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Pur alpha and Sp8 as opposing regulators of neural *gata2* expression

William Todd Penberthy[a,1], Chengtian Zhao[b,1], Yu Zhang[b], Jason R. Jessen[a], Zhongan Yang[a], Olivier Bricaud[c], Andres Collazo[c], Anming Meng[b], Shuo Lin[a,*]

[a]*Department of Molecular, Cell, and Developmental Biology, University of California Los Angeles, Los Angeles, CA 90095-1606, United States*
[b]*Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, China*
[c]*House Ear Institute, Los Angeles, CA 90057, United States*

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Abstract

Gata2 is an essential hematopoietic transcriptional factor that is also expressed prominently in the nervous system. The early lethality of knockout mice due to severe anemia has largely precluded studies of *gata2* neural regulation and function. In this report, we describe the identification of zebrafish Pur alpha and Sp8 orthologs as two factors that function to regulate neuronal expression of *gata2*. During embryogenesis, *Pur alpha* is expressed widely, whereas *Sp8* has an overlapping pattern of expression with *gata2* in the nervous system. Knockdown and ectopic expressions of Pur alpha and Sp8 indicate that these factors function, respectively, as a repressor and an activator of *gata2* gene expression in the nervous system. With consideration given to the previously established roles for these factors, we propose a model for how the transcriptional regulation of neural *gata2* expression may be involved in controlling cellular proliferation in the nervous system.

Keywords: *Gata2*; Zebrafish Pur alpha ortholog; Sp8 ortholog; Endometrial carcinoma

Introduction

GATA2 is a member of the GATA family of transcription factors which possess a conserved C4 zinc finger DNA binding domain that recognizes the nucleotide core sequence of WGATAR (Ko and Engel, 1993; Merika and Orkin, 1993). There are six different members of the gene family each with distinct functions and expression patterns. *Gata1* has been shown to be essential for development of erythroid cells, and its disruption in mice induces apoptosis in erythroid progenitors (Orkin, 1995). *Gata2* is necessary for mast cell development, as well as for maintenance/expansion of multipotential progenitors and hematopoietic stem cells (Fujiwara et al., 2004). *Gata2* and *-3* are the only GATA genes with extensively overlapping expression patterns in the nervous system with *gata3* expression entirely dependent on the expression of *gata2* (Nardelli et al., 1999; Pata et al., 1999). *GATA4*, *GATA5*, and *GATA6* have roles in heart formation (Charron and Nemer, 1999).

The anatomical expression pattern of *gata2* is highly conserved in evolution. In the hematopoietic lineage, it is expressed in ventral mesoderm and hematopoietic progenitor cells (Detrich et al., 1995; Kelley et al., 1994; Minegishi et al., 1999; Tsai et al., 1994). In the nervous system, *gata2* is expressed in mesencephalon, rhombomeres, and ventral neurons of the spinal cord in mice (Nardelli et al., 1999; Pata et al., 1999), chick (Bell et al., 1999), and zebrafish (as described in this paper).

Gata2 function in the hematopoietic system has been well studied. Although some embryonic red blood cells are initially detectable in the *gata2* knockout mice, multipotential stem cells proliferate poorly and generate small colonies with excess cell death resulting in embryonic lethality (Tsai and Orkin, 1997; Tsai et al., 1994). Thus *gata2* is required for proliferation of hematopoietic stem cells

* Corresponding author. Department of Molecular, Cell, and Developmental Biology, University of California Los Angeles, 621 Charles E.Young Dr. S., LS4325, Los Angeles, CA 90095-1606. Fax: +1 310 267 4971.
E-mail address: shuolin@ucla.edu (S. Lin).
[1] These authors contributed equally.

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but not for differentiation (Tsai and Orkin, 1997). Given the early lethality in the *gata2* knockout mouse, only limited studies have been done to address the function of *gata2* in neural tissues. It has been observed that the absence of *gata2* in embryonic mice (E9.5 and 10.5 dpc) leads to defects in neurogenesis suggesting involvement in maintenance of a ventral pool of neural progenitors (Nardelli et al., 1999). However, the extent of *gata2* function in nervous system and its neural-specific regulation are yet to be elucidated.

To study *gata2* expression and regulation, we produced germ line transgenic zebrafish carrying various *gata2* promoter constructs (*gata2*/GFP), as previously described in transient analysis by Meng et al. (1997) and Jessen et al. (1998). By expression cloning based on the binding activity of recombinant proteins to the previously defined *gata2 cis*-acting neural element (G2NE) (Meng et al., 1997), we isolated the zebrafish ortholog for Pur alpha. Pur alpha was originally isolated as a factor binding to a putative origin of replication site in the c-*myc* gene (Bergemann et al., 1992). Pur alpha is known to be involved in replication of the neurotropic JC virus (Chang et al., 1996). Since its original isolation, Pur alpha has been identified as having roles in the regulation of the several neural-specific genes including myelin basic protein MBP (Tretiakova et al., 1999b), FE65 (Zambrano et al., 1997), and neuronal nicotinic acetylcholine receptor β4 gene (neuronal nAch β4; Du et al., 1997; Melnikova et al., 2000). Other genes regulated by Pur alpha in a negative fashion include FAS (Lasham et al., 2000), CD43 (DaSilva et al., 2002), and vascular smooth muscle alpha-actin (VSM α-actin (Kelm et al., 1997, 1999a,b)). Knockout of Pur alpha in mice leads to neural-specific developmental abnormalities due to abnormal cellular proliferation (Khalili et al., 2003). It has been shown that Pur alpha exerts a negative effect on cellular proliferation in a wide variety of cells including several transformed lines (Barr and Johnson, 2001; Darbinian et al., 2001; Stacey et al., 1999). This stands in contrast to gata2, which is known to positively confer proliferative potential in both the hematopoietic cells (Jippo et al., 1996; Tsai and Orkin, 1997) and a subpopulation of cells involved in ventral neurogenesis (Nardelli et al., 1999).

By an alternative cloning approach involving in situ hybridization screening for specific cDNA clones with overlapping patterns of expression with *gata2*, we isolated Sp8. There are currently eight members of the Sp family of transcription factors (Ravasi et al., 2003). They all possess three zinc fingers in the DNA binding domain and typically bind GC-rich sequence. In mice, Sp8 is expressed in restricted portions of the nervous system. Knockout of Sp8 leads to defects in the closure of the anterior and posterior neuropore resulting exencephaly and spina bifida with deficits in the progenitor cells of the apical ectodermal ridge (Bell et al., 2003).

The Sp family members are known to have potent roles in growth, apoptosis, and regulation of proliferation depending on the cellular context (for a review of Sp1-4, see Black et al., 2001). We show that removal of Sp8 results in a loss of *gata2* gene expression in zebrafish. Pur alpha has already been observed to interact with Sp1 at closely spaced *cis*-elements to regulate two other neural genes, MBP and neuronal nAch β4 genes, respectively (Bigger et al., 1997; Melnikova et al., 2000; Tretiakova et al., 1999b). Our studies point again to another example of a neural gene coordinately regulated by Pur alpha and an Sp family member through the same *cis*-acting element. We have observed that Pur alpha functions as a general mediator of repression, while Sp8 functions as a requisite activator of *gata2* neural expression.

Materials and methods

Zebrafish transgenesis and microscopy

Zebrafish were maintained under standard conditions and staged according to Kimmel et al. (1995). Transgenic zebrafish prepared as described previously (Meng et al., 1997) were reared to adulthood followed by screening for germ line transgenic zebrafish using fluorescence microscopy. Embryos for confocal viewing were anesthetized and viewed using either a Bio-Rad Radiance 2000/2100 or a Zeiss LSM 410. Optical sections were collected along the *z*-axis for each embryo imaged using high numerical aperture water objectives (20× to 60×). Projections of all optical sections in a series to one plane were made using the Bio-Rad Lasersharp software or MetaMorph (Version 4.0; Universal Imaging). Usually, all sections were used in a projection, but to eliminate extraneous fluorescence, a subset of the sections was sometimes used.

Isolation of Sp8 and whole-mount in situ hybridization analysis

A cDNA library prepared from mRNA isolated from GFP-positive cells of *gata2*/GFP transgenic embryos was screened for clones with tissue-specific expression by RNA whole-mount in situ hybridization. This procedure was based on the protocols described by Long et al. (2000) and in *The Zebrafish Book* (Westerfield, 1995).

Preparation of nuclear extract

Respective organs were dissected from 10–15 adult zebrafish. Tissues were minced on ice with cold PBS. Cells were collected by centrifugation at 1000 × *g* for 5 min, and the packed cell volume (PCV) was measured. Cells were resuspended in two PCVs with 0.05% trypsin, 0.53 mM EDTA before incubation at 37°C for 2 min to obtain individual cells. Trypsin/EDTA was thoroughly washed twice with greater than 2000 PCVs using PBS followed and centrifugation at 1000 × *g* for 2 min. Cells were resuspended in two PCVs of hypotonic lysis buffer (10 mM

HEPES, pH 7.5, 10 mM KCl, 1 mM DTT, 0.5 mM EDTA, 1/20th × Sigma Protease Inhibitor Cocktail, PIC, catalog number P8340). Cells were vortexed for 15 s and left on ice for 1 min. This vortexing and incubation on ice were repeated four times before centrifugation at 20,000 × *g* for 10 min. Supernatants were removed, and nuclei were resuspended in 1.0 M NEB (20 mM HEPES, pH 7.5, 20% glycerol, 10 mM KCl, 1.5 mM $MgCl_2$, 1/5th × PIC) followed by 15 s vortexing and 3 min incubation at 4°C. This vortexing and incubation on ice were repeated three times for a total of 12 min. Then nuclei were thoroughly lysed with a tight-fitting dounce homogenization for at least 15 strokes. Nuclear extract was collected by centrifugation at 20,000 × *g* for 10 min saving the supernatant. This was dialyzed against over 500 volumes of dialysis buffer (25 mM HEPES, pH 8.0, 2 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 mM DTT) overnight followed by one more change of the buffer for at least two additional hours' dialysis. Protein concentrations were estimated by Bradford assays, and samples were stored at −80°C.

Electrophoretic mobility shift assay (EMSA)

DNA binding activity by EMSA reaction was performed as described by Pfaff and Taylor (1992). Double-strand oligonucleotides for the G2NE element (5′ TCTGCGCCGC TTTCTGCCCCCTCCTGCCCTCT) were used as probes. G2NE was ^{32}P-end labeled with polynucleotide kinase and [γ-^{32}P]-ATP. Unlabeled competitor double-stranded oligonucleotides included the G2NE itself, the G2NE mutant "M3" (5′ TCTGCGCCGCTTTCTGCCAACTCCTGCCCTCTT), HNF4α (TCGACTTGCTTGTACTTTGGTGCCTTTTC, or the p53 consensus sequences (5′ CTTTTCCGTTCCGGT CCGTTCAGGTCCGTTGAGCT) at 100× molar ratio greater than the labeled probe. Bovine serum albumin, 200 mg/ml, was included for binding reactions using recombinant proteins.

Isolation of cDNA encoding G2NE binding proteins by expression cloning

A zebrafish brain cDNA library in λZiplox (Gibco/BRL) was provided by John Ngai. Y1090 cells were grown overnight to stationary phase in LB broth with 10 mM $MgSO_4$ and 0.2% maltose before resuspension in equal volume of SM media. Titered library in 0.1 ml of SM media was mixed with 0.3 ml of Y1090 cells followed by 20-min incubation at 37°C to allow phage to absorb. Seven milliliters of molten top agarose (45–50°C) was added before plating onto 150-mm LB agar plates. After 3-h incubation at 42°C, plates were shifted to 37°C for 3 h at which point IPTG (10 mM)-soaked nitrocellulose filters were placed over the newly formed plaques. From this point, the protocol by Ausubel et al. (1997) was followed. Clones were excised from bacteriophage via site-specific recombination using the cre-lox system (Gibco/BRL).

Subcloning, expression, and purification of recombinant zebrafish Sp8 and Pur alpha

Zebrafish Sp8 and Pur alpha were cloned into pCR-T7/NT-Topo (Invitrogen) by PCR-TopoTA, such that the primer encoded an HA tag next to the first codon following the ATG. Primers for rSp8 were 5′-TATCCTTACGACGTTCCTGAC-TACGCGCTAGCTATGTTGGCTGCGACGTGT and 5′-GCTTCGCCTGTCGGTGTCA. For recombinant Pur alpha, the primers were 5′-TATCCTTACGACGTTCCTGACTAC GCGCTAGCTGCGGACAGAGACAGTGGAAGT and 5′-TCAATCCTCATCCCCGTCGT. Both purifications were achieved by nondenaturing protocols described in Qiaexpressionist (August 2002), using a column format with 1-ml bed volumes of nickel agarose. Samples were dialyzed in the same manner as described for the nuclear extract preparation.

Morpholino and mRNA injection studies

Morpholinos were obtained from Gene Tools, LLC, and resuspended at 2 mM in Danieau solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca $(NO_3)_2$, 5 mM HEPES, pH 7.6] (Nasevicius and Ekker, 2000). Zebrafish embryos were injected at the single cell stage into the yolk with approximately 2 nl of either 0.2 or 2.0 mM morpholino for a maximum of 4 pmol per embryo. In all cases, 4 pmol injections resulted in significant concentration-dependent phenotype. No overt effects on development were observed with injection of 4 pmol of control morpholino. mRNA was synthesized by in vitro transcription and suspended in RNase-free water. Approximately 25 pg of Pur alpha mRNA was injected into embryos. At least 50 embryos were injected per condition in all cases with experiments independently performed on at least two additional dates. Two morpholino sequences complementary to Pur alpha were 5′-CACTTCC ACTGTCTCTGTCCGCCAT and 5′-TCCGCCATGATGC CGGACGCG. A 4-mismatch morpholino P1 (−) 5′-CAgTT gCACTGTgTCTGTCaGCgAT was used as a control. The morpholino sequences for targeting Sp8 were Sp8-MO1, 5′-TCTGTGTCCTCGGTTTATTGATTGA; and Sp8-MO2, 5′-ATCTCGGCTCCTCCTGAAACAACAA. Again, a 4-mismatch morpholino Sp8 (−) 5′-ATgTCcGCTCCTgCTGAA AgAAgAA was used as a control. Other controls included morpholinos targeting eight unrelated previous candidate regulators of *gata2* expression, sequence against GFP, and sequence against heat shock protein (Wang et al., 2001).

Results

Gata2 regulatory cis-element analysis using germ line transgenic zebrafish

A 30-base pair *cis*-acting DNA element in the *gata2* promoter (G2NE) was identified through transient transgenic analysis by injecting a series of constructs carrying

deletions and point mutations in the zebrafish *gata2* promoter region followed by examination of GFP expression pattern at 48 h postfertilization (hpf) (Meng et al., 1997). Previously, we described that *gata2* bacterial artificial chromosome (BAC) constructs engineered with GFP were more efficient than plasmid-derived constructs for obtaining authentic expression patterns in zebrafish (Jessen et al., 1998). All these studies were carried out as transient embryonic assays. To examine the *gata2*/GFP expression pattern in detail and avoid mosaic transient expression, we produced germ line stable transgenic zebrafish carrying GFP reporter in BAC and plasmid-derived constructs. The BACs contain 70–80 kb of the *gata2* gene representing 61.4 kb (BAC4) or 20.7 kb (BAC1) 5′ of the ATG. The plasmid-derived constructs contain 7.3 kb 5′ of GATA-2 ATG (1040) or a 50 bp of the neural element (-3851 to -3801) linked to a minimal *gata2* promoter containing 381 bp (998) (Fig. 1A).

Gata2 expression detected by whole-mount in situ hybridization has been described previously (Detrich et al., 1995). At shield stage, it is expressed in ventral and posterior region. By 24 hpf, expression is confined to the hematopoietic tissues (arrow), neural tissues including the dorsal diencephalon, large areas in the ventral midbrain, a portion of the hindbrain, various ganglia (asterisks), the primordial lateral line ganglia, and spinal secondary motor neurons (Fig. 1B). The same expression pattern is evident in the *gata2*/GFP transgenic fish.

For germ line transgenic zebrafish carrying BAC1, BAC4, and 1040 constructs, *gata2*/GFP gene expression is first detectable at shield stage where GFP is present within the prospective ventral mesoderm and ectoderm (Fig. 1C). This expression is absent in 998-derived transgenic fish. In the mouse, the hematopoietic *cis*-acting element of the *gata2* gene is located at 100 to 150 kb upstream of the start site of *gata2* transcription (Zhou et al., 1998). In zebrafish, *gata2*/GFP gene expression in the intermediate cell mass, the site of hematopoiesis in fish before 24 hpf, was not detectable using plasmid-derived constructs, whereas the GFP expression was detectable in the BAC constructs (Figs. 1D, E). Thus, similar to the mouse, the hematopoietic *cis*-acting element is present in a distal region of the gene very far upstream of the *gata2* ATG start site of translation. For zebrafish, this is in the range of 20.7 to 7.3 kb, which corresponds to the upstream difference between constructs 1040 and BAC1 (Fig. 1A).

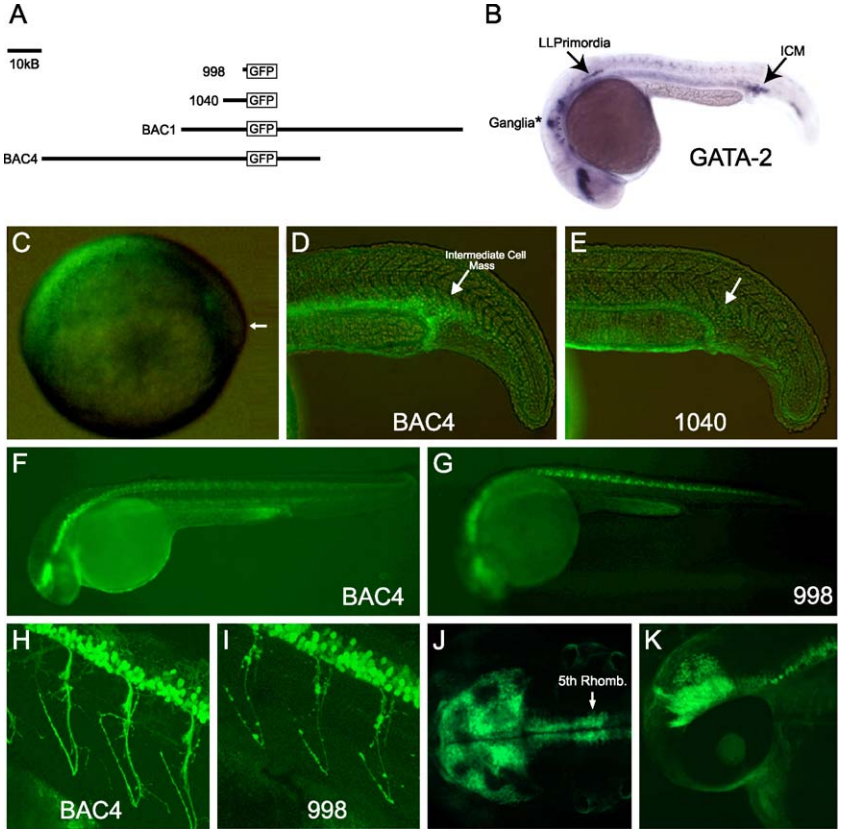

Fig. 1. *Gata2* gene expression analysis using zebrafish GFP transgenesis. (A) Constructs used for germ line *gata/2GFP* zebrafish. (B) *Gata2* mRNA expression detected by in situ hybridization at 24 hpf. (C) Expression of gata2/GFP at shield stage. Arrow denotes shield. (D) *Gata2/GFP* detection in the intermediate cell mass (arrow) at 19 hpf in artificial chromosome construct (BAC4, D) but not plasmid-derived construct (1040, E). (F–G) *Gata2* directed GFP expression in 24-hpf stage transgenic zebrafish comparing *gata2* promoter constructs BAC4 (F) and 998 (G) by lateral views. (H–K) Confocal microscopy images of 48-hpf embryos showing *gata/2GFP* expression similarly detectable in secondary motor neurons of BAC4 (H) and 998 (I) lines. GFP expression is shown in the developing brain of BAC1 (J–K).

The neural expression for all *gata2*/GFP constructs is observable at approximately 24 hpf and appears very similar in all four lines of transgenic fish including BAC1, BAC4, 1040, and 998. As shown in Figs. 1F, G, BAC- and 998-derived transgenic fish have nearly identical expression patterns in the nervous system. Along the length of the spinal cord, *gata2*/GFP expression is strongly detectable in cell bodies of the basal layer of the neural tube; some of which extend ventrally as motor neurons by 48 hpf (Figs. 1H, I). *gata2*/GFP expression is also strongly detectable in the midbrain including the tectum and tegmentum (Figs. 1J, K) as well as regions in the hindbrain, pineal gland, otic vesicles, and olfactory bulbs (data not shown). The observation that two different BAC constructs and two different plasmid constructs yielded generally similar expression patterns indicates that the essential regulatory element required for neural *gata2* gene expression is present in the linkage of the 50-bp enhancer to the 381 bp of *gata2* minimal promoter as represented by line 998. These germ line results verify that the G2NE as defined by transient analysis is sufficient in the context of *gata2* minimal promoter to confer *gata2* neural tissue expression.

DNA binding activity for G2NE in zebrafish brain extracts

Transcriptional regulators function in a sequence-specific manner to establish gene expression within distinct tissues. A lack of *cis*-acting G2NE did not result in fractional decrease in gene expression, but rather complete absence of gene expression of *gata2*/GFP within the nervous system (Meng et al., 1997). The G2NE contains two individual nucleotides that are required for the *trans*- activation seen by injection of *gata2*/GFP reporters. In light of these observations, we performed EMSA to determine if we could detect sequence-specific DNA binding activity with different tissues. Using the 30-bp G2NE as a probe, we detected sequence-specific G2NE binding activities in both a nearly ubiquitous and a neural-specific fashion (Fig. 2A). The binding activity was not competed by the M3 oligonucleotide with a two-base mutation that had previously been shown to prevent in vivo *trans*-activation when substituted for the wild-type sequence. This demonstrates that the sequence-specific recognition requirements for these DNA binding proteins is identical to the activity defined by the transient *gata2*/GFP transgenesis. Other cold oligonucleotide competitors such as the p53 consensus or HNF4α also did not compete for the binding affinity (data not shown).

Isolation of Pur alpha and Sp8 and their binding to G2NE

Next, we sought to isolate the gene(s) encoding the G2NE-specific binding protein(s) by cDNA expression cloning. A radioactively labeled double-stranded oligonucleotide for G2NE was used to screen a brain-derived adult zebrafish cDNA library, and positive clones were selected based on binding activity. Nine clones were sequenced after tertiary screening of 2×10^6 colonies. Four of these clones encoded zebrafish Pur alpha, while three corresponded to the related family member Pur beta. Other genes isolated included a neuronal-specific zebrafish BarH1-like protein that will be described separately.

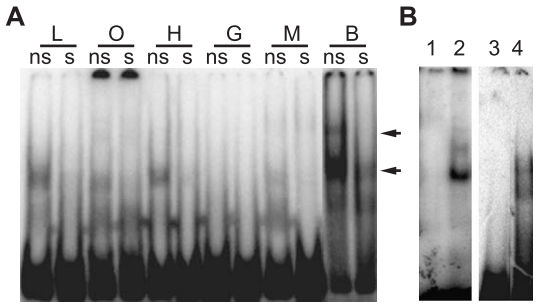

Fig. 2. Characterization of G2NE binding factor. (A) EMSA analysis using labeled G2NE as the probe and unlabeled competitors representative of the M3 mutation sequence (NS = nonspecific) or G2NE (S = specific) test for specificity (for sequence, see Meng et al., 1997). Specific G2NE binding activity restricted to the nervous system is noted by arrow. L indicates liver; O, ovary; H, heart; G, gut; M, muscle; B, brain. (B) Binding activity of recombinant zebrafish Pur alpha and Sp8 to G2NE. Five micrograms of nonspecific copurifying bacterial proteins (vector alone for bacterial growth) or 5 μg of purified bacterially expressed zebrafish Pur alpha or Sp8 was tested by EMSA for binding to the G2NE. Lanes 1 and 3: controls; lane 2: Pur alpha; and lane 4: Sp8.

Zebrafish Pur alpha is a highly conserved protein with 80% identity and 82% homology to human and murine Pur alpha (Supplement Fig. 1). The divergent part of the zebrafish protein is mostly confined to a distinct lack of a long glycine stretch typically associated with vertebrate Pur alpha. This notable absence is also seen in *C. elegans*. Because of the high homology to other Pur alpha proteins, we believe this isolated clone represents the zebrafish ortholog for Pur alpha.

An independent in situ hybridization screen was also performed using a strategy previously employed using *gata1*/GFP transgenic zebrafish (Long et al., 2000). In this case, *gata2*/GFP transgenic zebrafish were used for the generation of a specific cDNA library from FACS-sorted *gata2*/GFP-positive cells for randomly selecting clones with overlapping expression patterns with *gata2* itself. One of the clones showed expression that extensively overlaps with that of *gata2* in the embryonic nervous system (compare Figs. 1B, 3B, C, and 5G). The putative polypeptide encoded by this gene shares a sequence identity of 77.5% to human Sp8, but of less than 43.6% to the other known members of human Sp family, suggesting that it is the ortholog of human Sp8 (Supplement Fig. 2).

To determine if Pur alpha and Sp8 have any binding activity to G2NE, recombinant protein for each with

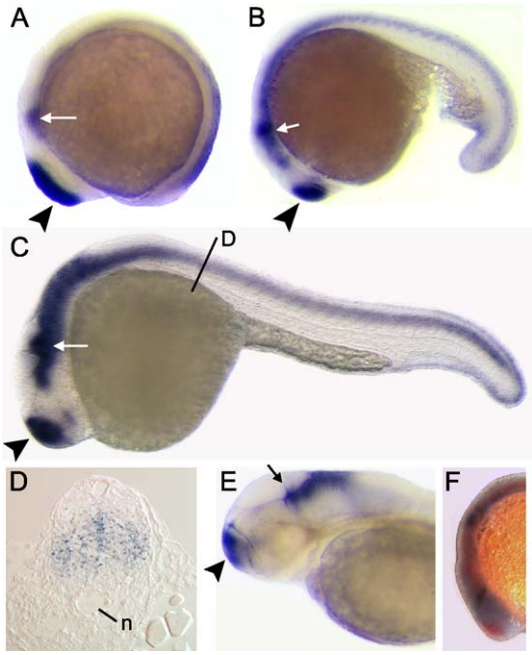

Fig. 3. Spatiotemporal expression pattern of Sp8, detected by in situ hybridization. Embryos are at the 10-somite (A), 20-hpf (B), 24-hpf (C, D), and 2-day (E) stages. D is a cross-section at the position indicated in C. F is a two-color RNA whole-mount in situ hybridization using Sp8 (red) and *gata2* (blue) probes. The telencephalon is indicated by arrowheads, and the midbrain/hindbrain boundary is indicated by arrows (n indicates notochord).

6xhis amino terminus tag was generated to perform binding assays to G2NE. As shown in Fig. 2B, both recombinant Pur alpha and Sp8 can bind to the G2NE as detected by EMSA.

Expression analysis of Pur alpha and Sp8

In order for the isolated transcription factor to be a regulator of GATA-2 neural gene expression, it should be expressed in the same tissues. During embryogenesis, Pur alpha is expressed ubiquitously during the first 48 h as determined by RNA whole-mount in situ hybridization (data not shown). For *Sp8*, a strong expression is detected in the telencephalon, the posterior midbrain, and the trunk neural tube (Fig. 3A) as early as 10-somite stage. During late segmentation, *Sp8* transcripts are detected in the telencephalon, the ventral midbrain with the highest level in the posterior border, the entire hindbrain, the medial neural precursors, and interneurons (Figs. 3B–D). The expression in the telencephalon and the posterior part of dorsal midbrain hindbrain junction continues through the end of the pharyngula period (by 48 hpf, Fig. 3E). Overall, *Sp8* expression is earlier and broader than *gata2* expression in neural tissues. Two-color double in situ hybridization using probes of *gata2* and *Sp8* also confirmed extensive coexpression in spinal neurons, hindbrain, and other neurons (Fig. 3F, spinal neuronal part is not shown due to extensive staining for expression of *Sp8* that overshadowed the *gata2* staining). All *gata2*-positive spinal neurons appear also positive for *Sp8* expression, whereas in the midbrain tegmentum, there is strong *gata2* expression (Fig. 1B) as well as expression in the tectum (data not shown) that are nonoverlapping with *Sp8*.

Pur alpha is required for gastrulation and involved in repression of neural gata2 expression

Gata2/GFP expression first becomes evident at the shield stage of development, thus it is easier to detect changes in GFP expression around this stage. Pur alpha antisense morpholinos were injected into *gata2*/GFP transgenic fish to prevent translation of Pur alpha. A significant increase in the expression of *gata2* promoter-driven GFP was detectable by 50% epiboly stage (Fig. 4A). Examination by RNA in situ hybridization also revealed a detectable increase in *gata2* mRNA. The earlier and increased expression of *gata2* is mostly observed in the ventral side of the embryos without any changes in overall expression pattern. Thereafter, epiboly dramatically slows down for the Pur alpha morphants with *gata2* expression pooling at the animal pole (Fig. 4B). This affect on development leads to a complete arrest in development during the gastrula stage of development. We observed the same effect using a second morpholino (Pur α.2) targeting a separate region of the Pur alpha mRNA, whereas a control morpholino with 4-mismatch morpholino showed no developmental delay.

Additionally, we examined the effect of overexpression of Pur alpha by injection of its mRNA and observed a loss of *gata2*/GFP gene expression early in the ventral ectoderm and later in the nervous system (Fig. 4C). Pur alpha mRNA overexpression also caused general developmental defects, and the embryos failed to develop beyond 30 hpf. However, the effect on *gata2* expression is highly specific and has not been observed in any other mRNA injection that causes general defects (data not shown). These results suggest that Pur alpha is involved in repressing *gata2* gene transcription and is also required to complete gastrulation.

Sp8 is required for gata2 gene expression

We used two morpholinos to target the endogenous *Sp8* mRNA; both of which can efficiently block the production of Sp8-GFP fusion protein (see an example shown in Fig. 5B). Microinjection of either morpholino causes similar abnormalities in neural patterning and neurogenesis. The major abnormalities in the head include reduction of telencephalon, missing or thinner midbrain/hindbrain boundary, and interruption of the hindbrain segmentation (Fig. 5D). The morphants have significantly reduced number of neurons in the spinal

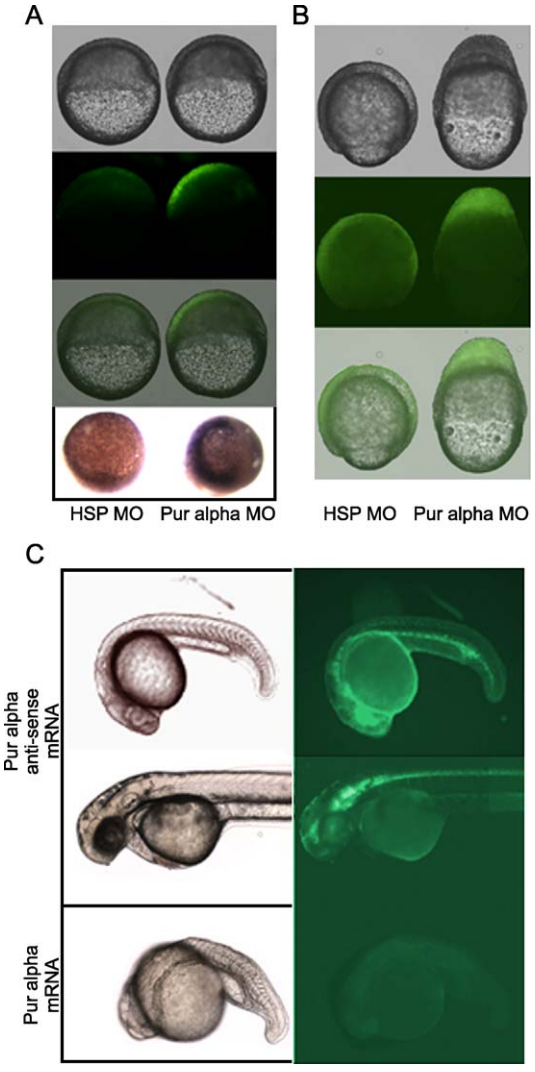

Fig. 4. Alterations in Pur alpha activity by morpholino or mRNA injection induce changes of *gata2* gene expression. (A) Injection of Pur alpha morpholino, but not controls, results in an increase in *gata2* promoter-driven GFP expression by 50% epiboly. All pictures were taken with control samples placed together on the same slide using identical exposure times and neutral filters. The bottom panel is a dorsal view of 50% epiboly embryos with *gata2* mRNA expression detected by in situ hybridization. (B) The Pur alpha morphants have dramatically slowed gastrulation compared to siblings injected with HSP morpholino. *gata/2GFP*-positive cells accumulate at the animal pole. The Pur alpha morpholino was highly penetrated with greater than 90% resulting in gastrulation stage arrest. (C) Injection of 25 pg of Pur alpha mRNA causes a repression of *gata/2GFP* gene expression. These results were observed for 80% of injected embryos ($n > 50$ in each case) after triplicate experiments. The upper two embryos (24 and 36 hpf) are controls injected with antisense mRNA of *Pur alpha*, which has effect neither on development nor expression of *gata2*.

cord, as revealed by examining GFP-positive neurons in *gata2*/GFP transgenic embryos (Fig. 5F, see enlarged insets) and mRNA in situ hybridization of *gata2* (Fig. 5H, see enlarged insets), suggesting a requirement for *Sp8* for the neural expression of *gata2* at least in trunk spinal regions. Injection of a control morpholino of Sp8 with 4-mismatch morpholino showed no repression of *gata2*/GFP expression.

Discussion

Transgenic zebrafish approach to study transcriptional regulation

We sought to determine the transcriptional regulatory mechanism of *gata2* expression using whole animal assays of transgenic zebrafish. By transient transgenesis, a *cis*-element was discovered that is absolutely required for all of

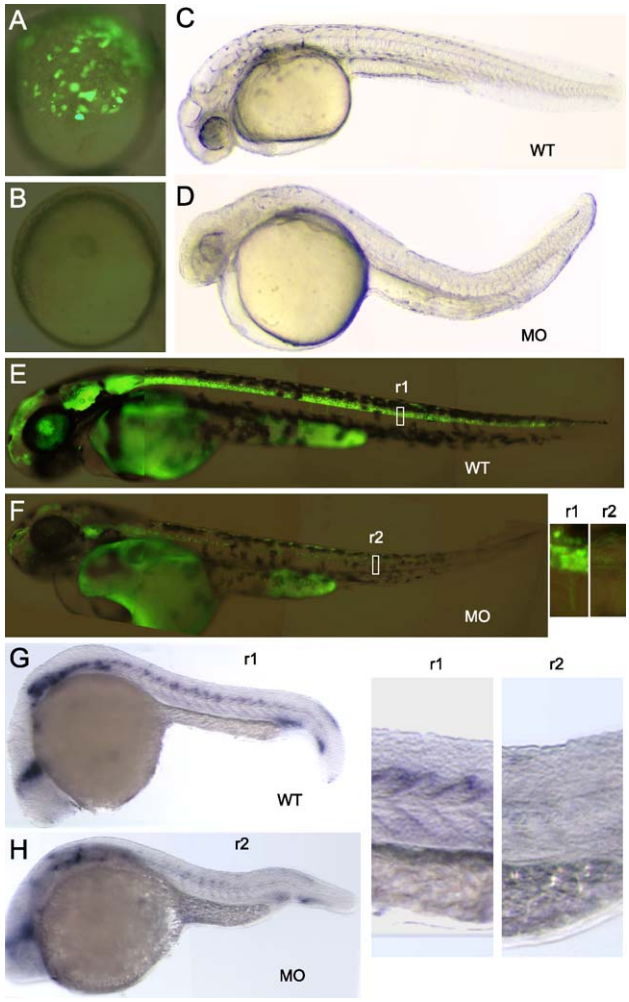

Fig. 5. Morpholino knockdown of Sp8 inhibits expression of *gata2*. (A) An embryo injected with 50 pg DNA of pCMV-Sp8-GFP fusion protein, which contains the 5′ UTR and adjacent coding sequence of Sp8 upstream of GFP coding sequence, showed GFP expression at 60% epiboly stage. (B) The embryo coinjected with the same amount of pCMV-Sp8-GFP DNA and Sp8 MO1 lacked GFP expression at the same stage, suggesting that the morpholino is functional. This was repeatedly observed for over 90% of injected embryos. The morpholino-injected embryos showed missing or size reduction of the midbrain/hindbrain boundary, small eyes, and curved posterior trunk at 24 hpf (C, D). Following morpholino injection, the GN2E-GFP transgenic fish lacked GFP in eyes and showed reduced GFP expression in other neuronal tissues at 48 hpf (E, F). The endogenous *gata2* mRNA level in the neurons was also lower in the morpholino-injected embryo at 24 hpf (H) as revealed by in situ hybridization. r1 And r2 regions (boxed in E, F, G, H) are enlarged to show more details of *gata2* reduction. All sample comparison pictures (A + B, C + D, E + F, and G + H) were taken under identical conditions and placed within the same slide.

the *gata2* neural expression (Meng et al., 1997). Detailed analysis revealed a sequence-specific requirement where just a two-base mutation of the *cis*-element prevented all detectable *gata2*/GFP expression in the nervous system. This information, directly obtained from rapid in vivo analysis, proved useful for the detection of sequence-specific endogenous binding proteins (Fig. 2A).

We then used four *gata2* promoter constructs to generate stable transgenic lines, allowing us to analyze the tissue specificity of the *gata2* neural promoter. Multiple GFP-expressing transgenic lines were generated for each construct. These lines allowed us to define a distal 13.4-kb stretch of the promoter where a hematopoietic *cis*-acting regulatory region resides (Figs. 1D, E). A long range regulatory element for hematopoietic regulation is consistent with the data obtained from mouse studies, but the element in zebrafish appears closer to the coding sequence of *gata2*. Interestingly, the two lines with the shorter constructs had broader neural expression than the two lines derived from BAC constructs. These results generally suggest that there are repressive regulatory element(s) that are missing in the two shorter 1040 and 998 constructs since we observed this in multiple lines. These elements seem to repress GFP expression in mainly peripheral neural structures such as the cranial ganglia, the olfactory bulb, and otic vesicle. The brain and motor neuron expression was the same in lines derived from all constructs.

This study presents for the first time the successful application of transient followed by germ line zebrafish GFP transgenesis for the mapping of a *cis*- element and the subsequent isolation and characterization of requisite *trans*-factors, respectively. This approach is particularly appealing since one can clearly visualize the effects of alterations in transcriptional regulation with respect to all vertebrate tissues in living zebrafish embryos.

Relationship between neural gata2 expression and Pur alpha/Sp8

The experimental observations described here support the hypothesis that Pur alpha and Sp8 are functionally related to act as opposing regulators of *gata2* gene expression. This interaction may come together through the *cis*-acting element of G2NE since Pur alpha was isolated as the gene product responsible for binding to the G2NE and both recombinant Pur alpha and Sp8 bind to the G2NE. Further studies using specific antibodies against Pur alpha and Sp8 in gel supershift assays could provide additional evidences for direct binding of the two proteins to G2NE. At the cellular level, *Sp8* has specific overlapping expression pattern with *gata2* (Fig. 3) in the nervous systems, while Pur alpha is expressed generally during embryogenesis. This at least is consistent with the hypothesis of coregulation of *gata2* by Pur alpha and Sp8 suggested by findings from functional assays. Analysis of the effects of altering Pur alpha expression revealed that Pur alpha functions as a negative regulator of *gata2* neural gene expression (Fig. 4) and morpholino knockdown showed a distinct requirement for Sp8 for the proper expression of *gata2* (Fig. 5), suggesting Pur alpha is involved in repression of *gata2* while *Sp8* is required to activate *gata2* gene expression. Evidence supporting a similar interaction for these factors has been seen in other organisms, which suggests evolutionary conservation. Pur factor interactions with Sp family members have been observed in studies on two other neural genes, mouse MBP (Tretiakova et al., 1999b), and rat neuronal nAch β4 (Bigger et al., 1997; Melnikova et al., 2000). The evidence for an evolutionally conserved Pur alpha:*gata2* link is strengthened based on *C. elegans* work where a Pur alpha-like factor (PLP1) mediates endoderm cell specification via regulation of the GATA family member END-1 (Joel H. Rothman, personal communication). Lastly, in mice, the expression pattern for *Sp8* (Bell et al., 2003) encompasses all described neural tissues with that of Gata2 (Nardelli et al., 1999). It is also possible that more factors are involved in Gata2 regulation. For instance, what activates *gata2* in ventral before *Sp8* is expressed has yet to be determined. In addition, we have preliminary data suggesting *gata2* neuronal expression in the brain may require BarH family of transcription factors. The complexity of temporal and spatial regulation of *gata2* expression therefore is achieved through a complicated regulatory network of transcription factors.

Possible roles of Pur alpha and Sp8 in neural development

Our morpholino analysis indicates that Pur alpha plays an important role in early development in zebrafish. As far as the effects of Pur alpha morpholinos on the nervous system, we generally see such a strong arrest during gastrulation after knockdown that the nervous system does not have time to form. However, overexpression of Pur alpha mRNA in zebrafish embryos blocked *gata2* gene expression early in the ventral ectoderm and later in the nervous system. It has now been determined that Pur alpha plays an essential role in vertebrate neural development that is coupled to cellular proliferation based on gene-targeted knockout of Pur alpha in mice (Khalili et al., 2003). We have functional data as to the apparent *trans*- activity for these transcription factors regulating *gata2*. Together, our data support the idea that Pur alpha can function as a repressor at later stages of development.

Neurons in general do not proliferate. Given that it is generally accepted that the Pur factors are involved in negatively controlling DNA replication, perhaps it is one of the primary roles of Pur alpha to maintain neural cells in a differentiated state. Pur alpha has been detected in neurons and glial cells in mice (Tretiakova et al., 1999a) with detection specifically in nonproliferating neurons (Yano et al., 1999). It has been shown that overexpression of *Pur alpha* in glioblastoma cells can inhibit the proliferation of this neural cell type (Darbinian et al.,

2001). JC virus late transcription is inhibited by Pur alpha in glial cells (Darbinian et al., 2001). It has been shown that Pur alpha associates with E2F-1 to prevent E2F-1 from activating a set of genes involved in S phase (Darbinian et al., 1999).

By contrast, *Gata2* is known to have a positive role in controlling the proliferation of a population of ventral neural progenitors (Nardelli et al., 1999), while Pur alpha is established as having a negative effect on cellular proliferation (Barr and Johnson, 2001; Darbinian et al., 2001; Stacey et al., 1999). It has been established that Sp8 is required for the maintenance of progenitor cells in the apical ectodermal ridge during limb development (Bell et al., 2003). This is similar to the role of Gata2 in the maintenance of progenitor cells of the nervous system and hematopoietic system (Nardelli et al., 1999; Tsai and Orkin, 1997; Tsai et al., 1994). Combined with our analysis of *Sp8* related to *gata2* expression, we favor a model that *Sp8* may be maintaining neuronal progenitor cells via the activation *gata2*, while Pur alpha functions as a negative regulator in nonproliferating or nonneuronal cells (Ishibashi et al., 2000; Wang et al., 1999; Xing et al., 2002).

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Appendix A. Supplemetary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.08.007.

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