

Temperature Dependence in Proliferation of Tetraploid Meth-A Cells in Comparison with the Parent Diploid Cells

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ABSTRACT. The temperature dependency for the growth of tetraploid Meth-A cells established from diploid cells was examined in comparison with the parent diploid cells. Proliferation of the tetraploid cells was markedly suppressed below 35°C. At above 40°C, both the diploid and tetraploid Meth-A cells ceased growing. Flow cytometry (FCM) analysis showed that the hyperploid cell fraction increased in the tetraploid Meth-A cell population at low temperatures. The fluidity of cell membranes at different temperatures was measured by means of electron spin resonance (ESR), and it was almost the same between the diploid and tetraploid Meth-A cells. It was suggested that the decreased proliferation below 35°C of the tetraploid Meth-A cells might be due to the increased volume of the cells.

Key words: Meth-A cells/tetraploid cell line/temperature dependency

Meth-A cells, a methylcholanthrene-induced sarcoma cell line, always have a small population of large cells which were spontaneously polyploidized and resulted in apoptosis (Fujikawa-Yamamoto *et al.* 1997a). The cells may be susceptible to polyploid transformation. Several important characteristics of polyploidized Meth-A cells have been studied, including DNA synthesis (Fujikawa-Yamamoto *et al.*, 1997b), apoptosis (Fujikawa-Yamamoto *et al.*, 1997a; Zong *et al.*, 1998), growth *in vivo* (Zong *et al.*, 1998), cell-surface hydrocarbon chains (Fujikawa-Yamamoto *et al.*, 2000) and involvement of protein kinase C (Zong *et al.*, 2000); however, the cells used were in a transient state of polyploidization.

A tetraploid Meth-A cell line was established from diploid Meth-A cells highly polyploidized by demecolcine (Fujikawa-Yamamoto *et al.*, 2001). They were tetraploid (4 homologous chromosomes) and had double the DNA content of the parent diploid cells. Their cell-cycle parameters were almost the same as those of the parent-diploid cells, differing from the results of artificially polyploidized cells (Fujikawa-Yamamoto *et al.*, 1997b). Because the tetraploid cell line could be reproducibly established from diploid Meth-A cell line, we used the diploid and tetraploid Meth-A

cell lines as a system to investigate cells. A comparison between diploid and tetraploid Meth-A cells may help to interpret why tetraploid cells were not selected as main constituents of mammals.

Polyploid cells in mammals are found in various organs, particularly in the aged or partially hepatectomized liver. However, they may be in a differentiated state. Tumor cells are generally in a state of growth *in vivo*, and they exhibit diploidy, aneuploidy, tetraploidy or a higher ploidy. It would be valuable to examine which characteristics of cells are changed when diploid cells become hyperploid.

In this study, the growth of diploid and tetraploid Meth-A cells was examined under changing medium temperatures. Distinct differences were observed between diploid and tetraploid Meth-A cells in the growth and DNA distribution. It will be concluded that temperature is an important factor in survival, particularly in tetraploid cells whose cell volume is double that of diploid cells.

Materials and Methods

Cells

Meth-A cells (a methylcholanthrene-induced mouse abdominal dropsy sarcoma cell line) were maintained in a humidified atmosphere of 5% CO₂ at 37°C as a suspension culture in a Leibovitz's L15 : Ham's F10 mixture (7:3) supplemented with 10% fetal bovine serum (CELLect GOLD, ICN Biomedicals, Aurora, OH, USA), streptomycin (100 µg/ml) and penicillin (50 units/ml). Tet-

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Abbreviations: c, haploid DNA content.

raploid Meth-A cells were cultured under the same conditions. The tetraploid Meth-A cell line was reproducibly established through polyploidization by demecolcine and the drug release (Fujikawa-Yamamoto *et al.*, 2001). The tetraploid Meth-A cell line has been maintained for over 1.5 years without any decrease in the DNA content.

Temperature dependency

Exponentially growing diploid and tetraploid Meth-A cells were plated in culture flasks (25 mm², Corning Costar Co., Acton, MA, USA) at a density of 5×10^5 cells/flask and cultured in a CO₂ incubator in a humidified atmosphere of 5% CO₂ at 37°C. The cell density was maintained at 5×10^5 – 1×10^6 cells/flask through subculturing. The cells obtained through subculture were used for FCM and cell number measurements. The temperature of the CO₂ incubator was changed to 36, 35, 34 and 37°C for one week each. The same experiments were performed at high temperatures above 37°C for 4 days each. The temperature of medium in culture flasks during the experiments was corrected by measuring medium temperature in a control flask.

Cell Preparation

The diploid or tetraploid Meth-A cells obtained through the experiments were fixed with 20% ethanol, resuspended in 0.5 ml of PBS⁽⁻⁾ (divalent cation-free phosphate-buffered saline) containing 0.25% RNase (Type II-A, Sigma, St. Louis, MO, USA) and incubated for 3 h at 4°C. Part of the cell suspension was used for counting by hemocytometers. Immediately before the measurements, the cells were stained with PI (propidium iodide, 7.5×10^{-5} M) and red fluorescence was examined by means of FCM. Under these staining conditions, the signal due to residual double stranded RNA is negligible and the relative intensity of the red fluorescence corresponds to the DNA content (Krishan, 1975).

Flow cytometry

The fluorescence from individual cells was measured using a FAC-Sort (Becton Dickinson Immunocytometry Systems, Franklin lake, ND, USA). The fluorescence of individual cells irradiated with a focused laser light at a wavelength of 488 nm was detected using a photomultiplier tube. The relative intensity of red fluorescence was measured and log scale-DNA histograms were obtained.

Cell cycle analysis

FCM data (signals of red-fluorescence intensity through a logarithmic amplifier) were input to CASL (a software for cell cycle analysis of DNA histograms on a log scale) and the DNA histograms were decomposed to cell fractions depending on the DNA content (Fujikawa-Yamamoto, 1999a). CASL is written in Mathematica software and can analyze DNA histograms with 2c to 128c DNA content. The algorithm is similar to Fried's method (Fried *et al.*, 1976; Fried, 1977) except that normal distribution functions having the same half-width instead of the same CV (coefficient of variation) value are used as components.

ESR measurements

Exponentially growing diploid and tetraploid Meth-A cells were harvested and condensed by centrifugation. The cell pellet was re-suspended in 4 ml of PBS⁽⁻⁾ containing 0.1% glucose. 16-doxyl-stearic acid was used as the spin probe to examine the cell membrane fluidity (Gaffney, 1976). The probe was dissolved in ethanol (0.1%) and aliquots of 20 µl were dried with a stream of dry N₂ gas in plastic test tubes. The cell suspension was then added to the test tube coated with a monolayered probe inside, and gently vortexed for 10 min at 37°C. The cells were washed three times and packed into a capillary-glass tube.

ESR spectra were recorded in a capillary cell at various temperatures set with a JEOL JES-FE2XG ESR spectrometer equipped with a variable temperature accessory. Samples were equilibrated for 5 min prior to taking the spectra. The microwave power was kept at 8 mW.

Cell volume distribution

Exponentially growing diploid and tetraploid Meth-A cells were fixed with 20 % ethanol, centrifuged and resuspended in PBS⁽⁻⁾. The distribution of cell volume (Coulter volume) was measured by a Coulter Counter (ZM/256, Coulter Electronics, Fullerton, CA, USA). Standard spheres (9.8 µm diameter, Coulter sphere, Coulter Electronics) were used as a control. Note that the Coulter volume depends on the materials being tested, because it is calculated based on the resistance of particles.

Photographs

Phase-contrast microphotographs of exponentially growing Meth-A cells in culture flasks were taken under a microscope (BX50, Olympus, Tokyo, Japan) with a digital camera (DC120 Kodak, USA). The microphotographs were printed out through a personal computer (8600/200, Macintosh).

Results

To examine the effects of low temperatures on the proliferation of diploid and tetraploid Meth-A cells, growth curves for both cell types were obtained by decreasing the temperature (Fig. 1). The growth of tetraploid Meth-A cells slowed down at 35 and 34°C. The cell growth did not completely recover when the temperature was returned to 37°C from 34°C, suggesting significant damage to the tetraploid cells.

To examine the cell cycle responses of diploid and tetraploid Meth-A cells, the DNA histograms of cell populations at 37 to 34°C were obtained (Fig. 2). At low temperatures, 8c and 16c peaks increased in the tetraploid cell populations, although the DNA histograms for diploidy were almost the same in this temperature span. The DNA histograms were analyzed with a cell cycle analysis program, CASL, and the fractions of polyploid cells are listed in Table 1. It was concluded that the growth of tetraploid Meth-A cells was decreased at low temperatures, and that the hyper-

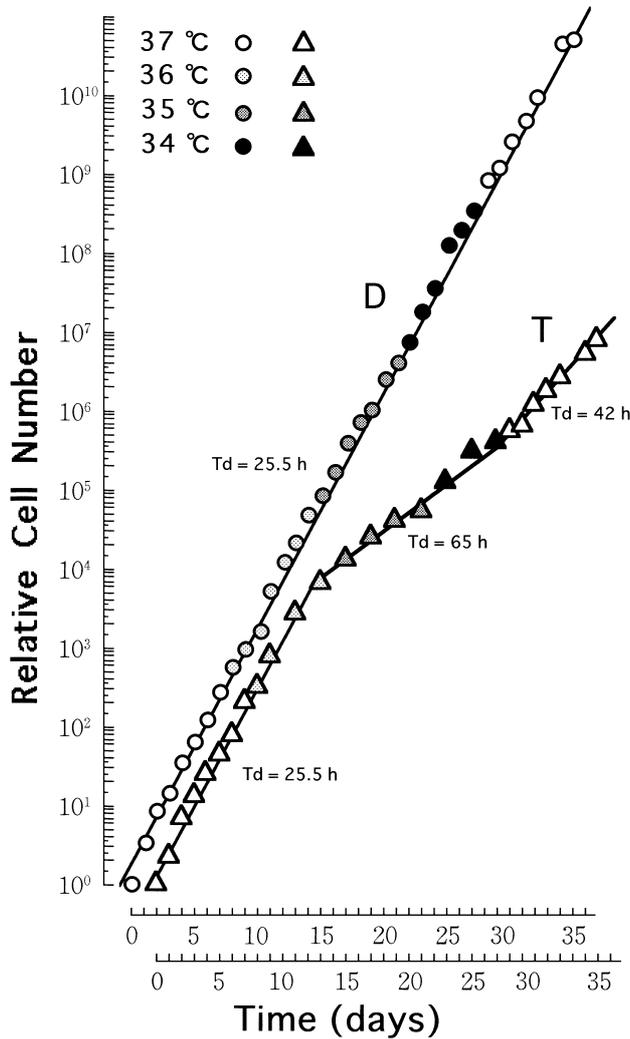


Fig. 1. Growth curves of diploid (D, circles) and tetraploid (T, triangles) Meth-A cells at various temperatures. Exponentially growing diploid and tetraploid Meth-A cells were cultured at 37, 36, 35, 34 and 37°C, in that order, for one week each with subculturing. Solid lines were drawn to facilitate understanding.

ploid population increased.

To examine the effects of high temperatures, growth curves for diploid and tetraploid Meth-A cell lines above 37°C were obtained (Fig. 3). The doubling time for both cell types at 37 to 39°C was about 26 h, slightly longer than the 24 h doubling time of diploid Meth-A cells at 37 to 34°C. The growth of both cell types decreased at 40.1°C. No distinct differences between the diploid and tetraploid cells were observed, suggesting a common cause for the slowed growth.

To examine the cell cycle responses of diploid and tetraploid Meth-A cells at high temperatures, DNA histograms were obtained (Fig. 4). The late S-phase population increased in the DNA histograms of diploid and tetraploid

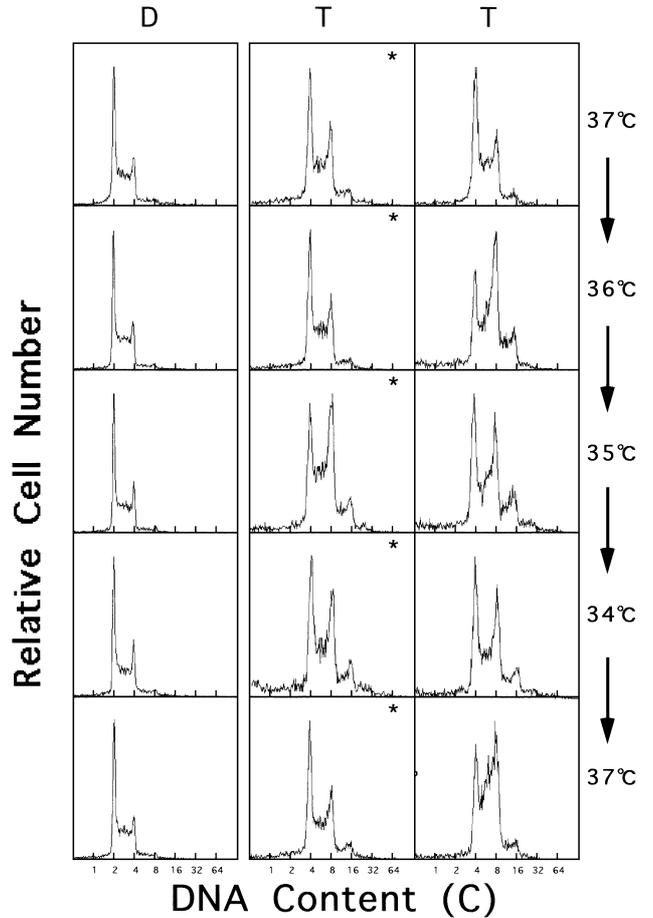


Fig. 2. Representative DNA fluorescence histograms of the diploid (D) and tetraploid (T) Meth-A cells in Fig. 1. The histograms in the right and left columns are for the last day at each temperature, and the histograms in the middle column (asterisk) are for the first day. The abscissa represents the relative DNA content (c, complement).

Meth-A cells at high temperatures, suggesting a delay of cell cycle progression in the late S phase. No increase in the hyperploid population was observed. It was concluded that tetraploid Meth-A cells showed decreased proliferation, as did the diploid cells, at high temperature, but the mechanism is not likely to be different from the growth regulation at low temperatures.

To examine whether or not the fluidity of cells at the molecular level was altered at low temperatures, cell membrane fluidity in molecular level was measured. The diploid and tetraploid Meth-A cells were labeled with 16-doxylstearic acid, a stable free-radical, and the ESR spectra were measured to obtain the order parameter, $S(T_{\perp})$, (Fig. 5 and Table 2). We selected 16-doxylstearic acid to examine the fluidity on the inside of the cell lipid bilayer, because it was difficult to specify a cell organelle whose fluidity would affect the cell growth. The order parameters of tetraploid Meth-A cells were smaller than those of the diploid cells at

Table I. CELL FRACTIONS AT VARIOUS TEMPERATURES.

Ploidy ^{a)}	2n	4n	8n	16n	32n	64n	>8n	>16n
Diploid cells								
37°C	0.79	0.18	0.04	0.00	0.00	0.00	0.00	-
36°C	0.80	0.16	0.03	0.01	0.00	0.00	0.01	-
35°C	0.77	0.17	0.05	0.01	0.00	0.00	0.01	-
34°C	0.69	0.23	0.07	0.01	0.00	0.00	0.01	-
Tetraploid cells								
37°C	0.10	0.57	0.26	0.05	0.01	0.00	-	0.01
36°C	0.06	0.44	0.39	0.06	0.01	0.00	-	0.01
35°C	0.11	0.46	0.31	0.10	0.02	0.00	-	0.02
34°C	0.06	0.51	0.32	0.09	0.02	0.01	-	0.03

a) 2n, 4n, 8n, 16n, 32n and 64n represent diploid, tetraploid, octaploid, hexadecaploid, dotriacontaploid and tetrahexacontaploid cells, respectively. The fractions were calculated based on the assumption that the G₂/M fraction is negligible, because the peaks above 4C DNA content cannot be divided into the hypoploid G₂/M and the hyperploid G₁ phases.

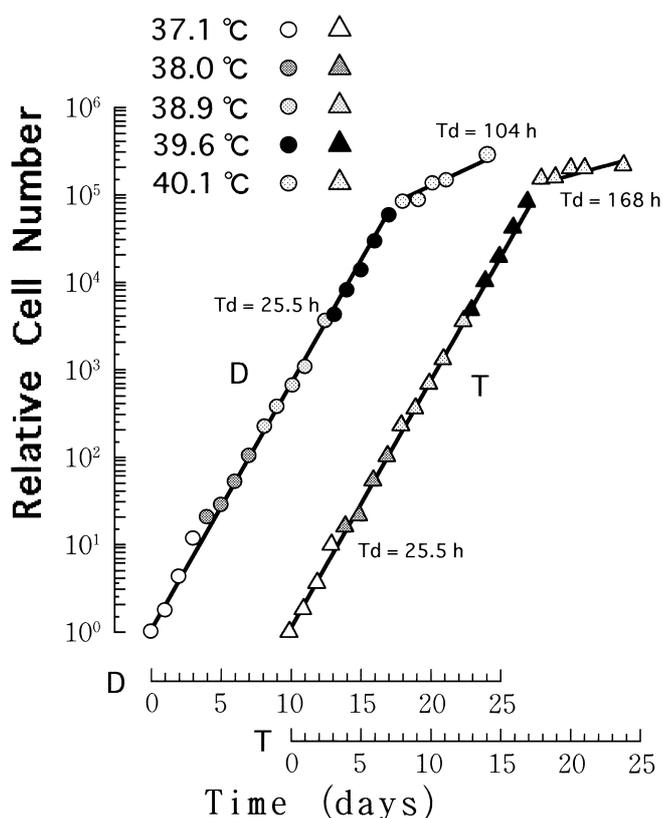


Fig. 3. Growth curves of diploid (D, circles) and tetraploid (T, triangles) Meth-A cells at various temperatures. Exponentially growing diploid and tetraploid Meth-A cells were cultured at 37.1, 38.0, 38.9, 39.6 and 40.1°C, in that order, with subculturing. Solid lines were drawn to facilitate understanding.

all of the temperatures except 40°C, meaning more rotation of the doxyl-radicals in the membrane. A slight increase in the order parameter with a decrease in temperature was ob-

served in both diploid and tetraploid Meth-A cells, indicating that the membrane fluidity of Meth-A cells decreased with temperature.

To examine cell morphology, phase contrast microphotographs and cell-volume distribution were measured (Fig. 6). The tetraploid Meth-A cells were spherical, as were the parent diploid cells, and their cell volume was about twice that of the diploid cells.

Discussion

Growth control of mammalian cells involves the interaction of several factors including temperature (Holley, 1974), and many changes to the cells are caused by temperature alterations (Cremisi and Duprey, 1986). We adopted a method of progressively changing the temperature to examine the effects on diploid and tetraploid Meth-A cells, because drastic changes in temperature may not occur *in vivo*. Cumulative effects would be expected by the method. Although the effects might be equally affect both cell types, it is not sure whether or not it may be excluded from the discussion of the differences in cell growth between diploid and tetraploid Meth-A cells.

The proliferation rate of tetraploid Meth-A cells was reduced at 35°C (low temperature) compared to that of diploid cells. Because diploid cells could normally grow at that low temperature, the possible decrease in activity of intracellular enzymes would not be likely to affect cell survival. The intracellular fluidity might be decreased at low temperatures, although the differences were negligibly small between diploid and tetraploid Meth-A cells. The cell volume of tetraploid cells was about twice that of diploid cells. This means about 1.26 times increase in length for the sphere-like tetraploid cells. This small-spatial increase of cells would cause a delay in the transport of materials particularly at low temperatures. Cumulative effects might assist to

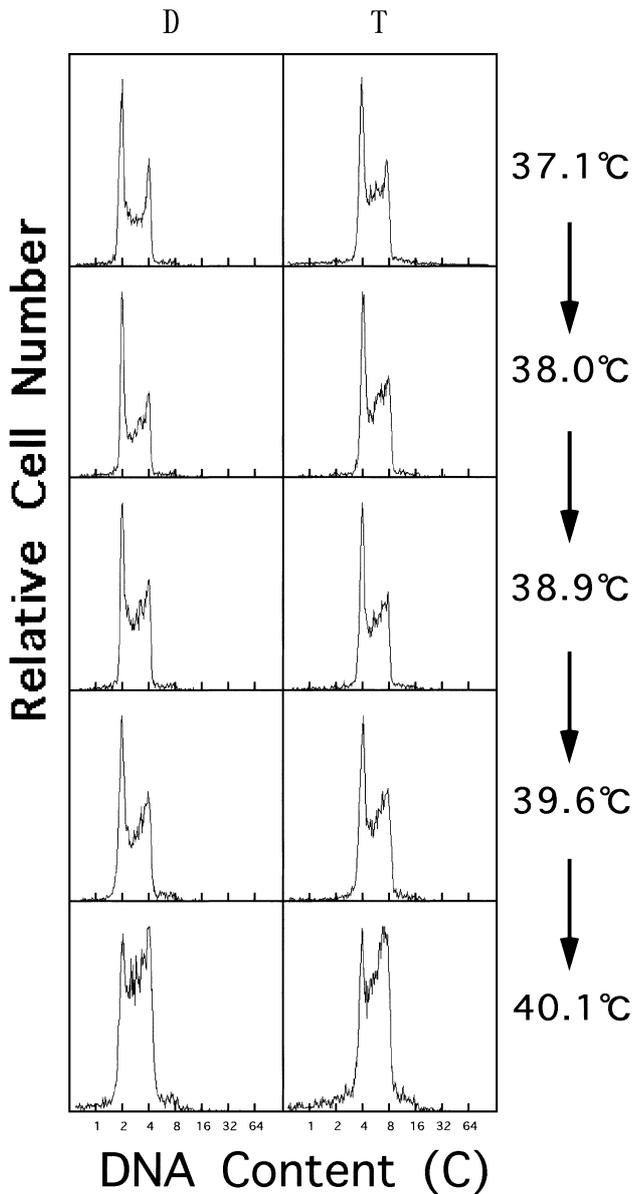


Fig. 4. Representative DNA fluorescence histograms of the diploid (D) and tetraploid (T) Meth-A cells in Fig. 3. All of the histograms are for the third day after the temperature change. The abscissa represents the relative DNA content (c, complement).

decelerate cell growth in the tetraploid Meth-A cells.

At 40°C (high temperature) the diploid and tetraploid Meth-A cells ceased growing and the fractions of late S and G₂/M phases increased in both cell lines. At high temperatures, the enzyme activity would be impaired, and the cells would not be able to maintain normal proliferation (Tanonaka *et al.*, 1986).

It is of interest to discuss the cell cycle responses of diploid and tetraploid Meth-A cells at low and high temperatures. A variety of types of cell cycle responses at

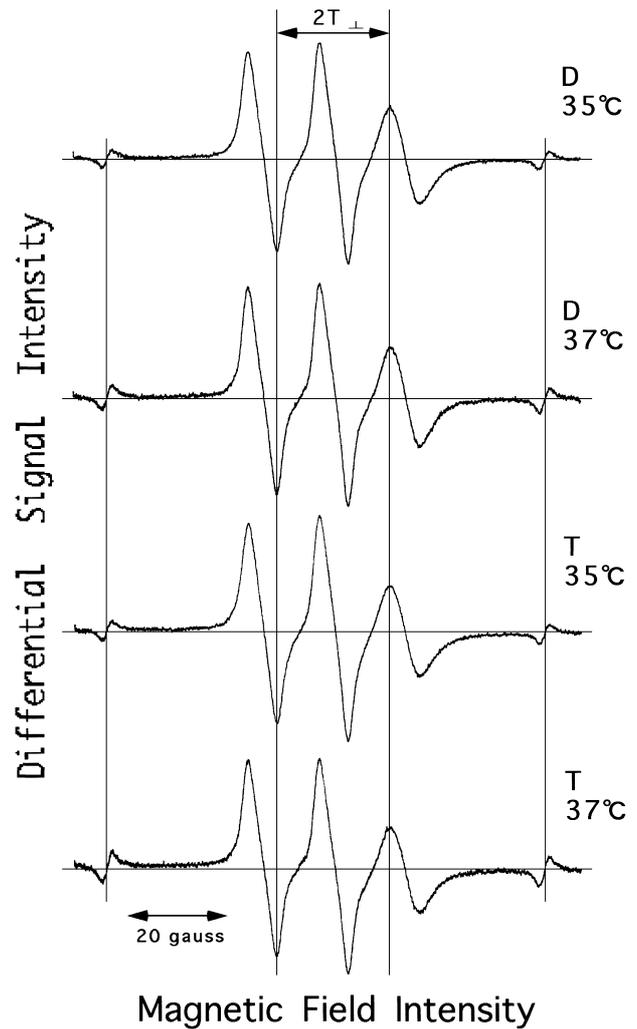


Fig. 5. Representative electron spin resonance (ESR) spectra of diploid (D) and tetraploid (T) Meth-A cells labeled with 16-doxylstearic acid. The temperature was increased from low to high. $2T_{\perp}$ represents the inner hyperfine splitting. Small signals on both sides originate from the manganese marker. Broken lines were drawn to facilitate understanding.

high temperatures were reported, such as G₁/G₀ (Tanonaka *et al.*, 1986) and G₂/M (Okuda *et al.*, 1986) accumulations. Diploid and tetraploid Meth-A cells responded as accumulation in the late S phases at high temperatures, meaning a low transit rate in the late S phase (Rice *et al.*, 1986). At low temperatures, a hyperploid cell population appears in the tetraploid cell line with growth arrest at the G₂/M phase, which differed from the response at high temperatures. The slowed progression of the cell cycle in the G₂/M phase may lead to a spontaneous transformation into a hyperploid cell cycle (Fujikawa-Yamamoto *et al.*, 1997).

We emphasize that the results of this study on the temperature dependency of tetraploid Meth-A cells are due to intracellular variations of the cells cultured under specific growth conditions.

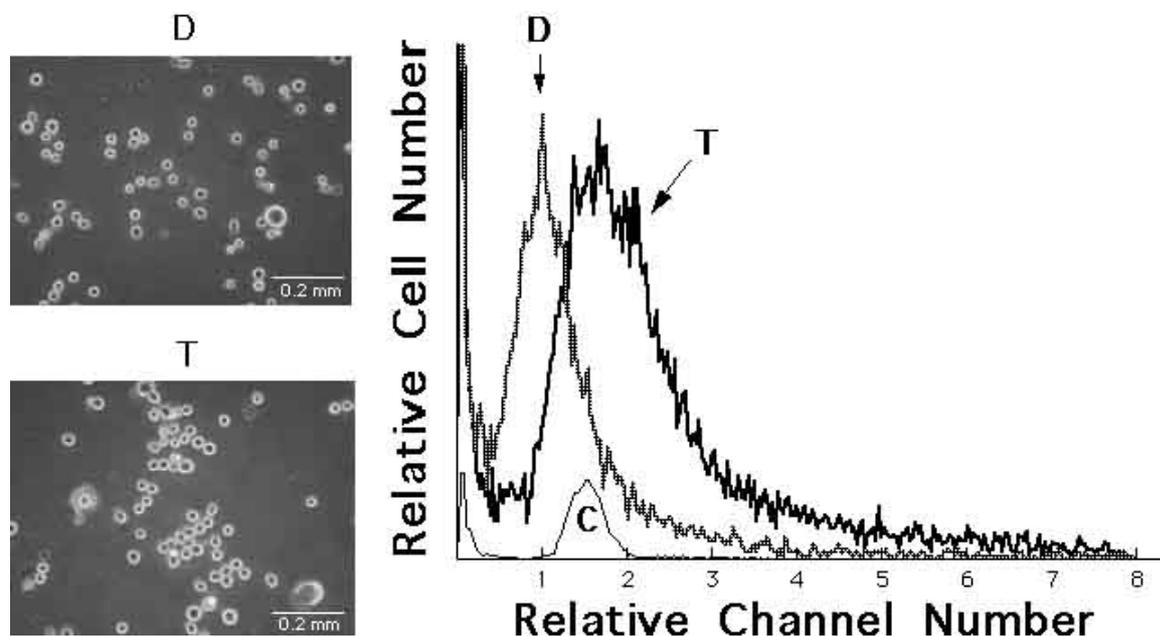


Fig. 6. Phase-contrast microphotographs (left panel) and volume distribution (right panel) of diploid (D) and tetraploid (T) Meth-A cells. For volume measurements, exponentially growing diploid and tetraploid Meth-A cells were fixed with ethanol and resuspended in PBS⁻. The cell volume (Coulter volume) was measured with a Coulter counter. C is of latex sphere with a diameter of 9.8 μ m.

Table II. HYPERFINE SPLITTING, T_{\perp} (GAUSS), AND THE ORDER PARAMETERS, $S(T_{\perp})$, OF DIPOID AND TETRAPLOID METH-A CELLS^{a)}

	Diploid Meth-A		Tetraploid Meth-A	
	$2T_{\perp}$ ^{b)}	$S(T_{\perp})$	$2T_{\perp}$ ^{b)}	$S(T_{\perp})$
25°C	22.3	0.3859	22.4	0.3775
30°C	22.4	0.3775	22.7	0.3635
35°C	22.5	0.3719	22.9	0.3523
37°C	23.0	0.3439	23.4	0.3214
40°C	23.5	0.3158	23.5	0.3158

^{a)} Results from a series of measurements. $S(T_{\perp})$ was calculated from the inner hyperfine splitting (T_{\perp}) of the ESR spectra by the following equation; $S(T_{\perp}) = 1.723(43.7 - 3T_{\perp})/46.1$ (Gaffney, 1975).

^{b)} The experimental errors were within 0.2 gauss.

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