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Regulatory Elements Directing Gut Expression of the GATA6 Gene during Mouse Early Development

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The GATA6 transcription factor, as well as GATA4 and GATA5, is expressed in a variety of mammalian tissues including the precardiac mesoderm and endoderm, and gut-related organs. Genetic studies have also implicated GATA factors as important regulators of gut endoderm development. Previously, we identified the promoter and a cardiac-specific enhancer of mouse GATA6 [Sun-Wada *et al.* (2000) *J. Biochem.* 127, 703–709], however, little is known about the regulatory elements that govern GATA6 expression in the primitive gut. Here, we identified a distal enhancer of the GATA6 gene directing expression in the gut by creating transgenic mice. A sequence of approximately 200 bp between –8.0 kb and –7.8 kb contains element(s) that enhance transcription in the gut during embryonic development, when linked to the *hsp68* promoter/*lacZ* fusion gene. Our results also show that GATA6 expression is controlled by multiple regulatory regions including cardiac-specific and gut-specific enhancers.

Key words: GATA6, gastrointestinal expression, *lacZ* reporter, transgenic mice, transcription.

Development of the gastrointestinal organs requires several sequential steps: differentiation of a definitive endoderm, formation and regional specialization of the primitive gut tube, followed by morphogenesis and differentiation of component cell lineages (*1*). Genetic studies have implicated GATA factors as important regulators of gut endoderm differentiation and development in a variety of organisms (*2–13*). *In vivo* footprinting analyses suggested that GATA-binding proteins, in conjunction with other transcription factors such as HNF-3/fork head factors, endow genes with the ability to be selectively activated in endodermal derivatives (*14–16*). In addition, trans-activation and electrophoretic mobility shift assays indicated that GATA4, 5, and 6 regulate genes known to be expressed in parietal cells [H^+/K^+ ATPase α and β subunit (*17–19*)], pit cells [trefoil factors 1 and 2 (*20*)], and neck/zymogenic cells [pepsinogen (*21*)].

The expression pattern of the GATA6 gene during mouse early development has been characterized (*22*, *23*). GATA6 is expressed within the primitive streak on embryonic day 7.0 (E7.0), and the cardiogenic mesoderm between E7.5 and E8.0 (*22*, *23*). In addition, high levels of hybridization of GATA6 RNA probes have been detected in the embryonic liver, midgut and hindgut region (*23*) on embryonic day 9.5. In late stage embryos, the expression of GATA6 is sequentially up-regulated in distinct segments of the gastrointestinal epithelium as they undergo terminal differentiation (*22*, *23*). Whole mount in situ hybridization analysis has also revealed that GATA6 is expressed in the developing heart and gastrointestinal tissues (*24*). The regulatory mechanisms inducing GATA6 transcription in the heart and gastrointestinal systems are of particular interest because GATA6 expression starts as early as E5.5, and is maintained in cardiac and extracardiac tissues throughout postnatal life. Previously, we showed that a 5′ flanking sequence (6.9 kb) including the two noncoding 5′ exons (*25*) is sufficient to trigger expression in the cardiac tissues and bronchial epithelium during embryonic development (*24*) (Fig. 1, construct 0). This region contains two alternative promoters for native GATA6 expression (*25*). However, this region could not induce expression in the gastrointestinal regions of transgenic mice. Thus, little is known about the mechanism controlling the expression of GATA6 in the gut. In this study, we identified a distal upstream element required specifically for the expression of GATA6 in gastrointestinal tissues during embryonic development.

To identify the regulatory region required for expression in the gut, we isolated a clone containing the GATA6 gene from a bacterial artificial chromosome (BAC) library (GenomeSystem Inc.). The 9.7 kb 5′-upstream region was cloned into pBluescript KS+ (Stratagene), the resulting plasmid being designated as pB-3-3, and sequenced with an EZ::TN™<KAN-2>Insertion kit (Epicentre Technologies). The genomic organization of the upstream region is shown in Fig. 1a (DDBJ/EMBL/GenBank nucleotide sequence databases, Accession Number AB119275).

The ability of upstream sequences to induce gene expression during embryogenesis was examined in transgenic mice. At 10.5 days post-coitum (dpc), embryos were genotyped and examined for *lacZ*-reporter gene expression by incubation with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). A summary of the results of transgenic analysis is presented in Fig. 1b. We found that a reporter (construct 1, –8.8 kb/+2.2 kb) containing 8.8 kb of the 5′ flanking sequence, two noncoding exons and a part of the coding exon 2 linked upstream to the reporter

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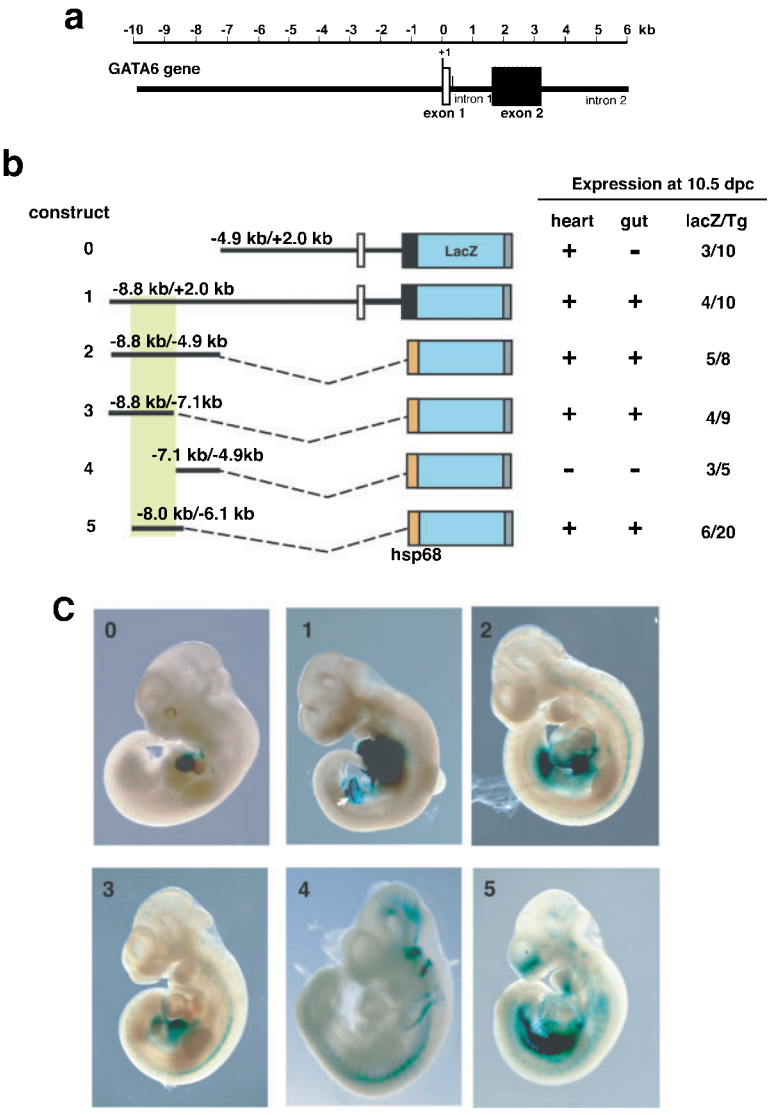

Fig. 1. **The –8.8 to –4.9 kb 5′ flanking region of the mouse GATA6 gene contains the enhancers required for gut-specific expression.** (a) Genomic organization of the mouse GATA6 gene locus and 5′ flanking region. (b) Structures of transgenes used to identify the regulatory region for the gut. The construct numbers are indicated on the left, and the corresponding expression patterns are summarized on the right. The far-right column indicates the fraction of F_0 transgenic embryos showing *lacZ* expression *(lacZ/Tg)*. Transient transgenic embryos were obtained from injected fertilized eggs as described previously (*33*), and genotyped by Southern analysis with *lacZ*-specific probes amplified with primers lacZFw and LacZRv (*33*). For construct 1, pB-3-3 was digested with *Age*I and *Not*I, and the resulting 8.0 kb fragment was inserted into the *Age*I–*Not*I gap of pCYG6P (*1*). The vector element was removed by digestion with *Sfi*I and *Not*I for injection. Constructs 2 to 5 were obtained by insertion of the *Sfi*I and *Nco*I (3.9 kb), *Pst*I (1.7 kb), *Pst*I (6.5 kb), and *Bbv*CI (1.9 kb) fragments of construct 1 into pASShsp68lacZ-pA (*26*, *34*), respectively. (c) Representative embryos with each construct stained with X-gal. Transgenes containing the overlapping region (–8.0 kb to –7.1 kb) were capable of generating specific expression patterns in the gut.

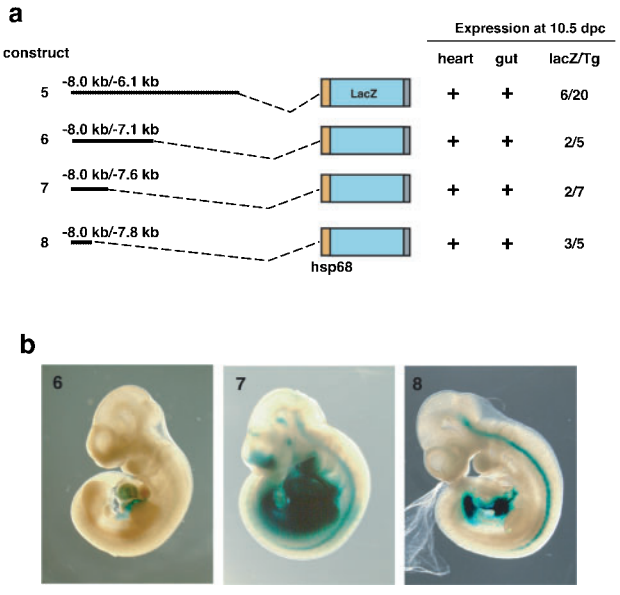

Fig. 2. **Fine mapping of enhancer elements directing expression in the gut.** (a) Deletions of the 1.9-kb region (contained in construct 5) were generated and fused with the hsp68/*lacZ* reporter. The construct numbers are indicated on the left, and their *lacZ* expression at 10 dpc is summarized on the right. Constructs 6, 7, and 8 were constructed by insertion of PCR products amplified with primer sets YK1 (5′-TCAGCTGGCTGGCTGGGGTGGGGGAC-3′) and YK2 (5′-GCAGAAC-CGAGATAGCAATCAATCTTGA-3′), YK1 and GH109 (5′-CATTGGAA-GGAGGGGGAAGAGAAGGGCC-3′), and YK1 and GH108 (5′-CACAG-CCCAGCCACAGCCCAGCCACAG-3′), respectively, into the *Pst*I and *Sma*I gap of pASShsp68lacZ-pA (*26*). The vector region for the constructs was removed by *Sal*I and *Not*I digestion. (b) Representative embryos carrying constructs 6, 7, and 8 were stained with X-gal. An approximately 200 bp element (construct 8, –8.0 kb/–7.8 kb) was responsible for the gut-specific enhancer activity.

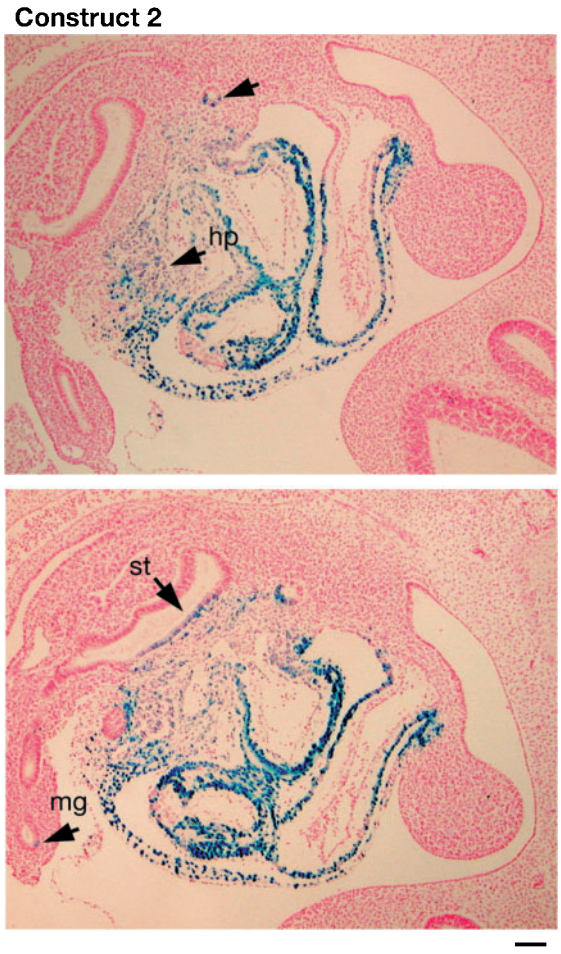

Fig. 3. **Sagittal histological sections of 10.5 dpc transgenic embryos with construct 2.** Reporter *lacZ* expression was found in the hepatic progenitor (hp), midgut (mg), and primitive stomach (st). The sections were counterstained with Nuclear Fast Red. Bar, 50 μm.

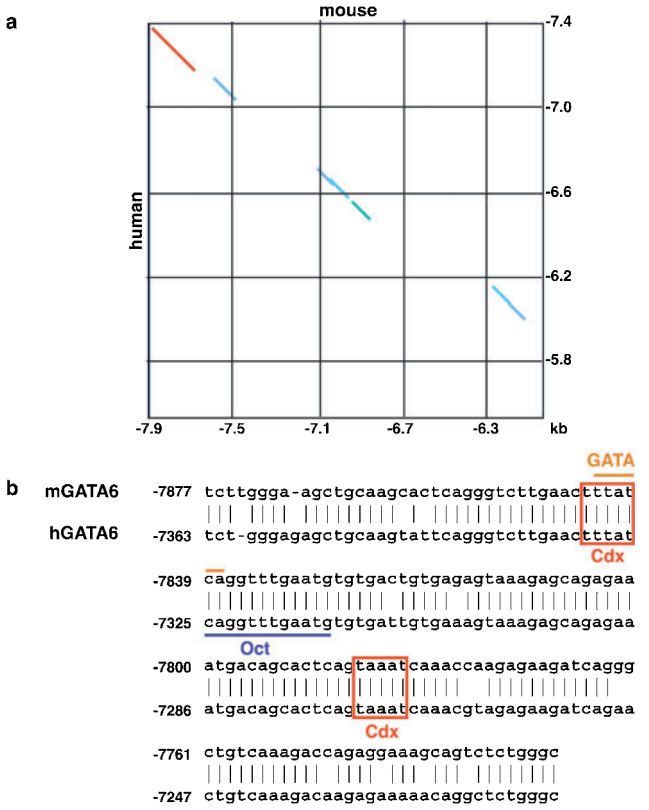

Fig. 4. **Nucleotide sequence of the regulatory region directing gut-specific expression of GATA6.** (a) Dot matrix analysis of 5′ upstream regions in the mouse and human GATA6 genes. The 1.8 kb murine (mGATA6, –7.9 kb/–6.0 kb) and 1.9 kb human (hGATA6, –7.4 kb/–5.5 kb) sequences were compared using the Lasergene 'Megalign' program. The red diagonal line indicates regions of extensive homology. (b) Alignment of the murine and human sequences (shown by the red diagonal line in a) revealed the conservation of several potential transcription factor-binding sites. The consensus Cdx binding sites are shown in red boxes, the GATA factor binding site by an orange bar, and Oct-1 by blue underlining.

gene induced reproducible expression of *lacZ* in both the developing heart and gut (Fig. 1, b and c, construct 1). Four of ten transgenic-positive embryos showed expression patterns consistent with that observed on whole mount in situ analysis (*24*). Since a region including the 5′ flanking sequence (6.9 kb) could only support expression in the cardiac region (*24*) (Fig. 1, b and c, construct 0), this result suggested that the enhancer elements required for gastrointestinal expression were included in the 3.9 kb (–8.8 kb/–4.9 kb) upstream region.

To determine if this region was sufficient to direct gut expression with a heterologous promoter, we examined its activity with the hsp68 (heat shock protein 68) basal promoter (*26*). We found that construct 2 (–8.8 kb/ –4.9 kb) could drive the expression in both heart and gastrointestinal tissues (Fig. 1, b and c), suggesting that the 3.9 kb region contained the enhancer activity for gut expression. A deletion series was constructed to narrow down the enhancer region. Construct 3 (–8.8 kb/–7.1 kb) and construct 5 (–8.0 kb/–6.1 kb) gave the same *lacZ* expression pattern as construct 1 (Fig. 1, b and c), whereas construct 4 (–7.1 kb/ –4.9 kb) induced no gut expression (data not shown). These results suggest that the overlapping region (–8.0 kb/–7.1 kb) may harbor the enhancer element(s).

We have examined further deletions of the segment (–8.0 kb to –7.1 kb) to narrow down the enhancer region (Fig. 2). Construct 6 (–8.0 kb/–7.1 kb), construct 7 (–8.0 kb/–7.6 kb), and construct 8 (–8.0 kb/–7.8 kb) produced strong expression of the reporter gene in primitive gut regions (Fig. 2b). Three of five transgenic-positive embryos with construct 8 exhibited the same *lacZ* expression pattern as those with the larger enhancer element, suggesting that an approximately 200 bp element (–8.0 kb/–7.8 kb) was responsible for the gut-specific enhancer activity. The *lacZ* expression patterns were also examined with paraffin sections of transgenic embryos. Reporter *lacZ* expression was detected in the stomach, midgut and hepatic primordial region (Fig. 3).

LacZ expression in the spinal cord was observed in almost all the transgenic embryos at different levels with the lacZ reporter gene driven by the hsp*68* promoter (Figs. 1 and 2). Recently, it was reported that GATA6 immunoreactivity was detected in the neural tube as

early as embryonic day 7.5 (*27*), and thus it is possible that the lacZ signal observed may represent the neural expression of GATA6. However, we found that this expression was specific to the hsp68 promoter used (*28*), no spinal cord expression being found in transgenic embryos with construct 1, which contained the authentic promoter of GATA6 (Fig. 1c). It has been reported that the hsp68 promoter contains an element that is capable of directing expression in the spinal cord and that such expression is only detectable when the promoter is flanked by a strong enhancer element (*28*). Similar expression has also been found in transgenic lines in which several copies of the retinoic acid response element were placed upstream of the reporter (*28*).

Inspection of the nucleotide sequence upstream of mouse GATA6 revealed that the minimal 200-bp enhancer directing expression in the gut is highly conserved when compared with the human GATA6 upstream region (Human genome resources) (Fig. 4a). Alignment analysis of the 200-bp region and the corresponding human counterpart revealed the conservation of several potential transcription factor-binding consensus sequences including a GATA-binding site, Cdx sites and an Oct-1 site (Fig. 4b). It has been reported that the four Cdx factors identified in mouse are homologues of caudal in fly (*29*), and mainly expressed in the intestine but not in the heart (*30*). The GATA-binding site may also play an essential role in transcription regulation. Transgenic reporter studies on mice regarding the regulation of hematopoietic GATA factors revealed that GATA2 binds to GATA sites at the onset of GATA1 expression in the early stage of erythroid differentiation, and GATA1 replaces GATA2 to promote an autoregulatory loop (*31*, *32*). Future studies with the gastrointestine-specific regulatory region will help us reveal the trans factors and mechanisms involved in the control of expression of genes during development of the gastrointestinal system.

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