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 \( V_n \) and \( V_c \) genes can be identified as rearranged bands by Southern hybridization of EcoRI- and HindIII-digested DNAs with \( J_n \) and \( J_c \) probes, respectively, and such fragments can be isolated in \( \lambda \)-EcoRI and \( \lambda \)-HindIII vectors, respectively. We constructed two plasmids: pSV2-HG\( \text{lg} \)gpt contains human \( C_c \) and \( \text{Ecogpt} \) genes, and only one EcoRI site upstream of the \( C_c \) gene; pSV2-HC\( \text{neo} \) contains human \( C_c \) and neo genes, and only one HindIII site upstream of the \( C_c \) gene. An isolated EcoRI fragment containing a \( V_n D_n J_n \) gene and a HindIII fragment containing a \( V_c J_c \) gene are inserted into pSV2-HG\( \text{lg} \)gpt and pSV2-HC\( \text{neo} \), respectively. Both resulting plasmid DNAs are cotransfected 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.

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 DNA, recombinant; Chimeric antibody; Anti-phosphorylcholine

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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-azobenzenarsanate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphorylcholine; V, variable
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 EcoRI 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

λphes[15] and λphes[16] were used as EcoRI and HindIII phage vectors, respectively. pSV2gpt [17] and pSV2neo [18] were provided by P. Berg (Stanford). Mouse J, gene-containing fragment (J, probe), was isolated from clone Isi146 [19]. Mouse J_H probe was isolated from MEP203 [20]. Mouse C_r-containing clone MEP10 was donated by S. Tonegawa (MIT) [21]. Human HaelIII-Ahl genomic library was donated by T. Maniatis (Harvard) [22]. Three oligonucleotides: GGAATTCC, CAAGCG, and CGGATCCG were used as EcoRI, HindIII and BamHI linkers, respectively. A myeloma TEPC15 was obtained from M. Potter (NIH). SP2/0 was obtained from ATCC.

2.2. Cloning of human C_r and C_s genes

Clone HuC_r,2 containing human J, genes, enhancer region and C, gene was obtained by screening the human genomic library [22] with mouse J, probe. Clone HG163 containing human C, gene was isolated as follows: human placenta DNA was digested with HindIII and fractionated by agarose gel electrophoresis. A library was constructed from 8.4-8.8 kb long DNA in λgt11 vector. Clone HG163 was obtained by screening with mouse C_r probe. Since the sizes of four different C, gene-containing fragments are similar to each other [23], we partially sequenced HG163 to ascertain C_r 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 HindIII and EcoRI-digested DNAs with J, and J_H probes, respectively. DNAs were eluted from the relevant regions separated on agarose gel, ligated with λgt11 and λphes 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 x 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 pSV2HgilVpc and pSV2HCxVpc 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. Microwell 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 x-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% α-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. Asciates were used in the following experiments.

2.6. Western blotting

Protein samples were suspended in 52 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 x-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 x-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

PM-binding activity of the chimeric Ab was assayed as follows: Microwell 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% (v/v) p-nitrophenyl phosphate, 9.7% diethanolamine, 0.01% (w/v) MgCl_2·6H_2O,
and 0.02% (w/v) NaN₃ 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-HGlgpt and pSV2-HC,neo

Fig.1 shows the plasmid construction scheme: pSV2-HGlgpt and pSV2-HC,neo. The 8.5 kb HindIII fragment containing human Cγ1 gene was isolated from clone HG163, treated with Klenow enzyme and ligated with an EcoRI linker. The fragment was then digested with EcoRI and BamHI, and inserted into the EcoRI-BamHI sites of pSV2gpt [17], resulting in pSV2-HGlgpt. It contains human Cγ1 and EcoGpt genes, and only one EcoRI site upstream of the Cγ1 gene. It does not contain an enhancer sequence.

The HindIII 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 HindIII 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 EcoRI site of the plasmid was converted into a HindIII linker. The 2.1 kb PvuII fragment (the 3' portion of this fragment was derived from Charon 4A vector) containing a human enhancer region and Cγ gene was isolated from clone HuCγ2, and ligated with a mixture of HindIII and BamHI linkers. After digestion of this fragment with both HindIII and BamHI, it was inserted into the HindIII-BamHI sites of the above-modified pSV2neo plasmid. We selected a clone which contains a HindIII site upstream of the Cγ gene and a BamHI site downstream of the Cγ gene, and named it pSV2-HC,neo.

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 Vₓ and four Jₓ genes; two Vₓ and three Jₓ genes; about 100 Vₓ, 12 Dₓ and four Jₓ genes (review [31]). More than 95% of Ig are x-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. EcoRI and HindIII digestions are practical for H- and x-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 Jₓ-µ and Jₓ-Cₓ introns [33,34]. Although pSV2-HGlgpt does not contain the enhancer sequence, a Jₓ gene-
containing EcoRI fragment includes the enhancer sequence. On the other hand, a J\, gene-containing \textit{HindIII} fragment does not contain the enhancer sequence, but is included in pSV2-HC\textsubscript{neo}. The enhancer sequence of human origin is effective in mouse cells. Based on the above principles, we isolated active \(V_H\) and \(V_\lambda\) genes from anti-PC myeloma, TEPC15. As shown in fig.2, \(J_H\) probe identified one rearranged band at 7 kb in EcoRI-digested TEPC15 DNA. We cloned this band (named \(\lambda V_H T15\)) as an active \(V_H\) gene-containing fragment [35]. \(J_\lambda\) probe identified one rearranged band at 5 kb in addition to a germline band at 3 kb in \textit{HindIII}-digested TEPC15 DNA. We cloned the 5 kb band (named \(\lambda V_\lambda T15\)). The restriction map of this clone is the same as for the published \(V_\nu\) 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 EcoRI fragment was isolated from \(\lambda V_H T15\) clone and inserted into the EcoRI site of pSV2-HG\textsubscript{1gpt}. We selected a clone in which the polarities of the \(V_H\) and \(C_H\) genes are the same, and designated it pSV2HG\textsubscript{1gpt}VC. The 5 kb \textit{HindIII} fragment was isolated from \(\lambda V_\nu T15\) clone, and inserted into the \textit{HindIII} site of pSV2C\textsubscript{neo}. A proper clone containing \(V_\lambda\) and \(C_\lambda\) of the same polarity was selected, and named pSV2HCV\textsubscript{PC}. Both plasmid DNAs were co-transfected into SP2/0 cells by electroporation as described in section 2. First, cells were selected in \textit{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 \(\mu g/ml\)) 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 \(x\)-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.

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 \(D_{O52}\) probe [37]. Active \(V_H D_H J_H\) gene does not
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 EcoRI fragment containing a $V_{H}D_{H}J_{H}$ gene and the HindIII fragment containing a $V_{\lambda}J_{\lambda}$ 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 $\lambda$-type, we cannot use these protocols. Also, if an EcoRI site exists in the promoter and coding regions of a $V_H$ gene, or if a HindIII site exists in those of a $V_\lambda$ gene, we need other devices. Using these protocols, it takes 2 months to obtain chimeric Ab.

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