Isolation and characterization of zebrafish NFE2

STEPHEN J. PRATT,1,2,* ANNA DREJER,2,* HELEN FOOTT,1,2,* BRUCE BARUT,1,2 ALISON BROWNlie,1,2 JOHN POSTLETHWAIrT,5 YASUTAKE KATO,6 MASAYUKI YAMAMOTO,6 AND LEONARD I. ZON1,2

Howard Hughes Medical Institute, 2Children’s Hospital, Division of Hematology/Oncology, 3Dana-Farber Cancer Institute, and 4Harvard Medical School, Boston, Massachusetts 02115; 5Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403; and 6Center for Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan

Received 3 December 2001; accepted in final form 9 August 2002

Pratt, Stephen J., Anna Drejer, Helen Foott, Bruce Barut, Alison Brownlie, John Postlethwait, Yasutake Kato, Masayuki Yamamoto, and Leonard I. Zon. Isolation and characterization of zebrafish NFE2. Physiol Genomics 11: 91–98, 2002. First published August 27, 2002; 10.1152/physiolgenomics.00112.2001.—Vertebrate hematopoiesis is regulated by distinct cell-specific transcription factors such as GATA-1 and SCL. Mammalian p45-NFE2 was characterized for its ability to bind the hypersensitive sites of the globin locus control region. NFE2 is a member of a cap’n’collar (CNC) and basic zipper (BZIP) superfamily that regulates gene transcription. It has been implicated in diverse processes such as globin gene expression, oxidative stress, and platelet lineage differentiation. Here, we have isolated the zebrafish ortholog of NFE2. The gene is highly homologous, particularly in the DNA-binding domain. Mapping the zebrafish NFE2 to linkage group 23 establishes a region of chromosomal synteny with human chromosome 12, further suggesting evolutionary conservation. During embryogenesis, the zebrafish gene is expressed specifically in erythroid cells and also in the developing ear. NFE2 expression is lacking in zebrafish mutants that have no hematopoietic cells. An analysis of the sauternes mutant, which carries a mutation in the ALAS-2 gene and thus has defective heme synthesis, demonstrates higher levels of NFE2 expression than normal. This further establishes the block to erythroid differentiation in the sauternes mutant. Our studies demonstrate conservation of the vertebrate genetic program for the erythroid lineage.

hematopoiesis; transcription factors; erythroid lineage; sauternes mutant

HEMATOPOIESIS involves the production of hematopoietic stem cells, with subsequent cell proliferation and differentiation. This process is regulated by transcription factors specifically expressed in hematopoietic cells (5). The erythroid program has been extensively characterized in vertebrate species, and the transcription factors GATA-1 and GATA-2 directly activate promoters and enhancers of many, if not all, erythroid genes. The factors SCL and LMO2 have recently been shown to regulate the earliest stage of the hematopoiesis. The recent finding of a cofactor of GATA-1, called FOG1, also demonstrates the regulation of transcription by complexes of proteins (45).

The factor p45-NFE2 is a member of the basic zipper (BZIP) transcription factor family and contains a cap’n’collar (CNC) motif (2, 3). p45-NFE2 was originally isolated due to its ability to bind a duplicated AP-1 site TGCTGA(G/C)TCA(T/C) in the hypersensitive site 2 (HS2) region of the globin locus control region (43). This factor was subsequently shown to heterodimerize with members of the maf family of transcription factors, which also encode BZIP proteins (4, 18). p45-NFE2 is a member of a family of factors that are also known as NRF1, NRF2, and NRF3 (7, 9, 11, 22). NRF1 and NRF2 are also known as LCRF1 and LCRF2. Bach1 and Bach2 are also new members of this family. These factors are able to bind similar sites of the globin HS2 locus (34). p45-NFE2 has been targeted by homologous recombination in mouse embryonic stem cells, and the mouse knockout demonstrates that the factor is critically required for platelet production (23, 24). Consistent with this, a hematopoietic-specific tubulin has been shown to be a target of p45-NFE2 (23). The NFE2−/− animals also have a mild microcytic anemia, suggesting p45-NFE2 does have a role in hemoglobin production (39). Further support for a role of this transcription factor in erythropoiesis comes from in vitro studies. An erythroid cell line deficient in p45-NFE2 fails to differentiate (25), whereas ectopic expression of NFE2 in a myeloid cell line induces erythroid differentiation (38). NRF1 and NRF2 have each been inactivated in mouse embryonic stem cells, and it is apparent that these factors do not participate in erythropoiesis in a cell autonomous manner (10, 11, 15, 28).

The zebrafish is a new developmental and genetic system for studying organ development and embryogenesis (17) and is well suited for the analysis of hematopoiesis. Several forward genetic screens have identified mutants with defective erythropoiesis (1). To date there are 26 complementation groups of hematopoietic mutants, each with defects in distinct stages of
There are mutants that affect dorsal-ventral patterning, hemangioblast formation, hematopoietic stem cell production, proliferation, and differentiation. In addition, there are several mutants with defects in the production of hemoglobin or with defects in heme biosynthesis. The cloning of the genes for some of these mutants has demonstrated the conservation of the hematopoietic program. For instance, the *sauternes* gene encodes ALAS-2 (6). The *sauternes* mutant has hypochromic anemia and is reminiscent of the human ALAS-2 deficiency syndrome, congenital sideroblastic anemia. A mutation in *β*-spectrin in the *rizo* mutant leads to hereditary spherocytosis (27).

We have begun to define the hematopoietic program in the zebrafish by isolating transcription factors known to regulate hematopoiesis in higher vertebrates. To date, we have isolated the genes encoding GATA-1, -2, and -3, SCL, LMO2, *fli1*, and *c-myb* (12, 44). In an effort to further characterize the program, we sought to isolate the NFE2 transcription factor in zebrafish. NFE2 is expressed in an erythroid-specific manner, and its expression is not detected in the yolk to the embryonic circulation (14).

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**METHODS**

**Isolation of cDNA clone for zebrafish NFE2.** A PCR fragment of 180 bp encoding the DNA-binding domain of mouse p45-NFE2 (a gift from Nancy Andrews) was used to screen a gridded adult zebrafish kidney cDNA library (Research Genetics, and Ressourcen Zentrum/Primar Datenbank (RZPD)). The library consisted of 92,160 oligo dT primed clones and 92,160 random primed clones in the pBK-CMV cloning vector (Stratagene). The mouse p45-NFE2 fragment was randomly primed with [32P]dCTP for 2 h and hybridized to the filters at 65°C overnight. The filters were then washed to 0.1× SSC at 65°C and exposed to autoradiography film overnight. The ends of the BAC were directly sequenced and primers (NFE2BACS6, F1-TC-CTATTTCACCAAGCAAGCTG; and NFE2BACSP6, R1-CCAAAGGAGGTTTCTCAGCGATGC) were designed for single-strand conformational polymorphism (SSCP) analysis. A 200-bp band was generated that segregated in the genetic mapping panels (16, 20).

**Other plasmids.** The cDNA plasmids encoding either mouse p45-NFE2, zebrafish NF-E2, or mouse Maf K were subcloned in pIK111 (18) and pBK-CMV-zp45 and pm-MafK15 (30), respectively.

**In vitro transcription/translation.** One microgram of each of the cDNA constructs encoding mouse p45-NFE2, zebrafish NF-E2, or mouse Maf K were transcribed and translated in vitro using the TNT T7 or T3 Coupled Wheat Germ Extract System (Promega).

**Gel mobility shift assay.** The binding mixture (10 μl) contained 250 pg of the radiolabeled oligonucleotide probe no. 25 (21), 20 mM HEPES-KOH (pH 7.9), 1 mM EDTA, 20 mM KCl, 5 mM DTT, 4 mM MgCl2, 1,000 g/ml of poly(dI-dC), 100 μg/ml of BSA, and 5 μl of in vitro translated material. The resultant protein-DNA complexes and free probe were electrophoresed on a 5% non-denaturing polyacrylamide gel. Filters were then washed to 0.1× SSC at 65°C and exposed to autoradiography film overnight. The library consisted of 92,160 oligo dT primed clones and 92,160 random primed clones in the pBK-CMV cloning vector (Stratagene). The mouse p45-NFE2 fragment was randomly primed with [32P]dCTP for 2 h and hybridized to the filters at 65°C overnight. The filters were then washed to 0.1× SSC at 65°C and exposed to autoradiography film overnight. The ends of the BAC were directly sequenced and primers (NFE2BACS6, F1-TC-CTATTTCACCAAGCAAGCTG; and NFE2BACSP6, R1-CCAAAGGAGGTTTCTCAGCGATGC) were designed for single-strand conformational polymorphism (SSCP) analysis. A 200-bp band was generated that segregated in the genetic mapping panels (16, 20).

**Mapping of zebrafish NFE2.** A single bacterial artificial chromosome (BAC) clone was obtained by hybridizing the zebrafish p45-NFE2 complete cDNA to BAC filters (Genome Systems). The cDNA was randomly primed with [32P]dCTP for 2 h and hybridized to the filters at 65°C overnight. The filters were then washed to 0.1× SSC at 65°C and exposed to autoradiography film overnight. The ends of the BAC were directly sequenced and primers (NFE2BACS6, F1-TC-CTATTTCACCAAGCAAGCTG; and NFE2BACSP6, R1-CCAAAGGAGGTTTCTCAGCGATGC) were designed for single-strand conformational polymorphism (SSCP) analysis. A 200-bp band was generated that segregated in the genetic mapping panels (16, 20).

**Genotyping of zebrafish mutants.** Zebrafish embryos stained for NFE2 expression were photographed before extracting DNA. Scored embryos were then genotyped using the previously described markers E9.T3F5/R3 (F5, 5′-CACCAATAATGGATGTTG-3′; R3, 5′-AAAAAAGACAGATCCCATCGTC-3′). Fifteen fish with increased NFE2 expression were genotyped as mutant with one mis-score.

**In situ hybridization.** In situ hybridization was performed as described (44). Antisense riboprobes for the zebrafish NFE2 were made by digesting the cDNA with the restriction enzyme EcoRI and utilizing T7 RNA polymerase as described (44).

**Fig. 1. Comparison of zebrafish and human NFE2 cDNAs.** Note the extensive degree of identity in the DNA binding domain in the COOH-terminal region of the sequence. Identity is shaded in gray.
through a 4% polyacrylamide gel in 0.5× TBE buffer at 25°C. A 400-fold molar excess of unlabeled oligonucleotide was added to the reaction as a specific competitor (30).

RESULTS

Isolation of zebrafish NFE2. Utilizing a mouse p45-NFE2 cDNA probe, we screened a gridded zebrafish kidney cDNA library and identified seven hybridizing clones. These clones were isolated and sequenced, and one clone (139 O4) was found to encode a protein related to mammalian p45-NFE2. A comparison of the zebrafish cDNA to human NFE2 revealed that the structure has largely been conserved during vertebrate evolution, particularly in the BZIP and CNC regions.
(Fig. 1), but the domains outside of the BZIP region are relatively divergent.

**Positioning of NFE2 to the zebrafish genome map.** To evaluate the map location of the zebrafish NFE2 gene and to determine whether NFE2 was a potential candidate for any of the currently mapped zebrafish hematopoietic mutants, a BAC clone (85 K11) was isolated, and its ends were mapped utilizing both a genetic backcross panel as well as a radiation hybrid panel. It was found that NFE2 mapped to linkage group 23 between RAPD marker 8A.800 and the HoxC cluster (20, 35) (Fig. 2 and Table 1). This map position does not correspond to any of our mutants. Interestingly, the region of LG23 on which NFE2 lies is highly syntenic to human chromosome 12 and mouse chromosome 15 (Fig. 2) (35) where human and mouse NFE2 map, respectively. The similar structure of the zebrafish cDNA with mammalian NFE2, taken together with the chromosomal synteny, indicates that this gene has been conserved throughout vertebrate evolution.

**Analysis of gene expression.** Expression pattern of NFE2 during embryonic development was examined by whole embryo in situ hybridization (Fig. 3). NFE2 initiates expression at 10 somites in the developing intermediate cell mass (ICM) in a pattern very similar to that of GATA-1 (12). As development continues from 18 somites to 24 h, the gene is more highly expressed in the developing ICM, the functional equivalent of the mammalian yolk sac. Thus NFE2 appears to be induced during erythroid differentiation rather than being expressed in the early hematopoietic cell population. After 36 h, NFE2 is expressed at a much lower level in circulating blood cells. In addition, we find NFE2 transcripts in the developing otic vesicle.

**Analysis of the NFE2 expression in mutant zebrafish.** We first analyzed NFE2 expression in the mutant cloche (Fig. 4). Expression of GATA-1 is deficient in cloche mutants (41), consistent with an absence of blood as previously described. NFE2 expression in the blood forming regions, similarly to GATA-1, is absent in cloche (44). This confirms the hematopoietic-specific expression of NFE2. In cloche the expression in the developing ear is still present, suggesting that cloche is not defective at the NFE2 locus.

We subsequently analyzed the expression of NFE2 in the sauternes mutant (ALAS-2 deficiency) (Fig. 5). We have previously demonstrated alterations of gene expression in the sauternes mutant (6). GATA-1 expression in wild type embryos is down regulated by day 3. However, in sauternes mutants, GATA-1 expression is

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**Table 1. An analysis of the genetic mapping for NFE2**

<table>
<thead>
<tr>
<th>Locus</th>
<th>M</th>
<th>P</th>
<th>X</th>
<th>N</th>
<th>Map Interval, cM</th>
<th>95% CI, cM</th>
<th>LOD</th>
</tr>
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<tbody>
<tr>
<td>hoxc5a</td>
<td>42</td>
<td>54</td>
<td>1</td>
<td>46</td>
<td>2.17 ± 2.15</td>
<td>0.1 to 11.5</td>
<td>11.8</td>
</tr>
<tr>
<td>nfe2</td>
<td>22</td>
<td>24</td>
<td>7</td>
<td>46</td>
<td>15.22 ± 5.30</td>
<td>6.3 to 28.9</td>
<td>5.3</td>
</tr>
<tr>
<td>z3157</td>
<td>45</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Locus, hoxc5a (Y14539), nfe2 (this work), z3157 (ZFIN ID: ZDB-SSLP-980528-342); M, maternal genotype; P, paternal genotype; X, cross-overs; N, number scored; Map Interval, map interval ± SE; 95% CI, 95% confidence interval; LOD, logarithm of the odds score.

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Fig. 3. Whole embryo in situ hybridization studies for NFE2 expression during development. The stages are indicated, from 5 somites to 48 h.
not downregulated by day 3 of development. We find that similar to GATA-1, NFE2 is also not downregulated by day 3 of development (Fig. 5). The downregulation of GATA-1 and NFE2 suggests the transcription of these genes is regulated by heme levels. This indicates a block to hematopoietic differentiation in the cells in circulation of sauternes mutants.

Characterization of zebrafish NFE2 protein. We sought to characterize whether zebrafish NFE2 could bind similar sites as the mammalian protein. Mammalian p45-NFE2 can bind to an NFE2 site in the presence of mafK (21). Zebrafish NFE2 was expressed in wheat germ extract. In the presence of expressed mafK, a gel mobility shift reminiscent of the mammalian p45-NFE2/mafK heterodimer is seen (Fig. 6, lane 7), whereas homodimers of p45 or mafK do not bind. The binding is specific since it was competed with excess unlabeled oligonucleotide. NFE2 sites have yet to be described in zebrafish genes involved in hematopoiesis, although only few genes have been examined. Our studies suggest NFE2 sites will be found in the globin locus and in thrombocyte-specific genes of the zebrafish.

DISCUSSION

The vertebrate hematopoietic program is highly conserved, particularly for the transcription factors involved in hematopoiesis (33). We believe that the zebrafish NFE2 gene is the true ortholog because the zebrafish NFE2 gene is adjacent to the HoxC cluster, similar to mapping studies in mouse and humans. As illustrated by the cloning of zebrafish NFE2, there is extensive conservation of the DNA-binding domain, whereas regions outside of these domains are less conserved. DNA-binding domains are important functional motifs, and recently these domains have also been shown to function as protein-protein interaction motifs. The lack of amino acid conservation in the putative activation domains of zebrafish and mammalian p45-NFE2 does not necessarily indicate lack of importance, and some structural characteristics may be conserved.

We find that p45-NFE2 expression is not detected in cloche mutant embryos. This is consistent with previous work showing that cloche is a gene that affects hematopoietic stem cells, perhaps acting at the heman-
gioblast level. Other mutants could be studied for NFE2 expression, and it is likely some of the 26 complementation groups of zebrafish mutants will affect expression of this factor. In this regard, we analyzed the expression of NFE2 in sauternes, a mutant with heme deficiency (6). Our previous analyses of this mutant indicated that GATA-1 expression is abnormally maintained at a time when wild-type siblings show a downregulation. In this report, we found expression of NFE2 is also maintained in sauternes mutants. This suggests that heme deficiency leads to a defect in hematopoietic maturation and that this delays the normal downregulation of expression of the erythroid-specific transcription factors, NFE2 and GATA-1.

The isolation of zebrafish NFE2 demonstrates the conservation of the erythroid program among the vertebrates and supports an important role for this factor throughout evolution. The essential role of NFE2 may be in the generation of the thrombocyte lineage in the fish (19), which is functionally similar to the platelet lineage in mammalian biology. We have not yet evaluated the thrombocyte lineage in zebrafish for p45-NFE2 expression. p45-NFE2 may also have a role in erythroid development as evidenced by the murine knockout, although this role appears to be subtle (40). The conservation of the genomic location of NFE2 as adjacent to the Hox C cluster suggests that during the ancient tetraploidation of the vertebrate genome, the genes adjacent to the Hox clusters were maintained and utilized for distinct purposes (22). nrf1 and nfr2 are utilized for oxidative stress, whereas p45-NFE2 appears to be more relevant to hematopoietic differentiation. The zebrafish has a globin gene structure that includes α- and β-globins in the same locus (8). This structure is distinctly different from the mammalian globin loci. It is possible that p45-NFE2 could regulate globin gene expression, although locus control region elements have not been defined yet in the zebrafish system. Future work could assess the role of p45-NFE2 in hemoglobin switching in the zebrafish.

The zebrafish is an excellent forward genetic system for studying hematopoiesis. We have isolated factors by homology that are conserved throughout vertebrate evolution and are important in gene transcription. We have also defined the program by the study of 26 complementation groups of mutants that affect various differentiation processes (1, 36, 46). The availability of hematopoietic mutants allows for a defined characterization of the critical regulators of the blood program. Recent experiments using morpholinos, an antisense technology, offers an additional method for evaluating gene function (31). Characterization of zebrafish NFE2 using antisense or overexpression studies will provide a greater understanding of the function of this transcription factor during vertebrate hematopoiesis and should reinforce studies done in mammalian species. It will be interesting to evaluate the thrombocyte lineage with directed morpholinos to NFE2. As human diseases are mapped to genetic loci, the chromosomal synteny of the zebrafish should provide candidate genes for the study of human disease. For instance, the iron transporter, ferroportin-1, was isolated as a zebrafish mutant gene (14). Recently, mutations in the orthologous gene were detected in human patients with a rare form of hemochromatosis (29, 32). With the sequence of the zebrafish genome being done by the Sanger Center over the next 2 yr, additional informa-

Fig. 6. Gel mobility shift analysis of zebrafish p45-NFE2 with mouse maf K. Lanes 1–10: labeled no. 25 DNA (250 pg) as a probe. *Lanes 5, 8, 10: plus nonlabeled no. 25 DNA (100 ng) as a competitor.
tion about the evolution of factors critical to hematopoiesis should be uncovered.

We thank Alan Davidson for reading this manuscript. This work was supported by National Institutes of Health Grants R01-HL-48801-11, R01-DK-55381, and F50-DK-49216, as well as by Legal Sea Foods. L. I. Zon is an Investigator of the Howard Hughes Medical Institute.

Editor S. L. Alper served as the review editor for this manuscript submitted by Editor L. I. Zon.

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