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Tumorigenesis in mice with a fusion of the leukaemia oncogene *Mll* and the bacterial *lacZ* gene

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Many different chromosomal translocations occur in man at chromosome 11q23 in acute leukaemias. Molecular analyses revealed that the *MLL* gene (also called *ALL-1*, *HRX* or *HTRX*) is broken by the translocations, causing fusion with genes from other chromosomes. The diversity of MLL fusion partners poses a dilemma about the function of the fusion proteins in tumour development. The consequence of MLL truncation and fusion has been analysed by joining exon 8 of *Mll* with the bacterial *lacZ* gene using homologous recombination in mouse embryonic stem cells. We show that this fusion is sufficient to cause embryonic stem cell-derived acute leukaemias in chimeric mice, and these tumours occur with long latency compared with those found in MLL–Af9 chimeric mice. These findings indicate that an MLL fusion protein can contribute to tumorigenesis, even if the fusion partner has no known pathogenic role. Thus, truncation and fusion of MLL can be sufficient for tumorigenesis, regardless of the fusion partner.

Keywords: ALL-1/cancer/chromosomal translocations/leukaemia/trithorax

Introduction

Chromosomal translocations are involved in the development of tumours by activation of oncogenes (reviewed in Rabbitts, 1994). Chromosome 11q23 is important as translocations occur here in both acute myeloid and lymphoblastic leukaemias (Rowley, 1993; Bernard and Berger, 1995; Rubnitz *et al*., 1996; Look, 1997; Gilliland, 1998) as well as in therapy-related leukaemias (DeVore *et al*., 1989; Pui *et al*., 1989; Cimino *et al*., 1997; Nasr *et al*., 1997; Rowley *et al*., 1997; Sobulo *et al*., 1997; Atlas *et al*., 1998; Felix, 1998). Molecular cloning of various translocation breakpoints revealed a gene called *MLL* (also called *ALL-1*, *HRX* or *HTRX*) in which the translocations occur (Ziemin-van der Poel *et al*., 1991; Djabali *et al*., 1992; Gu *et al*., 1992; Morgan *et al*., 1992; Tkachuk *et al*., 1992; Thirman *et al*., 1993). The affected region of the *MLL* gene is fairly small, around exon 8, but the chromosomal translocations result in fusion with a range of genes from other chromosomes. The diversity of the MLL fusion partners is very broad, ranging from putative transcription factors, ABL-binding proteins to septins (reviewed in DiMartino and Cleary, 1999). There is no obvious common feature of these fusion partners apparent from sequences alone, and in addition, some would be expected to be in the nucleus and others in the cytoplasm of cells, at least in their normal context.

Thus, the diversity of MLL partners raises questions about any possible function of the fusion proteins in dictating tumour phenotype. It is possible that the chromosomal translocations occur in committed cells, which become a tumour of that lineage as a result of the chromosomal translocations, or that they occur in early progenitors and thus contribute to (i.e. help specify) the phenotype of the final tumour by virtue of the fusion partner. An alternative possibility is that the chromosomal translocations usually occur in non-committed cells and the default pathway is to the myeloid lineage, which is the most common phenotype for MLL-associated tumours (DiMartino and Cleary, 1999). The occurrence of a lymphoid tumour may require a specific fusion for specificity of the lineage, as may be the case with the MLL–AF4 fusion. Some model systems have been established to assess the biological role of various *MLL* gene fusions. The HRX–ENL (MLL–ENL) fusion encoded by a retrovirus caused myeloproliferation and myeloid tumours (Lavau *et al*., 1997; Slany *et al*., 1998) and similarly homologous recombination 'knock-in' of *AF9* into *Mll* caused acute myeloid leukaemia (AML) (Corral *et al*., 1996) preceded by myeloproliferation (Dobson *et al*., 1999). These suggest gain-of-function features of MLL-mediated tumorigenesis. However, in the self-fusion of *ALL1* (*MLL*) there is a duplication of MLL, suggesting that the phenotypic effect might be production of a dominant-negative protein in this case (Schichman *et al*., 1994, 1995). In addition, an artificially created truncation of *Mll* at exon 8 (Mll–myc tag) in mice failed to show any influence on haematopoietic differentiation nor on tumour propensity (Corral *et al*., 1996). This, and the self-fusion of MLL, suggested that addition of material to the N-terminal portion of MLL might be able to elicit a tumorigenic effect, perhaps by stabilizing a truncated MLL protein or via protein interactions. In an attempt to assess diversity of the Mll fusions, we have used β-galactosidase as an Mll fusion partner *in vivo*. This enzyme has been extensively used in transgenic mouse and gene targeting experiments and has no demonstrated role in tumour formation. Accordingly, we have fused exon 8 of *Mll* with the *lacZ* gene by homologous recombination in embryonic stem (ES) cells (herein called *Mll–lacZ*) and used these ES cells to create chimeric mice. We found that this *Mll–lacZ* fusion gene was sufficient to cause leukaemia in chimeric mice and that ES cell-derived acute leukaemias arose among *Mll–lacZ* chimeric mice with long latency, compared with Mll–AF9 chimeric mice.

Results

Fusion of lacZ with Mll exon 8 by homologous recombination

We constructed a knock-in ES cell targeting vector designed to fuse the *lacZ* gene into exon 8 of the endogenous *Mll* gene, which would result in the synthesis of an Mll–β-galactosidase fusion protein. Figure 1A–C shows the predicted organization of the targeted knock-in allele (Figure 1A, Mll–exon8–lacZ) compared with Mll–exon3–lacZ (previously called AT–lac; Corral *et al*., 1996) and Mll–myc tag. Homologous recombination of the *Mll–lacZ* targeting vector was carried out in ES cells and filter hybridization analysis of DNA from three clones (clone 14 is shown in Figure 1D as an example) confirmed the integrity of the targeting events using external probes on the 5′ and 3′ side of the targeting region and confirmation of a single insertion with an internal probe (Figure 1D).

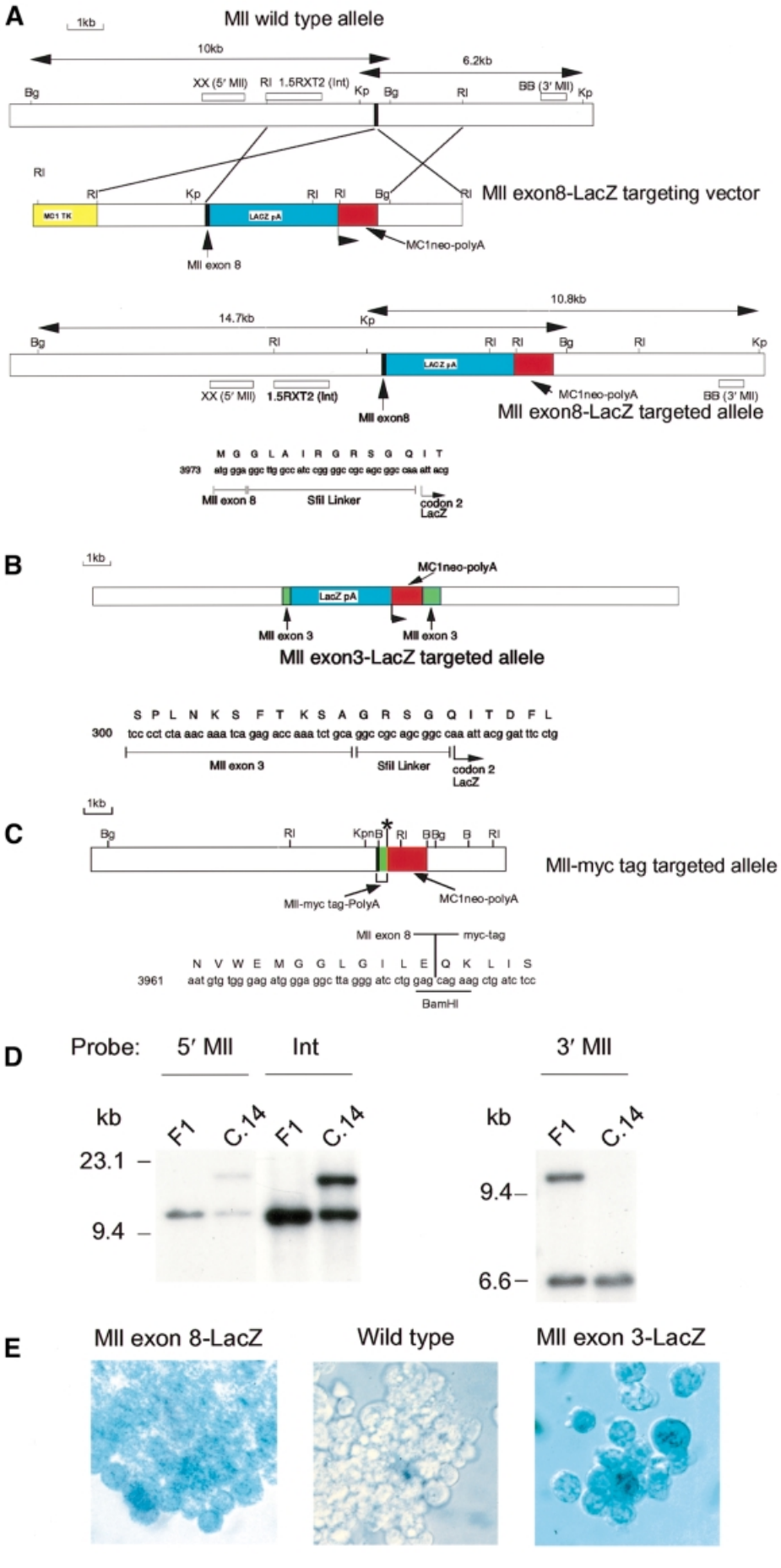
The integrity and function of the *Mll–lacZ* fusion gene were established by analysis of β-galactosidase enzyme activity in ES cell cultures. We compared the expression of the Mll–exon3–lacZ (Corral *et al*., 1996) and Mll–exon8–lacZ fusion genes in ES cells by histochemical analysis of β-galactosidase protein activity in comparison with wild-type ES cells. Both the Mll–exon8–lacZ and Mll–exon3–lacZ ES cells express β-galactosidase (Figure 1E). Mll–exon8–lacZ therefore represents a phenotypic truncation of the *Mll* gene in which functional Mll fusion protein is made. We used these *Mll–lacZ*-targeted ES cells to generate chimeric mice and these mice were monitored for the occurrence of tumours over a 20 month period. A cohort of 43 chimeras was established with the targeted *Mll–lacZ* clones. A full post-mortem examination was performed during a period of 20 months on all mice that developed symptoms. In those cases where leukaemia or lymphoma was found, histological analysis and fluorescent antibody cell sorter (FACS) analysis of surface antigen expression were conducted. At the designated end of the experiment, all surviving mice were examined to determine any pathological features. The control groups were of two different types: (i) a cohort of negative controls comprising C57/Bl6 mice or chimeric mice made by injecting E14 ES cells with a single Lmo2 null allele (C320 cells; Yamada *et al*., 1998); and (ii) a cohort of Mll–myc tag mice.

Mll–lacZ mice develop haematopoietic malignancies

Mll–lacZ fusion mice were found to be prone to developing haematopoietic malignancies (Figure 2A). About one-third of the mice developed ES cell-derived leukaemias prior to 14 months. Fifteen Mll–lacZ mice developed leukaemia, of which 12 had AML and three had acute lymphoblastic leukaemia (ALL) or lymphoma (Figure 2B; see below). Filter hybridization of spleen tumour DNA was conducted to determine the presence of the *Mll*-targeted allele to determine whether the tumours were of ES cell or blastocyst origin. This analysis demonstrated that all the Mll–lacZ mouse tumours were of ES cell origin as the tumour DNA samples had the targeted *Mll* allele plus germ-line allele (Figure 2C). In contrast, the spleen DNA of four Mll–lacZ mice lacking detectable tumours showed predominantly germ-line *Mll* alleles (Figure 2C). The *Mll–lacZ* chimeric mice that did not develop leukaemia were not low chimeras, as most of these *Mll–lacZ* mice had high levels of coat chimerism (Figure 3D). Furthermore, the Mll–lacZ tumour DNAs had variable ratios of targeted to germ-line alleles due to the chimeric origin of these mice. Interestingly, in two samples the targeted allele seemed more intense than the germ-line allele (Figure 2C, numbers 4 and 9), indicative of loss of chromosomal material including the normal *Mll* allele from the non-targeted chromosome in these tumours. Unfortunately, no karyotype data are available for these tumours and we cannot therefore assess the extent of this putative loss.

In confirmation of previous observations (Corral *et al*., 1996), we found no tumours arising in the Mll–myc tag mice during the 20 month experimental period (Figure 2A). However, four tumours did arise [all AML, Figure 2B] in 34 months. DNA filter hybridization analysis of these tumours failed to reveal targeted alleles in the tumour DNA, so we conclude that none of these tumours were of ES cell origin (Figure 2D) and these presumably represent the low, sporadic incidence of leukaemia in the C57/Bl6 strain over a 3-year period. It should be stressed

Fig. 1. *Mll* gene targeting lacZ fusion constructs and β-galactosidase expression. (**A**) The Mll–exon8–lacZ targeting construct. The top map represents the *Mll* wild-type allele, indicating the position of exon 8 (black box) and positions of probes used to evaluate the gene targeting. Below this is a map of the Mll–exon8–lacZ targeting construct indicating the location of the LacZ fusion (blue box) in Mll exon 8, together with the MC1-neo-pA resistance gene (red box) (located downstream of the Mll–lacZ fusion gene). The MC1-tk-pA cassette (Thomas and Capecchi, 1987) [encoding the HSV thymidine kinase gene (yellow box)] is upstream of the fusion gene to allow negative selection of non-homologous integrations. The regions of homology between the Mll–exon8–lacZ targeting construct and the germ-line *Mll* gene are indicated. The bottom map indicates the expected organization of the *Mll* locus in the targeted allele together with the junctional sequence between *Mll* exon 8 and the *lacZ* gene fusion, including the *Sfi*I linker, which was incorporated at the *Bam*HI site to facilitate insertion of the lacZ-MC1-neo-pA cassette. The positions of the three DNA probes used to assess the gene targeting are indicated [XX, 5′ Mll; 1.5RXT2, internal (Int); BB, 3′ Mll]. (**B**) Organization of the Mll–exon3–lacZ targeted allele (previously AT-lac; Corral *et al*., 1996) and the junctional sequence between *Mll* exon 3 and the *lacZ* gene fusion. (**C**) Organization of the Mll–myc tag targeted allele (previously Mll–myc; Corral *et al*., 1996) and the junctional sequence between *Mll* exon 8 and the myc epitope tag fusion. (**D**) Southern filter hybridization of DNA from ES cells with targeted Mll–exon8–lacZ alleles. Three independent targeted ES clones were derived, two made in E14 ES cells (clones 14 and 24) and one in CCB ES cells (clone 118). Filter hybridization of clone 14 is shown for representation, in comparison with wild-type (F1 mouse kidney) DNA. Genomic DNA was digested with either *Bgl*II or *Kpn*I. *Bgl*II-digested DNA was hybridized with the probe XX (5′ Mll) from outside the targeting vector and with the probe p1.5RXT2 (Int), which is located internally in the targeting vector. *Kpn*I-digested DNA was hybridized with the probe BB (3′ Mll) from outside the targeting vector. In each case, the lower band represents the germ-line band and the larger band the targeted allele). Confirmation that a single insertion had occurred in each cell line was obtained by re-probing with a neo probe (data not shown). (**E**) β-galactosidase staining of targeted ES cells. ES cells targeted with either Mll–exon8–lacZ clone 14, Mll–exon3–lacZ or wild-type E14 ES cells were prepared and fixed with 2% formaldehyde/0.2% glutaraldehyde before staining for 48 h with X-gal solution. Cells were transferred to glass slides for photography. Both Mll–exon8–lacZ- and Mll–exon3–lacZ-targeted ES cells stain blue with X-gal.


A
1kb
Mll wild type allele
10kb
6.2kb
XX (5′ Mll)
RI 1.5RXT2 (Int)
BB (3′ Mll)
Bg
Kp
RI
MC1 TK
LACZ pA
Mll exon8-LacZ targeting vector
MC1neo-polyA
Mll exon 8
14.7kb
10.8kb
Mll exon8-LacZ targeted allele
M G G L A I R G R S G Q I T
3973 atg gga ggc ttg gcc atc cgg ggc cgc agc ggc caa att acg
Mll exon 8
SfiI Linker
codon 2
LacZ
B
1kb
MC1neo-polyA
LacZ pA
Mll exon 3
Mll exon3-LacZ targeted allele
S P L N K S F T K S A G R S G Q I T D F L
300 tcc cct cta aac aaa tca gag acc aaa tct gca ggc cgc agc ggc caa att acg gat ttc ctg
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1kb
KpnI
BamHI
Mll-myc tag targeted allele
Mll-myc tag-PolyA
Mll exon 8
myc-tag
N V W E M G G L G I L E Q K L I S
3961 aat gtg tgg gag atg gga ggc tta ggg atc ctg gag cag aag ctg atc tcc
D
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5′ Mll
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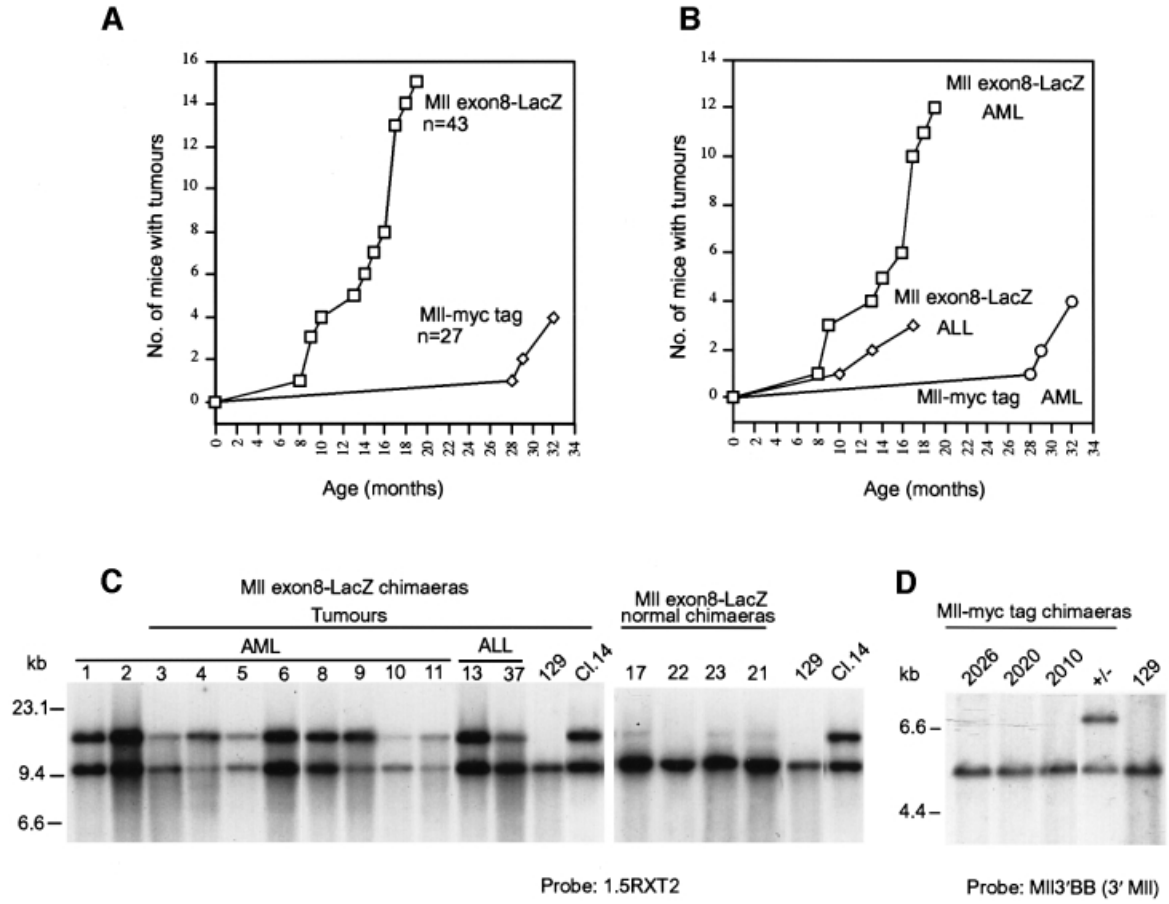

Fig. 2. Tumour incidence in Mll–lacZ mice. Tumour incidence was monitored in cohorts of mice and when signs of indolence or unhealthy coat appeared, animals were killed and post-mortems carried out. (**A**) Data showing incidence of Mll–lacZ mice with detectable tumours compared with a cohort of Mll–myc tag mice (plotted as numbers of mice with time in months). The cohort size of the Mll–lacZ mice was 43 and that of the Mll–myc tag mice was 27. (**B**) Data showing the incidence of AML versus ALL within cohorts of Mll–lacZ and Mll–myc tag mice. Distinct forms of disease were found to be AML or ALL and lymphoma in the groups of mice as indicated. (**C**) Southern filter hybridization of DNA from Mll–lacZ chimeric animals with acute leukaemia. DNA was prepared from the spleen of the chimeras indicated (1–6 and 8–11 diagnosed with AML and 13 and 37 diagnosed with ALL), or chimeras not afflicted with discernible disease (17, 22, 23 and 21). DNA was digested with *Bgl*II, separated on 0.8% agarose alongside 129 liver DNA and DNA from Mll–lacZ ES clone 14, both digested with *Bgl*II. After transfer to nylon membranes, hybridization was carried out with ^{32}P-labelled probe 1.5RXT2. The upper band represents the targeted allele (found in clone 14) and the lower band represents the germ-line allele. (**D**) Southern filter hybridization of DNA from Mll–myc tag chimeric animals with acute leukaemia. DNA was prepared from the spleen of the chimeras indicated (2026, 2020, 2010 diagnosed with AML) digested with *Kpn*I, separated on 0.8% agarose alongside 129 liver DNA and DNA from an Mll–myc tag heterozygous mouse. After transfer to nylon membranes, hybridization was carried out with ^{32}P-labelled probe BB (3′ Mll probe). The upper band represents the targeted allele (found in clone 14) and the lower band represents the germ-line allele.

that none of the Mll–myc chimeras developed leukaemia within the designated experimental period (20 months).

Longer latency of tumorigenesis in Mll–lacZ chimeras

We have compared the incidence and latency of tumours in *Mll–lacZ* chimeras with a cohort of chimeric mice developed from ES cells with an *Mll–AF9* knock-in gene fusion (Corral *et al*., 1996). All the *Mll–AF9* chimeras developed leukaemia (Figure 3A) with 50% having died by ~8 months. The penetrance for the Mll–lacZ fusion mice is lower, with only 35% developing leukaemia, starting at 8 months and ending at 20 months (Figure 3A). The difference in tumour occurrence in Mll–AF9 and Mll–lacZ mice is apparently not attributable to different levels of chimerism. The ES cell contribution to chimerism was assessed by coat colour difference between 129 and C57/Bl6 strains. Figure 3B and C shows histograms of coat colour chimerism compared with age of leukaemia detection. These data show that there is no relationship between level of coat colour chimerism and tumour onset in either Mll–AF9 or Mll–exon8–lacZ groups.

Although we could not assess bone marrow chimerism in the live animals, the difference in coat colour chimerism indicates that the difference in leukaemia latency in the two groups is due to the targeted alleles, rather than merely different chimeric levels in the bone marrow. In addition, this conclusion is supported by the finding that the majority of Mll–Af9 chimeras develop tumours before 12 months, irrespective of chimerism. Finally, examination of chimerism in Mll–lacZ mice with no signs of leukaemia showed that the survivors at 17 months of age or more were, with two exceptions, high chimeras (Figure 3D). As every tumour in the Mll–lacZ group had a high ES

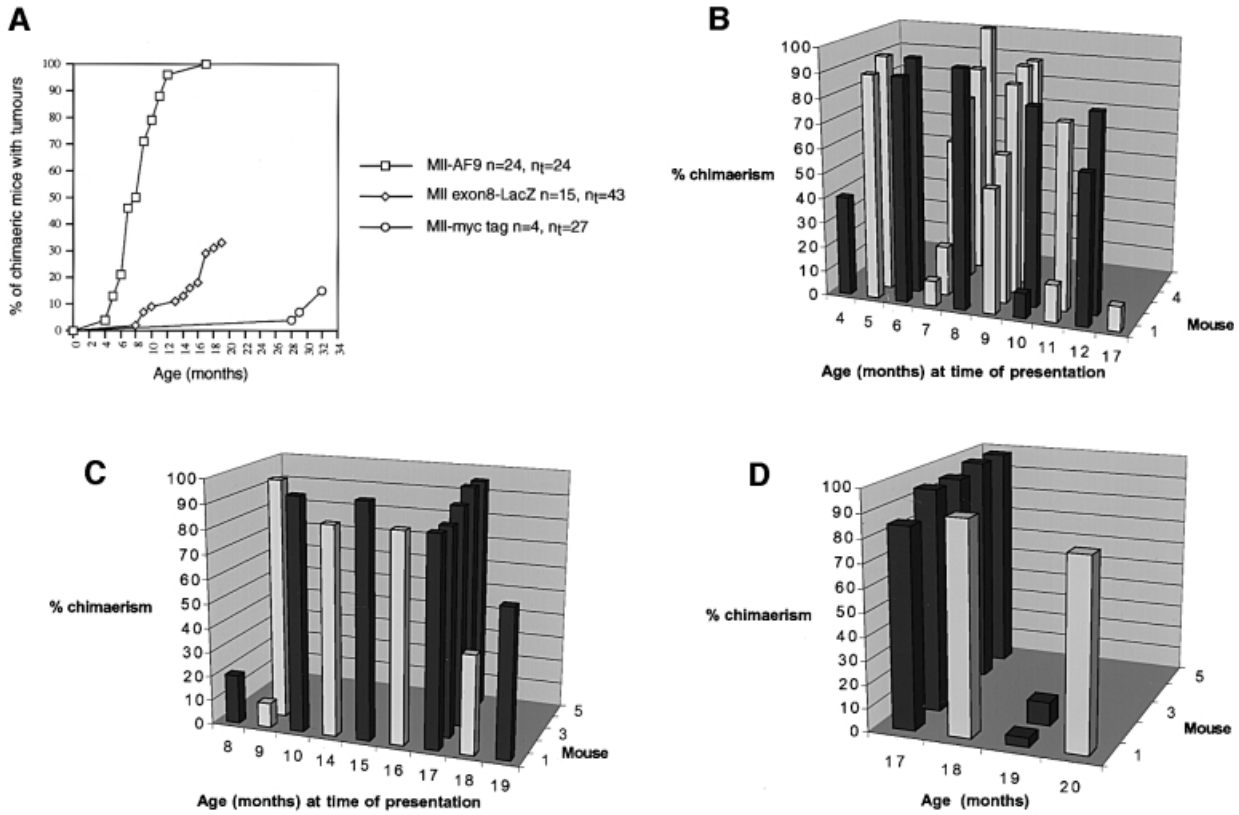

Fig. 3. Relationship between chimerism and tumour incidence in Mll–lacZ mice. Comparison of tumour incidence and coat colour chimerism in Mll–lacZ and Mll–AF9 mice. (**A**) Comparative tumour incidence in the cohort of chimeric mice made with the Mll–lacZ ES cells and cohorts of Mll–myc tag and Mll–AF9 chimeric mice. *n*, number of mice that developed tumours; n_t, number of mice in the cohort. (**B**) Histograms showing the estimated coat colour chimerism in mice derived from injection of targeted ES cells with Mll–AF9 and time of leukaemia incidence. (**C**) Histograms showing the estimated coat colour chimerism in mice derived from injection of targeted ES cells with Mll–lacZ and time of leukaemia incidence. (**D**) Histograms showing the level of estimated coat colour chimerism in those Mll–lacZ mice that were culled for post-mortem examination without symptoms or pathology of leukaemia.

cell contribution (Figure 2C), a direct effect of the *Mll–lacZ* allele is therefore implicated in tumorigenesis.

Mll–lacZ chimeric mice develop acute leukaemias

Mll–lacZ mice with haematological disease generally had an enlarged thymus, splenomegaly, hepatomegaly and pale kidneys. Twelve mice developed AML (Figure 2B). Three mice developed lymphoid disease, of which two were lymphoblastic lymphomas and one was ALL. In the control groups, the Mll–myc tag mice with late onset non-ES-derived tumours had AML (Figure 2B). The majority of Mll–lacZ tumours were Gr-1/Mac-1 double-positive cell type, indicating a myeloid lineage origin. FACS analyses were carried out with spleen and thymus cells of Mll–lacZ tumour-bearing mice and compared with phenotypically normal Mll–exon3–lacZ and wild-type mice (Figure 4). In the case shown, the Mll–lacZ mouse thymus is almost completely populated by Mac-1/Gr-1 double-positive tumour cells compared with the control thymi, which have few Mac-1/Gr-1 double-positive cells (Figure 4A). Similarly, the spleen of the Mll–lacZ mouse is infiltrated with Mac-1^+/Gr-1^+ cells (Figure 4B). From 10 Mll–lacZ tumour-bearing mice examined in detail, all but one had thymus cells expressing the Mac-1 antigen. We conclude, therefore, that the majority of tumours are in the myeloid lineage.

This classification of the malignancies was consolidated by histological analysis of the bone marrow, liver, spleen and peripheral blood. The bone marrow was typically found to have layers of malignant myeloid cells with increased numbers of circulating myeloblasts in blood (Figure 5). Most of the afflicted Mll–lacZ mice also had extensive perivascular infiltration of the abnormal myeloblasts in liver (e.g. Figure 5, mouse 2) and similar extensive infiltrates in the spleen. In all cases, we observed marked leukaemic cell infiltration in the bone marrow, liver and peripheral blood and, to a lesser extent, in the spleen (data not shown).

A small number of Mll–lacZ knock-in mice developed lymphoid tumours. Histology of these tumours revealed few circulating lymphoblasts in blood but pronounced swathes of abnormal cells in bone marrow and extensive perivascular deposits in liver (Figure 5, mouse 13). FACS analysis of tumours from two ALL-bearing mice (mice 13 and 37) showed that these expressed the B-cell marker antigen B220 in both spleen and thymus (Figure 6A). Unexpectedly, tumour cells in these mice also expressed the T-cell marker CD4 (Figure 6A) suggesting a mixed lineage cell type. This was further examined by clonality filter hybridization assays of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangement. Figure 6B shows the hybridization patterns of DNA from spleen and

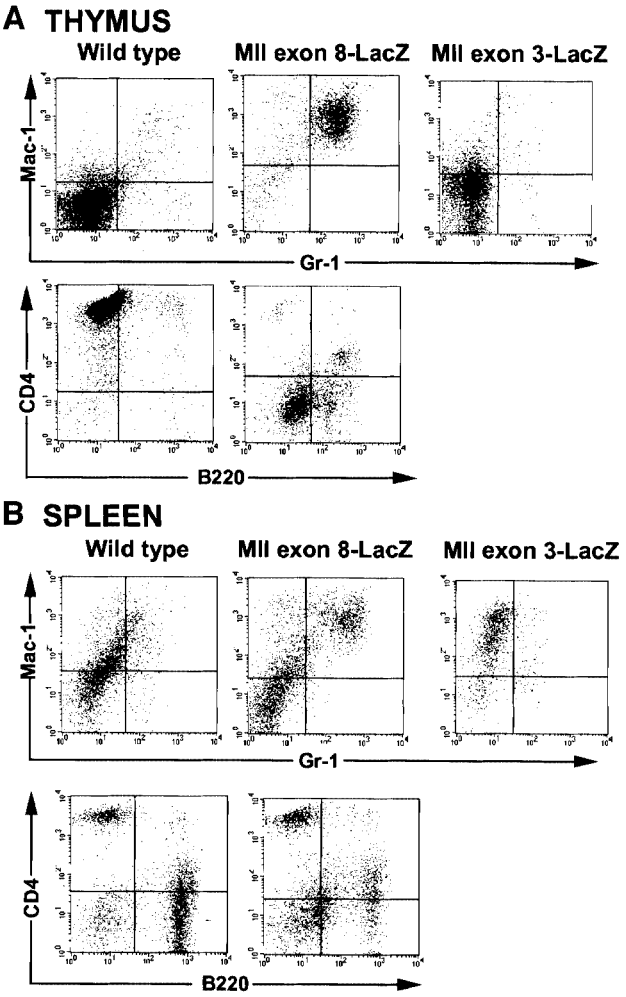

Fig. 4. FACS analysis of tumours from Mll–lacZ mice. Single-cell suspensions were made from spleen and thymus of an Mll–lacZ mouse (mouse number 6) with symptoms of disease and from Mll–exon3–lacZ or wild-type mice. Cells were stained with an anti-Mac-1 antibody coupled with PE together with an anti-Gr-1 antibody coupled with FITC or with an anti-CD4 antibody coupled with PE together with an anti-B220 antibody coupled with FITC. The markers were Gr-1, Mac-1 (alone or together) to detect myeloid populations, and CD4 or B220 to detect T- or B-cell populations, respectively. (**A**) Thymus cell populations; (**B**) spleen cell populations.

thymus from mice 13, 16 and 37, each of which had a diagnosed lymphoid tumour. All three tumour DNA samples exhibited clonal rearrangements of the Ig heavy chain gene on one (Figure 6B, mouse 37) or both alleles (mice 13 and 16). When these same DNA samples were examined with a TCR β-chain probe, mice 13 and 16 showed clonal TCR gene rearrangements, in addition to Ig rearrangements (Figure 6C). Mouse 37 did not exhibit rearranged TCR alleles (data not shown). Thus, all three of these Mll–lacZ tumours are clonal lymphoid tumours and two of the three samples have two attributes of mixed lymphocyte lineage. While we have no experimental evidence of clonality of the AML tumours, it is reasonable to assume that they are clonal as there is a long latency before their occurrence. It thus seems very likely that in both Mll–AF9 and Mll–lacZ, secondary mutations in other genes (e.g. undetermined tumour suppressor genes) are necessary for overt tumours to occur.

Discussion

Mll fusion protein variability and possible functions in leukaemogenesis

Our results show that the stable expression of a truncated *Mll* (truncated where many chromosomal translocations occur in man) contributes to the development of leukaemia in mice, regardless of the fusion partner. The latency period prior to tumour appearance in the *Mll–lacZ* fusion mice appears longer than that observed with the *Mll–AF9* fusions (Corral *et al*., 1996; Dobson *et al*., 1999). These data argue that there are at least two constituents to MLL-mediated tumorigenesis. One is the effect of the removal of *MLL* sequences after the truncation of the gene, which may create a dominant-negative form (Prasad *et al*., 1994; Schichman *et al*., 1994, 1995). The second is the positive contribution of gaining specific fusion partners. The faster rate at which tumours develop in mice with *Mll–AF9* fusion genes points to a further biological role for the Mll–AF9 protein fusion in tumorigenesis. These data are consistent with *in vitro* transfection studies with a construct encoding a truncated MLL protein (Joh *et al*., 1996), which produced an inhibition of differentiation and responsiveness to GCSF in 32Dc13 cells as well as gain-of-function features proposed for the ENL (chromosome 19) fusion partner of MLL (Lavau *et al*., 1997; Slany *et al*., 1998).

Clonality and latency of mouse tumours with Mll fusions

The mouse models of Mll-mediated tumorigenesis are characterized by long latency. In addition, the lymphoid tumours are clonal in origin, as presumably are the myeloid tumours, and thus may require secondary mutations to express the overt tumour phenotype. This may point to a difference in the situation with the human paediatric tumours, which arise early and may not require any somatically acquired mutation other than the chromosomal translocation. It is possible, however, that inherited predisposition gene products may synergize with the chromosomal translocation in these cases. In mice, the additional mutation(s) that seems to be required could be loss of heterozygosity of tumour suppressor genes or gain-of-function mutations in other oncogenes.

It is noteworthy that the spectrum of leukaemias found in the Mll–lacZ fusion mice is similar in phenotype to that of Mll–AF9 fusion mice (Corral *et al*., 1996; Dobson *et al*., 1999). It is possible, therefore, that the default pathway for Mll-mediated tumorigenesis is in the myeloid lineage. Thus, the development of a lymphoid phenotype may be due to other specific secondary mutations or, perhaps more likely, a specific Mll fusion partner that may normally have a specific role in the lymphoid pathway, such as AF4, since the MLL–AF4 fusion predominates in B-lymphoid tumours. In support of this is the fact that the mouse *Af4* gene is required for aspects of lymphopoiesis (Dr M.Djabali, personal communication). *In vivo* experiments to test the specificity of Mll–Af4 gene fusion should help to resolve these questions.

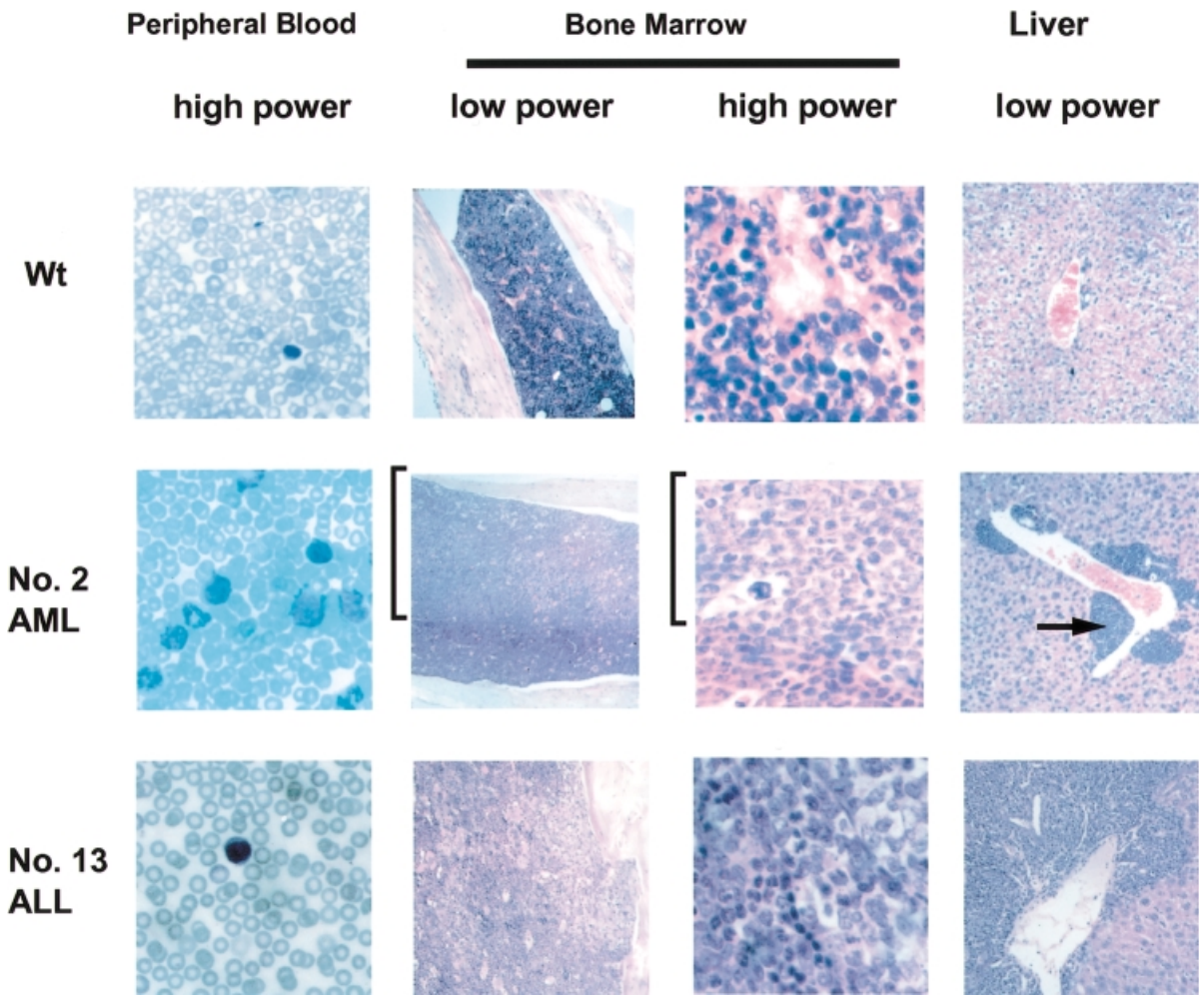

Fig. 5. Histology of haematological tumours arising in Mll–lacZ mice. Tissues were dissected and fixed in 10% formalin. Paraffin sections were prepared and stained with H&E. Blood films were stained with MGG. Comparison of tissues from Mll–lacZ mice with those from a normal C57/Bl6 wild-type mouse. Mll–lacZ mouse number 2 had AML and number 13 had ALL. The tissues from the Mll–lacZ mouse 2 show infiltration with myeloblastic tumour cells, e.g. the arrow indicates the location of the myeloblasts around a blood vessel in the liver of the Mll–lacZ mouse. These myeloid tumour cells are located next to and within the centrilobular vein. A band of myeloblasts (bracketed region) is seen adjacent to a layer of darker normal cells in the bone marrow. Mll–lacZ mouse number 13 had a lymphoblastic lymphoma with few lymphoid cells in peripheral blood, with patches of tumour cells in the bone marrow but large amounts of tumour cells surrounding the veins in liver. Low or high power magnifications are indicated.

A model of the Mll–β-galactosidase fusion protein in leukaemia

Our data on the Mll–lacZ fusion indicate that it may be possible to dissect different elements of MLL function in tumorigenesis. A possible explanation of some distinct components might be that the MLL fusion protein must be stable after chromosomal translocation and that the various fusion partners confer this stabilization. Thus, the β-galactosidase moiety may confer stability of the Mll truncation, yielding effectively a functional Mll-half protein. In the case of the Mll–lacZ fusion, the truncated Mll may function as a dominant-negative molecule, operating in the absence of C-terminal sequences, as suggested for the self-fusion of MLL (Schichman *et al*., 1994, 1995).

Alternatively, complementary functions of truncation and protein fusion of MLL may be important such that Mll–lacZ fusion exhibits the loss of function (i.e. C-terminal sequences) and gain of function. In the case of the *Mll–lacZ* gene some potentially important characteristics are shared between this fusion and some naturally occurring fusions that arise somatically in man by chromosomal translocations. Potentially the most pertinent is the protein–protein interaction feature of β-galactosidase. The crystal structure of the active enzyme shows a tetrameric structure, with the enzyme active site located around a pocket in the tetramer (Jacobson *et al*., 1994). For the Mll–β-galactosidase fusion to function as an enzyme, it is therefore presumably also necessary for the fusion molecule to assemble into a tetrameric form. In the structural model of β-galactosidase (Jacobson *et al*., 1994), the N-termini of the four β-galactosidase molecules are central in the tetramer and thus the Mll moieties would be located there in a putative Mll–β-galactosidase tetramer. Thus, the contacts in β-galactosidase, which allow the formation of the tetrameric active enzyme, might create a dimer of Mll dimers, potentially with the two Mll 'dimers' facing away from each other. A possible Mll dimerization effect has interesting parallels with possible dimerization of MLL fusion genes made by chromosomal translocations. In particular, two translocations have been described in which related genes are fused to *MLL*, namely *AF10* and *AF17*, which are fused to *MLL* after the chromosomal transloca-

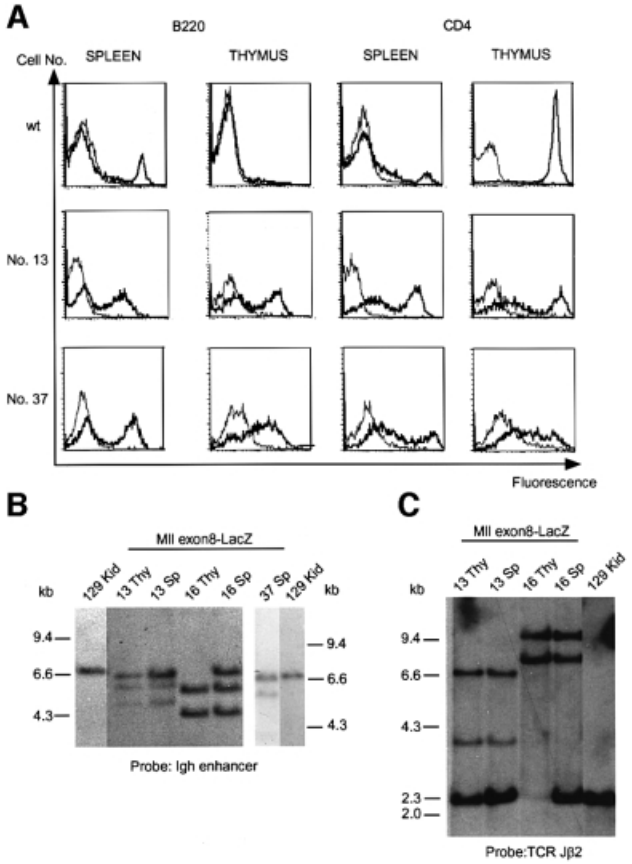

Fig. 6. Clonality of ALL tumours arising in Mll–lacZ chimeras. Three animals from the Mll–lacZ cohort developed ALL according to post-mortem examination and histological analysis. The spleen and thymus cells from these mice were isolated for FACS analysis of lymphoid surface marker analysis (A), and DNA prepared for hybridization analysis of the rearrangement status of Ig heavy chain genes (B) and TCR genes (C). (**A**) Spleen and thymus cells were prepared from a C57/Bl6 mouse (control) and Mll–lacZ tumour mice numbers 13 and 37. The cells were incubated with antibodies recognizing B220 and CD4 antigens. The stained cells were analysed on a FACSCALIBUR instrument. The thinner line represents the signal obtained with an isotype control for B220 or CD4, respectively. The thick line represents the signal obtained with stained mouse cells. (**B**) Autoradiograph of a filter hybridization using an IgH-chain enhancer probe (Neuberger *et al*., 1989). Spleen and thymus DNA from Mll–lacZ mice numbers 13, 16 and 37 (ALL tumours) were digested with *Eco*RI (compared with 129 kidney DNA) prior to gel separation, transfer to nylon and hybridization. The DNA shows similar rearrangement patterns between spleen and thymus in each mouse. Each of these ALL-bearing Mll–lacZ has clonal IgH rearrangements. (**C**) Autoradiograph of a filter hybridization using a TCR Jβ2 probe (Malissen *et al*., 1984). Spleen and thymus DNA from Mll–lacZ mice numbers 13 and 16 were digested with *Eco*RI (compared with 129 kidney DNA) prior to gel separation, transfer to nylon and hybridization. The DNA shows similar rearrangement patterns between spleen and thymus in each Mll–lacZ mouse. Mll–lacZ mouse 37 had no evidence of TCR rearrangement (data not shown). Autoradiography was for 16 h.

tions t(10;11)(p12;23) and t(11;17)(q23;q21), respectively (Prasad *et al*., 1994; Chaplin *et al*., 1995a,b; Saha *et al*., 1995). A prominent feature of the AF10 and AF17 proteins is the presence of a leucine zipper protein dimerization domain in the part retained by the MLL–AF10 and MLL–AF17 fusions, suggesting that a dimer of MLL–AF10 or MLL–AF17 could occur in the leukaemic cells after the chromosomal translocations, thereby eliciting the function of the MLL fusion protein (Prasad *et al*., 1994; Chaplin *et al*., 1995b). Thus, there is a potential similarity between the Mll–β-galactosidase and MLL–AF10 or MLL–AF17 fusions in terms of dimerization.

Materials and methods

Construction of targeting vectors and analysis of targeted ES cells

The Mll–exon8–lacZ plasmid for gene targeting was constructed from a λ phage genomic clone containing *Mll* exon 8, isolated from a library of *Sau*3A partial fragments made in λ 2001. A 5.5 kb *Eco*RI genomic fragment containing exon 8 was subcloned in pBlueScript and an *Sfi*I site added at the *Bam*HI site within exon 8. An *Sfi*I cassette carrying the bacterial β-galactosidase gene coding region upstream of the SV40 poly(A) site plus the MC1-neo-polyA (Thomas and Capecchi, 1987) was inserted into the modified *Sfi*I site yielding an open reading frame fusion with *Mll* exon 8 (Figure 1A). This fusion was confirmed by DNA sequencing. An MC1-tk cassette was cloned at the 5′ end of the *Eco*RI fragment and the final targeting construct was linearized at the 3′ end by *Sal*I digestion. The Mll–exon3–lacZ targeting construct was the AT–lac clone and Mll–myc tag was the Mll–myc clone previously described (Corral *et al*., 1996).

E14 cells were grown and transfected with DNA as described (Warren *et al*., 1994). Homologous recombination with the Mll–exon8–lacZ vector was assessed by filter hybridization (LeFranc *et al*., 1986) of *Bgl*II-digested genomic DNA using a 5′ end 1 kb *Xba*I fragment probe pXX (E.Collins and T.H.Rabbitts, unpublished data) or the *Eco*RI–*Xho*I fragment from p1.5RXT2 (Corral *et al*., 1996). Confirmation of correct targeting was obtained by hybridization of *Kpn*I-digested DNA with the 3′ *Mll* probe BB (derived from a *Bst*XI–*Bgl*II subclone) and the presence of a single insertion site was confirmed using the MC1-neo-pA fragment as a probe. Targeted ES clones were injected into C57/Bl6 blastocysts, which were transferred into recipient females. Chimeric mice thereby generated were estimated by coat colour compared with the black colour of the recipient strain.

β-galactosidase assay

Single-cell suspensions of ES cells were prepared and fixed with 2% formaldehyde/0.2% glutaraldehyde before staining for 48 h at 37°C with X-gal solution [0.2% X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6{\cdot}3H_2O$] and 5 mM $K_3Fe(CN)_6$. The cells were then fixed again with 2% formaldehyde/0.2% glutaraldehyde and washed with phosphate-buffered saline (PBS). Slides were prepared for photography on Kodak Tungsten 160 film.

Histological and pathological analysis of mouse tissues

Mice were culled as soon as signs of ill health appeared. Mice were killed and a post-mortem carried out. Acute leukaemia was characterized by at least 30% of the nucleated cells in the bone marrow being blasts and this disease was mainly AML. The peripheral blood in AML was characterized by circulating myeloblasts. Appropriate tissue samples were taken for FACS analysis and for histology. For the latter, tissue specimens were fixed in a large volume of 10% formalin at room temperature for at least 24 h and embedded in paraffin wax. The wax blocks were sectioned and stained with haematoxylin–eosin (H&E) by routine techniques. Blood films on glass slides were stained with May–Giemsa–Grünwald (MGG). Slides were photographed using Kodak Tungsten 160 film.

Clonality analysis was carried out using filter hybridization as described previously (LeFranc *et al*., 1986) using an Ig heavy chain enhancer probe (Neuberger *et al*., 1989) or TCR β-chain Jβ2 and Cβ1 probes (Malissen *et al*., 1984).

Flow cytometric analysis (FACS) of cell surface marker expression

Single-cell suspensions were prepared from the thymus, spleen and bone marrow of Mll–exon8–lacZ or control mice as appropriate. Cells (100 ml; 5×10^7/ml) were incubated at 4°C for 20 min in PBS with 5% fetal calf serum (FCS) with a 1 in 1000 dilution of the following antibodies: anti-Gr-1 and anti-Mac-1 (granulocytes), anti-B220 (B cells), anti-CD3 and anti-CD4 (T cells). Antibodies (Pharmingen, San Diego, CA) were conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Isotype-matched fluoresceinated antibodies were used as controls for non-specific immunofluorescence. After antibody labelling, cells were washed once with PBS plus 5% FCS and resuspended in 1 ml of

PBS plus 5% FCS. Flow cytometric analysis was performed using a FACSCALIBUR and data analysis was performed with Cell Quest (Becton Dickinson, San Jose, CA). Each phenotype was generated by analysis of 10 000 cells.

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