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A multisite-directed mutagenesis using T7 DNA polymerase: application for reconstructing a mammalian gene

(Recombinant DNA; porcine growth hormone; oligodeoxynucleotides; presequence; heterologous gene expression; deletions; insertions)

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SUMMARY

A method to introduce multiple mutations and to reconstruct genes, using a single oligodeoxyribonucleotide and DNA polymerase with high processivity, such as modified T7 DNA polymerase [Tabor and Richardson, Proc. Natl. Acad. Sci. USA 84 (1987a) 4767–4771], is described. A eukaryotic cDNA, coding for porcine growth hormone (pGH), was reconstructed in this study to delete 75 bp and to introduce a G → A transition. The deletion removes 75 bp and brings an ATG just upstream from the codon for the first amino acid in the mature protein. Moreover, the G → A substitution creates a new *PvuII* restriction site to facilitate further manipulation of the gene. Maximum mutation frequency with this multisite-directed mutagenesis is reached within 15 min with an efficiency approaching 50%, when using the modified T7 DNA polymerase. No multisite-directed mutants were obtained when T4 DNA polymerase or Klenow (large) fragment of DNA polymerase I were used. The described method is also applicable to simple single site-directed mutations as well as to more complex gene reconstruction strategies.

INTRODUCTION

In vitro mutagenesis, random or site-specific, provides a powerful tool to study the biological activity

of genes and their proteins (Smith, 1985). Another major potential application for site-directed mutagenesis is in gene reconstructions for the purpose of heterologous gene expression of eukaryotic genes in

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human; HPLC, high-performance liquid chromatography; ligation buffer, see MATERIALS AND METHODS, section a; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; pfu, plaque-forming units; pGH, porcine growth hormone; Polk, Klenow (large) fragment of *E. coli* DNA polymerase I; RF, replicative form; ss, single strand(ed).

Abbreviations: aa, amino acid(s); b, bovine; bp, base pair(s); ds, double strand(ed); DTT, dithiothreitol; GH, growth hormone; h,

prokaryotic cells. The application of site-directed mutagenesis to eukaryotic gene reconstruction for expression in bacteria has been very limited. Sollazzo et al. (1985) have constructed deletion mutants in ss vectors in *Escherichia coli*. Site-specific methods that employ phenotypic selection have improved the efficiency of in vitro mutagenesis (Kunkel, 1985; Kunkel et al., 1987). In this paper we report an improved and efficient way to do major gene reconstructions in a single step by site-directed mutagenesis using pGH cDNA as an example, and by employing modified T7 DNA polymerase (Tabor and Richardson, 1987a,b). Modified T7 DNA polymerase is stable, processive, has a high rate of DNA polymerization (> 300 nt/s), and is essentially 99% free of its 3'-to-5' exonuclease activity (Tabor and Richardson, 1987a,b; Tabor et al., 1987; Huber et al., 1987). These features suggested the use of modified T7 DNA polymerase for site-directed mutagenesis.

MATERIALS AND METHODS

(a) Plasmid constructions

The pMC1403 plasmid containing the full-length pGH cDNA was generously supplied by S.Z. Qi (Beijing Agricultural University). This clone was selected from pig pituitary gland cDNA library using two synthetic oligo probes (Xia, 1987). A *Bam*HI-*Eco*RI fragment containing the pGH coding sequence was removed from pMC1403 and subcloned in M13mp18 (Messing, 1983) in the corresponding sites. Plasmid pMC1403 and dsDNA of M13mp18 RF were mixed at a ratio of 4:1 and cleaved simultaneously with *Bam*HI + *Eco*RI. Completeness of the digestion was determined by 0.8% agarose gel electrophoresis. The DNA was extracted with phenol/chloroform and precipitated with 2.5 vols. of cold ethanol. After washing with 70% ethanol, DNA was dried and resuspended in 1 × ligation buffer (10 mM Tris · HCl pH 7.4, 10 mM MgCl₂, 50 mM NaCl, 0.5 mM DTT). T4 DNA ligase (0.5 unit) and ATP (to 1 mM) were added to ligate the resulting restriction fragments. The reaction mixture was then used to transfect JM101 (Yanisch-Perron et al., 1985). White plaques were selected and ssDNA was isolated and sequenced to verify the 5' terminus.

(b) DNA preparations

All plasmid DNA preparations were made using the mini alkaline lysis method (Birnboim and Doly, 1979). M13 dsDNA or ssDNA was prepared using Messing's (1983) method.

(c) Site directed mutagenesis

(1) *The oligodeoxynucleotide*

An oligo of 34 nt complementary to pGH coding gene in the 'plus' (+) strand of M13 was prepared by the phosphoramidite method using an Applied Biosystems 380A DNA synthesizer and then purified by HPLC. The oligo sequence is as follows: 5'-CAGCTCACCAGCTGTGATGTTCCCAGC-CATGCC-3'.

This oligo is homologous to two different regions in the pGH gene and also contains a single nt substitution of G → A at the nt position -7. The oligo was phosphorylated by T4 polynucleotide kinase (Maniatis et al., 1978) using a trace quantity of [γ -³²P]ATP in addition to the cold ATP to monitor the kinase reaction. Following the reaction the unincorporated ATP was removed by precipitation of the oligo with 3 vols. of cold 100% ethanol, and washed twice with cold 70% ethanol. The oligo was dried and then resuspended in sterile double-distilled water.

(2) *The procedures*

The recombinant bacteriophage M13mp18 containing pGH-coding cDNA (M13.pGH) was used to infect *Escherichia coli* strain RZ1032 (*ung*⁻*dut*⁻) (Kunkel et al., 1987). This step produces a DNA template containing a small number of uracil residues in place of thymine (Kunkel, 1985). After three propagative cycles of infection in the RZ1032 strain, M13.pGH ssDNA was prepared. Samples containing this DNA (500 ng/reaction) were used to test three different reactions to make the site-directed changes. Two reactions (**A** and **B**) served as controls. In all these protocols, the mutagenic oligo was first annealed to the DNA.

(A) One aliquot was treated according to Kunkel's (1985) procedure using T4 DNA polymerase and T4 DNA ligase for 30 min at 37°C.

(B) A second sample was incubated with PolIk at

0°C overnight followed by T4 DNA ligase. In another experiment requiring the introduction of a limited number of nt substitutions these modifications had increased the fraction of mutants recovered (El-Gewely et al., 1988).

(C) A modified T7 DNA polymerase (Tabor and Richardson, 1987) known commercially as Sequenase™ (United States Biochemicals Corporation, P.O. Box 22400, Cleveland, OH 44122) was

added to the final sample. The steps and buffers employing this enzyme are described below.

(3) Buffers and solutions

(i) 5 × T7 polymerase buffer: 200 mM Tris · HCl, pH 7.5, 100 mM MgCl₂, and 250 mM NaCl.

(ii) Deoxyribonucleotide mix (dNTP) for site-directed mutagenesis: 500 μM dATP, 500 μM dGTP, 500 μM dCTP, 500 μM dTTP, and 50 μM

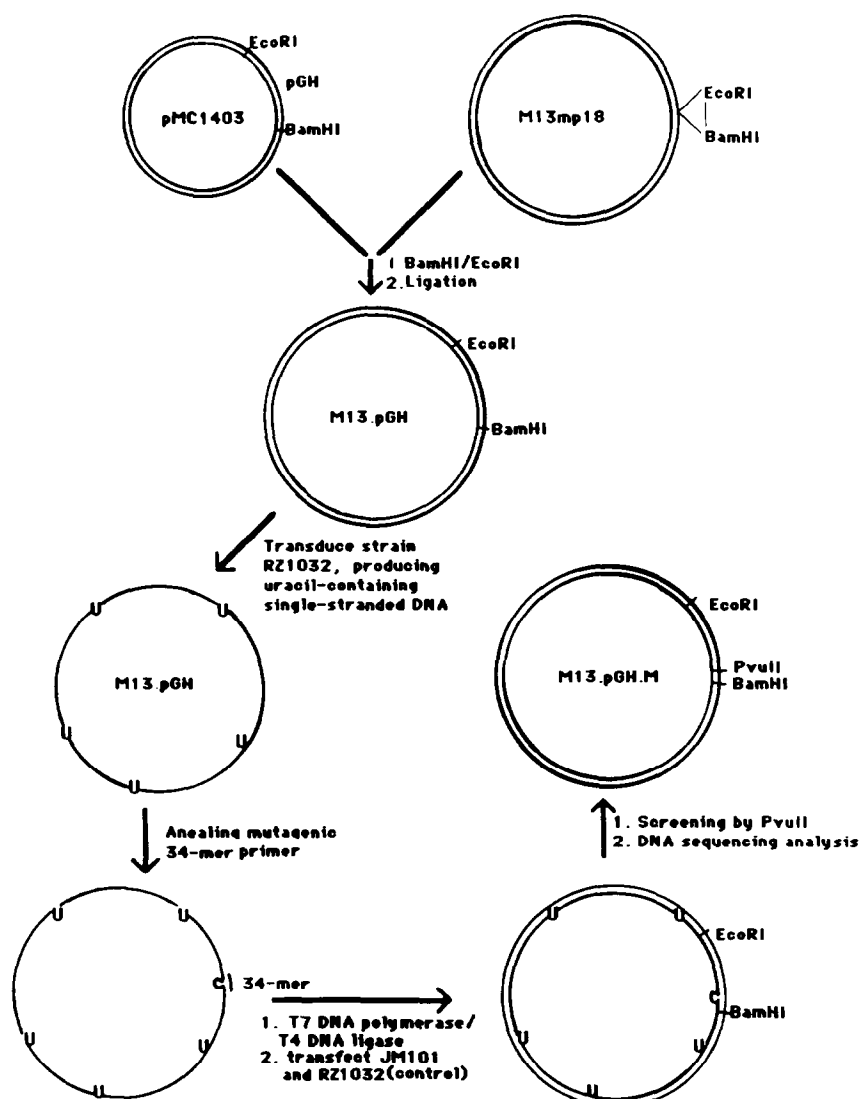


Fig. 1. The strategy for the multisite-directed mutagenesis of pGH-coding cDNA by T7 polymerase method. The *Bam*HI-*Eco*RI fragment containing pGH cDNA was inserted in M13mp18 and this recombinant bacteriophage was designated M13.pGH. A U-containing M13.pGH DNA template was prepared after growing in RZ1032 strain (*ung*⁻ *dut*⁻) for two or three cycles. The resulting template was used to anneal the mutagenic synthetic 34-mer (looping out 75 bp, see Fig. 2) and the complementary strand was made by modified T7 DNA polymerase and T4 DNA ligase, followed by transfecting strains JM101 (*ung*⁺ *dut*⁺) and RZ1032 (as control). The survivors on JM101 were screened for putative mutants by the appearance of a new *Pvu*II restriction site and sequencing analysis.

DTT. This mix was made in $1 \times$ T7 polymerase buffer.

(iii) ATP solution: 10 mM ATP, in sterile double-distilled water.

(iv) Primer/oligo solution: the phosphorylated primer/oligo was diluted with sterile double-distilled water to a final concentration of 0.5 pmol/ μ l.

(4) Annealing reaction

In a small eppendorf tube the following were mixed: 3 μ l of DNA template (approx. 500 ng, 0.19 pM), 4 μ l H₂O, 1 μ l primer (0.5 pM), 2 μ l $5 \times$ T7 polymerase buffer.

This mix was heated at 65°C for 5 min, then the temperature was allowed to cool slowly to room temperature over a period of 60 min. After incubation at room temperature for 15 min, the sample was incubated on ice for an additional 15 min.

(5) Extension and ligation reaction

The following were added to the above mix (on ice): 2 μ l of dNTP mix, 1.5 μ l of ATP solution, 1 μ l (14 units/ μ l) of modified T7 polymerase (SequenaseTM), and 2 units of T4 DNA ligase.

The reaction was allowed to proceed at 37°C for

5, 10, 15, 30, and 60 min to test the effect of time on mutation frequency. The reaction was stopped by heating at 65°C for 5 min. The effect of adding more ligase (5 units) in the reaction mix was also tested. Aliquots from each experiment were used to transfect RZ1032 or JM101 competent cells.

(d) Restriction enzyme analysis for the screening of putative mutants

Twelve different plaques from each reaction/experiment that survived on JM101, were picked and their DNA was isolated using the alkaline lysis method (Birnboim and Doly, 1979). The isolated ds DNA was then analyzed by *Pvu*II restriction enzyme digestion. The digested DNA was subjected to polyacrylamide gel electrophoresis (3.5%) and the DNA band were visualized by staining with ethidium bromide.

(e) Nucleotide sequencing

The dideoxy method (Sanger et al., 1977) was used to verify the sequence of the mutations after initial screening by restriction analysis with *Pvu*II.

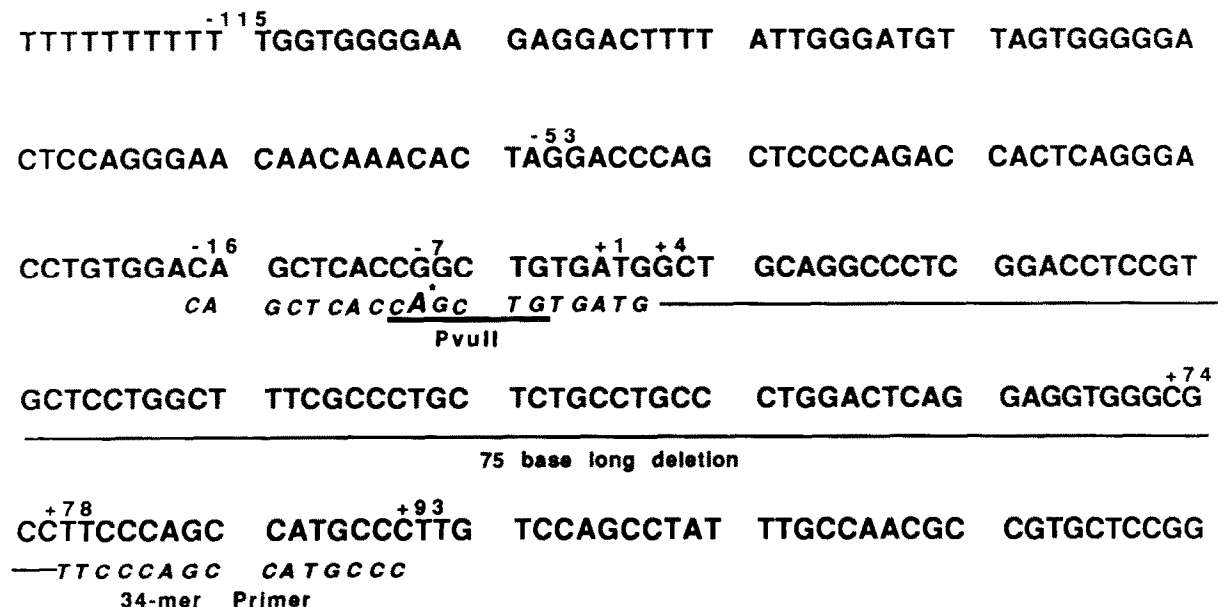


Fig. 2. Sequence of the 5'-terminus of pGH-coding cDNA, indicating the 34-mer mutagenic primer. The numbering is relative to the ATG start codon (A = +1). The primer overlaps the cDNA sequence from -17 to +4 and from +79 to +93, with the G → A substitution at -7. The 75-nt long deletion region is underlined. The ATG start codon of pre-pGH cDNA is positioned at the first codon of mature pGH (TCC) as a result of the deletion. A *Pvu*II restriction site (5'-CAGCTG) is generated by the G → A substitution as shown by an asterisk and large letter A.

Modified T7 DNA polymerase (SequenaseTM) was used according to the manufacturer's instructions. [³⁵S]dCTP (Amersham) was used (Biggins et al., 1983) and gel electrophoresis was performed as described by Sanger and Coulson (1978).

RESULTS AND DISCUSSION

(a) Strategy

The strategy to make the multiple changes in pGH gene is illustrated in Fig. 1. pGH cDNA was subcloned in M13mp18 to give the recombinant phage M13.pGH. Before any gene reconstruction, the 5' terminus of the cloned pGH coding gene was verified by sequencing. The nucleotide sequence at

this end was in agreement with published work (Seeburg et al., 1983; Vize et al., 1987) except for a silent substitution where A (nt 25 in the presequence) was replaced by C without changing the aa (Gly). The 5'-untranslated sequence upstream from position -54 was found to be different from the published sequence (Vize et al., 1987). The complete sequence of the 5' terminus of the pGH coding gene indicating these changes is shown in Fig. 2. DNA templates containing uridinyI residues were prepared by infecting RZ1032 strain with M13.pGH. As expected after two or three propagative cycles of infection in RZ1032 (Kunkel, 1985), the survival of this U-containing DNA in the wild-type strain (JM101) is greatly reduced. The U-containing DNA template was annealed with the synthetic primer (Figs. 1 and 2) causing the looping out of 75 nt, bringing the ATG immediately 5' to the first codon of the mature

TABLE 1

Effect of oligodeoxynucleotide extension and ligation time on the survival of M13.pGH in strain JM101 and on mutation frequency^a

Time ^b min	pfu/ng ^c		Survival % ^d	Mutation % ^e
	JM101	RZ1032		
5	0.32	9.4	3.4	31
10	0.68	8.8	7.7	45
15	6.4	12	53	42
30	2.6	13	20	38
60	0.34	11	3.1	8
Control (no enzymes were added)	0.01	9.1	0.13	0

^a Uracil-containing DNA template was prepared using RZ1032 strain (*ung⁻ dut⁻*) as described by Kunkel (1985). Cells were grown in YT medium (yeast extract, 5 mg/ml; tryptone, 8 mg/ml; NaCl, 5 mg/ml) supplemented with thymidine (20 µg/ml) and deoxyadenosine (100 µg/ml) until absorbance of 0.6 is reached (A_{600}). Cells were centrifuged for 15 min at 2000 × *g*, washed with YT medium, resuspended in the same volume of YT medium containing uridine (0.25 µg/ml), and then infected with the recombinant bacteriophage M13mp18 containing pGH cDNA (M13.pGH) at a multiplicity of infection of 1 to 10. Incubation at 37 °C was continued overnight (first cycle of growth). Cultures were centrifuged and the M13.pGH bacteriophage in the supernatant fraction was titrated and then 2nd and 3rd cycles of RZ1032 infection were repeated in uridine containing YT medium. Uracil-containing DNA was titrated on both JM101 (*ung⁺ dut⁺*) and RZ1032.

^b Time after adding modified T7 DNA polymerase and T4 ligase to the reaction mix. Samples were incubated at 37 °C (see MATERIALS AND METHODS, section c).

^c pfu/ng of U-containing DNA on JM101, and RZ1032. 10-µl portions, out of the reaction mix (30 µl) were used to transfect JM101 strain while 5-µl portions were used to transfect RZ1032.

^d Survival of U-containing DNA of M13.pGH on both strains JM101 and RZ1032. Survival % was calculated as (number of plaques on JM101 divided by number of plaques on RZ1032) × 100.

^e Mutation % is calculated as total number of mutants × 100 divided by total number of plaques (on JM101). Mutants were identified by the appearance of a new *Pvu*II restriction site when analyzed by 3.5% polyacrylamide gel electrophoresis (see Fig. 4, legend, map A and panel B).

protein. The same primer included a G → A substitution generating a *PvuII* restriction site.

(b) The use of T4 DNA polymerase (method A) and PolIk (method B) in multisite directed mutagenesis

Plaques obtained on JM101 using T4 polymerase or PolIk as described showed no mutagenic changes as judged by *PvuII* restriction enzyme analysis.

It can be concluded from these experiments that the use of T4 polymerase (Kunkel, 1985) or PolIk at 0°C did not result in efficient multisite-directed mutagenesis of the type described here. Simpler site-directed changes (two nt substitutions) were made previously using PolIk under these conditions which minimize the 3'-to-5' exonuclease activity (El-Gewely et al., 1988).

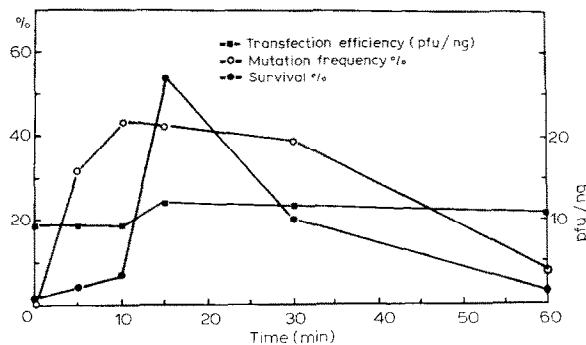


Fig. 3. The relationship between reaction time and mutation frequency. 500 ng of M13.pGH U-containing template was mixed with synthetic 34-mer at a molar ratio of 1:2–3 in 1 × T7 DNA polymerase buffer. The mixture was heated at 65°C for 5 min, then allowed to cool slowly to room temperature and then placed on ice. 1.5 μl 10 mM ATP, 1 μl of modified T7 DNA polymerase (14 units), and 1 μl of T4 ligase (5 units) were added. The reaction was incubated at 37°C for various times, and stopped by heating at 65°C for 5 min. Transfections were done in JM101 and RZ1032 to determine the survival percentage ([number of plaques on JM101/number of plaques on RZ1032] × 100) and transfection efficiency ([number of plaques on RZ1032/μg DNA] × 100). Samples of DNA from twelve different plaques that survived on JM101 were isolated and analyzed (see MATERIALS AND METHODS, section d) using 3.5% polyacrylamide gel electrophoresis. The mutation frequency was based on the *PvuII* analysis where the mutant constructions are cleaved by an additional *PvuII* site (see RESULTS AND DISCUSSION, section e, and Fig. 4, panel B).

(c) The use of modified T7 polymerase in multisite-directed mutagenesis

The use of modified T7 DNA polymerase in the oligo extension reaction have resulted in the multiple changes required for reconstruction as outlined in Figs. 1 and 2. In Table I, it is indicated that even after a 5-min reaction, 31% of surviving plaques on JM101 contained the change. The frequency of mutation increased with reaction time and then slowly decreased (Table I and Fig. 3). At these conditions, the optimum time for survival of the resulting DNA by forming plaques on JM101 was around 15 min. The mutation frequency was also the highest after 10–15 min of incubation. Unlike survival percentage, incubation time between 5–60 min did not drastically affect mutation rate. Transfection efficiency (pfu/ng) was not affected by the time of incubation after an initial increase in the first 10 min (Table I and Fig. 3).

(d) The effect of the concentration of T4 ligase on mutation frequency

The mutation and the survival percentages were improved by the use of higher concentrations of T4 DNA ligase (5 units per reaction instead of 2 units per reaction, Table II).

The higher concentration of ligase was added in the reaction to ensure ligation of the new strands as soon as they are formed thus reducing any possible second cycle read-through by T7 polymerase. Long incubation with the enzyme (60 min) reduced the survival percentage as well as the mutation frequency when the resulting DNA was transfected into JM101. It is possible that longer incubation time results in the displacement of the mutagenic oligo and the newly synthesized strand producing multiple rounds of DNA replication. Aberrant products may be subject to DNA repair mechanisms reducing both the survival percentage and the frequency of mutants. Since longer incubation time did not affect transfection efficiency, we can rule out the presence of residual molecules with 3'-to-5' exonuclease activity as an explanation for the reduction of both the survival of M13.pGH on JM101 and the mutation frequency. Modified T7 DNA polymerase preparation contains some molecules (0.01%) that still retain 3'-to-5' exonuclease activity (Tabor and Richardson, 1987b).

TABLE II

Effect of T4 DNA ligase concentration on mutation frequency

T4 ligase ^a (units)	pfu/ng ^b		Survival % ^b	Mutants % ^b
	JM101	RZ1032		
0	0.09	—	—	0
2	1.80	35	5.1	31
5	6.90	89	7.8	42
Control (no enzymes were added)	0.04	5.8	0.62	0

^a T4 ligase (0–5 units) per reaction were added simultaneously with modified T7 DNA polymerase (14 units) (see MATERIALS AND METHODS, section c).

^b See corresponding footnotes c–e to Table I.

(e) Restriction enzyme analysis for the screening of putative mutants

The results of *PvuII* restriction enzyme digestion of DNA from putative mutants are shown in Fig. 4. Mutant DNA could be identified by the absence of the 890-bp fragment and the appearance of the two DNA fragments of 568 and 247 bp. Together the two fragments add up to a total of 815 bp reflecting both the introduction of a new *PvuII* site in the 890-bp fragment and the deletion of 75-bp presequence (Fig. 4).

(f) Nucleotide sequence analysis of the reconstructed pGH gene

Sequence of the mutants, as judged by the restriction analysis, was confirmed by nucleotide sequencing (Fig. 4). All of the tested mutants had the predicted sequence including the G → A substitution creating a *PvuII* site (CAGCTG), and the 75-bp deletion from nt + 4 (G) to nt + 78 (C) (Figs. 2 and 4). This deletion introduced ATG just upstream from TTC coding for the initial N-terminal amino acid, phenylalanine, in the mature protein.

It appears that the described method of site-directed mutagenesis using modified T7 DNA polymerase has succeeded in introducing multiple, simultaneous changes using a single oligo.

This method has been useful in making simpler site-directed changes as well (M. Adams, personal

communication). Also, this method and procedure was used to introduce an insertion of 29 bp in pGH coding sequence that included *EcoRI* restriction enzyme site, ribosome-binding site, and additional codons for methionine, alanine, and glutamic using a single 59-nt oligo (unpublished). This method should also be applicable in cases where more than one mutagenic primer are used to introduce simultaneous changes in different domains of the gene.

The success of modified T7 DNA polymerase in the described multisite-directed mutagenesis procedures could be attributed to enzyme processivity, stability, and high rate of DNA polymerization (Tabor and Richardson, 1987a,b; Huber et al., 1987; Tabor et al., 1987). The expected probability of multiple-site mutagenesis is the product of probabilities at each site. The more complicated the required changes (especially for the introduction of deletions or insertions), the lower the final probability of the multiple mutant. It appeared that T4 DNA polymerase or PolIk in such experiments are not as efficient as modified T7 polymerase.

(g) Recommended conditions and procedures for site- and multisite-directed mutagenesis

Based on our results and using all the described solutions and buffers, we recommend the following protocol for 500 ng DNA reactions.

(1) The mutagenic primer is annealed to U-containing DNA templates in a molar ratio of 2–3 : 1. In a

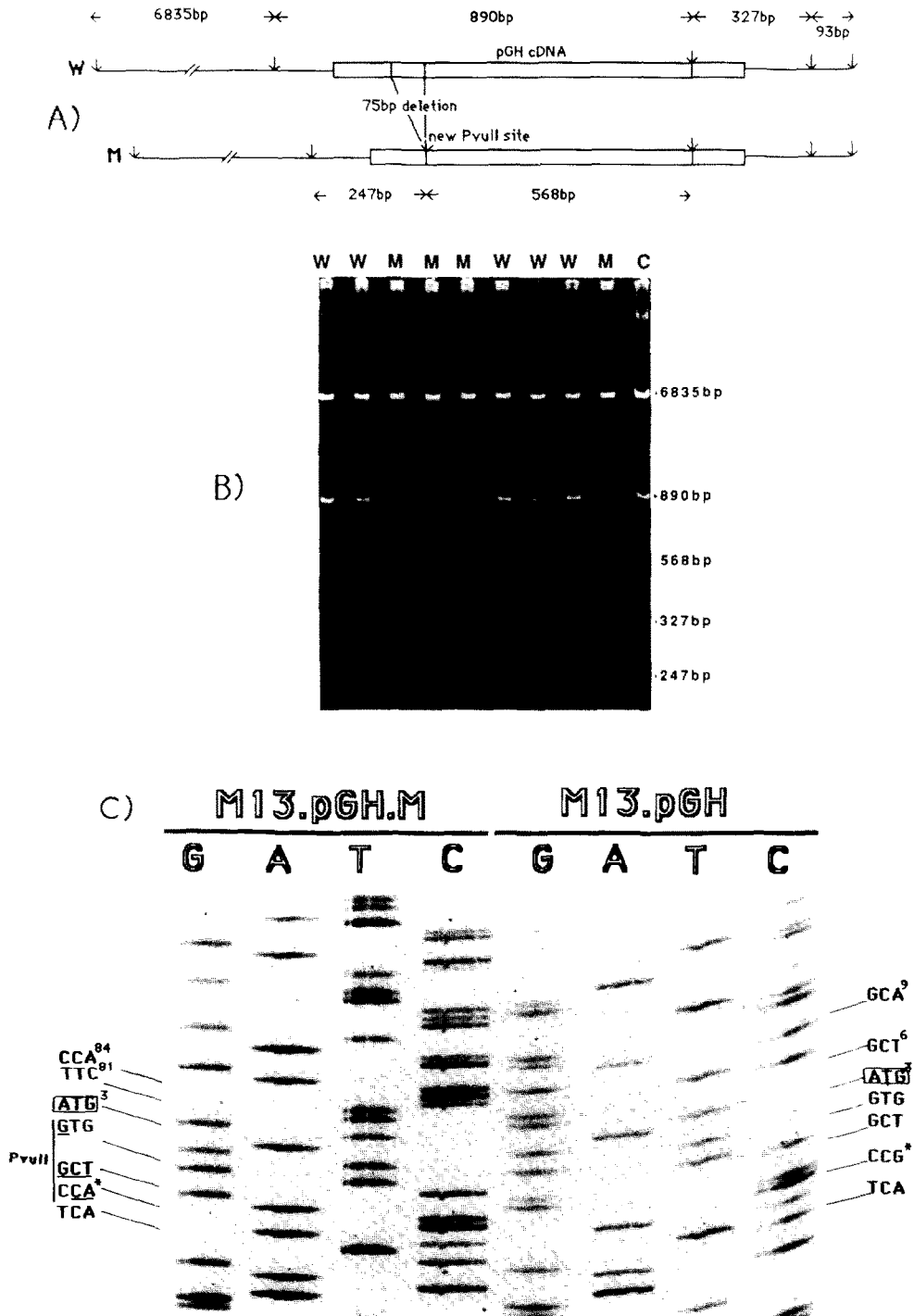


Fig. 4. Mutants verified by restriction enzyme analysis with *Pvu*II (A and B) and by nucleotide sequencing (C). Mutagenesis was made as described in MATERIALS AND METHODS, section c. (Map A) *Pvu*II restriction enzyme analysis of RF DNA from plaques with putative mutants (isolated on JM101). The restriction map of wild-type M13.pGH (W) DNA and the putative mutant M13.pGH.M (M) DNA. *Pvu*II restriction sites on the map are indicated by downward arrows. In mutants, the 890-bp fragment is further restricted at the new *Pvu*II site into two fragments, 568 bp and 247 bp. The 568-bp fragment indicating the 75-bp-long deletion helped in identifying the mutants. (Panel B) Polyacrylamide gel electrophoresis (3.5%) of *Pvu*II fragments after staining with ethidium bromide (see MATERIALS AND METHODS, section d). The fragments are from control DNA (wild type) prior to mutagenesis (C), non-mutated DNA after the mutagenesis (W), and mutant DNAs produced by mutagenesis (M); the letter is characterized by the presence of the

small eppendorf tube add the following:

7 μ l DNA template (0.2 pM), 1 μ l primer (0.5–0.6 pM), 2 μ l 5 \times T7 DNA polymerase buffer (see MATERIALS AND METHODS, section c3i). Heat the tube at 65°C for 5 min, then allow to cool slowly to room temperature (40–60 min). Leave at room temperature for 15 min, then place the tube on ice for 15 min.

(2) Extension/ligation reaction. To the above mixture add the following (on ice): 2 μ l dNTP, 1.5 μ l ATP, 1 μ l modified T7 DNA polymerase (14 units), and 1 μ l T4 ligase (5 units).

Incubate at 37°C for 15 min, then immediately stop the reaction by heating at 65°C for 5 min.

(3) After the completion of the reaction, transfect JM101 strain. As a control RZ1032 (*ung*⁻ *dut*⁻) is used. Number of plaques on JM101 (*ung*⁺ *dut*⁺) should be significantly lower than of those formed on RZ1032. The DNA can then be isolated and analyzed for the mutation(s).

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568-bp fragment. (Panel C) Autoradiogram of polyacrylamide gel (7.2%) showing nucleotide sequence of the region that has been reconstructed (M13.pGH.M) and the wild type (M13.pGH). Experimental details of nucleotide sequence are shown in MATERIALS AND METHODS, section e. The nt listed on both sides show the changes after the multisite-directed mutagenesis. (1) The G \rightarrow A substitution (indicated by an asterisk) generating a *Pvu*II restriction site. (2) A deletion in the presequence from nt + 4 to + 78 (inclusive) with respect to the start codon. (3) The ATG start codon, as a consequence of the deletion, is positioned just before the first codon (TTC, coding for phenylalanine) of the mature pGH gene. Number of nt, with respect to the start codon ATG, are shown superscript. The underlined sequence 5'–CAGCTG constitutes the newly generated *Pvu*II site in the mutants.