Importance of Receptor Usage, *Fli1* Activation, and Mouse Strain for the Stem Cell Specificity of 10A1 Murine Leukemia Virus Leukemogenicity[▽]

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Murine leukemia viruses (MuLV) induce leukemia through a multistage process, a critical step being the activation of oncogenes through provirus integration. Transcription elements within the long terminal repeats (LTR) are prime determinants of cell lineage specificity; however, the influence of other factors, including the Env protein that modulates cell tropism through receptor recognition, has not been rigorously addressed. The ability of 10A1-MuLV to use both PiT1 and PiT2 receptors has been implicated in its induction of blast cell leukemia. Here we show that restricting receptor usage of 10A1-MuLV to PiT2 results in loss of blast cell transformation capacity. However, the pathogenicity was unaltered when the *env* gene is exchanged with Moloney MuLV, which uses the Cat1 receptor. Significantly, the leukemic blasts express erythroid markers and consistently contain proviral integrations in the *Fli1* locus, a target of Friend MuLV (F-MuLV) during erythroleukemia induction. Furthermore, an NB-tropic variant of 10A1 was unable to induce blast cell leukemia in C57BL/6 mice, which are also resistant to F-MuLV transformation. We propose that 10A1- and F-MuLV actually induce identical (erythro)blastic leukemia by a mechanism involving *Fli1* activation and cooperation with inherent genetic mutations in susceptible mouse strains. Furthermore, we demonstrate that deletion of the *lcsbp* tumor suppressor gene in C57BL/6 mice is sufficient to confer susceptibility to 10A1-MuLV leukemia induction but with altered specificity. In summary, we validate the significance of the *env* gene in leukemia specificity and underline the importance of a complex interplay of cooperating oncogenes and/or tumor suppressors in determining the pathogenicity of MuLV variants.

Murine leukemia viruses (MuLV) are naturally occurring, replication-competent retroviruses with a high propensity to induce malignancies of the lympho-hematopoietic system, as their name implies. The relatively long latent period of tumor/leukemia development, compared to their acutely transforming counterparts that have incorporated oncogenic sequences into their genome, reflects the need for selection of multiple cooperative changes in growth control mechanisms. It is now well-established that proviral insertional mutagenesis, resulting in either activation of cellular “proto-oncogenes” or, more rarely, inactivation of tumor suppressor genes, is a critical mechanism by which normal regulatory processes are disrupted (reviewed in references 15 and 21). An increasing interest in MuLV pathogenicity has developed over the last 5 years due to the growing awareness of its utility in identifying cooperating oncogenes in both hematopoietic and non-hematopoietic cancers (1, 27, 56) and, more recently, due to two unexpected cases of leukemia in patients receiving MuLV-based gene transfer vectors in clinical gene therapy trials (16, 19).

Using MuLVs as tools to identify novel or cooperating oncogenes or as safe gene-transfer vectors requires a better understanding of the mechanisms that control leukemia induction. How can these tools be manipulated to transform (or avoid transforming) a specific hematopoietic cell lineage? A

central question is thus what viral and cellular determinants contribute to the lineage specificity of leukemia induction? Functional analysis of oncogenes implicated in acute leukemias of humans, mice, and chickens have clearly shown that oncogenes that regulate cell fate decision can determine the cell lineage specificity of the leukemia (17, 30, 35, 53, 55). However, the role of viral elements in determining lineage specificity is less clear. Are these elements necessary for targeting infection of specific cell types or are they necessary to induce oncogene expression to a critical level required to disrupt normal controls? The characterization of different MuLV isolates that induce distinct leukemia subtypes with varying kinetics has demonstrated the significance of enhancer elements within the long terminal repeats (LTR) in disease induction, in particular the T-lymphomagenic Moloney (Mo) and SL3-3 MuLV variants (2, 18, 51, 52, 61). However, although the importance of the LTR in disease specificity goes unquestioned, it is also clear that other viral elements contribute to disease kinetics and specificity. Furthermore, mouse strain differences are often ignored, although inherent mutations can be strong modifiers of both virus spread and disease induction.

To obtain better insight into the importance of sequences outside the LTR for determining cell lineage specificity in MuLV-induced leukemogenesis, we chose to investigate the viral determinants involved in the unique pathogenicity of 10A1-MuLV. 10A1-MuLV was generated upon in vivo recombination between an exogenous amphotropic MuLV and endogenous polytropic *env* sequences (25, 40, 43). Previous studies have shown that 10A1-MuLV induces early blast cell

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FIG. 1. (A) Schematic representation of 10A1-MuLV *env* recombinants used in this study. (B) Kaplan-Meier survival curves of mice infected with indicated virus recombinants.

leukemia in NIH Swiss mice, distinct to the lymphoid leukemia induced by the related amphotropic 4070A-MuLV (41, 42). In contrast to 4070A-MuLV, which uses the solute transporter PiT2 as a receptor, 10A1-MuLV also utilizes the closely related PiT1 for cell entry (32, 59). It has thus been postulated that the unique pathology of 10A1-MuLV may be due to its distinct receptor usage that enables targeting of an early hematopoietic stem cell (42). To investigate the importance of receptor usage in cell lineage specificity, we constructed retroviral chimeras using a 10A1 backbone with the *env* genes from either 4070A-MuLV or the T-cell-tropic Mo-MuLV, which uses the Cat1 receptor for cell entry. The results reported here underline the importance of cell targeting in disease specificity by showing that the unique blast cell pathology of 10A1 requires the use of either the PiT1 or Cat1 receptor. Strikingly, all mice developing blastic leukemia also showed integrations within the *Fli1* locus, originally identified as a common integration site in Friend MuLV (F-MuLV)-induced erythroleukemia (7, 8, 48). We show that this leukemia phenotype is mouse strain specific and suggest that critical levels of *Fli1* synergize with an inherent mutation in these mice and thereby induce the specific (erythro)blastic phenotype. These results have important implications in understanding cell lineage specificity in MuLV-induced leukemogenesis and manipulating these tools for insertional mutagenesis studies.

MATERIALS AND METHODS

Cells and viruses. SC1 mouse fibroblasts were used for production of virus from cloned proviral genomes, as previously described (34). The *env* recombinants were generated by exchanging the SphI-ClaI fragment containing the entire SU coding region of 10A1-MuLV with that of either Mo-MuLV or 4070A-

MuLV. Virus titers were determined by end-point dilution (1:5) and immunocytochemical detection of the CA_{p30}, as previously described (34).

Virus assays. Marker rescue assays were employed to verify the host range of the viral recombinants. SC1 cells expressing an MPEVneo retroviral vector (26) were infected with supernatant from virus-producing cells and cultured for a minimum of 10 days to ensure complete virus spread. Medium was replaced 24 h prior to harvesting of virus stocks from confluent monolayers and passed through a Millex-GP 0.22-μm filter (Millipore). Titers of virus stocks of MPEVneo pseudotypes were determined by end-point dilution. Human TE671 or murine SC1 cells expressing the indicated viruses were plated at 10⁴ cells per well of a 24-well plate on day 1. On day 2, medium was replaced with medium containing virus stock serial dilutions (1:5) and 8 μg of Polybrene per ml. After 12 h, medium was replaced, and on day 3 to 4, G418 (400 μg/ml; specific activity, 0.6) was added. Drug-resistant colonies were counted and were expressed as G418-resistant transfer units (GTU) after 10 to 14 days. All virus dilutions were analyzed in triplicate.

Mice, virus inoculations, and clinical evaluation. NIH/Ola and C57BL/6J mice were bred in the Heinrich-Pette-Institute animal quarters. C57BL/6 *Icsbp*^{-/-} mice (20) were kindly provided by Ivan Horak and maintained as homozygotes in our animal quarters. Mice were infected intraperitoneally within 24 to 48 h after birth with 50 μl of virus-containing supernatant with a virus titer of between 3 × 10⁵ and 8 × 10⁵ infectivity units/ml. Mice were regularly monitored for

TABLE 1. Receptor interference assays confirm the expected receptor usage for recombinant 10A1-E_{env}

Target cell	Titer (GTU/ml) of MuLV ^a :		
	10A1-E _{env}	10A1	Moloney
SC1	8.2 × 10 ⁵	2.5 × 10 ⁵	1.3 × 10 ⁵
SC1-10A1-E _{env}	0	9 × 10 ⁵	4
SC1-10A1	8.8 × 10 ⁵	1	3.9 × 10 ⁵
SC1-Moloney	0	4 × 10 ⁵	0
TE671	0	1 × 10 ⁵	0

^a Data are averages of results from two independent experiments.

TABLE 2. Receptor interference assays confirm the expected receptor usage for recombinant 10A1-*A_{env}*

Target cell	Titer (GTU/ml) of MuLV ^a :		
	10A1- <i>A_{env}</i>	10A1	4070A ^b
SC1	3.8×10^4	1.3×10^5	6.2×10^3
SC1-10A1- <i>A_{env}</i>	44	3.0×10^4	3.2
SC1-10A1	5	6	0
SC1-MoAmphoV	33	5.9×10^4	2.5

^a Data are averages of results from two independent experiments.^b For these experiments, the amphotropic recombinant MoAmphoV was used.

disease symptoms. Moribund mice were bled and then sacrificed and examined for hepatosplenomegaly, thymomas, and lymphomas.

Flow cytometry. For lineage marker analysis of diseased organs, single-cell suspensions were prepared from hematopoietic tissues after lysis of erythroid cells with PharmLysate (BD Pharmingen) and incubated at 4°C for 30 min in phosphate-buffered saline containing 2% bovine serum albumin with phycoerythrin-, allophycocyanin-, or CyChrom-conjugated monoclonal antibodies (BD Pharmingen). Nonspecific binding of monoclonal antibodies was prevented by preincubation with Fc Block (BD Pharmingen). Only vital cells, as determined by exclusion of propidium iodide, were measured. Cells were washed twice with phosphate-buffered saline containing 2% bovine serum albumin and applied for analysis on a FACSCalibur (BD Biosciences, Heidelberg, Germany).

Histopathology and immunohistochemistry of infected mice. Peripheral blood smears and organ sections were fixed and stained using the indicated stains obtained from Sigma (Taufkirchen, Germany). Immunohistological inspection of hematopoietic tissue was performed as previously described (47). Briefly, antigen retrieval was achieved by autoclaving deparaffinized sections for 30 min in a citrate-based demasking solution (Antigen Retrieval Citra solution; BioGenex, San Ramon, CA). After incubation with primary antibodies, specifically bound antibodies were detected using a highly sensitive phosphatase- and polymer-conjugated anti-rabbit immunoglobulin G (IgG) detection system (Histofine; Nuchirei, Tokyo, Japan). Phosphatase activity was revealed with naphthol-AS-BI-phosphate and New Fuchsin (DakoCytomation) as a substrate.

RESULTS

10A1-*env* recombinants display the expected receptor usage.

To determine the importance of the PiT1 receptor for the unique pathogenicity of 10A1, recombinants were constructed in which the *env* gene of 10A1-MuLV was replaced with either that from the ecotropic (E) Mo-MuLV or the amphotropic (A)

4070A-MuLV, generating 10A1-*E_{env}* and 10A1-*A_{env}*, respectively (Fig. 1A). To verify the receptor usage of the chimeric constructs, interference assays were performed in which retroviral vectors carrying the neomycin-resistance gene (*neo^r*) were pseudotyped with the different 10A1 *env* chimeras and then used to assess infectivity of different target cells expressing viruses of different interference groups. The 10A1-*E_{env}* recombinant displayed the same infectivity pattern as the ecotropic Mo-MuLV, including the ability to infect mouse cells (SC1) but not human cells (TE671) and being unable to infect cells expressing ecotropic viruses (Table 1). Similarly, 10A1-*A_{env}* displayed the same infection pattern as 4070A (Table 2). Cells expressing either amphotropic 4070A or 10A1 are resistant to 10A1-*A_{env}* infection, due to down-regulation of the PiT2 receptor, whereas cells expressing 10A1-*A_{env}* are still responsive to 10A1 infections, due to availability of the PiT1 receptor. In summary, both 10A1 chimeric virus constructs have the expected host range.

10A1-*E_{env}* and 10A1-*A_{env}* induce fatal diseases with kinetics similar to that induced by wild-type 10A1 and demonstrate the importance of sequences outside *env* in disease induction. Chimeric 10A1 viruses, as well as control viruses (10A1-, 4070A-, and Mo-MuLV), were used to infect newborn NIH/Ola mice with comparable titers. Ninety percent of the 10A1-*E_{env}*-infected mice ($n = 39$) succumbed to a hematopoietic malignancy with a median latency of 158 days. The disease kinetics were similar to that observed in 10A1-MuLV-infected mice but significantly slower than that observed in Mo-MuLV infected mice (Fig. 1B). Similarly, 10A1-*A_{env}* resulted in relatively rapid deterioration of infected mice, with mice surviving on the average for 162 days ($n = 26$) (Fig. 1C). This is in striking contrast to the rather slow disease induced by 4070A-MuLV, with a mean latent period of 228 days ($n = 24$). These results clearly show that regulatory sequences outside the *env* gene are important determinants of retroviral pathogenicity. However, despite the similar kinetics of disease induction by the two 10A1 chimeras, closer examination revealed distinct differences in the pathology.

TABLE 3. Summary of the leukemic phenotypes induced in MuLV-infected NIH/Ola mice

MuLV	No. of mice	Blood cell lineage	Incidence ^a	Hematocrit (%)	Mean leukocyte count (10 ⁶ /ml)	Mean spleen wt (g)	Mean liver wt (g)	Lymph nodes enlarged
None (uninfected)	19			44 ± 6.5	8.5 ± 1.2	0.37 ± 0.21	1.9 ± 0.4	
10A1- <i>E_{env}</i>	21	Blast	0.75	13 ± 6.0^c	78 ± 13	2.2 ± 0.5	3.9 ± 0.3	No
		B cell	0.10	27 ± 2.1	26 ± 56	1.8 ± 0.6	Normal ^b	Yes
		T cell	0.14	35 ± 3.5	6.8 ± 3.4	1.3 ± 0.1	Normal	Yes
10A1	26	Blast	0.64	15 ± 4.6	37 ± 53	2.0 ± 0.5	3.4 ± 0.3	No
		B cell	0.36	32 ± 9.8	66 ± 70	0.9 ± 0.4	Normal	Yes
		T cell	1.0	36 ± 6.3	26 ± 19	1.5 ± 0.6	Normal	Yes ^c
Moloney	10							
10A1- <i>A_{env}</i>	38	B cell	0.08 ^d	40 ± 5.0	14 ± 0.4	1.5 ± 0.02	Normal	Yes
		B cell	0.86	29 ± 9.8	28 ± 29	2.0 ± 0.5	Normal	Yes
4070A	15	T cell	0.14	40 ± 0.7	9.1 ± 4.5	0.9 ± 0.6	Normal	Yes
		Blast	0.50	16 ± 6.0	64 ± 70	2.4 ± 0.6	3.4 ± 0.3	No
		B cell	0.31	30 ± 7.2	13.2 ± 7.9	0.6 ± 0.2	Normal	Yes
10A1-Mo _{gag}	28	T cell	0.19	42 ± 5.7	16 ± 8	1.6 ± 0.7	3.1 ± 1.1	Yes

^a Mice that succumbed to a spongiform encephalomyelopathy before the clear onset of leukemia were not included in the leukemia incidence.^b Normal is defined as the absence of visible enlargement of the liver compared to uninfected controls.^c In addition to lymphomas, Mo-MuLV infection induced thymomas in 80% of the mice.^d In most cases, mice were sacrificed due to severe wasting symptoms before the onset of discernible hematopoietic neoplasia.^e Boldface type indicates shared characteristics of the blast cell leukemia.

FIG. 2. Histological analysis of indicated organs from mice infected with either 10A1-E_{env} or 10A1 at the time of death. Shown are the typical characteristics of mice with either a blastic leukemia (A to C) or a B-cell leukemia (D to F). (A) Spleen section stained with hematoxylin-eosin. Hematopoietic cells of the red pulp are replaced by invading tumor cells. (B) Liver section stained by periodic acid-Schiff reaction. Sinusoids between hepatocellular trabeculae are strikingly dilated through the infiltration of hematopoietic tumor cells, primarily composed of immature erythroid cells but also myelo- and lymphoblastic cells. The hepatocytes show a microvesicular fatty degeneration. (C) Blood smear stained by the method of Papanheim. Clear evidence of anemia is seen by the decreased number and the thorny form of the erythrocytes (acanthocytosis). In addition, the erythrocytes appear polychromatic. The presence of proerythroblasts, erythroblasts, and normoblasts in the blood reflects the transformation of the erythroid compartments. (D) Spleen section stained with hematoxylin-eosin. The accumulation of lymphoblastic tumor cells (lower left corner) leads to the expansion of the splenic white pulp. Hyperplastic erythropoiesis, presumably as compensation to impaired bone marrow erythropoiesis, is also observed (right side). (E) Liver section stained by the method of Giemsa. Depicted is a typical leukemic infiltrate in a portal field of the liver. The neoplastic cells show a high level of both mitosis and apoptosis. The liver tissue surrounding the tumor invasion is intact. (F) Blood smear stained by the method of Papanheim. The presence of lymphoblasts in the blood reflects the egression of differentiation blocked B cells from the bone marrow into the circulation. The polychromasia of erythrocytes is indicative of a secondary tumor anemia.

10A1-E_{env} induces an (erythro)blastic leukemia, similar to that induced by wild-type 10A1. Animals infected with 10A1-E_{env} developed two distinct diseases. The majority of the mice developed a hematopoietic neoplasia involving the erythroid/myeloid progenitor compartment; however, a significant proportion developed a lymphoid-derived malignancy (see below).

In the former case, the disease was strikingly uniform, clinically characterized by anemia and hepatosplenomegaly, but with no involvement of lymph nodes (Table 3). Histological analysis revealed that the normal architecture of the spleen was almost completely destroyed due to the accumulation of blastic tumor cells within the expanded red pulp, leading to a compression

FIG. 3. Representative FACS analysis of hematopoietic organs of moribund mice infected with either 10A1- E_{env} or 10A1-MuLV. Single-cell suspensions obtained from either enlarged spleens (A and B) or an enlarged axillary lymph node (C) were stained with antibody recognizing the indicated antigens to determine the hematopoietic lineage of the tumor cells. Mice with the characteristics of a blast leukemia were negative for all lineage markers and variably expressed the Sca-1 antigen (A) or the erythroid antigen TER119 (B). In contrast, mice with a characteristic B-cell leukemia/lymphoma were positive for the B-cell marker B220, but not for IgM, reflecting their blast morphology.

and breakdown of the white pulp (Fig. 2A). Similarly, tumor infiltrates destroyed the bone marrow structure, and both myelo- and erythropoiesis was drastically reduced. The liver was generally pale and greatly enlarged (up to 2.5-fold the normal weight) as a consequence of tumor cell invasion (Fig. 2B). Not surprisingly, due to the breakdown of the blood-forming tissue of both the bone marrow and spleen, only a very few mature erythroid cells were found in the blood, which were often distorted (acanthocytosis) (Fig. 2C). Disrupted erythropoiesis was also evidenced by the presence of immature erythroid forms, including normoblasts, in the blood. Importantly, blastic forms with either myeloid or lymphoid characteristics were also detected in blood smears. The leukocyte count varied greatly between mice (range, 4 to 211 cells/nl) (Table 3 and Fig. 2C), which probably reflects the fact that egression of the transformed cells into the blood was a late event.

A similar disease phenotype was observed in mice infected with wild-type 10A1-MuLV (Table 3) and is consistent with what was observed in earlier studies (41, 42). In these studies,

it was postulated that an early hematopoietic stem cell may be targeted, as the tumor cells did not express antigens specific for particular lineages. To better characterize the tumor cells, fluorescence-activated cell sorter (FACS) analysis was performed on cells dispersed from either the spleen or liver from 10A1- E_{env} -infected mice. Significantly, we also failed to detect any lineage-specific antigens on these cells, with the exception of the erythroid marker TER119, which was detected in some but not all tumors (Fig. 3A). The tumor cells were also negative for CD34 and either negative or low for CD117 (cKit) expression, two markers expressed on early multipotent stem cells. In contrast, the majority of tumors were positive for stem cell antigen 1 (Sca-1). Although the recurrent (but not consistent) TER119 expression would support the diagnosis of erythroblastic leukemia, the tumor cells often had morphological characteristics of myeloid or lymphoid progenitors. We thus support the conclusion of Ott and coworkers (41) that the primary target cell is a multipotent progenitor but the erythroid compartment is most strongly affected.

FIG. 4. Southern blot analysis of DNA isolated from the enlarged spleens of mice infected with the indicated virus and developing the typical blast leukemia. (A) DNA was digested with BamHI, size separated by gel electrophoresis, and transferred to a nylon membrane. Hybridization with a Fli1 probe detects both two endogenous bands (indicated by arrows on right side) and a rearranged allele (indicated by arrows on left side) which were not detected in DNA isolated from the spleen of an uninfected NIH 3T3 mouse. Numbers correspond to different infected mice. (B) DNA digested with BglII and hybridized with either an ecotropic (Mo-MuLV) or amphotropic (10A1-MuLV) *env* probe demonstrated that the virus in the blast tumors contained the expected *env* sequences. Each band denotes an independent provirus. Unlike the ecotropic *env* probe, which does not detect endogenous viral sequences, the 10A1 probe detects an endogenous virus, indicated by the arrow on the right.

Both 10A1-E_{env} and 10A1-MuLV wild-type also induce a B-cell lymphoma/leukemia distinct from the T-cell malignancy induced by Mo-MuLV. A smaller percentage of both 10A1-E_{env}- and 10A1-infected mice (27% and 36%, respectively) presented with a distinct disease phenotype, characteristically involving the spleen and other lymphoid organs (Table 3). The tissue architecture of the bone marrow and enlarged lymph nodes was completely effaced through the infiltration of lymphocytic tumor cells. In the spleen, tumor cells impinged on both the white and red pulp, the latter also being the site of compensatory erythropoiesis (Fig. 2D). In contrast to the blastic leukemia described above, tumor infiltrates in the liver were rare and locally restricted (Fig. 2E). Blood smears and hematocrit counts confirmed normal erythrocyte numbers in the blood, but increased leukocyte numbers were often observed (Fig. 2F and Table 3). The clinical presentation of this disease was very similar to that induced by Mo-MuLV in control mice (Table 3); however, FACS analysis confirmed that most of these tumors were of B-cell origin (i.e., CD19⁺ and B220⁺), in

contrast to the lymphoma and thymomas induced by Mo-MuLV, which are strictly of T-cell origin (CD3⁺ CD90⁺ CD4^{+/−} CD8^{+/−}) (Fig. 3C and data not shown). No surface IgM antigen was detected by FACS analysis, although recombination of the Ig heavy chain gene locus was readily detected by Southern blot analysis using a J_H probe (data not shown), confirming both a pro-B-cell phenotype and the oligoclonality of the tumor. Although earlier studies did not report a B-cell lymphoma/leukemia in 10A1-infected mice, this most likely reflects subtle differences in mouse strains (41). Interestingly, two of the 10A1-E_{env} tumors were of T-cell origin, but these tumors arose quite late (mean latency of 245 days), in contrast to the aggressive T-cell tumors induced by Mo-MuLV, which led to the death of the mice with a mean latency of 101 days.

In summary, the pathogenicity of 10A1-MuLV is not drastically altered when its *env* gene is replaced with that of the T-tropic Mo-MuLV. Although the disease penetrance is somewhat reduced, the mice succumbed to either a blastic leukemia

or B-cell malignancies, similar to that induced by 10A1 in this mouse strain.

10A1-*A_{env}* retains its capacity to induce a B-cell neoplasia but has lost its blast cell-transforming capacity. In striking contrast to both wild-type 10A1 and 10A1-*E_{env}* infections, mice infected with the 10A1-*A_{env}* recombinant, which carries the amphotropic *env* and thus solely uses the Pit2 receptor for cell entry, presented with clinical symptoms of a fatal wasting syndrome (cachexia) which was not due to advanced stages of a hematopoietic neoplasia, as assessed by normal spleen size, absence of lymphadenopathy, and normal blood counts (Table 3). However, in three mice (0.8%, $n = 38$) small B-cell lymphomas were observed and confirmed by FACS analysis (Table 3 and data not shown). Histological inspection of hematopoietic organs of six animals suffering from the wasting disease but without enlarged hematopoietic organs confirmed early signs of a lymphoid disorder, as evidenced by the expansion of the white pulp in the spleen due to increased lymphopoiesis (data not shown). Notably, there were no signs of the rapid dissemination of erythroid blasts in the liver and spleen, which was a consistent characteristic of the blastic leukemia observed as early as 9 weeks after infection with 10A1-MuLV or 10A1-*E_{env}* (M. Rodenburg and J. Löhler, unpublished results). In view of the fact that the 10A1-*A_{env}*-infected mice had approximately the same life span as 10A1-infected controls, we conclude that the 10A1-*A_{env}* virus has lost its ability to induce blast cell leukemia. However, the recombinant virus is still able to induce a B-cell neoplasia which is not fully manifested due to the development of a second disease entity that leads to the death of the mice. Histological analysis of different tissues from these animals has revealed several defects, including severe odontopathy, hepatitis, and uropathy, which contribute to their deaths. These findings will be published separately.

In accord with the theory that 10A1-*A_{env}* mice still have the potential to develop B-cell neoplasias, all mice infected with 4070A developed B-cell tumors (B220⁺, CD19⁺) with a mean disease latency of 228 days (Fig. 1C), a time point when most of the 10A1-*A_{env}*-infected mice have died. The disease resembled the B-cell neoplasia observed in 10A1-infected mice, characterized by moderate splenomegaly and consistent involvement of the mesenteric lymph node (Table 3). Ott et al. have previously reported that the tumors induced by 4070A in NIH Swiss mice with a similar latency were of T-cell origin, due to the expression of CD90 (Thy1.1). Although these tumors were reported to be B220^{neg}, rearrangements of the Ig heavy chain locus were observed in 6 of 7 tumors. We thus predict that these are indeed early pro-B cells with low levels of B220 expression, not detectable by the antibodies used in that study. Importantly, we have also observed CD90 expression in several murine B-cell tumors (unpublished results).

Taken together, restricting the receptor usage of 10A1 to the Pit2 receptor results in its inability to transform an early blast cell compartment, but not its ability to induce a B-cell hyperplasia (and presumably neoplasia), similar to the highly homologous 4070A virus. The mice, however, succumbed to a second disease entity that does not involve the hematopoietic system.

Retroviral integration within the *Fli1* gene locus is observed in all blastic leukemias, whether induced by 10A1-*E_{env}* or wild-type 10A1. Ott et al. have previously reported a striking correlation of 10A1-induced blast leukemia and integration

within the first coding exon of the *Fli1* gene, encoding an ETS transcription factor (41). To determine if transformation of the same cell compartment by 10A1-*E_{env}* also involved disruption of the *Fli1* regulatory regions, Southern blot analysis on tumor DNA was performed using an upstream *Fli1* probe. As shown in Fig. 4A, integrations clustered in the same locus as that observed in 10A1-induced tumors, indicating the importance of *Fli1* activation in this distinct disease phenotype. In one tumor from a total of 12 analyzed, another restriction digest pattern was observed. Isolation of the integration site by ligase-mediated PCR and sequence analysis showed that this integration had occurred in the same region. To verify that the tumors arose from 10A1-*E_{env}* and were not due to contaminating 10A1-MuLV, tumor DNA was also hybridized to *env* probes. This analysis confirmed that the tumors contained ecotropic *env* and not 10A1 *env*-containing provirus (Fig. 4B).

Consistent with the hypothesis that *Fli1* activation correlates with the blast cell leukemia, we found no *Fli1* integrations in 11 of 12 B-cell tumors from 10A1-infected mice. In one tumor, a faint rearranged band was detected, but as these tumors are generally polyclonal (as determined by hybridization with an *env* probe), this may represent a small subclone with blast cell morphology. Isolation of integration sites from five independent B-cell tumors revealed common integration sites upstream of the *Il7*, *Mef2c*, *Pim1*, and *Myc* gene loci, all of which have been found in other B-cell tumors induced by different MuLV isolates (1).

The blast cell transformation capacity of 10A1 is mouse strain specific. Our results show that a 10A1 variant that uses the Cat1 receptor induced the same disease phenotype as wild-type 10A1-MuLV. F-MuLV also uses the Cat1 receptor and induces an erythroleukemia, which strictly correlates with *Fli1* integrations. A review of the literature describing the F-MuLV disease (29, 50, 54) suggested that 10A1-MuLV induces the same disease. It is known that F-MuLV does not induce an erythroleukemia in C57BL/6 mice. To test the disease pathogenicity of 10A1-MuLV in this mouse strain, it was necessary to exchange *gag* sequences with an NB (or B)-tropic MuLV, such as Mo-MuLV, because C57BL/6 mice are *Fv1^{bb}*. This recombinant, designated 10A1-Mo_{gag}, was shown to induce blast cell leukemia with a similar latency and incidence as wild-type 10A1-MuLV in NIH/Ola mice (Table 3 and Fig. 5A and B, compare with Fig. 1B). In contrast, C57BL/6 mice infected with 10A1-Mo_{gag} generated B-cell leukemia at a very low incidence and with a long latent period (Fig. 5C and data not shown).

Two experiments provided evidence that the weak leukemogenic potential of 10A1-Mo_{gag} in C57BL/6 mice was not due to virus-host interactions that inhibited virus spread but rather to insufficient oncogenic hits. First of all, Mo-MuLV induced T-cell leukemia in these mice with close to 100% penetrance and with a latency similar to that induced in NIH/Ola mice, demonstrating that the mice were receptive to virus infections (Fig. 5C). Secondly, C57BL/6 mice homozygous for a deletion of the tumor suppressor *Icsbp* and infected with 10A1-Mo_{gag} were also susceptible to leukemia induction (Fig. 5D). *Icsbp* encodes the interferon factor 8, an important regulator of myeloid and lymphoid differentiation (20). Its deficiency leads to a myeloproliferative disease, thus acting as a first hit in leukemia induction. Consistent with this hypothesis, leukemia

FIG. 5. (A) Schematic representation of 10A1-MuLV *gag* recombinant (10A1-Mo_{gag}), in which the *gag* sequences from 10A1 were replaced with that from Mo-MuLV using the indicated conserved restriction sites. (B) Kaplan-Meier survival curves of mice of different strains infected with either 10A1-Mo_{gag} or, as a control, Mo-MuLV.

developing in *Icsbp*^{-/-} mice infected with 10A1-Mo_{gag} were primarily of myeloid origin (CD11b⁺/Gr1⁺). Indeed, circa 30% of leukemia/tumors in Mo-MuLV-infected C57BL/6 *Icsbp*^{-/-} mice were also of myeloid origin, although the majority was of T-cell origin, consistent with the strong T-cell tropism of this virus.

DISCUSSION

MuLVs induce lymphomas and leukemias by a complex mechanism involving multiple stages of cell pool expansion and clonal outgrowth, in which cooperating events disrupting

growth controls are selected. Although oncogene activation (or tumor suppressor inactivation) by proviral insertional mutagenesis is clearly a central mechanism, our understanding of the process is still incomplete. Many studies over the last two decades have been designed to identify viral sequences responsible for determining the cell lineage specificity in leukemia induction, primarily concentrating on the enhancer elements within the LTR. Here we sought to demonstrate the importance of receptor usage for leukemia induction and specificity. Interestingly, in the course of these studies, it became clear that several MuLV isolates (including 10A1, Friend, Cas-Br-E, and Graffi strain 1.2) actually induce the same leukemic phenotype, with the common denominator being the activation of the *Fli1* oncogene. Significantly, instead of defining viral factors that are important for such "lineage specificity," our results predict that inherent genetic mutations in susceptible mouse strains are the dominating determinants.

Role of receptor usage in MuLV pathogenicity. The ability of a retrovirus to efficiently infect hematopoietic cells within a specific cell lineage or during a distinct differentiation stage is an obvious prerequisite for transforming this cell compartment. Thus, it was predicted that the unique stem-cell-transforming properties of 10A1-MuLV may reflect its unique usage of Pit1 (42). Indeed, substitution of the 10A1 *env* gene with that from the B-lymphogenic and amphotropic 4070A-MuLV resulted in a virus recombinant that lost its ability to induce blast cell leukemia, supporting this hypothesis. Somewhat surprisingly, replacing the *env* gene of 10A1 with that of Mo-MuLV did not change its ability to induce blastic leukemia. These results are consistent with the hypothesis that the unique disease induction of 10A1-MuLV is not strictly related to its ability to use Pit1 as a receptor, although it does require a receptor, such as Cat1 but not Pit2, which enables efficient targeting of an early hematopoietic progenitor. In support of this hypothesis, retroviral vectors pseudotyped with ecotropic Env (using Cat1), but not amphotropic Env (using Pit2), can efficiently infect long-term repopulating cells of the bone marrow (24, 39). Similarly, retroviral vectors pseudotyped with gibbon ape leukemia virus, which recognizes human Pit1, are used routinely for transduction of human hematopoietic progenitors and have been shown to be more efficient than amphotropic pseudotypes (6, 22). These observations are also supported by the fact that transcript levels of Cat1 and Pit1 are relatively higher than Pit2 in early murine hematopoietic progenitors (39).

It is not surprising that *env* sequence variations that alter receptor usage or affinity may have a drastic influence on the cell types targeted during infection; however, few studies have addressed this issue. A prominent example of the importance of receptor usage in MuLV pathogenicity is the generation of polytropic (mink cell focus-forming virus) viruses (which use the Xpr receptor) during infections by the ecotropic Mo- and F-MuLV (15, 57). This is generally thought to be important for rapid spread of the virus during early stages of infection (and thus targeting rapidly proliferating cells) and for permitting multiple integrations. However, other roles, such as inducing hyperproliferation in a preleukemic stage by interaction with other host cell factors, may also be important (15). Interestingly, we found that recombinants with endogenous retroviral sequences were a rare event in 10A1 infections and were only

observed in B-cell tumors with a long latent period (M. Rodenburg, M. Fischer, and C. Stocking, unpublished results). Variations in the *env* gene have also been implicated in the altered leukemia specificity in feline leukemia viruses, although this may involve a receptor-independent mechanism (12, 14, 28).

Fli1: the common denominator in erythro(blastic) leukemia? The diseases induced by 10A1 and 10A1-*E_{env}* were strikingly similar, with most mice (>65%) succumbing to blast leukemia closely associated with severe anemia and hepatosplenomegaly, but not lymphadenopathy. In all cases analyzed, the distinct blast cell phenotype was strictly correlated with provirus insertion upstream of the coding sequence of the *Fli1* gene. Ott et al. also noted a 100% correlation between disease phenotype and *Fli1* integration. Notably, *Fli1* was first identified as a common integration site in F-MuLV-induced erythroleukemia (with >80% correlation) (7, 8, 48) and has since been found to be a common integration site in “non-B/non-T-cell” tumors induced by Cas-Br-E and “myeloid leukemia” induced by Graffi-MuLV (9, 13, 41). A review of the literature and methodology used to classify these tumors suggests that they most likely represent the same disease entity, characterized by anemia, splenomegaly, and frequent infiltration of the liver but not lymph nodes (29, 50, 54, 60). The target of transformation appears to be a very early progenitor, variably committed to the erythroid lineage (e.g., TER119 and/or cKit positive), probably explaining the discrepancies in the classification. The latent period of the disease differs between the various mouse strains, correlating with the strength of the LTR (see below). The strong association of this phenotype to a single oncogene suggests that the single most important criteria for MuLV induction of (erythro)blastic leukemia is its ability to activate the *Fli1* oncogene in an early progenitor. Interestingly, *Fli1* encodes an ETS transcription factor, closely related to the PU.1 transcription factor activated by integration of the Friend spleen focus-forming virus into the *Spil* locus in erythroleukemia induction (10, 23, 55). Transformation thus probably occurs by down-regulation of the GATA family of transcription factors, which are important regulators of early multipotent (GATA-2) and erythroid (GATA-1) differentiation (23, 33).

Interestingly, the integration sites of 10A1-*E_{env}* were consistently found in the first coding exon, similar to 10A1 and Cas-Br-E (with highly homologous LTR and *gag* sequences) but in contrast to Graffi- and F-MuLV-induced tumors, in which the integrations are generally found in an upstream intron (9, 13, 41). Strikingly, the permissive *Fli1* integration sites of Cas-Br-E and 10A1-MuLV, which do not have duplicated enhancer elements within the LTR, are much more limited than those of Graffi (1.2)-MuLV and F-MuLV, which have duplicated enhancer regions (9, 13). Similarly, the latent period is generally shorter for these latter two viruses. Importantly, growing evidence suggests that the expression level of a transcription factor required to induce leukemogenicity is defined by a very small window (46); thus, the two distinct integration clusterings probably reflect the limited number of permissive sites, dictated by the configuration of the enhancer/promoter or other *cis*-regulatory elements of the provirus, that enable critical *Fli1* expression levels. Transcriptional assays of Cas-Br-E, Graffi (1.2), and Friend LTRs have provided evidence that all are expressed at relatively high levels in ery-

throid cells, in contrast to the T-cell-tropic Moloney and SL3-3 MuLVs (5, 49). Significantly, the former LTRs contain binding sites for the GATA family of transcription factors (4). We thus predict that both high transcriptional activity of the LTR in this compartment and integration within defined regions of the gene are necessary to activate *Fli1* to levels required for transformation.

The results discussed below show that at least two criteria have to be met to ensure outgrowth of a transformed pool of (erythro)blasts after 10A1-MuLV infections: (i) targeting of a relatively early progenitor and (ii) integration within a relative small region of the small *Fli1* locus. It is thus not surprising that not all mice succumbed to the blast leukemia. Significantly, both 10A1 and 10A1-*E_{env}* also induced a second disease entity, namely a B-cell neoplasia, at a reduced incidence. On the whole, these malignancies had a longer latent period than the blast leukemias (mean latency of 167 days versus 132 days) and appeared to be oligoclonal, as assessed by Southern blotting (data not shown). In many respects they resembled the B-cell neoplasia induced by 4070A, with the exception that the latency was significantly shorter. We predict that these leukemias arise after a more random chain of cooperating oncogenic events. Indeed, several different common integration sites have been identified in these tumors.

Influence of mouse strain genetic factors on MuLV “lineage specificity.” Strikingly, although not unexpectedly, the typical blastic leukemia induced by 10A1-MuLV, which we observed in NIH/Ola and previously observed in NIH/Swiss mice, was not observed in C57BL/6 mice. This was previously also reported for F-MuLV but has not been evaluated for Cas-Br-E and Graffi-MuLV, which are N-tropic viruses.

An early study provided genetic evidence that the resistance to F-MuLV (erythro)blastic leukemia in C57BL/6 mice is governed by a single dominant resistance locus, which was coined *Fre*^r (50). A recessive locus sensitive for disease development was found in BALB/c- and NIH-related mouse strains (e.g., FVB/n, NFS, and NIH/Swiss). These studies also showed that the locus did not affect viral replication, consistent with our studies and in contrast to other known resistant loci that modulate leukemia formation (including *Fv1*, *Fv4*, and *Fv6/Rmcf*). Importantly, the *Fre*^r locus is also unlikely to be the same as the *Fv2*^r locus, also found in C57BL/6 mice, which confers resistance in a recessive fashion to disease induction by the Friend virus complex, composed of the replication-defective Friend spleen focus-forming virus and the helper virus F-MuLV. Interestingly, the recent identification of the *Stk/Ron* gene, encoding the stem cell tyrosine kinase, as the target gene of *Fv2* genetic variations, has resulted in a better understanding of Friend virus complex pathogenicity and erythroid regulation (36, 38, 44, 62). Studies are thus warranted to determine if *Fv2*^{ss} is also required for *Fli1*-associated erythroblastic leukemia induced by these numerous MuLV variants.

Unfortunately, there have been no follow-up publications on the *Fre* locus. It is thus not clear at what level the resistance locus may suppress the development of the (erythro)blast leukemia. Either *Fre*^{ss} may be required to provide sufficient target cells (early progenitors) to increase the likelihood of infection and thus *Fli1* gene activation or its product may directly be required to synergize with *Fli1* activity during transformation. It is important to note that as early as 6 weeks after 10A1

infection of NIH/Ola mice, we observed a hyperplasia in the spleen involving both the white pulp (with increased numbers of B cells) and the red pulp (with increased levels of erythroid cells) (J. Löhler and M. Rodenburg, unpublished results). The increased levels of erythropoiesis confirm the early results of Niho et al. (37), who demonstrated dramatic increases in the number of very early progenitors (blast-forming units-erythroid) but not later progenitors (CFU-erythroid) 7 weeks after F-MuLV infection. Whether this hyperproliferation is modulated by *Fre*, *Fli1*, or nonspecific effects of virus infection remains to be determined. Molecular identification of the *Fre* locus or other mouse strain variations that modulate the induction of *Fli1*-associated (erythro)blastic leukemia will be instrumental in understanding this unique pathology. The presence of disease modifiers in other mouse strains has been clearly demonstrated in a recent study of Graffi (1.2)-MuLV, in which "erythroleukemia" was observed in 72%, 45%, and 26% of mice from FVB/n, NFS, and BALB/c strains, respectively (60).

Conclusions and outlook. Although our in-depth analysis of 10A1-MuLV pathogenicity clearly points to the importance of *env* (i.e., receptor usage) and LTR (i.e., transcriptional activity) sequences for the induction of a unique blast cell pathology, we find that inherent genetic alterations in the mouse strain are equally important and have been too-often ignored. Somewhat to our surprise, our study has also led us to the conclusion that the erythroleukemia/blastic leukemias induced by several different viruses are exclusively linked to the ability of the virus to activate a single oncogene, *Fli1*. Thus, although the enhancer element of some MuLVs may be optimized for *Fli1* activation within a very early progenitor, inherent properties of the mouse strain are as important, if not more important, for lineage determination. Interestingly, the influence of mouse strain variations appears to be less relevant for the T-cell transformation capacity of Mo-MuLV (and probably SL3-3). This is probably due to the fact that a wider range of oncogenes can be activated to strongly transforming levels by these viruses, either due to the optimal expression levels of these viruses in the T-cell compartment, less rigorous constraints on optimal transforming levels of the oncogene, or the high infectivity of this compartment during fetal development (e.g., more hits). However, even the specificity of these T-tropic viruses can be completely changed when predisposing mutations for B-cell tumors are made in the mouse (58) or when viral mutations increase the frequency of activating an oncogene predisposing to myeloid transformation (3, 45). With the availability of the mouse genomic sequence, future work directed at deciphering differences between mouse strains should provide invaluable insight into MuLV pathogenicity and leukemia. Importantly, the fact that MuLVs generally have pleiotropic infectivity and transcriptional activity, and not lineage-specific activity, makes them excellent tools for delineating cooperating events in carcinogenesis (1, 11, 31).

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