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Defective DNA Replication and Repair Associated with Decreases in Deoxyribonucleotide Pools in a Mouse Cell Mutant with Thermolabile Ubiquitin-Activating Enzyme E1[1]

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Upon shift-up in temperature, mouse tsFS20 mutant cells with thermolabile ubiquitin-activating enzyme E1 immediately stopped DNA replication and showed cell cycle arrest in S-phase. In contrast, when the cells were permeabilized with lysolecithin after culture at the nonpermissive temperature, they exhibited a normal level of repliative DNA synthesis *in vitro*. In agreement with this, intracellular pools of deoxyribonucleoside triphosphates were significantly reduced in the cells cultured at the nonpermissive temperature. Even under the permissive conditions, tsFS20 cells were more sensitive to hydroxyurea and alkylating agents, and induced less mutation than the wild-type cells. These results suggest that the ubiquitin system affects DNA replication and repair.

Key words: DNA replication, mutation rate, nucleotide pool, temperature-sensitive mutant, ubiquitin-activating enzyme E1.

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Ubiquitin is a small protein found in eukaryotes, and its covalent binding to target proteins modulates various cellular functions. Ubiquitin activating enzyme (E1) catalyzes the first step of sequential transfer of ubiquitin by coupling ubiquitin through a thioester bond (*1, 2*). Ubiquitin on E1 is transferred to the second protein factors called ubiquitin carrier proteins (E2s). Finally, it is transferred to the targets directly or by aid of the third protein factors called ubiquitin isopeptide ligases (E3s).

In mammals, temperature-sensitive E1 mutants can arise from various cell lines, while no E2 or E3 mutant has yet been isolated. This may be related to the findings that E1 is involved in tolerance to heat (*3*) and its gene is located on X chromosome (*4, 5*). The E1 mutants isolated thus far can be classified into two groups. One group includes FM3A ts85 (*6*), Chinese hamster ts20 (*7*), and BHK tsBN75 (*8*). They are able to undergo normal DNA replication but unable to enter mitosis under the nonpermissive conditions (*9-11*). This phenotype is coupled with loss of chromosome condensation (*12*), an event prerequisite for entry into M-phase of the cell cycle. The other group includes FM3A tsFS20 (*3*), FM3A tsl3lb (*13*), and mouse L tsA1S9 (*14*). They are arrested in S-phase with an immediate decrease in DNA synthesis *in vivo* upon shift-up in temperature. Due to these phenotypes, they have long been thought as mutants of DNA replication machinery. Until now, a molecular basis for these phenotypes remains to be characterized.

Several explanations of the phenotypic differences in the E1 mutants are possible. The simplest one is that the heat sensitivity of E1 is involved (*3, 15*). Indeed, mutants of E1 with modest heat sensitivity tend to show G2 arrest, as cell cycle progression through this phase seems to be most sensitive to depletion of activated ubiquitin. Mutants of E1 with severe heat sensitivity are likely to lead to S phase arrest. Alternatively, sites of mutation on E1 may be responsible for the phenotypic diversity (*3*). A particular type of mutation abolishes an interaction between E1 and a particular member of the E2 family (Kaneda, S. *et al.*, unpublished). This may in turn lead to loss of ubiquitination of a target protein that works in S-phase or G2-phase. Ubiquitinated histone H2A and H2B are possible candidates, as they have been shown to modulate chromatin structures (*15, 16*). A decrease in ubiquitinated histone H2A and H2B is thought to affect DNA replication.

It is reasonable to postulate that E1 mutants exhibit multiple phenotypes, because the ubiquitin system is involved in various biological functions. Characterization of E1 mutants is thus expected to provide a clue to understanding further the function of ubiquitination in mammalian cells. In the present study, we examined the molecular basis for the defect in DNA synthesis in FM3A tsFS20. Relevant properties such as DNA repair and mutation induction were also examined, since the ubiquitin system has been shown to affect these functions in yeast.

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Abbreviations: dNTP, deoxyribonucleoside triphosphate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; rNTP, ribonucleoside triphosphate; TG, 6-thioguanine.

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MATERIALS AND METHODS

Cell Lines and Culture Conditions—tsFS20 and ts85 contain thermolabile ubiquitin-activating enzyme E1 as described previously (*3, 17*). tsFT20 contains temperature-sensitive DNA polymerase α catalytic subunit as described previously (*18*). These mutants are derived from F28-7, a subclone of mouse mammary carcinoma FM3A cells (*19*), which was used as the wild-type in the present study. Cells were grown in suspension in ES medium supplemented with 2% fetal calf serum as described previously (*20*) at the permissive (33.5°C) or nonpermissive temperature (39°C) unless otherwise stated. Colonies were formed on semi-solid ES medium solidified with 0.35% agarose as described previously (*19*).

Transfection with Plasmids—tsFS20 cells were seeded onto 60-mm plastic Petri dishes and allowed to attach to the bottom upon culture in 0.5% serum for 30 min. Then 5 μg of plasmid pcMUBA-1 encoding the wild-type mouse E1 (*21*) was co-precipitated with calcium phosphate and added to the cells as described previously (*3*). After incubation for 8 h, DNA was removed, and the cells were cultured overnight in normal growth medium. The cells were plated onto semi-solid agarose medium and incubated at 39°C for 2 weeks. Colonies formed were picked up and expanded for characterization. One of the colonies, TF1, that grew normally at 39°C was used in subsequent experiments.

Flow Cytometry—Cells ($2\text{-}3\times10^6$) were fixed and stained with propidium iodide according to the procedure recommended by Becton Dickinson (Cycle TEST Plus DNA Reagent Kit). Approximately 2×10^4 cells were analyzed with a flow cytofluorometer (FACScan, Becton Dickinson) as described (*3*).

Macromolecular Synthesis—Cells (1×10^5) exponentially growing in 35-mm dishes at 33.5°C were transferred to 39°C. At intervals, the cells were pulse-labeled with [methyl-^{3}H]thymidine (25 Ci/mmol, Amersham), [5-^{3}H]-uridine (25-30 Ci/mmol), or [4,5-^{3}H]leucine (45-85 Ci/mmol) for 30 min. An equal volume of cold 10% trichloroacetic acid was added to the dishes, and radioactivity in the acid-insoluble fractions was counted as described previously (*22*).

DNA Synthesis in Permeabilized Cells—Exponentially growing cells were suspended in cold solution A [150 mM sucrose, 80 mM KCl, 35 mM HEPES (pH 7.4), 5 mM KH_2PO_4/K_2HPO_4 (pH 7.4), and 5 mM $MgCl_2$] and permeabilized by addition of 1 mg/ml of lysolecithin as described previously (*22, 23*). To an aliquot of 2×10^6 permeabilized cells, 100 μM ATP, 25 μM each of dATP, dGTP, dCTP, and dTTP, and 0.25 μM [methyl-^{3}H]dTTP (40 Ci/mmol, Amersham) were added in a total volume of 100 μl. Reaction mixtures were incubated at 30 or 37°C, then 1 ml of cold 5% trichloroacetic acid plus 10 mM sodium pyrophosphate was added. The precipitates were washed several times with the acid solution by centrifugation. Radioactivity incorporated into acid-insoluble material was determined as described previously (*22*).

Measurement of Intracellular Nucleotide Pools—Cells grown to 2×10^5/ml were collected by centrifugation at 4°C, washed with cold phosphate-buffered saline containing 0.1% glucose, and extracted with 0.3 M cold trichloroacetic acid (*20, 24*). After centrifugation ($15{,}600\times g$, 30 s) at 4°C, the acid supernatant was separated and extracted with 1.1 volume of cold 0.5 M tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane. The aqueous extract was applied to chromatography on Partisil-10 SAX (4.6×250 mm, Whatman) to determine the amount of ribonucleoside triphosphates (rNTPs). HPLC analyses were performed using a Waters 6000A pump with a Waters 440 absorbance detector and a Hewlett-Packard 3390A integrator (*24*).

To determine the amounts of deoxyribonucleoside triphosphates (dNTPs), 80 μl of the above aqueous extract was incubated with 20 μl of 0.2 M deoxyguanosine and 20 μl of 0.2 M $NaIO_4$ at 37°C for 2 min. Then the reaction mixture was mixed with 2 μl of 1 M rhamnose and 30 μl of 4 M CH_3NH_2 (pH 6.5), incubated at 37°C for 30 min, and applied to chromatography as described above (*24*).

CDP Reductase Assay—Cells grown to $4\text{-}5\times10^5$/ml were collected by centrifugation at 4°C, washed with cold phosphate-buffered saline, suspended in 100 μl of 10 mM Tris-HCl (pH 7.2) containing 2 mM dithiothreitol and 0.2% Triton-X100, and passed 5-10 times through a needle (21G). The homogenate was centrifuged ($100{,}000\times g$, 30 min) at 4°C, and the resulting supernatant was desalted by passing through a Sephadex G-25 spin column (Boeringer) equilibrated with 10 mM Tris HCl (pH 7.2) containing 2 mM dithiothreitol to give a cell extract. The reaction mixture for CDP reductase assay (*19*) contained 20 mM Tris-HCl (pH 7.2), 1 mM ATP, 6 mM dithiothreitol, 5 mM $MgCl_2$, 0.09 mM [^{3}H]CDP (4.6 Ci/mmol, Amersham), and the cell extract (0.3-0.4 mg protein) in a final volume of 50 μl. The mixture was incubated at 37°C for 30 min, boiled for 5 min, and centrifuged ($15{,}600\times g$, 5 min). The supernatant was incubated at 37°C for 2 h with 15 μg of phosphodiesterase I (Sigma) and 0.02 unit of *Escherichia coli* alkaline phosphatase (Sigma), boiled for 6 min, and centrifuged. The resulting supernatant (15 μl) was spotted on a PEI-cellulose plastic sheet (borate form) (Merck, Germany) and developed for 2.5 h at room temperature with solvent [10 ml of 5.0 M ammonium acetate (pH 9.5), 40 ml of saturated sodium tetraborate, 110 ml of ethanol, and 0.25 ml of 0.5 M EDTA (pH 8.0)] (*25*). The plate was examined under UV light and spots corresponding to cytidine or deoxycytidine were cut out to determine their radioactivities in a scintillation counter.

Determination of Mutation Rate—To determine the induced mutation rate, cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at the indicated temperature for 2 h. An aliquot was plated on semisolid medium to determine survival (*26*). The rest were incubated at the same temperature for a further 14 h in fresh medium. Then the cells were cultured for mutation fixation at 33.5°C for 7-8 days and allowed to form colonies at 33.5°C on semisolid medium containing 1 μM 6-thioguanine (TG). The mutation frequency for the TG resistance marker was calculated by dividing the number of TG-resistant colonies formed by the number of cells plated after correcting for plating efficiency.

Spontaneous mutation rate was determined by Luria-Delbruck fluctuation test. A small number of cells were seeded in 25 dishes and expanded at the temperature indicated for 8 days. Cells were plated on semisolid medium containing TG to select resistant clones. Mutation rate was calculated according to equation 8 of Luria and Delbruck (*27*).

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RESULTS

Cell Cycle Analysis—When asynchronously growing tsFS20 cells were cultured at 39°C for 16 h, they were arrested in mid to late S-phase of the cell cycle (Fig. 1). Under the same conditions, the wild-type FM3A cells were not affected (Fig. 1). In contrast, the majority of ts85 cells were arrested at G_2-phase (not shown) as described (*9*). When tsFS20 cells were treated with aphidicolin, an inhibitor of DNA replication, for 20 h and then cultured at 39°C for 16 h, the cells were arrested in early to mid S-phase (not shown). In agreement with these observations, cdc2 kinase, a G_2 specific marker, was induced in ts85 (*28*) but not in tsFS20 (*3*) under the nonpermissive culture conditions.

Macromolecular Synthesis In Vivo—We monitored levels of macromolecular synthesis *in vivo* in tsFS20 and FM3A cells cultured at 39°C. The rate of DNA synthesis in tsFS20 was decreased to 50% of control by 2-3 h after temperature shift, and below a detectable level by 7-8 h (Fig. 2). FM3A cells showed no decrease in it. Further, we tested tsFT20 with thermolabile DNA polymerase α and obtained a time course similar to that of tsFS20 (not shown). The rates of total RNA and protein synthesis did not decrease in tsFS20 and FM3A under the nonpermissive conditions (Fig. 2). These results demonstrate that the ubiquitin system somehow regulates DNA replication.

DNA Synthesis In Vitro—We tried to understand the molecular basis for the defect in DNA replication in tsFS20. We employed a permeabilized cell system to examine an *in vitro* activity of DNA synthesis (Fig. 3). This system depends on exogenous supply of deoxyribonucloside triphosphates and can support an almost *in vivo* rate of semiconservative DNA replication in FM3A cells (*22, 23*). When tsFS20 cells were cultured at 39°C for 4 h, the activity in these cells was slightly higher than that of control cells maintained at 33.5°C, even though their rate of DNA synthesis *in vivo* had dropped to 30% of the control cells (Fig. 2). When cultured at 39°C for 16 h, the activity still retained a level approximately two-thirds that of the control cells. In these cells, the rate of DNA synthesis *in vivo* decreased to an undetectable level. In FM3A, the activity did not change significantly upon shift-up in temperature (Fig. 3).

From these results, we reasoned that the DNA replication machinery *per se* is not defective but, instead, that the supply of DNA precursors is somehow impaired in tsFS20.

Intracellular Nucleotide Pools—We measured intracellular nucleotide pools in tsFS20, TF1, one of its temperature-insensitive transformants isolated by transfection with plasmid encoding the wild-type mouse E1 (*4*), tsFT20, and FM3A. tsFT20 is a reference cell line unable to undergo DNA replication at the nonpermissive temperature (*6*). When cultured at 39°C for 16 h, tsFS20 showed significant decreases in all of the four dNTP pools, most remarkably the dTTP pool (Table I). FM3A and TF1 showed slight decreases in the dNTP pools. As these cell types grow more rapidly at 39°C than at 33.5°C, such changes may reflect their vigorous consumption of dNTP pools. It was also found that the decreases in the dNTP pools in tsFS20 are

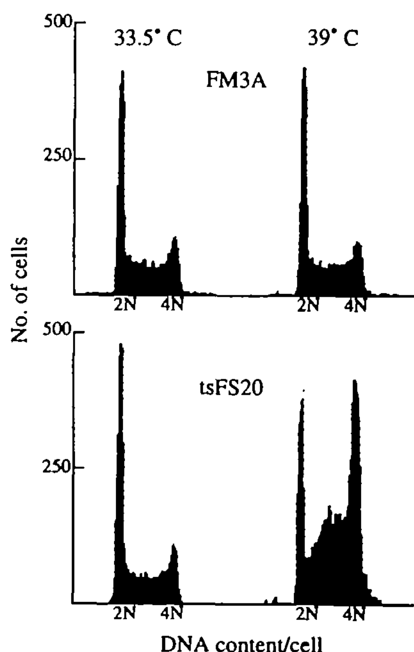

Fig. 1. **Cell cycle progression in FM3A and tsFS20 upon shift up in temperature.** Cells maintained at 33.5°C (left) were transferred to 39°C and cultured for 16 h (right). DNA contents in cells were analyzed by flow cytometry as described in "MATERIALS AND METHODS." 2N and 4N denote the positions of DNA contents in G_1 and G_2/M cells, respectively.

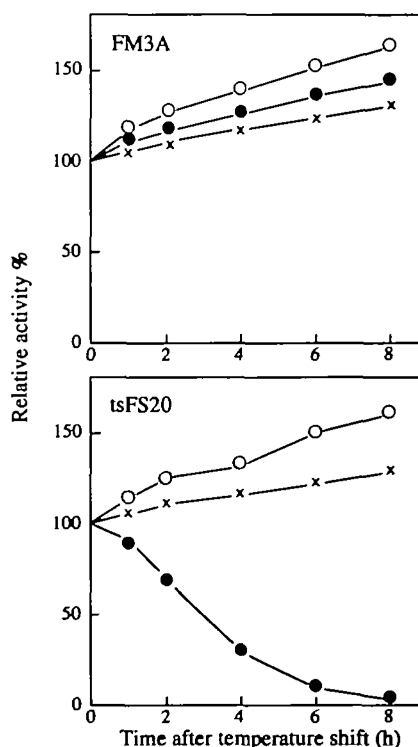

Fig. 2. **Changes in synthetic rates of macromolecules in FM3A and tsFS20 upon shift-up in temperature.** Cells maintained at 33.5°C were shifted up to 39°C, and the synthetic rates of DNA (●), RNA (○), and protein (×) were determined at intervals as described in "MATERIALS AND METHODS." Each point represents an average of duplicate assays. Three to four independent determinations gave similar results.

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caused by its defective E1. In contrast, tsFT20 showed increases in the dNTP pools when cultured at 39°C for 16 h, suggesting the occurrence of overflows of dNTP pools.

The levels of intracellular rNTP pools did not change or changed only slightly in the cell types examined (Table II). In FM3A and TF1, CTP and UTP pools were significantly decreased when cultured at 39°C. This may also represent stimulated use of the rNTP pools.

Assay of Ribonucleotide Reductase—We assessed ribonucleotide reductase, a key enzyme in the metabolic pathway of deoxyribonucleotides (*19, 29*), because thymidylate synthase, another key enzyme in the pathway, was found to be normal in tsFS20 (*3*). The activity of CDP reductase in tsFS20 was not temperature-sensitive as in FM3A. However, the activity of tsFS20 (403 units, expressed as nmol dCyd formed per mg protein per h) was approximately half of that of FM3A (725 units) when cultured at 33.5°C. When cultured at 39°C for 16 h, the

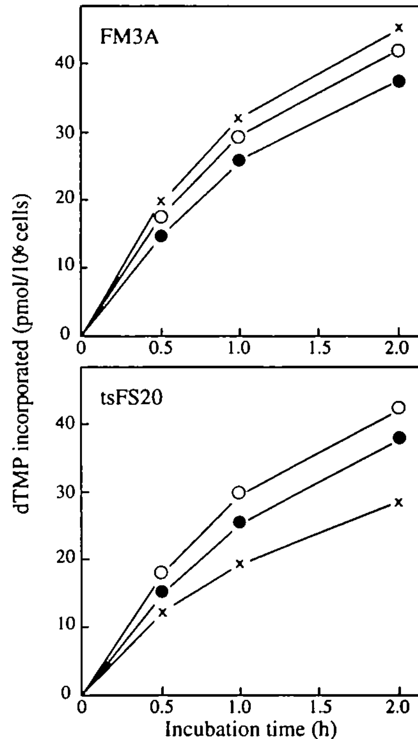

Fig. 3. **Activity of DNA synthesis in permeabilized cells cultured at the nonpermissive temperature in FM3A and tsFS20.** Cells were cultured at 39°C for 0 h (○), 4 h (●), and 16 h (×), permeabilized with lysolecithin and incubated at 30°C in a reaction mixture containing tritiated dTTP. At intervals, the labeled dTMP incorporated into DNA was determined as described in "MATERIALS AND METHODS."

TABLE I. **Intracellular dNTP pools in the wild-type FM3A, mutant tsFS20, its transformant TF1, and mutant tsFT20 when cultured at 33.5 or 39°C for 16 h.**

Cell type	dCTP[a]	dTTP	dATP	dGTP
FM3A				
33.5°C	21	46	29	2.8
39.0°C	18	37	27	2.2
	(86)[b]	(80)	(93)	(79)
tsFS20				
33.5°C	21	42	26	2.6
39.0°C	7.4	9.2	12	0.8
	(35)	(22)	(46)	(31)
TF1				
33.5°C	23	41	25	2.5
39.0°C	20	35	23	2.3
	(87)	(85)	(92)	(92)
tsFT20				
33.5°C	14	20	29	1.7
39.0°C	19	22	33	2.5
	(136)	(110)	(114)	(147)

[a]Picomoles per 10⁶ cells. Values are averages of three independent determinations. [b]The value at 39.0°C expressed as a percentage of that at 33.5°C.

TABLE II. **Intracellular NTP pools in the wild-type FM3A, mutant tsFS20, its transformant TF1, and mutant tsFT20 when cultured at 33.5 or 39°C for 16 h.**

Cell-type	CTP[a]	UTP	ATP	GTP
FM3A				
33.5°C	467	781	910	290
39.0°C	293	487	832	282
	(63)[b]	(63)	(92)	(97)
tsFS20				
33.5°C	551	773	1,785	444
39.0°C	560	731	2,767	756
	(102)	(95)	(155)	(170)
TF1				
33.5°C	460	778	955	314
39.0°C	345	552	907	335
	(75)	(71)	(95)	(107)
tsFT20				
33.5°C	289	520	747	448
39.0°C	297	583	805	596
	(103)	(112)	(108)	(133)

[a] Picomoles per 10⁶ cells. Values are averages of three independent determinations. [b]The value at 39.0°C expressed as a percentage of that at 33.5°C.

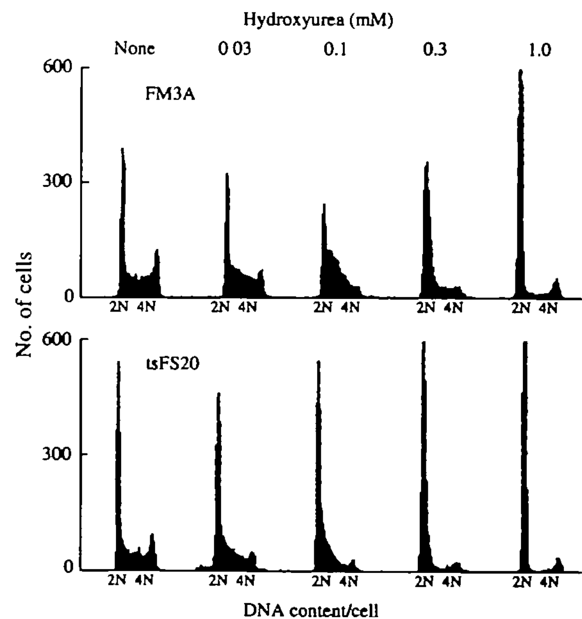

Fig. 4. **Cell cycle progression in the presence of hydroxyurea in FM3A and tsFS20 under the permissive conditions.** Cells were cultured at 33.5°C for 16 h in the presence of various concentrations of hydroxyurea as indicated. The cells were analyzed by flow cytometry as in Fig. 1.

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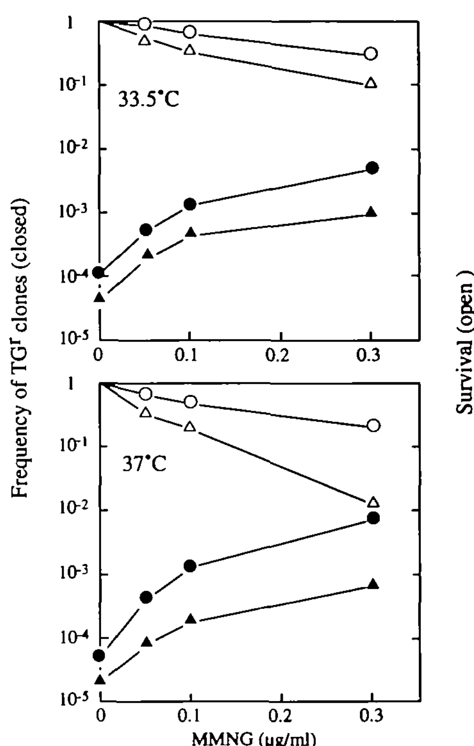

Fig. 5. **Survival and mutation induction in FM3A and tsFS20 following treatment with MNNG.** Cells were treated with MNNG at 33.5 or 37°C for 2 h at the concentrations indicated. An aliquot of the cells were plated on semi-solid medium to determine survival (open symbols). The rest were allowed to form TG-resistant colonies (closed symbols) on selective medium at 33.5°C as described in "MATERIALS AND METHODS." ● and ○, FM3A; ▲ and △, tsFS20.

activity of tsFS20 (181 units) was further decreased, although the activity of FM3A (441 units) was also decreased significantly.

We do not know at present to what extent the above decrease in CDP reductase activity contributes to the depletion of dNTP pools in tsFS20. However, it should be noted that ribonucleotide reductase is 10-fold higher in S-phase than in G_1 phase (*30, 31*) and tsFS20 cells are synchronized in S-phase under the nonpermissive conditions. Therefore, if we compare the activity in S-phase cells under the nonpermissive conditions, the difference between tsFS20 (synchronized in S-phase) and FM3A (asynchronously growing) should be greater than that calculated from the above values.

Sensitivity to Hydroxyurea—We cultured tsFS20 and FM3A cells at 33.5°C for 16 h in the presence of hydroxyurea, an inhibitor of ribonucleotide reductase (*32*), and analyzed the change in cell cycle progression by flow cytometry (Fig. 4). In FM3A, 1 mM hydroxyurea was required to arrest cells at the G1/S boundary. In tsFS20, 0.3 mM hydroxyurea was sufficient to arrest cells at the G1/S boundary. These results suggest that the activity of ribonucleotide reductase *in vivo* is somehow decreased in tsFS20 even under the permissive conditions.

Mutation Rate—We tested sensitivity to MNNG and induced mutation frequency in tsFS20 (Fig. 5). As expected, tsFS20 was significantly more sensitive to killing by MNNG and more resistant to induction of TG-resistant mutants than FM3A. Spontaneous mutation rate, as determined by the Luria-Delbruck fluctuation test (*26*), was almost normal in tsFS20. These results indicate that tsFS20 is defective in DNA repair and induction of mutation. Further, temperature-insensitive transformants of tsFS20 expressing ectopic wild-type mouse E1, including TF1, showed a normal level of sensitivity to MNNG and induced mutation frequency.

DISCUSSION

We have found that tsFS20 cells retain the activity of replicative DNA synthesis in the permeabilized cell system when they have lost the activity *in vivo* during culture under the nonpermissive conditions. The cells also showed marked reduction in dNTP pools, most notably in dTTP pool (22% of control) under the nonpermissive conditions. The reduction in dNTP pools can account for the interruption of DNA replication *in vivo*. For instance, when mutants with thermolabile thymidylate synthase were cultured under the nonpermissive conditions, dTTP pools dropped to 20-30% of control levels (*20*). In contrast, dNTP pools increased slightly when DNA replication was inhibited by shift-up in temperature in tsFT20 with thermolabile DNA polymarase α or by treatment of the wild-type FM3A with aphidicolin, an inhibitor of DNA replication (Wataya, unpublished data).

On the other hand, ubiquitination of histones is known to modulate chromatin structures and thought to affect DNA replication (*15, 16*). In tsFS20, the ubiquitination of histone H2A and H2B is expected to occur. It is therefore possible that a defect in histone ubiquitination might negatively affect DNA replication together with the deprivation of NTP pools. In *S. cerevisiae*, however, the absence of ubiquitination of histone H2A is not essential for growth, sporulation, or resistance to stresses. Nevertheless, *S. cerevisiae* has several gene products, such as RAD6 and CDC34, involved in transfer of ubiquitin to histone H2A as in higher eucaryotes (*33*).

We reasoned that ribonucleotide reductase is somehow altered in tsFS20, as this enzyme plays a major role in the metabolism of dNTPs together with thymidylate synthase. CDP reductase activity in the extract of tsFS20 cells was half of that of FM3A under the permissive conditions, in agreement with the observation that tsFS20 was more sensitive to hydroxyurea than FM3A. The activity in tsFS20 cells was further decreased by approximately 50% upon temperature shift. Under the nonpermissive conditions, tsFS20 cells are arrested in S-phase, while FM3A cells grow asynchronously. Also, ribonucleotide reductase has been shown to be active in S-phase (*30, 31*). Together, these findings suggest that the difference in the activity in S-phase cells should be more marked between tsFS20 and FM3A.

Ribonucleotide reductase consists of R1 regulatory subunit and R2 catalytic subunit (*34*). Mouse enzyme is localized in the cytosol (*35*), and recombinant mouse enzyme expressed in *E. coli* and free of ubiquitination has been shown to be active *in vitro* (*35*). While R1 is expressed constitutively during the cell cycle, R2 is expressed in S-phase with a very short half-life and in small quantity (*31*). Interestingly, R1 has been shown by immunocheminal analysis to be ubiquitinated in rat liver (*36*). Such an

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ubiquitinated form has also been shown to increase in parallel with the DNA synthetic activity in normal liver cells. In one experiment, we overexpressed ectopic mouse R2 subunit in tsFS20 cells, but were unable to rescue the temperature-sensitive phenotype. As ubiquitination plays a role in translocation of proteins with a chaperone-like function (*37, 38*), a defect in ubiquitination of R1 might impair the *in vivo* function of ribonucleotide reductase, especially since the metabolic pathways of dNTPs are channeled or compartmented in cells (*39, 40*).

Unfortunately, we were not able to prove that the decrease in the reductase activity is sufficient for the remarkable reduction in dNTP pools in tsFS20. It is possible that an enzyme other than ribonucleotide reductase may mediate between the alteration of E1 and the decreases in dNTP pools. Therefore, it remains to be determined what links the alteration of E1 to the decreases in dNTP pools. However, we speculate that the site of mutation on E1 in tsFS20 is responsible for its phenotypes, because mutation sites on E1 have been correlated with various phenotypes in E1 mutants (*3, 11*, and Kaneda, S. *et al.*, unpublished).

Finally, tsFS20 with reduced ribonucleotide reductase activity showed hypersensitivity to MNNG and a decrease in induced mutation frequency. Mutants of FM3A with altered R1 regulatory subunit have been shown to be hypersensitive to bleomycin (*25*). Therefore, ribonucleotide reductase is suggested to modulate DNA repair and mutagenesis in mouse cells. In yeast, ribonucleotide reductase is implicated in DNA repair and induced mutation. Certain types of R2 mutations show hypersensitivity to hydroxyurea and to killing by methyl methanesulfonate (*34*). Also, yeast rad6 mutant (*41*), which contains defective ubiquitin-conjugating enzyme UBC2, shows increased sensitivity to alkylating agents and induction of mutation (*42, 43*). Mice lacking the homolog of RAD6 in yeast display male infertility associated with chromatin modification (*44*). Our data suggest that DNA replication and repair are also under the control of the ubiquitin system through an as yet unidentified mechanism in mammals.

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