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Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor β

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ABSTRACT **Core binding factor β (CBFβ) is considered to be a transcriptional coactivator that dimerizes with transcription factors core binding factor α 1 (CBFA1), -2, and -3, and enhances DNA binding capacity of these transcription factors. CBFβ and CBFA2, which is also called acute myeloid leukemia 1 gene, are frequently involved in chromosomal translocations in human leukemia. To elucidate the function of CBFβ, mice carrying a mutation in the Cbfb locus were generated. Homozygous mutant embryos died between embryonic days 11.5–13.5 due to hemorrhage in the central nervous system. Mutant embryos had primitive erythropoiesis in yolk sac but lacked definitive hematopoiesis in fetal liver. In the yolk sac of mutant embryos, no erythroid or myeloid progenitors of definitive hematopoietic origin were detected, and the expression of flk-2/flt-3, the marker gene for early precursor cells of definitive hematopoiesis, was absent. These data suggest that Cbfb is essential for definitive hematopoiesis in liver, especially for the commitment to early hematopoietic precursor cells.**

The cytogenetic abnormality inversion 16 is the nonrandom translocation found in acute myelomonocytic leukemia with eosinophilia, and its frequency is ≈4-5% in acute myelocytic leukemia (AML) patients. Recently, the genes disrupted by inversion 16 were cloned, and one of the two genes involved was shown to be core binding factor β (CBFβ; ref. 1).

CBF was purified from the proteins binding to the conserved core site in mammalian type C retroviral enhancers (2), enhancers of T-cell receptors (3), and polyoma virus enhancer (4). It consists of two subunits, α and β (4), and the α subunits are transcription factors that have a DNA binding domain homologous to the *Drosophila* segmentation gene runt (5–7), whereas β subunit is considered to be a transcriptional coactivator that cannot bind DNA by itself (8, 9) but enhances the DNA binding capacity of the α subunits by forming heterodimers with them *in vitro* (5, 7, 10). Single β subunit and three α subunits have been cloned so far, and the α subunits are encoded by the distinct genes called Cbfa1, -2, and -3, respectively (5–7). One of the three α subunits, the human AML1 gene, which is a homologue of mouse Cbfa2, was also shown to be involved in AML with the 8;21 translocation (11), the 3;21 translocation found in myelodysplastic syndrome (12) and chronic myelocytic leukemia in blastic crisis (13), and the 12;21 translocation found in acute lymphoblastic leukemia (14).

In inversion 16, CBFβ is fused with the smooth muscle myosin heavy chain (SMMHC) gene in-frame (1), and it was shown that the encoded chimeric protein is capable of transforming NIH 3T3 cells *in vitro* (15). CBFβ–SMMHC was also shown not only to form heterodimers with α subunits via CBFβ domain, but also to form multimers by itself, via α-helical coiled-coil domain of SMMHC (16). Studies on deletion mutants of CBFβ–SMMHC revealed that both CBFβ and SMMHC domains are required for transformation of NIH 3T3 cells, suggesting that the interactions with α subunits as well as the formation of multimers are required for the transforming ability of CBFβ–SMMHC (15). Furthermore, the oncogenic property of CBFβ–SMMHC was suppressed by the overexpression of Cbfa2, suggesting that CBFβ–SMMHC interferes with the normal function of CBF (17). Therefore, it is suggested that dysregulation of CBF leads to transformation of cells.

Although *in vitro* function of Cbfb is to enhance the DNA binding capacity of the α subunits, as shown by electrophoretic mobility shift assay (8, 9), its function *in vivo* remains unknown. To elucidate the role of Cbfb *in vivo*, we generated mice in which Cbfb was knocked out by gene targeting.

MATERIALS AND METHODS

Construction of the Targeting Vector. We screened the 129/Sv mouse genomic library in the Lambda Fix II phage vector (Stratagene) with Cbfb cDNA, and obtained genomic fragments encompassing exon IV and V of Cbfb. After making a restriction map and carrying out sequencing, we disrupted exon V by replacing it with a neomycin resistant gene (neo) cassette driven by a phosphoglycerol kinase (PGK) promotor (18). We inserted a 4.5-kb *Eco*RI–*Sal*I* fragment 3′ of the Cbfb exon V and a 1.6-kb *Eco*RI–*Bgl*II fragment of PGK–neo cassette into an *Eco*RI–*Sal*I* site and an *Eco*RI–*Bam*HI site of the pBluescript SK, respectively, in which the *Ssp*I fragment was replaced with PGK–thymidine kinase cassette as described (18). To obtain the final targeting vector, we inserted a 5.8-kb *Not*I*–*Xba*I fragment 5′ of the Cbfb exon V into a *Not*I*–*Xba*I site of pBluescript SK containing 3′ of the Cbfb exon V, PGK–neo, and PGK–thymidine kinase. (Asterisk denotes a site in the cloning vector; see Fig. 1*A*.)

Generation of Mutant Mouse. The targeting vector was linearized by *Not*I digestion, electroporated into E14-1 embryonic stem (ES) cells (1×10^7), and selected with G418 (0.4 mg/ml) and gancyclovir (2 μM; refs. 19 and 20). Resistant colonies were selected, expanded, and screened for homologous recombination by Southern blot analysis, and ES cell clones heterozygous for Cbfb mutation were obtained. Independent clones were injected into blastocysts derived from C57BL6/J mice, and transferred into uteri of pseudopregnant Institute for Cancer Research (ICR) mice. Chimeric mouse were mated with C57BL6/J mice, and germ-line transmission of the ES cell-derived phenotype was assessed by coat color and further confirmed by the identification of heterozygotes using Southern blot analysis. After brother–sister mating, the

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Abbreviations: CBF, core binding factor; AML, acute myelocytic leukemia; SMMHC, smooth muscle myosin heavy chain; PGK, phosphoglycerol kinase; ES, embryonic stem; RT-PCR, reverse transcriptase–PCR; E, embryonic day.

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genotypes of embryos were identified by PCR and Southern blotting.

cDNA Synthesis and Reverse Transciptase–PCR (RT-PCR). Total RNA was prepared from fetus, yolk sac, or both together (whole embryo), at various stages of mouse development using LiCl/urea, and 1 μg of total RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) in a 20-μl reaction volume, using random hexamers. PCR amplifications were performed with GeneAmp 2400 (Perkin–Elmer) and Ampli*Taq* DNA polymerase (Perkin–Elmer), using 2.5 μl of cDNA solution in a 50-μl reaction volume. Thirty cycles of amplification were carried out (94°C for 30 sec, 50–65°C for 30 sec, and 72°C for 30–60 sec). Then 18 μl of the 50-μl reaction volume was analyzed by ethidium bromide-stained 1.5% agarose gel electrophoresis. The flk-2/flt-3 PCR product was alkaline-blotted to a nylon membrane after gel electrophoresis and hybridized with a specific probe encompassing amino acids 511–610. Primers for RT-PCR analysis were as follows: Cbfb exon IV sense primer, 5′-GCAAGGTATACTTGAAGGCT-3′; Cbfb exon VI antisense primer, 5′-TGAGATCATCACCGCCACCT-3′; Cbfa2 5′R primer, 5′-AGCAAGCTGAGGAGCGGCGA-3′; Cbfa2 3′R primer, 5′-AAACTCCCGGGCTTGGTCTG-3′; flk-2/flt-3 (21); embryonic globin ζ; εy^2 (22); and βh1(23), as described.

Methylcellulose Colony Forming Assays. Yolk sacs were obtained from embryonic day 9.5 (E9.5) embryos and dispersed completely by mechanical manipulation. Following resuspension in α minimal essential medium (αMEM; GIBCO/BRL), the viability of yolk sac hematopoietic precursor cells was examined by trypan blue staining. The cells were cultured with 20% selected fetal calf serum, methylcellulose, 2-mercaptoethanol (100 μM), and various cytokines as follows: human erythropoietin at a final concentration of 2 units/ml; human granulocyte-colony stimulating factor, 10 ng/ml; mouse granulocyte-macrophage colony stimulating factor, 100 ng/ml; human interleukin 6, 100 ng/ml; mouse stem cell factor, 100 ng/ml; and mouse interleukin 3, 500 units/ml. After incubation in a fully humidified atmosphere of 5% CO_2/95% air at 37°C for 7 days, erythroid, myeloid, and mixed colonies were counted under a dissecting microscope.

RESULTS

Disruption of Cbfb Gene Results in Embryonic Death. The Cbfb gene, which spans 50 kb and contains six exons (16, 24), does not have any known functional domains (8, 9). There are three splicing variants in Cbfb transcripts, called Cbfb1, Cbfb2, and Cbfb3 (8, 9). Cbfb2 is a full-length cDNA, including all coding exons from I to VI. Cbfb1 has a 31-bp deletion in exon V and Cbfb3 lacks the complete exon V as a result of alternative splicing (8). Cbfb1 and -2 were detected as major transcripts, whereas Cbfb3 was detected as a minor transcript (8). Because supershift of the CBF–DNA complex was observed in both CBFB1 and -2 but not in CBFB3 in electrophoretic mobility shift assay, CBFB3 was considered to have no ability to bind with CBFA (8, 9). Therefore, the deletion of exon V should abolish the function of Cbfb.

We constructed a targeting vector by replacing exon V of Cbfb with PGK–neo cassette (Fig. 1*A*), and it was electroporated into E14-1 ES cells. A total of 280 G418-resistant colonies were screened for homologous recombination by Southern blot analysis of *Spe*I-digested genomic DNA probed with the *Apa*LI–*Pst*I fragment (Fig. 1*A*), and 11 independent ES cell clones heterozygous for the Cbfb mutation were obtained. Homologous recombination was confirmed by digesting genomic DNA with *Xba*I and further probing with neo (data not shown). Five independent clones were injected into blastocysts, and three clones gave rise to chimeric mice capable of transmission of the targeted allele through the germ line.

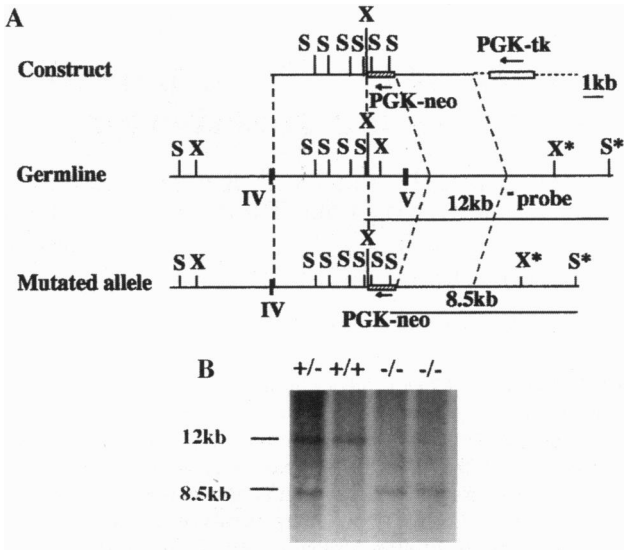

FIG. 1. Generation of mutant mice in which exon V of Cbfb was replaced by PGK–neo. (*A*) Structure of the targeting vector and partial restriction map of genomic Cbfb locus and mutated allele after homologous recombination. Exons IV and V are depicted as black boxes. Dotted line in construct represents pBluescript SK sequence. S, *Spe*I; X, *Xba*I. *Xba*I site between X* and S* was not determined. (*B*) Southern blot analysis of Cbfb fetal or yolk sac DNA. Genomic DNA was isolated from fetus or yolk sac at various stages of gestation. DNA was digested with *Spe*I and hybridized with the *Apa*LI–*Pst*I probe shown in *A*. Bands are indicated corresponding to wild-type (12-kb) and mutant (8.5-kb) genes. Representative data from littermates of E11.5 are shown.

After interbreeding of heterozygotes, we examined the genotypes of littermates at 4 weeks of age and found no homozygotes (Table 1). As we did not observe the death of mice until 4 weeks of age, Cbfb mutant homozygotes seemed to be lethal during gestation. To determine when embryonic lethality occurred, we analyzed fetuses at various stages of gestation (Table 1). The analysis of genotypes showed that all Cbfb mutant homozygotes died between E11.5 and E13.5 (Table 1).

To confirm that Cbfb is non-functional in mutant mice, we examined the expression of Cbfb in wild-type, heterozygote, and homozygote yolk sacs, fetuses, and whole embryos by RT-PCR (Fig. 2). In wild-type littermates, Cbfb was expressed in E8.5 whole embryos and in E9.5-12.5 fetuses and yolk sacs (data not shown). One major transcript and one minor transcript were detected in wild-type embryos. The major transcript is longer than the minor transcript and should corre-

Table 1. Genotype of Cbfb mice and embryos

Age	Total no. of mice	Genotype, no. of mice		
		+/+	+/−	−/−
4 weeks	41	14	27	0
E13.5	9	0	9	0
E12.5	29	7	19	3
E11.5	78	23	35	20
E10.5	78	19	41	18
E9.5	58	15	33	10
E8.5	14	4	5	5

Genomic DNA was isolated from the tail of mice at 4 weeks, and yolk sac or fetus at various stages of gestation after E9.5. Genotypes were determined by Southern blot analysis as described in Fig. 1*B*. Total RNA was isolated from the E8.5 whole embryo. Using Cbfb exon IV sense primer and exon VI antisense primer, the genotype of the E8.5 embryo was determined by RT-PCR, as shown in Fig. 2. Genotype was determined only in live fetuses, with viability ascertained by the presence of heartbeat at the time of dissection.

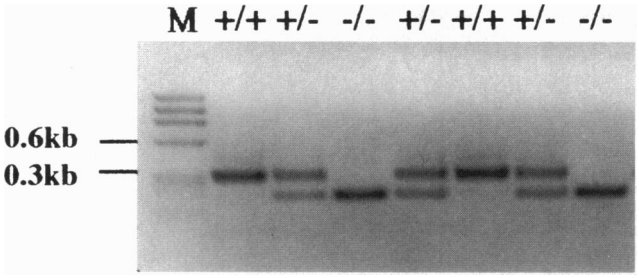

FIG. 2. RT-PCR analysis of the Cbfb transcripts. Total RNA was isolated from fetus or yolk sac after E9.5 and from the whole embryo at E8.5. PCR amplification was done for 30 cycles under the conditions of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec by using a Cbfb exon IV sense primer and an exon VI antisense primer. The lengths of the PCR products corresponding to the transcripts of Cbfb1, -2, and -3 are 276, 307, and 180 bp, respectively (8). Further analysis revealed that the long transcript in the figure contained two bands that correspond to Cbfb1 and -2 (data not shown).

spond to Cbfb1 and -2, which can dimerize with α subunits *in vitro*. The minor transcript, which was detected as a faint band, should correspond to Cbfb3, which does not bind with CBFA *in vitro* (8, 9). Both the long and short transcripts were detected in heterozygotes in similar amounts, but only the short transcript was detected in homozygotes (Fig. 2). We cloned the short transcript of homozygotes into a plasmid vector and carried out sequencing. Sequence analysis showed that exon IV was directly connected to exon VI of Cbfb, confirming that the short transcript represents Cbfb3 (data not shown). Because CBFB3 is not able to form heterodimers with the α subunits *in vitro* (8, 9), the function of Cbfb should have been abolished in the homozygotes .

Cbfb Knockout Mice Showed Hemorrhages in the Central Nervous System. Homozygotes appeared normal at E10.5, but intracranial hemorrhage and sometimes hemorrhage along the spinal cord were observed in most E11.5-12.5 homozygotes (Fig. 3*A*). The bodies and yolk sacs were pale compared with heterozygote littermates (Fig. 3*B*) and, at E11.5, livers could not be distinguished because of their pallor (Fig. 3*A*), suggesting severe anemia.

When E11.5 homozygote mice were serially sectioned, hemorrhage was seen in midbrain parenchyma (Fig. 4 *A* and *B*), forebrain ventricle (Fig. 4*C*), spinal canal (Fig. 4*D*), and the connective tissue of the head and below the spine above the second pharyngeal pouch. Moreover, hemorrhage was also observed in peripheral ganglia, such as dorsal root ganglia, and in the facio-acoustic ganglia of the head (Fig. 4 *E* and *F*). The ventricle and spinal canal were filled with nucleated red blood cells (Fig. 4*G*), which were thought to be generated during primitive hematopoiesis in yolk sac.

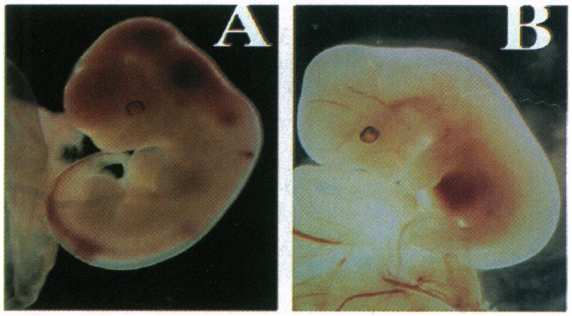

FIG. 3. Gross appearance of E11.5 homozygote fetus showing intracranial and spinal hemorrhage (*A*), which are not observed in the heterozygotes (*B*). The homozygote fetus is pale, and its liver cannot be discriminated because of its pallor.

Definitive Hematopoiesis in Fetal Liver Is Absent in Cbfb Homozygotes. The major organ of hematopoiesis is considered to shift from yolk sac to fetal liver at about E11.5-12.5 (25). When the livers of heterozygotes and homozygotes at E11.5 and E12.5 were serially sectioned and analyzed, few hematopoietic cells were observed in the sinusoids of the liver of homozygotes (Fig. 4*H*), in contrast to the livers of heterozygotes, where abundant hematopoietic cells were observed (Fig. 4*I*). Furthermore, no nonnucleated red blood cells, the presence of which would be a sign of definitive hematopoiesis, were observed in the liver sinusoids of homozygotes.

To detect the presence of definitive hematopoietic precursor cells in the yolk sac, methylcellulose colony forming assay was performed using E9.5 yolk sac (Fig. 5). Whereas abundant hematopoietic colonies were observed in the yolk sacs of wild-type and heterozygote littermates, no hematopoietic colonies were observed in homozygotes, including erythroid and myeloid lineages.

Further, we examined flk-2/flt-3 expression in the yolk sac by RT-PCR (Fig. 6). flk-2/flt-3 is a type III receptor tyrosine kinase that is preferentially expressed in early definitive hematopoietic precursor cells in fetal liver (26). In the yolk sacs of heterozygotes, flk-2/flt-3 expression increased through E9.5-11.5, then decreased at E12.5. However, it was absent in E9.5-12.5 yolk sacs of homozygotes (Fig. 6). Because flk-2/flt-3 expression is limited to early definitive hematopoietic precursor cells, including hematopoietic stem cells in the fetus, and is not expressed in lineage-committed precursor cells (26), our results indicate that hematopoietic precursor cells of definitive hematopoiesis are absent in the yolk sac of homozygotes.

To confirm that primitive hematopoiesis is intact in the yolk sac beginning at E7.5-8.5 (25), RT-PCR analysis of embryonic globin genes, ζ, εy^2, and $\beta h1$ was performed (22, 23, 27). All of these genes were expressed in E8.5 whole embryos and E9.5-12.5 yolk sacs of homozygotes, and there were no differences in expression compared with heterozygotes (data not shown). It is also clear that embryonic nucleated red blood cells were present in hemorrhagic regions of homozygotes. Thus, primitive hematopoiesis in Cbfb mutant homozygotes seemed to be normal.

On the other hand, adult heterozygotes appeared normal and showed no significant differences in the number of red blood cells and white blood cells, or in the appearance of terminally differentiated white blood cells in the periphery, when compared with wild-type littermates at 6–7 weeks of age. Furthermore, there were no significant differences in flow cytometric analysis of thymus, spleen, bone marrow, and lymph node between wild-type littermates and heterozygotes at 8 weeks of age (data not shown).

DISCUSSION

We generated Cbfb knockout mice, in which only Cbfb3 that cannot form heterodimers with the α subunits *in vitro* was transcribed. Cbfb knockout mice were lethal owing to intracranial hemorrhage. The mutant mice lacked definitive hematopoiesis in liver, and we could not detect early precursor cells of definitive hematopoiesis in yolk sac.

The phenotype of Cbfb knockout mice was similar to that of Cbfa2/AML1 knockout mice generated by us (unpublished data) and other investigators (28, 29), which resulted in embryonic death with brain hemorrhage and lack of fetal liver hematopoiesis. As Cbfa2 forms a heterodimer with Cbfb *in vitro* (10), Cbfa2 may be unable to function properly without forming a heterodimer with Cbfb *in vivo*. RT-PCR analysis demonstrated that Cbfa2 was expressed at similar levels in homozygotes of Cbfb mutant embryos as well as heterozygotes and wild-type embryos (data not shown). It is possible that

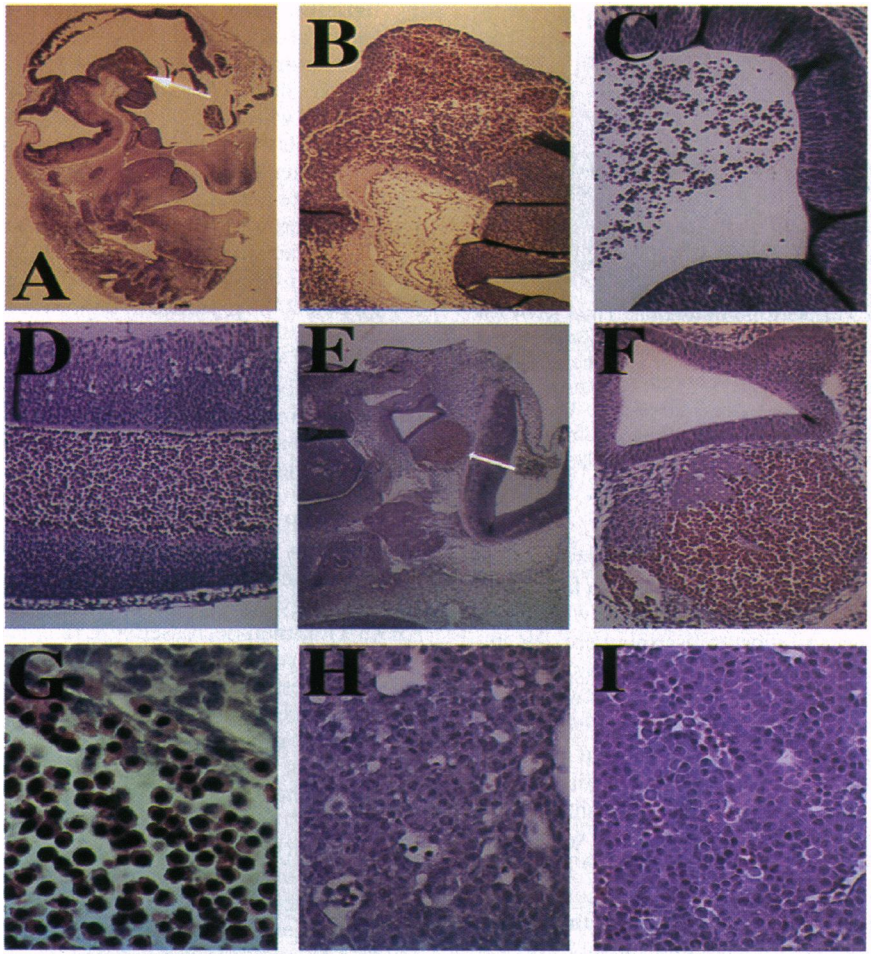

FIG. 4. Histological analysis of Cbfb mutants at E11.5. (*A*) Homozygote; hemorrhage in parenchyma of the midbrain. (×5.) (*B*) Higher magnification of midbrain hemorrhage in homozygote. (×25.) (*C*) Homozygote; hemorrhage in forebrain ventricle. (×25.) (*D*) Homozygote; hemorrhage fills the spinal canal in lumbar area. (×50.) (*E*) Homozygote; hemorrhage in the facio-acoustic ganglion complex, with adjacent otic vesicle. (×10.) (*F*) Higher magnification of hemorrhage in the facio-acoustic ganglion complex. (×50.) (*G*) Homozygote; nucleated red blood cells in the spinal canal adjacent to the spinal cord. (×400.) (*H*) Homozygote; liver showing very little hematopoiesis. (×100.) (*I*) Heterozygote; the liver shows more hematopoiesis in sinusoids than homozygote liver. (×100.)

Cbfb does not only enhance the DNA binding capacity of Cbfa2 but is also essential for its function *in vivo*.

Cbfb was also shown to form heterodimers with Cbfa1 and -3 *in vitro* (5, 7), and it is possible that Cbfa1 and -3 are unable to accomplish their function in Cbfb knockout mice. An explanation as to why the phenotype of Cbfb knockout mice resembles that of Cbfa2 knockout mice must await the outcome of studies on Cbfa1 and Cbfa3 knockout mice. One possibility, however, is that Cbfa2 may be the earliest of the three α subunits to be required during mouse development; Cbfa2 has been shown by *in situ* hybridization to be expressed in the fetus for the first time at E9.2 (30).

Intracranial hemorrhage penetrating the brain ventricles and spinal canal is one of the most prominent pathological features observed in Cbfb and Cbfa2 knockout mice. Two

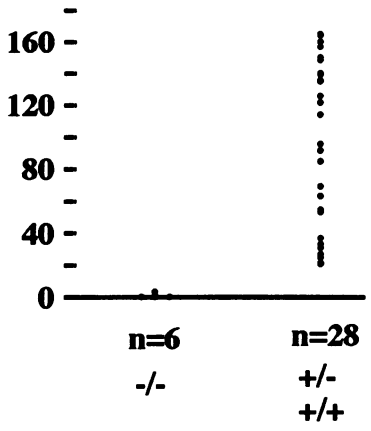

FIG. 5. Methylcellulose colony forming assay of yolk sac at E9.5. In normal littermates and heterozygotes, abundant colonies were observed in E9.5 yolk sac. However, no hematopoietic colonies were detected in homozygotes.

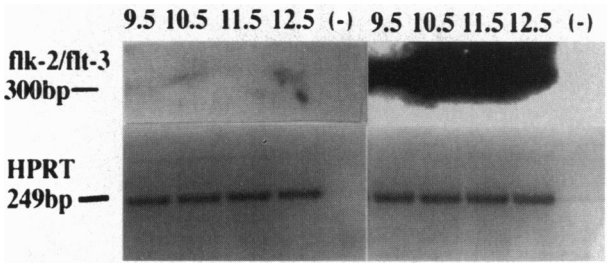

FIG. 6. RT-PCR analysis of flk-2/flt-3 in yolk sacs. In heterozygotes, flk-2/flt-3 expression increased from E9.5 to E11.5, then decreased at E12.5 (*right*). In contrast, no flk-2/flt-3 expression was observed in the yolk sacs of Cbfb homozygotes from E9.5 to E12.5 (*left*).

hypotheses have been put forward to explain the cause of intracranial hemorrhage in Cbfa2 knockout mice (28, 29). The first is a lack of platelets, since no platelets were observed in the peripheral blood of E12.5 homozygotes (28). However, this hypothesis is likely to be untenable because no similar cerebral hemorrhages were found in c-*myb* knockout mice, which lack definitive hematopoiesis in fetal liver and die by E15 (31), or in NF-E2 knockout mice, which have no platelets due to a disturbance in platelet development from megakaryocytes, yet have no cranial hemorrhage and live until the perinatal period (32), as described (29). The second hypothesis is that cellular necrosis found in endothelial cells in the central nervous system and neural crest cells at E10.5 precede hemorrhage (29). Although we found cells with nuclear fragmentation in the central nervous system of homozygote as well as in wild-type and heterozygote littermates of either Cbfb or Cbfa2 knockout mice, cellular necrosis did not seem to precede hemorrhage (data not shown). Further examination is necessary to clarify the reason for the central nervous system hemorrhage of Cbfb and Cbfa2 knockout mice.

flk-2/flt-3 is a type III receptor tyrosine kinase cloned from hematopoietic precursor cells in fetal liver (26) and adult testis (33). It is preferentially expressed in early hematopoietic precursor cells of fetal liver during embryonic definitive hematopoiesis; it is not expressed when they are committed to lineages (26). The flk-2/flt-3-positive population can reconstitute the bone marrow of lethally irradiated mice, but not all of the hematopoietic stem cells reside in the flk-2/flt-3-positive population, suggesting flk-2/flt-3 is not expressed in hematopoietic stem cells in G_0 phase of the cell cycle (34). Although flk-2/flt-3 expression was detected not only in fetal liver but also in fetal brain, the length of the transcript from brain was different from that of fetal liver (26), and flk-2/flt-3 expression was not recognized in other fetal tissues (26, 33). In the yolk sac of Cbfb mutant heterozygotes, flk-2/flt-3 expression increased from E9.5, reaching a maximum at E11.5, then decreased at E12.5, when primitive hematopoiesis in yolk sac ends and definitive hematopoiesis in fetal liver begins (Fig. 6). However, in homozygotes, flk-2/flt-3 expression was absent from E9.5 to E12.5 (Fig. 6), when both primitive and definitive hematopoietic precursor cells should be present in the yolk sac. Therefore, our data show that flk-2/flt-3 is expressed in definitive precursor cells but not in primitive precursor cells residing in the yolk sac because of the presence of primitive hematopoiesis in Cbfb mutant homozygotes, and early precursor cells of definitive hematopoiesis are absent in yolk sac of homozygotes. Thus, it was suggested that Cbfb is essential for the commitment to early hematopoietic precursor cells in yolk sac.

Definitive hematopoietic stem cells are assumed to colonize fetal liver both from yolk sac and AGM (aorta, gonad, mesonephros) region (25, 35). It is possible that homozygotes also lack hematopoietic precursor cells derived from the AGM region. RT-PCR analysis was performed on flk-2/flt-3 expression from RNA of the whole body from E9.5 to E12.5. Although the expression in homozygotes was greatly reduced in comparison with heterozygotes, we could not conclude that homozygotes of Cbfb mutant mice also lack hematopoietic stem cells of the AGM origin because of flk-2/flt-3 expression in nervous system (data not shown).

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