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Integration vectors for antibody chimerization by homologous recombination in hybridoma cells

Gene targeting in hybridoma cells provides a tool for generating chimeric antibodies with great ease and at high yield. We present an evaluation of integration vectors for the chimerization of the immunoglobulin heavy chain locus which are universally applicable to hybridomas of different isotypes and mouse strains. There are three problems arising with vector integration: (i) the frequent persistence of the parental isotype; (ii) an isotype-dependent aberrant replacement-like recombination giving rise to antibodies devoid of the C_H1 domain; and (iii) secondary recombinations leading to excision of the integrated sequence. To overcome these problems, we have systematically evaluated the consequences of extending the vector flank. Although the homology length clearly determines the recombination frequency, this effect is counteracted by the secondary recombination, which also correlates to the homology length. In contrast, the truncating recombination events are not dependent on the homology length and never lead to re-excision of the construct. To take advantage of the increased genetic stability obtained with short flanks, we constructed an enrichment vector which yields high recombination efficiencies despite using a short flanking sequence. In addition, irradiation of the cells enhanced homologous recombination. The problem of the co-production of two isotypes was overcome by a two-step targeting reaction.

1 Introduction

Homologous recombination has become a powerful tool for specifically modifying chromosomal genes (reviewed in [1]). However, in mammalian cells, homology-directed incorporation is a rare event compared to random integration [2]. The efficiency of homologous recombination correlates with the length of the homologous sequences [3–5]. At the Ig loci, gene targeting has mainly been used to introduce subtle, predetermined mutations (*e.g.* [3, 6, 7]), and to exchange C regions for the generation of mouse/human chimeric mAb [8–12]. Gene targeting permits the high-level production of recombinant Ab and overcomes the drawbacks of expression in nonproducing cell lines [13]. For the substitution of complete C regions in hybridomas, integration [8, 11, 12] as well as replacement constructs [9–11] have been applied. The former vector type is characterized by a homologous flanking sequence in which the construct is linearized [14]. The integration of the complete vector via a single crossover results in a duplication of the target sequence. In contrast, a replacement vector is cut outside or at the end of the homology [14] and requires two crossovers. Integration vectors were shown to be less sensitive to the presence of base pair mismatches and to yield higher recombination efficiencies and fidelities when non-isogenic sequences are targeted [5]. In addition, an integration vector does not need a 3′ flank that has to be matched to the isotype of the hybridoma, thus offering several advantages for mAb chimerization. Indeed, it should be possible to devise a unique integration vector which is applicable to hybridomas of all isotypes and from different mouse strains.

Problems encountered in this procedure are the frequent co-expression of the parental isotype [8, 10, 12] and aberrant recombinations which give rise to truncated H chains, which are often observed when the human IgG1 isotype is involved [12]. As this is the most promising candidate for therapeutic applications [15], it is necessary to improve the recombination efficiency. The dependence of recombination frequency on the homology length was demonstrated also for Ig targeting vectors, but solely for replacemnt constructs which were designed to introduce small mutations into the IgM isotype [3]. Here, we present a systematic evaluation of integration vectors for the chimerization of the IgH loci in hybridomas non-isogenic to the flanking sequence, and an effective frequency is defined on which homology length does not seem to exert the same influence as seen in other systems.

2 Materials and methods

2.1 Vector construction

The vectors pSVgpt-huγ1-A4, pSVgpt-huγ3-A4 and pSVgpt-huγ1-A5 were described elsewhere [12]. pSVneo-huγ1-A5 was constructed by transferring the *neo* gene as Pvu I-Bam HI fragment from pSV2neo into the Pvu I-Bam HI cut pSVgpt-huγ1-A5 vector. Insertion of the 1.4-kb m5′Sμ flank together with the IgG1 C exons into pSV2gpt gave rise to pSVgpt-huγ1-m5′Sμ. pSVgpt-huγ1-X5 was created by excision of the 1.0-kb Xba I fragment containing

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the μ enhancer from pSVgpt-huγ1-A5. A 4.0-kb PCR product spanning the μ intron 3′ of the Eco RI site was ligated via Eco RI and Sma I ends into pSVgpt-huγ1-A4 whose Sac I site was blunted. The resulting construct was named pSVgpt-huγ1-A6. To construct pSV232Agpt-huγ1-A4 the SV40 enhancer was removed from pSVgpt-huγ1-A4 by Pvu I-Hind III digestion and replaced with the truncated SV40 enhancer that was isolated from the plasmid pSV232Agpt [16] (a gift from P. Berg). The homology flank was extended to 3.0 kb by substituting the 0.7-kb EcoRi-Sac I fragment by m5′Sμ. Finally, the μ enhancer was eliminated by excision of the 1.0-kb Xba I fragment. This vector was named pSV232Agpt-huγ1-X5. All homology flanks are compiled in Fig. 1C.

2.2 Cell culture and immunological techniques

MmT1 is an AKR/J hybridoma (IgG2a/κ) producing an mAb against the murine T cell Ag Thy-1.2 [17]. It was propagated in RPMI 1640 (Gibco BRL, Eggenstein, Germany) supplemented with 10% FCS and 2 mM glutamine. CLB-CD19 (clone H12) is a mouse IgG1/κ hybridoma of BALB/c genetic background secreting mAb specific for the human B cell Ag CD19 [18]. It was grown in DMEM with 5% FCS. For transfection, 10^7 cells were suspended in 700 μl RPMI 1640. Plasmid (20 μg), which was linearized with Eco RI or Xba I, was added and a single pulse of 220 V, 500 μF (MmT1) or 240 V, 960 μF (CLB-CD19) was delivered in a genepulser apparatus (Bio-Rad, München, Germany). Cells were kept at 0 °C for 10 min and then plated in 96-well plates at a density of 10^4 cells/well. On day 3, selection with 1.0 or 1.5 mg/ml G418 or with increasing concentrations of mycophenolic acid was initiated [12]. ELISA plates were coated with goat anti-human IgGFc and blocked with 1% milk powder in PBS. Incubation with culture supernatant was followed by incubation with peroxidase-coupled goat anti-human IgGFc or, for examining the presence of the C_H1 domain, anti-human IgGFab (Dianova, Hamburg). Murine Ig was detected by goat anti-mouse IgGFc. The color reaction was initiated by H_2O_2 and *o*-phenylenediamine (Sigma, München). For protein quantitation, a purified mouse mAb or a human IgG plasma fraction was used as calibration standard. Alternatively, antibody concentrations were measured by inhibiting the binding of biotinylated MmT1 to an immobilized anti-idiotypic mAb [19]. For Western blotting, reduced mAb were run on 10% SDS-PAGE and electroblotted to nitrocellulose filters and revealed with peroxidase-conjugated goat anti-human IgGFc with H_2O_2 and 3,3′-diaminobenzidine as substrate.

2.3 DNA hybridization

DNA from cultured cells was digested with restriction enzymes, run on 0.7% agarose gels (10 μg per lane) and blotted to Genescreen membranes (Du Pont). The filters were hybridized with a ^{32}P-labeled probe, washed under stringent conditions and exposed to Kodak X-ray film. As hybridization probes, we used the 1.6 kb Hind III-Eco RI fragment MJ_H and the 238-bp PCR product huγ1 derived from the human IgG1 C_H3 exon (Fig. 1A).

3 Results

3.1 Characterization of targeting events at the IgH locus

To study the effector functions of human isotypes, the murine anti-Thy-1.2 mAb MmT1 [17] was partially chimerized by homologous recombination. The integration vector pSVgpt-huγ1-A4 or pSVgpt-huγ3-A4 with a 5′ flank of 2.3 kb was linearized and transfected into MmT1 (Fig. 1A). After selection for guanine phosphoribosyl transferase (gpt) gene expression, targeted transformants can be identified by ELISA.

Despite the non-isogenic source of the vector flank (BALB/c), homologous events occurred in the AKR/J hybridoma at a frequency of 0.46% (Table 1). As a control, a vector with the 2.3-kb flank was transfected into the BALB/c-derived anti-CD19 hybridoma CLB-CD19 (clone H12 [18]). Homologous recombination was as efficient in the AKR/J cells as in the isogenic hybridoma.

The genomic situation in the targeted clones was examined by Southern blotting. MmT1 contains three H chain alleles [20] (Fig. 2, lane 1). The 5.6-kb fragment detected by the probe MJ_H in the Bam HI digest originates from the fusion partner, the 6.6-kb band represents the aberrantly rearranged allele from the spleen cell, and the 9.8-kb band is the functional allele (unpublished). After targeting with the IgG1 vector, we distinguish four situations which are shown in Fig. 2. Correct integration results in a novel fragment of 5.6 kb, which co-migrates with that of the fusion partner and which also hybridizes to probe huγ1, and a 13.5-kb fragment resulting from the duplication (Fig. 2, lanes 2 and 3). The persistence of the 9.8-kb band in lane 2 is discussed in Sect. 3.3. Another situation was found in clones that were first identified as IgG1 producers, but have ceased to express the human isotype within 2 weeks after the initial positive ELISA. Since the pattern in the Southern blot is identical to the original MmT1 situation (lane 5) and no signal is produced by the huγ1 probe (lane 4), we argue that these clones have initially undergone correct integrations, but have subsequently lost the human sequence due to a secondary intrachromosomal recombination between the duplicated intron sequences (Fig. 1A, lower part). In addition to the predicted integrations, we observed replacement-like events involving illegitimate recombinations at one or both sides [12]. In the clones having undergone illegitimate recombinations at the 3′ side (crossovers **a** in Fig. 1B), the 5.6-kb band hybridizing to huγ1 and MJ_H is present, but the 13.5-kb fragment is missing (Fig. 2, lanes 8, 9). In other clones, illegitimate events have taken place at both sides (crossovers **b** in Fig. 1B). Since different fragments are detected by the probes MJ_H and huγ1, the C exons and the homology flanks have been separated from one another (lanes 6, 7). These latter events are only observed with the human IgG1 isotype and give rise to mAb with a deletion of the C_H1 domain, because the 5′ breakpoint is located in or 3′ to the C_H1 exon [12]. In transformants showing replacements events, secondary recombinations should not be possible, since no duplication occurred. To verify this prediction, we examined the size of the H chains secreted within 2 weeks following transfection and monitored ongoing or ceasing of IgG1 production. Since the amount of chimeric Ab secreted at such an early stage after transfection was not sufficient for Western blot

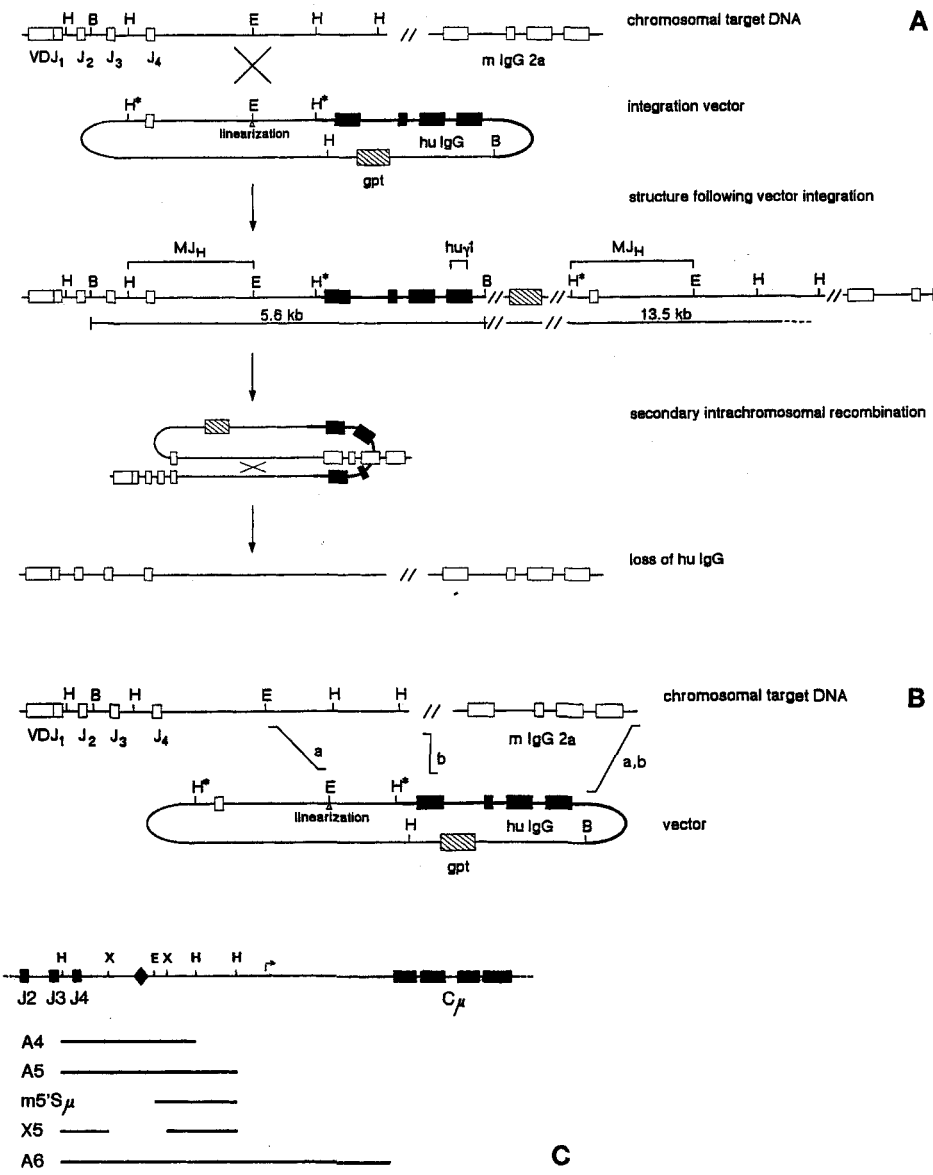

Figure 1. (A) Integration of the vector pSVgpt-huγ1-A4 into the functional IgH locus of the cell line MmT1 and secondary vector excision. (B) Replacement-like events induced by pSVgpt-huγ1-A4 using one (**a**) or two (**b**) illegitimate crossovers. Pathway **b** leads to the loss of the C_H1 domain [12]. Mouse sequences are depicted as thin lines and open boxes, human sequences as bold lines and closed boxes. Brackets represent hybridization probes, the bars hybridizing fragments. (C) Origin of the homology flanks. The arrow indicates the 5′ boundary of the switch region, the diamond the position of the Igμ enhancer. Restriction sites: B, Bam HI; E, Eco RI; H, Hind III; X, Xba I. Asterisks denote destroyed restriction sites.

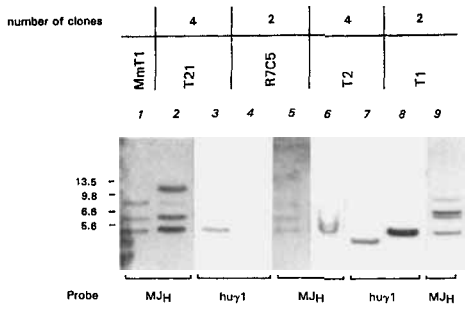

Figure 2. Four recombination patterns are found in the Southern blot following targeting with pSVgpt-huγ1-A4. Bam HI-digested DNA from the indicated clones was hybridized with MJ_H and huγ1. Lanes 2, 3: Predicted integration; lanes 4, 5: situation following vector re-excision; lanes 6, 7: replacement recombination with two illegitimate crossovers (**b** in Fig. 1B); lanes 8, 9: replacement recombination utilizing the 5′ flank but with an illegitimate 3′ breakpoint (**a** in Fig. 1B). A total of 12 clones was examined.

analysis, we examined the supernatants of the primary clones in the 96-well plates by ELISA using goat anti-human Fab which reacts exclusively with the C_H1 domain. All primary clones that secreted truncated H chains were stable over prolonged cultivation as well as in limiting dilution assays. In contrast, only 60% of the clones producing intact IgG1 retained the human isotype.

3.2 Effects of the homology length on recombination efficiency and stability

To facilitate chimerization with human IgG1, the recombination efficiency had to be increased. Therefore, the effect of different homology flanks (Fig. 1C) was systematically evaluated (Table 1). The minimal requirement for homologous recombination was 2.0 kb. By stepwise elongation, we obtained the relationship represented by the upper curve in Fig. 3. The frequency of the truncating recombination

Table 1. Results of targeting experiments with different IgH integration vectors in the hybridoma MmT1

Vector[a]	Homology length (kb)	Number of target events/ total number of drug resistant clones[b]	Recombination efficiency (% hu$^+$)	Clones producing two isotypes/clones tested	Clones secreting truncated Ab/clones tested[c]	Unstable clones/ clones tested[d]	Net efficiency (%)
pSVgpt-huγ1-A4/Xba I	1.3	0/ 546	0	–	–	–	–
pSVgpt-huγ1-m5'Sμ/Xba I	1.4	0/ 518	0	–	–	–	–
pSVgpt-huγ1-X5/Xba I	2.0	3/ 960	0.31	n. d.	n. d.	1/3	0.21
pSVgpt-guγ1-A4	2.3	10/2193	0.46	6/9	3/9	3/10	0.32
pSVgpt-huγ1-A5	3.0	11/1282	0.85	3/4	6/7	6/12	0.43
pSVgpt-huγ1-A6	5.6	31/ 836	3.71	n. d.	1/4	12/17	1.09
pSVgpt-huγ1-A5/irradiated	3.0	43/1539	2.79	17/17	5/13	19/34	1.23
pSV232Agpt-huγ1-X5/Xba I	2.0	4/ 88	4.55	2/4[e]	1/1	1/4	3.41

a) The cutting enzyme is Eco RI unless otherwise noted. For each vector, the results of three to six independent electroporations are compiled.
b) After transfection cells were plated at a density of 10^4/well (10^5/well for the enhancer-less vector). Given a transfection efficiency of 2.5×10^{-5} (1.3×10^{-6} for the enhancer-less vector), each positive well represents a monoclone.
c) As determined by Western blotting and by testing for the presence of the C_H1 domain by ELISA (Sect. 2.2).
d) Clones that are unstable lose the human isotype within 2 weeks following identification as targeted transformants. Stable clones retain the human isotype indefinitely.
e) Reduction to 1/4 after repeated limiting dilution cloning.

events did not show the clear dependence on the homology length as did the total frequency.

Lengthening the vector flank had another effect, in that the fraction of unstable producers was increased (Table 1). This may be ascribed to the fact that also the secondary recombination depends on the length of the duplicated sequence. Hence, lengthening the vector flank leads not only to the desired enhancement of recombination, but also to decreased stability of the clones. If the overall efficiencies are corrected with regard to the unstable clones, we obtain a relationship which is shown as the lower curve in Fig. 3.

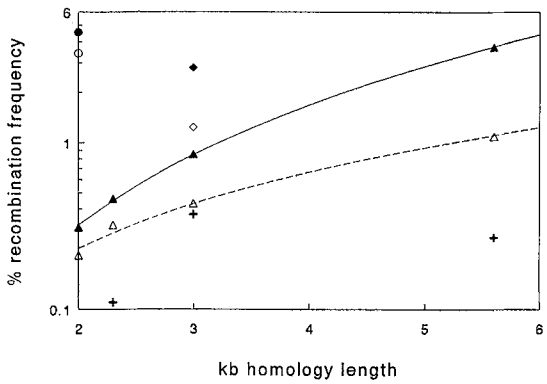

Figure 3. Dependence of recombination efficiency of IgH integration vectors on the length of the homologous flank. Closed symbols: primary recombination efficiencies following transfection; open symbols: efficiencies corrected for secondary vector excision. Cross symbols: frequencies of the truncating recombination mechanism. Diamonds: effect of irradiation. Circles: efficiency of the enrichment vector.

To bypass this difficulty, we devised systems yielding high recombination efficiencies despite the use of short vector flanks. Gamma irradiation induces double strand breaks in the genome and activates cellular repair mechanisms. We transfected the vector pSVgpt-huγ1-A5 into MmT1 cells which were irradiated at 36 cGy from a ^{137}Cs source, 1 h before or following electroporation. The recombination frequency was significantly increased, but secondary recombinations were as frequent as in the non-irradiated controls (Table 1 and Fig. 3). Next, we created a vector using the minimal homology requirement of 2.0 kb. Its design is based on the consideration that random insertions should be reduced if the selection marker is rendered inactive [21]. To this end, the SV40 enhancer which drives the gpt gene was truncated [16]. To preclude gpt expression under the control of the nearby μ enhancer in the vector sequence, the construct was linearized with Xba I. Thus, drug-resistant clones should arise preferentially by activation of the gpt gene by the endogenous μ enhancer, *i.e.* site-specific integration. Actually, homologous events were favored by a factor of up to 20 (Table 1 and Fig. 3). This vector provides a considerable simplification of the selection scheme: it is sufficient to plate the cells in only four 24-well plates (1.5×10^5 cells per well), and only few supernatants have to be screened by ELISA.

3.3 Co-expression of two isotypes and a novel Ig re-targeting system

A problem which severely hampers routine application of gene targeting is the co-expression of the parental isotype in about 80% of the targeted clones (Table 1). In these clones, the functional 9.8-kb Bam HI fragment does not disappear, but becomes weaker (Fig. 2, lanes 2, 9). This suggests the

existence of multiple copies of the functional IgH chain allele which are located on the same chromosome, as shown by *in situ* hybridization. To overcome this problem, we devised a system that retargets those clones which have undergone homologous recombination at one gene copy and retained the other one. The cell line T1 has a weakened 9.8-kb band (Fig. 2, lane 9) and shows the double-producer phenotype which could not be abrogated by two rounds of limiting dilution. T1 was re-transfected with the vector pSVneo-huγ1-A5, which is identical to pSVgpt-huγ1-A5, but bears the *neo* gene as a selection marker. After selection with mycophenolic acid and G418, clones were established that only expressed the human isotype, and in which the 9.8-kb Bam HI band completely disappeared (not shown). The secondary chimerization resulted in a substantially elevated expression of human IgG1 as compared to T1.

4 Discussion

Homologous recombination in hybridoma cells provides a means of generating chimeric mAb at high yield and with great ease. We argued that integration vectors might be superior to replacement constructs, because they only need a 5′ flank derived from the μ intron that is common to all hybridomas, irrespective of the isotype expressed. For replacement recombination at the IgH locus, a panel of constructs matched to the isotype of the hybridoma was used [11]. Wood et al. have employed a replacement vector only for chimerization in an IgM hybridoma [9]. In this regard, replacement of C_κ can more easily be done because of the unique C_κ segment [10]. Moreover, integration vectors yield higher recombination frequencies and fidelities at several loci when targeted to non-isogenic sequences [5, 22, 23]. While little is known about allotypic differences in the C_κ region, there is considerable variability at the IgH locus. Significantly, our integration construct is able to promote homologous recombination in a hybridoma of genetically different origin at the same frequency as in an isogenic hybridoma.

The persistence of the parental mouse isotype is a general problem of mAb chimerization by homologous recombination [8, 10, 12]. It is explained by the presence of multiple copies of the functionally rearranged allele. This problem could be overcome by retargeting with an integration vector bearing a second selection marker. For mAb chimerization, it is more advantageous to retarget the second expressed copy rather than to disrupt it, because retargeting further enhances the expression level. Another problem that might be specific to the human IgG1 isotype is the generation of H chains without C_H1 domain, resulting from "targeted illegitimate" replacement events [12] which do not seem to be induced by the double-strand break repair mechanism [14]. Two findings point to a novel mechanism: the frequency of these events does not show the clear dependence on the homology length (Fig. 3), and, despite a homology that did not activate the correct integration pathway, the murine flank was able to induce aberrant events even in rat cells [12]. Also the frequency of this inter-species recombination was independent of the homology length (unpublished).

The occurrence of the truncation event prompted us to increase the recombination efficiency. The dependence of recombination frequency on homology length has been demonstrated also at the IgH locus, albeit for replacement constructs introducing small mutations into the IgM isotype [3]. We systematically assessed the influence of homology length for mAb chimerization by integration vectors and found a non-linear correlation (Fig. 3), similar to that detected at the Hprt locus [5]. The recombination frequencies must be re-evaluated due to an effect which counteracts the positive influence of the homology length: in correctly targeted transformants, the duplicated μ intron leads to secondary recombinations and to re-excision of the integrated region. Clones that had undergone replacement events never lost the human isotype. Thus, the influence of the homology length on the net efficiency was attenuated compared to other loci (lower curve in Fig. 3).

Genetic instability was also observed after integration of vectors with two-sided homology [24], but has not yet been reported for mAb chimerization [8, 11]. Thus, Fell et al. argued that the lack of the switch region in the vector would preclude secondary recombinations [8]. Like the primary targeting frequency, the rate of secondary events was clearly correlated to the length of the vector flank. An internal deletion within the A5 flank, reducing the homology length to 2 kb (X5 flank), did not decrease the excision rate. This can be explained by a gap repair which takes place during the integration reaction. The loss of the human isotype may be related to the "hit-and-run" mechanism proposed in [25] where the integration of a mutated IgM exon with two-sided homology and its subsequent excision result in either the wild-type or the mutated situation. Intermediates with the transferred exons located 3′ of the endogenous region do not become detectable. As in our system, a single flank is used, the introduced exons are always situated in a 5′ position and are therefore readily detected.

Whereas for primary recombination, long homology flanks are favored, it is advantageous to use short ones to reduce secondary recombinations in targeted clones. Therefore, we applied an enhancer-less enrichment vector [21, 25] for the production of mouse/human chimeric Ab. Due to the lack of the SV40 enhancer driving the gpt gene and of the μ enhancer in the flank, illegitimate integrations are suppressed, thereby considerably increasing the net efficiency. Upon site-directed insertion, the endogenous Ig enhancer complements the defective SV40 enhancer. In the study by Wood et al. [9], only the promoter of the selection marker was deleted. We argue that, therefore, no enrichment effect was obtained. In our experiments, the μ enhancer in the vector restored the activity of the non-functional SV40 enhancer. Conversely, the lack of the μ enhancer alone had no effect (not shown). This was also observed in a replacement reaction involving a vector without a μ enhancer and the IgH locus of a rat hybridoma [12].

Taken together, our studies indicate that mAb chimerization by homologous recombination in hybridoma cells poses several problems which are surmountable. Given the ease of manipulation and the considerable production rates obtained, it is a valuable alternative to more traditional procedures.

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