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Upstream Regions Directing Heart-Specific Expression of the GATA6 Gene During Mouse Early Development

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The expression of murine transcription factor GATA6 is restricted to tissues including the heart and gastrointestinal systems during embryogenesis, and is maintained throughout postnatal life. We have characterized the 5′ upstream region (6.4 kb) of the mouse GATA6 gene, and identified two closely spaced transcription initiation sites. The flanking sequence lacks a typical TATA-box, and is rich in guanine and cytosine. The role of the 5′ upstream region was examined using the *lacZ* reporter gene in transgenic mice. A construct containing the 5′ flanking sequence (4.9 kb), untranslated exon 1 and 1.3 kb intron 1 could drive the gene expression in the embryonic and adult heart regions. Weak expression was also observed in the stomach, liver, and bronchial arch in addition to the cardiac region. Deletion of the 5′ upstream region (~1.2 kb) or intron 1 abolished all this expression, indicating that at least two *cis*-acting control elements are necessary for heart-specific expression of GATA6 *in vivo*.

Key words: *cis*-elements, GATA6, heart, transcriptional regulation, transgenic mice.

In vertebrates, the GATA family transcription factors play crucial roles in the differentiation of a number of tissues during development (for reviews, see *1–3*). These factors bind to the consensus sequence (A/T)GATA(A/G) through a conserved Cys-X_2-Cys-X_{17}-Cys-X_2-Cys zinc finger motif. Six members of the family have been identified in vertebrates, each of them being expressed in a developmentally regulated and lineage-restricted manner.

Members of the GATA4/GATA5/GATA6 subfamily show a partially overlapping expression pattern in the heart and intestinal tract (*1–6*). During mouse embryogenesis, GATA4 is expressed in the visceral and parietal endoderm at E5.5 (embryonic day 5.5) (*5*, *7*), and the prospective cardiac splanchic mesoderm at E 7.5 (*3*, *8*, *9*), and continues to be expressed in the endocardium and the myocardium of the folding heart. Consistent with this expression, GATA4-deficient embryos show defects in ventral morphogenesis and heart tube formation (*10*, *11*). The cardiac expression of murine GATA5 is first detected in the precardiac mesoderm, and then appears in the atrial and ventricular chambers, and becomes restricted to the atrial endocardium (*5*). Inactivation of the GATA5 gene did not result in embryonic lethality (*10*), suggesting that the GATA5 function may overlap with those of GATA4 and GATA6 in early development. Murine GATA6 is coexpressed with GATA4 in the primitive streak mouse embryo, and its expression is restricted to the precardiac mesoderm, embryonic heart tube and primitive gut. It is also expressed in the developing respiratory system, urogenital tracts and bladder (*5*, *12*, *13*). Several target genes in these tissues have been shown to be regulated by GATA6 (*14–16*). Targeted mutation of GATA6 leads to lethality within ~12 h of the onset of scheduled GATA6 expression (E6.5) (*7*, *17*), suggesting that GATA6 is required for the survival of the embryo in the early primitive streak stage.

The regulatory mechanisms inducing GATA6 transcription in the heart and gastrointestinal systems are of particular interest because its expression starts as early as E5.5, and is maintained in cardiac and extracardiac tissues throughout postnatal life. In addition, only the GATA6 gene is expressed in the developing bronchi, urogenital ridge, and bladder (*5*). Thus, an understanding of the temporal and spatial expression of the GATA6 gene should provide an insight into the regulatory networks involved in cardiac and endodermal lineage determination. In this study, we examined the *cis*-acting sequences controlling GATA6 expression during mouse early development. Our results showed that a region including the 5′ flanking sequence (4.9 kb), untranslated exon 1 and 1.3 kb intron 1 is sufficient to activate the expression in the cardiac tissues, bronchial epithelium, and stomach during embryonic development.

MATERIALS AND METHODS

Cloning and Sequencing of the Mouse GATA6 Gene—A 257 bp *Eco*RI–*Mlu*I rat GATA6 cDNA fragment (*1*) was used to probe a 129SVJ mouse genomic library constructed on the Lamda Fix II vector (Stratagene, La Jolla, CA). Approximately 2×10^6 recombinant phages were plated and then their DNAs were transferred to nitrocellulose filters using standard procedures (Sambrook *et al.*, 1989). Twenty-five positive clones were purified and subcloned into pBluescript for sequencing. All positive clones contained the same 16 kb insertion.

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Oligo-Capping Analysis of GATA6 mRNA—The transcriptional initiation sites were determined using the oligo-capping method that specifically replaces the 5′-cap structure of full-length mRNA with a synthetic RNA oligonucleotide (R-OLIGO) (*18–20*). CapSite cDNA (Nippon Gene) from mouse heart was used as a template for PCR with Primer 1RC (Nippon Gene) corresponding to the oligonucleotide sequence ligated at the cap site and a GATA6 gene–specific primer, MGGSP-7A (5′-GGCCGAGCTGCGGACAGCGAGCTGTAC-3′). The PCR product was then used as a template for the second round PCR with nested primers 2RC (Nippon Gene) and MGGSP-A5 (5′-GCCCCGGGAGCAGGAGGAGGACGAA-3′). The positions of the primers MGGSP-7A or MGGSP-5A are shown in Fig. 2B. The amplified products were purified, cloned and sequenced.

GATA6/lacZ Transgene Constructs—The 6.8 kb (−4861 to +2008 bp) fragments of the GATA6 gene were cloned into plasmid pBS-GAL1 with SV40 sequences at the 3′ end of the *lacZ* gene to provide a polyadenylation signal (*21*), and the resulting plasmid was designated pYCG6P. In plasmid pYCG6P, the *lacZ* gene was connected in frame with the GATA6. DNA fragments (Fig. 1B, GATA6/lacZ-1 and -2) containing the *lacZ* and 5′ flanking regions of GATA6 were generated by digestion of pYCG6P with restriction emzymes *Not*I and *Sca*I, respectively. GATA6/lacZ-3 was constructed by PCR mutagenesis to remove the intron region with primers MG6DintA (5′-CAACCTGACTTTTGATTCCTCGAGCGATGTGCGAGA-3′) and MG6Dint1S (5′-TCTCGCACATCGCTCGAGGAATCAAAAGTCAGGTTG-3′).

Production of Transgenic Mice and Embryos—DNA fragments were separated from the vector DNA on an agarose gel, purified using a Concert Rapid Gel Extraction System (GIBCO), and then microinjected into one-cell stage embryos obtained from BDF1 female mice mated with BDF1 male (*22*). Injected embryos were implanted into pseudo-pregnant ICR or BDF1 female mice. The founder animals were identified by PCR analysis of genomic DNA using primers in the *lacZ* gene (*23*).

β-Galactosidase Staining and Histology—The methods for the analysis of transgenic mice were described previously (*22*). Transgenic males were bred with non-transgenic C57B6/J females for timed matings. For the F_0 analysis of *lacZ* expression, embryos were isolated from implanted foster mothers 8–10 days after injection. Verification of transgenic animals was performed by PCR analysis of yolk sac DNA and primers as described above. Whole embryos or their tissues were fixed in ice-cold phosphate-buffered saline containing 4% paraformaldehyde for 5 to 30 min depending on the size of the embryos or tissues, washed twice in the same buffer containing 0.01% sodium deoxycholate and 0.02% NP-40, and then stained overnight in a solution comprising 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), 0.01% sodium deoxycholate, 0.02% NP-40, 20 mM Tris-HCl (pH 7.3), and 0.1 M sodium phosphate (pH 7.3). The specimens were postfixed overnight in 4% paraformaldehyde after washing off the X-gal. The embryos were dehydrated with ethanol, cleared in xylene or benzene, embedded in paraffin, sectioned at 5 μm, rehydrated, and then counterstained with Nuclear Fast Red (*24*).

Whole-Mouse In Situ Hybridization—Embryos were isolated after timed matings of C57B6/J at E8.5–9.5 and fixed overnight in 4% paraformaldehyde/PBS, dehydrated in methanol and stored at −20°C. Whole-mount *in situ* hybridization was as described (*22*). GATA6 antisense digoxigenin-labeled RNA probe was synthesized with T7 or SP6 RNA polymerase (Promega) from a subclone of GATA6 covering nucleotides (+1534 to +2061) in exon 2 (Fig. 2A).

RESULTS

Isolation and Characterization of the Mouse GATA6 Gene—A genomic clone for the mouse GATA6 gene was isolated using rat GATA6 cDNA (Tamura *et al.*, 1993) as a probe. The isolated clone had three coding exons (exon 2, 3, and 4) (Fig. 1A) covering the amino-terminal part of the GATA6 protein. The transcription initiation site(s) of adult mouse heart GATA6 mRNA was determined by oligo-capping analysis (*18–20, 25*). Sequence analysis of the products amplified with the primers corresponding to the oligonucleotide ligated at the 5′ cap site and GATA6 gene–specific primers (Fig. 2A) revealed that the GATA6 gene has two transcription initiation sites (I and II) (Fig. 2B). Transcription initiation site I is 89 bp upstream from site II, suggesting that GATA6 transcription starts from the same promoter. No TATA-box was found immediately upstream of the two initiation sites, indicating that GATA6 is a TATA-less gene. Comparison of the PCR products amplified with cap- and gene-specific primers with the corresponding genomic sequence revealed that the GATA6 gene has a

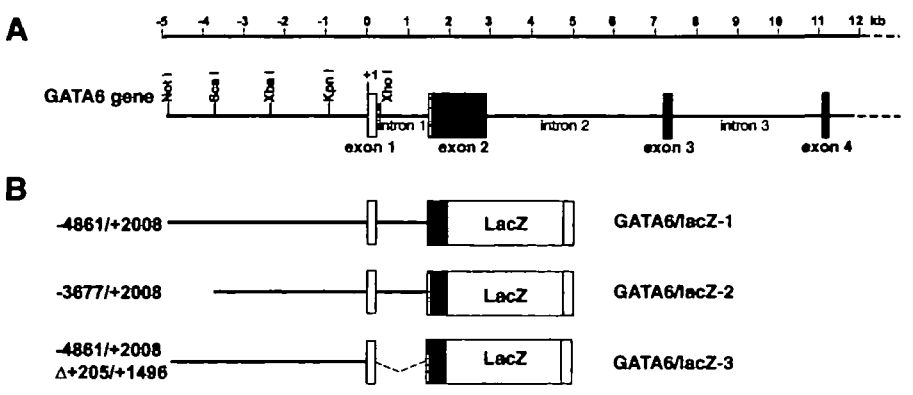


Fig. 1. **Structures of the mouse GATA6 gene and constructs used for transgenic experiments.** (A) Mouse GATA6 gene. The open box and closed boxes represent untranslated (exon 1) and coding (exons 2 to 4) sequences, respectively. (B) Transgenic constructs ligated with the *lacZ* gene. GATA6/lacZ 1 and 2 with different lengths of the 5′ flanking sequence linked to the *lacZ* reporter gene for the generation of transgenic mice were obtained by digestion of pYCG6P with *Not*I and *Sca*I, respectively. The 3′ end of the *lacZ* was ligated with the SV40 sequence (shadowed box) containing a polyadenylation signal.

non-coding exon 1 located 1.3 kb upstream of exon 2.

Comparing DNA sequence from the published mouse GATA6 cDNA (*5*) and the genomic DNA, we found that the nucleotide sequence of the published cDNA contains two extra guanine nucleotides (between +2708 and +2709 in exon 2) that change the reading frame for 10 amino acid residues and an additional insertion of a guanine (between +2736 and +2737) that shifted the reading frame back with an insertion of glycine. Therefore, the amino acid residues in this region are changed from "GRELHSRCHGGP" to "AGAPLPVPRGP." The amino acid sequence in this region "AGAPLPVPRGP" is completely conserved in that of rat (*1*) and human GATA6 (*13*, *26*), and is also similar to a RT-PCR sequence of mouse GATA6 directly submitted to the Genbank Database (AF179425).

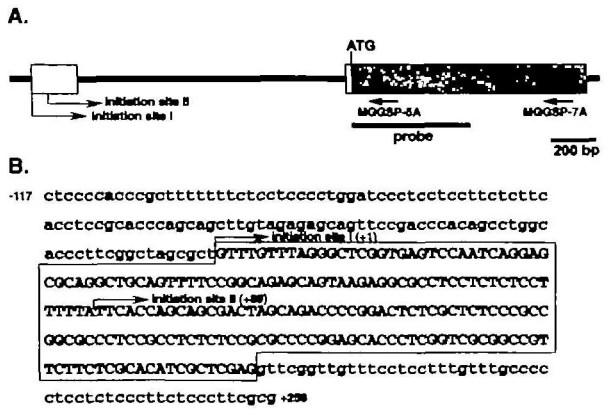


Fig. 2. **The 5′ end structure and transcription initiation of the GATA6 gene.** (A) Two antisense primers, MGGSP-5A and MGGSP-7A (indicated by arrows), that are located in the second exon, were used for oligo-capping analysis. The positions of initiation sites are shown. The region for generation of RNA probe used in *in situ* hybridizatin is also indicated by a closed bar. (B) The initiation sites determined by sequence analysis are indicated by arrows. The region of exon 1 is boxed.

The GATA6/lacZ-1 Drives lacZ Expression in the Heart, Bronchi, as Well as Liver and Stomach—The ability of upstream sequences to induce gene expression during embryogenesis was examined in transgenic mice. We first tested the GATA6/lacZ-1 reporter construct which includes 4.9 kb of 5′ flanking sequence, untranslated exon 1, intron 1 and 513 bp of exon 2 ligated upstream the *lacZ* gene (Fig. 1, GATA6/lacZ-1). Of the 60 mice born from foster mothers, 10 carried the transgene. Embryonic *lacZ* gene expression was analyzed after timed mating of the transgenic with nontransgenic mice, and three lines were noted to exhibit similar *lacZ* staining.

At E8.5–9.0, the *lacZ* expression was restricted to the primitive heart tube region (Fig. 3A), whereas no expression was observed in transgene-negative littermates (Fig. 3B). A sagittal section of the heart tube showed that ex-

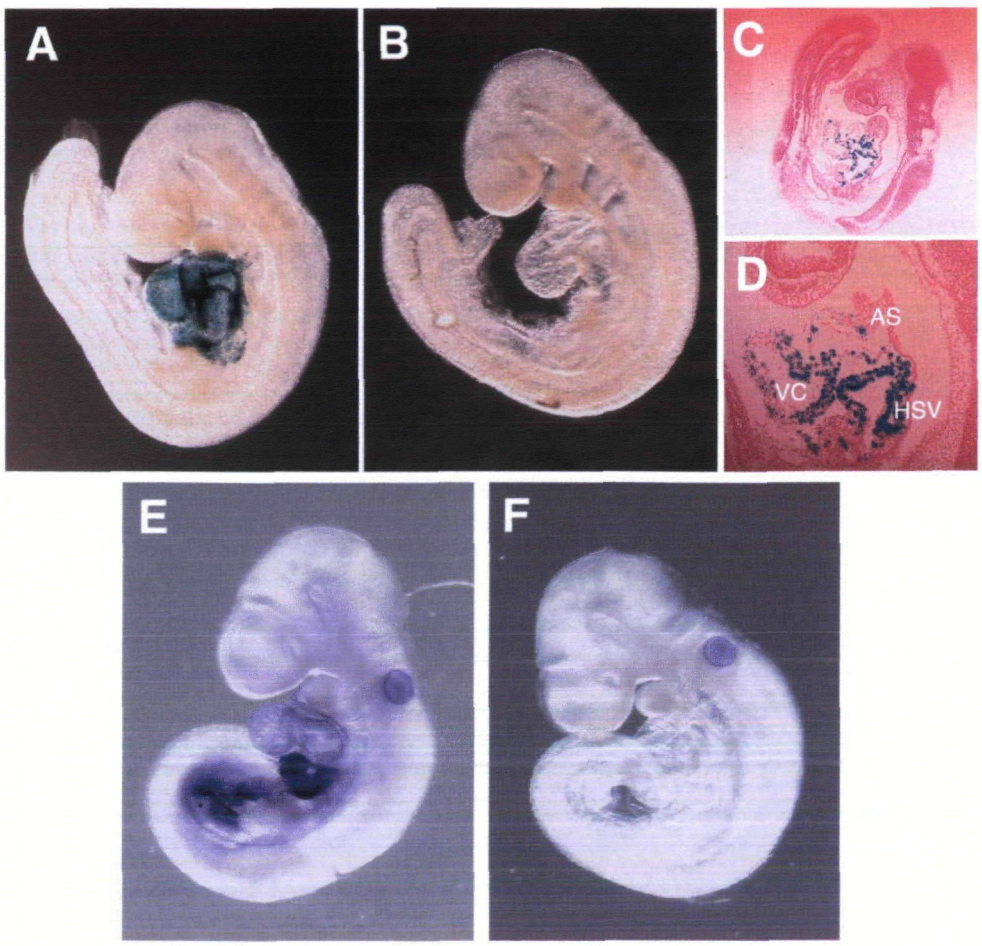


Fig. 3. ***lacZ* expression from the GATA6/lacZ 1 at E8.5–9.0.** Founder transgenic mice bearing a transgene fragment (4.9 kb of 5′ flanking sequence, untranslated exon 1, intron 1 and 513 bp of exon 2) linked to *lacZ* (GATA6/lacZ-1, Fig. 1) were analyzed at E8.5–9.0. Whole-mount staining of a transgenic embryo (A) and a non-transgenic littermate (B) is shown. *lacZ* expression is apparent in the heart tube region. Sagittal sections of the transgenic embryo (A) stained with Nuclear Fast Red (C, D). The *lacZ* expression is restricted to the myocardial wall of the ventricular chamber (VC), aortic sac (AS), and horns of the sinus venosus (HSV). The endogenous pattern of GATA6 gene expression was determined by *in situ* hybridization with digoxigenin-labeled GATA6 antisense (E) and sense (F) RNA probes.

pression was restricted to the myocardial wall of the ventricular chamber, aortic sac, and horns of the sinus venosus (Fig. 3, C and D). Low *lacZ* expression was detected in the midgut, but the expression was not detectable in hindgut where the expression of endogenous GATA6 was confirmed by *in situ* hybridization (*5*).

At E11.5–12.5, strong *lacZ* staining was observed in the heart region (Fig. 4A) and sections are shown in Fig. 4, B–F. *lacZ* expression was observed in the outflow tract of the heart (OT, Fig. 4B), thoracic wall overlying the pericardial cavity (TW, Fig. 4B), atrioventricular canal (AVC, Fig. 4B), and myocardial wall of both the ventricular chambers and atrial chambers (VC and AC, Fig. 4, B and E). The expression of *lacZ* was also observed in the lumen of the primitive stomach (ST, Fig. 4, C and D), hepatic sinusoids (HS, Fig. 4D), wall of the bladder (BL, Fig. 4D), lumen of the trachea (LT, Fig. 4E), and main bronchi (MB, Fig. 4F). The expression levels in these tissues were significantly lower than

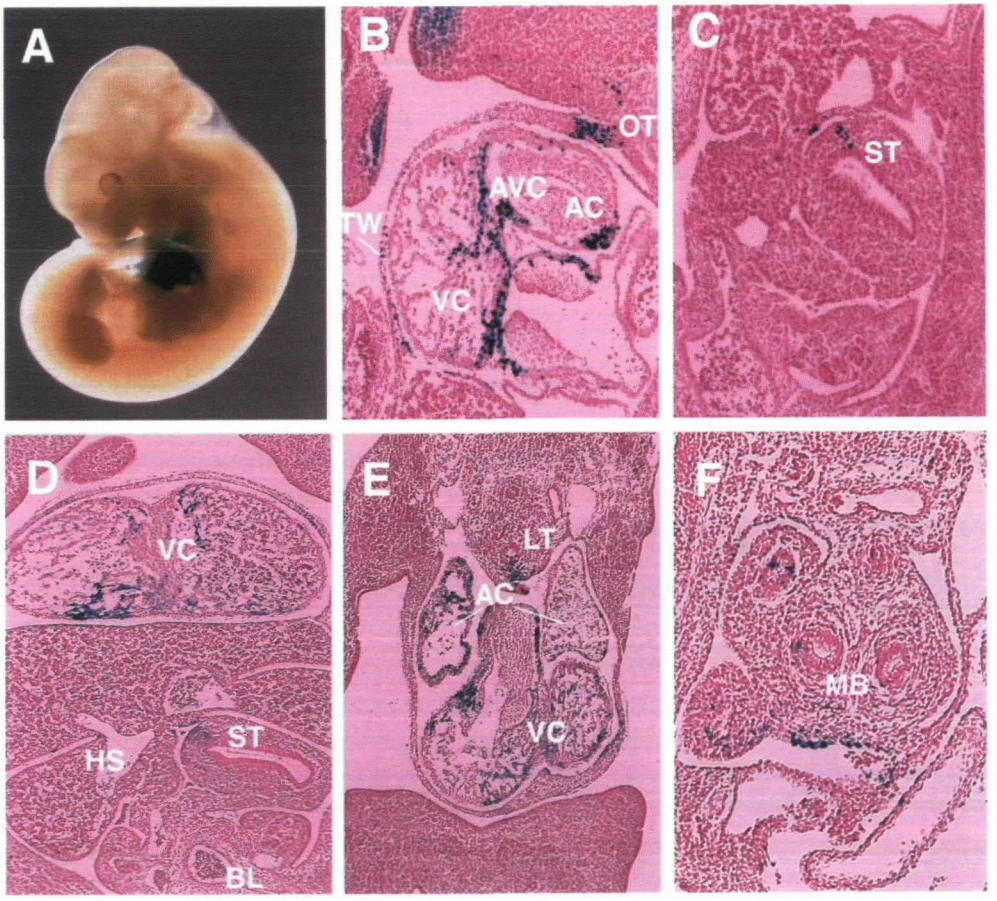


Fig. 4. ***lacZ* expression from the GATA6/lacZ 1 at E11.5–12.0.** (A) Whole-mount staining of transgenic embryo. Sagittal (B-C), longitudinal (D-E) and transverse (F) sections of whole-mount stained transgenic embryos were stained with Nuclear Fast Red. *lacZ* expression was observed in the outflow tract of the heart (OT), thoracic wall overlying the pericardial cavity (TW), atrio-ventricular canal (AVC), myocardial wall of both the ventricular chambers and atrial chambers (VC and AC). *lacZ* expression was also observed in the lumen of the primitive stomach (ST), lumen of the trachea (LT), main bronchi (MB), hepatic sinusoids within liver (HS), and wall of the bladder (BL) in addition to the cardiac region.

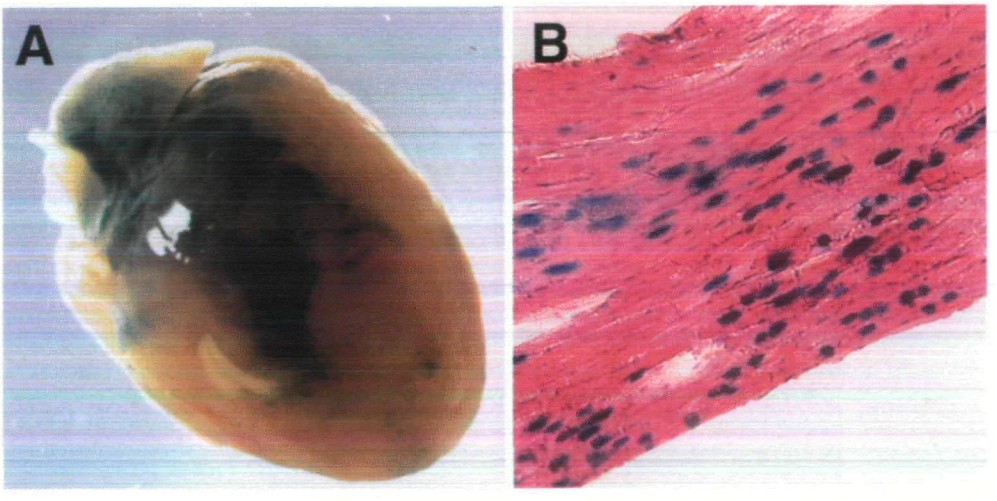


Fig. 5. **Expression of the GATA6/lacZ-1 transgene in the adult heart region.** The transgene expression was prominent in the myocardium surrounding the pulmonary and aortic valves in adult hearts (A). A section of the heart after *lacZ* staining revealed that the myocardiac muscle cells are strongly stained (B). No obvious staining was observed in the non-transgenic adult heart (data not shown).

those in the cardiac region. No evident *lacZ* expression was detected in the small intestine, large intestine or urogenital region. The *lacZ* expression patterns in the cardiac and extracardiac tissues were identical in the independent transgenic lines.

The GATA6/lacZ-1 Drives lacZ Expression in the Adult Heart—GATA6 is highly expressed in the heart, stomach, small intestine, and bladder in the adult mouse (*5*). To determine whether or not the 5′ flanking sequence is capable of supporting the postnatal expression of GATA6 in the heart and other tissues, the *lacZ* expression in tissues from adult transgenic mice was assayed: expression was observed in the heart, especially in the myocardium surrounding the pulmonary and aortic valves (Fig. 5A). Histological sections of the stained adult heart of transgenic mice confirmed that expression was confined to the myocardiac cells (Fig. 5B). However, no *lacZ* expression was detectable in the stomach and intestines of adult mice (data not shown), although very low expressions were observed at embryonic stages (Fig. 4). These observations suggest that regulatory sequences within the GATA6/lacZ-1 construct are sufficient for the GATA6 expression at early stages critical for lineage determination and maintain the expression in cardiac regions.

A cis Regulatory Element for Expression of GATA6 Exists between –4.9 and –3.7 kb of the 5′ Flanking Sequence—5′ deletion analysis was carried out to determine the location of *cis*-acting regulatory elements within the flanking region. GATA6/lacZ-2 containing 3.7 kb of the 5′ flanking sequence (Fig. 1) was examined in transgenic embryos. Fifty-eight F_0 embryos at E10.5 were analyzed and fourteen of them carried the transgene. None of these transgenic embryos showed *lacZ* expression in the heart or any other extracardiac tissues (Table I), suggesting that critical *cis* control elements are located within the 1.2 kb region between –4.9 and –3.7 kb.

Another cis Regulatory Element for Expression of GATA6 Exists in Intron 1—GATA6/lacZ-3 lacking the first intron (Fig. 1B) was constructed and introduced into mouse embryos. We examined twenty F_0 embryos at E10.5, and found that 10 of them contained the transgene. No GATA6-specific expression was observed in these embryos (Table I), indicating that a critical tissue-specific enhancer element is present within the first intron of the gene. As sequence comparisons among different species may facilitate the identification of regulatory elements, we cloned the human GATA6 gene and compared the 1.4 kb first intron sequence with that of the mouse. This comparison revealed at least four regions of significant homology (Fig. 6A). Multiple consensus transcription factor DNA-binding sites were found in this region: four GATA factor binding sites and one Nkx2.5 binding consensus sequences were completely conserved in both genes (Fig. 6B).

TABLE I. **Summary of transgene integration and *lacZ* expression.**

Construct	Number of embryos	Transgene (+)	*lacZ* (+) embryos among transgene (+) embryos
GATA6/lacZ-1[a]	60	10 (17%)	3
GATA6/lacZ-2[b]	48	14 (29%)	0
GATA6/lacZ-3[b]	20	11 (55%)	0

The structures of the transgenic constructs are shown in Fig. 1. Transgene (+), number of transgenic mice or embryos in which the *lacZ* sequence was detected by PCR; *lacZ* (+), number of transgenic mice or embryos expressing the *lacZ* transgene. [a]line analysis; [b]founder analysis.

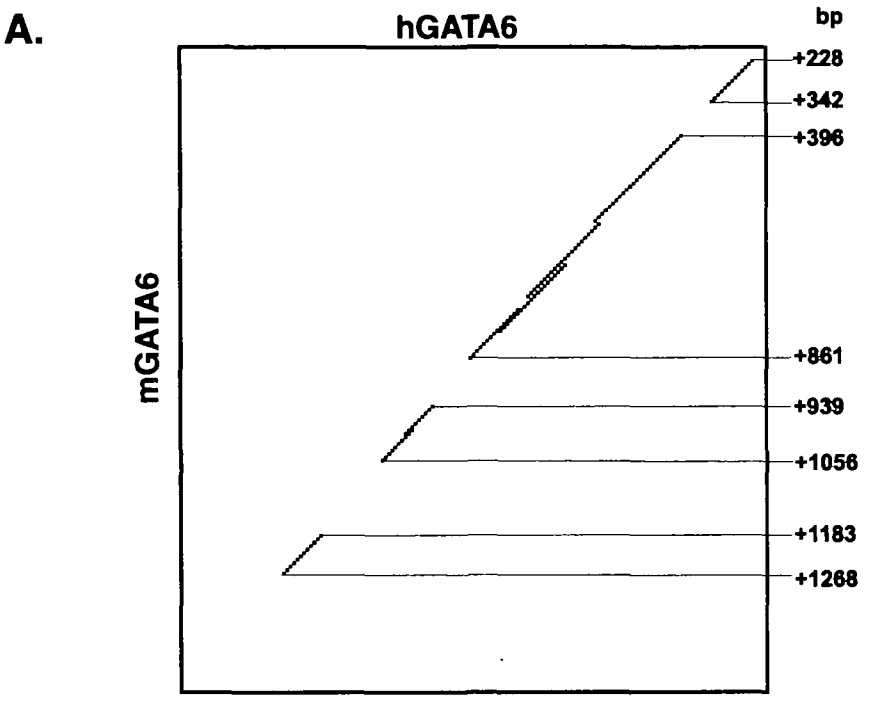
DISCUSSION

Precise regulation of tissue-specific transcription factors is essential for the development and maintenance of distinct cell types. The GATA-family transcription factors play important roles in regulating lineage-specific gene expression during development (for reviews, see *1–2*). Mouse GATA6 is expressed at an early developmental stage (E5.5), and is involved in the development of primitive endoderm-derived tissues, thus, greater interest has been focused on the mechanisms that might control GATA6 activity. Although the functions of the GATA6 protein have been examined from different aspects, little is known about how its transcription is regulated. In this study, we have analyzed the role(s) of the 5′ upstream region of the GATA6 gene in transgenic mice.

We have cloned the mouse GATA6 gene, and characterized its 5′ flanking region. The GATA6 gene has two closely spaced transcription initiation sites. Since no typical TATA-box is found upstream the transcription initiation, our results indicated that GATA6 contains multiple transcription initiations like many TATA-less genes. Transcripts from both sites contain same non-coding exon 1. Our oligo-capping method could not detect any other alternative 5′ non-coding exons as recently reported by Brewer *et al.* when using a 5′ RACE assay (*27*).

Using transgenic mice, we have clearly shown that the cloned region including 5′ flanking sequence (4.9 kb), untranslated exon 1 and intron 1 is sufficient to induce GATA6 gene expression in cardiac region at the early stages of development. The *lacZ* reporter expression driven by the upstream sequences is activated in the cardiogenic region at the stage when the heart has looped. In the differentiated heart, cardiac expression from these elements is restricted to the outflow tract, thoracic wall overlying the pericardial cavity, and myocardial wall of the four chambers during embryonic development. The expression in the cardiac region is also maintained during postnatal development. The activity of the upstream region was lost when 1.2 kb (–4973 to –3774) was deleted from the 5′ end. Sequence analysis of this region revealed two GATA motifs and four E-boxes (data not shown, but the motifs can be found in the sequence deposited, AB034243), suggesting that GATA factors and/or E-box binding transcription factors may be important for the activity of the upstream element. During our transgenic analysis, Molkentin *et al.* reported that a 1.8 kb cardiac-specific enhancer region exists between –4.3 and –2.4 kb, which contains an Nkx2.5 binding consensus critical for GATA6 expression (*28*). These observations are consistent with our results.

Deletion analysis also indicated that the first intron is required for the expression of GATA6. Transcription factor consensus binding sites are present within the intron 1 region: among them are several known regulators of cardiac gene expression including six GATA sites, three NKE sites, and three E-box (*29–32*). Four GATA sites and one NKE site were completely conserved between mouse and human genes. In this regard, analysis of the *cis*-acting element of the chicken GATA6 gene using transgenic mice



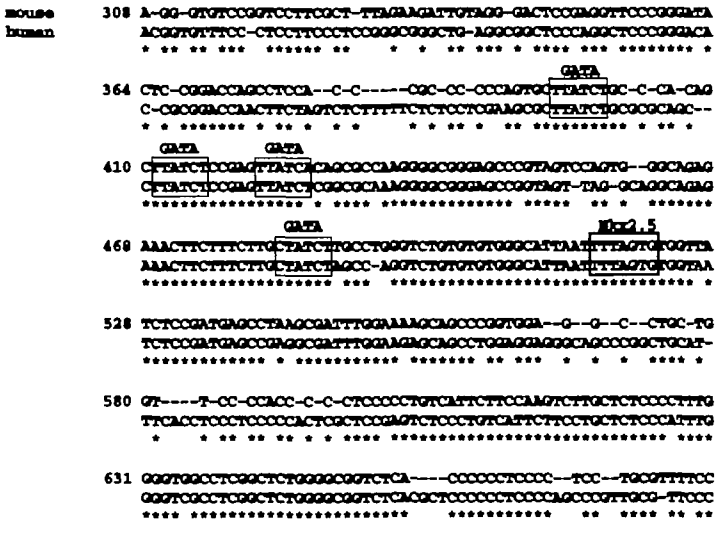


Fig. 6. **(A) Dot matrix analysis of intron 1 in the mouse and human GATA6 genes.** The 1.3 kb murine (mGATA6) and 1.4 kb human (hGATA6) sequences of the intron 1 region were compared using the Lasergene "Megalign" program. The diagonals indicate regions of extensive homology. **(B) Alignment of the murine and human intron 1 sequences.** The mouse (upper) and human (below) sequences are shown. Nucleotide identities are indicated by asterisks (*) and the GATA- and Nkx2.5-binding consensus sequences are boxed. The nucleotide positions of mouse intron 1 are indicated (numbered from the transcription initiation site I). The GenBank accession numbers for the mouse and human sequences are AB034243 and AB034207, respectively.

showed that the regulatory mechanism underlying cardiac-restricted expression is conserved (*33*). A DNase I hypersensitive (HS) site is located in the first intron of the chicken GATA6 gene (*33*).

In situ hybridization (Fig. 4E) and expression analysis of GATA6 using a *lacZ* reporter introduced into the GATA6 locus through homologous recombination revealed evident *lacZ* expression in the developing gut at E8.5, and very strong expression in the foregut and hindgut at E9.5 (*17*). Although the upstream sequences also induced the gene expression in the developing stomach, liver, trachea and main bronchi at developmental stages of E11.5–12.5, the *lacZ* expression in the stomach and intestine is considerablly low. In addition, we could not detect any expression in

the embryonic testis, where expression of GATA6 is revealed by Northern analysis (*34*) and *in situ* hybridization (*5*). These results suggest that there may be another regulatory region controlling GATA6 expression in these extra-cardiac tissues.

The results of the present study are an initial step for elucidating the exact mechanisms of cell lineage-specific expression of GATA6. Further work will be aimed at narrowing down the region necessary for this expression and finally the actual nucleotide sequence responsible for the activity. It will also be particularly interesting to identify element(s) which direct expression in the gastrointestinal and urogenital systems.

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