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A Novel and Stable Mouse Artificial Chromosome Vector

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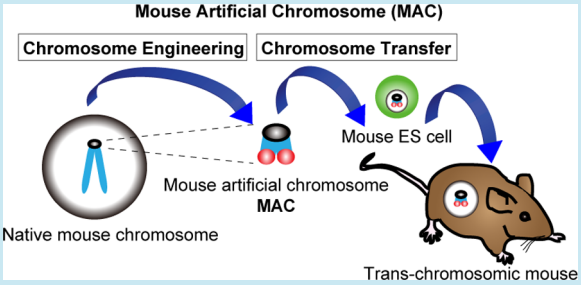
Supporting Information

ABSTRACT: Human chromosome fragments (hCFs) and human artificial chromosomes (HACs) can be transferred into mouse ES cells to produce trans-chromosomic (Tc) mice. Although hCFs and HACs containing large genomic DNAs can be autonomously maintained in Tc mice, their retention rate is variable in mouse ES cell lines and Tc mouse tissues, possibly because of centromere differences between the species. To improve the retention rate of artificial chromosomes in mouse cells, we constructed novel mouse artificial chromosome (MAC) vectors by truncating a natural mouse chromosome at a site adjacent to the centromeric region. We obtained cell clones containing the MAC vectors that were stably maintained in mouse ES cells and various tissues in Tc mice. The MACs possess acceptor sites into which a desired gene or genes can be inserted. Thus, Tc mice harboring the MAC vectors may be valuable tools for functional analyses of desired genes, producing humanized model mice, and synthetic biology.



KEYWORDS: *human artificial chromosome (HAC), mouse artificial chromosome (MAC), trans-chromosomic mouse, humanized model mouse, chromosome engineering*

Random integration of exogenous DNA into the host genome is a conventional technique for obtaining stable transgenic (Tg) mammalian cells and mice. Although the Tg technique has been used successfully to introduce genes of interest into mammalian cell lines and experimental animals, it presents some problems, i.e., the size of the DNA fragment to be transferred and its integration into the host genome, which may cause position-dependent effects.[1,2] Thus, the transgene is governed by unexpected position effects, and the genomic context of the host chromosome is abolished by integration of the transgene. Bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs) have a cloning capacity of hundreds of kilobase pairs of DNA in size,[3] but their copy number and integration into the host genome potentially affect both the transgene and the host genome.[4−7] Furthermore, it is difficult to transfer Mb-sized genomic regions with PACs and BACs. Although yeast artificial chromosomes (YACs) can carry large-sized fragments, i.e., approximately up to 1 Mb,[3] such large genomic inserts are often unstable and undergo recombination.[8]

To circumvent these limitations, the use of human chromosome fragments (hCFs) as vectors for introducing large genomic regions of human DNA into mice was demonstrated.[9,10] Transferred hCFs were maintained as extra chromosomes in somatic cells and Tc mice. The introduced human genes on hCFs were expressed under appropriate tissue-specific regulation. Thus, human artificial chromosomes (HACs) have been constructed for use as universal transgene vectors. The two major approaches for the construction of HACs are known as "top-down" and "bottom-up".[1,11−15] In the top-down approach, specific human chromosomes are successively truncated into mini-chromosomes by means of telomere-directed chromosome truncation at a specific region in homologous recombination-proficient chicken DT40 cells.[11,16,17] Such HACs have been used to introduce extremely large genomic DNAs into mammalian cells for the production of Tc mice, stem-cell therapy, and protein production.[9,18,19] In contrast, bottom-up HACs multimerize during chromosome formation, and because they can include both centromere and telomeric repeats as well as gene sequences,[1] they do not form predictable structures. However, the recent demonstration that single genes can be incorporated into *de novo* HACs comprising

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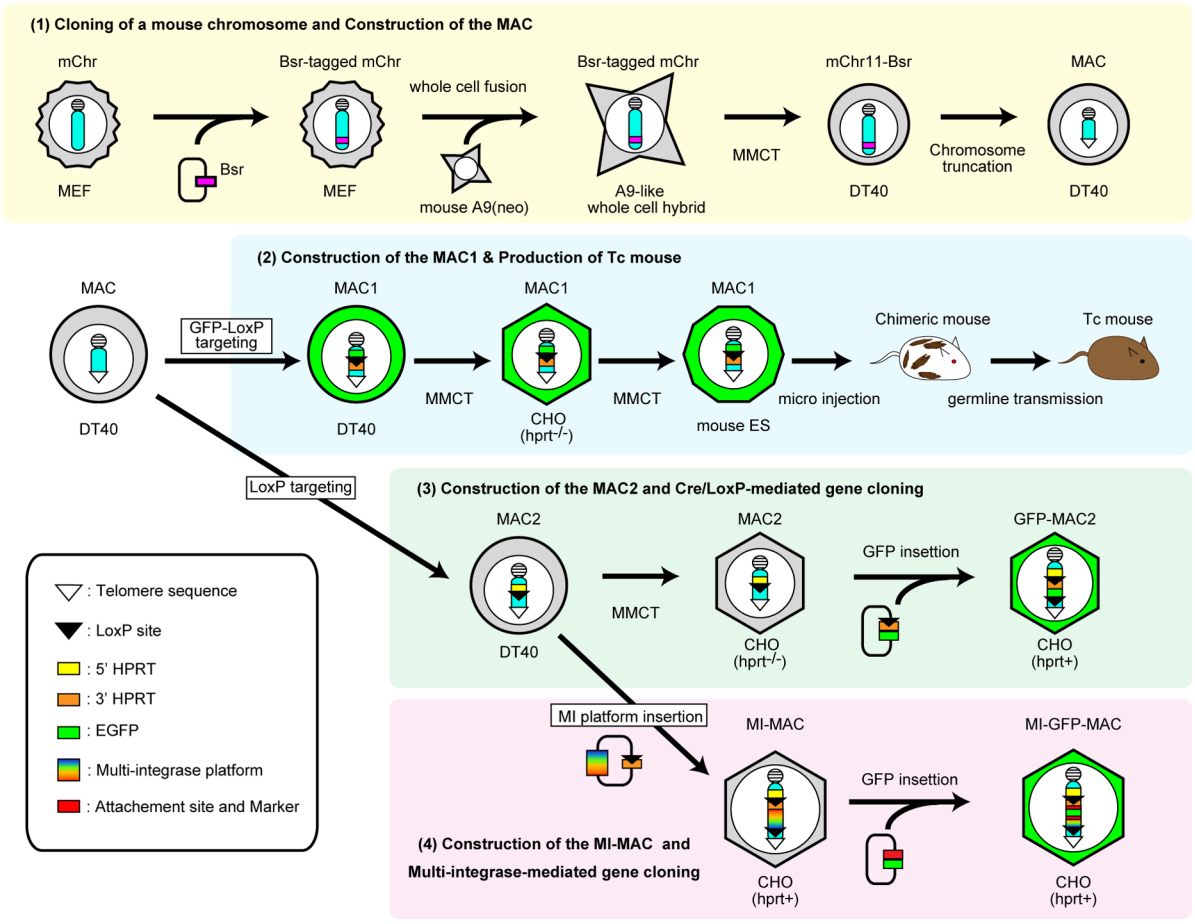

Figure 1. Schematic diagram outlining the construction of MAC vectors. (1) Cloning of a mouse chromosome and construction of the MAC vector. Mouse embryonic fibroblasts (MEFs) containing the BSr-tagged mouse chromosome (mChr-Bsr) were fused with mouse A9 cells. The mChr-Bsr was transferred from the whole cell hybrids into DT40 cells by microcell-mediated chromosome transfer (MMCT), and the DT40 microcell hybrid clones containing the BSr-tagged mouse chromosome 11 were designated DT40(mChr11-Bsr). Chromosome manipulation was performed in the homologous recombination-proficient DT40 microcell hybrids. The distal q-arm was deleted from the mChr11-Bsr by telomere-directed truncation. This mini-chromosome was designated as the mouse artificial chromosome (MAC). (2) Construction of the MAC1 vector and production of Tc mice. The desired gene can be sequentially cloned into a specific site of the MAC vector in DT40 cells by homologous recombination. The *EGFP* gene containing a loxP site was targeted in the MAC vector to monitor the existence of the MAC vector. The MAC vector containing a loxP site and the *EGFP* gene was designated as MAC1. The MAC1 was transferred into mouse embryonic stem (ES) cells via CHO cells for subsequent studies. To investigate the stability of the MAC vector, chimeric mice were produced from the ES cells containing the MAC1. The F1 mice were obtained by mating between chimeric and wild-type mice. (3) Construction of the MAC2 vector and Cre/loxP-mediated gene cloning. For site-directed insertion of a circular DNA using the Cre/loxP system, the 5′-*HPRT*-loxP plasmid was targeted to a proximal region of the q-arm at the BX572640 locus of the MAC vector in DT40 cells. The MAC vector containing a loxP site was designated as MAC2. The MAC2 vector was transferred into CHO cells, and the circular vector can be cloned into the MAC2 in CHO(hprt−/−) cells by Cre-loxP-mediated gene insertion and *HPRT* gene reconstitution. In this study, an *EGFP* gene was cloned into the MAC2 vector. (4) Construction of the MI-MAC vector and multi-integrase-mediated gene cloning. A multi-integrase (MI) platform was inserted into MAC2 with the Cre/loxP system. The MI-MAC vector was generated as a multiple gene delivery vector.

synthetic centromeric DNA has increased their utility and potential application.[20,21]

In some cases, the hCFs and the HACs can be transmitted through the mouse germline, resulting in the establishment of novel mouse strains (trans-chromosomic (Tc) mice) containing heritable hCFs and HACs.[9,22] Thus, such Tc mice are considered useful for overcoming the size constraints of cloned transgenes in conventional Tg mice and to facilitate functional studies of the human genome. The retention rates of hCFs and HACs were shown to be variable in Tc mouse tissues.[9,10,23] In particular, the retention rate in hematopoietic cells of the bone marrow and spleen was extremely low.[10] A high retention rate is important for accurately determining the function of a gene of interest in Tc mice. The stable artificial chromosomes are better suited for this purpose.

Hadlaczky's group generated a murine satellite DNA-based artificial chromosome (mSATAC) from mouse chromosome 7 by the targeted amplification of pericentric heterochromatin and centromeric DNA (50 to approximately 450 Mb in size). The mSATAC used in their study, for which they analyzed the stability and germline transmission in mice, contained approximately 60 Mb of DNA and was composed of a centromere, a telomere, blocks of murine satellite repeats, two regions of heterologous DNA including 6−10 copies of a reporter gene (*β*-galactosidase), and a marker gene (hygromycin phosphotransferase).[24,25] Mice were generated by pronuclear microinjection of the isolated mSATAC. The

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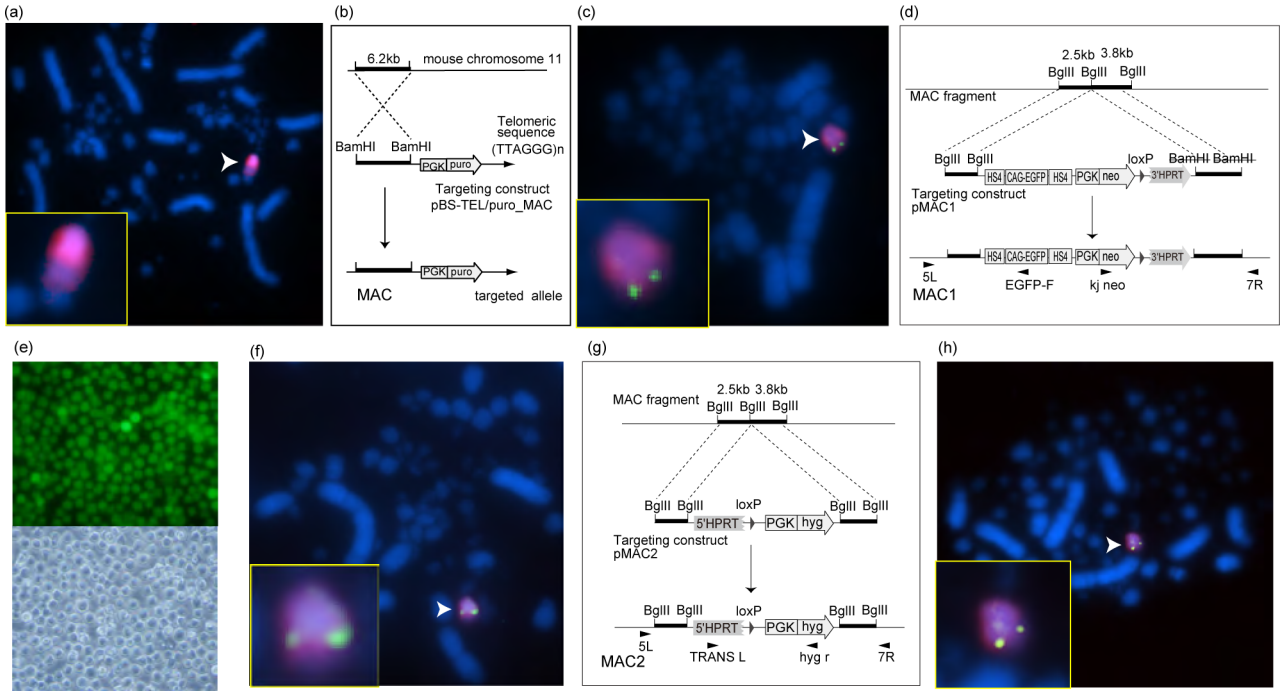

Figure 2. Strategy for the targeted truncation and construction of MAC vectors. (a) Fluorescence *in situ* hybridization (FISH) analysis with a digoxigenin-labeled mouse Cot-1 DNA (red) identified the intact mouse chromosomes in the DT40 cells. The arrowheads indicate an intact mouse chromosome. The inset shows an enlarged image of the intact mouse chromosome. The chromosomal DNA was counterstained with DAPI, and DAPI was used for the same purpose in the following FISH experiments. (b) Strategy for the targeted truncation of the distal region of mouse chromosome 11 at the AL671968 locus by the targeting vector, pBS-TEL/puro_MAC. The telomere-seeding vector was electroporated into DT40 cells containing mChr11-Bsr to yield puromycin-resistant transfectants. (c) A two-color FISH probe comprising the mouse Cot-1 DNA (red) that hybridizes with the MAC and puromycin-resistant gene (green) localized to the distal end of truncated MAC. The arrowhead indicates the MAC fragment, and the inset shows an enlarged image. (d) Strategy for the targeted integration of the *EGFP* gene with 3′ *HPRT*-loxP into the BX572640 locus, the locus proximal to the truncated site in the MAC vector, by the targeting vector, pMAC1. Arrowheads indicate genomic PCR primers. (e) Electroporation of the pMAC1 plasmid yielded GFP-expressing, G418-resistant transfectants from the DT40 (MAC)-1 cells. Fluorescence (top panel) and phase-contrast (bottom panel) micrographs are shown. (f) FISH analysis of the MAC1 vector in the DT40 (MAC1) cells using the digoxigenin-labeled mouse Cot-1 DNA (red) and the biotin-labeled EGFP gene (green). The arrowhead indicates MAC1, and the inset shows enlarged images of MAC1. (g) Strategy for the targeted integration of a loxP site and 5′ HPRT into the BX572640 locus, the locus proximal to the truncated site on the MAC vector, by the targeting vector, pMAC2. Arrowheads indicate genomic PCR primers. (h) FISH analysis of the MAC2 vector in the DT40 (MAC2) cells using the digoxigenin-labeled mouse Cot-1 DNA (red) and the biotin-labeled hygromycin gene (green). The arrowhead indicates the MAC2 vector, and the inset shows enlarged images of the MAC2 vector.

mSATAC was transmissible through the germline, and the retention rate of the mSATAC was approximately 60% in the lymphocytes of both the founder and its progeny.[26]

Brown's group reported that a mini-chromosome, ST1, derived from the human Y chromosome, is linear, has a molecular weight of approximately 4.5 Mb, and contains incidentally acquired mouse major and minor satellites as well as human DNA, including tandemly repeated alphoid DNA sequences.[12,27] The ST1 is stable in several cell lines, including mouse ES cells, mouse fibroblasts, chicken DT40 cells, and human fibrosarcoma HT1080 cells.[28] The ST1 was transmitted to progeny and a high retention rate was expected, but the retention rate was not uniform among tissues. In liver and prostate, the ST1 showed a high retention rate of >90%, while it was around or below 60% in testis and kidney.[23] From these studies, it was thought that the mouse centromere sequence was required to stabilize artificial chromosomes and hCFs in mouse cells. However, these studies also suggested that both mSATACs with an unpredictably amplified and rearranged mouse centromere and ST1 with incidentally acquired mouse centromere sequences may not exhibit uniform and high stability in mouse tissues.

A native centromere may be predicted to improve artificial chromosome stability in the target species, but an incidentally formed centromere structure did not function well in mouse tissues. Thus, we hypothesized that a vector with a mouse native centromere would be highly stable in mouse tissues. Although the stability of our HAC, which contains a native centromere from human chromosome 21, was unknown in human tissues, considerably high stability of the HAC in a variety of human cultured cells also led us to this hypothesis. For the first time, we report here the construction of a mouse artificial chromosome (MAC) vector derived from a natural mouse chromosome by a controlled top-down approach. To use the MAC as a functional gene delivery vector, we investigated whether the MAC vector faithfully segregated after its transfer to mouse ES cells and in Tc mice. We also developed several gene insertion systems on the MAC for functional analysis of desired genes and efficient animal transgenesis.

RESULTS AND DISCUSSION

Generation of the Mouse Artificial Chromosome (MAC) Vector. We performed the following experiments to construct MAC vectors by chromosome engineering. The

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overall strategy for vector construction is shown schematically in Figure 1. A mouse chromosome from mouse embryonic fibroblasts (MEFs) was cloned into DT40 cells as follows. First, a drug-resistance marker, the blasticidin S-resistance gene (*BSr*), was randomly integrated into the host chromosomes of MEFs by transfection. We produced whole cell hybrids between mouse A9 cells and MEFs containing a *BSr*-tagged chromosome. At present, microcell-mediated chromosome transfer (MMCT) is the most commonly used method to introduce a chromosome or a chromosome fragment into recipient cells.[29,30] Microcell formation in the donor cells is an important factor for the efficiency of MMCT. However, microcell formation in the primary cultured MEFs following colcemid treatment was negligible. Microcells can be efficiently generated in mouse A9 cells cultured in colcemid-containing medium.[30,31] Thus, MEFs containing a *BSr*-tagged mouse chromosome were fused with neomycin-resistant mouse A9 cells, and morphologically A9-like whole cell hybrids were obtained by double selection in medium containing both blasticidin S and G418. Then, the *BSr*-tagged mouse chromosome was transferred to DT40 cells by MMCT, because DT40 cells exhibit a high frequency of homologous recombination between an exogenous DNA template and its chromosome counterparts.[32] Fluorescence *in situ* hybridization (FISH) analysis with digoxigenin-labeled mouse Cot-1 DNA showed that a mouse chromosome was independently present in DT40 cells (Figure 2a). Multicolor FISH analysis with a MetaSystems probe indicated that the cloned mouse chromosome in DT40 cells was mouse chromosome 11 (mChr11-Bsr) (Supplementary Figure S1a,b).[33] The DT40 cells contained no other mouse chromosomes. Mouse chromosome 11 was confirmed by genomic PCR using DNA isolated from the DT40 cells with specific primers that amplified homologous regions for targeting vectors (data not shown). In this study, we performed multiple chromosome transfer experiments. Cytogenetic (FISH) and molecular (genomic PCR) analyses were performed to confirm that rearrangement did not occur after each chromosome transfer.

To remove all endogenous genes on the mouse chromosome, the distal q arm of mouse chromosome 11 was deleted in DT40 cells by telomere-associated chromosome truncation. The p arm on acrocentric mouse chromosome is short, and the satellites at the centromere are linked to the telomere. Thus, there was no need to truncate the p arm. We constructed a targeting plasmid containing the homologous sequence of an adjacent proximal region (AL671968; accessible in the NCBI database) of mouse chromosome 11, a puromycin-selectable marker gene, and 1 kb of TTAGGG telomeric sequence repeats (Figure 2b). The constructed plasmid, pBS-TEL/puro_MAC, was transfected into DT40(mChr11-Bsr) cells, and 115 puromycin-resistant transfectants were isolated. If the expected modification occurred, the BSr-resistant gene integrated on mouse chromosome 11 would be simultaneously removed, such that positive clones became sensitive to blasticidin S. Thus, we identified five blasticidin S-sensitive clones by subculturing cells with blasticidin S. FISH analysis performed with the digoxigenin-labeled mouse Cot-1 DNA probe and the biotin-labeled puro DNA probe indicated that the intact mouse chromosome 11 was truncated in the distal region of the mouse centromere following introduction of the pBS-TEL/puro_-MAC plasmid (Figure 2c). The digoxigenin-labeled probe was co-localized to the independently segregating truncated chromosome only, and no insertion or translocation was observed (Figure 2c). These results suggested that the majority of the q arm of mouse chromosome 11 was successfully deleted by telomere seeding. Two clones, no. 1 and no. 2, were obtained from these experiments. This mouse chromosome fragment was designated as the mouse artificial chromosome (MAC), and the DT40 cells containing the MAC fragment were designated as DT40(MAC), DT40(MAC)-1, and DT40-(MAC)-2.

Targeted Integration of *EGFP* and loxP Sites into the MAC. We constructed two types of targeting plasmid. The first plasmid contained the fluorescent marker, *EGFP*, to simplify further analysis of the stability of the MAC vector in mouse, and also a loxP site (Figure 2d). The second plasmid contained an insertion site that allows us to clone desired DNAs into the MAC vector via the Cre/loxP system (Figure 2g). Intra- and intermolecular recombination between two loxP sites is efficiently promoted by Cre recombinase.[34] For inserting an exact copy number of any exogenous sequence into the MAC vector, the Cre/loxP system was employed.[35] The Cre/loxP system enabled a circular DNA and the large genomic sequence of a chromosome region to be inserted or translocated into the MAC vector. The first targeting plasmid, pMAC1, containing the neomycin resistance gene, the *EGFP* gene, and the 3′ hypoxanthine phosphoribosyl transferase (*HPRT*) gene, was transfected into DT40(MAC)-1 cells (Figure 2d), and 14 G418-resistant transfectants that expressed EGFP were isolated and confirmed by genomic PCR analyses (Figure 2e). FISH analysis revealed that the mouse Cot-1 DNA-derived signal independently localized to the MAC fragment, and the neomycin-resistant gene-derived signal localized to the distal region of the MAC fragment (Figure 2f). From these analyses, the successful targeting event was identified in two clones (no. 52 and no. 58). The targeted MAC fragment carrying the *EGFP* gene with a loxP site was designated as the MAC1 vector. The DT40 cells containing the MAC1 vector were designated as DT40(MAC1). DT40(MAC1)-52 and DT40(MAC1)-58 cells were used for subsequent experiments. These results suggest that the desired gene can be cloned into the MAC by homologous recombination in DT40 cells. Previously, desired genes were inserted sequentially into the 21HAC using homologous recombination systems.[36] Thus, the sequential gene cloning using the system can be applied to the MAC.

The second targeting plasmid, pMAC2, contained a 5′ *HPRT* gene and the hygromycin drug-resistant marker gene (Figure 2g). The 5′-*HPRT*-loxP type plasmid has been used previously to insert a circular DNA or translocate a chromosome fragment during the construction of human artificial chromosome (HAC) vectors.[36−39] The 5′-*HPRT*-loxP type plasmid enables the MAC vector to use the same DNA or chromosome fragments as the HAC vector without modifying its construction. The constructed plasmid was transfected into DT40(MAC)-1 cells and 45 hygromycin-resistant transfectants were screened by genomic PCR analyses. Two clones were identified from eight randomly selected clones by FISH analysis with the digoxigenin-labeled mouse Cot-1 DNA probe and the biotin-labeled hygromycin gene probe (Figure 2h). The targeted MAC fragment carrying the 5′-*HPRT*-loxP site was designated as the MAC2 vector. DT40 cells containing the MAC2 vector were designated as DT40(MAC2). DT40-(MAC2)-5 and DT40(MAC2)-17 were used for subsequent experiments. By seeding an adjacent site of the centromeric region with the telomere as described above, the majority of the q arm of mouse chromosome 11 was deleted except for the

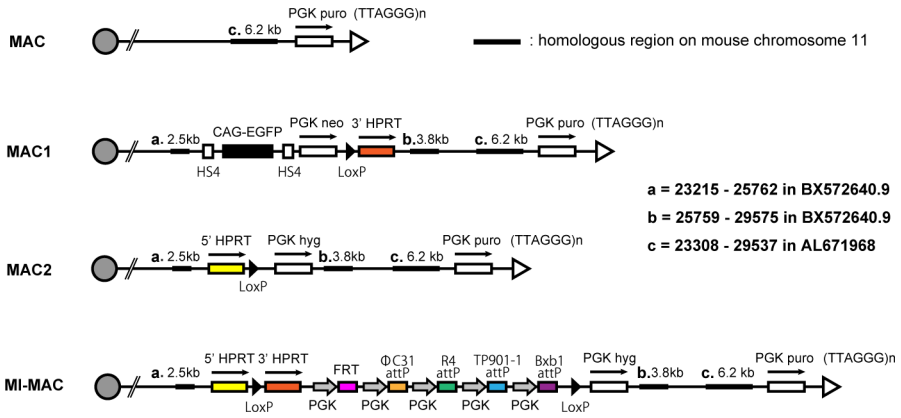

Figure 3. Detailed maps of the MACs. (a–c) Homologous regions on mChr.11 for gene targeting. *EGFP*-neo-3′ *HPRT*-loxP and 5′ *HPRT*-loxP-*hyg* were inserted into MAC1 and MAC2, respectively. The targeting vectors are described in Figure 2. The multi-integrase MAC (MI-MAC) vector is described in Supplementary Figure 2.

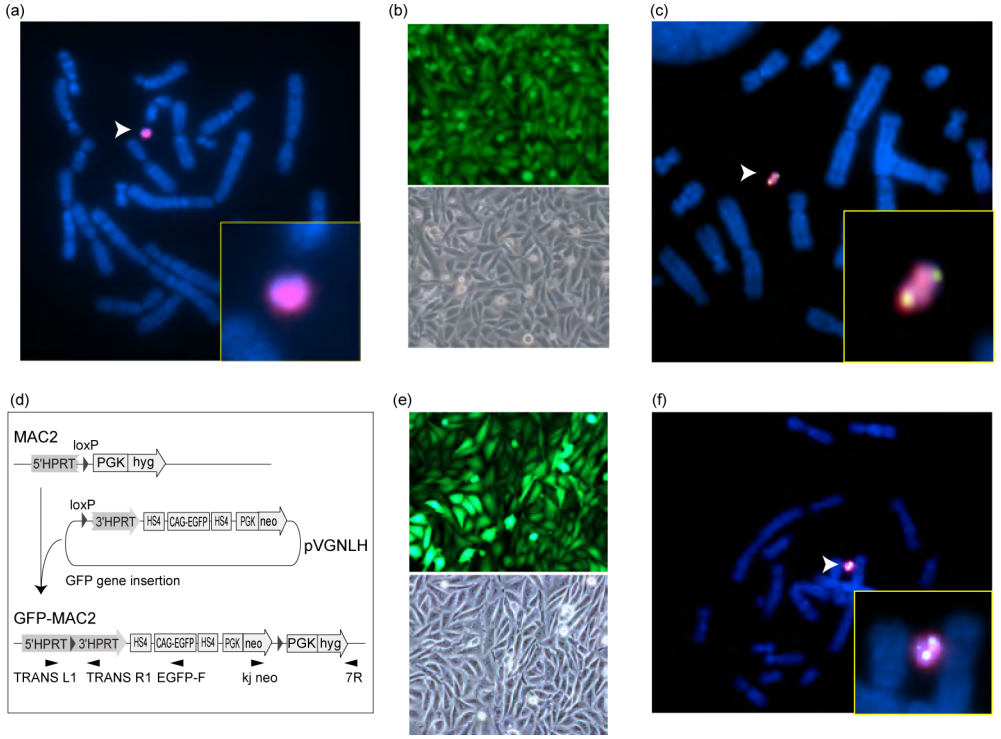

Figure 4. MMCT of the MAC1 and MAC2 vectors into CHO cells, and insertion of the *EGFP* gene into the MAC2. (a) FISH analysis of the MAC1 in HPRT-deficient Chinese hamster ovary (CHO) cells was performed with the digoxigenin-labeled mouse Cot-1 DNA (red). The arrowhead indicates MAC1, and the inset shows enlarged images. (b) CHO cells expressed EGFP on the MAC1 vector. (c) FISH analysis of the MAC2 vector in HPRT-deficient CHO cells was performed with the digoxigenin-labeled mouse Cot-1 DNA (red) and a biotin-labeled hygromycin gene (green). The arrowhead indicates the MAC2, and the inset shows enlarged images of the MAC2. (d) Strategy for the site-specific insertion of the *EGFP* gene into the MAC2 vector using the Cre/loxP-mediated system in CHO cells containing the MAC2. The arrowheads indicate genomic PCR primers. (e) Electroporation of the pVGNLH plasmid yielded GFP-expressing HAT-resistant transfectants from the CHO(MAC2) cells. Fluorescence (top panel) and phase-contrast (bottom panel) micrographs are shown. (f) FISH analysis of the GFP-MAC2 in the CHO(GFP-MAC2) cells using the digoxigenin-labeled mouse Cot-1 DNA (red) and the biotin-labeled *EGFP* gene (green).

pseudo gene, phosphatidylserine decarboxylase, pseudogene 1 (Pisd-ps1 in the NCBI database; http://www.ncbi.nlm.nih.gov/). The insertion of the loxP site for the construction of MAC1 and MAC2 disrupted the coding sequence of Pisd-ps1, and eventually the entire q arm of mouse chromosome 11 was deleted. Thus, MAC1 and MAC2 contain no known endogenous genes. Detailed genetic maps of MAC, MAC1, and MAC2 are shown in Figure 3.

Transfer of the MAC Vectors from DT40 Cells to Chinese Hamster Ovary Cells. The MAC1 vector containing the *EGFP* gene and a loxP site was transferred from DT40 cells into HPRT-deficient Chinese hamster ovary cells, which were designated as CHO(hprt−/−). Twenty-four G418-resistant CHO clones were obtained from two independent DT40 clones using DT40(MAC1)-52 and DT40(MAC1)-58 as donor cells. PCR analyses to detect a MAC1 sequence showed that 20 of the 24 CHO hybrids retained the MAC1 vector. FISH

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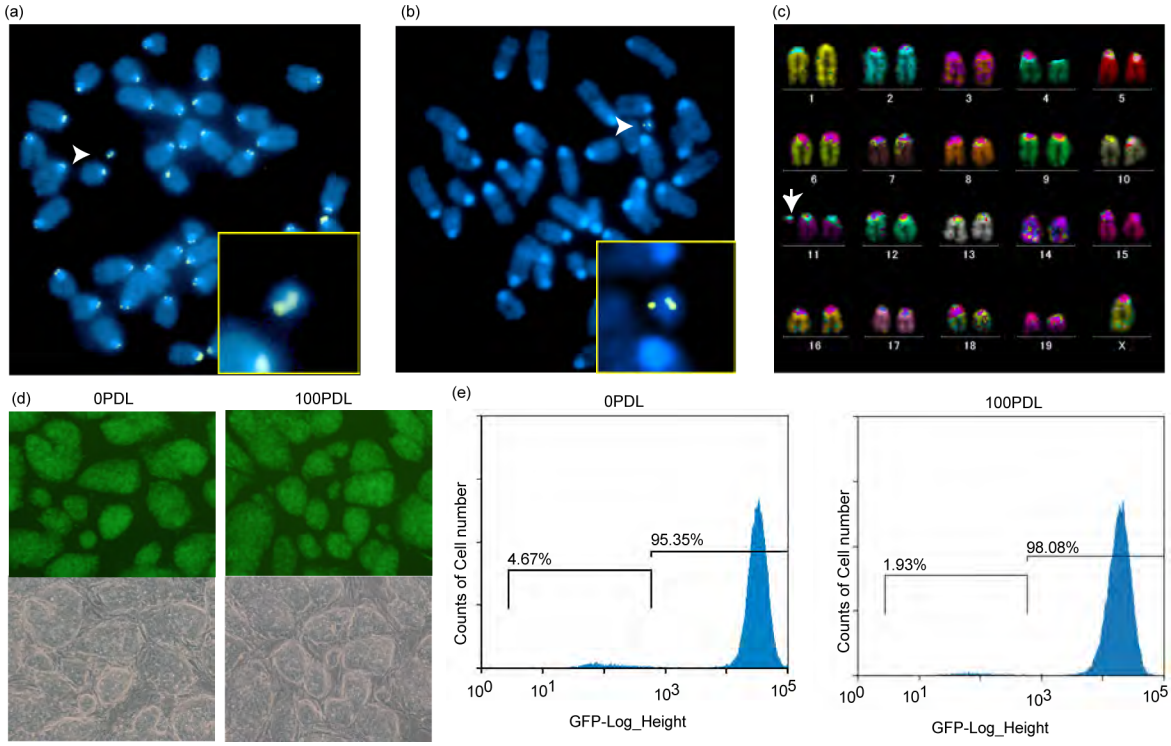

Figure 5. Analysis of mouse ES microcell hybrids containing MAC1. (a,b) FISH analyses of the MAC1 in mouse ES microcell hybrids. (a) The biotin-labeled mouse minor satellite probe (green) and (b) the biotin-labeled *EGFP* gene probe (green) detected the MAC1 and the localization of the *EGFP* gene in mouse ES cells, respectively. (c) Multicolor fluorescence *in situ* hybridization analysis (mFISH) indicated a normal karyotype of mouse ES cells containing MAC1. The arrowhead indicates MAC1. (d) Morphology of mouse ES cells containing the MAC1 at 0 PDL and 100 PDL during long-term cell culture. The top panels show fluorescence images, and the bottom panels show phase-contrast images. (e) Flow cytometry (FCM) analysis of mouse ES cells during long-term cell culture was performed to determine the EGFP expression level at 0 PDL and 100 PDL. Bars indicate the ranges of the EGFP negative level (left) and the EGFP positive level (right).

analysis with the digoxigenin-labeled mouse Cot-1 DNA probe showed that a single copy of MAC1 was independently present in 4 of 10 randomly selected clones (Figure 4a), and these clones expressed the EGFP gene on the MAC1 vector (Figure 4b). Thus, the MAC1 vector was successfully transferred into the CHO cells from the DT40 cells by MMCT. The CHO hybrid clones containing the MAC1 were designated as CHO(hprt−/−; MAC1), or briefly CHO(MAC1). The CHO hybrid clones, CHO(MAC1)-3, CHO(MAC1)-5, and CHO-(MAC1)-22, were used for subsequent experiments.

In this study, the Cre/loxP-mediated insertion of genes into the MAC2 in DT40 cells by *HPRT* gene reconstitution could not be performed because we used HPRT-positive DT40 cell lines. Therefore, the MAC2 was transferred into HPRT-deficient CHO cells by MMCT. Forty hygromycin-resistant CHO clones were obtained from two independent DT40 clones, DT40(MAC2)-5 and DT40 (MAC2)-17. PCR analyses using primers for the detection of MAC2 and FISH analyses with the digoxigenin-labeled mouse Cot-1 DNA probe and the biotin-labeled hygromycin-resistance gene DNA enabled two clones to be chosen from 10 randomly selected clones (Figure 4c). The CHO hybrid clones, CHO(hprt−/−; MAC2)-13 and CHO(hprt−/−; MAC2)-18, were used for subsequent experiments.

Site-Specific Insertion of an Exogenous Gene into the MAC2 Vector. To investigate the feasibility of inserting an exogenous gene into the MAC2 vector by Cre-mediated homologous recombination, gene-insertion experiments were performed in HPRT-deficient CHO hybrid cells. For this purpose, an *EGFP* gene was inserted into a plasmid carrying a loxP sequence and the 3′ *HPRT* exons, which recombine with the 5′ *HPRT* exons and lead to HPRT gene expression on the MAC2 vector (Figure 4d). The construct and the Cre-expression vector were co-transfected into CHO(MAC2)-13 and CHO(MAC2)-18 cells. The presence of HAT-resistant clones suggested that proper recombination occurred on the MAC2 vector. EGFP expression was detected in the HAT-resistant clones by fluorescence microscopy (Figure 4e). PCR and FISH analyses with the digoxigenin-labeled mouse Cot-1 DNA probe and the biotin-labeled *Hygro* gene DNA enabled two clones to be obtained from 10 GFP-expressing HAT-resistant clones (Figure 4f). These results showed that the site-specific insertion of a foreign DNA into the MAC2 vector containing 5′-*HPRT*-loxP could be achieved with the Cre/loxP system.

Transfer of the MAC1 Vector from CHO Cells to Mouse Embryonic Stem Cells. The MAC1 was transferred from CHO(MAC1) cells into mouse embryonic stem (mES) cells to investigate the stability of the MAC vector itself by monitoring EGFP expression *in vitro* and *in vivo*. Seventeen G418-resistant mES clones expressing EGFP were obtained from three independent CHO clones containing the MAC1 as donor cells. The MAC1 vector was detected in 16 clones from the 17 drug-resistant mES hybrids by PCR screening.

FISH analysis with the biotin-labeled mouse minor satellite DNA probe showed that a single copy of the MAC1 was present in 10 of 16 ES hybrids (Figure 5a). FISH analysis with the biotin-labeled *EGFP* gene DNA probe showed that the

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EGFP gene localized on the MAC1 without insertion into host genome (Figure 5b). Multicolor FISH analysis with a MetaSystems probe indicated that the MAC1 was transferred into mES cells with a normal karyotype, indicating that these cells are suitable for the production of chimeric mice (Figure 5c).

To investigate the stability of the MAC1 in mouse ES cells, we performed long-term culture of the mES(MAC1) cells. Even after long-term cell culture without antibiotic selection, i.e., at the 100 population-doubling level (PDL), EGFP expression was observed in the mES(MAC1) cells (Figure 5d). FISH analysis indicated that the MAC1 was independently present in mES cells and the retention rate of the MAC1 was 97% at the 100 PDL. Flow cytometry (FCM) analysis was performed to measure the ratio of cells expressing EGFP in the total cell population. The rate of GFP-expressing cells was consistent during long-term cell culture (Figure 5e). Thus, the EGFP gene in the MAC1 was stably expressed in mES cells during long-term culture, even at the 100 PDL, suggesting that the MAC1 vector is stable in mES cells and does not integrate into the host genome even after long-term culture *in vitro*. The FISH result was comparable to the expected rate of GFP-expressing cells in the total cell population. Therefore, we determined the retention rate of the MAC1 by measuring EGFP as MAC1 retention marker in further analyses.

Retention Rate of the MAC1 Vector in Mouse Tissues. To demonstrate that the MAC1 can be used for the generation of trans-chromosomic (Tc) mice for the functional analysis of specific genes, 12 mES(MAC1) clones were used to produce chimeric mice by microinjection into 8-cell stage embryos. Chimeras with various forms of coat-color chimerism were successfully obtained (Table 1). The MAC1 was transmitted through the germline from four of 14 chimeric mice with high coat-color chimerism (100%). The EGFP-positive trans-chromosomic F1 mice and their progeny (F2−F5) were used for the following analyses. The tail fibroblasts of Tc mice expressed EGFP (data not shown), and FISH analysis showed that the tail fibroblasts contained the MAC1 (100% in all spreads examined) as a single extra chromosome (Figure 6a). The *EGFP* gene on the MAC1 was expressed in all tissues examined (Figure 6b). The retention rate of the MAC1 in mouse tissues, including testis, liver, thymus, spleen, and brain, was analyzed in detail. Tissues were homogenized, and the isolated cells were analyzed by FCM to determine the ratios of EGFP-expressing cells (Figure 6c). Tc mice containing a human artificial chromosome (21HAC2) were used for the control group.[36] Both MAC1 and 21HAC2 contain the *EGFP* gene driven by the CMV early enhancer/chicken beta actin (CAG) promoter. The retention rate of the MAC1 was high and uniform among tissues (87−92%), while that of 21HAC2 varied in different tissues (19−87%). The retention rate of 21HAC2 was lower in highly proliferative tissues such as spleen and thymus, while brain, in which cell proliferation is low, showed a higher retention rate. Thus, we speculate that tissues with rapid turnover are more likely to lose the human artificial chromosome, possibly due to the heterocentromere, in mice. However, the factors determining the retention rate are still unknown. Detailed analysis is required to understand the reason for the variability in HAC retention rate among tissues. To investigate the retention rate of the MAC1 in mouse lymphocytes from spleen and bone marrow, FCM analysis was also performed to determine the GFP-expressing cell ratios. The ratios of GFP-expressing cells with the MAC1 in lymphocytes were clearly higher (>90%) than those with 21HAC2 (<20%) (Figure 6d,e). These data suggest that the MAC1 does not interfere with normal development, that genes inserted into MACs are stably expressed and that the MAC vector is consistently maintained in mice.

Table 1. Summary of the Production of Chimeric Mice

		coat color chimerism (%)				
clone no.	transplanted embryos	0	10−30	40−60	70−90	100
1	140	38	10			
3	140	15	4	5		
4[a]	140	25	1	1	1	9
5	270	39	10	7	5	5
6	140	38	2			
7	380	66	22	6		
8	60	17	5			
9	80	9	3	2		
10	80	15	14			
13	140	43	6			
15	40		2			
23[a]	100	42	3			

[a]HPRT+ line.

We have previously reported that the retention rate of transferred human chromosome 21 in Down syndrome (DS) model mice varied considerably among mice, and even among different tissues. In the present study, the stability of MAC in these tissues was striking. In the previous study on Down syndrome mouse models, the most significant problem was that the introduced human chromosome 21 was not mitotically stable in mice, making further analysis of its effects on phenotypes difficult. In general, more abnormalities of greater severity were seen in fetuses with a greater proportion of cells retaining the transferred chromosome 21.

Fisher's group produced the Tc mouse line, Tc1, which is a model of DS.[40] Mouse female ES cells containing a human chromosome 21 (hChr.21) fragment were generated by irradiation MMCT (XMMCT).[41] Germline transmission was achieved with this hChr.21 fragment, which harbored 75% of normal human chromosome 21 genes.[42] Although the Tc1 mice recapitulate several phenotypes of DS, the frequency of each phenotype varied between tissues even in the same mouse line, possibly because of variation in the retention rate in different tissues, i.e., mosaicism. Indeed, the retention rate of the hChr.21 fragment in Tc1 mice varied widely among tissues and was particularly low in spleen.[40] In this model, there are also concerns regarding the low germline transmission frequency, which may prevent further analysis. Thus, although previous reports with other artificial chromosomes have uncovered a number of biologically important phenomena, our new MAC may resolve these concerns and provide important insights.

Construction of MAC2 with Multiple Integration Sites (MI-MAC). The ability to carry multiple genes will significantly expand the utility of MACs. We previously reported the construction of a multi-integrase (MI)-HAC in which five recombination sites are available for the insertion of multiple genes.[38] We thought that the combination of the MI system and MAC (MI-MAC) may be useful in addition to the previously reported applications of the MI-HAC. Therefore, we attempted to construct a MI-MAC. The same MI platform plasmid used for the MI-HAC construction can be adapted to

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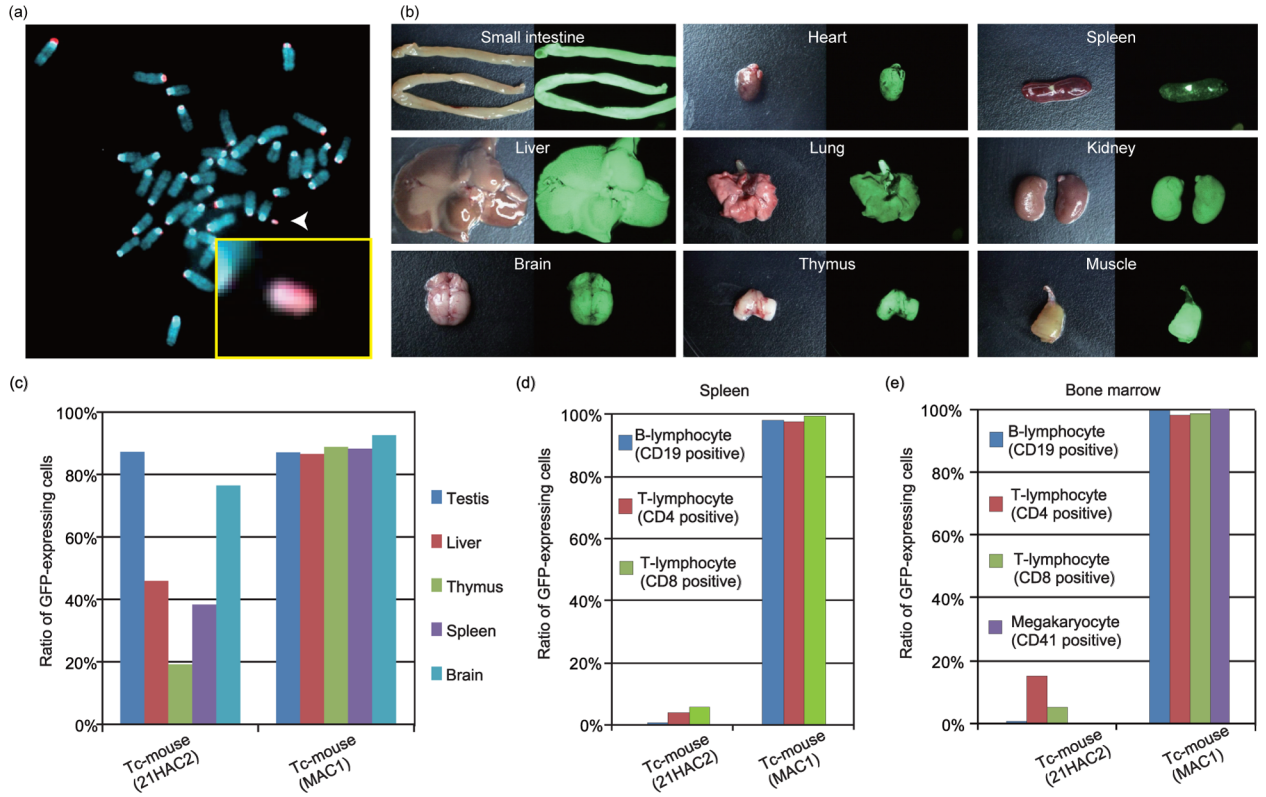

Figure 6. Stability of the MAC1 in the tissues of trans-chromosomic (Tc) mice. F1 mice were produced by crossing 100% chimeras produced from MAC1 ES cells with ICR mice. The F1 mice and their progeny (F2−F5) were analyzed. The reproducibility of the results was confirmed ($n = 3$). (a) FISH analysis of tail-fibroblasts from Tc mice containing the MAC1 using the digoxigenin-labeled mouse minor satellite probe (red). The arrowhead indicates the MAC1, and the inset shows an enlarged image of MAC1. (b) EGFP expression in various tissues from Tc mice containing the MAC1. Bright (left panel) and fluorescence (right panel) micrographs are shown. (c) FCM analyses show the ratios of GFP-expressing cells in various tissues from Tc mice containing the MAC1 and the 21HAC2. (d,e) FCM analyses show the ratios of GFP-expressing cells in lymphocytes from spleen (d) and bone marrow (e) of Tc mice containing the MAC1 and the 21HAC2, respectively.

the MAC2 for the MI-MAC construction by using the Cre/loxP system, leading to HPRT gene reconstitution (Supplementary Figure S2a). We obtained 32 HAT-resistant clones by Cre/loxP recombination in CHO (MAC2) cells. Genomic PCR showed that all clones were positive for the HPRT reconstitution junction (TransL1/R1), and three were positive for another junction (loxP4548/Hyg696). Three clones positive for both junctions were also PCR-positive for the MI platform (PhiC31F1/Bxb1R3) and were further analyzed by FISH. FISH analysis revealed that the MI platform plasmid was correctly inserted into the MAC2 without randomly integrating into the host chromosomes and that the MI-MAC was independently maintained in CHO cells (Supplementary Figure S2b). The detailed genetic map of the MI-MAC is shown in Figure 3.

To validate the site-specific recombination into each attachment site, PhiC31 attP, R4 attP, Bxb1 attP, TP901-1 attP, and the FRT site in the MI-MAC, we co-transfected each recombinant plasmid and their corresponding integrase expression plasmids in CHO (MI-MAC) cells (Supplementary Figure S2c). G418-resistant and GFP-positive clones were analyzed by genomic PCR using primers for the recombination junction (PGK5/G418 3AS). The recombination efficiency was comparable to that in a previous study using the MI-HAC (data not shown). The insertion of recombinant vectors into the MI-MAC was further confirmed by FISH analysis. Signals for the insert were detected in the MI-MAC without any signals in host chromosomes, and the MI-MAC was still maintained independently (Supplementary Figure S2d). This suggested that EGFP expression in CHO cells was derived from the *EGFP* gene on the MI-MAC (Supplementary Figure S2e). Taken together, these data demonstrated that the MI-MAC was correctly constructed, and the site-specific acceptor sites were functional.

Summary and Conclusion. The conventional technologies used to produce transgenic (Tg) mice are often associated with insertional disruption of the host genome, limitation of the size of DNA to be transfected, and unpredictable and irreproducible expression of the transgene due to random integration.[2] The term "MAC" has been often used to represent the Mammalian Artificial Chromosome, including artificial chromosomes in human, mouse, and possibly other mammalian species. These artificial chromosomes have been produced by either a top-down approach (engineered chromosome) or a bottom-up approach (*de novo* artificial chromosome).[43−45] In this study, we constructed the first mouse artificial chromosome vector from a natural mouse chromosome by the top-down approach.

We herein designated the mouse artificial chromosome vector as the MAC vector. We demonstrated the stability of the MAC1 in mouse ES cells, as well as in tissues in mice. The stability of the MAC1 in mouse cells was higher than that of other reported artificial chromosomes created using either top-down or bottom-up approaches.[10,23,26,46] Compared with previously reported artificial chromosomes, including HACs and mSATAC, the MAC vector was more stably maintained in

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mouse tissues. These results suggest that the MAC vector is a potentially powerful gene delivery vector for mouse cells that may facilitate the production of Tc mice for various functional studies. The availability of MAC vectors with high stability in mouse cells will be of great value in the production of mice carrying human genetic elements for modeling human diseases, as well as in the production of humanized model mice.

In this study, we also constructed MI-MAC, which possesses multiple different integration sites.[38] Thus, the MAC and MI-MAC developed in this study will facilitate a variety of studies, e.g., analyses of gene interactions and the monitoring of gene functions in mice and cultured cells. The MAC1, MAC2, and MI-MAC vectors should become powerful tools for future synthetic biology strategies.

METHODS

Cell Culture. The tail fibroblasts, mouse embryonic fibroblasts, and the whole cell hybrid cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. The DT40 hybrid cells were maintained in Roswell Park Memorial Institute medium 1640 (Invitrogen) containing 10% fetal bovine serum (FBS, Biowest), 1% chicken serum (Invitrogen), 50 μmol/L 2-mercaptoethanol (Sigma), and the appropriate antibiotics. The DT40 hybrids containing a single copy of mouse chromosome 11 were produced by MMCT from mouse A9 hybrid cells containing BSr-tagged mouse chromosome 11 and maintained with 15 μg/mL blasticidin S (BS, Funakoshi). The HPRT-deficient CHO (JCRB0218) hybrids containing either MAC1 or MAC2 were maintained in Ham's F-12 nutrient mixture (Invitrogen) containing 10% fetal bovine serum, 800 μg/mL G418 (Calbiochem), and 250 ng/mL hygromycin (Wako). The parental mouse ES cell line, TT2F, and the microcell hybrid clone, ES (MAC1), were maintained on mitomycin C (Sigma)-treated Jcl:ICR (CLEA) MEFs as feeder layers in Dulbecco's modified Eagle's medium containing 18% FBS (Thermo Scientific HyClone), 1 mmol/L sodium pyruvate (Invitrogen), 0.1 mmol/L nonessential amino acids (Invitrogen), 0.1 mmol/L 2-mercaptoethanol (Sigma), 2 mmol/L L-glutamine (Invitrogen), and 1,000 U/mL leukemia inhibitory factor (Funakoshi).

Construction of Targeting Vectors. The region described in Figure 3 shows the homologous regions on mouse chromosome 11 for the following targeting vectors. The primer sequence information is listed in Supplementary Table S1. For construction of the chromosome truncation vector, pBS-TEL/puro_MAC, a fragment (8.3 kb) of the mouse chromosome 11 region in AL671968 was amplified by PCR with the m11_17L/17R primers, digested with *Bam*HI (6.2 kb) and subcloned into the *Bam*HI sites of the pBS-TEL/Puro vector.[32] The targeting vector, pMAC1, for introducing EGFP/neo/3′ HPRT-loxP, was constructed as follows. Two 2.5 kb and 3.8 kb fragments for homologous arms corresponding to the mouse chromosome 11 peri-centromeric region in BX572640.9 were amplified by PCR using the m11_5L/5R primers (2.8 kb) and the m11_6L/7R primers (4.1kb), digested with *Bgl*II, and subcloned into the *Bgl*II and *Bam*HI sites of the pKO Scrambler V913 backbone vector (Lexicon Genetics), respectively (designated as the pVH21-12 vector). The neo-loxP-3′ HPRT cassette was introduced between the *Kpn*I and AscI sites of the V913 backbone vector (Lexicon Genetics), and the neo-loxP-3′ HPRT cassette was derived from the following two selection/insertion fragments: a 2.3 kb XbaI/AscI fragment from pKO SelectHPRT V820 (Lexicon Genetics) containing part of the human HPRT gene with loxP sites inserted at the XbaI site of intron B was introduced into the pKO Scrambler V907 backbone vector (Lexicon Genetics), and a 1.6 kb *Kpn*I/NotI blunt-end fragment from pPGKneo encoding the neo gene was inserted at the *Eco*RI/*Kpn*I sites of V907. *Sal*I/*Hin*dIII fragments of CAG-EGFP from pCX-EGFP (a gift from Dr. M. Okabe) were cloned into pJC5-4 (a gift from Dr. G. Felsenfeld) containing HS4 insulators, and the CAG-EGFP fragments flanked by the HS4 insulator were then cloned into the EcoRV site of the V913 vector containing the neo-loxP-3′ HPRT cassette (designated as the pVGNLH vector). The selection/insertion cassette was introduced between the XhoI and AscI sites of the pVH21-12 vector: a 9.4 kb *Sal*I/AscI fragment of the neo-loxP-3′HPRT/CAG/EGFP cassette including a 4.0 kb neo-loxP-3′ HPRT cassette and a 5.4 kb CAG-EGFP HS4 insulator cassette. The targeting vector, pMAC2, for introducing 5′ HPRT-loxP-PGK-hyg, was constructed as follows: a 3.3 kb XhoI/*Sal*I fragment of the 5′ HPRT-loxP/PGK-hyg cassette was introduced into the XhoI site of pVH21-12 containing two homologous arms, as described above. The 5′ HPRT-loxP/PGK-hyg vector contained the following two selection/insertion cassettes between the AscI and *Kpn*I sites: a 1.4 kb ClaI/AscI (5′-HPRT-loxP) fragment from pKO SelectHPRT V820 (Lexicon Genetics) containing part of the human HPRT gene with a loxP site inserted at the XbaI site of intron B and a 1.8 kb ClaI/*Kpn*I fragment (PGK-Hyg) from PGKhygrodetlaLT20 encoding the hygromycin gene. The pVGNLH vector containing the 3′ HPRT-loxP-CAG-EGFP plus HS4 insulators was used as the site-specific gene insertion vector for MAC2 containing the 5′ HPRT-loxP site.

MMCT. The MAC1 and MAC2 vectors were transferred from the DT40 cells into the CHO cells using MMCT technology.[19] Briefly, microcells were prepared by centrifugation of 1×10^9 DT40 cells attached to flasks (Nalge Nunc) coated with poly-L-lysine (Sigma) followed by fusion with 1×10^6 CHO cells using 47% poly(ethylene glycol) 1000 (WAKO). CHO hybrids containing the MAC1 were selected in 800 μg/mL G418 and picked for expansion. CHO hybrids containing the MAC2 were selected in 250 μg/mL hygromycin and picked for expansion. The transfer of the MAC1 from CHO cells to mouse ES cells was performed using standard procedures.[9] The mouse ES cell hybrids were selected in 200 μg/mL G418.

Transfection of DT40 and CHO Cells. The DT40-(mChr11-Bsr) hybrid was transfected with pBS-TEL/Puro_-MAC by electroporation of 1×10^7 cells with 25 μg of *Eco*RI-linearized plasmid at 25 μF and 550 V in a 4 mm cuvette using a Gene Pulser (Bio-Rad). The cells were resuspended in basic growth medium and aliquoted into four 96-well flat-bottomed microtiter plates (Becton-Dickinson). After 2 days, the cells were resuspended in selective medium containing puromycin. Fourteen days later, drug-resistant colonies were picked and expanded for subsequent analysis. The DT40(MAC)-1 cells were similarly transfected with pMAC1 and pMAC2 for targeting as described above. The CHO cells containing MAC2 were transfected by lipofection of 4×10^5 cells with 7 μg of pVGNLH and 1 μg of pBS185 plasmid using 20 μL of Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 24 h in basic growth medium, cells were cultured in medium containing HAT (Sigma). Fourteen days later, drug-resistant colonies were picked and expanded for further analysis as described below.

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Genomic PCR Analysis. Genomic DNA was extracted from cell lines and chimeric tissue specimens using a genomic extraction kit (Gentra Systems), and PCR was performed as follows. The primer pairs for the detection of the *EGFP* gene with a loxP site targeting the MAC1 were kj_neo/m11_7R (7 kb) and m11_5L/EGFP-F (5 kb). The primer pairs for the detection of a loxP site targeting the MAC2 were TransL1/m11_7R (6.5 kb) and m11_5L/hyg_r (5.2 kb). The primer pairs for the detection of HPRT gene recombination and GFP-MAC2 were TransL1/R1 (0.4 kb), kj_neo/m11_7R (7 kb), TransL1/m11_7R (16 kb), and EGFP-F/TransL1 (6 kb). DT40(mChr11-Bsr), DT40(MAC), DT40, CHO, and mouse ES cells were used as negative controls. The primer sequences are shown in Supplementary Table S1.

FISH Analysis. Preparation of metaphase chromosomes from exponentially growing cell cultures and FISH were performed according to standard methods. Briefly, FISH analysis was performed using fixed metaphase spreads of each cell hybrid using digoxigenin-labeled (Roche) mouse Cot-1 DNA (Invitrogen), digoxigenin-labeled mouse minor satellite DNA (a gift from Dr. Vladimir), biotin-labeled (Roche) DNA (mouse minor satellite DNA, PGK-*puro*, CAG-*EGFP*, PGK-*Hygro*), essentially as described previously.[9] Chromosomal DNA was counterstained with DAPI (Sigma). Images were captured using the NIS-Elements system (Nikon).

mFISH Analysis. Mouse mFISH probes were purchased from MetaSystems GmbH (Altlussheim). Procedures for the denaturation of metaphase chromosomes and mFISH probes, hybridization, post-hybridization washes, and fluorescent staining were performed using the methods recommended by the manufacturer. Microscopic analysis was performed using an AxioImagerZ2 fluorescence microscope (Carl Zeiss GmbH) with an HBO-103 mercury lamp and filter sets for FITC, Cy3, Texas Red, Cy5, DEAC, and DAPI. Metaphase images were captured digitally with a CoolCubeI CCD camera and the ISIS mFISH software program (MetaSystems), processed, and stored for subsequent analysis.

Generation of Chimeric Mice. Chimeric mice were produced from mouse ES (MAC1) cell lines. Chimera production was performed as described previously.[9] Briefly, mouse ES cells were injected into 8-cell stage embryos derived from ICR mice (CLEA) and then transferred into pseudopregnant ICR females; 100% coat-color chimeric mice were used for mating with ICR mice to obtain Tc mice. All Tc mice used for analysis were 8−12 weeks old. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University.

FCM Analyses of Mouse ES Cells. A total of 10^7 cells from each mouse ES hybrid were suspended in phosphate-buffered saline (PBS) supplemented with 5% fetal bovine serum (FBS; Gibco) and analyzed by Moflo XDP (Becton Dickinson). Three × 10^4 mouse ES cells of specific gate were analyzed by FCM, and the percentage of EGFP-positive cells was calculated. EGFP was excited with a 488 nm argon laser and was detected with band-pass filters of 530/40 nm. To set the parameters for FCM analysis, normal mouse ES cells were used as negative controls.

FCM Analyses for Tissues and Blood Cells. Samples were collected from wild-type mice, Tc mice containing the MAC1, and Tc mice containing the 21HAC2 as a control.[36] Each tissue was dissociated with the following enzyme cocktails: Cocktail I (collagenase 300 U/mL, elastase 0.3 U/mL and DNase 100 U/mL) for thymus and testis; Cocktail II (collagenase type I 0.25% and DNase 100 U/mL) for liver; and Cocktail III (papain 10 U/mL and DNase 200 U/mL) for brain. The dissociated cells were then washed, filtered, and finally suspended in HBSS containing 5% FCS, 1 mM EDTA. A mixture of antibodies for maturation markers, including antimouse CD4, CD8, CD19, TER119, CD11b, and Gr-1 antibody conjugated with biotin (Biolegend), was used to eliminate blood cells from brain and liver tissue. Streptavidin-Allophycocyanin (SA-APC; Beckman Coulter) was used as secondary antibody for detection.

For lymphocyte analyses, isolated cells were labeled with the following biotin-conjugated antibodies: anti-mouse CD4, CD8, and CD19. Anti-CD4, CD8, CD19, and TER119 antibodies were used for depletion of other cell lineages when CD41-positive megakaryocytes were selected with anti-mouse CD41 antibody conjugated to PE (BD Pharmingen).

Data for the ratio of cells expressing EGFP were acquired with a Moflo XDP and analyzed by using Summit software (Beckman Coulter). Approximately 20,000 events were acquired for the GFP expression in each sample. EGFP and PE were excited with a 488 nm argon laser, and APC was excited with a 633 nm argon laser. GFP, PE, and APC were detected with band-pass filters of 530/40 nm, 575/25 nm, and 670/30 nm, respectively. To set the parameters for FCM analysis, cells isolated from wild-type mice were used as negative controls.

Insertion of Multiple Integration Sites into MAC2. CHO cells harboring the MAC2 were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum, 200 μg/mL hygromycin, and 1% penicillin/streptomycin. Cells were seeded on 6-well culture plates and co-transfected with 1.0 μg Platform32, a multi-integrase platform plasmid carrying loxP and 3′ HPRT, and 1.0 μg Cre expression vector, using Lipofectamine 2000. The cells were seeded on five 10-cm dishes at 24 h post-transfection, and HAT-selection was started at 48 h post-transfection. Cells were selected for 11 days. Validation of the MI-MAC was performed by co-transfection of each plasmid containing a recombination site, neomycin-resistance gene, and CMV promoter driving EGFP with the corresponding integrase expression plasmid, as previously reported.[38]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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