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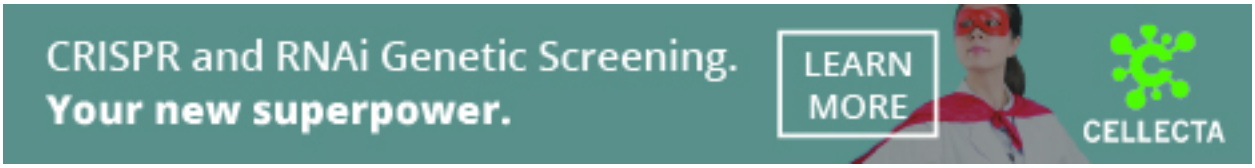

Rapid PCR Construction of a Gene Containing Lym-1 Antibody Variable Regions

Xu-Bao Shi,[1] Paul H. Gumerlock,[1] Arthur A. Wellman,[2] Claude F. Meares,[2] Gerald L. DeNardo,[1] Frederick J. Meyers,[1] and Sally J. DeNardo[1]

[1]Department of Internal Medicine, University of California, Davis, Medical Center, Sacramento, California 95817; [2]Department of Chemistry, University of California, Davis, California 95616

Clinical applications of monoclonal antibodies (mAbs) to tumor-associated antigens provide potential for targeted cancer therapy. Radiolabeled mAb Lym-1 has been used successfully to treat >50 patients with B-cell lymphoma.[1–3] The uptake of Lym-1 in lymphomas is rapid and efficient compared with solid tumors. However, the amount of the radioactive dose injected on these large molecules reaching each gram of tumor is usually <0.1%. When Lym-1 is labeled with radiometal chelates to enhance its tumoricidal effects, there is undesirable accumulation and slow clearance of radioactivity in the liver. Evidence suggests that this is related to receptors for components of the mAb Fc region.[4,5] Recent studies[6,7] have demonstrated that two recombinant radio-directed single-chain antigen-binding (SCA) proteins derived from monoclonal antibodies have in vivo tumor-targeting capacity. Although the percent injected dose per gram of tumor is low, the therapeutic index (tumor to normal organ radiation dose) is several times that of the intact mAb, $F(ab')_2$, or Fab' fragments.[7] This improvement in biokinetics properties of the radiolabeled SCA protein make it a promising agent for alternative therapeutic strategies. Furthermore, SCA proteins facilitate development of single or bispecific radioimmunoconjugates, humanized antibodies, or new versions of tumor-reactive molecules for immunotherapy. This may be particularly rewarding for a mAb such as Lym-1 that has already been demonstrated to be successful in clinical radioimmunoconjugate cancer therapy.

Gene engineering techniques have been used to produce the SCA protein, which consists of the heavy-chain variable region (V_H) and the light-chain variable region (V_L) of an antibody synthesized as a single polypeptide.[8] In this engineered protein, the carboxyl terminus of one variable region is linked by a linker peptide to the amino terminus of the other. Some are V_H–V_L and some are V_L–V_H.

Obtaining the V_H and V_L coding regions is the first step for production of an SCA protein. Several strategies to generate these coding regions are available. Variable-region sequences can be assembled using overlapping synthetic oligonucleotides designed from the protein sequence, and then transferred into vectors for expression.[9,10] Alternatively, cDNA libraries coding for the heavy and light chains can be constructed, and restriction sites can be introduced in the cDNAs by site-directed mutagenesis at the 5′ and 3′ ends of both variable regions.[11] These strategies require detailed knowledge of the antibody gene sequence. However, this information is not available for many antibodies and is not required for the strategy described here.

PCR has been used to amplify the sequences encoding the variable domains of antibodies.[12] The technique has also been used by some investigators to assemble different genes for expression.[13,14] Generally, two target DNA fragments are separately amplified by PCR with primers that create products that share a common sequence at one end of each fragment. These products are then mixed and denatured, and upon annealing, the positive strand of one fragment can hybridize with the negative strand of the other fragment (and vice versa) at the common sequence position. These partially double-stranded fragments become the templates for further PCR. Fusion of four PCR-generated DNA fragments requiring three separate reactions has been described in one report.[13] An alternative method has been described wherein all the overlapping oligonucleotides comprising the entire gene were synthesized.[15] These oligonucleotides were phosphorylated at the 5′ ends and ligated together to generate a target DNA template for PCR amplification. In this method, the requirement to synthesize the entire gene sequence and to phosphorylate the oligonucleotides is expensive and cumbersome.

In a different approach, Dillon and Rosen[16] were able to synthesize the small HIV-2 Rev protein gene of 303 bp by PCR using four overlapping synthetic oligonucleotides. We built on this concept and report here the generation of the 954-bp Lym-1 SCA protein gene. Our approach was to use PCR to amplify the V_H and V_L coding regions of Lym-1 heavy- and light-chain cDNAs separately. These products were then included in a PCR reaction with 9 synthetic oligonucleotides. Six of these oligonucleotides created a 5′ restriction site for cloning, a hybrid λ phage promoter (o_L/p_R), a Shine/Dalgarno (SD) sequence for ribosomal translation, and a signal peptide sequence (*ompA*). Two oligonucleotides created a 14-amino-acid peptide

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linker between the V_H and V_L fragments, and the ninth oligonucleotide added two translational stop codons and a 3′ restriction site. This gene fusion by PCR created a complete SCA protein gene (954 bp) for expression in a bacterial system. Also, discussed here are strategies for V_H and V_L amplification and single-stranded DNA sequencing.

MATERIALS AND METHODS

Primers and Probes for V_H and V_L Amplification

Table 1 lists the DNA oligonucleotides that were used to amplify and probe the Lym-1 mAb V_H and V_L gene transcripts that had been reverse-transcribed into cDNA. Primers HS-1 and κ-N′ were graciously provided by Dr. D. L. Robbins (Department of Rheumatology, University of California Davis Medical Center, Sacramento) and have been described previously.[17] These oligonucleotides were designed to amplify a wide range of human heavy- and light-chain genes and are targeted to the conserved leader sequence (HS-1) and the κ constant region (κ-N′) within the coding regions of the genes. HS-1 is a mixture of eight oligonucleotides due to variation at three positions (see Table 1). These primers were not further purified upon receipt and were used to amplify the cDNA from a mouse mAb. Oligonucleotides PO3, G2A, 1G, PO2, and 1A were synthesized in-house and purified by reverse-phase HPLC. These were the primers used to obtain the initial Lym-1 V_H and V_L sequences.[18] The oligonucleotides γ-15 and κ-17 were purchased from a commercial source (Pharmacia LKB Biotechnology, Piscataway, NJ), reconstituted according to the supplier's instruction, and not purified further.

Amplification of mAb V_H and V_L Coding Regions

RNA/PCR amplification was performed using a modification of the method described in our previous paper.[19] In brief, whole RNA was extracted from the Lym-1 hybridoma cell line using an acid guanidinium thiocyanate–phenol–chloroform extraction method.[20,21] Hybridoma cells (1×10^7) were harvested and dissolved in 3 ml of 5 M guanidinium thiocyanate. The lysate was centrifuged for 18 hr at 35,000 rpm through a cesium chloride density gradient. The RNA pellet was resuspended and subjected to two phenol/chloroform extractions. After the second extraction, the RNA in the aqueous phase was precipitated by adding two volumes of cold 100% ethanol. The RNA pellet was obtained by microcentrifugation at 4°C for 15 min. cDNA was generated by reverse transcription of ~1 μg of RNA in a 20-μl reaction mixture using a random hexadeoxynucleotide primer.[19]

Using the cDNA reaction mixture, the target DNA was amplified by *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The reaction was set up by mixing 10 μl of 10× PCR buffer [final buffer composition was 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 0.01% gelatin], 100 μM each of dNTPs, 20 pmoles of each primer, 2 units of *Taq* DNA polymerase, and 2 μl of cDNA in a volume of 100 μl. The amplification was performed in an automated

TABLE 1 The Primers and Probes Used for Amplification and Probing of Lym-1 V_H and V_L cDNAs

Primers for V_H amplification and binding positions:		
upstream:	HS-1. GGGAATTCATGGACTGGACCTGGAGG(AG)TC(CT)TCT(GT)C	(-55 - -21)
downstream:	PO3. ACACCACTGGACAGGGATCCAGAG	(537 - 560)
	G2A. GTTGTATCTCCACACAC	(451 - 467)
Probes for V_H:	γ-15. GGCCAGTGGATAGAC	(433 - 447)
	1G. GGCTCTTGGAGTTGTCC	(270 - 286)
Primers for V_L amplification and binding positions:		
upstream:	PO2. CTCAGGTCCTGGGGTTGCTGCTGC	(1 - 24)
downstream:	κ-N'. CCAAGCTTCATCAGATGGCGGGAAGAT	(396 - 442)
Probes for V_L:	κ-17. TGGATGGTGGGAAGATG	(395 - 410)
	1A. AGGAGCTGAGGAGATTT	(174 - 190)

All sequences are listed 5′ → 3′. The numbers in parentheses represent the primer-binding-positions as numbered in Fig. 4.

DNA thermal cycler (Perkin-Elmer Cetus) for 35 cycles, with each cycle consisting of 30 sec at 95°C, 30 sec at 65°C, and 30 sec at 72°C. The amplified products were electrophoresed in a 2% NuSieve/1% agarose gel.

Southern Blotting

The amplified mAb V_H and V_L cDNAs were confirmed by Southern blotting.[22] The PCR products were electrophoresed in a 1.5% agarose gel for 1.5 hr at 110 V. The DNA in the gel was transferred to a Biotrans nylon membrane (ICN Biomedicals, Irvine, CA) in 0.4 N NaOH. The probes, labeled with biotin–dUTP, were hybridized with the transferred V_H and V_L DNAs on the membrane. The hybridized probe was detected by an enzymatic chemiluminescence method.[23]

Production of Single-stranded DNA

The amplified DNA was purified using a low melting point (LMP) agarose gel.[21] First, the PCR product was electrophoresed in a 1.5% agarose gel. After separation of the target DNA band from other bands, a well was made in the same lane by cutting out a piece of gel in the positive direction close to the target DNA band. The well was filled with 1% LMP agarose syrup that was gelled at 4°C. The gel was subjected to a second run for 20 min to move the target DNA band into the LMP agarose gel. The piece of LMP agarose gel containing the DNA band was cut out and stored at 4°C until used.

The purified double-stranded DNA was used to generate single-stranded DNA by an asymmetric PCR amplification protocol.[24] Two to five microliters of melted LMP agarose gel containing the purified DNA and 2 μl of primer solution (10 pmoles/μl of upstream or downstream primer) was added to 100 μl of PCR reaction buffer containing 2 units of *Taq* DNA polymerase, and the reaction was run for 40 cycles as described above. The asymmetric amplified product (10 μl) was electrophoresed in a 1.5% agarose gel. The DNA was visualized after

TABLE 2 The Synthetic Oligonucleotides for PCR Fusion

1. EOP-L1
GAATTTATTTTTTGCAGGGGGGCATTGTTTGGTAGGTGAGAGAGATCCCGGAATTCCGG
2. EOP
CCGGAATTCCGGGATCTCTCTC
3. EOP-L2
CATACAACCTCCTTAGTACATGCAACCATTATCACCGCCAGAGGTAAAATAGTCAACACCGCCAGAGATAATTTATC
4. EOP-U
UCCCCTGCAAAAAATAAATTCATATAAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTG
5. ompA-L
TGGAGACTGAGTCATCTGGATGTCGGCCTGCGCTACGGTAGCGAAACCAGCCAGTGCCACTG
6. ompA-U
TGTACTAAGGAGGTTGTATGGATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGG
7. Link-L
GCCAGGTCCTGACTCCTTCAGCTGCACCTGACCTTTACCTTCAGAAGATTTACCAGAACC
8. Link-U
TCGGCTCGGGGACAAAGTTGGAAATAAAAGGTTCTACCTCTGGTTCTGGTAAATCTTCTG
9. PO3
ACACCACTGGACAGGGATCCAGAG
10. BSH
CGCGGATCCGCGTTATTATGCAGAGACAGTGACCAGAGTCCC

All sequences are listed 5′ → 3′.

staining with ethidium bromide and exposure to UV light. The single-stranded DNA was purified by Centricon-100 (Amicon, Beverly, MA) centrifugation to remove the extra dNTPs and primers. In addition, the double-stranded PCR products were cloned into the TA-cloning vector (Invitrogen, San Diego, CA) for sequencing according to the supplier's instructions.

DNA Sequencing

The purified single-stranded DNA was suitable for sequencing using the Sanger chain-termination method,[25] with S-35-dATP (DuPont NEN Research Products, Boston, MA) and T7 DNA polymerase (Sequenase 2.0, U.S. Biochemical, Cleveland, OH). Approximately 100 ng of the single-stranded DNA produced by PCR was incubated with 2.5 pmoles of sequencing primer at 94°C for 4 min, and the vial containing the mixture was placed in a dry ice–ethanol bath until used. The mixture was brought to room temperature and the cDNA sequenced according to the manufacturer's protocol. The synthesized DNA was electrophoresed in a denaturing 6% polyacrylamide gel. Autoradiographic exposure on Kodak XAR X-ray film varied from 1 to 5 days depending on the radioactivity in the gel.

Oligonucleotides for Gene Construction by PCR

Primer PO3 and 9 oligonucleotides (22–77 bp) coding the hybrid λ phage promoter o_L/p_R, the SD sequence, the *ompA* signal sequence, a 14-amino-acid linker, *Eco*RI and *Bam*HI cloning sites, and a pair of translational stop codons were synthesized in the University of California Davis Protein Structure Laboratory (Table 2). The deprotected single-stranded DNA products were supplied by the manufacturer in two different forms: trityl-on and trityl-off. All of the oligonucleotides were purified before use. The trityl-on products were purified using Poly-Pak cartridges (Glen Research Corporation, Sterling, VA) according to the supplier's instructions. The trityl-off oligonucleotides were purified by desalting on a G-50 resin (Bio-Rad Laboratories, Hercules, CA) column. These oligonucleotides were designed so that the last 16–21 nucleotides of each were complementary to the first part of the next strand. The oligonucleotides in close proximity to the PCR-amplified V_H and V_L coding regions shared 24- and 30-nucleotide overlaps with these regions, respectively.

Gene Fusion by PCR

The complete gene was constructed from the double-stranded V_H and V_L coding regions and a series of nine overlapping single-stranded DNA fragments by PCR, using an extension of the method of Dillon and Rosen.[16] The principle of the method is illustrated in Figure 1. When mixed together, these nucleic acid fragments could be assembled into a hybrid representing the complete SCA gene, as shown in Figure 1. The fused gene was constructed by a two-step PCR: fusion and amplification. Standard conditions using *Taq* DNA polymerase yielded products that contained sequence errors (data not shown). Therefore, we used $Vent_R$ DNA polymerase (New England Biolabs, Beverly, MA). The fusion step was set up according to the supplier's protocol, by mixing 5 μl of 10× $Vent_R$ buffer [final buffer composition was 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 4 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, and 0.1% Triton X-100], 100

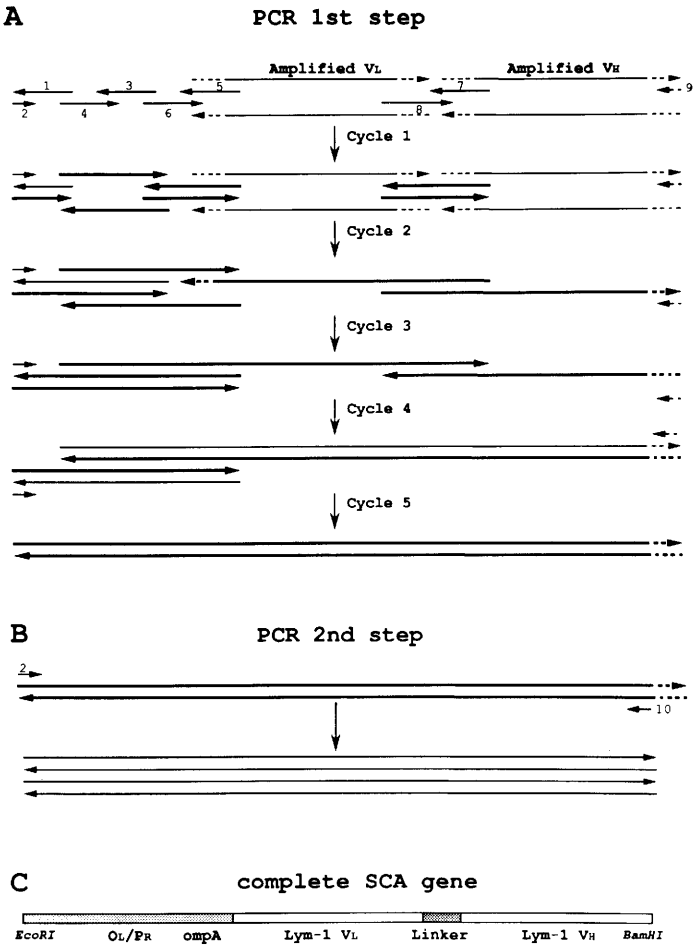

FIGURE 1 Strategy used to generate the Lym-1 SCA protein gene by a two-step PCR. (*A*) The PCR first step. The eight short lines (*1–8*) represent the synthetic oligonucleotides listed in Table 2. Number *9* (PO3) is a primer to the constant region of heavy chain and is used to generate the Lym-1 SCA gene with some of the antibody constant region. The PCR-produced V_H and V_L coding regions are shown by four long lines; the broken lines represent the signal sequences at 5′ ends and the constant regions at 3′ ends. The arrows indicate the 5′ → 3′ direction. The bold lines represent the products used as templates for the next cycle. The complete Lym-1 SCA protein gene containing a portion of the constant region of the heavy chain is assembled by the initial five cycles. The product is doubled by cycle 6 (not drawn). (*B*) The PCR second step. The DNA fused in step 1 is used as a template, and the SCA protein gene was amplified with primers EOP and BSH (*10*) listed in Table 2. (*C*) Diagram of the Lym-1 SCA protein gene.

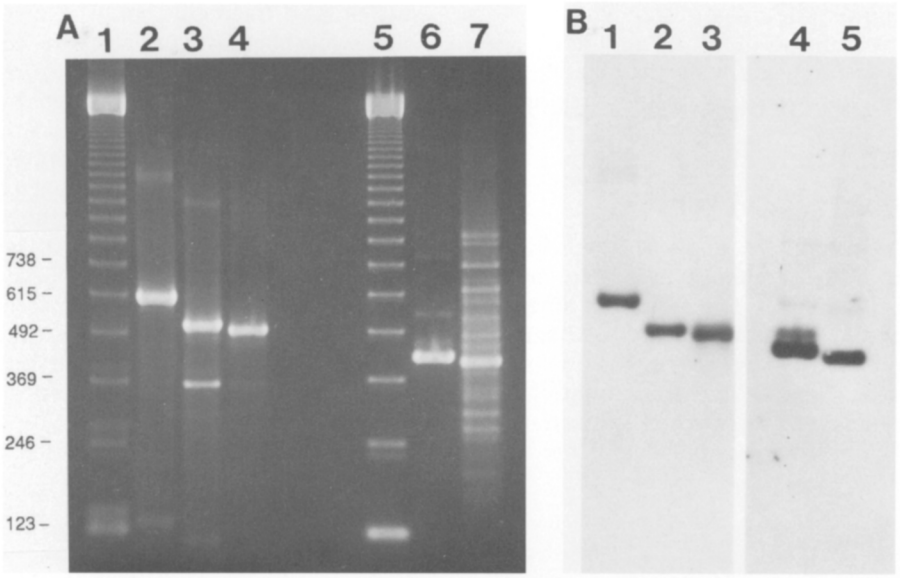

FIGURE 2 Gel electrophoretic analysis of PCR products of Lym-1 V_H and V_L cDNAs. (*A*) Ethidium bromide-stained 2% NuSieve/1% agarose gel with PCR-amplified products of Lym-1 V_H and V_L coding regions. (Lanes *1,5*) 123-bp DNA marker ladder (bp at *left*); (lanes *2–4*) V_H coding region amplified using HS-1/PO3, HS-1/G2A, and HS-1/γ-15, respectively; (lanes *6,7*) V_L coding region amplified using PO2/κ-N′ and PO2/κ-17, respectively. (*B*) Southern blot analysis of the amplified Lym-1 V_H and V_L cDNA products using probe 1G or 1A. (Lanes *1–3*) Amplified V_H coding region probed using 1G; (lanes *4,5*) amplified V_L coding region probed using 1A.

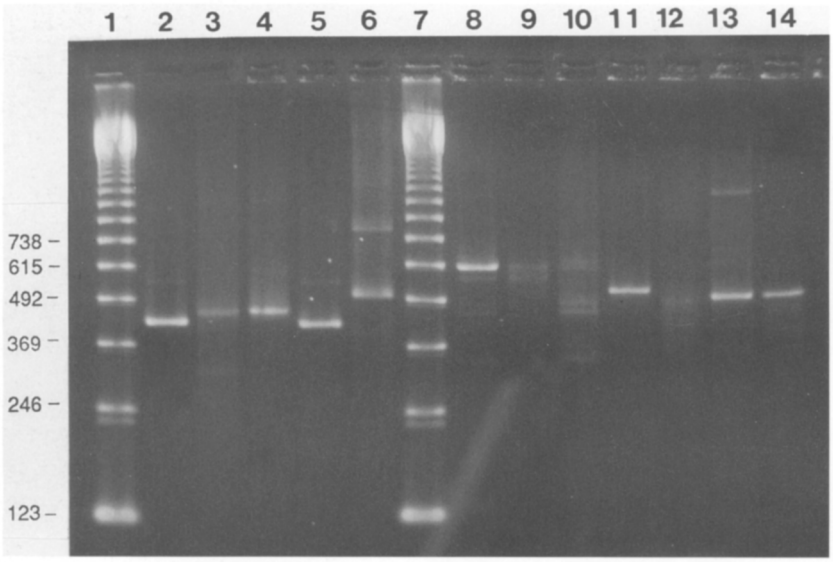

FIGURE 3 Gel electrophoretic analysis of double- and single-stranded DNA generated by PCR of Lym-1 V_H and V_L cDNAs. (Lanes *1,7*) 123-bp DNA marker ladder (bp at *left*). (Lane *2*) Products amplified with V_L primers PO2 and κ-N′ (422 bp); (lanes *3,4*) products generated by asymmetric PCR of lane *2* double-stranded products by primers PO2 and κ-N′, respectively; (lane *5*) products of V_L primers PO2 and κ-17 (410 bp); (lane *6*) products generated by asymmetric PCR of lane *5* products by primers κ-17; (lane *8*) products of V_H primers HS-1 and PO3 (615 bp); (lanes *9,10*) products generated by asymmetric PCR of lane *8* products by primers HS-1 and PO3, respectively; (lane *11*) products of V_H primers HS-1 and G2A (522 bp); (lane *12*) products generated by asymmetric PCR of lane *11* products by primer G2A; (lane *13*) products of V_H primers HS-1 and γ-15 (502 bp); (lanes *14*) products generated by asymmetric PCR of lane *13* products by primer γ-15.

μg/ml of BSA, 200 μM each of dNTPs, 20 pmoles each of the oligonucleotides, 2 μl each of the amplified V_H and V_L coding regions prepared above, and 1 unit of $Vent_R$ DNA polymerase, in a final volume of 50 μl. The mixture was subjected to 6 thermal cycles, each consisting of 30 sec at 95°C, 30 sec at 65°C, and 60 sec at 72°C. Primer PO3 was used in this step to generate a complete Lym-1 SCA gene that carried part of the constant region of the heavy chain on the 3′ end as a template, which could be amplified effectively by primer EOP and BSH in the heminested PCR second step. The PCR second step was amplification that was set up by mixing 10 μl of the PCR-fused DNA fragment mixture from step 1 with the two primers EOP and BSH (Table 2) and run for 30 cycles.

RESULTS

Amplification and Detection of mAb Variable Region Genes

As shown in Figure 2A, cDNAs generated by reverse transcription of RNA extracted from a hybridoma cell line were successfully amplified using the primers listed in Table 1. For the heavy chain of Lym-1, primers HS-1 and PO3 amplified a DNA fragment of 615 bp; primers HS-1 and G2A amplified a DNA fragment of 522 bp. For the light chain of Lym-1, a DNA fragment with 422 bp was generated with primers PO2 and κ-N′. The amplified variable regions were confirmed by Southern blotting (Fig. 2B).

Sequencing

The single-stranded DNA for nucleotide sequencing was generated by an asymmetric PCR amplification. Figure 3 illustrates the results of these asymmetric amplifications. In lanes 1 and 7 are the 123-bp DNA marker ladders. Double-stranded DNA products, as templates for asymmetric amplification, are shown from PCR of Lym-1 cDNA with these primers: V_L primers PO2 and κ-N′ (422 bp); V_L primers PO2 and κ-17 (410 bp); V_H primers HS-1 and PO3 (615 bp); V_H primers HS-1 and G2A (522 bp); V_H primers HS-1 and γ-15 (502 bp). Single-stranded DNA products of asymmetric PCR of the double-stranded templates listed above are these: template amplified with V_L primers PO2 and κ-N′, respectively; template amplified with V_L

Lym-1 Heavy Chain Variable Region DNA Sequence

```
                -51         -41         -31         -21         -11         -1
          5' GGGAA TTCATGGACT GGACCTGGAG GGTCCTCTTC TCATAGAGCC TCCATCAGAG

ATG GCT GTC CTG GGG CTG CTT CTC TGC CTG GTG ACT TTC CCA AGC TGT GTC CTG TCC CAG  60
Met Ala Val Leu Gly Leu Leu Leu Cys Leu Val Thr Phe Pro Ser Cys Val Leu Ser Gln

GTG CAG CTG AAG GAG TCA GGA CCT GGC CTG GTG GCG CCC TCA CAG AGC CTG TCC ATC ACA 120
Val Gln Leu Lys Glu Ser Gly Pro Gly Leu Val Ala Pro Ser Gln Ser Leu Ser Ile Thr

TGC ACC ATC TCA GGG TTC TCA TTA ACC AGC TAT GGT GTA CAC TGG GTT CGC CAG CCT CCA 180
Cys Thr Ile Ser Gly Phe Ser Leu Thr Ser Tyr Gly Val His Trp Val Arg Gln Pro Pro

GGA AAG GGT CTG GAG TGG CTG GTA GTG ATA TGG AGT GAT GGA AGC ACA ACC TAT AAT TCA 240
Gly Lys Gly Leu Glu Trp Leu Val Val Ile Trp Ser Asp Gly Ser Thr Thr Tyr Asn Ser

GCT CTC AAA TCC AGA CTG AGC ATC AGC AAG GAC AAC TCC AAG AGC CAA GTT TTC TTA AAA 300
Ala Leu Lys Ser Arg Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln Val Phe Leu Lys

ATG AAC AGT CTC CAA ACT GAT GAC ACA GCC ATA TAC TAC TGT GCC AGT CAC TAC GGT AGT 360
Met Asn Ser Leu Gln Thr Asp Asp Thr Ala Ile Tyr Tyr Cys Ala Ser His Tyr Gly SER

ACC CTT GCC TTT GCT TCC TGG GGC CAC GGG ACT CTG GTC ACT GTC TCT GCA GCC AAA ACA 420
Thr Leu Ala Phe Ala Ser Trp Gly His Gly Thr Leu Val Thr Val Ser Ala

ACAGCCCCAT CGGTCTATCC ACTGGCCCCT GTGTGTGGAG ATACAACTGG CTCCTCGGTG ACTCTAGGAT    490

GCCTGGTCAA GGGTTATTTC CCTGAGCCAG TGACCTTGAC CTGGAACTCT GGATCCCTGT CCAGTGGTGT 3' 560
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Lym-1 Light Chain Variable Region DNA Sequence

```
          5' CT CAG GTC CTG GGG TTG CTG CTG CTG TGG CTT ACA GTA GGT GTC AGA TGT  50
               Gln Val Leu Gly Leu Leu Leu Leu Trp Leu Thr Val Gly Val Arg Cys

GAC ATC CAG ATG ACT CAG TCT CCA GCC TCC CTA TCT GCA TCT GTG GGA GAA ACT GTC ACC 110
Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr

ATC ATA TGT CGA GCA AGT GTG AAT ATT TAC AGT TAT TTA GCA TGG TAT CAG CAG AAA CAG 170
Ile Ile Cys Arg Ala Ser Val Asn Ile Tyr Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Gln
                          ▼
GGA AAA TCT CCT CAG CTC CTG GTC TAT AAT GCC AAA ATC TTA GCA GAA GGT GTG CCA TCA 230
Gly Lys Ser Pro Gln Leu Leu Val Tyr Asn Ala Lys Ile Leu Ala Glu Gly Val Pro Ser

AGG TTC AGT GGC AGT GGA TCA GGC ACA CAG TTT TCT CTG AAG ATC AAC AGC CTG CAG CCT 290
Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln Phe Ser Leu Lys Ile Asn Ser Leu Gln Pro
                                                  ▼
GAA GAT TTT GGG AGT TAT TAC TGT CAA CAT CAT TAT GGT ACA TTC ACG TTC GGC TCG GGG 350
Glu Asp Phe Gly Ser Tyr Tyr Cys Gln His His Tyr Gly Thr Phe Thr Phe Gly Ser Gly

ACA AAG TTG GAA ATA AAA CGGGCTGATG CTGCACCAAC TGTATCCATC TTCCCGCCAT CTGATGAAGC  418
Thr Lys Leu Glu Ile Lys

TTGG 3'                                                                      422
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FIGURE 4 Nucleotide sequences of Lym-1 V_H and V_L coding regions. Included are some of the signal sequences at 5′ ends and part of the constant regions at 3′ ends expressed in italics. The hypervariable loops are underlined. The arrows indicate errors detected in the previously published Lym-1 V_L coding region.[18] These sequences have been deposited with the EMBL gene bank under accession numbers X53483 and X53484, respectively.

primer κ-17; template amplified with V_H primers HS-1 and PO3, respectively; template amplified with V_H primer G2A; template amplified with V_H primer γ-15. As expected, in most cases the single-stranded DNA product ran slightly behind its double-stranded DNA template.[26] Some samples displayed a smear of bands in the expected region of the gel.

Nucleotide sequencing of the V_H and the V_L coding regions of Lym-1 was performed using the primers listed in Table 1. To check for sequence errors introduced by reverse transcriptase and *Taq* DNA polymerase, the sequences of the V_H and V_L coding regions were determined using two independent PCR products from different batches of cDNA for each. Separately, the V_H and V_L coding regions inserted into plasmids were sequenced. The sequences were identical. Figure 4 shows the nucleotide sequences of the Lym-1 V_H and V_L regions, with some of the signal peptide sequences at their 5′ termini and part of the constant regions at their 3′ termini. We detected two errors in the previously published[18] V_L region: A→G (position 191) and C→A (position 330). The sequences of Lym-1 V_H and V_L have been submitted to the EMBL gene bank under accession numbers X53483 and X53484, respectively.

PCR-fused Lym-1 SCA Gene

Figure 5 shows the 954-bp PCR-fused Lym-1 SCA protein gene. After the first-step fusion, 10 μl of amplified product was run in 1.5% agarose gel; a weak DNA band of the expected 1088-bp size was visible and is indicated by an arrow. However, the product of the heminested second-step amplification was a very sharp and strong DNA band of the expected 954-bp size. To confirm the sequence of the gene, the PCR product was introduced into the TA cloning vector, which was used to transform *Escherichia coli* INVαF′. The cells were cultured, the plasmid was isolated, and the insert se-

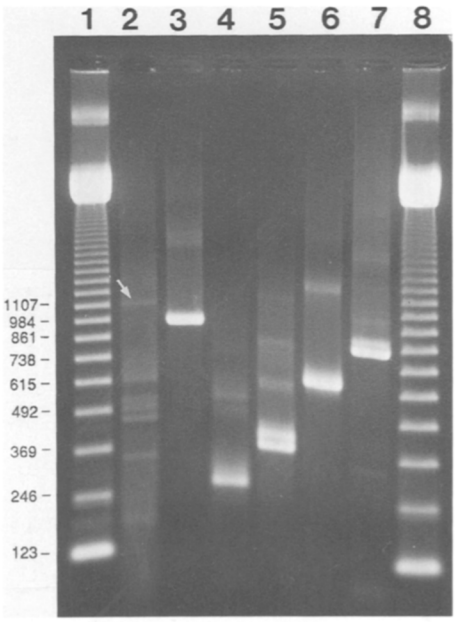

FIGURE 5 Gel electrophoretic analysis of the PCR-fused Lym-1 SCA protein gene. (Lanes *1,8*) 123-bp DNA marker ladder (bp at *left*). (Lane *2*) Products of the first-step PCR; the weak band of 1088 bp is indicated by an arrow. (Lane *3*) Products of the second-step PCR, which is a sharp DNA band of 954 bp. (Lanes *4–7*) PCR-amplified DNA fragments using the fused gene in lane *3* as the template and these heminested primers: EOP and ompA-L (lane *4*); EOP and 1A (lane *5*); EOP and link-L (lane *6*); EOP and 1G (lane *7*).

```
GAATTCCGGGATCTCTCTCACCTACCAAACAATGCCCCCCTGCAAAAAATAAATTCATATAAAAAACATACAGATAACCAT
EcoR1
      OL                                    PR                  +1       SD(cro)
CTGCGGTGATAAATTATCTCTGGCGGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTGCATGTACTAAGGAGGTTGT
ompA signal                                  -10                                  -1
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala
ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC
Lym-1 Light Variable                  10                                      20
Asp Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr
GAC ATC CAG ATG ACT CAG TCT CCA GCC TCC CTA TCT GCA TCT GTG GGA GAA ACT GTC ACC
                                      30                                      40
Ile Ile Cys Arg Ala Ser Val Asn Ile Tyr Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Gln
ATC ATA TGT CGA GCA AGT GTG AAT ATT TAC AGT TAT TTA GCA TGG TAT CAG CAG AAA CAG
                                      50                                      60
Gly Lys Ser Pro Gln Leu Leu Val Tyr Asn Ala Lys Ile Leu Ala Glu Gly Val Pro Ser
GGA AAA TCT CCT CAG CTC CTG GTC TAT AAT GCC AAA ATC TTA GCA GAA GGT GTG CCA TCA
                                      70                                      80
Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln Phe Ser Leu Lys Ile Asn Ser Leu Gln Pro
AGG TTC AGT GGC AGT GGA TCA GGC ACA CAG TTT TCT CTG AAG ATC AAC AGC CTG CAG CCT
                                      90                                     100
Glu Asp Phe Gly Ser Tyr Tyr Cys Gln His His Tyr Gly Thr Phe Thr Phe Gly Ser Gly
GAA GAT TTT GGG AGT TAT TAC TGT CAA CAT CAT TAT GGT ACA TTC ACG TTC GGC TCG GGG
                    106                 Linker
Thr Lys Leu Glu Ile Lys Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly
ACA AAG TTG GAA ATA AAA GGT TCT ACC TCT GGT TCT GGT AAA TCT TCT GAA GGT AAA GGT
Lym-1 Heavy Variable                  10                                      20
Gln Val Gln Leu Lys Glu Ser Gly Pro Gly Leu Val Ala Pro Ser Gln Ser Leu Ser Ile
CAG GTG CAG CTG AAG GAG TCA GGA CCT GGC CTG GTG GCG CCC TCA CAG AGC CTG TCC ATC
                                      30                                      40
Thr Cys Thr Ile Ser Gly Phe Ser Leu Thr Ser Tyr Gly Val His Trp Val Arg Gln Pro
ACA TGC ACC ATC TCA GGG TTC TCA TTA ACC AGC TAT GGT GTA CAC TGG GTT CGC CAG CCT
                                      50                                      60
Pro Gly Lys Gly Leu Glu Trp Leu Val Val Ile Trp Ser Asp Gly Ser Thr Thr Tyr Asn
CCA GGA AAG GGT CTG GAG TGG CTG GTA GTG ATA TGG AGT GAT GGA AGC ACA ACC TAT AAT
                                      70                                      80
Ser Ala Leu Lys Ser Arg Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln Val Phe Leu
TCA GCT CTC AAA TCC AGA CTG AGC ATC AGC AAG GAC AAC TCC AAG AGC CAA GTT TTC TTA
     82 82A 82B 82C                                 90                      97
Lys Met Asn Ser Leu Gln Thr Asp Asp Thr Ala Ile Tyr Tyr Cys Ala Ser His Tyr Gly
AAA ATG AAC AGT CTC CAA ACT GAT GAC ACA GCC ATA TAC TAC TGT GCC AGT CAC TAC GGT
        100 100A100B                                 110         113
Ser Thr Leu Ala Phe Ala Ser Trp Gly His Gly Thr Leu Val Thr Val Ser Ala End End
AGT ACC CTT GCC TTT GCT TCC TGG GGC CAC GGG ACT CTG GTC ACT GTC TCT GCA TAA TAA

CGCGGATCC
   Bam H1
```

FIGURE 6 The PCR-generated Lym-1 SCA protein gene sequence. Included and labeled are the o_L/p_R promoter, *ompA* signal sequence, Lym-1 V_L and V_H, and a 14-amino-acid linker (underlined).

quenced using Sequenase 2.0 according to the supplier's protocol. The sequence of the gene is shown in Figure 6.

DISCUSSION

Here, we describe a strategy for amplifying the V_H and V_L genes of Lym-1. The variable regions of antibody genes convey antibody specificity; thus, it is critical to accurately reproduce the variable regions of both the heavy and light chains in preparation for engineered antibodies. In agreement with other researchers,[12,27] we have demonstrated the success of using consensus primers in the amplification of antibody variable region genes. The genes were sequenced by three independent protocols and no discrepancies were detected.

The PCR technique has been used to construct and/or amplify genes by several approaches.[13–16,28] In this paper we extend the strategy to construct a 954-bp Lym-1 SCA protein gene using nine synthetic oligonucleotides and two PCR-produced V_H and V_L coding regions by a two-step PCR method. We demonstrate that the strategy greatly simplifies the time-consuming process of preparing a recombinant gene.

Taq DNA polymerase lacks 3′-exonucleolytic proofreading activity. In agreement with other researchers,[29,30] we found that the fused products produced by the *Taq* enzyme contained errors, including base substitutions, deletions, and insertions (data not shown). The fused gene prepared by *Vent*$_R$ DNA polymerase, which has the 3′ → 5′ exonuclease proofreading activity, was introduced into the vector by restriction cloning, and the correct nucleotide sequence was produced.

The advantages of this strategy include its simplicity, speed, efficiency, and low cost. Construction of a gene by PCR fusion eliminates the need for incorporation of internal restriction sites to assemble the gene. Except for insertion into the cloning vector, neither restriction enzymes nor ligase are needed. The two-step PCR recombination can be performed in a single day. DNA can be prepared in microgram quantities. No purification is required, and the product can be directly introduced into the TA cloning vector. Although we constructed a PCR fusion gene of 954 bp, it seems that longer DNA fragments can be generated by the same strategy. In addition, the technique can be applied to genes other than antibodies.

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