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Sequencing heavy- and light-chain variable genes of single B-hybridoma cells by total enzymatic amplification

(antibody/immunoglobulin/polymerase chain reaction/single cell)

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ABSTRACT **We have devised a protocol to obtain accurate and complete sequences of the immunoglobulin heavy- and light-chain variable-region (V_H and V_L) genes of single B-hybridoma cells that express defined V genes. The amplification achieved ranges from 2×10^{13}- to 1×10^{14}-fold. Only one potential *Taq* DNA polymerase error was observed in 7590 nucleotides of sequence, thus permitting the identification of naturally occurring somatic mutations. The two-step nature of the amplification protocol provides sufficient DNA for a minimum of 160 sets of sequencing reactions of both the V_H and V_L genes from one cell without cloning. The amplification of relatively long segments of DNA in the first step of the protocol permits second-step amplification and sequencing of regions that flank V_H and V_L codons. Fractionating cellular lysates prior to the first step of amplification permits the separate amplification of V genes on opposite sister chromatids and possibly on opposite strands of the same DNA duplex. Accurate sequencing of V_H and V_L genes of defined germ-line origin that are expressed by single B cells taken directly from the animal is thus made feasible by this approach.**

During an antibody immune response, two basic processes operate in concert to produce specific antibody of high affinity for the immunogen: clonal selection favors the proliferation of B lymphocytes with receptor antibodies that bind the antigen with highest affinity, and somatic hypermutation diversifies the immunoglobulin heavy- and light-chain variable (V_H and V_L) genes expressed by B cells. This simplified view of humoral immunity has been largely derived from results of sequencing studies of V genes expressed by B-cell hybridomas (reviewed in ref. 1). Immune responses to small haptens such as *p*-azophenylarsonate, phosphocholine, 2-phenyl-5-oxazolone, and (4-hydroxy-3-nitrophenyl)acetyl have been especially revealing in such studies, because in each response, the participating B cells frequently express defined V_H and V_L genes of known germ-line origins (2–9). Knowledge of the germ-line sequences of these V genes permits unambiguous identification of somatic mutations and junctional codons, and an evaluation of their influences on the affinities of encoded antibodies and on the selection of B cell clones that express them (10–15). Such reproducible anti-hapten model immune responses are currently the focus of research into the population dynamics behind memory and plasma B-cell differentiation and the somatic hypermutation process (16–26). However, knowledge of both of these processes has been limited by the nearly exclusive reliance on hybridoma sampling procedures to obtain V-gene sequence information. During B-cell hybridoma formation, one is limited by uncertainty regarding compatibility requirements between the fusion parent and the B cells participating in the immune response. Phenotypic characteristics such as size and surface antigens that mark defined stages of B-cell differentiation may be lost upon hybrid formation. Finally, the somatic hypermutation process is inactive in B-cell hybridomas (27). Thus, several laboratories have devised approaches to identify *in vivo* and/or to isolate B cells expressing defined V genes during or subsequent to their participation in model anti-hapten immune responses (21–23, 40, 41). If sequence information for expressed V genes could be directly obtained from single B cells, it should be possible to associate phenotypic characteristics of B cells with mutational differentiation as they participate in immune responses.

Since the discovery of rapid gene amplification through the polymerase chain reaction (PCR) (28), several investigators have developed methods of specific gene amplification from single cells, especially for haplotype analysis of genetic disease markers in oocytes and sperm and for the study of meiotic recombination frequencies (29–31). In these studies, amplified genes have been used primarily in conjunction with hybridization analysis to identify a gene or an allele of interest.

We have carried the single-cell amplification approach to its ultimate limit by directly sequencing single-copy genes amplified from chromosomes of individual cells. To demonstrate the feasibility and accuracy of this method, we have amplified and sequenced the V_H and V_L genes of single cells picked from an equal mixture of two B-cell hybridomas whose V genes differ at 43 nucleotide positions. The hybridomas used in this study, 45-49 and P65I4-2, produce anti-hapten (*p*-azophenylarsonate) antibodies encoded by rearranged V_HId^{CR} and $V_\kappa Id^{CR}$ genes (12, 32). These genes encode a predominating antibody population that is reproducibly elicited in A/J mice immunized with this hapten. We expect that this method will allow the acquisition of detailed sequence information for defined V genes expressed by B cells participating in this and other model immune responses.

MATERIALS AND METHODS

Hybridoma Cell Lines. Two B-cell hybridoma lines were used in the amplification and sequencing studies described. Hybridomas P65I4-2 and 45-49 both express V_H and V_κ genes of common germ-line origin. The V genes of P65I4-2, however, are unmutated, while those of 45-49 are heavily mutated (12, 32). In addition, the heavy-chain junctional (J) and diversity (D)-region sequences differ. In all, the expressed V genes of these two hybridomas are distinguishable by 43 nucleotides. The hybridomas were subcloned three times and

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Abbreviations: V, immunoglobulin variable region; V_H, immunoglobulin heavy-chain V region; V_L, immunoglobulin light-chain V region; NP-40, Nonidet P-40.

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maintained in RPMI 1640 with 15% calf serum (iron-supplemented) at 37°C in an atmosphere containing 5% CO_2.

Single-Cell Isolation. P65I4-2 and 45-49 hybridoma cells were washed and diluted with filtered phosphate-buffered saline to 8000 cells per ml, then mixed together in equal quantities. This mixed cell suspension was spotted in microdrops (0.1–0.2 μl) onto cleaned coverslips [ethanol-wiped and dusted with a pressurized lens cleaner (Tech Spray, Amarillo, TX)] that were previously coated with 0.1 μl of endonuclease-free bovine serum albumin (0.1 mg/ml in filtered 0.1× PBS) to prevent cell adherence to the coverslip. The albumin-containing microdrops were allowed to dry before application of the cells. Microdrops containing only one live cell, as identified by phase-contrast microscopy (Nikon Diaphot-TMD inverted microscope with an ELWD 0.3 phase-contrast addition, ×100–200 magnification), were flushed with 1 μl of phosphate-buffered saline and pipetted immediately into GeneAmp reaction tubes (Perkin–Elmer/Cetus) containing 3.5 μl of lysis buffer. Microdrops containing no visible cells were picked as negative controls. Lysis buffer was 0.68% Nonidet P-40 (NP-40)/0.68% Tween 20/26.2 mM Tris·HCl, pH 8.3/75 mM KCl/2.2 mM $MgCl_2$ with proteinase K (33) (International Biotechnologies) at 57 μg/ml. A stock solution of lysis buffer (without proteinase K) was stored at −20°C. A stock solution (1 mg/ml) of proteinase K in water was also stored at −20°C and added to the stock solution containing the remaining components of the lysis buffer immediately prior to use to achieve the final concentrations listed above. The lysates were covered with a drop of light mineral oil and incubated at 52°C for 30 min. Proteinase K was then heat-inactivated by incubating the tubes for 20 min at 95°C. Extreme care was taken throughout the procedure to minimize contamination by DNA or by particulate impurities that might obscure identification of single cells by microscopy. After heat inactivation, additional components needed for the first round of PCR amplification were added to achieve the final concentrations listed below in a total volume of 25 μl. Additional Tris, KCl, $MgCl_2$, gelatin, and dNTPs, from a mixed stock solution stored at −20°C, were added. For long-term storage, the oligonucleotide primers were kept lyophilized at −70°C. Stock solutions consisting of the four flanking primers, each at 30 μM in 10 mM Tris, pH 8.0/1 mM EDTA and stored at −20°C, were used for first-round amplifications (see below). Similar stocks consisting of "nested primers" for second-round amplifications of either the $V_\kappa Id^{CR}$ or the $V_H Id^{CR}$ genes were prepared. These oligonucleotide stock solutions were used within 6 weeks of their preparation. With longer storage in aqueous buffers, amplification efficiencies diminished.

Amplification of V_H and V_L Genes from Single-Cell Lysates by PCR. To generate enough V-gene copies for sequence analysis, lysed single-cell samples were subjected to two rounds of gene amplification with a thermostable DNA polymerase (AmpliTaq, Perkin–Elmer/Cetus) (34) and a DNA thermal cycler (model 480, Perkin–Elmer/Cetus). During the first round, both V_H and V_L genes from a single-cell lysate were amplified in the same tube by using four flanking primers (1.5 μM each). In the second round, 1.5-μl aliquots from the first tube were pipetted into paired tubes, and the V_H and V_L genes were amplified separately using V_H- or V_L-specific primers (1.5 μM each) located internally (nested) to those used during the first run. First- and second-round amplifications differed in their number of amplification cycles per round (first round, 35 cycles; second round, 30 cycles). First-round amplifications also required 2.5 mM $MgCl_2$, 0.09% Tween 20 (Sigma), and 0.09% NP-40 (Sigma); in the second round, 1.5 mM $MgCl_2$ and 0.05% Tween 20 (without NP-40) were used. Otherwise, each amplification was performed in a total volume of 25 μl and contained the following components in addition to those listed above: dNTPs (Pharmacia), 200 μM each; AmpliTaq DNA polymerase, 1 unit; Tris·HCl (pH 8.3), 17 mM; KCl, 50 mM; and gelatin, 0.001%.

Each thermal cycle consisted of a 94°C dissociation segment (30 sec), followed by a 62°C primer–template reannealing segment (1 min), then a ramp segment (2 min) to a 72°C extension segment (30 sec). The completion of all cycles for each run was followed by an extension period at 72°C (7 min). Amplified gene products were stored at 4°C. Samples (1 μl) of each second-round amplification product were visualized under ultraviolet illumination following electrophoresis in 2% agarose gels and staining with ethidium bromide.

Primers. In all, we used 8 oligodeoxynucleotides as amplification primers and four oligodeoxynucleotides as sequencing primers. Their sequences are listed below. In the first round of amplification, the $V_H Id^{CR}$ and $V_\kappa Id^{CR}$ genes were simultaneously amplified in the same tube by using outermost flanking primers located 5′ of the $V_H Id^{CR}$ and $V_\kappa Id^{CR}$ genes together with J_{H2} and $J_{\kappa4}$ primers. The 5′ flanking V_H primer is homologous to a sequence that runs from 330 to 353 bases on the 5′ side of the first $V_H Id^{CR}$ codon. The 5′ flanking V_κ primer matches a sequence between 79 and 97 bases on the 5′ side of the first $V_\kappa Id^{CR}$ codon. The flanking J_{H2} and $J_{\kappa4}$ primers were homologous to noncoding sequences located 3′ of these gene segments (35, 36). These outermost primers defined DNA fragments of 782 and 1440 base pairs that carried the $V_H Id^{CR}$ and $V_\kappa Id^{CR}$ genes, respectively. In the second round of amplification, $V_H Id^{CR}$ and $V_\kappa Id^{CR}$ genes were amplified separately with nested V_H and J_{H2} primers or with nested V_κ and $J_{\kappa2}$ primers. The nested 5′ V_H primer matched a sequence between 190 and 167 bases on the 5′ side of $V_H Id^{CR}$, and the nested 5′ V_κ primer matched a sequence located between 59 and 38 bases 5′ of the first $V_\kappa Id^{CR}$ codon. The nested J_{H2} and $J_{\kappa2}$ primers were located 3′ of the codons for these gene segments (35, 36). These nested primers defined DNA fragments of 647 and 782 base pairs that carried the $V_H Id^{CR}$ and $V_\kappa Id^{CR}$ genes, respectively. Two primers homologous to sequences located from 8 to 28 bases on the 3′ side of $J_{\kappa1}$ and codons specifying amino acids 67–74 in the third framework region were extended to obtain the coding sequence of the V_κ–$J_{\kappa1}$ genes. A primer homologous to a sequence located from 33 to 55 bases 5′ of the first V_H codon and a primer homologous to codons 69–75 for the third framework region were extended with T7 DNA polymerase to obtain the sequence of the amplified V_H–D–J_{H2} genes. Some of these primers contained additional nucleotides near their 5′ ends to complete recognition sequences for restriction enzymes (our results showed, however, that cloning was unnecessary, so these sites were never used).

Amplification primers: flanking 5′ V_H, 5′-CTTGGACCTGAGCACACTGCTGTC-3′; flanking 5′ V_κ, 5′-CAGTGATTCTTGTTGACTG-3′; flanking J_{H2}, 5′-GACTCCAAGCTTGTCCCTAGTCCTTCATGACC-3′; $J_{\kappa4}$, 5′-AAACAGGGATCCAACGTCACAAGTAAATGAGC-3′; nested 5′ V_H, 5′-CTATGATCAGTGTCCTCTCCACAC-3′; nested 5′ V_κ, 5′-AGGGGTGAGCTCTTTTCCTGCTTAACTCTGT-3′; nested J_{H2}, 5′-TCCTTCATGACCTGAAATTCAGAT-3′; $J_{\kappa2}$, 5′-AAAGAAAAGCTTAACAAGGTTAGACTTAGTG-3′. Sequencing primers: 5′ V_H, 5′-GGCCTGGGAATGTCACTCACATC-3′; V_H framework 3, 5′-ACACTGACTGTAGACAAATCC-3′; $J_{\kappa1}$, 5′-GACATAGGATCCGGAAGAAAGAGACTTTGGAT-3′; V_κ framework 3, 5′-GGTGAGAGAATAATCTGTTCCAGA-3′.

DNA Purification for Sequencing. Amplified products were extracted with phenol/chloroform (1:1) and then with chloroform and washed three times with 10 mM Tris, pH 8.0/1 mM EDTA in Centricon-30 microconcentrators (Amicon) to remove salts, nucleotides, and amplification primers. Sodium acetate was added (0.3 M) and the nucleic acids were precipitated with 3 volumes of ethanol. The precipitate was rinsed once in ethanol, dried, and resuspended in 5 mM Tris,

pH 8.0/0.5 mM EDTA. Amplified product was quantified by agarose gel electrophoresis and diluted to 50 μg/ml in the same buffer for sequencing analysis.

Double-Stranded DNA Sequencing with ^{32}P-Labeled DNA Primers. Primers for DNA sequencing were labeled with [γ-^{32}P]ATP (>7000 Ci/mmol, 167 mCi/ml, ICN; 1 Ci = 37 GBq) using T4 polynucleotide kinase (37) (United States Biochemical). After the labeling reaction, the enzyme was inactivated by heating at 65°C for 5 min. Specific activity of radiolabeling was determined by γ scintillation counting of DEAE-purified aliquots of radiolabeled primers. Incorporations of ≥7 × 10^8 cpm/μg of primer were considered adequate for sequencing. Purification of primers on DEAE was found to be unnecessary for sequencing, so primers were used in the sequencing reactions immediately following the heat-inactivation step.

Double-stranded amplification products were sequenced using Version 2.0 of the Sequenase kit (United States Biochemical), with some modifications to the manufacturer's recommendations. End-labeled primer (10 ng) was added to 50 ng of double-stranded DNA template together with the provided Sequenase reaction buffer (5×) and dithiothreitol. These template/primer samples were heated to 95°C for 1 min in 0.5-ml microcentrifuge tubes, then allowed to anneal at room temperature for 3 min. Sequenase 2.0 (2.5 μl of a 1:15 dilution in enzyme dilution buffer) was then added. The contents of the tubes were mixed and distributed in 3.5-μl aliquots to wells (of a round-bottomed 96-well tray, Corning) containing the provided Sequenase termination mixes, and the sequencing reaction was allowed to proceed for 10 min at 45°C before it was terminated with the provided "stop" solution.

Samples were then loaded onto 8% polyacrylamide gels and electrophoresed at 2000–2800 V to separate the extension products. Gels were fixed in 5% methanol/5% acetic acid, vacuum dried onto Whatman 3MM chromatography paper, and exposed to Kodak XAR-5 film for 0.5–4 days at room temperature without the use of an enhancing screen before developing.

RESULTS

Amplification of V_H and V_L Genes from Single Hybridoma Cells. The hybridomas used in this study, 45-49 and P65I4-2, produce anti-hapten (*p*-azophenylarsonate) antibodies encoded by rearranged V_HId^{CR} and $V_\kappa Id^{CR}$ genes of known sequences that differ at 43 nucleotide positions because of somatic mutations, use of different D segments, and junctional variability (12, 32). The two cell types were mixed together prior to selecting individual cells for lysis and amplification so that we could be certain that the final DNA sequences were derived from the amplified genes of single cells.

Fig. 1 illustrates the procedure we used to isolate, amplify, and sequence the variable genes of these cells. Mixed hybridoma cells were spotted in microdrops onto coverslips and examined under an inverted phase-contrast microscope for drops containing one cell or no cells. In two experiments, we picked 21 microdrops that visibly contained one cell each and 8 drops that contained no cells. The contents were subjected to PCR amplification using primers that matched sequences flanking both the V_HId^{CR} and $V_\kappa Id^{CR}$ genes. Following the first-round amplification, 1.5-μl aliquots from each tube were secondarily amplified in paired tubes with nested primers designed for either the V_HId^{CR} or the $V_\kappa Id^{CR}$ genes. Eleven of the original 21 microdrops that contained cells by microscopic visualization yielded PCR products that stained visibly with ethidium bromide following the second-round amplifications (Fig. 2). None of the 8 microdrops that lacked cells by microscopic visualization yielded visible amplified products by ethidium bromide staining.

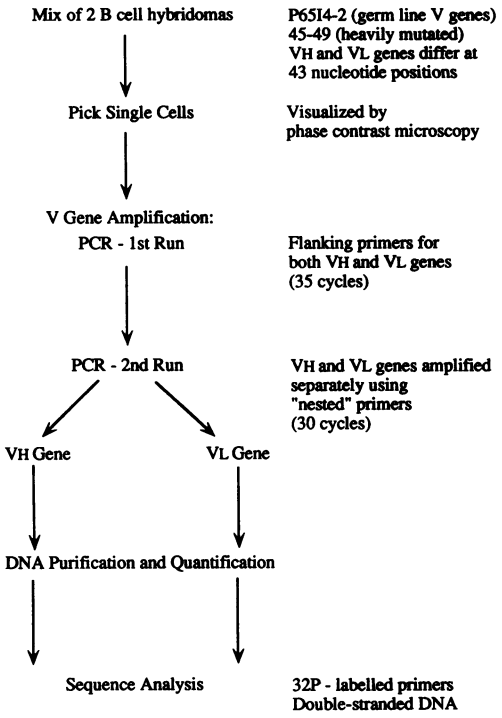

FIG. 1. Overview of V_H and V_L gene amplification and sequencing from single-cell isolates.

In all cases, the final amplified products were of the anticipated sizes given the locations of the primers used in the second-round amplifications: approximately 780 bases for $V_\kappa Id^{CR}$ and 650 bases for V_HId^{CR}. Most impressively, in each of the 11 instances where amplification products were observed, both $V_\kappa Id^{CR}$ and V_HId^{CR} products were seen in electrophoresed aliquots from the paired tubes. The quantities of amplified products varied from 2 to 5 μg (in 25 μl), giving a final amplification of 0.4–1 × 10^{14}-fold. This degree of amplification corresponds to an average amplification efficiency of ≈80% for each PCR cycle.

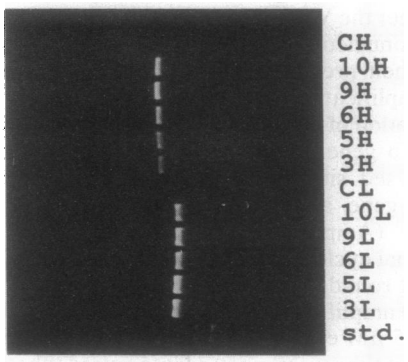

FIG. 2. Example of final (second-round) V_H and V_L amplification products derived from single-cell isolates. V_H and V_L genes were amplified separately in a two-step process (*Materials and Methods*). In each lane, a 1-μl sample of amplification product was electrophoresed through a 2% agarose gel. V_H and V_L amplification products are shown from five single-cell isolates (nos. 3, 5, 6, 9, and 10). The size standard (std.) is a *Hae* III digest of φX174 replicative-form DNA (1353, 1078, 872, 603, and 310 bp). From the known V-gene sequences of P65I4-2 and 45-49, the final amplified V_H gene product is expected to be 647 base pairs; the V_L gene product should be 782 base pairs. A control (C) microdrop containing no cell was subjected to the same amplification procedure.

Table 1. Summary of V-gene sequencing results obtained from single-cell isolates

Single-cell isolate	Sequences at 43 distinguishing positions			
	45-49		P65I4-2	
	V_H	V_κ	V_H	V_κ
6	+	+	−	−
5	−	−	+	+
10	−	−	−	−
8*	−	−	−	−

*Control microdrops (no cell visualized).

In a third experiment, we attempted to amplify V_H and V_L genes from separate chromatids or from the single strands of DNA duplexes. Seven microdrops containing microscopically visualized single cells were each pipetted into amplification tubes containing 28 μl of lysis buffer and 12 μl of water. Following the lysis and protease digestion step, the lysates were subjected to five rounds of freezing in a dry ice/ethanol bath for 1 min, heating at 65°C for 1 min, and vortex mixing for 30 sec to shear the DNA. Then the tubes were heated to 95°C for 20 min to dissociate DNA duplexes. From each original tube containing cellular lysate, 5-μl aliquots were distributed to each of eight tubes containing the remaining components needed for first-round amplification of V_HId^{CR} and $V_\kappa Id^{CR}$ genes. Following first-round amplification, 1.5-μl aliquots from each tube were distributed into paired tubes for second-round amplifications of the V_HId^{CR} or $V_\kappa Id^{CR}$ genes as described above.

Four of the original seven cells yielded amplification products (data not shown). Two of these cells gave V_H gene products in two separate (second-round) tubes and a V_κ gene product in one tube. One other cell yielded the reciprocal pattern. A final cell yielded only a V_κ gene product in one tube. Four microdrops that contained no visible cells were subjected to amplification (in a single tube during the first round) and yielded no visible products. In all cases, products from a first-round tube that were subsequently used to amplify a visible second-round product yielded only a V_H or V_κ product, but not both. This demonstrates that the V_H or V_κ genes were separated among the eight tubes containing fractions of a single-cell lysate. More importantly, the presence of twice-amplified V_H or V_L products from a single cell indicates either that sister chromatids carrying these genes were separately amplified or that the opposite strands of a single DNA duplex were separately amplified. The quantities of amplified products obtained were comparable to those obtained from whole single-cell lysates.

Sequencing of Amplified V_H and V_L Genes. We sequenced the 11 amplified V_H and V_κ genes from the first two experiments by extending end-labeled (^{32}P) primers that were located internally to those last used for amplification. Sequencing was performed by the dideoxy chain-termination method with T7 DNA polymerase on dissociated double-stranded amplified products. In each of the 11 cases, the pairs of V_H and V_κ sequences derived from the initial single-cell picks corresponded to the V_H and V_κ sequences of either the 45-49 cells (6 of 11 cases) or the P65I4-2 cells (5 of 11 cases) as determined at all 43 positions that distinguish the pairs of V genes expressed by the two hybridomas (Table 1). The sequences were clear, unambiguous, and readable for 270–325 bases. A representative sequence autoradiogram is shown in Fig. 3.

In all, we sequenced 7590 bases and observed only one change from the published V_HId^{CR} and $V_\kappa Id^{CR}$ sequences that might have been due to an amplification error by the *Taq* DNA polymerase. In this case, one of the $V_\kappa Id^{CR}$ sequences (a lysine codon, AAA, at position 45) was missing a single nucleotide. This extremely low *Taq* error frequency was probably due to the fact that PCR products were not cloned before sequencing, so that errors introduced after the first amplification cycle were not generally observed.

DISCUSSION

The data demonstrate that it is possible to obtain complete and accurate sequences of both the V_H and V_L genes expressed by single hybridoma cells without cloning. To our knowledge, this is the first report of DNA sequences derived from a single molecule that is amplified entirely in the test tube. The extremely low frequency of *Taq* errors seen here (1 in 7590), though far less than generally reported, is in fact comparable with results of other studies (34, 38). Our PCR products are not cloned, so that *Taq* errors only in the first cycle of amplification are expected to be visualized on sequencing autoradiograms. Thus, the *Taq* error *frequency* that we observe is essentially the same as the *Taq* error *rate*, which has been estimated to be 1 per 5000 bases per replication cycle (34, 38). The important point is that the frequency of *Taq* errors is far less than the frequency of naturally occurring somatic mutations in antibody V genes, which generally ranges from 1% to 7% (1). Identifying somatic mutations in V genes amplified from single cells is therefore possible by this procedure. Similarly, acquiring precise sequence information for the highly variable junctional regions of assembled antibody or T-cell receptor genes at the single-cell level is also possible.

We believe that the use of a high-temperature segment for hybridization to promote specificity, the use of a protracted ramp segment to promote the extension of nascently hybridized primers, and the use of two pairs of primers all contribute to the magnitude of the amplifications achieved. In pilot studies with cloned V-gene substrates, we have only rarely visualized (by ethidium bromide staining) amplified products derived from one DNA duplex when only one primer pair was used in the amplification. In all such cases, the product was relatively short, the quantity of amplified product was low (<5 ng), and product was contaminated with amplified artifacts. An additional advantage of the consecutive amplification approach used here is that relatively long fragments of DNA can be amplified in the first step. For example, the $V_\kappa Id^{CR}$ gene in this study was initially amplified on a fragment of DNA that was 1440 base pairs long and that spanned the J_κ locus. This capability is useful in cases where a defined V gene is assembled with an undefined J segment. Since only a small fraction of the product from the first round amplification is used for the second amplification round, regions flanking the coding sequences can also be secondarily am-

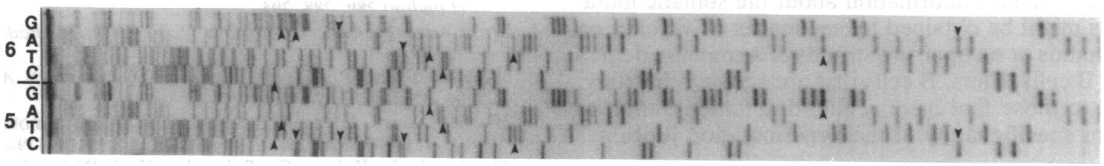

FIG. 3. Example of V_H gene sequences obtained from two single-cell isolates. Isolates 5 (45-49) and 6 (P65I4-2) differ in this segment at 10 nucleotide positions, identified by arrowheads.

plified and sequenced. This may prove useful for establishing lineage relationships among B cells (16, 17) because somatic mutations that occur in noncoding flanking regions should not influence clonal selection and are therefore useful markers to identify cells that are derived from a common precursor (16, 17).

We use 1.5 μl of product from the first amplification round (from a total of 25 μl) as template for the second-round amplification. Generally, at least 1 μg of product is recovered after secondary amplification, which is enough DNA for 20 sets of sequencing reactions. By extrapolating back to the 25 μl present in the initial tube, it is possible to obtain enough of the final product for 333 sets of sequencing reactions by the current protocol. We have not attempted to amplify less than 1.5 μl from the initial tube, so it is possible that this value could be extended significantly.

In our hands, ≈50% of the hybridomas selected following microscopic examination yielded specific amplification products. But in 11 of 11 cases, both V_H and V_κ amplification products were observed. This suggests that the major limitation in our procedure is successfully transferring the single cell (in the microdrop) to the first amplification tube. The remains of the microdrop that are left on the coverslip after it is aspirated crystallize quickly, so it is difficult to determine whether the cell was successfully transferred to the first amplification tube.

Though PCR and cloning procedures have been used to acquire sequences of V genes expressed by whole populations of B cells selected directly from immune animals (21, 26, 40, 41), critical information is lost in such analyses because V_H and V_L gene pairs are separated. In addition, sequence ambiguities due to scrambled amplifications of rearranged V genes from multiple B cells cannot be excluded. In the procedure described here, both of these problems are eliminated. However, this method shares a common limitation with the others, as it presently cannot be used to amplify immunoglobulin V genes from any B lymphocyte without some prior knowledge of their flanking sequences. Furthermore, although complete sequence information for expressed V_H and V_L genes of single cells may ultimately permit an analysis of antibody function, hybridoma technology already allows for this.

However, when used in combination with methods for cell sorting of antigen-specific B lymphocytes by flow cytometry (21, 23, 26, 39, 41), this method provides the opportunity to sequence and identify somatic mutations in the V genes of B cells taken directly from the animal. For example, a successful effort to select and expand in culture single memory B cells that were taken from immune mice and to obtain partial V region sequences from amplified cDNA was recently reported (23). The procedure described here complements this approach by eliminating the necessity of the culturing step, providing complete V_H and V_L sequences and the potential to acquire sequence information of flanking regions for lineage determinations. Its lack of bias toward activated B cells gives this procedure an advantage over conventional hybridoma technology for the study of resting B lymphocytes such as those of the memory compartment. The efficiency of the method may allow for the study of B lymphocytes participating in antigen-specific responses through peripheral blood sampling, without requiring the sacrifice of the immunized subject. Finally, information about the somatic mutation process might be obtained by sequences of V genes on sister chromatids or on opposite strands of a common duplex from single B cells. Thus, B cells that are actively mutating, as opposed to those that have acquired mutations, may be identified for specific study of the hypermutation process.

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