Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element

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Summary

The GATA family of zinc-finger transcription factors plays key roles in the specification and differentiation of multiple cell types during development. GATA4 is an early regulator of gene expression during the development of endoderm and mesoderm, and genetic studies in mice have demonstrated that GATA4 is required for embryonic development. Despite the importance of GATA4 in tissue specification and differentiation, the mechanisms by which *Gata4* expression is activated and the transcription factor pathways upstream of GATA4 remain largely undefined. To identify transcriptional regulators of *Gata4* in the mouse, we screened conserved noncoding sequences from the mouse *Gata4* gene for enhancer activity in transgenic embryos. Here, we define the regulation of a distal enhancer element from *Gata4* that is sufficient to direct expression throughout the lateral mesoderm, beginning at 7.5 days of mouse embryonic development. The activity of this enhancer is initially broad but eventually becomes restricted to the mesenchyme surrounding the liver. We demonstrate that the function of this enhancer in transgenic embryos is dependent upon highly conserved Forkhead and GATA transcription factor binding sites, which are bound by FOXF1 and GATA4, respectively. Furthermore, the activity of the *Gata4* lateral mesoderm enhancer is attenuated by the BMP antagonist Noggin, and the enhancer is not activated in *Bmp4*-null embryos. Thus, these studies establish that *Gata4* is a direct transcriptional target of Forkhead and GATA transcription factors in the lateral mesoderm, and demonstrate that *Gata4* lateral mesoderm enhancer activation requires BMP4, supporting a model in which GATA4 serves as a downstream effector of BMP signaling in the lateral mesoderm.

Key words: GATA4, GATA, BMP4, BMP, FOXF1, Forkhead, Transcription, Enhancer, Transgenic, Mouse, Liver, Mesenchyme, Lateral mesoderm, Septum transversum

Introduction

Mesoderm forms initially during vertebrate gastrulation when cells of the epiblast ingress through the primitive streak. Mesodermal cells accumulate and fill the space between the ectoderm and the endoderm, and migrate towards the anterior of the embryo to form distinct subpopulations, including axial, paraxial, intermediary and lateral plate mesoderm. The lateral mesoderm is continuously involved in reciprocal signaling interactions with the adjacent endoderm, and these interactions are crucial for the proper specification of different cell types within both lineages (Rawdon, 2001; Tam et al., 2003). Members of the bone morphogenetic protein (BMP) family, including BMP2, BMP4, BMP5 and BMP7, are highly expressed in the lateral mesoderm and septum transversum (Hogan, 1996; Zhao, 2003), and previous studies have shown that BMP is necessary to direct the specification of cardiomyocytes, hepatocytes and gut mesenchyme (Rossi et al., 2001; Schultheiss et al., 1997; Sukegawa et al., 2000; Zhang et al., 2004). In response to BMP induction, numerous genes are activated and repressed as cell fates become

increasingly restricted during embryonic development (Hogan, 1996; Zhao, 2003).

Members of the Forkhead and GATA families are among the earliest transcription factors that have been implicated downstream of BMP signaling in vertebrates and invertebrates (Klinedinst and Bodmer, 2003; Rossi et al., 2001; Schultheiss et al., 1997; Tseng et al., 2004; Zaffran et al., 2001). Forkhead domain proteins comprise a large family of transcription factors that are defined by the presence of a winged-helix DNA-binding domain, and members of this large superfamily are expressed in virtually all tissues derived from all three germ layers (Carlsson and Mahlapuu, 2002). Among the Forkhead proteins that are restricted to the mesoderm, FOXF1 (FOXF1A – Mouse Genome Informatics) is a key regulator of embryonic and extraembryonic mesoderm development, and FOXF1 null embryos die by 9.5 days post-coitum (dpc) because of defects in the extraembryonic mesoderm (Kalinichenko et al., 2004; Kalinichenko et al., 2002; Mahlapuu et al., 2001a; Mahlapuu et al., 2001b). Thus, it is clear that Forkhead proteins, including FOXF1, play crucial roles in the mesoderm during development. However, the transcriptional

pathways downstream of Forkhead factors in the developing mesoderm remain to be determined.

GATA transcription factors belong to an evolutionarily conserved family of zinc finger-containing proteins that recognize the consensus DNA sequence WGATAR (Molkentin, 2000; Patient and McGhee, 2002). There are six mammalian GATA factors, which play key roles in gene activation in multiple lineages including hematopoietic tissues, heart and liver (Burch, 2005; Molkentin, 2000; Shivdasani, 2002; Weiss and Orkin, 1995; Zaret, 1999). Among GATA family members, the *Gata4* gene is expressed early in the post-gastrula embryo and is a key regulator of mesodermal and endodermal development (Arceci et al., 1993; Grepin et al., 1997; Heikinheimo et al., 1994; Rossi et al., 2001). *Gata4*-knockout mice die around 9.5 dpc, and display defects in heart and foregut morphogenesis that result from severe defects in the ventral foregut endoderm (Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1997). GATA4 functions in a variety of transcriptional complexes with multiple other factors, including NK class homeodomain factors and Forkhead transcription factors such as FOXA2, to activate downstream genes associated with specification and differentiation in cardiac mesoderm, prehepatic endoderm and gut endoderm (Bossard and Zaret, 1998; Denson et al., 2000; Divine et al., 2004; Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998; Sepulveda et al., 2002). However, despite the importance of GATA4 in mouse embryonic development, the transcription factors upstream of *Gata4* have not been defined.

In this study, we identify for the first time a transcriptional enhancer from the mouse *Gata4* gene. This evolutionarily conserved distal enhancer is sufficient to direct expression to the lateral mesoderm in transgenic mouse embryos, beginning at 7.5 dpc. As development proceeds, this novel enhancer element directs robust expression throughout the visceral mesoderm and septum transversum mesenchyme, and, by 11.5 dpc, enhancer activity becomes restricted to the mesenchyme surrounding the liver. We show that the activity of the enhancer is inhibited by the BMP antagonist Noggin in embryo culture explants, and that the enhancer is not active in *Bmp4* null embryos, demonstrating that this *Gata4* lateral mesoderm enhancer is a downstream target of BMP4. This evolutionarily conserved *Gata4* enhancer element contains an essential FOX-binding site that is efficiently bound by the Forkhead transcription factor FOXF1, and this element is required for enhancer activity throughout development. The enhancer also appears to be a target for autoregulation via two perfect consensus GATA sites that are robustly bound by GATA4 and are also required for enhancer function in vivo. Thus, these studies identify *Gata4* as a direct transcriptional target of Forkhead and GATA transcription factors in the lateral mesoderm. Furthermore, these studies identify *Gata4* as a downstream target of BMP4 in the lateral mesoderm and septum transversum, and support a role for GATA4 as a transcriptional mediator of BMP signaling in those lineages.

Materials and methods

Cloning and mutagenesis

The 4368 bp G2 fragment of the mouse *Gata4* gene was generated by PCR using the following two primers: 5′-gtgatttatcagatatctccttcccaa-3′ and 5′-cagccctggatatcaacactcata-3′. This fragment was then cloned as an *Eco*RV fragment into the *Sma*I site of the transgenic reporter plasmid HSP68-*lacZ* (Kothary et al., 1989). Deletion fragments of the G2 *Gata4* lateral mesodermal enhancer were generated by digestion using the following restriction sites, followed by subsequent subcloning back into HSP68-*lacZ*: G2Δ1, *Sal*I-*Kpn*I; G2Δ2, *Pst*I; G2Δ3, *Stu*I-*Xmn*I; G2Δ4, *Pst*I-*Stu*I. G2Δ5 was generated by PCR using the primers 5′-cactgaggaagcttgtacctgg-3′ and 5′-atggatgcctgcagattggtg-3′, and subsequent cloning into HSP68-*lacZ* as a *Hin*dIII-*Pst*I fragment; G2Δ6 contains an internal deletion within G2 generated by cleavage with *Sph*I and religation. Mutations were introduced into the G2Δ2 enhancer fragment in HSP68-*lacZ* for analysis in transgenic mice. The following mutant sequences were created in the context of fragment G2Δ2: mFox I, 5′-tggggtagtctcg-agaagcatcttcagaaaag-3′; m-gata I, 5′-caagacagtagagctcagcagggctc-3′; m-gata II, 5′-ttacaagctccatgtgaaggcccttgtctttag-3′. To create the double gata I/gata II mutant, referred to as mGATA, the m-gata II sequence was introduced into a form of fragment G2Δ2 that already contained the m-gata I sequence. The sequence of each mutant fragment was confirmed by sequencing on both strands. The SMaa-*lacZ* reporter construct contains the mouse smooth muscle α-actin promoter cloned into plasmid AUG-β-gal and has been described previously (Anderson et al., 2004). The GenBank Accession number for the sequence of the *Gata4* G2 lateral mesoderm enhancer is AY763588.

Generation of transgenic mice and mouse embryo culture

Transgenic reporter fragments were digested from the plasmid backbone with *Sal*I (fragments G2, G2Δ1, G2Δ3, G2Δ4, G2Δ5 and G2Δ6), *Sal*I-*Pst*I (G2Δ2 and mutants in that context), or *Xho*I-*Sac*II (SMaa-*lacZ*), gel purified, and suspended in 5 mM Tris-HCl, 0.2 mM EDTA (pH 7.4) at a concentration of 2 μg/ml for pronuclear injection, as described previously (Hogan et al., 1994). Injected embryos were implanted into pseudopregnant CD-1 females, and embryos were collected at indicated times for F0 analysis or were allowed to develop to adulthood for the establishment of transgenic lines. DNA was extracted from the yolk sac of embryos, or from tail biopsies from mice by digestion in tail lysis buffer (100 mM NaCl, 25 mM EDTA, 1% sodium dodecyl sulfate, 10 mM Tris-Cl, 200 μg/ml of proteinase K, pH 8.0) at 56°C overnight. Digested samples were extracted once with phenol-chloroform and ethanol precipitated. The presence of the *lacZ* transgene was detected either by PCR or Southern blot. For PCR genotyping, the *lacZ* primers LACZ5 (5′-cggtgaatggtgctgcgttgga-3′) and LACZ3 (5′-accaccgcacgatagagattc-3′) were used. For determination of genotype by Southern blot, DNA samples were digested with *Sac*I, followed by blotting and hybridization using a radiolabeled *lacZ* probe. The *Bmp4*-knockout mice have been described previously (Liu et al., 2004).

For embryo explant culture experiments, embryos from a single stable transgenic line of G2-*lacZ* or SMaa-*lacZ* were collected at 9.5 dpc, and the heart, septum transversum and adjacent regions were dissected and cultured in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml) and 2 mM L-glutamine at 37°C in 5% CO_2 in 24-well tissue-culture plates. Recombinant mouse Noggin (R&D Systems) was prepared in PBS+0.1% BSA and added to the cultured embryonic tissues at a final concentration of 10 nM for 48 hours. Control tissues were treated with PBS+0.1% BSA without recombinant Noggin. The hearts continued beating throughout the 48-hour duration of the experiment. Following treatment, tissues were fixed and X-gal stained for β-galactosidase activity, as described previously (Dodou et al., 2003). All experiments using animals complied with federal and institutional guidelines, and were reviewed and approved by the UCSF Institutional Animal Care and Use Committee.

X-gal staining, immunohistochemistry and in situ hybridization

β-Galactosidase expression in *lacZ* transgenic embryos or tissues was

detected by X-gal staining, which was performed as described previously (Dodou et al., 2003). Transverse and sagittal sections from X-gal-stained embryos and tissues were prepared and counterstained with Neutral Fast Red, as described previously (Anderson et al., 2004). Whole-mount in situ hybridization was performed as described previously (Wilkinson and Nieto, 1993). Briefly, embryos were fixed overnight in 4% paraformaldehyde, then washed twice with phosphate-buffered saline (PBS) and dehydrated through a series of PBT (1×PBS+0.1% Tween20)-methanol washes. Embryos were then rehydrated through a reciprocal series of PBT-methanol washes and were treated at room temperature with 10 μg/ml proteinase K for varied times depending on age: 8.25 dpc embryos were incubated for 1 minute and 9.5 dpc embryos were incubated for 7 minutes. After proteinase K treatment, embryos were rinsed with 2 mg/ml glycine in PBT followed by two successive washes in PBT at room temperature. Embryos were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde for 20 minutes at room temperature, rinsed three times in PBT, and incubated in hybridization solution (50% formamide, 1% SDS, 5×SSC, 50 μg/ml yeast tRNA, 50 μg/ml heparin) for 16 hours at 70°C. Whole-mount in situ hybridization was carried out with digoxigenin-labeled antisense or sense RNA probes at 100 ng/ml in hybridization buffer.

For in situ hybridization on sections, embryos were fixed, embedded in paraffin wax, sectioned at a thickness of 7 μm, and dewaxed as described previously (De Val et al., 2004). Sections were then digested for 8 minutes in 40 μg/ml proteinase K, fixed in 4% paraformaldehyde for 20 minutes, dehydrated through a series of ethanol washes and allowed to dry. In situ hybridization was performed with digoxigenin-labeled antisense or sense RNA probes at a concentration of 1 μg/ml in 50 μl of hybridization buffer. Following hybridization, embryos or sections were washed and treated with RNaseA using previously described standard methods (Wilkinson and Nieto, 1993). Signal was detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody and BM Purple alkaline phosphatase substrate (Roche Pharmaceuticals). Following staining, sections were counterstained with Neutral Fast Red. The *Bmp4* in situ probe has been described (Jones et al., 1991). *Foxf1* antisense probe was generated from pGEM-FOXF1 (HFH-8), which was kindly provided by R. Costa and has been described (Peterson et al., 1997). *Gata4* antisense probe was generated from a pBluescript plasmid containing the mouse *Gata4* cDNA sequences from −100 to +350 (relative to the translational start site), linearized with *Eco*RI and transcribed with T3 polymerase.

Electrophoretic mobility shift assay (EMSA)

DNA-binding reactions were performed as described previously (Dodou et al., 2003). Briefly, double-stranded oligonucleotides were labeled with ^{32}P-dCTP, using Klenow to fill in the overhanging 5′ ends, and purified on a nondenaturing polyacrylamide-TBE gel. Binding reactions were pre-incubated at room temperature in 1×binding buffer [40 mM KCl, 15 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM DTT, 5% glycerol] containing recombinant protein, 1 μg of poly dI-dC, and competitor DNA for 10 minutes prior to probe addition. Reactions were incubated for an additional 20 minutes at room temperature after probe addition and electrophoresed on a 6% nondenaturing polyacrylamide gel. *Foxf1*, *Foxa2*, *Gata4*, *Gata5* and *Gata6* cDNAs were transcribed and translated using the TNT Quick Coupled Transcription/Translation Systems, as described in the manufacturer's directions (Promega).

FOXF1 protein was generated from plasmid pCITE-FOXF1. pCITE-FOXF1 was made by cloning the *Foxf1* cDNA from pGEM-FOXF1, which was kindly provided by R. Costa and has been described (Peterson et al., 1997), as a PspOMI insert into the *Not*I site in pCITE-2A (Novagen). FOXA2 protein was generated from plasmid pCDNA1-FOXA2, which was made by cloning the mouse *Foxa2* cDNA as an *Eco*RI-*Xba*I fragment into the *Eco*RI and *Xba*I sites of pCDNA1/amp (Invitrogen). GATA4 protein was generated from

plasmid pCITE-GATA4, which has been described previously (Dodou et al., 2004). GATA5 protein was generated from pRK5-GATA5, which was made by cloning the mouse *Gata5* cDNA from pCDNA1-GATA5 as a *Eco*RI-*Xho*I fragment into the *Eco*RI and *Sal*I sites in pRK5. GATA6 protein was generated from pCITE-GATA6, which was made by cloning the mouse *Gata6* cDNA from pCDNA1-GATA6 as a *Not*I-*Xba*I fragment into the *Not*I and *Xba*I sites in pCITE-2B (Novagen). The GATA pCDNA1 plasmids were kindly provided by Jeff Molkentin and have been described previously (Liang et al., 2001). The *Nkx2.5* gs1 control GATA4-binding site oligonucleotides have been described (Lien et al., 1999). The sequences of the control FOXF1- and FOXA2-binding sites have also been described previously (Overdier et al., 1994; Peterson et al., 1997). The sense-strand sequences of the *Gata4* G2 oligonucleotides used for EMSA were:

Fox I, 5′-gccctggggtagtctaaacaagcatcttcagaaaa-3′;
mFox I, 5′-gccctggggtagtctcgagaagcatcttcagaaaa-3′;
Fox II, 5′-aaaggggggtttattgccaagacagtagagtaagcaggg-3′;
Fox III, 5′-ggagaatatatattttgtttaaccaaacctgtctat-3′;
gata I, 5′-gagtagagataagcaggg-3′;
m-gata I, 5′-gagtagagctcagcaggg-3′;
gata II, 5′-ggctccagataaggccct-3′;
m-gata II, 5′-ggctccatggaaggccct-3′;
gata III, 5′-gttgtagtgatagtcgccactggagataaggagaat-3′.

Results

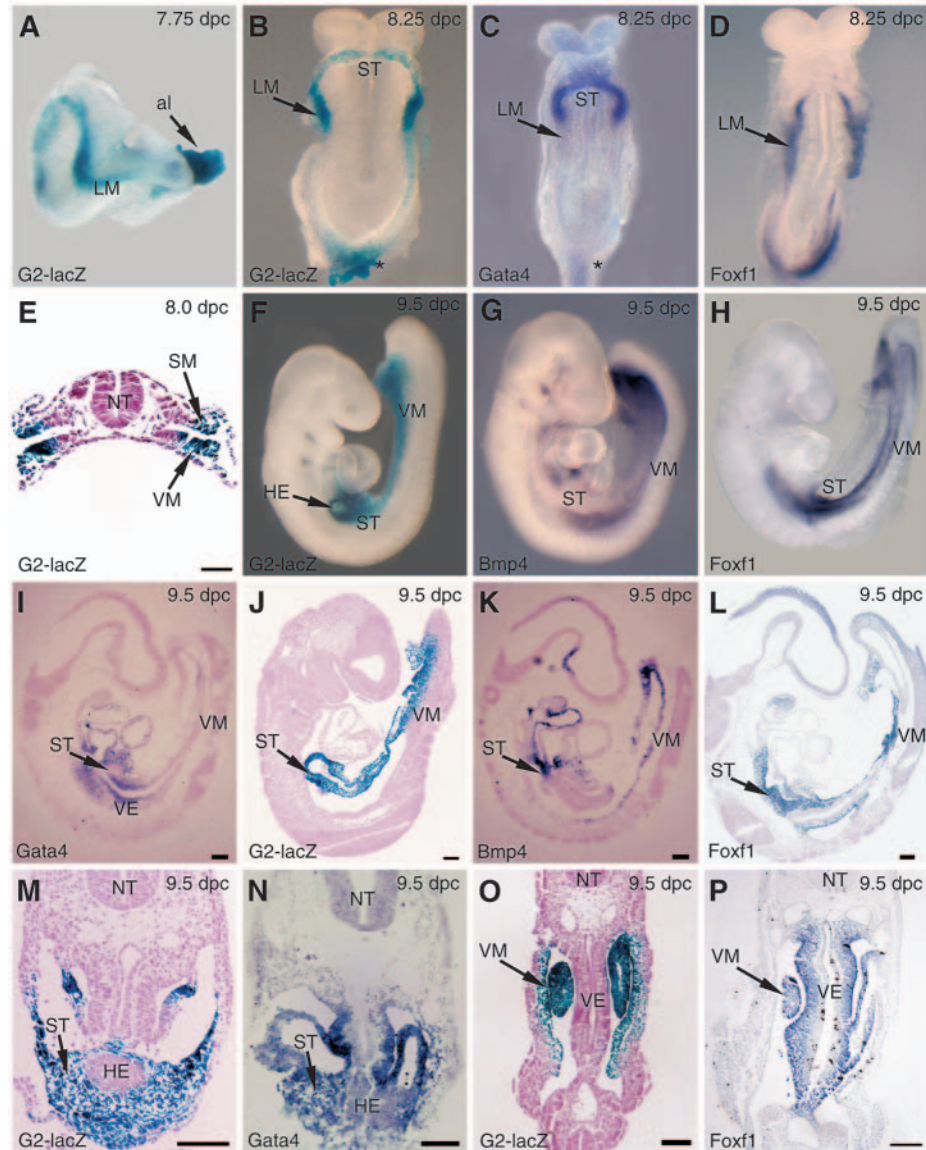### A novel mesoderm-specific *Gata4* transcriptional enhancer

The zinc finger transcription factor GATA4 is expressed broadly in the mesoderm and endoderm of the early mouse embryo (Arceci et al., 1993). Because GATA4 is among the earliest transcription factors expressed in these lineages, we sought to identify the upstream transcriptional regulation of the *Gata4* gene to define early pathways governing mesoderm and endoderm development. The expression of *Gata4* in multiple germ layers suggested that it might be regulated by multiple transcriptional enhancers that each control expression in a single lineage. Therefore, to identify *Gata4* transcriptional enhancers, we compared the sequences of the mouse and human *Gata4* loci for regions of conservation using BLAST and VISTA analyses (Altschul et al., 1990; Mayor et al., 2000). These comparisons identified five regions of strong conservation in noncoding sequences, referred to as G1-G5, within the *Gata4* locus (Fig. 1). Based on the notion that
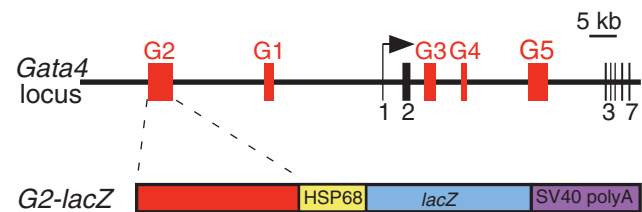
Fig. 1. Schematic representation of the *Gata4* locus and the *Gata4* G2-*lacZ* transgene. The top line represents a 103 kb region of the mouse *Gata4* locus, including its seven exons (black vertical lines). The arrow represents the transcriptional start; exon 2 is the first coding exon of the *Gata4* gene. The red boxes (G1-G5) represent five regions of strong conservation between human and mouse *Gata4* within noncoding sequences. The lower line depicts the transgene construct G2-*lacZ*, which contains the 4368 bp G2 fragment of *Gata4* subcloned into the transgenic reporter plasmid HSP68-*lacZ*.

conservation occurs preferentially in functionally important sequences, we tested each of these conserved noncoding sequences by cloning each into the transgenic reporter plasmid HSP68-*lacZ* (Kothary et al., 1989), as depicted in Fig. 1, and testing each for activity in transgenic embryos.

Among the five conserved noncoding sequences within the mouse *Gata4* locus, the G2 region represented a distal upstream transcriptional enhancer that was sufficient to direct *lacZ* expression to the lateral mesoderm in transgenic embryos, beginning at 7.5 dpc (Fig. 2). The G2 mesodermal enhancer spanned 4368 bp and was located between 45.3 and 40.9 kb upstream of the transcriptional start site (Fig. 1). At 7.75 dpc, this enhancer directed expression throughout the lateral mesoderm, including both somatic and splanchnic mesoderm, and it directed very robust expression in the allantois at this stage (Fig. 2A). This broad pattern of activity within the lateral mesoderm continued at 8.0 dpc and 8.25 dpc (Fig. 2B,E), and could also be detected in the septum transversum at this stage (Fig. 2B). By 9.5 dpc, expression directed by the *Gata4* G2 enhancer was strongly detected in the septum transversum and in visceral mesoderm, but it could no longer be observed in the somatic mesoderm (Fig. 2,J). Expression of the transgene was clearly restricted to the mesoderm and was completely absent from the endoderm. Within the septum transversum, staining could be seen in the mesenchyme, but not in the hepatic endoderm, where expression was noticeably absent (Fig. 2F,J,M). Similarly, expression in the gut was restricted to the mesenchyme and was absent from the endodermal component (Fig. 2O). Expression directed by the *Gata4* G2 enhancer overlapped endogenous *Gata4* expression at all stages (Fig. 2C,I,N). Expression of endogenous *Gata4* was broader than the expression of the G2-*lacZ* transgene, probably as a result of other enhancers controlling other aspects of *Gata4* expression. Transgene expression was often stronger than endogenous gene expression because of the long half-life and enzymatic activity of β-galactosidase (Fig. 2, compare panels B and C), but transgene expression was always contained within the weaker endogenous pattern.

Fig. 2. The *Gata4* G2-*lacZ* transgene is expressed in the lateral mesoderm and septum transversum during mouse embryonic development. Whole-mount (A,B,F), transverse (E,M,O) and sagittal (J) sections of X-gal-stained G2-*lacZ* transgenic embryos are shown. For comparison, whole-mount (C,D,G,H), sagittal (I,K,L) and transverse (N,P) section in situ hybridization with different mesodermal markers is shown. (D,H,L) *Foxf1*; (G,K) *Bmp4*; (C,I,N) *Gata4*. (A,B) At 7.75 dpc and 8.25 dpc, *lacZ* expression directed by the *Gata4* G2 enhancer is present in the lateral mesoderm (LM) and allantois (al). (E) A transverse section at midgut level at 8.0 dpc, β-galactosidase activity is evident in both the somatic mesoderm (SM) and visceral mesoderm (VM) of the transgenic embryo. (F,J,M,O) By 9.5 dpc, *lacZ* expression is very robust in the mesodermal component of the septum transversum (ST) and in the visceral mesoderm surrounding the gut. Note that transgene expression is completely absent in the hepatic endoderm (HE) within the ST (F,J), and in the visceral endoderm (VE) of the gut (O). The expression directed by the *Gata4* enhancer overlaps the expression of the mesodermal forkhead gene *Foxf1*, *Bmp4* and endogenous *Gata4* at all time points examined. NT, neural tube. Asterisks denote expression of the G2-*lacZ* transgene and endogenous *Gata4* in the allantois at 8.25 dpc. Scale bars: 100 μm.

Expression directed by the *Gata4* lateral mesoderm enhancer overlaps *Foxf1* and *Bmp4*

To define the *Gata4* lateral mesoderm enhancer in more detail, we compared the expression pattern directed by the enhancer with the expression pattern
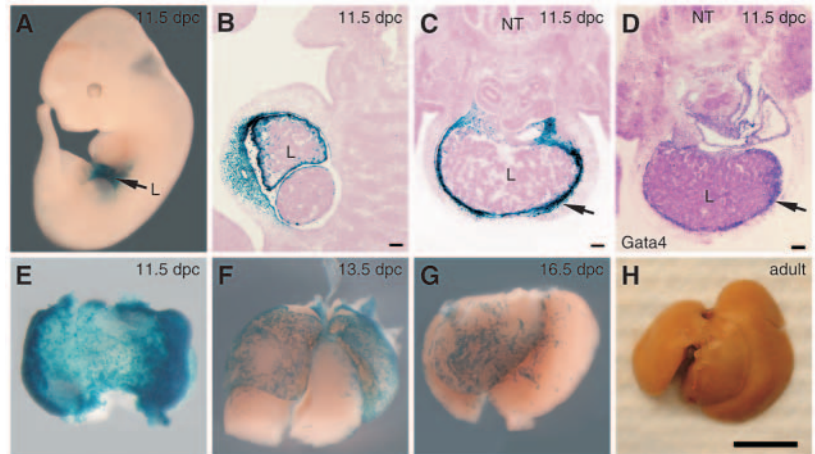
of the early mesodermal marker *Foxf1* (Peterson et al., 1997). The expression patterns of the *Gata4* transgene and *Foxf1* appeared to be nearly identical at 8.25 dpc (Fig. 2, compare B and D) and at 9.5 dpc (Fig. 2, compare F and H, and J and L). Expression directed by the *Gata4* G2 mesodermal enhancer was nearly identical to the expression of *Foxf1* in the septum transversum (Fig. 2, compare J and L), and was largely, but not completely, overlapping in the gut mesenchyme (Fig. 2, compare O and P). By contrast, the gut expression directed by the *Gata4* G2 enhancer did not overlap with the gut expression of *Foxa2*, which is restricted to the endodermal compartment within the developing gut (not shown). β-Galactosidase expression directed by the *Gata4* G2-*lacZ* transgene also overlapped the expression of *Bmp4* (Fig. 2, compare F and G, and J and K), but did not overlap the expression of the hepatic endodermal marker *Hex* (not shown).

Expression directed by the *Gata4* lateral mesoderm enhancer becomes restricted to the mesenchyme surrounding the liver

Later in embryonic development, expression directed by the *Gata4* G2 enhancer became tightly restricted to the mesenchyme surrounding the liver (Fig. 3). At 11.5 dpc, transgene expression could only be detected in the mesenchymal cells surrounding the liver (Fig. 3A-C), which are derived from septum transversum mesenchyme.


Fig. 3. Expression directed by the *Gata4* lateral mesoderm enhancer becomes restricted to the mesenchyme surrounding the liver. Representative X-gal-stained transgenic embryos (A-C) and dissected livers from transgenic animals (E-H) are shown. (A-C) Expression directed by the *Gata4* enhancer is present exclusively in the liver (L) of transgenic embryos at 11.5 dpc (A), and this expression is restricted to the mesenchyme surrounding the liver, as observed in sagittal (B) and transverse (C) sections. The liver mesenchyme expression directed by the enhancer is identical to the expression of endogenous *Gata4* in the liver, which was evident in transverse sections at 11.5 dpc (D). (E-G) Expression directed by the *Gata4* enhancer in the liver mesenchyme was strong at 11.5 dpc (E) but began to diminish by 13.5 dpc (F) and 16.5 dpc (G). No transgene expression was observed in the adult liver (H). NT, neural tube. The arrows in C and D denote expression of G2-*lacZ* and endogenous *Gata4* in the mesenchyme surrounding the liver at 11.5 dpc. Scale bars: in B-D, 100 μm; in H, 1 cm.

The expression directed by the *Gata4* G2 enhancer to the liver mesenchyme (Fig. 3C) was more robust than endogenous *Gata4* expression in the liver mesenchyme because of the longer half-life and enzymatic activity of β-galactosidase, but transgene expression completely mirrored the endogenous pattern (Fig. 3, compare C and D). Transgene expression was not detected in any other tissues outside of the liver at 11.5 dpc (Fig. 3A,C), or at any later stages in development or adulthood (not shown). Expression in the mesenchyme surrounding the liver was still fairly robust at 11.5 dpc (Fig. 3E) but began to diminish as development progressed. Expression in the mesenchyme surrounding the liver could still be detected weakly at 13.5 dpc (Fig. 3F) and 16.5 dpc (Fig. 3G), but was completely absent in the adult liver (Fig. 3H).

A small, evolutionarily conserved module is necessary and sufficient for *Gata4* lateral mesoderm enhancer function in vivo

We next wanted to define the minimal region of the *Gata4* G2 mesodermal enhancer that was required for expression in vivo. Comparison of the mouse *Gata4* gene sequence with the *Gata4* sequence from the opossum *Didelphus virginiana* identified three smaller regions of very high conservation within the G2 sequence, denoted CR1, CR2 and CR3 (Fig. 4A). We reasoned that one or more of the smaller regions of conservation was likely to be crucial for enhancer function in vivo because these sequences have been conserved for the approximately 150 million years since placental and marsupial mammals diverged (Graves, 1996). Accordingly, we designed a series of deletion constructs of the G2 region from the mouse *Gata4* gene to

define which of these three deeply conserved regions were important for mesodermal expression at 9.5 dpc (Fig. 4). The 4368 bp G2 fragment directed robust expression in the septum transversum and visceral mesoderm at 9.5 dpc (Fig. 4B). Deletion of the CR2 and CR3 regions from the 3′ end of G2 to create construct G2Δ1 (nucleotides 1-3186) completely abolished transgene activity in vivo (Fig. 4C). By contrast, a 1358 bp fragment, designated G2Δ2 (nucleotides 3011-4368), that included CR2 and CR3 was sufficient to direct robust transgene expression in a temporal and spatial pattern that was identical to that of the larger G2 construct (Fig. 4D).

We next created two deletion constructs designed to separate CR2 from CR3, to determine whether either or both of these regions were sufficient to direct mesodermal expression in vivo (Fig. 4A). G2Δ3 contained only the CR3 region of opossum homology and was unable to direct any detectable expression in transgenic embryos at 9.5 dpc (Fig. 4E). By contrast, the G2Δ4 construct, which contained the CR2 region but not the other deeply conserved regions within the G2 region was sufficient to direct the mesodermal expression pattern observed with the full-length construct (Fig. 4F). Further dissection of the CR2 region to create construct G2Δ5, a smaller 308 bp region that contained only the most highly conserved region of sequence within CR2, had a dramatic and deleterious impact on transgene expression in vivo, although the pattern of expression appeared similar (Fig. 4G). Construct G2Δ6, which contained an internal deletion from construct G2 that removed the CR2 region, was inactive in transgenic embryos (Fig. 4H). Taken together, the results of these deletional analyses demonstrate that the CR2 region of ancient conservation within

Fig. 4. Deletional analysis of the *Gata4* lateral mesoderm enhancer identifies a highly conserved element that is necessary and sufficient for enhancer function in vivo. (A) Schematic diagram of the deletion constructs of the *Gata4* lateral mesoderm enhancer. The genomic organization of the G2 region of *Gata4* is depicted at the top. Red boxes represent three regions of high sequence homology between the opossum and mouse *Gata4* genes, denoted as CR1, CR2 and CR3. The nucleotide positions of each deletion construct, relative to G2, are denoted on the left, and mesoderm expression directed by

each construct is summarized by a plus (mesodermal expression) or a minus (no detectable mesodermal expression) to the right of the line representing each construct. The column on the far right indicates the number of independent transgenic lines or F0 embryos that expressed *lacZ* in the septum transversum and lateral mesoderm as a fraction of the total number of transgene-positive F0 embryos or lines examined. (B-H) Representative transgenic embryos for each of the deletion constructs depicted in A were collected at 9.5 dpc and X-gal stained. G2, G2Δ2 and G2Δ4 directed strong expression in the septum transversum (ST) and visceral mesoderm (B,D,F). G2Δ4 encompasses only the region surrounding the highly conserved CR2. G2Δ5 contains only 308 bp of CR2 and was sufficient to direct only very weak expression in septum transversum and gut mesoderm (G). Deletion of CR2 (Δ2495-3738) from G2 to generate G2Δ6 completely ablated transgene activity (H). Arrowheads indicate visceral mesoderm.
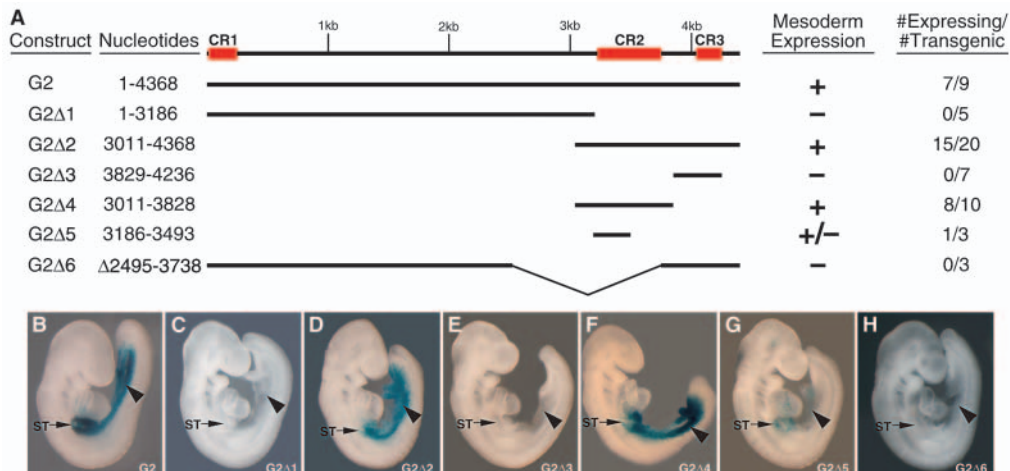
the *Gata4* G2 mesodermal enhancer is necessary and sufficient for enhancer function in vivo.

The *Gata4* lateral mesoderm enhancer contains deeply conserved Forkhead-, GATA- and SMAD-binding sites
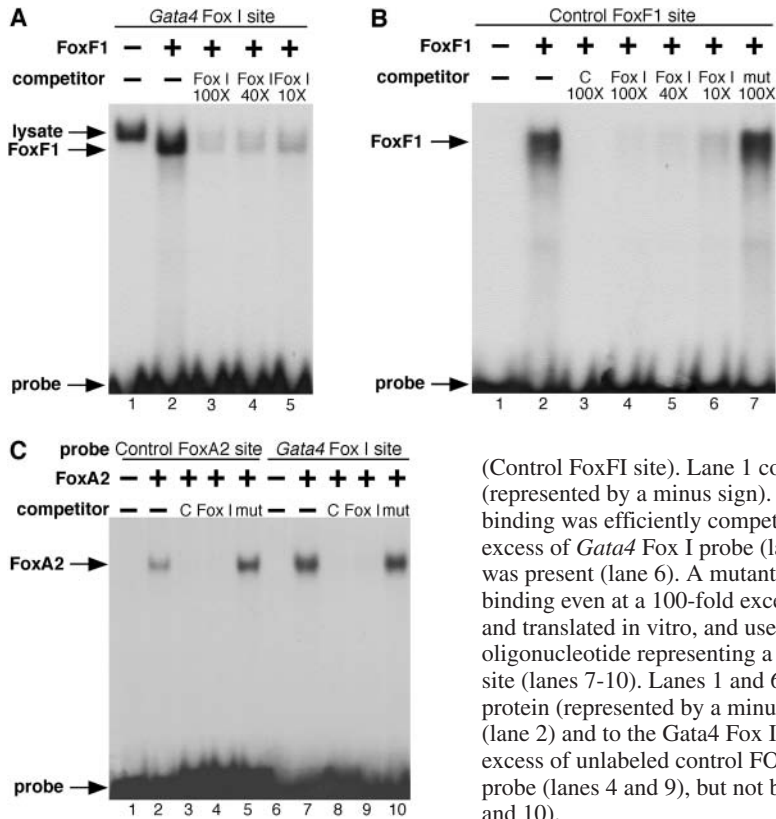
The deletional analyses shown in Fig. 4 identified the CR2 conserved region from the *Gata4* G2 mesodermal enhancer as being required and sufficient for activity in vivo. Therefore, we examined the CR2 region for candidate transcription factor binding sites and for cross species conservation (Fig. 5). A ClustalW analysis (Thompson et al., 1994) comparing the conserved region of the enhancer from mouse, human and opossum identified perfect conservation in three candidate Forkhead transcription factor (FOX)-binding sites and three candidate GATA transcription factor binding sites (Fig. 5).

These analyses also identified two conserved candidate SMAD-binding sites (Fig. 5). SMAD proteins are downstream transcriptional effectors of BMP signaling, and, as shown in Fig. 2, the expression of *Bmp4* largely overlaps with the expression of *Gata4* G2-*lacZ*. Similarly, the expression of the Forkhead transcription factor gene *Foxf1* almost completely overlaps the *lacZ* expression directed by the *Gata4* G2 enhancer (Fig. 2). Thus, the putative SMAD- and FOX-binding sites in the conserved region of the enhancer represented excellent candidate sites for a potential role in the regulation of the *Gata4* gene. Likewise, the candidate GATA-binding sites in the enhancer represented excellent potential sites for auto- and cross-regulation of the enhancer by GATA4 and other GATA family members.

To determine whether the candidate GATA-, FOX- and SMAD-binding sites in the *Gata4* mesodermal enhancer might

Fig. 5. The *Gata4* lateral mesoderm enhancer contains three conserved, candidate FOX-binding sites, two conserved, candidate SMAD-binding sites, and three perfectly conserved, candidate GATA factor binding sites. ClustalW analysis comparing the sequence of the conserved enhancer region from mouse, human and opossum, identified: three conserved candidate binding sites for FOX transcription factors (blue boxes), denoted as Fox I, Fox II and Fox III; two conserved, candidate binding sites for SMAD transcription factors (yellow boxes), denoted as Smad I and Smad II; and three perfectly conserved, candidate binding sites for GATA factors (red boxes), denoted as gata I, gata II and gata III. Asterisks denote nucleotides that have been perfectly conserved among all three species.

```
                        Fox I                        Fox II   Smad I
mouse     cc-ctgggggtagtctaaacaagcatcttcagaaaagggggg---tttattgccaagacagt-ag
human     tctcctgggggtagtctaaacaagcatcttaagaaaaagaca---tttattgccaagacaattgg
opossum   ttccagaggtagtctaaacaagcatcttaaaacaggaagacattttattgccaagacaactgg
          *   ******* ************** *  *  *    ****** **********       *

          gata I                Smad II
mouse     agataagcagggctcatctgtctgccacaatgactcgggctgggcctgggttgca-cattgtc
human     agataagggggaaaatcatctgtctgccaaaatggcttgcgctgggcctgagctgtggcattgtc
opossum   agataaggaaagatcatctgtctactaaaatgactccagttgagcctgacctgtggcattgac
          *******         ********** *  * **** **   * ** ***** **    *** *

                                                          gata II
mouse     agagtgattttcagcagag--cctgcaagccatgaaactcttacaagctccagataaggccctt
human     agagtgattttagcacagaccctgctagacatgagccctattacaaatgcagataaggccctc
opossum   agaatgacttttaatgtccactgagacctgtgcacaagtataatagtttttaagataaggacatt
          *** *** ** *        *           *         * * **  ******** * *

mouse     gtctttagagagcttgg-aggag---aggggagg-----ggggagttgtagtgatagtcgcca
human     gttttttatagagctcgg-aggagtagagggggagac-----gggggagttgcagtgatagtcgcca
opossum   gtttttatagagattgtcaggaggaatgggggagtcaaaggggacttggagcaatagtcacca
          ** **** **** * *  *****     ****    ***** *** ** ** ****** ***

          gata III             Fox III
mouse     ctggagataaggagaatatatatttttgtttaaccaaacctgtctattggctagagacaccaat
human     ctgcagataaggagaatatatattttgtttaaccaaacctgtccgttggctagagactccaat
opossum   ctgtagataaggagaatctatatttttgtttaaccatacctgtc--tcagctagaaaatcaaat
          *** ******* ***** ***** ************ ******      * ****** *  * ***
```
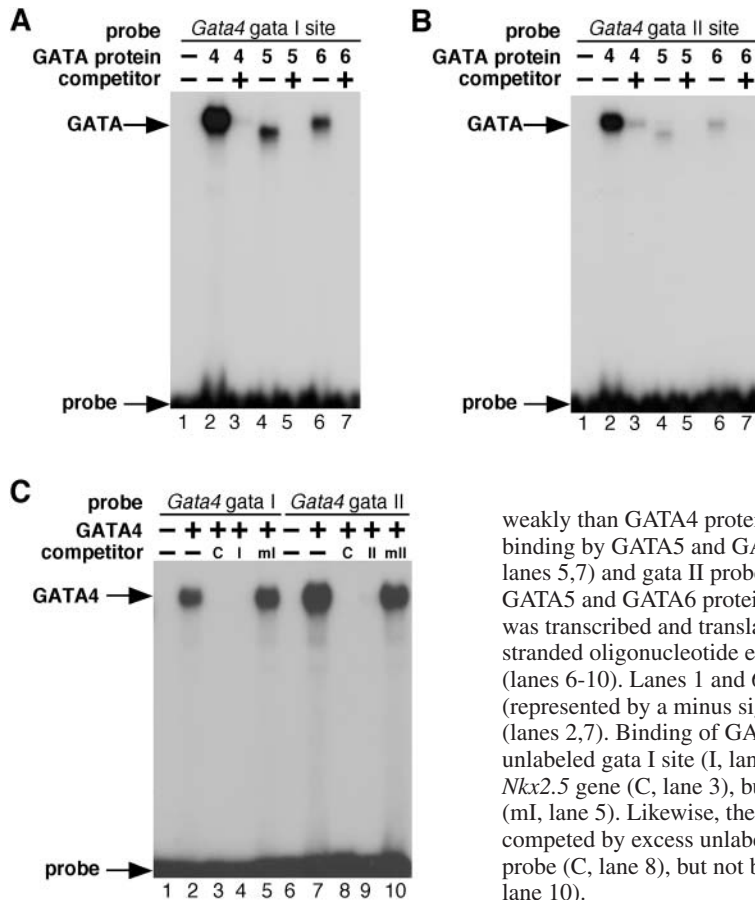
Fig. 6. The *Gata4* lateral mesoderm enhancer contains a high-affinity Forkhead-binding site. (A) Recombinant FOXF1 protein was transcribed and translated in vitro and used in EMSA with a radiolabeled double-stranded oligonucleotide encompassing the *Gata4* Fox I site (lanes 2-5). Lane 1 contains reticulocyte lysate without recombinant FOXF1 protein (represented by a minus sign). FOXF1 efficiently bound to the *Gata4* Fox I site (lane 2) and this binding was efficiently competed by a range of excess unlabeled *Gata4* Fox I oligonucleotides (lane 3-5). A nonspecific lysate-derived band is denoted. (B) Recombinant FOXF1 protein was transcribed and translated in vitro, and used in EMSA with a radiolabeled double-stranded oligonucleotide representing a canonical FOXF1 site (Control FoxFI site). Lane 1 contains reticulocyte lysate without recombinant FOXF1 protein (represented by a minus sign). FOXF1 efficiently bound to the control Fox I site (lane 2). This binding was efficiently competed by an excess of unlabeled control probe (lane 3) and by an excess of *Gata4* Fox I probe (lanes 4-6), even when only a 10-fold excess of the competitor was present (lane 6). A mutant version of the *Gata4* Fox I site failed to compete for FOXF1 binding even at a 100-fold excess (lane 7). (C) Recombinant FOXA2 protein was transcribed and translated in vitro, and used in EMSA with a radiolabeled double-stranded oligonucleotide representing a canonical control FOXA2 site (lanes 2-5) or the *Gata4* Fox I site (lanes 7-10). Lanes 1 and 6 contain reticulocyte lysate without recombinant FOXA2 protein (represented by a minus sign). FOXA2 efficiently bound to the control FOXA2 site (lane 2) and to the Gata4 Fox I site (lane 7), and this binding was efficiently competed by an excess of unlabeled control FOXA2 probe (lanes 3 and 8) and by an excess of *Gata4* Fox I probe (lanes 4 and 9), but not by an excess of unlabeled mutant *Gata4* Fox I probe (lanes 5 and 10).

represent bona fide cis-acting elements, we tested whether each of the sites was bound by its candidate factor by EMSA. FOXF1 bound to the *Gata4* Fox I site (Fig. 6A, lane 2), and it also bound to the Fox II and Fox III sites in the enhancer, but the binding to those sites was weaker than to the Fox I site (data not shown). Binding was specific because it was competed by excess unlabeled self probe over a range of concentrations (Fig. 6A, lanes 3-5). To examine the binding of FOXF1 to the *Gata4* Fox I site in more detail, we determined the ability of the Fox I site and a mutant version of that site to compete for FOXF1 binding to a canonical consensus control Forkhead-binding site (Fig. 6B), which has been described previously (Peterson et al., 1997). FOXF1 bound efficiently to the control FOXF1 site (Fig. 6B, lane 2) and this binding was specific, as it was efficiently competed by an excess of unlabeled control FOXF1 site probe (Fig. 6B, lane 3). Likewise, the binding of FOXF1 to the control FOXF1 site was abolished by the addition of unlabeled *Gata4* Fox I site probe at a 10-fold excess (Fig. 6B, lane 6) but not by a mutant version of the *Gata4* Fox I site, not even when added at a 100-fold excess (Fig. 6B, lane 7).

The expression pattern of G2-*lacZ* largely overlapped with the expression of *Foxf1*, making FOXF1 a likely potential regulator of *Gata4* expression in the lateral mesoderm via the Fox I site in the enhancer (Fig. 5). However, it is possible that other Forkhead factors may also bind to the *Gata4* enhancer through the FOX sites in the G2 enhancer. To test this, we tested the ability of FOXA2 to bind to the FOX sites in the *Gata4* G2 enhancer. As was the case with FOXF1, FOXA2 bound efficiently and specifically to the Fox I site (Fig. 6C), but was unable to bind to the Fox II and Fox III sites in the

Gata4 lateral mesoderm enhancer (data not shown). FOXA2 bound efficiently to a control FOXA2 site (Fig. 6C, lane 2), which has been described previously (Overdier et al., 1994). This binding was specifically abolished by the addition of excess unlabeled control site (Fig. 6C, lane 3) and by the addition of unlabeled G2 Fox I sites (Fig. 6C, lane 4), but not by the addition of unlabeled mutant Fox I site at a 100-fold excess (Fig. 6C, lane 5). FOXA2 also bound efficiently to the *Gata4* Fox I site itself (Fig. 6C, lane 7), and this binding was efficiently competed away by the addition of unlabeled FOXA2 control or G2 Fox I site oligonucleotides (Fig. 6C, lanes 8,9), but not by a 100-fold excess of the mutant Fox I site (Fig. 6C, lane 10). FOXA2 is expressed within the endoderm in the gut, but a recent paper reported that FOXA2 is also expressed in the mesoderm (Hu et al., 2004), suggesting that it or another related Forkhead factor could regulate *Gata4* expression in lateral mesoderm. Overall, the results presented in Fig. 6 demonstrate that the conserved Fox I site in the *Gata4* lateral mesoderm enhancer is a bona fide binding site for Forkhead transcription factors, including FOXF1.

We also tested the three conserved, putative GATA sites contained within the CR2 region of the enhancer to determine if they were bound in EMSA by GATA4, GATA5 or GATA6. The gata I and gata II sites were each efficiently bound by GATA factors (Fig. 7), while the gata III site exhibited only very weak binding by GATA factors in vitro (data not shown). GATA4 bound very robustly to the gata I site in the *Gata4* lateral mesoderm enhancer (Fig. 7A, lane 2), whereas GATA5 and GATA6 bound more weakly to that site (Fig. 7A, lanes 4 and 6, respectively). Binding to the gata I site by each of the three GATA factors was specific because the binding of each

Fig. 7. The *Gata4* lateral mesoderm enhancer contains two high-affinity GATA-binding sites.
(A,B) Recombinant GATA4, GATA5 and GATA6 proteins were transcribed and translated in vitro, and used in EMSA with radiolabeled double-stranded oligonucleotides encompassing the *Gata4* gata I site (A, lanes 2-7) or the gata II site (B, lanes 2-7). In A and B, lane 1 contains reticulocyte lysate without recombinant protein (represented by a minus sign). GATA4 efficiently bound to both the gata I and gata II sites (A and B, lane 2) and this binding was specifically competed by excess unlabeled gata I (A, lane 3) and gata II probes (B, lane 3). GATA5 and GATA6 proteins also bound, although more weakly than GATA4 protein, to the gata I (A, lanes 4,6) and gata II sites (B, lanes 4,6), and binding by GATA5 and GATA6 was specifically competed by excess unlabeled gata I (A, lanes 5,7) and gata II probes (B, lanes 5,7). Approximately equivalent amounts of GATA4, GATA5 and GATA6 proteins were used in each sample. (C) Recombinant GATA4 protein was transcribed and translated in vitro and used in EMSA with a radiolabeled double-stranded oligonucleotide encompassing the *Gata4* gata I site (lanes 1-5) or the gata II site (lanes 6-10). Lanes 1 and 6 contain reticulocyte lysate without recombinant GATA4 protein (represented by a minus sign). GATA4 efficiently bound to both the gata I and gata II sites (lanes 2,7). Binding of GATA4 to the *Gata4* gata I site was competed by an excess of unlabeled gata I site (I, lane 4) and by an excess of an unlabeled control GATA site from the *Nkx2.5* gene (C, lane 3), but not by an excess of a mutant version of the *Gata4* gata I site (mI, lane 5). Likewise, the binding of GATA4 to the *Gata4* gata II site was specifically competed by excess unlabeled gata II probe (II, lane 9) and by excess unlabeled control probe (C, lane 8), but not by an excess of a mutant version of the gata II probe (mII, lane 10).

was efficiently competed by the addition of excess unlabeled control gata I site (Fig. 7A, lanes 3,5,7). Similarly, GATA4 also bound very robustly to the *Gata4* G2 enhancer gata II site (Fig. 7B, lane 2), whereas the binding of GATA5 and GATA6 to that site was considerably weaker (Fig. 7B, lanes 4 and 6, respectively). As with the gata I site, binding of all three factors to the gata II site was specifically inhibited by the addition of excess unlabeled gata II probe (Fig. 7B, lanes 3, 5 and 7). Because the binding of GATA4 to both the gata I and gata II sites appeared to be stronger than GATA5 or GATA6, we examined the binding of GATA4 in additional detail by competing with a bona fide control GATA-binding site and with mutant versions of the *Gata4* gata I and gata II sites (Fig. 7C). Binding of GATA4 to the gata I and II sites in the *Gata4* enhancer was efficiently competed in both cases by a 100-fold excess of the *Nkx2.5* gs1 control site (Fig. 7C, lanes 3,8), which is a bona fide GATA4-binding site and has been described previously (Lien et al., 1999). The gata I site was also efficiently competed by excess self probe (Fig. 7C, lane 4) but not by mutant version of itself (Fig. 7C, lane 5). Similarly, the gata II site was competed by excess unlabeled self probe (Fig. 7C, lane 9) but not by a mutant form of itself (Fig. 7C, lane 10). These results demonstrate that the gata I and gata II sites in the *Gata4* lateral mesoderm enhancer both represent extremely robust and specific GATA-binding sites.

In addition to the FOX and gata sites in the enhancer, we also tested two candidate SMAD sites in the enhancer (Fig. 5) to determine if these putative sites might be targets for SMAD

protein binding and direct regulation by BMP signaling. Because SMAD proteins bind to DNA as oligomers with SMAD4 (Derynck and Zhang, 2003; von Bubnoff and Cho, 2001), we tested the ability of SMAD1/SMAD4, SMAD4/SMAD5, SMAD4/SMAD8, and SMAD4 alone to bind to either of the two candidate SMAD-binding sites in the enhancer. In no case were we able to detect any binding by SMAD factors to either of the sites in the enhancer under conditions in which a control SMAD site was bound by SMAD4 oligomers in EMSA (data not shown). Furthermore, we introduced mutations predicted to disrupt SMAD protein binding into the two candidate SMAD elements in the G2 lateral mesoderm enhancer and tested the effect of those mutations on enhancer function in transgenic embryos. Simultaneous mutation of both putative SMAD sites in the enhancer had no effect on transgene expression at any time point examined, including early time points at 7.75 dpc and 8.5 dpc (data not shown). Taken together, these data suggest that the two putative elements in the G2 enhancer are not bona fide SMAD-binding sites and that the *Gata4* G2 lateral mesoderm enhancer is not a direct target of BMP signaling.

Gata4 lateral mesoderm enhancer activity is dependent on Forkhead- and GATA-binding sites

To test the function of the *Gata4* FOX and GATA sites in vivo, we introduced mutations into the Fox I site and both of the functional GATA sites (gata I and gata II), in the context of the G2Δ2-*lacZ* transgene (Fig. 4), and determined the effect of

those mutations on enhancer function in transgenic embryos collected at 7.75 dpc, 9.5 dpc and 11.5 dpc (Fig. 8). The introduced mutations were identical to those used in the EMSA analyses, which were shown in Figs 6 and 7 to completely ablate FOXF1 and GATA4 binding, respectively. The wild-type G2Δ2-*lacZ* transgene directed robust expression throughout the lateral mesoderm and allantois at 7.75 dpc (Fig. 8A). The wild-type construct continued to direct expression broadly in the visceral mesoderm and septum transversum mesoderm at 9.5 dpc (Fig. 8D) and directed expression restricted to mesenchyme surrounding the liver at 11.5 dpc (Fig. 8G). Mutation of the Fox I site in the enhancer resulted in a complete disruption of enhancer activity in 15 out of 15 F0 transgenic embryos at all time points (Fig. 8B,E,H), indicating a key role for Forkhead factors in the activation of the enhancer. Likewise, double mutation of the two bona fide GATA sites in the enhancer completely ablated enhancer activity in 14 out of 14 independent F0 transgenic embryos at all time points (Fig. 8C,F,I). These results demonstrate that *Gata4* expression in the lateral mesoderm is subject to auto- and/or cross-regulation by GATA4 and other GATA factors.
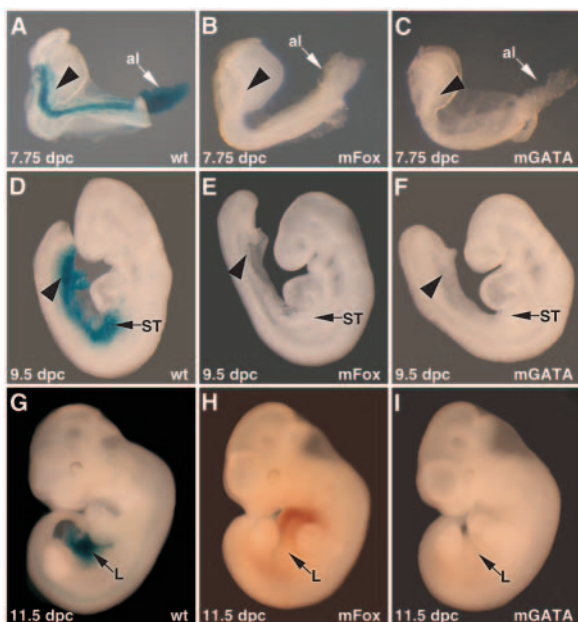
Fig. 8. The *Gata4* lateral mesoderm enhancer is dependent on conserved Forkhead and GATA sites for its function in vivo. The wild-type *Gata4* enhancer transgene construct G2Δ2 (wt; A,D,G) and transgenes containing mutations either in the Fox I site (mFox; B,E,H), or in both the gata I and gata II sites (mGATA; C,F,I), in the context of G2Δ2, were used to generate transgenic embryos. Representative transgenic embryos are shown at 7.75 dpc (A-C), 9.5 dpc (D-F) and 11.5 dpc (G-I). The wild-type construct directed strong expression in the lateral mesoderm (arrowhead) and allantois (al) at 7.75 dpc (A), in the septum transversum (ST) and visceral mesoderm (arrowhead) at 9.5 dpc (D), and in the mesenchyme surrounding the liver (L) at 11.5 dpc (G). Mutations in the Fox I site completely eliminated transgene expression in all of the fifteen independent transgenic embryos analyzed (B,E,H). Similarly, mutation of both GATA sites completely eliminated *lacZ* expression in the fourteen independent transgenic embryos analyzed (C,F,I). Arrowheads in A-C indicate lateral mesoderm; arrowheads in D-F indicate visceral mesoderm.

Activation of the *Gata4* lateral mesoderm enhancer is dependent on BMP4

Although we did not observe any bona fide SMAD-binding sites in the enhancer, several lines of evidence suggested the possibility that the *Gata4* G2 lateral mesoderm enhancer might be an indirect downstream target of BMP4. Previous studies indicated an interrelationship among Forkhead and GATA factors and BMP4 during the development of the endoderm (Rossi et al., 2001). Furthermore, the broad expression of the G2-*lacZ* transgene in the mesoderm and in the allantois, combined with the largely overlapping expression of *Bmp4* in those lineages or in nearby lineages where signaling could occur from one region to another, prompted us to test whether BMP4 might be an upstream regulator of *Gata4* enhancer activity (Fig. 9). To test this hypothesis, we crossed mice
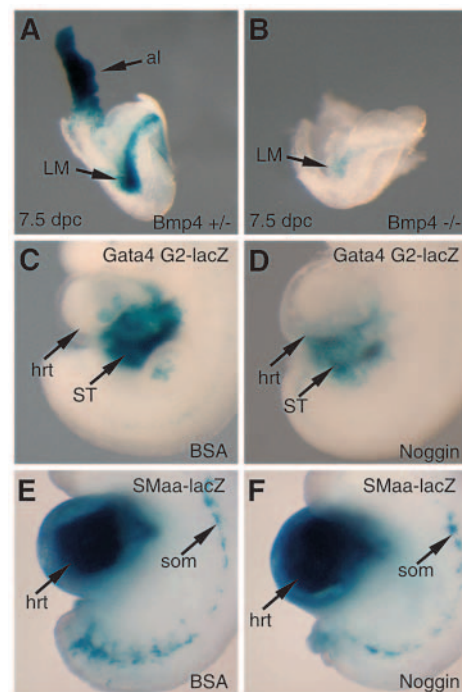
Fig. 9. Activity of the *Gata4* lateral mesoderm enhancer requires BMP4. (A,B) The G2-*lacZ* transgene was crossed into either a *Bmp4*^{+/−} or a *Bmp4*^{−/−} background, and embryos were collected at 7.5 dpc and stained with X-gal. Heterozygous *Bmp4*^{+/−}; G2-*lacZ* Tg/0 embryos displayed strong expression of *lacZ* in the lateral mesoderm (LM) and allantois (al; A). By contrast, expression directed by the *Gata4* lateral mesoderm enhancer in *Bmp4*^{−/−}; G2-*lacZ* Tg/0 embryos was dramatically attenuated in all embryos examined (B), indicating that transgene activity is dependent on BMP4. (C-F) Explanted tissue containing the heart (hrt) and septum transversum (ST) of embryos from a single G2-*lacZ* stable transgenic line (C,D) or a single SMaa-*lacZ* stable transgenic line (E,F) was collected at 9.5 dpc and cultured for 48 hours in the presence of BSA (C,E) or recombinant Noggin (D,F). Following incubation, explants were X-gal stained. *Gata4* lateral mesoderm enhancer activity was significantly reduced in all 15 viable embryo explants treated with Noggin when compared with the 15 viable embryo explants treated with BSA. By contrast, no differences were observed in X-gal staining in the heart or somites in SMaa-*lacZ* explants treated with BSA or Noggin (compare E and F), indicating that embryo explants were not in general crisis in the presence of Noggin. Viability of embryo explants was assessed by the observation of a continually beating heart.

Development

harboring a stably integrated G2-*lacZ* transgene with *Bmp4*^{+/–} mice to generate mice that were G2-*lacZ* Tg/0; *Bmp4*^{+/–} and crossed these mice to *Bmp4*^{+/–} mice to generate embryos that were positive for the *Gata4* enhancer transgene and null for *Bmp4*. Approximately half of the *Bmp4*-null embryos died prior to gastrulation because of the role of BMP4 in gastrulation (Winnier et al., 1995). However, about half of the *Bmp4* null embryos survived gastrulation and were clearly alive at 7.5 dpc, as has been previously reported (Winnier et al., 1995). At this stage, the activity of the *Gata4* enhancer was easily detectable in heterozygous *Bmp4* embryos, with strong expression of *lacZ* evident in the lateral mesoderm and allantois (Fig. 9A). By contrast, the activity of the *Gata4* enhancer was barely detectable in the lateral mesoderm of *Bmp4* null embryos (Fig. 9B), indicating that the *Gata4* lateral mesoderm enhancer requires BMP4 for activity in vivo. It was difficult to determine the requirement of BMP4 for activation of *Gata4* G2-*lacZ* in the allantois, as that structure was missing or severely reduced in *Bmp4* null embryos, as has been reported previously (Winnier et al., 1995). However, in *Bmp4*^{–/–} embryos where some allantoic tissue was present, we never observed transgene expression (data not shown), suggesting that BMP4 is required for *Gata4* activation in the allantois as well.

The BMP antagonist Noggin inhibits *Gata4* lateral mesoderm enhancer activity

Mice lacking *Bmp4* often die prior to gastrulation and those that do survive past gastrulation have defects in mesodermal development, although mesoderm is present in those embryos (Winnier et al., 1995). Therefore, we considered the possibility that the severe reduction in *Gata4* transgene expression in *Bmp4* null embryos (shown in Fig. 9B) might be secondary to an overall mesodermal defect. Although we considered this possibility unlikely because *Bmp4* mutants that survive gastrulation do have embryonic mesoderm, we wanted to define further the relationship between BMP signaling and the *Gata4* mesodermal enhancer. As an independent approach to examine the role of BMP signaling on *Gata4* enhancer activation, we tested whether the activity of the enhancer was sensitive to the BMP inhibitor Noggin.

Transgenic embryos from a single stable G2-*lacZ* transgenic line were explanted at 9.5 dpc and the region of the embryos containing the heart, septum transversum and portions of the visceral mesoderm were maintained in culture in the presence of BSA (Fig. 9C) or recombinant Noggin (Fig. 9D). As a control, transgenic embryos from a single stable transgenic smooth muscle α-actin line, SMaa-*lacZ*, which expresses β-galactosidase in the heart and somites at 9.5 dpc, were also explanted in the presence of BSA (Fig. 9E) or Noggin (Fig. 9F). Embryo explants were maintained in culture for 48 hours and the hearts continued to beat throughout the time course of the experiment, indicating the explants were viable for the duration of the experiment. A total of fifteen G2-*lacZ* transgenic embryo explants from six different litters were treated with BSA and fifteen were treated with Noggin, and we compared X-gal staining in the septum transversum of embryo explants from the two groups. In each case, Noggin-treated *Gata4* G2-*lacZ* transgenic embryos exhibited a clear attenuation of β-galactosidase activity (Fig. 9D) when compared with control-treated embryos (Fig. 9C). By contrast,

no change in β-galactosidase activity in SMaa-*lacZ* explants was observed in the presence of Noggin (Fig. 9F) when compared with BSA-treated control explants (Fig. 9E), indicating that embryo explants were not in general crisis as a result of Noggin treatment. Thus, these results show that the activity of the *Gata4* enhancer is inhibited by Noggin and further demonstrate that *Gata4* is a downstream target of BMP in the septum transversum and lateral mesoderm. Taken together, the results presented in Fig. 9 indicate that the *Gata4* mesodermal enhancer requires BMP signaling for activation, and suggest that GATA4 may serve as a downstream effector of BMP4 in the lateral mesoderm and septum transversum mesenchyme.

Discussion

Gata4 is among the earliest markers of the endoderm and the lateral mesoderm following gastrulation in the mouse embryo (Arceci et al., 1993; Heikinheimo et al., 1994; Nemer and Nemer, 2003), and targeted inactivation of the *Gata4* gene in mice results in delayed ventral morphogenesis and subsequent cardia bifida as a result of severe endodermal defects (Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1997). However, despite the importance of *Gata4* during development, it has been unclear how *Gata4* expression is activated. In this manuscript, we identified the first transcriptional enhancer from the mouse *Gata4* gene, and we show that this novel enhancer is active throughout the lateral mesoderm beginning at 7.5 dpc. As development proceeds, expression directed by the *Gata4* lateral mesoderm enhancer becomes successively restricted to the visceral mesoderm and to the mesodermal component of the septum transversum (Fig. 2), and finally becomes restricted to the mesenchyme surrounding the liver at 11.5 dpc (Fig. 3).

The *Gata4* lateral mesoderm enhancer requires an evolutionarily conserved Forkhead-binding site for activity in vivo (Fig. 8), and FOXF1 binds to the Forkhead-binding site in the enhancer with high affinity (Fig. 6). Thus, these results demonstrate that *Gata4* is a direct transcriptional target of Forkhead factors, and we believe that FOXF1 is a likely candidate based on the overlapping expression pattern of *Foxf1* and the G2-*lacZ* transgene (Fig. 2). The *Gata4* lateral mesoderm enhancer also requires two high-affinity GATA-binding sites for function in vivo (Fig. 8), suggesting an essential role for auto- and cross-regulation of *Gata4* expression by itself and other GATA factors.

GATA4 as a downstream effector of BMP signaling

BMP proteins are key mediators of tissue specification and of patterning in multiple developmental lineages. BMPs are known to play essential roles in bone and cartilage development, neural patterning, heart development, development of the extraembryonic mesoderm and in signaling from the mesoderm to the endoderm during endodermal specification (Hogan, 1996; Shivdasani, 2002; Tam et al., 2003; Zaret, 2001; Zhao, 2003). As members of the TGFβ superfamily of signaling molecules, BMP signaling modulates gene expression, at least in part, through the action of SMAD transcription factors (Derynck and Zhang, 2003; von Bubnoff and Cho, 2001). It has been proposed that BMP factors are able to exert such a diverse array of downstream effects through the

interactions of SMAD proteins with other transcription factors and through the resulting downstream activation of numerous transcription factor networks (Derynck and Zhang, 2003). In this regard, several studies have suggested that GATA transcription factors are downstream targets of BMP signaling. In response to BMP signaling, *Gata4* expression is upregulated in the precardiac mesoderm in the chick (Schultheiss et al., 1997). Similarly, BMP4 signaling from the mesoderm positively induces the expression of *Gata4* in prehepatic endoderm during liver development, demonstrating activation of *Gata4* by BMP4 over a range from mesoderm to endoderm (Rossi et al., 2001). However, the pathways through which *Gata4* is activated in response to BMP4 have not been defined in the endoderm or the mesoderm. Here, we show that the activity of the *Gata4* lateral mesoderm enhancer is attenuated by the BMP antagonist Noggin and is significantly inhibited in the absence of BMP4 (Fig. 9). Taken together with our analyses of the SMAD-binding sites in the enhancer, these observations demonstrate that *Gata4* is probably an indirect downstream target of BMP4 in the lateral mesoderm via the enhancer described here, and suggest that GATA4 functions as a downstream effector of BMP signaling in the mesoderm.

A reinforcing transcriptional pathway dependent on GATA4

In this study, we demonstrate that the *Gata4* lateral mesoderm enhancer requires an essential Forkhead-binding site that is efficiently bound by FOXF1 (Figs 6, 8), supporting the possibility that BMP activation of *Gata4* could be mediated by FOXF1 or other Forkhead proteins. Indeed, a recent study performed in *Xenopus* embryos has shown that *Foxf1* expression is reduced when BMP4 signaling is reduced, indicating that FOXF1 is also a downstream target of BMP4 (Tseng et al., 2004). Interestingly, however, *Bmp4* has been shown to be a potential target of FOXF1 during mouse development, as the level of *Bmp4* mRNA was significantly reduced in the posterior primitive streak, the lateral plate and the allantois in FOXF1 null embryos (Mahlapuu et al., 2001b). Taken together, these results suggest that FOXF1 and BMP4 may function in a reinforcing regulatory circuit designed to amplify a transcriptional response downstream of BMP4 signaling, possibly via GATA and other transcription factor families. GATA transcription factors also appear to activate *Bmp4* expression, further supporting the notion of a reinforcing transcriptional network (Nemer and Nemer, 2003; Peterkin et al., 2003). The mouse and *Xenopus Bmp4* regulatory sequences contain GATA-binding sites that are functional in studies performed in vitro, suggesting that *Bmp4* expression may be activated or maintained by GATA factors (Nemer and Nemer, 2003; Peterkin et al., 2003). A reinforcing transcriptional model is consistent with the work presented here, which suggests a model for *Gata4* activation in the lateral mesoderm in which BMP4 activates the expression of *Gata4* indirectly via FOXF1, and GATA4 functions in a positive-feedback loop to reinforce its own expression through essential GATA sites in its enhancer (Fig. 10).

Modular regulation of *Gata4* transcription

During embryonic development, *Gata4* is expressed in multiple tissues and exhibits a dynamic and distinct pattern of expression in each different lineage where it is expressed
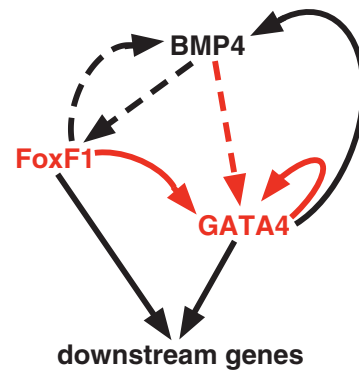
Fig. 10. A reinforcing transcriptional network for gene activation in the lateral mesoderm dependent on GATA4. In this model, GATA4 expression is activated indirectly by BMP4 (indicated by a dashed arrow) and directly by Forkhead factors, such as FOXF1. BMP4 and FOXF1 reciprocally activate the expression of one another, which further reinforces GATA4 expression. GATA4 then further reinforces the program by activating its own transcription and the transcription of downstream mesodermal genes. Red arrows represent evidence provided in the current study. Black arrows represent evidence provided by previously published studies. Dashed arrows indicate either a direct or indirect activation; solid arrows represent direct activation through direct enhancer binding.

(Arceci et al., 1993; Heikinheimo et al., 1994; Parmacek and Leiden, 1999). Notably, the *Gata4* enhancer described here directs expression only to a subset of the endogenous pattern of *Gata4* expression, suggesting that other distinct modular enhancers sufficient to direct expression to other lineages are also likely to be present within the *Gata4* locus. This type of modular regulation has been observed for other key transcriptional regulators of tissue specification and differentiation, including *Nkx2.5* (Schwartz and Olson, 1999) and *mef2c* (De Val et al., 2004; Dodou et al., 2004; Dodou et al., 2003), and modular regulation has been suggested as a mechanism for regulatory diversity (Firulli and Olson, 1997). Given the role of GATA4 as an early regulator of cardiac and endodermal development, it will be important to identify enhancers sufficient to direct expression to those lineages also. It will be particularly interesting to determine whether *Gata4* is a target of Forkhead proteins and is subject to auto-regulation in other lineages, as it is in the lateral mesoderm, or whether regulation in other lineages is via distinct transcriptional hierarchies.

The expression directed by the *Gata4* enhancer described in these studies shows a progressive restriction from an initially broad pattern within the mesoderm to a narrow pattern restricted only to the mesenchymal cells surrounding the liver. The presence of a distinct enhancer for this expression domain suggests a role for GATA4 in the specification of mesodermal cells and their derivatives early in mesoderm development, and it also suggests a potential role for GATA4 in regulating gene expression in the cells of the liver mesenchyme. Thus, it will be important to define the function of GATA4 in the early lateral mesoderm and in the development of the mesenchyme surrounding the liver. The rapid and progressive restriction of enhancer activity from 7.5 dpc to 11.5 dpc suggests that the activators of the *Gata4* lateral mesoderm enhancer also become restricted during development. Alternatively, the *Gata4*

enhancer may contain cis-acting elements that are responsive to active repression and that ultimately restrict the activation of the enhancer to the mesoderm of the septum transversum and, finally, to the liver mesenchyme.

While the importance of GATA4 during embryogenesis is clearly established, an understanding of its transcriptional regulation has remained elusive. Here, we show that the expression of *Gata4* in the lateral mesoderm and septum transversum is mediated by an enhancer located ~40 kb upstream of the start of transcription. Detailed analyses of this enhancer show functional FOX and GATA sites that are essential for transcriptional activation in vivo. These data suggest that transcriptional regulation of *Gata4* is modular, and that enhancer elements may be remote from the coding sequences of the gene. The use of deep evolutionary sequence conservation allowed the identification of its early mesodermal enhancer and suggests that enhancers driving cardiac and endodermal expression might be identified through a similar process. Indeed, we have recently identified a separate enhancer from the *Gata4* gene that is sufficient to direct expression to the endoderm (A.R. and B.L.B., unpublished). It will be interesting to determine whether pathways similar to those described here also regulate *Gata4* expression in the endoderm and other lineages where it is expressed.

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