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Homologous Recombination in Hybridoma Cells: Dependence on Time and Fragment Length

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Mutant hybridoma-myeloma cell lines that are defective in immunoglobulin production are expected to be useful for defining the molecular requirements of immunoglobulin gene expression. The analysis of such mutants would be greatly facilitated if they could be mapped by marker rescue, i.e., by identifying the segments of wild-type DNA that can restore the normal phenotype by homologous recombination with the mutant chromosomal immunoglobulin gene. To assess the feasibility of this type of mapping, we have measured the efficiency with which fragments of wild-type DNA recombine with a mutant hybridoma immunoglobulin gene and restore normal immunoglobulin production. We found that most if not all recombinants were detectable 2 days after DNA transfer and that the frequency of gene restoration increased with increasing length of the transferred μ gene fragments, between 1.2 and 9.5 kilobases. These results indicate that the available technology should be adequate to map mutations in the μ gene to within ~1 kilobase.

Homologous recombination between transferred and chromosomal DNA permits us to make predetermined changes in chromosomal genes and thus permits us to test the importance of specific sites for the expression of genes in their normal chromosomal location. However, the chromosomal DNA of mammalian cells is vast, and the technology for introducing mutations is time consuming and labor intensive. For these reasons, it is necessary to identify and locate candidate sites before undertaking this approach. Assays that measure expression from transferred rather than chromosomal genes have evidenced many candidate sites, such as enhancers and silencers, and it will be interesting to measure their effects on expression in the normal chromosomal context. It is also possible that important expression elements exist which will not be detected with this type of gene transfer-expression assay, either because the elements are far from the exons (14, 15) and are not tested or because their activity depends on a still undefined aspect of the normal chromosomal locus. If so, it will be advantageous to use the traditional microbial approach of identifying such sites by analyzing mutants that have been selected phenotypically, for example, because of a defect in gene expression.

The immunoglobulin genes of myeloma and hybridoma cells are amenable to this type of approach because (i) the immunoglobulin genes are haploid, highly expressed, and well characterized, (ii) the cell lines grow rapidly, and (iii) efficient mutant selection and screening protocols have been devised (5, 9). To identify such mutations, it will be useful to map them by marker rescue, whereby segments of the normal gene are tested for their capacity to undergo homologous recombination with the mutant chromosomal gene and restore the normal phenotype. This information would thus indicate which segment of the mutant DNA should be cloned and sequenced to identify the mutation.

Our previous work in this area concentrated on the use of homologous recombination to mediate the integration of vector DNA into the immunoglobulin genes (1, 2). In marker rescue mapping, however, it is important that the homologous recombination event restore the normal gene without incorporating extraneous vector sequences that might perturb gene expression. For this type of mapping, long gene segments are useful for the preliminary localization of the mutation; short segments serve to delimit the amount of mutant DNA that must be sequenced.

To test the feasibility of this approach, we have measured recombination between transferred DNA fragments and a previously defined mutant chromosomal μ heavy-chain gene. Our results suggest that isolated fragments of DNA can be used to map mutations by marker rescue to within 1 kilobase (kb) 2 days after gene transfer.

MATERIALS AND METHODS

Tissue culture. Techniques and media have been described previously (10).

DNA transfer. In the experiments shown in Table 1, the specific fragments of the μ gene were excised by digestion with the indicated restriction enzymes, after which they were isolated by electrophoresis in agarose, electroelution, and purification by phenol extraction and ethanol precipitation. In the experiment shown in Table 2, the fragments were excised from the vector with the indicated enzymes, and the mixture of vector and μ fragment was present during electroporation.

For electroporation, the hybridoma cells were grown to a density of 2×10^5 to 3×10^5 cells per ml, harvested by centrifugation, washed, and suspended in phosphate-buffered saline. Then 0.8 ml containing 2×10^7 to 4×10^7 cells were mixed with DNA in an electroporation cuvette (Bio-Rad Laboratories) and subjected to two 25-μF pulses at 750 V (1,750 V/cm) at ~0°C. After 30 min, the cells were transferred to culture medium and incubated for various times before measuring the frequency of plaque-forming cells (PFC). Cell survival was assessed microscopically by comparing the number of viable cells in the electroporated and nonelectroporated cultures the day after DNA transfer and ranged from 30 to 60%. The surviving cells were then expanded so that several plates each containing ~10^7 cells were assayed for plaques as described previously (1).

Analysis of recombinants. Isolation of PFC by sib selection has been described elsewhere (1). Immunoglobulin M (IgM) was biosynthetically labeled with [^{14}C]leucine, purified by

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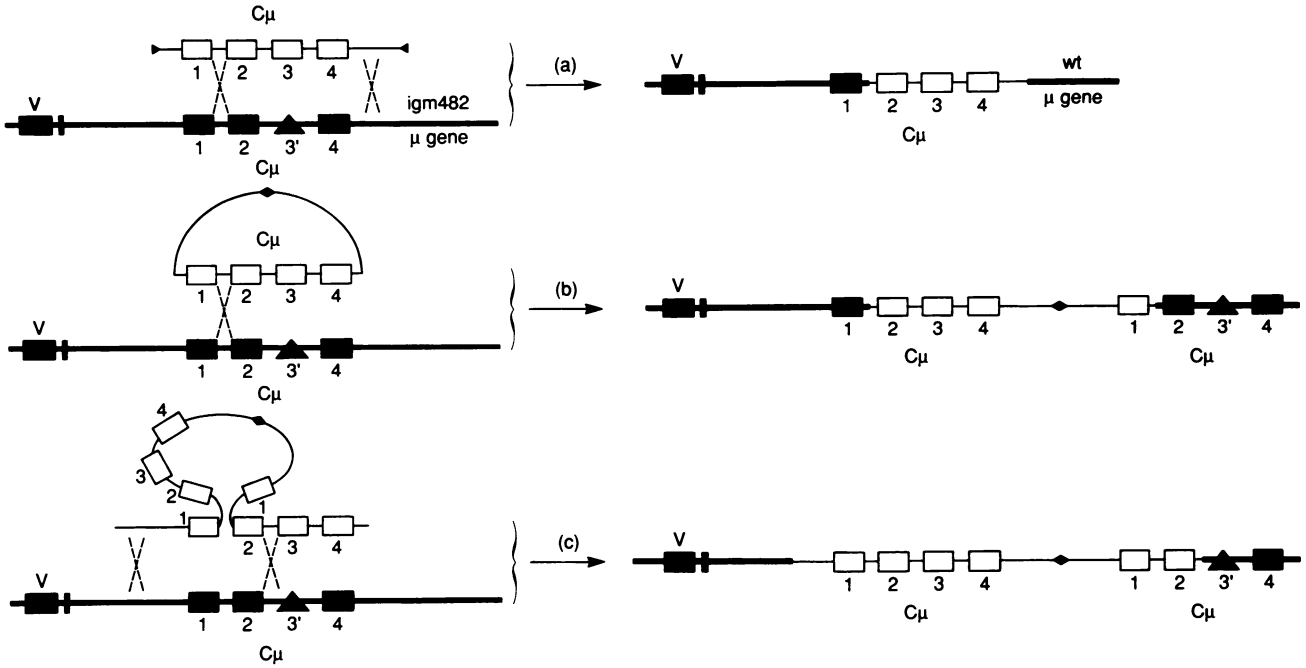

FIG. 1. Pathways of recombination. The diagram represents recombination between the mutant chromosomal μ gene (thick line) of igm482 and transferred DNA (thin line). In pathway a, a fragment of the μ gene including the four exons encoding the μ constant region replaces part of the chromosomal μ gene so as to restore the normal Cμ3 exon. In pathway b, the fragment has circularized before recombining with the chromosomal μ gene, so that a single reciprocal crossover upstream of the chromosomal mutation restores a normal μ structural gene. In pathway c, two DNA fragments have ligated intracellularly before recombination with the chromosomal μ gene. Gene conversion or double crossover can restore a normal μ sequence.

binding to DNP-Sepharose, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4). DNA blots were prepared as described by Southern (11). For karyotyping, cells were incubated in colcemid (0.01 μg/ml for 2 h at 37°C). Chromosomes were visualized by Giemsa staining.

RESULTS

We have previously described the system used here to measure homologous recombination between transferred and chromosomal immunoglobulin DNA (1). In brief, we make use of several mutant cell lines derived from the hybridoma Sp6, which makes IgM (κ) specific for the haptens dinitrophenol and trinitrophenol. The mutant hybridoma cell line, igm482, was derived from Sp6 and has a 2-base-pair (bp) deletion in the exon encoding the third constant region domain of the μ heavy chain. This mutation results in a truncated IgM that is unable to activate complement. We assay for homologous recombination by measuring the capacity of transferred DNA to restore the wild-type μ gene in igm482. Wild-type cells are conveniently detected by a plaque assay. That is, wild-type cells make plaques on trinitrophenol-coupled erythrocytes with an efficiency of ~0.8 plaques per cell, whereas igm482 cells plaque with efficiency of less than 10^{-7}.

Figure 1 shows three pathways by which the transferred DNA fragments could in principle restore μ gene function in igm482. Pathway a depicts a simple gene replacement event, such as might occur by gene conversion or double crossover. Other pathways are suggested by the finding that the ends of transferred DNA can ligate intracellularly (16). For example, in pathway b, the fragment circularizes and integrates by a single reciprocal crossover. Pathway c involves transferred fragments that catenate intracellularly and yield an integrated copy of the fragment, which, depending on the crossover sites, could be the same structure as in pathway b. The segments of DNA that were used in these experiments are shown in Fig. 2.

Kinetics of PFC formation. We measured the time course of the appearance of PFC after transfer of μ segment A. For this purpose, fragment A was excised from a pSV2neo-derived vector, and 15 pmol of digested DNA (15 pmol of fragment and 15 pmol of vector) was transferred to igm482 cells. The PFC frequency (plaques per 10^7 cells) was then measured at various intervals after DNA transfer: 5 h, 0 PFC; 23 h, 3 ± 2 PFC; 30 h, 10 ± 8 PFC; 47 h, 48 ± 25 PFC; 72 h, 54 ± 29 PFC; 96 h, 51 ± 13. These results indicate that most recombinants become apparent between 23 and 48 h after gene transfer. Reconstruction experiments indicated that electroporation does not cause a decrease in the plaquing efficiency of wild-type (Sp6) cells (data not shown). The significance of the delay in appearance of the wild-type recombinants is considered further in Discussion.

Dependence on length of homology. To assess the dependence of recombination frequency on fragment length, we isolated various subfragments of the μ constant-region gene. These fragments ranged from 1.2 to 9.5 kb (Fig. 2) and were chosen such that the mutation in the chromosomal gene corresponds to the middle of the fragment. It should be noted that in the course of molecular cloning, our (otherwise wild-type) μ gene lost a 3-kb segment from the switch region, which is therefore lacking in the 9.5-kb *Eco*RI fragment but present in the chromosomal μ gene of the hybridoma. The frequency of PFC increased with increasing fragment size over the range 1.2 to 9.5 kb (Table 1 and Fig. 3). No plaques

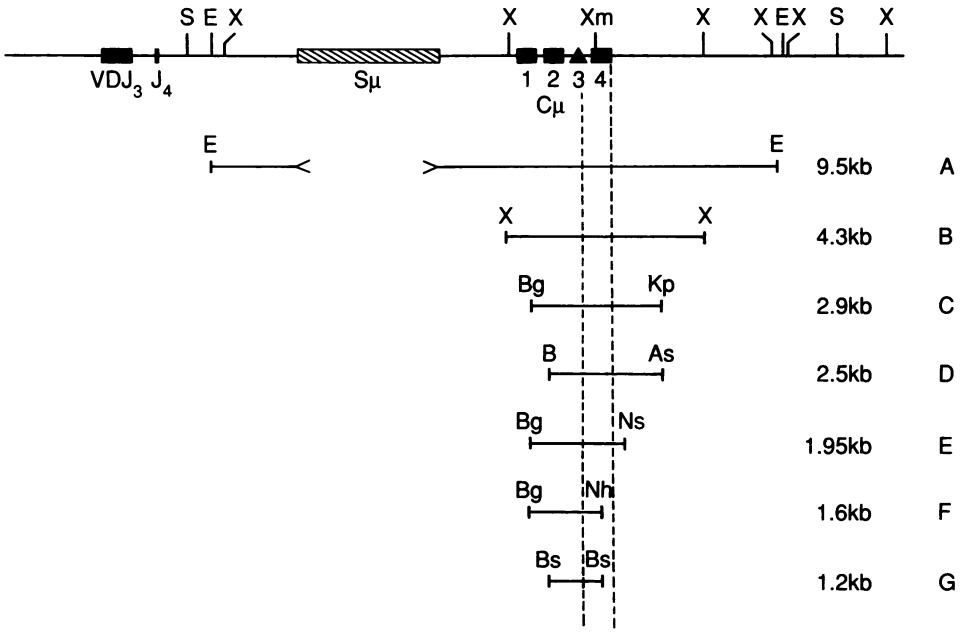

FIG. 2. Map of μ gene and transferred μ gene fragments. The top line shows the relevant restriction enzyme sites in the μ gene of igm482. The fragments, labeled A to G, that were transferred are shown along with their sizes. The break in fragment A corresponds to the deletion introduced in the cloning of the μ gene (see text). The vertical dashed line in Cμ3 indicates the site of the 2-bp deletion in igm482 (which destroys an *Xmn*I site present in the normal μ gene). The dashed line at the 3′ end of the Cμ4 exon is included to indicate fragments that must lack the 3′ end of the μ gene. Enzyme abbreviations: As, *Asp*I; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; K, *Kpn*I; Nh, *Nhe*I; Ns, *Nsi*I; S, *Sca*I; X, *Xba*I; Xm, *Xmn*I.

were obtained after transfer of a mutant fragment B bearing the same 2-bp deletion as is in the recipient igm482 cells.

A possible explanation for the finding that longer fragments yielded more recombinants was that the transferred DNA was subject to extensive exonucleolytic degradation and that the wild-type sequence in the middle of the longer fragments was better protected than the sequence in the shorter fragments. This possibility was tested by measuring whether recombination frequency was increased when nonhomologous DNA was added to the ends of the short fragments. We confirmed that nonhomologous DNA at the ends of the molecule does not impede the recombination process (7), i.e., that when the vector bearing fragment B was linearized in the region of nonhomology, such that the fragment was bounded by ~2 kb of nonhomologous DNA, this DNA preparation generated PFC at a frequency comparable to that of isolated fragment B (Table 1). Similarly, we found that the isolated fragment G gave PFC at approximately the same frequency as was obtained when this fragment was bounded by ~2 kb vector segments (Table 1). Taken together, the results for segments B and G imply that the higher recombination frequency of the longer fragments is not due to a protective effect of the extra DNA.

TABLE 1. Dependence of recombination on fragment length

Fragment	Mean frequency of PFC (plaques/10^7 cells [range])[a]	
	Excised[b]	Within linearized vector[c]
A	100 (92–109)	
B	20 (10–28)	13 (8–22)
C	12 (7.3–20)	
D	4 (2.2–7.7)	
E	8 (5.2–10)	
F	4 (3–7.5)	
G	3 (1–4)	3 (1–5.9)

[a] Calculated from two to four independent experiments.

[b] The indicated fragments of the μ gene (A to G; Fig. 2) were excised and purified by gel electrophoresis.

[c] The vectors (fragment B in pSV2neo; fragment G in pUC18) bearing fragments A to G were linearized so as to yield DNA in which the μ gene fragment was bounded on each side by approximately 2 kb of vector sequences. Then 8 pmol of each fragment was transferred to igm482 cells, and the resulting PFC were measured 2 to 3 days after electroporation as described in Materials and Methods.

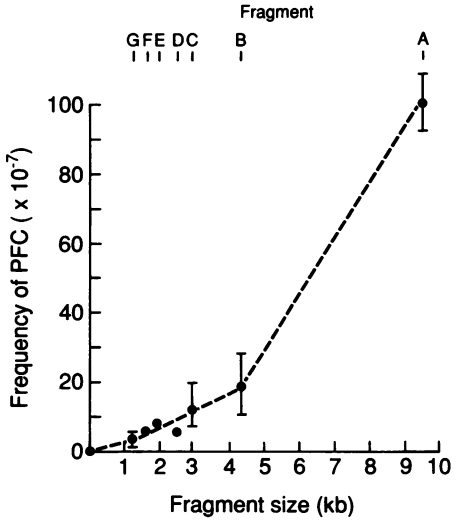

FIG. 3. Dependence of recombination frequency on fragment length. The data from Table 1 are plotted.

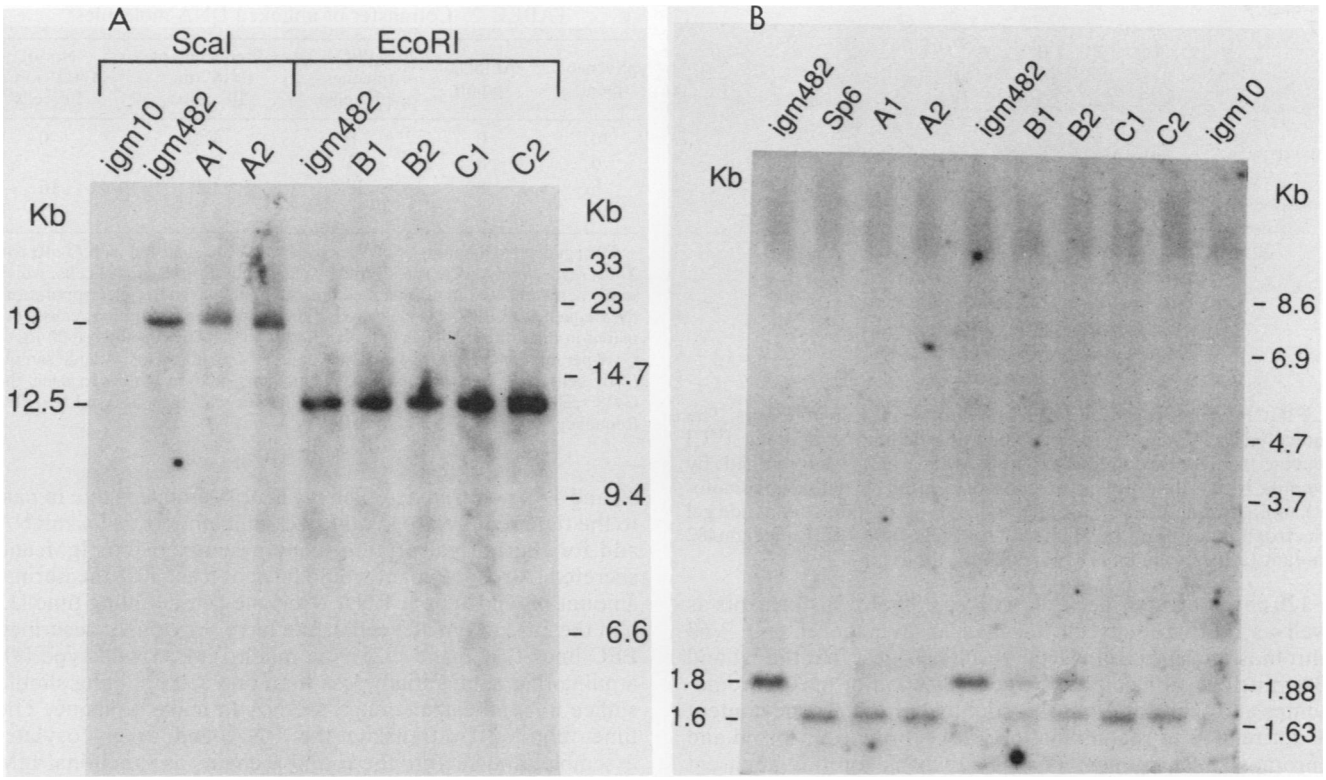

FIG. 4. Analysis of recombinant μ gene structure. (A) Evidence for replacement; (B) restoration of wild-type sequence. Two PFC generated by the transfer of fragments A, B, and C were isolated by sib selection (1) and designated A1, A2, B1, B2, C1, and C2. DNA was isolated from each, digested with the indicated restriction enzymes, and probed with radiolabeled fragment G. (A) DNA from A1 and A2 and appropriate controls was digested with *Sca*I; all others were digested with *Eco*RI. (B) All DNA samples were digested with a combination of *Xmn*I and *Xba*I. The mutant cell line, igm10, has lost the μ gene (5a) and is included as a negative control. The sizes of the μ gene fragments are indicated at the left. Probe G overlaps the 2.5-kb *Xmn*I-*Xba*I Cμ4 fragment by only ~200 nucleotides, and we presume that this overlap is too short to have detected this expected fragment in this experiment.

Recombination pathway. Another potential explanation for the relationship between PFC frequency and fragment size is that whereas larger fragments might restore the wild-type gene by pathway b or c as well as pathway a (Fig. 1), smaller fragments lacking the 3′ end of the gene are expected to restore only by pathway a. That is, if a fragment lacking an essential part of the 3′ end of the μ gene recombined by pathway b or c, the Cμ region proximal to V_H would lack this essential part and would probably be nonfunctional. To test whether recombination with the longer fragments predominantly followed pathways b and c, we isolated DNA from several representative wild-type transformants and analyzed the size of μ gene fragments generated with enzymes that do not cut within the transferred fragments. Integration of a circularized Cμ fragment (pathway b; Fig. 1) or incorporation of a tandem repeat (pathway c) is expected to increase the length of the μ gene by 9.5, 4.3, and 2.9 kb for fragments A, B, and C, respectively (Fig. 2). By contrast, gene replacement (pathway a) is expected not to change the size of the restriction fragments. The μ gene fragments of the recombinant transformants were of normal size (Fig. 4A). That is, DNA from fragment A recombinants was digested with *Sca*I and yielded ~19-kb Cμ fragments, the same size as from the recipient igm482 hybridoma. Similarly, DNA from fragment B or C recombinants was digested with *Eco*RI and yielded ~12-kb Cμ fragments, as in the case of igm482. We conclude from these results that recombination with fragment A, B, or C does not usually occur by pathway b or c or that if such recombinants occur as a major product, they do not express sufficient μ RNA to make a plaque. As mentioned above, fragment A lacks ~3 kb that is present in the normal μ gene. This deletion does not appear in the corresponding recombinant μ genes, suggesting that the 5′ end of this transferred fragment is not usually a crossover point.

We confirmed that the normal sequence had been restored to the μ gene in these transformants as follows. The 2-bp deletion in mutant igm482 destroys the *Xmn*I site normally present in the Cμ3 exon (Fig. 2). To test for this site, we digested the fragment with *Xmn*I in combination with *Xba*I; under these conditions, the probe detects 1.6- and 1.8-kb fragments from wild-type and mutant DNAs, respectively. The wild-type (1.6-kb) sequence was present in each plaque-forming transformant (Fig. 4B). The two transformants derived from fragment B also showed the mutant band. That the 1.6- and 1.8-kb bands were of comparable intensity suggests that individual cells contain one wild-type and one mutant copy of the μ gene. We confirmed this interpretation by showing that both mutant and wild-type μ proteins were synthesized by these transformants (Fig. 5) and by their subclones (results not shown). The presence of two copies of the μ gene in these transformants raises the question of whether a second copy arose as a product of the recombination process. Karyotyping of these and other transformants indicates that this is not the case. That is, the B1 and B2 transformants with two μ genes had an average of ~123 and

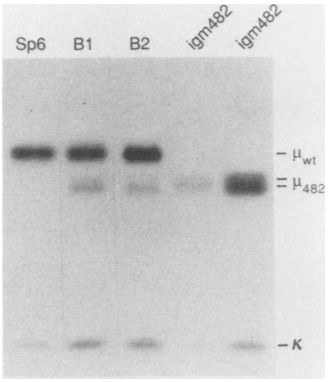

FIG. 5. Analysis of μ protein in recombinant transformants. The indicated cell lines were incubated in medium containing [^{14}C] leucine to label protein biosynthetically. IgM was purified by binding to 2,4-dinitrophenyl-Sepharose, reduced with 2-mercaptoethanol, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positions of the wild-type (μ_{wt}) and igm482 mutant (μ_{482}) μ chains are indicated on the right.

~120 chromosomes per cell, whereas other transformants as well as the parental cell line had an average of only ~68 chromosomes per cell. These results suggest that the B1 and B2 transformants contain two copies of all or most chromosomes and therefore that the doubling of the μ gene content is not related to recombination between the transferred and chromosomal μ genes. Possible reasons for this apparent doubling of chromosomal content are considered further in Discussion.

Cotransfer of unlinked DNA. The low frequency ($<10^{-5}$) of gene restoration makes it tedious to recover the PFC by sib selection. Markers on unlinked DNA molecules have been reported to be cotransferred at high frequency when introduced by electroporation (13). We have tested whether homologous recombinants could likewise be enriched by the cotransfer of pSV2neo and selection of G418-resistant (G418^r) transformants. The source of the Cμ fragment in these experiments was the vector pT18Cμ, which had been constructed by inserting the 4.3-kb fragment B at the *Xba*I site in pUC18. For these experiments, the Cμ fragment was excised (but not purified) by digesting pT18Cμ with *Xba*I. The vector psV2neo was cut with *Eco*RI, according to the rationale that linearization of the DNA enhances the frequency of stable G418^r transformants (data not shown) and that by maximizing the number of G418^r transformants, we might maximize the number of G418^r recombinants. These fragments (Cμ fragment B and pUC18/XbaI; pSV2neo/EcoRI) were transferred either separately or together to igm482 cells, and the frequency of PFC in both the G418^r and unselected populations was measured (Table 2). The frequency of PFC in the G418^r population was $\sim 15 \times 10^{-7}$, approximately threefold lower than the frequency of PFC in the (G418) unselected population. This negative enrichment notwithstanding, we note that DNA transfer generated $\sim 3 \times 10^4$ G418^r transformants and that this population consistently included at least one PFC, suggesting that initially the frequency of μ recombinants in the G418^r population was $\sim 1/3 \times 10^4 = \sim 3 \times 10^{-5}$, substantially higher than in the unselected population. The possible reasons for this discrepancy will be considered in Discussion.

TABLE 2. Cotransfer of unlinked DNA molecules[a]

pSv2neo (pmol)	pT18Cμ (pmol)	PFC (plaques/ 10^7 cells)	Frequency of G418^r (no./ 10^3 cells)	No. of G418^r PFC/ 10^7 cells
30	0	0	4.2	0
0	8	43	0	
8	8	38	3.1	16
30	8	26	3.8	13

[a] The indicated amounts of pSV2neo and pT18Cμ, digested with *Eco*RI and *Xba*I, respectively, were transferred by electroporation to igm482 cells, which were then incubated in normal medium. At 2 to 3 days after electroporation, the frequency of PFC was measured; 3 days after electroporation, cells were plated in microtiter wells at limiting dilution in selective medium (600 μg of G418 per ml) to measure the frequency of G418^r transformants. A large sample ($>10^7$ cells) was also incubated in G418 medium for 10 to 14 days to select the G418^r cells in batch. This selected population was then assayed for PFC frequency.

DISCUSSION

Recombinants begin to be detectable about 23 h after DNA transfer and become fully apparent some time between 30 and 47 h posttransfer. The lag in appearance is due in part to the time required to synthesize sufficient normal μ mRNA and for the normal protein to be assembled into IgM and secreted. A recombinant would have at least 50% the normal amount of wild-type μ RNA after one cell doubling time (12 h in the case of igm482 cells). We have previously described PFC lines that make ~25% as much IgM as wild type (9), arguing that substantially less than one cell doubling should suffice to synthesize enough μ RNA to make a plaque. The time required to translate the RNA and to glycosylate, assemble, and secrete the μ and κ chains as functional IgM is estimated to be 2 to 4 h (8). Thus, ~10 h should suffice to obtain adequate RNA synthesis and protein secretion for plaque formation. These considerations argue that there is an unaccounted delay of ~20 to 37 h between DNA transfer and full-level expression of the recombinant μ gene. Wong and Capecchi (17) concluded that homologous recombination between transferred DNA molecules occurs during S phase. Homologous recombination between transferred and chromosomal DNAs might also require a round of DNA synthesis, which would occur over a period of ~12 h for (exponentially growing) igm482 cells. the delay could be longer if the normal cell cycle has been perturbed in the recombinants. We do not know whether the delay represents the time required for the transferred DNA to recombine with the chromosomal μ gene or whether the recombinant is actually formed earlier but remains in a silent state. It is not surprising that the number of PFC does not rise detectably after 2 days. Bertling et al. (3) have observed that newly introduced DNA persists in the nucleus with a half-life of 15 to 24 h. Moreoever, the exponential growth of the early recombinants is expected to obscure PFC arising from later recombination events.

For the sake of discussion, we estimate the number, N, of independent recombinants that are generated in this type of experiment by the formula $N = (FS)\,(2^t)/p$, in which F is the frequency of PFC, S is the number of cells that survive electroporation, t is the number of doubling times that transpire between DNA transfer and the moment when the recombinants begin to grow (exponentially), and p is the plaquing efficiency of wild-type cells ($p = \sim 0.8$). The uncertainty about the cause of the lag in appearance of the recombinants makes the value of t uncertain. In the calculations considered below, we assume that one cell division (~ 12 h) intervenes between DNA transfer and the formation of exponentially growing recombinants.

Our procedure of selecting for expression of cotransferred, unlinked DNA did not enrich significantly for homol-

ogous recombinants. These experiments generated $\sim 3 \times 10^4$ G418^r transformants, so that the lowest non-zero frequency of G418^r PFC is expected to be $\sim 1/3 \times 10^4$, 15-fold higher than the observed frequency. We suggest that this discrepancy arises because independent G418^r transformants express the *neo* gene at different rates and grow at different rates in the presence of G418. When only a few G418^r PFC are generated, it is unlikely that these few transformants will be among the fastest growing, and thus it is expected that the PFC will ultimately be overgrown by faster-growing G418^r transformants. Although the uncertainty in the relative growth rates means that we cannot accurately assess the enrichment that cotransfer might initially have provided, our results place a lower limit on the enrichment. That is, if only one G418^r PFC was generated, its inclusion among the other $\sim 3 \times 10^4$ G418^r transformants represents a frequency of $\sim 3 \times 10^{-5}$, an enrichment of approximately sixfold over PFC frequency ($\sim 5 \times 10^{-6}$) in the unselected population. The 15-fold difference between the observed and expected minimum frequency of G418^r PFC after ~30 doublings could be accounted for by a difference of ~14% between the doubling times of the one G418^r homologous recombinant and other G418^r transformants. Inasmuch as these experiments generated ~100 PFC, a population which consistently included at least one G418^r PFC, the efficiency of cotransfer and coexpression of the unlinked DNAs must have been at least 1%. By way of comparison, Toneguzzo et al. (13) reported a value of ~50% for coexpression of unlinked DNA molecules transferred by electroporation. If, in fact, differential growth rates obscured an initial enrichment for PFC, it should be possible to preserve the enrichment by plating the cells at limiting dilution (one G418^r cell per well). Similarly, the overgrowth problem would probably be less severe in the case of adherent cells.

The occurrence of transformants that have apparently doubled their chromosome content is puzzling. Electric pulses can be used to fuse cells, although the optimum conditions for promoting cell fusion are thought to be quite different from those used here for electroporation (18). Although we did not detect any such cases of chromosome doubling in the igm482 culture, it should be noted that a sample of the igm482 culture as it existed at the time of DNA transfer was not preserved and therefore it was not possible to measure contemporary karyotypes. That the chromosome doubling was observed in two recombinants raises the question of whether chromosome doubling might be enhanced among recombinants. On the other hand, there is the trivial possibility that these two recombinants are sibs. We estimate that this experiment yielded ~50 transformants; thus, the probability that the two recombinants that were analyzed were sibs is low but not forbidding.

Our analysis of the DNA structure of recombinants obtained with the 9.5-kb fragment A, which has a 3.5-kb deletion, indicates that the crossover points in these recombinants did not occur at the 5′ end of the transferred fragment, similar to the findings of Koller and Smithies (6), who used much smaller mutations as markers. Our results confirm the report of Mansour et al. (7) that the presence of extensive regions of nonhomologous DNA at the ends of the fragments does not significantly influence recombination frequency.

The dependence of recombination on fragment length is expected to be complicated. To mention just a few of the potential variables, a longer region of homologous transferred DNA has a correspondingly larger number of matches with the chromosomal gene, and this effect is expected to increase the frequency of recombination. On the other hand, larger DNA molecules are expected to diffuse less rapidly, so that migration to the nucleus is expected to be slower and encounters with the chromosome are expected to be fewer. Our results indicate that the frequency of homologous recombination between the chromosomal μ gene and transferred fragments increases with increasing length of homologous DNA. Although the variability of the results precludes us from deducing the exact relationship between fragment length and recombination frequency, it appears that recombination frequency might increase more than linearly with fragment length. It might be important, however, that the aforementioned 3-kb deletion in fragment A interrupts the homology between the transferred and chromosomal μ genes; we do not know whether this interruption might influence recombination frequency. The ~4-fold difference in recombination that we observed for fragments of 4.3 and 9.5kb is less than the ~20-fold difference reported for the HPRT gene in ES cells by Thomas et al. (12), who used a vector with a *neo* insertion as well as an HPRT deletion.

There are two evident limitations in using this system to map immunoglobulin mutations. First, in order to measure sufficient PFC, it is necessary to electroporate $\sim 10^7$ cells and assay them for PFC; PFC can be detected under these circumstances only if the mutant recipients make very little or no cytolytic IgM. These considerations mean therefore that only tight mutations can be mapped with the methods described here. Second, the precision of the mapping is determined by the smallest fragment size that can be reliably tested for marker rescue. Our results can be used to estimate the likelihood of false-negatives, although it is important to note that we do not know whether recombination frequency will be the same throughout the immunoglobulin gene locus. The PFC frequencies from the 9.5- and 1.2-kb fragments (Fig. 3) correspond to ~250 and ~8 independent recombination events, respectively. The limit of detection is ~1 PFC per 10^7 cells per plate. Inasmuch as the 1.2-kb fragment G yielded only ~3 PFC per 10^7 cells, we feel that ~1 kb represents the smallest size fragment that can be practically assessed by marker rescue. If these same values obtain throughout the (mutant as well as wild-type) μ heavy-chain locus, it will be possible to map (tight) mutations in the μ gene to within ~1 kb quickly and conveniently by marker rescue.

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LITERATURE CITED

1. **Baker, M. D., N. Pennell, L. Bosnoyan, and M. J. Shulman.** 1988. Homologous recombination can restore normal immunoglobulin production in a mutant hybridoma cell line. Proc. Natl. Acad. Sci. USA **85**:6432–6436.
2. **Baker, M. D., and M. J. Shulman.** 1988. Homologous recombination between transferred and chromosomal immunoglobulin κ genes. Mol. Cell. Biol. **8**:4041–4047.
3. **Bertling, W., K. Hunger-Bertling, and M. J. Cline.** 1987. Intranuclear uptake and persistence of biologically active DNA after electroporation of mammalian cells. J. Biochem. Biophys. Methods **14**:223–232.
4. **Davis, A. C., K. H. Roux, and M. J. Shulman.** 1988. On the structure of polymeric IgM. Eur. J. Immunol. **18**:1001–1008.
5. **French, D. L., R. Laskov, and M. D. Scharff.** 1989. The role of somatic hypermutation in the generation of antibody diversity. Science **244**:1152–1157.

5a. **Köhler, G., M. J. Potash, H. Lehrach, and M. J. Shulman.** 1982. Deletions in immunoglobulin mu chains. EMBO J. **1**:555–563.
6. **Koller, B. H., and O. Smithies.** 1989. Inactivating the β2-microglobulin locus in mouse embryonic stem cells by homologous recombination. Proc. Natl. Acad. Sci. USA **86**:8932–8935.
7. **Mansour, S. L., K. R. Thomas, and M. R. Capecchi.** 1988. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature (London) **336**:348.
8. **Pfeffer, S. R., and J. E. Rothman.** 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and golgi. Annu. Rev. Biochem. **56**:829–852.
9. **Shulman, M. J., L. Bosnoyan, C. Collins, N. Pennell, and M. D. Baker.** 1989. Immunoglobulin molecular genetics: the prospects for mutational analysis of the chromosomal immunoglobulin genes. Genome **31**:175–181.
10. **Shulman, M. J., C. Heusser, C. Filkin, and G. Kohler.** 1982. Mutations affecting the structure and function of immunoglobulin M. Mol. Cell. Biol. **2**:1033–1043.
11. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
12. **Thomas, K. R., and M. R. Capecchi.** 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell **51**:503–512.
13. **Toneguzzo, F., A. Keating, S. Glynn, and K. McDonald.** 1988. Electric field-mediated gene transfer and pattern of integration in lymphoid cells. Nucleic Acids Res. **16**:5515–5532.
14. **Tuan, D. Y. H., W. B. Solomon, I. M. London, and D. P. Lee.** 1989. An erythroid-specific, developmental-stage-independent enhancer far upstream of the human "β-like globin" genes. Proc. Natl. Acad. Sci. USA **86**:2554–2558.
15. **van Assendelft, G. B., O. Hanscombe, F. Grosveld, and D. R. Greaves.** 1989. The β-globin dominant control region activates homologous and heterologous promoters in a tissue-specific manner. Cell **56**:969–977.
16. **Wilson, J. H., P. B. Berget, and J. M. Pipas.** 1982. Somatic cells efficiently join unrelated DNA segments end-to-end. Mol. Cell. Biol. **2**:1258–1269.
17. **Wong, E. A., and M. R. Capecchi.** 1987. Homologous recombination between coinjected DNA sequences peaks in early to mid-S phase. Mol. Cell. Biol. **7**:2294–2295.
18. **Zimmerman, U., and H. B. Urnovitz.** 1987. Principles of electrofusion and electropermeabilization. Methods Enzymol. **151**: 194–221.