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Human antibody expression in transgenic rats: Comparison of chimeric IgH loci with human V_H, D and J_H but bearing different rat C-gene regions

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ABSTRACT

Expression of human antibody repertoires in transgenic animals has been accomplished by introducing large human Ig loci into mice and, more recently, a chimeric IgH locus into rats. With human V_H, D and J_H genes linked to the rat C-region antibody expression was significantly increased, similar to wild-type levels not found with fully human constructs. Here we compare four rat-lines containing the same human V_H-region (comprising 22 V_HS, all Ds and all J_HS in natural configuration) but linked to different rat C_H-genes and regulatory sequences. The endogenous IgH locus was silenced by zinc-finger nucleases. After breeding, all lines produced exclusively chimeric human H-chain with near normal IgM levels. However, in two lines poor IgG expression and inefficient immune responses were observed, implying that high expression, class-switching and hypermutation are linked to optimal enhancer function provided by the large regulatory region at the 3' end of the IgH locus. Furthermore, exclusion of C δ and its downstream interval region may assist recombination. Highly diverse IgG and immune responses similar to normal rats were identified in two strains carrying diverse and differently spaced C-genes.

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1. Introduction

Successful strategies to produce human antibodies have involved humanization of rodent monoclonal antibodies (mAbs), selection of antigen-specific human sequences by display technology and the generation of transgenic animals carrying human Ig loci (Green, 1999; Lonberg, 2005, 2008;

Brüggemann et al., 2007). All these approaches provided an increasing number of valuable drugs, even though the manipulation of individual antibody H- and L-chains can be quite laborious (with case by case recurrence) while the production of human antibody repertoires in rodents relies on the assembly and integration of large gene constructs (Riechmann et al., 1988; Mendez et al., 1997; Nicholson et al., 1999). In transgenic animals, sizeable germline regions performed better in rearrangement and expression (Xian et al., 1998), nevertheless, rodents with fully human IgH transloci often failed to produce high affinity binders after multiple immunizations (Green and Jakobovits, 1998; Pruzina et al., 2011).

The suboptimal performance of a human IgH locus in transgenic mice, in respect of antibody yield and immune response, was attributed to the imperfect interaction of the human constant region of membrane Ig with the endogenous

Abbreviations: 3'RR, IgH 3' regulatory region; BAC, bacterial artificial chromosome; YAC, yeast artificial chromosome; ZFN, zinc-finger nuclease.

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rodent cellular signaling machinery (Pruzina et al., 2011). This was supported by work in transgenic rats, carrying human V_H , D and J_H gene segments linked to the rat C-region locus, which displayed IgG immune responses very similar to wild type controls (Osborn et al., 2013). In these transgenic animals a large part of the rat C-region was included in conjunction with ~30 kb downstream of C α containing the 3' enhancer regulatory region, termed 3'RR (Vincent-Fabert et al., 2010). It was reasoned that intergenic regions containing cis-acting control sequences might be important in their entirety to secure class-switch recombination and hypermutation.

The size of the complete human IgH locus, ~1.3 Mb with 38–46 V_H , ~23 D and 6 J_H segments (Hofker et al., 1989; Matsuda et al., 1998), provides an immense challenge for engineering of multi-gene constructs and their germline integration by DNA microinjection into fertilized eggs. This can be partly overcome by using a previous finding where co-injection of multiple DNA constructs with homologous overlaps frequently led to co-integration into the genome (Bruggemann et al., 1991; Wagner et al., 1996). The use of large restriction fragments from modified bacterial artificial chromosomes (BACs) with terminal homology sequence enabled a functional Ig locus to be assembled (Osborn et al., 2013). Successful homologous or tandem integration could be verified by transcript analysis, which showed productive rearrangement of diverse V_H -D- J_H -C γ products brought together from segments accommodated on several BACs.

Here we compare chimeric human IgH expression from four transgenic rat-lines with identical human V_H , D and J_H segments but different rat C-region and 3' enhancer sequence. We found that there is flexibility in the positioning of the C-genes but that the region downstream of C α containing multiple transcriptional enhancer elements resulted in optimal immune response, class-switch recombination and somatic hypermutation.

2. Materials and methods

2.1. Construction of chimeric human-rat IgH loci

For the construction of the HC10 translocus, the rat genomic region from BAC clone CH230-408M5 (Invitrogen), including C μ , C δ and the region up to the γ 2c switch region on a ~49 kb fragment, was extended with a 100 bp homology arm corresponding to the sequence immediately upstream of the rat γ 2b switch region using the Red/ET recombination method (Gene Bridges GmbH, Heidelberg, Germany). Briefly, the recombination reaction used the pACYC177 vector DNA amplified via PCR using long primers containing homology arms matching the 5' and 3' end of the gene loci of interest followed by NotI restriction sites. The rat genomic region encompassing C γ 2b, C ϵ , C α and 3'RR was isolated from BAC clone CH230-162108 (Invitrogen) as a ~76 kb NruI-fragment using the BAC Subcloning Kit from Gene Bridges. The rat γ 2b C H 1 region was replaced by human γ 1 C H 1 according to the instructions using the Counter Selection BAC Modification Kit (service provided by Gene Bridges). Finally, HC10 was assembled as a circular YAC/BAC (cYAC/BAC) construct in *Saccharomyces cerevisiae* using 6 overlapping fragments (oligos are listed below): a 6.1 kb fragment 5' of human V_H 6-1 (amplified using oligos 383 and 384, and human genomic DNA as template), a ~78 kb PvuII-PaCI fragment containing the

human V_H 6-1-Ds- J_H s region cut out from BAC1 (RP11645E6, Invitrogen), a 8.7 kb fragment joining human J_H 6 with the rat genomic sequence immediately downstream of the last J_H and containing part of the rat C μ coding sequence (using oligos 488 and 346, and rat genomic DNA as template), the ~49 kb NotI-fragment covering rat μ up to the γ 2c switch region as described above, the ~76 kb NruI-fragment from rat C γ 2b up to the 3'RR as described above, the pBelo-CEN-URA vector with URA3 joined with a homology tail matching the 3' end of the rat 3'RR, and CEN4 joined with a homology tail matching the 5' end of human V_H 6-1 (using long oligos 385 and 322, and pBelo-CEN-URA as template). Further details, including the purification of the constructs, and the methods for converting a cYAC into a BAC were published previously (Osborn et al., 2013).

For the construction of HC13 a 5.6 kb fragment encompassing the membrane exon 2 as well as 3' UTR of rat γ 2b was amplified from BAC clone CH230-162108 using primers 547 and 548 with PmlI and Ascl sites, respectively. This fragment was cloned into pGEM®-T Easy via TA cloning (Promega). The short 3' E region, 3'RR hs1,2, located ~17 kb downstream of rat C α (Pettersson et al., 1990) was amplified from BAC clone CH230-162108 using primers 549 and 252, and isolated as a 950 bp Ascl-SacII fragment. This fragment was cloned downstream of the γ 2b 3' UTR into the multiple cloning sites of pGEM®-T Easy. Finally, the γ 2b 3' region joined together with the 3'RR hs1,2 was isolated as a ~6.6 kb PmlI-SacII fragment. HC13 is an extension of the previously constructed BAC containing human V_H 6-1-Ds- J_H s followed by the authentic rat μ , δ , and γ 2c region on a single ~140 kb NotI fragment (Osborn et al., 2013). The following 5 fragments were used to assemble HC13 as a cYAC/BAC construct: the ~140 kb NotI fragment described above, a ~1.8 kb PCR fragment covering the γ 2c 3' UTR followed by a 65 bp homology tail matching the sequence 3.8 kb upstream of the γ 2b switch region (using primer 502 and 503, and rat genomic DNA as template), a ~16 kb SphI-fragment covering the entire modified γ 2b locus with rat γ 2b C H 1 replaced by human γ 1 C H 1 (cut out from the modified BAC clone CH230-162108 described under 'HC10'), the ~6.6 kb PmlI-SacII γ 2b 3' enhancer fragment described above, and amplified pBelo-CEN-URA vector with homology tail ends (using long oligos 385 and 560, and pBelo-CEN-URA as template).

HC17 is an extension from HC13. The region including human V_H 6-1-Ds- J_H s followed by the rat μ , δ , γ 2c and the modified γ 2b region, was cut out from HC13 as a single ~160 kb NotI-Ascl fragment. A cYAC/BAC construct was made from 4 fragments: the ~160 kb NotI-Ascl region, a ~1.7 kb PCR fragment containing a 58 bp 5' homology tail matching the sequence ~5 kb downstream of the γ 2b membrane exon 2 followed by the sequence located ~3.6 kb upstream of the α switch region (using primers 591 and 592, and rat genomic DNA as template), the ~40 kb FspI-SalI region with C α and the 3'RR from BAC clone CH230-162108, and amplified pBelo-CEN-URA vector with homology tail ends (using long oligos 385 and 322, and pBelo-CEN-URA as template). The following oligos have been used:

252 [TGGAACCTGCTTAGGTCAGC];

322 [TATACATCGTCTTAGTATCTGTCTGACCCACCACCATC
TTCCCTGCCTCC GTCCACTCACAGATCTCTGACGCGTCACCGC
AGGGTAATAACTG];

502 [GAAAGCTGAGCTGCAAGAGACCCATCG];
 503 [CTTCTACCACTTAGCACAGTGGTCTTATGCCATGGGT
 AGCCACTCTGAATATCAGATATCCTGGCTACAACAGCGGATA
 GCAGCACTCAG];
 547 [GATGCACGTGTCAACCTGCGATGTCCTCAC];
 548 [ACCTGGC GCGCCGTGGCTGCTCTCATGTACATCCTG];
 549 [CCACGGCGCGCCAGACACCATCTTG GAAGACC];
 560 [AGGGGCCATTAACTCATCACAGTCCAGGCCATCATGG
 TGAGCCCTAGCTGACCTAAGCAGGTTCCACGCGTACCGCA-
 GGTAATAACTG];
 591 [ATCTGCCCATAGGAGGAAGACCTCGGCCTCAAGTCAGG
 ATGTACATGA GAGCAGCCACGGCGGCCCTCAAGACATGAT
 CTCACATGAC];
 592 [GTCAGAAGACACCTCAGGACATCG]; or have been
 listed previously (Osborn et al., 2013).

2.2. DNA microinjection and breeding to homozygosity

Comprehensive details for all methods used have been described previously (Osborn et al., 2013). BAC inserts were purified after digests that released the vector DNA. For DNA microinjection BAC6-3, a 182 kb *Asi*SI-*Asi* fragment, and BAC3, a 173 kb *Not*I fragment, were pooled with the particular C-region BAC on a *Not*I fragment (Fig. 1). Equal amounts of DNA were mixed in microinjection buffer and injected into fertilized oocytes at concentrations from 0.5 to 10 ng/ μ l (INSERM UMR 1064 and Taconic Biosciences, Cranbury, NJ). Three to five separately derived founder rats for each injected construct or line were bred to homozygosity with the J_H KO heavy-chain knock-out strain (Menoret et al., 2010) at Charles River under specific pathogen-free conditions. All animal procedures involving care and use were in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals, available at <http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf> (the “Guidelines”), which are adapted from the requirements of the Animal Welfare Act or regulations concerning the ethics of science research in the INSERM UMR 1064 animal facility and approved by the regional ethics and veterinary commissions (N° F44011).

2.3. PCR and RT-PCR

Transgenic rats were identified by PCR from tail or ear clip DNA extracted using a Genomic DNA Mini Kit (Bioline). RNA was extracted from blood in RNeasy later using the RiboPure blood kit (Ambion). cDNA was made using Oligo dT and Promega Reverse Transcriptase at 42 °C for 1 h. Detailed PCR conditions have been provided (Osborn et al., 2013).

2.4. Ig analysis and flow cytometry

IgM was purified on anti-IgM affinity matrix (BAC B.V., Netherlands, CaptureSelect #2890.05) as described in the provided protocol. For rat IgG purification (Bruggemann et al., 1989) protein A resin was used (Innova, Cambridge, UK, #851-0024) with serum incubated with the resin and binding facilitated at 0.1 M sodium phosphate pH 8 under gentle mixing. Poly-prep columns (Bio-Rad) were packed with the

mixture and washed extensively with PBS pH 7.4. Elution buffer was 0.1 M Sodium Citrate pH 2.5 and neutralization buffer was 1 M Tris-HCl pH 9. Electrophoresis was performed on 4–15% SDS-PAGE and Coomassie brilliant blue was used for staining. MW standards were HyperPage Prestained Protein Marker (#BIO-33066, Bioline).

Multiple immunizations were carried out with 100–125 μ g β -galactosidase (β -gal) or human progranulin (hPG) or ovalbumin (OVA) or hen egg lysozyme (HEL) as described (Osborn et al., 2013).

For flow cytometry cell suspensions were washed and adjusted to 5×10^5 cells/100 μ l in PBS with 1% BSA and 0.1% Azide. Identification of B-cell subsets was with anti-rat IgM FITC-labeled mAb (MARM 4, Jackson Immunoresearch Laboratories) in combination with anti-B cell CD45R (B220)-PE-conjugated mAb (His 24, BD biosciences). FACS Cantoll flow cytometer and FlowJo software (Becton Dickinson, Pont de Claix, France) were used for the analysis (Menoret et al., 2010).

3. Results

3.1. Chimeric human-rat IgH loci

To provide an extensive human V_H repertoire, 2 BACs with 22 V_H genes were chosen and modified to facilitate homologous integration (Hu BAC6-3 and Hu BAC3, Fig. 1 top) (Osborn et al., 2013). The assembly of a BAC construct accommodating human V_H 6-1, all D and J_H segments linked to part of the rat C region, termed HC14 Hu-Rat Annabel, has been described recently (Osborn et al., 2013). Various difficulties were encountered in the assembly of the rat C-region; first, cloning into a BAC restricted the region selected to below 250 kb, second, to allow class-switch recombination several highly repetitive and unstable switch sequences had to be retained, and finally, it was unclear how much of the 3'RR was needed for appropriate expression.

In Fig. 1 the assembled BACs are illustrated with V_H -region BACs at the top, followed by C-region BACs with overlapping region in the middle part, and the rat C_H region in natural configuration shown at the bottom.

For the construction of HC10 Hu-Rat Emma, a region immediately 3' of rat J_H 4, including rat E_H , S_H , C_H , $C\delta$ and all sequences up to $S\gamma$ 2c was added to the human V_H 6-1, D and J_H sequence. A further addition of rat $S\gamma$ 2b, $C\gamma$ 2b, $C\epsilon$, $C\alpha$ and the 3'RR in natural configuration was made (Bruggemann et al., 1986). In this 202 kb construct $C\gamma$ 2b is in the position where normally $C\gamma$ 2c is located. In HC13 Hu-Rat Belinda, the authentic region from rat E_H to $C\gamma$ 2c was added, which is followed by $S\gamma$ 2b, $C\gamma$ 2b and the 3'RR $hs1,2$ (Pettersson et al., 1990) on a 160 kb fragment. For HC17 Hu-Rat Frieda, the Hu-Rat Belinda BAC was modified by adding $C\alpha$ with ~30 kb 3' region after $C\gamma$ 2b, which generated a 202 kb BAC. In HC10, HC13 and HC17 the rat $C\gamma$ 2b C_H 1 exon was exchanged for human γ 1 C_H 1.

Purified BAC clones with the same human V_H region but different rat C-regions were microinjected into fertilized oocytes. Random integration into a rat chromosome was in tandem, facilitated by overlapping ends. This generated 4 transgenic lines with several founders each, which all showed productive integration of 3 BACs carrying the same V_H region but different C-genes. In Fig. 1 the gray bar illustrates how

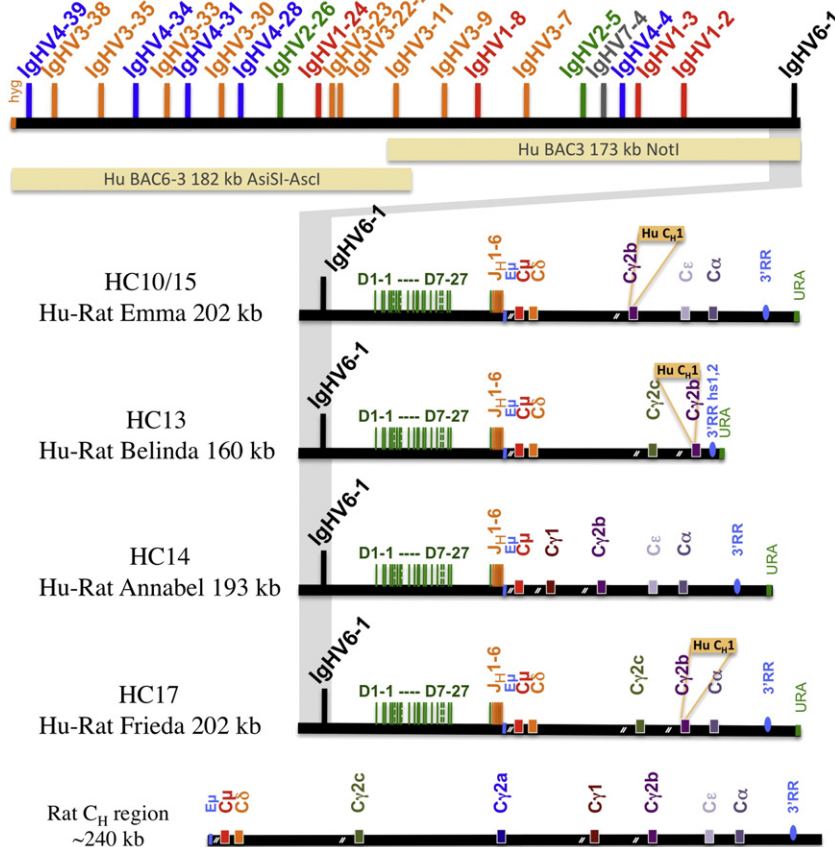


Fig. 1. Human V_H s, Ds and J_H s linked to different rat C-regions. Integration of large linear DNA segments of several hundred kb to generate human–rat IgH transloci was facilitated by overlapping regions of ~11 kb engineered to link the V_H gene BACs, Hu BAC6-3 an Asi SI- $Ascl$ fragment of 182 kb and Hu BAC3 a Not I fragment of 173 kb (top left) and, shown by the gray bar, the four chimeric C-region BACs all on Not I fragments, Emma of 202 kb, Belinda of 160 kb, Annabel of 193 kb and Frieda of 202 kb. The authentic rat IgH C-region is shown at the bottom. All engineered C-region BACs contain an identical part with human V_H6-1 , all human Ds and all human J_H s, followed by rat $E\mu$ and rat $C\mu$. Rat $C\mu$ is followed by rat $C\delta$ in natural configuration in all constructs, except Hu-Rat Annabel. Various rat C-region genes have been added downstream of $C\mu$ or $C\delta$, with some rat $C\gamma2b$ genes carrying human C_H1 . Switch regions are indicated (//) where analyzed. The 3'RR of ~30 kb, 3' of $C\alpha$, includes $hs3a$, $hs1,2$ and $hs3b$ ($hs4$ may not be present in the rat (Sepulveda et al., 2005)) and was either fully included or as a minimal 3'RR $hs1,2$ (Pettersson et al., 1990; Osborn et al., 2013).

tandem integration of the same human V_H6-1 , all D and J_H segments but with different rat C-regions might have been achieved. For HC10 only Hu BAC3 was included in conjunction with the C-region but in a separate experiment, generating HC15, both human V_H BACs, Hu BAC6-3 and Hu BAC3, were integrated together with Hu-Rat Emma. As we found no expression differences between these lines, except in the number of used V_H genes we have grouped the results together. Correct integration was identified by PCR and confirmed by human V_HDJ_H rearrangements to rat Cs.

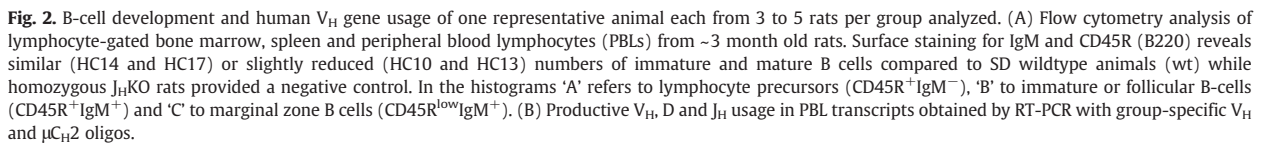
For the analysis several founders of each line were bred to homozygosity with IgH knock-out rats in which the endogenous J_H segments had been deleted (Menoret et al., 2010).

3.2. B-cells expressing diverse human V_H -D- J_H

The 4 transgenic lines were compared after breeding into the J_HKO/J_HKO background. Flow cytometry assessed if the introduced chimeric IgH loci could reconstitute normal B-cell development and RT-PCR analysis, using PBLs, determined if diverse human (V_HDJ_H)s were produced (Fig. 2). Staining cell

suspensions of bone marrow, spleen and PBLs for IgM and CD45R (B220) (Fig. 2A) revealed in HC10 and HC13 a slight reduction in the numbers of IgM^+CD45R^+ cells, while in HC14 and HC17 the numbers were very similar to wt controls. However, as we do see differences in cell populations between individual rats, from both transgenic and wt controls, this may suggest that all 4 lines, HC10, HC13, HC14, HC17, show near normal B-cell development with adequate numbers of immature and mature B-cells.

This is supported by the finding of highly diverse human V_HDJ_H rearrangement of $C\mu$ H-chain, when analyzing 50–100 random sequences for each line (Fig. 2B). Similar to wt controls these IgM sequences showed little hypermutation. Extensive diversity of rearranged V_HDJ_H transcripts was also found for $C\gamma$ sequences but only in HC14 and HC17, with few class-switch products obtained in HC10 and HC13. Many of the chimeric class-switch products were extensively mutated, but normal levels of IgG transcripts were only found in HC14 and HC17 while HC10 and HC13 produced little. As shown previously, B-cell development in HC14 is very similar to wt rats with mutational changes predominantly found in



The level of serum Ig from ~3 month old rats kept in isolators was compared in ELISA (not shown) and after purification on SDS-PAGE (Fig. 3A and B). The molecular weights of samples

fractionated under reduced conditions were for all lines as expected with ~75 kDa for μ -, ~55 kDa for γ - and ~25 kDa for L-chain. The yield for IgM was quite similar between the different lines (Fig. 3A); often up to 500 μ g/ml as identified in wt controls and only occasionally somewhat reduced but never less than half of the wt level analyzed in parallel. Thus, despite some variation, the IgM concentrations in all lines were in good agreement with the levels produced in wt rats kept under the same conditions. Near normal increase in IgM titer was also seen after immunization and in all lines specific IgM levels were similar to wt (not shown).

For IgG purification on protein A, the results were split as low and normal levels were identified (Fig. 3B). For HC14, HC17 and wt this revealed 500–1000 μ g IgG/ml serum; this level was established from several experiments and agrees with previous findings despite the suboptimal purification of rat IgG using protein A (Bruggemann et al., 1989; Osborn et al., 2013). A consistently lower amount, ~10% of normal levels was identified in HC10 and HC13 animals, where some rats had barely more than 50 μ g IgG/ml serum. In these rats specific IgG was lacking after immunization while HC14 and HC17 produced extensive immune responses frequently similar to wt rats (Fig. 3C). Immunizations were carried out using 4 different antigens, β -galactosidase (β -gal), human progranulin (hPG), ovalbumin (OVA) and hen egg lysozyme (HEL), all of which failed to work efficiently in HC10 and HC13.

4. Discussion

Previously we showed that a chimeric IgH locus with human V_H S, Ds and J_H segments linked to rat C-region genes and control sequences, produced highly diverse and near-normal expression

levels of antibodies with human idiotypes (Osborn et al., 2013). Here we assess the performance of 4 translocus rat-lines, with silenced endogenous IgH locus (Menoret et al., 2010), carrying the same human V_H -region but different rat C_H-regions. The comparison was aimed at identifying minimal C_H transloci, which would permit near normal expression.

In these lines, the IgM expression level with a diverse repertoire of human V_H -D- J_H rearrangement was very similar, with surface μ^+ B-cells and secreted IgM in serum comparable to wt rats. This suggests that DNA rearrangements with developmental stages from pro to pre to immature B-cells are adequately performed as described (Almqvist and Martensson, 2012). In previous transgenic IgH lines carrying only human C_H-genes reduced levels of serum IgM and IgM⁺ B-cells have been identified (Green and Jakobovits, 1998; Nicholson et al., 1999; Bruggemann et al., 2007), even with E μ , C μ and downstream regions analogous to our transgenic constructs (Lonberg et al., 1994; Mendez et al., 1997; Nicholson et al., 1999). The suboptimal performance of fully human IgH constructs is likely to reflect imperfect interaction of the human C-genes with the rodent cellular signaling machinery. Reconstitution of B-cell development may simply be more efficient, during progression of early lymphocyte differentiation events in the bone marrow, when C μ is of endogenous (rat) origin associated as BCR with matching co-receptor polypeptides. This suggests that transgenic H-chain constructs containing the genomic region including E μ and C μ , ideally of endogenous origin, can initiate normal antigen-independent B-cell differentiation events (Kurosaki et al., 2010; Dunnick et al., 2011b).

The rat 3'RR containing hs3a, hs1.2 and hs3b is similar to the mouse but it is unclear if there is an equivalent region to

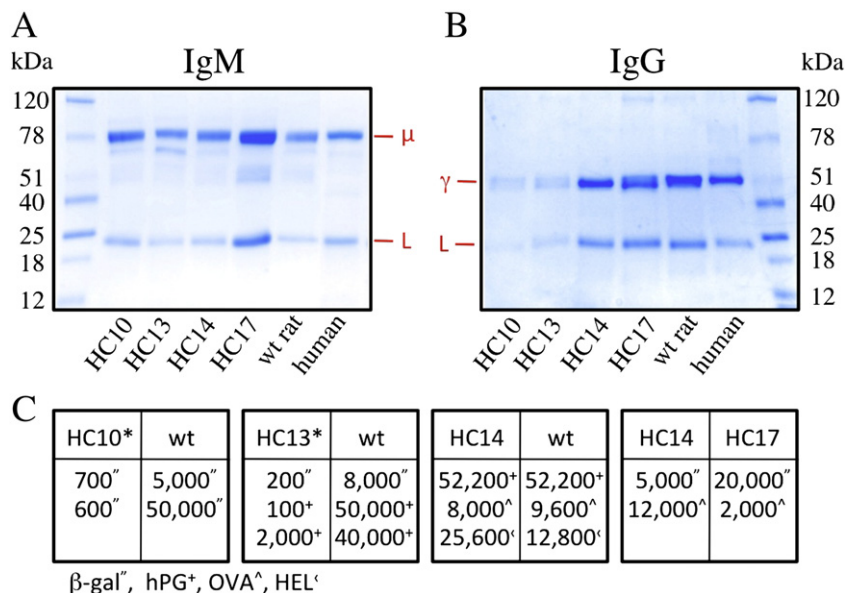


Fig. 3. Chimeric Ig and antibody response. (A) and (B) purified Ig separated under reducing conditions on 4–15% SDS-PAGE and stained with Coomassie brilliant blue. Ig from serum (100 μ l each with equal amounts from 4 to 5 randomly bled rats) was captured (A) with anti-IgM matrix, which identified 300–500 μ g/ml IgM in each of the samples, and (B) protein A, which produced a lower level of IgG in HC10 and HC13 (<100 μ g/ml) but normal levels in HC14 and HC17 compared to wt (500–1000 μ g/ml). Concentrations were determined by nanodrop and ELISA. (C) IgG antigen titers in HC rats and control animals at EC₅₀ binding dilutions in ELISA. Separate immunizations with β -gal, hPG, OVA and HEL were compared in HC rats and control animals in parallel as described (Osborn et al., 2013). *Frequently a lack or background level of antigen-specific IgG was found after immunization (not shown).

hs4 in the rat (Sepulveda et al., 2005). In our constructs either the potentially complete rat 3'RR, including hs3a, hs1,2 and hs3b located downstream of C α (Bruggemann et al., 1986), or a minimal 3'RR sequence with hs1,2 (Pettersson et al., 1990) was used. The 3'RR hs1,2 sequence has also been used in other, fully human, constructs (Harding and Lonberg, 1995) but no previous constructs contained the large 3'RR accommodating multiple transcriptional enhancer elements. It has been reported that a minimal 3'RR sequence, accommodating only one or possibly two hs regions, reduces germline transcription and class-switch recombination (Pinaud et al., 2001; Dunnick et al., 2011b), which agrees with our findings. The constructs Hu-Rat Belinda (HC13) and Hu-Rat Frieda (HC17) are identical except the former has only a 3'RR hs1,2, which is replaced later with the complete region including C α and the 3'RR. Animals expressing HC13 switched very inefficiently, while HC17 rats switched and underwent hypermutation normally. Separately derived animals, but carrying the same translocus, produced very similar results. This implies that the functionality of the full 3'RR appears to comprehensively mediate or control downstream expression events; from the transitional B-cell stage onwards when IgM⁺ lymphocytes exit the bone marrow and enter the blood to reach other lymphoid organs, such as spleen and lymph nodes, where they mature further (Kurosaki et al., 2010). Maturation is accompanied by class-switch recombination and somatic hypermutation, which leads to antigen-dependent cell expansions with differentiation into plasma or memory B-cells. This is supported by very recent results, which showed that the removal of the whole 3'RR in the mouse abrogated class-switch recombination and abolished somatic hypermutation in germinal centers (Vincent-Fabert et al., 2010; Rouaud et al., 2013). A summary of these events in our different transgenic lines is shown in Table 1.

In three of the chimeric constructs the ~30 kb 3'RR is present, but despite this, in the Hu-Rat Emma line, the first made, little switching occurs with only a few C γ 2b(Hu C μ 1) transcripts being isolated. Here C γ 2b is immediately downstream of the γ 2c germline promoter and I-exon, taking the position of C γ 2c. In wt rats the expression of this isotype is reduced compared to other IgGs (Bazin et al., 1974), which may

to some extent explain the low levels we find. Interchanging promoter and I exon between different C γ -genes can have a profound effect and poor class-switch recombination has been obtained with a particular combination (Dunnick et al., 2011a). An alternative explanation could be the lack of or wrong positioning of multiple control regions, possibly well separated in the IgH locus, but essential for optimal B-cell function. This may resemble the dynamic interplay of enhancer and repressor function identified for the β -globin locus (Sutter et al., 2003; Recillas-Targa et al., 2004). A modified C γ gene with human C μ 1 appears to be fully active as HC17 works fine.

Our final two lines contained very different IgH regions due to size limitations (inserts <220 kb) imposed by the BAC vector: Hu-Rat Annabel has the region from C δ to downstream of C γ 2a omitted and Hu-Rat Frieda has the region from C γ 2a to C γ 1 and a ~21 kb section containing C ϵ removed. Hu-Rat Annabel (termed OmniRat when expressing human L-chain and with endogenous IgH/K/L knock-out) has been published recently and we showed that B-cell development, expression, class-switch, hypermutation and immune responses were very similar to wt animals (Osborn et al., 2013). In this line only authentic rat C-genes have been assembled but C δ together with the large interval region (Mundt et al., 2001) and downstream C genes, γ 2c and γ 2a up to 4.4 kb 5' of S γ 1, has been removed. Expression results of this line are in agreement with knock-out mice deficient for IgD (Nitschke et al., 1993), which may express a somewhat higher level of surface IgM, but show normal serum Ig levels and no impairment of class-switching. In Hu-Rat Annabel both transgenic C γ genes are equally well expressed and it appears that class-switch recombination does not favor one or the other. Expression similar to wt was also obtained with Hu-Rat Frieda, which retained C δ with its downstream region followed by C γ 2c, C γ 2b(Hu C μ 1) and the full 3'RR. However, class-switching of this translocus favored C γ 2b(Hu C μ 1) and not C γ 2c the first C γ -gene downstream of C μ /C δ . Nevertheless, both, Hu-Rat Annabel and Hu-Rat Frieda, showed the expected 4- to 5-log titer increase of antigen-specific serum IgG after immunization.

It has been shown that the interval sequence between C δ and the first C γ has a significant effect on activation and

Table 1
Expression and class-switch of chimeric human-rat Ig loci.^a

Hu VDJ	IgM	IgG ^b	Class-switch	Hyper-mutation	Line	Founders ^c
+++	+++	+/-	— some trans	—	HC10/15 Hu-Rat Emma	3/3
+++	+++	+/-	— some trans	—	HC13 Hu-Rat Belinda	4
+++	+++	+++ γ 1, γ 2b	+ little trans	+++	HC14 Hu-Rat Annabel	5
+++	+++	+++ γ 2b Hu C μ 1 some γ 2c	+ little trans	+++	HC17 Hu-Rat Frieda	4

^a Summary of RT-PCR with strong bands indicated by +++ and faint bands by +/-.

^b RT-PCR and 5'RACE identified human V μ DJ μ -rat C γ products. Class-switch of the transgenic locus is indicated by + (normal) and — (ineffective) while some/little trans indicates trans-chromosomal recombination between transgenic and endogenous IgH locus. An extensive number of translocus V μ DJ μ -C γ transcripts were found for HC14 as described (Osborn et al., 2013) and similarly for HC17 with many V μ sequences extensively diversified by hypermutation.

^c Number of rats carrying a complete translocus and producing similar results in comparative analyses.

expression control of the IgH locus at the early stages of B-cell development before class-switching (Mundt et al., 2001), at which stage this sequence will be deleted. The function of particular sequences in this region mediated an increase of transcription in early B cells but much-reduced transcriptional activation of a reporter gene in mature or fully differentiated B-cells. Inducing transcription from germ line promoters upstream of switch-regions, which produce sterile RNAs of I-exons, determines the isotype or class-switch product of the B-cell (Perlot et al., 2008; Stavnezer et al., 2008). In three lines, Emma, Belinda and Frieda, little switch recombination to the first C γ next to the C δ 3' region could be identified while C γ -gene usage in Annabel, not carrying C δ with its interval region, was normal. It is possible that this DNA region which contains an extensive number of lymphocyte-specific as well as ubiquitous transcription factor-binding motifs may provide a control mechanism on Ig gene activation and repression (Mundt et al., 2001; Kurosaki et al., 2010). Switching and expression of C γ genes further downstream may not be affected as many diverse human V_HDJ_H-C γ 2b (with human C_H1) have been identified in Frieda. A reason that few switch products were identified in Belinda might be that the minimal 3'RR hs1,2 on this translocus is unable to counteract reduced S region transcription rates (Kaminski and Stavnezer, 2004).

Recombination between the translocus and the endogenous IgH locus termed trans-switching has been observed in all lines and appears to be at a level in HC14 and HC17, possibly up to 10%, similar to that described in mice (Reynaud et al., 2005; Dougier et al., 2006; Osborn et al., 2013). In HC10 and HC13, trans-switch products (e.g. human V_HDJ_H-rat C γ 1 or C γ 2a), although quite low, appear to be generated more easily than translocus switch products (e.g. human V_HDJ_H-rat C γ 2b with human C_H1). This confirms that although intra-chromosomal recombination is repressed in HC10 and HC13, possibly due to reduced accessibility of σ y in the constructs (Kaminski and Stavnezer, 2004), inter-chromosomal recombination or switching from transgenic C μ to endogenous C γ is perhaps independently attainable, which agrees with the conclusion derived from transgenic mouse models (Dunnick et al., 2009; Shansab et al., 2011).

In summary, DNA mixtures of several human and human-rat chimeric BACs with inserts of up to ~200 kb allowed tandem chromosomal integration when microinjected into oocytes. This resulted in high expression of a diverse antibody repertoire with human V_H-D-J_H linked to rat C_H. Modified C loci established that the ~30 kb C α downstream region containing the 3'RR was essential for normal immune development and that multiple C-genes provided an advantage. As observed in other species, a lack of C δ was not detrimental for class-switch recombination and expression (Chen and Cerutti, 2010).

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