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Telomere Change and Loss of Heterozygosity of Mouse Primary Tumors and Cell Lines

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Changes in the number of telomere repeat arrays were examined in mouse tumor cells. Telomeres that function for the protection of chromosomes were detected as bands and a smear by pulsed field gel electrophoresis and gel-hybridization using $(TTAGGG)_4$, as a probe. Of eight primary tumors induced in F_1 mice between C57BL/6 and C3H/He and between C57BL/6 and MSM, three showed telomere alteration, two having extra bands and one having lost several telomere bands. The others exhibited patterns similar to those of normal tissues. However, the change was detected in all four cell lines that were established from one of the tumors. One cell line was further cloned and examined. Two of the nine clones differed in the telomere pattern. The telomere change was also observed in two other cell lines, FM3A cells and nontransformed BALB3T3 cells. These results suggest that telomeres are highly mutable in tumor cells and cultured cell lines. Three of the tumors and one cell line were analyzed for loss of heterozygosity with 51 microsatellite probes covering all 19 autosomes. Also, karyotype analysis of the cell line was performed. No allelic loss was seen and chromosomal abnormality was rare, although aneupoloidy and imbalance in chromosomal number were observed. Possible involvement of the telomere changes observed here in chromosome impairment is discussed.

Key words: Telomere — Genomic instability — Allelic loss — Tumor progression

The unicellular origin of tumor cells is well documented.[1)] However, at the time of formation of a tumor, it is already composed of genetically altered subpopulations with different characteristics. A variant subpopulation(s) having a proliferative growth advantage dominates the primary site and eventually gives rise to metastasis.[2,3)] In the process of this tumor progression, tumor cells often show aneuploidy and rearrangement of chromosomes, some of which result in loss of genetic information and/or abnormal expression of genes in the tumor cells. These changes are thought to result from genomic instability of tumor cells, although the molecular basis for genomic instability is unclear.

It is known that alteration of telomeres, the ends of chromosomes, leads to chromosomal abnormality and aneuploidy in yeast and *Drosophila*, because telomeres are responsible for maintenance of the chromosomal integrity and partition of chromosomes.[4-6)] Mouse and human telomeres consist of tandem repeats of a six-base consensus sequence: $(TTAGGG)_n$.[7,8)] However, there are several differences. Mouse telomeres are many times larger than those of human chromosomes, and are detected as bands and a smear when analyzed by blot hybridization. The banding pattern is highly polymorphic within the population of inbred mice, reflecting frequent germline recombination of telomeres.[9,10)] Human telomeres appear only as a smear in blot patterns. Size-reduction in telomere repeat arrays is observed during the aging of normal cells[11)] and in most colorectal carcinomas.[12,13)] Therefore, the telomere shortening is ascribed to the loss of repeat arrays during cell divisions. On the other hand, such behavior of telomeres was not found in normal tissues of mice,[9,10)] and has not been examined in mouse tumors.

We induced primary tumors in F_1 mice consisting of two different sets of genomes and established cell lines. The telomere variability of those tumor cells was examined using a synthetic oligonucleotide, $(TTAGGG)_4$, as a probe. Loss of heterozygosity and karyotype were also examined. We found that telomere changes were frequent, while chromosomal abnormalities were rare in tumors.

MATERIALS AND METHODS

Tumor induction F_1 mice from C57BL/6 female and MSM male and from C3H/He female and C57BL/6 male crosses were used for tumor development. MSM mice are an inbred strain derived from Japanese wild mice, *Mus musculus molossinus*.[14)] Eight- to twelve-week-old mice were given a single subcutaneous injection of 1.0 mg of methylcholanthrene dissolved in olive oil. Mice were killed and tumors were collected on the appearance of sarcomas, generally 3–4 months after the injection. Mice were kept in plastic cages, in an air-conditioned

room. They were given commercial pellets and tap water *ad libitum*.

Cells and *in vitro* cloning Three cell lines were used in this experiment. MST cells were established from a methylcholanthrene-induced fibrosarcoma in the F_1 mice between C57BL/6 and MSM. Morphologically non-transformed BALB3T3 cells were obtained from Japanese Cancer Research Resources Bank (JCRB). These cells were cultured at 37°C in 5% CO_2 and 95% air in alpha-modified minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. MST cells and BALB3T3 cells were cloned by plating into 96-well flat-bottomed microtiter plates at an average concentration of 0.75 cell/well. Those clones were expanded *in vitro*, and one was subjected to subcloning in the same manner.

FM3A cells obtained from JCRB were a cell line derived from a mouse mammary tumor and cultured in ES medium with 5% calf serum. The cells were suspended at a density of 2×10^5 cells/ml and plated on 0.5% agarose gels containing ES medium with 10% fetal bovine serum. One colony, FM3A-S1, was further subjected to subcloning in the same manner. Each subclone was expanded *in vitro* and DNA was isolated.

DNA isolation and pulsed-field gel electrophoresis Cellular DNA was extracted as described previously.[15] Cells were lysed in a buffer containing 0.5% sodium dodecyl sulfate (SDS), 0.1 *M* NaCl, 1 m*M* EDTA, 10 m*M* Tris-HCl pH 7.6, and 100 μg/ml proteinase K. After incubation at 50°C overnight followed by phenol-chloroform extraction of the lysate, DNA was recovered by ethanol precipitation. Such DNA was used for polymerase chain reaction.

High-molecular-weight DNA was prepared in agarose plugs from liver and kidney of mice and from cultured cells as previously described, with a minor modification.[9] Each tissue was homogenized using a Potter homogenizer in 15 ml of 1×buffer A (60 m*M* KCl, 15 m*M* NaCl, 0.5 m*M* spermine, 0.15 m*M* spermidine, 15 m*M* Tris-HCl buffer [pH 7.5], 14 m*M* β-mercaptoethanol), 2 m*M* EDTA, 0.3 *M* sucrose. The homogenate was passed through three layers of gauze and layered onto 20 ml of 1×buffer A, 1.37 *M* sucrose, 1 m*M* EDTA. After centrifugation in an angle rotor for 15 min at 12,000 rpm and 4°C, the pellet of nuclei was resuspended in 0.5 to 2 ml of 1×buffer A, 1 m*M* EDTA at 2×10^7 nuclei/ml. An equal volume of 1% low-gelling-temperature agarose in 1×buffer A, 1 m*M* EDTA (maintained molten at 50°C) was added, and aliquots were transferred to plug moulds.

Cultured cells were collected by centrifugation for 5 min at 1,200 rpm at room temperature and were washed twice with PBS (0.14 *M* NaCl, 5 m*M* KCl and 20 m*M* phosphate [pH 7.5]). The pellet of cells was resuspended in PBS at 2×10^7 cells/ml. An equal volume of 1% low-gelling-temperature agarose in 1×TAFE (10 m*M* Tris base, 0.5 m*M* EDTA, 4.35 m*M* acetate) was added, and aliquots were transferred to plug moulds.

The plugs were removed and washed three times by incubating in 0.5 *M* EDTA, 1% N-lauroylsarcosine, 1 mg/ml proteinase K at 50°C for 48 h. Plugs were then stored at 4°C. After digestion with restriction enzymes in the manufacturer's recommended buffer as described, DNAs were separated on a 1% agarose gel using a Beckman Gene Line apparatus.[9, 10] Electrophoresis was run in 1×TAFE at 100 V for 48 to 60 h with a 30-s to 40-s switching interval, the running buffer being maintained at 12°C. The gel was vacuum-dried on Whatman No. 3 MM for 1 h at room temperature and 1 h at 60°C. The dried gel was freed from the paper backing by wetting in 6×SSC (1×SSC=0.15 *M* NaCl, 0.015 *M* sodium citrate) prior to use. The gel was directly hybridized to 1×10^6 cpm/ml labeled oligonucleotide in 5×SSPE (1×SSPE=10 m*M* sodium phosphate [pH 7.0], 0.18 *M* NaCl, 1 m*M* EDTA), 0.1% SDS, and 10 μg/ml sonicated denatured salmon sperm DNA at 37°C for 16 h.[16] The gel was washed with 6×SSC twice for 15 min each, followed by a 4-h wash at room temperature with 6×SSC, a 3-min wash at 37°C with 5×SSPE, and finally a 30-min wash with 6×SSC. The gel was then exposed to X-ray film (Fuji) for 18 to 36 h with an intensifying screen at −20°C.

Telomere and minor satellite probes The consensus sequence of mouse telomere of $(TTAGGG)_4$[7] and the minor satellite sequence of CACATTCGTTGGAAA-CGGGATTTGTAGAACAGT[17, 18] were made using an automated DNA synthesizer (Applied Biosystems Inc.) and purified by polyacrylamide gel electrophoresis. Each oligonucleotide (1 pmol) was labeled at the 5′-hydroxyl group with [γ-^{32}P]ATP (16 pmol at >5000 Ci/mmol, Amersham) and T4 polynucleotide kinase (4 units; Takara) in 10 μl of kination buffer (50 m*M* Tris-HCl [pH 7.6]), 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 0.1 m*M* spermidine, 0.1 m*M* EDTA). The kinase reaction was carried out at 37°C for 60 min. The specific activity was about 2×10^8 cpm/μg.

Karyotype analysis The cell clone MST3-C6 was allowed to incorporate 5-bromodeoxyuridine (0.1 mg/ml) for 8 h before harvest, including the last hour in the presence of Colcemid. Chromosome slides prepared by a routine air-drying method were stained with acridine orange and examined under a fluorescence microscope.

LOH analysis with microsatellites Microsatellites were used as probes for analysis of loss of heterozygosity (LOH). The names and chromosomal locations (location: cM) of microsatellites are as follows: D1Mit1; 1(1), D2Nds1; 2(28), I1-1b; 2(47), D3Nds1; 3(59), D3Nds3; 3(83), D4Jp2; 4(41), D4Nds2; 4(53), D4Mit13; 4(94),

D4Jp1; 4(49), D5Mit1; 5(10), I1-6; 5(11), D5Nds2; 5(37), D6Mit1; 6(10), D6Jp1; 6(32), Ngfg; 7(21), D7Nds2; 7(29), D7Mit14; 7(76), D8Nds1; 8(32), D8Jp1; 8(49), D8Jp2; 8(49), D9Nds2; 9(37), D9Mit18; 9(77), D10Nds1; 10(29), D10Nds2; 10(55), D10Jp1; 10(51), GLNS; 11(10), I1-5; 11(29), Csfgm; 11(30), D11Nds1; 11(39), p53; 11(41), D11Jp1; 11(37), Myla; 11(62), D11Mit10; 11(65), D12Mit2; 12(20), D12Jp1; 12(71), D13Nds1; 13(45), D13Mit9; 13(45), Plau; 14(1), D15Mit12; 15(1), Myc; 15(18), D15Jp1; 15(55), D16Nds1; 16(35), Tnfb; 17(19), D17Jp1; 17(79), D18Mit10; 18(32), Mbp; 18(57), D18Mit16; 18(57), D19Mit5; 19(21), D19Jp1; 19(37), D19Jp2; 19(1), DXJp1; X. Most of the microsatellite primers for polymerase chain reaction (PCR) were synthesized on an Applied Biosystems 380B synthesizer, as reported.[19–21] The other probes named DNJpN have been isolated by our group and the details will be published elsewhere.

PCR was carried out in a 10–20 μl volume essentially as described by Saiki *et al.*[22] Samples were processed through 30 cycles consisting of 1 min at 94°C, 1.5 min at 55°C, and 1.5 min at 72°C, with the last elongation step lengthened to 10 min. The products were analyzed by 4% NuSieve agarose gel or 12% polyacrylamide gel electrophoresis.[23] In some cases, they were subjected to PCR-SSCP analysis for the resolution of alleles.[24] Of 76 microsatellite loci examined, 51 loci described above showed polymorphisms and were used for this analysis.

RESULTS

Primary tumor cells having telomeres of different lengths

We investigated whether or not change of telomeres can be detected in mouse primary tumors. Five and three primary fibrosarcomas were induced in F_1 mice between C57BL/6 and MSM and between C57BL/6 and C3H/He, respectively, by treatment with methylcholanthrene. DNA was isolated from liver, kidney and tumor tissues in agarose plugs and digested with *Hae* III. The DNA digests were separated in an agarose gel by pulsed-field gel electrophoresis (PFGE). The gel was dried and directly hybridized to a labeled synthetic oligonucleotide, $(TTAGGG)_4$. This sequence constitutes the telomere repeat arrays in mouse chromosomes. Telomeres were detected as bands and a smear.

The patterns of liver and kidney derived from the same mouse were identical but were different from those of different individuals (Fig. 1). This is consistent with the previous finding that telomeres are highly changeable in germline cells but are stably maintained in somatic tissues.[9] Three of the eight tumors showed differences in the band pattern from those of liver and kidney. Two

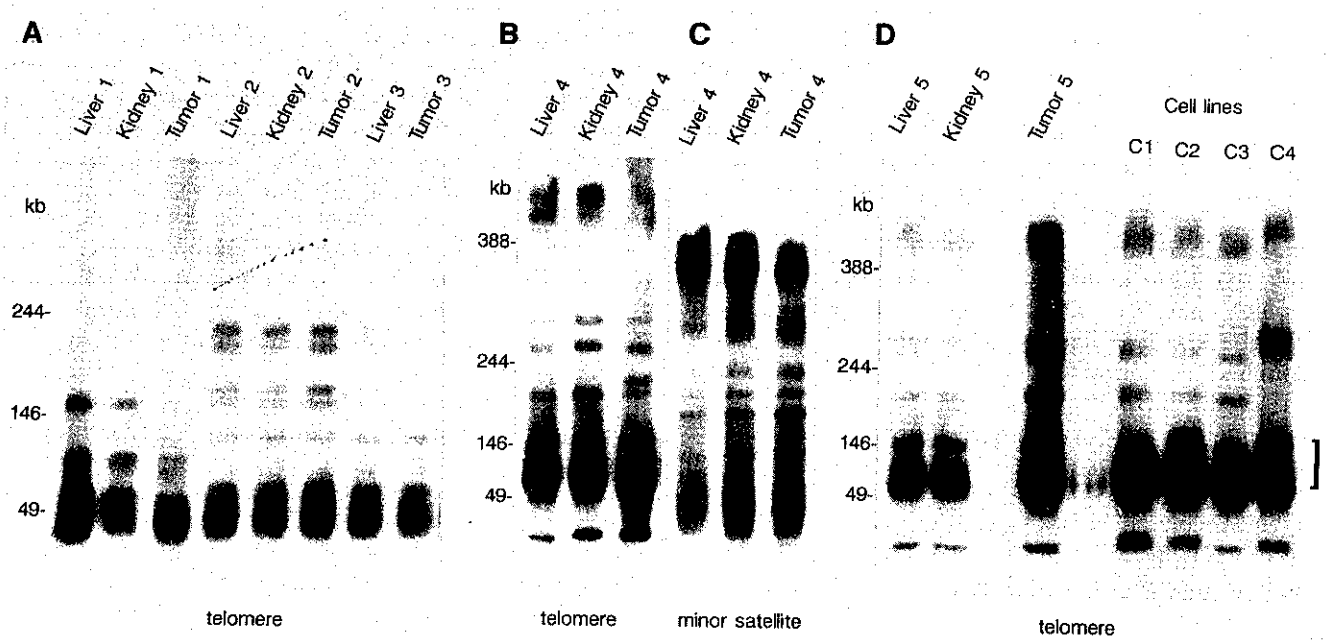

Fig. 1. Change of telomeres found in primary tumors. DNA was isolated from primary tumors, and liver and kidney of the host mice. *Alu* I digests were analyzed by PFGE and hybridization to the probes of telomere, $(TTAGGG)_4$ and minor satellite (see "Materials and Methods"). Panel A shows the results of the samples from F_1 mice between C57BL/6 and C3H/He, and panels B, C and D show those from F_1 between C57BL/6 and MSM. A bracket marks telomeres showing variation.

exhibited extra bands (Tumors 1 and 4 in panels A and B). This suggests that these tumors comprised at least two cell populations, one having telomeres similar to those of the normal tissues and the other having mutated telomeres. The other one showed a pattern different from that of liver; sizes of several telomere fragments were reduced (Tumor 3). *Hinf* I and *Alu* I digests gave essentially similar results (data not shown).

The five remaining tumors showed patterns similar to those of normal tissues (see Tumors 2 and 5). This result seems to indicate that telomeres were well maintained in these tumors. However, this does not exclude the possibility that there was a small subpopulation(s) having telomere changes. Therefore, four tumor cell lines, designated as MST cells, were established from one of the primary tumors (Tumor 5), and the length of telomeres was examined. Three lines, C1 (MST-1), C2 (MST-2) and C3 (MST-3), were similar to one another except for loss of the largest telomere band. The other line, C4 (MST-4), exhibited a pattern distinct from that of the tumor (panel D). In addition, telomere bands ranging from 150 kb to 70 kb (marked by a bracket) seem to have been altered in length relative to those of normal tissues. These results suggest that telomere changes accumulated in the tumor and some subpopulations with changed telomeres were subsequently selected to give rise to cultured cell lines. Alternatively, it is also possible that new mutations of telomeres were induced during the establishment of these cell lines.

To confirm complete digestion, the gel was washed out to remove the probe and hybridized again to a synthetic oligonucleotide homologous to the core sequence of mouse minor satellite. The minor satellite is located at the centromere region of chromosomes. Panel C shows an example of such results. The bands ranging from approximately 400 kb to 50 kb were similar to one another, indicating that the digestion was complete.

Change of telomere size among subclones of MST cells A cell line (MST-3) was subjected to cloning and nine clones were obtained. The clones were analyzed by PFGE and gel hybridization using the telomere probe (Fig. 2). Two clones exhibited telomere patterns that were distinct from that of the others: a band was elongated (lanes 1 and 4). The result suggests that the MST-3 line also consisted of a heterogeneous population. This heterogeneity may reflect pre-existing mutations in the primary tumor or mutations that had been newly induced during the establishment of the cell line. In any case, this strongly suggests that telomeres are unstable in the MST tumor cells. The gel was rehybridized to the minor satellite probe. The band patterns of all subclones were similar to one another (data not shown).

Telomere heterogeneity in other cell lines In order to know whether or not such telomere instability is observed

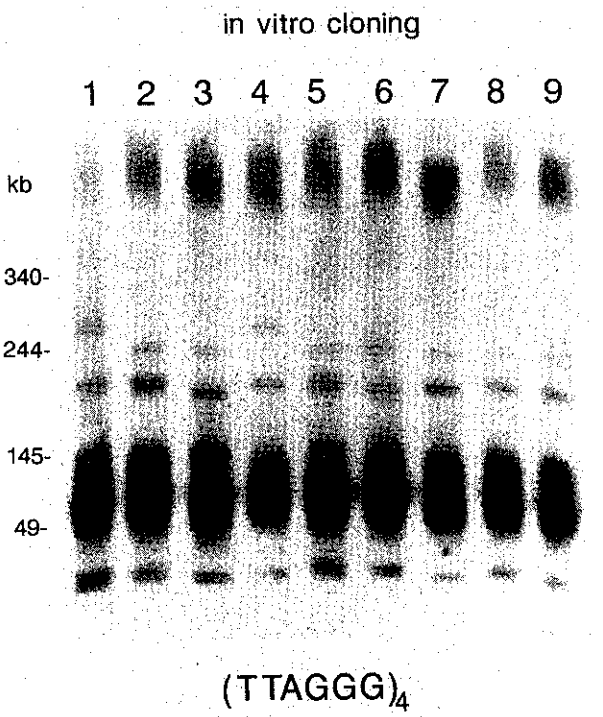

Fig. 2. Heterogeneity of MST-3 clones with regard to the telomere length. An MST-3 cell line was distributed into 96-well flat-bottomed microtiter plates and nine clones were obtained. DNA was isolated from each clone, digested with *Alu* I and analyzed by PFGE and gel-hybridization to $(TTAGGG)_4$.

in another tumor cell line, we examined FM3A cells derived from a mouse mammary tumor induced in a C3H/He mouse. FM3A cells were grown on soft agarose plates and one clone, FM3A-S1, was isolated. FM3A-S1 was further subjected to cloning *in vitro* and 9 subclones were obtained. DNA was isolated and the *Hae* III and *Alu* I digests were examined by pulsed-field gel electrophoresis (Fig. 3). The subclones of lanes 5 and 9 exhibited extra telomeric bands. This strongly suggests that telomeres of FM3A cells are also genetically unstable.

BALB/3T3 cells, a normal established line, was next examined. A fresh clone was further subjected to subcloning and eight clones were obtained (Fig. 4A). One subclone (lane 7) exhibited an extra band but the others showed only a smear, suggesting that the telomeres of morphologically nontransformed BALB/3T3 cells are also genetically unstable. The patterns probed with the minor satellite were very similar to one another (Fig. 4B).

Analyses of karyotype and LOH Changes in the telomere length observed here may result in chromosome instability (see "Discussion"). Hence, chromosomes were

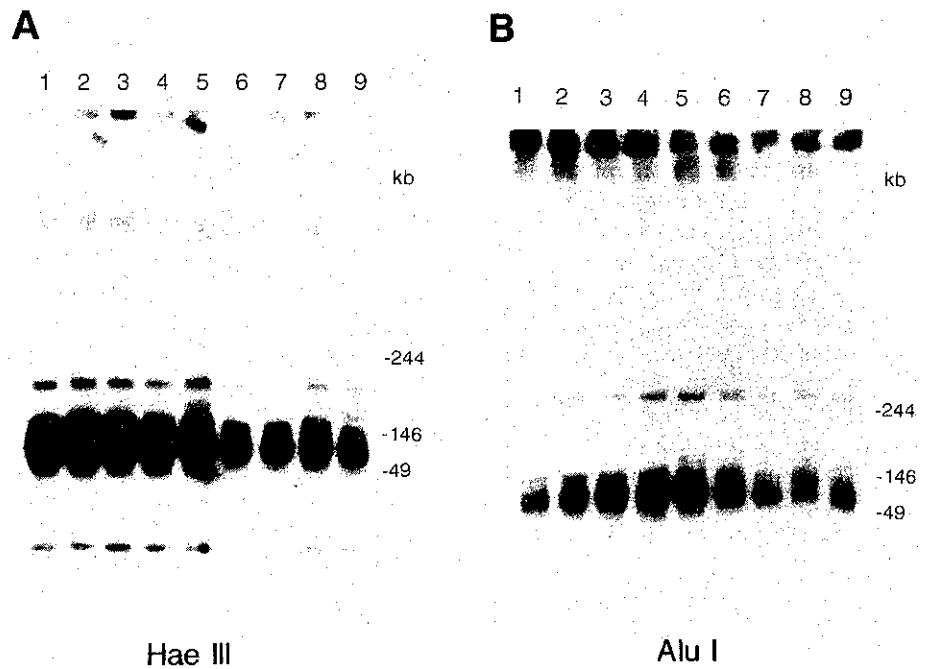

Fig. 3. Telomere instability observed in FM3A subclones. Freshly cloned FM3A-S1 cells were subjected to cloning and nine subclones were obtained. *Hae* III (A) and *Alu* I (B) digests were probed with $(TTAGGG)_4$.

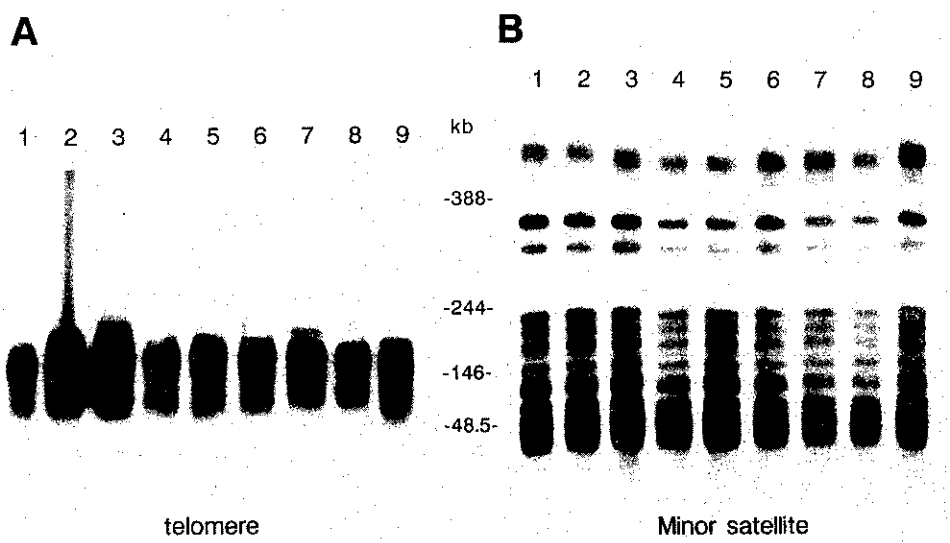

Fig. 4. Telomere (A) and minor satellite (B) patterns of BALB/3T3 cells. A fresh clone of BALB/3T3 cells was further cloned and eight subclones were obtained. *Hae* III digests were probed with the telomere and minor satellite sequences.

studied in one cell clone (MST3-C6) to examine this possibility. The most abundant cells were near-tetraploid with 68–82 chromosomes including four X chromosomes (Fig. 5). Octaploid or higher polyploid cells were not rare, and were probably directly derived from these near-tetraploid cells. Abnormality in chromosomes was rarely observed except for an MSM-derived X chromosome. Thus, the present observation did not provide us

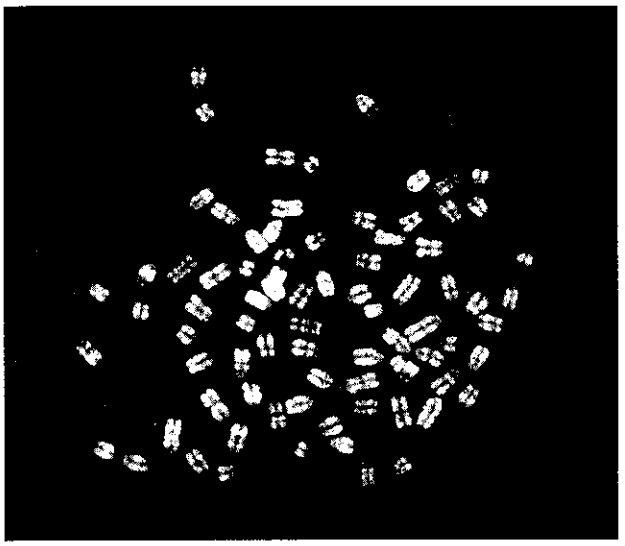

Fig. 5. An R-banded metaphase cell from MST3-C6 clone with 81 chromosomes including four X chromosomes, two from C57BL/6 and two from MSM. Chromosome loss and rearrangement are apparently rare.

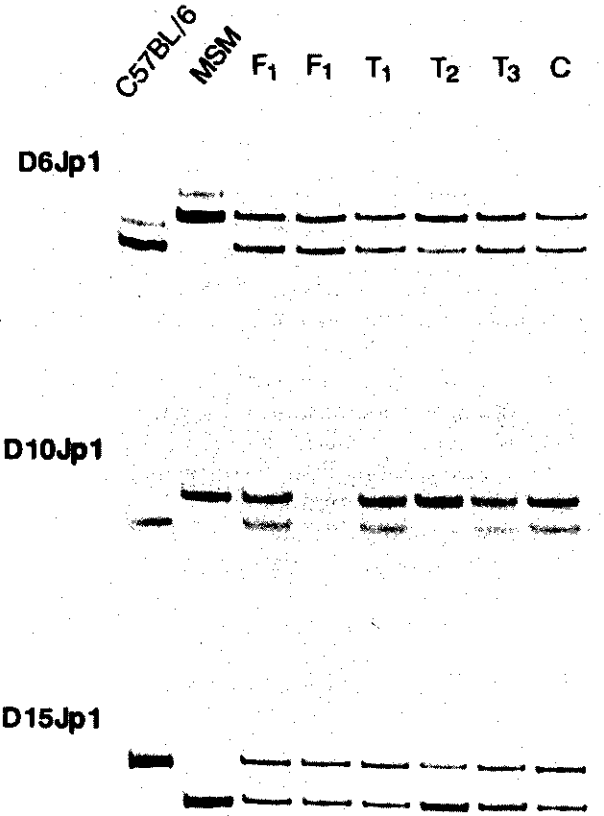

Fig. 6. LOH analysis of the D6Jp1, D10Jp1 and D15Jp1 loci. PCR products with the primers were each separated by 12% polyacrylamide gel electrophoresis and stained with a silver-staining kit. D6Jp1, D10Jp1 and D15Jp1 were located at chromosome 6 (41 cM), chromosome 10 (51 cM), and chromosome 15 (55 cM), respectively. T1 is the tumor 4 having changed telomeres shown in Fig. 1. T2 and T3 are tumors without alteration at telomeres. T2 is the tumor 5 and C is one cell line (MST-3) derived from the tumor 5.

with positive evidence that chromosomes are unstable in this particular tumor cell clone.

Analysis of LOH was also carried out for three MST tumors and one cell line (MST-3). These tumor cells were composed of two different haploid-genomes derived from F_1 mice between C57BL/6 and MSM. For probes, we used microsatellites consisting of 51 loci covering all 19 autosomes. We failed to observe allelic losses in these four tumor cells. Three representative examples of such analysis are given in Fig. 6. PCR products with D6Jp1, D10Jp1 and D15Jp1 primer pairs each gave two polymorphic bands. All four samples exhibited two bands derived from the C57BL/6 and MSM alleles, although one tumor (T2) showed differences in intensity between the two allelic bands. The results obtained from the LOH analysis suggested the absence of allelic losses in these samples.

DISCUSSION

In this study, the change of telomeres has been examined in normal mouse tissues, primary tumors, two tumor cell lines of the mouse, and nontransformed BALB3T3 cells. No difference was observed in the band pattern between DNAs from normal tissues, which is consistent with previous reports.[9, 10] This suggests that telomere alteration hardly occurs in normal cells within the detection limit of the method applied here; we may have failed to detect the telomere change in sample cells with a mosaicism, unless the alteration occurs sufficiently early in a stem cell lineage. Therefore, the possibility is not ruled out that normal tissues comprise cells with altered telomeres. On the other hand, shifts and/or losses of the telomere bands were clearly observed in three out of the eight primary tumors examined and some subclones of the cultured cell lines.

Telomeric DNA fragments detected by use of the $(TTAGGG)_n$ probe consist of the telomere sequence and the flanking subtelomeric DNA with a certain restriction site. The length of flanking sequences in the digests with 4-base recognition enzymes is probably short, but it would be long if they comprise simple tandem repeats. All or most mouse chromosome have the minor satellite arrays close to one end of the chromosomes.[25] Therefore, if the repeat sequences undergo recombination or muta-

tion, resulting in gain or loss of a new restriction site, the telomere fragment appears to change in length. Examination of repeat arrays of the minor satellite in the tumor cells, however, revealed that the change was sometimes observed in the digests with 4-base recognition enzymes but the frequency was much lower than that of telomeres (some data shown in Figs. 1 and 4). Therefore, change in subtelomeric regions can be a cause of apparent telomere mutations, though it seems unlikely. These results strongly suggest that the changes in telomeric fragments observed here resulted from alteration in telomeres, not in the flanking sequences.

Changes of telomeres suggest that tumor cells undergo recombination at telomeres at a considerably high frequency, probably during cell divisions. This is consistent with our previous finding that the elongation of telomeres occurs frequently in the tumor cells in metastatic nodules.[15] The changes may be due to the frequent recombination that was observed in tumor cells.[26, 27] The mutation or shortening of telomeres may impair the function of the telomeres. If this is the case, such telomere changes could be a cause of chromosomal instability (see the next section). Alternatively, they may represent a by-product or consequence of the genomic instability often observed in tumor cells.

The telomere is an essential structure located at the end of eukaryotic chromosomes. Two distinct roles are suggested. Firstly, telomeres protect the DNA from degradation and end-to-end joining, as suggested by cytological and genetic evidence.[4–6, 28] The protection by telomeres appears to be necessary for chromosome stability, since nontelomeric ends of broken chromosomes have a tendency to fuse with other broken ends, sometimes forming dicentric chromosomes. Dicentric chromosomes subsequently break to form new ends and this cycle continues.[4–6, 29] On the other hand, linear DNA with telomeres is protected from degradation.[30, 31] The second role is to anchor chromosomes in the nucleus by interactions with the nuclear envelope.[12] Cells with abnormally long, short or altered telomere sequences exhibit aberrant nuclear morphology, cell division and loss of chromosomes, probably because such cells may have topologically unstable chromosomes in the nucleus. This implies that telomeres interact with other nuclear structures in a way that influences the fidelity of chromosome transmission.[32]

The function of telomeres suggested above implies that some of the tumor cells with alteration in telomeres may well lose some chromosomes, resulting in aneuploidy, or carry aberrant chromosomes with rearrangement, deletion and breaks. In order to see whether this is the case, we have carried out karyotype and LOH analysis. Change in karyotype was observed (Fig. 5), and was basically consistent with previous results for mouse cell lines.[33] This is consistent with the result of LOH analysis, showing imbalance in copy number between paternal and maternal alleles in some loci (Fig. 6). Such aneuploidy may have resulted from the telomere alteration, but the relationship between the two changes is uncertain at present. On the other hand, allelic losses were not detected in the LOH analysis. Likewise, chromosomal loss and rearrangement were apparently rare in the karyotype analysis. The chromosomal changes observed here may be a result of hyperploidy and chromosomal loss occurring either independently or simultaneously. In any event, these results suggest that the frequency of chromosomal aberration was low in these tumors. Otherwise, this may be due to low sensitivity of the methods employed for detection of chromosome changes. In the present study, we were unable to define or estimate the effect of the observed telomere changes on chromosome stability. Further studies are required to examine the possibility that telomere change increases the level of chromosome instability in tumor cells.

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