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Genetics

Spectrum of spontaneous mutation at the *APRT* locus of Chinese hamster ovary cells: An analysis at the DNA sequence level

(spontaneous mutagenesis/mutational specificity/cytosine deamination)

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ABSTRACT **The spectrum of spontaneous mutation of an endogenous mammalian cell gene has been determined at the DNA sequence level. Thirty independent spontaneous *APRT*$^-$ mutations were cloned and subsequently completely sequenced. Twenty-seven contained single base substitutions. Of these, 22 were G·C to A·T transitions, suggesting a major role for the deamination of cytosine in spontaneous mutagenesis of Chinese hamster ovary cells. The remaining mutants included a tandem double substitution, a −1 frameshift, and a 17-base-pair deletion flanked by a 2-base-pair direct repeat. Many of the independently recovered mutants were clustered at sites of multiple occurrence (hot spots). One site accounted for >25% of all independently recovered events. Mutations were generally located within the coding sequence, although two mutations occurred within the consensus sequence for a 3′ splice site.**

A fundamental understanding of mutagenesis in mammalian cells ultimately requires specific knowledge of the DNA sequence alterations involved. A complete analysis would describe the spectrum of mutational alterations for a random collection of independent mutants at a given locus. Such analyses have recently been reported in bacteria (1–3), but logistical difficulties have prevented such an analysis of an endogenous mammalian cell gene. Other endpoints have, by necessity, been used to construct a preliminary view of mutation in mammalian cells. These include comparative mutation induction at several selectable loci (4, 5), two-dimensional gel electrophoresis analysis of mutant proteins (6), and Southern blot analysis of restriction site polymorphisms (7–10). Recently, recombinant shuttle vectors, which can be propagated in both prokaryotic and eukaryotic cells, have been developed for the analysis of mutant DNA sequences in mammalian cells (reviewed in ref. 11). Although the use of shuttle vectors facilitates the recovery and sequence analysis of mutations, this experimental approach has several drawbacks, often including an extraordinarily high mutation frequency and a high percentage of deletions and rearrangements among the recovered plasmids (12–16). This is true even under conditions in which the shuttle vector has been incorporated into the chromosome, although some inserts appear to approach the stability expected for natural genes (17, 18). These technical problems and the artificial nature of such constructs make it questionable whether these systems accurately represent mutagenesis at endogenous cellular genes.

Our efforts have therefore concentrated on the development of a practical approach to the cloning and sequencing of mutant alleles of an endogenous gene. Our system involves the analysis of mutants at the adenine phosphoribosyltransferase (*APRT*) locus of Chinese hamster ovary (CHO) cells. This locus is well suited for mutational studies. It encodes an enzyme in the nucleotide salvage pathway and is thus nonessential for cell survival in ordinary growth medium. Consequently, all classes of mutational events can in principle be expected. Simple single-step selections for both forward and reverse mutations have been well characterized (19, 20). Advantages of the CHO *APRT* locus for molecular analyses include the availability of hemizygous strains (6, 21), the apparent absence of pseudogenes (8, 9), and the small [2.6 kilobases (kb)] size of the functional CHO *APRT* gene, as determined by DNA sequencing analysis and DNA transfection studies (unpublished results). A potential limitation of the system is the possible exclusion of mutational events that would inactivate essential genes within the region of hemizygosity. However, APRT$^-$ mutants with substantial deletions and other chromosomal rearrangement events have been isolated (9).

We have recently reported (9) a Southern blotting analysis of 67 spontaneous APRT$^-$ mutants, none of which demonstrated large genomic alterations. In this report, the DNA sequence alterations of 30 of these spontaneous APRT$^-$ mutants are presented.

MATERIALS AND METHODS

Cell Culture Conditions and Mutant Selection. The CHO strain D422 used in this study is hemizygous at the *APRT* locus (21). The conditions used for the selection and phenotypic characterization of spontaneous APRT$^-$ mutants have recently been detailed (9). However, we will briefly describe some of the most pertinent aspects here. For routine growth, cells were maintained in α minimal essential medium supplemented with 2.5% fetal calf serum and 2.5% heat-inactivated horse serum. Each spontaneous APRT$^-$ mutant was obtained by establishing independent cultures at an initial inoculum of 500 cells, which were grown to $\approx 2 \times 10^6$ cells per culture. The cells were then reseeded at a density of 5×10^5 cells per 100-mm Petri dish in medium containing 0.4 mM 8-azaadenine, a toxic adenine analog (Sigma). Only one mutant clone was selected for further analysis from each initial culture.

Cloning Strategy. Our approach is based on the *in vivo* clone selection procedure designed by Seed (22) and adapted by us for the cloning and sequencing of the CHO *APRT* gene. Specific details on vector constructions and cloning protocols will be described elsewhere (B.W.G., A.J.G., E. A. Drobetsky, and P.J.D., unpublished data). In short, the procedure depends on the rescue of conditionally defective λ clones from genomic libraries by homologous recombination with a plasmid containing a suppressor gene. The resulting

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incorporation of the plasmid into the λ genome is only possible for clone/plasmid combinations sharing homologous sequences. In this particular case, the λ clone being sought contains a 4.3-kb *Hin*dIII/*Bgl* II insert, which includes the complete *APRT* allele as well as upstream and downstream flanking sequences. The plasmids are derived from pSDL12 (24), a ColE1 type miniplasmid of 1.9 kb carrying the 200-base-pair (bp) *supF* amber suppressor gene as well as a 700-bp CHO insert derived from sequence downstream of *APRT* on the 4.3-kb *Hin*dIII/*Bgl* II fragment.

Genomic λ libraries were constructed by ligation of size-fractionated genomic DNA, cut with *Hin*dIII and *Bgl* II, to compatible restriction ends of a λ insertion vector (λ PDJ11, 40.6 kb). This vector has unique *Hin*dIII and *Bam*HI sites separated by 0.5 kb of λ sequence as well as two essential genes (*A* and *B*), which each contain an amber mutation. Homologous recombination between the library phage containing the *APRT* locus and the plasmid occurs during the amplification of the primary genomic library on a host (MC1061.p3) harboring the pSDL12 derivative. Recombination occurs at an efficiency of ≈0.1% (unpublished results). Recombinants carrying the *APRT* gene and the integrated plasmid have a size of 47.0 kb, which is well within the packageable size limits of λ vectors (38–51 kb). The recombinants are selected by titering the amplified library on a nonsuppressor host, LG75 (22), which has an amber-mutated *lacZ* gene. Recombinants are distinguished from other library clones by the ability to form plaques on LG75 and by the suppression of the *lacZ* amber mutation on the host, as detected on indicator plates with the synthetic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside.

Homologous recombination occurs in a manner that allows the plasmid sequences and the entire *APRT* gene to be recovered on a single 5.7-kb *Bam*HI fragment. This fragment is circularized and used to transform an appropriate F′ host strain (XS127). Single-stranded plasmid DNA can be obtained for dideoxy sequencing by infecting the transformed bacteria with M13 phage since the pSDL12 plasmid carries an M13 origin of replication (24). A series of oligonucleotide primers which hybridize at chosen points along the *APRT* gene, but not to the M13 sequence, is used for sequence determinations.

RESULTS

The wild-type *APRT* allele from strain D422 was independently cloned and sequenced three times (data not shown) and was comparable to that recently reported by Nalbantoglu *et al.* (25). The entire coding sequence of each mutant allele was determined along with most, and in many cases all, of the intron and flanking sequences, to be certain that the observed alterations were unique. As an additional control, two of the mutants (nos. 59 and 60) were independently cloned and sequenced three times each. In both cases, each independent clone carried the same unique sequence alteration.

Single base substitutions occurred in 27 of the 30 mutated sequences (Table 1). Of these, 22 (≈82%) are G·C to A·T transitions. The A·T to G·C transition has not been observed.

Table 1. Sequence analysis of spontaneous APRT⁻ mutants

Mutant	Sequence alteration	Position	Predicted amino acid change	Target sequence
2	TS C → T	241	Ser → Phe	CGCCT C CTTCC
5	TS C → T	1324	Pro → Leu	TGGCC C CTCCC
13	TS G → A	1351	Gly → Asp	CCTGG G CTGTG
16	TS C → T	241	Ser → Phe	CGCCT C CTTCC
17	TV G → T	1422	Gly → Cys	AGTAT G GCAAG
20	TV C → G	242	Phe → Ile	GCCTC CT TCCGA
	TV T → A	243		
21	TS C → T	58	Pro → Ser	ACTTC C CCATC
27	TS G → A	210	Asp → Lys	CCAGG G ATATC
29	TS C → T	241	Ser → Phe	CGCCT C CTTCC
39	TS G → A	1309	Gly → Glu	CAGGG G ATTCT
43	TV G → T	1591	Glu → Stop	AACTA G AAATC
48	TV C → A	1780	Cys → Trp	ATGTG C GCTGC
55	TS C → T	222	Leu → Phe	CGCCC C TCCTG
57	TS C → T	241	Ser → Phe	CGCCT C CTTCC
59*	TS C → T	253	Ser → Phe	AGCTT C CATCC
60*	TS C → T	1786	Ala → Val	CGCTG C CTGTG
61	TS C → T	241	Ser → Phe	CGCCT C CTTCC
63	TV T → A	1573	Splice recognition site	†
65	TS C → T	1384	Pro → Leu	GCTGC C AGGCC
66	TS C → T	253	Ser → Phe	AGCTT C CATCC
68	TS C → T	1383	Pro → Ser	AGCTG C CAGGC
69	Deletion	1401–1418		†
70	TS C → T	1303	Ser → Phe	AGACT C CAGGG
75	TS C → T	222	Leu → Phe	CGCCC C TCCTG
84	TS C → T	241	Ser → Phe	CGCCTC CTTCC
86	Frameshift	1600		†
93	TS C → T	241	Ser → Phe	CGCCT C CTTCC
94	TS C → T	222	Leu → Phe	CGCCC C TCCTG
100	TS C → T	59	Pro → Leu	CTTCC C CATCC
104	TV G → T	1581	Splice recognition site	†

TV, transversion; TS, transition.
*These mutants were independently cloned three times and fully sequenced. Each clone of a given mutant strain demonstrates the same sequence alteration, hence confirming the methodology used.
†See Fig. 1 for target sequences.

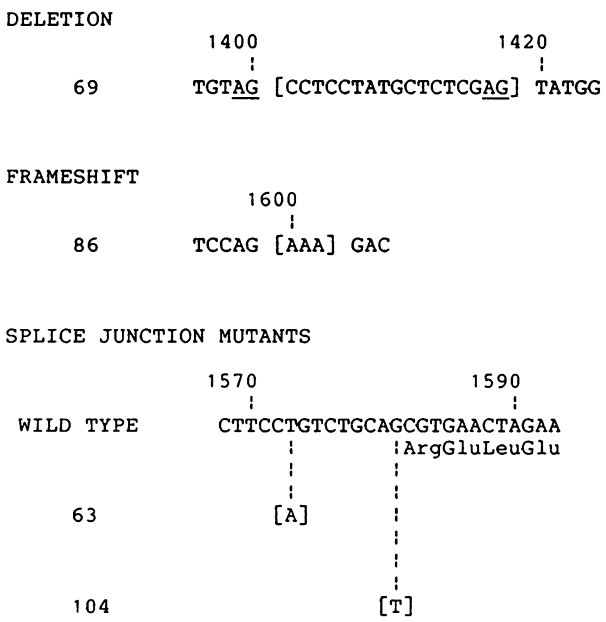

FIG. 1. Target sequences for *APRT* deletion mutant 69, frameshift mutant 86, and splice junction mutants 63 and 104. Mutant 69: the 17 deleted bases are bracketed and the 2-bp repeat is underlined. Five bases of surrounding sequence on either side are also shown. Mutant 86: The frameshift results from the loss of one adenine in the run of three adenines set off from the surrounding sequence. The target nucleotide in 86 is also indicated by spacing from the surrounding sequence. Splice junction mutants 63 and 104: These have both occurred within the consensus 3′ splice acceptor sequence of intron 3. The wild-type sequence and the altered nucleotide in each case are shown; the beginning of exon 4 is indicated by the accompanying amino acid sequence.

The 5 remaining single base substitutions are transversions. One tandem base substitution (mutant 20) was also observed.

With two exceptions, each base substitution yields an amino acid substitution. The exceptions (mutants 63 and 104) occurred within intron 3 near the 3′ splice site (Figs. 1 and 2). In mutant 104, a G·C to T·A transversion has destroyed the AG dinucleotide at the splice junction, whereas in mutant 63 an A·T to T·A transversion has resulted in the introduction of an AG dinucleotide 7 bp upstream of the normal splice junction. The changes observed in the splice region can be reasonably expected to account for the APRT$^-$ phenotype.

One deletion and one frameshift event have also been detected. In mutant 86, a frameshift of the coding sequence resulted from the deletion of a single adenine in a run of three adenines while a deletion of 17 bp was observed in mutant 69 (Fig. 1).

The distribution of mutations along the gene is depicted diagrammatically in Fig. 2. There are several positions of multiple occurrence or "hot spots" (Table 1, Fig. 2). The G·C to A·T transition at position 241 was independently observed 7 times, accounting for nearly 25% of all spontaneous *APRT* mutations. A G·C to A·T transition was also recovered at positions 222 and 253 two and three times, respectively. The occurrence of hot spots can be expected to be governed by both DNA sequence and protein function determinants; the results presented (Table 1, Fig. 2) likely reflect both parameters. In two cases, the same codon has been mutated in two independent strains (mutants 68 and 65, 21 and 100) by base substitution at the first or second codon position, respectively. In addition, we have observed two cases of independent mutations recovered in codons only one amino acid position apart (mutants 48 and 60, 39 and 70).

While at least one mutant was isolated for each exon (Fig. 2), the sites of multiple occurrence at a single nucleotide are clustered in exon 2. Exon 3 is evolutionarily conserved (26), suggesting that most sequence alterations within this region would result in a selectable phenotype. In fact, a relatively large number of mutant sites were identified within this exon. Two of the three closely neighboring amino acid sequence alterations fall within this exon, but there were no sites of multiple occurrence at a single nucleotide. Several mutational spectra will be required before a more detailed analysis of the available sites for mutation can be undertaken.

DISCUSSION

The spectrum of spontaneous mutation in CHO cells is shown here to be characterized by two major features: a predominance of G·C to A·T transitions and a prominent hot spot, which is the site of almost 25% of all mutational events. These results for mammalian cells can be contrasted with a recent study (2) in which the spectrum of spontaneous mutation at the *lacI* gene of *Escherichia coli* was reported. The *E. coli* spectrum was characterized by a major hot spot for a frameshift event at the site of three tandem repeats of a 4-base sequence. Approximately two-thirds of the characterized mutants were either insertions or deletions of one copy of the 4-base direct repeat. Among the remaining mutations were two major classes of events: deletions (37%) and base substitutions (34%). However, among the base substitutions the G·C to A·T transitions were no more frequent than many of the other types of substitution. The predominance of G·C to A·T transitions in CHO cells in part reflects the contribution of the hot spots, each of which involves this substitution. However, G·C to A·T transitions are also predominant among the non-hot-spot mutations since 10 of the remaining 18 were of this type.

G·C to A·T transitions may reflect cytosine deamination, the most commonly encountered spontaneous DNA lesion (27). The modified base 5-methylcytosine position has been found to be associated with spontaneous hot spots in the *lacI* gene of *E. coli* (28). The explanation for this is that the deamination of 5-methylcytosine produces a thymine residue that is not removed from DNA by uracil glycosylase as would be uracil, the normal product of cytosine deamination. In mammalian cells, the methylation of cytosine is thought to

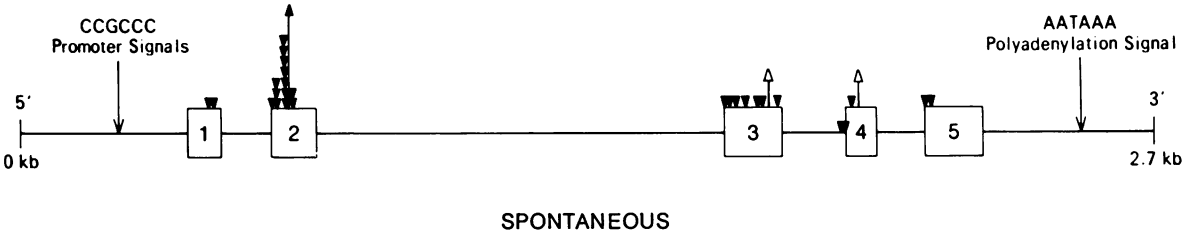

FIG. 2. The *APRT* gene is drawn to scale with the five exons represented by open boxes, the four introns lying between them, and the position of the promoter and polyadenylylation signals indicated. Mutations are indicated above the gene. ▼, Base substitutions; ▲, tandem base substitution; △, deletion and frameshift mutations. Multiple occurrences are indicated by stacked symbols.

occur largely at CpG dinucleotides (29). However, the G·C to A·T transitions described here did not arise at such sequences but rather in runs of pyrimidines (Table 1), although we note that the methylation of cytosine bases at sequences other than CpG has been suggested to occur in *Neurospora* (30). A defect in uracil *N*-glycosylase, such as in an *E. coli* ung$^-$ mutant, enhances the frequency of G·C to A·T transitions (31, 32). Mammalian cells, in general, have been reported to have lower levels of uracil glycosylase as compared with prokaryotes (33); CHO cells are particularly poor in this glycosylase activity. By analogy with ung$^-$ bacteria, a low level of this enzyme could account for the observed prevalence of the G·C to A·T transitions.

A strand bias may exist among the G·C to A·T transitions. Eighteen of the 22 independent mutants of this type (or 9 of 12 individual sites of G·C to A·T mutations) were read as cytosine to thymine changes in the single-stranded DNA used for dideoxy sequencing, which is, in this case, the nontranscribed strand. Such a preference was also observed in an Ung$^-$ strain of *E. coli* (34). The predominance of mutation in the nontranscribed strand in *E. coli* was thought to reflect the single-stranded nature of this strand during transcription, while the transcribed strand is complexed with RNA. The rate of deamination in single-stranded DNA occurs at a much greater rate than in double-stranded DNA (27). Alternatively, the strand bias might result from differences in the replication fidelity of the continuously and discontinuously replicated strands (35).

The predominance of G·C to A·T transitions may alternatively reflect the fidelity of eukaryotic polymerases (36). In a study that compared the sequence of several pseudogenes with that of the functional gene (37), transitions were revealed to be much more common than transversions and a preference was observed for G·C to A·T transitions as compared to transitions in the opposite direction. This trend seen among sequences that have diverged *in vivo* is consistent with the mutational spectrum generated in the *APRT* gene *in vitro*.

While most mutations altered the coding properties of the mRNA, two mutants were recovered within an intron at the 3′ splice site consensus sequence. This site is characterized by a canonical AG dinucleotide at the splice junction, preceded by a run of pyrimidines that may be interspersed by an occasional purine but not by an AG dinucleotide (38). In mutant 104, a G·C to T·A transversion results in the loss of the AG dinucleotide at the acceptor splice junction (Fig. 1). In contrast, the T·A to A·T transversion in mutant 63 creates an AG dinucleotide 7 bp upstream of the normal splice junction. The introduction of the dinucleotide AG within this region has been reported to impede recognition of the wild-type splice junction (39). The sequence alterations in each of these mutants readily account for their APRT$^-$ phenotype.

Our previous Southern blotting analysis of APRT$^-$ mutants (8) did not reveal any spontaneous deletion or chromosomal rearrangement events, in contrast to a parallel collection of γ-ray-induced mutants. The DNA sequencing analysis presented here confirms and extends these findings. The sequencing of 30 of these spontaneous "point mutations" revealed them to be largely attributable to base substitution events; only one frameshift and a single deletion too small to be detected by Southern blotting were recovered (Table 1).

Our single frameshift mutant (number 86, Fig. 1) is an example of a frameshift occurring within a run of identical bases. The now classical model for frameshift mutagenesis (40) predicts the slippage and misalignment of a base onto the complement of a nearby base within such runs. The slippage and misalignment model was later extended (41–43) to include a role for repeats in deletion and insertion events. A recent study has reported (23) the sequence alterations of six deletions of the CHO *APRT* gene originally detected by Southern blot analysis. These deletions were characterized by short repeated sequences (2–7 bp) at their termini. Four of the deletion termini were clustered in a 40-bp region of the gene. Our single deletion (mutant 69) does not fall within this region, but it is flanked by a short direct repeat of 2 bp. This was the only deletion among the 30 mutants sequenced.

An improved understanding of the molecular mechanisms of mutagenesis has resulted from studies of sequence specificity. In addition to providing insight into the mechanism of spontaneous and induced mutagenesis, such studies also provide information concerning the structure and expression of the target gene. Spectra derived from diverse treatments can in the future be expected to yield important insights into the mutational mechanisms operating in mammalian cells. The accumulation of a large data base and a characterized collection of mutants will represent invaluable resources for further studies of molecular cell genetics. To achieve this goal, experiments are currently under way to analyze spontaneous and induced mutation at the *APRT* locus in this and other genetic systems.

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