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Genetics

Targeting of the T-cell receptor ζ-chain gene in embryonic stem cells: Strategies for generating multiple mutations in a single gene

(gene targeting/homologous recombination)

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ABSTRACT **The T-cell receptor ζ chain is a member of a family of related proteins that play a critical role in coupling cell-surface receptors to intracellular signaling pathways. To study the role of ζ chain in T-cell ontogeny, we generated targeted mutations of the ζ-chain gene in murine embryonic stem cells. The mutant alleles are predicted to result either in a null phenotype or in the synthesis of a truncated protein capable of supporting T-cell-receptor surface expression but deficient in transmembrane signaling. Both of these targeting events were recovered in a single electroporation experiment with either coelectroporation or a combination deletion/truncation construct. Our results suggest that similar approaches could be used to generate multiple single mutations, modifications of more than one site within a gene, or subtle alterations that rely upon coconversion with the selectable marker gene.**

The T-cell receptor (TCR) ζ chain is the prototype of a family of related proteins (the ζ-family dimers) important for both signal transduction and cell-surface expression of receptor complexes (1–7). ζ chain is expressed in thymocytes and mature T cells, where it functions as a subunit in the multimeric T-cell antigen receptor, and in natural killer cells, where it associates with the IgG Fc receptor (1–4). Studies of altered or mutated forms of ζ chain have revealed that the sequences required for receptor assembly and transport are distinct from those required for signal transduction (8–10). Specifically, the short extracellular and transmembrane domains of ζ chain appear sufficient for reconstitution of surface TCR expression, whereas the cytoplasmic tail of ζ chain is required to elicit characteristic T-cell activation responses (8–10).

The dual functions of ζ chain in receptor expression and signal transduction suggest that this protein may play an important role in T-cell ontogeny. To explore this possible relationship, we were interested in generating targeted mutations of the ζ-chain gene in embryonic stem (ES) cells. In recent years, gene targeting has emerged as a powerful tool for creating defined modifications of the mammalian genome (11). When performed in ES cells, these alterations can be passed into the germ line, allowing the developmental effects of any predetermined mutation to be studied in detail (12–18).

So that both of the known activities of ζ chain could be examined, two targeted mutations were generated: one that should result in complete loss of function and one that should interfere with signaling, but not cell-surface expression of the TCR complex (8–10). In this report, we describe the use of coelectroporation and a specialized deletion/truncation vector to generate both mutations in a single electroporation experiment. In addition to offering a practical and expedient method for creating more than one modification of a single locus, our observations suggest that these methods could also be used to overcome positional silencing effects and to create subtle mutations in any gene of interest.

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MATERIALS AND METHODS

Targeting Vectors. Sequence replacement targeting vectors (see Fig. 1) were assembled in plasmid pGEM-11Zf(−) (Promega), using contiguous *Bam*HI ζ-chain genomic fragments (19) and the *neo* and *Tk* genes from plasmids pMC1-NEO [poly(A)⁻] and pMC1-TK [poly(A)⁺], respectively (20). To generate the gene disruption construct pζ-Δ, a 700-base-pair (bp) *Xba* I–*Bam*HI fragment containing the intron I splice acceptor and 22 bp of exon II (encoding part of the ζ-chain transmembrane domain; ref. 19) was removed so that the targeted gene would be rendered nonfunctional. In pζ-CT94, a 32-bp synthetic adapter containing three in-frame stop codons (UAA-UAG-UGA; corresponding to amino acids 95–97) followed by two polyadenylylation signals was introduced into the *Bam*HI site in ζ-chain exon IV (5′ of the *neo* gene). Homologous integration of pζ-CT94 should therefore result in a modified gene that produces both a truncated ζ transcript and protein (lacking the last 70 amino acids). Plasmid pζ-Δ/CT94 was generated by first inserting the 4.2-kilobase (kb) genomic fragment into plasmid pζ-CT94 and then deleting a 1.7-kb internal *Xba* I–*Hin*dIII fragment containing exon II.

ES Cells, Electroporation, and Selection. ES cells (D3; ref. 21) were propagated on mitomycin C (10 μg/ml)-treated mouse embryonic (STO) fibroblasts (22, 23). Culture medium (23) was supplemented with leukemia inhibitory factor at 1000 units/ml (ESGRO; GIBCO). Trypsinized cells (2.5×10^7) were washed and resuspended in 0.8 ml of Dulbecco's phosphate-buffered saline (GIBCO), mixed with 50 μg of linearized plasmid DNA, and electroporated by using a Bio-Rad gene pulser (240 V, 500 μF). Under these conditions, cell survival was reproducibly in the 50–60% range. After electroporation, ES cells were plated at a density of 2×10^5 cells per 60-mm plate on STO-NEO^r feeders (E. J. Robertson, Columbia University). Selection was started 24 hr after electroporation with medium containing G418 (active ingredient at 125 μg/ml; GIBCO) and ganciclovir (Ganc, 5 μM; Syntex, Palo Alto, CA), unless otherwise stated. Transformation efficiency was calculated by selection in the presence of G418 alone. After 10–12 days, individual colonies were transferred to 96-well plates and grown without further drug selection. After a second passage onto 24-well plates for expansion, cells were trypsinized and divided into aliquots for freezing and DNA extraction (24).

Abbreviations: ES cells, embryonic stem cells; Ganc, ganciclovir; TCR, T-cell receptor; Ganc^r, Ganc-resistant; G418^r, G418-resistant.

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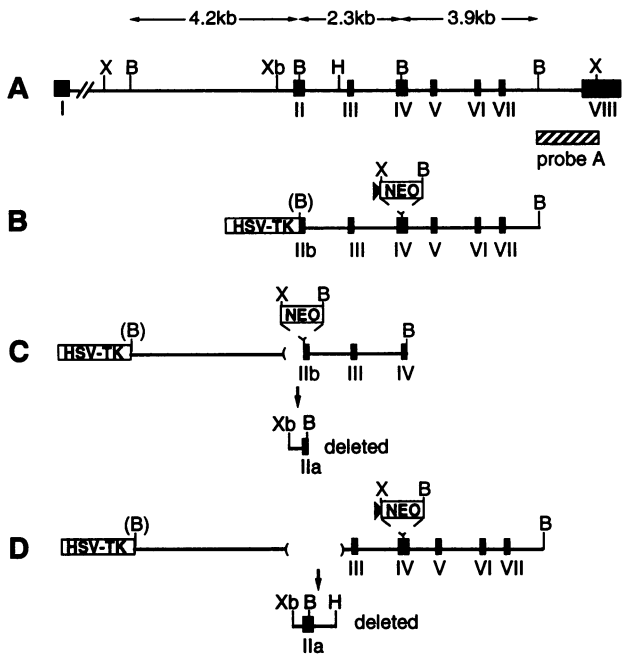

FIG. 1. Intron/exon organization of the murine TCR ζ-chain gene (*A*) and targeting constructs pζ-CT94 (*B*), pζ-Δ (*C*), and pζ-Δ/CT94 (*D*). Location and size of *Bam*HI genomic fragments used to generate the targeting constructs are depicted at top. Also shown are restriction fragments deleted during construction of pζ-Δ and pζ-Δ/CT94: B, *Bam*HI; H, *Hin*dIII; X, *Xho* I; and Xb, *Xba* I; sites in parentheses have been destroyed. For alignment purposes, deletions are indicated by parentheses. The small triangle located 5′ of MC1NEO in constructs pζ-CT94 and pζ-Δ/CT94 represents the 32-bp stop/polyadenylylation sequence (see text). Location of the probe (probe A) used to detect homologous integration is shown. HSV-TK, herpes simplex virus thymidine kinase; NEO, neomycin phosphotransferase.

Screening. DNA from individual colonies was digested to completion with *Xho* I and analyzed by Southern blotting using a probe lying outside of the targeting vector (Fig. 1, probe A). Positive samples were then analyzed further by either Southern blotting or PCR as described in text. DNA from each positive clone was also tested for random insertions of the targeting vector by probing with MC1NEO. PCR was performed in 50 μl of buffer containing each dNTP at 100 μM, 15 mM Tris·HCl (pH 8.4), 50 mM KCl, 1 mM $MgCl_2$, 2.5 units of *Taq* polymerase, and 0.4 μg of each primer. Cycling parameters were 94°C, 1 min; 63°C, 1 min; 72°C, 4 min × 35 cycles. Recombination between constructs pζ-Δ and pζ-CT94 was detected with primers NEO1, 5′-CAGCCGGAACACGGCGGCATC-3′, and NEO2, 5′-CTCCCCGATTCGCAGCGCATCGCCTT-3′, which generated a 2.3-kb fragment that hybridized to probe B (see Fig. 2). Integrations of construct pζ-CT94 were detected with NEO1 and a primer specific to the stop/poly(A) adapter (see above), 5′-GCTCGGGATCTCTAATAGTGAGCAATA-3′ (430-bp fragment). Integrations of construct pζ-Δ were detected with primer NEO3, 5′-GTCGTGACCCATGGCGATGCCTGCTTG-3′, and a primer specific to ζ-exon II, 5′-ATCTAGCAAGTAGCAGAGTTTGGGATC-3′ (275-bp fragment).

RESULTS

Coelectroporation of Constructs pζ-Δ and pζ-CT94. ES cells were coelectroporated with linearized pζ-Δ and pζ-CT94 to determine whether both the ζ-chain deletion and truncation genotypes could be generated in a single experiment and to directly compare targeting frequencies with the two constructs. Colonies resistant to both G418 and Ganc were screened for targeted events by blotting *Xho* I-digested total genomic DNA with probe A (see Fig. 1). Using this detection scheme, the endogenous ζ allele(s) result in a 13.4-kb fragment, and homologous integration of pζ-CT94 or pζ-Δ should result in a 6.6-kb or 8.9-kb fragment, respectively (see Fig. 1).

Of 200 G418-resistant ($G418^r$)–Ganc-resistant ($Ganc^r$) colonies individually screened by Southern blotting, 16 (8%) had modifications of one ζ-chain allele indicative of homologous integration. Each of these presumably contained a ζ-CT94 targeting event, as evidenced by the presence of equal-intensity bands of 13.4 and 6.6 kb (see Fig. 2*C*). Because no ζ-Δ targeting events had been generated, additional experiments were done in which only construct pζ-Δ was electroporated. A total of 97 $G418^r$–$Ganc^r$ colonies were screened, but, again, no targeting events were observed. One possible explanation for these results is that genomic sequences within pζ-Δ (particularly the 4.2-kb BamHI fragment composed entirely of intron I) exert a silencing effect on *neo* expression (25–28), thereby lowering the relative targeting frequency (i.e., number of positive colonies per number of $G418^r$–$Ganc^r$ colonies). Alternatively, the absolute targeting frequency (i.e., number of positive colonies per number of electroporated cells) of pζ-Δ may be relatively low, perhaps due to base-pair mismatches or repetitive sequences within intron DNA (29). To explore this observation further, we first ruled out the possibility that the MC1NEO gene within construct pζ-Δ had been mutated by isolating the intact MC1NEO genes from both constructs and demonstrating that they gave rise to equivalent numbers of ES colonies after electroporation (data not shown). We then analyzed 185 colonies from the coelectroporation experiment that had random integrations to determine which construct(s) had inserted into the genome. Of 162 colonies with insertion of either pζ-Δ or pζ-CT94, only 22 (14%) contained pζ-Δ. The frequency of cointegration (23/185; 12%) was also much lower than the expected 75% (30). These results are consistent with those of subsequent experiments, in which the number of $G418^r$ ES colonies obtained with construct pζ-Δ was 5- to 10-fold lower than that obtained with pζ-CT94 (data not shown). The difference was more pronounced still (50- to 80-fold) when the G418 concentration was increased to 200 μg/ml. Taken together, these findings suggest, but do not prove, that sequences within pζ-Δ act to silence MC1NEO expression.

Rescue of ζ-Δ Phenotype by Recombination Between Coelectroporated Constructs. Further examination of the positive ES colonies from the coelectroporation experiment revealed that in four instances (colonies 18, 31, 61, and 145) recombination had occurred between constructs pζ-Δ and pζ-CT94 either before or after chromosomal integration (Fig. 2 *B* and *C*). This event was detected during our screening for multiple insertions (using MC1NEO as a probe, see Fig. 2*C*) and lends additional support to our contention that localized silencing effects on MC1NEO, rather than low recombinogenic potential, explains the absence of ζ-Δ targeting events. From a practical viewpoint, this occurrence was also fortuitous because the ζ-Δ integration phenotype was in effect "rescued," presumably by coupling construct pζ-Δ to the more highly expressed MC1NEO gene within pζ-CT94. In two instances (colonies 18 and 145), the integration pattern was inconsistent with a simple replacement event, suggesting rather that a partial deletion of flanking sequence, accompanied by a duplication of MC1NEO had occurred (data not shown). Integration patterns of this type have been reported previously and appear specific to replacement vectors, particularly those in which the *neo* gene is poorly expressed (26, 31). Examination by PCR of DNA from colonies with nonhomologous integration of the vectors indicated that recombination between pζ-Δ and pζ-CT94 of the type shown in Fig. 2*B* had occurred in 21 of 250 (8%) of all colonies screened.

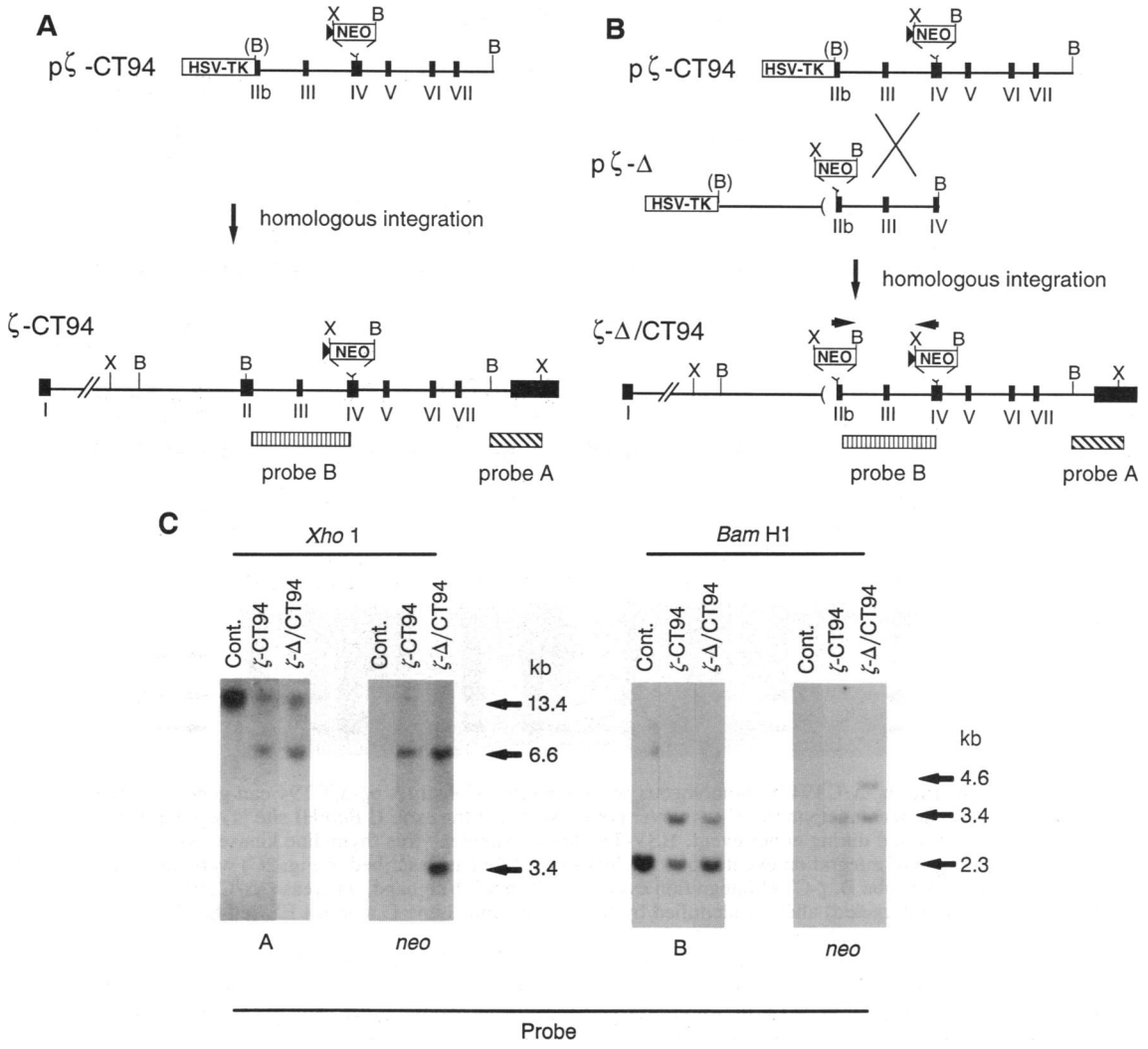

FIG. 2. (*A*) Expected structure of the TCR ζ-chain gene after integration of construct pζ-CT94 by homologous recombination. (*B*) Schematic representation of recombination between constructs pζ-CT94 and pζ-Δ followed by homologous integration. Note regeneration of the exon II *Bam*HI site in ζ-CT94 (*A*) and the intron I *Bam*HI site in ζ-Δ/CT94 (*B*). Small arrows represent PCR primers used to detect recombination between pζ-CT94 and pζ-Δ. Only one of two possible mechanisms is shown for simplicity, the other being integration of either pζ-Δ or pζ-CT94 into the genome followed by a second homologous integration involving the other construct. (*C*) Southern blots of DNA from control and targeted ES cell clones. ζ-CT94 (ES clone 73) and ζ-Δ/CT94 (ES clone 61) are representative of the targeted events depicted in *A* and *B*, respectively. HSV-TK, herpes simplex virus thymidine kinase; NEO, neomycin phosphotransferase.

This frequency, which probably is an underestimate because we did not screen for other possible recombination products, is nevertheless similar to that seen for recombination between cointroduced fragments in different mammalian cell lines (32, 33).

Generation of ζ-CT94 and ζ-Δ Targeting Events with Construct pζ-Δ/CT94. Our accumulated data suggested that it should be possible to directly generate ζ-Δ integration events if the MC1NEO gene was buffered from the localized effects of the flanking 4.2-kb genomic fragment. To test this hypothesis, we made construct pζ-Δ/CT94 by joining the 4.2-kb fragment in its proper genomic order to pζ-CT94 (Fig. 1*D*). To provide the deletion phenotype, a 1.7-kb *Xba* I–*Hin*dIII fragment containing exon II was removed, leaving 1.3 kb of sequence (derived from the 2.3-kb genomic fragment; Fig. 1) between the 4.2-kb genomic fragment and MC1NEO. Another approach would have been to attempt to protect MC1NEO by inserting an unrelated sequence into pζ-Δ. However, we were also interested in determining whether pζ-Δ/CT94 could be used to generate both the deletion and truncation modifications in a single electroporation, as illustrated in Fig. 3 *A* and *B*.

Pilot electroporation experiments were first conducted in which the frequency of G418^r colonies obtained with pζ-Δ/CT94 and pζ-CT94 was found equivalent. In the third experiment, G418^r–Ganc^r colonies were selected and individually screened for homologous integration of pζ-Δ/CT94, as described. Twenty-two of 232 colonies (10%) were positive; however, the initial screening did not distinguish ζ-CT94 (Fig. 3*A*) from ζ-Δ/CT94 (Fig. 3*B*) events. To determine which integration event had occurred, DNA samples were digested with *Bam*HI and hybridized with probe B (Fig. 3*C*). Although this probe hybridizes to sequences contained within both constructs, restriction fragments of the correct sizes should only result from homologous recombination events (see Fig. 3). Surprisingly, both events were detected at roughly equal frequencies (12 ζ-CT94 and 10 ζ-Δ-CT94), indicating that there had been no strong preference for the site of 5′ crossover (Fig. 3*C*). Further examination of these colonies by Southern blotting revealed no evidence of structural alterations (e.g., deletions or duplications) or random insertions of the targeting vector elsewhere in the genome. To see whether this approach could be used as general strategy or depended upon either the particular ES cell line or passage

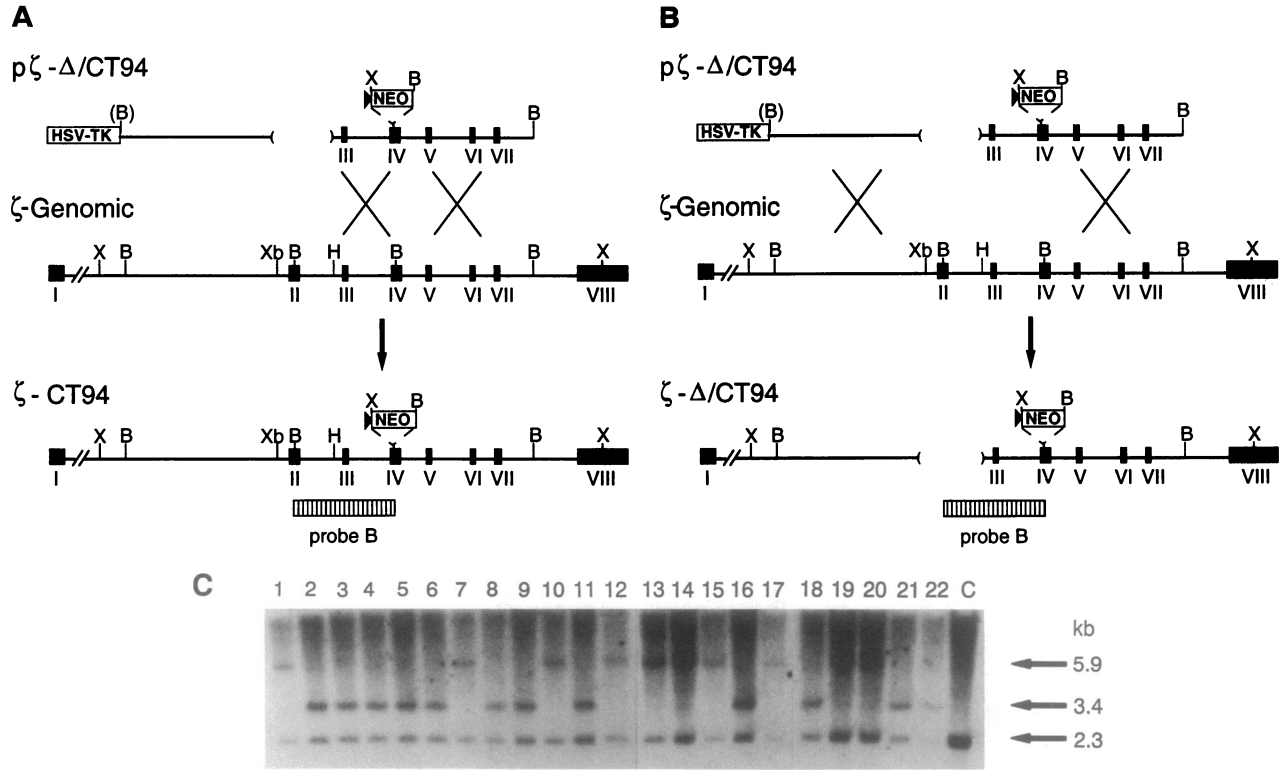

FIG. 3. Integration of construct pζ-Δ/CT94 by homologous recombination. Construct pζ-Δ/CT94 can generate either a ζ-CT94 (*A*) or a ζ-Δ/CT94 (*B*) integration event, depending upon the 5′ crossover point. Note that the exon II *Bam*HI site is regenerated in *A* but not in *B*, and the intron I *Bam*HI site is regenerated during either event. HSV-TK, herpes simplex virus thymidine kinase; NEO, neomycin phosphotransferase. (*C*) Distinguishing the type of integration event. Positive clones (identified as described in Fig. 2*C*) were analyzed further by digesting with *Bam*HI and hybridizing with probe B. ζ-CT94 integration events result in a 3.4-kb band, whereas ζ-Δ/CT94 integration events result in a 5.9-kb band. The endogenous (nontargeted) allele is identified by the 2.3-kb band. Lane C, control ES cell DNA.

number (27), we repeated the experiment using a low-passage E14 ES cell line (34). A total of 670 colonies obtained from G418 selection alone was screened by PCR (data not shown), and 10 positive colonies were detected. When these positive colonies were analyzed further, we found an equal frequency of each targeting event (i.e., 5 colonies had ζ-CT94 integrations, and 5 colonies had ζ-Δ/CT94 integrations). Because all positive colonies were derived from individual plates, we are confident that they represent unique targeting events.

DISCUSSION

We have generated, by gene targeting, deletion and truncation mutations within the TCR ζ-chain locus as an initial step toward examining the role of ζ chain in thymocyte development. During this study, we have also made several observations of a more general nature concerning gene targeting. First, we have shown that localized position effects can profoundly influence the relative targeting frequency. Combining the results of three independent experiments, the relative targeting frequency with construct pζ-Δ was <1 in 142. This result contrasts sharply with the 1 in 10 frequency seen with constructs pζ-CT94 and pζ-Δ/CT94, particularly in view of the fact that the targeting vectors differ by only 2.3 kb and 1.3 kb with respect to MC1NEO placement (Fig. 1).

The susceptibility of marker genes to position effects has been mentioned previously, but in most cases the silencing effects were severe enough to automatically preclude further use of the targeting vector (25–28). In this instance, these effects were apparent only when the transformation frequencies of different constructs were compared directly and were most evident when the G418 concentration was increased from 125 to 200 μg/ml. Undetected positional effects such as these may have contributed to the wide variation in reported targeting frequencies (20, 28, 35–38) and may also explain the inability to target certain genes. As a practical consideration, our results suggest that assessing gene-targeting constructs for positional effects is crucial to obtaining optimal results.

An interesting observation from our study is that coconversion of both the deletion and truncation mutations occurred at relatively high frequency with construct pζ-Δ/CT94 (data not shown). Similar rates of coconversion have been reported informally by others for even greater distances with gene-replacement vectors (11). We were surprised, however, to find that a 1.7-kb deletion within the region of homology did not strongly bias the integration toward ζ-CT94 events (see Fig. 3). Considerably larger deletions have been generated with replacement vectors; however, the effect of having homologous sequences directly flanking the deletion region was not assessed (39). It is probable that factors such as the size of deletion, relative lengths of sequences flanking the deletion, and degree of homology between donor and host DNA can influence the crossover site.

These experiments also show the feasibility of generating multiple genotypes in a single electroporation. Although our example underscores the application of this approach in overcoming problems from positional effects, it should, in theory, be applicable to any situation where more than one modification of a single gene is desired. As gene-targeting technology becomes more widely applied, the major interest will probably shift from creating null phenotypes to generating various subtle modifications of single genes. For genes such as ζ chain that are amenable to targeting at a relatively high frequency, these techniques may represent an efficient approach to creating multiple independent targeting events. In addition, gene-replacement vectors similar to pζ-Δ/CT94 could be specialized for a number of applications. By the placement of two mutations on the same arm of flanking

DNA, as we have done, ES lines with either the proximal or both mutations can be obtained. Alternatively, independent single modifications could be generated by incorporating separate mutations into each arm. Especially attractive is the idea of generating subtle modifications, such as changes in only a few base pairs, without altering the expression pattern of the gene. One way in which these mutations can be made is to place the selectable gene into a contiguous intron and rely on coconversion (40). Our data suggest that it should be possible to recover these events with reasonable frequency in ES cells.

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