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Genetics

Homologous recombination can restore normal immunoglobulin production in a mutant hybridoma cell line

(marker rescue/mammalian gene targeting/immunoglobulin engineering)

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ABSTRACT **We report here the occurrence of homologous recombination between transferred and chromosomal immunoglobulin genes. Specifically, we have corrected a chromosomal immunoglobulin gene mutation by transferring pSV2neo vectors encoding the constant region of the immunoglobulin μ heavy chain to mutant hybridoma cells that bear a 2-base-pair deletion in the third constant region exon of their chromosomal μ gene. After DNA transfer, we detected G418-resistant transformants that produce normal IgM. Analysis of the DNA structure of the μ gene in these transformants indicates that in four of five cases the μ gene has been restored as a result of the integration of a single copy of the transfer vector by a reciprocal homologous recombination event; the fifth case seems to have resulted from gene conversion or double crossover. These results suggest that this technology might be adapted for mapping immunoglobulin gene mutations by marker rescue and for more convenient engineering of specifically altered immunoglobulin.**

The usual methods for identifying the regulatory elements of the immunoglobulin genes have depended on assaying expression from transferred DNA. Several such elements have been defined by comparing expression from specifically altered immunoglobulin genes (1). A possible weakness of this approach is that the transferred genes are assayed in an abnormal context, either integrated at abnormal chromosomal sites or in an unintegrated form. If a regulatory element were to depend on the normal chromosomal context or to lie outside the gene segment that is assayed, the usual gene transfer methods would be inadequate.

In microbial systems, these problems are avoided because the chromosomal genes can be modified by homologous recombination with a segment of transferred DNA. Such a technique would be very useful for introducing precise changes into the chromosomal immunoglobulin genes as well as for mapping regulatory mutations, thus providing an important tool for analyzing immunoglobulin gene expression. To assess the feasibility of this approach, we have tested for homologous recombination between transferred and chromosomal immunoglobulin genes by using, as recipient cells, a mutant hybridoma with a frameshift mutation in the DNA encoding the constant region of the μ heavy chain (C_μ region) and vectors that bear DNA encoding the normal C_μ region. Here we report that homologous recombination can occur between transferred and chromosomal μ genes so as to regenerate a wild-type μ gene sequence and restore normal IgM production.

MATERIALS AND METHODS

General Techniques. The cell lines and methods for cell culture and DNA analysis have been described (2–4).

Gene Transfer. The construction of the pRCμ and pTCμ vectors carrying the subfragment of the wild-type μ gene is described in the text. The appropriate vector DNA (50 μg) was linearized by *Bam*HI digestion, extracted with phenol, precipitated with ethanol, and resuspended in 50 μl of phosphate-buffered saline (PBS) (2). Mutant igm482 cells were harvested by centrifugation and were washed, and 2×10^7 cells were resuspended in 0.45 ml of PBS. Prior to electroporation, the plasmid DNA and igm482 cells were kept on ice. The plasmid DNA and igm482 cells were mixed, subjected to a 700-V, 25-μF pulse, and placed on ice for 10 min. The cells were then transferred to 40 ml of Dulbecco's modified Eagle's medium (DMEM) containing 13% heat-inactivated fetal calf serum and 3.5×10^{-4}% 2-mercaptoethanol (2, 3) and were placed at 37°C. Cell viability was determined by trypan blue exclusion after ≈12 hr of incubation.

IgM Analysis. Plaque-forming cells (PFC) were detected by lysis of 2,4,6-trinitrophenyl (TNP)-coupled erythrocytes by using plaque assays (5, 6) or spot tests (2). IgM was biosynthetically labeled with [^{35}S]methionine and was purified by binding to 2,4-dinitrophenyl-Sepharose, and the μ and κ chains were visualized by Na-DodSO$_4$/PAGE after reduction of disulfide bonds as described (7). The concentration of wild-type IgM in culture supernatants was measured by an ELISA specific for the fourth domain of C_μ ($C_{\mu 4}$) (8, 9).

RESULTS

Detection of Homologous Recombination. The system that we have used to detect homologous recombination is based on the hybridoma Sp6, which bears a single copy of the μ gene (10) and makes IgM (κ chain) specific for the hapten TNP (2). The mutant hybridoma cell line igm482 was derived from Sp6 and bears a 2-base-pair (bp) deletion in the exon encoding $C_{\mu 3}$, the third domain of C_μ, and therefore produces a truncated μ chain lacking the $C_{\mu 4}$ domain (11). IgM bearing this mutant μ chain does not activate complement, which provides a convenient assay for distinguishing mutant and wild-type cells; that is, Sp6 cells make plaques (efficiency = 0.5) on TNP-coupled erythrocytes, whereas igm482 cells do not (efficiency $< 10^{-7}$). The 2-bp deletion destroys an *Xmn* I restriction site in the $C_{\mu 3}$ exon, a feature that can be used to test for the restoration of the wild-type nucleotide sequence.

To make the transfer vectors pRCμ and pTCμ (Fig. 1), a 4.3-kilobase (kb) DNA segment encoding only the C_μ region was inserted in both orientations in pSV2neo, which confers resistance to the antibiotic G418 (13). Because these vectors do not include DNA for the heavy-chain variable region, they cannot themselves encode a μ heavy chain. However, if the vectors integrate into the chromosomal DNA of igm482 so as to juxtapose the normal C_μ region of the vector and the chromosomal TNP-specific heavy-chain variable region, the

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Abbreviations: C_μ region, μ-chain constant region; PFC, plaque-forming cells; TNP, 2,4,6-trinitrophenyl; G418^R, G418 resistant (resistance).

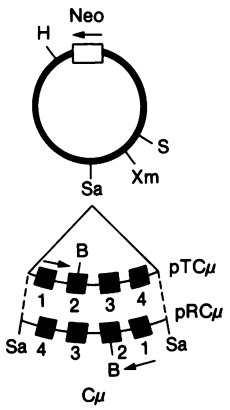

FIG. 1. Construction of transfer vectors. The segment of the μ gene bearing the exons (filled boxes) for the four domains of the C_μ region was inserted in both orientations in a derivative of pSV2neo that lacks the *Bam*HI site (12). For the construction of pRCμ and pTCμ, the *Eco*RI site of pSV2neo was converted to a *Sal* I site. The 4.3-kb *Xba* I fragment encoding the C_μ region was inserted in the modified pSV2neo by using *Xba* I/*Sal* I adapters generating 10-kb vectors in which the transcriptional orientation of the C_μ region is in tandem (pTCμ) or reversed (pRCμ) to the *neo* gene. These vectors have neither *Eco*RI nor *Xba* I sites. The unique *Bam*HI site in the $C_{\mu 2}$ exon of the insert was used to linearize the vector for electroporation. H, *Hpa* I; S, *Sca* I; Xm, *Xmn* I; Sa, *Sal* I; B, *Bam*HI.

resulting recombinant DNA will then encode a normal TNP-specific μ chain. We have assayed for homologous recombination of this type by measuring the frequency of PFC after transfer of these vectors to the igm482 cells (Table 1). The frequency of PFC among untreated igm482 cells is $<10^{-7}$. The frequency of PFC is at least 3- to 5-fold higher among cells that have been electroporated with pRCμ or pTCμ. In the G418-resistant ($G418^R$) populations, the frequency of PFC has increased to $\approx 10^{-3}$. Oligonucleotide-directed mutagenesis (12, 14) was used to introduce the 2-bp deletion present in the μ gene of igm482 into the pRCμ vector, thus generating the vector pRCμ482. The pRCμ482 vector generates $G418^R$ transformants, but does not yield PFC (i.e., none was detected among 2.5×10^4 independent $G418^R$ transformants), implying that the capacity to generate PFC depends on the normal C_μ region of these vectors. As described in the legend to Table 1, we estimate that this gene transfer protocol yields approximately 3 independent PFC from 2×10^7 recipient igm482 cells.

Analysis of μ Protein and DNA Structure. To examine the structure of the μ gene and protein in the PFC, representative transformants were cloned by sib selection from independent DNA transfer experiments. Transformants of igm482 that were derived from pTCμ and pRCμ are designated by the prefixes Im/TCμ and Im/RCμ, respectively (Table 1). In addition, we isolated two randomly selected $G418^R$ transformants, which are designated by R/RCμ and R/TCμ.

We have applied two other tests to verify that the IgM made by the PFC transformants has normal structure. The μ chains of the wild-type and igm482 cell lines differ in molecular mass by ≈15 kDa and are readily distinguished by their mobility in $NaDodSO_4$/PAGE (Fig. 2). All of the Im/RCμ and Im/TCμ transformants produce full-length μ chains indistinguishable from wild-type μ and have apparently ceased to make the mutant μ chain. We have also used a $C_{\mu 4}$-specific ELISA to quantify IgM production and to verify that the TNP-specific wild-type IgM made by these transformants includes the $C_{\mu 4}$ domain (8, 9). Compared to the wild-type Sp6 cells, the Im/RCμ transformants produced approximately the same amount of normal IgM (0.5–1.3 times as much), whereas the Im/TCμ transformants produced approximately 4 times less (0.1–0.4 times as much).

These results indicate that the $G418^R$ plaque-forming transformants make cytolytic IgM bearing a full-length, $C_{\mu 4}$-positive μ chain and thus suggested that the transferred DNA in the Im/TCμ and Im/RCμ transformants had integrated such that the wild-type C_μ region was now adjacent to the TNP-specific heavy-chain variable region. To test this possibility, and in particular to test whether the vectors had integrated by homologous recombination, we measured the size of μ gene and vector fragments generated from transformant DNA by various restriction enzymes. Fig. 3 shows

Table 1. Restoration of normal IgM production by DNA transfer to igm482 cells

DNA transferred	Frequency of $G418^R$ transformants	Frequency of PFC: Nonselected recipient cell	Frequency of PFC: $G418^R$ transformants	PFC isolated for DNA analysis
None	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	—	—
pTCμ	8.3×10^{-4}	5×10^{-7}	$0.2–6 \times 10^{-3}$	Im/TCμ-27.2 (Im/TCμ-38.15) Im/TCμ-3.13 (Im/TCμ-3.10)
pRCμ	9.5×10^{-4}	3×10^{-7}	$1–12 \times 10^{-3}$	Im/RCμ-5C3 Im/RCμ-2A2 Im/RCμ-D7 (Im/RCμ-G3)
pRCμ482	1.3×10^{-3}	$<1 \times 10^{-7}$	$<0.04 \times 10^{-3}$	—

The indicated DNAs (50 μg) were linearized as described in Fig. 1, mixed in PBS (2) with 2×10^7 igm482 cells, and subjected to a 25-μF, 700-V pulse. Cell survival averaged 22%. After incubation in DMEM (2, 3) for 3 or 4 days, cells were assayed for PFC (5) on TNP-coupled erythrocytes and were distributed at limiting dilution in microtiter wells in DMEM containing G418 at 0.6 mg/ml to measure the frequency of $G418^R$ transformants. The $G418^R$ cells were selected in batch culture and tested for PFC. Repeated transfer of pRCμ482 DNA to igm482 generated a total of 2.5×10^4 independent $G418^R$ transformants but no PFC (i.e., the frequency of PFC is less than 0.04×10^{-3} per $G418^R$ transformant). Reconstruction experiments indicated that wild-type (Sp6) cells yield detectable plaques with the same efficiency (≈0.5) in the absence and presence of up to 10^7 igm482 cells. The plaque-forming transformants were isolated by sib selection from the indicated gene transfer experiments. Transformants listed in parentheses are not necessarily independent of the one listed above, as they derive from the same gene transfer experiment. We can estimate the number (N) of independent PFC that these gene transfer protocols generate, subject to two assumptions—namely, that the plaquing efficiency of the PFC is 0.5, like the normal cells and that the PFC arise immediately after electroporation. Then $N = (2 \times 10^7) \times$ (fraction of surviving cells) × (frequency of PFC in nonselected recipient cells) × $(1/0.5) \approx 3$.

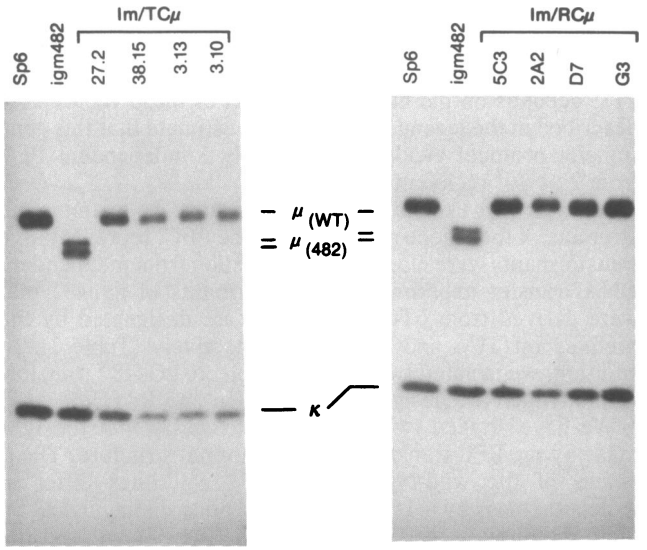

FIG. 2. Analysis of μ chains produced by plaque-forming transformants. The indicated cell lines were incubated in medium containing [^{35}S]methionine to biosynthetically label protein. IgM was purified by binding to 2,4-dinitrophenyl-Sepharose. The μ and κ chains were analyzed by NaDodSO$_4$/PAGE after reduction of disulfide bonds (7). We have found that μ chains made by igm482 in the presence of tunicamycin migrate as a single band (data not shown), suggesting that the double band seen here reflects alternative modes of glycosylation.

fragment sizes expected from the μ genes of igm482 and from the transformant DNA if the transfer vectors have integrated by homologous recombination. The μ genes of igm482 and Sp6 are identical except that the *Xmn* I site present in the $C_{\mu 3}$ exon of Sp6 is destroyed by the 2-bp deletion of igm482. The origins of the probes are shown in Fig. 3*A*: probes a, b, and e contain sequences that are present in the chromosomal μ gene but not in the transfer vectors; probe f contains sequences present in both the chromosomal and vector DNAs; and probe g is the 762-bp *Pvu* II fragment from the *neo* gene. Some of the μ-gene probes detect other (joining region or cross hybridizing) sequences in the hybridoma DNA. To indicate these irrelevant bands, we have included the cell line igm10, an Sp6-derived mutant cell line that has been shown to lack the TNP-specific μ gene (10).

Fig. 4 *A* and *B* present a *Sca* I digest analyzed with probes b and g, respectively. These results indicate that for all the Im/TCμ and Im/RCμ transformants, except Im/RCμ-5C3, the observed fragment sizes correspond with expectations for the case where a single copy of the vector has integrated into the chromosomal μ gene by homologous recombination. The bands detected for DNA from Im/RCμ-5C3 by probe b have the same mobility as for igm482. The low ($<10^{-7}$) frequency of PFC in the igm482 population argues that this transformant did not arise by reversion of the frameshift mutation and consequently suggests that the μ gene was in this case restored by a double crossover or gene conversion event, with the simultaneous acquisition of G418^R by an independent integration event. The same conclusions as to the structure of the recombinant μ genes in the plaque-forming transformants is supported by the analysis of *Eco*RI-digested DNA with probe b (Fig. 4*C*) and *Hpa* I-digested DNA with probes b and g (data not shown). The cell lines R/RCμ-1, R/RCμ-2, R/TCμ-1, and R/TCμ-2 were selected as G418^R transformants and do not produce normal IgM. They, as well as the transformant Im/RCμ-5C3, are expected to have integrated the transfer vector at more-or-less random chromosomal sites. As expected, the sizes of the bands differ among these transformants (Fig. 4*B*).

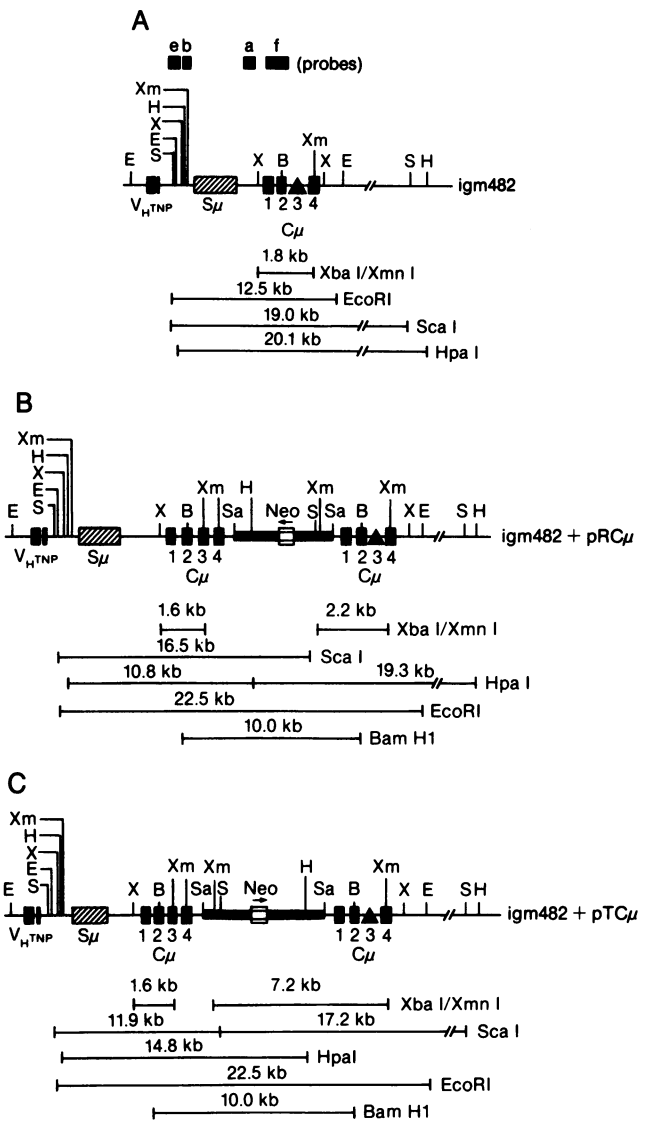

FIG. 3. DNA fragments predicted for the transformant μ genes. The sizes (in kb) that the indicated restriction enzymes should generate are shown for the μ gene of igm482 (*A*) and for transformants where a single copy of the pRCμ (*B*) or pTCμ (*C*) vector has integrated by homologous recombination into the chromosomal igm482 μ gene so as to restore the wild-type μ sequence (from ref. 15; F. Blattner, personal communication). Probe a is the 968-bp *Hin*dIII/*Xba* I fragment; probe b is the 915-bp *Sst* I fragment; probe e is a 1983-bp *Bam*HI/*Eco*RI fragment; probe f is an 870-bp *Xba* I/*Bam*HI fragment; and probe g is the 762-bp *Pvu* II fragment from the *neo* gene of pSV2neo (13). E, *Eco*RI; S, *Sca* I; X, *Xba* I; H, *Hpa* I; Xm, *Xmn* I; B, *Bam*HI; Sa, *Sal* I; V$_H$TNP, TNP-specific heavy-chain variable region.

The pTCμ and pRCμ transfer vectors were linearized before electroporation by digestion at the unique *Bam*HI site in the $C_{\mu 2}$ exon. The transformants that have integrated a vector into the chromosomal μ gene yield a 10-kb *neo*-containing *Bam*HI fragment (data not shown). As shown in Fig. 3, this result indicates that both copies of the C_μ region have their *Bam*HI site intact.

The 2-bp deletion of igm482 destroyed an *Xmn* I site, which would be restored if the vectors integrate by homologous recombination (Fig. 3). By using probe f on DNA that has been digested with a combination of *Xba* I and *Xmn* I, we therefore expected to detect fragments of 1.8-kb from the mutant μ gene compared to 1.6-kb from the wild-type. In addition, the integration of the pTCμ and pRCμ vectors generates downstream fragments that will be detected with

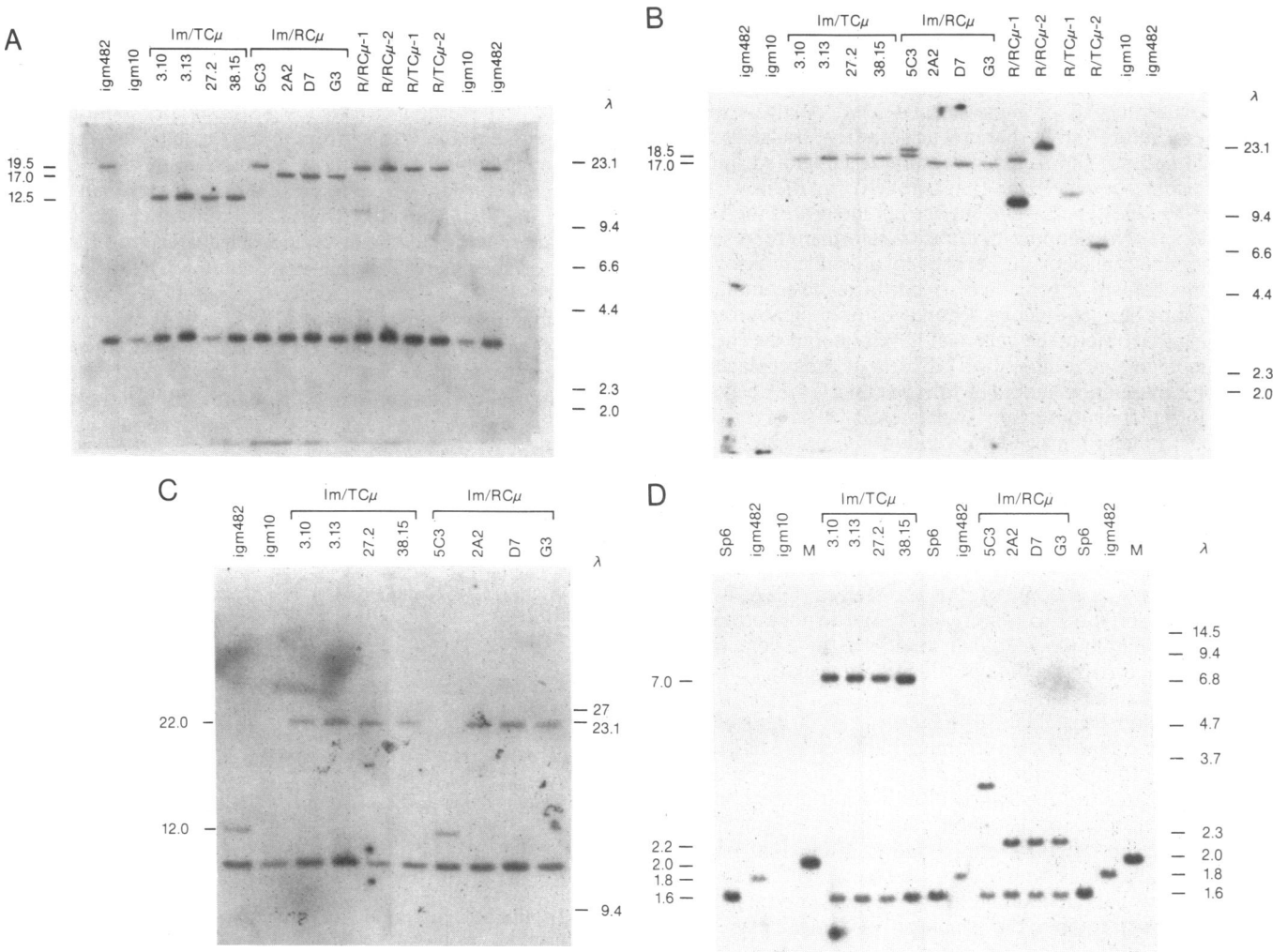

FIG. 4. Analysis of transformant DNA structure. DNA from the indicated cell lines was digested with *Sca* I (*A* and *B*), *Eco*RI (*C*), or *Xba* I/*Xmn* I (*D*) and was probed with probe b (*A* and *C*), probe g (*B*), or probe f (*D*). The size of each band (in kb) of interest is indicated to the left of the blot and was calculated by comparison with the position of the λ marker bands indicated to the right of the blot. M, position of 2.0-kb marker.

this probe. If these fragments have retained the 2-bp deletion of igm482, they are expected to be 7.2 and 2.2 kb from the Im/TCμ and Im/RCμ transformants, respectively, whereas they will be 7.0 and 2.0 kb if they have the wild-type sequence. As shown in Fig. 4*D*, all the Im/RCμ and Im/TCμ transformants show the 1.6-kb band, as expected for transformants that have acquired the wild-type μ gene sequence. The Im/RCμ transformants 2A2, G3, and D7 also show a 2.2-kb band clearly resolved from the 2.0-kb marker (lane M), implying that the downstream copy of the μ gene has retained the 2-bp deletion. The recombinant Im/RCμ-5C3, which is presumably a case of gene replacement, shows a 3-kb fragment in addition to the 1.6-kb band. We suppose that this band derives from the vector integration event that conferred $G418^R$. In addition to the 1.6-kb band, the Im/TCμ transformants also yield a fragment migrating at ≈7 kb. This gel did not resolve 7.2 kb from 7.0 kb and consequently does not indicate whether the downstream C_μ segment in these transformants has retained the 2-bp deletion.

In other digestions, we have confirmed that the chromosomal DNA segment bearing the TNP-specific heavy-chain variable region–C_μ intron has not been altered by the integration of the vectors. That is, by using probe e on *Eco*RI-digested DNA and probe a on *Xba* I- and *Xmn* I-digested DNA, we have shown that the transformant and Sp6 bands comigrate (data not shown). In summary, these results are in each case consistent with the hypothesis that the μ gene in the Im/RCμ and Im/TCμ transformants has been restored by homologous recombination with the transfer vector and that in the cases of integrative recombination the crossovers have occurred between the *Xba* I site just 5′ of $C_{\mu 1}$ and the *Xmn* I site in $C_{\mu 3}$.

DISCUSSION

We have shown that normal IgM production can be restored in the mutant hybridoma igm482 by the transfer of the pRCμ and pTCμ vectors, which encode a normal C_μ region, but not by the vector pRCμ482, which has the same 2-bp deletion as igm482 cells. We estimate that our standard gene transfer protocol, whereby 2×10^7 cells are electroporated in the presence of 50 μg of DNA, yields approximately three independent normal IgM-producing transformants, as detected by their plaque-forming capacity. By selecting for $G418^R$ transformants, the frequency of wild-type μ-gene recombinants is raised to $\approx 10^{-3}$. Other investigators have examined homologous recombination by using different genes, different methods of DNA transfer, and different requirements for gene replacement versus vector integration and have estimated that the ratio of homologous to nonhomologous integrative recombinations ranged from 10^{-2} to 10^{-3} (16–22).

We have concentrated, in this study, on the G418^R transformants because of the greater ease with which the PFC can be isolated and have analyzed five independent transformants of the frameshift mutant igm482, which were selected for their ability to produce normal IgM. Analysis of the μ-gene structure shows that in four of the five transformants, a single copy of the transfer vector has integrated into the chromosomal μ gene. One transformant, Im/RCμ-5C3, has acquired a wild-type μ gene but has not integrated the vector at that locus. We suppose that this transformant represents a case of gene replacement, perhaps by a double crossover or some mechanism of gene conversion. In such recombinants, G418^R must be acquired by a second vector integration event, implying that among the cells that have restored their μ gene by homologous recombination the ratio of gene replacement to vector integration should be lower among G418^R cells than in the unselected population. Inasmuch as we have estimated that our standard procedure yielded approximately three plaque-forming transformants, of which at least one occurred in each case by vector integration, we conclude that the frequency of G418-sensitive PFC in the unselected population is not enormously higher than G418^R PFC. The transformants that integrated the vector into the μ gene have acquired only one copy of the transferred DNA, which evidently is sufficient to confer G418^R. We do not know if the integration of multiple vectors in tandem in the μ gene would depress its expression, thus precluding the isolation of such transformants as PFC.

Thomas and Capecchi (20) have used a chromosomally integrated *neo* gene bearing an amber mutation as a target and have observed that transfer of a segment of wild-type *neo* DNA stimulates the generation of frameshift mutations that suppress the effects of the amber mutation. The igm482 cell line has a 2-bp deletion, and it seems likely that nearby frameshift mutations would suppress the igm482 mutation. However, we have found that in all five independent plaque-forming transformants the mutation of igm482 has been eliminated, suggesting that the stimulated generation of frameshift mutations might reflect peculiarities of the *neo* DNA sequence, such as were pointed out (20).

The Im/RCμ transformants produce, on the average, as much normal IgM as the wild-type hybridoma Sp6 and 4-fold more than the Im/TCμ transformants. The Im/RCμ and Im/TCμ transformants presumably differ only in the orientation of the integrated pSV2neo, thus suggesting that the vector can affect the expression of the nearby μ gene. The Im/RCμ and Im/TCμ transformants seem to be more uniform in their μ-gene expression than stable transformants that have integrated single or multiple copies of complete μ genes at different chromosomal sites and show variable and usually low expression (12, 23, 24). At least for the Im/RCμ transformants, this apparent uniformity of expression cannot be attributed to their having been selected for their plaque-forming capacity, as the (plaque-forming) Im/TCμ transformants make 4 times less IgM. The difference between the expression of the μ gene at its normal and other loci suggests the possibility that the cloned μ gene lacks as yet unidentified elements that are needed for the normal, high-level expression. Some of the hybridoma mutants that have been selected for low-level μ-gene expression (2–4) might be defective in such elements. The finding that homologous recombination can be used to correct and thus map chromosomal mutations in the μ gene suggests that this technology might be used to identify the mutations that affect μ-gene expression. Conversely it might be possible to extend this system to introduce well-defined mutations into the normal μ gene.

The integration of the vectors into the μ gene by homologous recombination has placed the vector-borne C_μ segment so as to separate the original chromosomal DNA segments for the variable and (mutant) C_μ regions. The NaDodSO$_4$/PAGE analysis (Fig. 2) indicates that these transformants have ceased to produce the mutant μ chain. It should, therefore, be possible to use this type of recombination as a much easier method of producing specifically modified immunoglobulin than is currently available. For example, immunoglobulin might be more effective in therapy if it had an altered constant region (e.g., of human rather than mouse origin or toxin linked). DNA encoding the appropriate constant region could be inserted in a transfer vector so as to lie 3′ of the major variable–constant (V–C) intron. Such vectors would be expected to integrate by homologous recombination so as to place the constant region of the transferred DNA in position to be expressed with the endogenous, chromosomal variable region, thus making it possible to produce optimized immunoglobulin without having to isolate, modify, and reexpress the endogenous variable-region genes.

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