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Manipulating the Mouse Genome to Engineer Precise Functional Syntenic Replacements with Human Sequence

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SUMMARY

We have devised a strategy (called recombinase-mediated genomic replacement, RMGR) to allow the replacement of large segments (>100 kb) of the mouse genome with the equivalent human syntenic region. The technique involves modifying a mouse ES cell chromosome and a human BAC by inserting heterotypic *lox* sites to flank the proposed exchange interval and then using Cre recombinase to achieve segmental exchange. We have demonstrated the feasibility of this approach by replacing the mouse α globin regulatory domain with the human syntenic region and generating homozygous mice that produce only human α globin chains. Furthermore, modified ES cells can be used iteratively for functional studies, and here, as an example, we have used RMGR to produce an accurate mouse model of human α thalassemia. RMGR has general applicability and will overcome limitations inherent in current transgenic technology when studying the expression of human genes and modeling human genetic diseases.

INTRODUCTION

Studies of how human genes are normally regulated and the effects of mutations that cause human genetic disease are limited by the availability of appropriate primary cells and tissues and the necessary constraints on experimental interventions. Consequently, over the past 20 years, transgenic mice have become important experimental models to understand how human genes are regulated.

Small transgenes, however, often do not contain all of the *cis*-acting elements required for fully regulated expression since such sequences may be found tens or even hundreds of kilobases from the gene in question (Kleinjan and van Heyningen, 2005). Even when large transgenes derived from PACs, BACs or YACs are used, they are frequently rearranged; it is often very difficult to fully analyze their structural integrity and copy number (Peterson et al., 1998). Furthermore, their expression is often influenced by their position of integration in the genome (Alami et al., 2000; Kaufman et al., 1999). Making and characterizing directed mutations for structure/function studies is also difficult when using such large molecules. Even when successful, the interpretation of these transgenic experiments is complicated by the fact that the endogenous mouse genes are still present unless bred against mice in which they have been deleted or inactivated by homologous recombination.

A complementary approach is to identify the mouse ortholog and alter this gene or its regulatory elements by homologous recombination using conventional gene-targeting methods (Nagy, 2003). Clearly, mutating a single gene at its normal chromosomal locus avoids many of the problems associated with transgenes (copy number differences, complex mapping problems, position effects), and, in this process, the normal endogenous gene does not remain intact to complicate the analysis. However, there is increasing evidence demonstrating considerable differences in the ways in which orthologous human and mouse genes are normally regulated. In many instances, mutations known to cause disease in human do not mimic the phenotype when introduced into the mouse ortholog (Colledge et al., 1995; Engle et al., 1996; Garrick et al., 2006). These include the α globin locus, where deletion of the major regulatory element causes a much milder condition in mouse than in human (Anguita et al., 2002). Therefore, although mouse models have provided many insights into human genetic disease, it is becoming increasingly clear that they are also beset by inherent problems and difficulties in interpretation resulting from differences in basic biological processes that have evolved over the 70 million years of evolution that separate humans and mice.

Clearly, the next step in developing mouse models of human disease is to replace large segments (100–1000s kb) of the mouse genome with the wild-type or mutated syntenic region of the human sequence, thereby including all remote regulatory elements and inserting the human segment as a single copy at a natural chromosomal position for the genes contained within it. A range of chromosome-engineering technologies are now available that, combined in a novel way, could make this feasible (Yu and Bradley, 2001; Copeland et al., 2001; Muyrers et al., 2001; Testa et al., 2003; Valenzuela et al., 2003; Yang and Seed, 2003), including application of various heterotypic site-specific recombination systems (Cre, FLP, ϕC31) to achieve efficient exchange of sequence cassettes at targeted chromosomal loci, a process known as recombinase-mediated cassette exchange (RMCE) (Baer and Bode, 2001).

In this study, we have combined various aspects of these technologies to develop what we believe to be a new, general strategy for replacing large segments of the mouse genome with wild-type or mutated syntenic regions of the human genome. We refer to this process as recombinase-mediated genomic replacement (RMGR). This allows precise, reproducible, genetically selectable genomic replacement on a scale not previously attained and should be generally applicable to large-scale modification of the mouse genome. We have established a "proof of principle" for its application in generating "humanized" mice by replacing the syntenic region encompassing the mouse α globin regulatory domain (the region containing all *cis*-acting sequences required for fully regulated expression of the α globin genes) with human BAC-derived sequence in ES cells and subsequently deriving viable mice homozygous for this syntenic substitution producing only human α-globin chains. In addition, we have demonstrated the potential of this approach for making authentic mouse models of human disease by replacing the mouse region with a recombineered human BAC in which the α globin major regulatory element has been deleted from the α globin regulatory domain. This created a model of α thalassemia that faithfully mimics the phenotype of patients with this common human genetic disease.

RESULTS

Genomic Replacement Strategy for Substitution of the α Globin Gene Domain

We set out to exchange an extensive part of the mouse genome with its human equivalent in a region where the consequences could be clearly analyzed. We chose the region encompassing the α globin gene cluster since this has been fully characterized both structurally (Flint et al., 2001; Hughes et al., 2005; Kielman et al., 1996) and functionally (Anguita et al., 2001, 2002, 2004) in both mouse and man. The gene order and transcriptional orientation of the globin genes and other widely expressed genes flanking them in this segment of the genome are highly conserved (Figure 1); a multispecies DNA comparison shows that the region of most conserved synteny extends from the *DIST* gene (a rhomboid-like gene also known as *c16orf8*, gene 5 in Figure 1) to the θ globin gene (Hughes et al., 2005). This region contains all known multispecies conserved regulatory elements (MCS-Rs), the corresponding erythroid-specific DNase1 hypersensitive sites and well-defined segments of chromatin that become acetylated specifically in erythroid cells (Anguita et al., 2001, 2002; Higgs et al., 1998). This region is therefore thought to encompass the entire α globin regulatory region (Figure 1).

In outline, the plan involved insertion of noninteracting (heterospecific) *lox* recombination sites (*loxP* and *lox511*) into positions (in the *Dist* and θ genes, respectively) flanking the α globin regulatory domain in the mouse ES cell chromosome and in the corresponding regions of a BAC (344L6) that spans the entire ($\sim$120 kb) human α globin region. In the presence of Cre recombinase, reciprocal exchange of sequence between the BAC and mouse chromosome should occur since only the compatible *loxP*/*loxP* and *lox511*/*lox511* sites will recombine (Bethke and Sauer, 1997) and thus result in replacement of the entire mouse regulatory region in the ES cell genome with the corresponding human region (Figure 1).

We chose the fourth intron of the *DIST* gene to insert the *loxP* sites (upstream exchange points) in both mouse (ES cells) and human (BAC) sequences and the second intron of the 3′ θ gene in mouse (ES cells) and the corresponding region of the second intron of the single human θ gene (BAC) (Figures 1–3) to insert the *lox511* sites (downstream exchange points). When recombination is activated, this should result in the exchange of 117 kb of human sequence for the equivalent 85 kb of mouse sequence, creating a hybrid human-mouse *DIST* gene upstream and a hybrid human-mouse θ gene downstream in the recombinant mouse chromosome.

Targeted Modifications of the α Globin Regulatory Domain in Mouse ES Cells

The strategy required the sequential insertion of a *loxP* site and then a *lox511* site (in inverted orientation) into the upstream and downstream positions of the mouse α globin cluster by conventional homologous recombination (Mansour et al., 1988). The vectors designed to achieve this also integrated genetic markers directly linked to the *loxP* and *lox511* sites to enable selection of the chromosome replacement in the second stage. Positive genetic selection for *loxP* × *loxP* recombination involved reconstruction of a functional hypoxanthinephosphoribosyltransferase (*Hprt*) minigene from defective 5′ and 3′ components (Smith et al., 1995), and negative selection for *lox511* × *lox511* recombination involved the use of HSV thymidine kinase (*tk*) genes.

For the mouse *Dist* gene, a targeting vector containing a *frt*/I-*Sce*I/*Hprt*$^{-\Delta 3'}$/*loxP*/MC1*neo*/*loxP* cassette (Figure 2A) was electroporated into HPRT-deficient (HPRT$^-$) E14-TG2a.IV ES cells, and correctly targeted clones were obtained (Figure 2B). One of these *Dist*-targeted

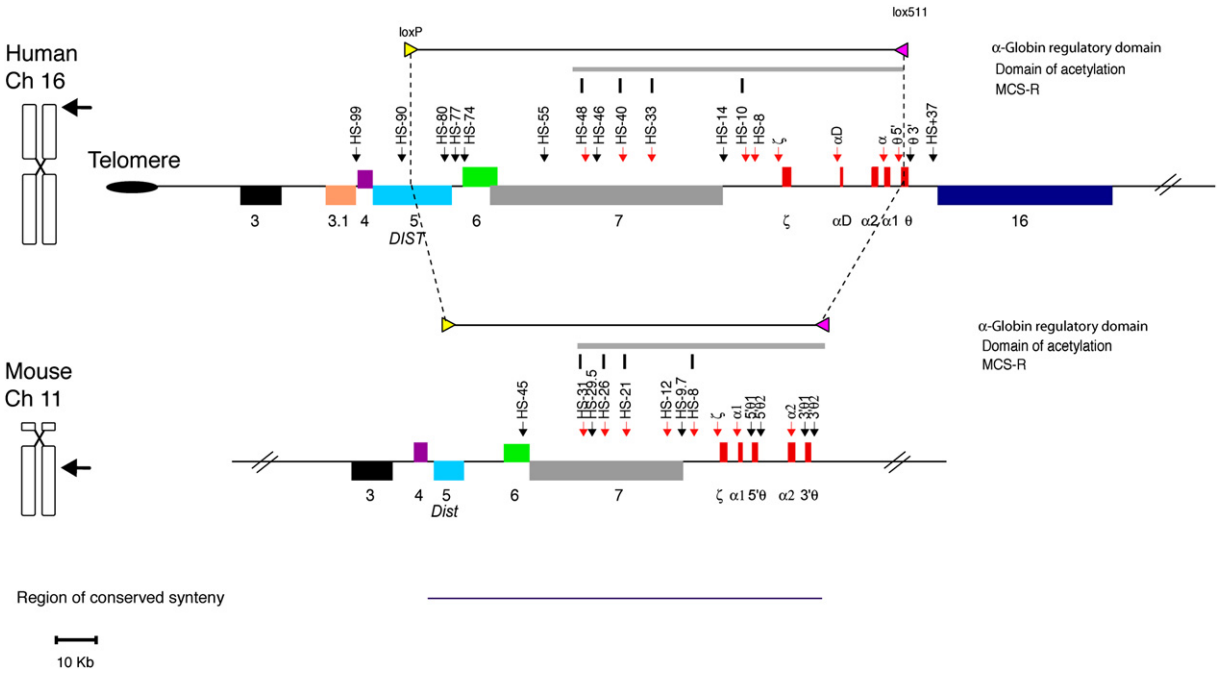

Figure 1. The Chromosomal Localization and Organization of the Human and Mouse α Globin Clusters

The human cluster (16p13.3) is located close to the telomere (oval), whereas the mouse cluster lies at an interstitial chromosomal position (11qA4). The globin genes are shown as labeled red boxes. Other human genes and their mouse orthologs are annotated as previously described (Flint et al., 1997) 3, *IL9RP3*; 3.1, *POLR3K*; 4, *c16orf33*; 5, *C16orf8* (*Dist*); 6, *MPG*; 7, *C16orf35*; and 16, *LUC7L*; and shown as colored boxes encoded by the top (above the line, 5′ to 3′ left to right) or bottom (below the line, 5′ to 3′ right to left) DNA strand. Note that two genes (*POLR3K* and α^D) are found only in the human cluster. *IL9RP3* is a pseudogene in man but a functional gene in mouse. A gray line above each cluster represents the domain of acetylation that appears only in erythroid cells. MCS-R refer to multispecies conserved regulatory elements. Below this in each cluster are known erythroid-specific (red arrows) and constitutive (black arrows) DNaseI hypersensitive sites. Above this in each cluster is the extent of the α globin region exchanged between man (BAC) and mouse (ES cells) delimited upstream by the *loxP* site (yellow triangles) and downstream by the *lox511* site (purple triangles). The orientation of the *loxP* and *lox511* sites is indicated by the directionality of the triangles.

clones was used for a second round of homologous recombination to target the θ gene using a vector with the PGK*puro*/MC1*tk*/*lox511*/I-*Sce*I cassette (Figure 2C), and several independently targeted clones were obtained. Double *Dist*-θ targeted clones should contain the integrated cassettes either on the same chromosome (in-*cis* targeting) or on each of the two homologs (in-*trans* targeting). We distinguished these based on the efficiency of Cre-mediated recombination when a plasmid pBS-*loxP*/*Hprt*$^{-\Delta 5'}$/*lox511*, coelectroporated with a Cre expression plasmid, interacted with the integrated cassettes reconstructing a functional *Hprt* minigene. Cells with sites in-*cis* should result in a higher frequency of recombination relative to those in-*trans*, as measured by the frequency of HPRT$^+$ colonies (see Supplemental Data Section S3, Figure S1, and Table S1, available with this article online). On this basis, three from 12 independent cell lines (2/B-6, 2/H-1, and 2/H-10) were assigned as having in-*cis*-targeted *Dist* and θ genes.

Targeted Modifications of the Human α Globin Cluster in a BAC

A human BAC clone, 344L6, covering the interval from 3 kb upstream of the *IL-9RP3* pseudogene to 18 kb downstream of the θ gene provided the human sequences. This BAC was modified by inserting *loxP* and *lox511* sites (in the equivalent positions and in the same orientations as in the mouse chromosome) using λ Red-mediated homologous recombination in the *E. coli* strain DY380 (Lee et al., 2001). The human *DIST* gene was targeted with the vector containing the I-*Sce*I/*loxP*/PGK-Tn903*hyg*/*loxP*/*Hprt*$^{-\Delta 5'}$/*frt* cassette (Figure 3A), and the θ gene was targeted with the vector containing the *lox511*/PGK-Tn903*neo*/MC1*tk*/I-*Sce*I cassette (Figure 3B). Finally, it was necessary to remove a second, pre-existing, endogenous *lox511* site present in the BACe3.6 vector; an additional recombination event was therefore carried out to replace the endogenous *lox511* site with a blasticidin selection cassette (Figure 3C), which consequently would also permit the detection of any potential random BAC backbone DNA integration in ES cells.

Selection and Characterization of the Genome Replacement Event

The vectors used to target the mouse and human *DIST* genes (Figures 2A and 3A) inserted the *loxP* sites and their linked *Hprt*$^{-\Delta 3'}$ and *Hprt*$^{-\Delta 5'}$ sequences, respectively, in the opposite transcriptional orientation to the *Dist* gene. This

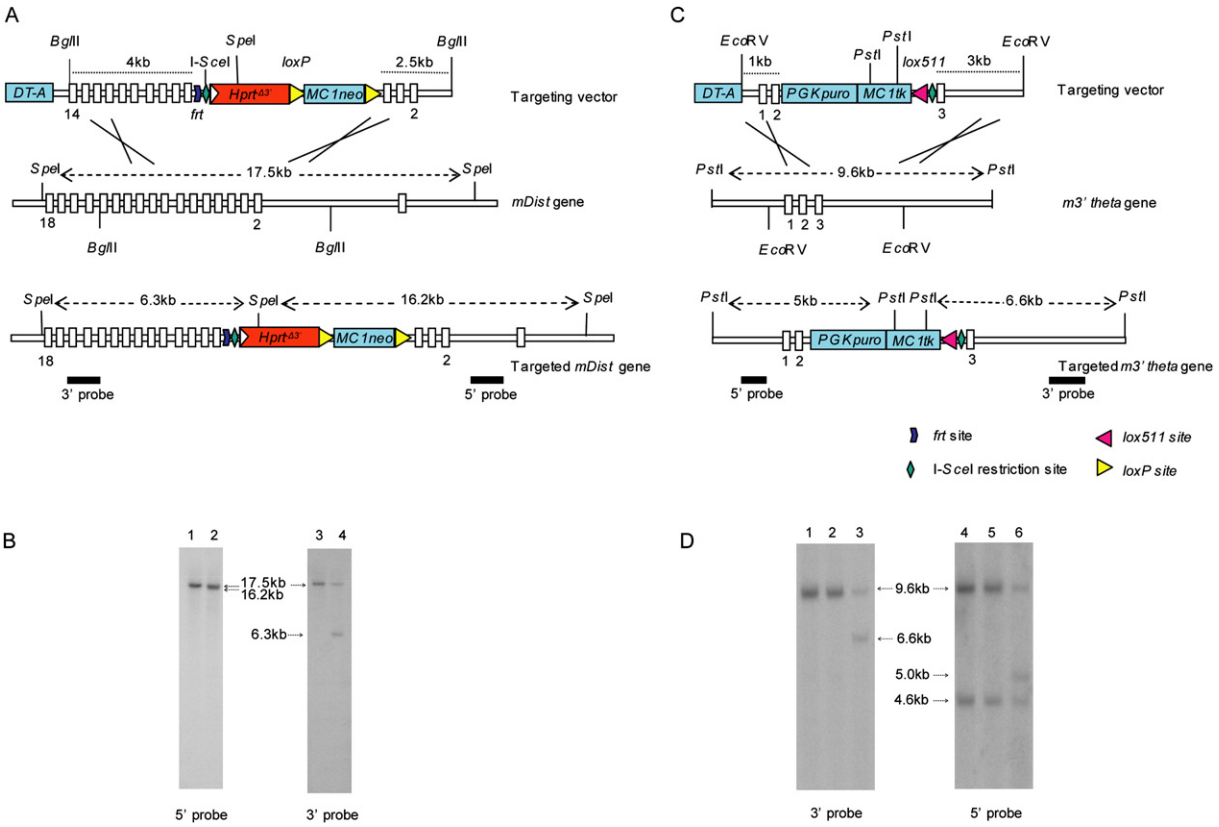

Figure 2. Targeting the Mouse *Dist* and 3′ θ Genes

(A) A cassette *frt*/I-*Sce*I/*Hprt*$^{-\Delta 3'}$/*loxP*/MC1*neo*/*loxP* was targeted to mouse *Dist* gene intron 4 using a linearized replacement vector with a diphtheria toxin (*DT-A*) gene for negative selection (Yagi et al., 1990). (B) Southern blot analysis of the parental ES cell line (lanes 1, 3) and a *Dist*-targeted cell line (lanes 2, 4) digested with *Spe*I (0.65% gel). The normal 17.5 kb fragment is reduced to 16.2 kb when hybridized with the *Dist* 5′ probe (lanes 1, 2) and to 6.3 kb when hybridized with the *Dist* 3′ probe (lanes 3, 4). (C) A cassette PGK*puro*/MC1*tk*/*lox511*/I-*Sce*I was targeted to mouse 3′ θ gene intron 2 in the *Dist*-targeted cell line using a linearized replacement vector as above. (D) Southern blot analysis of the parental ES cell line (lanes 1, 4), a *Dist*-targeted cell line (lanes 2, 5), and a *Dist*/θ double-targeted cell line (lanes 3 and 6) digested with *Pst*I (0.8% gel). The normal 9.6 kb fragment is reduced to a 6.6 kb fragment when hybridized with the 3′ θ gene 3′ probe (lanes 1, 3) and to a 5.0 kb fragment when hybridized with the 3′ θ gene 5′ probe (lanes 4, 6). N.B. the 4.6 kb restriction fragment seen with the 5′ probe corresponds to the upstream mouse 5′ θ gene sequences; all other restriction fragment sizes are derived from the 3′ θ gene. Symbols: exons, clear rectangles (some numbered); noncoding sequences and introns, narrow clear rectangles; selection markers, blue boxes; *Hprt*$^{-\Delta 3'}$ segment, red box (clear indent indicates promoter, blunt end indicates break in intron sequence); *lox*, *frt*, and I-*Sce*I sequences, see key, orientations indicated by directionality of triangles and chevrons; probes, black boxes; vector homology arms, dotted lines with sizes in kilobases (kb); predicted homologous recombination between vector and chromosome, crossed black lines; restriction sites, thin vertical lines; diagnostic restriction fragments, dashed lines with arrow heads, size in kilobases (kb). For clarity, the pBS component of the vectors and their site for linearization are not shown. Some components are not drawn to scale.

should create a reconstructed *Hprt* minigene after Cre-mediated recombination located in the recombinant mouse chromosome. The vectors used to target the mouse and human θ genes (Figures 2C and 3B) inserted the *lox511* sites in the same orientation but opposite to that of the *loxP* site. The relative order of the *lox511* and linked HSV thymidine kinase negative selection marker, MC1*tk*, should, after Cre-mediated recombination, result in a recombinant mouse chromosome lacking a negative selection marker.

This overall arrangement of targeted sequences in the ES cells and the BAC necessary to implement the desired Cre-mediated exchange is illustrated in Figures 4A and 4B. The reciprocal recombinant BAC product (not shown) should remain unintegrated and therefore be lost after cell division. ES cells with the resulting recombinant mouse chromosome will have an HPRT^{+}, ganciclovir-resistant phenotype.

Two cell lines, 2/B-6 and 2/H-1, with putative in-*cis*-targeted events at the *Dist* and θ loci were coelectroporated in an approximate 1:10 molar ratio with the modified BAC and a Cre recombinase expressing plasmid (pCAGGS-Cre-IRES*puro*) (Smith et al., 2002), and HPRT^{+} cells subsequently selected in medium with hypoxanthine, aminopterin, and thymidine (HAT). HAT-resistant (HAT^{R}) colonies were obtained at an approximate frequency of 1×10^{-8} (an average value determined from three independent electroporations). Two independent colonies

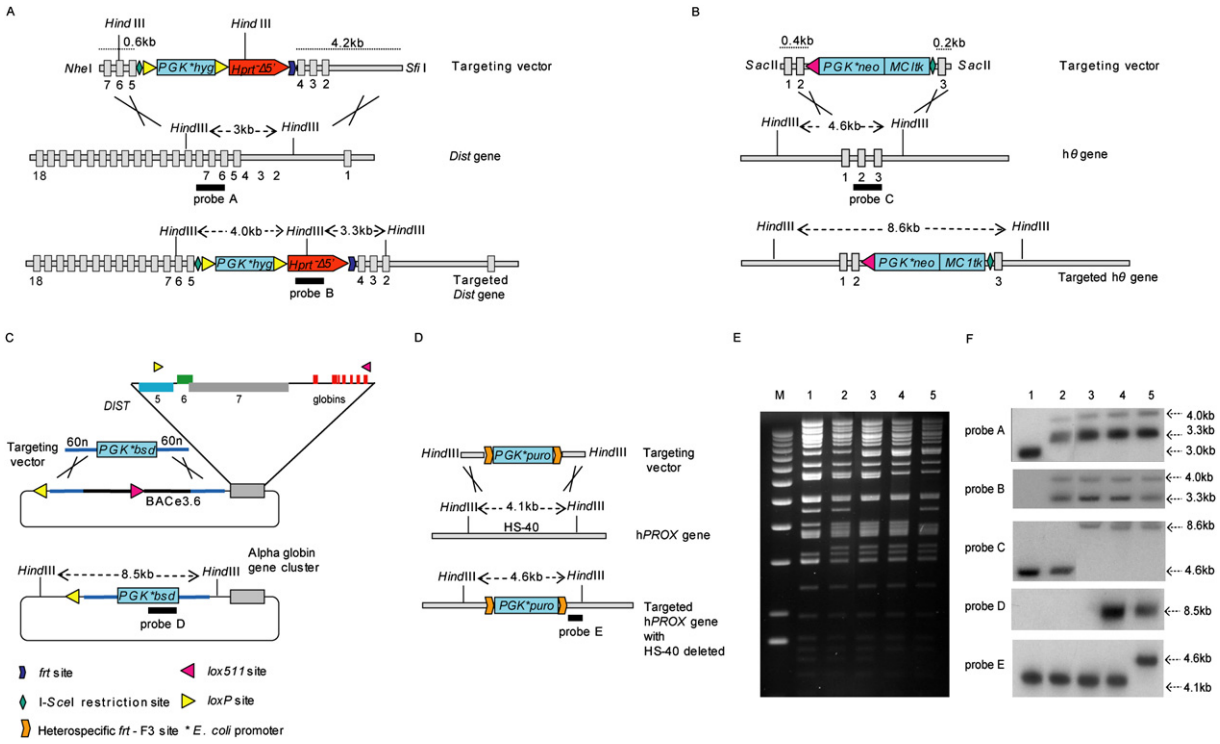

Figure 3. Targeting into BAC 344L6

(A) Vector used to insert a I-*Sce*I/*loxP*/PGK-Tn905*hyg*/*loxP*/*Hprt*$^{-\Delta 5'}$/*frt* cassette into human *DIST* gene intron 4. (B) Vector used to insert a *lox511*/PGK-Tn905*neo*/MC1*tk*/I-*Sce*I cassette into human θ gene intron 2. (C) Vector with 120 nucleotides total homology used to replace the *lox511* site in the BAC vector backbone with a PGK-EM7*bsd* cassette. The α globin cluster contained in the BAC is shown (not to scale). (D) Vector used to delete HS -40 within the *PROX* gene (*C16orf35*), replacing it with a *frt*-F3/PGK-EM7*puro*/*frt*-F3 cassette. (E) *Hind*III digests (0.8% gel) of BAC DNAs at each stage of retrofitting: BAC 344L6 (lane 1), *DIST* targeted (lane 2), *DIST* and human θ double targeted (lane 3), triple targeted in which BAC vector backbone *lox*511 site is deleted (lane 4), triple targeted with the HS -40 regulatory element deleted (lane 5). (F) Southern blot analysis of *Hind*III BAC digested DNA hybridized with probes A–E showing the expected size changes from each progressive modification. Symbols: exons, gray rectangles (some numbered); noncoding regions and introns, narrow gray rectangles; *Hprt*$^{-\Delta 5'}$ segment, red box (pointed end indicating polyadenylation addition site, blunt end the break in intron sequence); BAC sequences, black lines, or blue lines for the 60 nucleotide (n) homologous sequences. Other symbols as in Figure 2. Restriction enzymes used to generate the homologous ends of the targeting vectors are indicated in (A), (B) and (D); in (C) this was *Asc*I. Some components not drawn to scale.

derived from the 2/B-6 ES cell line and one from the 2/H-1 line were obtained. All three were resistant to ganciclovir, indicating that Cre-mediated exchange had also occurred via the *lox511* site at the θ gene (Figures 4A and 4B). In addition, the cells were sensitive to hygromycin, puromycin, and G418, indicating that the expected loss of these markers had occurred (Figure 4B). Sensitivity to blasticidin suggested that there was no random integration of the reciprocal BAC product of the exchange event nor had the BAC DNA integrated by nonhomologous recombination.

The reconstructed *Hprt* minigene embedded in the *Dist* gene and now flanked by directly repeated *frt* sites was subsequently excised by electroporating the FLPe-expressing plasmid pCAGGS-FLPe-IRES*puro* (Schaft et al., 2001) into the cells. Resistant clones were recovered at a high frequency after selection in media supplemented with 6-thioguanine (6-TG). This final modification resulted in a replacement event in which the only extraneous sequence remaining is an *frt* site at the *Dist* end of the interval and a *lox511*site and I-*Sce*I site at the θ end (Figure 4C).

To confirm the anticipated Cre and FLPe recombination events had occurred at the exchange end points, genomic DNA from the HAT^R and 6-TG^R clones was analyzed by extensive Southern blot analysis. Fragments of the predicted sizes (Figures 4D and 4E [lanes 1 to 6] and Figures S2 and S3) confirmed that the expected mouse-human junction sequences had been created at both ends of the exchange interval. All three of the independent HAT^R clones (and their 6-TG-resistant derivative clones) contained the Cre-mediated exchange event.

Generating Mice Containing the Human α Globin Gene Cluster Replacement

The HAT^R cell lines with replacements containing the wild-type human α globin gene cluster (2/B-6R1, 2/B-6R2, and 2/H-1R1, all of which retained the *Hprt* minigene) were used to generate chimeras. All three ES cell lines gave

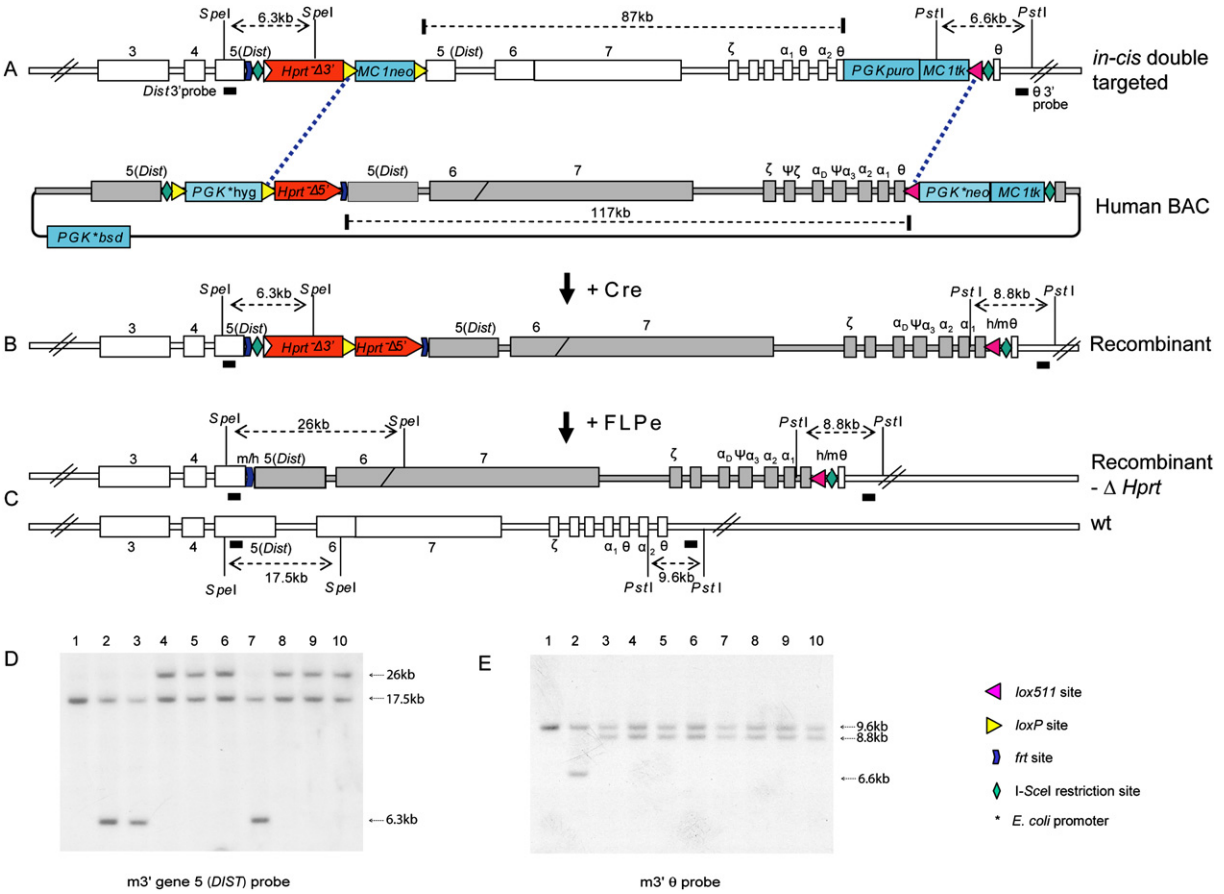

Figure 4. Replacement at the Mouse α Globin Locus

(A) Arrangement of sequences in double-targeted ES cells with in-*cis* chromosomal *loxP* and *lox511* sites and the modified human BAC, also showing potential Cre-mediated recombination between modified mouse chromosome and BAC. (B) Arrangement of sequences in the recombinant chromosome with human replacing mouse sequence and the embedded reconstructed *Hprt* minigene. (C) Arrangement of sequences in the recombinant chromosome after removal of the *Hprt* minigene (Δ *Hprt*) by FLPe recombinase. The nontargeted homolog is shown beneath for comparison. (D) Southern blot analyses following *Spe*I digestion (0.65% gel) and hybridization with mouse *Dist* 3′ probe (Figure 2). Lane 1, parental ES cells; lane 2, double-targeted cell line 2/B-6; lane 3, double-targeted cell line 2/B-6 HAT^R exchange clone # 1; lanes 4, 5, and 6, 6-TG^R clones derived from 2/B-6 HAT^R exchange clone # 1; lane 7, 2/B-6 HS -40 deletion HAT^R exchange clone # 1; lanes 8, 9, and 10, 6-TG^R puromycin-sensitive clones derived from 2/B-6 HS -40 deletion HAT^R exchange clone # 1. N.B. Lanes 3 and 7 show no alteration in the *Spe*I fragment size following the exchange because the *Hprt* gene is still present. (E) Southern blot analyses following a *Pst*I digestion (0.8% gel) and hybridization with mouse 3′ θ gene 3′ probe (Figure 2). Lanes 1 to 10 as above in (D). Symbols as follows: mouse genes, clear rectangles; mouse intergenic regions, narrow clear rectangles; human genes, gray rectangles (diagonal division indicates gene overlap); human intergenic regions, narrow gray rectangles; thin black dashed lines with bars, mouse and human regions delineated by the *lox* site integrations, size in kilobases (kb); blue dashed lines, predicted Cre recombination between BAC and chromosome resulting in the exchange. Other symbols as in Figures 2 and 3. Diagram not to scale.

rise to male chimeras that transmitted the recombinant chromosome.

Of the three mouse lines in which the human α globin regulatory domain replaced the mouse domain, one (line 1, derived from 2/B-6R1) was mapped in detail and a second (line 3, derived from 2/H-1R1) in less fine detail. In both lines, the α globin regulatory domain was shown to be intact and structurally normal (Figure S2). Line 2 (derived from 2/B-6R2) showed some anomalous bands, and further investigation (data not shown) demonstrated this line had an internal deletion of the BAC replacement region that encompassed at least some of the gene 7 sequence but did not extend into the sequence of the *MPG* or ζ genes. This deletion was subsequently found to be present in the ES cell line and presumably arose during the exchange event. This line was not analyzed further.

When heterozygotes established on a mixed (129/Ola)/(C57BL/6)/(CBA) genetic background from both lines (1 and 3) were mated, offspring homozygous for the recombinant chromosome were produced but not in the expected 1 in 4 Mendelian ratio (3 homozygotes: 20 heterozygotes: 12 wild-type). Surviving homozygotes developed normally and were fertile, albeit with significant hematological abnormalities (see below). From homozygote × heterozygote crosses, 6/16 homozygotes were

Table 1. Hematologic Analysis of Humanized Mice

	Hb (g/dl)	MCV (Fl)	MCH (pg)	Retics (%)	αH/αM mRNA Real-Time PCR	αH/αM mRNA RPA
A						
Human BAC line 1 (+H)	15.2 ± 1.0	43.2 ± 2.4	15.6 ± 0.4	1.7 ± 1.1	0.43 ± 0.07	0.39 ± 0.03
Human BAC line 1 (−H)	15.0 ± 0.8	45.8 ± 1.1	15.7 ± 0.6	1.9 ± 0.5		0.37 ± 0.07
Human BAC line 3 (+H)	15.5 ± 3.2	44.7 ± 3.1	15.8 ± 1.2	0.8 ± 0.7	0.39 ± 0.04	0.49 ± 0.05
Human BAC line 3 (+H)	14.4 ± 0.7	44.8 ± 1.5	15.3 ± 0.5	1.6 ± 0.6		0.49 ± 0.10
Δ HS -40 BAC (+H, +P)	13.6 ± 0.4	36.6 ± 1.3	14.4 ± 1.3	2.3 ± 0.5	0.016 ± 0.005	0.016 ± 0.003
Δ HS -40 BAC (−H, −P)	14.2 ± 1.5	39.5 ± 3.5	12.9 ± 0.8	2.8 ± 0.7		
Normal mouse	15.9 ± 1.1	45.7 ± 2.4	16.5 ± 0.3	1.4 ± 1.0		
–/αα	11.8 ± 0.9	36.7 ± 1.5	13.7 ± 0.7	3.9 ± 1.0		
B						
Human BAC line 1 (+H)	15.2 ± 0.6	39.8 ± 0.8	13.1 ± 0.3	5.8 ± 2.7		
Human BAC line 3 (+H)	14.4 ± 0.5	40.0 ± 0.1	13.3 ± 0.2	6.7 ± 1.8		
Human BAC line 3 (−H)	14.6 ± 2.0	40.0 ± 1.4	13.1 ± 0.3	7.0 ± 4.5		

(A) Hematologic data and ratios of human to mouse α globin mRNA levels in adult blood from mice heterozygous for either the intact human BAC-derived sequence (lines 1 and 3, with the *Hprt* gene present [+H] or after its removal [−H] with FLPe recombinase) or one from which the major regulatory element has been deleted (Δ HS -40), before (+H, +P) and after removal (−H, −P) of the *Hprt* and puromycin resistance genes. Equivalent results from normal mice and those carrying a deletion of the structural α globin genes are provided for comparison. n = 3–11 animals. (B) Hematologic data on adult blood from mice (lines 1 and 3) homozygous for the human BAC-derived sequence, with the *Hprt* minigene present [+H] or after it was removed [−H]. n = 2–5 animals. Hb, level of hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; Retics, reticulocytes; αH, human α globin; αM, mouse α globin.

obtained. Some homozygotes appeared, therefore, to be lost in utero, and of those that were born, 3/9 died in the neonatal period.

Mice from lines 1 and 3 were crossed with a FLPe recombinase germline deleter C57BL/6 strain (unpublished). Analyses of mice carrying the recombinant chromosome and the FLPe transgene confirmed deletion of the *Hprt* gene as predicted, and these were subsequently intercrossed. Southern blot analysis identified embryos and pups from the intercross that were homozygous for the now modified recombinant chromosome (Figure S3). Two viable homozygotes lacking the *Hprt* gene were produced and were hematologically indistinguishable from those that retained the *Hprt* gene, demonstrating that the presence of the *Hprt* gene and its regulatory elements had no apparent effects on the expression of genes within the α globin region (Table 1B). After further backcrossing onto a C57BL/6 background (three generations) heterozygotes with the *Hprt* gene deleted when intercrossed still produced a deficiency of viable homozygous offspring.

In Vivo Analysis of Mice Carrying a Human α Globin Gene Cluster Replacement

To visualize the exchange, splenic lymphocyte metaphases from a heterozygote with the recombinant chromosome (line 1) were hybridized with differentially labeled probes for the mouse and human α globin gene clusters together with a chromosome 11 paint. In each cell, one chromosome 11 carried a human hybridization signal, while its homolog bore a mouse signal, confirming that one of the mouse chromosomes had the human replacement event and that no additional insertions had occurred (Figure 5).

Expression of the genes from the region containing the human α globin domain was first checked by hybridization of RNA from the erythroblasts of a homozygous, humanized mouse to an Affymetrix tiled microarray containing all unique sequences in the terminal 300 kb of chromosome 16 (De Gobbi et al., 2006). The pattern of expression was very similar to that seen from normal human erythroblasts (Figure 6A). Using RT-PCR, transcripts from the *MPG*, *PROX*, α^D, and α^A genes were readily detected in peripheral blood and spleen (Figure 6B). The low levels of α globin RNA in brain was consistent with blood contamination. A hybrid RNA crossing the *DIST* gene breakpoint was detected using 5′ human and 3′ mouse primers in brain, spleen, and blood from a heterozygote from which the *Hprt* minigene had been excised. Similarly, we detected the hybrid RNA crossing the breakpoint that lies in the human and mouse θ gene (Figure 6C).

Expression of human and mouse α globin mRNA was measured in adult peripheral blood samples by both quantitative real-time PCR and by RNase protection assays (RPA). The results (Table 1 and Figures 6D–6F) were similar for both lines analyzed and by both methods and show that expression from the recombinant chromosome is

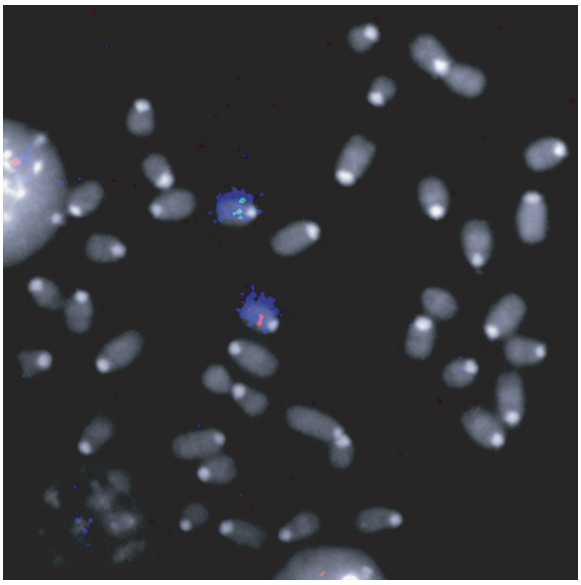

Figure 5. Fluorescence In Situ Hybridization Demonstrating the Exchange of the Human (Green) and Mouse (Red) α Globin Regulatory Domains within the Context of Mouse Chromosome 11 (Blue)

about 40% that of the mouse chromosome. At 9.5 days gestation, embryos expressed high levels of the embryonic ζ globin gene ($\zeta^H/(\alpha+\zeta)^H$ ~70%, ζ^H/ζ^M ~60%); the absence of ζ gene expression in adults (Figure 6E) confirms that the normal developmental pattern of globin gene expression is retained.

Blood films from the mice carrying one copy of the recombinant chromosome were largely normal in appearance, with a minor increase in anisopoikilocytosis (abnormally shaped cells). No difference was evident between the two lines. Red cells from the homozygotes were more markedly abnormal, with microcytic (small), hypochromic (pale) anisopoikilocytotic cells producing a typical thalassemic picture (Figure S4). Blood counts from an automated counter (ABX Pentra 60) confirmed that in heterozygotes the Hb level, mean cell volume (MCV), and mean cell hemoglobin (MCH) were normal, while the homozygotes had normal Hb levels but a raised reticulocyte count and reduced MCV and MCH values. These values were similar (Table 1) to those from thalassemic mice heterozygous for a targeted deletion (Pászty et al., 1995) of both α globin structural genes (--/αα).

Deletion of the HS -40 Regulatory Element in the α Globin Gene Cluster

The RMGR strategy was designed to facilitate rapid recursive introduction of mutations into the exchange region by additional rounds of BAC modification using λ Red-mediated recombination prior to Cre recombination in ES cells to allow systematic in vivo functional analyses of human regulatory elements. As a proof of principle, λ Red-mediated recombination was used to remove a 1.1 kb region containing the HS -40 regulatory site that lies in intron 5 of the *C16orf35* (also known as the *PROX* gene; gene 7 in Figure 1) gene (Figure 3D) in the triple targeted BAC. The 1.1 kb region was replaced with a PGK/EM*7puro* selection cassette flanked with direct repeats of the heterospecific *frt* site F3 (Schlake and Bode, 1994); to enable its subsequent excision by FLPe recombinase without intrachromosomal recombination occurring with the *frt* sites flanking the *Hprt* minigene. Figures 3D and 3F (lane 5) show the expected change of a *Hind*III fragment from 4.1 kb to 4.6 kb. The modified BAC was coelectroporated into the recipient 2/B-6 ES cells, in the presence of Cre, and a single HAT-resistant clone, resistant to ganciclovir and puromycin, was recovered, consistent with the anticipated replacement recombination event. Southern blot analysis of this clone, and of independent 6-TG^R derivative clones (which were also puromycin sensitive) obtained subsequent to electroporation with the FLPe-expressing plasmid, showed the identical restriction fragment size changes corresponding to the mouse-human junction sequences (Figures 4D and 4E [lanes 7 to 10]) obtained with the original replacement event. The frequency of puromycin-sensitive clones (approximately 50% of total 6-TG^R clones) and the identical restriction fragment size change consistently seen in 6-TG^R puromycin-sensitive clones across the mouse/human *Dist* junction indicated that efficient codeletion of the *Hprt* and PGK/EM*7puro* selection cassettes occurred without heterotypic *frt* × *frt*-F3 recombination events and consequent intrachromosomal rearrangement.

Characterization of Mice Carrying a Human α Globin Gene Cluster Replacement with the HS -40 Deletion

The HAT^R cell line containing the human α-globin gene cluster from which HS -40 had been deleted (but retaining the *Hprt* minigene) also generated a transmitting male chimera. Mapping of this line showed only the expected band sizes.

When these heterozygotes were crossed, homozygotes were phenotypically indistinguishable from littermates at 10.5 days gestation but by genotyping were detectable at normal frequencies. By 11.5 days gestation, homozygotes were identifiable as small, pale, dead embryos that had presumably died from severe anemia.

Expression of the human α globin genes in adult heterozygotes carrying the human cluster from which the major regulatory element (HS -40) had been deleted (Figure 6E) was reduced to barely detectable levels (<2%), in keeping with results from human patients in whom this element is missing (Higgs et al., 1998). In embryos, human RNA levels were again severely reduced, with ζ not as extremely affected as α (Figure 6F).

Red cell morphology was also abnormal in mice carrying the mutated human sequence (Δ HS -40); hypochromia, microcytosis, and target cells were prevalent as well as marked anisopoikilocytosis (Figure S4). These films were similar to those from thalassemic mice heterozygous for

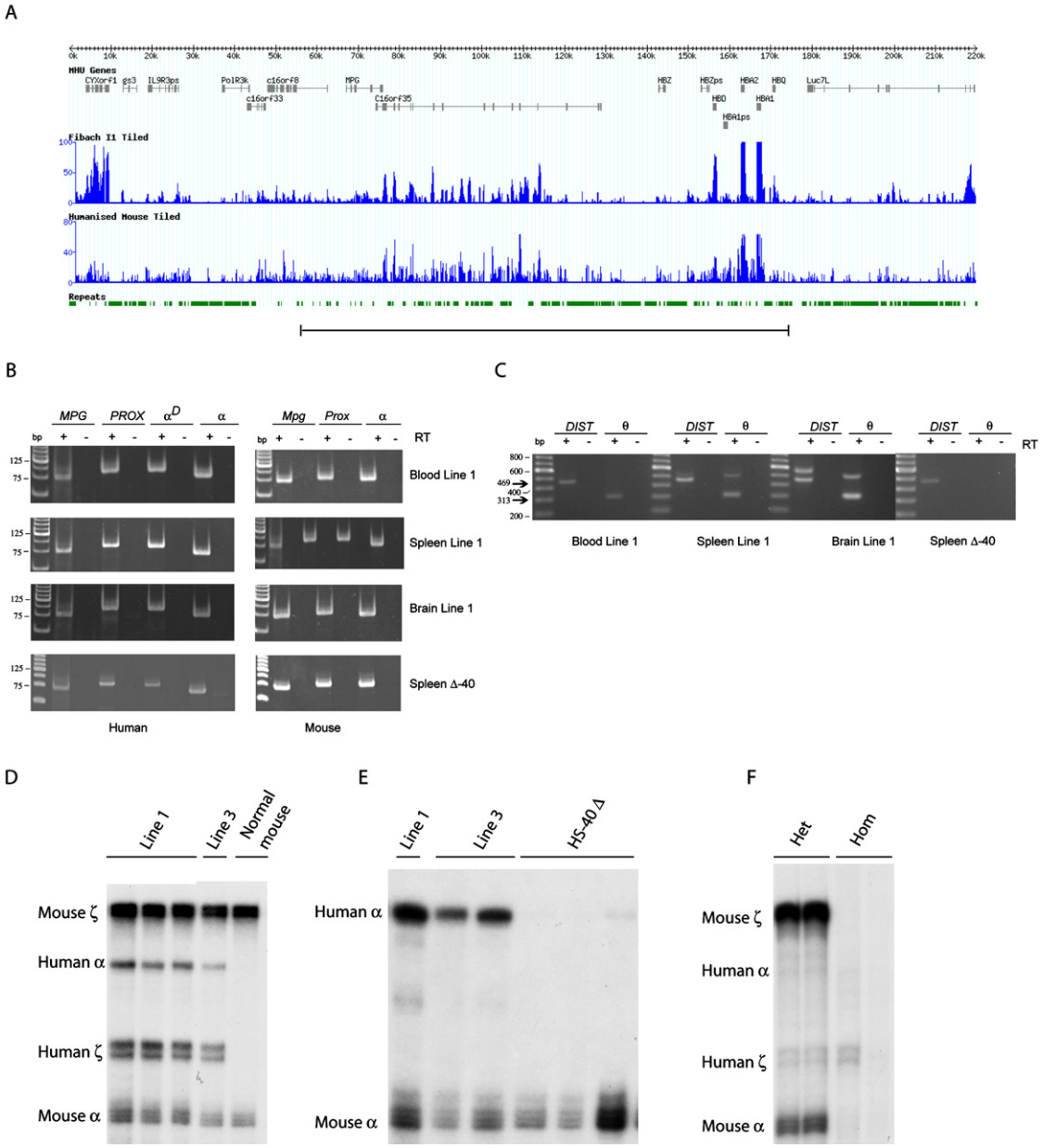

Figure 6. Expression from the Exchanged α Globin Regulatory Domain

(A) hybridization of cDNA from normal human erythroblsts (above) and erythroblasts from a mouse (line 1) homozygous for the replacement event on a chromosome 16 array (coordinates 1–300,000). (NB: the higher expression of *CYXorf1* seen in the human Fibach cells reflects the higher number of paralogs of this gene [on several chromosomes] in human than in mouse DNA.)

(B) Expression of *MPG*, *PROX*, α^D, and α (left panel) was detected in spleen, peripheral blood, and brain samples from a heterozygote from line 1 and in spleen from a Δ HS -40 heterozygote, using gene-specific primers. Likewise, expression of *Mpg*, *Prox*, and α was present in the murine locus (right panel). Low levels of expression of α globin in nonerythroid tissues is due to contamination with small amounts of blood.

(C) RT-PCR analysis of mRNA expression across the murine/human junction (*Dist* and θ genes) in spleen, peripheral blood and brain samples from a heterozygote line 1 or Δ HS -40 mouse in which the *Hprt* gene has been excised from the *Dist* gene. A correct size transcript is observed across the junction (arrows), showing that the gene expression is not affected. Note that in the absence of the HS -40 element, θ gene expression is severely downregulated.

(D) RNase protection assays of 10.5 day heterozygous embryos showing expression of mouse and human ζ and α genes.

(E) RNase protection assay of adult blood RNA from heterozygotes of lines 1 and 3 and the Δ HS -40 line.

(F) RNase protection assays of embryonic blood (9.5 days) from heterozygotes and homozygotes for the recombinant chromosome from which the HS -40 element was deleted.

a targeted deletion (Pászty et al., 1995) of both α globin structural genes (--/αα). Blood counts showed a reduced Hb level and low MCV and MCH, similar to those from --/αα mice (Table 1). The puromycin selection marker gene, which replaced the HS -40 element, and the *Hprt* minigene were excised by crossing this line with the FLPe recombinase deleter line. Hematological analyses of two adult mice derived from this cross carrying the now modified, mutated human sequence were indistinguishable from mice in which these selection marker genes had not been excised (Table 1).

DISCUSSION

Here we have shown that it is possible to precisely replace a large segment of the mouse genome containing multiple loci with the corresponding, syntenic segment of the human genome. Combining a variety of developments in chromosome engineering, RMGR has significantly extended the principles underpinning RMCE to enable extensive tracts (100s to 1000s kb) of mouse genomic DNA to be replaced by the orthologous sequences of other species cloned in BAC libraries. RMGR will therefore be widely applicable and offers several advantages over other approaches to large-scale genome modification. In particular, correct genomic replacement using RMGR is genetically selectable, precise, and reproducible. This reduces the effort of screening and greatly simplifies the characterization of the chromosomal breakpoints. Minimal extraneous DNA remains to affect the functional integrity of the engineered chromosomal region, provided the locations of the recombination sites are chosen carefully. Once correctly targeted, germline transmitting ES cells are established, RMGR allows iterative replacement with mutated BACs for detailed functional dissection of genomic sequences. Although the frequency of replacement is low, it is reproducible. General application may reveal locus-specific and/or BAC-specific variations in efficiency requiring adjustments to the electroporation parameters. The RMGR strategy will be adaptable for application with other (e.g., ϕC31) (Belteki et al., 2003) site-specific recombination systems to improve the overall efficiency and allow the Cre/*lox* system to be used to introduce conditional deletions within the human sequence. In its present form, the extent of sequence replacement is limited only by the size of the BAC inserts. Recent approaches to construct mega-BACs (Shen et al., 2005) in *E. coli* will therefore extend the size range of replacements possible.

By analyzing the well-characterized α globin regulatory domain by RMGR, we could be confident that the structurally intact segment contains all known *cis*-acting elements, at a single copy in a chromosomal position that is appropriate for globin gene expression. Furthermore, by carefully examining gene expression in erythropoiesis and evaluating the resulting, sensitive red cell phenotype, we could accurately assess the degree to which regulation of the human globin genes mimic their normal pattern and levels of expression in the mouse model. We found that under these conditions the human genes are expressed in an appropriate developmental stage- and tissue-specific manner, although the level of expression of the α genes is only about 40% of that of the endogenous mouse α globin genes. This leads to abnormal red blood cells, and homozygotes for the human synteny replacement may die prematurely even though they are not frankly anemic. These differences between expression of the human gene in man and mouse could result from changes in the chromosomal environment, although the relationship between transcription and nuclear sublocalization remains unclear (Brown et al., 2006). Perhaps more likely, changes in the structure or recognition sequences of the key transcription factors have altered during the evolution of the two species such that the binding or stability of mouse transcription factors on human sequences are suboptimal.

Knowing the phenotype resulting from expression of the normal human α globin cluster in mouse, it is now possible to compare and evaluate the effects of specific mutations introduced into the human sequence. Using RMGR, the only variable is the mutation itself. Such mutations could either be introduced by further rounds of homologous recombination in the humanized ES cells or, as demonstrated here, by recombineering the appropriate mutation into the modified BAC followed by a new round of RMGR at the target region. Again using the α globin cluster as the model we could show that removal of just 1.1 kb containing the major regulatory element (HS -40) virtually abolishes human α globin expression consistent with previous extensive observations (Higgs et al., 1998). In homozygotes, this deletion mimics the lethal clinical phenotype of *hydrops fetalis* seen in human patients with this degree of α globin deficiency. We have previously shown that removal of the mouse sequence (HS -26), which is the equivalent of the human major regulatory element (HS -40), from the endogenous locus only reduces mouse α globin expression to ~50% of normal, and homozygotes for this allele survive with only mild anemia rather than developing hydrops fetalis (Anguita et al., 2002). This clearly shows that the detailed regulation of human and mouse genes may be quite different and emphasizes the point that extrapolating conclusions from studying regulation of an orthologous gene may be misleading when one wishes to determine how a particular human gene is regulated.

It seems likely that the observations set out here represent general recurrent issues that arise when making and analyzing mouse models of human gene regulation and genetic disease. RMGR will exclude most of the variables associated with current transgenic experiments, but it is still likely that human genes will not be completely faithfully regulated in mouse systems. However, comparing wild-type and mutant chromosomal domains iteratively inserted into an appropriate chromosomal environment will allow one to determine more precisely the effect of specific mutations. This will not only be of value when studying previously well-characterized loci but will also

enable those studying complex traits to assess the functional role of mutations and SNPs localized within less well characterized large segments of the human genome.

EXPERIMENTAL PROCEDURES

ES Cell Gene Targeting Vectors

Replacement vectors were constructed in pBluescript (pBS) plasmid using 129/Ola strain cloned genomic DNA; (see Supplemental Data Section S1 for details). Vectors were linearized at a unique restriction site prior to electroporation.

BAC Retrofitting Vectors

Vectors were constructed in pBS plasmid using subcloned human BAC 344L6 DNA or duplex synthetic oligonucleotides, and selection cassettes with dual eukaryotic (PGK) and prokaryotic (Tn903 or EM7) promoters (Testa et al., 2003); (see Supplemental Data Section S2 for details). DNA restriction fragments used for electroporation were gel purified from the pBS backbone.

BAC Modifications

The BAC 344L6 was transferred into *E. coli* DY380 (gift from D. Court and N. Copeland) selected in chloramphenicol 12.5 μg/ml and electrocompetent cells then prepared for retrofitting (Lee et al., 2001). λ Red-mediated recombination was carried out mixing 50 μl of cells with 150 ng to 300 ng purified DNA in a 0.1 cm wide cuvette using a Bio-Rad gene pulser set at 1.8 kV. Immediately after electroporation, 1 ml of SOC media was added, and the cells were further grown at 32°C for 2 hr before being plated on appropriate selective agar media. Drug concentrations: puromycin, 62.5 μg/ml; kanamycin, 12.5 μg/ml; blasticydin, 100 μg/ml; hygromycin, 50 μg/ml.

ES Cell Gene Targeting

E14-TG2a.IV ES cells were grown as previously described (Nichols et al., 1990). 75 μg of *Not*I linearized *Dist* targeting vector was electroporated into 5×10^7 E14-TG2a.IV cells as previously described (Smith et al., 2002). Cells were selected in 160 μg/ml G418 and resistant colonies later picked into 96-well plates for freezing and DNA analysis. Targeted clones were identified by Southern blot analysis of *Spe*I digested DNAs and hybridization with 5′ and 3′ external probes (5′, 0.96 kb *BamH*I - *Hind*III fragment; 3′, 0.90 kb *Spe*I - *Bgl*II fragment). The *Dist*-targeted cell line was then used in a second round of electroporation to target the 3′ θ gene according to the procedure above. Cells were selected in 1.5 μg/ml puromycin. Targeted clones were identified by Southern blot analysis of *Pst*I-digested DNAs and hybridization with 5′ and 3′ external probes (5′, 0.22 kb *Xho*I/*Hind*III fragment; 3′, 0.45 kb *Bgl*II fragment).

Determination of in-*cis* Double Targeted Clones

Double-targeted ES cell clones were coelectroporated with 75 μg pBS vector containing a *loxP*/*Hprt*$^{-\Delta 5'}$/*lox511* cassette and 25 μg pCAGGS-Cre-IRES*puro* plasmid (Smith et al., 2002). 24 hr later, cells were plated at 5×10^6 cells per 10 cm dish. Cells were selected the following day in HAT supplemented medium (0.1 mM hypoxathine, 0.4 μM aminopterin, 0.016 mM thymidine). After 10 days, HAT^R colonies were counted, picked into 96-well plates, and subsequently replica 96-well plates made for G418 and puromycin resistance/sensitivity testing. Details are provided in Supplemental Data Section S3.

BAC Exchange

Approximately 80 μg of retrofitted supercoiled BAC DNA (see Supplemental Data Section S4 for DNA preparation method) and 30 μg pCAGGS-Cre-IRES*puro* plasmid DNA (~1:10 molar ratio) were mixed and coprecipitated with ethanol. The DNA pellet was dissolved in a final volume of 100 μl 90% phosphate-buffered saline (PBS), and the DNAs were very gently mixed with a 0.7 ml suspension of 5×10^7 to 1×10^8 double-targeted ES cells in PBS in a 0.4 cm wide Bio-Rad cuvette. Cells were electroporated at 0.25 kV, 960 μF using a Bio-Rad gene pulser and then plated into twenty 10 cm diameter dishes. 24 hr later, HAT supplemented media was added and left for 4 days. The medium was then changed to HT (0.1 mM hypoxanthine plus 0.016 mM thymidine) supplemented media, and any resistant colonies were picked 6–10 days later. The clones were subsequently expanded for freezing, DNA analysis, and testing resistance/sensitivity to the following: G418 (160 μg/ml), hygromycin (120 μg/ml), puromycin (1.5 μg/ml), blasticydin (5 μg/ml), ganciclovir (2.5 μM), 6-thioguanine (10 μM), HAT (0.1 mM hypoxathine, 0.4 μM aminopterin, 0.016 mM thymidine). DNA of potential replacement clones was first analyzed at the end points of the exchange event by *Spe*I digestion and hybridization with 5′ and 3′ mouse *Dist* probes and *Pst*I digestion and hybridization with 5′ and 3′ mouse 3′ θ probes.

Hprt Cassette Deletion

Clones with a confirmed exchange event were electroporated with 75 μg supercoiled pCAGGS-FLPe-IRES*puro* plasmid DNA (Schaft et al., 2001). The cells were grown nonselectively for 6 days and then plated out at 10^3 cells per 10 cm plate in medium with 10 μM 6-thioguanine. Resistant colonies were picked after 8 days into a 96-well plate for freezing and DNA analysis.

Generation of Chimeras and Breeding

All animal experiments were conducted under Home Office licenses after ethical review of the Universities of Edinburgh and Oxford. Chimeras were generated by injection of ES cells into C57/BL6 blastocysts and transfer into pseudopregnant recipients. Male chimeras were test-crossed with C57/BL6 mice. Agouti F1 test-cross offspring were genotyped by *Eco*RI digestion of tail biopsied DNA and hybridization with a human ζ gene probe. Test-cross positive heterozygotes were further backcrossed onto the C57BL/6 strain to maintain the lines. Test-cross positive heterozygotes were also bred with C57BL/6 × CBA F1 mice, and heterozygotes on this mixed genetic background intercrossed to derive homozygotes. To derive lines with the *Hprt* cassette removed, F1 test-cross positive heterozygotes were crossed with a C57BL/6 FLPe germline deleter, and these were subsequently maintained by backcrossing with C57BL/6 mice and heterozygotes intercrossed to derive homozygotes.

DNA and RNA Analysis

Genomic DNA analysis and RNase protection assays were as previously described (Anguita et al., 2002). DNA FISH studies were as described (Brown et al., 2006).

Total RNA was isolated from adult tissues or whole embryos using the Trizol reagent (Sigma) and analyzed by Affymetrix array as previously described (De Gobbi et al., 2006) and by quantitative real-time RT-PCR. Specific primers and TaqMan probes were designed with Primer Express software (ABI) and sequences used are provided in Table S2.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/128/1/197/DC1/.

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performed the chromosome engineering; F.M.-K., J.A.S., W.G.W., and D.R.H. performed the analysis of cells and transgenics. J.H. developed the human 16p microarray for analyzing gene expression. H.A.C.W., W.G.W., D.R.H., and A.J.H.S. wrote the paper. This work was supported by the UK Medical Research Council (MRC) and the award of a MRC project grant to H.A.C.W. and A.J.H.S.

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