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Homologous Recombination between Transferred and Chromosomal Immunoglobulin κ Genes

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Homologous recombination between transferred and chromosomal DNAs provides a means of introducing well-defined, predetermined changes in the chromosomal genes. Here we report that this approach can be used to specifically modify the immunoglobulin genes in mouse hybridoma cells. The test system is based on the Sp6 hybridoma, which synthesizes immunoglobulin M (κ) specific for the hapten 2,4,6-trinitrophenyl (TNP). As recipient cells, we used the Sp6-derived mutant hybridoma igk14, which has a deletion of the κTNP gene and consequently does not synthesize TNP-specific immunoglobulin M. igk14 retains the μTNP gene and two additional rearranged κ genes, denoted κM21B1 and κM21G. As a transfer vector, we used pSV2neo bearing the functionally rearranged TNP-specific Vκ segment. Following DNA transfer by electroporation, we isolated rare transformants which produced normal amounts of the functional κTNP chain. Analysis of the DNA of these transformants indicated that in all cases, a functional κTNP gene had been formed as the result of a homologous integrative recombination event with the igk14 κM21B1 gene. These results suggest that homologous recombination might be used for mapping and introducing immunoglobulin gene mutations and for more conveniently engineering specifically altered immunoglobulins.

The capacity of transferred DNA segments to undergo homologous recombination with chromosomal genes is a powerful tool for studying and manipulating gene structure and function. Homologous recombination between transferred and chromosomal mammalian genes has recently been reported (4, 19, 23) and may permit detailed molecular studies, as have been done in microbial systems, of mammalian genes in their native chromosomal context.

The immunoglobulin genes as they exist in mouse hybridoma cells provide an advantageous system for genetic and biochemical studies of immunoglobulin synthesis and function (9, 17). The use of homologous recombination to introduce predetermined genetic changes in the hybridoma immunoglobulin genes would have many applications, ranging from the production of immunoglobulin which is specifically optimized for diagnostic and therapeutic use to the detection and analysis of the molecular elements which determine immunoglobulin gene expression. We have examined the feasibility of this approach by using homologous recombination to change the specificity of an endogenous, rearranged κ gene in a mutant hybridoma cell line. In particular, we have targeted DNA encoding a 2,4,6-trinitrophenyl (TNP)-specific rearranged variable region to its normal position 5′ of the constant region of the chromosomal κ gene. The recombination event creates a functional κTNP gene that allows the production of TNP-specific immunoglobulin M (IgM) by the transformed cells.

MATERIALS AND METHODS

General techniques. The origin of the hybridoma cell lines Sp603, Sp6/HL, and igk14 and the methods used for their growth in cultures have been described previously (9, 17). 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. (5). Plasmid DNA was prepared by alkaline lysis, with the final purification step involving isopycnic CsCl-ethidium bromide gradient centrifugation. Restriction enzymes were purchased from New England BioLabs, Inc., Boehringer Mannheim Biochemicals, and Bethesda Research Laboratories, Inc., and used in accordance with manufacturer specifications. Following restriction enzyme digestion, DNA was electrophoresed through 0.7% agarose gels. The preparation of gels and subsequent blotting of DNA onto nitrocellulose were performed by the method of Southern (21). The preparation of ^{32}P-labeled probes and the conditions used for hybridization have been reported previously (27).

Gene transfer. The construction of the vector pVκTNP is shown in Fig. 1a. The vector DNA (50 μg) was digested with *Xba*I, which linearizes the vector within the major Jκ-Cκ intron. The cut vector DNA was phenol extracted, ethanol precipitated, and suspended in 50 μl of phosphate-buffered saline (9). The igk14 hybridoma cells were used as the recipients for DNA transfer. Exponentially growing igk14 cells were harvested by centrifugation and washed in phosphate-buffered saline, and 2×10^7 cells were suspended in 0.45 ml of phosphate-buffered saline. The plasmid DNA and igk14 cells were mixed in a sterile cuvette and subjected to a 700-V, 25-μF pulse at room temperature with a Bio-Rad Laboratories gene pulser. The cuvette was placed on ice for 10 min, and the cells were added to a tissue culture flask containing 40 ml of Dulbecco modified Eagle medium containing 13% heat-inactivated fetal calf serum (GIBCO Laboratories) and 0.0035% 2-mercaptoethanol. The flask was placed at 37°C for approximately 12 h, and cell survival was determined by trypan blue exclusion.

IgM analysis. Plaque-forming cells (PFC) were detected by their ability to lyse TNP-coupled sheep erythrocytes with agar plaque assays (7), spot tests (9), or Cunningham chambers (3). IgM was biosynthetically labeled with [^{35}S]methionine, purified by immunoprecipitation with rabbit anti-

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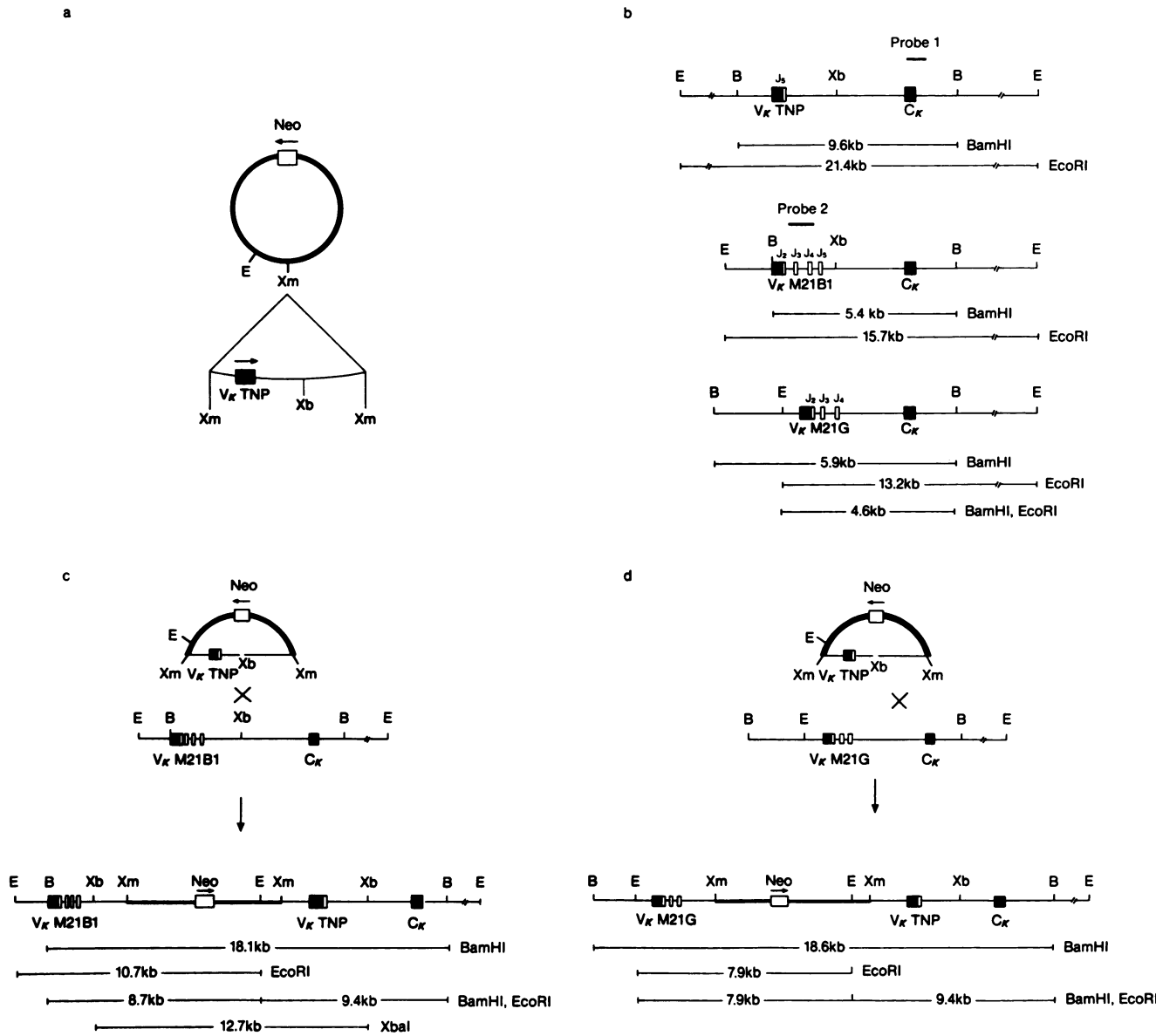

FIG. 1. Structure of the transfer vector and predicted sizes of the endogenous and recombinant κ genes. (a) The 12.7-kb transfer vector pVκTNP was constructed by cloning the 7.0-kb *Xmn*I fragment containing the TNP-variable region and major intron of the Sp6 κ gene (6) into the *Xmn*I site of a derivative of pSV2neo lacking the *Bam*HI site (18). The VκTNP subfragment lacks the Cκ coding region and is in the same transcriptional orientation as the *neo* gene. The unique *Xba*I site in the major Jκ-Cκ intron was used to linearize the vector for DNA electroporation. (b) The sizes (in kilobases) of the fragments which the indicated restriction enzyme(s) should generate are shown for the Sp6/HL and igk14 κ genes. As described in the text, Sp6/HL contains three distinct κ genes, whereas igk14 contains only the κM21B1 and κM21G genes. Cκ probe 1 is the 413-base-pair *Xmn*I fragment of the Cκ coding region; Jκ probe 2 is the 416-base-pair *Acc*I fragment encompassing the Jκ3 and Jκ4 regions; probe 3 is the 762-base-pair *Pvu*II fragment from the *neo* gene. (c and d) Fragments predicted if a single copy of the pVκTNP vector integrates by homologous recombination into either the κM21B1 or the κM21G gene of igk14 (6, 14, 29, 30). In panel c, the recombination site (×) is positioned between the vector and κM21B1 *Xba*I sites. In the case of the κM21G gene (panel d), in which a 1-kb deletion removes the chromosomal *Xba*I site (29, 30), we have shown homologous recombination occurring 3′ of this site (×). Abbreviations: E, *Eco*RI; B, *Bam*HI; Xb, *Xba*I; Xm, *Xmn*I.

mouse (κ) serum coupled to protein A-Sepharose beads, and eluted with 0.5% SDS. The μ and κ chains were visualized by SDS-polyacrylamide gel electrophoresis after reduction of disulfide bonds with 2-mercaptoethanol (9, 17).

RESULTS AND DISCUSSION

The test system is derived from the Sp6 hybridoma, which was originally generated by fusing the MOPC21 myeloma cell line, X63-Ag8, with spleen cells synthesizing IgM(κ) specific for the hapten TNP (9). In addition to the TNP-specific μ and κ chains (denoted μTNP and κTNP), Sp6 synthesizes the γ_1 heavy chain and κ chain from X63-Ag8 (9). Sp6 contains three distinct κ genes, the κTNP gene, the gene for the MOPC21 κ chain (denoted κM21B1), and a nonfunctionally rearranged κ gene (denoted κM21G) (6, 9, 29, 30). Two subclones of Sp6 were used in this study: the cell line Sp603, which does not make the MOPC21 γ_1 chain

TABLE 1. Production of TNP-specific IgM in PFC[a]

Transfer vector	Frequency of G418^r transformants	Frequency of PFC per:		Recombinants cloned for DNA analysis
		Nonselected recipient cell	G418^r transformant	
None	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$		
pVκTNP	0.6×10^{-3}	2×10^{-7}	0.4×10^{-2}	Ik/TVκ-A, (Ik/TVκ-F), Ik/TVκ-26, and (Ik/TVκ-34)

[a] Plasmid DNA (50 μg) was linearized by *Xba*I digestion, mixed with 2×10^7 igk14 cells in a 0.5-ml volume of phosphate-buffered saline (9), and subjected to a 700-V, 25-μF pulse at room temperature with a Biorad Laboratories gene pulser. Cell survival averaged 20%. After incubation in normal medium for 3 to 4 days, one portion of cells was assayed for PFC (7) on TNP-coupled erythrocytes (nonselected recipient cells) and distributed in microdilution wells at a limiting dilution in medium containing 600 μg of active G418 per ml to measure the frequency of wells containing G418^r transformants. The Poisson distribution was used to calculate the frequencies of G418^r transformants. The remaining cells were incubated in G418 for 2 weeks to select for G418^r transformants and then assayed for PFC (G418^r transformants). Reconstruction experiments indicated that Sp6/HL cells yielded detectable plaques with the same efficiency (~0.5) in the presence or absence of up to 10^7 igk14 cells. The frequency of PFC in the unselected cultures was stable over the 1-month study period, implying that the PFC were generated within 3 to 4 days of DNA transfer. Data from two independent experiments are presented. Particular lines were isolated by sib selection from the G418^r population (recombinants cloned for DNA analysis). Sib selection was done by plating G418^r cells in microdilution wells at a limiting dilution. Wells containing PFC were detected by testing cultures for cytolysis of TNP-coupled erythrocytes either by plaquing approximately 10^4 cells in Cunningham chambers (3) or by spot tests (9). Transformants listed in parentheses were not necessarily independent of those preceding them, as they were derived from the same gene transfer experiment. We can estimate the number of independent PFC that these gene transfer protocols generated subject to the assumptions that the PFC make plaques with the same efficiency (~0.5) as the Sp6/HL cells and that the PFC arise immediately after electroporation: $n = [(2 \times 10^7) \times (\text{fraction of surviving cells}) \times (\text{nonselected recipient cells}) \times 1]/0.5 \sim 2$.

(9), and the cell line Sp6/HL, which makes neither the MOPC21 γ_1 nor the MOPC21 κM21B1 chain (8). It is not known why these subclones have ceased to produce the MOPC21 immunoglobulin chains. The Sp603-derived mutant hybridoma igk14 has lost the κTNP gene and consequently produces IgM(κ) which does not bind to TNP (14). We used igk14 as a recipient cell line in these studies so that we could use a plaque assay to detect homologous recombination events which restore κTNP production. That is, Sp603 and Sp6/HL cells make plaques on TNP-coupled erythrocytes (efficiency, 0.5 PFC), whereas igk14 cells do not (efficiency, $<10^{-7}$PFC); transformants of igk14 which produce normal levels of κTNP are expected to make plaques at normal efficiency.

The transfer vector pVκTNP (Fig. 1a) contains the rearranged TNP-specific variable region and 3′ major intron sequences of the wild-type Sp6 κTNP gene inserted into a derivative of pSV2neo, which confers resistance to the antibiotic G418 (22). The absence of the Cκ coding region in pVκTNP precludes the formation of a functional κ light chain. The VκTNP subfragment contained in the transfer vector includes 2.3 kilobases (kb) which is homologous to the Jκ-Cκ intron of the κM21B1 gene. In the case of the κM21G gene, a 1-kb chromosomal deletion (29, 30) reduces this homology to only about 1.3 kb. The unique *Xba*I site in the Jκ-Cκ intron was used to linearize the pVκTNP vector before transfer to igk14 cells (Fig. 1a). If pVκTNP integrates by homologous recombination into either the κM21B1 or the κM21G gene of igk14, this recombination will place the vector-borne VκTNP region 5′ of the chromosomal Cκ coding region so as to form a functional VκTNP gene (Fig. 1c and d). Such a recombination event would be expected to restore normal TNP-specific IgM production, thus generating cells which plaque on TNP-coupled erythrocytes.

We assayed for homologous recombination by measuring the frequency of TNP-specific PFC generated following the transfer of the vector into igk14 cells. Whereas the frequency of PFC among untreated igk14 cells was undetectable ($<10^{-7}$), in igk14 cells electroporated with pVκTNP, the frequency of PFC was at least two times higher. We estimated that this gene transfer protocol generated approximately 2 independent PFC among 2×10^7 recipient igk14 cells (Table 1). The frequency of PFC was much higher (0.4×10^{-2}) in the G418-resistant (G418^r) population. Other investigators have examined homologous recombination in other cell types with different genes and different methods of DNA transfer and estimated that the ratio of homologous to nonhomologous integrations ranged from 10^{-2} to 10^{-3} (4, 10, 19, 20, 23, 24). To examine the structures of the IgM and of the κ genes in the PFC, we cloned representative G418^r plaque-forming transformants by sib selection from independent DNA transfer experiments. The transformants derived from the transfer of pVκTNP are denoted by the prefix Ik/TVκ (Table 1). One randomly picked G418^r transformant (R/T-1) which did not synthesize TNP-specific IgM was also cloned.

Figure 2 presents the analysis of intracellular and extracellular μ and κ chains in the various control and transformant cell lines. The κTNP chain present in Sp6/HL was distinguished from the κM21B1 chain present in igk14 by its faster mobility in SDS-polyacrylamide gel electrophoresis. In contrast to the recipient cell line igk14, all transformants synthesized normal amounts of full-length κTNP chain indistinguishable from those synthesized by Sp6/HL or Sp603 cells. Therefore, the ability to make a plaque on TNP-coupled erythrocytes was correlated with the presence of a functional, normally expressed κTNP chain. The transformants differed in their production of the κM21B1 chain. Transformants Ik/TVκ-A, Ik/TVκ-26, and Ik/TVκ-F continued to synthesize the κM21B1 chain, whereas no κM21B1 chain was detected in transformant Ik/TVκ-34. This difference will be discussed more fully below.

The presence of a normal κTNP chain in all transformants suggested that the plasmid-borne VκTNP region had recombined with either the κM21B1 or the κM21G gene in igk14, thus restoring a normal κTNP gene. To test whether the κTNP gene was restored by a homologous recombination event, we examined the structure of the κ genes in the various control and transformed cell lines. Figures 1b to d show the structure of the κ genes in Sp6/HL and igk14 as well as the predicted structures for the cases in which the pVκTNP vector has integrated by homologous recombination into either the igk14 κM21B1 or igk14 κM21G gene. The probe fragments 1 (Cκ) and 2 (Jκ) detect κ sequences present in the chromosome but not in the vector (Fig. 1b), while probe 3 is the 762-base-pair *Pvu*II fragment from the *neo*

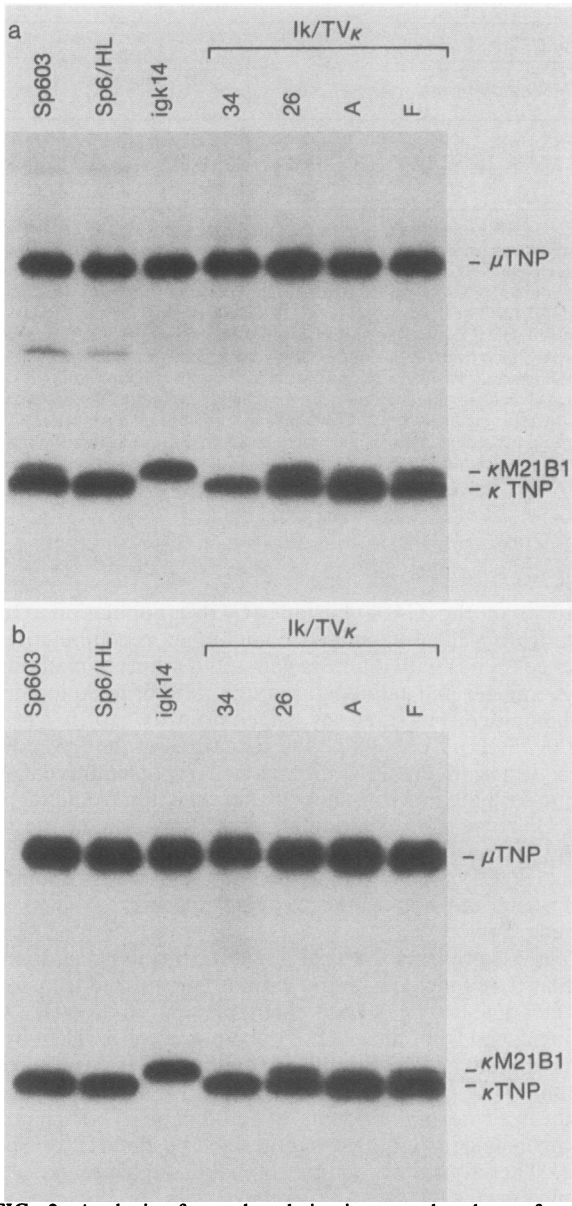

FIG. 2. Analysis of μ and κ chains in control and transformant cell lines. The indicated cell lines were incubated in medium containing [^{35}S]methionine to label protein biosynthetically. Both intracellular IgM(κ) (a) and extracellular IgM(κ) (b) were purified by immunoprecipitation with rabbit anti-mouse (κ) serum coupled to protein A-Sepharose beads. The immunoglobulin chains were eluted with 0.5% SDS and analyzed by SDS-polyacrylamide gel electrophoresis after the reduction of disulfide bonds (9, 17).

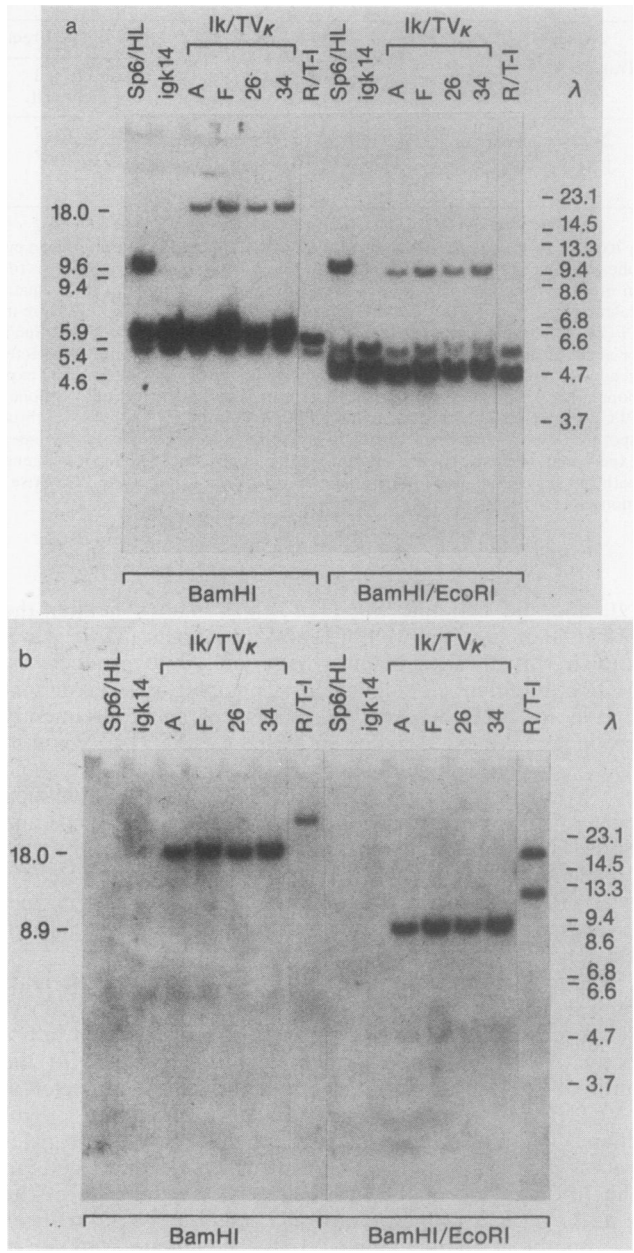

FIG. 3. Analysis of DNA structures in plaque-forming transformants. DNA from the indicated cell lines was digested with *Bam*HI or *Bam*HI-*Eco*RI and probed with Cκ fragment 1 (panel a). The material in the blot in panel a was washed (25) and probed with *neo*-specific fragment 3 to generate the blot shown in panel b. The size (in kilobases) of each band of interest is shown to the left of the blot and was calculated by comparison with the sizes (in kilobases) of the marker bands (λ digested with *Hin*dIII and *Ava*I) indicated to the right of the blot.

gene. The blots shown in Fig. 3 and 4 were designed to test the predictions inherent in Fig. 1.

Figure 3a presents results for *Bam*HI and *Bam*HI-*Eco*RI-digested DNAs probed with Cκ fragment 1. As reported previously, igk14 lacked the 9.6-kb *Bam*HI band corresponding to the κTNP gene present in Sp6/HL (6, 14). All cell lines contained *Bam*HI bands of 5.4 and 5.9 kb corresponding to the κM21B1 and κM21G genes, respectively (6, 14, 29, 30). The 12.7-kb vector, pVκTNP, has no *Bam*HI sites, so that its integration into the Jκ-Cκ intron of a κ gene of igk14 is expected to generate either an 18.1-kb (κM21B1) or an 18.6-kb (κM21G) band. The transformants all had an 18.0-kb band which, within the limits of gel resolution, was not significantly different in size from the predicted band of 18.1 or 18.6 kb (Fig. 3a). Thus, these results suggest that the κTNP gene has been restored by the integration of a single copy of the vector into either the κM21B1 or the κM21G gene. As expected, transformant R/T-1, which was isolated as a G418^r non-plaque-forming transformant, had no novel Cκ-containing fragments. Integration of the vector into a κ

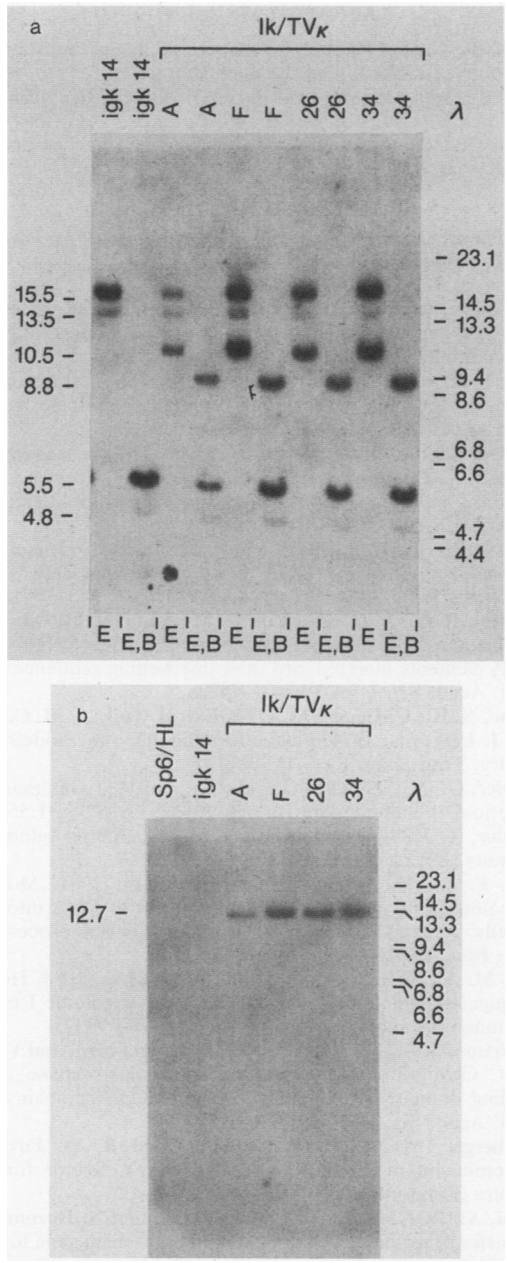

FIG. 4. Analysis of the site of homologous recombination. DNA from the indicated cell lines was digested with *Eco*RI (E) or *Eco*RI-*Bam*HI (E,B) and probed with Jκ fragment 2 (a) or digested with *Xba*I and probed with *neo*-specific fragment 3 (b). The size (in kilobases) of each band of interest is shown to the left of the blot and was calculated by comparison with the sizes (in kilobases) of the marker bands (λ digested with *Hin*dIII and *Ava*I) indicated to the right of the blot.

gene of igk14 should cause the disappearance of the corresponding band in the transformant. Possible reasons for the continued presence of the igk14 bands, as seen in the blots shown in Fig. 3a and 4a, will be discussed below.

The presence of the 18.0-kb *Bam*HI Cκ fragment in the DNA of the plaque-forming transformants indicated that the vector had inserted near Cκ. To test whether the insertion occurred by homologous recombination, we digested DNA with *Bam*HI and *Eco*RI together to generate fragments expected to span the vector-chromosome junctions (Fig. 1). The endogenous κM21B1 and κM21G genes yield 4.6- and 5.4-kb Cκ fragments. If the pVκTNP vector has integrated into either the κM21B1 or the κM21G gene by homologous recombination, *Bam*HI-*Eco*RI digestion should yield a 9.4-kb Cκ-bearing fragment. The Ik/TVκ transformants all had a novel band of this predicted size (Fig. 3a).

The material in the blot shown in Fig. 3a was washed and rehybridized with *neo*-specific probe fragment 3. A single *neo*-containing band was observed in all plaque-forming (Ik/TVκ) transformants, implying that a single vector integration event had occurred in these cells (Fig. 3b). This band migrated at 18.0 kb and was presumably the same band as that detected by the Cκ probe (Fig. 3a). The predicted sizes for the *neo*-containing *Bam*HI-*Eco*RI fragments were different for the κM21B1 and κM21G genes, namely, 8.7 and 7.9 kb, respectively (Fig. 1c and d). *Bam*HI-*Eco*RI digestion revealed a single *neo* band migrating at 8.9 kb in the transformants (Fig. 3b). This result suggests that the vector had integrated by homologous recombination into the κM21B1 gene rather than the κM21G gene. The G418^r (non-plaque-forming) transformant R/T-1 appeared to contain two copies of the vector integrated in tandem at one site.

Figure 4a presents results for *Eco*RI and *Eco*RI-*Bam*HI-digested DNAs probed with Jκ fragment 2 and also indicates that the κM21B1 gene was the site of homologous recombination. First, with respect to the endogenous κ genes of igk14, the Jκ probe should detect *Eco*RI fragments of 15.7 and 13.2 kb for the κM21B1 and κM21G genes, respectively (Fig. 1b). Furthermore, the sizes of the κM21B1 and κM21G genes should shift to 5.4 and 4.6 kb, respectively, following *Eco*RI-*Bam*HI digestion. Within the limits of gel resolution, the fragment sizes observed agreed with these predictions, although the reason for the different relative intensities of the κM21B1 and κM21G bands when Jκ and Cκ (Fig. 3) probes were used is unknown. With regard to the plaque-forming transformants, a homologous recombination event involving the κM21B1 gene would be expected to yield an *Eco*RI fragment of 10.7 kb (Fig. 1c) as compared with a 7.9-kb fragment for the κM21G gene (Fig. 1d). A novel *Eco*RI fragment of 10.5 kb was found in all transformants (Fig. 4a). Moreover, following *Eco*RI-*Bam*HI digestion, the 10.7-kb *Eco*RI fragment in a recombinant κM21B1 gene should shift to 8.7 kb (Fig. 1c), whereas the 7.9-kb *Eco*RI fragment in the κM21G gene should be unchanged (Fig. 1d). All plaque-forming transformants had an 8.8-kb *Eco*RI-*Bam*HI band (Fig. 4a), thus arguing that the κM21B1 gene is the site of recombination.

The pVκTNP vector was linearized at *Xba*I before gene transfer; therefore, in the absence of any net degradation, the integration of a single copy of the vector by a reciprocal recombination event involving the κM21B1 gene would be expected to yield two *Xba*I sites separated by the length of the vector (12.7 kb) (Fig. 1c). The presence of a 12.7-kb *Xba*I fragment in all transformants confirmed these expectations (Fig. 4b), which are analogous to the properties of homologous recombinants described by Smithies et al. (19) for the β-globin gene. The κM21G gene contains a 1-kb deletion which removes the region around the *Xba*I site of the Jκ-Cκ intron (29, 30). This deletion might have precluded homologous recombination between the pVκTNP vector linearized at the *Xba*I site and the κM21G gene.

The restriction enzymes and probe fragments used in these studies were chosen to detect restriction fragments spanning both the left and right junctions of the recombinant κ gene. All the enzyme sites which we tested for are present and generate fragments of the sizes predicted for the case in

which one copy of the vector has integrated by a single reciprocal crossover in the 2.3-kb region of homology shared by the vector and the κM21B1 gene. The conservation of the *Xba*I site which was used to linearize the transfer vector is of particular interest, as this region might be expected to be especially susceptible to degradation. While these results argue that the recombination process is conservative, we did not otherwise test whether minor nucleotide changes occurred elsewhere in the recombinant κ gene.

Vector integration is expected to inactivate the endogenous κM21B1 gene and result in the disappearance of the normal κM21B1 gene fragment. However, three of the four transformants continued to synthesize a normal κM21B1 chain (Fig. 2), and in all plaque-forming transformants a normal-sized κM21B1 gene still remained. One possibility is that igk14 has two copies of the κM21B1 gene, one functional and one nonfunctional. Thus, transformants Ik/TVκ-26, Ik/TVκ-A, and Ik/TVκ-F, which produced both κM21B1 and κTNP chains, may have arisen by homologous recombination in the nonfunctional κM21B1 gene, while a recombination event in the functional κM21B1 gene may have generated transformant Ik/TVκ-34, which produced only the κTNP chain. Another possibility is that the recombination event is associated with a duplication of the chromosomal target region and that somehow this duplication process is prone to mutation. Regardless of the origin of the remaining copy of the κM21B1 gene in the plaque-forming transformants, we conclude that homologous recombination can be used to modify chromosomal immunoglobulin genes in a predetermined fashion.

The high level of expression of the recombinant κ gene contrasts with the variable and generally low levels of expression seen for transferred (complete) genes which have been integrated elsewhere in the chromosome (14, 15) and suggests that this method is preferred for the production of modified immunoglobulins. Several extensions of this technology might be possible. The introduction by homologous recombination of specifically modified immunoglobulin variable regions could enable the production of novel abzymes (16, 26) as well as antibodies having new or improved antigen-binding characteristics. In related work, we have used homologous recombination to insert a vector-borne heavy-chain constant region 3′ of a functional variable-region gene (M. D. Baker, N. Pennell, L. Bosnoyan, and M. J. Shulman, Proc. Natl. Acad. Sci. USA, in press). The ability to manipulate the constant region should make possible the construction of hybridoma or myeloma cell lines secreting normal amounts of chimeric, toxin, hormone, or enzyme-linked antibodies (1, 2, 11–13, 28).

In addition to providing a convenient way of producing optimized immunoglobulins, homologous recombination may allow detailed studies of immunoglobulin gene regulation and expression. In this context, we have developed efficient methods for isolating mutant hybridomas defective in IgM gene structure and function (9, 17). Homologous recombination may prove to be a useful method for mapping these mutations, thus providing important information regarding the elements which regulate immunoglobulin gene expression in its natural chromosomal environment. Moreover, homologous recombination might be used to introduce well-defined mutations into immunoglobulin genes, thus permitting a rigorous analysis of immunoglobulin regulatory elements.

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