Transgenic Animals Derived by DNA Microinjection

Marianne Brüggemann, Michael J. Osborn, Biao Ma, Suzanne Avis, Ignacio Anegon, and Roland Buelow

4.1 Introduction

Antibodies are produced in all jawed vertebrates with diverse repertoires being generated by DNA rearrangement, first for the immunoglobulin heavy (IgH) V (variable), D (diversity), and J (joining) segments, then by IgL (light) V and J segments [1, 2]. In mammals, this rearrangement is initiated at the pre B-cell stage in bone marrow cells which subsequently express surface IgM and migrate via the cardiovascular system to other lymphatic organs such as spleen and lymph nodes. Upon antigen encounter, low affinity binders can be edited by somatic hypermutation and this may be followed by cellular expansion [3]. For therapeutic applications, monoclonal antibodies have been derived from rodents using spleen cells from immunized animals fused to myeloma cells and from human blood lymphocytes using phage and ribosome display. In addition, rodent antibodies have been “humanized” and transgenic animals have been generated to produce human antibody repertoires [4]. Figure 4.1 provides a list of FDA-approved monoclonal antibodies produced in the last ~20 years, which shows a recent increase of fully human antigen binders derived from transgenic mice.

Here, we summarize the production of fully human monoclonal antibodies in mice and rats generated by DNA microinjection and compare these strains with lines derived from manipulated embryonic stem (ES) cells. In the previous edition, we provided extensive details of the various transgenic constructs to express fully human Ig [5]. In this review, particular emphasis is on the efficiency of innovative transgenic constructs, which, in combination with new KO approaches, yield high expression levels and diverse monoclonal antibodies of sub-nanomolar affinity as effectively as endogenous loci in normal animals.
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Figure 4.1 FDA-approved therapeutic monoclonal antibodies from 1994 to 2013. (Underlined products have been withdrawn (www.immunologylink.com/FDA-APP-Abs.html and http://en.wikipedia.org/wiki/Monoclonal_antibody_therapy.)

4.2 Construction of Human Ig Transloci

The human Ig loci have been cloned in bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) libraries and all genes have been sequenced [6]. For the IgH locus 38–46 functional V_H genes have been identified followed by ∼23 D segments, 6 J_H segments, and 9 constant (C)-region genes on a region of ∼1.3 Mb [7, 8]. The Igκ locus accommodates up to 36 functional V_κ genes, 5 J_κ, and 1 C_κ, all on ∼2.6 Mb, but with V_κ genes in both transcriptional orientation and clustered on two well-separated regions [9, 10]. For the Igλ locus 29–33 functional V_λ genes have been identified, followed by 4–5 J_λ-C_λ genes on an ∼1 Mb region [11, 12].

Sizes and contents of fully human Ig loci introduced and expressed in transgenic animals have been summarized [5, 13] with many regions being quite small and incomplete. Here we focus on a new strategy to assemble, integrate, and express Ig loci on cointegrated BACs of several hundred kilobase pairs with extensive V and C regions.

4.2.1 IgH

The first germline configured construct to rearrange and express chimeric human H-chains in mice was a plasmid minilocus of ∼25 kb [14], which was followed
4.2 Construction of Human Ig Transloci

by fully human regions of ∼100 kb on cointegrated cosmids [15] or a YAC [16]. These constructs on plasmids, cosmids, and YACs contained human V-, D-, J-, and C-region genes, which after rearrangement would provide fully human antibody H-chains. In general, larger constructs with more genes allowed better expression in a transgenic animal when the natural configuration was maintained [17]. For example, XenoMouse animals [18] carry a substantially larger and more diverse IgH translocus and this led to a better expression when compared to smaller and less diverse transloci [19–21]. Nevertheless, it became clear that fully human IgH transloci were suboptimal regarding their efficiency in human antibody production and it has been suggested that this may be caused by the imperfect interaction of membrane-expressed human C regions with rodent cellular signaling components [21, 22]. It was also reasoned that inclusion of large parts of cis-acting control sequences might improve affinity maturation as sequences downstream of Cα in rat and mouse may play an important role in class-switch recombination as well as hypermutation [21, 23, 24].

To overcome the shortcomings in expressing fully human H-chains from transgenes, a rat strain was generated that carries a translocus with human V\textsubscript{H}, D, and J\textsubscript{H} genes in natural configuration but linked to the rat C-region locus [22]. Figure 4.2 shows the inserted IgH locus made up from three modified BACs, which provided the sequence, and homology regions for overlapping integration of human V\textsubscript{H}s, and human D and J\textsubscript{H} segments linked to rat C-region genes with the complete 3′ regulatory region. The three parts of the chimeric region were assembled from several BACs containing human and, separately, rat genomic sequences by purification of the relevant fragments, design of large oligos for joining up regions, and transformation into yeast to obtain circular yeast artificial chromosomes (cYACs). A shuttle (cYAC/BAC) vector allowed recombination in

![Figure 4.2](image)

**Figure 4.2** IgH BACs with human V\textsubscript{H}, D, J\textsubscript{H} segments and rat C-region locus [22]. (a) Homologous integration of three BACs (all near 200kb) by overlapping regions of ∼11 kb. (b) Head-to-tail tandem integration.
Figure 4.3 Igκ BACs containing human V\(_κ\), J\(_κ\), and C\(_κ\) genes [22]. (a) Homologous integration by overlapping regions of \(~14\) and \(~40\) kb on two IgKV BACs of \(~150\) kb and the KDE (kappa deleting element) on a \(~55\) kb BAC. (b) Head-to-tail tandem integration.

4.2.2 Igκ

The expression of human L-chain in transgenic mice was initially achieved with minigene constructs containing one or two V\(_κ\) genes. Later cosmids and BACs provided many different V\(_κ\) genes, which were well expressed and provided extensive V\(_κ\)J\(_κ\) diversity [5]. Figure 4.3 illustrates the integration of human Igκ sequences from three different BACs obtained by digests using rare cutting restriction enzymes, which produced overlapping fragments. A performance comparison of naturally spaced and closely assembled V\(_κ\) genes revealed poor expression of tightly spaced V\(_κ\) sequences on small DNA constructs [17], probably independent of the integrated copy number. Using larger regions on YACs and from multiple BACs showed impressive results with extensive transgene rearrangement and good expression even in a wildtype (Wt) background and thus successful competition with the endogenous locus.

4.2.3 Igλ

There are few reports on the expression of transgenic human Igλ constructs and perhaps the most successful approach was the use of a YAC extended by cosmids and containing the authentic region of the human Igλ locus with over
half of all V\(_{\lambda}\) genes (Figure 4.4) \[25, 26\]. As diverse use and good expression even in a Wt background was obtained, the human Ig\(_{\lambda}\) translocus must provide regulatory sequences to allow broad utilization in mice and rats despite these animals producing little endogenous Ig\(_{\lambda}\) \[20, 22, 26\].

4.3 BAC Integration

When purified YAC or BAC DNA was microinjected into fertilized eggs, a recurring problem was the instability of the large linear DNA with strand breakage occurring during the purification process or when forcing the DNA through the injection needle \[5\]. For the purification of large fragments obtained from restriction digests separated on agarose gels via conventional or pulsed field electrophoresis, electroelution using Elutrap™ worked efficiently \[27\]. DNA recovery was usually 20–50% of the starting material. For fragments up to 200 kb, the DNA could be precipitated and redissolved in microinjection buffer at the desired concentration. For the separation of ~10 kb vector DNA from >150 kb BAC insert, sepharose 4B-CL filtration columns using a microinjection buffer gave very good results \[28\] and avoided DNA precipitation \[22\].

Cointegration of several large fragments proved beneficial for the reconstitution of an authentic Ig locus. The purification of 400+ kb YAC fragments, which are prone to degradation and difficult to obtain at the required concentration, was avoided. For DNA injection, equal molar amounts of the different purified BAC fragments at concentrations of 0.5–3 ng \(\mu\)l\(^{-1}\) were mixed. In many cases, three large fragments were coinjected, which resulted in transgenic integration of at least 1% of injected eggs. Tandem insertion was frequently observed; either by homologous integration or head-to-tail integration as shown in Figures 4.2 and 4.3. Integration success was initially identified by an extensive genomic polymerase chain reaction (PCR) and then confirmed by the analysis of rearranged V(D)J transcripts where recombinations of the most 5' to the most 3' gene segments were readily found \[22\]. Germline transmission was frequently seen, with mosaicism being less of a problem compared with ES cell technology.
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4.4 Designer Zinc Finger Endonucleases to Silence Endogenous Ig Loci

In the mouse, gene targeting in ES cells allows the generation of heritable mutations or knockout animals. As suitable stem cells were lacking for the rat, an alternative method using zinc finger (endo)nucleases (ZFNs) was attempted recently producing several knockout lines with fully silenced Ig loci [22, 29, 30]. The targeting sites of a ZFN pair contain a unique DNA-homology region of \(2 \times (9–18)\) nucleotides on opposite DNA strands. Three to six zinc fingers bind on each strand with high specificity and a 6 bp spacer in between the targeting sites permits \(FokI\) cleavage (Figure 4.5) [31]. ZFN constructs are assembled on plasmids and the targeting efficiency can be assessed by transfection into cell lines before microinjection of the purified expression cassettes into one-cell embryos [29]. An interesting observation was that cleavage and DNA repair could lead to various mutations and sizable deletions. For example, disruption of the rat \(C_\mu\) gene, which silenced the IgH locus, was achieved in a number of rats carrying deletions ranging from a few bases to hundreds of nucleotides [29]. Targeting of the \(J_H\) locus produced a deletion of about 2.5 kb resulting in removal of all \(J_H\) segments [30] and it may be possible to use mixtures of ZFN pairs to remove larger regions by design.

Silencing of the IgH locus was also achieved in the rabbit using two ZFN pairs designed to target and replace \(C_\mu\) exons 1 and 2 [32]. It appears that gene targeting with designer ZFNs induces double-strand breaks at the desired target sites.

![Color Fig. 4.5](www.biochem.utah.edu/carroll/public_html/research/backgr.html). Targeted gene disruptions and germline modifications of all rat Ig loci using ZFNs have been accomplished [22, 29, 30].
and subsequently nonhomologous end-joining repair, which results in targeted replacement or deletion in a relatively high frequency of embryos born. In many cases, the breeding efficiency was little affected, which led to the conclusion that targeting of unique sequences may avoid detrimental deletions.

4.5 Expression Comparison of Fully Human and Chimeric IgH Loci

In many transgenic strains, mainly mice-derived in the last 20 years, improved cloning strategies allowed the addition of a large number of human V genes to D and/or J regions followed by one or more human C-region genes [5]. Such transloci contained only human genes, with expression control sometimes provided by short rat or mouse enhancer sequences. In these strains, human Ig loci are integrated in the germline and endogenous mouse Ig loci have been rendered nonfunctional by gene targeting [20, 33, 34]. Although fully human therapeutic antibodies have been derived from these transgenic mouse lines (e.g., from XenoMouse [35]), frequently difficulties have been encountered with eliciting diverse human antibody responses [21]. A possible reason for this observation is a compromised immune response because of the imperfect interaction of the human constant region with the mouse cellular signaling machinery.

A reduction in the numbers of surface IgM+ cells in fully human IgH transgenic mice compared to Wt animals is illustrated in Figure 4.6a, which shows reduced levels, from 14% to 3% for bone marrow cells and 30% to 19% for spleen cells [20]. A similar reduction in spleen cells has been observed in other transgenic strains [35].

For transgenic rats carrying the constructs shown in Figure 4.2, no significant B-cell reduction was observed when Wt and transgenic lines where compared (Figure 4.6b) [22]. B-cell numbers found in normal rats were very similar or indistinguishable when compared to rats carrying a chimeric human IgH locus bred with Ig KO lines (termed OmniRat when carrying a chimeric IgH locus, a human IgL [κ and/or λ] locus, and endogenous KOs of IgH, IgK, and IgL). In IgH (and IgH + IgL) KO strains obtained by ZFN targeting, no Ig+ cells were identified and B-cell development was abrogated (Figure 4.6c). Importantly, the OmniRat strain with human IgVH, D, and JH segments linked to germline-configured rat IgC regions produces chimeric antibodies and immune responses in a highly efficient manner [22].

Previous analyses by us and other laboratories showed much reduced IgM and IgG titers in transgenic human antibody mice [5, 20, 21, 33, 36–38]. In these animals, the 3′ regulatory region was incomplete and contained only one of the four enhancer elements. A problem was that class-switch recombination, changing Cμ to CGamma, was much less efficient than observed in normal animals, hence relatively little IgG was produced. Sometimes, a significant amount of trans-switching from human Cμ to endogenous mouse CGamma was found along with transgene switching [39]. For a rearranged transgenic IgH locus that lacks the 3′ enhancer region, a
Figure 4.6 Flow cytometry analysis of bone marrow (bm) and spleen lymphocytes stained with anti-IgM and anti-B220 (CD45R) using human, mouse, or rat-specific antibodies. (a) Comparison of a wild type (Wt) mouse line and a fully human transgenic mouse line carrying human V_{H}s, D_{s}, J_{H}s, and human C_{μ}, expressed in a KO background with silenced endogenous IgH and Igκ loci [20]. (b) A wild type rat and a translocus (chimeric) rat line carrying human V_{H}s, D_{s}, J_{H}s, part of the rat C region with several C genes, and disabled endogenous Ig loci [22]. Please note that fixed area settings (boxed cells) for Wt and transgenic lines stained with different reagents can slightly increase or decrease relevant populations. (c) Ig deficient rat-line (KO) obtained by ZFN technology. In bone marrow, A refers to pro/pre B cells (CD45R^{+}IgM^{-}) and B refers to immature B cells (CD45R^{+}IgM^{+}). In spleen, A refers to lymphocyte precursors (CD45R^{+}IgM^{-}), B to follicular B cells (CD45R^{+}IgM^{+}), and C to marginal zone B cells (CD45R^{low}IgM^{+}).

reduction in transgenic but an increase in interchromosomal class-switch has been identified [40]. In OmniRat, the levels of chimeric IgM and IgG in serum with fully human idiotypes but rat C regions and entirely human Igκ and Igλ loci were similar to that in Wt or normal rats (Figure 4.7a,b). Interestingly the level of IgG was not reduced in OmniRat despite the lack of Cγ2a in the translocus construct, which suggests that class-switching is similarly efficient but is using different C genes. Purification of Ig by capturing with either anti-human κ or anti-human λ L-chain affinity matrix (Figure 4.7c,d) also demonstrated that normal amounts are readily expressed; chimeric IgM and IgG levels with human L-chain from ~3-month-old transgenic rats were very similar compared to the Ig levels found in older children [22]. It is also interesting to note that the fully human L-chain transloci are generally well expressed in many transgenic lines with or without particular KO background [17, 26]. This suggests that expression of fully human L-chain is not biased or reduced by imperfect interaction with the rodent cellular machinery or impeded by association with chimeric H-chains.

The benefit of finding normal B-cell development and differentiation in a transgenic line, accompanied by high IgG expression, led to several essential questions: do class-switch and hypermutation function effectively in OmniRat and will immunization generate high affinity antibodies, ideally monoclonals by
cell fusion? In fully human transgenic IgH locus lines, reduced hypermutation and clonal expansion of particular V genes has been identified [21], which we do not see in OmniRat [22]. Indeed transcripts from nearly all human V, D, and J segments present in OmniRat have been identified in lymphoid tissue with up to 80% of V\textsubscript{H} genes linked to D-J\textsubscript{H}-rat C\textsubscript{\gamma} being hypermutated, many extensively. Successful immunization using various antigens resulted in a large number of high affinity IgG monoclonals and similar numbers of hybridomas were obtained from OmniRat and Wt control animals compared side by side [22].

### 4.6 Outlook

Significant improvements in the production of human antibody repertoires have been achieved by the expression of a chimeric IgH locus with human V\textsubscript{H}, D, and J\textsubscript{H} segments and rodent C genes. Extensive diversity and hypermutation was found and many high affinity antibodies were identified by cell fusion. It appears that optimal interaction with the rodent cellular-signaling machinery can be achieved when species-specific C\textsubscript{H} regions and control sequences are preserved. As found previously, extensive diversity was also seen for the introduced human Ig\textsubscript{\kappa} and Ig\textsubscript{\lambda} transloci. For therapeutic applications, a desired human C\textsubscript{H} region can easily replace the rat C\textsubscript{H} region in a monoclonal antibody without compromising antigen binding.

### References


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References


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Abstract

Human antibody expression in transgenic rodents has been significantly improved by the integration of large chimeric DNA constructs linking human VH, D, and JH segments to rat constant (C) regions and regulatory sequences. Light-chain constructs were fully human. The Ig loci were cloned and modified in artificial minichromosomes and added to the rat germline by DNA microinjection into fertilized oocytes. Silencing of all endogenous Ig loci was achieved using zinc finger nuclease constructs, which accomplished targeted gene disruption of sometimes several kilobases. DNA rearrangements provided extensive diversity, and near-normal or wild-type levels of transgenic IgM and IgG were produced. Hypermutation was readily found and many high affinity antibodies could be produced by cell fusion. For therapeutic applications, the rat C-region could be easily replaced by a desired human C without the loss of antigen binding.

Keywords

transgenic rodents; ZFN knockout; chimeric antibody repertoires; human epitopes; class-switch recombination; lymphocyte development

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