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Increased Rate of Base Substitution in a Hamster Mutator Strain Obtained during Serial Selection for Gene Amplification

MARIA ADELAIDE CALIGO,[1]† WENDY ARMSTRONG,[1] BELINDA J. F. ROSSITER,[2] AND MARK MEUTH[1]*

Imperial Cancer Research Fund, Clare Hall Laboratories, Blanche Lane, South Mimms, Hertfordshire EN6 3LD, United Kingdom,[1] and Institute for Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030[2]

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The pattern of mutations produced by a mutator gene (obtained during serial selection for amplification of the dihydrofolate reductase [*dhfr*] locus) shows a pronounced shift from that found in wild-type cells. The rate of certain types of base substitutions (particularly transitions) is dramatically increased, while gene rearrangements constitute a lower proportion of mutations. These data suggest a lower fidelity of the replication process in the mutator strain.

DNA replication is a highly accurate process dependent upon error correction mechanisms acting both at the replication fork (10, 11, 18) and postreplication (13). In *Escherichia coli*, the mechanisms maintaining fidelity have been defined through the use of strains with increased mutational rates (mutator strains [4]). To examine replication fidelity in mammalian cells, Chinese hamster ovary cell strains having increased mutation rates at multiple, independent loci were isolated from cultures exposed to the serial drug selections used to obtain amplified gene arrays (5). We suggested that the selection pressures of such protocols may enrich for cells able to initiate the DNA sequence rearrangements necessary for gene amplification and that the increased frequency of these events may generate mutations at independent loci. To define the alteration responsible for the increased mutation rate in these strains and the possible relationship between the mutator phenotype and gene amplification, mutations occurring at the X-linked hypoxanthine-guanine phosphoribosyl transferase (*hprt*) loci of these strains have been examined. Here we show a striking change in the pattern and rate of base substitution in the mutator strain, suggesting a lower level of replication fidelity.

The methotrexate-resistant (Mtx^R) strain of CHO used in these analyses is a derivative of one described previously (MP-2 [5]). This Mtx^R (2 μM methotrexate) strain is about 1,000-fold-more resistant to the toxic effects of methotrexate (with a D_{10} of 2 μM methotrexate, i.e., 10% of the cells survived 2 μM methotrexate) and has a similar (500- to 1,000-fold) increase in the number of copies of the *dhfr* gene as determined by slot blot analysis. The mutation rate at the *hprt* locus in the most resistant strain (determined by fluctuation analysis) is about 100-fold higher than the rate in the wild type: 1.6×10^{-8} 6-thioguanine-resistant (tg^r $HPRT^-$) mutations per cell per generation for the wild-type Pro^- CHO cells as opposed to 1.7×10^{-6} for the Mtx^R (2 μM) CHO cells.

To determine the nature of these mutations, independent tg^r mutants derived from both strains were isolated as described previously (1). DNA purified from 18 spontaneous $HPRT^-$ strains derived from the wild type and from 28 strains derived from the Mtx^R (2 μM) strain was digested with restriction endonucleases, fractionated by electrophoresis on agarose gels, and transferred onto nitrocellulose. These blots were probed with ^{32}P-labeled mouse *hprt* cDNA probe (9). A third of the mutations derived from the wild-type CHO strain had alterations of *hprt* fragments, indicating large deletions (Table 1); this figure is consistent with another study of spontaneous $HPRT^-$ mutations in CHO cells (23). Rearrangements were much less common among strains derived from the Mtx^R (2 μM) strain, as only 1 of the 28 mutants characterized (~4%) had an alteration detectable on Southern blots.

The remaining $HPRT^-$ mutations were examined at the nucleotide level by two approaches. First, RNA was purified from $HPRT^-$ strains and a cDNA copy was made by using an oligonucleotide complementary to the *hprt* message (9). Nested oligonucleotides were then used to amplify overlapping portions of the *hprt* cDNA (22) for sequencing from the double-stranded products (16). The entire cDNA was sequenced for each mutant gene, and the substitutions detected were confirmed in most cases by sequencing the opposite strand. Some of the mutations could not be identified by this approach, as a few $HPRT^-$ strains did not produce mRNA or splice signal modifications simply eliminated an entire exon from the message. These were

TABLE 1. Pattern of mutations at the *hprt* locus in wild-type and mutator strains

Strain	No. of:						
	Mutations in collection	Deletions		Duplications	Base substitutions		
		Large	Small (frameshifts)		Transitions	Transversions	Splice sites[a]
Wild-type	18	6	3	0	0	7	2
Pro^- Mtx^R (2 μm)	28	1	0	1	11	10	5

[a] These mutations lost exons from the cDNA but had no splice donor or acceptor consensus alterations when the appropriate region of the structural gene was sequenced. Other transitions or transversions also eliminated exons as a result of mutations at the donor or acceptor sites (see Table 2).

* Corresponding author.
† Present address: Istituto di Anatomia Patologica, Universita Degli Studi di Pisa, 45100 Pisa, Italy.

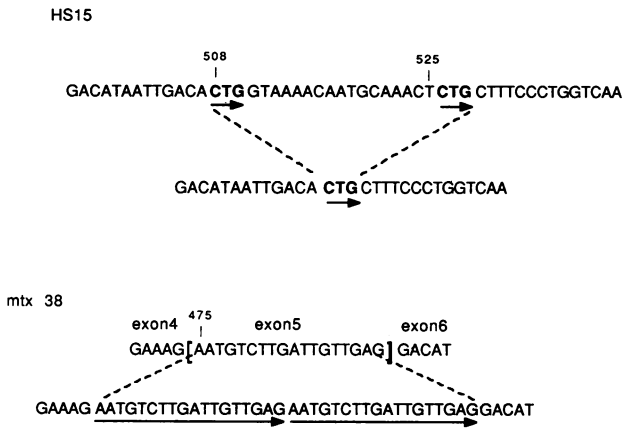

FIG. 1. Deletion and duplication mutations in the *hprt* locus of wild-type and mutator strains. Positions of the nucleotides in the cDNA sequence (9) are indicated by the numbers, as are positions of the exons in mtx 38. Short direct repeats are indicated by the short arrows in HS 15; the duplication of exon 5 in mtx 38 is denoted by the long arrows.

characterized further by sequencing mutant exons from genomic DNA by using oligonucleotide primers specific for the surrounding intron sequences (B. Rossiter et al., in press). The results of these analyses are presented in Table 2 and Fig. 1. Of the limited number of spontaneous *hprt* base substitutions analyzed in wild-type cells, the predominant class was transversions at G · C base pairs, with one site having three independent mutations. Additionally, two frameshifts and a small deletion in exon 6 were found (Fig. 1). Spontaneous base substitutions at the adenine phosphoribosyl transferase (*aprt*) locus of a closely related CHO cell strain grown in identical conditions show a similar bias towards G · C base pairs, although transitions constitute a significantly greater proportion of the mutations (16).

Base substitutions in the Mtx^R (2 μM) strain were considerably more varied, consisting of both transitions and transversions at A · T and G · C base pairs (Table 2). A high proportion (13 of 28) of mutations isolated from the Mtx^R (2 μM) strain affected splicing of the *hprt* mRNA. In several cases, these were the result of lost consensus splice donor or acceptor signals (including three independent transversions found at the exon 9 splice acceptor site [Table 2]). One mutant (mtx 28 [Table 2]) retained the normal splice acceptor for exon 7 but as a result of a mutation in IVS 6 gained a new

TABLE 2. Sequence determination of mutations of the *hprt* locus in wild-type and mutator strains

Strain and mutation type	Map site[a]	Exon	Substitution	Mutant sequence[b]	Amino acid substitution
Wild-type strains					
Transversions					
HS 12	298	3	G:C→T:A	TGAAG **T** GGGGC	Gly→Trp
HS 2,6,7	419	4	C:G→A:T	TCAGT **A** AACAG	Ser→Stop
HS 21	634	8	G:C→T:A	GATTT **T** AAATT	Glu→Stop
HS 16	664	8	G:C→C:G	GATAT **C** CCCTT	Ala→Pro
HS 11	724	9	G:C→T:A	AAACT **T** GGAAA	Gly→Trp
Frameshifts					
HS 19	110, 117	1	−G	CCCCACGTCGTG|TGAGC	
HS 10	562	6	+T	AATGG **T** TTAAG	
Mtx^R (2 μm) strains					
Transitions					
mtx 11,22	117 (+5)	1	G:C→A:T	G|GTGA **A** CCCCG	Splice
mtx 6	303	3	G:C→A:T	GGGGG **A** CTATA	Gly→Asp
mtx 29	391	3	A:T→G:C	TTATC **G** GACTG	Arg→Gly
mtx 9	449	4	G:C→A:T	GTGGG **A** ATGAT	Asp→Asn
mtx 13	484	5	A:T→G:C	TCTTG **G** TTGTT	Ile→Val
mtx 4	496	6	A:T→G:C	TTGAG **G** CATAA	Asp→Gly
mtx 27	515	6	A:T→G:C	GTAAA **G** CAATG	Thr→Ala
mtx 28	576 (−8)	7	A:T→G:C	TGTA **G** |TTAACAG|CTT[c]	Splice
mtx 15	680	8	A:T→G:C	TAATG **G** GTACT	Glu→Gly
mtx 24	688	8	A:T→G:C	ACTTC **G** GGGAT	Arg→Gly
Transversions					
mtx 8	117 (+2)	1	T:A→G:C	CGTG|G **G** GAGCC	Splice
mtx 3	302	3	G:C→T:A	AGGGG **T** GCTAT	Gly→Cys
mtx 37	382	3	G:C→T:A	CTGTA **T** ATTTT	Asp→Tyr
mtx 2	569	6	T:A→G:C	TAAGG **G** TGCAA	Val-Gly
mtx 1	623 (−1)	8	G:C→C:G	TTACA **C** |TTGTT	Splice
mtx 21	628	8	G:C→T:A	TTGTT **T** GATTT	Gly→Phe
mtx 12,18,31	700 (−1)	9	G:C→T:A	TTGCA **T** |CATAT	Splice
mtx 20	704	9	T:A→G:C	GCATA **G** TTGTG	Ile→Ser

[a] Map sites are according to the hamster *hprt* cDNA sequence of Konecki et al. (9). Those falling outside this sequence at splice junctions are noted by the nucleotide number of the splice site in the cDNA and the distance (in base paris) of the mutation from this site in the intervening sequence. A + indicates a mutation falling in the intervening sequence downstream of the splice donor; a − indicates a mutation upstream of the splice acceptor.

[b] Nucleotide sequences for the cDNA are those of Konecki et al. (9), and intron sequences are from B. J. F. Rossiter et al. (in press). The positions of the splice sites in the wild-type sequence are marked by vertical lines.

[c] In this mutant strain, the gained splice junction is indicated by the vertical line immediately to the right of the mutation; the wild-type junction is also indicated. The 7 base pairs between the two appear as an insertion in the mutant cDNA.

(apparently preferred) splice acceptor, and the mutant message contained a 7-bp insert originating from IVS 6 (much like a thalassemic β-globin gene [12]). Five other strains lost exons and have normal splice junction sequences, but we have not yet analyzed the intervening sequences for mutations. Given the greater number of exons of the *hprt* structural gene (compared with the number in *aprt*), it is not surprising that splice mutations are more prevalent, but two of the sites appear to be hot spots in the Mtx^R (2 μM) strain, with recurring mutations.

No frameshifts or small deletions were found among mutants from the Mtx^R (2 μM) strain, but an unusual rearrangement resulting in the precise duplication of exon 5 (and presumably some surrounding sequence) was detected (Fig. 1). This particular type of rearrangement has not been previously found among mutations of the *aprt* or *hprt* locus in CHO cells, but a Lesch-Nyhan patient with a similar *hprt* duplication was described previously (24).

These changes in the pattern of base substitutions at the *hprt* locus of the mutator strain (the increased frequency of both types of transitions and the broader range of transversions) indicate that the alteration in the Mtx^R (2 μM) strain affects the production or correction of a wide range of mispairs. Thus, replication fidelity could be directly affected in this strain, or error correction mechanisms (such as proofreading or mismatch correction) could be less efficient. The use of in vitro DNA replication systems (6) to examine the fidelity of replication of extracts from this cell strain may help resolve this issue. The increased mutation rate does not appear to be a consequence of metabolic imbalances induced by the amplification of the *dhfr* gene. No significant changes in intracellular deoxyribonucleoside triphosphate levels have been found in the Mtx^R (2 μM) strain, and the broad range of mutations contrasts with the very directed nature of substitutions induced by DNA precursor pool imbalances (17). Furthermore, serial cultivation of the wild-type strain in another drug (5-flurodeoxyuridine) yielded strains with similar increases in the mutation rate (5).

It is not obvious how the increased rate of base substitution relates to the complex rearrangements formed during gene amplification (20). Observations that this strain also has increased rates of amplification at loci other than *dhfr* (M. Perry and G. Stark, personal communication) together with the unusual duplication of exon 5 found in one $HPRT^-$ strain indicate that such a relationship may exist. It has been shown that a wide variety of agents (carcinogens, DNA-damaging agents, or inhibitors of DNA synthesis [2, 3, 7, 8, 21]) increases the frequency of amplification at several loci. However, these effects appear to be transitory and cell line dependent. An enhanced misincorporation rate in the Mtx^R (2 μM) strain may retard replication fork movement because of difficulties in replicational extension past mispaired nucleotides, for example (15). It has been proposed that such effects may lead to overreplication and -amplification (7, 19). An alternative explanation is that replication fork retardation may increase the frequency of strand switching, leading to the production of the inverted repeat structures found in amplified gene arrays (14).

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