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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...
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. DT40 Gene Disruptions

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