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Genetics

Chemically induced mutagenesis in a shuttle vector with a low-background mutant frequency

(Epstein–Barr virus/thymidine kinase/*N*-ethyl-*N*-nitrosourea)

NORMAN R. DRINKWATER AND DONNA K. KLINEDINST

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706

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ABSTRACT **We have developed a recombinant DNA shuttle vector that permits the molecular analysis of mutations induced in human cells by chemical or physical mutagens. The vector is able to replicate as a plasmid in *Escherichia coli* and in Epstein–Barr virus (EBV)-transformed human lymphoblastoid cell lines and contains the herpes simplex virus type 1 thymidine kinase gene (HSV *tk*) as the target for mutagenesis studies. After introduction of the vector into an EBV-transformed lymphoblastoid cell line (LCL-721) by electroporation, ≈2% of the transfected cells expressed the vector-encoded gene for hygromycin resistance. Plasmid DNA isolated from cells immediately after selection for hygromycin resistance (10 population doublings posttransfection) contained mutations in the HSV *tk* gene at a frequency of 6×10^{-5}. Treatment of plasmid-bearing LCL-721 cells with *N*-ethyl-*N*-nitrosourea resulted in a dose-dependent increase of up to 15-fold in the frequency of mutations in the HSV *tk* gene. The dose–response for the induction of mutations in the plasmid-encoded gene closely paralleled that for the induction of mutations in the cellular gene for hypoxanthine (guanine) phosphoribosyltransferase.**

Our understanding of the types of mutations induced by chemical or physical mutagens in mammalian cells and of the mechanisms by which such mutations are fixed is at present quite limited. Although it is now possible to determine DNA sequence changes induced in cellular genes, the complex structure of most mammalian genes presents difficulties in cloning and characterizing the mutant sequences. Recombinant DNA shuttle vectors that are able to replicate as plasmids in mammalian cells and bacteria can more readily be used to study the molecular details of mutagenesis. After fixation of mutations by replication of the vector in mutagen-treated mammalian cells, mutants may be efficiently selected and amplified in bacteria for subsequent analysis.

A stumbling block to the use of available shuttle vectors for the study of mutagenesis has been the observation that the process of transfection of DNA into mammalian cells is itself highly mutagenic (1–4). Plasmid DNA isolated from a variety of mammalian cells shortly after transfection of papovavirus-based shuttle vectors was found to contain a high frequency (*ca.* 1%) of mutations. An exception is the observation by Lebkowski *et al.* (3) that simian virus 40 (SV40)-based shuttle vector transfected into two human cell lines had a lower frequency (*ca.* 0.1%) of mutations. By using a *lacI*–SV40 shuttle vector in the human 293 cell line, Calos and coworkers (5, 6) observed a significant increase in the mutant frequency of the *lacI* gene after treatment of cells containing the vector with ultraviolet light or ethyl methanesulfonate.

Most transfection-induced mutations involve DNA sequence rearrangements (1–4, 7, 8) and are thought to result from damage to the DNA that occurs prior to replication of the vector (1, 3). The papovavirus-based shuttle vectors replicate as plasmids in nondividing cells for a relatively short period of time after transfection. Thus, one is constrained to isolate plasmid DNA from transfected populations of cells, many of which contain rearranged vectors. We reasoned that the use of a shuttle vector that was stably maintained in the nuclei of replicating cells would allow us to isolate clones of cells that contained only wild-type vector DNA. These plasmid-bearing cells would be an appropriate target for studies of induced mutagenesis.

Sugden and coworkers (9–11) have recently identified a *cis*-acting element (*oriP*) of Epstein–Barr virus (EBV) that allows replication and stable maintenance of recombinant plasmids in the nuclei of cells that express the viral nuclear antigen EBNA. We have constructed a shuttle vector based on the EBV *oriP* element for studying mutagenesis in EBV-transformed human lymphoblastoid cell lines. Other key elements of this shuttle vector include sequences derived from the bacterial plasmid pBR322 that are necessary for replication in *Escherichia coli*, antibiotic-resistance markers to allow selection of plasmid-bearing bacterial (*bla*, ampicillin resistance) or mammalian (*hph*, hygromycin resistance) cells, and, as a target for mutagenesis, the thymidine kinase gene from herpes simplex virus type 1 (HSV *tk*). As we anticipated, clones of plasmid-bearing cells provided plasmid DNA with a relatively low mutant frequency for the HSV *tk* gene. Moreover, the HSV *tk* mutant frequency was low when the vector was isolated shortly after selection of transfected populations of cells. We have also shown that the induction of mutations by *N*-ethyl-*N*-nitrosourea (EtNU) in the plasmid-encoded HSV *tk* gene and in the cellular gene for hypoxanthine (guanine) phosphoribosyltransferase (HPRT) follows a similar dose–response.

METHODS

Cell Culture. LCL-721 cells [an EBV-transformed lymphoblastoid cell line established by infection of peripheral blood lymphocytes obtained from a clinically normal female (12)] were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). Normal human fibroblasts (SL68, ref. 13) and HPRT-deficient human fibroblasts (GM3467, National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, NJ) were grown in Ham's F-10 medium containing 15% fetal bovine serum and gentamicin (50 μg/ml).

Construction of Shuttle Vector pHET and Its Introduction into Lymphoblastoid Cells. Construction of the plasmid pHET is described in the legend to Fig. 1. Supercoiled plasmid DNA was introduced into LCL-721 cells by electroporation as

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Abbreviations: EBV, Epstein–Barr virus; HSV *tk*, thymidine kinase gene of herpes simplex virus type 1; HPRT, hypoxanthine (guanine) phosphoribosyltransferase; FdUrd, 5-fluorodeoxyuridine; EtNU, *N*-ethyl-*N*-nitrosourea; bp, base pair(s); SV40, simian virus 40.

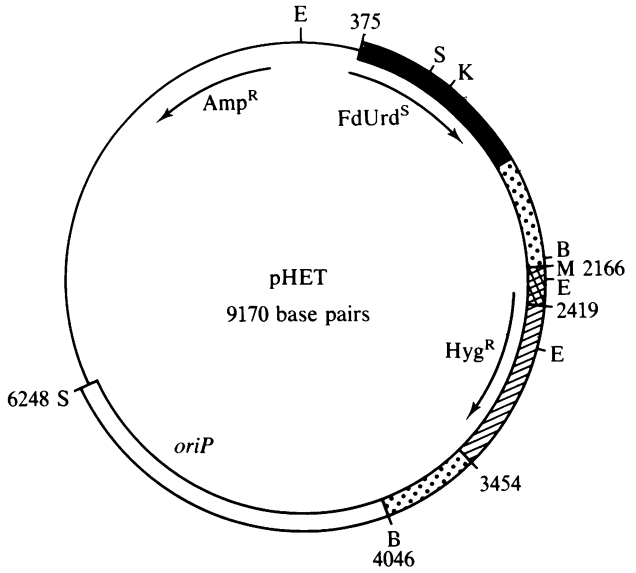

FIG. 1. Construction of shuttle vector pHET. The bacterial plasmid portion of the vector (thin line) is a deletion mutant of pBR322 (del 1420–2490) obtained from J. Yates (McArdle Laboratory, University of Wisconsin). Resistance to the antibiotic hygromycin is provided by fusion of the coding sequence of the bacterial *hph* gene (hatched box) (14) to the regions of the HSV *tk* gene that control initiation (cross-hatched box) and termination (stippled box) of transcription. The *oriP* element (open box) from EBV, which allows replication of the vector as a plasmid in EBV-transformed lymphoblastoid cells, was obtained from pHEBo (10) by digestion with *Sst* II and *Sph* I. The region of the HSV *tk* gene (*Bgl* II to *Pvu* II) that contains the coding (filled box) and 3′-noncoding (stippled box) sequences was inserted at the *Bam*HI site of the bacterial plasmid. Expression of this gene in *E. coli* is controlled by the promoter of the plasmid *tet* gene (15). The nucleotide positions of the junctions between the elements of the vector are indicated. The origin for the map is the *Eco*RI site at the top of the figure. Restriction enzyme cleavage sites are shown for *Eco*RI (E), *Sph* I (S), *Kpn* I (K), *Bst*EII (B), and *Bam*HI (M). The sequences conferring the following functions are indicated: resistance to ampicillin (Amp^R), resistance to hygromycin (Hyg^R), and sensitivity to 5-fluorodeoxyuridine ($FdUrd^S$).

described by Sugden *et al.* (10). LCL-721 cells ($1–4 \times 10^7$) were resuspended in 0.5 ml of phosphate-buffered saline, mixed with 5–10 μg of plasmid DNA, and subjected to a pulse of electric current (2000 V; 3–6 A; decay time constant, 30 msec). The cells were then diluted with nonselective medium to a density of 10^6 per ml and incubated at 37°C for 48 hr. Hygromycin-resistant cells were then selected by diluting the cells to a density of 10^5 per ml in medium containing hygromycin (300 μg/ml). Cultures were maintained at densities of $0.25–1 \times 10^6$ cells per ml in selective medium for 2 weeks, after which the cultures consisted entirely of hygromycin-resistant cells. Cultures were subsequently grown in medium containing hygromycin at 150 μg/ml. This lower level of hygromycin permitted the plasmid-bearing cells to grow at a more rapid rate than in medium containing 300 μg/ml while inhibiting the growth of cells that had lost the plasmid.

Isolation of Plasmid DNA from Lymphoblastoid Cells. Plasmid DNA was isolated from cultures of $0.5–2 \times 10^9$ lymphoblastoid cells by an alkaline extraction method (16). To isolate supercoiled vector DNA, the nucleic acid extract was first incubated with RNase A, and low molecular weight material was removed by ultrafiltration through a membrane filter (30,000 molecular weight cutoff). These samples were digested with *Xba* I, which cleaves supercoiled mitochondrial and EBV DNA. The pHET shuttle vector DNA, which is not cleaved by *Xba* I, was then purified on a CsCl/ethidium bromide density gradient. The yield of purified vector DNA was generally 50–100 ng of DNA per 10^9 cells.

Selection for Plasmids Containing Mutant HSV *tk* Genes. Mutant HSV *tk* plasmids were selected after transformation of a FdUrd-resistant derivative of *E. coli* HB101 (strain FT334; tdk^-, upp^-) (K. Eckert and N.R.D., unpublished data). Competent FT334 cells were prepared by the method of Hanahan (17) using a transformation buffer containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.2), 50 mM $CaCl_2$, 45 mM $MnCl_2$, 100 mM RbCl, and 3.5% (vol/vol) dimethyl sulfoxide. Competent cells (4×10^8 cells per 0.2 ml) were transformed with 100 ng of pHET DNA or a fraction (10–20%) of plasmid DNA purified from lymphoblastoid cells. After a 60-min expression period at 37°C, the cells were collected and resuspended in 1 ml of VBA medium [Vogel–Bonner minimal salts (18), 0.3 mM each of 19 of the common amino acids (no asparagine), 40 μg of thiamine per ml, 0.5% glucose, 40 μg each of cytidine, adenosine, and guanosine per ml, and 100 μg of uridine per ml]. Aliquots of the cells were plated in a VBA soft agar overlay containing ampicillin (0.25 mg/ml) to determine the number of transformed cells present in the culture. The remaining cells were plated in an overlay that contained ampicillin (0.25 mg/ml) and FdUrd (10 μM) in order to select for HSV *tk* mutants. The plates were incubated for 36 hr at 37°C. The HSV *tk* mutant frequency was determined as the ratio of the number of ampicillin-FdUrd resistant colonies to the number of transformants plated.

Mutagenesis. Plasmid-bearing LCL-721 cells ($10^7–10^8$ cells) were resuspended in serum-free RPMI medium containing 15 mM Hepes (pH 7.4) at a density of 10^6 per ml. The cells were then treated with EtNU dissolved in dimethyl sulfoxide (final concentration, 0.5%) for 2 hr at 37°C. Immediately after treatment, cell survival was determined by suspension at cloning densities over a feeder layer of SL68 cells in medium that contained 0.35% agarose. The remaining cells were allowed to grow for 14–15 days in complete medium to yield $\approx 10^9$ cells for each treated culture. An aliquot of the cells was then analyzed for the HPRT mutant frequency as described below. Plasmid DNA was isolated from the remaining cells for analysis of the HSV *tk* mutant frequency.

The mutant frequency for the cellular *HPRT* locus was determined as described by Thilly *et al.* (19). Cells (1.8×10^6) were suspended at densities of 5×10^4 per ml in medium containing 0.35% agarose and 40 μM 6-thioguanine over a feeder layer of GM3467 cells to select for HPRT-deficient mutants. The observed mutant frequency was corrected for the cloning efficiency of the cells in nonselective medium.

RESULTS

Characterization of Transfected Cells. As reported by Sugden *et al.* (10), electroporation is an efficient method for introducing plasmid DNA into human lymphoblastoid cells. After electroporation of LCL-721 cells with pHET DNA, between 0.1% and 2% (average, 1.1%) of the cells grew in the presence of hygromycin (300 μg/ml) when plated in multiwell dishes at a density of 100 cells per well. When plated in the absence of hygromycin, the cloning efficiency of the cells was ≈40%. Thus, we estimate the plasmid was expressed in 0.25–4.5% of the transfected cells.

Hygromycin-resistant cells were cloned in nonselective medium containing 0.35% agarose over a feeder layer of SL68 fibroblasts. Nine independent clones were isolated and grown in medium containing hygromycin (150 μg/ml). In duplicate experiments, total cell DNA was isolated from each clone and from the uncloned parental population. These samples (10 μg of DNA) along with reference standards (LCL-721 DNA to which known amounts of pHET DNA were added) were digested with *Bam*HI. After electropho-

Table 1. Spontaneous and transfection-induced mutations in plasmids isolated from human lymphoblastoid cells

Source of DNA*	Total plasmids assayed × 10^{-5}	HSV *tk* mutant frequency × 10^5
Transfection		
1	1.05	7.6
2	1.40	6.4
3	0.78	2.6
Clone		
7	0.42	4.8
8	0.58	3.4
23	1.07	15
FT334	17.2	1.7

Supercoiled pHET DNA was introduced into LCL-721 cells and plasmid-bearing cells were selected. Plasmid DNA was isolated from 0.5–2 × 10^9 cells and the mutant frequency for the plasmid HSV *tk* gene was determined.

*Transfections 1, 2, and 3 refer to three independent experiments in which plasmid DNA was isolated immediately after selection (10 population doublings posttransfection) of hygromycin-resistant cells. Clones 7, 8, and 23 were grown in the presence of hygromycin for approximately 34, 36, and 60 population doublings, respectively, between the time of cloning and the isolation of plasmid DNA. The value for FT334 refers to plasmid DNA isolated from bacteria.

resis of the products on a 0.7% agarose gel, the amounts of pHET DNA in the samples were determined by transfer of the DNA to a nylon membrane by the method of Southern (20) and hybridization to a radiolabeled probe consisting of the pBR322 and HSV *tk* sequences. An autoradiogram of the Southern blot was analyzed by microdensitometry, and the amounts of pHET DNA in the samples were estimated by comparison to the reference standards (data not shown). Among the clones tested, 9–29 copies of pHET DNA per cell were detected (average, 21 copies per cell). The parental population had 24 ± 4 copies per cell. There was no evidence (detection limit, 1 copy per cell) of integrated or rearranged plasmid in any of the clones or in the parental population.

We analyzed the plasmid DNA isolated from populations of hygromycin-resistant LCL-721 cells for the presence of unreplicated pHET DNA by taking advantage of the fact that the plasmid DNA used for transfection was obtained from an *E. coli* strain with a functional *dam* methylase. Plasmid DNA isolated from these bacteria is resistant to cleavage by the restriction endonuclease *Mbo* I and sensitive to digestion by *Dpn* I, whereas the converse is true of plasmid DNA that has undergone replication in the lymphoblastoid cells (11). Digestion of the plasmid DNA isolated from hygromycin-resistant LCL-721 cells with *Mbo* I eliminated its ability to transform *E. coli*, whereas these DNA samples were resistant to digestion by *Dpn* I (data not shown). Thus, all of the DNA isolated from the transfected cells had apparently undergone replication in the LCL-721 cells.

Frequencies of Spontaneous and Transfection-Induced Mutations. A low frequency of mutations in the HSV *tk* gene was observed when pHET DNA was isolated from transfected LCL-721 cells (Table 1). These cells had been grown in the presence of hygromycin for ≈10 population doublings after electroporation. In three independent experiments, the frequency of HSV *tk* mutants averaged 6 × 10^{-5}. This frequency was ≈4-fold higher than that observed for the input pHET DNA, which was purified from plasmid-bearing *E. coli* FT334.

Three clones of hygromycin-resistant LCL-721 cells (clones 7, 8, and 23) were continuously cultured in the presence of hygromycin for 34, 36, and 60 population doublings from the time of cloning. The HSV *tk* gene in plasmid DNA isolated from clone 23 cells, which were passaged for the longest time, had a mutant frequency of 1.5 × 10^{-4} (Table 1).

Mutagenesis by EtNU. Frequencies of mutations in the plasmid HSV *tk* gene and in the cellular *HPRT* locus were compared after treatment of plasmid-bearing LCL-721 cells with EtNU at doses from 0.25 to 1 mM (Table 2). Survival of cells treated with the highest dose of EtNU was 12% relative to solvent-treated cells. A dose-dependent increase in HSV *tk* mutant frequency was observed for plasmid DNA isolated 14–15 days after treatment. In two experiments, the HSV *tk* mutant frequency increased ≈15-fold from an average of 2.5 × 10^{-5} for solvent-treated cells to 4.2 × 10^{-4} for cells treated with 1 mM EtNU. Although the mutant frequency at the *HPRT* locus in solvent-treated cells was lower by a factor of ≈10 than that for the HSV *tk* gene, the yields of mutants after treatment with EtNU were quite similar for these two genes. For example, the induced mutant frequencies after treatment with 1 mM EtNU were 5.4 × 10^{-4} and 4.2 × 10^{-4} for the *HPRT* and HSV *tk* genes, respectively.

Characterization of HSV *tk* Mutant Plasmids. A small fraction of the colonies obtained on FdUrd-selective plates was chosen for further analysis. Plasmid DNA was isolated from 52 independent FdUrd-resistant colonies obtained after transformation of FT334 cells with plasmid DNA purified from lymphoblastoid cells. When these DNA samples were used to retransform FT334 cells, survival of the transformants on FdUrd-selective plates was 85–100%—i.e., each of the isolated plasmids lacked a functional HSV *tk* gene.

We studied the structures of the HSV *tk* mutant plasmids by digestion of the DNA with *Kpn* I, *Ava* I, and *Xho* II followed by separation of the products by electrophoresis on a 2% agarose gel. Digestion of pHET DNA with these three enzymes cleaves the coding sequence of the HSV *tk* gene into

Table 2. EtNU-induced mutations in the HSV *tk* and *HPRT* genes of plasmid-bearing human lymphoblastoid cells

Experiment	EtNU dose, mM	Surviving fraction	HPRT mutant frequency × 10^5	Total plasmids assayed × 10^{-5}	HSV *tk* mutant frequency × 10^5
1	0	1.0	0.3	0.72	1.4
	1.0	0.09	58	0.92	38
2	0	1.0	0.4	2.31	3.5
	0.25	0.73	10	2.00	7.5
	0.5	0.54	20	0.24	29
	1.0	0.15	51	1.50	46

Plasmid-bearing LCL-721 cells (12–16 population doublings posttransfection) were treated with EtNU in serum-free medium containing 15 mM Hepes (pH 7.4) for 2 hr at 37°C. Immediately after treatment, an aliquot of the cells was plated in agarose to determine the cloning efficiency. Survival is expressed as the ratio of the cloning efficiency for EtNU-treated cells relative to that for solvent-treated cells (32% and 26% for experiments 1 and 2, respectively). After 14–15 days of growth in culture, 1.8 × 10^6 cells were selected for mutations in the *HPRT* locus by growth in 6-thioguanine. Plasmid DNA was isolated from the remaining (10^9) cells and analyzed for mutations in the HSV *tk* gene.

four fragments of 356, 301, 283, and 223 base pairs (bp). This method would allow us to detect deletions within the HSV *tk* coding sequence as small as 30 bp. The structures of the mutant plasmids depended on the source of the DNA (Fig. 2). Thus, 9 of 11 mutants obtained from plasmid DNA isolated from LCL-721 cells shortly after transfection were deletion mutants, whereas only 1 of 7 of the mutants obtained from plasmid DNA isolated from clone 23 cells carried deletions. Approximately 30% (11/34) of the mutants obtained from plasmid DNA isolated from cells that were treated with 1 mM EtNU contained detectable deletions.

The observed deletions ranged in size from 50 to 5400 bp, with a median of 2350 bp. The approximate 5′ ends of the deletions varied from position 8800 in the plasmid (between the *bla* and HSV *tk* genes) to position 1300 (in the middle of the HSV *tk* coding sequence). With one exception, the approximate 3′ ends of the deletions lay between position 1350 and position 4480 (upstream of the essential sequences of the *oriP* element). An exception was the single deletion mutant isolated from clone 23 plasmid DNA; this deletion extended from position 400 to position 5800 and eliminated the HSV *tk* and *hph* genes and nearly all of the *oriP* element. Because this plasmid lacks the sequences in the *oriP* element required for replication in lymphoblastoid cells (11), it may have arisen either immediately prior to isolation of plasmid DNA from clone 23 cells or during the transformation of FT334.

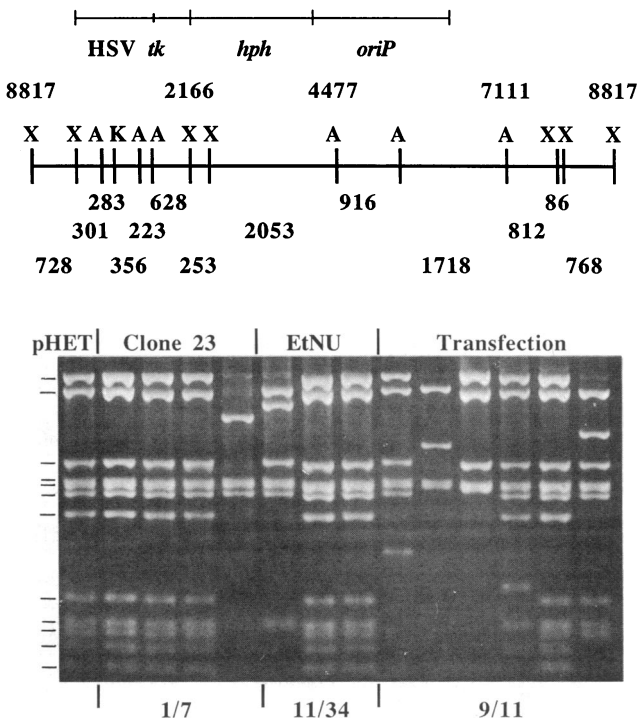

FIG. 2. Characterization of mutant plasmids isolated from human lymphoblastoid cells. DNA samples from 11 transfection-induced mutants, 7 mutants isolated from clone 23 cells, and 34 mutants obtained from cells treated with 1 mM EtNU were analyzed for deletions or rearrangements by restriction mapping. HSV *tk* mutant DNA was digested sequentially with *Kpn* I, *Ava* I, and *Xho* II and the products were separated by electrophoresis on a 2% agarose gel. (*Upper*) Map indicating the positions of restriction enzyme cleavage sites in pHET. The map shows the 9170-bp plasmid linearized at the *Xho* II site at position 8817 (the plasmid map is numbered from the *Eco*RI site derived from pBR322). The cleavage sites for *Kpn* I (K), *Ava* I (A), and *Xho* II (X) are indicated by the vertical bars. Four cleavage sites that generate small DNA fragments (17, 12, 11, and 5 bp) are not shown on the map. These sites are at nucleotide positions 8817, 8032, 7934, and 2424, respectively. The numbers above the map indicate the nucleotide positions of some of the cleavage sites. The numbers below the map are the sizes of the fragments in base pairs. The positions of the HSV *tk*, *oriP*, and *hph* sequences are also indicated. The HSV *tk* gene is shown from 5′ to 3′, and the small tick in the middle of the sequence indicates the end of the coding sequence. (*Lower*) Ethidium bromide-stained gel obtained for mutant plasmids digested with restriction endonucleases. The sources of the DNA samples are indicated above the gel photograph. The leftmost lane contained purified wild-type pHET DNA. The positions of the restriction fragments for pHET DNA are indicated at the left edge of the photograph. The ratios given below the photograph are the number of observed deletions per total mutants tested for each category of DNA sample. The 86-bp *Xho* II fragment is not visible.

DISCUSSION

We have demonstrated that a shuttle vector (pHET) containing the *oriP* element of EBV permits the molecular analysis of chemically induced mutagenesis in human cells. Introduction of the shuttle vector into human lymphoblastoid cells by electroporation resulted in a low background of transfection-induced mutants. The vector was stably maintained in cells grown under selective conditions and was subject to a low spontaneous mutation rate. Treatment of plasmid-bearing lymphoblastoid cells with the mutagen EtNU resulted in a dose-dependent increase of up to 15-fold in the mutant frequency for the plasmid-encoded HSV *tk* gene.

When pHET DNA was isolated immediately after selection of plasmid-bearing lymphoblastoid cells, the frequency of mutations in the HSV *tk* gene was $\approx 6 \times 10^{-5}$, a 4-fold increase over the mutant frequency in the input DNA. This result contrasts with those of Calos and coworkers (2, 3) and Seidman and coworkers (1, 4), who studied induction of mutations in the *lacI* or *galK* genes, respectively, after transfection of shuttle vectors that replicate transiently in mammalian cells. Transfection of vectors based on the replication origin of SV40 or other papovaviruses into human, monkey, or mouse cells resulted in induced mutant frequencies ranging from 5×10^{-4} to 10^{-2} (1–4), one to two orders of magnitude higher than observed in our studies.

There are several possible explanations to account for the difference in the frequencies of transfection-induced mutations. (*i*) The introduction of pHET into lymphoblastoid cells by electroporation may result in less damage to the DNA than the methods used for transfection in previous studies. (*ii*) The frequency of transfection-induced mutations may depend on the type of cells. Lebkowski *et al.* (3) have shown that transfection of a SV40-based shuttle vector into human 293 or HeLa cells was 10% as mutagenic as transfection into several monkey cell lines. The low mutant frequencies observed in our studies could be a property of LCL-721 cells or human lymphoblastoid cells in general. (*iii*) We observed that most of the HSV *tk*-deficient mutants isolated from transfected cells contained deletions that included part or all of the hygromycin gene. Cells containing these deleted plasmids may have been at a selective disadvantage during the 10 generations of growth in hygromycin prior to isolation of plasmid DNA.

A large fraction (85%) of the transfection-induced mutations observed in our studies were deletions. Previous studies have shown that the proportion of deletions among transfection-induced mutants depends on the distance between essential sequences flanking the target gene (7). The *oriP* element and the ampicillin-resistance gene are both required for a HSV *tk* deficient mutant to be scored. Thus, pHET has a large target size ($\approx$4.5 kilobases) for deletion events.

Once established in human lymphoblastoid cells, pHET is genetically stable. When we isolated clonal lines of plasmid-bearing cells and grew them in culture for up to 60 population doublings, the vector accumulated mutations in the HSV *tk*

gene at a rate of $\approx 2 \times 10^{-6}$ mutations per plasmid per cell generation. Although these data are limited, the apparent spontaneous mutation rate for the plasmid-encoded HSV *tk* gene is similar to that observed for the *HPRT* locus in cultured human cells—i.e., $\approx 10^{-6}$ mutations per cell per generation (21). Nearly all of the spontaneous HSV *tk* mutants observed in clonal lines of pHET-bearing cells contained putative point mutations as defined by restriction mapping of mutant DNA to a precision within 30 bp. These results differ from those of Ashman and Davidson (22), who observed deletion mutations at a frequency of $\approx 10^{-2}$ in a bovine papillomavirus shuttle vector isolated from clonal lines of transfected C127 cells.

The HSV *tk* gene has a coding sequence of ≈1100 bp and is a sensitive target for studying the induction of mutations in the shuttle vector. The mutant phenotype is characterized by the loss of functional enzyme activity for the gene product. Thus, use of this target gene should allow the detection of a broad range of mutational events, including base substitutions, frameshift mutations, insertions, or deletions. We observed 10- to 15-fold increases in the frequency of mutations in the HSV *tk* gene when plasmid-bearing lymphoblastoid cells were treated with EtNU at a dose allowing 10% survival of the treated cells relative to solvent-treated cells. Only a small fraction (30%) of the induced mutants contained detectable deletions. This result is consistent with studies done in bacteria (23) and in mammalian cells (24) that demonstrated that EtNU efficiently induced base-substitution mutations. The proportion of deletion mutations observed in our studies was slightly larger than would be expected if all of the deletions were derived from the background of transfection-induced mutations. The hypothesis that EtNU may induce deletion mutations in the shuttle vector awaits testing by treating cloned plasmid-bearing cell lines; these cells have a lower background of deletion mutations than the transfected populations of cells used in the current studies.

The use of shuttle vectors provides a facile approach to studying mutagenesis in mammalian cells. The selection and amplification of mutants in *E. coli* allow ready determination of the DNA sequences of a large number of induced mutations. Analysis of a large number of mutations is crucial to addressing questions related to the mutational specificity of chemical or physical mutagens and to understanding the mechanisms by which induced mutations are fixed. The *oriP* shuttle vector has several favorable attributes as a model for studying mutagenesis in mammalian cells. (*i*) The vector is stably maintained in human lymphoblastoid cells and probably replicates synchronously with normal cell replication (25). (*ii*) The vector's stability in transfected cells and its low background of transfection-induced mutations allow treatment with mutagen under conditions in which the induction of mutations in cellular genes such as *HPRT* may also be studied. (*iii*) The vector may be introduced into EBV-transformed lymphoblastoid cell lines derived from humans known to respond abnormally to DNA damage (26). Analysis of mutagenesis in plasmid-bearing cells derived from these human mutants may provide important clues to the molecular details of the process of mutagenesis in normal mammalian cells.

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