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A Hormone-Encoding Gene Identifies a Pathway for Cardiac but Not Skeletal Muscle Gene Transcription

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In contrast to skeletal muscle, the mechanisms responsible for activation and maintenance of tissue-specific transcription in cardiac muscle remain poorly understood. A family of hormone-encoding genes is expressed in a highly specific manner in cardiac but not skeletal myocytes. This includes the A- and B-type natriuretic peptide (ANP and BNP) genes, which encode peptide hormones with crucial roles in the regulation of blood volume and pressure. Since these genes are markers of cardiac cells, we have used them to probe the mechanisms for cardiac muscle-specific transcription. Cloning and functional analysis of the rat BNP upstream sequences revealed unexpected structural resemblance to erythroid but not to muscle-specific promoters and enhancers, including a requirement for regulatory elements containing GATA motifs. A cDNA clone corresponding to a member of the GATA family of transcription factors was isolated from a cardiomyocyte cDNA library. Transcription of this GATA gene is restricted mostly to the heart and is undetectable in skeletal muscle. Within the heart, GATA transcripts are localized in ANP- and BNP-expressing myocytes, and forced expression of the GATA protein in heterologous cells markedly activates transcription from the natural cardiac muscle-specific ANP and BNP promoters. This GATA-dependent pathway defines the first mechanism for cardiac muscle-specific transcription. Moreover, the present findings reveal striking similarities between the mechanisms controlling gene expression in hematopoietic and cardiac cells and may have important implications for studies of cardiogenesis.

The discovery of the MyoD family of myogenic factors has resulted in great advances in the understanding of the mechanisms of skeletal muscle commitment and differentiation (reviewed in references 48 and 68). In contrast, the mechanisms controlling cardiac determination and differentiation remain essentially unknown (49). So far, no MyoD-like factors have been detected in cardiac muscle (54), and mice homozygous for inactivated MyoD (53), Myf-5 (13), or myogenin (27) loci have a normal cardiac phenotype. These observations suggest that tissue-specific transcription and cell differentiation are controlled by distinct regulatory pathways in the two striated muscles. This would be consistent with the fact that skeletal and cardiac myocytes have distinct spatial and temporal origins in the developing embryo, although they both arise from mesoderm.

Skeletal muscle cells originate from the somites of the dorsal (paraxial) mesoderm, whereas cardiac muscle cells are derived from the splanchnic mesenchyme of the anterior lateral plate mesoderm (10, 32). Commitment of mesodermal cells to the cardiac lineage occurs very early, when cells migrate to form the cardiogenic area at the beginning of the third week (days 16 to 18) of human embryonic development (or at 18 to 20 h in chicken embryogenesis [35]). By the end of the third week (days 21 to 22), the tubular—or primitive—heart is formed and joined by blood vessels. Thus, the heart is functional (blood is circulating and the heart is beating) well before commitment of somites to the skeletal lineage, which does not take place until the end of the fourth week (days 26 to 28) of human development. In addition, the two striated muscles develop differently, since skeletal muscle develops by fusion of undifferentiated myoblasts to form multinucleated muscle cells, whereas cardiac muscle arises after proliferation of single differentiated cells, which lose their proliferative potential soon after birth (36). Thus, the growth and differentiation of cardiac and skeletal muscles are very different throughout embryonic development and in postnatal life.

Despite these important differences, cardiac and skeletal muscles express a common subset of contractile proteins which result in similar mechanical and electrophysiological properties between the two tissues (11, 64). This fact has led many investigators to postulate the existence of similar mechanisms of gene expression in heart and skeletal muscles. Consistent with this hypothesis, studies from several laboratories revealed that transcription of many of these muscle-specific genes in heart and skeletal muscle requires similar AT-rich regulatory elements termed MEF-2 (myocyte-specific enhancer-binding factor 2) (1, 26, 41, 42). A family of transcription factors which interact with this element and are present in mesodermal tissues, including the heart, have recently been cloned (52, 73). However, these proteins cannot account for cardiac muscle-specific transcription, and pathways specific to cardiac but not skeletal muscle must exist to account for restricted expression of cardiac muscle-specific genes and functional differentiation of the heart. Indeed, the heart is an important endocrine organ with a crucial role in regulation of blood flow and cardiovascular homeostasis. This functional differentiation is the result of tissue-specific expression in cardiac but not in skeletal myocytes of a family of biologically active peptide hormones

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TABLE 1. GATA oligonucleotides used in gel shift assays

Oligonucleotide	Sequence[a]	Reference
rBNP −90	GATCCCAGGAATGTGTCTGATAAATCAGAGATAACCCA	This study
rBNPmut −90	GATCCCAGGAATGTGTCTGGTAAATCAGAGGTAACCCA	This study
rBNP −30	GATCCGACCCCAGATAAAAGGCAG	This study
Mouse α-globin	GACCGGGCAACTGATAAGGATTCCCA	51
Chicken β-globin mutant	GATCCCGGCGGAGGCGAGGAAAGTGGGGACACAG	25
Human T-cell receptor δ	TCGACACTTGATAACAGAAAGTGATAACTCT	33
Human endothelin 1	GATCTGGCCTTATCTCCGG	69

[a] GATA motifs in the sense and antisense orientations are overlined and underlined, respectively.

with natriuretic, diuretic, and vasorelaxant properties (19, 43, 57). These cardiac hormones have been conserved throughout evolution, as they are present in the heart of virtually all species examined (14), ranging from the most primitive heart in the animal kingdom (65) to fishes (20), reptiles (15), amphibians (46, 59), birds (62), and mammals (19).

Since cellular differentiation is largely a consequence of tissue-specific gene expression, we have used the cardiac B-type natriuretic peptide (BNP) gene as a probe to elucidate the mechanisms underlying differentiation of the heart. In this report, we show that the mechanisms of BNP gene transcription are highly similar to the mechanisms underlying erythroid, particularly globin, gene transcription. This is evident from the structural organization of the BNP promoter and the presence of a cardiac muscle-specific GATA factor which is sufficient to activate fully the natural BNP promoter in heterologous cells. The involvement of a tissue-restricted member of the GATA family of transcription factors in cardiac but not skeletal muscle transcription identifies the first pathway for cardiac muscle-specific gene expression. Moreover, given the important role of GATA proteins in differentiation of the erythroid lineage, the present findings suggest closely related pathways for cardiac and hematopoietic cell differentiation.

MATERIALS AND METHODS

Plasmids. Rat BNP (rBNP)-luciferase plasmids containing various rBNP promoter fragments were obtained by subcloning appropriate 5′ deletions of the BNP promoter (generated by restriction or by PCR) in PXP-2 (38). The heterologous rBNP plasmids were obtained by inserting the 2.1-kbp *Hin*dIII-*Sst*I BNP fragment into the *Hin*dIII and *Sst*I sites upstream of the bp −109 or bp −81 herpes simplex virus thymidine kinase (TK) promoter. All deletions were confirmed by sequencing. Rous sarcoma virus (RSV)-luciferase and rat atrial natriuretic peptide (rANP)-luciferase plasmids were described previously (38). The expression vectors used for human GATA-1 and GATA-3 were kindly provided by P.-H. Roméo and are described by Joulin et al. (30). The mouse GATA-1 vector was a gift of B. Emerson. Rat GATA-4 (rGATA-4) expression vectors were prepared by subcloning an *Xba*I fragment containing the entire GATA-4 coding region in the *Xba*I site of pCG, a cytomegalovirus-driven eukaryotic expression vector generously provided by W. Herr (60).

Cell cultures and transfections. Primary cardiocyte cultures were prepared from 1- to 4-day-old Sprague-Dawley rats and kept in serum-free medium as described previously (38). L, L_6, and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. DNA was transfected by the calcium phosphate precipitation technique 24 h after plating. RSV-human growth hormone (hGH) (2 μg per dish) was included as an internal control to normalize transfection efficiencies. The amount of reporter plasmid was kept at 3 μg per dish, and the total amount of DNA used (maximum, 12 μg per dish) was kept constant in all dishes by addition of plasmid SP64 or pCG. Cells were harvested, and the medium was collected 36 h after transfection. Luciferase activity was assayed with an LKB luminometer, and hGH in the medium was measured by radioimmunoassay as described previously (38). The results reported were obtained from several independent experiments, each carried out in duplicate.

In vitro DNA-binding assays. Nuclear extracts were prepared from cells by the procedure of Schreiber et al. (55). Since L cells were devoid of GATA activity, nuclear extracts overexpressing cloned rGATA-4 were prepared from 400,000 L cells transfected with 80 μg of the control pCG or rGATA-4 antisense or sense expression vector. The sequences of the double-stranded oligonucleotides used as probes or as unlabeled competitors are shown in Table 1. Binding reactions were performed in 20-μl reaction mixtures containing 2 to 4 μg of nuclear extracts in a buffer containing 4 mM Tris-HCl (pH 7.9), 24 mM KCl, 0.4 mM EDTA, 0.4 mM dithiothreitol, 5 mM $MgCl_2$, 1.6% Ficoll, and, when appropriate, various molar excesses of unlabeled double-stranded oligonucleotides. Binding was allowed to proceed for 15 min at 4°C. The reaction mixes were then analyzed by electrophoresis on 4% polyacrylamide gel in 0.5× TBE (Tris-borate-EDTA buffer) at 250 V for 3 h at 4°C.

RNA extraction and reverse transcription-PCR. Total cellular RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method (16). Northern (RNA) blots were made by the glyoxal method as described previously (9). After transfer, the Nytran membranes were prehybridized and hybridized with random-primed ^{32}P-labeled rGATA-4 DNA probes. Blots were washed for 15 min at 65°C in 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After autoradiography, the membranes were stripped and reprobed with the rBNP or rat β-actin probe as described previously (18). For PCR amplification of GATA transcripts, oligonucleotides 29-17 (amino acids 199 to 207) and 26-23 (amino acids 435 to 442) were used on cDNAs made from various rat tissues and cultured cells as described by Dagnino et al. (17). PCR products were separated by agarose gel electrophoresis, and Southern blots were probed with radiolabeled internal oligodeoxynucleotides corresponding to amino acids 206 to 214 of the sequence. The same cDNA samples were subjected to BNP and tubulin amplification with the sense and antisense oligonucleotides described previously (18).

Library screening and DNA sequence determination. A directional cDNA library (superscript kit from Bethesda Research Laboratories) was prepared with mRNA from primary cardiocyte cultures derived from total neonate (1 to 3 days old)

rat hearts. As described previously (9), these cultures are enriched (over 90%) with cardiac myocytes; contamination with endothelial cells is minimal, as evidenced by the failure to detect factor VIII immunoreactivity by immunocytochemistry. The cDNAs were size fractionated by column chromatography, and the high-molecular-weight fraction was cloned into λgt22 phage. All subsequent manipulations were done by standard protocols (37). This cDNA library contained 4×10^6 independent clones, and the average insert size was about 2,000 bp. A total of 2×10^6 recombinant clones were screened at low stringency (2× SSC–0.1% SDS at 50°C). The probe used was the *Eco*RI fragment of mouse GATA-1 (63), which contains the zinc finger region (donated by B. Emerson). Positive cDNA clones were subcloned in pKS plasmids for sequencing and further manipulation.

For rapid amplification of cDNA ends (RACE)-PCR, 5 μg of total RNA extracted from ventricular cells of 3-day-old rats were reverse transcribed with 20 U of avian myeloblastosis virus reverse transcriptase. The sequence of the specific antisense primer was GGCTGGCCGAGAGCAG. The cDNA equivalent of 1 μg of total RNA was polyadenylated and amplified further. PCR products were subcloned in the *Eco*RV site of pKS, and colonies were analyzed by filter hybridization with the ^{32}P-labeled oligonucleotide 29-20 (CCTCTAGAGC CCGGGAAGCTGCAGCCT). A positive clone containing a 470-bp insert was fully sequenced and contained the N-terminal extension of the GATA-4 cDNA, including the initiator methionine. The full-length cDNA clones were reconstituted by inserting the *Not*I fragment obtained from RACE-PCR into the unique *Not*I site of the partial clone. DNA sequence analysis was performed by the dideoxy chain termination method.

Genomic Southern blots. Genomic DNA was extracted from the tails of adult rats by standard procedures. DNA (10 μg) was digested with appropriate restriction enzymes, separated by agarose gel electrophoresis, and subjected to Southern blot hybridization with the partial rGATA-4 clone insert as the probe. Hybridization and washing were carried out at high stringency (65°C for 15 min in 0.1× SSC–0.1% SDS).

In situ hybridization and immunocytochemistry. Atrial and ventricular cardiocytes maintained in monolayer culture as described above were fixed in situ for 30 min in 4% paraformaldehyde (buffered in 0.1 M phosphate buffer [pH 7.5]). After being washed in the same buffer, the cells adhering to the culture dishes were processed for immunocytochemistry (3) or for in situ hybridization (4). In some experiments, immunocytochemistry was followed by in situ hybridization on the same cultured dish or tissue section. Immunocytochemistry and in situ hybridization were also carried out on tissue sections from whole hearts. In this case, adult male Sprague-Dawley rats (Charles River) weighing 250 to 300 g were used. Rats were perfused through the aorta with buffered 4% paraformaldehyde (see above). Tissues were subsequently excised, further fixed in the same solution for up to 4 h, and then conventionally dehydrated and embedded in paraffin. Histological sections (3 to 4 μm) were cut and processed for in situ hybridization with ^{35}S-labeled cRNA probes as described previously (45). Monospecific polyclonal antibodies against rANP and rBNP were purchased from Peninsula Laboratories.

RESULTS

Proximal region of rBNP promoter required for cardiac gene expression. Previous work indicated that expression of the BNP gene is largely restricted to cardiac cells (17), suggesting that the BNP gene would be an ideal marker with which to probe the mechanisms of transcriptional activation in the heart. In order to determine whether regulatory elements necessary for targeting transcription to the heart were contained in the 5′-flanking region of the rBNP gene, a genomic fragment containing sequences between kbp −2.2 and bp +75 was fused to the luciferase reporter gene and transiently transfected into cardiac and noncardiac cells. The rBNP reporter construct containing 2.2 kbp upstream of the transcription initiation start site is expressed at a very high level (activity similar to that of the RSV-luciferase construct) in cardiac cells, whereas this construct is significantly less active in other cell lines (Fig. 1A). These results indicate that the 2.2-kbp BNP promoter fragment displays cardiac muscle-specific activity in cultured cells.

Sequence analysis of this fragment revealed no sequence homology to previously characterized skeletal or cardiac muscle promoters; in particular, regulatory elements that have been implicated in the transcription of some muscle genes in the heart, such as the MEF-2 (26) and CArG/serum response element (40) motifs, were not found within the 2.2 kbp of BNP upstream sequences. Deletion of sequences between kbp −2.2 and bp −114 did not affect the high-level activity of the BNP-luciferase vectors in cardiac cells (Fig. 1B), suggesting that the determinant for cardiac specificity resides within the first 114 bp.

Replacing the 114-bp proximal BNP promoter with the homologous region of the herpes simplex virus TK promoter resulted in dramatic loss of transcriptional activity in cardiocytes but had little or no effect on BNP promoter activity in non-BNP-expressing cells, such as HeLa, L fibroblasts, and L6 skeletal myoblasts (Fig. 1B). These data confirmed that the proximal promoter region contains elements necessary for high-level transcription in the heart. Sequence analysis of this region (Fig. 2A) revealed a structural organization reminiscent of erythroid cell-specific promoters (23, 24, 67). It contains a specialized TATA box at bp −30 (a GATA motif), a CACC box at bp −70, two repeated GATA motifs at bp −90, and an AP-1-like sequence at about bp −110. The sequence and position of the GATA and AP-1-like motifs are highly conserved in the rat, dog, and human BNP genes (Fig. 2B).

In order to define the roles of those elements, nested 5′ deletions were generated and tested in cardiocytes by transient transfection (Fig. 3A). Deletion of the AP-1-like motif decreased promoter activity fourfold, and deletion of the distal GATA motifs reduced promoter activity another fourfold. The contribution of the GATA box at bp −90 to the cardiac muscle-specific activity of the BNP promoter was further confirmed by the fact that a double point mutation that destroyed both GATA motifs (rBNPmut −90, Table 1) eliminated binding of GATA proteins to the site (Fig. 4B). Compared with the wild-type promoter, this mutation resulted in a 10-fold decrease in BNP promoter activity in differentiated cardiocytes and, paradoxically, in a 3- to 8-fold increase in BNP promoter activity in HeLa cells (data not shown). Deletion and linker-scanning mutations of the CACC box had a small but consistent stimulatory effect on promoter function (Fig. 3A and data not shown). These results indicate that, similar to their activity in erythroid cell transcription, GATA motifs are positive regulators of cardiac cell transcription. In agreement with this, a 38-bp oligonucleotide containing the distal bp −90 GATA motifs displayed transcriptional activity when inserted upstream of the minimal BNP −60 or TK −80 promoter in cardiac myocytes but not in fibroblasts or skeletal myoblasts (Fig. 3B).

Cardiac myocytes contain GATA-binding proteins. Electro-

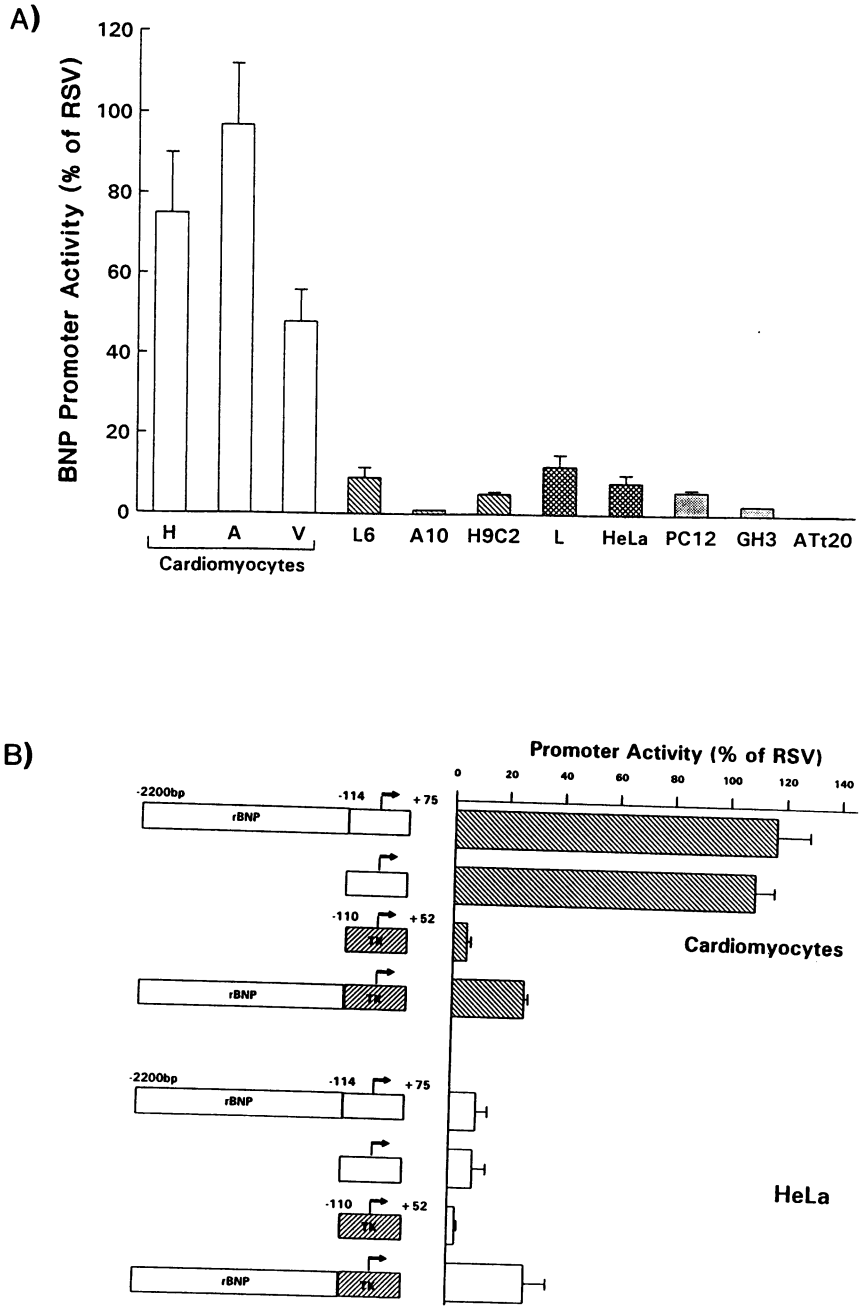


FIG. 1. (A) Transfection of rBNP-luciferase vectors in primary cardiocyte cultures derived from total heart (H) or dissected atria (A) or ventricles (V) and in various cell lines which do not express the BNP gene. The RSV-hGH construct was included in all transfections as an internal control to correct for transfection efficiencies. The results are expressed relative to the activity of an external RSV-luciferase construct standard and represent the mean ± standard deviation (SD) for at least six independent determinations. PC12 is a rat adrenal cell line, GH3 and ATt20 are pituitary cell lines, L6 cells are rat skeletal myoblasts, A10 is a rat smooth muscle cell line, H9C2 is derived from a rhabdomyosarcoma, L is a mouse skin fibroblast cell line, and HeLa is a human epithelial cell line. The results were identical when the 2.2-kbp BNP fragment was fused to the hGH reporter gene (not shown). (B) The BNP proximal promoter region is necessary for cardiac activity. The constructs used are described in Materials and Methods. Transfections were carried out as described for panel A. The data are the means ± SD for 6 to 12 independent determinations with at least two different plasmid preparations for each construct. The activity of the BNP-TK construct was also tested in L6 cells, in which it was identical to that of the native BNP promoter, and in L cells, in which replacement of the BNP proximal region with the TK fragment reduced transcriptional activity threefold (not shown). Similar results were obtained in all cells tested whether the proximal BNP region was replaced by TK −81 or TK −109 (not shown). Activity is expressed as a percentage of that obtained with the RSV-luciferase construct.

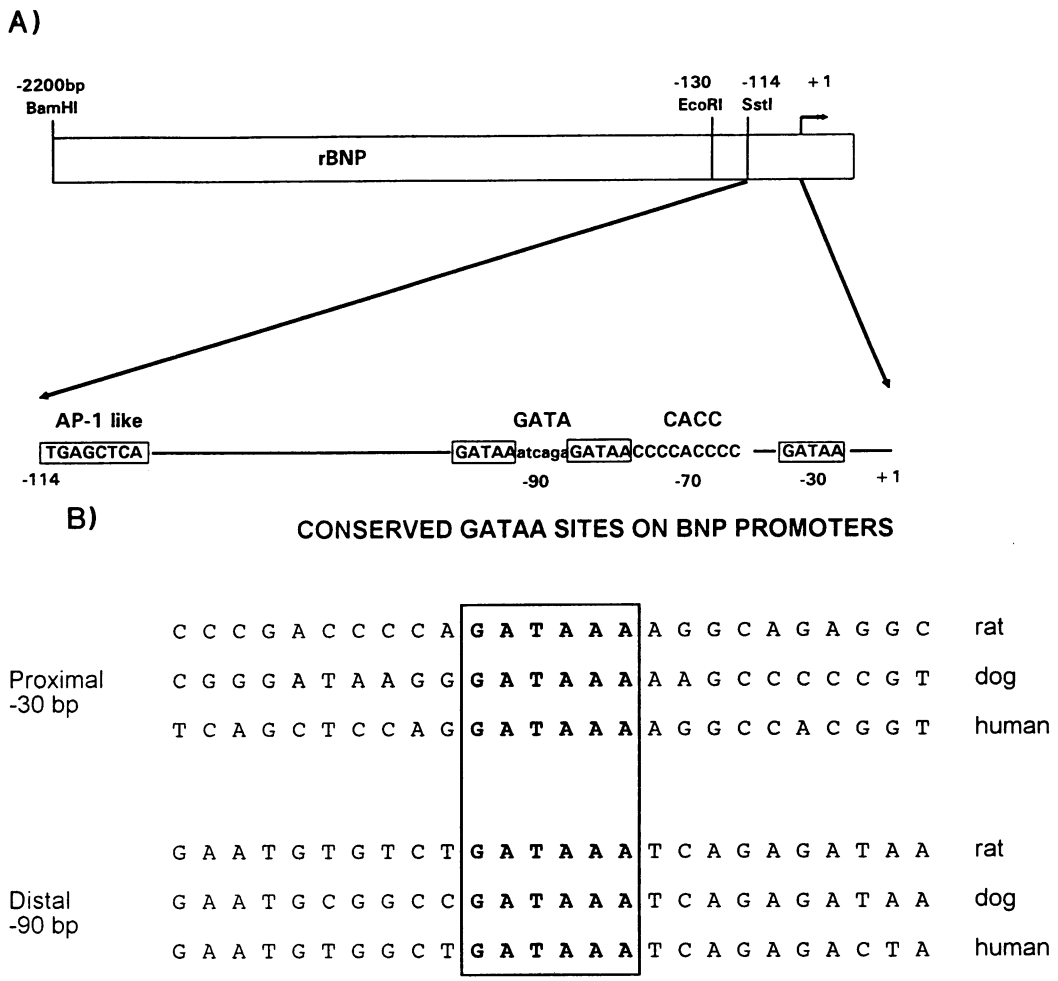


FIG. 2. (A) Structural organization of the rBNP promoter. A 4-kbp genomic fragment isolated from a male buffalo rat liver genomic library was subcloned in a Bluescript plasmid (Stratagene) and sequenced by the dideoxy chain termination method. The sequence of the rBNP gene, including the upstream flanking region, has been deposited in the GenBank/EMBL data bank (accession number M60266). The position of the transcription start site is based on primer extension data in Dagnino et al. (17). (B) Conserved GATA motifs in BNP promoters from other species. The GATA motifs and the AP-1-like sequence are the only conserved motifs within the first 120 bp of 5'-flanking sequences.

phoretic mobility shift assays (EMSAs) were used to analyze the DNA-binding activities which interact with the GATA motifs of the rBNP promoter. The 33-bp double-stranded oligonucleotide containing the bp −90 BNP GATA sites (Table 1) did not form any specific complexes with nuclear extracts from L cells (Fig. 4A) or L6 cells (data not shown). However, a specific complex was obtained with nuclear extracts from either ventricular or atrial cardiocytes (Fig. 4A); this complex had the same electrophoretic mobility as the faint but specific complex seen with GATA-2-containing HeLa nuclear extracts (Fig. 4A) and GATA-1-containing K562 cell extracts (Fig. 4B). The formation of cardiac or erythroid cell complexes with the rBNP −90 GATA probe was effectively inhibited by unlabeled excess oligonucleotides corresponding to the GATA-1, GATA-2, or GATA-3 site (Fig. 4B) or to a 25-bp element containing the bp −30 BNP GATA motif (see Fig. 6B). However, binding was unaffected by excess oligonucleotides corresponding to the mutated BNP GATA (rBNPmut −90) or β-globin GATA-1 site (Table 1), which are no longer recognized by GATA-1 proteins (25), or by unrelated competitors, such as cyclic AMP and tetradecanoyl phorbol acetate (TPA) response elements (data not shown). Similarly, when a 25-bp double-stranded oligonucleotide containing the −30 GATA motif was used as the probe, a single complex formed with cardiocyte nuclear extracts; this complex was effectively eliminated by excess unlabeled GATA-containing oligonucleotides but not by the mutant rBNPmut −90 GATA site (not shown). Thus, two conserved regulatory elements of the cardiac BNP promoter contain GATA motifs and interact with GATA-binding proteins present in cardiac myocytes. It is noteworthy that GATA-binding activity was not restricted to neonatal cardiocytes in cultures but was also present in nuclear extracts prepared from adult atrial and ventricular tissues, as assessed by EMSA (data not shown). Therefore, atrial and ventricular myocytes contain nuclear proteins that are related—at least with respect to their DNA-binding properties—to the GATA family of transcription factors.

At the time that this work was being carried out, three GATA proteins with distinct tissue distribution had been identified. GATA-1 is specifically expressed in erythroid cells (22, 63), whereas GATA-3 is present predominantly in T cells (28, 30, 33). GATA-2 has a more widespread distribution, at least in embryonic chicken tissues (70) and in various mammalian cell lines (34). Neither GATA-1 nor GATA-3 tran-

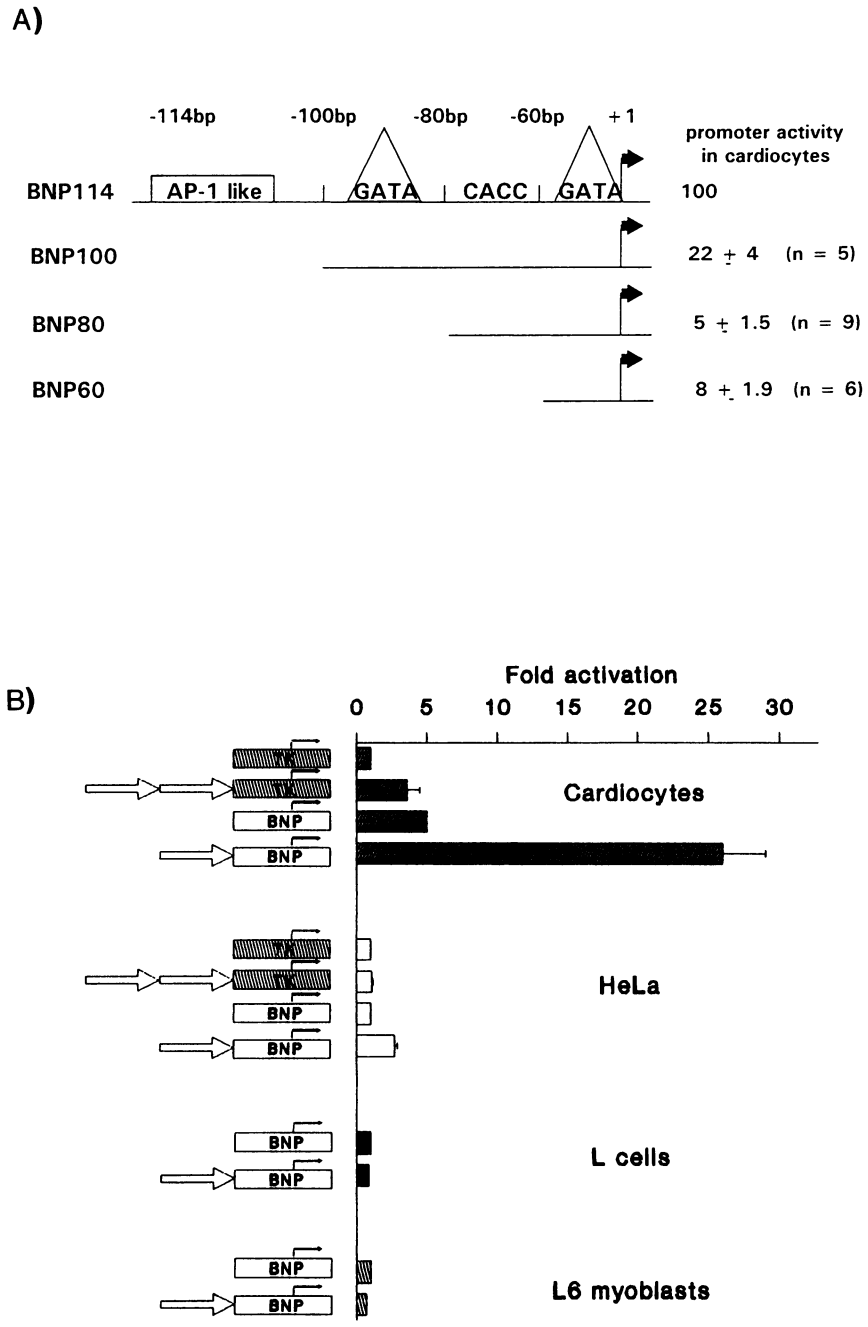


FIG. 3. (A) Functional analysis of the BNP proximal promoter in cardiomyocytes. 5′ deletions were generated by PCR and fully sequenced. The results represent the means ± SD for five to nine independent determinations in three or four different ventricular cultures. The effect of the deletions was the same in atrial cells. The data are expressed relative to that obtained with the BNP 114 construct. As usual, the RSV-hGH construct was included as an internal control. (B) The activity of the −90 GATA motif was tested by inserting oligonucleotide rBNP −90 (shown in Table 1) in one or two copies in the *Bam*HI polylinker site upstream of the minimal TK (bp −81) or BNP (bp −60) promoter. The results (means ± SD for four to six determinations) are expressed relative to the activity of the TK −81 promoter in each cell line. The low activity of the short BNP promoter containing three GATA sites in HeLa cells probably reflects the presence of low levels of GATA-2 activity in these cells (see Fig. 4A).

scripts are found in the heart (references 33 and 70 and unpublished observations), but low levels of GATA-2 transcripts are present in embryonic chicken hearts (70), although it is not clear which cells within the heart express GATA-2 proteins.

Cross-linking studies were carried out to analyze the electrophoretic properties of the GATA-binding proteins in ventricular cardiomyocytes. These studies revealed the presence of a single specific band corresponding to a protein(s) of approximately 48 to 50 kDa, which had the same mobility as the GATA-binding species present in extracts from the erythroleukemic cell line K562, which contains GATA-1 (data not

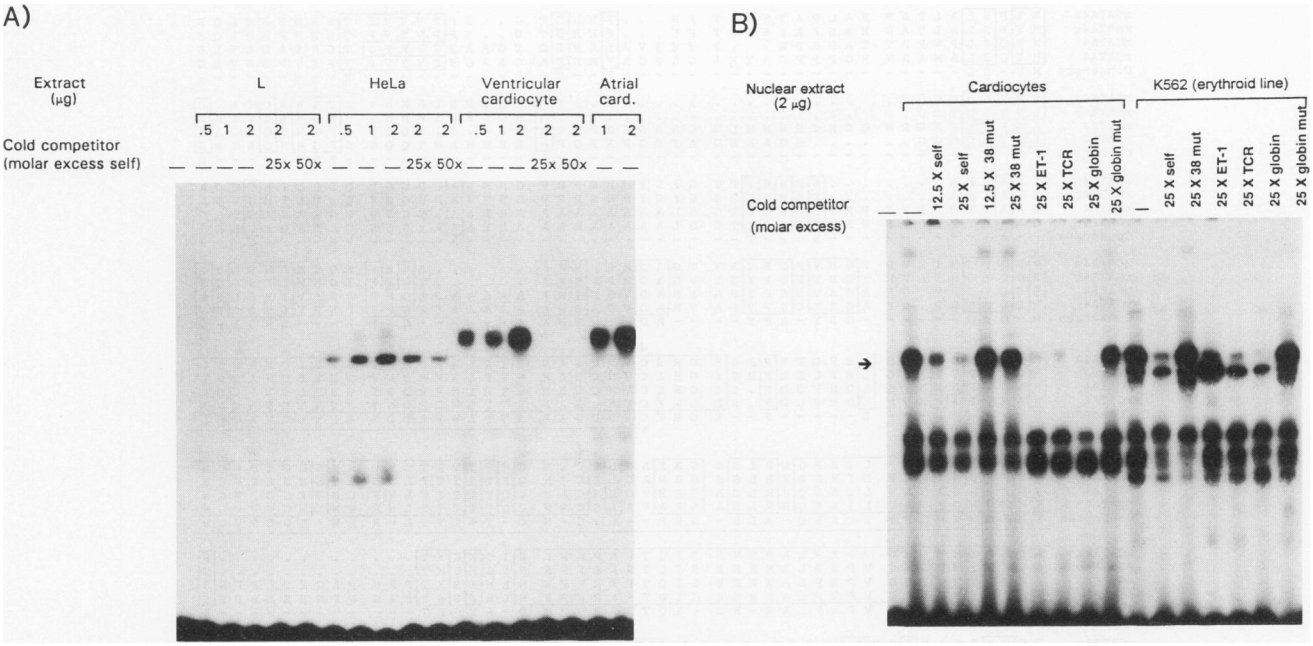


FIG. 4. Interaction of the BNP GATA element with nuclear extracts from cardiac and noncardiac cells. The probe used (rBNP −90) is a 33-bp double-stranded oligonucleotide corresponding to the double −90 GATA element (Table 1). Nuclear extract preparation, binding reactions, and electrophoresis conditions are described in Materials and Methods. The undetectable binding in L cell extracts and the lower binding activity in HeLa cell extracts are not due to a general low activity of the extracts, as determined by using probes containing serum, cyclic AMP, or TPA response elements (not shown). The sequences of all of the competitor oligonucleotides used in panel B are shown in Table 1. Self, rBNP −90; 38 mut, rBNPmut −90; ET-1, human endothelin-1; TCR, human T-cell receptor δ; globin, mouse α-globin; globin mut, chicken β-globin mutant. The arrow indicates the specific GATA-containing complexes.

shown). Together, these results suggested the presence of a novel 50-kDa GATA-binding protein in the myocytes.

In order to characterize the GATA-binding proteins in cardiac myocytes, we screened a cardiocyte cDNA library at low stringency with the zinc finger region of mouse GATA-1 as the probe (63). Four positive clones were obtained from 2×10^6 phage. Their inserts, which varied between 2 and 2.5 kbp in length, were subcloned into pKS and sequenced by the dideoxy procedure. No open reading frame could be found in three of the four clones which appeared to contain repetitive 3′ untranslated sequences. However, the 2.5-kbp insert from λ75 had an open reading frame encoding a predicted polypeptide of 323 amino acids and a 3′ untranslated region of 1.5 kbp, including the polyadenylation signal and the poly(A) tail. The deduced peptide sequence contained a zinc finger region 80% homologous to those of the three other members of the GATA family but highly divergent C- and N-terminal regions. However, the cDNA clone appeared to be partial at the 5′ end, since it was missing a 5′ untranslated region and because the first amino acid was not a methionine.

With an antisense oligonucleotide corresponding to positions 77 to 93 of the partial DNA sequence as a primer, we performed RACE-PCR on mRNA from ventricular cardiocyte cultures and obtained a 470-bp cDNA clone which contained the initiator methionine. With this N-terminal extension, the entire coding sequence spanned 1,320 bp, corresponding to a protein of 440 amino acids with a predicted molecular mass of 49 kDa, which is consistent with the size of the 50-kDa GATA-binding protein detected by cross-linking studies in cultured cardiocytes and very similar to the sizes of the three other members of the family (GATA-1 and GATA-3, 49 kDa; GATA-2, 47 kDa). The zinc finger domain lies between amino acids 210 and 300, and each side of the finger region contains a proline-rich (12%) domain, which may be important for the transcription activation function. Extensive computer-assisted sequence analysis revealed no obvious homology to the GATA-1, -2, or -3 proteins outside the zinc finger domain. Therefore, this protein likely represented a new member of the GATA transcription factor family and will be referred as rGATA-4.

Southern blot hybridization of rat genomic DNA with labeled rGATA-4 DNA produced a pattern consistent with the presence of a unique rGATA-4 gene (not shown). As this work was being finalized, Arceci et al. (5) reported the sequence of a novel mouse GATA protein whose cDNA clone was isolated from a 6.5-day-old mouse embryo cDNA library, and Kelley et al. (31) published the deduced amino acid sequences of two *Xenopus* cDNA clones, which they referred to as xGATA-4a and xGATA-4b. Alignment of our amino acid sequence with those of the mouse and *Xenopus* clones revealed high homology to the product of the mouse GATA-4 clone (Fig. 5). Since the rat and mouse genes have a similar pattern of tissue distribution, the two cDNA clones likely encode the mouse and rat homologs of the same new GATA-4 protein. The homology between mouse, rat, and *Xenopus* GATA-4 was most evident in the zinc finger region, and the four proteins showed limited conservation outside the DNA-binding domain (Fig. 5). The tissue distribution of the *Xenopus* genes also appears to differ from that of the putative rat and mouse GATA-4 homologs, especially with respect to their expression in endocardial rather than cardiac muscle cells (discussed below). Whether the differences in primary sequence and in tissue distribution between the rodent and *Xenopus* GATA-4 genes reflect species

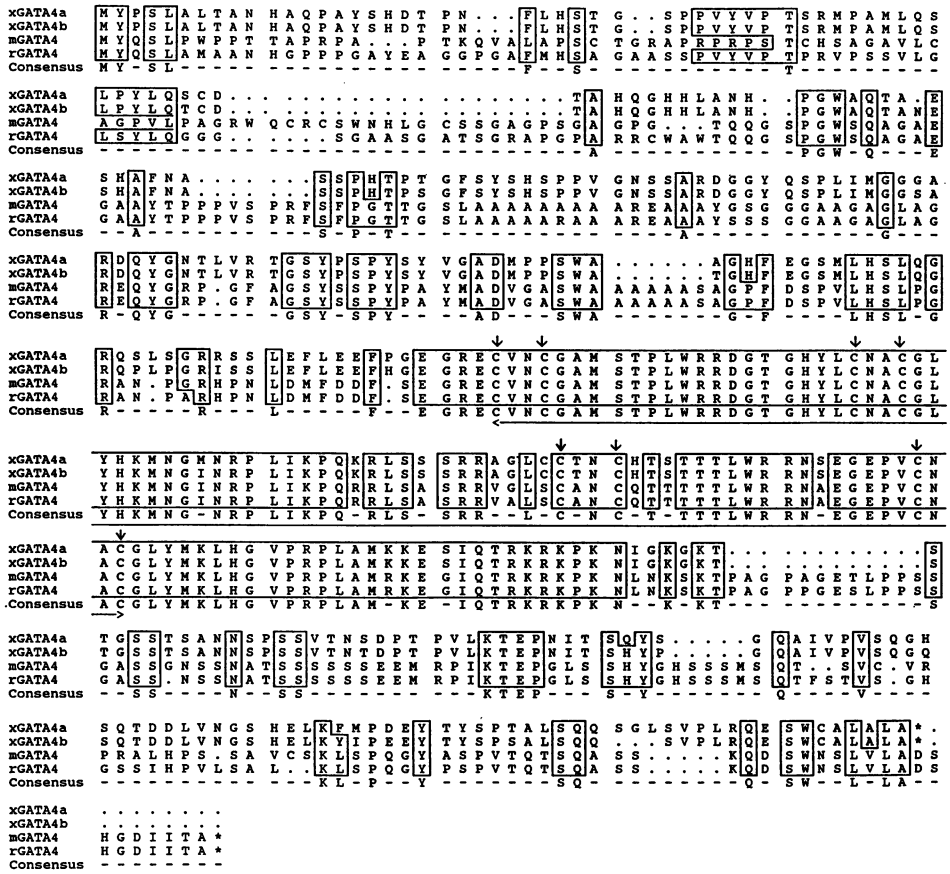


FIG. 5. Comparison of amino acid sequences of rat, mouse, and *Xenopus* GATA-4 proteins. Computer-assisted sequence analysis was carried out with the software package from the Genetics Computer Group, Inc. Identical amino acids and conservative substitutions are boxed. Dots correspond to gaps. The zinc finger domain is underlined.

differences or whether the *Xenopus* genes correspond to another (GATA-5?) GATA member has yet to be determined.

Cardiac BNP promoter is a target for rGATA-4. In order to analyze the functional properties of cloned rGATA-4 on the native BNP promoter, an *Xba*I fragment of the rGATA cDNA containing the open reading frame was subcloned in a eukaryotic expression vector (pCG) downstream of the cytomegalovirus promoter (60). Since L cells did not contain any GATA-binding activity, they were transiently transfected with the GATA-4 expression vector, and nuclear extracts were prepared and tested for the presence of functional GATA-4 protein by EMSA. As a control, nuclear extracts were also prepared from untransfected cells and from cells transfected with the background pCG vector or the antisense rGATA-4 expression vector. The rBNP −90 GATA probe did not produce any specific complex when incubated with nuclear extracts from control L cells or from L cells transfected with the antisense rGATA-4 expression vector (Fig. 6A). In contrast, a specific complex was formed with the rBNP −90 GATA probe and nuclear proteins extracted from L cells transfected with the rGATA-4 expression vector. The mobility of the complex formed with extracts containing recombinant GATA-4 protein was identical to that of the complex present in the ventricular extracts (Fig. 6A); in fact, when nuclear extracts were prepared from cardiocytes transfected with the GATA-4 vector, the abundance of the GATA complex was greatly increased (Fig. 6B). Both endogenous and recombinant GATA complexes were eliminated by the presence of excess unlabeled rBNP −90 oligonucleotides but not by their mutated rBNPmut −90 counterparts (Fig. 6). These results indicate that functional GATA-4 protein is produced from the GATA-4 expression vector and that the cloned rGATA-4 cDNA encodes a DNA-binding protein with GATA specificity.

The effect of forced expression of cloned rGATA-4 on BNP promoter activity was then assessed by cotransfecting the GATA-4 expression vector with various BNP promoter constructs. Forced expression of GATA-4 in L and HeLa cells increased the activity of the full-length BNP promoter by more than 10-fold and reconstituted its cardiac activity (Fig. 7A). Shorter BNP promoter fragments containing the −30 GATA motif (BNP60) with one copy of the rBNP −90 oligonucleotide containing the two −90 GATA sites were activated 30-fold by GATA-4 but the BNP-TK heterologous promoter, which does not contain any GATA site, was marginally affected by GATA-4 expression (Fig. 7A and B). The RSV and TK promoters were marginally responsive to GATA-4; however, the cardiac muscle-specific promoter of the ANP gene, which contains conserved GATA motifs (Fig. 8), was significantly activated by GATA-4 (Fig. 7A).

When dose-response assays were carried out with various concentrations of rGATA-4, mouse GATA-1, or human GATA-1 or GATA-3 expression vectors, interesting differences in the ability of the various GATA proteins to transactivate the BNP promoter were noted. For example, the maxi-

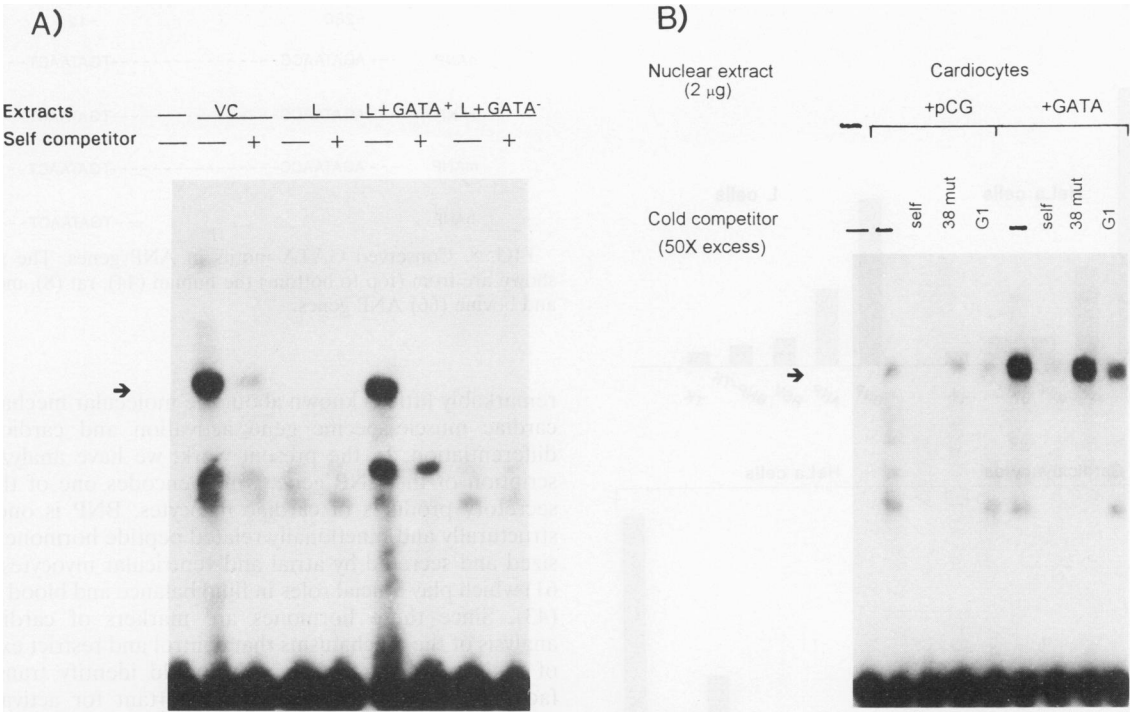


FIG. 6. DNA-binding properties of cloned GATA-4. (A) Gel shift analysis was carried out with the ^{32}P-labeled rBNP −90 probe and nuclear extracts prepared from ventricular cardiocyte (VC) cultures containing endogenous GATA-4 and extracts from control untransfected L cells or from L cells transiently transfected with sense (GATA$^+$) or antisense (GATA$^-$) expression vectors. One microgram of extract was used in binding reactions with and without a 50-fold molar excess of unlabeled BNP −90 GATA oligonucleotide (self) as a competitor. Similar results were obtained with two independent transfections and extract preparations. rBNPmut −90 (38 mut) did not eliminate the GATA complexes (indicated by arrows) or form any specific complex with GATA-containing extracts (not shown). (B) Cardiac extracts prepared from cells transiently transfected with the GATA-4 expression vector also show increased GATA-binding activity. Self, rBNP −90; 38 mut, rBNPmut −90; G1, rBNP −30.

mal activation achieved with human GATA-1 or GATA-3 never exceeded 3-fold, compared with a maximal 30-fold induction achieved with rGATA-4 (Fig. 7C). These differences are not due to differences in the amount of protein produced, as assessed by EMSA with nuclear extracts prepared from transfected L cells (data not shown). Therefore, at least at the level of the BNP gene, rGATA-4 was a more potent transcriptional activator than mouse or human GATA-1 or human GATA-3. It is noteworthy that expression of a single transcription factor was sufficient to induce transcription from the cardiac BNP promoter and recapitulate its cardiac activity.

GATA-4 transcripts are present in BNP-expressing cardiac myocytes. RNA blot analyses were performed to investigate the pattern of cellular expression of the rGATA-4 gene. A radiolabeled rGATA-4 fragment was used as probe to screen total RNA from various tissues and from primary atrial and ventricular tissue cultures. A unique rGATA-4-hybridizing species of approximately 4,500 nucleotides was found in the atrial, ventricular, and testis tissues from neonate and adult rats (Fig. 9A and B). The transcripts were also detectable by Northern analysis in the ovary but not in any other rat tissues examined, including brain, lung, liver, kidney, and skeletal muscle (Fig. 9A and B and data not shown). rGATA-4 transcripts appeared to be more abundant in primary atrial and ventricular cell cultures than in whole cardiac tissue, suggesting that they may be specific to cardiac myocytes.

Reverse transcription-PCR was also used to investigate further the pattern of expression of the rGATA-4 gene. Sense and antisense oligodeoxynucleotides which amplified a 730-bp fragment corresponding to coding sequences from positions 595 to 1324 on the rat cDNA sequence (Fig. 9C) were used in PCR on cDNAs prepared from various adult or newborn rat tissues. The PCR products were analyzed by Southern blotting after hybridization with a ^{32}P-labeled internal oligonucleotide. A 546-bp rat tubulin fragment was amplified as an internal control. GATA-4-specific transcripts were again detectable in newborn and adult atria and ventricles and in adult ovary and testis; weak signals were detectable upon longer exposure only in adult lung and intestine. No GATA-4 transcripts could be detected in a variety of other newborn or adult tissues, including the hypothalamus, pituitary gland, adrenal gland, kidney, liver, or submaxillary and parotid glands. This tissue distribution is closer to the distribution in the mouse than it is to that of the *Xenopus* GATA-4 transcripts (5, 31). The localization of GATA-4 transcripts in cardiac muscle cells was investigated further by in situ hybridization of primary cardiomyocyte cultures and rat heart tissue sections. These studies clearly showed that GATA-4 transcripts are localized within ANP- and BNP-expressing myocytes in atrial and ventricular cells (Fig. 10).

DISCUSSION

Tissue-specific gene expression is an important determinant of cellular differentiation, and analysis of cell-specific transcription factors has greatly increased our understanding of the early steps of differentiation. Although the heart is the first organ to reach a functional state in the developing embryo,

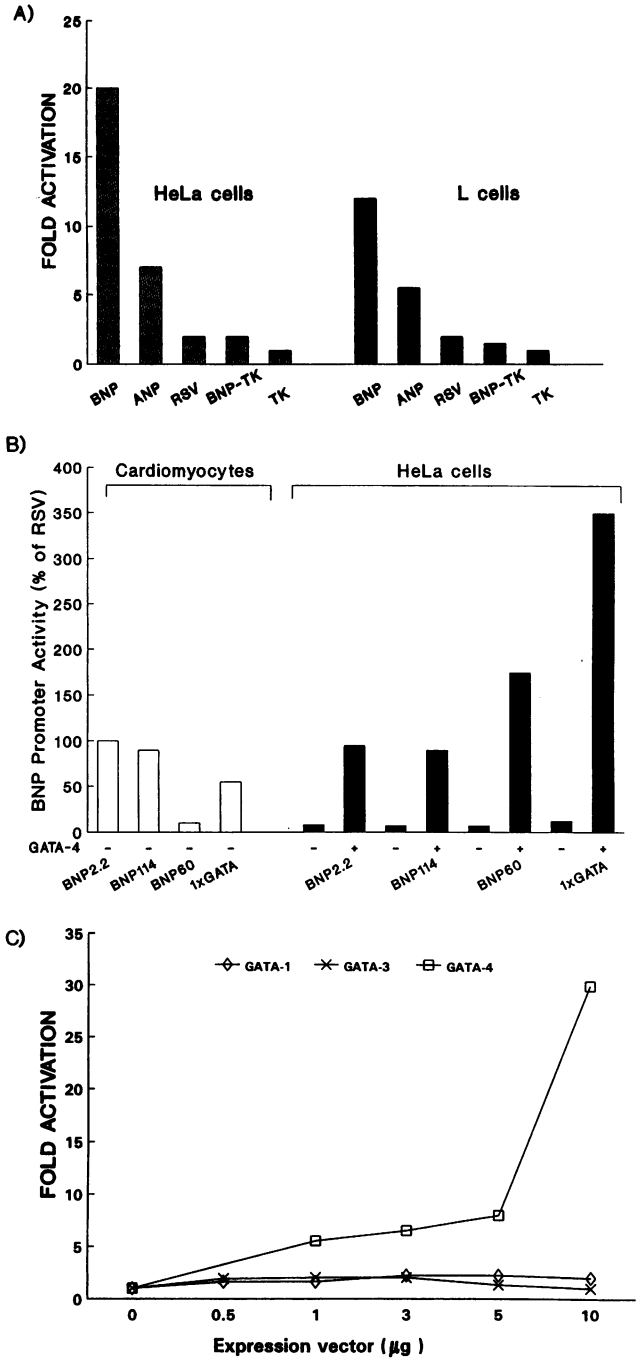


FIG. 7. Transactivation properties of recombinant GATA-4. (A) Transactivation of various natural and synthetic promoters by GATA-4. In all cotransfections, the amount of reporter DNA was kept constant at 3 μg and the amount of expression vector was varied. The data shown are the means of two to four independent experiments, each carried out in duplicate, with 5 μg of GATA-4 expression vector (standard error was less than 15%). The results are expressed as fold activation relative to cotransfection with the background or antisense vector. The BNP construct contains the entire 2,200-bp promoter; the ANP promoter used corresponds to the 640-bp rANP promoter (9). BNP-TK is a heterologous promoter containing the proximal TK region (diagrammed in Fig. 1B); TK contains 109 bp of upstream sequences; RSV is the RSV-luciferase plasmid. (B) Forced expression of GATA-4 in HeLa cells recapitulates the high-level cardiac activity of the BNP promoter. Promoter activity with (+) or without (−) GATA-4 is expressed relative to that with the RSV promoter to allow comparisons between cardiac and HeLa cells. The data are from at least three experiments carried out in duplicate with 3 μg of reporter DNA and 3 μg of expression vector. HeLa cell control lanes (−) contained equivalent amounts of background or antisense GATA-4 vector. BNP2.2 is a full-length (2.2-kbp) promoter construct; the other BNP promoter fragments are shown in Fig. 3, including 1×GATA, which contains one copy of the rBNP −90 oligonucleotide fused to BNP60. (C) Effect of cotransfecting various amounts of GATA expression vectors with BNP promoter constructs. The data shown represent fold activation of the BNP −114 construct in HeLa cells (averages of two independent experiments carried out in duplicate). The total amount of DNA per petri dish was kept constant by adding appropriate amounts of control background vector. Similar activation patterns were also obtained with the longer 2.2-kbp BNP promoter fragment (not shown). The results shown for GATA-1 and GATA-3 were obtained with the human GATA constructs.

```
                -280                    -122
hANP   ---AGATAACC----------TGATAACT---
rANP   ---AGATAACC----------TGATAACT---
mANP   ---AGATAACC----------TGATAACT---
bANP                        ---TGATAACT---
```

FIG. 8. Conserved GATA motifs in ANP genes. The sequences shown are from (top to bottom) the human (44), rat (8), mouse (56), and bovine (66) ANP genes.

remarkably little is known about the molecular mechanisms of cardiac muscle-specific gene activation and cardiomyocyte differentiation. In the present work, we have analyzed transcription of the BNP gene, which encodes one of the major secretory products of cardiac myocytes. BNP is one of two structurally and functionally related peptide hormones synthesized and secreted by atrial and ventricular myocytes (47, 58, 61) which play crucial roles in fluid balance and blood pressure (43). Since these hormones are markers of cardiac cells, analysis of the mechanisms that control and restrict expression of their genes to cardiac cells should identify transcription factors and pathways that are important for activating the cardiac genetic program and responsible for generating the cardiac phenotype. As described in the present work, studies of the BNP promoter have led to the isolation of a cardiac tissue-specific transcription factor which is not present in other muscle cells and is not related to transcription factors associated with skeletal muscle gene expression, such as the basic helix-loop-helix (bHLH) and MEF-2 proteins (49). In fact, the studies of the BNP promoter and the isolation of a cardiac member of the GATA family of transcription factors revealed unexpected similarities between the mechanisms controlling cardiac and hematopoietic gene expression.

Isolation and sequence analysis of the BNP gene 5′-flanking region revealed a structural organization more typical of erythroid cell than of muscle cell promoters, particularly in the proximal promoter region. Indeed, mutational analysis of various erythroid cell promoter/enhancers has suggested that, in addition to GATA motifs, CACC and AP-1-like elements play important roles in erythroid cell-specific transcription (21, 67, 72), and tissue-specific transcription factors that interact with these elements have recently been isolated (2, 39). The BNP promoter contains, in addition to the GATA motifs, both CACC and AP-1-like elements (Fig. 2A). The AP-1-like element is highly conserved across species, and its deletion

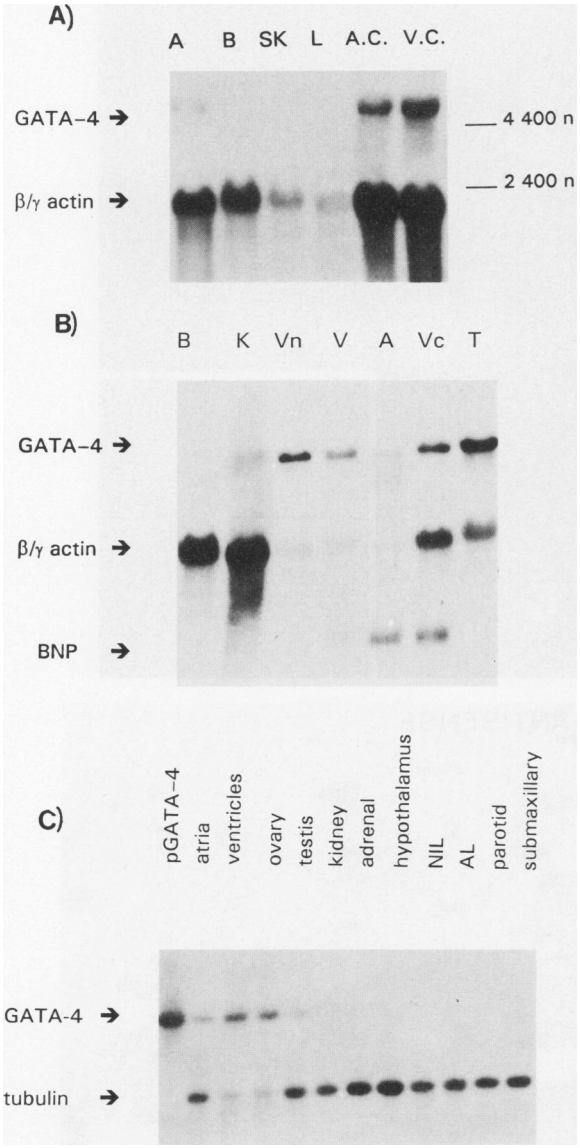


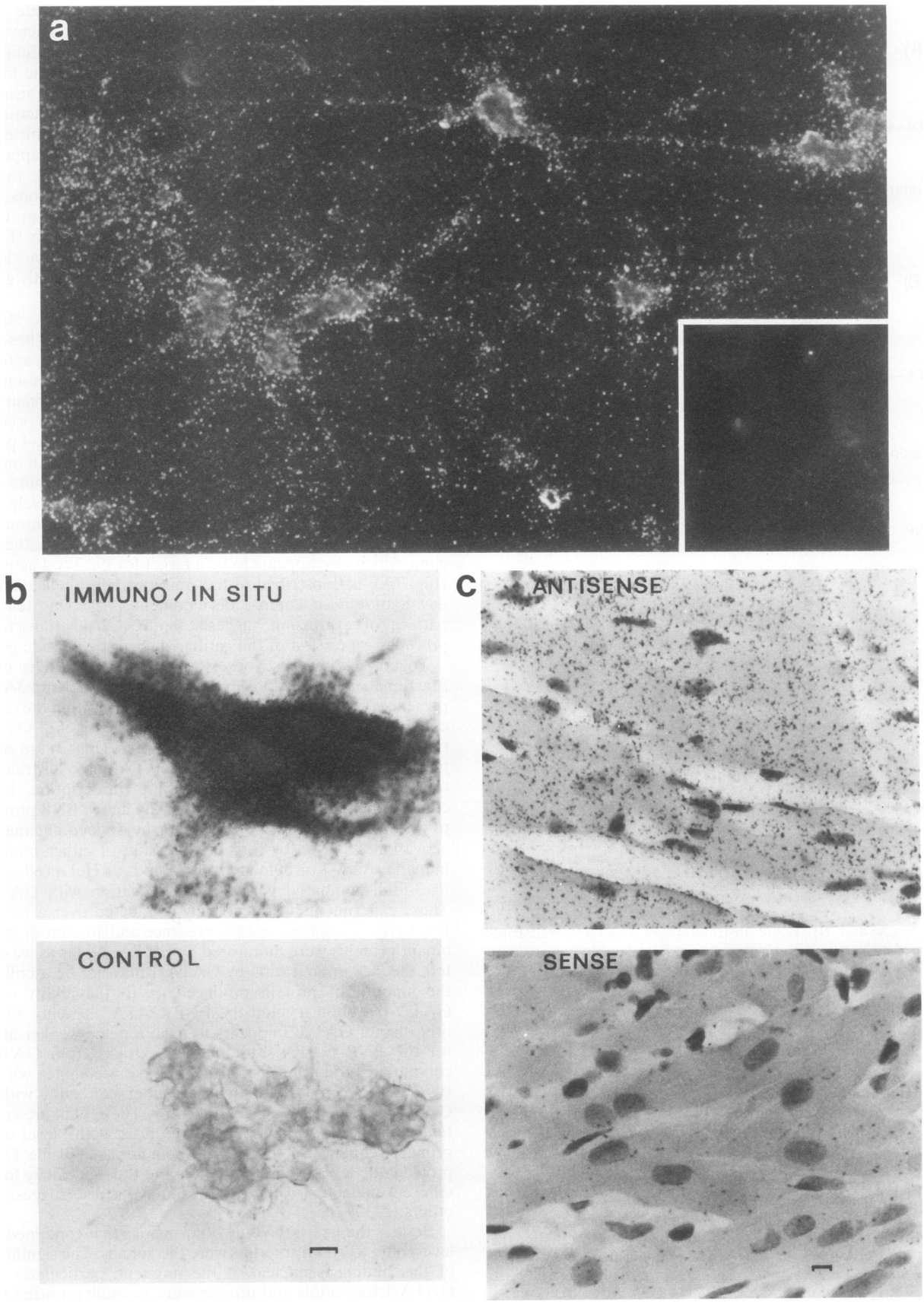
FIG. 9. (A and B) Tissue distribution of GATA-4 transcripts determined by Northern blot hybridization. Each lane contained 20 μg of total RNA from 1- to 3-day-old neonatal (A) or adult (B) rat tissues unless otherwise specified. A, atria; B, brain; SK, skeletal muscle; L, liver; K, kidney; T, testis; Vn, neonatal ventricle; A.C., atrial cardiocytes; V.C., ventricular cardiocytes; V, ventricle; Vc, primary ventricular myocyte cultures. The same blots were rehybridized sequentially with the rat β-actin or BNP probe as described in Materials and Methods. Sizes are shown to the right of panel A (in nucleotides). (C) PCR amplification of GATA-4 transcripts in various tissues. PCR-amplified transcripts were separated by agarose gel electrophoresis, blotted on a nylon membrane, and hybridized to a specific internal oligonucleotide. The primers, amplification conditions, and internal hybridization probes used are described in Materials and Methods. pGATA-4 is the recombinant Bluescript plasmid containing the λ75 insert which was used as a control. NIL, neurointermediate lobe; AL, anterior pituitary lobe. Please note that the PCR was not done under quantitative conditions; as determined from Northern blots, the amounts of GATA-4 in ovary and testis are equivalent.

reduces cardiac promoter activity fourfold (Fig. 3A). In a heterologous promoter context, an oligonucleotide corresponding to this element is a strong transcriptional activator in cardiac and, to a lesser extent, other cell types; preliminary evidence suggests that ubiquitous as well as cardiac muscle-specific complexes form over this element (26a), raising the possibility that yet another class of tissue-specific factors may be common to cardiac and erythroid cells. In contrast, the CACC element is less conserved across species and appears to have a negative role, if any, in BNP transcription; this was observed in experiments with 5′ and internal deletions of the BNP promoter in cardiocytes (Fig. 3A) and also in the transactivation assays with GATA expression vectors (Fig. 7B and data not shown), in which promoter constructs lacking the CACC box are consistently activated to a significantly greater extent than the wild-type promoter.

The identification of the double GATA site at bp −90 as an important positive element for cardiac transcription has led to the isolation of a GATA transcription factor whose expression is largely restricted to cardiac myocytes. The presence of GATA-4 transcripts at day 6.5 of embryo development (5), well before the formation of the tubular heart, which occurs on day 8, would be consistent with a role for the GATA-4 protein in the early events of cardiogenesis. While the time of onset of BNP gene expression is not known yet, our results (18a) indicate that BNP transcripts are present constitutively in rat hearts from day 14 of embryo development and throughout pre- and postnatal development. On the other hand, the ANP gene, which also appears to be a target for GATA-4 activation (Fig. 7A), is transcribed in the primitive heart cells on about day 8 of mouse embryo development (74). This temporal pattern of expression suggests that GATA-4 transcription precedes expression of the cardiac natriuretic peptide genes.

Together with these observations and the results of the functional analysis of the BNP promoter (Fig. 1B and 3A), the presence of conserved GATA motifs (Fig. 8) within the cardiac enhancer of the ANP gene (38) and the ability of GATA-4 to transactivate the BNP and ANP promoters (Fig. 7) are consistent with an important role of the GATA family of transcription factors in cardiac muscle-specific transcription. In this context, the differential sensitivity of the native BNP promoter to various GATA proteins is noteworthy. Indeed, the maximal activation achieved by forced expression of either mouse or human GATA-1 or human GATA-3 in L or HeLa cells was 2- to 3-fold, compared with 30-fold activation with GATA-4. These experiments were carefully conducted over a range of DNA concentrations, and the presence and amount of recombinant proteins were monitored by EMSA. As far as we could tell, the lack of activation by GATA-1 may not be ascribed to the amount of protein produced or to the ability of the GATA-1 protein to bind the BNP GATA elements. On the other hand, GATA-3 appears to have a much weaker affinity for the BNP GATA sites; in fact, extracts from GATA-3-containing Jurkat T cells fail to produce a complex with the BNP GATA probe, although they interact readily with the GATA element of the T-cell receptor. These data are consistent with the view that subtle differences at the level of the primary sequence of the GATA elements (see Table 1) may provide one level of discrimination and thus specificity for the different members of the GATA family, as also suggested by others (23, 71).

Besides the heart, the only other tissues that contained high levels of GATA-4 transcripts were the gonads. The significance of this finding is unclear at the moment, particularly since GATA-1 transcripts and protein were recently reported to be abundantly present in mouse testis (29). Nevertheless, it is


a
b
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CONTROL
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SENSE

FIG. 10. In situ localization of GATA-4 transcripts in cardiac myocytes. (a) Representative low-magnification photomicrograph of 1-day-old ventricular myocyte cultures hybridized to ^{35}S-labeled GATA-4 cRNA; the hybridization signal seen corresponds to myocytes organized in typical spontaneously beating clusters. The inset shows control hybridization of RNase-treated myocytes. (b) High-magnification double-label immunohistochemistry-in situ hybridization confirms the colocalization of GATA-4 transcripts and BNP peptide within atrial and ventricular myocytes. (Top) rBNP staining of 1-day-old atrial myocytes in culture followed by hybridization to the ^{35}S-labeled antisense GATA-4 probe; the cells also stain positively with anti-ANP antibody (not shown). (Bottom) Cells were processed similarly except that the first antibody was normal rabbit serum and the hybridization was carried out with a sense GATA-4 cRNA probe. (c) In situ hybridization of GATA-4 with tissue sections from adult rat ventricles. The ^{35}S-labeled probe used was either antisense (top) or sense (lower). Note the numerous grains overlying cardiac myocytes in the top panel, whereas very few grains are observed in the lower panel. A similar specific signal is observed with atrial tissue sections (not shown). For experimental details, refer to Materials and Methods. Bar, 10 μm.

notable that the GATA proteins identified so far are essentially specific to cells and tissues derived from the lateral plate mesoderm, including the gonads, heart, and hematopoietic and endothelial cells. Given the important role of GATA-1 in erythroid cell differentiation (50), it is tempting to speculate on the role of other cell-restricted GATA proteins in the commitment and differentiation of other lateral-mesoderm-derived cell lineages and particularly on the role of GATA-4 in the commitment and differentiation of the cardiac cell lineage.

Finally, in considering the mechanisms controlling cardiac muscle-specific transcription, it is important to mention that additional cardiac muscle-specific transcription factors are likely required to set the proper spatial and developmental pattern of gene expression within the heart. For example, unlike the BNP gene, which is constitutively expressed at similar levels in atrial and ventricular cells (Fig. 9) (17), the ANP gene presents a more complex pattern of cardiac expression. Indeed, transcription of the ANP gene is differentially regulated in atrial and ventricular cells during heart development (12, 45, 74) and would require more complex regulatory pathways than the apparently simpler situation with the BNP gene. Consistent with this view, analysis of the regulatory elements of the ANP gene has identified several distinct tissue-specific elements within the rat ANP promoter and enhancer that are differentially active in atrial versus ventricular cells in a development stage-specific manner (6, 7, 38). From these results and on the data in this article suggesting that GATA-4 transcripts are constitutively expressed in neonatal and adult atrial and ventricular cells (Fig. 9), we propose that the GATA pathway may be important for the initiation and maintenance of a "basal" level of cardiac muscle-specific transcription and that additional cardiac factors are likely involved in the complex development phenotype of the heart.

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