MOLECULAR AND CELLULAR BIOLOGY, Mar. 1997, p. 1484–1489 Vol. 17, No. 3
0270-7306/97/$04.00+0

Incubation at the Nonpermissive Temperature Induces Deficiencies in UV Resistance and Mutagenesis in Mouse Mutant Cells Expressing a Temperature-Sensitive Ubiquitin-Activating Enzyme (E1)

HIRONOBU IKEHATA,[1]* SUMIKO KANEDA,[2] FUMIAKI YAMAO,[2] TAKESHI SENO,[2] TETSUYA ONO,[1] AND FUMIO HANAOKA[3]

Department of Radiation Research, Tohoku University School of Medicine, Sendai 980-77,[1] Laboratory of Mutagenesis, National Institute of Genetics, Mishima 411,[2] and Cellular Physiology Laboratory, The Institute of Physical and Chemical Research (RIKEN), Wako 351-01,[3] Japan

Received 8 July 1996/Returned for modification 29 July 1996/Accepted 17 December 1996

In temperature-sensitive (ts) mutants of mouse FM3A cells, the levels of mutagenesis and survival of cells treated with DNA-damaging agents have been difficult to assess because they are killed after their mutant phenotypes are expressed at the nonpermissive temperature. To avoid this difficulty, we incubated the ts mutant cells at the restrictive temperature, 39°C, for only a limited period after inducing DNA damage. We used ts mutants defective in genes for ubiquitin-activating enzyme (E1), DNA polymerase α, and p34^{cdc2} kinase. Whereas the latter two showed no effect, E1 mutants were sensitized remarkably to UV light if incubated at 39°C for limited periods after UV exposure. Eighty-five percent of the sensitization occurred within the first 12 h of incubation at 39°C, and more than 36 h at 39°C did not produce any further sensitization. Moreover, while the 39°C incubation gave E1 mutants a moderate spontaneous mutator phenotype, the same treatment significantly diminished the level of UV-induced 6-thioguanine resistance mutagenesis and extended the time necessary for expression of the mutation phenotype. These characteristics of E1 mutants are reminiscent of the defective DNA repair phenotypes of *Saccharomyces cerevisiae rad6* mutants, which have defects in a ubiquitin-conjugating enzyme (E2), to which E1 is known to transfer ubiquitin. These results demonstrate the involvement of E1 in eukaryotic DNA repair and mutagenesis and provide the first direct evidence that the ubiquitin-conjugation system contributes to DNA repair in mammalian cells.

Mutations that lead to temperature-sensitive (ts) phenotypes usually occur in genes essential for cell viability. Since DNA repair plays a critical and basic function to preserve the genetic information of cells against external and internal DNA-modifying agents, it is logical to assume that some ts mutations fall within DNA repair genes. We isolated ts mutants from the mouse mammary carcinoma cell line FM3A and classified them into 11 genetic complementation groups (8) comprising three groups of defective genes, each of which encoded DNA polymerase α (Pol α), ubiquitin-activating enzyme (E1), or p34^{cdc2} kinase. There are indications that these three gene products contribute to cellular DNA repair mechanisms. First, a study with DNA polymerase inhibitors suggested that Pol α is a repair synthesis enzyme, in addition to its role in DNA replication (44). Second, a *rad6* DNA repair mutant of *Saccharomyces cerevisiae* is defective in UBC2 (14), a ubiquitin-conjugating enzyme (E2), to which E1 transfers ubiquitin. This suggests relevance to DNA repair of the ubiquitin-conjugation system in which E1 and E2s cooperate to mark proteins with ubiquitins as substrates for intracellular nonlysosomal protease complexes (10). Third, a number of cellular responses to DNA-damaging agents in eukaryotes are mediated through cell cycle arrest or delay (7, 25), which is regulated by the cyclin-cdk systems in which p34^{cdc2} kinase is a key enzyme (30).

In the present study, we attempted to evaluate the contribution of these three enzymes to mammalian DNA repair by estimating the levels of cell survival and induced mutation frequency in ts cells with mutations in these enzymes' genes. However, the study of DNA repair using ts mutant cells is not easy for the following reason. Cell survival and mutation frequency cannot be estimated by colony formation assays at the temperature used to induce the mutant phenotype of ts cells, because the cells are not viable at this limiting temperature. In fact, until now, clonogenic assays for estimating the DNA repair capacity of ts mutants at the limiting temperature have not succeeded for mammalian cells, although some results concerning yeast cells have been published elsewhere (3, 19, 43). To overcome this problem, we incubated cells for only a limited period at the nonpermissive temperature after exposure to DNA-damaging agents. Using this approach, we were able to show that E1 mutants are deficient with respect to UV resistance and mutability, thus directly demonstrating the relevance of E1 to eukaryotic DNA repair.

MATERIALS AND METHODS

Cells and culture. The following ts mutant cell lines were used: ts85 and tsFT5 defective in E1 (9, 22, 23), tsFT20 defective in Pol α (11, 13, 24), and tsFT210 defective in p34^{cdc2} kinase (21, 41, 45) at the nonpermissive temperature 39°C. All of these mutants were derived from the wild-type clone 28 of the mouse mammary carcinoma suspension cell line FM3A (26). Transformed cell lines of ts85, including ts85YMTA1, were isolated by transfection with a mouse E1 cDNA (12) cloned downstream of the simian virus 40 promoter in the pSG5 expression vector (Stratagene) and established by selection for viability at the limiting temperature (29). The proficiency of E1 activity at 39°C in the transformants was confirmed by assaying for ubiquitin-protein conjugation activity in crude cell extracts (9). All cell lines except the E1 cDNA transformants were cultured at the permissive temperature of 33°C. The transformants were grown at 33°C only during the last two subcultures before experiments but were otherwise maintained at 39°C. The basic culture medium was RPMI 1640 (Nissui) supplemented with 5% calf serum (HyClone). Medium for colony formation assays was RPMI 1640 agar medium containing 10% calf serum.

* Corresponding author. Mailing address: Department of Radiation Research, Tohoku University School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai 980-77, Japan. Phone: 81-22-717-8134. Fax: 81-22-717-8136.

Irradiation of UV and X rays. Cells suspended in phosphate-buffered saline at a density of 6×10^5 (survival assays) or 1.2×10^6 (mutation induction assays) cells per ml were irradiated with UV at a dose rate of 0.2 J m^{-2} s^{-1} from 254-nm germicidal lamps (Hitachi). Cells suspended in medium at a density of 6×10^5 cells per ml were irradiated with 0.722 Gy of X rays per min with a PANTAK HF-320 X-ray source (200 kV, 10 mA; Shimadzu).

Survival assays. Cells in the growth phase were suspended in phosphate-buffered saline or in medium, irradiated if necessary, harvested, and resuspended in fresh culture medium. After dilution with molten agar medium (final concentration, 0.33% agar), 1-ml portions were plated onto 60-mm culture dishes containing a 4-ml base of 0.5% agar medium. Unless otherwise indicated, half of the plates were directly placed at 33°C, while the other half were incubated at 39°C for 20 h before being transferred to 33°C. The incubation at 33°C was continued until the 14th day after plating, at which point the colonies were counted and the level of survival was calculated.

Assay of UV-induced mutation frequency. Cells were maintained at 33°C in medium supplemented with HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) to suppress propagation of cells resistant to 6-thioguanine (6TG) that might preexist in the cell population. After a passage through medium supplemented with HT (100 μM hypoxanthine, 16 μM thymidine), exponentially growing cells were suspended in phosphate-buffered saline, UV irradiated if necessary, and harvested. A portion of the cells was used for the survival assay, and the remainder was transferred to nonselective medium and divided into two cultures, one of which was directly placed at 33°C and the other of which was incubated at 39°C for 20 h before being placed at 33°C. (At this step, it was critical to process cells in a population large enough to include the potentially mutating cells which should later express 6TG resistance. If this requirement were not satisfied, the apparent mutation frequencies would be found to fluctuate widely from experiment to experiment, because the population of the ts cells deficient in UV resistance decreases additively or synergistically by killings following exposure to UV and incubation at the restrictive temperature.) The incubation at 33°C in nonselective medium was continued for 2 weeks, to fully express 6TG resistance ($6TG^r$) mutations, with subculturing every 3 or 4 days. To determine plating efficiencies, a portion of the culture was plated on nonselective agar medium in the same way as for the survival assay. From the remainder, another portion was removed and resuspended at a density of 3.3×10^5 cells per ml in molten 0.33% agar medium containing 2.73 μg of 6TG per ml (Wako, Osaka, Japan). Three-milliliter portions, each containing 10^6 cells, were plated onto 100-mm culture dishes containing a 10-ml base of 0.5% agar medium to select $6TG^r$ cells. There was no apparent metabolic cooperation (31) in FM3A cells under these plating conditions. After a further 2 weeks at 33°C, colonies were scored and mutation frequencies were calculated by dividing the $6TG^r$ colony number by the plated cell number and the plating efficiency. Spontaneous mutation frequency was the mutation frequency of unirradiated cells, and induced mutation frequency was calculated by subtracting the spontaneous frequency.

Expression time assay for $6TG^r$ mutation. Cells were exposed to UV with or without a transient postirradiation incubation period at 39°C as described above for the induced mutation assay. Thereafter, they were maintained in 25 ml of nonselective medium at 33°C. Portions were removed on the day of irradiation, 4 days later, and thereafter every other day, and the UV-induced mutation frequency was assayed as described above. The reduced volume of the expression culture was brought up to 25 ml by adding fresh medium after every removal, so that the subsequent cultures were always in the growth phase.

RESULTS

Temperature sensitivity of ts mutants. Our basic strategy to study DNA repair in FM3A ts mutant cells was to inactivate the ts gene products only for the period necessary for the establishment of the DNA damage as a lethal hit. This was achieved by incubating the cells transiently at the nonpermissive temperature, 39°C. Before starting the experiments with the DNA-damaging agents, we evaluated the feasibility of this strategy by studying the survival kinetics of the ts mutant cells during incubation at 39°C, by colony formation assays (Fig. 1). Depending on the incubation period, the survival of all the ts mutants decreased in a similar shape of curves, though the sensitivities were quite different among the mutants. The results showed that a 1-day incubation at 39°C decreased survival of the ts mutants to 10 to 80%, resulting in an adequate number of survivors for further investigation with colony formation assays of the cell killing and mutations induced by DNA-damaging agents. Therefore, we decided to use a 20-h incubation period at the nonpermissive temperature, 39°C, in the studies with the DNA-damaging agents as this time length also fitted well with the experimental schedule.

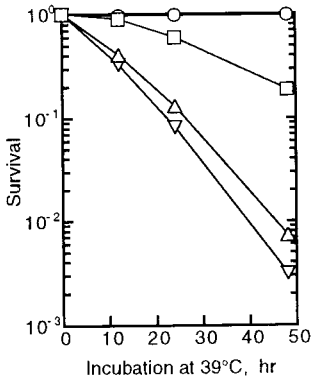

FIG. 1. Cell survival of FM3A ts mutants following nonpermissive-temperature incubation. Cells exponentially growing at 33°C were plated on agar medium, incubated at 39°C for the indicated times, and then returned to 33°C for colony formation. The survival of each cell line was calculated as the ratio of the plating efficiency to that of the non-39°C-treated control. Circles, wild-type FM3A clone 28; triangles, E1 mutant ts85; inverted triangles, Pol α mutant tsFT20; squares, $p34^{cdc2}$ kinase mutant tsFT210.

UV survival. The colony formation assay including the transient incubation at 39°C was used to examine the UV survival of the parental FM3A clone 28 and its ts mutants ts85, tsFT20, and tsFT210 (Fig. 2A and B), which are known to be defective at 39°C in E1, Pol α, and $p34^{cdc2}$ kinase activities, respectively. Cells were UV irradiated, plated onto agar medium, and incubated at 39°C for 20 h and then at 33°C to form colonies. The E1 mutant ts85 clearly expressed greater UV sensitivity than the others by the 20-h incubation at 39°C (Fig. 2A). The acquisition of UV sensitivity by the ts85 strain was not a general effect of the 39°C treatment but was specific to this strain, since the parental clone 28 and the other ts mutants were not significantly sensitized by the same treatment. A result similar to that for the ts85 mutant was observed for another E1 mutant, tsFT5 (data not shown), indicating that UV sensitization by the 39°C treatment is a common feature of E1 mutants, and not a cell-line-specific characteristic of ts85. A comparison of the survival curves of clone 28 with those of ts85 showed that the survival of these two clones differed significantly only if they were incubated at 39°C, irrespective of the UV doses used to irradiate the cells (Fig. 2A). This suggests that heat inactivation of E1 expected to occur specifically in ts85 cells enhances UV killing.

As ts85 cells are known to be arrested in late S and G_2 phases (8, 22), it was possible that the UV sensitivity induced in the ts85 cells was not a direct effect of inactivation of ts gene products but rather a result of growth arrest at a specific cell cycle phase(s). However, this appears unlikely as little UV sensitivity was induced for tsFT20 and tsFT210 (Fig. 2B), which are arrested at 39°C in the same S and G_2 phases of the cell cycle, respectively (8, 21, 24), as the ts85 clone.

Complementation by E1 cDNA transfection. To confirm that the UV sensitivity of ts85 induced by transient 39°C treatment is really the result of a ts mutation in the E1 gene, we performed a complementation test using clonal transformants of ts85 which were transfected with mouse E1 cDNA. A typical result is shown in Fig. 2A, where the temperature-induced UV sensitivity of the ts85 clone was completely abolished in the transformant, ts85YMTA1, to recover a level comparable to that of the wild-type clone 28. On the other hand, without the 39°C treatment, the UV resistance of the transformant did not exceed those of clone 28 and ts85. This ruled out the possibility of a nonspecific stimulatory effect on cell survival due to the

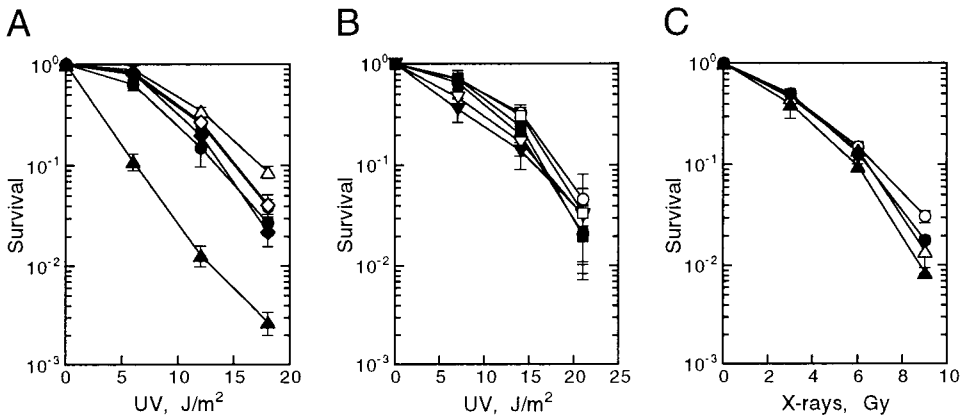

FIG. 2. Cell survival of FM3A ts mutants following UV or X-ray irradiation. (A) UV survival of the E1 mutant and its E1-cDNA transformant; (B) UV survival of the other mutants; (C) X-ray survival of the E1 mutant. Cells exponentially growing at 33°C were irradiated and plated on agar medium, followed by an incubation for colony formation at 33°C with (closed symbols) or without (open symbols) a prior 20-h incubation at 39°C. Survival was calculated for each experimental group of each cell line with or without the 39°C incubation as the ratio of their plating efficiencies to that of the unirradiated control. Error bars indicate standard deviations determined from at least three independent experiments. Circles, wild-type FM3A clone 28; triangles, E1 mutant ts85; inverted triangles, Pol α mutant tsFT20; squares, $p34^{cdc2}$ kinase mutant tsFT210; diamonds, an E1 cDNA transformant of ts85, ts85YMTA1.

exogenous E1. Similar results were obtained for the other clonal transformants of the E1 mutant cells (data not shown).

Dependence of UV survival on 39°C incubation time. Although 20 h was used as the 39°C incubation period, experimental confirmation of its virtue was necessary to precisely evaluate the methodology used in our study. Therefore, we investigated the kinetics of UV sensitization of ts85 at 39°C by changing variously the length of the 39°C incubation period in the UV survival assay (Fig. 3). Although the results showed time-dependent decreases in the viability of both irradiated and unirradiated ts85 cells at 39°C, the extent of heat inactivation of the irradiated cells was significantly greater than that of the unirradiated cells at every time point examined (Fig. 3A). These results indicate that killing by UV irradiation and incubation at 39°C is synergistic, probably reflecting the heat lability of some cellular activity mediating to remove or avoid UV-induced lethal damage. This activity is in all likelihood E1 in the ts85 clone. To extract only the synergistic effect, we divided the surviving fractions of the irradiated cells by those of the corresponding unirradiated cells. The surviving fractions of the unirradiated cells reflect the killing effect of the 39°C incubation alone. The calculated values are plotted in Fig. 3B and reveal that ts85 cells were killed quickly during a period of 12 h after the postirradiation shift to 39°C, running up to 85% of the overall sensitization to UV finally achieved by incubation at 39°C. More than 36 h at 39°C, however, did not produce any further sensitization for ts85. Therefore, the 20-h incubation at 39°C is sufficient for assaying the cell survival of E1 mutants. Longer incubation periods would create experimental difficulties with loss of cell population although little UV sensitization should be added. In addition, we found that cell inactivation at 39°C of the irradiated clone 28 was also moderately synergistic (Fig. 3B), suggesting that some normal cellular processes related to UV resistance are slightly susceptible to inactivation at 39°C although they remain unknown.

X-ray survival. Since the yeast *rad6* mutant is sensitive to X rays as well as UV (6), X-ray survival of the E1 mutant ts85 was also examined by the same method applied to the UV survival assay and compared with that of clone 28 (Fig. 2C). Unexpectedly, ts85 was not appreciably more sensitive than clone 28 irrespective of the 39°C treatment. Although this failure in detecting X-ray sensitivity for ts85 might suggest a special function for yeast *RAD6*-encoded E2 in X-ray resistance, it is also possible that our protocol did not allow ts85 to express X-ray sensitivity sufficiently because E1 inactivation was only transient. However, a prolonged 48-h incubation at 39°C failed to induce any sensitization to X rays in ts85 above the level of the non-39°C-treated cells (data not shown).

UV-induced mutation frequency. Our success in detecting UV sensitivity in mammalian E1 ts mutant cells prompted us to assay the UV-induced mutation frequency in these mutants with a 20-h postirradiation incubation period at 39°C. As shown in Fig. 4, the 39°C treatment enhanced the induced $6TG^r$ mutant frequencies of the wild-type clone 28 (Fig. 4B),

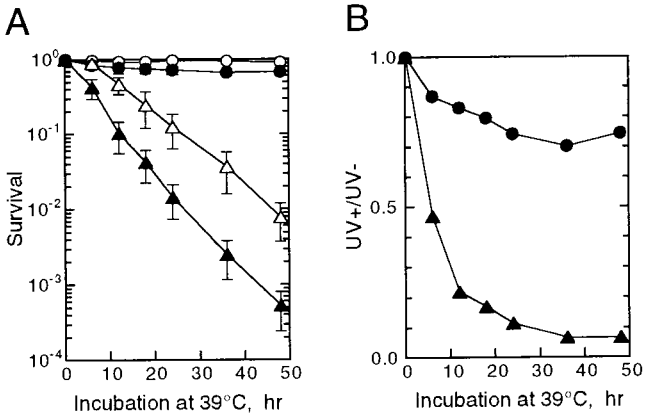

FIG. 3. Kinetics of UV sensitization of the E1 mutant, ts85, at 39°C. (A) Cell inactivation kinetics of UV-irradiated and unirradiated FM3A clone 28 and ts85 at 39°C. Cells exponentially growing at 33°C were irradiated with (closed symbols) or without (open symbols) 6 J of UV per m^2, plated on agar medium, incubated at 39°C for periods of 0 to 48 h, and then returned to 33°C for colony formation. Survival was calculated for each experimental group of each cell line with or without irradiation as the rate of their plating efficiencies to that of the corresponding non-39°C-treated cells. Error bars indicate standard deviations determined from at least four independent experiments. Some error bars of clone 28 are hidden behind the symbols. The survival at 33°C following 6-J/m^2 irradiation of UV was 0.76 ± 0.08 for clone 28 and 0.68 ± 0.21 for ts85. Circles, wild-type FM3A clone 28; triangles, E1 mutant ts85. (B) Synergistic cell killing of clone 28 and ts85 by UV irradiation and 39°C incubation. Surviving fractions of the irradiated cells shown in panel A were divided at every time point of the 39°C incubation by those of the corresponding unirradiated cells, and the values were plotted. Circles, clone 28; triangles, ts85.

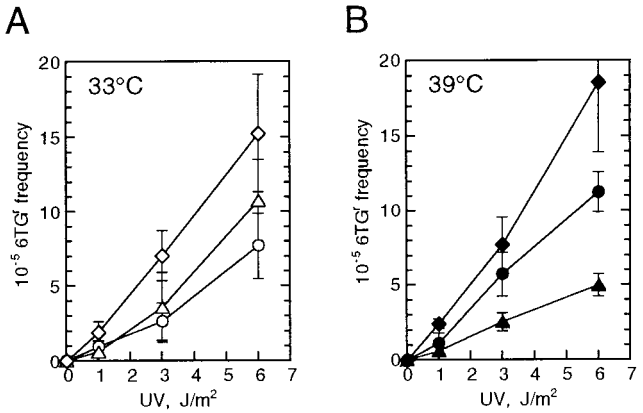

FIG. 4. UV induction of 6TG^r mutations in the E1 mutant, ts85. Cells exponentially growing at 33°C were UV irradiated with (closed symbols [B]) or without (open symbols [A]) a 20-h postirradiation incubation at 39°C, grown at 33°C in nonselective medium for 2 weeks, and then plated on agar medium containing 6TG. Induced mutation frequencies were calculated as described in Materials and Methods. Error bars indicate the standard deviations determined from at least four independent experiments. Circles, wild-type FM3A clone 28; triangles, E1 mutant ts85; diamonds, an E1 cDNA transformant of ts85, ts85YMTA1.

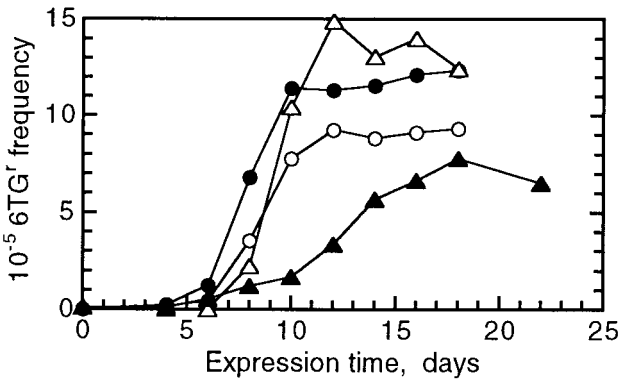

FIG. 5. Expression time for UV-induced 6TG^r mutation. Cells were irradiated with 6 J of UV per m^2, transferred to nonselective medium, subjected (closed symbols) or not (open symbols) to a 20-h incubation at 39°C, and then grown at 33°C. A portion was removed at the indicated postirradiation time points (expression time), and the frequency of 6TG^r mutations was assayed. A representative result is shown. Five independent experiments were performed, and similar results were obtained. Circles, wild-type FM3A clone 28; triangles, E1 mutant ts85.

compared to the control incubated only at 33°C (Fig. 4A). On the other hand, the same treatment rather suppressed the mutation induction of ts85 and resulted in a significant decrease of the induced mutation frequencies of ts85 compared with those of clone 28 (Fig. 4B), although there were no significant differences in the frequencies of the non-39°C-treated cells (Fig. 4A). The same results were obtained for another E1 mutant, tsFT5 (data not shown). These findings indicate that E1 inactivation results in inefficiency in the stable conversion of DNA lesions into mutations. This was confirmed by the remarkable recovery of the UV-induced mutagenesis in ts85 after transfection with E1 cDNA, as shown for ts85YMTA1 in Fig. 4B. Notably, the degree of recovery in the transformant exceeded the wild-type level whether the irradiated cells had been temperature shifted or not (Fig. 4), suggesting that an activity that promotes the induction of mutations is associated with E1.

The spontaneous mutation frequencies in all cell lines were 3×10^{-6} to 5×10^{-6} irrespective of the temperature, with the exception of ts85 treated with 39°C, which showed about three to five times more than the normal level (Table 1). Significant differences in the spontaneous mutation frequency of the 39°C-treated ts85 were apparent when compared to those of clone 28, ts85YMTA1, and non-39°C-treated ts85 cells. Thus, E1 inactivation might induce a moderate mutator phenotype in cells, as in the case of the yeast *rad6* mutant (34).

TABLE 1. Spontaneous mutation frequencies[a]

Cell type	No. of expts	39°C treatment	Spontaneous 6TG^r frequency[b] (10^{-6})
FM3A	9	−	3.5 ± 1.5
		+	4.2 ± 2.3
ts85	9	−	4.9 ± 3.4
		+	16.2 ± 6.0
ts85YMTA1	7	−	2.7 ± 1.8
		+	3.6 ± 1.9

[a] Cells maintained in HAT medium were passaged once through HT medium, harvested at exponential growth phase, resuspended in fresh nonselective medium, incubated or not at 39°C for 20 h, and then placed at 33°C. After 2 weeks, cells were plated onto agar medium containing 6TG and left to form colonies at 33°C for 2 weeks.

[b] Values are means ± standard deviations of the means.

Expression time of the UV-induced 6TG^r mutation. When clone 28 was mutagenized with UV and grown at 33°C, full expression of 6TG resistance took at least 12 days to appear (see Fig. 5). During this period, the preexisting wild-type hypoxanthine phosphoribosyltransferase activity should have been decreased and lost through dilution by cell division or degradation by proteolysis. However, the acquisition of maximal 6TG resistance in E1 mutants might be delayed by the 39°C treatment compared to wild-type cells, since E1 mutants arrest cell growth and are defective in protein degradation at the nonpermissive temperature (5). In other words, 2 weeks in the nonselective medium used for the mutation induction assay might be too short a time for the E1 mutants to acquire the complete 6TG^r mutant phenotype. To test this notion, we investigated the expression kinetics of 6TG resistance in clone 28 and ts85 by varying the nonselective incubation period available for expression of the mutant phenotype (Fig. 5). Without the 20-h postirradiation incubation at 39°C, ts85 expressed full 6TG resistance in 11 or 12 days like the wild-type clone 28. When clone 28 was subjected to the postirradiation 39°C treatment, the full expression of 6TG resistance was hastened by a day or two. This was in contrast to ts85, for which this treatment had the opposite effect of delaying the acquirement of the full 6TG resistance by 3 to 5 days compared with the non-39°C-treated controls. However, the maximum level of mutation frequency achieved by the 39°C-treated ts85 after the extended expression time was still much lower than those of the 39°C-treated clone 28 and the untreated ts85. Moreover, since the 39°C-treated ts85 shows a relatively high spontaneous mutation frequency as shown in Table 1, if the components derived from spontaneous mutations were subtracted from the mutation frequencies presented in Fig. 5, the differences between the 39°C-treated ts85 and the others should be larger than those actually appearing in Fig. 5. These results confirmed that E1 deficiency not only prolongs the time for the full expression of mutant phenotypes like 6TG resistance but also represses the conversion of lesions in cellular DNA into mutations.

DISCUSSION

Methodology. In order to estimate the surviving and mutagenic potentials of mammalian ts mutant cells exposed to DNA-damaging agents, we used a method that relied on the inactivation of the ts gene products for a limited period. This was achieved by transient incubation of the mutant cells at the nonpermissive temperature. We succeeded in detecting both UV sensitivity and a mutagenic deficiency in mouse E1 mutant cells, proving the suitability of this method. However, it should be pointed out that the phenotypes of DNA repair deficiency detected in this manner should be leaky, because inactivation of the mutant gene products might be delayed or even incomplete after the shift to 39°C. Moreover, it is possible that some repair activities recover or survive and work effectively even after the 20-h incubation at 39°C. In fact, more than 10% of the activity of histone ubiquitination remains in ts85 after an 8-h incubation at 39°C (23), and the UV sensitization and mutagenic deficiency found in ts85 are less evident than those of the budding yeast *rad6* mutant (18). Thus, the data obtained by the method used here may underestimate the contribution of the ts gene products to DNA repair processes. It is probable that E1 plays a more critical role in DNA repair of mammalian cells than that indicated by this study. Despite this possible underestimation, this method provides an effective tool for the detection of DNA repair defects in mammalian ts mutant cells.

E1 mutants. The defects in UV resistance and induced mutagenicity of E1 mutants, as well as their mutator phenotype, directly demonstrate the involvement of E1 in eukaryotic DNA repair pathways. These phenotypes of the mouse E1 mutant cells are similar to, though less remarkable than, those of the *S. cerevisiae rad6* mutants defective in a ubiquitin-conjugating enzyme, E2 (17, 18, 34). E1 and E2s catalyze the first and second steps, respectively, of the ubiquitin-conjugation pathways in a closely interactive manner (10), which strongly suggests that the ubiquitin system itself, but not E1 or E2 alone, contributes to the eukaryotic DNA repair process. Actually, the ubiquitin-mediating activity of the RAD6 protein, which is catalyzed by E1, is essential in yeast cells for UV resistance and mutagenicity (39, 40). Also, a ubiquitin mutant of yeast deficient in an alternative multiubiquitination site shows defective UV resistance and mutability (38). In addition, structural and functional homologs of the *RAD6* gene have been isolated from *Schizosaccharomyces pombe* (33), *Drosophila* (15), mouse (35), and human (16). Therefore, the involvement of the ubiquitin-conjugation system in DNA repair may be an important characteristic conserved among eukaryotic cells from yeasts to mammals.

Mechanisms implicating the ubiquitin-conjugation system in DNA repair. It has been suggested that the presence of many kinds of E2 species in yeast and mammalian cells implies that these enzymes, through their activation by E1, function in different cellular reactions and that they share multiple cellular functions mediated by the ubiquitin-conjugation system (10). Therefore, it is likely that some E1-E2 pathways specifically contribute to certain eukaryotic DNA repair reactions. Actually, their relevance in postreplicative DNA repair has been shown by a study of a yeast *rad6* mutant (32), although the precise molecular mechanisms remain unclear.

One possible mechanism whereby the ubiquitin system might participate in DNA repair is through a direct interaction between E1 or E2s and damaged regions of cellular DNA or repair enzymes working therein. In fact, yeast RAD6/UBC2 E2 forms a specific complex with the product of the DNA repair gene *RAD18*, which is able to bind single-stranded DNA (1). In addition, products of the human DNA repair genes *HHR23A* and *HHR23B* and their yeast homolog *RAD23* have a ubiquitin-like domain at their amino termini (20, 42), suggesting a physical association of these gene products with ubiquitin-binding proteins such as E1 or E2s. Actually, the ubiquitin-like domain has been shown to be essential for the DNA repair functions of the yeast RAD23 protein (42). An alternative mechanism by which the ubiquitin pathways work in DNA repair could be mediated indirectly through the proteolysis-promoting function of the ubiquitin system (10), for example, by removing stalled and dysfunctional enzyme complexes for replication, transcription, or repair from damaged DNA or by metabolizing repair and mutagenesis proteins for activation or degradation.

We also found that most of the UV sensitization of E1 mutant cells appeared within the first 12 h after the shift to 39°C. This suggests that the DNA repair pathway involving the ubiquitin-conjugation system processes UV-induced DNA lesions that would otherwise be lethal for cells if they were not repaired within half a day. Cyclobutane-pyrimidine dimers, the major type of DNA damage induced by UV irradiation, are removed by two distinctive nucleotide excision repair pathways, one working at actively transcribed DNA regions and the other covering the entire genome (2). The former pathway functions relatively faster than the latter and follows a time course that is similar to the sensitization kinetics of UV-irradiated E1 mutants at 39°C (2) (Fig. 3B). Therefore, the ubiquitin system might be more involved in the former, referred to as transcription-coupled DNA repair.

Mammalian cells induce cell-cycle arrest mechanisms called checkpoint controls in response to DNA damage (7). A tumor-related protein, p53, is a key factor functioning in checkpoint control, and its cellular level is thought to be regulated partly through the ubiquitin-mediated proteolytic pathway, according to studies using oncogenic human papillomavirus-infected cells (36). Chowdary et al. reported that p53 proteins are stabilized in a ts E1 mutant of mouse BALB/c 3T3 cells through a reduction in their breakdown after a shift to a restrictive temperature (4). This might suggest that alterations in the checkpoint controls induced by a deficiency of p53 metabolism cause the DNA repair defects observed in E1 mutants.

Pol α and p34^{cdc2} kinase mutants. The UV sensitivity of the Pol α mutant tsFT20 and the p34^{cdc2} kinase mutant tsFT210 was not higher than that of the wild-type clone 28, irrespective of the transient postirradiation incubation at 39°C (Fig. 2B). In addition, we did not detect any alterations in the X-ray sensitivity of tsFT20 with or without the 20-h treatment of 39°C (unpublished data). These results might suggest that Pol α and p34^{cdc2} kinase make little contribution to at least some DNA repair pathways that intervene rapidly after the occurrence of DNA damage. Although Pol α has been suggested to be relevant to DNA repair as a repair synthesis enzyme (44), recent reports about its role in repairing UV-damaged DNA are contradictory (3, 28). Studies with specific chemical inhibitors (28), antibodies (46), in vitro-reconstituted systems (27, 37), and a genetic approach (3) support the notion that the eukaryotic DNA polymerases δ and ε are repair synthesis enzymes of the eukaryotic nucleotide-excision repair system. The results presented in this study are consistent with this notion.

ACKNOWLEDGMENTS

This study was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, from the Special Researchers' Basic Science Program and the Biodesign Research Program of the Institute of Physical and Chemical Research (RIKEN), and from the Mitsubishi Foundation.

We thank T. Kato and C. Lawrence for helpful comments on the manuscript.

REFERENCES

1. **Bailly, V., J. Lamb, P. Sung, S. Prakash, and L. Prakash.** 1994. Specific complex formation between yeast RAD6 and RAD18 proteins: a potential mechanism for targeting RAD6 ubiquitin-conjugating activity to DNA damage sites. Genes Dev. **8:**811–820.
2. **Bohr, V. A., C. A. Smith, D. S. Okumoto, and P. C. Hanawalt.** 1985. DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell **40:** 359–369.
3. **Budd, M. E., and J. L. Campbell.** 1995. DNA polymerase required for repair of UV-induced damage in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:** 2173–2179.
4. **Chowdary, D. R., J. J. Dermody, K. K. Jha, and H. A. Ozer.** 1994. Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. Mol. Cell. Biol. **14:**1997–2003.
5. **Ciechanover, A., D. Finley, and A. Varshavsky.** 1984. Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell **37:**57–66.
6. **Cox, B., and J. Parry.** 1968. The isolation, genetics, and survival characteristics of ultraviolet light-sensitive mutants in yeast. Mutat. Res. **6:**37–55.
7. **Cox, L. S., and D. P. Lane.** 1995. Tumor suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. Bioessays **17:**501–508.
8. **Eki, T., T. Enomoto, A. Miyajima, H. Miyazawa, Y. Murakami, F. Hanaoka, M. Yamada, and M. Ui.** 1990. Isolation of temperature-sensitive cell cycle mutants from mouse FM3A cells. J. Biol. Chem. **265:**26–33.
9. **Finley, D., A. Ciechanover, and A. Varshavsky.** 1984. Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85. Cell **37:**43–55.
10. **Harshko, A., and A. Ciechanover.** 1992. The ubiquitin system for protein degradation. Annu. Rev. Biochem. **61:**761–807.
11. **Ikehata, H.** 1994. Comparison of DNA polymerases α, δ, and ε of mouse FM3A and its temperature-sensitive mutant tsFT20. Tohoku J. Exp. Med. **172:**65–81.
12. **Imai, N., S. Kaneda, Y. Nagai, T. Seno, D. Ayusawa, F. Hanaoka, and F. Yamao.** 1992. Cloning and sequencing of a functionally active cDNA encoding the mouse ubiquitin-activating enzyme E1. Gene **118:**279–282.
13. **Izumi, M., H. Miyazawa, S. Harakawa, F. Yatagai, and F. Hanaoka.** 1994. Identification of a point mutation in the cDNA of the catalytic subunit of DNA polymerase α from a temperature-sensitive mouse FM3A cell line. J. Biol. Chem. **269:**7639–7644.
14. **Jentsch, S., J. P. McGrath, and A. Varshavsky.** 1987. The yeast DNA repair gene *RAD6* encodes a ubiquitin-conjugating enzyme. Nature (London) **329:** 131–134.
15. **Koken, M., P. Reynolds, D. Bootsma, J. Hoeijmakers, S. Prakash, and L. Prakash.** 1991. *Dhr6*, a *Drosophila* homolog of the yeast DNA-repair gene *RAD6*. Proc. Natl. Acad. Sci. USA **88:**3832–3836.
16. **Koken, M. H. M., P. Reynolds, I. Jaspers-Decker, L. Prakash, S. Prakash, D. Bootsma, and J. H. J. Hoeijmakers.** 1991. Structural and functional conservation of two human homologs of the yeast DNA repair gene *RAD6*. Proc. Natl. Acad. Sci. USA **88:**8865–8869.
17. **Lawrence, C.** 1994. The *RAD6* DNA repair pathway in *Saccharomyces cerevisiae*: what does it do, and how does it do it? Bioessays **16:**253–258.
18. **Lawrence, C. W., J. W. Stewart, F. Sherman, and R. Christensen.** 1974. Specificity and frequency of ultraviolet-induced reversion of an iso-1-cytochrome *c* ochre mutant in radiation-sensitive strains of yeast. J. Mol. Biol. **85:**137–162.
19. **Longhese, M. P., P. Plevani, and G. Lucchini.** 1994. Replication factor A is required in vivo for DNA replication, repair, and recombination. Mol. Cell. Biol. **14:**7884–7890.
20. **Masutani, C., K. Sugasawa, J. Yanagisawa, T. Sonoyama, M. Ui, T. Enomoto, K. Takio, K. Tanaka, P. J. van der Spek, D. Bootsma, J. H. J. Hoeijmakers, and F. Hanaoka.** 1994. Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast RAD23. EMBO J. **13:**1831–1843.
21. **Mineo, C., Y. Murakami, Y. Ishimi, F. Hanaoka, and M. Yamada.** 1986. Isolation and analysis of mammalian temperature-sensitive mutant defective in G2 functions. Exp. Cell Res. **167:**53–62.
22. **Mita, S., H. Yasuda, T. Marunouchi, S. Ishiko, and M. Yamada.** 1980. A temperature-sensitive mutant of cultured mouse cells defective in chromosome condensation. Exp. Cell Res. **126:**407–416.
23. **Mori, M., T. Eki, M. Takahashi-Kudo, F. Hanaoka, M. Ui, and T. Enomoto.** 1993. Characterization of DNA synthesis at a restrictive temperature in the temperature-sensitive mutants, tsFT5 cells, that belong to the complementation group of ts85 cells containing a thermolabile ubiquitin-activating enzyme E1. J. Biol. Chem. **268:**16803–16809.
24. **Murakami, Y., H. Yasuda, H. Miyazawa, F. Hanaoka, and M. Yamada.** 1985. Characterization of a temperature-sensitive mutant of mouse FM3A cells defective in DNA replication. Proc. Natl. Acad. Sci. USA **82:**1761–1765.
25. **Murray, A. W.** 1992. Creative blocks: cell-cycle checkpoints and feedback controls. Nature (London) **359:**599–604.
26. **Nakano, N.** 1966. Establishment of cell lines in vitro from a mammary ascites tumor of mouse and biological properties of the established lines in a serum containing medium. Tohoku J. Exp. Med. **88:**69–84.
27. **Nichols, A. F., and A. Sancar.** 1992. Purification of PCNA as a nucleotide excision repair protein. Nucleic Acids Res. **20:**2441–2446.
28. **Nishida, C., P. Reinhard, and S. Linn.** 1988. DNA repair synthesis in human fibroblasts requires DNA polymerase δ. J. Biol. Chem. **263:**501–510.
29. **Nishitani, H., H. Goto, S. Kaneda, F. Yamao, T. Seno, P. Handley, A. L. Schwartz, and T. Nishimoto.** 1992. tsBN75 and tsBN423, temperature-sensitive X-linked mutants of BHK21 cell line, can be complemented by the ubiquitin-activating enzyme E1 cDNA. Biochem. Biophys. Res. Commun. **184:**1015–1021.
30. **Norbury, C., and P. Nurse.** 1992. Animal cell cycles and their control. Annu. Rev. Biochem. **61:**441–470.
31. **O'Neill, J. P., P. A. Brimer, R. Machanoff, G. P. Hirsch, and A. W. Hsie.** 1977. A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): development and definition of the system. Mutat. Res. **45:**91–101.
32. **Prakash, L.** 1981. Characterization of postreplication repair in *Saccharomyces cerevisiae* and effect of *rad6*, *rad18*, *rev3*, and *rad52* mutations. Mol. Gen. Genet. **184:**471–478.
33. **Reynolds, P., M. H. M. Koken, J. H. J. Hoeijmakers, S. Prakash, and L. Prakash.** 1990. The *rhp6*$^+$ gene of *Schizosaccharomyces pombe*: a structural and functional homolog of the *RAD6* gene from the distantly related yeast *Saccharomyces cerevisiae*. EMBO J. **9:**1423–1430.
34. **Roche, H., R. D. Gietz, and B. A. Kunz.** 1995. Specificities of the *Saccharomyces cerevisiae rad6*, *rad18*, and *rad52* mutators exhibit different degrees of dependence on the *REV3* gene product, a putative nonessential DNA polymerase. Genetics **140:**443–456.
35. **Roest, H. P., J. van Klaveren, J. de Wit, C. G. van Gurp, M. H. M. Koken, M. Vermey, J. H. van Roijen, J. W. Hoogerbrugge, J. T. M. Vreeburg, W. M. Baarends, D. Bootsma, J. A. Grootegoed, and J. H. J. Hoeijmakers.** 1996. Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. Cell **86:**799–810.
36. **Scheffner, M., J. M. Huibregtse, R. D. Vierstra, and P. M. Howley.** 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell **75:**495–505.
37. **Shivji, M. K. K., V. N. Podust, U. Hübscher, and R. D. Wood.** 1995. Nucleotide excision repair DNA synthesis by DNA polymerase ε in the presence of PCNA, RFC, and RPA. Biochemistry **34:**5011–5017.
38. **Spence, J., S. Sadis, A. L. Haas, and D. Finley.** 1995. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. Mol. Cell. Biol. **15:**1265–1273.
39. **Sung, P., S. Prakash, and L. Prakash.** 1990. Mutation of cysteine-88 in the *Saccharomyces cerevisiae* RAD6 protein abolishes its ubiquitin-conjugating activity and its various biological functions. Proc. Natl. Acad. Sci. USA **87:**2695–2699.
40. **Sung, P., S. Prakash, and L. Prakash.** 1991. Stable ester conjugate between the *Saccharomyces cerevisiae* RAD6 protein and ubiquitin has no biological activity. J. Mol. Biol. **221:**745–749.
41. **Th'ng, J. P. H., P. S. Wright, J. Hamaguchi, M. G. Lee, C. J. Norbury, P. Nurse, and E. M. Bradbury.** 1990. The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive *CDC2* gene product. Cell **63:**313–324.
42. **Watkins, J. F., P. Sung, L. Prakash, and S. Prakash.** 1993. The *Saccharomyces cerevisiae* DNA repair gene *RAD23* encodes a nuclear protein containing a ubiquitin-like domain required for biological function. Mol. Cell. Biol. **13:**7757–7765.
43. **Wilcox, D. R., and L. Prakash.** 1981. Incision and postincision steps of pyrimidine dimer removal in excision-defective mutants of *Saccharomyces cerevisiae*. J. Bacteriol. **148:**618–623.
44. **Yamada, K., F. Hanaoka, and M. Yamada.** 1985. Effect of aphidicolin and/or 2′, 3′-dideoxythymidine on DNA-repair induced in HeLa cells by four types of DNA-damaging agents. J. Biol. Chem. **260:**10412–10417.
45. **Yasuda, H., M. Kamijo, R. Honda, M. Nakamura, F. Hanaoka, and Y. Ohba.** 1991. A point mutation in C-terminal region of cdc2 kinase causes a G2-phase arrest in a mouse temperature-sensitive FM3A cell mutant. Cell Struct. Funct. **16:**105–112.
46. **Zeng, X.-R., Y. Jiang, S.-J. Zhang, H. Hao, and M. Y. W. T. Lee.** 1994. DNA polymerase δ is involved in the cellular response to UV damage in human cells. J. Biol. Chem. **269:**13748–13751.