Human neocentromeres are functional centromeres that are devoid of the typical human centromeric \(\alpha\)-satellite DNA. We have transferred a 60-Mb chromosome 10-derived neocentric marker chromosome, mardel(10), and its truncated 3.5-Mb derivative, NC-MiC1, into mouse embryonic stem cell and have demonstrated a relatively high structural and mitotic stability of the transchromosomes in a heterologous genetic background. We have also produced chimeric mice carrying mardel(10) or NC-MiC1. Both transchromosomes were detected as intact episomal entities in a variety of adult chimeric mouse tissues including hemopoietic stem cells. Genes residing on these transchromosomes were expressed in the different tissues tested. Meiotic transmission of both transchromosomes in the chimeric mice was evident from the detection of DNA from these chromosomes in sperm samples. In particular, germ line transmission of NC-MiC1 was demonstrated in the F1 embryos of the chimeric mice. Variable (low in mardel(10)- or NC-MiC1-containing embryonic stem cells and chimeric mouse tissues and relatively high in NC-MiC1-containing F1 embryos) levels of missegregation of these transchromosomes were detected, suggesting that they are not optimally predisposed to full mitotic regulation in the mouse background, particularly during early embryogenesis. These results provide promising data in support of the potential use of neocentromere-based human marker chromosomes and minichromosomes as a tool for the study of centromere, neocentromere, and chromosome biology and for gene therapy studies in a mouse model system. They also highlight the need to further understand and overcome the factors that are responsible for the definable rates of instability of these transchromosomes in a mouse model.

Centromeres are chromosomal loci that ensure the correct segregation of chromosomes and inheritance of genetic information. In humans, the centromeres consist of the 171-bp \(\alpha\)-satellite DNA that is tandemly repeated up to several megabases in size. In contrast, human neocentromeres belong to a new class of centromeres that are devoid of \(\alpha\)-satellite DNA formed epigenetically following cytogenetic rearrangements (reviewed in Ref. 1). These neocentromeres associate with all of the functionally essential centromere proteins and heterochromatin proteins to form active kinetochores conferring full mitotic stability (2, 3). The first described case of a neocentromere was found at the q25 region of a chromosome 10-derived marker chromosome mardel(10) (4, 5).

Human engineered chromosomes (HECs)\(^1\) are autonomously replicating entities that can function and segregate as stable episomal entities. Such HECs serve as a useful model system for the study of centromere and chromosome properties and as gene expression vectors to complement genetic deficiencies in human cells. Early studies in the construction of HECs have involved either the transfection of \(\alpha\)-satellite DNA into human cells (6–12) or the use of telomere-associated chromosomal truncation to remove the arms of endogenous chromosomes to produce minichromosomes (13–17). Another strategy has involved the amplification of pericentric satellite DNA followed by the breakage of chromosomes to produce satellite DNA-based HECs (18, 19).

The discovery of neocentromeres has provided an alternative approach to the construction of HECs. We have described previously the production of mitotically stable neocentromere-based minichromosomes (NC-MiCs) through the telomere-mediated truncation of the mardel(10) chromosome (20). These NC-MiCs are amenable to full sequence characterization to provide a well defined tool. In addition, we have examined the expression status of coding genes within our 1q25 neocentromere on the mardel(10) chromosome and demonstrated transcriptional competency within the CENP-A and CENP-H domains and throughout the domain of enhanced scaffold/matrix attachment region, indicating that the process of neocentromere formation that results in the assembly of highly specialized centromeric chromatin has no measurable effect on gene expression (21).

Mice containing HECs or human chromosomal fragments have been generated to provide animal models for HEC studies; however, little is known regarding the stability and expression status of these exogenous chromosomes in the different mouse tissues (22–29). In this study, we have generated transgenic mice containing a neocentromere-based mardel(10) or NC-MiC and studied the in vivo stability and expression of coding genes within the 1q25 neocentromeric region.
gene expression status of these chromosomes in a range of tissues and cell types.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**HT1080 cell line containing NC-MiC1 and CHOK1-derived ZB30 cells containing mardel(10) tagged with zeocin resistance gene were cultured in DMEM (Invitrogen) with 10% FCS or KAO-modified Hams' medium, respectively, as described previously (20). Zeocin (Invitrogen) was added into both cultures at a final concentration of 100 μg/ml. Mouse embryonic stem cell line ES129.1 was cultured in DMEM with 15% heat-inactivated FCS and 103 units/ml leukemic inhibiting factor (Chemicon International) and 0.1 mM β-mercaptoethanol. The mouse ES129.1 cell line expressing green fluorescent protein (GFP) was generated by the transfection of a plasmid pEF-GCF-C1 containing the GFP and neomycin-resistant genes. 5 × 10⁷ cells were electroporated (1.2 kV/25 microfarads, Bio-Rad Gene Pulser electroporator) with 15 μg of linearized construct. Cells were plated 24–48 h posttransfection, and 200 μg/ml neomycin G418 (Invitrogen) was added into the culture for selection of positive clones.

**Microcell-mediated Chromosome Transfer (MMCT)—**Microcell fusion was carried out to transfer mardel(10) and NC-MiC1 tagged with zeocin resistance gene from the CHOK1 and HT1080 respective back-grounds into neomycin-resistant mouse ES129.1 cells expressing GFP (ES129.1GFP). Log-phase donor ZB30 cells were arrested in colcemid (Invitrogen) for 48 h and resuspended in Percoll/serum-free DMEM (1:1) supplemented with 20 μg/ml cytochalasin B (Sigma). The cell suspension was then subjected to centrifugation at 18,000 rpm for 90 min at 32°C. Both bands of cell mixture were pelleted, washed with serum-free DMEM, and filtered through isopore membranes of 30, 8, and 5 μM (Millipore Corp.). Microcells were then fused with recipient

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**FIG. 1. Formation and characterization of NC-MiC1.**

*a,* Mardel(10) was formed following a complex rearrangement in which the central part of chromosome 10 was deleted to form a ring structure with joining of the ends and neocentromere formation producing the mardel(10) marker chromosome. NC-MiC1 was formed as a result of unknown rearrangements following transfer of this marker from a CHO-based somatic cell hybrid line (20) into human HT1080 cells. *b,* FISH analysis using BAC clones of known position indicates the estimated size of NC-MiC1 to be ~3.5 Mb. + and − denote presence and absence of a BAC signal, respectively. *c,* examples of FISH experiments used to characterize NC-MiC1 are as follows: *i,* dual-color FISH analysis using BACs BA153G5 (green) and BA373K12 (red; q' arm) mapping to 10q25 and 10p15, respectively, demonstrating separation of signals on chromosome 10 (open arrow) and co-localization of signals on NC-MiC1 (closed arrowhead); *ii,* dual-color FISH analysis using BACs BA153G5 (green) and BA69k10 (red; p' arm) both mapping to 10q25, demonstrating positive signals on NC-MiC1; *iii,* dual-color FISH analysis using BACs BA153G5 (green) and BA5N23 (red; q' arm) mapping to 10q25 and 10p15, respectively, demonstrating the presence of signals on chromosome 10 (open arrow) and the absence of B5N23 signal on NC-MiC1 (closed arrowhead); and *iv,* reverse painting of NC-MiC DNA on human chromosomes, demonstrating that NC-MiC1 was exclusively derived from 10p15 and 10q25 DNA.
Mardel(10) and NC-MiC1 Remain Structurally Intact and Stable following Transfer into Mouse ES Cells—In this study, both ZB30 and HT1080-NC-MiC1 lines were used as donors for cloning experiments on ES GFPmar(10)-1 and ES GFPNC-MiC1-2 were cultured for 80 divisions in the presence (100 \mu g/ml) or absence of zeocin. The cells were harvested at various intervals to determine the stability of mardel(10) or NC-MiC1. Retention rates of 80–90% and 55–70% were observed for the respective marker chromosomes after 80 cell divisions with and without selection, implying a small loss rate over time in the mouse ES cell background.

## Table I

**Stability of ES GFPmar(10)-1 and ES GFPNC-MiC1-2**

| Cell line | Passage no. | Division no. | Drug selection | No. of cells scored | Percentage of retention | Loss per division |
|-----------|-------------|--------------|----------------|--------------------|------------------------|------------------|
| ES GFPmar(10)-1 | 5 | 20 | Zeocin | 20 | 20/20 (100) | 0 |
| | 10 | 40 | Zeocin | 20 | 19/20 (95) | 0.13 |
| | 15 | 60 | Zeocin | 20 | 18/20 (90) | 0.17 |
| | 20 | 80 | Zeocin | 20 | 18/20 (90) | 0.13 |
| ES GFPmar(10)-1 | 5 | 20 | Zeocin | 20 | 20/20 (100) | 0 |
| | 10 | 40 | Zeocin | 20 | 19/20 (95) | 0.13 |
| | 15 | 60 | Zeocin | 20 | 15/20 (75) | 0.42 |
| | 20 | 80 | Zeocin | 20 | 15/20 (75) | 0.42 |
| ES GFPNC-MiC1-2 | 5 | 20 | Zeocin | 20 | 20/20 (100) | 0 |
| | 10 | 40 | Zeocin | 20 | 17/20 (85) | 0.38 |
| | 15 | 60 | Zeocin | 20 | 15/20 (75) | 0.42 |
| | 20 | 80 | Zeocin | 20 | 15/20 (75) | 0.63 |
| | 20 | 13/20 (65) | 0.58 |
| | 20 | 11/20 (55) | 0.56 |

For reverse painting of NC-MiC1, total genomic DNA from ES GFPNC-MiC1 was subjected to Alu-PCR amplification (30) followed by standard FISH analysis. Fluorescence in Situ Hybridization (FISH)—Standard procedures were followed for FISH analysis. Mouseubat-uolymerizing agent colcemid was added at 10 \mu M for 1 h before the harvesting of the cells. These cells were then subjected to hypotonic treatment in 0.075M of KCl followed by fixation in 3:1 methanol/acetic acid prior to spreading onto slides. The slides were then dehydrated in an ethanol series, denatured in 70% formamide/2\% SSC at 70 °C, and hybridized at 37 °C overnight with the relevant labeled probes. After three washes in 0.1× SSC at 60 °C, the probes were detected with fluorescence-conjugated reagents according to the manufacturer’s instruction. Images were collected using a fluorescence microscope linked to a CCD camera system. For reverse painting of NC-MiC1, total genomic DNA from ES GFPNC-MiC1 was subjected to Alu-PCR amplification (30) followed by standard FISH analysis.

**Blastocyst Injection and Generation of Chimeric Mice—ES129.1GFp**

The injected blastocysts were then transferred into recipient pseudopregnant mice. Chimeric mice were selected by coat color. Chimeric mice were also crossed with C57BL/6 mice to generate embryos and mice containing germ-line transmitted mardel(10) or NC-MiC1.

**Tissue Collection and Genotyping of Chimeric Mice and Embryos—**

Tissues collected from chimeric mice were subjected to DNA isolation using QIAamp tissue purification kit (Qiagen) according to manufacturer’s protocols. In addition, certain tissues including lung, kidney, and mice containing germ-line transmitted mardel(10) or NC-MiC1 were subjected to DNA isolation and mice containing germ-line transmitted mardel(10) or NC-MiC1 were subjected to Alu-PCR amplification (30) followed by standard PCR analysis.

**Characterization of NC-MiC1—**

We have previously produced a somatic hybrid cell line (designated ZB30) containing mardel(10) tagged with a zeocin resistance gene in a Chinese hamster ovary background (20). The transfer of the mardel(10) chromosome via MMCT into human HT1080 cells followed by telomere-associated chromosome truncation resulted in a number of lines containing truncated minichromosome derivatives of mardel(10) (20). A cell line, NC-MiC1 (zeoR), was identified by FISH as carrying a small minichromosome containing the 10q25-derived neocentromere region and the zeocin resistance gene from a separate region of the mardel(10) chromosome formed through unknown rearrangements during the MMCT procedure (Fig. 1n). Reverse painting demonstrated that this minichromosome was derived solely from DNA of the 10q25 and 10p15 regions of chromosome 10 (Fig. 1c). As we had previously characterized the 10q25 region contained in this minichromosome (20), sequence-tagged site PCR analysis was performed to determine the content of the 10p15-derived DNA of this minichromosome. A total of 47 primer pairs spanning 15 Mb of the 10p region (Supplementary Table I) were used in amplification experiments on ES GFPNC-MiC1 genomic DNA that contained NC-MiC1 alone in a mouse ES background (see below). Of these primer sets, five produced positive signals in the PCR amplification. BAC clones in this region were then used in FISH experiments to directly visualize the 10p15 DNA content of NC-MiC1 (Fig. 1, b–c). From these combined analyses, the structure of NC-MiC1 was determined and the total size was calculated as 3.5 Mb.

**Mardel(10) and NC-MiC1 Remain Structurally Intact and Stable following Transfer into Mouse ES Cells—**

In this study, both ZB30 and HT1080-NC-MiC1 lines were used as donors for cloning experiments on ES GFPmar(10)-1 and ES GFPNC-MiC1-2 were cultured for 80 divisions in the presence (100 \mu g/ml) or absence of zeocin. The cells were harvested at various intervals to determine the stability of mardel(10) or NC-MiC1. Retention rates of 80–90% and 55–70% were observed for the respective marker chromosomes after 80 cell divisions with and without selection, implying a small loss rate over time in the mouse ES cell background.

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| | 20 | 80 | Zeocin | 20 | 18/20 (90) | 0.13 |
| ES GFPmar(10)-1 | 5 | 20 | Zeocin | 20 | 20/20 (100) | 0 |
| | 10 | 40 | Zeocin | 20 | 19/20 (95) | 0.13 |
| | 15 | 60 | Zeocin | 20 | 18/20 (90) | 0.25 |
| | 20 | 80 | Zeocin | 20 | 18/20 (90) | 0.25 |
| ES GFPNC-MiC1-2 | 5 | 20 | Zeocin | 20 | 20/20 (100) | 0 |
| | 10 | 40 | Zeocin | 20 | 17/20 (85) | 0.38 |
| | 15 | 60 | Zeocin | 20 | 15/20 (75) | 0.42 |
| | 20 | 80 | Zeocin | 20 | 15/20 (75) | 0.63 |
| | 20 | 13/20 (65) | 0.58 |
| | 20 | 11/20 (55) | 0.56 |

For reverse painting of NC-MiC1, total genomic DNA from ES GFPNC-MiC1 was subjected to Alu-PCR amplification (30) followed by standard FISH analysis. Fluorescence in Situ Hybridization (FISH)—Standard procedures were followed for FISH analysis. Mouseubat-uolymerizing agent colcemid was added at 10 \mu M for 1 h before the harvesting of the cells. These cells were then subjected to hypotonic treatment in 0.075M of KCl followed by fixation in 3:1 methanol/acetic acid prior to spreading onto slides. The slides were then dehydrated in an ethanol series, denatured in 70% formamide/2\% SSC at 70 °C, and hybridized at 37 °C overnight with the relevant labeled probes. After three washes in 0.1× SSC at 60 °C, the probes were detected with fluorescence-conjugated reagents according to the manufacturer’s instruction. Images were collected using a fluorescence microscope linked to a CCD camera system. For reverse painting of NC-MiC1, total genomic DNA from ES GFPNC-MiC1 was subjected to Alu-PCR amplification (30) followed by standard FISH analysis.

**Blastocyst Injection and Generation of Chimeric Mice—ES129.1GFp**

The injected blastocysts were then transferred into recipient pseudopregnant mice. Chimeric mice were selected by coat color. Chimeric mice were also crossed with C57BL/6 mice to generate embryos and mice containing germ-line transmitted mardel(10) or NC-MiC1 were subjected to Alu-PCR amplification (30) followed by standard PCR analysis.
Chimeric Mice Retained Mardel(10) and NC-MiC1 Episomally and Relatively Stably in Many Tissue Types—Microinjection of neomycin-resistant ESGFPmar(10)-1 or ESGFPNC-MiC1-2 into mouse blastocysts followed by reimplantation into foster female mice resulted in 19 and 65 high grade chimeric mice, respectively. An analysis of tissue samples from adult mice by PCR using human chromosome 10q25-specific primers demonstrated the presence of both mardel(10) and NC-MiC1 in a variety of tissues (examples from two mice for each of mardel(10) and NC-MiC1 are shown in Tables II and III). In total, 85.7 and 84.6% chimeric mice were found to be positive for mardel(10) and NC-MiC1, respectively. Of the positive chimeric mice, we asked the question of what percentage of ES129.1GFP-derived tissues had retained the transchromosomes. The presence of parental ES129.1GFP cells was identified by PCR using primers corresponding to a part of the neomycin resistance gene that was present in these cells. The

MMCT into mouse embryonic stem cell line ES129.1GFP expressing GFP (see “Experimental Procedures”). Thirteen positive fusion cell lines containing mardel(10) and seven containing NC-MiC1 were isolated. One cell line containing mardel(10) (ESGFPMar(10)-1) and another containing NC-MiC1 (ESGFPNCMiC1-2) were analyzed further. Mitotic stability of ESGFPmar(10)-1 over 80 cell divisions was 90% with selection and 80% without selection, suggesting that mardel(10) was largely stable but carried a very low rate (Table I). All of the other cell lines containing mardel(10) showed similar mitotic stability (with average loss rates of 0.15 and 0.25%/division with and without selection). NC-MiC1 was found to be slightly less stable in mouse ES 129.1 cells with retention rates of 70 and 55% with and without selection after 80 cell divisions, respectively. Likewise, all of the other NC-MIC1 containing cell lines were relatively similar in terms of their mitotic stability with the exception of two clones that demonstrated some degree of DNA amplification (with retention rates ranging from 50 to 75% after 80 divisions). Immunofluorescence using CREST6 autoimmune anticentromere serum (5) and specific antiserum to centromere proteins CENP-A and CENP-E was positive on both mardel(10) and NC-MiC1 (Figs. 2 and 3). Detailed FISH analysis using human COT1, total genomic mouse DNA, mouse centromeric major and minor satellite DNAs, and various BAC probes at and surrounding the 10q25 neocentromere demonstrated that both the mardel(10) and NC-MiC1 remained structurally intact following MMCT transfer and had not acquired any mouse genomic sequences including centromeric elements (Figs. 2 and 3). Thus, notwithstanding the low measurable loss rate, the results demonstrate the overall function of human neocentromeres to support mitotic stability of intact mardel(10) and NC-MiC1 minichromosome in the mouse ES cell background.

**Fig. 2.** Microcell-mediated chromosome transfer of mardel(10) into mouse ES129.1GFP cells. I, FISH analysis of ESGFPmar(10)-1 cell line using 10q25 neocentromere-specific and flanking BAC probes (20). Mardel(10) is indicated by an arrow. a, i–iii, combined and split images for BAC probe (green) and DAPI staining. FISH using 10q25 neocentromere-specific probe B153g5 (ii, green) showing the presence of mardel(10) in ESGFPmar(10)-1. b–c, i–iii, combined and split images for dual-color FISH signals (green and red). Positive FISH signals using

BAC probes B5402 on proximal p’ arm (bii, green), B119p3 on proximal q’ arm (ciii, green), and neocentromeric E8 probe (biii and ciii, red) show intactness of the neocentromere region on mardel(10) in mouse ES cells. d–e, dual-color immunofluorescence and FISH analysis using centromere-specific CREST6 (diii, red) and CENP-E (eiii, red) antibodies and NC-specific probe E8 (ii, green), showing the presence of a functional centromere on mardel(10). II, FISH analysis of ESGFPmar(10)-1 using human Cot1 and mouse DNA probes. Mardel(10) is indicated by an arrow. a, i–iii, combined and split images for green and red, respectively. a, FISH using NC-specific probe E8 (ii, green) and human Cot1 DNA (iii, red), showing the sole presence of mardel(10) and the absence of other human chromosomes in ESGFPmar(10)-1. b, FISH using E8 (ii, green) and mouse centromeric major satellite DNA (iii, red), showing the absence of mouse major satellite in mardel(10) in ESGFPmar(10)-1. c, FISH using E8 (ii, green) and mouse centromeric minor satellite DNA (iii, red), showing the absence of mouse minor satellite in mardel(10) in ESGFPmar(10)-1. d, FISH using E8 (ii, green) and mouse genomic DNA paint (iii, red), showing the absence of mouse genomic DNA in mardel(10) in ESGFPmar(10)-1. The
PCR results (some examples shown in Tables II and III) indicated that 74.6 and 69.5% ES129.1GFP-containing tissues were positive for mardel(10) and NC-MiC1, respectively. Together, the above analyses indicated a 15% loss rate for the transchromosomes during chimeric mouse production and 25–30% loss rate within the ES cell-positive tissues of the transchromosomal mice.

Tissues such as skin, kidney, lung, and tail (other tissues were not viable in cultures) were collected from the transchromosomal chimeras for FISH analysis to determine the structural status of the introduced chromosomes. A combination of human and mouse cot1 DNA, whole human chromosome 10 paint, and neocentromere-specific BACs were used as FISH probes. The presence of both mardel(10) or NC-MIC1 in the cells derived from these cultured tissues varied significantly (ranging from 1 in 1000 to 1 in 25 cells); however, in all of the positive tissues, the transchromosomes were shown to be epigenomically intact with no detectable mouse se-
quences (some examples are shown in Figs. 4 and 5). In a small proportion (8.9%) of cells derived from NC-MiC1 chimeric tissues, two transchromosomes were observed, suggesting a certain level of missegregation.

Specific experiments were performed to isolate hematopoietic stem cells from two chimeric mice, one carrying mardel(10) and the other carrying NC-MiC1. The ES129.1GFP-derived hematopoietic stem cells were isolated by FACS of bone marrow cells expressing both GFP and Sca-1 markers. Despite our attempts to culture the FACS-purified cells, no metaphase spreads were found to enable a more detailed chromosomal analysis. However, FISH studies on cells at metaphase cultured from other tissues (such as kidney, lung, and spleen) from both mice have demonstrated that the transchromosomes in these animals were episomal and structurally intact.

**Table III**

| Tissues | NC-MiC1 | ES129.1 |
|---------|---------|---------|
| Left lung | +ve | +ve |
| Right lung | +ve | +ve |
| Left kidney | -ve | -ve |
| Right kidney | +ve | +ve |
| Small intestine | -ve | -ve |
| Large intestine | -ve | -ve |
| Heart | +ve | +ve |
| Brain | +ve | +ve |
| Stomach | +ve | +ve |
| Thymus | +ve | +ve |
| Adrenal | -ve | +ve |

**A** Chimeric mouse THO

**B** Chimeric mouse JN

![Figure 4](image1.png)

**Figure 4.** Characterization of mardel(10) in various tissues of chimeric mice. FISH analysis of tissues derived from chimeric mice PL, CH, and KM. Mardel(10) is indicated by an arrow. a, i–iii, FISH using E8 probe (green) and human cot1 DNA (red), showing presence of mardel(10) in cells cultured from PL lung (i), PL spleen (ii), and CH tail (iii). b, i–iii, FISH using E8 probe (green) and mouse cot1 DNA (red), showing the absence of mouse DNA on mardel(10) in cells established from PL lung (i), PL spleen (ii), and CH skin (iii). c, i–iii, FISH using E8 probe (red) and human chromosome 10 paint (green), showing positive painting solely on mardel(10) in cell cultures established from PL skin (i), CH tail (ii), and KM tail (iii).

![Figure 5](image2.png)

**Figure 5.** Characterization of NC-MiC1 in various tissues of chimeric mice. FISH analysis of tissues derived from chimeric mice TT and TEQ. NC-MiC1 is indicated by an arrow. FISH using B153g5 probe (i, green) and DAPI (ii) shows the presence of NC-MiC1 in cells cultured from TT skin (a), TEQ lung (b), and TEQ skin (c).
Expression of Genes on Mardel(10) and NC-MiC1 in Chimeric Mouse Tissues—RT-PCR analysis was also performed on RNA samples collected from the chimeric mice to determine the expression status of a number of genes present in the 10q25 DNA contained within mardel(10) and NC-MiC1. A wide variety of tissues from ESGFPmar(10) (e.g. mouse KM) and four ESGFPNC-MiC1 (e.g. mouse AIR) mice were obtained and subjected to analysis using previously optimized RT-PCR assays (20). The primer pairs are listed in Supplementary Table II. From this analysis, it was clear that many of the genes present on mardel(10) and NC-MiC1 are expressed within the chimeric mouse tissues (Table IV).

Germ Line Transmission and Mitotic Instability of NC-MiC1 in F1 Mouse Embryos—Although several studies have now demonstrated germ line transmission of human chromosomal fragments containing α-satellite-based centromeres, to date no such analysis has been performed on neocentromere-based marker chromosomes or minichromosomes. To test for the capability of such chromosomes to undergo germ line transmission, we first carried out PCR analysis to test chimeric mice for the presence of the transchromosomal DNA in sperm samples. A total of 14 and 67% chimeric mice were found to have positive sperm containing mardel(10) and NC-MiC1 DNA respectively, indicating the transmission of neocentromere-based DNA markers through meiosis to the male gametes. Mice containing these chromosomes were subsequently crossed with C57BL/6 mice to generate F1 mouse progeny to determine the germ line transmissibility of the transchromosomes. The mice were mated continuously over a period of 12 months to optimize the number of progeny from each mouse. From the small number of chimeric mice that were positive for mardel(10) DNA in sperm samples, no mardel(10)-positive embryo or live born progeny was produced, suggesting either mitotic dysfunction and/or a developmental incompetence of mardel(10)-containing embryos.

Despite the use of a significantly larger number of sperm-positive chimeric mice carrying NC-MiC1 (in the F1-production experiment), again no live born F1 progeny containing NC-MiC1 was observed. This prompted us to undertake a more detailed examination of early gestation embryos harvested from some of the pregnant mice on day 2.5 post coitum (38 embryos in total; 11 of which were positive) and day 9.5 post coitum (18 embryos in total; 4 of which were positive). FISH analysis using human cot1 probe performed on these embryos showed that NC-MiC1 was detected at the early stages but was lost at a relatively high rate during embryogenesis even at 2.5 days post coitum (examples are shown in Fig. 6 and Table V). Furthermore, the older embryos (9.5 days post coitum) showed a greatly increased number of cells containing more than one copy of NC-MiC1 in each cell (Fig. 6 and Table V). However, the NC-MiC1 remained as an episomal entity in all of the embryos that contained the transchromosome (Fig. 6).

**DISCUSSION**

HECs, similar to autonomous entities that can function and segregate like their normal chromosome counterparts, provide a useful tool to study centromere and chromosome biology and a potential novel strategy for the ex vivo gene therapy of a variety of clinical conditions. Neocentromeres that are fully functional and formed spontaneously on non-centromeric regions offer an alternative source of centromere function for the construction of HECs. In this study, we have characterized the properties of a HEC (NC-MiC1) generated from the neocentric mardel(10) chromosome in mouse background. Both mardel(10) and NC-MiC1 were found to be relatively stable in mouse ES cells with a
TABLE V

| Embryo    | Percentage of cells positive for NC-MiC1 (actual no. of cells scored) | NC-MiC1 copy no. in positive cells |
|-----------|------------------------------------------------------------------------|-----------------------------------|
| JL 1.1 (9.5 days) | 1.6 (16/1000)                                                       | 1 copy 38 15                       |
| JL 1.48 (9.5 days) | 4 (40/1000)                                                          | 1 copy 36 14                       |
| JL 1.61 (2.5 days) | 75 (15/20)                                                           | 1 copy 11 0                        |
| JL 1.66 (2.5 days) | 50 (4/8)                                                              | 1 copy 0 50                        |
| JL 1.73 (2.5 days) | 66.7 (4/6)                                                            | 1 copy 100 0                       |
| JL 1.82 (2.5 days) | 100 (5/5)                                                            | 1 copy 100 0                       |

respective retention rate of ~85 and 65% without selection after 60 cell divisions, implying a small loss rate over time. The initial loss rate appears to slow down after 40–60 divisions as the human transchromosomes “adapt” to the mouse genetic background, suggesting that there may be slight differences in the properties associated with mitotic activity between a human and mouse chromosomes. The instability of human chromosomes, particularly during early divisions, in a somatic mouse hybrid background has been described previously (14, 15, 33, 34); however, the mechanism underlying such instability is still unclear. The loss rate of these neocentromere-based transchromosomes compares favorably to the rates seen in other *α*-satellite DNA-based HECs in mouse background where loss rates as high as 4.5%/division have been described (14, 34, 35). Furthermore, in some previous studies, HECs have been shown to have acquired mouse centromeric/pericentric satellite sequences following transfer to a mouse cell line (15, 25). Immunofluorescence using CREST6 autoimmune antiserum and specific antibodies to CENP-A and CENP-E confirmed neocentromere activities on both mardel(10) and NC-MiC1. No structural modifications to the mardel(10) and NC-MiC1 (with the exception of two clones) transchromosomes were detected in our study in the various ES cell lines produced. These results suggest the mitotic and structural stability of the human neocentric transchromosomes not only in human cells (20) but also in mouse cells. This observation, in conjunction with the previously reported stability of the mardel(10) chromosome in CHO cells and mouse F9 teratocarcinoma cells (20), provides further evidence in support of the dispensability of the normal centromeric repetitive DNA in the propagation of centromeric activity across mammalian species.

We have analyzed the behavior of the 60-Mb neocentromere-based mardel(10) and its 3.5-Mb derivative, NC-MiC1, in mice. Chimeric mice carrying either of these chromosomes were successfully generated. The analysis of tissue samples from adult mice by PCR using mardel(10) and NC-MiC1-specific primers has demonstrated their presence in many different mouse tissues including lung, kidney, heart, brain, skin, adrenal, uterus, testis, liver, bone marrow, spleen, pancreas, tail, and others. Furthermore, these transchromosomes appear to be structurally intact and remain as episomal entities as indicated by the FISH results. The presence of an extra human neocentromere-based transchromosome does not appear to significantly affect the differentiation of the mouse ES cells into many different functional cell types. Specifically, we have detected the presence of mardel(10) and NC-MiC1 in the hemopoietic stem cells isolated from the bone marrow. These results illustrate that both mardel(10) and NC-MiC1 are structurally functional and that their presence does not lead to any observable detrimental effect on the development of the mice.

In addition to mitotic competence, we have examined the meiotic function of mardel(10) and NC-MiC1 in the mice. The detection of the transchromosomal DNA by PCR in the sperm samples of 14 and 67% mardel(10) and NC-MiC1-positive chimeric mice, respectively, is suggestive of a germ line transmission of the DNA from these neocentric markers and a certain degree of meiotic competence associated with these neocentric markers. The difference in the percentage of positive sperm samples between these two groups of chimeric mice may be a result of a higher possibility of incorrect pairing during meiosis associated with mardel(10), because it is much bigger in size as compared with NC-MiC1. The proof of germ line transmission of NC-MiC1 comes from the direct demonstration of intact episomal NC-MiC1 in F1 embryos. However, it is also evident from the rapid decline in the proportion of NC-MiC1-positive cells and the sharp increase in the number of cells carrying more than one copy of NC-MiC1 from the 2.5–9.5-day-old embryos that NC-MiC1 is relatively unstable in the mouse embryo background. This rapid loss of NC-MiC1 due to postzygotic mitotic missegregation provides an explanation for our failure to observe NC-MiC1-positive F1 mice despite a continuous effort to breed the relatively large number of sperm-positive chimeric animals (>40). No evidence for the presence of mardel(10) in either F1 embryos or mice has been obtained, but the small number of sperm-positive chimeric mice (three) has precluded a detailed analysis and firm conclusion.

We have previously demonstrated the expression of many genes within the modified centromeric chromatin of the 10q25 neocentromere present on mardel(10) in a CHO cell-derived somatic cell hybrid line (20). In this study, we have extended these observations by demonstrating the expression of numerous genes from both mardel(10) and NC-MiC1 in a variety of mouse tissues. In addition, tissue-specific expression for several genes was observed, indicating that the introduced trans-
The relatively higher rate of mitotic missegregation of NC-MiC1 in mouse ES cells and chimeric mouse tissues. Previous studies of repetitive centromeric DNA-based HECs in mouse cell lines (15, 25, 34, 35) have suggested that the instability may be due to differences in species. There may be a requirement of some yet undefined trans-acting factors that are essential for chromosomal segregation in a mouse background. The slightly increased instability of NC-MiC1 compared with that of mardel(10) in the ES cells may be attributed to a significant difference in chromosome size, a factor that has recently been shown by Rudd et al. (36). An alternative explanation may be the requirement of distinct telomere structures for chromosomes in both human and mouse given that telomeres play a significant role in the maintenance of chromosome stability. For example, a previous study (37) has demonstrated the significant difference in telomere length between a human and mouse cell line. The DNA amplification observed in two of the ES GFPNC-MiC1 clones may be the results of the compromised telomere structures leading to subsequent fusion of the chromosomal ends. The mitotic instability of NC-MiC1 appears to be more acute in the early mouse embryos compared with that seen in cultured mouse ES cells and chimeric mouse tissues. The relatively higher rate of mitotic missegregation of NC-MiC1 in the F1 mouse embryos suggests that the minichromosome may be unfavorably predisposed to the normal mitotic regulatory mechanism of early mouse embryogenesis.

Notwithstanding the measurable variable rate of mitotic error, this study provides the evidence that human neocentromere and its associated marker chromosome and derivative minichromosome are mitotically (and meiotically) functional in mouse ES cells and in mice. We have shown that the transchromosomes can be transmitted in many different tissue types including hemopoietic stem cells, suggesting a potential utility of the neocentromere-based minichromosomes for somatic cell therapy through the stem cell route. We have also shown that genes can be positively and differentially expressed from our minichromosomes in a large spectrum of mouse tissues. These results provide promising support for the potential use of these minichromosomes for therapeutic gene replacement strategies. Future studies should aim to determine and address the factors that are responsible for the observed definable rate of instability of the neocentromere-based human marker chromosome and minichromosome in a mouse model system. This will allow the further development of these entities into a useful tool for the study of centromere, neocentromere, and chromosome biology and for gene therapy applications.

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Analysis of Mitotic and Expression Properties of Human Neocentromere-based Transchromosomes in Mice
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