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High-Frequency Homologous Recombination between Duplicate Chromosomal Immunoglobulin μ Heavy-Chain Constant Regions

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Homologous recombination was used in a previous study to correct a 2-base-pair deletion in the third constant domain ($C\mu_3$) of the haploid chromosomal μ gene in a mutant hybridoma cell line by transfer of a pSV2neo vector bearing a subfragment of the normal Cμ region (M. D. Baker, N. Pennell, L. Bosnoyan, and M. J. Shulman, Proc. Natl. Acad. Sci. USA 85:6432–6436, 1988). In these experiments, both gene replacement and single reciprocal crossover events were found to restore normal, cytolytic 2,4,6-trinitrophenyl-specific immunoglobulin M production to the mutant cells. In the cases of single reciprocal recombination, the structure of the recombinant μ gene is such that the normal Cμ region, in its correct position 3′ of the expressed 2,4,6-trinitrophenyl-specific heavy-chain variable region, is separated from the mutant Cμ region by the integrated vector sequences. I report here that homologous recombination occurs with high frequency between the duplicate Cμ regions in mitotically growing hybridoma cells. The homologous recombination events were easily detected since they generated hybridomas that were phenotypically different from the parental cells. Analysis of the recombinant cells suggests that gene conversion is the most frequent event, occurring between 60 and 73% of the time. The remaining events consisted of single reciprocal crossovers. Intrachromatid double reciprocal recombination was not detected. The high frequency of recombination, the ability to isolate and analyze the participants in the recombination reactions, and the capacity to generate specific modifications in the immunoglobulin Cμ regions by gene targeting suggest that this system will be useful for studying mammalian chromosomal homologous recombination. Moreover, the ability to specifically modify the chromosomal immunoglobulin genes by homologous recombination should facilitate studies of immunoglobulin gene regulation and expression and provide a more convenient method of engineering specifically modified antibody molecules.

The immunoglobulin genes as they are presented in mouse hybridoma and myeloma cell lines have features which provide an advantageous system for the study of homologous recombination in mammalian cells. First, they are normally present in a haploid state; second, they are expressed at high levels; and third, both the DNA and protein have been extensively characterized (1, 10, 33). These properties expedite the detection of the recombination event and facilitate the characterization and analysis of the recombinant cells. Baker and Shulman (3) and Baker et al. (2) have recently shown that both the μ heavy-chain and κ light-chain immunoglobulin genes in mouse hybridoma cells can be specifically modified by homologous recombination with transferred immunoglobulin gene subfragments. In the case of the μ heavy-chain gene (2), homologous recombination was studied by using a mutant hybridoma (igm482) (5, 16) bearing a frameshift mutation in the DNA encoding the third constant domain of the μ gene ($C\mu_3$) as recipient cells and DNA transfer vectors bearing the normal Cμ region. Recombination between homologous Cμ regions in the transfer vector and the chromosomal μ gene resulted in correction of the chromosomal mutation and the restoration of normal, cytolytic 2,4,6-trinitrophenyl (TNP)-specific immunoglobulin M (IgM) production in the mutant cells (2). In these experiments, both single reciprocal recombination and gene replacement (gene conversion or double reciprocal crossover) events were observed. In all cases of single reciprocal recombination, the integration of the transfer vector into the igm482 chromosomal μ gene generates a G418-resistant ($G418^r$) cell line bearing a recombinant μ gene whose structure is such that the normal Cμ region, in its correct position 3′ of the endogenous TNP-specific heavy-chain variable region, is separated from the mutant Cμ region by the integrated pSV2neo vector sequences (2). I report here that this recombinant μ gene is unstable in mitotically growing hybridoma cells. The instability occurred at a high frequency and resulted from homologous recombination between the duplicated chromosomal Cμ regions.

MATERIALS AND METHODS

Cell lines. The origins of the hybridoma cell lines Sp6/HL, igm482, igm10, and Im/TCμ-3.10 and the methods used for cell culture have been described previously (2, 5, 16, 17).

General DNA techniques. High-molecular-weight DNA was prepared from the various hybridoma cell lines by the sodium dodecyl sulfate (SDS)-proteinase K method of Gross-Bellard et al. (8). Restriction enzymes were purchased from New England BioLabs, Inc., Boehringer Mannheim Biochemicals, and Bethesda Research Laboratories, Inc., and were used in accordance with the specifications of the manufacturers. After restriction enzyme digestion, DNA was electrophoresed at 2 V/cm through agarose gels of the consistency indicated in the figure legends. The preparation of gels and subsequent blotting onto nitrocellulose was performed by the method of Southern (26). The preparation of ^{32}P-labeled probes and the conditions used for hybridization have been reported previously (34).

Isolation and assay of recombinant cells. Hybridoma cul-

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tures making cytolytic wild-type TNP-specific IgM were cultivated in Dulbecco modified Eagle medium (DMEM) (17) in the presence or absence of 600 μg of G418 per ml. To screen for the presence of noncytolytic segregants making mutant, monomeric IgM, the cultures were cloned in 96-well tissue culture plates at a mean density of 1.0 cell per well. A total of 480 wells were plated per culture. The number of colonies arising from single cells (*S*) was calculated from the fraction of growth-negative wells according to the Poisson distribution. To screen culture wells for colonies containing only noncytolytic hybridomas, approximately 1 μl of culture supernatant was transferred, by using a polyvinyl chloride replicator plate (Dynatech Laboratories, Inc.), onto the surface of agarose plates containing TNP-coupled sheep erythrocytes and complement. The agarose plates were placed in a 7% CO_2 incubator at 37°C for 1 h. Culture wells containing noncytolytic hybridomas were identified by the inability of their supernatants to lyse the TNP-coupled erythrocytes. These isolates were grown and retested by TNP-specific plaque assay (7) to confirm their noncytolytic phenotype. The Poisson distribution was then used to calculate the number of these noncytolytic hybridoma cultures that arose from single cells (*N*). In culture wells receiving a single cell, there is a random chance that the cell will be either cytolytic or noncytolytic; thus, the frequency of noncytolytic hybridomas (*F*) can be calculated by the equation: $F = N/S$. Noncytolytic hybridomas isolated from the cloning experiments were tested both for their ability to grow in DMEM containing 600 μg of G418 per ml and for production of TNP-specific monomeric IgM by hemagglutination assays using α-μ serum (17). Representative isolates were saved for DNA and IgM analysis.

In other experiments, the frequency of G418-sensitive ($G418^s$) hybridoma cells making either mutant monomeric or wild-type polymeric IgM was determined. To screen for $G418^s$ cells, cultures were grown in medium lacking G418 and then cloned in 96-well tissue culture plates at a mean density of 1.0 cell per well. When colonies appeared, the wells were mixed, and 10 μl from each well was replicated by using a multichannel pipetter into the corresponding wells of duplicate 96-well tissue culture plates, one containing DMEM and the other containing DMEM supplemented with 600 μg of G418 per ml. After growth, the wells from the duplicate culture plates were compared, and the $G418^s$ cells were isolated, grown, and retested for their $G418^s$ phenotype. $G418^s$ cells were tested for production of TNP-specific mutant monomeric or wild-type polymeric IgM by hemagglutination assays and spot tests (17).

IgM analysis. IgM was biosynthetically labeled with [^{35}S] methionine and purified by binding to 2,4-dinitrophenyl-Sepharose, and the μ and κ chains were visualized by SDS-polyacrylamide gel electrophoresis after reduction of disulfide bonds with 2-mercaptoethanol (17, 24).

RESULTS

Test system. The system used to study homologous recombination is based on the hybridoma Sp6, which bears a single copy of the μ gene and makes cytolytic, wild-type polymeric IgM(κ) specific for the hapten TNP (16, 17). The mutant hybridoma cell line igm482 was isolated from Sp6 and bears a 2-base-pair deletion in the third constant domain of the μ gene ($C\mu_3$) (Fig. 1C). Consequently, igm482 produces a μ chain lacking the $C\mu_4$ domain (5, 16). IgM bearing this mutant μ chain is monomeric and does not activate complement, thus failing to lyse TNP-coupled sheep erythrocytes. These features allow the IgM of wild-type and mutant cells to be distinguished on the basis of TNP-specific hemagglutination and hemolysis assays and SDS-polyacrylamide gel electrophoresis (2). Also, the 2-base-pair deletion destroys an *Xmn*I restriction enzyme site, a feature which can therefore be used to discriminate between mutant and wild-type μ genes (2).

As shown previously, homologous recombination between the mutant igm482 μ gene and the normal Cμ region contained on a pSV2neo transfer vector restores normal, cytolytic, TNP-specific IgM production to the mutant cells (2). In the case of single reciprocal recombination, the recombination event generates a $G418^r$ hybridoma cell bearing a duplication of the Cμ-coding region. Thus, as shown in Fig. 1A, the structure of the recombinant μ gene is such that the normal Cμ region, in its correct position 3′ of the TNP-specific heavy-chain variable region, is separated from the mutant Cμ region by the integrated pSV2neo sequences (2). Both Cμ regions are in the same transcriptional orientation, and the distance between corresponding exons is 10 kilobases (kb) (2). With the exception of the 2-base-pair deletion in the mutant igm482 $C\mu_3$ exon, both Cμ regions are expected to share 4.3 kb of homology.

The duplicate Cμ regions serve as potential substrates for studying homologous recombination in mammalian cells. Thus, a single reciprocal crossover event (occurring either within a chromatid [intrachromatid] or unequally between sister chromatids) on the 5′ (Fig. 1C) or 3′ (Fig. 1B) side of the igm482 mutation would excise the vector sequences, generating $G418^s$ cell lines making noncytolytic, mutant monomeric IgM and cytolytic, wild-type polymeric IgM, respectively. On the other hand, a gene replacement event (gene conversion or double reciprocal recombination) (Fig. 1D through F) would yield $G418^r$ recombinants which, depending on their mode of recombination, differed in the arrangement of mutant and wild-type Cμ sequences. For example, an intrachromatid gene conversion or one occurring unequally between sister chromatids is expected to generate either a hybridoma cell line that bears the igm482 mutation in both Cμ regions and produces noncytolytic, mutant monomeric IgM (Fig. 1D) or a cell line which contains two wild-type Cμ regions and hence produces cytolytic, wild-type polymeric IgM (Fig. 1E). A double reciprocal recombination event occurring unequally between sister chromatids would also be expected to generate cell lines with the μ genes depicted in Fig. 1D and E. On the other hand, an intrachromatid double reciprocal crossover should produce a cell line making the noncytolytic, mutant monomeric IgM characteristic of the cells shown in Fig. 1D but with the μ gene structure depicted in Fig. 1F. With the exception of the gene replacement which changes the mutant Cμ region to the wild type (Fig. 1E), all recombinations alter the parental cell phenotype either with respect to cytolytic activity or $G418^r$, thus providing a method for identifying cell lines representing the various products of the recombination reactions.

Characterization of recombinant cells. To screen for hybridomas making noncytolytic mutant, monomeric IgM, the $G418^r$ hybridoma cell line Im/TCμ-3.10 bearing the μ gene structure shown in Fig. 1A was grown for 60 generations in medium lacking G418 and then cloned at 1.0 cell per well. The cytolytic activity of the IgM in supernatants from growth-positive culture wells was assessed by spot tests using TNP-coupled sheep erythrocytes (17). The frequency of noncytolytic hybridomas was calculated as described in Materials and Methods. The results of this initial screening

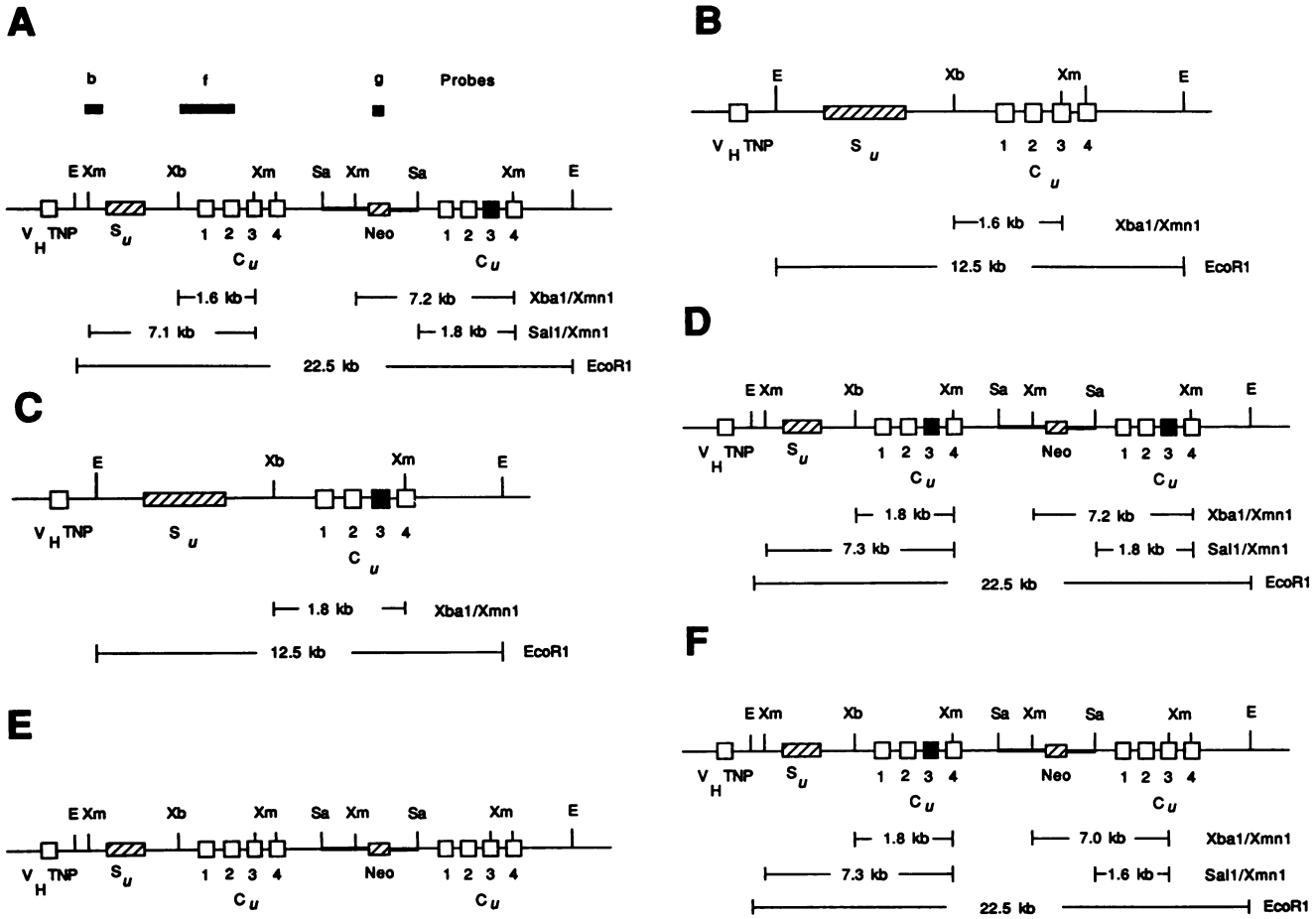

FIG. 1. DNA structures and fragment sizes predicted for homologous recombination between the duplicated immunoglobulin Cμ regions. The sizes of fragments (in kilobases) that the indicated restriction enzymes should generate are shown for the Im/TCμ-3.10 μ gene (A) and for those μ genes arising from either single reciprocal recombination (B and C) or gene replacement (gene conversion or double reciprocal recombination) events (D through F). The structure of the μ gene in Sp6/HL and igm482 is identical to that shown in panels B and C, respectively (2, 16). Cell lines with the μ gene structure depicted in panel E have the same phenotype as the parental cells shown in panel A and were not isolated and analyzed in this study. The mutant igm482 $C\mu_3$ exon is indicated (■). The heavy line between the *SalI* sites in panels A and D through F represents the pSV2neo-derived transfer vector, pTCu (2). The μ and *neo* genes are in the same transcriptional orientation. The probe fragments used in Southern blotting experiments are shown in panel A; probe b is a 915-base-pair *SstI* fragment, probe f is an 870-base-pair *XbaI-BamHI* fragment, and probe g is the 762-base-pair *PvuII* fragment from the *neo* gene of pSV2neo (27). Abbreviations: E, *EcoRI*; Sa, *SalI*; Xb, *XbaI*; Xm, *XmnI*; V_HTNP, TNP-specific heavy-chain variable region; C_μ, μ gene constant region; S_μ, μ gene switch region; Neo, neomycin phosphotransferase gene from pSV2neo (27).

experiment showed that noncytolytic hybridomas were present in the Im/TCμ-3.10 culture at a frequency of 0.03. To determine whether the generation of the noncytolytic hybridoma cells was a recurring phenomenon, three independent $G418^r$ subclones (started from a single cell) were grown for 60 generations in medium lacking G418 and then tested in the screening procedure. These experiments revealed that noncytolytic hybridomas were also present in the Im/TCμ-3.10 subclones at a frequency of 0.08 ± 0.03 (mean frequency ± the standard error of the mean). Noncytolytic hybridomas were present at the same high frequency (0.01) in Im/TCμ-3.10 subclones grown for only 16 generations in medium lacking G418. The instability of hybridoma cells bearing the duplicated Cμ region is not simply a reflection of their having been grown in medium lacking G418 because Im/TCμ-3.10 subclones grown only in medium containing G418 also contained noncytolytic variants at approximately the same frequency. Moreover, the instability that leads to the generation of the noncytolytic variants in these cultures is dependent on the presence of the duplicated Cμ region, since after >100 generations of growth noncytolytic cells were not detected in a culture of the Sp6 hybridoma (subclone Sp6/HL) (16) which bears a single copy of the TNP-specific chromosomal μ gene (frequency, <0.0057).

In principle, several mechanisms can account for the generation of the noncytolytic hybridomas in these experiments: loss of the μ gene, mutation resulting in loss of μ gene expression, switch recombination to a different constant region isotype, or homologous recombination between the duplicated Cμ regions. Of these possibilities, only homologous recombination is expected to place the igm482 mutation in the $C\mu_3$ exon of the expressed Cμ region (Fig. 1), thus generating noncytolytic hybridomas making the mutant monomeric IgM characteristic of igm482. A TNP-specific hemagglutination test was used to assay for cells of this type among the noncytolytic hybridomas. This analysis revealed that, from a total of 73 noncytolytic hybridoma cells obtained from two cloning experiments, 72 made mutant mo-

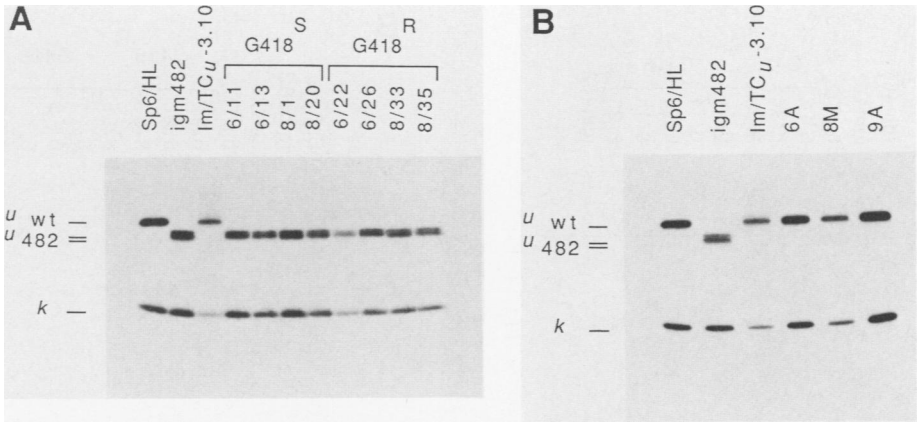

FIG. 2. Analysis of μ and κ chains produced in recombinant cell lines. The cell lines indicated above the lanes making TNP-specific mutant monomeric (A) and wild-type polymeric (B) IgM were incubated in medium containing [^{35}S]methionine to label protein biosynthetically. The IgM(κ) was purified by binding to 2,4-dinitrophenyl-Sepharose. The immunoglobulin chains were eluted with 0.5% SDS and analyzed by SDS-polyacrylamide gel electrophoresis after reduction of disulfide bonds (17, 24). The μ chains made by igm482 in the presence of tunicamycin migrated as a single band (data not shown), suggesting that alternative modes of glycosylation resulted in the double band seen here.

nomeric IgM, suggesting that they were generated by homologous recombination between the duplicate Cμ regions (Fig. 1). The remaining isolate failed to react in the hemagglutination assay and was not analyzed further. As shown in Fig. 1, cell lines making mutant monomeric IgM can be generated by either gene replacement or single reciprocal recombination. In the case of gene replacement, the recombinant cell line is expected to be G418^r, whereas a single reciprocal recombination event will generate a G418^s cell line. Thus, it should be possible to estimate the frequency with which gene replacement and single reciprocal crossovers contribute to the generation of cell lines making mutant monomeric IgM by measuring their resistance or sensitivity to the drug G418. This analysis suggests that recombinations consist of both gene replacement and single reciprocal crossover events, with gene replacement accounting for between 60 and 73% of the monomer-producing cell lines.

A single reciprocal recombination event occurring on the 5′ or 3′ side of the igm482 mutation is expected to generate a G418^s cell line making mutant monomeric (Fig. 1C) or wild-type polymeric (Fig. 1B) IgM, respectively. Therefore, information regarding the frequencies and positions of reciprocal recombination events can be obtained by analyzing the IgM produced by G418^s cells. Hybridoma cells sensitive to G418 were isolated from the Im/TCμ-3.10 subclones and tested for IgM production. Of 13 independent G418^s cell lines, 7 produced noncytolytic, mutant monomeric IgM and 6 produced cytolytic, wild-type polymeric IgM. This suggests that there is an approximately equal chance (frequency, ~0.5) for a single reciprocal crossover to occur on either the 5′ or 3′ side of the igm482 mutation despite the fact that there is ~1.1-kb-greater homology on the 3′ side.

Analysis of μ protein and DNA structure in recombinant cells. To determine whether homologous recombination was responsible for the generation of hybridoma cell lines producing either mutant monomeric or wild-type polymeric IgM, the μ protein and DNA from representative cell lines were analyzed. In the case of cell lines making mutant monomeric IgM, two G418^r and two G418^s isolates were chosen from two different subclones of Im/TCμ-3.10. The high frequency with which the monomer-producing isolates arise during mitotic growth argues strongly in favor of their being independent isolates. The μ chains of the wild-type Sp6/HL and mutant igm482 differ in molecular mass by ~15 kilodaltons and are readily distinguished by their mobilities in SDS-polyacrylamide gel electrophoresis (Fig. 2). The G418^r cell line Im/TCμ-3.10 synthesizes, as shown previously (2), a normal-length μ chain indistinguishable from wild-type μ. As expected, the independent G418^r cell lines 6/22, 6/26, 8/33, and 8/35 and the G418^s cell lines 6/11, 6/13, 8/1, and 8/20 have apparently ceased making the wild-type μ chain and now make the mutant μ chain characteristic of the igm482 cell line (Fig. 2A). Figure 2B presents the analysis of μ chains from independent G418^s cell lines that, according to TNP-specific hemolysis tests, make cytolytic, wild-type polymeric IgM. This analysis revealed that the cell lines 6A, 8M, and 9A produce the normal-length μ chain characteristic of Im/TCμ-3.10 and the wild-type Sp6/HL hybridoma.

The sizes of the μ gene and vector fragments generated by various restriction enzymes were also measured in cell lines producing either mutant monomeric or wild-type polymeric IgM (Fig. 1). The μ genes of Sp6/HL and igm482 are identical to those depicted in Fig. 1B and C, respectively, and differ only in that the *Xmn*I site present in the Cμ3 exon of Sp6/HL is destroyed by the 2-base-pair deletion in igm482 (2). The origin of the probe fragments is shown in Fig. 1. Probe b contains sequences present only in the chromosomal μ gene (2), probe f contains sequences present in both chromosomal and vector DNAs (2), and probe g is the 762-base-pair *Pvu*II fragment from the *neo* gene of pSV2neo (27). Because of its position 5′ of the μ gene switch region (Sμ), probe b detects γ gene sequences present within the hybridoma DNA (2). To indicate this irrelevant fragment, I have included the cell line igm10, an Sp6-derived mutant, which lacks the TNP-specific μ gene (4, 16).

To ascertain the structure of the μ gene in G418^r and G418^s cell lines making the mutant igm482 μ chain, an *Eco*RI digest of cellular DNA was analyzed with probe b (Fig. 3A). As shown previously (2), the μ gene of igm482 is present on a 12.5-kb *Eco*RI fragment, while the recombinant μ gene of the G418^r cell line Im/TCμ-3.10 is present on a 22.5-kb *Eco*RI fragment. The results showed that the G418^r isolates 6/22, 6/26, 8/33, and 8/35 also contain the μ gene on a 22.5-kb *Eco*RI fragment, while the μ gene in the G418^s cell lines 6/11, 6/13, 8/1, and 8/20 is present on a 12.5-kb *Eco*RI fragment. The blot shown in Fig. 3A was washed (32) and reprobed

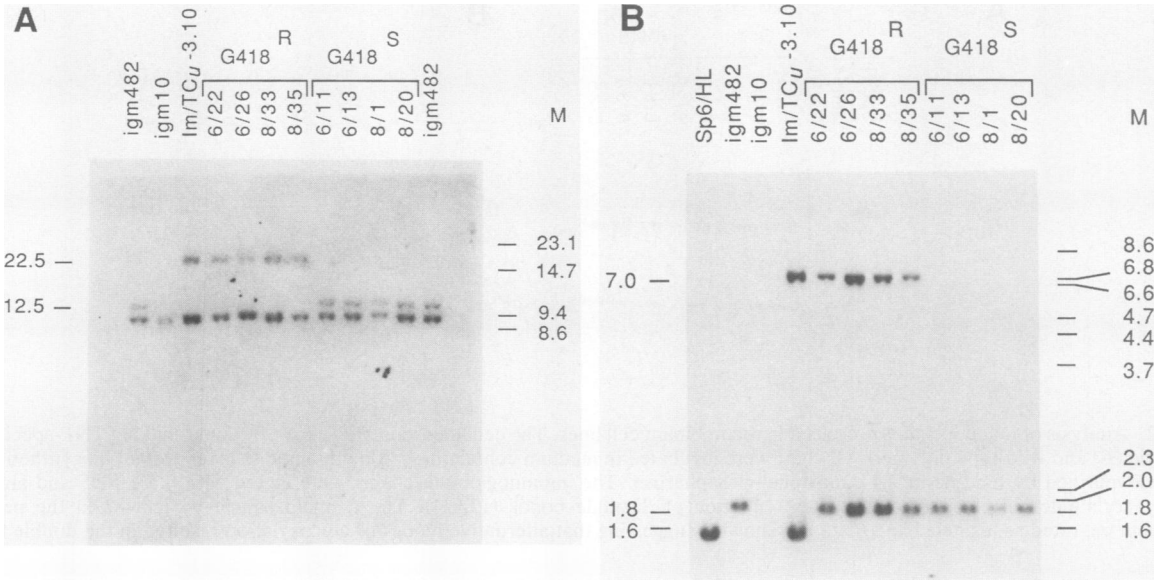

FIG. 3. Analysis of DNA structures in cell lines producing TNP-specific mutant monomeric IgM. DNA from the cell lines indicated above the lanes was digested with *Eco*RI (A) or *Xba*I-*Xmn*I (B) and electrophoresed through 0.7 and 1.0% agarose gels, respectively. Probe b was used to obtain the blot shown in panel A, while probe f was used to obtain the blot shown in panel B. The size (in kilobases) of each band of interest is shown to the left of the blot. The sizes (in kilobases) of the marker bands (M) are indicated to the right of the blot; marker bands consist of λ DNA digested with *Hin*dIII and *Ava*I.

with the *neo*-specific probe g. The results (data not shown) revealed that the *neo* gene was present only in the G418^r cell lines. Moreover, the *Eco*RI fragment containing the *neo* gene was 22.5 kb in size, implying that the μ and *neo* genes in 6/22, 6/26, 8/33, and 8/35 are physically linked, as they are in the parental Im/TCμ-3.10 cell line (Fig. 1A). These results are consistent with the interpretation that G418^r and G418^s cell lines making mutant monomeric IgM arise by different recombination mechanisms. The data suggest that G418^r cell lines are generated by gene replacement (gene conversion or double reciprocal crossover) as shown in Fig. 1D and/or F, while the G418^s isolates arise by a single reciprocal recombination event 5′ of the igm482 mutation, resulting in the excision of the vector sequences (Fig. 1C). The G418^r and G418^s cell lines making mutant monomeric IgM are therefore expected to contain the mutant igm482 $C\mu_3$ exon in its correct position to be expressed with the TNP-specific heavy-chain variable region. Thus, the use of probe f on DNA digested with the combination of *Xba*I and *Xmn*I should lead to the appearance of a 1.8-kb band for the mutant igm482 $C\mu_3$ exon, compared with a 1.6-kb band for the wild type. In addition, probe f will also detect a 7.2-kb *Xmn*I fragment for G418^r cell lines bearing a downstream mutant $C\mu_3$ exon (Fig. 1D), whereas the corresponding fragment bearing the wild-type $C\mu_3$ exon will be 7.0 kb in size (Fig. 1F). As shown in Fig. 3B, all G418^r and G418^s cell lines making the mutant igm482 μ chain contain a 1.8-kb band clearly resolved from the 1.6-kb band of the wild type, thus indicating that these cell lines all contain the mutant igm482 $C\mu_3$ exon and hence were derived by the recombination reactions described above. In addition, the G418^r isolates contain a fragment migrating at ~7.0 kb. From this blot, it is not possible to determine whether this band is the 7.2-kb fragment containing the mutant igm482 $C\mu_3$ exon found in Im/TCμ-3.10 (Fig. 1A) and in the recombinant shown in Fig. 1D or the 7.0-kb fragment containing the wild-type $C\mu_3$ exon found in the recombinant μ gene of Fig. 1F. An experiment to distinguish these recombinant μ genes will be presented below. I have examined the μ genes in an additional 17 independent G418^r and G418^s cell lines making mutant monomeric IgM, and in each case the results were identical to those shown in Fig. 3A and B (data not shown).

The μ gene shown in Fig. 1D could arise either by intrachromatid gene conversion or possibly by gene conversion or double reciprocal crossover occurring unequally between sister chromatids. On the other hand, the μ gene with the structure shown in Fig. 1F could only be generated by intrachromatid double reciprocal crossover. Cell lines bearing the μ genes shown in Fig. 1D and F, while phenotypically identical, are genetically distinct because of the presence of the different downstream Cμ regions. Thus, with probe f, only cell lines with the μ gene structure shown in Fig. 1D should yield a 1.8-kb *Sal*I-*Xmn*I fragment, while in cell lines with the μ gene depicted in Fig. 1F, a 1.6-kb band should be present. The analysis of 11 independent G418^r cell lines making mutant igm482 μ chains (including cell lines 6/22, 6/26, 8/33, and 8/35) revealed that all contained the downstream 1.8-kb mutant Cμ region and hence were not derived by the intrachromatid double reciprocal crossover shown in Fig. 1F. In addition to the 1.8-kb *Sal*I-*Xmn*I band, probe f also detects the upstream *Xmn*I fragment containing the expressed mutant Cμ region (data not shown).

The DNAs from the independent G418^s cell lines 6A, 8M, and 9A, which make the cytolytic, polymeric IgM characteristic of the wild-type Sp6/HL hybridoma, were also analyzed. The use of probe b on *Eco*RI-digested DNA revealed that the μ gene in these cell lines was present on a 12.5-kb fragment which comigrated with the Sp6/HL wild-type μ gene. This result was in marked contrast to the 22.5-kb μ gene fragment present in the parental cell line Im/TCμ-3.10 (Fig. 4A). When the blot shown in Fig. 4A was washed (32) and reprobed with the *neo*-specific fragment g, only the 22.5-kb *Eco*RI fragment present in Im/TCμ-3.10 was evident (data not shown). Moreover, as shown in Fig. 4B, the use of

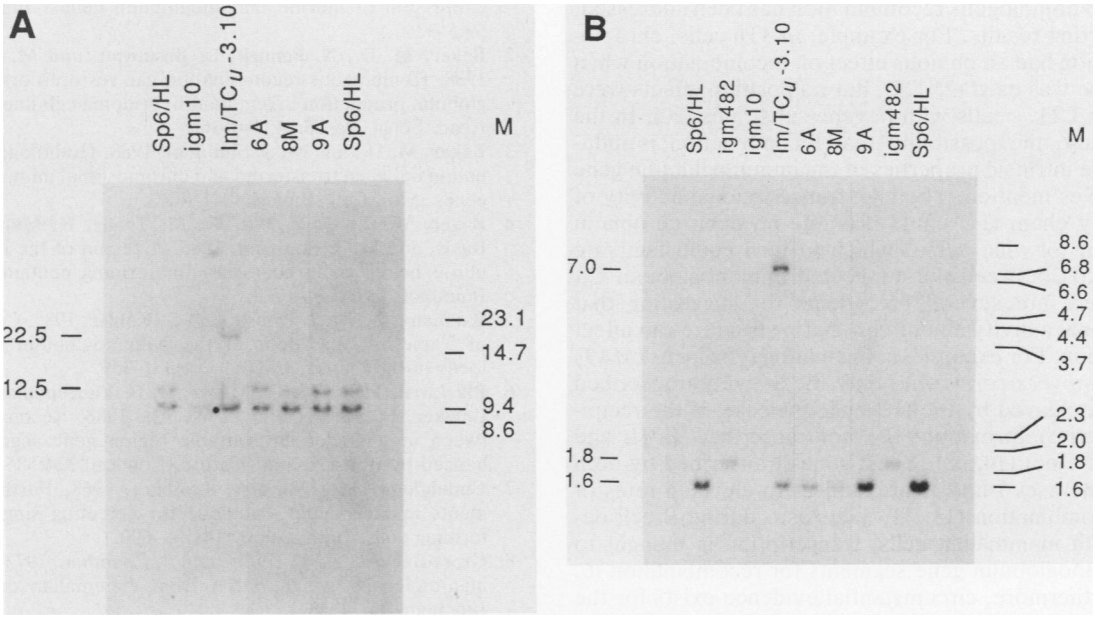

FIG. 4. Analysis of structures in cell lines producing TNP-specific wild-type polymeric IgM. DNA from the cell lines indicated above the lanes was digested with *Eco*RI (A) or *Xba*I-*Xmn*I (B) and electrophoresed through 0.7 and 1.0% agarose gels, respectively. Probe b was used to obtain the blot shown in panel A, while probe f was used to obtain the blot shown in panel B. The size (in kilobases) of each band of interest is shown to the left of the blot. The sizes (in kilobases) of the marker bands (M) are indicated to the right of the blot; marker bands consist of λ DNA digested with *Hin*dIII and *Ava*I.

probe f on cellular DNA digested with *Xba*I-*Xmn*I revealed that the G418^s isolates all bear the 1.6-kb fragment characteristic of the wild-type Cμ region in Sp6/HL and Im/TCμ-3.10, in contrast to the 1.8-kb fragment in the mutant igm482. Therefore, these results are in each case consistent with the idea that G418^s cell lines making cytolytic, polymeric IgM have the μ gene structure of the Sp6/HL hybridoma (Fig. 1B) and hence are generated by a single reciprocal recombination event occurring 3′ of the igm482 mutation.

DISCUSSION

The present study has shown that hybridoma cells bearing the μ gene structure shown in Fig. 1A are unstable during mitotic growth and undergo homologous recombination between the duplicate immunoglobulin Cμ regions. Two classes of recombinant products were detected. One class consisted of G418^r cells making the TNP-specific mutant monomeric IgM characteristic of the igm482 hybridoma. In these cells, the recombinant μ gene contains two mutant igm482 Cμ regions (Fig. 1D) and has the same flanking chromosome structure (i.e., *Eco*RI sites) as the parental cells (Fig. 1A). Recombinants of this type could be generated by gene conversion occurring either within or between sister chromatids or possibly by double reciprocal recombination after the unequal pairing of sister chromatids. The failure to detect the products of intrachromatid double reciprocal exchange events suggests that G418^r hybridoma cell lines making mutant monomeric IgM are generated by gene conversion. Consequently, in the following discussion, gene conversion will be referred to as the mechanism responsible for generating cell lines with these properties. Gene conversion accounts for between 60 and 73% of the recombinations leading to the generation of cell lines making IgM monomers. Other investigators working with duplicated genes in yeast and mammalian cells have also reported a higher frequency of gene conversion relative to reciprocal recombination (12, 15, 20), lending support to the proposal that it is an important mechanism controlling the sequence homogeneity of various multigene families (30). The second class of recombinants are G418^s and according to the structures of their μ genes can be divided into two groups. One group has the μ gene structure characteristic of the mutant igm482 hybridoma (Fig. 1C) and produces a truncated μ chain indistinguishable from that of igm482. The second group has the Sp6/HL μ gene structure (Fig. 1B) and produces a normal-length wild-type μ chain. Thus, in both groups the duplicated Cμ region and intervening vector sequences are lost. The generation of hybridoma cell lines bearing either the igm482 or Sp6/HL μ genes is consistent with single reciprocal recombination events occurring on the 5′ or 3′ side of the igm482 mutation, respectively. The frequency of the two groups of recombinants is similar (~0.5) despite the fact that, of the 4.3 kb of homologous DNA available for recombination, 1.6 kb lies 5′ and 2.7 kb lies 3′ of the igm482 mutation. Apparently, this twofold difference in homology does not affect the recombination frequency, suggesting not only that a minimum homology requirement for recombination has been satisfied but also the absence of a hot spot for recombination within the immunoglobulin Cμ region.

The frequency of recombination between chromosomal immunoglobulin Cμ regions measured here is approximately 1,000 times higher than the frequency of recombination between duplicate copies of *neo* and *tk* genes located on vector sequences integrated into the mammalian genome (20, 29). The reason for this significant difference is not clear. Recombination is affected by the length of homology shared by the recombining genes and the distance between them (18–20, 29), and although some differences do exist between the experimental systems, it is likely that other factors contribute to the large discrepancy in recombination frequencies. The possible influence of different chromosomal

positions on homologous recombination has been addressed, with conflicting results. For example, in 3T6 cells, chromosomal position had an obvious effect on recombination when the *neo* gene was used (25, 29), but no position effects were observed in LTk− cells with *tk* gene substrates (20). In the present study, the possibility that recombination is influenced by the intrinsic properties of the immunoglobulin gene itself deserves mention. The high transcriptional activity of the μ heavy chain (11) could promote an open chromatin environment (or vice versa) which in turn could facilitate recombination between the duplicated immunoglobulin Cμ region. There are several precedents for suggesting that transcriptional activity and/or chromatin structure can affect recombination. For example, in the mating type locus (*MAT*) of *Saccharomyces cerevisiae*, only the actively transcribed *MAT* locus, cleaved by the HO endonuclease, is the recipient of genetic information; the nontranscribed *HML* and *HMR* loci are not (14, 28). Yeast genes transcribed by both RNA polymerases I and II are subject to elevated rates of genetic recombination (13, 31, 35). Also, during B-cell development in mammalian cells, transcription is thought to target immunoglobulin gene segments for recombination (6, 36–38). Furthermore, circumstantial evidence exists for the integration of retroviral and other foreign DNAs into transcriptionally active sites (9, 22, 23). In view of these findings, further experiments will be necessary to determine what influence, if any, transcription and/or chromatin environment have on recombination between the duplicate immunoglobulin Cμ regions.

The system described here has several advantages for studying mammalian homologous recombination. The recombinant immunoglobulin μ gene in the parental cells is haploid; thus, recombination reactions are limited to a single pair of chromosomal Cμ regions. Moreover, hybridoma cell lines bearing specific modifications in the duplicate Cμ regions can be constructed by targeting genetic changes to the immunoglobulin μ gene (2). These features, along with the high frequency of recombination and the ability to isolate and analyze the recombination products, suggest that this system will be useful for addressing various questions regarding the substrate requirements and the role of chromosomal immunoglobulin sequences in homologous recombination. The ability to modify chromosomal immunoglobulin genes by homologous recombination, both as shown here and as reported previously (2, 3), should also permit studies of immunoglobulin gene regulation and expression as well as provide a more convenient method of producing specifically modified immunoglobulin. However, chromosomal immunoglobulin genes modified by single reciprocal recombination will contain a sequence duplication at the site of reciprocal crossover. The results of the present study suggest that these immunoglobulin genes will be highly unstable. Consequently, the immunoglobulin produced by such hybridoma or myeloma clones will be impure and probably unsuitable for certain diagnostic and therapeutic applications. Thus, for obtaining antibodies with these applications in mind, chromosomal immunoglobulin genes would probably best be modified by gene replacement reactions (2, 21).

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