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Genetics

Detection and analysis of UV-induced mutations in mammalian cell DNA using a λ phage shuttle vector

(gene rescue/mutagen specificity/*supF* gene)

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ABSTRACT **In order to study mutagenesis in mammalian cells, stable mouse L-cell lines were established with multiple copies of a λ phage vector that contains the *supF* gene of *Escherichia coli* as a target for mutagenesis. Rescue of viable phage from high molecular weight mouse cell DNA using λ *in vitro* packaging extracts was efficient (5 phage per μg of cell DNA per copy) and yielded a negligible background of mutant phage (0 out of 54,605). From mouse cells exposed to 254-nm ultraviolet light (12 J/m^2), 78,510 phage were rescued, of which 8 were found to have mutant *supF* genes. DNA sequence analysis of the mutants suggests that the primary site of UV mutagenesis in mammalian cells is at pyrimidine-cytosine (Y-C) sequences, and that the most frequent mutation at this site is a C→T transition.**

The molecular nature of mutagenesis in mammalian cells has been difficult to study. This difficulty has been due in part to the complexity of genetic analysis in mammalian cells. It is a formidable task to identify, isolate, and analyze the products of mutagenic events occurring in the mammalian genome. As one approach, we and others (1–3) have sought to exploit the power of prokaryotic genetics in the study of mutagenesis in mammalian cells. Previous work using this approach has employed simian virus 40 (SV40)-related shuttle vectors which replicate independently of the mammalian cell genome, and which are prone to high levels of spontaneous mutagenesis that are atypical for mammalian genes (1–5). Instead, we have developed a vector based on the bacteriophage λ, λsupF-neo. This vector carries the neomycin-resistance gene (*neo*), allowing selection for stable integration of the vector into the mammalian cell genome. The phage vector DNA should therefore behave with respect to replication and mutagenesis as does chromosomal DNA. The vector also carries *supF*, the amber suppressor tyrosine tRNA gene of *Escherichia coli* (6), serving as a target gene for the study of mutagenesis. The *supF* gene is not expected to exhibit any phenotype in mammalian cells, but upon retrieval of the gene from mammalian cells and its introduction into the appropriate *E. coli* host, forward mutations to the non-suppressor phenotype can be scored. To achieve efficient rescue of the target gene from mammalian cells, we have established mouse cell lines, by DNA-transfection procedures, that carry as many as 100 copies of the λsupF-neo vector DNA. When high molecular weight DNA from these cell lines is used as a substrate for λ *in vitro* packaging extracts, viable phage particles bearing the λsupF-neo genome are produced. These phage can be propagated and analyzed in *E. coli*, and once rescued supF genes are identified as mutant, they can be further analyzed by DNA sequencing techniques.

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The utility of this system for the study of mutagenesis depends on its ability to detect mutants generated by specific mutagenic agents above the background level of mutagenesis. The high frequency of background mutations found with SV40-based shuttle vectors has been of serious concern (1–5). We present evidence here that the background level of mutagenesis of the target gene, following stable transformation of the vector into mouse cells, is $<10^{-4}$ in the absence of mutagenic treatment.

We have used this system to study the molecular nature of mutations induced by ultraviolet light in mammalian cells. Mouse cells carrying multiple copies of the λsupF-neo vector DNA in their genome were exposed to 254 nm UV light, and rescued phage with mutations in the *supF* gene were isolated and analyzed. The data suggest that the major site of UV mutagenesis in mammalian cells is at pyrimidine-cytosine (Y-C) sequences, and that the predominant mutation at these sites is a C→T transition.

MATERIALS AND METHODS

Cells. The construction of *E. coli* SY204 *lacZ125* (Am) *trp-49*, *hsdR2*::Tn*10* was described previously (1). *E. coli* SY301 was constructed by introduction into SY204 of a mutation in ultraviolet mutability at the *umuC* locus by P1 transduction with Tn*5* kanamycin resistance from *E. coli* GW2100, which carries the *umuC122*::Tn*5* mutation (7). Mouse LTK$^-$cells (8) were grown in modified Eagle's medium supplemented with 10% fetal bovine serum.

Phage. λsupF-neo was constructed from the phage vector λgt2 (9). The *supF* gene was isolated as a 200-base-pair (bp) *Eco*RI fragment from plasmid p3AC (1). Following attachment of *Sal* I linkers, the *supF* gene was ligated into the unique *Sal* I site of the plasmid pdBPV-MMTneo (10) from which the 8-kbp *Bam*HI fragment containing bovine papilloma virus sequences had been eliminated. The resulting plasmid, pPG2, was linearized at the *Eco*RI site and ligated into the *Eco*RI cloning site of λgt2 (Fig. 1).

DNA Transfection. LTK$^-$ cells were transformed with λsupF-neo DNA in the absence of carrier by the calcium phosphate method (11). Before transfection, the phage DNA was self-ligated at a concentration of 300 μg/ml in order to generate concatamers. Transformed cells were selected at an initial density of 4000 cells per cm^2 in the presence of the neomycin analogue G418 at 400 μg/ml.

Phage Rescue. High molecular weight DNA prepared from transformed cells (12) was used as a substrate for λ *in vitro* packaging extracts, which were prepared as described by Hohn (13). Only extracts efficient enough to package at least 5×10^8 plaque-forming units (pfu) per μg of purified λ DNA were used. For cell DNA, up to 1 μg of DNA in up to 5 μl of water was added per 50-μl packaging reaction. Packaged

Abbreviations: SV40, simian virus 40; pfu, plaque-forming units; bp, base pair(s).

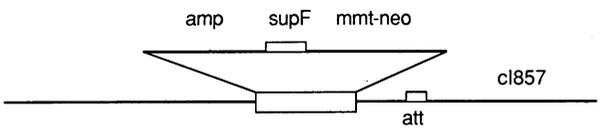

FIG. 1. Diagram of the λsupF-neo vector genome. This phage is a derivative of λgt2 (9), into which was inserted a pBR322-derived plasmid containing the *E. coli* ampicillin-resistance gene (*amp*), the *supF* gene (in the unique *Sal* I site of the plasmid), and the mouse metallothionein-I promoter attached to the *E. coli* coding sequence for neomycin resistance (*mmt-neo*). For orientation purposes, the lambda *att* site is indicated along with the locus of the *c*I857 gene from λgt2.

phage were plated on Luria-broth plates in top agar containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) at 200 μg/ml and isopropyl β-D-thiogalactopyranoside (IPTG) at 75 μg/ml. Either SY204 or SY301 was used as a host. Since both strains contain *lacZ125*, an amber nonsense mutation in the β-galactosidase gene, phage carrying a functional *supF* gene produce blue plaques in this assay, whereas colorless plaques arise from phage that have lost suppressor activity.

DNA Blots. DNA dot blots were performed using a Schleicher and Schuell microsample filtration manifold as described by the manufacturer. Southern blots and filter hybridizations were performed by standard methods (14).

DNA Sequencing. Mutant *supF* genes were subcloned from λsupF-neo into the *Sal* I site of phage M13 mp10. Sequencing by the chain-termination method (15) was carried out using 5′-[α-[^{35}S]thio]dATP (Amersham) and buffer-gradient gels (16).

RESULTS

Transfection of λ Phage Shuttle Vector. Fig. 1 schematically depicts the λ shuttle vector used in these studies. This vector carries a marker selectable in mammalian cells, the neomycin-resistance gene driven by the mouse metallothionein-I promoter. It also carries the *supF* gene of *E. coli* as the target gene for mutagenesis experiments. This phage can lysogenize *E. coli* and confers on such lysogens resistance to ampicillin. Hence phage that lack suppressor activity can be screened quickly to determine whether they represent background from the *in vitro* packaging extract, in which case they cannot form ampicillin-resistant lysogens, or whether they are *bona fide* mutants rescued from mammalian cell DNA. This construct carries no origin of replication that can function in mammalian cells, and so it is unlikely to be maintained outside of the chromosomes.

Prior to transfection, the phage DNA was ligated to form concatamers to protect the λ cohesive ends, which are necessary for efficient packaging of the DNA into phage particles (13, 17). Transfection of LTK$^-$ cells and selection in the presence of G418 (400 μg/ml) yielded 15 colonies of resistant cells, designated LN1 through LN15. Fig. 2 shows a DNA dot blot done to determine the number of copies of the λsupF-neo genome per cell. As standards for quantitation, DNA from the parental cell line, LTK$^-$, was mixed with appropriate amounts of purified λsupF-neo DNA to approximate 0, 1, 5, 10, and 50 phage-genome copies per cell. For each standard or cell line, aliquots containing 1 and 5 μg of DNA were applied to the nitrocellulose filter, which was exposed to the ^{32}P-labeled internal 6.8 kbp *Eco*RI fragment of λsupF-neo DNA under hybridization conditions. As can be seen, there is a wide range in copy number. Southern blot analysis (not shown) indicates that the internal λ DNA fragments are essentially unrearranged in most of the cell lines regardless of copy number.

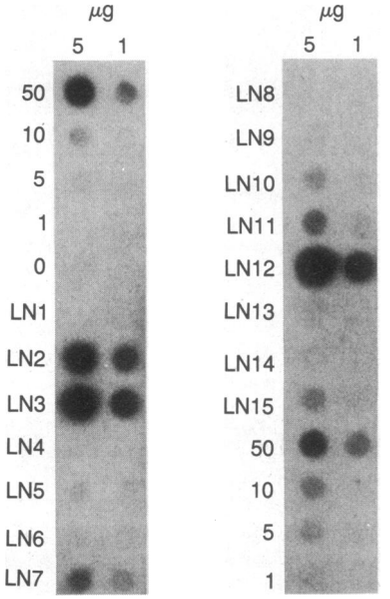

FIG. 2. DNA dot blot analysis of the number of copies of the λsupF-neo vector genome contained in transfected mouse LTK$^-$ cells. DNA was prepared from 15 G418-resistant cell lines (LN1–LN15) made by transfection of LTK$^-$ cells with λsupF-neo vector DNA. As standards for quantitation, DNA from untransfected LTK$^-$ cells was mixed with purified λsupF-neo DNA to approximate 0, 1, 5, 10, and 50 phage genome copies per cell. For each sample as indicated, aliquots of 1 and 5 μg of total DNA were applied to a nitrocellulose filter and then hybridized to the ^{32}P-labeled, 6.8-kbp internal *Eco*RI fragment of λsupF-neo DNA and autoradiographed.

Phage Rescue. High molecular weight DNA from each cell line was used as a substrate in λ *in vitro* packaging reactions. In Fig. 3, the number of pfu rescued from 1 μg of cell DNA is plotted on a logarithmic scale against the number of copies of λsupF-neo per cell. As expected, there is a direct relationship between these variables. On average, the yield is 1–5 pfu per μg of cell DNA per copy of λ.

Analysis of Rescued Phage. The cell line yielding the greatest number of rescued phage was LN12. From this cell line, which carries about 100 copies of the phage genome per cell, 500 phage could be rescued per μg of cell DNA. Phage

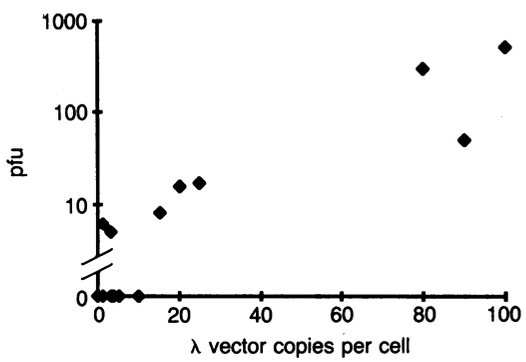

FIG. 3. Efficiency of phage rescue. High molecular weight DNA from LTK$^-$ cells and from cell lines LN1–LN15 was used as a substrate in λ *in vitro* packaging reactions. The resulting number of pfu rescued per μg of cell DNA for each cell line is plotted on a logarithmic scale against the number of copies of the λsupF-neo genome estimated to be present per cell on the basis of the DNA dot blot shown in Fig. 2. Note that the vertical axis is broken and that the horizontal axis denotes the value zero. Points displayed on the horizontal axis represent the seven cell lines (LN1, LN5, LN6, LN9, LN10, LN13, and LN14) plus the LTK$^-$ cells from which no phage have been rescued.

rescued from this cell line were analyzed for *supF* activity by growth on a *lac*(amber) *E. coli*, either SY204 or SY301, in the presence of the chromogenic β-galactosidase indicator 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). A total of 54,605 phage were screened, and all were found to produce the blue plaques indicative of a functional *supF* gene in the assay used. No mutant phage were rescued from cells in the absence of exposure to mutagens. Therefore, the background level of mutants in this system is less than one in 54,605.

Mutagenesis by Ultraviolet Light. LN12 cells at a density of 2–4 × 10^5 per cm^2 were exposed to 254-nm UV light (12 J/m^2). This dose reduced the survival of the cells, with respect to their ability to form viable colonies, to about 10% that of untreated cells. Forty-eight hours after exposure to UV, DNA was harvested from the cells. Screening was performed using two *lac*(Am) *E. coli* hosts that differed only in that one (SY301) was a UmuC-deficient derivative of the other (SY204). UmuC-deficient strains show decreased UV-mutability with respect to both their own genome and that of UV-irradiated phage for which they are host (18). If the phage rescued from irradiated LN12 cells carry photochemical lesions in their DNA that have not been processed into mutations within the mouse cells (possibly because of radiation damage to the cells), then screening of phage on SY301 should yield a lower frequency of mutants. If, however, the mutations in the rescued phage are preexisting, having been fully processed in the mouse cells, then the mutation frequency should be the same as measured in the two *E. coli* strains. Table 1 lists the number of phage screened and the number of mutants detected using the two stains. As can be seen, the mutation frequency is independent of the strain used for screening, implying that the mutations arose from the processing of UV-induced lesions within the mouse cells. The total mutation frequency of 8/78,510 is significantly higher than that derived from untreated LN12 cells (0/54,605), and so it is reasonable to assume that the mutants collected are the result of exposure of the cells to UV light and are representative of the spectrum of UV-induced mutations.

The *supF* genes from the eight mutant phage were subcloned into M13 for sequence analysis. Table 2 presents the results of this analysis. Seven of the eight mutations consisted of single base changes. One involved two base changes separated by a single nucleotide. No rearrangements, insertions, or deletions were detected. Of the nine base changes, eight occurred at the 3′ cytosine of a Y-C sequence. Seven of these eight were C→T transitions, while one was a C→G transversion. The remaining mutation consisted of a T→C transition at a CTC triplet, which may be interpreted as a mutation at the 5′ pyrimidine of a Y-C sequence. All the mutations occurred within runs of 4–10 pyrimidines. These conclusions are summarized in Fig. 4.

The position of each of the mutations can be seen better in Fig. 5, in which the mutated nucleotides are identified on the cloverleaf *supF* tRNA structure. The mutations seem to cluster in two regions, and there is some overlap among them.

Table 2. Sequences of *supF* gene mutations in rescued phage

Mutant number	*supF* sequence	Mutant sequence
1,2,8	ACTTCGAA TGAAGCTT	ACTTTGAA TGAAACTT
3	AAAGGGAG TTTCCCTC	AAAGAGGG TTTCTCCC
4	ACTTCGAA TGAAGCTT	ACTTCAAA TGAAGTTT
5	AATCCTTC TTCGGAAG	AATTCTTC TTAAGAAG
6	AAAGGGAG TTTCCCTC	AAAGAGAG TTTCTCTC
7	AGACTCTA TCTGAGAT	AGACTGTA TCTGACAT

The *supF* genes of mutant phage rescued from irradiated mouse LN12 cells were isolated and sequenced. For each *supF* mutant as indicated, an 8-bp region of DNA sequence, including the actual mutation, is listed opposite to the corresponding sequence of the normal *supF* gene.

One of the two changes in mutant 3 constitutes the single change in mutant 6, and mutants 1, 2, and 8 involve identical mutations. Mutants 1 and 2 came from the same mutagenesis experiment, but this experiment and the ones that yielded mutants 3, 6, and 8 were all different. Thus all except possibly mutants 1 and 2 were independent mutants.

DISCUSSION

Much of the current understanding of DNA repair and mutagenesis in mammalian cells depends on extrapolations from mechanisms elucidated in bacteria. We have developed a system to study directly mutagenesis in mammalian cells by exploiting the power of prokaryotic genetics. Using a λ phage vector that is stably integrated into the mammalian cell genome, we have avoided the problem of a high background level of mutagenesis that has been observed in other systems (1–5). Also, our results may be more representative of the processes that affect the normal cellular DNA, in contrast to the results obtained with extrachromosomal, independently replicating SV40-related vectors. The use of a phage vector is preferable to the use of a cosmid, since Lau and Kan (19) report a high frequency of rearrangements among cosmids rescued from mammalian cells.

The efficiency of gene rescue with this system renders it suitable for the study of mutagen specificity. Preliminary experiments indicate further that an assay based on a system of phage rescue is sensitive enough to detect the products of recombination events between phage genomes transfected into mammalian cells. The use of phage and cosmid rescue in the cloning of mammalian genes has been reported (17, 19, 20).

Table 1. Frequency of mutant phage rescued from mouse LN12 cells

E. coli host	Control cells			UV-irradiated cells		
	Phage, no.	Mutants, no.	Mutation frequency	Phage, no.	Mutants, no.	Mutation frequency
SY204	41,105	0	$<0.2 \times 10^{-4}$	47,510	5	1.1×10^{-4}
SY301 (*umuC*)	13,500	0	$<0.7 \times 10^{-4}$	31,000	3	1.0×10^{-4}
(Total)	54,605	0	$<0.2 \times 10^{-4}$	78,510	8	1.0×10^{-4}

Phage were rescued by *in vitro* packaging of DNA isolated from either unirradiated mouse LN12 cells or from LN12 cells treated with UV radiation (12 J/m^2). Phage were screened for mutations in the *supF* gene by an assay for suppression using two *lac*(amber) *E. coli* hosts, either SY204 or its UmuC-deficient derivative, SY301.

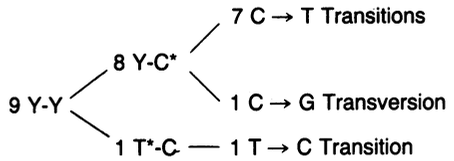

FIG. 4. Summary of UV-induced mutations in LN12 cells. The *supF* genes of mutant phage rescued from LN12 cells were isolated and sequenced. A total of nine single base-pair changes were observed in the eight mutant genes sequenced. The diagram indicates the number of times a mutation was found at each particular kind of site. An asterisk marks the nucleotide involved in the mutation. Nucleotide sequences are written in the 5'-to-3' direction. Y, pyrimidine.

In this study, it was demonstrated that the mutation frequency is independent of the *umuC* mutation in the *E. coli* strain used for growth of the rescued phage. This suggests that the UV-induced mutations detected in this system are actually produced in mammalian cells and do not result from bacterial repair of unprocessed lesions persisting in mouse cell DNA.

The use of the *supF* gene as a target gene in these experiments provides the benefit not only of a small gene that can be easily sequenced, but also of a gene that has been well-studied with regard to the functional implications of a wide range of mutations (21). Previous work has shown that a variety of mutations can inactivate *supF* and other bacterial tRNA genes. Hence, there are no particular constraints on the types of mutations that can be detected by use of this system.

The analysis of UV-induced mutations in mouse cells presented here suggests that such mutations tend to occur at Y-C sequences and that the predominant change is a C→T transition at these sites. This specificity is similar to that reported in *E. coli* (22–25), supporting the validity of cautious extrapolation from *E. coli* to mammalian cells. Interestingly, no mutations were found at T-T sequences even though T-T dinucleotides are well-represented in the *supF* gene, with 31 T-T sites in addition to 59 Y-C sites in the 200-nucleotide-long gene. Brash and Haseltine (23) suggest that for *E. coli* the

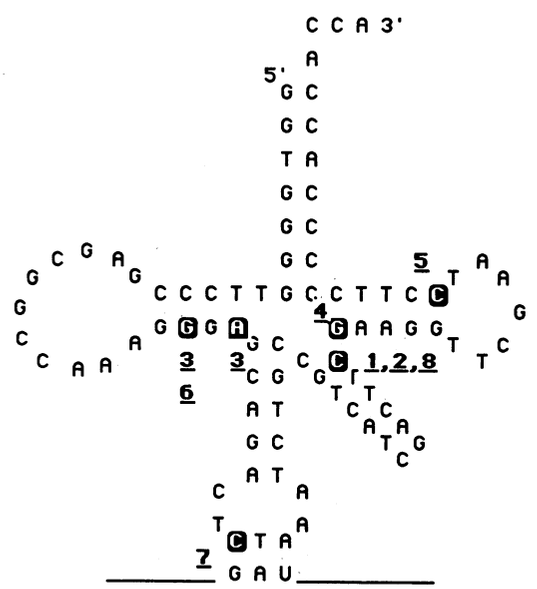

FIG. 5. Location of the UV-induced mutations on the clover-leaf structure of the *supF* tRNA. Shadowed letters indicate the position on the tRNA structure of nucleotides observed to be mutated in the *supF* genes of mutant phage rescued from UV-irradiated LN12 cells. Also noted are the numerical designations of the mutant phage that bear the particular mutations. Note that mutant 3 involves two nearby base changes.

main mutagenic lesion may be the pyrimidine-pyrimidone-(6-4) photoproduct rather than thymidine dimers, even though thymidine dimers may occur more frequently in response to UV light. Our data indicate that such may be the case for mammalian cells as well.

We have recently learned of the work of Sarasin *et al.* (26), who studied a collection of UV-induced reversion mutations in a temperature-sensitive strain of SV40. About half of these mutations were transversions and half were transitions, in contrast to our results, although, as we observed, most mutations occurred at Y-C sites. The reason they detected relatively more transversions may be that they were looking for reversion mutations rather than forward mutations. Further, none of their revertants involved the actual tsA58 mutant codon, and so the spectrum of mutations they observed may be biased by the particular requirements for intragenic suppression of the initial mutation. In addition, SV40, as an extrachromosomal replicon, may not precisely mimic the cellular genome in regard to DNA damage and repair.

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