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Genetics

Mouse transgenes in human cells detect specific base substitutions

(adenine phosphoribosyltransferase/mutagenesis/splice junction/ethyl methanesulfonate/2-aminopurine)

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ABSTRACT **We describe a system of transgenic human cell lines that detects and identifies specific point mutations at defined positions within a gene. The target transgenome is a mouse adenine phosphoribosyltransferase (APRT) gene rendered nonfunctional by introduction of a substitution at either of two bases that comprise a splice acceptor site. Reversion at a mutated site results in the expression of wild-type mouse APRT and consequent growth of APRT$^+$ transgenic cell colonies. Site-specific reversion to wild-type sequence is confirmed by regeneration of a previously destroyed diagnostic *Pst* I site. Two independent cell clones, each with mutant transgenomes bearing an A → G transition, exhibited an up to 7500-fold, dose-dependent induction of reversion following treatment with ethyl methanesulfonate. Treatment of these clones with 2-aminopurine resulted in no induction of revertants. In contrast, another transgenic cell clone, bearing a G → A transition, reverted as a consequence of 2-aminopurine, but not ethyl methanesulfonate, treatment. These data confirm for human cells the proposed mechanisms of action of these mutagens and provide evidence for the utility of our site-specific reversion method for mutagenesis studies.**

There are no mammalian cell assays that precisely define, without the aid of DNA sequencing, the type(s) of mutation that an exogenous agent can produce. In contrast to the Ames *Salmonella* test (1–3), most mammalian cell mutagenesis assays measure forward mutation at a target locus whose altered phenotype is drug resistance (4). Although useful for detecting agents that cause mutations, such assays provide little information regarding the type(s) of mutation produced, unless the nucleotide sequence of the mutant target gene is determined in each case (5, 6). To circumvent the necessity for DNA sequencing (e.g., refs. 7 and 8), we have developed a detection system that relies on site- and sequence-specific reverse mutation. First, a defined point mutation is introduced into a wild-type mouse adenine phosphoribosyltransferase (APRT) gene. Then, the mutated gene is transfected into a human recipient cell with a stable APRT$^-$ phenotype. Only reverse mutation to the wild-type mouse gene sequence is detected by expression of the selectable mouse APRT$^+$ phenotype and growth of cell colonies, thereby defining the precise mutation that has occurred. The site within the target gene at which mutations have been introduced was chosen so that second-site mutations would be unlikely to produce phenotypic reversion, and none have been detected.

The mouse APRT gene was chosen as the target transgenome primarily because the expression or absence of the encoded enzyme provides the basis for sensitive forward and backward chemical selection of cultured cells (9). This mammalian gene is also small [≈3 kilobases (kb)], which facilitates its *in vitro* manipulation; its nucleotide sequence and its structural organization are known (10); and its encoded product (mouse APRT) is electrophoretically distinguishable from the human enzyme (11, 12).

The two nucleotide targets for mutagenesis are the terminal AG of the third intron of the mouse APRT gene. Both are components of the splice acceptor sequence (13) and mutation of either should disrupt RNA splicing (14, 15) and interfere with APRT production. Furthermore, the likelihood of second-site reversion is remote since this junction sequence is highly conserved in all species. Of 1432 AG splice acceptor sites surveyed, only 4 exceptions, all in immunoglobulin genes, are known (13). These nucleotides also comprise part of a *Pst* I restriction site that is lost when either nucleotide is altered and that is diagnostically regenerated only upon reversion to the original sequence. In this report we demonstrate the specificity and high efficiency of the transgenic cells in detecting G → A transitions following exposure to ethyl methanesulfonate (EMS) but not 2-aminopurine (2AP). Conversely, we have detected A → G transitions after 2AP, but not EMS, exposure. Ultimately, we plan to develop six transgenic cell lines, each with a different base substitution at one of the two aforementioned adjacent nucleotides. Together, these cell lines will contain all possible DNA base substitutions and will provide an assay for determining the relative efficiencies of any given mutagen to induce specific base changes at this site.

MATERIALS AND METHODS

Mutant Construction. For introduction of specific point mutations into the mouse APRT gene, the internal *Bam*HI fragment was cloned into the polylinker of bacteriophage M13mp18 replicative form, and site-directed mutagenesis performed as described by Kunkel (16). Recombinant bacteriophage were used to infect *Escherichia coli* BW313 (*dut, ung, thi-1, relA, spoT*/F′ *lysA*) grown in TY medium supplemented with uridine at 0.25 mg/ml. Following overnight growth, uracil-containing template DNA was isolated from polyethylene glycol (PEG 8000)-precipitated phage. An 18-nucleotide primer, containing the designated A → G or A → G substitution, was hybridized to the template and extended by T4 polymerase in the presence of all four dNTPs, ATP, and T4 DNA ligase (16). The resultant duplex DNA was used to transfect *E. coli* JM109 [*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA2,* Δ(*lac-pro*AB)/F′ *traD36, proAB, lacI^{q}Z*ΔM15], individual plaques were picked, and their DNAs were screened (17) for the presence or absence of the mutation by cleavage, or lack thereof, with *Pst* I.

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Abbreviations: APRT, adenine phosphoribosyltransferase; EMS, ethyl methanesulfonate; 2AP, 2-aminopurine; AAA, alanosine/azaserine/adenine.

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DNA Sequencing and Plasmid Reconstruction. Sequencing of the mutant *Bam*HI insert was performed according to Sanger *et al.* (18). The *Bam*HI fragment of plasmid pSAM4.4, which contains a functional mouse APRT gene (19), was replaced by a mutant *Bam*HI fragment and the resultant construct was designated pSAM4.4M4 (M4 has an A substituted for a G) or pSAM4.4M1 (M1 has a G substituted for an A). The plasmids are composed of an *Eco*RI–*Hin*dIII fragment containing a mutant mouse APRT gene that extends 872 base pairs (bp) upstream of the translation start site and 1.3 kb downstream of the polyadenylylation signal, cloned into the polylinker of pUC19.

Cell Culture and Transfections. Human APRT$^-$ HT 1080 fibrosarcoma cells, designated HTD 114, were grown as described (20). Selection for G418-resistant cells was in medium containing G418 (GIBCO) at 400 μg/ml, and selective medium [alanosine/azaserine/adenine (AAA) medium] for APRT$^+$ cells was as described (9, 20). Transient or stable transfections were conducted as described (11, 19).

Mutagen Treatment. Subconfluent cultures at a density of 2.6–3.9 $\times$ 10^4 cells per cm^2 were treated with EMS concentrations of 50–200 μg/ml for 18 hr. Higher concentrations were not used, as these cells are unusually sensitive to EMS. For 2–10 mM 2AP treatment of 48 hr, cell densities were reduced to 8–16 $\times$ 10^3 per cm^2. Treatment with 2AP at lower densities resulted in higher levels of cell death, possibly as a consequence of greater 2AP incorporation. Controls were subjected to mock treatment. After mutagen exposure, cells were rinsed twice in serum-free medium, counted, and inoculated into growth medium at one-third their previous density. At this point, mutagen-induced cell death was determined by inoculating cells into growth medium. Cell survival after mutagen treatment is expressed as the fraction of treated cells that formed colonies, corrected for the fraction of cells that formed colonies in untreated controls. Cells were maintained for 4 days in growth medium, after which time they were removed and inoculated into AAA selective medium at a density of 1.3–2.6 $\times$ 10^4 cells per cm^2. In some experiments the concentration of alanosine in AAA medium was decreased to 2 μg/ml after 7 days to reduce its nonspecific cytostatic effects. Colonies were picked after 10–16 days. Each data point represents at least four independent experiments in which a total of 4–23 $\times$ 10^6 viable cells were tested for reversion.

RESULTS

Fig. 1 shows the organization of the mouse APRT gene, the position of the targeted nucleotides, and the predicted restriction fragment sizes following digestion with *Xmn* I plus *Pst* I for genes containing or lacking the diagnostic *Pst* I site. All permutations of base substitutions involving the terminal AG of intron 3 have been produced (21). Each mutated 1-kb *Bam*HI fragment was sequenced to confirm the mutation and to ensure against the introduction of adventitious mutations elsewhere in the gene. Furthermore, each of the six mutant genes and the wild-type gene were discriminated by denaturing gradient gel electrophoresis, providing additional evidence against the introduction of adventitious mutations (21).

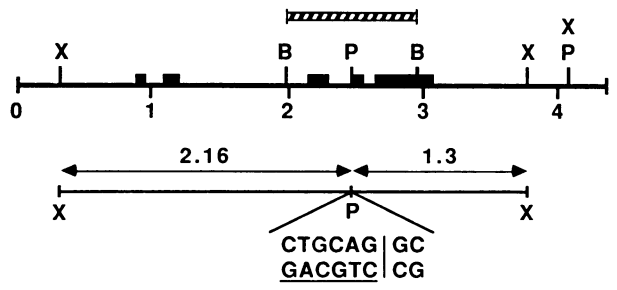

FIG. 1. Organization of the 4.4-kb genomic fragment bearing the mouse APRT gene. Solid blocks represent exons. The scale, in kilobases, is below the map. The *Xmn* I (X) and *Pst* I (P) restriction sites are indicated as are the predicted DNA fragment sizes produced by double digestion with these enzymes. The diagnostic *Pst* I site at the wild-type intron 3 splice acceptor site is indicated, separated from exon 4 by a vertical line. The last two nucleotides of the intron 3 splice acceptor site are the targets for mutagenesis. The *Bam*HI (B) fragment, used as a probe for Southern blots, is indicated by the diagonally striped bar.

Human APRT$^-$ HTD 114 (20) cells were cotransfected with pSG272 (22), which contains a G418-resistance marker, and either plasmid M4 or plasmid M1, which contain an A $\rightarrow$ G or G $\rightarrow$ A transition, respectively, at the target sites. These mutations prevent APRT expression in either a transient or a stable transfection assay (data not shown). The M4 and M1 mutants were selected as test models because it is well established that EMS causes primarily G $\rightarrow$ A transitions in bacteria (23) and in shuttle-vector systems (24–26) whereas 2AP is believed to induce primarily A $\rightarrow$ G transitions in prokaryotes and cultured mouse cells (27, 28). Three independently derived, G418-resistant colonies contained at least one apparently full-length, mutant mouse APRT gene, as demonstrated by an intact *Xmn* I fragment (Fig. 1), but did not express APRT activity. These clones, designated DM4, FM4, and AM1, exhibited a low spontaneous reversion frequency ($<10^{-7}$; see Table 1), and retained their APRT transgenes over a period of several months in the absence of G418 selection. Following exposure to EMS at 50 or 100 μg/ml the reversion frequencies of DM4 and FM4 increased in a dose-related manner to $>10^{-4}$. Even higher rates of reversion were observed when the cells were treated with EMS at 200 μg/ml, but these rates can only be estimated as this dose was extremely toxic, producing highly variable corrected cloning efficiencies as low as 0.04% (data not shown). Similar levels of EMS toxicity have been observed with human lymphoblasts (29). In five experiments, exposure of DM4 or FM4 cells to 2, 6, or 10 mM 2AP reduced survival but did not induce reversion (Table 1). Reversion of AM1 cells after exposure to 2AP is indicated in Table 1. Exposure of AM1 cells to EMS in four experiments produced no revertants (Table 1).

The APRT activities of several independent revertants exhibited an electrophoretic mobility characteristic of the mouse enzyme (11, 12), indicating reversion of a mouse transgene rather than reactivation or reversion of a human gene (Fig. 2). To confirm that reversion of the mouse M4 transgene was due to a G $\rightarrow$ A transition at the target site, DNAs from parental clones DM4 and FM4, and from their EMS-induced revertants, were digested with both *Xmn* I and

Table 1. Spontaneous and induced reversion of transgenic cells

Clone	EMS, μg/ml	2AP, mM	Survival, %	APRT$^+$ colonies, no. per 10^7 viable cells
DM4	0	0	100	0.8
	50		42	200
	100		10	3900
		2	90	0.3
		6	64	0.5
		10	10	1.5
FM4	0	0	100	1.3
	50		20	650
	100		2	7500
AM1	0	0	100	0.3
	50		31	<2*
		2	84	3.6
		6	69	30

*No revertants among 5 $\times$ 10^6 surviving cells.

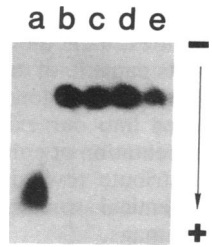

FIG. 2. Autoradiograph showing mouse-type APRT activity in EMS-induced revertants of HTD 114 cells. Cell extracts were electrophoresed in starch gels, which were then incubated to show the migration of APRT activity (11, 12). Lanes: a, human HT 1080 cells; b, mouse L cells; c–e, three independent EMS-induced APRT$^+$ revertants of cell line FM4.

Pst I. The parental DNAs produced the expected single 3.5-kb fragment, while the revertants each displayed additional 2.2-kb and 1.3-kb bands, indicative of site-specific reversion and regeneration at the diagnostic *Pst* I site (Fig. 3). Similar results have been obtained with digestion of polymerase chain reaction-amplified DNAs (31) from 21 clone AM1 revertants (data not shown). Retention of the 3.5-kb band is expected in revertants if one or more of several integrated mouse genes remains mutant. With the assumption of a single reversion event, the relative intensity of this 3.5-kb band in clone F (Fig. 3) suggests that each cell contains three or four mouse APRT genes (see below). We have analyzed DNAs and APRT enzymes from more than 30 independent, EMS-induced revertants derived from clones DM4 and FM4, and all have regenerated the *Pst* I site and expressed an APRT with mouse-type electrophoretic mobility.

In clones DM4 and FM4, pSAM4.4M4 has probably integrated in a tandem arrangement of multiple copies as a consequence of homologous recombination during transfection (32). A perfect tandem array of pSAM4.4M4 digested with *Pst* I, which cuts at a unique site at the 3′ end of the plasmid, will yield only 7.1-kb and junctional fragments (see Fig. 1). Clones DM4 and FM4 (Fig. 4, lanes C and D) exhibit a 7.1-kb band as well a 5.3- and a 5.7-kb band, respectively. These latter bands represent 5′ junctional fragments and display an intensity consistent with a single-copy sequence.

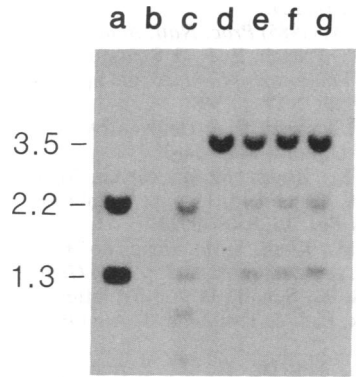

FIG. 3. Restoration of the diagnostic *Pst* I site in transgenomes of EMS induced APRT$^+$ revertants. Genomic DNAs (10 μg) were digested with *Xmn* I and *Pst* I, electrophoresed through a 0.8% agarose gel, blotted, and hybridized with the radiolabeled internal *Bam*HI fragment (Fig. 1). Under the conditions used, this probe does not hybridize to the human APRT gene (11). Lanes: a, HTD 114 plus 40 pg of plasmid pSAM4.4, containing the wild-type mouse APRT gene; b, HTD 114; c, clone EM4, an APRT$^+$ transfectant of HTD 114 carrying at least one functional mouse APRT gene (additional low molecular weight bands reflect a rearranged mouse gene); d, clone FM4, an Aprt$^-$ human HTD 114 clone bearing three or four copies of the mutant mouse APRT transgenome; e–g, three independent EMS-induced APRT$^+$ clone FM4 revertants.

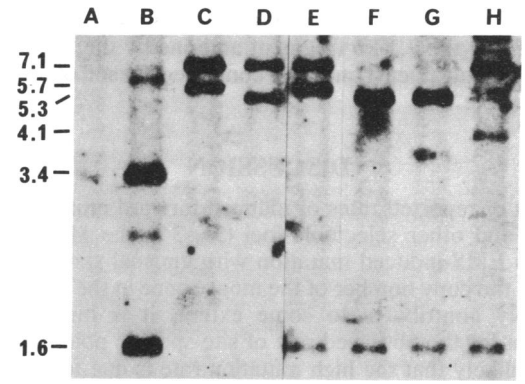

FIG. 4. *Pst* I digestions of parental and revertant transgenomes. Southern blots of *Pst* I-digested DNAs were hybridized to the internal *Bam*HI fragment (Fig. 1). Lanes: A, HTD 114; B, CAK, a wild-type mouse cell line [faint bands indicate mouse pseudogenes (12)]; C, clone FM4; D, clone DM4; E, clone FM4 revertant R2; F, clone DM4 revertant R1; G, clone DM4 revertant R2; H, clone FM4 revertant R1 (incomplete digestion).

The patterns generated by integration in clones DM4 and FM4 are illustrated in the deduced restriction maps of revertant clones (Fig. 5). Two EMS-induced revertants of clone DM4 display a loss of the 7.1-kb band and the appearance of a 1.6-kb band (Fig. 4, lanes F and G, and Fig. 5*A*), the latter indicative of restoration of the mutated *Pst* I site. Based upon densitometry, the remaining band is actually a doublet, probably containing the original 5.3-kb band and a second 5.5-kb band derived from the remainder of the revertant gene (Fig. 5*A*). The 7.1- and 5.3-kb bands in clone DM4 are approximately equal in intensity (Fig. 4, lane D, and data not shown), suggesting that two copies of pSAM4.4M4 have integrated in tandem and that the 3′ copy has reverted (Fig. 4, lanes F and G, and Fig. 5*A*). Revertants of clone FM4 exhibit two distinct patterns, consistent with three tandem integrants. The first type displays the expected 1.6-kb fragment. The 7.1-kb fragment is diminished in intensity relative to the 5.7-kb fragment (Fig. 4, lane E, and Fig. 5*B*). The broad band of about 5.7 kb is actually a doublet comprised of the 5.7-kb band seen in clone FM4 and a 5.5-kb band derived from the 5′ half of the reverted gene. The second type (Fig. 4, lane H) also exhibits the expected 1.6-kb fragment but shows loss of the 5.7-kb fragment and appearance of a fragment of about 4.1 kb. This pattern is consistent with reversion of the 5′ gene copy, resulting in a change in size of the 5′ junctional fragment (Fig. 5*C*). Thus, clone FM4 prob-

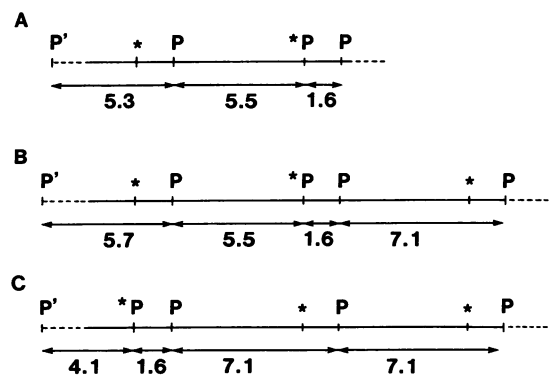

FIG. 5. Proposed *Pst* I restriction maps of revertant transgenomes. P, *Pst* I site in revertant transgenome; P', *Pst* I site in flanking human genomic DNA; *, mutated site at which *Pst* I no longer cuts; *P, *Pst* I site restored by reversion. The predicted fragment sizes (kb) are indicated below each map. (*A*) Clone DM4 revertants R1 and R2 (Fig. 4, lanes F and G). (*B*) Clone FM4 revertant R2 (Fig. 4, lane E). (*C*) Clone FM4 revertant R1 (Fig. 4, lane H).

ably contains at least three transgenes in tandem, two represented by the 7.1-kb fragment and one by the 5.7-kb *Pst* I fragment, and at least one gene contained in each of the bands is revertible.

DISCUSSION

In light of reported rates of induced forward mutation at the APRT and other selectable loci (33, 34), the M4 construct detects EMS-induced mutation with unusual sensitivity. Although the copy number of the mouse gene in the human cells probably contributes to some extent, it is insufficient to account for the observed rate of site-specific point mutation. It is unlikely that the high mutation rate is due to a chromosomal position effect, since the integration sites in clones DM4 and FM4 are different. However, the intragenic site of mutagenesis, mandated by the position of the inactivating mutation, may reflect a hot spot for EMS-induced mutation. At least nine independently derived, spontaneous or EMS- or UV-induced mutations at the homologous position within the very similar Chinese hamster APRT gene have been reported (refs. 5, 6, and 25; G. M. Adair, personal communication). This hot-spot hypothesis could be examined by testing the same mutation introduced into other critical areas of the gene.

The mutagen sensitivity of HTD 114 cells may also be relevant. Both HT 1080 and HTD 114 exhibit the same level of cell death after treatment with EMS at 100 μg/ml as rodent cell lines treated with EMS at 400–500 μg/ml (unpublished data). In contrast, HTD 114 cells were relatively refractory to 2AP-induced toxicity. Levels of cell death after 2AP treatment were the same for HTD 114 and its APRT$^+$ parent HT 1080, consistent with reports that 2AP is not a substrate for APRT (35). Reports concerning the mutagenicity of 2AP have been variable. It is an effective mutagen in mouse lymphosarcoma cells (27) and mouse lymphoma cells (36) and a weak mutagen in Syrian hamster cells (37).

This report suggests the utility of our assay for detecting specific types of point mutations at defined positions in a mammalian gene. In particular, we demonstrate induction of specific G → A and A → G transitions by EMS and 2AP, respectively, in human cells, indicating mutagenic mechanisms similar to those observed in prokaryotes (23). Experiments utilizing each of the six mutant transgenes would provide a basis for defining, at two particular nucleotides, the spectrum of mutations induced by any mutagen, and the relative frequencies with which each occurs. The frequency of a particular base change that restores a wild-type DNA sequence can be measured by the frequency of phenotypic revertants, permitting the collection of large amounts of data about molecular events in the absence of marker recovery and DNA sequencing. An assay measuring phenotypic reversion of a bacterial transgene within mouse cells has been described, but determination of the molecular basis of reversion entailed both transgene recovery and sequencing, since second-site reversion was observed and no readily assayed markers for molecular events were described (8, 25). Since this report describes mutation at a specific nucleotide pair, one cannot generalize from these data about the frequency at which the same mutations might occur at other sites. Nucleotide sequence context, differential repair capacity, and chromatin organization may all contribute to differences in mutagenesis. In any event, mutation in specific sequences is of particular importance in carcinogenesis. For example, chemically induced rodent tumors in different tissues exhibit different yet characteristic mutations in Ha-*ras* genes (30).

A significant aspect of our assay is its potential for characterizing mutagens that require metabolic activation. Some previously described assays may introduce extraneous variables inherent in the utilization of liver cell supernatants (38) or cocultivation with hepatocytes (39) to provide promutagen activation. By starting with human cell lines that retain some of these metabolic activities, it should be possible to create transgenic cell constructs capable of endogenous activation of some mutagens. Alternatively, cloned cytochrome P-450 genes may be introduced into our existing transgenic cell lines. Successful implementation of either of these strategies would significantly contribute toward the elusive goal of defining categories of chemical structures that cause specific types of mutations in humans.

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