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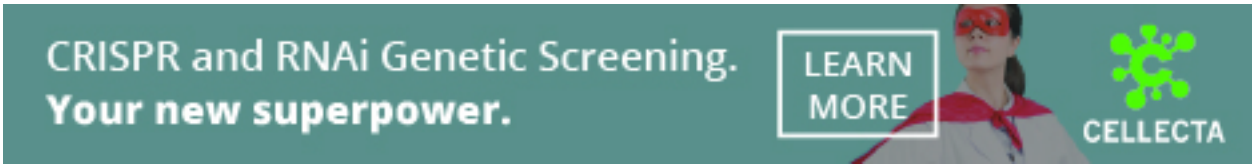

Specific Immunoglobulin cDNA Clones Produced from Hybridoma Cell Lines and Murine Spleen Fragment Cultures by 3SR Amplification

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The isothermal 3SR amplification method has been employed to assist in cloning the V_L and V_H genes from cells of hybridomas and splenic fragment cultures expressing antibodies for phosphorylcholine (PC) and estradiol (E2), respectively. As a first step, pools of degenerate primer pairs were identified complementary to immunoglobulin light and heavy chain variable (*V*) genes and capable of amplifying immunoglobulin RNA specifically at 42°C. To evaluate the functionality of the 3SR-cloned immunoglobulin genes, anti-PC V_H and V_L cDNAs were joined together to form a single chain (sc) antibody construct and were expressed in *Escherichia coli* under the regulation of the alkaline phosphatase (*pho*A) promoter. Similarly, the combination of a murine spleen fragment and 3SR methodologies were employed to clone a selected pool of cDNAs for cultures producing anti-estradiol antibodies. This approach of using the murine spleen fragment and 3SR isothermal amplification offers the advantages of B-cell follicle architecture for antigen-driven B-cell maturation and proliferation and RNA-specific amplification, respectively. The potential utility of these advantages for the production of monoclonal antibodies and for providing the capability of studying memory B-cell development are discussed.

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Rearrangements of the variable (*V*)-gene segments of immunoglobulin heavy (V_H, *D*, and J_H) and light (V_λ, J_λ and V_κ, J_κ) chains, coupled with the pairing of different rearranged V_H and V_L genes constitute the genetic repertoire available to mammalian immune systems in responding to foreign antigens. In mice this immunologic repertoire has been estimated to be $>5\times10^7$ combinations.[1,2] On its surface, each B cell presents only one pair of heavy and light chain combinations that bind the stimulating antigen. It is hypothesized that B cells are selected for expansion based on both the affinity and kinetic properties of the antigen–cell surface antibody interaction.[3] It is in this manner that the immune system sorts through the combinations of V_H and V_L gene pairings.

Hybridoma technology allows for the isolation of B cells containing specifically paired V_H and V_L genes and, in turn, provides a source of secreted antibodies of unique specificity.[4] The application of the PCR amplification to the *V* genes present in hybridoma cell lines[5] or single hybridoma cells,[6] followed by subsequent cloning and expression in mammalian cells[5] or bacteria,[7] permits the isolation and retention of the original V_H and V_L chains in a highly engineered environment. However, when heterogeneous populations of circulating lymphocytes are used as a source of V_H and V_L genes, specific pairings are lost after the PCR amplification and subsequent cloning and expression. In an effort to recover the originally paired V_H and V_L genes, as well as to test novel synthetic *V* gene combinations, a bacteriophage display approach has been used. Antibodies engineered as single chain (sc)[8] or heterodimeric Fab[9] constructs have been displayed on the surface of a filamentous phage (fd) as a fusion of the phage pIII protein. Fusions of the phage pVIII protein also have been used to display antibody fragments on the surface of the fd phage.[10–12] Binding of the displayed antibody on the surface of the phage with antigen can be used as a way to recover antibodies composed of the original or novel V_H and V_L gene pairings. However, because each phage contains only one such V_H and V_L pairing, the frequency of the occurrence of any single V_H and V_L pairing is $<1/10^8$. Consequently, it is necessary to construct phage display libraries of 10^{11} copies in order to detect specific V_H and V_L combination. To recover antibodies of especially high affinity or unique specificity, the size of a phage display library may have to be $>10^{11}$ copies.

In this report we describe the use of an alternative to hybridoma cell lines or circulating lymphocytes as a source of antibodies of monoclonal specificity. A spleen fragment culture method combined with the self-sustained sequence replication (3SR) protocol have been used to clone V_H and V_L cDNAs from cultures expressing anti-estradiol (E2) antibodies. The isothermal 3SR method of nucleic acid amplification was employed because of its capability to amplify RNA specifically.[13,14]

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MATERIALS AND METHODS

Murine Splenic Fragment Culture: Production of Anti-Estradiol Antibodies

The splenic fragment culture method has been described previously.[15–18] The combination of this method and the 3SR procedure is depicted in Figure 1. Briefly, donor BALB/c mice were each injected twice with 100 μg of estradiol b (E2) coupled to *Limulus polyphemus* hemocyanin (Hy) (gift from F. Boches, Baxter Diagnostics, Inc., Miami, FL) at 2-month intervals. One to two months after the second injection, ~1×10^8 to 2×10^8 whole spleen cells were collected from the donor mouse. MHC syngeneic, Hy (carrier)-primed recipient mice were lethally irradiated with 1300 rads, and ~4×10^6 donor spleen cells were transferred intravenously. Within 24 hr of cell transfer, the recipient's spleen was removed and dissected into 1-mm^3 fragments. Each spleen fragment was cultured in individual wells of a microtiter dish in the presence of E2–Hy for 2–3 days. Five to seven days later, culture fluids from the wells were screened by an ELISA assay[16] for antibodies specific for E2 (anti-E2). When limiting numbers of B cells are transferred, subsequent responses can be monoclonal. However, in this case, most of the microtiter wells were positive, indicating that responders were likely to be polyclonal. Antibodies obtained from positive fragments were analyzed further for relative affinity by an inhibition ELISA assay wherein 10^{-5} to 10^{-8} molar dilutions of competing E2 were added to the culture-produced antibodies during the ELISA assay.[16]

Culture fragments that were positive for anti-E2 antibody were subjected to extraction of total nucleic acids (DNA and RNA) by a modified procedure first described by Stallcup and Washington.[19] This total nucleic acid was used for 3SR amplification and subsequent cloning of immunoglobulin light and heavy chain cDNAs.

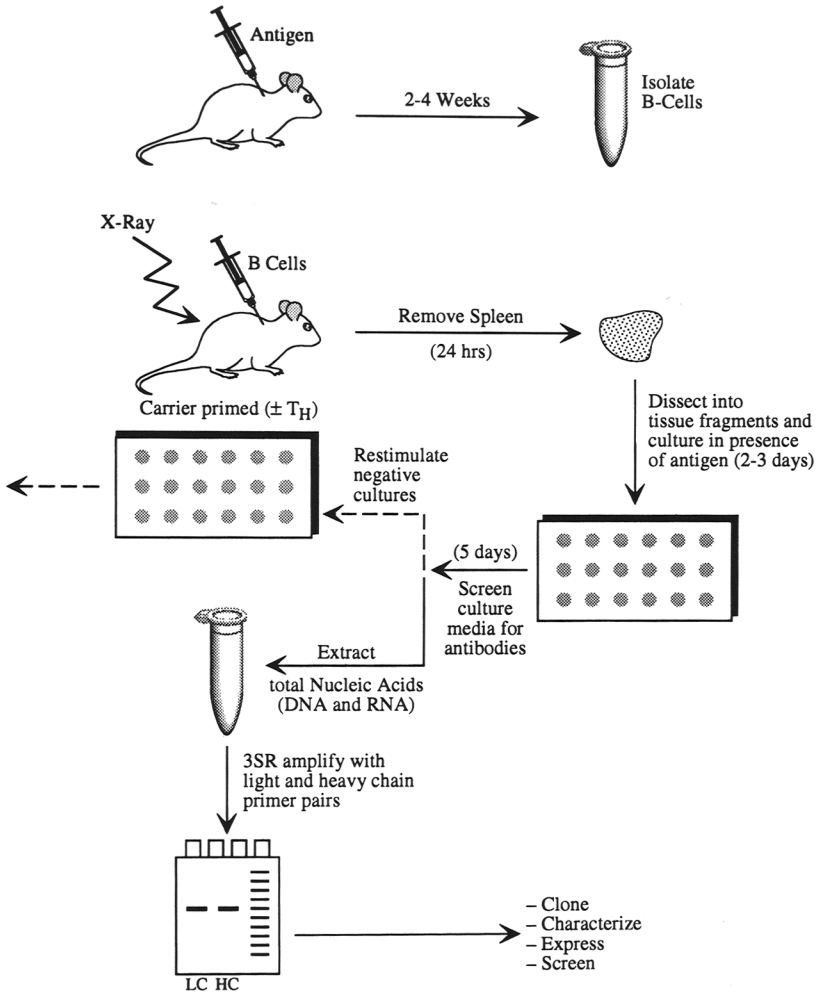

FIGURE 1 Scheme for splenic fragment culture/3SR methodologies. Steps in the scheme are described in Materials and Methods (see also Refs. 15–18). Conditions for 3SR reaction, cloning expression, and ELISA testing of produced monoclonal antibody are described in Materials and Methods.

Hybridoma Cell Line R2-09: Production of Anti-Phosphorylcholine (PC) Monoclonal Antibody

The hybridoma cell line R2-09, which synthesizes an IgG anti-PC monoclonal antibody, was used as a source of mRNA. This hybridoma was generated by the fusion[4] of SP20 hybridoma cells with spleen cells from a BALB/c mouse that had been primed and boosted with PC–Hy.[20] The antibody produced was an anti-PC IgG that shared idiotype and DNA sequence with the TEPC-15 myeloma protein variable region.[21] Because the nucleotide sequence and the antigenic specificities of the light and heavy chains of this antibody were determined previously[22] the mRNA from R2-09 served as a control to test whether the 3SR generic primers (Fig. 2) could produce the same amplification products as the light and heavy chain-specific primers (see sequences below) that were complementary to the V_κ T15 light chain and V_H S107 (T15) heavy chain. Total nucleic acid was extracted from 1×10^7 cells using the AGPC method of extraction.[23]

3SR and PCR Amplification Reactions

The 3SR reactions were conducted as described previously,[24] with the exception that each primer (Fig. 2) was present at 0.25 μM and the reaction time was extended to 90 min. The 3SR generic heavy chain primers used to amplify both the anti-PC and anti-E2 mRNAs are shown in Figure 2A. The 5′-end generic primers were composed of two pools of degenerate oligonucleotides targeted to either the leader (92–107/92–108) or variable (92–109/92–110) regions of the heavy chain mRNAs. Additionally, an oligonucleotide primer [(5′-*AATTTAATACGACTCACTATA*GGGAGAGGTGAAGC-

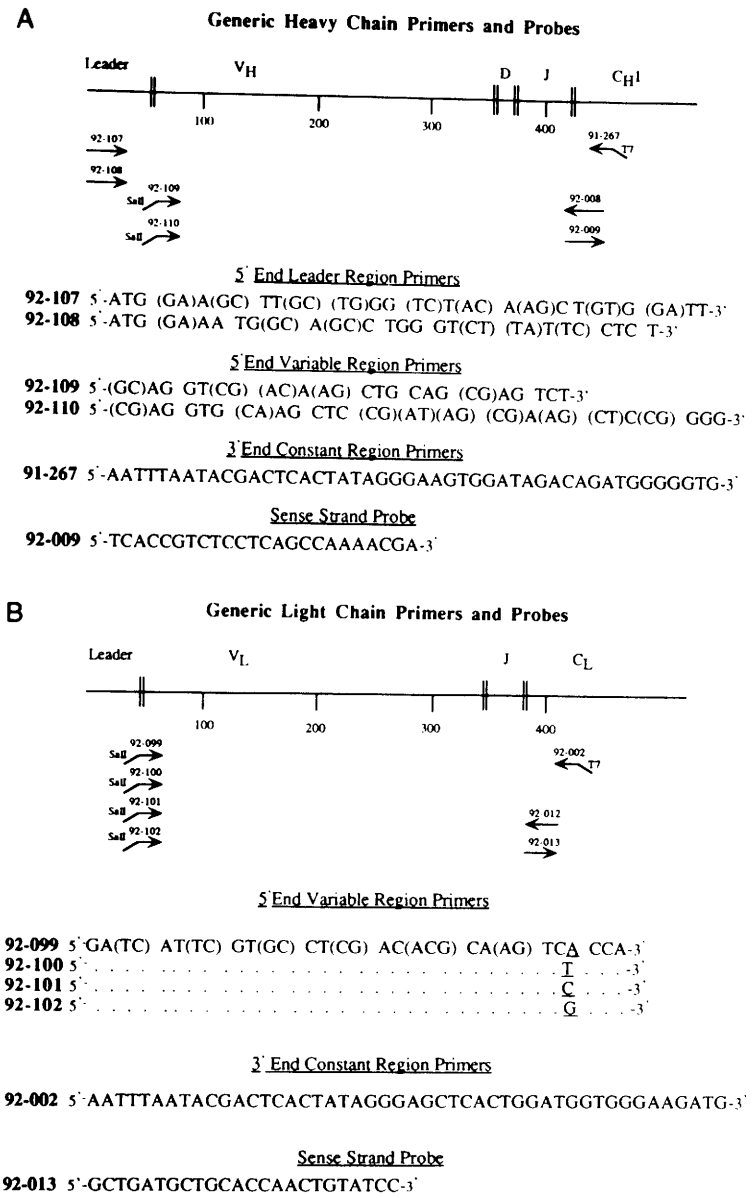

FIGURE 2 Maps and sequences for generic 3SR primers and probes used to amplify immunoglobulin heavy (*A*) and light (*B*) chain mRNAs. (*A*) Scheme of heavy chain mRNA, depicting the leader, variable (V_H), diversity (*D*), junction (*J*), and constant (C_H1) regions with the approximate distance in nucleotides listed below the map. The positions and sequences of 3SR primers and probes are noted. The primer 91–267 contains noncomplementary T7 RNA polymerase (T7) promoter and transcriptional initiation sequences. For the sequence of 92–267 the T7 promoter and transcription initiation involves the first 21 and the next 4 nucleotides, respectively. Positions of degeneracy are noted within parentheses. Two pools of degenerate primers each were made for the 5′ leader and variable regions of the mRNA. Oligonucleotides 92–008 and 92–009 were used as hybridization probes for the sense and antisense 3SR products, respectively. (*B*) Scheme of a light chain mRNA. Oligonucleotide 92–002 contains T7 RNA polymerase promoter sequence. Four pools of degenerate primers were made for the 5′ variable region. The underlined nucleotides in pools 92–099, 92–100, 92–101, and 92–102 distinguish each pool.

TGGTGGAGTCTGGA-3′) italic and underlined sequences specify the T7 RNA polymerase-specific promoter and transcription initiation sequences, respectively] specific for the 5′ variable region of the anti-PC heavy chain was used in a 3SR reaction as a comparison with the 3SR heavy chain generic primers. The 3′-end primer used with both generic and specific 5′-end primers was 91–267, which hybridizes to the C_{H1} region of the heavy chain mRNA. The 3SR generic light chain primers are shown in Figure 2B. Primers 92–099 to 102 hybridize to the 5′ end of the light chain variable region and are composed of four pools of degenerate oligonucleotides. Primer 92–002 hybridizes to the C_L region.

To convert the 3SR RNA amplification products to DNA, 2 μl of each 50-μl 3SR amplification reaction was amplified by the reverse transcriptase (RT)/PCR protocol recommended by the manufacturer (Perkin-Elmer Cetus, GENEAmp RNA kit). Heavy chain oligonucleotide primers 92–107/92–108 (leader region) and 92–109/92–110 (variable region) (Fig. 2A) were used with AMV RT to synthesize the first strand of cDNA. Following the RT reaction, primer 92–140 (5′-ACT-A*GAATTCA*GTGGATAGACAGATGGGGG-TG-3′), which contains an inserted *Eco*RI site (italics), was used to complete the PCR primer pairs. For the light chain PCR reaction, a primer pool of 92–099 to 102 was used to create the first-strand cDNA. Primer 92–012 was then used to complete the PCR primer pair. During the RT reaction, the primer concentration was set at 1.25 μM; during the PCR reaction, the final concentration of each 5′- and 3′-end primer was 0.25 μM and 1.0 μM, respectively. The conditions used for the thermal cycling were 30 cycles, each cycle consisting of 1 min at 94°C (denaturation), 1 min at 55°C (annealing), and 2 min at 72°C (primer extension), followed by 10 min at 72°C and a 4°C termination step.

Northern and Southern Hybridization Analyses

Northern hybridization analyses were performed on the 3SR RNA amplification products with a NuPAGE 8% RNA Gel Kit (Novex, San Diego, CA) according to the manufacturer's instructions. Aliquots (10 μl) of 3SR amplification products were denatured by diluting each sample with an equal volume of 2× NuPAGE urea sample buffer, followed by heating for 2 min at 85°C before electrophoresis. Following electrophoresis, transfer of the nucleic acids onto a Zeta Probe nylon filter (Bio-Rad, Richmond, CA) was carried out for 45 min at 0.4 A in 1× NuPAGE running buffer on a TE22 transfer apparatus (Hoefer Scientific, San Francisco, CA). The DNA was cross-linked to the filter by UV irradiation at

0.15 J/cm^2. The filters were probed with ^{32}P-labeled oligonucleotides 92–009 or 92–013 (Fig. 2). An *Msp*I digest of pBR322 DNA (New England Biolabs, Beverly, MA) that had been prelabeled with ^{32}P was included on each blot as molecular weight markers.

Southern blot analyses were performed on aliquots (1–5 μl) of PCR amplification products separated on precast 6% TBE polyacrylamide gels (Novex, San Diego, CA) as described previously.[25] Following electrophoresis, the gel was soaked for 10 min in 0.05 M NaOH and 5 min in 1× TBE. Transfer of the nucleic acids onto Zeta Probe nylon filter was carried out for 45 min at 0.4 A in 1× TBE buffer on a TE22 transfer apparatus. After the transfer, the nylon membrane was washed for 5 min each in 0.1 M NaOH and H_2O. The DNA was cross-linked to the filter by UV irradiation at 0.15 J/cm^2. The filters were probed with ^{32}P-labeled oligonucleotides 92–009 or 92–013 (Fig. 2).

Cloning, Nucleic Acid Sequencing, and Computer Analysis of Anti-E2 Heavy and Light Chain Clones

Because generic primers were used to amplify heavy and light chain immunoglobulin mRNAs, the amplification products were cloned and the clones were individually analyzed by sequencing. A 10-μl aliquot of the anti-E2 leader heavy chain amplification reaction was treated with Klenow DNA polymerase[26] and digested with *Eco*RI. The blunt end/*Eco*RI-cleaved fragments were gel purified and ligated into pUC18 that had been digested previously with *Sma*I and *Eco*RI and treated with calf alkaline phosphatase (CAP).[26] The anti-E2 heavy chain variable region amplification reaction (10 μl) was digested with *Sal*I and *Eco*RI because both restriction endonuclease sites were engineered into primers 92–(109 to 110) and 92–140, respectively, for cloning purposes. The digested PCR fragments were gel purified and ligated into pUC18, which had been digested previously with *Sal*I and *Eco*RI and treated with CAP. The anti-E2 light chain amplification reaction (10 μl) was treated with Klenow DNA polymerase and digested with *Sal*I. The PCR fragments were gel purified and ligated into *Sma*I- and *Sal*I-digested pUC18 or pBR322, which had been treated with CAP. Dilutions of each ligation were used to transform competent[27] *Escherichia coli* MC1061 (recA$^+$) or HB101 (recA$^-$ cells).[28]

Nucleotide sequence analyses of pUC18 and pBR322 heavy and light chain clones were performed by the modified dideoxy nucleotide chain termination method described by Hsiao et al.[29] Computer analysis of the derived sequence was performed with MacVector (IBI, New Haven, CT) and Line-Up from the University of Wisconsin Genetics Computer Group (UWCG).

Synthesis of Anti-PC sc Antibody

The 3SR amplification products of anti-PC light and heavy chains from hybridoma R2-09 were cloned into pBR322 as described above for anti-E2 antibodies. Plasmids pSHC-5 and pSLC-2 contained heavy and light chain anti-PC clones, respectively (Fig. 3). pSHC-5 and pSLC-2 were linearized with *Pst*I, and the heavy and light chain inserts were joined using a two-step PCR reaction. The first PCR reaction employed primer pairs 92–194 and 92–188 and 92–195 and 92–201 (Table 1) to amplify the heavy and light chain inserts, respectively (Fig. 3). Primers 92–188 and 92–201 contain overlapping linker sequences based on the Genex 212 linker[30] (5′-GGCTCTACTTCCGGTAGTGGTAAGAGCTCTGAAGGTAAAGGT- 3′). The PCR fragments produced by the first PCR step were then joined by a second PCR reaction with the primer pair 92–194 and 92–195 (Fig. 3; Table 1). The resulting PCR fragment contained *Sal*I and *Xma*I cloning sites at the termini (Fig. 3).

Construction of pT15-110 Expression Vector

Plasmid pT15-110 was constructed for the expression of anti-PC sc antibody in *E. coli*. In vector pT15-110, the gene for PC sc antibody is under the regulation of the alkaline phosphatase (*pho*A) promoter region and the *Bacillus thuringunsis cry* transcription terminator.[31] The *pho*A leader sequence is used to direct secretion of the antibody. Additionally, in pT15-110, a nucleotide sequence encoding five histidine residues is fused to the 3′ end of the PC sc antibody gene. These histidine residues function as a metal binding site (MBS) and can be used in a rapid partial purification of the antibody. A nucleotide sequence encoding the 13 amino acids from the carboxyl terminus of the human c-Myc protein[32] is also fused to the 5′ end of the antibody gene. These amino acids provide an immunological tag useful for monitoring the expression and purification in Western blots and functionality in ELISA assays.

Expression vector PT15-110 was constructed as follows (Fig. 3). The *pho*A promoter and leader sequence and the *cry* terminator were obtained in plasmid pSYC 1087 (gift of H. Wong, Baxter Diagnostics, Inc.). Oligonucleotide 92–183 (5′-*GGCGCC*GTCGCCCCGGGCATCACCATCATCACTAG*GGATCC*-3′) was inserted into the *Nar*I–*Bam*HI sites (italics) of pSYC 1087 to form vector pMBS4. This resulted in the insertion of the codons for five histidine residues as well as restriction enzyme sites *Nar*I, *Sal*I, and *Sma*I for subsequent cloning steps.

The original polylinker positioned 5′ to the *pho*A promoter in pSYC 1087 was removed by digestion of pMBS-4 with *Cla*I and *Xba*I. The ends were repaired by Klenow DNA polymerase and religated to form plasmid pMBS-101.

To form pPHO 101, oligonucleotide 93–033 (5′-GAACAAAAACTCATCTCAGAAGAGGATCTGGGTGCAGTCGAC-3′) was inserted into pMBS 101, which had been digested with *Nar*I, treated with Klenow DNA polymerase, and digested with *Sal*I. This resulted in the addition of the sequence encoding the c-Myc tag.

The anti-PC sc antibody gene, constructed by a series of PCR reactions described previously (Fig. 3), was then inserted into the *Sal*I–*Xma*I sites of pPHO 101 to form expression vector pT15-110.

Expression of Anti-PC sc Antibody

Cell Culture Conditions

E. coli strain MM294 was transformed with pT15-110 and used as a host for the expression of anti-PC sc antibody. Cells transformed with plasmid pPHO-101 served as a negative control. Individual *E. coli* transformants were grown at 30°C to ~4–5 OD_{600} in phosphate medium (1× MOPS, 0.4% glucose, 0.15% vitamin-free casamino acids, 10 μg/ml of B1, and 100 μg/ml of ampicillin)[33] containing 10 mM KH_2PO_4. This medium represses *pho*A expression. The cells were washed with phosphate me-

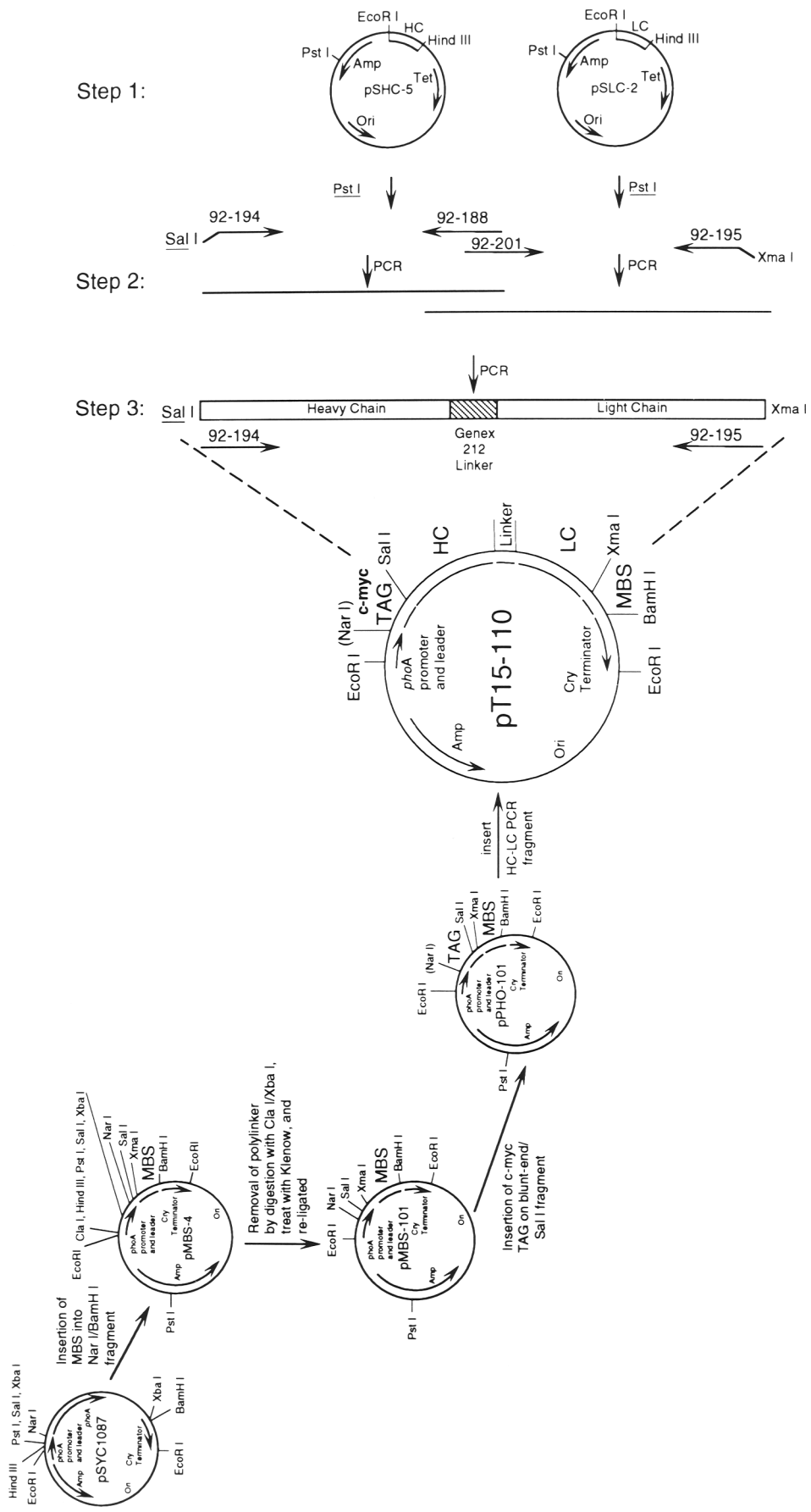

FIGURE 3 Scheme for the construction of plasmid pT15-110, which contains the sc antibody form of the anti-PC antibody. The MBS and the c-Myc tag (TAG) sequences are defined in Materials and Methods. Light chain (LC) and heavy chain (HC) are joined together by Genex 212 linker sequence as described in Materials and Methods. PCR reaction conditions used to join the HC and LC fragments are similar to that described in Materials and Methods to convert 3SR products to cDNA fragments.

TABLE 1 Oligonucleotides Used for the Synthesis of Anti-PC sc Antibody

Primer	Sequence	Strand[a]	Target gene[b]	Restriction sites[c]
92–188	AGAGCTCTTACCACTACCGGAAGTAGATGAGGAGACGGTGACCGTGGTCCCTGC	AS	V_H	none
92–194	ACTAGTCGACGAGGTGAAGCTGGTGGAATCTGGAGGA	S	V_H	*Sal*I
92–195	ACTACCCGGGCCGTTTCAGCTCCAGCTTGGTCCCAGCA	AS	V_L	*Xma*I
92–201	GGTAGTGGTAAGAGCTCTGAAGGTAAAGGTATTGTGATGACTCAGTCTCCAACTT	S	V_L	none

[a]S (sense) refers to sequences that are identical to the target RNA; AS (anti-sense) refers to the sequences that are complementary to the target RNA.
[b]Heavy chain variable region (V_H), light chain variable region (V_L).
[c]*Sal*I and *Sma*I sites are underlined in sequence.

dium and then suspended in phosphate medium containing 0.1 mM KH_2PO_4 at an OD_{600} of ~0.08. *E. coli* cultures were grown under low phosphate conditions for 7 hr to achieve maximal anti-PC sc antibody expression.

Cell Lysis and Antibody Purification

Cell lysis was accomplished by resuspending transformed cells (20 OD_{600}) in 1.0 ml of sonication buffer [50 mM Na phosphate (pH 8.0), 300 mM NaCl, 0.25% Tween 20, 0.1 mM EGTA, and 1 mM phenylmethylsulfonylchloride]. The cells were frozen on dry ice/ethanol, thawed, and sonicated on ice (10 cycles of 10-sec bursts with 1 min cooling, at 20 W with a Bronson sonifier, model 450, Danbury, CT). The lysed cells were centrifuged at 11,000 rpm for 20 min at 4°C. The supernatant represented the total soluble protein. The total protein concentration in the supernatant was measured by the Lowry method[34] with a DC protein assay kit (Bio-Rad, Richmond, CA).

Partial Purification of Anti-PC sc Antibody

Anti-PC sc antibody was partially purified from cell lysates using a nickel (Ni)–NTA resin (Qiagen, Chatsworth, CA) according to the manufacturer's recommendations. Briefly, a 50% slurry of Ni–NTA resin (previously equilibrated in sonication buffer) was added to an aliquot of supernatant cell lysate and agitated for 1 hr at 4°C. The resin was centrifuged at 14,000 rpm, and the unbound fraction was collected. The resin was then eluted with 1.0-ml aliquots of 10–100 mM imidazole dissolved in sonication buffer (above).

Western Blot and ELISA Assays

Aliquots (5 μl) of unbound or imidazole-eluted fractions were diluted in equal volumes of 2× sample buffer [0.9 M Tris-HCl (pH 8.45), 24% glycerol, 8% SDS, 0.1% Coomassie brilliant blue R-250, and 200 mM dithiothreitol] and boiled for 5 min before resolution by electrophoresis on a 10–20% tricine gradient gel (Novex, San Diego, CA). Transfer to S&S Nytran nylon membranes (Schleicher & Schuell, Keene, NH) was carried out for 1.5 hr at 0.2 A. Western blot detection was carried out according to recommendations of ELC Western Blot Analysis System (Amersham, Arlington Heights, IL). Nylon membranes were blocked first with 50 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM NaCl, 5% dry milk, 0.2% NP-40, and 0.02% thimerosal. Subsequent washes and the antibody detection buffer contained 1× PBS, 0.1% dry milk, 0.1% NP-40, and 0.02% thimerisol. The primary antibody reaction step consisted of incubating the membranes overnight at 4°C in a 1/500 dilution of monoclonal antisera specific to c-*myc* (Oncogene Sciences, Uniondale, NY). During the secondary antibody step, the membranes were incubated at room temperature for 1 hr in a 1/50,000 dilution of sheep anti-mouse immunoglobulin–horseradish peroxidase detection complex.

For the ELISAs each well of a 96-well microtiter plate was coated with 50 μl of 50 μg/ml phosphorylcholine conjugated to bovine serum albumin (PC–BSA) in 0.05 M carbonate buffer (pH 9.6, containing 0.02% NaN_3) and incubated at 4°C overnight. The coated plates were rinsed with 1× PBS and incubated for 1 hr with 100 μl per well of blocking solution (1× PBS, 1% BSA, 0.02% NaN_3). The blocking solution was removed and the wells were washed with 1× PBS. Aliquots (50 μl) of *E. coli* lysates or fractions eluted from the Ni–NTA resin were added and reacted at room temperature for 4 hr. The samples were removed and the wells washed three times with 1× PBS. The secondary antibody reaction, in which mouse anti-c-Myc binds to the anti-PC sc antibody, was allowed to incubate 4 hr at room temperature. After washing with PBS, 50 μl of 0.5 μg/ml alkaline phosphatase-labeled goat anti-mouse immunoglobulin antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well and incubated at 4°C overnight. The plates were washed four times with 1× PBS following which 100 μl of *P*-nitrophenylphosphate (NPP) solution (3 mM NPP, 0.05 M carbonate buffer (pH 9.6), 1 μM $MgCl_2$] was added to each well. Color development was allowed to proceed for 1 hr at room temperature. Plates were read at 405 nm on a Dynatech (Chantilly, VA) MR 5000 microtiter plate reader.

RESULTS

Generic 3SR Primers for Immunoglobulin Genes and Cloning of Anti-phosphorylcholine Monoclonal Antibody from Hybridoma Line R2-09

Degenerate oligonucleotide primers useful for the amplification of immunoglobulin light and heavy chain genes have been described previously.[5,6,35,36] These degenerate oligonucleotides were used in PCR amplification reactions in which the specificity of the priming reactions was, in part, dependent on conducting the primer extension reaction at a temperature close to the melting point of the primer–template duplex. Unlike PCR reactions, the 3SR reaction proceeds at a constant temperature of 42°C, thereby providing lower stringency for primer hybridization than used for PCR reactions. Consequently, it was neces-

sary to design and test alternative degenerate oligonucleotide primer pairs capable of providing immunoglobulin specificity for the 3SR reaction at 42°C. The 3SR primers selected were designed to function generically for the amplification of κ light chain and most heavy chain families of immunoglobulin genes.

To test the utility of the 3SR generic primers, heavy (Fig. 2A) and light (Fig. 2B) chain mRNAs were amplified from the total nucleic acid extracted from the anti-PC hybridoma cell line R2-09. For amplification of the light chain mRNA, the 3SR generic primers were complementary to V_L sequences and were comprised of 96 oligonucleotides synthesized as four degenerate groups (92–099 to 92–102). For amplifications of the heavy chain mRNA, sequences in the leader or V_H regions were selected as areas for hybridization of the 5′ primers. A total number of 30 oligonucleotides, synthesized as two groups (92–107 and 92–108), was used for the leader region, whereas 28 oligonucleotides were synthesized as two groups (92–109 and 92–110) and were used as V_H region primers.

The RNA products produced by the 3SR amplification of the anti-PC light and heavy chain mRNA and analyzed by Northern blot corresponded to the expected sizes (see legend to Fig. 4A). Southern hybridization analysis of the DNA products produced from the 3SR amplification products by PCR revealed fragments of the expected sizes of ~400 bp for the light chain amplification and ~465 and 420 bp for the leader and V_H amplifications, respectively (Fig. 4B). As determined by hybridization (Fig. 4B) and direct sequence analyses (data not shown), the DNA produced with the generic light chain variable (LC–G_v) and the generic heavy chain variable (HC–G_v) primers were identical to the products produced by those derived from the published sequence of the light (LC-S) and heavy (HC-S) chains of the anti-PC antibody in the R2-09 hybridoma cell line.[20] The sequence analyses of the clones produced from the 3SR generic heavy and light chain primers revealed that several mismatches could be tolerated. For the degenerate heavy chain primers, only the 3′-terminal six nucleotides were common among all of the clones. At each of the other degenerate positions within the primer, either of the alternative bases were observed. For the light chain primers, only the three 3′-terminal nucleotides were found to be common among the clones.

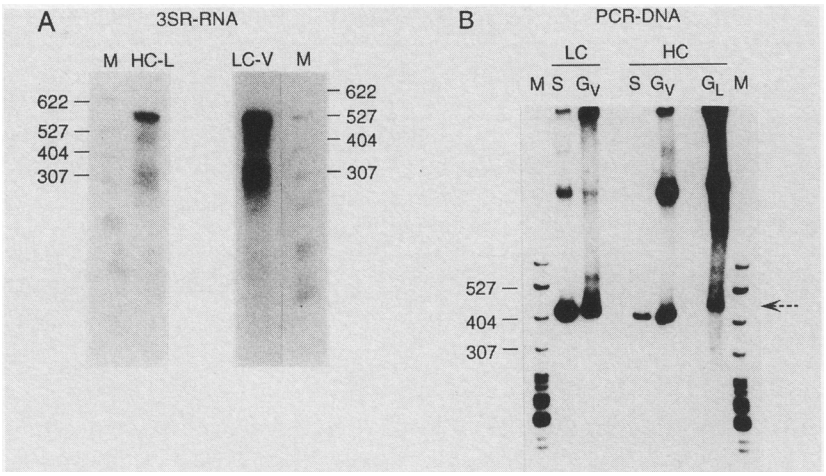

FIGURE 4 Northern (*A*) and Southern (*B*) blot analyses of 3SR and PCR amplification products. (*A*) 3SR RNA products using heavy chain leader (HC-L) and light chain variable (LC-V) 5′-end primers. *Msp*I digested pBR322 fragments serve as size markers (M). The expected size products of 550 and 520 nucleotides were observed for the HC-L- and LC-V-primer reactions, respectively. (*B*) The 3SR RNA products were converted to cDNA using the PCR and conditions described in Materials and Methods. The light chain (LC) 3SR reactions carried out using anti-PC specific (S) or generic variable (G_V) region 5′-end primers produced identical 405-bp fragments after the PCR reactions as observed with Southern hybridization. The heavy chain (HC) 3SR reactions carried out using specific (S) or generic variable (G_V) and generic leader (G_L) region 5′-end primers were converted to cDNA by PCR. The identical 420-bp DNA fragments are observed for both the S and G_V primed reactions. The expected larger 465-bp product (arrow) is observed from the use of the G_L primers. Larger products observed above the expected cDNA products most likely represent chimeric products derived from each of the expected cDNA products.

Cloning and Sequence Analysis of Light and Heavy Chain Genes Derived from Cells Expressing Anti-E2 Antibodies in Splenic Fragments Culture

Monoclonal antibodies specific for E2 have been problematic to produce (F. Boches, pers. comm.). Such a problematic antigen provides an opportunity to evaluate the effectiveness of the spleen fragment/3SR approach for the production of an antibody of monoclonal specificity. During the initial screening step depicted in Figure 1, cells in a splenic fragment culture, D_1A_6, were identified as producing antibody specific for E2 with an affinity constant of $\sim 10^{-7}$ M. Total nucleic acid from cells of this fragment was amplified by 3SR with the light and heavy chain generic primers shown in Figure 2. The 3SR RNA products of the heavy and light chain amplifications were converted to DNA, digested with the appropriate restriction endonucleases, and cloned into either pUC 18 or 19.

A total of 39 heavy and 36 light chain productive clones derived from fragment D_1A_6 were sequenced. Comparative sequence analysis of the clones using the UWGCG LineUP program revealed the presence of four alleles each for the heavy and light chain groups (Table 2). The nucleotide sequence representative of clones from each allelic group is presented in Figure 5A. The heavy chain clones from groups 2, 3, and 4 exhibited 81–86% similarity with the J558 V_H heavy chain family,[22,37] whereas clones from group 1 were marginally similar to the S107 (79.7%) V_H family. However, comparison of the sequences of clones of group 1 to sequences in GenBank revealed a 96% similarity to a mouse anti-DNA rearranged heavy chain variable region.[38] Only in clones from groups 2 and 4 could the family of the D_H minigenes be determined unambiguously as being from DFL16.1 and DST4 minigenes, respectively.[22] The J_H minigenes utilized were $J_H2,3$ and J_H4. The light chain clones from fragment D_1A_6 also consisted of

TABLE 2 Heavy and Light Chain Clones Obtained from Spleen Fragment Culture D_1A_6

Alleles	No. of clones analyzed	Minigene families: *V* (% homology)	*D*	*J*
		V_H clones		
1	11	S107 (79)	DSP 2.3, 2.4, 2.6	J_H2
2	6	J558 (81)	DFL 16.1	J_H3
3	15	J558 (86)	DSP 2.3, 7	J_H4
4	7	J558 (83)	DST 4	J_H3
		V_L clones		
1	16	V_κ 4/5 (92)	—	$J_\kappa4$
2	10	V_κ 19/28 (96)	—	$J_\kappa5$
3	6	V_κ 10 (88)	—	$J_\kappa5$
4	3	V_κ 19/28 (97)	—	$J_\kappa5$

four V_κ alleles (Fig. 5B). Clones from groups 2 and 4 were highly similar (96–97%) to the 19/28 V_κ family,[39] whereas clones from groups 1 and 3 were similar to the V_κ 4/5 (92%) and V_κ 10 (88%) families, respectively. Light chain clones used the J_κ 5 minigene while clones from group 1 used the J_κ 4 minigene.

Unlike the variable region degenerate primers, the leader region primers allowed for unambiguous determination of the sequences present at the amino terminus of the V_H minigene. In group 1 clones, derived from the leader region primers, the nucleotide sequences predicted amino acids Val-5, Glu-6, and Thr-7 in place of Gln-5, Gln-6, and Ser-7 as predicted by using the generic variable region primers. Likewise, in group 4, clones derived from the leader primers contained the codon for the amino acid Leu at position 3 in place of Lys as predicted by the variable region primers. Because none of the clones of groups 2 and 3 were derived using the leader region primers, the sequences produced using the variable region primers could not be confirmed.

Expression of sc Anti-PC Antibody

To determine whether the clones generated by the 3SR amplification method produced functional antibodies, the anti-PC V_H and V_L genes were joined into an sc antibody construct and expressed in *E. coli*. Initially, the anti-PC heavy and light chain 3SR products generated by the generic primers were converted into DNA by PCR and cloned into pBR322 to produce plasmids pSHC-5 and pSLC-2, respectively (Fig. 3). The genes from the heavy and light chain inserts in pSHC-5 and pSLC-2 were linked together with the Genex 212 linker sequence and inserted into pPHO 101 to form the expression vector pT15-110. Transformants of *E. coli* strain MM294 with pT15-110 were grown either under inductive low phosphate (0.1 mM) or uninductive high phosphate (10 mM) conditions. The *pho*A promoter in pT15-110 expresses the anti-PC sc antibody only under low phosphate (Fig. 6). Processed (~32-kD) and unprocessed (~33-kD) monomeric as well as dimeric (~60-kD) and trimeric (~90-kD) forms of sc anti-PC antibody were observed on Western blot analyses of whole cell lysates (Fig. 6A). However, Western blot analysis of a periplasmic space fraction extracted with Tween 20 and EGTA revealed that ~75% of the total sc antibody expressed by *E. coli* is processed and transported into the periplasmic space where it apparently is associated with the inner membranes requiring release by nonionic detergent (data not shown).

Partial purification of the anti-PC sc antibody was achieved by absorption through the metal-binding polyhistidine tract inserted at the 3′ end of the sc antibody (Fig. 6B). Denaturation conditions of 6 M urea was required for the sc antibody to bind quantitatively to the resin; otherwise the majority of the sc antibody was observed in the unbound fraction (Fig. 6B). The peak of the elution profile of sc antibody occurred at 40 mM imidazole.

Detection of Anti-PC sc Antibody Activity by ELISA

The specificity of sc anti-PC antibody expressed by the plasmid pT15-110 and partially purified by the Ni–NTA resin was analyzed in a quantitative ELISA-based assay with the anti-PC sc and anti-c-Myc antibodies as the primary and secondary antibodies, respectively. The nondenatured unbound and the 40 mM imidazole fractions (Fig. 6B) were tested for binding to the PC–BSA immobilized on the microtiter well. The fraction eluted with 40 mM imidazole from Ni–NTA resin was the only material that demonstrated quantitative binding to the immobilized PC (Fig. 7). Neither lysate from the *E. coli* host strain nor the sc antibody present in the unbound fractions was capable of binding quantitatively to PC. The amount of the sc anti-PC antibody produced by pT15-110 in *E. coli* was estimated, using silver-stained SDS-PAGE, to be <0.1% of the total cell protein (data not shown).

DISCUSSION

Development of hybridoma cell lines producing monoclonal antibodies of a desired specificity requires multiple experimental steps that may extend over a significant time period. The steps involved in production of a monoclonal antibody include immunization of the animal, screening of the hybridoma, and antibody production,[40] any or all of which may be problematic. Additional difficulties are frequently encountered even after a hybridoma cell line is identified and clonally expanded. Expanded hybridoma lines can lose their ability to produce a specific monoclonal antibody after prolonged growth in culture. This loss of antibody expression is also observed in cell lines that have been frozen and stored after clonal selection. Thus,

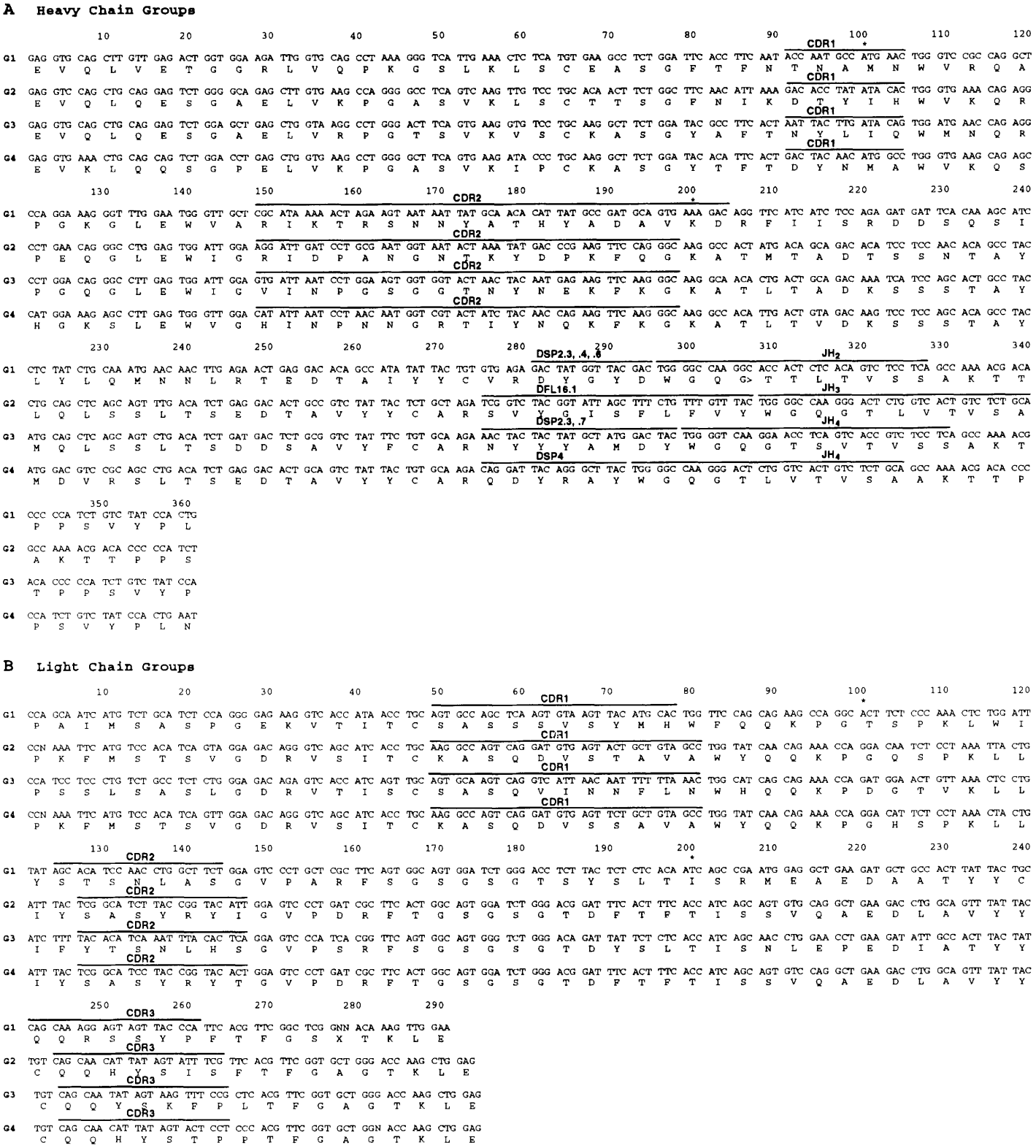

FIGURE 5 Nucleotide and amino acid sequences of the four heavy (*A*) and light (*B*) chain groups (G_{1-4}) cloned from spleen fragment culture D_1A_6. The CDR regions are noted in the heavy and light chain groups by overlining the appropriate sequences. The heavy chain sequences include the 5′-end variable region primers (Fig. 2); the light chain sequences include only the last three nucelotides of the 5′ end variable region primers (Fig. 2).

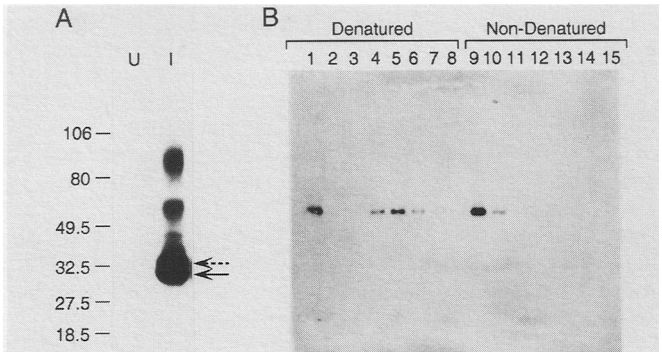

FIGURE 6 Western blot analyses of anti-PC sc antibody. (*A*) The anti-PC sc antibody present in whole cell (*E. coli*) lysate. Aliquots from uninduced (U) and induced (I) cultures are presented. Monomeric forms of the processed (broken arrow) (~32 kD) and unprocessed (solid arrow) (~33 kD) sc antibody are the prominent products detected by anti-c-Myc antibody, but dimeric (~60 kD) and trimeric (~90 kD) forms are observed. (*B*) The elution profile of Ni–NTA resin. The *E. coli* lysates were either denatured with 6 M urea prior to addition to the Ni–NTA resin or left non-denatured. The elutions were performed stepwise using 10 (lanes *4,12*), 40 (lanes *5,13*), 60 (lanes *6,14*), 80 (lanes *7,15*), and 100 (lane *8*) mM imidazole. The unbound (lanes *1,9*) and pre-elution buffer wash (lanes *2,3,10,11*) aliquots were also analyzed.

the stability of valuable hybridoma cell lines is not assured.

The combination of splenic fragment/3SR methodologies provide solutions to several of the problems associated with hybridoma cell line production, including the ability to preselect for (1) specific isotypes, (2) antibodies with binding affinities of desired levels, and (3) defined specificity as determined by competitive analog screening. All of these parameters can be selected during the screening of the splenic fragment culture media before confronting the cloning, characterization, and recombinant expression challenges.

The utility of the spleen fragment/3SR approach was dependent on identifying generic primer pairs for immunoglobulin genes that would hybridize specifically under the low stringency conditions of the 3SR isothermal reaction (i.e., 42°C). The successful use of such generic primers was demonstrated by the 3SR amplification of the mRNAs present in the total nucleic acid extract from anti-PC hybridoma cell line R2-09. The cloned products derived from PC-specific and generic primers pairs were identical for both heavy and light chain genes.

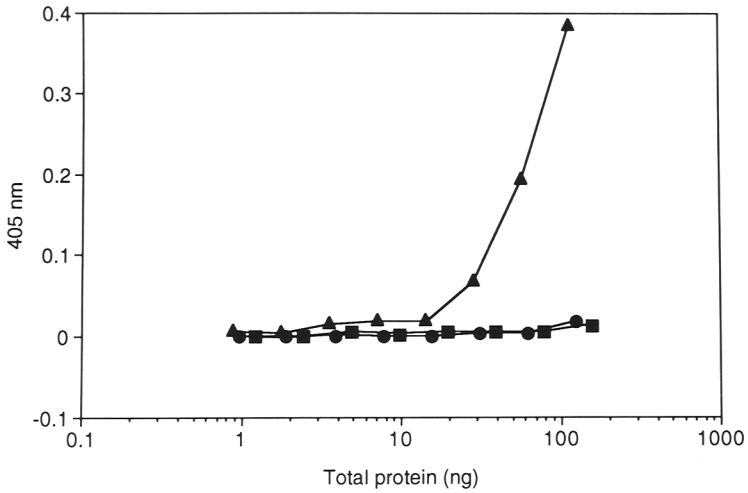

FIGURE 7 ELISA to detect affinity of Ni–NTA resin-purified anti-PC sc antibody. Aliquots (50 μl) from ~1–150 ng of total protein contained in the *E. coli* host strain MM 294 lysate containing pPHO-101 (●), the unbound fraction from the Ni–NTA resin (■) (Fig. 6B, lane *9*), and the fraction eluted from the Ni–NTA resin at 40 mM imidazole (▲) (Fig. 6B, lane *13*). The ELISA assay was performed as described in Materials and Methods.

These generic 3SR primer pairs were used to clone four groups each of light and heavy chain variable region genes from the cells in splenic fragment culture D_1A_6. The clones sequenced in each of the groups were productive, indicating that more than one memory B cell colonized the splenic fragment D_1A_6. To avoid obtaining multiple sequences, limiting dilution of transferred lymphocytes can be performed to obtain only one colonizing antigen-responsive B cell. However, even in this case with multiple heavy and light chain alleles represented, it is relatively straightforward to join together the four light and heavy chain clones into all 16 possible anti-E2 sc antibody combinations. One of the combinations will reflect the original V_H and V_L pairings. Even in this case, the need to create and screen larger phage display libraries is avoided.

A functional sc anti-PC antibody was expressed from 3SR-generated heavy and light chain clones derived from the hybridoma R2-09. Interestingly, the nature of the folded protein of the *E. coli*-expressed anti-PC sc antibody required that the antibody be denatured prior to binding to the Ni–NTA resin. This suggests that the metal binding sequence located at the carboxyl end of the sc antibody was not available for binding to the resin. This is not the case with the c-Myc peptide positioned at the amino terminus of the sc antibody. Detection of the anti-PC antibody bound to the immobilized PC in the ELISA was achieved with native anti-PC sc antibody. However, because the undenatured anti-PC sc antibody that was present in the unbound fraction from the Ni–NTA resin did not react in the ELISA assay, it may be concluded that the majority of the anti-PC sc antibody made in *E. coli* is folded such that it does not recognize the immobilized PC and/or the c-Myc tag is also unavailable for the second antibody. Such improperly folded sc antibody can be denatured and refolded into a functional form as demonstrated previously for an anti-PC sc antibody.[41]

Additionally, the spleen fragment/3SR methodologies have been useful for other types of studies. One such application involves the analysis of the origin of memory B cells. Mechanistically, both

somatic mutation and affinity-based antigen selection are poorly understood. We have recently employed the spleen fragment/3SR methodologies to study the origin of primary and secondary B cells in response to multiple exposures of stimulating antigen.[42] These studies present the first in vitro clonal analysis of hypermutation during memory B-cell generation. This was possible because unlike cell suspension cultures, fragment cultures retain sufficient splenic architecture (i.e., germinal centers) that allows for memory B-cell generation and somatic mutation. Using the spleen fragment/3SR methodologies, we confirmed that the progenitors of memory B cells differ from primary B cells, as originally proposed by Linton et al.[17]

The applications of the spleen fragment/3SR methodologies have been investigated only recently. Other applications that take advantage of the germinal center-like architecture of the spleen fragments and the elevated levels of amplification capable with the RNA-specific 3SR methodology are currently being explored.

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