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Activation of a novel proto-oncogene, *Frat1*, contributes to progression of mouse T-cell lymphomas

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Acceleration of lymphomagenesis in oncogene-bearing transgenic mice by slow-transforming retroviruses has proven a valuable tool in identifying cooperating oncogenes. We have modified this protocol to search for genes that can collaborate effectively with the transgene in later stages of tumor development. Propagation of tumors induced by Moloney murine leukemia virus (M-MuLV) in Eμ-*Pim1* or H2-K-*myc* transgenic mice by transplantation to syngeneic hosts permitted proviral tagging of 'progression' genes. Molecular cloning of common proviral insertion sites that were detected preferentially in transplanted tumors led to the identification of a novel gene, designated *Frat1*. The initial selection for integrations near *Frat1* occurs in primary tumor cells that have already acquired proviruses in other common insertion sites, yielding primary lymphomas that contain only a minor fraction of tumor cells with an activated *Frat1* allele. Transplantation of such primary lymphomas allows for a further expansion of tumor cell clones carrying a proviral insertion near *Frat1*, resulting in detectable *Frat1* rearrangements in 17% of the transplanted Eμ-*Pim1* tumors and 30% of the transplanted H2-K-*myc* tumors, respectively. We have cloned and sequenced both the mouse *Frat1* gene and its human counterpart. The proteins encoded by *Frat1* and *FRAT1* are highly homologous and their functions are thus far unknown. Tumor cell lines with high expression of *Myc* and *Pim1* acquired an additional selective advantage *in vivo* upon infection with a *Frat1*-IRES-*lacZ* retrovirus, thus underscoring the role of *Frat1* in tumor progression, and the ability of *Frat1* to collaborate with *Pim1* and *Myc* in lymphomagenesis.

Keywords: insertional mutagenesis/Moloney leukemia virus/oncogenes/T-cell lymphoma/transgenic mice

Introduction

Cancer arises via a multistep process in which a normal cell progresses to a fully malignant tumor via a recurrent mechanism of clonal expansions triggered by (epi)genetic lesions (Vogelstein and Kinzler, 1993). Such progression steps may involve reduced growth control, acquired invasiveness (Habets *et al.*, 1994) or increased metastatic potential (MacDonald and Steeg, 1993). Slow-transforming retroviruses, lacking viral oncogenes, are capable of activating cellular proto-oncogenes by insertional mutagenesis, and these agents have been shown to contribute directly to different stages of tumorigenesis (Jonkers and Berns, 1996). Retrovirus-induced neoplasms acquire increasing numbers of proviruses in their genome during progression *in vivo* or passaging *in vitro* and, in various systems, more than one provirally activated proto-oncogene was found within a single cell (Tsichlis *et al.*, 1985; Cuypers *et al.*, 1986; Peters *et al.*, 1986). Since the probability of simultaneous integration near two or more proto-oncogenes is negligible, consecutive integrations have to be assumed. Indeed, in Friend murine leukemia virus-induced erythroleukemias, proviral integrations in p53 appear to be preceded by retroviral activation of *Fli1* (Howard *et al.*, 1993).

The multistep nature of Moloney murine leukemia virus (M-MuLV)-induced lymphomagenesis has been studied initially by monitoring clonal selections that occur after transplantation of primary tumors to syngeneic hosts. Proviral activation of *Myc* and *Pim1* was frequently found in the same cell clone, suggesting cooperation between these genes in tumorigenesis (Cuypers *et al.*, 1986). In addition, prolonging the life span of M-MuLV-induced tumors by serial transplantations often results in the outgrowth of subclones carrying additional proviral integrations. Such integrations might mark genes that are associated with the growth advantage of these subclones. Molecular cloning of such an additional proviral integration site has led to the identification of the common insertion site *Tic1* (previously named *Pim2*) (Breuer *et al.*, 1989). Thus far, no gene has been identified that was affected by integrations in *Tic1* (J.Jonkers and M.Breuer, unpublished data)

More recently, oncogene-bearing transgenic mice have been exploited in the experimental analysis of multistep tumorigenesis (Adams and Cory, 1992; Cardiff and Muller, 1993). Retroviral infection of such mice was shown to be a particularly powerful strategy to identify genes that effectively collaborate with the transgene in tumorigenesis. Infection of Eμ-*Pim1* transgenic mice with M-MuLV revealed *Myc* or *Nmyc1* rearrangements in almost 100% of the resulting T-cell lymphomas (van Lohuizen *et al.*, 1989) and, in M-MuLV-induced B-cell lymphomas in Eμ-*myc* transgenic mice, frequent proviral integrations were observed near *Pim1*, *Bmi1*/*Bla1* and *Pal1* (Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991). Employing oncogene-bearing transgenic mice instead of normal mice may have additional advantages. Proviral tagging in transgenic animals allows focusing on a specific cell lineage and/or

stage of differentiation, as these mice are predisposed to specific tumors by virtue of the nature and expression pattern of the trans-oncogene. Furthermore, this approach may facilitate the identification of additional hits, since the transgene constitutes the 'initiating' mutation in the transforming process. It is conceivable that these additional hits may affect not only oncogenes that cooperate with the transgene, but also genes that are more specifically involved in tumor progression.

Combining provirus tagging in oncogene-bearing transgenic mice with transplantation of primary lymphomas provides an opportunity to uncover genes primarily contributing to tumor progression. Here we describe such an approach with Eμ-*Pim1* or H2-K-*myc* transgenic mice, and report on the identification of a novel gene, designated *Frat1* (for frequently rearranged in advanced T-cell lymphomas), that is involved in progression of M-MuLV-induced T-cell lymphomas from these mice.

Results

Transplantation of MuLV-induced Eμ-Pim1 T-cell lymphomas as a tool to monitor tumor progression

Infection of Eμ-*Pim1* transgenic mice with M-MuLV results in an accelerated onset of T-cell lymphomas which invariably harbor proviral insertions in *Myc* or *Nmyc1* (van Lohuizen *et al.*, 1989). To generate primary and transplanted virus-induced Eμ-*Pim1* lymphomas, newborn offspring from crossings between heterozygous Eμ-*Pim1* mice and normal mice were infected with 10^4–10^5 plaque-forming units (p.f.u.) of M-MuLV. Mice were sacrificed when moribund, and single-cell suspensions from tumor tissue (mesenteric lymph node) containing 10^7 viable cells were injected intravenously (i.v.) or intraperitoneally (i.p.) into three recipients each. Nearly all tumors (93%) were of T-cell origin, as was apparent from the rearrangements of the T-cell receptor (TCR) β chain gene (data not shown). The proviral integration patterns of the resulting sets of primary and transplanted tumors were determined by Southern blot analysis. In several tumor sets, all transplanted tumors were derived from the predominant primary tumor cell clone, as judged by the essentially identical integration patterns. In some of these tumor sets, the transplanted tumors invariably had acquired an additional proviral insertion, illustrating that: (i) in the primary tumor, a novel subclone had emerged from the predominant tumor cell clone upon integration of a provirus; and (ii) the subclone with the extra provirus had expanded selectively after transplantation (Figure 1). Hence, the additional proviral insertions might mark genes that are directly involved in the selective outgrowth of the affected cell clone. To investigate this possibility, three tumor sets of which the transplanted tumors had acquired one or two extra proviruses in loci distinct from *Tic1* were selected for molecular cloning of host DNA flanking the newly acquired proviral insertion.

Identification of a new common insertion site in transplanted Eμ-pim1 T-cell tumors

Southern blot analysis of *Eco*RI-cleaved DNA from tumor set 35 with a probe specific for the U3 long terminal repeat (LTR) of M-MuLV revealed two fragments of 9.6

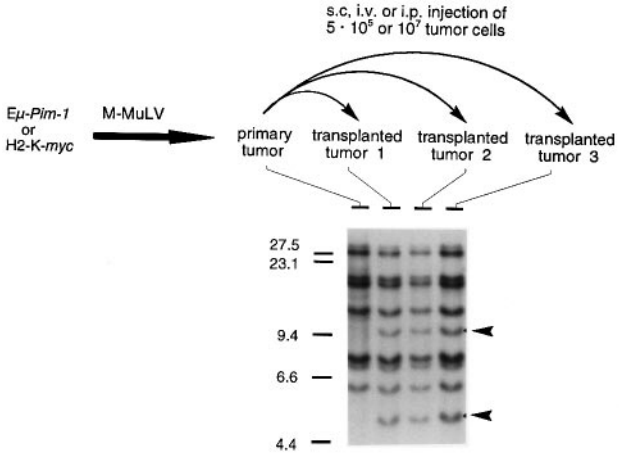

Fig. 1. Proviral integration pattern of the primary tumor from Eμ-*Pim1* mouse #35, and the tumors that were obtained after i.p. transplantation of 10^7 primary tumor cells to three different syngeneic recipients. A Southern blot containing *Eco*RI-digested tumor DNA was hybridized with a probe specific for the M-MuLV U3 LTR. The additional proviral insertions in the transplanted tumors are marked by arrowheads. Sizes of *Hin*dIII-digested λDNA fragments are indicated in kb.

and 4.7 kb that were present in all transplanted tumors but not in the primary tumor. Both restriction fragments were cloned from size-selected libraries of *Eco*RI-digested tumor DNA. Restriction enzyme analysis showed that the 9.6 and 4.7 kb fragments represented the 5′ and 3′ provirus-host junctions of an integrated MCF provirus. A single copy probe from flanking cellular sequences (probe P6 in Figure 2B) detected proviral insertions in the transplanted tumors from 10 out of 70 Eμ-*Pim1* tumor sets. In three of these tumor sets, subclonal integrations could also be detected in the primary tumor, indicating that initial selection for integration in this locus had occurred in the primary tumor, prior to transplantation. The P6 probe was subsequently used to isolate a 37 kb genomic clone from a cosmid library, and a physical map of the locus, which we named *Frat1*, was constructed (Figure 2A). An interspecific mouse backcross mapping panel generated from crosses of C57BL/6J and *Mus spretus* mice (Copeland *et al.*, 1993) was used to map the *Frat1* locus to chromosome 19, 1.7 cM distal to the common integration sites *His2* and *Gin1*. No recombination was found between *Frat1* and *Tdt*, suggesting that *Frat1* maps to human 10q23–q24 (N.Copeland and N.Jenkins, personal communication).

Proviral insertion in the Frat1 locus is a late event in lymphomagenesis

The frequencies of proviral integrations near the *Myc*, *Nmyc1*, *Pal1*, *Tiam1*, *Tic1* and *Frat1* loci were monitored by Southern blot analysis of DNA isolated from 31 primary and 78 transplanted M-MuLV-induced Eμ-*Pim1* tumors. To measure clonal expansion upon transplantation, these frequencies were corrected for clonality of the proviral insertions by estimating the relative hybridization intensity of DNA fragments corresponding to the rearranged allele and the germline allele. The resulting frequencies of proviral insertion are depicted in Figure 3A. While for the proviral occupancy of *Myc*, *Nmyc1* and *Pal1* no differences were observed between the primary and trans-

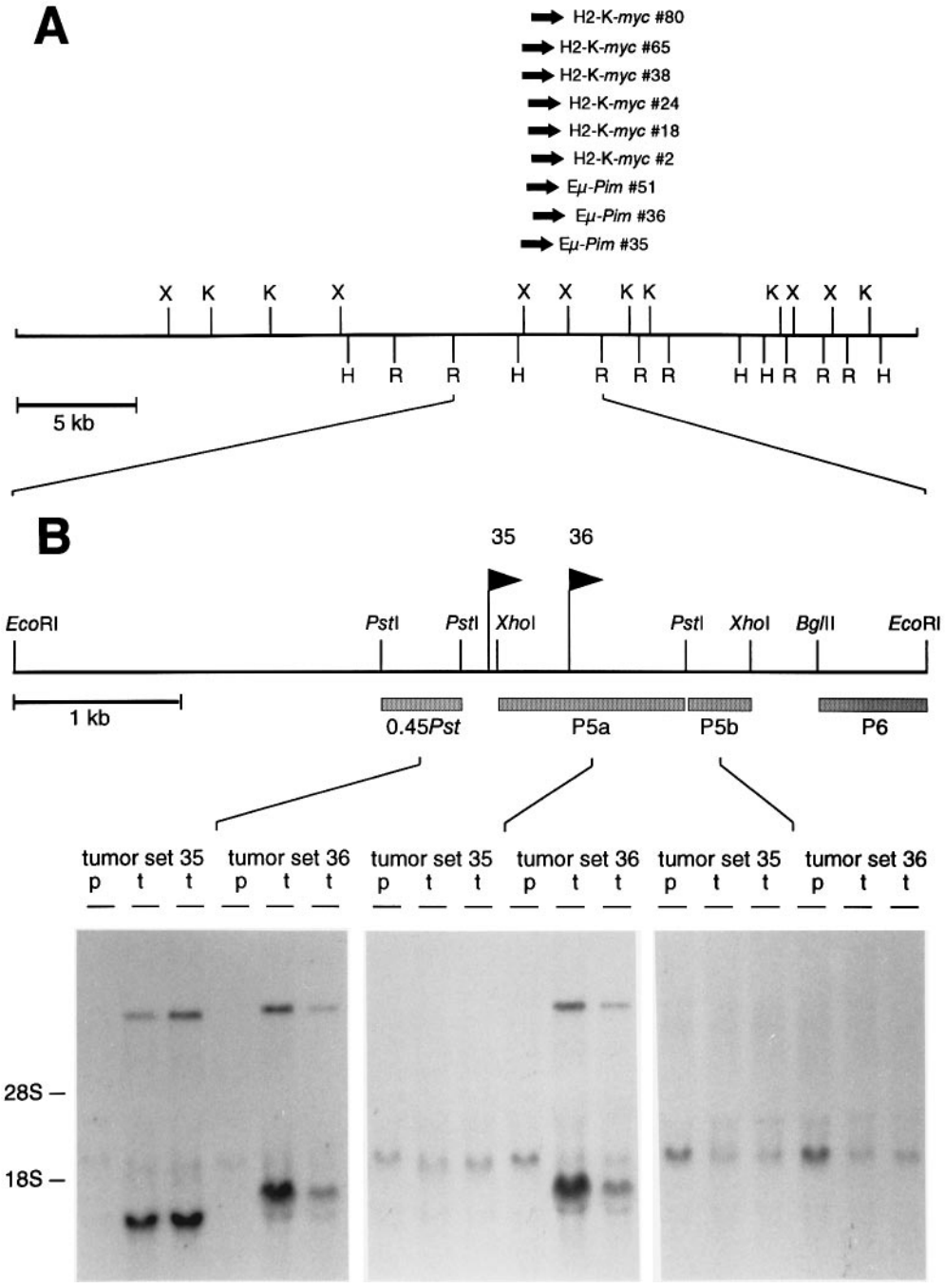

Fig. 2. (**A**) Restriction map of the cloned *Frat1* locus. *Xho*I (X), *Kpn*I (K), *Hin*dIII (H) and *Eco*RI (R) restriction sites are indicated. Proviral insertions as found in transplanted Eμ-*Pim1* and H2-K-*myc* tumors are indicated by arrows. (**B**) Enlargement of the 5.5 kb *Eco*RI fragment encompassing the proviral insertion cluster. The borders of the integration cluster are marked by two flags, representing the proviral insertions in Eμ-*Pim1* tumor sets 35 and 36, respectively. The probes are indicated as hatched bars. Three identical Northern blots containing 20 μg of total RNA from primary (p) and transplanted (t) tumors were hybridized with probes 0.45*Pst*, P5a and P5b, respectively. The 18S and 28S ribosomal bands are indicated as size markers.

planted Eμ-*Pim1* tumors, increased frequencies of proviral insertions near *Tic1* and *Frat1* were found in the transplanted tumors, compared with the primary tumors. Since initial selection for integrations near *Tic1* or *Frat1* occurs in the primary tumor, the observed increase reflects a clonal expansion of the fraction of tumor cells carrying a rearranged *Tic1* or *Frat1* allele upon transplantation. We also screened for proviral activation of *Tiam1*, a gene which confers invasiveness to a lymphoma cell line *in vitro* (Habets *et al.*, 1994). Although proviral insertions in *Tiam1* were found rather frequently (15 out of 31 primary tumors; 23 out of 75 transplanted tumors), these integrations do not confer a strong selective advantage to the lymphoma cells in the protocol used (Figure 3A). Moreover, a number of primary tumors that harbored a provirus in *Tiam1* in a nearly clonal fashion resulted in transplanted tumors with no detectable proviral insertions in *Tiam1*.

Since *Pim1*/*Pim2* and *Myc*/*Nmyc1* are strong collaborators in M-MuLV-induced T-cell lymphomagenesis (van Lohuizen *et al.*, 1989; van der Lugt *et al.*, 1995), we

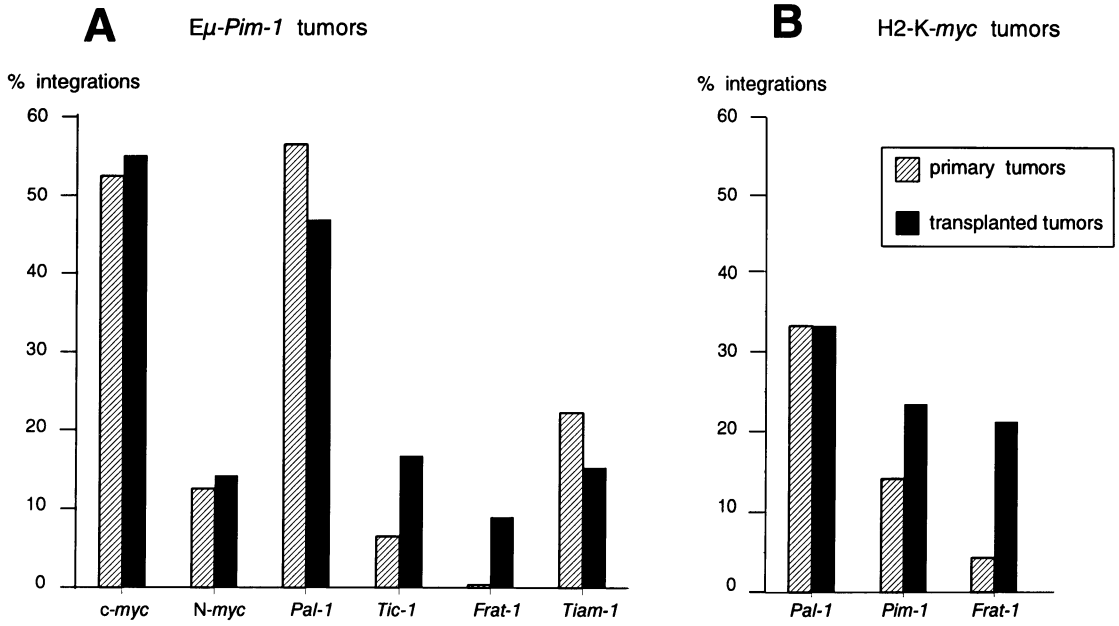

Fig 3. Frequency of common proviral insertion sites in primary and transplanted tumors from (**A**) Eμ-*Pim1* and (**B**) H2-K-*myc* transgenic mice. All frequencies were corrected for clonality of the proviral integrations, as judged by the relative hybridization intensity of DNA fragments corresponding to the rearranged allele and the germline allele. For example, a tumor of which 50% of the cells contain a provirally mutated allele (as indicated by a relative hybridization intensity of 1:3) was scored as 0.5 tumor. Consequently, the frequencies of integrations near M*yc* and N-*myc1* in the Eμ-*Pim1* tumors are somewhat lower than those reported previously (van Lohuizen *et al.*, 1989).

determined whether proviral activation of *Frat1* might also contribute to later stages of tumorigenesis in H2-K-*myc* transgenic animals. These mice overexpress the *myc* gene predominantly in hematopoietic organs. H2-K-*myc* transgenic mice develop primarily T-cell lymphomas, in contrast to Eμ-*myc* transgenic animals, which are predisposed to B-cell tumors. Newborn offspring from crosses between heterozygous H2-K-*myc* transgenic mice and normal FVB mice were infected with 10^4–10^5 infectious units of supF-M-MuLV (Reik *et al.*, 1985). SupF-M-MuLV infection reduced the latency period of lymphoma induction from an average of 105 days to an average of 65 days. Flow cytometric analysis of 95 primary M-MuLV-induced lymphomas revealed that 93 tumors were of T-cell origin (67 TCR$\alpha\beta^+$CD4$^+$CD8$^-$, 8 TCR$\alpha\beta^+$CD4$^+$CD8$^+$, 5 TCR$\gamma\delta^+$CD4$^+$CD8$^-$ and 13 oligoclonal T-cell lymphomas of mixed phenotype). Transplantation of primary lymphomas was performed by subcutaneous (s.c.) injection of 5×10^5 viable tumor cells into three syngeneic FVB/N recipients. Southern blot analysis of 23 primary and 46 transplanted H2-K-*myc* T-cell lymphomas with probes for *Pim1*, *Pal1* and *Frat1* showed that also in these tumors proviral activation of *Frat1* is a relatively late event in tumorigenesis, and that cell clones carrying integrations near *Frat1* expanded rapidly upon transplantation (Figure 3B).

Several tumor sets of monoclonal origin had sequentially acquired a provirus in two common insertion sites, thereby revealing both the order and the identity of three collaborating oncogenic events in a single cell clone, e.g. the transgene and two cellular proto-oncogenes. In a subset of Eμ-*Pim1* tumor sets with clonal integrations in either *Myc* or *Nmyc1*, integrations near *Frat1* were detected in the transplanted tumors, suggesting that activation of *Frat1* can confer an additional selective advantage to lymphoma cells that overexpress *Pim1* and *Myc*. Likewise, consecutive activation of *Pim1* and *Frat1*, respectively, was observed in H2-K-*myc* tumor sets. In other H2-K-*myc* tumor sets, sequential activation of *Pal1* and *Frat1* suggested collaboration between *Myc*, *Pal1* and *Frat1*. While no significant variation in the frequency of *Pim1* activation was observed between primary and transplanted H2-K-*myc* lymphomas (8 out of 23 primary tumors; 18 out of 46 transplanted tumors), the frequencies that are calculated after correction for the clonality of the proviral insertions suggest that activation of *Pim1* in these tumors may be preceded by other events (Figure 3B). The observation that integrations in *Pal1* occasionally occur prior to activation of *Pim1* is in agreement with this notion.

Proviral insertion at Frat1 causes enhanced expression of the Frat1 gene

As shown in Figure 2A, all proviruses in *Frat1* had integrated in the same transcriptional orientation in a small genomic region of ~0.5 kb. In order to identify the gene affected by the proviral insertions at *Frat1*, Northern blots containing RNA from two Eμ-*Pim1* tumor sets with clonal insertions in *Frat1* (set 35 and 36) were hybridized with genomic probes derived from the *Frat1* locus (Figure 2B). All three probes used in this experiment recognized a 2.5 kb transcript in all tumors, including the primary tumors without a *Frat1* rearrangement. In addition to the normal 2.5 kb messenger, the transplanted tumors with a proviral insertion in *Frat1* showed high levels of truncated transcripts of 1.7 (tumor set 35) or 2.2 kb (tumor set 36). The truncated transcripts in the transplanted tumors could only be detected with probes located upstream of the proviral integration site, indicating that these mRNAs contain the 5′ end of the parental 2.5 kb messenger and were terminated prematurely by the polyadenylation signal

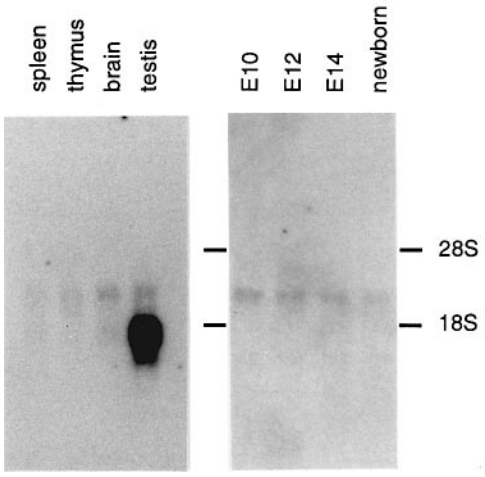

Fig. 4. Expression of *Frat1* mRNA in normal mouse tissues and normal mouse embryos. Northern blots containing poly(A)-selected RNA from various adult mouse tissues (5 μg) and total RNA from embryos (20 μg) were hybridized with probe 0.45*Pst*.

in the 5′ LTR of the inserted provirus. Also, larger transcripts of 10–11 kb were found. They probably represent readthrough RNAs that have terminated in the 3′ proviral LTR.

In normal mouse tissues, low levels of *Frat1* mRNA were detected in spleen, thymus and brain (Figure 4). High expression was observed in testis where, in addition to the normal *Frat1* transcript, a mRNA of 1.7 kb is very prominent. *Frat1* is also expressed at low levels during embryonic development.

Structure and sequence of Frat1

An oligo(dT)-primed cDNA library was constructed using poly(A)-selected RNA from tumor 35.1, which carries a proviral insertion in *Frat1*. Screening with probe 0.45*Pst* yielded two cDNA clones of 0.9 and 1.0 kb, respectively. Restriction enzyme analysis showed that these clones consist of *Frat1* sequences fused to the 5′ LTR. In both cDNA clones the 5′ LTR sequence terminates at the U3 polyadenylation site, demonstrating that the presence of a provirus in the *Frat1* locus causes premature polyadenylation of *Frat1* transcripts. Both cDNA clones were sequenced, and a gene-specific primer (GSP1) was used to construct a GSP-primed tumor cDNA library that yielded two *Frat1* cDNA clones of 0.4 and 0.7 kb, respectively. The 5′ end, as present in the longest cDNA clone, was confirmed by a 5′ RACE protocol (Frohman *et al.*, 1988) and by RNase protection (data not shown). The 3′ *Frat1* cDNA sequences were cloned by PCR amplification on cDNA from tumor 36.1, and by screening an oligo(dT)-primed murine thymus cDNA library with probe P5b. This screen yielded two cDNA clones of 0.9 and 1.2 kb, respectively, which both start at a poly(A) tail.

The nucleotide sequence of the *Frat1* cDNA reveals a long 3′ untranslated region (UTR) (1469 nucleotides), an 822 nucleotide long open reading frame (ORF) and a short 5′ UTR of 50 nucleotides (Figure 5A). The 3′ UTR contains multiple copies of the ATTT motif thought to contribute to mRNA instability (Shaw and Kamen, 1986). All insertions in *Frat1* cluster in a 500 bp region of DNA directly downstream of the protein-encoding domain, resulting in the production of 3′-truncated transcripts that encode an unaltered *Frat1* protein, while lacking a varying number of the mRNA-destabilizing motifs. The *Frat1* cDNA sequence proved to be completely co-linear with the genomic *Frat1* sequence, demonstrating that *Frat1* is an intronless gene. This feature allowed us to determine the human *FRAT1* ORF via sequence analysis of a genomic clone. Comparison of the mouse *Frat1* and human *FRAT1* amino acid sequences revealed that the ORF is conserved between man and mouse, with an amino acid identity of 78.5% and a similarity of 87.6% (Figure 5B). Likewise, hybridization of zoo blots with probe 0.45*Pst* indicated that *Frat1* is well conserved among vertebrates (not shown).

The *Frat1* ORF encodes a polypeptide of 274 amino acid residues with a predicted M_r of 29 kDa. The protein is largely hydrophilic, and an acidic domain is present in the N-terminal region (amino acids 6–22). Comparison of the mouse *Frat1* and human *FRAT1* nucleotide and protein sequences with sequences present in the SwissProt or GenBank database did not show homologous proteins or common motifs. *In vitro* transcription and translation of *Frat1* proved to be very inefficient, even after addition of an N-terminal hemagglutinin tag with an AUG surrounded by an optimal Kozak sequence. Therefore, the integrity of the ORF was verified by transient expression of a GST–*Frat1* fusion construct in COS cells (Figure 5C).

Infection of Eμ-Pim1 tumor cells with a Frat1 retrovirus results in a selective advantage in vivo

To test the effect of enhanced *Frat1* expression on the proliferation and metastatic potential of lymphoma cells *in vivo*, we constructed a retrovirus expressing both *Frat1* and β-galactosidase via a bicistronic messenger. The use of a retroviral vector permitted an efficient introduction and overexpression of *Frat1* in lymphoma cells. The presence of a β-galactosidase reporter gene permitted monitoring of the infected tumor cells at the cellular level. Two T lymphoma cell lines, derived from spontaneous tumors in Eμ-*Pim1* transgenic FVB mice, were used in this experiment. In addition to the *Pim1* transgene, *Myc* was found to be highly expressed in these cell lines, named DNPTHY and DNP13642 (data not shown). Upon s.c. injection into syngeneic recipients, these lymphoma lines gave rise exclusively to the formation of local tumors. This reproducible behavior allowed us to investigate whether overexpression of *Frat1* might induce the formation of metastases.

FVB mice were injected s.c., either with mock-infected tumor cells or with a mixture of mock-infected cells and 1 or 2% transduced cells, and the resulting local tumors were analyzed as depicted in Figure 6. Clonal expansion of the transduced fraction of tumor cells was observed in four out of six DNPTHY tumors and in six out of eight DNP13642 tumors, demonstrating that overexpression of *Frat1* can confer an additional selective advantage *in vivo* to lymphoma cells that already overexpress *Pim1* and *Myc* (Table I). In addition to the local tumor, a number of other organs (liver, kidney, lung, heart, thymus, spleen or peripheral lymph nodes) were analyzed for the presence of tumor cells. No (micro)metastases were found in any of these tissues, indicating that *Frat1* does not induce any metastatic behavior in DNPTHY or DNP13642 cells after s.c. inoculation. A parallel experiment, in which we used MFG-*lacZ* instead of MFG-*Frat1*-IRES-*lacZ*, confirmed

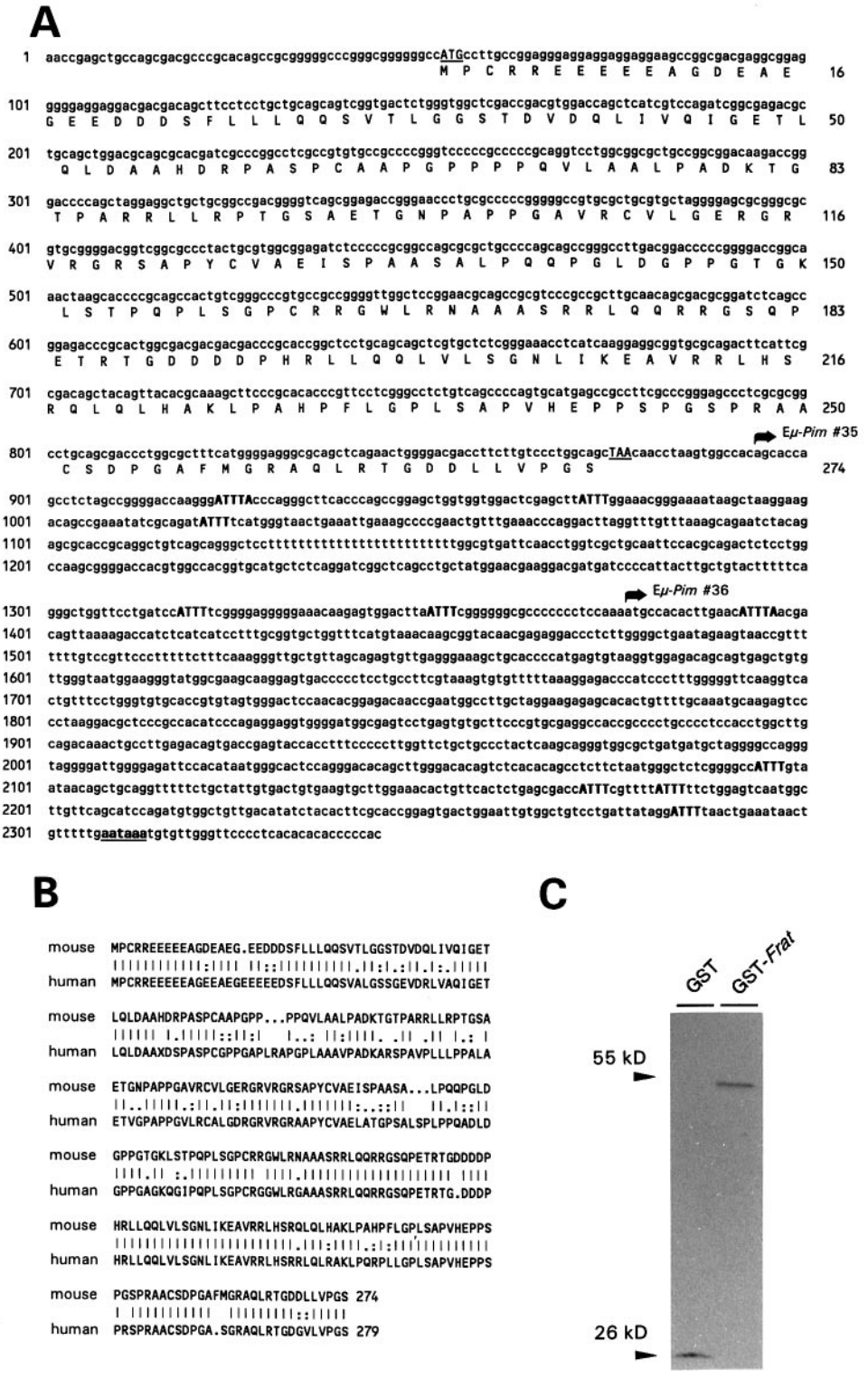

Fig. 5. (**A**) Mouse *Frat1* cDNA and encoded polypeptide sequence. The provirus–host junctions of the cDNA clones corresponding to the truncated *Frat1* transcripts in the transplanted tumors from Eμ-*Pim1* mouse #35 and #36, respectively, are indicated by arrows. In the 3′ UTR, AT degradation motifs are in upper case and the polyadenylation site consensus sequence is underlined. The predicted amino acid sequence is shown below the nucleotide sequence in single-letter code. (**B**) Comparison of the mouse Frat1 and human FRAT1 amino acid sequences. Alignment was performed with the GCG program BESTFIT (Devereux *et al.*, 1984). Identical residues are indicated by vertical lines, well conserved replacements that score better than 0.5 in the PAM-250 matrix by colons, and replacements scoring better than 0.1 by dots. (**C**) Immunodetection of GST–Frat1 fusion protein. Western blot analysis of total cell lysates of COS-7 cells, transiently overproducing GST or GST–Frat1, with a monoclonal anti-GST antibody.

that no retroviral sequences, other than *Frat1*, were responsible for the observed growth advantage.

We found significant variations in the contribution of MFG-*Frat1*-IRES-*lacZ*-infected cells to the end-stage tumors and, in four out of 14 local tumors, no outgrowth of the transduced fraction of cells could be detected. These

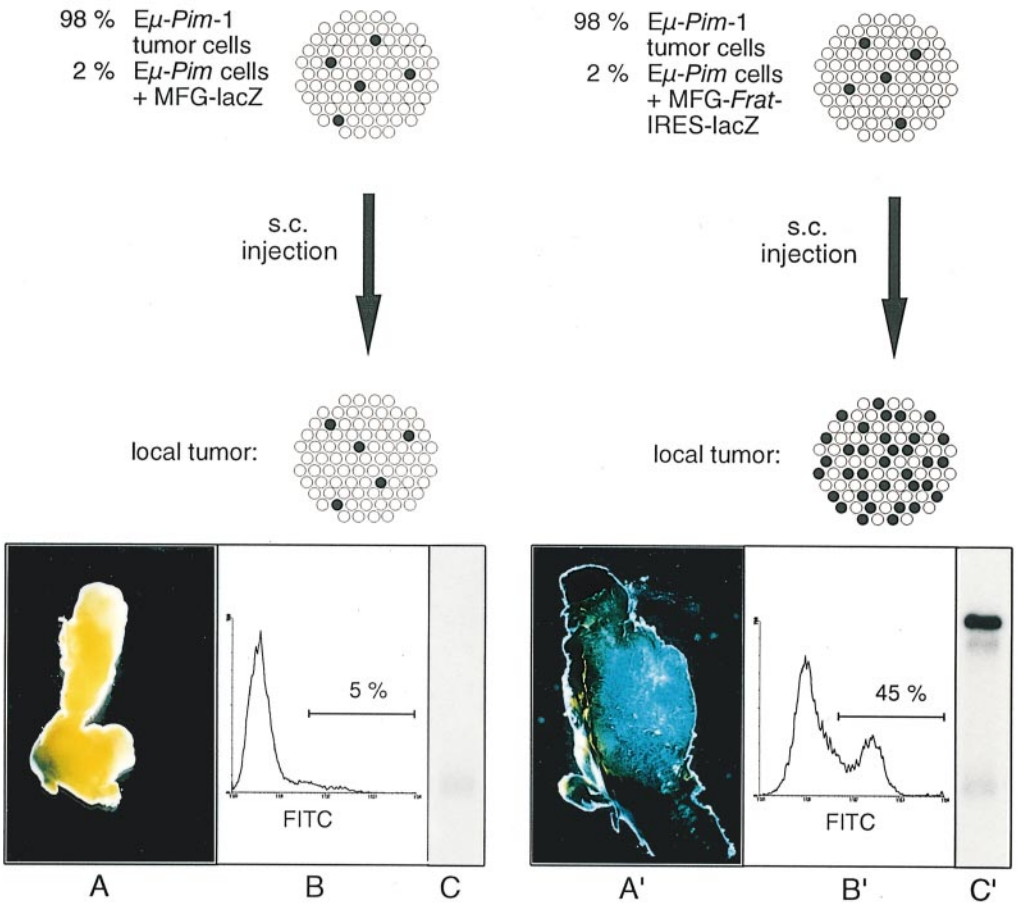

Fig. 6. Selective outgrowth of Eμ-*Pim1* tumor cells transduced with MFG-*Frat1*-IRES-*lacZ*. Mixtures of Eμ-*Pim1* lymphoma cells and tumor cells transduced with MFG-*lacZ* or MFG-*Frat1*-IRES-*lacZ* were injected s.c. into syngeneic recipients. The resulting local tumors were analyzed by staining tumor tissue for β-galactosidase activity (left panels), flow cytometry (middle panels) and Northern analysis, using a *Frat1* cDNA probe (right panels). The transcript, detected in both local tumors, corresponds to the normal *Frat1* mRNA. The larger transcript found in the local tumor derived from the mixture containing MFG-*Frat1*-IRES-*lacZ*-infected cells corresponds to the bicistronic retroviral mRNA.

differences could not be attributed to the injected tumor cells, since variations were also observed between mice that were injected with the same cell suspension. Apparently, the fate of the transduced tumor cells was also influenced by host factors. This notion was supported by experiments in which the same cell suspensions were injected i.p. or i.v. None of the resulting tumors contained an increased percentage of transduced lymphoma cells.

Discussion

To identify genes involved in progression of T-cell lymphomas, we have combined proviral tagging in oncogene-bearing Eμ-*Pim1* and H2-K-*myc* transgenic mice with transplantations of the primary tumors to syngeneic recipients as a means of allowing progression to higher malignancy. Molecular cloning of proviral insertion sites that were additionally and reproducibly found after transplantation of primary tumors has led to the identification of a novel locus, *Frat1*, that is frequently mutated by proviral insertions in the transplanted lymphomas from M-MuLV-infected Eμ-*Pim1* or H2-K-*myc* transgenic mice. Integrations near *Frat1* constitute a relatively late event in T-cell lymphomagenesis, since several primary tumors of monoclonal origin were found to contain, in addition to the predominant cell clones lacking a provirus near *Frat1*, direct derivatives of these clones carrying proviral integrations near *Frat1*. Transplantation of such tumors resulted in a further expansion of the fraction of tumor cells with the rearranged *Frat1* allele, implying that activation of *Frat1* confers an additional selective advantage.

Primary tumors of monoclonal origin, and the resulting transplanted tumors, have been instrumental in the identification of up to three collaborating oncogenes in T-cell lymphomagenesis: (i) the 'initiating' transgene; (ii) a common proviral insertion site that is clonal in both the primary tumor and the transplanted tumors; and (iii) a common proviral insertion site that is clonal in transplanted tumors but subclonal in primary tumors. In this way, a number of favorable combinations can be defined: *Myc*, *Pal1* and *Pim1*; *Myc*, *Pim1* and *Frat1*; or *Myc*, *Pal1* and *Frat1*. The fact that *Frat1* does not fall into any of the known complementation groups for lymphoid transformation (*Myc*/*Nmyc1*, *Pim1*/*Pim2* and *Pal1*/*Bmi1*/*Bla1*) suggests that *Frat1* acts in a distinct signal transduction pathway. Whether *Frat1* and the previously identified 'transplantation-specific' common insertion site *Tic1* belong to different complementation groups remains to be established.

Mapping of the proviral insertion sites near the *Frat1*

Table I. Selective outgrowth of Eμ-*Pim1* lymphoma cells transduced with MFG-*Frat1*-IRES-*lacZ* upon subcutaneous transplantation

Cell line	% transduced cells	No. of injected cells	Mean latency (days)	% of transduced cells in local tumor			
				mouse 1	mouse 2	mouse 3	mean
DNPTHY							
	2	10^5	23	25	15	20	20
	2	10^4	29	2	ND	no tumor	
	1	10^5	29	1	ND	no tumor	
	1	10^4	26	25	ND	no tumor	
	0	10^5	23	0	0	0	0
	0	10^4	33	0	no tumor	no tumor	
DNP13642							
	2	10^5	27	30	2	ND	15
	2	10^4	32	40	ND	no tumor	
	1	10^5	27	35	10	15	20
	1	10^4	33	5	1	ND	3
	0	10^5	27	0	0	0	0
	0	10^4	30	0	0	0	0

locus revealed that all proviruses had integrated in the same transcriptional orientation, in a small genomic region of ~500 nucleotides. Using single copy probes from the genomic region upstream of the proviral insertions, we detected enhanced levels of truncated transcripts in all tumors carrying a proviral insertion near *Frat1*. Sequence analysis of *Frat1* cDNA clones showed that the truncated transcripts terminate at the polyadenylation site present in the 5′ LTR of the inserted provirus. Consequently, these transcripts lack most or all of the 3′-untranslated *Frat1* sequences, suggesting that both up-regulation of transcription by the viral enhancer and mRNA stabilization by the removal of negatively regulating elements in the 3′-untranslated region may contribute to increased levels of *Frat1* mRNA. All proviral insertions leave the coding domain of *Frat1* intact, indicating that overexpression of normal Frat1 protein contributes to transformation.

Apart from the N-terminal acidic region, the Frat1 protein contains no apparent structural features that might provide us with clues about its function. The acidic regions of the yeast transcription factors GAL4 and GCN4 and the herpes simplex virus factor VP16 have been shown to be involved in transcriptional activation and protein–protein interaction (Ma and Ptashne, 1987; Sigler, 1988), and several proto-oncogenes, such as *Evi1* (Perkins *et al.*, 1991), c-*myb* (Weston and Bishop, 1989) and *Myc* (Blackwell *et al.*, 1990), encode transcription factors containing an acidic region. It remains to be established whether Frat1 has similar activities.

To examine the role of Frat1 in tumor progression, we have developed a sensitive assay to monitor the selective advantage conferred by *Frat1* overexpression. DNPTHY and DNP13642 lymphoma cells, which represent stable cell lines derived from spontaneous lymphomas in Eμ-*Pim1* transgenic mice, were transduced with an MFG-*Frat1*-IRES-*lacZ* retrovirus followed by injection into syngeneic recipients, and outgrowth of the marked cell population was measured. This protocol allowed us to examine the effects of enhanced *Frat1* expression on cell proliferation and metastatic behavior *in vivo*. The data presented here demonstrate that overexpression of *Frat1* following retroviral transduction with MFG-*Frat1*-IRES-*lacZ* can confer an additional selective advantage to lymphoma cells that already overexpress *Pim1* and *Myc*. No selective outgrowth was observed when MFG-*lacZ* was used instead of MFG-*Frat1*-IRES-*lacZ*, confirming that the observed growth advantage of MFG-*Frat1*-IRES-*lacZ*-infected lymphoma cells can only be attributed to overexpression of *Frat1*. However, the selective outgrowth appears to be influenced by host factors, as significant variations were found when the same cell suspension was injected s.c. into different mice. Also, no selective outgrowth of the transduced subpopulation was observed after i.p. or i.v. inoculation. These results would be in accordance with a model in which activation of *Frat1* obviates the need for one or more specific growth factors that can contribute to the proliferation or survival of the lymphoma cells. In this case, the selective advantage of the *Frat1*-overexpressing subpopulation would depend on the local concentration of these growth factors. Alternatively, the variations in selective outgrowth and the absence of detectable micrometastases might be caused by an immune response against the β-galactosidase produced by the MFG-*Frat1*-IRES-*lacZ*-infected tumor cells. In line with the latter, it was reported recently that expression of the *lacZ* gene in the highly tumorigenic murine mastocytoma cell line P815 frequently caused a strong immune response in syngeneic recipients, resulting in the rejection of the tumor cells (Abina *et al.*, 1996). Since the MFG-*Frat1*-IRES-*lacZ*-infected lymphoma cells produce a bicistronic messenger encoding both Frat1 and LacZ, the selective growth advantage conferred by *Frat1* might be counteracted effectively by the selective removal of β-galactosidase-expressing tumor cells by the immunocompetent host. While this potential complication might be circumvented by performing the transplantations in nude mice, the experiments described here strongly support the notion that *Frat1* can function as a tumor progression gene in lymphomagenesis.

In summary, we have used a tumor progression protocol based upon transplantation of M-MuLV-induced lymphomas from oncogene-bearing transgenic mice to identify a novel tumor progression gene, *Frat1*. We have shown that this gene can confer an additional selective advantage *in vivo* to lymphoma cells that already overexpress *Myc* and *Pim1*. While we do not yet understand the nature of

this selective advantage, we expect that testing the effects of *Frat1* on the growth properties of a series of defined cell lines *in vitro* might provide clues about the function of this gene.

Materials and methods

Transgenic mice, lymphoma induction and transplantation of primary lymphomas

The generation of Eμ-*Pim1* transgenic strains ppG66 and ppG64 has been described previously (van Lohuizen *et al.*, 1989). Briefly, the transgene consists of a genomic *Pim1* clone containing two copies of the immunoglobulin Eμ enhancer located upstream of the *Pim1* promoter, and a single M-MuLV LTR within the 3′ UTR. The transgene was introduced into (CBA/BrA×C57BL/LiA)F1 zygotes and the resulting mice were backcrossed with (CBA/BrA×C57BL/LiA)F1, C57BL/LiA or C57BL/6 mice.

The H2-K-*myc* construct was generated by fusing the H2-K promoter (Morello *et al.*, 1986) to a 5.5 kb *Xba*I–*Bam*HI genomic mouse *Myc* fragment containing exons 2 and 3, including the *Myc* polyadenylation signal. The transgene was microinjected into the pronuclei of FVB/N zygotes (Taketo *et al.*, 1991). Genotyping was performed by Southern analysis of tail tip DNA according to Laird *et al.* (1991).

Lymphomas were induced by injecting 1-day-old mice with 10^4–10^5 infectious units of M-MuLV clone 1A as described (Jaenisch *et al.*, 1975). Mice were sacrificed when moribund, and tumor tissues (spleen, thymus, mesenteric/peripheral lymph nodes, liver) were frozen at –80°C. Transplantations and FACS analysis were performed with single-cell suspensions from mesenteric lymph node. Lymphomas from Eμ-*Pim1* transgenic animals were transplanted to (CBA/BrA×C57BL/LiA)F1 sex-matched recipients by i.v. or i.p. injection of 10^7 viable cells. Transplantation of lymphomas from H2-K-*myc* transgenic animals was performed by s.c. injection of 5×10^5 viable cells into syngeneic FVB/N females.

DNA and RNA analysis

High molecular weight DNA from lymphoma tissues was prepared as described (van der Putten *et al.*, 1979). For Southern analysis, 10 μg of total genomic DNA of each tumor were digested with restriction enzymes as recommended by the supplier, separated on a 0.6% agarose gel, transferred to nitrocellulose and hybridized to ^{32}P-labeled probes. For Northern analysis, 20 μg of total RNA, prepared by the LiCl–urea method, or 5 μg of poly(A)-selected RNA were separated on 1% agarose formaldehyde gels (Sambrook *et al.*, 1989) and transferred to nitrocellulose membranes. The following probes were used: *Myc*, 3 kb *Xba*I–*Hin*dIII fragment (Shen-Ong *et al.*, 1982); *Nmyc1*, 3.5 kb *Pst*I fragment (Taya *et al.*, 1986); *Pim1* probe A, 0.9 kb *Bam*HI fragment (Cuypers *et al.*, 1984); *Pal1* probe A, 1 kb *Bgl*II–*Eco*RI fragment (van Lohuizen *et al.*, 1991); *Tic1* probe MB20, 1.5 kb *Pst*I–*Eco*RI fragment (Breuer *et al.*, 1989); *Tiam1* probe D22.3, 0.8 kb *Sal*I fragment (Habets *et al.*, 1994); M-MuLV U3 LTR, 180 bp *Hpa*II fragment (Cuypers *et al.*, 1984); and TCR β chain probe 86T5 (Hedrick *et al.*, 1984). The *Frat1* probes used in this study were: 0.45*Pst*, 0.45 kb *Pst*I fragment; P5a, 1.2 kb *Xho*I–*Pst*I fragment; P5b, 0.4 kb *Pst*I–*Xho*I fragment; and P6, 0.7 kb *Bgl*II–*Eco*RI fragment.

Cloning

Cloning and subcloning of cDNAs and genomic DNAs were done according to established procedures (Sambrook *et al.*, 1989). Clones were obtained from the following libraries: oligo(dT)-primed thymus cDNA (Stratagene), oligo(dT)-primed testis cDNA (Clontech) and 129/Sv genomic cosmid library (Stratagene).

For cDNA synthesis, 5 μg of poly(A)-selected RNA from tumor 35.1 was used in a $(dT)_{15}$-primed or GSP-primed (GSP1, 5′-CTCCTTGATG-AGGTTTCCC-3′) cDNA reaction according to Gubler and Hoffman (1983), with a cDNA synthesis kit from Boehringer Mannheim. cDNA was treated with T4 polymerase, size selected, and *Not*I linkers (Biolabs) were added. After digestion with *Not*I, cDNA was cloned in λZAPII. Libraries of 2–5×10^5 primary plaques were obtained.

PCR amplification of RNA was performed by a first strand reverse transcriptase reaction (Boehringer Mannheim) with a gene-specific RT primer (GSP2, 5′-CGCTGCGTCCAGCTGCAGCGTCTCG-3′) followed by 5′ end cloning with the primers GSP3, 5′-CTGGACGATG-AGCTGGTC-3′, and GSP4, 5′-CTCGAGCCACCCAGAGTCACC-3′. RACE conditions were essentially as described (Frohman *et al.*, 1988).

PCR amplification on cDNA from tumor 36.1 was performed with a M-MuLV-specific U3 LTR primer (AB949, 5′-CGCGTCGACCTTG-CCAAACCTACAGGT-3′) and a *Frat1*-specific primer (GSP5, 5′-ACGACCTTCTTGTCCCTGGCAG-3′).

Sequencing of *Frat1* cDNA and genomic clones was performed on both double-stranded templates and M13 single-stranded templates, using a Pharmacia T7 polymerase sequencing kit.

Construction of a Frat1 retrovirus

The following restriction fragments were used in a three-point ligation reaction: (i) a 950 bp *Nco*I–*Not*I fragment, containing an in-frame fusion between an N-terminal hemagglutinin epitope tag and the *Frat1* coding sequences; (ii) a 1.7 kb *Not*I–*Cla*I fragment from IRES-βgeo (Pilipenko *et al.*, 1992), containing an internal ribosomal entry site (IRES) and the N-terminal portion of *Escherichia coli* β-galactosidase; and (iii) a 9 kb *Nco*I–*Cla*I fragment containing the MFG retroviral vector (Dranoff *et al.*, 1993) and the C-terminal portion of β-galactosidase. Introduction of the ensuing retroviral construct into producer cells (see below) resulted in the production of a bicistronic viral messenger encoding both *Frat1* and β-galactosidase.

Tissue culture and transduction experiments

Cell suspensions from spontaneous Eμ-*Pim1* lymphomas were seeded in tissue culture flasks containing subconfluent BALB/c3T3 fibroblast feeder layers, at several dilutions. After a crisis, non-adherent tumor cells emerged and expanded rapidly. Lymphoma cells were grown in RPMI-1640 (Gibco-BRL) with 10% fetal calf serum (FCS; Gibco-BRL), 5×10^{-5} M β-mercaptoethanol and antibiotics. ψ-CRE packaging cells (Danos and Mulligan, 1988) were cultivated in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% FCS and antibiotics. To generate ψ-CRE ecotropic virus-producing cell lines, ψ-CRE cells were co-transfected with MFG-*Frat1*-IRES-*lacZ* and pRc/CMV (Invitrogen). After G418 selection, individual colonies were picked and analyzed for their transduction capacity.

Subconfluent producer cells (clone A1) were incubated with a minimal volume (10 ml/T75 flask) of medium. The supernatant was collected after 16 h and filtered through 0.45 μm membrane filters. Infection was achieved by cultivating lymphoma cells for 2 h in freshly collected supernatant supplemented with polybrene (10 μg/ml). The supernatant was replaced with RPMI-1640 medium and, 24 h after infection, the efficiency of transduction was determined by staining the cells for β-galactosidase activity (Hogan *et al.*, 1994).

FVB mice were injected with mock-infected tumor cells or with a mixture of mock-infected cells and 2–3% transduced cells. S.c. injections were performed with 10^4 or 10^5 viable cells, i.p. injections with 10^4 viable cells and i.v. injections with 10^2 or 10^3 cells. Mice were sacrificed and analyzed when moribund, or when they had developed a local tumor. The resulting tumors were stained for β-galactosidase activity as described elsewhere (Hogan *et al.*, 1994).

Flow cytometric analysis

A total of 10^6 cells were incubated in 96-well plates for 30 min at 4°C in 20 μl PBA (phosphate-buffered saline with 1% bovine serum albumin and 0.1% sodium azide) and saturating amounts of monoclonal antibody. Cells were washed twice with PBA and incubated with streptavidin–phycoerythrin for biotinylated antibodies or PBA. The following antibodies were used: CD45R/B220 (clone 6B2), CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-6.7), TCRαβ (clone H57-597) and TCRγδ (clone GL3), all from PharMingen (San Diego, CA). Intracellular β-galactosidase activity was measured using the fluorogenic substrate fluorescein-di-β-D-galactopyranoside (FDG) in conjunction with flow cytometry (Berger *et al.*, 1994). In brief, 10^6 cells were incubated with a hypotonic solution containing 1 mM FDG for 50 s at 37°C. After addition of excess RPMI medium to restore iso-osmotic conditions, the enzymatic reaction was allowed to proceed on ice for 2 h. All flow cytometric analyses were performed on a FACScan (Becton Dickinson).

Database accession numbers

The GenBank accession numbers for the sequences reported in this paper are U58974 and U58975.

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