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MoMuLV proviral integrations identified by Sup-F selection in tumours from infected *myc*/*pim* bitransgenic mice correlate with activation of the *gfi-1* gene

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ABSTRACT

Infecting mice with a mutant Moloney murine leukemia virus which contains the bacterial suppressor tRNA *supF* in its LTR allows rapid cloning of proviral integration sites from genomic tumour DNA. In a previous study Eμ *pim-1*/Eμ *L-myc* bitransgenic mice had been inoculated neonatally with MoMuLV *supF* virus. The retroviral infection led to acceleration of lymphomagenesis indicating the proviral activation of further oncogenes cooperating with *myc* and *pim-1* in tumour development. Using a functional *supF* screen for analysis of genomic mouse tumour DNA libraries which had been constructed in the phage vector EMBL3A, a common proviral integration site on mouse chromosome 5 was cloned and found to be identical to the proviral integration site *evi-5* which has recently been identified in an AKXD T-cell lymphoma and which is located 18 kb upstream of the *gfi-1* gene. Tumours bearing *evi-5* integrations showed an enhanced *gfi-1* expression level suggesting that *gfi-1* is the target gene for insertions at the *evi-5* locus. Together with three other previously described Moloney integration clusters all responsible for enhanced *gfi-1* expression the number of tumours from infected double transgenic Eμ L-*myc*/Eμ *pim-1* transgenic mice with retrovirally activated *gfi-1* added up to 53% underscoring the role of GFI-1 as an effective collaborator for MYC and PIM-1 in the process of lymphomagenesis.

INTRODUCTION

Infection of mice with the non-acute transforming retrovirus Moloney murine leukemia virus (MoMuLV) has become an established method to search for new genes implicated in the process of lymphomagenesis (1,2; for review see 3). Genomic DNA from the tumours that arise upon infection is analysed for proviral integrations, and genes that are located in the vicinity of viral sequences can be identified. Several mechanisms lead to the activation of genes that are hit by proviral integrations: by promoter and enhancer insertions, the expression of the host target gene becomes elevated by the retroviral transcription control elements residing within the LTR sequences. Alternatively, the proviral integration could either remove RNA destabilising elements (3) or it could lead to a differently spliced gene products with new properties (4). Proviral integration sites can be isolated either by inverse PCR reaction or by screening a genomic DNA library constructed from DNA of infected tissue or cells with a virus specific probe. With this method, phage clones containing genomic mouse sequence in their inserts that flank the proviral DNA can be isolated (2).

Transgenic mice expressing activated oncogenes under cell type specific promoter and enhancer elements have been proven to be valuable tools to investigate oncogene cooperation *in vivo* (5–7). A well studied system have been Eμ *c-myc* transgenic mice where the *c-myc* gene is linked to an immunoglobulin heavy chain enhancer (8,9). Here, the lymphocyte specific expression of the transgene leads to development of pre-B or B-cell lymphoma (8,10). To search for oncogenes that cooperate with *c-myc*, a retroviral infection of newborn Eμ *c-myc* transgenic mice had been performed and led to identification of the cytoplasmic serine/threonine kinase PIM-1 and the zinc finger protein BMI-1 as strong collaborators of c-MYC in the development of B-cell lymphoma (1,2). Another retroviral integration site, *pal-1*, that was hit in a large proportion of tumours (2,11), was identified in this experiment. Subsequent studies showed that proviral activation within the *pal-1* locus lead to the activation of a gene termed *gfi-1* (for growth factor independence 1) located several kb downstream of *pal-1* (11,12). The *gfi-1* gene had previously been identified in a search for genes that confer T-cells the independence of IL-2 (13). The efficient collaboration between *myc* and the *pim-1* gene was not only evident in MoMuLV infection experiments but could also be shown by creating various Eμ *myc/pim-1* double transgenic mice which show a dramatic acceleration in lymphomagenesis compared to the single transgenic parent strains (14,15).

The analysis of tumours arising in Eμ *myc/pim-1* bitransgenic mice also showed that all malignancies were still of clonal or oligoclonal origin. This indicated that additional events were

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required for full malignant transformation even in the presence of two activated *trans*-oncogenes. To search for these additional cooperating oncogenes that synergize in the process of lymphomagenesis with both *myc* and *pim-1*, a MoMuLV infection of Eμ L-*myc/pim-1* bitransgenic mice was carried out. The infection resulted in a profound acceleration of tumour formation in these animals and the analysis of the emerging tumours showed that in 37% of the cases the *pal-1/gfi-1* locus was affected by retroviral integration resulting in high level expression of the *gfi-1* gene (12). This finding underscored the potential of the *gfi-1* gene to efficiently cooperate with both *myc* and *pim-1* genes.

In the infection experiment with Eμ *pim-1*/Eμ *L-myc* bitransgenic animals a replication competent mutant MoMuLV virus strain was used which contains a 200 bp *supF* suppressor tRNA in its LTR sequence (16). In this paper we present the identification of proviral integration sites from tumours that arose in infected bitransgenic mice by taking advantage of a *supF* selection procedure. To this end, we used the EMBL3A phage cloning vector to construct genomic DNA libraries from DNA isolated from tumours of infected animals. The EMBL3A vector contains two amber stop codons in genes essential for its replication (17) so that plating the libraries on the *supF*$^-$ bacteria strain MC1061 allowed exclusive isolation of phage clones with retroviral *supF* and flanking mouse sequences in their inserts. Using this functional *supF* screen we could clone and analyse a common integration site which was tentatively named *tmi-1* (for T-cell Moloney integration site 1) and which was affected by proviral insertions in 22% of the tumours in the Moloney infected Eμ *pim-1*/Eμ *L-myc* bitransgenic mice. Detailed analysis revealed that *tmi-1* is identical to the recently discovered Moloney integration site *evi-5* on mouse chromosome 5 (18) and probably activates the expression of the *gfi-1* gene located ~18 kb downstream of *evi-5*.

MATERIALS AND METHODS

Transgenic mice and MoMuLV infection

Transgenic Eμ *pim-1* and Eμ *L-myc* animals and the generation of doubly transgenic animals have already been described (14,25). Mice originating from breedings between Eμ *pim-1* and Eμ *L-myc* parents were infected until 48 h after birth intraperitoneally with 50–100 μl sterile MoMuLV *supF* containing supernatant from the producer cell line MoMuLV^{sup}-1 (16). The supernatant of subconfluently growing virus producing cells was concentrated 100-fold under N_2 pressure in a stirring cell (Amicon) to obtain a virus titre of 10^4–10^5 p.f.u./ml. Virus containing supernatants were titrated by seeding 1×10^5 XC cells per well into a 24-well plate. The wells were inoculated with serial dilutions of virus containing cell culture supernatant and the titre was determined from the number of syncitia 24 h later (26). XC cells were maintained in DMEM with 10% FCS and antibiotics.

Library construction and *supF* screening

Genomic tumour DNA (100–500 μg) was partially cleaved with *Sau*3A and enriched for fragments of 10–20 kb by a 10–40% sucrose gradient. The size-selected inserts were ligated to the *Bam*HI cleaved vector arms of the lambda replacement vector EMBL3A. For preparation of the phage arm 100 μg EMBL3A DNA were cleaved to completion with *Bam*HI and *Eco*RI. The original insert and the 'stuffer' polylinker fragment were separated from the cloning phage arms by a 10–40% sucrose gradient. The ligation reaction was packaged by using a lambda *in vitro* packaging kit from Amersham. An aliquot of the packaging reaction was plated on the *supF*$^+$ bacterial strain LE392 to determine the titre of the library. Phage particles (10^6) were then plated with the *supF*$^-$ bacterial strain MC1061 which only allowed propagation of phage clones containing retroviral *supF* sequence in their insert. To molecularly clone the unrearranged genomic DNA at the *tmi-1/evi-5* locus a genomic mouse library (Stratagene, liver, agouti, λ FixII vector) was screened for overlapping clones.

Bacterial strains

For library construction and *supF* screening the following bacterial strains were used: LE 392 *supE*44 *supF*58 *hsdR*514 *galK*2 *galT*22 *metB*1 *trpR*55 *lacY*1; MC1061 *hsdR mcrB araD*139 Δ*(araABC-leu)*7679 Δ*lacX*74 *galU galK rpsL thi.*

Characterisation of tumours

Based on rearrangements of the T-cells receptor β locus, the immunoglobulin heavy and light chain genes and on the presence of characteristic surface markers (B220, Thy 1.2, CD4, CD8) as determined by FACS analysis (Becton-Dickinson FACSCAN) the tumours were classified as either of the T- or the B-cell lineage. The probes used to detect distinct rearrangements of either the T-cell receptor gene locus or immunoglobulin genes in Southern blot analysis with genomic tumour DNA have already been described (27).

Molecular analysis and probes

Preparation of genomic DNA from mouse tail tips and tumour samples was performed as previously described (27). Cleavage of DNA by restriction enzymes and DNA blotting procedures were performed as described elsewhere (28). The *supF* probe was a 200 bp *Bam*HI–*Bam*HI fragment containing the complete sequence of the *supF* suppressor tRNA and had been kindly provided by Dr Stocking, Heinrich-Pette-Institut, Hamburg. The probes E2.1 and D22 that were used to detect proviral insertions within the *tiam-1* locus were 1 kb *Eco*RI and *Sal*I genomic DNA fragments, respectively (4). The probe for GAPDH has already been described (25). To detect *gfi-1* integrations a 2.0 kb *gfi-1* mouse cDNA fragment was used which contained almost the complete coding sequence with only the first five amino acids missing. The *evi-5* probe was a 1.4 kb mouse genomic fragment (18). As a *bla-1* probe a genomic 1.9 kb *Eco*RI–*Sal*I fragment and as a *pal-1* probe a genomic 1.3 kb *Bgl*II–*Eco*RI fragment from the respective locus were used (2). Preparation of RNA was as described (29) and Northern blotting was performed according to established procedures (28). For hybridisation of the *evi-5* probe with poly(A)$^+$ RNA from different mouse organs a Northern blot membrane from Clontech (Multiple Tissue Northern Blot) was used.

Chromosome localisation by FISH hybridisation

Chromosomal localisation of the *tmi-1* locus and the mouse cell lines used for FISH analysis was performed as described elsewhere (19).

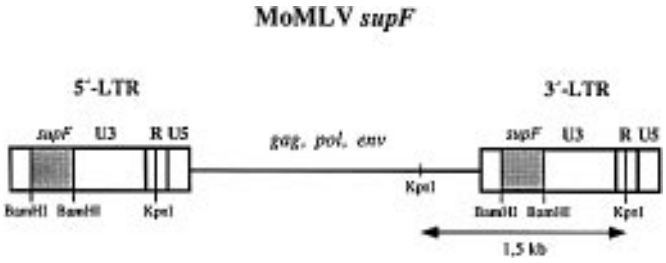

Figure 1. Schematic map of the MoMuLV *supF* retrovirus used for the infection of Eμ *pim-1*/Eμ *L-myc* bitransgenic mice in this experiment. In the U3 region of the MoMuLV LTR the 200 bp bacterial suppressor tRNA *supF* was inserted (16). A 1.5 kb internal viral *Kpn*I fragment containing the 3′-LTR *supF* sequence is indicated. The sizes of the LTRs and of the viral genes *gag*, *pol* and *env* are not to scale. (*Kpn*I is an isoschizomer of *Asp*718.)

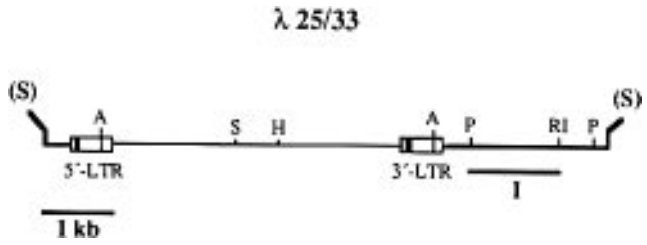

Figure 2. Restriction map of the insert of phage clone 25/33 from tumour 33. The insert contains a complete proviral MoMuLV *supF* genome plus 4 kb flanking genomic mouse DNA sequence, from this insert the 2.2 kb *Pst*I–*Eco*RI fragment was isolated as probe I. A, Asp718; S, *Sal*I; H, *Hin*dIII; P, *Pst*I; (S), *Sal*I site in the EMBL3A polylinker.

RESULTS

Cloning of MoMuLV *supF* proviral integration sites from genomic tumour DNA using a functional *supF* screen

To identify novel oncogenes cooperating with *myc* and *pim-1* in the process of lymphomagenesis we infected Eμ *pim-1*/Eμ *L-myc* double transgenic mice with a mutant replication competent Moloney murine leukemia virus carrying the bacterial suppressor tRNA *supF* in the proviral LTR sequence (Fig. 1; 12,16). The infected bitransgenic animals succumbed to 100% with lymphoid malignancies after an average latency period of 66 days. This was significantly shorter compared to the onset of tumourigenesis in uninfected *pim-1/L-myc* transgenic control mice indicating the proviral activation of further oncogenes cooperating in the process of lymphomagenesis (12,14). For cloning and analysis of the retroviral integration sites we made use of the fact that the *supF* suppressor tRNA cloned into the LTR of the Moloney virus used in this infection experiment helps to override amber stop codons in the *Aam32* and *Bam1* genes of the lambda phage vector EMBL3A (17) thereby allowing its replication and propagation. By using a *supF* probe for hybridisation of Southern blots with genomic DNA from mouse tumours we confirmed that the integrated proviruses still contained the *supF* sequence and had not lost the suppressor tRNA due to recombination events (data not shown). The Southern blots also showed that each tumour carried in average four to five proviral integrations. We chose five tumours from MoMuLV *supF* infected Eμ *pim-1*/Eμ *L-myc* double transgenic mice with no virus insertions in genes known to be frequent target sites for MoMuLV integrations (i.e. *myc*, *pim-1*, *pal-1*, *bla-1* and *bmi-1*) to isolate DNA for the construction of genomic DNA libraries with the EMBL3A phage cloning vector. The titre of each library was evaluated by growth on the *supF*$^+$ bacterial strain LE392 which allows replication of all phage particles. Plating the libraries on the *supF*$^-$ bacterial strain MC1061 led to replication of only those phage particles which contained in their DNA inserts a MoMuLV *supF* LTR providing the essential suppressor tRNA for propagation. The numbers of phage clones obtained with MC1061 bacteria (*supF*$^-$) are shown in Table 1. The average size of the DNA inserts was in the range of between 12 and 17 kb so that a reasonable portion of mouse DNA flanking the retroviral integration site was isolated. All phage clones isolated by this screen contained retroviral LTR with its *supF* sequence in their inserts as could be confirmed by Southern blots using a *supF* specific probe (data not shown).

Table 1. Number of phage clones after *supF* selection

Genomic phage library from tumour no.	Total no. of phage clones plated (Le392 *supF*$^+$ host)	Phage clones growing on MC1061 (*supF*$^-$)
20	1×10^6	3
33	1×10^6	34
84	1×10^6	9
128	1×10^6	10
154	1×10^6	12

Titre of EMBL3A phage libraries with genomic tumour DNA and number of phage clones with *supF* sequence in their inserts obtained from these libraries. To estimate the titre an aliquot of each library was plated on LE 392 bacteria which contain endogenous *supF* suppressor tRNA molecules. Phage particles of each library (1×10^6) were then plated on MC1061 bacteria which contain no endogenous *supF* so that only the indicated numbers of phage clones with *supF* sequences in their inserts could be isolated.

Identification of common proviral integration sites

To search for common integration sites which were affected by MoMuLV insertions in several tumours, Southern blot hybridisation of tumour DNA with probes obtained from the genomic mouse DNA sequences flanking the proviral insertion sites in the *supF* phage clones was performed. For this purpose, a restriction map of several phage clones was established. Figure 2 shows a partial restriction map of *supF* phage clone 25/33. The phage insert contained the complete proviral MoMuLV *supF* sequence an in addition 500 bp flanking sequences upstream and ~4 kb downstream of the 5′- and the 3′-LTR, respectively (Fig. 2). The 2.2 kb *Pst*I–*Eco*RI fragment downstream of the 3′-MoMuLV *supF* LTR was designated probe I (Fig. 2) and was used in Southern blot analysis with DNA from all tumours of the infected *L-myc/pim-1* bitransgenic mice. Fourteen out of 63 tumours (22%) showed retroviral integrations at this locus. The insertions were detected by Southern blot hybridisation using three different restriction enzymes (Fig. 3). The membranes were then stripped off the probe and rehybridised with the 200 bp *supF* fragment confirming that all signals representing alleles occupied by proviral DNA also contained *supF* sequences (data not shown). We concluded that the genomic locus covered by the insert of phage clone 25/33 contains a common proviral integration site which was tentatively termed *tmi-1* (for T-cell Moloney integration site 1).

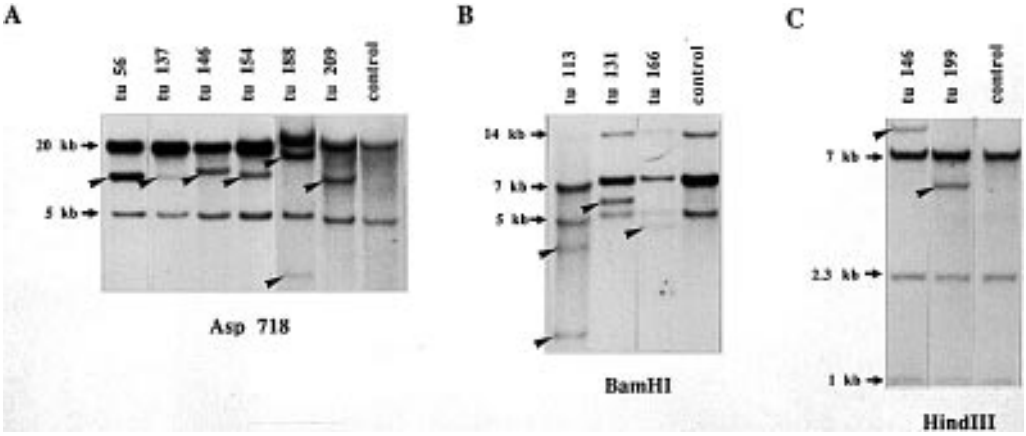

Figure 3. Southern blot analysis of genomic tumour DNA from MoMuLV *supF* infected *pim-1/L-myc* bitransgenic mice. The 2.2 kb *Pst*I–*Eco*RI DNA fragment from phage clone 25/33 (probe I; see Fig. 2) was used as a ^{32}P-labelled probe. The genomic DNA was cut with the restriction enzymes *Asp*718 (A), *Bam*HI (B) or *Hin*dIII (C). DNA from an uninfected mouse served as a control. The signals marked by an arrow represent rearranged DNA fragments due to proviral insertions.

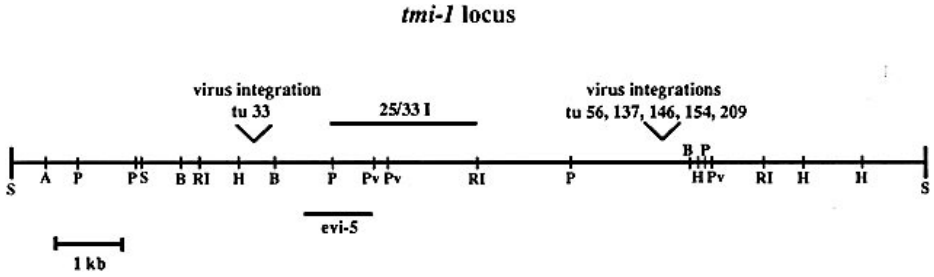

Figure 4. Restriction map of the *tmi-1/evi-5* locus. Two nearly identical lambda phage clones from a genomic mouse DNA library were identified with the 2.2 kb *Pst*I–*Eco*RI DNA fragment from phage clone 25/33 (probe I; see Fig. 3) and digested with the indicated restriction enzymes. S,*Sal*I; A, *Asp*718; P, *Pst*I; B, *Bam*HI; RI, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*II. Integration sites for tumour 33, 56, 137, 146, 154 and 209 are indicated.

Characterisation of the genomic *tmi-1* locus

For further characterisation of the *tmi-1* locus a genomic mouse DNA lambda phage library (Stratagene, agouti, liver, λ-FixII vector) was screened with the 2.2 kb *Pst*I–*Eco*RI fragment from phage clone 25/33 (probe I). Two overlapping clones could be isolated and were used to establish a partial restriction map and to localise the proviral integrations of several tumours (Fig. 4). The positions of proviral *tmi-1* integrations were found to span a region of ~6–7 kb (Fig. 4). Data from genomic Southern blots indicated in a *Hin*dIII digest the existence of two germline fragments of 7 and 2.3 kb (Fig. 4). However, the restriction map derived from genomic phage clones isolated with probe I indicated only one germline *Hin*dIII fragment (Fig. 4). A similar situation is found for *Bam*HI and *Asp*718 digests suggesting that probe I contains sequences that recognise another locus besides *tmi-1*. Indeed, FISH experiments to determine the chromosomal localisation of *tmi-1* confirmed this conclusion. In order to identify loci on mouse chromosomes by FISH we had previously established two mouse lymphoma cell lines WMP-1 and WMP-2 by MoMuLV infection (19). These lines were obtained from a wild mouse strain (WMP) that carries Robertsonian translocations which allow an easy morphological distinction between different chromosomes (19). As a result, *tmi-1* was clearly localised to chromosome 5, however, some of the mitotic figures revealed hybridisation to chromosome 14 (Zörnig and Möröy unpublished).

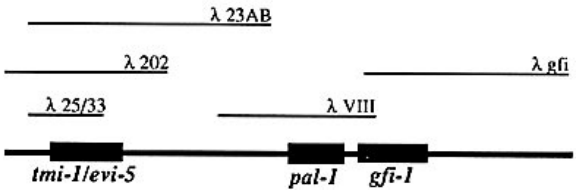

Figure 5. Schematic representation of 40 kb genomic DNA on mouse chromosome 5. The map shows the relative positions of the proviral integration loci *tmi-1/evi-5* and *pal-1* and of the MoMLV target gene *gfi-1*, respectively. Indicated are the isolated overlapping genomic lambda phage clones which cover the complete chromosomal region.

Therefore, it is possible that the additional 2.3 kb DNA fragment that is recognized by probe I represents another genomic locus with sequence homology to *tmi-1*. Alternatively, this fragment could be the result of a restriction fragment length polymorphism. This is not unlikely as the mouse strain that was used for the MoMuLV experiment is different from the strain used for the construction of the genomic library.

The finding that *tmi-1* is localised on chromosome 5 prompted us to examine the distance to the *pal-1/gfi-1* locus which is also localised on this chromosome (18). Probes from the phage cone 25/33 were used to screen DNA genomic phage libraries and to perform a genomic walking experiment (Fig. 5). Several clones were obtained that contain overlapping sequences and stretched

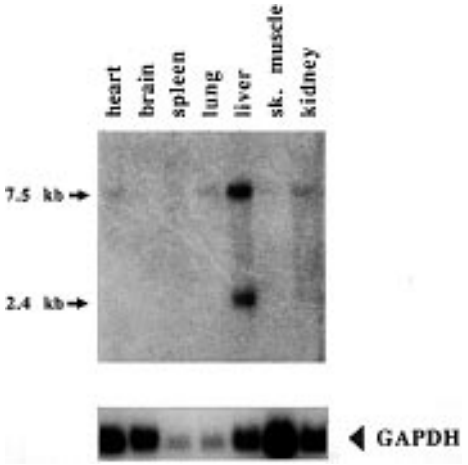

Figure 6. Northern blot hybridisation of mouse organ poly(A)$^+$ RNA with the *evi-5* probe shown in Figure 5. Besides a strong 7.5 kb signal there is an additional 2.4 kb message in liver RNA which is absent in other organs. The transcript of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a measure of mRNA loaded.

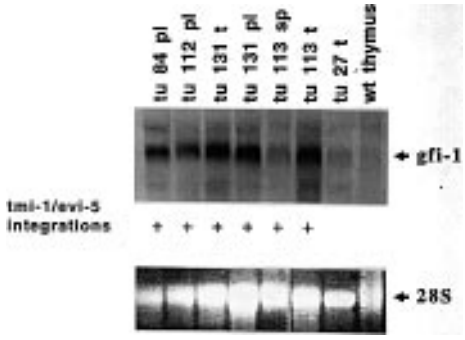

Figure 7. Northern blot hybridisation of total RNA isolated from tumours bearing proviral *tmi-1/evi-5* integrations. Tumour number and organ of which the tumour material was prepared is indicated. As a control, RNA from tumour 27 that does not bear integrations in the *tmi-1/evi-5* locus and RNA isolated from thymus of an uninfected wild-type mouse are loaded. RNA amounts were checked by ethidium bromide staining of 28S ribosomal RNA. pL, peripheral lymph node; t, thymus; sp, spleen.

until the *pal-1/gfi-1* locus (Fig. 5). Hybridisation of tumour DNA harbouring MoMuLV *supF tmi-1* integrations with a *gfi-1* cDNA however showed that *tmi-1* and *gfi-1* are distinct loci.

Recently, another common proviral insertion site in close neighbourhood to *pal-1/gfi-1* on mouse chromosome 5 has been identified in tumours that arose in the inbred mouse strain AKXD (18). Mice from this background have a high rate of spontaneous lymphoma due to the mutagenic effect of their endogenous retroviruses (18). The novel integration site *evi-5* turned out to cosegregate with *gfi-1* and detailed analysis of the locus revealed that *evi-5* and *gfi-1* are ~18 kb apart (18). Hybridisation of tumour DNA from MoMuLV *supF* infected *pim-1/L-myc* double transgenic mice with an *evi-5* probe (generously provided by A. Berns, Amsterdam) showed the same signals on Southern blots as probe I and thus clearly indicating that *evi-5* is identical to *tmi-1* (data not shown). The 2.9 kb *evi-5* probe overlapped with the 2.2 kb *Pst*I–*Eco*RI fragment (probe I) in the genomic *tmi-1* phage clones (Fig. 4). With several overlapping genomic phage clones we could confirm the notion from Liao *et al*. that *tmi-1/evi-5* is located ~18 kb upstream of *gfi-1* (Fig. 5; 18).

MoMuLV integrations at the *tmi-1/evi-5* locus enhance *gfi-1* mRNA level

By using the 2.9 kb *evi-5* probe for a Northern blot hybridisation with total RNA from tumours bearing proviral *tmi-1/evi-5* integrations no signal could be obtained (data not shown) raising the question what target gene is affected by proviral insertion at the *tmi-1/evi-5* locus. Northern blot with poly(A)$^+$ RNA isolated from different mouse organs (Stratagene) the *evi-5* probe detected a 7.5 kb message in several organs, especially abundant in liver where an additional 2.4 kb band appeared (Fig. 6). These data suggest that although there is a transcribed sequence in close vicinity to the *tmi-1/evi-5* integration, it is very unlikely that the expression of this *evi-5* associated gene is transcriptionally activated by proviral integrations in lymphoid tumours.

For further investigation of a possible target gene activated by *tmi-1/evi-5* integrations we next analysed *gfi-1* expression in a northern blot hybridisation with total tumour RNA. Interestingly, when the murine *gfi-1* cDNA was used as a probe all tumours harbouring a MoMuLV *supF tmi-1/evi-5* insertion showed an increased *gfi-1* mRNA level compared with thymus and spleen from wild-type mice (Fig. 7). Increase in *gfi-1* expression in these tumours is to the same extent as in tumours which exhibited elevated *gfi-1* RNA amounts due to proviral insertions at two other integration loci immediately 5′ and 3′ of the *gfi-1* gene affecting its expression (12). These results suggest that *evi-5* is another common proviral integration site that activates *gfi-1* expression and thereby participates in the process of lymphomagenesis.

Further retrovirally activated oncogenes in tumours from *pim-1/L-myc* bitransgenic mice harbouring *tmi-1/evi-5* integrations

Table 2 lists second-site mutations at known common proviral integration sites in tumours with *tmi-1/evi-5* insertions from MoMuLV *supF* infected Eμ *pim-1*/Eμ *L-myc* double transgenic mice. Five of the tumours bearing a *tmi-1/evi-5* integration also show viral activation of the *tiam-1* gene (4). Five tumours show a further MoMuLV integration at the *pal-1* locus. Tumour 154 shows in addition to its *tmi-1/evi-5* integration a further direct activation of *gfi-1* by a MoMuLV *supF* insertion upstream of and close to the *gfi-1* gene. One tumour bearing a retroviral *tmi-1/evi-5* integration also exhibits an insertion in the *bla-1* locus which probably leads to activation of the zinc finger oncoprotein BMI-1 (1,2; A. Berns, personal communication).

DISCUSSION

In a previous study we reported retroviral activation of the *gfi-1* and the *tiam-1* gene in T-cell lymphoma in an infection experiment with Eμ *pim-1*/Eμ *L-myc* bitransgenic mice (12). For the infection of these animals a mutant Moloney virus strain was used which contains the bacterial suppressor tRNA *supF* in its LTR. We attempted to isolate proviral integration sites in a functional *supF* screening procedure: genomic DNA libraries were constructed with the EMBL3A phage cloning vector which contains amber stop codons in two of the viral genes essential for

phage replication (17). When plated with the *supF*$^-$ bacterial strain MC1061 only those phage clones could replicate that contained the retroviral *supF* sequence in their insert. All 68 clones obtained from five different lambda phage libraries which were constructed with the EMBL3A vector and genomic tumour DNA contained retroviral *supF* sequences in their insert representing MoMuLV *supF* integration sites. This procedure allowed to identify a common proviral integration site and demonstrated that MoMuLV retroviral insertion sites can be isolated from tumour tissue quite efficiently by using the MoMuLV *supF* mutant and the appropriate selection procedure.

Table 2. Second site MoMuLV proviral insertions in tumours from infected double transgenic mice

Tumour number	Cell type	Additional identified proviral MoMLV supF integration sites
12	T	*bla-1*
21	T	*pal-1*
55	T	*pal-1*
84	preB	*tiam-1*
112	preB	*tiam-1*
113	T	*tiam-1*
129	preB	*pal-1*
146	Preb	*pal-1*
154	T	*gfi-1*
188	preB	*tiam-1*
199	T	*tiam-1*
209	T	*pal-1*

Further identified MoMuLV *supF* integration sites in tumours bearing a Moloney virus insertion in the *tmi-1/evi-5* locus. Retroviral integrations were identified by Southern blot hybridisation. The tumour cell type was determined by FACS analysis using antibodies against surface marker or by Southern blot analysis with immunoglobulin gene or T-cell receptor specific probes establishing the rearrangement status of the genes.

The integration site identified in the *supF* selection experiment was found to be identical to *evi-5* which was originally discovered as a novel common site of retroviral integration in AKXD T-cell lymphoma and is located 18 kb upstream of *gfi-1* on mouse chromosome 5 (18). Although it was also reported that *evi-5* cosegregated with *gfi-1* it remained unclear what gene is activated and is therefore involved in the process of tumourigenesis by the proviral insertion at *evi-5* (18). To address the question we used different genomic probes from the locus to search for coding sequences. None of these probes revealed signals on Northern blots with total RNA from tumours bearing *tmi-1/evi-5* integrations. It therefore seems very unlikely that proviral *tmi-1/evi-5* insertions lead to an elevated expression level of coding sequences nearby the integration site.

When we analysed lymphoid tumours with *tmi-1/evi-5* MoMuLV *supF* integrations for *gfi-1* RNA amounts we found a significantly enhanced mRNA expression level which was comparable to the *gfi-1* RNA amount found in lymphomas harbouring MoMuLV *gfi-1* insertions. Thus it seems very likely that proviral integrations in the *tmi-1/evi-5* locus lead to *gfi-1* overexpression whereby the retroviral LTR sequences would have to act over a distance of 18 kb. This action over large distances is not unprecedented. Proviral integrations at *Mlvi-1* and *Mlvi-4* map between 30 and 270 kb 3′ of the *c-myc* gene and upregulates its expression (20). Another example is the transcriptional activation of the cyclin D1 gene (21). Here, retroviral integrations at the *Fis-1* locus activated expression of cyclin D1 over a distance of 50–300 kb (21). On the other hand, these findings indicate that besides the activation of the *gfi-1* gene another target could be activated by tmi-1/evi-5 integrations that is located several hundred kb apart.

Three other proviral insertion clusters have been described which also lead to *gfi-1* overexpression in Moloney virus induced murine lymphoma (11,12): virus integrations immediately upstream or downstream of the *gfi-1* coding sequence as well as provirus insertions at the *pal-1* locus enhance *gfi-1* transcription and thus are very likely to contribute to T- and B-cell lymphomagenesis. Taking all these *gfi-1* affecting integration sites together, the total percentage of tumours with retrovirally activated *gfi-1* in the MoMuLV *supF* infected Eμ *pim-1*/Eμ *L-myc* bitransgenic mice adds up to 53% excluding double integrations (12). This high percentage confirms the important role of *gfi-1* as an effective collaborator of *myc* and *pim-1* in lymphomagenesis. Originally, *gfi-1* had been identified as a retroviral integration site in rat T-cell lines in a screen for IL-2 independence (13). Overexpression of the *gfi-1* gene together with other yet unidentified events leads to IL-2 independent growth of former strictly IL-2 dependent growing T-cells (12,13). Transfection experiments with CTLL cells have indicated that *gfi-1* expression is involved in an increase in proliferation rate (12) although it is very well possible that GFI-1 acts also by inhibiting apoptosis of IL-2 deprived T-cells. As the independence from growth factors like IL-2 is a prerequisite to tumour progression towards a more malignant state it would be a reasonable model to assume that *myc*, a transcription factor important in the regulation of cell proliferation, and *pim-1*, a cytoplasmic serine/threonine kinase, would cooperate in the onset of tumourigenesis while engagement of *gfi-1* in a tripartite cooperation with *myc* and *pim-1* leads to tumour progression.

T- and B-cell lymphomagenesis is a multistep process in which activation of several cooperating oncogenes and inactivation of certain tumour suppressor genes contribute to malignant transformation of the lymphocytes. Therefore, it is not surprising to find MoMuLV integrations at several common integration sites such as *tiam-1* and *pal-1* or *bla-1/bmi-1* in tumour DNA from the same infected mouse. *Tiam-1* has recently been identified in a search for genes that coordinate the invasive behaviour of already transformed cells (4). Moreover, the TIAM-1 protein is thought to act as a GDP/GTP exchanger for the *rho* family of GTPases (4,22). Five tumours show a further MoMuLV integration at the *pal-1* locus. While transcriptional activation of a candidate gene within the *pal-1* locus has not been observed in tumours with *pal-1* integrations all these tumours express high levels of *gfi-1* mRNA confirming that retroviral insertions into the *pal-1* locus also activate the *gfi-1* gene (11,12). MoMuLV integrations at the *tmi-1/evi-5* and the *pal-1* locus in the same tumour might further upregulate *gfi-1* expression. BMI-1 belongs to the polycomb family (2) and seems to be important in regulating the proliferation of a number of hematopoietic cells throughout pre- and postnatal life as well as for morphogenesis during embryonic development (23). Deregulated overexpression of BMI-1 in

lymphocytes of Eμ *bmi-1* transgenic mice provokes a predisposition for lymphoma and strongly collaborates with c-MYC in tumourigenesis (24).

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