Isolation of Temperature-sensitive Cell Cycle Mutants from Mouse
FM3A Cells

CHARACTERIZATION OF MUTANTS WITH SPECIAL REFERENCE TO DNA REPLICATION*

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A large number of mutants that are temperature sensitive (ts) for growth have been isolated from mouse mammary carcinoma FM3A cells by an improved selection method consisting of cell synchronization and short exposures to restrictive temperature. The improved method increased the efficiency of isolating DNA ts mutants, which showed a rapid decrease in DNA-synthesizing ability after temperature shift-up. Sixteen mutants isolated by this and other methods were selected for this study. Flow microfluorometric analysis of these mutants cultured at a nonpermissive temperature (39°C) for 16 h indicated that five clones were arrested in the G1 to S phase of the cell cycle, six clones were in the S to G2 phase, and two clones were arrested in the G2 phase. The remaining three clones exhibited 8C DNA content after incubation at 39°C for 28 h, indicating defects in mitosis or cytokinesis. These mutants were classified into 11 complementation groups. All the mutants except for those arrested in the G2 phase and those exhibiting defects in mitosis or cytokinesis showed a rapid decrease in DNA synthesis after temperature shift-up without a decrease in RNA and protein synthesis. The polyomavirus DNA cell-free replication system, which consists of polyomavirus large tumor antigen and mouse cell extracts, was used for further characterization of these DNA ts mutants. Among these ts mutants, only the tsFT20 strain, which contains heat-labile DNA polymerase α, was unable to support the polyomavirus DNA replication. Analysis by DNA fiber autoradiography revealed that DNA chain elongation rates of these DNA ts mutants were not changed and that the initiation of DNA replication at the origin of replication was impaired in the mutant cells.

Temperature-sensitive (ts) mutants have provided a powerful tool for the analysis of the complex processes in the cell cycle including DNA replication. It is well known that in prokaryotic cell systems, especially in Escherichia coli, ts mutants related to DNA replication (DNA ts mutants) have contributed greatly to the identification of replication enzymes and proteins as well as the understanding of the molecular mechanism of DNA replication (1–3).

As for lower eukaryotic cells, many cell division cycle (cdc) ts mutants have been isolated from Saccharomyces cerevisiae or Schizosaccharomyces pombe and characterized to clarify the molecular mechanism of the progression of cell cycle (4, 5). With several cdc ts mutants of yeast cells, the ts proteins were identified as the proteins required for DNA replication. These mutants and cell-free DNA replication systems using a plasmid DNA containing an autonomously replicating sequence (6) will provide valuable tools for understanding the molecular mechanism of DNA replication in yeast cells.

Many cdc ts mutants have been isolated up to the present from various cultured mammalian cell lines (7–10). Although those cdc ts mutants exhibit DNA ts phenotype, only a small number of DNA ts mutants have been proved to have ts protein, which is required for DNA replication (11–13).

To understand the mechanism that controls the cell division cycle of mammalian cells, we have isolated many cdc ts mutants from mouse FM3A cells (12, 14–17) and characterized the properties of some of the mutants such as ts85 (15, 18–21), tsFT20 (12, 22–26), tsFT101 (16), and tsFT210 (17). ts85 cells, which have been initially characterized as a G2 mutant (15) and whose uH2A protein disappears at the restrictive temperature (21), have been proved to have a thermolabile ubiquitin-activating enzyme (E1) (27, 28). tsFT20 cells were isolated as DNA ts mutants and were revealed to have thermolabile DNA polymerase α. tsFT101 cells are defective in cytokinesis and consequently form multinuclei. tsFT210 cells are arrested in the G2 phase, exhibiting a defect in hyperphosphorylation of histone H1.

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1 The abbreviations used are: ts, temperature-sensitive; cdc, cell division cycle; CMF-PBS, calcium- and magnesium-free phosphate buffered saline; FdUrd, 5-fluoro-2'-deoxuryridine; PyV, mouse polyomavirus; T antigen, large tumor antigen; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MEM, Eagle’s minimal essential medium.
In this study, we have improved the selection method for isolating DNA ts mutants and have isolated many of them. These mutants were characterized with a newly developed method, a polyomavirus DNA cell-free replication system in which all of the replication factors except for polyomavirus-encoded large tumor antigen were supplied from mouse cell extracts (29), as well as with the methods used in the previous studies such as flow microfluorometry and DNA fiber autoradiography.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—All ts mutants used in this study were isolated from FM3A clone 28 cells (wild type), which were originally established from a spontaneous mammary carcinoma in a C3H/He mouse (30), and maintained in suspension culture in RPMI 1640 medium supplemented with 10% calf serum, 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G potassium. Cells were cultured at 33 °C (permissive temperature) or at 39 °C (restrictive temperature). The cells used in these experiments were free of Mycoplasma contamination.

**Isolation of ts Mutants and Improvement of Selection Method**—A typical isolation procedure is as follows. Exponentially growing FM3A cells (5 × 10^6) were inoculated in a culture flask with 45 ml of the growth medium and cultured for 1 day at 33 °C. 1-Methyl-β-nitro-β-nitroso-2-nitrobenzene (MNNG, Aldrich) was added to the culture at a final concentration of 0.7 μg/ml, and then the cells were incubated for 3 h. The cells were washed three times with ice-cold Ca^2+ and Mg^2+-free phosphate-buffered saline (CMF-PBS) by centrifugation at 1,400 rpm for 5 min. Under these conditions of mutagenization, the surviving fraction was reduced to approximately 10%. Mutagenized cells were further incubated for 3 days to fix mutations. The cells were washed twice with CMF-PBS. Approximately 1 × 10^7 cells were inoculated with 80 ml of the growth medium to be incubated for 1 day and shifted up to 39 °C. The cells were incubated for 2 h to decrease the DNA-synthesizing ability of DNA ts mutants and added to [3H]dTd at a final concentration of 5 μCi/ml and further incubated at the same temperature for 16 h. The cells were washed with ice-cold CMF-PBS containing 1 × 10^−6 M nonlabeled dThd and cultured at 33 °C for 3 days in the growth medium containing 1 × 10^−3 M dThd. The cells were treated once more with [3H]dTd according to the procedure described above. The cells treated twice with [3H]dTd were cultured in HAT medium (the medium containing 1 × 10^−3 M hypoxanthine, 1 × 10^−6 M amethopterin, and 5 × 10^−3 M dThd) for 3 days at 33 °C to kill thymidine kinase-negative mutants, and then cultured for 1 day in HT medium (HAT minus amethopterin). The cells were diluted with the growth medium containing 1 × 10^−6 M dThd to 1 × 10^−1-1 × 10^−6 M. One volume of the diluted cell suspension was mixed with 2 volumes of 0.5% agar solution in the growth medium containing 5 ml of the HAT medium, and was poured on 0.5% underlayer agar in a glass dish (inner diameter of 60 mm). Fifty plates were further incubated for 2 weeks at 33 °C in a CO_2 incubator. More than 10^4 clones were picked up with bamboo sticks and inoculated on replica plates (50 clones/plate). After incubation for 1 day at 33 °C, each of the replica plates was shifted to 39 °C. The plates were further incubated for 10 days. Putative ts mutants were picked up and inoculated in 24-well multiplates. The improved method is essentially the same as the method described above except that mutagenized cells were cultured for 13 h with 3 × 10^−6 M FdUrd in the growth medium containing 10% dialyzed calf serum before temperature shift-up and at 2 h after the temperature shift-up. FdUrd was washed out. In addition, the length of incubation time with [3H]dTd at the restrictive temperature was shortened from 16 to 4-6 h.

**Measurements of Macromolecular Synthesis**—Exponentially growing cells (1 × 10^6) were inoculated with the growth medium containing 1 × 10^−6 M dThd and 10 mM HEPES buffer, pH 7.5, and incubated for 1 day at 33 °C. The cells were labeled with [3H]dTd (50 Ci/mmol) or [3H]FUDR (25 Ci/mmol) for 30 min after various lengths of incubation at 30 °C. Protein synthesis was measured by the incorporation of [3H]leucine (2 μCi/ml, 138 Ci/mmol) for 30 min into the cells cultured in the leucine-free RPMI 1640, which was added with 0.03% dialyzed bovine serum albumin and 0.1% dialyzed calf serum. After being pulse-labeled, cells were lysed, and acid-insoluble materials were collected on a Whatman glass-fiber filter (GF/C) as described previously (18).

**Cell Cycle Analysis by Flow Microfluorometry**—Cells (2-3 × 10^6) were fixed with 90% (v/v) ethanol. After washing once with ice-cold CMF-PBS, the fixed cells were treated with 300 units/ml ribonuclease A (from bovine pancreas, Sigma) in CMF-PBS for 45 min at 37 °C and then washed with CMF-PBS. The cells were treated with 280 units/ml pepsin (from porcine stomach mucosa, Sigma) in 2% (w/v) HCI for 15 min at 37 °C. Pepsin treatment was omitted in the analysis of ts mutants that formed multinuclei or micronuclei. Cells were then washed with CMF-PBS and stained with 20 μg/ml ethidium bromide. Approximately 2 × 10^6 cells were analyzed with a flow cytofluorometer (cytowej Floograph, Ortho Laboratories).

**Preparation of Cell Extracts**—Approximately 4 × 10^8 cells cultured at 33 °C for the indicated times were washed twice with ice-cold CMF-PBS and suspended in 10 mM HEPES buffer, pH 7.5, 5 mM dithiothreitol, and 0.25 mM phenylmethylsulfonyl fluoride (PMSF). The cell suspension was centrifuged at 1,500 rpm for 5 min, and the precipitate was resuspended in an equal volume of the hypertonic buffer, stood at 0 °C for 10 min, and was homogenized with 25 strokes in a Potter-Elvehjem-type Teflon-glass homogenizer. The homogenate was added to 5 mM NAC (cytofluorograph, Ortho Laboratories). The final concentration of NAC 0.2 mM and centrifuged at 20,000 rpm for 20 min in a Beckman TLA 100.2 rotor at 2 °C. The supernatant was dialyzed against buffer A (20 mM Hepes/KOH, pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 0.25 mM phenylmethylsulfonyl fluoride) and centrifuged at 55,000 rpm for 15 min in the same rotor. The supernatant containing approximately 20 mg of protein/ml was stored at −80 °C until use. A 20% (v/v) cell extract was prepared as described previously (23).

**Assay of PyV DNA Replication in Cell-free System**—The improved method is essentially the same as the method described previously (18). The improved method is essentially the same as the method described previously (18). The improved method is essentially the same as the method described previously (18). The improved method is essentially the same as the method described previously (18).
RESULTS

Improvement of Selection Method for the Isolation of DNA Temperature-sensitive Mutants—Temperature-sensitive mutants in DNA replication have been isolated with a variety of selective agents. The selection method depends on the arrest of DNA synthesis of ts mutants in the mass population after up-shift to a restrictive temperature. Although longer incubation with [3H]dThd kills more wild-type cells, prolonged incubation at the restrictive temperature also reduces the viability of ts mutants. For example, 95% of exponentially growing wild-type cells were killed by the incubation of the cells with [3H]dThd for 16 h at 39°C (standard selection condition), and only 10% of tsFT173 cells (whose characteristics will be described in this paper) survived after a 16-h incubation at 39°C even in the absence of [3H]dThd (data not shown). Thus, we tried to shorten the incubation time at 39°C without decreasing the efficiency to kill wild-type cells.

Cells were synchronized at the G1/S boundary by the treatment of FdUrd for 13 h at 33°C and then incubated for 2 h at 39°C to arrest DNA synthesis of DNA ts mutants. After release from the FdUrd block, the cells were further incubated for 4 h at 39°C with [3H]dThd. The viability of thus treated wild-type cells was decreased to less than 0.5%. The above procedure indeed increased the efficiency of isolating DNA ts mutants. Table I shows the results obtained by the previous method and by the improved method. The improved selection method enriched ts mutants 6.5-fold more than the previous one. In addition, 13 clones isolated by the improved method lost their ability to synthesize DNA more rapidly after temperature up-shift than did the two clones that were isolated by the previous method (Table II).

Characterization of Phenotypic Properties of ts Mutants—We selected the ts mutants that exhibited clear temperature sensitivity for growth or apparent phenotypes from over 200 ts mutants isolated by the improved method. These mutants were characterized by their properties together with the mutants, which were isolated by other selection methods, some properties of which had been already characterized, such as tsFT20, ts85, tsFT101, and tsFT210 (12, 15-26).

Fig. 1 shows the growth curves of the ts mutants and wild-type cells cultured at the restrictive temperature. Almost all the mutants showed clear temperature sensitivity for growth, especially tsFT5, tsFT20, tsFT21, ts85, tsFT101, tsFT144, tsFT173 and tsFT210. They either formed no colony in a soft agar at 39°C or formed microcolonies only.

Arresting points of the mutants in the cell cycle were examined by flow microfluorometry. Fig. 2 shows the distribution pattern of individual cells of each mutant in the cell cycle after an incubation at 39°C for 16 h (approximately one doubling time). Five clones (tsFT20 (B), tsFT48 (H), tsFT101 (I), tsFT111 (J), and tsFT117 (K)) were arrested at the G1/S boundary and in the S phase, and two clones (tsFT210 (M) and tsFT666 (data not shown)) were in the G2 phase. Six other clones (tsFT5 (C), tsFT21 (D), ts85 (E), tsFT144 (F), tsFT173 (G), and tsFT141 (L)) were arrested mainly in the S to G2 phase.

Table I Comparison of selection methods

<table>
<thead>
<tr>
<th>Selection method</th>
<th>No. of colonies examined (A)</th>
<th>No. of ts mutants (B)</th>
<th>R/A x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsynchronized selection</td>
<td>1176</td>
<td>5</td>
<td>0.43</td>
</tr>
<tr>
<td>Synchronized selection</td>
<td>1580</td>
<td>44</td>
<td>2.76</td>
</tr>
</tbody>
</table>

Table II Temperature sensitivity of DNA-synthesizing ability of ts mutants isolated by two selection methods

<table>
<thead>
<tr>
<th>Selection method</th>
<th>Level of DNA-synthesizing ability*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-40%</td>
</tr>
<tr>
<td>Nonsynchronized selection</td>
<td>0</td>
</tr>
<tr>
<td>Synchronized selection</td>
<td>2</td>
</tr>
</tbody>
</table>

* The incorporation of [3H]dThd for 30 min by the cells cultured at 39°C for 5 h was compared with that of the same cells cultured at 33°C (incorporation at 39°C/incorporation at 33°C x 100).
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FIG. 2. Flow microfluorometric analysis of ts mutants (I). Exponentially growing cultures of ts mutants and wild-type cells were shifted to 39 °C and cultured at the same temperature for 16 h. The cells were fixed and processed as described under “Experimental Procedures.” 2C and 4C in the figure indicate the amount of DNA of the cells in the G1 phase and that of the cells in the G2/M phase, respectively. The figures indicate the distribution pattern of wild-type cells (A), tsFT20 (B), tsFT5 (C), tsFT21 (D), ts85 (E), tsFT144 (F), tsFT173 (G), tsFT48 (H), tsFT107 (I), tsFT111 (J), tsFT117 (K), tsFT141 (L), and tsFT210 (M) cells.

requires one virus-encoded protein, large tumor antigen, and other proteins derived from host mouse cells. Thus, by using this system, a ts protein necessary for cellular DNA replication might be more readily identified if the protein were used in viral DNA replication as well.

Mutant and wild-type cells were cultured at 39 °C for the indicated times, and then cell extracts were prepared at each time point. Fig. 5 shows the ability of cell extracts to support PyV DNA replication (closed circles) as well as the levels of cellular DNA synthesis when the extracts were prepared (open circles). As expected, the extract from tsFT20 cells that have ts DNA polymerase α lost its ability to support PyV DNA replication, corresponding closely with the decrease in cellular DNA synthesis. On the other hand, the extracts from other mutants supported PyV DNA replication even after the mutant cells lost their ability to synthesize their own DNA, indicating that these mutants had no defect in the proteins required for PyV DNA replication.

Complementation Analysis—Pairwise complementation analysis among the 16 ts mutants were performed as described under “Experimental Procedures.” Under these conditions, 20-90 colonies appeared/1 × 10^3 inoculated cells in the case of positive complementation, and no colony appeared from the same number of cells in the negative complementation. Table III summarizes the results of the complementation analysis and phenotypic properties of these ts mutants. The 16 ts mutants were classified into 11 complementation groups. Five clones (tsFT5, tsFT21, ts85, tsFT144, and tsFT173), which had been isolated in independent experiments belonged to the same complementation group. TsFT210 and tsFT666 belonged to one group. Other ts mutants were revealed to be genetically independent of each other.

Analysis of DNA Synthesis in DNA ts Mutants by Autoradiography and DNA Fiber Autoradiography—Nine mutants exhibiting clear temperature sensitivity in DNA synthesis, which belong to seven independent complementation groups (tsFT21, ts85, and tsFT173 belong to the same group), were chosen for further analysis. The mutant cells were examined in the change of labeling index, which is defined as the percentage of labeled nuclei, during incubation at the restrictive temperature (Fig. 6). The labeling indexes of these mutants exhibited little decrease during the incubation up to 8 h.

In contrast to the labeling index, the number of silver grains/nucleus, which represents the amount of synthesized DNA in an individual nucleus, decreased greatly after temperature shift-up (data not shown).

We next performed DNA fiber autoradiography in order to know whether the decrease in the grain number in the individual nucleus is due to the decrease in the rate of DNA chain elongation or to the decrease in the frequency of the initiation of DNA replication at the origin of replicons. Mutant cells were cultured for 4 or 6 h at 39 °C and were then pulse labeled with [3H]dThd for 10, 20, or 30 min. As shown in Fig. 7, a linear relationship was observed between the duration of labeling time and the mean of track lengths obtained from distribution histograms of track length. From the slopes in the figure, DNA chain elongation rates were estimated to be 0.83 ± 0.05 μm/min for tsFT20, 0.62 ± 0.03 for tsFT21, 0.83 ± 0.02 for tsFT48, 0.75 ± 0.01 for tsFT107, 0.75 ± 0.01 for tsFT111, 0.80 ± 0.01 for tsFT117, 0.61 ± 0.01 for tsFT141, 0.76 ± 0.01 for tsFT173, and 0.70 ± 0.02 for wild-type cells. These values do not so much differ from those of the mutant cells cultured at 33 °C.

Fig. 8 shows distribution histograms of the center-to-center
Characterization of Mouse cdc ts Mutants

FIG. 4. Macromolecular synthesis in ts mutants after temperature shift-up. Cultures of each cell line were incubated at 39 °C for the indicated periods, and a portion of the culture was taken and pulse labeled with [3H]dThd (O), [3H]Urd (△), or [3H]leucine (□) for 30 min at the indicated times as described under "Experimental Procedures." The values are expressed as the percentage relative to each initial level.

distance observed in the mutant and wild-type cells cultured at 39 °C for 4 or 6 h. Because the center-to-center distance indicated the distance between initiation sites of DNA replication along DNA, the larger value indicates lower frequency of initiation. Similar distribution histograms were obtained between the mutant strains and wild-type cells at 33 °C (data not shown). On the other hand, the distribution histograms of the mutant cells cultured at 39 °C were quite different from that of wild-type cells. The distribution of center-to-center distances became broader, and the arithmetic means of the center-to-center distances became larger after temperature shift-up, indicating that the initiation frequency/unit length of DNA at a given time was reduced in the mutant cells. The levels of [3H]dThd incorporation in these mutants were 20% (tsFT20), 62% (tsFT21), 48% (tsFT48), 46% (tsFT107), 56% (tsFT111), 49% (tsFT117), 52% (tsFT141), 41% (tsFT173), and 125% (wild-type cells) as compared with the incorporation of each cell strain just before temperature shift-up.

FIG. 5. Measurement of polyomavirus DNA-replicating activity of mutant cell extracts. Cells were cultured at 39 °C for the indicated periods, and a portion of the culture was taken and pulse labeled with [3H]dThd for 30 min to measure DNA-synthesizing ability (O—O). Cell extracts were prepared from the rest of the culture and assayed with the polyomavirus DNA replication system as described under "Experimental Procedures" (△—△). The values are expressed as the percentage relative to each initial level.

DISCUSSION

We have improved the method for the selection of ts mutants and have isolated many mutants that show rapid decrease in DNA-synthesizing ability after temperature shift-up. For example, eight mutant clones exhibited DNA-synthesizing ability less than 20% of the initial level after incubation of the restrictive temperature for 8 h (Fig. 4). Although previous mutant isolations from various mammalian cell lines have resulted in several collections of cell division cycle ts mutants (7–10), there are few ts mutants that are proved to be defective in DNA replication. Furthermore, most DNA ts mutants reported previously retained their DNA-synthesizing ability by more than 50% of the initial level after incubation of the restrictive temperature for 8 h, exhibiting a leaky temperature-sensitive phenotype for DNA synthesis. A possible reason for the inefficiency in isolating good DNA ts mutants is that rare DNA ts mutant cells are killed by the
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Table III

Genetical complementation groups of cell division cycle ts mutants isolated from mouse FM3A cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Phenotype</th>
<th>ts Protein</th>
<th>Arresting point</th>
<th>Refs.</th>
</tr>
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<tr>
<td>A</td>
<td>tsFT20</td>
<td>DNA ts</td>
<td>DNA polymerase α</td>
<td>G1/S-S</td>
<td>12, 22-26</td>
</tr>
<tr>
<td>B</td>
<td>tsFT5</td>
<td>DNA ts</td>
<td>Ubiquitin-activating enzyme (E1)</td>
<td>S-G2</td>
<td>15, 21, 27, 28</td>
</tr>
<tr>
<td></td>
<td>tsFT21</td>
<td>Disappearance of uH2A protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ts85</td>
<td>DNA ts</td>
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</tr>
<tr>
<td></td>
<td>tsFT144</td>
<td>DNA ts</td>
<td></td>
<td></td>
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<td></td>
<td>tsFT173</td>
<td>DNA ts</td>
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<td>C</td>
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<td>DNA ts</td>
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<tr>
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<td>tsFT101</td>
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<td>E</td>
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<td>F</td>
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<td>H</td>
<td>tsFT50</td>
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<td>M*</td>
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<td>K</td>
<td>tsFT210</td>
<td>Defect in hyperphosphorylation</td>
<td>Protein kinase related to G2</td>
<td>G2</td>
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<tr>
<td></td>
<td>tsFT666</td>
<td>DNA ts</td>
<td>Unknown</td>
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</table>

* These cells were not arrested in the M phase but were defective in M phase function.

Eleven mutants exhibiting apparent DNA ts phenotype were classified into seven complementation groups (Table III). The complementation group of tsFT20, which has a heat labile DNA polymerase α, includes this strain only. It must be noted that many to mutants (tsFT5, tsFT21, tsFT144, and tsFT173) belong to a single complementation group including ts85 cells that have a heat-labile ubiquitin-activating enzyme, E1, as reported by Varshavsky and co-workers (27, 28). Thus, the E1 locus of FM3A cells is very sensitive to somatic mutation. Nishimoto and colleagues (38) reported that approximately 50% of their collection of baby hamster kidney
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Polyomavirus DNA replication system... 

Figure 8. Histograms of center-to-center distance. Cells were cultured at 39°C as described under Fig. 7 and then pulse labeled with 250 μCi/ml [3H]dThd (115 Ci/mmol) for 20 min and chased with [3H]dThd (115 Ci/mmol) for 80 min by the addition of nonradioactive dThd. The labeling of cells, the preparation of samples for DNA fiber autoradiography, and the measurement of center-to-center distances were performed as described previously (23).

cell ts mutants were members of a single complementation group, which was corrected by the introduction of the wild-type X chromosome. Then, the E1 locus may locate on the X chromosome or functionally hemizygous regions of autosomes. Our preliminary experiment indicated that the ts phenotype of ts85 cells is complemented by the human X chromosome.

It has been proven in prokaryotic systems that the cell-free DNA replication system of phage DNA is a powerful tool in understanding the molecular mechanism of DNA replication and characterizing DNA ts mutants. The cell-free system for polyomavirus DNA replication, which we have established (29), may serve as a mammalian counterpart to the prokaryotic cell-free system. This system requires polyomavirus large tumor antigen and mouse cell extracts as a source of DNA replication proteins. By the analogy to the cell-free system for simian virus 40 DNA replication, it is expected that the polyomavirus DNA replication system requires DNA polymerase α, DNA primase, DNA topoisomerases I and II, the single-stranded DNA-binding proteins, and other unidentified proteins. If DNA ts mutant cells have a defect in these proteins, the extract prepared from the cells cultured at the restrictive temperature will show defects in the cell-free system for polyomavirus DNA replication. As expected, the extract from tsFT20 cells cultured at the restrictive temperature did not support the polyomavirus DNA replication, and DNA-replicating activity was restored by the addition of DNA polymerase α-primase purified from wild-type cells (data not shown). Although other mutants examined in this experiment do not show defects in the polyomavirus DNA replication, several DNA ts mutants that do not support intracellular polyomavirus DNA replication have been isolated from mouse Balb/c ST3 (39, 40) and Chinese hamster cells (38). Thus, the cell-free system for polyomavirus DNA replication may facilitate the characterization of such mutants and the identification of ts proteins.

ts85 cells were initially characterized as G2 mutants. However, precise analysis of this mutant cell revealed that the cells were arrested also in the S phase, and the initiation of replicons was impaired in these cells. In addition, the decrease in DNA synthesis correlated well with the decrease in the amount of uH2A protein. uH2A protein is observed to be enriched in transcriptionally active chromatin regions (41) and to disappear in the M phase (42). Thus, these observations seem to suggest that uH2A protein maintains chromatin structure to permit the initiation of replicons. Although the possibility cannot be excluded that another unknown protein, which is activated by the conjugation with ubiquitin, is required for the initiation of replication at origins of replicons, the mutants that belong to the ts85 group may become a very valuable tool for the analysis of the chromatin structure required for the initiation of replicons.

Two types of DNA ts mutants are known in E. coli with respect to cessation of DNA synthesis when the temperature condition is shifted to restrictive (2). The elongation mutants, which have defects in the proteins required for DNA chain elongation such as dna G products, quickly stop or slow down DNA synthesis. The initiation mutants such as the dna A mutant sustain synthesis until completion of the ongoing round of replication.

All the mutants examined in this study were phenotypically initiation mutants (Figs. 7 and 8). Several possibilities can be considered for the above observation. First, DNA replication in mammalian cell may be catalyzed by a multienzyme complex (43), and once accommodated into DNA replication machinery, the ts enzyme required for DNA chain elongation retain its stability or are protected from temperature inactivation.

Second, proteins or the modification of proteins required to change the chromatin structure to permit the initiation of replication are temperature sensitive in some of these ts mutants as the ts85 group.

Third, some of these ts mutants are truly the initiation mutants in which proteins required for the initiation of DNA replication are temperature sensitive. The last possibility can be confirmed by developing a cell-free DNA replication system containing a mammalian DNA replication origin. We are now trying to clone mammalian DNA replication origins. The isolation of initiation mutants and the development of the cell-free DNA replication system will establish the molecular mechanism of the initiation of DNA replication in mammalian cells.

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Characterization of Mouse cdc ts Mutants

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