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Targeted disruption of the ubiquitous CNC-bZIP transcription factor, Nrf-1, results in anemia and embryonic lethality in mice

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The CNC-basic leucine zipper (CNC-bZIP) family is a subfamily of bZIP proteins identified from independent searches for factors that bind the AP-1-like *cis*-elements in the β-globin locus control region. Three members, p45-Nf-e2, Nrf-1 and Nrf-2 have been identified in mammals. Expression of p45-Nf-e2 is largely restricted to hematopoietic cells while Nrf-1 and Nrf-2 are expressed in a wide range of tissues. To determine the function of Nrf-1, targeted disruption of the *Nrf-1* gene was carried out. Homozygous *Nrf-1* mutant mice are anemic due to a non-cell autonomous defect in definitive erythropoiesis and die *in utero*.

Keywords: anemia/bZIP gene/knockout mouse/Nrf-1

Introduction

A number of major protein-binding sites including GATA, NF-E2/AP-1 and CACC sequences, have been identified within Dnase I hypersensitive sites (HS) of the α- and β-globin locus control region (LCR). We and others have sought to identify proteins other than AP-1 which activate transcription by binding the NF-E2/AP-1 motif of HS-2 in the β-globin LCR (Orkin, 1990). Several such proteins including p45-Nf-e2, Nrf-1(LCR-F1/NFE2L1/TCF11) and Nrf-2(ECH) have been identified to date (Andrews *et al.*, 1993; Chan *et al.*, 1993a; Ney *et al.*, 1993; Caterina *et al.*, 1994; Moi *et al.*, 1994; Chui *et al.*, 1995; Itoh *et al.*, 1995; McKie *et al.*, 1995). These proteins are members of a subset of bZIP genes sharing a conserved structural domain, termed the 'CNC' domain, first noted in the *Drosophila cap and collar* (CNC) gene which is required for labial and mandibular development (Mohler *et al.*, 1991, 1995). The CNC domain is situated immediately N-terminal to the bZIP DNA-binding and its function is unknown. In addition to containing the CNC domain, members of this family show strong similarity in their basic DNA-binding domain, suggesting that they bind the NF-E2/AP-1 element with similar specificity. Available evidence suggests that Nrf-1 functions as a heterodimer with members of the NRL/small-maf family of proteins (Motohashi *et al.*, 1997).

Previous studies indicate that AP-1 is not likely to be the key regulator of the NF-E2/AP-1 binding sites since point mutations which abolish NF-E2 binding, but not AP-1 binding, inhibit HS-2 activity (Moi and Kan, 1990; Francastel *et al.*, 1997). Moreover, overexpression of cJUN in K562 cells down regulates HS-2 β-globin reporter constructs (Moi and Kan, 1990). As p45-Nf-e2 is abundantly expressed in hematopoietic cells, but not in other cells, it was predicted to play a major role in globin gene expression and erythroid development (Andrews *et al.*, 1993; Chan *et al.*, 1993b; Ney *et al.*, 1993). Indeed, the disruption of the *p45-Nf-e2* gene in a murine erythroleukemia cell line by retroviral insertion, as well as the loss of p45-Nf-e2 function in cell lines containing a transdominant mutant of p18-maf, the partner subunit for p45-Nf-e2, result in the loss of globin gene expression (Lu *et al.*, 1994; Kotkow and Orkin, 1995). However, homozygous disruption of the *p45-Nf-e2* gene in mouse results in defective megakaryopoiesis and thrombocytopenia, but erythroid development is only minimally affected, as red cells only show mild microcytosis with a slight decrease in hemoglobin content (Shivdasani *et al.*, 1995a,b). Whether there is compensation by neighboring *cis*-acting elements in the LCR or compensation by other *trans*-acting factors, specifically Nrf-1, is not clear. A role for Nrf-1 in globin gene regulation has been suggested, given its high level of expression in erythroid cell lines, and its ability to preferentially activate reporter genes linked to the β-globin LCR in erythroid cells (Ney *et al.*, 1993; Caterina *et al.*, 1994). Moreover, the coexpression of Nrf-1, Nrf-2 and p45-Nf-e2 in erythroid cells suggests that there may be overlap in their function in addition to the unique functions that each protein may have. On the other hand, Nrf-1 is ubiquitously expressed. To determine the function of Nrf-1, we disrupted the mouse gene by homologous recombination. We show that the *Nrf-1* gene is essential for development. Mice homozygous for the *Nrf-1* mutation suffer from anemia as a result of abnormal fetal liver erythropoiesis and die *in utero*. No defect in globin gene expression was detected however. Abnormal red cell production appears be the result of a defect in the fetal liver microenvironment specific for erythroid cells. These data suggest that target genes regulated by *Nrf-1* play an essential role during fetal liver hematopoiesis.

Results

Targeted disruption of the mouse Nrf-1 gene

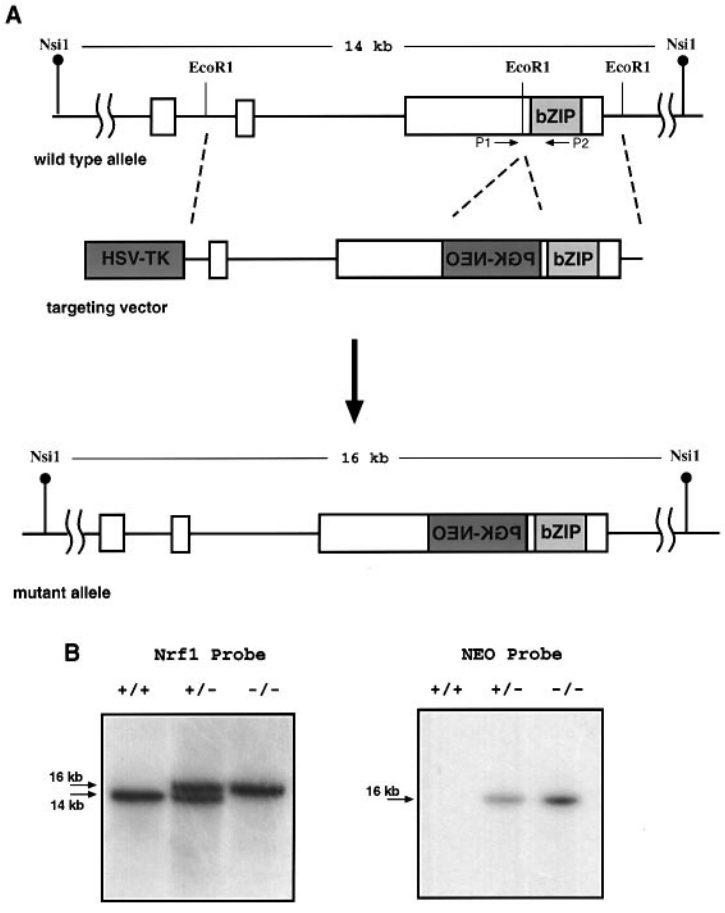
To disrupt the mouse *Nrf-1* gene, a targeting vector was constructed in which the neomycin cassette was inserted in the opposite transcriptional orientation into the terminal exon which encodes the DNA binding and leucine zipper domains (Figure 1A). Correctly targeted ES cell clones were identified by Southern blot analysis. Genomic DNA was digested with *Nsi*I and probed with an *Nrf-1* gene fragment external to the targeting vector. The wild type

Nrf-1 allele gave a 14 kb band, while the targeted allele resulted in a 16 kb band (Figure 1B). A neomycin-cassette probe detected a 16 kb band only, thus random integration did not occur in the targeted ES cells. Positive ES cells were injected into C57BL/6 blastocyst to generate chimeric animals and germline transmission by male chimeras were achieved for two of the ES cell clones. Identical results were obtained from these two lines, therefore combined data will be presented.

To characterize the effects of the neo insertion on *Nrf-1* expression, embryos were isolated for mRNA and protein analysis. Poly(A)$^+$ RNA was isolated from day 12.5 or 13.5 embryos and analyzed by Northern blotting. As expected, the murine *Nrf-1* cDNA probe detected a 5.0 kb transcript in wild type and heterozygote embryos. The probe also detected a 7.0 kb readthrough transcript from mRNA isolated from heterozygote and homozygote knockout embryos (Figure 1C). Based on the structure of the targeted mutation, the readthrough transcript should contain sequences from the non-coding strand of the neo cassette, and this was indeed the case as a neo probe hybridized to the same 7.0 kb transcript in both the heterozygotes and homozygous mutant samples (data not shown). While these results indicate that the mutant allele is transcribed and is stable, readthrough translation into the bZIP domain to generate a functional Nrf-1 protein capable of DNA-binding and protein dimerization would not be possible due to the neo insertion. Translation which terminates in the stop codons of the PGK-neo insert would result in a truncated peptide of ~40 kd. To address this possibility, we performed immunoblot experiments using an affinity purified rabbit antibody against a segment of Nrf-1 which is N-terminal to the PGK-neo cassette insertion. Figure 1 shows that the anti-Nrf-1 antibody recognized the protein products derived from *in vitro* transcription and translation of the human Nrf-1 cDNA. The p60 product represents translational starts from AUGs located internally. Nrf-1 immunoblot analysis on whole embryo lysates revealed expression of Nrf-1 protein in wild type embryos, but not in homozygous mutant embryos (Figure 1D). Figure 1D (lane 2) shows that the antibody efficiently recognized *in vitro* derived peptide corresponding to the truncated form which has the potential of being expressed by the mutant allele. However, we were unable to detect the production of the putative truncated peptide in our assay. Although unlikely, it is conceivable that the PGK-neo insert or portions of it can be spliced out at low levels to regenerate wild type *Nrf-1* transcript and protein below the detection sensitivity of Northern or Western analysis. To ensure that this was not the case, reverse transcription–polymerase chain reaction (RT–PCR) analysis was carried out on total RNA isolated from homozygous mutant and control embryos. No evidence of an intact transcript in mutant RNA samples was detected (Figure 1E). Based on these results, we conclude that the engineered mutation described here represents a null allele.

Nrf-1$^{-/-}$ animals die at mid-late gestation

Heterozygous mice developed normally, were fertile and showed no obvious abnormalities. To determine the effect



of the targeted *Nrf-1* allele in the homozygous state, heterozygous mice were intercrossed and the offspring were genotyped by Southern and PCR analysis (Figure 1B and data not shown). No homozygotes were found at weaning, and the ratio of wild type to heterozygous progeny was ⊤1:2 (Table I). These findings indicate that *Nrf-1* is an essential gene, disruption of which results in perinatal death.

Timed matings were done to analyze embryos of various gestational age. The relative proportion of homozygous mutant embryos obtained before day 12.5 was ~25%. Thereafter the recovery of viable homozygous *Nrf-1*$^{-/-}$ embryos steadily decreased (Table I). Although the proportion of viable homozygous mutant embryos was reduced by day 13.5, a significant proportion was still observed at day 16.5. Few homozygous mutant embryos were present by 17.5–18.5 days of gestation, indicating that most had already undergone complete resorption at late stages. On very rare occasions, homozygous mutant animals were found immediately postpartum among all the progenies from numerous litters analyzed (data not shown). These animals are markedly pale and died within hours after birth. Results obtained from matings done on a 129/SvJ background are similar, thus the wide range in time of death is not due to the hybrid 129/C57 genetic background of the animals (data not shown).

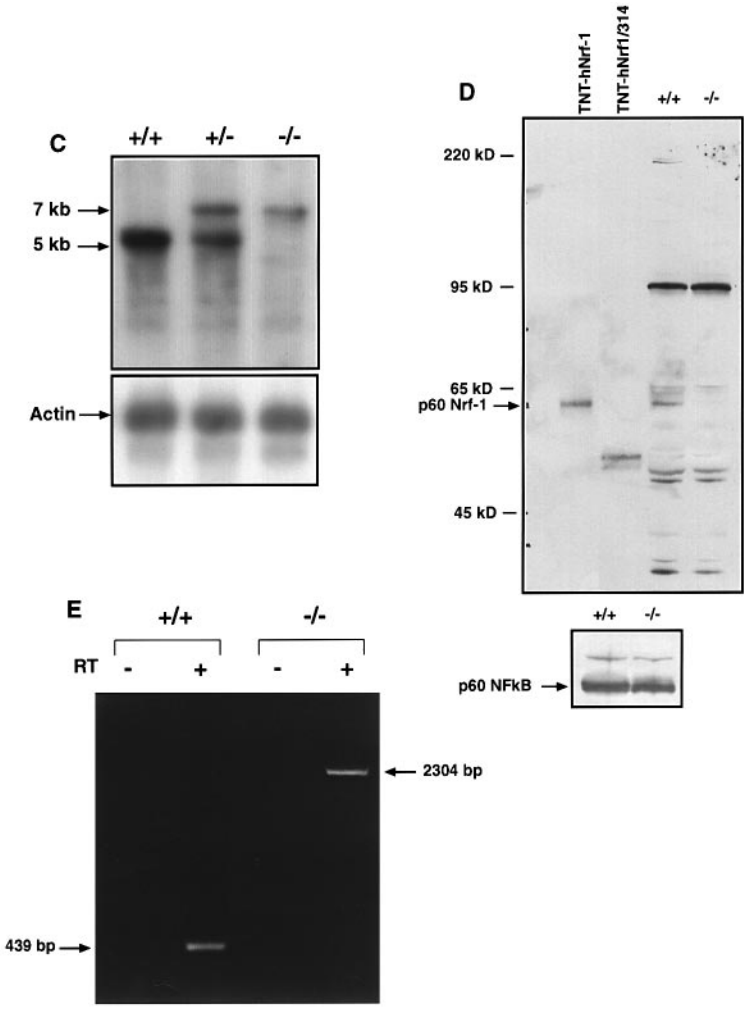


Fig. 1. Targeted disruption of the *Nrf-1* gene. (**A**) The top drawing represents the 3′ end of the *Nrf-1* gene with the last three exons represented by boxes, and the bZIP domain is indicated. Disruption of *Nrf-1* is achieved by targeting the phosphoglycerate kinase-neomycin cassette 5′ to the bZIP domain. The predicted sizes of the *Nsi*I fragments of the wild type and mutant *Nrf-1* alleles are indicated. (**B**) Southern blot analysis of tail DNA digested with *Nsi*I and analyzed using a 5′ external probe or neo probe. The 5′ external *Nrf-1* probe detects a 16 kb (targeted allele) or a 14 kb (wild type allele); the neo probe detects the 16 kb targeted band. (**C**) Northern blot analysis of wild type, heterozygous and homozygous *Nrf-1*$^{-/-}$ embryos. A 5 kb transcript corresponding to the normal Nrf-1 mRNA is detected in the wild type and heterozygote samples. A larger transcript of ~7 kb corresponding to the mutant mRNA is detected in the heterozygote and mutant samples. β-actin control is shown below. (**D**) Western blot analysis of lysates from wild type and homozygous mutant embryos. Molecular weight markers are indicated on the left. Lane 1 contains the p60 Nrf-1 peptide derived from *in vitro* transcription and translation of a plasmid encompassing the coding region of the human Nrf-1 cDNA beginning from the second AUG initiation codon. Lane 2 contains a truncated Nrf-1 derived from *in vitro* transcription and translation of a plasmid corresponding to the protein that is potentially expressed from the mutant *Nrf-1* allele. Lanes 3 and 4 contain wild type mutant extracts, respectively. The large arrow indicates the p60 Nrf-1 product detected in the TNT-hNrf-1 and +/+ lanes. A very prominent non-specific band of 95 kDa is also detected in embryo extracts with this antibody. Loading control, shown below, was determined with an antibody against the p60-NFkB peptide. (**E**) RT–PCR analysis of mouse embryo mRNA. The P1 and P2 primers (see 1a) detect a 439 bp band for the wild type transcript and a 2.3 kb targeted transcript.

Table I. Genotype distribution of progenies from heterozygous matings

Age	Litters	+/+	+/–	–/–	Total
E8.5–11.5	20	37	73	41	141
E12.5	16	29	62	29	120
E13.5	11	27	38	18	83
E14.5	11	23	49	11	83
E15.5	20	47	98	15	160
E16.5	17	28	59	20	107
E17.5	6	10	15	4	29
E18.5	5	11	19	1	31
Weaning	26	60	105	0	165

Nrf-1$^{-/-}$ mice have impaired fetal liver erythropoiesis

In spite of the variability in time of death, the overall characteristics of the mutant embryos were quite constant. At 10.5–12.5 days, the most obvious finding was that mutants were smaller in comparison with wild type and heterozygous littermates (Figure 2). By 13.5–15.5 days, mutant animals were also pale in comparison with their normal littermates. No ostensive abnormalities were detected in the development of mutant embryos. Gross and histologic examinations revealed no abnormalities in the major organs, including heart, muscle, liver and lung, where NRF-1 would normally be expressed at high levels.

Hematocrits of viable mutant embryos obtained at day 16.5 were reduced by ~50% compared with wild type and heterozygote controls (Table II). Moreover, the peripheral blood smear of mutants at this stage showed a high percentage of nucleated red blood cells (NRBC ⊤20%) (Figure 3B). In contrast, wild type and heterozygous animals contained mostly enucleated RBC (<1% NRBC) which is characteristic of the peripheral blood at this time (Figure 3A). RNase protection analysis of total RNA from liver showed comparable levels of β-globin transcripts between control and homozygotes (data not shown). Thus, there is no evidence for down regulation of globin gene expression. To examine the nature of the anemia further, total red cell counts were done at various days of development. In mouse, hematopoiesis begins in the yolk sac at around day 7 of development. The yolk sac blood islands give rise to primitive nucleated red blood cells that express embryonic globin. At around day 10 and 11 of development, hematopoiesis shifts to the fetal liver and give rise to definitive red blood cells. Unlike primitive erythrocytes, definitive red cells contain adult globin proteins and undergo enucleation prior to release into the circulation. After birth, hematopoiesis occur largely in the bone marrow and spleen. At day 11.5, the number of yolk-sac derived nucleated red blood cells was indistinguishable between controls and homozygous mutants (Table II). While the number of nucleated red cells was comparable at day 13.5, definitive enucleated red cells were decreased in mutants compared with controls. Analysis of globin chains present in peripheral blood of E15.5 mutants showed increased amounts of embryonic globin compared with normal and heterozygote littermates (data not shown). This finding is consistent with primitive nucleated red cells persisting in the circulation, rather than the premature release of nucleated red cells of definitive origin into the circulation secondary to anemia.

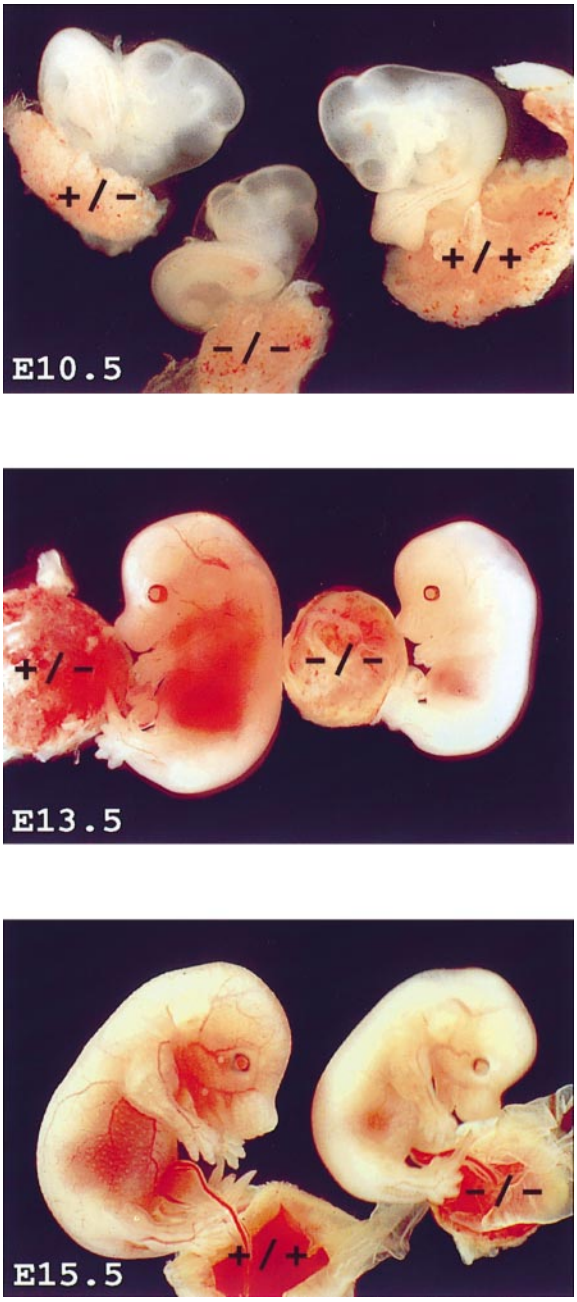


Fig. 2. Comparison of embryos at different developmental time points. The genotypes and gestational ages of the embryos are indicated. Note that the gross appearance of the homozygous mutant is normal compared with their normal control littermates except for their pallor and reduction in size.

In mutant embryos, the livers were noticeably smaller and lighter in color (Figure 4A). The reduction in size was noted at E12.5, and was prominent by E13.5–14.5. Liver sections of E15.5-E16.5 mutant embryos stained with hematoxylin–eosin showed a normal architecture and cellular composition with hepatocyes and megakaryocytes (Figure 4B and C). Consistent with findings in the peripheral blood, there was a paucity of enucleated erythrocytes in the livers of mutant embryos. While erythropoietic islands were clearly discernable in the mutant livers, touch preparations showed a disproportionate abundance of immature erythrocytes, and a relative lack of more mature

cells such as reticulocytes and enucleated red blood cells (Figure 4D and E). Flow cytometry revealed similar proportions of $Gr1^+$ myeloid cells and $Ter119^+$ mononuclear cells representing erythroid progenitors in controls and mutants (data not shown). Measurement of cell death using the TUNEL assay for DNA fragmentation did not show increased number of apoptotic cells in mutants compared with normal liver (data not shown). These results suggest that the defect is specific to erythroid cells, and that a delay or arrest in their maturation as a cause of impairment of fetal liver erythropoiesis in the mutant animals. In the absence of other discernable defect, we propose the resulting anemia and ultimately hypoxia as the cause of death.

Impaired erythropoiesis may result from the inability of precursor cells to mature due to either an intrinsic defect or a failure in the microenvironment to support maturation. To determine the nature of the impaired erythropoiesis in the mutants, *in vitro* progenitor assays were carried out. Single cell suspensions prepared from E13.5 mutant and control embryos were plated in methylcellulose-based medium supplemented with a cocktail of growth factors optimized to support multilineage colony growth. Plates were seeded with equal number of nucleated cells, and colonies were enumerated for CFU-E and BFU-E at 2 and 7 days, respectively, after plating. Consistent with the smaller liver size, the numbers of nucleated cells recovered from livers of E13.5 mutant embryos was 20–30% of normal livers (Table II). Interestingly, a slight increase in numbers of CFU-E and BFU-E colonies per 10^5 nucleated cells plated from mutant embryos was detected in comparison with wild type and heterozygote controls (Table II and data not shown). CFU-E and BFU-E colonies from wild type and mutant mice were indistinguishable with similar hemoglobinization (based on the degree of red color attained by the colonies) and cytologic findings. Mutant and control colonies contained large numbers of late normoblast and some enucleated red cells. The number of CFU-GM were comparable in both mutants and controls (data not shown). These results indicate that erythroid progenitors are slightly enriched in the fetal liver of mutant embryos proportional to the total liver cellularity, but their absolute numbers are decreased. Taken together with the previous data, it appears then the anemia observed in Nrf-1 mutant embryos results from the abnormal maturation of these cells in the fetal liver microenvironment and not as a consequence of a reduction in hematopoietic progenitor cells in the fetal liver.

Table II. Hematologic findings from heterozygous matings

Gestation	Parameters	Nrf-1 genotype	
		(+/+; +/–)	(–/–)
11.5	Primitive nucleated rbc ($\times 10^6$/embryo)	3.0 ± 0.9	2.5 ± 0.9
13.5	Primitive nucleated rbc ($\times 10^6$/embryo)	2.2 ± 0.5	2.2 ± 0.6
	Definitive enucleated rbc ($\times 10^6$/embryo)	1.9 ± 0.7	0.4 ± 0.1
16.5	Hematocrit (%)	37.6 ± 2.4	22.3 ± 4.2
13.5	Nucleated cells/liver (10^6)	2.2 ± 0.9	0.5 ± 0.2
	CFU-E ($\times 10^3/10^5$nucleated cells)	2.5 ± 1.0	4.2 ± 0.8
	CFU-E ($\times 10^4$/liver)	3.8 ± 1.8	1.0 ± 0.4

Extensive contribution of NRF-1 deficient ES cells to adult chimeric blood cells

It remained possible that the defect observed may not be entirely cell-extrinsic as *in vitro* culture conditions, which usually contain growth factors in excess, do not mimic the situation *in vivo*. Thus, ES cells bearing disruption in both *Nrf-1* alleles were examined for their ability to contribute to blood cells in chimeric animals. *Nrf-1* homozygous mutant ES cells were generated by a two-step targeting approach. Heterozygous ES cells were retargeted using a vector similar to the targeting vector described above except that the neomycin resistance gene has been replaced with a hygromycin resistance gene. Following selection in G418, gancyclovir and hygromycin, double knockout ES cells were identified by Southern blot and PCR analysis (data not shown). Homozygous knockout ES cells were then injected into wild type C57BL/6 blastocyst to generate chimeras. Glucose phosphate isomerase (GPI) isoform analysis was used to determine the extent of contribution by the injected ES cells to blood of chimeric animals. The ES cells used contains the GPI-A isoform, whereas C57BL/6 host embryo contains the GPI-B isoform. GPI analysis clearly shows that ES cells bearing two disrupted *Nrf-1* alleles contributed efficiently

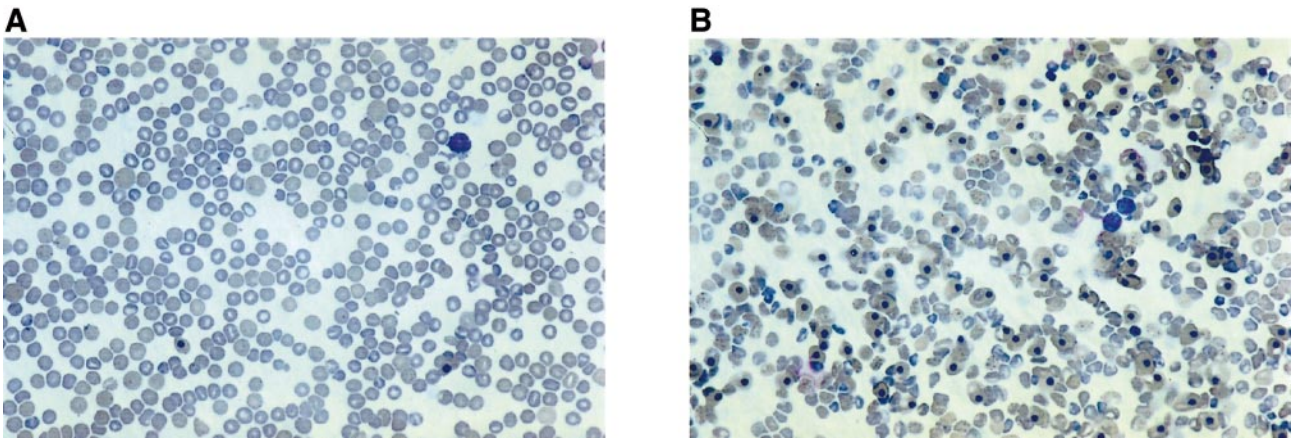


Fig. 3. Persistence of yolk-sac derived primitive erythrocytes in homozygous mutant embryos. Peripheral blood smear from control d 16.5 embryos revealed rare nucleated red blood cells (**A**), while blood from mutant embryos showed an abundance of nucleated red blood cells (**B**).

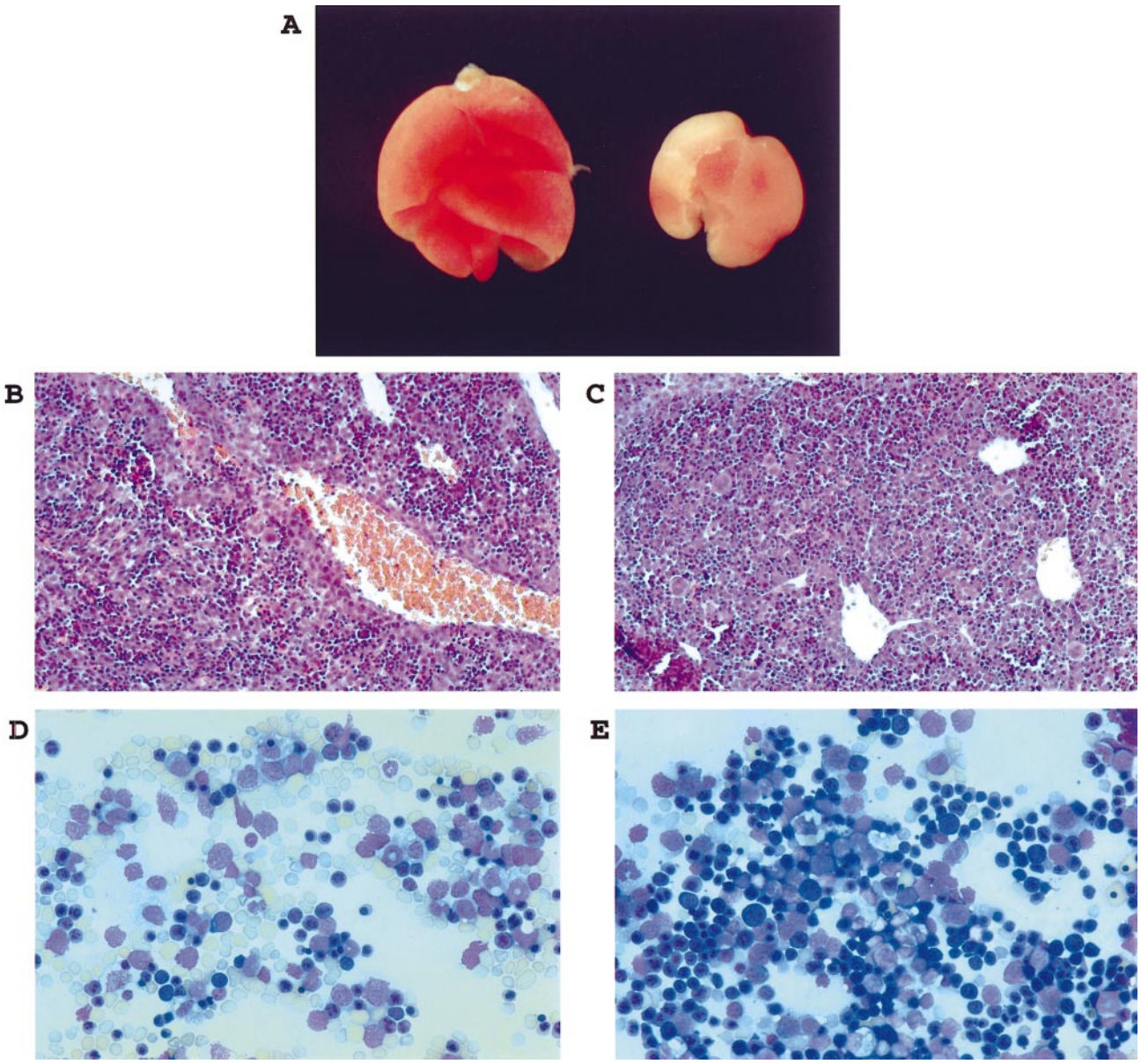


Fig. 4. Arrested development of erythroid cells in homozygous mutant fetal livers. (**A**) Wild type and mutant livers from E15.5 embryos. Note the pallor and small size of the mutant liver. (**B** and **C**) Hematoxylin–eosin stained liver sections from control and mutant E16.5 embryos revealed numerous hematopoietic cells. Mutant embryos, however, showed very few enucleated red blood cells within the vessels and sinusoids, with only rare nucleated red blood cells present. (**D**) Wright–Geimsa stained touch preparations of liver from control embryos revealed erythroid cells at various stages of maturation and an abundance of mature enucleated red cells. (**E**) In contrast, mutant livers revealed few erythroid cells beyond the normoblast. Few normoblasts, reticulocytes and mature enucleated red cells were seen.

to blood cells of the chimeric animals tested (Figure 5). This result is consistent with the *in vitro* progenitor assays and demonstrates that the defect in erythropoiesis in *Nrf*-1 mutant animals is not cell autonomous.

Discussion

In this paper, we have demonstrated a specific role of *Nrf*-1 in fetal liver hematopoiesis, in that the disruption of the gene leads to anemia and embryonic lethality in homozygous mutant mice. With the exception for anemia and growth retardation, no obvious defects in development were seen in the homozygous mutant animals prior to their death. This was unexpected given that *Nrf*-1 is expressed in a large number of tissues in mice. As the

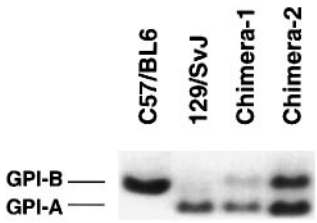


Fig. 5. Nrf-1 deficient ES cells contribute to blood cells of chimeric animals. ES cell contribution to the blood of two adult chimeras was estimated by the ratio of GPI-A (C57BL/6-blastocyst specific isoform) to GPI-B (129-ES specific isoform) isoenzyme. Positions of C57BL/6 and 129 GPI isoforms are shown in lanes 1 and 2.

growth retardation was evident prior to the onset of anemia, we believe that this may reflect a general role for *Nrf*-1 in cellular metabolism or proliferation. It is apparent from the phenotype that *Nrf*-1 is not essential, at least up to mid- and late-gestational stages, for the basic specification and development of major organ systems, including, heart, skeletal muscle and kidney, where it is normally expressed at high levels. Death at mid-late gestation could occur as a result of stage-specific general cellular function(s) where *Nrf*-1 becomes absolutely essential. Alternatively, it is likely that the impaired erythroid maturation, with the ensuing anemia, from a functional defect in the stromal compartment which is normally dependent on *Nrf*-1, was the cause of death. Primitive erythropoiesis in the yolk sac appears unaffected as survival of homozygous mutant embryos is not compromised until past day 12 of embryonic development when the site of hematopoiesis has already shifted to the fetal liver. Indeed, the number of red cells present in the peripheral blood in mutant animals at day 11.5 was comparable with their wild type and heterozygote littermates. Thus, failure in erythropoiesis is at the fetal liver stage. The phenotype described here is similar in some respects to that in mice containing targeted disruption of the *Rb* gene which results in anemia and death at midgestation due to a failure of fetal liver hematopoiesis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). While neural development was also affected in the $Rb^{-/-}$ knockout, the defect in *Nrf*-1 mutant mice appears to be restricted to the erythroid cells. Like the $Rb^{-/-}$ knockout mice, the defect here is not cell autonomous as well (Maandag *et al.*, 1994; Williams *et al.*, 1994). Indeed, the developmental potential of these progenitors appears to be normal under *in vitro* culture conditions, and homozygous mutant ES cells can efficiently contribute to the formation of blood in chimeric animals. It is interesting that a block late in hematopoietic maturation also occurs in the p45Nf-e2 knockout mice, except that the block is in the megakaryocytic lineage (Shivdasani *et al.*, 1995b).

It is possible that the failure in erythropoiesis is the result of the high proliferative pressure in this compartment during development. However, the finding that the defect here is not cell autonomous and that *Nrf*-1 is expressed in other tissues also undergoing rapid proliferative rates would argue against this possibility. The lack of cell-autonomy is consistent with an indirect role for *Nrf*-1 in erythropoiesis. It is known that blood cell formation occurs within specific microenvironments in hematopoietic tissues (Papayannopoulou and Abkowitz, 1995). Within this microenvironment, hematopoiesis occurs in close association with stromal cells and extracellular matrix proteins (ECM). The importance of cell–cell and cell–ECM interactions for erythropoiesis have been demonstrated. For example, work done *in vitro* has shown that fibronectin is necessary for the terminal differentiation of murine erythroleukemia cells (MEL) (Patel and Lodish, 1987). When attached to fibronectin coated plates, MEL cells were able to undergo maturation with enucleation of the nucleus to become reticulocytes. The subsequent finding that primitive yolk-sac derived erythrocytes were normal in fibronectin knockout mice does not necessarily refute the *in vitro* studies done with MEL cells (George *et al.*, 1993). Fibronectin mutants die early at E10 prior to fetal liver development, thus precluding analysis of definitive erythropoiesis. Moreover, an important role for VLA-4, a known fibronectin receptor, has also been shown to play an important role for erythropoiesis *in vivo (*Hamamura *et al.*, 1996).

Another function of the microenvironment is the production of cytokines by stromal cells and other accessory cells to sustain hematopoiesis (Papayannopoulou and Abkowitz, 1995). While EPO null animals show a defect in erythroid maturation, the block occurs earlier at the proerythroblast stage (Wu *et al.*, 1995). Thus, *Nrf*-1 may regulate the expression of other factors, novel or known, which have yet to demonstrate a role in erythroid maturation. While the lack of cell autonomy suggest an indirect role for *Nrf*-1 in erythropoiesis, erythroid cells also express high levels of *Nrf*-1 as well as *Nrf*-2 and p45-Nf-e2 (Chan *et al.*, 1993a; Moi *et al.*, 1994). It remains a possibility that *Nrf*-1 may also have a more direct role in erythroid cells not evident here due to compensatory functions provided by the other two CNC-bZIP proteins.

During the completion of this work, Farmer and Townes published their data on mice with a similar knockout of the *Nrf-1* (LCRF1) gene (Farmer *et al.*, 1997). Several findings are in agreement in the two knockouts. Double knockout ES cells contributed efficiently to blood formation in both mutations. Erythropoiesis appears to be normal as determined by *in vitro* differentiation assays; albeit that globin expression was decreased in the Farmer paper, this was attributable to reduced mesoderm formation in their ES differentiation and not erythropoiesis. Therefore, neither of the mutations is cell autonomous. While both mutations result in death *in utero*, the major difference, however, is that the homozygous animals derived from their mutation die at a very early stage during gastrulation, and fail to form the mesoderm layer. The basis for the phenotypic difference between the mutant alleles is not known, but there are several possibilities. First, the fact that heterozygous animals described here are normal would argue against a mutation resulting in a gain of function protein. This raises the possibility that we have generated a hypomorphic mutation. In contrast to the targeting strategy described here, their animals were generated employing a targeting construct which deleted nearly the entire gene. While a readthrough RNA transcript was detected in mutant embryos, we were not able to detect Nrf-1 protein product from our mutant allele. Moreover, we did not detect a truncated form of Nrf-1 peptide from our mutation. Thus, we conclude that our mutation is not leaky. Another possibility is that the genomic orientation of the neo gene cassette or the large deletion engineered in the mutation by Farmer *et al.* interfere with expression of a neighboring gene. Distinct phenotypes resulting from different targeting strategies have been reported for the myogenic bHLH gene *MRF4*. The differences in phenotypes resulted from influences of the different targeted allele on expression of the nearby *myf5* gene (Olson *et al.*, 1996). Alternatively, another gene may be embeded within intronic sequences of the *Nrf-1* gene, e.g. the *Evi-2* gene located within an intron of the *Nf-1* gene (Cawthon *et al.*, 1990). Finally, the difference may be attributed to different genetic backgrounds of the mice used in the studies. However, both mutations have been introduced into three different strains without any

difference in the results observed. Whether an influence as a result of the different ES lines used will require further testing. In this regard, it is curious that we were able to detect Nrf-1 expression in our ES cell (unpublished data) line in contrast to Farmer *et al.* (1997). These possibilities must be strongly considered, and may account for the lethality at day 6.5 reported by Farmer *et al.*, thus precluding analysis at later time points when the effects of the Nrf-1 mutation becomes manifest.

Several other issues remain unresolved and require further experimentation. First, as lethality occurs *in utero* (for the two mutant alleles), the requirement for Nrf-1 in adult life cannot be determined. Conditional knockouts may overcome this limitation. Secondly, downstream targets of Nrf-1 have yet to be determined, and a future challenge is to determine the molecular basis for the maturation arrest in erythroid cells in Nrf-1 deficient embryos. Fetal livers from mutant embryos would be a useful source in identifying some of the downstream targets regulated by Nrf-1. In this regard, it is interesting that we have found that several genes encoding phase-II detoxifying enzymes appear to be downregulated in Nrf-1 mutant animals (preliminary data). Both Nrf-1 and -2 have been implicated in the regulation of expression of phase-II detoxifying genes such as, NAD(P)H: quinone oxidoreductase (NQO1) and glutathione *S*-transferase (GST), which protect cells from oxidative damage and chemical carcinogens. Essential to the induction of these genes, in response to antioxidants and xenobiotics, is the antioxidant response element (ARE) whose core sequence is similar to the NFE2/AP1 binding site (Rushmore and Pickett, 1993; Xie *et al.*, 1995). While AP-1 does not appear to play a role in the induction of these genes, Nrf-1 and Nrf-2 have been shown to upregulate the human NQO1 gene promoter via the ARE sites in transfection experiments (Venugopal and Jaiswal, 1996). While this study points to the importance of Nrf-1 and -2, genetic evidence has emerged from a Nrf-2 knockout reported recently (Itoh *et al.*, 1997). Itoh *et al.* showed that homozygous mutant animals were impaired in the induction of GST and NOQ1 expression. Whether altered expression of these genes play a role in the phenotype remains to be determined. Furthermore, a role for detoxifying enzymes in erythropoiesis, and possibly contributing to the phenotype here, deserves further experimentation in the future as antioxidants have been shown to have a positive effect on erythroid colony growth (Ono and Alter, 1995). Finally, the data here show that Nrf-1 plays no role in globin gene expression. It is possible that other members of the CNC-bZIP proteins substitute for Nrf-1 in this function. Although RT–PCR failed to show a compensatory increase in expression of Nrf-2 and p45-Nf-e2 (unpublished results), redundancy provided by these factors have not been ruled out. This possibility can now be tested by generating mice missing various combinations of these three genes.

Materials and methods

Targeting of the Nrf-1 gene

Nrf-1 genomic clones were isolated from a 129/Sv mouse library using standard techniques. A mouse *Nrf-1* targeting plasmid was constructed using the pPNT vector (Tybulewicz *et al.*, 1991). The right arm was generated by cloning a 2.2 kb *Eco*RI genomic fragment encompassing the bZIP domain and the 3′ untranslated region of the *Nrf-1* gene into the *Eco*RI site of pPNT. A 4 kb *Not*I–*Xho*I genomic fragment, encompassing intronic sequences and the coding region immediately upstream of the bZIP domain, was then cloned upstream of the PGK-neo^r cassette to generate the left arm. The resulting vector has 4 kb of homology to *Nrf-1* upstream and 2.2 kb of homology downstream of the PGK-neo^r insertion. The direction of the neo transcription is opposite to that of *Nrf-1*. JM-1 ES cells (gift from Dr Roger Pedersen) were transfected with linearized targeting vector DNA. Transfected cells were cultured on feeder layers and selected in 200 μg/ml of G418 and 0.2 μM of Gancyclovir for 7 days. Double-resistant colonies were isolated and expanded for freezing and Southern analysis. From 45 clones analyzed, four homologous recombinants were identified. The targeting efficiency of selected clones is therefore ~1/10. Positive clones were injected into 3.5 day blastocysts from C57BL/6J mice and transferred into pseudopregnant (B6×DBA) recipients. Male chimeras were bred with C57BL/6J female mice. Germline transmission was determined by transmission of the dominant agouti coat color in the F_1 animals and presence of the targeted allele was screened by either Southern blot or PCR analysis on DNA isolated from tail biopsies.

PCR, Southern, Northern and Western analysis

DNA was digested with *Nsi*I for Southern blots using standard protocols. PCR genotyping were done using a set of common primers specific for sequences located 5′ and 3′ of the neo cassette (Primer 1-GACAAGATCATCAACCTGCCTGTAG, Primer 2-GCTCACTTCCTCCGGTCCTTTG) in conjunction with a primer specific for the PGK poly(A) site of the neo cassette (Primer 3-GATTAGATAAATGCCTGCTCTTTAC). The products specific for the mutant and wild type alleles are 340 and 440 bp, respectively. Reactions were carried out in 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 pmol of each of the three primers and 2.5 U of Amplitaq polymerase (Perkin Elmer). Northern blot hybridization was performed on Poly(A)$^+$ RNA prepared whole embryos using Fasttrack RNA isolation kit (Invitrogen). Northern blot hybridization was performed using standard procedures. For RT–PCR, first-strand cDNA was synthesized using random hexamer primers according to manufacturer's protocol (Pharmacia). PCR was carried out P1 and P2 primers described above. *In vitro* transcription and translation was done using TnT reticulolysate system from Promega. p60-Nrf-1 control peptide was derived from pSK517 plasmid which contained a 2.1 kb insert missing the first AUG codon of Nrf-1. Plasmid for the truncated Nrf-1-314 product was derived from truncating pSK517 at an *Eco*RI site site corresponding to the neo insertion site in the targeting vector. For protein analysis, E13.5–15.5 embryos were harvested and quick frozen. After establishing the genotype, frozen tissues were homogenized using standard protocols. Equivalent amounts of extracts were separated on 8% SDS–PAGE gel and transferred to nitrocellulose filters. Antibody incubations were done at room temperature in TBS (0.5% milk, 10 mM Tris 7.5, 150 mM NaCl, 0.5% Tween-20). Antibodies are against synthetic peptides corresponding to amino acid residue 274–293 (KGSKEKQADFLDKQMSRDEHR) of the mouse Nrf-1 protein sequence (numbering for peptides begins from the second AUG codon as we have not been able to detect a product corresponding to initaition from the first AUG codon). Visualization was done using goat anti-rabbit-HRPO conjugated antibody and the ECL detection system (Amersham).

Hematological analysis and in vitro progenitor cell assays

Blood for hematocrit determinations was collected using heparinized capillary tubes on decapitated embryos. Blood for red cell counts were collected by bleeding decapitated embryos into calcium/magnesium free phosphate buffered saline solution, and counts were done using a hemocytometer chamber. For *in vitro* colony assays, fetal livers from day 13.5 or day 14.5 embryos were used. Single cell suspensions were prepared and non-nucleated mature erythrocytes were lysed in buffered NH_4Cl solution. An aliquot of the recovered cells are counted in Trypan Blue solution to assess percentage of viable cells. Cells were seeded in triplicates at 5×10^4 per 3.5 cm dish in methylcellulose media supplemented with fetal calf serum, transferrin, insulin, recombinant mIL-3, hIL-6, mSCF and EPO. Colonies were maintained at 37°C under humidified conditions with 5% CO_2. Colonies were monitored for 7 days, CFU-E and BFU-E colonies were enumerated at 1 or 2 days and 4–7 days, respectively after benzidine staining.

GPI analysis

Blood obtained from the tail vein was diluted with water. Three cycles of freeze–thawing was done to lyse the cells completely. Supernatants were electrophoresed on cellulose acetate membranes in 100 mM Tris/

750 mM glycine (pH 8.5) running buffer for 45 min at 150 V in 4°C. GPI activities was detected by overlaying the membrane with 10 ml 0.4% agarose at 55°C containing 80 mM Tris pH 8, 5 mM magnesium acetate, 20 mg fructose 6-phosphate, 0.25 mg phenazine methosulfate, 2 mg methylthiazolium tetrazolium, 2 mg nicotinamide dinucleotide phosphate, 1.4 units glucose 6-phosphate. Incubations at room temperature was carried out in the dark for 1 min prior to photographing the GPI isoenzyme bands.

Histology

Embryos or tissues were fixed in 10% phosphate-buffered neutral formalin overnight in room temperature, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Blood smears and liver touch preparations were stained with Giemsa–Wright stain.

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