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Cloning immunoglobulin variable domains for expression by the polymerase chain reaction

(chimeric antibodies/MBr1)

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ABSTRACT **We have designed a set of oligonucleotide primers to amplify the cDNA of mouse immunoglobulin heavy and light chain variable domains by the polymerase chain reaction. The primers incorporate restriction sites that allow the cDNA of the variable domains to be force-cloned for sequencing and expression. Here we have applied the technique to clone and sequence the variable domains of five hybridoma antibodies and to express a mouse–human chimeric antibody that binds to the human mammary carcinoma line MCF-7. The technique should also lead to the cloning of antigen-binding specificities directly from immunoglobulin genes.**

For serotherapy, human monoclonal antibodies (mAbs) would be preferred to mouse mAbs because the foreign immunoglobulin can elicit an anti-globulin response that may interfere with the therapy (1) or cause allergic or immune complex hypersensitivity (2). However, there are considerable difficulties in making human mAbs of the required specificity by hybridoma technology (3). Recently, protein engineering has been used to convert mouse mAbs into "human" mAbs by joining the entire immunoglobulin variable (V) domains from mouse mAbs to human constant (C) domains (4–6) or by transplanting the complementarity-determining regions (CDRs) of the mouse mAbs into human myeloma proteins (7–9). Thus, the rearranged immunoglobulin V genes provide the raw material for engineering and have been derived by cloning from genomic DNA (10, 11) or cDNA (12).

The polymerase chain reaction (PCR) (13) has been used recently for genomic (14) and cDNA cloning (15). It involves repeated rounds of extension from two primers specific for regions at each end of the gene. The primers need not match the gene sequence exactly (16), and restriction sites can be incorporated within the primers to allow the forced cloning of the amplified DNA (14). In principle, the restriction sites within the PCR primers could be devised to clone a gene directly for expression, although this has not been described. Therefore, we sought to apply the PCR to the cloning and expression of immunoglobulin V genes.

First, we identified conserved regions at each end of the nucleotide sequences encoding V domains of mouse immunoglobulin heavy chain (V_H) and κ light chain (V_κ). Second, we designed primers for the amplification, which incorporated restriction sites for forced cloning. Third, we constructed vectors that allow the amplified cDNA to be expressed, while retaining the amino acid sequence typical of V domains. We then applied the technique to clone and sequence cDNA encoding five mouse mAbs of therapeutic potential: MBr1 (17), BW 431/26 (18), BW 494/32 (19), BW 250/183 (18, 20), and BW 704/152. MBr1 has been raised against a human mammary carcinoma line MCF-7 and recognizes a saccharide epitope (21). The cDNA of the MBr1 V_H and V_κ domains (mouse μ and κ chains) was expressed as a simple chimeric antibody (human γ1 and κ chains). The human γ1 isotype was chosen, as it should mediate cell killing by both complement lysis and cell-mediated routes (9, 22).

METHODS

Comparison of Nucleotide Sequences. The aligned entries of the nucleotide sequences of the V genes were extracted from the Kabat data base (23): the beginning of the nucleotide sequences correspond to the mature N terminus of the protein, and nucleotides encoding the protein signal sequences are not included. To allow manipulation of the data with the computer program DBUTIL (24) and analysis with the FAMNS and PLOTD programs (R. Staden, personal communication), the entries were entered into a shotgun sequencing data base (24). By using DBUTIL, each V_H or V_κ block was trimmed to a "core alignment" by removing positions encoding extra amino acid residues (A to K in ref. 23) except for residues 82A, 82B, and 82C of the V_H domains. By using FAMNS, the frequency of the most common nucleotide was scored for each site, and the information was used to design the amplification primers (see *Results*). By using PLOTD, the 14 nucleotides at the 3′ end of the forward and back primers (see below) were matched with each entry in the data base. (Entries with nucleotides missing in this section of sequence were excluded from the comparison.) Likewise, PLOTD was used to match up the nucleotide sequences encoding CDR1 and CDR2 of MBr1 V_H and V_κ domains with each of the entries in the data base.

cDNA Synthesis and Amplification. RNA was prepared from about 5×10^8 hybridoma cells grown in roller bottles, and mRNA was selected on oligo(dT)-cellulose (25). First-strand cDNA synthesis was based on ref. 26. A 50-μl reaction mixture containing 10 μg of mRNA, 20 pmol of VH1FOR primer [5′-d(TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG)] or VK1FOR primer [5′-d(GTTAGATCTCCAGCTTGGTCCC)], 250 μM of each dNTP, 10 mM dithiothreitol, 100 mM Tris·HCl (pH 8.3), 10 mM $MgCl_2$, and 140 mM KCl was heated at 70°C for 10 min and cooled. Reverse transcriptase (Anglian Biotec, Colchester, U.K.) was added (46 units) and incubated at 42°C for 1 hr. For amplification with a thermostable DNA polymerase (15), a 50-μl reaction mixture containing 5 μl of the cDNA·RNA hybrid, 25 pmol of primers VH1FOR or VK1FOR and VH1BACK [5′-d(AGGTSMARCTGCAGSAGTCWGG) in which S = C or G, M = A or C, R = A or G, and W = A or T] or VK1BACK [5′-d(GACATTCAGCTGACCCAGTCTCCA)] as appropriate, 250 μM

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Abbreviations: mAb, monoclonal antibody; V_H, heavy chain variable region; V_κ, κ light chain variable region; PCR, polymerase chain reaction; CDR, complementarity-determining region; C, constant; J, joining.

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of each dNTP, 67 mM Tris chloride (pH 8.8), 17 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 200 μg of gelatine per ml, and 2 units of thermus aquaticus (Taq) polymerase (Cetus) was overlaid with paraffin oil and subjected to 25 rounds of temperature cycling with a Techne PHC-1 programable heating block. A typical cycle was 1 min at 95°C (denature), 1 min at 30°C (anneal), and 2 min at 72°C (elongate). The sample (and oil) was extracted twice with ether, once with phenol, and then with phenol/$CHCl_3$, followed by ethanol precipitation. The sample was taken up in 50 μl of water and frozen.

Vector Construction. To make the phage M13-VHPCR1 vector, a *Bst*EII site was introduced into the M13-HuVHNP vector (7) by site-directed mutagenesis (27, 28). To make the M13-VKPCR1 vector, *Pvu* II and *Bcl* I sites were likewise introduced into a version of M13-HuVKLYS (J. Foote and G.W., unpublished data) in which the *Pvu* II sites had been removed from the M13 backbone as follows: M13mp18 (29) was cut with *Pvu* II, and the vector backbone was blunt-ligated to a synthetic *Hin*dIII–*Bam*HI polylinker. The HuVKLYS gene was then introduced as a *Hin*dIII–*Bam*HI fragment.

Cloning of Amplified cDNA. The amplified cDNA was digested with the restriction enzymes *Pst* I and *Bst*EII for the V_H gene or *Pvu* II and *Bgl* II for the V_κ gene. The fragments were phenol-extracted, purified on 2% low-melting-point agarose gels, and force-cloned into M13-VHPCR1 (digested with *Pst* I and *Bst*EII) or into M13-VKPCR1 (digested with *Pvu* II and *Bcl* I). Note that there is a short [4 base pair (bp)] region of complementarity between the 3′ ends of the VH1FOR and VH1BACK primers, and the two primers can prime on each other to give a short duplex, which is readily cloned (J. Gamble and G.W., unpublished data). Therefore, it is important to gel-purify the amplified cDNA. The vector backbone was purified on 0.8% agarose gels and, in the case of the M13-VKPCR1 vector, was also treated with calf intestinal phosphatase. The M13-VKPCR1 vector was prepared in *Escherichia coli* JM110 (29) to avoid Dam methylation at the *Bcl* I site. Clones containing V gene inserts were identified directly by sequencing (30) with primers based in the 3′ noncoding region of the V gene in the M13-VHPCR1 and M13-VKPCR1 vectors.

Antibody Expression. The *Hin*dIII–*Bam*HI fragment carrying the MBr1 V_H gene in M13-VHPCR1 was recloned into a pSV-gpt vector with human immunoglobulin heavy chain γ1 C regions (9). Likewise, the MBr1 V_κ gene in M13-VKPCR1 was recloned as a *Hin*dIII–*Bam*HI fragment into pSV vector with a hygromycin-resistance marker and human κ chain C domains [pSV-hyg-HuCK] (J. Foote and G.W., unpublished data). Vectors were linearized with *Pvu* I and cotransfected into the nonsecreting mouse myeloma line NS0 (31) by electroporation (32). Cells were selected in the presence of mycophenolic acid at 0.3 μg/ml after 1 day and at 1 μg/ml after 7 days. After 14 days, four wells, each containing one or two major colonies, were screened by incorporation of [^{14}C]lysine (33), and the secreted antibody was detected after precipitation with protein A-Sepharose (Pharmacia) on Na-DodSO$_4$/PAGE (34). The gels were stained, fixed, soaked in fluorographic reagent Amplify (Amersham), dried, and autoradiographed on preflashed film at −70°C for 2 days. Supernatant was also tested for binding to the mammary carcinoma line MCF-7 and the colon carcinoma line HT-29 essentially as described (17), either by indirect immunofluorescence assay on cell suspensions (using fluorescein-labeled goat anti-human IgG; Amersham) or solid-phase RIA on monolayers of fixed cells (using ^{125}I-labeled protein A; Amersham). The MCF-7 and HT-29 cell lines were provided by J. Fogh.

RESULTS

Design of Amplification Primers and Vectors. The nucleotide sequences of V genes in ref. 23 were aligned as described

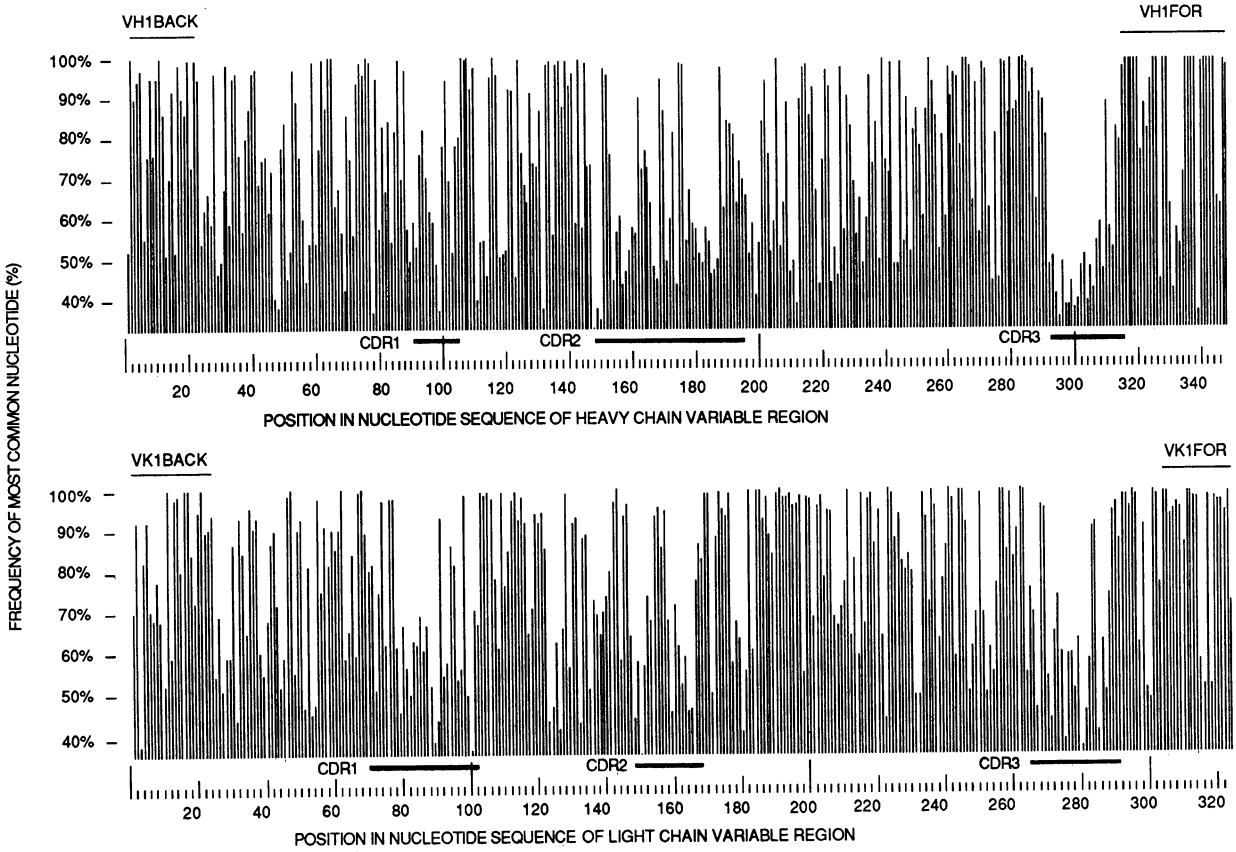

FIG. 1. Frequency of the most common nucleotides in V_H and V_κ gene sequences in ref. 23. CDR1, CDR2, and CDR3 are located, respectively, at nucleotide positions 91–105, 148–195, and 292–315 of V_H genes and positions 70–102, 148–168, and 265–291 of V_κ genes.

Table 1. Checking primers for mismatches with the data base entries

Primers	Entries with none, one, or two mismatches		
	0	1	2
VH1FOR	50/131	71/131	
VH1BACK	22/141	56/141	43/141
VK1FOR	38/61	20/61	
VK1BACK	19/115	54/115	26/115

For each primer, the number of entries with zero, one, or two mismatches with respect to the 14 nucleotides at the 3′ end of each of the primers are given, as well as the total number of eligible entries. For the mixed VH1BACK primer, each of the possible variants was scored and the results summed.

in *Methods*. The frequency of the most common nucleotide was plotted for each position in the aligned V_H and V_κ gene domains (Fig. 1). As expected, the nucleotide sequences encoding the protein CDRs are variable, and those corresponding to the joining (J)-region segments are conserved. However, there are several other conserved regions, in particular those encoding the mature N terminus of both V_H and V_κ domains.

Amplification primers VH1FOR and VK1FOR were designed to be complementary to the mRNA in the J regions; and primers VH1BACK and VK1BACK, to be complementary to the first-strand cDNA encoding the conserved N-terminal region. VH1BACK primer has a mixed sequence. The restriction sites for forced cloning were incorporated toward the 5′ end (the least conserved part) of the primer, and mismatches were minimized near the 3′ end.

The degree of complementarity of the 3′ end of each primer to each of the entries in the Kabat data base was checked with PLOTD. For most entries, this region of the primer proved to be perfectly matched or with only one or two mismatches (Table 1). The 3′ ends of the forward primers were also checked against each of the mouse heavy and light chain J regions, and the 3′ ends of the back primers with consensus sequences derived from each of the mouse V_H and V_κ families (as listed in ref. 23). The primers match well to each J region or mouse V gene family, apart from the VK1BACK primer, which has several mismatches with the mouse V_κ II family.

The scheme for cloning of V_H and V_κ sequences from mouse Ig mRNA is shown in Fig. 2. The restriction sites at each end of the mature V domains allow the existing domain to be excised and the amplified cDNA to be inserted. At the

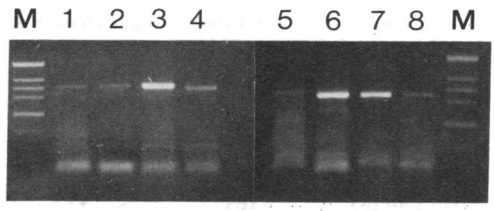

FIG. 3. Amplified cDNA from heavy and light chain V regions of four hybridomas. Each sample (5 μl) was checked on 2% agarose gels and stained with ethidium bromide. Lanes: M, 1 μg of pBR322 *Hin*fI fragments (517 and 506) bp, 396 bp, 344 bp, 298 bp, (221 and 220) bp, 154 bp, and 75 bp as markers; 1–4, V_H cDNA of BW 704/152, BW 250/183, BW 494/32, and BW 431/26, respectively; 5–8, separate gel for the corresponding V_κ cDNA.

cloning junction and at both ends of the V genes, the cloned cDNAs encode amino acid sequences that are typical of V_H or V_κ domains.

Amplification and Cloning. cDNA was prepared from each of five hybridoma lines: MBr1, BW 704/152, BW 250/183, BW 494/32, and BW 431/26 (17–20) by using the amplification primers. A typical result is shown in Fig. 3, in which the amplified cDNA gives a major band of the expected size (about 320–350 bp), although its intensity varies and there are also smaller bands. For the V_κ cDNA of MBr1, the band was very weak and was excised from the gel and reamplified in a second round (not shown). After digestion with restriction enzymes, the cDNA was gel-purified and cloned into the M13-VHPCR1 and M13-VKPCR1 vectors. The clones containing V_H or V_κ gene inserts were identified directly by sequencing, as the majority carried correct inserts. (There is an internal *Pst* I site in the V_H gene of BW 431/26, and the gene was therefore assembled in two steps: the 3′ *Pst* I–*Bst*EII fragment was first cloned into M13-VHPCR1, followed in a second step by the 5′ *Pst* I fragment.)

A comparison of the sequences of V_H and V_κ domains from the five antibodies with the Kabat protein data base (23) revealed that there were five different mouse V_H families (IA, IB, IIA, IIB and IIIA) and two different mouse V_K families (I and VI) (Table 2). To ensure accuracy, the sequences of the V genes for each antibody were determined from two independent amplifications from the cDNA·mRNA hybrid or, in the case of MBr1, from two independent batches of mRNA. With the exception of two single nucleotide discrepancies and a deletion of three nucleotides beyond the 3′ end of the VK1BACK primer (presumably due to slippage of the

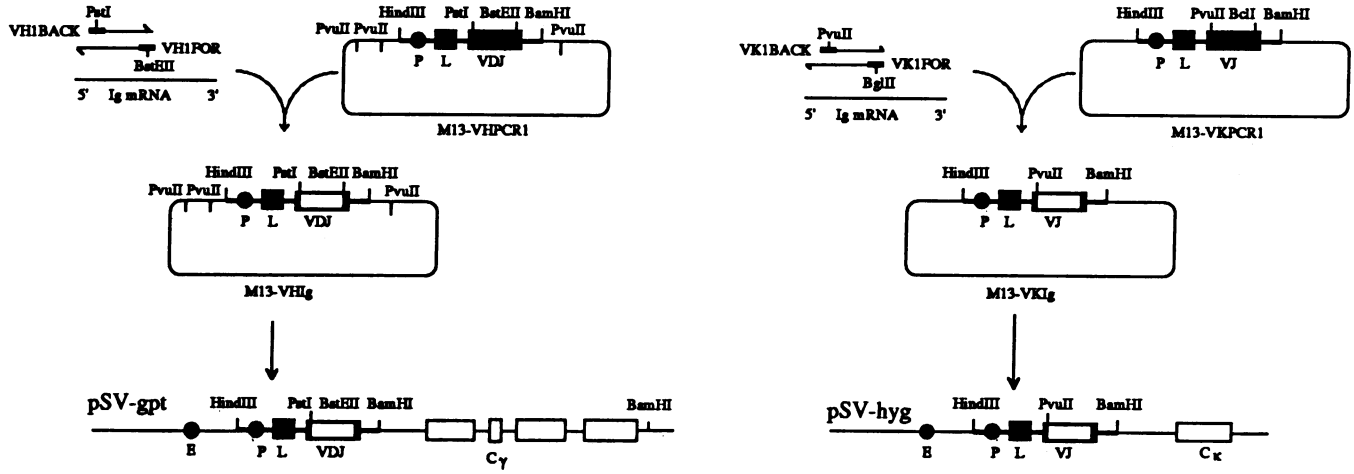

FIG. 2. Scheme for amplification of cDNA and cloning into phage M13 vectors to hook up V region genes for expression. The vectors M13-VHPCR1 and M13-VKPCR1, for cloning the amplified cDNA, contain introns: transcription is driven from the immunoglobulin heavy chain promoter (P), and the signal sequence (L) and leader intron are taken from the mouse V47 unrearranged V_H gene (12). The noncoding sequence to the 3′ end of the V_H gene is described in ref. 12 and of the V_κ gene in ref. 9.

Table 2. Identification of mouse heavy or light chain V gene families cloned from hybridoma mRNA

Hybridoma	V_H family	V_κ family
MBr1	IIB	I
BW 431/26	IA	VI
BW 494/32	IIIA	VI
BW 250/183	IIA	VI
BW 704/152	IB	I

primer), the sequences of the independent clones were identical.

Sequencing and Expression of MBr1. The nucleotide sequence of the MBr1 heavy and light chain V genes is shown in Fig. 4 with part of the flanking regions of the M13-VHPCR1 and M13-VKPCR1 vectors. There are some unusual features of the MBr1 sequence—for example, CDR3 of the V_H domain is very short (35), with a diversity (D) segment encoding apparently only three amino acids. However, these features proved identical in two independent clones. The nucleotide sequences of CDR1 and CDR2 of MBr1 V genes were screened against each of the entries in the Kabat data base using PLOTD. This showed that the sequence of the MBr1 V_H gene was most closely related to that of 119.1, a member of the 4-hydroxy-3-nitrophenylacetyl (NP) major idiotypic family (36).

The V_H and V_κ gene regions of MBr1 were assembled together with human genes $C_{\gamma 1}$ and C_κ for expression of simple mouse–human chimeric antibodies in myeloma cells NS0 (31). The transfected cells were screened for secretion of antibody by precipitation of ^{14}C-labeled heavy and light chains with protein A-Sepharose: one of the four wells

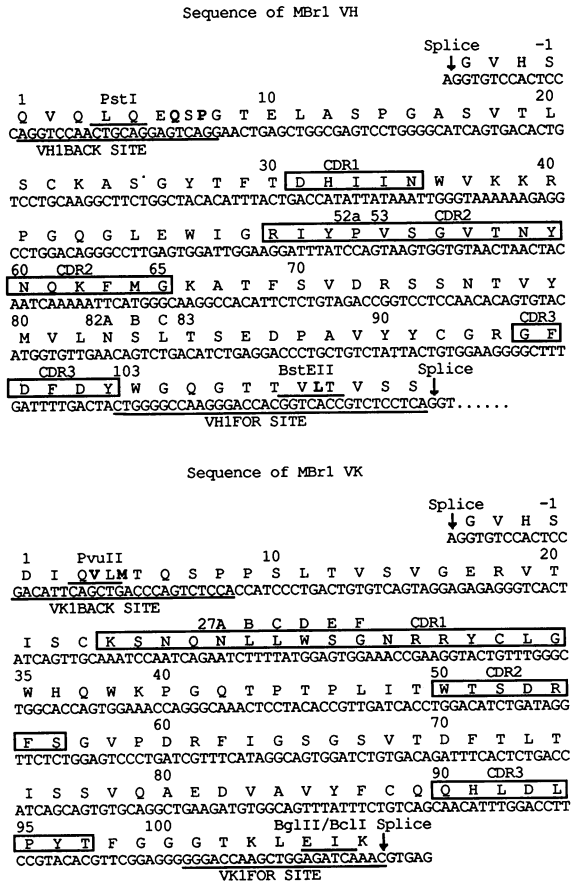

FIG. 4. Nucleotide sequences of MBr1 heavy and light chain V genes and adjacent flanking regions of the M13-VHPCR1 and M13-VKPCR1 vectors. Amino acids encoded within the amplification primers, which are likely to differ in MBr1, are marked in bold type.

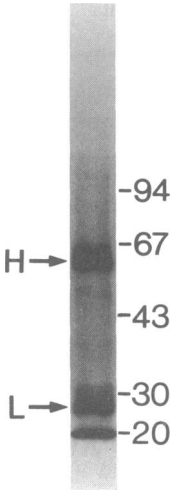

FIG. 5. Secretion of MBr1 chimeric antibody (human $C_{\gamma 1}$ and C_κ) from the myeloma NS0. Cells were labeled with [^{14}C]lysine, and the secreted antibody was subjected to Na-DodSO$_4$/PAGE (10% acrylamide). The heavy (H) and light (L) chain bands are marked, and the molecular weights of the protein markers phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor are shown $\times 10^{-3}$.

secreted antibody (Fig. 5). The chimeric antibody in the supernatant, like the parent mouse MBr1 antibody, was found to bind to the mammary carcinoma MCF-7 but not to the colon carcinoma HT-29.

DISCUSSION

The sequencing and cloning of immunoglobulin V genes is the first and often rate-limiting step in making simple chimeric or reshaped human antibodies. We have devised a simple and rapid way of cloning these genes via the PCR. By making a systematic comparison of the aligned sequences of V_H and V_κ genes, we have identified nucleotide sequences at the 5′ ends of both V_H and V_κ genes that are relatively conserved. Since the J_H and J_κ regions at the 3′ ends of the genes are also conserved, we were able to design primers for PCR amplification based on these sequences and to include restriction sites for forced cloning. In general, the clones could be screened directly by sequencing, with almost all of the recombinants carrying the correct inserts.

From the five mouse hybridomas, we succeeded in preparing mRNA and amplified cDNA and in cloning heavy chain genes from five V_H gene families and light chain genes from two V_κ gene families. This suggests that our primers might amplify most immunoglobulin mRNA of the mouse repertoire. We have also used the same primers for amplification and cloning of immunoglobulin V_H and V_κ genes from mouse hybridoma mRNA and spleen genomic DNA (D.H.G. and G.W., unpublished data). Nevertheless, we might anticipate some problems—for example, the poor match of the mouse V_κ II gene family with VK1BACK primer. As an alternative to the "universal" amplification primers, we have also used mixtures of back primers based on the concensus sequences of each V_H and V_κ gene family and mixtures of forward primers based on individual J region sequences or mixtures of forward primers based in the CH_1 or C_κ gene regions (D.H.G. and G.W., unpublished data). However, the siting of both primers within the rearranged V genes is probably necessary for their cloning from genomic DNA.

Errors in the amplified V_H and V_κ genes were readily identified by sequencing clones from two independent amplifications. We observed only two single nucleotide discrepancies on comparing 10 pairs of genes of about 300 nucleotides. Thus, the overall error frequency (1/1500) for 25 cycles of PCR is in the same range as the previous estimates of 1/400 (15) and 1/4000 (37) for 30 cycles and is comparable to that expected for a single round of copying of cDNA from RNA by reverse transcriptase (38). As alternative to sequencing independent clones, the amplified cDNA could be sequenced directly (37).

Given our choice of priming sites, it is not possible to determine the exact sequence at both ends of the V genes, as it is dictated by the primer. However, to some extent, we can reconstruct the sequence in these regions (Fig. 4). For example, MBr1 uses heavy chain J_{H2} and light chain $J_{\kappa 2}$: assuming no somatic mutation, we would expect that residue 109 of the V_H MBr1 product is probably leucine and not valine. The V genes of MBr1 belong to the mouse V_H IIB and V_κ I families. For the MBr1 V_H gene, we might expect its product to have Gln-6 and Pro-7, rather than Glu-6 and Ser-7; for the MBr1 V_κ gene, we similarly might expect Val-3 and Met-4, rather than Gln-3 and Leu-4. Nevertheless, the chimeric MBr1 antibody binds to the antigen, and in any case, these uncertainties in the framework regions are unlikely to affect the antigenic specificity, since the specificity can be transplanted from one antibody to another by transfer of the CDRs only (7–9). For the construction of therapeutic antibodies, the sequence of the original mouse hybridoma antibody scarcely matters if the antigenic specificity is retained.

We envisage many other applications of this technique, ranging from the analysis of mouse immunoglobulin gene repertoire to the "rescue" of mAbs from unstable human hybridomas (3). (In view of the over-representation of the mouse immunoglobulin V genes in the Kabat nucleotide data base, these primers may not be optimal for the PCR amplification of human immunoglobulin genes.) However, the most exciting applications may come from the construction of antibody expression libraries. Our design of PCR primers allows the V genes to be joined in frame to a specialized vector, which directs the expression of antibody. Thus, from the mRNA of the mouse hybridoma MBr1, we readily derived a mouse–human chimeric antibody of the same specificity, which was expressed and secreted from the mouse myeloma NS0. In principle, the amplified cDNA could also be expressed and secreted as Fv or Fab fragments from *E. coli* (39, 40). Furthermore, we anticipate that antigenic specificities could be cloned directly from genomic DNA or the mRNA from stimulated spleen or peripheral blood lymphocytes, particularly if the sequence of either heavy or light chain partner is already known. For example, the products of the germ line V_H genes for the major mouse NP^b idiotypic family are associated with λ light chains (41). Alternatively, antigenic specificities might be derived directly from single cells, if both V_H and V_κ genes could be amplified (42). Thus, ultimately this technique could provide an alternative to hybridoma technology (43) for the cloning of antigenic specificities.

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