Immunology 1996 **89** 309–315

Genetic stability of gene targeted immunoglobulin loci. I. Heavy chain isotype exchange induced by a universal gene replacement vector

C. KARDINAL, M. SELMAYR & R. MOCIKAT *GSF-Institut für Immunologie, München, Germany*

SUMMARY

Gene targeting at the immunoglobulin loci of B cells is an efficient tool for studying immunoglobulin expression or generating chimeric antibodies. We have shown that vector integration induced by human immunoglobulin G1 (IgG1) insertion vectors results in subsequent vector excision mediated by the duplicated target sequence, whereas replacement events which could be induced by the same constructs remain stable. We could demonstrate that the distribution of the vector homology strongly influences the genetic stability obtained. To this end we developed a novel type of a heavy chain replacement vector making use of the heavy chain class switch recombination sequence. Despite the presence of a two-sided homology this construct is universally applicable irrespective of the constant gene region utilized by the B cell. In comparison to an integration vector the frequency of stable incorporation was strongly increased, but we still observed vector excision, although at a markedly reduced rate. The latter events even occurred with circular constructs. Linearization of the construct at various sites and the comparison with an integration vector that carries the identical homology sequence, but differs in the distribution of homology, revealed the following features of homologous recombination of immunoglobulin genes: (i) the integration frequency is only determined by the length of the homology flank where the cross-over takes place; (ii) a 5′ flank that does not meet the minimum requirement of homology length cannot be complemented by a sufficient 3′ flank; (iii) free vector ends play a role for integration as well as for replacement targeting; (iv) truncating recombination events are suppressed in the presence of two flanks. Furthermore, we show that the switch region that was used as 3′ flank is non-functional in an inverted orientation.

INTRODUCTION

For the study of immunoglobulin gene expression homologous recombination in B cells is a suitable system. Also for the generation of recombinant antibodies (Ab) gene targeting in hybridoma cells can be successfully applied thereby overcoming the low productivity of the usual mammalian expression systems. We and other groups have devised an expression system which is based on site-specific integration of the desired human constant (C) gene segments into the immunoglobulin loci of hybridoma cell lines expressing the desired specificities.[1–5] Similar gene targeting strategies have been used for introducing subtle mutations into the immunoglobulin heavy (IgH) chain locus of hybridoma cells.[6,7] Since the endogenous regulatory elements remain unaltered, one can obtain expression rates in the range of the parental hybridoma.[2,4,8] Furthermore, this system is very convenient in as much as the variable (V) genes have not to be isolated from the hybridoma.

In general, two types of recombination vectors are feasible. An integration vector is characterized by a linearization site situated within the homology region.[9,10] Free adjacent ends produced by vector cutting within the homology lead to a dramatic increase of the recombination frequency, as was shown in mammalian cells and in yeast.[11–13] The incorporation into the genome of the whole vector via a single cross-over gives rise to a duplication of the target sequence. By contrast, a replacement vector usually bears homology flanks on both sides of the sequence to be introduced. It is linearized outside or at either or both ends of the homology.[9] The incorporation involves double reciprocal recombination or gene conversion and is likely to be mediated by a mechanism different from the integration pathway.

The exchange of C regions for monoclonal antibody (mAb) chimerization has been performed by integration[1–3] as well as replacement vectors.[3–5] Due to the requirement of a 3′ flank, IgH chain replacement vectors have the disadvantage that they are not universally applicable to hybridomas of all isotypes. Thus, a set of constructs had to be designed with 3′ flanks matched to the different H chain subclasses.[3] By contrast, integration vectors only need a 5′ homology derived from the μ intron which is identical in all hybridomas. However, integration vectors suffer from the drawback that the duplication of the target sequence confers genetic instability which at high frequency leads to a secondary excision of the introduced C region and to restoration of the parental murine locus.[14,15] Furthermore, we have shown that

Received 29 April 1996; revised 15 July 1996; accepted 21 July 1996.

Correspondence: Dr R. Mocikat, GSF-Institut für Immunologie, Marchioninistr. 25, D-81377 München, Germany.

integration vectors carrying the human IgG1 sequence often induce aberrant replacement-like events which partly result in a loss of the C_H1 domain.[15,16] These latter events – termed as 'targeted illegitimate' recombinations – utilize breakpoints which are not located in the homology flank despite the presence of free adjacent homologous vector ends.[16]

To take advantage of the genetic stability achieved by replacement targeting and to obviate the need of using an isotype-matched 3′ flank, we have designed a novel type of a replacement vector that is universally applicable to all hybridomas irrespective of the isotype expressed. Although this vector also promotes integration events, the genetic stability obtained is markedly improved by virtue of a considerably increased replacement frequency. Surprisingly, replacements resulting in C_H1 truncation, as they were observed with integration vectors,[15,16] are now significantly suppressed. This gives hints to the mechanistics of homologous recombination at the IgH locus.

MATERIALS AND METHODS

Vector construction

The construction of the integration vectors pSVgpt-huγ1-A5 and pSVgpt-γ1-A6 has been described previously.[15] To create the 3′ flank for the replacement constructs parts of the mouse Igμ intron were amplified by PCR from the plasmid pSVμm5 which was kindly provided by M. Reth (Freiburg). A 2·8-kb fragment including the switch (Sμ) region was then cloned via *Bam*HI and *Sac*I ends into pSP72 whose *Eco*RI site had been destroyed. The fragment was excised by *Bam*HI/*Bgl*II digestion and ligated into the *Bam*HI site of pSVgpt-huγ1-A5 in the physiological (pSVgpt-huγ1-A5-S5′) or in the inverted (pSVgpt-huγ1-A5-S3′) orientation, respectively.

Cell culture techniques

MmT1 is an AKR/J-derived hybridoma (γ2a/κ) with anti-Thy-1.2 specificity.[17] MPC11 is an IgG2b expressing murine myeloma cell line from BALB/c.[18] The cells were propagated in RPMI-1640 supplemented with 10% fetal calf serum (FCS) at 37° in a 5% CO_2 atmosphere. For transfection 10^7 exponentially growing cells were mixed with 20 μg of DNA which had been cut with various enzymes (see Results) and precipitated with isopropanol. The electroporation was performed in 700 μl RPMI-1640 using a Bio-Rad (München, Germany) Genepulser apparatus at a voltage of 220 V and a capacitance of 500 μF. Cells were kept on ice for 10 min and then plated on 96-well dishes at a density of 10^4 cells per well. Selection began after 48 hr with 250 μg/ml xanthine, 15 μg/ml hypoxanthine and increasing concentrations of mycophenolic acid, up to a final concentration of 2 μg/ml.

Enzyme-linked immunosorbent assay (ELISA) and Western blotting

Stable transformants secreting human immunoglobulins were detected by ELISA using goat anti-human IgGFc as coating Ab and horseradish peroxidase-conjugated goat anti-human IgGFc as detecting Ab. To test for the presence of the C_H1 domain goat anti-human IgGFab was used as detecting Ab. All Ab were purchased from Dianova (Hamburg). The colour reaction was initiated by adding H_2O_2 and *o*-phenylenediamine (Sigma, München). For Western blotting, immunoglobulin was precipitated from culture supernatants by protein G beads, reduced, run on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted on nitrocellulose filters which were treated with peroxidase-coupled goat anti-human IgGFc and H_2O_2 and 3,3′-diaminobenzidine as substrate.

Southern blotting

Genomic DNA was isolated according to ref. 19 and digested with restriction enzymes. Ten micrograms per lane were loaded on 0·7% agarose gels and blotted to Genescreen filters (Du Pont, Boston, MA) which were subsequently hybridized with a 32P-labelled probe, washed under stringent conditions and exposed to preflashed Kodak X-ray films. The hybridization probes were the 1·6-kb *Hin*dIII/*Eco*RI fragment MJ_H from the murine J_H region and the 238-bp PCR product huγ1 from the IgG1 C_H3 exon (Fig. 1).

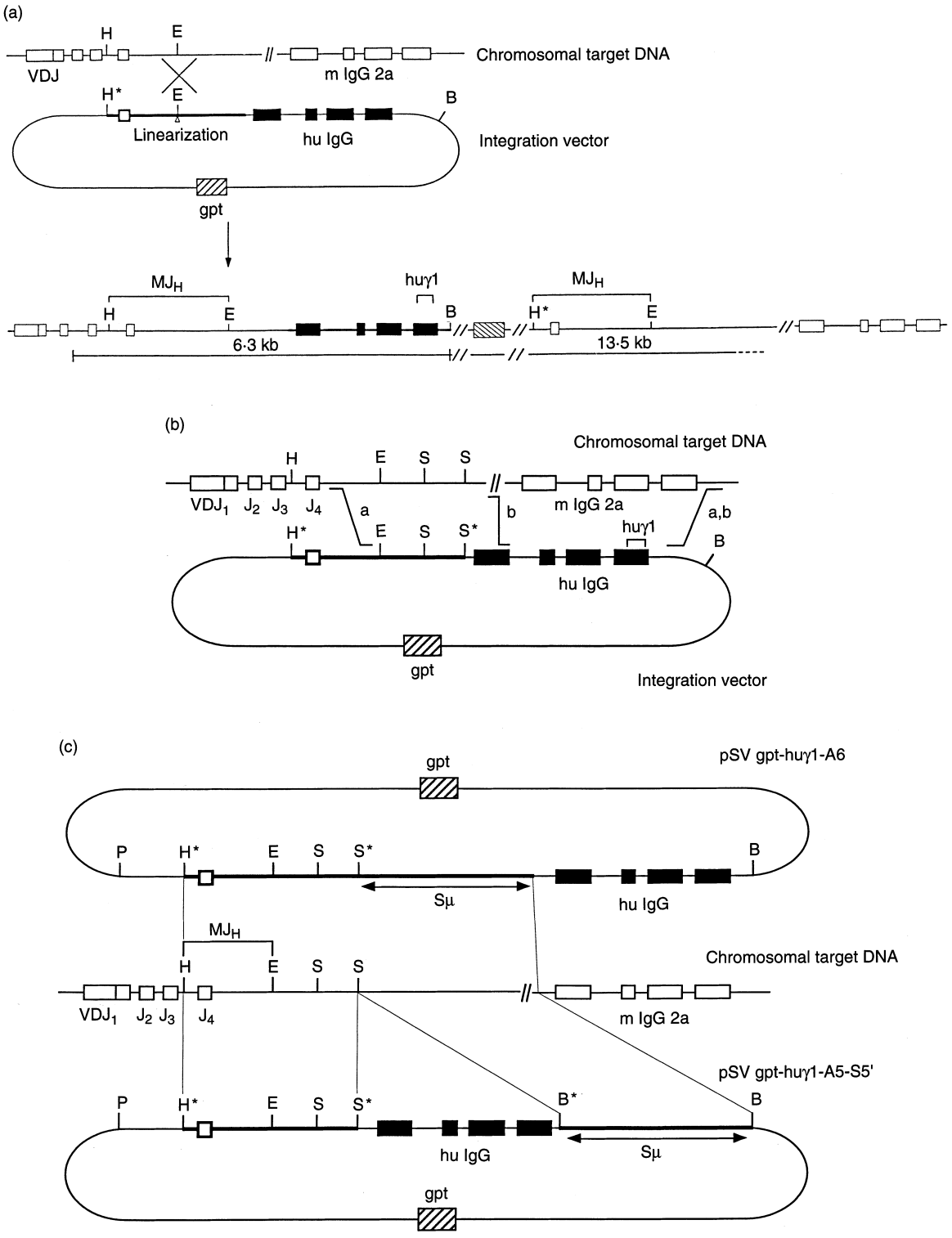
RESULTS

Generation of a stable chimeric situation at the IgH locus by gene replacement

In the past, we have used integration vectors to target the IgH locus of hybridoma cell lines for the generation of chimeric mAb. Targeted clones are enriched by selecting for *gpt* activity and identified as human immunoglobulin producers in the ELISA. As we have shown, a human IgG1 integration vector does not only undergo integration events, as shown in Fig.1a, but also promotes replacement reactions despite the presence of free homologous DNA ends.[15,16] These replacement events are due to illegitimate cross-overs at the 3′ side (as shown for the vector pSVgpt-huγ1-A5 in Fig. 1b; pathway a) or at both sides (pathway b). Pathway b gives rise to immunoglobulin devoid of the C_H1 domain. The location of the illegitimate breakpoints is variable, as we could show by hybridization of *Sac*I/*Bam*HI digested DNA from several clones with the huγ1 probe which should detect a 3·6-kb fragment if the vector-borne human C region remained intact upon incorporation (Fig. 2a).

We showed[15] that following replacement, the introduced human exons are always stably retained. Integration events, however, lead to secondary vector excision and loss of the human phenotype at high incidence, as detected in the ELISA after

Figure 1. (See page 311.) (a) Expected vector integration pathway of the integration construct pSVgpt-huγ1-A5 at the IgH locus. The predicted hybridizing fragments are indicated by bars. (b) Replacement recombination induced by the integration vector pSVgpt-huγ1-A5 at the IgH locus. Targeting involves one (pathway a) or two (pathway b) illegitimate cross-overs. The latter pathway gives rise to truncation of the C_H1 domain. (c) Distribution of homology sequences in the integration vector pSVgpt-huγ1-A6 (upper part) and in the replacement vector pSVgpt-huγ1-A5-S5′ (lower part). Mouse exons are depicted as open boxes, human exons as blackened boxes. The homology flanks are indicated as bolt lines. Restriction sites: E, *Eco*RI; S, *Sac*I; H, *Hin*dIII; B, *Bam*HI; P, *Pvu*I. Asterisks denote destroyed restriction sites. Brackets indicate the location of hybridization probes. The plasmids are not drawn to scale.


(a)
H
E
VDJ
m IgG 2a
Chromosomal target DNA
H*
E
B
Linearization
hu IgG
Integration vector
gpt
MJH
huγ1
MJH
H
E
B
H*
E
6·3 kb
13·5 kb
(b)
Chromosomal target DNA
H
E
S
S
VDJ1 J2 J3 J4
a
b
m IgG 2a
a,b
H*
E
S
S*
huγ1
B
hu IgG
gpt
Integration vector
(c)
gpt
pSV gpt-huγ1-A6
P
H*
E
S
S*
B
Sμ
hu IgG
MJH
Chromosomal target DNA
H
E
S
S
VDJ1 J2 J3 J4
m IgG 2a
pSV gpt-huγ1-A5-S5'
P
H*
E
S
S*
B*
B
hu IgG
Sμ
gpt

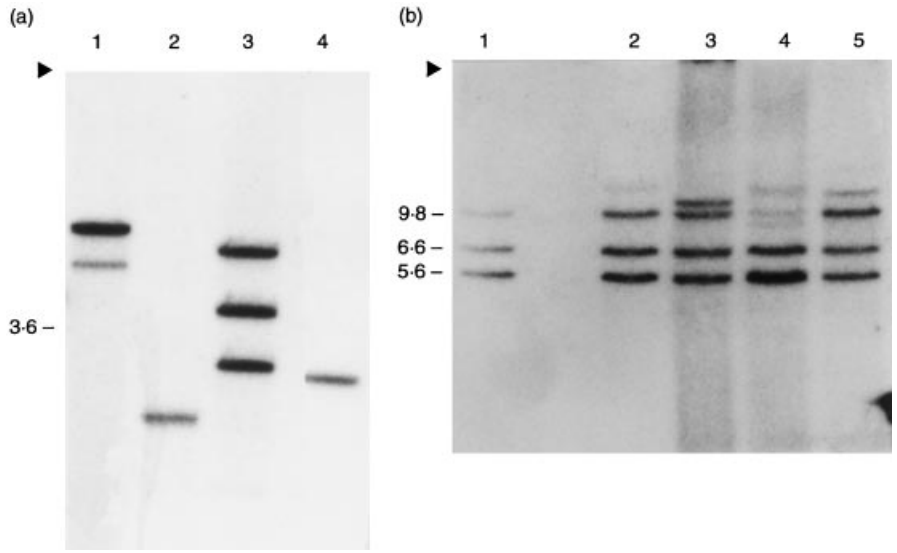


Figure 2. (a) Representative Southern blot showing replacement events induced by the integration vector pSVgpt-huγ1-A5 in the hybridoma MmT1. *Sac*I/*Bam*HI digested DNA from recombinants was hybridized with the probe huγ1. The theoretical fragment size of 3·6 kb, as it is present in the targeting vector, is indicated. (b) Southern blot analysis of representative MmT1 clones targeted with the replacement construct pSVgpt-huγ1-A5-S5′. DNA was digested with *Bam*HI and hybridized with the probe MJ_H. Lane 1, pattern of the parental cell line MmT1; lanes 2–5, typical stably targeted human IgG1 producers. The results were verified by other restriction digests and hybridization probes. Multiple bands in both blots can be explained by additional random integrations. The arrow heads indicate the migration start points. Sizes are given in kb. The location of the hybridization probes is depicted in Fig. 1.

about two weeks of cultivation.[15] Maintaining selection pressure with mycophenolic acid was not able to prevent the excision of the human C region. This can be explained by additional random *gpt* gene integrations into the genome which were consistently observed when stable clones were examined by Southern analysis. A characteristics of vector integration is the occurrence of a novel 13·5-kb *Bam*HI fragment detected by the probe MJ_H. This band originates from duplication of the intron sequence (Fig. 1a). Up to now we have examined 53 transfectants in the Southern blot (not shown), 15 of which had lost human immunoglobulin secretion before Southern blotting and therefore showed the hybridization pattern of the parental cell line. All 38 stable IgG1 producers were found to be replacement recombinants lacking the 13·5-kb band. This implies that, when incorporated by vector integration, the IgG1 construct is highly excision-prone and has segregated at the time of Southern analysis, i.e. after about 20 cell doublings following the initial positive ELISA. During these early stages after transfection, integration cannot be examined by Southern blotting because of the cell numbers being too low.

As human IgG1 chimeric mAbs are of considerable clinical relevance, we set out to improve the stability of gene targeted hybridomas. We constructed an IgG1 vector of the classical replacement type with two-sided homology which was expected to exclusively undergo correct replacement events. To this end, the insertion vector pSVgpt-huγ1-A5 was endowed with an additional 2·8-kb flank 3′ of the human C region (Fig. 1c). This flank was derived from μ intron sequences immediately downstream of the homology flank of pSVgpt-huγ1-A5. Therefore, the resulting replacement vector pSVgpt-huγ1-A5-S5′ has a total homology of 5·8 kb which is identical to the insertion vector pSVgpt-huγ1-A6,[15] but is interrupted by the heterologous region (Fig. 1c). Since the C_H downstream region is not utilized, this configuration allows for replacement targeting of virtually all isotypes. The recombination substrate was released from the vector by *Pvu*I/*Bam*HI digestion (Fig. 1c) and cotransfected along with the *gpt* selection marker into the IgG2a hybridoma MmT1. As shown in Table 1, the overall recombination frequency was increased as compared with the integration vector pSVgpt-huγ1-A6. This was accounted for by an about five-fold enhanced yield of stable producers which could be identified as replacement recombinants by hybridization with the probes MJ_H and huγ1. Examples are depicted in Fig. 2b. The parental cell harbours a 9·8-kb *Bam*HI fragment which represents the functional allele and a 6·6- and a 5·6-kb band which is derived from the fusion partner (lane 1). Also when targeted with pSVgpt-huγ1-A5-S5′, the stable IgG1 producers never exhibited the 13·5-kb band which would be indicative for vector integration. Instead we observed additional fragments of about 12 kb (lanes 2–5) which result from replacement reactions, as they also hybridized with the huγ1 probe (not shown). However, only the clone in lane 5 displayed the expected 12·7-kb *Bam*HI band. The heterogeneity among the other clones may be explained by the repetitive motifs included in the 3′ vector flank giving rise to varying breakpoints at the 3′ side. Despite its design as a replacement vector, however, pSVgpt-huγ1-A5-S5′ also induced unstable integrations, albeit at a markedly reduced level.

The expression levels of chimeric Ab exhibited the same variability (not shown) as observed for the transformants modified by an integration vector which included the selection marker.[2] This underscores our notion that the *gpt* gene does not interfere with immunoglobulin transcription.[2]

To assess the recombination efficiency of our construct in a cell line of a different isotype we transfected MPC11 cells which express the IgG2b subclass. Essentially the same results were obtained as for the IgG2a hybridoma (Table 1, last line).

Table 1. Efficiency of IgH targeting vectors in the hybridoma MmT1. All experiments were carried out at least in duplicate

Cell line	Vector	Homology length (kb) 5′/3′	Cutting enzyme	hu^+ clones/ clones tested*	Targeting frequency (% hu^+)	% Stable hu^+ clones (replacements)†	% Unstable clones†	Ratio intact/ C_H1-deficient IgG1‡
MmT1	pSVgpt-huγ1-A5	3·0/–	*Eco*RI	11/746	1·47	1·03	0·44	0·87
	pSVgpt-huγ1-A6	5·8/–	*Eco*RI	31/836	3·71	1·09	2·62	0·98
	pSVgpt-huγ1-A5-S5′	3·0/2·8	*Pvu*I/*Bam*HI	29/510	5·69	5·30	0·39	7·98
	pSVgpt-huγ1-A5-S5′	1·4/2·8	*Eco*RI/*Bam*HI	6/609	0·99	0·99	0·00	4·82
	pSVgpt-huγ1-A5-S5′	3·0/2·8	*Eco*RI	15/492	3·05	2·44	0·61	1·39
	pSVgpt-huγ1-A5-S5′	3·0/2·8	none	8/462	1·73	1·44	0·29	0·25
	pSVgpt-huγ1-A5-S3′	3·0/2·8	*Eco*RI	10/855	1·17	0·97	0·20	1·49
MPC11	pSVgpt-huγ1-A5-S5′	3·0/2·8	*Pvu*I/*Bam*HI	26/507	5·13	4·14	0·99	6·00

* Human IgG producers as identified in the primary ELISA and total number of mycophenolic acid-resistant transformants.

† Stable clones retained the human isotype indefinitely and were identified as replacement recombinants in the Southern blot. Unstable clones lost human Ig secretion within two weeks after the initial positive ELISA.

‡ As determined by Western blotting and by testing for the presence of the C_H1 domain by ELISA.

Integration events induced by the replacement vector

Integration events induced by pSVgpt-huγ1-A5-S5′ might be explained by recircularization and subsequent insertion[11,20] or concatemerization followed by replacement.[21] We wanted to know whether these events also occur in a situation which provides sufficient homology, but precludes primary integration via the 5′ flank. Flanks shorter than 2 kb were shown to be non-functional in the IgG1 integration vector.[15] By cutting with *Eco*RI/*Bam*HI (Fig. 1c) a replacement fragment was generated from pSVgpt-huγ1-A5-S5′ in which the 5′ flank is reduced to 1·4 kb. Despite the presence of a 4·3-kb overall homology the 5′ flank could not be used for vector integration, as all recombinants remained stable and displayed a replacement pattern in the Southern blot (Table 1). Obviously, the additional 3′ flank is not able to stabilize intermediates leading to vector integration. However, also the replacement events were markedly reduced and were just as frequent as in the case of the 3-kb flank of pSVgpt-huγ1-A5. This indicates that the recombination frequency is exclusively determined by the 2·8-kb 3′ flank and that an insufficient 5′ flank cannot even enhance the replacement efficiency.

pSVgpt-huγ1-A5-S5′ can also be linearized as an integration vector by *Eco*RI. This enables us to assess the role of the distribution of homology in direct comparison to pSVgpt-huγ1-A6. As outlined in Table 1, the interruption of the homology suppressed vector integration in favour of gene replacement despite the presence of free adjacent homologous ends. The comparison to the *Pvu*I/*Bam*HI replacement fragment, however, showed that the free adjacent ends do exert some effect, since the frequency of unstable producers was not as low as found with the true replacement fragment.

If secondary vector excision is associated with integration events which are due to intracellular recircularization of the replacement fragment, a circular plasmid too is predicted to undergo such reactions. Transfection of pSVgpt-huγ1-A5-S5′ without linearization did indeed result in secondary vector excision (Table 1). The reduced number of stable clones with a replacement situation suggests that free vector ends may also play some role in initiating the replacement mechanism.

Whereas the number of clones secreting C_H1-deficient and intact human IgG1 was roughly equal when integration vectors were used, the ratio was clearly biased towards intact IgG in the case of the replacement fragments. This effect was reversed when the replacement construct was linearized like an integration vector (Table 1, last column).

A replacement construct with an inverted 3′ flank

The 3′ flank of pSVgpt-huγ1-A5-S5′ is provided by sequences from the switch region which consists of highly repetitive motifs. It is not known whether the function of the Sμ region is strictly dependent on its physiological orientation (A. Radbruch, personal communication). To shed light on this question we transfected the replacement vector pSVgpt-huγ1-A5-S3′ which differs from pSVgpt-huγ1-A5-S5′ by an inversion of the 3′ flank. As shown in Table 1, the recombination frequency, in particular the replacement frequency, was markedly reduced and was in the range of the integration vector pSVgpt-huγ1-A5. Thus, the inverted Sμ region is not functional. The extended heterology provided by the inverted 3′ flank may account for the fact that the integration efficiency is slightly reduced as compared with pSVgpt-huγ1-A5.

DISCUSSION

Homologous recombination at the immunoglobulin loci is a rapid and highly efficient method for mAb chimerization. A shortcoming of IgH integration vectors is the frequent secondary loss of the introduced isotype which can be explained by intrachromosomal recombination between the duplicated sequences.[14,15] On the other hand, replacement constructs which yield a stable situation suffer from the drawback that they have to be matched to the isotype expressed by the hybridoma of interest.[3] We have developed a replacement vector that is universally applicable to virtually all subclasses, because it has a homology whose genomic counterpart is a contiguous sequence, but which is divided by the heterology in the vector (Fig. 1c). Therefore, vectors individually designed for the downstream regions of different subclasses are not necessary.

On examination of the incorporation pattern of a vast array of transformants by Southern analysis, we observed correct integration events solely in the case of an integration vector carrying the human IgG3 isotype.[2] By contrast, all human IgG1 producers which survived the first two weeks after the initial positive ELISA were replacement recombinants. Hence, IgG1 vector integration must always have given rise to secondary excision. The particular propensity of IgG1 for vector excision may be related to the high similarity between human IgG1 and the endogenous mouse IgG2a which may provide extended regions of microhomologies.

In another system, replacement vectors displayed higher recombination frequencies than integration vectors at the IgH locus.[3] In this study, however, the replacement construct carried more homology than the integration vector. We have for the first time compared immunoglobulin replacement and integration vectors which have exactly the same homology sequence and only differ by the distribution of homology thereby excluding the influence of the homology lengths and the particular sequences involved. In fact, the recombination frequency of the replacement construct was increased as compared with pSVgpt-huγ1-A6. This increase was totally accounted for by replacement events, since the frequency of unstable clones was significantly reduced to the level obtained with the integration vector pSV-gpt-huγ1-A5. This latter finding indicates that only the 5′ flank where the cross-over takes place is the essential determinant for vector integration and that the search for homology which should be facilitated by the 3′ flank is not rate limiting in this case. This view is corroborated by the finding that integration is no longer possible when the 2·8-kb 3′ flank is present, but the length of the 5′ flank is below 2 kb which was shown to be the minimum requirement for vector integration.[15] Conversely, the replacement frequency is exclusively determined by the length of the 3′ flank if the 5′ flank is insufficient.

pSVgpt-huγ1-A5-S5′ differs from pSVgpt-huγ1-A6 in that the recombination fragment and the selection marker are separately transferred so that some gpt positive clones may not have received the immunoglobulin fragment. However, this may lead to an underestimation of the recombination frequency and cannot explain the difference observed. Taken together, the efficiencies of integration and replacement vectors endowed with the same homology sequences are dependent on whether the homology constitutes a contiguous stretch or is separated into two halves.

We have previously shown that an immunoglobulin integration vector is capable of initiating replacement events.[16] Conversely, the occurence of unstable producers suggests that our replacement construct can also be incorporated by vector integration. Such events have also been observed at other loci[20] and have been ascribed to allotypic differences between vector and target sequence.[22] In an isogenic situation, replacement targeting took place at high fidelity.[22] However, allotypic differences are not responsible for the unpredicted behaviour of our replacement construct, because integration events also occurred in the isogenic MPC11 cell line. Thus, these events may reflect a particular recombinogenicity of the IgH locus. Integration of a replacement construct may be explained by recircularization or concatemerization prior to incorporation.[11,20,21] Our finding of secondary vector excision following integration of an undigested plasmid is in accordance with the former possibility.

Some replacement events result in a loss of the C_H1 domain.[16] A surprising finding was the suppression of the truncating events by means of IgG replacement constructs. This may be a result of stabilizing the recombination intermediates at either side of the fragment. The effect could be overcome by the free adjacent homologous ends which could be produced by cutting the replacement construct like an integration vector. These findings are important for routine mAb chimerization. Thus, the IgG replacement vector not only provides insights into the mechanisms of insertion and replacement recombination in hybridoma cells, but also is a powerful tool for mAb chimerization.

ACKNOWLEDGMENTS

We are indebted to Dr S. Thierfelder for his support. Furthermore we wish to thank Dr M. Reth for the clone pSVμm5, Dr E. Kremmer for the MmT1 and Dr J. Johnson for the MPC11 cell line. We thank K. Bauer and Dr H. Lindhofer for critically reading the manuscript and A. Kardinal for excellent technical assistance.

REFERENCES

1. Fell H.P., Yarnold S., Helström I., Hellström K.E. & Folger K.R. (1989) Homologous recombination in hybridoma cells: heavy chain chimeric antibody produced by gene targeting. *Proc Natl Acad Sci USA* **86,** 8507.
2. Mocikat R., Kardinal C., Lang P. Zeidler R. & Thierfelder S. (1995) Unaltered immunoglobulin expression in hybridoma cells modified by targeting of the heavy chain locus with an integration vector. *Immunology* **84,** 159.
3. Yarnold S. & Fell H.P. (1994) Chimerization of antitumor antibodies via homologous recombination conversion vectors. *Cancer Res* **54,** 506.
4. Wood C.R., Morris G.E., Alderman E.M., Fouser L. & Kaufman R.J. (1991) An internal ribosome binding site can be used to select for homologous recombinants at an immunoglobulin heavy-chain locus. *Proc Natl Acad Sci USA* **88,** 8006.
5. Sun W., Xiong J. & Shulman M.J. (1994) Production of mouse V/human C chimeric κ genes by homologous recombination in hybridoma cells. *J Immunol* **152,** 695.
6. Baker M.D., Pennell N., Bosnoyan L. & Shulman M.J. (1988) Homologous recombination can restore normal immunoglobulin production in a mutant hybridoma cell line. *Proc Natl Acad Sci USA* **85,** 6432.
7. Shulman M.J., Nissen L. & Collins C. (1990) Homologous recombination in hybridoma cells: dependence on time and fragment length. *Mol Cell Biol* **10,** 4466.
8. Baker M.D., Karn H.A. & Read L.R. (1994) Restoration of a normal level of immunoglobulin production in a hybridoma cell line following modification of the chromosomal immunoglobulin μ gene by gene replacement. *J Immunol Methods* **168,** 25.
9. Thomas K.R. & Capecchi M.R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51,** 503.
10. Deng C., Thomas K.R. & Capecchi M.R. (1993) Location of crossovers during gene targeting with insertion and replacement vectors. *Mol Cell Biol* **13,** 2134.
11. Hasty P., Rivera-Pérez J. & Bradley A. (1992) The role and fate of DNA ends for homologous recombination in embryonic stem cells. *Mol Cell Biol* **12,** 2464.
12. Valancius V. & Smithies O. (1991) Double-strand break repair in a mammalian gene targeting reaction. *Mol Cell Biol* **11,** 4389.
13. Orr-Weaver T.L., Szostak J.W. & Rothstein R.J. (1981) Yeast transformation: A model system for the study of recombination. *Proc Natl Acad Sci USA* **78,** 6354.
14. Baker M.D. (1989) High-frequency homologous recombination between duplicate chromosomal immunoglobulin μ heavy-chain constant regions. *Mol Cell Biol* **9,** 5500.

15. KARDINAL C., HOOIJBERG E., LANG P., ZEIDLER R. & MOCIKAT R. (1995) Integration vectors for antibody chimerization by homo--logous recombination in hybridoma cells. *Eur J Immunol* **25,** 792.
16. LANG P. & MOCIKAT R. (1994) Replacement-like recombination induced by an integration vector with a murine homology flank at the immunoglobulin heavy-chain locus in mouse and rat hybridoma cells. *Mol Gen Genet* **242,** 528.
17. KREMMER E., THIERFELDER S., KUMMER U., LEDERER R. & MYSLIWIETZ J. (1989) Neutralization of immunosuppression by antibodies against variable as well as constant regions of monoclonal anti-Thy-1 xenoantibodies and their ability to be suppressed by initial T cell depletion. *Transplantation* **47,** 641.
18. LASKOV R. & SCHARFF M.D. (1970) Synthesis, assembly and secretion of gamma globulin by mouse myeloma cells. I. Adaptation of the Merwin plasma cell tumor-11 to culture, cloning, and characterization of gamma globulin subunits. *J Exp Med* **131,** 515.
19. BOWTELL D. (1987) Rapid isolation of eukaryotic DNA. *Anal Biochem* **162,** 463.
20. HASTY P., RIVERA-PÉREZ J., CHANG C. & BRADLEY A. (1991) Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. *Mol Cell Biol* **11,** 4509.
21. THOMAS K.R., DENG C. & CAPECCHI M.R. (1992) High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. *Mol Cell Biol* **12,** 2919.
22. DENG C. & CAPECCHI M.R. (1992) Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol Cell Biol* **12,** 3365.