Chromosomal manipulation by site-specific recombinases and fluorescent protein-based vectors

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Modern genetic engineering depends on DNA-modifying enzymes including restriction endonucleases, ligases and polymerases. This technology has been applied to manipulation of purified DNA less than a few hundred kilobases. Broadening the range of manipulatable DNA to megabase scale would be fundamental to deepen the understanding of gene regulation in the chromosomal context. Here we developed a Cre-mediated chromosomal recombination system using fluorescent proteins and various site-specific recombinases. These techniques enabled quick construction of targeting vectors, easy identification of chromosome-rearranged cells, and rearrangement leaving minimum artificial elements at junctions. Applying this system to a human cell line, we successfully recapitulated two types of pathogenic chromosomal translocations in human diseases: MYC/IgH and BCR/ABL1. By inducing recombination between two loxP sites targeted into the same chromosome, we could mark cells harboring deletion or duplication of the inter-*loxP* segments with different colors of fluorescence. In addition, we demonstrated that the intrachromosomal recombination frequency is inversely proportional to the distance between two recombination sites, implicating a future application of this frequency as a proximity sensor. Our method of chromosomal manipulation can be employed for any cell type, including ES cells, in which gene targeting is possible. Experimental use of this system would open up new horizons in genome biology, including the establishment of cellular and animal models of diseases caused by translocations and copy-number variations.

INTRODUCTION

Feasibility of chromosomal manipulation in mammalian cells was first reported 14 years ago. Although this technique is useful for precise understanding of gene regulation in the chromosomal context, a limited number of laboratories have used it in actual practice because of associated technical difficulties. These studies relied exclusively on the selection of cells expressing hypoxanthine phosphoribosyltransferase (HPRT) as an indicator of recombination, and therefore, the use of HPRT-deficient cells was a prerequisite. In this study, we introduced two improvements to this technology. First, we utilized fluorescent proteins as rearrangement markers to broaden the range of cells this technology can be applied to. Second, we adopted Gateway cloning system to facilitate the otherwise cumbersome construction of targeting vectors.

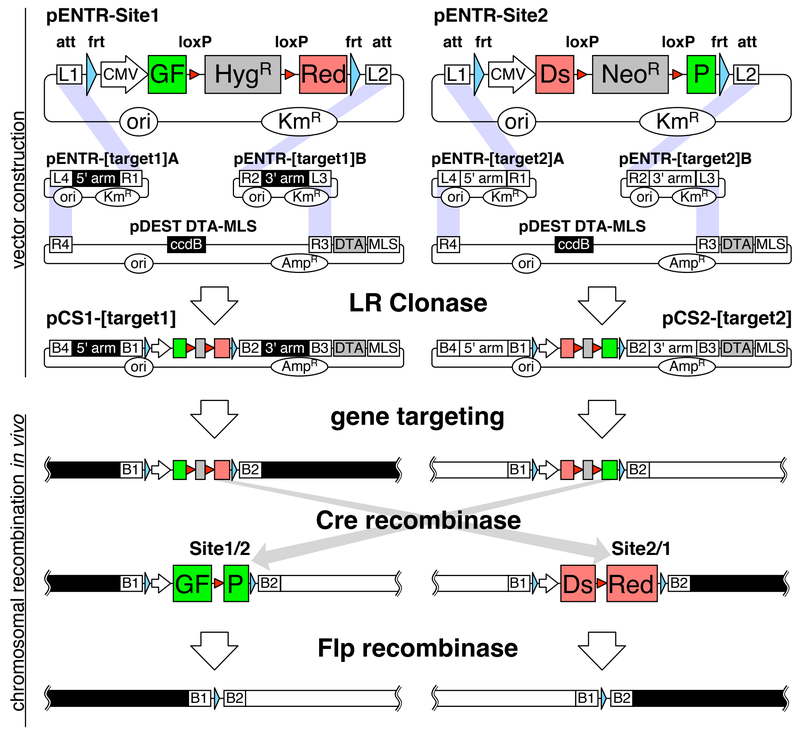

Figure 1. Scheme for vector construction and chromosomal recombination

(upper) pENTR-Site1 plasmid contains cytomegalovirus (CMV) promoter-driven fusion gene, the first half consisting of GFP (green box) and the last half of dimer2 (a DsRed variant, red box) with an intervening hygromycin-resistance gene (grey box) flanked by *loxP* sites (red triangles), outer two *frt* sites (blue triangles) and *att* L1 and L2 sites (open box). pENTR-Site2 expresses a fusion gene complementary to pENTR-Site1 harboring a neomycin-resistance gene (grey box). These vectors in addition to 5'- and 3'-targeting homology arm vectors and the pDEST DTA-MLS destination vector were assembled into targeting vectors by LR Clonase. Purple shadows connect *att* sites to be recombined. (lower) After gene targeting into homologous chromosomal regions, Cre first removes drug-resistance genes and then recombines distant *loxP* sites. After recombination, GFP and dimer2 mRNAs are spliced and expressed. Expression of Flp excises fluorescent protein genes to achieve clean rearrangement, leaving a 103-bp element. DTA, diphtheria toxin A; MLS, multiple linearization sites (PmeI, AscI, I-SceI, SwaI, PacI); ori, replication origin; Kmᴿ, kanamycin-resistance gene; Ampᴿ, ampicillin-resistance gene; ccdB, bacterial *ccdB* gene.

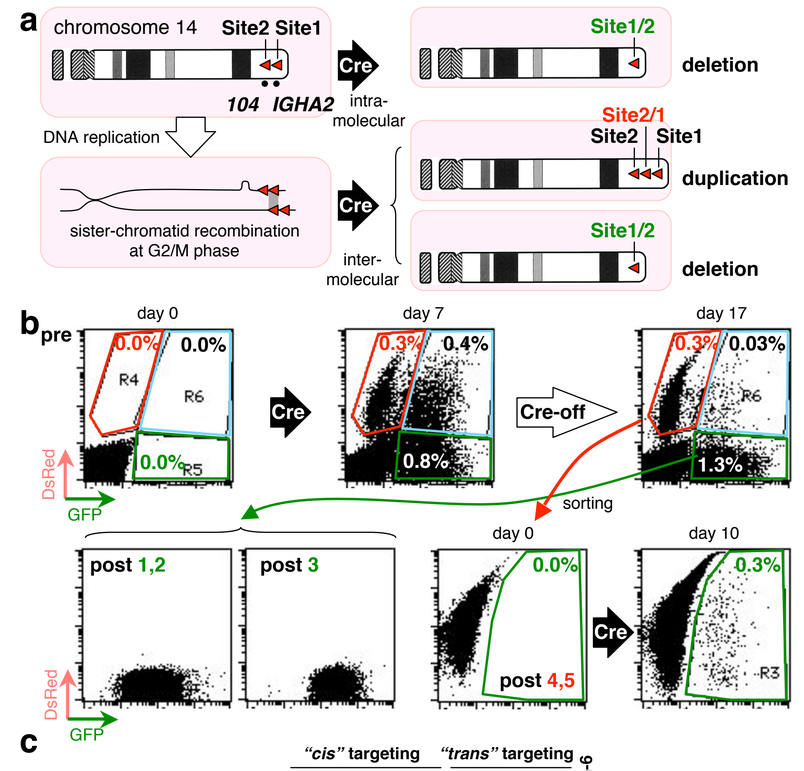

Figure 3. Induction of copy-number variation

a, Possible outcome of Cre-mediated recombination between two *loxP* sites with an identical orientation in the same chromosome 14. Site1 was targeted to the *IGHA2* locus within the IgH gene cluster. Site2 was targeted to a region tentatively designated *104*, 1.1 Mb centromeric to the *IGHA2* loci. b, Flow cytometric profiles of double-targeted cells, Cre-activated cells, and Cre-terminated cells kept OHT-free for 10 days (top). Flow cytometric profiles of sorted cell clones and a DsRed-positive clone 10 days after Cre activation (bottom). c, PCR confirmation of rearrangement for clones from cis- and trans-targeting cells. Clone numbers from 1 to 5 correspond to rearranged clone numbers (post) in b. Clones 6 to 9 are derived from trans-targeted cells. Cells before Cre activation (pre), Nalm-6 and the no-template control (H_2O) are included. Triangles above the transgene schemes represent primer positions.

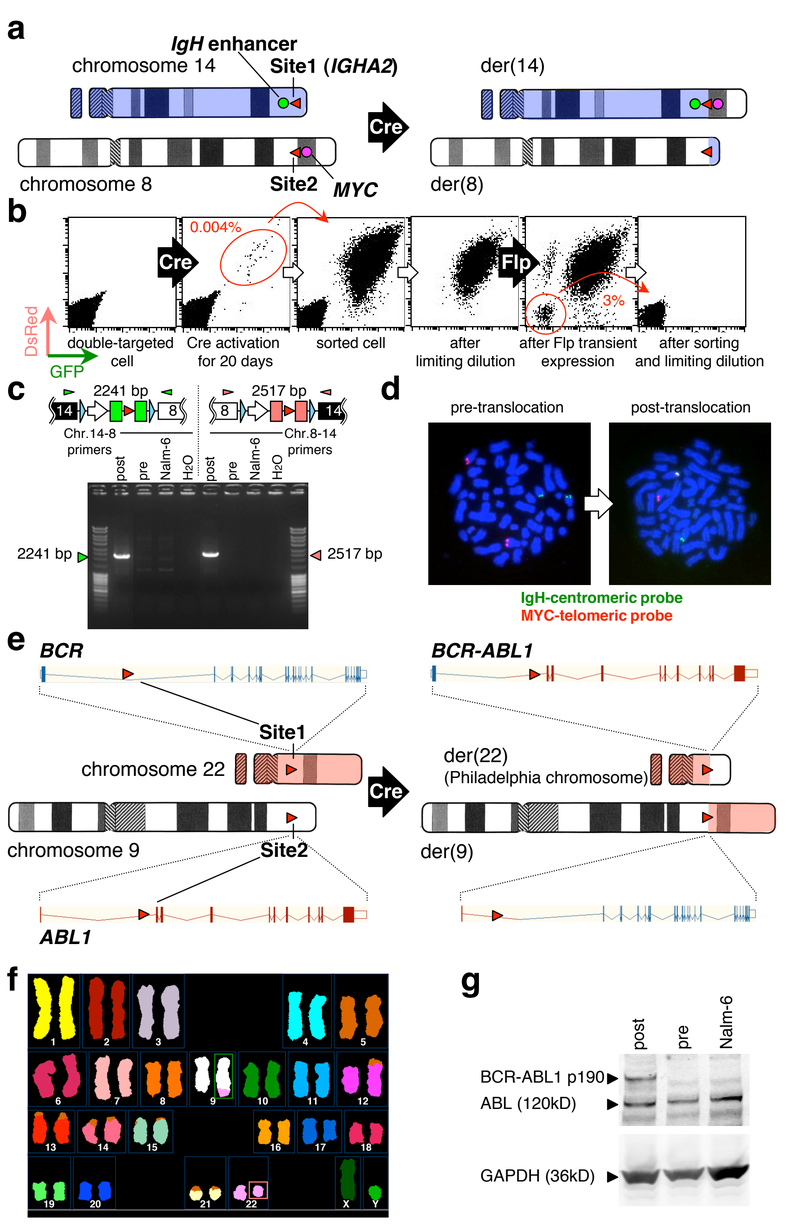

Figure 2. Recapitulation of *MYC/IgH* and *BCR/ABL1* translocations

a, Site1 vector (red triangle) was targeted to the *IGHA2* locus of Nalm-6 cells expressing tamoxifen-regulated Cre. Site2 vector was subsequently targeted to the *MYC* locus (red circle). The IgH enhancer is indicated by a green circle. b, Flow cytometric profiles of the indicated stages. Red circles indicate the sorted cell populations. c, Confirmation of translocation by PCR. PCR products of expected sizes were observed for cells after translocation (post) but not for cells before Cre expression (pre). H_2O lanes show the no-template controls. Triangles above the transgene schemes represent primer positions. d, FISH analysis demonstrating *MYC/IgH* translocation for cells after cleanup by Flp, using BAC probes. e, Site1 and Site2 vectors containing *loxP* sites (red rectangle) were targeted into the first intron of the *BCR* gene and the *ABL1* gene, respectively. Vertical bars and boxes connected with v-shaped lines indicate exon–intron structure of genes. The derivative chromosome 22 [der(22)] recapitulates the Philadelphia (Ph) chromosome. f, SKY analysis of cells with artificial *BCR/ABL1* translocation. The derivative chromosome 9 [der(9)] in the green box contains the material (pink) translocated from chromosome 22. The amount of chromosome 9 material that was translocated to chromosome 22 was too small to be resolved by this SKY analysis (Ph chromosome, pink box). The previously reported translocation between chromosomes 5 and 12 in Nalm-6 cells could be also detected. g, Western blot confirming expression of the BCR-ABL1 fusion protein (190 kD) after clean translocation (post) with constitutive expression of ABL and GAPDH proteins.

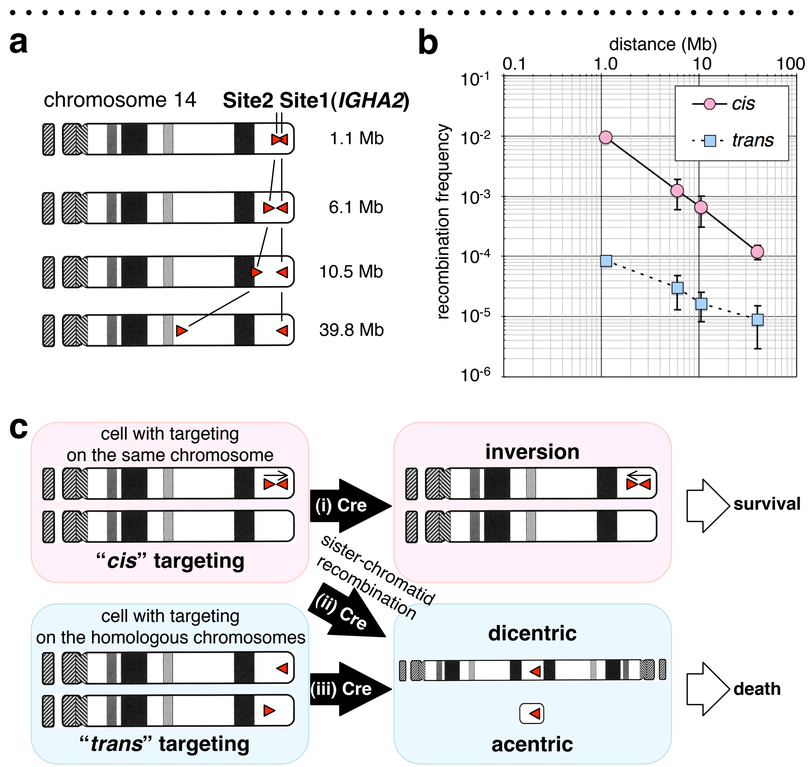

Figure 4. Application to proximity sensor

a, Scheme of chromosome 14 after cis targeting with the indicated Site1–Site2 intervals. b, Plots of physical distance in the chromosome on the x axis and frequency of recombination on the y axis for *cis*- and *trans*-targeted cells. c, Outcome of inter-loxP recombination in *cis*- and *trans*-targeted cells.

CONCLUDING REMARKS

In this study, we demonstrated the use of our novel system in studying artificial chromosomal translocation, duplication, deletion, and inversion as well as in determining gene proximity in a human cell line. At the same time, this system may be useful for screening of recessive mutations and induction of specific chromosomal loss in cell lines or animal models. The technical improvements reported here will facilitate mammalian chromosome engineering and better understanding of human diseases caused by chromosomal translocations and copy-number variations.