Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3084–3087, April 1991
Genetics

Creation of a large genomic deletion at the T-cell antigen receptor β-subunit locus in mouse embryonic stem cells by gene targeting

(homologous recombination)

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ABSTRACT **Recently it has become possible to introduce predesigned mutations into a given gene in the mouse germ line by homologous recombination in embryonic stem cells. The mutations are usually introduced by inserting the neomycin phosphotransferase gene into an exon of a particular gene. Here we describe an extension of this method that can result in at least a 15-kilobase-long deletion. The deletion created in the present work encompasses one of the two diversity gene segments of the mouse T-cell receptor β-subunit locus, 10 out of the 12 joining gene segments, and both constant gene segments. This strategy is a valuable alternative to sequential targeting of multiple genes forming a gene cluster, could simplify the construction of plasmids to be used for targeting, and could be the solution for inactivating small genes that have eluded conventional targeting approaches.**

Gene targeting—i.e., homologous recombination between chromosomal DNA and altered input DNA sequences (1–6) in embryonic stem (ES) cells (7–9)—followed by injection of the genetically altered ES cells into blastocysts has proven to be an effective way to create mice with a designed mutation in a given gene (10–17). However, for those genes that are present in multiple copies and in a cluster, an alteration of the phenotype would be expected only after sequential targeting of the multiple gene copies, using different selectable markers. This requires repeated cloning of ES cells, which increases the possibility of overgrowth of aneuploid variants that reduce the germ-line-colonizing potential of the genetically altered ES cells. An alternative approach would be to design an input plasmid in which a selectable marker gene [e.g., the neomycin phosphotransferase (*neo*) gene] is sandwiched by the DNA sequences derived from the flanking regions of the gene cluster. Double homologous recombination in the flanking sequences would result in the replacement of the gene cluster by the selectable gene. In this report, we describe an application of this method to the mouse T-cell receptor (TCR) β-subunit locus. A complete TCR β gene, like immunoglobulin and other TCR genes (18), is generated by somatic rearrangement of gene segments. An inactivation of the TCR β locus requires an alteration involving two homologous C_β (constant) gene segments that are about 7 kilobases (kb) apart (19). Using the replacement approach outlined above, we have generated ES cell lines in which both of these C_β gene segments are deleted from one chromosome.

MATERIALS AND METHODS

Cells. The 129/Ola blastocyst-derived male ES cell line E14 was cultured as described before (20), with the exception that Buffalo rat liver cell-conditioned medium (21) was replaced by medium supplemented with a 1:2000 dilution of conditioned medium from COS-7 cells transfected with pC10-6R cDNA as a source of human leukemia inhibitory factor (LIF; ref. 22; gift from Austin G. Smith and John K. Heath).

Selection Protocol. ES cells ($0.5–1 \times 10^8$ at a time) were electroporated with a Bio-Rad Gene Pulser (800 V, 3 μF; electrode distance, 0.4 cm). The cells were resuspended in 600 μl of phosphate-buffered saline in which had been dissolved beforehand 75–150 μg of linearized plasmid DNA. The electroporated cells were plated in ten 10-cm dishes. G418 (GIBCO) at 200 μg/ml was added 24 hr after electroporation, and ganciclovir [gift from Syntex (Palo Alto, CA)] (23) at 2 μM was added after 4–7 days to most of the plates. Colonies were picked at day 12–14 of selection into wells of 24-well dishes and further expanded.

Plasmid Construction. As a source of germ-line sequences of the TCR β locus we used the cosmid 2.3W7 (ref. 19; gift from Leroy Hood). The fragments flanking the D–J–C (diversity–joining–constant) β gene-segment clusters are, respectively, a 6-kb *Bam*HI fragment upstream of $J_{\beta1.3}$ and a 2.5-kb *Hin*dIII–*Bam*HI fragment downstream of $C_{\beta2}$ (Fig. 1). The 5′ probe was a 0.3-kb *Eco*RI–*Bam*HI fragment, the $C_{\beta2}$ probe was a 0.8-kb *Sac* I fragment, the 3′ probe was a 1.2-kb *Bam*HI–*Sac* I fragment, and the *neo* probe was a 0.65-kb *Pst* I fragment. pgk-neo (24) was a gift from Michael A. Rudnicki (Whitehead Institute, Cambridge, MA), pMCl-tk (23) was obtained from Mario Capecchi, and pBS/tk (25) was a gift from Randall S. Johnson. The vector pPMKO-17 has the herpes simplex virus 1 tk gene, and pPMKO-18 has MC1-tk as well. The vectors were linearized at artificial polylinker sites of the Bluescript (Stratagene) plasmid.

Screening. Individual clones were analyzed by Southern blotting, using *Eco*RI and the 3′ probe. Targeted events were confirmed with the 5′ probe on *Eco*RI-digested genomic DNA. Quantitative hybridization on clone X39 was carried out with the 0.8-kb *Sac* I fragment as a probe for $C_{\beta2}$ and a 0.6-kb *Bam*HI–*Bgl* II genomic fragment as a probe for C_α. The Southern blots were exposed and developed with the use of a Fuji BA 100 Bio-Image analyzer, which allows direct quantitation of the intensity of the radioactive bands from the digitalized data.

RESULTS

The structure of the targeting plasmid used for transfection of E14 ES cells, a map of the TCR β locus of E14 cells, and a map of the relevant region of E14-homologous recombinants (E14-HR) are illustrated in Fig. 1. The lengths of the DNA fragments that would be detected by the hybridization probes (see Fig. 1) upon digestion of the E14 and E14-HR genomic DNA with chosen restriction enzymes are summarized in

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Abbreviations: ES cell, embryonic stem cell; neo, neomycin phosphotransferase; tk, thymidine kinase; TCR, T-cell receptor.

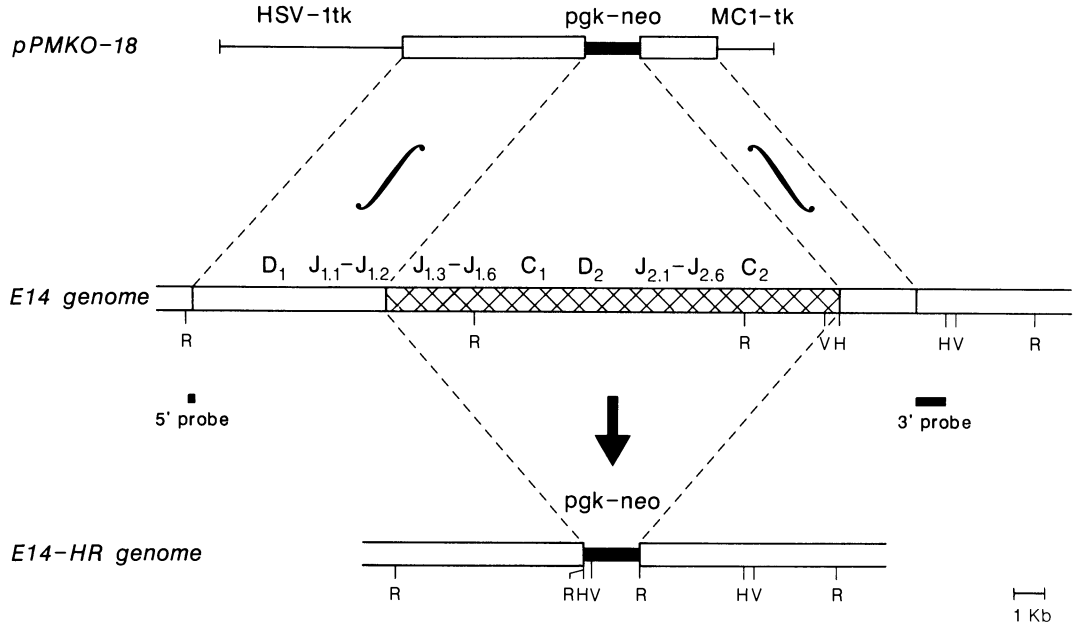

FIG. 1. Schematic diagram of the homologous recombination resulting in a 15-kb deletion. (*Top*) Targeting construct pPMKO-18. The transcriptional orientation of the *pgk-neo* gene is opposite to that of the various gene segments in the target locus. The plasmid is linearized at the *Xho* I site in the polylinker of pBluescript. HSV-1, herpes simplex virus 1; tk, thymidine kinase. (*Middle*) TCR C_β locus in the genome of E14 cells. The cross-hatched area is the region that will be deleted after homologous recombination. The deletion breakpoint at the 5′ end is the *Bam*HI site between the $J_{\beta1.2}$ and $J_{\beta1.3}$ gene segments. Only the relevant restriction sites are indicated. The location of each of the two external probes is indicated by a bar. (*Bottom*) TCR C_β locus in the E14 clones after homologous recombination (E14-HR). R, *Eco*RI; V, *Eco*RV; H, *Hin*dIII.

Table 1. We performed several independent electroporation experiments with pPMKO-17 and pPMKO-18 and obtained transfection efficiencies of 1/5000 to 1/50,000 electroporated cells. The enrichment obtained by negative selection with ganciclovir was at the most 2-fold. We isolated 249 G418-resistant, ganciclovir-resistant E14 clones and screened them individually by Southern blotting using *Eco*RI and the 3′ probe (see Fig. 1 for the location of the probe sequence in the E14 genome).

If homologous recombination had taken place in the 3′ flanking region of the C_β locus in one of the two homologous chromosomes, one would expect a novel 6.5-kb *Eco*RI fragment, in addition to the 9.5-kb band representing the unaltered chromosome (Table 1). In the initial screening we identified five clones that gave this pattern (data for clone X39 are shown in Fig. 2*A*). We then analyzed four of these clones for homologous recombination in the 5′ flanking region by using *Eco*RI and the 5′ probe. Two clones (clones 59 and X39) gave the predicted additional 6.5-kb band (Table 1 and Fig. 2*B*). The two other clones did not show this or any other additional band and were therefore excluded from further analysis.

To confirm that the DNA rearrangement indeed occurred as illustrated in Fig. 1 in clones 59 and X39, we analyzed the hybridization pattern of their DNA by using *Hin*dIII and the 3′ and *neo* probes (Table 1 and Figs. 1 and 3). The novel restriction fragment of 5.3 kb indicative of homologous recombination at the 3′ end hybridized to the *neo* probe. Similarly, the expected pattern was obtained using *Eco*RV and the 3′ and *neo* probes (data not shown). With either enzyme no other bands were observed when the *neo* probe was used, suggesting that no other copies of the plasmid had integrated stably in the genome of these clones. The results of further analysis of the 3′ end with *Pst* I, *Nco* I, and *Hpa* I are consistent with the interpretation that correct homologous recombination had occurred at the 3′ flanking region of the TCR β locus (data not shown). Taken together, these data suggest that the expected modification had taken place in two of the four clones.

For clone X39, we carried out further analysis aimed at demonstrating the deletion of C_β sequences on one of the two homologous chromosomes. This was achieved by hybridizing *Eco*RI/*Hin*dIII-digested E14 and X39 DNA with a mixture of the $C_{\beta2}$ and C_α probes (Fig. 4). The former probe tested whether the intended deletion has taken place and the latter probe served as an internal control for variations in amount of DNA loaded and Southern transfer efficiency. The relative intensity of the $C_{\beta2}$ band was 40% in clone X39 as compared to E14 cells (Fig. 4). As 13 out of 20 metaphase spreads of this clone contained the diploid number, 40, of

Table 1. Lengths of expected restriction fragments of the TCR β locus in E14 cells and in E14 clones with homologous recombination (E14-HR)

	Fragment length, kb						
	3′ probe			5′ probe,	*neo* probe		$C_{\beta2}$ probe,
Cells	*Eco*RI	*Hin*dIII	*Eco*RV	*Eco*RI	*Hin*dIII	*Eco*RV	*Eco*RI/*Hin*dIII
E14	9.5	3.5	4.2	9.5	—	—	3.0
E14-HR	9.5	3.5	4.2	9.5			
	6.5	5.3	5.4	6.5	5.3	5.4	3.0*

—, No fragment expected.
*Intensity reduced by a factor of 2.

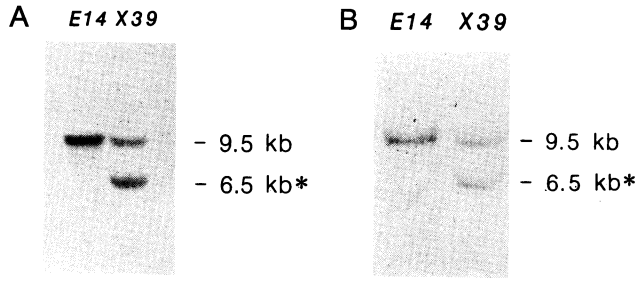

FIG. 2. Southern blot of *Eco*RI-digested genomic DNA of E14 cells and clone X39, hybridized with the 3′ probe (*A*) or with the 5′ probe (*B*). The band representing the allele that has undergone homologous recombination is indicated with an asterisk. Hybridizations were performed on separate blots.

chromosomes of normal gross morphology, this confirmed our expectation that the intensity of the $C_{\beta 2}$ band would be reduced by about half due to deletion of these sequences on one of the two homologous chromosomes.

DISCUSSION

Even with the use of two tk genes as negative selectable markers, we were not able to achieve an enrichment greater than 2-fold. This low enrichment factor is in sharp contrast with the value of up to 2000-fold as reported in refs. 23 and 25. In our experience these low enrichment factors are rather the rule than the exception: with similarly low enrichment, we have been able to target TCR C_α and TCR C_δ, and similar observations were made when the hypoxanthine phosphoribosyltransferase gene and adipocyte P2 gene were targeted with control vectors, pRV9.1/tk and paP2/tk, described respectively in ref. 23 and ref. 25 (P.M., Yoshiaki Ichikawa, Shigeyoshi Itohara, and S.T., unpublished results). The overall targeting efficiency—about 1/250 G418-resistant colonies—is comparable to the values obtained previously for targeted replacement without deletion of sequences (2). It remains to be seen whether larger deletions can be achieved at comparable frequencies.

Previously, small deletions, of up to 2 kb have been reported (4, 15, 26, 28). However, those experiments were not intended to mutate more than one gene at once and the size of the deletion was considerably smaller. In contrast, in this study we have deleted a 15-kb DNA fragment containing one D_β, ten J_β, and both C_β gene segments. A fragment of similar size (12 kb) has been inserted into the hypoxanthine phosphoribosyltransferase gene (27). We have been able to produce a deletion of 4 kb in TCR C_δ at a frequency of about 1/30 G418-resistant clones (P.M., Shigeyoshi Itohara, and S.T., unpublished results). If these two cases can be generalized, the deletion strategy opens a number of interesting applications.

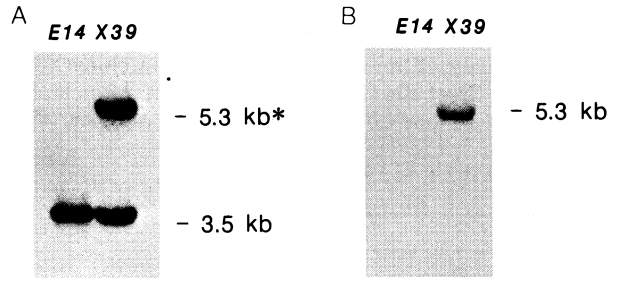

FIG. 3. Southern blot of *Hin*dIII-digested genomic DNA of E14 cells and clone X39, hybridized with the 3′ probe (*A*) or with the *neo* probe (*B*). The band representing the allele that has undergone homologous recombination is indicated with an asterisk. The samples were run in duplicate in the same gel; two separate blots were used for the hybridizations. Note that in *A* the two bands given by clone X39 are of similar intensity.

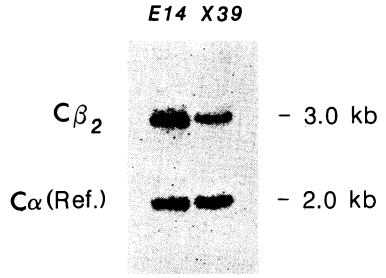

FIG. 4. Southern blot of genomic DNA of E14 cells and clone X39. DNA was digested with *Eco*RI and *Hin*dIII. The blot was hybridized simultaneously with a $C_{\beta 2}$ probe and a C_α probe. The 3.0-kb band represents $C_{\beta 2}$ sequences that are deleted from one chromosome in clone X39. The 2.0-kb band represents C_α sequences and its intensity serves as an internal reference (Ref.) standard.

First, it can be applied to targeting clustered genes serving a related function, such as the major histocompatibility complex, tumor necrosis factor α/β, RAG-1 and RAG-2, globin and homeobox genes. In these instances creating a deletion is preferable to sequential targeting of the individual genes, as it requires the construction of only one plasmid and the application of only one selection procedure. It also has advantages over the approach of introducing mutations in the individual genes independently into the germ line and crossing the resulting mice to obtain mice with mutations in multiple genes, since such recombinant chromosomes will arise only very rarely in crosses when the genes are closely linked. Second, this approach speeds up plasmid construction because it requires only a rough map of the relevant region of genomic DNA and because it does not necessitate inserting the gene into a unique restriction site of a coding region.

Finally, this approach could be the solution for inactivating small genes that are refractory to standard targeting approaches. A large variability in the ease of targeting various loci has been observed (2). One could speculate that those loci that seemingly elude targeting consist of (*i*) sequences of an intrinsically low recombinogenic nature or (*ii*) sequences that silence expression of the *neo* marker. If the gene is large, different parts of the gene can be utilized to construct targeting plasmids. However, when the gene is relatively small, the choices of insertion of the selectable marker can be limited. The alternative to disrupting such a gene by inserting a *neo* marker into an exon is to try to create a partial or complete deletion of the gene by utilizing upstream and downstream sequences that may recombine at higher frequencies or that remove an intervening sequence with a negative effect on transcription of the positive selectable marker. In this way the genomic region surrounding the gene can be scanned until appropriate sequences are included.

We wish to thank Allan Bradley for reviewing the manuscript and the Fuji Film Co. for the use of the BA-100 Bio-Image analyzer. P.M. is a Howard Hughes Medical Institute Predoctoral Fellow in Biological Sciences and partly supported by a short-term fellowship from the Human Frontiers Science Program. This work was supported by grants from the Howard Hughes Medical Institute, the Human Frontiers Science Program, and the National Institutes of Health.

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