An Improved Method for Isolation of Mutator Mutants from Mouse FM3A Cells and Their Characterization

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ABSTRACT. An improved method to select mutator mutants was developed. By this new method, mutator mutants were isolated efficiently, and 7 mutants were obtained from cultured mouse FM3A cells. These mutator mutants have an elevated rate of spontaneous mutation at 3 genetic loci (resistance to ouabain, blasticidin S, and tunicamycin). The sensitivity of these mutants to aphidicolin and arabinofuranosylcytosine was the same as in the wild-type cells. Determination of the size of the cellular dNTP pool revealed that there was no large imbalance in the precursor pool in the mutator mutants. These results suggested that the mutator character may be due to alteration in some factor(s) correlated directly to DNA replication. Also, there was no change in the sensitivity of all these mutator mutants to DNA damaging agents.

Mutation in the cellular genes occurs either spontaneously or is induced by a variety of mutagens. One form of spontaneous mutation, which could be due to the incorporation of mismatching nucleotides into DNA, occurs during DNA replication or repair of spontaneously occurring DNA damage such as the apurinic sites (10). Spontaneous mutation may also be caused by other changes in DNA, such as deletion or insertion of some sequences (13), but the detailed molecular mechanism is unknown.

One of the approaches to this is to study mutator mutants where spontaneous mutation occurs at a higher rate than in the wild-type cells. In *E. coli*, there are more than 10 genetic loci which affect the spontaneous mutation rate (3). Recent studies with some *E. coli* mutants have revealed that mutation in the subunits of DNA polymerase III holoenzyme affects the fidelity of DNA replication (4, 14). From these results it was suggested that there are mechanisms to control the faithfulness of DNA replication.

Mutator mutants have also been isolated from several mammalian cell lines, and some of them have been characterized (1, 2, 12, 18). In most of them, however, mutator activity has been shown to be associated with an imbalance in the cellular dNTP pool. Thus studies with these mutants have identified mutator genes which code for such enzymes as ribonucleoside diphosphate reductase (1, 2, 18), dCMP deaminase (18), thymidylate synthetase (1), and dCTP synthetase (17). These cell clones were first isolated as mutants with altered sensitivity to inhibitors of DNA

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replication, such as arabinofuranosylcytosine (AraC) or aphidicolin.

In addition to these mutator mutants, Liu *et al.* (9) recently reported that one of the mutants resistant to aphidicolin had altered DNA polymerase α . They showed that the mutant DNA polymerase was resistant to aphidicolin and had a decreased K_m for dCTP.

Studies with these mammalian mutator mutants should help clarify the mechanism of somatic cell mutation, and this will also lead us to understand the mechanism of regulation of DNA replication. For this purpose, we previously developed a method to isolate mutator mutants from mouse FM3A cells which was based directly on the alteration in mutation rate (5). An improved method for this isolation is described here.

MATERIALS AND METHODS

Cell culture. A clone termed F9 was selected from several spontaneous mutants lacking hypoxanthine phosphoribosyl transferase (HPRT⁻ mutants) which were isolated as 6-thioguanine-resistant cells from a mouse FM3A cell clone F28-7 (7). This clone was used as the parental cell for the isolation of mutator mutants. F9 cell produced revertants spontaneously at a frequency of approximately 10^{-7} , and the frequency increased about 20–50 times after treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (our unpublished results). These facts suggest that the reversion from HPRT⁻ to HPRT⁺ occurred by a mutation.

Cells were grown in suspension either in Eagle's essential medium (MEM) supplemented with 5% calf serum and 0.1% bactopectone or in ES medium, a slightly modified MEM (7), supplemented with 5% calf serum at 34°C in an atmosphere of 5% CO₂ and moist air.

Isolation and identification of mutants. To isolate mutator mutants from mouse FM3A cells, we applied the selection system of the HPRT locus. The advantage of using this system is that HPRT⁺ cells or HPRT⁻ cells can be selected from the mixed population by HAT (hypoxanthine/aminopterin/thymidine) medium or medium containing 6-thioguanine, respectively.

The new protocol for isolation of mutator mutants is shown in Fig. 1. For the isolation procedure, ES medium was used.

Cells were mutagenized by treatment with 0.5 μ g/ml of MNNG for 4 h at 34°C. With this treatment, mutation at the mutator gene is expected to occur, while the gene for the enzyme HPRT of this cell may not be affected at this stage; thus the phenotype of the mutator mutant would be HPRT⁻. Treatment with MNNG also caused reversion of the other HPRT⁻ cells to HPRT⁺. To eliminate these revertants, 3 or 5 μ g/ml of 6-thioguanine was added to the culture medium after 6 days of expression time, and the cells were incubated for 4 more days. Then the cells were resuspended in ES medium, and one day later the cells were exposed to 39.5°C for 8 h. This was performed to obtain mutator mutants with temperature dependency, but none of the mutator mutants isolated by this new procedure had this characteristic.

During the following 6 days of incubation at 34°C, the phenotype of the mutator mutants may turn from HPRT⁻ to HPRT⁺, which would be isolated by plating the cells onto agar plates containing HAT medium (5). The colonies were selected 2 weeks later, and then they were screened several times by determining if they contained an increased number of ouabain resistant mutants (5).

The culture was first split into two, and 3 mM ouabain was added to one of the cultures.

After 3–4 days of incubation, the culture was inspected microscopically to see if they contained any ouabain-resistant (Oua^r) cells. Then the Oua^r mutation frequency of the positive clones was determined every 3–4 weeks by plating 10^6 cells on soft agar plates containing 1.5 mM ouabain. The clones which showed an increase in mutation frequency during incubation were left as the possible mutator mutants, and the mutation rate was determined by the fluctuation analysis described below. By this method, 7 mutator mutants were isolated.

Characterization of mutator mutants. The rate of spontaneous mutation of the isolated cell clones was determined by fluctuation analysis (11) at the 3 genetic loci. They were resistance to ouabain (Oua^r; an inhibitor of membrane Na-K ATPase (19)), blasticidin S (Bla^r; an inhibitor of peptide-chain elongation (18)), and tunicamycin (Tun^r; an inhibitor of oligosaccharide synthesis of cell glycoproteins (6, 16)). Mutation of each of these loci was reported to be either dominant or co-dominant (6, 8, 19).

For the fluctuation analysis, 100 cells were plated to each dish of a series which contained 5 ml of culture medium and incubated for 9–14 days at 34°C until the cell number reached to approximately $2-5 \times 10^6$ per dish. Then the cells were collected by centrifugation, resuspended in the culture medium, and plated on soft agar plates. These plates were made in 9 cm plastic dishes which contained 0.5% purified agar in ES medium supplemented with 10% dialysed fetal calf serum and the selective agents. The concentration of the drugs in the soft agar plates and the cell number plated were: 1.5 mM ouabain and 10^6 cells, 5 µg/ml blasticidin S and 10^6 cells, or 1 µg/ml tunicamycin and 5×10^5 cells. The plates were incubated in a CO₂ incubator, and the colonies formed were counted after 2 weeks.

The mutation rate was determined by the equation (8) of Luria and Delbruck (11):

$$r = aN_t \ln(N_t/C)$$

where r is the average number of mutant cells in the culture dishes, a is the mutation rate/cell/division, N_t is the number of cells per dish at the time of plating, and C is the number of dishes. To solve this equation, a computer program was developed and run on a personal computer.

The sensitivity of the isolated mutants to 6-thioguanine or inhibitors of DNA replication such as aphidicolin and AraC was determined by counting and number of cells grown in a plastic multiwell dish containing these agents, as described previously (5). The sensitivity of cells to several DNA damaging agents was determined by the same method.

The cellular dNTP concentration in HClO₄ extracts was measured by high-pressure liquid chromatography (HPLC) as described previously (5).

RESULTS AND DISCUSSION

A method of selecting mutator mutants from cultured mouse FM3A cells was described previously (5). However, the efficiency of selection was low and from 670 cell clones which survived the selection procedure, only 3 clones were finally identified as mutator mutants. There may have been several reasons for this inefficiency. One reason may have been the inability of the HAT medium to eliminate all the HPRT⁻ mutants in the cell population. Secondly, spontaneous HPRT⁻ mutation in the non-mutator cells may have occurred during the selection procedure. Also the treatment with MNNG may have produced mutants resistant to both 6-thioguanine and to the HAT medium, so that even after selection of HPRT⁺ cells by the HAT medium, a number of non-mutator HPRT⁻ cells could have survived.

Therefore, to lower the background of non-mutator cells which survive the selection procedure and to increase the efficiency of isolation, we reversed the whole process

Fig. 1. Protocol for the isolation of mutator mutants. Procedure for treatments of cells, medium changes, the period of incubation (days, the number above the line) and expected cell types are shown. The details are described in MATERIALS AND METHODS.

TABLE 1. FLUCTUATION ANALYSIS OF MUTATOR MUTANTS

Cells	Number of dishes	Final cell number per dish ($\times 10^{-6}$)	Number of mutants per dish	Mutation rate per cell per division ($\times 10^7$)
<i>Ouabain-resistant mutation</i>				
F28-7*	17	2.86	0.41	0.94(1.0)
F9	25	2.07	0.96	1.99($\times 2.1$)
Fmut-11	18	5.04	52.7	20.1 ($\times 21$)
Fmut-12	18	6.25	201	50.7 ($\times 54$)
Fmut-13	23	2.98	89.6	51.3 ($\times 55$)
Fmut-14	26	2.07	7.19	8.96($\times 9.5$)
Fmut-15	23	1.62	22.0	29.0 ($\times 31$)
Fmut-16	16	5.21	40.0	15.7 ($\times 17$)
Fmut-17	12	3.09	7.0	6.97($\times 7.4$)
<i>Blasticidin S-resistant mutation</i>				
F28-7*	26	1.10	0.62	2.74(1.0)
F9	24	2.99	2.04	2.40($\times 0.87$)
Fmut-11	13	4.44	71.0	30.9 ($\times 11$)
Fmut-12	22	3.13	137	71.0 ($\times 26$)
Fmut-13	23	3.45	24.0	14.6 ($\times 5.3$)
Fmut-14	18	3.21	23.7	16.3 ($\times 5.9$)
Fmut-15	13	4.74	41.0	18.3 ($\times 6.7$)
Fmut-16	19	2.69	94.3	61.0 ($\times 22$)
Fmut-17	25	2.88	81.5	48.4 ($\times 18$)
<i>Tunicamycin-resistant mutation</i>				
F28-7*	19	3.95	9.94	6.48(1.0)
F9	23	3.70	7.91	5.55($\times 0.85$)
Fmut-11	21	2.23	259	173 ($\times 27$)
Fmut-12	23	3.03	381	177 ($\times 27$)
Fmut-13	12	4.51	710	222 ($\times 34$)
Fmut-14	19	4.16	251	91.8 ($\times 14$)
Fmut-15	16	4.64	1205	332 ($\times 51$)
Fmut-16	16	6.63	976	193 ($\times 30$)
Fmut-17	15	2.11	308	223 ($\times 34$)

Mutation rates were calculated using an equation derived by Luria and Delbrück (11). The method of fluctuation analysis was described in MATERIALS AND METHODS. *Data from our previous report (5).

of the protocol. In the new procedure, we changed to using HPRT⁻ cells (termed the F9 clone) as the parent cell line and isolated HPRT⁺ revertants from these cells, as shown in Fig. 1. It was found that this new protocol worked much better to reduce the background of non-mutator cells. By this new method, 55 clones which survived the selection procedure were found and 7 cell clones (designated as Fmut-11, -12, -13, -14, -15, -16, and -17), isolated independently, were finally identified as mutator mutants by the fluctuation analysis. In some experiments, several mutator mutants were obtained from the same MNNG-treated culture dish, but these might have been the progeny of a mutant cell, so only one clone was selected from each dish.

Table 1 shows the results of fluctuation analysis of the isolated mutator mutants. All the mutants had an increased rate of spontaneous mutation for the 3 genetic loci: resistance to ouabain, blasticidin S, and tunicamycin. The rate varied from 5.3-fold (Fmut-13, Bla^r) to 55-fold (Fmut-13, Oua^r) that of the wild-type cells.

Since the mutator mutants isolated by the new protocol shown in Fig. 1 were finally selected on the agar plates containing HAT medium, the phenotype of these clones should be HPRT⁺. When the HPRT enzyme activity of these cells was determined, they indeed contained 65–130% of that of the wild-type cell (F28-7 clone) (data not shown). The parental F9 clone had no detectable enzyme activity. When the sensitivity of these mutants to 6-thioguanine was tested, however, some of them (Fmut-11, -13, -14, and -17) were found to be even more resistant than the parental F9 cells (Fig. 2). The other clones (Fmut-12, -15, and -16) showed some degree of reversion of sensitivity to 6-thioguanine, but still they were more resistant than the wild-type HPRT⁺ cells (F28-7 clone). Although the reason for this is not known at present, these results may indicate that the enzyme in the revertants had altered the K_m for 6-thioguanine. Such a mutation in the HPRT gene in L cells has been reported by Sharp *et al.* (14).

Fig. 2. Sensitivity of cell clones to 6-thioguanine. Cells were plated in a plastic multiwell dish (10^4 /ml) containing various concentrations of 6-thioguanine. The cell number was determined after 6 days of incubation. The relative cell number is indicated taking the cell number in culture medium without this agent as 100%. F28-7 (○), F9 parental cell (*), Fmut-11 (●), Fmut-12 (△), Fmut-13 (▽), Fmut-14 (□), Fmut-15 (■), Fmut-16 (◇), and Fmut-17 (◆).

Fig. 3. Sensitivity of cell clones to aphidicolin (A) and AraC (B). Cell growth was determined in the presence of these agents as described in the legend of Fig. 2. F28-7 wild-type cell (○), Fmut-11 (●), Fmut-12, (△), Fmut-13 (▽), Fmut-14 (□), Fmut-15 (■), Fmut-16 (◇), and Fmut-17 (◆).

Sensitivity of the mutator mutants to aphidicolin and AraC was determined since some of the mammalian mutator mutants reported previously were first isolated as mutants resistant to these agents (1, 2, 12, 18). Fig. 3 shows the results and indicates that none of these mutator mutants had altered sensitivity to these agents. The results suggest that there might be no large imbalance in the cellular dNTP pool in these cells.

The size of the dNTP pool of the mutator mutants was determined by a HPLC method. Table 2 shows the amount of dNTPs in the mutator mutants relative to the wild-type F28-7 cells. The results show that in one of them, Fmut-17, there was an increase of dCTP to about 1.9-fold that of the wild-type cells. The same degree of increase in dCTP or dATP was also observed in the mutator mutants isolated by the previous method (5), but these differences were much smaller than the other mutator mutants with the dNTP pool imbalance (12, 18).

There was no large change in the dNTP pool of the other mutant clones; they remained similar to the wild-type cells. These results suggest that these mutator mutants were different from most other mammalian mutator mutants with a large imbalance in the dNTP pool.

TABLE 2. RELATIVE INTRACELLULAR dNTP CONCENTRATIONS (%)

Cell line	dCTP	dTTP	dATP	dGTP
Fmut-11	88	106	93	101
Fmut-12	49	63	78	77
Fmut-13	72	94	60	91
Fmut-14	58	76	100	100
Fmut-15	104	95	83	93
Fmut-16	60	94	71	99
Fmut-17	189	97	93	109

The concentrations were determined using the HPLC method and the amount relative to that in wild-type F28-7 cells is shown.

Fig. 4. Sensitivity of cell clones to UV (A) and MNNG (B). (A) Cells were suspended in colorless Hanks' solution at a cell density of $2.5 \times 10^5/\text{ml}$ and exposed to UV light at the incident flux of $0.4 \text{ J/m}^2\text{-sec}$. Then 10^4 cells were plated in a plastic multiwell dish. (B) Cell growth in the presence of MNNG. In both cases the cell number was determined as described in the legend of Fig. 2. F28-7 wild-type cell ($\circ$), Fmut-11 ($\bullet$), Fmut-12 ($\triangle$), Fmut-13 (∇), Fmut-14 ($\square$), Fmut-15 ($\blacksquare$), Fmut-16 ($\diamond$), and Fmut-17 ($\blacklozenge$).

The sensitivity of these cells to some of the DNA-damaging agents were also tested. The results with UV and MNNG are shown in Fig. 4. None of the cell clones had any alteration in sensitivity to these agents. The results suggested that these mutator mutants were different from the mutator mutants reported by Liu *et al.* (9), which showed hypersensitivity to UV.

Although the gene(s) which was affected in these mutator mutants has not yet been identified, it seems to be related to some aspects of DNA replication; therefore, studies with these mutator mutants should provide a new approach to understanding the correlation of DNA replication and somatic cell mutation.

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