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Genetics

Homologous recombination at c-*fyn* locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection

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ABSTRACT **In attempting to produce a mutant mouse with embryonic stem cells, the critical step is the efficient isolation of homologous recombinants; the frequency of the homologous recombination is usually low and the potency of the cells to differentiate into germ cells is unstable in culture. Here, we report an efficacious method for such isolation in which the diphtheria toxin A-fragment gene is used to negatively select nonhomologous recombinants. In contrast to the use of the herpes simplex virus thymidine kinase gene, the selection can be made singly by the neomycin analog G418 without using a drug such as ganciclovir, a nucleoside analog. At the c-*fyn* locus, the diphtheria-toxin negative selection enriched the recombinants about 10-fold, and half of the cells integrating with the neomycin phosphotransferase gene were homologous recombinants.**

Embryonic stem cells (ES cells) cultured from 4-day embryos have been successfully used to establish mutant mice (1–7). The leukemia inhibitory factor (LIF) has greatly improved their stable culture (8, 9), but the germ-line colonization of the ES cells is still a critical matter in this system. Since the frequency of homologous recombination is on the order of 10^{-3} to 10^{-4} among nonhomologous recombinants (10), a long period of culture is required to identify the recombinants by the standard method utilizing the polymerase chain reaction (PCR) (3, 11). Several efforts to minimize the interval of culture have been reported, the most sophisticated among them being the use of herpes simplex virus thymidine kinase gene (*tk*) for the negative selection of nonhomologous recombinants (12). The use of a nucleoside analog such as ganciclovir for the selection, however, has the potential of causing random mutations. Negative selection that works at the translational level, as positive neomycin selection does, is preferable to avoid this problem. A second proposed means of enriching the homologous recombinants is the use of the neomycin phosphotransferase gene (*neo*) in the absence of the transcriptional regulatory unit; the *neo* gene is expressed as the fusion gene directed by the transcriptional regulatory sequences of the target gene when homologously integrated (5). However, the extent of the enrichment is not yet high enough with this strategy. In this paper, we report the efficient isolation of homologous recombinants at the c-*fyn* locus through use of the diphtheria toxin A-fragment (DT-A) gene for the negative selection.

MATERIALS AND METHODS

Isolation of Mouse Genomic c-*fyn* Gene. Mouse genomic c-*fyn* DNA was isolated from an EMBL3 library containing DNAs partially digested with *Mbo* I (Clontech) by screening with human *fyn* cDNA [the 256-base-pair (bp) *Sac* I–*Ssp* I fragment of pSN-Mlu I; ref. 13]. A recombinant phage (pFYN15.5) containing the 15.5-kilobase (kb) fragment covering exons 2 and 3 was identified and subcloned into pBluescript (BSK) SK(+) plasmid (Stratagene) after digestion into 4.7-, 4.5-, 3.0-, 1.7-, 0.9-, and 0.7-kb fragments by *Eco*RI. The subclones containing exon 2 (pGFYN3.0) at the *Eco*RI site and exon 3 (pGFYN4.7) at *Eco*RI and *Sal* I sites were sequenced by the dideoxy method of Sanger *et al.* (14).

Construction of the Vector for Homologous Recombination. By use of an oligonucleotide-directed *in vitro* mutagenesis system (version 2, Amersham), the GGATAATG sequence including the translation initiation codon in the second exon was converted into GGATCCTG, thereby disrupting the initiation codon and creating a *Bam*HI site (pGFYN3.0Bm). The 3.0-kb *Eco*RI fragment of pGFYN3.0Bm was then inserted into pGFYN4.7 at the *Eco*RI site, yielding pGFYN7.7Bm. The 5′ side of the *Xba I–Acc I* fragment of the first intron was deleted as follows by changing the *Acc* I site to an *Xba* I site: the *Acc* I–*Bam*HI fragment was separated from pGFYN7.7Bm and ligated with an *Xba* I linker at the *Acc* I site. The resulting 0.8-kb *Xba* I–*Bam*HI fragment was reinserted into pGFYN7.7Bm at the *Xba* I and *Bam*HI sites, yielding pGFYN6.3Bm.

The *neo* gene was obtained from pMC1NeopolyA (ref. 15; referred to as pMC1NeopA here; Stratagene), leaving enhancer and promoter sequences behind by *Mlu* I and *Bam*HI digestions, introduced into pSN-MluI (13) at the *Mlu* I–*Sal* I site, and released as the *Bam*HI fragment. The fragment was inserted into pGFYN6.3Bm and pGFYN7.7Bm at the *Bam*HI site, yielding pHF-NeopA and pHF7.7-NeopA, respectively.

The DT-A gene was obtained from a DT-A cassette (kindly provided by I. H. Maxwell; ref. 16) after digestion with *Pst* I and *Bgl* II leaving the poly(A) signal behind, then combined with the 0.3-kb enhancer–promoter sequences of pMC1NeopA (*Xho* I–*Pst* I) at the polylinker site of the pBSK·SK$^+$ vector, and released as the *Xho* I–*Sal* I fragment. The fragment was introduced into pHF-NeopA at the *Sal* I site, yielding pHF-NeopA-DT. Plasmid pMC1NeopA-DT was constructed by inserting the DT fragment from the cassette into pMC1NeopA at the *Sal* I site, and pMC1NeopA-DTpA by inserting the poly(A) signal of the pMC1NeopA (blunted 0.2-kb *Dde* I fragment) into pMC1NeopA-DT at the *Hinc*II site. For electroporation, these DNAs were linearized by digestion with the enzymes indicated later in the text.

Culture of ES Cells. The ES cells, E14, used throughout the present study were the gift of M. Hooper (17). The cells were

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Abbreviations: DT, diphtheria toxin; DT-A, DT fragment A; ES cells, embryonic stem cells; LIF, leukemia inhibitory factor; PCR, polymerase chain reaction.

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cultured on mitomycin C-treated feeder cells in Dulbecco's modified Eagle's medium (high glucose) supplemented with 20% fetal bovine serum, 0.1 mM 2-mercaptoethanol, nonessential amino acids (Flow Laboratories), 1 mM sodium pyruvate, and LIF (1000 units/ml; kindly provided by M. N. Gough; ref. 8), as described (18, 19). A transgenic mouse line (TGNeo1) was established by injecting pSTNeo (generously provided by H. Kondoh; ref. 20) into mouse zygotes. When available, secondary cultures from 16-day embryos of the transgenic mice served as the source of feeder cells; otherwise, STO-Neo15, a subline of STO cells harboring pSTNeo, served this purpose. Feeder cells were prepared on gelatin-coated dishes by mitomycin treatment (18). Cells cultured for 6 days and 9 days after thawing of the original stock with two and three passages, respectively, were supplied for electroporation. Aliquots of cells from the same culture were kept for 2 months and tested for multipotency at 6–13 days and at 40–50 days in a parallel experiment.

About 10^7 cells were harvested from each flask, suspended in 25 mM Hepes, pH 7.05/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM dextrose with 12 nM linearized DNA, and electroporated at 1.35×10^7 cells per 0.2 ml in a cuvette of 0.4-cm path length with a Bio-Rad Gene Pulser (25 μF, 1.35 kV), yielding 10% cell survival. Then 10^6 cells were seeded per 6-cm dish; G418 (180 μg/ml) was added 48 hr later and cells were selected for 7 days as described (18). Aliquots of the electroporated cells were plated at 1000 cells per dish and cultured in normal medium to assess their viability.

PCR and Southern Analyses. DNA was isolated (21) and 1 μg was dissolved in 100 μl of 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.001% gelatin/0.2 mM each dNTP with 5 units of *Thermus aquaticus* DNA polymerase and 100 pmol of each of the two primers. Primer 1 was derived from the *neo* gene, GAACCTGCGTGCAATCCATCTTGTTCAATG, and primer 2 from the *fyn* sequence that was not included in the targeting vector, GTGATGCACAGCTTCCAGGTCATCCTAGTC. The PCR was carried out (22) for 35 cycles in a thermal cycle (Perkin–Elmer/Cetus). Each cycle consisted of denaturation for 1.5 min at 94°C, annealing for 2 min at 62°C, and extension for 5 min at 72°C. A 20-μl reaction sample was run in a 1% agarose gel by standard procedures. Southern analysis was done as described (21) with the probes and restriction enzymes indicated later in the text.

Determination of Multipotency of the ES Cells. A karyotype of the cells was examined as described (19). Multipotency was determined by histologically examining the subcutaneous grafts of the cells into CD-1 nude mice and by producing chimeric mice after injection into host blastocysts (19). The cells were injected into 4-day embryos from C57BL/6 mice and the embryos were fostered in the uterus of CD-1 pseudopregnant females. Male chimeras were mated with BALB/c females. Mice were purchased from Charles River Breeding Laboratories.

RESULTS

Structure of Mouse Genomic c-*fyn* DNA. The protooncogenes of the *src* family encode proteins consisting of the tyrosine kinase domain, the *src* homology (SH) domain, and a domain unique to each member of the family (23). The unique domain is located at the 5′ side of each gene, and the 15-kb fragment containing this region was identified by screening the BALB/c mouse genomic DNA library with the 5′ portion of human *fyn* cDNA as a probe (13). The 15-kb fragment (Fig. 1*A*) included exon 2 (containing the translation initiation codon ATG) and exon 3, both of which cover the c-*fyn* unique region; the presence or absence of the noncoding first exon was not determined. Amino acid and nucleotide sequences are highly conserved in these exons between human and mouse: 99.1% and 94.3%, respectively (data not shown). The Z-DNA-motif poly[d(GT)] stretch was present at introns 2 and 3. The gene was confirmed to be single-copy in the haploid mouse genome by Southern blot analysis using various restriction enzymes (data not shown).

Strategy of the Selection with the DT-A Gene for Homologous Recombination. To construct the targeting vector, c-*fyn* expression was first examined in the ES cell line E14. The c-*fyn* mRNA was present at approximately the same level as

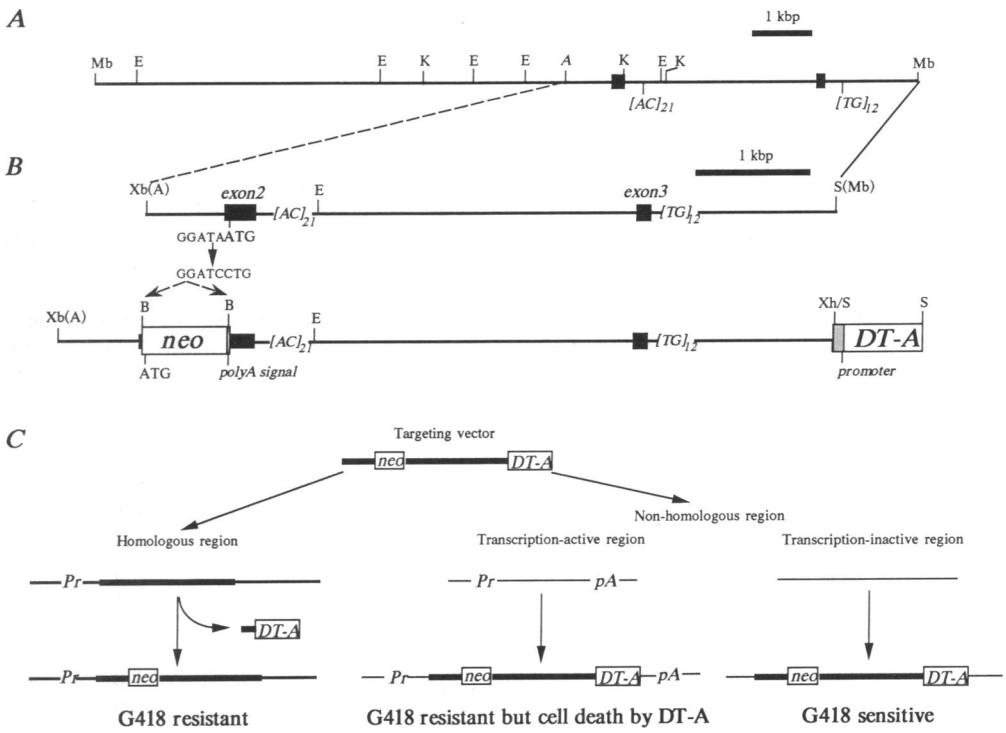

FIG. 1. (*A*) The c-*fyn* gene in pGFN15.5. (*B*) The targeting vector, pHF-NeoA-DT. (*C*) Strategy for selection. *Pr*, transcriptional regulatory sequences; *pA*, polyadenylylation signal; A, *Acc* I; E, *Eco*RI; K, *Kpn* I; Mb, *Mbo* I; S, *Sal* I; Xb, *Xba* I; Xh, *Xho* I; kbp, kilobase pair.

in adult thymus (data not shown). Then the *neo* gene was introduced, without promoter and enhancer sequences, into exon 2, disrupting the ATG initiation codon of the c-*fyn* gene and resulting in pHF-NeopA (Fig. 1*B*). For this, the GGATAATG sequence was mutagenized into GGATCCTG, thereby creating a *Bam*HI site, and the *neo* gene was introduced at this site. The *neo* gene is transcribed only when properly targeted into the gene which is expressed in ES cells. At the same time, for negative selection of random recombinants, the DT-A gene was added at the 3′ end, in the absence of the poly(A) signal, with the transcriptional regulatory unit from the polyoma enhancer and the herpes simplex virus *tk* promoter (15), thus producing our targeting vector, pHF-NeopA-DT (Fig. 1*B*). Fig. 1*C* outlines our experimental design for enriching for rare homologous recombinants with this construction; in nonhomologous recombination, which usually occurs through the ends of the introduced DNA, the DT-A gene will, in most cases, receive the poly(A) signals for its proper transcription when the DNA is inserted in such a way that the *neo* gene is expressed. DT-A expression will then cause the death of these nonhomologous recombinants. When the DNA is integrated into sites that are not expressed in ES cells, the *neo* gene will not be expressed and these random recombinants will not survive the G418 selection. Upon homologous recombination, the DT-A gene is lost and the cells will survive the G418 selection through the expression of the *neo* gene directed by the transcription machinery of the c-*fyn* gene, with translation initiated at the ATG codon of the *neo* gene. Disruption of the ATG codon of the c-*fyn* gene by the *neo* gene in the construct does not yield any new meaningful ATG initiation codon by the end of the third exon. The total length of the homologous region in the targeting vector is 6.3 kb, with 0.8 kb on the 5′ side of the *neo* gene and 5.5 kb on the 3′ side.

Feasibility of the Strategy. The basic question in using the poly(A)-signal-less DT-A gene for negative selection is whether this gene actually causes cell death only when properly integrated into a gene. This question was tested by examining the frequency of G418-resistant cells when pMC1NeopA (15) was introduced into E14 cells in the presence and absence of DT-A sequences. The frequency of G418-resistant cells was 1.5×10^{-6} after electroporation with pMC1NeopA. G418-resistant colonies were obtained at about two-thirds of this frequency ($\approx 10^{-6}$), after E14 cells received pMC1NeopA-DT, which lacks the poly(A) signal at the 3′ end of the DT-A gene and carries the MC1 promoter at the 5′ end of the gene. These results indicate that the DT-A gene does not cause cell death in the absence of the poly(A) signal by leaky expression prior to integration.

Recombination at the c-*fyn* Locus. By electroporating pHF-NeopA-DT (linearized with *Xba* I and *Sal* I) into ES cells, we obtained 16 cells (out of 5×10^8 electroporated cells) in which the *neo* gene had been integrated as shown by selection with G418 (180 μg/ml) over a 7-day period (Table 1). These 16 ES

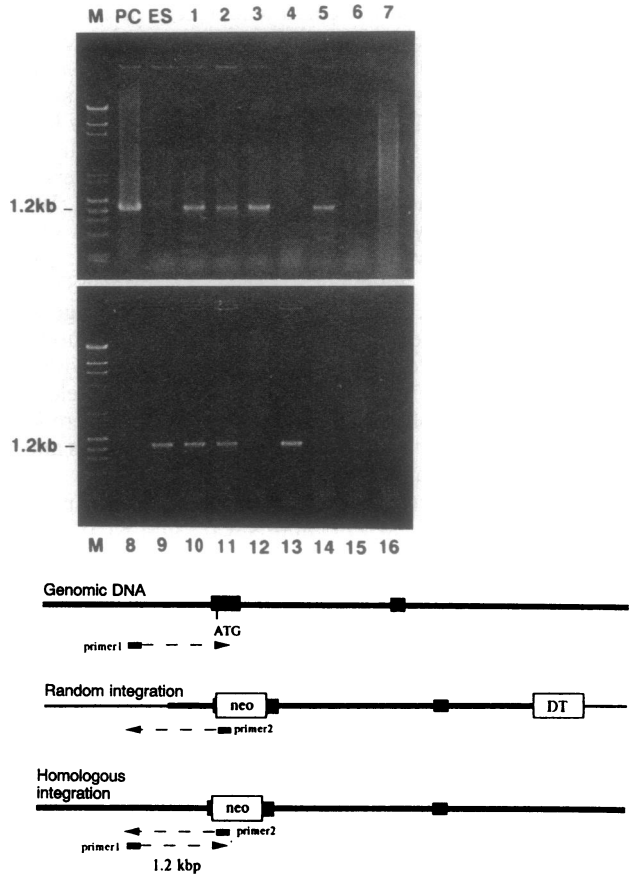

FIG. 2. PCR identification of homologous recombinants. Lanes: M, size markers; PC, E14 cells plus 10^{-6} μg of pHF7.7-NeopA DNA; ES, E14 cells; 1–16, G418-resistant clones. The strategy is shown below the gel.

clones were then screened for homologous recombination by the PCR technique (Fig. 2). A 30-bp sequence within the *neo* gene served as primer 1 and a 30-bp sequence within the first intron of the c-*fyn* gene, which is absent from the introduced DNA, as primer 2. No amplification was expected from the endogenous c-*fyn* gene because of the absence of the *neo* gene. Similarly, no amplification could occur from the nonhomologously integrated transgene because of the absence of the primer 2 sequence. Only the homologous recombinants could be expected to yield the amplification of the 1.2-kb DNA fragment. Among the 16 ES clones isolated above, 8 yielded the amplification and thus could be considered as homologous recombinants. As controls, we used pHF-MC1NeopA, carrying a promoted *neo* gene, and pHF-NeopA (linearized with *Xba* I and *Sal* I), carrying a promoterless *neo* gene. Neither of these plasmids contains the DT-A gene.

Table 1. Frequency of targeting at the c-*fyn* locus

Exp.	Vector*	No. of cells electroporated	No. of *neo*-positive cells	No. of homologous recombinants
1	pHF-MC1NeopA	1×10^8	65	0
	pHF-NeopA	1×10^8	34	0
	pHF-NeopA-DT	1×10^8	4	1
2	pHF-NeopA-DT	2×10^8	6	4
3	pHF-NeopA-DT	2×10^8	6	3
Total	pHF-MC1NeopA	1×10^8	65	0
	pHF-NeopA	1×10^8	34	0
	pHF-NeopA-DT	5×10^8	16	8

*pHF-MC1NeopA, *neo* gene carrying MC1 promoter and poly(A) signal; pHF-NeopA, *neo* gene carrying poly(A) signal without promoter; pHF-NeopA-DT, see Fig. 1*B*.

Electroporation of pHF-MC1NeopA and of pHF-NeopA into E14 cells yielded 65 and 34 ES clones integrating the *neo* gene per 10^8 electroporated cells, respectively (Table 1). None of these yielded the PCR amplification and thus all were considered to be nonhomologous recombinants.

Southern Identification of Recombinants. To confirm the nature of the recombination in one allele of the diploid genome, clones HRfyn-1 and HRfyn-12 were analyzed by Southern blotting (Fig. 3). When DNA from the parent ES cells was digested with *Kpn* I, the *fyn* probe identified a 3.7-kb fragment; the patterns of HRfyn-1 and HRfyn-12 were different, with additional fragments of 4.5 kb and 7.1 kb, respectively. In homologous recombinants, since the *neo* gene has no *Kpn* I site, the size of the genomic *Kpn* I fragment should be increased 0.8 kb by the *neo*-gene insert in the recombined allele. In nonhomologous recombinants, since the sequences containing the 5′ *Kpn* I site are eliminated in the targeting vector, the size of the fragment depends on the *Kpn* I site that happens to locate at the 5′ side of the integration site, and is >2.4 kb. These fragments should also be identified with the *neo* probe, while the normal c-*fyn* allele should not be identified by this probe. The results of the analysis were precisely as expected for HRfyn-1 as a homologous recombinant and for HRfyn-12 as a nonhomologous one. On the other hand, the DT-A probe was expected to isolate a fragment in nonhomologous recombinants when the transgene was integrated through the ends. Since the transgene does not contain the *Kpn* I site at the 3′ side of the DT-A gene, the size of the fragment depends on the *Kpn* I site that happens to locate at the 3′ side of the DT-A gene. The probe identified these fragments (5.7 kb in NHRfyn-12) in two of eight nonhomologous recombinants, but not in the remaining six, suggesting a significant frequency of DT-A gene elimination upon random integration. Similar results were also obtained for each type of recombination after digestion with *Eco*RI. Thus, these results confirmed the results of the PCR analyses.

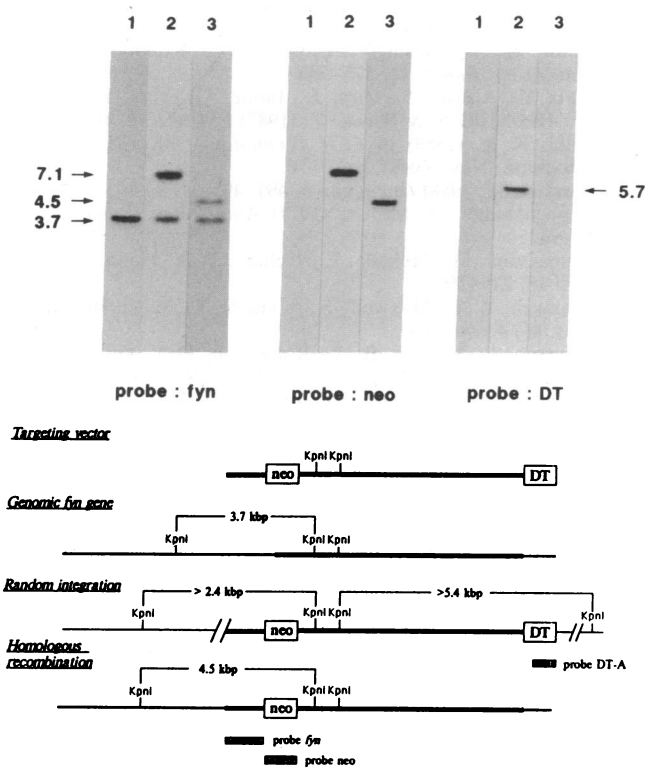

FIG. 3. Southern blot analysis of *Kpn* I digests of genomic DNA from parent ES cells (lanes 1), nonhomologous recombinant NHRfyn-12 cells (lanes 2), and homologous recombinant HRfyn-1 cells (lanes 3) with the indicated probes. The expected fragments as well as the probes used are indicated schematically below the autoradiograms.

Multipotency of Clone HRfyn-1. In a parallel experiment on the isolation of the homologous recombinant, we checked the germ-line colonization of the parent cells at hand. Using cells cultured for 6–13 days, we obtained 52 male chimeras, 41 of which were fertile and 16 of which yielded offspring derived from the ES cells (ES offspring). Two of these gave rise to ES offspring at 100% frequency, five at 10–50%, and nine at 1% frequency. Thus the parent cells in the culture from which we started the recombinant isolation had the potency to differentiate into germ cells as efficiently as reported (17). However, this frequency decreased when the cells were cultured for 40–50 days; only 3 of 20 fertile male chimeras yielded ES offspring, at 1–10% frequency. The frequency of diploid cells in these cultures was about 75%.

Among the eight homologous recombinants isolated, HRfyn-1 had diploid cells at about 90% frequency, HRfyn-5 at 75%, HRfyn-6 at 80%, and the others at <50%; we are, therefore, currently focusing our examinations on HRfyn-1 cells. It took 22 days to isolate, propagate, and freeze HRfyn-1 cells. Their subcutaneous transplants into nude mice showed histologic features composed of a variety of cell types and indistinguishable from those of the parent cells. To date we have obtained 23 pups after injection into C57BL/6 blastocysts. Seven of these were apparently chimeric as judged from coat color, being at almost the same frequency as the parent cells (23 of 78), and a male predominance was noted (15 males).

DISCUSSION

In our experience with the E14 cells used in this study, LIF enhanced their karyotypic stability, but not absolutely: 95% and 75% of the cells were diploid after culture for 30 and 50 days, respectively, in contrast with 50% and 5% in the absence of LIF. The frequency of the germ-line colonization decreased markedly after 40–50 days of culture. Thus the culture conditions, even with LIF, are still not adequate to maintain the cells stably in culture. To produce mutant mice, therefore, it is critical to efficiently isolate homologous mutants, which occur at low frequency (10^{-6} to 10^{-7}), under a condition that does not harm the germ-line differentiating potency of the cells.

DT is composed of A and B fragments. The B fragment is involved in binding to cell surfaces and penetrating cells and the A fragment exerts toxicity by inhibiting ADP-ribosylation of elongation factor 2 upon protein synthesis (24). A single molecule of DT-A has been reported to be enough to kill L cells (25). As the source of the DT-A gene, we used the DT-A cassette originally reported by Maxwell *et al.* (26), which was modified and successfully used to ablate cells in transgenic mice by Palmiter *et al.* (16). The question of whether this gene might kill the cells when present in an extrachromosomal transient state upon transfection was ruled out in the absence of the poly(A) signal.

The frequency of *neo*-positive (G418-resistant) cells was 65 per 10^8 electroporated cells when the *neo* gene was introduced with the MC1 promoter inserted at the same position of the *fyn* gene (pHF-MC1NeopA). The frequency was less than half of that with pMC1NeopA and only about twice that with the promoterless pHF-NeopA. Though several factors could account for this, some sequences of the *fyn* gene fragment might reduce the transcriptional activity of the MC1 promoter in ES cells. In any event, the enrichment by the promoter selection was incidentally low at the *fyn* gene. On the other hand, the advantage of using the DT-A gene for negative selection was apparent. The decrease in the number of *neo*-positive cells by the DT-A selection, the enrichment factor, was about 10, which is on the same order as the *tk*

negative selection. The frequency of the homologous recombinants among the random recombinants isolated was 10^{-3}–10^{-4} by the *neo* gene transfection with promoter, 10^{-2} by the *neo* gene transfection without promoter, and 10^{-1} by the *neo–tk* selection, though the frequencies varied significantly from one gene to another (10, 27). Thus, even in frequency the present *neo*–DT selection is not at all inferior to the *neo–tk* selection.

The frequency of homologous recombinants per electroporated cell was $\approx 1.6 \times 10^{-8}$ at the *fyn* locus. Since the frequency of DNA integration was $\approx 6.5 \times 10^{-7}$ with the pHF-MC1NeopA under the same conditions, the frequency of homologous recombinants per integration appears to be high, $\approx 2 \times 10^{-2}$. One possible explanation for this is the presence of a purine-pyrimidine stretch in introns 1 and 2. Stretches of alternating purines and pyrimidines are known to form a Z-DNA structure and to comprise the binding sites of the recombination enzymes Rec-1 from *Ustilago maydis* and RecA from *Escherichia coli* (28). Recently, the possibility that the Z-DNA structure enhances homologous recombination has been suggested by work on mammalian cells (29, 30). Obviously, it should be examined whether or not the DT-A selection is effective on other loci and whether the Z-DNA structure indeed enhances the frequency. Furthermore, use of the DT-A gene for negative selection can be expected to be useful in making mutant mice by the direct injection of DNA into mouse zygotes; this method is free from the problem of successfully obtaining offspring, which is inherent in the method using ES cells. However, to get a single homologously recombined mouse, one has to produce, maintain, and examine 1,000 transgenic mice (31). If the number can be decreased by an order of magnitude, the approach is practical and the possibility for negative selection with the DT-A gene at the putative highly recombinable *fyn* locus can be examined.

Proteins encoded by the *src* family of protooncogenes are located at the inner surface of the plasma membrane (32) and possibly interact with cell surface receptors—as is known for the *lck* gene product, which interacts with CD4 and CD8 molecules (33)—thereby playing a role as an intermediate stimulus accepted by cell surface receptors into the cytoplasm. Seven protooncogenes in this family are known to date. The expression of this family of genes seems to cover cells in neuronal and hematopoietic lineages, and each is expressed uniquely among these cells (32). The *fyn* gene of the family is most conserved among human, mouse, and frogs, as far as examined (34), but no homologous gene has been detected in an invertebrate. In adults, the gene is expressed ubiquitously at a low level, but at a high level in brain and thymus (13, 35). With the long-range goal of elucidating the compatibility and compensability of the protooncogenes of this family and their role in normal development and differentiation, the production of mice lacking the *fyn* gene is highly desired. The multipotency of pHRfyn1 cells offers encouragement that they will be capable of forming germ-line chimeras.

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1. Zijlstra, M., Bix, M., Simister, E. N., Loring, M. J., Raulet, H. D. & Jaenisch, R. (1990) *Nature (London)* **344,** 742–746.
2. Thomas, K. R. & Capecchi, M. R. (1990) *Nature (London)* **346,** 847–850.
3. Joyner, L. A., Skarnes, C. W. & Rossant, J. (1989) *Nature (London)* **338,** 153–156.
4. DeChiara, M. T., Efstratiadis, A. & Robertson, J. E. (1990) *Nature (London)* **345,** 78–80.
5. Schwartzberg, L. P., Goff, P. S. & Robertson, J. E. (1989) *Science* **246,** 799–803.
6. Zimmer, A. & Gruss, P. (1989) *Nature (London)* **338,** 150–153.
7. Johnson, S. R., Sheng, M., Greenberg, M. E., Kolodner, R. D., Papaioannou, V. E. & Spiegelman, B. M. (1989) *Science* **245,** 1234–1236.
8. Williams, L. R., Hilton, J. D., Pease, S., Willson, A. T., Stewart, L. C., Gearing, P. D., Wagner, F. E., Metcalf, D., Nicola, A. N. & Gough, M. N. (1988) *Nature (London)* **336,** 684–687.
9. Smith, G. A., Heath, K. J., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M. & Rogers, D. (1988) *Nature (London)* **336,** 688–690.
10. Jasin, M. & Berg, P. (1988) *Genes Dev.* **2,** 1353–1363.
11. Zijlstra, M., Li, E., Sajjadi, F., Subramani, S. & Jaenisch, R. (1989) *Nature (London)* **342,** 435–438.
12. Mansour, L. S., Thomas, R. K. & Capecchi, M. R. (1988) *Nature (London)* **336,** 348–352.
13. Semba, K., Nishizawa, M., Miyagima, N., Yoshida, C. M., Sukegawa, J., Yamanashi, Y., Sasaki, M., Yamamoto, T. & Toyoshima, K. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5459–5463.
14. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
15. Thomas, R. K. & Capecchi, M. R. (1987) *Cell* **51,** 503–512.
16. Palmiter, R. D., Behringer, R. R., Quaife, C. J., Maxwell, F., Maxwell, I. H. & Brinster, R. L. (1987) *Cell* **50,** 435–443.
17. Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. (1987) *Nature (London)* **326,** 292–295.
18. Suda, Y., Hirai, S., Suzuki, M., Ikawa, Y. & Aizawa, S. (1988) *Exp. Cell Res.* **178,** 98–113.
19. Suda, Y., Suzuki, M., Ikawa, Y. & Aizawa, S. (1987) *J. Cell. Physiol.* **133,** 197–201.
20. Katoh, K., Takahashi, Y., Hayashi, S. & Kondoh, H. (1987) *Cell Struct. Funct.* **12,** 575–580.
21. Suda, Y., Aizawa, S., Hirai, S., Inoue, T., Fruta, Y., Suzuki, M., Hirohashi, S. & Ikawa, Y. (1987) *EMBO J.* **6,** 4055–4065.
22. Saiki, K. R. (1989) in *PCR Technology*, ed. Erlich, A. H. (Stockton, New York), pp. 7–16.
23. Pawson, T. (1988) *Oncogene* **3,** 491–495.
24. Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem.* **46,** 69–94.
25. Yamaizumi, M., Mekada, E., Uchida, T. & Okada, Y. (1979) *Cell* **15,** 245–250.
26. Maxwell, I. H., Maxwell, F. & Glode, L. M. (1986) *Cancer Res.* **46,** 4660–4664.
27. Charron, J., Malynn, B. A., Robertson, E. J., Goff, S. P. & Alt, F. W. (1990) *Mol. Cell. Biol.* **10,** 1799–1804.
28. Holliday, R. (1989) *Trends Genet.* **5,** 355–356.
29. Wahls, P. W., Wallace, J. L. & Moore, D. P. (1990) *Mol. Cell. Biol.* **10,** 785–793.
30. Wahls, P. W. & Moore, D. P. (1990) *Mol. Cell. Biol.* **10,** 794–800.
31. Brinster, L. R., Braun, E. R., Lo, D., Avarbock, R. M., Oram, F. & Palmiter, D. R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7087–7091.
32. Vellette, A. & Bolen, J. B. (1989) in *Oncogenes*, eds. Benz, C. & Liu, E. (Kluwer, Boston), pp. 121–142.
33. Vellette, A., Bookman, M. A., Horak, E. M. & Bolen, J. B. (1988) *Cell* **55,** 301–308.
34. Steele, E. R., Deng, C. J., Ghosn, R. C. & Fero, B. J. (1990) *Oncogene* **5,** 369–376.
35. Kawakami, T., Pennington, C. Y. & Robbins, K. C. (1986) *Mol. Cell. Biol.* **6,** 4195–4201.