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Source: Zoological Science, 14(5) : 747-752

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.747

ZOOLOGICAL SCIENCE **14**: 747–752 (1997)

Changes in UV Sensitivity with Cell Cycle, Clonal Age, and Cultural Age in *Paramecium tetraurelia*

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ABSTRACT—*Paramecium tetraurelia* cells age with repeated cell cycles and finally die after a specific number of cell divisions. In addition to this clonal aging, starvation results in cultural aging in division-arrested cells. They are reset to time zero to initiate a new clonal life cycle, if autogamy occurs when starved. Increased UV sensitivity is one of the representative markers of clonal aging in *P. tetraurelia*. We studied changes in UV sensitivity with the advance of cell cycle, clonal age, and cultural age. In this study, we made the following observations: 1) cells of any clonal age became less sensitive to UV as the cell cycle advanced; 2) cells became more sensitive to UV as clonal age advanced; 3) cells became temporarily hypersensitive to UV through an unknown mechanism at the clonal ages of less than 80 fissions; 4) cells at the stationary phase were more sensitive to UV than those at the log-phase; and 5) both autogamy-mature and immature cells became more sensitive to UV as cultural age advanced and became less sensitive with similar kinetics when transferred to culture medium. The fact that UV sensitivity increased with cultural age irrespective of the occurrence of autogamy suggests that this is caused by a different mechanism from that associated with clonal age because the latter could be canceled by autogamy.

INTRODUCTION

Paramecium tetraurelia cells have a clonal life cycle that begins with sexual reproduction (autogamy or conjugation) and terminates in clonal death (Sonneborn, 1954, 1957; Smith-Sonneborn, 1981, 1985; Takagi, 1988). They age as they repeat cell cycles and finally die after 150-300 fissions. If this process is interrupted by autogamy, i.e. sexual reproduction in a single cell, the aging cells are reset to time zero to initiate a new clonal life cycle. *P. tetraurelia* cells are unable to undergo autogamy during the period up to about 20 fissions after the preceding autogamy; this period is called autogamy-immaturity. Autogamy is thereafter induced by starvation. The cells undergo physiological changes called cultural aging if they remain unfed, and this process occurs in cell cycle-arrested cells under nutrient-poor conditions irrespective of the occurrence of autogamy. Clonal aging is related to the rounds of cell cycle under nutrient-rich conditions, whereas rejuvenation is related to the alternation of generations through autogamy with starvation. If well-fed mature cells are once starved and then refed, the latter cells are of the next generation if autogamy occurs during starvation, but both remains identical if autogamy does not occur. The aim of this study was to have an insight into the relationship among the cell cycle, clonal aging and cultural aging, by monitoring the changes in UV sensitivity during the whole life of *P. tetraurelia*.

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MATERIALS AND METHODS

Stocks

Stock 51 and F_1 clones of a cross between d4N-131 (mating type VII) and d4N-527 (mating type VIII) of *Paramecium tetraurelia* were used. Stocks of d4N-131 and d4N-527 are trichocyst non-discharging mutants homozygous for *nd242* and *nd169*, respectively, which are closely linked with a distance of only 0.5 centimorgans (Nyberg, 1978). The F_1 clones are trichocyst discharging because they are heterozygous for these two loci. The occurrence of autogamy is detectable at a probability of 99.5% by the appearance of trichocyst non-discharging cells, monitored by dropping a saturated solution of picric acid on sampled cells (Takagi *et al.*, 1987). When trichocyst non-discharging cells were detected in subclones cultured in daily reisolation lines, they were discarded.

Culture medium and handling of culture

Cells were cultured in 0.5% phosphate-buffered Wheat Grass Powder (Pines Int., USA) medium that had been inoculated with *Klebsiella pneumoniae* 2 days before use. Culture vessels for most of the experiments were 3-well depression slides housed in moist chambers: Petri dishes or Erlenmeyer flasks capped with silicon sponge closures (Sigma) were also used. Cultures were handled on a clean bench and incubation was at 25.5 ± 1°C. Single cells were treated with a micropipette under a dissecting microscope.

Advance of cell cycle

Dividing cells (dividers) were selected from log-phase cultures and pooled in fresh culture medium. One time-selection, usually comprising hundreds of dividers, was limited to 10 min to synchronize the cell cycle. Six different cell cycle groups, with 1 hr-intervals, were prepared because the duration of cell cycle was 6~8 hr under our experimental conditions. Each group was comprised of 30 cells, and 3 sets of 10 cells were UV-irradiated at the same time.

Advance of clonal age

Exconjugant and exautogamous cells were aged by daily transfer of single cells into fresh culture medium. The number of cells derived from a single cell was counted daily; $\log_2$ of the number was the number of fissions per day, and the sum of the daily fissions was the clonal age. The remaining cells after daily transfers were allowed to grow to a population of several thousand in Petri dishes in 10 ml of medium. Immature cells 5 and 9 fissions old as shown in Figs. 5 and 6, respectively, were prepared from mass cultures of ca. 100% autogamy. Aliquots of the culture were transferred to a known volume of fresh culture medium to allow a definite number of fissions on average.

Advance of cultural age

The cessation of growth in mass culture was detected when the culture medium became transparent because of the exhaustion of food bacteria. Cultures in this state were denoted as the early stationary phase, and the cultural age is indicated relative to the stationary phase as day 0. To determine the percentage of autogamy, cells were sampled from the 1-day-old cultures and those with fragmented macronuclei were counted after staining with Dippell's solution (10 parts acetocarmine, 4.5 parts 45% acetic acid, 2 parts 1N-HCl, and 1 part 1% solution of fast green FCF dissolved in 95% alcohol) (Dippell, 1955). The cultures in which more than 95% of cells were in autogamy were called "autogamy-mature" or simply "mature"; those in which less than 5% cells were in autogamy are referred to as "autogamy-immature" or "immature".

UV irradiation and judgment of UV sensitivity

For each experimental and control group, 3 sets of 10 cells (30 cells) were used. Each set (10 cells) was washed by transferring twice to Dryl's solution (Dryl, 1959) and finally placed in 0.1 ml of Dryl's solution. Three sets were irradiated concomitantly with UV in a dark box held in a dark room. Non-irradiated controls were treated in the same way except that they were put under UV light with a thick black-colored cover glass. A germicidal lamp emitting mainly at 253.7 nm was used for UV irradiation at a dose rate of 3.8 J/m^2/sec or 2.9 J/m^2/sec as measured by a Topcon radiometer.

After irradiation, the cells were transferred with a micropipette as single isolates under a dissecting microscope equipped with a red filter into wells of depression slides each containing 0.6 ml of culture medium. The slides were kept in the dark and examined after 4 days (Method I) or 14 days (Method II). In Method I, depressions containing 0 or 1 cell were scored as UV-sensitive, and UV sensitivity was expressed as a percentage relative to these. In Method II, depressions containing 0 cell were scored as UV-sensitive because the result on the 14th day was all (more than 1,000 cells) or nothing (0 cell). As cells which did not divide for 4 days could recover to produce a clone of 1,000 cells or more after 10 more days, Methods I and II represent a tentative measurement of repairable UV damage and a permanent non-repairable UV damage, respectively. In this study, the results are shown in either of them.

RESULTS

Cells became less sensitive to UV with advance of the cell cycle

Cells that were dividing were pooled, the cell cycle was allowed to continue, and cells were irradiated with UV every hour from the last division for 6 hr. Cells of different clones at different clonal ages showed decreased UV sensitivity with advance of the cell cycle (Fig. 1). The gradual decrease in UV sensitivity during the cell cycle was a general pattern also observed in the other experiments (data not shown). The degree of sensitivity was, however, not consistent among the cells of the same clonal ages if different clones were used, because of sporadic occurrence of UV hypersensitivity as mentioned below.

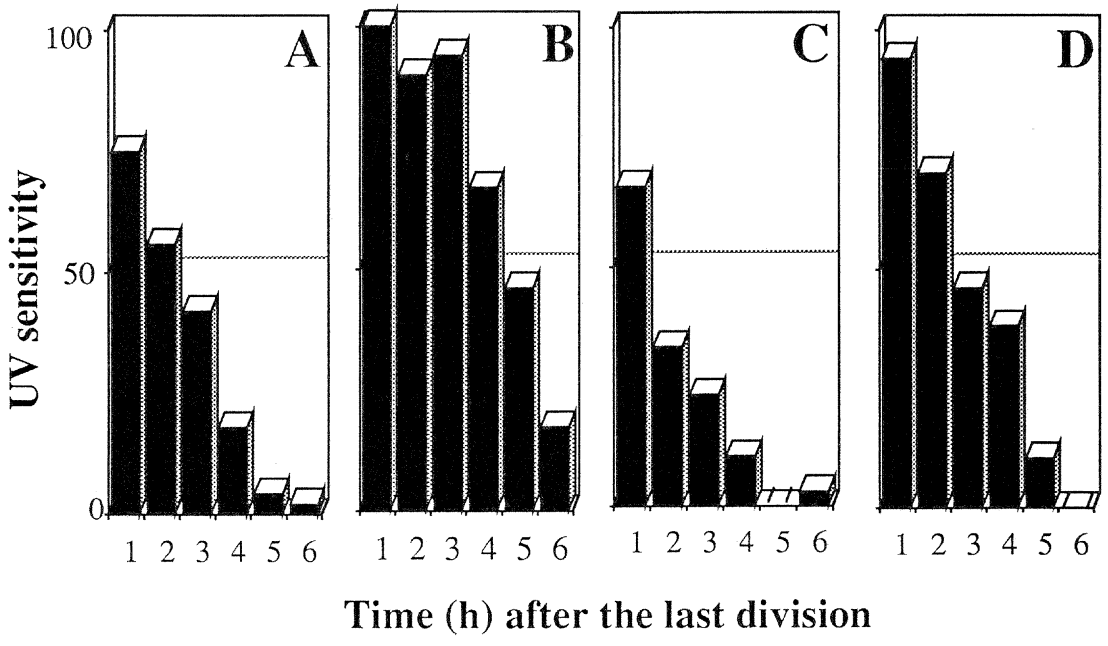

Fig. 1. Changes in UV sensitivity with cell cycle. **A**, **B**, **C** and **D** are different F_1 clones derived from a cross between d4N-131 and d4N-527; their clonal ages were 16, 30, 44 and 76 fissions old, respectively. For each clone, dividing cells were pooled in culture medium to advance the cell cycle synchronously, and were irradiated with UV of 456 J/m^2 with 1-hr intervals. The UV sensitivity was judged by Method I (percentages of dead and non-divided cells 4 days after irradiation).

Cells became more sensitive to UV with advance of clonal age, but became temporarily hypersensitive at a young age

Exautogamous clones were monitored for UV sensitivity at various clonal ages. In dividing cells of a given clonal age, the cell cycle was allowed to continue for 1 hour and then cells were irradiated with UV. At UV doses of 609 J/m^2 (2.9 J/m^2/sec, 3.5 min) or more, almost all cells of 44 fissions or older died, while at UV doses of 174 J/m^2 (1 min) or less, more than half of the cells survived at all clonal ages. When UV dose was 522 J/m^2 (3 min) or 348 J/m^2 (2 min), cells showed increasing sensitivity with clonal age, except for those of 20 fissions that showed hypersensitivity (Fig. 2). Using the same data, UV sensitivity is depicted as a function of UV dose in Fig. 3. The curves of UV sensitivity shifted to the left with increasing clonal age, indicative of increasing sensitivity with clonal age. However, the curve for the cells at 20 fissions overlapped that of those at 105 fissions. Fig. 3 indicates the existence of a threshold dose of UV for cells of each clonal age, suggesting that every kind of the lesions caused by UV less than the threshold would all be repaired.

The hypersensitivity of the cells at 20 fissions was not an artifact because a similar phenomenon was observed in almost all of the clones studied. The hypersensitivity, however, was not fixed to a specific number of fissions; it appeared in some cases several times at younger clonal ages of less than 80 fissions (data not shown).

Cells at the stationary phase were more sensitive to UV than those at the log-phase

A population of cells at the clonal age of 52 fissions was starved to advance cultural age. Daily cell samples were irradiated with UV at 171 J/m^2 (3.8 J/m^2/sec, 45 sec) and 228 J/m^2 (1 min). Although the dose of 171 J/m^2 was far less than

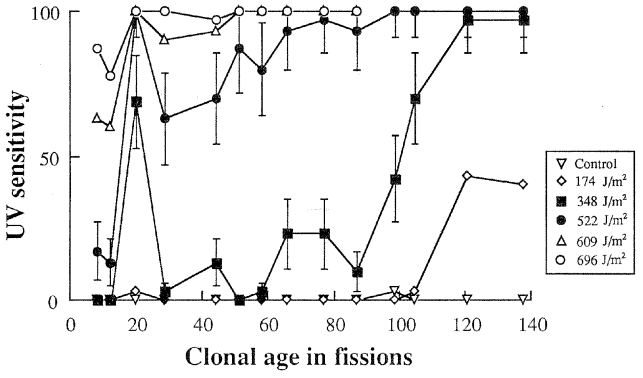

Fig. 2. Changes in UV sensitivity with clonal age. Cells of stock 51 at various clonal ages were irradiated with various doses of UV. The cells were at G_1 phase of the cell cycle (1 hr after division). Bars indicate mean 90% confidence limits. The UV sensitivity was judged by Method II (percentages of dead cells 14 days after irradiation).

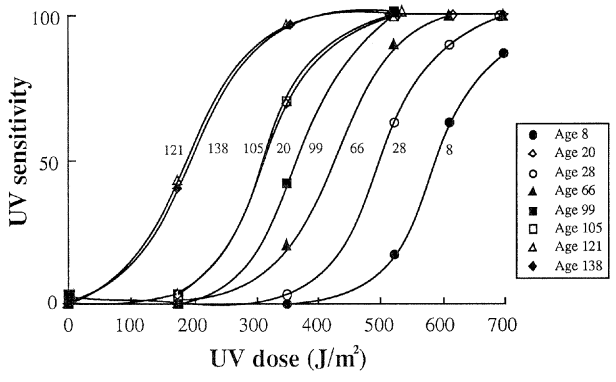

Fig. 3. Dose-dependence pattern of UV sensitivity at different clonal ages. Based on the data shown in Fig. 2, UV sensitivity of the cells at 8 clonal ages was depicted as a function of UV dose. The numbers indicate clonal ages in fissions.

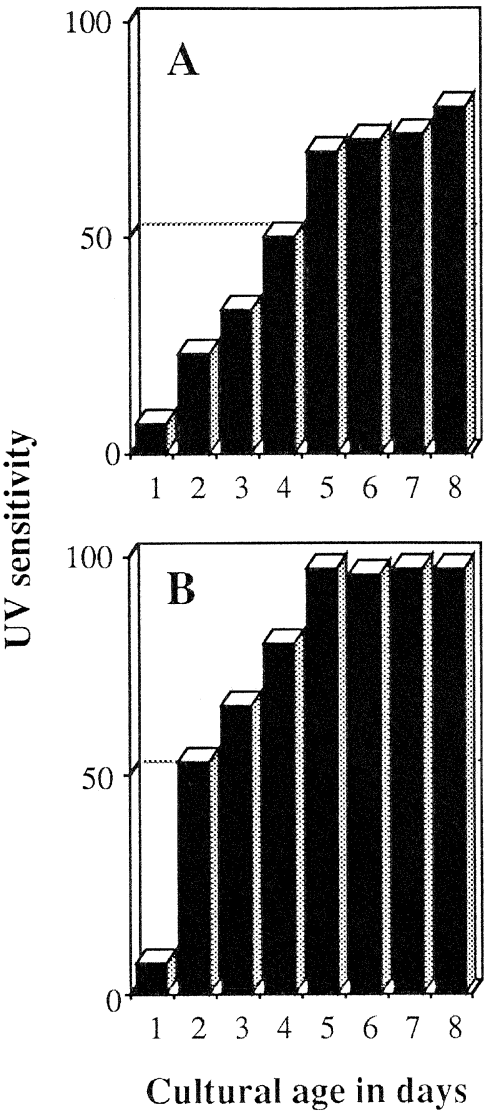

Fig. 4. Changes in UV sensitivity with cultural age. The clone **C** in Fig. 1 at the age of 52 fissions old was used. A mass culture at this age was allowed to age in culture and cells (96% were in autogamy) were irradiated daily with UV of 171 J/m^2 (**A**) and 228 J/m^2 (**B**). The UV sensitivity was judged by Method I.

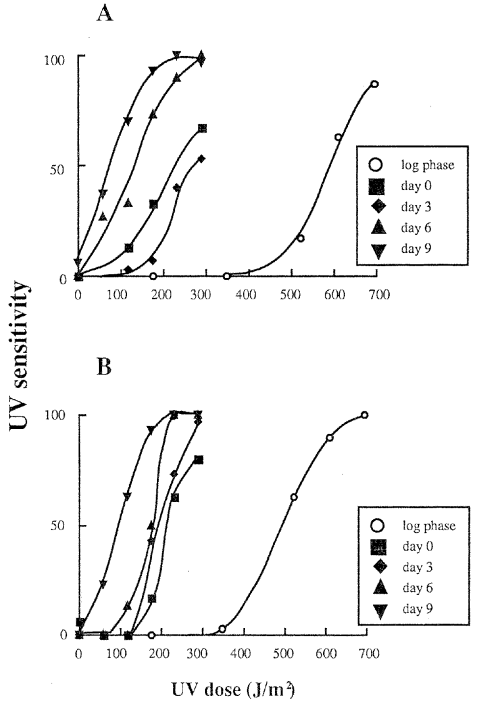

Fig. 5. Dose-dependence of UV sensitivity at different cultural ages of day 0, day 3, day 6 and day 9 in immature (**A**) and mature (**B**) cells. Cells of stock 51 at the clonal ages of 9 fissions old (immature cells) and 30 fissions old (mature cells 98% of which were in autogamy) were used. UV doses at the stationary phase were 58, 116, 174, 232 and 290 J/m^2. Cells of the log phase (G_1) of the clonal ages of 8 (immature) and 29 fissions old (mature, but no autogamy because of cell cycling) were also studied; UV doses were 348, 522, 609 and 696 J/m^2.

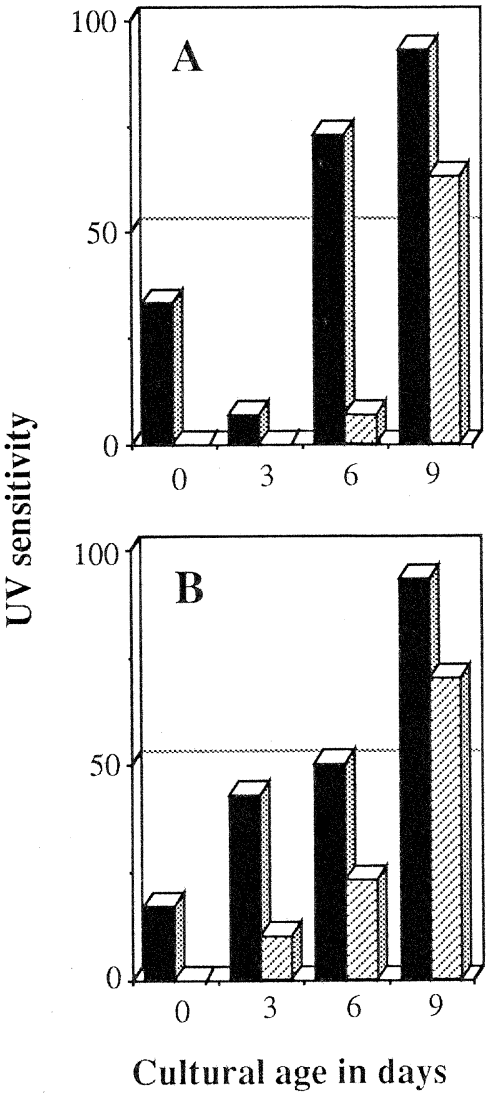

Fig. 6. Comparison of changes in UV sensitivity between immature (**A**) and mature (**B**) cells, in which cultural age is advancing and reverting. Cells of stock 51 at the clonal ages of 5 fissions old (immature cells) and 80 fissions old (mature cells 99% of which were in autogamy) were used. Some of the cells of advancing cultural age were irradiated daily with UV of 171 J/m^2 (closed column) and their aliquots were fed and irradiated with the same dose of UV 5 hr later (hatched column). The UV sensitivity was judged by Method II.

the threshold for G_1 cells of the corresponding clonal age (Fig. 3), cells of the 1-day-old culture were UV-sensitive to some extent and became more sensitive with the advancing cultural age (Fig. 4). The UV sensitivity leveled off at cultural age 5 (Fig. 4) as we used Method I here. If the Method II was applied, the UV sensitivity would continue to increase until the cultural age 8 as suggested in the following experiments. The increase in UV sensitivity with cultural age was commonly observed in cells of all clonal ages examined (data not shown).

To test the effect of autogamy on UV sensitivity, we studied autogamy-immature cells in parallel with autogamy-mature cells. As shown in Figs. 5 and 6, in both types of cells, the UV sensitivities were far greater when starved than well fed and increased with cultural age with similar kinetics. The increases in UV sensitivity continued until the cultural age of day 9, because Method II was used. When cells of different cultural ages were transferred to culture medium and their UV sensitivities were studied 5 hr later, they showed decreased UV sensitivities in both immature and mature cells, the rate of the recovery being dependent on cultural age (Fig. 6).

DISCUSSION

UV sensitivity has been studied in many organisms, but there have been few such studies in paramecia. In the present study, we examined UV sensitivity at different stages in the life cycle of *P. tetraurelia* cells, including the advancing cell cycle, clonal aging, and cultural aging. In this study, we used 10-fold higher doses of UV than used in other organisms. The macronucleus of *Paramecium* is polygenic, most if not all of the micronuclear genes being amplified during the differentiation process of the new macronucleus (Freiburg, 1988; Raikov, 1995). This is considered to be why paramecia are generally more resistant to UV than other diploid cells.

The cell cycle of *P. tetraurelia* consists of G_1, S and D at the macronuclear level (there is no G_2), where D stands for division and is used instead of M because the macronuclear

division is not mitotic but amitotic. The G_1 phase occupies one quarter of the total cell cycle (Berger, 1988). In this study, the total cell cycle was 6-8 hr, and therefore the first irradiation of UV at 1 hr after division was regarded to target the G_1 phase cells. The present results showed that the UV sensitivity was highest in the early G_1 phase and decreased gradually during the S phase (Fig. 1). We used Method I for this experiment (UV-irradiated cells that died or failed to divide during the first 4 day-incubation were scored as UV-sensitive), but we would have reached the same conclusion with Method II. Even if all of the survivors in the 1 hr group on the 4th day (those which failed to divide) would be recovered on the 14th day, and all of the survivors in the 6 hr group on the 4th day would die on the 14th day, the UV sensitivity of the former group was still higher than that of the latter group.

The decrease in UV sensitivity with the advance of cell cycle is consistent with the results in other cells such as L cells (Rauth and Whitmore, 1966), yeast (Chanet *et al.*, 1973), chinese hamster ovary cells (Burg *et al.*, 1977), and cyanobacterium (Amla, 1983). The increasing nuclear DNA during the S phase may make the cells less vulnerable to UV. However, the situation is not so simple. In HeLa cells, for example, UV sensitivity is maximal in the mid S phase and then decreases in the rest of the S phase (Djordjevic and Tolmach, 1967; Watanabe and Horikawa, 1974). Why UV sensitivity varies with different cell cycle stages remains unclear. Different repair mechanisms may function at different cell cycle stages. It also should be considered that DNA lesions in transcriptionally active genes would be repaired earlier and more effectively than those in transcriptionally inactive genes (Terleth *et al.*, 1990; Hoeijmakers, 1993).

UV sensitivity of G_1 cells in *P. tetraurelia* increased with the advance of clonal age (Figs. 2 and 3). It is consistent with the previous report by Smith-Sonneborn (1971) at least in the clones older than 80 fissions. She assessed the UV sensitivity by death rate in F_1 progeny after autogamy, while we assessed the UV sensitivity by death rate in asexual progeny, representing UV damage in the somatic macronucleus. The death rate in F_1 progeny would be represented by UV damage primarily in the germinal micronucleus, but the macronucleus is essential for the proper execution of sexual reproduction (Mikami, 1992, 1996). Therefore, the increase in UV sensitivity with clonal age may be attributed to macronuclear functional decline; errors accumulated unrepaired in aged macronuclei would exceed the lethal threshold by a low dose of UV irradiation. The macronuclear DNA content decreases with clonal age in various species of *Paramecium* (Schwartz and Meister, 1973, 1975; Klass and Smith-Sonneborn, 1976; Takagi and Kanazawa, 1982). This is, however, insufficient to explain the clonal age-related increase in UV sensitivity, because the decreases in macronuclear DNA in *P. tetraurelia* (Klass and Smith-Sonneborn, 1976) were inconsistent with the increases of UV sensitivity.

A peculiar and interesting phenomenon found in this study was the UV hypersensitivity at the age of 20 fissions (Figs. 2 and 3). Since *P. tetraurelia* cells become capable of undergoing autogamy at about this age, we first supposed that the phenomenon may be related to the transition from autogamy-immaturity to maturity. Thus, we repeated the experiments and found that UV sensitivity did become very high at clonal ages younger than 80 fissions, but the hypersensitive ages varied among clones and there were several peaks in some cases (unpublished). These observations raise questions about the previous explanation for the age-related increase in UV sensitivity, i.e. gradual accumulation of unrepairable errors with age (Smith-Sonneborn, 1979). As the high UV sensitivities at young ages were neither age-related nor irreversible, they should be distinguished from those at ages older than 80 fissions, for which the earlier explanation is reasonable.

We demonstrated that the stationary phase (G_0) cells were much more sensitive to UV than G_1 cells, which were more sensitive than S and D phase cells, and that they became more sensitive to UV with the advance of cultural age (Figs. 4-6). This is contrast to previous studies in normal human fibroblasts (Chan and Little, 1979) and in yeast cells (Siede and Friedberg, 1990) in which the stationary (G_0) phase cells were most resistant to UV. It is notable that the increase in UV sensitivity with the advance of cultural age in *P. tetraurelia* occurred in a similar way in both mature and immature cells (Figs. 5 and 6). Immature cells retained their normal state of one macro- and two micronuclei during the experimental period. In mature cells, 96% of which were in autogamy, the macronuclei were broken into fragments and finally disappeared while the micronuclei were at drastic stages of meiosis, fertilization, and differentiation to the new macro- and micronuclei. In both types of cells, when refed, UV sensitivity decreased in a similar way (Fig. 6), indicating that the increase in UV sensitivity with cultural age is elicited by a mechanism different from the increase in UV sensitivity with clonal age, which can be canceled only in the mature cells through autogamy. It appears that the cell membrane rather than DNA may be a major target of UV in cell cycle-arrested cells.

There are reasons that the UV sensitivity should be considered in relation with the state of the cell membrane, in addition to the DNA level and repair mechanism. Exposure of UV to mammalian cells triggers the UV signaling cascade that finally activates some transcription factors of the UV-sensible genes. The cascade is initiated on the plasma membrane, and not on nuclear DNA; for example, NF-κB, one of the transcription factors, is activated in enucleated cells by UV (Devary *et al.*, 1993). The elicitor of UV signaling would be the growth factor receptor on the plasma membrane in mammalian cells (Devary *et al.*, 1992; Sachsenmaier *et al.*, 1994). Since there is evidence that ciliates including *Paramecium* have receptors that can respond to mammalian growth factors (Andersen *et al.*, 1984; Hide *et al.*, 1989; Csaba and Kovacs, 1990, 1991; Christensen, 1993; Sternberg and McGuigan, 1994; Christopher and Sundermann, 1995; Tokusumi *et al.*, 1996), the difference of UV sensitivity between clonal aging and cultural aging in *P. tetraurelia* should also be considered in this context.

ACKNOWLEDGMENTS

We thank Dr. Hiroshi Utumi of Kyoto University for his reading the manuscript and helpful suggestions. This research was supported in part by the Sasakawa Scientific Research Grant of The Japan Science Society to N.Y.

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(Received April 18, 1997 / Accepted June 17, 1997)