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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 Cδ 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.

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 Cα 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-Cγ 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 Cα 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 Cμ, Cδ and the region up to the γ2c switch region on a ~49 kb fragment, was extended with a 100 bp homology arm corresponding to the sequence immediately upstream of the rat γ2b 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 Cγ2b, Cε, Cα and 3′RR was isolated from BAC clone CH230-162I08 (Invitrogen) as a ~76 kb NruI-fragment using the BAC Subcloning Kit from Gene Bridges. The rat γ2b C_H1 region was replaced by human γ1 C_H1 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_H6-1 (amplified using oligos 383 and 384, and human genomic DNA as template), a ~78 kb PvuI–PacI fragment containing the human V_H6-1–Ds–J_Hs region cut out from BAC1 (RP11645E6, Invitrogen), a 8.7 kb fragment joining human J_H6 with the rat genomic sequence immediately downstream of the last J_H and containing part of the rat Cμ coding sequence (using oligos 488 and 346, and rat genomic DNA as template), the ~49 kb NotI-fragment covering rat μ up to the γ2c switch region as described above, the ~76 kb NruI-fragment from rat Cγ2b 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_H6-1 (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 γ2b was amplified from BAC clone CH230-162I08 using primers 547 and 548 with PmlI and AscI 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 Cα (Pettersson et al., 1990) was amplified from BAC clone CH230-162I08 using primers 549 and 252, and isolated as a 950 bp AscI-SacII fragment. This fragment was cloned downstream of the γ2b 3′ UTR into the multiple cloning sites of pGEM®-T Easy. Finally, the γ2b 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 humanV_H6-1-Ds-J_Hs followed by the authentic rat μ, δ, and γ2c 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 γ2c 3′ UTR followed by a 65 bp homology tail matching the sequence 3.8 kb upstream of the γ2b switch region (using primer 502 and 503, and rat genomic DNA as template), a ~16 kb SphI-fragment covering the entire modified γ2b locus with rat γ2b C_H1 replaced by human γ1 C_H1 (cut out from the modified BAC clone CH230-162I08 described under 'HC10'), the ~6.6 kb PmlI-SacII γ2b 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 humanV_H6-1-Ds-J_Hs followed by the rat μ, δ, γ2c and the modified γ2b region, was cut out from HC13 as a single ~160 kb NotI-AscI fragment. A cYAC/BAC construct was made from 4 fragments: the ~160 kb NotI-AscI region, a ~1.7 kb PCR fragment containing a 58 bp 5′ homology tail matching the sequence ~5 kb downstream of the γ2b 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 Cα and the 3′RR from BAC clone CH230-162I08, 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 [TATACATCGTCTTAGTATCTGTCTGACCCCACCACCATC TTCCCTGCCTCC GTCCACTCACAGATCTCTGACGCGTCACCGC AGGGTAATAACTG];

502 [GAAAGCTGAGCTGCAAGAGACCCATCG];
503 [CTTCTACCCACTTAGCACAGTGGTTCTTATGCCATGGGTAGCCACTCTGAATATCAGATATCCTGGCTACAACAGCGGATAGCAGCACTCAG];
547 [GATGCACGTGTCAACCCTGCGATGTCCTCAC];
548 [ACCTGGC GCGCCGTGGCTGCTCTCATGTACATCCTG];
549 [CCACGGCGCGCCCAGACACCATCTTG GAAGACC];
560 [AGGGGCCCATTAACTCATCACAGTCCAGGCCATCATGGTGAGCCCTAGCTGACCTAAGCAGGTTCCACGCGTCACCGCA-GGGTAATAACTG];
591 [ATCTGCCCATAGGAGGAAGACCTCGGCCTCAAGTCAGGATGTACATGA GAGCAGCCACGGCGCGCCCCTCAAGACATGATCTCACATGAC];
592 [GTCAGAAGACACCCTCACAGGACATCG]; 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 AsiSI-AscI fragment, and BAC3, a 173 kb NotI fragment, were pooled with the particular C-region BAC on a NotI 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/μl (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_HKO 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 RNAlater 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 μg 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 μl 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 CantoII 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_H6-1, 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_H4, including rat Eμ, Sμ, Cμ, Cδ and all sequences up to Sγ2c was added to the human V_H6-1, D and J_H sequence. A further addition of rat Sγ2b, Cγ2b, Cε, Cα and the 3′RR in natural configuration was made (Bruggemann et al., 1986). In this 202 kb construct Cγ2b is in the position where normally Cγ2c is located. In HC13 Hu-Rat Belinda, the authentic region from rat Eμ to Cγ2c was added, which is followed by Sγ2b, Cγ2b 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 Cα with ~30 kb 3′ region after Cγ2b, which generated a 202 kb BAC. In HC10, HC13 and HC17 the rat Cγ2b C_H1 exon was exchanged for human γ1 C_H1.

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

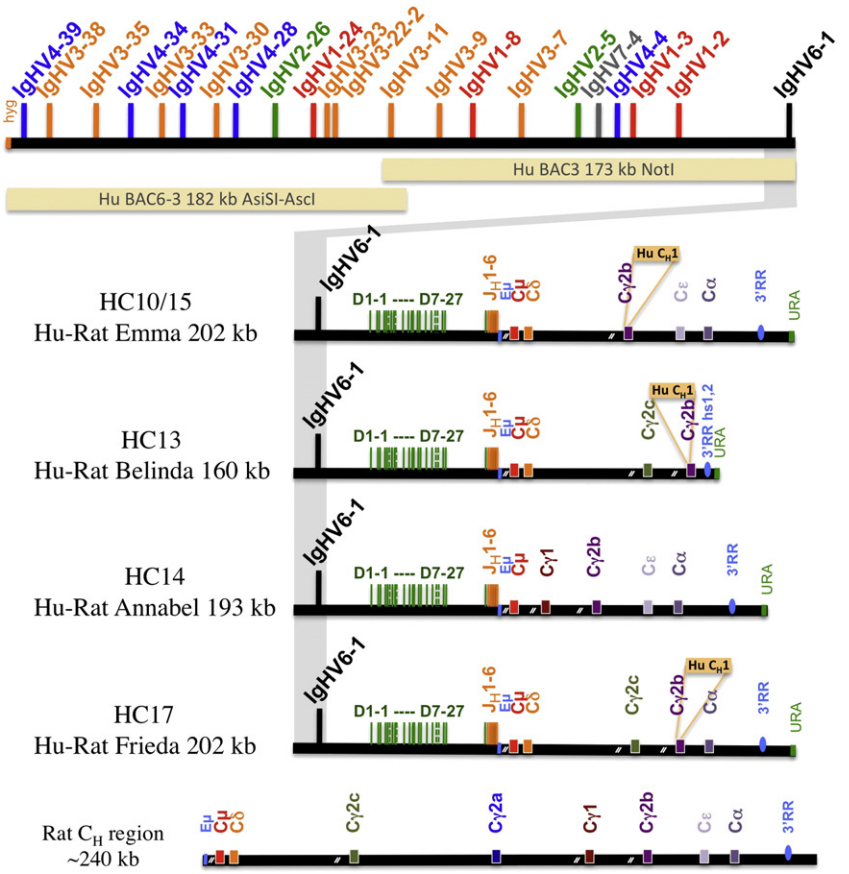


Fig. 1. Human V_Hs, Ds and J_Hs linked to different rat C-regions. Integration of large linear DNA segments of several hundred kb to generate human-rat IgH transloci was facilitated by overlapping regions of ~11 kb engineered to link the V_H gene BACs, Hu BAC6-3 an AsiSI-AscI fragment of 182 kb and Hu BAC3 a NotI fragment of 173 kb (top left) and, shown by the gray bar, the four chimeric C-region BACs all on NotI 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_Hs, followed by rat Eμ and rat Cμ. Rat Cμ is followed by rat Cδ in natural configuration in all constructs, except Hu-Rat Annabel. Various rat C-region genes have been added downstream of Cμ or Cδ, with some rat Cγ2b genes carrying human C_H1. Switch regions are indicated (///) where analyzed. The 3′RR of ~30 kb, 3′ of Cα, 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).

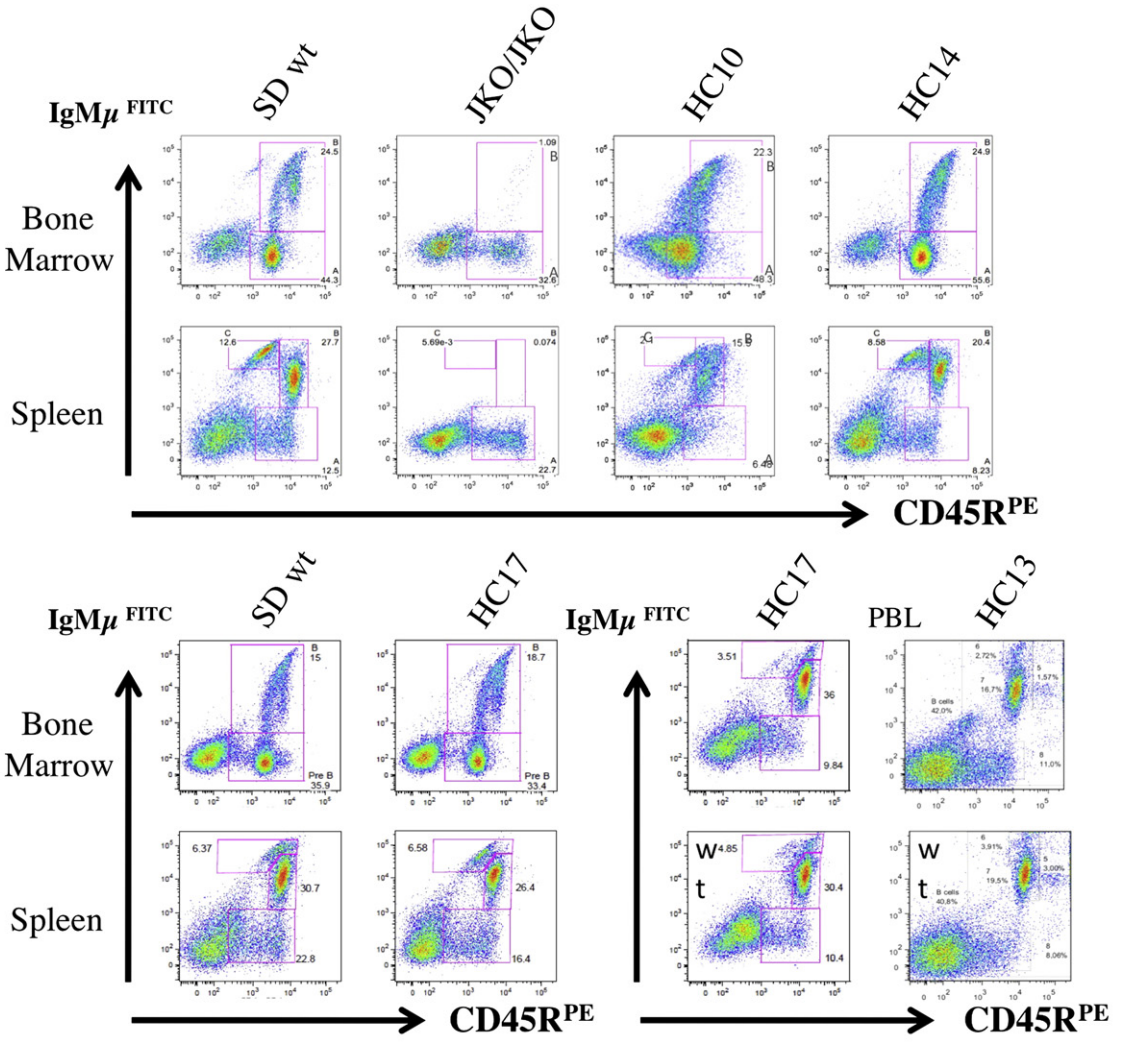
tandem integration of the same human V_H6-1, all D and J_H segments but with different rat C-regions might have been achieved. For HC10 only Hu BAC3 was included in conjunction with the C-region but in a separate experiment, generating HC15, both human V_H BACs, Hu BAC6-3 and Hu BAC3, were integrated 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 Cμ 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 Cγ 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



B

	IgH V																							IgH D																											IgH J					
	4-39	4-38	3-35	4-34	3-33	4-31	3-30	4-28	2-26	1-24	3-23	3-22	3-11	3-9	1-8	3-7	2-5	7-4	4-4	1-3	1-2	6-1	1-1	2-2	3-3	4-4	5-5	6-6	1-7	2-8	3-9	3-10	4-11	5-12	6-13	1-14	2-15	3-16	4-17	5-18	6-19	1-20	2-21	3-22	4-23	5-24	6-25	1-26	7-27	1	2	3	4	5	6
HC10/15	√			√	√	√	√		√	√	√		√	√	√	√	√	√	√	√	√	√	√	√	√		√	√	√	√	√	√		√	√	√	√	√	√		√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
HC13	√			√	√	√	√	√	√	√	√		√	√	√	√	√	√	√	√	√	√	√	√		√	√	√		√	√	√		√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
HC14	√			√	√	√	√	√	√	√	√		√	√	√	√	√	√	√	√	√	√	√	√	√		√	√	√	√	√	√		√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
HC17	√			√	√	√	√		√	√	√		√	√	√	√	√	√	√	√	√	√	√	√				√		√	√	√	√	√	√	√	√	√	√	√	√		√	√	√			√	√	√	√	√	√	√	√

Fig. 2. B-cell development and human V_H gene usage of one representative animal each from 3 to 5 rats per group analyzed. (A) Flow cytometry analysis of lymphocyte-gated bone marrow, spleen and peripheral blood lymphocytes (PBLs) from ~3 month old rats. Surface staining for IgM and CD45R (B220) reveals similar (HC14 and HC17) or slightly reduced (HC10 and HC13) numbers of immature and mature B cells compared to SD wildtype animals (wt) while homozygous J_HKO rats provided a negative control. In the histograms 'A' refers to lymphocyte precursors ($CD45R^+IgM^-$), 'B' to immature or follicular B-cells ($CD45R^+IgM^+$) and 'C' to marginal zone B cells ($CD45R^{low}IgM^+$). (B) Productive V_H, D and J_H usage in PBL transcripts obtained by RT-PCR with group-specific V_H and μC_H2 oligos.

V_HDJ_H-Cγ transcripts (Osborn et al., 2013). As comparable results were obtained for HC17 we can conclude that both these lines allow B-cell development, while in HC10 and HC13 B-cell maturation stages following IgM expression appear to be suboptimal.

3.3. Serum Ig and immune response

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 μg/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).

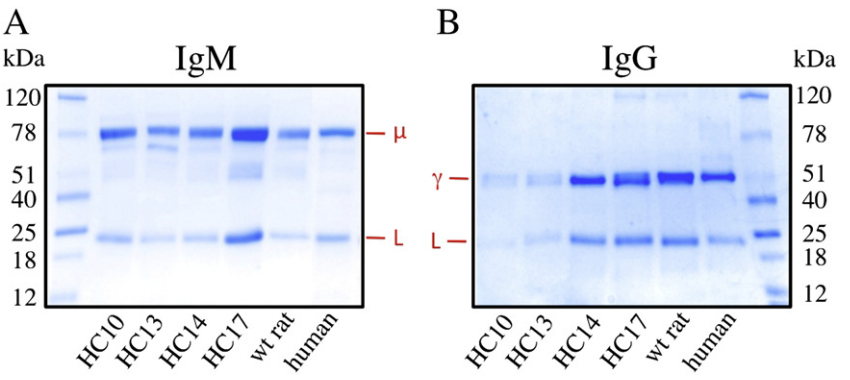
For IgG purification on protein A, the results were split as low and normal levels were identified (Fig. 3B). For HC14, HC17 and wt this revealed 500–1000 μg IgG/ml serum; this level was established from several experiments and agrees with previous findings despite the suboptimal purification of rat 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 μg 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_Hs, 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; Brüggemann et al., 2007), even with Eμ, Cμ 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 Cμ 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 Eμ and Cμ, 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



C

HC10*	wt	HC13*	wt	HC14	wt	HC14	HC17
700″	5,000″	200″	8,000″	52,200⁺	52,200⁺	5,000″	20,000″
600″	50,000″	100⁺	50,000⁺	8,000^	9,600^	12,000^	2,000^
		2,000⁺	40,000⁺	25,600‘	12,800‘		

β-gal″, hPG⁺, OVA^, HEL‘

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 μl each with equal amounts from 4 to 5 randomly bled rats) was captured (A) with anti-IgM matrix, which identified 300–500 μg/ml IgM in each of the samples, and (B) protein A, which produced a lower level of IgG in HC10 and HC13 (<100 μg/ml) but normal levels in HC14 and HC17 compared to wt (500–1000 μg/ml). Concentrations were determined by nanodrop and ELISA. (C) IgG antigen titers in HC rats and control animals at EC_{50} 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 Cα (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 Cα 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 Cγ2b(Hu C_H1) transcripts being isolated. Here Cγ2b is immediately downstream of the γ2c germline promoter and I-exon, taking the position of Cγ2c. 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 Cγ-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 Cγ gene with human C_H1 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 Cδ to downstream of Cγ2a omitted and Hu-Rat Frieda has the region from Cγ2a to Cγ1 and a ~21 kb section containing Cε 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 Cδ together with the large interval region (Mundt et al., 2001) and downstream C genes, γ2c and γ2a up to 4.4 kb 5′ of Sγ1, 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 Cγ 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 Cδ with its downstream region followed by Cγ2c, Cγ2b(Hu C_H1) and the full 3′RR. However, class-switching of this translocus favored Cγ2b(Hu C_H1) and not Cγ2c the first Cγ-gene downsteam of Cμ/Cδ. 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 Cδ and the first Cγ 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
+++	+++	+++ γ1, γ2b	+ little trans	+++	HC14 Hu-Rat Annabel	5
+++	+++	+++ γ2b Hu C_H1 some γ2c	+ 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_HDJ_H-rat Cγ 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_HDJ_H-Cγ transcripts were found for HC14 as described (Osborn et al., 2013) and similarly for HC17 with many V_H 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 Cγ next to the Cδ 3′ region could be identified while Cγ-gene usage in Annabel, not carrying Cδ 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 Cγ genes further downstream may not be affected as many diverse humanV_HDJ_H-Cγ2b (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 Cγ1 or Cγ2a), although quite low, appear to be generated more easily than translocus switch products (e.g. human V_HDJ_H-rat Cγ2b with human C_H1). This confirms that although intra-chromosomal recombination is repressed in HC10 and HC13, possibly due to reduced accessibility of sγ in the constructs (Kaminski and Stavnezer, 2004), inter-chromosomal recombination or switching from transgenic Cμ to endogenous Cγ 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 Cα 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 Cδ was not detrimental for class-switch recombination and expression (Chen and Cerutti, 2010).

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