Human artificial chromosomes (HACs) are alternative vectors that promise to overcome problematic transgene expression often occurring with conventional vectors in mammalian cells and bodies. We have successfully generated HACs by multimerization of a cloned long alphoid stretch in a human cell line, HT1080. Furthermore, we developed technologies for cloning large genomic regions into HACs by means of co-transfection of clones with the alphoid array and clones encoding the genomic region of interest. The purpose of this study was to investigate the mitotic and meiotic stability of such HACs in mouse cells and bodies. We transferred a circular HAC containing the guanosine triphosphate cyclohydrolase I gene (GCH1-HAC) and a linear HAC containing the human globin gene cluster (globin-HAC) from HT1080 cells into mouse embryonic stem (ES) cells by microcell-mediated chromosome transfer. The HACs were stably maintained in mouse ES cells for 3 months. GCH1-HACs in every ES cell line and globin-HACs in most ES cell lines maintained their structures without detectable rearrangement or acquisition of mouse genomic DNA except one globin-HAC in an ES cell line rearranged and acquired mouse-type centromeric sequences and long telomeres. Creation of chimeric mice using ES cells containing HAC and subsequent crossing showed that both the globin-HAC that had rearranged and acquired mouse type centromeric sequences/long telomeres and GCH1-HACs were retained in tissues of mice and transmitted to progeny. These results indicate that human artificial chromosomes constructed using the bottom-up strategy based on alphoid DNA are stable in mouse bodies and are transmissible.

Many types of viral and episomal shuttle vectors that do not cause insertional mutagenesis or silencing in host chromosomes have been developed for gene delivery and expression in mammalian cells. Adenoviruses and adeno-associated viruses have high infection efficiencies, and EBV and herpes simplex virus have been employed successfully for the delivery of large DNA fragments (1–3). However, effective episomal vectors that are able to be maintained stably in the nuclei of mammalian cells in defined copy numbers and provide natural and long-term gene expression are still under development. In this respect, chromosomal vectors containing genomic components for replication and segregation in the host cell are among the safest and most useful episomal vectors in terms of low toxicity and stable maintenance.

Over the past 10 years, many groups have tried to engineer human mini-chromosomes, attempting to define elements required for chromosomal maintenance (4–6). The primary focus has been to delimit a functional centromeric sequence that is able to assemble the kinetochore de novo (7–11).

Two major techniques have been used to engineer human mini-chromosomes. One is telomere-associated chromosome fragmentation (top-down construction), which truncates human host chromosomes into smaller sizes by using targeting vectors containing telomeric sequences. Mini-chromosomes derived from the X and Y chromosomes by this method segregated equally into sister cells (7, 8). Recently, chicken DT40 cells, which have a high level of homologous recombination activity, were utilized as host cells to efficiently produce truncated chromosomes (12).

The other technique is de novo construction of human artificial chromosomes (HACs, bottom-up construction) by introducing synthetic or cloned centromeric alpha satellite (alphoid) DNA into human HT1080 cells (9–11). Many studies have demonstrated that linear alphoid DNA in yeast artificial chromosomes (YACs) and circular alphoid DNA in P1-derived artificial chromosomes (PACs) and bacterial artificial chromosomes (BACs) are able to generate HACs ranging in size from 1–10 megabases. Circular HACs without telomeres can be generated with circular alphoid DNA cloned in PACs and BACs (13–15). HACs have been applied as gene expression vectors to complement a gene deficiency in human cell lines. The generation of a HAC containing a 40-kg gene encoding hypoxanthine

ref: 1. This work was supported by a special grant for Core Research for Evolutional Science and Technology from the Japan Science and Technology Corp., a grant for establishment of transgenic agro-biofarm systems from the Ministry of Agriculture, Forestry, and Fisheries of Japan, and a grant-in-aid for Scientific Research from The New Energy and Industrial Technology Development Organization, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2. The abbreviations used are: HAC, human artificial chromosome; YAC, yeast artificial chromosome; PAC, P1-derived artificial chromosome; BAC, bacterial artificial chromosome; GCH1, guanosine triphosphate cyclohydrolase I; ES, embryonic stem; MMCT, microcell-mediated chromosome transfer; DMEM, Dulbecco’s modified Eagle’s medium; kb, kilobase(s); FISH, fluorescence in situ hybridization; TRITC, tetramethylrhodamine isothiocyanate.
guanine phosphoribosyltransferase (HPRT1) was accomplished by two methods based on bottom-up construction; 1) transfection of a BAC clone containing both the HPRT1 gene and alphoid DNA sequences (16) and 2) co-transfection of two PACs, one encoding the HPRT1 gene and the other encoding alphoid DNA (17). We previously demonstrated that HACs bearing the large human genomic region containing guanosine triphosphate cyclohydrolase I (GCH1) or the globin gene cluster were generated in HT1080 cells after co-transfection of the cloned genes and alphoid DNA in BACs or YACs, respectively (14). The HACs mimicked the response of the gene expression from the authentic chromosomal gene (14).

Recently, natural and truncated human chromosomes were introduced into mice, and their stabilities during mitosis/meiosis and transgene expression were studied. A Down syndrome model mouse was created from an embryonic stem (ES) cell line harboring human chromosome 21 in addition to the normal mouse karyotype (18, 19). In another study, truncated chromosomes derived from human chromosomes 14, 2, and 22 that contained the immunoglobulin heavy chain, κ, and λ light chain sequences were transmitted through the germ line and showed a functional antibody response (20, 21). In contrast, another study showed that a truncated Y chromosome required mouse centromeric DNA for mitotic stability in mouse ES cells and transmission to progeny (22). These results indicate that although natural and truncated human chromosomes can be maintained and human transgenes can be expressed in mice, the mitotic and structural stabilities of human chromosomes in mouse ES cells and bodies and the efficiency of germ line transmission are variable among experiments (18–20, 22, 23). Thus, the general stability of human chromosomes in mouse cells and bodies remains unclear. The establishment of a bottom-up-constructed HAC in mouse ES cells and bodies has not yet been reported. Information from several types of HACs constructed by the bottom-up method as well as from truncated chromosomes based on native chromosomal elements enables better understanding of their mitotic/meiotic stabilities and behaviors in mice and will pave the way for future HAC applications in animal models.

Here we address the mitotic/meiotic stabilities of the circular GCH1-HAC and the linear globin-HAC after transfer from the human fibroblast cell line HT1080 to mouse ES cells by microcell-mediated chromosome transfer (MMCT). The results we obtained indicate that bottom-up constructed HACs derived from alphoid DNA are mitotically stable. Circular HACs could be efficiently transmitted in mice, whereas linear HACs without rearrangement were not transmissible to progeny.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HT/GCH2-10 and HT/globin29 were cultured in DMEM supplemented with 10% fetal calf serum and selected with 4 μg/ml blasticidin S (Funakoshi) or 400 μg/ml G418 (Sigma), respectively. The mouse ES cells lines TT2 and TT2F were cultured in DMEM supplemented with 20% knock-out serum replacement (Invitrogen), 10^3 units/ml ESGRO (Chemicon), and 0.1 mM β-mercaptoethanol. TT2/GCH2-10 and TT2F/GCH2-10 were selected with 4 μg/ml blasticidin S. TT2/globin29 and TT2F/globin29 were selected with 300 μg/ml G418.

**MMCT**—Microcell-mediated chromosome transfer was conducted as follows. Forty 9-cm dishes of HT-A9/GCH2-10 and HT-A9/globin29 cells were grown to 80% confluency, then colcemid was added to 0.05 μg/ml. The cells were cultured for 72 h and harvested by trypsinization. Cells were resuspended in prewarmed serum-free DMEM containing cytochalasin B at a final concentration of 20 μg/ml. The suspension was incubated for 5 min at 37 °C, and then an equal volume of Percoll (Amerham Biosciences) was added. The suspension was centrifuged in a Hitachi R20A2 rotor at 15,000 rpm for 90 min at 37 °C.

Microcells were harvested by aspiration and collected by centrifugation at 2000 rpm for 5 min in a swinging bucket rotor. Microcells were resuspended in serum-free DMEM and collected by centrifugation. ES cells were harvested and suspended in serum-free DMEM. Microcells suspended in serum-free DMEM were mixed with ES cells and centrifuged at 2000 rpm for 5 min. The resulting pellet was suspended in 1 ml of serum-free DMEM and left for 10 min at room temperature. The mixture was centrifuged at 1500 rpm for 5 min. The pellet was suspended in 1 ml of 50% PEG1500 (Roche Diagnostics) and incubated at room temperature for 90 s. Five ml of serum-free DMEM was added, and the mixture was centrifuged at 1000 rpm for 5 min. The fusion product was washed twice in serum-free DMEM and plated onto three 9-cm dishes layered with feeder cells. After 24 h, the medium was changed to ESM supplemented with 300 μg/ml G418 and selected for 7 days. Resistant colonies were isolated and analyzed by fluorescence in situ hybridization (FISH).

**Pulsed-field Gel Electrophoresis and Southern Analysis**—Agarose-embedded DNA was prepared as previously described (24). DNA in agarose blocks was digested for 4 h and size separated in a 1% agarose gel (Cambrex) on the CHEF mapper system (Bio-Rad). The running conditions were dependent on the auto algorithm from 5 to 500 kb. DNA in the gel was transferred to a nylon membrane and hybridized with 32P-labeled DNA probe prepared from either 11-4 alphoid DNA (24), 1 kb of GCH1 exon 1, or 2 kb of Aγ globin DNA produced by PCR (primers were 5’-GTGACTAATGTTTGTTATCC-3’ and 5’-TGAGGCGGCTAGAGCTTAAGAGAAGAG-3’).

**FISH**—Metaphase chromosomes spread from mouse ES cell lines and primary tail fibroblasts from F1 mice were prepared on glass slides after methanol/acetate (3:1) fixation, and FISH was carried out as previously described (24). For detection of GCH1-HAC or globin-HAC, digoxigenin-labeled phelo-BAC or pacYACs, respectively, were used as probes. The probe for the GCH1 gene was a biotin-labeled 13-kb fragment containing exon 1 (14), and the probe for the globin gene was a biotin-labeled 9-kb fragment produced by PCR (primers were 5’-TGCTAATGTTTGTTATCC-3’ and 5’-ATCAGCCAAACAGGGTTCGCGAGGTC-3’).

The alphoid, mouse minor satellite, mouse major satellite, telomeric, and mouse B1 repeat DNA sequences were detected with biotin-labeled α 21-I alphoid DNA (24), a 1.2-kb fragment consisting of 10 repeats of a mouse minor satellite unit, a 2.4-kb fragment of 12 repeats of a mouse major satellite unit, a 1-kb fragment consisting of (TTAGGG)n, and a 206-bp fragment of mouse B1 repeat DNA, respectively. Biotin-labeled DNA was visualized with fluorescein isothiocyanate-conjugated avidin (Vector), and digoxigenin-labeled DNA was analyzed by fluorescence in situ hybridization (FISH).
visualized with TRITC-conjugated anti-digoxigenin (Roche Diagnostics). Photographs were taken using a CCD camera mounted on a Zeiss microscope AxioPlan2. Images were processed using AxioVision.

Mitotic Stability—The mitotic stabilities of GCH1-HAC and globin-HAC in ES cells were measured by culturing cells for 200 or 120 divisions under non-selective growth conditions. At 10-day (20 divisions) intervals during the culturing, spreads of metaphase chromosomes were prepared, and the presence of HACs was determined by FISH. The percentage of metaphase cells with HACs was measured. The rate of HAC loss (R) was calculated by the formula $N_n = N_0 (1 - R)^n$, where $N_0$ is the number of metaphase spreads containing a HAC under selective conditions, and $N_n$ is the number of metaphase spreads containing a HAC after $n$ divisions of culture under non-selective conditions.

RESULTS

Transfer of HACs into Mouse ES Cells by MMCT—We have previously constructed HACs containing the large human genomic region that encompasses GCH1 or the globin gene cluster. These HACs were constructed in HT1080 cells by cotransfection of an alphoid precursor and a large genomic sequence. The HT/GCH2-10 cell line possessed a single GCH1-HAC that carried three copies of the GCH1 gene and a CMV-bsd selection marker (Fig. 1A, Table 1) (14). The HT/globin29 cell line possessed a single globin-HAC that carried 10 copies of the globin cluster and a PGK-neo selection marker (Fig. 1B, Table 1). GCH1-HAC, constructed from a BAC-based precursor, is circular in form and lacks telomeres. In contrast, the globin-HAC, constructed from a YAC-based precursor, is linear in form with a telomere at each end.

To examine the mitotic and structural stabilities of bottom-up-constructed HACs in mouse cells and the germ line transmission of HACs in mouse bodies, the HACs were transferred from HT1080 cells to the male and female mouse ES cell lines TT2 and TT2F by MMCT. The HT/GCH2-10 and HT/globin29 cells were fused with mouse A9 cells to establish HT1080-A9 hybrid cell lines that possessed single HACs. Subsequently, the HACs in microcells from HT1080-A9 hybrid cells were transferred into ES cell lines. Mouse ES cell lines screened by blasticidin S or G418 were analyzed by FISH to determine whether or not the HACs were maintained extrachromosomally (Fig. 2). Nine ES cell lines possessing GCH1-HACs and 14 ES cell lines possessing globin-HACs were established from $3 \times 10^7$ ES cells. All cell lines examined carried single copies of HACs with no integration of HAC components into the mouse chromosomes. The ES cell lines that had normal karyotypes (TT2/GCH2-10, TT2F/GCH2-10, TT2/globin29-2, and TT2F/globin29) were used for further studies.

To examine the structures of the HACs in ES cells in detail, genomic DNA from HAC-possessing cells was digested with restriction enzymes, and the HAC components were detected by Southern blot analysis (Fig. 3). The 100- or 90-kb fragments of the alphoid components were excised from GCH1-HACs or globin-HACs by BstEII or Scal digestion, respectively. The 110-kb exon 1 region of the GCH1 gene in GCH1-HAC was detected

![FIGURE 1. Construction of HACs.](Image)

A. The HACs were generated by multimerization of transfected precursor DNAs in HT1080 cells. A, alphoid-BAC and GCH1-BAC were precursors of GCH1-HAC. The 100-kb alphoid-BAC contains 90 kb of alphoid array and CMV-bsd (closed circle). The 180-kb GCH1-BAC contains 6 exons (1–6). The 110-kb fragments of GCH1 gene or 100-kb alphoid fragments were detected by Pael or BstEII digestion of GCH1-HAC, respectively. The probes for FISH analyses are indicated as closed bars, and the probes for Southern analyses are shadowed bars. B, alphoid-YAC and globin-YAC were precursors of globin-HAC. The 90-kb alphoid-YAC contains 80 kb of alphoid array and YAC contains 80 kb of alphoid array and SV-bsr (open circle) and human telomere sequence (open arrows). The 150-kb globin-YAC (A20I1F4.3) contains locus control region (LCR), e, GY, AY, δ, and β globin genes and the PGK-neo selection marker (open rhombus). The 40-kb fragments of the globin gene or 90-kb alphoid fragments were detected by Kpnl digestion or Scal digestion of globin-HAC, respectively. The probes for FISH analyses are indicated as closed bars, and the probes for Southern analyses are indicated as shadow bars. The compositions of the HACs were estimated based on Southern analyses.

### TABLE 1

| Characteristics of HACs in HT1080 cells |
|----------------------------------------|
| HT1080 cell line with HAC  | Component | Selection | Gene | Telomere | Chromosome loss rate |
|-----------------------------|-----------|-----------|------|----------|----------------------|
| HT/GCH2-10                  | Alphoid-BAC (100 kb) GCH1-BAC (180 kb) | CMV-bsd | 3 copies of GCH1 gene | − | 0.5 |
| HT/globin29                 | Alphoid-YAC (90 kb) Globin-YAC (150 kb) | PGK-neo | 10 copies of globin cluster | + | 0.3 |
by PacI digestion, and a 40-kb fragment of the globin gene in globin-HACs was excised by KpnI digestion, indicating that the size of all the fragments detected by probes were the same as in the precursor DNAs (Figs. 1 and 3).

**Structural and Mitotic Stabilities of HACs in Mouse ES Cells**—In mouse cells centromeric sequence is not alphoid DNA but mouse minor satellite DNA, and the average telomere length is 10 times longer than that of human chromosomes. We questioned whether bottom-up-constructed HACs composed of human sequences could maintain their original structures in a mouse ES cell line. To examine possible chromosomal rearrangements in HACs caused by acquisition of mouse genomic DNA during MMCT, HACs in ES cells were analyzed by FISH (Fig. 4). Mouse centromeric minor satellite, pericentromeric major satellite, and interspersed B1 repeat DNA sequences were used as probes. FISH analysis showed that signals for the minor satellite, major satellite, and B1 sequences were not detected in any of the nine GCH1-HACs or in 13 of 14 globin-HACs in mouse ES cells. One globin-HAC in the TT2/globin29-2 cell line contained minor satellite and major satellite DNA (Fig. 4, Table 2). These results indicate that all circular HACs and most linear HACs maintained structural stability in mouse ES cells without incorporation of mouse DNA.

Both the GCH1-HACs and the globin-HACs were stably maintained in HT1080 cells with a chromosome loss rate of 0.5 and 0.3%. The mitotic stability of the HACs in mouse ES cells was investigated by culturing under non-selective conditions for 200 or 120 divisions. The mitotic chromosome loss rate of GCH1-HACs in ES cells was 0.4 – 0.6%, whereas that of globin-HACs in ES cells was 1.0 – 3.6% (Table 2). These results indicate...
that circular-type GCH1-HACs can be maintained stably in mouse ES cells at the same level as in HT1080 cells, whereas the stability of linear-type globin-HACs was decreased severalfold in ES cells compared with HT1080 cells.

We next focused on the presence and lengths of the telomeres of HACs in ES cells. FISH analysis with telomeric probes showed that no signals were detected in GCH1-HACs in any of the nine cell lines including TT2/GCH2-10 and TT2F/GCH2-10 (Fig. 5, panel a and c). Weak telomeric signals were detected in globin-HACs in 13 cell lines including TT2/globin29-1 and TT2F/globin29 (Fig. 5, panel d). These results indicate that the circular HACs remained as telomere-less structures and that the linear HACs retained short telomere lengths. Interestingly, the telomeres of the globin-HAC in TT2/globin29-2, which acquired minor satellite DNA, became as long as those of mouse chromosomes (Fig. 5, panel b). The mitotic chromosome loss rate of the globin-HAC in TT2/globin29-2 was 1.0%, indicating that it was more stable than the other globin-HACs with short telomeres (Table 2).

Chimeric Mice Carrying the HACs—Chimeric mice carrying the HACs were created by injection of ES cell lines TT2/GCH2-10, TT2F/GCH2-10, TT2/globin29-2, and TT2F/globin29 into ICR(CD-1) blastocysts. One male chimera derived from TT2/GCH2-10, 2 male chimeras from TT2/globin29-2, 13 female chimeras from TT2F/GCH2-10, and 5 female chimeras from TT2F/globin29 were obtained. To investigate the presence of the HACs in chimeric mice, genomic DNA derived from primary tail fibroblasts was analyzed by PCR using HAC-specific primers. The HAC-specific signal was detected in each chimeric mouse. After crossing the chimeric mice with C57BL/6,
Transmissible Human Artificial Chromosomes in Mice

HAC-positive F1 offspring were obtained from three female chimeras derived from TT2F/GCH2-10 and one male chimera from TT2F/globin29-2 (Table 3). To investigate the presence and characteristics of the HACs in F1 mice, metaphase chromosomes derived from primary tail fibroblasts were analyzed by FISH using alphoid, mouse minor satellite, and telomeric sequences as probes (Fig. 6). All HAC+ F1 mice possessed one copy of the HAC as an independent chromosome. The HACs in the F1 cells appeared to be correctly maintained in that GCH1-HAC remained in a telomere-less circular form, and acquisition of mouse minor satellite DNA was not detected. The globin-HAC remained in a telomere-less circular form, and acquisition of mouse minor satellite DNA and long telomeres was the same as that of circular HACs without telomeres. The female germ line transmission of globin-HACs was not determined.

Mitotic Stability of HACs in Somatic Tissues of Mice—To investigate the stability of HACs after germ line transmission, the retention of GCH1-HAC in cells from brain, heart, liver, kidney, spleen, and bone marrow of F3 offspring at 93–113 weeks old from TT2F/GCH2-10 was analyzed by FISH (Fig. 7A). The GCH1-HACs were retained in all tissues examined. The stability of the HAC was high in the brain (41.7–89.1%), heart (45.5–77.3%), and kidney (43.3–84.2%), moderate in the liver (34.6–62.8%), and low in the spleen (7.6–26.2%) and bone marrow (10.2–18.2%) (Fig. 7B).

DISCUSSION

Mitotic and Structural Stabilities of Bottom-up-constructed HACs in Mouse ES Cells—Native and truncated human chromosomes can be maintained in somatic vertebrate cell lines, indicating that the human sequences of the centromere, replication origin, and telomere can function in vertebrate cells (12, 18–23, 25, 26). However, the mitotic and meiotic stabilities of the native and truncated human chromosomes measured by FISH analyses of mouse ES cells and bodies or by mouse germ line transmission are not high when compared with those of native mouse chromosomes (18–20, 22, 23, 25). The present study was the first to demonstrate the transfer of bottom-up-constructed circular and linear HACs into mouse ES cells and bodies. It has been reported that chromosomes transferred by MMCT often undergo chromosomal rearrangements of an unknown molecular mechanism. A truncated Y chromosome, ST1, was stabilized by rearrangement with mouse minor and major satellite DNA in mouse ES cells (27). We examined the acquisition of mouse minor satellite, major satellite, and B1 repeat DNAs by GCH1-HACs and globin-HACs in mouse ES cells. All GCH1-HACs and most globin-HACs did not acquire detectable mouse sequences, suggesting that the bottom-up-constructed HACs derived from human sequences were fully stable during mitotic growth in mouse ES cells.

The chromosome loss rates in mitotic segregation of GCH1-HACs and globin-HACs in mouse ES cells were 0.4–0.6 and 1.0–3.6%, respectively. This result indicated that circular

FIGURE 5. Structural analyses of HACs using telomeric probes. ES cells possessing HACs were analyzed by FISH, probing for telomeric DNA. Telomere signals (red) were not detected on GCH1-HACs in the metaphase chromosomes of TT2/GCH2-10 (a) or TT2F/GCH2-10 (c). In contrast, obvious telomere signals were detected on the chromosome ends of the globin-HAC in TT2/globin29-2 (b), and a weak telomere signal was observed on the globin-HAC in TT2F/globin29 (d) cell lines. The green signals (arrowheads) indicate the GCH1-HACs or globin-HACs detected by BAC vector (a and c) or YAC vector probes (b and d), respectively.

TABLE 3

| HAC+ pups/Total pups | Parents | F2 | F3 |
|----------------------|---------|----|----|
|                      | Male    | Female | Total |
| TT2F/GCH2-10         | 3 Females | 4 Males | 9/57 (16) | 5/35 (14) |
|                      | 6 Females |       | 35/122 (29) | 19/73 (23) |
| TT2F/globin29-2      | 1 Male   | 2 Males | 2/10 (20) | NT* |
|                      |         |       | 2/10 (20) | NT |

* NT, not tested.

which retained short telomeres and possessed no detectable mouse DNA, were not transmitted even with high chimeras or after several crossings. In contrast, we were able to obtain F1 offspring from a male TT2F/globin29-2 chimeric mouse. The 20% transmission rate of the globin-HAC in TT2F/globin29-2 indicated that the male germ line transmission of HACs with mouse centromeric DNA and long telomeres was the same as that of circular HACs without telomeres. The female germ line transmission of globin-HACs was not determined.
HACs in ES cells were as stable as those in human HT1080 cells (0.5%), whereas the stability of linear HACs in ES cells was slightly decreased compared with that in HT1080 cells (0.3%) (Table 2). To further examine the difference in stability between circular and linear HACs, the structure of the HACs was analyzed in detail. The major structural difference identified between globin-HACs and GCH1-HACs was the presence of telomeres at the chromosomal ends. Telomere-FISH indicated that BAC-based GCH1-HACs remained in the circular form, and most of the YAC-based globin-HACs remained in the linear form in mouse ES cells. The telomere length of 13 globin-HACs was substantially shorter than that of native mouse chromosomes. The only exception was that the telomere length of a globin-HAC in the TT2/globin29-2 cell line was the same as that of native mouse chromosomes. Furthermore, the chromosome loss rate of this globin-HAC was 1.0%, indicating that it was several times more stable than other globin-HACs. The reason why most of the globin-HACs were able to retain short telomeres remains unknown. Telomeric epigenetic factors or sub-telomeric sequence may be involved in the formation and maintenance of long telomeres in mice. Long telomere in TT2/globin29-2 could be acquired from mouse chromosomes at the same time as mouse minor and major satellite DNA.

These results suggest that the stability of linear chromosomes depends on the length of the telomeres, which can be altered by acquisition of telomeric DNA from the host cell. In terms of chromosome loss rate, both circular and linear HACs were slightly less stable than the native mouse chromosomes in mouse ES cells. Probably unknown factors such as the total length and composition of the chromosome influence HAC stability in each cell line.

**Germ Line Transmission of HACs**—The germ line transmission of human chromosomes in mice has been investigated using several types of chromosomes. Truncated chromosomes derived from human chromosomes 2, 14, 21, and 22 containing native centromeres naturally generated ring chromosomes isolated from a patient, and a truncated Y chromosome (ST1) stabilized with mouse centromeric DNA were all shown to be transmissible through the germ line, whereas the mardel (10) chromosome containing a neo-centromere was non-transmissible (18, 20, 22, 25, 28). However, the germ line transmission efficiency of human chromosomes in mice varies with the size and content of the chromosome. Therefore, the critical parameters that govern meiotic stability of human chromosomes in mice have not yet been established.

In this study germ line transmission of HACs was examined using circular (GCH1-HAC) and linear (globin-HAC) gene-containing HACs. The chimeric mice derived from TT2F/GCH2-10 successfully transmitted GCH1-HACs to their offspring at an average rate of greater than 20%. The transmission rate of GCH1-HACs from F1 offspring to F2 was 29% and from F2 offspring to F3 was 23%. The globin-HAC in TT2/globin29, which acquired mouse minor satellite DNA and long telomeres, was transmitted at a rate of 20%. It was believed that pairing of homologous chromosomes and telomeres is essential for the progression of meiosis because chiasmas are generated by crossing-over between homologous non-sister chromosomes, and telomere clustering and bouquet formation play an important role in homologous chromosome alignment (29). Mutations affecting telomere function in Schizosaccharomyces pombe lead to meiotic asynapsis, improper chromosome segregation, and sporulation defects (30). However, we demonstrated in this study that similar to human truncated chromosomes containing native centromeres, bottom-up-constructed HACs can be transmitted through meiosis even if the HACs are single-copy and do not possess telomeres. Cytogenetic studies indicated that ring
chromosomes in mouse meiotic division were associated with mouse pericentric heterochromatin (31). We were unable to determine whether the bottom-up-constructed HACs divided independently or divided through the centromeres of mouse chromosomes in meiosis I.

The small difference in chromosome loss rate between the linear globin-HACs in TT2/globin29−1 (2.4%) and TT2/globin29−2 (1.0%) suggested that the acquisition of long telomeres and minor satellite DNA did not always determine the mitotic stability of the linear HACs. However, the globin-HACs in TT2F/globin29, which maintained short telomeres, failed to be transmitted even after several crossings, suggesting that the meiotic stability was associated with the telomere length and/or the mouse centromeric sequence. However, the meiotic stability of chromosomes probably depends upon the structure and/or length of the chromosome in addition to distinct functional elements in the centromere and telomere, since the globin-HAC that acquired long telomeres did not always have high transmission efficiency compared with mouse chromosomes.

A previous study has indicated that ring chromosomes show no sex bias in germ line transmission (32). In contrast, we observed a sex bias in germ line transmission of the circular GCH1-HAC, with the transmission efficiency from females being higher than that from males. Transmission rates approaching 50% from females are as high as theoretically possible because GCH1-HAC was present in each cell as a single copy. Sex bias in chromosome segregation in mice was also observed in studies of dicentric chromatids and the chimeric chromosome ST1 (25, 33). In our study we obtained the interesting result that the transmission rate from females was 40% at every crossing, whereas the transmission rate from males decreased from 50 to 0% with successive crossings. The difference in the transmission pattern of the circular HACs between males and females may depend on differences in the mechanism of meiotic division in spermatogenesis and oogenesis. The results suggest that the circular HACs were not recognized as chromosomes, or circular HACs moved with the mouse chromosomes. In contrast, linear structures or telomeres of HACs were recognized by the meiotic mechanism.

Development of HACs as Gene Expression Vectors—The construction of HACs containing large genomic regions using a co-transfection strategy might be applicable to any genomic DNA cloned into BACs, PACs, or YACs. Compositional characteristics of HACs are simple compared with that of naturally derived ring chromosomes and mini-chromosomes produced by processing natural chromosomes, which may involve the retention of unknown genes. Although the several mini-chromosomes were stable in mouse ES cells and chimeric mice, moderate instability was observed after germ line transmission (23). In particular, the retention in dividing cells, spleen and bone marrow, was low in 11–35-week-old mice. The GCH1-HAC in 93–113-week-old F3 offspring was retained in 60% of the cells from the brain, heart, liver, kidney, spleen, and bone marrow. The percentage of HAC-retaining cells was calculated from about 300 cells.

Gene expression from HACs is predominantly regulated by the promoter that drives the genes, whereas the expression of transfected genes in mammalian stable transfectants is often repressed by positional effects of chromosomes at the points of gene insertion (34). Expression of the GCH1 gene from GCH1-HAC in HT1080 cells was shown to be elevated by induction of interferon-γ (14). Unfortunately, the expression of the GCH1 from GCH1-HAC was barely detected in mouse ES cells and in adult mouse tissues by reverse transcription-PCR. However, the human STAT3 gene cloned in another circular HAC was

FIGURE 7. Mitotic stability of GCH1-HAC in somatic tissues of mice. A, interphase nuclei from brain (a) and kidney (b) and metaphase chromosomes from spleen (c) of 93–113-week-old F3 mouse progeny were analyzed by FISH, probing for alphoid and BAC DNA. B, the retention of GCH1-HAC in brain, heart, liver, kidney, spleen, and bone marrow. The percentage of HAC-retaining cells was calculated from about 300 cells.
fully expressed in mouse ES cells for long periods in culture.\(^3\) The sequence variation in regulatory elements and transcriptional factors between mice and humans might induce transcriptional incompatibility of the large genomic GCH1 gene on the HAC rather than the position effect of HAC structure. In contrast, the expression of G\(\gamma\)-globin from a globin-HAC was shown to be repressed in fibroblast HT1080 cells but activated in the leukemia cell line K-562.\(^4\) The \(\beta\)-globin gene in a globin-HAC was expressed in the blood cells of mice (unpublished data).\(^4\) Regulated gene expression has been accomplished both by the use of large genomic regions containing regulatory elements in addition to the gene sequences and by prevention of positional effects or insertional mutagenesis of host chromosomes. Development of the HAC technology has enabled us to achieve this regulated gene expression.

In this study we demonstrated that gene-carrying HACs could be maintained in mouse ES cells and mouse somatic tissues and that they were transmissible in mice via the germ line. The mitotic and meiotic stabilities of HACs demonstrated the feasibility of regulated gene expression in mice using bottom-up-constructed HACs. MMCT using HAC could make stable transfectants with uniform characteristics, whereas conventional transfection using large genomic regions containing regulatory elements in addition to the gene sequences and by prevention of positional effects or insertional mutagenesis of host chromosomes. Development of the HAC technology has enabled us to achieve this regulated gene expression.

Acknowledgment—We thank Shin-ichi Aizawa for gift of TT2F.

REFERENCES

1. Mineta, T., Rabkin, S. D., Yazaki, T., Hunter, W. D., and Martuza, R. L. (1995) Nat. Med. 1, 938–943
2. Pfeifer, A., and Verma, I. M. (2001) Annu. Rev. Genomics Hum. Genet. 2, 177–211
3. Wade-Martins, R., White, R. E., Kimura, H., Cook, P. R., and James, M. R. (2000) Nat. Biotechnol. 18, 1311–1314
4. Willard, H. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6847–6850
5. Lin, Z., and Meija, J. E. (2002) Trends Genet. 18, 313–319
6. Grimes, B. R., and Mnaco, Z. L. (2005) Trends Genet. 21, 313–319
7. Farr, C. J., Bayne, R. A. L., Kipling, D., Mills, W., Critcher, R., and Cooke, H. J. (1995) EMBO J. 14, 5444–5454
8. Heller, R., Brown, K. E., Burgtorf, C., and Brown, W. R. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7125–7130
9. Harrington, J. I., Van Bokkelen, G., Mays, R. W., Gustashaw, K., and Willard, H. F. (1997) Nat. Genet. 4, 345–355
10. Ikeno, M., Grimes, B., Okazaki, T., Nakano, M., Saitoh, K., Hoshino, H., McGill, N. L., Cooke, H., and Masumoto, H. (1998) Nat. Biotechnol. 16, 431–439
11. Henning, K. A., Novotny, F. A., Compton, S. T., Guan, X. Y., Liu, P. P., and Ashlock, M. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 592–597
12. Kuroiwa, Y., Tomizuka, K., Shinohara, T., Kazuki, Y., Yoshiha, H., Ohguma, A., Yamamoto, T., Tanaka, S., Oshimura, M., and Ishida, I. (2000) Nat. Biotechnol. 18, 1086–1090
13. Ebersole, T. A., Ross, A., Clark, E., McGill, N. L., Schindelhauer, D., Cooke, H. I., and Grimes, B. R. (2000) Hum. Mol. Genet. 9, 1623–1631
14. Ikeno, M., Inagaki, H., Nagata, K., Morita, M., Ichinose, H., and Okazaki, T. (2002) Genes Cells 7, 1021–1032
15. Ohzeki, J., Nakano, M., Okada, T., and Masumoto, H. (2002) J. Cell Biol. 159, 765–775
16. Mejia, J. E., Willmott, A., Levy, E., Earnshaw, W. C., and Linar, Z. (2001) Annu. Hum. Genet. 69, 315–326
17. Grimes, B. R., Schindelhauer, D., McGill, N. L., Ross, A., Ebersole, T. A., and Cooke, H. I. (2001) EMBO Rep. 2, 910–914
18. Kazuki, Y., Shinohara, T., Tomizuka, K., Kato, M., Ohguma, A., Ishida, I., and Oshimura, M. (2001) J. Hum. Genet. 46, 600–603
19. O’Doherty, A., Ruf, S., Mulligan, C., Hildreth, V., Errington, M. L., Cooke, S., Sesay, A., Modino, S., Vanes, L., Hernandez, D., Linehan, J. M., Sharpe, P. T., Brandner, S., Bliss, T. V., Henderson, D. J., Nizetic, D., TyeBulewicz, V. L., and Fisher, E. M. (2005) Science 309, 2033–2037
20. Tomizuka, K., Yoshiha, H., Uejima, H., Kugoh, H., Sato, K., Ohguma, A., Hayasaka, M., Hanaoka, K., Oshimura, M., and Ishida, I. (1997) Nat. Genet. 16, 133–143
21. Kuroiwa, Y., Kasinathan, P., Choi, Y. J., Naeem, R., Tomizuka, K., Sullivan, E. J., Knott, J. G., Duteau, A., Goldsby, R. A., Osborne, B. A., Ishida, I., and Robl, J. M. (2002) Nat. Biotechnol. 20, 889–894
22. Shen, M. H., Mees, P. J., Nichols, J., Yang, J., Brook, F., Gardner, R. L., Smith, A. G., and Brown, W. R. A. (2000) Curr. Biol. 10, 31–34
23. Shinohara, T., Tomizuka, K., Takehara, S., Yamauchi, K., Katao, M., Ohguma, A., Ishida, I., and Oshimura, M. (2000) Chromosome Res. 8, 713–725
24. Ikeno, M., Masumoto, H., and Okazaki, T. (1994) Hum. Mol. Genet. 3, 1245–1257
25. Mee, P. J., Shen, M. H., Smith, A. G., and Brown, W. R. A. (2003) Chromosome (Berl.) 112, 183–189
26. Mills, W., Critcher, R., Lee, C., and Farr, C. J. (1999) Hum. Mol. Genet. 8, 751–761
27. Shen, M. H., Yang, J., Lopour, M. L., Smith, A., and Brown, W. R. A. (1997) Hum. Mol. Genet. 6, 1375–1382
28. Wong, L. J., Saffery, R., Anderson, M. A., Earle, E., Quach, J. M., Stafford, A. J., Fowler, K. J., and Choo, K. H. A. (2005) Nature 431–439
29. Cooper, J. P., Watanabe, Y., and Nurse, P. (1998) Nature 392, 828–831
30. Nimmo, E. R., Pidoux, A. L., Perry, P. E., and Allshire, R. C. (1998) Nature 392, 825–828
31. Voet, T., Liebe, B., Labaere, C., Marynen, P., and Scherthan, H. (2003) J. Cell Biol. 162, 795–807
32. Voet, T., Vermeesch, J., Carens, A., Durr, J., Labaere, C., Duhamel, H., David, G., and Marynen, P. (2001) Genome Res. 11, 124–136
33. Kohler, K. E., Millie, E. A., Cherry, J. P., Burgoyne, P. S., Evans, E. P., Hunt, P. A., and Hassold, T. J. (2002) Genetics 162, 1367–1379
34. Karpen, G. H. (1994) Curr. Opin. Genet. Dev. 4, 281–291

---

\(^3\) N. Suzuki, T. Okazaki, and M. Ikeno, unpublished data.

\(^4\) N. Suzuki, T. Itou, K. Nishii, T. Okazaki, and M. Ikeno, unpublished data.