Plasmid mediated mutagenesis of a cellular gene in transfected eukaryotic cells

Curtis R.Brandt, Franco M.Buonaguro, James K.McDougall and Denise A.Galloway

Fred Hutchinson Cancer Research Center, 1124 Columbia St, Seattle, WA 98104, USA

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ABSTRACT

NIH3T3 cells are widely used in transformation assays and readily take up transfected DNA. A system has been devised using NIH3T3 cells to measure the mutagenic effect of transfected DNA on recipient cell genes. NIH3T3 cells can be mutated to 6-thioguanine resistance at a frequency which suggests that at least a portion of the cells have only one functional copy of the HGPRT gene. They have a low spontaneous background mutation frequency ($\approx 1 \times 10^{-7}$). Transfection of three different plasmids into NIH3T3 cells induced 6-thioguanine resistant mutants at frequencies ranging from 3 to 11 fold above background. The mutant phenotype is stable and reversion frequencies of several mutants are $\leq 1 \times 10^{-7}$. Southern blot analysis of the HGPRT gene in several mutants showed that 4 of 26 mutants (15.4%) had detectable alterations in the structure of the HGPRT gene. Interestingly 3 of the 4 mutants showing rearrangements were obtained by transfection of the HSV-2 morphological transforming region.

INTRODUCTION

Transfection of DNA into eukaryotic cells is a useful technique in the study of gene function, gene regulation, and oncogenesis. Despite the extensive use of DNA transfection, little is known about how transfection works. Integration of the introduced DNA is usually selected for and occurs at a frequency of about 1×10^{-4} depending on the method of transfection and the DNA sequence being introduced (1). Mutation of the introduced DNA is also a common result (2-5).

Since transfected DNA can be integrated into the host cell genome, one might expect to find, at a low frequency, mutations in cellular genes resulting from integration. In addition, the high mutation frequency in the transfected DNA population leads one to ask if mutations are also occurring in host cell genes. Very little is known about the effect DNA transfection protocols have on the recipient cell genome. Lau et al. (6) have shown that transfected plasmid DNA induces transformation of hamster cells in culture.

Previously, we have used transfection of DNA into eukaryotic cells to define the regions of the herpes simplex type 2 (HSV-2) and human

cytomegalovirus (HCMV) genomes that are involved in morphological transformation (7-10). Transformation by HSV-2 and HCMV appears to occur through an unconventional mechanism in that neither of these viruses appear to encode a transforming protein and defined fragments of viral DNA are not necessarily retained in transformed cells (11). The term "Hit and Run" transformation has been used to describe transformation by these viruses (12, 13). The lack of retention of HSV-2 or HCMV sequences in transformed cells has made it difficult to determine the mechanism of transformation. One possible mechanism is that the HSV-2 and CMV transforming regions induce mutations, however the cellular target is unknown. We therefore set out to devise a system whereby we could study the effect of transfected DNA sequences on genes of the recipient cells. Resistance to the purine 6-thioguanine has many of the desired characteristics: a) the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene (EC2.4.2.8) encodes resistance to 6-thioguanine (14); b) the 6-thioguanine is toxic when incorporated into cellular DNA allowing selection for resistant mutants; c) the HGPRT gene is not essential; d) growth of cells in HAT medium forces cells to use HGPRT, allowing for selection of revertants; e) the HGPRT gene is well characterized, both the human and murine genes have been cloned (15, 16) and cDNA probes are available for analysis of the gene (17).

The results presented below describe the transfection mutagenesis protocol we have devised and show that NIH3T3 cells can be mutated to 6-thioguanine resistance. The mutation frequencies obtained suggest that at least a subpopulation of NIH3T3 cells have only one functional copy of the HGPRT gene. Using this system, we show that transfection of plasmid DNAs into eukaryotic cells can induce mutations in recipient cell genes.

MATERIALS AND METHODS

Cells and Media

NIH3T3 cells were grown in Dulbecco's Modification of Eagles Medium (DME, GIBCO) containing 10% Fetal Calf Serum (FCS, Hyclone) as described previously (9). HAT medium contained DME with 10% FCS, 0.1mM hypoxanthine, 16 μ M thymidine, 0.4 μ M aminopterin and 3.0uM glycine. Six-thioguanine was added to DME with 10% FCS at a concentration of 0.1mM for selection of mutants. The optimal concentration of 6-thioguanine was determined from toxicity curves of the drug on NIH3T3 cells (data not shown).

MNNG Mutagenesis

NIH3T3 cells were maintained in HAT medium for two weeks prior to mutagen treatment to reduce spontaneous background; the cells were trypsinized and

seeded at a density of 1.5×10^6 cells per 100mm plate 24 hours prior to treatment. Two plates each were treated with 20 μ M and 80 μ M MNNG (N-methyl-N'-nitroso- N-nitrosoguanidine) for four hours. The medium containing MNNG was removed and the cells were washed with normal medium. After passage for 8 days in nonselective medium, the cells were trypsinized, counted and plated at a density of 2×10^5 cells per plate (15 plates total) in medium containing 0.1mM 6-thioguanine. Selective medium was replaced every three days during the selection. Resistant colonies were counted 2-3 weeks after plating. Plating efficiencies were determined by plating $1-2 \times 10^2$ cells in normal medium. Plates were stained and counted two weeks after plating. Stock MNNG was prepared by dissolving the MNNG in DMSO at a concentration of 160mM. The final DMSO concentration was less than 5%. Control plates were treated with DMSO in normal medium. Six-thioguanine and MNNG were purchased from Sigma Chemical Co., St. Louis.

Transfection mutagenesis protocol

NIH3T3 cells were passed for two weeks in HAT medium prior to transfection. Twenty four hours before transfection, NIH3T3 cells were trypsinized and seeded into 100mm plastic dishes (1.5×10^6 cells per plate). The cells were kept in HAT medium for 12 hours, then the HAT medium was replaced with normal DME with 10% FCS. Four hours later DNA was added as a calcium phosphate precipitate (18). Four hours after adding the DNA, the medium was removed. The cells were rinsed with DME containing 10% FCS and "shocked" with DME containing 10% FCS and 10% DMSO for one minute. After rinsing with DME + 10% FCS twice, fresh medium with 10% FCS was added. The following day the cells were trypsinized, seeded into roller bottles and passed for 8 days to allow for expression of the mutant phenotype. During this time the cells were split twice when they reached 80% confluency. At the end of the expression time, the cells were trypsinized, counted and seeded at a density of 5×10^5 cells/100mm dish in DME with 10% FCS and 0.1mM 6-thioguanine. One hundred 6-thioguanine resistant cells (6-thio-1) were mixed with varying numbers of NIH3T3 cells and plated in 100mm culture dishes in 0.1 mM 6-thioguanine to determine the maximum number of cells which could be plated without crossfeeding. The optimum number of cells was found to be 5×10^5 cells per 100mm dish. Thirty plates were used per sample (1.5×10^7 total cells). At the same time, three plates containing $1-2 \times 10^2$ cells were plated in DME with 10% FCS (no inhibitor) to determine the plating efficiency. All plates were fed twice weekly. Plates were examined 3-4 weeks after plating for 6-thioguanine resistant mutants. Several mutants

were picked using cloning cylinders and grown up for further study. Plating efficiencies were determined by staining plates 2-3 weeks after plating cells in nonselective medium. Plating efficiency was calculated as: number of stained colonies/no. of cells plated. The mutation frequency was calculated as follows:

$$M_f = \frac{M}{CE}$$

Where M_f is the mutation frequency, M is the number of mutants obtained, C is the number of cells plated and E is the plating efficiency. Figure 1 shows a schematic diagram of the transfection mutagenesis protocol.

Plasmids

The plasmids containing the HSV-2 transforming fragment (pBC24) and the human cytomegalovirus transforming region (p4127) have been described previously (9,10). The plasmid pHPT5 (a gift of T. Caskey) is a cDNA clone of the mouse HGPRT gene and hybridizes to all coding exons (17).

Southern blot analysis of DNA

High molecular weight cellular DNA was isolated as described previously (21). DNA was digested with restriction enzymes as described by the manufacturer (BRL), electrophoresed in 0.7% agarose and transferred either to nitrocellulose or Gene Screen Plus (NEN). Blocking and hybridization conditions for nitrocellulose have been described (22).

Gene Screen Plus was used as follows. Transfer of the DNA to the filters was carried out using 10X SSC as described by the manufacturer (New England Nuclear). Filters were prehybridized in 50% formamide, 1.0M NaCl, 100 μ g/ml denatured salmon sperm DNA, 1% SDS and 10% dextran sulfate at 42°C for 6-12 hours. The prehybridization solution was replaced with hybridization solution (prehybridization solution with 1×10^6 cpm per ml of probe). Probes were labelled with α [32 P] dTTP and dCTP by nick translation. Specific activities ranged from 4 to 8×10^8 cpm/ μ g. The 1.3kb PstI insert from the cDNA plasmid pHPT5 was used as probe for the HGPRT gene (17).

RESULTS

NIH 3T3 cells can be used for mutation experiments

NIH3T3 cells have not been previously used for mutation experiments involving 6-thioguanine selection. A preliminary experiment was carried out to determine if 6-thioguanine resistant mutants could be induced at an appreciable rate. NIH 3T3 cells were passed for two weeks in HAT medium to reduce background by eliminating HGPRT⁻ cells. The cells were trypsinized and plated at a density of 1.5×10^6 cells per 100mm dish. A total of 3×10^6

TABLE 1. FREQUENCIES OF 6 THIOGUANINE RESISTANT MUTANTS

EXPERIMENT 1					
SAMPLE	AMOUNT OF MUTAGEN	MUTANTS	SURVIVING ^b FRACTION	MUTATION FREQUENCY	ENHANCEMENT OVER CONTROL
MNNG	20 μ M	20	.03	2.22×10^{-4}	-
MNNG	50 μ M	0	$< 6 \times 10^{-5}$	-	> 150
CONTROL	-	0	0.31	$\leq 1.48 \times 10^{-6}$	
EXPERIMENT 2					
SAMPLE	DNA TRANSFECTED PER PLATE	MUTANTS	SURVIVING ^b FRACTION	MUTATION FREQUENCY	ENHANCEMENT OVER CONTROL
CONTROL	-	1	0.45	1.4×10^{-7}	-
pBR322	8	3	0.30	6.7×10^{-7}	4.8
pBC24	25	3	0.20	1×10^{-6}	7.1
pBC24	50	5	0.20	1.6×10^{-6}	11.4
pBC24	200	3	0.21	9.5×10^{-7}	6.8
pCM4127	6	3	0.44	4.5×10^{-7}	3.2
pCM4127	30	5	0.38	8.8×10^{-7}	6.3
EXPERIMENT 3					
SAMPLE	DNA TRANSFECTED PER PLATE	MUTANTS	SURVIVING ^b FRACTION	MUTATION FREQUENCY	ENHANCEMENT OVER CONTROL
MOCK TRANSFECTION	-	0	0.25	$\leq 1.39 \times 10^{-7}$	-
pBR322	50	2	0.27	4.7×10^{-7}	3.4
pCM4127	50	4	0.23	1.06×10^{-6}	7.6
pBC24	50	2	0.22	5.5×10^{-7}	4.0

^a Micrograms of DNA transfected^b Equivalent to plating efficiency in non selective media after treatment

cells were then treated with two different doses of the mutagen N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG). The results for one experiment are shown in Table 1, Experiment 1. In control (untreated) cells the mutation frequency was less than or equal to 1.48×10^{-6} . When cells were treated with 20 μ M MNNG, the frequency of mutation rose to 2.22×10^{-4} and 1-4%

Figure 1. Schematic diagram showing the transfection mutagenesis protocol.

of the cells survived. At a dose of 80uM MNNG, all of the cells were killed. These results indicate that NIH 3T3 cells can be used in mutation experiments selecting for 6-thioguanine resistance. The karyotype of NIH3T3 cells is aneuploid and it is not possible to be sure that the cells have only a single copy of the X chromosome or the portion of the chromosome encoding HGPRT. However, the mutation frequency suggests that in at least a subpopulation of the cells only a single functional copy of the HGPRT gene is present.

Transfected plasmids are mutagenic. To determine if transfected plasmid DNA was mutagenic to recipient cells, plasmid DNAs were transfected into NIH3T3 cells and 6-thioguanine resistant mutants isolated as described in Materials and Methods and outlined in Figure 1. Carrier DNA was not used in any of the experiments. In general 80% of the cells remained attached after transfection and the plating efficiency of the treated cells was 20-45%. The transfection efficiency was not measured, firstly because no dominant selectable marker was used, and secondly because that only measures expression of a transfected gene. The results of one experiment are shown in Table 1, Experiment 2. Three mutants were found in the sample treated with pBR322 which gave a mutation frequency of 6.7×10^{-7} . This is a 4.78 fold increase over the mutation frequency of 1.4×10^{-7} in cells containing no plasmid DNA. All three doses of pBC24 were mutagenic varying from 6.78 fold to 11.4 fold over the background mutation frequency. An increased mutation frequency was also seen in the p4127 treated samples (3.2 to 6.3 fold). These results show that plasmid DNAs when transfected into cells can induce mutations in cellular genes. Increasing the dose of pBC24 twofold (25 μ g to 50 μ g) resulted in an almost two fold increase in the number of mutants. The effect was not seen with a dose of 200ug/plate, however this amount of DNA

may be toxic to the cells. Since the increases are not linear and the numbers of mutants are small, we cannot say conclusively that there is a dose response relationship. To confirm the results and to directly compare the mutagenic efficiencies of the three plasmids, the mutagenesis assay was repeated using a dose of 50ug per plate for all three plasmids (Table 1, experiment 3). Mock transfection resulted in a mutation frequency of $\leq 1.39 \times 10^{-7}$, indicating that the transfection protocol per se (no DNA) is not mutagenic. In other experiments control (untreated) and mock transfected cells were compared directly and no significant differences in the mutation frequencies were found (data not shown).

Reversion frequency and stability of the mutant phenotype

The reversion frequencies of several mutant cell lines were measured by plating cells in HAT medium. Cells resistant to 6-thioguanine i.e. that have mutated HGPRT genes, are unable to grow in HAT medium allowing revertants to 6 thioguanine to be selected. Mutant cell lines were grown in medium containing 6-thioguanine for 2 weeks and then transferred to non-selective medium. After two weeks in regular medium, the cells were plated in medium containing HAT. Plating efficiencies in regular medium were measured and used to correct the reversion frequencies. A total of 14 mutants including 5 spontaneous mutants, two pBR322 mutants, 4 p4127 mutants and three pBC24 mutants and 3MNG induced mutants were tested. After correction for plating efficiency, the total number of cells tested for each mutant was approximately 1×10^7 . No HAT resistant colonies grew indicating that the reversion frequency for the mutants tested was less than approximately 1×10^{-7} (data not shown). The mutants induced by transfection of plasmid DNA into NIH3T3 cells therefore have a low spontaneous reversion rate.

To determine if the 6-thioguanine resistant phenotype was stable in the absence of selection several mutants were grown in 6 thioguanine containing media for two weeks and then subcultured in the absence of selection. After 8 weeks in non-selective media, the number of cells able to grow in selective media was determined. If the phenotype is stable, then cells grown for long periods in the absence of selection should have plating efficiencies similar to cells grown without selective pressure for short times. A total of 14 6-thioguanine resistant mutants were tested for stability of the mutant phenotype. This analysis showed that almost all of the mutants isolated were stable. Furthermore, the growth rate of the mutant vs. the unselected treated populations was seen (data not shown) confirming the validity of the stability analysis.

Figure 2. Southern blot analysis of mutant DNAs. High molecular weight DNA was digested with *EcoRI*, electrophoresed through agarose, transferred to nitrocellulose or Gene Screen Plus, and hybridized to the indicated probes. Southern blots probed with the HGPRT, 4127 or BC24 insert DNAs were washed at 68°C in 0.5X SSC. Southern blots using pBR322 as probe were washed at 68°C in 0.1 SSC. Panel A. Control and mock transfected mutants probed with HGPRT insert. Panel B. pBR322 mutants probed with HGPRT insert. Panel C. BC24 and 4127 induced mutants probed with HGPRT insert. Reconstructions (lanes marked R) contained pHPT5 DNA mixed with NIH3T3 DNA at single copy amounts and digested with enzymes to release the insert. The pHPT5 reconstruction was cut with *PstI* and the extra bands results from the *PstI* fragments of the NIH3T3 DNA. Bacteriophage DNA digested with *HindIII* was used as marker. Panel D. Restriction map of the murine HGPRT gene adapted from Melton et al. (15).

Southern blot analysis of mutants

The mutants were further analysed by Southern blot analysis of the HGPRT gene. High molecular weight DNA was digested with EcoRI, electrophoresed in 0.7% agarose, transferred to a membrane support and hybridized to a cDNA probe for the HGPRT gene. The results are shown in Figure 2. The 1.3kb cDNA insert from pHPT5 (17) which hybridizes to all of the coding exons of the HGPRT gene allowing examination of almost 80% of the gene was used as a probe. EcoRI restriction fragments of 9.3, 6.3, 5.5, 5.0 and 1.3 kb are detected with the pHPT5 probe. A pseudogene of 3.6 kb is also detectable. A restriction map of the HGPRT gene (mouse) is shown in Figure 2D. The pHPT5 probe does not hybridize to EcoRI fragments of 1.9, 0.9 and 0.2 kb, which map in the intervening sequence between the first and second exons, and a 4.3 kb EcoRI fragment which maps between exons 3 and 4.

A total of 6 mutants isolated from mock transfected or control cultures were analysed (Figure 2A). Five of the six mutants had normal EcoRI restriction patterns. One mutant, C8-2 was missing the 6.3kb EcoRI fragment which contains Exon 1. Figure 2B shows a Southern blot of several pBR322 mutants. A total of nine mutants were analysed, none of which showed altered EcoRI restriction patterns when probed for the HGPRT gene. Although the 5.0 kb EcoRI fragment appears to be missing from the pBR322/1-1 mutant, it is present but faint on this autoradiogram.

Figure 2C shows the results of Southern blot analysis of six pBC24 mutants and four p4127 mutants. None of the five p4127 mutants tested show any alterations in the EcoRI digestion patterns of the HGPRT gene (4127/30-1 not shown). In contrast, three of the six pBC24 mutants analysed had altered EcoRI restriction patterns. The mutant BC24/50-1 is missing the 5.0 kb and the 9.3 kb EcoRI fragments and contains a new band of 11.5 kb. The mutant BC24/50-2 is missing the 5.0 kb EcoRI fragment and the mutant BC24/50-4 has a normal pattern except that the 9.3 kb EcoRI fragment has been replaced by a slightly larger fragment of 10.5 kb. Thus, 3/6 of the pBC24 induced mutants have gene rearrangements (deletions, insertions, or fusion fragments) whereas none of the pBR322 or pCM4127 induced mutants have an altered HGPRT gene structure. This difference is statistically significant ($p=0.044$) and suggests that although both pBR322 and pBC24 induce mutations with similar efficiencies, they tend to induce different kinds of mutations.

Because transfected DNAs can be efficiently integrated into cellular DNA (1), one might expect to find mutations due to integration of the plasmid DNA into the HGPRT gene. To determine if plasmid DNA had been integrated into

the cellular DNA of the mutants, Southern blots were probed with nick translated pBR322. None of the BC24 mutants tested contains integrated plasmid DNA although single copy reconstructions were clearly visible (data not shown). When the pBR322 mutants were probed we found that none of them had integrated pBR322 sequences (data not shown); none of the five p4127 mutants analysed contained integrated plasmid sequences. These results show that none of the mutations are due to retention of integrated plasmid DNA. We cannot rule out the possibility that plasmid DNA was integrated into the HGPRT gene and was excised subsequently. Mutant DNAs were also probed for retention for the presence of the BC24 insert and the 4127 insert and no insert DNA was detectable in any of the mutants (data not shown).

DISCUSSION

The results presented here show that we have devised an experimental system that should prove useful in studying the effect of transfected DNA sequences on the structure and function of genes in the recipient cell. Three different plasmid DNAs have been shown to be mutagenic when introduced into NIH3T3 cells. Mutation frequencies varied from threefold to elevenfold above background depending on the experiment and the amount of DNA added to the cells. These results also show that the mutations are stable and that they have a low spontaneous reversion rate.

The mutation frequency in plasmid treated cells was 100fold lower than in cells treated with the potent mutagen MNNG (20 μ M) suggesting that the plasmid DNAs are only weakly mutagenic. However, we cannot compare MNNG directly with the plasmids since we do not know what fraction of the transfected cells took up DNA and were therefore susceptible to plasmid mediated mutagenesis. For this reason, our mutation frequencies for the plasmid mutagens are conservative estimates. The mutation frequencies we observed are low but are similar to reported mutation frequencies when viruses are used as mutagenic agents (22).

The structure of the HGPRT gene was analysed in several mutants. Out of 26 mutants analysed, 22 showed no detectable alterations in the structure of the HGPRT gene. These mutants presumably contain point mutations. Alternatively, they could be small deletions or insertions or have alterations in restriction fragments not detectable with the cDNA clone used as probe. Analysis of the mRNA and HGPRT protein in these mutants could be useful in further defining the alterations responsible for 6-thioguanine resistance. The four mutants which showed alterations in the structure of the HGPRT gene were one control mutant and three BC24 mutants.

Previous studies on spontaneous and mutagen (UV light, methylcholanthrene derivatives) induced 6 thioguanine resistant mutants have shown that gene deletion or rearrangement accounts for only a fraction of the mutants analysed. Fuscoe et al., (24), found only 2 of 18 (11%) 6-thioguanine resistant mutants analysed were due to major DNA alterations. In other studies, 2 of 33 (6.7%) 6 thioguanine resistant mutants examined by Southern blotting had major DNA alterations (25,26). Our results are similar (4 of 26 or 15.4%) however, three of the 4 mutants with gene alterations were isolated from pBC24 treated cells. This raises the intriguing possibility that the presence of the HSV-2 sequence (BC24 fragment) leads to an increased frequency of gene rearrangements.

Integration is one result of transfection of DNA into eukaryotic cells. Therefore one might expect to find that some of the mutants would be the result of integration of plasmid sequences into the HGPRT gene. This is not the case for the mutants analysed in this study. A total of twenty mutants isolated from plasmid transfected cultures were examined but none contained plasmid DNA sequences. We cannot rule out integration and subsequent excision of the plasmid sequences however precise excision leaving the HGPRT gene intact would be an unlikely event.

These results, showing that integration of the mutagenic DNA is uncommon is similar to results with viruses. Adenoviruses are mutagenic in cultured cells (23,27-29) but when 18 adenovirus induced 6-thioguanine resistant mutants were analysed by Southern blotting, none of them contained adenovirus DNA (30). In similar experiments using Moloney Murine Leukemia Virus, only 2.9% of the mutants analysed had insertions of the viral DNA (31). King et al. (32) found that 28.6% of the HGPRT mutants were the result of proviral insertion into the affected gene. These results show that mutation events other than insertion are clearly more prevalent in these assays and that the presence of foreign DNA whether introduced by virus infection or transfection is mutagenic in cultured cells.

Are these results relevant for morphological transformation? Carcinogenic agents are known to be mutagens and these results show that the plasmids are mutagenic. The BC24 plasmid transforms NIH3T3 cells in culture while pBR322 does not (9). The finding that 3 of 6 pBC24 mutants have rearranged HGPRT genes while 9 of 9 pBR322 mutants have normal HGPRT restriction patterns may be significant in this regard. Chromosomal aberrations and rearrangements are increasingly being associated with human cancer and cellular transformation. All carcinogens that have been

rigorously tested induce chromosomal rearrangements (33) and plasmids have been shown to induce hit and run transformation in hamster cells and cause chromosomal aberrations (6). We have not analysed our mutants for chromosomal aberrations since NIH3T3 cells are highly aneuploid and because we were interested in examining the effect of the transfected plasmid DNA on a specific target gene.

The transfection mutation system described here should be useful for further studies on mutation. One advantage is that the assay uses NIH3T3 cells which are commonly used in morphological transformation assays and readily take up DNA. A further advantage is the low spontaneous mutation frequency ($\approx 1 \times 10^{-7}$) which is 10 to 100 fold lower than most systems commonly used for mutation analysis and may be useful for analysis of weakly mutagenic agents. The system needs to be characterized further, e.g. mutation frequencies vary from experiment to experiment, a problem that has been observed in other systems, and more experiments with plasmids will be helpful in determining how much variation exists. We do not know the mechanism or mechanisms of mutagenesis. We do know that insertional mutagenesis is not a common event. One possibility is that transfection may activate or stimulate error prone repair systems. Further work to define the types of mutations that are induced may provide information on the mechanism of mutation. Studies to determine if any DNA is mutagenic and if damaged DNA (UV-light, chemical mutagens) has any effect on genes in recipient cells are also needed.

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