Bypass of lethality with mosaic mice generated by Cre–*loxP*-mediated recombination

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Background: The analysis of gene function based on the generation of mutant mice by homologous recombination in embryonic stem cells is limited if gene disruption results in embryonic lethality. Mosaic mice, which contain a certain proportion of mutant cells in all organs, allow lethality to be circumvented and the potential of mutant cells to contribute to different cell lineages to be analyzed. To generate mosaic animals, we used the bacteriophage P1-derived Cre–*loxP* recombination system, which allows gene alteration by Cre-mediated deletion of *loxP*-flanked gene segments.

Results: We generated nestin–cre transgenic mouse lines, which expressed the Cre recombinase under the control of the rat nestin promoter and its second intron enhancer. In crosses to animals carrying a *loxP*-flanked target gene, partial deletion of the *loxP*-flanked allele occurred before day 10.5 *post coitum* and was detectable in all adult organs examined, including germ-line cells. Using this approach, we generated mosaic mice containing cells deficient in the γ-chain of the interleukin-2 receptor (IL-2Rγ); in these animals, the IL-2Rγ-deficient cells were underrepresented in the thymus and spleen. Because mice deficient in DNA polymerase β die perinatally, we studied the effects of DNA polymerase β deficiency in mosiac animals. We found that some of the mosaic polymerase β-deficient animals were viable, but were often reduced in size and weight. The fraction of DNA polymerase β-deficient cells in mosaic embryos decreased during embryonic development, presumably because wild-type cells had a competitive advantage.

Conclusions: The nestin–cre transgenic mice can be used to generate mosaic animals in which target genes are mutated by Cre-mediated recombination of *loxP*-flanked target genes. By using mosaic animals, embryonic lethality can be bypassed and cell lineages for whose development a given target gene is critical can be identified. In the case of DNA polymerase β, deficient cells are already selected against during embryonic development, demonstrating the general importance of this protein in multiple cell types.

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Received: 18 June 1996
Revised: 6 August 1996
Accepted: 6 August 1996

Current Biology 1996, Vol 6 No 10:1307–1316

Background

The generation of mice containing defined mutations has contributed considerably to the understanding of gene function in mammals [1]. If gene disruption leads to embryonic lethality, gene function can be assessed only at early developmental stages. To overcome this limitation, a method for the conditional inactivation of endogenous genes has been developed [2,3], which is based on the bacteriophage P1-derived Cre–*loxP* recombination system [4–7]. A critical region of a target gene is flanked by *loxP* sites, introduced by homologous recombination in embryonic stem (ES) cells, leaving gene function undisturbed. In mice carrying *loxP*-flanked alleles and expressing the Cre recombinase in a specific cell type, gene inactivation results from Cre-mediated deletion of the *loxP*-flanked gene segment in a cell type-specific manner [2]. In order to extend this kind of analysis to cells of all lineages it would be desirable to analyze mosaic animals that lack the gene of interest in some cells of all types. Depending on the proportion of cells carrying the 'lethal' mutation, mosaic animals would be able to develop to adults, allowing one to study the ability of mutant cells to contribute to different cell lineages. If the gene of interest is important for the formation or maintenance of certain tissues or cell types, mutant cells will be absent or underrepresented in those tissues. Unexpectedly, transgenic mice that expressed the

Cre recombinase under the control of the rat nestin [8] promoter and its second intron enhancer were suitable for the Cre–*loxP*-mediated generation of mosaic mice; however, in mice expressing β-galactosidase under the same regulatory elements, reporter activity was restricted to the developing nervous system [9].

In the present work, we first assess the validity of this approach in a situation where clear predictions can be made with regard to the ability of mutant cells to contribute to different cell lineages. This is the case for mice deficient in the γ chain of the interleukin-2 receptor (IL-2Rγ) [10]. IL-2Rγ is the common subunit of cytokine receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 [11]. In IL-2Rγ-deficient mice T- and B-cell development is severely impaired, there is a 25-fold reduction in the number of thymic cells, and a 10-fold reduction in the number of T and B cells in peripheral lymphoid organs; in addition, natural killer (NK) cells, T cells expressing the γδ T-cell receptor and intra-epithelial lymphocytes are undetectable [11]. In humans, mutation of the gene encoding IL-2Rγ results in X-linked severe combined immunodeficiency (XSCID) [12]. In the T, B and NK cells from female carriers of XSCID, the non-mutant X-chromosome is used as the active X [13].

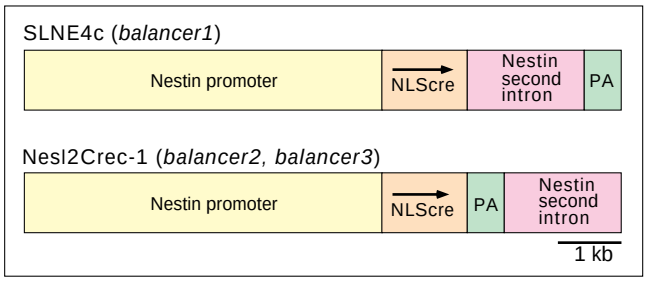
In contrast to IL-2Rγ, the effects of DNA polymerase β (Polβ) deficiency *in vivo* are not well understood. Polβ is a ubiquitously expressed DNA repair enzyme [14] and mice deficient in Polβ are not viable [2]. Polβ-deficient embryos are small than their littermates and die perinatally for unknown reasons (G. Texido, unpublished observations; [15]). Fibroblast cell lines established from Polβ-deficient embryos are more sensitive to DNA-alkylating agents than wild-type cells, and are defective in uracil-initiated base excision repair [15]. By generating and analyzing mosaic Polβ-deficient animals we wanted to bypass the lethal phenotype and find out for which cell lineages Polβ function is critical.

Results

Generation of nestin–cre transgenic lines

Originally, we intended to generate a nestin–cre transgenic mouse for the deletion of *loxP*-flanked target genes specifically in the developing central nervous system (CNS). We therefore established nestin–cre transgenic lines using the constructs NesI2Crec-1 and SLNE4c (Fig. 1; see Materials and methods for details). The expression control elements, the rat nestin promoter and second intron enhancer, had previously been shown to be active only in the developing CNS [9]. We obtained seven transgenic founders, which gave rise to three independent nestin–cre transgenic lines — *balancer1* (*bal1*; SLNE4c construct), *balancer2* (*bal2*; NesI2Crec-1 construct) and *balancer3* (*bal3*; NesI2Crec-1 construct) — each with one head-to-tail transgene integration cluster containing three to five copies of the transgene, as assessed by Southern blot analysis (not shown).

Figure 1



Constructs used to generate transgenic mice. In SLNE4c (top), Cre is expressed under the control of the rat nestin promoter and its second intron enhancer, followed by a SV40 polyadenylation sequence (PA). The transcriptional orientation of the cre recombinase gene containing a nuclear localization signal (NLS) is depicted by an arrow. In NesI2Crec-1 (bottom), the polyadenylation signal precedes the second intron enhancer.

In nestin–cre transgenic *polβ^{flox}*/+ mice, Cre-mediated deletion is detectable in all organs examined

Cre expression was analyzed by crossing nestin–cre transgenic mice to mice carrying *loxP*-flanked alleles of the gene encoding Polβ (*polβ^{flox}*) [2], producing nestin–cre transgenic *polβ^{flox}*/+ mice. This allele can be inactivated by Cre-mediated deletion of its promoter and first exon, producing *polβ^{Δ}*. We prepared DNA from various organs from nestin–cre transgenic *polβ^{flox}*/+ mice and used Southern blotting to determine the percentage deletion of the *loxP*-flanked allele, as described [2,3]. The results of this quantification are expressed as the deletion index ((intensity of band representing deleted allele / (intensity of band representing *loxP*-flanked allele + intensity of band representing deleted allele)) × 100) (Table 1). In *bal1;polβ^{flox}*/+ mice, deletion of the *loxP*-flanked allele of *polβ* was detectable in all organs examined (Fig. 2). The highest deletion indices were seen in brain, testis and gut; the lowest index was in liver (Table 1). The variability in the deletion index between individuals can be seen in Figure 3. In *bal2;polβ^{flox}*/+ mice, Cre-mediated deletion occurred to about the same extent in all organs examined (Fig. 2), except for testis, where the deletion index was much higher, and liver, where some individuals had a reduced deletion index (Table 1). The deletion index varied considerably between individuals (Fig. 3). We analyzed a single *bal3;polβ^{flox}*/+ mouse and found that Cre-mediated deletion was again detectable in all organs examined (Table 1), and that the highest deletion indices were in the brain and testis. CNS-specific deletion of the *loxP*-flanked target genes did not occur in any of the nestin–cre transgenic lines. The mice are, however, suitable for the generation of mosaic mice by Cre–*loxP*-mediated recombination.

Table 1

Deletion index in nestin–cre transgenic *polβ*^{flox}/+ mice.

Line	*bal1*	*bal1*	*bal1*	*bal2*	*bal2*	*bal2*	*bal2*	*bal2*	*bal2*	*bal3*
Mouse	10m	132-6	135-2	159-3	130b3	11-1	12b2	61b2	142b	171–1
Adipose tissue	82	n.d.	n.d.	78	78	52	57	59	19	88
Bone + marrow	n.d.	n.d.	69	83	57	n.d.	45	46	23	75
Brain cortex	87	89	84	69	70	49	46	44	24	87
Cerebellum	98	100	95	66	66	55	54	50	40	96
Ear conch	79	66	60	77	55	n.d.	42	n.d.	14	79
Gut	93	94	92	70	62	49	34	35	14	74
Heart	53	47	47	n.d.	52	49	35	30	16	73
Kidney	75	65	71	71	59	54	49	48	27	71
Liver	33	27	45	56	57	59	17	21	13	67
Lung	83	76	72	79	63	55	44	42	28	70
Midbrain	90	82	85	73	64	51	45	44	25	89
Muscle	80	80	79	85	64	47	52	50	21	78
Pancreas	68	51	55	81	49	45	40	24	16	68
Skin	76	47	68	82	57	38	26	49	23	70
Spleen	75	74	66	79	66	48	47	47	30	79
Stomach	48	49	52	76	69	51	49	41	24	77
Tail	71	57	63	82	53	51	43	41	15	78
Testis	97	100	n.d.	99	87	n.d.	82	66	n.d.	91
Thymus	72	72	63	77	60	49	49	49	29	68
Uterus	n.d.	n.d.	72	n.d.	n.d.	48	n.d.	n.d.	22	n.d.
Generation	F1	F2	F2	F3	F2	F1	F1	F1	F3	F3

DNA was prepared from various organs from nestin–cre transgenic *polβ*^{flox}/+ mice and the deletion index was determined as described in Materials and methods. Each column shows the data obtained from the organs of one individual. The generation number with respect to the founder is given for each animal analyzed. n.d., not determined.

Deletion of a *loxP*-flanked *polβ* allele is detectable in 10.5 dpc embryos

When does the Cre-mediated target gene deletion take place in nestin–cre transgenic mice? Deletion of the *polβ*^{flox} allele was already detectable in 10.5 dpc (days *post coitum*) embryos with the genotype *bal1*;*polβ*^{flox}/+ or *bal2*;*polβ*^{flox}/+ (see below). The deletion index in extra-embryonic tissue from *bal2*;*polβ*^{flox}/+ 10.5 dpc embryos

Figure 2

Southern blot analysis of Cre-mediated deletion in various organs from a *bal1;polβ*^{flox}/+ mouse (10m) and a *bal2*;*polβ*^{flox}/+ mouse (11-1). The positions of the fragments derived from the *polβ*$^{+}$, *polβ*^{flox} and *polβ*$^{\Delta}$ alleles are indicated. Each lane represents DNA derived from one organ sample. The band derived from the wild-type allele is lower in intensity because of the reduced blotting efficiency of large-sized fragments.

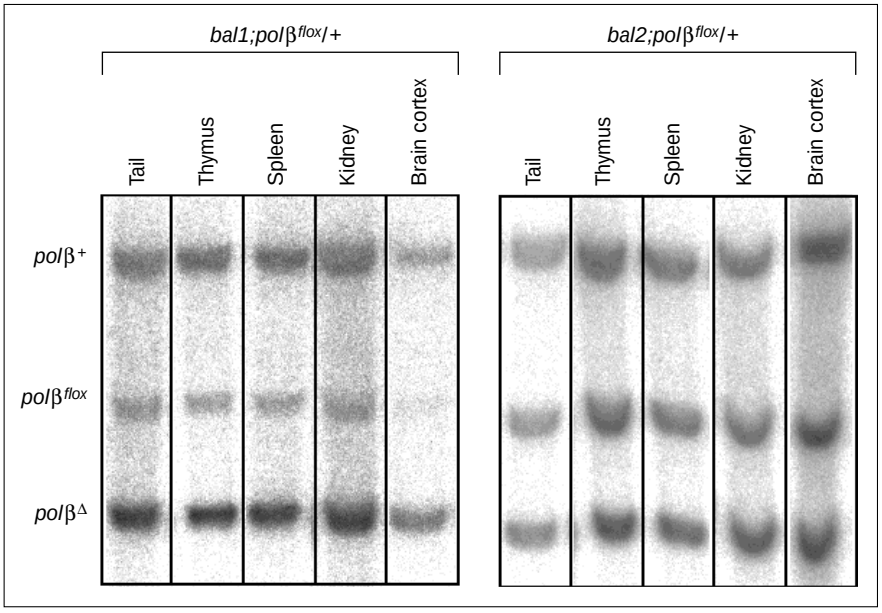
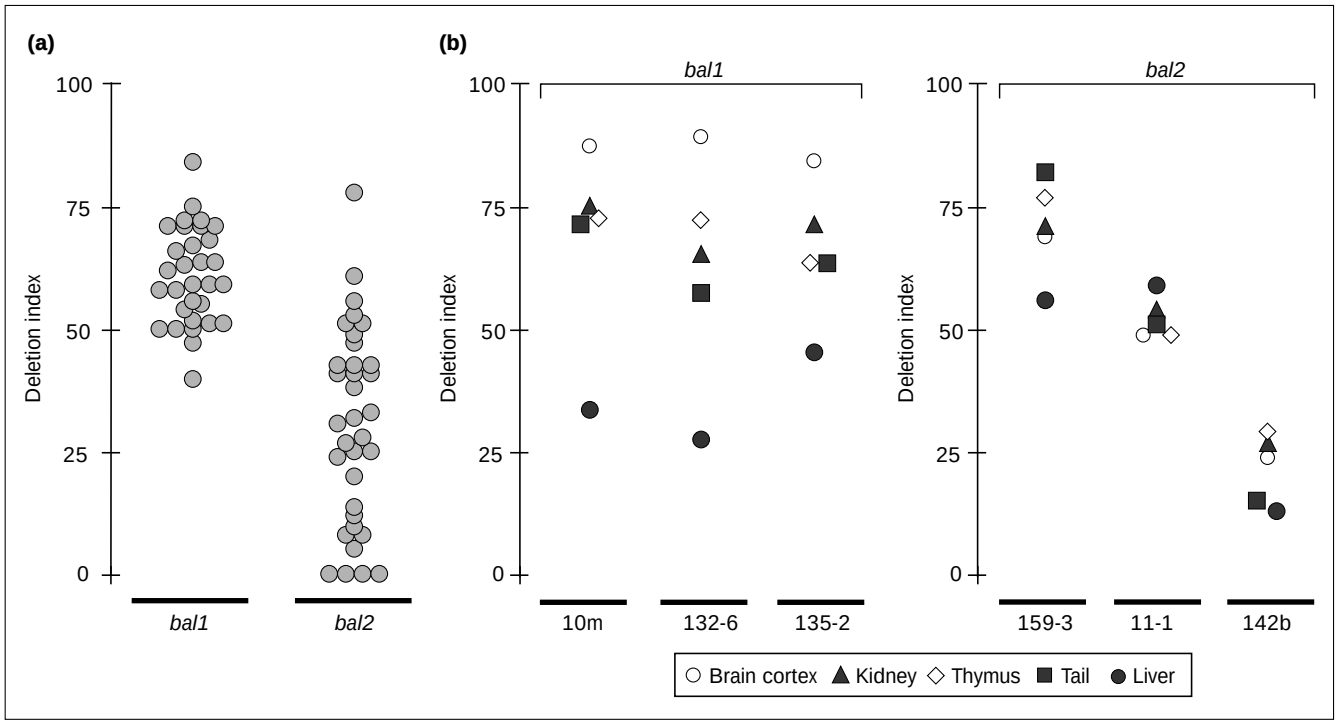


Figure 3



(a) Variation in the deletion index in tail DNAs from various individuals with the *bal1*;*polβ*^{flox}/+ (*bal1*) and *bal2*;*polβ*^{flox}/+ (*bal2*) genotype. DNA was prepared from tail biopsies from various individuals and the deletion index was determined as described in Materials and methods. One circle represents one individual. (b) Diagram showing the deletion index in various organs prepared from three *bal1*;*polβ*^{flox}/+ and *bal2*;*polβ*^{flox}/+ individuals. The individuals from which the organs were taken are shown below (see Table 1).

was about one third of that found in the embryos themselves (data not shown). Polymerase chain reaction (PCR) analysis of individual 3.5 dpc *bal2*;*polβ*^{flox}/+ embryos did not lead to the amplification of the mutant allele generated by Cre-mediated deletion; however, the *loxP*-flanked allele was amplified in the same reaction using the same primers (data not shown). The deletion of a *loxP*-flanked allele therefore starts between 3.5 dpc and 10.5 dpc in *bal2*;*polβ*^{flox}/+ mice.

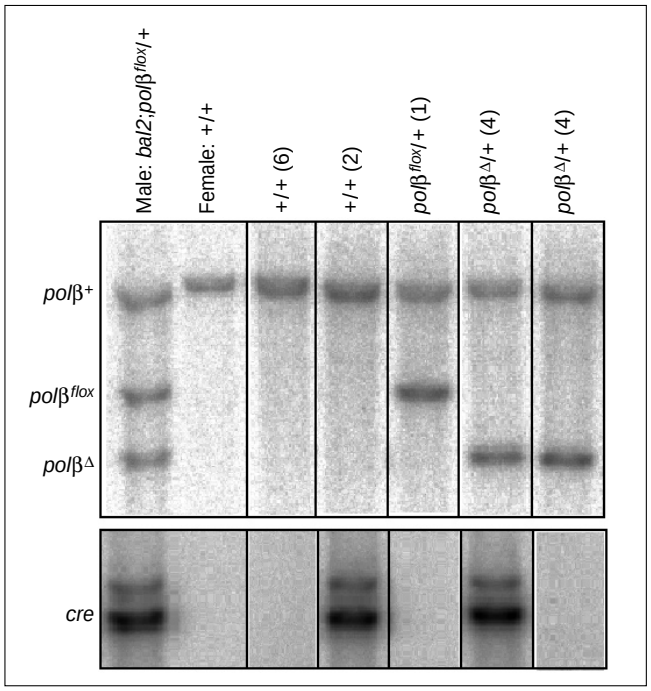
Nestin–cre transgenic mice delete a *loxP*-flanked gene in the germ line

In crosses of male (Fig. 4) and female (data not shown) nestin–cre transgenic *polβ*^{flox}/+ mice to wild-type mice, 16 out of 17 offspring inherited the *polβ*$^{\Delta}$ allele rather than the *polβ*^{flox} allele. Accordingly, nestin–cre transgenic mice delete a *loxP*-flanked target gene in the male and female germ lines. Cre expression in oocytes would lead to a maternal effect — apparent in the progeny of a Cre-positive female. To assess this possibility, we crossed male *polβ*^{flox}/*polβ*^{flox} mice to female *bal1*;+/+ mice. In the 20 F1 offspring tested, deletion of the *loxP*-flanked target gene occurred only in the cre transgenic mice (data not shown). Thus, if there is any Cre protein or Cre-encoding mRNA present in oocytes, it is not sufficient to lead to the deletion of the paternal *loxP*-flanked target gene upon fertilization. Also, reverse transcription PCR (RT–PCR) analysis of RNA prepared from different organs of *bal2* mice, including testis, did not lead to the amplification of a band corresponding to *cre* mRNA, whereas RT–PCR using RNA prepared from individual 10.5 dpc *bal2* embryos did. Actin message, which was used as a positive control for mRNA integrity, was amplified from embryos and the organs of adults in all cases (data not shown). Therefore, Cre is expressed early in ontogeny but could not be detected in the adult *bal2* mouse.

IL-2Rγ-deficient cells are underrepresented in thymus and spleen from *bal2*;*IL-2Rγ*^{flox}/Y mice

bal2 mice were crossed to mice carrying a *loxP*-flanked allele of the X-linked gene encoding IL-2Rγ (*IL-2Rγ*^{flox}; [10] and C.V., J. DiSantoa and W.M., unpublished results). This allele can be inactivated by the Cre-mediated deletion of exons 2–6. Cre-mediated deletion occurred in all organs examined from *bal2*;*IL-2Rγ*^{flox}/+ females to about the same extent (Table 2). In *bal2*;*IL-2Rγ*^{flox}/Y males, Cre-mediated deletion generated *IL-2Rγ*-deficient cells during mouse ontogeny. In *bal2*;*IL-2Rγ*^{flox}/Y mice, the deletion index in thymus, spleen and, to a lesser extent, bone marrow was reduced compared with other organs (Fig. 5),

Figure 4



A *bal2*;*polβ*^{flox}/+ male was crossed to a wild-type female and tail DNA from the offspring was analyzed by Southern blotting as described in Materials and methods. The blot was stripped and rehybridized with a *cre* DNA probe. The genotypes of the offspring (and the number of pups with that genotype) are shown above the lanes. It can be seen that the *loxP*-flanked allele was efficiently deleted in the germ-line cells of the male animal.

Table 2

Deletion index in *bal2*;*IL-2Rγ*^{flox}/+ and *bal2*;*IL-2Rγ*^{flox}/Y mice.

Genotype	*IL-2R*^{flox}/Y	*flox*/Y	*flox*/+	*flox*/+
Bone marrow	53	75	64	87
Brain cortex	82	100	78	95
Cerebellum	74	90	72	95
Ear conch	60	85	67	n.d.
Gut	67	87	60	n.d.
Heart	84	83	59	81
Kidney	77	98	70	100
Liver	60	80	50	96
Lung	74	99	61	87
Midbrain	82	100	85	96
Muscle	77	83	76	100
Pancreas	77	82	45	93
Skin	66	100	73	90
Spleen	37	39	69	88
Stomach	88	85	n.d.	97
Tail	69	83	66	90
Testis	62	100	n.d.	n.d.
Thymus	0	5	64	84
Uterus	n.d.	n.d.	65	99
Sex	Male	Male	Female	Female
Generation	F2	F2	F2	F2

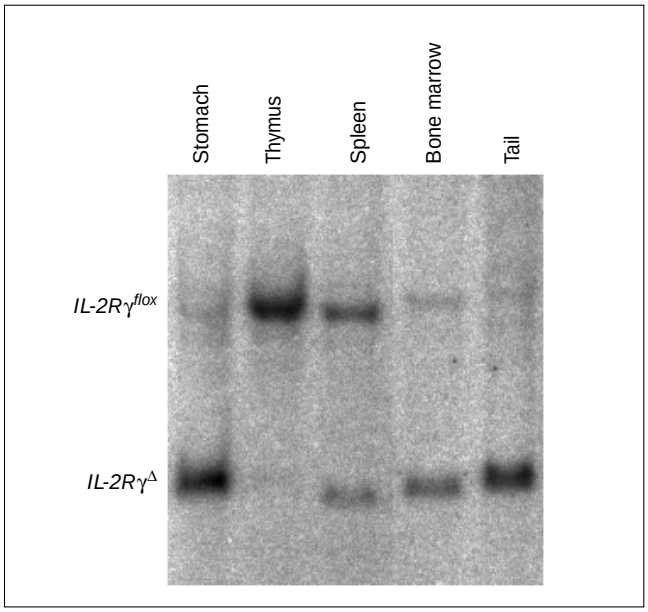
DNA was prepared from various organs from *bal2*;*IL-2Rγ*^{flox}/Y male mice and *bal2*;*IL-2Rγ*^{flox}/+ female mice and the deletion index was determined as described in Materials and methods. Each column shows the data obtained from the organs of one individual. The generation number with respect to the founder is given for each individual analyzed. n.d., not determined.

indicating that *IL-2Rγ*$^{\Delta}$/*Y* cells were underrepresented in these lymphoid organs (Fig. 6a and Table 2). Thus, IL-2Rγ-deficient cells did not efficiently contribute to the lymphoid lineage, a finding that is in accordance with the phenotype of IL-2Rγ-deficient mice [10,11]. Likewise, a slight reduction in the deletion index in thymi from *bal2*;*IL-2Rγ*^{flox}/+ female mice (Fig. 6a), can be explained by the generation of IL-2Rγ-deficient cells if the deleted, formerly *loxP*-flanked allele, is located on the active X chromosome. These results show that cell lineages affected by the introduced mutation can be identified in mosaic animals generated by Cre–*loxP*-mediated recombination.

Generation of mosaic Polβ-deficient animals

The Cre-mediated deletion of the *loxP*-flanked allele of *polβ* in nestin–cre transgenic *polβ*$^{\Delta}$/*polβ*^{flox} mice generates *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ cells during embryonic development. According to the results obtained for nestin–cre transgenic *polβ*^{flox}/+ animals, nestin–cre transgenic *polβ*$^{\Delta}$/*polβ*^{flox} individuals should be mosaic, and should contain a certain percentage of Polβ-deficient cells in all organs. *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ embryos die perinatally and, likewise, it was not possible to obtain viable *bal1*;*polβ*$^{\Delta}$/*polβ*^{flox} individuals. Apparently, the fraction of *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ cells in these mosaics was too high for survival. However, in the case of *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} embryos, some animals survived beyond birth and developed to adults. The deletion index (see Materials and methods) in viable newborn *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} pups was low compared with the deletion index in *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} littermates that died perinatally (Fig. 7). Thus, the ability to circumvent lethality seems to be related to the proportion of polβ-deficient cells. The deletion index in all organs from adult *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} mice was strongly reduced compared with *bal2*;*polβ*^{flox}/+ animals (Fig. 6b and Table 3). The reduction was most pronounced in thymus and least pronounced in brain. Many adult *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} mice were reduced in size and weight compared with their littermates (Table 3). The deletion index in the brains of weight-reduced *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} mice was significantly increased compared with normal weight *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} animals (cerebellum $p \leq 0.019$; brain cortex $p \leq 0.050$; midbrain $p \leq 0.001$) (Table 3). The weight reduction is in accordance with the phenotype of *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ embryos, which are reduced in size compared with wild-type and heterozygous littermates (G. Texido, unpublished observations). Apart from the weight reduction, *bal2*; *polβ*$^{\Delta}$/*polβ*^{flox} individuals appeared normal. No increased mortality was observed within nine months and no tumors were

Figure 5



Southern blot analysis of Cre-mediated deletion in various organs from a *bal2;IL-2Rγ^{flox}/Y* mouse. The positions of the fragments derived from the *IL-2Rγ^{flox}* and *IL-2RγΔ* alleles are indicated. Each lane represents DNA derived from one organ sample. The average deletion index in this individual is high compared with the distribution in *bal2;polβ^{flox}*/+ mice (Fig. 3).

detectable in these mice. Histological examination indicated that the liver, gut and kidney appeared to be normal (J. Löhler, unpublished observations).

Polβ-deficient germ cells are functional

It has been shown that *polβ* mRNA is most abundant in testis [14]. As *loxP*-flanked target gene deletion was most efficient in germ cells (Fig. 4), it was tempting to speculate that *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} individuals might be sterile because of a Polβ deficiency in their germ cells. However, we found male and female Polβ-deficient individuals to be fertile in crosses to wild-type mice. Litter size and offspring appeared normal. Of 11 offspring tested, 10 inherited a *polβ*$^{\Delta}$ allele (data not shown). This result demonstrates that Polβ-deficient germ cells are functional.

The proportion of *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ cells in *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} mice decreases during ontogeny

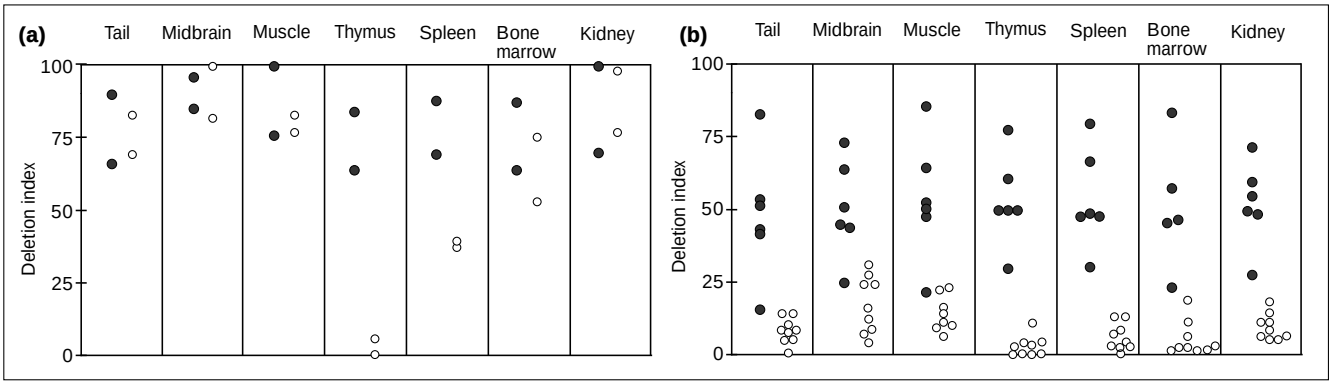
The reduction in the deletion index in *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} compared with *bal2*;*polβ*^{flox}/+ individuals could simply be the result of the perinatal death of all *bal2*; *polβ*$^{\Delta}$/*polβ*^{flox} individuals with a high deletion index. We therefore compared embryos with the genotypes *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} and *bal2*;*polβ*^{flox}/+, with regard to the deletion index detectable in DNA prepared from total embryos at different gestational stages. Although there was a high variation in the deletion index between individual embryos of the same genotype, the average deletion index in *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} embryos was reduced compared with *bal2*; *polβ*^{flox}/+ embryos (Fig. 7). This reduction was much more pronounced in 13.5 dpc and older embryos compared with 10.5 dpc embryos (Fig. 7). As *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ embryos are found in the expected Mendelian ratios up to 18.5 dpc (G. Texido, unpublished observations), this cannot be explained by intrauterine death of mosaic embryos with a high deletion index. We interpret the decreased proportion of Polβ-deficient cells at later stages of development as reflecting a competitive disadvantage of *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ cells in ontogeny.

Discussion

Cre-mediated deletion in nestin–cre transgenic mice is not specific for the CNS

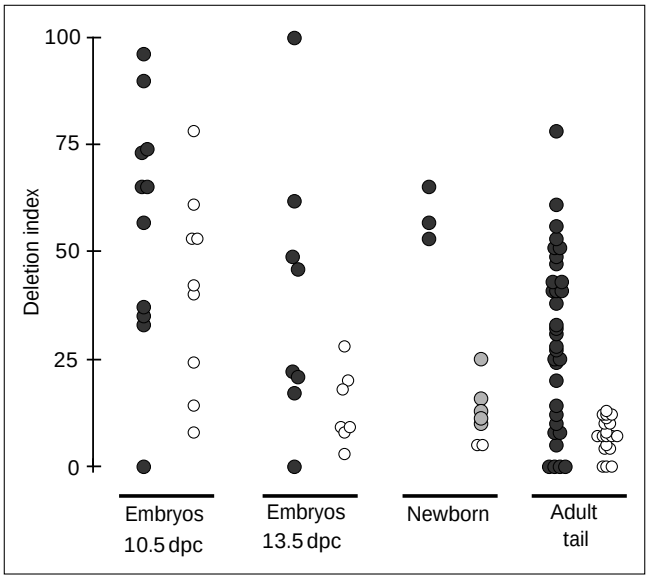
Although transgenic mouse embryos expressing β-galactosidase under the control of the rat nestin promoter and its second intron enhancer contained β-galactosidase

Figure 6



(a) The deletion index in various organs of *bal2; IL-2Rγ^{flox}/Y* males (white circles) and *bal2;IL-2Rγ^{flox}*/+ females (black circles). The deletion index of all organs analyzed is shown in Table 2. (b) The deletion index in various organs of *bal2;polβ^{flox}*/+ (black circles) and *bal2;polβ*$^{\Delta}$/*polβ^{flox}* (white circles) individuals. The deletion index for all the organs analyzed is shown in Tables 1 and 3.

Figure 7



The deletion index in *bal2;polβ^{flox}*/+ (black circles) and *bal2; polβ^{Δ}/polβ^{flox}* (white circles) individuals at different developmental stages. The deletion index remained virtually unchanged in *bal2;polβ^{flox}*/+ embryos but dropped in *bal2; polβ^{Δ}/polβ^{flox}* embryos. Grey circles denote dead *bal2;polβ^{Δ}/polβ^{flox}* newborn mice.

activity exclusively in the developing CNS [9], we found that in three independent transgenic lines expressing *cre* under the same control elements, Cre-mediated deletion of a *loxP*-flanked target gene was detectable in all tissues examined (Fig. 2 and Table 1). However, crossing nestin–cre transgenic mice to animals carrying a *loxP*-flanked gene segment allows a comprehensive analysis of transgene expression because all cells whose progenitors have gone through a stage where Cre was expressed carry an inheritable marker generated by the Cre-mediated deletion of the *loxP*-flanked gene segment. It is not yet clear whether the ubiquitous Cre-mediated deletion that we observe corresponds to the ubiquitous expression of the intermediary filament protein nestin in early embryos. Nevertheless, this result demonstrates that expression patterns determined by conventional techniques may not hold true if tested using the Cre–*loxP*-mediated recombination technique in transgenic mice.

Comparison of mosaic mice generated by Cre–*loxP*-mediated recombination with chimeric mice generated from homozygous mutant ES cells

As shown in this paper, mosaic mice generated by Cre–*loxP*-mediated recombination using nestin–cre transgenic animals can be used to bypass lethality and to identify cell lineages affected by the introduced mutation. An

Table 3

Deletion index in *bal2;polβ^{Δ}/polβ^{flox}* mice.

Mouse	92*	87b3*	178-1	149-1	160-6	178-5	142-2	148b4	148b1
Adipose tissue	6	24	6	5	n.d.	11	5	0	5
Bone + marrow	2	6	19	2	1	11	2	1	1
Brain cortex	8	20	34	23	13	12	7	2	0
Cerebellum	38	20	24	19	7	15	10	1	4
Ear conch	0	8	10	15	11	11	5	8	5
Gut	10	6	13	7	7	9	0	6	10
Heart	6	28	22	31	9	17	3	6	12
Kidney	6	14	18	8	11	11	5	5	6
Liver	0	6	15	18	8	25	1	3	7
Lung	6	16	26	9	9	11	13	8	10
Midbrain	24	24	30	27	12	15	8	4	7
Muscle	10	16	24	n.d.	14	23	9	6	11
Pancreas	0	10	19	8	4	8	2	2	8
Skin	0	n.d.	18	13	n.d.	14	2	7	4
Spleen	0	8	13	4	3	13	2	2	7
Stomach	10	16	20	15	9	12	7	8	11
Tail	0	8	14	10	8	14	7	5	5
Testis	n.d.	64	n.d.	n.d.	48	n.d.	n.d.	1	52
Thymus	0	0	10	4	3	4	0	0	3
Uterus	0	n.d.	6	4	n.d.	7	6	n.d.	n.d.
Weight	Reduced	Reduced	Reduced	Reduced	?	Normal	Normal	Normal	Normal
Generation	F2	F2	F3	F3	F3	F3	F3	F3	F3

DNA was prepared from various organs from *bal2; polβ^{Δ}/polβ^{flox}* mice and the deletion index was determined as described in Materials and methods. Each column shows the data obtained from organs of one individual. Weight-reduced animals are indicated; the question mark indicates that no sex-matched littermates were available for comparison. The generation number with respect to the founder is given for each individual analyzed. *Genotype provided that the *polβ^{flox}* allele was deleted in the germ line of the *bal2;polβ^{flox}*/+ parent. n.d., not determined.

alternative approach is to determine the contribution that homozygous mutant ES cells make to different tissues in a chimeric mouse [16–19]. ES cell chimeras, however, often show a bias in the ability of the ES cells to contribute to different cell lineages [20,21], regardless of the mutation introduced. This might be because genetic or epigenetic modifications acquired during ES cell derivation or maintenance can affect the developmental potential of normal ES cells. Strain incompatibilities between ES cells and blastocysts [22] might also play a role. In contrast, Cre–*loxP*-mediated gene inactivation generates mutant cells whose sole difference compared to wild-type cells is the designed gene deficiency. Finally, this approach is mandatory in cases where homozygous mutant ES cells cannot be obtained, because the gene in question is essential for the ES cells themselves.

Pol β-deficient cells have a competitive disadvantage compared with wild-type cells

The proportion of Polβ-deficient cells decreased in *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} embryos after 10.5 dpc, demonstrating the general importance of Polβ for multiple cell types. A disadvantage of Polβ-deficient compared with wild-type cells can also explain the results obtained with mice that specifically lack Polβ in T cells (*cre*^{lck};*polβ*$^{\Delta}$/*polβ*^{flox}) [2]. The *cre* transgene in these mice is driven by the *lck* proximal promoter which is active only at early stages of T-cell development. The deletion index observed in splenic T-cells from *cre*^{lck};*polβ*$^{\Delta}$/*polβ*^{flox} mice was significantly lower than in the same cells from *cre*^{lck};*polβ*^{flox}/+ animals. The competitive disadvantage of Polβ-deficient cells could result from a delayed cell cycle or an increased susceptibility to cell death resulting from the accumulation of mutations or other structural alterations in the DNA. In this respect it is interesting to note that the underrepresentation of Polβ-deficient cells in mosaic mice was most obvious in thymus and least obvious in brain (Fig. 6b). This might be related to the fact that continuing cell proliferation and apoptosis play a dominant role in thymus, but not in brain.

Many adult *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} mice (Table 3), and all *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ embryos (G. Texido, unpublished observations), were reduced in size and weight compared with littermates. Growth retardation and a decrease in body weight can also be induced by X-ray treatment of embryos after 6.5 dpc; irradiation treatment at earlier stages has no effect on the body weight of adult mice because of compensatory growth [23,24]. Compensatory growth is obviously not sufficient in *bal2*;*polβ*$^{\Delta}$/*polβ*^{flox} and *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ mice. This argues that the cell loss resulting from the reduced proliferation or increased cell death of Polβ-deficient cells occurs after 6.5 dpc in mosaic and *polβ*$^{\Delta}$/*polβ*$^{\Delta}$ mice. Compromized DNA repair (Polβ-deficiency) and exposure to mutagenic stimuli (irradiation) may well result in the same growth-inhibiting effect.

Future applications for nestin–cre transgenic mice

Apart from being able to delete a *loxP*-flanked gene in the germ line (see also [25]), nestin–cre transgenic mice can be used to generate mosaic individuals, consisting of wild-type and mutant cells, whose proportions vary between individuals. On one hand, this variability and instability, particularly in the *bal2* line (Fig. 3), creates a potential problem — to maintain that line, deletion indices have to be monitored so that individuals in which Cre-mediated deletion of *loxP*-flanked target genes does not occur can be eliminated. On the other hand, the variability of the *bal2* line is a welcome feature of the system if the gene of interest is vital and mosaic individuals are used to bypass lethality. In this case, it is desirable to generate many mosaic individuals carrying different proportions of mutant cells, as the percentage of mutant cells that can be tolerated without causing lethality is unpredictable for a given mutation. In the mosaic animals, cell lineages can be identified for whose development a given target gene is critical. Studies of chimeric mice showed that absence of mutant cells in certain tissues can result from, for example, a block in differentiation [17], impaired migration [18] or a competitive disadvantage [19]. As Cre-mediated deletion in nestin–cre transgenic mice takes place early in development, the function of genes in embryonic development and adulthood can be assessed. This approach is not limited to vital genes, and in many cases it might be interesting to compare the phenotypes of homozygous mutant and mosaic mice. For example, it is conceivable that, in mosaic animals, mutant cells will be able to develop into a certain cell type, although that cell type is absent in homozygous mutant mice. This would be the case if the cell type itself is not directly affected by the mutation and its absence in the homozygous mutant mice is caused by secondary effects. Likewise, it is conceivable that the presence of competing wild-type cells will reveal deficiencies in mutant cells which remain unrecognized in homozygous mutant mice.

Materials and methods

Construction of pNesI2Crec-1

The *lacZ* gene from the plasmid pNesPlacZ/3introns [9] was replaced by a polylinker containing *Xho*I, *Eco*RI and *Nhe*I sites, generating the plasmid pNes/vector (U. Lendahl, unpublished results). A 1.4 kb fragment, which contains the modified coding region of *cre*, including a nuclear localization signal [3,26], was isolated from pTZ19RcreNLS-1 and inserted into the *Sal*I site of pNES/vector, generating pNEScre. A 1.8 kb fragment encompassing the second intron and adjacent exon sequence from the rat nestin gene was PCR-amplified from the plasmid pNesPlacZ/3introns using the primers 5′–ATCCTCGAGAAAGCCAAGAGAAGCCT–3′ and 5′–ATCCTCGAGATCCTGGAAGGTGGGCA–3′, and inserted in the *Xho*I site of pNEScre. A fragment isolated by *Sma*I and *Nhe*I digestion (NesI2Crec-1) was used to inject the pronuclei of fertilized oocytes (Fig. 1).

Construction of pSLNE4c

pNes/vector was digested with *Bst*YI and a 1.8 kb fragment containing the second intron and adjacent exon sequence of the nestin gene was isolated and ligated into the *Bam*HI site of pGC1 [27], regenerating a *Bam*HI site upstream of intron 2, and creating convenient restriction sites. The 600 bp SV40 polyadenylation sequence of pNes/vector was removed by *Sal*I and

*Xho*I and inserted downstream of the second intron of the nestin gene, using the *Sal*I and *Xho*I sites present in pGC1, thereby generating pGC1UB2. A 5.8 kb *Not*I (blunt-ended) *Sal*I fragment containing the nestin promoter was cut out of pNes/vector and ligated between the *Eco*RI (blunt-ended) and *Sal*I sites of pSL1190 (Pharmacia, Uppsala, Sweden), generating pSLNE1. A 1.4 kb fragment, which contained the modified coding region of *cre,* including a nuclear localization signal [3,26], was isolated from pTZ19RcreNLS-1 and inserted into the *Sal*I site of pSLNE1. The plasmid was then cut with *Nhe*I (blunt-ended) and *Xho*I, and a 2.4 kb fragment, generated by *Bam*HI (blunt-ended) and *Xho*I digestion of pGC1UB2, was inserted. After the internal *Sma*I site had been deleted, a fragment isolated by digestion with *Sma*I and *Xho*I (SLNE4c) was used to inject the pronuclei of fertilized oocytes.

Generation of nestin–cre transgenic mice

Mice were maintained in standard conditions and provided with food and water *ad libitum*. Transgenic mice were produced by injecting the pronuclei of fertilized (C57BL/6 × CBA) F2 eggs [28]. The presence of the *cre* transgene was detected by PCR analysis (40 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min) of mouse tail DNA, using the primers 5′–TAATCGCCATCTTCCAGCAG–3′ and 5′–CAATTTACTGACCGTACAC–3′ in PCR buffer (GibcoBRL, Eggerstein, Germany) containing 1.5 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase; the PCR resulted in a band of about 1 kb. Subsequently, transgenic mice were characterized by digesting mouse tail DNA with *Bam*HI (Nesl2Crec-1) or *Xba*I (SLNE4c), which was subjected to Southern blotting and hybridization [29] with a *cre* DNA probe. Six transgenic founders for Nesl2Crec-1 and one transgenic founder for SLNE4c were identified. Only three out of seven transgenic founders produced nestin–cre transgenic lines. In the *bal2* and *bal3* lines, some transgenic mice developed a severe hydrocephalus and survived at most four weeks after birth. Occasionally, individuals which had lost some copies of the transgene emerged. No strict correlation between a reduced deletion index and the loss of transgene copies could be found. These individuals, however, were not used for further crossings. In the *bal1* line, we observed a slightly increased mortality, which had no obvious explanation.

Determination and quantification of Cre-mediated deletion

DNA was prepared from various organs of transgenic mice (the brain was split in three parts, referred to as cerebellum, brain cortex and midbrain) or from total embryos [28,30] and subjected to Southern blot analysis as described [2,3,10]. Quantification of band intensity was carried out using a Bio-Imaging analyzer (Fuji Bas1000, Fuji Photo Film Co., Japan) and lPLabGel software (Signal Analytics Corporation, Vienna, Virginia). Window size-corrected background radioactivity was subtracted as measured between the two respective bands. Comparatively, quantification was carried out by calculating the background-subtracted peak intensity in a profile plot (lPLabGel). The extent of Cre-mediated deletion was determined by comparing the intensity of the band representing the Δ allele to the intensity of the band representing the *flox* allele. For *flox*/+ mice, the deletion index was calculated as follows: deletion index = (intensity of band representing deleted allele / (intensity of band representing *loxP*-flanked allele + intensity of band representing deleted allele)) × 100. For *Δ/flox* mice, the deletion index was calculated as follows: deletion index = (((intensity of band representing deleted allele / (intensity of band representing *loxP*-flanked allele + intensity of band representing deleted allele)) × 100) – 50) × 2. For *IL-2Rγ*^{flox}/+ females, the deletion index was calculated as follows: deletion index = ((2 × intensity of band representing deleted allele) / (intensity of band representing *loxP*-flanked allele and wild-type allele + intensity of band representing deleted allele)) × 100. This was necessary because in this case the band representing the wild-type allele and the band representing the *loxP*-flanked allele could not be efficiently separated.

To exclude the possibility that Cre-mediated deletion takes place *in vitro* during the DNA isolation procedures, a spleen from a CD19–cre mouse [31], which contains active Cre recombinase in B cells, was homogenized together with a spleen from a *lacZ*^{flox} transgenic mouse (A. Ayral and F. Sablitzky, data not shown) and the mixture was subjected to the DNA isolation procedure. PCR analysis using primers specific for the *lacZ* gene failed to amplify a band characteristic for the *lacZ*^{Δ} allele but readily amplified the *lacZ*^{flox} allele in the same reaction. In DNA prepared from nestin–cre transgenic *lacZ*^{flox} mice, the primers were able to amplify the *lacZ*^{flox} and *lacZ*^{Δ} alleles simultaneously. Therefore no Cre-mediated deletion occured during the DNA isolation procedures *in vitro*.

Statistics and weight reduction

The significance of the differences in deletion indices in various organs from weight-reduced compared with normal-weight *bal2*; *polβ*^{Δ}/*polβ*^{flox} individuals was calculated using a two-sample two-tailed Student's *t*-test. We identified weight-reduced *bal2*;*polβ*^{Δ}/*polβ*^{flox} mice by monitoring their weight gain and comparing it to their littermates over a period of several weeks.

Acknowledgements

We thank U. Lendahl for the plasmids pNesPlacZ/3 introns and pNes/vector, anti-nestin antiserum and for discussions and advice. We are grateful to J. Löhler for histological examinations, J. DiSanto for *IL-2Rγ*^{flox}/+ ES cells, A. Ayral and F. Sablitzky for providing *lacZ*^{flox} transgenic mice, C. Redies for a Nestin-positive cell line and R. Torres, R. Rickert and A. Tarakhovsky for critical reading of the manuscript. Supported by the Deutsche Forschungsgemeinschaft through SFB 243, the Bundesministerium für Forschung und Technologie (ZMMK), the Land Nordrhein-Westfalen, the Human Frontier Science Program and the EU Biotechnology Program.

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