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Chimerization of antibodies by isolation of rearranged genomic variable regions by the polymerase chain reaction

(Hybridoma; transfectoma; force cloning; cassette vectors; PCR; recombinant DNA)

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SUMMARY

We describe a new method for amplification, by polymerase chain reaction (PCR), of rearranged segments encoding the variable part of light and heavy chains of an antibody (Ab) from the chromosomal DNA of hybridoma cells for the chimerization of Abs. A fundamental prerequisite for this is the knowledge of the exact sequences in the 5′-untranslated region of light and heavy chain mRNA, and of the joining segment used for rearrangement. This allows the design of nondegenerated oligodeoxyribonucleotides for PCR. The primer design permits directional cloning of the amplified, promoterless fragments into cassette vectors, in which they will be linked to the appropriate human constant domains and immunoglobulin (Ig) promoter/enhancer elements. The method is illustrated for chimerization of an Ab directed against the human T-lymphocyte antigen, CD4. The chimerized Ab is secreted in abundant quantities after transfection of the engineered plasmids into non-Ig-producing myeloma cells.

INTRODUCTION

The benefits of mAbs in the treatment of human diseases, such as autoimmune diseases, graft rejections and cancer are now widely accepted. A major progress toward therapeutic applicability was the construction of chimeric Abs with variable rodent and constant human domains (Verhoeyen and Riechmann, 1988). Substantially lower immunogenicity has been found for chimeric Abs compared to their murine counterparts in colon cancer patients (Lo Buglio et al., 1989). With the advent of PCR technology (Saiki et al., 1985; 1988) cloning of cDNAs for L and H chains of Ig genes has been dramatically facilitated by making use of degenerate oligos (LeBœuf et al., 1989; Orlandi et al., 1989; Huse et al., 1989; Larrick et al., 1989). For expression, the V_L and V_H regions have to be cloned into vectors carrying the appropriate constant human region thus mimicking *Ig* genes. In contrast to Ig cDNAs, *Ig* genes are well expressed in Ig nonproducer hybridoma cells (Weidle et al., 1987). For this purpose, the isolated *V* regions have to be mutagenized to match the reading frame of the *Ig* gene on the vector. Alternately, restriction sites have to be incorporated into the primers for directed cloning

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Abbreviations: aa, amino acid(s); Ab, antibody; Ap, ampicillin; bp, base pair(s); C, constant region; cDNA, DNA complementary to mRNA; D, diversity region; ELISA, enzyme-linked immunosorbent assay; Fab fragments, antigen-binding fragments of Abs that are derived by papain digestion and contain the light chain and part of the heavy chain (variable region and first constant domain); H, heavy chain of Ab; Ig, immunoglobulin; J, joining region; kb, kilobase(s) or 1000 bp; L, light chain of Ab; mAb, monoclonal Ab; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PA, polyacrylamide; PCR, polymerase chain reaction; ss, single strand(ed); *UT*, untranslated region; V_H, variable heavy chain; V_L, variable light chain.

and expression (Orlandi et al., 1989). In this paper we present a different approach for cloning the relevant $V_L J$ and $V_H DJ$ regions with non-degenerate primers. Our technique includes PCR amplification of specific genomic DNA fragments and their subsequent force-cloning into cassette vectors. To our knowledge, this is the first report on the cloning of rearranged $V_L J$ and $V_H DJ$ regions from genomic DNA by PCR. In contrast to the procedure described by Orlandi et al. (1989), the expressed *Ab* gene retains its authentic signal peptide sequence (including introns) and does not differ at the N-terminal aa from the original murine Ab.

Primer **a**: 5'-GATCGTCGACAAGACTCAGCCTGGACATGATGTCC
SalI 5'UT

Primer **b**: 5'-ATCAGCGGCCGCACTTAACAAGGTTAGACTTAGTG
NotI Intron J_2

Primer **c**: 5'-CATAGTCGACGTTAGTCTTAAGGCACCACTGAGCC
SalI $5'UT_{\gamma 2a}$

Primer **d**: 5'-GAGCGCGGCCGCTTAAAAATAAAGACCTGGAGAGGCCA
NotI Intron $J4_{\gamma 2a}$

Fig. 1. Primers used for amplification of $V_L J$ and $V_H DJ$ regions of mAb MT151 by PCR. Primers **a** and **b** were used for amplification of the $V_L J$ region, **c** and **d** for the amplification of the $V_H DJ$ region of mAb MT151. Primers **a**, **b** and **c** are 35-mers, and primer **d** is a 38-mer. The numbers of matching nt for primers **a**, **b**, **c** and **d** are 25, 23, 25 and 26. Primers were synthesized on an Applied Biosystems (Foster City, CA) oligo synthesizer. The 5' primers contain in their overhanging part four nt and a *SalI* recognition site, the matching part corresponds to nt in the 5'*UT* region of κ and the γ_{2a} mRNA. The 3' primers contain in their non-matching part four random nt and a *NotI* recognition site, in their matching part 23 nt corresponding to the J_2 intron of the L-chain encoding gene and to the J_4 intron of the H-chain encoding gene. Restriction sites are underlined. Dashed lines represent Ig gene sequences.

EXPERIMENTAL AND DISCUSSION

(a) Principle of the method for chimerization

The most important prerequisite for the new method of chimerization is the knowledge of the exact sequence of the 5'UT region of the mRNA and of the *J* region used for the $V_L J$ and $V_H DJ$ rearrangement for the L and H chain of the Ab. This information allows the design of nondegenerate primers for the isolation of the relevant $V_L J$ and $V_H DJ$

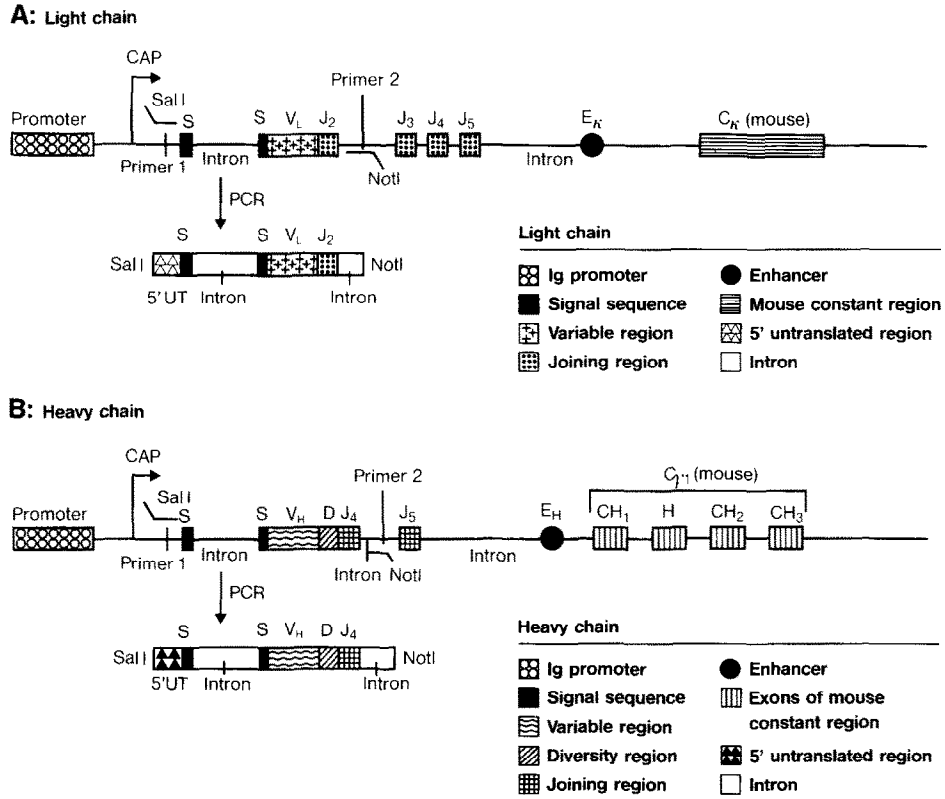

Fig. 2. Schematic representation of the chimerization method. The procedure is shown for the L chain (**A**) and for the H chain (**B**). PCR amplification with primers complementary to the 5'UT region of the mRNA and to intron sequences 3' to the *J* segment used for rearrangement leads to $V_L J$ and $V_H DJ$ fragments with a *SalI* site at their 5' ends and a *NotI* site at their 3' ends for directed cloning into cassette vectors with Ig promoter elements and the appropriate human constant regions. A general outline of the procedure is described in section **a**. *C*, constant region; *CAP*, initiation site for mRNA; *D*, diversity region; E_κ and E_H, enhancers for light and heavy chain; *H*, hinge region; *J*, joining region; *S*, signal sequence; V_L and V_H, variable region of *L* and *H* Ig genes.

regions from DNA of the hybridoma cell line by PCR. The primers (Fig. 1) are designed as follows: (**a**) and (**c**) 5′ primers matching to the 5′UT regions of L and H chain mRNA. They include at their 5′ end a restriction enzyme cleavage site (*Sal*I) useful for force cloning. Primers (**b**) and (**d**) are the 3′ primers matching the intron sequences downstream from the *J* region used for the rearrangement. This is possible because the J loci for the L and H chains of the mouse are completely sequenced (Max, 1981; Sakano et al., 1980). The 3′ primers include a *Not*I cleavage site at their 5′ ends for force cloning of the amplified fragments. As a result of the amplification procedure promoterless V_LJ and V_HDJ fragments are obtained including its intron, the V_LJ or V_HDJ region and intron sequences 3′ from the *J* region used for rearrangement including the splice donor consensus GT (Fig. 2). The resulting amplified V_LJ and V_HDJ fragments are endowed with a *Sal*I site at their 5′ ends and a *Not*I site at their 3′ ends for directed cloning into a cassette vector containing Ig promoter elements and appropriate human constant regions.

(b) Application of the technique to the chimerization of an anti-CD4 antibody

The mAb MT151 (IgG_{2a}) binds to an epitope in the first extracellular loop of the human T lymphocyte surface antigen CD4 (Reinherz et al., 1986). The murine Ab has been successfully used in the treatment of patients with rheumatoid arthritis (Herzog et al., 1987). Therefore, a chimerized, less immunogenic version of this Ab is of great importance for clinical applications. The sequence of the 5′ *UT* and the V regions of the κ and γ_{2a} chains of mAb MT151 was determined as described in the legend to Fig. 3. We identified rearrangement of V_L with the J_2 segment of the L chains and of V_H with J_4 of the H chains. This information enabled us to synthesize 5′ and 3′ primers for amplification of the rearranged *VJ* and *VDJ* segment by PCR (Fig. 1). For the light chain, a 600-bp V_LJ fragment, and for the heavy chain, an approx. 700-bp *VDJ* fragment was amplified (Fig. 3). The fragments with *Sal*I and *Not*I sites were subcloned and the sequences of five isolates for each chain were determined (Sanger et al., 1977). Compared to the cDNA sequence we detected an exchange in one of the five V_LJ isolates as well as in one of the five V_HDJ isolates due to the high error rate of *Taq* polymerase. We have applied the described technique to isolate the V_LJ and V_HDJ segments of the two additional mAbs, one directed against an epitope on human CD4 different from that of mAb MT151 and another binding to the α-chain of the human interleukin-2 receptor. Also for these two mAbs fragments in the range of 600–700 bp were amplified (data not shown). Sequencing of five isolates of subcloned V_LJ and V_HDJ regions for each Ab revealed that the error rate of *Taq* polymerase was comparable to that obtained for mAb

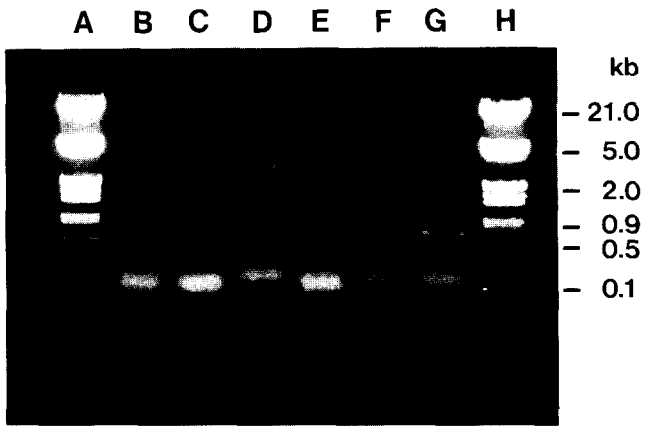

Fig. 3. Amplification of V_LJ and V_HDJ regions encoding mAb MT151 by PCR. Lanes: **A** and **H**, marker fragments; **B** and **F**, control PCR (minus DNA); **C**, amplification with primers **c** and **d**; **D** and **E**, amplification with γ primers different from those shown in Fig. 1; **G**, amplification with κ primers **a** and **b**. The sequences of primers **a**, **b**, **c** and **d** are shown in Fig. 1. **Methods.** For cDNA cloning and sequence determination RNA was isolated from 2×10^7 cells (Chirgwin et al., 1979). The RNA pellet (250 μg) was dissolved in 12.5 μl H_2O, incubated with 375 μl dimethylsulfoxide for 20 min at 45°C, ethanol-precipitated and dissolved in H_2O. Synthesis of cDNA was performed with 200 μg RNA and 20 ng ^{32}P-labelled primers with the first-strand buffer of the BRL cDNA synthesis kit according to the instructions of the manufacturer. κ primer: 5′-GAAGATGGATACAGTTGGTGC. γ primer: 5′-GGCCAGTGGATAGACAGATGG. These primers match the 5′ part of the constant regions of the κ and the γ chain. After treatment of the ss DNA with 50 μg RNase A for 30 min, phenolization and ethanol precipitation, the ssDNAs were size-fractionated on a 5% PA gel and the position of the V_L and V_H fragments visualized by autoradiography. The bands were excised, DNA eluted with 2 M NH_4 · acetate at 37°C, precipitated, and tailed with G residues with terminal deoxynucleotidyl-transferase (Maniatis et al., 1982). After phenol extraction and ethanol precipitation, the ss DNA was dissolved in 10 μl H_2O. 3 μl were used for the following PCR reaction. 10 pmol of 5′ and 3′ oligos were used in a 20 μl reaction in 10 mM Tris · HCl pH 8.4/50 mM KCl/1 mM $MgCl_2$/0.1 mM dNTPs and *Taq* polymerase. 5′ primer: 5′-GCATGCGCGCGGGCCGCGGAGGCCCCCCCCCCCCC (contains sites for *Sac*II, *Sph*I, *Not*I and *Sfi*I; Loh et al., 1989). The 3′ primer contains an *Eco*RI cleavage site near the 5′ end and matches the nt of the 5′ part of the constant regions of κ and γ mRNA. 3′ primer κ: 5′-GGGAATTCTGGTGCAGCATCAGCCC. 3′ primer γ: 5′-GGGAATTCGATAGACAGATGGGGGTG. The reaction was carried out in a Perkin-Elmer cycler with the following conditions: 5 min at 94°C, 2 min at 42°C, five cycles (1 min each) at 72°C, 1 min at 94°C, 1 min at 55°C, 20 cycles at 72°C (1 min each), 10 min at 72°C. An aliquot of the PCR products was size-fractionated on a 1% low-melting-agarose gel, the amplified DNA was eluted, cleaved with *Eco*RI + *Not*I, subcloned and the sequence determined by the dideoxy method (Sanger et al., 1977). Reaction conditions for PCR amplification with primers **a**, **b**, **c** and **d**: 10 mM Tris · HCl pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.1% (w/v) gelatine/1 μg chromosomal DNA/5′ and 3′ primer 1 μg each/dATP, dGTP, dCTP, dTTP 200 μM each/*Taq* polymerase (Boehringer-Mannheim) 2.5 units in a reaction volume of 100 μl. The reaction mixture was overlayered with 100 μl mineral oil (Sigma). The reaction mixture was kept at 92°C for 5 min, then for 2 min at 55°C and 2 min at 72°C. Before the following 26 cycles at 72°C (2 min each) the mixture was kept for 2 min at the temperatures indicated above. 10% of the mix was size-fractionated on a 1.5% low-melting-point agarose gel (0.5 μg ethidium bromide/ml), the amplified DNA was isolated, cleaved with *Sal*I + *Not*I and ethanol-precipitated.

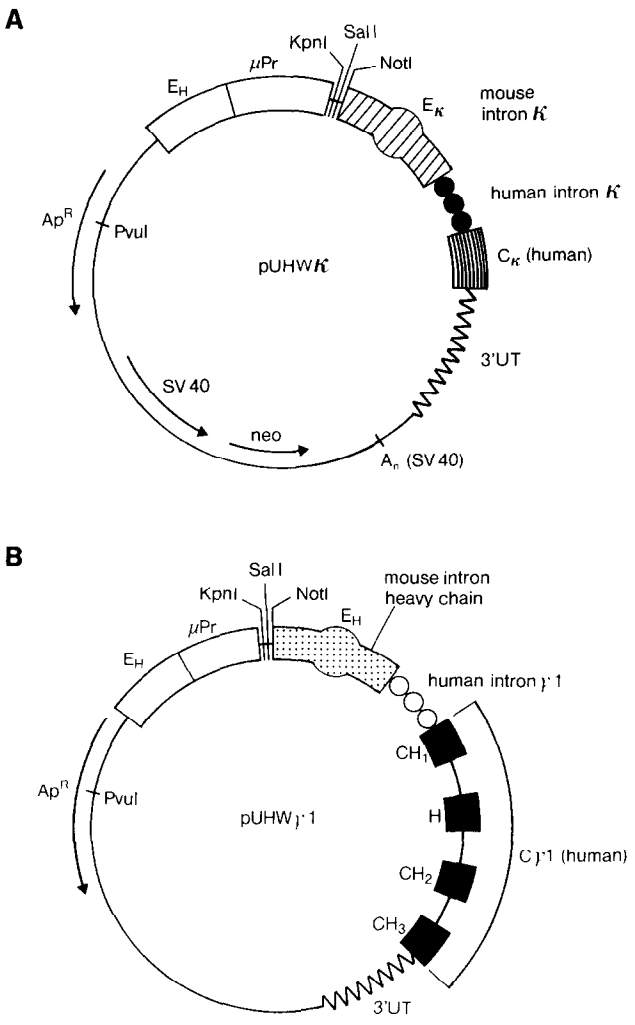

Fig. 4. Expression vectors for chimeric Ab in non-Ig producer hybridoma cells. Amplified V_LJ and V_HDJ fragments encoding mAb MT151 (Fig. 3) were cloned as *Sal*I-*Not*I fragments into cassette vectors pUHWκ (**A**), and pUHWγ1 (**B**) (which were cleaved with *Sal*I + *Not*I) to give rise to vectors pUHW-CD4κ and pUHW-CD4γ1 by forced cloning. As promoter elements both vectors contain a combination of a heavy-chain enhancer (E_H), and a μ gene promoter (μPr) (Weidle et al., 1987). Both vectors contain a polylinker including unique *Kpn*I, *Sal*I and *Not*I sites. Plasmid pUHWκ contains the constant region of a human κ gene and is based on pSV2neo (Southern and Berg, 1982). The plasmid contains a hybrid light-chain intron with mouse and human elements. Plasmid pUHWκ contains the mouse light-chain enhancer region as a 2-kb *Eco*RI-*Asp*700 fragment (Picard and Schaffner, 1984) and a part of the human light chain intron, the human κ exon and 3′*UT* region as a 2.8-kb fragment from pC1 (Klobeck et al., 1984). Plasmid pUHWγ1 is the cassette vector for the insertion of amplified V_HDJ fragments. It contains the mouse heavy-chain enhancer region as a 1.6-kb *Hin*dIII-*Eco*RI fragment (Neuberger, 1983) and part of the human heavy-chain intron, the four exons and three introns and 3′*UT* region of the human γ1 gene as a 7-kb *Hin*dIII fragment (Takahashi et al., 1984). Plasmids pUHW-CD4κ and pUHW-CD4γ1 were linearized at their unique *Pvu*I sites and an equimolar mixture of both plasmids (5 μg per 10^6 cells) was transfected into Sp2/0 cells (Ochi et al., 1983) by electroporation. Stable transfectants were isolated by selection with G418 (1000 μg/ml) as described (Lenz and Weidle, 1990) and reconstituted Ab was quantified by ELISA. μPr, promoter of a mouse μ gene; E_κ and E_H, enhancers of light and heavy chains of the mouse, respectively; 3′*UT*, 3′ untranslated region; A_n(SV40), polyadenylation signal derived from simian virus 40; H, exon coding for hinge region; Ch_1, CH_2, CH_3, exons of the human γ1 gene. Arrows indicate the direction of transcription.

MT151. The size of the intron which separates the signal sequences was in the range between 60 and 120 nt, when the six isolates of the three mAbs were compared (data not shown). All three mAbs make use of different *J* segments. From these observations we conclude that the method is of general applicability. After sequencing, the amplified *Sal*I-*Not*I fragments (V_LJ and V_HDJ) of mAb MT151 were force-cloned into cassette vectors pUHWκ (with a human κ constant region) and pUHWγ1 (with a human γ1 constant region). The vectors are displayed in Fig. 4. Both vectors make use of heavy-chain enhancer and promoter elements at the 5′ end of the V_LJ and V_HDJ fragments to be inserted by force-cloning and can be linearized at their unique *Pvu*I sites in the Ap^R gene before transfection into hybridoma cells. Plasmids pUHWκCD4 and pUHWγCD4 obtained after ligation of the appropriate V_LJ and V_HDJ fragments into *pUHW*κ and *pUHW*γ1 were transfected into Sp2/0 cells (Ochi et al., 1983) and stable transformants were isolated by G418 selection. Transformants expressing up to 10 μg/ml of chimerized Ab per 10^6 cells and 24 h were obtained.

(c) Conclusions

(*1*) We demonstrated for the first time the feasibility of cloning rearranged genomic V_LJ and V_HDJ segments of Ab with PCR and nondegenerate oligos.

(*2*) After the PCR, the amplified promoterless fragments can be directionally cloned into cassette vectors carrying the appropriate human constant regions.

(*3*) The method seems to be of general applicability because it has been applied successfully to the chimerization of Ab from three different hybridoma cell lines.

(*4*) Contrary to previous approaches making use of cDNA cloning, mutation and insertion into expression vectors, authentic V_LJ and V_HDJ genomic segments are isolated by our method.

(*5*) High-level synthesis of chimerized Ab was monitored after transfection of expression vectors with human κ and γ1 constant regions and the V_LJ and V_HDJ regions of the murine Ab into non-Ig-producer myeloma cells.

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