Site specific mutagenesis: insertion of single noncomplementary nucleotides at specified sites by error-directed DNA polymerization

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ABSTRACT

We have utilized infidelity of DNA synthesis as a basis for site-directed mutagenesis. Both an endonuclease restriction fragment and a synthetic oligonucleotide were used as primers. DNA polymerase from bacteriophage T4 was used to elongate primer termini to a position immediately adjacent to two different preselected positions on ϕ X174 DNA templates. Then, the error-prone DNA polymerase from avian myeloblastosis virus was used to insert single non-complementary nucleotides at the designated positions at high efficiency. DNA sequence analysis confirmed that the mutant phage produced as a result of each site-specific mutagenesis reaction contained the nucleotide that was complementary to the one provided during the DNA copying reaction. The general applicability of this methodology to cloned DNAs will be discussed.

INTRODUCTION

The ability to manipulate the primary sequence of a DNA molecule *in vitro* should greatly facilitate studies of gene expression and mutagenesis¹. Three *in vitro* approaches have been used to generate specific base substitutions at designated sites in DNA. The first involves the introduction of a change into a DNA molecule via the insertion of a synthetic oligonucleotide which contains a preselected change in its nucleotide sequence (2,3). This method, which has been used to generate novel DNA sequences with viral DNA templates and with recombinant DNAs (4) it is highly efficient and can produce any type of base substitution mutation (5,6). However, each different mutation that is introduced requires the synthesis of a unique oligonucleotide. A second approach involves the introduction of a small single strand gap in a DNA molecule followed by mis-repair DNA synthesis. Starting with specifically gapped template molecules, Shortle, *et al.* (7), have produced site specific changes by polymerization in the presence of only three of the four dNTPs and sealing the gaps with DNA ligase. Since the gaps had been formed such that all four nucleotides were present in the template strand, closed circular product molecules could be formed only by the mis-incorporation of a non-complementary nucleotide in place

of the missing complementary nucleotide. These same authors (7) also showed that by utilization of α -thiol nucleotides (8) excision of non-complementary nucleotides could be minimized. The third method is based on infidelity of DNA polymerases (8) and involves extension of a DNA restriction primer by a non-proofreading DNA polymerase in the presence of a single non-complementary deoxynucleotide triphosphate (10). Thereafter, synthesis is completed by a highly accurate procaryotic DNA polymerase with all four deoxynucleotide substrates. Using this approach, Traboni *et al.* (11) have made a number of different site-specific modifications in the RNA Pro gene from *C. elegans* cloned into bacteriophage M13 derivative vectors (12). In this paper, we will consider the extension of this method to oligonucleotide primers and to procedures for obtaining specified single-base substitutions in high yield.

We previously demonstrated that the mis-insertion of a non-complementary nucleotide could be directed by specifying which nucleotides were available in the reaction mixture starting from a restriction endonuclease fragment primer (10). Mis-insertion could be carried out at a preselected position following stepwise elongation of the primer. In these previous experiments, several nucleotides were present in a DNA copying reaction that combined primer elongation with mis-insertion of a non-complementary nucleotide. In the present analysis, we demonstrate that single specified nucleotides can be mis-inserted at two different sites in DNA template molecules from bacteriophage ϕ X174. We further show that in addition to using restriction fragment primers, a synthetic oligonucleotide can be elongated in a controlled fashion to provide a 3'OH primer terminus adjacent to a template position chosen as the target for site specific mutagenesis. Applications of this approach allows the formation of a series of single base mutations across a DNA sequence.

MATERIALS

The *Escherichia coli* strains used in these experiments are the same as those described (13,14). Viral template DNA was isolated from ϕ X174 bacteriophage am3 (a gift of J. Weiner and A. Kornberg) and am18 (a gift of P. Weisbeek) as previously described (14-16). The restriction endonuclease fragment primer (HpaI, 2; ϕ X174 DNA sequence positions 31-1294) (17,18) was isolated from a 1% agarose gel following the digestion of am18RF DNA with the restriction enzyme HpaI (BRL, Inc., Gaithersburg, MD) and then it was hybridized to a single-stranded am18 DNA template. The synthetic oligodeoxynucleotide containing the sequence 5'-d (GGAAAGCGAGGGTAT)-3' (P-L Biochemicals, Inc., Milwaukee, WI) is complementary to ϕ X174 DNA sequence positions 590-604. The oligonucleotide was

hybridized to am3 DNA templates in a volume of 0.05 ml at a primer:template molar ratio of 50:1 carried out in 2 X SSC by heating at 80°C for 5 min, following by incubation at 65°C for 10 min. The mixture was allowed to cool slowly to 37° and remain at that temperature for 15 min, then at room temperature for 1 hr. Prior to hybridization, the 5' end of the oligonucleotide was phosphorylated in a 0.5 ml reaction containing 100 ng of the oligonucleotide, 35.4 units of T4 polynucleotide kinase (P.L. Biochemicals, Inc.), 2 μ M rATP, 50 mM Tris-HCl (pH 7.6) 10 mM MgCl₂ and 5 mM dithiothreitol. Radioactive labelled oligonucleotide primer was prepared in the same way except that 0.5 μ M ³²P-ATP was included in the reaction. This primer was mixed with the unlabeled primer at a ratio of 1:10 (labelled: unlabelled) when used for mis-insertion reactions. Only unlabeled primer was used for DNA sequencing reactions. T₄ DNA polymerase was either a gift of M. Goodman, Sp. Act. 14,000 units/mg or purchased from P.L. Biochemicals Sp. Act. 25,000 units/mg. E. coli DNA polymerase I was that previously described (15), Sp. Act. 22,000 units/mg protein. One unit catalyzed the incorporation of 10 nmoles of total nucleotides using activated DNA as a template.

METHODS

Elongation Reactions. Limited elongation of the 3' OH terminus of the primer was carried out with T4 DNA polymerase. Reactions were incubated at 37°C for 5 min. and contained in a volume of 0.5 ml: 6 μ g of primed template DNA; 25 units T4 DNA polymerase; 50 mM Tris HCl (pH 7.6); 10 mM MgCl₂; 5 mM dithiothreitol; and 50 μ M each of the designated complementary deoxynucleoside triphosphates, one of which was labelled with [α -³²P]. The reaction was stopped by adding EDTA to a final concentration of 10 mM; the mixtures were heated at 65°C for 10 min and extracted once with an equal volume of redistilled phenol equilibrated with 50 mM Tris HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl. To separate the DNA from the unincorporated nucleotides, the aqueous phase was loaded onto a Sephadex G-100 column (1 X 60 cm) and the column eluted with 50 mM Tris-HCl (pH7.8), 0.15 mM KCl. Fractions (1 ml) were collected and aliquots (20 μ l) processed for acid-insoluble radioactivity. The peak fractions were pooled, 1/10th volume of 3 M sodium acetate was added, and the DNA was precipitated with 2.5 volumes of ethanol. The DNA was pelleted, washed and resuspended in 100 μ l of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA. Elongation with E. coliPol I was carried out as described above in a reaction containing 3 μ g of the primed DNA template and 5 μ g of Pol I instead of the T4 DNA polymerase.

Site Specific Mis-insertion Reactions; Reaction mixtures (0.05 ml) contained

0.2 μ g of ϕ X174 DNA template with the elongated primer, 10 units of avian myeloblastosis virus (AMV) DNA polymerase (Life Science, Inc., St. Petersburg, FL), 50 mM Tris-HCl (pH 8.1), 6 mM $MgCl_2$, 2 mM dithiothreitol and 10 μ M for each of the deoxynucleoside triphosphate(s) designated in the individual experiments. Incubation was for 5 min. at 37°C after which all four deoxynucleoside triphosphates were added to yield a final concentration for each of 100 μ M. Further incubation of 5 min at 37°C was used to seal the mis-incorporated nucleotide into the newly replicated DNA. Reactions were stopped by adding 0.1 vol of 100 mM EDTA and then used for transfection directly or further processed to enrich for closed circular molecules.

Enrichment for closed circular molecules After copying with AMV DNA polymerase the reaction mixtures were extracted with equilibrated phenol and the DNA was precipitated in ethanol, centrifuged and resuspended in 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA. For completion of DNA synthesis around the template circles, incubation was for 1 hr at 37°C in a 0.025 ml reaction mixture containing the DNA, 60 units T4 DNA polymerase, 50 mM Tris-HCl (pH 8.1), 10 mM $MgCl_2$ and 2 mM dithiothreitol, 400 μ M rATP, 50 μ M each of the four deoxynucleoside triphosphate substrates. In addition, 1 u of T4 ligase (P.L. Biochemical) was included to close the double-stranded circles. Following circle completion and ligation, the reaction mixtures were extracted with phenol, precipitated in ethanol and resuspended as described above. In the indicated experiments, S_1 nuclease was included to inactivate the biological activity of incompletely copied templates and unprimed single-stranded DNA circles. The resuspended DNA was incubated for 1 hr at 37°C with 2 units of S_1 nuclease (BRL, Inc.) in a reaction containing 30 mM Na acetate (pH 4.6) 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol in a final volume of 0.01 ml. The reaction was stopped by the addition of 0.1 vol of 100 mM EDTA and the DNA was used directly for transfection. Under these conditions, 100% of the biological activity of single-stranded ϕ X174 DNA template molecules is destroyed as documented in control tables.

Transfection assay. Uncopied control DNA and copied DNA molecules from each reaction mixture were transfected into *E. coli* spheroplasts to produce progeny phage. The reversion frequencies of the amber mutations in uncopied and copied DNA molecules were determined by measuring the titer of progeny phage on bacterial lawns that were either permissive or nonpermissive for the amber mutations. The details of this methodology have been published previously (16).
DNA sequence analysis. Single-stranded phage DNA was obtained from the *am3* revertants taken from independently isolated nonpermissive infective centers. Isolation of the revertant phage and preparation of the viral template DNA for

sequencing were performed as described elsewhere (15,16). Using the oligonucleotide labelled at the 5' terminus as a primer, DNA sequence analysis was performed by the chain terminator method of Sanger (19).

RESULTS

Mis-insertion of a single nucleotide using a restriction fragment primer.

The experimental strategy for the mis-insertion of a single nucleotide from an elongated restriction endonuclease fragment primer is depicted in Figure 1. We chose the thymidine at template position 23 (18) in the am18 locus of ϕ X174 DNA as a target for site-specific change since all three substitutions at this position yield viable wild type revertants (10). A restriction fragment from a HpaI digest of ϕ X174 RF DNA which ends at template position 31 was hybridized on to single-stranded ϕ X174 DNA. Faithful elongation was carried out in a series of stepwise DNA copying reactions, (Fig 1, steps 1 and 2) to yield a 3'OH terminus at position 24, next to the target for site-specific mis-insertion. Using this elongated primer, each of the three non-complementary nucleotides were incorporated opposite template position 23 in individual DNA copying reactions using AMV DNA polymerase (Fig. I, part II). As shown in Table I, the reversion frequency of progeny phage produced by DNA molecules copied in the presence of the different nucleotides increased significantly when each of the non-complementary nucleotides were substituted for dATP, the nucleotide complementary to the thymidine at position 23. The reversion frequencies were increased by 32-, 150- and 340-fold for the dTTP, dCTP and dGTP substrates, respectively.

In contrast to the procedure that we reported previously (10), in which Pol I was used for a single step elongation starting at a restriction fragment primer, bacteriophage T4 DNA polymerase was used in the experiments now reported. The relative fidelity, as reflected by the reversion frequency of templates containing the elongated primers, of these two enzymes, both of which possess 3'→5' exonuclease proofreading activity, is shown in Table II. When dATP, the nucleotide complementary to the thymidine at position 23 is present, the reversion frequency is similar to that exhibited by uncopied DNA. With only dTTP and dCTP, the reversion frequency of product molecules elongated in the Pol I catalyzed reaction (57×10^{-5}) is much greater than that observed with T4 DNA polymerase (3×10^{-5}). In a separate experiment using this same template-primer combination and all from deoxynucleotides, each of these polymerases supported DNA synthesis to an equal extent suggesting that the lower reversion frequency for the T4 polymerase elongated molecules was not due to lack of synthesis.

Figure 1: Use of a DNA endonuclease restriction fragment primer for site directed mutagenesis. The starting primer template combination consisted of the restriction endonuclease fragment Hpa I 2 from ØX174 hybridized to a single stranded ØX174 aml8 DNA template molecule.

I. Limited elongation of the 3'OH terminus was carried out using DNA polymerase from bacteriophage T4 in a stepwise series of two synthetic reactions as described in Materials and Methods. In each reaction, only two complementary deoxynucleoside triphosphate substrates were included in order to limit the extent of the elongation. Polymerization is terminated when the enzyme reaches a position on the template for which the complementary nucleotide substrate is missing. Following each elongation step, the DNA and the unincorporated nucleotides were separated by gel filtration. Following elongation Step 2, the 3- hydroxyl terminus of the primer is at position 24, adjacent to position 23 of the ØX174 aml8 template, the site preselected as the target for mutagenesis.

II. Site specific mis-insertion of a single nucleotide was carried out using AMV DNA polymerase in the presence of a single deoxynucleoside triphosphate (dXTP) where X corresponds to the nucleotide chosen for insertion opposite the thymidine at position 23. Following site specific mis-insertion, all four deoxynucleoside triphosphates were added to the AMV DNA polymerase reaction mixture to allow synthesis to continue in order to seal the mis-inserted nucleotide into place.

Mis-insertion of a single nucleotide from an oligonucleotide primer.

The protocol for inserting a single base starting with an oligonucleotide primer of 15 bases in length is depicted in Figure 2. The oligonucleotide primer was elongated by the incorporation of two deoxycytidines with T4 DNA polymerase so as to provide a 3'OH terminus adjacent to the template site (position 587) selected for modification. Starting with an oligonucleotide that had been radioactively labelled on the 5' terminus using T4 polynucleotide kinase, we observed after elongation with T4 DNA polymerase a complete shift in the position of the 15mer to correspond to that of a 17mer following electrophoresis on a polyacrylamide gel. Only a small (ie., <2-fold) increase in reversion frequency was detected following primer elongation using the T4 DNA polymerase (data not shown).

TABLE I: Reversion frequency of DNA produced by site-specific insertion of single specified nucleotides into the am18 locus of ϕ X174 DNA

Added dNTPs	Reversion frequency ($\times 10^{-5}$)
None	1
dATP	2
dTTP	32
dCTP	150
dGTP	340
dATP, dCTP, dGTP, dTTP	10

The reversion frequency of ϕ X174 am18 DNA template containing an elongated HpaI-2 primer (Figure 1, Elongation Step 2) and copied with AMV DNA polymerase in the presence of the indicated deoxynucleoside triphosphates was determined as described in Materials and Methods. The reversion frequency of 10×10^{-5} of DNA copied with all four dNTP's reflects the high error-rate of the viral AMV DNA polymerase.

The reversion frequency for am3 DNA increased when DNA molecules containing the elongated oligonucleotide primer were copied using AMV DNA polymerase with each different non-complementary nucleotide in the absence of dTTP, the nucleotide complementary to the adenine in the template at position 587 (Table III). The relative increase in reversion frequency is different among the non-complementary nucleotides, but each is at least 40-fold greater than that of

TABLE II: Effect of DNA polymerases on the fidelity of primer elongation

DNA Polymerase	Reversion frequency ($\times 10^{-5}$) of ϕ X174 <u>am18</u> DNA elongated with	
	dTTP	dTTP
	dCTP	dCTP
	dATP	
<u>E. coli</u> DNA Polymerase I	2	57
Bacteriophage T4 DNA Polymerase	2	3

To monitor the fidelity of elongation synthesis, the reversion frequency of ϕ X174 am18 template containing the elongated primer, shown as the product in Step 1 of Figure I was assayed following a second elongation synthesis for each DNA polymerase in the presence of the two indicated combinations of the deoxy-nucleoside triphosphate substrates.

Figure 2 Use of an oligonucleotide primer for site directed mutagenesis. A synthetic oligonucleotide of 15 bases in length complementary to nucleotides 590-604 of the ϕ X174 genome (18) was hybridized to a single stranded ϕ X174 am3 circular template. Primer elongation, site specific mis-insertion, and synthesis to seal the mis-inserted nucleotide into place, were carried out as described in Figure 1 and in Materials and Methods. The "A" at template position 587 in the am3 locus was the target site preselected for mutagenesis.

the uncopied DNA. A 300-fold increase was obtained by substitution of dCTP in both this and in one other experiment (data not shown). A small increase in reversion frequency was also observed when dTTP, the complementary nucleotide at position 587, was the only nucleotide included in the copying reaction. This increase could be due to the presence of a small amount of dCTP that remained following the elongation reaction with deoxynucleoside triphosphates despite the fact that the DNA had been passed over a Sephadex column.

Sequence analysis of DNA isolated from wild type revertant phage obtained from independent infective centers from each copying reaction showed, in all cases that the substitution present in each revertant corresponded to that predicted on the basis of the single nucleotide present during the DNA copying reaction (Table III). For example, the two revertants sequenced in which dATP was the only nucleotide present in the copying reaction both contained an A at position 587 of the complementary plus strand. The same relationship is indicated by substitutions with dCTP and dGTP. In all cases, the substitution occurred at template position 587, and no other substitutions were observed when up to 50 nucleotides per revertant were read. The results of the sequence analysis along with that previously reported for am18 (10) support the hypothesis that the mutants obtained by site-specific mis-insertion can be directed by the selection of nucleotides which are experimentally provided in the copying reaction. The specific insertion of the biased non-complementary nucleotide has been confirmed in a large number of sequences with dATP (B. Preston, unpublished

TABLE III: Reversion frequency at the am3 locus and DNA sequence of revertants copied by AMV DNA polymerase in the presence of different deoxynucleoside triphosphates.

Added dNTPs	Reversion frequency ($\times 10^{-5}$)	Sequence
None	0.1	C (2)
dATP	17	A (2)
dCTP	32	C (2)
dGTP	4	G (2)
dTTP	0.8	--

The reversion frequency of ϕ X174 am3 DNA templates containing the elongated oligonucleotide primer shown in Figure 2 was assayed following synthesis by AMV DNA polymerase in the presence of the indicated deoxynucleoside triphosphates as described in Materials and Methods. The results of sequence analysis of duplicate independent isolates DNA obtained from wild type revertant phage from each copying reaction are also shown. The number of independent revertants that were sequenced is given in parenthesis.

results) and with M-13 single-stranded DNA at different sites (Kunkel, T.A., N.I.E.S.H., unpublished results).

The efficiency of mis-insertion

In these experiments the efficiency of mutagenesis depends on the percent of primer molecules hybridized to the template and on the percent correctly elongated prior to adding a single non-complementary nucleotide. In order to obtain an estimate of the maximum frequency of site specific mis-insertion, the reaction mixture was enriched for the subpopulation of molecules containing the mutagenic change and conditions were chosen which allowed the maximum expression of the mutation. After the site-specific mis-insertion reaction, DNA synthesis was continued with all four nucleotides using AMV DNA polymerase and then followed by T4 DNA polymerase to complete synthesis around the circular template. Thereafter, closed circular DNA molecules were formed using T4 DNA ligase, and any incompletely copied molecules were cleaved with nuclease S_1 . The circle completion and ligation procedure minimized the problems of expression of an incomplete complementary strand (20). A comparison of the reversion frequencies at each of these different steps for am3 and am18 is shown in Table IV. For both loci, the same general trends are observed. A large increase, approximately 250 to 300-fold, was observed following the site-specific mis-incorporation of non-complementary nucleotides. Circle completion and ligation produced a further increase in reversion frequency of about 8 to 10-fold. After S_1 treatment, the final reversion frequency was 33% for am18 and 9% for am3.

TABLE IV: Efficiency of mis-insertion mutagenesis

	Reversion Frequency of DNA ($\times 10^{-5}$)			
	Uncopied	partially double-stranded	closed circular double-stranded	after S_1 Nuclease
am18	1.0	340	3,300	32,500
am3	0.1	25	180	8,700

am18 and am3 ϕ X174 DNAs primed as described in Figures 1 and 2, respectively, were copied with AMV DNA polymerase as described in Materials and Methods in the presence of the three non-complementary nucleotides for each DNA (dCTP, dGTP and dTTP for position 23 of am18 and dATP, dCTP, dGTP for position 587 of am3). Synthesis of the partially double-stranded product was completed with T4 DNA polymerases and DNA ligase was used to form closed circles. The DNA after T4 DNA polymerase and ligase treatment was incubated with S_1 nuclease to remove any incompletely copied molecules.

If all molecules contained a single base mis-insertion at the designated position, a reversion frequency of 50% would be expected in the absence of mismatch correction in vivo. These results indicate that it is possible to eliminate most of the non-mutagenized molecules. Thus, designated mutations can be produced in vitro without screening on the basis of mutant phenotype.

DISCUSSION

We have designed a method for the incorporation of a single nucleotide to yield a single substitution mutation at a specified site on a cloned DNA molecule based on infidelity of DNA polymerases. In our earlier publication (10) DNA synthesis was initiated at a known position by the use of a purified restriction fragment; elongation was carried by limited reactions with E.coli DNA polymerase I, and mutagenesis was achieved in reactions with AMV DNA polymerase in which one complementary nucleotide was deleted. Each of these steps has been modified in order to increase the versatility of the method, to enhance the efficiency of mutagenesis, and to incorporate a specified single base at designated sites.

The use of a synthetic oligonucleotide as a primer for the mis-insertion of a non-complementary nucleotide provides an alternative to using restriction endonuclease fragment primers. Having established the sequence of a DNA template, it should be possible to synthesize an oligonucleotide whose 3'OH terminus is adjacent to the site desired for mutagenesis. Thus, one is not limited by the proximity of available restriction cleavage sites to a target sequence. In this paper we have demonstrated that an oligonucleotide primer can

be sequentially elongated and used to produce mutations at an efficiency similar to that obtained with restriction fragments. Using these methods it should also be possible to prepare a series of primers by multiple single step elongations from a single oligonucleotide. Such an array of primers, each obtained from an elongation step, could be used to produce a series of molecules each of which contain a single-base substitution at a different position. Furthermore, an oligonucleotide primer that contains an internal base-substitution should allow one to potentially synthesize multiple specified mutations, one of which being introduced by the oligonucleotide and others produced by enzymatic mis-insertion. A number of different sites that yield color changes on indicator plates using M13mp 2 phage (21,22) contain single base substitutions which if within the starting primer can be used as a basis for preselection of the newly synthesized product strand. Traboni et al (11) have used a selectable phenotype provided by the sequence of a modified primer to select for mis-insertion mutants at very high efficiency in DNA segments cloned into an M13 mp2 vector.

To provide a 3'OH terminus adjacent to a preselected site on ϕ X174 DNA templates, we have in a series of in vitro DNA synthesis reactions selectively elongated both a restriction endonuclease fragment primer and a synthetic oligonucleotide primer using T4 DNA polymerase in the presence of different combinations of deoxynucleotide triphosphate substrates. We have substituted T4 DNA polymerases for E. coli Pol I since the ratio of 3'→5' exonuclease to polymerase activity with T4 DNA polymerase is approximately 100 times greater than that exhibited by Pol I (23). This presumably accounts for its higher fidelity during DNA synthesis in vitro (20). The weaker proof reading activity of Pol I provides a likely explanation for the increased reversion frequency observed for this enzyme at the am18 locus when we compared the products from the elongation reactions that used different enzymes (Table II). Thus, the use of T4 DNA polymerase with its strong proofreading activity allows for the faithful elongation of primers. The position of the 3' OH terminus of a primer can also be modified by taking advantage of the 3'→5' exonuclease activity of a proofreading enzyme as described by Traboni, et al. (11) using the Klenow fragment of E. coli DNA polymerase I. Likewise, T4 DNA polymerase could be used to shorten the length of a primer; however, this approach is limited to primers in which stability of the hybrid is not lost following the exonuclease reduction.

In a previous report (10), we had elongated a restriction primer several nucleotides distant from a site selected for mutagenesis. Elongation to the site selected for mutagenesis was combined with incorporation of one of the noncomple-

mentary nucleotides present in the elongation reaction. In this approach, control over which non-complementary nucleotide mis-inserted is limited to those which were required for elongation. This problem was overcome by elongation to produce a 3'OH terminus one nucleotide away from a preselected position on a DNA template chosen for site specific mutagenesis. This allowed mis-insertion mutagenesis using a single specified nucleotide. The specificity of mis-insertion was confirmed by sequencing products of the reaction (Table II). By screening a population enriched for copied molecules, we obtained each specified single-base mis-insertion at high frequency. Using AMV DNA polymerase, product phage populations were isolated which contained a mis-insertion frequency greater than 50% for selected targets in the lacZ region cloned into bacteriophage M13mp2 (Kunkel, T.A. personal communication). Deletions and insertions that could also occur during copying were eliminated by S1 treatment. AMV DNA polymerase lacks a 3'→5' exonuclease and exhibits low fidelity (24) which presumably facilitates mis-insertion. Since the error-rate of a polymerase is proportional to the ratio of non-complementary to complementary nucleotides in the reaction mixture (9), we have termed the presence of only a single nucleotide as an "infinite pool bias" (10). Given sufficient time, any non-complementary nucleotide should be incorporated and not excised by AMV DNA polymerase. Multiple mis-incorporations should be very infrequent since elongation would require synthesis on a non-base paired primer.

Enzymatic methods for the introduction of single base substitution mutations into a DNA molecule combined with the procedures for selective elongation of a primer terminus provide several advantages for the site-specific modification of DNA. First, any type of base substitution mutation can be introduced. We have directed the formation of both transition and transversion mutations at two different sites in a genome. Second, starting from a single primer, provided either from a restriction endonuclease fragment or from a synthetic oligonucleotide, the biological effects of a cluster of different specified single base substitution mutations can be studied. Using the enrichment procedures described here and previously (20), it should be possible to generate a library of different mutants that could be screened by DNA sequence analysis. Third, selective elongation of specifically gapped molecules, obtained by procedures similar to those described by Shortle, et al, (7), could be used to generate a primer terminus which would allow one to insert single specified nucleotides into a double stranded DNA molecule. The use of AMV DNA polymerase for gap mis-repair mutagenesis could perhaps also be more efficient than the long incubations that were reported for the procedures using Micrococcus

luteus DNA polymerase I and might perhaps diminish the number of small deletions observed (7). Finally, mis-insertion mutagenesis may provide an alternative to other procedures for site-directed mutagenesis in certain situations. For example, when a secondary structure prevents the annealing of an oligonucleotide selected for site specific mutagenesis, base substitutions may be introduced by mis-insertion from an elongated restriction fragment primer. It is becoming increasingly apparent that the choice of protocol for site-specific mutagenesis, synthetic oligonucleotides, gap-misrepair or mis-insertion during primer elongation is dictated by the nature of the problem. For a single specified substitution, a chemically synthesized oligonucleotide is frequently the most advantageous approach. Gap misrepair is presently limited to sequences obtained by restriction enzymes, while mis-insertion is particularly advantageous for obtaining a series of specific mutations across a gene starting from a single primer.

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