Short communication

til-1: a novel proviral insertion locus for Moloney murine leukaemia virus in lymphomas of CD2–myc transgenic mice

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Moloney murine leukaemia virus (MoMLV) markedly accelerates thymic lymphoma development in mice carrying a transgene in which the human c-myc gene is linked to the CD2 locus control region. To investigate the mechanism of synergy and identify the genes which collaborate with myc in these clonal tumours, we analysed the sites of MoMLV insertion. Analysis of known viral integration loci revealed only a small number of insertions at bmi-1, pim-1 and ahil-1. Further cloning and hybridization analysis revealed a new common integration locus, designated til-1, which was occupied in 25 out of 77 lymphomas examined, with evidence of multiple clonal insertions in some cases. Mapping relative to established chromosomal markers in interspecific backcross mice located til-1 to mouse chromosome 17, distal to pim-1 and tic-1. These results suggest that the til-1 locus may harbour a novel myc-collaborating gene which acts as a target for activation in T cell lymphomas.

Proviral insertional mutagenesis is a pivotal event in oncogenesis by murine leukaemia virus (MLV) and the study of viral insertion sites in MLV-induced tumours has led to the identification of a large number of host cell genes relevant to this process (Kung et al., 1991). Moreover, the discovery that transgenic mice carrying a dominant oncogene develop tumours much more rapidly if infected early in life with MLV has provided a powerful approach to the identification and complementation mapping of the host cell genes which drive the multi-step evolution of lymphomas (Berns, 1994). As an example, Eu–myc mice developed B cell lymphomas very rapidly after infection with Moloney MLV (MoMLV), with frequent insertions at several distinct host cell loci including pim-1, pal-1, bmi-1 and bila-1 (van Lohuizen et al., 1991). The importance of these insertions to lymphoma development has been amply confirmed for pim-1 and bmi-1, both of which can act independently as dominant oncogenes and are strongly synergistic with myc in lymphomagenesis (Verbeek et al., 1991; van Lohuizen et al., 1989; Haupt et al., 1993). Moreover, a reciprocal pattern of gene activation was seen in pim-1 transgenic mice which developed low incidence lymphomas that could be accelerated by neonatal infection with MoMLV, with concomitant insertions at c-myc, N-myc and pal-1 loci (van Lohuizen et al., 1989).

To test the effects of overexpressing c-myc in the T cell lineage, we developed a transgenic model in which the human c-myc oncogene was linked to a T cell-specific regulatory element, the locus control region from the human CD2 gene. We found that CD2–myc transgenic lines developed thymic lymphomas at low frequency and that neonatal infection with MoMLV dramatically accelerated this process, leading to 100% tumour incidence within 80 days of birth (Stewart et al., 1993). The tumours were of a homogeneous T cell phenotype (CD3+, CD8+, CD4+) and invariably expressed the transgene and contained clonal integrations of MoMLV. Surprisingly, an initial screen of a panel of MoMLV-accelerated CD2–myc tumours showed no evidence of proviral insertions at any of the loci previously identified in Eu–myc lymphomas (Stewart et al., 1993).

These observations suggested that the acceleration of tumour onset in CD2–myc mice was due to the activation of a novel set of myc-collaborating genes by MoMLV. To test this hypothesis, we undertook the cloning and characterization of proviral integration sites from a representative tumour (tumour 10i; Fig. 1a) with a small number of exogenous virus insertions (3–4) as revealed by analysis with a U3 LTR probe (Cuypers et al., 1986). The integrated proviruses were cloned along with their flanking sequences from a tumour DNA library genera-
Fig. 1. (a) Clonality and low proviral copy number in MoMLV-induced tumours of CD2–myc transgenic mice. Southern blot analysis was performed on EcoRI-digested control (lane C, C57) and tumour DNAs as indicated (7i–27i) and the filters were probed with an exogenous MLV-specific LTR probe (Cuypers et al., 1986). Blots were washed at high stringency (0.1 x SSC, 0.5 % SDS, 60 °C) and exposed to Kodak X-Omat AR film. Molecular size markers (kbp) were HindIII-digested λ DNA (lane M). The asterisk indicates the tumour (10i) which was used to generate the phage library. (b) Virus-induced rearrangements of the til-1 locus. Southern blot hybridization analysis and washing conditions were as described in (a). Tumour (lanes 1–10) and control (C) DNA were digested with EcoRI and filters screened with the til-1A probe. (c) Partial restriction endonuclease map and sites of proviral integration in the til-1 locus. The map of the unoccupied site was determined by Southern blot analysis of normal mouse genomic DNA with the til-1A and til-1D probes which are indicated by blocks above the map. Arrowheads indicate the location and orientation of integrated proviruses. The open arrowheads denote the locations of two separate insertions in the reference tumour 10i. Key to restriction enzyme sites: B, BamHI; S, SstI; P, PstI; RV, EcoRV; RI, EcoRI; K, KpnI.

The technique used to detect proviral insertion at the til-1 locus by complete digestion with EcoRI, which has no recognition site within the MoMLV provirus, and ligation to λEMBL4 arms. U3-positive clones were selected and plaque purified. Provirus–host junction sequences were then sub-cloned into plasmid vectors and sequenced. Flanking sequence-specific probes free of repetitive DNA elements were generated and used to screen a panel of similar tumours for evidence of a common insertion locus. Of three U3 hybridization-positive recombinants isolated, one was unique to the tumour of origin, while the other two contained related flanking sequences. Analysis by restriction mapping and DNA sequencing at the insertion site (not shown) showed that these represented two separate viral insertions 535 bp apart at the same locus. Furthermore, screening of a panel of 77 MoMLV-induced lymphomas in CD2–myc mice showed insertions at the same locus in 25 independent tumours. This insertion locus was provisionally designated til-1 (T cell integration locus).

Further examples of virus-induced rearrangements of til-1 are shown in Fig. 1(b). The unoccupied site corresponding to probe til-1A was detected in all cases as a 10 kb EcoRI fragment. In some cases, submolar levels of the rearranged allele were detected (see lane 1 in Fig. 1b), consistent with its presence in a subset of tumour cells, and more than one clonal insertion could be discerned in several other tumours (Fig. 1b, lane 3). Since the virus-accelerated tumours were often oligo-clonal, the frequency with which multiple clones carrying insertions at til-1 were observed in an individual tumour was no greater than would be expected from its high rate of occupancy. In the rare examples where proviral insertion ablates gene function in tumours, loss of the rearranged locus is a common occurrence (Ben-David
et al., 1988; Lu et al., 1994). In contrast, it appears that each insertion at til-1 affected only a single allele, suggesting that these were selected by virtue of a gain rather than loss of function.

Mapping of integrated proviral elements at the til-1 locus showed that with one possible exception all of these were clustered within the 10 kb EcoRI fragment of mouse DNA. Typically, the EcoRI fragment was increased to around 18 kb as a result of proviral insertion (Fig. 1b). A minority of rearranged EcoRI fragments was of smaller size which could have resulted from the insertion of a defective provirus or a variant which had acquired a novel EcoRI site (Fig. 1b, lane 4). An EcoRI site in env is a hallmark of recombinant mink cell focus-forming (MCF) viruses (van der Putten et al., 1981). However, the rarity of U3-hybridizing EcoRI fragments under 9 kb (Fig. 1a) suggested that most insertions were due to ecotropic MLV and that the integration of MCF viruses in turnouts was not an obligatory step in the development or acceleration of lymphomas in CD2-myc mice. Proviral orientation was established by digestion with PstI, XhoI/EcoRI and KpnI/EcoRI; the corresponding map of the til-1 locus is shown in Fig. 1(c). As can be seen, all proviruses at til-1 were found to be inserted in the same orientation. The two distinct insertions cloned from tumour 10i are indicated by open arrowheads.

The next priority was to discover whether til-1 corresponds to a known proviral insertion locus. Towards this goal, we established the chromosomal location of til-1 using the European Interspecific Backcross (Breen et al., 1994). An SstI polymorphism was established by probing parental DNAs (C57BL/6 and Mus spretus) with the til-1A probe and used to follow the segregation of the locus in 64 random backcross progeny. This analysis located til-1 on chromosome 17 close to the D17Mit9 anchor marker. Chromosome 17 harbours two known MoMLV insertion loci [pim-1 and tic-1, formerly designated pim-2; (Hilkens et al., 1986; Breuer et al., 1989)] and for this reason we analysed the segregation of all three markers in a panel of mice recombinant for chromosome 17. This analysis placed til-1 distal to both loci, with the marker order pim-1-D17Mit61, 28, 103-tic-1-D17Mit125, 176, 137, 108-til-1. In the chromosome 17 panel, 35% (21/60) had recombinations between pim-1 and tic-1, and 31% (13/42) between tic-1 and til-1. By extrapolation of the observed recombination frequency to the entire backcross, the genetic distance between tic-1 and til-1 was estimated to be 6 cM (see Fig. 2). These results demonstrate that til-1 is not equivalent to pim-1 or tic-1 but rather represents a novel insertion locus for MoMLV in T cell lymphomas.

Although no known insertion loci were found to be occupied in our previous studies of CD2–myc lymphomas (Stewart et al., 1993), further screening of the extended panel of 77 lymphomas revealed two tumours with insertions at ahi-1 (Poirier et al., 1988), and one at each of bmi-1 (van Lohuizen et al., 1991) and pim-1 (Cuypers et al., 1984) (Table 1). The pim-1 insertion and one of the ahi-1 insertions occurred in tumours with proviruses at til-1. However, in both of these cases the til-1 rearrangements were submolar and we cannot conclude that these insertions co-existed in the same malignant clone.

These results suggest that til-1 may harbour a novel myc-collaborating gene of relevance for T cell lymphoma development. Furthermore, the large fraction of

Table 1. Moloney MLV-induced rearrangement in CD2–myc lymphomas

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<thead>
<tr>
<th>Locus</th>
<th>No. of tumours with rearrangement (n = 77)</th>
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<tr>
<td>til-1</td>
<td>25</td>
</tr>
<tr>
<td>pim-1</td>
<td>3*</td>
</tr>
<tr>
<td>ahi-1</td>
<td>2*</td>
</tr>
<tr>
<td>bmi-1</td>
<td>1</td>
</tr>
<tr>
<td>pal-1</td>
<td>0</td>
</tr>
<tr>
<td>tic-1</td>
<td>0</td>
</tr>
<tr>
<td>p53</td>
<td>0</td>
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* One of each of these tumours also showed a rearrangement of til-1.
CD2–myc tumours with no insertions at any known locus (64%) suggests that further uncharacterized genes may be affected in CD2–myc mouse lymphomas. The marked difference between CD2–myc and Eμ–myc transgenic mice in the pattern of MoMLV gene activations cannot be explained by a simple B cell/T cell difference, since pim-1 and bmi-1 can be activated in tumours of either lineage (Levy et al., 1993; Tsatsanis et al., 1994). While c-myc plays a central role in regulating proliferation in many cell types, its over-expression may induce cell death instead of proliferation under unfavourable conditions (Evans et al., 1992). These observations provide a rationale for the requirement for further genetic events in myc-mediated transformation and oncogenesis. The range of potential myc-collaborating genes is broad, including ras, raf, bcl-2 and p53 in addition to the viral insertion loci already discussed (Berns, 1994; Blyth et al., 1995). At present we favour the hypothesis that the CD2–myc transgene requires a specific set of myc-collaborating genes due to its unique properties of developmental activation and tissue-specific expression. Our future studies will concentrate on the search for a transcription unit affected by the insertions at til-1.

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References


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