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Evidence for the involvement of *pim*-2, a new common proviral insertion site, in progression of lymphomas

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We have compared proviral integrations near (putative) proto-oncogenes in Moloney murine leukemia virus-induced primary and transplanted T cell lymphomas. We previously found proviruses integrated near c-*myc*, *pim*-1, and N-*myc* in primary tumors (Selten *et al.*, 1984; Van Lohuizen *et al.*, 1989a; Van Lohuizen *et al.*, 1989b). We have now identified an additional common proviral integration site, called *pim*-2, that carries somatically acquired proviruses in the majority of transplanted tumors. In primary tumors integration near *pim*-2 is usually undetectable or present in only a minor fraction of the tumor cells. This subpopulation selectively grows out upon transplantation. Insertion near *pim*-2 is a relatively late event in tumorigenesis and is often preceded by proviral insertions in other common insertion sites, yielding tumor clones which carry proviruses in up to three different common insertion sites within the same cell (c-*myc*, *pim*-1 and *pim*-2). The data suggest that *pim*-2 plays an important role in tumor progression.

Key words: *pim*-2/provirus/tumor/progression/oncogene

Introduction

It is generally accepted that tumorigenesis is a multistep process in which a normal cell progresses in a step-by-step fashion to a fully malignant tumor cell (Pitot, 1979; Farber and Cameron, 1980; Farber, 1984; Klein and Klein, 1985, 1986; Peters *et al.*, 1986). The various stages in this process are characterized by the repeated clonal outgrowth of rarely occurring cells which are selected on the basis of reduced growth control, acquired invasiveness or increased metastatic potential (Poste and Fidler, 1980; Nowell, 1976). This ultimately results in highly malignant cells, as found in rapidly metastasizing tumors (Cairns, 1975).

Although a number of *in vitro* assay systems have been described which can be used to isolate and characterize oncogenes involved in cell proliferation and transformation, few *in vitro* systems are available to isolate and characterize genes involved in later stages of the tumorigenic process (Roos *et al.*, 1985). More likely, such tumor progression genes can confer a selective growth advantage in *in vivo* systems only, where the intact immune system, the humoral and cell-mediated growth controls, and the physical barriers to invasion act directly on the tumor cells. Here we show that slow-transforming retroviruses can be used *in vivo* to uncover genes contributing to different stages of the tumorigenic process. Tumorigenesis by these viruses depends on host proto-oncogenes, that are transcriptionally activated or otherwise mutated as a consequence of proviral integration (reviewed by Nusse, 1986; Nusse and Berns, 1988). In the present study we tested whether Moloney murine leukemia virus (MuLV), which induces T cell lymphomas after inoculation in newborn mice (Jaenisch *et al.*, 1975; Reddy *et al.*, 1980), is not only instrumental in inducing the initial neoplasia but can also contribute directly to later stages of the tumorigenic process. It has been shown previously that transplantation of primary tumor cells will often lead to the selective outgrowth of clones with more malignant characteristics. If a provirus can also act as a causative agent in later steps of tumorigenesis, one might then expect that subclones growing out preferentially upon transplantation of primary virus-induced tumors, are marked by (one or more) common proviral insertions, that are specific for these later tumor stages. Here we report the cloning of a common proviral insertion site, *pim*-2, which fulfils these predictions.

Results

Lymphomagenesis induced by Moloney MuLV

Transplantation of primary lymphomas, induced by inoculation of newborn BALB/c or C57BL10 mice with Moloney MuLV, frequently results in the selective outgrowth of clones marked by additional proviral insertions (Cuypers *et al.*, 1986). Transplantation of primary tumor cells of mouse #9 into five syngeneic mice resulted in the reproducible outgrowth of a subclone which, besides retaining a set of proviral insertions (including proviral integrations near c-*myc* and *pim*-1), was marked by new proviral insertion sites, which were not detectable in the primary tumor. The independent outgrowth of a single subclone marked by a specific set of proviral integrations in five independent transplantation experiments indicates that these integrations pre-existed in a minor subclone within the primary tumor, and that this subclone was far better adapted to grow out after transplantation than was the major constituent of the primary tumor. The integration pattern of four transplants is shown in Figure 1. This observation encouraged us to clone these additional insertion sites.

Characterization of the pim-2 locus

DNA obtained from the outgrown tumor obtained after transplantation of primary lymphoma cells of mouse #9 was cleaved with *Eco*RI, and a fragment of ~9.4 kb, corresponding to a 'transplant-specific' virus–host junction, was cloned. Single copy probes were derived from the flanking cellular sequences and used to determine whether this proviral locus represented a common integration site. A probe from this locus did detect alterations in the corresponding region of DNA of independently induced lymphomas, and thus identified this locus as a common insertion site. The

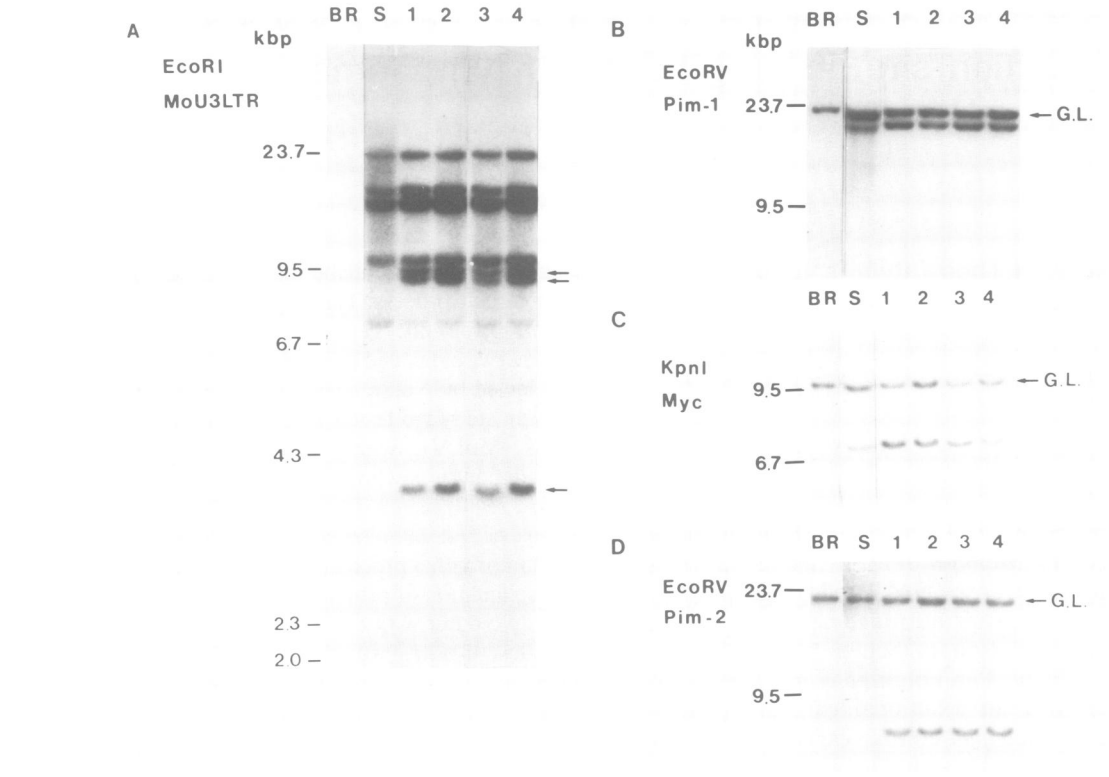

Fig. 1. Proviral integration pattern and rearrangements in the c-*myc*, *pim*-1 and *pim*-2 locus of primary and transplanted tumors of mouse #9. Abbreviation used for the indicated fragments: g.l., germ line. (**A**) **Lane Br**, proviral integration pattern in DNA of brain; **lane S**, primary tumor cells (DNA was isolated from a viable cell suspension which was also used for transplantation); **lanes 1–4**, transplanted lymphomas, as visualized by the specific U3LTR probe. (**B**) Same DNAs hybridized with a c-*myc* probe. (**C**) Same DNAs hybridized with a *pim*-1 specific probe. (**D**) Same DNAs hybridized with a *pim*-2 specific probe.

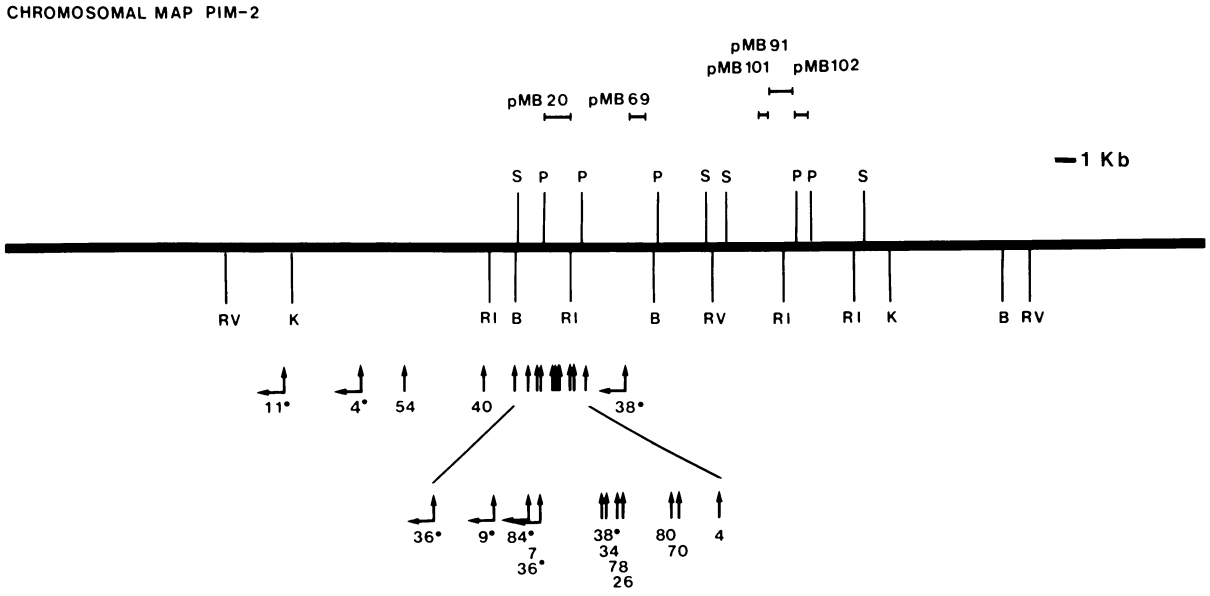

Fig. 2. Chromosomal map of the *pim*-2 locus. A partial restriction map of the *pim*-2 chromosomal region was derived by Southern blotting using probes pMB20, pMB69 and pMB102. The position of restriction endonuclease sites for *Bam*HI, *Eco*RI, *Eco*RV, *Kpn*I, *Pst*I and *Sac*I are indicated. The site of insertion and transcriptional orientation of the proviruses are indicated by arrows. The figures beneath the arrows refer to the mouse # harboring that particular proviral insertion. Transplanted tumors are marked with an asterisk. The relevant probes used in this study are indicated by the upper bars. Note that the proviral insertion site in the primary tumor of mouse 4 differs from that of the transplanted tumors. The transplanted tumor of mouse 36 consists of two subclones, each carrying an independent provirus near c-*myc* and *pim*-2. The transplanted tumor of mouse 38 consists of three subclones, two of which carry an insertion at *pim*-2, that was also present in the primary tumor (see Figure 4).

provirus specific band of 4 kb (Figure 1A) also corresponds to this locus and represents the 3′ junctional fragment. A 1.5 kb *Pst*I/*Eco*RI cellular subclone (pMB20) was used as probe to clone the corresponding region from normal BALB/c liver DNA. The physical map of this region, which we named *pim*-2, is depicted in Figure 2.

Pim-2 is located on mouse chromosome 17

Using a panel of 28 mouse–Chinese hamster somatic cell hybrids, *pim*-2 was assigned to mouse chromosome 17. DNA from hybrid cells, which also served to assign the *pim*-1 to mouse chromosome 17 (Hilkens *et al.*, 1986) was digested with *Bam*HI and hybridized to probe MB20. The correlation between the presence of the *pim*-2 specific band and the presence of chromosome markers was as described for *pim*-1 (Hilkens *et al.*, 1986). The *pim*-2 map differs from the other common insertion sites on chromosome 17: *pim*-1 (Cuypers *et al.*, 1984; Selten *et al.*, 1986) and *int*-3 (Gallahan and Callahan, 1987). Furthermore, insertion in the *pim*-2 locus is not associated with transcriptional activity of either *pim*-1 or *int*-3 (data not shown). Human genomic Southern blot analysis revealed that the sequences present in the probes pMB20 and pMB69 were highly conserved between mouse and man.

Pim-2 is a common insertion site

DNAs from 71 virally induced primary tumors and from 12 transplanted lymphomas were analyzed for the presence of a provirus in the *pim*-2 region using three different restriction endonucleases (*Eco*RV, *Kpn*I or *Bam*HI). Fifteen out of 71 primary tumors revealed novel bands (Figure 3 and Table I). Analysis of the DNAs from transplants of 12 primary tumors showed alterations in the restriction enzyme pattern in seven cases (Figure 3 and Table I). The low hybridization intensity of the modified *pim*-2 allele in primary tumors, as compared to the *pim*-2 germ-line allele, indicated that in the vast majority of these tumors only a small fraction of the cells carried a provirus in *pim*-2 (< 10% of the tumor cells, indicated by +/− in Table I). In contrast the altered alleles of c-*myc* and *pim*-1 were found in the vast majority of the cells of both the primary and transplanted tumors (indicated by +++ or ++++ corresponding with 75–100% of the tumor cells). Summated over all tumor cells within the 71 primary tumors analyzed we estimated the frequency of the proviral insertions in the *pim*-2 locus to be in < 10% of the cells.

Structural organization of the proviruses integrated in the pim-2 region

To show that the observed alterations in the *pim*-2 locus are due to proviral insertions, blot-hybridization with the Moloney-specific U3LTR probe was performed. Novel bands detected with probe pMB20 in the *Bam*HI digest also hybridized with the MoU3LTR probe (data not shown).

Various double digests provided a more accurate determination of the sites of insertion and orientation of the proviruses (Figure 2). Proviruses were detected within a region of 10 kb, while the majority was clustered within 4 kb of host DNA. All proviruses analyzed showed the same transcriptional orientation (see Figure 2).

By making use of an *Eco*RV site in the U3 region of the viral LTR the orientation of the provirus was determined from the hybridization pattern to a U3LTR probe located 5′ of the *Eco*RV site (Cuypers *et al.*, 1984). Differently sized fragments, hybridizing with a *pim*-2 probe and also hybridizing with the U3LTR probe represent the junctional fragment with the 5′ region of the provirus.

Proviral insertion in the pim-2 locus is a relatively late event in lymphomagenesis

Analysis of the common insertion sites, c-*myc*, *pim*-1 and

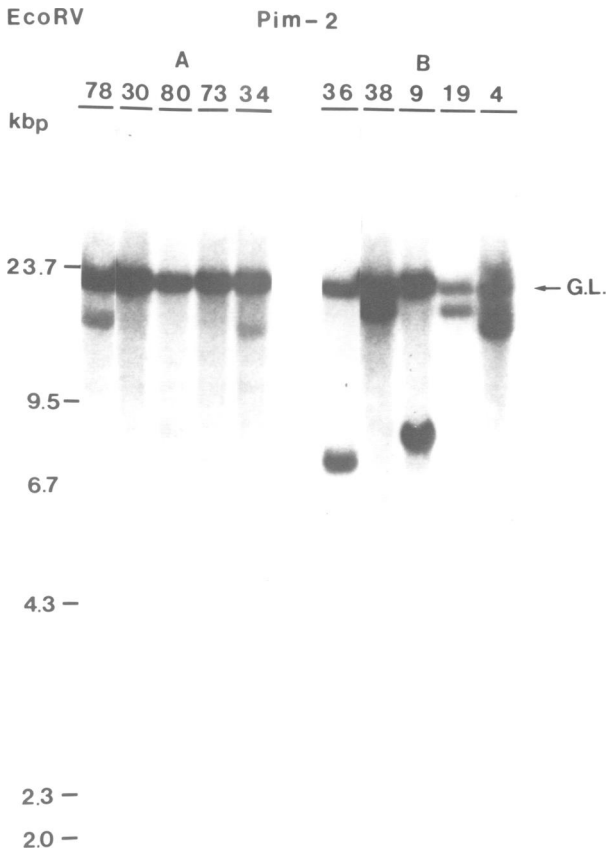

Fig. 3. Proviral insertion in *pim*-2 within primary and transplanted tumors. Procedures were as described in the legend to Figure 1. (**A**) DNA from independent primary tumors hybridized with the *pim*-2 probe. (**B**) DNA from transplanted tumors hybridized with the *pim*-2 probe. Note the difference in the hybridizing intensity of the altered *pim*-2 alleles in the primary and transplanted tumors, respectively.

pim-2, in primary lymphomas showed that several tumors harbor proviruses in more than one of these loci, as was also observed previously for a variety of common insertion sites (Selten *et al.*, 1984; Tsichlis *et al.*, 1985; Peters *et al.*, 1986; Sola *et al.*, 1986; Mester *et al.*, 1987; Mucenski *et al.*, 1987). In most cases the fraction of cells within a single tumor carrying proviral insertions near c-*myc* and/or *pim*-1 was significantly higher than the fraction harboring a provirus within *pim*-2. We wanted to know whether the cells with insertions in *pim*-2 were derived from cell clones which already harbored proviruses near c-*myc* and/or *pim*-1, or represented independently arisen tumor cell clones. If the former is the case this would indicate that integration near *pim*-1 and/or c-*myc* often precedes insertion in *pim*-2. Transplantation of the primary tumors provided a direct answer to this question. The analysis of primary and transplanted tumors of mouse #9 shows that both the primary and the transplanted tumors are monoclonal with respect to proviral insertions near c-*myc* and *pim*-1. In contrast, proviral occupancy of the *pim*-2 locus in the primary tumor is undetectable, whereas in the transplanted tumors all cells carry a provirus in *pim*-2 (Figure 1). Therefore, in the course of tumor development in mouse #9, proviruses integrated subsequently into three different common insertion sites within the same cell lineage, conforming to a multistep process of tumorigenesis via insertional mutagenesis. In the primary and transplanted tumors derived from mouse #38

Table I. Shifts in proviral occupancy of the loci *pim*-1, *pim*-2, N-*myc* and c-*myc* in primary and corresponding transplanted tumors

Tumor #	Primary tumor				Transplanted tumor			
	pim-1	c-*myc*	N-*myc*	*pim*-2	*pim*-1	c-*myc*	N-*myc*	*pim*-2
04	+/−	+	−	+	−	++++	−	++++[a]
07	+/−	+	nd	nd	+/−	+++	−	+++
08	+/−	+	++	−	+/−	++	++	−
09	++++	++++	−	−	++++	++++	−	++++
10	+++	−	+/−	−	++	−	−	−
11	++	−	nd	++	++++	−	−	++++
17	+	+/−	−	−	++	++	−	−
19	++	−	−	−	Non-transplantable			
36	+	++	nd	++	−	++++	−	++++[b]
37	+/−	++++	−	−	−	++++	−	−
38	−	++++	−	+	−	++++	−	+++[c]
85	−	−	nd	nd	Non-transplantable			
86	−	−	nd	−	Non-transplantable			
87	−	−	−	−	−	−	−	−
88	−	−	−	−	−	−	−	+++

Proviral occupancy of *pim*-1, c-*myc*, N-*myc* and *pim*-2 in 15 primary tumors, transplanted in syngeneic hosts. Proviral insertions in the three common insertion sites are listed. Of every primary tumor 5×10^6 viable cells were inoculated (i.v. and some s.c.) in five different syngeneic recipients as described in the method section. These data were obtained by estimating the relative hybridizing intensity of DNA fragments corresponding to the germline allele and the provirally modified allele. Occupancy of proviruses at *pim*-1, c-*myc*, N-*myc* or *pim*-2: −, no proviral insertion; +/−, <10% of tumor cells; +, ~25%; ++, 50%; +++, 75%; ++++, 100%.

[a]Insertion site in transplanted tumor differs from that of primary lymphoma (see Figure 2).

[b]Transplanted tumor consists of two subclones, each carrying an independent provirus near both c-*myc* and *pim*-2 (see Figure 4).

[c]Transplanted tumor consists of three subclones, two of which carry an insertion at *pim*-2, that was also present in the primary tumor.

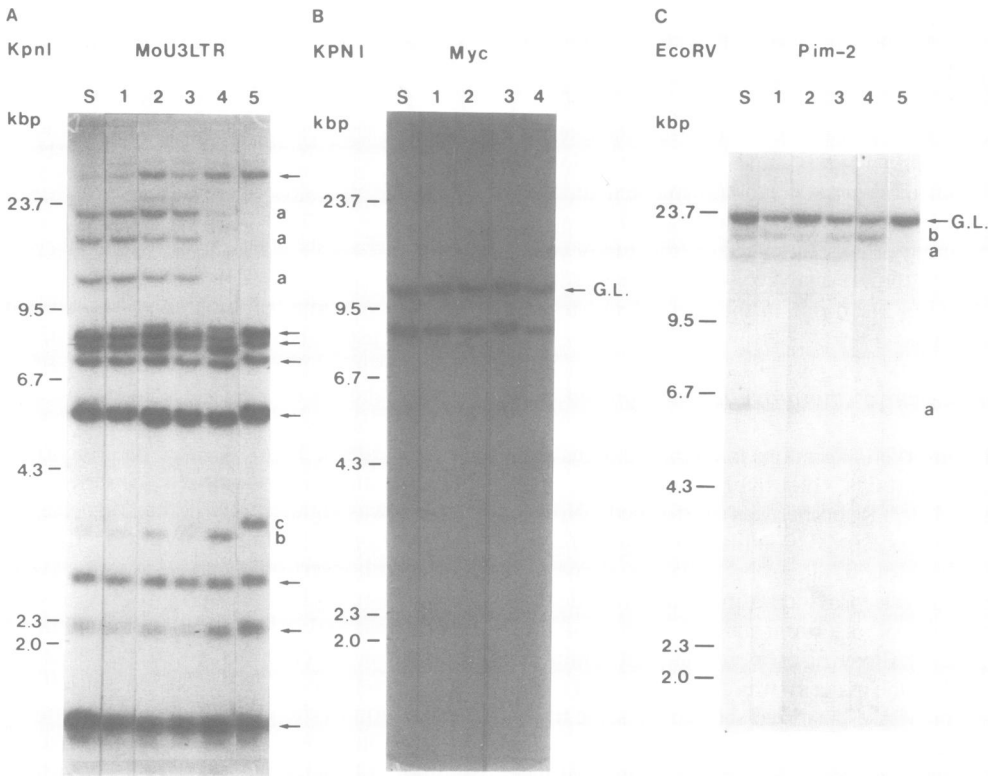

Fig. 4. Proviral integration pattern and rearrangements near *pim*-2 in primary and transplanted tumors of mouse #38. Procedures were as described to the legend of Figure 1. (**A**) DNA of primary, lane S, and transplanted tumors, either inoculated i.p. (**lanes 1–3**), or s.c. (**lanes 4** and **5**), hybridized with the Moloney MuLV U3LTR probe. The proviral insertion sites common to all clones are marked with arrows whereas subclone specific bands are indicated by a, b and c, respectively. (**B**) Same DNAs hybridized with c-*myc*. (**C**) Same DNAs hybridized with *pim*-2 probe pMB20. The proviral integration in *pim*-2 in subclone a is located within the fragment corresponding to probe pMB20, which therefore reveals two bands.

the situation is more complex. Both the primary and transplanted tumors are monoclonal with respect to a distinct proviral insertion in one of the c-*myc* alleles (see Figure 4, panel b). Depending on the route of transplantation and the tissue analyzed, different subclones grow out that are marked by a specific set of proviral integrations. These subclones

pre-existed in the primary tumor and varied both in their sensitivity for different transplantation barriers and in their homing characteristics (Figure 4a). Two of the three discernible subclones show a unique proviral insertion in the *pim*-2 region (Figure 4c).

The majority of transplanted tumors are monoclonal with respect to proviral insertions in pim-2

We have compared the frequency of proviral insertions near c-*myc*, N-*myc*, *pim*-1 and *pim*-2 in primary and transplanted lymphomas. In total 15 primary tumors were transplanted. Tumor cells of 12 primary tumors grew out upon transplantation, whereas three did not. Table I shows the proviral occupancy of the common insertion sites c-*myc*, N-*myc*, *pim*-1 and *pim*-2 in these 15 primary tumors and in the corresponding transplanted tumors. The extent of oligoclonality was estimated from the hybridizing intensities of the normal and altered alleles in the different tumor DNAs. Overall, no selective outgrowth of cells harboring a provirally activated *pim*-1 was found upon transplantation. All transplantable primary tumors bearing an insertion near c-*myc* retained those integrations in the transplanted tumors. After transplantation the fraction of cells carrying a provirus near c-*myc* increases from 30 to 55%. Strikingly, there was an even more significant enrichment from 10 to over 50% for insertions near *pim*-2 in the tumor transplants as compared to the primary tumors (see Table I). All the transplants derived from *pim*-2 positive primary tumors, showed insertion near *pim*-2 in the vast majority of their cells, although integration in *pim*-2 was often found in only a small fraction of the cells of the primary tumor. None of the non-transplantable tumors that were analyzed, carried an insertion near c-*myc*, N-*myc* or *pim*-2.

Search for transcriptional activity in the pim-2 region

The high frequency of proviral integrations within *pim*-2, the undirectional orientation of the inserted proviruses and the evolutionary conservation of sequences in this locus are suggestive of the presence of an oncogene within *pim*-2. So far, Northern blot analysis of a variety of tumors, cell lines and control tissue, using a set of 14 different single copy probes dispersed over a region of 35 kb of *pim*-2, have not revealed the presence of discrete transcripts. S1 analysis, using conserved single copy probes located adjacent to the region in which most proviruses had integrated, has also failed to provide indications of the presence of a genuine transcript near the proviral insertions. We have started chromosome walking both up and downstream from the cluster of proviruses within *pim*-2 in order to search for genes that are transcriptionally affected by proviral integration at *pim*-2.

Discussion

This study illustrates that provirus tagging can reveal genes contributing to different stages of tumorigenesis. Slow-transforming retroviruses can function as persisting mutagenic agents, which are able to activate and mark oncogenes throughout the tumorigenic process. They not only allow the identification of oncogenes that act synergistically in this process (e.g. by integration in loci-like c-*myc*, *pim*-1 and *pim*-2 within the same tumor cell), but they also provide information on the order in which these loci are modified during tumorigenesis and the selective advantage conferred by each of them. Using transplantation of primary MuLV-induced T cell lymphomas as a means of selecting for cells which have further progressed towards high malignancy, we have followed the involvement of the c-*myc* and *pim*-1 oncogenes in primary and transplanted tumors and we have shown that in transplanted tumors a new common insertion site, *pim*-2, is occupied with high frequency.

Pim-2 is probably instrumental in tumor progression, as concluded from the following. (i) Integration at *pim*-2 occurs relatively late in the neoplastic process, often in cells already carrying proviruses in one or more common insertion sites. This was apparent from transplantation of primary tumors that were clonal with respect to an insertion near c-*myc*, but subclonal with respect to insertions near *pim*-2. The transplants were clonal for both. (ii) A strong selective advantage is associated with a proviral insertion in *pim*-2. Transplantation of several primary tumors in which insertions near *pim*-2 were not detected, to independent recipient mice, resulted in tumors that all carried the same unique proviral insertion in *pim*-2 in the vast majority of their cells. This indicates that the major constituent of the transplanted tumor pre-existed in the primary tumor as a minor subclone. All primary tumors in which an insertion at *pim*-2 was observed, were transplantable and retained a provirus at *pim*-2. None of the three non-transplantable tumors harbored a provirus at *pim*-2 or c-*myc*.

Primary tumor cell clones carrying a proviral insertion near c-*myc* but not at N-*myc* showed preferential outgrowth upon transplantation (see Table I). However, proviral integrations near N-*myc* were found in only two out of 10 primary tumors analyzed for N-*myc*. A larger panel of lymphomas will be required to reveal possible differences between the involvement of c-*myc* and N-*myc* in these T cell tumors. The number of cells carrying an insertion near *pim*-1 does not increase upon transplantation, suggesting that these cells have no selective advantage over other cells at this stage of tumorigenesis (see Table I).

Sixty percent of the transplanted tumors in this study carry insertions in the *pim*-2 locus. This raises questions about the remaining *pim*-2 negative tumors. In those tumors, proviruses might have inserted outside the region that was screened for insertions, the gene might have been altered by another mechanism, or mutations might have occurred in or near other gene(s), which confer a similar growth advantage. We could not demonstrate a transcriptionally active gene at the *pim*-2 locus. Perhaps, the gene(s) influenced by integrations at *pim*-2 are located at larger distance from the integration cluster, or only a small exon is located in the region of the proviral cluster, which has escaped detection. Determination of the gene(s) that are transcriptionally activated or otherwise mutated by proviral insertion at *pim*-2, will hopefully provide insight in the molecular mechanisms underlying later steps in tumorigenesis.

Materials and methods

Mice, viruses, lymphomas and transplantation

Lymphomas were induced by injection of newborn BALB/c or C57/BL10 mice with Moloney MuLV clone 1A as described previously (Jaenisch *et al.*, 1975; Cuypers *et al.*, 1984). Transplantation of lymphomas was performed

by i.p. or s.c. injection of ~5 × 10^6 viable primary lymphoma cells into syngeneic mice, which developed lymphomas in 2–4 weeks.

DNA and RNA analysis

For Southern blot analysis 10 μg of total genomic DNA of each mouse was digested with restriction enzymes as recommended by the supplier, separated on agarose gels and transferred to nitrocellulose. Filters were hybridized to ^{32}P-labelled probes and washed as described (Cuypers *et al.*, 1984). Final wash was at 0.1 × SSC, 42°C. Probes used for DNA analysis were as follows. The Moloney MuLV-specific U3LTR probe, the c-*myc* and the *pim*-1 probe A have been described earlier (Cuypers *et al.*, 1984; Selten *et al.*, 1984). The N-*myc* probe has been described by Van Lohuizen *et al.* The positions of the *pim*-2 specific probes pMB20, pMB69 and pMB102, are as depicted in Figure 2. Screening of mouse–hamster somatic cell hybrids was performed as described previously (Hilkens *et al.*, 1986).

RNA analysis was performed as described previously (Selten *et al.*, 1984).

Molecular cloning

Molecular cloning of a proviral insertion site was performed by ligating the 9–10 kb gel-purified junctional *Eco*RI fragment from DNA of tumor transplants of mouse # 9 in Charon30 and plaques were screened with the Moloney MuLV-specific U3LTR probe (Cuypers *et al.*, 1984). Plaques hybridizing to the probe were processed following standard procedures (Maniatis *et al.*, 1982). The inserts were transferred to the plasmid pSP64 and characterized further. Probe pMB20 was used to screen a partial *Sau*3A λ library of BALB/c liver DNA.

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