Gap misrepair mutagenesis: Efficient site-directed induction of transition, transversion, and frameshift mutations in vitro

(site-specific mutagenesis/base substitutions/DNA polymerase/fidelity of DNA synthesis/thiophosphate nucleotides)

DAVID SHORTLE*, PAULA GRISAFI*, STEPHEN J. BENKOVIC†, AND DAVID BOTSTEIN*

*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and †Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Contributed by David Botstein, November 13, 1981

ABSTRACT Short single-stranded gaps can be constructed by limited exonuclease action at single-stranded breaks (nicks) placed at predetermined sites on closed circular DNA molecules. As efficient primer-templates for DNA polymerase, single-stranded gaps can be repaired in vitro to regenerate an intact DNA duplex. In this report two in vitro reaction schemes are described that produce a high frequency of errors during repair ("misrepair") of gaps and thereby allow the efficient recovery of mutations limited to the nucleotide sequence at or near the original gap. In the first of these misrepair schemes, nucleotide misincorporations are stimulated by omission of one of the four deoxynucleoside triphosphates; the misincorporations are trapped by the presence of excess DNA ligase in the reaction mixture. The second misrepair scheme involves the misincorporation of an excision-resistant α-thiophosphate nucleotide, followed by gap filling in the presence of all four conventional deoxynucleoside triphosphates. When applied to short gaps constructed at one of several unique restriction sites on the small plasmid pBR322, both gap misrepair methods yielded mutations within the targeted restriction site at high frequency (6–49%). A majority of the sequence changes identified were base substitutions; transversions and transitions are approximately equally represented. The remaining sequence changes were an insertion of a single base pair and deletions of one to four base pairs.

In the past few years, several methods of site-specific mutagenesis have been developed to facilitate recovery of mutations in genes carried on small circular DNA molecules (1–3). Perhaps the most direct of these methods involves the use of synthetic oligonucleotides as primers for DNA synthesis after annealing to a single-stranded circle of wild-type DNA (2, 4). Because the nucleotide sequence of the synthetic oligonucleotide replaces the homologous wild-type sequence, in principle any mutation—base substitution, insertion, or deletion—can be induced at any site within the appropriate oligonucleotide. However, when the exact nucleotide sequence change necessary to create a mutant allele with a particular phenotype is unknown, synthesis of the many different oligonucleotides required to cover all possibilities becomes inefficient and laborious. In such situations, the optimal strategy for mutagenesis is one that induces a variety of point mutations scattered over a specified region of the gene.

Methods have been developed (5–7) that allow the direction of mutagenesis to unique nucleotide positions or to sites distributed within a defined segment of DNA. These methods are based on the introduction of a single-stranded break (nick) into a DNA molecule, followed by enlargement to a small gap that can serve as a specific target for an in vitro mutagenic reaction. Because the position of the nick determines the region of mutagenesis to within a few base pairs, the degree of site-specificity is determined by which of several endonuclease reactions are used to generate the nick (5–11).

MATERIALS AND METHODS

Strains. The Escherichia coli strains HB101 (thr− leu− pro− recA thi− hisD46 trpE supE95) BD-1528 (F′ met− hisD46 trpE supE95 ung-1 nadB7; obtained from B. Duncan), and DS409 (thr− leu− thi− supE lacY tonA ara− hisD46 trpE supE95) were used as recipients for transformation with the small plasmid pBR322.

Materials. Micrococcus luteus DNA polymerase I was obtained from Miles; restriction endonuclease Cla I was obtained from Boehringer Mannheim; all other enzymes were obtained from New England Biolabs. Units of enzyme activity are those of the manufacturer. Deoxynucleoside triphosphates were purchased from Schwarz/Mann. The α,β,γ,δ-diastereomers of thymidine 5′-O-(1-thiophosphosphate) (dTTP[αs]) and 2′-deoxyadenosine 5′-O-(1-thiophosphosphate) (dATP[αs]) were prepared according to Bryant and Benkovic (12).

Restriction Enzyme Nicking Reactions. Covalently closed circular pBR322 DNA (13) was nicked with restriction endonucleases HindIII, Cla I, or BamHI by incubating 10 μg of plasmid DNA in a 100-μl solution of 20 mM Tris-HCl, pH 7.8/7.7 mM MgCl₂/7 mM 2-mercaptoethanol/gelatin (100 μg/ml) and a concentration of ethidium bromide (150 μg/ml, 75 μg/ml, or 100 μg/ml, respectively) determined by titration to give an optimal level of nicking. An amount of restriction endonuclease was added sufficient to convert =50–90% of the input DNA to an open circular form on incubation at room temperature for 2–4 hr. The nicking reaction with the EcoRI enzyme consisted of 100 μM Tris-HCl, pH 7.6/50 mM NaCl/5 mM MgCl₂/gelatin (100 μg/ml)/ethidium bromide (150 μg/ml). Reactions were stopped by addition of excess EDTA followed by phenol extraction and ethanol precipitation.

Gapping Reaction and Purification of Gapped-Circular DNA. To convert restriction enzyme-induced nicks into short single-stranded gaps, 5 μg of nicked pBR322 was added to 25 μl of 70 mM Tris-HCl, pH 8.0/7 mM MgCl₂/1 mM 2-mercaptoethanol containing 0.5 unit of M. luteus DNA polymerase I per μg of DNA (10). After incubation at room temperature for 60 min, the reaction was stopped with excess EDTA, phenol extraction, and ethanol precipitation. From this mixture of DNA forms, circular DNA with single-stranded gaps was selectively purified by first ligating linear and nicked-circular molecules to a relaxed, covalently closed form in a 50-μl reaction consisting of 66 mM Tris-HCl, pH 7.6/6.6 mM MgCl₂/5 mM dithiothreitol/gelatin (100 μg/ml)/0.5 mM ATP/150 μM units of T4 DNA ligase; the reaction was incubated overnight at 0°C. After phenol extraction and ethanol precipitation, this DNA mixture was dissolved in 0.2 ml of 10 mM sodium citrate,
pH 6.0/0.5 mM EDTA/0.2 M NaCl/ethidium bromide (2 µg/ml) and passed over a 0.2 ml column of acridine yellow ED beads (Boehringer Mannheim). After washing the column—first with the loading solution and then with 10 mM sodium citrate, pH 6.0/0.5 mM EDTA/0.2 M NaCl without ethidium bromide—the open-circular (i.e., gapped) DNA fraction was eluted with the same buffer containing 0.5 M NaCl and was concentrated by ethanol precipitation in the presence of carrier tRNA.

**Gap Misrepair: Method 1.** A typical reaction consisted of 100–200 ng of gapped pBR322 DNA in 20 µl of 60 mM Tris-HCl, pH 8.0/20 mM 2-mercaptoethanol/1 mM MgAc2/2 mM MnCl₂/gelatin (100 µg/ml)/0.5 mM ATP and three deoxynucleoside triphosphates at a concentration of 125 µM each. To this mixture was added 150 units of T4 DNA ligase plus 0.2 unit of M. luteus DNA polymerase I, and incubation was carried out at 26°C for 16–18 hr. The percentage of molecules that had been converted to a covalently closed form was assayed by electrophoresis of an aliquot on a 1.2% agarose gel containing ethidium bromide at 0.5 µg/ml in both the gel and the running buffer. On such gels, circular DNA closed by ligation runs as a faintly fluorescent band with a mobility slightly greater than that of negatively supercoiled pBR322.

**Gap Misrepair: Method 2.** In the first reaction, an α-thiophosphate deoxyribonucleoside was misincorporated onto the 3’ terminus of a single-stranded gap in a 15-µl reaction consisting of 130 mM Hepes, pH 7.5/0.2 mM MnCl₂/2 mM 2-mercaptoethanol/gelatin (100 µg/ml)/100 µM αTTP[αS] (or αdTTP[αS])/50–100 ng of DNA. In some reactions, either dTTP or dGTP was present with a concentration of 20 µM. After addition of 0.3 unit of Klenow fragment polymerase, incubation was carried out at 26°C for 14–16 hr. The reaction was stopped by making the mixture 10 mM in EDTA, followed by phenol extraction and ethanol precipitation. The precipitate was dissolved in a few microliters of 2 mM Tris-HCl, pH 8.0/0.2 mM EDTA. To fill in the remaining of the single-stranded gap, the same M. luteus DNA polymerase I reaction used in method 1—as described above except for the addition of all four deoxynucleoside triphosphates—was carried out. DNA recovered from this reaction was used directly for transformation.

**Recovery and Analysis of Mutant Plasmids.** Several nanograms of mutagenized pBR322 were used to transform E. coli strain HB101 by the protocol of Dagert and Ehrlich (14). Transformants were selected on LB agar plates containing ampicillin at 250 µg/ml. To allow for segregation of mutant plasmids from wild-type, 24–48 independent colonies were picked and streaked twice. A small amount of plasmid DNA was prepared from each isolate by a modification of the method of Holmes and Quigley (15) and was digested with the cognate restriction endonuclease for the site that had been mutagenized. In most experiments, plasmid DNA was also prepared from a pool of 1000 or more transformed cells. The percentage of this DNA pool that had lost the restriction site was estimated by comparison of the circular pBR322 band intensity before and after exhaustive restriction enzyme digestion. For several mutant plasmids identified among the independent isolates, a large preparation of DNA was made and the nucleotide sequence surrounding the lost restriction site was determined (16).

**RESULTS**

The basic steps of the gap misrepair mutagenesis methods described above are illustrated in Fig. 1. A short single-stranded gap was generated at a unique restriction site on the small plasmid pBR322 in two steps. First, the DNA was nicked with the restriction endonuclease in the presence of ethidium bromide (6, 8) and then a limited gap was formed by exonuclolytic digestion with DNA polymerase I from M. luteus (8). Under the conditions used, an average of five or six nucleotides were removed from the nicked strand, predominantly in the 5’ → 3’ direction. Circular DNA gapped at a particular restriction site was purified and used as substrate for an in vitro "gap misrepair" reaction in which the short stretch of DNA missing at the single-stranded gap was synthesized by DNA polymerase under conditions that promote a high frequency of nucleotide misincorporation.

Because the bacterial DNA polymerases used for repair synthesis possess a 3’ → 5’ "excision" exonuclease, the occasional misincorporated nucleotide will be efficiently excised. To eliminate this undesired competing reaction, two different approaches were taken. In method 1 (gap misrepair via nucleotide omission), misincorporation is promoted by omitting one of the four deoxynucleoside triphosphates from the reaction mixture (17) and by adding manganese ion (18) plus T4 DNA ligase and ATP. Under these conditions, only noncomplementary nucleotides are available for incorporation at those positions where the omitted nucleotide would otherwise be correctly incorporated. Infrequently, DNA synthesis proceeds past such a point by misincorporation; if the remainder of the single-stranded gap is filled in and the strand immediately closed by DNA ligase, the misincorporated nucleotide will be trapped in a nonexcisable state. In method 2 (gap misrepair via nonexcisable nucleotides), an "excision-resistant" α-thiophosphate analogue of a deoxynucleoside triphosphate is misincorporated onto the 3’ terminus of a single-stranded gap as a first step. Because these nucleotide analogues are efficient substrates for DNA polymerases (19, 20) but are resistant to 3’ → 5’ exonuclease activity once incorporated into a DNA strand (ref. 21; unpublished results), their misincorporation onto a 3’ terminus becomes effectively irreversible. As a consequence, misincorporation events accumulate over time. In a second reaction, the remainder of the single-stranded gap is filled in by DNA synthesis from this stable, mismatched 3’ terminus, and the strand is sealed by DNA ligase.

With both methods, the product of a completed reaction is a covalently closed circular DNA with one or more mismatched base pairs at the site of the original single-stranded gap. On
transformation of such DNA into an appropriate host cell, the misincorporated nucleotide(s) on one strand should segregate from the wild-type sequence on the other strand at the first round of replication (or by random mismatch repair), resulting in a fixed base substitution mutation in half of the progeny molecules. For method 1, the expected result is that base substitutions should only occur at positions in the single-stranded gap where the omitted nucleotide would otherwise have been correctly incorporated, the actual base change being determined by the nucleotide misincorporated in its place. For method 2, base substitutions are expected at positions where the α-thiophosphate nucleotide has been misincorporated—i.e., the position of the 3′ terminus when misincorporation occurred. In this case, although the mutational site may be somewhat indeterminate due to movement of the 3′ terminus by the 3′ → 5′ exonucleolytic activity of DNA polymerase, the mutation should be a base substitution determined by the α-thiophosphate nucleotide used.

**Method I (Gap Repair via Nucleotide Omission).** In a test experiment, purified pBR322 containing a single-stranded gap at the HindIII site was used as substrate for repair synthesis in the presence of excess DNA ligase (as described in Materials and Methods) with either all four nucleotide triphosphates added or with dATP alone omitted. For both reactions, the extent of gap filling as assayed by conversion of the input DNA to a closed circular form was 90% or greater. This DNA was used to transform *E. coli* strain HB101 to ampicillin resistance. Of 40 independent transformants with DNA repaired in the reaction with all four dNTPs present, none yielded plasmid DNA resistant to cleavage by the HindIII endonuclease. However, 5 of 36 transformants (14%) with DNA repaired in the absence of dATP contained mutant plasmids that had lost the HindIII site.

Similar experiments were carried out at three other unique restriction sites (Cla I, BamHI, and EcoRI) on the plasmid pBR322. In each experiment, the deoxyxynucleoside triphosphate omitted from the misrepair reaction represented the first nucleotide that would normally be incorporated at the 3′ hydroxyl end at restriction enzyme-induced nicks—that is, for pBR322 DNA molecules gapped at the Cla I site, dCTP was omitted. For molecules gapped at the BamHI or EcoRI sites, misrepair was carried out in the absence of dGTP or dATP, respectively. In each of these three misrepair reactions, greater than 50% of the input DNA was converted to a covalently closed form. After transformation with DNA misrepaired at the Cla I site and single colony isolation, 12 of 36 transformants (33%) contained mutant plasmids lacking the Cla I site. Of 36 transformants obtained with DNA misrepaired at the BamHI site, 5 (14%) contained BamHI site mutants, whereas only 3 EcoRI site mutants were identified among 48 transformants (6%) with DNA mutagenized at this site.

Control DNA polymerase reactions with all four dNTPs included were run in parallel with each of the three misrepair reactions, and a pool of transformants (>1000) obtained with this "correctly" repaired DNA was screened in *masse* to determine the frequency of restriction-site mutants. In each case, no restriction enzyme-resistant DNA was detected, whereas similar screening of the corresponding misrepaired DNA revealed a percentage of restriction site mutant DNA in close agreement with the values obtained from screening of 36–49 transformants individually. Therefore, most, if not all, of the recovered restriction site mutants were induced as a result of omitting one dNTP from the polymerase reaction. Because the DNA undergoing mutagenesis at a restriction site represents molecules that had been nicked by the cognate restriction enzyme, preexisting mutations were, if anything, selected against.

The nucleotide sequence surrounding the lost restriction site was determined by the method of Maxam and Gilbert (16) for several mutants from each experiment. As shown in Fig. 2, most of the mutants induced by misrepair at the HindIII, Cla I, and BamHI sites have each acquired a single base substitution at the position expected based on the nucleotide omission—i.e., the first nucleotide position beyond the restriction enzyme-induced nick in one of the two strands. One exception is H103, where the mutation is shifted one position away from a nick. However, four of the mutant plasmids contain from one to three additional base substitutions in the nucleotide sequences flanking the mutant restriction site. Every base substitution can be explained as resulting from a misincorporation event at positions where the omitted nucleotide could have been correctly incorporated during 5′ → 3′ DNA synthesis from a restriction enzyme-induced nick. For this reason, we presume that the mutations located outside the restriction site arose by limited nick translation occurring prior to strand closure by DNA ligase or by misrepair of a gap longer than six nucleotides.

Unlike the situation at the HindIII, Cla I, or BamHI sites, DNA polymerase proceeding 5′ → 3′ from a nick in the EcoRI site encounters two adjacent positions where the omitted nucleotide dATP would otherwise be incorporated. For DNA synthesis to advance beyond this point in the absence of dATP requires two consecutive misincorporations. Such double aberrant events would be expected to occur at significantly lower frequencies than single misincorporations. The frequency with which EcoRI site mutants were recovered from DNA mispaired in the absence of dATP was lower (6%) than that at the HindIII site (14%) where only a single misincorporation is required. The nucleotide sequence changes in three EcoRI site mutants shown in Fig. 2 occur only at the second of the two A positions. To reach this position starting from a 3′ terminus at a nick, DNA polymerase must have incorporated an A residue at the first position, either as dATP contaminating one of the other three dNTPs or as dATP (22) which is present in the reaction as cofactor for T4 DNA ligase. This result suggests that, at sites in DNA where one type of base pair occurs in runs of two or more, all nucleotide positions might not be readily mutagenized.
able by using gap misrepair via nucleotide omission.

The pattern of base alterations from the nucleotide omission gap misrepair experiments can be summarized by noting that the 17 sequenced mutant plasmids contained 26 single-base changes, with transversions as frequent as transitions (12 vs. 13); one deletion mutation was found. Of the 10 mutational events induced by DNA synthesis in the absence of dATP, G substitution occurred six times, T substitution occurred twice, C substitution occurred once, and deletion of single A-T base pair occurred once. The 13 mutational events in the absence of dCTP were all base substitutions—6 T's, 4 G's, and 3 A's; in the absence of dGTP there were 2 substitutions of T and 1 of A.

Method 2 (Gap Misrepair via Incorporation of Nonexcisable Nucleotides). This method consists of two sequential reactions: misincorporation of an α-thiophosphate nucleotide onto the 3' terminus of a single-stranded gap, followed by gap filling and ligation. These two reactions were carried out with dATP[aS] by using as substrate pBR322 DNA gapped at the HindIII site, the Cla I site, or the EcoRI site. After the second reaction, the plasmid DNA was used to transform E. coli strain HB101 to ampicillin-resistance, and 24 independent transformants were screened for mutant plasmid DNA with the cognate restriction enzyme. Three HindIII-resistant mutants (13%), ten Cla I-resistant mutants (42%), and five EcoRI-resistant mutants (20%) were identified.

The nucleotide sequence changes are shown in the top part of Fig. 3. Of the 10 mutants sequenced, 7 contained single-base substitutions consistent with the misincorporation of a T residue onto the 3' terminus of a gap generated from a restriction enzyme-induced nick on one of the two strands. In the case of mutants H301, C304, and E302, the 3' terminus was presumably moved one or two positions in the 3' → 5' direction from the nick by the editing exonuclease of DNA polymerase prior to the misincorporation event. The three exceptional mutants—H302, H303, and C301—have acquired deletions of one or two base pairs.

To explain the origin of these deletions at sites where misincorporation might have been expected, two types of mechanisms can be invoked. Either nucleotide positions in the template strands are skipped over by an abortive event during the in vivo polymerase reactions, or deletions are formed in vivo after transformation by an abortive attempt at excision repair, possibly as a consequence of the fact that thiophosphate-containing phosphodiester linkages are resistant to a variety of nuclease (21, 23-25). With regard to the second mechanism, it seemed significant that the host cell for DNA transformation in these experiments was a recA strain; therefore, DNA lesions generated by incomplete excision, such as apurinic sites, might not be repairable via recombination with the wild-type sequence present on the other strand or via the inducible error-prone repair pathway (26).

To test the possible influence of the recA+ lesion carried by strain HB101, the preparation of mutagenized pBR322 DNA from which the deletion mutants H302 and H303 were recovered was used to transform two different recA+ E. coli strains—DB1528 and DB4906. On screening a total of 48 independent transformants, 7 additional HindIII resistant mutants were identified. On sequence analysis, all seven were found to have acquired the same base substitution as mutant H301 with no deletions. Although the numbers are small, this result suggests that the frequency of deletion mutations induced by this method can be reduced by use of a recA+ strain for transformation.

In a series of mutagenesis experiments with the nucleotide dATP[aS], an effort was made to fix the position of the 3' terminus of the single-stranded gap by adding one conventional

![Fig. 3. Results with method 2 (gap misrepair via nonexcisable nucleotide): restriction site mutants induced in plasmid pBR322 by misincorporation of an α-thiophosphate nucleotide in a two-step reaction series. A dashed line indicates the wild-type base pair was present in the mutant; a black rectangle indicates deletion of the corresponding base pair; a caret indicates insertion of a base pair. * Mutants H202, H203, and C201 were induced by gap misrepair reactions with dATP[aS] to which dGTP (for H202 and H203) or dTTP (for C201) had also been added (see text). Presumably, these mutations resulted from misincorporation of the conventional nucleotide.](image-url)
of the type expected from misincorporation of one analogue residue constitute 50–60% of the total. The remainder of the mutants consisted predominantly of either very small deletions or unexpected types of base substitutions.

**DISCUSSION**

From the results presented in this report (summarized in Table 1), we conclude that a variety of base substitutions can be induced at relatively high rates by using DNA polymerase to catalyze the misincorporation of nucleotides at specific sites. The gap misrepair methods allow site-directed induction of transversion mutations without chemical synthesis of oligonucleotides. Like previous versions of in vitro mutagenesis directed to sites by specific nicking (5–7, 9), gap misrepair has the advantages and disadvantages of highly localized mutagenesis without complete specification of the mutant sequence in advance. Therefore, this approach may be particularly useful for genetic investigations, whereas constructions of predetermined variant sequences may still be best accomplished by using chemical synthesis (2, 4). With gap misrepair mutagenesis, the frequency is high enough to encourage screening of individual isolates one by one, even by biochemical methods.

The two methods (misrepair via nucleotide omission and misrepair with α-thiophosphate nucleotides) have properties that make them differentially advantageous in different circumstances. The nucleotide omission method appears to allow the misincorporation of all three remaining dNTPs at roughly equal frequency. It should be noted that this result is in contrast to the failure to observe high levels of pyrimidine–pyrimidine mispairing under other, possibly more physiological, conditions (28, 29). It may be possible to control the pattern of misincorporation by varying the relative concentrations of the three deoxyribonucleotide triphosphates in the misrepair reaction. However, two properties of this mutagenic DNA polymerase reaction may limit its use in the *in vitro* construction of point mutations. First, when the template for DNA synthesis is longer than a few nucleotides multiple base substitutions can occur. More important, sites where the omitted nucleotide would be correctly incorporated at two or more adjacent positions may not always be efficiently mutable by this reaction. Addition of lower concentrations of the “omitted” nucleotide may reduce this problem.

Gap misrepair via misincorporation of an α-thiophosphate nucleotide can also induce point mutations at high frequency. In this case, there appears to be a greater measure of predictability because a majority of the mutations recovered are single-base substitutions of the type expected from the nucleotide used. Among the unexpected mutations, the predominant types are deletions of one to four base pairs. It is not yet clear whether the frequency of these deletions can be controlled by modifications in the reaction conditions or variation in the bacterial hosts used for transformation—or both. Nor is it clear whether all kinds of point mutations can be readily induced. Such a determination will depend upon experiments using α-thio dCTP and α-thio dGTP. Nevertheless, given the results to date plus the relatively straightforward chemistry involved, it seems reasonable to expect that α-thiophosphate nucleotide misincorporation may provide a simple, efficient method of inducing all types of single-base substitutions.

We thank Scott Putney, Tom Kunkel, and Paul Schimmel for providing us with data prior to publication; we are also indebted to Douglas Kosholthand Dan Nathans for helpful discussions. This research was supported by grants to D.B. from the American Cancer Society (M91111) and the National Institutes of Health (GM18973 and GM21533) and to J.R. from the National Institutes of Health (GM13306). D.S. is a fellow of the Helen Hay Whitney Foundation.


**Table 1. Summary of nucleotide sequence changes induced by gap misrepair mutagenesis**

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>Method 1</th>
<th>Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Transversion</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>Deletion</td>
<td>1</td>
<td>6*</td>
</tr>
<tr>
<td>Insertion</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total lesions</td>
<td>26</td>
<td>30</td>
</tr>
<tr>
<td>Total mutant plasmids</td>
<td>17</td>
<td>29</td>
</tr>
</tbody>
</table>

Method 1 and method 2 are described in the text. Numbers given are the simple sum of mutations analyzed and cannot (particularly with method 2) be construed to have more than a very limited statistical significance.

*All four possible transversions are represented.
† Of these, four had more than one adjacent base pair deleted.