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Leukemia initiated by hemopoietic stem cells expressing the v-*abl* oncogene

(Abelson murine leukemia virus/chronic myelogenous leukemia/hemopoietic stem cells/tyrosine kinase)

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ABSTRACT **We report a mouse model with which to study leukemogenesis initiated by a specific genetic change introduced into a primary lymphoid–myeloid pluripotent stem cell. Fetal liver hemopoietic cells were infected with a high titer of helper-free Abelson murine leukemia virus (A-MuLV) and were used to reconstitute lethally irradiated mice. Two weeks later, progenies of a single primitive hemopoietic stem cell carrying a specifically integrated A-MuLV proviral DNA could be detected in both colony-forming units in spleen and myeloid colony-forming cells in the bone marrow. Beginning at 3 weeks after transplantation, the recipients developed elevated leukocyte counts, splenomegaly, and increase of blast cells in the peripheral blood. Multiple clones of A-MuLV-infected cells were infused into each recipient. However, in the same animal, DNA extracted from various affected organs and from factor-independent lymphoid and myeloid immortalized cells all contained an identical, specifically integrated proviral genome. The A-MuLV-infected stem cells differentiated into various lineages of hemopoietic cells. Our data show that the expression of the v-*abl* oncogene in a primary lymphoid–myeloid hemopoietic stem cell directly initiates leukemogenesis by stimulating factor-independent growth. The monoclonal-type disease development seen in these animals may require the occurrence of an additional genetic event.**

Abelson murine leukemia virus (A-MuLV) is a naturally occurring retrovirus carrying the v-*abl* oncogene. The virus was originally isolated from lymphoma of B-cell origin (1). Subsequently, A-MuLV-transformed T cells (2), macrophages (3), erythroid precursors (4), mast cells (5), plasmacytoma (6), and fibroblasts (7) have been reported. The ways these transformed cells were generated appear to be a function of the specific route of virus inoculation. Therefore, accessibility of virus to the appropriate cell types appears to be an important determining factor.

Transduction of the c-*Abl* sequences into the retroviral genome generated the v-*abl* oncogene, which is the fusion product of part of the *gag* sequences of the Moloney viral genome and the truncated c-*Abl* sequences from the host genome. The protein of this *gag–Abl* fusion gene has enhanced tyrosine kinase activity. These characteristics are very similar to those of a fused breakpoint cluster region (*BCR*)-*ABL* gene found in leukemic cells of patients with chronic myelogenous leukemia (CML) (8–11). The *BCR–ABL* fusion gene is located on the Philadelphia chromosome, which originates from a balanced, reciprocal translocation between chromosomes 22 and 9. This Philadelphia chromosome was found in leukemic cells of lymphoid and myeloid origin (12, 13), and thus the initial lesion of CML is considered to have occurred at the hemopoietic stem-cell level. Given the similar structural alteration and enhanced tyrosine kinase activity in both v-*abl* and *BCR–ABL* genes, determination of the effect of v-*abl* in hemopoietic stem cells would be important. For understanding multistep leukemogenesis, it would also be important to determine the basic alterations in a hemopoietic stem cell expressing only a single genetic change.

Unlike previous experimentation on A-MuLV and following the protocol of retrovirus-mediated gene transfer, hemopoietic stem cells are fully accessible to virus infection. Our hypothesis is that expression of the v-*abl* oncogene in a primary hemopoietic stem cell is sufficient to initiate leukemogenesis. This hypothesis is tested by transplanting lymphoid–myeloid stem cells infected with A-MuLV into lethally irradiated mice. We document that lymphoid–myeloid pluripotent hemopoietic stem cells expressing the v-*abl* oncogene can develop *in vivo* a disorder that resembles a myeloproliferative disease in humans. Despite introduction of multiple clones of hemopoietic stem/progenitor cells, clonal predominance always occurred. This result also implies the involvement of additional events in v-*abl*-mediated stem-cell leukemogenesis.

MATERIALS AND METHODS

Preparation of A-MuLV Virus Stock. High-titer virus stock of $5–10 \times 10^6$ focus-forming units per ml was prepared as follows. Nonadherent, A-MuLV-transformed ψ 2 cells from our previous stock (14, 15) were collected and expanded in Dulbecco's modified Eagle's medium/10% fetal calf serum. Exponentially growing cells at one million per ml were incubated with fresh medium overnight. The next day, viral supernatant was harvested and filtered, and aliquots were stored at −70°C until used. When tested on XC plaque assay (16), this stock of viral supernatant did not contain any helper virus.

Animals and Cells. Eight- to ten-week-old BALB/c inbred mice were obtained from the National Cancer Institute (Frederick, MD). For transplantation, recipient animals received 9 Gy of total body irradiation. The preparation, dissection, and collection of day 12 fetal liver cells used for retrovirus infection were done as described (14, 17).

Retrovirus Infection. About two million day 12 fetal liver cells were incubated with 1 ml of virus stock in the presence of Polybrene at 4 μg/ml overnight at 37°C. The next day, one million washed cells were injected into each lethally irradiated recipient.

Spleen Focus Assay. Colony-forming units in the spleen (CFU-S) spleen focus assay was done as described by Till and McCulloch (18) with the following modifications. Fourteen days after transplantation, individual spleen foci were dissected, and a single-cell suspension was prepared. The cells

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Abbreviations: CFU-S, colony-forming units in the spleen; A-MuLV, Abelson murine leukemia virus; CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; IL-3, interleukin 3.

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were used for either DNA extraction or methylcellulose clonogenic assay or for both.

Long-Term Reconstitution. Irradiated recipient mice were maintained in a sterile laminar flow hood and given autoclaved food and acidified water. Peripheral blood was obtained by drawing blood through the retroorbital plexus. At sacrifice, various tissues were dissected out, and a small piece from each organ was fixed in 10% (vol/vol) formaldehyde; the tissues were subsequently transferred to 70% ethanol. After fixation, thin sections were prepared, stained with hematoxylin, and counterstained with eosin. Cytoprep bone-marrow smears were stained with Wright–Giemsa stain. Methylcellulose clonogenic assay was done as described (14).

Southern Blot Analysis. Frozen tissues were pulverized in liquid nitrogen. Extraction of high-molecular-weight DNA from various tissues was done by treatment with SDS followed by proteinase K digestion. DNA samples were usually digested with *Eco*RI or *Bam*HI, enzymes that generate junctional fragments with respect to the integrated A-MuLV proviral genome.

RESULTS

Experimental Design. In our experimental protocol, fetal liver cells from 12-day-old mouse embryos were exposed to A-MuLV virus supernatant overnight. The next day, these cells were used to reconstitute lethally irradiated mice. Because we had success with our high-titer N2-IL3 virus in delivering the interleukin 3 (IL-3) gene into stem cells (17, 19), we explored ways to prepare high-titer A-MuLV virus stock. We improved the 3T3-transforming titer from our original p160 A-MuLV-transfected $\psi 2$ cells by 50- to 100-fold after we collected nonadherent, transformed virus producer cells to prepare new virus stock. All experiments in this report were done by using the new virus stock, which had a titer of 1×10^7 focus-forming units per ml. The helper-free status of the A-MuLV virus stock was confirmed by the XC plaque assay as reported (15, 20).

Multiple Clones of Stem/Progenitors Were Infected with A-MuLV. Before transplanting the infected donor cells into recipient mice, a portion of them were plated into methylcellulose culture. As we had documented (14, 15), numerous multilineage factor-independent myeloid colonies were observed. Cell lines were established from individual colonies, and DNA was extracted from them. By digesting the DNA samples with *Eco*RI or *Bam*HI, restriction enzymes that yield junction fragments, the number of stem-cell clones infected with A-MuLV could be determined. Fig. 1 shows the Southern blot analysis of five of these independent clones (NA3–NA8). Four of four analyzable DNA samples (NA6 DNA was incompletely digested) indicated the presence of a single copy of A-MuLV proviral DNA, and the provirus integrated randomly into the host genome at four different sites. These data indicate that multiple clones of A-MuLV-infected stem/progenitor cells were infused into each recipient.

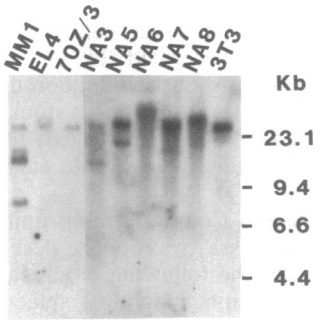

FIG. 1. Junction fragment analysis of A-MuLV-induced cell lines from factor-independent colonies. Ten micrograms of DNA samples (NA3–NA8) was digested with *Eco*RI, an enzyme that cuts externally with respect to A-MuLV proviral DNA. MM1, DNA from A-MuLV-transformed mast cell line; EL4, 7OZ/3, and 3T3, DNA from T-cells, B-cells, and fibroblast cells, respectively.

Expression of the v-*abl* Oncogene in Stem Cells Earlier than CFU-S. Donor stem cells in lethally irradiated mice were shown to develop individual colonies or foci in the recipients' spleens 10–14 days after transplantation (18). We examined 12 mice for such CFU-S spleen focus analysis. Southern blot analysis of DNA from 9 of 37 analyzable foci contained the v-*abl*-specific sequences (data not shown). In particular, more detailed analysis of one recipient, A5, revealed the following results (Fig. 2). One of three foci analyzed had a distinct 16-kilobase (kb) v-*abl*-specific fragment in addition to the 30-kb endogenous c-*abl* fragment (CFU-S2). Hemopoietic colony assay of bone marrow from the same animal, A5, revealed many progenitor cells, which developed colonies without hemopoietic growth factors, such as IL-3 and erythropoietin. Fifty-three factor-independent macroscopic colonies from four dishes containing a total of 400,000 plated bone-marrow cells were scored. Cytoprep smears of individual colonies indicated colony-forming-unit-granulocyte, erythroid, macrophage, megakaryocyte, colony-forming-unit-macrophage, colony-forming-unit-mast, and immature types of colonies. Twelve of 15 factor-independent colonies were selected and passaged into cell lines. DNAs extracted from five such cell lines were analyzed by Southern blot. Each clone showed a 16-kb v-*abl*-specific fragment (Fig. 2*B*), identical to that of CFU-S2 (Fig. 2*A*). These data indicate that v-*abl* was successfully delivered into a primitive stem cell, which gave rise to both a day 14 spleen focus (CFU-S2) and several progenitor cells simultaneously detected in the bone marrow.

Monoclonal Disease Development. Beginning 3 weeks after transplantation, pathological symptoms were noticeable in some recipients. Organ enlargement was commonly detected in the spleen and lymph node (Fig. 3). In animals A8 and A19,

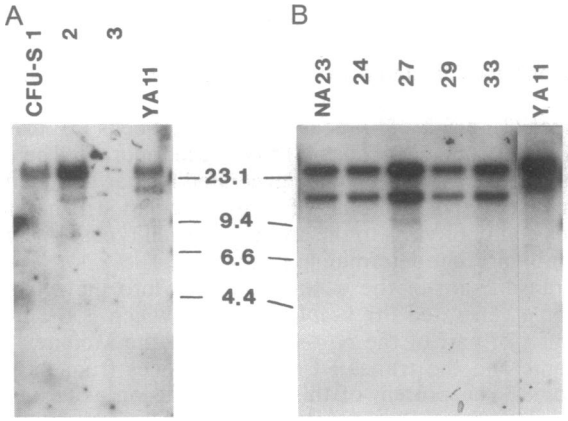

FIG. 2. (*A*) Fourteen days after transplantation with A-MuLV-infected fetal liver cells, recipient A5 was sacrificed, and DNA was extracted from individual spleen foci, CFU-S1, -2, and -3; these DNAs were then digested with *Eco*RI, electrophoresed, and transferred to Nytran nylon filter. One to 10 μg of each sample was loaded into each lane. The filter was hybridized with a ^{32}P-labeled 1.6-kb *Bgl* II v-*abl*-specific fragment. Labeling was done by using the Amersham multiprime kit. YA11, A-MuLV-transformed mast cell line (14). Note the 16-kb band in CFU-S2 DNA. (*B*) At sacrifice, bone-marrow cells from animal A5 were plated for methylcellulose hemopoietic colony assay (21). Seven days later, 15 individual factor-independent colonies were selected and passaged in suspension culture. DNAs from five independent lines (NA23, -24, -27, -29, and -33) were extracted, and Southern analysis was done. Note the same 16-kb v-*abl*-specific band in all samples.

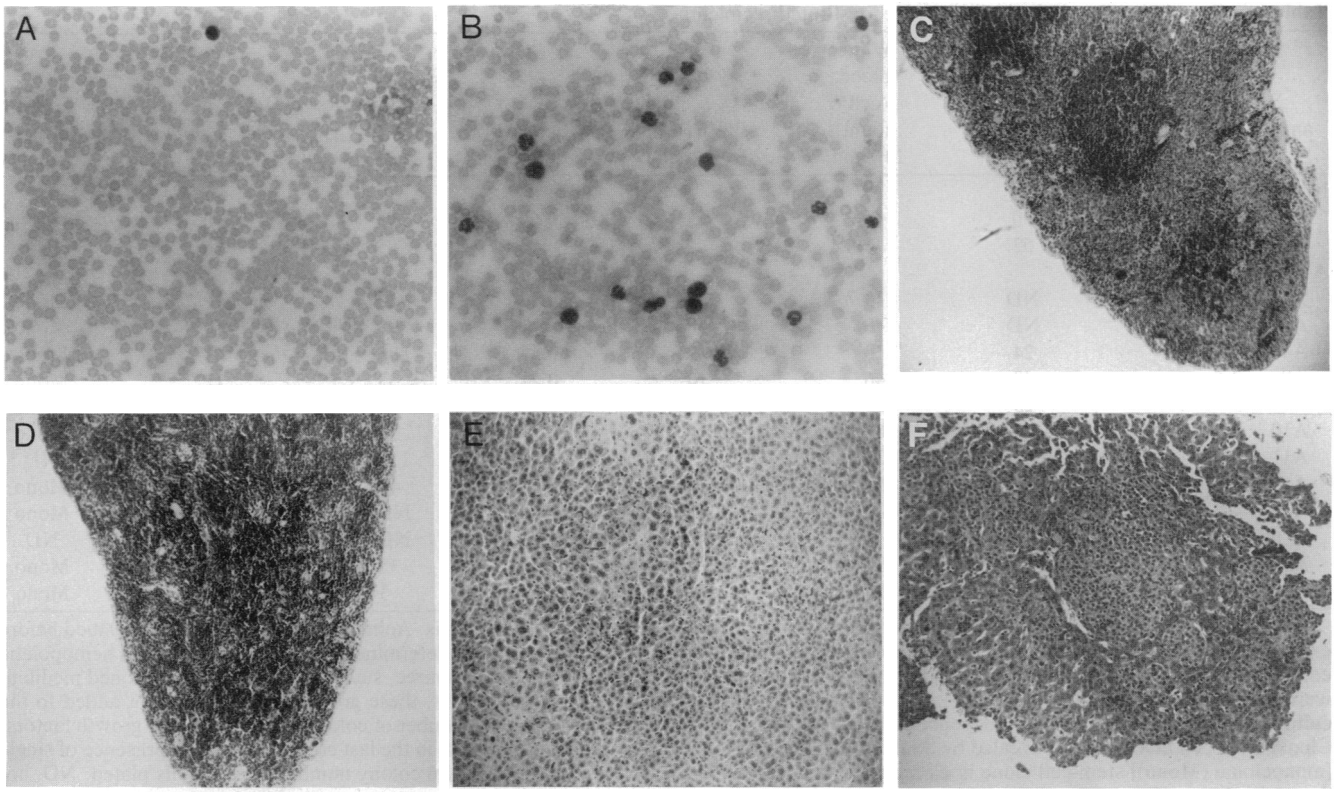

FIG. 3. Giemsa–Wright stain of a peripheral blood smear of normal mouse (*A*), leukemic recipient (*B*) (×280). Note the elevated numbers of neutrophils and the presence of blast cells in *B*. Hematoxylin/eosin-stained cross sections of normal spleen (*C*), leukemic spleen (*D*), normal liver (*E*), and leukemic liver (*F*) (×70). Disappearance of the lymphoid follicle and presence of multinucleated megakaryocytes in the leukemic spleen (*D*) were noticeable. The homogenous sheet of hepatocytes in normal mouse (*E*) was, to a large degree, infiltrated with leukemic cells resembling monocytes (*F*).

thymus enlargement was also observed. Histological sections of lymph nodes, spleens, and livers from three animals (A2, A4, and A19) revealed the following similar results. The enlarged lymph node was filled with a homogeneous population of undifferentiated leukemic cells. In the enlarged spleen, the normal architecture was destroyed (Fig. 3*D*). The cells were replaced by a type of leukemic cells similar to those in the lymph node; yet many megakaryocytes could be identified among them. In the infiltrated liver, cells resembling monocytes were present among many leukemic cells (Fig. 3*F*). These data suggest local maturation of the undifferentiated leukemic cells.

Table 1 contains the cumulative data from the reconstituted animals analyzed. The hematocrit was either normal or low. Elevation of leukocyte count and splenomegaly were common. Increase of neutrophils and blast cells in the peripheral blood was the primary cause of the elevation of leukocyte count (Table 1). An increase in blast-cell number in the bone marrow was also noted. In recipients A2 and A19, blast cells were the predominant leukocytes in the peripheral blood. In recipient A21, there were numerous mast cells present in the bone marrow, which were easily passaged *in vitro*.

To determine the number of A-MuLV-infected stem-cell clones involved in disease development, we performed restriction fragment analysis. Fig. 4 indicated that in addition to the endogenous 30-kb c-*Abl* fragment, a distinct v-*abl*-specific fragment of 19.2 kb was present in the bone marrow, thymus, lymph nodes, spleen, lung, but not in the liver. For all animals analyzed to date, except one (which contained two copies of proviral DNA), a single, specifically integrated A-MuLV proviral DNA was invariably found in the affected organs. The disease was, thus, monoclonal in all animals analyzed. This monoclonality was also confirmed by Southern blot analysis of DNA digested with *Bam*HI. Factor-independent growth of various types of myeloid progenitor cells was frequently detected and was associated with the presence of integrated A-MuLV genome (Table 1). Cell lines could be obtained easily from colonies of such factor-independent cultures. Again, a corresponding specifically integrated proviral DNA was present in the leukemic cell lines when compared with the DNA from the affected organs of the same animal.

High levels of v-*abl* mRNA were easily detected in all cell lines examined by using Northern (RNA) blot analysis (data not shown). The v-*abl* oncoprotein was expressed in all the transfected cells, as the A-MuLV RNA genome in all cell lines analyzed could be rescued by the replication-competent Moloney M-MuLV. Upon infection of 3T3 cells with the rescued virus supernatant, transformed foci characteristic of A-MuLV were observed (data not shown). These analyses in the reconstituted recipients showed that the development of leukemia is the result of v-*abl* expression in a single clone of hemopoietic stem cell.

A-MuLV-Infected Stem Cells Can Differentiate into Various Lineages of Hemopoietic Cells. Several immature cell lines were obtained from various factor-independent colonies (14, 21). Southern blot analysis of these lines from recipient A1 indicated monoclonality with respect to A-MuLV integration (Fig. 5*A*). However, when the same filter was probed with the J segment of T-cell receptor δ-chain gene, aside from those lines with germ-line configuration (NA44, NA39), different rearranged patterns were observed in NA42, -34, -35, and -37 (Fig. 5*B*). By Wright–Giemsa staining of these cell lines, we could identify lymphocytes, monocytes, and large granular lymphocytes among the predominant immature cells. Our preliminary fluorescence-activated cell sorter analysis indicated that these cell lines were positive for a natural killer cell marker asialo-GM1, a pre-B marker B220, Fc RII (22), and a

Table 1. Cumulative hematological data of reconstituted recipients

Sacrificed animal, wk after transplant	Hemato-crit, %	White blood cell count, $10^3/mm^3$	Blast cells, % in blood	Spleen wt, g	Control colonies per 10^5 cells	Factor-independent colonies, % control	Clonality
Normal mouse							
C1	50	4.2	3	0.09	95	0	
C2	52	3.8	2	0.1	85	0	
A-MuLV recipients							
A1 5	ND	13.7	31	0.22	43	5	Mono
A2 5	ND	49	80	0.31	93	10	Mono
A4 5	24	10	14	0.35	66	41	Mono
A8 3.5	40	46	10	0.21	33	12	Mono
A9 3	23	50	57	0.3	26	173	Mono
A10 4.5	45	62	ND	0.2	50	12	Mono
A11 8	46	28	55	0.45	112	6	ND
A14 4	41	57	ND	0.15	46	13	Mono
A15 4.5	ND	ND	ND	ND	ND	(17)	Mono
A16 6.5	11	3.6	ND	0.1	ND	ND	ND
A19 4.5	42	56	73	0.36	35	91	Mono
A21 17	43	16	ND	0.19	3*	25*	Mono

Animals A5, A6, A7, A12, and A13 were sacrificed 14 days after transplant for CFU-S analysis. Animals A3, A17, A18, and A20 died before examination could be done. Presence of hemopoietic progenitor cells in the bone marrow was determined by using the semi-solid hemopoietic colony assay as described (21). Under normal culture conditions, erythropoietin and an IL-3 source, such as spleen cell-conditioned medium, were added. To determine whether factor-independent growth of progenitor cells was present, these growth factors were not added to the cultures. The frequency of factor-independent colonies was expressed as percentage of total number of colonies in culture with growth factors. Clonal stem-cell involvement revealed by Southern blot analysis on junction fragments is shown in the last column. Note the presence of single [monoclonal (Mono)] stem-cell clone in all recipients analyzed. Number in parentheses is actual colony number per 10^5 cells plated. ND, not determined.

*Spleen cells were used instead of bone-marrow cells.

macrophage marker, F4/80 (23). They were negative for Thy 1.2, Mac-1, and a granulocytic marker RB6 (24). These data indicate that A-MuLV-infected stem cells could differentiate into various lineages of myeloid and lymphoid cells.

DISCUSSION

The A-MuLV-induced hematological abnormalities observed in our studies differ sharply from other reports (1–6). We attribute that difference to important changes in methods. In our studies, fetal liver cells were infected *in vitro* with a helper-free virus stock, a method we routinely used to infect hemopoietic stem/progenitor cells (14, 17, 19, 21). This procedure was followed by infusion of infected cells into lethally irradiated mice in which pluripotent stem cells are pressured to reconstitute the recipients. All leukemias induced by A-MuLV previously reported were done by inoculation with virus supernatant. Depending on the route of inoculation, plasmacytoma, thymoma, B-cell lymphoma, and mastocytoma can be observed (1–6). Normally, stem cells represent <0.1% of all marrow cells or fetal liver cells and are not in cycle. Thus stem cell-induced leukemia from i.v. inoculation with a viral supernatant is unlikely. Our studies therefore represent a protocol in which infection of stem cells with A-MuLV results in a disease reflecting the nature of the target cells.

From the animals used in this work, we could readily generate immortalized cells that further differentiate *in vitro*. *In vivo*, although blast cells were frequently present, the peripheral blood was composed mainly of neutrophils. Interestingly, recipient A2 and A19 contained many more blast cells in the peripheral blood (Table 1), and A21 had a striking

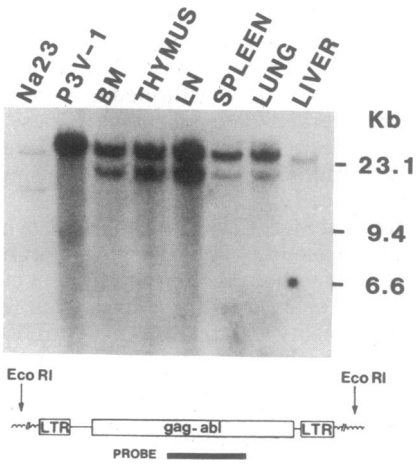

FIG. 4. Southern blot analysis of *Eco*RI digestion of DNA from various organs of recipient A8. Note the 19.2-kb v-*abl*-specific band in all organs except liver. NA36, A-MuLV-transformed line from a different recipient; P3V-1, myeloma cell line not infected with A-MuLV. LTR, long terminal repeat.

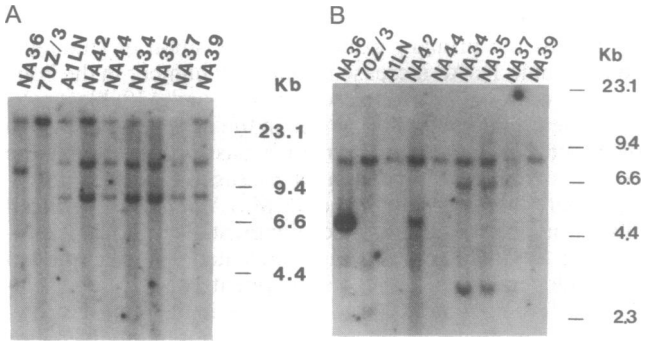

FIG. 5. Southern blot analysis on DNA of cell lines from recipient A1 (NA42, -44, -34, -35, -37, and -39). DNA samples were digested with *Eco*RI. The filter was first hybridized with *Abl*-specific probe (*A*). The DNA was then stripped and rehybridized with a probe made from J segment of T-cell receptor δ-chain gene (*B*). NA36, cell line from another recipient; 7OZ/3, pre-B cell line.

increase of mast cells in the bone marrow. Thus, although abnormalities in factor-independent growth of hemopoietic progenitor cells could be detected immediately after A-MuLV infection, expansion of a particular abnormal cell type may require additional genetic or environmental factors. This system will be useful in identifying factor(s) that influence lineage development or expansion when the initial genetic lesion occurs at the level of pluripotent hemopoietic stem cells. Growth factor production in cells expressing v-*abl* or *BCR–ABL* genes has been reported (21, 25–27). Thus, one additional factor in the development of a particular cell lineage from the infected stem cell could be the activation and production of hemopoietic growth factors. This concept is consistent with the findings that a similar type of profound myeloproliferative disorder can be demonstrated in mice having received hemopoietic progenitor or stem cells expressing granulocyte–macrophage colony-stimulating factor, IL-3, and granulocyte colony-stimulating factor genes (19, 28–31).

Junction fragment analysis of integrated A-MuLV proviral DNA indicated that the disorder involved the expansion of a single clone of pluripotent hemopoietic stem cell. It is worthwhile to consider the reason for such monoclonal development, as our *in vitro* hemopoietic colony assay indicated that a frequency as high as 50% of colony-forming cells could be infected with A-MuLV to yield factor-independent growth (data not shown; ref. 14). Most of the *in vitro* colony-forming cells are hemopoietic progenitors but not pluripotent stem cells. It is therefore conceivable that only a single pluripotent stem cell was infected in each recipient, as they are mostly dormant and represent a much smaller cell population. The A-MuLV-infected hemopoietic progenitor cells apparently did not contribute significantly to disease development. Alternatively, a number of pluripotent stem cells could have been infected with A-MuLV. Competition between various clones exist such that one predominates at a specific time. Expression of v-*abl* genes in such a clone, presumably in cooperation with other factor(s), leads to monoclonal leukemic development. We favor the latter possibility, as monoclonal stem-cell predominance related to cell cycling status has been demonstrated (32, 33). The implication is that the A-MuLV-infected stem cells maintain the property of being able to compete with one another, as normal stem-cell clones do. We think this is quite likely, as immature immortalized cell lines obtained from either hemopoietic colony cultures or directly generated from organs manifest a continuous program of differentiation (Table 1 and Fig. 5).

There are two different forms of Philadelphia-positive leukemias, CML and acute lymphocytic leukemia (ALL). These result from different breakpoints within the same gene on chromosome 22, giving rise to the expression of distinctive *BCR–ABL* gene products (34), p210 and p190 for CML and ALL, respectively. These activated p210 *BCR–ABL* and p190 *BCR–ABL* products have been suggested to account for the specific disease development of CML and ALL, respectively. Indeed, during the final preparation of our work, Daley *et al.* (35) and Elefanty *et al.* (36) reported that retroviral-mediated transfer of the p210 *BCR–ABL* gene of the Philadelphia chromosome into hemopoietic cells resulted in CML and multiple hemopoietic neoplasms in mice. The oncogene v-*abl*, a truncated and activated *Abl* gene with changes similar to *BCR–ABL*, was used in the present study. As in these two recent reports (35, 36), we document a type of hemopoietic neoplasm induced as the result of expression of the *gag–Abl* (v-*abl*) oncogene in primitive lymphoid–myeloid hemopoietic stem cells. Thus, no significant difference appears between *BCR–ABL* and *gag–Abl* with respect to leukemia induction. These results imply that the development of the type of leukemia is not due to differences between the 5′ sequences fused with the truncated c-*Abl*. In this regard, it is of interest to note that transgenic mice expressing the p190 *BCR–ABL* from ALL developed acute leukemia that affects both lymphoid and myeloid cells (37).

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