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Engineering Synthetic Chromosomes by Sequential Loading of Multiple Genomic Payloads over 100 Kilobase Pairs in Size

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Gene delivery vehicles currently in the clinic for treatment of monogenic disorders lack sufficient carrying capacity to efficiently address complex polygenic diseases. Thus, to engineer multifaceted genetic circuits for bioengineering human cells as a therapeutic option for polygenic diseases, we require new tools that are currently in their infancy. Mammalian artificial chromosomes, or synthetic chromosomes, represent a viable approach for delivery of large genetic payloads that are mitotically stable and remain independent of the host genome. Previously, we described a mammalian synthetic chromosome platform, termed the ACE system, that requires a single unidirectional integrase for the introduction of multiple genes onto the ACE platform chromosome. In this report, we provide a proof of concept that the ACE synthetic chromosome bioengineering platform is amenable to sequential delivery of off-the-shelf large genomic fragments. Specifically, large genomic clones spanning the human solute carrier family 2, facilitated glucose transporter member 1 (*SLC2A1* or *GLUT1*, 169 kbp), and human monocarboxylate transporter 1 (*SLC16A1* or *MCT1*, 144 kbp) genetic loci were engineered onto the ACE platform and demonstrated to express and correctly splice both gene transcripts. Thus, the ACE system provides a facile and tractable engineering platform for the development of gene-based therapeutic agents targeting polygenic diseases.

INTRODUCTION

Progress in cell bioengineering for gene-based therapies has been held back by the absence of the one indispensable tool required to address polygenicity and/or delivery of large genetic payloads (>100 kbp): a stable, non-integrating, self-replicating, and biocompatible (i.e., mammalian) intracellular synthetic chromosome that ensures controlled expression. Synthetic chromosomes provide the breakthrough in biological bandwidth required to manage such complex polygenic challenges and introduction of large genetic payloads. Synthetic chromosomes can be generated by several means, including (1) co-transfection of defined chromosomal elements (i.e., telomere elements, centromeric alpha-satellite DNA multimers, and mammalian replication origins) along with a drug-selectable marker into a permissive cell line, which then assembles the components into an artificial chromosome (e.g., mammalian artificial chromosome [MAC]); (2) dissection of individual host cell chromosomes down to minichromosomes consisting of minimal functional centromere regions or neocentromeres by a process of targeted telomere integration and excision; (3) stable maintenance of centric fragments or small accessory chromosomes modified to accept foreign genes; and (4) generation of satellite DNA-amplified chromosomes (SATACs) by targeted amplification and fragmentation of pericentromeric sequences from acrocentric chromosomes into stably maintained chromosome vectors.[1] The overriding principle common to all of these methods is the recapitulation of functional mammalian centromeres and telomeres in a form suitable for downstream engineering. To date, engineering of synthetic chromosomes required multi-integrase systems (Cre, ΦC31, and ΦBT1 recombinases) to enable multigene loading.[2–6]

Previously, we have described a derivation of the SATAC methodology, termed the ACE synthetic chromosome, as a means to introduce large payloads of genetic information into the cell.[7] The ACE system can be reproducibly generated *de novo* in mammalian cell lines and readily purified from the host cell's chromosomes by flow cytometry and chromosome sorting. In turn, purified mammalian ACE synthetic chromosomes can be easily introduced into a variety of mammalian cell lines by transfection and maintained for extended periods without enforced genetic selection.[8–11] The ACE system consists of a synthetic chromosome (ACE synthetic chromosome), ACE targeting vectors, and the ACE integrase.[7] The ACE synthetic chromosome contains more than 50 copies of the 245-bp bacteriophage lambda *attP* site-specific recombination acceptor sequence, all of which are equal in availability. The multiple copies of the acceptor site allow the ACE synthetic chromosome to be engineered to carry single or multiple copies of genes of interest using ACE targeting vectors that contain the donor *attB* recombination site.[7] The ACE integrase is a derivative of bacteriophage lambda integrase (λINTR) engineered to direct site-specific, unidirectional recombination in mammalian cells. In contrast to multi-recombinase systems required for other synthetic chromosome engineering platforms, the ACE

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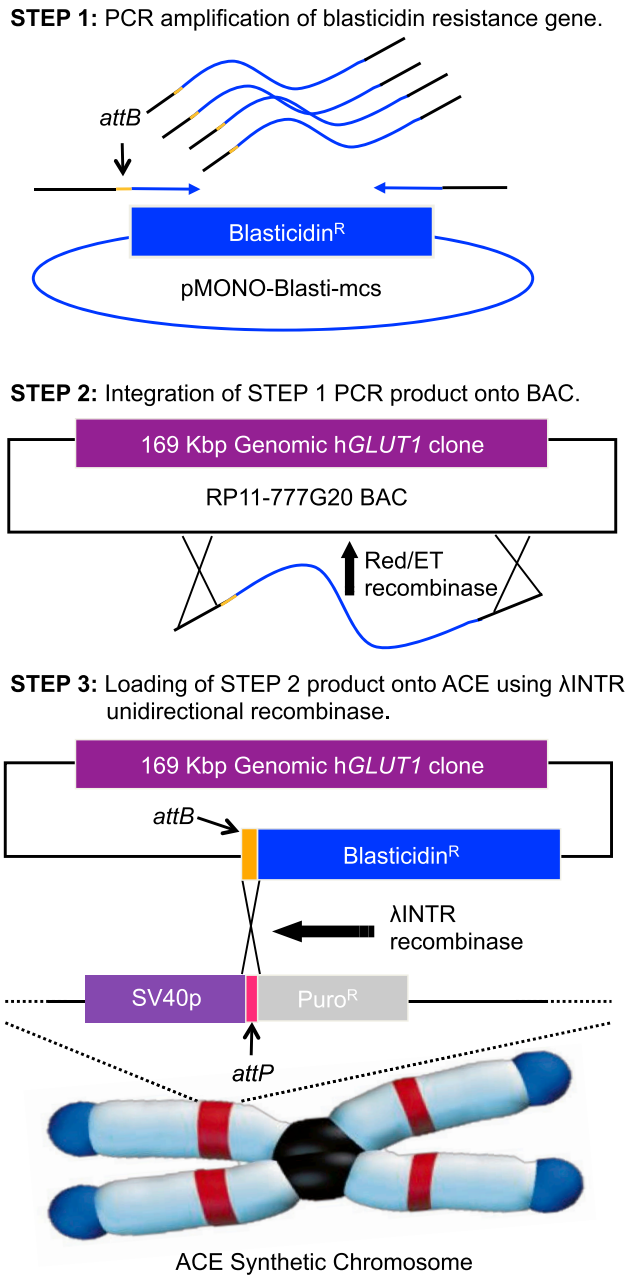


Figure 1. Genomic Hg*GLUT1* BAC Retrofitting and ACE Integration

The conversion of a BAC clone for loading onto a synthetic chromosome requires three steps. In the first step, the desired selectable marker gene (in this case, blasticidin resistance [Blasticidin^R] from pMONO-Blasti-mcs) is PCR-amplified with primers containing homology to the BAC/PAC vector backbone (black line) and the *attB* sequence (orange segment). Second, the BAC is retrofitted to carry the amplified PCR product containing the *attB* recombination site and the selectable marker gene from step 1 using Red/ET recombination. In the third step, the λINTR recombinase is used to site-specifically load the retrofitted BAC clone containing the *attB* site and Blasticidin^R resulting from step 2 onto the ACE synthetic chromosome.

system requires only the single ACE integrase and ACE loading vector to engineer multiple genes onto the ACE synthetic chromosome, alleviating the need for complicated synthetic chromosome designs incorporating multiple targeting vectors, each of which require unique site-specific recombination sites and recombinases.

In this report, we demonstrate successful engineering of the ACE synthetic chromosome with multiple large genetic payloads. The ACE synthetic chromosome was initially engineered to contain and express a copy of the human genomic *GLUT1* (*SLC2A1*) locus (Hg*GLUT1*), nearly 169 kbp in size and spanning both 5′ and 3′ regulatory elements, designated ACE$^{\mathrm{Hg}GLUT1}$. Moreover, a second large payload, the human genomic *MCT1* (*SLC16A1*) locus (Hg*MCT1*; approximately 144 kbp in size), was delivered onto ACE$^{\mathrm{Hg}GLUT1}$ for a combined total of over 300 kbp of genomic DNA delivered to the parent ACE synthetic chromosome. We confirmed the presence of all 10 Hg*GLUT1* exons and all 4 Hg*MCT1* exons on ACE$^{\mathrm{Hg}GLUT1/HgMCT1}$ as well as expression and correct splicing of the full-length Hg*GLUT1* and Hg*MCT1* transcripts. These results provide a proof-of-principle demonstration of loading multiple large genetic payloads onto the ACE synthetic chromosome with subsequent expression of engineered gene products.

RESULTS

The ACE synthetic chromosome was engineered to contain and express a genomic copy of the human *GLUT1* locus in a three-step process outlined in Figure 1. Bacterial artificial chromosomes (BAC/PACs) as well as vector controls, pBACe3.6 and pCYPAC2, were purchased from the BACPAC Resources Center, Children's Hospital Oakland Research Institute (BPRC, CHORI; Oakland, CA). RP11-777G20 is a 169-kbp BAC clone spanning the Hg*GLUT1* locus on chromosome 1 and includes 39 kbp upstream and 87 kbp downstream of the Hg*GLUT1* transcribed region. The sequences required for λINTR recombination between the ACE's *attP* site and an *attB* site on the BAC/PAC as well as the blasticidin resistance gene were PCR-amplified as a single product from pMONO-blasti-mcs (InvivoGen, San Diego, CA) using the primers BacRFitFor and BacRFitRev (Table S1; Figure 1, step 1). The PCR primer BacRFitFor encodes the *attB* sequence. The gel-purified 1.448-kbp PCR product was incorporated into the BAC/PACs using Red/ET recombination (Red/ET Recombination Kit, Gene Bridges, Heidelberg, Germany; Figure 1, step 2). Blasticidin-resistant bacterial colonies were selected and screened for the expected Red/ET recombination junctions, as shown in Figure 2A, using the primer sets BamHIFOR and *attB*REV for junction 1 (Figure 2B) and SV40polyAnFOR and SacBREV for junction 2 (Figure 2C). The sequences homologous to the SacB_REV and BamHI_FOR primers reside in the BAC/PAC vector, whereas the *attB*_REV and SV40PolyAn_FOR homologous sequences reside in the amplified *attB*BSR PCR product. Two of four candidates for the retrofitted Hg*GLUT1* BAC (RP11-777G20$^{attB\mathrm{BSR}}$) produced the expected PCR products for the novel Red/ET recombination junctions (Figures 2B and 2C, lanes 1–4), whereas 4 of 4 pBACe3.6$^{attB\mathrm{BSR}}$ candidates (Figures 2B and 2C, lanes 5–8) and 3 of 4 pCYPAC2$^{attB\mathrm{BSR}}$ candidates (Figures 2B and 2C, lanes 9–12)

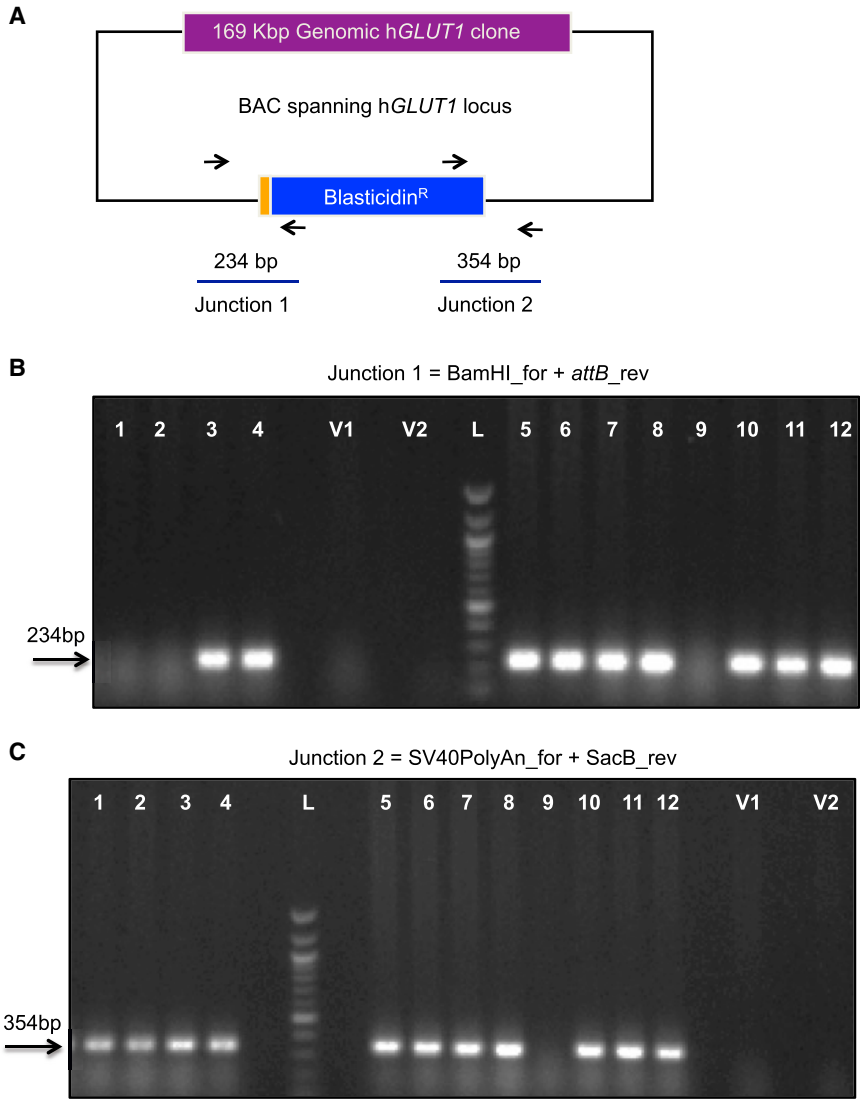


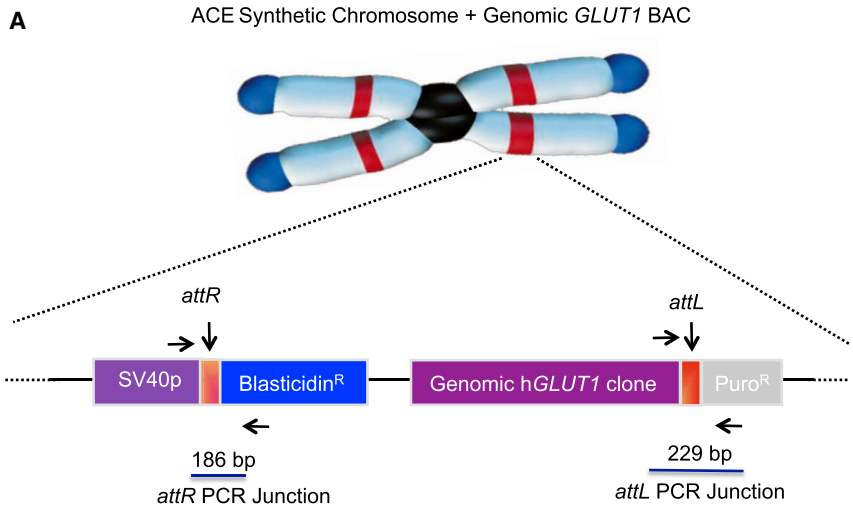
Figure 2. Analysis of BAC Red/ET Recombination Junctions

PCR analysis of the Red/ET recombination junctions to confirm incorporation of the *attB*-Blasticidin cassette into the BAC clones. (A) Location of primers used and predicted PCR products. (B) The primer pair used for PCR analysis of junction 1 was BamHI_FORand *attB*_REV with a 234 bp band expected. (C) The primer pair used for PCR analysis of junction 2 was SV40PolyAn_FOR and SacB_REV with a 354 bp band expected. DNA templates for the PCR reactions in (B) and (C) were as follows: lanes 1–4 were four RP11-777G20^{attBBSR} candidates; lanes 5–8 were four pBACe3.6^{attBBSR} candidates; lanes 9–12 were four pCYPAC2^{attBBSR} candidates. V1 and V2 represent parent vectors pBACe3.6 and pCYPAC2, respectively, as negative controls. L is the 100 bp ladder molecular weight marker. RP11-777G20^{attBBSR} candidates 3 and 4, all four pBACe3.6^{attBBSR} candidates, and pCYPAC2^{attBBSR} candidates 10–12 are positive for both junctions. RP11-777G20^{attBBSR} candidate 3 was loaded onto the ACE. Primers are listed in Table S1.

also displayed correct junctions. These retrofitted BACs/PACs were then amenable to loading onto the ACE via the *attB* recombination site and subsequent selection by the blasticidin drug-selectable gene.

One RP11-777G20^{attBBSR} BAC clone (Figures 2B and 2C, lane 3) and one pBACe3.6^{attBBSR} BAC (Figures 2B and 2C, lane 5) were chosen for expansion in liquid culture to produce adequate DNA for loading onto the ACE synthetic chromosome. Each BAC was co-transfected with a plasmid expressing the λINTR integrase (pCXLamIntROK[7]) into Y29-13D-SFS cells, a DG44 Chinese hamster ovary (CHO)-derived cell line containing the parental ACE synthetic chromosome, to site-specifically load each BAC onto an ACE synthetic chromosome (Figure 1, step 3). Four blasticidin-resistant clones from each of four independent transfections were expanded in culture to isolate genomic DNA and screened by PCR for site-specific recombination events (*attP* × *attB* recombination events), resulting in *attR* and *attL* junctions, as diagrammed in Figure 3A. PCR products from the *attR* and *attL* junctions (186 bp and 229 bp, respectively) were present in five of the 16 clones screened, confirming successful engineering of the ACE synthetic chromosome with RP11-777G20^{attBBSR} at a frequency of 31.25%. Junction PCR analysis of two of the five Hg*GLUT1* engineered clones is shown in Figures 3B and 3C, lanes 1 and 2. Both of the ACE candidates containing RP11-777G20^{attBBSR} in Figure 3 were analyzed further and also shown to contain all ten Hg*GLUT1* exons by PCR analysis using Hg*GLUT1* exon primer sets (listed in Table S1; Figure 4A, lane 3, depicts one of the two candidates for each exon). Finally, the presence of RP11-777G20^{attBBSR} on the ACE synthetic chromosome was confirmed by fluorescence *in situ* hybridization (FISH) colocalization of probes hybridizing to the Hg*GLUT1* exons and elements of the ACE synthetic chromosome (puromycin resistance gene). Figure 5 depicts the FISH analysis of the candidate in Figures 3B and 3C, lane 2. These results demonstrate that RP11-777G20^{attBBSR} correctly recombined onto the ACE synthetic chromosome; i.e., both end junctions were correct and contained the intervening sequences (all exons were present). Furthermore, RP11-777G20^{attBBSR} was only present on the ACE synthetic chromosome. We conclude that RP11-777G20^{attBBSR} was loaded onto the ACE synthetic chromosome in its entirety. This ACE synthetic chromosome containing RP11-777G20^{attBBSR} was named ACE^{HgGLUT1}, and the resultant cell line was designated DG44-ACE^{HgGLUT1}.

To demonstrate that multiple genomic payloads can be delivered sequentially onto an ACE synthetic chromosome, the ACE^{HgGLUT1}



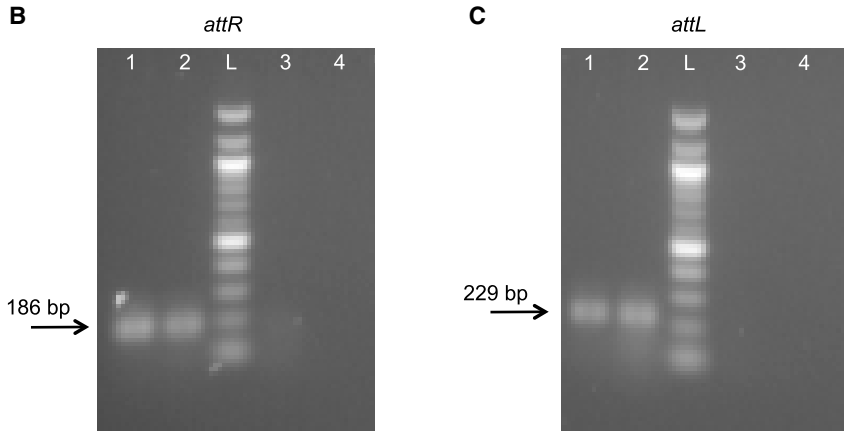


Figure 3. Analysis of λINTR Recombination Junctions between ACE and BAC

Shown is junction PCR for ACE loading of Hg*GLUT1* onto the synthetic chromosome with λINTR. (A) Diagram of PCR confirmation of λINTR-directed ACE integration of RP11-777G20^{attBBSR}. (B) The primer pair used for PCR analysis of the left (*attR*) recombination junction was 2010*attR*_R and 2010*attR*_L with a 186-bp PCR product expected. (C) The primer pair used for PCR analysis of the right (*attL*) recombination junction was 2010*attL*_F and 2010*attL*_L with a 229 bp PCR product expected. Template DNAs for the PCR reactions were as follows: lanes 1 and 2, genomic DNA from two independent candidates of DG44-ACE loaded with RP11-777G20^{attBBSR}; lane 3, Y29-13D-SFS; lane 4, water control. L is the 100 bp ladder molecular weight marker. The candidates are positive for both junctions, indicating correct loading of RP11-777G20^{attBBSR} onto the ACE. Primers are listed in Table S1.

synthetic chromosome was engineered to contain a second large payload for a combined total of over 300 kbp of genomic DNA delivered to the ACE synthetic chromosome. A BAC clone spanning the Hg*MCT1* locus, RP11-1151D18, was purchased from BPRC, CHORI. RP11-1151D18 is 144 kbp and contains 52 kbp upstream and 48 kbp downstream of the Hg*MCT1* transcribed region, respectively, in addition to the Hg*MCT1* coding sequences. In a manner similar to that shown in Figure 1 for Hg*GLUT1*, the Hg*MCT1* BAC and the pBACe3.6 parent vector were retrofitted by Red/ET recombination to incorporate the *attB* site necessary for integration onto ACE$^{Hg GLUT1}$ and, in this case, zeocin resistance for positive selection following loading in the DG44-ACE$^{Hg GLUT1}$ cell background. PCR amplification of a fragment containing the *attB* recombination site and a GFP-zeocin resistance gene fusion transcript was performed using linearized pSELECT-GFPzeo (InvivoGen, San Diego, CA) as a template and the PCR primers Hg*MCT1*_RfitLower and Hg*MCT1*_RfitUpper (Table S1). The *attB* sequence was incorporated into the Hg*MCT1*_RfitUpper primer used in the PCR amplification. Following Red/ET recombination and zeocin selection in *E. coli*, integration of the *attB* recombination site and zeocin resistance gene on the BACs was confirmed using PCR amplification with primers specific to the novel junctions formed by Red/ET recombination with the *attB*-GFP-zeocin construct: BamHIFor and attB_Rev for junction 1 and BetaGlo_pAnFor and 3′SacBRev for junction 2 (Table S1; data not shown). The retrofitted BAC candidates of RP11-1151D18^{attBGFPzeo} were also analyzed for the presence of the four Hg*MCT1* exons using PCR primers specific to the Hg*MCT1* exons (Table S1). Figure 4B depicts one of four candidates tested for the four *MCT1* exons by PCR analysis and confirms their presence.

E. coli carrying BAC candidates of RP11-1151D18^{attBGFPzeo} or pBACe3.6^{attBGFPzeo} were selected and expanded in liquid culture for isolation of DNA and subsequent ACE$^{Hg GLUT1}$ loading by co-transfection into DG44-ACE$^{Hg GLUT1}$ of pCXLamIntROK[7] and BAC DNA from either RP11-1151D18^{attBGFPzeo} or pBACe3.6^{attBGFPzeo}. Following selection on zeocin and expansion in culture, genomic DNA was isolated from 12 drug-resistant candidates and screened by PCR for site-specific recombination, resulting in two novel junctions formed: *attR*, 2010_*AttR*_L, and GFP_Rev and *attL*, AQ751307.1For, and Puro_Rev for RP11-1151D18^{attBGFPzeo} or BamHI_For and Puro_Rev for pBACe3.6^{attBGFPzeo} (Table S1). Two of the 12 clones had both *attL* and *attR* junctions for a loading efficiency of 16.67%. Figure 6 depicts the expected PCR products for the *attR* and *attL* junctions at 365 and 904 bp, respectively, for one of the clones. Genomic DNA prepared from zeocin-resistant RP11-1151D18^{attBGFPzeo} transfected clones with correct *attR* and *attL* junction PCR products (designated DG44-ACE$^{Hg GLUT1/Hg MCT1}$ candidate clones) were further screened for the presence of Hg*MCT1* and Hg*GLUT1* exons using the appropriate PCR primer sets (Table S1; Figure 4). This analysis confirmed that one candidate maintained

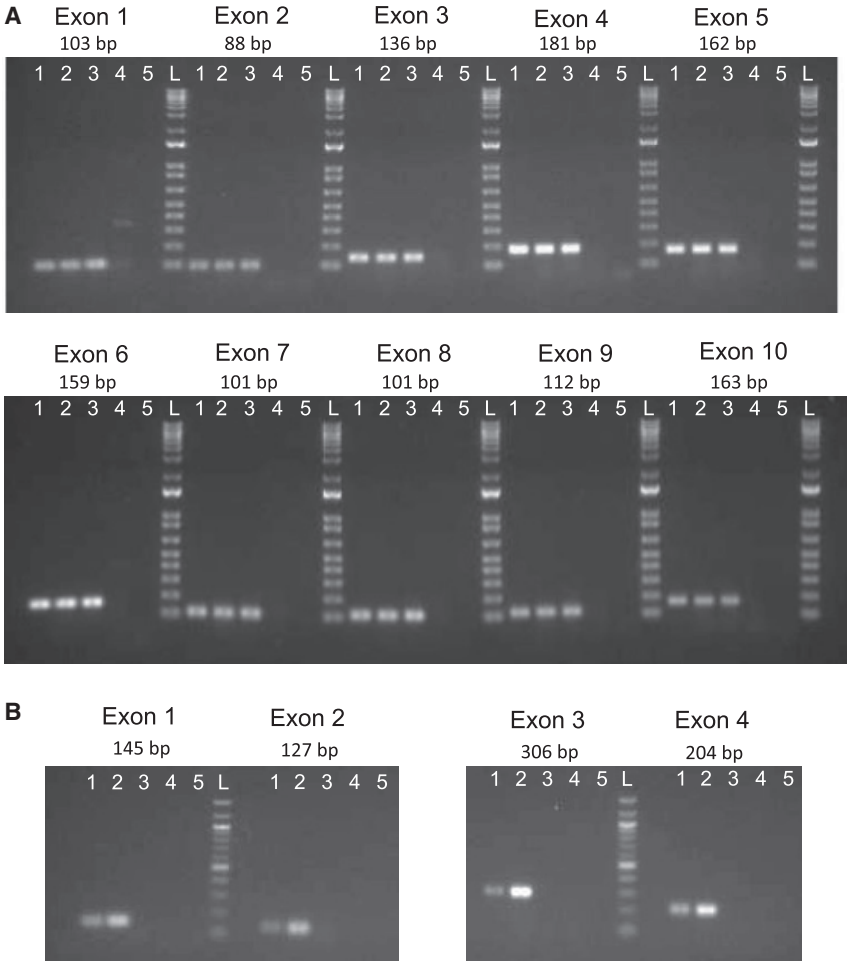


Figure 4. Retention of All Hg*GLUT1* and Hg*MCT1* Exons Confirmed by PCR

(A) Hg*GLUT1* exon PCR analysis for the presence of exons 1–10. Each primer set for each exon was used with the following templates: RP11-777G20^{attBBSR} BAC DNA (1), DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$ candidate genomic DNA (2), DG44-ACE$^{Hg\mathit{GLUT1}}$ genomic DNA (3), Y29-13D-SFS genomic DNA (4), and distilled water (dH_2O) (5). L1, Invitrogen 1 Kbp TrackIt Ladder; L2, NEB low-molecular-weight ladder. Primers are listed in Table S1. (B) Hg*MCT1* exon PCR for exons 1–4. Each Hg*MCT1* exon primer set was used with the following templates: RP11-1151D18^{attBGFPzeo} BAC DNA (1), DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$ candidates genomic DNA (2), DG44-ACE$^{Hg\mathit{GLUT1}}$ genomic DNA (3), Y29-13D-SFS genomic DNA (4), and dH_2O control (5). L is the 100 bp molecular weight marker. Primers are listed in Table S1.

the ten Hg*GLUT1* exons and incorporated the four Hg*MCT1* exons, indicating that the loading of the 144-kbp retrofitted Hg*MCT1* BAC onto ACE$^{Hg\mathit{GLUT1}}$ was achieved for a total payload of over 300 kbp. Engineering of this double loaded ACE, ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$, successfully demonstrates the ability to load multiple large DNA fragments, each greater than 100 kbp in size, onto a single ACE.

To further confirm the presence of both Hg*GLUT1* and Hg*MCT1* on the ACE synthetic chromosome of DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$, FISH was used to assess the localization of the two genomic BAC sequences. Probes to Hg*MCT1* and Hg*GLUT1* were applied to metaphase spreads of the DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$ cell line. The colocalization of FISH probe signals for the Hg*GLUT1* and Hg*MCT1* genes to a single chromosome, shown in Figure 7, further demonstrates that dual loading was accomplished. These results indicate that the capacity of the ACE synthetic chromosome exceeds 300 kbp, although its upper capacity limit remains to be determined.

To demonstrate correct expression from the Hg*GLUT1* and Hg*MCT1* loci on the engineered ACE, total RNA was isolated from DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$ and Y29-13D-SFS, the parental ACE synthetic chromosome-containing cell line, and used to prepare cDNA. This cDNA was used as template in two PCR reactions to amplify the complete coding sequence from both Hg*GLUT1* and Hg*MCT1* using primers homologous to the 5′ and 3′ UTRs (Hg*GLUT1*: *GLUT1*xpt_For, *GLUT1*xpt_Rev; Hg*MCT1*: *MCT1*xpt_For, *MCT1*xpt_Rev; Table S1). PCR products corresponding to the correct sizes (1.667 kbp and 1.833 kbp, respectively) were obtained from DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$ (Figure 8), demonstrating correct splicing of the Hg*GLUT1* and Hg*MCT1* transcripts. The PCR products amplified with PrimeStar, a high-fidelity DNA polymerase, were sequenced (Hg*GLUT1*: h*SLC2A1*Ex5_F, h*SLC2A1*Ex5_R, h*SLC2A1*Ex8_F, h*SLC2A1*Ex8_R; Hg*MCT1*: h*SLC16A1*Ex2_For, h*SLC16A1*_Ex2Rev, h*SLC16A1*_Ex3For, h*SLC16A1*_Ex3Rev; Table S1, see footnote) and shown to contain wild-type Hg*GLUT1* and Hg*MCT1* sequences. The Hg*MCT1* sequence contained the common T1470A SNP (rs1049434). [12,13] These results confirm that neither gene acquired mutations during the engineering process and that both were properly spliced from ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$. Taken together, these results demonstrate that both RP11-777G20^{attBBSR} and RP11-1151D18^{attBGFPzeo} retained their original genomic organization throughout the engineering process.

DISCUSSION

Cell and gene therapy research directed toward monogenic disorders and select cancers have resulted in ascertainable, curative therapeutic endpoints. [14–16] However, based on historical research in human genetic diseases and codified by the information gleaned from the Human Genome Project, it is apparent that multifactorial (polygenic and environmentally influenced) disorders are much more frequent than monogenic disorders. [17] Although the viral and non-viral delivery vehicles utilized to treat monogenic disorders have provided a valuable engineering blueprint for gene- and cell-based therapies, these tools

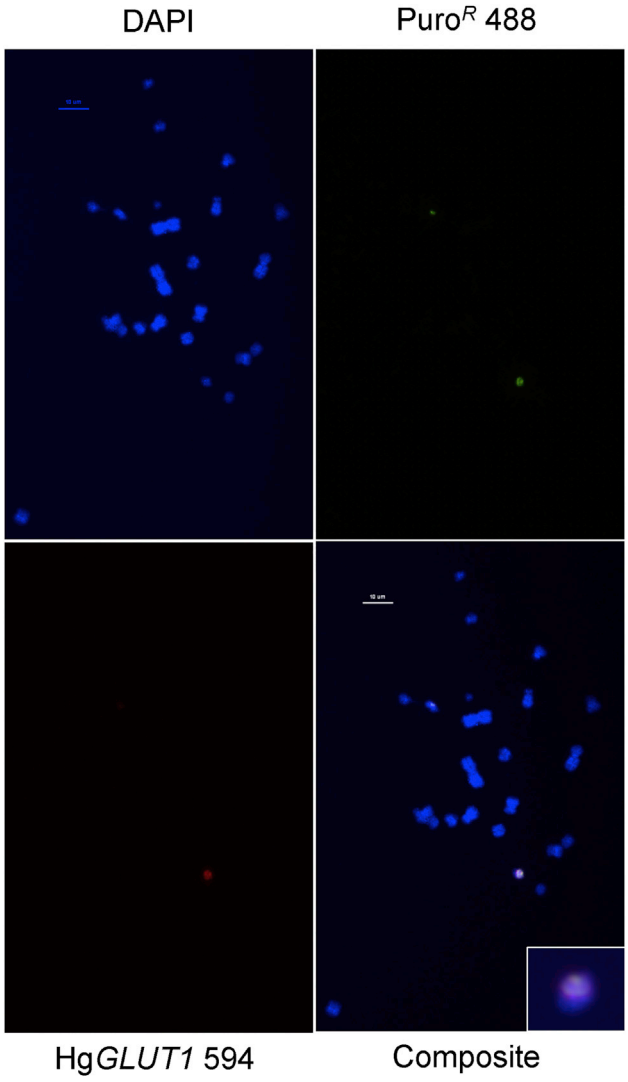


Figure 5. Hg*GLUT1* BAC Colocalizes to the ACE

Shown is fluorescent *in situ* hybridization analysis of the ACE synthetic chromosome engineered with the retrofitted Hg*GLUT1* BAC clone RP11-777G20$^{attB\text{BSR}}$ in the DG44 engineering cell line (DAPI). Hybridization probes were generated to the puromycin resistance gene (PuroR; Alexa Fluor 488 signal) and Hg*GLUT1* BAC, RP11-777G20$^{attB\text{BSR}}$ (Hg*GLUT1*; Alexa Fluor 594 signal). Composite is the overlay of the three panels, indicating colocalization of the Hg*GLUT1* BAC RP11-777G20$^{attB\text{BSR}}$ with the PuroR located on the ACE. The resultant engineered chromosome was named ACE$^{\text{Hg}GLUT1}$, and the resultant cell line was designated DG44-ACE$^{\text{Hg}GLUT1}$. Two-color hybridization and signal capture via fluorescence microscopy were performed as described previously.[7] Scale bar, 10 μm.

are insufficient to address complex, polygenic diseases because of the limited amount of genetic payload that can be delivered as well as the need to find "safe" genomic sites of integration to maintain stability.[18]

For coordinated production and expression of multiple proteins directed to the treatment of polygenic disorders, polycistronic vector systems incorporating internal ribosome entry site (IRES)[19,20] elements or self-cleaving peptides[21,22] have been utilized to build combinatorial arrays of multigene constructs.[23–25] Although these systems are capable of producing a polycistronic mRNA, their utility is limited to the production of only a few proteins under the control of synthetic promoters. Furthermore, the delivery of these combinatorial arrays is reliant on current viral and non-viral delivery vehicles with their associated limitation of payload capacity and, in the case of integrating viral vectors, the liability of insertional mutagenesis.

In this report, we demonstrate for the first time that the ACE platform chromosome can be sequentially engineered with extra-large genomic fragments in a predictable manner as a first step toward the construction of complex synthetic gene circuits incorporating native regulatory elements. For this, the ACE chromosome was engineered to contain a genomic copy of Hg*GLUT1*, approximately 169 kbp in size, including 5′ and 3′ regulatory regions with a loading frequency of 31.25%. Moreover, a second large payload, Hg*MCT1*, was delivered onto ACE$^{\text{Hg}GLUT1}$ for a combined total of over 300 kbp of genomic DNA bioengineered onto the ACE synthetic chromosome, with a loading efficiency of 16.67%. In previous work, we demonstrated a loading efficiency of 100% for a 5-kbp ACE targeting vector. Here the loading vectors were more than 100 kbp in size; thus, the stoichiometry between the size of the DNA fragment and *att*B sites is significantly different. Future work will focus on determining the factors that affect loading efficiency. However, the efficiencies demonstrated here are easily managed for screening to identify clones with correct integration junctions. Any clones not meeting integration junction screening criteria were not analyzed further. We confirmed the presence of all Hg*GLUT1* (10) and Hg*MCT1* (4) exons on the ACE$^{\text{Hg}GLUT1/\text{Hg}MCT1}$ synthetic chromosome by PCR as well as expression of the h*GLUT1* and h*MCT1* transcripts. These results provide a proof-of-principle demonstration of loading multiple large genetic payloads onto the ACE synthetic chromosome with subsequent expression of engineered gene products. Importantly, both large payloads retained their organization, and neither acquired mutations during the engineering process. In addition, loading of the Hg*MCT1* BAC clone introduced an allele of the Hg*MCT1* locus (A1470T) associated with endurance and blood lactate levels after intensive exercise.[12,13,26]

The ACE engineering system provides a tractable engineering platform for transferring large (>100 kbp) genetic payloads with native genetic architecture onto a synthetic chromosome. Although other MAC platforms have been developed for multiple loadings onto a synthetic mammalian chromosome,[2,4,6,27,28] these previously described platforms required the creation of multiple vectors with different site-specific recombination systems to achieve multiple loadings and have not reached the engineerable carrying capacity demonstrated here. For example, Honma et al.[28] have demonstrated loading of genomic DNA up to 39 kbp in size. Alternatively, Hoshiya et al.[29] have used homologous recombination-proficient chicken DT40 cells to engineer a human chromosome limited to the Duchenne muscular dystrophy locus (2.4 Mbp) by repeated

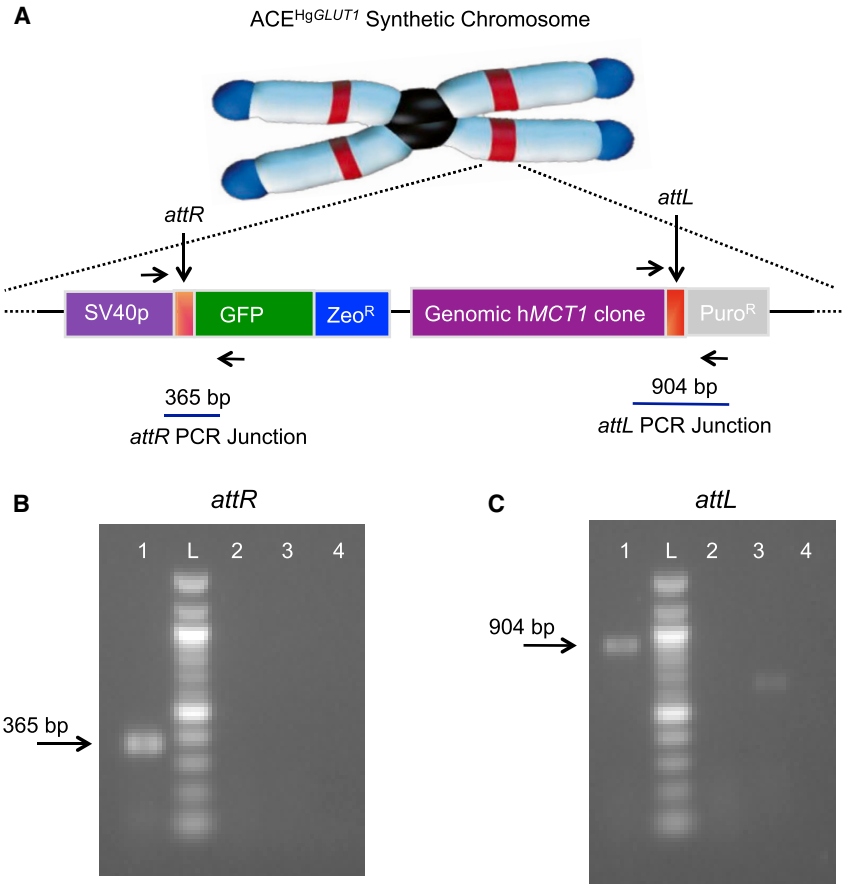


Figure 6. PCR Confirmation of ACE Integration of the *attB*GFPzeo-Retrofitted Hg*MCT1* BAC

(A) Diagram of ACE$^{attB\text{GFPzeo}}$ λINTR recombination junctions. RP11-1151D18 (144 kbp) was purchased from BPRC, Chori and engineered to contain *attB*-GFPzeo to confer selection in DG44-ACE$^{Hg\textit{GLUT1}}$ cells. (B) The primer pair used for PCR analysis of the left (*attR*) recombination junction was 2010*attR*_L and GFPRev with a 365 bp PCR product expected. (C) The primer pair used for PCR analysis of the right (*attL*) recombination junction was AQ741307.1_For and PuroRev with a 904 bp PCR product expected. Template DNAs for PCR cycling were DG44-ACE$^{Hg\textit{GLUT1}/Hg\textit{MCT1}}$ candidate genomic DNA in lane 1 and negative controls in lanes 2–4, DG44-ACE$^{Hg\textit{GLUT1}}$ genomic DNA, Y29-13D-SFS genomic DNA, and dH_2O, respectively. L is the 100 bp molecular weight marker. Primers are listed in Table S1.

truncation of the chromosome. In the approach presented here, the use of a single uni-directional, site-specific ACE integrase system mitigates the need for an admixture of multiple recombination and/or integration systems. Because the *attP* recombination acceptor site is 245 bp, the presence of a pseudosite in the human genome is less probable than recombination systems using minimal *attP* sites (e.g., ΦC31 minimal *attP* sites have over 100 pseudosites in the human genome[30]). Furthermore, a BLAST search of the human, mouse, and CHO genomes with the 245-bp *attP* site returned no matches. Thus, the probability of off-target integration events is extremely unlikely. Furthermore, the ability to flow-sort-purify ACE chromosomes to high purity allows the subsequent transfer of engineered ACE chromosomes into a variety of cell types, circumventing the need for microcell transfer methodology.[7,8] Finally, by engineering the ACE in the host DG44 cell background prior to flow sorting and transfer to the recipient (e.g., patient) cell background, any off-target effects and resulting toxicities are nullified. The upper limit of the capacity of the ACE platform, in both numbers of sequential loadings as well as final combined payload, remains to be tested.

MACs circumvent many of the limitations associated with plasmid- and viral-based gene expression systems and provide an alternative means to introduce large payloads of genetic information into cells as an autonomously replicating, non-integrating, chromosome-based vector platform. Furthermore, MACs allow the engineering of large segments of genomic DNA, such as fragments containing long-range genetic elements required for appropriate regulation of gene expression, developmentally regulated multigene loci, or multiple copies of two or more genes in fixed stoichiometry. In contrast to current viral and non-viral delivery vehicles, MACs provide the scalability (a predictable manner to construct complex genetic circuits) and orthogonality (the ability to alter engineering system components without influencing the performance of the therapeutic components[18]) necessary for the development of a multi-therapeutic approach to target complex genetic disease. In addition, MACs provide a genetic focal point by which unlinked mammalian genetic loci can be brought into linkage disequilibrium. Here we demonstrate that the ACE platform chromosome provides a predictable designer therapeutic approach for efficient, tractable bioengineering of large genomic fragments onto a portable chromosome platform as a tool to address polygenic disorders.

MATERIALS AND METHODS

BAC Retrofitting with Red/ET Recombination

BACs were retrofitted to include an *attB* recombination site and a drug resistance gene cassette within the BAC vector backbone for site-specific loading onto the synthetic chromosome and drug selection as diagrammed in Figure 1, steps 1 and 2. RP11-777G20 was retrofitted with the blasticidin resistance gene (BSR), amplified from NotI-linearized pMONO-blasti-mcs, using primers containing BAC homology, *loxP511*, and *attB* sequences (primers BACRFitFor and BACRFitRev; Table S1). The zeocin resistance gene used to retrofit RP11-1151D18 was amplified from PacI linearized pSELECT-GFPzeo using primers containing BAC homology, *loxP511*, and *attB* sequences (primers Hg*MCT1*_RfitUpper and Hg*MCT1*_RfitLower;

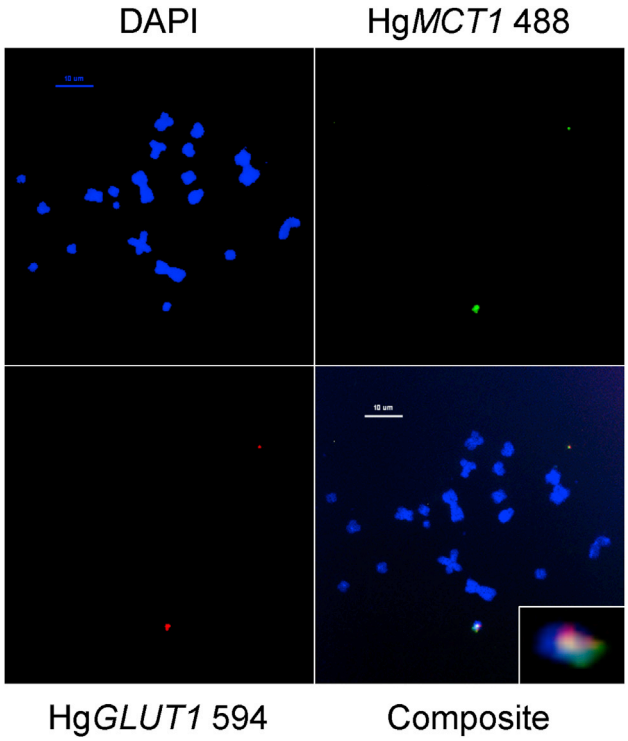


Figure 7. Fluorescence *In Situ* Hybridization Analysis of DG44-ACE$^{Hg\mathit{GLUT1}}$ Cells Engineered with the Retrofitted Hg*MCT1* BAC RP11-1151D18$^{\mathit{attB}GFPzeo}$

Shown is mitotic spread of the DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$ cell line (DAPI). Hybridization probes were generated to the Hg*MCT1* BAC RP11-1151D18$^{\mathit{attB}GFPzeo}$ (Hg*MCT1* exons; Alexa Fluor 488 signal) and the Hg*GLUT1* BAC RP11-777G20$^{\mathit{attB}BSR}$ (Hg*GLUT1*; Alexa Fluor 594 signal). Composite is the overlay of the three panels, indicating colocalization of Hg*GLUT1* with Hg*MCT1*. The inset is a magnified view of the colocalization of the signals. Two-color hybridization and signal capture via fluorescence microscopy were performed as described previously.[7] Scale bar, 10 μm.

Table S1). A two-step PCR reaction was performed with 3 cycles at the initial annealing temperature (Ta; 60°C for pMONO-blasti-mcs and 59°C for pSELECT-GFPzeo) and 32 cycles at the final Ta (68°C for pMONO-blasti-mcs and 72°C for pSELECT-GFPzeo) using Phusion High Fidelity DNA Polymerase (New England Biolabs). The 1.448-kbp *attB*-IRESBSR PCR product was gel-purified (QIAGEN QIAEX II Gel Extraction Kit; Hilden, Germany) according to the manufacturer's protocol and then used to retrofit RP11-777G20 as outlined below using Red/ET recombination. The 1.77-kbp *attB*-GFPzeo PCR product was gel-purified (Promega, Madison, WI; Wizard SV Gel and PCR Clean-Up System) according to the manufacturer's protocol and then used to retrofit RP11-1151D18 as outlined below using Red/ET recombination.

Gene Bridges' Red/ET Kit (Quick and Easy BAC Modification Kit, K001) was used according to the manufacturer's protocol. In brief, electroporation was used to transform each BAC-containing *E. coli* strain with the Gene Bridges' pRed/ET vector containing the tetracycline resistance gene and the Red/ET protein expression gene. Transformed cells were selected by tetracycline resistance (3 μg/mL tetracycline) at 30°C. Two tetracycline-resistant (Red/ET vector-containing) transformants for each BAC-containing *E. coli* strain were subsequently transformed in duplicate with the appropriate PCR fragment containing the *attB* recombination site and BAC-specific drug resistance gene by electroporation as follows. Cells for the duplicate transformations were grown with or without 10% arabinose because the presence of arabinose induces production of the bacteriophage lambda Red/ET proteins, which direct homologous recombination between the PCR product and the BAC vector backbone. Duplicate transformation without arabinose served as a negative control because the Red/ET recombination proteins were not present. Drug selection on Luria broth (LB) agar plates with both blasticidin (100 μg/mL) and chloramphenicol (12.5 μg/mL) was used to obtain colonies with proper integration of the 1.448-kbp *attB*-IRESBSR PCR product onto the RP11-777G20 BAC backbone. Zeocin-resistant bacterial colonies containing RP11-1151D18 with proper integration of the 1.718-kbp *attB*-GFP-zeocin PCR product were selected by plating the transformation to LB agar plates with zeocin (25 μg/mL; InvivoGen, San Diego, CA) and chloramphenicol (12.5 μg/mL). All transformation plates were incubated at 37°C. Four drug-resistant colonies for each retrofitted BAC-containing strain were selected for expansion in liquid culture for further testing.

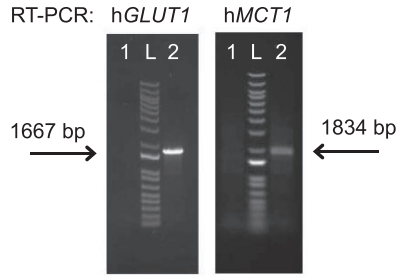


Figure 8. Hg*GLUT1* and Hg*MCT1* Expression from the ACE Synthetic Chromosome

cDNA prepared from the DG44 cell line containing the unaltered ACE (1) or the DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$ cells (2) was used as template in PCR reactions using specific primer sets for Hg*GLUT1* or Hg*MCT1* that span each gene from the respective 5′ UTR to the 3′ UTR. Primers are listed in Table S1. Hg*GLUT1* and Hg*MCT1* products of the correct size were detected in the DG44-ACE$^{Hg\mathit{GLUT1}/Hg\mathit{MCT1}}$ cDNA template but not in the parental Y29-13D-SFS cDNA template. These results indicate expression of both human Hg*GLUT1* and Hg*MCT1* transcripts from the ACE synthetic chromosome. L is the 1 kbp TrackIt ladder.

PCR Confirmation of Red/ET Recombination Junctions

Proper Red/ET recombination junctions were confirmed by colony PCR using Promega (Madison, WI) 2X GoTaq Master Mix with the following primer sets at the designated Ta for 35 cycles: RP11-777G20$^{\mathit{attB}BSR}$, 5′ junction (BamHIFor + *attB*rev, Ta = 57°C) and 3′ junction (SV40polyAnFor + 3′SacBrev, Ta = 53°C); RP11-1151D18$^{\mathit{attB}GFPzeo}$, 5′ junction (BamHI_For + *attB*_Rev, Ta = 57°C) and 3′ junction (BetaGlopAnFor + 3′SacBRev, Ta = 57°C). Primers are listed in Table S1. PCR was carried out in a Bio-Rad T100

Thermocycler using the following conditions: step 1: 95°C for 2 min; step 2: cycles 1–35, 95°C for 30 s, Ta for 30 s, and 72°C for 25–30 s; step 3: 72°C for 2 min. Correctly retrofitted BACs were expanded in LB liquid culture supplemented with 12.5 μg/mL chloramphenicol and either 25 μg/mL blasticidin for RP11-777G20$^{attB\text{BSR}}$ or 25 μg zeocin for RP11-1151D18$^{attB\text{GFPzeo}}$. BAC DNA was isolated using a Sigma-Aldrich (St. Louis, MO) PhasePrep BAC DNA Kit for use in ACE loading (Figure 1, step 3).

Cell Culture and Transfections

Cells were maintained in a 37°C incubator in the presence of 5% CO_2. Y29-13D-SFS is a DG44 CHO-derived cell line containing the parental ACE synthetic chromosome provided by Calyx Bio-Ventures (https://calyxbio.com/).[7] This cell line and cell lines derived thereof were grown in Gibco MEM Alpha medium with nucleosides and glutamine (Gibco catalog number 12571-063; Thermo Fisher Scientific, Waltham, MA) supplemented with 5% HyClone FetalClone III serum (GE Healthcare, Chicago, IL), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Corning, Corning, NY; 100× penicillin and streptomycin), subsequently referred to as "complete MEM Alpha" cell culture medium. Complete MEM Alpha culture medium was also supplemented with the appropriate antibiotics as required for ACE synthetic chromosome selection: Y29-13D-SFS, 5 μg/mL puromycin (InvivoGen, San Diego, CA); DG44-ACE$^{\text{Hg}GLUT1}$, 5 μg/mL puromycin and 3 μg/mL blasticidin (InvivoGen, San Diego, CA); DG44-ACE$^{\text{Hg}GLUT1/\text{Hg}MCT1}$, 5 μg/mL puromycin and 500 μg/mL zeocin (InvivoGen, San Diego, CA).

For targeted integration onto the ACE synthetic chromosome in the DG44 cell line, the cells were plated at a density of 500 to 1,000 cells per well of a 24-well culture dish (so that the cell density reaches 50%–80% confluency by the time of transfection) 1 day prior to transfection with Lipofectamine LTX with Plus Reagent (Invitrogen, Carlsbad, CA). On the day of transfection, the medium was aspirated from the cells and replaced with antibiotic-free Alpha MEM medium supplemented with 5% HyClone FetalClone III. For each well transfected, 0.5 μg of BAC DNA and 0.2 μg of the ACE Integrase expression vector, pCXLamIntROK,[7] were complexed with 1 μl PLUS reagent in 100 μL Opti-MEM reduced serum medium (Gibco, Thermo Fisher Scientific, Waltham, MA), followed by addition of 3 μL of Lipofectamine LTX, and the complex was incubated with cells according to the manufacturer's recommendations. Each transfected well was subsequently expanded to a 10-cm^2 culture dish 24 h post-transfection. Selection medium containing 3 μg/mL blasticidin plus 5 μg/mL puromycin or 250 μg/mL zeocin plus 5 μg/mL puromycin (depending on the version of BAC used) was added the following day (i.e., 48 h post-transfection), and the medium was changed every 2–3 days thereafter. After 14 days of incubation under selection, drug-resistant colonies were harvested via a cloning ring and further expanded for analysis.

PCR for Assessment of Loading a BAC onto the ACE

To assess proper λINTR recombination junctions (*attL* and *attR*), genomic DNA was prepared from 50,000 to 200,000 cells of each candidate cell line using either the Promega (Madison, WI) Wizard SV Genomic DNA Purification System or the QIAGEN DNeasy Blood and Tissue Kit (Hilden, Germany) for use in the PCR assay. A 10 μL PCR reaction mix consisting of 1× GoTaq Green Master Mix (Promega, Madison, WI), 0.25 μM each forward and reverse primers, and 20–100 ng template was used to analyze the junctions of candidate cell lines following the targeted integration event. The primer sets and Ta used to screen the recombination junctions are listed in Table S1. PCR was carried out using the following conditions in a Bio-Rad (Hercules, CA) T100 Thermocycler: step 1: 95°C for 3 min; step 2, cycles 1–35, 95°C for 30 s, Ta°C for 30 s and 72°C for 30 s; step 3: 72°C for 2 min.

PCR was used to assess the presence of the exons, associated with the targeted integration event, in a 10 μL reaction mix consisting of 1× GoTaq Green Master Mix (Promega, Madison, WI), 0.5 μM each forward and reverse primers, and 50–150 ng template. The primer sets and Ta for each exon are listed in Table S1. For the Hg*GLUT1* exons, PCR was carried out using the following conditions in a Bio-Rad (Hercules, CA) T100 Thermocycler: step 1: 95°C for 2 min; step 2: cycles 1–40, 95°C for 30 s, Ta°C for 30 s, and 72°C for 20 s; step 3: 72°C for 5 min. For the Hg*MCT1* exons, PCR was carried out using the following conditions in a Bio-Rad (Hercules, CA) T100 Thermocycler: step 1: 98°C for 5 min; step 2: cycles 1–40, 95°C for 30 s, Ta for 15 s, and 72°C for 30 s; step 3: 72°C for 5 min.

FISH

Conventional single-color and two-color FISH analyses and high-resolution FISH were carried out using PCR-generated probes from Hg*GLUT1* exons 1–10, pPUR*attP*, and Hg*MCT1* exons 1–4 as follows.

For PCR generation of FISH probes, the following 25-μL reaction mix was used: 20 pg linearized DNA template (RP11-777G20 for Hg*GLUT1*, RP11-1151D18 for Hg*MCT1*, or pPUR*attP* for the ACE); 1× Taq ThermoPol buffer (NEB); 200 μM each of dATP, dCTP, and dGTP; 130 μM of dTTP, 70 μM biotin-16-2'-deoxyuridine 5'-triphosphate (dUTP) or DIG-11-dUTP (Roche Biosciences); 200 nM forward and reverse primers; and 4.5 U of Taq DNA polymerase (NEB). The primer sets and Ta for amplifying FISH probes are listed in Table S1. PCR was carried out using the following conditions: step 1: 95°C for 2 min; step 2: cycles 1–35, 95°C for 30 s, Ta°C for 30 s, and 72°C for 20 s; step 3: 72°C for 10 min. The extension time was increased to 1 min to produce the 618 bp product from the pPUR*attP* template. Following PCR production of a probe, unincorporated nucleotides were removed using a QIAquick PCR Cleanup Kit (QIAGEN) or Monarch PCR & DNA Cleanup Kit (NEB).

Actively dividing cells were treated with 10 μg/mL Karyomax for 10–18 h for metaphase arrest and then harvested by centrifugation at 200 relative centrifugal force (RCF) for 7 min in preparation for metaphase spreads. Pelleted cells were resuspended in PBS, pelleted by centrifugation at 200 RCF (7 min), resuspended in 5–10 mL of 75 mM KCl, and incubated at 37°C for 4 min. The swollen cells

were then collected by centrifugation at 100 RCF, KCl was removed, and the pellet was resuspended in 5 mL cold fixative (3:1 methanol: acetic acid). Fixed cells were collected again by centrifugation at 100 RCF, the fixative was removed, and the pellet was resuspended in fresh cold fixative and stored at −20°C. For metaphase spreads, 30 μL of cells in fixative were dropped to an angled (∼45°), cold glass slide prewet with fixative. The slide was then placed in a humidified chamber for 1–3 min and air-dried for 10 min. Finally, the slides were "aged" by placing them in a 65°C slide chamber (MJ Research) overnight prior to initiating probe hybridization.

Hybridization and detection of sequence-specific DNA probes complementary to the ACE backbone and/or λINTR targeted genomic sequences were performed as follows. Prepared slides containing metaphase spreads were treated for 20 min with 10 μg/mL RNaseA at 37°C and then rinsed twice for 2 min each time in PBS at room temperature (RT). The slides were then dehydrated through a series of ethanol solutions (70%, 85%, and 100%) for 2 min each at RT, followed by denaturation for 2 min at 70°C in 70% formamide/2× saline sodium citrate (SSC). Finally, the slides were again denatured in a cold ethanol series (70%, 85%, and 100%) for 2 min each and air-dried prior to applying the probe.

Approximately 100 ng of each labeled probe (Hg*GLUT1* (digoxigenin; detected with Alexa Fluor 595 nm) plus either pPUR*attP* or Hg*MCT1* (biotin; detected with Alexa Fluor 488 nm)) was applied to metaphase spreads on glass slides, which were then coverslipped and incubated overnight at 37°C in a humidified chamber. TSA signal amplification (Invitrogen, Carlsbad, CA) was carried out prior to visualization according to the manufacturer's protocol in the following manner to allow for 2-color FISH. Post-hybridization, each slide was washed twice for 8 min each time in 2× SSC at 42°C, washed twice for 8 min each time in 50% formamide/2× SSC at 42°C, washed for 2 min in PBS at RT, blocked by application of 100 μL 1% blocking reagent (TSA kit #5; Invitrogen, catalog number T20915) in 1× PBS, and coverslipped and incubated for 30 min at RT. The coverslip was removed and the blocking reagent was drained, and then 100 μL of a 1/400 dilution of mouse anti-digoxigenin antibody (Roche, catalog number 11 333 062 910) in 1% blocking reagent was applied. The coverslip was applied and incubated at 30°C in a humid chamber, washed 3 times for 5 min each time in 1× PBS at 42°C, and excess wash solution was drained. 100 μL of a working solution of goat anti-mouse-horseradish peroxidase (HRP) conjugate (TSA kit #5) was applied, and the coverslip was applied and incubated for 30 min at RT in a humid chamber. It was washed 3 times for 5 min each time in 1× PBS at 42°C (the remaining steps and storage of the finished slides were carried out in a darkened environment). 100 μL tyramide (594 nm) in amplification buffer with 0.0015% H_2O_2 (TSA kit #5) was applied. The coverslip was applied and incubated for 5–10 min at RT in a humid box, washed 3 times for 5 min each time in 1× PBS at 42°C, and excess wash solution was drained and 1% peroxide was applied. The coverslip was applied and incubated for 15 min at RT in a humid box, washed 3 times for 5 min each time in 1× PBS at RT, and excess wash solution was drained and 1% blocking reagent in 1× PBS was applied. The coverslip was applied and incubated for 30–60 min at RT in a humid box. Excess Blocking Reagent was drained, and 100 μL working solution of HRP-streptavidin conjugate was applied (TSA kit #22; Invitrogen, catalog number T20932). The coverslip was applied and incubated for 30 min at RT in a humid chamber, washed 3 times for 5 min each time in 1× PBS at 42°C, and 100 μL tyramide 488 nm in amplification buffer with 0.0015% H_2O_2 was applied (TSA kit #22). The coverslip was applied and incubated for 10 min at RT in a humid box, washed 3 times for 5 min each time in 1× PBS at 42°C, and the slide was air-dried (∼10 min). 30 μL Vectashield Antifade Mounting Medium with DAPI was applied (Vector Laboratories, Burlingame, CA; catalog number H1200. The coverslip was applied, and the slide was stored at 4°C. Slides were viewed with a Nikon 80i fluorescence microscope, and images were acquired with Nikon Elements BR imaging software on a Nikon DS-QI1 camera.

RT-PCR and Sequencing

Total RNA was isolated (RNeasy Mini Kit, QIAGEN) from Y29-13D-SFS control cells, a DG44 CHO-derived cell line containing the ACE synthetic chromosome, and the DG44-ACE$^{\mathrm{Hg}GLUT1/\mathrm{Hg}MCT1}$ cell line containing the Hg*GLUT1* and Hg*MCT1* loci. First-strand cDNA was prepared according to the manufacturer's protocol using the Applied Biosystems (Foster City, CA) High Capacity cDNA Reverse Transcription Kit with random primers or the LunaScript RT SuperMix Kit (NEB, Waltham, MA) with random hexamer and poly-dT primers following the manufacturers' recommendations. Gene-specific PCR products were amplified from the cDNA with gene-specific primers to Hg*GLUT1* (1.667 kbp) spanning from the 5′ UTR through exon 10 to the 3′ UTR (*GLUT1*xpt_For and *GLUT1*xpt_Rev; Table S1) or to Hg*MCT1* (1.833 kbp) spanning from the 5′ UTR through exon 4 to the 3′ UTR (*MCT1*xpt_For and *MCT1*xpt_Rev; Table S1) using 1 μL of first-strand cDNA template. For confirmation of the expected PCR product size from correctly spliced transcripts, Promega GoTaq DNA polymerase was used with an Ta of 55°C and the following cycling conditions: step 1: 95°C, 2 min; step 2: 35 cycles (95°C, 30 s; 55°C, 30 s, 72°C, 2 min); step 3: 72°C, 5 min. PrimeStar HS DNA Polymerase (Clontech, Mountain View, CA) was used to amplify the transcripts for sequence analysis. *MCT1* amplification required 35 cycles (98°C for 10 s, 55°C for 5 s, 72°C for 2 min), whereas *GLUT1* amplification required only 30 cycles (98°C for 10 s; 55°C for 5 s; 72°C for 2 min) to produce sufficient product for gel purification. The resulting PrimeStar HS Polymerase PCR products were assessed by gel electrophoresis for product presence and size, gel-purified using the NEB (Ipswich, MA) Monarch Gel Purification Kit, and sequenced (University of Texas Austin DNA Sequencing Facility, Austin, TX) using the primers indicated in Table S1 (see footnotes a and b).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.04.006.

AUTHOR CONTRIBUTIONS

Conceptualization, A.G. and E.P.; Methodology, A.G. and E.P.; Validation, A.G., E.P., D.B., and K.P.; Formal Analysis, A.G., E.P., D.B., and K.P.; Investigation, A.G., E.P., D.B., and K.P.; Writing – Original Draft, A.G., E.P., and D.B.; Writing – Review & Editing, A.G., E.P., D.B., and K.P.; Visualization, A.G., E.P., D.B., and K.P.; Supervision, A.G. and E.P.; Project Administration, A.G. and E.P.; Funding Acquisition, A.G. and E.P.

CONFLICTS OF INTEREST

A.G. and E.P. each own over 10% interest in SynPloid Biotek, LLC.

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