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Reviews and Protocols in DT40 Research

Edited by

Jean-Marie Buerstedde
and Shunichi Takeda

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FOREWORD

Jean-Marie Buerstedde

The DT40 B cell line was established in the laboratory of Eric H. Humphries from an ALV induced B cell lymphoma. However, it was only after the demonstration of high ratios of targeted to random integration of transfected gene constructs that DT40 gained wide-spread popularity. Shunichi Takeda and I have been working with DT40 for the last 15 years and I remember Tatsuko Honjo once told us that we had a love affair with this cell line. If it is love, it was not love without doubts and crisis. It would be unfair to blame this on DT40 as it proved to be a reliable and robust companion with fast doubling time, easy clonability and a relatively stable karyotype. The problem was rather that some of the early knock-outs were technically demanding due to the lack of good chicken cDNA and genome resources. In addition, it is difficult to predict gene disruption phenotypes as seen by genes which are needed for DT40 proliferation, but whose homologues in yeast are not essential. All this would have been much harder to bear without the nice spirit in the DT40 research community. This is still a small, friendly world and many reagents in form of vectors and assays are freely shared among the laboratories even before publications.

It is with this in mind that the DT40 handbook has been perceived by Mike van den Bosch from the Springer publishing house. The intention is to give an up to date overview about the different facets of research, but also to help newcomers get started and avoid looming pitfalls. The collection of protocols which have been kindly provided by a number of laboratories will be particularly useful in this regard.

Research is fast paced and advances in RNA interference have recently opened up new opportunities for genetic experiments in human cell lines. However the possibility to easily modify the genome still remains a powerful tool to investigate the function of coding and regulatory sequences in the vertebrate genome. DT40 has never been a quick and easy road to

fame. If this model system is going to flourish over the next 15 years, it will be thanks to ingenious and original researchers. They may feel as if they work outside the mainstream, but they can take heart by the fact that only the clever exploitation of diversity and conservation makes biological research both elegant and rewarding.

Chapter 1

DT40 GENE DISRUPTIONS: A HOW-TO FOR THE DESIGN AND THE CONSTRUCTION OF TARGETING VECTORS

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Abstract: Genome projects have provided comprehensive gene catalogs and locus maps for many model organisms. Although sequence comparison and protein domain searches may suggest evolutionary conserved gene functions, genetic systems are still needed to determine the role of genes within living cells. Due to high ratios of targeted to random integration of transfected DNA constructs, the chicken B cell line DT40 has been widely used as a model for gene function analysis by gene knockout. Targeting vectors need to be carefully designed to introduce defined mutations and to ensure high targeting rates. In this review we summarize general guidelines for the design of targeting vectors which can be used for single, multiple or conditional gene knockouts, as well as site-directed genome mutagenesis in DT40.

Key words: DT40, knockout, targeting vector, mutant loxP.

1. INTRODUCTION

Targeted integration of DNA constructs by homologous recombination enables the inactivation of genes by disruption or deletion as well as the introduction of more subtle gene mutations. This approach, first pioneered for the yeast *S. cerevisiae* has been used extensively for gene modification in murine embryonic stem (ES) cells, which are subsequently used to produce mutant mouse strains. However, genes essential for cell proliferation and tissue development are often difficult to study, as a homozygous deletion

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causes early embryonic lethality. Gene disruption in a cell line is an alternative to knockouts in murine ES cells, if the mutant phenotype can be studied in cell culture. The chicken B cell line DT40 is popular for these studies due to unusual high ratios of targeted to random integration (Buerstedde and Takeda, 1991).

The design of targeting vectors for DT40 studies requires information about the chicken target gene locus in form of restriction and exon-intron maps. The ideal situation, in which the sequence of the entire locus is available, is now often encountered, because more than 90% of chicken genomic sequence has been released to the public databases (International Chicken Genome Sequencing Consortium, 2004). In addition, large EST and full length cDNA sequence database from bursal cells (Abdrakhmanov et al., 2000; Caldwell et al., 2005) and other chicken tissue (Boardman et al., 2002) help to reveal the precise exon-intron structure of many loci (Caldwell et al., Chapter 3, this issue).

A series of versatile plasmid vectors have been developed to assist genetic engineering of DT40. Mutant loxP vectors enable the excision of the drug-resistance gene by Cre/loxP recombination for drug-resistance marker recycling (Arakawa et al., 2001). In this way vectors including the same drug resistance gene can be repeatedly used for the selection of stable transfectants. Other vectors allowing the cloning of cDNA's into a loxP flanked expression cassette can be useful for the complementation of knockout phenotypes and conditional gene expression (Arakawa et al., 2001). These tools are freely distributed through DT40 web site (http://pheasant.gsf.de/DEPARTMENT/dt40.html), and have been commonly used by the DT40 community.

1.1 How to determine the exon-intron structure of the target locus

Information about the genomic locus of the gene of interest is critical for the design of the gene targeting construct. To determine the exon-intron structure of a gene, you can simply use the chicken cDNA sequence as input for a genome BLAT search (http://genome.cse.ucsc.edu/cgi-bin/hgBlat). If the sequence of the genomic locus is available, the cDNA sequence will be aligned along the genomic sequence, showing the location of the exons. If the chicken cDNA sequence is not available, it is still worth trying to use either the cDNA or the protein sequence of the gene ortholog from other species like human or mouse as input. Depending on the degree of sequence conservation this may indicate the locations of homologous exons. More detailed information on how to use the available genome resources is provided in another review in this issue (Randy Caldwell et al., Chapter 3, this issue).

1.2 How to design a gene knockout construct

Gene knockout constructs need to be carefully designed to ensure the introduction of the desired mutation at high ratios of targeted to random integration. The inactivation of a gene (null mutation) can either be achieved by gene deletion or gene disruption. If feasible, the deletion of the complete coding sequence is preferred, since this precludes interference of left over gene sequences with the mutant phenotype. Since the size of the deletion is determined by the positions of the 5' and 3' target arms within the genome sequence, it is not difficult to design constructs for large deletions, if the arm sequences are available. However, a large distance between the 5' and 3' target arms in the genome sequence most likely decreases the ratios of targeted to random integration of the construct. Although more than 20 kb of the immunoglobulin light chain locus could be deleted using a conventionally designed targeting construct (Arakawa et al., 2004), we have the feeling that the ratios of targeted to random integration are difficult to predict a priori for deletions of this size. We usually try complete gene deletions only, if the size of the target locus does not exceed 5 kb.

If the target locus covers large genomic distances, one may attempt to create a null mutation either by the deletion of exons encoding a critical domain of the protein or by deleting as much of the gene coding region as possible. We usually combine a partial gene deletion with the introduction of an in-frame stop codon near the 5' end of the gene coding sequence. This has the advantage that the translation of the remaining transcript will terminate at a defined position and produce a truncated peptide which is unlikely to have a function. However, the effects of the partial gene deletions must be carefully considered on a case by case basis, because it is difficult to predict the phenotypes with certainty due to possible variation in mRNA translation and splicing. Especially if a mutation does not produce a measurable effect, it becomes difficult to determine whether this is due to the incomplete inactivation of the gene or due to redundant gene function. In certain situations, the partial inactivation of a gene will be more informative than a null mutation and is the intended outcome of the gene targeting as shown below for the example of a PCNA mutation.

The size of target arms may influence targeting efficiency, and in general, longer target arms are believed to increase targeting efficiency. However, there is a trade-off as plasmids of larger size are more difficult to handle and possess a lower number of unique restriction sites. We usually design our constructs in such a way that the sizes of the individual 5' and 3' arm of are more than 1 kb, the combined size of the 5' and the 3' arms is more than 3 kb and a total plasmid size is less than 12 kb. Targeting vectors made according

to these rules will usually give targeting efficiencies of 20-80% among stable transfectants.

We recommend amplification of the target arms by long range PCR using primers with attached restriction sites and genomic DNA from DT40 as template. This will produce arm sequences isogenic to at least one allele of DT40 and should enhance the targeting efficiency. A systematic design of knockout constructs enables the easy exchange of drug resistance marker cassettes. All of our drug resistance marker cassettes are flanked by *Bam*HI sites and can be conveniently cloned into *Bam*HI or *Bgl*II sites located between the 5' and the 3' arms. If feasible, we try to clone the 5' arm into the *Xho*I-*Bam*HI sites and the 3' arm into the *Bam*HI-*Spe*I sites of pBluescript plasmid vector. If one of those restriction sites is present internally in the arm sequences, other compatible sites for example *Sal*I instead of *Xho*I; *Xba*I, *Nhe*I and *Avr*II instead of *Spe*I; *Bgl*II and *Bcl*I instead of *Bam*HI might be chosen. The resulting targeting vector can usually be linearized in the plasmid polylinker by the rare cutter *Not*I, which is unlikely to cleave the target arms.

Efficient targeting of the first allele, but no recovery of homozygous mutant clones after transfection of the heterozygous clone may indicate an critical role of the target gene for cell growth. However, difficulties to recover clones targeted for both alleles in the second transfection cannot be taken as proof that the target gene is essential, because we have experienced situations in which the second allele could eventually be inactivated by a differently designed targeting construct. The most likely explanation for this phenomenon is that the original knock-out construct targeted preferentially the already targeted allele due to a stronger degree of sequence homology and/or due to the difference of deletion size. We therefore recommend to redesign the position of one target arm, if homozygous mutant are not recovered after transfection of heterozygotes and there remains doubt about whether the target gene is essential. The best way to prove that a gene is required for DT40 cell survival or proliferation is by conditional gene knockouts as described below.

1.3 Drug resistance marker recycle by Cre/loxP recombination

The generation of a homozygous mutant usually requires two different drug resistance markers for the stepwise targeting of both alleles and another drug resistance marker is needed if the mutant phenotype is complemented by the re-introduction of the gene. Modifications of the genome by additional transfections are limited by the number of available drug resistance markers. This problem can be solved by the excision of the drug

resistance marker using a site-specific recombination system like Cre/loxP. We previously described floxed drug-resistance marker cassette vectors which can be recycled after Cre expression (Figure 1) (Arakawa et al., 2001).

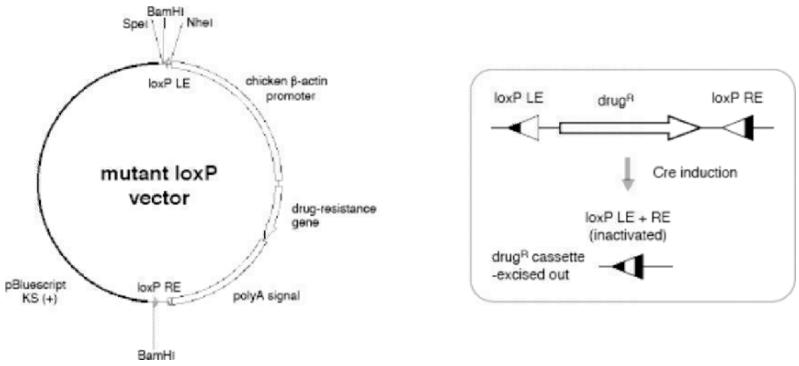

Figure 1-1. Drug resistant marker cassette flanked by mutant loxP sites. Left, Five vectors are available including different drug-resistance genes: pLoxNeo, pLoxPuro, pLoxBsr, pLoxGpt and pLoxHygro. Right, The principle of the mutant loxP system Rearrangement of mutant loxP sites creates a new site which is poorly recognized by Cre.

To prevent genetic instability due to the cutting of the loxP sites left-over after marker excision, the drug resistant markers are flanked by mutant loxP sites (loxP_RE and loxP_LE) (Albert et al., 1995). The Cre recombinase efficiently recombines the loxP_RE and loxP_LE sites leading to the deletion of the drug resistance marker gene. However, the new variant loxP_RE+LE site generated after the excision is only poorly recognized and cleaved by Cre. At this moment five of mutant loxP vectors are available: three vectors, pLoxNeo (neomycin resistance), pLoxPuro (puromycin resistance) and pLoxBsr (blasticidin S resistance) which have been reported previously (Arakawa et al., 2001), and two new vectors, pLoxGpt (mycophenolic acid resistance) and pLoxHygro (hygromycin resistance) which have been recently completed (unpublished results). All these vectors contain drug resistance genes driven by the chicken β-actin promoter. The selectable marker cassettes can be easily cloned into the *Bam*HI or *Bgl*II sites of targeting constructs and are freely distributed as part of the DT40 web site.

1.4 Conditional gene knockout

Mutants homozygous for genes which are essential for cell survival or proliferation cannot be produced by standard targeted gene disruption. One way to study the function of these genes is by stable transfection of conditional cDNA expression constructs followed by the knockout of both alleles. When the expression of the cDNA is terminated, such cell line will be converted to the homozygous mutant stage.

A vector, pExpress, has been designed for the conditional expression of cDNA inserts (Figure 2) (Arakawa et al., 2001). The expression of the cloned cDNA is controlled by the chicken β-actin promoter and a SV40 poly A signal. The cDNA of interest can be inserted into multiple cloning sites (*Hin*dIII, *Nhe*I, *Eco*RV, *Bgl*II, *Nco*I, *Sma*I sites). The pExpress vector can be combined with the mutant loxP vectors by cloning the cDNA expression cassette (*Spe*I cassette) either outside (*Spe*I site) or inside (*Nhe*I site: *Spe*I-compatible) of the marker cassettes. pExpress offers additional flexibility, because any one of five different drug-resistance marker genes (*neoR*, *puroR*, *bsr*, *gpt* and *hygroR*) can be chosen and expression constructs can be designed either for random or targeted integration.

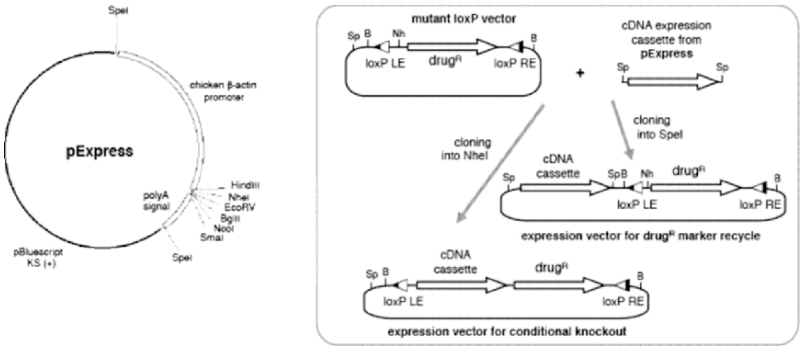

Figure 1-2. The cDNA expression vector, pExpress. After inserting a cDNA coding sequence into pExpress, the expression cassette can be excised as a *Spe*I cassette. Cloning of the expression cassette (*Spe*I cassette) into the *Spe*I site of mutant loxP vectors produces an expression vector whose drug-resistance marker can be recycled. Cloning of the expression cassette (*Spe*I cassette) into the *Nhe*I site of mutant loxP vectors produces a vector in which the drug-resistance marker and the expression cassette are located between mutant loxP sites and can be excised together. B: *Bam*HI, Nh: *Nhe*I, Sp: *Spe*I.

In addition, a new generation of conditional expression vectors is being developed which allows cDNA expression to be turned on (pTurnOn) or turned off (pIresGfp and pIresDrugR) by mutant-loxP based inversion or deletion. Multiple cloning sites are followed either by an internal ribosome entry site (IRES)-GFP sequence or an IRES-drug resistance gene enabling an estimation of the cDNA expression levels by measuring green fluorescence or drug-resistance. These vectors are currently being tested in pilot experiments. They will be released to the DT40 community as soon as their function is confirmed and documented.

1.5 Site-directed mutagenesis

Site directed mutagenesis of genes is possible in DT40 by the expression of mutant cDNA's after the disruption of the endogenous gene copies (Morrison et al., 1999). However, difficulties to fine tune gene expression using artificial cDNA expression vectors can be a problem, if a certain level of gene expression or cell cycle controlled gene expression is required. In these cases, it is preferable to introduce the desired mutation into the endogeneous loci of DT40 thereby preserving the transcriptional and post-transcriptional regulation of the mutated gene. Cre/loxP marker cassette recycle can be adapted to introduce subtle mutations into a locus accompanied by minimal additional sequence modifications. We tested this strategy in DT40 by studying the role of post-translational modification of the proliferating cell nuclear antigen (PCNA) whose expression is synchronized with DNA replication. Since this cell cycle dependent regulation of PCNA expression is likely to be required for normal proliferation rates of DT40 cells, a point mutation of codon 164 was introduced into the *PCNA* locus using a construct which carried the mutation in its 5' target arm (Figure 3).

Targeted integration of the PCNA mutagenesis construct accompanied by a crossing-over upstream of the mutation in the 5' target arm introduced the point mutation into the locus. Since the bsr marker gene cassette may perturb the splicing and transcription of *PCNA* gene, it was excised out by Cre induction leaving only a remnant loxP site in an intronic position. This procedure was repeated to modify the second allele. The presence of the desired mutations was confirmed by sequencing of *PCNA* locus.

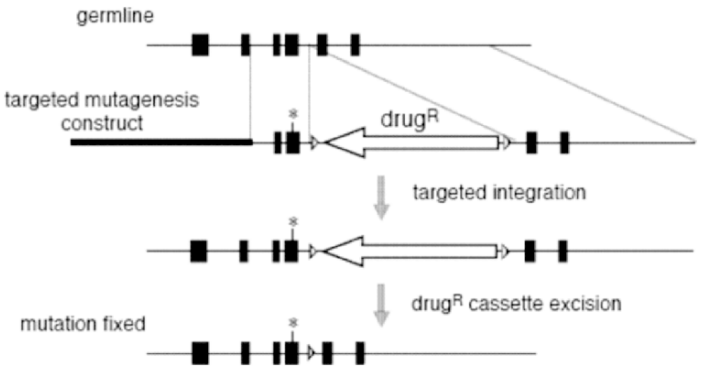

Figure 1-3. Site-directed mutagenesis of a target gene locus using PCNA as an example. Physical maps of the PCNA locus and the PCNA mutagenesis construct are shown together with the targeting strategy.

1.6 Future perspective

Work with murine embryonic stem cells has been a driving force to develop a wide range of technologies for targeted genome modifications. Most of these techniques could be successfully adapted to DT40. We are currently working on a new series of cloning vectors which should further facilitate studies in DT40. Among these are i) a new generation of user-friendly, conditional expression vectors, ii) a loxP system using mutant loxP sites for conditional gene knockout without cDNA complementation, iii) expression vector carrying tags for biochemical purification of the protein complexes, iv) a new version of mutant loxP vectors based on Gateway technology (Invitrogen) for restriction-site independent cloning of targeting vectors. Some of these futuristic vectors already exist and they will be released for general use as soon as quality checks and proper documentation are completed.

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Chapter 2

IMMUNOGLOBULIN GENE CONVERSION OR HYPERMUTATION: THAT'S THE QUESTION

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Abstract: Chicken B cells develop their primary immunoglobulin (Ig) gene repertoire by pseudogene templated gene conversion within the bursa of Fabricius. The DT40 cell line is derived from bursal B cells and continues to diversify its rearranged Ig light chain in cell culture. Ig gene conversion of DT40 requires expression of the AID gene which was earlier shown to be needed for Ig hypermutation and switch recombination in mammalian B cells. Interestingly, Ig hypermutation can be induced in DT40, if Ig gene conversion is blocked by the disruption of RAD51 paralog genes, the deletion of the nearby pseudogene locus or the disruption of the UNG gene. The ease of gene targeting and the compactness of the chicken Ig light chain locus makes DT40 an ideal model to study the molecular mechanism of AID induced gene conversion and hypermutation.

Key words: Immunoglobulin, gene conversion, somatic hypermutation, DT40, B cell, AID, UNG, DNA repair.

1. INTRODUCTION

The immune system of vertebrates is unique, as B lymphocytes activate and diversify their Ig genes by recombination and hypermutation. Somatic modification of the Ig loci accomplishes the following tasks at different stages of B cell development: i) allelic exclusion assuring the expression of only one Ig light and heavy chain in each B cell, ii) development of a diverse primary antibody repertoire, iii) affinity maturation of antibodies leading to

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tighter antigen binding and iv) isotype switching to produce antibodies with different heavy chain isotypes. These biological phenomena can be explained by four types of sequence alterations in the Ig loci: V(D)J recombination, gene conversion, hypermutation and switch-recombination.

Ig genes are not encoded in a functional form in the germline, but are assembled from gene segments by site-specific recombination. In humans and mice, functional V, D and J gene segments exist in large clusters and the random assortment of individual V, D and J segments generates considerable combinatorial diversity for the primary repertoire (Tonegawa, 1983).

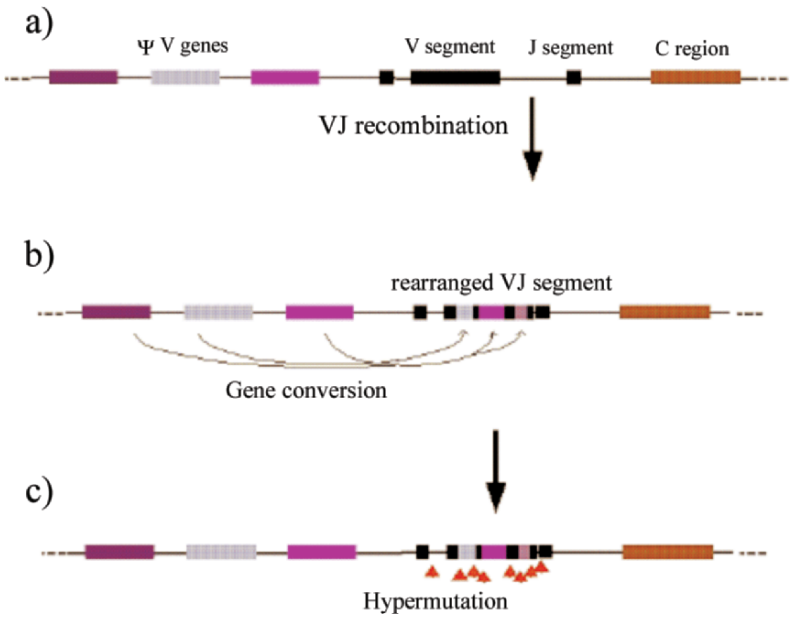

Figure 2-1. Development of a diverse Ig light chain gene repertoire by gene conversion and hypermutation (see plate 1).

In other vertebrate species, V(D)J recombination only ensures allelic exclusion. This was first demonstrated for the chicken where only a single functional V and J segment are rearranged in the light chain gene locus (Fig.2-1a). The rearranged chicken light chain V segment is diversified by gene conversion using nearby pseudo V (ψV) genes as donor sequences (Reynaud et al., 1987) (Fig.2-1b). A gene conversion scheme, in which the boundaries and the lengths of the conversion tracts vary and multiple

conversion events superimpose on each other, is ideally suited for the development of a diverse primary Ig repertoire from a germline sequence pool. Not only avian species, but also rabbits, cattle, swine and horses create their primary Ig gene repertoire by this elegant mechanism (Butler, 1998).

Following antigen stimulation the rearranged V(D)J segments are further modified by the introduction of single untemplated nucleotide substitutions (Fig.2-1c). Some of the mutations increase the affinity of the encoded antibodies leading to the proliferation of the respective B cells (Jacob et al., 1991). All vertebrate species including chicken (Arakawa et al., 1996) employ Ig hypermutation for affinity maturation of antibodies. In parallel to hypermutation, class switch recombination introduces deletions between so-called switch regions in the Ig heavy chain locus resulting in the expression of a new isotype (Dudley et al., 2005).

1.1 Ig hypermutation and switch recombination in mice and men

A number of approaches have been tried to identify genes involved in Ig gene repertoire formation in mammals. B cell lines undergoing Ig gene diversification and other cell lines in which this phenomenon is induced by gene transfections are valuable for cell culture experiments. Mice and human patients suffering from hereditary immunodeficiencies can be searched for mutations in genes involved in Ig recombination or hypermutation. The function of candidate genes can be tested by disruption in murine embryonic stem cells and the analysis of lymphoid developments in animals derived from the embryonic stem cells.

The outlined studies were successful to define the lymphoid specific and general DNA repair genes and the mechanism of V(D)J recombination in molecular detail (Jones and Gellert, 2004, Dudley et al., 2005). In comparison, the genetics of Ig hypermutation and switch recombination remained poorly understood for a long time (Jakobs et al., 1998). However, the cloning of the AID gene from switch recombination active cells (Muramatsu et al., 1999) and the subsequent demonstration that Ig hypermutation and switch recombination is abolished in AID knockout mice (Muramatsu et al., 2000) and AID deficient patients (Revy et al., 2000) was a breakthrough. The hypothesis that the AID protein, which contains an evolutionary conserved cytosine deamination motif, directly deaminates cytosines to uracils inspired further genetic studies. Most important were the findings that inactivation of the UNG gene inhibited switch recombination and changed the spectrum of Ig hypermutations at G/C bases in mice (Rada et al., 2002) and human patients (Imai et al., 2004). Although hypermutations at A/T bases were not affected by UNG deficiency, these mutations were reduced in MSH2 and MSH6

deficient mice (Wiesendanger et al., 2000). UNG/MSH2 double knock-out mice showed no hypermutations at A/T bases and no switch recombination (Rada et al., 2004) consistent with the hypothesis that MSH2 is needed for A/T hypermutations and for the low level of switch recombinations still present in UNG deficient mice.

It remains still unresolved how hypermutations are targeted to the Ig loci. The analysis of hypermutations in B cells indicated that mutations are limited to a 2-kb region from the Ig transcription start site and that apart from rare exceptions, other transcribed genes are not mutated (Kotani et al., 2005). It was furthermore demonstrated that artificial Ig locus constructs in transgenic mouse lines required the presence of Ig enhancer sequences for hypermutation activity (Neuberger et al., 1998). Nevertheless, new results from AID expressing non B cell and even B cell lines suggest that hypermutations may not be strictly limited to Ig loci and that AID can act as a global genome mutator (Yoshikawa et al., 2002; Wang et al., 2004).

1.2 Ig gene conversion in DT40

Ig gene conversion in bursal B cells and the DT40 cell line has recently been reviewed (Arakawa and Buerstedde, 2004) and emphasis is placed on new insights and certain technical issues. Ig gene conversion is difficult to investigate in primary B cells from chicken or other farm animals, because these cells can be maintained only for short time in cell culture. In contrast, the ALV induced lymphoma cell line DT40 which seems to be arrested at the stage of bursal B cells continues gene conversion during in-vitro cell culture (Buerstedde et al., 1990; Kim et al., 1990). A high ratio of targeted to random gene integration (Buerstedde and Takeda, 1991) makes DT40 an ideal system to study the mechanism of Ig gene conversion by candidate gene disruptions and the modifications of cis-acting regulatory sequences.

Ongoing gene conversion can be detected in DT40 by sequencing rearranged light chain VJ segments from the progeny of a single cell after subcloning. In most cases, the VJ sequence of the progenitor cell is still dominant among the sequences and can be established as a consensus sequence to which all divergent sequences are compared. This approach has the advantages that conversion events can be detected within the whole VJ segments and the length of the tracts as well the likely pseudogene donors can be determined. However, an exact quantification of the Ig conversion activity in different genetic backgrounds is difficult by direct sequencing due to fluctuation effects within individual subclones. This problem can be addressed by using the surface Ig (sIg) reversion assay which relies on the repair of an Ig light chain frameshift by gene conversion (Buerstedde et al.,

1990) (Fig. 2-2). Wild-type DT40 cells are dominantly sIg(+), but spontaneously arising subclones have been isolated which have lost sIg expression due to frameshifts in the rearranged light chain V segment. Overlapping gene conversion events can repair these frameshifts leading to re-expression of sIg. The reversion from sIg(-) to sIg(+) status can be easily measured by fluorescence activated cell sorter (FACS) analysis in a large number of subclones. Furthermore, sIg(+) revertants from individual subclones can be isolated by preparative FACS sorts and their rearranged light chain V segments can be sequenced to confirm that the frameshift was indeed repaired by gene conversion.

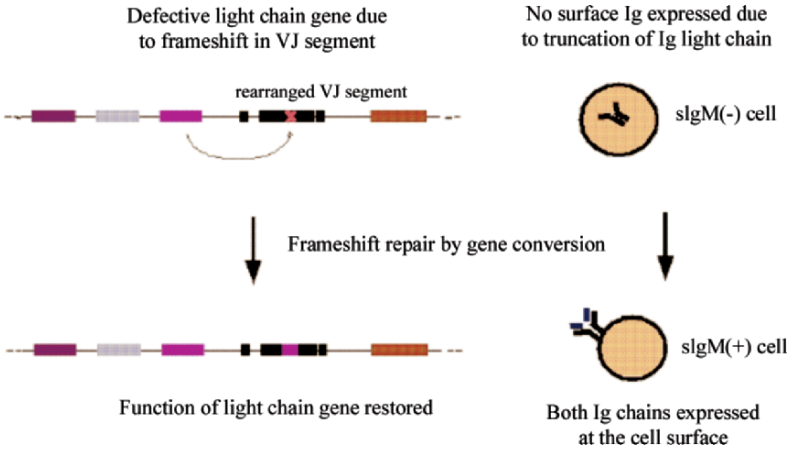

Figure 2-2. Surface Ig reversion assay to quantify Ig gene conversion activity (see plate 2).

The level of Ig gene conversion has been tested in many DT40 mutants in which general DNA repair and recombination factors had been inactivated. Reminiscent of Ig hypermutation, the deletion of the AID gene had most striking effect abolishing all detectable Ig gene conversion activity of DT40 (Arakawa et al., 2002; Harris et al., 2002). This result demonstrated that all three B cell specific mechanisms of Ig diversification - hypermutation, switch recombination and gene conversion - are tightly regulated by AID. Surprisingly, no evidence for Ig gene conversion events were detected in $AID^{-/-}$ cells by the Ig reversion assay even after prolonged culture given that most cells seem to perform intrachromosomal gene conversion at low frequency (Liskay and Stachelek, 1983). This suggests that Ig gene conversion is completely suppressed in the absence of AID expression

despite the unusual density of potential conversion donor sequences in the neighborhood.

A reduction, but not a complete block in Ig gene conversion frequencies was demonstrated for a number of other DT40 mutants by the Ig reversion assay or by direct sequence analysis. Most of these mutants are either members of the RAD52 recombination repair pathway which is known to mediate DNA repair by homologous recombination (Bezzubova et al., 1997; Sale et al., 2001; Hatanaka et al., 2005) or are associated with this pathway (Hatanaka et al., 2005). Inactivation of two members of the Fanconi anemia pathway, FANCC and FANCD2, also reduced Ig gene conversion activity a few fold (Niedzwiedz et al., 2004; Yamamoto et al., 2005). Interestingly, all mutants mentioned above showed not only reduced Ig gene conversion, but also lower ratios of targeted to random gene integration suggesting that the same recombination factors participate in both phenomena. This is consistent with the hypothesis that bursal B cells and DT40 have up-regulated homologous recombination to facilitate Ig gene conversion and that the high ratios of targeted to random integration are a convenient side-effect of this hyper-recombination activity.

Ig gene conversion was also decreased after transfection of the dominant negative uracil glycosylase inhibitor Ugi (Di Noia and Neuberger, 2004) and after UNG gene disruption (Saribasak et al., submitted) indicating that UNG is not only required for normal Ig switch recombination and Ig hypermutation, but also for Ig gene conversion. It was recently reported that disruption of the REV1 gene, which encodes an error prone polymerase, also decreases Ig gene conversion as measured by the Ig reversion assay (Okada et al., 2005). However, second group did not observed a decrease in Ig gene conversion in $REV1^{-/-}$ cells by direct light chain gene sequencing (Simpson and Sale, 2003). Another finding which needs to be confirmed is a report that over-expression of the NBS1 gene stimulates Ig gene conversion in DT40 (Yabuki et al., 2005). No correlation between the amount of NBS1 over-expression and the stimulatory effect on Ig gene conversion were observed and it would be interesting to test how NBS1 gene disruption affects Ig gene conversion (Tauchi et al., 2002). Ig gene conversion is significantly enhanced after treating DT40 with the histone deacetylase inhibitor, trichostatin A, and this phenomenon could be exploited for the production of antigen specific antibodies in DT40 (Seo et al., 2005) .

1.3 Ig hypermutation in DT40

Wild-type DT40 diversifies its Ig gene predominantly by ψV templated gene conversions similar to bursal B cells. Nevertheless, single nucleotide substitutions, which cannot be accounted for by the known ψV genes of the

chicken CB strain, are occasionally found in light chain VJ sequences of the same subclone. These apparently untemplated nucleotide substitutions have been considered Ig hypermutations (Simpson and Sale, 2003; Niedzwiedz et al., 2004), although it cannot be ruled out that they are templated by polymorphic ψV genes of DT40 or that they are a by-product of gene conversion events (Reynaud et al., 1997). Good evidence for a new Ig hypermutation activity is however found in certain DT40 mutants in which AID is expressed and Ig gene conversion is compromised at an early stage. A shift from Ig gene conversion to hypermutation were first reported for disruptions of the RAD51 paralog genes, XRCC2, XRCC3 and RAD51B, which are members of the RAD52 recombination repair pathway (Sale et al., 2001). Direct sequencing of the light chain VJ segments of mutant subclones revealed a decreased frequency of gene conversion tracts and high frequencies of single untemplated nucleotide substitutions at G/C bases.

This hypermutation activity is reflected by the fast appearance of sIg negative cells during the culture of subclones (Sale et al., 2001). In this Ig loss assay (Fig. 2-3), the precursor cell is sIg(+), and deleterious Ig hypermutations lead to the loss of sIg expression in some of its progeny which can be easily measured by FACS. Wild-type DT40 and non-hypermutating mutants generate only low numbers of sIg(-) negative cells, because Ig gene conversion events are usually accurate and the resulting sequence modifycations rarely interfere with sIg expression. A correlation of sIg status and light chain VJ sequences in a situation, where only hypermutations of the light chain gene are expected, suggests that most sIg expression defects are not due to premature stop codons, but to mis-sense mutations which impair Ig light and heavy chain pairing (Arakawa et al., 2004). The sIg loss assay can be used to quantify Ig hypermutation activity easily in a large number of subclones and thereby minimize fluctuation effects. However, this assay is of questionable value for the quantification of Ig gene conversion activity (Yabuki et al., 2005) and the sIg reversion assay should be used instead for this purpose.

Truncation of the BRCA2 gene also decreased Ig gene conversion and activated Ig hypermutation indicating that BRCA2 acts in a similar way to the RAD51 paralogues. However, the RAD54 (Bezzubova et al., 1987), the FANCC (Niedzwiedz et al., 2004) and the FANCD2 (Yamamoto et al., 2005) disruption mutants showed no evidence for an increase of untemplated Ig mutations despite a reduced Ig gene conversion activity.

A complete block in Ig light chain gene conversion and a high frequency of hypermutations at G/C bases within and surrounding the light chain VJ segment were seen after the deletion of all ψV genes in the rearranged light chain locus (Arakawa et al., 2004). This demonstrated that only the ψV genes on the same chromosome are used as donors sequences for conversion

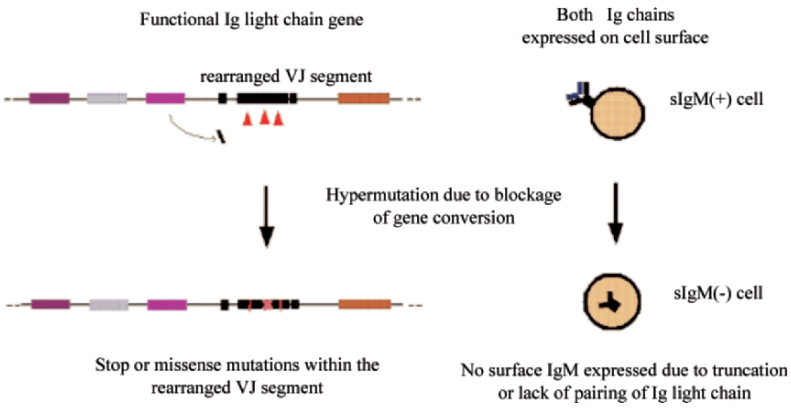

Figure 2-3. Surface Ig loss assay to quantify Ig hypermutation activity (see plate 3).

events. The hypermutation activity induced by the pseudogene deletion was shown to be AID dependent, Ig locus specific and focused to known Ig hypermutation hotspot sequences within the light chain V sequence. In contrast to the other hypermutating DT40 mutants, the ψV deleted variant of DT40 is DNA repair proficient and devoid of any background Ig gene conversion activity and therefore represents an ideal system to study the mechanism of Ig hypermutation in more detail.

1.4 The relationship of Ig gene conversion and hypermutation

The results cited above support a simple model explaining how Ig hypermutation and recombination may be initiated and regulated (Arakawa et al., 2004) (Fig.2-4). At the top of the events is a modification of the rearranged V(D)J segment induced by AID. The default processing of this alteration in the absence of nearby donors or high homologous recombination activity leads to Ig hypermutation in form of a single nucleotide substitution (Fig.2-4, right side). However, if donor sequences and homologous recombination factors are available, processing of the AID induced lesion can be divided into a stage before strand exchange, when a shift to Ig hypermutation is still possible and a stage after strand exchange when a commitment toward Ig gene conversion has been made (Fig.2-4, left side). Whereas completion of the first stage requires the participation of the RAD51 paralogues and BRCA2, the second stage involves other recombination factors like the Rad54 protein and the Fanconi anemia pathway.

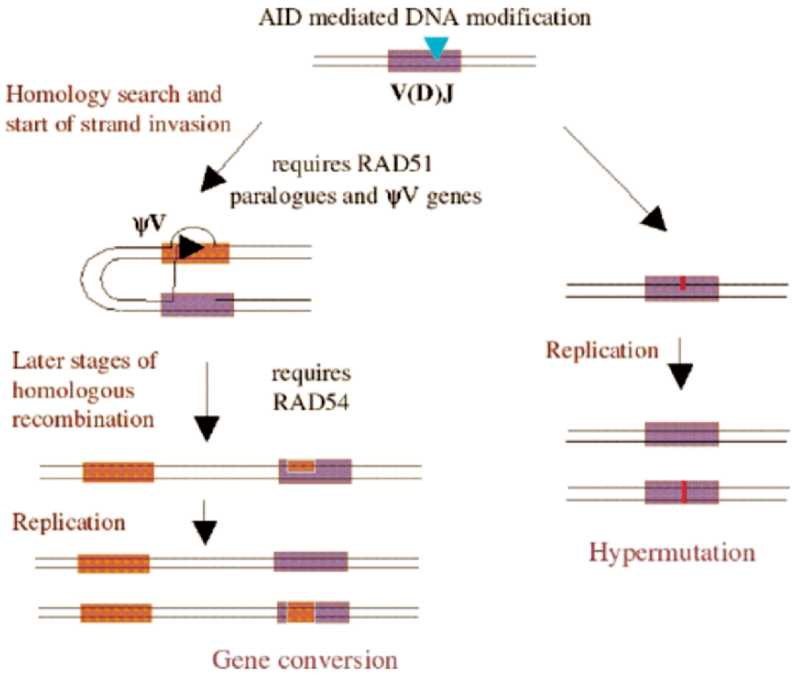

Figure 2-4. Model for Ig gene conversion and hypermutation (see plate 4).

The difference in commitment explains why disruptions of the RAD51 paralog and BRACA2 genes not only decrease Ig gene conversion, but also induce Ig hypermutation (Sale et al., 2001; Yabuki et al., 2005) whereas disruption of the RAD54 gene only decreases Ig gene conversion (Bezzubova et al., 1997). The model also predicts that a low homologous recombination activity precludes Ig gene conversion even in the presence of conversion donors. Such a lack of recombination proficiency might be the reason why human and murine B cells never use Ig gene conversion despite the presence of nearby candidate donors in form of unrearranged V segments and why chicken germinal center B cells have shifted the balance from Ig gene conversion to Ig hypermutation (Arakawa et al., 1996).

1.5 Insight into the mechanism of Ig hypermutation

The exciting discovery of Ig hypermutation in DT40 mutants of the RAD51 paralogues triggered further studies about the hypermutation