Site-specific mutagenesis using asymmetric polymerase chain reaction and a single mutant primer

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ABSTRACT

A method is described for preparing site-specific mutants using a polymerase chain reaction (PCR) based protocol. The protocol requires a single mutant primer, and has been used to introduce mutations into DNA fragments ranging in size from 200 bp to 1569 bp in length in the GM-CSF, β-actin, human growth hormone and erythropoietin genes. Sequence analysis of PCR derived mutant fragments shows an error rate of less than one bp change per 1500 bp incorporated. Single base pair mutations have been introduced into these genes which create unique restriction sites. We demonstrate that these mutant templates may be used for competitive PCR to quantitate mRNA and DNA. This method thus offers a rapid means for producing competitive templates for use in quantitative PCR.

INTRODUCTION

Higuchi *et al.* (1) have recently described a method utilizing polymerase chain reaction (PCR) to create site-specific mutant templates. Although this technique is rapid and reliable compared with standard methods (2), limitations include necessity for using two mutant primers, and difficulty in generating mutants greater than 500 bp in length. We have modified this technique so that (i) only one mutant primer is required, and (ii) mutations can be introduced into DNA fragments as long as 1500 bp. In brief, a large, single stranded mutant primer (100−1400 bp) is synthesized through PCR by means of a short mutant primer (<30 bp) and wildtype 5′ primer. After removal of unincorporated primers, the large mutant primer is added to a standard PCR mix with wildtype 3′ primer and a catalytic amount of wildtype template. Subsequent PCR generates a mutant DNA fragment of the desired length.

We demonstrate that mutants constructed using this technique can be used as templates for competitive PCR to quantitate mRNA and DNA species by PCR (3, 4).

MATERIALS AND METHODS

Nucleotide triphosphates were HPLC grade 0.1 M stocks (US Biochemicals): dATP # 314244; dCTP # 314279; dGTP # 14314; TTP # 22324 and diluted to final concentrations of 2.5 mM. 10×PCR Buffer contained 500 mM KCl, 100 mM Tris-EDTA pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin. Taq polymerase (Perkin Elmer Cetus #N801-0046-01) was used at a final concentration of 5 units/ml. Oligonucleotide primers were synthesized by the β-cyanoethylphosphoramidite method on an Applied Biosystems 380B automated synthesizer, purified on NAP-10 columns (Pharmacia # 17-0854-01) diluted into stock solutions of 5 pmoles/μl in dH_2O. All flanking primers were 30 mers with an approximate G-C content of 50%. Mutagenic primers were 15 to 30 mers with one to four base pair changes which create a new unique restriction enzyme site and/or ablate an existing site, without changing the amino acid sequence of the translated product. Restriction enzymes were purchased from Boehringer Mannheim Biochemicals.

Site specific mutagenesis using two mutant primers

The method of Higuchi *et al.* (1) requires two separate amplifications utilizing two mutant primers (Fig. 1). In one reaction 1.2 nmoles of plasmid containing the DNA fragment of interest was mixed with: 10×PCR buffer (10 μl), dNTP (8 μl), 5′ flanking primer (4 μl), antisense mutant primer (4 μl) and Taq polymerase (0.1 μl) in a final volume of 100 μl. In a second reaction 1.2 nmoles of plasmid containing the DNA fragment of interest was mixed under identical conditions with a 3′ flanking primer and the sense mutant primer. Eighteen cycles of amplification at 37°C annealing were performed, generating one mutant fragment with the mutation at the 3′ end and another mutant fragment with the mutation at the homologous 5′ end. Removal of excess primers and nucleotides was achieved by dilution of the PCR products in 2 mls of dH_2O, transfer to a Centricon-100 centrifugal microconcentrator (Amicon #S0347) and centrifugation at 2K for 10 minutes in a Sorvall RT6000B centrifuge. The two mutant fragments were mixed in a 1:1 molar ratio in the presence of flanking primers under standard PCR conditions and amplified for 20 additional cycles to give the full length mutant fragment.

Site specific mutagenesis using a single mutant primer

Using asymmetric PCR, a single-stranded mutagenic fragment was prepared with the mutation at the 3′ end by means of a single mutant primer (primer 3, Fig. 2) and the corresponding flanking primer (primer 1, Fig. 2). This single stranded mutant fragment

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served as a primer and, in combination with the 3′ flanking primer (primer 2, Fig. 2), permitted amplification of full length double stranded mutant product from a catalytic amount of the wildtype DNA template. The initial amplification was performed with 0.05 pmoles of plasmid containing the DNA fragment of interest in 100 μl volume containing 10×PCR buffer (10 μl), dNTP (8 μl), 5′ flanking primer (100 pmoles), antisense mutant primer (1 pmole) and 0.5 units Taq polymerase. Asymmetric amplification was performed at 37°C−42°C annealing temperature for 30 cycles. Single stranded mutant primer was purified by gel electrophoresis (1% agarose for fragments > 800 bp or 1% agarose/2% Nusieve for fragments < 800 bp) with Geneclean (Bio 101 #3105), as recommended by the manufacturer, in a final volume of 60 μl of dH_2O per 0.5 μg of DNA. Agarose gel electrophoresis was performed on an aliquot of the Geneclean product to estimate the concentration of mutant fragments.

The second amplification to generate full length mutant fragment was performed using 3′ flanking primer (5 pmoles), amplified single stranded mutant fragment (5 pmoles), 10×PCR buffer (10 μl), dNTP (8 μl), wildtype DNA (0.001−0.01 attomoles), and Taq polymerase (0.1 μl) in a final reaction volume of 100 μl. 30 cycles of amplification were performed at 52°C−62°C annealing temperature.

Restriction digest of mutant fragments

A 20 μl aliquot of the PCR product was digested with 20 units of the appropriate restriction enzyme for 3−4 hours using recommended assay conditions. 100 ng of wildtype DNA was digested under identical reaction conditions. Digested products were gel electrophoresed as previously described.

Sequence analysis of PCR products

Mutant fragments were subcloned into Bluescript KS (+) plasmid (Stratagene) and sequenced by a modification of the method of Sanger (5) using Sequenase (US Biochemicals).

RESULTS AND DISCUSSION

Use of a single mutant primer to prepare site-specific mutants

Site specific mutagenesis by PCR is a versatile and reliable tool. Using the method of Higuchi *et al.* (1) we readily synthesized mutant DNA fragments up to 600 base pairs with few background bands. However we found that efficiency of amplification declines and product yield is markedly decreased when fragment size exceeds 600 bp or when the desired mutation is close to one end of the fragment.

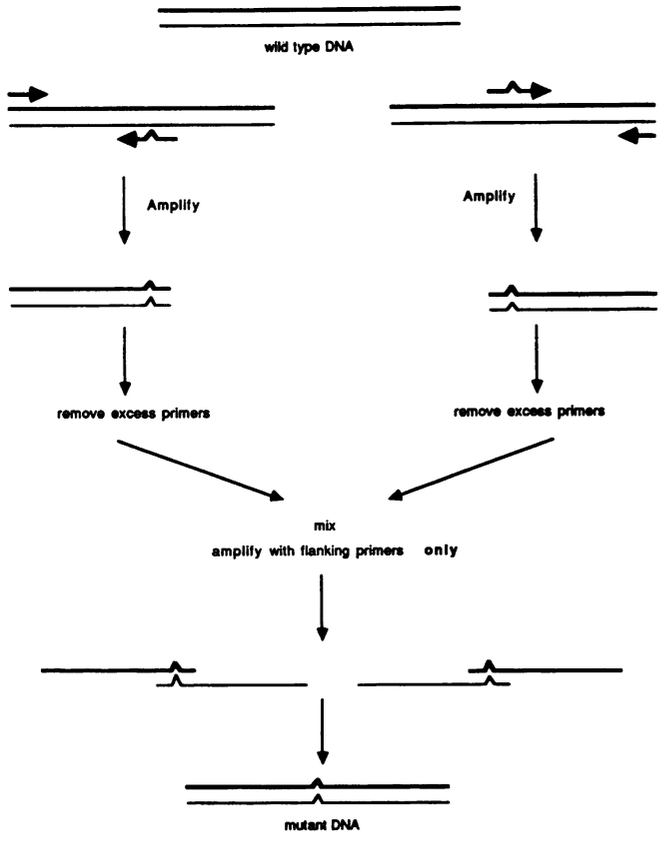

Figure 1. Schematic representation of site specific mutagenesis using overlap extension with two mutant primers and two flanking primers, as previously described (1).

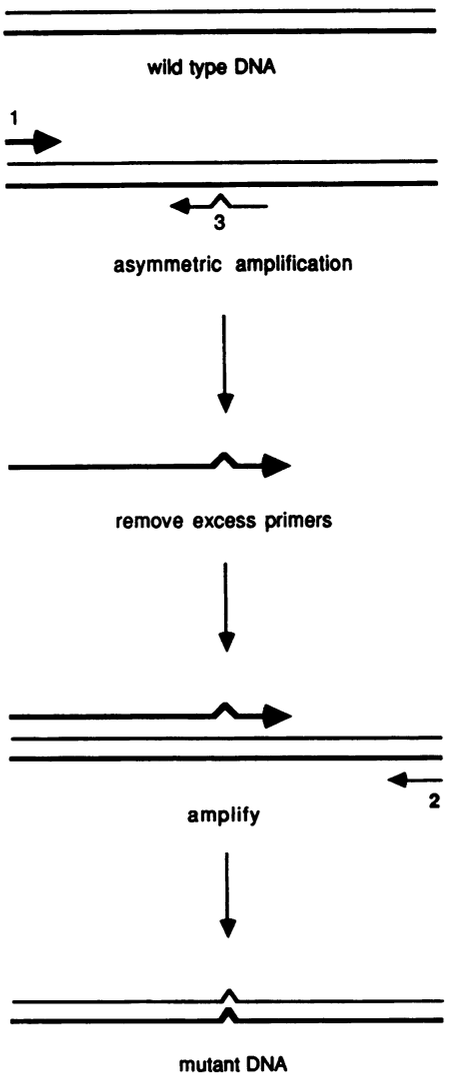

Figure 2. Schematic representation of site specific mutagenesis with a single mutant primer and two flanking primers. A primary mutant fragment is used as a primer on a wildtype template to generate full length mutant product.

The basis for low product yield with long fragments or fragments with offset mutations may be lack of stability of overlapped heteroduplexes (Fig. 1). To obtain full length mutants with this technique, the two primary mutant fragments must reanneal in an overlapping fashion in the area of the mutation such that the recessed 3′ OH ends can be extended by Taq polymerase in a 5′ to 3′ direction (overlap extension). The complementary overlapping region is produced from the homologous sense and antisense mutant primers used in the first round of amplification, and is therefore limited by the length of the mutant primers. The most stable reannealed products during amplification are the primary mutant templates themselves, rather than a product arising from the overlap of homologous mutated 5′ and 3′ ends. This problem is accentuated when long or offset mutant fragments are being synthesized.

We have circumvented this problem by preparing a long single stranded mutant primer using asymmetric PCR and a single mutant primer. The mutant primer anneals with high efficiency to a 'catalytic' amount of wildtype DNA template, and the desired mutation in the primer is incorporated into PCR product in the

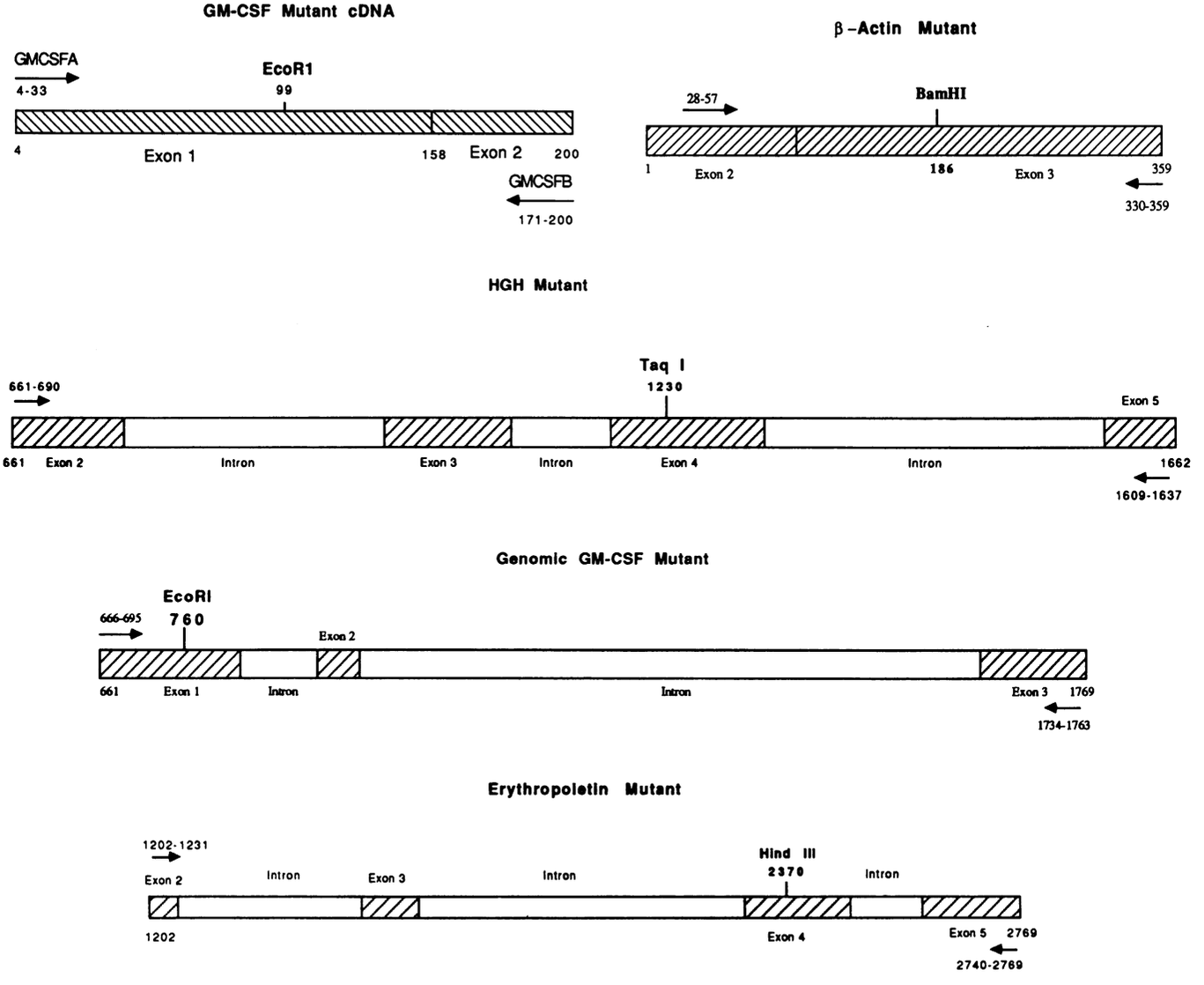

Figure 3. Diagram showing location of primers and mutations in GM-CSF, β-actin, HGH and epo genes. Primer 1 designates the 5′ sense primer, primer 2 is the 3′ antisense primer, and primer 3 is the antisense mutant primer. In experiments where two mutant primers were used, an additional complementary mutant primer was prepared. Mutant bases are shown in bold print. The four bp changes in the erythropoietin gene create a HindIII site and ablate an AccI site.

A) GM-CSF:	Primer 1	5′GGCTGCAGAGCCTGCTGCTCTTGGGCACTG 3′
	Primer 2	5′TTGGAGGGCAGTGCTGCTTGTAGTGGCTGG 3′
	Primer 3	5′GCCGGGCCTCCTGGATGG**A**ATTCACATGCT 3′
B) β-actin:	Primer 1	5′GTCGACAACGGCTCCGGCATGTGCAAGGCC 3′
	Primer 2	5′GGTCATCTTCTCGCGGTTGGCCTTGGGGTT 3′
	Primer 3	5′GTGAGGAT**C**CCTCTCT 3′
C) HGH:	Primer 1	5′GCAGTGCCTTCCCAACCATTCCCTTATCCA 3′
	Primer 2	5′AGATCTGCCCAGTCCGGGGGCTGCCATCTT 3′
	Primer 3	5′CACGGGCTC**G**AGCCACGACTG 3′
D) epo:	Primer 1	5′ATGTCCTGCCTGGCTGTGGCTTCTCCTGTC 3′
	Primer 2	5′TCTGTCCCCTGTCCTGCAGGCCTCCCCTGT 3′
	Primer 3	5′GCTGGGAAGA**A**TTGACCAACA**AA**GC**T**TGGC 3′

presence of a 3′ flanking antisense primer (primer 2, Fig. 2). This approach precludes problems with forming duplexes with short overlaps, and is less expensive since only one mutant primer is necessary.

Using this approach, we have prepared site-specific mutants ranging from 200−1600 bp. As shown in Fig. 3, mutations are 1−3 bp changes which generate and/or ablate unique restriction sites in the genes for erythropoietin (epo), human growth hormone (HGH), β-actin, and granulocyte-macrophage colony stimulating factor (GM-CSF). A G-T substitution at bp 103 in the GM-CSF gene creates a unique EcoRl site at bp 99. Amplified wildtype GM-CSF is not cleaved by digestion with EcoRl, but the mutant GM-CSF product gives two bands at 99 and 103 bp which comigrate (Fig. 4, Lanes 1−4). In another example, a C-G substitution at bp 189 in the β-actin gene generates a unique BamHl site. Digestion of the mutant fragment with BamHl yields two fragments of 171 bp and 162 bp (Lanes 5−8, Fig. 4).

A single mutant primer was also used to prepare mutant fragments greater than 600 bp and offset mutations. A G-C substitution at bp 1232 in the HGH gene creates a unique Taql site (Fig. 3). The mutant HGH fragment is 978 bp and gives fragments of 571 and 407 bp on digestion with Taql (not shown). A G-T substitution at bp 762 of genomic GM-CSF creates a unique EcoRl site (Fig. 3). Although this mutation is close to the 5′ end of the gene, single primer mutagenesis readily produced the desired mutant. Synthesis of the offset 1097 bp GM-CSF mutant using double and single stranded mutant primers is shown in Fig. 5. Finally, a 1569 bp mutant erythropoietin has been prepared with 4 bp substitutions (G-A at 2371, C-T at 2374 and 2375, C-T at 2386 in a single mutant primer) which create a HindIII site at 2370 and ablate an Accl site at 2382 without changing coding (not shown).

There are small amounts of contaminating bands present in these unpurified preparations. For example, in Lane 4 a small amount of uncleaved (wildtype) product is seen. For this reason, mutant products are purified from gels using Geneclean, subcloned, and sequenced before further use. In addition, errors in wildtype sequence occur rarely using this approach, at a frequency less than one error per 1600 bp of incorporated.

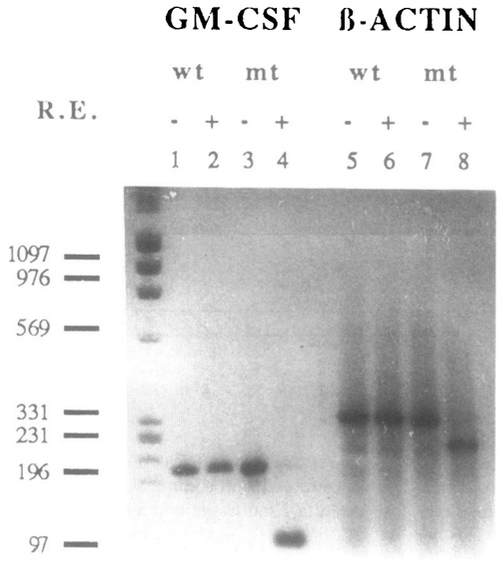

Figure 4. Preparation of site specific mutants using a single mutant primer. Mutations in GM-CSF (Lanes 1−4) and β-actin (Lanes 5−8) are shown. Each set of four lanes shows (i) amplified wildtype DNA, (ii) amplified wildtype DNA with appropriate restriction enzyme added, (iii) amplified mutant DNA and (iv) amplified mutant DNA with restriction enzyme added. As described in the text, wildtype DNA is not cleaved by restriction enzyme, but mutant DNA is cleaved at the expected location. The mutant fragment was purified from gels, subcloned and sequenced as described in Materials and Methods.

Use of a double stranded mutant 'primer' for site specific mutagenesis

Although use of asymmetric PCR to produce single stranded mutant primers is effective, the yield of mutant single stranded product is more variable in our hands than production of ds mutant templates using standard 'symmetric' PCR. One would predict that ds mutant DNA would function equally well in site specific mutagenesis, since the antisense mutant strand should not participate in PCR. Therefore, we tested ds mutant GM-CSF to determine whether it would be as effective as ss mutant GM-CSF in preparing site specific mutants.

ss mutant GM-CSF DNA migrates more slowly on agarose gels than does ds mutant DNA, with an apparent molecular weight of approximately 1200 bp (Lanes 1−2, Fig. 5). However, a comparison of the ss and ds mutant GM-CSF DNA shows that these two forms of mutant GM-CSF are equally effective for preparing site specific mutants (Lanes 3−6, Fig. 5). The expected bands are produced with each template, and there is no apparent difference in background bands or wildtype DNA template after digestion with restriction enzyme. As expected, wildtype GM-CSF does not digest with EcoRl (Lanes 7−8, Fig. 5). Based on these results, we routinely use ds mutant DNA templates for preparation of site specific mutants.

Optimization of PCR based site-specific mutagenesis reactions

We have examined several reaction variables in order to optimize single mutant priming. One factor that decreases final product yield is the concentration of contaminating mutant primers remaining after Centricon-100 centrifugation. Although repetitive centrifugation in microconcentrators may remove greater than 99% of the mutant primers from the primary amplifications, even small amounts of these primers will interfere with formation of long mutant products. For this reason, we purify our primary

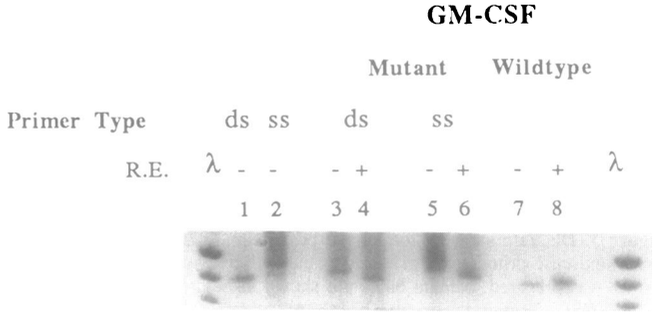

Figure 5. Comparison of ds mutant DNA vs. ss mutant DNA templates for PCR-based site specific mutagenesis. Single and double stranded forms of an 121 bp mutant genomic GM-CSF fragment were prepared as described in Materials and Methods. Lanes 1−2: electrophoretic mobility of *ss* and *ds* GM-CSF mutant fragments. Lanes 3−4: Mutant GM-CSF (1102 bp) prepared using *ss* mutant GM-CSF primer, with and without addition of EcoRl. Lanes 5−6: Mutant genomic GM-CSF (1102 bp) prepared using *ds* mutant GM-CSF primer, with and without addition of EcoRl. lanes 7−8: Wildtype GM-CSF with and without addition of EcoRl.

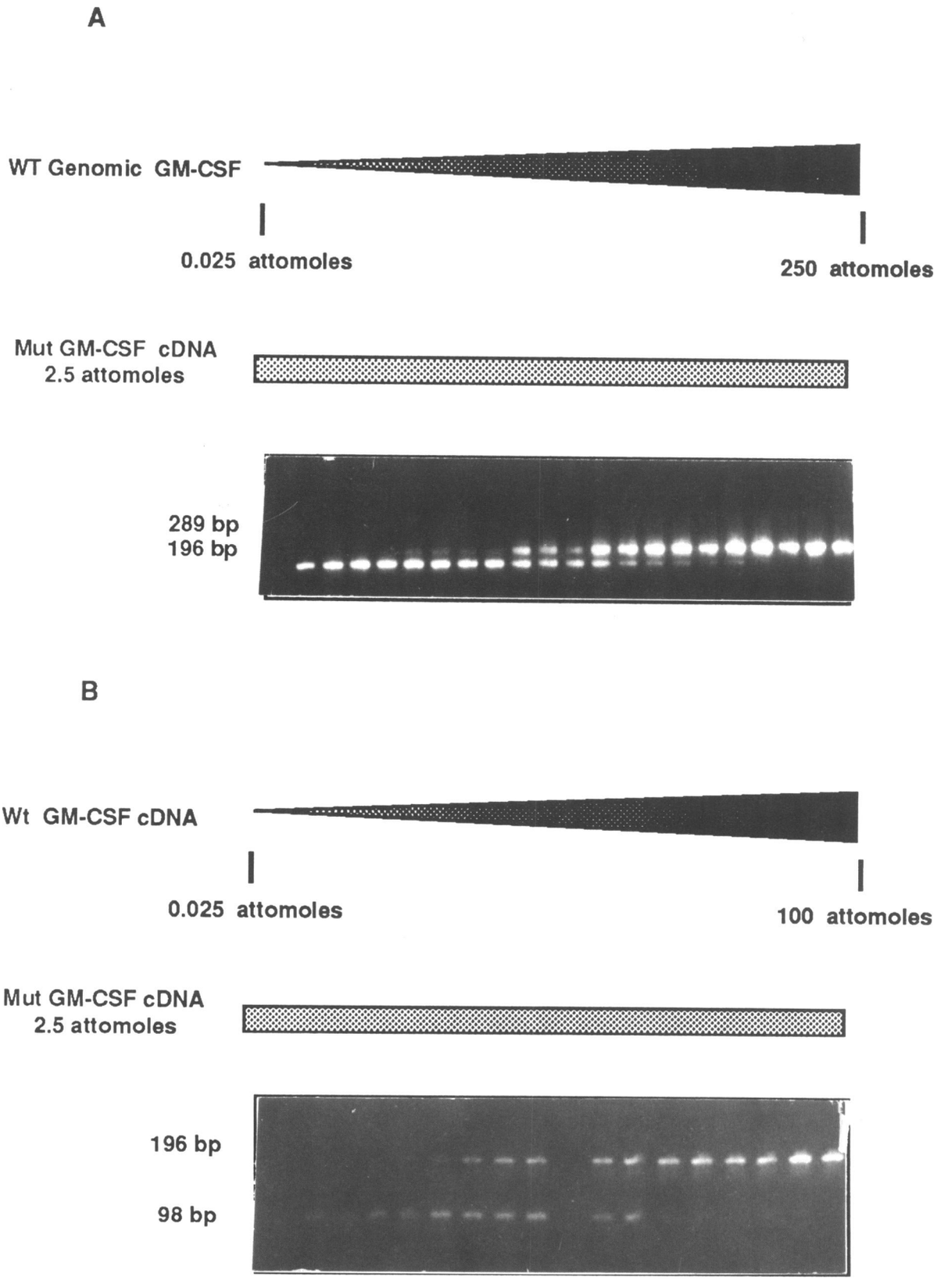

Figure 6. Use of mutant GM-CSF template in competitive PCR. A fixed concentration of mutant GM-CSF (2.5 attomoles/tube) was added to a master mix which contained dNTP (200 μM in each, including [α-^{32}P]dCTP at 50 μCi/ml), GM-CSF primers 1 and 2 (20 pmoles/tube), 1×PCR buffer, Taq polymerase (0.1 μl/tube). The master mix containing the mutant GM-CSF cDNA was aliquoted into separate tubes containing (A) genomic GM-CSF DNA ranging in concentration from 0.025 to 250/tube or (B) wildtype GM-CSF cDNA ranging in concentration from 0.025 to 100 attomoles/tube. Samples were amplified for 40 cycles of PCR as described above, and electrophoresed on 2% Nusieve/1% agarose gels. Input mutant GM-CSF cDNA is calculated as described (4). Briefly, the ratio of mutant GM-CSF cDNA/genomic GM-CSF is determined for each tube by excising and counting the appropriate bands, and plotted as a function of input genomic GM-CSF in the titration series. Where the ratio is 1.0, input genomic GM-CSF from the titration series is equivalent to the amount of input mutant GM-CSF cDNA.

mutant fragments by gel electrophoresis and Geneclean rather than by Centricon-100 in order to assure complete removal of all mutant primers before secondary amplification.

Experiments were performed to determine the optimal amount of wild type DNA to use as a 'catalyst' for production of site specific mutants, since too high a concentration of wildtype DNA in the reaction mixture would result in undesired products. Titration experiments showed that the optimum range for mutant production with minimal contaminating wildtype amplification was 0.001−0.01 attomoles of wildtype template.

It is important that primers be added in equimolar ratios to prevent asymmetric PCR and generation of single stranded product in the final reaction mix. When using a long mutant fragment as a primer in secondary amplifications, use of equimolar ratios of primers is more difficult because the long mutant fragments have significantly higher molecular weights than do the 30 bp flanking primers. We have performed experiments titrating the concentration of mutant primer fragments against a fixed concentration of flanking primer. We found a significant decrease in product yield and increase in background bands if molar concentrations of mutant and flanking primer vary by more than two fold.

Application of site-specific mutants for competitive PCR

Site-specific mutants prepared by single primer mutagenesis may be used for competitive PCR to quantitate DNA and mRNA species (2−4). To demonstrate this application, a mutant GM-CSF cDNA template containing a unique EcoRl site (2.5 attomoles/tube) was co-amplified with a dilution series of genomic GM-CSF plasmid ranging from 0.025 to 250 attomoles/tube (Fig. 6A). As would be predicted, the mutant template acted as a competitive inhibitor of the production of genomic GM-CSF during the PCR reaction, and allowed quantitation of input mutant GM-CSF cDNA as described in the legend to Fig. 6. In this example, the amount of input mutant GM-CSF cDNA (2.5 attomoles/tube) correlated well with a calculated value of 2.4 attomoles/tube.

Because of the presence of a restriction site, mutant GM-CSF cDNA can also be used for competitive PCR with wildtype GM-CSF cDNA. A dilution series of wildtype GM-CSF cDNA (0.025−100 attomoles/tube) was co-amplified with mutant GM-CSF cDNA (2.5 attomole/tube, Fig. 6B). After amplification, wildtype GM-CSF cDNA was distinguished from mutant GM-CSF by digestion with EcoRl. The ratio of mutant GM-CSF cDNA to wildtype GM-CSF cDNA was determined, and the amount of input GM-CSF cDNA was calculated to be 2.4 attomoles. Mutants prepared using this method can thus be used as competitive templates for quantitation of DNA as well as cDNA reverse transcribed from mRNA.

Use of a single primer PCR mutagenesis is an effective, rapid and cost effective method for preparing mutants. The technique offers all the advantages and opportunities of standard site specific mutagenesis techniques, but is far less time consuming and labor intensive. Once primers are available, the entire mutagenesis process takes approximately 24 hours. Subcloning and sequencing product is still required, but is far less time consuming than with standard protocols in that virtually every transformed colony will contain the appropriate mutant. Under these reaction conditions, sequence errors apart from the desired mutation are rare. Site-specific mutants may be used for a broad range of applications, including rapid production of competitive templates for quantitative PCR.

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REFERENCES

1. Higuchi,R., Krummel,B. and Saiki,R.K. (1988) *Nucleic Acids Res.* **16**, 7351−7367.
2. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
3. Gilliland,G., Perrin,S. and Bunn,H.F. (1990) In Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (ed.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 60−69.
4. Gilliland,D.G., Perrin,S.P., Blanchard,K.B. and Bunn,H.F. (1990) Analysis of cytokine mRNA and DNA: Detection and Quantitation using Competitive Polymerase Chain Reaction. *Proc. Natl. Acad. Sci. USA* (in press).
5. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463−5467.