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Resistance of Cloned 1F5 Chimeric Anti-CD20 Antibody Heavy-Chain Gene to DNA Polymerase due to a Predicted Hairpin Structure

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Abstract

Background: Formation of secondary structures such as DNA hairpins or loops may influence molecular genetic methods and PCR-based approaches necessary for genetic engineering as well as gene regulation.

Materials and Methods: A polymerase chain reaction with splice overlap extension (SOE-PCR) was used to create fully synthetic 1F5 chimeric anti-CD20 heavy- and light-chain genes. The chimeric genes were cloned into the pCR-Blunt II-TOPO vector followed by cloning into the pBudCE4.1 expression vector. Prediction of secondary structure was performed with the Vienna RNAfold webserver. PCR and sequencing across the predicted secondary structure of chimeric 1F5 heavy-chain gene were performed with multiple protocols for standard and GC-rich templates.

Results: In an attempt to design vectors which aimed at generating mouse-human chimeric antibody against CD20 (1F5), we found that the coding sequence of 1F5 chimeric heavy-chain gene constructed by SOE-PCR was resistant to polymerase during both PCR and sequencing reactions. Furthermore, we were also unable to analyze some positive transformants by restriction enzyme digestion. Encountering such difficulties to identify the cloned anti-CD20 chimeric heavy-chain gene, we found that the chimeric heavy-chain sequence is highly GC-rich and predicted to form a stable secondary structure.

Conclusion: In conclusion, this is the first report on several difficulties with production of chimeric 1F5 anti-CD20 antibody due to a predicted hairpin cluster which correlates with barriers to PCR, sequencing and possibly restriction analysis. Our findings provide a probable note for researchers experiencing technical difficulties with construction of chimeric anti-CD20 antibody 1F5 gene vectors and also with other genes and molecular biology techniques requiring PCR-based method or restriction enzyme analysis.

Keywords: Chimeric gene; Monoclonal antibody; Secondary structure; SOE-PCR; 1F5

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Introduction

The growing success of monoclonal antibodies (mAbs) in the treatment of cancer and other diseases in human owes much to the recombinant DNA technology. Monoclonal antibodies against CD20 have been most successful in the treatment of patients with B-cell non-Hodgkin's lymphoma (1-3). B-lymphocyte antigen CD20 is a transmembrane phosphoprotein that has been validated as a superior biomarker for immunotherapies targeting B-cell lymphoma (4). CD20 is expressed on normal and most malignant B cells but not on stem cells and plasma cells (4).

Chimeric mAbs are produced by replacing the constant regions of mouse antibodies with those of human antibodies by genetic engineering techniques (5, 6). The splice overlap extension (SOE) PCR is an

approach for recombining DNA molecules without the use of restriction and ligase enzymes (7). It allows the fusion of a mouse variable segment with a human constant segment utilizing the J-region similarity between the mouse and human immunoglobulin genes without the addition of complementary tags (8). Here, we initially aimed to use the SOE-PCR method to generate 1F5 anti-CD20 mAb chimeric gene constructs with a goal of producing recombinant antibody against CD20. In our attempt to construct the subcloning and expression vectors, we found that the coding sequence of antibody 1F5 chimeric heavy-chain gene was resistant to polymerase during both PCR and sequencing reactions. Furthermore, we encountered difficulties to analyze positive transformants by restriction enzyme digestion.

Regions with high guanine-cytosine content within DNA molecule can cause the formation of stable secondary structures such as hairpins that have been known to promote polymerase read-through under normal conditions, resulting in prohibiting PCR amplification and sequencing (9-13). Also the plasmid supercoiling structure can induce cruciform and other secondary structures, favoring deletions or rearrangements (14, 15). It is important to detect the GC-rich sequences or secondary structures in DNA constructs, especially if it has the potential to interfere with amplification of these sequences by PCR or recombination across these regions (16). Nelms and Labosky (16) discovered a 370 bp segment of DNA resistant to polymerase read-through, resulting in disruptions of PCR and sequencing in addition to interfering with Bacterial Artificial Chromosome (BAC) recombineering. It was shown that proofreading DNA polymerases can be inhibited by certain primers containing guanine-rich sequences (17). Montgomery et al. (18) demonstrated that polymerase activity is influenced by the GC content of the template, and secondary structure which is difficult to be predicted interferes with extension.

1F5 was the first mouse anti-CD20 monoclonal antibody used in serotherapy of human B-cell lymphoma (19). Despite the efficacy of murine 1F5 mAbs in lymphoma patients, the 1F5 chimeric antibodies with human effector functionality are yet to be approved and widely used in the treatment of lymphoma. In the present study, we report several difficulties with the production of therapeutic 1F5 chimeric anti-CD20 antibody. The 1F5 chimeric heavy-chain sequence is predicted to form a cluster of DNA hairpins. Here we demonstrate that such a hairpin cluster, while causing difficulty for PCR or sequencing may also interfere with restriction enzyme analysis. Barriers to PCR-based methods caused trouble in screening and identification of the cloned 1F5 chimeric heavy-chain gene and subsequently selection of the appropriate clones. However, we describe the chimeric 1F5 anti-CD20 antibody expression vector can be constructed successfully in spite of such difficulties.

Materials and methods

Amplification of the VH and VL genes and the CH and CL genes

The cDNA of mouse variable regions of heavy and light chains (VH and VL) was amplified from hybridoma, clone 1F5 (ATCC HB-9645). The human heavy and light chain constant region genes (CH and CL) were amplified from human peripheral blood mononuclear cells (PBMC) cDNA. The cDNAs of 1F5 variable gamma heavy chain (VH) and variable kappa light chain (VL) were amplified using two sets of primers. The 1F5 VH gene (GeneBank AY058907.1) was amplified using the forward primer VHF and the reverse primer JHR. The 1F5 VL gene (GeneBank AY058906.1) was amplified using the forward primer VKF and the reverse primer JKR. The sequences of primers are listed in Table 1. Specific forward primers VHF and VKF were designed based on each of the sequences of the 1F5 VH and VL genes. The J-region reverse primers JHR and JKR were made, which contained complementary sequence to the immunoglobulin J-regions for the heavy and light chains.

Human IgG1 constant heavy-chain (CH) and constant kappa light-chain (CL) genes were amplified from human PBMC cDNA with two sets of primers—the forward JHF and reverse IgGHR primers for the IgG1 CH gene, and the forward JKF and reverse IgGKR primers for the IgG1 CL gene. The sequences of primers are listed in Table 1. The J-region forward primers JHF and JKF were made, which contained complementary sequence to the J-regions, for heavy and kappa chains. The class-specific reverse primers IgGHR and IgGkR were similar to those described previously (8).

In all cases, PCR reactions were set up containing 5 µL of 10× Pfx Ampli¬fication buffer (Invitrogen), 1 µL of 50 mM MgSO4, 2 µL of 10 mM dNTPs, 1 µL of the appropriate forward and reverse primers (10 µM), 2 µL of cDNA as template, 0.4 µL (1 U) Platinum® Pfx DNA polymerase (Invitrogen), and nuclease-free water to yield a total volume of 50 µL. The PCR was performed as follows: 1 cycle at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C (VH, VL and CL), or 60 °C (CH) for 45 seconds, extension at 68 °C for 1 minute, and a final cycle of 15 minutes at 68 °C. The amplified products of VH, VL, CH, and CL genes were analyzed using agarose gel-electrophoresis to confirm successful amplification of

individual genes. The PCR products were purified from agarose gel with a Qiaquick gel extraction kit (Qiagen, Valencia, CA).

Table 1. The table details sequences of each primer used for construction of anti-CD20 chimeric genes (heavy and light chains) using SOE-PCR.

Primer Name	Sequence
J_HF	5'-GACTACTGGGGCCAAGGGAC-3'
J_HR	5'-CCTTGGCCCCAGTAGTCAAAG-3'
J_kF	5'-GGGACCAAGCTGGAAATCAACG-3'
J_kR	5'-CGTTGATTTCCAGCTTGGTCCC-3'
V_HF	5'-ATGGCCCAGGTGCAACTGCG-3'
IgG_HR	5'-TCATTTACCCGGAGACAGGGAG-3
V_LF	5'-ATGGCCCAAATTGTTCTCTCC-3'
IgG_kR	5'-CTCCCTCTAACACTCTCCCCTG-3'

Construction of chimeric heavy- and light-chain genes

A polymerase chain reaction with splice overlap extension (SOE-PCR) was used to create fully synthetic human–mouse chimeric heavy-chain and light-chain genes (8). Chimerization of mouse 1F5 variable genes to the human IgG1 constant genes was performed using immunoglobulin J-region similarity. The J-region primers, JKR for the amplification of 1F5 VL domain and JKF for the amplification of IgG1 CL domain, are reverse complements, similar to those reported by Jones and Barnard (8), which allows subsequent fusion of the sequences by SOE. However, we designed new J-region primers (JHR and JHF) for the amplification of 1F5 VH and IgG1 CH domains.

SOE-PCR was then carried out using the primers VHF and IgGHR for the creation of chimeric heavy-chain (VH-CH) and the primers VkF and IgGkR for the light-chain (VL-CL) sequences. PCR was performed using 5 µL of 10× Pfx Ampli¬fication buffer, 1 µL of the appropriate forward and reverse primers, 1 µL of VH and 1 µL of CH or 1 µL of VL and 1 µL of CL (20 ng/µL), 2 µL dNTPs, 1 µL MgSO4, 0.4 µL (1 U) Platinum® Pfx DNA polymerase, and sterile water to final volume of 50 µL. PCR conditions were as follows: 1 cycle at 94 °C for 5 minutes, followed by 35 cycles (94 °C for 45 seconds, 60 °C [VH-CH] or 55°C [VL-CL] for 45 seconds, 68 °C for 1 minute 30 seconds), and a final extension for 15 minutes at 68 °C. The full-length chimeric products were verified by agarose gel electrophoresis and purified from the agarose gels using the Qiaquick gel extraction kit.

Construction of plasmids

After gel purification, chimeric heavy- and light-chain genes were each cloned into the pCR-Blunt II-TOPO vector (invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The plasmid DNA from the resulting colonies was analyzed by the colony PCR and restriction enzyme digestion followed by agarose gel electrophoresis. Finally, the chimeric heavy and light genes were excised from the TOPO vector using vector-derived restriction sites and then both were inserted into the mammalian expression vector pBudCE4.1 (Invitrogen) at the KpnI and XhoI sites for chimeric heavy gene and at the HindIII and XbaI sites for chimeric light genes according to the Thermo Scientific protocols.

Polymerase chain reaction

Polymerase chain reaction across the predicted secondary structure of anti-CD20 chimeric heavy-chain gene was performed with multiple protocols using different polymerases including Platinum Pfx DNA Polymerase (Invitrogen), Platinum PCR SuperMix High Fedility (Invitrogen) and Taq DNA polymerase (Vivantis). In an attempt to improve amplification through the GC rich template, PCRx Enhancer Solution was used with Platinum Pfx DNA Polymerase.

Restriction enzyme analysis

The DNA molecule was digested with appropriate restriction enzymes according to the instruction of the manufacturer (Thermo Fisher Scientific, Waltham, MA). Briefly, 1 µg of miniprep DNA from TOPO and pBudCE4.1 plasmids carrying VH-CH and VL-CL gene fragments were digested with a mixture of KpnI and XhoI restriction enzymes for VH-CH and HindIII and XbaI for VL-CL. In addition, we used Thermo scientific double digest tool and recommendation to determine optimal reaction conditions for our double digest reactions. The digests were incubated for 16 h at 37 °C.

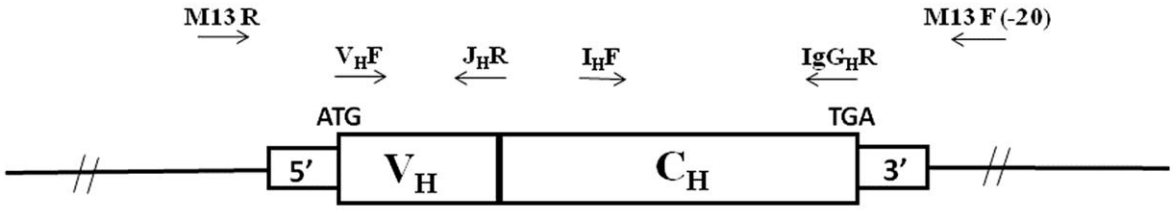
Sequencing

All DNA was sequenced using ABI 3730xl DNA Analyzer. Two protocols were used for sequencing including standard sequencing protocols and protocols for GC-rich templates. Primer sequences are listed in Figure 1. Templates used for sequencing included chimeric anti-CD20 heavy- and light-chain genes cloned into the pCR-Blunt II-TOPO or pBudCE4.1 bicistronic expression vector.

Prediction of secondary structure

RNA fold analysis was performed with the Vienna

RNAfold webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (20, 21) on the 1,635 nucleotides comprising the 1,365 bp polymerase resistant region (1F5 chimeric heavy-chain gene) and 135 bp flanking sequence of the pCR-Blunt II-TOPO or pBudCE4.1 on either side.



Sample Name	Primer Name	Primer Start	Primer End	Sequence	Use
TOPO 1365	M13 Forward (-20)	1477	1462	5'-GTAAAACGACGACGGCCAG-3'	Seq/PCR
	M13 Reverse	-197	-181	5'-CAGGAAACAGCTATGAC-3'	Seq/PCR
TOPO 1365 &	V_HF	1	20	5'-ATGGCCCAGGTGCAACTGCG-3'	Seq/PCR
pBud 1365	IgG_HR	1365	1344	5'-TCATTTACCCGGAGACAGGGAG-3	Seq/PCR
	J_HR	350	330	5'-CCTTGGCCCCAGTAGTCAAAG-3'	Seq/PCR
	I_HF	688	707	5‘-CTCACACATGCCCACCGTG-3'	Seq
TOPO 653 &	V_LF	1	21	5'-ATGGCCCAAATTGTTCTCTCC-3'	Seq/PCR
pBud 653	IgG_kR	653	632	5'-CTCCCTCTAACACTCTCCCCTG-3'	Seq/PCR

Figure 1. Schematic representation of the chimeric anti-CD20 heavy chain gene locus shows the location of primers used for PCR and sequencing (arrows). The analysis of transformant TOPO and pBudCE4.1 plasmid containing chimeric heavy-gene via PCR and sequencing using various primers failed, even under optimum conditions suitable for GC-rich templates. However, the chimeric heavy-gene amplicon was obtained via PCR only with M13 Reverse and IgG_HR. The table details location and sequences of each primer used for PCR or sequencing. abbrevations: ATG, start codon; Seq, sequencing; TGA, stop codon.

In addition, RNA fold analysis was performed on the 1F5 chimeric light-chain gene (653 bp) and 135 bp flanking sequence of the two studied vectors on either side. Input consisted of a single stranded DNA sequence. DNA parameters provided by David Matthews were selected, and the temperature was adjusted at 68 °C. The minimum free energy (MFE) algorithm for the prediction of a single optimal structure, and the partition function algorithm for the calculation of base-pairing probabilities in the thermodynamic ensemble were used (22). The outputs were visualized as an interactive secondary structure plot.

Results

Construction of plasmids containing chimeric genes

The method for constructing 1F5 chimeric antibody genes involves the following: (1) Primary PCR amplification of VH and VL, and CH and CL genes from 1F5 hybridoma and human PBMC cDNAs, respectively; (2) Joining of the VH and CH, and VL and CL sequences via overlap PCR to create chimeric heavy-chain and chimeric light-chain genes, respectively.

Analysis of the PCR products in a 1% agarose gel showed bands for the VH and VL genes at about 350 bp and 325 bp, respectively, in agreement with the expected sizes. Agarose gel electrophoresis of the PCR products showed the CH and CL genes at 1,032 bp and 350 bp, respectively, in agreement with the expected sizes. Figure 2 shows agarose gel electrophoresis of amplified VH and CH genes. The

VH, VL, CH, and CL genes were subsequently gel-purified to a final concentration of 20 ng/µL.

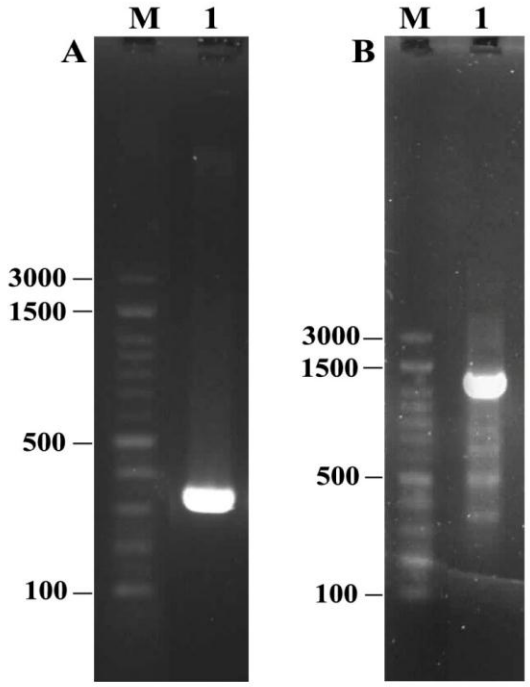


Figure 2. Agarose gel electrophoresis of the amplified mouse variable and human constant heavy genes. (A) Primary PCR amplification of variable heavy genes from 1F5 hybridoma cDNA. Lanes 1, 1F5 variable heavy gene (V_H, 350 bp). (B) Primary PCR amplification of constant heavy genes from human PBMC cDNAs. Lane 1, human IgG constant heavy gene (C_H, 1032 bp). M indicates molecular weight marker (100 bp DNA ladder, Morganville Scientific).

We adopted the SOE-PCR method for construction of chimeric heavy-chain and chimeric light-chain genes. The chimeric heavy and light genes were cloned into the pCR-Blunt II-TOPO vector. The TOPO recombinant vectors containing chimeric heavy- and light-genes were separately transformed into *E. coli* cells. Transformed cells were analyzed for the presence and correct orientation of the chimeric heavy and light genes. Insertion and correct orientation of the chimeric light-gene (653 bp) into the TOPO vector was successfully confirmed by colony PCR and restriction enzyme analysis, followed by conformation of the construct sequence by DNA sequence analysis. However, we run into difficulties with screening and identifying positive clones containing chimeric heavy-chain gene vector. First, the PCR amplification of 1F5 chimeric heavy-chain domain generated a 1365 bp fragment corresponds to the chimeric heavy-chain gene besides truncated variant of it, probably because of secondary structure formation (Figure 3). After gel purification and cloning of the chimeric heavy-gene into the pCR-Blunt II-TOPO, the identification of positive clones via colony PCR method was thoroughly failed using primers including M13 Forward (-20) and VHF, M13 Forward (-20) and M13 Reverse (vector primers), and VHF and IgGHR (specific primers).

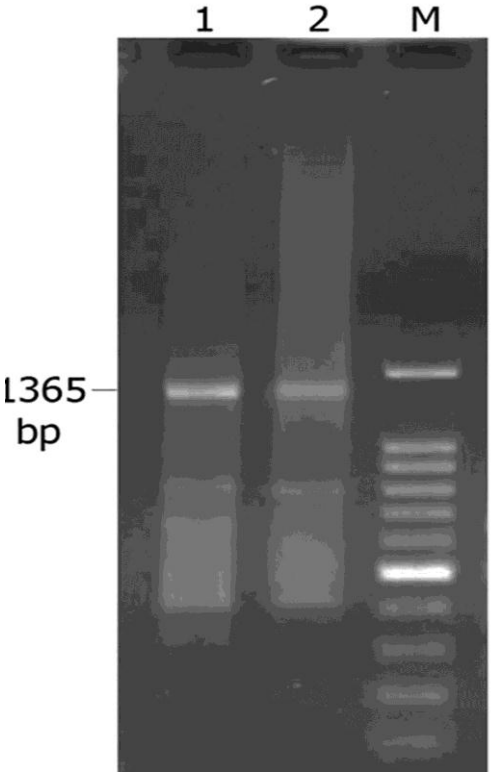


Figure 3. Construction of 1F5 anti-CD20 chimeric heavy chain gene by splice overlap extension (SOE) PCR. Lanes 1 and 2 show 1,365 nucleotide chimeric heavy-gene and truncated PCR products, probably because of the inability of the DNA polymerase to read through regions of strong secondary structure. M indicates molecular weight marker (100bp DNA ladder RTU, GeneDirex).

However, the colony PCR was only successful with M13 Reverse (a primer that hybridizes within vector) and IgGHR (a primer specific for insert). Moreover, restriction enzyme analysis of the TOPO plasmid containing chimeric heavy-gene was undesirable. Agarose gel electrophoresis of the isolated chimeric heavy gene from TOPO vector through digestion with KpnI and XhoI showed a faint DNA band (1,365 bp), despite multiple attempts (Figure 4). However, this weak band was purified from the agarose gel and was cloned into the expression vector pBudCE4.1.

Finally, the chimeric 1F5 heavy- and light-chain genes were excised from the TOPO vector and then both were cloned into the pBudCE4.1 expression vector. The colony PCR, and sequencing using specific primers (VHF, IgGHR), and restriction enzyme analysis of the pBudCE4.1 containing chimeric heavy-chain fragment were thoroughly

unsuccessful. However, the insertion of the chimeric heavy-chain gene into the pBudCE4.1 could be only confirmed by comparing the electrophoretic mobility shift of the empty vector (4,595 bp) and the vector containing 1,365 bp sequence (5,960 bp).

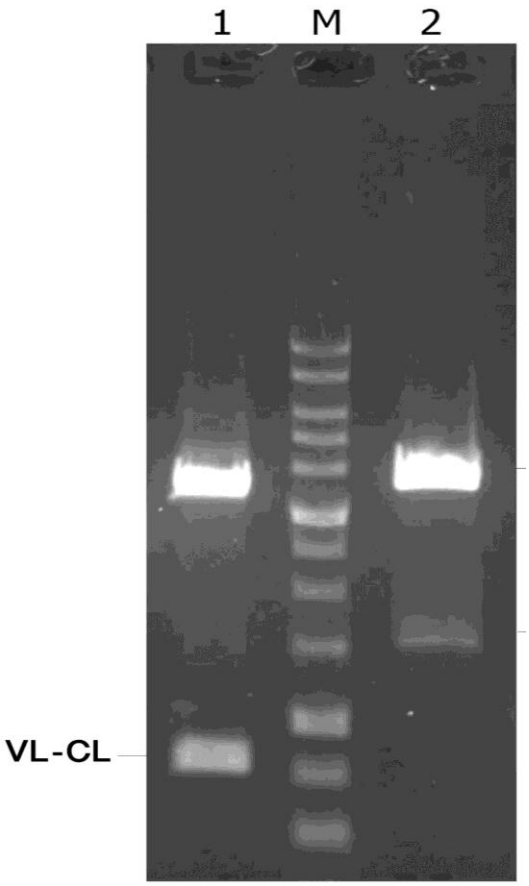


Figure 4. Restriction enzyme analysis of TOPO plasmid containing 1F5 chimeric heavy- and light-chain genes. Restriction enzyme digestion of TOPO plasmid containing anti-CD20 chimeric light chain with *Hind*III and *Xba*I released the chimeric light-gene as a strong band (lane 1) but restriction enzyme digestion of TOPO plasmid containing 1F5 chimeric heavy chain with *Kpn*I and *Xho*I released the chimeric heavy gene as a very faint band (lane 2). M indicates molecular weight marker (1Kb DNA ladder RTU, GeneDirex).

After transformation into E. coli cells, the presence of chimeric light-gene was successfully confirmed by colony PCR and restriction enzyme analysis, followed by DNA sequence analysis. Altogether, despite such difficulties in producing 1F5 chimeric heavy-gene vectors, the expression vector with chimeric heavy- and light-chain genes was successfully constructed as discussed above.

Detection of the resistance of 1F5 anti-CD20 chimeric heavy-chain coding sequence to polymerase and restriction enzyme analysis

In an attempt to produce chimeric 1F5 anti-CD20 antibody, we found that the chimeric heavy-chain gene cloned into the pCR-Blunt II-TOPO or pBudCE4.1 as expression vector is resistant to polymerase read-through and in some cases to restriction enzyme digestions. We attempted PCR across the heavy chain segment, in addition to light chain-containing plasmids. The PCR was carried out on the two distinct pCR-Blunt II-TOPO vectors containing chimeric heavy- and light-chain genes, and pBudCE4.1 containing both heavy- and light-chain genes using specific vector or insert primers. In contrast to the light-chain gene, the PCR of the heavy chain-containing plasmid was unsuccessful under different conditions; since this coding sequence is GC-rich (58% GC content). Therefore, the PCR amplification of this region was performed with different polymerases and kits under conditions suitable for GC-rich templates. However, using such conditions, no chimeric heavy-gene amplicon was obtained from pBudCE4.1 expression vector using VHF, IgGHR primers and from pCR-Blunt II-TOPO vector using M13 Forward (-20) and VHF, M13 Forward (-20) and M13 Reverse, and VHF and IgGHR primers.

In parallel, sequencing of the plasmids containing the chimeric heavy-chain coding region (under both normal conditions and conditions for GC-rich templates) was performed with M13 Forward (-20), M13 Reverse, VHF, IgGHR, JHR and IHF primers for pCR-Blunt II-TOPO and VHF, IgGHR, JHR and IHF primers for pBudCE4.1 (primers listed in Figure. 1). But sequencing reads extending across the chimeric heavy-gene were never obtained.

We also found that the coding sequence of the 1F5 chimeric heavy-chain gene interfered with restriction enzyme analysis. We performed the restriction analysis to confirm the presence of the insert in the plasmids. Both TOPO and pBudCE4.1 vectors (miniprep purified) were digested with restriction endonucleases KpnI and XhoI to excise the heavy chain and HindIII and XbaI to excise the light chain genes. Since the TOPO vector includes flanking EcoRI sites, we also used EcoRI to excise the cloned heavy and light chain sequences. No chimeric heavy-gene band was observed after EcoRI digestion of the TOPO plasmid or double digestion of the pBudCE4.1 expression vector using agarose gel electrophoresis, despite the multiple attempts. In addition, the agarose gel electrophoresis revealed the excised chimeric heavy-chain gene as a faint band at 1,365 bp after double digestions of pCR-Blunt II-TOPO compared with the strong band of the excised chimeric light-chain gene (653 bp) under the same conditions (Fig. 4). However, this weak band was extracted from the agarose gel and was used for being cloned into the

expression vector. Despite the confirmation of the presence of chimeric heavy- gene in the pBudCE4.1vector by comparing the size of the vector containing 1,365 bp sequence and the empty vector, our attempts for restriction analysis of the vector was unsuccessful. Altogether, the expression vector was successfully constructed and the chimeric 1F5 anti-CD20 antibody was expressed and correctly folded in CHO-K1 cells (data will be reported).

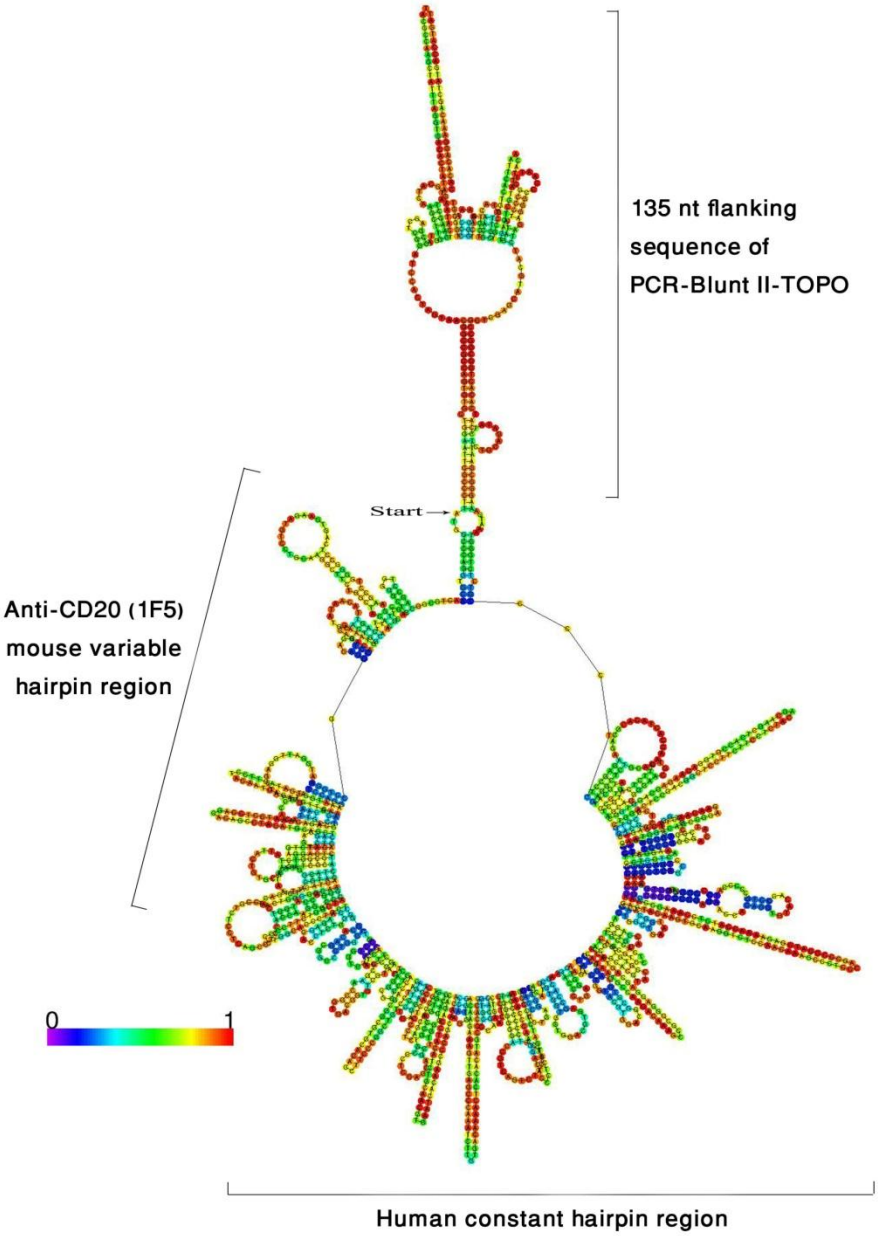


Figure 5. A predicted, stable DNA secondary structure (hairpin loop) exists in the 1F5 anti-CD20 chimeric heavy-chain coding sequence which corresponds to resistance of DNA to polymerase read-through. Both the mouse variable region and the human constant region were predicted to form a series of hairpins, however, the base-pairing probability of the constant region was stronger and higher than the variable region. The rainbow scale indicates a range of base-pairing probability from 0 to 1, violet to red.

The secondary structure prediction of the polymerase resistant chimeric heavy-gene coding sequence

The inability of the DNA polymerase to read and polymerization through chimeric 1F5 heavy-chain gene fragment suggested secondary structures such as DNA hairpins. Since secondary structures that inhibit sequencing or PCR reactions occur in regions with

high GC-content, since, we analyzed the GC-content of the chimeric heavy-chain fragment compared to the chimeric light-chain segment. We found that the chimeric heavy-gene has high GC content (58%) and the chimeric light-gene has relatively lower GC content (54%). However, the cloned chimeric light-gene was neither resistant to polymerase read-through nor interference with restriction enzyme digestion. We used RNA fold analysis for DNA to determine if the inability of DNA polymerase to negotiate through polymerase resistance region was due to the presence of secondary structure. The anti-CD20 chimeric heavy-chain coding sequence (with 135 nt flanking sequence of pCR-Blunt II-TOPO or pBudCE4.1 on either side) was predicted by minimum free energy to form a tight hairpin structure at the extension temperature (68 °C) (Figure 5, data of pBudCE4.1 not shown). Both the mouse variable region and the human constant region were predicted to form a series of hairpins, however, the base-pairing probability of the constant region was stronger and higher than the variable region. While the anti-CD20 chimeric light chain was also predicted to form a series of hairpins, it lacked any strong base-pairing probability hairpins.

Discussion

Herein, we report a predicted hairpin cluster in the coding sequence of 1F5 anti-CD20 chimeric heavy-chain gene which probably causes problems including resistance to polymerase read-through, especially after being cloned into the plasmids, resulting in disruptions of sequencing and prohibiting PCR amplification across this fragment. PCR screening of transformant TOPO and pBudCE4.1 plasmid containing chimeric heavy-gene completely failed using various primers even under optimum conditions suitable for GC-rich templates. However, several positive clones were selected by colony PCR using M13 reverse (a primer that hybridizes within vector) and IgGHR (a specific primer). In addition, sequencing of the chimeric heavy-chain gene cloned into both plasmids TOPO or pBudCE4.1 (under both normal conditions and conditions for GC-rich templates) was unsuccessful using various primers listed in Table 1. We also found that the cloned anti-CD20 1F5 chimeric heavy-chain gene interferes with restriction enzyme digestion. Our multiple attempts for restriction analysis of the pCR-Blunt II-TOPO and pBudCE4.1 vectors resulted in releasing of the chimeric heavy chain gene as a weak band from the pCR-Blunt II-TOPO and no band from the pBudCE4.1 vector. However, we confirmed the presence of the chimeric heavy-gene in the pBudCE4.1 expression vector by comparing the size of the empty vector with the vector containing chimeric heavy-gene. Altogether, the expression vector was successfully constructed and the chimeric 1F5 anti-CD20 antibody was expressed and correctly folded in CHO-K1 cells (data will be reported).

Encountering such difficulties in screening and identifying the cloned 1F5 anti-CD20 chimeric heavy-chain gene, we found that the sequence is highly GC-rich (58%) and predicted to form a stable secondary structure using RNA fold analysis. The cloned chimeric light-chain gene fragment which is screened and identified without difficulties was analyzed as well. It was also predicted to form a series of hairpins, but it lacked any strong base-pairing probability hairpins.

It was shown that proofreading DNA polymerases can be inhibited by certain primers containing guanine-rich sequences (17). Montgomery et al. (18) demonstrated that polymerase activity is influenced by the GC content of the template, and secondary structure which is difficult to be predicted interferes with extension. Nelms and Labosky (16) discovered a 370 bp segment of DNA resistant to polymerase read-through, resulting in disruptions of PCR and sequencing in addition to interfering with BAC recombineering. The presence of this type of constructs or secondary structure may be important to be considered in other genes (16). According to the significant role of the gene cloning in the antibody engineering techniques and including numerous main variables in this process to get appropriate results, it is important to discover the secondary structure in the DNA constructs. Especially, if the formation of the secondary structure has the potential to interfere with the amplification of these sequences by PCR-based methods. We also acknowledge that the predicted secondary structure could interfere with the enzymatic digestion of DNA by restriction enzymes in addition to the DNA polymerase.

The use of chimeric monoclonal anti-CD20 antibodies, such as rituximab for the treatment of B-cell malignancies, has greatly improved overall patient survival (23). Rituximab has provided important impetus to the biotechnology industry in its search for mAb-based drugs (24). 1F5 is one of the panels of monoclonal antibodies that have been produced recognizing the CD20 pan B cell antigen (25), and it was the first anti-CD20 monoclonal antibody used in serotherapy of human B cell lymphoma (19). Despite the success of murine 1F5 in the treatment of lymphoma, 1F5 chimeric antibodies with human effector functionality have not been approved yet and are not widely used in the treatment of lymphoma.

1F5 mAbs and rituximab as Type I anti-CD20 mAbs are both similar in their efficacy in antibody-dependant cellular cytotoxicity (ADCC) (26).

Rituximab and 1F5 mAbs are able to activate complement efficiently as a result of translocating CD20 to lipid rafts in the cell membrane (27). So, it appears that chimeric 1F5 mAbs with human effector functionality may activate complement more efficiently compared to rituximab or mouse 1F5 mAbs. In a future study, our aim is to purify and characterize 1F5 chimeric anti-CD20 mAbs in order to be used as potentially therapeutic monoclonal antibodies.

Conclusion

In conclusion, for the first time, we reported several difficulties with the production of chimeric 1F5 anti-CD20 antibody due to a predicted hairpin cluster which correlates with barriers to PCR, sequencing and possibly restriction analysis. Our findings provide a cautionary note for researchers experiencing technical difficulties with construction of chimeric 1F5 anti-CD20 antibody gene vectors and provide a basis for further studies with other genes and molecular biology techniques requiring polymerase activity or restriction enzyme analysis.

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Conflict of interest

The authors report no conflicts of interest.

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