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Homeobox gene expression plus autocrine growth factor production elicits myeloid leukemia

(hematopoietic differentiation/*Hox-2.4*/interleukin 3 growth factor/leukemogenesis)

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ABSTRACT In the murine myelomonocytic leukemia WEHI-3B, proviral insertions have induced expression of the *Hox-2.4* homeobox gene and the gene for the myeloid growth factor interleukin 3 (IL-3). To assess their potential oncogenic role, normal bone marrow cells were infected with retroviruses bearing the genes for IL-3 or IL-3 plus *Hox-2.4*. Unlike the IL-3 virus, the IL-3/*Hox-2.4* virus was highly leukemogenic. Infected cells expressing both genes exhibited retarded differentiation *in vitro*, generated myelomonocytic cell lines, and provoked a rapid, transplantable myeloid leukemia *in vivo*. The oncogenic action of *Hox-2.4* appears to derive from its ability to impede the IL-3-driven terminal differentiation of myeloid cells. The results suggest that homeobox genes can regulate key differentiation processes such as self-renewal capacity and that their inappropriate expression can be oncogenic.

The numerous vertebrate homeobox genes (1, 2) appear to encode DNA-binding transcription factors that may have managerial roles not only in embryogenesis (3) but also in subsequent cellular differentiation processes (4–7). While exploring the proposition that homeobox genes help to govern hematopoiesis, we noted (4) that the murine myelomonocytic leukemia cell line WEHI-3B (8, 9), unlike other myeloid lines, expressed the *Hox-2.4* gene, a member of the *Hox-2* cluster of homeobox genes on chromosome 11 (10). Studies by Blatt *et al.* (11) and our work (12) revealed that an intracisternal A particle (IAP) genome, an endogenous retrovirus-like element, had integrated into the 5′ noncoding region of *Hox-2.4* and induced its expression. Hence, deregulated *Hox-2.4* expression might have contributed to development of the WEHI-3B leukemia. Intriguingly, another IAP insertion in WEHI-3B has induced expression of the myeloid growth factor interleukin 3 (IL-3) (13), but that alteration alone cannot account for leukemic transformation since enforced autocrine IL-3 production provokes a gross overproduction of mature myeloid cells rather than a true leukemia (14, 15).

By retroviral delivery, we have evaluated the impact of concerted expression of *Hox-2.4* and IL-3 in normal hematopoietic cells and report here that the combination is highly leukemogenic. Whereas IL-3 renders myeloid cells autonomous, *Hox-2.4* apparently impedes their programed terminal differentiation. Thus homeobox genes can control cellular maturation. Moreover, together with recent evidence implicating a human homeobox gene in a lymphoid leukemia (16, 17), these findings indicate that somatic mutations affecting homeobox gene expression can be oncogenic.

METHODS

Retroviruses. To make a retrovirus bearing the IL-3 and *Hox-2.4* cDNAs, a *Hox-2.4* expression cassette was introduced into an MPZenIL-3 retroviral construct (15). First, an 812-base-pair (bp) *Mlu* I/*Ava* I fragment of *Hox-2.4* cDNA from WEHI-3B that includes 66 bp of 5′ and 17 bp of 3′ untranslated sequences (12) was fused to the *Hin*dIII–*Pst* I region of pcDL-SR296 (kindly provided by N. Arai [DNAX, Palo Alto, CA]), which includes the SRα enhancer/promoter and a small simian virus 40 intron (18). The SRα (*Hox-2.4*) cassette was then introduced into MPZenIL-3 at the *Cla* I site, generating MPZenIL-3/*Hox-2.4* plasmid. To produce helper-free virus, this construct was electroporated into the ψ-2 cell line (19) together with pSV2Neo at a ratio of 10:1. Transfectants were selected after 10–14 days in the antibiotic G418 (400 μg/ml) and expanded, and Southern blots were examined for intact provirus. The ψ-2 clones were titered for virus production by their ability to convert cells of the IL-3-dependent line FDC-P1 (20) to factor independence (15, 21). The best clone rendered 100% of cells factor independent; by parallel assays with a previously titered MPZipNeo producer line, its titer was estimated to be 10^6 plaque-forming units (pfu)/ml. The MPZenIL-3-producing line M5 has a titer of 2×10^5 pfu/ml (15).

Infection and Cell Culture. Cells from femurs of 12 DBA/2 mice treated 4 days earlier with 5-fluorouracil (22, 23) were cocultivated in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum, 0.05 mM 2-mercaptoethanol, and 10% pokeweed mitogen-stimulated spleen-conditioned medium (PWM-SCM) (24) with virus-producing ψ-2 cells plated 24 hr previously at 5×10^4 cells per 10-cm dish (15, 21, 23). After 5 days, nonadherent marrow cells were plated in 0.3% Bacto-agar in DMEM containing 20% fetal calf serum with or without 10% PWM-SCM. Agar colonies were scored after 7 days. Individual colonies were dispersed in DMEM containing 10% fetal calf serum and 0.05 mM 2-mercaptoethanol and expanded in this medium when appropriate. Cytochemistry was performed on fresh cytocentrifuged specimens as described (24). Flow cytometry utilized a FACScan (Becton Dickinson), and unfixed cells were labeled with directly fluorescein isothiocyanate-conjugated monoclonal antibodies produced by the following hybridomas (see refs. 25 and 26): AR3-6B2 (B220), RB6-8C5 (Gr-1), MS/114 (class II), 53-2.1 (Thy-1), M1/70 (Mac-1), and E13-161.7 (Sca-1). Dead cells were excluded by propidium iodide uptake.

***In Vivo* Tests.** Lethally irradiated (9.0 Gy) DBA/2 mice were injected with 5×10^5 to 10^6 DBA/2 marrow cells infected with MPZenIL-3 or MPZenIL-3/*Hox-2.4*. Controls were marrow cells infected with MPZipNeo virus (23) or mock-infected by cocultivation with the parental ψ-2 cells; as no differences emerged, those results are pooled in Table 1. Terminally ill mice were sacrificed, and tumorigenicity tests were performed by subcutaneous injection of 10^6 cells from their bone marrow or spleen into unirradiated or sublethally

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Abbreviations: IL-3, interleukin 3; IAP, intracisternal A particle; PWM-SCM, pokeweed mitogen-stimulated spleen-conditioned medium.

irradiated (4.5 Gy) DBA/2 recipients, which gave identical results.

RESULTS

A retroviral vector that works efficiently in hematopoietic cells, MPZen (15, 21, 23), was used to construct a retrovirus bearing the IL-3 and *Hox-2.4* genes, denoted MPZenIL-3/*Hox-2.4* (Fig. 1*a*), and its effects were compared with those of similar viruses expressing the IL-3 (MPZenIL-3; ref. 15) or an irrelevant gene (MPZipNeo; ref. 23). All were packaged in ψ-2 cells (19) to obviate the need for a helper virus. The integrity of the MPZenIL-3/*Hox-2.4* virus was confirmed by its ability to render the IL-3-dependent myeloid cell line FDC-P1 (ref. 20) factor independent, by blot analysis of DNA from infected cells (not shown), and by the presence of the expected three viral transcripts (Fig. 1*b*, lane 2). To introduce the viruses into diverse primitive hematopoietic cells, we infected bone marrow cells from DBA/2 mice previously treated with 5-fluorouracil to enrich for precursors (22) and monitored their ability to generate colonies in agar and to elicit leukemia in syngeneic mice.

***In Vitro* Growth.** The *in vitro* growth properties of the infected marrow cells suggest that *Hox-2.4* expression impedes terminal myeloid differentiation. As expected (14, 15), soft agar cultures of marrow cells infected with either IL-3-bearing virus developed colonies in the absence of added factor, but their morphology was strikingly different. Like normal uninfected colonies supported by factor, all factor-independent colonies generated by the IL-3 virus were diffuse (Fig. 2*a*) due to the migration of terminally differentiated granulocytes and macrophages. In contrast, like WEHI-3B colonies (Fig. 2*d*), a high proportion (50–60%) of the autonomous colonies generated by the IL-3/*Hox-2.4* virus were large and compact (Fig. 2*b*), some with a faint halo of migrating cells. Their cellular composition was also very similar, since stained agar cultures (24) and cytocentrifuged colony cells revealed large granulated cells resembling myeloid precursors plus a few mature granulocytes and monocytes. In three experiments, the frequency of autonomous colonies generated by the IL-3/*Hox-2.4* virus (86, 135, and 17 per 10^4 cells plated) was comparable to the frequency of clonogenic cells in control, mock-infected cultures supplemented with factors (71, 230, and 13 per 10^4 cells plated). The lower frequencies of factor-independent colonies obtained with the IL-3 virus (<1, 23, 4) probably reflect its severalfold lower titer.

Clonal longevity appears to be enhanced by *Hox-2.4* expression, because cells from the compact colonies generated by the MPZenIL-3/*Hox-2.4* virus frequently could be established in liquid culture. From three experiments, 14 of 66 such colonies tested (21%) readily yielded cell lines, whereas none of 12 factor-independent MPZenIL-3 virus-infected colonies did so. The lines were composed of round nonadherent cells with a tendency to clump, and they proliferated rapidly, doubling every 10–15 hr. Some mature neutrophils and monocytes were apparent, but most cells were large and granulated, with prominent nucleoli and

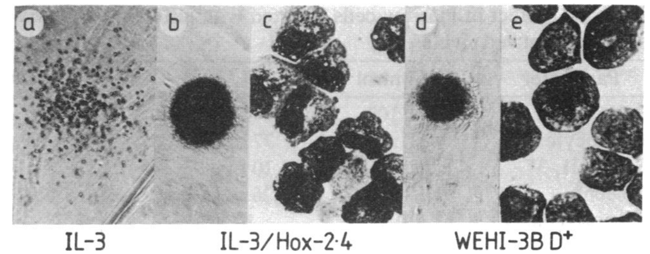

FIG. 2. Morphology of factor-independent colonies and of cells expressing IL-3 or IL-3 plus *Hox-2.4*. (*a*, *b*, and *d*) Typical colonies generated in agar in the absence of exogenous growth factor by culturing marrow cells infected with MPZenIL-3 (*a*), MPZenIL-3/*Hox-2.4* (*b*), or WEHI-3B D^+ cells (*d*). (*c* and *e*) Cytocentrifuge preparations, stained with May/Grünwald/Giemsa, of cells from a line derived from an MPZenIL-3/*Hox-2.4* virus-infected colony (*c*) or from the WEHI-3B D^+ line (*e*).

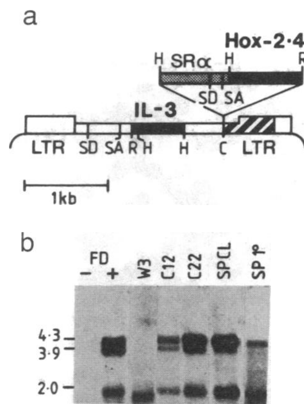

FIG. 1. MPZenIL-3/*Hox-2.4* retrovirus and blot analysis of viral RNA. (*a*) MPZen (21, 23) contains sequences from myeloproliferative sarcoma virus (crosshatched), and the hybrid long terminal repeat (LTR) drives IL-3 expression in infected cells. *Hox-2.4* mRNA initiates at an efficient derivative (SRα) of the simian virus 40 early region promoter (18). Restriction endonuclease sites are *Eco*RI (R), *Hin*dIII (H), and *Cla* I (C), whereas SD and SA mark the retroviral splice sites and that from simian virus 40 within the SRα cassette. (*b*) Poly(A)$^+$ RNA derived from uninfected (−) FDC-P1 (FD) and WEHI-3B D^+ cells (W3); FDC-P1 cells infected with MPZenIL-3/*Hox-2.4* virus (+); two myeloid cell lines derived from agar colonies of MPZenIL-3/*Hox-2.4*-infected marrow cells (C12 and C22); a line from the spleen (SP CL) of a sick animal transplanted with infected cells; and the spleen of another transplanted mouse (SP 1°). The *Hox-2.4* probe (an 800-bp *Sac* I fragment) detects the 2-kilobase (kb) transcript from the SRα-driven cassette as well as the LTR-directed 4.3-kb genomic and 3.9-kb spliced transcripts.

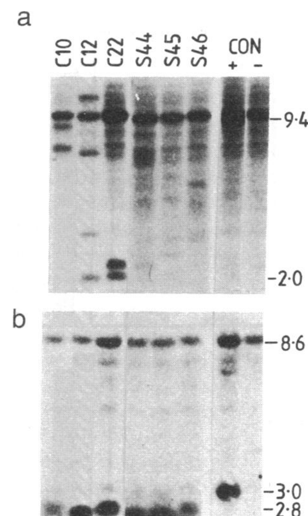

FIG. 3. Southern blots with a *Hox-2.4* probe of DNA from infected cells and tissues. (*a*) *Hin*dIII digests. (*b*) *Eco*RI digests. DNA samples were from cell lines established from agar clones of marrow cells infected with MPZenIL-3/*Hox-2.4* virus (C10, C12, and C22) or spleens of sick transplanted animals (S44, S45, and S46); the control samples (CON) were DBA/2 liver DNA with (+) or without (−) added MPZenIL-3/*Hox-2.4* plasmid. The proviral *Eco*RI fragment presumably is smaller than that from the plasmid because the small SRα intron was excised during transcription in the ψ-2 producer line. Sizes are given in kb.

Table 1. Effect of marrow cells infected with different viruses on transplant recipients

Parameter	Control	IL-3	IL-3/*Hox-2.4*
Survival*	>100	45 (30–85)	19 (16–21)
Leukocytes†	5.1 ± 3.5	77 ± 37	390 ± 130
Serum IL-3‡	0.1 ± 0.1	10 ± 9	72 ± 23
Spleen§	0.15 ± 0.05	0.26 ± 0.10	0.58 ± 0.07
Tumorigenicity¶	0/2	0/3	7/7

Sixteen irradiated mice were engrafted with cells infected with MPZenIL-3/*Hox-2.4*; five were engrafted with cells infected with MPZenIL-3; four controls were infected with MPZipNeo and one was injected with uninfected cells.
*Mean time (days) and range (in parentheses) to terminal illness.
†Leukocytes, normally 4–7 × 10^6 per ml (mean ± SD).
‡Serum IL-3 (10^3 units/ml); assayed on 32Dc13 cells and converted to bone marrow units as in refs. 15 and 24.
§Grams of tissue (mean ± SD).
¶Numbers denote individual donor animals tested. The subcutaneous tumors arose after 19–57 days.

variable nuclear indentation (Fig. 2*c*), virtually indistinguishable from WEHI-3B cells (Fig. 2*e*). Most cells stained for myeloperoxidase and chloroacetate esterase (two markers of the granulocyte lineage) as well as nonspecific esterase, which is expressed in the monocytic lineage. None stained with astra blue, which is specific for mast cells. Their myeloid character was confirmed by flow cytometry (see refs. 25 and 26): the cells expressed Mac-1, Mac-2, and Gr-1 (a marker present on granulocytic but not monocytic cells) but lacked H2 and the lymphoid surface antigens Ly5 (B220) and Thy-1 as well as Sca-1, which is borne by some B and T cells as well as multipotential stem cells.

The cell lines generated by the IL-3/*Hox-2.4* virus secreted IL-3 at a level (5000–20,000 units/ml per 10^5 cells in 72 hr) within the range of those produced by FDC-P1 cells bearing this virus or the MPZenIL-3 virus (15). Thus its transforming ability for myeloid cells cannot be ascribed merely to an unusually high level of autocrine IL-3 production. The presence of viral sequences in the myeloid cell lines was confirmed by RNA and DNA blot analysis. Clones C12 and C22, for example, expressed the three expected viral transcripts, and the level of the 2.0-kb *Hox-2.4* mRNA was comparable to that engendered in WEHI-3B by the IAP insertion (Fig. 1*b*). Southern blot analysis with *Hin*dIII (Fig. 3*a*) revealed that most clones contained one to three proviral inserts. Certain clones derived from the same infection had very different proviral insertion patterns (e.g., C10 and C22 in Fig. 3*a*), indicating that more than one precursor cell had been transformed by the virus, but other clones appeared to share a common insert and may represent reinfected siblings of a single transformed cell (not shown). Hence further analysis will be required to establish the frequency of transformation.

***In Vivo* Behavior.** The IL-3/*Hox-2.4* virus provoked an aggressive and distinctive myeloid disease (Table 1). All 16 animals transplanted with infected bone marrow cells became terminally ill within 16–21 days. Their pathology, which included an enlarged pale spleen, distended mesenteric lymph node, congested lungs, and a high IL-3 level in the serum, was similar to that of mice reconstituted with MPZenIL-3 virus-infected cells, but the latter survived longer (30–85 days; Table 1 and ref. 15). Both cohorts of sick mice had a great excess of mature myeloid cells (primarily neutrophils, monocytes, and eosinophils) in the blood (Fig. 4 *a*, *c*, and *d*) and the alveolar walls of the lungs (not shown) as well as increased numbers of mast cells in the marrow and spleen. Only IL-3 plus *Hox-2.4*, however, provoked an elevated level of *immature* myeloid cells in the bone marrow. Myeloblasts and myelocytes were notably elevated (Fig. 4 *e* and *h*) and frequently infiltrated the adjacent muscle (not shown), sometimes in association with hind limb paralysis. Immature myeloid cells were also prominent in the spleen, lymph nodes, and periportal areas of the liver. Autonomous myelomonocytic cell lines were readily obtained from the spleen and bone marrow of these mice, whereas mice reconstituted with MPZenIL-3 virus-infected cells yielded only slowly growing mast cell lines, in agreement with previous studies (14, 15).

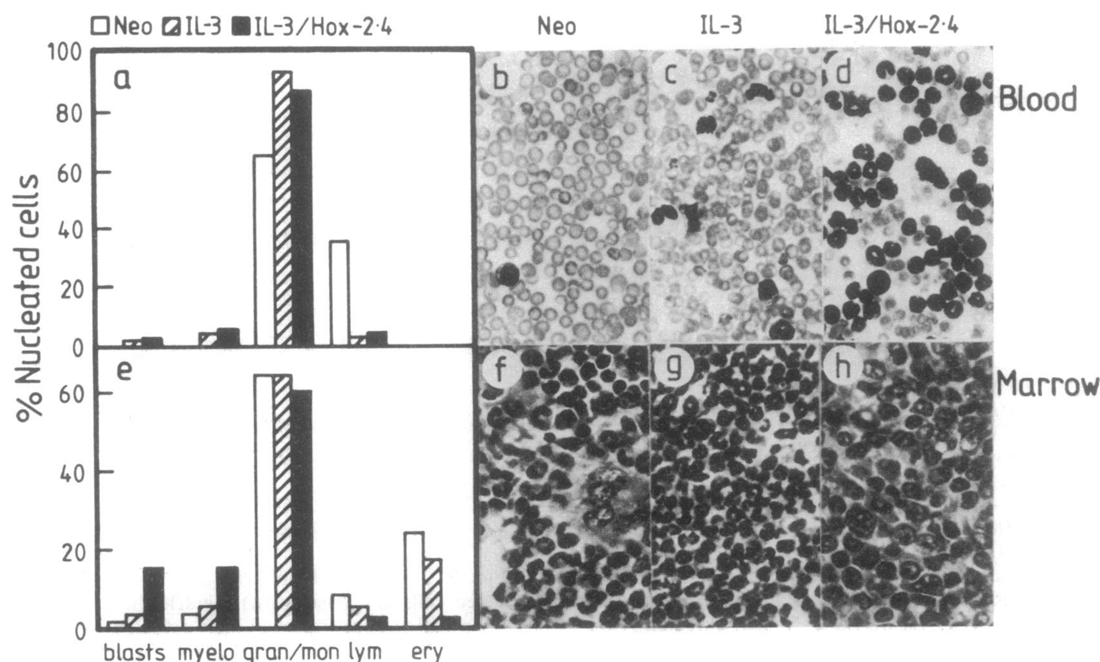

FIG. 4. Impact of viruses expressing neomycin resistance (Neo) (control), IL-3, or IL-3 plus *Hox-2.4* on the composition of the blood and bone marrow of reconstituted mice. The blood films and cytocentrifuged specimens, stained with May/Grünwald/Giemsa, were derived from mice reconstituted with marrow cells infected with MPZipNeo (*b* and *f*), MPZenIL-3 (*c* and *g*), and MPZenIL-3/*Hox-2.4* (*d* and *h*). In the quantitative analysis of leukocytes (*a* and *e*), "blast" includes promyelocytes and myeloblasts; "myelo" represents myelocytes; "gran/mon" includes "bands", neutrophils, eosinophils, and monocytes; "lym" refers to lymphocytes; and "ery" refers to erythroid elements.

The proliferative disorder induced by the MPZenIL-3/*Hox-2.4* virus appears to be at least oligoclonal, since *Hin*dIII digests of splenic DNA rarely revealed dominant proviral inserts (e.g., S44, S45, and S46 in Fig. 3*a*), despite the high concentration of proviral DNA evident in *Eco*RI digests (Fig. 3*b*). Thus *in vivo* as well as *in vitro* results suggest that multiple clones were transformed. Viral RNA was evident in the spleens of transplant recipients (e.g., SP 1°, lane 7 in Fig. 1*b*), although, curiously, the spliced (3.9 kb) transcript was not prominent.

The myeloid disease elicited by the MPZenIL-3/*Hox-2.4* virus was aggressively tumorigenic, in marked contrast to the nontransplantable myeloid hyperplasia induced by viruses producing IL-3 alone (refs. 14 and 15 and Table 1). In all seven cases tested, bone marrow or spleen cells from sick mice elicited tumors in syngeneic mice within 19–57 days (Table 1) as did seven of eight cell lines established from agar clones. The large subcutaneous tumor deposits contained abundant proviral DNA and were composed of immature myeloid cells that infiltrated neighboring tissues. High levels of serum IL-3 (25,000 units/ml) were accompanied by a marked increase in mature myeloid cells in the blood, hematopoietic organs, liver, and lungs. This disease profile recapitulates that of mice transplanted with WEHI-3B cells (8).

Tests on Fibroblasts. To determine whether *Hox-2.4* is also oncogenic for nonhematopoietic cells, we tested the MPZen-IL-3/*Hox-2.4* virus on the established NIH 3T3 and BALB/c 3T3 fibroblast lines. Infection of 4×10^5 cells with 10^4 or 10^5 infectious units of virus yielded no morphologically transformed foci under conditions where H-*ras*-transformed foci were readily apparent. As a sensitive tumorigenicity test, pools of these infected cells, and also of NIH 3T3 cells infected at 2000 infectious units per cell, which secreted abundant IL-3, were injected into nude mice (2×10^6 cells per mouse). Only 2 of 25 injected mice developed fibrosarcomas, and these appeared after 15 weeks and did not contain proviral DNA. Although the IL-3/*Hox-2.4* virus was not tumorigenic for fibroblasts, the level of the SRα-driven *Hox-2.4* mRNA in infected NIH 3T3 cells unexpectedly proved to be severalfold lower than in myeloid cell lines. Hence oncogenic effects on fibroblasts at much higher levels of expression cannot be excluded.

DISCUSSION

The inference that the activated *Hox-2.4* and IL-3 genes found in the WEHI-3B myelomonocytic tumor contribute to leukemia development has been confirmed here by retroviral delivery of the two genes. Their concerted expression in bone marrow cells was highly tumorigenic, whereas enforced expression of IL-3 alone elicited a florid myeloproliferative syndrome that was not transplantable (refs. 14 and 15 and Table 1). Since multiple transformed clones were obtained *in vivo* and *in vitro*, it seems likely that unrestrained proliferation follows directly from expression of *Hox-2.4* and IL-3 and does not require rare mutational events. Hence concerted expression of *Hox-2.4* and IL-3 appears to be sufficient to provoke leukemia in certain myeloid precursors, although their frequency remains to be determined.

The critical effect of *Hox-2.4* appears to be to retard differentiation, as evidenced by the elevated proportion of immature myeloid cells observed *in vitro* (Fig. 2 *b* and *c*) and in hematopoietic organs (Fig. 4 *e* and *h*). A concomitant increase in longevity of myeloid clones could be inferred from the substantial proportion of colonies that readily engendered cell lines. It will be interesting to test the effects of *Hox-2.4* alone, but the titers of the viruses bearing only *Hox-2.4* that we have so far made have been too low to allow infection of normal hematopoietic cells.

Although autocrine growth factor production is implicated in a proportion of leukemias, the hallmark of leukemia is an altered differentiation program that allows indefinite persistence and hence inexorable expansion of the affected clone (27). Since *Hox-2.4* appears to augment self-regenerative divisions at the expense of terminal maturation, its action is reminiscent of that of nuclear oncoproteins such as *myc* or *myb* (28–31), and the collaboration with IL-3 may parallel their synergy with *ras*. Factor-dependent myeloid cell lines such as FDC-P1, which can be transformed simply by enforced IL-3 expression (15, 32), presumably harbor a mutation enhancing self-renewal. In some such lines, the Zn finger gene *evi-1* has been induced (33), so its action may be akin to that of *Hox-2.4*. *Hox-2.4* might be oncogenic because it mimics the action of a homeobox gene product that normally maintains ability to self-renew or because it inhibits the function of one needed for terminal differentiation. The similar structures and shared DNA recognition sequences of many Antennapedia-like homeobox genes, such as *Hox-2.4*, favor the view that their gene products can replace or compete with one another (2).

These results suggest that a new reservoir of oncogenes may be provided by the large vertebrate homeobox gene family. Although *Hox-2.4* itself was expressed in only 2 of 31 leukemia cell lines surveyed, many homeobox genes are very similar to it (12). Moreover, the human *Hox-2* locus maps to the long arm of chromosome 17 near breakpoints implicated in hematologic malignancies (34–36). Recent findings indicate that the t(1;19) translocation common to human acute lymphoblastic (pre-B) leukemia generates a chimeric polypeptide bearing a very divergent homeodomain (16, 17). Thus certain homeobox genes may be rendered oncogenic either by deregulated expression, as shown here for *Hox-2.4*, or by an altered coding region, as inferred for the t(1;19).

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