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Error-Prone Mutagenesis Detected in Mammalian Cells by a Shuttle Vector Containing the *supF* Gene of *Escherichia coli*

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When a shuttle vector containing a tyrosine suppressor tRNA (*supF*) gene as a target for mutagenesis replicated in a monkey kidney cell line, the frequency of $SupF^+$ mutations was $2.3 \pm 0.5 \times 10^{-3}$. When the host cells were treated with ethyl methanesulfonate 40 h before transfection, a 10-fold increase in $SupF^+$ mutation frequency was observed. These results supported the hypothesis that a damage-inducible mutagenic pathway exists in mammalian cells and also demonstrated the utility of this shuttle vector for the study of mutagenesis in mammalian cells.

The molecular basis of mutagenesis in mammalian cells remains obscure in part because of the difficulty in analyzing the products of the mutagenic events. In the absence of the elegant genetic and biochemical systems of procaryotes or fungi, progress in the study of mammalian-cell mutagenesis has been slow. As one approach, we and others (3, 15) have reasoned that a system that exploits the power of procaryotic genetic analysis might be useful in the study of mutations in mammalian cells. Since several so-called shuttle vectors have been described (7, 14, 17) which allow replication of bacterial plasmid DNA in mammalian cells, we used a recombinant plasmid which contains two origins of replication, one from simian virus 40 (SV40) which drives replication in mammalian cells, and one from the bacterial plasmid which allows replication in *Escherichia coli*. To provide a target gene for mutagenesis, we inserted a DNA fragment that encodes the *supF* (amber suppressor tyrosine tRNA) gene (2). This gene was not expected to exhibit any phenotype in mammalian cells, but upon reintroduction into the appropriate *E. coli* host, forward mutations to Sup^+ (that is, nonsuppressor phenotype) could be scored. Isolation of the plasmid DNA permitted rapid sequence analysis of the small target gene to determine the molecular nature of the mutations.

Cells. The COS-7 derivative of the African green monkey kidney cell line CV-1 contains the T antigen gene of SV40 (10). This protein activates replication of the plasmid at the SV40 origin and promotes many rounds of replication. *E. coli* SY204 was constructed by introduction into *E. coli* of the host restriction mutation *hsdR2* by P1 transduction with Tn*10* tetracycline resistance (donor, *E. coli* LCK8, (Coli Genetic Stock Center [CGSC; Yale University] number 6515); recipient, *E. coli* CA274 *lacZ125 trp-49* (CGSC 4990).

Plasmids. Plasmid p3AC (Fig. 1) was constructed by ligation of the 200-base-pair *Eco*RI fragment, which contains the *supF* gene (2) from the plasmid πVX, into the unique *Eco*RI site on plasmid $pATpCC_2$. This latter plasmid was constructed by J. Tournow and C. Cole and contained the *Bam*HI to *Hpa*II early-region fragment of SV40 inserted at the *Cla*I site of PBR322 which contained a deletion of the "poison sequences" (14) contained in *Hae*II fragment B.

Gene transfer method. COS cells were propagated and described by Gluzman (10). Cells were subcultured at a dilution of 1:4 in Dulbecco modified Eagle (DME) medium supplemented with 5% fetal calf serum on plates (diameter, 10 cm) 2 days before transfection. We prepared calcium phosphate-DNA coprecipitates by the method of Chu and Sharp (4) by using 20 μg of plasmid p3AC per ml. After trypsin treatment, cells from four 10-cm plates were suspended in DME medium and pelleted by centrifugation at 1,000 rpm for 10 min in a Sorval GLC-2 centrifuge. The cells were then suspended in 2.5 ml of calcium phosphate-DNA precipitate and incubated at room temperature for 15 min. DME medium (40 ml) supplemented with 12.5 mM $CaCl_2$ and 0.1× HEPES-buffered saline (4) was then added to the tubes, and the cells were transferred to dishes. The cells were allowed to adhere to the dishes in the incubator for 5 to 6 h. The medium was aspirated, and the cells were shocked with 1 ml of 25% glycerol (8) for 1 min at room temperature and rinsed twice with warm medium. Fresh medium was added to the plate, and cells were grown further for 40 to 46 h. In the case of ethyl methanesulfonate (EMS) treatment, the cells in a monolayer were first exposed to different concentrations of freshly prepared EMS in DME medium at room temperature for 90 min. EMS was removed, and the cell layer was washed twice with medium and then covered with 10 ml of fresh medium. After 36 to 40 h of incubation at 37°C, the cells were trypsinized and transfected with plasmid P3AC as described above.

Mutation assay. DNA was recovered from the COS cells by the Hirt method (12) and was used in the transformation of *E. coli* SY204 to ampicillin resistance. The frequency of $SupF^+$ mutations in the plasmid DNA before and after replication of COS cells was measured. *SupF* plasmids (i.e., nonmutated gene) convert the *E. coli* SY204 (*lac*[Am]) to a Lac^+ phenotype by suppression of the *lacZ125*(Am). These colonies are red on MacConkey agar medium. By contrast, $supF^+$ (i.e., mutated plasmids which have lost the SupF function) will not convert *E. coli* SY204 to Lac^+, so these colonies are white on MacConkey agar medium. We plated transfection mixtures on MacConkey agar with ampicillin to select for bacteria with a plasmid and to score the mutation frequencies of Sup^+ to total mutations. Whole plasmid DNA prepared in *E. coli* consistently gave values of $5 \times 10^{-4} \pm 3 \times 10^{-4}$ for the mutation frequency of Sup^+ (Table 1); a plasmid which had replicated in COS cells gave mutation frequencies of $2 \times 10^{-3} \pm 0.5 \times 10^{-3}$ (Table 2).

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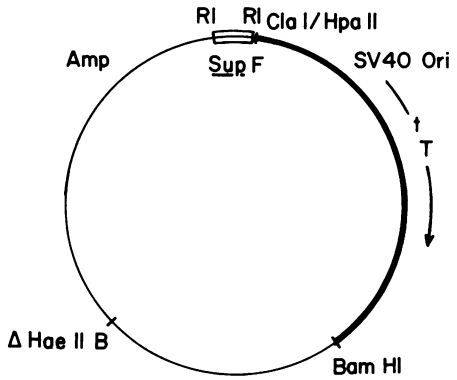

FIG. 1. Structure of the plasmid p3AC.

To test whether the system is sufficiently sensitive and reproducible, we subjected purified plasmid DNA to UV light in vitro and also transfected *E. coli* which had been exposed to UV light before transfection. In both cases, a significant increase in Sup^+ mutation frequency was observed (Table 1). Similar results were obtained with plasmids made with and without exposure to CsCl-ethidium bromide during its purification. This increase in Sup^+ mutation frequency confirmed the work of Fuchs et al. (9) and was a variance with the conclusions of Schmid et al. (18) who did not find eroor-prone mutagenesis of plasmid DNA in *E. coli*.

We determined that the plasmid p3AC replicated in COS cells after transfection. At various times after transfection, DNA was harvested by the Hirt method (12), digested with endonuclease *Mbo*I, and subjected to electrophoresis in agarose gels. Since *Mbo*I digestion was inhibited by the presence of the methylated cytosine residues which are put on in *E. coli*, the bacteria-grown plasmid was resistant to *Mbo*I. After replication in mammalian cells, this methylation pattern was lost, and the DNA became sensitive to *Mbo*I (Fig. 2).

We tested the influence of prior exposure of the cells to the DNA-damaging agent EMS on the mutation frequencies that occur in a plasmid replicating in such treated cells. The purpose of such a protocol was to test for the ability of EMS to induce an error-prone replication environment (6, 13, 16). The effect of a 90-min exposure of COS cells to EMS at various concentrations 40 h before introduction of p3AC DNA is shown in Table 2. Two results were apparent: EMS seemed to reduce the yield of plasmid DNA as measured by the recovery of ampicillin resistance-transforming plasmids in the Hirt supernatant, and even the lowest EMS concentration tested (20 mM) resulted in a 10-fold stimulation in the frequency of Sup^+ mutations in the recovered plasmid.

TABLE 1. Frequency of mutant *supF* genes in plasmid p3AC subjected to replication in *E. coli* SY204[a]

Treatment (ergs/mm²) of:		No. of transformants	No. of mutants	Frequency ± SD[a]
Cell	Plasmid			
None	None	6,072	3	0.0005 ± 0.0003
UV (250)	None	5,600	17	0.003 ± 0.0007
UV (500)	None	1,450	10	0.007 ± 0.002
UV (500)	UV (1,800)	2,980	13	0.004 ± 0.001
None	UV (1,800)	1,680	3	0.002 ± 0.001

[a] Estimated standard deviations were calculated on the assumption that mutations occurred randomly.

TABLE 2. Frequency of mutant *supF* genes in plasmid p3AC subjected to replication in mammalian cells

Treatment of:		No. of transformants	No. of mutants	Frequency ± SD[a]
Cell (EMS concn [mM])	Plasmid (erg/mm²)			
None	None	7,200	17	0.0023 ± 0.0005
None	UV (1,800)	638	8	0.012 ± 0.005
EMS (80)	UV (1,800)	120	5	0.04 ± 0.02
EMS (20)	None	477	10	0.021 ± 0.007
EMS (40)	None	182	9	0.05 ± 0.02
EMS (60)	None	50	4	0.08 ± 0.04
EMS (80)	None	288	11	0.04 ± 0.01

[a] Estimated standard deviations were calculated on the assumption that mutations occurred randomly (Poisson hypothesis).

The mutations produced in the p3AC DNA were classified in a preliminary way by gel electrophoretic analyses of the mutant plasmid DNAs. Analyses of 15 randomly selected spontaneous mutants and 15 randomly selected mutants from the prior EMS exposure protocol were carried out. Data for 14 of these mutants are shown in Fig. 3. The major class of mutations in the spontaneous group were those associated with gross DNA rearrangements. When the whole plasmid was considered, 12 out of 15 mutations were of this type. In contrast, only 5 of 15 plasmids of the error-prone

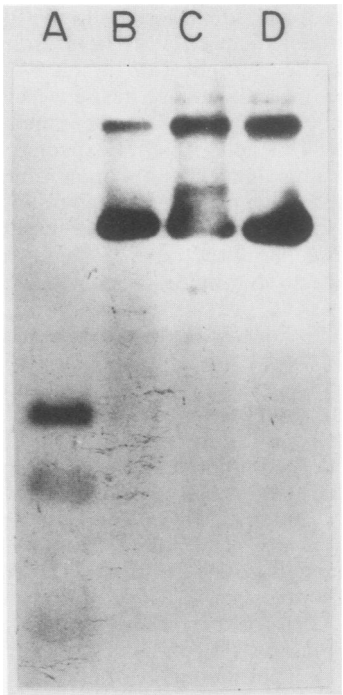

FIG. 2. Analysis of replication of p3AC in COS-7 cells. Lanes: A and B, Electrophoresis of DNA extracted by the Hirt procedure (11) from COS-7 transfected 40 h previously with 20 μg of p3AC DNA that was digested (A) with *Mbo*I or was undigested (B); C and D, electrophoresis of p3AC DNA prepared from *E. coli* before (D) and after (C) digestion with *Mbo*I; After electrophoresis the DNA was transferred to a nitrocellulose filter and hybridized to ^{32}P-labeled p3AC DNA. The autoradiograph of the hybridized filter is shown.

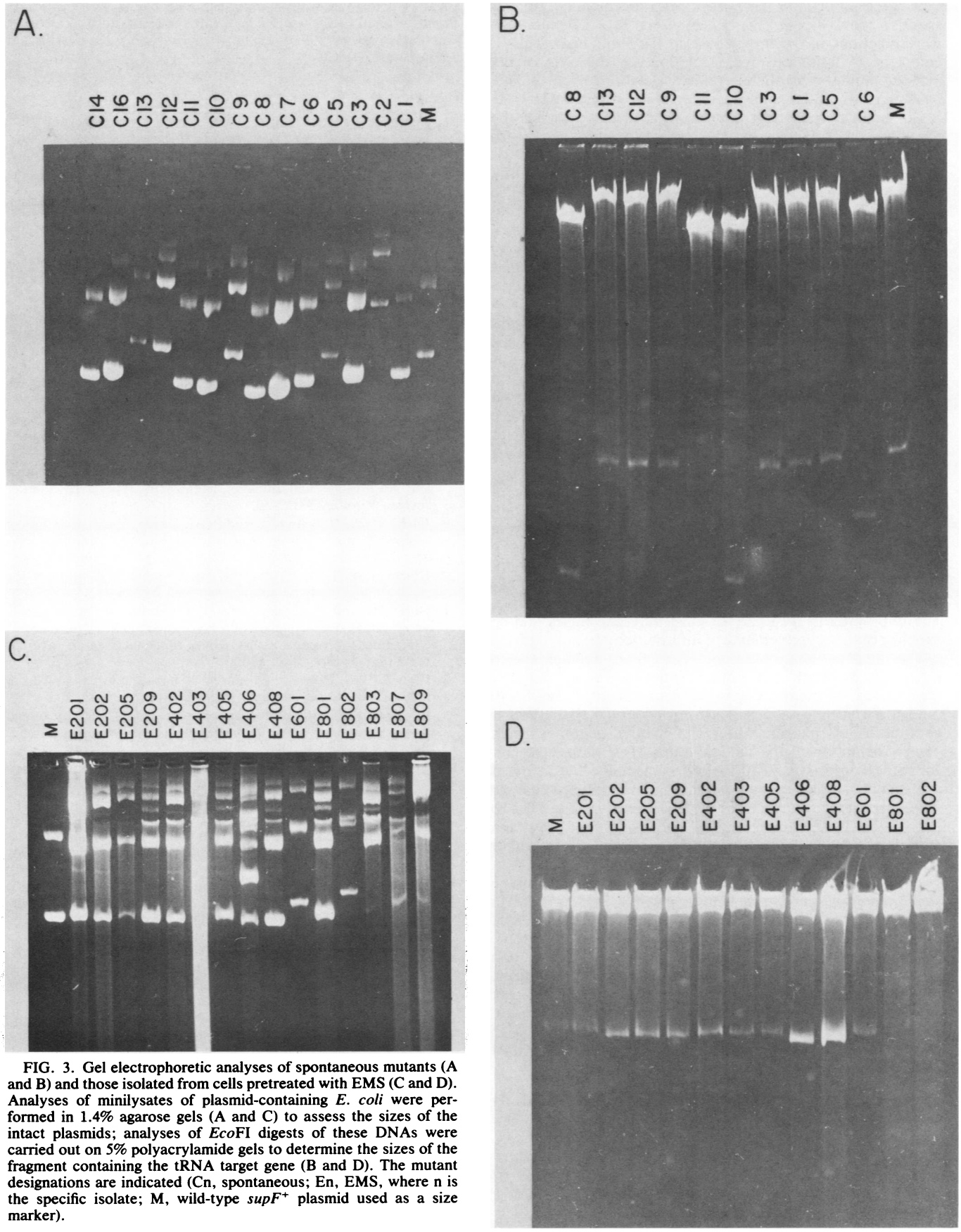

FIG. 3. Gel electrophoretic analyses of spontaneous mutants (A and B) and those isolated from cells pretreated with EMS (C and D). Analyses of minilysates of plasmid-containing *E. coli* were performed in 1.4% agarose gels (A and C) to assess the sizes of the intact plasmids; analyses of *Eco*FI digests of these DNAs were carried out on 5% polyacrylamide gels to determine the sizes of the fragment containing the tRNA target gene (B and D). The mutant designations are indicated (Cn, spontaneous; En, EMS, where n is the specific isolate; M, wild-type $supF^+$ plasmid used as a size marker).

type were associated with gross rearrangements. With respect to the 200-base-pair tRNA insert, 6 of 15 mutations were deletions in the target gene in the spontaneous class, and 2 of 15 mutations were deletions in the case of the induced mutants. Small insertions or deletions of less than 5 base pairs would have escaped detection in this analysis.

Our main purpose in this work was to test whether the phenomenon of damage-induced error-prone mutagenesis in mammalian cells, which we described previously for the herpes simplex virus thymidine kinase gene (6), could be studied at the gene structure level with a shuttle vector. The results presented here show that a significant level of damage-induced, indirect mutagenesis occurs in a plasmid replicating in EMS-treated COS-7 cells. Although replication of p3AC in normal COS-7 cells results in mutations, mainly by rearrangements (3, 15), prior treatment of the cells with EMS enhances mutagenesis at least ten-fold.

Since the EMS treatment of the COS-7 cells is for 90 min, after which it is washed away by several medium changes, and since 40 h elapse before plasmid DNA is added, it is unlikely that the observed mutagenesis resulted from direct EMS-induced damage to the plasmid DNA. Instead, it appears that the COS-7 cells, in response to the EMS, are converted to a state in which p3AC replication is more error prone. The molecular basis for this error-prone state is as yet unclear.

The system described here has the advantages of ease of genetic manipulation and rapid sequence analysis of mutants; in addition, it represents a different class of genes from those recently employed in similar shuttle vectors (3, 15); that is, p3AC has a tRNA gene rather than a protein-encoding gene as its target for mutagenesis. Each system has inherent biases because of the possibility of silent mutations. The two systems are likely to have different biases and so should provide complementary information.

The high frequency of spontaneous rearrangements seen in the plasmid when grown in untreated COS-7 cells was not unexpected. In essence, the system involves the SV40 replication system, and it has been observed that SV40 is very prone to the generation of defective genomes when complementation of the defective genomes is possible (11, 19). Since defective SV40 DNA is detectable by chemical analysis in samples prepared from high-multiplicity passage (19), such defective genomes must represent at least 0.1 to 1% of the DNA. Indeed, it is surprising that this high mutation frequency does not totally obscure any other mutagenic phenomena.

We conclude from our study of the mutagenesis of the *supF* gene contained in a shuttle plasmid, p3AC, that, in support of previous experiments with infectious virus (1, 5, 6), mammalian cells can respond to genomic damage in some way which results in an apparently error-prone replication apparatus. In our previous work with herpes simplex virus, we could not eliminate the role of many possible virus gene products in the error-prone replication process. In the present experiments, a much simplier replicon has been used in which the replication dependence on the host cell is almost complete.

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