into the mechanisms involved in these processes.

REFERENCES AND NOTES

1. L. M. Kemp, S. G. Sedgwick, P. A. Jeggo, *Mutat. Res.* **132**, 189 (1984); A. Giaccia, R. Weinstein, J. Hu, T. D. Stamato, *Somatic Cell Mol. Genet.* **11**, 485 (1985); G. F. Whitmore, A. J. Varghese, S. Guylas, *Int. J. Radiat. Biol.* **56**, 657 (1989); G. M. Fulop and R. A. Phillips, *Nature* **374**, 479 (1990); K. A. Biedermann, J. Sun, A. J. Giaccia, L. M. Tosto, J. M. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1394 (1991); E. A. Hendrickson *et al.*, ibid, p. 4061.
2. P. A. Jeggo, J. Tesmer, D. J. Chen, *Mutat. Res.* **254**, 125 (1991).
3. P. A. Jeggo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6423 (1992).
4. J. Thacker and R. E. Wilkinson, *Mutat. Res.* **254**, 135 (1991).
5. A. J. Giaccia *et al.*, Am. J. Hum. Genet. **47**, 459 (1990); M. Itoh *et al.*, Radiat. Res. **134**, 364 (1993); K. Komatsu, T. Ohta, Y. Jinno, N. Nikawa, Y. Okumura, *Hum. Mol. Genet.* **7**, 1031 (1993); C. U. Kirchgessner *et al.*, Cancer Res. **53**, 6011 (1993).
6. G. E. Taccioli, H.-L. Cheng, A. J. Varghese, G. Whitmore, F. W. Alt, *J. Biol. Chem.* **269**, 7439 (1994).
7. F. W. Alt *et al.*, Immunol. Today **13**, 306 (1992); M. Gellert, *Annu. Rev. Genet.* **22**, 425 (1992).
8. F. Pergola, M. Z. Zdzienicka, M. R. Lieber, *Mol. Cell. Biol.* **13**, 3464 (1993).
9. B. A. Malynn *et al.*, Cell **54**, 453 (1988); M. R. Lieber *et al.*, ibid. **55**, 7 (1988); T. K. Blackwell *et al.*, EMBO J. **8**, 735 (1989).
10. G. E. Taccioli *et al.*, Science **260**, 207 (1993).
11. C.-L. Hsieh *et al.*, *J. Biol. Chem.* **268**, 20105 (1993); J. Thacker *et al.*, Mutagenesis **9**, 163 (1994).
12. P. A. Jeggo and L. M. Kemp, *Mutat. Res.* **112**, 313 (1983); M. Z. Zdzienicka, Q. Tran, C. P. Van Der Schans, J. W. I. M. Simons, *ibid.* **194**, 239 (1988).
13. M. Hafezparast *et al.*, Somatic Cell Mol. Genet. **19**, 413 (1993).
14. Q.-Q. Cai *et al.*, Cytogenet. Cell Genet. **65**, 221 (1994).
15. T. Mimori, J. A. Hardin, J. A. Steitz, *J. Biol. Chem.* **261**, 2274 (1986).
16. E. de Vries *et al.*, *J. Mol. Biol.* **208**, 65 (1989); W. Zhang and M. Yaneva, *Biochem. Biophys. Res.* **186**, 574 (1992).
17. XR-V15B/H22 (D2) is a complementing hybrid that contains two closely located but independent regions of chromosome 2, mapping to 2q36 (including the marker GNT1) and to 2q33-35 (including the marker TNP-1 (*13*). Subclones derived from D2 segregated the markers GNT1 and TNP-1 independently, and in every subclone examined (more than 20), radioresistance and ability to carry out V(D)J recombination cosegregated with the TNP-1 marker. D2-X-3 is a complementing subclone retaining both fragments. D2-X-5 is noncomplementing and contains only the GNT1 fragment. D2-X-38 is complementing and contains only the TNP-1 fragment. D2-X-38D is a noncomplementing derivative of D2-X-38 and does not contain detectable human sequences. Microcell transfer hybrids H22 and H27 were isolated and characterized previously (*13*). H22, a complementing hybrid, contains the distal third of chromosome 2, including GNT1 and TNP-1; H27, a noncomplementing hybrid, contains three small fragments, including GNT1 but excluding TNP-1. Other microsatellite markers present in D2, D2-X-3, and D2-X-38 are D2S301, D2S164, and D2S137. Markers analyzed and absent in all D2 hybrids are D2S143, D2S128, D2S334, D2S371, D2S317, D2S155, D2S154, D2S157, D2S153, D2S173, D2S163, D2S120, and those listed in (*13*). Primer sequences for the Ku80 3′ UTR are 5′-ACATCACAAGGGCTGCAACTGTCA-3′ and 5′-TCGCTGTGATGCTGGGAGTTCTAA-3′, and PCR conditions were as described for D2S137 (*13*).
18. W. K. Rathmell and G. Chu, *Mol. Cell. Biol.* **14**, 4741 (1994); R. C. Getts and T. D. Stamato, *J. Biol. Chem.* **269**, 15981 (1994).
19. T. M. Gottlieb and S. P. Jackson, unpublished data.
20. J. E. Hesse *et al.*, *Cell* **49**, 775 (1987).
21. M. A. Oettinger *et al.*, *Science* **248**, 1517 (1990).
22. The V(D)J recombination assay was done as described (*10*). Substrates pJH200 and pJH290 allowed the analysis of RS and coding joins, respectively (*20*). The percentage of V(D)J recombination was measured by the ratio of rearranged substrate (based on gain of chloramphenicol resistance, Cam^R) to the total amount of recovered substrate (based on ampicillin resistance, Amp^R). The accuracy of join formation and the junction modifications were analyzed by specific restriction endonuclease digestion or by direct DNA sequencing as described (*10*) clones were analyzed. A minimum of 20 clones were analyzed, and 100 for *xrs-6* samples containing Ku80. The results of complementation of *xrs-6* with Ku80 were reproducible in three independent experiments.
23. A 2200–Base pair sequence containing the Ku80 ORF was cloned into the expression vectors pcDNA1/Amp and p220LTR, where it was under the control of the cytomegalovirus and Rous sarcoma virus long terminal repeat (LTR) promoters, respectively. The p220LTR vector also contained a hygromycin gene (*hyg*), which is a dominant selectable marker in mammalian cells.
24. D. S. Thaler, M. M. Stahl, F. W. Stahl, *EMBO J.* **6**, 3171 (1987); J. E. Akroyd, E. Clayson, N. P. Higgins, *Nucleic Acids Res.* **14**, 6901 (1986).
25. W. K. Rathmell and G. Chu, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7623 (1994).
26. T. M. Gottlieb and S. P. Jackson, *Cell* **72**, 131 (1993); A. Dvir *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11920 (1992).
27. C. W. Anderson and S. P. Lees-Miller, *Crit. Rev. Eukaryotic Gene Expression* **2**, 283 (1992); N. Finnie, T. Gottlieb, K. Hartley, S. P. Jackson, *Biochem. Soc. Trans.* **21**, 930 (1993); S. J. Froelich-Ammon, K. C. Gale, N. Osheroff, *J. Biol. Chem.* **269**, 7719 (1994).
28. P. A. Jeggo and J. Smith-Ravin, *Mutat. Res.* **218**, 75 (1989).
29. M. W. Knuth *et al.*, *J. Biol. Chem.* **265**, 17911 (1990).
30. H. R. Scholer *et al.*, *EMBO J.* **8**, 2543 (1989).
31. R. Pruzan, P. K. Chatterjee, S. J. Flint, *Nucleic Acids Res.* **20**, 5705 (1992).
32. J. J. Lafaille *et al.*, *Cell* **59**, 859 (1989).

33. We thank A. Kuhn for purified mouse Ku; M. Knuth, N. Thompson, R. Burgess, and J. Flint for mAbs; and W. Reeves for Ku80 cDNA. The substrates of pJH200 and pJH290 were provided by J. Hesse and M. Gellert, and the XR-1 cell line was a gift from T. Stamato. We thank G. Silverman, J. Wagstaff, A. Beggs, R. Swirski, and T. Lindahl for discussions. Research in S.P.'s laboratory is funded principally by grant SP2143/0101 from the Cancer Research Campaign (CRC). T.M.G. is supported by a CRC studentship. Research in the MRC Cell Mutation Unit was supported in part by Commission of European Communities grants F13PCT920007 and ERBSC1CT920823. Research in F.W.A.'s laboratory is supported by NIH grant A.I. 20047, the Howard Hughes Medical Institute, and a postdoctoral fellowship from Irvington Institute (G.E.T.). G.E.T. is a special fellow of the Leukemia Society of America.

16 June 1994; accepted 5 August 1994

Thrombocytopenia in c-*mpl*–Deficient Mice

Austin L. Gurney, Karen Carver-Moore, Frederic J. de Sauvage, Mark W. Moore*

Thrombopoietin (TPO) is a cytokine that is involved in the regulation of platelet production. The receptor for TPO is c-Mpl. To further investigate the role and specificity of this receptor in regulating megakaryocytopoiesis, c-*mpl*–deficient mice were generated by gene targeting. The c-$mpl^{-/-}$ mice had an 85 percent decrease in their number of platelets and megakaryocytes but had normal amounts of other hematopoietic cell types. These mice also had increased concentrations of circulating TPO. These results show that c-*mpl* specifically regulates megakaryocytopoiesis and thrombopoiesis through activation by its ligand TPO.

The proto-oncogene c-*mpl* is a member of the cytokine receptor superfamily with sequence similarity to the erythropoietin receptor and to the granulocyte colony-stimulating factor (G-CSF) receptor (*1*, *2*). Expression of c-*mpl* in normal mice appears to be restricted to hematopoietic tissue, primitive hematopoietic stem cells, megakaryocytes, and platelets, although low levels of expression have been detected in endothelial cells (*2*, *3*). Antisense oligodeoxynucleotides to c-*mpl* selectively inhibit in vitro megakaryocytic colony formation without affecting the growth of erythroid or granulomacrophage colonies, which suggests that c-Mpl and its putative ligand may function in regulating megakaryocytopoiesis (*3*). This hypothesis has been reinforced by the recent purification and cloning of the ligand for c-Mpl (*4*, *5*). This ligand has an NH_2-terminal domain homologous to erythropoietin and a COOH-terminal glycosylated domain unrelated to any known protein. In vitro and in vivo experiments with recombinant Mpl ligand indicate that it has both megakaryocyte colony-stimulating activity and thrombopoietin activity and therefore corresponds to the long-sought platelet growth factor thrombopoietin (TPO) (*4*, *6*, *7*).

To substantiate the involvement of

A. L. Gurney and F. J. de Sauvage, Department of Molecular Biology, Genentech, South San Francisco, CA 94080, USA.
K. Carver-Moore and M. W. Moore, Department of Cell Genetics, Genentech, South San Francisco, CA 94080, USA.

*To whom correspondence should be addressed.

c-*mpl* and TPO in the control of platelet production, we generated mice deficient in c-*mpl*. A targeting vector containing a neomycin-resistance (*neo*^{r}) cassette inserted into the third exon of a 6.6-kb c-*mpl* mouse genomic clone (Fig. 1A) was electroporated into embryonic stem (ES) cells. Gene targeting was detected in 8 of 288 ES colonies screened, and 5 colonies were selected for microinjection into blastocysts (Fig. 1B). Four clones gave germline transmission, and two separate lines were interbred to generate homozygous gene-targeted mice (c-*mpl*$^{-/-}$) (Fig. 1C). These mice were viable, healthy, and displayed no overt abnormalities.

Complete blood cell counts performed on c-*mpl*$^{-/-}$ and c-*mpl*$^{+/+}$ mice revealed an 85% decrease in platelet counts in the gene-targeted animals, with 100% penetrance in mice derived from two independent ES cell clones ($P < 0.0001$) (Fig. 2A). There was also a significant increase in platelet volume, with a mean of 6.25 μm^3 for c-*mpl*$^{-/-}$ mice compared to 4.7 μm^3 for c-*mpl*$^{+/+}$ mice ($P < 0.0001$). Histopathology also showed a loss of megakaryocytes in spleen and bone marrow in these mice (Fig. 2B). There was no significant alteration in platelet counts, platelet volume, or megakaryocyte numbers in heterozygous animals. These data suggest that the c-*mpl*$^{-/-}$ mice are unable to produce normal amounts of megakaryocytes and platelets, because of the loss of a functional c-*mpl* gene. The c-*mpl*$^{-/-}$ mice are not completely devoid of platelets, which suggests that although c-*mpl* is a key regulator of megakaryocyte and platelet production, alternative mechanisms may be triggered in the c-*mpl*$^{-/-}$ mice to promote megakaryocytopoiesis. Alternatively, low ex-

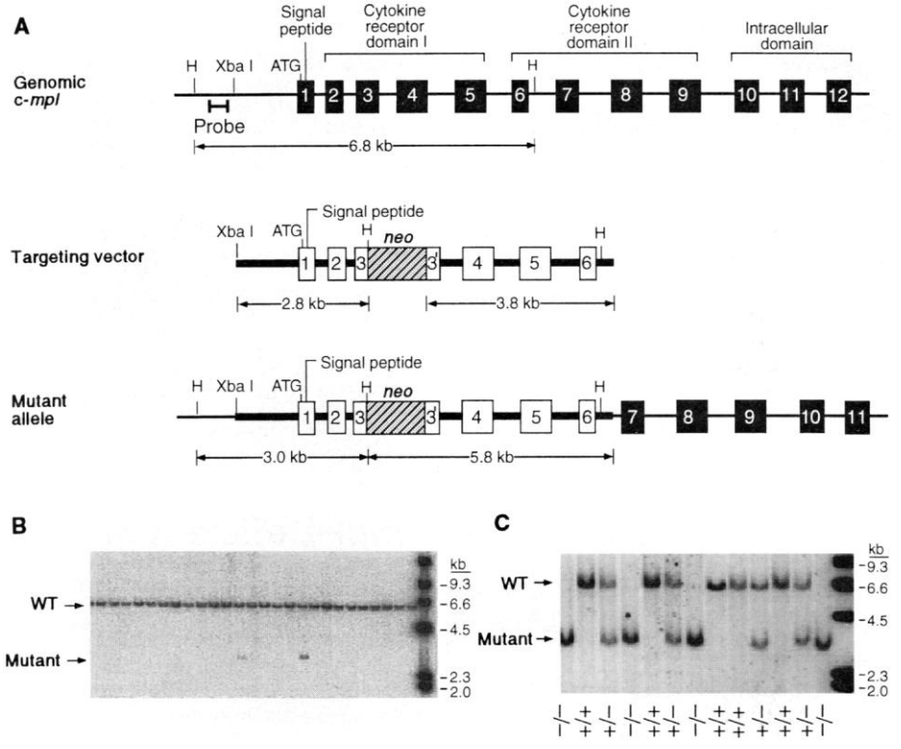

Fig. 1. Targeting of the c-*mpl* gene by homologous recombination. (**A**) Structure of the c-*mpl* gene (*13*) and gene-targeting vector. The restriction enzyme sites are those from mice, whereas the intron-exon organization is taken from humans. Exons are numbered. A *neo*^{r} gene under control of the *pgk* promoter (*14*) was inserted into a synthetic Not I site engineered into the third exon of c-*mpl* by site-directed mutagenesis. An oligonucleotide probe was designed from sequences 5′ to those present on the targeting vector. After homologous recombination, the mutated allele was detected by the addition of a Hind III (H) site from the *neo*^{r} gene. (**B**) Targeting of the c-*mpl* gene in ES cells. (**C**) Analysis of mouse genomic DNA that was isolated from tail snips of the offspring of interbreeding heterozygous mice, digested with Hind III, and analyzed by Southern blot hybridization with the oligo probe indicated in (A). Wild-type (WT) (+/+), heterozygous (+/−), and homozygous receptor-ablated (−/−) samples are indicated.

Table 1. White cell counts in whole blood of c-*mpl*–deficient mice. Blood collected by retro-orbital venous puncture was analyzed in a Serono-Baker Diagnostics System 9000 Diff Model Hematology Analyzer (n = 4 mice per group). Blood smears were stained with hematoxylin and eosin for differential cell counts performed microscopically. The total number of each indicated cell type was determined by multiplying the total white cell count by the percentage of each cell type indicated in the differential counts. All values are thousands per microliter, except for red blood cells, which are millions per microliter.

Cells	Counts in c-*mpl*–deficient mice			*P* (+/+ versus −/−)
	+/+	+/−	−/−	
Red blood cells	8.86 ± 0.70	8.98 ± 1.11	9.90 ± 0.38	0.11
Total white cells	5.08 ± 1.61	5.52 ± 2.33	6.40 ± 2.41	0.33
Lymphocytes	3.86 ± 1.11	3.71 ± 1.00	4.19 ± 1.02	0.64
Neutrophils	0.67 ± 0.14	0.79 ± 0.32	0.85 ± 0.42	0.39
Bands	0.13 ± 0.16	0.09 ± 0.07	0.06 ± 0.07	0.35
Eosinophils	0.09 ± 0.07	0.16 ± 0.05	0.18 ± 0.22	0.31

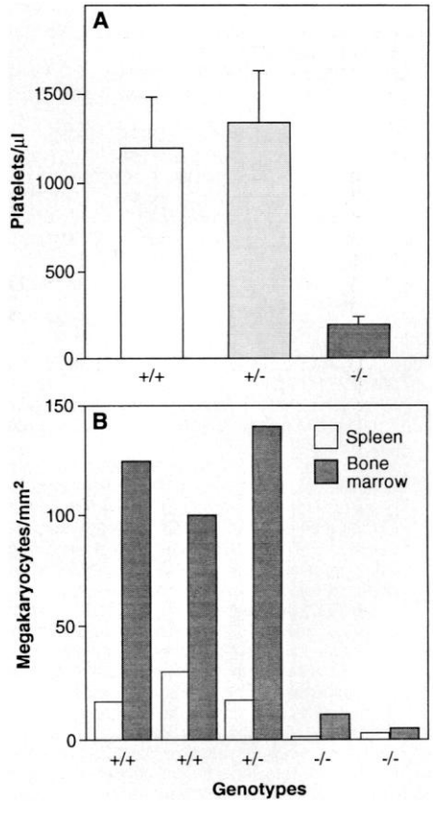

Fig. 2. Platelet and megakaryocyte counts in c-*mpl*$^{-/-}$ mice. (**A**) Platelet counts in blood collected by retro-orbital venous puncture and analyzed in a Serono-Baker Diagnostics System 9000 Diff Model Hematology Analyzer (Allentown, Pennsylvania); n = 5 mice per group. (**B**) Animals were killed for necropsy, and blood and bone marrow smears were stained with hematoxylin and eosin for differential cell counts performed microscopically. Megakaryocytes were counted in bone marrow smears and 5-μm spleen sections. Genotypes of individual mice are indicated.

pression of a truncated version of c-Mpl may be responsible for the presence of a small amount of platelets. However, no c-*mpl* transcripts were detectable by reverse transcriptase–polymerase chain reaction (RT-PCR) in the spleens of c-*mpl*$^{-/-}$ mice (*8*).

There was no significant difference in the numbers of red blood cells, total white blood cells, neutrophils, bands, and eosinophils in the peripheral blood of c-*mpl*$^{+/+}$ and c-*mpl*$^{-/-}$ mice, as determined by differential cell counts (Table 1). There was also no significant alteration in the size and cellularity of lymphoid organs in the c-*mpl*$^{-/-}$ mice. To further investigate the specificity of action of TPO through c-Mpl in hematopoiesis, we analyzed spleen, bone marrow, and thymus cells for cell surface markers identifying T and B cells. No alteration was detected in either maturation markers or cell type ratios (Fig. 3). Thus, disruption of the c-*mpl* gene results in a specific loss of both megakaryocytes and platelets, leaving other cell lineages unaffected.

The sera of thrombocytopenic animals have been shown to contain elevated levels of TPO (*4*, *7*, *9*–*11*). Therefore, we tested sera from c-*mpl*$^{-/-}$ mice for the ability to stimulate the proliferation of Ba/F3 cells that were engineered to express human c-*mpl* (Ba/F3-*mpl*) (*4*). Sera from both wild-type and heterozygous mice do not contain any stimulatory activity. Sera from c-*mpl*$^{-/-}$ mice were able to stimulate the proliferation of Ba/F3-*mpl* cells but not the parental Ba/F3 cells (Fig. 4). Half-maximal stimulation was observed at a 1:100 dilution and could be neutralized by the addition of *mpl*-IgG (immunoglobulin G) to the serum (*8*). The activity detected in the sera of c-*mpl*$^{-/-}$ mice was comparable to that present in aplastic porcine plasma (*4*). No increase in interleukin-3 (IL-3), IL-6, or granulocyte-macrophage colony-stimulating factor (GM-CSF), which are other cytokines with a weak megakaryocytopoietic ability, could be detected by enzyme-linked immunosorbent assay (ELISA) based assays (*12*). The increase in TPO serum concentrations provides further evidence that this cytokine is a major regulator of megakaryocytopoiesis and thrombopoiesis and suggests that in response to thrombocytopenia, TPO levels are up-regulated. The mechanisms responsible for this apparent feedback loop are unknown at this time.

These data demonstrate at the genetic level that c-Mpl is directly responsible for regulating the proliferation and maturation of megakaryocytes. The involvement of TPO and c-Mpl at an early stage of megakaryocyte formation would be consistent with the detection of c-Mpl expression in primitive stem cells (*3*). The phenotypic alteration in c-*mpl*$^{-/-}$ mice is specific, as it appears to affect only megakaryocytes and platelets. We anticipate therefore that the effects of TPO, the ligand for c-Mpl, will be restricted to these cell types, which is an important consideration for its potential therapeutic use.

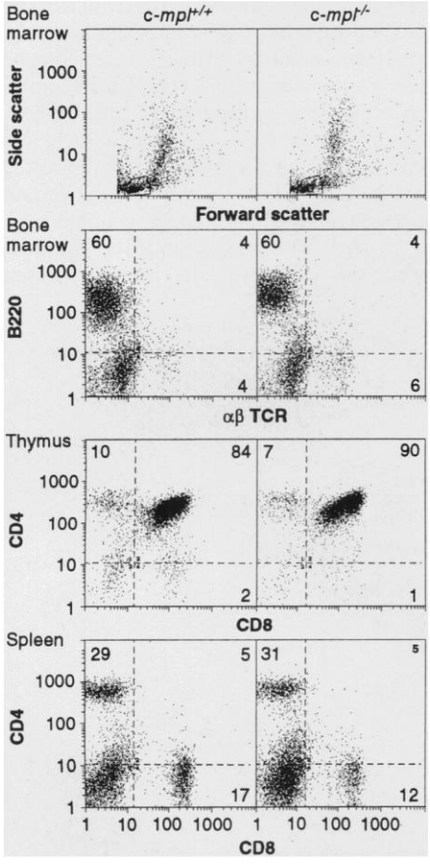

Fig. 3. Flow cytometric analysis and histology of c-*mpl*$^{-/-}$ mice. Analysis of spleen, bone marrow, and thymus for T cell– and B cell–specific cell surface markers. Single-cell suspensions were prepared from spleen, thymus, and bone marrow in Hanks' balanced salt solution with 2% fetal calf serum. There was no significant difference in cellularity between c-*mpl*$^{+/+}$ and c-*mpl*$^{-/-}$ mice in any of the indicated organs. Samples were treated with the indicated antibodies [phycoerythrin (PE)-conjugated antibodies to CD4 (Becton Dickinson), fluorescein isothiocyanate (FITC)–conjugated antibodies to CD8 (Becton Dickinson), FITC-conjugated antibodies to T cell receptors α and β (αβ-TCR), and PE-conjugated antibodies to B220 (Boehringer Mannheim)] and analyzed on a FACScan (Becton Dickinson).

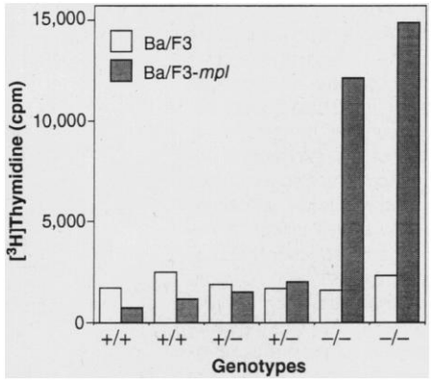

Fig. 4. Effect of c-*mpl*$^{-/-}$ mouse sera on Ba/F3-*mpl* cell proliferation. Proliferation assays were conducted essentially as described (*4*). Ba/F3 or Ba/F3-*mpl* cells were cultured in the absence of IL-3 for 16 hours in RPMI 1640 media supplemented with 10% fetal calf serum. Cells were then washed twice with phosphate-buffered saline and plated in 96-well plates (20,000 cells per well) in the presence of 5% of sample mouse serum. Each sample was tested blind and in duplicate. After 22 hours at 37°C, 1 μCi of [^{3}H]thymidine was added per well and incubation continued for six additional hours. After filtration of the cultures on glass fiber filters, incorporation of [^{3}H]thymidine was measured with a top count counter (Packard Instruments).

REFERENCES AND NOTES

1. M. Souyri *et al.*, *Cell* **63**, 1137 (1990).
2. I. M. Vigon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5640 (1992).
3. N. Methia, F. Louache, W. Vainchencker, F. Wendling, *Blood* **82**, 1395 (1993).
4. F. J. de Sauvage *et al.*, *Nature* **369**, 533 (1994).
5. S. Lok *et al.*, *ibid.*, p. 565.
6. K. Kaushansky *et al.*, *ibid.*, p. 568.
7. F. Wendling *et al.*, *ibid.*, p. 571.
8. A. L. Gurney, K. Carver-Moore, M. W. Moore, F. J. de Sauvage, unpublished observations.
9. T. T. Odell, T. P. McDonald, T. C. Detwiker, *Proc. Soc. Exp. Biol. Med.* **108**, 428 (1961).
10. A. Nakeff and K. J. Roozendaal, *Acta Haematol.* **54**, 340 (1975).
11. R. B. Hoffman *et al.*, *N. Engl. J. Med.* **305**, 533 (1981).
12. K. Carver-Moore, A. L. Gurney, M. W. Moore, F. J. de Sauvage, unpublished observations.
13. V. Mignotte *et al.*, *Genomics* **20**, 5 (1994).
14. M. W. McBurney *et al.*, *Nucleic Acids Res.* **19**, 5755 (1991). Genomic clones were isolated from a library from mouse strain 129. The targeting vector was constructed from a 6.6-kb subclone. The targeting vector (20 μg) was linearized with Sal I and electroporated at 275 V and 200 μF with a BTX (San Diego, CA) 300 electroporator into ES-D3 C-12, a subclone of D3 ES cells [A. Gossler, T. Doetschman, R. Korn, E. Serfling, R. Kemler, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9065 (1986)]. Cells were subjected to G418 selection at a concentration of 400 μg/ml for 10 days. Single-colony wells were expanded for DNA isolation and Southern (DNA) blot analysis. Five gene-targeted clones were selected to generate chimeric mice by microinjection into the blastocoel cavity of 3.5-day-old C57BL/6J blastocysts [A. Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, Ed. (IRL Press, Oxford, 1987), pp. 113–152]. Chimeric males were mated with C57BL/6J females, and agouti-colored offspring were screened for germline transmission by PCR analysis for the *neo*^{r} gene and confirmed by Southern blot analysis of tail DNA. Heterozygous mice were interbred to obtain homozygous animals.

15. We thank S. Pitts-Meek, M. Dowd, and M. Bauer for blastocyst injection; L. Loverro for animal care; T. Doetschman, University of Cincinnati, for D3 ES cells; J. Hooley for flow cytometry; T. Terrel for pathology; G. Hatami and P. Serarne for the blood analysis and differential cell counts; D. Giltinan for statistical analysis; W. Anstine for professional graphics assistance; and D. L. Eaton and D. V. Goeddel for advice and comments. All animal care was in accordance with NIH institutional guidelines.

24 June 1994; accepted 10 August 1994