Oligonucleotide directed mutagenesis of the human β -globin gene: a general method for producing specific point mutations in cloned DNA

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ABSTRACT

A nonadecanucleotide has been used both as a site specific mutagen to introduce an T→A transversion mutation in the human β -globin gene cloned in pBR322 as well as a probe to screen transformed colonies for the desired mutant. The specificity of the oligonucleotide as a mutagen and as a hybridization probe provide a general method for producing site specific mutations in DNA cloned in plasmid vectors such as pBR322.

INTRODUCTION

Synthetic oligonucleotides have the potential to direct virtually any mutational change in DNA when used as a specific primer for DNA polymerase I (large fragment) on a single stranded circular DNA template (1 - 7). Oligonucleotides have been used to introduce both transition (1 - 4) and transversion (5) point mutations into λ X174 DNA. Recently, a T→G transversion mutation has been introduced into the TATA box of the chicken covalbumin gene cloned into a bacteriophage fd vector (7). In both cases the DNA to be mutagenized was single stranded circular phage DNA.

Recently we have described a procedure for mutagenizing DNA cloned in the plasmid vector pBR322 (6). Single stranded circular template DNA was produced by nicking the plasmid DNA with *Eco*RI endonuclease in the presence of ethidium bromide (8) followed by removal of the nicked DNA strand with exonuclease III. An oligonucleotide was used to direct a 14 b.p. (base pair) deletion of the yeast suppressor tRNA gene intervening sequence (IVS).

The greatest obstacle to the general application of oligonucleotide directed mutagenesis is in identifying the desired mutant DNA from a background of unaltered DNA. This is particularly true for cloned DNAs which do not have an assayable phenotype in *E. coli*. Wasylyk, *et al* (7), used the fact that mutagenesis of the TATA box of the covalbumin DNA (TCTATA→TCTAGA) formed a new *Xba*I recognition site. Screening for the desired mutant clones involved

inspection of individual clones for the presence of the *Xba*I site. In the oligonucleotide directed deletion of the 14 b.p. IVS of the yeast tyrosine tRNA gene (6), mutant colonies were identified by virtue of hybridization of the oligonucleotide used for the deletion specifically to mutant DNA. This was possible because the mutant plasmid DNA was completely complementary to the oligonucleotide (a henicosamer) while the wild type DNA contained an extra 14 nucleotides, interrupting the region of complementarity. We have previously shown that conditions can be established where oligonucleotides forming a single base pair mismatch will not hybridize to DNA while those forming perfect matches will (9, 10). In this report, we show that this specificity of oligonucleotide hybridization can be used to identify colonies containing a specific point mutation produced in the human β -globin gene cloned in pBR322.

MATERIALS AND METHODS

Oligonucleotide Synthesis

The two nonadecanucleotides (Table I) were synthesized by the triester approach on a solid support as described previously (11). The oligonucleotides were purified after deprotection by high performance liquid chromatography on a μ Bondapak C₁₈ column (Waters) using a linear gradient of acetonitrile (10-20%) at pH7.8, as described elsewhere (12).

³²P Labeling of Oligonucleotides

Oligonucleotides were labeled by transferring the ³²P from γ [³²P]ATP using T4 polynucleotide kinase (New England Nuclear) as described previously (9). Labeled oligonucleotide was separated from unreacted γ [³²P]ATP by chromatography on Whatman DE52 cellulose. After kinase labeling, the reaction mixture was diluted ten fold with 10 mM Tris-HCl pH8.0, 1 mM EDTA (TE) and applied to a small DE52 column equilibrated with TE. The column was then washed with 4 column volumes of TE, the γ [³²P]ATP eluted with 4 column volumes of 0.2 M NaCl in TE and the oligonucleotide recovered by elution with 0.5 M NaCl in TE. The labeled oligonucleotide was used directly for hybridization.

Preparation of DNA

The pBR322-H β *Pst* plasmid contains a 4.4 kb *Pst*I fragment of the human β -globin gene subcloned in the *Pst*I site of pBR322 (13). The DNA was originally obtained from Dr. T. Maniatis and was used to transform *E. coli* K12 MC1061 (14) by the procedure of Kushner (15).

Plasmid DNA was prepared from chloramphenicol amplified cultures by scaling up the procedure of Birnboim (16). Plasmid DNA was further purified by

isolating the covalently closed circular DNA (CCC-DNA) after equilibrium centrifugation in a CsCl-ethidium bromide gradient (17).

Preparation of Nicked Circular Plasmid DNA

The pBR322-HgPst DNA was randomly nicked by incubating 600 µg of CCC-DNA in 1 ml of *Hin* buffer [7 mM NaCl, 7 mM Tris-HCl pH7.5, 7 mM Mg(OAc)₂, 7 mM DTT] in the presence of ethidium bromide (100 µg/ml) and pancreatic DNase I (150 ng/ml) as described by Shortle and Nathans (8). Approximately 80-90% of the CCC-DNA was nicked by this procedure. The nicked DNA was purified by centrifugation in a CsCl-ethidium bromide gradient (17).

Oligonucleotide Directed Mutagenesis

10 µg of nicked pBR322-pHgPst was incubated in 0.1 ml of 10 mM Tris-HCl pH7.2, 2 mM MgCl₂, 1 mM β-mercaptoethanol with 60 units of exonuclease III (Bethesda Research) for 90 minutes at 37°C. The reaction was extracted 2 times with phenol/chloroform and the single stranded circular DNA recovered by ethanol precipitation after the addition of sodium acetate to 0.3 M.

The single stranded circular DNA was resuspended in 5 µl of TE. 64 pmoles of 5' phosphorylated nonadecanucleotide designated Hg19S (Table I) was added, the reaction heated to 90°C for 3 minutes and then cooled to 0°C. The reaction (final volume 10 µl) was then adjusted to 100 µM in each of the deoxynucleoside triphosphates, 0.4 mM ATP, 90 units DNA polymerase I/ml (large fragment, BoehringerMannheim) and 30,000 units T4 DNA ligase/ml (New England Biolabs) and incubated at 12°C for 1 hour.

Transformation and Screening for Mutants

The DNA from the oligonucleotide primed reaction was used to transform MC1061 by the procedure of Kushner (15). The transformed bacteria were plated onto 10 nitrocellulose filters (Millipore HATF090) placed on 100 mm L-broth agar plates containing 20 µg/ml tetracycline. After overnight growth, approximately 1300 colonies appeared. The colonies on the nitrocellulose filters were replica-plated onto a second nitrocellulose filter as described previously (6, 18). The replica filters were incubated overnight on L-broth agar plates containing 20 µg/ml tetracycline and the colonies amplified for 5 hours on L-broth agar plates containing 250 µg/ml chloramphenicol. The filters were prepared for hybridization as described by Hanahan and Meselson (18), and hybridized with 5' ³²P-labeled Hg19S in 6 X NET, (NET = 0.15 M NaCl, 0.015 M Tris-HCl pH7.5, 0.001 M EDTA), 5 X Denhardts, 10% Dextran sulfate (Sigma), 250 µg/ml tRNA, 0.5% NP40 (Shell Oil), 2 ng/ml probe at 55°C, overnight. The filters were washed at 0°C in four changes of 6 X SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate pH7.2) for a total of 20 minutes. The filters were

then washed in 6 X SSC at 55°C for one minute and exposed to Kodak XR-5 X-ray film between two intensifier screens (DuPont Lightning Plus) for five hours at -70°C. Longer washes at 55°C tend to reduce the amount of probe hybridized without improving the background. Approximately 0.5% of the colonies were identified as mutants by virtue of hybridization to the H β 19S probe.

Plasmid DNA was prepared from one of the mutant colonies and used to transform *E. coli* K12 LS1. All of the colonies obtained from the transformation were found to hybridize to the H β 19S probe, indicating that the primary isolate did not contain a mixture of wild type and mutant plasmid DNAs. Mutant plasmid DNA was prepared from one of the LS1 transformants for further analysis.

DNA Sequence Analysis

DNA sequencing was performed by the chain termination method of Sanger and coworkers (19) using the decanucleotide TCTTCAGACG as a specific primer as described elsewhere (20) and by Smith, *et al* (21).

Southern Blot Analysis

pBR322-H β Pst DNA or mutant DNA (0.5 μ g) was cleaved with *Bam*HI endonuclease (10 units) in 20 μ l of Hin buffer. Samples were subjected to electrophoresis in a 1% agarose gel, the DNA denatured *in situ*, and transferred to nitrocellulose as described previously (9). Filters were baked *in vacuo* for 2 hours at 65°C and hybridized with ³²P-labeled nonadecanucleotide (H β 19A or H β 19S, Table I) in 6 X NET, 5 X Denhardt's, 250 μ g/ml tRNA, 0.5% NP40, 2 ng/ml probe at 55°C, overnight. Filters were washed with four changes of 6 X SSC at 0°C for a total of 20 minutes and exposed to Kodak XAR-5 film between two intensifier screens (DuPont Lightning Plus) at -70°C for 24 hours.

RESULTS

Priming Specificity of Oligonucleotide

Two nonadecanucleotides (Table I) complementary to either the normal β -globin gene (H β 19A) or sickle cell β -globin gene (H β 19S) were chemically synthesized. We have previously shown that these oligonucleotides hybridize specifically to their respective genes (Conner, Reyes and Wallace, in preparation). Oligonucleotide H β 19S was used for the site specific mutagenesis of the normal β -globin gene (β^A) to a sickle cell β -globin gene (β^S). In order to show that H β 19S would prime specifically on the cloned β^A gene, the oligonucleotide was labeled with ³²P and used as a primer for DNA polymerase I (large fragment) on the cloned β^A DNA digested with *Bam*HI endonuclease. From the DNA sequence of the β^A gene (22), the oligonucleotide should give a primer

TABLE I.

Oligonucleotides Complementary to the Human β -Globin Gene

Amino Acid Sequence β^A	4	5	6	7	8	9	10
	Thr	Pro	Glu	Glu	Lys	Ser	Ala
H β 19A	C	T	C	C	T	G	<u>A</u> G G A G A A G T C T G C
Amino Acid Sequence β^S	4	5	6	7	8	9	10
	Thr	Pro	Val	Glu	Lys	Ser	Ala
H β 19S	C	T	C	C	T	G	<u>T</u> G G A G A A G T C T G C
The underlined nucleotide represents the position of the point mutation.							

extension product 413 nucleotides in length. Figure 1 shows the results of this primer extension reaction. The major product is a DNA fragment which is between 400 and 450 nucleotides long with respect to marker DNA fragments when electrophoresed on a denaturing polyacrylamide gel.

Isolation of β^S Point Mutant

For the oligonucleotide directed mutagenesis of the β^A gene to a β^S gene, single stranded circular DNA was prepared from randomly nicked plasmid DNA by exonuclease III digestion as described in the Methods. Oligonucleotide H β 19S was used as a primer for the large fragment of DNA polymerase I on the single stranded circular DNA template. The oligonucleotide was 5' phosphorylated such that, in the presence of T4 DNA ligase and ATP, a closed circular heteroduplex DNA would be formed containing a single T-T base pair. After transformation into *E. coli* the heteroduplex would be resolved into mutant and/or wild type DNA molecules, by DNA repair or DNA replication.

Transformed cells were plated onto nitrocellulose filters and grown in the presence of tetracycline. A filter replica of the colonies was made on a second filter, the colonies were grown in the presence of tetracycline, the plasmid DNA of the colonies amplified on chloramphenicol containing agar plates, and the DNA of the colonies immobilized on the filters (18). The filters were then hybridized with ^{32}P labeled H β 19S at 55°C in 0.9 M Na $^{+}$. Under these conditions the H β 19S probe hybridizes very poorly with the β^A gene due to the single T-T mismatch. A typical colony screening filter is shown in Figure 2. It can be seen that the DNA of a single colony (out of 125 on this

FIGURE 1. Primer Extension.

The nonadecanucleotide Hg19S was labeled with ^{32}P as described in the Methods. The DE52 purified oligonucleotide was precipitated with 2 volumes of ethanol after the addition of 21 μg tRNA. 1 pmole of ^{32}P labeled Hg19S was mixed with 0.36 μg of *Bam*HI digested pBR322-Hg*Pst* in 80 mM NaCl, 67 mM Tris-HCl pH7.5, 9 mM $\text{Mg}(\text{OAc})_2$, 9 mM DTT, boiled 3 minutes and cooled to 0°C . The four deoxynucleoside triphosphates were added to 100 μM , the large fragment of DNA polymerase I (1 unit) added and the reaction incubated at 37°C for 30 minutes. After phenol/chloroform extraction, the DNA was recovered by ethanol precipitation. The sample (lane b) was dissolved in 80% formamide, 4.5 mM Tris-boric acid pH8.3, 1 mM EDTA, 0.1% xylene cyanol FF, 0.1% bromophenol blue, boiled, and subjected to electrophoresis on an 8% acrylamide, 8 M urea gel as described (24). As DNA fragment size markers, ^{32}P labeled *Hinf*I fragments of pBR322 were electrophoresed on the gel in parallel (lane a). The numbers to the left indicate the size of the marker fragments in nucleotides (25). The arrow indicates the position of the major primer extension product.

filter) hybridizes with the probe. This colony was isolated, plasmid DNA prepared, and the plasmid DNA used to retransform *E. coli*. Screening of the resultant transformants showed that all colonies contained mutant plasmid DNA.

This indicates that the primary transformant (Figure 2) contained only mutant DNA, not a mixture of mutant and wild type DNA (6).

Characterization of the Mutant Plasmid

Mutant plasmid DNA and pBR322-Hg^{Pst} DNA were isolated and sequenced by the method of Sanger and coworkers (19). The primer for the sequencing was the decamer TCTTCAGACG, which is complementary to both the β^A and the β^S gene 3 nucleotides from the site of the point mutation. The sequences of the two DNAs are shown in Figure 3. It can be seen that, indeed, a single T→A transversion mutation has been introduced.

In order to confirm the hybridization specificity of the two nonadecanucleotides Hg19A and Hg19S (Table I) to the β^A and the β^S genes, the two DNAs were digested with *Bam*HI endonuclease, subjected to electrophoresis in a 1% agarose gel and the DNA fragments transferred to nitrocellulose by the blotting technique of Southern (23). Figure 4 shows the result of such a blot hybridized with either ³²P labeled Hg19A or ³²P labeled Hg19S. It can be seen

FIGURE 2. Colony Screening for Point Mutation.

A typical colony screening filter showing a single point mutant (arrow) amongst the 125 colonies on the filter.

FIGURE 3. Sequence of pBR322-H β Pst (1) and Mutant (2) DNAs.

Sequencing was done as described in the Methods, the point mutation is indicated by the difference in the second band from the bottom of the gel.

that under these conditions H β 19A only hybridizes to the β^A gene and H β 19S only to the β^S gene.

DISCUSSION

In this manuscript we describe two roles for the oligonucleotide in site specific mutagenesis. First, the oligonucleotide directs defined changes in the cloned DNA when used as a primer on single stranded circular DNA. These changes are fixed by propagation of the heteroduplex DNAs in *E. coli*. Second, the hybridization specificity of the oligonucleotide is used to identify colonies containing the desired mutated plasmid DNAs (see Figures 2 and 4). The ease of screening colonies for the desired mutants, together with the fact that mutagenesis can be done on DNAs cloned in pBR322, provides a general method for oligonucleotide directed mutagenesis of cloned DNA.

The oligonucleotide used in this mutagenesis was 19 bases long. It is likely that a shorter oligonucleotide could have been used (3). However, with

FIGURE 4. Southern Blot Analysis of Mutant DNA.

Mutant DNA (a) and pBR322-HgPst DNA (b) were digested with *Bam*HI endonuclease and blotted onto nitrocellulose filters as described in the Methods. Filters containing both DNAs were hybridized with ³²P labeled Hg19A (2) or Hg19S (3) as described in the Methods. The ethidium bromide stained gel is shown in panel 1, the arrow indicates the position of the 2.0 Kb *Bam*HI fragment containing probe complementary sequences (13).

the rapid progress being made in the field of solid-phase synthesis of synthetic DNA, the synthesis of longer oligonucleotides is not a practical limitation (12). Starting from trimer blocks, a 19 base long oligonucleotide can be synthesized and purified in one week. Indeed, synthetic DNA greater than 30 bases long can routinely be made (12). The availability of longer oligonucleotides should make site specific insertion mutations possible by the methodology described previously (6) and in this paper.

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