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The gapped duplex DNA approach to oligonucleotide-directed mutation construction

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ABSTRACT

A simple and efficient method is described to introduce structurally pre-determined mutations into recombinant genomes of filamentous phage M13. The method rests on gapped duplex DNA (gdDNA) molecules of the phage M13 genome as the key intermediate. In this gdDNA, the (+) and the (shorter) (-)strand carry different genetic markers in such a way, that a rigorous selection can be applied for phage carrying the markers of the (-)strand. For introduction of the mutation, a synthetic oligonucleotide with partial homology to a target site within the single stranded DNA region is annealed to the gdDNA. The oligonucleotide subsequently becomes part of the (-)strand by enzymatic DNA gap filling and sealing. This physical linkage is preserved at the genetic level after transfection of a recipient E.coli strain deficient in DNA mismatch correction, so that the synthetic marker can be selected from the phage progeny independent from its potential phenotype. It is demonstrated that by this method mutants can be constructed with marker yields in excess of 70%.

INTRODUCTION

In recent years, oligonucleotide-directed mutation construction has become an important tool in molecular genetics since it allows structurally pre-determined mutations to be obtained at high frequency without any bias for a putative phenotype of the mutation. Elegant applications to enzyme engineering have been described (e.g., see Carter et al.[1] and literature cited there). The most frequently used method to date starts with annealing of a synthetic oligonucleotide to a target region on a single stranded recombinant genome of phage M13 with one or several mismatched nucleotides present in the DNA/DNA hybrid. The oligonucleotide is then elongated in vitro by DNA polymeraseI,large fragment and the resulting full-length duplex sealed with DNA ligase and enriched by sedimentation centrifugation through an alkaline sucrose gradient[2,3]. The isolated cova-

lently closed circular heteroduplex DNA is used to transfect recipient E.coli cells and mutant phage clones are produced principally as outlined in figure 1.

In a previous publication[4] we had pointed out three drawbacks of this procedure:

1. Potentially ambiguous primer annealing[5,6].
2. Unefficient cccDNA synthesis by DNA polymerase reaction "all the way round" (analogous to findings of Grosse and Krauss[7]).
3. Loss of synthetic marker by unfavourably oriented DNA mismatch correction acting in vivo on the transfecting DNA heteroduplex.

In an attempt to overcome all three difficulties by a single modification of the experimental procedure, we had demonstrated the utility of hemimethylated gapped duplex DNA molecules which have all the N^6-me adenine bases of GATC-sites residing in the (-)strand[4]. This last feature proved to be of special importance, since it forces the DNA mismatch repair system of the recipient cell to act in favour of the desired synthetic marker[8,9], which also becomes part of the (-)strand. Since our original suggestion, gdDNA has successfully been used for mutation construction in our laboratory[9] and others[10-12].

A recent systematic study carried out in our laboratory on DNA mismatch repair showed different types of base/base mismatches to be repaired with strikingly different efficiencies[9]. For mutation construction this means that by the approach we originally introduced, one will always prevent unfavourably oriented DNA mismatch repair to occur; the added advantage, however, of marker enrichment by forced DNA mismatch repair in the favourable direction can only be realized in cases of base/base mismatches with high repair efficiency. This finding prompted us to search for a more generally applicable method of obtaining pre-determined mutations in high marker yield. In the present paper we describe a method that is independent of repair efficiencies and rests on two essential features: 1. Physical linkage of the synthetic mutagenic oligonucleotide to a gdDNA (-) strand that carries selectable genetic markers not present in the (+)strand. 2. Conservation of this linkage at the genetic

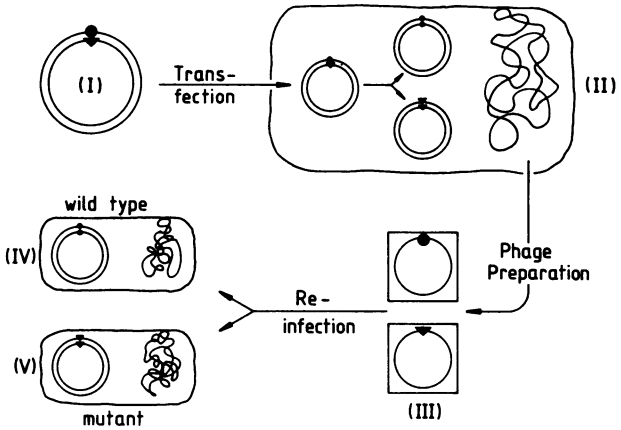

fig 1: General scheme of mutation construction using filamentous phage as the cloning vehicle.
An in vitro constructed heteroduplex DNA molecule (I) is used to transfect E.coli. The symbols ● and ▼ represent different genetic markers. For further explanations refer to text.

level by suppression of marker scrambling during transfection, i.e. by use of a transfection host that is deficient in DNA mismatch repair.

Ideas similar to the one underlying the first feature were expressed in literature before [11,13,14]. To our knowlegde, however, the necessity to suppress DNA mismatch repair in order to maintain marker linkage has not been realized previously.

For a statistically meaningful evaluation of the new method, a simple model experiment was chosen, that allowed convenient scoring of large numbers of phage clones exhibiting different plaque colour phenotypes.

MATERIALS AND METHODS

Bacterial strains: The following E.coli strains were used: BMH 71-18 (Δ(lac-proAB), thi, supE; F' laciq, ZΔM15, proA+B+) (source: B. Müller-Hill). MK 30-3 (Δ(lac-proAB), recA, galE, strA; F' laciq, ZΔM15, proA+B+) (source: M. Koenen). Construction of BMH 71-18mutS (BMH 71-18, mutS215::Tn10) was described[9].

Phage strains: The phages used were M13mp9[15] and M13mp9am16, which carries an amber-mutation in codon 16 (TGG) of the original lacZ-gene[16,17]. (This phage was derived from M13mp9 by

oligonucleotide-directed mutation construction in a procedure analogous to the one elaborated on here but via a hemimethylated gdDNA intermediate as described earlier[4,9].) Furthermore, M13mp9rev was used which has those two amber codons[18], that render propagation of M13mp9 dependent on the presence of a host-encoded suppressor, reverted to sense codons. For construction of this phage, ssDNA of M13mp9 was mixed with the HaeIII restriction fragments of M13mp2Z1[4] RF-DNA (molar ratio 6.5:1); the mixture was denatured and renatured (see below), then used for transfection of strain MK 30-3 (su^-) and plated on IPTG/x-gal indicator plates. Blue plaques were picked and the presence of the polylinker region of M13mp9 in these phages was confirmed by DNA restriction analysis. (Concomitantly with reversion of the amber codons M13mp9rev has gained a second HincII-site, most likely the original HincII-site of the phage M13 genome[19].)

Enzymes and chemicals: All enzymes (unless otherwise indicated) were purchased from Boehringer as well as deoxynucleotide triphosphates, ATP and x-gal. IPTG was from Serva. For agarose gels "Seakem-Agarose" from Marine Colloids was used, for polyacrylamide gels premixed "Acrylamide/BIS" 29:1 or 19:1 from BioRad. Microbiological growth media were purchased from Difco, salts and buffers from either Merck or Serva.

Synthesis of oligonucleotides: The two oligonucleotides d(pGGTTTTCCTAGTCACG) (for amber construction) and d(pGGTTTTCCCAGTCACG) (for reversion of the amber mutation) were synthesized by the phosphoramidite method[20] manually[21] or using a DNA synthesizer (Applied Biosystems). The oligonucleotides were purified by high pressure liquid chromatography[22] or preparative gel electrophoresis[23]. The oligonucleotides were enzymatically phosphorylated[9]. The extent of 5'-phosphorylation (≥97%) was determined by paired-ion chromatography[22]. Addition of 5'-terminal phosphate groups to the hexadecanucleotides lead to a pronounced shift to longer retention times on C_{18} stationary phase.

Media: Microbiological growth media were as described[4], but instead of 2YT, "Antibiotic Medium 3" from Difco and, in addition, minimal-medium was used (39mM Na_2HPO_4, 22mM KH_2PO_4, 19mM NH_4Cl; after autoclaving made up to 0.5% glucose, 0.1mM $CaCl_2$, 1mM $MgSO_4$, 0.5µM $FeCl_3$ and 5µg/ml thiamine).

Plating techniques: a) Titration of a phage suspension: Two drops of an overnight culture of the host strain and 100µl of phage suspension (various dilutions in 20mM sodium phosphate pH 7.2, 0.002% gelatine) were added to 2.5ml thoroughly melted standard top agar[4] of 45°C. The mixture was poured onto EHA[4] or IPTG[4] plates. In the latter case, 50µl 2% (w/v) x-gal solution in dimethylformamide were added in addition. The top agar was allowed to solidify for about 20min at room temperature. The plates were then incubated upside down at 37°C (at least 6h for EHA-plates, overnight for IPTG/x-gal indicator plates).
b) Determination of transfection efficiency: 20µl suspension of transfected cells (see "Transfection and segregation") were diluted in 180µl 100mM $CaCl_2$, 100µl of this suspension were added to the prepared top agar (see above) and plated as above. 20µl of the dilution were again diluted in 180µl $CaCl_2$ and plated. (If undiluted transfected cells were plated, no additional cells were added to the top agar).
Preparation of phage virion DNA: For preparation of M13 virion DNA (ssDNA), a modified procedure according to Heidecker et al.[24] was used. 200ml of "Antibiotic Medium 3" were simultaneously inoculated with 1ml overnight culture of strain BMH 71-18 and with phage (m.o.i. $\sim 10^2$), then shaken overnight at 37°C. The phages were precipitated essentially as described[24]. The final phage pellet was resuspended in 3ml 50mM NaCl, 10mM Tris-HCl, 1mM EDTA, pH 8.0 and extracted with a) phenol, b) phenol/chloroform 1:1 (v/v), c) chloroform and, d) three times with diethylether. The aqueous phase was adjusted to 0.3M sodium acetate and the DNA precipitated by addition of three volumes of ethanol and storage at -20°C for 1h. The yield, determined by UV-spectroscopy ($\varepsilon_{260} = 2.86 \times 10^{-2} cm^2/\mu g$), was typically about 300-1000µg ssDNA/200ml culture.
Preparation of RF-DNA: For the preparation of RF-DNA the "cleared lysate" technique of Clewell and Helinski[25] was adapted. 3x1l of "Antibiotic Medium 3" were each inoculated with 25ml overnight culture of strain BMH 71-18 and shaken at 37°C until an $O.D._{546}$ of 0.5-0.6 was reached. At this point, the cultures were infected with phage (m.o.i. of about 10^3) and shaken for another 3-4 hours. The cells were collected by centrifugation and resuspended at 0°C in 22.5ml 25% sucrose, 50mM

Tris-HCl, pH 8.0. Lysozyme (Millipore) was added (6ml, 5mg/ml in 50mM Tris-HCl, pH 8.0). After incubation at 0°C for 10min 7.5ml 250mM EDTA, pH 8, were added, and, after 10min at 0°C, 27.5ml 50mM Tris-HCl, 1% "Brij 58" (polyethyleneglykolmonostearylether, Serva), 0.4% sodiumdeoxycholate, 62.5mM EDTA, pH 8.0 (all volumes given per 3 liters of culture). The mixture was left at 0°C for another 10min. The lysate was then centrifuged (60min, 30,000rpm, rotor 30 (Beckman)). Per ml supernatant, 0.97g CsCl and 20µl ethidiumbromide solution (10mg/ml) were added. The mixture was centrifuged for at least 14h in a rotor VTi50 (Beckman) at 45,000rpm and the RF-DNA containing band in each tube was isolated and recentrifuged for at least 8h in a rotor VTi65 (Beckman) at 45,000rpm. Again, the RF-DNA containing band was saved, the CsCl was removed by dialysis against 1000 volumes 50mM NaCl, 10mM Tris-HCl, 1mM EDTA pH 8.0. The DNA solution was extracted three times each with phenol and diethylether and extensively dialysed (same buffer as above). The yield, determined by UV-spectroscopy (ε_{260}= 2 x $10^{-2}cm^2/\mu g$), was typically about 100-400µg RF-DNA/liter culture.

Preparation of the large EcoRI/PvuI-fragment of M13mp9rev DNA: RF-DNA of M13mp9rev was cleaved with EcoRI. This linear DNA was now cleaved with PvuI. Completeness of this second cleavage was tested by cutting an aliquot of this DNA with ClaI, which should yield only the 480bp PvuI/ClaI fragment and none of the 600bp EcoRI/ClaI fragment[15,16,17,19,26]. The large fragment was separated from the 120bp-fragment[15,16,17,26] by sedimentation through a linear sucrose gradient (10-30% sucrose in 100mM Tris-HCl, 10mM EDTA, 100mM NaCl, 20µg ethidium bromide/ml, pH 8.0; 30,000rpm, 15°C, 16h, rotor SW41 (Beckman); capacity: ca. 30µg DNA per centrifuge tube).

Construction of gapped duplex DNA (gdDNA): 0.1pmole (0.5µg) of the large EcoRI/PvuI-fragment of M13mp9rev were mixed with 0.5pmole (1.25µg) of virion DNA of either M13mp9 or M13mp9am16 in a total volume of 40µl of 187.5mM KCl/12.5mM Tris-HCl pH 7.5. The mixture was kept at 100°C for 3min and incubated at 65°C for 5min. Formation of gdDNA was checked by agarose gel electrophoresis of an aliquot of the DNA mixture (see figure 3).

Annealing of the mutagenic primer and DNA polymerase/DNA ligase reaction: 8µl hybridization mixture (containing 20fmole -100ng- of the input linear DNA) were mixed with 4pmole (2µl of aqueous solution) of 5'-phosphorylated oligonucleotide. This mixture was heated to 65°C for 3min and then allowed to cool to room temperature. The mixture was now adjusted to the following concentrations: 100mM KCl, 30mM Tris-HCl pH 7.5, 15mM $MgCl_2$, 2mM DTT, 50µM ATP and 25µM of each of the four dNTPs with 1u DNA polymeraseI,large fragment and 2u T4 DNA ligase added (final volume: 40µl). The mixture was incubated at room temperature for 45 minutes. The reaction was stopped by addition of 1µl 0.5M EDTA, pH 8 and heating to 65°C for 10min. The mixture was then extracted with 40µl phenol/chloroform (v:v/1:1) followed by three extractions with 100µl diethylether. Residual traces of ether were evaporated at 65°C and the mixture immediately used for transfection. In some experiments the enzymatic reactions in vitro were omitted.

Transfection and segregation: Competent cells were prepared according to Cohen et al.[27] with modifications. 0.5ml overnight culture of strain BMH 71-18mutS grown in minimal-medium containing 20µg/ml tetracycline was used to inoculate 50ml "Antibiotic medium 3". The culture was grown with shaking at 37°C to an $O.D._{546}$ of 0.4-0.6. The cells were collected by centrifugation, washed twice with about 20ml ice-cold 100mM $CaCl_2$, resuspended in 2ml 100mM $CaCl_2$ and kept on ice for 30min. 200µl of this cell suspension were added to the polymerase/ligase reaction mixture (or to the 10µl annealing mixture, respectively) that had been made up to 100µl with 100mM $CaCl_2$. The resulting mixture was kept on ice for 90min. After heating the suspension for 3min to 45°C (for annealing mixtures without enzyme treatment: 37°C), an aliquot of 20µl was plated (see paragraph "plating techniques") to determine the efficiency of transfection. The remaining 280µl were used to inoculate 25ml "Antibiotic medium 3" and cells were grown overnight with shaking at 37°C. The supernatant was isolated and phages were plated with strain MK 30-3 at an m.o.i. of about 10^{-5} on IPTG/x-gal indicator plates[4].

RESULTS AND DISCUSSION

The genetic assay

Filamentous phage of the M13mp family carries genetic information for an amino-terminal fragment of the β-galactosidase ("α-fragment")[16]; hence, this type of phage is able to complement host bacteria carrying the lacZΔM15 mutation to a LacZ$^+$ phenotype (α-complementation[16,28]). In previous studies[4,9] we had exploited point mutations within the phage-borne lacZ gene fragment in a genetic assay to follow the fate of heteroduplex DNA molecules during transfection of host bacteria. This generally applicable test is based on very easily discernable phenotypes (blue and colourless plaques, respectively, on IPTG/x-gal indicator plates[4]). This makes it possible to score conveniently large numbers of phage clones and thus to arrive at a sound statistical basis for the calculation of marker distributions.

Using a very similar genetic assay, we focussed in the present model study on codon 16 of the lacZ gene fragment of phage M13mp9 (TGG: trp; numbering derived from the wild type lacZ gene[17]). By means of a synthetic hexadecanucleotide, this codon was converted to TAG: amber, causing a change in plaque phenotype from blue to colourless. In a second experiment, this amber codon was reverted to TGG, also directed by a synthetic hexadecanucleotide. The two experiments are complementary but not equivalent: The structural requirements at the DNA level for reversion of the LacZ$^-$ phenotype to LacZ$^+$ are much more stringent than for the forward mutation; i.e. if a significantly higher efficiency of the latter process would be observed, this could be taken as an indication that concomitantly with the controlled mutation construction, other, unwanted DNA rearrangements occur (see below).

Preparation of gapped duplex DNA

Gapped duplex DNA (gdDNA) was prepared as schematically outlined in figure 2 and documented in figure 3. RF-DNA of phage M13mp9rev (figure 2, structure I; figure 3, lane 2; see "Materials and Methods" for description of phage) was cleaved with EcoRI and PvuI and the purified large DNA restriction fragment (figure 2, structure II; figure 3, lane 3) was mixed with an

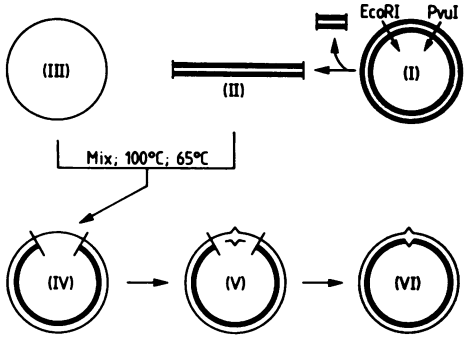

fig.2: Schematic outline of the in vitro construction of heteroduplex DNA.
RF-DNA (I) of M13mp9rev is cleaved with EcoRI and PvuI. The long restriction fragment (II) and ssDNA (III) of M13mp9 or M13mp9am16 are mixed. Denaturation/renaturation yields gapped duplex DNA (IV). Annealing the mutagenic primer leads to ternary DNA duplex (V). The remaining gaps are filled and sealed in a DNA polymerase/DNA ligase catalysed reaction to yield fully double stranded heteroduplex DNA (VI).

excess of single stranded virion DNA of either M13mp9 or M13mp9am16 (figure 2, structure III; figure 3, lane 4; for description of phages see "Materials and Methods"). This DNA mixture (figure 3, lane 5), was thermally denatured and renatured (total reaction time: 10min), which resulted in the formation of gapped duplex DNA molecules (figure 2, structure IV)[29]. This DNA species, besides remaining ssDNA and reformed double stranded linear DNA, can be seen in figure 3, lane 6 as the band with the lowest electrophoretic mobility[29]. The product gdDNA has the following characteristics:

1. The (-)strand contains a precisely defined gap of 117 nucleotides[15,16,17,26] spanning from codon 5 to codon 45 of the (original) lacZ gene, thus exposing the target region of mutation construction (codon 16) in single-stranded form.
2. The gapped (-)strand of the gdDNA (derived from M13mp9rev) contains wild type codons at two places where amber mutations are present in the (+)strand. These mutations render propagation of M13mp9 dependent on the presence of a host encoded suppressor. They had been introduced into the phage genome by Messing, Crea and Seeburg[18].

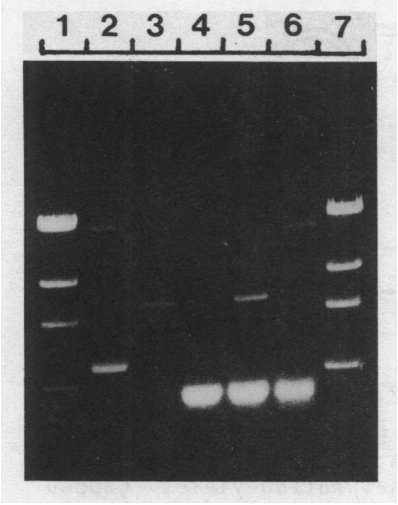

fig. 3: Gel electrophoretic analysis of different intermediates in the construction of gdDNA. lane 1 and 7: λ DNA, digested with HindIII; lane 2: RF-DNA of M13mp9rev; lane 3: RF-DNA of M13mp9rev digested with EcoRI and PvuI; lane 4: ssDNA of M13mp9; lane 5: M13mp9rev, large EcoRI/PvuI fragment and ssDNA of M13mp9; lane 6: same DNAs as in lane 5 but after thermal denaturation/renaturation.

Annealing of synthetic oligonucleotide and in vitro gap-filling reaction

The crude hybridization mixtures (see figure 3, lane 6) were used for annealing reactions with 40 equivalents (based on input ssDNA) in each case of 5'-phosphorylated oligonucleotides. The hexadecanucleotide d(pGGTTTTCCTAGTCACG) was used with the hybridization mixture containing the ssDNA of M13mp9 and d(pGGTTTTCCCAGTCACG) with the mixture containing the ssDNA of M13mp9am16 (mismatched nucleotides underlined). Two products are expected from primer annealing: A ternary duplex as depicted in figure 2, structure V and a binary duplex consisting of single stranded virion DNA and oligonucleotide (not shown). Products of the annealing reactions were then subjected to gap-filling and sealing with DNA polymeraseI,large fragment and T4-DNA ligase. The reaction time can be kept short (45min), since only two small DNA gaps have to be filled in. Starting from ternary duplex DNA, the oligonucleotide has at this point become an integral part of the (-)strand, which is derived from M13mp9rev, containing two selectable wild type codons (figure 2, structure VI).

In a second set of experiments, the annealing mixtures were used directly for transfection (see below) without any enzymatic in vitro manipulations.

Transfection, segregation and determination of marker yield

The DNA mixtures resulting from the previous step were used to transfect strain BMH 71-18mutS which is deficient in DNA mismatch repair[9,30]. Exclusion of DNA mismatch repair is an essential part of the procedure for the following reasons: The crucial component of the transfecting DNA mixture is fully double stranded heteroduplex DNA derived from input gdDNA. This heteroduplex DNA contains (at least) four mismatched positions: The target site of mutation construction, the two sites where amber codons in the (+)strand are juxtaposed to wild type codons in the (-)strand, and the additional HincII recognition site of the (-)strand (compare "Materials and Methods").

Upon transfection, the DNA mismatch repair system of the recipient cell could act on any of these base/base mismatches and correct them individually, thus providing for efficient marker scrambling.

While the mismatch at the additional HincII site of the (-) strand has no relevance for the genetics of the experiment, it is of utmost importance to preserve the linkage between the synthetic marker and the two selectable wild type codons of the (-)strand. Transfection of a host bacterium deficient in DNA mismatch repair with fully double stranded heteroduplex DNA leads to a mixed burst[9] (compare figure 1) under preservation of marker linkage. In addition, the mixed phage population produced as the result of thousands of initial transfection events (compare table 1) plus subsequent phage propagation contains progeny of the excess single stranded virion DNA present in the transfecting DNA mixture. This part of the population is genotypically characterized by the presence of two amber codons in vital phage genes.

Complete marker segregation was achieved by using the mixed phage population for re-infection of host bacteria at very low multiplicity[4]. This was done with strain MK 30-3 as the recipient. This strain is su^- and therefore not able to propagate phage M13mp9. This eliminates the progeny of all virion DNA irrespective of whether it was part of a gdDNA molecule or not. Remaining double stranded linears of M13mp9rev were lost already in the transfection step. (The molar transfection efficiency of

Table 1: Marker yields of mutation construction experiments

Experiment number		Plaque score colour blue	less	Yield of synthetic marker (%)	Average marker yield (%)
A	1	252	694	73	76
	2	285	885	76	
	3	254	962	79	
B	1	572	282	67	70
	2	989	377	72	
	3	970	392	71	
a	1	758	202	21	24
	2	867	324	27	
b	1	357	1451	20	20
	2	207	812	20	

A,a: Starting phage: M13mp9; plaque phenotype of the synthetic marker: colourless. B,b: Starting phage: M13mp9am16; plaque phenotype of the synthetic marker: blue. A,B: Experiments including polymerase/ligase reactions in vitro. a,b: Experiments without polymerase/ligase reactions in vitro.

Individual experiments listed were carried out with independent preparations of gdDNA. At least 5000 primary transfectants were obtained in each individual experiment. In separate experiments (data not shown) it was demonstrated that at least 40% of the transfectants are due to gdDNA.

the double stranded DNA linear is about 200 times lower than that of the final DNA mixture; data not shown.) Thus, the two-stage procedure applies two successive selections and any phage surviving the re-infection step must be a descendent of the (-) strand of a gdDNA molecule. This is why no purification of gdDNA from the crude hybridization mixture is required.

Table 1 shows under A and B the results of the experiments. Re-infection mixtures were plated on IPTG/x-gal indicator plates for analysis of the marker distribution. The expected enrichment for the synthetic marker is demonstrated by marker yields of 70% or more for both experiments.

Under a and b in table 1, the results of experiments using the crude annealing mixture without enzymatic treatment in vitro for transfection of strain BMH 71-18mutS are given. Marker yields of about 20% are obtained. This approach may offer a biochemically even simpler option for experiments without strong requirements for high marker yields. Additionally, the enzymatic DNA manipu-

lations may have some disadvantages due to the potentially decreased fidelity of the DNA polymerase reaction in vitro. Indeed, in one intance we have observed an additional mutation within the region of the original DNA gap but outside the synthetic oligonucleotide sequence (B. Kramer, unpublished observation).

Why does the marker yield achieved fall short of the theoretical value of 100%? We think there are two reasons to this fact, a biochemical and a biological one:

1. The gap-filling and sealing reaction is not perfect. A significant percentage of the mutagenic primer annealed to the DNA gap seems to be lost by peeling off in a strand displacment reaction after the growing 3'-terminus of the (-)strand (i.e. of the original EcoRI/PvuI fragment) has reached its 5'-end (W. Kramer, unpublished experiments). Furthermore, a nick remaining at the 5'-end of the mutagenic primer can lead to marker loss by nick-translation in vivo.
2. The host bacterium, BMH 71-18mutS, is over-proficient in genetic recombination ("hyper-rec")[31]. Since transfection of this strain by heteroduplex M13 DNA leads to a mixed burst (see above), some marker scrambling due to homologous recombination is expected. No attempt was made yet to quantitate this effect and - if necessary - to suppress it.

CONCLUSION

We have devised a method of oligonucleotide-directed mutation construction that leads to marker yields of 70% or more as judged from a simple and realistic model experiment. Two crucial features of the method are:

1. A gapped duplex DNA intermediate of the phage M13 genome built from two DNA strands differing in selectable genetic markers.
2. Use of an E.coli strain that is deficient in DNA mismatch repair as a transfection recipient for the heteroduplex DNA.

Besides marker enrichment, the method has the additional advantage of simplifying in vitro DNA manipulations. Previously published procedures necessitated sedimentation centrifugation

through an alkaline sucrose gradient[3], column chromatography on hydroxylapatite[4] or preparative agarose gel electrophoresis[9] for purification of intermediates in heteroduplex DNA construction. Such purification steps can now be bypassed by the selection used which results in significant savings of material, time and effort. Furthermore, even transfection with the annealing mixture (gdDNA hybridisation mixture plus mutagenic oligonucleotide) without any enzymatic DNA manipulation in vitro still results in marker yields of 20%.

To introduce mutations into DNA cloned in M13mp9, the gdDNA can be prepared from ssDNA of the recombinant M13mp9 and RF-DNA of M13mp9rev cleaved with the restriction enzyme(s) used for cloning the DNA fragment. In this case, no purification of the ds linear DNA is necessary.

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ABBREVIATIONS

cccDNA: covalently closed circular DNA; dNTPs: deoxyribonucleotide triphosphates; gdDNA: gapped duplex DNA; IPTG: Isopropyl-thio-β-D-galactopyranoside; m.o.i.: multiplicity of infection; O.D.: optical density; RF-DNA: replicative form DNA; ssDNA: single stranded DNA; x-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

REFERENCES

1. Carter, P.J., Winter, G., Wilkinson, A.J. and Fersht, A.R. (1984) Cell 38, 835-840
2. Zoller, M.J. and Smith, M. (1982) Nucl. Acids Res. 10, 6487-6500
3. Zoller, M.J. and Smith, M. (1983) Methods in Enzymology 100, 468-500
4. Kramer, W., Schughart, K. and Fritz, H.-J. (1982) Nucl. Acids Res. 10, 6475-6485
5. Baas, P.D., Teertstra, W.R., Van Mansfeld, A.D.M., Jansz, H.S., Van der Marel, G.A., Veeneman, G.H. and van Boom, J.H. (1981) J. Mol. Biol. 152, 615-639
6. Osinga, K.A., van der Bliek, A.M., van der Horst, G., Groot Koerkamp, M.J.A., Tabak, H.F., Veeneman, G.H. and van Boom, J.H. (1983) Nucl. Acids Res. 11, 8595-8608
7. Grosse, F. and Krauss, G. (1984) Eur. J. Biochem. 141, 109-114
8. Pukkila, P.J., Peterson, J., Herman, G., Modrich, P. and Meselson, M. (1983) Genetics 104, 571-582
9. Kramer, B., Kramer, W. and Fritz, H.-J. (1984) Cell 38, 879-887
10. Marmenout, A., Remaut, E., van Boom, J. and Fiers, W. (1984) Mol. Gen. Genet. 195, 126-133
11. Hirose, S., Takeuchi, K., Hori, H., Hirose, T., Inayama, S. and Suzuki, Y. (1984) Proc. Natl. Acad. Sci. USA 81, 1394-1397
12. Kozak, M. (1984) Nature 308, 241-246
13. Traboni, C., Cortese, R., Ciliberto, G. and Cesareni, G. (1983) Nucl. Acids Res. 11, 4229-4239
14. Messing, J. (1983) Methods in Enzymology 101, 20-78
15. Messing, J. and Vieira, J. (1982) Gene 19, 269-276
16. Messing, J., Gronenborn, B., Müller-Hill, B. and Hofschneider, P.H. (1977) Proc. Natl. Acad. Sci. USA 74, 3642-3646
17. Kalnins, A., Otto, K., Rüther, U. and Müller-Hill, B. (1983) EMBO J. 2, 593-597
18. Messing, J., Crea, R. and Seeburg, P.H. (1981) Nucl. Acids Res. 9, 309-321
19. van Wezenbeek, P.M.G.F., Hulsebos, T.J.M. and Schoenmakers, J.G.G. (1980) Gene 11, 129-148
20. Mateucci, M.D. and Caruthers, M.H. (1981) J. Am. Chem. Soc. 103, 3185-3191
21. Winnacker, E.-L. and Dörper, T. (1982) in Chemical and enzymatic synthesis of gene fragments, Gassen, H.G. and Lang, A. Eds., pp. 97-102, Verlag Chemie, Weinheim
22. Fritz, H.-J., Eick, D. and Werr, W. (1982) in Chemical and enzymatic synthesis of gene fragments, Gassen, H.G. and Lang, A. Eds., pp 199-223, Verlag Chemie, Weinheim
23. Gait, M.J., Matthes, H.W.D., Singh, M., Sproat, B.S. and Titmas, R.C. in Chemical and enzymatic synthesis of gene fragments, Gassen, H.G. and Lang, A. Eds., pp 1-42, Verlag Chemie, Weinheim
24. Heidecker, G., Messing, J. and Gronenborn, B. (1980) Gene 10, 69-73
25. Clewell, D.B. and Helinski, D.R. (1969) Proc. Natl. Acad. Sci. USA 62, 1159-1166
26. Gronenborn, B. and Messing, J. (1978) Nature 272, 375-377

27. Cohen, S.N., Chang, A.C.Y. and Hsu, L. (1972) Proc. Natl. Acad. Sci. USA 69, 2110-2114
28. Langley, K.E., Villarejo, M.R., Fowler, A.V., Zamenhof, P.J. Zabin, I. (1975) Proc. Natl. Acad. Sci. USA 72, 1254-1257
29. Courage-Tebbe, U. and Kemper, B. (1982) Biochim. Biophys. Acta 697, 1-5
30. Siegel, E. C. and Kamel, F. (1974) J. Bact. 117, 994-1001
31. Glickman, B.W. and Radman, M. (1980) Proc. Natl. Acad. Sci. USA 77, 1063-1067