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Convenient plasmid vectors for construction of chimeric mouse/human antibodies

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Chimeric antibodies composed of mouse-derived variable regions and human-derived constant regions have been developed for clinical use. However, construction of chimeric mouse/human genes in expression vectors is time-consuming work. In this study, we developed convenient vectors for construction of chimeric mouse/human antibodies. The protocols are as follows: In mouse hybridomas and B cells, most active V_H and V_κ genes can be identified as rearranged bands by Southern hybridization of *Eco*RI- and *Hin*dIII-digested DNAs with J_H and J_κ probes, respectively, and such fragments can be isolated in λ-*Eco*RI and λ-*Hin*dIII vectors, respectively. We constructed two plasmids: pSV2-HG1gpt contains human $C_{\gamma 1}$ and *Ecogpt* genes, and only one *Eco*RI site upstream of the $C_{\gamma 1}$ gene; pSV2-HC$_\kappa$neo contains human C_κ and *neo* genes, and only one *Hin*dIII site upstream of the C_κ gene. An isolated *Eco*RI fragment containing a $V_HD_HJ_H$ gene and a *Hin*dIII fragment containing a $V_\kappa J_\kappa$ gene are inserted into pSV2-HG1gpt and pSV2-HC$_\kappa$neo, respectively. Both resulting plasmid DNAs are co-transfected into SP2/0 cell, a non-Ig-secreting mouse myeloma. Transformants are selected by both mycophenolic acid and G418. With this procedure, it takes only 2 months to obtain chimeric antibodies.

DNA, recombinant; Chimeric antibody; Anti-phosphorylcholine

1. INTRODUCTION

Since hybridoma technology became available [1], many tumor-specific antibodies (Ab) have been developed for the diagnosis and treatment of cancer [2]. However, since many of these Ab are derived from mouse, they have clinical limitations. Ab consist of two structurally and functionally different portions. Variable (V) regions bind antigens (Ag) and constant (C) parts bear effector functions such as binding and activation of complements, stimulation of phagocytosis by macrophages, and triggering of granule release by mast cells. Utilizing the dual characters in the structure of Ab, two research groups [3,4] succeeded in constructing chimeric molecules consisting of mouse-derived V regions and human-derived C regions. These chimeric Ab retained specificity for the haptens phosphorylcholine (PC) [3] and trinitrophenyl [4]. Since these studies several groups have reported production of similar chimeric Ab showing antitumor activities [5–11].

Since three fragments, a mouse-derived active V gene-containing fragment, a human-derived C gene-containing fragment and an expression vector, must be properly connected to construct chimeric genes of heavy (H) and light (L) chains, this work is time-consuming. In fact, various restriction sites have been utilized in the published cases [3–11]. One of the authors (Y.K.) isolated

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Abbreviations: Ab, antibody; Ag, antigen; C, constant; D, diversity; ELISA, enzyme-linked immunosorbent assay; H, heavy; Ig, immunoglobulin; J, joining; L, light; PAB, *p*-azobenzenarsonate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphorylcholine; V, variable

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fifteen different active V_H genes in Dr Tonegawa's laboratory (MIT) [12,13], and noticed that the sizes of active V_H gene-containing *Eco*RI fragments ranged from 2 to 20 kb in most myeloma cells [14]. Here, we constructed convenient vectors for construction of chimeric mouse/human Ab. Our protocols avoid a time-consuming step in obtaining chimeric Ab.

2. MATERIALS AND METHODS

2.1. *Vectors, clones, probes, linkers and cells*

λ_{WES} [15] and λ_{788} [16] were used as *Eco*RI and *Hin*dIII phage vectors, respectively. pSV2gpt [17] and pSV2neo [18] were provided by P. Berg (Stanford). Mouse J_κ gene-containing fragment (J_κ probe) was isolated from clone Ig146 [19]. Mouse J_H probe was isolated from MEP203 [20]. Mouse $C_{\gamma 1}$-containing clone MEP10 was donated by S. Tonegawa (MIT) [21]. Human *Hae*III-*Alu*I genomic library was donated by T. Maniatis (Harvard) [22]. Three oligonucleotides: GGAATTCC, CAAGCTTG and CGGATCCG were used as *Eco*RI, *Hin*dIII and *Bam*HI linkers, respectively. A myeloma TEPC15 was obtained from M. Potter (NIH). SP2/0 was obtained from ATCC.

2.2. *Cloning of human C_κ and $C_{\gamma 1}$ genes*

Clone HuC_κ2 containing human J_κ genes, enhancer region and C_κ gene was obtained by screening the human genomic library [22] with mouse J_κ probe. Clone HG163 containing human $C_{\gamma 1}$ gene was isolated as follows: human placenta DNA was digested with *Hin*dIII and fractionated by agarose gel electrophoresis. A library was constructed from 8.4–8.8 kb long DNA in λ_{788} vector. Clone HG163 was obtained by screening with mouse $C_{\gamma 1}$ probe. Since the sizes of four different C_γ gene-containing fragments are similar to each other [23], we partially sequenced HG163 to ascertain $C_{\gamma 1}$ gene in the clone [24].

2.3. *Cloning of size-fractionated DNA*

Active V gene-containing fragments were identified as rearranged bands in Southern hybridization of *Hin*dIII- and *Eco*RI-digested DNAs with J_κ and J_H probes, respectively. DNAs were eluted from the relevant regions separated on agarose gel, ligated with λ_{788} and λ_{WES} arms, and packaged into λ phage. Plaque hybridization was carried out according to Benton and Davis [25].

2.4. *Introduction of DNA into myeloma cells by electroporation*

SP2/0 cells, a non-Ig-secreting mouse myeloma, were used as host for transfection [26]. Electroporation was carried out essentially as in [27]. In brief, approx. 2×10^7 cells were subjected to an electric field of 1.5 kV/cm twice in 0.5 ml phosphate-buffered saline (PBS) containing 50 μg DNAs of pSV2HG1V_{PC} and pSV2HCκV_{PC} on ice. Conditions were set to cause 50% cell death after electroporation. After incubation on ice for 5 min, the cells were transferred to RPMI 1640 medium supplemented with 10% fetal calf serum. After incubation at 37°C for 72 h, the cells were transferred into selection medium (RPMI 1640 medium containing 10% fetal calf serum, 5 μg/ml mycophenolic acid and 250 μg/ml xanthine). Transformants were further analyzed for resistance to G418 (400 μg/ml). The cells showing resistance to both mycophenolic acid and G418 were grown.

2.5. *Enzyme-linked immunosorbent assay (ELISA)*

Production of chimeric Ab by the transformed cells was assayed by ELISA. Microtiter plates (96 wells) were coated with 100 μl anti-human immunoglobulin (Ig) per well by incubation at room temperature for 1 h, and plates were washed three times with PBS containing 0.5% BSA (buffer). 50 μl of each supernatant were added to each well, and incubation carried out at 40°C for 1 h. Plates were washed three times with buffer, and 100 μl peroxidase-conjugated goat anti-human IgG (Fc fragment) Ab or goat anti-human κ-chain Ab was added and incubated at 40°C for 1 h. Plates were washed four times with buffer and 100 μl of a mixture containing 0.04% *o*-phenylenediamine, 0.033% H_2O_2, 25 mM citric acid, and 50 mM Na_2HPO_4 (pH 5.0) were added to each well. The reaction was stopped with 2.5 M H_2SO_4, and the absorbance (A) at 492 nm was measured. Purified human IgG was used as a control. SP2-PC Chimera-1 cells were injected into Balb/c mouse bellies, the mice having received 500 μl/mouse of pristane (2,6,10,14-tetramethylpentadecane; Wako, Osaka) 2 weeks previously. Ascites were used in the following experiments.

2.6. *Western blotting*

Protein samples were suspended in 32 mM Tris-HCl, pH 6.8, 1% SDS, 5% glycerol, 0.005% bromophenol blue, and 2.5% β-mercaptoethanol. After incubation at 100°C for 2 min, the proteins were separated by SDS-PAGE on an 8–18% linear gradient containing 0.1% SDS [28], and electrophoretically transferred to nitrocellulose membrane as described by Towbin et al. [29]. The membrane was blocked with 3% gelatin in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) at room temperature for 1 h, and incubated for 3 h with a mixture of rabbit anti-human IgG and κ-chain Ab diluted 300 times in TBS containing 1% gelatin. The membrane was washed three times with TBS containing 0.05% Tween20 for 10 min, and was incubated for 1 h with peroxidase-conjugated goat anti-rabbit second Ab diluted 3000 times in TBS containing 1% gelatin. The membrane was washed and reacted with 4-chloro-1-naphthol-H_2O_2 color-producing solution and rinsed with water. Rabbit anti-human IgG Ab, rabbit anti-human κ-chain Ab and peroxidase-conjugated goat anti-rabbit Ab were purchased from Cappel. 4-Chloro-1-naphthol was purchased from Wako.

2.7. *Antibody-binding assay*

PC-binding activity of the chimeric Ab was assayed as follows: Microtiter plates (96 wells) were coated with 25 μl PC-OVA (100 μg/ml) per well by overnight incubation at 4°C, 150 μl of buffer was added and incubation was performed at room temperature for 0.5 h. Plates were washed three times with buffer and 25 μl ascites was added. The plates were then incubated at room temperature for 1 h, washed three times with buffer, and 40 μl alkaline phosphatase-conjugated anti-human Ig Ab was added. Subsequently, they were incubated at room temperature for 1 h, washed four times with buffer, and 100 μl of a mixture (pH 9.8) containing 0.1% (w/v) *p*-nitrophenol phosphate, 9.7% diethanolamine, 0.01% (w/v) $MgCl_2 \cdot 6H_2O$,

and 0.02% (w/v) NaN_3 was added per well. The reaction was stopped with 3 M NaOH and A at 405 nm was measured.

3. RESULTS AND DISCUSSION

3.1. *Construction of plasmids: pSV2-HG1gpt and pSV2-HC$_\kappa$neo*

Fig.1 shows the plasmid construction scheme: pSV2-HG1gpt and pSV2-HC$_\kappa$neo. The 8.5 kb *Hin*dIII fragment containing human $C_{\gamma 1}$ gene was isolated from clone HG163, treated with Klenow enzyme and ligated with an *Eco*RI linker. The fragment was then digested with *Eco*RI and *Bam*HI, and inserted into the *Eco*RI-*Bam*HI sites of pSV2gpt [17], resulting in pSV2-HG1gpt. It contains human $C_{\gamma 1}$ and *Ecogpt* genes, and only one *Eco*RI site upstream of the $C_{\gamma 1}$ gene. It does not contain an enhancer sequence.

The *Hin*dIII site located at the junction between SV40 promoter and *neo* gene in the original pSV2neo plasmid [18] was destroyed as follows: pSV2neo DNA was linearized by *Hin*dIII digestion, and both ends were converted into blunt ends with Klenow enzyme, and re-ligated. The resulting plasmid kept the phenotype of G418 resistance (not shown). The *Eco*RI site of the plasmid was converted into a *Hin*dIII site using a *Hin*dIII linker. The 2.1 kb *Pvu*II fragment (the 3′ portion of this fragment was derived from Charon 4A vector) containing a human enhancer region and C_κ gene was isolated from clone HuC_κ2, and ligated with a mixture of *Hin*dIII and *Bam*HI linkers. After digestion of this fragment with both *Hin*dIII and *Bam*HI, it was inserted into the *Hin*dIII-*Bam*HI sites of the above-modified pSV2neo plasmid. We selected a clone which contains a *Hin*dIII site upstream of the C_κ gene and a *Bam*HI site downstream of the C_κ gene, and named it pSV2-HC$_\kappa$neo.

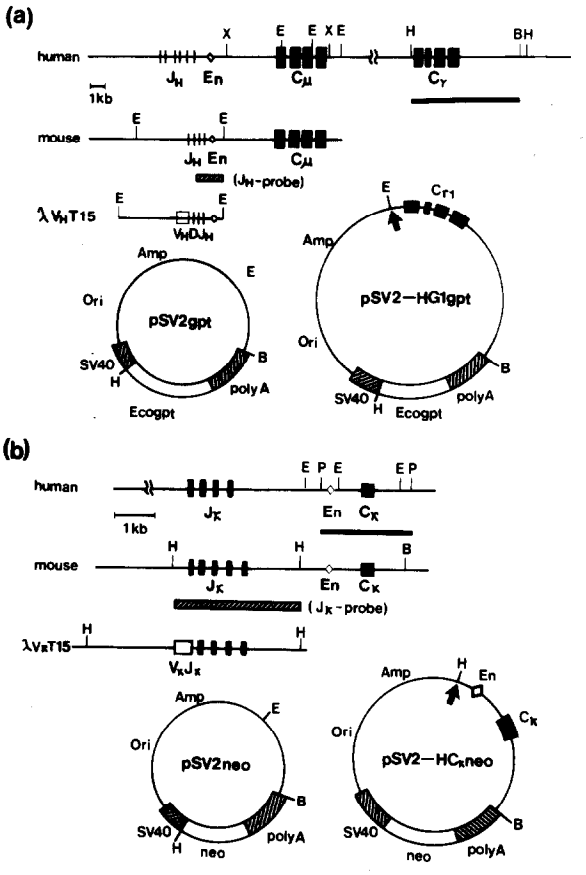

Fig.1. Construction of plasmids: pSV2-HG1gpt (a) and pSV2-HC$_\kappa$neo (b). Restriction maps of the immunoglobulin J_H, C_μ and C_γ gene loci as well as J_κ and C_κ gene loci in both human and mouse are shown. λVHT15 and λV$_\kappa$T15 are active V_H and V_κ genes from myeloma TEPC15. DNA regions involved in constructed plasmids are indicated by thick bars. Thick arrows indicate the position where active V genes should be inserted. J_H and J_κ probes are indicated by hatched bars. En, enhancer; Ori, pBR322 ori; Amp, β-lactamase; SV40, SV40 promoter; polyA, poly(A) attachment signal. E, *Eco*RI; P, *Pvu*II; B, *Bam*HI; H, *HindIII*.

3.2. *Isolation of active V genes*

In germline genome, V regions of Ig are encoded by two split genes: V and J (joining) genes for L chains, and three split genes: V, D (diversity) and J genes for H chains [30]. In mouse, there exist 200–300 V_κ and four J_κ genes; two V_λ and three J_λ genes; about 100 V_H, 12 D_H and four J_H genes (review [31]). More than 95% of Ig are κ-type and the rest λ-type. Since these genes undergo DNA rearrangements such as V–(D)–J during B-cell ontogeny, active V genes can be identified on rearranged fragments by Southern hybridization with J gene-containing DNAs as probes. *Eco*RI and *Hin*dIII digestions are practical for H- and κ-chains, respectively, since the sizes of rearranged fragments range from 2 to 20 kb in most cases [14]. Maintaining a high level expression of Ig genes in B cells requires characteristic octamer sequences located upstream of V genes [32] and enhancer elements located in J_H-μ and J_κ-C_κ introns [33,34]. Although pSV2-HG1gpt does not contain the enhancer sequence, a J_H gene-

containing *Eco*RI fragment includes the enhancer sequence. On the other hand, a J_κ gene-containing *Hin*dIII fragment does not contain the enhancer sequence, but is included in pSV2-HC_κneo. The enhancer sequence of human origin is effective in mouse cells. Based on the above principles, we isolated active V_H and V_κ genes from anti-PC myeloma, TEPC15. As shown in fig.2, J_H probe identified one rearranged band at 7 kb in *Eco*RI-digested TEPC15 DNA. We cloned this band (named $\lambda V_H T15$) as an active V_H gene-containing fragment [35]. J_κ probe identified one rearranged band at 5 kb in addition to a germline band at 3 kb in *Hin*dIII-digested TEPC15 DNA. We cloned the 5 kb band (named $\lambda V_\kappa T15$). The restriction map of this clone is the same as for the published V_κ clone [36]. A general protocol for identification of active V gene-containing fragments is described later.

3.3. *Production of anti-PC chimeric Ab*

The 7 kb *Eco*RI fragment was isolated from $\lambda V_H T15$ clone and inserted into the *Eco*RI site of pSV2-HG1gpt. We selected a clone in which the polarities of the V_H and C_H genes are the same, and designated it pSV2HG1V_{PC}. The 5 kb *Hin*dIII fragment was isolated from $\lambda V_\kappa T15$ clone, and inserted into the *Hin*dIII site of pSV2-$C_\kappa$neo. A proper clone containing V_κ and C_κ of the same polarity was selected, and named pSV2HCκV_{PC}. Both plasmid DNAs were co-transfected into SP2/0 cells by electroporation as described in section 2. First, cells were selected in *Ecogpt* selection medium. The frequency of transformants was approx. 10^{-5}. One-third of the transformants also showed G418 resistance. Transformants resistant to both mycophenolic acid and G418 were grown, and amounts of Ab secreted into the culture supernatants were measured by ELISA. One of the stable transformants (SP2-PC Chimera-1) producing a high level of Ab (5 μg/ml) was cloned by limiting dilution. SP2-PC Chimera-1 cells were injected into Balb/c mouse belly. After growth of the tumor, the ascites was analyzed. Fig.3 shows the results of Western blotting. Chimeric antibodies were reacted with a mixture of anti-human Ig and κ-chain Ab (lane 1). They discriminate between human and mouse Ab (lanes 2,3). Moreover, the chimeric Ab bound to PC-OVA, but not PAB-OVA as shown in fig.4. The chimeric Ab clearly retained the original antigen specificity.

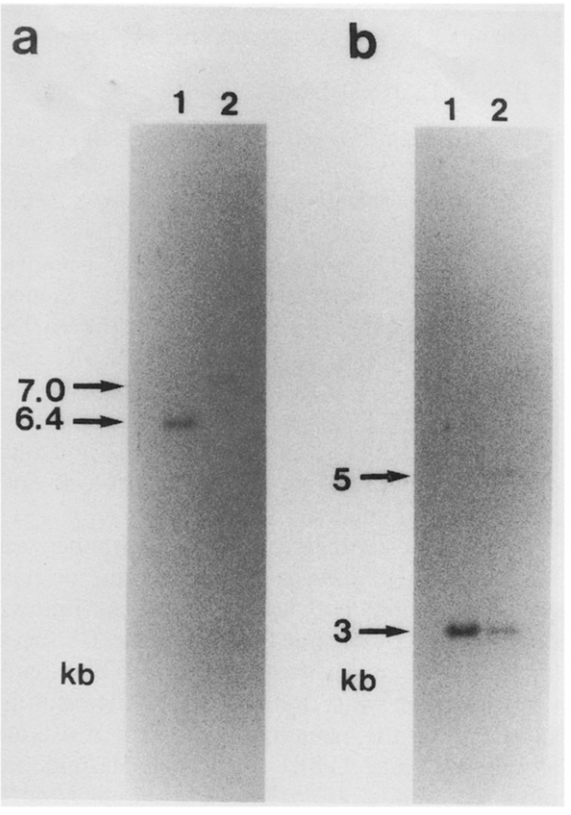

Fig.2. Southern hybridization of TEPC15 DNA. (a) Balb/c mouse kidney DNA (lane 1) and TEPC15 DNA (lane 2) were digested with *Eco*RI. Probe was J_H probe. (b) Balb/c mouse kidney DNA (lane 1) and TEPC15 DNA (lane 2) were digested with *Hin*dIII. Probe was J_κ probe.

3.4. *Standard protocols for chimeric Ab production*

In the case of hybridomas, we adopted the following criteria for identification of active V gene-containing fragments. First, active V genes should be on rearranged fragments which are identified only in hybridoma DNA, not in the fusion partner's DNA. If there are two candidate bands, we clone both of them. In the case of H chains, since many abortive rearranged bands contain the D_H-J_H structure without V_H genes [14], they can be differentiated from active V gene-containing fragments by Southern hybridization with D_H probes. Although 12 D_H genes have been identified in mouse, 11 of them can be identified with one D_H probe, the D_{SP2} probe, and the remaining one with the D_{Q52} probe [37]. Active $V_HD_HJ_H$ gene does not

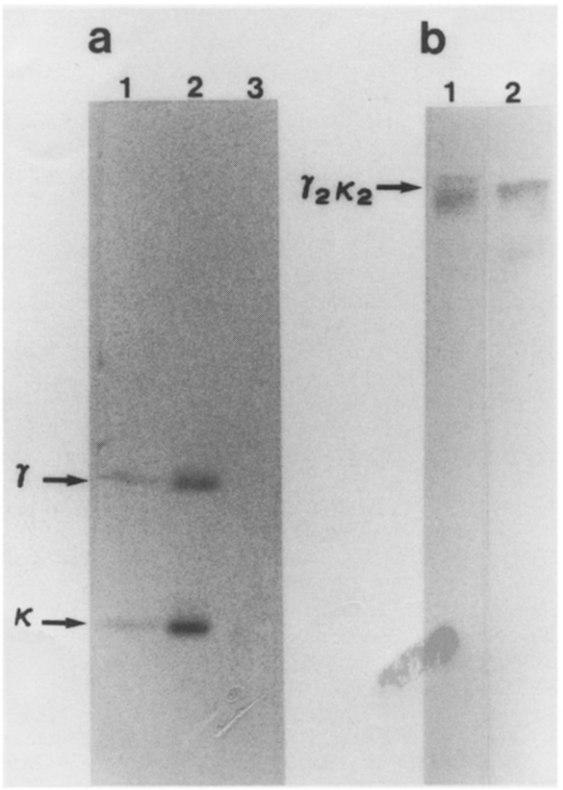

Fig.3. Western blotting of chimeric, human and mouse Ab. (a) 2 μl of 10 times diluted ascites from SP2-PC chimera-1 cell-injected mouse (lane 1), 5 μl of 1000 times diluted normal human serum (lane 2) and 1 μl of 10 times diluted normal mouse serum (lane 3) were applied to SDS-PAGE. (b) 5 μl of 100 times diluted ascites (lane 1) and 1 μl of 1000 times diluted normal human serum (lane 2) were applied without mercaptoethanol treatment.

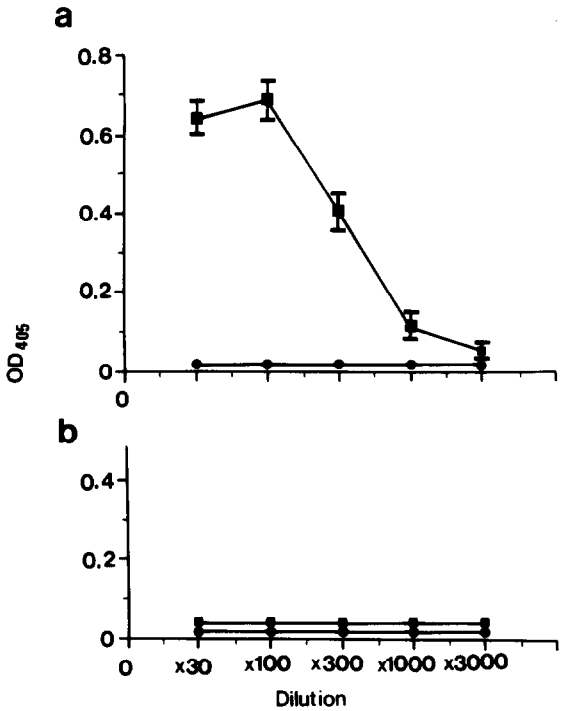

Fig.4. Specific PC-binding activity of chimeric antibody. Anti-PC chimeric antibody (■—■) in ascites in various dilutions reacted with PC-OVA (a) or PAB (*p*-azobenzenarsonate)-OVA (b) coated plates as described in section 2. Mouse/human chimeric antibody constructed with anti-GM3 mouse IgV and human Igh·$C_{\gamma 1}$ under the same conditions as described here was used as a control chimeric antibody (●—●).

give a positive signal to the D_H probes, since a D_H-coding region is too short to be hybridized with the D_H probes. Second, active V genes should be expressed in the hybridomas. This can be examined by Northern hybridization of mRNA from the hybridoma with the isolated fragments as probes. In most cases, these two criteria are sufficient for identification of active V gene-containing fragments. The isolated *Eco*RI fragment containing a $V_HD_HJ_H$ gene and the *Hin*dIII fragment containing a V_xJ_x gene are treated in the same way as TEPC15 DNA described in this paper. We made chimeric antibodies according to these protocols by using monoclonal antibody M2590, which shows preferential reactivity with various types of melanomas [38] (details to be published elsewhere). If the Ig is λ-type, we cannot use these protocols. Also, if an *Eco*RI site exists in the promoter and coding regions of a V_H gene, or if a *Hin*dIII site exists in those of a V_x gene, we need other devices. Using these protocols, it takes 2 months to obtain chimeric Ab.

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