Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1761–1765, March 1985
Genetics

Characterization of a temperature-sensitive mutant of mouse FM3A cells defective in DNA replication

(DNA polymerase α/somatic cell genetics/*dna*^{ts}/cell cycle)

YASUFUMI MURAKAMI*, HIDEYO YASUDA, HIROSHI MIYAZAWA, FUMIO HANAOKA†, AND MASA-ATSU YAMADA

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Tokyo 113, Japan

Communicated by Theodore T. Puck, November 7, 1984

ABSTRACT **The characterization of a temperature-sensitive mutant (tsFT20 strain, *dna*^{ts}) of mouse FM3A cells is reported. After incubation of tsFT20 cells at the nonpermissive temperature (39°C), DNA synthesis ceased with little change in either RNA or protein synthesis. Flow-microfluorometric analysis revealed that the cell cycle of tsFT20 cells grown at 39°C for 16 hr was similar to that of wild-type cells that were synchronized at the G_1/S boundary and at S phase by treatment with aphidicolin, a specific inhibitor of DNA polymerase α. The DNA polymerase α activity of tsFT20 cells measured in crude cell extracts or in purified preparations was inactivated more rapidly at 39°C than the activity of wild-type cells. In the growth revertants of the tsFT20 cell strain, the heat lability of DNA polymerase α decreased. These data suggest that tsFT20 is a temperature-sensitive mutant of DNA polymerase α or of a factor associated with DNA polymerase α that is essential for its activity.**

Conditionally lethal mutants have proven essential in analyzing the events in the replication cycle of prokaryotic cells. In identifying the true replication enzyme in *Escherichia coli*, for example, the most crucial proof was the isolation of two clones, one markedly deficient in DNA polymerase I (1) and the other in which DNA polymerase III was temperature sensitive (2).

In the past 10 years, many temperature-sensitive mutants of cultured mammalian cells have been isolated (for review, see ref. 3). However, only a small number of these mutants have had mutations related to DNA replication (4–6), and the characterization of these mutants at the molecular level has been difficult. As a corollary, the elucidation of the molecular mechanism of mammalian DNA replication has not proceeded as far as the microbial one.

This report describes a temperature-sensitive mutant of mouse cells in which DNA synthesis largely ceases at the nonpermissive temperature. The temperature-sensitive clone, designated tsFT20, was found to have a heat-labile DNA polymerase α activity that became more heat stable with reversion of temperature-sensitive growth.

MATERIALS AND METHODS

Cell Culture. Mouse mammary carcinoma cell line FM3A clone 28 cells (7) were maintained in suspension culture in RPMI 1640 medium (Flow Laboratories) supplemented with 10% calf serum (GIBCO).

Assay of Macromolecular Synthesis Ability. Cells were inoculated at 1×10^5 cells per ml, incubated at 33°C for 1 day, and then labeled with [^{3}H]thymidine (1 μCi/ml, 24 Ci/mmol), [^{3}H]leucine (1 μCi/ml, 40 Ci/mmol), or [^{3}H]uridine (1 μCi/ml, 46 Ci/mmol) (1 Ci = 37 GBq). Tritiated compounds were purchased from Amersham. Acid-insoluble radioactivity was determined by the glass filter method and liquid scintillation counting (Aloka LSC-700).

Cell Cycle Analysis by Flow Microfluorometry. Cells in suspension culture were fixed with 90% (vol/vol) ethanol, treated with RNase A (60 units/mg, Sigma) at 0.5 mg/ml and 37°C for 1 hr and with pepsin (1:60,000, Sigma) at 0.1 mg/ml in 2% (wt/vol) HCl for 15 min at 37°C. Cells were then stained with ethidium bromide at 20 μg/ml in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. The stained cells were subjected to flow microfluorometry (Cytofluorograph, Ortho Instruments).

Isolation of the Revertant Clones from tsFT20 Cells. tsFT20 cells (8×10^7) were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 0.5 μg/ml for 24 hr and cultured at 33°C for 3 days without the mutagen. They were then plated in soft agar plates and incubated at 39°C for 2 weeks, and the resultant colonies were tested for growth potency at 39°C. All of the colonies tested showed slightly slower growth rate than wild-type cells. The frequency of the growth-revertant clones was about one clone per 1.5×10^5 mutagenized cells.

Preparation of Crude Cell Extract. FM3A cells stored at −80°C were thawed quickly, suspended in buffer A (20 mM Tris·HCl, pH 7.5/2 mM $MgCl_2$/2 mM 2-mercaptoethanol/0.5 mM Na_3EDTA/0.25 mM phenylmethanesulfonyl fluoride/10 mM $NaHSO_3$/2 μg of antipain per ml) at 2.5×10^8 cells per ml and kept at 0°C for 8 min and then homogenized in a loose-fitting Potter–Elvehjem type Teflon/glass homogenizer. The salt concentration of the homogenate was adjusted to 0.5 M KCl by the addition of ⅓ vol of 2 M KCl in buffer A. The suspension was allowed to stand at 4°C for 60 min, after which it was centrifuged at 105,000 × *g* for 60 min. The supernatant was designated the crude cell extract.

Partial Purification of DNA Polymerase α. From 10^9 each of tsFT20 and wild-type cells, crude cell extracts were prepared as described above. Both extracts were subjected to the following procedures individually. A solution containing 50 mM KCl in buffer A (0.8 vol) was added to the extract with stirring to make a final concentration of 0.3 M KCl. The extract was loaded onto a DEAE-cellulose (Whatman DE-52) column (20 ml; 1.2 × 18 cm) equilibrated with 0.3 M KCl in buffer A. The flow-through fraction (nucleic acid-free fraction) was dialyzed against 50 mM KCl in buffer B [buffer A plus 20% (vol/vol) ethylene glycol minus $MgCl_2$]. The dialysate was loaded on a second DEAE-cellulose column (15 ml; 1.2 × 13 cm) equilibrated with 50 mM KCl in buffer B. Proteins were eluted from the column with 8 bed volumes of a linear gradient of KCl from 50 mM to 0.4 M in buffer B. The active fractions eluting at about 180 mM KCl were pooled and dialyzed against 0.1 M KCl in buffer C (20 mM

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*Present address: Memorial Sloan-Kettering Cancer Center, New York, NY 10021.
†To whom correspondence and reprint requests should be addressed.

potassium phosphate, pH 7.5/0.1 mM Na_3EDTA/1 mM 2-mercaptoethanol/0.25 mM phenylmethanesulfonyl fluoride/0.01% Triton X-100/10 mM $NaHSO_3$/20% ethylene glycol/0.1% ethanol). The dialysate was chromatographed on a phosphocellulose (Whatman P11) column (4 ml; 1.2 × 3.5 cm) equilibrated with 0.1 M KCl in buffer C. The proteins bound to the column were eluted with 8 bed volumes of a linear gradient of KCl from 0.1 to 0.6 M KCl in buffer C. DNA polymerase α activity was eluted from the column at 0.3–0.4 M KCl.

Assay of DNA Polymerase Activity. The assay mixture (150 μl) contained 50 mM Tris·HCl at pH 7.5, 5 mM $MgCl_2$, 100 μM each of dATP, dCTP, and dGTP, 10 μM [^{3}H]dTTP (0.5 Ci/mmol), activated DNA (8) at 250 μg/ml, 2 mM 2-mercaptoethanol, and the extract from 6 × 10^3 cells or an aliquot of column fraction. The reaction mixture was preincubated without the 4 dNTPs and activated DNA when indicated. The reaction was started by immersing the assay tubes in 33°C water bath and was stopped by chilling the tubes and by the addition of 650 μl of chilled water. Acid-insoluble material was collected after addition of 200 μg of salmon sperm DNA (as carrier) and 5% trichloroacetic acid by filtration through glass fiber filters (Whatman, GF/C). The filters were washed successively with 5% trichloroacetic acid, ethanol, and acetone. Radioactivity was determined in a liquid scintillation spectrometer (Aloka LSC-700).

RESULTS

General Growth Properties. A temperature-sensitive clone, designated tsFT20, was isolated from mouse mammary carcinoma FM3A clone 28 cells after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 33°C and selection by the [^{3}H]thymidine suicide method, in which non-temperature-sensitive clones incorporated [^{3}H]thymidine at 39°C (9, 10). Fig. 1 shows the growth curves of wild-type and tsFT20 cells. At 33°C, both cells grew at the same rate, with a doubling time of 16 hr. Wild-type cells showed a higher growth rate at 39°C than at 33°C, while tsFT20 cells did not increase in cell number at 39°C. The number of viable cells gradually decreased in the culture of tsFT20, beginning 24 hr after the temperature shift from 33°C to 39°C.

Macromolecular Synthesis. Fig. 2 shows the rate of incorporation of radioactive precursors into acid-precipitable fractions after logarithmically growing cultures were shifted to 39°C. The incorporation of [^{3}H]thymidine decreased to 70% of the control at 39°C during the initial labeling period (0 to 30 min after the temperature shift), and to only 16% at 2 hr after the shift (Fig. 2*A*). In a more detailed kinetic analysis, the incorporation decreased to 70% and 50%, 30 min and 1 hr after the temperature shift, respectively (Fig. 2*B*). In contrast to the results with [^{3}H]thymidine, the incorporation of [^{3}H]uridine doubled after the temperature shift. This increase paralleled the behavior of wild-type cells, in which the incorporation rate of macromolecular precursors was higher at 39°C than at 33°C. This high incorporation rate continued for 8 hr after the temperature shift. The incorporation of [^{3}H]leucine began to decrease gradually 8 hr after the temperature shift.

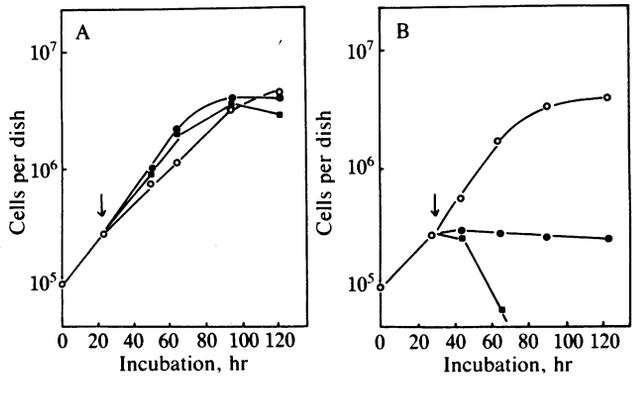

FIG. 1. Growth curves of wild-type cells (*A*) and tsFT20 cells (*B*). Cells (1 × 10^5) were inoculated into 30-mm Petri dishes with 2 ml of the growth medium and incubated at 33°C for 1 day. Half of the dishes were then incubated at 39°C and the total cell number was determined in duplicate cultures. Viability of the cells was determined by the dye exclusion test using 0.04% erythrosine. The arrow indicates the time of the temperature shift. ○, Total cell number at 33°C; ●, total cell number at 39°C; ■, viable cell number at 39°C.

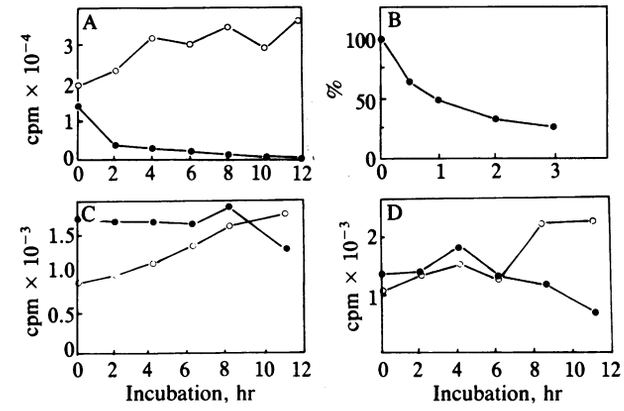

FIG. 2. Macromolecular synthesis in tsFT20 cells at 33°C and 39°C. Exponentially growing tsFT20 cells were labeled with [^{3}H]thymidine (*A*), [^{3}H]uridine (*C*), and [^{3}H]leucine (*D*) for 30 min at 2-hr intervals after temperature shift, as described in *Materials and Methods*. At 33°C, the cell number increased during this experiment. The values are shown per tube and not per cell number. (*A*, *C*, and *D*) Radioactivity incorporated at 33°C (○) and 39°C (●). (*B*) Exponentially growing tsFT20 cells were labeled with [^{3}H]thymidine for 10 min at 30-min intervals after temperature shift. The percentage of radioactivity incorporated at 39°C, relative to the initial value, is shown. Mean values of triplicate cultures are shown in all experiments.

Cell Cycle Analysis. Logarithmically growing tsFT20 cells were shifted from 33°C to 39°C and cultured for 16 hr. The cells were then fixed and analyzed by flow microfluorometry. As shown in Fig. 3*E*, the percentage of mutant cells with DNA content of tetraploid (G_2–M phase cells; 4*C* peak) decreased and cells with DNA content of diploid (G_1 phase cells; 2*C* peak) increased. The peak near the 2*C* region is skewed towards 4*C* and is not identical to the peak at 2*C* of cells obtained at 33°C. This pattern was similar to the pattern of cells that were synchronized at the G_1/S boundary and at S phase by treatment with aphidicolin, a specific inhibitor of DNA polymerase α, or excess thymidine (Fig. 3 *H* and *I*). In contrast, tsFT20 cells at 33°C and wild-type cells at both 33°C and 39°C showed distributions typical of exponentially growing cells (Fig. 3 *F*, *C*, and *B*, respectively). From these data, it appeared that tsFT20 cells at 39°C ceased cell cycle progression at the G_1/S boundary and S phase.

Heat Inactivation of DNA Polymerase α in Crude Cell Extracts. Since the flow-microfluorometry revealed that tsFT20 cells at 39°C showed a distribution pattern similar to that of wild-type cells treated with aphidicolin, we examined the temperature sensitivity of DNA polymerase α in the crude cell extracts of these cells. Crude cell extract was also prepared from one of the revertant clones of tsFT20, R4C, and the temperature sensitivity of its DNA polymerase α activity was examined. The relationship between the concentration of extract and DNA polymerase activity was determined at 33°C (Fig. 4*A*). Judging from aphidicolin and *N*-ethylmaleimide sensitivity, more than 98% and 94% of the activity, in wild-type and tsFT20 cells, respectively, was due to DNA polymerase α. Under these conditions, the reaction

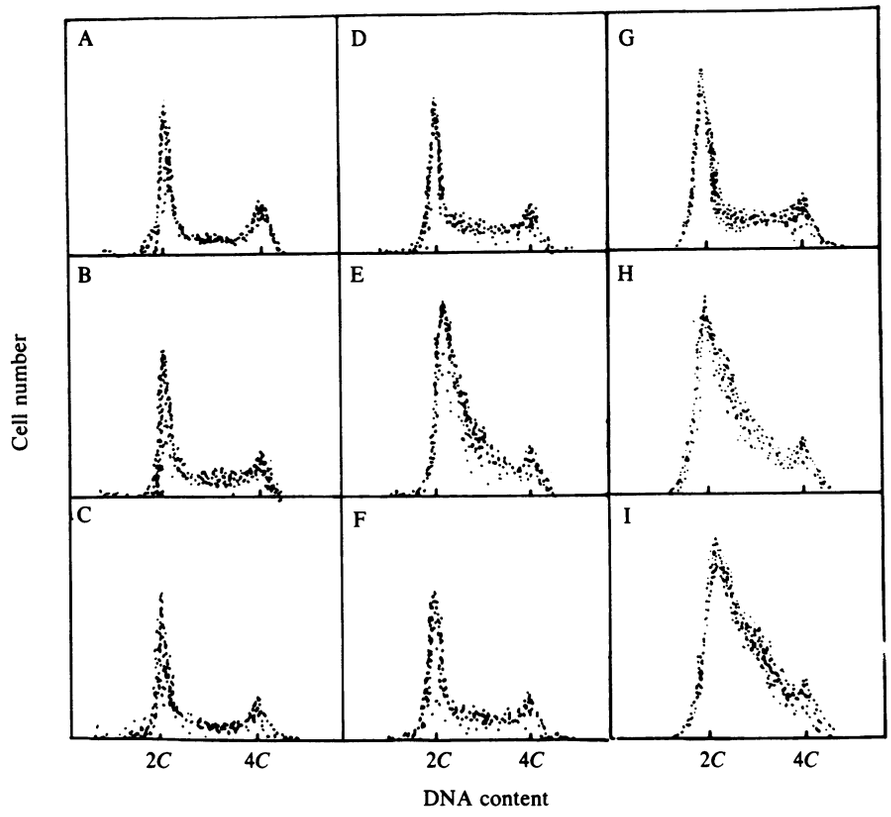

FIG. 3. Cell cycle analysis by flow microfluorometry. Wild-type cells and tsFT20 cells were inoculated at 1.5×10^5 cells per ml and incubated at 33°C for 1 day, followed by incubation for 16 hr at 33°C or at 39°C with or without drug. (*A*) Wild-type cells, before temperature shift; (*B*) wild-type cells, 39°C for 16 hr; (*C*) wild-type cells, 33°C for 16 hr; (*D*) tsFT20, before temperature shift; (*E*) tsFT20, 39°C for 16 hr; (*F*) tsFT20, 33°C for 16 hr; (*G*) wild-type cells, logarithmically growing phase (control pattern of *H* or *I*); (*H*) wild-type cells, aphidicolin at 1 μg/ml, for 16 hr; (*I*) wild-type cells, 2 mM thymidine, for 16 hr.

was linear with the highest level of the extract used. With these concentrations of protein, extracts were incubated at 39°C for various lengths of time in the absence of dNTPs and activated DNA. After this incubation, activated DNA and the 4 dNTPs were added, and the mixture was incubated at 33°C for 30 min. As shown in Fig. 4*B*, DNA polymerase activity (mostly DNA polymerase α as described above) in the tsFT20 extract was more heat-labile than that in the wild-type extract. Incubation of a mixture of the extracts from tsFT20 cells and from wild-type cells at 39°C resulted in a heat inactivation approximately the mean value of both activities (Fig. 4*B*), indicating the absence of an inhibitor in the heat-inactivated extract of tsFT20 cells. In addition, the rate of heat inactivation of the DNA polymerase activity of the revertant clone decreased.

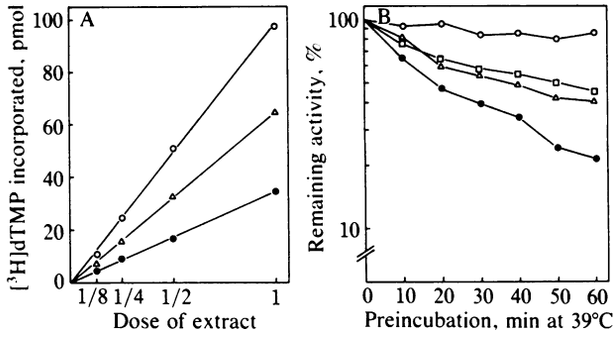

FIG. 4. Heat inactivation of DNA polymerase α in crude cell extract. (*A*) Influence of concentration of extract on activity. Crude cell extracts were prepared from wild-type (○), tsFT20 (●) and a growth-revertant clone, R4C (△), and DNA polymerase α activity was measured. The highest concentration of extract (from 6×10^5 cells) was used per assay (150 μl), and the mixtures were incubated at 33°C for 30 min. (*B*) Heat inactivation experiments. Crude cell extracts from wild type (○), tsFT20 (●), a mixture of these (□), and R4C (△) were preincubated at 39°C for various lengths of time in the reaction mixture (without activated DNA and the dNTPs). The highest concentration of extract in *A* was used; in a mixing experiment half of this amount of wild-type and half of the highest concentration of extract from tsFT20 were mixed (□). The activity of DNA polymerase α was measured by incubating the reaction mixture for 30 min at 33°C in the presence of activated DNA and the substrates.

Heat Lability of Partially Purified DNA Polymerase α. To further establish that DNA polymerase α itself was temperature sensitive in tsFT20, DNA polymerase α was partially purified from the mutant as well as from the wild-type cells by DEAE- and phosphocellulose column chromatography. The elution of DNA polymerase activity of tsFT20 and wild-type cells from a DEAE-cellulose column was examined (Fig. 5*A*). The activities eluted from DEAE-cellulose were identified as DNA polymerase α by their sensitivity to aphidicolin and the sedimentation coefficient of 6–8 S (data not shown). The elution position of the putative mutant DNA polymerase α was about the same as that obtained from wild-type cells. The total activity of DNA polymerase α in the mutant, however, was about 30% of that found in the wild-type cells. Active fractions were pooled and subjected to heat inactivation as described for the experiments with crude cell extracts. As shown in Fig. 5*B*, DNA polymerase α from tsFT20 cells was more heat-labile than the polymerase activity from wild-type cells.

The pooled DEAE-cellulose column fractions were further purified by phosphocellulose chromatography (Fig. 6*A*). Again, DNA polymerase α from tsFT20 yielded lower activity than that from the wild-type cells. Active fractions were divided into two portions, named α1 and α2. As shown in Fig. 6*B*, both α1 and α2 from tsFT20 were more heat labile

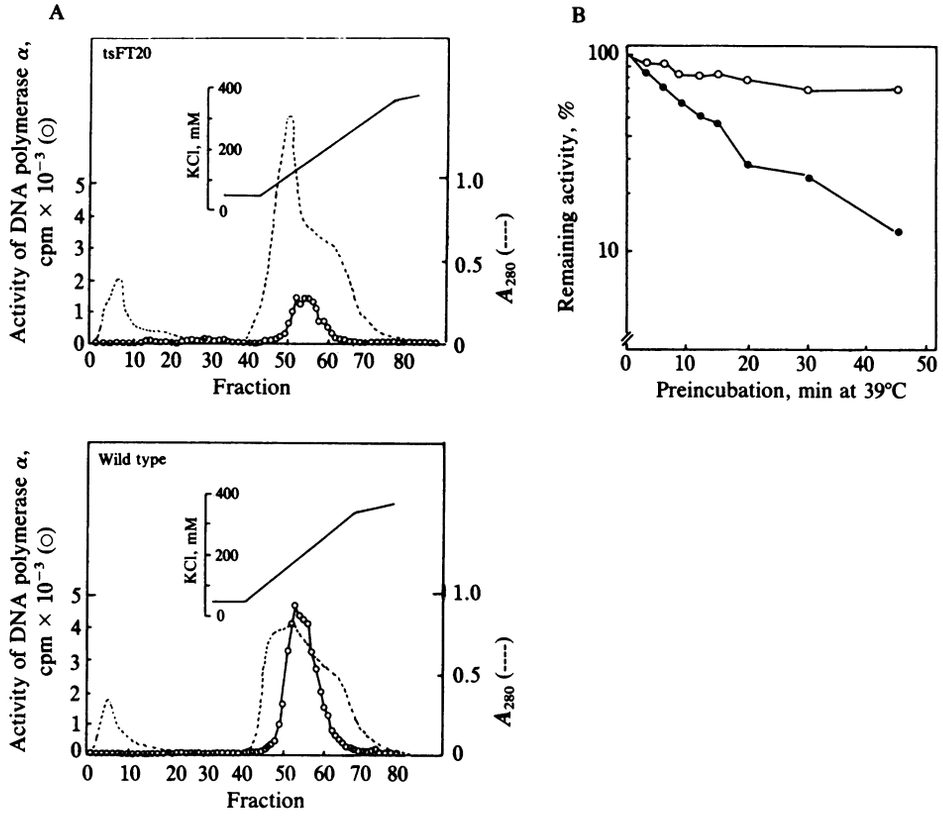

FIG. 5. DEAE-cellulose column chromatography. (*A*) Column patterns. The first DEAE-cellulose column flow-through fractions from tsFT20 and wild-type cells were applied to second DEAE-cellulose columns and the columns were developed. An aliquot of each fraction was assayed for DNA polymerase α activity (○) and absorbance at 280 nm (---). (*B*) Heat inactivation experiments. Peak fractions obtained from the second DEAE-cellulose column chromatography were preincubated at 39°C, and the remaining activity was determined as described in the legend of Fig. 4. ○, Wild-type cells; ●, tsFT20 cells.

than the corresponding fractions isolated from wild-type cells.

DISCUSSION

We have described the characteristics of a temperature-sensitive mutant of mouse FM3A cells. These cells (tsFT20) ceased DNA replication after the temperature shift to 39°C; as shown by flow microfluorometry, the cells, at 39°C, appeared to be largely arrested at the G_1/S boundary and/or in S phase of the cell cycle, although the 4*C* peak did not disappear completely (Fig. 3*E*). The cell cycle distribution pattern observed with tsFT20 after 16 hr of incubation at 39°C was similar to that found with wild-type cells after treatment with aphidicolin (a specific inhibitor of DNA polymerase α) or after treatment of wild-type cells with excess thymidine (Fig. 3 *H* and *I*). These two patterns also had relatively small 4*C* peaks, probably due to the cessation of the cell cycle in late S phase, because these drugs are thought to affect only S phase. DNA replication that occurs in late S phase may be more sensitive to these drugs or to temperature in tsFT20 cells than replication that occurs during other periods of the S phase. Excess thymidine inhibits the supply of the precursors for DNA replication. The temperature-sensitive defect in tsFT20 cells does not, however, involve the concentration of precursor necessary for DNA synthesis; DNA synthesis in nuclei isolated from tsFT20 cells was also temperature sensitive. These reactions were carried out in the presence of a large excess of dNTPs (data not shown).

Heat inactivation experiments revealed that the DNA polymerase α activity of tsFT20 was more heat labile than that of wild-type cells. Furthermore, each revertant cell line yielded DNA polymerase α preparations that were more resistant to heat than DNA polymerase α isolated from the tsFT20 cells. Since DNA polymerase α was not purified to homogeneity, it is possible that the temperature sensitivity of growth of tsFT20 cells is due to a mutation in a factor associated with DNA polymerase α rather than to a mutation in DNA polymerase α itself. This point could be clarified by further purification of DNA polymerase α from tsFT20.

The reversion frequency of the mutant suggests that the mutation of tsFT20 cells is on a single gene. In the parental clone, FM3A clone 28 cells, dominant mutations such as resistance to ouabain occurred with a frequency of about 10^{-4} to 10^{-5} when the cells were treated with mutagens [an observation that coincides with the published results with other rodent cell lines (12, 13)]. Revertant clones of tsFT20 cells were isolated at a frequency of one clone per 1–5 × 10^5 mutagenized cells. If the temperature-sensitive defect of tsFT20 cells was derived from two or more mutational events, the reversion frequency would be 10^{-8} to 10^{-10} or lower, by analogy with the ouabain system. In addition, spontaneous revertants were isolated from the mutant with the frequency of 1–5 × 10^{-7}, which is consistent with the reversion frequency observed at the thymidylate synthase locus of FM3A cells (14). Furthermore, all revertant cells thus far obtained showed an increase in heat stability of DNA polymerase α activity and in the enzyme activity itself compared to tsFT20 cells; in contrast, no changes in DNA polymerase β or γ activity were detected (Fig. 4*B* and unpublished observations). These facts are consistent with a single mutational event leading to the formation of the tsFT20 clone.

Recently, several laboratories have reported that DNA primase activity is associated with certain forms of DNA polymerase α (11, 15–21). More recently, primase activity

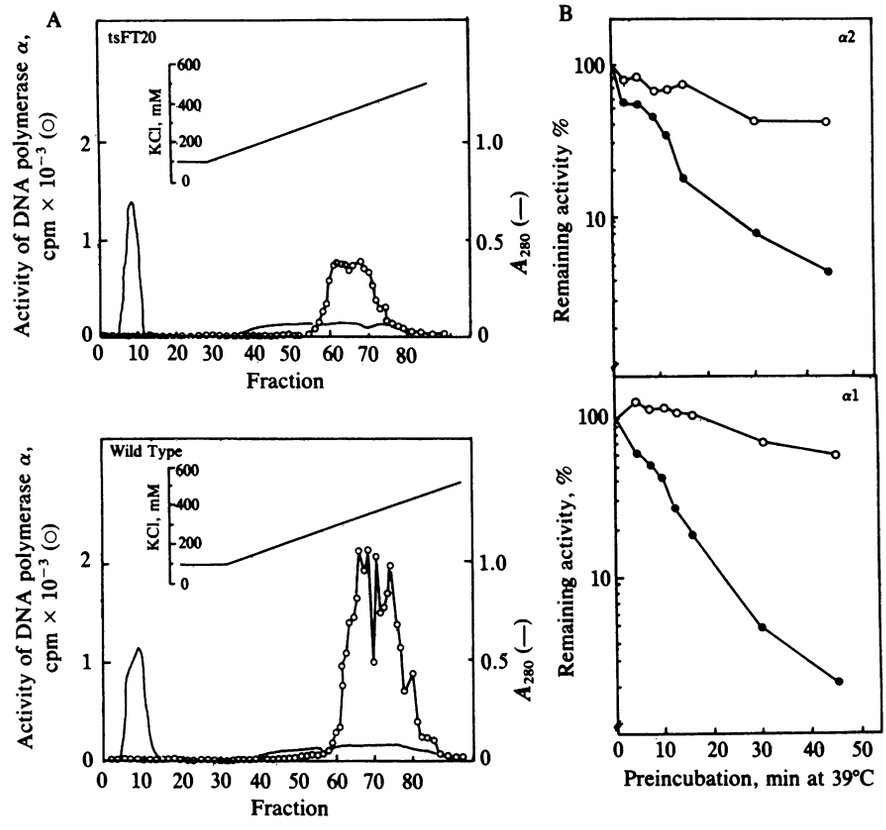

FIG. 6. Phosphocellulose column chromatography. (*A*) Column patterns. The peak fractions obtained from the second DEAE-cellulose column were applied to a phosphocellulose column and eluted. An aliquot of each fraction was assayed for DNA polymerase α activity (○) and absorbance at 280 nm (—). (*B*) Heat inactivation experiments. Active fractions obtained from the phosphocellulose columns were divided into two portions, α2 (eluting in fractions 57–65 for tsFT20 and 60–70 for wild-type cells) and α1 (eluting in fractions 66–75 for tsFT20 and 71–80 for wild-type cells) (11). These fractions were preincubated at 39°C, and the remaining activity was determined as described in the legend of Fig. 4. ○, Wild-type cells; ●, tsFT20 cells.

was separated from DNA polymerase activity by certain treatments (22, 23). We tested the primase activity (mainly associated with α1 fraction obtained by phosphocellulose chromatography) for temperature sensitivity in tsFT20 cells; the primase activity was not heat labile (unpublished results). The mutant reported here will be useful for studying the mechanism of DNA polymerase α, and hopefully, for cloning the DNA polymerase α gene.

We thank Dr. A. Sato for his generous help with the cytofluorometric analysis. We also thank Dr. E. M. Bradbury and Dr. J. Hurwitz for their critical reading of this manuscript and Ms. K. Logan and Ms. H. Ohno for their help in preparing it. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, and by a Life Science Grant from the Institute of Physical and Chemical Research, Tokyo, Japan.

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