Development 127, 3829-3838 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV6455

GATA2 is required for the generation of V2 interneurons

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Accepted 21 June; published on WWW 9 August 2000

SUMMARY

During embryogenesis, transcription factor GATA2 is expressed in a variety of distinct cell types, and earlier experiments showed that GATA2 is a vital regulator of both hematopoiesis and urogenital development. Despite the fact that GATA2 is expressed early and abundantly in the nervous system, there has been no demonstration of its direct participation in neurogenesis. We show here that GATA2 is expressed in the ventral spinal cord exclusively in newly generated V2 interneurons, suggesting that GATA2 might be required for the generation of this discrete neuronal population. Proof for this hypothesis was provided by showing that the number of cells expressing V2 neuronal markers was drastically diminished in *gata2* null mutant embryos. The tissue-specific enhancer that directs *gata2* transcription specifically in V2 neurons was localized to a 190 bp intragenic element lying within *gata2* intron 5, and this element is both necessary and sufficient to confer GATA2 spinal cord expression. The identification of a V2-specific enhancer should allow fundamental new insight into the genetic hierarchy of regulatory events that govern neurogenesis in a well-defined cell lineage.

Key words: GATA2, V2 interneuron, Transgenic mice

INTRODUCTION

The pervasive central question in embryology is how determined cell lineages are generated from the initial uncommitted diploid cell created by fertilization. This process is thought to represent a consequence of a highly orchestrated series of temporally regulated extracellulae and intracellular events. In *Drosophila*, interactions at compartment boundaries induce the establishment of one or more early morphogen gradients, which indirectly or directly induce the expression of an overlapping array of inhibitory and activating transcription factors. The activation of a unique combination of target transcription factor activities then determines the developmental fate of specific subpopulations of cells within the compartment, thus leading to developmental determination of specific cell lineages.

The developing vertebrate spinal cord provides an excellent model system for studying early mammalian patterning events. Precisely arranged along the dorsoventral axis, distinct neuronal populations form longitudinal columns that are generated at precisely controlled locations at highly reproducible intervals during embryogenesis. Dorsally localized neurons include both sensory neurons and interneurons involved in the processing of sensory information, while ventral interneurons as well as motor neurons control motor output.

The dorsal and ventral spinal cord neurons are generated during embryogenesis by the interplay of distinct inductive signals. Dorsal signals originate from the epidermal ectoderm and roof plate and are generally mediated by bone morphogenetic proteins (BMPs; Liem et al., 1995), whereas ventral signals are mediated by sonic hedgehog (Shh), produced from the notochord and floor plate (Ericson et al., 1996; Marti et al., 1995). In addition to Shh, an independent retinoic acid-mediated signal from the presomitic and somitic mesoderm is able to specify the two most dorsally located ventral interneuron populations (Pierani et al., 1999). In response to graded expression of Shh, ventral progenitors give rise to motor neurons as well as to at least four distinct classes (based on their expression of distinguishable molecular markers) of ventral interneurons. V0 neurons express Evx1 and are most dorsally localized in the ventral neural tube (Burrill et al., 1997). V1 neurons express En1 and are localized ventrally to V0 (Ericson et al., 1996). V2 neurons are located between V1 and motor neurons, and co-express Lim3 and Chx10 (Ericson et al., 1997), while V3 interneurons are located ventrally to motor neurons and express Nkx2.2 (Briscoe et al., 1999).

GATA2 is a C_4 zinc finger protein and was one of the original proteins to define the GATA transcription factors as a related family. It was originally cloned from a chicken embryo erythroid cDNA library but later shown to be expressed in a broad spectrum of different tissues and cell types (Yamamoto et al., 1990). Early attention focused on the role of GATA2 in hematopoiesis. Numerous studies demonstrated that expression of GATA2 is not only closely associated with the proliferation of hematopoietic progenitor cells, but also with lineage specification (Briegel et al., 1993; Leonard et al.,

1993). Murine embryos bearing an inactivating targeted deletion of *gata2* in the germline die at around 10 days post coitus (dpc) from pan-hematopoietic failure (Tsai et al., 1994).

Little is known about the requirement for GATA2 in developmental processes outside the hematopoietic system because of the early embryonic lethality exhibited by the *gata2* germline mutants. When this developmental block was overcome by complementing the germline mutation with a *gata2* YAC transgene, a second critical role for GATA2 in urogenital development was revealed (Zhou et al., 1998). Recently, it was reported that *gata2* responds to BMP signals and, in collaboration with Pit1, determines the fate of two ventral pituitary cell types (Dasen et al., 1999).

We previously found that GATA2 is transiently expressed in the neural tube between 10 and 12 dpc (Zhou et al., 1998), when many neuronal subtypes are born (Nornes and Carry, 1978). This early neuronal expression pattern indicated that GATA2 might be involved in the determination of specific central nervous system (CNS) cell lineages. We found that GATA2 is expressed in only a single, specific cluster of ventrally localized spinal cord neurons, the majority of which are postmitotic. This transient population of GATA2-expressing cells is dorsal to motor neurons but ventral to V1 interneurons. A majority of the GATA2-postive spinal cord neurons express the Lim3 and Chx10 markers, supporting the notion that they are V2 interneurons. Consistent with this hypothesis, the number of V2 interneurons expressing Lim3 and Chx10 is significantly reduced in germline-targeted *gata2* homozygous mutant embryos, thus providing direct evidence that GATA2 is required for the existence of the V2 interneurons.

Since it is currently impossible to analyze the functional consequences of V2 loss on neurogenesis because of *gata2* mutant early embryonic lethality, we set out to establish a strategy by which we might investigate the consequences of tissue-specific *gata2* loss-of-function mutations. We describe here the identification and characterization of a 190 bp enhancer that is both necessary and sufficient for GATA2 expression in the V2 population. Targeted deletion of this element from a *gata2* YAC specifically ablated GATA2 expression in those neurons, suggesting that the generation of mice bearing such a deletion in the germline could provide new insight into the role GATA2 plays in the generation of a specific class of CNS neurons, as well as the role that this class of neurons play in murine neurophysiology.

MATERIALS AND METHODS

Genotyping

Embryos were genotyped by PCR using DNA prepared from individual yolk sacs. PCR primers and conditions for genotyping *gata2* mutant embryos were described (Tsai et al., 1994). Transgenic embryos carrying either YAC or plasmid transgenes were genotyped using primers specific for the bacterial *lacZ* gene (Lieuw et al., 1997).

YAC mutagenesis

YAC d18Z (Zhou et al., 1998) was modified by homologous recombination in yeast cells to generate YAC d18.1Z and d18.2Z. The targeting construct for generating d18.1Z was created by a three-piece ligation of YIP5 (containing URA3, digested with *Cla*I and *Sph*I), a 150 bp yeast telomere-containing fragment from pWJ528 (digested with *Cla*I and *Sac*II), and a 1.8 kb fragment cloned from a position lying 20 kb upstream of the *gata2* gene 1S promoter (digested with *Sac*II and *Sph*I). The *gata2* sequence in this plasmid was next replaced with a 3 kb *Xba*I fragment from the *gata2* distal promoter region to generate the d18.2Z targeting construct. After transforming cells harboring YAC d18Z with each of the targeting constructs, Ura3+Lys2− transformants were selected and characterized by Southern blotting.

Gap repair

A 4 kb *Xho*I-*Bam*HI fragment from *gata2* distal promoter and a 1.7 kb *Bam*HI-*Not*I fragment from the 3′ of the *gata2* cDNA were subcloned into vector pRS316 (containing URA3 and CEN/ARS). This construct was linearized with *Bam*HI and used to transform yeast cells containing YAC d18Z. The transformants were selected on uracil plates and two clones containing the complete *gata2* locus (called GR17 and GR22, respectively) were identified by Southern blot. Total yeast DNA was prepared from clone GR22 and used to transform bacteria. The recovered plasmid GR22 was 26 kb in size with 6 kb of vector sequence and 20 kb of insert. Extensive restriction mapping showed that the plasmid contained the complete *gata2* locus from –4 kb through the end of exon 6, with the *lacZ* gene inserted into the translational start site in exon 2, which is identical to the corresponding region in the YAC. No rearrangements were detected.

Construction of plasmid transgenes

A 1.5 kb *Eco*RI-*Kpn*I fragment flanking *gata2* exon 5 was inserted into a *Kpn*I site located 1 kb 5′ to the *gata2 Ib* promoter in a construct driving *lacZ* (fused to the translational initiation site, precisely as in the YACs) to generate RKβ. The SKβ fragment was released by digesting RKβ with *Sal*I. Since the transcription potential of RKβ and SKβ were identical, expression of RKβ was not shown. The *Sal*I-*Kpn*I fragment was then cloned into pGEM7 to make plasmid SK0.6 for sequencing. The insert of SK0.6 was excised with *Sph*I and *Hin*dIII and inserted into TKMAX (Lieuw et al., 1997), a pGEMEX2-based plasmid harboring TKβ (Clonetech), to generate SKTKβ. The two intron segments in SK0.6 flanking both 3′ and 5′ to exon 5 were amplified by PCR. The 230 bp 5′ fragment, SKu, was generated using upstream primer SK+1: 5′-TCGCATGCCTCCTCTAGACTCG-3′ and downstream primer SK–2: 5′-TTAAGCTTCTGGCAGCAGACT-AGG-3′. The 190 bp exon 5 3′ fragment, SKd, was generated using upstream primer SK+3: 5′-AAGCATGCACAATGTGAGTGC-3′ and downstream primer SK–4: 5′-CCAAGCTTATCGATTTCGAACC-3′. SKu and SKd were digested with *Sph*I and *Hin*dIII and then ligated to TKMAX to generate plasmid subclones SKuβ and SKdβ.

X-gal staining

The procedure for X-gal staining of whole-mount embryos is the same as described previously. For sections, 10-15 μm frozen sections were dried at room temperature for 30 minutes, washed with PBS and incubate with X-gal staining solution at 37°C in the dark for 4 hours to overnight. They were then washed again with three changes of PBS and counterstained with nuclear fast red.

Immunohistochemistry

10.5 dpc embryos were dissected in ice-cold PBS and fixed in freshly prepared 4% paraformaldehyde in PBS for 35 minutes at 4°C. They were then washed three times with PBS and incubated in PBS at 4°C for 1 hour, followed by overnight incubation in 30% sucrose at 4°C. The embryos were then embedded in OCT compound and frozen in dry ice/ethanol bath. 10-15 μm sections were collected on a cryostat, dried at room temperature, blocked with 5% normal goat serum in PBS containing 0.1% Triton X-100 at 4°C for 1 hour and incubated with primary antibody overnight. The following day, the sections were washed with PBS and incubated with secondary antibody for 2 hours at room temperature. They were then washed again with three changes of PBS.

Signals were visualized either by direct color development or by indirect immunofluorescence. Pax2, Engrailed 1 and Chx10 were detected using rabbit polyclonal antibodies (at dilutions: anti-Pax2: 1/2000, anti-Engrailed 1: 1/500, anti-Chx10: 1/3000). Islet1, Lim3, MNR2, Nkx2.2 and BrdU were detected with monoclonal antibodies from the Developmental Studies Hybridoma Bank at U. Iowa (at dilutions: Islet1, 1/100; Lim3, 1/200-1/1000; MNR2, 1/20; Nkx2.2, 1/200). β-gal was detected with either a rabbit polyclonal (1/2000) or a monoclonal antibody from the D. S. H. B. (1/20). The anti-Pax2 antibody was purchased from Zymed.

The data shown in Table 1 were quantified by counting the number of each immunofluorescent labeled neuron in three independent 10.5 dpc d16Z5 transgenic embryos; four consecutive thoracic cross sections were used for this quantification. For Table 2, three independent 10.25 dpc GATA2 homozygous mutant embryos and three wild-type littermates were examined, also at the thoracic level.

BrdU pulse labeling

BrdU was administered to pregnant mice by peritoneal injection at 100 μg/gram of body weight. 2 hours later, the female was sacrificed and embryos were processed as described above. Sections were treated with 2 M HCl in PBS containing 0.1% Triton X-100 for 30 minutes at room temperature and then washed three times with PBS to expose the epitope.

RESULTS

GATA2 is transiently expressed in the CNS during embryogenesis

We previously described lines of *gata2* YAC transgenic mice that were able to recapitulate the proper spatial and temporal expression pattern of GATA2 in the developing CNS (Zhou et al., 1998). In the mouse CNS, GATA2 is first expressed in rhombomeres 2 and 4 beginning about day 9, shortly after embryonic turning (Nardelli et al., 1999). Shortly thereafter, GATA2 expression intensifies in the forebrain, midbrain, hindbrain and spinal cord. In the forebrain, GATA2 is expressed in the pretectal area (prosomere 1) and the zona limitans, which separates the ventral from the dorsal thalamus (Fig. 1A,E,I). In the midbrain, GATA2 is first expressed in a group of ventrolateral cells at 10.5 dpc (Fig. 1B), which begins to expand dorsally by 11.5 dpc (Fig. 1F), and essentially fills the circumference of the midbrain by 12.5 dpc (Fig. 1J). In the hindbrain, two populations of cells express GATA2, one located ventromedially, while the other is more dorsolaterally disposed (Fig. 1C,G,K); unlike expression in the midbrain and forebrain, the dorsolateral hindbrain expression ceases by 12.5 dpc (Fig. 1K). In the spinal cord, GATA2 is expressed in a group of ventral neurons at 10.5 and 11.5 dpc (Fig. 1D,H), but this level declines to the lower limits of detection by 12.5 dpc (Fig. 1L). In addition to those sites of cranial and spinal expression, GATA2 is also abundant in the ventral pituitary, the otic vesicles and inner ear and the olfactory epithelium (Fig. 1M-O, respectively).

GATA2 is expressed in V2 interneurons but not in motor neurons.

In the ventral spinal cord, GATA2 is restricted to expression in only a small group of cells, originally suggesting that it might play a role in the proliferation and/or differentiation of a specific cell type in the ventral neural tube. Based on their position, we initially suspected that these cells might belong to a specific subset of interneurons (Fig. 2). The onset of GATA2 expression in the neural tube also coincides with the initial generation of interneurons (Nornes and Carry, 1978). However, there was a recent suggestion that GATA2 might also be expressed in motor neurons (Nardelli et al., 1999).

To determine the identity of the GATA2-expressing cells, we examined the cellular co-localization of GATA2 with established neuronal lineage markers at 10.5 dpc. When sections of line d16Z5 YAC transgenic embryos (Zhou et al.,

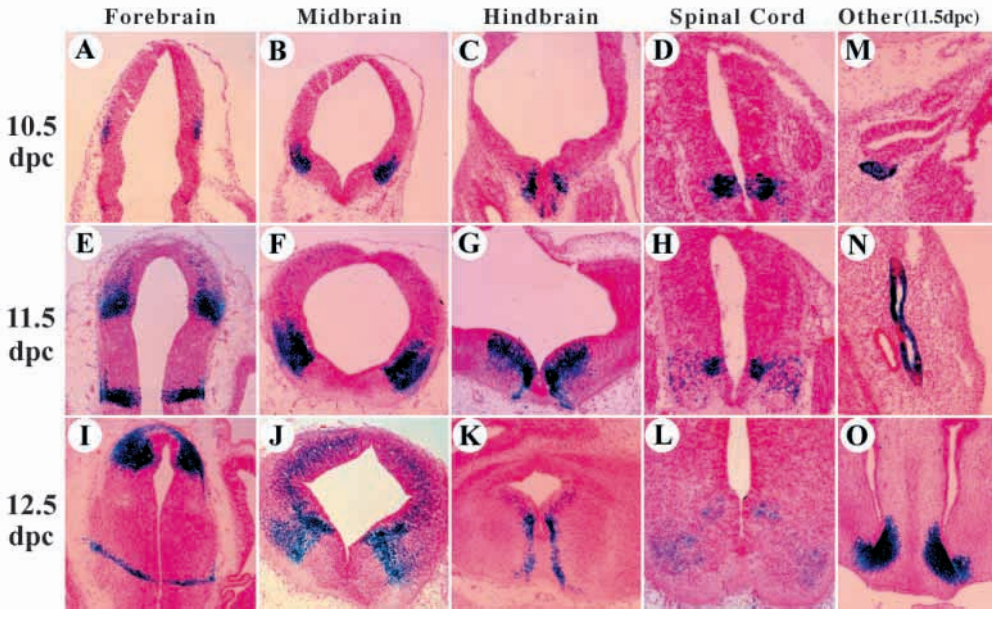

Fig. 1. Expression of GATA2 in the developing CNS. Expression of GATA2 in the embryonic central nervous system at embryonic days 10.5 (A-D), 11.5 (E-H,M-O) and 12.5 (I-L)was examined using YAC transgenic line d16Z5, which was previously shown to recapitulate GATA2 expression in many tissues (Zhou et al., 1998). (A,E,I) Frontal sections through the diencephalon revealed GATA2 expression in two domains: the pretectal area (dorsal domain) and the zona limitans (ventral domain). (B,F,J) In the mesencephalon, GATA2 expression was (B) initially restricted to a group of ventrally localized cells at 10.5 dpc, (F) appears on the dorsal side at 11.5 dpc and (J) becomes very strong at 12.5 dpc. (C,G) In the myencephalon, two GATA2-expressing domains were seen at (C) 10.5 dpc and (G) 11.5 dpc. (K) One day later, at 12.5 dpc, the expression in the dorsolateral group became undetectable. (D,H) Cross sections through the spinal cord showed that GATA2 was strongly expressed in a ventral cell population at (D) 10.5 dpc and (H) 11.5 dpc. (L) Similar to the dorsolateral domain in the hindbrain, the expression in the spinal cord also disappears at 12.5 dpc. (M-O) Expression of GATA2 is also detected in (M) the ventral pituitary, (N) the otic vesicle and (O) in olfactory epithelium.

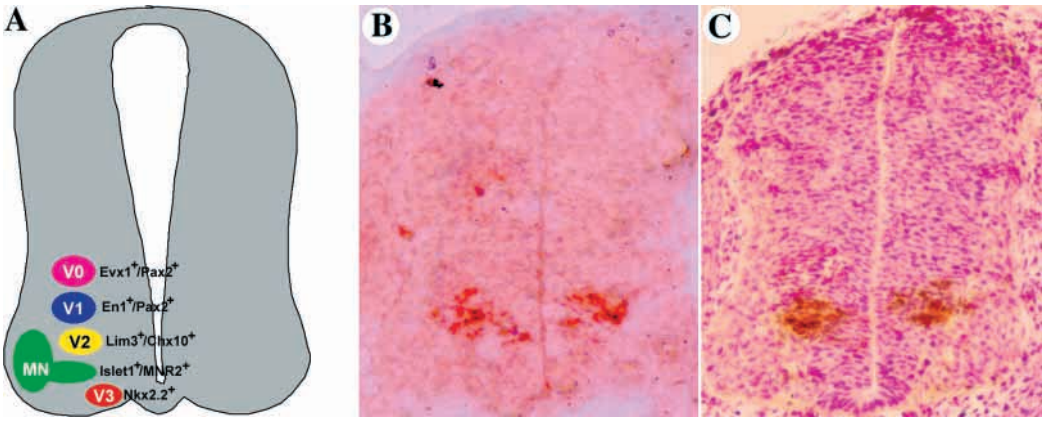

Fig. 2. GATA2 patterning in the spinal cord. (A) Diagram of distinct neuronal populations in the murine ventral spinal cord. (B) Expression of GATA2 in the ventral spinal cord of 11.5 dpc d16z5 transgenic embryo detected by in situ hybridization with an anti-sense probe from the murine GATA2 3′ UTR. (C) An adjacent section incubated with an antibody for β-gal, which detected the same population of cells as the GATA2 in situ hybridization.

1998) were hybridized to a *gata2* antisense RNA probe, with adjacent sections separately incubated with an antibody against β-gal, identical cells were detected in the ventral spinal cord (Fig. 2B,C), proving that the *lacZ* transgene perfectly coincides with the expression of endogenous *gata2*. Therefore, sections of 10.5 dpc embryos bearing the d16Z5 transgene were stained with both X-gal and then separately with antibodies defining well-characterized neuronal molecular markers. GATA2-positive cells, stained in blue, are located immediately dorsal to motor neurons that express Islet1/2 (Figs 2, 3C,D). Immunofluorescence co-localization of GATA2 and Islet1/2 showed that the expression of both proteins is mutually exclusive (Figs 2, 3M), and thus GATA2 is not expressed in motor neurons (Table 1). The same result was observed when we assayed for expression of a second motor neuron marker, MNR2 (Fig. 3N), confirming that GATA2 is expressed only in ventral interneurons, but not in motor neurons. Co-staining with other neuronal markers showed that V1 interneurons (which co-express En1 and Pax2; Fig. 2) are located dorsally to the GATA2-expressing cells (Fig. 3I-L,S,T; Table 1). Sandwiched between the V1 interneurons and the motor neurons are the V2 interneurons, which are defined by the co-expression of Chx10 and Lim3 (Fig. 3R). Although Chx10 is expressed exclusively in V2 cells, Lim3 expression is also found in a subset of motor neurons. Co-staining of GATA2 with Chx10 and Lim3 revealed that all Chx10-positive cells, as well as the slightly more dorsally localized Lim3 cells, express GATA2 (Fig. 3E-H,P,Q; Table 1). Thus among all of the previously defined interneuron subtypes, only V2 neurons express GATA2. Reciprocally, however, not all GATA2-positive cells express both Chx10 and Lim3 (Fig. 3P,Q). Detailed examination revealed that most of these Lim3/Chx10-negative cells are more medially located, suggesting that GATA2 is likely activated prior to Lim3 and Chx10 during V2 neuronal differentiation, and therefore these GATA2-positive, Lim3/Chx10-negative cells might represent less-mature V2 neurons.

Table 1. Co-expression of GATA2 with products of lineage-specific genes

	$En1^+$ (V1)	$Lim3^+$ (V2+MN)	$Chx10^+$ (V2)	$Islet1^+$ (MN)	$Nkx2.2^+$ (V3)
Total	56	45	21	88	33
$GATA2^+$	0	18	19	0	0
%	0	40	90	0	0

Most GATA2-expressing V2 interneurons are postmitotic

Within the hematopoietic system, GATA2 is expressed most abundantly in proliferating progenitor cells (Yamamoto et al., 1990; Leonard et al., 1993; Tsai et al., 1994). Once erythroid progenitor cells commit to terminal differentiation, GATA2 is downregulated and diminishes through dilution or through direct repression as early progenitors mature and divide. It was therefore of interest to determine if analogous observations held true for GATA2-expressing cells in the developing neural tube. In the ventral neural tube, GATA2-expressing cells are localized in a relatively medial position, raising the possibility that they represent dividing progenitor cells. To discriminate between the expression of GATA2 in mitotic versus postmitotic cells, 10.5 dpc embryos of YAC line d16Z5 were labeled with BrdU, an indicator of DNA replication, for 2 hours. Sections through the spinal cord were then stained with antibodies against β-galactosidase and BrdU.

As anticipated, cells that had incorporated BrdU were located adjoining the ventricular zone, while GATA2-positive cells were located more laterally, adjacent to the BrdU-positive cells. Although a small fraction of cells on the boundary between the proliferative and GATA2-expressing domains are positive for both, there is little overlap between expression of GATA2 and mitotic neurons (Fig. 3U). We therefore conclude that the vast majority of GATA2-positive cells represent postmitotic neurons. The small fraction of neurons that both express GATA2 and are actively dividing may represent cells that have just completed their last cell division and are in transition to terminal neuronal differentiation. This result, considered in concert with the fact that GATA2 is expressed only briefly in V2 interneurons during embryogenesis, suggests that GATA2 is either involved in initiating the genetic program for V2 neuronal differentiation or, alternatively, functions as part of a molecular switch instructing cells to progress from proliferative to differentiation phases.

Inhibition of V2 interneurons in embryos lacking GATA2

The evidence thus far suggests that GATA2 could be a crucial determinant in the initial formation of V2 interneurons. To test this hypothesis, we examined the number of V2 interneurons in *gata2*$^{-/-}$ embryos. Embryos from *gata2* heterozygous intercrosses were collected at early 10.5 dpc since most *gata2*

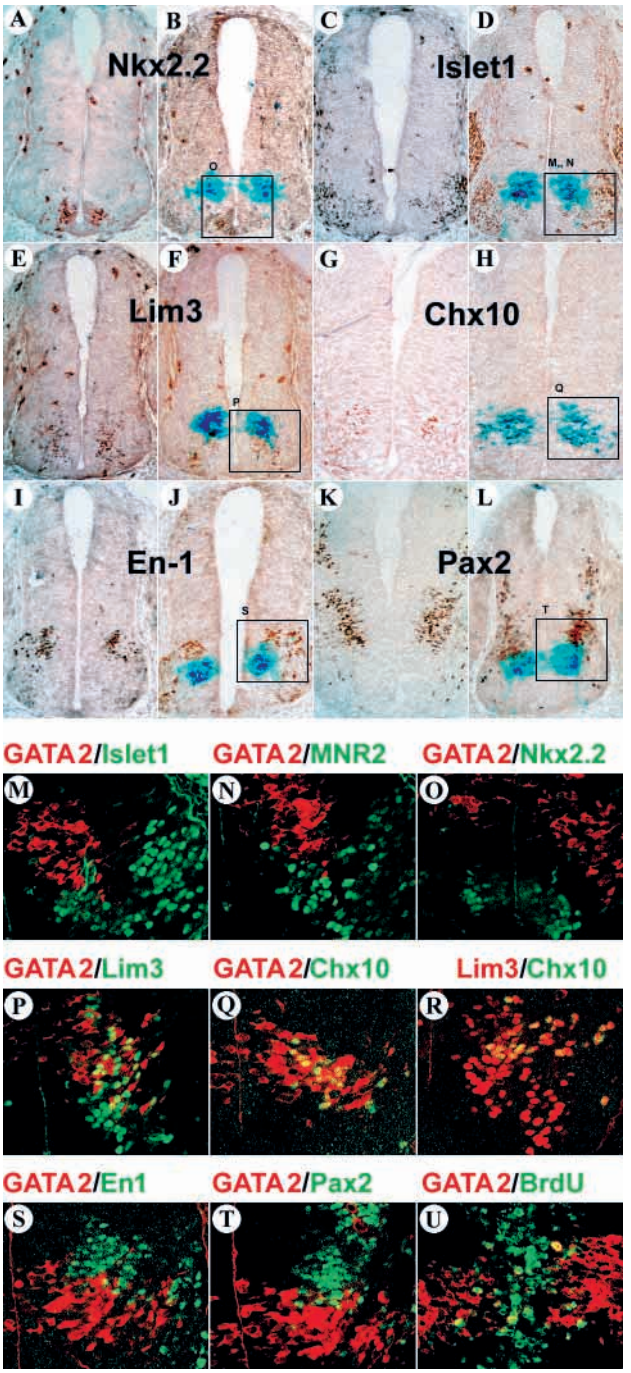

Fig. 3. GATA2 expression in ventral spinal cord is restricted to V2 interneurons. (A-L) Immunohistochemical staining of spinal cord sections from 10.5 dpc YAC d16Z5 transgenic embryos with antibodies specific for different neuronal types (A,C,E,G,I,K) or co-staining with antibodies and X-gal (B,D,F,H,J,L). Blue GATA2-positive cells are localized dorsally to V3 interneurons expressing Nkx2.2 (A,B) and motor neurons expressing Islet1 (C,D), ventral to V1 interneurons expressing En1 (I,J) and Pax2 (K,L), but in an overlapping pattern with V2 interneurons expressing Lim3 (E,F) and Chx10 (G,H). (M-U) Immunofluorescent co-localization of GATA2 with ventral markers. GATA2-positive cells were detected with an antibody against β-gal and colored red (Texas Red) while all other markers were green (FITC, except in R). GATA2 and motor neuron markers, Islet1 (M) or MNR2 (N), are expressed in adjacent cells but are never co-expressed. (O) Nkx2.2-positive cells are located most ventrally, away from the GATA2-expressing cells. (R) V2 interneurons that co-express Lim3 and Chx10 (Lim3, red; Chx10, green) also express GATA2. (P) The more dorsal Lim3-positive cells (V2 interneurons) express GATA2, but the more ventral Lim3-positive cells (motor neurons) do not. (Q) All Chx10-expressing cells also express GATA2. V1 interneurons express Engrailed 1 (S) and Pax2 (T), but do not express GATA2. When d16Z5 transgenic embryos were labeled with BrdU and then stained with both anti-BrdU and anti-β-gal antibodies, most of the GATA2-expressing cells were BrdU-negative (U), indicating they are postmitotic neurons.

mutant embryos die soon thereafter. Genotypes were determined by PCR of yolk sac DNA. *gata2*$^{-/-}$ embryos as well as wild-type controls were sectioned and incubated with the antibodies described above that discriminate among the various neuronal subtypes.

As anticipated, we found that the number of V2 interneurons is significantly reduced in *gata2*$^{-/-}$ embryos (Fig. 4E-H,Q-S). In thoracic level sections, occasionally a couple of Lim3/Chx10-positive cells were found (Fig. 4R), although many fewer than were observed in wild-type embryos (Fig. 4Q). At the lumbar level, in contrast, we never detected cells expressing both Chx10 and Lim3 in the mutant embryos (Fig. 4S). On average, the number of V2 interneurons is reduced by more than 90% in *gata2*$^{-/-}$ embryos, which demonstrates that GATA2 is vital for the generation of V2 cells (Table 2).

This neurogenic defect caused by the loss of GATA2 is specific to the V2 lineage and cannot be explained by growth retardation. Although the shape of the spinal cord is somewhat distorted in the mutant embryos, none of the other neuronal subtypes are severely affected. We found clear evidence for the formation of V0 (Pax 2$^+$ En1$^-$; Fig. 4K-N), V1 (Pax2$^+$ En1$^+$; Fig. 4I,J,O,P) and V3 (Nkx2.2$^+$; Fig. 4A,B) populations as well as for motor neurons (Fig. 4C,D,T,U). The numbers of both V1 and motor neurons, the two lineages directly adjacent to the missing V2 population, are relatively normal in the mutant animals when compared with wild-type controls (Table 2). Therefore, inactivation of GATA2 led to a severe and specific loss of V2 interneurons from the ventral spinal cord.

Defective generation of V2 interneurons in *gata2* mutant mice could be due to a lack of proliferation, altered cell fate or increased apoptosis. As has been demonstrated previously, loss of an essential gene can lead cells to assume an altered identity, usually by conversion to a neighboring cell type (Dasen et al., 1999). Theoretically, the missing V2 neurons could have been converted to motor neurons or to other classes of interneurons in the *gata2* mutant mice. To test this hypothesis, we co-stained *gata2* mutant and wild-type spinal cord sections with Pax2 and Islet1 to determine whether either of these markers now expanded into the V2 domain. As shown in Fig. 4T,U, there

Table 2. Expression of products of lineage-specific genes in 10.5 dpc *gata2* mutant embryos

	En1$^+$ (V1)	Lim3$^+$Chx10$^+$ (V2)	Lim3$^+$Chx10$^-$ (MN)	Islet1$^+$ (MN)
gata2$^{+/+}$	21	15	26	58
gata2$^{-/-}$	19	1	24	54
%	90	6.7	92	93

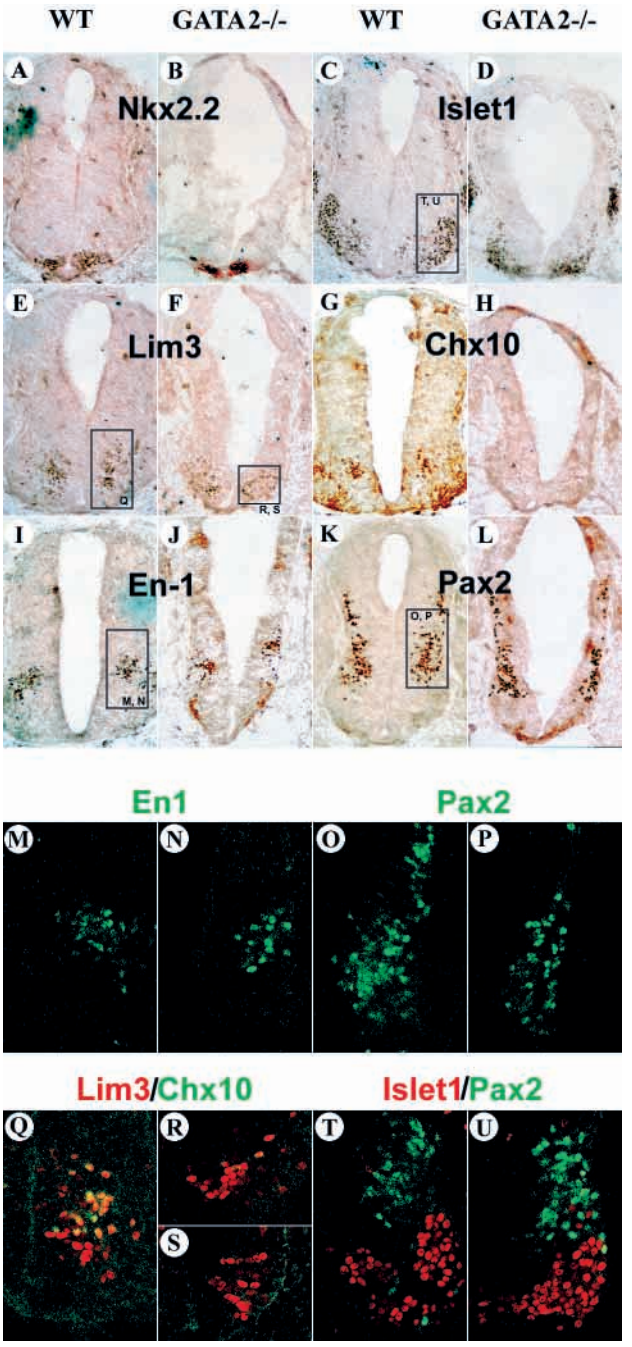

Fig. 4. V2 interneurons are absent in the spinal cord of *gata2*$^{-/-}$ germline mutant embryos. Ventral cell lineages in 10.5 dpc wild-type (A,C,E,G,I,K and M,O,Q,T) and *gata2* mutant (B,D,F,H,J,L, and N,P,R,S,U) embryos were examined by both immunohistochemistry (A-L) as well as immunofluorescence (M-U). Although the spinal cord sections from the *gata2*$^{-/-}$ mutant embryos were slightly distorted, most of the ventral cell types could be readily detected. (A,B) V3 neurons were recognized by an antibody against Nkx2.2, (C,D) motor neurons were detected by Islet1 antibody, and (I-P) V1 neurons express Engrailed 1 (I,J,M,N) and Pax2 (K,L,O,P). The expression of all those markers was relatively normal in the *gata2*$^{-/-}$ mutant embryos. However, expression of the V2 neuron marker Chx10 was virtually undetectable (G,H), as was the dorsal domain of Lim3 expression (E,F). (Q-S) Immunofluorescent co-localization with Lim3 (red) and Chx10 (green) showed that, although the number of Lim3+Chx10- cells in GATA2 homozygous mutant embryos (motor neurons) was comparable to that observed in wild-type embryos, the number of Lim3+Chx10+ cells was significantly lower in mutant embryos. (R) At the thoracic level, few double-positive cells were detected; (S) at the lumbar level, no double-positive cells were seen. (T,U) Double staining with anti-Pax2 and anti-Islet1 showed that neither gene product expanded into the V2 domain.

was no significant alteration in the spacing between Pax2 cells and Islet1 cells in *gata2* mutant versus wild-type embryo spinal cords, indicating that loss of V2 neurons is not due to an alteration in ventral cell fate in the absence of GATA2. We cannot distinguish between the possibilities that V2 loss is due to inhibited proliferation or increased apoptosis since *gata2* mutant embryos die by 10.5 dpc, and therefore detection of a higher than usual rate of apoptosis in the spinal cords of the mutant animals would probably be meaningless. Consequently, in order to understand the function of GATA2 in V2 neuronal generation, we must either prolong the lifespan of *gata2* mutant embryos or perform spinal cord-specific gene inactivation of *gata2*.

GATA2 expression in the brain and spinal cord are controlled by discrete tissue-specific enhancers

To study the function of GATA2 in V2 neurons in a tissue-specific manner, we next decided to identify the enhancer element(s) regulating GATA2 expression in the embryonic CNS. We recently reported that a 120 kb YAC, called d18Z, contains all the regulatory information required for GATA2 expression in the CNS (Zhou et al., 1998). This YAC has approximately 40 kb of sequence 5′, and approximately 80 kb of sequence 3′, to the 14 kb *gata2* structural gene. We subsequently generated two smaller YACs from d18Z, called YAC d18.1Z and YAC d18.2Z, containing either 20 kb or 4 kb of 5′ flanking sequence, respectively, with both retaining the original 80 kb of 3′ sequence from d18Z. We found that all three YACs conferred the same CNS expression pattern in transgenic mice (e.g. Fig. 5C).

We next examined a 20 kb DNA fragment containing the entire *gata2* structural gene (with *lacZ* inserted at the translation initiation site) plus 4 kb of 5′ sequence (Fig. 5A: GR22, isolated from YAC d18Z using gap repair; Materials and methods). Since there was no convenient restriction site that would excise the whole insert of this recombinant, we separately tested two overlapping fragments (Fig. 5A). First, when a 17 kb *Xho*I/*Sal*I fragment isolated from GR22 (GR22/XS) was microinjected into fertilized ova, transgenic F_0 embryos analyzed at 11.5 dpc showed tissue-specific nervous system staining only in the brain (7/9), but not in the spinal cord (Fig. 5B; 0/9). When we next tested an overlapping 3′ 11 kb *Kpn*I fragment from GR22 (GR22/K) in the same assay, the pattern was complementary to that detected with GR22/XS. Expression of the GR22/K reporter gene was observed only in the hindbrain and spinal cord (Fig. 5D; 5/5), but not in the forebrain or midbrain (0/5). Thin sectioning of GR22/K embryos confirmed that the stained cells in the spinal cord were V2 interneurons.

To our surprise, although both of the GR22 fragments direct expression of *lacZ* to the hindbrain, each of them regulates expression in a different neuronal population. The expression directed by the ‘brain’ enhancer (in the more 5′ fragment,

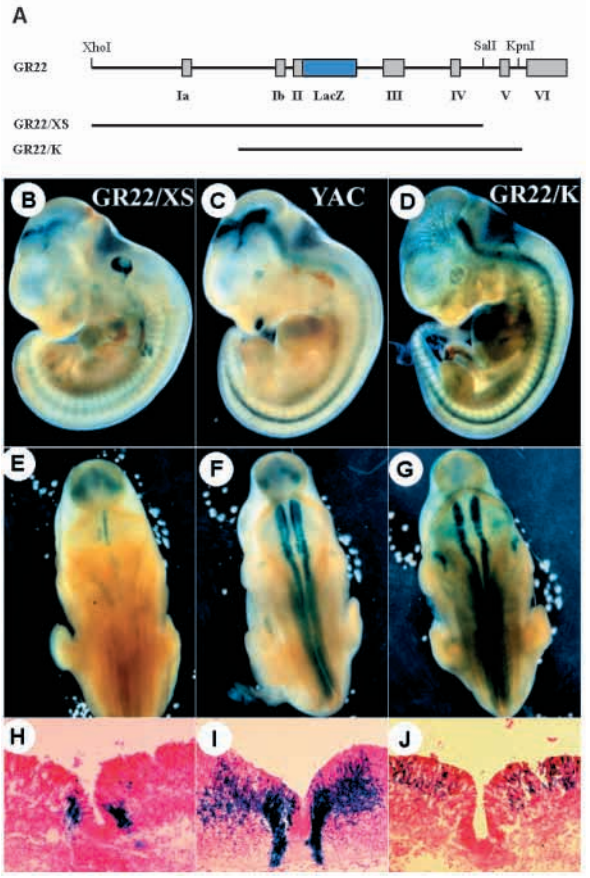

Fig. 5. Distinct tissue-specific enhancers regulate GATA2 expression in the brain and spinal cord. (A) A diagram of GR22, containing complete GATA2-coding sequence with a *lacZ* reporter gene inserted into the translational start site in exon 2, and two restriction fragments that we assayed by transgenic experiments, GR22/XS and GR22K. (C) YAC d18.2Z is capable of directing *lacZ* expression in both the brain and the spinal cord; (F,I) expression in the hindbrain was detected in two domains. Construct GR22/XS contains enhancer activity conferring expression in the forebrain and midbrain, but not in the spinal cord (B); expression in the hindbrain was restricted to the ventromedial cells (E,H). In contrast, GR22K displays the complementary activity: the *lacZ* gene is exclusively expressed in the spinal cord and the dorsolateral domain in the hindbrain (D,G,J).

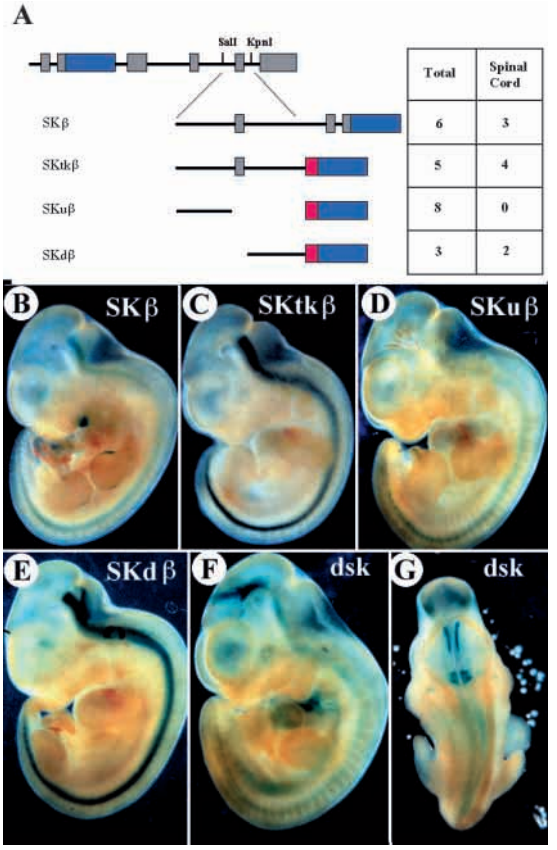

Fig. 6. A 190 bp enhancer in intron 5 is necessary and sufficient for directing GATA2 expression in V2 interneurons. (A) Diagram of plasmid transgenic constructs for further localization of the GATA2 V2 interneuron enhancer (see Materials and Methods). All transgenic embryos were analyzed at 11.5 dpc. The *Sal*I-*Kpn*I fragment can direct *lacZ* expression in the hindbrain and spinal cord with either the endogenous *gata2* Ib promoter (B, SKβ) or a heterologous *hsv-tk* gene promoter (C, SKtkβ). The 230 bp fragment 5′ to exon 5 has very little enhancer activity (D, SKuβ), whereas the downstream 190 bp fragment drove robust expression in the spinal cord and hindbrain (E, SKdβ). Deletion of the *Sal*I-*Kpn*I fragment from YAC d18Z abrogated expression in the spinal cord (F) but revealed continued expression in rhombomere 4 in the hindbrain (G).

GR22/XS) is limited to a ventromedial group of hindbrain cells (Fig. 5E,H), whereas expression from the presumptive 'spinal cord' enhancer (in GR22/K) is specific to a grouping of dorsolateral hindbrain cells (Fig. 5G,J). Together these two comprise the composite hindbrain expression pattern seen in all of the YAC/*lacZ* transgenic mice (e.g. Fig. 5F,I). Thus these data show that transcription of *gata2* in the CNS is controlled by a minimum of two distinct and separable regulatory elements described uniquely by two overlapping DNA fragments flanking and within (GR22/XS), or entirely within (GR22/K), the *gata2* structural gene. Thus the enhancer conferring *gata2* expression in the spinal cord must be located inside the structural gene.

A 190 bp intron enhancer is necessary and sufficient for GATA2 expression in V2 interneurons

Deductively, the V2 interneuron element must be located within the boundaries described by the *Sal*I-*Kpn*I fragment present in GR22/K, but outside the limits of the GR22/XS fragment. We tested this hypothesis directly by linking the GR22/SK fragment (Fig. 6A) to a *lacZ* gene directed by either the *gata2* proximal Ib promoter (Minegishi et al., 1998) or to the HSV tk gene promoter. Both promoters conferred proper expression in both the hindbrain and spinal cord of transgenic mice, whereas neither the *gata2* minimal *1b* gene promoter nor the *tk* gene promoters alone were able to direct expression to those sites (Fig. 6 and data not shown). This experiment demonstrated that this *Sal*I-*Kpn*I fragment contains a classical enhancer that can independently activate a heterologous gene promoter, and that the tissue-specific expression of the reporter gene is controlled solely by this enhancer.

Since the fragment conferring expression in the hindbrain and spinal cord spans *gata2* exon 5, we next attempted to determine the precise location of the element. Both fragments flanking exon 5 were synthesized by PCR and then linked in

cis to a *tk*/*lacZ* reporter gene. In a small fraction of transgenic embryos linked to the exon 5 5′ PCR product (SKuβ; 2/8), we were able to detect extremely weak expression in the caudal part of the spinal cord (Fig. 6D). However, expression in the hindbrain and rostral part of the spinal cord was completely missing, demonstrating that sequences immediately 5′ to *gata2* exon 5 appear to have very limited transcription potential. In contrast, an intron fragment lying immediately 3′ to exon 5 (SKdβ) displayed robust enhancer activity in the same assay (Fig. 6E). This fragment was able to direct strong expression throughout the entire hindbrain-spinal cord continuum. Therefore, the major *gata2* V2 enhancer activity is localized to this 190 bp fragment lying between exons 5 and 6.

In order to determine whether this V2 enhancer is also necessary for *gata2* expression in V2 interneurons, we deleted the 542 bp *Sal*I-*Kpn*I fragment containing the enhancer from the *gata2* d18Z YAC. When 11.5 dpc founder embryos were stained for β-gal activity (dsk, Fig. 6F), we found that expression in the spinal cord was completely ablated, demonstrating that this enhancer is not only sufficient, but also necessary, for GATA2 expression in V2 interneurons. Surprisingly, in transgenic embryos in which the spinal cord enhancer had been deleted, we now observed staining in rhombomere 4 of the hindbrain (Fig. 6G). This observation suggested that expression of GATA2 in rhombomere 4 is controlled by yet a third CNS-specific transcriptional regulatory element.

Next, to test whether the V2 interneuron enhancer was conserved between species, we cloned human *GATA2* intron 5 by PCR and sequenced it. Alignment of the mouse and human sequences revealed very high homology between the two over the V2 enhancer region. Most remarkably, the sequence conservation within the first 108 bp is over 98% (Fig. 7A).

Within this highly conserved region, we identified several putative transcription factor binding sites (Fig. 7A). However, most of these factors are either ubiquitously expressed or have not previously been correlated with expression unique to the spinal cord. We focused our attention on the palindromic sequence with high homology to the consensus sequence of the OLF binding motif in both sense and antisense orientation (Fig. 7B). Three OLF factors have been identified, and all three are expressed in the spinal cord (Garel et al., 1997). To investigate whether the OLF motif is important for the V2 enhancer activity, we introduced a mutation that has been shown to abolish OLF binding (Kudrycki et al., 1993) into plasmid SKTKβ. This mutation failed to alter the enhancer activity (Fig. 7C), suggesting that the transcription factors that regulate GATA2 expression in V2 interneurons lie elsewhere within this domain.

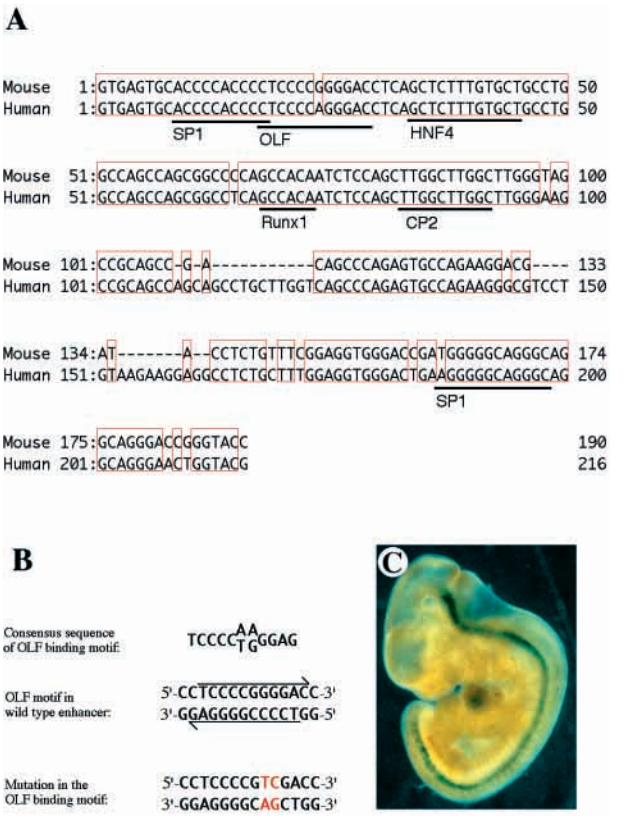

Fig. 7. Sequence conservation of the GATA2 V2 interneuron enhancer and mutagenesis of the OLF binding motif. (A) Alignment of the murine V2 enhancer sequence and the corresponding sequence from the human *GATA2* gene. Putative binding sites are underlined and identified. (B) Mutagenesis of the OLF binding motif. (C) Disruption of OLF binding did not affect V2 enhancer activity.

DISCUSSION

From embryonic days 9 to 13, different neuronal subtypes are generated in a ventral-to-dorsal order in the developing murine spinal cord (Nornes and Carry, 1978). These neurons eventually form a network that processes sensory input and control muscle movement. Generation of this multitude of different cell types in a precisely controlled spatial and temporal pattern requires coordinated expression of multiple morphogens as well as the cooperative and coordinate, sequential induction of a large number of transcription factors. Many of the best-studied transcription factors that play discrete roles in the patterning and development of the neural tube are homeodomain proteins.

The GATA factor family was cloned a decade ago, and GATA factors 1, 2 and 3 have each been shown to be key contributors to various aspects of hematopoiesis (Fujiwara et al., 1996; Hendriks et al., 1999; Pandolfi et al., 1995; Takahashi et al., 1997; Tsai et al., 1994; Yamamoto et al., 1990). Additionally, GATA2 and GATA3 were implicated through loss- or gain-of-function studies to be important regulatory molecules for other developmental processes as well (e.g. in CNS, cardiovascular, thymic and/or urogenital development). It was recently reported that GATA3 expression in the spinal cord is dependent on the presence of GATA2, and thus we are beginning to establish the epistatic relationships between other well-characterized regulatory molecules and the GATA factors in the murine CNS (Nardelli et al., 1999; Pata et al., 1999). In the present studies, we showed that GATA2 is expressed exclusively in newly generated V2 interneurons in the spinal cord and, in support of the hypothesis that GATA2 plays an important role in the generation or maintenence of those cells, we found that *gata2* homozygous mutant embryos have a markedly reduced number of V2 neurons. Furthermore, we found that most GATA2-expressing cells are also postmitotic.

Finally, we showed that an intragenic enhancer is both necessary and sufficient for conferring the GATA2-specific expression pattern to a subset of cells in the hindbrain as well as to the entire spinal cord.

It was previously reported that GATA2 expression overlaps that of Islet 1, and further that expression of Islet 1 and Nkx2.2 was reduced in *gata2*$^{-/-}$ embryos (Nardelli et al., 1999). However, we were unable to detect neurons that co-express GATA2 and Islet 1 or GATA2 and Nkx2.2. Furthermore, *gata2*$^{-/-}$ embryos did not appear to display any significant deviation of Islet 1 or Nkx2.2 expression patterns from the pattern observed in wild-type embryos. We are unable to account for the disparity between the two sets of observations, except for the possible explanation that determination of cell identity by whole-mount in situ hybridization is far more challenging than immunohistochemical co-localization.

The properties of GATA2 in V2 neurons mimic the apparent function of MNR2 in motor neurons (Tanabe et al., 1998). MNR2 is a homeodomain protein expressed in newly generated motor neurons, and like GATA2 in the spinal cord, its expression is developmentally transient. Most MNR2-positive cells are postmitotic, but a small fraction of mitotic cells also express this factor. It has been demonstrated that intermediate levels of Shh administration can induce MNR2, and that forced expression of MNR2 in the dorsal spinal cord induces ectopic expression of motor neuron markers. Since the generation of V2 interneurons is also dependent on Shh signaling (Ericson et al., 1996), it remains to be determined if GATA2 is induced by Shh. In this regard, it would also be important to determine whether or not ectopic expression of GATA2 in other parts of the spinal cord was able to induce ectopic expression of V2 neuronal markers.

In *gata2*$^{-/-}$ embryos, we found that the number of V2 interneurons expressing the Lim3 and Chx10 markers is significantly reduced. We do not feel that this reduction is due to general growth retardation because this loss of expression was specific to V2 neurons, and neither motor neurons nor other classes of interneurons appeared to be affected. Thus we next attempted to address the question of what happened to V2 neurons in the *gata2*$^{-/-}$ embryos. We previously proposed the possibilities that: (a) these cells had apoptosed, (b) the transition from proliferation to differentiation might have been delayed, or (c) V2 progenitors in the mutants had lost the ability to differentiate along the V2 developmental pathway. Thus clarifying this question will not only lead to a better understanding of how GATA2 contributes to the regulatory hierarchy specifying V2 interneuron function, but may also shed new light on the more general question of how a specific neuronal cell type is generated. Such an analysis, however, will require the generation of healthy *gata2* mutant embryos at later stages of development and, unfortunately, the early hematopoietic failure in the *gata2* loss-of-function mutant embryos does not permit such an analysis. However, we have recently shown that *gata2*$^{-/-}$ mutants complemented by a *gata2* YAC transgene are capable of completing embryogenesis, suggesting that this kind of genetic manipulation may be extremely useful for continued analysis of this question.

In order to explore an alternative strategy to study GATA2 loss of function in the spinal cord, we searched for, and identified, a GATA2 V2 interneuron-specific enhancer. Starting from quite large YAC genomic fragments, this top-down approach effectively narrowed the position of this V2 interneuron enhancer from an initial 240,000 bp to the final 190 bp. We showed that this enhancer was necessary for V2-specific expression by targeted deletion of the element from a YAC clone followed by observing its specific loss of expression in the spinal cord of mutant YAC transgenic mice. Therefore targeted deletion of this enhancer in the genomic *gata2* locus will almost certainly generate an allele lacking expression specifically in V2 interneurons. It will be of interest to then determine whether or not the anticipated early V2 neuron reduction will be compensated later on, or if not, what neurological defect might result as a consequence of this specific deficiency.

Examination of the spinal cord enhancer sequence revealed only one potentially interesting protein binding site that might be important for the V2 neuronal enhancer activity. However, mutation of this binding site did not alter either the tissue specificity or the potency of the enhancer. The failure to identify known neuronally restricted transcription factors that recognize binding sites within an element that we have shown functionally to be necessary and sufficient for V2 activity might indicate that *gata2* transcription in the spinal cord could be regulated by novel transcription factors. We have therefore recently initiated screens for such novel proteins that may be involved in ventral neural tube patterning.

We thank Jie Fan and Weimin Song for excellent technical support in generating transgenic mice. We are grateful to our colleagues in the laboratory for discussions, to T. Jessell for the anti-Chx10 antibody, to A. Joyner for anti-Engrailed 1 antibody and to R. H. Holmgren for polyclonal anti-β-gal antibody and fluorescent secondary antibodies. This work was supported by a research grant from the NIH (GM28896) as well as by core facility support from a NIH Center of Excellence award (CA60553) to the Robert H. Lurie Comprehensive Cancer Center of Northwestern University.

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