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A general method to select for M13 clones carrying base pair substitution mutants constructed *in vitro*

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ABSTRACT

In this paper we describe a method to select base pair substitution mutants constructed in vitro. The mutagenesis is performed by forcing mistakes during in vitro synthesis from a primer annealed to a single stranded DNA template. The selection is based on the fact that, following transformation, the progeny of the strand elongated in vitro and the template strand have different phenotypes. The method is general and applicable to any DNA segment; the type of base pair substitution and its position can be chosen at will. The combined efficiency of mutagenesis and selection allows for 85% frequency of mutants in all analyzed clones.

INTRODUCTION

In a previous publication (1) we have described a method to select mutants carrying insertions and deletions in any segment of DNA cloned in the mp family of M13 derivatives vectors (2). The method is based on the selection of insertions and deletions capable of restoring the correct translational frame of the beta-galactosidase alpha peptide, which had been shifted by the insertion of the DNA segment to be mutagenized. For most purposes however, in addition to deletions and insertions, it is desirable to construct in vitro base pair substitution mutants. In this paper we present a method to construct in vitro and select in vivo base pair substitution mutants at practically any preselected site. Our procedure relies upon the same principle of selection which we have previously described (1). Mutations are generated by mispairing induced with reverse transcriptase in conditions of "infinite bias pool" (3).

MATERIALS AND METHODS

Chemicals. Isopropyl-beta-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-galactoside (BCIG) were purchased from Sigma. Deoxyribonucleoside triphosphates were purchased from PL Biochemicals or Sigma. Dideoxynucleotides and sequencing primers were purchased from Bethesda

Research Laboratories. DNA polymerase Klenow fragment was from Boehringer Mannheim. Reverse transcriptase was obtained from Dr. Beard. Restriction endonucleases were purchased from Bethesda Research Laboratories. P32-deoxy-ATP was purchased from Amersham.

Bacterial strains and cloning vectors. E.coli K12 (strain 71-18) was used for transformation with M13 derivative recombinants (2). Dam- E.coli strain GM99-F2, used for the preparation of demethylated single stranded DNA template was a gift of Dr. H. Fritz and its use has been described (4). For experiments with B78R (1) it was necessary to introduce an amber suppressor in strain GM99-F2: this was done by lysogenization with Phi 80 psuIII. Transformation was done as described (5).

Single stranded DNA preparation. Isolated phage plaques were toothpicked and inoculated in 1.5 ml of L- broth containing 5 x 10^7 cells/ml and incubated for 6 hours at 37°C; bacteria were spun down and 800 microliters of the phage supernatant was precipitated (15 min at room temperature) in the presence of 200 microliters of 20% polyethylen glycol, 2.5 M NaCl, and then centrifuged in an Eppendorf microfuge for 5 min. The pellet was resuspended in 100 microliters of 10 mM Tris, pH 8, 1 mM EDTA and extracted with an equal volume of phenol saturated with 100 mM Tris, pH 9, 1 mM EDTA. The DNA was precipitated from the acqueous phase by addition of 20 microliters of 5 M sodium perchlorate and 50 microliters of isopropanol, and centrifuged for 15 min in an Eppendorf microfuge. The pellet was resuspended in 100 microliters of 0.3 M ammonium acetate and precipitated with three volumes of ethanol. After 30 min at -80°C, the DNA was spun down and resuspended in 10 mM Tris, pH 8, 0.1 mM EDTA.

Annealing conditions. 20 microliters containing 50 mM NaCl, 6.6 mM $MgCl_2$, 6.6 mM Tris pH 8, 6.6 mM beta-mercaptoethanol, 2 micrograms of single stranded DNA template and 5 fold molar excess of double stranded primer were sealed in a siliconized capillary tube, boiled for three minutes in a water bath and then slowly cooled at room temperature.

Modification of the length of the primer. 5 microliter aliquots (containing about 0.5 micrograms of template DNA) of the annealed reaction mixture were incubated with 0.5 units of DNA polymerase, Klenow fragment, in annealing buffer, containing each desired deoxynucleotide at a concentration of 50 uM, in a final volume of 20 microliters. Incubation was performed at room temperature for 15 min or 60 min for lengthening or shortening of the primer respectively. At the end of the reaction, DNA polymerase was inactivated for 20 min at 75°C, and deoxynucleotides were removed by gel filtration on

Sephadex G- 50 column.

Mispairing reaction. 0.5 micrograms of the primer-template annealed complex were incubated with 6 units of reverse transcriptase in 40 mM Tris, pH 8, 40 mM MgCl, 1.6 mM dithiothreitol, 0.5 mM of each desired nucleotide, in a final volume of 50 microliters, for 1 hour at 37°C. 0.5 mM of all four deoxynucleotides were then added, and incubation was continued for another hour. At the end of the reaction deoxynucleotides were removed by gel filtration.

DNA sequence. DNA sequence was performed using the dideoxy chain termination method (6).

RESULTS

Borrias et al. (7) have shown that it is possible to select wild-type Phi X phage particles following transformation of E.coli with single stranded Phi X DNA carrying an amber mutation, annealed with a short Phi X DNA segment containing the corresponding wild-type sequence; inside the cell the primer is elongated so as to complete the complementary strand. The resulting double stranded DNA is replicated, yielding a progeny of wild-type and mutant viruses. A short DNA segment annealed to the template can be used as primer for DNA synthesis in vitro, using a DNA polymerase. During the elongation in vitro, a mispairing reaction can occur, if the correct nucleotide is not available. Furthermore this mistake cannot be corrected if the polymerase used, for instance reverse transcriptase, lacks a 3' exonuclease proof reading activity. Zakour and Loeb (3) have used this mispairing reaction to synthesize a wild type Phi X strand on to a template carrying an amber mutation. In their experiment the 3' OH end of the primer was adjacent to the amber triplet present in the template sequence. By omitting the correct nucleotide from the reaction mixture, it was possible to force a mispairing reaction such that the amber triplet was incorrectly copied into a sense triplet. After transformation it was possible to select for wild type phage particles: the overall yield of revertants was rather low (3-4%), suggesting that this method cannot be used to construct mutants without a selectable marker. Zakour and Loeb (3) mention the possibility to enrich for mutants by completing the in vitro synthesis of the complementary strand and subsequent treatment with S1 nuclease.

A selectable phenotype can however depend on the sequence of the primer used, as in the case of Borrias et al. (7), and not generated by the mispairing reaction. In this way the selectable marker is associated to the

strand synthesized in vitro from the primer, and therefore linked to any mistake made during in vitro elongation. A general protocol to introduce base pair substitutions in any DNA segment and to recognize the mutant phenotype is thus the following: i) the wild-type DNA segment to be mutagenized is cloned in a single stranded mp phage vector, within the coding sequence of beta-galactosidase alpha peptide, so as to shift the frame and give white plaques; ii) the wild-type single stranded DNA is annealed with a short DNA segment carrying insertions or deletions of one or two bases so as to restore the beta-galactosidase frame and ending at its 3' OH in the proximity of the region where base pair substitutions are to be introduced. It is important to realize that the frame restoring insertions or deletions can be outside the gene to be mutagenized, and conveniently placed in the adjacent vector sequences. iii) The primer is elongated in the presence of reverse transcriptase and a subset of deoxynucleoside triphosphates to force the incorporation of a mispairing nucleotide at the desired position. A full complement of deoxynucleoside triphosphates is then added to continue elongation. The annealed complex is then introduced in E.coli by transformation: the progeny of the template strand and the primed strand will yield white and blue plaques respectively. Blue plaques should contain the desired mutations. An outline of this procedure is shown in fig. 1.

Isolation of base pair substitution mutants in a tRNApro gene

The procedure described in the preceding paragraph was used to mutagenize the coding region of the tRNApro gene isolated from C. elegans (1,8,9,10). The tRNApro coding region was cloned either as a 74 bp EcoRI-EcoRI DNA segment into the vector mp2, to give the recombinant W.S., or as an 83 bp EcoRI-BamHI DNA segment into the vector mp701 to give the recombinant B78R (1). Both recombinants form white plaques. From plasmid W.S. a new plasmid, DN3, was constructed (1), carrying a single base pair insertion within the region coding for the anticodon of tRNApro and forming blue plaques. From plasmid B78R a new plasmid was constructed, Bo78R, by filling in the BamHI site, which resulted in the insertion of four bases in the polylinker sequence flanking the tRNApro gene. Bo78R forms blue plaques.

W.S. or B78R single stranded circular DNA were used as templates and short DNA segments from DN3 or Bo78R were used as primers. We were interested in isolating mutants in regions of the tRNApro gene which had been shown to play an important role in transcription. One of the components of the internal promoter, the box B (10,11,12) is approximately comprised between nucleotide 50 and nucleotide 60 of the tRNApro coding sequence. For this purpose

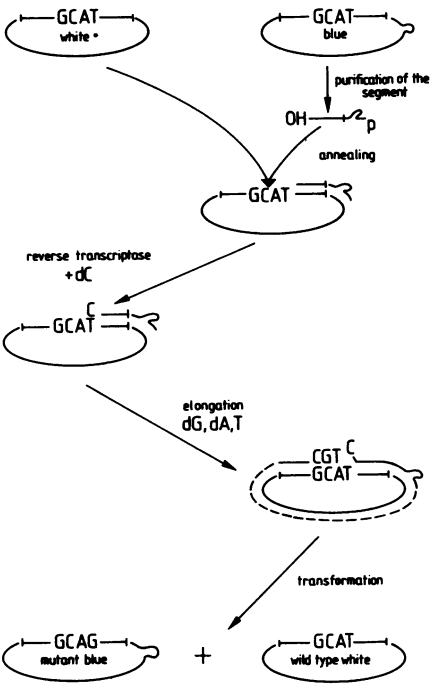

Fig. 1: Outline of the site directed mutagenesis method described in this paper. Circles are single stranded DNA from M13 recombinants. The segment between slashes represents the DNA insert; the sequence GCAT is an arbitrary sequence, exemplifying a preselected target for mutagenesis. The small loop on the blue circle represents the frame restoring insertion, located outside the insert, in the vector sequence.

(fig. 2a) we used as template W.S. DNA and as primer a 53 bp EcoRI-SmaI segment ending at its 3' terminus at nucleotide 50. Another region of interest was the anticodon stem: to mutagenize this region we used as template B78R DNA and as primer the 39 bp PstI-HinfI segment ending at its 3' OH terminus at nucleotide 23 (fig. 2b). These combinations of primer and template allow the distinction between the progeny derived from the template (white plaques) and the progeny derived from the primer (blue plaques). The reverse transcriptase induced mispairing reactionswere carried out in the presence of various combinations of nucleotides. A number of blue plaques were analyzed by DNA sequencing and several base pair substitution mutants were found, as indicated in table I. In all cases the predicted misincorporations were found, even though sometimes unexpected mutations were also found.

In order to direct the error of the reverse transcriptase it is

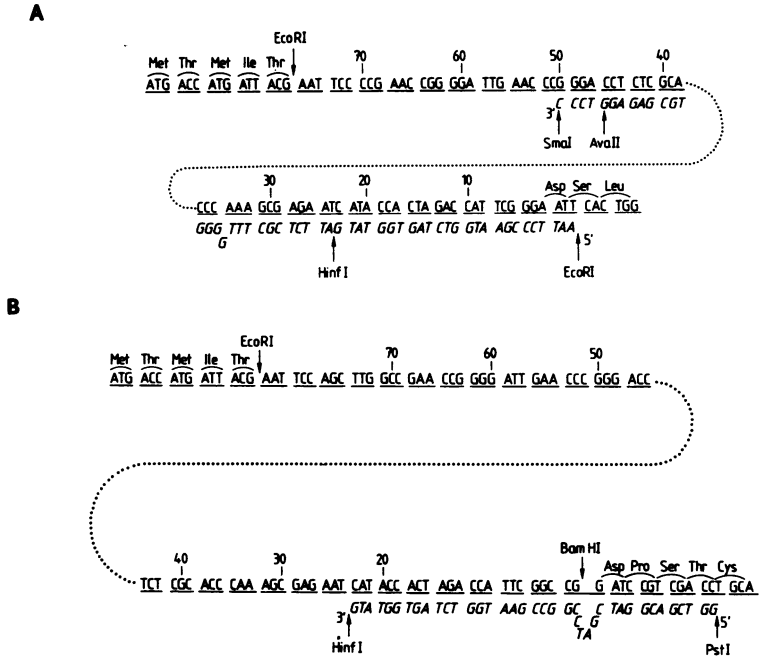

Fig. 2: The coding region of tRNApro gene, cloned as an EcoRI-EcoRI segment, in plasmid W.S. (panel a) and as an EcoRI-BamHI segment in plasmid B78R (panel b) (1), showing the combinations of primer and template used in most of our experiments. Nucleotides belonging to the beta-galactosidase gene are grouped with arcs in triplets representing the correct codons for the aminoacids indicated above them. Under them, and all along the inserted DNA, nucleotides are grouped with straight lines in triplets representing the shifted codons.

necessary to make an opportune choice of the deoxynucleotide mixture. The primer however, deriving usually from restriction enzymes digests, not always has its 3'OH end in perfect position for the mispairing reaction. In this case it is necessary to modify it by lengthening or shortening. An example of this situation is shown in fig. 3. We wished to substitute the G residue in position 60 with an A residue. The presence of a G residue at position 56, between the 3'OH end of the primer (position 50) and the pre-selected site, made this substitution difficult. We therefore incubated the primer-template complex with E.coli DNA polymerase Klenow fragment and a mixture of dG, dC and T to add six bases to the primer so as to pass the G56 residue. The Klenow was then inactivated at 75°C for 20 min and the unincorporated nucleotides were removed by gel filtration. At this point the 3'OH end of the primer was complementary to nucleotide G56 and, in order to

TABLE I

Exp. No.	primer used	combination of nucleotides	predicted mutations	no. of clones analysed	mutations obtained
1	EcoRI-SmaI	dG	G54	4	2x G54 1x G57 1 wild-type
2	EcoRI-SmaI	dG+T	T56, G56	8	6x T56 1x T56 G57 1x wild-type
3	EcoRI-SmaI	T+dC	T51, T51	10	3x T51 1x T53 C55 4x C57 2x T57 T58
4	EcoRI-SmaI	dA,dG +T*	A55,G55 A56,G56	4	2x A55 2x A56
5	PstI-HinfI	dA+T	T27,A27	1	1x T26
6	PstI-HinfI	dA,dC, T	A30,T30 C30	3	1x T30 1x A30 T36 1x wild-type

Site directed mutagenesis of tRNApro coding region. For these experiments EcoRI-Sma or PstI-HinfI segments were used as primers (see fig. 2), on W.S. or B78R respectively. The deoxynucleotides incubated in the presence of reverse transcriptase are indicated for each experiment in the third column. In the fourth column is indicated for each experiment the predicted base substitution on the strand elongated from the primer assuming only one mispair. In the fifth column are the total number of clones which were analyzed by DNA sequence. In the sixth column are the type and the number of mutants found, indicated as the substituting base on the strand elongated from the primer.

In experiment 1 the unpredicted G57 could be due to the presence of small amounts of dC and T in our dG preparation. In experiment 2 there is an example of double mispair. In experiment 3 the unpredicted mutations could be due to trace contaminations of dG in either dC or T. In experiment 6 the double mutant A30T36 could be due to trace contamination of dG in either dA or T.

* used at 0.05 µM

obtain the A60 mutation it was sufficient to add reverse transcriptase in the presence of dA and T to direct the misincorporation of either A or T in position 60. After 5 min the remaining complement of deoxynucleotides was added to continue DNA synthesis for 30 more minutes. Two blue plaques were analyzed by DNA sequencing and in both cases the desired mutation was present. The opposite case of the necessity to shorten the primer is illustrated in

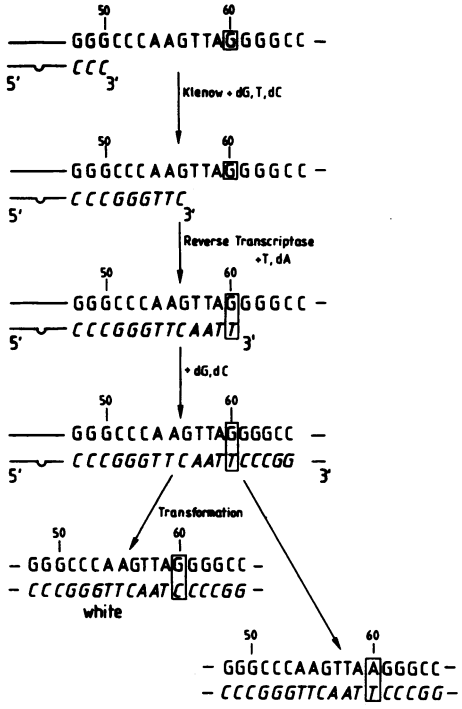

Fig. 3: Outline of an experiment in which the primer was lengthened before mutagenesis. The small loop indicates the insertion responsible for the blue phenotype of the primed strand.

the experiment shown in fig. 4. Here we wished to substitute residue T in position 42 with a C. The 3'OH end of the primer used in this case (an EcoRI-AvaII segment) was beyond the preselected site; we shortened it by incubating the primer-template complex with E.coli Klenow DNA polymerase and dC, exploiting the 3' exonuclease activity of the Klenow to remove five nucleotides from the primer. After inactivation of the Klenow and removal of unincorporated dC, we then added reverse transcriptase and dG to force misincorporation of G in position 42. After 5 min the other three deoxynucleotides were added and the reaction was allowed to continue for 30 more minutes. One blue plaque was analyzed by DNA sequencing and showed to contain the desired base pair substitution.

DISCUSSION

A variety of methods to construct site directed base pair mutants have been described. The general strategy of many of them is based either on the

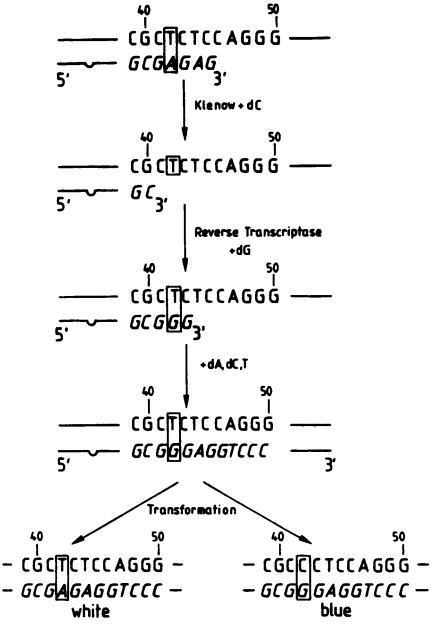

Fig. 4: Outline of an experiment in which the primer was shortened before mutagenesis. The small loop indicates the insertion responsible for the blue phenotype of the primed strand.

forced incorporation of the wrong nucleotide during DNA synthesis in vitro (3,13) or on the incorporation of base analogs, for instance OH-dC, with the potential for mispairing (14). Alternatively an oligodeoxynucleotide containing the desired sequence is chemically synthesized in vitro and then annealed to the region of DNA to be mutagenized, which must be in the single stranded form. Then the complementary strand is synthesized in vitro. Following transformation, some clones should contain the desired mutation (15).

In general the efficiency of these reactions is such that, following transformation, only a few percent of all clones contain mutations, and therefore more work must be done to identify the interesting clones. The method presented in this paper uses the Zakour and Loeb procedure (3) to generate base pair substitution mutants, and it is designed so that any mutation is linked to a detectable marker. In this way, following transformation, the mutant clones are immediately identified by their phenotype. The frequency of mutants found among the blue plaque forming clones depends solely on the efficiency of the mutagenic event and on the hypothetical repair activity of E.coli. We have minimized the effect of the latter by

using an undermethylated DNA template, as suggested by Kramer et al. (4). According to our results therefore, the efficiency of the reverse transcriptase induced error in our experimental conditions must be not far from 100% because about 85% of all blue plaques contained mutations.

In this paper we present the results of several experiments of site directed mutagenesis showing that in all cases we obtained the mutants we wished. It must be stressed however that mutants were constructed on the basis of our specific interest in the promoter of tRNApro gene and therefore we have not systematically explored all possible experimental conditions and combinations of nucleotides. At its present stage of development, the precision of our method is satisfactory but not perfect. We discovered, for instance, that a double mistake, that is the incorporation of two mispairing nucleotides in adjacent positions, though rarely, can occur without eliminating the possibility of further elongation. Occasionally we also observed, though never in the mispairing position, the incorporation of nucleotides which had been omitted in the in vitro reaction mixture. It is most likely that trace contaminations of our nucleotide samples were responsible for this phenomenon. We have in fact found that the frequency and the type of unpredicted incorporations were different in different commercial preparations. We obtained more predictable results using dC, dA and T from PL Biochemicals and dG from Sigma.

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