Toward an animal model of cystic fibrosis: Targeted interruption of exon 10 of the cystic fibrosis transmembrane regulator gene in embryonic stem cells

(homologous recombination/gene targeting/*CFTR* gene)

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ABSTRACT   A gene-targeting construct was made containing 7.8 kilobases of DNA spanning exon 10 of the mouse cystic fibrosis transmembrane regulator (*CFTR*) gene in which part of the exon has been replaced by two neomycin-resistance (*Neo*) genes driven by different promoters. (This replacement introduces a chain-termination codon at amino acid position 489 in the CFTR sequence.) A herpes simplex thymidine kinase gene was on each end of the construct, which was electroporated into embryonic stem (ES) cells. Colonies resistant to G418, or to G418 plus ganciclovir, were selected and screened by Southern blotting or by PCR amplification. Five pools of G418-resistant cells gave PCR products diagnostic of targeting. Four independent clones of ES cells with a disrupted *CFTR* gene have been isolated from these pools. The frequency of targeting was 1/2500 G418-resistant colonies. This low frequency is not the consequence of marginal expression of the *Neo* genes in the targeted cells. The CFTR targeting events were clustered among our experiments in a manner suggesting that some unidentified factor(s), possibly passage number, influences the recovery of CFTR-targeted cells.

The most common mutation associated with cystic fibrosis is the deletion of the three bases coding for phenylalanine-508 in exon 10 of the cystic fibrosis transmembrane regulator (*CFTR*) gene (1). Homozygotes for this deletion have a severe form of the disease, involving lung and pancreatic dysfunction. The effects of homozygosity for other types of mutant *CFTR* alleles are now beginning to be described as a result of the availability of PCR methods for their detection (2, 3). A Soviet family with cystic fibrosis causing early death appears to provide an example (4). Three infants died in this family within months of birth (two with pneumonia and one with presumed meconium ileus). The parents were shown to be heterozygotes for a normal *CFTR* gene and a mutant allele with a chain-termination codon at amino acid position 515, as a result of deletion of two nucleotides in exon 10. While a direct association between homozygosity for this mutant *CFTR* gene and the occurrence of cystic fibrosis was not established in this family (the genotypes of the deceased infants were not determined), the data suggest that chain termination of the CFTR protein within exon 10 is likely to cause a severe form of cystic fibrosis. Similar conclusions can also be reached by examining the phenotypes of individuals having the phenylalanine-508 deletion mutation combined with several different chain-termination mutants (Lap-Chee Tsui, Toronto, personal communication).

No naturally occurring mutations are known that model human cystic fibrosis. But mice have a single *CFTR* gene that codes for a protein identical to the human protein in >78% of its amino acids (5). We have therefore commenced studies aimed at generating mice of two types: animals homozygous for a *CFTR* gene disrupted by a chain-termination mutation and animals homozygous for the phenylalanine-508 deletion. Inactivation of a gene can be achieved by a single gene targeting event involving homologous recombination between the target gene and exogenous DNA (6, 7). More subtle changes, such as the deletion of nucleotides in a chosen position within a coding sequence, currently require an initial gene targeting step and then a second step in which sequences introduced during the targeting step can undergo spontaneous excision to generate the desired change (8, 9).

We describe here experiments demonstrating that the *CFTR* gene can be targeted in mouse embryonic stem cells in a manner expected to lead to the introduction of a chain-termination mutation at amino acid position 489 in exon 10. Pluripotent embryonic stem (ES) cells modified in this way, when injected into blastocysts, should allow the production of animals with a genotype similar to that described in the Soviet family.

MATERIALS AND METHODS

Targeting Plasmids. λ bacteriophages containing DNA from the *CFTR* gene of a strain 129 mouse were isolated, using a mouse *CFTR* cDNA probe (5), from a library in bacteriophage EMBL3 of genomic DNA partially digested with *Sau*3AI. From one of these bacteriophages, a 14-kilobase (kb) *Bam*HI fragment containing exon 10 was isolated by hybridization to a probe specific for human *CFTR* exon 10 sequences. A plasmid KK50 was constructed that contains the neomycin-resistance gene (*Neo*) from pMC1Neo (7), together with its promoter and enhancer, flanked at both its 5′ and 3′ ends by a copy of the herpes simplex thymidine kinase gene driven by the same promoter and enhancer (7). The targeting plasmid pCF Neo was constructed from KK50 in two steps. First, a 700-base-pair (bp) fragment from the 14-kb *Bam*HI fragment of the mouse *CFTR* gene, extending from a *Sty* I site in intron 9 to the *Xmn* I site in exon 10, was cloned into an *Nhe* I site located immediately 5′ to the pMC1Neo sequences in KK50. Then a 7.1-kb fragment also from the 14-kb *Bam*HI fragment, extending from a *Stu* I site

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Abbreviations: CFTR, cystic fibrosis transmembrane regulator; ES cell, embryonic stem cell; PGK, phosphoglycerokinase; LIF, leukemia inhibiting factor.
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just 3' to exon 10 to an *Nco* I site in intron 10, was cloned into a *Sac* II site located immediately 3' to the pMC1Neo sequences in KK50. A second targeting plasmid, pCF Neo^2 (see Fig. 1), was constructed by codirectionally inserting an additional *Neo* gene, driven by the phosphoglycerokinase (PGK) promoter (10), into a *Sal* I site located 3' to the pMC1Neo sequences in pCF Neo.

ES Cells. The ES cell line E14TG2a (11) was cultured in some experiments on primary embryonic fibroblast feeder layers that had been irradiated with 3000 rads (1 rad = 0.01 Gy); the fibroblast feeder cells were from (129/0la × C57BL/6J)F$_2$ embryos carrying a copy of a neomycin-resistance transgene in the β_2-microglobulin locus (12) and can grow in medium containing G418. In other experiments, no feeder layers were used, but leukemia-inhibiting factor (LIF) (13) was added that had been produced from COS-7 cells transfected with the LIF-expressing plasmid pC106R (Genetics Institute, Cambridge, MA); in these experiments, the culture dishes were pretreated with 0.1% gelatin to facilitate cell adhesion.

Electroporation. Electroporation in culture medium containing 3–5 nM targeting plasmid was as described (14) except with a 1-sec discharge from a 150- to 250-μF capacitator charged to 250–400 V. Prior to most of the electroporations, the targeting sequences were excised from their plasmids by digestion with *Not* I (see Fig. 1); excised sequences are identified below by the same names as the plasmids but without the letter p. In some experiments, the targeting plasmids were linearized by cutting at a restriction site within the vector plasmid sequences.

Selection. After electroporation, ES cells were plated onto feeder layers (or cultured in the presence of LIF) in either 100-mm Petri dishes or in 24-well plates at dilutions that gave between 10 and 60 drug-resistant colonies per 16-mm-diameter culture well. For positive selection experiments, G418 (Sigma) was present in the medium at a final concentration of 200 μg/ml. For the positive–negative selection experiments (15), ganciclovir (a gift from Syntex, Palo Alto, CA) was present at a final concentration of 1 μM in addition to the G418. After 10–14 days, colonies were tested for targeting by Southern blotting or by PCR amplification.

Southern Blot Analysis. Colonies were expanded to a level sufficient for Southern blot analysis, which was carried out conventionally using either a *Bam*HI/*Sty* I probe specific for *CFTR* sequences (see Fig. 1) or one specific for the *Neo* gene.

PCR Primers. Primers 1 and 3 (see Fig. 1) are specific to the incoming DNA; their sequences are as follows: primer 1, 5'-ACACTGCTCGAGGGCTAGCCTCTTC-3'; primer 3, 5'-ACACTGCTCGACATTGGGTGG-3'. Primers 2 and 4 (see Fig. 1) are specific to the target locus; their sequences are as follows: primer 2, 5'-CAGTGAAGCTGAGACTGTGAGC-TT-3'; primer 4, 5'-CCACAATGAGAGGCTGAGACA-3'.

PCR-Positive Control Cell Line. A PCR-positive control cell line having primers juxtaposed in the way they occur in correctly targeted cells (see Fig. 1C) was made by transforming E14TG2a ES cells with plasmid F141.1. This plasmid was constructed by cloning into the *Nhe* I site located 5' to the *Neo* sequences in KK50 a 1.2-kb fragment from the 14-kb *Bam*HI fragment of the mouse *CFTR* gene DNA, extending from the 5' *Bam*HI site (in intron 9) to the *Xmn* I site in exon 10 (see Fig. 1A). Lysates from ES cells targeted at the *CFTR* locus in the desired manner should give the same PCR signals after amplification with primers 1–4 as do lysates from this control cell.

PCR Amplification. Cell lysates were prepared as described (16). PCR amplification was typically in 50 μl of buffer containing each dNTP at 50 μM, 0.4 μg of each primer, 50 mM Tris·HCl (pH 8.5), 1.5 mM MgCl$_2$, 20 mM (NH$_4$)$_2$SO$_4$, 10% dimethyl sulfoxide, and 0.2 unit of *Taq* polymerase. Denaturation was for a minimum of 30 sec at a maximum

temperature of 92°C. Extension was for 10 min at 65°C. Cycle number was 35. Pools of cells or pools of colonies were considered PCR positive when an amplified lysate equivalent to 50,000 cells gave, after electrophoresis, visible stained bands of the sizes diagnostic of targeting (see Figs. 1 and 2): 1050 bp for primers 1 and 2, and 1100 bp for primers 3 and 4. Lysates from 1:10 and 1:100 mixtures of the PCR-positive control cells with the starting ES cells were used to check amplifications. When lysates from single targeted colonies were being amplified, stained bands were readily seen with ≈500 cells.

RESULTS AND DISCUSSION

Targeting Strategy. Fig. 1 shows the targeting strategy. The targeting constructs contain ≈7.8 kb of mouse genomic DNA that spans exon 10 of the *CFTR* gene but with one (CF Neo) or two (CF Neo^2) copies of the positively selectable *Neo* gene in place of the 3' two-thirds of exon 10 and 200 bp of the 5' end of intron 10. Homologous recombination between the target *CFTR* locus (Fig. 1A) and the targeting construct CF Neo or CF Neo^2 (Fig. 1B) leads to a modified gene (Fig. 1C) that now contains the positively selectable gene(s) and should code for a truncated CFTR protein with amino acid 488 changed to alanine (from isoleucine) and with a stop codon (UAG) at position 489 (Fig. 1D). Cells containing the modified gene are expected to be resistant to the antibiotic G418. The targeting constructs also have a copy of the negatively selectable herpes simplex thymidine kinase gene (TK in Fig. 1) on their 5' and 3' ends to allow use of the positive–negative selection protocol of Mansour *et al.* (15). Screening for targeting can be carried out directly by Southern blotting the DNA from candidate colonies. As shown in Fig. 1, several fragments increase in length by ≈3 kb after targeting. Alternatively, screening can be by PCR amplification using pairs of primers that yield fragments of a predicted size only with DNA from targeted colonies (16). Primers 1 and 3 are specific for the incoming DNA and primers 2 and 4 are specific for the target locus; only when the odd-numbered primers are juxtaposed to the even-numbered primers by targeting will amplification give the correctly sized fragments (1050 with primers 1 and 2; 1100 with primers 3 and 4).

Detection of Targeted Colonies in Pools of ES Cells. ES cells were electroporated in the presence of the targeting constructs CF Neo or CF Neo^2 and plated under the appropriate selective conditions on embryonic fibroblast feeder layers or in the presence of LIF.

Table 1 summarizes all our experiments and their results. Experiment 1 was with the targeting construct CF Neo, which is the same as CF Neo^2 shown in Fig. 1 but without the PGK Neo insert. The treated cells were exposed to G418 and ganciclovir in the positive–negative selection protocol of Mansour *et al.* (15). Over 300 doubly resistant colonies were picked individually and tested directly for targeting by Southern blotting. None was targeted.

To allow comparison of experiments using positive–negative selection with those using only positive selection, we determined an "enrichment factor" (Table 1) for each doubly selected experiment by measuring, on convenient aliquots of the electroporated ES cells, the ratio of colonies resistant to G418 alone to colonies resistant to both G418 and ganciclovir. The number of colonies that would have been found had we used only G418 selection can then be calculated by multiplying the number of (G418 + ganciclovir)-resistant colonies by the enrichment factor. This value is also presented in Table 1 in the column G418 equivalents.

As a precaution against the possibility that the single copy of the *Neo* gene in CF Neo might not be expressed at a sufficiently high level to confer G418 resistance when embedded in the *CFTR* locus, we next introduced into the
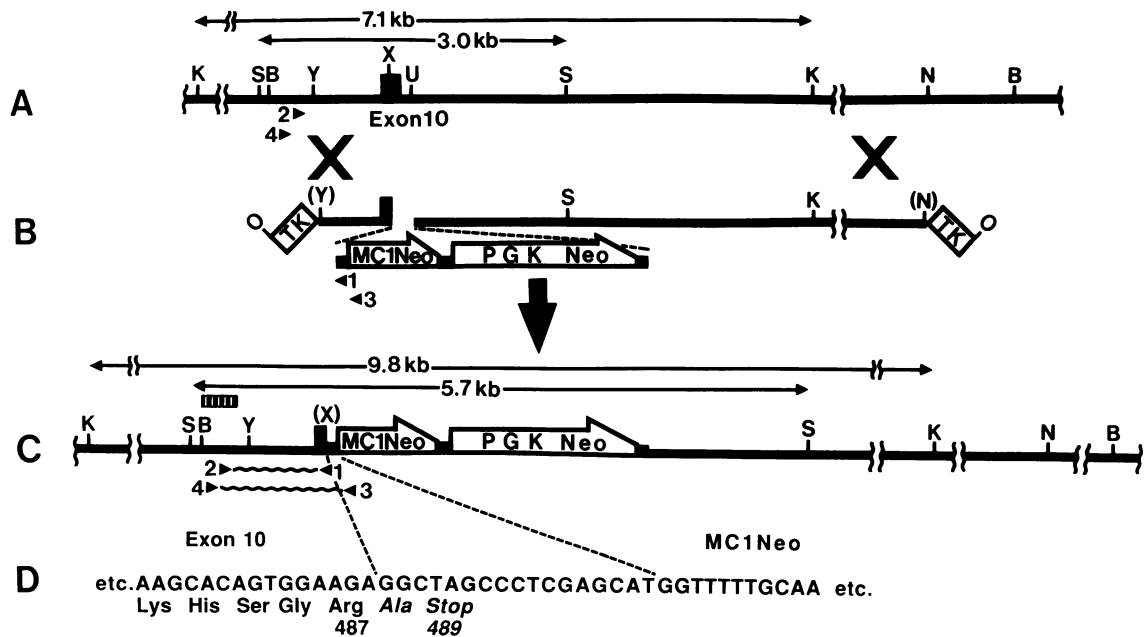
FIG. 1. (A) Exon 10 and its surroundings in the mouse *CFTR* gene. (B) The targeting construct CF Neo² (see text for details). (C) The *CFTR* gene expected after targeting. (D) Details of the nucleotide and deduced amino acid sequences (numbered) predicted for the junction between the 5' part of exon 10 and MC1Neo after targeting; italicized amino acids are not coded by the normal *CFTR* gene. Restriction sites important in construction of the targeting DNA or used for analysis of targeted colonies are shown. B, *Bam*HI; K, *Kpn* I; N, *Nco* I; O, *Not* I; S, *Sst* I; U, *Stu* I; X, *Xmn* I; Y, *Sty* I; sites in parentheses were destroyed during ligation. Sites and directions of primers 1–4 used for PCR analysis are indicated. Wavy lines show the amplified fragments that are characteristic of targeting. The sizes of some restriction enzyme fragments used for Southern analysis are indicated; vertically hatched box shows the *Bam*HI/*Sty* I CFTR-related probe used for hybridization.

targeting construct a second *Neo* gene, driven by the strong and ubiquitously expressed PGK promoter (10). The resulting construct, CF Neo² (shown in Fig. 1), was used in all subsequent experiments.

In experiments 2, 3A, 4A, 5, 6A, and 7, a total of 1501 doubly resistant colonies were individually picked and PCR tested (corresponding to 5995 G418 equivalents) together with 212 individually picked colonies resistant to G418 alone

Table 1. Screening of drug-resistant colonies and isolation of targeted colonies

Experiment	Protocol	G418^R colonies screened	(G418 + ganciclovir)^R colonies screened	Enrichment factor	G418 equivalents	PCR-positive pools	Targeted colonies isolated
1	F, I		303	8.1	2454	*	0
2	F, I		606	3.8	2303	0	0
3A	F, I		55	3.8	209	0	0
4A	F, I		54	5.0	270	0	0
5	F, I		48	6.8	326	0	0
6A	F, I		344	5.3	1823	0	0
7	L, I		394	2.7	1064	0	0
3B	F, I	87				0	0
4B	F, I	125				0	0
6B	F, P	578				1	1
8	L, P	580				2	1
9	F, P	475				1	1
10A	F, I	294				0	0
10B	F, P	2,098				0	0
11	F, P	1,146				0	0
12	F, P	452				0	0
13	L, P	875				0	0
14	L, P	1,890				0	0
15	L, P	3,738				1	1
Total		12,338	1804		8449	5	4

G418^R, resistant to G418 alone; (G418 + ganciclovir)^R, resistant to both G418 and ganciclovir. Experiments are numbered according to their time sequence but have been grouped by screening protocol; experiment 1 was with CF Neo, all others were with CF Neo²; colonies in experiments 3, 4, 6, and 10 were screened in one of two ways as indicated by A and B. Protocol is designated as follows: using cells on feeder layers (F) or LIF-adapted (L), with screening individually picking colonies (I) or by testing while still in pools (P). Enrichment factor, separately measured on suitable aliquots, is number of G418^R colonies divided by number of (G418 + ganciclovir)^R colonies. G418 equivalents are number of (G418 + ganciclovir)^R colonies screened × enrichment factor.
*In experiment 1, Southern blots were used directly to look for targeted colonies.

(in experiments 3B and 4B). Again, none was targeted, even though we found, as expected, that CF Neo^2 gave more (by a factor of $\approx$3) G418-resistant colonies than were obtained with CF Neo.

We next considered the possibility (disproved below) that even the two *Neo* genes in construct CF Neo^2 might not be expressed when integrated into the target *CFTR* locus. If this were so, it might still be possible to isolate targeted cells without depending on expression of the *Neo* gene in the *CFTR* gene. Thus, Reid *et al.* (17) have recently demonstrated that selecting for the expression of a *Neo* gene integrated into the genome nonhomologously gives survivors that are enriched for targeting, although the enrichment is much less than with conventional G418 selection. We therefore removed ganciclovir from the selection medium, so that nonhomologous integrants with CF Neo^2 could be tested for CFTR targeting. To enable the screening of larger numbers of colonies, PCR testing was carried out directly on small pools of colonies without picking them individually.

In experiments 6B, 8, and 9, respectively, 578, 580, and 475 G418-resistant colonies in pools of $\approx$8 visible colonies were tested by PCR. The ES cells for experiments 6 and 9 were cultured on feeder layers; the cells for experiment 8 were cultured in LIF-containing medium without feeder layers. A pool giving a positive PCR signal was identified in the G418-resistant pools from experiments 6B and 9, and two positive pools were identified from experiment 8. Fig. 2 illustrates the detection of these PCR-positive pools.

At this point, E14TG2a ES cells with a lower passage number (around 20) were used, and six more experiments, 10–15, were carried out with the construct CF Neo^2. A total of 10,493 G418-resistant colonies were screened in these six experiments, mostly in pools of 15–60 visible colonies. Not until experiment 15 was another PCR-positive pool identified.

Thus, we identified a total of five pools giving PCR signals indicating successful targeting (of which four are certainly independent since they were from different electroporations) from a total of >12,000 G418-resistant colonies, screened in pools, and >1800 individually picked colonies resistant to

both G418 and ganciclovir (corresponding to >8400 G418 equivalents). Some clustering of positive pools is apparent in the data summarized in Table 1.

Isolation of PCR-Positive Clones. ES cells from the five PCR-positive pools were treated with trypsin and replated onto fibroblast feeder layers in the presence of G418. Individual colonies were picked and divided into two portions—one as a replica and one for further PCR tests. In this manner, we were able to isolate PCR-positive colonies from four of the five PCR-positive pools identified in the course of our experiments.

Analysis of the Isolated Clones. PCR-positive clones isolated in the previous step were expanded to a level sufficient for testing their genomic DNA by Southern blot analysis. The simplest characteristic of correctly targeted cells (Fig. 1C) is that the size of restriction fragments spanning exon 10 should increase by 2.7 kb. Fig. 3 shows the results of digesting DNA from four colonies isolated in experiments 6B and 8 in Table 1 with *Sst* I or *Kpn* I and comparing Southern blots of these digests to those from the starting ES cells (E14TG2a). Hybridization was to a probe specific for sequences in intron 9 of the *CFTR* gene (see Fig. 1). The correctness of targeting in both colonies was established by the blots, which showed that one of the two *CFTR* alleles has now increased in size by 2.7 kb. Identical results were obtained with other isolates of PCR-positive cloned cells. Tests with other restriction enzymes, including *Bam*HI, *Eco*RV, and *Eco*RI, gave patterns (data not shown) that completely agreed with the correctness of the targeting. The exogenous DNA had only inserted into the target locus, as judged by the absence of any hybridizing bands (other than the targeted fragment), when the Southern blots were probed with a *Neo* gene probe specific for the incoming DNA (data not shown). The absence of any nonhomologously integrated copies of the *Neo* gene excludes the possibility that cotransformation played any role in isolating the targeted colonies.

Factors Affecting Targeting. Although our experiments demonstrate that the *CFTR* gene in mouse E14TG2a ES cells can be modified by gene targeting, the frequency of targeting is low—$\approx$1/2500 cells transformed to G418 resistance. We have attempted to identify a feature(s) responsible for this low frequency. One possibility is that expression of the *Neo* gene(s) when incorporated into the *CFTR* gene is too low to ensure survival of all targeted cells in medium containing G418. Against this possibility is our observation (data not shown) that cells targeted at the *CFTR* locus with CF Neo^2 survive perfectly well in medium containing 400 μg of G418 per ml, which is twice that used during selection.
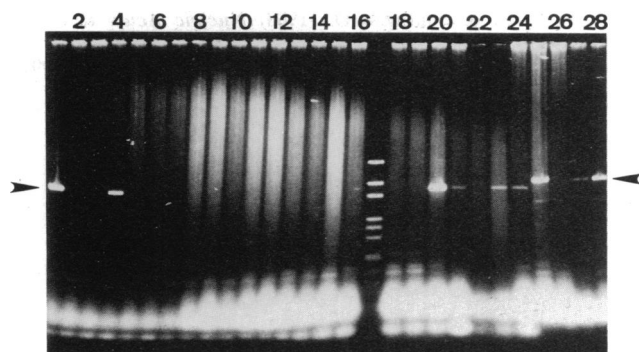

FIG. 2. Agarose gel electrophoresis of PCR-amplified lysates from pools of colonies of G418-resistant ES cells. Lanes 1–16, samples from pools 8.34–8.49 from experiment 8 in Table 1 (containing $\approx$10 colonies per pool) after PCR amplification with primers 1 and 2 (see Fig. 1). Pools 8.34 (lane 1) and 8.37 (lane 4) show the amplified fragment (right pointing arrowhead) characteristic of targeting. Lanes 18 and 19, samples from pools previously giving negative results. Lanes 20, 21, and 24, samples from a pool (6.35) that had given positive results in experiment 6B in Table 1. Lanes 22 and 23, samples of the positive control cells (see text) mixed with untargeted ES cells at ratios of 1:100 and 1:10. Lanes 25 and 26 and lanes 27 and 28, samples of pool 6.35, and of the 1:100 and 1:10 mixes of the positive control cell, respectively, after PCR amplification with primers 3 and 4; the size of the amplified fragment (left pointing arrowhead) is that expected for a targeted cell with these two primers. Lane 17, size marker.
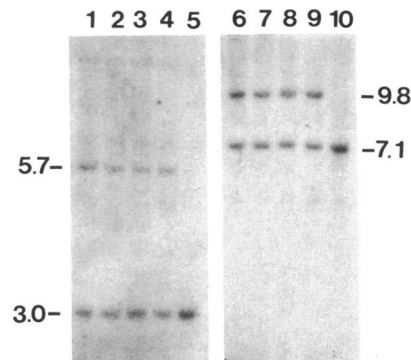

FIG. 3. Southern blots of targeted ES cells with a probe that hybridizes to CFTR sequences from intron 9 (see Fig. 1). Genomic DNA from two colonies from experiment 6B (lanes 1, 2, 6, and 7), two from experiment 8 (lanes 3, 4, 8, and 9) and from the starting ES cells (lanes 5 and 10) digested with *Sst* I (left blot), and *Kpn* I (right blot). The sizes of the fragments are indicated in kb.

The CFTR-targeting events detected were clustered in a manner suggesting that the ES cells were easier to target at some times than at others. Thus, a cluster of targeting events was observed in experiments 6, 8, and 9, and none was detected again until experiment 15. A variable that appears to parallel this distribution is the passage number of the ES cells being treated. The ES cells at the beginning of our work were early passage cells (passage number, $\approx$20); by experiments 6–9 the passage number had exceeded 50. Experiment 10 was with freshly thawed low-passage ES cells (passage number, $\approx$16); it was negative after screening more G418-resistant colonies than gave four PCR-positive pools in experiments 6–9. Not until experiment 15 with LIF-adapted cells (passage number, >50) was targeting again observed. These data suggest but do not prove that obtaining CFTR targeted cells is easier with later passage ES cells. Unfortunately, the frequency at which ES cells can contribute to the germline when injected into blastocysts decreases with increase in passage number. Comparison of targeting at a different locus, the hypoxanthine phosphoribosyltransferase (*HPRT*) locus, reveals a similar trend (data not shown). Thus, the ratio of targeting the *HPRT* locus with pMP8 (17) to transformation with pMC1 Neo (7), when both plasmids were introduced simultaneously, changed from $\approx$0.1 with LIF-adapted ES cells at passage number 25 to $\approx$0.9 at passage number >50. Determining the reproducibility and significance of these trends requires additional studies.

Our failure to detect any CFTR-targeting events in >1800 (G418 + ganciclovir)-resistant colonies (equivalent, when corrected for enrichment, to >8000 G418-resistant colonies) requires comment in light of our detection of $\approx$1 event for every 2500 colonies selected only in G418. It could be a reflection of the effect of passage number, since the double-selection experiments were with lower passage cells than the G418 experiments. It could be because small colonies may be missed when they are individually picked and pooled before PCR testing (all doubly selected colonies were individually picked), while comparable colonies give a detectable PCR signal when screened in a preexisting pool (essentially all singly selected colonies were in pools). It is not due to some unexpected sensitivity of the CFTR-targeted cells to ganciclovir, since we found them fully resistant to ganciclovir at twice the concentration used in selection.

Conclusion. Although there are some complicating features in targeting the CFTR gene in ES cells by homologous recombination, our data show unequivocally that targeting exon 10 of the gene is possible and is reproducible.

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