promoter required the proximal YY1 binding site, because 5' or internal deletions, as well as a point mutant that removes the YY1 site, significantly reduced transactivation (Fig. 3). Other potential YY1 binding sites are present further upstream (−209, −220, −374, and −636), which might explain why the −2.2-kilobase pair BNP construct is maximally induced by YY1.

To delineate the regions of YY1 required for transactivation, the effects of several deletion mutants of YY1 on the BNP promoter were assayed (Fig. 4). The results indicate that an intact zinc finger DNA binding is essential but not sufficient for maximal transactivation (Fig. 4B, DM4 and DBD mutants, respectively). Surprisingly, deletions of the putative N-terminal activation domains of YY1 did not completely abolish transactivation as these mutants retained the ability to consistently induce a 2-fold increase in promoter activity. These observations are consistent with studies indicating that, although the N-terminal region contains an autonomous activation domain (amino acids 70–143) (48), it is often dispensable for activation by YY1. For example, activation of the c-Myc promoter by YY1 is not affected by deletion of amino acids 89–220 (DM3) (10), and activation of a heterologous promoter construct containing the YY1 initiator element of the adeno-associated P5 promoter was maximal when amino acids 154–198 or 69–85 were deleted (49). Together, these data suggest that a YY1 protein containing essentially the zinc finger region, as found in cardiomyocytes, can activate target promoters possibly through recruitment or interaction with coactivators.

### YY1 Cooperates with GATA Transcription Factors to Activate the BNP Promoter

The BNP promoter is a target for both GATA-4 and YY1, and the YY1 site at −70 is flanked by functional GATA elements centered at −90 and −30 (1). We tested whether the two factors functionally cooperate. Cotransfection of YY1 with limiting amounts of YY1 on the BNP promoter were assayed (Fig. 4A). The results indicate that an intact zinc finger DNA binding is essential but not sufficient for maximal transactivation (Fig. 3). Other potential YY1 binding sites are present further upstream (−209, −220, −374, and −636), which might explain why the −2.2-kilobase pair BNP construct is maximally induced by YY1.

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specialized TATA box) cannot support YY1 cooperation.

Next, we tested whether YY1 binding was required for synergy. For this, we generated the YY1 mutant in the context of the BNP \(^2\) promoter. As shown in Fig. 5B, this construct was no longer activated by YY1 but retained GATA-4 inducibility. Remarkably, the YY1 mutation did not alter the ability of YY1 to cooperate with GATA-4 and activate the BNP promoter to nearly maximal levels (Fig. 5B). Thus the binding of GATA-4 to DNA appears to be sufficient to recruit YY1 into a transcriptionally productive complex.

Next, we tested whether a truncated YY1 protein can functionally synergize with GATA-4, and we performed a structure-function analysis to determine the GATA-4 domains required for YY1 interaction. Wild type and mutant GATA-4 were epitope-tagged as described under “Experimental Procedures” and were produced in HeLa or CV1 cells at similar levels as evidenced by Western blotting with the hemagglutinin antibody. Ctl are control extracts prepared from cells transfected with the empty vector. D, gel shift analysis using nuclear extracts prepared from HeLa cells transfected with empty vector (Control), wild type YY1, or a YY1 deletion containing only the DNA binding zinc finger domain (shown in Fig. 4A). Note that both protein forms are expressed at similar levels.

FIG. 6. Domains of GATA-4 required for activation and synergy. A, 6 \(\mu\)g of the −114-bp BNP-luciferase construct was cotransfected with wild type or mutant GATA-4 (1 \(\mu\)g/dish) expression vectors in the absence or presence of the cmv-YY1 vector. The results are expressed as fold-activation of the BNP promoter relative to the control antisense GATA-4 vector and represent a mean of three independent experiments each carried out in duplicate. B, the same BNP-luciferase construct was cotransfected with the various YY1 expression vectors (1 \(\mu\)g/dish) in the presence or absence of the GATA-4 vector. The results shown in A and B are the mean of two independent experiments each carried out in duplicate. Note that removal of the GATA-4 C-terminal activation domain eliminates synergy and that the DNA binding domain of YY1 is sufficient for synergy. C, schematic representation of the GATA-4 constructs used. The two black squares represent the two zinc fingers. All GATA-4 proteins were epitope-tagged as described under “Experimental Procedures” and were produced in HeLa or CV1 cells at similar levels as evidenced by Western blotting with the hemagglutinin antibody. Ctl are control extracts prepared from cells transfected with the empty vector. D, gel shift analysis using nuclear extracts prepared from HeLa cells transfected with empty vector (Control), wild type YY1, or a YY1 deletion containing only the DNA binding zinc finger domain (shown in Fig. 4A). Note that both protein forms are expressed at similar levels. CBP Potentiates Synergy between YY1 and GATA-4 on the BNP Promoter—To determine whether the synergy between GATA-4 and YY1 reflects physical interaction between the two proteins, we performed a series of coimmunoprecipitation experiments using \(\text{in vitro}\)-translated YY1 and GATA-4 proteins. No detectable interaction between GATA-4 and YY1 could be observed, although in the same experiments, we were able to detect other documented interactions (2) including that between GATA-4 and GATA-6 proteins (data not shown). These results suggest that either the interaction between the two proteins was not stable under these conditions or that it was mediated via a third protein.

Both YY1 and members of the GATA family interact with several other nuclear proteins (35, 50–53) including some coactivators (33). For example, it was recently demonstrated that

slightly but consistently enhanced the synergy with YY1 (Fig. 6C). Consistent with this, the DNA binding domain of GATA-4 was not sufficient for YY1 synergy. In contrast, the YY1 DNA binding domain is sufficient for maximal synergy with GATA-4, and the YY1 N terminus is dispensable in the presence of GATA-4 (Fig. 6D). Thus, in presence of GATA-4, the endogenous cardiac YY1 protein would be a potent transcriptional activator.
of one or more component of the complex. In the absence of complex assembly, YY1 may still activate the BNP promoter through sequence-specific binding to its site and via its own activation domains. Productive interaction among GATA factors, YY1, and CBP may be only observed under stimulated conditions such as stress or growth response or in a cell-specific manner, e.g., in cardiac but not brain cells, both of which express the BNP gene.

Finally, it is noteworthy that both GATA-4 and YY1 have been linked to growth responses. The role of YY1 in cell growth was confirmed by inactivation of the YY1 gene in mice (7) as discussed earlier. Furthermore, YY1 has been implicated in mediating interleukin-1β-induced hypertrophy in cardiomyocytes (58). More recently, YY1 has also been implicated in endoplasmic reticulum stress-induced transcription (59); whether in cardiomyocytes YY1 is involved with GATA-4 in mediating transcriptional regulation in response to stress or growth stimulation deserves to be investigated. On the other hand, GATA-4 has been implicated in the activation of several genes during hypertrophy, including the angiotensin II type 1a receptor and the α-myosin heavy chain genes in response to pressure overload hypertrophy in rats (32, 60, 61). The role of GATA-4 in the hypertrophic response may reside, in part, in GATA-4 association with other factors including NFAT-3 (62) or the MADS protein MeF2 and serum response factor (50, 63).

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Suparna S. Bhalla, Lynda Robitaille and Mona Nemer

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