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Generation and Analysis of Partially Haploid Cells with Cre-mediated Chromosome Deletion in the Lymphoid System*[S]

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The fast accumulation of mutant mouse strains in recent years has provided an invaluable resource for phenotype-based genetic screens. However, study of lymphoid phenotypes can be obscured or impractical if homozygous mutations cause early embryonic defects. To aid phenotype screening of germ line mutations in the lymphoid system, we developed a method to induce loss of heterozygosity (LOH) in developing lymphocytes through chromosome deletion. Chromosome deletion was triggered by Cre/loxP-mediated inverse sister chromatid recombination in the G_2/M phase of the cell cycle, leading to the generation of daughter cells missing part of or the entire recombinant chromosome. We show that the resulting cells were viable and capable of additional rounds of cell division, thus providing raw materials for subsequent phenotypic assessment. We used the recombination system to induce LOH at the *E2A* locus in developing B cells. A significant loss of pro-B and pre-B cells was observed when the wild-type allele was removed by chromosome deletion from the *E2A* heterozygous mice, a result consistent with the required role for E2A in B cell development. We also demonstrated the effectiveness of Cre-mediated chromosome deletion in the LOH assay for HEB function in T cell development. Thus, the Cre-mediated chromosome deletion provides a new and effective method for genome-wide assessment of germ line mutations in the lymphoid system.

Over 4000 protein-coding genes have been mutated and studied in the mouse by gene targeting, gene trapping, or other mutagenic methods in the past 2 decades (1, 2). This number is expected to increase dramatically following the recent calls for genome-scale coverage of germ line mutations by several international consortia (3). Studies of the existing germ line mutations have implied that approximately one-third of null mutations cause embryonic or neonatal lethality (4). Consequently, the effect of germ line mutations on postnatal life cannot be easily assessed for the genes playing important roles in both embryonic and postnatal life. Further analysis of tissue-specific gene function often requires a completely independent effort to establish a new allele for Cre/lox-mediated conditional gene knock-out. Although the Cre/lox system can effectively address the embryonic problem and permit the study of tissue-specific gene function, the experimental system is not practical for large-scale genetic analysis. Thus far, there is no simple method available for systematic evaluation of lymphoid phenotypes of lethal mutations accumulated from a large-scale mutagenesis approach.

Mitotic recombination provides one possible way to assess somatic phenotypes of pre-existing mutations. A recombination between homologous chromosomes during mitosis can lead to clonal segregation of heterologous alleles. The mosaics resulting from mitotic recombination allow for functional analysis of homozygous clones in an otherwise heterozygous background (5, 6). The feasibility of mosaic analysis in mice was demonstrated in the loss of heterozygosity assay of tumor suppresser genes *p27* and *p53* with the Cre/lox-mediated (7) and the Flp/FRT-mediated (8) mitotic recombination systems, respectively. Although these recent studies provide a proof of principles for mitotic recombination in mice, the general application of this technique is still pending on the development of mitotic recombination systems to cover each one of the 19 pairs of mouse chromosomes. In addition, the reported mitotic recombination frequency is generally below 1% (5, 6, 9), and each test typically requires three generations of breeding to introduce an existing mutation onto the test background. These technical issues prevent the technique from being used as a practical tool in a high-throughput analysis of germ line mutations in the mouse.

To facilitate high-throughput analysis of existing mouse mutations in defined somatic tissues, we propose a loss of heterozygosity (LOH)[2] assay based on tissue-specific chromosome deletion. LOH in somatic tissues has been commonly used in cancer studies to link gene function to tumor phenotypes. For example, tumors resulting from loss function of a tumor suppressor gene often arise in heterozygous carriers when the wild-type copy of the gene is inactivated due to gene conversion, silencing, or deletion of the wild-type allele (10). Chromosome deletion in defined somatic tissue has the potential to be used as a generic method in LOH analysis of mutations on the entire chromosome. In this study, we tested the utility of sister chromatid

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S8.

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[2] The abbreviations used are: LOH, loss of heterozygosity; FACS, fluorescence-activated cell sorting; CFSE, carboxyfluorescein succinimidyl ester; ISCR, inverse sister chromatid recombination; EGFP, enhanced green fluorescent protein; hCD2, human CD2; DN, double-negative; DP, double-positive.

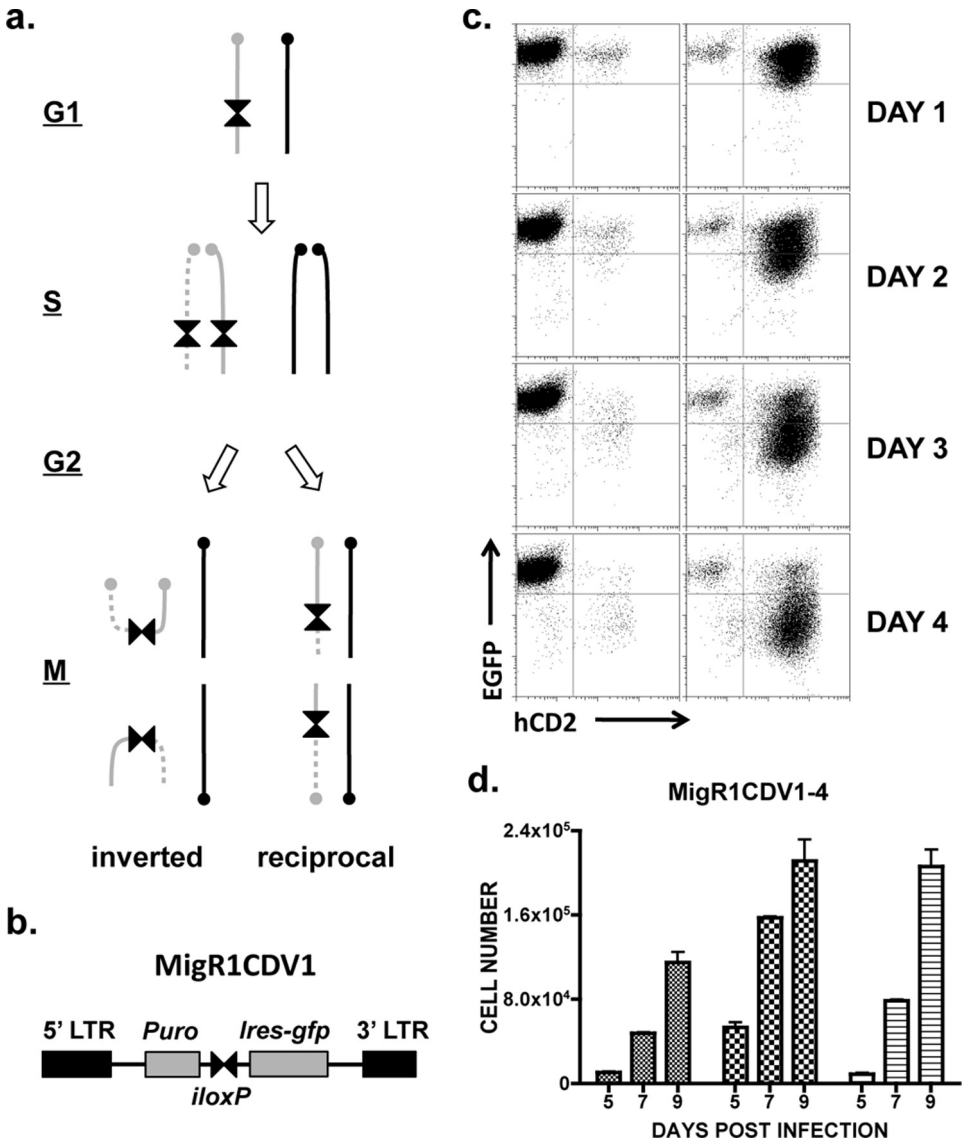

FIGURE 1. *a*, Cre/lox-mediated sister chromatid recombination. *Gray* and *black bars* represent a pair of homologous chromosomes with the round ends as centromeres. A pair of inverted loxP sites (*black triangles*) separates the chromosome into proximal and distal parts. Cre-mediated recombination between the duplicated sister chromatids (*gray*) during G_2 phase could result in either inverted or reciprocal recombination. *b*, diagram of the recombination cassette in the MigR1CDV1 retroviral vector (where CD is chromosome deletion). The MigR1 retroviral vector was modified by inserting the puromycin resistance gene (*Puro*) between the 5′-long terminal repeat (*5′ LTR*) and the internal ribosome entry site-EGFP (*Ires-gfp*). The iloxP site was inserted between the puromycin and internal ribosome entry site-EGFP sequences. *c*, Cre-dependent loss of EGFP expression in a representative clone from MigR1CDV1-infected NIH-3T3 cell lines. Retrovirus carrying Cre-internal ribosome entry site-hCD2 was used in the second round of infection of MigR1CDV1 stable cell lines. $hCD2^-$ (*left panels*) and $hCD2^+$ (*right panels*) cells were sorted into separate culture wells at day 0 with a purity exceeding 95%. EGFP expression was checked from day 1 to 4 and is displayed in two-dimensional plots for hCD2 and EGFP expression. *d*, cell counts of the same culture in *c* from day 5 to 9 at 2-day intervals. *Bar groups* from *left* to *right* represent $EGFP^+/hCD2^+$, $EGFP^-/hCD2^+$, and $EGFP^+/hCD2^-$ subsets from sorted $hCD2^+$ cultures.

recombination-induced deletion in developing lymphocytes. Our study establishes a simple genetic system for functional evaluation of heterozygous mutations in the lymphoid system.

EXPERIMENTAL PROCEDURES

Mice—pbch transgenic lines were generated by co-injection of the PBCH construct and the Act-PBase plasmid, which expresses the piggyBac transposase (11). All of the other mice have been described previously in cited references.

Fluorescence-activated Cell Sorting (FACS) and Analysis—Freshly isolated bone marrow, spleen, thymus, or lymph node cells were stained with antibodies (BioLegend and eBioscience) in phosphate-buffered saline supplemented with 5% bovine calf serum on ice. Annexin V (Pharmingen) staining was carried out according to the manufacturer's protocol. FACS analyses were performed on a FACSCalibur or FACSCanto (BD Biosciences). Statistic analysis was performed with Prism (GraphPad Software).

Karyotyping—Bone marrow cells were cultured for 24 h with 10 ng/ml interleukin-7 in RPMI 1640 medium (Invitrogen 11875-093) with 10% fetal bovine serum before treatment with 100 ng/ml colcemid (Invitrogen 15212-012) for 1 h. Cells were collected, resuspended in 0.075 M KCl solution, kept at room temperature for 5 min, and fixed with methanol/acetic acid (3:1). Chromosome spread was performed on poly-L-lysine-coated slides.

Carboxyfluorescein Succinimidyl Ester (CFSE) Analysis and B Cell Culture—Sorted B cells were stained with 5 μM CFSE (Invitrogen) in serum-free medium in the dark for 10 min before culture. Stained B cells were cultured on the S17 stromal layer either with or without 10 ng/ml interleukin-7 in RPMI complete medium for 3 days before analysis.

RESULTS

The natural paring of sister chromatids during mitosis provides a window of opportunity for Cre/lox-mediated mitotic recombination between the sister chromatids. If a pair of loxP sites are present in reverse orientation, Cre-mediated recombination could induce reciprocal or inverted sister chromatid recombination during mitosis (Fig. 1*a*) (12, 13). Although reciprocal sister chromatid exchange does not alter genetic information among the recombination products, an inverse sister chromatid recombination (ISCR) generates a dicentric and an acentric chromosomal fragment. The acentric chromosome will be segregated asymmetrically into daughter cells, whereas the dicentric chromosome is subject to additional breakage and asymmetric segregation during subsequent cytokinesis. Consequently, a fraction of the daughter cells will become partially haploid and may be used in LOH analysis.

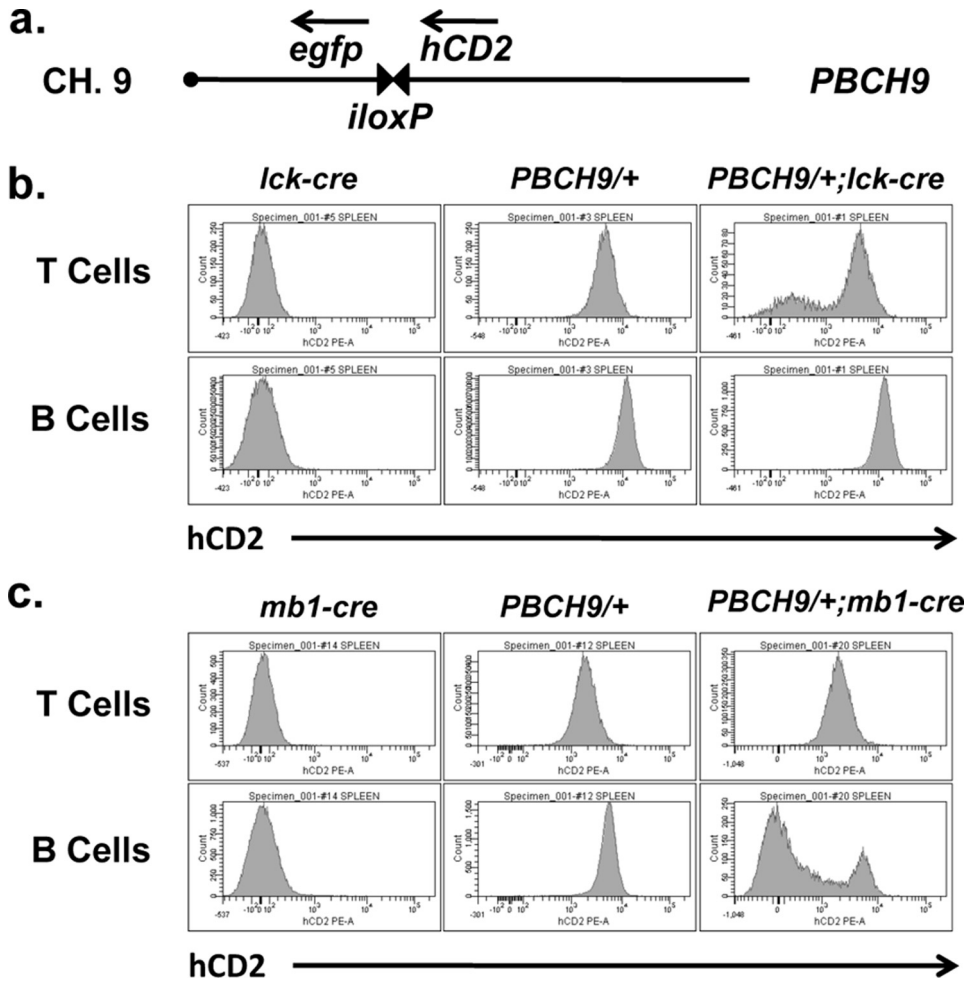

FIGURE 2. *a*, diagram of the iloxP recombination cassette on chromosome 9 in *pbch9* transgenic mouse. The recombination cassette containing EGFP, iloxP, and hCD2 was integrated into chromosome 9 via piggyBac transposition. EGFP is located on the proximal side of chromosome 9, and hCD2 is on the distal side. *Arrows* indicate the direction of transcription. *b*, effect of *LckCre* on hCD2 expression from the *pbch9* chromosome. The *pbch9* transgenic line was crossed onto the *LckCre* background. Splenic T and B cells from representative mice of the indicated genotypes were gated with the T cell receptor β and B220 markers, respectively, prior to histogram analysis for hCD2 expression ($n = 8$). *c*, effect of *mb1Cre* on hCD2 expression. The experiment was performed as described for *b* ($n = 4$). *d*, karyotyping analysis of mitotic events in *ex vivo* bone marrow culture. Mitotic spreads representing the euploid state, chromosome 9 (*Chr.9*) breaks, and chromosomal 9 distal deletions are shown. Four *pbch9;mb1Cre* animals were analyzed by scoring 20–22 mitotic nuclei for each sample. All chromosomal abnormalities identified involve chromosome 9, which accounts for 15 ± 4% of the total score. *PE-A*, phycoerythrin area.

Detection of ISCR in Culture—To explore the possibility of applying ISCR to LOH assay in the lymphoid system, we built a new recombination system for easy detection of the recombination events between designated sister chromatids. The recombination cassette includes a pair of inversely positioned loxP sites with 2-bp overlap (named iloxP) to prevent intrachromosomal recombination (supplemental Fig. S1*a*). We first tested this recombination cassette in 3T3 fibroblasts for efficiency of ISCR. The recombination cassette was placed in a retroviral vector between the 5′-long terminal repeat-driven puromycin marker and the internal ribosome entry site-driven enhanced green fluorescent protein (EGFP) marker (Fig. 1*b*). Individual 3T3 cells harboring this viral vector and expressing EGFP were sorted to establish clonal cell lines carrying a single integration event. A second retroviral vector carrying the Cre recombinase gene and the human CD2 (hCD2) marker was used to induce sister chromatid recombination at the iloxP site. Cells were separated into $hCD2^+$ and $hCD2^-$ fractions 1 day after viral transduction and analyzed daily thereafter for EGFP expression (Fig. 1, *c* and *d*). Although EGFP expression was maintained among $hCD2^-$ fractions, a progressive loss of EGFP expression was observed in $hCD2^+$ fractions. Both $hCD2^-$ and $hCD2^+$ cells continued to expand within the first week in culture, although the latter slowed down significantly in comparison with the former after 1 week (Fig. 1*d*). The fast conversion from EGFP-positive to EGFP-negative cells suggests that Cre/loxP-mediated recombination can induce efficient ISCR in cultured fibroblasts. We next tested ISCR by separating the two loxP sites and allowing intrachromosomal inversion as well as interchromosomal recombination. We moved one of the loxP sites between the 5′-long terminal repeat and the puromycin coding sequence so that the inverse loxP pair was separated by an ~600-kb sequence. 3T3 cells carrying this recombination cassette were tested for Cre-mediated recombination. Similar to the iloxP design, efficient EGFP loss was observed upon expression of the Cre recombinase (supplemental Fig. S1, *b* and *c*). For simplicity, we chose the iloxP design in the subsequent test of ISCR in live animals.

Detection of ISCR in T and B Lymphocytes—We chose piggyBac transposon as the transgenic vector to produce single-copy integrations of the iloxP site into the mouse genome (11). The iloxP site was placed between the EGFP and hCD2 markers in the piggyBac vector (supplemental Fig. S2*a*). With this design, upon ISCR, both the proximal and distal parts of the iloxP chromosome will be individually tagged with a visible marker for subsequent tracing of the dicentric and acentric chromosomes, respectively (supplemental Fig. S2*b*). The *pbch9* strain (piggyBac insertion in chromosome 9) contains an iloxP site mapped to chromosome 9 at a position approximately two-fifths of the chromosomal length to the centromere (Fig. 2*a*). FACS analysis demonstrated uniform hCD2 expression in both B and T cells (Fig. 2*b*). In contrast, GFP expression was extremely low (supplemental Fig. S3), presumably due to transcription interference from the upstream pCX-hCD2 cassette. When this strain was crossed with mice carrying the T cell-specific *LckCre* transgene (14), $hCD2^-$ cells appeared among T cells but not B cells in the spleen (Fig. 2*b*). Likewise, introduction of the B

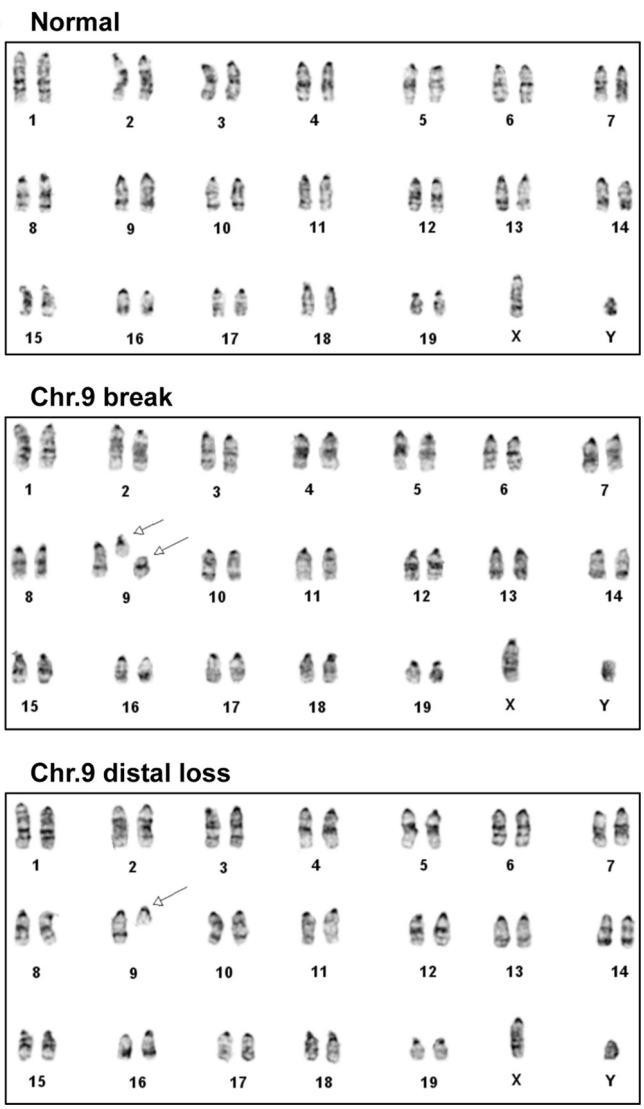

FIGURE 2—*continued*

cell-specific *mb1Cre* knock-in allele (15) resulted in the appearance of hCD2$^-$ cells in the gated B cell fraction but not in the T cell fraction (Fig. 2*c*). A small fraction of EGFP$^+$hCD2$^-$ cells were also detected in T and B cells in the presence of *LckCre* and *mb1Cre*, respectively (supplemental Fig. S3). High-level expression of the GFP marker in these cells is consistent with the idea of formation of the dicentric chromosomal fragment.

Detection of ISCR-induced Chromosomal Breaks—To further verify that loss of hCD2 expression is indeed due to ISCR, we performed karyotyping analysis with bone marrow cells isolated from the *pbch9* and *mb1Cre* double-positive mice. Chromosomal break or fragment loss involving chromosome 9 was found at a frequency of 9–19% of mitotic cells in four *pbch9;mb1Cre* mice analyzed (Fig. 2*d*). This result demonstrates that Cre-mediated recombination is capable of inducing efficient ISCR via a predetermined chromosomal site.

Effect of ISCR on Proliferation—We further tested the effect of ISCR on the proliferation ability of daughter cells. Because most mature lymphocytes are in a quiescent state under non-immunizing conditions, we analyzed the proliferation capacity of developing B cells from bone marrow in an *ex vivo* culture system. Bone marrow cells from *pbch9;mb1Cre* and control mice were labeled with CFSE, sorted into hCD2$^+$ and hCD2$^-$ B lineage fractions by FACS, and then cultured on the S17 stromal layer in the presence of the proliferation-stimulating cytokine interleukin-7 (16). In this culture system, S17 is sufficient to support proliferation of developing B cells, although exogenously added interleukin-7 can further stimulate cell proliferation (Fig. 3). Similar to what was observed in 3T3 cells, we found that both CD2$^+$ and CD2$^-$ B lineage cells isolated from bone marrow of *pbch9;mb1Cre* mice were capable of proliferation under these culture conditions. Most of the hCD2$^-$ cells had gone through up to seven rounds of cell division based on CFSE dilution after 4 days in culture. A substantial fraction of the hCD2$^+$ cells and the *Cre*-negative *pbch9* controls had completed a few more cell cycles in comparison with hCD2$^-$ cells in the window of analysis. As expected, the hCD2$^+$ cells continuously gave rise to hCD2$^-$ cells under these proliferative conditions (Fig. 3*b*, *right panel*).

Induction of ISCR Involving Different Chromosomes—The use of the transposon vector allowed us to generate multiple recombinant chromosomes. Four additional transgenic lines were established with the transposon vector mapped to different chromosomes (supplemental Fig. S4*a*). We tested these strains by crossing them with *LckCre* mice for chromosome deletion in the T cell lineage. Conversion from hCD2$^+$ cells to hCD2$^-$ cells was observed in all cases in the presence of *LckCre*, although the frequency varied among individual recombinant chromosomes (supplemental Fig. S4*b*). The ability to detect a substantial amount of live hCD2$^-$ cells involving five different chromosomes suggests that Cre-mediated ISCR and subsequent chromosomal loss may be used as a generic method for LOH assay in the lymphoid system.

Cre-mediated ISCR Can Be Detected Throughout Lymphocyte Development—We predict that ISCR represents only a fraction of the mitotic recombination events. Cre-mediated recombination may also lead to reciprocal sister chromatid exchange, which allows development to proceed to the next step prior to any genetic alteration. However, when these cells re-enter the cell cycle in a subsequent developmental window, they can give rise to new ISCR products at this later developmental stage. We followed the ISCR during B cell development in the mouse strain carrying the *pbch10* allele (Fig. 4*a*) and the *mb1Cre* transgene. B cell development in the bone marrow can be phenotypically separated into sequential prepro-B, pro-B, pre-B, and B cell stages based on sequential up-regulation of CD19 and down-regulation of CD43 in combination with the pan-B lineage marker B220 (17, 18). The CD43$^+$CD19$^-$B220$^+$ prepro-B cells are thought to be the immediate precursors of B lineage cells, which do not express the B lineage-specific *mb1* gene. CD19 expression marks the entry into the B cell lineage. The CD43$^+$CD19$^+$B220$^+$ pro-B cells express *mb1* and thus are subject to *mb1Cre*-mediated ISCR. CD43 down-regulation correlates with transition into the pre-B cell stage, and surface IgM expression indicates the completion of V(D)J recombination and successful generation of B cells. The *mb1Cre* knock-in allele supports Cre expression throughout B cell development (15). Consistent with the pattern of *mb1* expression, we found

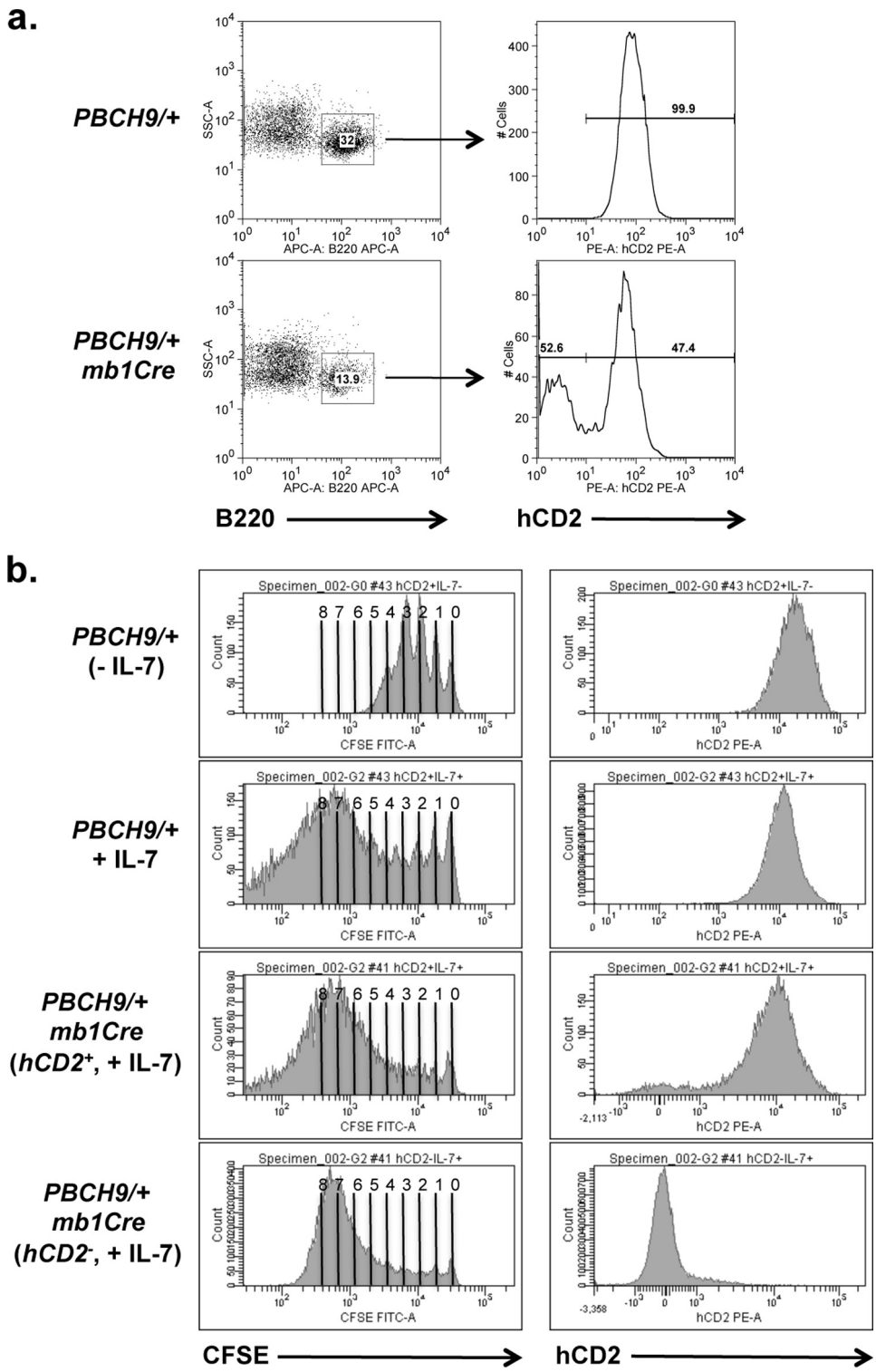

FIGURE 3. *a*, sorting criteria for bone marrow B cells used for CFSE staining. Cells were pre-gated on 7AAD$^-$ and B220$^+$ populations. hCD2$^+$ cells were sorted from a Cre$^-$ mouse, and both hCD2$^+$ and hCD2$^-$ cells were sorted from the same Cre$^+$ mouse. *b*, CFSE analysis of cell proliferation. Cells were pre-gated on 7AAD$^-$B220$^+$CD19$^+$ populations and checked for CFSE dilution and hCD2 expression after 3 days in culture. Data are representative of two independent experiments. *FITC-A*, fluorescein isothiocyanate area; *IL-7*, interleukin-7; 7AAD, 7-amino-actinomycin D; *SSC*, side scatter; *APC*, allophycocyanin; *PE-A*, phycoerythrin area.

that hCD2$^-$ cells were detected in both developing B cells in the bone marrow and mature B cells in the spleen (Fig. 4*b*). A substantial fraction of hCD2$^-$ cells expressed EGFP, suggesting their retention of the dicentric chromosomal fragment (supplemental Fig. S5). Analysis of total cell numbers showed a significant reduction of cell number at each developmental stage in the presence of *mb1Cre* (supplemental Fig. S6, *a* and *b*). Annexin V staining of bone marrow B cells from *mb1Cre* mice detected an increase of apoptotic cells in the hCD2$^+$ fraction but not in the hCD2$^-$ fraction (supplemental Fig. S6*c*). Therefore, the drop in total cell numbers could be partially explained by an increase in cell death associated with ISCR.

LOH Analysis of E2A Function in B Cell Development—The existence of live hCD2-negative cells at each stage of B cell development permits the system to be used in LOH analysis. Although these hCD2-negative cells are not the same as wild-type cells, the unique genetic background can help reveal recessive phenotypes of any mutation present on the remaining haploid chromosome. We chose the *E2A* gene located on chromosome 10 for such a LOH test. The E2A transcription factor is known to play essential roles throughout the early phase of B lymphocyte development (19, 20). *E2A* germ line knock-out demonstrated a complete block of B cell development at the prepro-B stage. Using an $E2A^{flox}$ allele, we determined that this developmental block could be induced by *mb1Cre*-mediated *E2A* conditional knock-out (supplemental Fig. S7). We performed a LOH test by crossing an $E2A^{flox}$ allele with the *pbch10* transgenic line. Both the hCD2 marker and *E2A* were located on the distal end of the chromosome relative to the iloxP recombination site (Fig. 4*a*). Therefore, the hCD2 marker is expected to be co-segregated with *E2A* upon *Cre*-induced ISCR. We compared *mb1Cre*-mediated ISCR between mice carrying either a wild-type or an $E2A^{flox}$ allele on the background of *pbch10*. The numbers of bone marrow hCD2$^-$ cells were dramatically reduced when the wild-type allele was replaced with the $E2A^{flox}$ allele. Quantitative analysis revealed that both the total (Fig. 4*c*) and hCD2$^-$ (Fig. 4*d*) fractions of B lineage cells in the bone marrow were reduced when the wild-type *E2A* allele was replaced with the

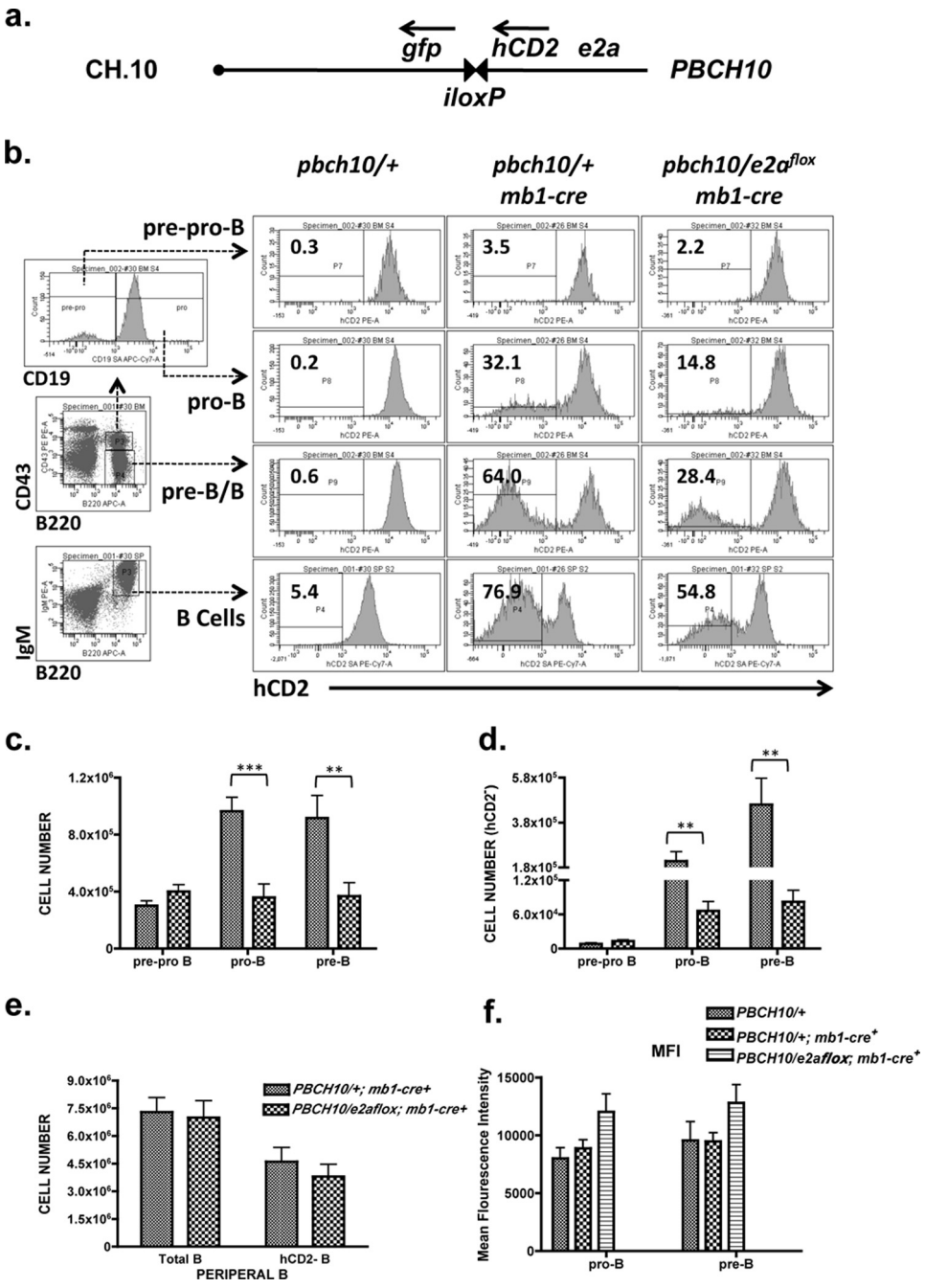

FIGURE 4. *a*, diagram of the *pbch10* allele. The EGFP-iloxP-hCD2 cassette was integrated at ~3 megabases upstream of the *E2A* gene on chromosome 10 (*CH.10*). hCD2 and E2A are both on the distal side of the iloxP site. *b*, hCD2 expression in B cells from different stages of development in the bone marrow and spleen of the indicated genotypes. A sequential gating of bone marrow cells from *pbch10* mice separated the B cell population into prepro-B ($B220^+CD43^+CD19^-$), pro-B ($B220^+CD43^+CD19^+$), and a combination of pre-B and B ($B220^+CD43^-$) cells. B cells are defined as $B220^+IgM^+$ cells isolated from the spleen. These gating criteria were used to analyze hCD2 expression in the three genotype groups shown on the *right*. Histograms of hCD2 expression in B cell subsets are shown, with the *vertical line* indicating gating criteria to separate $hCD2^+$ and $hCD2^-$ cells. The relative percentage of the $hCD2^-$ fraction is indicated in the plot. *SA APC-Cy7-A*, streptavidin-allophycocyanin-cyanine 7 area; *PE-A*, phycoerythrin area. *c*, total cell counts of B cell subsets in the bone marrow of *pbch10;mb1Cre* mice (*fine dot bars*, $n = 6$) and *pbch10/E2A^{flox};mb1Cre* mice (*coarse dot bars*, $n = 8$). *d*, cell counts of the $hCD2^-$ population in the corresponding groups in *c*. *e*, total cell counts and $hCD2^-$ cell counts of splenic B cells using the same mice analyzed in *b–d*. *f*, mean fluorescent intensity (*MFI*) of $hCD2^+$ fractions in pro-B and pre-B/B cells of the indicated genotypes. **, $p < 0.01$; ***, $p < 0.001$.

E2A^{flox} allele. The *E2A^{flox}* allele had a minimal negative effect on the numbers of both $hCD2^+$ and $hCD2^-$ splenic B cells (Fig. 4*e*). The significant loss of developing B cells in the bone marrow of *pbch10/E2A^{flox};mb1Cre* mice is consistent with the idea that both the *E2A^{flox}* allele on the non-recombinant chromosome and the wild-type *E2A* allele on the recombinant chromosome are compromised by *mb1Cre*-induced recombination. Similar results were obtained when a *E2A*-null germ line mutation, instead of the *E2A^{flox}* allele, was used in the LOH test (supplemental Fig. S8).

The level of hCD2 expression in $hCD2^+$ cells can reflect copy numbers of the hCD2 marker. If the acentric chromosomal fragment were retained after ISCR, we would expect to see an increase in the mean fluorescent intensity of hCD2. However, hCD2 expression was comparable between *pbch10/+* and *pbch10/+;mb1Cre* mice, indicating insignificant contributions of cells carrying the acentric chromosomal fragment. In contrast, we observed a 25–30% increase in the mean fluorescent intensity for the remaining $hCD2^+$ cells after replacing the wild-type allele with the *E2A^{flox}* allele (Fig. 4*f*). This result indicates that cells carrying the acentric chromosome are preferentially retained in *pbch10/E2A^{flox};mb1Cre* mice.

LOH Analysis of HEB Function in T Cell Development—To further evaluate the utility of ISCR in LOH analysis, we next examined a previously established *Heb* gene on chromosome 9 against *pbch9*-induced deletion. The *Heb* locus is located on the distal side of the *pbch9* iloxP site on chromosome 9. HEB is a structural and functional homolog of E2A. In contrast to B cell development, where E2A plays a predominant role, T cell development is regulated by the combined dosage of E2A and HEB (21). Thus, conditional knock-out of both *E2A* and *Heb* genes is required to induce severe developmental defects in the T cell lineage (22, 23). It has been shown that *LckCre*-mediated conditional knock-out of *E2A* and *Heb* at the double-negative (DN; $CD4^-CD8^-$) stage of thymocyte development blocks further development of the DN cells into the double-positive (DP; $CD4^+CD8^+$) stage (22). Therefore, DN-to-DP transition in thymocyte development can be used as a phenotypic assay for HEB function. To perform the LOH test

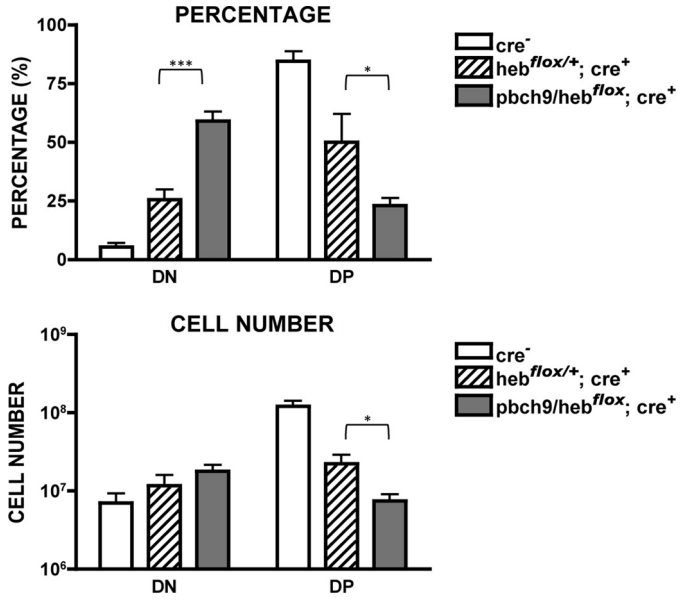

FIGURE 5. **LOH test of the *Heb* locus with the *pbch9* chromosome.** The bar graphs summarize the percentage (*upper graph*) and absolute numbers (*lower graph*) of DN and DP thymocytes in the thymus. Total thymocytes were analyzed by co-staining with the CD4 and CD8 markers, which separate cells into DN, DP, and CD4 or CD8 single-positive fractions. Three groups of mice 1–2 months old were included in this analysis. cre^-, *LckCre* transgene-negative controls ($n = 5$); $heb^{flox/+}$;cre^+, $Heb^{flox/+}$;$E2A^{flox/flox}$;*LckCre* mice ($n = 4$); *pbch9/*heb^{flox};cre^+, *pbch9/*Heb^{flox};$E2A^{flox/flox}$;*LckCre* mice ($n = 5$). *, $p < 0.05$; ***, $p < 0.001$.

under similar conditions, we used the same conditional knockout system to produce heterozygous *Heb* on an *E2A*-deficient background ($Heb^{flox/+}$;$E2A^{flox/flox}$;*LckCre*). These mice exhibited a partial block in the development from the DN to DP stage in comparison with the *Cre*-negative controls. This was evident by a reduction in both the percentage and numbers of DP cells (Fig. 5, compare Cre^- with $Heb^{flox/+}$;Cre^+ *samples*) and a proportional increase in DN cells in the thymus. Replacement of the wild-type *Heb* allele with the *pbch9* chromosome (*pbch9/*Heb^{flox};$E2A^{flox/flox}$;*LckCre*) resulted in a further loss of DP cells and a proportional increase in DN cells in the thymus. Deletion of the *pbch9* chromosome was confirmed by the appearance of hCD2-negative cells (data not shown). Thus, ISCR-mediated removal of the wild-type chromosome on an *Heb* heterozygous background induced a phenotype consistent with the loss of HEB function. This result indicates that *pbch9* can be used as a genetic tool in LOH assay involving the *Heb* locus.

DISCUSSION

Cre-mediated chromosomal loss in mice was first described 1 decade ago. Male mice with a pair of inverted loxP sites targeted on the Y chromosome produced very few male pups compared with female pups when mated with Cre-expressing females, indicating that the Y chromosome was eliminated (13). Recently, the same model was tested for chromosome 2 in limb development. Chromosome 2 was deleted in Cre-positive proliferating cells. The recombination event appeared to be cytotoxic in the specific tissue analyzed (24). The same model has also been successfully utilized in chromosome deletion in embryonic stem cells (25–27). It was not known whether these detrimental effects apply to all adult tissue types and chromosomes. Prior to the study described here, the utility of chromosome deletion in lymphoid system had not been tested.

We reasoned that the lymphoid system might be particularly suitable for mitotic recombination studies because of the availability of a large number of mitotic events throughout life. Our study demonstrated that Cre-mediated ISCR can induce efficient chromosomal loss at various stages of lymphocyte development. Chromosomal lesions during mitosis are thought to trigger cell cycle arrest at the mitotic checkpoint (28). In our test involving five individual chromosomes, viable lymphocytes were detected in each case, indicating that a fraction of cells after ISCR can still exit mitosis and remain viable. The result is in contrast to the previous reports and clearly demonstrates that chromosome deletion or fragmentation does not necessarily lead to complete cell cycle arrest and cell lethality at least in the lymphoid system. In general, we saw a reduction rather than an elimination of cells after ISCR in developing lymphocytes. The viability after ISCR could be due to a unique feature of lymphocytes, the use of the iloxP cassette, or the chromosomes used in our test. Although factors influencing cell survival after ISCR still require further investigation, the appearance of these partially haploid cells provides windows of opportunities for phenotypic analysis of gene functions.

The ISCR test could be extended to other chromosomes by moving the recombination cassette to different genomic locations. The use of the piggyBac transposon vector allows easy mobilization of the recombination cassette by transposase-mediated transpositions in the germ line (11). This will permit fast generation of a panel of mouse strains with the iloxP cassette inserted into each chromosome at varying positions. It remains possible that deletion of certain chromosomes may lead to complete cell death in lymphocytes. A genome scan with the ISCR cassette may help uncover chromosome regions that exhibit haploid insufficiency. This type of analysis alone should enrich our knowledge of genome function in different tissue types when the tissue-specific *Cre* transgene is used in the assay.

The LOH tests with the *E2A* mutation in B cell development and the *Heb* mutation in T cell development provide proofs of principle for general application of this recombination technique in the mouse. Several unique features need to be emphasized in regard to the application of ISCR for the LOH test. First, each ISCR event may produce daughter cells with different compositions of the recombinant chromosome. Although useful for scoring the recombination events, the markers used in our assay may not be sufficient to indicate the status of the entire chromosomal arm if additional recombination or translocation events occur involving the fragmented chromosome. However, these random genetic alterations should not alter the outcome of LOH studies in any significant way unless these events lead to clonal expansion. Second, a fraction of cells apparently do not go through ISCR at each cell cycle and therefore can serve as substrates for ISCR at a later stage of development. This feature allows *mb1Cre* to induce ISCR events throughout B cell development. A continuing induction of ISCR with *LckCre* was also observed in T cell development (data not shown). Thus, the effect of chromosome deletion on cell survival can be evaluated throughout lymphocyte development.

Third, we emphasize that chromosome deletion creates a special experimental condition in which gene function on the homologous chromosome can be easily evaluated. In the LOH test, the base-line control is from the ISCR test against a single copy of the wild-type chromosome. This base line is clearly different from that in the wild-type cells and may allow only limited assessment of the physiological features of the cells subjected to this test. For general application of the LOH assay, it is critical to evaluate and establish the base-line features for each ISCR chromosome at each developmental stage. Once these base-line features are established with the genetically marked populations, one can use the system to carry out LOH testing of relevant mutations provided by the homologous chromosome.

Our study of the *E2A* locus indicated the possibility that the ISCR system could detect cells carrying from 0 to three copies of the wild-type allele of the test gene. B cell development is known to be highly sensitive to *E2A* gene dosage. It is not surprising that cells retaining three copies of the wild-type *E2A* allele are selected against. The preferential retention of cells carrying two wild-type *E2A* alleles and one mutant *E2A* allele suggests that *E2A* may be the only gene on this chromosomal fragment particularly sensitive to triploidy. This result also suggests that certain chromosome regions may be more tolerable than others to alteration in copy numbers. Indeed, we found that the percentage of hCD2-negative cells varied in a test of five recombinant chromosomes. It will be interesting to further investigate this phenomenon by testing ISCR in different cell lineage. A severe loss of $hCD2^-$ cells in one cell type but not the others would imply lineage-specific functions associated with the chromosomal fragment. This type of analysis may be further refined by moving the recombination cassette to varying distance relative to the telomere in the test chromosome.

Perhaps the most significant application of the ISCR method established in this study would be a general LOH survey of lymphoid phenotypes of pre-existing germ line mutations. Large-scale gene knock-out (29) and insertional mutagenesis projects (30, 31) are currently under way and have produced a large quantity of germ line mutations. In contrast to other existing methods for evaluation of recessive phenotypes in adult tissues, ISCR can be performed with one generation of crossing. Thus, the ISCR method described here provides an alternative method of a cost-effective primary screen of germ line mutations for lymphoid phenotypes. It remains to be further tested whether the ISCR approach is generally applicable to other tissue types in which cell proliferation continues in adult life.

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