Characterization of the Murine A1 Adenosine Receptor Promoter, Potent Regulation by GATA-4 and Nkx2.5

(Received for publication, October 10, 1998, and in revised form February 10, 1999)

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Adenosine acts via A1 adenosine receptors (A1ARs) in the heart and brain to potently influence mammalian physiology. A1ARs are expressed very early in embryonic development, and A1ARs are among the earliest expressed G protein coupled receptors in the heart and brain. To understand the biologic basis of A1AR expression, a genomic fragment containing the murine A1AR promoter was cloned. Reporter assay studies using DDT1 MF2 cells that express A1ARs revealed that 500 bp base pairs of the proximal A1AR promoter contained essential elements for A1AR gene expression. Transgenic mice with A1AR proximal promoter coupled with the β-galactosidase reporter gene had heavy labeling of the brain and atria, consistent with normal patterns of A1AR expression. Within the proximal A1AR promoter, putative binding sites for cardiac transcription factors GATA and Nkx2.5 were identified. Co-expression studies revealed that GATA-4 and Nkx2.5 could individually drive A1AR promoter activity and act synergistically to activate A1AR expression. These observations suggest that embryonic A1AR expression involves activation of the A1AR promoter by GATA-4 and Nkx2.5.

The purine nucleoside adenosine exerts potent biological effects through specific G protein coupled receptors (GPCRs) that include A1, A2a, A2b, and A3 adenosine receptors (1). Each adenosine receptor subtype has distinct ligand binding properties and different patterns of tissue expression (1). A1 adenosine receptors (A1ARs) are widely distributed in the central nervous system and act to influence neuronal function, neurotransmitter release, and protect against seizure activity and cerebral ischemia (2, 3). A1ARs are also expressed in the heart, where their activation protects the myocardium against ischemia and can terminate supraventricular arrhythmias (4, 5).

Recent evidence shows that A1AR expression begins at very early stages of development (6). In the central nervous system, A1ARs are expressed in neurons during periods of active neurogenesis and neuronal migration (6). Brain regions with high levels of A1AR expression at early stages include the hippocampus, cerebellum, and hindbrain (6). A1ARs are expressed at even earlier stages in the myocardium when the heart is a primitive cardiac cylinder that has not begun beating, making A1ARs the earliest known expressed GPCR in the heart (6, 7).

Presently, our understanding of the factors that regulate A1AR gene expression is at early stages. The human A1AR gene promoter has been isolated and examined (8–10). The 5′-untranslated region of the human A1AR gene contains two promoter elements, designated “A” and “B,” that are separated by an intron (9). The A and B promoters are believed to have nonclassical TATA boxes (A, TTAAGAC; B, TTAAAA), and the B promoter is more active than the A promoter (9). It has been suggested that nuclear proteins bind to AGG motifs in the promoter A region, although the identity of these proteins is unknown (10).

Recognizing the unique temporal and spatial patterns of A1AR expression, there is considerable interest in identifying the factors that influence A1AR gene expression. To provide additional insights into the factors that regulate A1AR expression, we have isolated and characterized the murine A1AR promoter (mA1ARp). We now show that 500 bp of the proximal mA1ARp contains motifs responsible for A1AR expression in the brain and heart and that the transcriptional activating factors GATA-4 and Nkx2.5 potentely induce mA1ARp activation.

MATERIALS AND METHODS

Library Screening

A murine genomic 129/SVJ library (CLONTECH, Palo Alto, CA) was screened with a 32P-labeled probe generated from a 400-bp fragment from the 5′-end of the rat A1AR cDNA (11). A positive clone of 4.5 kb was isolated and purified using the polyethylene glycol (M, 8000) precipitation method. The phage insert was subcloned into a β-galactosidase reporter plasmid, pNLAC (Stratagene; La Jolla, CA), by restriction digestion, and sequenced in both directions.

Production and Identification of Transgenic Mice

The −502 to +35 mA1ARp fragment was subcloned into the pNLAC vector (12). The construct was then digested with KpnI and PstI, and the mA1AR-pNLAC DNA was gel purified (Qiagen; Santa Clarita, CA). The purified fragment was microinjected into the pronuclei of fertilized eggs (C57/BL6) at the Yale Transgenic Center. Injected eggs were implanted into pseudopregnant recipient mice. Offspring were screened for the presence of the transgenes by PCR amplification of DNA from tail biopsies using oligonucleotide primer pairs CCCTGTCATCTGG-CAGTTTAG (mA1ARp) and TGGGGCGATCGATCGTAGATT (mA1ARp). PCR conditions consisted of 30 cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 2 min. PCR products were then separated on a 1.2% agarose gel.

Whole Mount, β-Galactosidase Staining

β-Galactosidase staining was examined in whole-mount embryo specimens from timed pairings of male founders with wild-type female mice as described (12, 13). Embryos were dissected from the uteri and placed in individual wells in 12-well plates that contained ice-cold phosphate-buffered saline (PBS). Corresponding amniotic membranes were saved for reconstruction.
for PCR genotyping. Embryos were fixed in 0.25% glutaraldehyde for 30 min on ice. Specimens were then washed three times for 30 min in PBS. Specimens were then incubated in PBS staining solution containing 2 mM MgSO4, 5 mM KCl, 5 mM FeCl3, 5 mM KCl, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, and 1.0 mg/ml X-gal. Specimens were then incubated overnight at 37 °C. The next day, freshly prepared luciferase solution was added in PBS and stored at 4 °C until microscopic examination. After staining, some specimens were frozen in chilled (−20 °C) 2-methylbutanone, stored at −80 °C, and sectioned in a cryostat (10 μm), and tissue sections were examined.

**Determination of Transcription Start Sites**

5′-RACE—cDNA libraries were constructed from DDT1 MF-2 cell mRNA using the 5′-RACE system (Life Technologies, Inc., Gaithersburg, MD) (14). Single-stranded cDNAs were synthesized using the antisense gene-specific primer 1 (GSP1; ATAAGGATGGCCAGTGAGG-GATGAC) located 190 bp 5′ of the initiator methionine ATG codon. The cDNAs were tailed at the 3′-end with poly(A) using terminal transferase and then amplified by the PCR reaction using the specific anchor primer 2 (GSP2; ATATAAGCTTATCCTGCAGTAC; reverse primer, CTATATAAGCTTATCCTGCTCGTCTGCAACCGGTA) was subcloned into Bluescript SKII+

**RNase Mapping—Ribonuclease mapping was performed with an RPA II kit from Ambion (Houston, TX) (15). A 210-bp fragment of mA1AR genomic DNA that was 720–510 bp upstream of the initiator methionine was amplified by PCR (forward primer GACCACGCACTTACCTGATTACAGAAAGG; reverse primer, CTATATAAGCTTATCCTGCTCGTCTGCAACCGGTA) was subcloned into Bluescript SKII+ (Stratagene), linearized, and transcribed in vitro with T7 RNA polymerase (Amersham Pharmacia Biotech) to yield a 32P-labeled riboprobe that was gel purified. Twenty-five micrograms of total RNA from DDT1 MF-2 cells were hybridized with the riboprobe at 45 °C for 20 h and digested 95% 100-fold diluted RNase solution at 37 °C for 70 min. The protected products were analyzed by 8M urea, 7% polyacrylamide gel electrophoresis with 32P-labeled and HaeIII-digested X174 RF DNA to determine the sizes of the products.

**mA1AR Promoter–Luciferase Constructs**

All constructs were prepared by ligation of PCR-generated DNA fragments into the pGL3-Basic expression vector (Promega, Madison, WI). PCR products were generated using the full-length mA1ARp construct as a template. PCR conditions consisted of 25 cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 2 min using Roche Molecular Biochemicals Taq polymerase (Indianapolis, IN). PCR products were then separated on a 1.2% agarose gel and gel-eluted (QIAGEN II kit; Qiagen) before digestion with restriction endonucleases and ligation into pGL3. For isolation of the −502 to +35 construct, the forward primer TAAAATTCTGGGGATACTTGGCTAGAC; mutated GATA, TCTGGGGTTACTTGGCTAGAC; and the reverse primer was TCCCGAGCCCCGCTTTCC.

Specific mutations were made by the PCR overlap-extension method of Ho et al. (16). To generate the front part of mutant promoters, oligonucleotide primer pairs (primers A and B) were designed to generate a 5′-fragment of the mA1ARp. Another set of oligonucleotide primer pairs (primers C and D) were designed to generate a 3′-fragment of the mA1ARp receptor. B and C primers contained sequences that encoded for the desired mutations. PCR reactions were performed to generate A-B and C-D fragments, which were gel-eluted. Receptor fragments (A-B and C-D) were then combined in a third PCR reaction to generate a full-length mA1AR using flanking primers (A and D). Flanking PCR primers contained restriction endonuclease sites for subcloning into PGL3. Mutant constructs were then sequenced.

**Cell Culture and Transient Transfection**

DDT1 MF-2, MDCK, HELA, and HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in minimal essential medium containing 10% fetal bovine serum. All media were supplemented to final concentrations of 50 μg/ml penicillin and 50 μg/ml streptomycin. The cells were maintained in a humidified 5% CO2, 95% air atmosphere at 37 °C. On the day before transfection, the cells were passaged into 12-well plates (22-mm plate) and incubated in 300 μl of cell lysis solution (Promega). The supernatant obtained by centrifugation for 5 min was used to measure firefly and Renilla luciferase activities. Luciferase activity was measured on 15 μl of cell extract using a TD-2020 luminometer (Turner Designs, Sunnyvale CA) using a dual luciferase reporter assay system (Promega). Firefly luciferase activity was expressed relative to Renilla luciferase activity for all test constructs. Each sample was tested in quadruplicate. Each study was repeated at least four separate times.

**Radioreceptor Assays**

Radioligand binding studies were performed using intact cells as described (6, 11), using [3H]DPCPX (NEN Life Science Products; specific activity, 100 Ci/mmol). All determinations were done in quadruplicate.

**Preparation of Nuclear Extracts**

Nuclear extracts were prepared as described (17). Cultured cells were suspended in 200 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The homogenates were chilled on ice for 15 min, and then 25 μl of 10% Nonidet P-40 were added. After vigorous vortexing for 10 s, the nuclear fraction was precipitated by centrifugation at 15,000 × g for 5 min and suspended in 100 μl of buffer B (20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). The mixture was left on ice for 15 min with frequent agitation. Nuclear extracts were prepared by centrifugation at 15,000 × g for 5 min and stored at −80 °C. The protein concentration was determined using bicinchoninic acid (Pierce).

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assays (EMSAs) were performed as described (18, 19). DNA-protein reactions were performed for 30 min at 30 °C in a mixture (20 μl) containing 20 mM HEPES (pH 7.9), 0.3 mM EDTA, 0.2 mM EGTA, 80 mM NaCl, 1 mM dithiothreitol, 2 μg of poly(d(Deoxyinosine-deoxycytidine) (dI-dC), 0.1–0.4 ng 32P-labeled oligonucleotide probe, and nuclear miniextracts (2–8 μg of protein). Where indicated, the reaction was performed in the presence of unlabeled oligonucleotide competitors.

DNA-protein complexes were electrophoresed on 4% PAGE containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, 2.5% glycerol, and 0.1 mM EDTA. After electrophoresis, the gel was dried and exposed to x-ray film.

**Supershift assays using antibody to GATA 4 (sc-1237x; Santa Cruz Biotechnology; Santa Cruz, CA) were preincubated by incubating 0.5–2.0 μg of the antibody with 100 μg of the nuclear extract for 1 h at 4 °C. Nuclear extracts were incubated with 32P-labeled oligonucleotide probes as described above.

**Statistical Analysis**

ANOVA was used to test for differences among groups in luciferase reporter studies.

**RESULTS**

**Isolation of a Marine A1AR Promoter—To isolate the mA1Arp, a 125SVJ murine genomic library (CLONTECH) was screened using a probe generated from the 5′-coding region of the rat A1AR. Library screening resulted in the isolation of a 4.5-kilobase genomic fragment that was sequenced in full. The 3′-region of the genomic fragment contained 700 bp that was identical to the reported sequence of the murine A1AR (250 bp noncoding and 300 bp coding) (20). At 742 and 685 bp upstream of the initiator methionine site, sequences that were identical to the human A1AR promoter “A” (TTAAGA) and “B” (TT-TAAA) motifs were identified (Fig. 1).**

**Identification of a Transcription Start Site—We next determined the transcription start site of the mA1Arp using complementary methods of 5′-RACE and RNase mapping. mRNA from DDT1 MF-2 cells, which is a Syrian hamster myocyte cell line that expresses A1ARs at high levels (21), was used in these**
studies. In our hands, the concentration of A1ARs in DDT1 MF-2 cells is 253 ± 12 fmol/mg whole cell protein. Using 5'-RACE and RNase mapping, we identified a similar transcription start site 560 bp upstream of the initiation codon. Only one transcription start site was identified with each method.

**Studies of A1ARp Truncation Mutants**—To identify regions of the mA1ARp involved in control of A1AR gene expression, the expression of a series of mA1ARp-luciferase constructs was examined. The mA1ARp fragments were subcloned into the pGL3 reporter vector (Promega; Madison WI); different cell types were transfected with LipofectAMINE (Life Technologies, Inc.). To control for transfection efficiency, cells were co-transfected with a Renilla control reporter vector (pRL-CMV; Promega).

To examine the specificity of the observed responses, studies were performed in HeLa and HEPG2 cells that do not express A1ARs and in DDT1 MF-2 and MDCK cells that do express A1ARs (DDT1 MF-2, 236 ± 19 fmol/mg protein; MDCK, 45 ± 9 fmol/mg protein). Although we saw reporter expression in all cell types in which luciferase activity was driven by a control CMV-luciferase promoter (CMV-PGL3; Promega), no reporter expression was seen for any of the mA1ARp fragments tested in HEPG2 or HeLa cells (n = 4 separate studies using reporter constructs shown in Fig. 2). In contrast, we saw specific reporter activity after we transfected the DDT1 MF-2 and MDCK cells with mA1ARp gene fragments (Fig. 2).

Next, the expression of a broad series of mA1ARp truncation constructs was examined in DDT1 MF-2 cells. With progressive deletion of the 5' region of the A1ARp, reporter activity increased (Fig. 2). Additional truncation studies showed that, when base pairs from −500 to −250 were deleted, reporter expression declined (Fig. 3).

**Generation of A1ARp-nlacZ Transgenic Mice**—Because in *vitro* truncation reporter studies indicated that the proximal 500 bp of the mA1AR promoter resulted in high levels of reporter expression in cells that contain A1ARs, we next tested if this region played a role in A1AR expression in *vivo*. The proximal mA1ARp (−502 to +35) fragment was thus linked with a previously characterized β-galactosidase reporter construct for generation of transgenic mice (12, 13). The construct was injected into 100 murine oocytes that were then implanted into the uteri of pseudo-pregnant female mice. Forty mice were subsequently born, four males of which were positive for the mA1ARp-nlacZ transgene and were studied.

To analyze patterns of reporter gene expression during early development, founder males were mated with wild-type females. β-Galactosidase activity was examined in embryos, which were genotyped by PCR. In the embryos that were positive for the transgene (from two lines 5163 and 5174), a blue reaction product was present over the brain, spinal cord, and atria between PC 8.5–13 (Fig. 4). In contrast, no color reaction was seen in littermates that did not express the transgene (Fig. 4). This pattern of expression is identical to that seen by *in situ* hybridization or receptor-labeling autoradiography (6).

**Mutagenesis Studies of Putative GATA, Nkx2.5 Binding Sites, and TTAAAX Box Motif**—Next, we attempted to identify motifs within the proximal mA1ARp (−502 to +35) where transcription factors might bind. When we examined sequences within this region, we identified putative GATA, Nkx2.5 binding sites, and a potential TATA box. At position −434, a GGATAC motif was identified. This motif differs from the classical GATA binding motif of (A/T)GATA(A/G), but is shown to bind GATA proteins (22, 23). At position −243, the sequence TTAAAGA was identified, which is similar to the Nkx2.5 binding motif TNAAGTA (24, 25). This motif is similar to the “A"
promoter of the human A1ARp. At −85, the motif TTAAA was identified that corresponds with the “B” promoter of the human A1ARp.

To test the roles of these motifs on promoter activity, each was mutated and reporter assays were performed. Following conversion of the GATA motif to GTTA, reporter activity was markedly reduced (Fig. 5). Following conversion of the TTAAA to TTATGAA, reporter activity was reduced by 50% (Fig. 5). Following conversion of TTAAA to TCTACA, reporter activity was reduced by 70% (Fig. 5).

**Influence of GATA-4 and Nkx2.5 on A1ARp Expression**—Because GATA-4 and Nkx2.5 are important for heart development (26), and the mA1ARp contains putative GATA and Nkx2.5 binding sites, we next tested if GATA-4 and Nkx2.5 influence mA1ARp expression. A1ARp activity was thus examined after co-transfection with constructs driving the expression of GATA-4 or Nkx2.5 (provided by Dr. Robert Schwartz). These studies were performed in DDT1 MF-2 and HeLa cells.

In each cell line, co-expression of GATA-4 with −502 to +35 mA1ARp reporter constructs resulted in 20–40-fold increases in receptor expression for the constructs containing the GATA binding motif (Fig. 6). However, when the GATA motif was mutated to GTTA, there was no increased reporter activity (Fig. 6).

Following co-expression of Nkx2.5 with the mA1ARp reporter construct, mA1ARp reporter activity increased 15-fold (Fig. 6). However, when the Nkx2.5 motif was mutated to TCTACA, increased reporter activity was not seen (Fig. 6).

We also tested if Nkx2.5 and GATA-4 acted synergistically to drive A1AR expression, similar to that observed for the atrial natriuretic factor promoter (27, 28). Co-transfection studies were therefore performed by transfecting cells with amounts of Nkx2.5 and A1ARp expression constructs that individually did not induce reporter expression (Fig. 7). The studies showed that Nkx2.5 and GATA-4 acted synergistically to induce mA1ARp expression (Fig. 7).

**EMSA of Nuclear Extracts Interacting with GATA and Nkx2.5 Sites**—EMSA assays were next performed to test if the putative GATA and Nkx2.5 binding motifs interact with nuclear proteins. When a radiolabeled 20-bp oligonucleotide containing the GATA binding motif was incubated with DDT-MF2 cell nuclear extracts, one prominent band was seen (Fig. 8). When studies were performed with increasing concentrations of unlabeled oligonucleotides, the amount of radioactivity of the band decreased (Fig. 8). When the GATA site was mutated to GTTA, the amount of labeling of the band was markedly reduced (Fig. 8). When antibody against GATA-4 was added to the nuclear extracts, the size of the band representing the protein-DNA complex was shifted to a higher molecular weight (Fig. 8).

When a radiolabeled 20-bp oligonucleotide containing the Nkx2.5 binding motif was incubated with DDT-MF-2 cell nuclear extracts, one prominent band was seen (Fig. 9). When studies were performed with increasing concentrations of unlabeled oligonucleotides, the amount of radioactivity of the band decreased (Fig. 9). When the Nkx2.5 site in the competitor oligonucleotide was mutated to TCTACA, the amount of labeling was not reduced (Fig. 9). Because Nkx2.5 antibody was not available to us, we did not perform Nkx2.5 supershift studies.

**DISCUSSION**

To begin to identify the factors that regulate the expression of A1ARs, we isolated the murine A1AR promoter. Showing that the genomic fragment isolated contained a murine A1AR promoter, patterns of β-galactosidase expression in mA1ARp-<sup>lacZ</sup> mice were temporally and spatially similar to patterns of A1AR expression seen in rodents (6). When we compared human and murine A1AR promoter sequences, we detected sev-
eral similarities among the genes, further supporting the notion that we isolated a murine A1AR promoter.

In the human A1ARp, two promoter motifs designated “A” and “B” have been identified and shown to represent distinct transcriptional start sites (9). In the murine A1AR promoter, we identified identical motifs that were separated by 160 bp, whereas these motifs are separated by 650 bp in the human gene (9). Based on our mA1ARp truncation, mutation, and gel-shift studies, we believe that the “A” motif is a Nkx2.5 binding site. The “B” motif appears to be an unconventional TATA box similar to that reported for other genes (29, 30). Whereas there is evidence for two transcriptional start sites in the human A1ARp, we only observed one transcriptional start site for the murine A1AR promoter.

To characterize the mA1ARp, reporter assays were performed using Syrian hamster smooth muscle DDT1 MF-2 cells since they express A1ARs at high levels (21). We had hoped to examine murine A1AR promoter expression in murine cell lines that express endogenous A1ARs. However, we are unaware of murine cell lines expressing A1ARs at levels detectable by radioligand binding studies. Fortunately, although DDT1 MF-2 cell lines are not of murine origin, mA1ARp expression was readily apparent in these cells.

Studies of murine A1AR promoter truncation mutants showed that the reporter constructs spanning the region from −502 to +35 had the highest levels of activity. In contrast, when the distal regions of the mA1ARp were present in reporter constructs, receptor expression was greatly reduced, raising the possibility that this region contains binding domains for repression elements. Sequence analysis of the −502 to +35 fragment suggested the presence of GATA and Nkx2.5 binding sites; no other GATA or Nkx binding motifs were found

**FIG. 6.** GATA-4 and Nkx2.5 regulate A1AR promoter activity. HeLa cells were transfected with wild-type or mutated −502- to +35-bp A1AR-luciferase promoter constructs (0.5 μg/22-mm dish), and with GATA-4, Nkx2.5, or control (CON) expression vectors (0.5 μg/dish). Bars are averages of quadruplicate determinations and are representative of four such studies. *, p < 0.05; **, p < 0.01 ANOVA. Standard errors were less than 5% of mean values.

**FIG. 7.** Synergistic effects of GATA-4 and or Nkx2.5 on A1AR promoter expression. HeLa cells were transfected with the wild-type A1AR promoter construct (0.2 μg/22-mm dish), and with GATA-4, Nkx2.5, or control (CON) expression vectors (0.05 μg/dish). Bars are averages of quadruplicate determinations and are representative of four such studies. *, p < 0.05; **, p < 0.01 ANOVA. Standard errors were less than 5% of mean values.

**FIG. 8.** Gel shift assays showing interaction between GATA-4 and mA1ARp. Assays were performed with a 32P-labeled oligonucleotide (40 fmol, 3 × 10⁴ cpm/reaction). Lanes 1–4, DNA plus 2 μg of nuclear extract without competitor (lane 2), with GATA-4 binding site competitor (lane 3), and with mutated GATA-4 binding site competitor (M). For supershift studies, 2 μg of nuclear extract was preincubated with GATA-4 antisera for 30 min. F, free DNA; B, DNA bound by nuclear extract; S, DNA bound by nuclear extract incubated with GATA-4 antisera.

**FIG. 9.** Gel shift and assays showing interaction between Nkx2.5 and the mA1ARp. Assays were performed with a 32P-labeled oligonucleotide (40 fmol, 3 × 10⁴ cpm/reaction). Lanes 1–7, DNA plus 2.5 μg of nuclear extract without competitor (lane 1), with mutated Nkx2.5 oligonucleotide competitor (lane 2), and with different concentrations of NKX competitor (lanes 3 and 4); lane 5, no DNA extract. F, free DNA; B, DNA specifically bound by nuclear extract.
within this region. Mutation of the GATA or Nkx2.5 motifs resulted in reduction in promoter expression, suggesting that these sites play important roles in endogenous A1AR gene expression. When co-transfection studies were performed using GATA-4 and Nkx2.5 expression vectors, increased promoter activity was observed, supporting the notion that GATA-4 and Nkx2.5 can activate A1AR gene expression.

When cells were transfected with both GATA-4 and Nkx2.5, synergistic effects on mA1ARp activity were observed. In mice, GATA-4 and Nkx2.5 are expressed in the heart as early as postconceptional day 6.5 and play a role in driving cardiac gene expression (31–33). Recently, Nkx2.5 and GATA-4 have been shown to directly interact to drive the expression of the atrial natriuretic factor (ANF) promoter (27, 28), as we observed for the murine A1AR promoter.

Both ANF and A1ARs are expressed in the heart at early developmental stages (27, 28), although cardiac A1AR expression occurs at even earlier ages than ANF cardiac expression (6). It is thus tempting to speculate that GATA-4 and Nkx2.5 play a role in the early cardiac expression of A1ARs and ANF. Interestingly, Nkx2.5 is also expressed in the tongue during early gestation (33); we also observed A1AR gene expression in the tongue at the same developmental stages (6).

Whereas GATA-4 and Nkx2.5 may play a role in cardiac A1AR expression, these factors are not expressed in the central nervous system (31–33). Thus, other transcriptional activating factors will play a role in A1AR expression in the brain. Currently, the identity of these factors is unknown.

Overall, we now show that the proximal promoter of the murine A1AR contains critical elements for A1AR gene expression in the brain and heart. Murine A1AR promoter activity also appears to be potently regulated by the transcriptional activating factors GATA-4 and Nkx2.5, which interact at a specific site in the proximal A1AR promoter. Future studies are indicated to identify additional promoter regions and transcriptional regulators that influence A1AR expression in other important sites of adenosine action.

Acknowledgments—Dr. Jean Lachowicz is thanked for assistance in some of these studies. Dr. Robert J. Schwartz is thanked for providing GATA-4 and Nkx2.5 expression constructs. Dr. Patrick Gallagher is thanked for technical suggestions.

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