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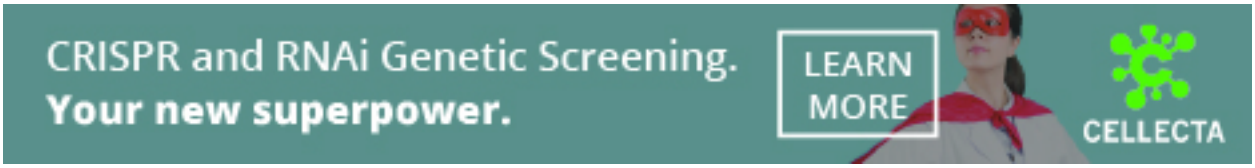

Specific Amplification by PCR of Rearranged Genomic Variable Regions of Immunoglobulin Genes from Mouse Hybridoma Cells

José Berdoz,[1] Thomas P. Monath,[2] and Jean-Pierre Kraehenbuhl[1]

[1]Swiss Institute for Experimental Cancer Research and Institute of Biochemistry, University of Lausanne, Switzerland; [2] OraVax Inc., Cambridge, Massachusetts 02139

We have designed a novel strategy for the isolation of the rearranged genomic fragments encoding the L-V_H-D-J_H and L-$V_{\kappa/\lambda}$-$J_{\kappa/\lambda}$ regions of mouse immunoglobulin genes. This strategy is based on the PCR amplification of genomic DNA from mouse hybridomas using multiple specific primers chosen in the 5′-untranslated region and in the intron downstream of the rearranged $J_H/J_{\kappa/\lambda}$ sequences. Variable regions with intact coding sequences, including full-length leader peptides (L) can be obtained without previous DNA sequencing. Our strategy is based on a genomic template that produces fragments that do not need to be adapted for recombinant antibody expression, thus facilitating the generation of chimeric and isotype-switched immunoglobulins.

The development of mouse hybridoma technology has allowed the production of antibodies specific to a wide range of antigens. Mouse monoclonal antibodies (mAbs) have been used extensively for diagnosis, and some have proven useful for treatment of human diseases. Generally, murine antibodies are cleared rapidly from the circulation and often do not interact effectively with the host immune system. Moreover, administration of murine antibodies to humans often induces a human anti-mouse antibody (HAMA) response after single or repeated treatments.[1] Attempts to replace murine mAbs have been limited by the difficulties in generating human hybridomas, which are generally unstable and secrete low amounts of antibodies (frequently IgMs). Thus, considerable efforts have been made to render murine mAbs more similar to those of the human host. Alternatives to human hybridoma-derived antibodies have been developed in which most of the rodent-derived sequences of mouse immunoglobulins are replaced with sequences derived from human immunoglobulins. Two approaches have been used: (1) chimeric mAbs, in which the xeno-V regions are combined with human constant domain regions (CDRs);[2,3] and (2) humanized mAbs, in which only the xeno-CDRs are linked onto human sequences.[4,5] These engineered antibodies retain their target specificity and show reduced HAMA responses when injected into patients.[6,7] In addition, the correct effector function of the antibody as required for certain clinical applications can be obtained by selecting the corresponding immunoglobulin isotype.

In spite of these advances, cloning of the variable region sequences has been a limiting step in the rapid construction of chimeric and isotype-switched antibody molecules. Amplification by PCR of the immunoglobulin heavy- and light-chain variable regions has facilitated this step greatly. However, the high degree of DNA sequence polymorphism in the leader and variable sequences of both heavy and light chain genes has required the preparation of complex sets of degenerate primers.[8–11] These primers are usually within the first framework of the variable region (FR1), and in a few cases in the leader peptide sequence (L). The 3′ primers are usually within framework 4 (FR4), which displays a limited polymorphism, or in the constant region, in which conserved, isotype-specific sequences can be identified easily. Therefore, most of the current PCR amplification reactions of the immunoglobulin H and κ/λ chain variable regions generate fragments containing incomplete V_H and V_L sequences, linked or not linked to part of the constant region. When cloned into cassette expression vectors, such fragments generate chimeric immunoglobulins directly when PCR primers are within the FR1 and FR4 regions. Additional modifications such as the reconstruction of the original splice donor site of the V region or the mutagenesis of sites in the V and C regions that create a common restriction sequence are required before cloning when the fragments contain C regions.[12,13] Other disadvantages of the methods currently

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in use arise from the observation that although complex sets of 5′ and 3′ primers have been designed, they do not always match the DNA template completely.[14,15] Native sequences of the immunoglobulin heavy and light chains may therefore be altered in the FR1 and/or in the FR4 regions during the amplification process. This can be a problem because modifications of the amino-terminal structure have been shown to reduce drastically the affinity of the immunoglobulin for its antigen. This is particularly true for the light-chain variable region, in which the amino acid at position 2 is part of the predicted canonical structure for CDR1.[16] Moreover, expression levels of the recombinant antibody may be altered when mutations occur in the leader peptide.

Other current technologies such as inverse or anchored PCR circumvent some of the problems described above. For example, one can determine the 5′ and 3′ sequences of the variable regions, and therefore PCR primers that match the template exactly can be prepared.[17,18] Variable regions with unaltered amino-terminal sequences can be generated. However, these procedures are time-consuming.

If DNA sequences of the V_H and V_L genomic regions from the parental hybridoma are known, including the 5′-untranslated regions and the introns downstream of the rearranged J segments, PCR-amplified fragments can be inserted into expression vectors within noncoding sequences.[19] This procedure generates complete, original variable regions that can be linked without modifications to any other constant region, provided that the reading frame of the V region is maintained.

We have developed a general method for PCR amplification of the rearranged genomic fragments encoding full-length variable regions of mouse immunoglobulin genes. This method does not require previous DNA sequencing. Amplified fragments can be characterized readily using a set of J-specific oligonucleotide probes that are used also for typing of the V_H-D-J_H and V_L-J_L rearrangements on hybridoma RNA.

MATERIALS AND METHODS

Sources of DNA and RNA

Mouse hybridoma cells HNK-20 (IgA, κ) and 3G3 (IgM, λ) were obtained from OraVax, Inc. (Cambridge, MA) and from American Type Culture Collection (ATCC, Rockville, MD) no. HB8516, respectively. Genomic DNA was prepared as described.[20] Total RNA was prepared from frozen cell pellets according to the guanidine/cesium chloride method.[21,22]

Synthesis of Oligonucleotide Primers and Probes

Oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method and purified by reverse-phase HPLC (MWG-Biotech, Ebersberg, Germany).

DNA Amplification by PCR

In vitro DNA amplification was performed in 100-μl final volume in a Perkin-Elmer 9600 thermal cycler (Norwalk, CT). Reagents were added to the reaction to yield the following final composition: 10 mM Tris-HCl (pH 8.3) at 25°C; 50 mM KCl; 2.5 mM $MgCl_2$; 0.001% gelatin (Sigma, cat. no. G2500, St. Louis, MO); 200 mM dNTP; 150 nM of each amplification primer; 1 μg of genomic DNA; and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). The cycling profile was as follows: 5 min at 94°C, 3 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, 27 cycles of 1 min at 94°C, 1 min at 62°C, 1 min at 72°C, and one final incubation at 72°C for 10 min.

Northern and Southern Blots

Total RNA (10 μg) was denatured with glyoxal and subjected to electrophoresis on an agarose gel.[23] The sample was transferred onto a GeneScreen Plus membrane (Du Pont, Wilmington, DE) according to the procedure described by the manufacturer. Products of PCR amplification of genomic DNA prepared from hybridoma cells were analyzed on agarose gels and transferred onto a GeneScreen Plus membrane as described.[24]

Northern and Southern blots were hybridized under identical conditions with ^{32}P-labeled oligonucleotide probes specific for each of the J_λ, J_κ, and J_H segments. Temperatures [melting temperature (T_m)] of prehybridization and hybridization were at $T_m - 4°C$, and washings at $T_m - 2°C$. The T_ms of the oligonucleotides were estimated by the formula: $T_m = 4$ (number of G+C) + 2 (number of A+T). The T_ms of the J-specific oligonucleotide probes are as follows: $J_{\lambda 1} = 58°C$; $J_{\lambda 2} = 62°C$;

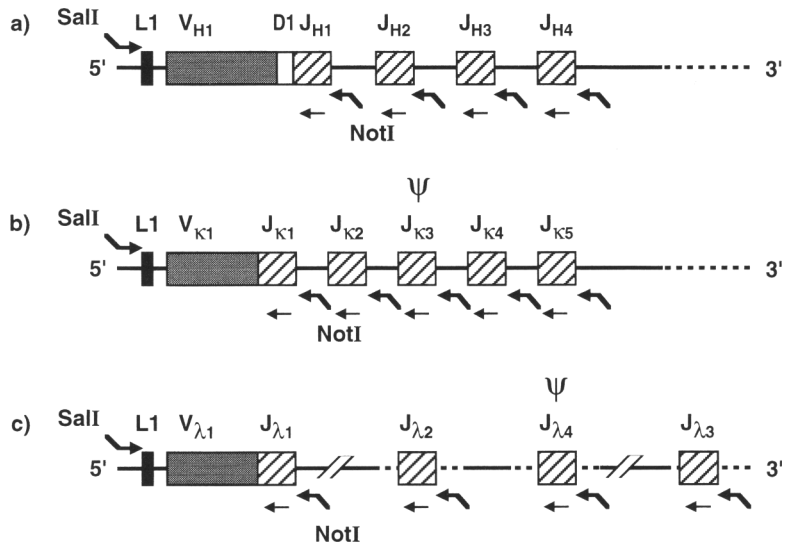

FIGURE 1 Map of the rearranged genes encoding the variable regions of mouse immunoglobulin heavy (*a*), κ (*b*), and λ (*c*) chains showing the location of the PCR primers and probes used for the amplification of V_H and V_L regions. Pseudogenes are indicated by Ψ. Bold, bent arrows indicate the PCR primers located in the 5′-untranslated regions and in the introns downstream of the J segments. *Sal*I and *Not*I restriction sites have been added at the 5′ ends of the PCR primers. Straight arrows below the J segments indicate the oligonucleotide probes used in Northern and Southern blot hybridization.

$J_{\lambda 3}$ = 60°C; $J_{\lambda 4}$ = 58°C; $J_{\kappa 1}$ = 64°C; $J_{\kappa 2}$ = 64°C; $J_{\kappa 3}$ = 56°C; $J_{\kappa 4}$ = 64°C; $J_{\kappa 5}$ = 62°C; J_{H1} = 68°C; J_{H2} = 64°C; J_{H3} = 64°C; and J_{H4} = 60°C.

Prehybridization was done for 3–4 hr in 2× SSC, 5× Denhardt's solution, 0.1% SDS, and 5 mM EDTA. Hybridization was for 14–18 hr in 5× SSC, 10× Denhardt's solution, 20 mM sodium phosphate buffer (pH 7.0), 7% SDS, 100 μg/ml of denatured herring sperm DNA, and ^{32}P-labeled oligonucleotide (10^6 cpm/ml). The membranes were then washed twice for 30 min in 3× SSC, 10× Denhardt's solution, 5% SDS, 70 mM sodium phosphate buffer (pH 7.0); and twice for 30 min in 1× SSC and 1% SDS.

As an internal control for total RNA loading, Northern blots were hybridized with a glyceraldehyde-3-phosphate-dehydrogenase cDNA probe.[25] Prehybridization was done for 3–5 hr at 42°C in 50% deionized formamide, 5× Denhardt's solution, 5× SSPE [30× SSPE = 4.5 M NaCl, 0.3 M NaH_2PO_4, 30 mM EDTA (pH 7.7)], 1% SDS, and 200 μg/ml of denatured salmon sperm DNA. Hybridization was for 14–18 hr at 42°C in the same mixture with ^{32}P-labeled cDNA probe (10^6 cpm/ml). The membranes were then washed twice for 5 min in 2× SSPE at room temperature, once for 15 min in 2× SSPE, and 0.5% SDS at 65°C, and once for 15 min in 0.5× SSPE at 65°C.

DNA Sequencing

DNA sequences were derived by subcloning the L-V_H-D-J_H and L-V_L-J_L PCR fragments in M13mp18 or in the expression vector pING,[16] followed by sequencing using the dideoxy chain termination method.[26]

RESULTS AND DISCUSSION

Design of Primers for Mouse V_H and V_L Amplification

Because most strategies used to amplify mouse V_H and V_L genes generate fragments that are truncated or that may contain mutations in their 5′ and/or 3′ ends, we have designed new sets of 5′ and 3′ PCR primers that preserve the entire Fv sequences. These primers were chosen in the 5′-untranslated region and in the intron downstream of the rearranged J segment of the λ, κ, and heavy-chain genes (Fig. 1).

A) PCR primers for Vλ :

(a) 5′λ1 : GATC**GTCGAC**CITGGTTTGTGAATTATG
(a) 5′λ2 : GATC**GTCGAC**AGTAGTACCTGCATTATG
3′λ1 : GATC**GCGGCCGC**AAAGGAGGIAGGAGTIAC
3′λ2 : ATC**AGCGGCCGC**AAGAAGCATTAAAGCCAC
3′λ3 : ATC**AGCGGCCGC**AAGAAGCTTTGAAACTAC

B) PCR primers for Vκ :

(a) 5′κ1 : GATC**GTCGAC**AAATTCAAAG/TACAA/CAATG
(f) 5′κ2 : GATC**GTCGAC**AAGACTCAGCCTGACATG
(a) 5′κ3 : GATC**GTCGAC**AAGTTCAAAGACAAAATG
(d) 5′κ4 : GATC**GTCGAC**AGACTCAGCCTIGACATG
(h) 5′κ5 : GATC**GTCGAC**AGCAGGGGGAGCAGGATG
(b) 5′κ6 : GATC**GTCGAC**AGGGAAAGTTTGAAGATG
(d) 5′κ7 : GATC**GTCGAC**ATACATCAGACCAGCATG
(d) 5′κ8 : GATC**GTCGAC**ATCTAGC/TTCTCAGAGATG
(f) 5′κ9 : GATC**GTCGAC**ATGCATCACACCAGCATG
(d) 5′κ10: GATC**GTCGAC**CACCAAGTTCTCAGAATG
(h) 5′κ11: GATC**GTCGAC**CAGAGCAGCAGGGACATG
(h) 5′κ12: GATC**GTCGAC**CAGGGACAAGTGGGAATG
(e) 5′κ13: GATC**GTCGAC**CATTCAGAACTCAGCATG
(h) 5′κ14: GATC**GTCGAC**GCGAGTCAGACCAGCATG
(b) 5′κ15: GATC**GTCGAC**GGACACAGTTTAGATATG
(i) 5′κ16: GATC**GTCGAC**GGACTCAGCATGGACATG
(e) 5′κ17: GATC**GTCGAC**GGAGACGTTGTAGAAATG
(f) 5′κ18: GATC**GTCGAC**GGATACACCATCAGCATG
(i) 5′κ19: GATC**GTCGAC**GGCAAA/GGGCATCAAGATG
(i) 5′κ20: GATC**GTCGAC**GGCAGG/TGGA/GAGCAAGATG
(g) 5′κ21: GATC**GTCGAC**GGTCACAGCACAAACATG
(g) 5′κ22: GATC**GTCGAC**GGTTGCCTCCTCAAAATG
(b) 5′κ23: GATC**GTCGAC**GTTCATTTCCTCAAAATG
(a) 5′κ24: GATC**GTCGAC**TATCAAGTTCICAGAATG
(c) 5′κ25: GATC**GTCGAC**TCTCAAGTTCTCAGAATG
(a) 5′κ26: GATC**GTCGAC**TCTTGTGAATTAATCATG
(c) 5′κ27: GATC**GTCGAC**TGAAAACACACAGACATG
(c) 5′κ28: GATC**GTCGAC**TGATAAAGCCAAGGAATG
(e) 5′κ29: GATC**GTCGAC**TGATCACACACAGA/TCATG
(g) 5′κ30: GATC**GTCGAC**TTCCAGCTCTCAGAGATG
3′κ1 : ATC**AGCGGCCGC**AGAGAG/CTTTGGATTCTAC
3′κ2 : ATC**AGCGGCCGC**AAGAGTTGAGAAGACTAC
3′κ3 : ATC**AGCGGCCGC**AGTTGAGCAAAAATGTAC
3′κ4 : ATC**AGCGGCCGC**AAATGAGCAAAAA/GTCTAC
3 κ5 : ATC**AGCGGCCGC**AAGATGAGAAAAGTGTAC

D) Jλ oligo probes :

Jλ1 : GTCAGTTTGGTTCCTCCAC
Jλ2 : GTGACCTTGGTTCCACCGC
Jλ3 : GTGACCTTGGTTCCACTGC
Jλ4 : GTCAATCTGGTTCCACCTC

F) JH oligo probes :

JH1 : GACCGTGGTCCCTGCGCCC
JH2 : GAGAGTGGTGCCTTGGCCC
JH3 : GACCAGAGTCCCTTGGCCC
JH4 : GACTGAGGTTCCTTGACCC

C) PCR primers for VH :

(j) 5′H1 : GATC**GTCGAC**ACACAGACTCACACCATG
(g) 5′H2 : GATC**GTCGAC**ACACAGGACCTCACCATG
(j) 5′H3 : GATC**GTCGAC**ACACAGGATCTCACCATG
(g) 5′H4 : GATC**GTCGAC**ACACAGGGCATTGCCATG
(b) 5′H5 : GATC**GTCGAC**ACACTGACTCAAAACATG
(c) 5′H6 : GATC**GTCGAC**ACACTGACTCAAACCATG
(j) 5′H7 : GATC**GTCGAC**ACACTGACTCACACCATG
(j) 5′H8 : GATC**GTCGAC**ACACTGACTCCAACCATG
(c) 5′H9 : GATC**GTCGAC**ACACTGACTCTAACCATG
(k) 5′H10: GATC**GTCGAC**ACACTGACTCTCACCATG
(c) 5′H11: GATC**GTCGAC**ACACTGACTTTCACCATG
(b) 5′H12: GATC**GTCGAC**ACATAGACTCTAACCATG
(b) 5′H13: GATC**GTCGAC**ACATTGACTCAAACCATG
(g) 5′H14: GATC**GTCGAC**AGCCTCCATCAGAGCATG
(e) 5′H15: GATC**GTCGAC**AGCCTCCGTCAGAGCATG
(a) 5′H16: GATC**GTCGAC**ATTATAACATTGAACATG
(b) 5′H17: GATC**GTCGAC**CAAGTCTTAGACATCATG
(k) 5′H18: GATC**GTCGAC**CACACATCCCTTACCATG
(h) 5′H19: GATC**GTCGAC**CACAGACACCTCACCATG
(e) 5′H20: GATC**GTCGAC**CACAGACCA/CCTCACCATG
(k) 5′H21: GATC**GTCGAC**CACAGACCTGTCAACATG
(h) 5′H22: GATC**GTCGAC**CACAGACCTGTCACCATG
(e) 5′H23: GATC**GTCGAC**CACGGAACCCTCACCATG
(f) 5′H24: GATC**GTCGAC**CACGGACCCCTCACCATG
(f) 5′H25: GATC**GTCGAC**CACGGACCCCTCACGATG
(k) 5′H26: GATC**GTCGAC**CACTCGACTCTAACCATG
(h) 5′H27: GATC**GTCGAC**CACTGGTGTGCAGTCATG
(a) 5′H28: GATC**GTCGAC**CACTTCTTAGACATCATG
(f) 5′H29: GATC**GTCGAC**CAGAGTCCACTCA/GCCATG
(l) 5′H30: GATC**GTCGAC**CCTGTCACTGACTTCATG
(c) 5′H31a:GATC**GTCGAC**CTCAAGGTCCTTACAATG
(l) 5′H31b:GATC**GTCGAC**CTCCAGGTCCTTACAATG
(i) 5′H32: GATC**GTCGAC**CTCAGTCCTGTCACCATG
(l) 5′H33: GATC**GTCGAC**CTCAGTCCTGTCACTATG
(i) 5′H34: GATC**GTCGAC**GCAGAGGACCTCACAATG
(d) 5′H35: GATC**GTCGAC**GCCTTTACAGACTTCATG
(f) 5′H36: GATC**GTCGAC**GGACCTCACCATGGGATG
(i) 5′H37: GATC**GTCGAC**GGGTGTTGCCTAAGGATG
(d) 5′H38: GATC**GTCGAC**GGTGTA/TGCCTAAAAGATG
(l) 5′H39: GATC**GTCGAC**GGTGTTGCCTAAAGGATG
(a) 5′H40: GATC**GTCGAC**GTTGTAGCCTAAAAGATG
(d) 5′H41: GATC**GTCGAC**TCAGTCCTTGTCACTATG
3′H1 : ATC**AGCGGCCGC**AAAGAAAAAAGCCAGCTTAC
3′H2 : ATC**AGCGGCCGC**GAGGTTT/GTAAGGACTCAC
3′H3a: ATC**AGCGGCCGC**GGAGAAA/GTTAGGACTCAC
3′H3b: ATC**AGCGGCCGC**GGAGAAGT/GTAGGACTCAC
3′H4 : ATC**AGCGGCCGC**TGGAGAGGCCATTCTTAC

E) Jκ oligo probes :

Jκ1 : GTGCCTCCACCGAACGTCC
Jκ2 : GTCCCCCCTCCGAACGTGT
Jκ3 : GTCCCATCACTGAATGTGA
Jκ4 : GTCCCCGAGCCGAACGTGA
Jκ5 : GTCCCAGCACCGAACGTGA

FIGURE 2 Nucleotide sequences of the PCR primers and probes used for the amplification of mouse V_λ, V_κ, and V_H regions. *Sal*I and *Not*I sites are in bold. (I) Inosine. The 5′ primers for the PCR amplification of the variable regions were grouped according to melting temperature, and the groups are designated (*a–l*) as indicated by the letters preceding each entry.

DNA sequence comparisons of the 5′-untranslated region and of the intron downstream of the J segments were performed using data from Kabat,[27] and from the GenBank and European Molecular Biology Laboratory (EMBL) libraries. Sequences were analyzed with the Genetics Computer Group (GCG, Madison, WI) package programs. Alignment of the DNA sequences of the 5′-untranslated region showed that the polymorphism in this region is globally identical to that of the leader peptide sequences (data not shown). However, in the 5′-untranslated region, a sequence of ~20 nucleotides located immediately upstream of the start codon (ATG) was less polymorphic. Therefore, we designed multiple 5′ PCR primers (18-mers) that terminated with the ATG sequence. This ensured a perfect match of the 3′ end of each primer with the template, which was shown to be crucial to initiate consistent priming of the *Taq* polymerase extension reaction. A *Sal*I restriction site and four additional nucleotides were added to the 5′ end of the primers to facilitate cloning of the PCR fragments. Complex sets of 5′ primers were synthesized consisting of two primers for λ, 30 primers for κ, and 42 primers for the heavy-chain genes, some of which contained inosine residues or were degenerate (Fig. 2).

In contrast with the 5′-untranslated region, alignments of the 5′ end of the intron sequences downstream of the J segments did not reveal significant polymorphism. Moreover, alignments showed that 3′ PCR primers specific for each of the J_λ, J_κ, and J_H segments could be designed in this intronic region. For this reason, a single PCR primer was prepared downstream of each J segment, except for the J_{H3} segment, where two primers were needed because of DNA sequence polymorphism. The 3′ PCR primers were 18-mers, except for the primer downstream of the J_{H1} segment which was a 20-mer, reflecting a high content of A/T nucleotides. A *Not*I restriction site and 4 additional nucleotides were added to the 5′ end of these oligonucleotides to allow directional cloning of the PCR fragments into expression vectors (Fig. 2).

To ensure specificity of the PCR amplifications and to characterize better the V_H-D-J_H and V_L-J_L rearrangements, sets of oligonucleotides (19-mers) specific for each of the J_λ, J_κ, and J_H segments, which display a very low level of polymorphism, were synthesized. These were used as probes in both Southern blot analysis of the PCR fragments and Northern blots of total RNA extracted from the mouse hybridoma cells.

Northern Blot Analysis of RNA from Mouse Hybridoma Cells Using J Segment-specific Oligonucleotide Probes

The specificity of the oligonucleotides that hybridize to each of the J segments was demonstrated by Southern blot using a variety of cloned V_L and V_H PCR fragments of known sequences (data not shown). The specificity of these probes was also shown with Northern blots. RNA from the hybridoma cells 3G3 [IgM (λ)] and HNK-20 [IgA (κ)] were hybridized with the J_λ, J_κ, and J_H oligonucleotides (Fig. 3). Probes $J_{\lambda1}$, $J_{\kappa2}$, and J_{H3} generated signals at the expected size for 3G3 and HNK-20, respectively. The signal generated by hybridization of HNK-20 RNA with the $J_{\kappa2}$ probe was much stronger than with the J_{H3} probe, although these two probes had similar specific activities and identical calculated melting temperatures. This observation suggests that either two or even three κ-chain transcripts hybridized with the

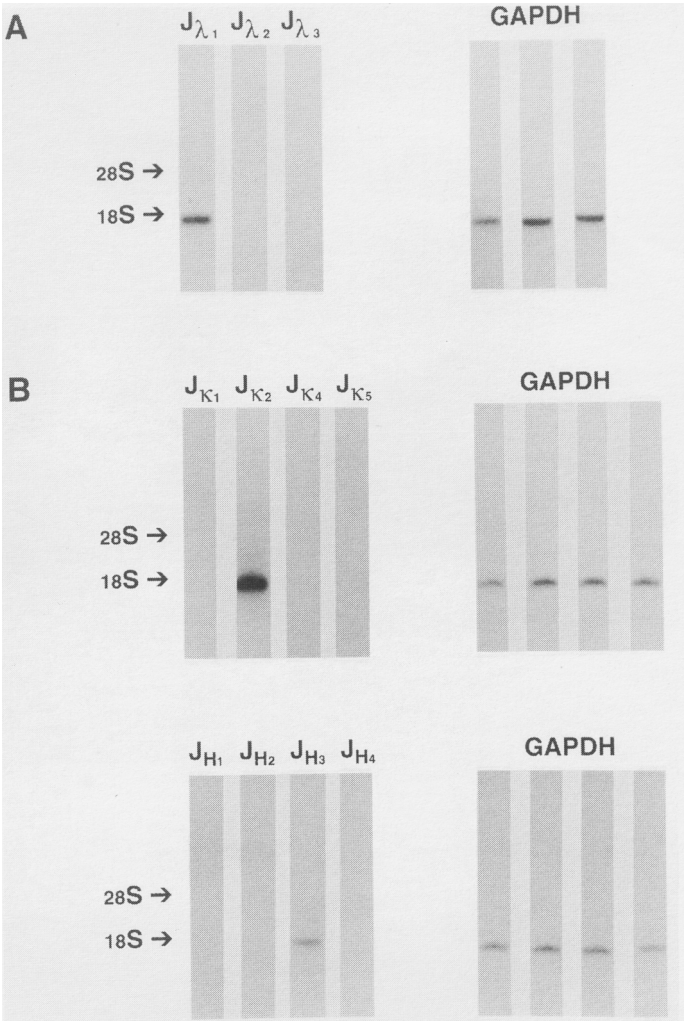

FIGURE 3 Northern blots of RNA isolated from hybridoma cells 3G3 and HNK-20 probed with oligonucleotides homologous to the J_λ, J_κ, and J_H segments. (*A*) Total RNA (10 μg per lane) from 3G3 [IgM (λ)] was hybridized with the oligonucleotides $J_{\lambda1}$, $J_{\lambda2}$, and $J_{\lambda3}$. (*B*) Total RNA (10 μg per lane) from HNK-20 [IgA (κ)] was hybridized with $J_{\kappa1}$, $J_{\kappa2}$, $J_{\kappa4}$, and $J_{\kappa5}$, or with J_{H1}, J_{H2}, J_{H3}, and J_{H4} (see Fig. 2 for sequences of the oligonucleotide probes). The oligonucleotides specific for the pseudogenes $J_{\kappa3}$ and $J_{\lambda4}$ were not used in this experiment. As an internal control for total RNA loading, Northern blots were stripped and rehybridized with a full-length glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA probe.[18] Positions of 18S and 28S rRNAs are indicated.

$J_{\kappa 2}$ probe. One of these transcripts, corresponding to a pseudogene rearranged with the $J_{\kappa 2}$ segment, originated from the immortalizing fusion partner X63Ag8.653.[28,29] For this reason, at least one κ pseudogene transcript and one κ functional gene transcript, both of them rearranged with the $J_{\kappa 2}$ segment, contributed to the strong signal observed. PCR amplification of the V_κ genes from HNK-20 genomic DNA supports the hypothesis that three κ-chain genes were rearranged with the $J_{\kappa 2}$ segment. All three of these rearrangements were transcribed using reverse transcriptase–PCR (RT–PCR) (data not shown). Consequently, the signal observed on Northern blots hybridized with the $J_{\kappa 2}$ oligonucleotide probe is composed of three superimposed signals. In all cases, the Northern blots were rehybridized with a probe specific for the housekeeping enzyme gene glyceraldehyde-3-phosphate-dehydrogenase[25] to control for the amount of RNA loaded (Fig. 3).

Strategy for Mouse V_H and V_L PCR Amplification

Several immunoglobulin variable region genes were amplified by PCR using genomic DNA prepared from mouse hybridoma cells. As an example, we describe the amplification of V_λ of the 3G3 hybridoma, V_κ, and V_H regions of the HNK-20 hybridoma.

From the Northern blot data shown in Figure 3, it was established that the λ chain gene was rearranged with $J_{\lambda 1}$, the κ chain genes with $J_{\kappa 2}$, and the heavy-chain gene with J_{H3}. Therefore, the 3′ primers required for amplification were 3′λ1, 3′κ2, and 3′H3a or 3′H3b, respectively. Because of the high degree of polymorphism seen in the 5′-untranslated region, all 5′ primers had to be included in the PCR reactions in combination with these 3′ primers. This would result in two PCR reactions for λ, 30 for κ, and 42 for the heavy-chain genes, if each PCR reaction contained one 5′ primer and one 3′ primer. To reduce the number of combinations of 5′ and 3′ primers and, consequently, the number of PCR reactions, the 5′ primers were divided into small groups of 2, 3, or 4 primers based on their melting temperature (Fig. 2). This resulted in 1 group for λ (group a), 9 for κ (groups a–i), and 12

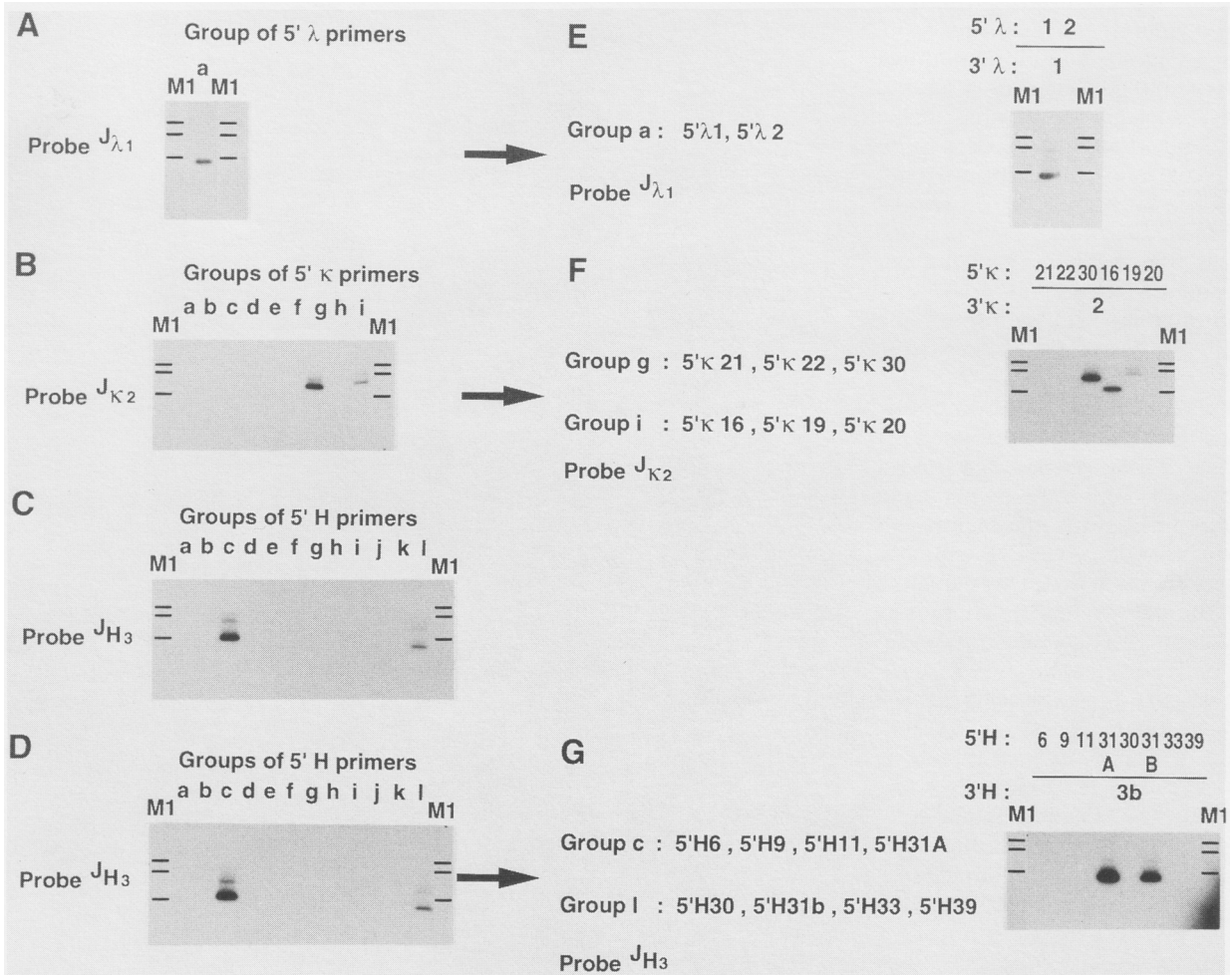

FIGURE 4 Southern blot analysis of the amplified 3G3 V_λ, HNK-20 V_κ, and V_H genomic fragments following a two-step PCR amplification. In the first step (*A–D*), PCR amplifications were performed with groups of 5′ primers (see Fig. 2) combined with a single 3′ primer. The 3′ primer for V_λ was 3′λ1; for V_κ 3′κ2; and for V_H 3′H3a or 3′H3b. In the second step (*E–G*), PCR amplifications were performed separately with each of the 5′ primers from the positive groups, allowing the 5′ primers that generate the signal in the first reaction to be identified. PCR amplification with 3′H3a was omitted in the second step, as 3′H3b generates stronger signals. Positions of size markers of 564, 831, and 947 bp are indicated (lane *M1*).

for the heavy-chain gene primers (groups a–l). A first set of PCR reactions contained one group of 5′ primers and one 3′ primer, thereby reducing the number of PCR reactions to 1 for V_λ, 9 for V_κ, and 12 for V_H. Southern blot analyses of these PCR reactions, using the $J_{\lambda1}$-, $J_{\kappa2}$-, and J_{H3}-specific oligonucleotide probes, allowed the identification of the combinations that generated positive signals: 5′λ group a with 3′λ1; 5′κ groups g and i with 3′κ2; and 5′ heavy-chain groups c and l with 3′H3a or 3′H3b (Fig. 4A–D). These combinations were reanalyzed in a second set of PCR reactions that each contained one 5′ and one 3′ primer, to identify the pairs of primers that generated positive signals. These PCR reactions were analyzed again by Southern blot using the $J_{\lambda1}$, $J_{\kappa2}$, and J_{H3} oligonucleotide probes. Results showed that the positive signals were generated by the following combinations: 5′λ1 with 3′λ1 (Fig. 4E); 5′κ30, 5′κ16, or 5′κ19 with 3′κ2 (Fig. 4F); and 5′H31A or 5′H31B (which have very closely related sequences) with 3′H3b (Fig. 4G). These results indicate that one λ-chain gene was rearranged in 3G3, and three κ-chain genes and one heavy-chain gene were rearranged in HNK-20 (Fig. 5).

With another mouse hybridoma cell (PCG-4), secreting IgG2a with a κ chain, three different rearrangements of the κ-chain genes, and a single rearrangement of the heavy-chain gene were also observed using the same PCR strategy (data not shown).

The presence of three V_κ rearrangements in HNK-20 and PCG-4 is consistent with both a functional V_κ and an aberrant V_κ in all hybridoma cells obtained by fusion with cell lines derived from the original MOPC-21 tumor.[28,29] A third κ-chain rearrangement was detected in both HNK-20 and PCG-4 hybridomas, although it involved different J_κ segments. RT–PCR showed that this third rearrangement was transcribed in HNK-20 and silent in PCG-4 (data not shown). Because the only κ-chain gene that the fusion partner could have contributed is the nonfunctional MOPC-21 κ gene,[30] this third rearrangement probably originated from the mouse B cell that served as a fusion partner. Contamination of the HNK-20 hybridoma cell by another hybridoma cell is improbable because a single heavy-chain rearrangement was detected in both Northern and Southern blots.

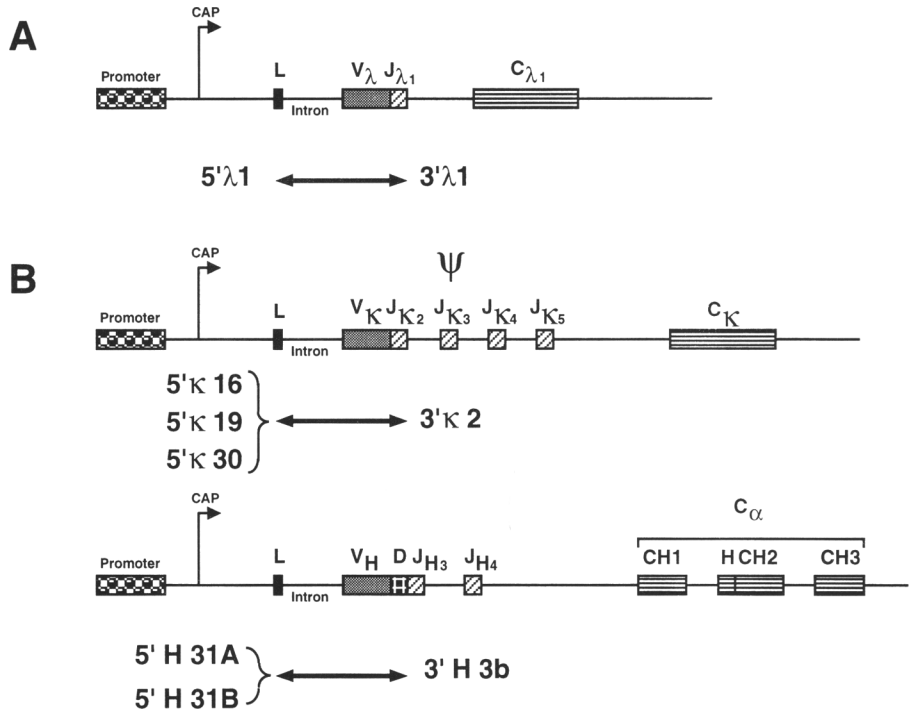

FIGURE 5 Map of the rearranged 3G3 V_λ, and HNK-20 V_κ, and V_H regions. (*A*) Rearrangement of the λ locus from 3G3; (*B*) rearrangements of the κ and heavy-chain loci from HNK-20. Three different κ-chain gene rearrangements were observed with the $J_{\kappa2}$ segment. For the heavy-chain genes, 5′H31a and 5′H31b generated the same PCR product; hence, a single rearrangement was observed for the heavy chain. The maps are not to scale. The pseudogene is indicated by Ψ.

Sequence Analysis of the V_λ, V_κ, and V_H PCR Fragments

The PCR-amplified variable regions of 3G3 V_λ, HNK-20 V_κ(s), and HNK-20 V_H were cloned into the pING expression vector[16] and sequenced using the dideoxy chain termination technique.[26] Sequences showed that the 3G3 V_λ (5′λ1-3′λ1), HNK-20 V_κ (5′κ16-3′κ2), HNK-20 V_κ (5′κ19-3′κ2), and HNK-20 V_H (5′H31B-3′H3b) fragments encode functional variable regions (Fig. 6A–D). The sequence of the HNK-20 V_κ (5′κ19–3′κ2) fragment is not reported here because its coding sequence was identical to a published immunoglobulin V_κ cDNA sequence.[31] The deduced amino acid sequences of the functional V_λ, V_κ, and V_H fragments correspond to open reading frames that are highly homologous to other mouse immunoglobulin variable regions. In these four PCR amplified fragments, the positions of the cysteines involved in the intramolecular disulfide bridge were conserved. The presence of conserved stretches of amino acids corresponding to framework sequences allowed the CDRs to be positioned (Fig. 6). Taken together, these structural features suggest that the amplified and cloned Fv fragments should be functional. However, it was not possible to determine by computer modeling which of the two κ fragments, shown by sequencing to be functional, is bound to the HNK-20 V_H (data not shown). Consequently, the two functional HNK-20 V_κs and the HNK-20 V_H have been inserted into expression vectors containing human heavy- and light-chain constant genes and cotransfected into myeloma cells. Currently, the secreted chimeric antibodies are being tested for their ability to recognize their corresponding antigen.

The cDNA sequence of the HNK-20 V_κ nonfunctional rearranged gene has been reported[28,29] and was shown to contain a tyrosine instead of the invariant cysteine in position 23. Moreover, at the site of VJ recombination, 4 nucleotides appear to have been deleted, leading to a frame shift. This results in a premature termination codon at the 3′ end of the *J* segment at position 105. We report here the genomic sequence of this V_κ pseudogene (Fig. 6C) in which the cod-

A

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GATCGTCGACCGTGGTTTGTGAATTATG GCC TGG ATT TCA CTT ATA CTC TCT CTC   55
                             Met Ala Trp Ile Ser Leu Ile Leu Ser Leu  10

CTG GCT CTC AGC TCA G GTCAGCAGCCTTTCTACACTGCAGTGGGTATGCAACAATGCGCAT 116
Leu Ala Leu Ser Ser G                                               15

CTTGTCTCTGATTTGCTACTGATGACTGGATTTCTCATCTGTTTGCAG GG GCC ATT TCC CAG 178
                                                 ly Ala Ile Ser Gln  20

GCT GTT GTG ACT CAG GAA TCT GCA CTC ACC ACA TCA CCT GGT GAA ACA GTC 229
Ala Val Val Thr Gln Glu Ser Ala Leu Thr Thr Ser Pro Gly Glu Thr Val  37

ACA CTC ACT TGT CGC TCA AGT ACT GGG GCT GTT ACA ACT AGT AAC TAT GCC 280
Thr Leu Thr Cys Arg Ser Ser Thr Gly Ala Val Thr Thr Ser Asn Tyr Ala  54

AAC TGG GTC CAA GAA AAA CCA GAT CAT TTA TTC ACT GGT CTA ATA GGT GGT 331
Asn Trp Val Gln Glu Lys Pro Asp His Leu Phe Thr Gly Leu Ile Gly Gly  71

ACC AAC AAC CGA GCT CCA GGT GTT CCT GCC AGA TTC TCA GGC TCC CTG ATT 382
Thr Asn Asn Arg Ala Pro Gly Val Pro Ala Arg Phe Ser Gly Ser Leu Ile  88

GGA GAC AAG GCT GCC CTC ACC ATC ACA GGG GCA CAG ACT GAG GAT GAG GCA 433
Gly Asp Lys Ala Ala Leu Thr Ile Thr Gly Ala Gln Thr Glu Asp Glu Ala 105

ATA TAT TTC TGT GCT CTA TGG TAC AGC AAC CAT TGG GTG TTC GGT GGA GGA 484
Ile Tyr Phe Cys Ala Leu Trp Tyr Ser Asn His Trp Val Phe Gly Gly Gly 122

ACC AAA CTG ACT GTC CTA G GTGAGTCACTCCTCCCTCCTTTGCGGCCGCTGAT       537
Thr Lys Leu Thr Val Leu                                           128
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B

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GATCGTCGACGGACTCAGCATGGACATG AGG ACC CCT GCT CAG TTT CTT GGA ATC   55
                             Met Arg Thr Pro Ala Gln Phe Leu Gly Ile  10

TTG TTG CTC TGG TTT CCA G GTAAAATGAACTAAAATGGGAATGTCACTGTGATTAGTGTTG 116
Leu Leu Leu Trp Phe Pro G                                           16

ATTGGCATTTGGGAGATTTTATCTTTTATGATGCTTACCTATGTAGATACTCATTATGTCTCCATTC 183

CTAG GT ATC AAA TGT GAC ATC AAG GTG ACC CAG TCT CCA TCT TCC ATG TAT 234
     ly Ile Lys Cys Asp Ile Lys Val Thr Gln Ser Pro Ser Ser Met Tyr  32

GCA TCT CTA GGA GAG AGA GTC ACT ATC ACT TGC AAG GCG AGT CAG GAC ATT 285
Ala Ser Leu Gly Glu Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile  49

AAT AAC TAT TTA AAC TGG TTC CAG CAG AAA CCA GGG AAA TCT CCT AAG ACC 336
Asn Asn Tyr Leu Asn Trp Phe Gln Gln Lys Pro Gly Lys Ser Pro Lys Thr  66

CTG ATC TAT CGT GCA AAC AGA TTG CTA GAT GGG GTC CCA TCA AGG TTC AGT 387
Leu Ile Tyr Arg Ala Asn Arg Leu Leu Asp Gly Val Pro Ser Arg Phe Ser  83

GGC AGT GGA TCT GGG CAA GAT TAT TCT CTC ACC ATC AGC AGC CTG GAG TAT 438
Gly Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr Ile Ser Ser Leu Glu Tyr 100

GAA GAT ATG GGA ATT TAT TAT TGT CTA CAG TTT GAC GAG TTT CCG TAC ACG 489
Glu Asp Met Gly Ile Tyr Tyr Cys Leu Gln Phe Asp Glu Phe Pro Tyr Thr 117

TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA C GTAAGTAGTCTTCTCAACTCTTGCG 545
Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys                             127

GCCGCTGAT                                                          554
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C

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GATCGTCGACTTCCAGCTCTCAGAGATG GAG ACA GAC ACA CTC CTG TTA TGG GTA   55
                             Met Glu Thr Asp Thr Leu Leu Leu Trp Val  10

CTG CTG CTC TGG GTT CCA G GTGAGAGTGCAGAGAAGTGTTGGATGCAACCTCTGTGGCCA 115
Leu Leu Leu Trp Val Pro G                                           16

TTATGATACTCCATGCCTCTCTGTTCTTGATCACTATAATTAGGGCATTTGTCACTGGTTTTAAGTT 182

TCCCCAGTCCCCTGAATTTTCCATTTCCTCAGAGTGATGTCCAAAATTCTTCTTAAAAATTTAAATC 249

AAAAGGTCCTCTGCTGTGAAGTCTTTTATACATATATAACAATAATCTTTGTGTTTATCATTCCAG 315

 GT TCC ACT GGT GAC ATT GTG CTG ACA CAG TCT CCT GCT TCC TTA GCT GTA 365
 ly Ser Thr Gly Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val  33

TCT CTG GGG CAG AGG GCC ACC ATC TCA TAC AGG GCC AGC AAA AGT GTC AGT 416
Ser Leu Gly Gln Arg Ala Thr Ile Ser Tyr Arg Ala Ser Lys Ser Val Ser  50

ACA TCT GGC TAT AGT TAT ATG CAC TGG AAC CAA CAG AAA CCA GGA CAG CCA 467
Thr Ser Gly Tyr Ser Tyr Met His Trp Asn Gln Gln Lys Pro Gly Gln Pro  67

CCC AGA CTC CTC ATC TAT CTT GTA TCC AAC CTA GAA TCT GGG GTC CCT GCC 518
Pro Arg Leu Leu Ile Tyr Leu Val Ser Asn Leu Glu Ser Gly Val Pro Ala  84

AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT 569
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Asn Ile His Pro 101

GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT CAG CAC ATT AGG GAG CTT 620
Val Glu Glu Glu Asp Ala Ala Thr Tyr Tyr Cys Gln His Ile Arg Glu Leu 118

ACA CGT TCG GAG GGG GGA CCA AGC TGG AAA TAA AAC GTAAGTAGTCTTCTCAACT 675
Thr Arg Ser Glu Gly Gly Pro Ser Trp Lys ***                         128

CTTGCGGCCGCTGAT                                                    690
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D

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GATCGTCGACCTCAAGGTCCTTACAATG AAA TGC AGC TGG GTC ATC TTC TTC CTG   55
                             Met Lys Cys Ser Trp Val Ile Phe Phe Leu  10

ATG GCA GTG GTT ACA G GTAAGGAGCTCCCAAGTCCCAAACTTGAGGGGCCATACACTCTGT 116
Met Ala Val Val Thr G                                               15

 GACAGTGGCAGTCACTTTGCCTTTCTTTCTACAG GG GTC AAT TCA GAG GTT CAG CTG 173
                                    ly Val Asn Ser Glu Val Gln Leu  23

CAG CAG TCT GGG GCT GAG CTT GTG AGG CCA GGG GCC TTA GTC AAG TTG TCC 224
Gln Gln Ser Gly Ala Glu Leu Val Arg Pro Gly Ala Leu Val Lys Leu Ser  40

TGC AAA GCC TCT GGC TTC AAC ATT AAA GAC TAC TAT ATG TAC TGG GTA AAA 275
Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr Tyr Met Tyr Trp Val Lys  57

CAG AGG CCT GAA CAG GGC CTG GAG TGG ATT GGA TGG ATT GAT CCT GAA AAT 326
Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile Gly Trp Ile Asp Pro Glu Asn  74

GGT AAT ACT GTT TAT GAC CCG AAG TTC CAG GGC AAG GCC AGT ATA ACA GCA 377
Gly Asn Thr Val Tyr Asp Pro Lys Phe Gln Gly Lys Ala Ser Ile Thr Ala  91

GAC ACA TCC TCC AAC ACA GCC TAC CTG CAG CTC AGC AGC CTG GCA TCT GAG 428
Asp Thr Ser Ser Asn Thr Ala Tyr Leu Gln Leu Ser Ser Leu Ala Ser Glu 108

GAC ACT GCC GTC TAT TAC TGT GCT TAC TAC GGT ACT AGC TAC TGG TTT CCT 479
Asp Thr Ala Val Tyr Tyr Cys Ala Tyr Tyr Gly Thr Ser Tyr Trp Phe Pro 125

TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA G GTGAGTCCTACCTTCTC 533
Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala                     137

CGCGGCCGCTGAT                                                      546
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FIGURE 6 Sequences of the PCR-amplified 3G3 V_λ, HNK-20 V_κ, and V_H regions. (*A*) 3G3 V_λ fragment (5′λ1-3′λ1); (*B*) HNK-20 V_κ fragment (5′κ16-3′κ2); (*C*) HNK-20 V_κ (pseudogene) fragment (5′κ30-3′κ2); (*D*) HNK-20 V_H fragment (5′H31B-3′H3b). These sequences have been deposited with the EMBL GenBank under accession numbers X82687 (3G3 V_λ), X82688 (HNK-20 V_κ), X82689 (HNK-20 V_κ pseudogene), and X82690 (HNK-20 V_H). The sequence of the PCR primers is shown in boldface type, the CDR regions are underlined, and the stop codon is noted by stars. Regions homologous to the J_λ, J_κ, and J_H oligonucleotide probes are double underlined.

ing sequence is in complete agreement with the reported cDNA sequences. This observation showed that the V_κ pseudogene was not affected by recombination events, at least in its coding region, during the PCR amplification process. Such PCR artifacts generating hybrid DNA molecules previously have been shown to occur when amplifying target sequences that are members of a gene family.[32] If the sequences of the PCR fragment and the corresponding cDNA or genomic clone, prepared without amplification techniques, are available, these artifacts can be ruled out. Consequently, because these sequences are not available for the functional V_λ, V_κ, and V_H PCR fragments shown in Figure 6, the possibility that they are hybrid molecules cannot be excluded. However, this hypothesis will be indirectly evaluated by comparison of the specificity and affinity of the original mouse antibody and the chimeric mouse–human antibodies.

In conclusion, we have designed a novel strategy that allows the cloning of the full-length, rearranged variable regions of mouse immunoglobulin genes. This strategy requires a small number of PCR amplifications; however, the large number of 5′ primers and the specificity of both 5′ and 3′ primers greatly increase the probability of amplifying the rearranged V_λ, V_κ, and V_H region genes. In addition, the amplified Fv genes can easily be inserted into expression vectors containing murine or human light- and heavy-chain constant genes into intron sequences. Thus, a serious impediment for the rapid construction of isotype-switched and chimerized mouse immunoglobulins has been overcome.

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