Inter-chromosomal recombination of *Mll* and *Af9* genes mediated by cre-*loxP* in mouse development

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Received March 23, 2000; revised May 4, 2000; accepted May 22, 2000

Chromosomal translocations are crucial events in the aetiology of many leukaemias, lymphomas and sarcomas, resulting in enforced oncogene expression or the creation of novel fusion genes. The study of the biological outcome of such events ideally requires recapitulation of the tissue specificity and timing of the chromosomal translocation itself. We have used the Cre-*loxP* system of phage P1 to induce *de novo* *Mll*-*Af9* chromosomal recombination during mouse development. *loxP* sites were introduced into the *Mll* and *Af9* genes on chromosomes 9 and 4, respectively, and mice carrying these alleles were crossed with mice expressing Cre recombinase. A resulting *Mll*-*Af9* fusion gene was detected whose transcription and splicing were verified. Thus, programmed inter-chromosomal recombination can be achieved in mice. This approach should allow the design of mouse models of tumorigenesis with greater biological relevance than those available at present.

INTRODUCTION

Chromosomal translocations are involved in the formation of many types of tumours (Rabbitts, 1994). There are two major outcomes involving either enforced expression (activation) of oncogenes near the translocation breakpoints, or fusion of genes on each chromosome resulting in the production of a fusion mRNA and protein. Animal models have been used to create a situation in which the consequences of the chromosomal translocation can be studied to identify biological pathways controlled by these translocation genes. The use of homologous recombination to create a gene fusion in one allele of a target gene, as described for the *Mll* gene (Corral *et al.*, 1996), comes close to the reconstruction of the gene fusion resulting from

chromosomal translocations. However, this approach has a number of drawbacks such as dominant lethality in some transgenics (Heisterkamp *et al.*, 1991). In addition, a transgenic or knock-in experiment only produces the equivalent of one reciprocal translocation product.

An ideal model would be the *de novo* creation of chromosomal translocations during mouse development and within specific cell types. A potential approach to this has been described using the Cre-*loxP* system in embryonic stem cells, whereby *loxP* sites introduced on two different chromosomes were used to facilitate inter-chromosomal translocations by transient expression of Cre recombinase (Smith *et al.*, 1995; van Deursen *et al.*, 1995). These results suggest that inter-chromosomal events might be possible between *loxP* sites introduced in the mouse genome if Cre recombinase were expressed during mouse development. We have applied this strategy to produce inter-chromosomal recombination between *Mll* and *Af9* (*Mllt3*) genes in mice. Translocations involving the *MLL* gene on chromosome 11, band q23, are found in >15% of acute leukaemias in man. The *MLL* gene spans the breakpoint region and >40 different translocations have been identified (Look, 1997). Each *MLL* fusion tends to associate with a particular leukaemia phenotype, a frequent fusion partner being *AF9* involved in the reciprocal translocation t(9;11)(p22;q23) (Iida *et al.*, 1993; Nakamura *et al.*, 1993). We have used homologous recombination to introduce *loxP* sites into the introns of mouse *Mll* and *Af9* genes in ES cells, corresponding to the breakpoint regions of human leukaemias. These mutations have been carried into the germ-line of mice and we have found that inter-chromosomal recombination occurs during development under the control of Cre recombinase. Thus, the Cre-*loxP* system is powerful for the

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Fig. 1. Strategy for Cre-*loxP*-mediated recombination between the *Mll* and *Af9* genes on chromosomes 9 and 4. (A) Targeted *Mll* and *Af9* alleles indicating *loxP* sites (large arrowheads) within the introns corresponding to the breakpoint regions in humans. Cre-mediated recombination between *loxP* sites results in inter-chromosomal recombination and the generation of two fusion genes. Following transcription and RNA splicing, an in-frame *Mll-Af9* mRNA is made. (B) Nested PCR primers for the detection of the *Mll-Af9* fusion gene. (C) Nested PCR primers for RT-PCR detection of *Mll-Af9* fusion mRNA. (D) Expected sequence at the junction of the *Mll-Af9* fusion chromosome. (E) Expected sequence at the junction of the *Mll-Af9* fusion mRNA.

induction of a site-specific inter-chromosomal recombination event in mice. This should allow further studies of the role of translocation genes in cancer.

RESULTS

Orientation of mouse *Mll* and *Af9* genes permits chromosomal translocation

The aim of this study was to evaluate the creation of chromosomal translocations of *Mll* and *Af9* genes in mouse using the Cre-*loxP* system. Our strategy for Cre-*loxP*-mediated recombination between murine *Mll* and *Af9* is schematically outlined in Figure 1. First, *loxP* sites were targeted into the *Mll* and *Af9* genes by homologous recombination in mouse ES cells, into the introns corresponding to the breakpoint regions in human leukaemia patients with t(9;11)(p22;q23) (Figure 1A). Cre-mediated recombination was subsequently attempted either by transient expression of Cre recombinase in ES cells carrying both the *Mll-loxP* (*Mlox*) and *Af9-loxP* (*Alox*) targeted alleles, or in mice with both targeted alleles and transgenic for Cre recombinase [*deleter*

mice (Schwenk et al., 1995)]. Successful recombination was assayed by genomic PCR analysis using *Mll*- and *Af9*-specific primers (Figure 1B), and expression of the fusion gene by reverse transcription-polymerase chain reaction (RT-PCR) with *Mll*- and *Af9*-specific coding region primers (Figure 1C). The sequences of genomic and mRNA junctions are shown in Figure 1D and E.

Before starting this analysis, it was necessary to determine the chromosomal location of mouse *Af9* (*Mllt3*) to ensure that inter-chromosomal translocation between *Mll* and *Af9* was feasible. The mouse homologue of *MLL* has previously been mapped to chromosome 9 (Ma et al., 1993). We mapped the murine *Af9* gene to the central region of chromosome 4, by inter-specific backcross analysis (E.C. Collins, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, and T.H. Rabbitts, unpublished). *Af9* co-segregates with *Ifna*, located 42.6 cM from the centromere of chromosome 4, in a region of synteny with human chromosome 9, band p21–p22. Thus, insertion of *loxP* sites in the same relative orientation in the *Mll* and *Af9* genes should allow a Cre-dependent translocation between mouse chromosomes 4 and 9.

The targeted alleles of *Mll* and *Af9* were made as shown in Figure 2. A *loxP* site was inserted between exons 8 and 9 of *Mll* (Figure 2A) and between the breakpoint exons, which we designated exon a and exon b of *Af9* (Figure 2C). DNA from ES cell clones with targeted *Mll* was assessed by filter hybridization using a 3' *Mll* probe and a hygromycin probe (Figure 2B). Similarly, *Af9* gene targeting was assessed with a 3' *Af9* probe and with a puromycin probe (Figure 2D). Mice were derived from the *Mll*- and *Af9*-targeted clones. In addition, a doubly transfected ES clone (MALX10) was used for transient expression of Cre recombinase as a preliminary assessment of inter-chromosomal recombination between *Mll* and *Af9 loxP*-targeted alleles. A pool of MALX10 ES cells was harvested after Cre transfection, and genomic DNA from 10⁵ cells was tested for the presence of the *Mll-Af9* translocation product by PCR (Figure 3A, left panel). A band of ~364 bp was observed in the MALX10 cells expressing Cre (MALX-Cre) but not in MALX10 cells or CCB control PCR reactions. The MALX-Cre fragment was subcloned for sequence analysis and a nucleotide sequence was obtained corresponding to the genomic junction expected for a Cre-mediated recombination between *loxP* sites in the *Mll*- and *Af9*-targeted alleles (Figure 1D). The presence of the reciprocal translocation product was also confirmed by PCR using puromycin and hygromycin-specific primers (data not shown) indicating that inter-chromosomal recombination had indeed occurred. The transient Cre expression assay was repeated and in this case RNA was extracted for evaluation of the generation of in-frame *Mll-Af9* fusion mRNA by RT-PCR. A 301 bp fragment was obtained from the MALX-Cre cDNA, but was absent from the MALX10 cDNA and CCB control cDNA (Figure 3A, right panel). The MALX-Cre RT-PCR fragment was sequenced and the resulting data showed the *Mll-Af9* mRNA junction expected from fusion of *Mll* exon 8 with *Af9* exon b (Figure 1E).

Mll-Af9 gene fusion during mouse development mediated by Cre recombinase

The PCR results on the ES cells carrying both *Mll* and *Af9* alleles with *loxP* sites show Cre-mediated recombination is possible between chromosomes 4 and 9, consistent with chromosomal translocation having taken place. The ability of this recombination

Fig. 2. Targeting strategy for the insertion of *loxP* sites into *Mll* and *Af9* genes via homologous recombination in mouse ES cells. **(A)** Genomic map of mouse *Mll* around exon 8 (line 1). Targeting vector pMll-loxP-hygro, with a *loxP*-*hygro* cassette inserted at a *Bgl*II site (line 2). Mutant *Mll* allele after homologous recombination (line 3). **(B)** Filter hybridization of targeted ES cell DNA. The 3' *Mll* probe detected a 6 kb *Kpn*I fragment wt allele and an 8 kb fragment mutant allele (left). The latter hybridized in targeted DNA with a probe for the inserted *hygro* gene (right); only a single *hygro* band was observed. **(C)** Genomic *Af9* segment containing the intron analogous to the human *AF9* translocation breakpoint region (line 1). Targeting vector pAf9-puro-loxP has a *puromycin-loxP* cassette inserted into an *Xba*I site (line 2). Mutant *Af9* allele after homologous recombination (line 3). **(D)** Filter hybridization analysis of ES clone DNA. The 3' *Af9* probe detected an 11 kb *Sph*I wt allele, and an 8 kb fragment mutant allele (left). The same band hybridized in targeted DNA with a probe for the inserted *puromycin* fragment (right); a single *puromycin* insertion was detected. wt, wild type; Mlox, *Mll*-targeted ES cell DNA; Alox, *Af9*-targeted ES cell DNA; tk, HSV thymidine kinase; B, *Bam*HI; Bg, *Bgl*II; Bx, *Bst*XI; H, *Hind*III; Kp, *Kpn*I; N, *Not*I; R, *Eco*RI; RV, *Eco*RV; S, *Sac*I; Sp, *Sph*I; Xb, *Xba*I. Filled boxes represent exons. loxP sites are shown as arrowheads.

Fig. 3. Detection of Cre-loxP-mediated recombination between murine *Mll* and *Af9* genes. **(A)** Detection of recombination in ES cells. Cells carrying both *Mlox*- and *Alox*-targeted alleles (MALX10) were subjected to transient expression of Cre recombinase (MALX-Cre). Nested PCR on genomic DNA (left) or RT-PCR on cDNA (right) was carried out with primers spanning the *Mll-Af9* recombination junctions (Figure 1). Control reactions were carried out with either no DNA template (H_2O) or on genomic DNA from untransfected CCB cells or from the untransfected MALX10 cells. **(B)** Detection of recombination between *Mll* and *Af9* genes in mouse tissues. Nested PCR analysis was carried out on genomic DNA from a variety of tissues from an *Mlox-Alox* control mouse (left) and from an *Mlox-Alox-Cre* animal with the *deleter* genotype (right). Reactions lacking template (H_2O) served as negative controls. Fragment sizes were estimated by co-electrophoresis of ϕ X174 DNA cleaved with *Hae*III.

event to occur in mice was assessed using mouse strains with germ-line transmission of the *Mlox* and *Alox* alleles (designated *Mlox-Alox* mice). These mice were crossed with transgenic mice expressing Cre recombinase under the control of a ubiquitous promoter [*deleter* mice (Schwenk *et al.*, 1995)]. The resulting strains were inter-bred to generate a pool of animals carrying both the *Mlox* and *Alox* alleles and the *deleter* transgene (designated *Mlox-Alox-Cre* mice). The possibility that inter-chromosomal translocations had occurred was assessed in *Mlox-Alox-Cre* and *Mlox-Alox* mice between the ages of 6 and 8 months. Genomic DNA was extracted from a number of tissues and PCR amplifications were performed (Figure 3B). A PCR product (~364 bp), corresponding to inter-chromosomal recombination between *Mll* and *Af9*, was visible when using brain DNA as a template but not in any other tissue DNA. The sequence of this product was obtained to confirm its origin from the *loxP* recombination (data not shown). The occurrence of the recombination product in brain implies that the *Mll* and *Af9* genes are accessible for inter-chromosomal Cre-mediated recombination and, although RT-PCR results were only semi-quantitative, the levels of Cre enzyme in brain appear compatible with these events (see below).

The presence of the fused chromosomal segment between *Mll* and *Af9* genes in the *Mlox-Alox-Cre* mouse was confirmed by analysing expression of the in-frame fusion mRNA by RT-PCR using cDNA templates prepared from the various tissues. This yielded similar results to the genomic PCR. An RT-PCR product

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Fig. 4. Detection of *Mll-Af9* fusion RNA from tissues of *Mlox-Alox-Cre* mice. (A) *Mll-Af9*-specific nested RT-PCR analysis of cDNA generated from tissue RNA samples of *Mlox-Alox* or *Mlox-Alox-Cre* mice. (B) Detection of Cre expression by RT-PCR using cDNA template. (C) Actin-specific RT-PCR on the various cDNA populations.

Fig. 5. Detection of *Mll-Af9* fusion in brain RNA of *Mlox-Alox-Cre* mice. (A) *Mll-Af9*-specific RT-PCR on cDNA generated from brain RNA of 10 *Mlox-Alox-Cre* (numbered 1–10) and two *Mlox-Alox* mice (numbered N1 and N2). (B) Actin-specific RT-PCR on the various cDNA populations.

from *Mll-Af9* mRNA was produced from the brain cDNA template of the *Mlox-Alox-Cre* mouse and not from that of the *Mlox-Alox* mouse (Figure 4A). All cDNA templates were verified with an actin-specific RT-PCR (Figure 4C). The expression of Cre recombinase was confirmed in all tissue RNA samples using RT-PCR (Figure 4B). Cre expression appeared highest in the brain.

The presence of Cre-mediated recombination between *Mll* and *Af9* chromosomes was investigated using brain RNA samples from 10 further *Mlox-Alox-Cre* mice compared with two *Mlox-Alox* control mice. All the brain cDNA templates from the *Mlox-Alox-Cre* mice generated the fusion mRNA product by RT-PCR amplification (Figure 5A, samples 1–10) but the *Mlox-Alox* controls did not (Figure 5A, lanes N1 and N2). The quality of cDNA templates was verified by amplification of an actin RT-PCR product (Figure 5B). Thus we conclude that inter-chromosomal recombinations, presumably translocations, can be induced by the Cre-*loxP* system in mice.

DISCUSSION

The Cre-*loxP* system of page P1 has been applied to induce site-directed chromosomal translocations in mouse ES cells (Smith *et al.*, 1995; van Deursen *et al.*, 1995) resulting in the generation of ES cells constitutively carrying the induced translocation. We have now demonstrated that *de novo* Cre-*loxP*-mediated inter-chromosomal recombination (presumably translocation) between non-homologous chromosomes is possible in mice. This approach has potential applications in the field of chromosome engineering and should provide insights into the consequences of large-scale DNA rearrangements in animals.

A limitation that remains is the apparent low efficiency of the inter-chromosomal recombination. Presumably, the chance of synapsis between the two *loxP* sites, a prerequisite for Cre-mediated strand exchange, is much lower for inter-chromosomal

than intra-chromosomal events. Indeed, the *deleter* mice used in our experiments have previously shown efficient recombination between *loxP* sites in close proximity, in a wide variety of tissues (Schwenk *et al.*, 1995). However, the *cre* gene appears to be only weakly transcribed in these mice. Our semi-quantitative RT-PCR results suggest stronger *cre* expression in the brain than in other organs, which may account for the presence of the recombination signal in the brain samples only. Nonetheless, the occurrence of inter-chromosomal recombination may also be influenced by the accessibility of the target genes, which in turn may depend on their level of transcription in a particular organ. While the expression of mouse *Mll* is ubiquitous (Yu *et al.*, 1995), our results with *Af9* gene expression show strong expression during brain development (E.C. Collins and T.H. Rabbitts, unpublished), which may also partly explain the propensity for rearrangements in brain rather than in other tissues.

An important potential use of *in vivo* Cre-mediated chromosomal translocations is in the study of the specificity and biological role of translocation genes. In particular, the *in vivo* function of fusion genes may be studied by this approach. The use of tissue-specific promoters to control the site and timing of Cre expression, either by homologous recombination knock-in or transgenesis, is a potentially powerful strategy. For instance, to gain insight into the role of MLL fusion partners in determining the tumour phenotype after 11q23 translocations, it would be useful to compare the consequences of the *Mll-Af9* fusion in myeloid and lymphoid lineages. Induction of *Mll-Af9* translocation in lineage-committed cells or in progenitors would remove the obligate myeloproliferative advantage conferred by the constitutive *Mll-Af9* knock-in fusion gene (Dobson *et al.*, 1999). The subsequent incidence of leukaemia of lineage-specific phenotype would provide important evidence about the timing of translocation and role of the fusion proteins.

Finally, the *Mll-Af9* knock-in mouse model showed that the fusion gene alone is sufficient for the development of leukaemia (Corral *et al.*, 1996; Dobson *et al.*, 1999), but the situation in

human patients is almost certainly more complex. The chromosomal translocation may have consequences ranging beyond the creation of one fusion gene; for example it has been shown that both PML-RARA and RARA-PML products are important in the phenotype of acute promyelocytic leukaemia in mice (Pollock *et al.*, 1999). [PML-RARA and RARA-PML products are made from the reciprocal chromosomal translocations der(15;17) and der(17;15) where PML = promyelocytic leukaemia gene; RARA = retinoic acid receptor α gene.] In addition, embryonic lethality resulting from the expression of fusion genes may require creation of *de novo* translocations for a full read-out of the pathogenic consequences of chromosomal rearrangements. Other factors, such as changes in chromatin structure and gene regulation near the breakpoints of the chromosomal translocation, may also be important for leukaemogenesis. The strategy of Cre-*loxP*-mediated recombination between chromosomes in specific mouse tissues should provide insights into these features.

METHODS

Gene targeting. A puromycin-*loxP* cassette was obtained by inserting a 115 bp *EcoRI*–*SalI* fragment from PL2XR into pPGK-puro. A *loxP* fragment from pPGK-puro-*loxP* was cloned into *EcoRV* of pPGK-hygro to generate a *loxP*-hygromycin cassette. Targeting vector pMll-*loxP*-hygro has a 5.5 kb *EcoRI* genomic fragment, containing the region of the *Mll* gene corresponding to the human breakpoint region, in the *EcoRV* site of pBluescript. The *loxP*-hygro cassette was cloned into a genomic *BglII* site in *Mll* intron 8. HSV thymidine kinase (tk), from pMC1-tk (Thomas and Capecchi, 1987), was cloned into the *XbaI* site. Targeting vector pAf9-puro-*loxP* has an 8.5 kb genomic *SphI*–*EcoRV* fragment, containing the breakpoint region of 129 mouse *Af9*, in the *XbaI* site of pBluescript. The *puro-loxP* cassette was cloned into an *XbaI* site in the *Af9* breakpoint intron (designated intron a). The tk fragment was introduced into the *NotI* site.

ES cells were transfected as described (Warren *et al.*, 1994). CCB ES cells were grown on drug-resistant mouse embryonic feeders during selection. pMll-*loxP*-hygro was introduced into ES cells grown on hygromycin-resistant feeders (Johnson *et al.*, 1995) to make Mlox cells and pAf9-puro-*loxP* into ES cells grown on puromycin-resistant feeders [C57BL/6J-TgN(pPWL88puro) 2Ems JR2355, E.M. Simpson, unpublished data] to make Alox cells. Finally, Mlox cells grown on puromycin-resistant feeders were re-transfected with pAf9-puro-*loxP* to make doubly targeted cells, MALX10. These were transiently transfected with Cre recombinase expression vector, pPGKCrebpA. Cells were grown for 72 h without selection and harvested for DNA and/or RNA extraction (MALX-Cre).

DNA preparation and filter hybridization analysis. DNA from ES cells and from mouse tissues was made using Puregene (Gentra Systems). Ten micrograms of DNA were digested with restriction enzyme and resolved on 0.8% agarose gels. After electrophoresis, the DNA was transferred to nylon membranes and hybridized to radioactive probes (LeFranc *et al.*, 1986).

RNA extraction and cDNA preparation. Extraction of total RNA was carried out with Trizol (Gibco-BRL). cDNA was generated using SuperRT reverse transcriptase (HT Biotechnology). cDNA from 5 μ g of total RNA was diluted to 100 μ l.

Detection of Cre-mediated translocations by PCR. Genomic DNA (0.5 μ g) was subjected to 35 cycles of PCR amplification with primers 1a (*Mll* intron primer 5'-gtcccataacacccagagtagtg-3') and 1b (*Af9* intron primer 5'-cctcattctgacagaccagagcca-3') (Figure 1B) at 95°C for 1 min, 65°C for 1 min and 72°C for 1 min. Each PCR sample (1 μ l) was used as a substrate for 35 further cycles, with nested primers 2a (*Mll* intron primer 5'-gggcatgtagaggttaagagcctg-3') and 2b (*Af9* intron primer 5'-atctccaggactgaatctagggc-3') (Figure 1B). PCR fragments were resolved on 2% agarose gels and cloned for sequence analysis.

RT-PCR was carried out as above, with 1 μ l of cDNA as template, and primers 3a (*Mll* exon primer 5'-ggagtccacaggatcagagtgac-3') and 3b (*Af9* exon primer 5'-tcaggatgttccagatgtttcag-3') (Figure 1B), and then with nested primers 4a (*Mll* exon primer 5'-cctgacctctgtcccataacacc-3') and 4b (*Af9* exon primer 5'-gactgtgtttgtccagcagca-3'). RT-PCR with actin-specific primers (5'-atggcactgccgcatcctctcc-3' and 5'-cacgatggagggcgactcatc-3') was carried out using 20 cycles as above. The touch-down method was used from 63 to 55°C (Don *et al.*, 1991) for RT-PCR with Cre-specific primers (5'-cgatagccgaaatgc-3' and 5'-caatcgatgagtgcttc-3').

ACKNOWLEDGEMENTS

We thank Andrew McKenzie for *loxP* and Cre plasmids, Anton Berns for pPGK-hygro and Peter Laird for pPGK-puromycin and pPWL88puro. We also thank Kevin A. Johnson, Charles P. Lerner, Thomas R. Ulrich, Craig A. Leach, Elizabeth R. Linnell and William C. Rafferty for development of mice for puromycin-resistant feeders. E.C.C. was a recipient of an LRF Gordon Pillar Studentship. E.M.S. was supported by NIH grant 1R01MH/HD57465.

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DOI: 10.1093/embo-reports/kvd021