의학 박사학위 논문

A Visible Phagemid System for the Estimation of Cre-mediated Recombination Efficiency

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A visible phagemid system for the estimation of Cre-mediated recombination efficiency

by

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- ABSTRACT -

A visible phagemid system for the estimation of Cre-mediated recombination efficiency

Purposes: The Cre-lox system is generally employed to increase the size of phage antibody libraries. However, estimation of library sizes after Cre-mediated recombination is difficult since time-consuming nucleotide sequence analyses are required. We aimed to develop a visible phagemid vector system that facilitates the estimation of recombination efficiency between V_H and V_L genes.

Materials & Methods: Two phagemids (pRGA and pRGB) were constructed. To induce recombination BS1365 bacterial cells expressing Cre recombinase were co-infected using the two phagemids. Cre-mediated recombination between two phagemids was verified by PCR, analysis by restriction enzymes, and DNA sequencing. To confirm the function of scFv proteins expressed from phagemids, total bacterial cell fractions were used for Western blotting and ELISA. Recombination efficiency was calculated simply by counting the number of blue colonies on X-gal-containing medium.

Results: It was found that intermolecular recombination between V_H and V_L genes and acquirement of β-galactosidase (β-gal) activity occurred simultaneously in BS1365 bacterial cells. Molecular analyses of plasmids isolated from blue colonies verified a novel V_H/V_L combination. Recombination efficiency was calculated from the frequency of a novel recombinant phagemid, and expressed as a percentage difference between the number of blue and total colonies. The calculated recombination efficiency was revealed to be 24.2%. ScFv-g3p proteins expressed

from phagemids were detected as a band of 97 kDa and showed specific binding activity to corresponding antigens.

Conclusions: We developed a visible phagemid system to measure the recombination efficiency of V_H/V_L genes without sequence analysis. Our results suggest that this newly developed visible phagemid system may be reliably used for the measurement of recombination efficiency, which would enable precise evaluation of the diversity of phage antibody libraries.

Keywords: Antibody library size, Visible phagemid system, Cre-mediated recombination, Recombinase efficiency, X-gal staining, β-galactosidase, scFv

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I. INTRODUCTION

Phage display is a technique used to establish antibody libraries *in vitro* without immunization and select antigen-specific antibodies, usually single chain Fv or Fab.[1, 2] It is difficult to obtain an antibody library size over $1x10^{10}$ using traditional phage display whereby antibody diversity is created by random combinatorial linkage of V_H and V_L genes and cloning, due to the limited efficiency of bacterial transformation (10^9 cfu/ml). Evidence showing that a larger initial phage antibody library results in significantly higher possibility of selection for high-affinity antigen-specific antibodies has encouraged further attempts to increase initial antibody library sizes.[8, 17, 21]

Site-specific recombination is an alternative to generating antibody libraries, circumventing the limitations of transformation in traditional phage display. To date, a single lambda recombinase-based technique[4] and several Cre recombinase-based strategies have been employed to generate combinatorial phage antibody libraries.[3, 5, 20, 22, 23] Cre recombinase derived from bacteriophage P1 catalyzes recombination between two 34 bp *loxP* recognition sites. The *loxP* site consists of two 13 bp inverted repeats separated by an 8 bp spacer region involved in cleavage and re-ligation of each strand of DNA.[6, 7] Depending on the orientation and location of the *loxP* sites, Cre-mediated recombination leads to different results. Intramolecular recombination between two direct repeats of *loxP* sites results in excision of the

DNA sequence flanked by the sites, and that between two *loxP* sites of inverted orientation leads to inversion. Intermolecular recombination entails the reciprocal exchange of DNA sequences flanked by mutant *lox* sites (Sauer, 1998).

In approaches using the *Cre/lox* system as a tool to generate diverse phage antibody libraries, the assembly of each V_H and V_L library created by molecular cloning has been exploited in two different ways, specifically, two-vector and one-vector systems.[16] In the two-vector system, V_H and V_L genes were separately cloned into two vectors, followed by induction of combinatorial assembly of both genes by Cre. However, this system was limited by remaining non-functional byproducts that did not participate in recombination, and irreversibility of the procedure.[5] In the more recent one-vector system, both V_H and V_L genes were cloned in a single phagemid, resulting in a library of primary functional scFv. This was followed by *Cre/lox* recombination in Cre-expressing bacteria, and intermolecular shuffling of V_L or V_H genes between phagemids to obtain a novel V_L-V_H combination. The one-vector system has advantages over the two-vector technique, due to the absence of non-functional byproducts and reversibility of the recombination procedure.[15] Despite the advantages of the one-vector system, it is difficult to estimate the final library sizes following recombination using this technique. Library size may be roughly estimated only by calculating the rate of appearance of novel recombinant phagemids through intensive sequence analyses of several hundred regions of the V gene.[15] Sequence analysis of a large number of DNA samples is both time- and labor- consuming.

Here, a new phagemid technique was described. That was based on the one-

vector system to measure the recombination efficiency of V_H/V_L genes without sequence analysis. Our vector system was designed so that intermolecular recombination between V_H and V_L genes and acquirement of β-galactosidase (β-gal) activity occurred simultaneously. We detected recombinants by X-gal staining, and measured recombination efficiency in Cre-expressing bacteria. Recombination efficiency was calculated by simply counting the number of colored bacterial colonies. By using this method to measure recombination efficiency, increase in final antibody library size after Cre-mediated recombination would be readily evaluated.

II. MATERIALS AND METHODS

A. Construction of phagemid vectors

Phagemids, pRGA and pRGB (Fig.1), are constructed by modifying pCANTAB-5E (Amersham Biosciences, Piscataway, NJ). In both phagemids, the linker fragment $(Gly_4Ser_1)_3$ within pCANTAB-5E was replaced with the loxP511 peptide-$(Gly_4Ser_1)_1$ sequence. Following PCR using overlapping and complementary oligonucleotides (5' primer, 5'- ATA ACT TCG TAT AAT GTA TAC TAT ACG AAG TTA TCT GGA GGT GGC GGT TCT AGA GAC CTT GTG ATG TCA CAG TCT CC -3'; 3' primer, 5'- ACC TCC AGA TAA CTT CGT ATA GTA TAC ATT ATA CGA AGT TAT GCT AGC AGA GGA GAC GGT GAC TGA GGT TCC- 3') a scFv containing the loxP511 peptide-$(Gly_4Ser_1)_1$ sequence was cloned into the pIg20 vector as a *Xma*I/*Nco*I fragment. The scFv was subcloned into pCANTAB-5E using *Sfi*I/*Not*I restriction sites. Next, two synthetic sequences containing $(His)_6$ tag-thrombin cleavage site and E tag are added to the carboxy-terminus of the scFv and g3p genes respectively, resulting in pRG. In pRG, contiguous *Bgl*II/*Eco*RI and *Spe*I/*Mlu*I sites are cloned as a *Nde*I/*Dra*III fragment downstream of the E-tag by overlapping PCR, leading to the generation of pRG1 and pRG2, respectively. To construct pRGA from pRG1, an *Eco*RI/*Dra*III overlap PCR fragment containing the loxP wt sequence was cloned into pRG1 digested with the corresponding restriction

enzymes. Next, the structural β-gal gene lacking N-terminal 5 amino acids (derived from pGEM-3Zf (+) (Promega Co, Madison, WI) was introduced by cloning between *Bgl*II and *Eco*RI sites. To construct pRGB, an *Nde*I/*Spe*I overlap PCR fragment containing the *loxPwt* sequence was cloned into the corresponding restriction sites of pRG2, and a *tac* promoter region plus 15 bases encoding the N-terminal 5 amino acids of β-gal was inserted between *Spe*I and *Mlu*I sites of pRG2. Finally, the 3D8 (D) V_H - S2C11 (S) V_L gene was subcloned into scFv to generate pRGA with D-S scFv, and the S2C11 V_H - 3D8 V_L gene was inserted to obtain pRGB with S-D scFv. Variable region genes originated from mouse monoclonal 3D8 antibody-specific DNA and S2C11 antibody specific for the surface antigen of hepatitis B virus (HBs).

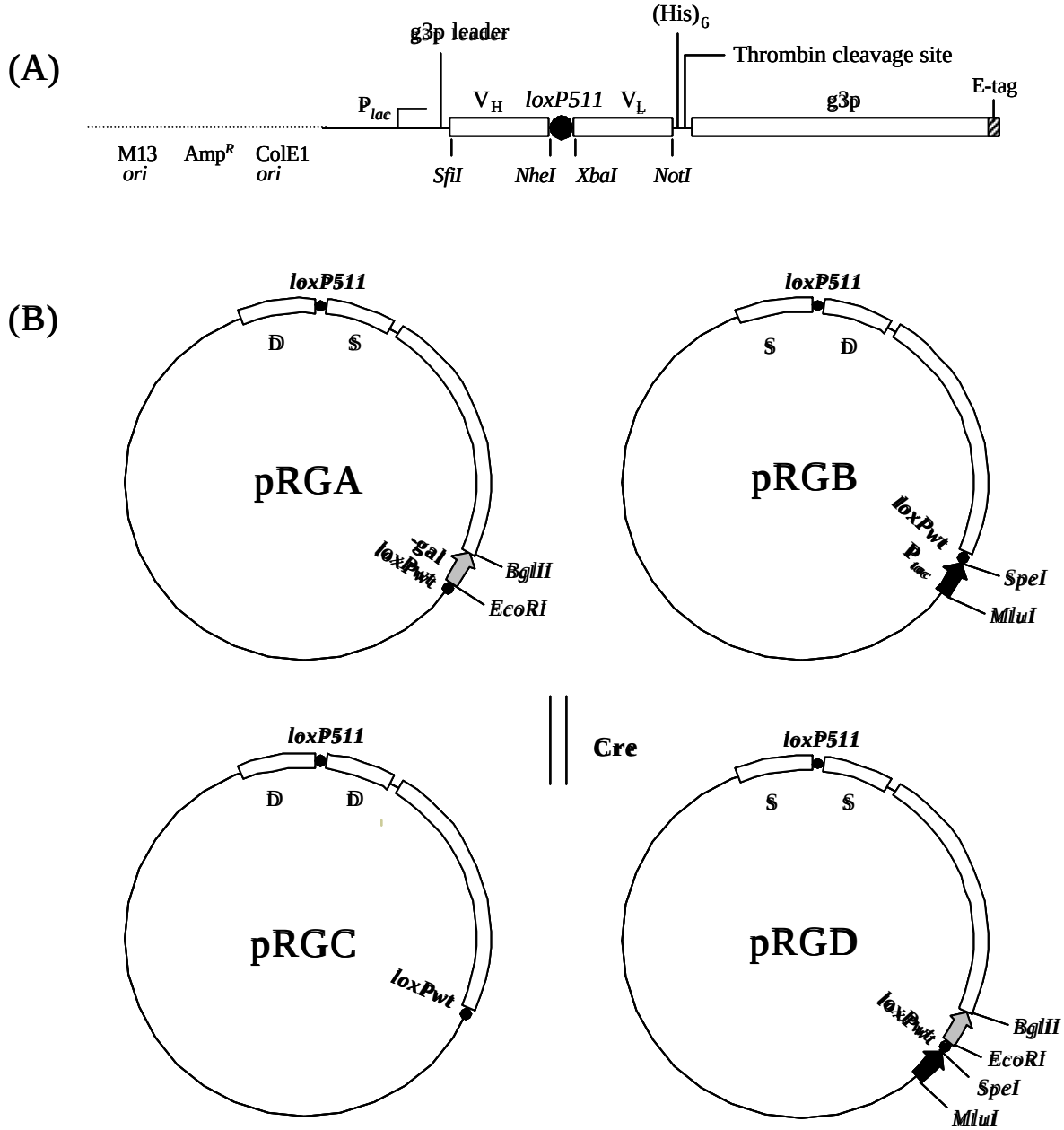

Fig. 1. Phagemid structure and Cre-mediated recombination between phagemids. (A) Shared structure with phagemids. **(B)** Scheme for reciprocal exchange of the V_L- g3p region by Cre-mediated recombination. Recombination of

parental pRGA and pRGB via compatible *loxP* sites leads to the generation of pRGC and pRGD. pRGD permits *lacZ'* fragment translation from the opposite-oriented *tac* promoter. D and S refer to 3D8 and S2C11, respectively.

B. Cre-mediated recombination

TG1 cells harboring pRGA or pRGB are separately superinfected by M13KO7 helper phage (Amersham Biosciences, Piscataway, NJ) to package phagemid DNA. Titers of the rescued phage containing pRGA or pRGB are measured as Amp^R colony forming units (CFU). To induce *in vivo* recombination, *E. coli* BS1365 cells (a gift from Dr. Brian Sauer) constitutively expressing Cre recombinase are used. BS1365 cells are grown at 37^0C in 10 ml LB containing 100 μg/ml kanamycin and 1% glucose to an OD_{600} of 0.5. Phages containing pRGA and pRGB are added to midlog BS1365 cells at a multiplicity of infection (MOI) of 100. Cells are left for 1 h at 37°C without shaking for infection, and ampicillin was added (100 μg/ml). Recombination was allowed for 2 h at 37 °C with shaking. Next, plasmid DNA was isolated from BS1365 using spin columns (Qiagen Inc, Stanford Valencia, CA), and used to transform *E. coli* JM109 cells.

C. Transformation and X-gal staining

JM109 bacterial cells which utilize α-complementation of *lacZ'* are transformed with DNA isolated from BS1365. Transformed bacterial cells are spread onto nitrocellulose membranes that are placed on the surface of SOB agar plates and incubated at 37^0C overnight. Glucose (2%) was added to SOB agar to suppress

cytotoxic g3p expression during bacterial growth. Colonies grown on the membrane are transferred onto an LB agar plate containing X-gal (20 μg/ml), IPTG (1 mM) and ampicillin (100 μg/ml), and left for several hours at room temperature to allow the blue color to develop. Blue and white colonies are separately harvested and grown in LB broth. Plasmid DNA was isolated using a Qiagen kit and analyzed for recombination.

D. PCR analysis

Cre-mediated recombination between two phagemids was verified by PCR using 4 primers. Backward DV_H (5'- CTT ACA ATG ATG GTA CTA AG -3') and forward DV_L (5'- ACT GTT GAA CAG ACT CTG -3') are designed to hybridize to CDR of $3D8V_H$ and $3D8V_L$ respectively. Backward SV_H (5'- CTT CAT TTA TTA CTC CGA TG -3') and forward SV_L (5'- CGT GAA TGG GTT ACT ACT CC -3') are employed for hybridization to CDR of S2C11 V_H and S2C11 V_L. Four primer combinations (DV_H-SV_L, SV_H-DV_L, DV_H-DV_L and SV_H-SV_L) are used for amplification of the scFv gene. Recombinant plasmid DNA isolated from BS1365 and blue and white colonies (JM109) are used as templates for PCR (30 cycles of 1 min at 94^0C, 1 min at 55^0C, and 1 min at 72^0C).

E. Isolation of recombinants

pRGC and pRGD plasmids resulting from recombination between pRGA and pRGB are isolated for confirmation of the correct sequence. To isolate pRGC, recombinant DNA extracted from BS1365 was digested with three restriction enzymes (*Bgl*II, *Mlu*I and *Spe*I) which linearize plasmids except pRGC. Among these, *Bgl*II linearizes pRGA and pRGD, *Mlu*I linearizes pRGB and pRGD, and *Spe*I linearizes pRGD as Ill as a small plasmid generated by intramolecular recombination between incompletely non-compatible *loxP* sites (*loxP* and *loxP511*). After bacterial transformation with a plasmid mixture treated with the three enzymes, several colonies are cultured in broth, and aliquots (1 μl) of cell suspension are employed for PCR analysis, using the DV_H - DV_L primer set. Plasmid DNA was prepared from cultures containing bands of expected sizes. Recombinant pRGD was isolated by culturing blue colonies after X-gal staining. The integrity of pRGC and pRGD was confirmed by sequencing, using a cycle sequencing ready reaction kit (Applied Biosystems, Foster, CA).

F. Expression and functional analysis of scFv proteins

Expression of scFv proteins generated from pRGA, pRGB, pRGC and pRGD was induced in JM109 with 1 mM IPTG at 25^0C overnight. To prepare total

cell protein, cells are lysed in resuspension buffer (20 mM Tris, pH 8.0, 50 mM NaCl, 2 mM EDTA) by sonication. Proteins are subjected to SDS-PAGE on a 10% gel, transferred to a nitrocellulose membrane and probed with rabbit anti-E tag antibody and secondary antibody goat anti-rabbit alkaline conjugate. Proteins are visualized with BCIP/NBT (Sigma-Aldrich Co, Milwaukee, MI). To confirm the function of scFv proteins, total bacterial cell fractions are used for ELISA, performed according to standard protocols. Briefly, Ills of an ELISA plate (Costar, High Wycombe, UK) are coated with 100 µl ssDNA (2 µg/ml) or HBs antigen (5 µg/ml). After blocking Ills with 3% BSA and incubation with the diluted total cell fraction, bound scFv are detected using primary rabbit anti-g3p (1:2,000) (MoBiTec, Göttingen, Germany) and secondary anti-rabbit antibody conjugated to alkaline phosphatase (1: 10,000) (Pierce, Rockford, IL). Reaction revealed with p-NPP substrate (Sigma-Aldrich Co, Milwaukee, MI) was read 495 nm.

III. RESULTS

A. Construction of phagemid vectors

To establish a novel system for the proficient estimation of recombination efficiency, two phagemid vectors are constructed. In both the vectors, a polypeptide of loxP511 (ITSYNVYYTKL)-$(Gly_4Ser_1)_1$ was used as a linker between V_H and V_L. Restriction enzyme sites flanking the loxP511 peptide-$(Gly_4Ser_1)_1$ are added to facilitate further V_H or V_L cloning. The first phagemid (pRGA) contains a β-gal gene lacking N-terminal MTMIT amino acids upstream of the *loxPwt* site. The second phagemid (pRGB) contains a *tac* promoter and a DNA fragment encoding the N-terminal MTMIT amino acids of β-gal downstream of the *loxPwt* site. In *E. coli*, β-gal expression is 5-fold more active under control of the *tac* promoter, compared to the *lac* promoter.[9] Different scFv genes are cloned into each vector to verify recombination between V_H/V_L genes.

B. Recombination in Cre-expressing bacteria

The 3D8V_H - S2ClIV_L (D-S) and S2C11V_H - 3D8V_L (S-D) genes are introduced into pRGA and pRGB, respectively. BS1365 cells expressing Cre recombinase are co-infected using the two phagemids containing scFv genes to

induce recombination. Recombination was analyzed by PCR, using plasmids isolated from BS1365 as templates. PCR analyses to identify scFv genes created by shuffling of the V_L genes revealed amplified bands of 551 bp (DV_H - SV_L primer set), 226 bp (SV_H - DV_L), 359 bp (DV_H - DV_L), and 423 bp (SV_H - SV_L), corresponding to the scFv formats within pRGA, pRGB, pRGC, and pRGD, respectively. The data indicate that four different scFv gene formats (D-S, S-D, D-D, and S-S) coexist in the BS1365 bacterial pool, as expected from the consideration that reversible recombination event by Cre recombinase results in coexistence of parent and recombined DNA at equilibrium state (Fig. 2A: lanes 9 - 12).

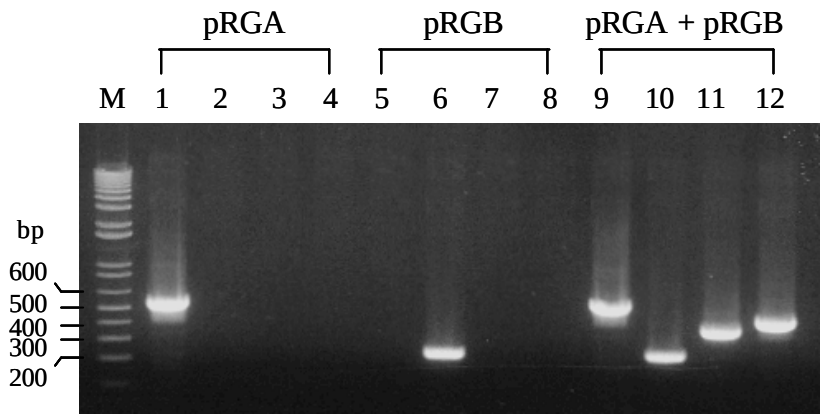

Fig. 2. Analysis of recombination in Cre-expressing bacterial cells. Recombination between V_H and V_L genes. Following recombination by infection of BS1365 with pRGA and/or pRGB at 37^0C for 2 h, plasmid DNA was isolated and used as templates for PCR. M: 1 kb plus ladder (BRL); Lanes 1, 5, 9: DVH - SVL primer; Lanes 2, 6, 10: SV_H-DV_L primer; Lanes 3, 7, 11: DV_H -DV_L primer; Lanes 4, 8, 12: SV_H-SV_L primer. Lanes 1 - 4: infection with pRGA; Lanes 5 - 8: by infection with pRGB; Lanes 9 - 12: infection with both pRGA and pRGB.

C. Analysis of plasmids following recombination

After transformation of JM109 cells with DNA purified from BS1365, blue colonies are observed on X-gal-containing agar plates. Digestion of twenty plasmids purified randomly from these cells by *Bgl*II and *Mlu*I revealed a 350 bp insert band corresponding to the restored β-gal gene in pRGD. Using the above plasmids as templates and the SV_H - SV_L primer set, PCR products of 423 bp are observed. An analysis of six representatives of these twenty plasmids is depicted in Fig. 2B. All plasmids isolated from the blue colonies indeed contained recombinant pRGD comprising S2C11V_H - S2C11V_L and an active β-gal gene (Fig. 2B). Correct recombination was confirmed by sequencing of the V_H-V_L and β-gal genes. Furthermore, when plasmid DNA isolated from a pool of cells collected by scraping several hundreds of white colonies on a plate was used for PCR with the SV_H - SV_L primer set, no DNA band was observed. However, DNA bands of expected sizes are amplified using the three primer sets, DV_H - SV_L, SV_H - DV_L and DV_H - DV_L. This indicates that pRGA, pRGB, and pRGC that are unable to develop blue color did not contain the S2C11V_H - S2C11V_L gene. Therefore, the frequency of blue colonies darectly represents the presence of pRGD. Analysis of individual white colonies disclosed that 25% contained an unknown 3.2 kb band, distinguishable from four unimpaared plasmids of around 5.3 kb (data not shown). Deletion of the 1.9 kb fragment corresponding to the V_L - g3p region by recombination between *loxPwt* and

loxP511 sites on a single phagemid would result in the small 3.4 kb band.

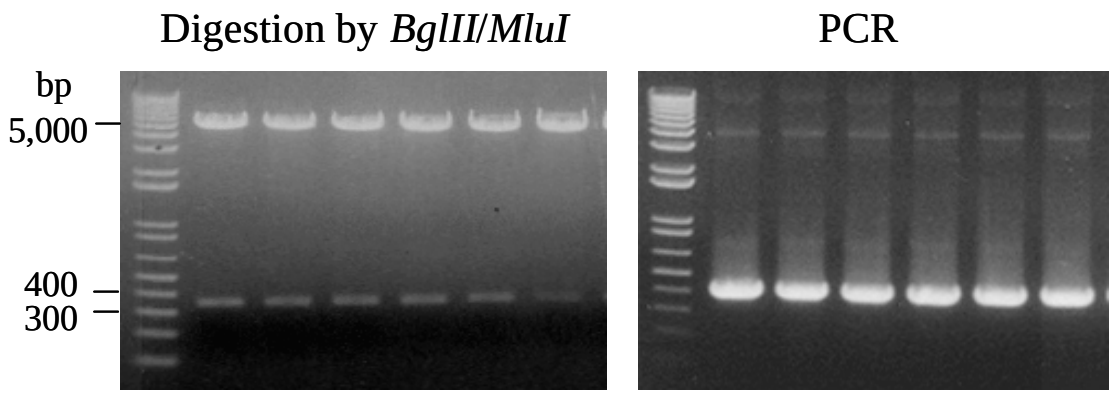

Fig. 3. Analysis of plasmids prepared from blue colonies. Twenty colonies showing blue color on LB plates containing X-gal were randomly cultured and DNA was prepared for analysis by PCR, using the SV_H - SV_L primer set (left panel) and the restriction enzymes, *Bgl*II/*Mlu*I (right panel). Six of these are shown.

D. Recombination efficiency

In the presence of Cre, recombination between V_H/V_L of two phagemids simultaneously resulted in the generation of a phagemid capable of expressing functional β-gal. In other words, expression of β-gal from a recombinant phagemid was indicative of V_H/V_L recombination. Recombination efficiency was calculated from the frequency of a novel recombinant phagemid, and expressed as a percentage difference between the number of blue and total colonies. If the recombination reaction was fully successful and reached equilibrium (denoted as 100% efficiency), pRGD (equivalent to blue colonies) comprised approximately 25%. Based on this assumption, if the frequency of blue colonies was 10%, this would mean that the recombination efficiency was 40% (10% multiplied by 4). In the case of recombination followed by co-infection of BS1365 with pRGA and pRGB, the percentage of blue colonies was 6.1%, compared to total colonies. Here, recombination efficiency was calculated as approximately 24.4%. When BS1365 cells are infected with either pRGA or pRGB alone, efficiency of recombination was not evaluated (Table 1).

Table 1. Recombination efficiency in BS1365 at 37 ºC for 2 hr

Phagemids participated in recombination	Frequency of blue colony (number of blue colony /total colony)	Efficiency of recombination
pRGA	0% (0/1,200)	Unknown
pRGB	0% (0/1,200)	Unknown
pRGA and pRGB	6.1% (158/2,600)	24.4%

E. Expression and functional analysis of recombinant scFv proteins

Expression of scFv-g3p fusion protein was induced from the two parental plasmids (pRGA and pRGB) and two novel recombinant plasmids (pRGC and pRGD). After induction with IPTG, scFv-g3p proteins in total cell extracts are detected by Western blotting using anti-E tag antibody (Fig. 3A). A band with a molecular mass slightly below 97 kDa was detected. This was significantly larger than the size calculated based on the amino acid sequence (64 kDa). A protein size of 64 kDa may be subdivided into 32 kDa for scFv plus additional sequences, and 42 kDa for the g3p sequence. The discrepancy between the calculated molecular weight of scFv-g3p and the Western blot is possibly due to the unusual three-dimensional structure of g3p. The g3p protein (calculated as approximately 42 kDa) was observed in a Western blot at 60 – 65 kDa, consistent with earlier reports.[19]

To ensure that scFv with a loxP511 peptide-$(Gly_4Ser_1)_1$ linker is functional, JM109 cells harboring each vector are grown and IPTG-induced cells are lysed. Total cell extracts are used for ELISA (Fig. 3B). Both 3D8 scFv (expressed from pRGC) and S2C11 scFv protein (expressed from pRGD) should specific binding to corresponding antigens; the former bound ssDNA while the latter bound HBs antigen. Furthermore, 3D8 scFv bound to ssDNA with a signal comparable to the standard $(Gly_4Ser_1)_3$ linker (data not shown). The linker used in this study served as a flexible polypeptide that allowed association between V_H and V_L domains to form a functional antigen-binding site. The binding signal of 3D8V_H - S2C11V_L to ssDNA

is due to 3D8 V_H, which was shown to bind ssDNA without associating with V_L in a previous assay.

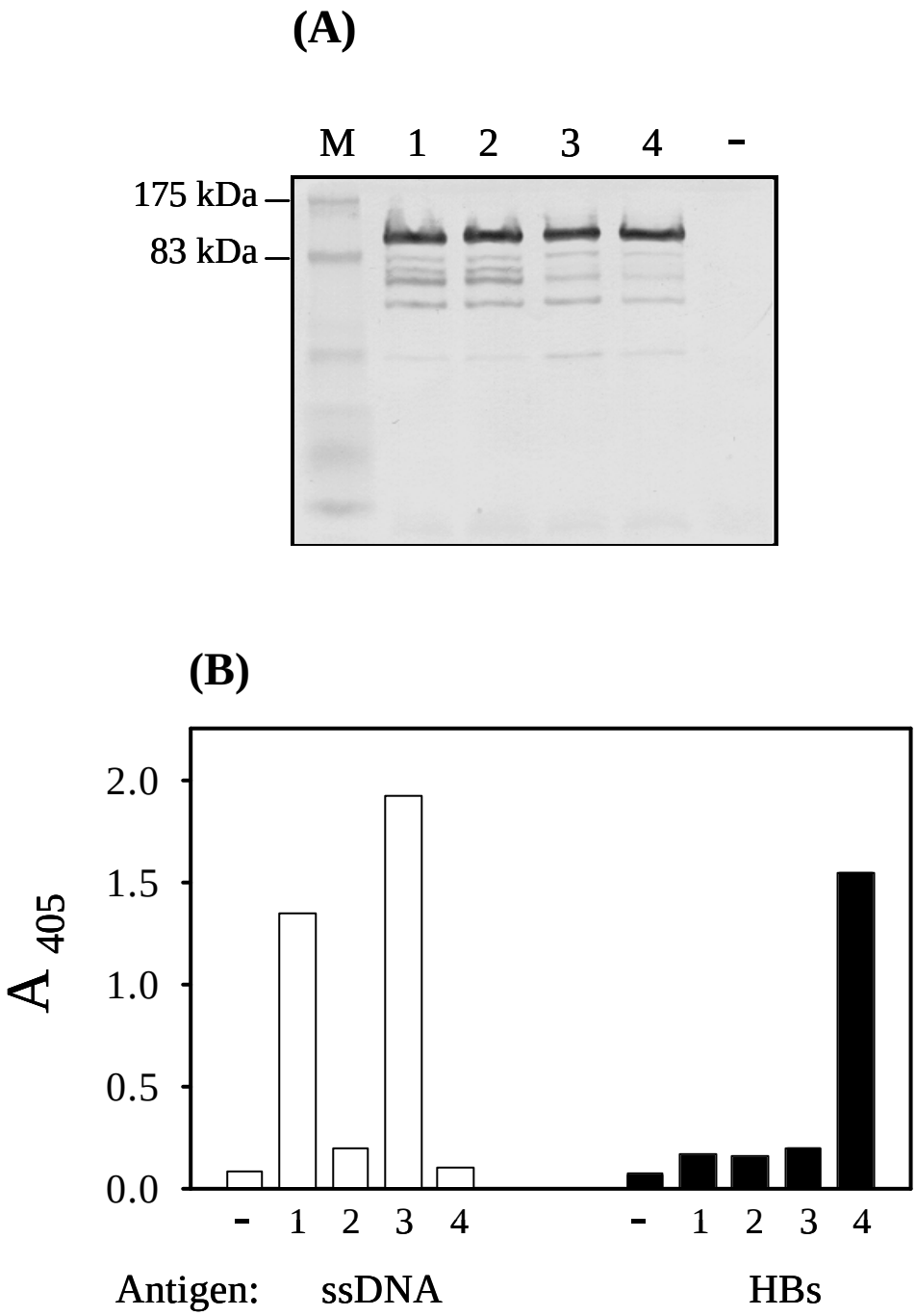
(A)
M 1 2 3 4 -
175 kDa
83 kDa
(B)
A 405
2.0
1.5
1.0
0.5
0.0
- 1 2 3 4 - 1 2 3 4
Antigen: ssDNA HBs

Fig. 4. Expression and functional analysis of scFv proteins. (A) Western blot of expressed scFv proteins. **(B)** ELISA for analysis of binding of scFv proteins. Polystyrene microtiter wells were coated with ssDNA and HBs antigen, followed by blocking of wells with 3% BSA. After IPTG induction, total cell extracts prepared from JM109 bacterial cells harboring pRGA, pRGB, pRGC and pRGD were added to each well. ScFv proteins binding to ssDNA or HBs antigen were detected using a mouse anti-E tag and a goat anti-mouse immunoglobulin conjugated with alkaline phosphatase. As a negative control (-) of scFv gene expression, JM109 cells alone were used. ScFv proteins expressed in bacterial cells transformed with each plasmid vector; 3D8 V_H - S2C11 V_L in pRGA (1), S2C11 V_H – 3D8 V_L in pRGB (2), 3D8 V_H – 3D8 V_L in pRGC (3), and S2C11 V_H – S2C11 V_L in pRGD (4).

IV. DISCUSSION

I have developed a visible phagemid system that permits the measurement of recombination efficiency between V_H/V_L genes by simple counting of the number of blue colonies. The ultimate purpose of this new system is to estimate the size of antibody libraries produced using *Cre-lox* recombination under the one-vector system.[15, 16] Recombination efficiency by Cre recombinase is readily measured using our visible phagemid system.

A key factor of our system was the graft of β-gal expression into the *Cre-lox* system. In the presence of Cre recombinase, assembly of β-gal fragments into the functional gene was accompanied by intermolecular recombination between V_H and V_L genes derived from two separate phagemid vectors. In constructing two novel vectors, I targeted the *loxPwt* site to a specific region within the β-gal gene, since the N-terminal region of the alpha peptide of *lacZ* (*lacZ'*) is amenable to disruption by foreign DNA fragments, such as multiple cloning sites up to 11-18 amino acids long.[13] In fact, replacement of multiple cloning sites of the β-gal gene with the *loxPwt* sequence did not interfere with β-gal function. In the established visible phagemid system, only bacterial cells harboring recombinant pRGD are stained with X-gal, signifying undetectable levels of background for false staining. Therefore, it is possible to determine precise recombination efficiency using this system.

The recombination efficiency was unable to be more than 30% (recombinant

frequency < 7%) through recombination in BS1365 (37^0C, 2 h). This was much lower, compared to other studies utilizing BS1365 as an *in vivo* recombination reactor. This may be attributed to the fact that experimental conditions are not identical. The first possible explanation is low intermolecular recombination frequency between the two vectors via *loxPwt* and *loxP511* sites. The Cre-mediated reaction includes recognition of the *loxP* sites by Cre, synapsis to bring the recombining strands into proximity, and formation of a covalent protein-DNA intermediate (tetramer composed of *Cre2-Lox* dimers).[12] Thus, the appropriate physical positioning of *lox* sites located on different DNA molecules may be unfavorable, compared to *cis*-linked *lox* sites. This possibility is supported by a recent report in which inter-chromosome recombination is shown to occur at a lower frequency than intra-chromosome recombination.[14]

The second possibility for reduced recombination efficiency is high excision rate due to intramolecular recombination between *loxPwt*/*loxP511* in a single vector. In BS1365, intramolecular recombination may occur within all four vectors (pRGA, pRGB, pRGC and pRGD), which would reduce the yield of pRGD (an indicator for recombination) despite high occurrence of the recombination reaction. In particular, early intramolecular recombination in either pRGA or pRGB may prevent intermolecular recombination between the two phagemids. In this study, 25% plasmids isolated from white colonies are less than 5.3 kb, probably due to excision of a 1.9 kb DNA fragment. When recombination in BS1365 was performed at 30^0C overnight, a lower frequency (1.2%) of blue colonies was observed with a three-fold

higher excision rate (data not shown) than that at 37^0C for 2 h (6.1%). This finding supports the second theory for low recombination efficiency.

Several studies on recombination between *loxPwt* and *loxP511* have reported different degrees of recombination (1~10%).[7, 11, 18] Though the differences are reasonable in view of the slightly different experimental conditions, evident compatibility exists between the *loxPwt* and *loxP511* sites to some extent. For practical use of this visible phagemid system in the generation of antibody libraries, two mutant *lox* sites more incompatible than *loxP511* are required to prevent excision by intramolecular recombination in a single phagemid vector, which would lead to loss of functional scFv genes. Systematic screening to identify *lox* mutants incompatible with *loxPwt* has been reported in both *in vitro* and *in vivo*, including Cre-expressing bacterial and mammalian cells.[10, 18] A number of *lox* mutants, such as FAS and 2272, that display a frequency of less than 1% with *loxPwt* in Cre-expressing bacterial recombination, may be used in lieu of *loxP511*.

This visible phagemid system may be rationally employed to generate antibody libraries. A critical advantage of this system is that the recombination efficiency value obtained by simple counting of blue colonies is applicable for accurate estimation of the size increase of secondary scFv libraries by *Cre-lox* recombination without laborious analysis of the scFv sequences. In addition, our system may potentially be employed in future studies that require the determination of efficiency of intermolecular recombination in the *Cre-lox* system.

V. CONCLUSIONS

I have developed a visible phagemid system that permits the measurement of recombination efficiency between V_H/V_L genes by simple counting of the number of blue colonies. The ultimate purpose of this new system is to estimate the size of antibody libraries produced using *Cre-lox* recombination under the one-vector system. Recombination efficiency by Cre recombinase is readily measured using our visible phagemid system.

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- 국문요약 -

Cre 유도 재조합 효율 향상을 위한 육안 판정 phagemid 방법의 확립

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목적: Cre-lox 시스템은 일반적으로 phage 항체 라이브러리의 크기를 증가시키는 데 이용되어 왔다. 그러나 Cre에 의해 유도된 재조합 후 라이브러리의 크기를 판정하는 것은 뉴클레오타이드 염기서열 분석에 의존하기 때문에 어렵다. 이 연구의 목적은 V_H와 V_L간의 재조합을 육안으로 판정할 수 있는 phagemid 벡터를 제작하는 것이다.

재료 및 방법: 두개의 phagemid(pRGA와 pRGB)를 제작하였다. 두개의 phagemid들을 함께 Cre 재조합 효소를 발현하는 BS1365 대장균내에 감염시켜 재조합을 유도하였다. 벡터간의 재조합 유발 여부는 PCR, 제한효소 처리, DNA 염기서열 분석으로 확인하였다. phagemid에서 발현된 scFv의 기능을 확인하기 위하여, 대장균 세포 전체 단백질을 Western blot과 ELISA에 사용하였다. 재조합 효율은 X-gal을 포함한 배지에서 청색 집락을 세어 계산하였다.

Results: BS1365 대장균에서 분자간 V_H와 V_L간의 재조합과 â-galactosidase (â-gal) 활성 획득이 함께 이루어졌다. 청색 집락에서 얻은 플라스미드의 분석으로 새로운 V_H/V_L 조합을 확인하였다. 재조합 효율은 새로 형성된 재조합

phagemid의 빈도로 계산되었고, 전체 집락과 청색 집락간의 백분율로 표시되었다. 계산된 재조합 효율은 24.2%였다. Phagemid에서 발현된 ScFv-g3p 단백질은 97 kDa 크기로 확인되었고, 특정 항원에 대한 결합 능력을 보였다.

결론: 본 연구에서는 염기서열 분석 없이 V_H/V_L 유전자간의 재조합 효율을 측정할 수 있는 육안 판정 phagemid 시스템을 개발하였다. 본 연구의 결과는 새로 개발된 이 phagemid 시스템이 재조합 효율을 측정하는데 이용될 수 있고, 이것은 phage 항체 라이브러리에 대한 정확한 평가를 가능하게 할 것이다.

핵심되는 말: 항체 라이브러리 크기, 육안 phagemid 시스템, Cre 유도 재조합, 재조합 효율, X-gal 염색, â-galactosidase, scFv