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Specificity of mutations induced in transfected DNA by mammalian cells

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DNA transfected into mammalian cells is subject to the high mutation frequency of ~1% per gene. We present data bearing on the derivation of the two main classes of mutations detected, base substitutions and deletions. The DNA sequence change is reported for nearly 100 independent base substitution mutations that occurred in shuttle vectors as a result of passage in simian cells. All of the mutations occur at G:C base pairs and involve either transition to A:T or transversion to T:A. To identify possible mutational intermediates, various topological forms of the vector DNA were introduced separately. Supercoiled and relaxed DNA are mutated at equal frequencies. However, linearized DNA leads to a greatly elevated frequency of deletions. Nicked and gapped templates stimulate both deletions and base substitutions. We discuss a model involving intracellular degradation of the transfected DNA which explains these observations.

Key words: base substitution/deletion/mutation/shuttle vector/transfection

Introduction

Plasmid DNA introduced into *Escherichia coli* by transformation becomes established without sequence alteration. In contrast, transfection of DNA into mammalian cells often results in mutation of the introduced DNA. Mutations incurred in the mammalian cell can be scored and characterized in bacteria by using bacterial genes such as *lacI* (Calos *et al.*, 1983) and *galK* (Razzaque *et al.*, 1983) as targets for mutation on vectors that can replicate in both bacterial and mammalian cells. When SV40-based vectors containing *lacI* are transfected into simian cells, allowed to replicate, then returned to *E. coli*, a high mutation frequency of ~1% is observed in the 1000-bp *lacI* gene. Approximately half of the mutant plasmids show no apparent size change in *lacI* and presumably contain I^- point mutations. The other half show rearrangements in *lacI*, predominantly deletions (Calos *et al.*, 1983).

Transfected DNA is mutated at a high frequency in all mammalian cells examined (Lebkowski *et al.*, 1984), and also in yeast (Clancy *et al.*, 1984). Since transfection has assumed an important role in experimentation with eukaryotic cells, we sought insight into the molecular mechanism of formation of the mutations. Through application of the *lacI* genetic system (Miller, 1978) the DNA sequence change has been determined for nearly 100 transfection-induced mutations. Furthermore, to identify possible intermediates in mutation formation, DNA was introduced as supercoiled, relaxed, linear, nicked or gapped molecules. The mutational behavior of these forms, as well as the mutagenic specificity of the point mutations, suggest possible mechanisms for the formation of the mutations.

Results

A series of vectors which contain *lacI*, the SV40 origin of replication, the pBR322 origin of replication and its ampicillin resistance gene were used to collect point mutations (Figure 1, b–f). These vectors can replicate in COS7 simian cells, which synthesize T antigen constitutively from an origin-defective SV40 provirus (Gluzman, 1981). T antigen, in the simian cell environment, activates replication at the SV40 origin located on the plasmids. The vectors were transfected into COS7 cells and by 48 h had replicated to high copy number in the mammalian nucleus. At this point the plasmid DNA was harvested and introduced into an *Escherichia coli* indicator strain where I^- mutants were scored. Each of the plasmids shown in Figure 1 yields a characteristic frequency of I^- mutants when tested in this protocol. The frequencies range from 0.3% to 2.6% (Lebkowski *et al.*, 1984). The differences are largely correlated with the amount of non-selected DNA adjacent to *lacI* (see Calos *et al.*, 1983, for a fuller discussion of this point).

The nature of the mutations was determined for each of a large collection of I^- mutants using restriction analysis. Each

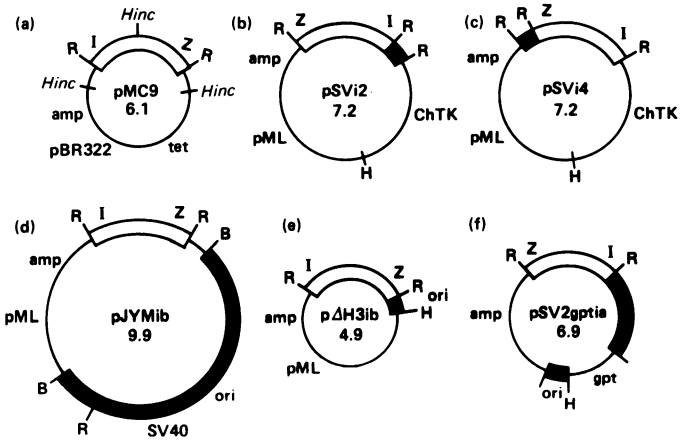

Fig. 1. Plasmid vectors. The *lacI* 1.7-kb fragment is indicated in each case by the open segment. The I and Z labels indicate the disposition of *lacI* and the beginning of *lacZ* within the 1.7-kb fragment. Sequences derived from SV40 are shown as filled-in segments and prokaryotic vector sequences are represented as a thin line. **(a)** pMC9. The three *Hinc* cuts are noted. The SV40 sequences present on **(b)** pSVi2 and **(c)** pSVi4 contain the SV40 origin of replication. **(d)** pJYMib contains all of SV40, pML (Lusky and Botchan, 1981), and the *lacI* 1.7-kb fragment. **(e)** pΔH3ib was derived from pSVi2 by removing a *Hind*III fragment. **(f)** pSV2gptia contains the SV40 origin and other SV40 sequences (Mulligan and Berg, 1982). B = *Bam*HI, *Hinc* = *Hinc*II, H = *Hind*III, R = *Eco*RI, amp = ampicillin resistance gene, tet = tetracycline resistance gene, Chtk = chicken thymidine kinase gene, pML = a deletion derivative of pBR322 (Lusky and Botchan, 1981). The a and b forms of a vector refer to the orientation of the *lacI* fragment (compare **e** and **f**). Both the a and b forms of plasmids **d**, **e**, and **f** were used to collect point mutations for this study. The size of each plasmid in kb is noted.

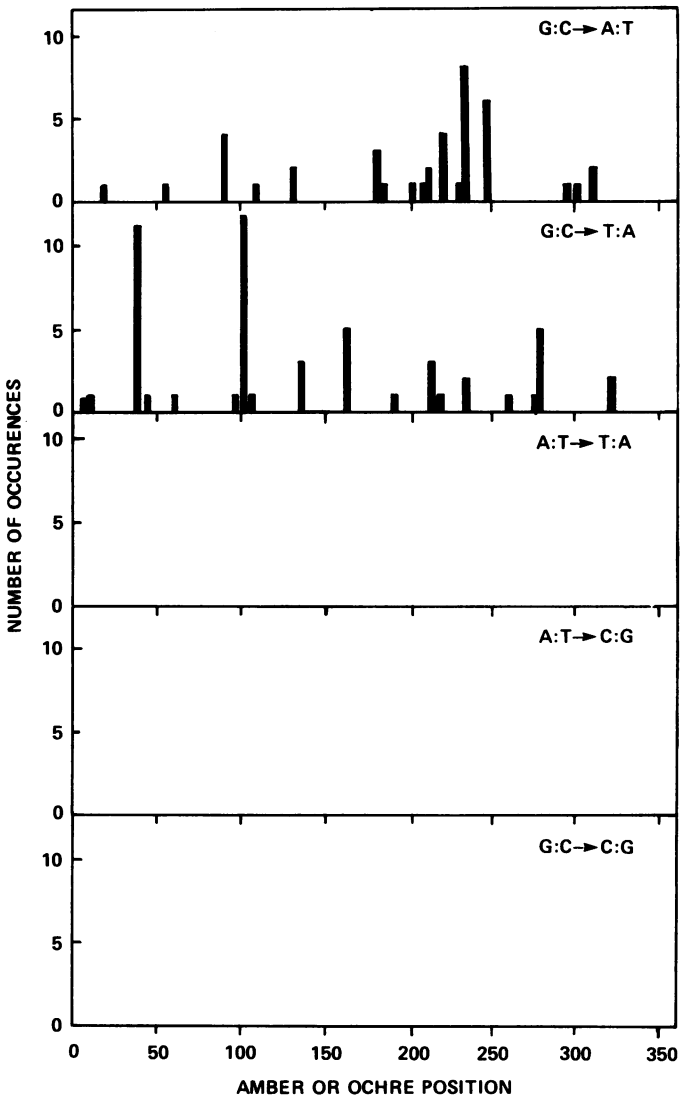

Fig. 2. Spontaneous amber and ochre mutations. The distribution of nonsense mutations collected in COS7 cells is shown. Each mutation is of independent origin. The height of each bar represents the number of independent occurrences in a collection of 93 nonsense mutations. The position of sites in the *lacI* gene is indicated on the horizontal axis by the number of the corresponding amino acid in the *lac* repressor. 81 of the mutations were detected in a *recA*$^+$ *E. coli* background, and 12 were detected in a *recA*$^-$ background (see text).

I$^-$ plasmid was digested with *Eco*RI and run on a 1% agarose gel. The wild-type *lacI* fragment is 1.7 kb long; if this fragment was unaltered in length in an I$^-$ mutant, the mutant was considered a candidate for containing a point mutation. The mutation could be a base substitution or an insertion or deletion too small to be resolved by the gel. 516 candidates were analyzed in further detail. The mutations were first crossed by genetic recombination from the plasmid to an F′ *lacproB* episome (see Farabaugh *et al.*, 1978). Each I$^-$ episome was then transferred to a series of nonsense suppressor strains (Coulondre and Miller, 1977) and the mutants that generated a nonsense codon were identified. All such mutations are necessarily base substitutions. Over 100 of the mutations generated an amber or an ochre codon, and were further characterized. Each of these mutations was assigned to one of the 77 amber and ochre mutations at 69 different sites in *lacI* using a combination of deletion mapping and an analysis of the pattern of nonsense suppression (Coulondre and Miller, 1977). Since the wild-type DNA sequence of *lacI* and the sequence of each nonsense codon is known, assignment of the position of the nonsense codon unambiguously identifies the DNA sequence change involved in the mutations. The high fraction of nonsense mutations (143/516, 28%) found among the putative point mutants indicates that the majority of the mutants assigned to this class contain base substitutions. The nonsense mutations that we analyzed represent ~15% of all the mutations that resulted in an I$^-$ phenotype.

Figure 2 shows the positions of the 93 independent mutations in *lacI* obtained in this study, classified according to the nature of the base substitution involved. The *lacI* forward nonsense system detects all possible base pair substitutions except the A:T to G:C transition. Figure 2 shows the distribution of the amber and ochre mutations we detected, categorizing the mutations according to the type of base substitution required to generate the mutation. Only G:C to A:T and G:C to T:A changes were found. The composition of the non-*lacI* portion of the vector did not influence the position of the base substitutions or the nature of the sequence change involved (data not shown).

It remained possible that the base substitutions were created in *E. coli* by the SOS error-prone replication system (Witkin, 1976; Radman, 1975; see review by Little and Mount, 1982), in response to some modification acquired in the mammalian cell. Therefore, a *recA* derivative of MC1061 F′ 150 kan (kind gift of T. Baker) was used as the recipient

Table I. Mutagenic behavior of topological forms

Type molecule	pSVi4					pSVi2				
	No. of colonies	% I$^-$	No. of muts. analyzed	% pt. mutations	% deletions[b]	No. of colonies	% I$^-$	No. of muts. analyzed	% pt. mutations	% deletions[b]
Supercoiled	83 465	3.5	96	12.5	87.5	26 750	0.92	225	57	43
Relaxed	7915	3.4	–[a]	–	–	–	–	–	–	–
Nicked	7355	7.8	36	13.9	86.1	1887	5.25	15	73.3	26.7
Gapped	4443	15.1	83	9.6	90.4	11 573	3.22	46	63.0	37.0
Linear										
*Hpa*I	6270	27.3	46	0	100	1735	20.6	46	4.3	95.6
*Kpn*I	16 467	52.0	–	–	–	–	–	–	–	–
*Sma*I	30 756	45.5	–	–	–	1253	0.40	–	–	–
*Xba*I	10 474	36.3	32	0	32	1798	0.17	–	–	–

[a]Hyphen means not done.
[b]The set of mutations classified as deletions include a few molecules that contain insertions from the simian genome.

strain since the SOS system does not operate in a *recA* background (Witkin, 1976). Vectors passaged in COS7 cells showed the same frequency of I^- mutations when either the *recA* derivative of MC1061 F′ 150 kan or the *recA*$^+$ strain was used as the recipient. The same fraction of the I^- mutations were putative point mutations in both backgrounds. All 12 of the I^- mutations detected in the *recA* background were G:C to A:T or G:C to T:A substitutions. There is no obvious difference in the distribution of mutations obtained in the wild-type and *recA* strains (data not shown). Furthermore, half of the plasmid DNA derived from a single plate of mammalian cells was assayed in the wild-type strain and the other half in the *recA* strain. In several cases the same rare mutation was identified in both cultures, strongly indicating that the mutation was generated and fixed in the mammalian cell. These data eliminate the argument that the SOS system of *E. coli* is responsible for creating the base substitutions.

To examine whether damage pre-existing in the vector DNA could account for the mutations and to identify possible intermediates in the mutagenesis, we manipulated the nature of the input DNA. Supercoiled, relaxed, nicked, gapped and linear DNA substrates were prepared (see Materials and methods). These populations of molecules were transfected and assayed as before; the results are shown in Table I. The vectors pSVi2 and pSVi4 were used in this study (Figure 1, b and c). These plasmids differ only in the position of the SV40 origin, a selected sequence in the experiment. pSVi2 has a lower mutation frequency than pSVi4. Furthermore, a smaller fraction of the mutations are deletions because *lacI* is flanked by selected or screened sequences. We saw no difference in the mutation frequency of supercoiled *versus* relaxed DNA. However, in pSVi2 and pSVi4 both nicked and gapped DNA showed mutation frequencies several times higher than those observed with supercoiled DNA. Point mutations and deletions were affected approximately equally.

Linearization led to profound effects on mutation frequency. For pSVi4 linearization at the *Kpn*I site, which is 240 bp from *lacI*, resulted in a mutation frequency of >50%. This frequency dropped somewhat as the distance of the cut from *lacI* increased (*Sma*I 380 bp, *Xba*I 1300 bp). All three of these sites are in the chicken thymidine kinase gene. Linear molecules with either blunt or staggered ends both showed elevated mutation frequencies. The mutation frequency of the molecules linearized at the *Hpa*I site, which is in *lacI*, was somewhat lower, probably because many deletions originating at this site also affected the *lacZ* portion of the plasmid. Under these circumstances an I^- mutation could not be scored. This hypothesis was substantiated by the finding that 25.1% of the colonies resulting from *Hpa*I linearization of pSVi4 contain a mutation in *lacZ*, which is identified as a pure white colony in our indicator systems. Linearization of pSVi2 with *Sma*I and *Xba*I did not lead to an increased frequency of I^- mutations, since the selected SV40 origin intervenes between the cut site and *lacI*. Essentially all of the mutations derived from linearized vectors were deletions, and the restriction site used for linearization was eliminated in every case. To ensure that the increased mutation frequency observed with nicked, gapped, and linear DNA was not caused by damage occurring in *E. coli* as a result of transformation with these altered forms of DNA, plasmid DNA extracted from the mammalian cells was treated with *Dpn*I to eliminate input DNA (see Lebkowski *et al.*, 1984). Thus, only DNA which had replicated in the mammalian cell was analyzed.

It remained possible that the high mutation frequency of transfected DNA was due to some aspect of the prokaryotic modification pattern of the DNA. In the mammalian cell, such modification might render prokaryotic DNA subject to attack or refractory to repair. To test this hypothesis we passaged plasmid DNA sequentially through two types of mammalian cells, without intervening bacterial passage. Plasmid pJYMib DNA was transfected into human 293 cells, where this vector has a mutation frequency of 0.05% (Lebkowski *et al.*, 1984). Plasmid DNA was extracted from the cells after 3 days, treated with *Dpn*I, and transfected directly into COS7 cells. A mutation frequency of 0.9% was obtained, which is similar to the mutation frequency of 0.5% which is normally observed for pJYMib in COS7 cells (Lebkowski *et al.*, 1984). The mutations induced included both base substitutions and deletions. We conclude from these experiments that the prokaryotic modification pattern of the transfected DNA does not play a major role in triggering mutagenesis.

Discussion

The mutation frequency affecting transfected DNA is approximately four orders of magnitude higher than the spontaneous mutation frequency in either mammalian or bacterial cells. Presumably this mutagenesis is confined to the transfected species. Experiments of Razzaque *et al.* (1984) fortify this contention: the mutation rate of a chromosomal gene was followed during transfection experiments and found to remain at 10^{-6}, while the transfected DNA suffered mutation at a frequency of 1%. We have previously shown through time course experiments that the mutations appear to occur shortly after arrival of the DNA into the nucleus, and that neither replication nor viral sequences are required for formation of point mutations or deletions (Lebkowski *et al.*, 1984). Thus, the bulk of the mutations cannot be ascribed to faulty viral replication. Instead, the preceding facts are consistent with the idea that the mutations are completed in the nucleus and involve as a substrate transfected DNA which has acquired damage.

Our study of the most subtle class of mutations, the base substitutions, reveals that the mutations have a distinct specificity. All 93 independent mutations examined occur at G:C base pairs and involve either the G:C to T:A transversion or the G:C to A:T transition. Prokaryotic studies argue that mutations are generally targeted by pre-mutagenic lesions, even when the recovery of mutations is dependent on the inducible SOS system (Miller, 1982; Miller and Low, 1984). Therefore it is reasonable to suggest that guanine and/or cytosine may be particularly susceptible to damage during transfection. One possibility is that depurination of guanine and deamination of cytosine are the two principle mutagenic reactions which produce the observed specificity. The sugar-base glycosyl bond of deoxyguanosine residues is particularly susceptible to hydrolysis. This reaction, leading to depurination, is acid-catalysed and occurs more readily on single-stranded DNA (Lindahl, 1982; Singer and Grunberger, 1983). Removal of the exocyclic amino group from cytosine is also readily observed under acidic conditions, and is strongly stimulated in single-stranded DNA. As we have summarized (Lebkowski *et al.*, 1983), the bulk of transfected DNA enters the cell by endocytosis and is delivered to lysosomes. These vesicles contain nucleases and maintain a pH of 5 (de Duve *et al.*, 1974; Helenius *et al.*, 1980). DNA subjected to these acidic conditions would acquire damage in the form of

depurination and deamination. This damage could constitute pre-mutational lesions if any of the DNA subsequently travelled to the nucleus. In *E. coli* a depurination site stops the replication fork, which induces the SOS system. Under SOS conditions the replication apparatus can continue, preferentially inserting adenine opposite the site of depurination (Strauss *et al.*, 1982; Schaaper *et al.*, 1983). Thus, the G:C to T:A transversion is the most common mutation found in depurinated DNA (Kunkel, 1984; see also Foster *et al.*, 1983). Replication past sites of gaunine depurination therefore represents an attractive possibility as an explanation of the G:C to T:A transversions observed. Mammalian DNA polymerases have been shown to misincorporate at apurinic sites *in vitro* (e.g., Shearman and Loeb, 1979; Schaaper *et al.*, 1983). It is not known if mammalian DNA polymerases will insert adenine across from a depurination site *in vivo*, either as an inducible or an endogenous response. We plan to transfect DNA which we have depurinated *in vitro* to see if the G:C to T:A transversion is specifically stimulated.

The deamination of cytosine converts cytosine to uracil. DNA polymerase would insert adenine opposite uracil, producing a G:C to A:T transition at the next round of replication (Lindahl, 1982). Thus deamination, which would also be stimulated by the lysosomal environment, could provide a plausible explanation for the transitions we observe.

Introduction of a double-stranded break into the transfected DNA molecules led to a sharp increase in the incidence of deletions recovered. We interpret this finding to mean that a double-stranded break may be an intermediate in deletion formation, probably by providing a substrate for exonucleolytic digestion. If a double-stranded break formed intracellularly, presumably a similar course of events would ensue. Nuclear ligase activity would restore a circular conformation to a degraded molecule, making it a substrate for replication. Razzaque *et al.* (1984) have also observed the stimulation of deletions in transfected DNA by a double-stranded break, and have used this finding to argue that the bulk of deletion formation must precede replication. The studies of Wake *et al.* (1984) indicate that fragmentation of transfected DNA is common, and that the subpopulation that reaches the nucleus suffers about one double-stranded break per 5–15 kb.

Stimulation of deletions by introduction of a single-stranded gap into the transfected plasmids may be accounted for by the increased susceptibility of a single-stranded region to breakage compared with a double-stranded region. The increase in base substitutions on a gapped molecule probably reflects the provision of a better substrate for base damage (single-stranded DNA) and an immediate substrate for repair replication. We view the similar behavior of nicked molecules as suggestive that they are predecessors to gapped molecules. It is unlikely that the minor amounts of linear, nicked or gapped molecules in the supercoiled DNA preparations we transfect are responsible for all the mutations observed. We note that plasmid molecules introduced into mammalian cells by protoplast fusion, so that they come directly from the bacterium and presumably enter the cell completely intact, also undergo mutation at a frequency of 1% (Razzaque *et al.*, 1983).

Our evidence suggests that the driving force in the formation of mutations in transfected DNA is the acquisition of intracellular damage. The nucleus appears to be required to complete a mutation (Lebkowski *et al.*, 1984). Though we have suggested the lysosome as a potential site of base damage, it is unclear whether the relevant DNA damage takes place in the nucleus, the cytoplasm or both. However, in preliminary studies with M.R. Capecchi, we observed deletions in DNA directly microinjected into the nucleus, while DNA injected into the cytoplasm gave rise to both deletions and point mutations. Unusual conditions such as the degradative enzymes and low pH of the lysosome could damage DNA in the cytoplasm, while the initial lack of chromatin structure could render the DNA susceptible to attack in the nucleus. We plan to attempt to transfect isolated nuclei with DNA to distinguish more clearly the roles of nucleus and cytoplasm and to bring us closer to the enzymes involved in mutagenesis.

Materials and methods

Plasmids

The 1.7-kb *lac* fragment of pMC9 (Figure 1a) was the source of *lacI* in all the vectors used. pMC9 was constructed as follows: an I^- missense mutation, *T63*, which removes the single cutting site for *Hin*cII within *lacI* was crossed onto the *lacI*-containing plasmid pMC1 (Calos, 1978). A 1.7-kb *Hin*cII fragment of pMC1 *T63* now contains all of *lacI*, the *lac* control region, and the beginning of *lacZ*, up to the *Hin*cII site corresponding to amino acid 146 of β-galactosidase. The I^Q promoter mutation carried by the *T63* donor episome was probably also transferred to the plasmid during the cross. The 1.7-kb *lac Hin*cII fragment was isolated from pMC1 *T63*, and *Eco*RI linkers were attached to it. The fragment with linkers was ligated into the *Eco*RI site of pBR322. The correct recombinant was identified as a red colony on MacConkey lactose plates upon transformation into strain CSH 35, △(*lacproB*) *supE thi* F′ *lac*I^s *proB* (Miller, 1972).(The *lac* operator on the plasmid titrates the *I* repressor of CSH 35, and β-galactosidase is synthesized.) The plasmid, pMC9 *T63* was returned to an I^+ state by transforming it into strain GM1, *ara*△(*lacproB*) *thi* F′ *lacproB* I^Q *L8*; (Miller *et al.*, 1977) to allow recombination with the I^+ episome. The I^+ pMC9 was isolated by transforming plasmids grown in GM1 into strain △196, *ara val* △(*lacproB*) *galE strA thi* (φ80d*lac* △*lacI tonB trp*; (Schmeissner *et al.*, 1977). When plated on agar containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), an I^+ plasmid will give a white colony in the I^-Z^+ △196 background. Construction of the other plasmids used in the study has been described (pSVi2, pSVi4 in Calos *et al.*, 1983; pJYMia, pJYMib, p△H3ia, p△H3ib, pSV2gptia, pSV2gptib in Lebkowski *et al.*, 1984).

Transfection and detection of I^- mutants

COS7 simian cells (Gluzman, 1982) were transfected with 20–100 ng of DNA per 60 mm dish by the DEAE-dextran procedure (McCutchan and Pagano, 1968). After 48 h plasmid DNA was collected from the COS7 cells by the Hirt (1967) procedure. The plasmid DNA was returned to *E. coli* and I^- colonies were scored as blue colonies on X-gal plates as described (Calos *et al.*, 1984).

Genetic analysis

Putative point mutations in *lacI* were crossed to the GM1 episome by genetic recombination. Amber and ochre nonsense mutations were identified and specifically assigned by mapping the mutations and determining their suppression pattern by genetic techniques that have been described in detail (Coulondre and Miller, 1977; Miller, 1978). Occasionally several point mutations derived from the same plate of mammalian cells were analyzed. To assure independence of the mutations, only one example of a given mutation per plate was saved.

Preparation of vector DNA

Plasmid DNA was prepared by the alkaline lysis procedure and was purified on cesium chloride gradients as described by Maniatis *et al.* (1982). This procedure yielded plasmid DNA that was at least 95% supercoiled, as evaluated by agarose gel electrophoresis. Relaxed closed circular DNA was prepared by incubating 500 ng of supercoiled plasmid DNA with 20 U of topoisomerase I (Bethesda Research Labs) for 5 h at 37°C. Singly nicked plasmid molecules were prepared by the procedure of Greenfield *et al.* (1975). Supercoiled plasmid DNA at a concentration of 200 μg/ml was mixed with ethidium bromide to a final concentration of 3 μg ethidium bromide/μg DNA. DNase I was added to 1 μg/ml and the mixture was incubated for 90 min at room temperature. After digestion, the mixture was extracted twice with phenol: chloroform:isoamyl alcohol (25:24:1 v/v), twice with 1-butanol, and once with ether. To purify the nicked molecules, the plasmid DNA was run on two strips of a 1% agarose gel. One strip was stained with ethidium bromide and used as a template to cut out the nicked plasmid DNA from the unstained gel strip. The unstained nicked plasmid DNA was electroeluted and concentrated

by ethanol precipitation. S1 nuclease (Boehringer Mannheim) digestion of the nicked molecules was used to verify that the molecules were predominantly singly nicked.

To prepare plasmid molecules with single-stranded gaps, singly-nicked molecules (not purified by gel electrophoresis) at 100 ng/μl were digested with 0.08 U/μl exonuclease III (New England Biolabs) at 37°C for 10 min. SDS was added to a final concentration of 0.1% to stop the reaction. To purify the resulting gapped molecules from contaminating linear plasmids, the mixture was electrophoresed on two lanes of a 0.6% low-melting point agarose gel (FMC Sea Plaque). One lane was ethidium bromide stained and used as a template to cut out the gapped molecule band. The gel band was dissolved by dilution with buffers prior to use in further procedures. This method yielded plasmid molecules with ~1000 bp single-stranded gaps as determined by S1 nuclease digestion. Supercoiled plasmid DNA was linearized with an excess of either *Kpn*I, *Xba*I, *Hpa*I or *Sma*I (New England Biolabs). The completeness of digestion was assayed by agarose gel electrophoresis. Following digestion, the plasmid DNA was extracted once with phenol:chloroform:isoamyl alcohol, twice with ether, and was ethanol precipitated. The final DNA pellet was resuspended in double distilled water and used in transfection experiments.

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