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Retrovirus-Mediated Transfer and Expression of the Interleukin-3 Gene in Mouse Hematopoietic Cells Result in a Myeloproliferative Disorder

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A high-titer, recombinant retroviral vector produced in ψ2 packaging cells has been used to introduce the murine interleukin-3 (IL-3) gene into mouse hematopoietic cells. Integration and expression of the IL-3 gene was observed in spleen foci from which could be derived factor-independent, continuously proliferating cell lines. Irradiated or genetically anemic W/W^v recipients of infected hematopoietic cells developed a myeloproliferative syndrome characterized by a marked elevation in leukocyte count, bone marrow hyperplasia, and enlargement of the liver and spleen. The syndrome reflected proliferation of one or more stem cell clones, the progeny of which were capable of repopulating secondary recipients. One animal developed the syndrome primarily by a paracrine mechanism. Endogenous IL-3 production caused amplification of hematopoietic cells but did not appear to alter the maturational or self-renewal potential of these cells.

Neoplastic cell proliferation reflects acquired somatic mutations in genes that control cell growth and differentiation (5). The dysregulated expression of growth factor genes can lead to uncontrolled proliferation of cells bearing the cognate receptors by autostimulatory mechanisms (33). A potential role for autocrine effects in human leukemia has been inferred from the observed production of hematopoietic growth factors by leukemic cells of many patients (12, 13, 43, 44). Growth factor independence correlated with endogenous growth factor production has been reported in Abelson-virus-transformed hematopoietic cell lines (8, 9, 40); autocrine-stimulated cells may be tumorigenic (32).

The use of retroviral vectors provides an efficient means to introduce growth factor genes, freed of normal regulatory constraints, into hematopoietic cells. Both interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) support proliferation of early hematopoietic progenitors of multiple lineages in addition to stimulating the function of more mature cells (25). Introduction of the mouse gene for GM-CSF into a factor-dependent hematopoietic cell line (FDC-P1) resulted in autonomous growth and tumorgenicity (20). Analogous results have been obtained with the murine IL-3 gene (14, 15, 38). In some cases, autonomous growth appeared to depend on secretion of the growth factor (14, 15, 18), whereas cell proliferation of other growth factor-producing lines was insensitive to cell density and neutralizing antibody (6, 20).

We have previously used a retroviral vector (N2-IL3) to introduce the IL-3-coding sequences into primary hematopoietic progenitors (38). Fetal liver cells were infected and cultured in vitro in semisolid culture medium. The requirement for exogenous growth factors for colony formation in such clonal hematopoietic cultures was eliminated. Mast cell lines, derived from multilineage hematopoietic colonies, were shown to contain the integrated proviral genome, to produce IL-3, and to exhibit factor-independent growth. These cells were not tumorigenic in animals, however, consistent with the need for additional mutations to fully transform primary hematopoietic cells.

In the present investigation, we used the N2-IL3 retroviral vector to introduce and express the IL-3 gene in multipotential hematopoietic progenitors and stem cells in mice. Retrovirus-infected hematopoietic cells were transplanted into irradiated or genetically anemic W/W^v mice. Many of the animals developed a myeloproliferative syndrome characterized by a 10- to 100-fold increase in the leukocyte count and enlargement of the spleen and liver due to infiltration and proliferation of myeloid cells.

MATERIALS AND METHODS

Animals and cells. All animals used were 6 to 10 weeks old. NIH/Swiss and BALB/c or NIH/Swiss nude mice were obtained from either the small animal facility of the National Institutes of Health, Bethesda, Md., or from the National Cancer Institute, Frederick, Md. The WBB6F1, +/+ and W/W^v mice, and C57BL/6J mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. The preparation, dissection, and suspension of embryonic 12-day fetal liver cells used for retrovirus infection was done as previously described (8, 39). Bone marrow cells were flushed from femurs and tibias in a small volume of Dulbecco modified Eagle medium supplemented with 2% fetal calf serum.

Retrovirus infection. The N2-IL3 virus was produced by ψ2 packaging cells that were designed to yield helper-free recombinant retrovirus particles (24). Serial dilutions of culture medium from the producer clone used in these experiments were assayed for helper virus by using an S^+ L^- cell line (3). Medium from two separate passages was assayed; each yielded only two foci on S^+ L^- cells, whereas the Neo^r titer on 3T3 cells was 2×10^7/ml, giving a ratio of recombinant-to-helper virus of 10^7. Then, 5×10^6 12-day fetal liver cells or 1×10^7 adult bone marrow cells were cocultured overnight in 100-mm-diameter tissue culture

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dishes (Falcon; Becton Dickinson Labware, Oxnard, Calif.) with nearly confluent virus-producer cells in 10 ml of Iscove modified Dulbecco medium containing 10% fetal calf serum and 6 μg of Polybrene (Sigma Chemical Co., St. Louis, Mo.) per ml. The next day, nonadherent cells were carefully aspirated and washed three times in fresh medium and they were finally suspended in 2% alpha medium at a cell concentration of 2×10^6/ml. In certain experiments, bone marrow cells were preselected in G418 (2 mg/ml) for 48 h before injection (10).

Spleen focus assay. The spleen focus assay for CFU-S was performed essentially as described by Till and McCulloch (34). Before injection of 10^6 infected fetal liver or 2×10^5 infected bone marrow cells, the animals received 900 rads of total body irradiation (cesium source, gamma cell 40). At 12 to 14 days after injection of infected cells, the animals were sacrificed. Individual spleen foci were dissected, and a single-cell suspension was made in alpha medium. Then, 15% of the cell suspension was used for clonogenic culture in methylcellulose, and the remainder was used for extraction of DNA and RNA.

Long-term reconstitution. Two types of animals were used for long-term reconstitution studies. Irradiated recipients of 10^6 fetal liver cells were maintained in autoclaved cages and received sterilized food and acidified sterile water. The W/W^v recipients of 2×10^6 infected bone marrow cells were injected without prior irradiation and were housed under standard conditions. Peripheral blood was obtained by drawing blood through the retroorbital plexus. Naphthol chloroacetate esterase (specific) and α-naphtyhl acetate esterase (nonspecific) staining of peripheral blood (41) was done according to the specifications of the manufacturer (Sigma). Differential cell counts (100 cells) were performed on Wright's stained peripheral blood smears. Hemoglobin phenotype was determined by cellulose-acetate electrophoresis of peripheral blood as previously described (36).

At the time of sacrifice, various tissues were removed and fixed in 10% formaldehyde. The next day, the specimens were transferred to 70% ethanol. After fixation, thin sections were cut and stained with hematoxylin for 20 to 30 min and then counterstained with eosin for 1 min. Cytospin preparations of bone marrow cells were made and stained with Wright-Giemsa. Clonogenic assay of hematopoietic progenitors was performed in methylcellulose culture containing erythropoietin (1.0 U/ml) as previously described (8, 40). Individual colonies were plucked and maintained in suspension culture (Iscove modified Dulbecco medium with 10% fetal calf serum). Karyotype analysis was performed as previously described (40).

Southern blot and S1 nuclease analysis. High-molecular-weight DNA from various tissues was obtained by lysis in sarcosyl followed by proteinase K digestion (23). DNA and RNA were extracted from individual spleen foci by lysing the suspended cell pellet in 4 M guanidine thiocyanate. This solution was overlaid on a cushion of 2.5 ml of 2 g of cesium chloride per ml in 0.1 M EDTA (pH 7.0) in polyallomer tubes (14 by 89 mm) (Beckman Instruments, Inc., Fullerton, Calif.). After centrifugation at 36,000 rpm for 16 h in an SW41 rotor, the DNA at the interface was collected and dialyzed against 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. The RNA pellet was dissolved in 0.1 M sodium acetate (pH 5.0) and precipitated with ethanol for storage. Southern blot and S1 nuclease analyses were done as previously described (21, 23).

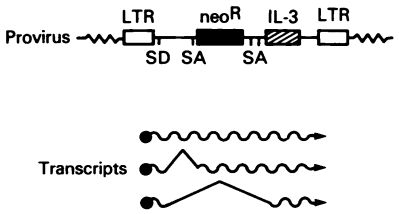

FIG. 1. Diagrammatic representation of the proviral integrated form of the N2-IL3 recombinant retrovirus. The N2 retroviral genome retains a portion of the *gag* coding sequences of the Moloney retrovirus, including the splice donor site; the presence of a cryptic site 5′ to the inserted phosphotransferase Neo^r coding sequences allows splicing at that position (1). The inserted IL-3 coding sequences are immediately preceded by a segment of the simian virus 40 early region that contains two potential splice acceptor sites (38). The primary transcript may be spliced as shown at the bottom.

RESULTS

Experimental design. By virtue of alternative splicing of the retroviral RNA transcript of the N2-IL3 provirus (Fig. 1), various mRNA species are generated that can be translated either into the phosphotransferase that confers resistance to G418 or into the IL-3 growth factor. A high-titer retroviral stock, generated in the ψ2 packaging cell line (24), has previously been used to transfer and express the IL-3 gene in established cell lines and primary hematopoietic progenitors in vitro (38). Fetal liver or bone marrow cells were infected by coculture with producer cells and injected into irradiated or W/W^v recipients, respectively. In a short-term assay, spleen foci were analyzed at 12 to 14 days to detect transfer into multipotential progenitors (CFU-S). To assess long-term reconstitution, animals were studied periodically for 15 or more weeks after transplantation.

Genetically anemic W/W^v mice (31) were used for certain of the long-term experiments, because these animals have previously proved valuable for gene transfer experiments (10). The need for lethal irradiation is eliminated; transplanted bone marrow cells competitively repopulate W/W^v mice due to their functional stem cell deficiency (2, 16). The W/W^v mice used in these experiments are heterozygous at the β-globin locus, producing three bands (50% β^s, 40% $\beta^{d\ maj}$, and 10% $\beta^{d\ min}$) on hemoglobin electrophoresis, whereas the histocompatible C57BL/6J donor animals are homozygous for the single β haplotype (31). Reconstruction with C57BL/6J cells causes a progressive increase to 100% of single hemoglobin in the recipient W/W^v animals. DNA analysis of the β-globin locus (35) can also be used to distinguish host and donor nucleated cells in transplant recipients.

Transfer and expression of the IL-3 gene in multipotential progenitors (CFU-S) in vivo. Hematopoietic cells from either bone marrow or fetal liver were used in these and subsequent experiments; similar results were obtained with cells from either hematopoietic tissue. Twenty to sixty percent of spleen foci were positive for the N2-IL3 proviral genome on DNA analysis after injection of infected bone marrow or fetal liver, and expression of the transferred IL-3 gene in individual foci was documented by S1 nuclease analysis (data not shown). Production of biologically active IL-3 and phosphotransferase in cells expressing the retroviral genome was suggested by the emergence of factor-independent, G418-resistant hematopoietic colonies in methylcellulose

TABLE 1. Factor-independent colony formation from spleen foci derived from cells infected with the N2-IL3 retrovirus[a]

Culture condition[b]	Avg no. of colonies from 5% of each focus		No. of foci analyzed		No. of foci giving colonies	
	N2-IL3[c]	Control[d]	N2-IL3	Control	N2-IL3	Control
With IL-3, without G418	—[e]	29 ± 17	—	6	—	6
With IL-3, with G418	45 ± 79	3 ± 1	7	6	5	4
Without IL-3, without G418	11 ± 9	0	19	6	14	0
Without IL-3, with G418	5.2 ± 5.8	0	27	6	16	0

[a] Individual spleen foci were dissected from spleens removed 12 to 14 days after injection of retrovirus-infected hematopoietic cells. The values given represent the mean and standard deviation of the total colonies observed in cultures of the individual foci.
[b] A cell suspension was made, and 5% of the total cells were plated in methylcellulose culture under standard conditions, including erythropoietin either with or without spleen-cell-conditioned medium (with IL-3) and/or G418.
[c] Experimental foci were derived from four animals.
[d] Control foci were derived from one animal.
[e] —, No measurement was made.

culture (Table 1). Five percent of the total cells recovered from each of several spleen foci were cultured in the presence or absence of a source of hematopoietic growth factors (spleen-cell-conditioned medium) with or without G418. Fourteen of nineteen foci from three animals gave rise to factor-independent colonies, and an additional 16 of 27 foci from four animals gave rise to colonies that were both factor independent and G418 resistant (Table 1). Approximately 10% of the colonies were mixed, containing both erythroid and myeloid cells; the remaining colonies contained only myeloid cells. No colonies were observed under these conditions from foci present in a control animal injected with cells infected with the N2 virus that contains the Neo^r marker but lacks the IL-3 coding sequence (Table 1).

Mixed hematopoietic colonies were plucked and propagated in suspension culture. Continuously proliferating, factor-independent cell lines with mast cell morphology emerged. Integration of the proviral genome in cells of seven such clones was documented by Southern blot analysis (Fig. 2). In this experiment, four clones were derived from four individual mixed hematopoietic colonies present in the methylcellulose culture of one spleen focus and the other three clones were derived from three individual mixed colonies from the culture of a second spleen focus. More than one proviral insertion site was revealed in each clone, but the patterns of insertion sites were identical for the four clones from the one focus and for the three clones from the second focus (Fig. 2). These data suggest that a single spleen focus progenitor (CFU-S) had three integrated proviral genomes in one case and two in the other. The mast cell lines had a normal mouse karyotype on cytogenetic analysis (data not shown).

Development of a myeloproliferative syndrome in recipients of N2-IL3-infected hematopoietic cells. A striking elevation in leukocyte count developed after transplantation of infected fetal liver or bone marrow cells in many recipients (Fig. 3 and Table 2). Morphological analysis of peripheral blood smears revealed that the majority of cells were of granulocytic lineage, with both mature forms, bands, and myelocytes but no myeloblasts (Fig. 4). An absolute and relative increase in eosinophils and, to a lesser extent, monocytes and basophils was also seen. The granulocytic lineage of the majority of cells was confirmed on peripheral blood and marrow smears by positive microgranular staining with naphthyl chloroacetate esterase and negative staining with α-naphthyl acetate esterase (41). Several animals were polycythemic, with hematocrits of >60%, but other animals had normal hematocrits or were anemic. Platelet counts were not done, but inspection of peripheral smears indicated normal or increased numbers of platelets. Many animals developed palpable splenomegaly and were less active than control animals. Several animals with the syndrome became semimoribund, necessitating sacrifice.

The bone marrow in the animals that had an elevated leukocyte count was intensely hyperplastic (Fig. 4). Hyperplasia was mainly of granulocytic lineage; the myeloid-to-erythroid cell ratio was vastly increased, ranging from

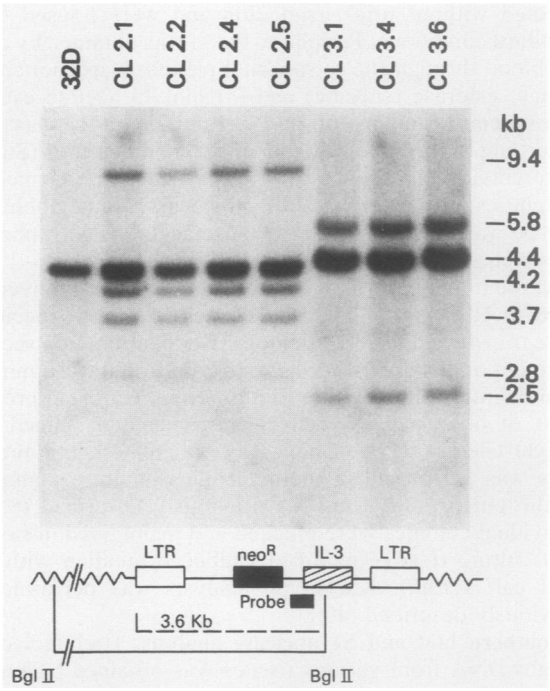

FIG. 2. Southern blot analysis of DNA extracted from factor-independent Neo^r cell lines derived from hematopoietic colonies that developed in culture of individual spleen foci. DNA (10 μg) was digested with *Bgl*II. The probe used was a *Pst*I-*Bgl*II fragment containing the IL-3 coding sequences diagrammed at the bottom. The endogenous mouse IL-3 gene, contained at a 4.4-kilobase *Bgl*II fragment, is visible in all lanes. The leftmost lane contains DNA from control 32D cells. Proliferating cells derived from four colonies of one foci (CL 2.1, CL 2.2, CL 2.4, and CL 2.5) and three colonies from a second focus (CL 3.1, CL 3.4, and CL 3.6) were analyzed. The distance between the *Bgl*II site and the 5′ end of the proviral genome is 3.6 kilobases; bands containing integration sites of less than that length are presumed to be derived from a rearranged proviral genome. Size markers (in kilobase pairs) are at the right.

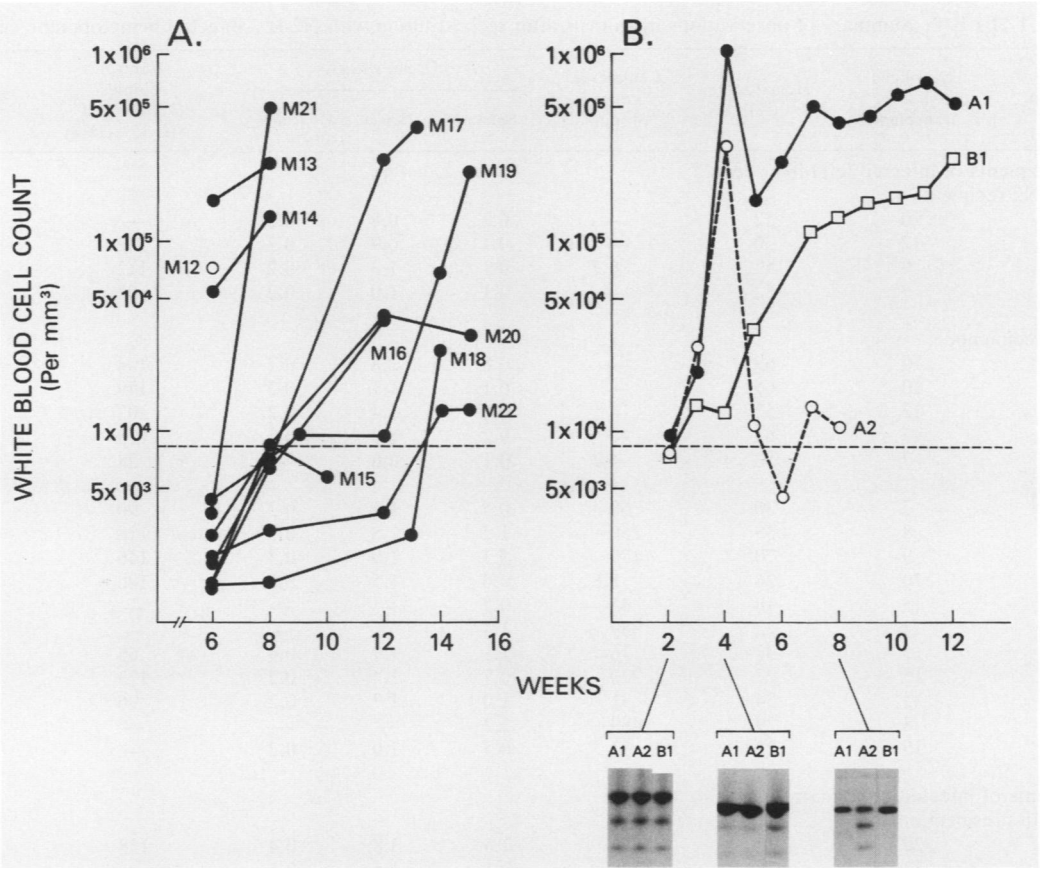

FIG. 3. Serial leukocyte counts in animals transplanted with hematopoietic cells infected with the N2-IL3 retrovirus. (A) Irradiated recipients of infected fetal liver cells. (B) Genetically anemic W/W^v recipients of infected bone marrow cells. Each line depicts data obtained from analysis of a single animal; the animal numbers correspond to those used for the data displayed in Table 2. The insets shown below panel B depict the results of hemoglobin electrophoresis at the indicated times after bone marrow transplantation. The hemoglobins in order of migration (top to bottom) are β^s and the two diffuse bands containing β^{maj} and β^{min}, respectively.

12:1 to 30:1 in affected animals (normal, 4:1). Normal maturational progression was observed, with no increase in the proportion of myeloblasts. A large increase in the number of basophil precursors was observed in many animals. All animals with elevated leukocyte counts exhibited major enlargement of the spleen and liver. Effacement of splenic architecture was observed, with a diffuse infiltrate of mature granulocytic cells (Fig. 4). In addition, megakaryocytes were regularly recognized. Striking infiltrates of mature cells of granulocytic lineage were observed in the portal areas of the liver. In addition, small foci composed of less mature cells were scattered throughout the hepatic lobules. Animals with the highest leukocyte counts also had infiltration of other parenchymal organs, including the heart, lungs, and kidneys, although normal organ architecture was preserved. The 35 animals with leukocytosis were monitored for periods of 8 to 28 weeks without evidence of progression of acute leukemia. The leukocyte differentials remained as described above, and the number of blast cells in bone marrow, spleen, and other tissues remained low.

Southern blot analysis of DNA extracted from several tissues of one animal (Table 2, M7) is shown in Fig. 5. Two retroviral insertion sites were present; the relative intensities of the two bands were equivalent in all tissues so examined. Furthermore, the intensities of the proviral bands relative to that of the endogenous IL-3 gene were consistent with the extent of tissue infiltration by hematopoietic cells as determined microscopically.

Table 2 provides a summary of the data on the animals transplanted with retrovirus-infected hematopoietic cells. Five separate experiments were performed involving 37 mice (M7 to M11, M12 to M22, A1 to A9, B1 to B3 and B7 to B12, and B4 to B6). Of 33 animals in which the leukocyte count was monitored, 17 exhibited a count of more than 10,000/mm^3 on one or more occasions (Fig. 3 and Table 2). Leukocytosis was shown to be unique to the animals that had integrated proviral DNA in their hematopoietic cells. Southern blot analysis, performed on DNA from peripheral blood, bone marrow, or spleen of animals M7 (Fig. 5), M12, M13, M14, M15, and M19 (data not shown). A1 (see Fig. 7A), B1 (see Fig. 6), and B2 to B6 (data not shown) revealed one or more integrated proviral fragments. In contrast, animals A3 to A9 and B7 to B12 lacked proviral sequences on DNA analysis and never exhibited a leukocyte count over 10,000/mm^3 (data not shown). There appeared to be a rough correlation between leukocyte elevation and organomegaly.

A total of 20 additional transplanted animals were studied to further examine the latency with which the myeloproliferative syndrome developed. W/W^v recipients received bone marrow cells cocultured with N2-IL3-producer cells. Of these, 18 animals developed leukocytosis (leukocyte count >25,000) between 3 and 6 weeks posttransplantation; in 16, the leukocytosis was sustained while two animals reverted to a normal leukocyte count by 8 weeks. In these and other animals studied (Table 2, B1 and B4 to B6), the progressive elevation in leukocyte count correlated with the conversion

TABLE 2. Summary of observations in animals after reconstitution with N2-IL3-infected hematopoietic cells[a]

Animal group	Sacrificed (wk after transplant)	Hematocrit (%)	Leukocyte count (10^3/mm^3)	Organ wt (g)			Control colonies/ 7×10^4 cell (+IL-3, −G418)	% of control	
				Spleen	Liver	Lungs		−IL-3, −G418	−IL-3, +G418
Irradiated recipients of infected fetal liver cells									
Control N2 recipients									
C1	10	52	—[b]	0.1	0.8	0.2	—	—	—
C2	12	50	—	0.1	0.9	0.2	142	<0.5	<0.5
C3	9	55	6.7	0.1	1.3	0.2	112	0.5	<0.5
C4	9	52	—	0.1	1.0	0.2	93	0.5	<0.5
N2-IL3 recipients									
M7	10	69	—	0.8	2.8	0.7	104	80	45
M8	10	65	—	0.1	1.1	0.2	149	13	8
M9	12	52	—	0.2	1.4	0.2	103	0.5	<0.5
M10	15	61	—	0.2	1.2	0.2	115	10	4
M11	7	50	4.2	0.1	0.6	—	28	<0.5	<0.5
M12	7	30	68	0.5	1.5	0.2	90	49	13
M13	8	39	250	1.2	1.8	0.3	118	59	25
M14	9	70	170	1.3	1.9	0.3	146	39	19
M15	10	26	5.6	0.6	1.5	0.2	100	18	6
M16	13	18	45	0.5	—	—	—	—	—
M17	13	22	389	1.4	—	—	—	—	—
M18	15	37	26	1.1	1.9	0.4	66	3	—
M19	15	27	220	0.7	1.4	0.2	123	10	2
M20	15	34	31	1.0	1.7	0.2	96	86	59
M21	8	29	480	1.2	—	—	—	—	—
M22	15	22	13	0.5	1.0	0.2	—	—	—
W/W^v recipients of infected bone marrow cells									
With G418 preselection									
A1	20	35	1076	1.5	3.8	0.4	158	21	7
A2	12	38	324[c]	0.1	1.5	0.3	—	—	—
A3-A9[d]	8–12	37	6.6	0.12	—	—	—	—	—
Without preselection									
B1	13	40	285	2.4	2.2	0.3	69	29	29
B2	7	42	49	0.7	1.3	0.2	60	8	6
B3	8	47	29[c]	0.4	1.4	0.3	—	—	—
B4	10	35	800	2.4	4.8	0.6	434	48	35
B5	12	40	531	2.7	3.8	0.4	235	28	25
B6	12	37	432	2.5	4.8	0.6	305	45	32
B7–B12[d]	8–12	50	7.8	0.17	—	—	—	—	—

[a] Each animal in this study was sacrificed at the indicated times, and the organ weights were determined as specified in the text. Bone marrow was cultured in vitro in methylcellulose. The plating efficiency was determined independently for each animal, and the relative numbers of colonies formed in the absence of IL-3 either with or without G418 was determined as described in the text.

[b] No measurement was made.

[c] Maximal value. Leukocyte count had returned to normal by the time of sacrifice.

[d] These animals showed no hematological abnormalities. Quantitative values are averages.

to donor type hemoglobin (Fig. 3D and data not shown). Serum obtained from these 20 animals at 11 weeks posttransplantation was analyzed for IL-3 activity by testing the ability of serial dilutions to stimulate growth of 32D cells (38). All animals with leukocytosis had high concentrations of IL-3 activity, whereas sera from animals with normal leukocyte counts had no stimulatory activity. There was no correlation between the serum IL-3 activity and the rate at which the syndrome developed, but those animals with the highest serum IL-3 activity had the highest leukocyte counts.

RNA extracted from peripheral blood, bone marrow, and spleen cells of three animals (A1, B1, and B2) was analyzed for proviral spliced IL-3 transcripts by S1 nuclease mapping. The predicted spliced species were demonstrated in each case (see Fig. 7; data not shown).

Mechanism of the myeloproliferative syndrome. (i) Gene transfer into stem cells. Three lines of evidence support integration of the N2-IL3 proviral genome into stem cells in several transplant recipients. The first is based on the development of factor-independent hematopoietic colonies in methylcellulose culture. The bone marrow cells of each animal were placed in culture with spleen-cell-conditioned medium as a source of growth factors to establish the colony-forming efficiency of that sample (Table 2). Concurrently, two additional aliquots of each sample were plated in the absence of added spleen-cell-conditioned medium (−IL-3), one without and the second with added G418 (Table 2). The number of factor-independent and the number of factor-independent, G418-resistant colonies for each animal were expressed as the percentage of the number of colonies observed in the control culture of bone marrow cells of that animal (Table 2). Growth factor-independent and G418-resistant colonies were consistently observed in cultures of bone marrow cells from animals that exhibited leukocytosis and organomegaly. Approximately 5 to 10% of the colonies

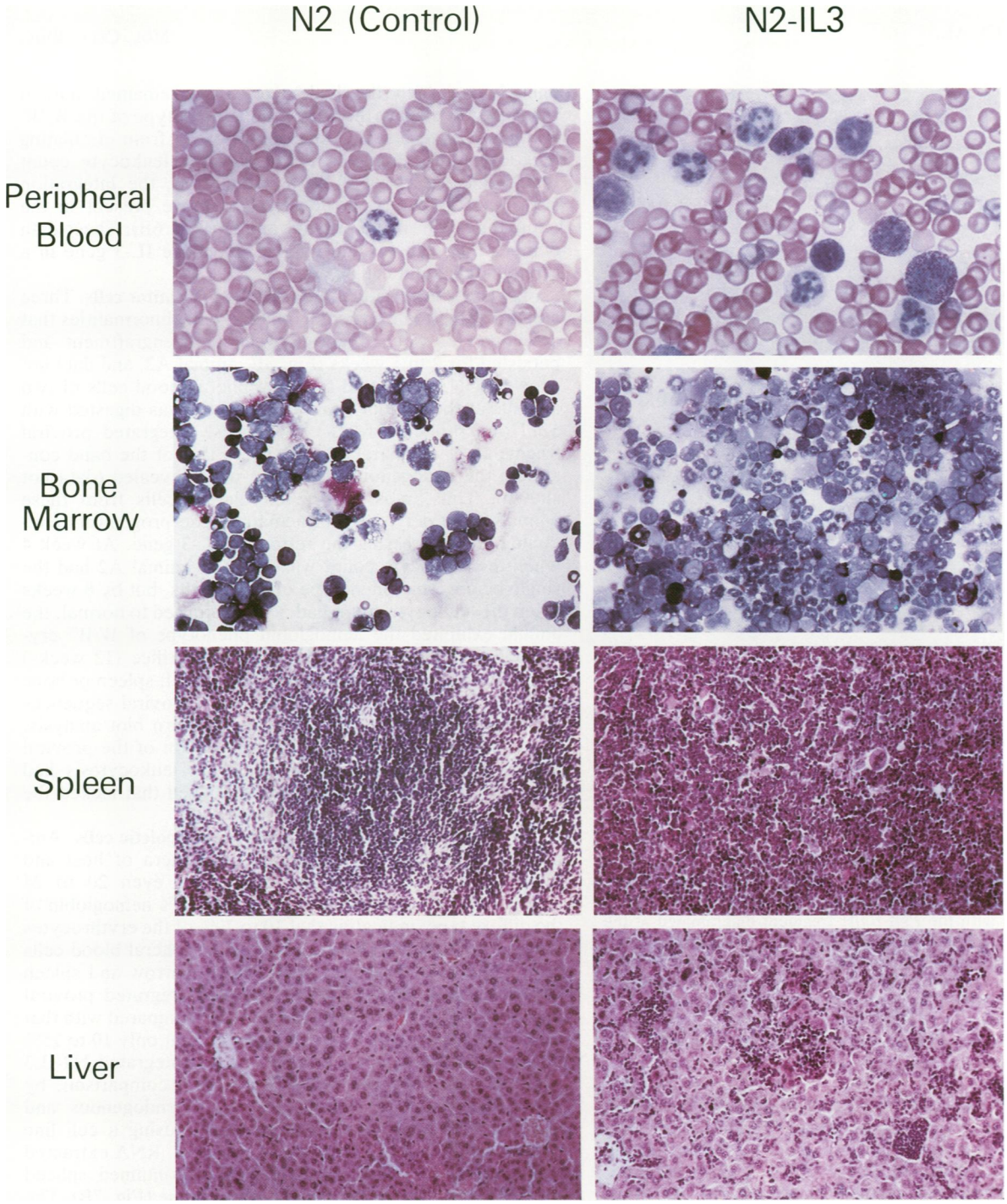

FIG. 4. Comparison of morphologic infections of peripheral blood, bone marrow, spleen, and liver from irradiated animals reconstituted with cells infected with the N2 (control) or N2-IL3 retroviruses are shown. Peripheral blood of the animal whose hematopoietic cells were expressing the IL-3 gene contain numerous mature neutrophils as well as immature forms and undergranulated basophils. The bone marrow was intensely hyperplastic, with predominantly myeloid cells that show normal maturational progression. The normal architecture of the spleen was distorted by active hematopoiesis; note the numerous megakaryocytes. The overall architecture of the liver was preserved, but numerous neutrophils were present in the portal areas and immature myeloid forms are clearly evident within the hepatic lobules.

were mixed, containing erythroid and myeloid elements, indicating derivation from a multipotential progenitor closely related to hematopoietic stem cells. Three control recipients that received bone marrow cells infected with the N2 virus (Table 2, animals C2 to C4) lacked factor-independent, G418-resistant colonies as did the bone marrow of control C57BL/6J and W/W^v animals (data not shown).

Four mixed colonies and three of seven nonerythroid colonies derived from the bone marrow of animal M7 generated continuously proliferating, factor-independent cell lines. The integrated proviral genome was demonstrated in each (data not shown). Cytogenetic analysis revealed a normal karyotype in the two lines so studied.

The second line of evidence for stem cell transfer is based on DNA analysis. As described above, evidence of retroviral insertion was found in cells of all animals studied that exhibited hematological abnormalities. The number of proviral integration sites generally provides an indication of the

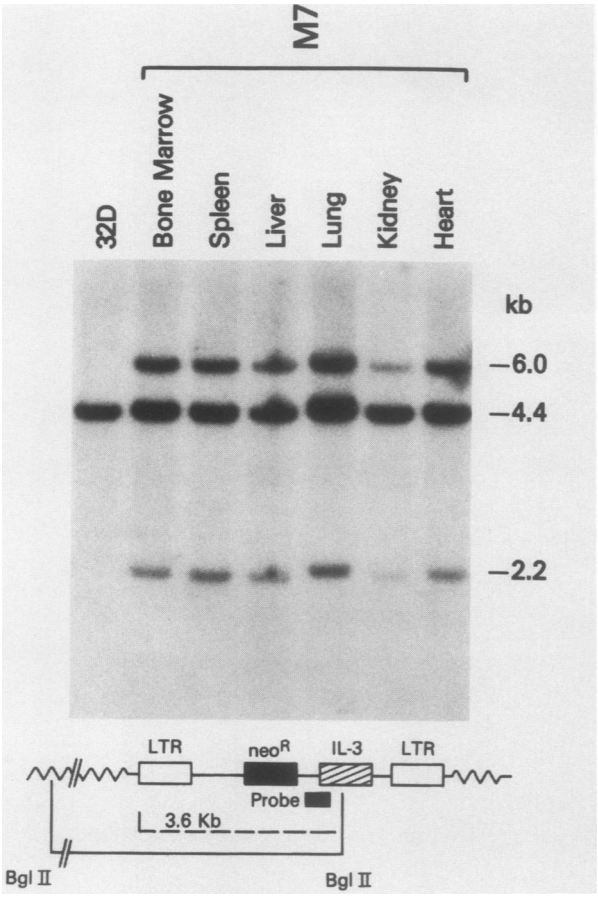

FIG. 5. Southern blot analysis of DNA extracted from several tissues of a mouse that exhibited the myeloproliferative syndrome (Table 2, animal M7). Ten micrograms of DNA was digested with *Bgl*II and probed with the *Pst*I-*Bgl*II fragment diagrammed at the bottom. The leftmost lane contains DNA from control 32D cells. The 4-kilobase fragment containing the IL-3 gene is visible in each lane. Tissues from mouse M7 contain two integrated proviral genomes, one of which is rearranged, because its length (2.2 kilobases) is less than the distance between the internal *Bgl*II site in the 5′ end of the proviral genome as shown in the diagram.

number of stem cells that are contributing blood cells in individual animals (10, 22). Five animals (M12, M14, B2, B5, and B6) had a single integrated provirus, verifying that the circulating blood cells were derived from a single hematopoietic stem cell (data not shown). In others, e.g., M7 (Fig. 5) and B1 (Fig. 6), there were two proviral insertion sites, consistent with a contribution from two stem cells or two integration events in a single stem cell.

The third line of evidence for stem cell transfer was the ability to repopulate secondary animals with cells from one of the primary recipients. Bone marrow cells (2×10^6) from animal B1 were injected into four secondary W/W^v recipients. Two animals developed a persistently high leukocyte count within 4 to 6 weeks after injection of cells from the primary animal. The leukocyte counts ranged from 60,000 to 400,000/mm^3 thereafter until one animal died spontaneously and the other was sacrificed at 12 and 14 weeks posttransplantation, respectively. Hematopoietic reconstitution, as judged by a switch to the single hemoglobin phenotype characteristic of donor erythroid cells, coincided with the increase in leukocyte count in both animals. The other two animals in which the leukocyte count remained normal continued to display the hemoglobin phenotype of the W/W^v recipient (three bands). Analysis of DNA from circulating leukocytes of animals with the elevated leukocyte count revealed evidence of proviral integration; the integration sites were shown to be identical to those present in the circulating cells of the primary animal (Fig. 6B). These data demonstrate transfer and expression of the IL-3 gene in a repopulating stem cell.

(ii) Gene transfer into multipotential progenitor cells. Three animals exhibited transient hematopoietic abnormalities that developed between 3 and 6 weeks postengraftment and persisted for 2 to 5 weeks (Fig. 3B, animal A2, and data not shown). DNA extracted from peripheral blood cells of two animals at the time of peak leukocytosis was digested with *Sac*I and probed for IL-3 sequences. Integrated proviral fragments of comparable intensity to that of the band containing the endogenous IL-3 gene were revealed (data not shown). Thus, most of the circulating cells from these animals were derived from hematopoietic progenitors containing and expressing the retroviral IL-3 gene. At week 4 when the leukocyte count was 315,000, animal A2 had the single hemoglobin phenotype of donor cells, but by 8 weeks when the leukocyte count had nearly returned to normal, the animal exhibited the hemoglobin phenotype of W/W^v erythroid cells (Fig. 3B). At the time of sacrifice (12 weeks) when the leukocyte counts were still normal, spleen or bone marrow DNA or both was obtained. No proviral sequences were detected in these samples on Southern blot analysis. We infer from these results that integration of the proviral genome in these animals with transient leukocytosis had occurred in a multipotential progenitor cell that lacked the potential for long-term reconstitution.

(iii) Paracrine stimulation of host hematopoietic cells. Animal A1 was a mixed hematopoietic chimera of host and donor cells. Hemoglobin electrophoresis even 20 to 24 weeks posttransplantation revealed 5 to 10% hemoglobin of the diffuse type, indicating that 10 to 20% of the erythrocytes were of host origin. DNA analysis of peripheral blood cells at 8, 12, 20, and 24 weeks and of bone marrow and spleen DNA at the time of sacrifice revealed integrated proviral sequences (Fig. 7A). The band intensity, compared with that of the endogenous IL-3 gene, suggested that only 10 to 15% of the hematopoietic cells contained the integrated N2-IL3 genome. This estimate was derived from comparison, by densitometry, of the intensities of the endogenous and inserted IL-3 bands on Southern bands, using a cell line containing the N2-IL3 provirus as a control. RNA extracted from peripheral blood cells and spleen contained spliced species transcribed from the proviral genome (Fig. 7B). The serum from this animal was shown to stimulate the proliferation of factor-dependent 32D cells; the addition of a neutralizing anti-IL-3 antibody to the assay reduced stimulation to 25% of that obtained with the serum alone (data not shown).

We interpreted these data to indicate that N2-IL3-infected donor cells were secreting IL-3 in amounts sufficient to stimulate other hematopoietic cells, including those of host origin, leading to bone marrow hyperplasia, an elevated leukocyte count, and organ infiltration. This interpretation was substantiated by DNA analysis of the β-globin locus in circulating leukocytes at 20 weeks (35). The ratios of band intensities of *Eco*RI fragments corresponding to the β^s, $\beta^{d\ maj}$, and $\beta^{d\ min}$ genes were nearly identical to that of control W/W^v DNA (data not shown), indicating that most of the circulating leukocytes were of host origin. The discrep-

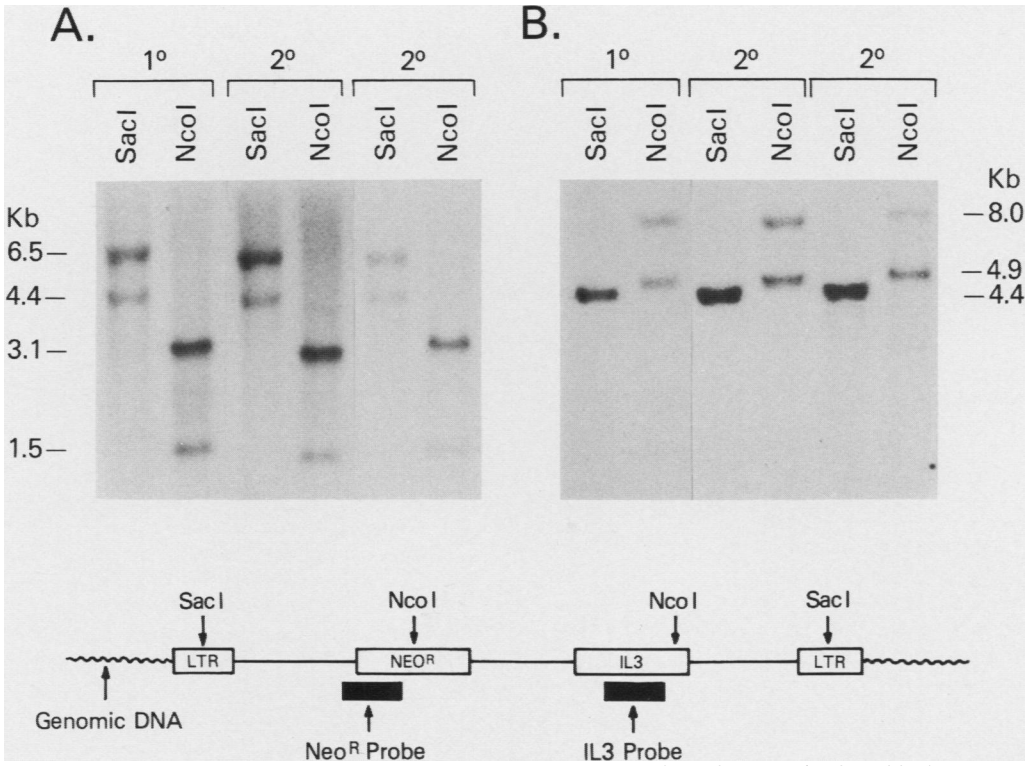

FIG. 6. Southern blot analysis of DNA extracted from circulating leukocytes of a primary animal (Table 2, B1) or from secondary recipients of B1 bone marrow cells was digested with *Sac*I or *Nco*I. Twenty micrograms of DNA was analyzed in each case. (A) The blot was probed with a *Pst*I-*Bgl*II fragment containing IL-3 sequences. The *Sac*I and *Nco*I fragments are internal to the proviral genome. (B) The same filter was probed with a *Bgl*II-*Nco*I fragment, as shown at the bottom, containing Neo^r coding sequences. The *Nco*I digestion reveals junction fragments; two fragments present in the primary animal (1°) are also present in the hematopoietic cells of the two secondary recipients (2°).

ancy between the apparent percentage of erythrocytes and leukocytes of host origin (10 to 20% compared with 80 to 90%) may be due to the relatively short survival of W/W^v erythrocytes compared with those of the donor.

DISCUSSION

These experiments were designed to define the effect of IL-3 synthesis in hematopoietic stem and progenitor cells in vivo. Insertion of the IL-3 gene into these cells was achieved by retrovirus-mediated gene transfer as documented by analysis of DNA extracted from the cell progeny. Expression of the proviral genome in very early hematopoietic cells was suggested by the ability of multipotential hematopoietic progenitors to give rise to mixed colonies in culture without added hematopoietic growth factors and in the presence of G418. Division of hematopoietic stem and early progenitor cells may result in self-renewal, with the formation of two cells that retain the full potential of the original cells. However, most often division of hematopoietic cells occurs in the context of maturational progression in which proliferation results in the increase in the production of functional, end-stage cells. From in vitro analysis, hematopoietic growth factors in general and IL-3 specifically, are thought to support proliferation in the context of maturational progression and not to influence lineage determination or self-renewal directly. Our in vivo data are consistent with this interpretation, because we observed expansion of hematopoietic tissue without a disturbance in maturation as a consequence of endogenous IL-3 synthesis. Specifically, there was no evidence for accumulation of early, undifferentiated cells due to enhanced self-renewal or a disturbance in maturation. None of the animals developed acute leukemia during the 8 to 28 weeks of observation after transplantation.

Exogenous administration of IL-3 to mice causes an increase in leukocyte count, splenic enlargement, and mast cell proliferation (26–28), although the magnitude of these changes has been far less than that observed in the animals reported here. These quantitative differences may reflect the short period of IL-3 administration in earlier studies or the difficulty of reproducing with exogenous growth factor the levels of IL-3 achieved in the bone marrow microenvironment and without hematopoietic progenitor and stem cells by the retrovirally inserted and expressed gene. The disturbances in hematopoiesis in our animals seem similar to those described in mice transplanted with malignant T cells that produce hematopoietic growth factors (42). Indeed, one of our animals (Table 2, A1, and Fig. 7) appeared to have developed the syndrome primarily by a paracrine mechanism.

IL-3 and GM-CSF have very similar activities in vitro (25). Nonetheless, the syndrome we have described differs strikingly from that observed in transgenic animals overexpressing the GM-CSF gene (19). The pathological accumulation of activated monocytes and macrophages in tissues of GM-CSF transgenic animals appeared to reflect preferential expression of the transgene in cells of the monocyte-macrophage lineage. Despite elevation in the circulating levels of GM-

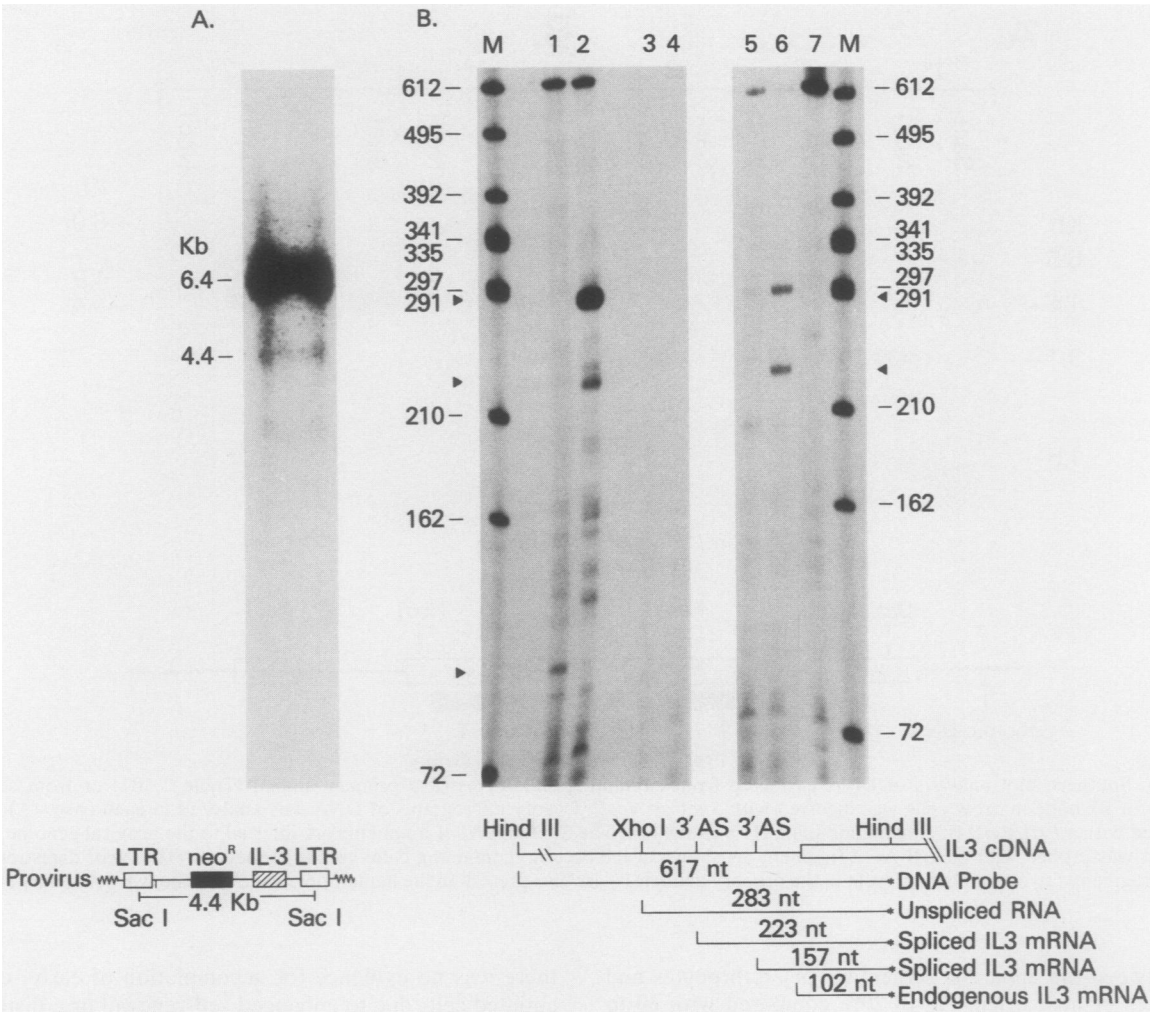

FIG. 7. Analysis of DNA and RNA extracted from the circulating blood cells of animal A1 at 25 weeks. (A) DNA was digested with *Sac*I and was hybridized to a *Pst*I-*Bgl*II probe containing IL-3 coding sequences. The intense 6-kilobase band contains sequences of the endogenous IL-3 gene, while the faint 4.4-kilobase band is the proviral genome. (B) S1 nuclease analysis of RNA extracted from control and peripheral blood cells of animal A1. In each case 20 μg of RNA was annealed to a 617-nucleotide *Hin*dIII fragment, 5′ labeled at the *Hin*dIII site. As indicated in the diagram at the bottom, an unspliced transcript protects a probe fragment of 283 nucleotides. Spliced IL-3 mRNA species derived from the proviral genome or endogenous genes are as indicated in the diagram. Two other potential spliced species that give protected fragments of 265 and 118 nucleotides with this probe (6) were not detected in these experiments. Lanes: 1, RNA from Wehi 3D cells that express the endogenous IL-3 gene; 2, RNA extracted from a 32D cell clone containing the integrated N2-IL3 provirus; 3 and 4, RNA extracted from peripheral blood and spleen, respectively, of a nontransplanted control animal; 5 and 6, RNA extracted from spleen and peripheral blood, respectively, of animal A1 at 25 weeks; 7, tRNA; M, ^{32}P-labeled ϕX DNA markers.

CSF, the bone marrow histology and circulating blood counts were normal in the transgenic animals.

The disease exhibited by the mice carrying N2-IL3 falls within the spectrum of human disorders referred to as myeloproliferative syndromes (37). These are clonal hematopoietic disorders characterized by excessive proliferation and accumulation of mature hematopoietic cells that have relatively normal morphology and function in contrast to the acute leukemias in which there is a maturational block with accumulation of immature blast cells. There are clues that the pathogenesis of myeloproliferative syndromes might involve excessive production of, or altered sensitivity to, hematopoietic growth factors. The bone marrow of patients with myelofibrosis often contains numerous megakaryocytes and monocytes, which are potential sources of platelet-derived growth factor, a fibroblast mitogen (30). Erythroid progenitors in patients with polycythemia vera have increased sensitivity to erythropoietin (11, 29). Transfer and expression of the v-*fms* gene in early hematopoietic cells may also be sufficient for the induction of a myeloproliferative process (17). Our animal model provides direct evidence that a potentially fatal myeloproliferative syndrome in mice can be caused by dysregulated growth factor production. Furthermore, the autostimulatory mechanism involving IL-3 does not interfere with cell maturation and therefore is insufficient for the development of acute leukemia.

Many features of this syndrome resemble human chronic myelogenous leukemia (7). A consistent genetic abnormality in human chronic myelogenous leukemia is the translocation between chromosomes 9 and 22 that leads to formation of the *bcr-abl* fusion gene and synthesis of an abnormal protein with tyrosine kinase activity (4). Certainly the molecular pathogenesis and evolution of the human disease is very different from that of the mouse syndrome. Nonetheless it is

interesting to speculate that the functional consequences of the presence of the *bcr-abl* protein in hematopoietic cells are directly analogous to those of endogenous production of IL-3 in the mouse model.

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