STUDIES ON SOMATIC CELL MUTATIONS
I. RADIATION- AND CHEMICAL-INDUCTION OF NUTRITIONALLY
DEFICIENT AND SUFFICIENT MUTANTS IN CHINESE
HAMSTER *HAI* CELLS *IN VITRO*

SADAYUKI BAN¹⁾, FUMIO SUZUKI AND MASAKATSU HORIKAWA

Division of Radiation Biology, Faculty of Pharmaceutical Sciences,
Kanazawa University, Kanazawa 920

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The establishment of sensitive assay systems for the mutations using mammalian cells *in vitro* is necessary for the detection of various potential mutagens present in the human environment and for the elucidation of the mechanisms of somatic mutation and phenotypic expression in cultured mammalian cells. Since genetic and biochemical studies of somatic mammalian cells *in vitro* were developed by Puck and his co-workers (Puck and Marcus 1955; Puck and Fisher 1956; Puck *et al.* 1956), many kinds of mutant cell lines, such as nutritional mutants (Kao and Puck 1967, 1968, 1969; Chu *et al.* 1969; Morrow 1971), drug-resistant cell lines (Lieberman and Ove 1959; Atkins and Gartler 1968; Chu and Malling 1968; Biedler and Riehm 1970; Kessel and Bosmann 1970; Orkin and Littlefield 1971; Shapiro *et al.* 1972) and temperature-sensitive mutants (Naha 1969; Thompson *et al.* 1970; Meiss and Basilico 1972; Willingham *et al.* 1973; Wittes and Ozer 1973; Wang 1974), have been isolated from various cultured mammalian cell lines, and studies of the mutations induced by radiations and chemicals using these various mutant cells have been conducted by many workers. However, there still remain many problems to be solved on account of the complexity of the mutations in cultured mammalian cells, especially because the induced mutation frequency in cultured cells is affected by the mutation expression time, the size of cell inoculum, the concentration of the selective agent, etc.

In the present study, we examined the frequency of the forward and reverse mutations induced by radiations (X-rays and UV) and chemicals (4-nitroquinoline 1-oxide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ethyl methanesulfonate), by using prototrophic CH-*hai* Cl 23 cells and auxotrophic CH-*hai* Cl 3 cells (Suzuki and Horikawa 1975) isolated from an original Chinese hamster *hai* cell line, and compared the sensitivity as the assay systems for mutagens. It was demonstrated by using these systems that the antimicrobial food additiv, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylic acid amide (furylfuramide: FF or AF2), has powerful mutagenic activity so far as it was tested by the sensitive forward mutation system using prototrophic CH-*hai* Cl 23 cells.

1) Present address: Department of Experimental Radiology, Faculty of Medicine, Kyoto University, Kyoto 606.

MATERIALS AND METHODS

Cells and medium.

Alanine-, asparagine-, proline-, aspartic acid-, and hypoxanthine-prototrophic clone (CH-*hai* Cl 23 cells (Suzuki and Horikawa 1975)) and thymidine-auxotrophic clone (CH-*hai* Cl 3 cells (Suzuki and Horikawa 1975)) isolated from an original Chinese hamster *hai* cells by our replica plating method (Suzuki and Horikawa 1973) were used in the present studies. The culture procedures and properties of these clones have been described previously (Suzuki and Horikawa 1975). Briefly, auxotrophic CH-*hai* Cl 3 cells were maintained in a complete medium composed of 90% Eagle's MEM+N18 medium (Suzuki and Horikawa 1973) and 10% dialyzed calf serum (Suzuki and Horikawa 1973) after the isolation. On the other hand, it was found by the subsequent analyses that CH-*hai* Cl 23 cells were also prototrophic for glutamic acid. Hence, this clone was maintained in a deficient medium composed of 90% Eagle's MEM+N12 medium (which lacked alanine, asparagine, proline, aspartic acid, hypoxanthine, and glutamic acid from Eagle's MEM+N18 medium) and 10% dialyzed calf serum. The average doubling times (and the average plating efficiencies) of a prototrophic CH-*hai* Cl 23 cells in a complete medium and deficient medium at 37°C were 13.2 hours (and 60.1%) and 15 hours (and 58.8%) which were determined by the cell growth and colony-forming ability respectively, whereas those of an auxotrophic CH-*hai* Cl 3 cells in a complete medium were 13.2 hours and 56.1%.

Additionally, thymidine-prototrophic CH-*hai* Cl 7 cells (colone 7 in Table 2 (Suzuki and Horikawa 1975)) isolated from the original Chinese hamster *hai* cells by the replica plating method were used as a control for the assay of thymidylate synthetase activity. These cells were maintained in a medium composed of 90% Eagle's MEM+N17 medium (which lacked thymidine from Eagle's MEM+N18 medium) and 10% dialyzed calf serum.

Radiations and chemicals.

The agents used as the mutagens were X-rays, UV-radiation, 4-nitroquinoline 1-oxide (4-NQO; Daiichi Pure Chemicals Co. Ltd., Tokyo), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Tokyo Kasei Kogyo Co. Ltd., Tokyo), ethyl methanesulfonate (EMS; Nakarai Chemicals, Ltd., Kyoto) and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylic acid amide (furylfuramide: FF or AF2; provided by Dr. A. Tonomura, Tokyo Medical and Dental University). X-rays and UV were irradiated with various doses, at a dose rate in air of 75 R/min as measured with a Victoreen condenser chamber and at a dose rate of 20 ergs/mm²/sec at the surface of glass petri dishes respectively.

EMS was dissolved in Hanks' balanced salt solution. 4-NQO and MNNG were dissolved in a small amount of absolute ethanol and diluted with Hanks' solution. On the other hand, FF was dissolved in a small amount of dimethyl sulfoxide (DMSO) and diluted with Hanks' solution. In some cases, FF was autoclaved at 120°C (15 lbs) for 20 min after being dissolved in DMSO. One ml aliquot of each Hanks' solution prepared in this manner was added to the cell suspensions (9 ml each) to be treated. In the cases of treatment with 4-NQO, MNNG and FF, maximum final concentrations of ethanol and DMSO in the cell suspensions were 0.1%, 0.2% and 5% respectively. There-

fore, Hanks' solution containing ethanol or DMSO was always added to the cell suspensions of the control group in each experiment, so as to make 0.1%, 0.2% or 5% in a final concentration.

Induction of nutritionally deficient (auxotrophic) mutants and their selection.

One ml suspensions containing 1×10^6 prototrophic CH-*hai* Cl 23 cells in the complete medium were suspended in short test tubes (Pyrex glass, inner diameter 10 mm, length 100 mm), and irradiated with various doses of X-rays. For UV-irradiation, 1×10^6 cells suspended in 1 ml of Eagle's MEM+N18 medium free of phenol red were spread over the surface of a 90-mm glass petri dish, and irradiated with various doses of UV. For chemical-treatment, 1 ml of Hanks' solution containing various concentrations of each chemical was added to 9 ml suspensions containing 1×10^6 CH-*hai* Cl 23 cells in complete medium, and incubated by shaking slowly in a 37°C water bath for 2 hours. After the treatment, cells were washed twice with Hanks' solution and suspended in 1 ml of a complete medium.

Each unirradiated (untreated) or irradiated (treated) cell suspension was transferred to a 200 ml square culture bottle containing 9 ml of a complete medium, and the bottles were incubated at 37°C for the mutation expression for various lengths of time. In order to select auxotrophic mutants from prototrophic CH-*hai* Cl 23 cells, a modification of the 5-bromodeoxyuridine (BUdR)-visible light method originally described by Puck and Kao (1967) was used. Being based on various fundamental experiments, we have established the procedure for selecting auxotrophs, as shown in Fig. 1.

Namely, after the treatment with radiation or chemical and incubation for the mutation expression (a), 1×10^5 cells from each bottle were distributed into 90-mm petri dishes containing 10 ml of a deficient medium composed of 90% (Eagle's MEM+N12-thymidine (TdR)) medium and 10% dialyzed calf serum, and the dishes were incubated in a 5% CO₂ incubator for 24 hours as the starvation time (b). (Thymidine was omitted from Eagle's MEM+N12 medium for stimulating the incorporation of BUdR into cellular DNA at the next step). After the incubation, BUdR was added to each dish so as to make 3×10^{-6} M at a final concentration and again incubated for 24 hours (c). After

Fig. 1. Schematic diagram of the procedure for selecting auxotrophs from prototrophic CH-*hai* Cl 23 cells.

two more washings, cells of dishes were exposed to visible light for 1 hour (d). This was accomplished by exposing cells attached on glass petri dishes containing Eagle's MEM medium free of phenol red to a depth of approximately 3 mm, to 120-W light (three 40-W fluorescent lamps, Hitachi "Sun Line Ace" tubes, 117.5 cm long), which were mounted in a fixture with an approximately parabolic reflector, so that the lamps were 5.5 cm apart, and 10 cm above the dishes.

By these procedures, prototrophic cells which are able to grow in a deficient medium, may take BUdR into their DNA and be killed by visible light. Only the colonies formed by auxotrophic mutants induced may appear in the dishes by the incubation in a complete medium. However, there are some possibilities of appearance of the colonies formed by prototrophic cells which passed through the BUdR-visible light treatments as mentioned above. Therefore, after the exposure with visible light and removal of Eagle's MEM medium free of phenol red, the dishes (containing cells treated with identical doses of radiation or chemical) were always divided into two groups (3 dishes in each), as shown in Fig. 1. Each dish of one group was added by 10 ml of a complete medium (A) and the other by a deficient medium (which lacked alanine, asparagine, proline, aspartic acid, hypoxanthine, and glutamic acid from a complete medium) (B). After 10 days of incubation in a CO₂ incubator, the colonies in each dish were fixed, stained, and the colonies containing more than 50 cells were counted. The colonies appearing in the dishes of the latter (B) should be ones formed by the original prototrophic cells. Hence, the number of the colonies appearing in each dish of the latter (B) subtracted from the number of the colonies in each dish of the former (A) was decided as the correct number of the colonies formed by auxotrophic cells respectively.

Induction of nutritionally sufficient (prototrophic) revertants and their selection.

Auxotrophic CH-*hai* Cl 3 cells maintained in a complete medium were dissociated by trypsinization. After the irradiation with X-rays or UV (or the treatment with chemical for 2 hours) and incubation in a complete medium for various lengths of time, as described above, 5×10^4 cells from each bottle were distributed into 90-mm petri dishes containing 10 ml of a selection medium composed of 90% Eagle's MEM+N17 medium (which lacked thymidine from Eagle's MEM+N18 medium) and 10% dialyzed calf serum, and the dishes were placed in a CO₂ incubator for 10 days. Then, the colonies in each dish were fixed, stained, and counted. In this case, 5×10^4 cells were particularly distributed into each petri dish, because it was found by another experiment that the maximum frequency of the mutations induced in CH-*hai* Cl 3 cells occurred in the inoculation of 5×10^4 cells per dish.

Survival measurements of the cells treated with radiations or chemicals.

Immediately after treatment of the cells with various doses of radiations or chemicals and after incubation for various lengths of time, followed by the treatment as described above, each 1 ml cell suspension containing 500 cells was placed in a 60-mm glass petri dish with an additional 4 ml of a complete medium. The dishes were incubated for 10 days, and the colonies were counted.

Determination of the mutation frequencies.

The number of auxotrophic mutant and prototrophic revertant colonies induced by

radiations or chemicals was corrected for the decrease in survival of cells incubated for various lengths of time following the treatment with radiations or chemicals; i.e., the mutation frequencies were expressed as the number of auxotrophic and prototrophic colonies per 10^5 surviving colonies.

Preparation of cell extracts and assay of thymidylate synthetase.

$2-3 \times 10^7$ cells in the logarithmic phase of growth were collected by centrifugation at 2,000 rpm for 5 min, and washed twice with phosphate buffered saline free of CaCl_2 and MgCl_2 . The cells were suspended in 1 ml of ice-cold 0.01M Tris-HCl buffer (pH 7.0) containing 0.25M sucrose, and disrupted by an ultrasonic disruptor (UR-200P, Tomy Seiko Co. Ltd., Tokyo) at 20 Kc for 15 sec in an ice bath. The supernatant fluid was collected by centrifugation at 30,000 g for 60 min, and used for the assay of thymidylate synthetase activity and protein content (Lowry *et al.* 1951).

Thymidylate synthetase activity was assayed by a modified combination of the methods described by Roberts (1966) and Orkin and Littlefield (1971). First, a solution A consisting of 8.6×10^{-4} M tetrahydrofolate, 0.075M 2-mercaptoethanol, 0.235M Tris-HCl buffer (pH 7.5), and 0.43% formaldehyde was prepared freshly each week and stored frozen. Next, a solution B consisting of 250 μl of 1.0M NaF, 100 μl of 2.8×10^{-3} M deoxyuridine 5'-monophosphate (dUMP), 500 μl of 1.0M Tris-HCl buffer (pH 7.5), 120 μl of $\text{d}[5\text{-}^3\text{H}]\text{UMP}$, 15 μCi (11 Ci/m mole; The Radiochemical Centre Amersham), and 500 μl of the solution A was prepared freshly just prior to use.

A 50 μl aliquot of cell extract was added to the substrate mixture consisting of 10 μl of the solution B and 20 μl of 1.25% bovine plasma albumin in Tris-sucrose solution (pH 7.0). After incubation at 37°C for 60 min, the reaction was terminated by the addition of 1 ml of 2% Norit A in 0.01N HCl solution. After vigorous mixing, each reaction tube was allowed to stand 15 min before its contents were filtered through a Sartorius Millipore filter (pore size 0.45 μm and 25 mm in diameter). 0.4 ml of the filtrate was counted in 10 ml Tritol scintillation fluid (Triton X-100, toluene, PPO and POPOP). A control from which enzyme was omitted was subtracted in each assay. Specific activity of thymidylate synthetase was expressed as the number of n mole of substrate converted (the amount of tritium released to water from position 5 of the pyrimidine ring of dUMP upon its conversion to thymidine 5'-monophosphate (dTMP)) per mg of cell extract protein per 60 min at 37°C.

RESULTS

Mutation expression time.

It is known that the length of the mutation expression time markedly affects the number of mutant colonies that arise. Therefore, the optimal expression time (defined as the time prior to challenge necessary for the maximum number of mutant colonies to arise) for 800R of X-rays was determined by using a forward mutation system from prototrophic CH-*hai* Cl 23 cells to auxotrophic cells. The results are shown in Fig. 2, in which the mutation frequency seems to increase with time up to 48 hours. It is clear that the maximum number of mutations could be recovered 48 hours after the

Fig. 2. The effect of the mutation expression time on the mutation frequency of prototrophic CH-*hai* Cl 23 cells following irradiation with 800R of X-rays. The bars indicate standard deviations of the mean for three independent experiments.

irradiation. However, the mutation frequency seems to decrease again after 48 hours.

On the other hand, spontaneous auxotrophic cells requiring any one of alanine, asparagine, proline, aspartic acid, hypoxanthine, and glutamic acid for their growth could not be found at any time of the mutation expression, as seen in Fig. 2. This may be due to the fact that the prototrophic characteristics of CH-*hai* Cl 23 cells is stable as described previously (Suzuki and Horikawa 1975). Being examined by the BUdR-visible light method as described in MATERIALS AND METHODS, the mean spontaneous mutation frequency of this characteristics is calculated to be 2.0×10^{-7} . From the above findings, 48 hours as the mutation expression time which followed the treatment with radiations or chemicals was used for the following experiments.

The frequency of the forward and reverse mutations induced by radiations in prototrophic and auxotrophic cells.

First, we compared the sensitivity between the forward and reverse mutation assay systems in order to detect the mutations induced by X-rays and UV. The dose-survival curves of prototrophic CH-*hai* Cl 23 cells and auxotrophic CH-*hai* Cl 3 cells irradiated with various doses of X-rays are shown in Figs. 3a and b. The forward and reverse mutation frequencies per 10^5 survivors of CH-*hai* Cl 23 cells and CH-*hai* Cl 3 cells incubated for the mutation expression of 48 hours following the irradiation with various doses of X-rays are also shown in Figs. 3a and b respectively. As seen in these figures, a significant non-linear increase in the frequency of forward mutations from prototrophs to auxotrophs was caused with increasing exposure to X-rays, while the increase of the reverse mutation frequency from auxotrophs to prototrophs was not remarkable. Especially, in the case of the latter, the increase of the mutation frequency was not observed at doses higher than 600R.

Similarly, the dose-survival curves of prototrophic CH-*hai* Cl 23 cells and auxotro-

Fig. 3. a) X-rays dose-survival curve of prototrophic CH-hai Cl 23 cells and induction of auxotrophs after X-irradiation. The bars indicate standard deviations of the mean for four independent experiments. b) X-rays dose-survival curve of auxotrophic CH-hai Cl 3 cells and induction of prototrophs after X-irradiation. The bars indicate standard deviations of the mean for three independent experiments.

phic CH-hai Cl 3 cells irradiated with various doses of UV, and the forward and reverse mutation frequencies per 10^5 survivors of CH-hai Cl 23 cells and CH-hai Cl 3 cells after the irradiation with various doses of UV are shown in Figs. 4a and b respectively. There was again a significant non-linear increase of the forward mutation frequency with increasing exposure to UV, that is, the frequency of the forward mutations from prototrophs to auxotrophs caused by UV increased significantly with increasing exposure from 0 to 150 ergs/mm², but it decreased at doses higher than 150 ergs/mm². On the other hand, the increase of the reverse mutation frequency from auxotrophs to prototrophs caused by UV-irradiation was not so remarkable, similarly in the case of X-rays. The increase of mutation frequency was not especially observed at higher ranges of dose. These findings indicate that the forward mutation system from prototrophs to auxotrophs established in this study is much more sensitive than the reverse mutations system from auxotrophs to prototrophs, for the detection of the mutation induced by X-rays and UV.

The frequency of the forward and reverse mutations induced by chemicals in prototrophic and auxotrophic cells.

Next, we compared the sensitivity between the forward and reverse mutation systems for detecting the mutations induced by chemical carcinogens or mutagens such as 4-NQO, MNNG and EMS. In this case, the cells were treated with each chemical ranging in the concentration which gave dose-survival curves near to that of X-rays

Fig. 4. a) UV dose-survival curve of prototrophic CH-hai Cl 23 cells and induction of auxotrophs after UV-irradiation. The bars indicate standard deviations of the mean for four independent experiments. b) UV dose-survival curve of auxotrophic CH-hai Cl 3 cells and induction of prototrophs after UV-irradiation. The bars indicate standard deviations of the mean for four independent experiments.

or UV, as measured by the colony-forming ability. Figs. 5a and 6a show the dose-survival curves of prototrophic CH-hai Cl 23 cells and auxotrophic CH-hai Cl 3 cells treated with various concentrations of 4-NQO, MNNG and EMS at 37°C for 2 hours respectively. On the other hand, the induced forward and reverse mutation frequencies per 10⁵ survivors of CH-hai Cl 23 cells and CH-hai Cl 3 cells incubated for 48 hours, following the treatment with various concentrations of these chemicals at 37°C for 2 hours are shown in Figs. 5b and 6b. (The dose-survival curves of CH-hai Cl 23 cells and CH-hai Cl 3 cells irradiated with various doses of X-rays and UV, and the induced forward and reverse mutation frequencies per 10⁵ survivors of CH-hai Cl 23 cells and CH-hai Cl 3 cells after the irradiation with various doses of X-rays and UV are also shown in Figs. 5b and 6b respectively as the references.) As seen in Fig. 5b, the frequencies of the forward mutations caused by 4-NQO and MNNG increased significantly according to the increasing concentrations, but it decreased at concentrations higher than $6 \times 10^{-7} \text{ M}$, in the case of 4-NQO. The increase of the forward mutation frequencies induced by EMS was not so remarkable as compared with those by 4-NQO and MNNG. On the other hand, it is difficult to conclude something from the results shown in Fig. 6b, because the frequencies of the reverse mutations induced by these chemicals were very low as a whole. In any case, it can be concluded from these figures that the forward mutation system is much more sensitive than the reverse mutation system, in detecting the mutations induced by these chemical carcinogens or mutagens, similarly

Fig. 5. a) Dose-survival curves of prototrophic CH-hai Cl 23 cells plotted against doses of X-rays, UV, 4-NQO, MNNG and EMS. Each point shows the mean of three to four independent experiments. b) Induced mutation frequency of auxotrophs *versus* doses of X-rays, UV, 4-NQO, MNNG and EMS for prototrophic CH-hai Cl 23 cells. Each point shows the mean of three to four independent experiments.

Fig. 6. a) Dose-survival curves of auxotrophic CH-hai Cl 3 cells plotted against doses of X-rays, UV, 4-NQO, MNNG and EMS. Each point shows the mean of three to four independent experiments. b) Induced mutation frequency of prototrophs *versus* doses of X-rays, UV, 4-NQO, MNNG and EMS for auxotrophic CH-hai Cl 3 cells. Each point shows the mean of three to four independent experiments.

Fig. 7. a) Dose-survival curves of prototrophic CH-hai Cl 23 cells plotted against doses of X-rays, UV and FF. Each point shows the mean of three to four independent experiments. $\square$, FF unautoclaved after dissolved in DMSO; $\blacksquare$, FF autoclaved after dissolved in DMSO. b) Induced mutation frequency of auxotrophs *versus* doses of X-rays, UV and FF for prototrophic CH-hai Cl 23 cells. Each point shows the mean of three to four independent experiments. $\square$, FF unautoclaved after dissolved in DMSO; $\blacksquare$, FF autoclaved after dissolved in DMSO.

in the cases of X-rays and UV.

Examination of the mutagenicity of food additive, furoylfuranamide (FF).

Finally, we examined the mutagenicity of the antimicrobial food additive, FF, by the use of the sensitive forward mutation system from prototrophs to auxotrophs. Fig. 7b shows the frequencies of the forward mutations induced by FF ranging in the concentration which gave a dose-survival curve near to that of X-rays or UV, as shown in Fig. 7a. From these figures, it can be concluded that FF has a powerful mutagenic activity as well as X-rays and UV. In addition, it was found that the mutagenic activity of FF decreases considerably (about one fourth of that of the unautoclaved) by autoclaving at 120°C (15 lbs) for 20 min after it was dissolved in DMSO as described in MATERIALS AND METHODS, although the killing activity of this chemical, as measured by the colony-forming ability of the cells, is not so affected by autoclaving.

Thymidylate synthetase activities in auxotrophic CH-hai Cl 3 cells and their revertants.

Thymidylate synthetase activities in auxotrophic CH-hai Cl 3 cells and their revertants (CH-hai Cl 3-R2, Cl 3-R7 and Cl 3-R15) which were obtained from auxotrophic CH-hai Cl 3 cells after the irradiation with 600R and 1,000R of X-rays or 100 ergs/mm² of UV were respectively assayed. Table 1 shows the specific activities of thymidylate synthetase in soluble, cell-free extracts of thymidine-prototrophic CH-hai Cl 7 cells

Table 1. Thymidylate synthetase activities in soluble, cell-free extracts of thymidine-prototrophic CH-*hai* Cl 7 cells, auxotrophic CH-*hai* Cl 3 cells and their revertants

Cell line	Spec. Act. (n mole/mg protein/h)	Relative activity (%)
TdR-prototrophic CH- <i>hai</i> Cl 7	1.00±0.31	100
TdR-auxotrophic CH- <i>hai</i> Cl 3	0.10±0.09	10
Revertants		
CH- <i>hai</i> Cl 3-R2 (600R)	0.91±0.33	91
CH- <i>hai</i> Cl 3-R7 (1000R)	0.98±0.26	98
CH- <i>hai</i> Cl 3-R15 (100 ergs/mm ²)	0.56±0.11	56

Activities are the mean and standard deviation of five independent determinations.

(see MATERIALS AND METHODS), thymidine-auxotrophic CH-*hai* Cl 3 cells and their revertants. Three revertants used here were randomly chosen from 18 revertant clones which were obtained from auxotrophic CH-*hai* Cl 3 cells after the irradiation with various doses of X-rays or UV.

As seen in this table, the enzyme activity of auxotrophic CH-*hai* Cl 3 cells was low, that is, 10% of that of thymidine-prototrophic CH-*hai* Cl 7 cells. However, in three clones (CH-*hai* Cl 3-R2, Cl 3-R7 and Cl 3-R15) which were reverted from auxotrophic CH-*hai* Cl 3 cells by X- or UV-irradiation, their enzyme activities reverted to 91%, 98% and 56% of that of CH-*hai* Cl 7 cells respectively.

DISCUSSION

Considerable efforts have been made in the direction of the study of mutagenesis in somatic mammalian cells, by using various cell lines such as mouse, rat, Chinese hamster, monkey and human cells, and also various genetic markers. 8-azaguanine resistant mutations have been especially used by many workers as the most popular system. However, there are still many complexities in the mutations of cultured mammalian cells, since it is known that the mutation frequency in cultured cells induced by chemicals and radiations is affected by the mutation expression time, the size of cell inoculum and the concentration of the selective agent and so on (Chu and Malling 1968; Bridges and Huckle 1970; Arlett and Harcourt 1972; Shapiro *et al.* 1972; Duncan and Brookes 1973).

In this experiment, we chose 1×10^5 to prototrophic CH-*hai* Cl 23 cells and 5×10^4 to auxotrophic CH-*hai* Cl 3 cells per 90-mm petri dish as the inoculum sizes. These inoculum sizes are based on the results of another fundamental experiments, in which we found that the maximum frequencies of induced mutations in CH-*hai* Cl 23 cells and CH-*hai* Cl 3 cells occurred in the inoculation of 1×10^5 cells and 5×10^4 cells per dish respectively. Furthermore, we chose 48 hours as the mutation expression time

for both CH-*hai* Cl 23 cells and CH-*hai* Cl 3 cells following the treatment with radiations or chemicals. This mutation expression time is based on the fact that the maximum frequency of the forward mutations in prototrophic CH-*hai* Cl 23 cells occurred 48 hours after the irradiation with a relatively high dose (800R) of X-rays (Fig. 2). This time roughly corresponds to three to four generations of the normal CH-*hai* Cl 23 cells.

The tests under these conditions indicate that the forward mutation system from prototrophic CH-*hai* Cl 23 cells (Ala⁺, Asn⁺, Pro⁺, Asp⁺, Hyp⁺, Glu⁺) to auxotrophs which were established in this study was much more sensitive than the reverse mutation system from auxotrophic CH-*hai* Cl 3 cells (TdR⁻) to prototrophs, for detecting mutations induced by chemical carcinogens or mutagens (4-NQO, MNNG and EMS), as well as by X-rays and UV. It is generally accepted that forward mutations are more sensitive than reverse mutations as indicators for screening the mutagenicity of agents, such as radiations and chemical mutagens. As an explanation of this, the suggestion by Chu *et al.* (1969) and Chu (1971) may be applicable that forward mutations represent the whole spectrum of mutational events, while reverse mutations to the wild-type allele require specific genetic alteration at the mutation site. However, in this study, another possibility may be considered as the reason why the forward mutation system is much more sensitive than the reverse mutation system, that is, there is the difference in the number of genetic markers between prototrophic CH-*hai* Cl 23 cells and auxotrophic CH-*hai* Cl 3 cells used here. In the forward mutation system, the changes in any one of alanine-, asparagine-, proline-, aspartic acid-, hypoxanthine-, and glutamic acid-sufficient characteristics of CH-*hai* Cl 23 cells can be detected as mutants, whereas in the reverse mutation system, only the changes in thymidine-deficient characteristic of CH-*hai* Cl 3 cells can be detected as mutants. The further study of determining whether this possibility is correct or not is now in progress.

Although it is difficult to conclude something from the results obtained from the reverse mutation system because the frequencies of reverse mutations induced by radiations and chemicals were generally very low and had rather complicated dose-effect relationships (Figs. 3b, 4b and 6b), it seems to be certain that the reverse mutations in this system are associated with the reverted activity of thymidylate synthetase (Table 1). Namely, it can be seen in Table 1 that thymidylate synthetase activities in the revertant clones which were induced in auxotrophic CH-*hai* Cl 3 cells by X- or UV-irradiation reverted to that or about half of that in thymidine-prototrophic CH-*hai* Cl 7 cells. On the other hand, it can be concluded from the results obtained by the forward mutation system that 4-NQO and MNNG are highly mutagenic, similar to X-rays and UV. In EMS whose high mutagenicity in cultured mammalian cells was first reported by Chu and Malling (1968) and Kao and Puck (1969), we could not observe remarkable induction of the forward mutations by this chemical, compared with 4-NQO and MNNG. We do not yet have any definite solution to this discrepancy. However, (a) the differences in Chinese hamster cell line used by each group, and (b) the insufficient mutation expression time after EMS-treatment, may be the reasons for this discrepancy, although their concentrations in this study were always adjusted by dose-survival curve.

It was furthermore found by the forward mutation system that the antimicrobial

food additive, FF, has a high mutagenic activity. This result agrees well with those found by Wild (1975) and Kuroda (1975), who used cultured Chinese hamster cells and human diploid cells respectively. In addition, it was found that the mutagenic activity of FF decreases considerably (about one fourth of that of the unautoclaved) by autoclaving at 120°C (15 lbs) for 20 min, whereas the killing activity of this chemical is not so affected by this treatment (Figs. 7a and 7b). This may be partly due to the geometrical isomerization of FF (conversion of *trans*-FF to *cis*-FF) by heating, as reported by Saikachi *et al.* (1966) or due to the partial decomposition of FF by heating and high pressure.

In contrast to the cases of X-rays and MNNG, the frequencies of the forward mutations induced by UV, 4-NQO, EMS and FF decreased clearly at higher doses, although the doses of each radiation or chemical used here were adjusted by each dose-survival curve, as mentioned above. Of course, the reason for this is still unknown at the present time. However, it can be supposed that such a decrease in the mutation frequencies at higher doses of UV, 4-NQO, EMS and FF may result from the lethal character of the mutations induced by relatively higher doses of these agents.

In any case, a sensitive forward mutation system from prototrophic CH-*hai* Cl 23 cells to auxotrophs established in this study can serve as a useful system for detecting environmental mutagens and for elucidating the mechanisms of somatic cell mutation. But the characteristics of mutant cells selected in this forward mutation system remains to be analysed.

SUMMARY

A forward mutation system using prototrophic CH-*hai* Cl 23 cells (Ala⁺, Asn⁺, Pro⁺, Asp⁺, Hyp⁺, Glu⁺) isolated from an original Chinese hamster *hai* cell line was found to be much more sensitive than a reverse mutation system using auxotrophic CH-*hai* Cl 3 cells (TdR⁻), for detecting the mutations induced by chemical carcinogens or mutagens (4-nitroquinoline 1-oxide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ethyl methane-sulfonate), as well as by X-rays and UV. It was found that the antimicrobial food additive, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylic acid amide (furylfuramide: FF or AF2) has highly mutagenic activity so far as it was tested by the forward mutations. The forward mutation system, from prototrophic CH-*hai* Cl 23 cells to auxotrophs, established in this study can be applied as a useful system for detecting environmental mutagens and for elucidating the mechanisms of somatic cell mutation.

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