Volume 16 Number 17 1988 Nucleic Acids Research

Oligonucleotide site-directed mutagenesis in Xenopus egg extracts

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Received April 12, 1988; Revised and Accepted July 6, 1988

ABSTRACT

Addition of M13mp18 single-stranded DNA annealed with an oligonucleotide to a Xenopus egg extract results in a rapid and efficient incorporation of the oligonucleotide in a complete double-stranded supercoiled molecule. Both the efficiency of DNA synthesis and the recovery of complete double-stranded molecules are increased relative to the reaction carried out by the classical technique using the *E. coli* Klenow DNA polymerase, DNA ligase, dNTPs, ATP and ions. Site specific mutagenesis was assayed by reverting a point mutation in the *lacz* region of M13mp18. The color assay described by Messing (1) and sequencing of the DNA extracted from isolated plaques was used to check for the reversion. A 2hr incubation of the heteroduplex carrying the mutagenic oligonucleotide in the Klenow-ligase-dNTP mixture allows a recovery of 6% mutant phage after transformation of competent cells with the reaction products. Using the Xenopus egg extract, 83% mutant phage were recovered after the same incubation time, in reactions entirely performed in parallel. The Xenopus extract is stable and contains all components required for the assay, including all ionic and protein factors; thus the only addition is the annealed DNA. Such an eukaryotic system is therefore an attractive alternative to the reconstituted prokaryotic DNA polymerase-DNA ligase system for site specific mutagenesis.

INTRODUCTION

Oligonucleotide site-directed mutagenesis is now widely used to produce defined point mutations in DNA fragments cloned into M13 vectors (2). The basic principle involves the extension by *E. coli* DNA polymerase (large fragment) of an oligonucleotide primer hybridized to a single-stranded circular template. The oligonucleotide, 8-20 nucleotides long, is complementary to a region of the template except for the mismatch that directs the mutagenesis. The products of the *in-vitro* reaction, which contains a mixture of fully and partially extended DNA chains annealed to the single-stranded circular template, are then used to transform competent cells, and a mixed population of mutant and wild type molecules are

obtained. Mutant molecules are distinguished from wild type by one of several screening procedures (2).

At least three points are essential to obtain a maximum number of mutant phages. The first one concerns the *in-vitro* extension reaction, the second and third points are relative to the *in-vivo* step. In the *In-vitro* extension reaction, since the 5' end of the primer is exposed, the mismatch may be edited out by 5'→3' exonuclease *in-vivo*. Therefore, protection of the 5' end of the primer is carried out by ligation with T4 DNA ligase in different techniques such as the "all the way round" (3) , the "double priming" (4) and the "gapped duplex" (5). In priming "all the way round" with mutagenic oligonucleotide, reaction products are a mixture of single-stranded DNA, incomplete heteroduplex (with unprotected 5' ends close to the mismatch) and double-stranded closed circular heteroduplex. Purification of the closed circular DNA is performed by sucrose gradient centrifugation (3), or agarose gel electrophoresis (6), and the contaminating single-stranded DNA molecules can be eliminated by S1 nuclease treatment (7). The efficiency of the elongation reaction can be improved by addition of T4 protein 32 in the reaction medium (8). However, despite these improvements the frequency of mutant phages is often low (5 - 10%). To increase this frequency, the *in-vivo* parameters have been worked up in the last years and this brings out the second point. *In-vivo*, after transfection of bacteria, repair of the mismatch may occur by correction of the unmethylated minus strand in favor of the methylated plus strand (5). The use of mismatch repair deficient strains of *E.Coli* allows one to enhance the yield of mutants (9,10). The third point is relative to the fact that progeny phages are derived from both the plus and minus strands of M13. Selection for phage progeny derived from replication of only the minus strand is a strategy recently developped using the "gapped duplex" technique and an amber marker (11), or a "double priming" technique and the restriction markers *EcoK* and *EcoB* (10) or by direct elimination of the plus strand (12,13). The combination of the three parameters: optimization of the in-vitro extension-ligation reaction, use of mismatch repair deficient strains and selection of minus strand leads to the best yields of mutants.

In this paper is presented an alternative biological system derived from Xenopus eggs. Cell-free systems derived from Xenopus eggs support complementary strand synthesis on single-stranded templates with high efficiency

(14). The enzymatic reactions observed mimic the mechanism of DNA synthesis at the replication fork of eukaryotic chromosomal DNA: a DNA primase activity synthesizes RNA primers of 10 nucleotides and DNA polymerase-α elongates DNA chains (14, 15). The overall reaction leads to double-stranded closed circular DNA. We show here that the enzymatic machinery present in an egg extract, without any addition, can replace the mixture of *E. coli* polymerase I, ligase, NTP's and dNTP's necessary for oligonucleotide site-directed mutagenesis, and gives a 20 fold increase in the efficiency of the elongation-ligation *in-vitro* reaction. This is the first eukaryotic system described that supports oligonucleotide site-directed mutagenesis. Our method very simple to use, when combined with the use of mismatch repair deficient strains can give yields of mutants comparable to the most efficient methods developped to date (10, 11, 12, 13).

MATERIALS AND METHODS

Bacterial strains :

The *E. coli* K12 strains BMH71-18 (Δ[*lac*- proAB], supE, thi; F'*lac* i^q zΔM15 $proA^+B^+$) and BMH71-18 mut L::Tn10 were provided by H. Bedouelle (Institut Pasteur).

Phage strains :

The original sequence of *lacz* present on M13mp18 was modified at position 6313, C was replaced by T. This mutant was obtained by classical oligonucleotide site-directed mutagenesis, as described in Results.

Preparation of phage DNAs :

M13mp18 double-stranded RF-DNA was prepared as described (16). Single-stranded M13mp18 DNA was from M13mp18 phage purified by CsCl buoyant density centrifugation (17).

Preparation of Xenopus egg extracts :

Xenopus laevis animals originally imported from South Africa (South African Farms, Fish Hoek) were accomodated and fed as described by Gurdon (18). Unfertilized eggs were obtained and dejellied as previously described (14). The extracts were prepared by a modification of a method described by Lohka and Masui (19). The preparation of the extract was carried out at 4°C. The eggs were washed in extraction medium (20mM HEPES pH 7.5, 70mM potassium acetate,

1mM dithiotreitol, 5% sucrose), and packed in centrifuge tubes. Excess medium was removed, leaving only the interstitial buffer between the cells. The cells were broken by direct centrifugation (12.000 g for 30 min) and the supernatant collected. The supernatant was then recentrifuged at 40.000 rpm for 60 min in a Beckman 50Ti rotor. The extract was stored in small aliquots at -80°C. DNA synthesis activity in the extract remains stable for at least six months, under these conditions. With this procedure the eggs are crushed directly by centrifugation, without any added solution except the interstitial buffer around the eggs, which is about half the volume of the packed eggs. Half the volume of an egg is composed of insoluble yolk (20), and therefore the packed eggs are composed of 1/3 volume cytoplasm, 1/3 volume yolk, and 1/3 volume interstitial buffer. After elimination of the yolk by centrifugation the resulting extract contains the egg cytoplasm exudated from the crushed cells with the yolk component of the egg replaced by buffer.

Oligonucleotides :

Oligonucleotides were synthesized by a Biosearch Model 8600 DNA synthesizer. All oligonucleotides were purified by preparative gel electrophoresis in 20% acrylamide, 7M urea and eluted as described by Maxam and Gilbert (21). The 16mer (5' GGTTTTCCTAGTCACG 3') was used to construct a mutation at position 6313 within the M13mp18 gene *lacz* , which may be removed using the selection primer 20mer (5'AGGGTTTTCCCAGTCACGAC3'). The 17 mer (5' GGTGCGGCCTCTTCGC3') located at position 6389 within the M13mp18 sequence was used to check the sequence of the revertants for the *lacZ* mutation at position 6313. Specific priming at the target site was tested for each oligonucleotide by dideoxy sequencing (22).

Labelled oligonucleotides were obtained by extension of the phosphorylated universal primer (BRL) annealed with single-stranded M13 DNA using *E. coli* DNA polymerase Klenow enzyme (Pharmacia) in the presence of 20µl [α^{32}P]-dATP (Amersham), and 200µM dCTP and dGTP, without dTTP. The dominant product in the reaction, the 25 mer (5' G TAAAACGACGGCCAGTGCCA*A*GC3'), was purified by gel electrophoresis in a 20% urea-polyacrylamide gel and the DNA was eluted and dialysed against TE buffer (10mM Tris HCl pH7,5; 1mM EDTA). Oligonucleotides were kinased for mutagenesis as previously described (23) using T4 polynucleotide kinase

(Pharmacia). When the primer used was non phosphorylated the efficiency of mutagenesis decreased to 3 - 4% in either the classical Klenow-ligase system or in Xenopus egg extract.

Oligonucleotide-directed mutation construction.

The kinased oligonucleotide was annealed as described (10 picomoles of primer/ µg of single-stranded DNA)(23). Unless specified, the reaction using the Klenow enzyme and T4 DNA ligase was as described by Zoller and Smith (3). With the egg extract, the reaction was performed with 10µg/ml of single-stranded DNA annealed to the oligonucleotide (500ng of single-stranded DNA in 50µl of egg extract), without any other additions, and the incubation was for 2hrs at 22°C. The reaction was stopped by 30mM EDTA, 0.1% SDS and treated with proteinase K (600µg/ml) for 1hr at 37°C. After phenol and chloroform extraction the DNA was ethanol precipitated and dissolved in TE buffer (25µl for 500ng of single-stranded DNA). Nacs Prepacs (BRL) or Gene Clean (Bio101) can be used in a further purification step to increase transformation efficiency, but does not increase the proportion of mutants obtained. Small aliquots were used to transform competent cells BMH71-18 *mutL*, a mismatch repair $^-$ strain (review, 24). Competent cells were prepared according to the Hanahan procedure (25). Lawn cells were provided by a wild type strain (BMH71-18) in order to minimize the exposure of the phage to the mutator phenotype of the *mutL*-strain, as described by Carter *et al.* (10) (a summary of all of these steps is presented in Table III).

Mutant phages were plaque purified by picking from the plate into 1ml of 2XTY (16g/l Bacto Tryptone, 10g/l Bacto Yeast extract, 10g/l NaCl). The mixture was spread on a fresh plate. Isolated plaques were then used to prepare single-stranded phage DNA (17). Mutations were verified by dideoxy sequencing (22) using the appropriate 17 mer primer located at position 6389 within the M13mp18 sequence.

RESULTS

Efficiency of complementary DNA strand synthesis and utilisation of the oligonucleotide primer with the Klenow-ligase system or with the Xenopus egg extract.

A major problem in oligonucleotide site-directed mutagenesis can be the inefficient conversion of single-stranded template DNA to the double-stranded form

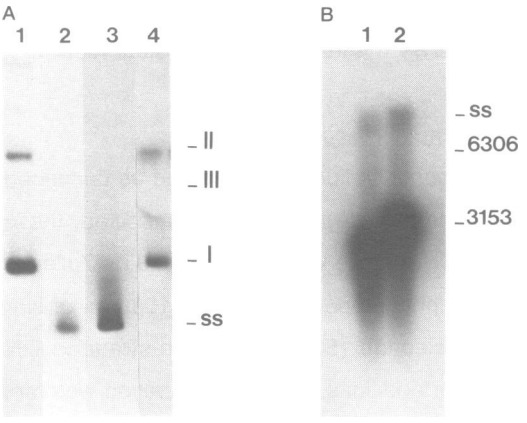


Figure 1 : Comparison of the *in-vitro* reaction in the Klenow-ligase mixture and in the Xenopus egg extract.
The *in-vitro* reactions were as described in Materials and Methods.
A. M13 single-stranded DNA (200ng) with no primer is incubated in the egg extract (20µl) in presence of [^{32}P] dATP (1). M13 single-stranded DNA annealed (200ng) with a [^{32}P] labelled 25 mer (2 picomoles)(2), is incubated either in the Klenow-ligase mixture (3) or in 20µl of the egg extract (4) for 2h at 22°C. The reaction were processed in parallel and analysed on the same 1% agarose gel.
B. Reactions were performed with single-stranded M13 DNA (200 ng) annealed to a kinased 17 mer (2 picomoles), with Klenow, ligase, dNTPs, NTPs, [^{32}P] dATP for 2h at 15°C (1) or 24h at 22°C (2). Products were analysed on a 1% alkaline agarose gel (16)

(2, 26). To carry out the oligonucleotide site-directed mutagenesis, two main conditions must be fullfilled. First, the rate of DNA synthesis should be sufficient to provide an adequate yield of double-stranded molecules. Second, the mutagenic primer should be present in the final DNA product. Both aspects have been investigated and compared using either the Xenopus egg extract or the classical Klenow-ligase system.

When single-stranded M13 mp18 DNA is incubated in a Xenopus egg extract in the presence of [α ^{32}P] dATP, full length double-stranded molecules are produced. Moreover, supercoiling of a substantial fraction of the double-stranded molecules occured, as shown by the formation of form I M13mp18 DNA (Figure 1A, lane 1). Such synthesis is initiated from RNA primers synthesized in the extract and is supported by a mechanism that mimics replication on the lagging strand at the eukaryotic replicating fork (14, 15, 27). The efficiency of DNA synthesis, expressed as DNA (µg) synthesized per µg of input single-stranded DNA reproducibly

Table I : Comparison of DNA synthesis efficiency and primer utilisation in egg extract and in the Klenow-ligase system.

	% DNA synthesized after 2hrs incubation	% primer maintained	% primer in a complete double-stranded form
Egg extract	95 to 100%	40 to 90%	~100% (a)
Klenow ligase	25 to 40%	75 to 85%	<1% (b)

Total DNA synthesized (µg) was determined from acid-precipitable radioactivity (14), and the efficiency was calculated as a percentage of total input single-stranded DNA (µg). % primer maintained is % acid-precipitable radioactivity at the end of reaction compared to the initial amount when the labelled primer 25mer (as described in Materials and Methods) was used. The variations observed with different egg extracts could be due to variable levels of exonuclease activities (unpublished results). As the labelling was localised at the 3' end of the molecule, the % primer maintained was probably underestimated. An overnight incubation in the Klenow-ligase mixture can increase the amount of primer incorporated in a complete double-stranded form to 5%, as previously described by Zoller and Smith (2). % primer in a complete double-stranded form is determined by scan densitometry on the agarose gel for egg extract or on alkaline gel for Klenow-ligase system (Figure 1). (a) in five independent assays the variations observed were inferior to 1%. (b) values were below the detection level in our assays.

approached 95 to 100% after 2hrs (Table I). The amount of synthesis in the Klenow-ligase system provided with an exogenous primer only gave a value of 25 to 40% (Table I) and furthermore complete double-stranded molecules were barely detected (Figure 1, lane 3, and unpublished results). A direct estimation of the extension of the primer was obtained by analysis of the products of this reaction on alkaline gels. Pauses in synthesis occurred at 2.5 kb in a reaction incubated 2hrs at 22°C and at 3.5 kb in a reaction incubated 24 hrs (Figure 1B). The proportion of complete molecules obtained was less than 5% of the reaction products after 24hr of incubation as measured by scan densitometry.

The maintenance of the primer was estimated by using a 25 mer primer labeled at its 3' end (Materials and Methods) annealed to single-stranded M13mp18 DNA (Figure 1A, lane 2). The duplex was incubated with either DNA polymerase I Klenow enzyme, T4 DNA ligase and the four dNTP's (Figure 1A, lane 3) as described by Zoller and Smith (4), or with the Xenopus egg extract without additions (Figure 1, lane 4). Measurement of TCA acid-precipitable radioactivity at

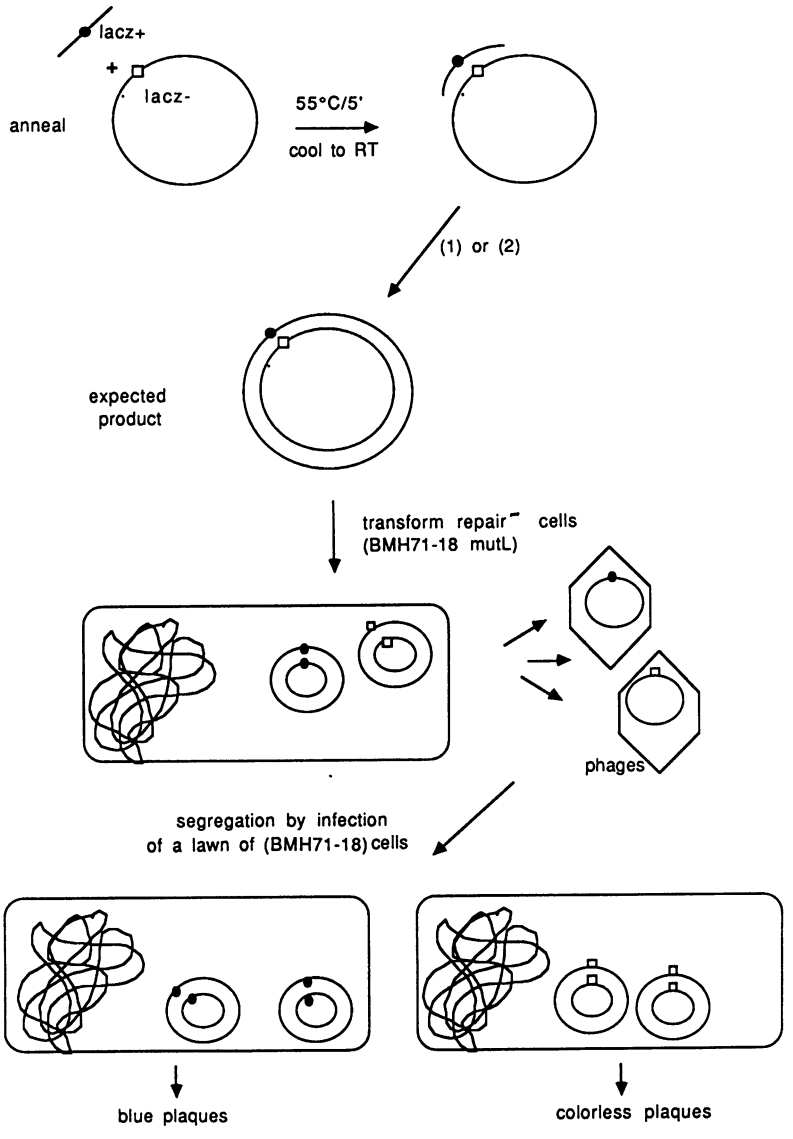


Figure 2 : General scheme used for the mutagenesis test.
M13 mp18 *lacz*⁻ DNA was annealed with an oligonucleotide wild type sequence to restore the wild type phenotype by a single base substitution T to C at position 6313. The heteroduplex was incubated either in the Klenow-ligase-dNTPs mixture (1) or in the Xenopus egg extract (2). The reaction products were used to transform competent cells as described in Materials and Methods. Original mutant phages give colorless plaques whereas revertant phages give blue plaques when spread on X-gal indicator plates (17).

the end of the reaction showed that with the Xenopus egg extract, 90% of the labelled primer was present on the double-stranded DNA (Table I and Figure 1A, lane 4). With the Klenow-ligase-dNTP mixture the maintenance of the primer was 85% (Table I) although the elongation process was much less efficient (Figure 1A; compare lanes 3 and 4). Table I summarizes the values and the variations obtained in different assays (five independent experiments) using the Xenopus egg extracts or the Klenow-ligase-dNTP system.

In-vitro mutagenesis in Xenopus egg extracts.

We used site-specific mutagenesis in the *lacz* region of M13mp18 together with the color assay described by Messing (1, 17), to screen for wild type and mutant phages (Figure 2). A M13mp18 mutation in the coding sequence of the ∝ -peptide of ß-galactosidase was constructed by substituting a cytidine with thymidine at position 6313 of the DNA, thus introducing a stop codon TGA in the sequence to give a colorless plaque (Materials and Methods). The single-stranded DNA of the mutant was annealed to the 20-mer oligonucleotide wild type sequence (Materials and Methods) to restore the wild type phenotype (blue plaque) by reverting T to C at position 6313. Such reversion by site-specific mutagenesis is a more reliable indicator of mutagenesis efficiency than forward mutagenesis.

The heteroduplex carrying the mutagenic oligonucleotide was incubated for 2hrs at 22°C either in the Klenow-ligase dNTP system or in different Xenopus egg extracts (Table II). The reaction products were then used to transform competent cells according to Carter *et al.* (11) in experiments entirely performed in parallel. With the Klenow-ligase dNTP system, 6% revertant plaques (blue) were observed after 2hrs synthesis at 22°C, whereas up to 40% revertants were recovered after 24hrs at 15°C which is in agreement with published results.With the Xenopus extract, for an incubation time of 2hrs at 22°C, the percentage of mutants recovered ranged from 50% with egg extract 4 (EE4) to 83% with egg extract 1 (EE1). With each extract the value indicated was reproducibly obtained for several independent experiments (five) with a fluctuation of about 5%. Efficiency of mutagenesis with the different extracts seems to be related to the percentage of primer maintained as shown in Table II. Egg extract 1 (EE1) with 83% revertants gave the best yield and this yield was not increased by further incubation. When completely double-stranded closed circular molecules are used to transfect *E. coli*

Table II : Genetic assay of mutagenesis with templates DNA obtained by elongation of the mutagenic primer in the Klenow-ligase system or in different Xenopus egg extracts.

In vitro reaction	% primer maintained double-stranded form	% mutant
EE 1	90%	83%
EE 2	47%	55%
EE 3	nd	54%
EE 4	41%	50%
Klenow-ligase	85%	6%

In each case, % primer maintained was determined using the labelled primer as described in Figure 1 and Table I. For the genetic assay, M13 DNA with the mutation at position 6313 was annealed with the 20mer selection primer (10 picomoles primer / µg DNA) as described (23). The *in vitro* elongation reactions were performed with an aliquot from the annealed reaction corresponding to 500 ng of single-stranded DNA, either with 50µl of egg extract (EE 1, EE 2, EE 3, EE 4) or with the klenow-ligase system as described by Zoller and Smith (3). Incubation was for 2hrs at 22°C. The reactions were stopped and processed as described in Materials and Methods. The DNA was dissolved in 25µl 10mM Tris-HCl, 1 mM EDTA. Competent cells BMH 71-18 mut L were transfected with 0, 1; 1 and 10 µl of the sample. Lawn cells were provided by repair+ strain BMH 71-18. Results indicated are an average of five independent experiments.

cells, there is around a 2 : 1 bias in favor of the minus strand as a template for amplification of the replicating double-stranded form (28, 9, 10). Thus, the yield of mutants recovered with the Xenopus egg extract mutagenesis system (EE1) approaches the theoretical maximum. In all assays, the mutant M13 DNA template was also added with the extract without hybridization to the reverting oligonucleotide. In all cases double-stranded supercoiled DNA was synthesized but no blue plaques were detected after transformation of repair $^-$ competent cells. In constrast, when M13 single-stranded wild type DNA was added to the extract, all the plaques resulting from transformation of *E. coli* cells were blue. We concluded that, in our experimental conditions, the Xenopus extract by itself did not exhibit detectable mutagenic activity.

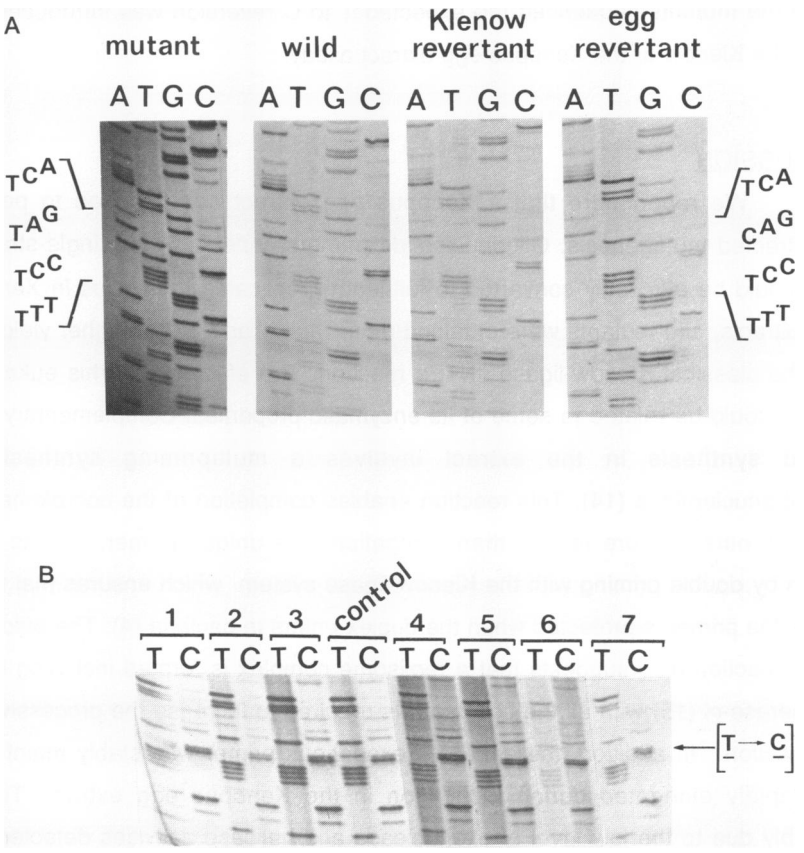


Figure 3 : Sequence analysis of the mutants obtained using Xenopus egg extract.
A. Analysis on sequencing gels of the four sequencing reactions are shown for the mutant (*lacz*$^-$), the wild type (*lacz*$^+$), the revertant obtained with Klenow-ligase (*lacz*$^+$), the revertant obtained with the egg extract (*lacz*$^+$).
The sequence is presented by triplets of nucleotides according to the genetic code. TGA corresponds to the codon stop of the mutant , reverted to CGA to produce the functional *lacz* protein.
B. Analysis on sequencing gels of the T and C reactions are shown for different revertants obtained with the egg extract (*lacz*$^+$). For comparison, the control shows the sequencing gel analysis before mutagenesis with the egg extract.

Sequence analysis of the revertants.

To confirm that the desired site-specific mutation was introduced by the egg extract, we purified the DNA from several isolated plaques, including the M13 mutant, the M13 wild type, and the reverting blue plaques obtained either with the Klenow-ligase dNTPs mixture or with the Xenopus egg extract. Figure 3 shows that

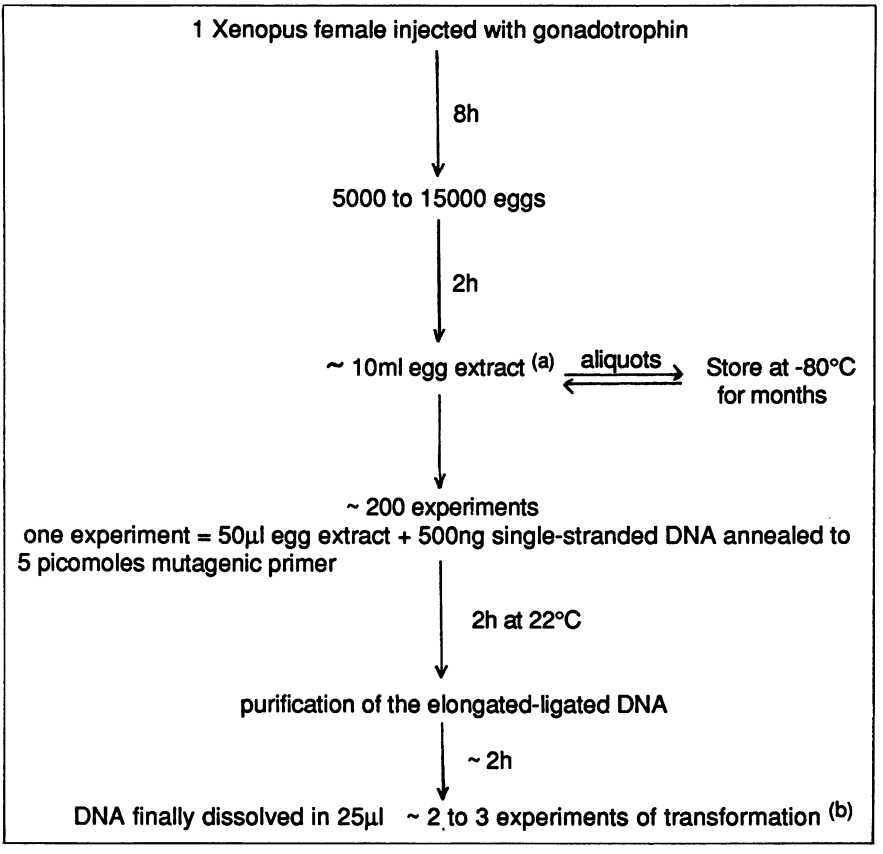
for all the mutants sequenced the expected T to C reversion was introduced with either the Klenow or the Xenopus egg extract assay.

DISCUSSION

We report here that a Xenopus egg extract can be used to perform site-directed mutagenesis. Oligonucleotide primers annealed with single-stranded DNA could be efficiently converted to full length replicated molecules in Xenopus egg extracts, and mutants were obtained more rapidly and with a higher yield than with the classical Klenow-ligase-dNTPs reaction. The efficiency of this eukaryotic system could be related to some of its enzymatic properties. Complementary DNA strand synthesis in the extract involves a multipriming synthesis of decaribonucleotides (14). This reaction enables completion of the complementary strand synthesis more readily than elongation of a unique primer, as has been shown by double priming with the Klenow-ligase system which ensures that the 5' end of the primer is protected when the duplex enters in bacteria (4). The efficiency of the reaction also suggests that a replisome complex is formed including DNA polymerase-α (15) with all the components required to increase the processivity of the reaction. In addition, the annealed exogenous primer was stably maintained and rapidly elongated during incubation in the Xenopus egg extract. This is probably due to the low level of exonuclease and helicase activities detected with these reaction conditions (unpublished data).

It is interesting to note that the system described here allows in a very simple way to obtain high yield of mutants comparable to those obtained with methods like those commercialized by Amersham and relying on selection of the mutated strand developped by Eckstein and coll. (7). This method includes the synthesis of phosphorothioate M13 DNA which permits the introduction of enzyme directed nicks lying exclusively in the (+) strand of the heteroduplex molecules. After digestion of the unwanted (+) strand sequence by exonuclease III, repolymerisation *in vitro* using *E.Coli* DNA polymerase I and T4 DNA ligase generates a homoduplex mutant molecule which is then used to transform bacteria. As incorporation of phosphorothioate analogs of nucleotides can be achieved by DNA polymerase-α (29, 30) and in the Xenopus system (not shown), it is possible

Table III : Estimation of the potentialities of the Xenopus egg extract to perform mutagenesis experiments.



(a) % primer maintenance can be tested and used as a criterium at this stage to determine extract potential.
(b) for each transformation experiment, three quantities of DNA are used: 0,1µl - 1µl -10µl

to perform a selection of the mutated strand in this system. This method was developed to increase efficiencies of mutagenesis in the classical system using Klenow DNA polymerase and T4 DNA ligase, however it can be dispensed within our simple system which directly yields high proportion of mutants.

The use of the Xenopus extract has the following advantages as summarized on Table III. First, the whole extract preparation is usually performed in one day and the extract is stable for at least six months at -80°C (unpublished

results). Second, the extract contains the source of the DNA replication machinery, including DNA polymerase-α, ligase, dNTPs and ATP, and all ionic as well as protein factors required for full length double-stranded DNA synthesis on single-stranded DNA (14). Thus, the only addition necessary is the annealed DNA. The classical method requires addition of the Klenow enzyme, DNA ligase, the four dNTPs, ATP and ions required for DNA synthesis. Third, the whole double-stranded DNA is synthesized without significant pauses in the reaction. Fourth, the *in-vitro* reaction is complete after 2hrs and thus both mutagenesis and transformation can be performed in one day. Therefore, the use of the DNA synthesis activity of the Xenopus egg extract offers an attractive simple and efficient alternative to perform oligonucleotide site-directed mutagenesis.

AKNOWLEDGEMENTS

We are grateful to P. Brooks, P. Hugues and P. Lecomte for critical reading of this manuscript and C. Michon for synthetic oligonucleotide synthesis.

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