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A MAMMALIAN DNA$^-$ MUTANT DECREASING NUCLEAR DNA POLYMERASE α ACTIVITY AT NONPERMISSIVE TEMPERATURE

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SUMMARY

A temperature-sensitive Dna$^-$ mutant (ts T244) of the mouse FM3A cell line was characterized. ts T244 showed no increase in cell number and rapid decrease in the rate of DNA synthesis after temperature upshift (33°C→39°C). The activity of nuclear DNA polymerase α decreased as rapidly as the rate of DNA synthesis, whereas the activities of thymidine kinase and thymidylate kinase decreased more slowly. The results suggest that the level of nuclear DNA polymerase α has close relation to the ts defect of this Dna$^-$ mutant.

INTRODUCTION

Temperature-sensitive Dna$^-$ mutants have proved of great value in the analysis of the mechanism of DNA replication in microbial systems. In the past several years, a number of laboratories have isolated and characterized temperature-sensitive (ts) mutants in different mammalian cells in culture. The ts defects have been reported to exist in DNA synthesis in several cases [1-5], however, the precise location of the biochemical defect hasn't been determined.

We have previously reported that ts mutants can be isolated in the mouse FM3A cells [6]. In order to obtain a ts mutant which has a defect in a component of putative DNA replication complex, we have tried to isolate a mutant which shows rapid cessation of DNA synthesis after temperature upshift. We describe here the preliminary characterization of a FM3A ts mutant, designated ts T244, which is defective in DNA synthesis at nonpermissive temperature, and provide evidence that the reduction of the activity of nuclear DNA polymerase α is an early event in the defect of DNA synthesis.

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MATERIALS AND METHODS

Cell cultures: A parental strain in this study, EM-6TG cl.13 [7], is a derivative of C3H mouse FM3A cells selected for the resistance to 6-thioguanine after mutagenesis with ethylmethane sulfonate, and thus, has a hypoxanthine-guanine phosphoribosyl transferase minus ($HGPRT^-$) phenotype. Several ts mutants have been isolated from EM-6TG cl.13 cells by using the procedures described below. All the different cell types were propagated as suspension cultures in RPMI 1640 medium containing 10% calf serum.

Induction and isolation of ts mutants: EM-6TG cl.13 cells were mutagenized by 0.25μg/ml of MNNG (N-methyl-N'-nitro-N-nitrosoguanidine, Dai-ichi Chemical Co., Tokyo) for 16h at 33°C (permissive temperature). After mutagenesis, the cultures were resuspended with the fresh medium and incubated at 33°C for 2 days in order to allow the expression of the induced mutant phenotypes. Thereafter, the cultures were shifted up to 39°C (nonpermissive temperature) for about 2-16h, and then selected by adding arabinofuranosyl cytosine (araC, Sigma Chemical Co.) and/or 5-fluoro-2'-deoxyuridine (FUdR, Sigma Chemical Co.) as killing (selective) agents at 39°C for about 2 days. This selection cycle was repeated one or two more times. After selection, the remained cells were allowed to form colonies between the two layers (0.33% agar for upper layer, and 0.5% for lower one) of soft agar medium in a CO_2 incubator at 33°C. The individual colonies were isolated and were tested for their temperature sensitivity.

Measurements of macromolecular synthesis: The synthesis of DNA, RNA and proteins were measured by the incorporation of radioactive precursors, 0.5μCi/ml of [^{3}H]thymidine(5Ci/mmol), 1μCi/ml of [^{3}H]uridine (29Ci/mmol) and 1μCi/ml of [^{3}H]leucine (32Ci/mmol), into 10% TCA-insoluble materials. In the case of the incorporation of [^{3}H]uridine, 10μM of thymidine was added to minimize the incorporation of the precursor into DNA. Sodium azide at final concentration of 100μg/ml was added to stop the pulse-labeling reaction, and then the cells were lysed with 0.1% SDS solution. After 10min at room temperature, acid-insoluble materials were collected on glass fiber filters, and the radioactivity was counted in liquid scintillation spectrometer. To measure the incorporation of [^{3}H]leucine, the medium containing one-tenth of the normal concentration of leucine was used.

Assay of thymidine kinase and thymidylate kinase: Cells ($1\text{-}2\times10^7$) were homogenized by 15 strokes in a Potter-Elvehjem type Teflon-glass homogenizer with 1ml of 0.05M Tris-HCl (pH 8.0), containing 1mM 2-mercaptoethanol and 1mM Na_3EDTA. The homogenate was further subjected to ultrasonication for 1min, and stood at 0°C for 30-60min. The sonicated suspension was then centrifuged at 105,000xg at 4°C for 60min. The supernatant was used as a source of crude enzyme extract.

Thymidine kinase activity was measured by the procedure of Bresnick and Karjala [8] with slight modification. Each reaction mixture (50μl) consisted of 0.2M Tris-HCl (pH 8.0), 5mM ATP, 5mM $MgCl_2$, 1μCi/ml [^{3}H]thymidine (27Ci/mmol) and about 100μg of the extract protein. Mixtures were incubated at 33°C for 30min and terminated by chilling in an ice-water bath. Aliquots of the reaction mixture were applied to DEAE-cellulose paper disks (Whatman type DE81). These disks were washed immediately with 1mM ammonium formate for three times (about 10ml per disk) and then washed with water for 15min. By this procedure, [^{3}H]-thymidine was effectively removed whereas [^{3}H]TMP was retained on the disk. Finally the disk was immersed in 95% ethanol and dried at 80°C. The radioactivity was determined in liquid scintillation counter.

The reaction mixture for the assay of thymidylate kinase activity was identical to that used in the thymidine kinase assay as described above, except that 10μCi/ml [^{3}H]TMP (54Ci/mmol) was used as substrate instead of [^{3}H]thymidine. After incubation at 33°C for 30min, the reaction was stopped by adding 20μl of 50mM EDTA. DEAE-cellulose paper (25x20cm) was washed successively with 0.1M formic

acid, 0.5M ammonium formate, and water before use. Ten to twenty microliters of the reaction mixture were applied to the bottom of the dried paper. Mixtures of TMP, TDP and TTP were applied as markers with the samples. The paper chromatogram was developed with 0.01M formic acid until the solvent front reached almost to the top of the paper. The paper was dried, and then developed again with 0.06M ammonium formate to about two-thirds from the origin. The positions of TDP and TTP were identified under an ultraviolet lamp. These areas were cut off and the radioactivity was measured.

Assay of DNA polymerase: Nuclear and cytoplasmic fractions were obtained as described previously [9]. DNA polymerase was extracted from the nuclei with 0.3M KCl. Under this condition, most of DNA polymerase α was extracted, whereas negligible amount of β polymerase was extracted. The reaction mixture (150µl) of the assay of DNA polymerase contained 50mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 200µg/ml bovine serum albumin, 300µg/ml activated calf thymus DNA, 100µM dATP, dCTP, dGTP and 10µM [^{3}H]dTTP (0.5Ci/mmol). After 30min of the incubation at 33°C, the reaction mixture was placed in an ice-water bath and TCA-insoluble materials were collected on a glass fiber filter paper disk. The radioactivity was counted in liquid scintillation spectrometer. Activated calf thymus DNA was prepared according to Aposhian and Kornberg [10].

RESULTS AND DISCUSSION

Cell growth at permissive and nonpermissive temperature: A clone, designated ts T244, was isolated at 33°C after mutagenesis of C3H mouse FM3A EM-6TG cl.13 cells with MNNG and selection with araC and/or FUdR at 39°C, as described previously [6]. ts T244 had a slightly larger cell size, and a larger number of chromosomes (64-66 compared to 42) than the parental strain. Parental cells required approximately 20h for a doubling of cell number at 33°C, and 16h at 39°C. In contrast, ts T244 cells grew with a slightly longer doubling time (24h) at 33°C, whereas they exhibited drastically altered growth characteristics at 39°C. As shown in Fig. 1, there was almost no cell division after shifting the randomly growing cultures from 33°C to 39°C. At that time the viability of ts T244 cells at 39°C was no less than 70% as determined by the dye exclusion method.

Macromolecular synthesis at 33°C and 39°C: Fig. 2 shows the rate of incorporation of the radioactive precursors into TCA-precipitable macromolecules after a culture at 33°C was shifted to 39°C. There was a rapid decrease in the incorporation of [^{3}H]thymidine at 39°C. The rate was reduced to less than 10% of the initial value within 6h, whereas a slight increase in the rate of DNA synthesis at 39°C was observed with parental cells. On the other hand, no significant change in the incorporation of [^{3}H]uridine or [^{3}H]leucine into RNA or proteins

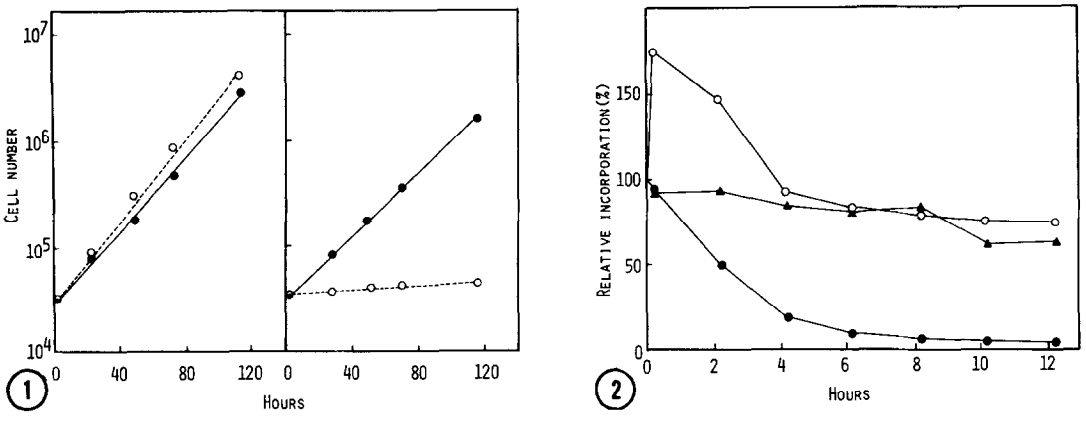

Fig. 1. *Growth curves of parental EM-6TG cl.13 cells and ts T244 cells.* EM-6TG cl.13 cells ($3x10^4$) and ts T244 cells ($3.4x10^4$) were inoculated in 30mm petri dishes at 0h and the cell number was counted with a hemocytometer.
left panel: EM-6TG cl.13, right panel: ts T244,
●——●: 33°C, o-----o: 39°C.

Fig. 2. *Macromolecular synthesis of ts T244.* Test tubes containing $1.5x10^5$ cells were incubated at 33°C for a day, and then half of the tubes were transferred from 33°C to 39°C. At various times, duplicate cultures were incubated for 30min with [^{3}H]thymidine (●——●), [^{3}H]uridine (o——o), and [^{3}H]leucine (▲——▲), and lysed. TCA-insoluble radioactivity was measured. The rates are expressed as the percentage of the incorporation at 39°C to that of controls maintained at 33°C.

respectively was found at 39°C up to 12h, except that [^{3}H]uridine incorporation increased rapidly immediately after the temperature upshift. ts T244 is termed a Dna$^-$ mutant because of the preferential reduction in the rate of DNA synthesis at 39°C.

Changes in the enzyme activities related to DNA synthesis: In order to determine the biochemical defect in DNA synthesis, we have examined several enzyme activities, which are involved in DNA synthesis. These enzymes are, DNA polymerase α which is a candidate for the replication enzyme [11], and thymidine kinase and thymidylate kinase, both are required for the synthesis of DNA precursors.

Crude cell extracts, or nuclear and cytoplasmic fractions, were prepared from the cells collected at 2h intervals after temperature upshift to 39°C, and from the control cultures (33°C). Gradual decrease in the activities of thymidine kinase and thymidylate kinase was observed in the cell extract from 39°C culture as compared to that of the control (Fig. 3). At 2h both activities had retained about 80-85% of their original activities. After 6h at 39°C, the activities were reduced to about one half of those at 33°C. Therefore the decrease of

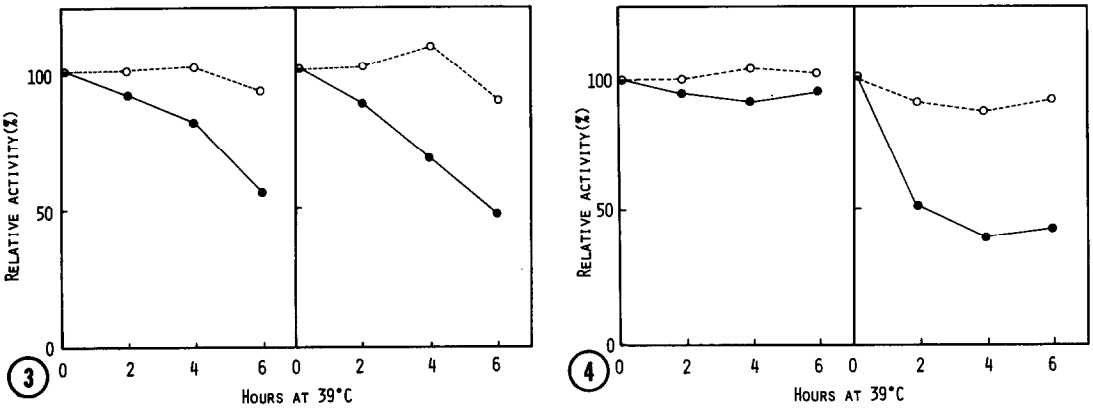

Fig. 3. *Changes in the activities of thymidine kinase and thymidylate kinase at 39°C.* EM-6TG cl.13 cells and ts T244 cells were incubated at 33°C for a day and then parts of the cultures were transferred from 33°C to 39°C. At various times, 1-2x10^7 cells were harvested, and thymidine kinase (left panel) and thymidylate kinase (right panel) activities were measured as described in Materials and Methods. Activities are expressed as the percentage of the activity at 39°C to that of controls maintained at 33°C.
o-----o: EM-6TG cl.13, ●———●: ts T244.

Fig. 4. *Changes in the activities of DNA polymerase α at 39°C.* EM-6TG cl.13 cells (left panel) and ts T244 cells (right panel) were harvested as described in the legend of Fig. 3. Cytoplasmic (o-----o) and nuclear (●———●) fractions were obtained and the activity of DNA polymerase α was measured at 33°C as described in Materials and Methods. The nuclear-cytoplasmic distribution of DNA polymerase α was about 2:3 both in parental EM-6TG cl.13 cells and ts T244 cells at 33°C. Relative activities are expressed as described in the legend of Fig. 3.

the activities of these enzymes is probably not the cause of the rapid decrease of the rate of DNA synthesis, but is the result of it.

Neither increase nor decrease in the activity of DNA polymerase α was noted in the cytoplasmic fraction, even after the culture was incubated at 39°C for 6h (Fig. 4). On the other hand, nuclear DNA polymerase α at 39°C was significantly reduced in the enzyme activity. It should be noted that the activity decreased to about 50% of the control within 2h. Considering that the activity in the assay system reflects the amount of the enzyme, the results suggest that the amount of nuclear DNA polymerase α decreased during the incubation at 39°C. The observed decrease in the activity of nuclear DNA polymerase α is coincident with the decrease of the rate of DNA synthesis (Fig. 2), suggesting that the reduction of the enzyme activity is, at least, one of the factors for the preferential inhibition of DNA synthesis, although the possibility that other replication proteins are also involved cannot be excluded. Such a coincidental decrease of the activity of

nuclear DNA polymerase α with the rate of DNA synthesis was not found in another Dna$^-$ mutant, designated ts T240, indicating that the phenomenon is rather specific to ts T244. Preliminary experiment shows that ts T244 neclear DNA polymerase α itself is not temperature sensitive (polymerase assay in Fig. 4 was performed at permissive temperature). Therefore the reduction of the activity of nuclear DNA polymerase α could be interpreted by three possibilities: (i) decay of *de novo* synthesis of nuclear DNA polymerase α, (ii) degradation of the enzyme, and (iii) appearance of an inhibitor of the enzyme. We are now going to test these possibilities.

Recently we reported that there are at least two forms of DNA polymerase α (P-I and P-II) with different degree of affinity to DNA in HeLa cells [12]. P-I is recovered from 0.3M KCl nuclear extract, and P-II from the cytoplasmic fraction. We also found that the variation in the activity of P-I, which possesses higher binding affinity to DNA, is correlated with the rate of DNA synthesis *in vivo* [13]. Studies on DNA polymerase α in ts T244 at permissive and nonpermissive temperatures could serve as a basis for more detailed elucidation of α polymerase in mammalian DNA replication, and the relationship between these multiple forms of this enzyme.

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