THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 279, No. 24, Issue of June 11, pp. 24986–24993, 2004
Printed in U.S.A.

Roles for c-Myc in Self-renewal of Hematopoietic Stem Cells*

Received for publication, January 14, 2004, and in revised form, March 18, 2004
Published, JBC Papers in Press, April 2, 2004, DOI 10.1074/jbc.M400407200

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Notch and HOXB4 have been reported to expand hematopoietic stem cells (HSCs) *in vitro*. However, their critical effector molecules remain undetermined. We found that the expression of c-*myc*, cyclin D2, cyclin D3, cyclin E, and E2F1 was induced or enhanced during Notch1- or HOXB4-induced self-renewal of murine HSCs. Since c-Myc can act as a primary regulator of G_1/S transition, we examined whether c-Myc alone can induce self-renewal of HSCs. In culture with stem cell factor, FLT3 ligand, and IL-6, a 4-hydroxytamoxifen-inducible form of c-Myc (Myc/ERT) enabled murine $Lin^-Sca\text{-}1^+$ HSCs to proliferate with the surface phenotype compatible with HSCs for more than 28 days. c-Myc activated by 4-hydroxytamoxifen augmented telomerase activities and increased the number of CFU-Mix about 2-fold in colony assays. Also, in reconstitution assays, HSCs expanded by c-Myc could reconstitute hematopoiesis for more than 6 months. As for the mechanism of c-*myc* induction by Notch1, we found that activated forms of Notch1 (NotchIC) and its downstream effector recombination signal-binding protein-J κ (RBP-VP16) can activate the c-*myc* promoter through the element between −195 bp and −161 bp by inducing the DNA-binding complex. Together, these results suggest that c-Myc can support self-renewal of HSCs as a downstream mediator of Notch and HOXB4.

Hematopoietic stem cells (HSCs)[1] are characterized by two distinct abilities: self-renewal ability and pluripotentiality. With these activities, HSCs supply all lineages of hematopoietic cells throughout their life. In a murine experimental model, only a single HSC with the $CD34^{low/-}c\text{-}Kit^+Sca\text{-}1^+$ phenotype can reconstitute whole hematopoiesis *in vivo* (1, 2). To maintain homeostasis of hematopoiesis and protect exhaustion of HSC population, most of the HSCs are kept quiescent, and only a limited number of cells enter the cell cycle to supply mature blood cells (3). As external regulatory factors, various cytokines such as stem cell factor (SCF), Flt3 ligand (FL), thrombopoietin, IL-3, and IL-6 promote the growth of HSCs (4–6). The combination of SCF, FL, thrombopoietin, and IL-6, in particular, was reported to efficiently induce the *in vitro* cell division with characteristics of self-renewal in HSCs (7). In contrast, transforming growth factor-β is known to inhibit the growth of HSCs (8, 9). Besides these cytokines, the direct interactions with stromal cells and extracellular matrix in the bone marrow microenvironment also influence the fates of HSCs (10, 11). As one mechanism responsible for this effect, Bernstein and co-workers (12) showed that the activation of Notch transmembrane receptors expressed on HSCs by their ligand (Jagged 1 or Jagged2) on stromal cells promotes the self-renewal of HSCs.

Furthermore, accumulated evidence indicated that the cell cycle state of HSCs is regulated by intrinsic transcription factors such as c-Myb and GATA-2. c-Myb promotes the growth of HSCs, and c-Myb-deficient mice die at embryonic day 15.5 due to the defect of definitive hematopoiesis (13). Similarly, $GATA\text{-}2^{-/-}$ mice are embryonic lethal around embryonic day 11.5 because of the defect in the development and/or maintenance of HSCs (14), whereas functional roles of GATA-2 in the growth of HSCs are still controversial (15, 16). Also, HOXB4, a member of Hox family of transcription factors, was reported to induce the marked *in vitro* expansion of HSCs (17, 18).

During the last decade, a number of cell cycle regulatory molecules such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors have been identified and characterized in various types of cells. As for the roles of these molecules in primitive hematopoietic cells, $p21^{WAF1}$ and $p27^{Kip1}$ keep the quiescence of HSCs and of progenitor cells, respectively, thereby governing their pool sizes (19, 20). Meanwhile, inactivation and/or deletion of $p16^{INK4A}$ and $p15^{INK4b}$ genes are supposed to contribute to leukemogenesis in a substantial proportion of *all* cases (21, 22). These results imply that appropriate cell cycle control, particularly at the early stage of stem/progenitor cells, is required for maintaining normal hematopoiesis.

c-Myc belongs to a family of transcription factors containing basic, helix-loop-helix, and leucine zipper domains. The expression of c-Myc is absent in quiescent cells and is rapidly induced by growth factors in normal tissues, whereas it is continuously overexpressed in a variety of neoplasms (23–25). In addition, c-Myc is highly expressed in various tissues during embryogenesis, and $c\text{-}myc^{-/-}$ mice die at embryonic day 10.5, indicating that c-Myc is essential for normal embryonic development (26). c-Myc, in conjunction with its heterodimer partner Max, regulates gene transcription through the element called the E box (CACGTG) (27, 28). c-Myc induces a number of target molecules involved in G_1/S transition such as E2Fs, CDC25A, CDK2, CDK4, Cul1, Id2, and Rb (29–35). Also, c-Myc activates cyclin E/CDK2 complexes alone or in combination with Ras-

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[1] The abbreviations used are: HSC, hematopoietic stem cell; 4-HT, 4-hydroxytamoxifen; SCF, stem cell factor; FL, Flt3 ligand; RBP, recombination signal-binding protein; CDK, cyclin-dependent kinase; Ab, antibody; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; BM, bone marrow; h, human; m, murine.

mediated signals (36–38). With these activities, c-Myc works at several points during the cell cycle. In fact, fibroblasts isolated from c-*myc*-deficient rats revealed a markedly prolonged cell doubling time accompanied by the drastic reduction of CDK4/6 and CDK2 activities (32, 33). Furthermore, ectopic expression of c-Myc in quiescent cells under some conditions is sufficient for inducing S phase entry (39). These lines of evidence indicates that c-Myc plays a central role in G_1/S transition as an upstream regulator of cell cycle regulatory molecules (40).

Once HSCs are induced to enter cell cycle under certain conditions, HSCs are obliged to select either self-renewal or differentiation during cell division. In this process, Notch signals and HOXB4 promote self-renewal of HSCs rather than differentiation as described above. Therefore, it is speculated that there must be a set of genes induced by Notch signals or HOXB4 contributing to self-renewal of HSCs. However, at present, their critical target molecules remain to be determined. Moreover, it remains unknown how this process is regulated by cell cycle regulatory molecules.

To clarify the roles of cell cycle regulatory molecules in the cell division of HSCs, in this study, we investigated the changes of their expression during Notch- or HOXB4-induced self-renewal of HSCs. We found that c-Myc was transcriptionally induced by Notch and HOXB4. In addition, we found that its ectopic expression induced the growth of HSCs without disrupting their biologic properties in terms of surface phenotypes, colony-forming activities, and reconstituting activities. Together, these results suggest that c-Myc plays a major role in the self-renewal of HSCs as an effector molecule of Notch signals and HOXB4.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human (h) interleukin-6 (hIL-6), murine (m) IL-3, and murine stem cell factor (mSCF) were provided by Kirin Brewery Company (Tokyo, Japan). Human flt3-ligand (hFL) was purchased from PEPROTECH (London, UK). Fluorescein isothiocyanate-conjugated rat IgG1 and biotinylated rat IgG2b were purchased from IMMUNOTECH (Marseilles, France). Biotinylated antilineage antibodies (Abs) against Gr-1 (RB6-8C5), B220 (RA3-6B2), CD3 (145-2C11), Mac1 (M1/70), and Ter119 (TER119); a fluorescence-labeled anti-Sca-1 Ab (D7), an allophycocyanin-labeled anti-c-Kit Ab (2B8), a fluorescein isothiocyanate-conjugated anti-Ly5.1 Ab (A20), a biotinylated anti-Ly5.2 Ab (104), and streptavidin-phycoerythrin were purchased from PharMingen. A fluorescein isothiocyanate-conjugated anti-CD34 Ab was kindly provided by Dr. T. Hirano (Osaka University, Osaka, Japan).

Plasmid Constructs—An expression vector encoding murine-activated Notch 1(NotchIC) (pSG5mNotchIC) was provided by Dr. G. W. Bornkamm (Institute for Clinical Molecular Biology, Munich, Germany); Notch1/ERT (rNERTneo) was provided by Dr. U. Just (Institute for Clinical Molecular Biology, Munich, Germany) (41); FLAG-tagged RBP-VP16 (pSG5FLAG-RBP-VP16) was provided by Dr. E. Manet (Unite de Virologie Humaine, Lyon, France); HOXB4 (pMSCVneoHOXB4) was provided by Dr. R. K. Humphries (British Columbia Cancer Agency, Vancouver, Canada). A retrovirus expression vector for Myc/ERT was generated by subcloning its cDNA into pMSCVneo (Clontech).

Flow Cytometry—The DNA content of cultured cells was examined by staining with propidium iodide and analyzed on FACSort (BD Biosciences). Cell cycle analysis was performed with the program Modfit LT2.0 (BD Biosciences). Surface phenotypes of cultured cells were examined with FACSCalibur (BD Biosciences).

Semiquantitative Reverse Transcription (RT)-PCR Analyses—Semiquantitative RT-PCR analyses were performed as reported previously (42). Briefly, total cellular RNA was extracted from cultured cells (about 10^5 cells) and reverse-transcribed into cDNA with oligo(dT) primers (Amersham Biosciences) using SuperScript II reverse transcriptase (Invitrogen). PCR was performed in a total volume of 30 μl using 1 μl of the cDNA product as a template. The primer sets to amplify c-*myc*, cyclin D1, cyclin D2, cyclin D3, cyclin E, E2F1, and β-actin are as follows: c-*myc*, 5′-TCACCAGCACAACTACGCCG-3′ and 5′-CAGGATGTAGGCGGTGGCTT-3′; cyclin D1, 5′-AGGCGGATGAGAACAAGCAGA-3′ and 5′-CAGGCTTGACTCCAGAAGGG-3′; cyclin D2, 5′-AAGGAGAAGCTGTCCCTGATC-3′ and 5′-GAACTGCTGCAGGCTGTTCAG-3′; cyclin D3, GCGTCCCCACCCGAAAGGCG-3′ and CCAGGAAGTCGTGCGCAATC-3′; cyclin E, 5′-CCCAGCAGTAAGAAGGCAGAG-3′ and 5′-CAGCTTCTGGAGCACTCAGTG-3′; E2F1, 5′-GACTGTGACTTTGGGACC-3′ and 5′-TGTTCACCTTCATTCCC-3′; β-actin, 5′-CATCACTATTGGCAACGAGC-3′ and 5′-ACGCAGCTCAGTAACAGTCC-3′. The samples were denatured at 94 °C for 10 min followed by 20–35 cycles of amplification (94 °C, 30 s for denaturation; 56 °C, 30 s for annealing; 72 °C, 30 s for extension). At first, we adjusted the amount of cDNA products among several samples based on the amount of β-actin PCR products. Then, an equal amount of cDNA products was subjected to the PCR. The amount of each mRNA was evaluated after 29–35 cycles of PCR, during which PCR products were exponentially amplified in all of the samples. The PCR products were electrophoresed on agarose gels, and their amount was evaluated by staining with SYBR GREEN I (BioWhittaker Molecular Applications, Rockland, ME).

Luciferase Assays—To construct reporter genes containing various fragments of murine c-*myc* promoter, we performed PCR and subcloned PCR products into the luciferase plasmid. Luciferase assays were performed with a Dual-Luciferase reporter system (Promega, Madison, WI) as described previously (42). In short, 293T cells (2×10^6 cells) cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum were seeded in a 60-mm dish and transfected with the appropriate effector gene (2 μg) and reporter gene (2 μg) together with 5 ng of pRL-CMV-Rluc, an expression vector of *renilla* luciferase, by the calcium phosphate coprecipitation method. After 12 h, the cells were washed and then serum-deprived for 24 h. Then, the cells were lysed and subjected to the measurement of the firefly and *renilla* luciferase activities on a luminometer LB96P (Berthold Japan, Tokyo, Japan). The relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the *renilla* luciferase activities. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments.

Electrophoretic Mobility Shift Assay (EMSA)—Seven types of double-stranded oligonucleotides were used as probes or competitors, and their sequences are shown in the legend for Fig. 6.

Assays for Telomerase Activities—Telomerase activities were measured by the stretch PCR method with a TeloChaser system (TOYOBO, Osaka, Japan) according to the manufacturer's instructions.

Isolation of Murine Bone Marrow Hematopoietic Stem/Progenitor Cells—We isolated murine bone marrow hematopoietic stem/progenitor cells as reported previously (42). Briefly, bone marrow cells were harvested from 8–10-week-old Ly5.2 mice pretreated with 150 mg/kg of 5-fluoruracil for 4 days. Mononuclear cells were isolated by density gradient centrifugation. Then, Lin (CD3, B220, Ter119, Mac1, and Gr-1)$^-$ Sca-1$^+$ cells were collected using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). At this step, we confirmed that more than 95% of the separated cells were Lin$^-$Sca-1$^+$ cells by flow cytometric analysis.

Retrovirus Transfection into Murine Bone Marrow Cells—At first, we prepared conditioned medium containing high titer virus particles using Plat-E cells as described previously (42). The isolated Lin$^-$Sca-1$^+$ cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in the presence of mIL-3 (10 ng/ml), mSCF (50 ng/ml), hIL-6 (50 ng/ml), and hFL (30 ng/ml) for 72 h. Then, the cultured cells were cultured with conditioned medium containing high titer retrovirus in the presence of polybrene (8 μg/ml). After 24 h, the cells were washed and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing the same cytokines and 1 mg/ml G418 for 72 h. After the selection with G418, retrovirus-infected cells were cultured under the conditions as indicated.

Colony Assays—Retrovirus-infected cells (1×10^6 cells/35-mm dish) were cultured in the methylcellulose media containing human erythropoietin (3 units/ml), mIL-3 (10 ng/ml), hIL-6 (10 ng/ml), and mSCF (50 ng/ml) (Stem Cell Technologies, Vancouver, BC, Canada). G418 selection was continued during methylcellulose cultures. The number of colonies was counted after 12 days.

Transplantation Assays—Ly5.2 mice were lethally irradiated (950 radians) 24 h before the transplantation. For transplantation assays, we prepared Myc/ERT-transduced bone marrow cells from congenic C57BL/6 (B6-Ly5.1) mice. Myc/ERT-transduced Ly5.1 cells (4×10^6 cells) were injected intravenously in combination with 1×10^5 normal bone marrow cells with Ly5.2 phenotype.

Statistical Analysis—Statistical analysis was performed with Student's *t* test.

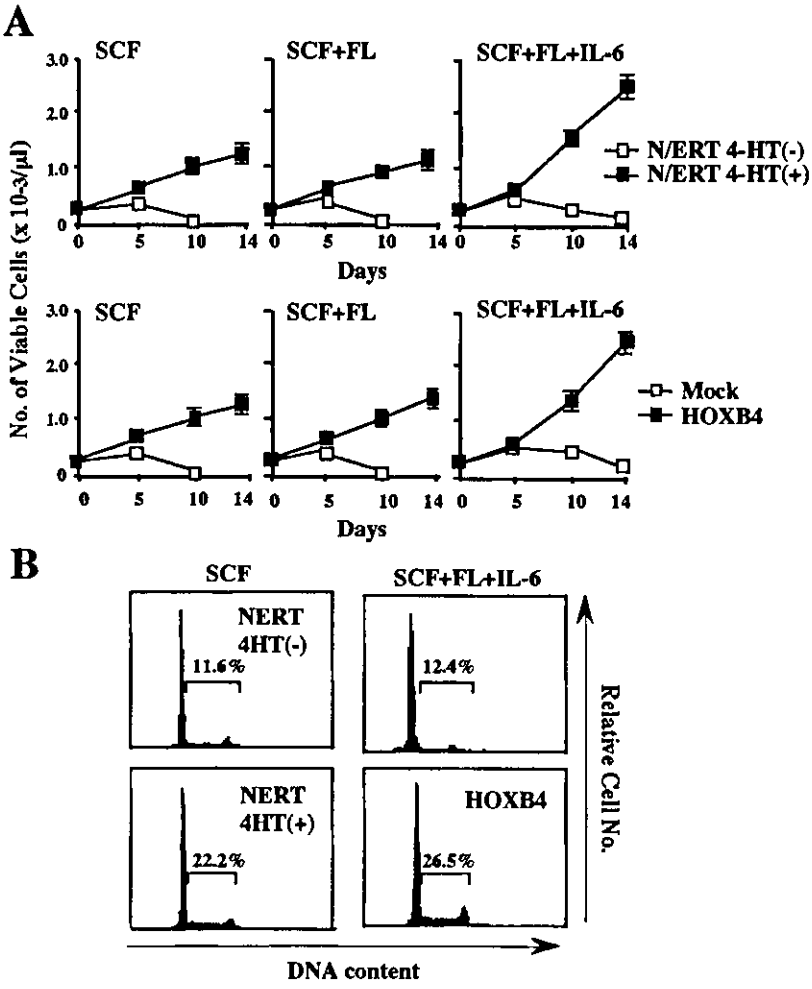

FIG. 1. **Effects of Notch1 and HOXB4 on the growth of murine Lin$^-$Sca-1$^+$ cells.** *A*, murine Lin$^-$Sca-1$^+$ cells were infected with retroviruses each containing the indicated gene and cultured for up to 2 weeks. The total number of viable cells was counted by the trypan blue dye exclusion method at the times indicated. The results are shown as mean ± S.D. of triplicated cultures. *B*, after 7-day cultures under the conditions indicated, the DNA content of the cultured cells was examined by propidium iodide staining. The proportion of the S-G_2/M phase is indicated.

RESULTS

The Effects of Notch1 and HOXB4 on the Expression of Cell Cycle Regulatory Molecules in Murine Lin$^-$Sca-1$^+$ Cells—First, we analyzed how self-renewal is regulated by cell cycle regulatory molecules in HSCs. For this purpose, we introduced a 4-HT-inducible form of Notch1 (N/ERT, a chimeric molecule consisting of the intracellular domain of Notch1 fused to the estrogen receptor) and HOXB4 into murine Lin$^-$Sca-1$^+$ cells since both molecules were reported to induce self-renewal in HSCs (17, 43). In the absence of 4-HT, N/ERT-transduced cells could not keep proliferating for 14 days under any cytokine combinations (*i.e.* SCF alone, SCF + FL, or SCF + FL + IL-6) (Fig. 1*A*, *upper panel*). In contrast, 4-HT-treated cells kept growing under the same cytokine combinations during 14 days. Similarly, HOXB4-transduced cells could proliferate for up to 14 days, whereas mock (an empty vector)-transduced cells could not (Fig. 1*A*, *lower panel*). In DNA content analysis, the 4-HT treatment increased the proportion of the cells in S-G_2/M phase from 11.6 to 22.2% in N/ERT-transduced cells under the culture with SCF (Fig. 1*B*, *left panel*). Also, HOXB4 augmented the proportion of the cells in S-G_2/M phase from 12.4 to 26.5% under the culture with SCF + FL + IL-6 (Fig. 1*B*, *right panel*).

Next, we examined the effect of Notch1 and HOXB4 on the expression of cell cycle regulatory molecules by semiquantitative RT-PCR analysis (Fig. 2). With or without the 4-HT treatment, total cellular RNA was extracted from N/ERT-transfected cells after a 7-day culture with SCF. Also, HOXB4-transfected cells were subjected to RT-PCR analysis after a 7-day culture with SCF. At first, we adjusted the amount of cDNAs according to the amounts of the β-actin PCR products (Fig. 2, *bottom panel*). Then, we evaluated the expression levels of c-*myc*, cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1. After 29–35 cycles of PCR, all of these PCR products were amplified exponentially. As shown in Fig. 2, the 4-HT treatment markedly induced or enhanced the expression of c-*myc*, cyclin D2, cyclin D3, cyclin E, and E2F1, whereas it could not

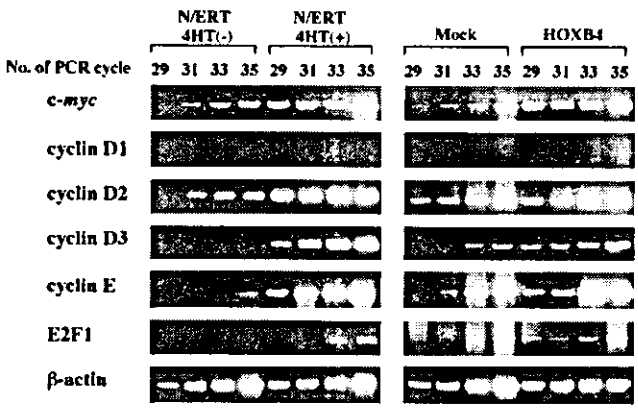

FIG. 2. **Effects of Notch1 and HOXB4 on the expression of cell cycle regulatory molecules.** Murine Lin$^-$Sca-1$^+$ cells were infected with retroviruses each containing the indicated gene, cultured for 7 days, and subjected to semiquantitative RT-PCR analyses on the expression of c-*myc*, cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1 mRNA. The PCR products were electrophoresed on agarose gels and visualized by staining with SYBR GREEN I.

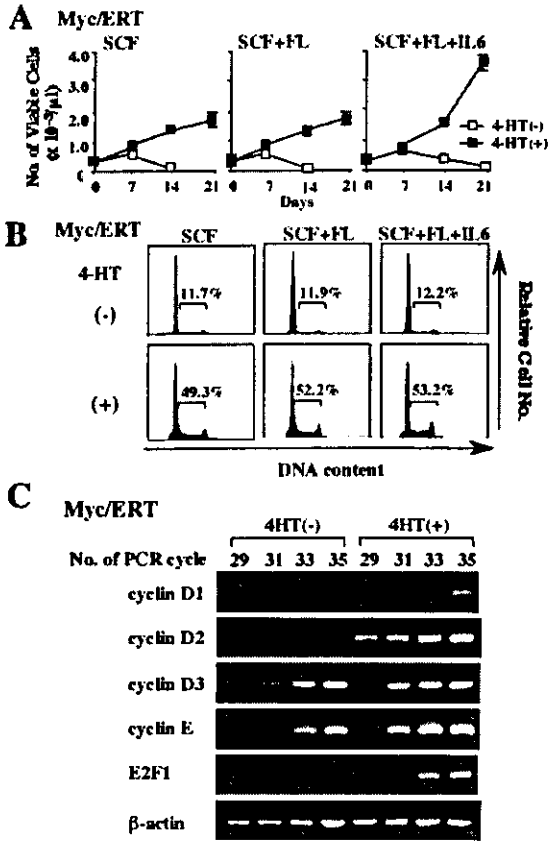

FIG. 3. **Effects of c-Myc on the growth of murine Lin⁻Sca-1⁺ cells.** *A*, murine Lin⁻Sca-1⁺ cells were infected with retroviruses containing Myc/ERT and cultured with or without 4-HT for up to 3 weeks. The total number of viable cells was counted by the trypan blue dye exclusion method at the times indicated. The results are shown as mean ± S.D. of triplicated cultures. *B*, after 7-day cultures under the conditions indicated, the DNA content of the Myc/ERT-transduced cells was examined by propidium iodide staining. The proportion of the S-G_2/M phase is indicated. *C*, after 7-day cultures with or without 4-HT, the Myc/ERT-transduced cells were subjected to semiquantitative RT-PCR analyses on the expression of cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1 mRNA.

induce the expression of cyclin D1. Similarly, we found that HOXB4 enhanced the expression of c-*myc*, cyclin D2, cyclin D3, cyclin E, and E2F1 but not of cyclin D1.

c-Myc Enhances and Supports the Growth of Murine Lin⁻Sca-1⁺ Cells—c-Myc is a primary regulator of G_1/S transition, and its ectopic expression can drive quiescent cells to S phase in fibroblasts (as described under the Introduction). Therefore, we examined the effect of c-Myc on the growth and biologic properties of HSCs. We retrovirally transduced a 4-HT-inducible form of Myc (Myc/ERT) to murine Lin⁻Sca-1⁺ cells. When treated with 4-HT, Myc/ERT-transduced cells proliferated for more than 21 days under the culture with SCF, SCF + FL, or SCF + FL + IL-6 (Fig. 3*A*). In contrast, 4-HT-untreated cells died within 21 days under the same cytokine combinations. In DNA content analysis, the 4-HT addition prominently increased the cells in S-G_2/M phase in Myc/ERT-transduced cells after 7 days (SCF, from 11.7 to 49.3%; SCF + FL, from 11.9 to 52.2%; SCF + FL + IL-6, from 12.2 to 53.2%) (Fig. 3*B*). Next, we examined the effects of c-Myc on the expression profile of cell cycle regulatory molecules. With or without the 4-HT treatment, we isolated total cellular RNA from Myc/ERT-transfected cells after a 7-day culture with SCF and performed semiquantitative RT-PCR analysis. In Myc/ERT-transduced cells, 4-HT-activated c-Myc induced or enhanced the expression of cyclin D1, cyclin D2, cyclin D3, cyclin E, and E2F1 mRNA (Fig. 3*C*), suggesting a possibility that c-Myc would be a primary regulator of Notch- or HOXB4-mediated cell growth among various cell cycle regulatory molecules.

Characterization of the Cells Expanded by Myc/ERT Using in Vitro Assays—The 4-HT treatment was able to keep Myc/ERT-transduced cells viable even after a 28-day culture with SCF or SCF + FL + IL-6 (data not shown). To investigate the biologic properties of these cells, we initially examined their surface phenotypes by fluorescence-activated cell sorter. Before the addition of 4-HT, 77.2% of the Myc/ERT-transduced cells were Sca-1⁺c-Kit⁺, and 94.7% (64.9 + 29.8) were Lin⁻ (including CD34-positive and negative cells). After a 28-day culture with SCF, more than 50% of the cultured cells still revealed immature surface phenotypes: Sca-1⁺c-Kit⁺ cells, 56.6%; Lin⁻ cells, 63.4% (24.5 + 38.9) (Fig. 4*A*). Similar results were also observed after a 28-day culture with SCF+FL+IL-6: Sca-1⁺c-Kit⁺ cells, 47.7%; Lin⁻ cells, 81.3% (32.0 + 49.3). Next, we analyzed the effects of c-Myc on the colony-forming ability of HSCs. After the 3-day selection with G418, Myc/ERT-transduced Sca-1⁺Lin⁻ cells were seeded into methylcellulose media (containing IL-3, IL-6, SCF, and erythropoietin) and cultured with or without 4-HT for 12 days. As shown in Fig. 4*B*, 4-HT-activated Myc/ERT increased the number of CFU-Mix with a statistically significant difference $p < 0.05$. Also, Myc/ERT significantly increased burst-forming unit-E ($p < 0.05$) and colony forming units-granulocyte/macrophage ($p < 0.01$). We also analyzed changes of telomerase activity in the Myc/ERT-transduced cells. Quiescent HSCs have been reported to exhibit a low level of telomerase activity, which is up-regulated in response to cytokine stimulation (44, 45), whereas it declines during the maturation process (46). After a 7-day culture in the presence of SCF with or without the 4-HT treatment, we isolated cell extracts from Myc/ERT-transduced cells and evaluated telomerase activity by the stretch PCR method. As observed in a positive control, Hela, the 4-HT treatment induced telomerase activity in Myc/ERT-transduced cells, of which specificity was confirmed by its heat instability. In contrast, Myc/ERT-transduced cells cultured without 4-HT did not have telomerase activity (Fig. 4*C*). Together, these results suggest that c-Myc-induced cell growth does not spoil the immature characteristics of HSCs.

Reconstitution Assays Using HSCs Expanded by Myc/ERT—Next, we evaluated the *in vivo* function of Myc/ERT-expanded HSCs with reconstitution assays. After 12-day cultures, 4-HT-treated or untreated Myc/ERT-transduced cells (4×10^6 cells) with Ly5.1 phenotype were transfused into lethally irradiated Ly5.2 mice in combination with 1×10^5 Ly5.2⁺ normal bone marrow cells. When 4-HT-treated cells were transplanted, 11.3% of the peripheral blood cells were Ly5.1⁺ in the recipient mice after 4 weeks (Fig. 4*D*, *upper left panel*). In contrast, Ly5.1⁺ cells were hardly detected in the mice transplanted with 4-HT-untreated cells after 4 weeks (Fig. 4*D*, *upper right panel*). Furthermore, Ly5.1⁺ cells were still detected in 7.9% of the peripheral blood cells in the mice transplanted with 4-HT-treated cells even after 12 weeks (Fig. 4*D*, *lower left panel*). In this Ly5.1⁺ fraction, 15% of the cells were Mac1/Gr-1⁺, 37.1% were CD4/8⁺, and 50.7% were B220⁺, indicating that HSCs expanded by Myc/ERT has an ability of multilineage reconstitution. Also, these cells were able to supply hematopoietic cells in the recipient mice for more than 6 months (data not shown). Collectively, these results suggested that ectopically introduced c-Myc can induce self-renewal of HSCs.

The Effects of Notch Signaling and HOXB4 on c-myc Promoter Activities—Since c-Myc was considered to be important for self-renewal of HSCs, we next tried to clarify the mecha-

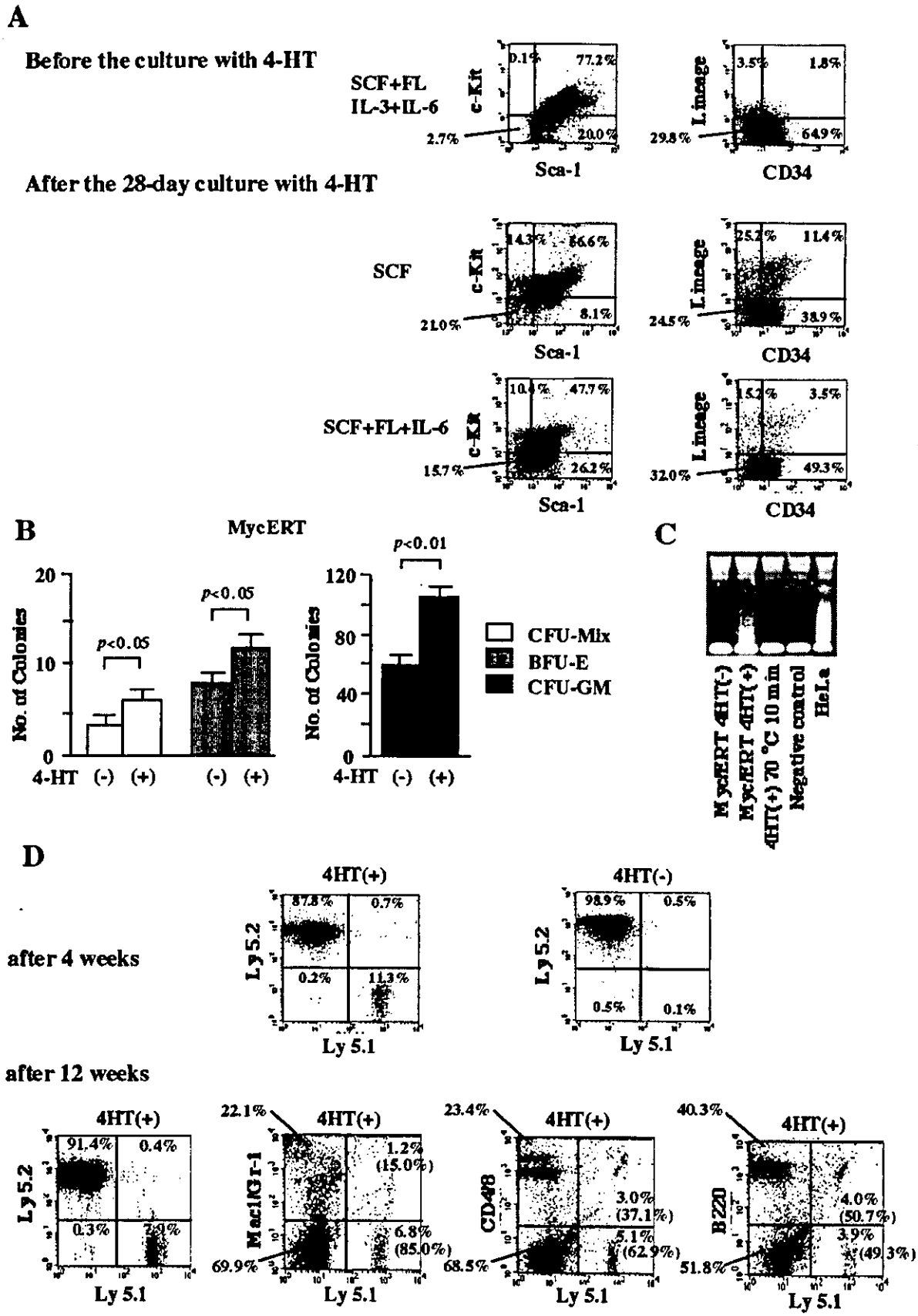

FIG. 4. **Characterization of the cells expanded and maintained by c-Myc for 28 days.** *A*, the surface phenotype of Myc/ERT-transduced cells was examined by flow cytometric analyses before and after the 28-day culture with 4-HT in the presence of SCF or SCF + FL + IL-6. The antilineage Abs recoguize Gr-1, B220, CD3e, Mac1 and Ter119. *B*, Myc/ERT-transduced murine $Lin^{-}Sca\text{-}1^{+}$ cells were cultured with G418 for 3 days, and the living cells were seeded into methylcellulose media (containing IL-3, IL-6, SCF, and erythropoietin). The number of the indicated

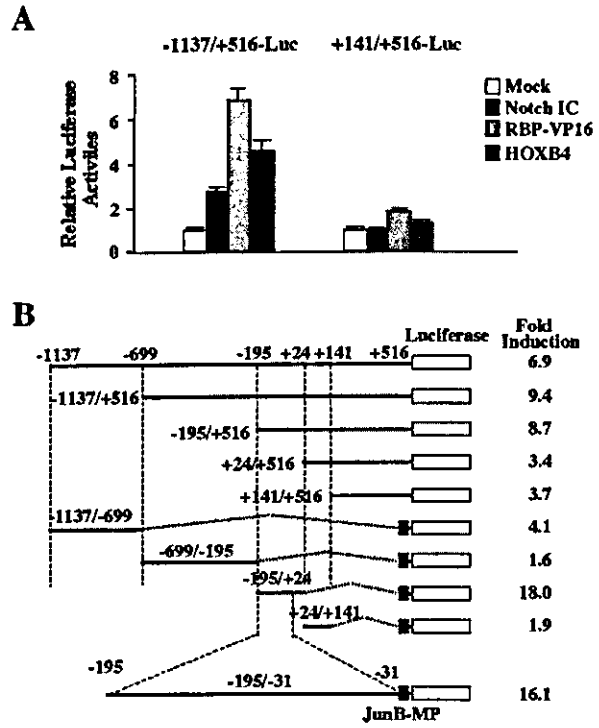

FIG. 5. **The effects of Notch signaling and HOXB4 on the activity of the c-*myc* promoter.** *A*, 293T cells seeded in 60-mm dish were transfected with 2 μg of the appropriate effector genes and reporter gene together with 5 ng of pRL-CMV-Rluc by the calcium phosphate coprecipitation method. After 12 h, the cells were washed and cultured without serum for 24 h and then subjected to luciferase assays. After normalizing transfection efficiencies according to the *renilla* luciferase activities, the relative firefly luciferase activities were calculated. The results are shown as the mean ± S.D. of triplicate cultures. *B*, structures of c-*myc* luciferase reporter genes are indicated in the *left panel*, and the effect of RBP-VP16 on the respective reporter gene is indicated as fold induction in the *right panel*.

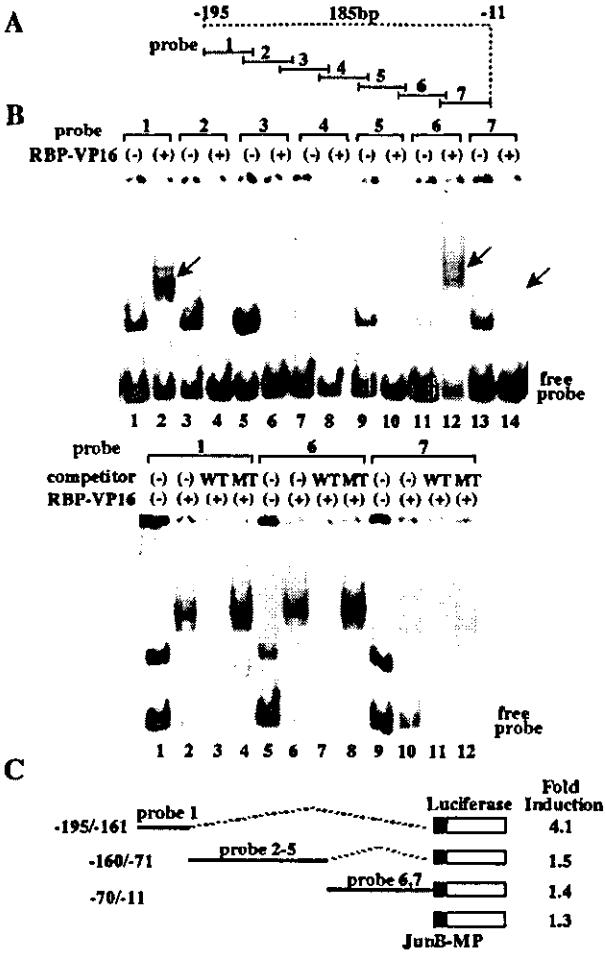

FIG. 6. **Analysis of the responsive element to RBP-VP16 in the c-*myc* promoter.** *A*, EMSA was performed with seven overlapping probes spanning between −195 bp and −11 bp in the c-*myc* promoter. The sequences of their probes are as follows: *probe 1*, 5′-ACTGGACOGCGGGCTGAGGCTCCTCCTCCTCTTTC-3′; *probe 2*, 5′-CTCCTCTTTCCCCGGCTCCCCACTAGCCCCCTCCC-3′; *probe 3*, 5′-GCCCCCTCCCGAGTTCCCAAAGCAGAGGGCGGGGA-3′; *probe 4*, 5′-AGGGCGGGGAAACGAGAGGAAGGAAAAAAATAGAG-3′; *probe 5*, 5′-AAAAATAGAGAGAGGTGGGGAAGGGAGAAAGAGAG-3′; *probe 6*, 5′-AGAAAGAGAGGTTCTCTGGCTAATCCCCGCCCACC-3′; *probe 7*, 5′-CCCGCCCACCCGCCCTTTATATTCCGGGGGTCTGC-3′. *B*, nuclear extracts were isolated from 293T cells transfected with FLAG-RBP-VP16 or a mock vector and subjected to EMSA. In competition assays, a 1000-fold molar excess of unlabeled wild-type or mutant competitor oligonucleotide was added to the binding mixture. *C*, 293T cells were transfected with the indicated reporter gene together with FLAG-RBP-VP16 as an effector gene. After 12 h, the cells were washed and cultured without serum for 24 h and then subjected to luciferase assays. The results are shown as the mean ± S.D. of triplicate cultures.

nism through which Notch1 and HOXB4 induce the c-*myc* mRNA expression. For this purpose, we performed luciferase assays with a reporter gene −1137/+516-Luc containing the murine c-*myc* promoter (numbered from the transcription initiation site) with NotchIC (an activated form of Notch1), RBP-VP16 (α transcriptionally active derivative of RBP-J), and HOXB4 as effectors. In 293T cells, NotchIC, RBP-VP16, and HOXB4 activated −1137/+516-Luc by 2.9-, 6.9-, and 4.7-fold, respectively, whereas these effector genes hardly stimulated its backbone vector +141/+516-Luc (Fig. 5*A*). Similar results were obtained from a murine fibroblast cell line NIH3T3 (data not shown). To determine which region is responsive to Notch in the c-*myc* promoter, we generated several reporter genes and performed luciferase assays with RBP-VP16 as an effector gene. As shown in Fig. 5*B*, −195/+24-Luc was most responsive to RBP-VP16 (18-fold induction). Also, the similar level (16.1-fold) of activation was induced by RBP-VP16 in −195/−31-Luc. To further identify which element is responsive to RBP-VP16, we isolated nuclear extracts from 293T cells transfected with FLAG-RBP-VP16 or a mock vector and performed EMSA with seven overlapping probes spanning between −195 and −11 of the c-*myc* promoter (Fig. 6*A*). As compared with DNA binding patterns formed from mock-transfected cells, nuclear extracts isolated from FLAG-RBP-VP16-transfected cells bound to the

colonies was counted after 12 days. The results are shown as mean ± S.D. of quintupled cultures. *BFU-E*, burst-forming unit-E. *CFU-GM*, colony forming units-granulocyte/macrophage. *C*, after 7-day cultures, the cultured cells were subjected to the stretch PCR method for the evaluation of telomerase activities. *Lane 1*, Myc/ERT-transduced cells cultured without 4-HT; *lane 2*, Myc/ERT-transduced cells cultured with 4-HT; *lane 3*, the heat-treated sample of *lane 2*; *lane 4*, negative control (PCR was performed without any template.); *lane 5*, Hela cells as a positive control. *D*, reconstitution assays using the cells expanded by Myc/ERT. MycERT-transduced Ly5.1 cells (4×10^6 cells) cultured with (*upper left panel*) or without (*upper right panel*) 4-HT were transplanted into lethally irradiated mice in combination with 1×10^5 normal bone marrow cells with Ly5.2 phenotype. The contribution of Myc/ERT-transduced Ly5.1 cells in the peripheral blood of the recipient mice was examined by flow cytometric analyses after 4 (*upper two panels*) and 12 weeks (*lower four panels*) from transplantation. The lineage distribution of Ly5.1 cells was examined with the indicated Abs. The relative frequency of the each fraction in the $Ly5.1^+$ cells is shown in the parenthesis.

probes 1, 6, and 7 (Fig. 6*B*, *upper panel*, *lanes 2*, *12*, and *14*, DNA-binding complexes are indicated by *arrows*), whereas a significant difference was not detected with the other probes. These binding complexes were abolished by non-labeled wild-type competitors (Fig. 6*B*, *lower panel*, *lanes 3*, *7*, and *11*) but not by mutated competitors (Fig. 6*B*, *lower panel*, *lanes 4*, *8*, and *12*), indicating that these complexes were formed in a sequence-specific manner. To further characterize the role of these sites in Notch signals, we performed luciferase assays with reporter genes each containing the sequence of the probe 1 (−195/−161-Luc) and probes 6–7 (−70/−11-Luc). As shown in Fig. 6*C*, RBP-VP16 activated −195/−161-Luc by 4.1-fold, whereas it was hardly effective on −70/−11-Luc, implying that the protein that binds to the probe 1 would contribute to the activation of the c-*myc* promoter by Notch signaling. However, this complex reacted neither to the anti-FLAG Ab nor to the anti-RBP-J Ab (data not shown). Also, the probe 1 did not contain the typical RBP-J-binding sequence. These results suggest that RBP-VP16 would activate this element indirectly. In addition, since the reactivity of −195/−161-Luc was not as prominent as that of −195/−31-Luc, it was speculated that an additional element would be necessary for the c-*myc* promoter to achieve the maximal response to Notch signals.

DISCUSSION

With regard to the roles for Notch and HOXB4 in cell cycle regulation, Notch1 activation was reported to shorten the G_1 phase of cell cycle, thereby delaying the differentiation of human HL-60 cells and primary $CD34^+$ cells (47). Also, Ronchini and Capobianco (48) showed that the intracellular domain of Notch directly activated cyclin D1 promoter in a human kidney cell line, 293T. In addition, Krosl and Sauvageau (49) reported that overexpression of HOXB4 led to the induction of Jun-B and Fra-1 expression and subsequent up-regulated expression of cyclin D1 in Rat-1 fibroblast. To identify a critical target molecule in Notch- and HOXB4-induced self-renewal of HSCs, we analyzed their effects on the expression of cell cycle regulatory molecules and found that both molecules up-regulated the expression of c-*myc*, cyclin D2, cyclin D3, cyclin E, and E2F1. However, neither Notch 1 nor HOXB4 up-regulated the expression of cyclin D1, whereas its expression was up-regulated by these molecules in other cell types (as described above). These findings were consistent with the previous report that cyclin D1 was not detected in the proliferating normal hematopoietic stem/progenitor cells (50). Although cyclin D1 expression did not accompany the proliferation of normal hematopoietic stem/progenitor cells, our results suggest that G_1/S transition in self-renewal of HSCs is not so unique but rather common to other cell types. In addition, we found that Myc/ERT efficiently induced the expression of cyclin D2, cyclin D3, and cyclin E and promoted the growth of HSCs. In addition, previous reports suggested that c-Myc would transcriptionally repress the expression of $p21^{WAF1}$, which plays a crucial role in the quiescence of HSCs (19, 51), and of $p15^{INK4b}$, which is induced by transforming growth factor-β signaling (52, 53). Together, these results imply that the c-*myc* could be a master regulator of the cell cycle in both Notch1- and HOXB4-induced self-renewal of HSCs.

In this study, promoter assays revealed that both Notch signaling and HOXB4 activated the c-*myc* promoter. However, the element most responsive to RBP-VP16 in the c-*myc* promoter did not contain the putative RBP-J-binding sequence. Also, we confirmed that the nuclear protein, which bound to this element, did not contain RBP-VP16 itself in EMSA with supershift assays, suggesting RBP-VP16 could activate the c-*myc* promoter indirectly. To understand the mechanism of Notch-induced self-renewal of HSCs, we are now trying to purify this molecule using nuclear extracts from RBP-VP16-transfected 293T cells.

In this study, reconstitution assays revealed that cells expanded by Myc/ERT could contribute to hematopoiesis for more than 6 months, indicating that these cells still possess characteristics of HSCs in terms of long term reconstitution. In addition, these cells developed $Mac1/Gr\text{-}1^+$ cells, $CD4/8^+$ cells, and $B220^+$ cells, implying that the cells expanded by Myc/ERT have a multilineage reconstitution ability. When we compare the reconstitution efficiency of Myc/ERT-expanded cells with that of Notch1-expanded cells (43), Myc/ERT-expanded cells showed similar or a little more potent reconstitution abilities than Notch-expanded cells. (120-fold excess of Notch1-expanded cells (*i.e.* transplantation of 120×10^5 Notch1-expanded cells in combination with 1×10^5 normal BM cells) contributed to hematopoiesis in about 23% of BM cells after transplantation *versus* 40-fold excess of Myc/ERT-expanded cells (*i.e.* transplantation of 40×10^5 Myc/ERT-expanded cells in combination with 1×10^5 normal BM cells) participated in hematopoiesis in about 10% of the peripheral blood cells.) However, even if sufficient numbers (1×10^7) of Myc/ERT-expanded cells were transplanted, co-injection of normal supporting BM cells was required for radioprotection in the recipient mice (data not shown). These results indicate that the cells expanded by Myc/ERT lack some ability to fully or rapidly reconstitute hematopoiesis *in vivo*. The similar defect was observed in HSCs expanded by Notch1 (43). Like Myc/ERT-expanded cells, Notch1-expanded cells alone could not reconstitute hematopoiesis in lethally irradiated mice, whereas these cells could participate in hematopoiesis for more than 6 months in combination with normal supporting BM cells. One possible explanation is that the *ex vivo* culture of HSCs or modulation of cell cycle by Notch1 or c-Myc might result in the reduced homing abilities after intravenous transplantation. For example, Szilvassy *et al.* (54) showed that HSCs expanded by growth factors *in vitro* expressed little or no β_1 integrin, and this change was associated with a failure of radioprotection. Alternatively, HSCs induced to proliferate by Notch or c-Myc *in vitro* might not be able to rapidly undergo terminal differentiation after transplantation, resulting in the failure to supply a sufficient number of mature cells required for radioprotection.

In summary, our results indicate that c-Myc is, at least in part, capable of supporting self-renewal of HSCs as a downstream mediator of Notch and HOXB4. Further analyses on cell cycle regulation would undoubtedly provide more informative findings to expand HSCs *in vitro*.

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Identification of a Cytokine-induced Antiapoptotic Molecule Anamorsin Essential for Definitive Hematopoiesis

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Abstract

Many growth factors and cytokines prevent apoptosis. Using an expression cloning method, we identified a novel antiapoptotic molecule named Anamorsin, which does not show any homology to known apoptosis regulatory molecules such as Bcl-2 family, caspase family, or signal transduction molecules. The expression of Anamorsin was completely dependent on stimulation with growth factors such as interleukin 3, stem cell factor, and thrombopoietin in factor-dependent hematopoietic cell lines, and forced expression of Anamorsin conferred resistance to apoptosis caused by growth factor deprivation in vitro. Furthermore, Anamorsin was found to act as an antiapoptotic molecule in vivo because Anamorsin$^{-/-}$ mice die in late gestation due to defective definitive hematopoiesis in the fetal liver (FL). Although the number of hematopoietic stem/progenitor cells in the FL did not decrease in these mice, myeloid, and particularly erythroid colony formation in response to cytokines, was severely disrupted. Also, Anamorsin$^{-/-}$ erythroid cells initiated apoptosis during terminal maturation. As for the mechanism of Anamorsin-mediated cell survival, a microarray analysis revealed that the expression of Bcl-xL and Jak2 was severely impaired in the FL of Anamorsin$^{-/-}$ mice. Thus, Anamorsin is considered to be a necessary molecule for hematopoiesis that mediates antiapoptotic effects of various cytokines.

Key words: antiapoptosis • cytokine signal • expression cloning • definitive hematopoiesis • gene targeting

Introduction

Growth, differentiation, and survival of hematopoietic cells are positively or negatively regulated by a number of cytokines. Among these factors, several cytokines such as erythropoietin (EPO), thrombopoietin (TPO), G-CSF, and stem cell factor (SCF) are considered to play crucial roles in hematopoiesis because the targeted disruption or spontaneous loss of function mutation of these factors or their receptors leads to severe defects in mutant mice (1–4).

Upon binding to their receptors, cytokines initially evoke phosphorylation and activation of cell surface tyrosine kinases such as receptor tyrosine kinases or JAK family tyrosine kinases. Activated tyrosine kinases transmit mitogenic and antiapoptotic signals through simultaneous activation of downstream signaling molecules including Ras/MAPK, PI3-K/Akt, and signal transducers and activators of transcription (STATs; 5, 6). During the last decade, accumulated experimental evidence has suggested that each of the Ras/MAPK, PI3-K, and STATs can mediate both cell growth and survival. In the case of Ras/MAPK, dominant negative Ras inhibits IL-3–dependent growth of the murine IL-3–dependent cell line Ba/F3 (7), and oncogenic Ras enables Ba/F3 cells to survive under conditions of IL-3 deprivation (8). In this system, Ras/MAPK was found to induce expression of antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-xL (8, 9). However, the induction of these molecules by Ras/MAPK seems to be indirect and it remains unknown which molecule(s) are involved in their induction. In addition, Ras promotes cell survival, at least in part, through the activation of PI3-K, which is observed in several, but

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The online version of this article contains supplemental material.

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Abbreviations used in this paper: BFU-E, burst-forming unit-erythroid; EPO, erythropoietin; ES, embryonic stem; FL, fetal liver; IPTG, isopropyl-β-D-thiogalactopyranoside; SCF, stem cell factor; STAT, signal transducer and activator of transcription; TPO, thrombopoietin.

not all, cell types (10). The PI3-K/Akt pathway exerts antiapoptotic effects by phosphorylating and inhibiting the function of proapoptotic molecules, BAD and FKHRL1. Also, the PI3-K/Akt pathway degrades the death effector, caspase-9 (11). Both Ras/MAPK and PI3-K/Akt pathways inhibit expression of the proapoptotic Bcl-2 family member, Bim, which plays a pivotal role in factor-deprived apoptosis (12). On the other hand, similarly to oncogenic Ras, a constitutively active form of STAT5A enables Ba/F3 cells to proliferate and survive under IL-3–starved conditions (13). Regarding the mechanism of STAT-mediated cell survival, STAT5 and STAT1 were found to induce the expression of Bcl-xL mRNA through direct binding to its promoter as transcription factors (14, 15). Supporting these findings, hematopoietic cells obtained from STAT5A$^{-/-}$5B$^{-/-}$ mice were more likely to undergo apoptosis due to a defect in induction of Bcl-xL (15, 16). Moreover, STAT3 and STAT5 were reported to induce expression of Bcl-2 mRNA, but its induction seems to be indirect (9, 17). Although great advances have been made in understanding signal transduction pathways mediated by cytokines, several critical points still remain unelucidated. In particular, it remains largely unknown how Ras/MAPK induces expression of Bcl-2 family members, and thus it has been speculated that some critical, intermediary signaling molecules might be missing in this pathway.

Factor-dependent cell lines such as Ba/F3 (pro–B cells), FL5.12 (pro–B cells), and 32D (myeloid cells) are useful tools for analyzing the mechanism of cytokine-dependent cell growth and survival. Indeed, most previous studies were performed with these cell lines. Moreover, recent studies identified two apoptosis regulatory molecules with cDNA microarray analyses by using FL5.12 cells cultured with or without IL-3. One is a proapoptotic molecule, 24p3, encoding a lipocalin, whose expression is up-regulated after IL-3 depletion (18). By contrast, the expression of a serine/threonine kinase, Pim-2, which confers resistance to a variety of apoptotic stimuli, was down-regulated by IL-3 deprivation in a microarray analysis (19). These cell lines have also been used to evaluate oncogenic properties of uncharacterized molecules and find activating mutations of certain genes. For example, activating mutants of STAT5 and c-*mpl* were both originally identified as molecules that confer factor-independent growth and survival of Ba/F3 cells by using retrovirus-mediated expression cloning (13, 20).

By using a retrovirus expression library prepared from Ba/F3-Ad, an IL-3–independent sub-line established from Ba/F3 (unpublished data), we cloned a novel antiapoptotic molecule, Anamorsin (ana-mors-in was designated to mean an anti-death molecule in Latin), which can confer resistance to IL-3 deprivation apoptosis in Ba/F3 cells. In addition to antiapoptotic activities of Anamorsin in in vitro assays, we also demonstrated its antiapoptotic function in vivo by generating KO mice. The Anamorsin$^{-/-}$ genotype is lethal at late gestation due to apoptosis of hematopoietic cells in the fetal liver (FL). Thus, Anamorsin appears to play a crucial role in definitive hematopoiesis as an antiapoptotic molecule.

Materials and Methods

Cell Culture and cDNA Transfection. The murine IL-3–dependent cell lines, Ba/F3 and 32D, were cultured in RPMI 1640 supplemented with 10% FCS in the presence of 0.1 ng/ml mIL-3 (Kirin Brewery Company). EPO receptor, c-kit, and c-mpl were individually engineered to be expressed in Ba/F3 cells, and their clones were able to proliferate in the presence of the corresponding cytokines. To construct expression vectors for Anamorsin and H-Ras^{G12V}, these cDNAs were subcloned into pcDNA3 (Invitrogen). Ba/F3 cells or 32D cells were transfected with the Anamorsin or H-Ras^{G12V} expression vector by electroporation and selected by culturing in media containing 1.0 mg/ml G-418. Out of several G-418–resistant clones, two clones, in which the transgene was most intensely expressed, were used for further experiments.

DNA Content Analysis. The DNA content of cultured cells was examined by staining with propidium iodide and then analyzed on a FACSort™ (Becton Dickinson) as previously described (7).

Assays for Caspase-3 Activities. Caspase-3 activities were measured with the PhiPhiLux-G1D2 kit (OncoImmunin). In this system, caspase-3 activities are measured by fluorescence that is derived from the cleaved substrate specific for caspase-3.

Northern Blot Analysis. Northern blot analysis was performed as previously described (21). Membranes were hybridized with ^{32}P-labeled Anamorsin probe (nucleotides 255–564 of Anamorsin cDNA: BamHI fragment) and β-actin probe (as a loading control) in rapid hybridization buffer (Amersham Biosciences).

Anti-Anamorsin mAbs. Three kinds of rat mAbs against murine Anamorsin (named KM3048, KM3052, and KM3056) were generated according to methods previously described (22). The sequences of the synthesized peptide antigens were CLFLKEPVETAEVNNDKMKTASKL (Anamorsin 1, amino acid residues 92–115), CRVTGKKPNFEVGSSSQ (Anamorsin 2, amino acid residues 158–173), and CGLAEELEREQSKAQSSQPKSA (Anamorsin 3, amino acid residues 249–270), respectively. All antibodies used were appropriate for immunoblotting, immunoprecipitation, and immunofluorescence staining.

Immunofluorescence Staining. Ba/F3 cells were attached to Silane-coated slide glasses (DakoCytomation) by cytospin centrifugation (Shandon, Inc.). Cytospin preparations were fixed with methanol at 4°C for 10 min, washed three times in PBS, blocked in PBS containing 1% BSA and 10% mouse serum for 30 min, and then incubated with 10 μg/ml of an anti-Anamorsin mAb for 1 h. These slides were next incubated with FITC-conjugated goat anti–rat IgG (1:50 dilution; ICN Biomedicals) for 1 h, washed twice in PBS containing 0.2 μg/ml DAPI, and observed by fluorescent microscopy.

Lac-inducible System. To express the dominant negative H-Ras gene (H-Ras^{S17N}), we used a LacSwitch II–inducible expression system (Stratagene) as previously described (7). In this system, isopropyl-β-D-thiogalactopyranoside (IPTG) is added to the culture medium causing the Lac repressor to be released from the lactose operon and transcription of the targeted cDNA (H-Ras^{S17N} in this case) is initiated.

Genotypic Analyses. High molecular weight genomic DNA was extracted from tails or whole embryos, digested with SacI, and subjected to electrophoresis on 0.8% agarose gels. Southern blot analysis was performed with a 5′ flanking probe. The sequences of primers used for PCR analysis were as follows: α, 5′-ACCTTCGGAAAAGTAGTCGGGTGCTCTTAC-3′; β, 5′-CGCATCGCCTTCTATCGCCTTCTTGACGAG-3′; γ, 5′-GTGTCTAAAACCCATGACCTTTCACCAG-3′. Genomic DNA from the wild-type allele yields a 310-bp fragment with a

primer pair α/β, and that from the targeted allele yields a 730-bp fragment with primer pair α/γ.

Treatments of the Embryos. Embryos of the stated age were dissected free of maternal tissues and photographed, and then fixed in 10% buffered formalin and embedded in paraffin. 4-μm thick sections were cut and stained. Cell suspensions were isolated from FLs and subjected to flow cytometric analysis, morphologic analysis of cytospin preparations, and methylcellulose colony assay analysis.

Colony Assays. 10^4 FL cells were obtained from Anamorsin$^{+/+}$ (n = 10), Anamorsin$^{+/-}$ (n = 36), and Anamorsin$^{-/-}$ (n = 11) embryos at E14.5, and cultured in methylcellulose medium containing the cytokine cocktail, SCF, IL-3, IL-6, and EPO (MethoCult; StemCell Technologies Inc.). The numbers of hematopoietic colonies were counted under phase contrast light microscopy after 8 d in culture. Also, 10^5 FL cells isolated from Anamorsin$^{+/+}$ (n = 11), Anamorsin$^{+/-}$ (n = 40), and Anamorsin$^{-/-}$ (n = 6) embryos at E14.5 were cultured with SCF and EPO for 8 d, and the numbers of burst-forming unit-erythroid (BFU-E) colonies were counted.

Flow Cytometry Analysis. 5×10^5 cells were incubated with 2 μl avidin-conjugated anti–mouse Ter-119 mAb (BD Biosciences; reference 23) at 4°C for 30 min and washed twice in PBS. Cells were incubated with 2 μl biotin-PE (Becton Dickinson) and annexin V–FITC (Immunotech; reference 24) at 4°C for 30 min, washed twice in PBS, and then analyzed on a FACSort™ (Becton Dickinson). Cells were also stained with PE-conjugated anti–mouse CD44 mAb (BD Biosciences). For CD34, c-kit, and Ter-119 staining, cells were first incubated with rat anti–mouse CD34 mAb (Caltag Laboratories), biotin-conjugated rat anti–mouse c-kit mAb (Immunotech), or avidin-conjugated anti–mouse Ter-119 mAb, and then incubated with FITC-conjugated anti–rat IgG (BD Biosciences), streptavidin-PE (Becton Dickinson), or biotin-FITC (Becton Dickinson), respectively. Appropriate isotype control antibodies were used. Cell surface expression of the different markers were analyzed on a FACSort™ (Becton Dickinson).

Immunoblotting. Isolation of total cellular lysates, gel electrophoresis, and immunoblotting were performed according to methods previously described (25). In brief, the embryo limbs were lysed in lysis buffer containing protease and phosphatase inhibitors. Insoluble fractions were removed by centrifugation. Whole cell lysates were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon; Millipore). Membranes were blocked in TBS-T with 2% BSA for 1.5 h and incubated with 10 μg/ml of an anti-Anamorsin mAb for 1.5 h. Immunoreactive proteins were visualized by a horseradish peroxidase–conjugated anti–rat IgG Ab with ECL system (Amersham Biosciences). To reprobe with an anti-GAPDH Ab (American Research Products), membranes were incubated in stripping buffer at 70°C for 1 h, washed, and reused.

cDNA Microarray Analysis. We performed a cDNA microarray analysis with IntelliGene II Mouse CHIP (TaKaRa Shuzo Co.). In brief, poly(A)$^+$ mRNA was prepared from total RNA using Oligotex-dT30 Super mRNA Purification Kit (TaKaRa Shuzo Co.). 1-μg aliquots of mRNA from E14.5 Anamorsin$^{+/+}$ and Anamorsin$^{-/-}$ FL cells were labeled with Cy3-dUTP and Cy5-dUTP (Amersham Biosciences), respectively, using an RNA Fluorescence Labeling Core Kit (M-MLV version; TaKaRa Shuzo Co.). Labeled probes were mixed with hybridization solution (6× SCC, 0.2% SDS, 5× Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA). After hybridization for 16 h at 55°C, the slides were washed twice in 2× SSC and 0.1% SDS for 5 min at 55°C, washed in 2× SSC and 0.1% SDS for 5 min at 65°C, and washed in 0.05× SSC for 5 min at room temperature. The slides were scanned using the Affymetrix 428 scanner (Affymetrix, Inc.). The signal intensity of hybridization was evaluated photometrically by the ImaGene computer program (BioDiscovery) and normalized to the averaged signals of housekeeping genes. A cut off value for each expression level was calculated according to the background fluctuation. The fluctuation can be estimated as the variance of the ratio of Cy5/Cy3.

RT-PCR Assays. Total RNA was isolated from E14.5 Anamorsin$^{+/+}$ FL cells and Anamorsin$^{-/-}$ FL cells with TRIsol reagent (GIBCO BRL). 1.5 μg total RNA was reverse transcribed to first strand cDNA with Moloney murine leukemia virus transcriptase (MMLV-RT; Invitrogen) in the presence of dNTPs, oligo(dT) (Roche), RNasin (Promega), and DTT in a total volume of 30 μl for 60 min at 42°C, followed by heating for 10 min at 75°C. PCR was performed in a total volume of 50 μl with 5 μl reverse-transcribed product, 2.5 U Taq-polymerase, dNTPs, and 0.5 μM primers in 1× PCR buffer. The sequences of primer sets are as follows: Jak2, (sense) 5′-GTTCTTACCGAAGTGCGTGCGA-3′ and (antisense) 5′-GGTAATGGTGTGCATCGCAGTT-3′); Bcl-xL, (sense) 5′-CACTGTGCGTGGAAAGCGTA-3′ and (antisense) 5′-AAAGTGTCCCAGCCGCC-3′; GAPDH, (sense) 5′-AATGTGTCCGTCGTGGATCTGA-3′ and (antisense) 5′-GATGCCTGCTTCACCACCTTCT-3′.

Online Supplemental Material. Table S1 shows the data of cDNA microarray analysis concerning the apoptosis-related genes within IntelliGene II Mouse CHIP. Table S1 is available at http://www.jem.org/cgi/content/full/jem.20031858/DC1.

Results

Isolation of Anamorsin by Expression Cloning from IL-3–independent Subline Ba/F3-Ad. We have established a subline named Ba/F3-Ad that can grow and survive under IL-3–deprived conditions from a murine IL-3–dependent cell line, Ba/F3 (unpublished data). To identify the molecule(s) that conferred the observed resistance to factor-deprived apoptosis on Ba/F3, we performed expression cloning by constructing a retroviral cDNA library from IL-3–starved Ba/F3-Ad according to the procedure previously described (26). In short, we infected the retrovirus library into parental Ba/F3 (5×10^5 clones, the average size of the inserts, 1.3 kb; the infection efficacy was estimated to be ~35%) and screened the clones that survived under IL-3–starved conditions for more than 1 wk and then started to proliferate in the medium containing IL-3. After isolation of several clones that survived under IL-3–deprived conditions, we isolated the integrated cDNA from genomic DNA extracted from one clone by the PCR method.

cDNA and Amino Acid Sequence of Anamorsin. By sequencing the integrated cDNA, we found that the coding region of a murine protein, Anamorsin, cDNA consisted of 930 bp (Fig. 1 a). Comparison with a DNA database search revealed that the sequence of Anamorsin does not exhibit homology with any known antiapoptotic molecules, including Bcl-2 family proteins, caspase inhibitors, or signal transduction molecules. Also, we found a human homologue of Anamorsin in GenBank data libraries, which revealed 82.6% similarity to murine Anamorsin at the DNA level and 81.9% similarity at the amino acid level. The hu-

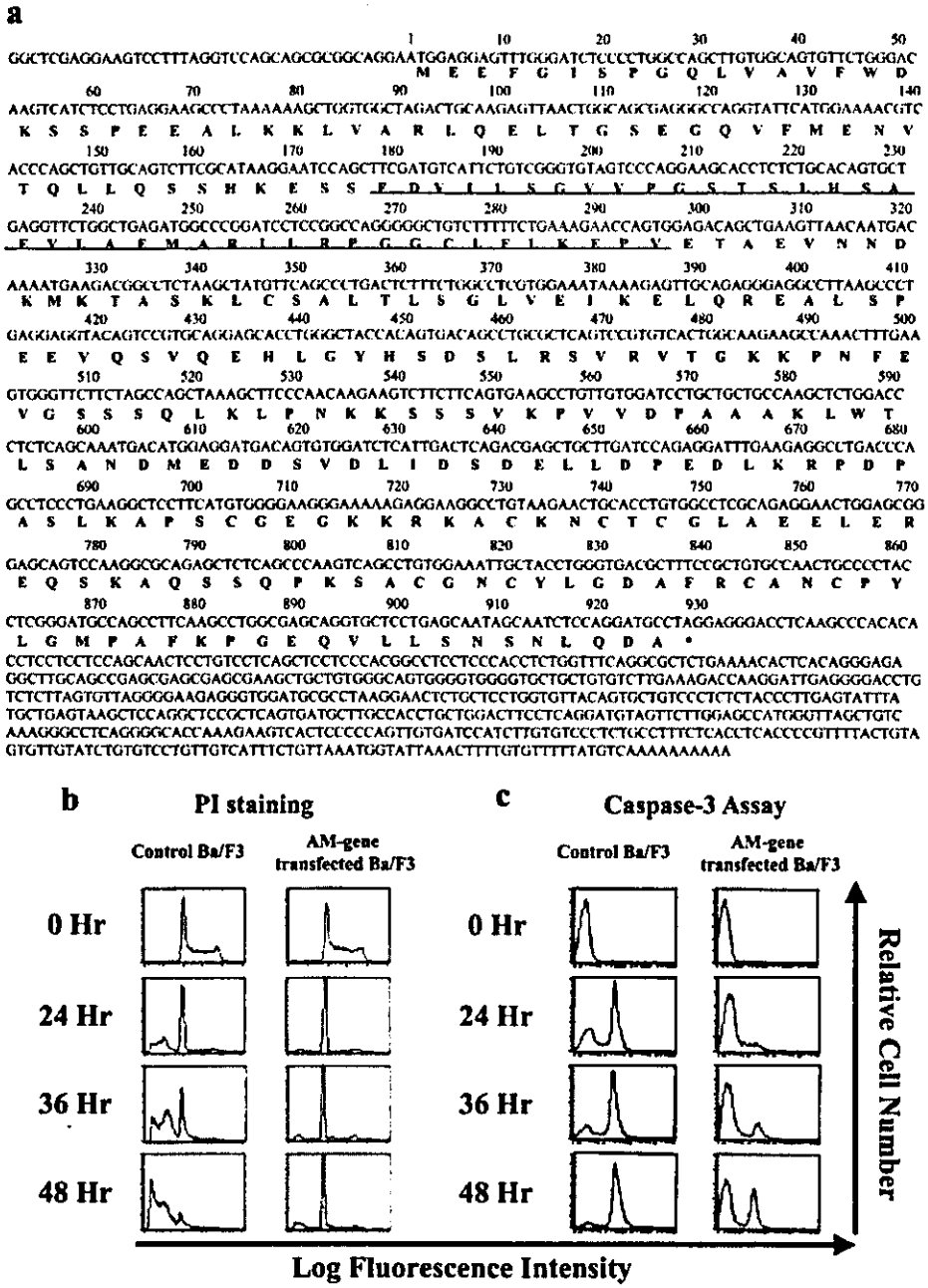

Figure 1. Anamorsin confers resistance to apoptosis caused by IL-3 depletion in Ba/F3 cells. (a) Anamorsin cDNA sequence and corresponding amino acid sequence. The underlined amino acid sequence shows generic methyltransferase motif. (b and c) Ba/F3 cells transfected with an empty vector or Anamorsin (AM) cDNA were deprived of IL-3 for the time indicated and the apoptotic cells were subjected to DNA content analysis (b) and caspase-3 assays (c).

man homologue of Anamorsin was originally found by Loftus et al. (27) as a molecule with unknown function on chromosome 16 (sequence data are available from GenBank/EMBL/DDBJ under accession no. AC004382). Anamorsin encodes a protein with a molecular weight of ~37 kD and the protein sequence database indicates that Anamorsin has a generic methyltransferase motif around amino acids 60–99 (Fig. 1 a).

Anamorsin Confers Resistance to Apoptosis Induced by IL-3 Depletion. First, we examined whether Anamorsin was actually involved in resistance to factor-deprived apoptosis of Ba/F3-Ad. For this purpose, we stably expressed murine Anamorsin cDNA in parental Ba/F3 cells and investigated sensitivity to IL-3–deprived apoptosis. In control Ba/F3 cells, the subdiploid fraction formed from apoptotic cells emerged as early as 24 h after IL-3 depletion, but this fraction was effectively reduced in Anamorsin-transfected Ba/F3 cells (percent of apoptotic fraction: control Ba/F3, 37% at 24 h, 64% at 36 h, and 86% at 48 h; Anamorsin-transfected Ba/F3, 7% at 24 h, 11% at 36 h, and 20% at 48 h; Fig. 1 b). In agreement with this finding, the activation of caspase-3, which is detected as a shift in fluorescent inten-

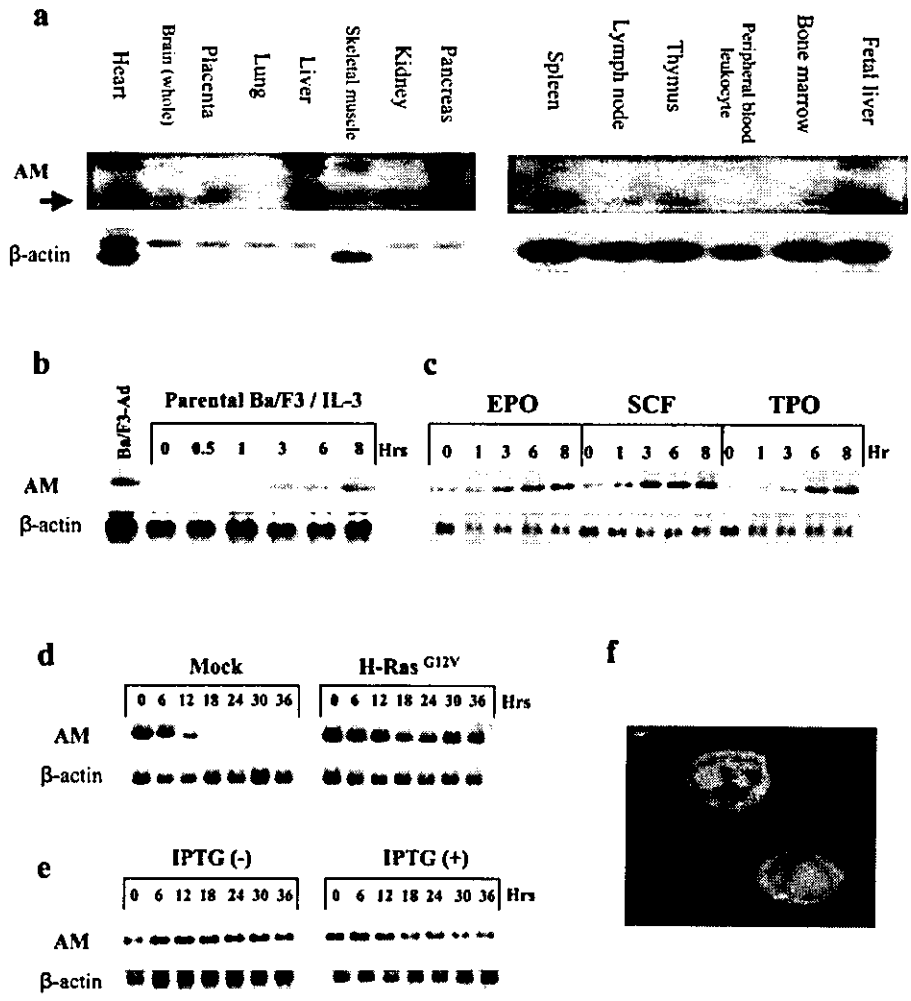

Figure 2. Anamorsin (AM) was expressed ubiquitously in various tissues and its expression was induced by various cytokines through the Ras signaling pathway. (a) The expression of the human Anamorsin homologue in various human organs (MTC panels, CLONTECH Laboratories, Inc.) was examined by Northern hybridization. (b) Ba/F3 cells were IL-3 depleted for 15–18 h and then cultured with 0.1 ng/ml mIL-3 for the time indicated. Induction of Anamorsin gene was examined by Northern blot analysis. Ba/F3-Ad, Ba/F3-Ad in the absence of IL-3. (c) Ba/F3 cells each transfected with EPO receptor, c-kit, and c-mpl were deprived of IL-3 for 15–18 h and cultured with 5 u/ml mEPO, 100 ng/ml mSCF, and 30 ng/ml mTPO, respectively, for the time indicated. Induction of Anamorsin gene was examined by Northern blot analysis. (d) Ba/F3 cells transfected with an empty vector (Mock) or oncogenic H-Ras (H-Ras^{G12V}) were deprived of IL-3 for the time indicated. The expression levels of Anamorsin were examined by Northern blot analysis. (e) Ba/F3 cells, in which dominant negative H-Ras (H-Ras^{S17N}) was inducibly expressed by a LacSwitch-II–inducible expression system, were cultured with IL-3 in the presence or absence of IPTG for the time indicated. The expression levels of Anamorsin were examined by Northern blot analysis. (f) The localization of Anamorsin was examined by an immunofluorescence staining with an anti-Anamorsin mAb. Nucleus was visualized with staining with DAPI. Green, Anamorsin; blue, nucleus.

sity, was significantly suppressed in Anamorsin-transfected Ba/F3 cells as compared with that seen in control Ba/F3 cells (Fig. 1 c). However, it should be noted that Anamorsin alone could not support the growth of Ba/F3 cells because the number of proliferating cells dramatically decreased in Anamorsin-transfected Ba/F3 cells under IL-3–deprived conditions (percent of the cells in S-G2/M phase before and after IL-3 depletion: 56% at 0 h vs. 6% at 48 h; Fig. 1 b, right). Furthermore, Anamorsin-transfected cells were found to become small and enter G0 phase after IL-3 depletion (unpublished data). We also confirmed that Anamorsin confers resistance to apoptosis caused by IL-3 depletion in another murine IL-3–dependent cell line, 32D (unpublished data).

The Expression Profile of Anamorsin and Its Regulation by Cytokines. Next, we examined the expression profile of the human Anamorsin homologue in various organs using MTA panels (CLONTECH Laboratories, Inc.). As shown in Fig. 2 a, left, the homologue was expressed ubiquitously in various tissues, with especially high expression levels detected in heart, liver, and pancreas. As for hematopoietic tissues, the homologue was abundantly expressed in FL and spleen (Fig. 2 a, right). Also, we found that expression of Anamorsin was detectable in early stages (7 d) of embryogenesis by the PCR method (not depicted).

Because Anamorsin was supposed to exert antiapoptotic effects in Ba/F3-Ad under IL-3–deprived conditions, we examined its expression levels in parental Ba/F3 and Ba/F3-Ad after IL-3 depletion. As expected, Anamorsin was still expressed in Ba/F3-Ad after 18 h of IL-3 deprivation, whereas its expression was hardly detectable in parental Ba/F3 cells (Fig. 2 b, lane 1 vs. 2). However, the addition of IL-3 recovered expression in parental Ba/F3 cells after 3 h (Fig. 2 b, lanes 2–7). Next, we investigated whether expression of Anamorsin was also regulated by other cytokines in Ba/F3 cells. After IL-3 depletion, clones of Ba/F3 that expressed receptors for EPO, SCF (c-Kit), and TPO (c-Mpl) were cultured with their corresponding cytokines for the time indicated. As shown in Fig. 2 c, EPO, SCF, and TPO individually induced expression of Anamorsin as

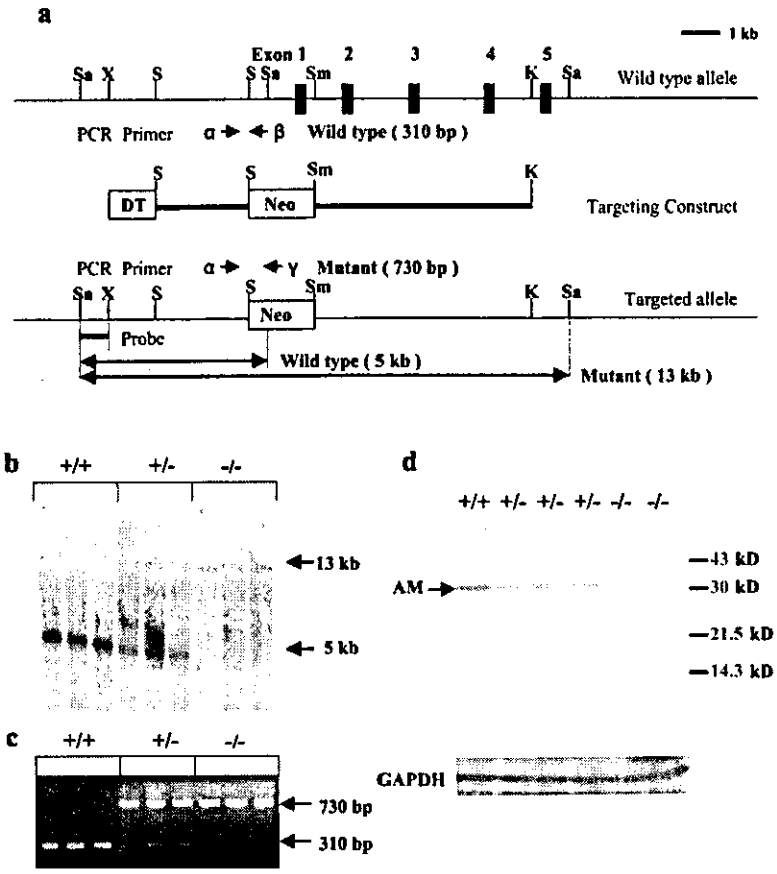

Figure 3. Targeted disruption of the murine Anamorsin locus. (a) Partial restriction map of the murine Anamorsin locus and the targeting construct. The first to fifth exons of Anamorsin gene are indicated as closed boxes in the top diagram. K, KpnI; S, SpeI; Sa, SacI; Sm, SmaI; X, XhoI. (b) Southern blot analysis was preformed with genomic DNA extracted from tails of embryos by hybridizing with a 5′ flanking probe. The wild-type allele is detected as a 5-kb SacI fragment and the targeted allele as a 13-kb SacI fragment. (c) PCR analysis was performed with primer pairs α/β and α/γ. Genomic DNA from the wild-type allele yields a 310-bp fragment with a primer pair α/β, and that from the targeted allele yields a 730-bp fragment with a primer pair α/γ. (d) Western blot analysis was performed on total cellular lysates obtained from limb of embryos with the indicated genotypes using an anti-Anamorsin mAb.

efficiently as IL-3, suggesting that expression of Anamorsin would be regulated by the common signaling molecule(s) shared by various cytokines. Based on the fact that Ras is activated by various cytokines and was constitutively activated in Ba/F3-Ad (unpublished data), we speculated that Ras might control expression of Anamorsin in Ba/F3 cells. To examine this possibility, we stably expressed oncogenic H-Ras (H-Ras^{G12V}) in Ba/F3 cells. Also, we prepared a clone from Ba/F3, in which dominant negative H-Ras (H-Ras^{S17N}) was inducibly expressed by the IPTG treatment. As expected, the Ba/F3 cells expressing constitutively active H-Ras survived under IL-3–starved conditions better than control Ba/F3 cells. In contrast, the Ba/F3 cells expressing dominant negative H-Ras died more rapidly after IL-3 withdrawal than control Ba/F3 cells. In Ba/F3 cells transfected with H-Ras^{G12V}, expression of Anamorsin was maintained after IL-3 depletion for up to 36 h, whereas its expression declined to undetectable levels in a mock clone transfected with empty vector (Fig. 2 d). Furthermore, induced expression of H-Ras^{S17N} led to reduction of Anamorsin expression, even in the presence of IL-3 (Fig. 2 e). Collectively, these results suggest that expression of Anamorsin is completely dependent on cytokine stimulation and, at least partially, regulated by Ras signaling in Ba/F3 cells. Next, we investigated the intracellular localization of Anamorsin using immunofluorescence staining with an anti-Anamorsin mAb. As shown in Fig. 2 f, Anamorsin was exclusively localized in the cytoplasm of Ba/F3 cells, regardless of stimulation with IL-3.

Generation of Anamorsin-null Mice. To assess in vivo roles of Anamorsin, we tried to generate Anamorsin-null (Anamorsin$^{-/-}$) mice. We constructed a targeting vector, in which the first exon was replaced by the neomycin-resistant cassette (a positive selection marker). The vector also included diphtheria toxin as a negative selection marker

Table I. *Genotypes of Embryos from Timed Pregnancies*

Stage	AM$^{+/+}$[a]	AM$^{+/-}$	AM$^{-/-}$	Total No.
E10.5	6	17	5	28
E12.5	7	14	4	25
E14.5	17	68	16 (3[b])	101 (3[b])
E16.5	15	16	11 (4[b])	42 (4[b])
E18.5	12	32	9 (4[b])	53 (4[b])
At birth	15	45	6 (6[c])	66 (6[c])

[a]AM, Anamorsin.
[b]Number of embryos absorbed in uterus.
[c]Number of dead babies.

(Fig. 3 a). The targeting vector was introduced into an embryonic stem (ES) cell line R1 by electroporation and transfected cells were cultured with 150 μg/ml G418. We confirmed homologous recombination in selected ES cell lines by Southern blot and PCR analyses. As expected from Fig. 3 a, the wild-type allele was detected as a 5-kb SacI fragment in Southern blot analysis with a 5′ flanking probe, whereas the targeted allele was detected as a 13-kb SacI fragment (unpublished data). Also, in PCR analyses, the 310-bp fragment was amplified from the wild-type allelic genomic DNA with a primer pair α/β, whereas the 730-bp fragment was amplified from the targeted allelic genomic DNA with a primer pair α/γ (unpublished data). To generate chimeras, we injected three ES cell lines that were confirmed to contain the homologous recombination into blastocysts of C57BL/6J mice. Male offspring with a high degree of chimerism were crossed with C57BL/6J females to generate Anamorsin$^{+/-}$ mice. Genotyping was performed by Southern blot and PCR analyses using tail- and embryo-derived DNA (Fig. 3, b and c). Finally, we confirmed that expression of Anamorsin protein in the limb was partially reduced in Anamorsin$^{+/-}$ embryos and com-

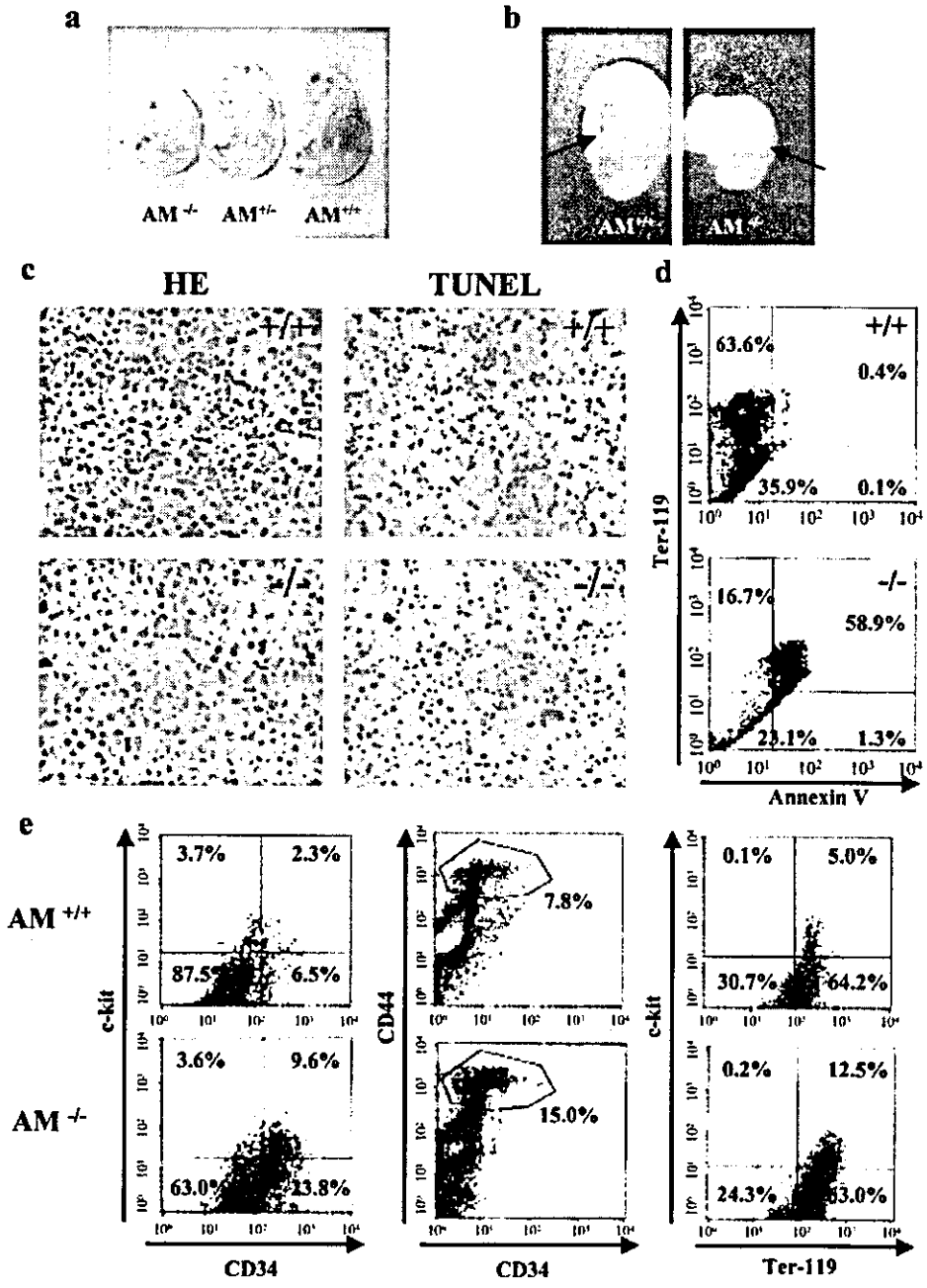

Figure 4. Phenotypes of Anamorsin (AM)$^{-/-}$ embryos, spleens, and FLs. (a) E16.5 embryos of Anamorsin$^{-/-}$ and the littermate control are shown. (b) The spleens (arrows) with stomach of Anamorsin$^{+/+}$ and Anamorsin$^{-/-}$ mice at birth are shown. (c) Transverse sections of FL at E14.5 obtained from Anamorsin$^{+/+}$ (top) or Anamorsin$^{-/-}$ (bottom) embryos were stained with hematoxylin and eosin for histological examination (left) and with TUNEL to detect apoptotic cells (right). Apoptotic cells are stained with brown in this TUNEL assay. ×400. (d) Cell suspensions isolated from Anamorsin$^{+/+}$ (top) and Anamorsin$^{-/-}$ (bottom) FL cells at E14.5 were stained with anti–Ter-119 and annexin V, and subjected to flow cytometric analysis. (e) The proportions of CD34^{+} c-kit^{+}, CD34^{low} CD44^{high}, and Ter-119^{+} c-kit^{+} cells in Anamorsin$^{+/+}$ and Anamorsin$^{-/-}$ FL cells at E14.5. Cell suspensions isolated from Anamorsin$^{+/+}$ (top) and Anamorsin$^{-/-}$ (bottom) FL cells at E14.5 were stained with anti–c-kit, anti-CD34, anti-CD44, or anti–Ter-119, and subjected to flow cytometric analysis.

pletely lost in Anamorsin$^{-/-}$ embryos by Western blot analysis using an anti-Anamorsin mAb (Fig. 3 d).

Anamorsin-null Mice Are Lethal at Late Gestation Due to the Defect of Hematopoiesis. Genotypic analysis of embryos from Anamorsin$^{+/-}$ mice intercrosses revealed that Anamorsin$^{-/-}$ embryos began to die between E12.5 and E14.5. The rate of dead Anamorsin$^{-/-}$ embryos increased after E14.5 (0% until E12.5, 18.8% at E14.5, 36.4% at E16.5, and 44.4% at E18.5) and all Anamorsin$^{-/-}$ mice died at birth, suggesting that Anamorsin$^{-/-}$ mice expire in late gestation (Table I). Anamorsin$^{-/-}$ embryos were smaller in body size than Anamorsin$^{+/+}$ embryos, whereas Anamorsin$^{+/-}$ embryos displayed phenotypes similar to Anamorsin$^{+/+}$ embryos (Fig. 4 a). Despite no significant difference in the formation of blood islands in yolk sacs, the FLs and spleens of Anamorsin$^{-/-}$ embryos were remarkably smaller than those of Anamorsin$^{+/+}$ embryos. The size of FL of Anamorsin$^{-/-}$ embryos was about one third of that of Anamorsin$^{+/+}$ embryos, and the spleen of Anamorsin$^{-/-}$ embryos appeared scarce (Fig. 4 b). Furthermore, Anamorsin$^{-/-}$ embryos later than E14.5 looked anemic (Fig. 4 a). Anamorsin$^{-/-}$ embryos at E18.5 showed nearly half of RBCs, hemoglobin, and hematocrit in the peripheral blood (RBC: 365 ± 50.8, 335.5 ± 50.6, and 210 ± 67.0×10^4/mm^3; hemoglobin: 11.7 ± 1.5, 10.6 ± 1.3, and 6.7 ± 2.3 g/dl; hematocrit: 39.7 ± 5.8, 37.8 ± 5.3, and 26.3 ± 7.6% in Anamorsin$^{+/+}$ [n = 10], Anamorsin$^{+/-}$ [n = 22], and Anamorsin$^{-/-}$ [n = 8] embryos, respectively). Moreover, RBCs of Anamorsin$^{-/-}$ embryos were macrocytic (mean corpuscular volume in Anamorsin$^{+/+}$, Anamorsin$^{+/-}$, and Anamorsin$^{-/-}$ embryos were 108.8 ± 4.3, 113.1 ± 9.5, and 128.2 ± 16.6, respectively). In addition to defects in hematopoietic organs, the heart walls of Anamorsin$^{-/-}$ embryos were thinner than those in Anamorsin$^{+/+}$ or Anamorsin$^{+/-}$ embryos, whereas Anamorsin$^{-/-}$ embryos displayed no apparent macroscopic or histological abnormalities in other organs (not depicted). It was unlikely that the abnormality in the heart walls caused death in Anamorsin$^{+/-}$ embryos.

Erythroid Cells Undergo Apoptosis in the FL of Anamorsin$^{-/-}$ Mice. To clarify the mechanism of anemia seen in Anamorsin$^{-/-}$ embryos, we analyzed the FL, the main he-

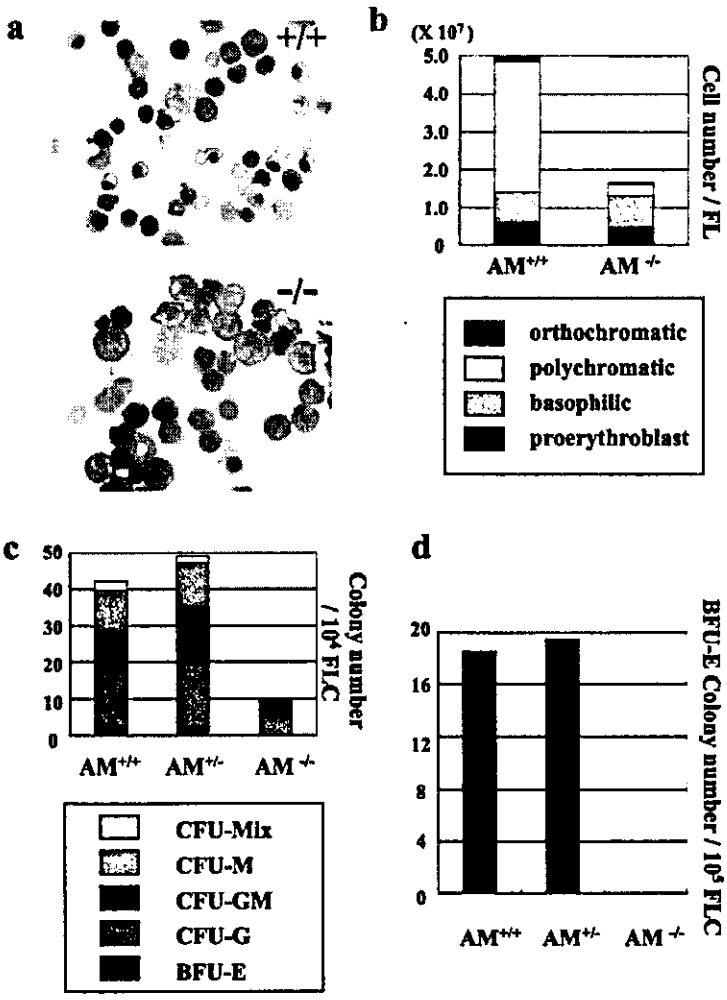

Figure 5. Anamorsin$^{-/-}$ erythroid progenitors could not differentiate in vivo and in vitro. (a and b) Cell suspensions isolated from Anamorsin (AM)$^{+/+}$ and Anamorsin$^{-/-}$ FL at E16.5 were subjected to cytospin centrifugation, stained with May-Grunwald-Giemsa, and analyzed under light microscopy. ×1,000 (a). The number of erythroid cells at each maturation stage was counted (b). (c and d) 10^4 FL cells at E14.5 obtained from Anamorsin$^{+/+}$, Anamorsin$^{+/-}$, and Anamorsin$^{-/-}$ mice were cultured in the methylcellulose containing SCF, IL-3, IL-6, and EPO, and the number of hematopoietic colonies were counted after 8-d cultures (c). Also, 10^5 FL cells at E14.5 were cultured with SCF and EPO for 8 d, and the number of BFU-E colonies was counted (d).

matopoietic organ of the late embryonic stage (28). The number of morphologically identifiable small-sized erythroid cells (erythroblasts) with condensed chromatin nucleus was apparently reduced in FL of Anamorsin$^{-/-}$ embryos compared with that of controls (Fig. 4 c). Furthermore, erythroblasts were larger in Anamorsin$^{-/-}$ embryos than in Anamorsin$^{+/+}$ embryos, and more mature erythroblasts (i.e., polychromatic and orthochromatic erythroblasts) markedly decreased in Anamorsin$^{-/-}$ embryos (Fig. 5, a and b). In addition, TUNEL assays showed that a substantial fraction of FL cells were apoptotic (stained by brown) at E14.5 in Anamorsin$^{-/-}$ embryos, whereas these apoptotic cells were hardly detected in Anamorsin$^{+/+}$ embryos (Fig. 4 c). To determine which type of cells undergo apoptosis, we performed flow cytometric analysis using Anamorsin$^{+/+}$ and Anamorsin$^{-/-}$ FL cells at E14.5. Although annexin V^{+} apoptotic cells were scarcely (only 0.5%) detected in Anamorsin$^{+/+}$ FL cells, 60.2% of isolated cells were positive for annexin V in Anamorsin$^{-/-}$ FL (Fig. 4 d). Most importantly, almost all of these apoptotic cells were Ter-119^{+} erythroid cells, but not Ter-119^{-} cells, mainly composed from hepatocytes (Fig. 4 d). Furthermore, although the cell number of Anamorsin$^{-/-}$ FL cells at E14.5 was approximately one third of Anamorsin$^{+/+}$ FL cells (unpublished data), the proportions of CD34^{+} c-kit^{+} cells, CD34^{low} CD44^{high} cells, or Ter-119^{+} c-kit^{+} cells in Anamorsin$^{-/-}$ FL cells at E14.5 was two to four times higher than those in Anamorsin$^{+/+}$ FL cells (CD34^{+} c-kit^{+}, 9.6 vs. 2.3%; CD34^{low} CD44^{high}, 15.0 vs. 7.8%; Ter-119^{+} c-kit^{+}, 12.5 vs. 5.0%; Fig. 4 e), indicating that neither the absolute number of hematopoietic stem/progenitor cells (CD34^{+} c-kit^{+} or CD34^{low} CD44^{high}) nor that of very immature proerythroblasts (Ter-119^{+} c-kit^{+}) decreased in Anamorsin$^{-/-}$ FL. Based on these data, it was speculated that immature hematopoietic cells of Anamorsin$^{-/-}$ mice may succumb to apoptosis and fail to attain terminal differentiation.

Colony Assays of the Anamorsin-null FL Cells. Next, we examined whether hematopoietic stem/progenitor cells of Anamorsin$^{-/-}$ FL cells could survive, proliferate, and differentiate in response to cytokines in vitro. When E14.5 Anamorsin$^{+/+}$, Anamorsin$^{+/-}$, or Anamorsin$^{-/-}$ FL cells were cultured in methylcellulose with the combination of cytokines SCF, IL-3, IL-6, and EPO, the number of myeloid (granulocyte/macrophage and granulocyte) colonies formed from Anamorsin$^{-/-}$ FL cells was one third to one fourth of those formed by Anamorsin$^{+/+}$ FL cells. Notably, Anamorsin$^{-/-}$ FL cells gave rise to little or no mixed (myeloid/erythroid) or erythroid colonies, whereas such colonies developed from Anamorsin$^{+/-}$ and Anamorsin$^{+/+}$ FL cells (Fig. 5 c). Furthermore, BFU-E colonies did not develop when E14.5 Anamorsin$^{-/-}$ FL cells were cultured with SCF and EPO (Fig. 5 d). These results suggest that Anamorsin is essential for cytokine-dependent survival and growth of hematopoietic stem/progenitor cells in vitro, especially those of erythroid lineage.

The Expression of Jak2 and Bcl-xL mRNA Was Down-regulated in Anamorsin-null FL Cells. To characterize the antiapoptotic function of Anamorsin, we compared gene expression profiles between Anamorsin$^{-/-}$ and Anamorsin$^{+/+}$ FL cells at E14.5 using a cDNA microarray. Among 4289 genes, including Bcl-2 family, caspases, cytokines, and signal transduction molecules, 184 genes were significantly down-regulated and 40 were up-regulated in Anamorsin$^{-/-}$ FL. Among apoptosis-related genes, Bcl-xL and Jak2 were down-regulated most significantly. We also confirmed that expression of these two molecules was decreased in Anamorsin$^{-/-}$ FL cells compared with Anamorsin$^{+/+}$ FL cells by semiquantitative RT-PCR assays (Fig. 6 and Table S1, available at http://www.jem.org/cgi/content/full/jem.20031858/DC1).

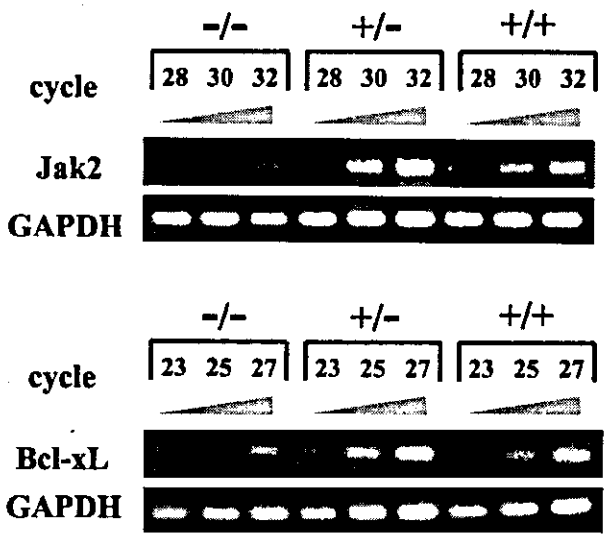

Figure 6. RT-PCR analysis on the expression of Jak2 and Bcl-xL mRNA in Anamorsin$^{-/-}$, Anamorsin$^{+/-}$, and Anamorsin$^{+/+}$ FL cells. Total RNA was isolated from E14.5 Anamorsin$^{-/-}$, Anamorsin$^{+/-}$, and Anamorsin$^{+/+}$ FL cells. 1.5 μg total RNA was reverse transcribed to first strand cDNA and subjected to PCR reactions. After the indicated cycles of PCR reaction, PCR products were electrophoresed on agarose gels and visualized with ethidium bromide staining.

Discussion

We have identified a novel antiapoptotic molecule, Anamorsin, by using expression cloning with the cytokine-independent subline Ba/F3-Ad. Although Anamorsin was constitutively expressed in Ba/F3-Ad, its expression was completely dependent on cytokine stimulation in Ba/F3 cells. It has been reported that IL-3, TPO, and SCF simultaneously activate the Ras/Raf/MEK/ERK pathway, (Ras)/PI3K/Akt pathway, and STATs in Ba/F3 cells, and that each of these signaling pathways can transmit antiapoptotic signals from these cytokines (5, 6). Among these pathways, cytokine-activated Ras was involved, at least partially, in the induction of Anamorsin. Although expression of Anamorsin mRNA was supposed to be transcriptionally regulated by cytokine stimulation, this expression was only detectable at relatively late phases (from after 3 h), suggesting that this induction is not an immediate early response.

Therefore, it is important to identify the molecule(s) that connects Ras signals to expression of Anamorsin. It is also necessary to examine whether other signaling pathways such as STATs and PI-3K/Akt pathway might contribute to induction of Anamorsin.

Anamorsin expression profiles showed that besides hematopoietic organs, Anamorsin was abundantly expressed in liver, heart, and skeletal muscle. Because Anamorsin was expressed in heart tissue, we compared hearts of Anamorsin$^{+/+}$, Anamorsin$^{+/-}$, and Anamorsin$^{-/-}$ embryos, and found that heart walls of Anamorsin$^{-/-}$ embryos were thinner than those of Anamorsin$^{+/+}$ or Anamorsin$^{+/-}$ mice. These results suggest that cells in these organs might also receive cytokine stimulation and Anamorsin might play some roles in cell survival of these cells. In fact, hepatocyte growth factor acts as a crucial regulator of cell growth and survival for hepatocytes (29), IL-6–type cytokines (i.e., cardiotrophin-1, leukemia inhibitory factor, and oncostatin M) for cardiac myocytes (30), and insulin-like growth factor 1, leukemia inhibitory factor, and hepatocyte growth factor for skeletal muscle cells (31). Therefore, it might be interesting to investigate whether expression of Anamorsin is also regulated by these growth factors in non-hematopoietic cells. In contrast to hematopoietic cells and cardiac myocytes, however, FL hepatocytes did not undergo apoptosis. Also, no apparent abnormality was detected in skeletal muscle of Anamorsin$^{-/-}$ mice. These findings suggest that the antiapoptotic roles of Anamorsin might be replaced by other molecule(s) in these cells. Furthermore, it is possible that Anamorsin might be a member of a new family of proteins and other family members might exist in these tissues.

Although expression of Anamorsin was detectable in the early stage of embryos, Anamorsin was not required for survival and development of early embryos. Moreover, primitive hematopoiesis in the yolk sac of Anamorsin$^{-/-}$ embryos seemed normal as judged from its histology. In contrast, Anamorsin$^{-/-}$ erythroid progenitor cells in FL could not proliferate or differentiate in response to cytokine stimulation in colony assays, and underwent apoptosis after the basophilic erythroblast stage in vivo. However, Anamorsin did not affect transcription of εy2-globin (embryonic-type), β-major-globin (adult-type), or α-globin (embryonic- and adult-type) because all these globins were normally expressed in Anamorsin$^{-/-}$ FL erythrocytes (unpublished data). From these data, it was assumed that in spite of its essential role as a survival factor, Anamorsin would not be involved in differentiation of erythrocytes.

We speculate that the main cause of death in Anamorsin$^{-/-}$ embryos was anemia because we could not find severe abnormalities in any organs except FL and spleen. By generating KO mice, a number of molecules were shown to be required for primitive and/or definitive hematopoiesis: cytokines and their receptors, EPO/EPO receptor (3), Angiopoietin-1/Tie-2 (32), and Flk1 (VEGF2 receptor; reference 33); signal transduction molecules, Jak2 (34) and p38αMAPK (35); transcription factors, c-Myb (36), GATA-1 (37), GATA-2 (38), GATA-3 (39), FOG-1 (40), AML-1 (41), SCL (42), LMO-2 (43), CBFβ (44), and EKLF (45); Bcl-2 family members, Bcl-xL (46) and DNaseII (47). As was seen in Anamorsin$^{-/-}$ mice, defects in Angiopoietin-1, Jak2, p38αMAPK, c-Myb, GATA-3, AML-1, CBFβ, EKLF, Bcl-xL, or DNaseII genes disrupted definitive hematopoiesis but not primitive hematopoiesis. To determine which molecule is responsible for the observed antiapoptotic effects of Anamorsin, it might be useful to measure expression levels of these molecules in Anamorsin$^{-/-}$ mice. At present, we have found that expression of Jak2 and Bcl-xL was down-regulated in Anamorsin$^{-/-}$ mice by using cDNA microarray and RT-PCR analyses. Because the Jak2/Stat5 pathway was reported to regulate expression of Bcl-xL (15, 16, 48), it was speculated that Jak2 might be a primary mediator of the antiapoptotic effects of Anamorsin. To examine this possibility, it would be effective to recover Jak2 and/or Bcl-xL expression in Anamorsin$^{-/-}$ hematopoietic cells in in vitro and in vivo assays.

In this study, we have shown that Anamorsin is a critical molecule in cytokine-dependent cell survival of hematopoietic cells. In addition, our preliminary studies suggested that Anamorsin expression may not only protect cells against cytokine withdrawal, but also against other apoptotic stimuli because Anamorsin-expressing Ba/F3 cells were less susceptible to apoptosis after treatment with etoposide, γ radiation, or staurosporine than IL-3–starved Ba/F3 cells in which Anamorsin expression was scarcely observed. Further studies on Anamorsin would indubitably reveal a new mechanism underlying antiapoptotic effects of hematopoietic growth factors and might be useful to establish novel therapeutic strategies to correct ineffective hematopoiesis.

We thank Dr. T. Kitamura (Tokyo University, Tokyo, Japan) for generous advice on the retrovirus-mediated expression cloning method, and Drs. A. Furuya and K. Shitara (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) for their help in making anti-Anamorsin mAbs. We also thank Drs. Y. Uno and S. Mizuno (Osaka University, Osaka, Japan) for injection of Anamorsin$^{+/-}$ ES clones into blastocysts, and Dr. G. Kondoh (Osaka University, Osaka, Japan) for dissection of mice.

This work was supported in part by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Japanese Ministry of Health, Labor and Welfare, the Japan Society for the Promotion of Science, and Kanae Foundation for Life and Socio-Medical Science.

Submitted: 27 October 2003
Accepted: 29 December 2003

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