An internal ribosome binding site can be used to select for homologous recombinants at an immunoglobulin heavy-chain locus
(chimeric antibody/homologous recombination/translation initiation/class switching)

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ABSTRACT The encephalomyocarditis virus (EMCV) leader sequence is responsible for efficient, cap-independent translation initiation from the viral RNA. It has been used to increase the expression of internal coding regions on polycistronic mRNA encoded by recombinant DNA constructs. We have designed a sequence-replacement-type vector for targeting to immunoglobulin heavy-chain loci in hybridoma cells. Homologous recombination of this vector introduces a human γ1 constant-region sequence linked to the EMCV leader and a neomycin phosphotransferase (neo) gene. The resulting cells express a bicistronic mRNA encoding at the 5’ end a chimeric murine VDJμ-human Cμ heavy chain, followed by neo linked to the internal ribosome binding site provided by the EMCV leader. These homologous recombinants express the chimeric heavy chain at levels equivalent to the heavy chain in the parental hybridoma. This strategy of using an EMCV-neo cassette to obtain efficient selectable marker gene expression has potential application to a range of gene targeting vectors.

The alteration of specific chromosomal sequences in mammalian cells by homologous recombination with transfected DNA has proven to be a powerful tool in biology (1–6). Many of these studies have aimed to inactivate an allele of a specific target gene by inserting a selectable drug marker such as the neomycin phosphotransferase gene (neo) in the same transcriptional frame. This can allow selection for expression of the product of the neo-target gene fusion. Such neo fusion products have frequently been found to confer drug resistance, but the target gene is interrupted and rendered dysfunctional. In some instances, homologous recombination has been used to introduce or correct point mutations in target genes or to create specific fusion genes (7–10).

The nontranslated leader sequences of the picornaviruses, including poliovirus and encephalomyocarditis virus (EMCV), function as sites for efficient cap-independent initiation of translation (11, 12). These sequences have been exploited to generate mammalian cell expression vectors, with an EMCV leader–selectable marker gene cassette placed downstream of a particular gene on a single bicistronic transcription unit (13). We reasoned that an EMCV leader–neo cassette could be used to construct sequence-replacement-type vectors for homologous recombination. The EMCV sequence could be used to permit the independent translation of the marker gene on the same transcription unit as the target gene. Therefore, the interruption of the target gene would not be required, and, for example, point mutations could be introduced into the target gene. An independent promoter for the marker gene would also not be required, thereby possibly lowering the background of non-homologous recombinant transfecants.

We have designed a sequence-replacement vector incorporating an EMCV leader–neo cassette that can be used to class-switch immunoglobulin heavy-chain genes. We have found efficient targeting of this vector and the expression of a mouse–human chimeric heavy chain at levels equivalent to the original murine heavy chain in the homologous recombinants.

MATERIALS AND METHODS

Cells and Electroporation. The F8/6.4.6 cell line (provided by P. J. Sztkut and W. B. Foster, Genetics Institute) is a murine hybridoma derived from BALB/c spleen cells and the P3X63-Ag8.653 plasmacytoma fusion partner (14). The IgM(κ) antibody produced by this hybridoma is directed against the light chain of human factor VIII. The cells were maintained in RPMI 1640 (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Flow Laboratories) and 1 mM glutamine. The 10A1C6 Chinese hamster ovary cell line, which expresses recombinant human factor VIII, was grown as described (15). Electroporation was carried out with 2 × 10⁶ cells in 1 ml as described (16). After ≈48 hr of growth, the neomycin analogue G418 (GIBCO) was added to a final concentration of 1 mg/ml.

Nucleic Acid Manipulations. All DNA manipulations were carried out as described (17). The human γ1 constant-region (Cγ1) sequence was cloned from a human lung fibroblast genomic library in λFIX (Stratagene). The predicted constant-region amino acid sequence of this clone was found to be identical to that previously reported (18), with only three exceptions: at position 97 of Cγ1, a change from lysine to arginine, and at positions 15 and 17 of Cγ3, a change from glutamic acid to aspartic acid and from methionine to leucine, respectively. A fragment derived from this genomic clone containing the complete constant-region sequence, extending from 211 base pairs (bp) upstream of the Cγ1 exon to 11 bp downstream of the stop codon, was cloned into Sal I-digested pBluescript KS+ (Stratagene) to form pγX12. This clone was oriented such that the EcoRI site of the vector polylinker was located upstream of the γ1 insert, and the vector Xho I site was located adjacent to the γ1 stop codon. The EMCV–neo cassette used was derived from p2BlSneo (C.R.W., M. Davies, and R.J.K., unpublished work). This cassette was excised on a Sal I–BamHI fragment consisting of the EMCV leader sequence, as used previously (13), and an ≈0.9-kilobase (kb) neo sequence derived from the Mlu I–BamHI neo fragment of pMC1neo (19). The genomic murine μ sequence was derived from pμ20. An EcoRI–Xho I μ fragment from pμ was cloned into EcoRI/Xho I-digested pBluescript KS+ (Stratagene) to form pμ15. The ≈1.1-kb Nae I–EcoRI fragment downstream of the Jμ heavy chain genomic sequence was cloned into pμ15 that had been digested with Not I ("filled-

Abbreviations: EMCV, encephalomyocarditis virus; SV40, simian virus 40.

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in" with the Klenow fragment of *Escherichia coli* DNA polymerase I) and EcoRI, to form pV3.

Plasmid pV3 was digested with EcoRI and ligated with three DNA fragments: (i) a 1.82-kb EcoRI–XhoI fragment of the human *Cγ1*, derived from pYX12; (ii) the EMCV-neo cassette on a 1.47-kb SalI–BamHI fragment; and (iii) a 206-bp BamHI–EcoRI fragment containing the simian virus 40 (SV40) polyadenylation signal sequence. The product of this ligation, pHRI, is shown in Fig. 1a. Prior to electroporation, pHRI was digested with *Xho*I.

The polymerase chain reaction (PCR) was carried out with reagents and a thermal cycler from Perkin-Elmer/Cetus. PCR was performed on genomic DNA, using the oligodeoxynucleotide primers USNAEMU (5'-GCAAGCTTGCAGTATTTGCCC-3') and HGAM-DS (5'-CATGATCTGGCCCAAGGGTCCAGGCG-3') for 30 cycles of 1 min at 94°C, 2 min at 69°C, and 3 min at 72°C.

**Enzyme-Linked Immunosorbent Assays.** ELISAs were carried out as described (16, 21, 22). The murine *μ*–specific ELISA (16) was calibrated with purified F8/6.4.6 hybridoma IgM. The human *γ*-specific ELISA used goat anti-human IgG1 (γ-chain-specific) conjugated to hors eradish peroxidase (Zymed Laboratories) and was calibrated with purified human IgG1 (Chemicon).

**RESULTS**

The homologous recombination vector designed for these experiments, pHRI (Fig. 1a), contains an ~3.55-kb fragment consisting of human *Cγ1*, the EMCV leader, the neo, marker gene, and the SV40 poly(A)-addition signal, flanked by genomic *μ* sequences that can act as targets for homologous recombination. The upstream target homology consists of 1.1 kb 5' of the EcoRI site in the *Jμ*-*Cμ* intron, and the downstream target homology extends from this EcoRI site to 3' of the *μ* membrane exons. Upon sequence-replacement-type integration of this construct into a rearranged *μ* heavy-chain allele, a single bicistronic mRNA transcript should be expressed from the endogenous *νμ* promotor. This *VDJHμ* rearrangement will be able to splice to the human *Cγμ* exons. A second coding region, encoding the neo gene product, will
be contained on the transcript. Translation of the neo sequence will be efficiently initiated by the EMCV leader (12).

To test the ability of pHR1 to target a rearranged heavy-chain allele, an IgM-producing hybridoma, F8/6.4.6, was selected. The construct pHR1 was digested with Xho I, which cuts immediately downstream of the genomic Cμ sequence, and then electroporated into the F8/6.4.6 hybridoma. G418-resistant stable transfectants were observed at a frequency of $4 \times 10^{-4}$. Culture supernatants of transfectants were assayed by ELISA for the presence of human γ1-chain-immunoreactive material. Two transfections were carried out with pHR1. In the first, 1 well (designated HR1-21) out of 94 wells proved positive in the human γ-specific ELISA. In the second, 2 (HR1-141 and HR1-445) out of 478 wells were positive.

The conditioned supernatants of these three positive transfectants were also examined with a murine μ-specific ELISA. Each supernatant was found to be negative, whereas the supernatants of all other G418-resistant wells examined were found to contain a large amount of murine μ heavy chain. The HR1-21 cells were subjected to limiting dilution cloning, and of 24 clones examined by ELISA all were found to produce human γ1-immunoreactive material and no murine μ was detected. Western blotting of HR1-21 conditioned medium revealed a γ1-reactive band that approximately comigrated with the human γ1 chain observed in human IgG1 myeloma serum (Fig. 2a).

Conditioned media from HR1-21, F8/6.4.6, and P3X63-Ag8.653 cells labeled with L-[35S]methionine was examined by precipitation with rabbit anti-murine μ and protein A-Sepharose and with protein A-Sepharose alone. Neither treatment precipitated labeled material from P3X63-Ag8.653 conditioned medium (Fig. 2b, lanes 1 and 4). However, consistent with a human IgG1-related antibody, an $\approx 55$-kDa species was precipitated from HR1-21 conditioned medium with protein A-Sepharose alone (lane 5) and, therefore, was also found in the precipitate of this medium sample with rabbit anti-murine μ and protein A-Sepharose (lane 2). The labeled IgM from the F8/6.4.6 hybridoma was not precipitated with protein A-Sepharose alone (lane 6), as expected, but was precipitated with rabbit anti-murine μ and protein A-Sepharose (lane 3). No murine μ chain was precipitated from the HR1-21 sample with rabbit anti-murine μ (lane 2). In lane 3 a specifically biotinylated Fc portion of the hybridomas therefore it is significant that the $\approx 30$-kDa F8/6.4.6 light chain was coprecipitated with the F8/6.4.6 μ chain (lane 3) and the 55-kDa species from HR1-21 conditioned medium. This indicates assembly of the human γ-chain-related polypeptide with the F8/6.4.6 light chain.

The antibody present in the conditioned medium of HR1-21 cells retained the binding specificity of the IgM(α) synthesized by the F8/6.4.6 hybridoma. Conditioned media from HR1-21 cells (Fig. 2c, lane 3) and F8/6.4.6 cells (lane 2) immunoprecipitated radiolabeled human factor VIII light chain, when a rat monoclonal antibody against murine κ light chain was added as an immunoadsorbent.

RNA expression in HR1-21 and F8/6.4.6 cells was examined by Northern blot hybridization. A probe specific for the human Cγ1-EMCV-neo sequence hybridized to a single species in RNA from HR1-21 cells (Fig. 2a, lane 2), but no hybridization was observed in F8/6.4.6 hybridoma RNA (lane 1). In contrast, a probe specific for the murine Cμ was hybridized to the expected species in F8/6.4.6 hybridoma RNA (lane 3), but no hybridization was observed in RNA from HR1-21 cells. These data indicate that no transcripts containing the murine Cμ are present in the steady-state RNA of the HR1-21 cells. In addition, a single novel RNA transcript was found in HR1-21 cell RNA hybridizing with the human Cγ1-EMCV-neo probe. Presumably, this novel transcript is the bicistronic mRNA encoded by the murine VDJγ-human Cγ1-EMCV-neo transcription unit generated by homologous recombination of pHR1 DNA with the functionally rearranged heavy-chain allele of F8/6.4.6. No other RNA transcripts were observed that hybridized to this neo-sequence-containing probe.

Southern blot hybridization analysis was used to examine the Jγ alleles of the F8/6.4.6 hybridoma and its HR1-21 derivative. Genomic DNA was digested with HindIII and hybridized with a Jγ3 probe consisting of the $\approx 680$-bp Xba I–EcoRI fragment downstream of Jγ3 (Fig. 1). All unarranged and Jγ1–Jγ3 rearrangements of this locus were identified by this analysis as an $\approx 2.27$-kb fragment, as seen in liver DNA (Fig. 3b, lane 12) and in P3X63-Ag8.653 DNA (lane 11). In F8/6.4.6 DNA there was an additional HindIII fragment of $\approx 10$ kb (lane 9). Presumably, this corresponded to the functional Jγ6 rearrangement used to encode the F8/6.4.6 μ chain. In HR1-21 DNA, this 10-kb fragment was not found, but a novel band of $\approx 11.6$ kb was found (lane 10). A duplicate blot of HindIII-digested genomic DNA was hybridized with a human Cμ,EMCV-neo probe. No hybridization was observed with this probe in F8/6.4.6 DNA (lane 7), but in HR1-21 DNA, a band that comigrates with the 11.6-kb Jγ1 band was found, in addition to an $\approx 2$-kb band (lane 8). The expected genomic rearrangements of the functional F8/6.4.6 heavy-chain allele before and after a sequence-replacement event with pH1 DNA are shown in Fig. 1. The loss of the 10-kb Jγ1-hybridizing fragment and the generation of 11.6- and 2.0-kb fragments in HR1-21 are consistent with the predicted result. The 2.0-kb band is internal to the pH1 construct. However, the upstream HindIII site of the 11.6-kb band is derived from 5′ sequence flanking the F8/6.4.6 Vμ. The absence of any other band overlapping with flanking DNA is

\[ \text{Fig. 2.} \ (a) \text{ Western blot analysis of conditioned media. Samples (1 ml) of conditioned media from HR1-21 and HR1-20 cultures were immunoprecipitated with sheep anti-human IgG1 (The Binding Site, San Diego) and protein A-Sepharose. The precipitated material was subjected to SDS/PAGE under reducing conditions, and the Western blot was probed with sheep anti-human IgG1 and [35S]methionine-labeled protein A (23). Lane 1, human IgG1 myeloma serum; lane 2, HR1-20; lane 3, HR1-21. (b) Immunoprecipitation analyses of radiolabeled conditioned media. Approximately 2 \times 10^5 cells were pulsed with L-[35S]methionine (500 μCi/ml; 1 μCi = 37 kBq) for 30 min and then supplemented with fresh medium containing unlabeled L-methionine and incubated for 4 h (16). Conditioned media from P3X63-Ag8.653, HR1-21, and F8/6.4.6 cells were immunoprecipitated with rabbit anti-murine μ antisera (Zymed) and protein A-Sepharose (lanes 1–3) or with protein A-Sepharose alone (lanes 4–6). (c) Immunoprecipitation of radiolabeled factor VII with hybridoma conditioned media. Radiolabeled factor VII from the 10A1C6 CHO cell line was mixed with conditioned media from P3X63-Ag8.653 (lane 1), F8/6.4.6 (lane 2), and HR1-21 cells (lane 3). Precipitation was completed by addition of a rat anti-murine κ monoclonal antibody. The precipitated material was examined by SDS/PAGE and fluorography. \]
Fig. 3. (a) Northern blot analysis of HR1-21 cells. Total cellular RNA from the F8/6.4.6 hybridoma and HR1-21 transfectant was electrophoresed in a 1% (wt/vol) agarose/formaldehyde gel (10 μg of RNA per lane) and examined by Northern blot hybridization. (Upper) Lanes 1 and 2 were hybridized with the 3.6-kb EcoRI fragment of pHRI that comprises the human Cγ1-EMC–neo (Huyl-EMCneo) sequence. Lanes 3 and 4 were hybridized with a murine Cγ1 fragment. The migration positions of 28S and 18S rRNA are indicated. (Lower) These lanes were later hybridized with a murine Cγ1 fragment. (b) Southern blot analysis of HR1-21 genomic DNA. Genomic DNA from F8/6.4.6, HR1-21, or P3X63-Ag8.653 cells or from BALB/c mouse liver was digested with EcoRI (lanes 5 and 6) or HindIII (lanes 7–12) and examined by Southern blot hybridization with the Huyl-EMCneo fragment (lanes 5–8) or the 680-bp XbaI–EcoRI fragment (JH, 3′) located downstream of murine JH4 (lanes 9–12). The migration positions of DNA marker fragments are indicated in kilobase pairs.

indictative of a single copy of the pHRI-derived sequence in the HR1-21 genomic DNA. The complete 3.6-kb human Cγ1-EMC–neo EcoRI fragment was also found in HR1-21 DNA (Fig. 3b, lane 6). These data indicate that the transfected pHRI DNA has been targeted to the functionally rearranged F8/6.4.6 heavy-chain allele in HR1-21 cells, to give the predicted recombination product shown in Fig. 1b.

The murine JH4–Cγ1 intron of the genomic μ clone used to construct pHRI has an ~2.5-kb deletion between the intron EcoRI site and the Cμ1 exon (data not shown). In generating pHRI, the human Cγ1-EMC–neo sequence was inserted into this EcoRI site, resulting in this deletion being located between the neo sequence and Cμ1. This deletion permits the JH4–Cγ1 intron of pHRI to be distinguished from the endogenous μ sequences in transfected cells. However, in a homologous recombinant clone such as HR1-21, the smaller JH4–Cγ1 intron will be present only if the 3′ site of the sequence replacement is downstream of this intron. Southern blot analysis of the genomic DNA of HR1-21 cells demonstrated that the smaller JH4–Cγ1 intron had been retained, indicating that the 3′ recombination occurred downstream of the JH4–Cγ1 deletion in pHRI (data not shown).

The homologous recombination of the pHRI sequence into a JH allele was confirmed in HR1-21, HR1-141, and HR1-445 by PCR with genomic DNA. Primers were used that anneal to the sequence immediately upstream of the 5′ homology of the construct (primer USNAEU) and at the 5′ end of the human γ1 sequence used (primer HGAM-DS) (see Fig. 1). As predicted, PCR with these primers generated an ~1235-bp band that also hybridized with the JH3 probe by Southern blotting (data not shown). No band was generated by PCR with these primers in F8/6.4.6 DNA.

The specific cellular productivity of the F8/6.4.6 hybridoma and its HR1-21, HR1-141, and HR1-445 derivatives was determined with conditioned media from exponentially growing cultures. The murine μ-specific ELISA showed that the parental F8/6.4.6 hybridoma produced ~12 μg of IgM per 10^6 cells per 24 hr. The human γ1-specific ELISA showed that HR1-21, -141, and -445 secreted 20, 13, and 28 μg of chimeric heavy chain per 10^6 cells per 24 hr, respectively.

**DISCUSSION**

The transfected pHRI construct was shown to target specifically a rearranged μ allele in a murine hybridoma and to introduce a human Cγ1-EMC–neo cassette. The resulting cell lines express the chimeric heavy chain at a high level, presumably using the intact promoter and enhancer elements of the murine gene to direct the expression of a bicistronic murine VDJH–human Cγ1-EMC–neo mRNA. The specific cellular productivity of the homologous recombinants is equal to or greater than that of the parental F8/6.4.6 hybridoma. The chimeric heavy chain assembles with the murine light chain to form an antibody that retains the binding specificity of the parental antibody, as judged in an antigen-specific ELISA and by immunoprecipitation of radiolabeled antigen.

No μ mRNA or polypeptide was detected in the homologous recombinants. This may be due to the efficient splicing of the murine VDJH exon to the human Cγ1 exon, perhaps enhanced by the close proximity of these donor and acceptor splice sites. In addition, the SV40 polyadenylation signal placed downstream of the neo sequence will lead to cleavage and polyadenylation of the nascent RNA and the probable degradation of the downstream Cμ transcript. The HR1-21 cells have maintained chimeric heavy-chain expression, with no μ polypeptide production, for over 6 months in culture (data not shown). These data contrast with previous work attempting to class-switch a murine heavy chain by homologous recombination using a sequence-insertion vector (24). Most transfecants in that study were found to have multiple copies of the transfected DNA, which can lead to genetic
instability (10); cells were cloned from chimeric heavy-chain-positive transfecteds that produced the chimeric heavy chain, the murine heavy chain, or both.

In the present study, the frequency of homologous recombinants detected was 1/191 G418-resistant clones. This compares favorably with other reports of, for example, 1/25 and 1/117 for β2-microglobulin (25, 26), 1/34 for c-abl (27) and 1/950 for hypoxanthine phosphoribosyltransferase (19). However, unlike the diploid embryonic stem cells used in much previous work, the potentially tetraploid hybridoma used here contains up to four target copies of the JH allele. This may decrease our apparent targeting to the transfectants detected.

The EMCV-neo cassette. The background of G418-resistant transfecteds was probably increased by incorporating the immunoglobulin heavy-chain enhancer on pH1 (see Fig. 1). This enhancer is known to act as a promoter element, being responsible for initiating sterile μ transcripts (28). In addition, the nonspecific integration of this enhancer may activate other promoter elements, causing neo expression. The efficiency of this selection system might be improved by omitting this enhancer element from the homologous recombination construct.

There are multiple reasons for genetically engineering the constant regions of monoclonal antibodies (29–33). These include the replacement of murine constant-region sequences with human equivalents for purposes of reducing the potential immunogenicity of a therapeutic monoclonal antibody, and the opportunity to modulate its effector-function sequences, such as those responsible for antibody-dependent cellular cytotoxicity. The conventional approaches to generating such chimeric antibodies require the cloning of the functionally rearranged immunoglobulins of a hybridoma, their in vitro manipulation, and the creation of a cell line expressing these genetically engineered immunoglobulins. Such transfected cell lines have generally been found to have specific productivity levels of monoclonal antibody substantially lower than that of the original hybridoma. Although techniques have been developed for increasing this stable production level, these can take a number of months to reach high expression levels (16).

The pH1 construct and future derivatives should greatly improve the ease with which chimeric antibodies can be constructed and expressed at high level. Given the sequences downstream of the JH locus that are common to all heavy-chain alleles, it should prove possible to class-switch heavy-chain rearrangements of any isotype with a construct such as pH1. This approach can be readily adapted for light-chain loci.

The EMCV-neo cassette has been shown to function efficiently in this work. It is ideal for selecting for recombination events designed not to disrupt completely the functional expression of the target gene or for causing gene fusions such as artificial class switching.

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