

Adrenomedullin Gene Expression Is Developmentally Regulated and Induced by Hypoxia in Rat Ventricular Cardiac Myocytes*

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Adrenomedullin is a recently discovered hypotensive peptide that is expressed in a variety of cell and tissue types. Using the technique of differential display, the adrenomedullin gene was observed to be differentially expressed in developing rat heart. Reverse transcription-polymerase chain reaction analysis revealed that the level of adrenomedullin mRNA was significantly higher in adult ventricular cardiac muscle as compared with embryonic day 17 ventricular cardiac muscle. Adrenomedullin receptor mRNA was constitutively expressed throughout development of the ventricular heart. Two potential hypoxia-inducible factor-1 (HIF-1) consensus binding sites were identified in the mouse adrenomedullin promoter at -1095 and -770 nucleotides from the transcription start site. Exposure of cultured adult rat ventricular cardiac myocytes to hypoxia (1% O₂) resulted in a significant, time-dependent increase in adrenomedullin mRNA levels. Transfection studies revealed that the 5'-flanking sequence of adrenomedullin was capable of mediating a hypoxia-inducible increase in transcription. Mutation of the putative HIF-1 consensus binding sites revealed that the major regulatory sequence that mediates the hypoxia-inducible transcriptional response is located at -1095. These data demonstrate that the adrenomedullin gene is developmentally regulated in ventricular cardiomyocytes, that adrenomedullin transcription can be induced by hypoxia, and that this response is primarily mediated by HIF-1 consensus sites in the adrenomedullin promoter.

Adrenomedullin (Adm)¹ is a recently discovered hypotensive peptide that was first identified in human pheochromocytoma tissue (1). The Adm peptide is expressed in a variety of rat tissues including the heart, adrenal medulla, brain, kidney, pancreas, lung, spleen, thyroid, and liver (2, 3). The rat Adm peptide consists of 50 amino acids and shows slight structural

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¹ The abbreviations used are: Adm, adrenomedullin; AdmR, adrenomedullin receptor; CGRP, calcitonin gene-related peptide; Epo, erythropoietin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; HIF-1, hypoxia-inducible factor-1; RT-PCR, reverse transcription-polymerase chain reaction; VEGF, vascular endothelial growth factor; kb, kilobase(s); bp, base pair; CMV, cytomegalovirus.

homology to the calcitonin gene-related peptide (CGRP) family (4). It is capable of acting through the CGRP receptor and the recently cloned Adm receptor (AdmR) (5).

The Adm peptide has been implicated as an important regulator in the renal and cardiovascular systems, where it has been observed to produce a dose-dependent increase in vasodilation (6, 7). The increased vasodilation was associated with a slight increase in glomerular filtration rate and natriuresis in the renal system (8). In the cardiovascular system, Ishiyama *et al.* (9) reported that the Adm peptide, in addition to producing a fall in blood pressure, elicited an increase in cardiac index and stroke volume without a subsequent change in heart rate.

Using the differential display technique (10, 11), we analyzed differential gene expression in the developing rat heart. The RNA samples analyzed were isolated from embryonic day 17; neonatal days 10, 17, and 21; and adult ventricular cardiac muscle. We observed 23 differentially expressed genes in this developmental series, and 15 were successfully reamplified and cloned. One of these clones was identified as the Adm gene.

In this study, we have determined that the expression of the Adm gene increases significantly in the developing rat heart, while the AdmR mRNA levels remained relatively constant throughout development. Furthermore, Adm mRNA levels increased in cultured adult ventricular cardiac myocytes in response to hypoxia as a function of time. Studies using an Adm promoter-luciferase reporter construct indicated that the increase in Adm mRNA occurred as a result of increased transcription in response to hypoxia. These studies suggest a potential role for Adm in the development of the heart and in the response of cardiomyocytes to hypoxic stress.

EXPERIMENTAL PROCEDURES

Vectors and Probes—The pGL2BmgAM5'-3' plasmid containing 3.2 kb of the mouse Adm promoter was obtained from Dr. E. J. Taparowsky (12) (Purdue University). The 3.2-kb Adm promoter fragment was removed by an *Xho*I and *Hind*III digest and subcloned using the same sites into pGL3Basic (Promega) upstream of the luciferase coding sequence, pAM/LUC.

Mutation of the putative HIF-1 binding sites was carried out by oligonucleotide-directed site mutagenesis (13). The pAM/LUC vector was used as the DNA template for PCR mutagenesis to produce mutated Adm promoter constructs, which lack one (pMutA or pMutB) or both of the potential HIF-1 binding sites (pMutAB). In pMutA, the core HIF-1 site (5'-ACGT-3') at -1095 upstream of the transcription binding site was replaced with 5'-AAAT-3'. In pMutB, the core HIF-1 site (5'-ACGT-3') at -770 upstream of the transcription binding site was replaced with 5'-AAAT-3'. In pMutAB, both the -1095 and the -770 HIF-1 sites were mutated to 5'-AAAT-3'. All mutated constructs were subcloned into the pGL3Basic vector and the mutations confirmed by sequence analysis of the final constructs.

To document specificity of the hypoxic response in our culture system, the pBT (positive control) and pBTmut (negative control) plasmids were used. These constructs were generously provided by Dr. Jawed Alam (Alton Ochsner Medical Foundation) (14). The plasmid pBT contained a *Bsr*BI/*Taq*I subfragment (residues 324–486) of the mouse heme oxygenase-1 promoter, which has been documented to contain two

HIF-1 consensus sites and to be responsible for hypoxia induction of transcription of the heme-oxygenase-1 gene (14). The two HIF-1 core sequences (5'-ACGT-3') within the BT fragment were mutated to 5'-AAAA-3' to produce the pBTmut plasmid. The mutated heme oxygenase-1 promoter (pBTmut) transfected into rat aortic vascular smooth muscle cells produced an 85% reduction in transcriptional activity in response to hypoxia (14). Other control plasmids were the pGL3Basic and pCMV/ β -gal (15) vectors.

Isolation of Ventricular Cardiac Tissue and Cells during Development—Holtzman-timed pregnant Sprague-Dawley rats were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN) on the 15th day of gestation. Ventricular cardiac tissue was isolated from embryonic day 17; neonatal days 10, 17, and 21; and adult (200–250 g) rats as described previously (16, 17).

Culture of Adult Cardiomyocytes—Adult rat ventricular cardiac myocytes were isolated and cultured exactly as described previously (16, 18). Proliferating, non-cardiomyocytes were eliminated from the cultures by addition of 10 μ M cytosine 1- β -D-arabino-5-ribose for 7 days. After 7 days in culture, the cardiac myocytes were fed with cytosine arabinoside-free medium, and the medium was replaced every other day. On day 12 of culture, the cardiomyocytes were used for the described experiments.

RNA Isolation—Total RNA was isolated from the developmental series of rat tissues using TriZOL reagent (Life Technologies, Inc.) following the protocol of the manufacturer. Total RNA was then treated with RNase-free DNase I according to standard protocol (19).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction—Total RNA from ventricular heart tissue and cells was reverse transcribed using an oligo(dT)_{12–18} primer (Life Technologies, Inc.) and MMLV-RT in a 20- μ l reaction as described previously (20). For Adm, the 50- μ l PCR reaction contained 2 μ l of the reverse transcribed RNA, 0.2 μ M of sense Adm primer_{51–74} (5'-CTC GGC TTC TCA TCG CAG TCA GTC-3'), 0.2 μ M of antisense Adm primer_{1242–1219} (5'-CAC ACG GGG AAC CAA ACA ACC TTA-3'), 0.2 mM dNTPs, 1.5 mM MgCl₂, 2.5 units of Taq DNA polymerase (Promega), and 1 \times supplied polymerase buffer (Promega). Thirty cycles of amplification were performed at 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min in a Perkin Elmer 9600 thermocycler. A final extension at 72 °C was carried out for 7 min. The expected 1192-bp PCR product was subcloned into pGEM5 and double-strand sequenced. For the AdmR, the PCR components were the same except that the following primers were used: AdmR sense_{709–730} (5'-AGCGCCACCAGCACCGAATACG-3') and AdmR antisense_{1179–1156} (5'-AGA GGA TGG GGT TGG CGA CAC AGT-3') (21). Thirty-five cycles of amplification were performed at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. A final extension at 72 °C was carried out for 7 min. This generated the expected 471-bp PCR product. For the GAPDH control, the PCR components were the same as above except that the primers used were as follows: sense GAPDH_{550–569} (5'-ACC ACA GTC CAT GCC ATC AC-3') and antisense GAPDH_{1001–982} (5'-TCC ACC ACC CTG TTG CTG TA-3'). Twenty cycles of amplification were performed at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. A final extension at 72 °C was carried out for 7 min. This generated the expected 452-bp PCR product. All PCR products were analyzed by agarose gel electrophoresis, visualized by staining with ethidium bromide, and verified by Southern blot analysis.

Southern Blot Analysis—The Adm RT-PCR amplified cDNA products were electrophoresed through a 0.5% agarose gel, and AdmR RT-PCR products were electrophoresed through a 1.5% agarose gel. All RT-PCR amplified cDNA products were blotted onto Zeta-Probe membrane (Bio-Rad). The DNA was then cross-linked to the membrane using ultraviolet light at 30 mJ. Three hundred ng of a nested oligo Adm_{227–204} (5'-AAC GGC GAG CGA ACC CAA TAA CAT-3') and AdmR_{1020–1044} (5'-GGT AGG GCA GCC AGC AGA TGA AA-3') were 5' end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase following standard protocols (19). These were then used for probing the appropriate Southern blots. Fifty nanograms of the GAPDH DNA fragment were labeled using the random prime protocol with [α -³²P]dCTP according to standard procedures (19). Blots were pre-hybridized for a minimum of 1 h at 63 °C for Adm blots, 77 °C for AdmR blots, and 63 °C for GAPDH blots in hybridization solution (10 \times Denhardt's reagent, 5 \times SSPE (0.9 M sodium chloride, 0.05 M sodium phosphate, and 5 mM EDTA pH 7.2), and 0.5% SDS). Each probe was denatured by boiling for 5 min, added to its appropriate blot, and allowed to hybridize overnight at the same temperature used for the pre-hybridization.

The membranes were washed under stringent conditions (0.1 \times SSPE, 0.1% SDS at 65 °C for 10 min), exposed to a phosphor screen overnight, and analyzed using the Storm 840 Imaging System and ImageQuant Software (Version 4.2, Molecular Dynamics).

For semi-quantitation of the data, Adm and AdmR mRNA levels were normalized to the GAPDH mRNA levels from the same RT reactions. Three independent RT-PCR analyses were performed, and the means \pm S.E. of each triplicate reaction were calculated.

Culture of HL-1 Cells—HL-1 cells are a differentiated and dividing cardiac myocyte cell line derived from mouse AT-1 cells (22). They were cultured as described (22) in HL-1 medium (ExCell 320 medium part A and B (JRH Biosciences), 10% fetal bovine serum (Whittaker lot number 4M1953), 10 μ g/ml insulin (Life Technologies, Inc.), 1 \times non-essential amino acids (Life Technologies, Inc.), 50 μ g/ml endothelial cell growth supplement (Upstate Biotechnology), 1 μ M retinoic acid (Sigma), and 0.1 mM norepinephrine (Sigma)).

Transfection of HL-1 Cells—Transient transfection of HL-1 cells was performed using LipofectAMINE Reagent (Life Technologies, Inc.). Cells were cultured in 6-well plates at a density of 1.6×10^6 cells/well in HL-1 medium. Twenty-four h later, 10 μ l of LipofectAMINE was added to 90 μ l of DMEM (pre-warmed to 37 °C) and allowed to incubate 30 min at room temperature. To this solution, 3 μ g of pCMV/ β -gal and 7 μ g of pAM/LUC, pGL3Basic, pMutA, pMutB, pMutAB, pBT, or pBTmut were resuspended in 100 μ l of DMEM and allowed to incubate for 30 min at room temperature. To this solution, 800 μ l of DMEM were then added to create the transfection solution. The cells to be transfected were rinsed once with DMEM, overlaid with the transfection solution, and incubated in a humidified tissue culture incubator at 37 °C in 95% air, 5% CO₂ for 6 h. One ml of HL-1 medium containing 20% fetal bovine serum and lacking antibiotics was then added. After 16 h, the solution was replaced with HL-1 medium, and the cells were placed at 30 °C in 95% air, 5% CO₂ for 8 h. Cells were then treated to normoxic (95% air, 5% CO₂) or hypoxic (1% O₂, 5% CO₂, 94% N₂) conditions at 30 °C for 6 or 12 h. The cells were incubated at 30 °C to increase the stability of the luciferase enzyme.

Luciferase and β -Galactosidase Assays—The 6-well plates containing the transfected HL-1 cells were placed on ice. The cells were washed twice with phosphate-buffered saline and lysed for 5 min with 250 μ l of cell lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100, and 0.5 mM dithiothreitol). The lysed cells were removed from the plate with the use of a cell scraper and centrifuged briefly at 12,000 \times g to pellet cell debris. Ten μ l of cell lysate were then used to perform the luciferase and β -galactosidase assays according to the Dual Light protocol (Tropix; Bedford, MA) with the exception that there was no delay between the addition of substrates and the measurement of relative light units. Each sample was counted for 10 s. After assaying for luciferase activity, the samples were incubated at room temperature for 30 min prior to assaying for β -galactosidase activity. Each sample was analyzed in triplicate and the mean data for all transfections were derived from at least three independent transfection studies. The β -galactosidase values were used as indicators of transfection efficiency and did not change significantly in response to hypoxia. The relative luciferase activity (mean \pm S.E.) was calculated as the luciferase/ β -galactosidase ratio (23). The luminometer used for detecting luciferase and β -galactosidase activities was manufactured by Dynatech Laboratories, model ML-2250.

Statistical Analysis—All data are expressed as the mean \pm S.E. (except where noted). Comparisons between and among the groups were made using either a one-way or two-way analysis of variance (ANOVA) with replication or a Student's *t* test. If significant differences were observed, either a Tukey's highly significant difference or a Student-Newman-Keuls test was performed post-hoc to determine which means were different. Data were statistically evaluated using the Excel (Version 7.0) and SigmaStat (Version 1.0) software packages. Differences were considered significant at a value of *p* < 0.05.

RESULTS

The Adm Gene Is Differentially Expressed during Heart Development while Levels of Its Receptor mRNA Remain Constant—The differential expression of a subset of mRNAs from a developmental series of rat ventricular heart tissues was analyzed using the differential display technique (10, 11). Sequence analysis of one of the cloned differential display products identified it as rat Adm, GenBank™ accession number D15069.

The differential expression of the Adm gene in a developmental series of ventricular cardiac muscle samples was confirmed using semi-quantitative RT-PCR analysis. The resulting 1192-bp PCR product was confirmed as being Adm by Southern blotting using a nested Adm_{227–204} oligonucleotide probe. The

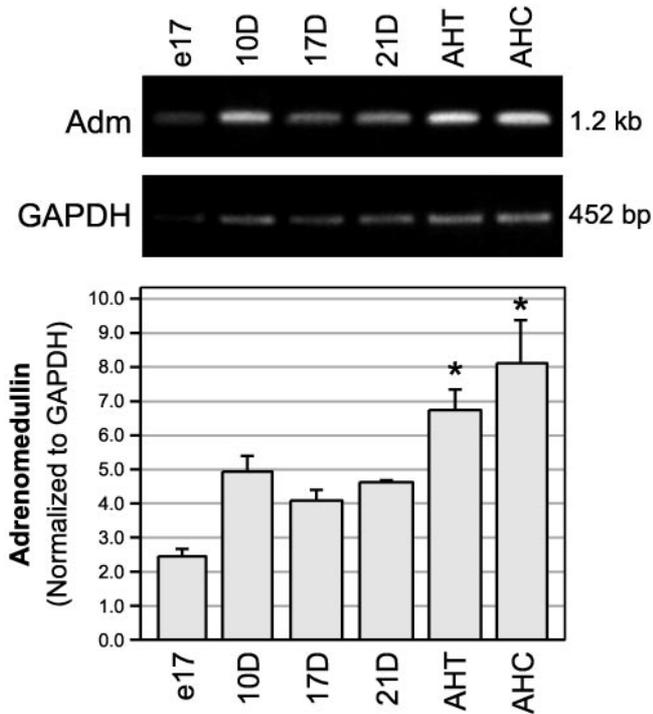


FIG. 1. Semiquantitative RT-PCR analysis of Adm mRNA levels during development of the rat heart. Adm mRNA is expressed in embryonic day 17 ventricular heart muscle (*e17*); neonatal ventricular heart muscle from days 10 (*10D*), 17 (*17D*), and 21 (*21D*); adult ventricular heart muscle (*AHT*); and isolated adult ventricular heart cells (*AHC*). Adm mRNA levels were normalized to GAPDH mRNA levels present in the same reverse transcription reactions. *Top panel*, representative ethidium bromide-stained agarose gels of the Adm and GAPDH RT-PCR products. *Bottom panel*, mean \pm S.E. of the Adm mRNA levels normalized to GAPDH mRNA levels (*, significantly different from E17; $p < 0.035$); each reaction was performed in triplicate on independent RNA samples.

data were normalized to the level of GAPDH mRNA in the same reverse transcription samples and the means \pm S.E. were plotted (Fig. 1). Adm mRNA was expressed at low levels in embryonic day 17 ventricular heart muscle. As the heart developed, an increase in the level of Adm mRNA was observed. This increase in expression was approximately 4-fold greater in isolated adult cardiac myocytes as compared with embryonic day 17 ventricular heart muscle ($p < 0.035$).

Expression of the adrenomedullin receptor was also analyzed by semi-quantitative RT-PCR analysis (Fig. 2). This data was normalized to GAPDH mRNA levels, and the means were calculated and plotted. There was a slight decrease in the level of AdmR mRNA as the heart developed, but this was not significantly different at the 90% confidence level, indicating that the AdmR mRNA is expressed at similar levels throughout cardiac development.

Temporal Expression of Adm and AdmR mRNA in Response to Hypoxia in Cultured Adult Rat Ventricular Cardiac Myocytes—A previous study using differential display to examine the expression of genes in the ischemic rat brain identified the Adm gene as being up-regulated in response to ischemia (28). Hypoxia has been reported to regulate the transcriptional expression of many genes, including the vascular endothelial growth factor (VEGF) and erythropoietin (Epo) genes (24). A HIF-1 element was identified in either the 5'- or 3'-untranslated region (UTR) of these hypoxia-inducible genes and found to be essential for induction by hypoxia (24). Upon analysis of the human Adm gene sequence, we identified three potential HIF-1 consensus binding sites in the 5'-flanking region (Fig. 3). Through analysis of the mouse Adm promoter sequence (Gen-

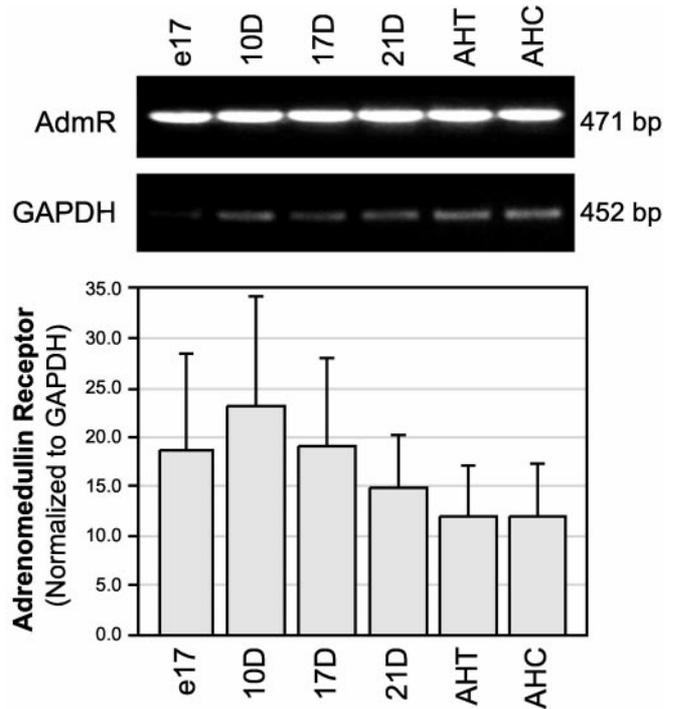


FIG. 2. Semiquantitative RT-PCR analysis of AdmR mRNA levels during development of the rat heart. AdmR mRNA is expressed in embryonic day 17 ventricular heart muscle (*e17*); neonatal ventricular heart muscle from days 10 (*10D*), 17 (*17D*), and 21 (*21D*); adult ventricular heart muscle (*AHT*); and isolated adult ventricular heart cells (*AHC*). AdmR mRNA levels were normalized to GAPDH mRNA levels present in the same reverse transcription reactions. *Top panel*, representative ethidium bromide-stained agarose gels of the AdmR and GAPDH RT-PCR products. *Bottom panel*, mean \pm S.E. of the AdmR mRNA levels normalized to GAPDH mRNA levels; each reaction was performed in triplicate on independent RNA samples. No significant difference between the AdmR mRNA levels at the various developmental time points was observed.

BankTM accession number D78349) (12), we were able to identify two potential HIF-1 consensus binding sites at -1102 to -1095 and -777 to -770 from the transcription start site. These sequences are 100% identical to the HIF-1 site over the core ACGT sequence and are significantly similar at the four other nucleotide positions (Fig. 3). We, therefore, determined whether expression of the Adm mRNA and/or peptide could be influenced by hypoxia. Preliminary radioimmunoassays revealed that hypoxia was capable of stimulating the secretion of Adm peptide from cultured adult rat ventricular cardiac myocytes.² Furthermore, this response was blocked by both actinomycin D and cyclohexamide, indicating that Adm peptide synthesis occurs by increased *de novo* transcription followed by translation rather than by secretion from stored pools. Therefore, we analyzed the levels of Adm mRNA in cultured adult ventricular cardiac myocytes. We observed a linear increase in Adm mRNA expression levels as a function of time of exposure to hypoxia, and this increase was significantly different from the normoxic controls at the 6- and 12-h time points (Fig. 4). In contrast, AdmR mRNA expression increased initially at 3 h of hypoxia and then decreased by 12 h of hypoxia (Fig. 5). The observed changes in Adm and AdmR mRNA levels were normalized to GAPDH mRNA levels, which remained constant during these studies (Figs. 4 and 5, respectively).

Activity of the Adm Promoter Increases in HL-1 Cells During Hypoxia—The increased levels of Adm mRNA in cultured adult

² D. M. Smith, S. Cormier-Regard, and W. C. Claycomb, unpublished data.

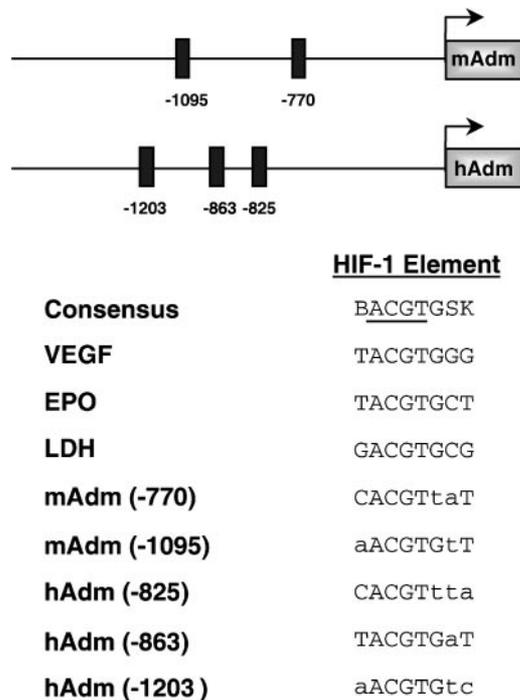


FIG. 3. Identification of potential HIF-1 sites in the mouse and human Adm promoter regions. *Top*, localization of potential HIF-1 sequences present within the 5'-flanking region of the mouse (*mAdm*) and human (*hAdm*) Adm gene and their distance in nucleotides from the transcriptional start site. *Bottom*, comparison of known HIF-1 elements with the potential HIF-1 elements in the mouse and human promoter regions. The HIF-1 consensus sequence is shown, and the HIF-1 core sequence is *underlined*.

ventricular cardiac myocytes suggested that Adm transcription was increased by hypoxia. To confirm that the increased expression of Adm mRNA was due to increased promoter activity, mouse Adm promoter activity was assayed in HL-1 cardiomyocytes (22) under normoxic and hypoxic conditions using a luciferase reporter construct.

To correct for variable transfection efficiencies, HL-1 cells were co-transfected with the pAM/LUC and pCMV/ β -gal plasmids (15). For pAM/LUC, the 3.2-kb Adm promoter was subcloned into the pGL3-Basic vector, which contains the firefly luciferase coding sequence and lacks eukaryotic promoter and enhancer elements. The pCMV/ β -gal plasmid contains the *Escherichia coli* β -galactosidase coding sequence under the control of the cytomegalovirus promoter. Cell lysates were assayed for luciferase and β -galactosidase levels, and the luciferase/ β -galactosidase ratios were determined. The assays were performed in triplicate on a minimum of least three independent transfection experiments, and the mean luciferase/ β -galactosidase values were obtained and then normalized to the results obtained with the pGL3-Basic vector. Results from these assays revealed a temporal increase in activity of the Adm promoter in response to hypoxia up to 12 h (Fig. 6). This increase was 1.8-fold greater than that observed in the normoxic controls. This level of induction of Adm mRNA in response to hypoxia is similar to that observed with the VEGF gene, where a 2–4-fold increase in VEGF transcription was observed (25).

Site-directed Mutagenesis of the Hypoxia-Responsive Elements in the Mouse Adm Promoter—To determine whether the cis-acting HIF-1 elements were responsible for mediating transcriptional activation of Adm in response to hypoxia, HL-1 cells were transiently transfected with plasmids containing mutations in the putative HIF-1 consensus sites. A 1.7-kb Adm promoter fragment (–1891 to –159 upstream of the transcrip-

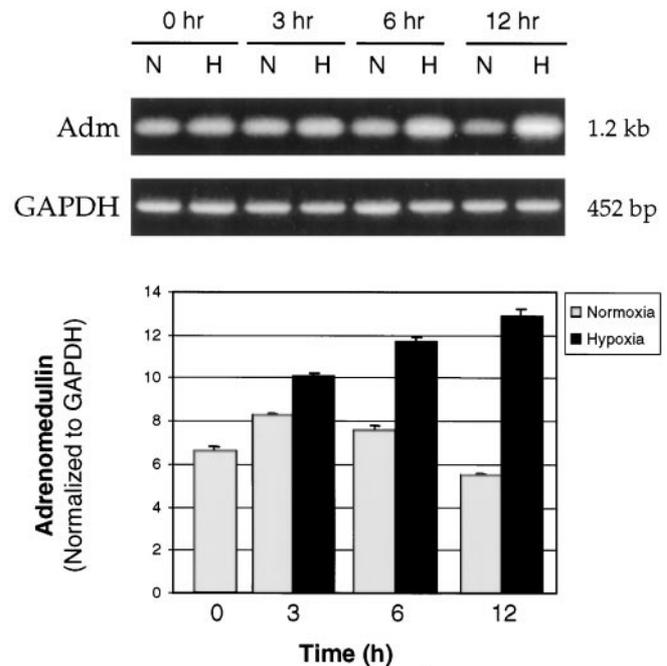


FIG. 4. Semiquantitative RT-PCR analysis of Adm mRNA levels in cultured adult rat ventricular cardiac myocytes in response to normoxia and hypoxia. Adm mRNA expression at 0, 3, 6, and 12 h of normoxia (N) and hypoxia (H). Adm mRNA levels were normalized to GAPDH mRNA levels present in the same reverse transcription reactions. *Top panel*, representative ethidium bromide-stained agarose gels of the Adm and GAPDH RT-PCR products. *Bottom panel*, mean \pm S.E. of the Adm mRNA levels normalized to GAPDH mRNA levels in cultured, adult ventricular cardiomyocytes under normoxic and hypoxic conditions for the indicated times; each reaction was performed in triplicate on independent RNA samples. Adm mRNA levels at 6 and 12 h of hypoxia are statistically significant from the 0 h normoxia control ($p < 0.05$).

tion start site) was subcloned into the pGL3Basic vector. This vector was then used as the DNA template in PCR mutagenesis to produce mutated Adm promoter constructs, which contained mutated sites at one (pMutA or pMutB) or both of the potential HIF-1 binding sites (pMutAB). In pMutA, the core HIF-1 site (5'-ACGT-3') at –1095 upstream of the transcription binding site was replaced with 5'-AAAT-3'. In pMutB, the core HIF-1 site (5'-ACGT-3') at –770 upstream of the transcription binding site was replaced with 5'-AAAT-3'. In pMutAB, both the –1095 and the –770 HIF-1 sites were mutated to 5'-AAAT-3'. Previous studies using other hypoxia responsive genes have demonstrated that similar mutations within the 5'-ACGT-3' core would attenuate HIF-1 enhancer function under hypoxic conditions (14, 26). All mutated constructs were subcloned into the pGL3Basic vector, and the mutations were confirmed by sequence analysis of the final constructs.

These constructs were transfected into HL-1 cells, and their ability to regulate transcription in response to hypoxia was assessed. A 54% reduction in hypoxia-induced transcription was observed when both putative HIF-1 responsive elements were mutated in the mouse Adm promoter. Mutation of the HIF-1 site at –1095 (pMutA) is primarily responsible for this result since a 52% decrease in transcription is observed with this construct alone and less of a decrease (23%) is observed with the pMutB construct (Fig. 7). In order to verify this hypoxic response in the HL-1 cells, we used the mouse heme oxygenase-1 gene construct, pBT, and its mutated construct, pBTmut, (14) as controls. The pBT construct has been shown previously to respond to hypoxia in rat aortic vascular smooth muscle cells, and mutation of the HIF-1 sequences in the pBTmut construct substantially decreased (85%) HIF-1 binding

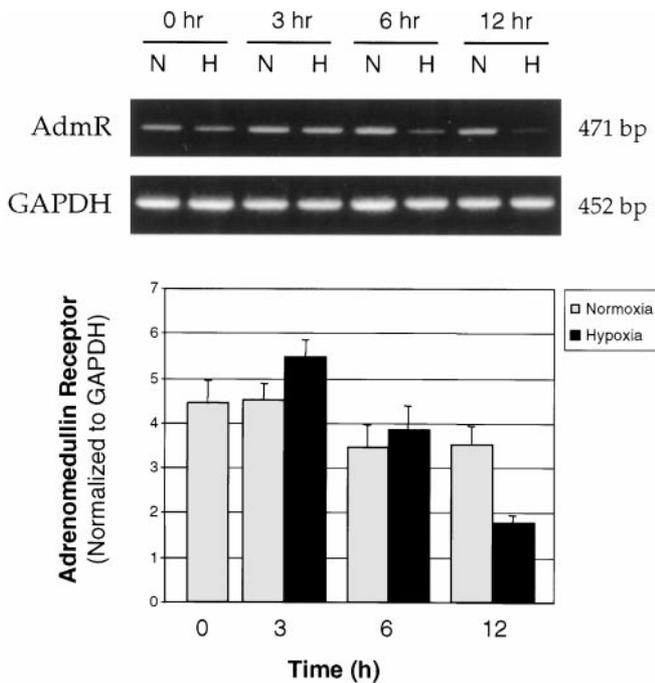


FIG. 5. Semiquantitative RT-PCR analysis of AdmR mRNA levels in cultured adult rat ventricular cardiac myocytes in response to normoxia and hypoxia. AdmR mRNA expression at 0, 3, 6, and 12 h of normoxia (N) and hypoxia (H). AdmR mRNA levels were normalized to GAPDH mRNA levels present in the same reverse transcription reactions. *Top panel*, representative ethidium bromide-stained agarose gels of the AdmR and GAPDH RT-PCR products. *Bottom panel*, mean \pm S.E. of the AdmR mRNA levels normalized to GAPDH mRNA levels in cultured adult ventricular cardiomyocytes under normoxic and hypoxic conditions for the indicated times. Each reaction was performed in triplicate on independent RNA samples. No significant difference was observed between the AdmR mRNA levels as compared with the 0 h normoxia control.

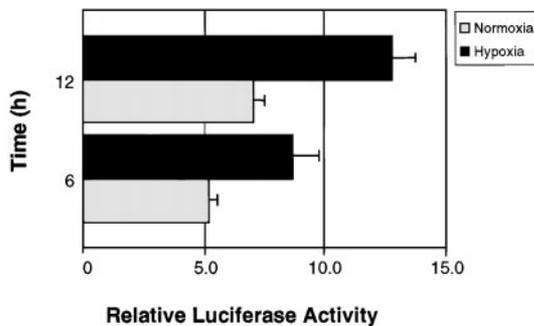


FIG. 6. Transcriptional activation of the Adm promoter in response to hypoxia. The 3.2-kb mouse Adm 5'-flanking sequence was cloned upstream of a luciferase reporter (pAM/LUC). This construct was transfected into HL-1 cells, and the relative luciferase activity was determined in response to 6 or 12 h of hypoxia. Data are shown as mean \pm S.E. of three independent transfection experiments, with each transfection experiment being analyzed in triplicate.

and hypoxia dependent gene activation in these cells (14). In the HL-1 cells, we observed a 49% reduction in transcription activity with the mutated heme oxygenase fragment (pBTmut) in response to hypoxia as compared with the pBT control (Fig. 7).

DISCUSSION

Using differential display and semiquantitative RT-PCR analysis, we have determined that the expression of the Adm gene is developmentally regulated in the rat heart. Our observation is in accordance with previous immunocytochemical data presented by Montuenga *et al.* (27). Interestingly, they show that the heart is the first organ to express the Adm

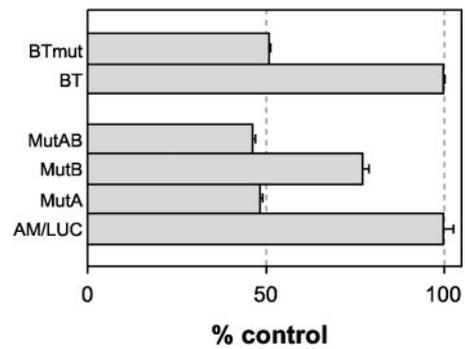


FIG. 7. Mutational analysis of the HIF-1 consensus sites in the Adm promoter. The pAM/LUC, pMutA, pMutB, pMutAB, pBT, and pBTmut plasmids were transfected into HL-1 cells, and their ability to promote transcription of a luciferase reporter was measured after 12 h of hypoxia. The pMutA, pMutB, and pMutAB data are plotted as percent of pAM/LUC. The pBTmut was plotted as percent of the pBT. Data are shown as percent of control \pm S.E. of three independent transfection experiments, with each transfection experiment being analyzed in triplicate.

peptide in both the mouse and rat, with expression first being observed as early as embryonic day eight (27). Furthermore, they show that localization of the Adm peptide corresponds to the degree of cellular differentiation in several organs (27). In our studies, Adm mRNA expression increases during cardiac muscle development, indicating a possible role for the Adm peptide in growth and differentiation. Whether Adm plays a role in heart development and, if so, what that role might be remains to be determined.

The increased expression of Adm in hypoxic brain tissue (28) suggested, to us, a role for Adm in the hypoxic response of the heart. Recent studies have shown an increase in blood levels of Adm peptide in human congestive heart failure (29, 30) and, furthermore, that this Adm peptide secretion originates from the heart and is correlated with the severity of the heart disease (31). In addition, cardiac Adm peptide synthesis and secretion has been demonstrated to be induced in a rat heart failure model (31). Cumulatively, these studies suggest a possible involvement for Adm in the response of the heart to ischemic stress. Our preliminary studies showed that cultured adult ventricular cardiomyocytes secrete the Adm peptide in response to hypoxia.² In addition, we show here that cardiomyocyte Adm mRNA levels increase temporally in response to hypoxia. This response reached a maximum at 12 h of hypoxia and was approximately 2.3-fold greater than the normoxic control (Fig. 4). Results from our transfection studies indicate that this increase is regulated by the Adm promoter. We found Adm promoter activity to increase 1.8-fold over control after 12 h of hypoxia, as assessed using a luciferase reporter. Although this increase in transcription in response to hypoxia appears to be modest, our data are consistent with that of other hypoxia-responsive genes such as VEGF (25), Src kinase (32), Fyn kinase (32), and Epo (33), where 1.7–4.0-fold increases in transcription have been observed. In contrast, the AdmR mRNA levels decreased somewhat with increasing time of exposure to hypoxia.

Our studies provide the first evidence that the Adm promoter contains HIF-1 sites that are capable of responding to hypoxia. Hypoxic regulatory elements have been identified in both the 5'- and 3'-flanking regions of numerous hypoxia-inducible genes (24). We have demonstrated that the 5'-flanking region of the Adm gene was capable of conferring hypoxia responsiveness in transient transfection assays performed in HL-1 cells. Sequence analysis of the mouse Adm 5'-flanking region has revealed the presence of two potential HIF-1 binding sites at -1095 and -770 upstream from the transcription start site.

These two sites are 100% identical to the HIF-1 core binding site, 5'-ACGT-3' (24), and similar to the defined HIF-1 consensus binding site, 5'-BACGTGSK-3' (23, 24) (B = C, G, or T; S = C or G; K = G or T). Mutation analyses of the HIF-1 sites in the mouse Adm 5'-flanking region revealed that there are two functional hypoxia-responsive sites (Fig. 7) and that their disruption leads to a substantial decrease (54%) in the transcriptional response of Adm to hypoxia. We found that the HIF-1 binding site at -1095 was largely responsible for the observed hypoxic response of Adm and that the HIF-1 binding site at -770 was only responsible for a small portion of the hypoxic response. Interestingly, mutation of HIF binding sites at -1095 and -770 were not additive and failed to abolish all transcriptional activity in response to hypoxia. The lack of a complete attenuation of the transcriptional hypoxia response suggests that other as yet unidentified cis-acting elements may be responsible for the full transcriptional response of Adm. A tripartite hypoxia-inducible enhancer has recently been proposed in which elimination of one of these sites in the Epo promoter resulted in a decrease but not a total elimination of the hypoxic response (34). This tripartite enhancer has been proposed for the two other well characterized hypoxia-responsive genes (VEGF and lactate dehydrogenase), and further functional studies may elucidate the presence of such an enhancer in the Adm promoter.

The physiological reason for the increase in cardiomyocyte Adm mRNA expression and peptide secretion in response to hypoxia is not clear, but this response may provide a compensatory mechanism for the ischemic heart. Adm is a potent hypotensive peptide (1) and has been shown to be important in the regulation of both the renal and cardiovascular systems (35). In addition to decreasing blood pressure, the Adm peptide is capable of increasing the cardiac index and stroke volume without a subsequent change in the heart rate (9). Further studies are needed to clarify the exact physiological role of the Adm peptide in the response of the heart to hypoxia.

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