

# Manipulation of human minichromosomes to carry greater than megabase-sized chromosome inserts

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For introducing regions of human chromosomes greater than a megabase into cells or animals, we have developed a chromosome-cloning system in which defined regions of human chromosomes can be cloned into a stable human minichromosome vector in homologous recombination-proficient chicken DT40 cells. The stable minichromosome vector allowed a 10 Mb-sized region of the mitotically unstable human chromosome 22 to be stably maintained in mouse embryonic stem (ES) cells, and in mice. Furthermore, we demonstrated functional expression of human genes from the HAC in mice. This study describes a stable cloning and expression system for greater than megabase-sized regions of human chromosomes.

Keywords: chromosome cloning, human artificial chromosomes

Bacterial artificial chromosomes (BACs)<sup>1</sup>, P1 phage-derived artificial chromosomes (PACs)<sup>2</sup>, and yeast artificial chromosomes (YACs)<sup>3</sup> have been used for cloning of large segments of genomic DNA. Although YACs have a cloning capacity of 1–2 Mb (ref. 3), such large genomic inserts are sometimes unstable and undergo recombination<sup>4</sup>. Recently, mammalian artificial chromosomes (MACs) have been developed for stable introduction of large segments of genomic DNA into cells or animals<sup>5–12</sup>. However, there has been no report showing the cloning of greater than megabase-sized genomic DNA into MACs.

Human chromosomes (hChrs) or their fragments have been used to introduce large segments of human genomic DNA into mice<sup>13,14</sup>. However, it has been reported that the mitotic stability of hChrs in mice varies among hChrs<sup>14</sup>. Thus, it is difficult to stably maintain any type of hChr in order to perform functional analyses in mice. Furthermore, it is difficult to introduce defined regions of hChrs into mice because the fragmentation of hChrs can occur randomly.

We report the development of a chromosome-cloning system in which a defined human chromosomal region was cloned into a stable human minichromosome vector by a combination of Cre/loxP-mediated chromosome translocation<sup>15</sup> and telomere-directed chromosome truncation<sup>16</sup> in homologous recombination-proficient chicken DT40 cells<sup>17</sup>. By cloning into the minichromosome vector, a 10 Mb human chromosome region defined by a loxP-integration site and telomere-truncation site containing the immunoglobulin  $\lambda$ -light chain (*Ig* $\lambda$ ) locus on the mitotically unstable hChr22 fragment was stabilized in mouse embryonic stem (ES) cells. We produced chimeric mice stably maintaining this human artificial chromosome (HAC) carrying the hChr22-derived insert, and showed that the human *Ig* $\lambda$  gene on the chromosomal insert in the HAC was functionally expressed. This HAC construction system will enable us to clone, stabilize, and functionally analyze greater

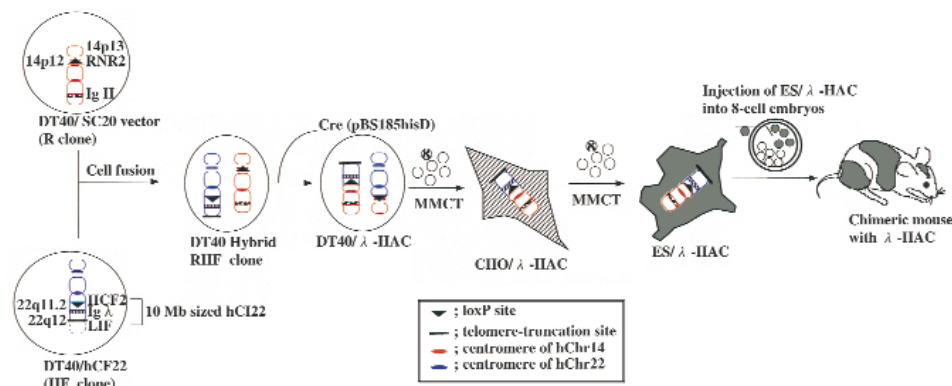
than megabase-sized defined human chromosomal regions in vitro and vivo.

## Results and discussion

**Generation of HACs carrying the defined hChr inserts.** A schematic diagram of the chromosome-cloning approach is shown in Figure 1. DT40 cells containing hChr14-derived SC20 fragment with the immunoglobulin heavy-chain (*IgH*) locus, a potential human minichromosome vector<sup>14</sup>, were transfected with pRNR2loxPbsr vector (Fig. 2A) to integrate the loxP sequence into the *RNR2* locus at 14p12 (ref. 18), thereby creating a cloning site for chromosomal inserts (R clone). To construct a HAC carrying a defined human chromosomal insert, we cloned the region on hChr22 between *HCF2* and *LIF* loci, which contains the *Ig* $\lambda$  locus<sup>19</sup>. This 10 Mb insert was designated hCI22. DT40 cells harboring hCF22 truncated at the *LIF* locus<sup>16</sup> at 22q12 (distal to *Ig* $\lambda$  locus) were transfected with the pHCF2loxPHYg vector. This integrated the loxP sequence into the *HCF2* locus at 22q11.2 (proximal to *Ig* $\lambda$  locus) (HF clone).

The R and HF clones were fused, resulting in RHF hybrids containing both SC20 and hCF22. The RHF hybrids were transfected with pBS185hisD vector to stably express Cre recombinase. This allowed cloning of the hCI22 region between the loxP-integration site (*HCF2*) and telomere-truncation site (*LIF*) into the loxP-cloning site on SC20 vector, by recombining the two exogenous nonhomologous human chromosomal fragments (hCFs). Stable transfectants derived from RHF hybrids were subjected to nested polymerase chain reaction (PCR)<sup>20</sup>, and recombination between the two loxP sites on the hCFs and the cloning of the hCI22 into the loxP-cloning site on SC20 vector was confirmed (Fig. 2B). This HAC was designated  $\lambda$ -HAC.

To determine whether this strategy works for other human chromosomal regions, we also examined cloning of the hChr2-derived insert (hCI2) defined by *yWHZ30-4* (ref. 21) and *CD8A* (ref. 22) loci



**Figure 1.** Schematic diagram of the chromosome-cloning approach. R clone: DT40 clone with SC20 vector where loxP sequence is integrated at the *RNR2* locus. HF clone: DT40 clone with hCF22 truncated at *LIF* locus where loxP sequence is integrated at the *HCF2* locus. RHF clone: DT40 hybrid with both SC20 and hCF22. In RHF-derived transfectants with pBS185hisD vector,  $\lambda$ -HAC is generated by recombination between SC20 and hCF22.  $\lambda$ -HAC is transferred from DT40 cells to mouse ES cells by means of CHO cells. Chimeric mouse mice are produced from ES cells containing  $\lambda$ -HAC.

(about 5 Mb) into SC20 vector. This insert was selected because recombination frequency between two exogenous nonhomologous chromosomes was suspected to be low<sup>15,23</sup>. Similarly, hChr2 was truncated by integrating human telomeric repeats into *CD8A* locus with pTELPuroCD8A vector (Fig. 2C) in DT40 cells, and confirmed by fluorescent in situ hybridization (FISH) and PCR analysis (data not shown). Next, a loxP sequence was integrated into *yWHZ30-4* locus with pYH2loxPhyg vector (Fig. 2C), creating hCF2. The DT40 hybrids with both SC20 and hCF2 were transfected with pBS185hisD vector. The cloning of the hCI2 into the loxP-cloning site on SC20 vector was similarly confirmed by nested PCR analysis (Fig. 2D). The second HAC carried the defined human chromosome insert hCI2. These results suggest that various human chromosomal regions defined by loxP-integration and telomere-truncation sites can be cloned by this method. The cloning capacity is at least 10 Mb greater than in conventional cloning methods.

**Structural analyses of  $\lambda$ -HAC in vitro.** To characterize the HAC carrying the human chromosomal insert in vitro and vivo, we isolated cells containing  $\lambda$ -HAC with the 10 Mb-sized human chromosomal insert from the nested PCR-positive pools. We then performed fluorescence-activated cell sorting (FACS), because the cells containing  $\lambda$ -HAC were expected to express the green fluorescent protein (GFP) gene (see Fig. 2B). FISH analysis of the sorted cells with both hChr14- and hChr22-specific probes demonstrated successful isolation of cells containing  $\lambda$ -HAC generated from the reciprocal translocation between SC20 and hCF22 (Fig. 3). For structural analyses of  $\lambda$ -HAC in several cell lines, we transferred  $\lambda$ -HAC to CHO cells from original DT40 cells by microcell-mediated chromosome transfer (MMCT)<sup>13</sup>, followed by transfer to mouse ES cells in which endogenous *IgH* and immunoglobulin  $\kappa$ -light chain (*Igk*) genes had been inactivated<sup>14</sup> (see Fig. 1).  $\lambda$ -HAC existed as a single-copy independent chromosome in each cell line, and apparent rearrangements and deletions in  $\lambda$ -HAC did not occur during MMCT, as shown by FISH, human L1 repeat-probed Southern hybridization, and PCR analyses (data not shown). In addition,  $\lambda$ -HAC was stably transferred into bovine fetal fibroblasts and several cell lines across several species (data not shown).

**Mitotic stability of  $\lambda$ -HAC in vitro.** Some hChrs have been reported to be mitotically unstable in mouse ES cells although they are stably maintained in other cultured cell lines<sup>6,14,24</sup>, prompting us to examine mitotic stability of  $\lambda$ -HAC in ES cells (Fig. 4). Whereas hCF22 was lost after 15-days in culture (~20 doublings) under nonselective conditions,  $\lambda$ -HAC carrying this unstable hCF22-derived insert (hCI22), as well as SC20 vector itself, was maintained at 99.8% retention per cell division throughout 45-days in nonselective culture (~70 doublings). The fact that we could stabilize the mitotically unstable hCF22-derived insert by cloning it into the SC20 vector suggests that the centromere of the SC20 vector dominantly contributed to the mitotic stability of  $\lambda$ -HAC.

**The function of  $\lambda$ -HAC in vivo.** Healthy chimeric mice were generated from ES cells containing  $\lambda$ -HAC exhibiting varying degrees of chimerism (20–80% of coat color). To determine the functionality of  $\lambda$ -HAC in vivo, we examined expression of human IgH and Ig $\lambda$  proteins. In the sera of  $\lambda$ -HAC chimeras, human Ig $\mu$  (h $\mu$ ),  $\gamma$  (h $\gamma$ ), and  $\lambda$  (h $\lambda$ ) chains were detected by enzyme-linked immunosorbent assay (ELISA; Table 1). h $\lambda$  Expression was almost equal to that of h $\mu$  and h $\gamma$  combined, and the ratio of h $\lambda$  to endogenous mouse Ig $\lambda$  (m $\lambda$ ) (h $\lambda$  : m $\lambda$  = 22 : 1) almost corresponded to that of mouse Ig $\kappa$  (m $\kappa$ ) to m $\lambda$  (m $\kappa$  : m $\lambda$  = 19 : 1) in wild-type mouse<sup>25</sup>. In contrast, in “conventional chimeras” created from ES cells containing both SC20 and hCF22 individually, human IgH and m $\lambda$  were detected but h $\lambda$  was not in some cases (data not shown), because of the mitotic instability of hCF22. Even in the case where h $\lambda$  was detected, m $\lambda$  was significantly expressed (h $\lambda$  : m $\lambda$  = 1 : 1) in compensation for the lost hCF22. Next, we immunized  $\lambda$ -HAC chimeras with granulocyte colony-stimulating factor (G-CSF) and observed an immune response of G-CSF-specific h $\gamma$ /h $\lambda$  in the sera (Fig. 5), leading to the production of hybridomas stably secreting G-CSF-specific human monoclonal IgG/ $\lambda$  antibodies (data not shown). These results suggest that  $\lambda$ -HAC was stably maintained during development of B cells lacking in endogenous *IgH* and *Igk* expression and that both human *IgH* and *Igk* loci on  $\lambda$ -HAC were fully functional even after the HAC construction.  $\lambda$ -HAC was also observed in tail fibroblasts of chimeras by PCR, Southern hybridization, and FISH analyses (data not shown). These data indicate that  $\lambda$ -HAC was stably maintained in somatic cells during development in vivo.

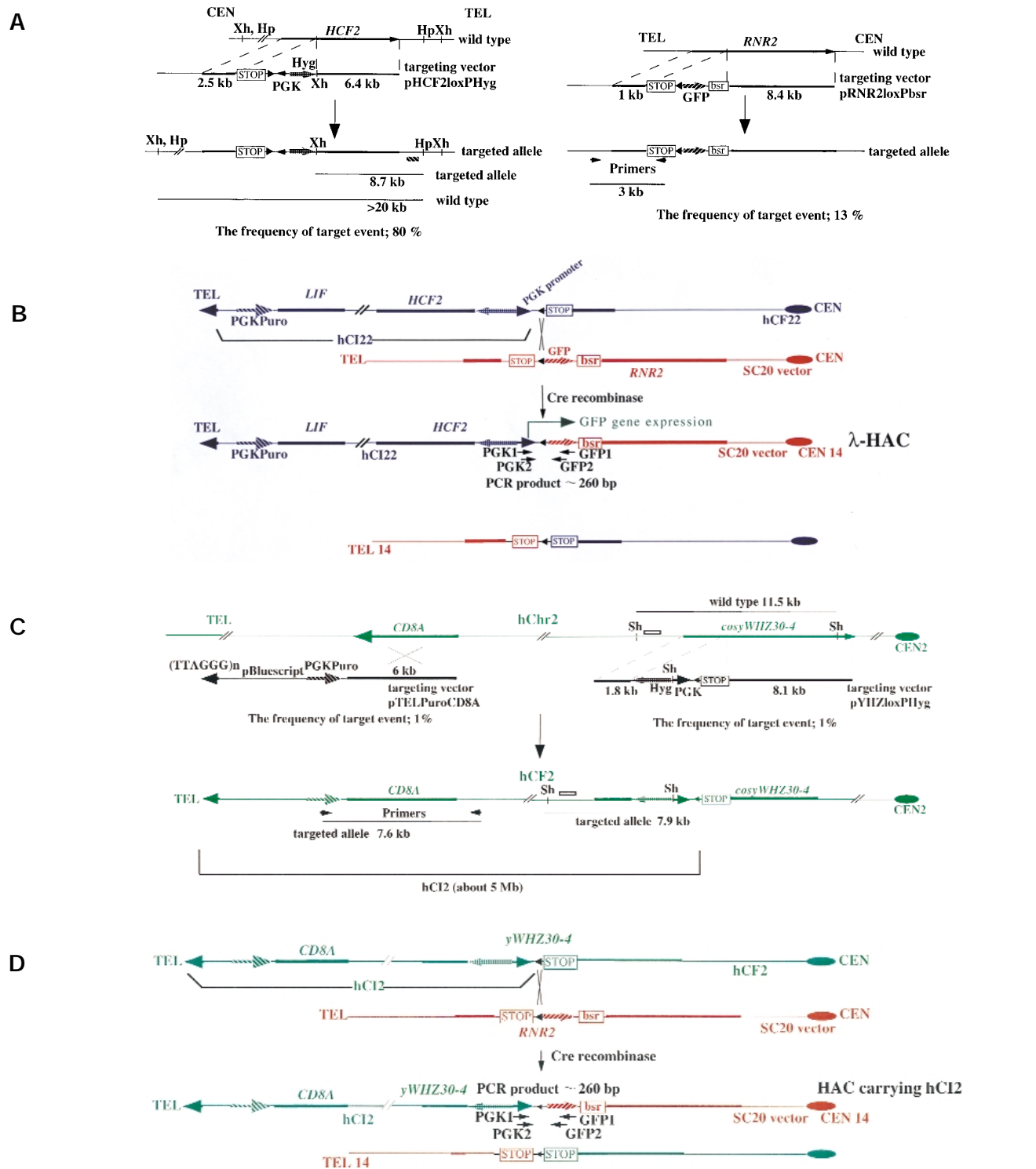
We have described a system to clone defined human chromosomal regions into a stable human minichromosome vector in DT40 cells. The HAC carrying a 10 Mb-sized human chromosomal insert was functional in vitro and vivo. Using our cloning system, human chromosomal regions previously difficult to clone by conventional cloning methods due to large size and complex structure (e.g., dystrophin, T-cell receptors, *MHC* cluster, *P450* cluster, and multigenetic disease regions) may be cloned into SC20 vector, stabilized, and functionally analyzed in vitro and vivo.

**Table 1.** Concentration of immunoglobulins in the sera of chimeras<sup>a</sup>

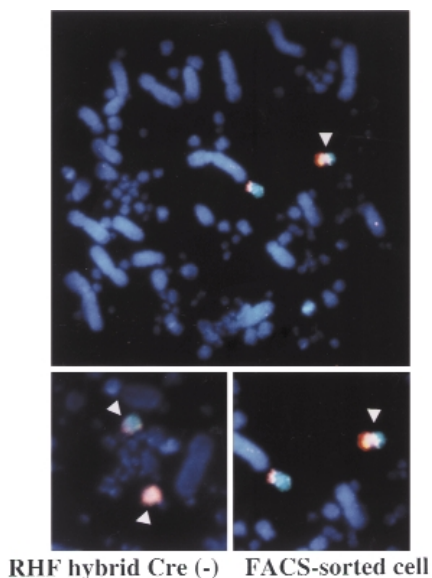
	Concentration ( $\mu$ g/ml)			
	h $\mu$	h $\gamma$	h $\lambda$	m $\lambda$
$\lambda$ -HAC chimera	610	140	933	41
Conventional chimera	440	160	200	160

<sup>a</sup>Conventional chimera represents a chimeric mouse containing both SC20 and hCF22 individually. h $\mu$ , h $\gamma$ , h $\lambda$ , and m $\lambda$  represent human Ig $\mu$ , Ig $\gamma$ , Ig $\lambda$ , and mouse Ig $\lambda$ , respectively.

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**Figure 2.** Cloning of the hCI2 and hCI2 into SC20 vector. (A) Targeting vectors, pRNR2loxPbsr and pHCF2loxPHYg. pRNR2loxPbsr: In targeted clones (R clones), 3 kb of PCR product is detected with indicated primers (arrows). pHCF2loxPHYg: In targeted clones (HF clones), 8.7 kb of band digested with *Hpa*I and *Xho*I is detected in Southern hybridization probed with HCF2 probe, compared with >20 kb in wild type. (B) Verification of cloning of the hCI2 defined by *HCF2* (loxP-integration site) and *LIF* (telomere-truncation site) into the loxP-cloning site on SC20 vector by nested PCR with indicated primers in RHF clones. In addition, PGK promoter on hCF2 drives the transcription of GFP gene on SC20 vector as a result of the recombination. (C) Targeting vectors, pTELuroCD8A and pYHZloxPHYg. pTELuroCD8A: In targeted clone (CD10), 7.6 kb of PCR product is detected with indicated primers (arrows). pYHZloxPHYg: In targeted clone (Y100), 7.9 kb of band digested with *Sph*I is detected in Southern hybridization probed with YHZ probe, compared with 11.5 kb in wild type. (D) Verification of cloning of the hCI2 defined by *yWHZ30-4* (loxP-integration site) and *CD8A* (telomere-truncation site) into the loxP-cloning site on SC20 vector by the nested PCR with indicated primers in DT40 hybrids containing both SC20 and hCF2. bsr, blasticidin S-resistant gene; GFP, promoter-less GFP gene; Hp, *Hpa*I site; Hyg, hygromycin-resistant gene; PGK, PGK promoter; Puro, puromycin-resistant gene; Sh, *Sph*I site; Xh, *Xho*I site.



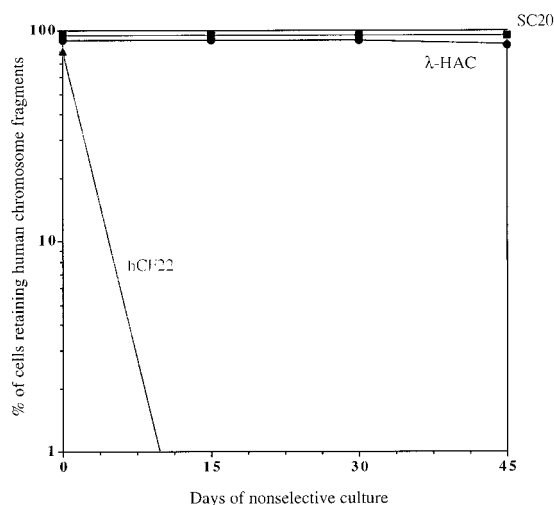
RHF hybrid Cre (-) FACS-sorted cell

**Figure 3.** Verification of successful isolation of the cells containing  $\lambda$ -HAC. FISH analysis of the FACS-sorted cells (lower right panel) using hChr14 (red) and hChr22 (green)-specific probes is shown. Arrowhead indicates  $\lambda$ -HAC. Lower left panel shows the FISH in RHF hybrids before transfection with pBS185hisD vector. Top panel is a lower magnification of the lower right panel.

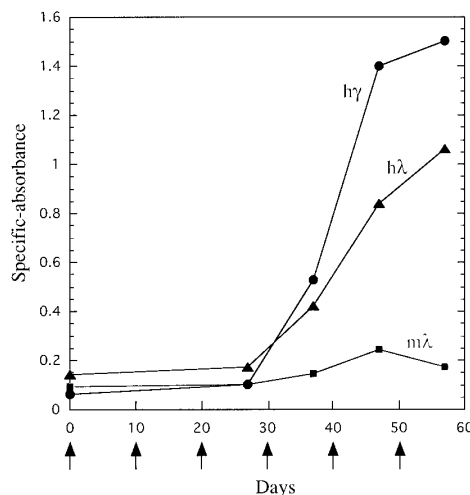
### Experimental protocol

**Plasmid construction.** Targeting vectors were constructed as follows. For pRNR2loxPbsr: the genomic sequence of *RNR2* was amplified with primers, 5'-AGTAGCTGGCACTATCTTTTGGCCATC-3' and 5'-GGA-GAAAGAACACACAAGGACTCGGTC-3' using LA-Taq (Takara, Kusatsu, Siga, Japan) in 35 cycles of 98°C for 10 s, 68°C for 15 min, and then cloned into *EcoRI* site in pBluescript II SK(-). GFP-loxP-bsr cassette was cloned into *KpnI* site in the *RNR2* genomic sequence. For pHCF2loxPHyg: the genomic sequence of *HCF2* was amplified with primers, 5'-GTGAGAACAAGACAGAGAATGAGGGAGG-3' and 5'-TAATGCAGAGGCTCTTTGGTGTACTTGG-3' and cloned into *KpnI* site in pBluescript II SK(-). Hyg-PGK-loxP cassette was cloned into *SnaBI* site in the *HCF2* genomic sequence. For pYHZloxPHyg: the genomic sequence of *yWHZ30-4* was amplified with primers, 5'-GATAGAGA-GATTGTCTTAAATGGGTGG-3' and 5'-AACAGCTGGAACATCAA-AAGCATAGC-3' and cloned into *EcoRI* site in pBluescript II SK(-). Hyg-PGK-loxP cassette was cloned into *Tth111I* site in the *yWHZ30-4* genomic sequence. For pTELuroCD8A: the genomic sequence of *CD8A* was amplified with primers, 5'-CTTTAGTGAAGGCAAAGGAAGGGA-CATC-3' and 5'-TGTAAGGGGTAGCCTGTCTCTTTCATG-3' and cloned into *BamHI* site in pTELuro cassette vector<sup>15</sup>. The pBS185hisD was constructed by cloning hisD cassette into *EcoRI* site in the pBS185 (Gibco BRL, Grand Island, NY).

**Target modification of hCFs.** The vectors listed above were electroporated to DT40 cells containing hCFs at 550 V, 25  $\mu$ F. Transfectants were selected under hygromycin B (1 mg/ml), blasticidin S (10  $\mu$ g/ml), puromycin (0.3  $\mu$ g/ml), or histidinol (0.5 mg/ml) for two weeks. Target events were determined by PCR or Southern hybridization as follows. For pRNR2loxPbsr: genomic DNA was extracted from transfectants and then subjected to PCR analysis with primers, 5'-TGGATGTATCTGTCAAGA-GACC-3' and 5'-CAGACTCTATGCCTGTGTGG-3' in 35 cycles of 98°C for 10 s, 65°C for 5 min. In targeted clones (R clones), an ~3 kb PCR product was detected. The frequency of the target event was 13%. For pHCF2loxPHyg: genomic DNA extracted from transfectants was digested with both *HpaI* and *XhoI* and then subjected to Southern hybridization probed with PCR product amplified with primers (HCF2 probe), 5'-CACATGACAAGAGCTCAGCG-3' and 5'-TCTGACTTCTCATGA-GAGCC-3' in 35 cycles of 98°C for 10 s, 65°C for 30 s, 72°C for 1 min. In targeted clones (HF clones), an 8.7 kb band was detected, compared with >20 kb in wild type. The frequency of the target event was 80%. For pTELuroCD8A: genomic DNA extracted from transfectants was subjected



**Figure 4.** Mitotic stability of  $\lambda$ -HAC in mouse ES cells. Mitotic stability was determined by FISH using human COT1 DNA probe.



**Figure 5.** The function of  $\lambda$ -HAC in chimeric mice. Immune response of G-CSF-specific  $h\gamma$ ,  $h\lambda$ , and  $m\lambda$  in the serum of  $\lambda$ -HAC chimera. Immunization was carried out at day 0, 10, 20, 30, 40, and 50 (indicated with arrows).

to PCR with primers (CD8A), 5'-GCCCTCATGGAATCTCTGGG-3' and 5'-GCAGCAACAGATGGAAGGCCTC-3' in 35 cycles of 98°C for 10 s, 68°C for 10 min. In targeted clone (CD10), an ~7.6 kb PCR product was detected. The frequency of the targeting event was 1%. For confirmation of chromosomal truncation at *CD8A* in the CD10 clone, PCR with primers, D2S373, FABP1, Vk3, Ck, D2S113, CD8A, D2S388, D2S1331, D2S134, D2S171 and TPO<sup>13</sup> and two-color FISH with human COT1 DNA and pGKPuro probes<sup>16</sup> were carried out. For pYHZloxPHyg: genomic DNA extracted from transfectants was digested with *SphI* and then subjected to Southern hybridization and probed with PCR product amplified with primers (YHZ probe), 5'-GTCTGGGGTTGGAGATCTG-3' and 5'-ATCCATCATAAGGGCTCG-3' in 35 cycles of 98°C for 10 s, 57°C for 30 s, 72°C for 1 min. In targeted clone (Y100 clone), a 7.9 kb band was detected, compared with 11.5 kb in wild type. The frequency of the targeting event was 1%.

In each targeting experiment, integration of the targeting vectors into the targeted locus only was confirmed by Southern hybridization using internal probes, bsr, Hyg or Puro in the vectors.

**Whole-cell fusion.**  $10^7$  cells of R and HF or Y100 clones were fused with polyethylene glycol (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's protocol and selected under both hygromycin B and blasticidin S for three to four weeks. Maintenance of the two hCFs was confirmed by PCR with primers, IGHV3 and IGLC or Ck (refs 13, 14) and FISH with COT1 DNA probe<sup>16</sup>.

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FACS.  $10^7$  cells of RHF hybrids transfected with pBS185hisD vector were suspended in phosphate-buffered saline (PBS) supplemented with 5% fetal bovine serum (FBS; Gibco) and 1  $\mu$ g/ml of propidium iodide (Sigma, St. Louis, MO), and analyzed by FACS Vantage (Becton Dickinson, Franklin Lakes, NJ). GFP-positive fraction was sorted and cultured for several days.  $10^7$  cells were reanalyzed by FACS, and GFP-positive cells were sorted. Four rounds of FACS sorting were performed in total.

**Confirmation of  $\lambda$ -HAC generation.** Genomic DNA was extracted from RHF hybrids transfected with pBS185hisD vector and subjected to nested PCR with primers as follows. First primers (PGK1-GFP1): 5'-ATAGCAGCTTTGCTCCTTCG-3' and 5'-TTCTCTCCTGCACATAGCCC-3'; second primers (PGK2-GFP2): 5'-TGTCTCTCCTTCTACTCTCC-3' and 5'-TGAAGGTAGTGACCAGTGTGG-3', in 35 cycles of 98°C for 10 s, 61°C for 30 s, 72°C for 1 min, and 98°C for 10 s, 59°C for 30 s, 72°C for 30 s, respectively. Two-color FISH using hChr14- and hChr22-specific probes (TAKARA) was carried out, according to manufacturer's protocol.

**Confirmation of cloning of the hC12 into SC20 vector.** Genomic DNA was extracted from DT40 hybrid containing both SC20 and hCF2 transfected with pBS185hisD vector and subjected to the nested PCR analysis, as described above.

**Structural analyses of  $\lambda$ -HAC in vitro and in vivo.** Genomic DNA was extracted from DT40, CHO, ES, bovine fetal fibroblast clones and tail fibroblasts of chimeras, and then subjected to PCR or Southern hybridization. Primers used for PCR were IGHV3, IGLC, AKT1, D14S543, D22S315, D22S275, CRYBA4<sup>13,14</sup>, Puro1-LIF1<sup>16</sup>, PGK1-GFP1, and PGK2-GFP2. Southern hybridization was performed with HCF2 or human L1 repeat probe<sup>13</sup>. Two-color or human COT1 DNA-FISH were carried out, as described above.

**Determination of mitotic stability of  $\lambda$ -HAC in mouse ES cells.** FISH was carried out using human COT1 DNA probe as described<sup>14</sup>. Fifty metaphase and 100 interphase spreads were counted and averaged in three independent experiments. The retention rate of  $\lambda$ -HAC from day 0 to day 45 (70 doublings) was calculated by the following formula:  $N_{70} = N_0R^{70}$ , where  $N_0$ ,  $N_{70}$  and  $R$  represent the percentage of cells containing  $\lambda$ -HAC at day 0 and 45, and the retention rate, respectively.

**Generation of chimeric mice.** Microcell fusion, ES cell manipulation, and chimera production were performed as described<sup>13,14</sup>, except that host eight-cell embryos were prepared from female mice whose endogenous *IgH* gene was inactivated<sup>14</sup>. Construction of mouse ES cell line in which both alleles of endogenous *Ig* heavy and  $\kappa$  chain genes are inactivated was carried out as follows. We employed a combination of homologous and Cre-loxP recombination<sup>26</sup> to generate ES cell lines carrying four mutations and devoid of selectable markers. Modified vectors containing the *neo<sup>r</sup>* (pSTneoB, ref. 13) or *puro<sup>r</sup>* (pPGKpuro, ref. 13) cassettes, in which the marker cassettes lies between the loxP sites, were used for the preparation of marker cassettes suitable for Cre-mediated excision. We used diphtheria toxin A-fragment gene as a negative selection marker<sup>27</sup>. The targeting construct for the *IgH* deletion contains 13kb of *Ig*  $\mu$  chain genomic DNA in which a *Bam*HI-*Xho*I segment (3.7kb) including a portion of  $C\mu 2$ ,  $C\mu 3$ - $C\mu 4$  and  $M\mu 1$ - $M\mu 2$  exons was replaced by a *neo<sup>r</sup>* cassette. Heterozygous mutant cell lines were then cultured in high concentration of G418 (3 mg/ml) to obtain homozygous mutants<sup>28</sup>. Homozygous *IgH* deletion mutant devoid of selectable marker obtained by Cre-mediated excision of the *neo<sup>r</sup>* cassette<sup>26</sup> was used for further genetic modification. The targeting construct for *Igk* deletion contains 13.5kb of *Ig*  $\kappa$  chain genomic DNA in which a *Sac*I-*Bgl*II segment (2kb) including the  $C\kappa$  exon was replaced by a *neo<sup>r</sup>* or *puro<sup>r</sup>* cassette. Heterozygous *Igk* deletion mutant obtained by the targeting construct containing *neo<sup>r</sup>* cassette was used for the second targeting experiment using the construct containing *puro<sup>r</sup>* cassette, leading to the generation of double drug-resistant homozygous *Igk* deletion mutants. Finally, Cre-mediated excision of both markers resulted in the ES cell line sensitive to both G418 and puromycin. The absence of  $C\mu$  and  $C\kappa$  sequences in the drug-sensitive, double homozygous deletion ES cell line was confirmed by southern blot. The HAC was introduced into this double homozygous deletion ES cell line, and then the ES cells containing the HAC were injected into host eight-cell embryos prepared from B cell-deficient *IgH*-KO female mice<sup>14</sup>. In resultant chimeric mice, B cells were derived only from ES cells containing the HAC because *IgH* gene is necessary for development of B cells and *IgH* gene of host cells was inactivated.

**ELISA.** Concentration of immunoglobulins in the sera of chimeras was determined by ELISA<sup>14</sup>. About 10 chimeric mice were examined respectively,

for two types of chimeras, " $\lambda$ -HAC chimera" and "conventional chimera," and averaged.

**Immunization and hybridoma production.**  $\lambda$ -HAC chimeras were immunized six times with G-CSF 50  $\mu$ g/injection in Titer Max Gold (CytRx Corp., Norcross, GA). At day 0, 28, 37, 47, and 57 after immunization, immune response of G-CSF-specific *h $\gamma$* , *h $\lambda$* , and *m $\lambda$*  in the sera of chimeras was determined by ELISA on G-CSF-coated plates, and then hybridoma production was performed as described<sup>14</sup>.

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