Leukaemogenesis by the ΔMo + SV Moloney murine leukaemia virus (M-MuLV) variant in Eu pim-1 transgenic mice: high frequency of recombination with a solo endogenous M-MuLV LTR in vivo

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We previously described an enhancer variant of Moloney murine leukaemia virus (M-MuLV), ΔMo + SV M-MuLV, in which the enhancers of MuLV have been deleted and replaced with the enhancers of the simian virus 40 (SV40). When this virus is injected into neonatal NIH Swiss mice, pre-B and B-lymphoblastic lymphomas develop with a latency of 17 months. Van Lohuizen et al. (1989) described a line of transgenic mice that carry an activated pim-1 proto-oncogene transgene (Eu pim-1). They also reported that Eu pim-1 transgenic mice show greatly accelerated lymphoma development when infected with wild-type M-MuLV at birth. In these experiments, neonatal Eu pim-1 transgenic mice were infected intraperitoneally with ΔMo + SV M-MuLV. Marked acceleration of T-lymphoid leukaemia was seen. However, 10 of the 11 tumours analysed were found to be negative for the SV40 enhancers, but they still contained M-MuLV DNA as measured by Southern blot analysis. The LTRs on viruses cloned from two such tumours (as well as on virus recovered by infection onto NIH 3T3 cells) were characterized by PCR amplification, molecular cloning and sequence analysis. The LTR's from the two tumours were identical to each other and were distinct from both the ΔMo + SV M-MuLV and wild-type M-MuLV LTRs. However, they were identical to a rearranged solo M-MuLV LTR present in the Eu pim-1 transgene. These results indicate that the recombination in vivo between ΔMo + SV M-MuLV and the Eu pim-1 transgene yielded a replication-competent and pathogenic virus at high efficiency. This is the first report of in vivo recombination between an exogenous MuLV and a solo endogenous LTR.

Introduction

Moloney murine leukaemia virus (M-MuLV) is a replication-competent retrovirus that lacks an oncogene. Like most non-acute transforming retroviruses, M-MuLV causes disease (T lymphoma) with a significant latency. A hallmark of carcinogenesis by non-acute transforming retroviruses is insertional activation of cellular proto-oncogenes (Cuypers et al., 1984; Fan, 1990; Hayward et al., 1981; Selten et al., 1984). In addition, MuLVs induce other events during leukaemogenesis, including events before outgrowth of the tumour cells (Fan, 1990) as well as events involved in tumour progression (Bear et al., 1989; Patriotis et al., 1993).

Several investigators have shown that the disease specificity for different M-MuLV strains is governed by the tissue specificity of the viral enhancer sequences (Chatis et al., 1983; DesGroseillers et al., 1983; Vogt et al., 1985). This reflects the requirement of an active LTR for insertional activation of proto-oncogenes in the target cell for tumorigenesis. The MuLV enhancers may also affect other steps in the leukaemogenic process as well. We have generated a number of variants of M-MuLV driven by chimeric LTRs with heterologous enhancer sequences (Brightman et al., 1991, 1993; Davis et al., 1985; Feuer & Fan, 1990; Fan et al., 1986; Hancek et al., 1986, 1988, 1991; Kitado & Fan, 1989; Overhauser & Fan, 1985). Several of these viruses show altered pathogenic potentials, and they have been useful in analysing steps in the leukaemogenic process.

We previously described an M-MuLV enhancer variant, ΔMo + SV M-MuLV, in which the M-MuLV enhancers were replaced by enhancer sequences (the 75 bp and 21 bp repeats) from simian virus 40 (SV40) (Hancek et al., 1988). This virus was found to induce B lymphomas in newborn NIH Swiss mice with a very long latency - 17 months for 50% mortality.

The proto-oncogene pim-1 was originally isolated as a common integration site in M-MuLV-induced tumours. Pim-1 encodes a serine/threonine kinase (Cuypers et al., 1984) similar to many previously isolated oncogenes (Racker, 1989), but initially there was no conclusive
evidence that pim-1 had oncogenic potential. Eμ pim-1 transgenic mice were developed by van Lohuizen et al. (1989). These mice carry a pim-1 transgene with a duplicated immunoglobulin heavy chain gene enhancer upstream of the pim-1 promoter and a single M-MuLV LTR within the 3'-untranslated region. Eμ pim-1 mice that are heterozygous for the transgene formed spontaneous T lymphomas in only 5 to 10% of uninoculated mice by 7 months. However, when these mice were injected at birth with M-MuLV they developed T cell lymphoblastic lymphoma with a mean latency of 7 to 8 weeks, as compared to 22 weeks in the parental strain C57BL/6 (van Lohuizen et al., 1989). Given the long latency of disease associated with the ΔMo+SV M-MuLV variant, as well as its induction of B lymphoma, it was interesting to infect this virus into Eμ pim-1 mice to test for acceleration of disease. The results of such experiments are described here.

Methods

Viruses, cells and animals. The wild-type M-MuLV and ΔMo+SV M-MuLV used in these experiments were previously described (Haneck et al., 1988). Procedures for growth and titration of viruses were also described previously (Haneck et al., 1988). Heterozygous Eμ pim-1 mice were obtained by crossing homozygous Eμ pim-1 males (generously provided by Anton Berns) with C57BL/6 females. Eμ pim-1 mice were inoculated intraperitoneally with approximately 10^5 XC p.f.u. of virus per animal within 48 h of birth. Moribund animals were sacrificed, gross pathology was noted and tissue sections were fixed in B5 fixative for histopathology.

Analysis of tumour DNAs. High molecular weight DNA was extracted from tumour tissues as described previously (Haneck et al., 1988). Tumour DNAs were digested with the appropriate restriction enzymes, and analysed by gel electrophoresis and Southern blot hybridization (Southern, 1975) with specific probes. The probes were random primer-labelled restriction fragments. A 0.18 kb M-MuLV LTR-specific Xbal–SmaI fragment from nucleotide -150 to +30 was used as a probe. Probes for the following sequences were used as described previously (Haneck et al., 1988): the T cell receptor β (TCRβ) gene, the immunoglobulin μ heavy chain (IgH) gene, the immunoglobulin kappa light chain (Igκ) gene, SV40 sequences and M-MuLV env sequences (Bosselman et al., 1982).

Infectious-centre assays of haematopoietic tissues and isolation of infectious virus. These assays were carried out as described previously (Davis et al., 1986). Briefly, isolated single cell suspensions from the spleen, thymus and bone marrow of ΔMo+SV M-MuLV-infected mice were co-cultivated for 24 h with NIH 3T3 cultures (5 x 10^5 NIH 3T3 per 10 cm dish) at concentrations ranging from 10^2 to 10^5 haematopoietic cells, in Dulbecco’s modified Eagle medium containing 10% calf serum and 1.5 μg of polybrene per ml. After this time, the haematopoietic cells were removed by washing twice with PBS. After the NIH 3T3 cells reached confluence, they were either scored for foci of M-MuLV infection by the UV-XC assay (Rowe et al., 1970), or the cells were passaged in the presence of 2 μg/ml of polybrene and assayed by UV-XC overlay until they were confluent infected. DNA was then isolated from the confluently infected cells as described previously (Haneck et al., 1988).

PCR cloning of M-MuLV LTRs from tumours. Forward and reverse oligonucleotides complementary to M-MuLV and spanning the ClaI site in env and the SacI site in the LTR at positions 7674 and 8229 (5'-ATTCTTAATCGATTGTTCA-3' and 5'-TTATTTGACTCGGGAGGC-3', respectively) were synthesized for PCR. Of each primer 50 pmol was used in a 100 μl volume containing 0.2 μM of nucleotide triphosphates and 2.5 U Taq polymerase (Perkin-Elmer Cetus) and 1 μg of tumour cell DNA. After denaturation at 94 °C for 5 min, samples were incubated for 35 cycles in a thermal cycler (94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1 min per cycle). Amplified fragments were digested with ClaI plus SacI, and cloned into similarly digested pBluescript SKII. These clones were then sequenced by the dideoxynucleotide chain termination method using a Pharmacia automated laser fluorescent (ALF) sequencer and sequencing kit, which included fluorescently labelled universal primers. A fluorescently labelled oligonucleotide complementary to the U3 region position 7861 of the M-MuLV LTR (5' - CCATTTTGCAAGGCGCATG-3') was used for sequencing.

Results

Leukaemogenicity of ΔMo+SV M-MuLV in Eμ pim-1 transgenic mice

Heterozygous Eμ pim-1 transgenic mice were injected with ΔMo+SV M-MuLV within 48 h of birth, to test if there was cooperation between the activated pim-1 transgene and the weakly leukaemogenic ΔMo+SV M-MuLV. The mice were then monitored for signs of disease and sacrificed when they appeared moribund. Leukaemic tissues were also saved for histopathology and molecular analysis. The time course of disease is shown in Fig. 1. As reported previously, the latency of B lymphoma was 17 months in ΔMo+SV M-MuLV-infected NIH Swiss mice (Haneck et al., 1988). However, 50% of ΔMo+SV M-MuLV-infected Eμ pim-1 transgenic mice developed lymphoma in 6 months, a marked acceleration. In contrast only 6% of heterozygous uninfected Eμ pim-1 transgenic mice developed T cell lymphoblastic lymphoma within 7 months. Furthermore, no C57BL/6 mice (the genetic background for transgensics) infected with ΔMo+SV M-MuLV developed disease by 9 months. Therefore, ΔMo-SV M-MuLV infection was responsible for accelerated leukaemogenicity in Eμ pim-1 transgenic mice.

Characterization of tumours in Eμ pim-1 transgenic mice infected with ΔMo+SV M-MuLV

It was important to determine the type of leukaemia occurring in the ΔMo+SV M-MuLV-infected transgenic mice, since ΔMo+SV M-MuLV-infected mice develop B lymphoma, whereas Eμ pim-1 transgenic mice spontaneously develop T lymphoma (van Lohuizen et al., 1989), and wild-type M-MuLV infection of these mice leads to high incidence of T lymphoma. To determine the tumour types, tumours from leukaemic mice were characterized by gross pathology, histopathology and molecular analysis for T cell TCRβ and immunoglobulin μ heavy chain (IgH) and kappa light chain (Igκ) gene
M-MuLV leukaemogenesis in transgenic mice

Fig. 1. Acceleration of Eμ pim-1 leukaemogenesis by ΔMo+SV M-MuLV. Two-day-old Eμ pim-1 mice were inoculated intraperitoneally with $1 \times 10^5$ XC p.f.u. of ΔMo+SV M-MuLV per animal. The time courses for appearance of moribund animals are shown. Symbols: ΔMo+SV M-MuLV infected Eμ pim-1 mice (○); uninfected Eμ pim-1 mice (●) and ΔMo+SV M-MuLV infected C57BL/6 mice (■). The numbers of animals in each group are indicated.

Table 1. Diagnosis of tumours from ΔMo+SV M-MuLV-inoculated and uninoculated Eμ pim-1 transgenic mice

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<th>JH</th>
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| Uninfected Eμ-pim-1            | R R ND G | LL, T cell type | - |

* Histological diagnosis as determined by histopathology of fixed tumour tissues.
† Tumour DNAs were analysed for the presence of ΔMo+SV M-MuLV LTR by Southern blot analysis of SmaI-digested tumour DNAs and Southern blot hybridization with an SV40-specific probe. After autoradiography, the blots were stripped and re-probed with an envelope gene specific probe Fig. 2 (a and b). Symbols: +, positive for SV40 or envelope hybridization; -, no detectable SV40 or envelope sequences present in the tumours.
‡ Data for ΔMo+SV M-MuLV-induced tumours in NIH Swiss mice was previously reported (Hanecak et al., 1988).
§ LL, Lymphoblastic Lymphoma; FCC lymphoma, Follicular Centre Cell lymphoma; AML, Acute Mylogenous Leukemia.
∥ Represents results of Southern blot analysis as follows: R, rearranged; G, germ line; G/R, primarily germ line configuration but showing a subpopulation of cells in the tumour carrying rearranged loci.
ND, not done.

rearrangements on Southern blots as described previously (Hanecak et al., 1988).

Lymphoblastic lymphomas shown to have TCRγ rearrangements (with or without the IgH gene rearrangements) were classified as T lymphoid, whereas those with no TCRγ gene rearrangements but rearrangements of IgH (with or without Igκ rearrangements) were classified as B or pre-B.

We reported previously that ΔMo+SV M-MuLV induces predominantly B or pre-B lymphomas (Hanecak et al., 1988). A summary from the previous studies is shown in Table 1: lymphoblastic lymphomas of B or pre-
When tumours from heterozygous Eu pim-1 transgenic mice neonatally infected with ΔMo + SV M-MuLV were analysed, 11 out of 11 were classified as T lymphoid by the molecular analyses as also shown in Table 1. Consistent with this, gross pathology of these mice showed extensive tumour involvement in the thymus as evidenced by greatly enlarged thymuses. Spleen and lymph nodes of these mice were also usually enlarged. The one control unoinculated heterozygous Eu pim-1 transgenic mouse that was analysed was also found to have a T lymphoid tumour.

**Presence of virus in tumours**

To assure that the acceleration of disease in the transgenic mice was indeed due to viral infection, high molecular weight DNAs from thymic or splenic tumours of moribund animals were tested by Southern blot hybridization for the presence of ΔMo + SV M-MuLV provirus using an SV40 enhancer-specific probe as described previously (Hanecak et al., 1988). Smal digestion will liberate an internal 2.09 kb fragment that hybridizes with an SV40 enhancer-specific DNA probe. In addition, although Smal is a methylation-sensitive restriction enzyme that will not cut well in the methylated mouse genomic DNA (since it contains the dinucleotide CpG in its recognition site), some viral host-junction fragments would also be expected (Fig. 2a). As illustrated in Fig. 2b none of the 11 tumours contained ΔMo + SV M-MuLV proviral DNA that could be detected by the SV40 enhancer sequence probe. This unexpected result raised the possibility that the tumours arising in the transgenic mice did not contain virus sequences. Alternatively, the tumours might have been infected with a variant of M-MuLV that did not contain SV40 sequences. To test this possibility, the blot of Fig. 2b was stripped and re-hybridized with an M-MuLV env gene probe (Fig. 2c). Ten of the 11 tumours analysed yielded the diagnostic M-MuLV fragment when hybridized with the envelope gene probe, indicating that they were M-MuLV infected, but that a variant virus was present in the tumours. Presumably, some form of rearrangement had occurred in the infected transgenic mice giving rise to the tumorigenic viruses. The data also indicated that the rearrangements involved the enhancer sequences in the LTR, with loss of the SV40 sequences. When tumour cells from two of these mice were co-cultivated with NIH 3T3 fibroblasts, infectious XC-positive viruses were recovered. They showed the same hybridization patterns with both the SV40 enhancer-specific, and M-MuLV env probes as shown in Fig. 2(b and c) lane 10. The one tumour that was also M-MuLV-negative by the env probe probably did not harbour virus; the animal may have died of a spontaneous T lymphoma characteristic
of uninfected Eμ pim-1 transgenic mice (van Lohuizen et al., 1989).

One possible explanation for the high frequency of LTR rearrangements observed in the tumours was that ΔMo + SV M-MuLVs may have been unable to replicate in C57BL/6 mice (the background strain for the transgenics); this could exert strong selective pressure for any rearrangements that improve replication. However, ΔMo + SV M-MuLV-infected C57BL/6 mice that were sacrificed at 16 weeks of age and analysed for the presence of virus showed detectable virus with the SV40 enhancer-containing probe (lane 11 of Fig. 2b). On the other hand, these mice harboured very low levels of infectious virus as measured by infectious centre assay (data not shown).

Cloning the variant LTRs

In order to determine the exact sequence of the rearrangements that had occurred in the LTR in the tumours, M-MuLV-specific LTR sequences were cloned out of the tumours of two mice using PCR. In addition, to ensure that the cloned LTRs were reflective of replication-competent virus, LTR sequences were also PCR cloned from virus recovered from one of the tumours by infection onto NIH 3T3 fibroblasts. In order to avoid cloning out any endogenous retroviral sequences, two oligonucleotide primers were used that had previously been employed to preferentially clone M-MuLV LTRs from virus-induced tumours in NIH Swiss mice (Brightman et al., 1993). Positive clones were characterized with respect to insert size, and representative clones with the predicted sized inserts were sequenced. The sequence of the LTRs cloned from both tumours and also from infectious virus from one tumour were identical, and they contained new sequences not present in the ΔMo + SV M-MuLV LTR, as shown in Fig. 3(a). Moreover, the SV40 sequences were absent. This was consistent with the Southern blot analysis of Fig. 2(b and c).

The identity of the LTRs from two independently arising tumours was striking, and suggested a common mechanism for generation of the rearrangement. Furthermore, analysis of these clones indicated that they resembled a rearranged wild-type M-MuLV LTR, as shown in Fig. 3(b). In particular, the new sequences present in the rearranged LTRs from the tumours could be found in the enhancer sequences of the wild-type M-MuLV LTR. The clones all contained a characteristic rearrangement relative to the wild-type M-MuLV LTR in which there was a deletion between base pairs 8008 and 8028 and an insertion at this site of a duplication of base pairs 8083 to 8161 and then a 6 bp non-M-MuLV sequence (GAAAACA) (Fig. 4). Furthermore, from one of the tumours a second smaller clone was isolated that contained a deletion between base pairs 7946 and 8028 and an insertion of only the 6 bp sequence GAAAACA (Fig. 5). When DNA from this tumour was digested with SmaI and probed with the MuLV env gene probe, two bands were seen, consistent with this tumour containing viruses with two different LTRs (Fig. 2c, lane 7). The most likely explanation for this was that LTR #2 arose from LTR #1 by deletion of the duplicated sequences as well as the sequences between 7946 and 8008.

Initially, the appearance of wild-type M-MuLV-related enhancer sequences in the rearranged LTR was puzzling, since ΔMo+SV M-MuLV completely lacks these sequences. One possible source could be the M-MuLV LTR present in the pim-1 transgene. The organization of the pim-1 transgene is shown in Fig. 5. The transgene contains a solo M-MuLV LTR derived from previously cloned integrated M-MuLV proviruses as described in the figure legend. The absence of any other M-MuLV sequences in the transgene was confirmed by direct sequencing of the transgene plasmid prior to generation of the transgenic animals (M. van Lohuizen & A. Berns, personal communication). Dr. Anton Berns provided us with the sequence of the M-MuLV LTR present in the transgene of Eμ pim-1 mice (Berns et al., 1983), which had previously been found to contain a rearrangement (Berns et al., 1983). The sequence of the transgene LTR was identical to the sequences contained within the rearranged LTRs in the tumours, as shown in Fig. 3(c). These results strongly suggested that the transgene was the source of the new LTRs present in the tumours, and that recombination between the transgene LTR and ΔMo + SV M-MuLV had occurred in vivo, giving rise to a tumorigenic M-MuLV variant.

Discussion

In these experiments we describe the acceleration of T lymphoid leukaemia when neonatal Eμ pim-1 transgenic mice were inoculated with ΔMo + SV M-MuLV. Eμ pim-1 mice infected with ΔMo + SV M-MuLV developed disease with a mean latency of 6 months. In contrast only 6% of uninfected heterozygous Eμ pim-1 mice developed T lymphoma within 7 months.

Furthermore, no C57BL/6 mice (the genetic background for the transgenics) infected with ΔMo + SV M-MuLV developed disease by 10 months. When tumours were analysed they were found to contain proviruses with variant LTRs lacking SV40 sequences. The sequence data of Fig. 3 indicated that the variant LTRs arose by recombination between ΔMo + SV M-MuLV and the rearranged solo M-MuLV LTR in the pim-1 transgene. Presumably, the resulting pseudo-wild-type M-MuLV
Fig. 3. For legend see opposite.
then induced the leukaemia by mechanisms equivalent to wild-type M-MuLV (activation of proto-oncogenes, etc.). The fact that independent tumours showed the identical rearrangements indicated that this recombination was a relatively common occurrence. Moreover, in the Southern blot data of Fig. 2(c), the new LTR-containing restriction fragments from different tumours comigrated, suggesting that the same recombinants were present in all of the virus-positive tumours studied. The recombination observed in these experiments could reflect selective advantage of the LTR recombinant virus.

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**Fig. 3.** Sequence of PCR clone #1 LTR. (a) Comparison with the ΔMo + SV LTR. The U3 regions of the LTRs cloned from viruses in two tumours and also rescued from an infected leukaeic mouse were sequenced by ALF automated sequencing as described in Methods. Clones from both tumours and the recovered virus all had the same sequence, typified by PCR clone #1. The numbers shown correspond to the nucleotide sequence of the wild-type M-MuLV LTR; the numbers are shown where there is sequence identity with wild-type M-MuLV. The U3 region, beginning at the first nucleotide of the U3 region (7816/−447) and ending at the SstI site (8229/−35), of clone #1, compared to that of the ΔMo + SV M-MuLV LTR is shown. (b) Comparison to the wild-type M-MuLV LTR. The U3 region of clone #1 and wild-type M-MuLV are shown. (c) Comparison with the transgene LTR. The U3 region of clone #1 and the pim-1 transgene LTR are shown. Key: capital letters, identical sequence; lower case, differing sequence; italics, SV40 sequence; underlined italics, SstI site; bold, deleted M-MuLV sequence; underline, duplicated sequence. Gaps in the alignment are shown by dashes.

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**Fig. 4.** Diagram of LTRs. The LTRs of ΔMo + SV M-MuLV, wild-type M-MuLV, the pim-1 transgene and both clones isolated from ΔMo + SV M-MuLV-induced tumours are shown. Clones with sequences identical to clone #1 were isolated from both tumours and from virus rescued; clone #2 was isolated from one of the two tumours; and the sequence of the transgene was provided by Dr Anton Berns (Berns et al., 1983) (see also Fig. 5).

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**Fig. 5.** Structure of the Eμ pim-1 transgene. The general structure of the pim-1 transgene as previously described (van Lohuizen et al., 1989) is shown in the upper portion of the figure. The pim-1 exons are shown as boxes on the main axis; the filled boxes represent the translated regions. The immunoglobulin enhancers and the solo M-MuLV LTR are also indicated. The lower portion of the figure shows details of the region surrounding the solo M-MuLV LTR. This was constructed from two fragments obtained from cloned M-MuLV proviral insertions. The U3 portion of the LTR was obtained as a partial BamHI–KpnI fragment from a clone of a proviral insertion from a tumor (Cuypers et al., 1984). This contained upstream adjacent cell sequences (marked by the cross-hatching) and upstream LTR sequences up to the KpnI site at +30 bp in the R region. The remaining portion of the LTR was derived from a different proviral plasmid; a small KpnI–HindIII fragment from a downstream LTR junction fragment supplied the remainder of the R and U5, as well as downstream adjacent cell sequences (marked by shading). Details of the construction were kindly provided by Drs Martin van Lohuizen and Anton Berns (personal communication). BamHI (B), KpnI (K) and HindIII sites surrounding the solo LTR are indicated.
for enhanced virus replication in vivo, or for enhanced tumorigenicity. We have previously found that although ΔMo+SV M-MuLV has near-wild-type infectivity in tissue culture, it establishes very low levels of infection in vivo in NIH Swiss mice (E. Suchman, R. C. Hanecak, P. K. Pattengale & H. Fan, unpublished results). Indeed, when virus was recovered by co-cultivation with NIH 3T3 cells from an 11 month-old ΔMo+SV M-MuLV-infected Eμ pim-1 transgenic mouse that had not developed leukaemia yet, the only virus detectable was the LTR-recombinant (data not shown). Presumably not all ΔMo+SV M-MuLV-infected transgenic mice developed leukaemia because LTR recombinant formation would have taken time. Leukaemogenesis by wild-type M-MuLV is most efficient when mice are inoculated at birth, and is less efficient when adults are infected.

Recombination between retroviral genomes during mixed infections with genetically marked viruses is well documented in the avian (Blair et al., 1976; Wyke & Beamand, 1979), murine (Wong & McCarter, 1973; Faller & Hopkins, 1978), and human (Clavel et al., 1989) viral systems. In addition, exogenous retroviruses can also recombine with endogenous retroviral sequences (Bosselman et al., 1982). The mink cell focus-inducing (MCF) recombinants of MuLVs result from recombination in vivo between ecotropic MuLVs and endogenous polytropic retroviral sequences; MCF recombinants play an important role(s) in tumour formation. Recombination between integrated retroviral DNA sequences and infecting retrovirus has also been described in vitro (Olson et al., 1992). However, this is the first documented case where in vivo recombination between an exogenous retrovirus and an endogenous ‘solo LTR’ (an LTR surrounded by non-viral sequences) has been shown. As shown in Fig. 5, the only viral sequences present in the pim-1 transgene are the LTR sequences. In other cases where LTR recombination between an exogenous and endogenous retrovirus occurs (e.g. generation of pathogenic MCF MuLV recombinants in AKR mice) the endogenous LTR is part of a complete endogenous provirus (Stoye et al., 1991). This allows for efficient co-packaging of an endogenous viral RNA with exogenous viral RNA for recombination (see below).

The recombination between ΔMo+SV M-MuLV and the transgene LTR could have occurred either during reverse transcription, or, potentially by DNA:DNA homologous recombination. The prevailing view is that retroviral recombination occurs via copy-choice reverse transcription from RNAs packaged into heterozygous virus particles (Stuhlmann & Berg, 1992; Coffin, 1979), and that co-packaging of the RNAs participating in recombination is required for the process (Hu & Temin, 1990). Packaging of RNA into virions is most efficient when viral cis packaging signals are present (e.g. the Ψ site) (Linial et al., 1978). The putative packaging of transgene RNA (that contains the rearranged LTR in the 3' end) into virus particles to allow for recombination involving the LTR was therefore somewhat unexpected. However, packaging of cellular RNAs that lack retroviral packing signals into retroviral virions has also been demonstrated (Aronoff & Linial, 1991). It has also been shown that transgene expression is high in both B lymphoid and T lymphoid cells as well as in many other organs in these transgenic mice (van Lohuizen et al., 1989). Thus, significant transgene RNA packaging into virions is plausible. In addition, the presence of the M-MuLV LTR in the 3'-untranslated region of the pim-1 RNA transcript might also enhance packaging of this transcript, although the LTR does not contain the classical Ψ sequences.

We cannot rule out the possibility that the recombination occurred through DNA:DNA homologous recombination. It has been suggested that rearrangements in retroviral LTRs (that do not encode viral protein) occur by DNA recombination or gene conversion (Olson et al., 1992; Goodrich & Duesberg, 1990a, b). These tumours were also tested for traditional MCF recombinants by Southern blot hybridization as described previously. However, no MCF recombinants were detected, even at the level of one MCF provirus per tumour cell (data not shown). This was interesting, because during infection with wild-type M-MuLV, MCF recombinants are almost always detected, beginning soon after infection (Evans, 1986). Moreover, recombinant MuLVs isolated from these tumours closely resembled wild-type M-MuLV. We have also shown that ΔMo+SV M-MuLV can form MCF recombinants in vivo, although apparently at relatively late times (E. L. Suchman, R. C. Hanecak, P. K. Pattengale & H. Fan, unpublished results). Thus, high frequency of MCF formation might have been expected. However, the endogenous retroviruses that contribute the env sequences to MCF recombinants are probably not expressed at the high levels of the pim-1 transgene, so less opportunity for recombination with these sequences might have existed. This still does not explain the lack of MCF formation, once the pseudo-wild-type M-MuLV had been generated. Perhaps the lack of detectable MCF provirus formation might be related to the timing. MCF recombinants have generally been detected in mice that have been infected by MuLVs (exogenous or endogenous) at or near birth. We have shown that initial levels of ΔMo+SV M-MuLV viraemia in neonatally infected animals are relatively low, so the pseudo-wild-type M-MuLV generated in the infected transgenic mice might have arisen at relatively late times, after which formation
of MCF recombinants might have been less efficient, or at which MCFs did not enhance tumour formation.

These results suggest that this system may serve as an interesting model for the study of retroviral recombination in vivo. Furthermore, the results show that other MuLV infections in Eμ pim-1 mice might result in the appearance of equivalent recombinants. This may be important for interpretation of further leukaemogenesis studies in these mice.

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References


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