B-myb Is Required for Inner Cell Mass Formation at an Early Stage of Development*

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Yasunori Tanaka‡§, Nikos P. Patetos†‡, Toshio Maekawa‡**, and Shunsuke Ishii***‡‡

From the ‡Laboratory of Molecular Genetics, RIKEN Tsukuba Life Sciences Center, and the **Core Research for Evolutional Science and Technology (CREST) Project, Japan Science and Technology Agency, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan

The myb gene family has three members, c-myb, A-myb, and B-myb, which have distinct expression patterns. Analyses of c-myb and A-myb mutant mice have indicated that c-myb and A-myb are important for hematopoiesis and spermatogenesis, respectively. However, there has been no evidence for a role for B-myb in development. To examine the role of B-myb in development, we generated B-myb-deficient mice by gene targeting. Although the heterozygous mutants were healthy, the homozygous mutants died at an early stage of development, around E4.5-E6.5. Consistent with the important role of B-myb in development, around E4.5-E6.5.

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The c-myb proto-oncogene is the cellular progenitor of the v-myb oncoproteins carried by the chicken retroviruses AMV and E26, which transform myelomonocytic hematopoietic cells (1, 2). The mammalian myb gene family contains two other members, A-myb and B-myb, in addition to c-myb (3). These three members display notable homology toward each other in three regions: the N-terminal DNA-binding domain and two regions in the C-terminal region (3, 4). The DNA-binding domain in the N-terminal region of the myb protein (Myb) consists of three imperfect tandem repeats of 51–52 amino acids, each of which contains a helix-turn-helix variation motif (4–7). The second and third repeats are closely packed into the major groove of DNA, and the two recognition helices in these repeats bind to the specific base sequence, AACNG, cooperatively (8, 9).

In addition to the N-terminal DNA-binding domain, c-Myb has two functional domains responsible for transcriptional activation and negative regulation, respectively (4). The transcriptional activation domain of c-Myb, which is rich in acidic amino acids, is adjacent to the DNA-binding domain. Deletion of the negative regulatory domain (NRD), located in the carboxyl-proximal portion of the molecule, increases both transcription and transformation capacities, implying that this domain normally represses c-Myb activity (4, 10, 11). A-Myb has a very similar functional domain structure to c-Myb and is the strongest transcriptional activator among the three members of the myb gene family (12–15). Compared with c-Myb and A-Myb, B-Myb is a weaker transcriptional activator (16–18). Although B-Myb has an acidic transcriptional activation domain located downstream of the DNA-binding domain, the C-terminal portion appears to be the region required for transcriptional activation (19, 20). One possible regulatory mechanism mediated by the C-terminal portion is phosphorylation by cyclin A-dependent kinase. CyclinA/Cdk2 phosphorolyses the C-terminal region of B-Myb and stimulates its trans-activation capacity (21–24).

Although the three members of myb gene family recognize the same DNA sequence, they have distinct expression patterns. The level of c-myb expression is predominantly, although not exclusively, high in immature hematopoietic cells, and its expression is turned off during terminal differentiation (25–27). Constitutive expression of a transfected c-myb gene blocks the induced differentiation of immature erythroid cells (28), and a block of c-myb expression reduces the proliferation of hematopoietic precursor cells (29). Furthermore, homozygous c-myb mutant mice are severely anemic and die in utero because of defective fetal hematopoiesis (30). In addition to the important role of c-myb in hematopoietic progenitor cells, c-myb is also critical for the mitogen-induced proliferation of certain types of cells such as T lymphocytes (31–33). Unlike c-myb, A-myb is highly expressed in the developing central nervous system, male germ cells, female breast ductal epithelium, and B lymphocytes (34). Consistent with its expression in adult testis and female breast, A-myb-deficient males are infertile because of a block in spermatogenesis, and null A-myb females show underdevelopment of breast tissue following pregnancy (35).

In contrast to the restricted expression of c-myb and A-myb, the cell-type specificity of B-myb expression is broader in both adult tissues and embryos (3, 36). Like that of c-myb, the expression of B-myb correlates with cellular proliferation. B-myb mRNA is not expressed in resting cells but is induced late in G1 phase, and the resulting high levels are maintained through S phase (37, 38). B-myb mRNA levels decrease when HL-60 or U937 cells are induced to differentiate (38, 39). Inhibition of B-myb expression by the introduction of a B-myb antisense construct diminished the cell proliferation of hematopoietic cells and fibroblasts, but constitutive expression of B-myb induced a transformed phenotype (39, 40). However, there has been no indication of a role for B-myb in development.

To understand the physiological role of B-myb, we have generated B-myb-deficient mice. Analysis of B-myb-deficient embryos indicates that B-myb is essential for inner cell mass (ICM) formation in an early stage of development.

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† Supported by the Special Researcher’s Basic Science Program.
‡‡ To whom correspondence should be addressed. Tel.: 81-298-36-9031; Fax: 81-298-36-9030; E-mail: sishii@rtc.riken.go.jp.

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‡‡‡ The abbreviations used are: ICM, inner cell mass; bp, base pair(s); PCR, polymerase chain reaction.
### Materials and Methods

**Construction of the Targeting Vector**—The mouse B-myb genomic clones were isolated from a library derived from C57BL/6 mice by the standard plaque hybridization procedure. A 15.0-kb genomic DNA sub-fragment which contains the five exons encoding amino acids 8–200 was used to generate the targeting vector. The BgII fragment containing the exon encoding amino acids 94–167 was replaced by a neomycin (neo) cassette driven by the phosphoglycerate kinase gene promoter. To increase the frequency of gene targeting, the DT-A (diphtheria toxin polypeptide A) signal cassette for negative selection was fused to the short arm as described previously (41).

**Generation of B-myb-deficient Mutant Mice**—The ES cells used were TT2 cells which had been derived from an F1 embryo resulting from a cross between C57BL/6 and CBA mice (42). The NotI-linearized targeting vector (100 μg) was electroporated into 1.0 × 10^6 TT2 cells. Targeted clones were selected after 7–10 days growth in the presence of G418 (150 μg/ml) and were then expanded in duplicate 24-well plates. The homologous nature of the recombination was confirmed by Southern blot analysis using several restriction enzymes and several probes located either inside or outside the targeting vector. In addition, three different primers were used to amplify a 175-bp fragment from the wild-type allele or a 236-bp fragment from the mutant allele. Chimeras were produced by injecting about ten ES cells into 40 ICR 8-cell embryos and transplanting the embryos into the uterus of pseudopregnant females as described (43). Six- to eight-week-old male progeny with a high degree of chimerism were derived from three clones and were bred with C57BL/6 females to produce heterozygous mice capable of transmitting the targeted allele through the germ line. The mice were maintained by the Division of Experimental Animal Research, RIKEN.

**Genotyping of ES Cells, Embryos, and Animals**—Genomic DNA was isolated from cultured ES cells, embryos, and tail clippings by digestion overnight at 55 °C in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K) followed by phenol-chloroform extraction, and ethanol precipitation. For Southern blot analysis, genomic DNA (about 20 μg) was digested with EcoRI and resolved on 0.8% agarose gels. For genotyping of cultured blastocysts, genomic DNA was isolated from yolk sacs by digestion for 60 min at 55 °C in lysis buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl_2, 0.5% Tween 20, 0.5% Nonidet P-40, 0.5 mg/ml proteinase K), and then at 94 °C for 10 min to inactivate proteinase K just prior to PCR analysis. PCR was carried using two of the following primers: forward B-myb primer, 5'-TGCCCTGTGTCGATGACCACTGTTGAT3'-; reverse B-myb primer, 5'-GCTGACGCTCATTAGTCTGTTACTGTTTGAT3'-; neo primer, 5'-CGTACGGGTAGTCTGGAATGTGTGGACG3'-; EcoRI probes (Fig. 1A, B and C). Conditions for PCR were 94 °C for 45 s, 55 °C for 25 s, and 72 °C for 3 min for 30 cycles.

**Detection of B-Myb Protein**—Splenocytes were disrupted in the hypotonic buffer (10 mM Hepes, pH 7.4, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM dithiothreitol) using Dounce homogenizer, and the nuclei were prepared by centrifugation. The nuclear extract was prepared by incubation of the nuclei with the extraction buffer (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl_2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). Western blots were performed using the anti-B-Myb polyclonal antibody (19) and ECL detection reagents (Amersham Pharmacia Biotech).

**Blastoctyst Outgrowth Cultures**—Blastocysts from heterozygous and control mating were flushed from the uterus on 3.5 days post-coitus as described by Hogan et al. (44). Blastocysts were grown on 4-well culture plates (Nunc) coated with 0.1% gelatin in CMRL1066 medium (Life Technologies, Inc.) supplemented with 15% fetal calf serum, 1 mM L-glutamine, and 1 mM sodium pyruvate at 37 °C in 5% CO_2 incubator as described by Gonda and Hsu (45) and Wu et al. (46). Embryo morphology was observed each day, and after 5 days blastocyst outgrowths were photographed. Cells were lysed off the bottom of the well, and their genotypes were determined by PCR as described above.

### Results

The B-myb mutant mice were generated by homologous recombination in TT-2 ES cells. Exon 5 encoding amino acids 94–167 of B-Myb, which corresponds to a part of repeats 2 and 3 required for binding to the Myb-recognition sequence, was replaced with a neo cassette in the gene-targeting vector (Fig. 1A). Therefore, removal of this exon should lead to a complete loss of DNA-binding activity of B-Myb. Homologous recombinants were characterized by the appearance of a 7.0-kb EcoRI fragment with the 3'-probe and a 236-bp PCR-amplified fragment with the B-myb forward primer and neo primer (Fig. 1, B and C). Chimeras were normally obtained from two independent mutant ES clones and mated with C57BL/6 females to generate F1 heterozygous mutant mice. Heterozygous (B-myb+/−) mice were identified by Southern blot and PCR analysis (Fig. 1B). Western blot analysis showed that the expression level of the 92-kDa B-Myb protein in spleen was similar between the wild-type and the B-myb+/− mice (Fig. 1C). This was confirmed by using the three different anti-B-Myb antibodies (data not shown). This suggests that a compensatory mechanism plays a role in maintaining the level of B-Myb protein. Similar observations were reported so far in other genes (47). The B-Myb protein fragment lacking the DNA-binding domain encoded by the mutated allele, which can act as a dominant negative form, was not detected in the B-myb+/− mice. 

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**FIG. 1.** Generation of B-myb-deficient mice. A, diagrammatic representations of the B-myb locus and targeting construct. Five exons are shown. Exon 1 encodes the initiating methionine, and exons 2–6 encode amino acids 8–32, 39–62, 63–93, 94–167, and 168–200, respectively. The BgII fragment containing exon 5 was replaced by the neomycin (neo) cassette and is flanked by 9.0- and 2.5-kilobase homologous sequences on its 5' and 3' side, respectively. The location of the probe used for Southern blot analyses is given along with the expected sizes of the hybridizing fragments. Bg, BgII; E, EcoRI; EV, EcoRV; BSK, pBlueScript II SK vector. B, genomic Southern blots (B) and PCR (C) analyses of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant mice. Genomic DNAs were isolated from the tails of mice and digested with EcoRI for Southern blot analysis. C, immunodetection of B-Myb protein. Indicated amounts of nuclear extracts from wild-type (+/+ or B-myb+/−) or B-myb−/− (+/−) mouse spleen were used for Western blotting with anti-B-Myb antibody.
mice (Fig. 1C). Heterozygous males and females were phenotypically normal and fertile.

Heterozygous mice were intercrossed, and the genotypes of resulting 3-weeks-old offspring were determined. Among 212 offspring derived from two independent germ-line chimeras, no B-myb−/− homozygotes were detected, and the ratio of B-myb+/− to B-myb−/− genotypes was 1:2 (data not shown). The fact that the ratio of B-myb+/− and B-myb−/− genotype in the progeny population of the cross of B-myb+/− and B-myb+/− (either male or female) was 1:1 indicated that B-myb−/− germ cells were not eliminated during spermatogenesis or oogenesis. Because 98 of the progeny were from intercrosses between two independent ES cell line-derived heterozygotes, the lack of B-myb-null progeny could not have been because of homozygosity of an undetected linked mutation inadvertently generated in either of the targeted ES cell lines. We conclude that homozygosity for the B-myb null allele is lethal during embryonic development. B-myb is expressed at similar levels during all stages of embryogenesis (36). As many of these expression sites may be required for development to birth, we examined embryos of heterozygotes intercrosses on different days of gestation (Table I). Among 32 phenotypically normal embryo day 3.5 (3.5 days post-coitus) blastocyst, 6 (19%) were homozygous for the mutant allele, indicating that B-myb is not essential for embryogenesis before uterine implantation. At embryo day 6.5–7.5, all decidua were externally indistinguishable, but dissection revealed that 8 of 51 (16%) implantation sites lacked discernible embryos, yolk sacs, and ectoplacental cones. Of 65 phenotypically normal embryo day 6.5–7.5 embryos subjected to genotyping, none were homozygous mutants. Consistent with this, none of the phenotypically normal embryo day 6.5–10.5 embryos were homozygous mutants, whereas an additional nine implantation sites (12% of the total) were undergoing resorption. The high frequency of abortive postimplantation development and lack of older B-myb null embryos indicate that B-myb null embryos degenerate shortly after uterine implantation.

Because B-myb is expressed in both the preimplantation embryos and maternal deciduim (36), the abortive development of B-myb mutant embryos could result from defects intrinsic to the embryo or from a breakdown in the signaling between the embryo and the uterus. To distinguish these two possibilities, embryo day 3.5-blastocysts isolated from heterozygotes intercrosses were grown in vitro, examined for growth abnormalities, and subsequently genotyped. By optimizing growth conditions, blastocysts grown in vitro develop to the egg cylinder stage and can even be taken to the 8-somite stage (45, 46). The series of developmental changes that occur during this process have been clearly delineated. Forty-three blastocysts were individually cultured for 5 days and then photographed and lysed for genotyping. Mutant homozygotes, heterozygotes, and wild-type embryos were indistinguishable as embryo day 3.5-blastocysts, attached blastocysts, or lipid vesicle-containing attached blastocysts (Fig. 2, A–C), indicating that the B-myb mutation does not affect development prior to implantation. After 5 days in culture, all the cultured blastocysts of each genotype (B-myb+/−, B-myb−/−, and B-myb+/−) gave rise to an adherent sheet of trophoderm-like cells, including trophoblastic giant cells (Fig. 2, D–F). However, B-myb−/− blastocysts showed severely impaired proliferation of the ICM. As summarized in Fig. 2G, the null genotype was never associated with robust outgrowths of the ICM (as in Fig. 2, D and E) and was the only genotype associated with undetectable ICM outgrowth (as in Fig. 2F). The developmental defects of B-myb null embryos in vivo and in vitro demonstrate that B-myb is essential for growth of the ICM in the postimplantation phase of development.

For the purpose of generating chimeras using B-myb−/− ES cells, we tried to generate B-myb−/− homzygous mutant ES cells by increasing G418 concentration but failed. These results suggest that B-myb is essential for the proliferation of ES cells. We examined the expression of the three members of myb gene family in ES cells by Northern blotting. The 2.6-kb B-myb mRNA was highly expressed in ES cells, whereas neither the c-myb nor A-myb mRNAs was detected (Fig. 3). Thus, only B-myb among the three members of myb gene family is expressed in ES cells, which is consistent with the speculation that B-myb is required for ES cell propagation.

### Table I

**Genotypes of litters from heterozygous intercross**

| Embryo day | +/+ | +/- | –/– | Resorption |
|------------|-----|-----|-----|------------|
| 8.5–10.5   | 21  | 44  | 0   | 9          |
| 6.5–7.5    | 16  | 27  | 0   | 8          |
| 3.5 (blastocyst) | 9   | 17  | 6   |            |

**TABLE I**

**Role of B-myb in Development**

**FIG. 2. In vitro outgrowth cultures of intercross embryos.** Blastocysts were flushed out of the uterus of a B-myb+/− female 3.5 days after mating with a B-myb+/− male and were grown in vitro. Embryo day 3.5 wild-type (A), heterozygous (B), and homozygous (C) blastocysts appear normal. Outgrowth of polyploid trophoderm (TE) cells was observed in all the cultures of wild-type (D), heterozygous (E), and homozygous (F) blastocysts. A well developed ICM is evident in the wild-type and heterozygous cultures and is composed of an outer primitive endoderm layer and an inner core ectoderm cells. No distinct ICM is seen in the homozygous culture. G, histogram summary of blastocyst outgrowth. The number of blastocysts with each genotype is shown by a bar graph.
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