Oligonucleotide-directed construction of mutations: a gapped duplex DNA procedure without enzymatic reactions in vitro

Hans-Joachim Fritz*, Joachim Hohlmaier†, Wilfried Kramer‡, Andrea Ohmayer and Jürgen Wippler

Max-Planck-Institut für Biochemie, Abteilung Zellbiologie, Am Klopferspitz 18a, D-8033 Martinsried bei München, FRG

Received March 2, 1988; Revised and Accepted June 14, 1988

ABSTRACT

The gapped duplex DNA approach to oligonucleotide-directed construction of mutations (Kramer et al. 1984, Nucl. Acids Res. 12, 9441-9456) has been developed further. A procedure is described that makes in vitro DNA polymerase/DNA ligase reactions dispensable. Direct transfection of host bacteria with gdDNA molecules of recombinant phage M13 plus mutagenic oligonucleotide results in marker yields in excess of 50% (gap size 1640 nucleotides). An important feature incorporated into the mutagenic oligonucleotide is the presence of one or two internucleotidic phosphorothioate linkages immediately adjacent to the 5'-terminus. Automated preparation and biochemical properties of such compounds are described as well as their performance in oligonucleotide-directed mutagenesis. A systematic study of the following parameters influencing marker yield is reported: Gap size, length of oligonucleotide, chemical nature of oligonucleotide termini and heatshock temperature during transformation.

INTRODUCTION

The precise construction of mutations directed by synthetic oligonucleotides has become a widely used tool in the analysis of many biological problems. Recent reviews covering this field are available1-3. In addition, the same technique is of prime importance in the rational design and synthesis of proteins with unprecedented functions. Clearly, there is great demand for simple and reliable procedures that yield the desired mutations at high frequency. Previously we have introduced the gapped duplex DNA method4,5 (see figure 1) and developed it to give reproducible marker yields of up to ca. 80%6. Starting materials for the gdDNA approach to the construction of mutations are two phages: M13mp9 and M13mp9rev (figure 1, 1 and II). The former phage carries two amber mutations in genes I and II7-8 which are reverted to wild type in the latter9 (open and filled boxes, respectively, in figure 1, I and II).

A generally applicable procedure of mutation construction consists of cloning the DNA fragment under study into the poly-linker region of M13mp9 (A). The recombinant phage genome (figure 1, III) is isolated in single stranded form from virion particles (A). From this, gdDNA (figure 1, VI) is constructed by preparative DNA/DNA hybridization (C), the partner being denatured RF-DNA of M13mp9rev previously cleaved with the same restriction enzyme(s) that was (were) used
for cloning the exogenous DNA fragment (A,B). In the resulting hybrid (figure 1, VI), the single stranded gap comprises exactly the cloned DNA insert. The mutation is introduced by annealing to the gdDNA a synthetic oligonucleotide containing the desired structural change (D), followed by enzymatic gap-filling/sealing reactions in vitro (figure 1, E) plus transfection of a recipient E. coli strain carrying a mutation in either gene mutS or mutL. (F). Such bacteria are deficient in the repair of any type of base/base mismatch and thus preserve the genetic linkage between the oligonucleotide-borne marker and the wild type genes I and II of M13mp9rev. Infection of suppressor-free host bacteria with the resulting mixed phage population (at very low multiplicity) leads to marker segregation and elimination of the progeny derived from the M13mp9 DNA strand. Consequently, the synthetic marker is enriched by virtue of its residence in the M13mp9rev genome.

For a number of reasons, the DNA polymerase reaction in vitro is a particularly critical step in this and similar procedures:

1. In our hands, the quality of commercial DNA polymerase I, large fragment, has shown dramatic variation depending on supplier and individual batch (unpublished).
2. An intrinsic problem of the reaction is finding conditions which balance two mutually linked and counteracting parameters: the tendency of the various DNA polymerases to stall at hairpin-loop structures in the template and their ability to promote strand displacement which can lead to loss of the synthetic oligonucleotide from its target hybridization site.
3. DNA polymerase reactions in vitro produce copying errors at low but significant frequencies thus occasionally introducing unwanted additional mutations outside the target site (this laboratory, unpublished observations).
4. Certain miscoding DNA lesions (such as sites of hydrolytic deamination of cytosine residues) introduced into the single stranded part of the gdDNA during in vitro handling of the phage genome are copied into the minus strand and consequently fixed as mutations (also see Discussion).
Together with the method outlined above, we also had described a considerably simplified protocol that eliminates all enzymatic manipulations in vitro and, instead, uses a mixture of gdDNA and mutagenic oligonucleotide directly for transfection\(^5\). Here we report results of a systematic effort to improve the moderate efficiency observed with the simplified co-transfection procedure in the earlier study\(^6\). Marker yields in excess of 50% are now achieved. This makes sequence analysis of a few candidate clones picked at random a rapid and economic means of screening for and verifying the desired mutant.

**MATERIALS AND METHODS**

**Bacterial and phage strains** were as described\(^5\). In addition, strain WK 6 (\(\Delta{lac-proAB}\), \(galE\), \(strA\); \(F^+\) \(lacI\), \(Z\Delta M15\), \(proA^+B^+\)) was used. WK 6 was isolated as a RecA\(^-\)-revertant of strain MK 30-3\(^9\) by virtue of its higher resistance to UV-irradiation. Phage M13mp9revaml6 is a derivative of M13mp9rev, carrying an amber mutation in codon 16 (TGG) of the original \(lacZ\)-gene\(^5\).

**Enzymes and chemicals:** Restriction endonucleases BanII, BglII and PvuII were purchased from New England BioLabs, other enzymes and chemicals were as described\(^5\).

**Media, plating techniques, and preparation of phage M13 virion and RF-DNA** were as described\(^5\).

**Preparation of RF-DNA fragments:** The preparation of the large EcoRI/PvuI fragment of RF-DNA derived from phage M13mp9revaml6 was as described\(^5\). For preparation of the large BanII/BglII fragment, RF-DNA derived from phage M13mp9revaml6 was cleaved with BanII and BglII. Completeness of the digestion was monitored by agarose gel electrophoresis. Subsequently, the mixture of DNA fragments was subjected to PvuII cleavage to further break down the smaller DNA fragment. The largest fragment was purified by centrifugation through sucrose gradients\(^5\). These were prepared by dispensing the following solution into centrifuge tubes, freezing and storage at -20°C, followed by thawing immediately prior to use: 100mM NaCl, 100mM Tris-HCl pH 8.0, 10mM EDTA, 20µg/ml ethidium bromide, 185mg/ml sucrose.

**2’-Deoxy-oligonucleotide synthesis** was carried out according to the phosphoramidite method essentially as described\(^9\). An “Applied Biosystems” Model 380A DNA synthesizer was used throughout. The introduction of internucleotide phosphorothioate linkages was adapted from Connolly et al.\(^13\) and Stec et al.\(^15\): A solution of 5% elemental sulfur in carbodiimide/2.4-lutidine (1:1) was used for oxidation (4 x 15min, delivery of 2.3ml of sulfur solution each time, ambient temperature). Before and after this step, the support was thoroughly washed with carbodiimide/2.4-lutidine (1:1) to avoid sulfur precipitation within the reagent delivery system. After the wash, the standard elongation cycle was resumed. This chemistry was implemented on the Model 380A synthesizer by using solvent port no. 13 for the sulfur solution, port no. 16 for the mixed solvent, plus making the appropriate changes in the control software.

**Purification and enzymatic phosphorylation of oligonucleotides:** Synthetic 2’-deoxy-oligonucleotides with all protecting groups except the 5’-terminal dimethoxytrityl group removed, were
purified by reversed phase high pressure liquid chromatography\textsuperscript{16}. After detritylation, the products were enzymatically phosphorylated essentially as described\textsuperscript{5}. 5'-Phosphorylated oligonucleotides were further purified by preparative polyacrylamide gel electrophoresis\textsuperscript{17}. UV shadowing was used for product detection\textsuperscript{18}. This step removed any short chain contaminants and, in particular, unphosphorylated starting material, if remaining. In some cases, the h.p.i.c. step was omitted from the work-up scheme.

**Construction of gapped duplex DNA (gdDNA)** was as described\textsuperscript{5}.

**Annealing of mutagenic primers**: 8μl hybridization products mixture (containing gdDNA and other DNA species equivalent to 20 fmol of input linear DNA) were mixed with 2μl aqueous solution of 5'-phosphorylated oligonucleotide at a concentration of 2 pmol/μl or more. Final buffer concentration: 150 mM KCl, 10 mM Tris-HCl pH 7.5. (Increased amounts of oligonucleotide had no significant effect on the marker yield, data not shown). The mixture was heated to 65°C for 5 min, kept at room temperature for 10 min and was then used for transfection.

**Transfection and segregation** were carried out as described\textsuperscript{5} with the following modifications:

Heatshock of the DNA/cell mixture after incubation on ice for 90 min was generally at 30°C (in few cases indicated also at 37°C or 45°C) for 3 min. For determination of the transfection efficiency, either strain BMH 71-18 or strain WK 6 was used as plating bacteria. At least 1000 primary transfectants were obtained in each individual experiment. In the reinfecation step, strain WK 6 was used in most experiments instead of strain MK 30-3\textsuperscript{5}. For determination of the marker yield, at least 1000 plaques were scored in each individual experiment (except for two experiments in which only 700-800 plaques were counted). Even in cases of extremely asymmetric marker distribution, no less than 57 plaques of the less abundant phenotype were scored.

**RESULTS**

The model experiment

The genetic assay on which this present investigation rests was essentially taken from our previous study\textsuperscript{5}. Single stranded virion DNA of phage M13mp9am16\textsuperscript{5} was the starting material for two different gapped duplex DNAs; the hybridization partner was a fragment of (double stranded) RF-DNA of phage M13mp9revam16 - in one case the large EcoRI/PvuI fragment leading to a single stranded gap of 120 nucleotides, in the other the large BanII/BglII fragment leading to a gap of 1640 nucleotides (see figure 2). Both gdDNAs, when used for transfection of indicator strains elicit colourless plaques exclusively ("X-gal" indicator plates). A series of oligonucleotides was synthesized, each carrying the information to revert lac-codon 16 of M13mp9am16 (TAG, amber) to wild type (TGG, trp) by oligonucleotide-directed mutagenesis under a variety of experimental conditions. The resulting transition mutation can be scored by the concomitant change of plaque phenotype from colourless to blue. Large numbers of individual plaques are conveniently classified and scored by their colour thus providing a sound statistical basis for the calculation of marker distribution. The one experimental parameter kept fixed throughout this study was the omission of all enzymatic in
Figure 2: Preparation of gapped duplex DNA. A: Schematic representation of the M13mp9 genome. The boxed insert represents lac DNA, the hatched part the fragment of the lacZ gene. Codon 16 of lacZ is situated between the EcoRI and the PvuI sites (compare earlier work). B: Agarose gel electrophoretic analysis of different DNA species. Lanes 1 and 10: Marker DNA (phage λ genome, cleaved with HindIII). Lane 2: RF-DNA of phage M13mp9revaml6. Lane 3: ssDNA of phage M13mp9aml6. Lane 4: Large EcoRI/PvuI fragment of DNA shown in lane 2. Lane 5: Mixture of DNAs shown in lanes 3 and 4. Lane 6: As in lane 5, but after heating to 100°C for 3 min. Lane 9: large BanII/BglII fragment of DNA shown in lane 2. Lane 8: Mixture of DNAs shown in lanes 3 and 9. Lane 7: As in lane 8, but after heating to 100°C for 3 min. The slowest moving band in lanes 6 and 7, respectively, corresponds to gDNA. Note the slightly greater mobility of the gDNA with the larger gap.

*in vitro* reactions. In the following, we refer to this direct co-transfection procedure as the "mix-heat-transfect protocol".

**Influence of oligonucleotide size on marker yield**

The most obvious parameter expected to influence the marker yield in a simple mix-heat-transfect procedure is the stability of the hybrid between the gDNA and the oligonucleotide. To investigate this influence, a series of oligonucleotides with increasing chain lengths (figure 3A; 16C, 24C, 32C, 40C, 48C) was synthesized and tested with the gDNA containing the small gap (120 nucleotides). Results are displayed in table 1A and can be summarized as follows: 16C is much less efficient in bringing about the TAG to TGG transition than all the longer oligonucleotides. From a chain length of 24 nucleotides upward one observes a saturation value of roughly 40% with significant deviations: marker yields achieved with 32C are lower, those with 40C higher. Tentatively, we correlate this variation with the base composition near the 5'-termini of these oligonucleotides (see figure 3A). The data seem to suggest that tight hybridization at the very 5'-end helps to increase the marker yield - possibly a reflection of reduced strand displacement and/or reduced nucleolytic oligonucleotide degradation *in vivo*.
Figure 3: Nucleotide sequences of the oligonucleotides used and their respective hybridization position. Part of the (+)strand DNA sequence of phage M13mp9aml6 is shown in lower case print (5' to 3' direction running from left to right). Names and nucleotide sequences of the various mutagenic oligonucleotides are given in capital letters. The residue unpaired in the DNA hybrid is printed in bold face. A: Series of oligonucleotides containing natural phosphodiester linkages exclusively. B: Series of oligonucleotides containing phosphorothioate linkages and their respective locations. Natural phosphodiester linkages are indicated by hyphens. Phosphorothioate linkages are marked "x".

The same series of oligonucleotides was also tested with the gdDNA containing the large gap (1640 nucleotides). Results are shown in table 1B. A number of features are evident:

1. The yields achieved are generally lower than with the small gap (the maximum value has dropped from 46% to 23%).
2. Mutation construction starting from gdDNA with a large gap is more demanding with respect to chain length of the oligonucleotide: the marker yield achieved with 24C is far from the saturation value (in contrast to the result of the previous experiment).
3. As before, 32C lies outside the trend.

A possible explanation for the much lower maximum yield obtained with the large gap may lie in a greater chance of the oligonucleotide being nucleolytically degraded before cccDNA synthesis in vivo is completed. Guided by this working hypothesis, we started to search for ways to protect the

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>16C</td>
<td>TGCAGACACTGACCCTTTTGGGACCp 5'</td>
</tr>
<tr>
<td>24C</td>
<td>GCAGGAATGTTGCGACACTGACCGCTTTTGGGACCp 5'</td>
</tr>
<tr>
<td>32C</td>
<td>TGCAGACACTGACCCTTTTGGGACCp 5'</td>
</tr>
<tr>
<td>40C</td>
<td>CAAAATGTTGCGACACTGACCGCTTTTGGGACCp 5'</td>
</tr>
<tr>
<td>48C</td>
<td>GCAGGAATGTTGCGACACTGACCCTTTTGGGACCp 5'</td>
</tr>
<tr>
<td>A</td>
<td>3' GCACCTGACCCTTTTGGp 5'</td>
</tr>
<tr>
<td>22C</td>
<td>G-T-T-(N16,T)-G-Gp 5'</td>
</tr>
<tr>
<td>28C</td>
<td>G-T-T-(N16,G)-C-G-Gp 5'</td>
</tr>
<tr>
<td>22C-I</td>
<td>G-T-T-(N16,T)-T-G-Gp 5'</td>
</tr>
<tr>
<td>22C-II</td>
<td>G-T-T-(N16,T)-C-G-Cp 5'</td>
</tr>
<tr>
<td>28C-II</td>
<td>G-T-T-(N16,G)-T-GxGp 5'</td>
</tr>
<tr>
<td>28C-III</td>
<td>G-T-T-(N16,G)-C-GxGp 5'</td>
</tr>
<tr>
<td>28C-IV</td>
<td>G-T-T-(N16,G)-C-GxGp 5'</td>
</tr>
<tr>
<td>B</td>
<td>G-T-T-(N16,G)-C-GxGp 5'</td>
</tr>
</tbody>
</table>
flanking regions of the synthetic oligonucleotides against nuclease attack. This seemed only the more attractive since the gap filling reaction in vivo (brought about by wild type enzymes!) is expected to cause primer loss by nick-translation irrespective of the size of the DNA gap. Below, we report results achieved with phosphorothioate functions introduced into the mutagenic oligonucleotide.

### Automated synthesis and chromatographic properties of 2'-deoxy-oligonucleotides containing phosphorothioate residues

In recent years, phosphorothioate residues introduced into nucleic acids and related molecules have played a major role in the analysis of a variety of biological problems, most notably the stereochemical characterisation of numerous reactions in DNA and RNA enzymology (for a recent review see\textsuperscript{18}). In the context of this study, the most interesting property of the internucleotidic

---

**Table 1: Influence of oligonucleotide length on marker yield**

<table>
<thead>
<tr>
<th>oligonucleotide(^\text{a})</th>
<th>A</th>
<th></th>
<th>B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>y</td>
<td>Y</td>
<td>N</td>
<td>y</td>
</tr>
<tr>
<td>16C</td>
<td>13%</td>
<td>16%</td>
<td>13</td>
<td>4%</td>
</tr>
<tr>
<td>24C</td>
<td>38%</td>
<td>39%</td>
<td>4-5</td>
<td>15%</td>
</tr>
<tr>
<td>32C</td>
<td>34%</td>
<td>31%</td>
<td>6</td>
<td>12%</td>
</tr>
<tr>
<td>40C</td>
<td>48%</td>
<td>46%</td>
<td>3-4</td>
<td>23%</td>
</tr>
<tr>
<td>48C</td>
<td>41%</td>
<td>38%</td>
<td>39%</td>
<td>4-5</td>
</tr>
</tbody>
</table>

\(\text{A: Results obtained with 120 nucleotides gap; B: Results obtained with 1640 nucleotides gap. y: Marker yield (results from individual experiments). Y: Average marker yield. N: Number of randomly picked candidate clones which have to be characterized for a 90% probability of finding at least one bearing the desired mutation (calculated according to Kramer et al.\textsuperscript{4}).}\)

\(\text{\textsuperscript{a}See figure 3A.}\)
phosphorothioate linkage is its resistance against a variety of endo- and exo- nucleases\textsuperscript{19}. With the advent of the phosphoramidite method of oligonucleotide synthesis\textsuperscript{13} it has become particularly convenient to synthesize oligonucleotides containing phosphorothioate functions; all that is required is replacing at the appropriate step of the elongation cycle the normally used oxidizing agent (iodine/water) by elemental sulfur\textsuperscript{14,15} (see figure 3A). Note that this reaction yields two stereoisomeric products (diastereomers). As described under Materials and Methods, we have adapted described procedures\textsuperscript{14,15} for use with an automated DNA synthesizer and have prepared the series of oligonucleotides containing phosphorothioate linkages listed in figure 3B. Each of the diastereomeric pairs of compounds 22C-II and 28C-II could be separated by reversed phase h.p.l.c. at the stage with all protecting groups except the 5'-O-dimethoxytrityl function removed from the oligonucleotides (see figure 4). Presence of a second phosphorothioate function near the 5'-terminus (28C-III) leads to a broadening of the two relevant h.p.l.c. peaks - not to four distinct peaks as possible in principle. Detritylation resulted in decrease or loss of stereoisomer resolution. Both observations together suggest prime importance of close proximity between the asymmetric phosphorus center and the dimethoxytrityl group for the observed separation. In accord with this notion is the fact, that two additional phosphorothioate functions near the 3'-terminus of the oligonucleotide (28C-IV) did not significantly affect the shape of the relevant peaks (data not shown). These findings confirm and extend similar observations by Stec et al.\textsuperscript{20}. For each set of compounds 22C-II and 28C-II, -III, -IV the earlier eluting fraction was designated “a”, the later eluting one “b”. Oligonucleotides containing phosphorothioate functions consistently travelled more slowly on the reversed phase column than the corresponding compound with exclusively natural phosphodiester linkages - compare the two panels of figure 4. The same data also show that the oxidation with elemental sulfur is quite efficient - side reactions to yield the normal phosphodiester linkage are insignificant, at least under the contained inert-gas conditions of the DNA synthesizer. The absolute configuration at the phosphorothioate center of compounds 28C-IIa and 28C-IIb, respectively, was determined (h.p.l.c. analysis of hydrolysis products obtained by treatment with snake venom phosphodiesterase\textsuperscript{21,22} and P1 nuclease\textsuperscript{23} respectively, experiments carried out
Table 2: Influence of internucleotidic phosphorothioate linkages on marker yield

<table>
<thead>
<tr>
<th>oligonucleotide*</th>
<th>A</th>
<th></th>
<th></th>
<th>B</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>22C-I</td>
<td>26%</td>
<td>27%</td>
<td>7-8</td>
<td>12%</td>
<td>11%</td>
<td>20</td>
</tr>
<tr>
<td>22C-IIa</td>
<td>29%</td>
<td>32%</td>
<td>6</td>
<td>16%</td>
<td>15%</td>
<td>14</td>
</tr>
<tr>
<td>22C-IIb</td>
<td>27%</td>
<td>28%</td>
<td>7</td>
<td>10%</td>
<td>9%</td>
<td>24</td>
</tr>
<tr>
<td>28C-I</td>
<td>45%</td>
<td>46%</td>
<td>3-4</td>
<td>19%</td>
<td>17%</td>
<td>12</td>
</tr>
<tr>
<td>28C-IIa</td>
<td>63%</td>
<td>62%</td>
<td>2-3</td>
<td>39%</td>
<td>38%</td>
<td>5</td>
</tr>
<tr>
<td>28C-IIb</td>
<td>53%</td>
<td>53%</td>
<td>3</td>
<td>29%</td>
<td>28%</td>
<td>7</td>
</tr>
<tr>
<td>28C-IIIa</td>
<td>59%</td>
<td>61%</td>
<td>2-3</td>
<td>39%</td>
<td>41%</td>
<td>5</td>
</tr>
<tr>
<td>28C-IIIb</td>
<td>51%</td>
<td>55%</td>
<td>3</td>
<td>36%</td>
<td>35%</td>
<td>5-6</td>
</tr>
<tr>
<td>28C-IVa</td>
<td>55%</td>
<td>57%</td>
<td>2-3</td>
<td>32%</td>
<td>33%</td>
<td>6</td>
</tr>
<tr>
<td>28C-IVb</td>
<td>50%</td>
<td>51%</td>
<td>3</td>
<td>29%</td>
<td>30%</td>
<td>6-7</td>
</tr>
</tbody>
</table>

* see figure 3B. For explanation of symbols see legend to table 1.

essentially as described, data not shown). Fraction 28C-IIa which eluted fast in the 5'-dimethoxytritylated state, proved to have the $S$-configuration, 28C-IIb the $R_p$-configuration in accord
In addition, we tested the mobility of all the compounds listed in table 2 in electrophoresis through denaturing polyacrylamide gels and found the phosphorothioate functions to have no or insignificant influence. Within each family of compounds (22C and 28C) no differences in mobility could be detected (data not shown). All oligonucleotides tested containing phosphorothioate functions near their termini were smoothly phosphorylated at their respective 5'-OH function by ATP and T₄ polynucleotide kinase.

**Influence of internucleotide phosphorothioate linkages on marker yield**

The series of oligonucleotides containing phosphorothioate linkages (see figure 3B) was first tested with the gdDNA containing the small gap (table 2A). Results obtained with the two control compounds containing natural phosphodiester linkages only (22C-I, 28C-I) are in close agreement with the expectation derived from their respective chain lengths and base compositions near their 5'-termini (see table 1A and discussion above). In the series of 28-mers, the introduction of phosphorothioate linkages leads to a significant increase in marker yield - in contrast to the 22-mer series. Within each set of stereoisomeric oligonucleotides, the early eluting fraction consistently gives higher marker yields than the late eluting one. Substitution of the 5'-terminal phosphodiester bridge for a phosphorothioate has by far the greatest influence; additional phosphorothioate functions seem to be of minor importance or - in the case of the two 3'-terminal ones - even lead to slightly lower yields. The lack of drastic influence of internucleotidic phosphorothioate functions in the 22-mer series seems to hint at low hybrid stability as the limiting factor with these rather short oligonucleotides.

A qualitatively similar picture - yet much more pronounced - emerges from the study with the gdDNA containing the large gap (table 2B). Again, the results obtained with control oligonucleotides 22C-I and 28C-I fit well into the trend observed before (compare table 1B). The effect of a 5'-terminal phosphorothioate is again marginal in the case of the 22-mer series. Within the 28-mer series, however, introduction of one or two phosphorothioate linkages adjacent to the 5'-termini of the oligonucleotides is of considerable practical importance: The number of clones that have to be screened for a 90% probability to find the correct mutant is now reduced by a factor of about 2 (Table 2B, right column, lines 4 - 8). Furthermore, the difference in yield between the early and the late eluting fractions is much smaller for the family of isomers containing two 5'-terminal phosphorothioate linkages. Both compounds 28C-IIIa and 28C-IIIb (two phosphorothioate functions) are of roughly equal efficiency as 28C-IIa (one phosphorothioate function, S₈ configuration).

Thus, stereochemistry at phosphorus is of no practical concern if two phosphorothioate functions are introduced.

**Effect of heatshock temperature**

The effect of different heatshock temperatures during transfection was tested with the oligonucleotide 28C-IIa (early eluting fraction; for structure see figure 3B) and the gdDNA with the large gap (table 3). Whereas no difference is found between 30°C and 37°C, a significant increase in marker yield can be observed when the heatshock temperature is raised to 45°C. In this case, the marker yield
is comparable to those achieved with an additional enzymatic in vitro gap-filling/sealing reaction. This increase seems rather surprising at first sight, especially since one would expect decreased hybrid stability at higher temperature. Thermal deactivation of intracellular nucleases is one possible explanation of the phenomenon.

**DISCUSSION**

Direct co-transfection of *E. coli* with a synthetic oligonucleotide and gapped duplex DNA of a recombinant phage M13 genome can be used to introduce structurally pre-determined mutations into that phage genome at frequencies in excess of 50%. This marker yield is comparable to that achieved with similar procedures encompassing in vitro gap filling/sealing reactions (see Kramer et al.). Thus, the most obvious advantages of the newly developed method lie in its experimental simplicity and the fact that a notorious source of variability is eliminated (i.e. the quality variations of commercial DNA polymerases; Baas et al. and this laboratory, unpublished observations). The model experiment that was subject of this study necessitated preparative purification of the double stranded DNA fragment of M13mp9revam16. Note that this step is not required in the usual situation, in which an exogenous DNA fragment is inserted into the poly-linker region of M13mp9 (compare figure 1).

The mix-heat-transfect protocol also abolishes another source of unwanted background: in vitro "polishing" of protruding ends of the double stranded linear DNA by Klenow DNA polymerase, followed by ligation. For two independent reasons the mix-heat-transfect procedure may, in addition, be less prone to introduce additional, hence unwanted mutations: First, inside the living cell, the gap-filling reaction may well be more accurate than one that is carried out in vitro with purified DNA polymerase. Second, during the heat denaturation step of gdDNA preparation, hydrolytic cytosine deamination may occur at random sites in the DNA molecules that make up the reaction mixture. The resulting DNA lesion (a 2'-deoxy-uridine residue) is pre-mutagenic and a C/G to T/A transition is fixed by the in vitro DNA polymerase reaction if such a lesion occurred within the single stranded region of the gdDNA. In vivo, however, a DNA molecule carrying such a lesion within the gap would be expected to be cleaved by the combined action of the Ung-glycosylase and an apurinic/apyrimidinic site endonuclease such as *E. coli* endonuclease VII. Such a DNA molecule would thus lose its transfecting activity and hence not be able to produce progeny carrying the unwanted additional mutation.

| Table 3: Influence of heatshock temperature on marker yield |
|-----------------|---|---|---|
| temperature     | y | Y | N  |
| 30°C            | 41% | 41% | 4.5 |
| 37°C            | 40% | 40% | 4.5 |
| 45°C            | 53% | 53% | 3  |

Oligonucleotide: 28C-IIa (see figure 3B). gdDNA: 1640 nucleotides gap. For explanation of symbols see legend to table 1.
The mix-heat-transfect protocol requires oligonucleotides of greater chain length than the conventional method and the presence of two phosphorothioate internucleotidic linkages, though not strictly necessary, has a distinct beneficial effect on the marker yield. Thus, the new method is at present somewhat more demanding with respect to organic synthesis. For oligonucleotide purification, polyacrylamide gel electrophoresis is most appropriate since as far as can be deduced from our experience, the different stereoisomers of a given phosphorothioate-containing oligonucleotide comigrate in a sharp band. This simplifies the isolation of the product; note that separation of stereoisomers is of no importance if two internucleotidic phosphorothioate functions are introduced immediately adjacent to the 5'-terminus of the oligonucleotide.

Preparation of gdDNA and annealing of the mutagenic oligonucleotide has routinely been carried out in two consecutive steps with the idea in mind to use the viral minus DNA strand to cover all potential secondary hybridization sites within the phage genome before the oligonucleotide is added and thus to suppress side reactions. If, however, a computer-assisted sequence comparison does not suggest a very strong such secondary hybridisation site, the oligonucleotide may already be added to the starting denaturation/renaturation mixture. Evidence has been brought to our attention which suggests that this variant of the procedure is successful in overcoming problems of insufficient oligonucleotide hybridisation to a target region which forms part of the stem in a hairpin DNA structure. (M. Szardenings et al., Gesellschaft für Biotechnologische Forschung, Braunschweig; manuscript in preparation). Alternative ways to deal with this and other problems are discussed in detail elsewhere28.

The results presented here confirm and extend our previous observations with the mix-heat-transfect procedure5. Recently a direct co-transformation procedure has also been described for use with yeast as the host organism29. Ott and Eckstein30 have studied the in vitro protection of oligonucleotide primers against degradation by DNA polymerase I. In contrast to the results reported here, these authors find little or no effect associated with a phosphorothioate function present at the first internucleotide position from the 5'-end. This discrepancy seems to suggest that the unknown sequence of in vivo reactions acting on the heteroduplex DNA in the mix-heat-transfect procedure is not closely reflected by an in vitro assay employing purified DNA polymerase I and DNA ligase.

In addition to the model experiment reported here, the mix-heat-transfect protocol has been used in a variety of different mutation constructions including adaptation of the method to its use with different cloning vectors (this laboratory, unpublished).

ACKNOWLEDGEMENT

This work was supported by the German Bundesminister für Forschung und Technologie through Schwerpunkt "Grundlagen und Anwendungen der Gentechnologie".

*To whom correspondence should be addressed

‡ Present address: Dr. Müller-Lierheim AG, Behringstraße 6, D-8033 Planegg / München, FRG

§ Present address: University of California, Department of Genetics, 345 Mulford Hall Berkeley, CA 94720, U.S.A
ABBREVIATIONS
cccDNA: covalently closed circular DNA; EDTA: ethylenediaminetetraacetic acid; gdDNA: gapped duplex DNA; h.p.l.c: high pressure liquid chromatography; RF-DNA: replicative form DNA; ssDNA: single stranded DNA; UV: ultraviolet light; x-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

REFERENCES
2. Leatherbarrow, R.J. and Fersht, A.R. (1986) Protein Engineering 1, 7-16