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Increased Mutational Rates in Chinese Hamster Ovary Cells Serially Selected for Drug Resistance

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Chinese hamster ovary cell populations exposed to the pressures of prolonged serial cultivation in cytotoxic drugs have increased mutational rates at independent genetic loci. Evidence suggests that the alterations generating these mutations may be independent of the lesions conferring drug resistance.

Mutator genes have been widely used to study the processes by which cells maintain the integrity of the structure of their genes. Several mutator genes have been identified in procaryotes (for review, see reference 4) and characterized as alterations of proteins directly involved in DNA replication (3, 7, 9) or repair (6). Mutator strains of *Drosophila* have been described with increased frequencies of gene rearrangements (8). Considerably less is known of the processes controlling mutation in mammalian somatic cells. Strains with increased mutational rates have been observed among mutants with altered DNA precursor pool balance or metabolism (13, 20). However, it is clear that new approaches must be developed to examine other cellular genes involved in controlling mutational rates. In this respect, one property of bacterial mutator strains which could be exploited for the isolation of somatic cell mutator strains is their competitiveness. In chemostat experiments, bacterial mutator strains overgrow wild-type strains (5). In an attempt to find an analogous system for cultured mammalian cells, we examined cell populations exposed to the pressures of prolonged serial cultivation in cytotoxic drugs. Presumably, cells which are able to evolve more rapidly owing to their increased mutational rates might predominate. In this communication, we show that strains of Chinese hamster ovary (CHO) cells obtained by serial selection in two different agents, 5-fluorodeoxyuridine (FUdR) and methotrexate (Mtx), indeed have higher rates of mutation at independent genetic loci.

FUdR-resistant ($FUdR^r$) strains of CHO cells were evolved by serial cultivation in increasing concentrations of the drug over a period of 6 months. At various points in this process, three cell strains were isolated and characterized (Table 1). FP-1, FP-2, and FP-3 (resistant to 8-, 20-, and 1,000-fold concentration of FUdR, respectively) were all initially maintained in FUdR for many generations and screened before being transferred to nonselective medium. As reported by other laboratories (17, 19), thymidylate synthetase (TS) activity was increased in each of the isolates 9- to 11-fold, although it is clear that the increase in enzyme activity bears little relationship to the degree of resistance to FUdR in FP-2 or FP-3. The resistance was stable since transfer to nonselective medium did not alter drug sensitivity or TS activity.

During the selection process, all $FUdR^r$ strains were periodically passed through HAT (hypoxanthine, aminopterin, thymidine) medium to eliminate thymidine kinase (TK^-) derivatives which otherwise predominated owing to their extreme resistance to FUdR. This measure was only partially successful as the most resistant strain, FP-3, plated with 80% efficiency in HAT but had a fourfold reduction in TK activity. FP-1 and FP-2 plated with 100% efficiency, and no alterations of TK activity were detected (Table 1). The FP-3 population was also subcloned to determine whether heterogeneity existed among individual cells for TK and TS activity. Of 10 FP-3 subclones isolated, 1 appeared to be TK^- as judged by its inability to grow on HAT medium. The others had plating efficiencies ranging from 60 to 100%. Elevated TS levels similar to those in the heterogeneous FP-3 population were found in all subclones (data not shown).

The properties of somatic cell hybrids between an FUdR-sensitive (GlyA) mutant and

TABLE 1. TS and TK activities in wild-type and FUdR^r strains

Cell strain	Enzyme activity[a]		D_{10} (nM)[b]
	TS	TK	
Pro$^-$	5.5	22.7	1
FP-1	52.5	28.0	8
FP-2	54.6	18.7	20
FP-3	57.0	5.2	1,000
Pro × GlyA.4	5.1	ND[c]	2
FP-3.1 × GlyA	29.2	ND	40

[a] Enzyme activities are expressed as picomoles per microgram of protein per hour. TS activities were determined as described by Roberts (18) and modified by Navalgund et al. (15). TK activities were determined on log-growing suspension cultures basically as described by Ives et al (10).

[b] Concentration of FUdR required to reduce cell survival to 10%.

[c] ND, Not determined.

FP-3 further confirmed the complexity of the resistance phenotype. Cell hybrids were selected on the basis of their amino acid requirements (FP-3 is auxotrophic for proline, and the FUdR-sensitive strain used requires glycine) after fusion in polyethylene glycol and dimethyl sulfoxide as described previously (14). Five such FP-3 × GlyA hybrid clones were picked and screened for FUdR sensitivity. Karyotype analysis revealed that all were tetraploid. They showed FUdR sensitivity similar to that of FP-2, i.e., hybrids retained some FUdR resistance, although not as much as the parental strain (Table 1). One hybrid, GlyA × FP-3.1, was tested for TS activity and was found to retain a fivefold increase in enzyme activity (Table 1).

Mtx-resistant strains were isolated by serial selection over a period of several weeks. Two Mtx^R populations were isolated from these cultures, MP-1 (10-fold resistant) and MP-2 (100-fold resistant). MP-2 was stably resistant to Mtx since on transfer to nonselective medium it maintained its resistance to Mtx. In contrast, MP-1 (10-fold resistant) lost its resistance to the drug after propagation in nonselective medium. This result is consistent with other Mtx^R-gene amplified systems in which cells in earlier stages of resistance are unstable with the presence of autonomously replicating, extrachromosomal elements (double minutes) (11), whereas more highly resistant isolates are stable and exhibit localized integrated amplified units (homogenous staining regions) (16). Southern blot analysis of *Hin*dIII-digested DNA from the wild type and the Mtx^R isolates indicates that the above may hold true for our strains (Fig. 1). MP-2, grown in the presence or absence of selective pressure, clearly contains multiple copies of the dihydrofolate reductase (DHFR, the target enzyme) gene as indicated by the increased intensity of bands representing *dhfr*-containing sequences. *dhfr* sequences in MP-1 appeared slightly amplified when maintained in Mtx but were comparable to wild-type when grown in regular medium.

To determine whether our strains serially selected in cytotoxic drugs had altered mutational rates, we examined events at independent genetic loci (Table 2). The rate of mutation to 6-thioguanine resistance (6-Tg^r; 2) is essentially unchanged in FUdR^r cells compared with wild-type cells, whereas the rate to ouabain resistance (Oua^R; 1) is increased 10-fold in FP-3 and 7-

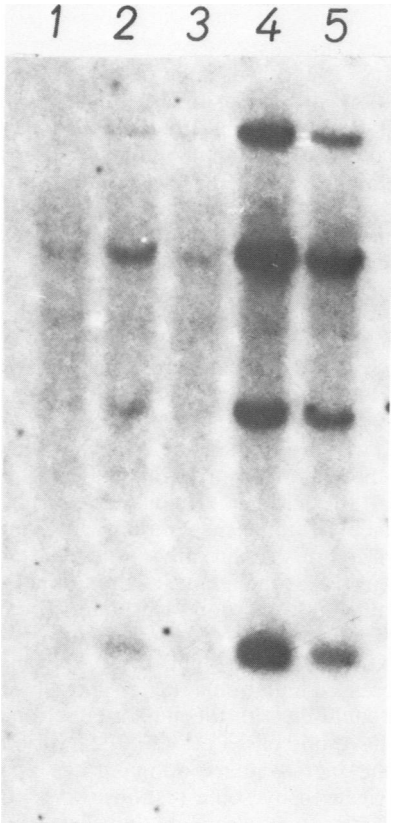

FIG. 1. Southern blot analysis of *dhfr* sequences in wild-type and Mtx-resistant strains. In each case, 12 μg of genomic DNA was digested with *Hin*dIII (New England Biolabs) and run on 0.7% agarose gels. DNA preparation, enzyme digestion, agarose gel electrophoresis, blotting, and hybridization were carried out as described previously (12). The probe used to detect *dhfr*-containing sequences was the murine pdhfr 11 kindly provided by Robert Schimke. Track 1, wild type; track 2, MP-1 maintained in 2×10^{-8} M Mtx; track 3, MP-1 maintained in nonselective medium; track 4, MP-2 maintained in 2×10^{-7} M Mtx; track 5, MP-2 maintained in nonselective medium.

TABLE 2. Spontaneous mutational rates in wild-type, FUdR^r, and Mtx^r strains[a]

Cell line	Mutational rate		
	Oua^r	6-Tg^r	Emt^r [b]
Pro$^-$	$(1.0 \pm 0.5) \times 10^{-7}$	$(1.0 \pm 0.46) \times 10^{-7}$	$(1.4 \pm 0.4) \times 10^{-8}$
FP-3	$(1.1 \pm 0.31) \times 10^{-6}$	$(1.8 \pm 0.66) \times 10^{-7}$	ND[c]
FP-2	$(6.9 \pm 1.8) \times 10^{-7}$	$(1.3 \pm 0.59) \times 10^{-7}$	ND
FP-1	$(1.6 \pm 0.69) \times 10^{-7}$	ND	ND
FP-3 × GlyA.4	$(1.3 \pm 0.40) \times 10^{-6}$	ND	ND
Pro × GlyA.1	$(2.2 \pm 0.67) \times 10^{-7}$	ND	ND
MP-1	$(6.6 \pm 3.3) \times 10^{-7}$	$(7.0 \pm 1.0) \times 10^{-7}$	$(7.5 \pm 2.8) \times 10^{-8}$
MP-2	$(1.6 \pm 0.5) \times 10^{-6}$	$(1.1 \pm 0.39) \times 10^{-6}$	ND

[a] Mutational rates were determined by growing replica cultures from an inoculum of 100 cells (to ensure that there were no preexisting mutants) in medium containing 5% dialyzed serum followed by plating as described previously (13). Mutational rates were calculated on the basis of the fraction of cultures containing no mutants (13). Values are expressed as mutations per cell per generation.

[b] Emt^r, Emetine resistance.

[c] ND, Not determined.

fold in FP-2. Two subclones of the FP-3 population also had similarly increased mutational rates. Mutational rates in FP-1 were not significantly altered. In somatic cell hybrids between GlyA and FP-3, the mutational rate to Oua^R remains high. Similarly, mutational rates were increased 5- to 16-fold in Mtx^R strains, although in these strains all loci examined were affected.

These observations demonstrate the usefulness of this approach for the isolation of mammalian somatic cell strains with increased mutational rates. We have as yet no indication of the lesions responsible for the altered mutational rates. Two observations suggest that the mutator phenotype of these strains may be independent of the primary lesions conferring drug resistance: (i) In the case of the FUdR^r mutants, cell-cell hybrids retain only minor resistance to FUdR, whereas the increased mutational rate to Oua^R appears to be dominant, and (ii) unstable Mtx^R strains transferred to nonselective medium lose extra *dhfr* copies and drug resistance but not increased mutational rates. A possible explanation could lie with the similar effect that both drugs have on cells, i.e., dTTP starvation. Perhaps the increased mutational rates observed were triggered by some response to protracted dTTP starvation, although they are no longer dependent upon continued presence of the drug (mutational rates were determined in the absence of FUdR or Mtx and were stable upon cultivation in the absence of the selective agent).

The types of mutations generated at an independent locus can now be determined by isolating mutator-induced adenine phosphoribosyl transferase- deficient strains and analyzing *aprt* gene sequences by Southern blotting with a cloned genomic probe (12). Such analyses may well allow elucidation of the types of gene structural alterations induced at independent loci and evaluation of the role these alterations play in the amplification process.

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