BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 172–179 (1996)
ARTICLE NO. 1003

Analysis of CSK Homologous Kinase (CHK/HYL) in Hematopoiesis by Utilizing Gene Knockout Mice[1]

Isao Hamaguchi,* Naoto Yamaguchi,* Junko Suda,* Atsushi Iwama,* Atsushi Hirao,* Motohiro Hashiyama,* Shin-Ichi Aizawa,† and Toshio Suda*,[2]

*Departments of *Cell Differentiation and †Morphogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto, Japan*

Received June 2, 1996

CHK/HYL is a non-receptor tyrosine kinase that belongs to CSK (C-terminal Src kinase) family. Northern blotting and RT-PCR analyses showed that CHK/HYL was expressed in large spectrum of hematopoietic cells except for erythroid cells and brain. To explore the function of CHK/HYL in hematopoietic cells, we generated CHK/HYL deficient mice. The mutant mice were apparently normal and fertile, while CSK knockout mice died until E11.5 from a defect in the neural tube formation. Hematological observations including blood counts and FACS analysis showed no significant abnormalities in CHK/HYL mutant mice. CHK/HYL did not affect the activity of Src, Hck, and Fgr in cultured bone marrow cells, although CSK negatively regulates Src family kinases. These results suggest that CHK/HYL might not have the same function as CSK.

Non-receptor tyrosine kinases play important roles in signal transduction. (1) The CSK-related kinases including HYL have been independently reported, and are found to be identical to each other or the mouse/rat counterparts (2-7). CSK homologous kinase (CHK) is proposed as a new nomenclature (McVicar, personal communication, 1995). The structure of CHK/HYL genomic DNA showed that the exon organization of CHK/HYL is identical to that of CSK (8). However the expression of CHK/HYL in brain increases postnatally while the expression of CSK decreases with age (9). This implies that CHK/HYL and CSK may not have the same function. Recently, it was also indicated that CHK/HYL might play some roles in monocytes (10), megakaryocytes (11), and T cells (5).

We elucidated the expression of CHK/HYL in hematopoietic cells, and in order to clarify the *in vivo* function of the CHK/HYL in hematopoietic cells, we generated CHK/HYL deficient mice.

MATERIALS AND METHODS

Northern blotting. Total RNA was isolated from tissues and cell lines by means of AGPC extractions (12) and poly(A)$^+$RNA was selected using Oligotex-dT30 (Takara Shuzo, Kyoto, Japan). Two micrograms of poly(A)$^+$RNA, electrophoresed on a formaldehyde gel, was then blotted to a Zeta-Probe membrane (Bio-Rad, Richmond, CA). Hybridization was proceeded according to the manufacturer's instructions. The 253 bp of PstI-PstI fragment of the mouse CHK/HYL cDNA was [^{32}P]-labeled for hybridization.

Antibodies. Fluorescein isothiocyanate (FITC)-conjugated streptavidin, anti-F4/80, anti-CD4, and anti-IgM were purchased from GIBCO BRL, Serotech (Oxford, UK), PharMingen (San Diego, CA), Zymed (South San Francisco, CA), respectively. Phycoerythrin (PE)-conjugated anti-Mac-1 was from PharMingen. Biotinylated anti-Gr-1, anti-B220, and anti-CD8 were from PharMingen. Allophycocyanin (APC)-conjugated streptavidin was from Becton Dickinson (San Jose, CA). APC-conjugated anti-c-kit and biotinylated anti-Fms was given by Dr. Shinichi Nishikawa (Kyoto

[1] The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X83972.

[2] To whom correspondence should be addressed. Fax:81-96-373-5332.

0006-291X/96 $18.00

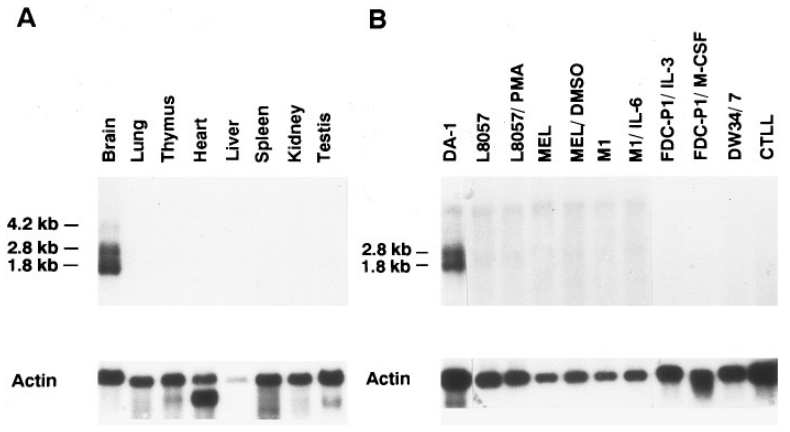

FIG. 1. Expression of mouse CHK/HYL mRNA. Northern blots of mouse CHK/HYL mRNA in adult tissues (A) and hematopoietic cell lines (B). CHK/HYL mRNA are both 1.8 and 2.8 kb. DA-1 (IL-3-dependent undifferentiated myeloid), L8057 (megakaryoblastic), MEL (erythroid), M1 (myeloid), FDC-P1 (myeloid), DW34/7 (B cell), CTLL (T cell), L8057/PMA: L8057 was differentiated into megakaryocytic lineage by an incubation with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) for 3 days. MEL/DMSO: MEL was differentiated into erythroid lineage by an incubation with 1.5% dimethyl sulfoxide (DMSO) for 3 days. M1/IL-6: M1 was differentiated into macrophage lineage by an incubation with human recombinant IL-6 (20 ng/mL) for 3 days. FDC-P1/M-CSF: FDC-P1 was differentiated into myeloid lineage by exposure to M-CSF (1000 U/mL) for 4 days. Loading was normalized using a β-actin probe. The blot was exposed for 2 days.

University) (13). FITC-conjugated anti-CD34 was given by Dr. Hirofumi Hamada (Cancer Institute) (14). Anti-Src (clone 327) was purchased from Oncogene Science (Uniondale, NY) and anti-actin (clone C4) was from Chemicon International (Temecula, CA), and rabbit antibodies against CHK, Hck, and Fgr were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against CHK/HYL were generated (Hirao et al., submitted).

FACS analysis and cell sorting. Bone marrow cells were stained with antibodies, and sorted on a FACSvantage (Becton Dickinson) as previously described (14). 4,000 cells each were prepared for RT-PCR. Bone marrow, spleen,

TABLE 1
The Expression of CHK in Hematopoietic Cells

FACS-sorted cells	CHK expression
Bone Marrow	
Mac-1$^+$ (Myeloid)	+
Gr-1$^+$ (Myeloid)	+
F4/80$^+$ (Myeloid)	+
TER119$^+$ (Erythroid)	−
B220$^+$ (B cell)	+
c-kit$^+$ (Progenitor cell)	+
Spleen	
CD4$^+$ (T cell)	+
CD8$^+$ (T cell)	+
B220$^+$IgM$^+$ (B cell)	+
Tymus	
CD4$^+$CD8$^-$ (T cell)	+
CD4$^-$CD8$^+$ (T cell)	+
CD4$^+$CD8$^+$ (T cell)	+
CD4$^-$CD8$^-$ (T cell)	+

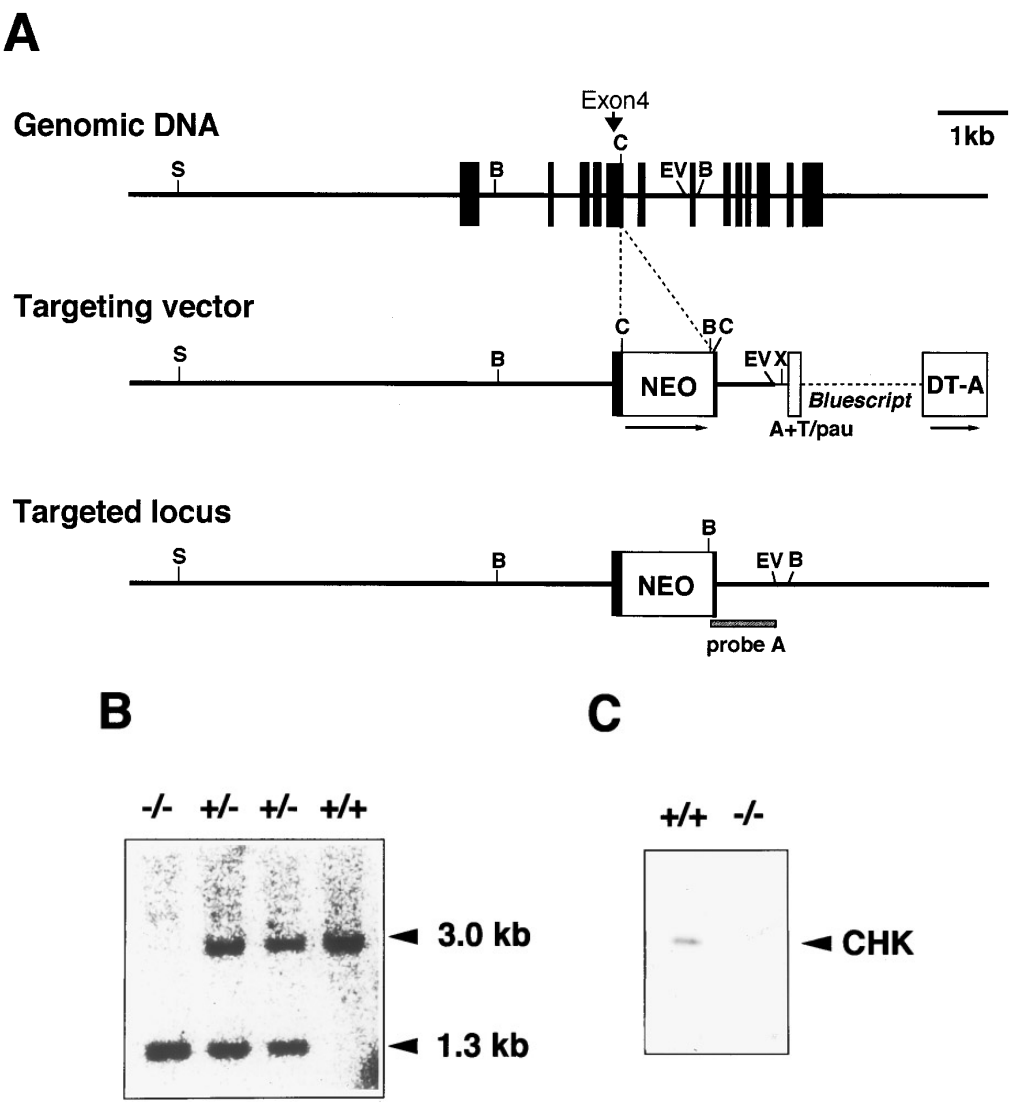

FIG. 2. Targeted mutation of CHK/HYL locus. (A) Top: restriction map of CHK/HYL locus. S, *Sal*I; B, *Bam*HI; C, *Cla*I; EV, *Eco*RV. Black boxes indicate 12 exons (exon 1a-12). Middle: the map of targeting vector. *Pgk-1 neo*^{r} cassette lacking a poly(A)$^+$ signal was inserted into the *Cla*I site of exon 4. A+T/pau, mRNA destabilizing sequence and pausing signal; DT-A, diphtheria toxin cassette. Arrows indicate orientations of the inserted cassettes. Bottom: the predicted map of the mutated allele following homologous recombination with targeting vector. Shaded boxes indicate location of fragments used as a probe in Southern blot analysis (probe A). (B) Southern blot analysis using a probe outside of the targeting vector (probe A). Genomic DNA from the tails of CHK/HYL+/+ and −/− mice was digested with *Bam*HI. *Bam*HI digestion generates a 3.0 kb product in the wild type allele and a 1.3 kb product in the targeted allele. (C) Brain lysates of CHK/HYL+/+ and −/− mice were immunoprecipitated with a polyclonal anti-CHK/HYL antibody and were immunoblotted with anti-CHK/HYL MoAb.

and thymic cells from CHK/HYL−/− and +/+ mice were stained with each antibody, and were analyzed using a FACScan. Bone marrow cells were cultured with 100 U/ml GM-CSF for 6 days, and were analyzed using a FACScan.

RT-PCR analysis of fractionated hematopoietic cells. Fractionated bone marrow, spleen, and thymic cells were prepared as described above, and total RNA was purified. cDNA was then synthesized. Cycling parameters were

TABLE 2
Blood Counts of CHK+/+ and −/− Mice

	WBC ($\times 10^2/\mu$l)	RBC ($\times 10^4/\mu$l)	PLT ($\times 10^4/\mu$l)
+/+	73.1 ± 27.4	939.0 ± 75.0	45.2 ± 19.4
−/−	60.0 ± 35.9	960.1 ± 77.3	45.8 ± 21.1

(mean ± S.D.)

1 minute at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C for 40 cycles. Primer sequences were: sense, 5′-TGGTTTCATGGCAAGATCTC-3′; antisense, 5′-AGTCAGATGCTGCAGGTCGAGTA-3′; PCR product (345 bp) was detected by electrophoresis on a 2% agarose gel.

Generation of CHK/HYL knockout mice. 12kb CHK/HYL genomic DNA was isolated from a mouse genomic library of TT2 ES cells (15). The CHK/HYL targeting vector consisted of a long arm of 7.5 kb of CHK/HYL genomic sequence, a *pgk-1 neo*^{r} cassette lacking a polyadenylation signal, a short arm of 1.3 kb from the 3′ region of mouse CHK/HYL, and a diphtheria toxin A (DT-A) gene. The *pgk-1 neo*^{r} cassette was inserted into the *Cla* I site located in exon 4. Negative selection for homologous recombinant was made using the DT-A gene at the 3′ end of a short arm. The TT2 ES cells were cultured, electroporated with the *Not*I-linearized targeting vector, and selected against

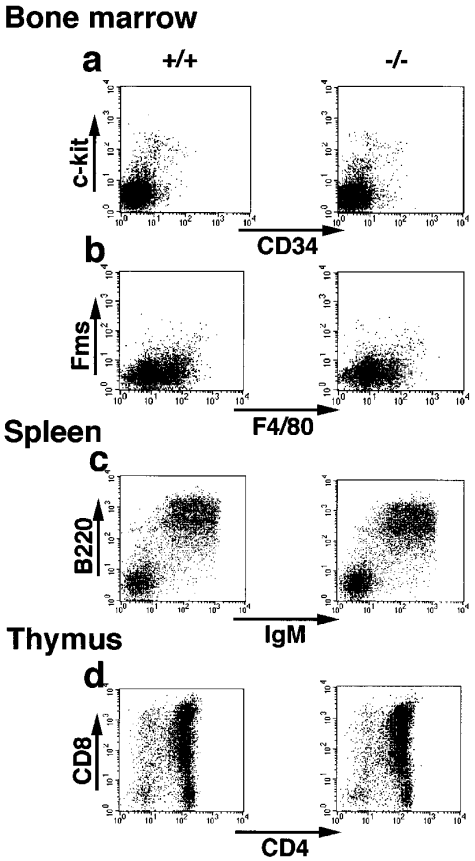

FIG. 3. Hematopoietic populations in the bone marrow, spleen, and thymic cells. Cells from the bone marrow, spleen, thymus of 8-week-old CHK/HYL mutant mice and wild-type littermates were examined with the following combinations of MoAb: anti-c-kit/anti-CD34 (a); anti-Fms/anti-F4/80 (b); anti-B220/anti-IgM (c); anti-CD8/anti-CD4 (d).

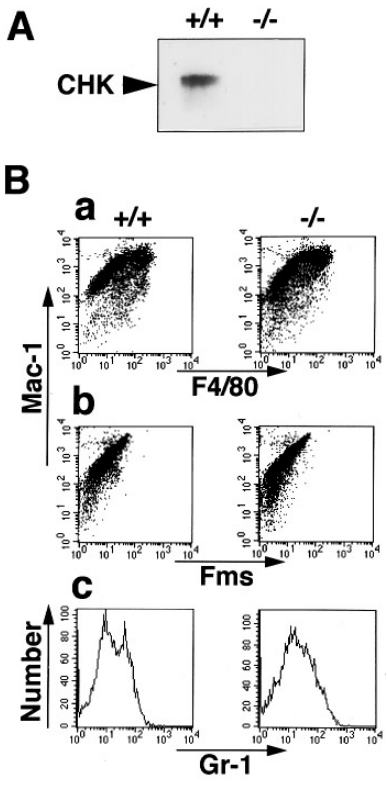

FIG. 4. The population of cultured bone marrow cells and CHK/HYL expression. (A) Lysates of cultured bone marrow cells were immunoprecipitated with a polyclonal anti-CHK/HYL antibody and were immunoblotted with anti-CHK/HYL MoAb. (B) Cultured bone marrow cells of CHK/HYL mutant mice and wild-type littermates were examined with the following combinations of MoAb: anti-Mac-1/anti-F4/80 (a); anti-Mac-1/anti-Fms (b); anti-Gr-1 (c).

G418 (GIBCO BRL, Gaithersburg, MD) as described (16,17). Homologous recombinants were screened by Southern blot analysis using a probe outside of the targeting vector (probe A) after digestion with *Bam*H1. Two independent germ-line chimeras were crossed with C57BL/6 females to produce hybrid animals, and these F1 offsprings were backcrossed one generation to C57Bl/6 to establish mutant strains for subsequent analysis. Two independent murine lines containing the CHK/HYL mutation were established from independent chimeras.

Immunoblot analysis and in vitro kinase assays. Brain and cultured bone marrow cells were lysed (18), and were immunoprecipitated with the specific antibody at 4°C for 1hr, followed by incubation with protein G-coupled beads (Pharmacia, Uppsala, Sweden) for 30min at 4°C. Beads were washed and incubated in kinase reaction buffer [50 mM HEPES (pH7.4), 10mM $MnCl_2$, 0.01% Triton X-100] plus 2.5 mg/mL enolase with 0.25 mCi/mL of [γ-^{32}P]ATP and 1μM ATP at 30 °C for 10 min. Phosphorylated proteins were run on 10% SDS-polyacrylamide gels, and were analyzed by BAS2000 II (Fuji Photo Film, Japan).

RESULTS AND DISCUSSION

Expression of CHK/HYL. We analyzed the expression of mouse CHK/HYL mRNA by Northern blotting and RT-PCR. As shown in Figure 1, CHK/HYL mRNA was evidently expressed in the brain and DA-1 cells (undifferentiated myeloid). Using PCR techniques, the expression of CHK/HYL was found in large spectrum of hematopoietic cells except for erythroid cells (Table 1.) CHK/HYL might play an important role in hematopoietic cells.

Generation of mice with mutated CHK/HYL gene. The CHK/HYL gene was disrupted in

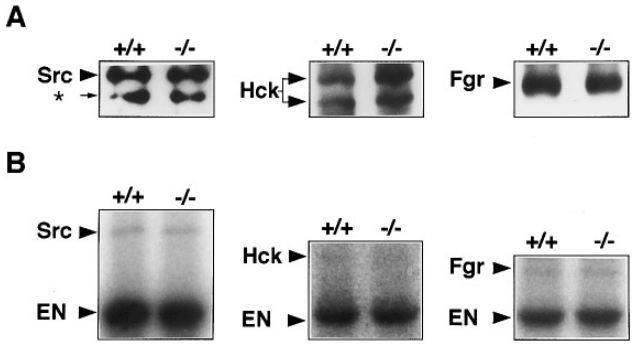

FIG. 5. The expression and activity of Src family kinases in cultured bone marrow cells. Src, Hck, and Fgr were immunoprecipitated with each MoAb from bone marrow cultured cells of CHK/HYL mutant mice and wild-type littermates. An equal part of these immunoprecipitates were immunobloted with each MoAb (A). Aliquots of those were subjected to kinase assay by enolase as a substrate (B). Asterisk and EN indicate a degradation product of Src and enolase, respectively.

TT2 ES cells by homologous recombination (Figure 2a). Four hundred G418-resistant ES colonies were screened by Southern blot analysis using probe A. As shown in Figure 2b, a 3.0 kb product in the wild type allele and a 1.3 kb in the targeted allele were detected in selected ES cells. Three independent homologous recombinant clones were obtained. Of these, two independent lines of homozygous mice were generated from heterozygous parental mice. The birth rate of homozygous mice coincided with the predicted Mendelian frequency. Mice with carrying the mutated CHK/HYL allele were healthy under specific pathogen-free conditions at least until one year without retardation in size and behavior, and were normally fertile. As shown in Figure 2c, the lack of CHK/HYL protein in mutant mice was confirmed by immunoprecipitation analysis.

Although it is reported that CSK deficient mice died until E11.5 with a defect in the neural tube formation (16,19), CHK/HYL deficient mice were apparently healthy, and histological findings of CHK/HYL−/− brain showed no significant abnormalities (data not shown).

Normal hematopoiesis in mutant mice. With regard to the peripheral blood of CHK/HYL−/− mice, blood counts were within normal range (Table 2) and white cell differential counts were comparable to that of normal littermates (data not shown). No increased bleeding tendency of mutant mice were seen, after their tails were cut. Macroscopically, the sizes of thymus and spleen in mutant mice were comparable with those of control littermates (data not shown).

Bone marrow, spleen, and thymic cells of the mutant mice or normal littermates (6 week-old) were analyzed by FACS. Hematopoietic stem cells, monocytes/macrophages, B-cells, and T-cells were fractionated with c-kit$^+$CD34$^+$, Fms$^+$F4/80$^+$, B220$^+$IgM$^+$, and CD8$^+$CD4$^+$, respectively. As shown in Figure 3, the distribution patterns of mutant cells were normal. Methylcellulose culture of CHK/HYL−/− bone marrow and spleen cells with each factor (SCF, IL-3, Epo, G-CSF, GM-CSF, M-CSF, and IL-7) demonstrated normal colony formation (data not shown). With regard to the function of CHK/HYL in megakaryopoiesis, CHK/HYL has been reported to participate in megakaryocyte progenitor proliferation (11). CHK/HYL−/− bone marrow cells contained a similar number of megakaryocytes compared with control (data not shown). Taken together, significant differences in hematopoiesis between CHK/HYL−/− and +/+ mice were not observed. The lack of an intact CHK/HYL gene did not appear to affect normal hematopoiesis *in vivo*. However, we found that CHK/HYL was translocated from the plasma membrane to the

cytoskeleton in platelets upon thrombin stimulation (Hirao et al., submitted), suggesting a role of CHK/HYL in platelet activation.

Analysis of Src family kinases in CHK/HYL deficient hematopoietic cells. To further analyze a role of CHK/HYL in hematopoietic cells, we prepared cultured bone marrow cells. CHK/HYL was expressed in these cells (Figure 4a) which were composed of macrophages and granulocytes (Figure 4b). To determine whether the defect of CHK/HYL could alter Src family kinases in the cells, we examined the expression level of Src, Hck, and Fgr proteins and their kinase activities. As shown in Figure 5a, the expression of Src kinase in CHK/HYL−/− cells was much the same as that in +/+ cells. With regard to the expression of Hck or Fgr, there was also little difference between CHK/HYL−/− and +/+ cells. Autophosphorylated Src was detected at 60 kDa, and phosphorylated exogenous substrate, enolase, at 45 kDa (Figure 5b). The phosphorylations of enolase were nearly the same between CHK/HYL−/− and +/+ cells. Thus, the Src kinase activity of CHK/HYL−/− cells were comparable with that of CHK/HYL+/+ cells. Also, the kinase activities of both Hck and Fgr in CHK/HYL−/− cells was the same as those in +/+ cells (Figure 5b). It is, however, reported that the kinase activity of Src is greatly increased in CSK−/− cells (16,19). The results presented here suggest that CHK/HYL may not modulate these Src kinases or may be compensated by CSK in macrophages and granulocytes.

ACKNOWLEDGMENTS

We thank Naoki Takeda for his technical assistance and Dr. Daniel G. Tenen for his critical reading of our manuscript. This work was supported by Grants-in-aid from the Ministry of Education, Science and Culture, Japan, and Research Fellowhsips of the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

1. Bolen, J. B. (1993) *Oncogene* **8,** 2025–2031.
2. Bennett, B. D., Cowley, S., Jiang, S., London, R., Deng, B., Grabarek, J., Groopman, J. E., Goeddel, D. V., and Avraham, H. (1994) *J. Biol. Chem.* **269,** 1068–1074.
3. Sakano, S., Iwama, A., Inazawa, J., Ariyama, T., Ohno, M., and Suda, T. (1994) *Oncogene* **9,** 1155–1161.
4. Klages, S., Adam, D., Class, K., Fargnoli, J., Bolen, J. B. and Penhallow, R. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2597–2601.
5. Chow, L. M. L., Jarvis, C., Hu, Q., Nye, S. H., Gervais, F. G., Veillette, A. and Matis, L. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4975–4979.
6. McVicar, D. W., Lal, B. K., Lloyd, A., Kawamura, M., Chen, Y.-Q., Zhang, X., Staples, J. E., Ortaldo, J. R., and O'Shea, J. J. (1994) *Oncogene* **9,** 2037–2044.
7. Kuo, S. S., Moran, P., Gripp, J., Armanini, M., Phillips, H. S., Goddard, A., and Caras, I. W. (1994) *J. Neurosi. Res.* **38,** 705–715.
8. Hamaguchi, I., Iwama, A., Yamaguchi, N., Sakano, S., Matsuda, Y., and Suda, T. (1994) *Oncogene* **9,** 3371–3374.
9. Brinkley, P. M., Class, K., Bolen, J. B., and Penhallow, R. C. (1995). *Gene* **163,** 179–184.
10. Musso, T., Varesio, L., Zhang, X., Rowe, T. K., Ferrara, P., Ortaldo, J. R., O'Shea, J. J., and McVicar, D. W. (1994) *J. Exp. Med.* **180,** 2383–2388.
11. Avraham, S., Jiang, S., Ota, S., Fu, Y., Deng, B., Dowler, L. L., White, R. A., and Avraham, H. (1995) *J. Biol. Chem.* **270,** 1833–1842.
12. Chomczynski, P., and Sacchi, N. (1987) *Anal Biochem.* **162,** 156–159.
13. Sudo, T., Nishikawa, S., Ogawa, M., Kataoka, H., Ohno, N., Izawa, A., Hayashi, S., and Nishikawa, S. (1995) *Oncogene* **11,** 2469–2476.
14. Ohbo, K., Suda, T., Hashiyama, M., Mantani, A., Ikebe, M., Miyakawa, K., Moriyama, M., Nakamura, M., Katsuki, M., Takahashi, K., Yamamura, K., and Sugamura, K. (1996) *Blood* **87,** 956–967.
15. Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., and Aizawa, S. (1993) *Anal. Biochem.* **214,** 70–76.
16. Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M., and Aizawa, S. (1993) *Cell* **73,** 1125–1135.

17. Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y., and Aizawa, S. (1993) *Anal. Biochem.* **214,** 77–86.
18. Okada, M., Nada, S., Yamanashi, Y., Yamamoato, T., and Nakagawa, H. (1991) *J. Biol. Chem.* **226,** 24249–24252.
19. Imamoto, A., and Soriano, P. (1993) *Cell* **73,** 1117–1124.