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Difference in Chromatin Packaging between Active and Inactive X Chromosomes by Fractionation and Allele-Specific Detection

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Using a novel method consisting of chromatin fractionation and allele-specific detection, chromatin packaging is compared between active X (Xa) and inactive X (Xi) chromosomes for five tumor cell clones that were derived from inter-subspecific F_1 female mice. Separation of heterochromatic (H) and euchromatic (E) fractions is monitored by hybridization with subtelomeric satellite DNA and ribosomal RNA gene and by PCR amplification of p53 gene/pseudogene with one primer set. The H fraction was enriched with satellite and p53 pseudogene probably existing in heterochromatic regions while the E fraction showed inverse, suggesting fair separation. Analysis with seven marker and three gene loci revealed concentration of alleles on Xi in the H fraction and those on Xa in the E fraction, though the concentration levels varied. This implies that the packaging level of Xi is higher than that of active or inactive euchromatin on Xa. Intriguingly, one cell line showed biallelic expression and chromatin relaxation of the *Pgk-1* locus, suggesting that the relaxation occur regionally on X chromosome.

Female mammals inactivate one of their two X chromosomes to achieve a level of X-linked gene expression equal to that of males (1, 2). The inactive X chromosome (Xi) is characterized by the cytologically obvious heterochromatic Barr body in interphase nuclei that is formed by condensation of all or part of Xi chromatin (3, 4). The chromatin and constitutive heterochromatin resemble in many respects including DNA replication late in S phase (5, 6), but differ in that its condensation is facultative, one of the two X chromosomes in female cells being randomly selected due to *Xist* expression *in cis* (7, 8). It has been assumed that the facultative and constitutive heterochromatins are much more condensed than active or inactive euchromatin in interphase nuclei. This packaging in higher-level chromatin is however little studied at the genomic or biochemical level, though many polymorphic markers have become available (9, 10). To address this issue, we have developed a novel method which is based on the chromatin fractionation by centrifugation and allele-specific detection using microsatellites. Analysis of tumor cell lines derived from F_1 hybrid mice allows quantitative measurement of the relative amounts of allelic DNA on X chromosome in heterochromatic and euchromatic fractions. This paper presents comparison of the chromatin condensation state between active and inactive X chromosomes in the tumor cells. Also, we show regional chromatin relaxation of Xi in a cell line and discuss the relation of *Xist* expression with the relaxation.

MATERIALS AND METHODS

Cells. Fibrosarcomas were induced in inter-subspecific F_1 mice obtained by mating C57BL/6(B6) females with MSM males and subjected to establishment of cell lines (11, 12). Isolation of DNA and RNA and synthesis of cDNA were carried out as described previously (11, 12).

PCR analysis of DNA and RNA. PCR was carried out for cellular DNA and cDNA in a 10-20 μl volume under conditions as described (13). One primer was end-labeled with ^{32}P and used for amplification. The reaction was processed through 30-35 cycles of amplification consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, with the last elongation step lengthened to 10 min. For single strand conformation polymorphism (SSCP) analysis, the products were heat-denatured and separated by 8% polyacrylamide gel electrophoresis. We certified no contamination of cellular DNA in RNA samples by control PCR without the reverse transcriptase treatment; the re-

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actions did not give any detectable PCR products. Primer sequences for *Xist* and Mit markers were synthesized according to sequences reported (10, 14). Primers for *Pgk-1*, *Fmr-1* and *Smcx* were as follows: 5′-GGTGGATGCTCTCAGCAATG and 5′-GGCCCTCTACAATGCACAATG; 5′-TCTGAGAAACATTGAAAGCAGG and 5′-TCAGAGTGAAGGTCCTTCACA; 5′-CTCCCAGAGGTCCAGTCTTC and 5′-CCTTCGGCATATTGCCAGTG, respectively.

Analysis of the chromatin status. Cells (10^7 cells) were washed thoroughly with phosphate-buffered saline (PBS) and the nuclei were isolated by mild detergent treatment. Subnuclear fractions were obtained according to Frenster *et al.* (15). In brief, the nuclear pellet was washed with 0.25 M sucrose and 3.3 mM $CaCl_2$. and resolved in 0.2 ml of buffer A (0.1875 M sucrose, 20 mM glucose, 24 mM TrisHCl (pH 7.1), 12.8 mM NaCl, 3.3 mM $CaCl_2$). The suspension was allowed to incubate at 37 °C for 30 min and centrifuged after addition of 0.8 ml of cold buffer A. The washed nuclear pellet was extracted three times with 1 ml of buffer B (10 mM TrisHCl, pH 7.1, 3.3 mM $CaCl_2$). The pellet obtained by centrifugation was resuspended in 1 ml of cation-free 0.25 M sucrose and subjected to sonication: a maximum power of for 10 min. The suspension was centrifuged at 1,400 rpm for 5 min after addition of 0.5 ml of cation-free 0.25 M sucrose. The supernatant was further centrifuged at 4,500 rpm for 10 min. The pellet was the heterochromatin (H) fraction. The supernatant fraction was centrifuged at 7,700 rpm for 30 min. The pellet was the intermediate (I) fraction. The resultant supernatant was again centrifuged at 44,000 rpm for 60 min and the pellet fraction was the euchromation (E) fraction. Those pellets were subjected to DNA extraction. The ratio of DNA amounts recovered in the H, I, and E fractions was 1:1:2.

Southern hybridization was carried out using mo-2/Mov-15 for the satellite probe (16, 17) and a 6.6 kb fragment containing most part of 28S RNA for mouse rDNA.

RESULT

RNA was isolated from five cell lines, designated as Y1 to Y5, that originated from F_1 female mice between C57BL/6(B6) and MSM, and analyzed for *Xist* and *Pgk-1* gene expressions with RT-PCR (Fig. 1). Allele-specificity of *Xist* was determined by using the *Hind*III recognition site polymorphism within the amplified region (11). Y1, Y2, Y3 and Y4 cells gave transcripts of *Xist* from B6 allele whereas Y5 cells showed expression from MSM allele. The polymorphism of *Pgk-1* was detected with SSCP analysis. Y1, Y2, Y3 and Y4 cells exhibited *Pgk-1* expression from the MSM allele opposite to the *Xist*-expressing allele. However, Y5 cells showed expression mainly from the B6 allele but weak expression also from the MSM allele (marked by arrow). This biallelic expression suggested chromatin relaxation in this particular cell line. These results indicated that the former four cell lines contained B6-derived inactive X chromosome(s) and Y5 had MSM-derived inactive X.

DNA amounts of B6-derived and MSM-derived X chromosomes in the cells were monitored by gel electrophoresis of the PCR products with *Pgk-1* primers. Y1 and Y2 provided patterns consisting of two polymorphic bands similar to that of F_1, indicative of having equal amounts of B6-derived and MSM-derived X chromosomes. On the other hand, Y3 and Y4 showed a signal on the MSM bands more than that on the B6 bands while Y5 exhibited an opposite pattern. Consistent results were provided by chromosome analysis. Fig. 2 shows an example of Y1 cells that had 68-82 chromosomes including two synchronously replicating MSM X chromosomes and two late replicating B6 X chromosomes.

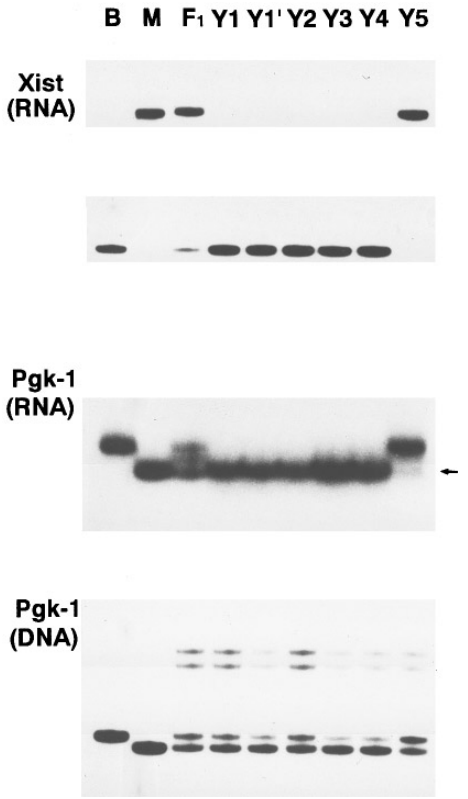

FIG. 1. Expression of *Xist* and *Pgk-1* genes. Sources of six RNA samples are indicated on the top of the panels; B, M and F_1 indicate liver RNA of B6, MSM and their F_1, respectively. Y1′ is another preparation from Y1. Arrow shows the presence of the MSM-derived band. The bottom panel: PCR products for cellular DNA.

In order to examine chromatin condensation state, we fractionated chromatin of sonicated cell nuclei. Heterochromatic (H) fraction was obtained by centrifugation of chromatin fragments into a pellet at a low speed, and euchromatic (E) fraction containing chromatin of open structure was recovered by further centrifugation of the supernatant at a high speed. Fig. 3A shows gel electrophoretic patterns of DNA in the two fractions. The H fraction contained more and longer DNA than the E fraction; DNA in the H fraction ranged between 20 kb to 2 kb whereas the E fraction exhibited a smear comprising DNA of 10 kb to 0.5 kb. The DNA were transferred to a filter and hybridized with the mouse satellite DNA and ribosomal DNA (Fig. 3B). The former is present in the subtelomeric heterochromatin region, and the latter probably exists in euchromatic regions near the short arm acrocentric chromosomes because the ribosomal RNA genes are constitutively transcribed in all cells. The satellite probe hybridized to

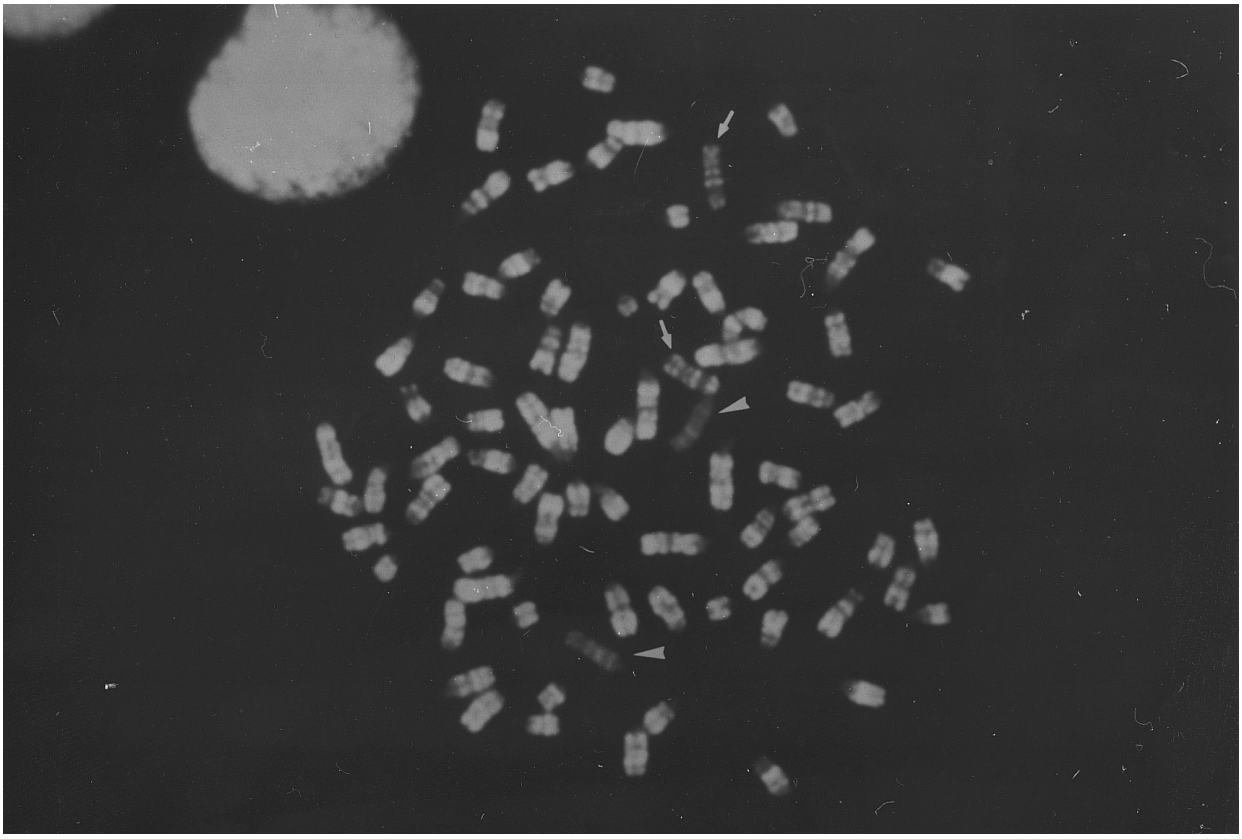

FIG. 2. An R-banded metaphase cell from Y1 clone. Y1 cells had 78 chromosomes, including two active X and two inactive X chromosomes. The arrowheads indicate inactive X chromosomes, and the arrows denote active chromosomes.

DNA in the H fraction more than DNA in the E fraction, whereas the ribosomal DNA probe showed similar intensities. The result indicated that the H/E fractionation separated heterochromatin and euchromatin though the separation was incomplete. This was confirmed by PCR analysis using a set of primer that amplified both of the p53 functional gene on chromosome 11 and the p53 processed pseudogene near the middle of chromosome 17 (18). The functional gene gave a 660 bp fragment containing introns 7 and 8 whereas the pseudogene provided the smaller 260 bp fragment without the introns (Fig. 3C). The MSM genome does not carry the pseudogene (18). The gene band signal was concentrated in the E fraction relative to the F_1 and H fractions although the pseudogene was favored over the p53 gene for amplification. This bias could be

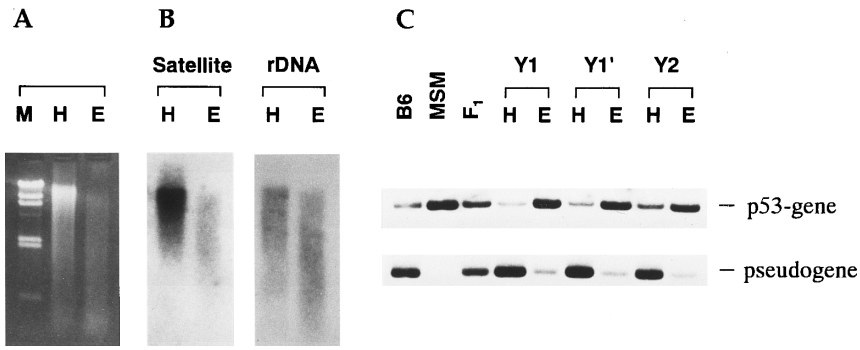

FIG. 3. Monitoring the H and E fractions with heterochromatic and euchromatic DNA probes. (A) Staining pattern of DNA in the H and E fractions. (B) Hybridization patterns of the satellite and ribosomal DNA. DNA in (A) were transferred onto a filter and hybridized to a satellite probe and then rehybridized to rDNA after washing. (C) PCR products given by the p53 primers were separated by polyacrylamide gel electrophoresis. Sources of DNA samples are indicated on the top of lanes.

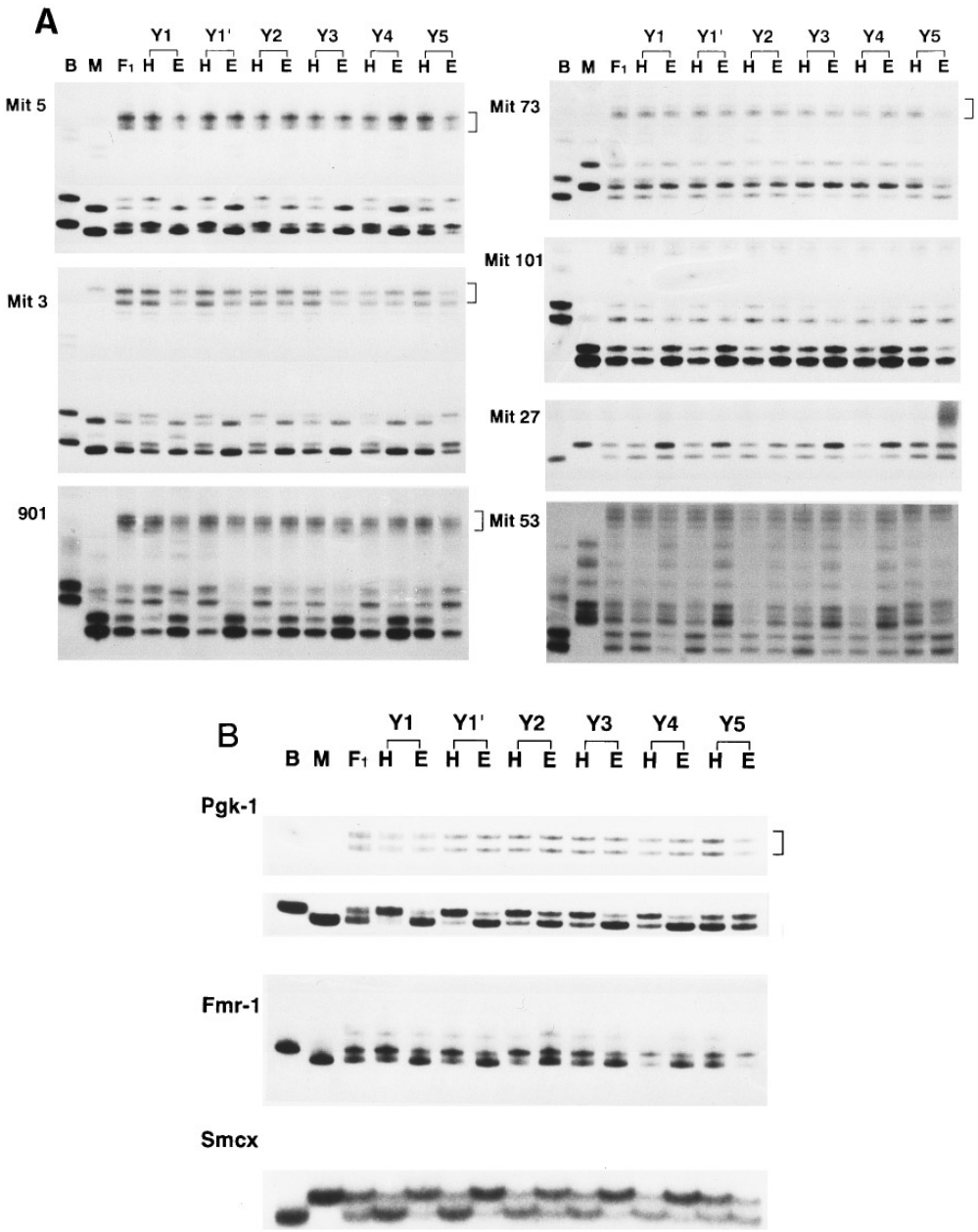

FIG. 4. Chromatin condensation state of seven microsatellite loci (A) and three gene loci (B) on X chromosome. The locus analyzed with the H/E assay is marked at the left side of each panel. Sources of DNA samples are indicated on the top. Small vertical bars at the right of panels indicate heteroduplexes formed between complementary B6 and MSM fragments. PCR products containing microsatellite often provide two or more bands from one allele, the reason being unclear.

overcome or minimized by comparison with the pattern of F_1 (see Fig. 5). The results indicated that the p53 pseudogene probably constituting heterochromatic configuration was yielded in the H fraction more than in the E fraction while the functional p53 gene was inverse.

H and E fractions obtained from the five cell lines were then analyzed with PCR using polymorphic markers on X chromosome (Fig. 4A). The *DXMit5* locus gave two bands representing one allele for both B6 and MSM strains, and four bands and heteroduplexes between their complementary fragments for F_1. In Y1 and Y2 cells, the H fraction showed the B6 bands more intense than the MSM bands (B6>M pattern), whereas the E fraction showed an inverse B6<M pattern. Such a reciprocal relation is consistent with the monoallelic expression of *Xist* from B6 allele, indicating that the B6 allele on Xi was more compact than the MSM allele on Xa. Y3 and Y4 cells provided patterns similar to those of Y1 and Y2, although B6-derived band-signals

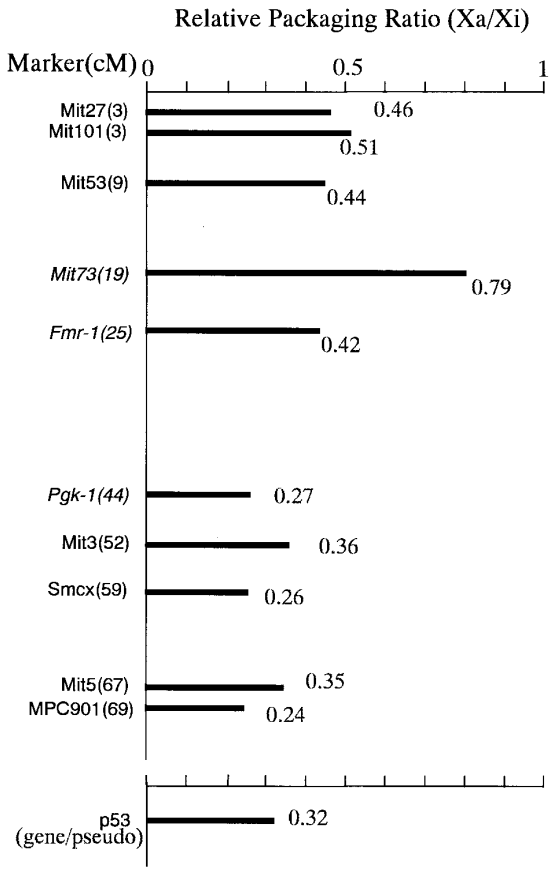

FIG. 5. Relative packaging ratios of alleles between Xa and Xi. Position of marker and gene loci on X chromosome is indicated at the left side. Relative packaging ratios of Xa to Xi are mean values of Y1, Y1′ and Y2 cells calculated as follows. B6-derived and MSM-derived band signals were counted using a molecular image analyzer (BIO-RAD) in each F_1, H or E fraction, and the MSM signal (Xa) was divided by the B6 signal (Xi). The obtained ratio was normalized by the ratio of the respective F_1 sample. Most numbers thus calculated were less than 1 in the H fraction and more than 1 in the E fraction except for *DXMit73*. The packaging ratios are displayed as a fraction of the ratio in H divided by the ratio in E in each locus.

were less intense due to about a half dose of B6-derived X chromosome relative to MSM-derived X chromosome (see Fig. 1). Y5 cells showed B6<M pattern in the H fraction and B6>M pattern in the E fraction, consistent with *Xist* expression from the MSM allele. *DXMit3* and *MPC901* marker loci showed similar patterns though amplification bias was remarkable. The *DXMit73* locus was exceptional and exhibited little difference between the H and E fractions probably because this region was condensed in both active and inactive X chromosomes. *DXMit101*, *DXMit27* and *DXMit53* gave patterns intermediate between those of *DXMit5* and *DXMit73*. Fig. 4B shows H/E patterns of three gene loci on X chromosome, *Pgk-1*, *Fmr-1* and *Smcx*, which were essentially similar to the pattern of *DXMit5* except for Y5. Relative packaging ratios are summarized in Fig. 5.

Y5 cells exhibited a unique fractionation pattern. The five loci, *DXMit5*, *DXMit3*, *MPC901*, *Smcx* and *Fmr-1*, showed M>B6 pattern in the H fraction and M<B6 pattern in the E fraction. This indicated that the MSM alleles on Xi were more compact than the B6 alleles on Xa. However, the *Pgk-1* or possibly *DXMit27* locus show little difference between the H and E fractions suggestive of chromatin relaxation.

DISCUSSION

The H/E fractionation used in this paper was originally developed to separate transcriptionally active chromatin from transcriptionally repressed chromatin, and chemical and physical properties of the two chromatin components were studied (15, 19). We infer that the H fraction is enriched with heterochromatin and a part of inactive euchromatin, whereas the E fraction consists mainly of active euchromatin and a part of inactive euchromatin. The implication is supported by the following results. First, the H fraction contained DNA fragments larger on the average than the E fraction (Fig. 3A). This suggests that the H fraction contains the chromatin of compact configuration resistant to sonication, which is consistent with a much larger sedimentation constant. Second, the mouse satellite DNA was more concentrated in the H fraction, whereas ribosomal DNA was in the E fraction (Fig. 3B). The third one is provided by probing the functional p53 gene and the p53 pseudogene which are probably present in active euchromatin and in inactive euchromatin, respectively; a clear difference in fractionation is seen between the two (Fig. 3C). H/E assay is also applicable to normal tissues. Analysis of liver of F_1 mice between MSM and the wild strain (T(X; 16)16H) with the Robertsonian translocation between X and 16 (20), cells of which comprised the inactive X chromosome of MSM and expressed the MSM-derived *Xist*, showed that the *Pgk-1* and *DXMit5* loci on Xi underwent chromatin compaction relative to those on Xa (unpublished data).

Difference in chromatin packaging between Xi and Xa was detected in Y1, Y2, Y3 and Y4 cells for almost all marker loci examined except the *DXMit73* locus. The unique *DXMit73* region is presumed to constitute inactive euchromatin, but even in this region chromatin structure is more compact on Xi than on Xa (Fig. 5). This suggests that the level of Xi compaction may be higher than that of the inactive euchromatin. Intriguingly, the packaging level in H/E assay appears to vary among loci, representing different degrees of chromatin packaging on Xa (Fig. 5).

Intriguingly, Y5 cells expressed the *Pgk-1* gene from both B6 and MSM alleles, though expression of the MSM allele being very weak. This biallelic expression is exceptional in tumor cell lines of monoclonal origin and therefore suggests an impairment of transcriptional suppression of the MSM allele of *Pgk-1* on Xi. It

is likely that relaxation of the suppression occurred during tumor development or establishment of the cell line. As for chromatin packaging state, little difference was detected between Xi and Xa (Fig. 4B). This suggests that the chromatin compaction of the *Pgk-1* gene region on Xi was loosened and the level of packaging was the same as that of the *Pgk-1* region on Xa. On the other hand, other regions on X chromosome except for the *DXMit27* locus seemed to retain packaging state in Y5 cells. These results suggest that the chromatin relaxation of Xi can occur regionally in some tumors.

Xist is present in all adult female tissues and associates with the whole inactivated interphase X chromosome, therefore suggesting that *Xist* continues to play a role in the maintenance of the inactive state (21, 22). The chromatin loosening observed in Y5 cells, which expressed *Xist* only from the allele on Xi, implies that the *Xist* gene is not strictly required for maintenance of transcriptional inactivity on Xi. This is consistent with that the inactive state is stably maintained after loss of regions containing the *Xist* gene on the human X chromosome (23).

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