Vectors for the expression of PCR-amplified immunoglobulin variable domains with human constant regions

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ABSTRACT
Cassette vectors have been constructed for mammalian expression of complete Immunoglobulin heavy and light chain genes whose variable regions are produced by the polymerase chain reaction (PCR). The light and heavy chain vectors have promoter, leader, partial intron, enhancer and constant region segments within modified pSV2-gpt and pSV2-neo plasmids, respectively. Variable (V) regions are obtained by PCR using a two step process: 1) the V gene is amplified from genomic or cDNA, cloned into an intermediate vector and sequenced; 2) the first PCR product serves as the template for a second amplification in which restriction enzyme recognition sites and limited flanking intron sequence are added. The second PCR product is inserted into the expression vector, which is then transfected into mouse myeloma cells. These vectors contain human constant regions and may be used to express chimeric, humanized or human Ig genes. This report describes the design of these vectors and their application for the expression of chimeric 60.3, an anti-CD18 antibody.

INTRODUCTION
Recombinant monoclonal antibodies are currently being clinically tested as therapeutic agents. However, repeated clinical treatment with non-human antibodies induces an adverse anti-immunoglobulin response (e.g., human anti-mouse antibody or HAMA [1,2]). To reduce this type of response, it becomes necessary to express potential therapeutic antibodies as either chimeric, humanized, or human monoclonal [3,4]. This usually requires the cloning of variable (V) region genes. To ensure the proper folding of recombinant immunoglobulin (Ig) molecules for applications where whole antibody is preferred, it is beneficial to utilize vectors suitable for eucaryotic expression over those that employ prokaryotic cells [5]. Although there are several examples of transient expression vectors available for use in eucaryotic cells [6–8], stable expression systems are most appropriate for manufacturing applications.

Previously, most vectors for stable expression of Ig genes required cloning relatively large genomic DNA fragments [9,10]. We have designed new vectors that accept V genes obtained by a rapid PCR based approach, thus avoiding the time consuming preparation and screening of a cDNA or genomic DNA library. When V genes are amplified from cDNA, they are deficient in promoter and intron sequences, which can be important for efficient expression. Depending on the primers used, amplified V genes also may lack all or part of leader (signal peptide) sequence. Our new cassette vectors are designed to provide all of these essential elements: promoter, leader, intron, enhancer and constant (C) region gene. To use these vectors, V genes are amplified from cDNA, the PCR product is cloned into an intermediate vector and sequenced. Prior to cloning into the expression vectors, V genes are modified by PCR to include restriction sites and splice recognition sequences.

This methodology was used to clone and express 60.3 as a chimeric Ab. 60.3 is a mouse monoclonal antibody (IgG2a, x) which recognizes a functional epitope on the CD18 component of leukocyte integrins [11,12]. It prevents adherence and aggregation of polymorphonuclear leukocytes, thus blocking neutrophil mediated damage during shock and reperfusion injury. Because of its clinical value, it would be advantageous to express 60.3 in chimeric (chi60.3) and humanized forms. This report describes the cloning of 60.3 heavy (H) and light (L) chain V regions into our cassette vectors and their stable expression by murine myeloma cells.

MATERIALS AND METHODS
Reagents
Unless specifically noted in the text, the chemical and biological reagents used in this study were purchased from Boehringer Mannheim (Indianapolis, IN).

Cells
P3X63Ag8.653 (Ag8.653) is an established mouse myeloma cell line (ATCC CRL 1580) that does not produce detectable H chain Ig. Ag8.653 cells produce a mRNA encoding an aberrant L chain.

* Z17330, Z17336 and Z17370
When using an Ag8.653 derived hybridoma as the source of mRNA, the deduced sequence should be compared with that of the Ag8.653 L chain.

Construction of pGx.11 (Figure 1)

Hind III and Bgl II sites were deleted from the 5' end of the Ecopt gene in pSV2-gpt [13] by restriction with Hind III and Bgl II, filling in with Klenow polymerase, and religation (this 121 bp fragment is not required for expression of Ecopt gene). The Eco I to Pvu II fragment containing pBR322 sequences was substituted with the analogous portion of pUC18 (Pvu II—Pvu II fragment; the Eco I overhang was filled in with Klenow polymerase prior to ligation with a Pvu II site, thus regenerating the Eco I site) to form pG.3. This was done to increase the yields of plasmid DNA [14].

pG.5 was constructed from pG.3 by replacement of a 750 bp Eco I—BamH I fragment with the 64 bp Eco I—BamH I multiple cloning site (MCS) from plC20R [15]. To create pG.12, a Not I site was inserted at the Nde I site of pG.5 using oligonucleotide linkers:

5'-TAGCGGCCGCA-3'
3'-CGCCGGCGTAT-5'

The Not I site was used for linearization of plasmid DNA prior to transfection. A 2.75 kb Eco R I fragment containing the human Cx gene was placed into the Eco R I site of the MCS of pG.12 to form pGx.3.

pG.9 was assembled from pG.3 by removal of a 140 bp portion of the SV40 enhancer from pG.3. This was achieved by fill in and ligation of Sp H I and Pvu II sites. (pG.9 was constructed for unrelated experiments; as is apparent from Figure 1, the final
Figure 2. Construction of heavy chain vector pNγ1.12. See Materials and Methods for details of construction. Below the name of each construct are the distinguishing characteristics of that vector. The direction of transcription is indicated by arrows. Restriction site abbreviations are: A, Asp718 I; B, BamH I; C, Cla I; E, EcoR I; Ev, EcoR V; H, Hind III; S, Sac I; Sa, Sal I; Xh, Xho I. Regions of the vectors are abbreviated as follows: amp, ampicillin resistance gene; neo, G418 and neomycin resistance gene; SV40 E, SV40 enhancer; MCS, multiple cloning site; Cyl, exons and introns encoding human Cγ1 gene, residing in a 2.8 kb Hind III—BamH I fragment; MHE, mouse heavy chain enhancer, 0.7 kb Xba I—EcoR I fragment; ori, bacterial origin of replication; Pro/L, PCR fragment containing promoter, leader and a portion of the L-V intron from L6 heavy chain.

vector, pGx.11, could have been more simply constructed without removal of the SV40 enhancer.) Subsequently, a Not I site was inserted at the Nde I site of pG.9 to form pG.10. The 195 bp Not I to BamH I fragment from pG.12 was put into the same sites of pG.10 to make pG.11. This replaced an 879 bp fragment by the 195 bp fragment consisting of the pIC20R MCS. The 3 kb Nar I—Cla I fragment of pGx.3, containing human Cγ1, was directionally subcloned into the same sites of pG.11 to create pGx.4. A 1 kb fragment containing the mouse heavy chain enhancer (MHE) was transferred from pICMHEXX to pGx.4 as a Cla I to Hind III fragment, thus producing pGx.5. pICMHEXX was made by insertion of the 1 kb Xba I fragment (filled in with Klenow polymerase) from RBL 216 [16] into the filled in Bgl II site of pIC19R [15]. A 579 bp Sau3A I fragment that included the 4B9 (human Group B Streptococcus Ab) promoter [17] from pGxA1.9 [18] was placed into the BamH I site of the MCS in pGx.5 to construct pGx.11. The following unique restriction sites between the leader sequence and the MHE are available for addition of Vγ genes: Sal I, Sse8387 I, Hind III, Nru I, and Xho I.

Construction of pNγ1.12 and pNγ1.16 (Figure 2)

The Hind III site in pSV2-neo [19] was destroyed by digestion with Hind III, filling in with Klenow polymerase and religation. The 750 bp EcoR I—BamH I fragment was replaced by the 64 bp EcoR I to BamH I polyn linker from pIC20R to form pN.5.

The 695 bp Xba I to EcoR I region of the MHE was amplified by PCR using primers which deleted EcoR I and Xba I sites, but provided restriction sites for EcoR V, Sac I, Asp 718 I, and Xho I at the 5' end and sites for Hind III, Sal I and BamH I at the 3' end. This PCR product was cloned into pIC20R to produce pMHE.pcr. The 723 bp EcoR V to BamH I fragment from pMHE.pcr was ligated into the same sites in the MCS region of pN.5 creating pN.8.

A 699 bp fragment comprised of promoter, leader, and intron sequences [20] from the L6 (a murine anti-tumor mAb [21]) H chain locus was amplified by PCR using primers which added recognition sequences for EcoR V and Sac I at the ends. After DNA sequence verification in an intermediate vector, the promoter/leader fragment was introduced into EcoR V and Sac I sites of pN.8 to produce pN.11.

A 2.8 kb Hind III to BamH I fragment containing the human γ1 gene [22] was inserted into the same sites of pN.11 to construct pNγ1.12. Vector pNγ1.16 is identical to pNγ1.12 except several mutations were introduced into the promoter (see Results and Discussion). The H chain vectors have the following unique restriction sites for insertion of VH genes: Asp718 I/Kpn I, EcoRI/Xba I, and Xho I. Potential sites for linearization of plasmids are EcoR I, Cla I, and EcoR V.

Amplification of V genes by PCR

Total cellular RNA was extracted from the 60.3 hybridoma [11] according to the method of Davis et al [23]. First strand cDNA was synthesized according to the manufacturer's instructions by using an oligo(dT) primer (Invitrogen, San Diego, CA). cDNAs of 60.3 H and L chains were amplified by PCR using degenerate primers complimentary to the framework 1 and constant regions [24,25], which were modified to facilitate cloning. Xho I sites
were used at the 5' ends of the sense heavy (MH-SP-ALT.1 or MH-SP-ALT.2) and light (EcoRI/FRI-ML(k)) chain primers. Pst I sites were utilized for antisense primers (MH-gamma-CONST and HindIII/ML(k)CONST).

For both H and L chains, PCR products were cloned into pUC18 using either the Xho I and Pst I sites or by blunt ended ligation into the Smal site. Ligation products were used to transform competent E.coli DH5α. Clones containing 0.5 kb Ecor I–Sal I fragments were subjected to double stranded DNA sequencing [26] with Sequenase (U.S. Biochemicals, Cleveland, OH). Products obtained by blunt ended ligation were used in the next step.

Insertion of V genes into expression vectors

In order to clone the amplified V genes into pGx.11 and pNγ1.16, respectively, they became templates for a second L chain cloning. Clones containing 0.5 kb EcoR I–Sal I fragments were subjected to double stranded DNA sequencing and used as templates for a second L chain cloning. Clones containing 0.5 kb EcoR I–Sal I fragments were subjected to double stranded DNA sequencing [26] with Sequenase (U.S. Biochemicals, Cleveland, OH). Products obtained by blunt ended ligation were used in the next step.

**RESULTS AND DISCUSSION**

**Design of vectors**

High level expression of Ig from genomic DNA constructs transfected into murine myeloma cell lines has been well documented [5], while Liu et al [28] and others [29] have reported weak expression of antibody using cDNA constructs. It is known that an Ig promoter/enhancer pair can elicit efficient expression in mouse myeloma cells. Furthermore, there is considerable evidence that introns are essential for the efficient expression of Ig genes [30,31]. Recently, one group described a kappa vector that omitted both the leader-V and J-C introns [32]; expression was not observed until the J-C intron was incorporated in their vector. Since non-coding intragenic sequences may have a positive influence on expression, our vectors were designed to mimic the genomic structure of rearranged Ig genes. The arrangement of these genes in a 5' to 3' direction is: promoter, leader exon 1, leader-V (V-D-J) exon, J-C intron, either a C gene exon (ι and λ genes) or exons and introns (μ, δ, γ, ε, and α genes), and 3' untranslated region. These components are transcribed as a unit, with the precursor mRNA being processed and translated into protein.

As shown schematically in Figure 3, genomic Ig DNA fragments have been assembled into modified versions of pSV2-gpt (containing EcoR gene [13]) and pSV2-neo (encoding neomycin and G418 resistance [19]) for our light and heavy chain
Figure 3. Components of the cassette vectors and amplified V genes. Schematic representation of the amplified V gene, the cassette vector, and the expression vector. The parts of the cassette vector contained within modified pSV2-gpt and pSV2-neo plasmids are: the Pro/L section [•]; a MCS [III] for insertion of the V gene; the MHE [II]; and the C region [□]. The component which includes the amplified L2-V(D)J exon [□] is inserted into the MCS of the cassette vector to form the final expression vector.

Figure 4. Comparison of the Pro/L sections of pNγ1.12 and pNγ1.16. Schematic representations of the Pro/L regions of pNγ1.12 (containing the wild type promoter) and pNγ1.16 (mutated promoter). A base in the pNγ1.16 sequence that is identical to one found in pNγ1.12 is depicted by a single dot (•). The names of significant regions are given above the pNγ1.12 sequence. Restriction sites and sequence motifs are enclosed within boxes. Consensus sequences of the motifs are presented below the boxes. Abbreviations for the nucleotides: A: adenine; C: cytosine; G: guanine; T: thymine; W: A or T; S: C or G; M: A or C; R: A or G. The leader sequence (LI) is underlined.

vectors, respectively. The components grouped 5' to 3' within the modified pSV2 vectors are: 1) an Ig promoter/leader sequence region (Pro/L; which includes partial intron sequence); 2) a MCS for subcloning in the V gene of interest; 3) the MHE [33] and 4) a human C region gene (C or C7).

The component farthest upstream is an Ig promoter. It has been shown that the combination of octamer motif (ATTGTCAT and ATGCAAT for the light and heavy chain, respectively) and TATA box element in the promoter are minimal requirements for the expression of Ig genes in lymphoid cells [34,35]. The H chain promoter frequently contains a heptamer motif immediately 5' of the octamer [36]. The heptamer has been shown to enhance Ig H promoter activity in combination with the octamer and TATA box motifs [37]. All of these elements are within 100 nt of the RNA start site; this value was chosen as the minimal Ig promoter length necessary for our expression vectors.

The Ig promoter is followed by the leader sequence that encodes the signal peptide. Although signal sequences lack sequence homology between each other, they do share structural similarity and are capable of facilitating the export of heterologous proteins [38]. In Ig genes, the leader sequence is composed of two exons. Leader exon 1 (L1) encompasses all but the last 11 nucleotides that encode the signal peptide. These 11 nucleotides (L2) follow the L-V intron and form the initial part of the L2-V(D)J exon. In our design, L1 is incorporated as a component of the vectors, whereas L2 is added as part of of the V(D)J gene.

In our cassette vectors the promoter, L1, and the 5' portion of the L-V intron are integral parts of the Pro/L region (Figure 3). Sequences for Pro/L sections were taken from genomic constructs whose expression resulted in high levels of Ab production (> 100 μg/ml after cloning). The Pro/L segments from L6 [20,21] and 4B9 [17,18] were used in H and L chain cassette vectors, respectively.

Restriction sites were added by PCR to both ends of the L6 Pro/L region, which included 607 nt upstream of the initiator methionine. In reviewing the sequence of this region, we noticed several mismatches to consensus sequences that may influence expression levels. We chose to 'correct' these mismatches and determine whether the mutated promoter (in vector pNγ1.16) performed differently than the wild type L6 promoter (in vector pNγ1.12). Bases were altered by the PCR based SOEing (splicing
by overlap extension) technique [39]. The mutations are shown schematically in Figure 4 and include addition of a matrix association region (MAR), alterations to the TATA box, splice donor signal and heptamer motifs, and removal of an inverted TATA box and its associated initiator methionine. MARs (often located within or near a topoisomerase II site) have been shown to stimulate several-fold the transcription of transfected Ig genes [40]. The role of the TATA box [34,35,41] in transcription has been well documented. Furthermore, the presence of an inverted TATA box upstream of the octamer may interfere with transcription in the forward direction [42]. A heptamer motif is frequently found upstream of the octamer and may positively influence expression [36,37]. Modifications to the splice donor signal [43] could alter both the rate of RNA splicing and the rate of signal peptide cleavage [44] since the mutated sequence encodes a Gly instead of Ser at the —4 position.

The 4B9 Pro/L includes 402 nt upstream of the initiator methionine and is in a Sau3A I fragment containing L1 and some intron sequences. Interestingly, there is a single nt mismatch within the octamer consensus motif (CTTTCGAT vs ATTTTCGAT). The CTTTCGAT is characteristic of the human Vκ region subgroup. The significance of this disparity is not known, but it does not appear to interfere with transcription of transfected Ig genes [45]. As with the heavy chain promoter, a mutated version of this promoter has been prepared; however, it has not yet been tested.

In the L-V intron there are three important sequence motifs involved in splicing: a splice donor site, branch point lariat motif and a splice acceptor site. There is evidence that a minimum of 50 bp of intron sequence between the splice donor site and the branch point lariat signal is necessary for efficient splicing [46]. More than 50 bp of L-V intron sequence has been included in both of our expression vectors. In the L and H chain cassette vectors, following L1 are the 5' region of the L-V intron and the 3' segment of the J-C intron. The two partial introns are joined by a polylinker that accepts the amplified V region; provided on this PCR product are the L2-V(D)J exon and the remaining portions of flanking introns.

In both of our vectors, the MHE follows a MCS and is included in the 3' part of the partial J-C intron component. The 1 kb Xba I fragment is used for the L chain vector, and the 0.7 kb Xba I to EcoR I fragment for H chain cassette vectors; this truncates unnecessary intronic sequence flanking the enhancer [47]. These fragments are functionally equivalent in myeloma cells and were chosen for compatible restriction sites.

In our cassette vectors, the enhancer is followed by 3' intron sequence that includes a branch point consensus sequence and an acceptor site for splicing to the C region. This intron sequence is provided by the DNA fragment containing either the x or γ1 genomic C region gene. This fragment also has 3' untranslated sequence including the polyadenylation signal [48]. The C gene in H chain vectors can be switched to g2, γ3, or γ4 by substituting the Hind III—BamH I fragments, whereas the C regions of other heavy chain isotypes can be modified to have flanking Hind III and BamH I sites, and then inserted into this location.

**Preparation of V genes for cloning into expression vectors**

For insertion into our expression vectors, the V gene must be flanked by L-V and J-C intron sequences on the 5' and 3' sides, respectively. In an ideal case, this would be accomplished with a single round of PCR using a cDNA template and degenerate primers. The sense primer would contain a restriction enzyme site, intron sequence, and degenerate sequence for annealing to the 5' part of the L2-V(D)J region. The antisense primer would include a restriction site, intron sequence, and degenerate sequence for the 3' end of the J regions. However, the low efficiency in the synthesis of long degenerate oligonucleotides makes this approach impractical. Moreover, a second PCR is necessitated by the use of either signal peptide or constant region primers, since intron sequences must be provided 5' and 3' of the L2-V(D)J exon.

With our method, two PCR steps are used to obtain the V gene insert. In the first step, the V(D)J region is amplified using degenerate primers from either the leader or framework regions (sense orientation) and from the constant region (anti-sense orientation). The use of sense primers from the leader region is preferable because oligonucleotides homologous to framework region 1 (FR1) may cause mutations in the V gene. Following sequence determination, a set of non-degenerate primers can be designed to provide intron and coding sequences, as described above. Either the original cDNA or the product of the first PCR can be used as template for the second PCR.

The cDNAs of 60.3 V genes were made from mRNA, and then amplified by PCR with degenerate framework primers as described in Materials and Methods. Amplified V genes were cloned into an intermediate vector for sequencing. Initially, this information was used to select clones containing V-gene like open reading frames. The sequences of several independent clones were then compared to each other to eliminate those with misincorporations caused by reverse transcriptase [49] and/or Taq polymerase I [50]. After obtaining the DNA sequences, the deduced amino acid sequences of 60.3 V genes were compared to known murine Ig sequences [51] and to the N-terminal amino acid sequence obtained by Edman degradation. The 60.3 V and Vκ domains belong to subgroups IIb and III, respectively. Sequences will be reported in another publication (Hsiao et al, manuscript in preparation).

After the initial sequence determination, the V genes were amplified again, with the addition of flanking intron sequences that are required by the expression vectors. The sense oligonucleotide includes in a 5' to 3' direction: (1) an enzyme site for cloning into the MCS of the cassette vector; (2) intron sequence containing the branch point lariat motif and a polypuridine tract [52]; preceding (3) a splice acceptor site [43]; (4) the last 11 nucleotides that encode the signal peptide (L2); and (5) the 5' portion of the V(D)J gene. The 11 nucleotides in (4) are derived from 4B9 and L6 (for L and H chains, respectively) and encode amino acids which conform to the rules for signal sequence cleavage sites (e.g., -3, -1 rule [44]). Only section 5(4) of the primer is homologous to and anneals with the template.

In a 5' to 3' direction, the anti-sense primer supplies: (1) a second cloning site; (2) intron sequence that has a splice donor site for joining to the C region gene; and (3) a portion of the 3' end of the V(D)J gene. Only region (3) anneals to the template. Partial introns included in the primer sequences are derived from either genomic 4B9 DNA or L6 DNA for the L and H chain constructs, respectively. A stretch of 4 to 10 extra nucleotides can be found at the 5' end of each primer to allow the enzymes to cut more efficiently. The sequences of PCR products from the second step were verified prior to their insertion into expression vectors. Alternatively, these PCR products can be cloned directly into the expression vectors and sequence can be confirmed using primers from the expression vector.
mutations could have enabled the transfectomas to express more
mutations to the heavy chain promoter in pNyl.16. Further experiments
splicing, or cleavage of the signal peptide. Further experiments
up to 10 ng/ml of humanized 60.3 Ab.

In a separate experiment, the same light chain vector and the
promotion of 3 heavy chain vector were used for the
expression of humanized 60.3 (Hsiao et al, manuscript in
preparation). After two rounds of limiting dilution cloning and
expression of humanized 60.3 Ab (Figure 5).

Expression

Plasmid constructs with chimeric H (with either the wild type
or mutated promoter) and L chain were cotransfected by
electroporation into Ag8.653 mouse myeloma cells, and then
selected for expression of the neomycin resistance gene (as
described in Materials and Methods). Two weeks post
transfection, the presence of the chimeric Ab in culture
supernatants was determined by ELISA. As seen in Table I, the
heavy chain vector with the mutated promoter (pNyl.1.16) gave
significantly better results (more master well supernatants
consisting of 20 ng of reduced Ab were analysed by SDS-PAGE on
a 4–20% gradient gel. Proteins were transferred to nitrocellulose membranes
and reacted with specific HRPO-conjugated goat antibodies as follows: A) anti-
murine IgG2a heavy chain; B) anti-human IgG heavy chain; C) anti-murine x
light chain; and D) anti-human x light chain. Lanes 1: culture supernatant from
Ag8.653 cell line; lanes 2: culture supernatant from murine 60.3 hybridoma; lanes
3: culture supernatant from transformants produced with light chain vector
(pGx.11) and mutated (pNyl.1.16) heavy chain vector; lanes 4: culture supernatant
from transformans produced with light chain vector (pGx.11) and wild type
(pNyl.1.12) heavy chain vector.

Table I. Comparison of vectors with mutated and wild type promoters

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<th>Promoter</th>
<th>Antibody Concentration (ng/ml)</th>
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<td>wild type</td>
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<td>mutated</td>
<td>852</td>
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Ag8.653 cells were transfected as described in Materials and Methods with 10
µg pGx.11 and either 10 µg pNyl.1.12 (heavy chain vector with wild-type promoter)
or 10 µg pNyl.1.16 (heavy chain vector with mutated promoter). Cells were plated
at 2 x 10^5/well in media containing G418. Two weeks post transfection, culture
supernatants (diluted 1 to 50) were screened for Ig by ELISA. Numbers indicate
the quantity of master wells (out of 960) with the indicated antibody concentration.

In similar experiments where the cells were plated at lower density, outgrowth
with the two heavy chain vectors was equivalent. Control, Ag8.653 cells
electroporated in the absence of DNA did not survive the selection with G418.
These experiments were repeated three times with similar results.

The size and immunoreactivity of the chimeric antibody in
culture supernatants was determined by Western Blot analysis
(Figure 5). Reduced chimeric proteins were detected by specific
reagents (HRPO labelled anti-human x and HRPO labelled
goat anti-human γ). Only one band each was detected with the
H and L chain reagents. These bands were similar size to the
murine controls (detected with reagents specific for murine
antibodies). In addition, FACS assays indicated that chimeric 60.3
Ab binds as well as the murine 60.3 Ab to CD18 positive cells
(Hsiao et al, manuscript in preparation).

Comparison with other vectors

Many vectors have been developed for the expression of Ig genes
where the V region was obtained in the absence of a promoter
(i.e., from cDNA or by PCR). These vectors fall into two general
classifications: 1) transient expression vectors [6–8] that can
generate in a short time sufficient antibody for experimentation;
and 2) stable expression vectors which are more appropriate for
manufacturing purposes. Most of the vectors currently available
for stable expression require the investigator to provide a leader
sequence along with the V region [53–58]. This may be achieved
when degenerate leader region primers are employed to amplify
the V gene. However, in cases where the V gene can not be
obtained with leader sense primers, it may be necessary to take
advantage of degenerate primers homologous to FRI. To use
these primers with certain vectors, the signal sequence, with or
without L-V intron, must be added during amplification of the
V gene. Two groups [59,60] describe vectors containing leader
sequences, but they clone V genes into their vectors using
restriction sites in FR1. This causes several amino acids to be
encoded by the vector and/or primer and may result in
modifications of the original sequence. Since our vectors include
the leader sequence plus a partial L-V intron, the V gene is
inserted without alteration, by using restriction sites in the intron.
Equally important, our vectors permit the flexibility of using
degenerate sense primers from either the leader sequence or from
FR1 of the V gene. If FR1 primers are required, the deduced
amino acid code can be compared with the actual sequence
information obtained by N terminal analysis. When necessary,
the nucleotide sequence is modified to correct any discrepancies.

Figure 5. Western blot analysis of murine and chimeric 60.3 antibodies. Culture
supernatants containing 20 ng of reduced Ab were analysed by SDS-PAGE on
a 4–20% gradient gel. Proteins were transferred to nitrocellulose membranes
and reacted with specific HRPO-conjugated goat antibodies as follows: A) anti-
murine IgG2a heavy chain; B) anti-human IgG heavy chain; C) anti-murine x
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Ag8.653 cell line; lanes 2: culture supernatant from murine 60.3 hybridoma; lanes
3: culture supernatant from transformants produced with light chain vector
(pGx.11) and mutated (pNyl.1.16) heavy chain vector; lanes 4: culture supernatant
from transformants produced with light chain vector (pGx.11) and wild type
(pNyl.1.12) heavy chain vector.
SUMMARY

The vectors presented in this paper represent a rapid method for the construction of chimeric, humanized, or human antibodies. Our vectors can be used for expressing Ig without the loss of antibody affinity or specificity (Hisao et al, manuscript in preparation). The L and H chain expression vectors preserve the genomic organization of rearranged Ig genes by utilizing an Ig promoter plus enhancer and by including intron sequences. cDNAs were prepared from mRNA templates derived from the 60.3 hybridoma cell line. Resultant cDNAs were amplified by PCR, their products sequenced, amplified again with the addition of introns and sequenced a second time, then subcloned into our mammalian expression cassette vectors for subsequent transfection into a mouse myeloma cell line. The rate limiting step of this methodology is the requirement to sequence several independent clones after each PCR. This ensures that the fidelity of the V gene sequence has not been compromised due to possible errors introduced either from single round copying of cDNA from mRNA with reverse transcriptase, or from amplification rounds with Taq polymerase I. However, this sequencing effort is minimal relative to the classical preparation of chimeric antibodies which involved first generating then screening a genomic or cDNA library.

Without changing the overall structure of the heavy chain vector, we have introduced several mutations into the promoter/leader region: addition of a matrix associated region, removal of a reverse TATA box and associated initiator methionine, and mutations resulting in sequences which better conform to the consensus for TATA box, heptamer motif, and splicing recognition signals. One or more of these mutations led to an increase in the number of transformants secreting higher levels of Ig (>300 ng/ml in master well culture supernatants). We are investigating the mechanism and specific mutation(s) which allowed this increase.

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