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Construction of 700 human/mouse A9 monochromosomal hybrids and analysis of imprinted genes on human chromosome 6

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Abstract As an in vitro assay system for the identification of human imprinted genes, a library of human/mouse A9 monochromosomal hybrids containing a single, intact bsr-tagged human chromosome of known parental origin, derived from normal human fibroblasts, has been previously generated by microcell-mediated chromosome transfer (MMCT). To supplement this assay system, we constructed additional 700 A9 monochromosomal hybrids, using a pSTneo or pPGKneo selection marker. To validate the A9 hybrids, we screened them with chromosome-specific polymorphic markers, and identified the hybrids containing either human chromosome 6, 7, 14, 18, or 21 of known parental origin. Matching paternal and maternal chromosome pairs of A9 hybrids were identified for chromosomes 6, 7, 14, and 18. The paternal-specific expression of ZAC (zinc finger protein, which regulates apoptosis and cell cycle arrest) and HYMAI (hydatidiform mole-associated and imprinted transcript), and the maternal-specific methylation of a CpG island within an imprinted domain on human chromosome 6q24, were maintained in A9 hybrids. For an example, we profiled the expression of expressed sequence tags (ESTs) and the methylation of CpG islands in the 300-kb imprinted domain around 6q24, which may be associated with cancers and transient neonatal diabetes mellitus (TNDM). Thus, the 700 A9 hybrids should be useful for various aspects of imprinting studies.

Key words Genomic imprinting · Human/mouse A9 monochromosomal hybrid · In vitro assay system · Human chromosome 6 · EST · CpG island

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Introduction

Genomic imprinting is a specific example of an epigenetic phenomenon in which parental alleles are genetically marked, causing differential expression of the two alleles in somatic cells (Surani et al. 1986; McGrath and Solter 1984). Aberrant imprinting is responsible for a number of diseases, including embryonic abnormalities, congenital diseases, and various tumors (Solter 1988; Hall 1990). Functional analyses have identified several features associated with imprinted genes and regions, including allele-specific methylation, chromatin structural modifications, asynchronous DNA replication timing, and uniparental disomy (UPD) in genetic diseases (Neumann and Barlow 1996; Kitsberg et al. 1993; Hurst and McVean 1997; Feil and Kelsey 1997). Approximately 30 human genes that are regulated by imprinting have been reported thus far, and it has become clear that genomic imprinting is involved in a number of human genetic disorders, as well as cancers (Feinberg 1999).

To investigate further the processes of diseases that are associated with imprinting and the regulatory mechanism, it is essential to identify additional imprinted genes. However, the identification of sequence polymorphisms is absolutely indispensable for analyses of genomic imprinting. In order to circumvent this significant barrier, a library of human/mouse A9 monochromosomal hybrids containing a human chromosome of known parental origin was generated previously by MMCT, as an in vitro assay system for the identification of human imprinted genes (Kugoh et al. 1999). This system is based on the separation of parental alleles by construction of A9 hybrids with a single human chromosome of known parental origin. A comparison between hybrids containing either a paternal or maternal chromosome enables the examination of parental-specific expression and differential methylation of CpG islands of human imprinted genes, without the requirement for identifying sequence polymorphisms. Therefore, this assay system using human/mouse A9 monochromosomal hybrids provides a valuable resource for the isolation of novel imprinted genes and the

analysis of the biological significance and mechanisms of genomic imprinting.

Imprinted genes are known to cluster on some human chromosomes, for example in the Beckwith-Wiedemann region on chromosome 11 and the Prader-Willi/Angelman syndrome region on chromosome 15 (Reik and Maher 1997). Recently, *ZAC* and *HYMAI*, which were localized on human chromosome 6q24, have been described as paternally expressed, imprinted genes, and have defined the positions of a new imprinted cluster (Kamiya et al. 2000; Arima et al. 2000). Although matching paternal and maternal pairs of A9 hybrids have been identified for chromosomes 5, 10, 11, and 15 thus far, the generation of additional matched hybrids for other chromosomes will facilitate the isolation of novel imprinted genes.

Therefore, we constructed 700 new A9 monochromosomal hybrids containing a human chromosome, of which parental origin can be known. For an example, we profiled the expression of expressed sequence tags (ESTs) and methylation of CpG islands, which were localized on the 300-kb imprinted domain around 6q24, using matched A9 hybrids with a paternal or maternal chromosome 6, which further demonstrated the usefulness of this assay system.

Materials and methods

Cell lines

Human fibroblasts were obtained from a normal adult, and cultured. A mouse A9 cell line, a hypoxanthine guanine phosphoribosyl transferase (HGPRT)-deficient derivative of mouse L cells, was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Human fibroblasts and mouse A9 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA).

Construction of human/mouse A9 monochromosomal hybrids

Human fibroblasts were transfected with pSTneo (Katoh et al. 1987) or pPGKneo (McBurney et al. 1991) plasmid DNA (neo) using the calcium phosphate precipitate method. Human fibroblasts (5×10^5 cells seeded per 100-mm dish) were incubated for 24h. The cells were transfected with 20 µg neo plasmid per dish, and incubated in nonselective medium at 35°C for 24h with 3% CO₂. After two washes with phosphate-buffered saline (PBS), the cells were cultured in nonselective medium at 37°C for 24h with 5% CO₂. Next, the cells were plated into five 100-mm dishes, and incubated in nonselective medium at 37°C. After 1 day, the medium was changed to selective medium containing 200 µg/ml of G418 (Sigma). Whole cell fusion between neo-resistant fibroblasts and mouse A9 cells was performed as described previously (Koi and Barrett 1986). Neo-resistant

clones of 50–100 were pooled, and 7×10^5 cells were seeded together with 5×10^5 mouse A9 cells per 60-mm dish, and incubated for 24h. The fusion was mediated by treatment with 3ml of 41.7% (w/v) polyethylene glycol (PEG, mw 1000; Baker Chemical, Phillipsburg, NJ, USA) containing 15% dimethyl sulfoxide (DMSO) for 1min. The cells were then incubated with 25% (PEG) solution for 1min, and were then washed extensively with serum-free DMEM. After 1 day of incubation in nonselective medium, the cells were plated into three 100-mm dishes, and incubated in nonselective medium. After 1 day, the medium was changed to selective medium containing 800 µg/ml of G418 and 10^{-5} M ouabain. After 3 to 4 weeks, double-resistant colonies were isolated as donor cells for microcell fusion.

The method for microcell-mediated chromosome transfer (MMCT) was performed as described previously (Koi et al. 1989). Whole cell hybrids were pooled into groups of ten clones, and were cultured in 25-cm² flasks (no 3025; Costar, Cambridge, MA, USA) for 2 days. The cells were treated with 0.05 µg/ml of colcemid (Sigma) for 48h to induce the formation of microcells. After flasks were filled with serum-free DMEM containing 10 µg/ml of cytochalasin B (Sigma), enucleation was performed by centrifugation of the flasks at 10,000g for 1h. The microcells were resuspended in 12 ml of serum-free DMEM, and filtered sequentially through 8-, 5-, and 3-µm polycarbonate filters (Nucleopore, Corning, NY, USA). The purified microcells were collected by centrifugation at 400g for 10min, and resuspended in 2ml of serum-free DMEM containing 100 µg/ml of phytohemagglutinin (PHA; Difco, Franklin Lakes, NJ, USA). The microcells were attached to prewashed A9 cell monolayers by incubation for 15min at 37°C. The cells were fused by treatment with 3ml of 47% PEG solution for 1min, followed by extensive washing with serum-free DMEM. After 1 day of incubation in nonselective medium, the cells were plated into three 100-mm dishes containing DMEM supplemented with 10% FBS and 800 µg/ml of neomycin. After 2 weeks of incubation in selective medium, microcell hybrids were selected individually. These clones were usually maintained with the selective medium.

Chromosome analysis

Conventional chromosome preparations were made as described previously (Kugoh et al. 1990). Chromosomes were stained with Quinacrine plus Hoechst 33258 (Sigma), and the karyotypes of at least ten well banded metaphases were analyzed.

Two-color fluorescence in situ hybridization (FISH)

In some of the hybrid clones, the position of the neo marker on the introduced human chromosome was determined by two-color FISH analysis, as described previously (Uejima et al. 1998). Chromosome preparations were hybridized with neo plasmid DNA labeled by nick-translation with biotin-16-deoxyuridine triphosphate (dUTP) (Boehringer, Indianapolis, IN, USA) and human Cot-1 DNA (GIBCO-BRL,

Fig. 1A–C. Chromosomal analysis and identification of parental origin. Chromosome preparations of A9 microcell hybrids containing a human chromosome 6 (43-7) were analyzed by Quinacrine plus Hoechst 33258-staining (**A**) and two-color fluorescence in situ hybridization (FISH) analysis using a digoxigenin-labelled COT-1 probe (red) and a biotin-labelled pSTneo probe (yellow) (**B**). The introduced human chromosome 6 is indicated with an arrow. **C** Identification of the parental origin of an introduced human chromosome 6 in A9 microcell hybrids. Genomic DNAs from peripheral lymphocytes of the donor's parent (Fa, Father; Mo, mother), donor fibroblasts (Fi), four A9 microcell hybrids (P, M), and mouse A9 cells were amplified using a tetranucleotide repeat polymorphic marker (D6S1279). nt, Nucleotide

Grand Island, NY, USA) labeled with digoxigenin (Boehringer). The hybridized probes were detected with fluorescein isothiocyanate (FITC)-avidin (Boehringer) and anti-digoxigenin-rhodamine (Boehringer), respectively. Chromosomes were counterstained with diamidino phenylindole (DAPI) (Sigma).

PCR-based polymorphic analysis

The parental origin of the introduced human chromosome in A9 microcell hybrids was identified, using polymorphic markers. Genomic DNAs were purified by standard procedures. Primer sequences for microsatellite markers were obtained from the Genome Data Base (Table 1) and primers were 5' end-labeled using T4 polynucleotide kinase (Toyobo, Tokyo, Japan) and [γ - 32 P] dATP (Amersham, Piscataway, NJ, USA). Polymerase chain reaction (PCR) amplifications were performed through 30–35 cycles of 94°C for 30s, 56–60°C (depending on the primer combination) for 30s, and 72°C for 30s. The products were resolved by electrophoresis on 7M urea-8% polyacrylamide gels and autoradiography.

Mapping and screening of ESTs on chromosome 6q24

Sequence of the 6q24 region, generated by the Sanger Centre, was obtained from the NCBI server (<http://www.ncbi.nlm.nih.gov/>), as a genomic contig (accession no. NT_002260). Repetitive elements were filtered using Repeat Masker programs (<http://ftp.genome.washington.edu/cgi-bin/RepeatMasker>), and ESTs were identified using the BLAST programs on the NCBI server. After the correction of sequence ambiguities that were present in the human ESTs, PCR primers were synthesized and used to amplify cDNA fragments. Total RNA was prepared using a guanidine isothiocyanate-based method, and first-strand cDNA synthesis was carried out with an oligo-(dT)₁₅ primer and M-MLV reverse transcriptase (Life Technologies, Grand Island, NY, USA). The reaction mixture for PCR analysis included 0.5 μ M of each primer, 200 μ M of each dNTP, 1 $\times$ PCR buffer, and 0.5 U of Taq polymerase (Perkin Elmer, Foster City, CA, USA) in a reaction volume of 10 μ l. PCR amplifications were performed through 28–35 cycles of 94°C for 30s, 56–60°C for 30s, and 72°C for 30s, depending on the region analyzed. PCR products were resolved by electrophoresis on 3% agarose gels, and stained with ethidium-bromide or SYBR Green I (Molecular Probes, Eugene, OR, USA).

DNA methylation analysis

The method of methylation sensitive-PCR (MS-PCR) was used to analyze the methylation status of four CpG islands, which were identified on the NT_002260 genomic contig by GRAIL programs (<http://compbio.ornl.gov/Grail-1.3/>). Genomic DNAs (2 μ g) were digested with 10 U of the methylation-sensitive endonuclease *HpaII*, at 37°C in a reaction volume of 40 μ l. The digested DNAs were amplified

Table 1. List of new A9 microcell hybrid clones containing a single human chromosome and microsatellite markers

Microcell hybrid clones	Human chromosome number in microcell hybrids	Parental origin of chromosome	Marker	Primer sequences\ (5'-3')
4-7	6	Maternal	D6S1279	TAAGGAGCTACAGTGGCAGG
48-9	6	Maternal		TACGTTCTGACAAATTGCTCC
43-7	6	Paternal		
31-11	6	Paternal		
18-5	7	Maternal	D7S500	CCAGAATTGAAAACCTACAGCA
70-4	7	Maternal		ATTGATTGAGGAACTGAACTTACCT
20-5	7	Paternal		
38-7	7	Paternal		
53-6	14	Maternal	D14S587	AAGAAGTTTATTTTAAAAGCAAGG
53-7	14	Maternal		CGGTAACAAGTGCAGATTCC
38-1	14	Paternal		
55-4	14	Paternal		
67-9	14	Paternal		
28-7	18	Maternal	D18S51	CTGTCCTCTAGGCTCATTTAGC
48-4	18	Paternal		TTATGAAGCAGTGATGCCAA
42-6	21	Maternal	L18360	GAGACGGTAGGAAAAGGAG
				AGCCAAGTTCGAGCCACTG

using primer combinations for each CpG island. In Fig. 2, the four CpG islands are indicated for convenience as CpGA, CpGB, CpGC, and CpGD. Primer sequences were: CpGA (forward, 5'-CGGAGAGGGAACCTGAAACT-3' and reverse, 5'-CGGCAGTCACCAGCTTTTA-3'); CpGB (forward, 5'-TCCATAAGAGACGAAAGTGC AA-3' and reverse, 5'-CTACCCTTTCTGCGTCTTCG-3'); CpGC (forward, 5'-AGTTTATTTTCGGGGCTGCT-3' and reverse, 5'-CACCAGCACAGTTCTCAGGA-3'); CpGD (forward, 5'-CGCCCCTGGATAGGAATTAT-3' and reverse, 5'-AATGCCACGACCCTTGAATA-3'). The methylation status of human IGF2R CpG island region 2 (CpG2), which has been reported to be maternally methylated (Smrzka et al. 1995; Riesewijk et al. 1996), was also analyzed. Primer sequences were: IGF2R CpG2 (forward, 5'-CACATTATGAGGGACCCGTA-3' and reverse, 5'-GTGCCCTATGACCTAGTTAC-3'). The reaction mixture included 1M GC Melt (Clontech, Palo Alto, CA, USA), 50ng of the digested DNA, 0.5μM each primer, 40mM Tris-HCl (pH 9.5, at 25°C), 85mM potassium acetate (KOAc), 5% dimethyl sulfoxide (DMSO), 0.1% Triton X-100, 200μM of each dNTP, 1.1mM Mg (OAc)₂ and 0.2μl of Advantage-GC Genomic Polymerase Mix (×50; Clontech), in a reaction volume of 10μl. PCR amplifications were performed through 35 cycles of 94°C for 30s, 58–62°C (depending on the primer combination) for 30s, and 72°C for 3min. PCR products were analyzed on 2% agarose gels, and stained with ethidium-bromide to test for the presence or absence of a post-digestion PCR product.

Southern blot analysis was performed to verify the methylation status of CpGB. Genomic DNAs (5μg) were digested with 25U of the indicated enzymes, *EcoRI* alone or *EcoRI* plus *NotI*, at 37°C. The digested DNAs were resolved by electrophoresis on 1% agarose gels, and then transferred to Hybond-N⁺ membranes (Amersham). The probe was labeled by random priming, using [α -³²P] deoxycytidine triphosphate (dCTP) (Amersham), hybridized to the membranes at 65°C for 18h, and washed with 2 × standard saline citrate (SSC) at room temperature and

twice with 0.1 × SSC plus 1% sodium dodecylsulfate (SDS) at 65°C for 15min. Autoradiography was carried out for 3 days at –80°C, using BioMax MS film (Kodak, Rochester, NY, USA). Probe DNA was generated by PCR from the region lying from nucleotide positions 49,723–51,235 on the genome sequence (accession no. AL109755). The primer sequences were: forward, 5'-CATTGCCTCAAAAGTCT GTGA-3' and reverse, 5'-CCGGAGAATGCTGTGTGT GATA-3'.

Results

Construction of A9 microcell hybrids containing an introduced human chromosome, and identification of the parental origin

Human fibroblasts were transfected with either the pST*neo* or pPGK*neo* plasmid, and 7000 independent stable transfectants were isolated. The direct transfer of human chromosomes from fibroblasts to mouse A9 cells was not successful, due to difficulties in generating sufficient donor microcells. Therefore, neo-resistant fibroblasts were pooled into groups of 50–100 clones and were fused with mouse A9 cells by treatment with PEG. After double selection with neomycin and ouabain, 1000 whole cell hybrids were isolated. However, because several human chromosomes could be present in individual fibroblast/A9 whole cell hybrids (data not shown), microcell-mediated chromosome transfer (MMCT) was performed to construct mouse A9 cells containing a single human chromosome. Whole cell hybrids were pooled into groups of 10–20 clones, and microcells were isolated and were fused with mouse A9 cells by treatment with PEG. After 2 weeks, 700 A9 microcell hybrids were isolated and stored in liquid nitrogen.

Thirty microcell hybrids containing either human chromosome 6, 7, 14, 18, or 21 were identified by PCR screening,

Fig. 2A–C. Expression and methylation analyses using A9 cells containing a single human chromosome 6. **A** Physical map of the 6q24 region. The extent of overlapping genomic contig is shown by the horizontal bars, with accession numbers listed below. Expressed sequence tags (ESTs) mapped using the BLAST program on the NCBI server are represented by dots (closed dots, paternally expressed ESTs; open dots, biallelically expressed ESTs). Four CpG islands identified by the GRAIL program are represented by the gray box for the maternally methylated CpG island and white boxes for the unmethylated CpG islands. The lower panel shows a detailed map of the ESTs in the imprinted domain. Exons of the three genes (*KIAA0680*, *ZAC*, *HYMAI*) are represented by black boxes. Arrows indicate the deduced transcriptional orientation. **B** Reverse transcription-polymerase chain reaction (RT-PCR) analysis using human ESTs on the 6q24 region.

cDNA from human chromosome donor fibroblasts (*Fi*), A9 microcell hybrids containing a paternal (*P*) or maternal (*M*) allele of chromosome 6, and mouse A9 cells were amplified using primers for 26 ESTs localized on the 6q24 region, and for AI910308, an EST of the *IGF2R* gene. **C** Methylation analysis by methylation-sensitive PCR. Genomic DNAs from human chromosome donor fibroblasts (*Fi*), A9 microcell hybrids containing a paternal (*P*) or maternal (*M*) allele of chromosome 6, and mouse A9 cells were digested with the methylation-sensitive endonuclease, *HpaII* (*H*). The digested DNAs were amplified using primers for CpGA, CpGB, CpGC, CpGD, and *IGF2R* CpG2. The non-digested DNA (*N*) from donor fibroblasts is shown as the control template. Amplification of nondigested DNAs from A9 microcell hybrids containing a human chromosome 6 was confirmed for each set of primers (data not shown).

using chromosome-specific markers (data not shown), and the state of the introduced chromosome was examined by double-staining with Quinacrine plus Hoechst 33258 (Fig. 1A). A9 microcell hybrids containing either a single intact human chromosome 6 (four clones), 7 (four clones), 14 (five clones), 18 (two clones), or 21 (one clone) were identified, as summarized in Table 1. Furthermore, A9 microcell hybrids containing either a single fragmented human chromosome 1, 11, or 19 were identified (data not shown). One hundred and seventy nine clones of the 700 A9 microcell hybrids did not contain either intact or fragmented human chromosome 1, 6, 7, 11, 14, 18, 19, or 21, suggesting that other human chromosomes could be present in the remaining clones. The introduced human chromosome was maintained stably in each clone, under selective conditions.

Two-color FISH analysis was performed to determine the position of the selection cassette on the human chromosome. For clone 43-7, which contained human chromosome 6, the neo marker was mapped on 6q22–24 (Fig. 1B). The integration sites in clones 48-9 and 31-11, which contain a chromosome 6, were mapped to 6q25 and q22, respectively, and clone 42-6, which contained chromosome 21 was mapped to 21q21 (data not shown).

The parental origin of the introduced human chromosome in A9 microcell hybrids was identified by PCR analysis using microsatellite markers (Table 1). For chromosomes 6, 7, 14, and 18, matching paternal and maternal pairs of A9 microcell hybrids were identified. Of the four independent A9 microcell hybrids containing a human chromosome 6, two were of maternal origin (4-7, 48-9) and two were of paternal origin (43-7, 31-11) (Fig. 1C). For hybrids containing a human chromosome 7, two were maternal (18-5, 70-4) and two paternal (20-5, 38-7); for chromosome 14, two were maternal (53-6, 53-7) and three paternal (38-1, 55-4, 67-9); and for chromosome 18, one was of paternal (48-4) and the other of maternal origin (28-7). The one clone containing a chromosome 21 (42-6) was of maternal origin.

EST screening on the 6q24 region

To identify novel imprinted genes on the 6q24 region as an example, we performed a search of differentially expressed transcripts, using A9 microcell hybrids containing a paternal or maternal chromosome 6. The structural characteristics of this region are presented in Fig. 2A. A database search of the 6q24 region revealed a genomic contig (NT_002260), which contained *ZAC* and *HYMAI*, part of the gene for the KIAA0680 protein, and a pseudogene similar to pantophysin, and represented the combined data of genome sequences (accession no. AL049844, AL109755, and AL031390) as reported previously (Fig. 2A) (Kamiya et al. 2000; Arima et al. 2000; Gardner et al. 2000). Twenty-six ESTs were mapped to NT_002260, and the transcripts represented by these were examined by reverse transcription (RT)-PCR for the presence of differential expression between hybrids with a paternal or maternal allele (Fig. 2A). R02646, which corresponded to *ZAC*, and AI635382, which corresponded to *HYMAI*, were expressed only from the

paternal allele, as reported previously (Fig. 2B) (Arima et al. 2000). AA161034, representing KIAA0680, seven ESTs (AA336115, R58726, AA947691, AW082730, AI669226, AI808626, AA757186) on AL049844, and four ESTs (AI253287, AF150406, AI052521, AI359320) on AL031390 indicated transcripts that were expressed equally from both alleles (Fig. 2A,B). Three ESTs (AI085760, R09553, AI953762) on intronic regions of the *ZAC* gene, and seven ESTs (AA410539, AI159943, AI453291, AI436373, AA339545, AA333879, AA525794) which were mapped within 13 kb upstream of the *ZAC* gene indicated paternally expressed transcripts (Fig. 2A). In addition, one of the paternally expressed ESTs (AA410539) was connected with an exon of *ZAC* transcripts by RT-PCR and sequencing analysis, using cDNA from a human pituitary gland (data not shown). Z36999, which was mapped to 15 kb downstream of the *HYMAI* gene, and AA346811, which was mapped to an exon of the *HYMAI* gene, were also expressed only from the paternal allele (Fig. 2A). AI910308, which represented *IGF2R* on human chromosome 6q27, which has been previously reported to be expressed from both alleles in humans (Kalscheuer et al. 1993; Ogawa et al. 1993), was biallelically expressed in mouse A9 cells, as expected (Fig. 2B).

DNA methylation analysis

Differential methylation between the parental alleles is one of the common landmarks at imprinted loci. We identified four CpG islands on NT_002260 (Fig. 2A), and then examined the methylation status for these CpG islands in mouse A9 cells by methylation-sensitive (MS)-PCR. *HapII* and *MspI* restriction enzymes both cleave at CCGG sites. However, *HapII* does not cleave when the internal C is methylated. DNAs were digested with these two enzymes independently, and amplified using primers for each CpG island, to test for the presence or absence of a PCR product, which is visible after *HapII* digestion only if the internal C is methylated. For the CpG island lying at nucleotides 52,444–53,374 on AL109755 (CpGB), which has been reported to be maternally methylated (Kamiya et al. 2000; Arima et al. 2000; Gardner et al. 2000), PCR products were detected only in hybrids with a maternal allele, as expected (Fig. 2C). However, for the other three CpG islands (CpGA, lying at nucleotides 25,044–25,626 on AL049844; CpGC, lying at nucleotides 81,956–83,074 on AL031390; and CpGD, lying at nucleotides 50,516–51,675 on AL031390), there was no difference in the methylation pattern between both hybrids (Fig. 2C). A CpG within the second intron of the human insulin-like growth factor type 2 receptor gene (*IGF2R*), CpG2, has been reported to be maternally methylated (Smrzka et al. 1995; Riesewijk et al. 1996). PCR products for this CpG island were detected only in hybrids with a maternal allele, as expected (Fig. 2C). *MspI* digestions were not amplified using each primer pair for the CpG islands, and confirmed the presence of the CCGG site (data not shown).

Subsequently, the maternal-specific methylation of CpGB was confirmed by Southern analysis. DNAs were

digested either with *EcoRI* alone or with *EcoRI* in combination with the methylation-sensitive restriction endonuclease, *NotI*. The blotted membranes were hybridized with a PCR product as a probe, which was generated by amplification using primers downstream of the CpG island (Fig. 3A). Human fibroblasts contained both alleles and exhibited bands of 6.7kb and 3.4kb after digestion with *EcoRI* plus *NotI*. In hybrids with a maternal allele, the 6.7-kb fragment was detected as the nondigested, or methylated allele, as in digestions with *EcoRI* alone for human fibroblasts (Fig. 3B). In contrast, in hybrids with a paternal allele, the 3.4kb fragment was detected as the digested, or methylated allele (Fig. 3B).

Fig. 3A,B. Methylation analysis of the differentially methylated CpG island by Southern blot. **A** Restriction map of the CpG island region. Numbers correspond to the genome sequence (accession no. AL109755) containing the differentially methylated CpG island (black box). The probe generated by PCR amplification is indicated by the white box. E, *EcoRI* site; N, *NotI* site. **B** Methylation status of the CpG island. Genomic DNAs from donor fibroblasts (Fi), A9 microcell hybrids with a paternal (P) or maternal (M) allele of chromosome 6, and mouse A9 cells were digested with *NotI* plus *EcoRI* (N/E). A 6.7-kb fragment was detected in digestions of hybrids with an allele exhibiting methylation at the *NotI* sites, as in digestions with *EcoRI* alone (E) from donor fibroblasts. A 3.4-kb fragment was detected in digestions of hybrids with an allele that was not methylated at the *NotI* site

Discussion

In this study, we constructed 700 A9 monochromosomal hybrids containing a human chromosome by MMCT, and identified matched hybrids containing either a paternal or maternal chromosome 6, 7, 14, and 18. A library of human/mouse A9 monochromosomal hybrids with either intact or fragmented human chromosomes has been generated previously by MMCT (Koi et al. 1989). It is possible to transfer a human chromosome tagged with a dominant selectable marker from A9 hybrids into other mammalian cells via MMCT, for genetic analyses of cellular phenotypes (Kugoh et al. 1990; Goyette et al. 1992). Therefore, this library has been used as a resource for the mapping and identification of genes that function during cellular aging, telomerase regulation, metastasis, tumor suppression, and DNA repair, or for the generation of transgenic mice with introduced human chromosomes and the establishment of animal models of human disease (Uejima et al. 1998; Ichikawa et al. 1994; Kurimasa et al. 1994; Tanaka et al. 1998; Tomizuka et al. 1997). In addition to this, a library of A9 monochromosomal hybrids containing human chromosomes of known parental origin has been generated, to develop an assay system for the identification of preferential allelic expression in vitro (Kugoh et al. 1999). This assay system has demonstrated that the expression pattern and methylation status of human imprinted genes, such as *KVLQT1* and *H19* on chromosome 11p15.5 (Zhang and Tycko 1992; Rainier et al. 1993), or *SNRPN* and *IPW* on chromosome 15q11-q13 (Glenn et al. 1993; Nakao et al. 1994; Reed and Leff 1994; Wevrick et al. 1994) have been maintained appropriately in mouse A9 cells (Kugoh et al. 1999; Meguro et al. 1997; Mitsuya et al. 1998). In addition, LIT1, which is a novel imprinted antisense RNA within the *KVLQT1* locus, has been identified by the screening of differential gene expression, as defined by EST sequences, on the 11p15 region, using A9 hybrids with a paternal or maternal chromosome 11 (Mitsuya et al. 1999). Therefore, the assay system using A9 monochromosomal hybrids has provided a valuable resource for the efficient identification of imprinted loci in humans, and the additional A9 monochromosomal hybrids, which we report here, will enhance this approach for the investigation of genomic imprinting and its underlying epigenetic mechanisms.

We demonstrated that the maternal-specific methylation of CpG2 and the biallelic expression of the human *IGF2R* gene on human chromosome 6 was maintained in mouse A9 cells. The imprinting status of the human *IGF2R* gene has yet to be fully resolved. Biallelic expression, suggesting an absence of imprinting, has been demonstrated in studies of embryonic and adult tissues (Kalscheuer et al. 1993; Ogawa et al. 1993), but one study identified sporadic monoallelic expression and raised the possibility that this locus was subject to polymorphic imprinting (Xu et al. 1993). In the contrast, the mouse *Igf2r* gene is expressed exclusively from the maternally inherited allele (Barlow et al. 1991). The human *IGF2R* gene contains two CpG islands, as in the mouse. CpG1, encompassing the transcription start site, is

completely unmethylated on both parental alleles, but is methylated paternally in the mouse, and another CpG island within the second intron, CpG2, is methylated only on the maternal allele, as in the mouse (Smrzka et al. 1995; Riesewijk et al. 1996). Therefore, our results suggested that human-specific epigenetic modifications could function effectively in mouse cells, which may exhibit different modifications of homologous regions.

ZAC, which was localized on human chromosome 6q24 and proposed to be a tumor suppressor gene with anti-proliferative properties through the regulation of cell cycle progression (G1 arrest) and apoptosis, has been described as a paternally expressed, imprinted gene (Abdollahi et al. 1997; Varrault et al. 1998; Bilanges et al. 1999; Kamiya et al. 2000). In addition, *HYMAI*, which was localized 70kb upstream of the *ZAC* gene, has also been identified as an imprinted gene that was expressed from the paternal allele, by screening transcripts identified by ESTs using A9 microcell hybrids with a human chromosome 6 of known parental origin (Arima et al. 2000). Both imprinted genes have been proposed as candidates for transient neonatal diabetes mellitus (TNDM), which shows an imprinting effect, because it appears to result from the upregulation of a paternally expressed gene in 6q24 according to paternal UPD 6 or paternal duplications of 6q24 (Temple et al. 1995; Temple et al. 1996; Gardner et al. 1998). Furthermore, Gardner et al. (2000) recently narrowed the TNDM critical region to 300–400kb of 6q24, which contained both the *ZAC* and *HYMAI* genes, and identified the loss of methylation of a maternally methylated CpG island within the TNDM critical region for patients without UPD 6 or duplications of 6q24. Therefore, this subset within the 6q24 region has defined the position of a novel imprinted domain on human chromosome 6q24. Major imprinting domains on human chromosomes 11 and 15 contain a number of imprinted genes that may be controlled by long-range regulatory elements. However, we did not identify novel allele-specific transcript expression or differential methylation of CpG islands within a surrounding 300-kb region of the imprinted domain of 6q24, and suggest that this domain might be formed with only two imprinted genes and a maternally methylated CpG island. Therefore, TNDM may be caused by the overexpression of *ZAC* and/or *HYMAI*, according to the loss of methylation of a maternally methylated CpG island, paternal UPD 6, or paternal duplication of 6q24. In addition, three variants of human *ZAC* transcripts have been reported: *ZAC* (accession no. AJ006354) (Varrault et al. 1998), *PLAGLI* (pleomorphic adenoma of the salivary gland gene like 1, accession no. NM_002656) (Kas et al. 1998), and *LOTI* (lost on transformation 1, accession no. U72621) (Abdollahi et al. 1997). These transcripts differ at their 5' ends, but are highly homologous in the zinc-finger domain encoded by downstream exons (Kamiya et al. 2000). Paternally expressed transcripts within the imprinted domain, which we identified by screening using A9 monochromosomal hybrids, on the basis of detailed EST mapping, suggested the presence of the novel alternate splicing variants of *ZAC/PLAGLI/LOTI* and/or *HYMAI*.

Finally, the human chromosomes in A9 monochromosomal hybrids that we report here were tagged with pST*neo* or pPGK*neo*, and are therefore suitable for conferring G418-resistance to mouse embryonic stem (ES) cells. Therefore, by direct transfer from A9 hybrids to mouse ES cells, trans-chromosomal mice carrying a human chromosome 21 were generated (unpublished data), and these may be useful for the functional analysis of Down's syndrome caused by trisomy of human chromosome 21. Recently, this monochromosomal hybrid system was combined with the targeting and precise modification of human chromosomal alleles in chicken DT40 cells, for the identification of regulatory elements that define epigenetic states of gene activity within chromosomal domains (Horike et al. 2000). These approaches are unique, and have the potential to contribute significantly to the understanding of the molecular basis of genomic imprinting. The 700 hybrids are available on request to anyone.

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