trophysiological and neurochemical changes that occur in the olfactory bulb after birth represent such an adaptive specialization in response to a behavioral requirement of the selective recognition of their own offspring.

REFERENCES AND NOTES

1. P. Poindron and P. Le Neindre, *Adv. Study Behav.* 11, 75 (1980).
2. F. Lévy, P. Poindron, P. Le Neindre, *Physiol. Behav.* 31, 687 (1983).
3. F. V. Smith, C. Van Toller, T. Boyes, *Anim. Behav.* 14, 120 (1966); B. A. Baldwin and E. Shillito, *ibid.* 22, 220 (1974).
4. E. B. Keverne, F. Lévy, P. Poindron, D. R. Lindsay, *Science* 219, 81 (1983); K. M. Kendrick, F. Lévy, E. B. Keverne, *Physiol. Behav.* 50, 595 (1991).
5. K. M. Kendrick, E. B. Keverne, C. Chapman, B. A. Baldwin, *Brain Res.* 439, 1 (1988); *ibid.* 442, 171 (1988); K. M. Kendrick, F. Lévy, E. B. Keverne, *Curr. Sep.* 10, 96 (1991).
6. The method for recording the activity of single cells in the brain of the conscious sheep is as described [K. M. Kendrick and B. A. Baldwin, in *Methods in Neuroscience*, P. M. Conn, Ed. (Academic Press, San Diego, 1991), vol. 4, pp. 3–14].
7. General anesthesia was induced with an intravenous injection of 400 mg of sodium methohexitone and then with an injection of halothane. Full sterile precautions were taken during surgery, and the animals were allowed 3 to 4 weeks to recover.
8. A differential response to odors was defined as a significant neuronal response to some, but not all, of the odor stimuli tested. A total of 107 cells did not fulfill this criterion (60 before birth and 47 after birth). Odor stimuli included three foods (oats, hay, and nuts), amniotic fluid from an alien lamb, a human hand, wool shorn from an unfamiliar adult female sheep, amyl acetate, and lamb's wool, either shorn or on the lamb. After birth, the wool fibers of both alien lambs and a ewe's own lambs were used as stimuli. The stimuli were presented for 10 s on two or three occasions and in random order (for statistics the firing rate during the previous 10 s (20 0.5-s bins) was compared to that during the odor stimulus (20 0.5-s bins). At least 1 min was allowed to elapse between successive odor stimuli.
9. Although firing rate changes in response to odors were based only on the duration of a 10-s stimulus, the responses of the cells to food odors before birth, and to lamb odors after birth, could be maintained for several minutes or more without significant habituation.
10. G. M. Shepherd, *Physiol. Rev.* 52, 864 (1972).
11. K. Mori, *Prog. Neurobiol. (Oxford)* 29, 275 (1987).
12. M. T. Shipley, F. G. Halloran, J. de la Torre, *Brain Res.* 329, 294 (1985).
13. D. A. Godfrey, C. D. Ross, F. M. Matschinsky, *Neuroscience* 5, 273 (1980).
14. R. Gervais, S. Araneda, J. F. Pujol, *Electroencephalogr. Clin. Neurophysiol.* 57, 462 (1984).
15. K. M. Kendrick, in *Microdialysis in the Neurosciences*, T. Robinson and J. Justice, Eds. (Elsevier, Amsterdam, 1991), pp. 327–348.
16. J. G. Taylor and E. B. Keverne, *Biol. Cybern.* 64, 301 (1991).
17. A. E. Rosser and E. B. Keverne, *Neuroscience* 15, 1141 (1985).
18. R. M. Sullivan and M. Leon, *Dev. Brain Res.* 35, 301 (1987).
19. W. J. Freeman and W. Schneider, *Psychophysiology* 19, 44 (1982).
20. E. B. Keverne and C. de la Riva, *Nature* 296, 148 (1982); R. A. Sullivan, D. A. Wilson, M. Leon, *J. Neurosci.* 9, 3998 (1989); C. Gray, W. J. Freeman, J. E. Skinner, *Behav. Neurosci.* 100, 585 (1986).
21. D. Pissonier *et al.*, *Physiol. Behav.* 35, 361 (1985).
22. R. G. M. Morris, E. Anderson, G. S. Lynch, M. Baudry, *Nature* 319, 774 (1986).
23. G. Buzsaki, *Neuroscience* 31, 551 (1989).
24. M. Higaskima, *Exp. Brain Res.* 72, 37 (1988).
25. P. K. Stanton and J. M. Sarvey, *J. Neurosci.* 5, 2169 (1985).
26. A. Artola and W. Singer, *Nature* 330, 649 (1987).
27. P. Rozin, in *Neural Mechanisms of Learning and Memory*, M. R. Rozenweig and E. L. Bennet, Eds. (Cambridge Univ. Press, Cambridge, 1976), pp. 3–48.

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Initiation of Deregulated Growth of Multipotent Progenitor Cells by *bcr-abl* in Vitro

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Expression of the *bcr-abl* oncogene in multipotent progenitor cells (MPPCs) is implicated as a key event in the development of chronic myelogenous leukemia. Bone marrow enriched for MPPCs was infected with a retrovirus that carried *bcr-abl*. The mixed-lineage colonies that resulted were responsive to growth factors and could differentiate. These cells later became growth factor–independent but, when injected into severe combined immunodeficient mice, were not leukemogenic. Thus, the presence of *bcr-abl* alone does not cause growth factor independence, although it initiates a stepwise process. This system may prove useful in the study of other oncogenes that cause leukemia.

The Philadelphia chromosome (Ph^1) is the molecular hallmark of human chronic myelogenous leukemia (CML) (*1*, *2*). A consequence of this interchromosomal translocation is the formation of the chimeric *bcr-abl* oncogene (*3*); the product of this *bcr-abl* gene (p210) is an activated form of the c-*abl* protein tyrosine kinase (*4*). Human CML exhibits a biphasic clinical course that originates with a clonal expansion of a pluripotent or multipotent hematopoietic progenitor cell (MPPC) (*1*). During the disease's initial chronic phase, which may last for several years, committed myeloid progenitor cells that retain their ability to differentiate increase in number. Progression of the disease is marked by accelerated growth of immature myeloid or lymphoid cells that no longer differentiate. The presence of Ph^1 and the $p210^{bcr\text{-}abl}$ protein in cells during both chronic phase and acute blast crisis suggests that *bcr-abl* expression is an important factor in CML pathogenesis (*5*).

It is not known if *bcr-abl* can directly stimulate the growth of MPPCs or if other genetic alterations that precede the formation of Ph^1 are responsible for the expansion of the MPPC clone observed in CML (*6*). In vivo murine studies have shown that *bcr-abl* can induce various hematopoietic malignan-

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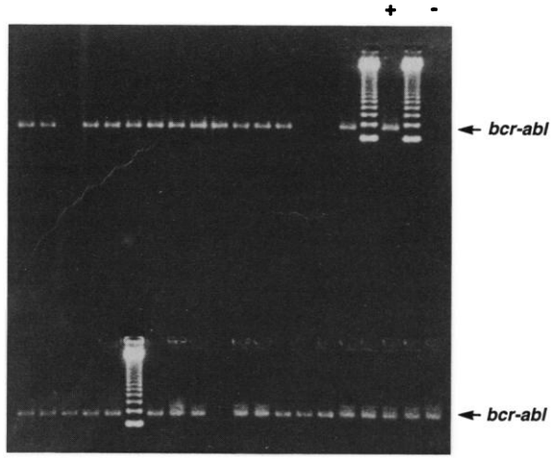

Fig. 1. Detection of *bcr-abl* in agar colonies using PCR. Each lane represents analysis of an individual agar colony. DNA from each agar colony was prepared and analyzed by PCR as described (*22*). The presence of the *bcr-abl* gene was evaluated with specific oligonucleotides that amplify a unique 200-bp fragment of this gene. Samples were subjected to 45 cycles of amplification according to the following protocol: 30 s at 93°C, 30 s at 59°C, and 15 s at 72°C. One-fifth of each reaction was run on a 2.5% agarose gel, and the specific amplified product was detected as a single ethidium bromide–stained band. The gel contains two rows of samples; arrows mark the migration of the *bcr-abl*–specific band. The identity of the band was confirmed by DNA hybridization analysis with a ^{32}P-labeled oligonucleotide specific to an internal region of the amplified *bcr-abl* segment. +, 10 ng of genomic DNA from a *bcr-abl*–transformed lymphoid cell line; −, 10 ng of genomic DNA from a nontransformed mast cell line.

cies, including myeloproliferative disorders similar to those observed in human CML (*7*). These observations support the critical role of *bcr-abl* in the etiology of CML. However, because of the extended time required to develop the murine pathology and the complexity of the cell populations in vivo, these studies could not determine if *bcr-abl* stimulated the growth of MPPCs or committed progenitors nor could they identify discrete stages during the initial development of pathology.

We used a single-step in vitro colony assay to investigate the direct effect of *bcr-abl* expression on development and growth of MPPCs. Prior work with lymphoid and myeloid cell lines suggested that *bcr-abl* may stimulate growth by altering cell sensitivity to specific growth factors (*8*). We reasoned that incubation of progenitor cells that contained *bcr-abl* in the presence of cytokines, at concentrations below those required to stimulate the growth of normal bone marrow cells, could positively select for the effect of the oncogene.

Bone marrow elements from mice pretreated with 5-fluorouracil (5-FU), for the enrichment of progenitor cells, were infected with helper-free retroviruses that expressed constructs that contained *bcr-abl* or the neomycin resistance gene (mock) (*9*). After infection, cells were cultured in soft agar (*10*) in the absence or presence of threshold concentrations of murine interleukin-3 (IL-3) or rat steel locus factor (SLF) (also known as the stem cell factor, mast cell growth factor, or kit ligand). Alone or in combination with other factors, IL-3 or SLF stimulates growth of MPPCs (*11*). Eight to 10 days after seeding, the colonies were counted and assessed for lineage phenotype with Wright-Giemsa stain, and the presence of *bcr-abl* was determined by *bcr-abl*–specific polymerase chain reaction (PCR) gene amplification.

Mock-infected 5-FU bone marrow did not form colonies in the absence of growth factors added exogenously. At low concentrations of IL-3 (1 to 30 pg/ml) or SLF (0.3 to 30 ng/ml), only a few small colonies (<0.4 mm) could be observed (Table 1). Approximately 90% of these colonies exhibited a mast cell morphology, and monocytic and mixed-phenotype colonies were also present. Cells from most of these colonies could not adapt to growth in liquid culture. Of the 120 monocytic and mixed-phenotype agar colonies studied, only four established growth factor–dependent cell lines. In all four cases, the cell lines had a differentiated mast cell morphology.

Bone marrow infected with retroviruses that contained *bcr-abl* formed growth factor–independent and growth factor–dependent soft agar colonies (Table 1). Growth factor–independent colonies [those that grew in media supplemented with fetal calf serum (FCS) and no additional growth factors] were large (>0.5 mm) and contained cells that exhibited differentiated mast cell or lymphoid morphology. Cell lines established from the large agar colonies were monoclonal and exhibited an end stage–differentiated phenotype (mast cell or lymphoid, respectively). This result suggested that the parental cell for these large colonies was probably not an MPPC but a committed progenitor cell; transformation of these cell types by *bcr-abl* has been reported (*12*).

To study the effect of *bcr-abl* on MPPCs, we characterized the factor-dependent agar colonies that exhibited the more diverse phenotypes. These factor-dependent colonies were small (<0.4 mm) and required threshold amounts of IL-3 (1 to 30 pg/ml) or SLF (0.3 to 30 ng/ml) for growth. The actual number of colonies varied between individual experiments, but we consistently observed a 2.5- to 10-fold greater number of small agar colonies in wells that contained *bcr-abl*–infected bone marrow than in mock-infected controls at the same concentration of cytokine (Table 1). Using PCR and oli-

Table 1. The *bcr-abl* gene stimulates hematopoietic cell growth in a growth factor–supplemented soft agar colony assay (*10*). The reported number of colonies for each experiment represents the mean number of colonies counted in three wells that contained 2×10^5 cells per well at the same concentration of growth factor. The colony number was determined between days 8 and 10 after seeding in agar. Colony size was estimated with a calibrated microscopic ocular. "Small" corresponds to a colony diameter size <0.4 mm; "Large" corresponds to a colony diameter of >0.5 mm. TK, thymidine kinase.

Experiment	Agar colonies (*n*)			
	TK-*neo*		*bcr-abl*	
	Small	Large	Small	Large
1*	7	0	36	5
2†	4	0	43	6
3†	16	0	47	5
4‡	8	0	51	3
5§	15	0	58	7

*IL-3 (30 pg/ml). †IL-3 (10 pg/ml). ‡SLF (1 ng/ml). §SLF (30 ng/ml).

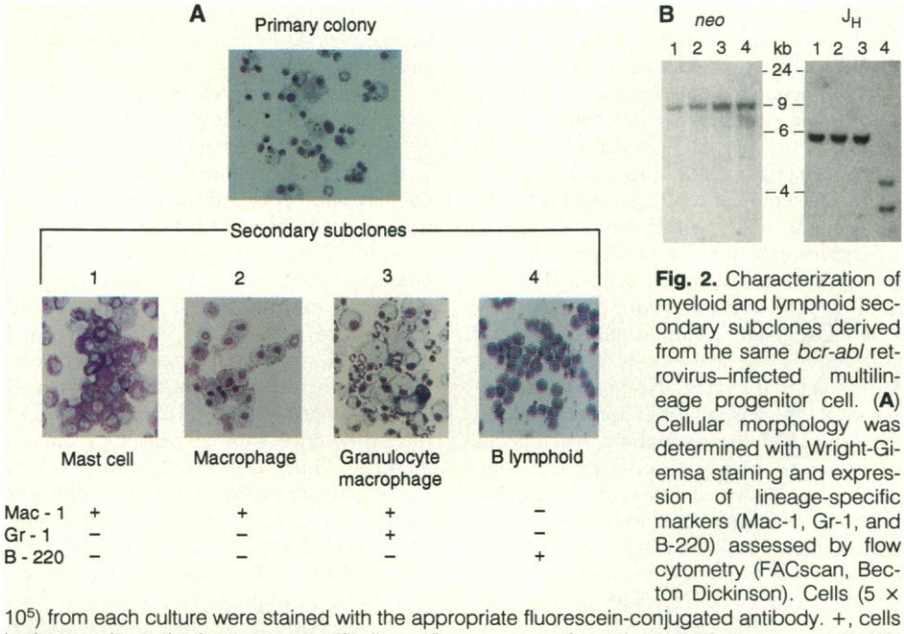

Fig. 2. Characterization of myeloid and lymphoid secondary subclones derived from the same *bcr-abl* retrovirus–infected multilineage progenitor cell. (**A**) Cellular morphology was determined with Wright-Giemsa staining and expression of lineage-specific markers (Mac-1, Gr-1, and B-220) assessed by flow cytometry (FACscan, Becton Dickinson). Cells (5×10^5) from each culture were stained with the appropriate fluorescein-conjugated antibody. +, cells in these cultures had a mean specific linear fluorescence of one log over isotype controls. We derived cultures 1 and 2 by subcloning cells from a single agar colony in Iscove's medium, 10% FCS, 50 μM 2-ME, and IL-3 (30 pg/ml) supplemented with SLF (50 ng/ml) for culture 1 and murine GM-CSF (1.0 ng/ml) for culture 2. We derived culture 3 from the same agar colony by growing cells in semisolid methylcellulose media in Iscove's medium, 20% FCS, 50 μM 2-ME, IL-3 (30 pg/ml), and human G-CSF (1.0 ng/ml). We established culture 4 by growing cells in the presence of an established S-17 bone marrow stroma (*21*) in RPMI 1640, 10% FCS, 50 μM 2-ME, IL-3 (30 pg/ml), and human IL-7 (50 ng/ml). Cells from each culture were harvested when they reached a density of $>2.5 \times 10^5$ cells per milliliter. Murine IL-3 was purchased from Biosource International (Westlake Village, California). (**B**) Twelve micrograms of DNA from each culture were digested with Eco RI restriction endonuclease, run on a 0.8% agarose gel, and transferred onto a nitrocellulose filter. Under these digest conditions, hybridization of the blot with a radioactive labeled probe of the neomycin resistance gene (*neo*) showed unique integration sites of the *bcr-abl–neo* retroviral vector. We determined Ig gene rearrangement by hybridizing the same blot with a probe toward the Ig heavy-chain–joining region (J_H) (*23*). Similar cultures were also derived from two other MPPCs noted in text.

Table 2. Cellular morphlogy of *bcr-abl*–containing agar colonies. Individual agar colonies were picked and transferred to 96-well plates that contained 100 μl of Iscove's medium, 20% fetal calf serum (FCS), and 50 μM 2-ME. Colonies were dispersed by repeated pipetting in each well. Slides were prepared, and cellular morphology was evaluated with Wright-Giemsa staining. The presence of the *bcr-abl* genome was evaluated with PCR and oligonucleotides specific for *bcr-abl* (Fig. 1).

Morphology	Number	Percent of total
Mast cell	86	15
Lymphoid	161	28
Macrophage monocyte	317	55
Mixed	11	2

gonucleotides specific for *bcr-abl*, we determined that all of the large colonies and up to 90% of the small colonies (range = 65 to 90%) (Fig. 1) contained the *bcr-abl* oncogene. In five independent experiments, a total of 768 small colonies were screened, 575 of which contained the *bcr-abl* gene. The predominant cellular morphology of the small colonies that contained *bcr-abl* was a macrophage or monocyte cell type (Table 2). In addition, some of the small colonies exhibited lymphoid, mast cell, and mixed-lineage phenotypes. These data demonstrate that *bcr-abl* can directly exert dominant but subtle effects on the growth of cells in different hematopoietic lineages.

We determined the differentiation potential of cells in the small agar colonies by subculturing them in the presence of specific growth factors that included IL-2, IL-3, IL-4, IL-6, IL-7, erythropoietin, SLF, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF). No growth was observed in the absence of exogenously added cytokines. Cells from lymphoid, macrophage or monocyte, and mast cell–type agar colonies were limited in their differentiation potential and only produced pre–B cells, macrophages, and mast cells, respectively. Colonies that had a mixed cellular morphology produced subcultures with diverse phenotypes, which suggested that they contained MPPCs.

In three independent experiments, we derived clonal outgrowths of B lymphocytes and cells of different myeloid lineages (mast cells, macrophages, granulocytes) from the same mixed-phenotype colony (Fig. 2A). Cellular phenotype was confirmed by flow cytometry analysis with lineage-specific markers. To determine the origin of individual cultures, we prepared and analyzed genomic DNA for the presence of a unique site-specific retroviral integration site (Fig. 2B). All secondary subclones contained the identical retroviral integration site. This result was confirmed by Southern (DNA) blot analysis of the same DNA after digestion with two other restriction enzymes (*13*). Thus, these cultures originated from the same *bcr-abl*–infected MPPC. Southern blot analysis to detect immunoglobulin (Ig) gene rearrangement demonstrated that only the B lymphoid cultures had rearranged both Ig gene alleles, whereas myeloid cultures retained the Ig genes in the germline configuration (*14*). Similar cultures were derived from MPPCs in two other experiments (*13*). Thus, MPPCs that express the *bcr-abl* oncogene can undergo lineage commitment and are not blocked in their ability to differentiate.

Cultures established from *bcr-abl*–expressing MPPCs progressed through two stages. Initially, the subcultures required the presence of specific growth factors and grew slowly. With extended passage in vitro (>6 weeks), most of the cultures became overgrown with cells that were growth factor–independent and had lineage-specific phenotypes. The acquisition of cytokine-independent growth in vitro by these cell lines was not associated with an increase in $p210^{bcr\text{-}abl}$ protein kinase activity (*13*). This result indicates that mechanisms other than increased *bcr-abl* expression are involved in this progression.

To test whether these murine factor–independent cell lines were tumorigenic, we injected groups of sublethally irradiated C.B-17 severe combined immunodeficient (SCID) mice (*15*) intravenously with cells from either myeloid or B lymphoid cultures established from the same MPPC. As a positive control for tumor growth, other mice were injected with a pre–B lymphoid cell line (MG 35.3) (*16*), which expressed *bcr-abl* and was growth factor–independent. Only mice injected with the control cell line developed tumors (Table 3); none of the other mice showed pathology after 3 months. Thus, factor independence and tumorigenicity probably represent different stages in the development of *bcr-abl*–induced pathology; additional genetic alterations are probably necessary for the development of a malignant phenotype.

On the basis of the clinical presentation of CML, the initiating event for this disease would be predicted to have a subtle effect on the growth of MPPCs and would not abrogate their ability to differentiate. Previous work suggested that several genetic alterations, including the Ph^1 chromosome, may be required to initiate the CML phenotype (6). Our results show that expression of *bcr-abl* alone can induce these types of effects in MPPCs in vitro. The fact that the cytokine-independent cell lines established in these studies were nonmalignant supports the hypothesis that other events are also required for progression of CML.

Table 3. Growth of tumors in C.B-17 SCID mice inoculated with growth factor–independent *bcr-abl*–expressing cell lines. After a sublethal dose of radiation (275 rads), 8- to 12-week-old C.B-17 SCID mice were injected intravenously with 1×10^6 cells of each cell line. Cultures of mast cell, macrophage, and B lymphoid cell lines were derived from two independent multipotent progenitor cell agar colonies (Table 1, experiments 2 and 4). Each of the outgrowths derived from either experiment 2 or experiment 4 was maintained in culture for 2.5 to 3 months. The control cell line MG 35.3 was a *bcr-abl*–expressing, growth factor–independent pre–B lymphoid cell line that was maintained in culture for over a year. At the time of inoculation, all cell lines were growing in media supplemented with 10% FCS alone. We evaluated these mice for the presence of leukemia by monitoring cell number in the peripheral blood at monthly intervals. Data reported were collected 3 months from the date of inoculation. B lym, B lymphoid colony; Mast, mast cell colony; Mono, monocytic cell colony. The latency for the disease in mice inoculated with the MG 35.3 cells was 6 to 10 weeks.

Clone	Type	Mice injected (*n*)	Tumors (*n*)
MG 35.3	B lym	3	3
Exp 2-9A	Mast	3	0
Exp 2-9B	Mono	5	0
Exp 2-9C	B lym	3	0
Exp 4-87A	Mast	4	0
Exp 4-87B	Mono	5	0
Exp 4-87C	B lym	3	0

Studies on clinical samples have focused on identification of oncogenes and tumor suppressor genes that may be affected by secondary cytogenetic abnormalities commonly observed in CML blast crisis. Potential candidates for such genes include *myc* (which may be affected by the trisomy 8 abnormality) (*17*) and *tp53* (located on the short arm of chromosome 17) (*18*). In addition, the recent observation that the retinoblastoma susceptibility gene protein is absent in patients with megakaryocytic CML blast crisis shows that the development of specific subtypes of blast crisis may be dictated by defined genetic lesions (*19*).

Studies that attempt to trace the cellular origin of other human leukemias suggest that such leukemias too may originate from a multipotent progenitor cell (*20*). The phenotype of each leukemia is determined by the cumulative effect of genetic lesions that occur with the progression of the individual disease. As our data demonstrate, the effect of a single genetic alteration may be subtle and could serve to either heighten the cellular response to positive stimuli or attenuate the response to negative effectors of growth. The experimental strategy described in this study can follow the temporal progression of leukemia from a single multipotent progenitor cell.

REFERENCES AND NOTES

1. R. Kurzrock, J. U. Gutterman, M. Talpaz, *N. Engl. J. Med.* **319**, 990 (1989).
2. P. C. Nowell and D. A. Hungerford, *Science* **132**, 1497 (1960); J. D. Rowley, *Nature* **243**, 290 (1973).
3. J. Groffen *et al.*, *Cell* **36**, 93 (1984); E. Shtivelman, B. Lifshitz, R. P. Gale, E. Canaani, *Nature* **315**, 550 (1985); J. B. Konopka, S. M. Watanabe, O. N. Witte, *Cell* **37**, 1035 (1984)
4. T. G. Lugo, A.-M. Pendergast, A. J. Muller, O. N. Witte, *Science* **247**, 1079 (1990); A. M Pendergast *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5927 (1991).
5. S. J. Collins, in *Chronic Myelogenous Leukemia· Molecular Approaches to Research and Therapy*, A. B. Deisseroth and R Arlinghaus, Eds. (Dekker, New York, 1990), p. 253.
6. P. J. Fialkow *et al.*, *Blood* **58**, 158 (1981).
7. G. Q. Daley, R. A. Van Etten, D. Baltimore, *Science* **247**, 824 (1990); A. G. Elefanty, I K Hariharan, S. Cory, *EMBO J.* **9**, 1069 (1990), M. A. Kelliher, J. McLaughlin, O. N. Witte, N. Rosenberg, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6649 (1990); N. Heisterkamp *et al*, *Nature* **344**, 251 (1990).
8. G. Q. Daley and D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9312 (1988), I. K. Hariharan and J. M. Adams, *Oncogene Res.* **3**, 387 (1988); J. C. Young, M. L. Gishizky, O. N. Witte, *Mol. Cell. Biol.* **11**, 854 (1991).
9. Bone marrow from 11- to 13-week-old BALB/c mice, treated with 5-FU (150 mg per kilogram of body weight) 6 days before harvest, was cultured at 1 × 10^6 cells per milliliter in Iscove's medium, 10% FCS, 50 μM 2-mercaptoethanol (2-ME), murine IL-3 (0.5 ng/ml), and human IL-6 (50 ng/ml). After 24 hours, media were removed, and cells were resuspended in helper-free retroviral stocks prepared in the same media. Retroviral stocks were prepared as described [A. J. Muller *et al.*, *Mol. Cell. Biol.* **11**, 1785 (1991)] and were supplemented with polybrene (4 μg/ml) and maintained in the same concentrations of IL-3 and IL-6. Fresh media that contained retroviruses were added every 6 hours for up to 48 hours. After a total of 72 hours in liquid culture, cells were washed three times and seeded at 1 × 10^5 cells per milliliter in soft agar.
10. Agar assay was performed as described [T. Lugo and O. N. Witte, *Mol. Cell. Biol.* **9**, 1263 (1989)] with the following modifications: After infection, cells were washed and resuspended at 1 × 10^5 cells per milliliter, and 2 ml of cell suspension was seeded per well in soft agar supplemented with either IL-3 or SLF. The threshold concentration of growth factor necessary to stimulate agar colony formation varied between individual experiments. In each experiment, we determined the optimal concentration of growth factor necessary to preferentially support the growth of *bcr-abl*–expressing cells by supplementing cultures with serial dilutions of either murine IL-3 (1 to 500 pg/ml) or rat SLF (0.3 to 100 ng/ml). Triplicate cultures were established at each concentration of growth factor. Each experiment also included cultures that contained no exogenously added cytokines.
11. D. M. Bodine, P. S. Crosier, S. C. Clark, *Blood* **78**, 914 (1991); P. de Vries, K. A. Brasel, J R. Eisenman, A. R. Alpert, D. E. Williams, *J. Exp. Med.* **173**, 1205 (1991); G. Migliaccio *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7420 (1991)
12. M. L. Gishizky, J. McLaughlin, A. M. Pendergast, O. N. Witte, *Oncogene* **6**, 1299 (1991); J. C. Young and O. N. Witte, *Mol Cell Biol.* **8**, 4079 (1988).
13. M. L. Gishizky and O. N. Witte, unpublished observations.
14. Despite repeated attempts, we were unable to derive erythroid outgrowths from cells in the mixed-phenotype colonies; the *bcr-abl* oncogene may have had a limited effect on the growth of cells in the erythroid lineage, which is correlated with no increase in the erythroid pool during the chronic phase of CML (*1*).
15. G. C. Bosma, R P Custer, M. J. Bosma, *Nature* **301**, 527 (1983).
16. The MG 35.3 cell line was established by infecting bone marrow, which was taken from mice pretreated with 5-FU (150 mg per kilogram of body weight) 6 days before harvest, with helper-free retroviral stocks that expressed $p210^{bcr\text{-}abl}$. After infection, cells were seeded onto S-17 bone marrow stromal cell layers (*21*). After 4 weeks in culture, cells were subcloned by limiting dilution, in the absence of stromal cell layers, in RPMI 1640 media that contained only 5% FCS and 50 μM 2-ME. The MG 35.3 subclone was maintained in this media for over a year before inoculation into mice
17. E. Liu, B. Hielle, J. M. Bishop, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1952 (1988); D. M. McCarthy, J. M. Goldman, F. V. Rassool, S V. Graham, G. D. Birnie, *Lancet* ii, 1362 (1984).
18. H. Ahuja *et al.*, *J. Clin. Invest.* **87**, 2042 (1991), E. Feinstein *et al.*, *Proc Natl. Acad. Sci. U.S.A.* **88**, 6293 (1991)
19. M. Towatari, K Adachi, H. Kato, H. Saito, *Blood* **78**, 2178 (1991).
20. J. Epstein, H. Xiao, X.-Y He, *N. Engl. J. Med.* **322**, 664 (1990), M Keinanen *et al.*, *ibid.* **318**, 1153 (1988).
21. L. S. Collins and K. Dorshkind, *J. Immunol.* **138**, 1082 (1987).
22. E. S. Kawasaki, in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, 1990), p. 21; E. S. Kawasaki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5698 (1988).
23. P. Early *et al.*, *Cell* **19**, 981 (1980).

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Primary Structure and Functional Expression of the β_1 Subunit of the Rat Brain Sodium Channel

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Voltage-sensitive sodium channels are responsible for the initiation and propagation of the action potential and therefore are important for neuronal excitability. Complementary DNA clones encoding the β_1 subunit of the rat brain sodium channel were isolated by a combination of polymerase chain reaction and library screening techniques. The deduced primary structure indicates that the β_1 subunit is a 22,851-dalton protein that contains a single putative transmembrane domain and four potential extracellular N-linked glycosylation sites, consistent with biochemical data. Northern blot analysis reveals a 1400-nucleotide messenger RNA in rat brain, heart, skeletal muscle, and spinal cord. Coexpression of β_1 subunits with α subunits increases the size of the peak sodium current, accelerates its inactivation, and shifts the voltage dependence of inactivation to more negative membrane potentials. These results indicate that the β_1 subunit is crucial in the assembly, expression, and functional modulation of the heterotrimeric complex of the rat brain sodium channel.

Voltage-sensitive sodium channels are the membrane proteins responsible for the rapid influx of Na^+ during the rising phase of the action potential in excitable cells (*1*). The major form of the Na^+ channel in rat brain is a heterotrimeric complex of an α subunit (260 kD), a noncovalently associated β_1 subunit (36 kD), and a disulfide-linked β_2 subunit (33 kD) (*2*). Sodium channels in eel electroplax and chicken heart are thought to consist of a single α subunit (*3*), whereas Na^+ channels in rat skeletal muscle and rat heart are heterodimeric, composed of α- and β_1-like subunits (*4*). Four rat brain Na^+ channel α-subunit isoforms have been identified by cloning and sequencing (*5*, *6*). There are also multiple subtypes of β_1 subunits expressed in rat brain, sciatic nerve, skeletal muscle, and heart (*7*). Expression of rat brain Na^+ channel α subunits alone in *Xenopus* oocytes (*6*, *8–10*) or in mammalian cells (*11*) yields functional Na^+ channels that activate and inactivate in response to depolarization. However, brain Na^+ channels expressed in *Xenopus* oocytes from RNA encoding α subunits inactivate slowly compared to Na^+ channels in neurons (*6*, *8*,

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