[31] Directed Mutagenesis with Sodium Bisulfite

By DAVID SHORTLE and DAVID BOTSTEIN

With the development of recombinant DNA technology and the many analytic techniques based on restriction endonucleases, it has become relatively routine to isolate a segment of genomic DNA carrying a gene of interest, to define the limits of the gene within this DNA segment, and then to determine the gene's complete nucleotide sequence. In this way, a number of structural questions can be directly answered. To extend the analysis of a gene further, particularly with regard to functional and regulatory phenomena, requires the isolation and systematic study of a sizable collection of mutant alleles of the gene. In the initial stages of such a mutational analysis, deletion and insertion mutations are often useful to identify important functional elements of the gene, particularly those at the 5′ and 3′ ends. When base substitution mutations are required to extend the analysis, directed mutagenesis with sodium bisulfite can provide an *in vitro* method for efficiently inducing C to T transition mutations at sites in a DNA molecule specified in advance by the experimenter.

Principles

The mutagen sodium bisulfite catalyzes the deamination of cytosine to form uracil under mild conditions of temperature and pH.[1] Cytosine residues in single-stranded DNA react at nearly the same rate as the mononucleotide. However, because of the stereochemistry involved in the bisulfite ion's attack on the cytosine ring, residues embedded within the Watson–Crick helix of double-stranded DNA are essentially unreactive. From the data available, the rate of cytosine deamination in duplex DNA appears to be less than 0.1% of the rate in single-stranded DNA.[1,2] Consequently, sodium bisulfite is, in effect, a single-strand specific mutagen; and a particular nucleotide sequence can be "targeted" for bisulfite mutagenesis by exposing it in a stretch of single-stranded DNA.

Segments of a circular, duplex DNA can be converted to a single-stranded, unpaired form in one of two ways. One approach, described in detail below, is to introduce a single nick at a specific site with an endonu-

[1] H. Hayatsu, *Prog. Nucleic Acid Res. Mol. Biol.* **16,** 75 (1976).
[2] D. Shortle and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2170 (1978).

ISBN 0-12-182000-9

clease and then to convert the nick into a short gap by exonucleolytic removal of a limited number of nucleotides.[2-4] In addition to serving as targets for bisulfite mutagenesis, short single-stranded gaps can be used as specific sites for construction of small deletions with S1 nuclease[5] or as sites for mutagenesis by nucleotide misincorporation.[6] Alternatively, single-stranded components of a circular DNA can be annealed to generate a duplex molecule in which a specific segment of one strand remains unpaired. Examples of this second approach that have been used to construct unique targets for bisulfite mutagenesis are (*a*) reannealing of separated strands of full-length linear plasmid DNA with separated strands of a large restriction fragment[7]; (*b*) annealing of plus strand DNA of an M13 phage carrying a DNA fragment cloned into a unique restriction site with denatured linears generated by cleavage of RFI DNA from the parental M13 (without the insert) at the same restriction site (William Folk, personal communication); (*c*) construction of a unique deletion loop by heteroduplex formation between wild-type DNA and DNA from a deletion mutant of known sequence[7a,7b]; and (*d*) formation of a displacement loop, or D loop, by annealing a unique single-stranded restriction fragment to a covalently closed circular DNA (Maria Jason and Paul Schimmel, personal communication). It should be noted that, when the resultant single-stranded region is relatively long, care must be taken to reduce the level of bisulfite mutagenesis in order to avoid multiple, widely separated C to T mutations.

Nicking Reactions

Restriction Endonuclease plus Ethidium Bromide

When incubated with duplex DNA containing one or more restriction sites, type II restriction endonucleases cleave at, or near, their recognition sequences by generating two nicks, one in each strand. With some type II enzymes, the cleavage reaction on negatively supercoiled circular

[3] D. Shortle, D. Koshland, G. M. Weinstock, and D. Botstein, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 5375 (1980).

[4] D. Shortle and D. Botstein, *in* "Molecular and Cellular Mechanisms of Mutagenesis" (J. F. Lemontt and W. M. Generosa, eds.), p. 147. Plenum, New York, 1982.

[5] D. Shortle, J. Pipas, S. Lazarowitz, D. DiMaio, and D. Nathans, *in* "Genetic Engineering" (J. K. Setlow and A. Hollaender, eds.), Vol. 1, p. 73. Plenum, New York, 1979.

[6] D. Shortle, P. Grisafi, S. J. Benkovic, and D. Botstein, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 1588 (1982).

[7] P. E. Giza, D. M. Schmit, and B. L. Murr, *Gene* **15,** 331 (1981).

[7a] D. Kalderon, B. A. Oostra, B. K. Ely, and A. Smith, *Nucleic Acids Res.* **17,** 5161 (1982).

[7b] K. W. C. Peden and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 7214 (1982).

DNA can be inhibited after only one nick has been induced by including in the reaction mixture the intercalating agent ethidium bromide.[2,8] Using purified supercoiled DNA and ethidium bromide at an optimal concentration, it is sometimes possible to nick 50–90% of the input DNA, yielding open circular molecules with a single nick at the restriction enzyme's normal cleavage site. Since open circular DNA binds a greater amount of ethidium bromide than does the negatively supercoiled form, inhibition of double-strand cleavage presumably results from the increased binding of ethidium bromide to the restriction site after the enzyme has made the first single-strand break.

Procedure. The concentration of ethidium bromide that will stimulate a maximal level of nicking with a given restriction enzyme must first be determined by titrating the amount of ethidium bromide (over a range from 20 to 200 μg/ml) added to a series of 10-μl reactions containing 0.5–1.0 μg of circular DNA in the enzyme's standard buffer. After adding an amount of restriction enzyme sufficient to cleave completely the DNA in 30–60 min in the absence of ethidium bromide, the reaction mixtures are incubated at room temperature for 2–4 hr; then the reaction is stopped by addition of 10 μl of 10% sucrose–25 m*M* EDTA, pH 8.0–0.4% sodium dodecyl sulfate (SDS)–bromophenol blue. The relative rate of nicking versus double-strand cleavage is estimated by electrophoresis of this reaction mixture on an agarose gel system that provides efficient separation of open circular DNA from closed circular and linear DNA forms. Figure 1 illustrates the results of titration of the ethidium bromide concentration on the cleavage of pBR322 by *Cla*I. Above a concentration of 75–100 μg/ml, the ratio of open circular to linear forms does not change significantly, although the overall reaction proceeds more slowly. This titration behavior is typical for enzymes that display the nicking reaction in the presence of ethidium bromide; for those enzymes that do not, little or no open circular DNA is generated at any ethidium bromide concentration.

Once the optimal conditions are determined, a large-scale reaction is carried out to prepare the necessary quantity of nicked circular DNA. It is important that the concentration of input DNA be kept close to the value used in the titration. To terminate the nicking reaction and remove the ethidium bromide and restriction enzyme, the mixture is made 25 m*M* EDTA, pH 8.0–0.25 *M* NaCl and extracted with phenol, followed by ethanol precipitation.

Comments. Two parameters that may slightly increase the efficiency of nicking are lower temperature and higher salt concentration. In the

[8] R. C. Parker, R. M. Watson, and J. Vinograd, *Proc. Natl. Acad. Sci. U.S.A.* **74,** 851 (1977).

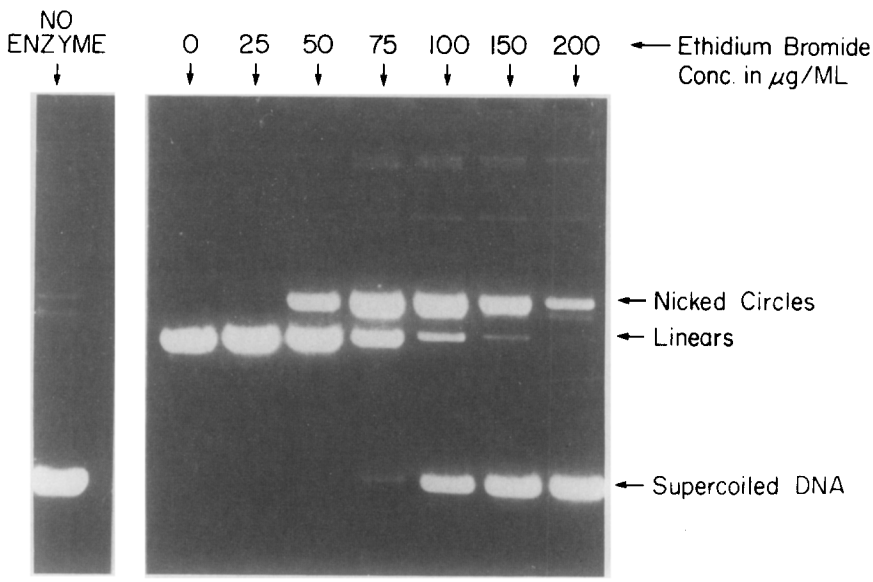

FIG. 1. Assay of the *Cla*I plus ethidium bromide-catalyzed nicking of covalently closed circular pBR322 DNA.

authors' experience, however, at least 5–10% of the input DNA is linearized under the best of circumstances. A few of the restriction enzymes that display an efficient nicking reaction are *Bgl*I, *Hpa*II, *Eco*RI, *Hin*dIII, *Bam*HI, *Cla*I, *Hin*cII, and *Bst*NI. However, with the conditions described above, little or no nicking was observed with the enzymes *Kpn*I, *Pvu*I, *Pvu*II, *Sal*I, or *Taq*I. With restriction enzymes that cleave at more than one site on a circular DNA, nicking may occur preferentially at a subset of sites.[9] To evaluate the extent of possible deviation from randomness, restriction enzyme-generated nicks can be labeled by carrying out a limited nick translation with an [α-^{32}P]dNTP, and the labeled sites can be localized by cleavage with one or more restriction enzymes, followed by autoradiography of gel-separated fragments.[3] In any event, a mutation

[9] K. W. C. Peden, J. M. Pipas, S. Pearson-White, and D. Nathans, *Science* **209**, 1392 (1980).

induced in one of the recognition sites of a multicut enzyme can be readily mapped by digestion with the cognate enzyme and identification of the two missing bands.

DNase I plus Ethidium Bromide

When the objective is to obtain mutations anywhere within a circular DNA, a simple approach is to generate a single bisulfite-sensitive site per molecule by nicking with DNase I in the presence of ethidium bromide.[10] Since this reaction induces one nick essentially at random per DNA circle, the percentage of molecules that acquire a nick in a given DNA segment will be equal to the percentage length of the DNA circle comprised by that segment. In principle, it should be possible to induce a C to T mutation at every cytosine residue without the limitation of mutational hot spots observed with other methods of random mutagenesis. Obviously, this approach will be most useful when an easily scorable phenotype is available to detect mutants of interest.

Procedure. To a solution of 50 m*M* Tris-HCl, pH 7.2–5 m*M* $MgCl_2$–0.01% gelatin–100 μg/ml ethidium bromide–50–100 μg/ml purified superhelical DNA is added an amount of pancreatic DNase I (typically 20–200 ng/ml) sufficient to convert 95–99% of the input DNA to an open circular form upon incubation at room temperature for 60 min. The minimal concentration of DNase I should be determined by titration with a series of small reactions and assaying the extent of nicking by agarose gel electrophoresis, as described earlier. For large-scale preparations, the reaction is stopped by adding EDTA to a final concentration of 20 m*M*, phenol extraction, and ethanol precipitation.

Comments. If a large excess of DNase I is used in this reaction, more than one nick will be induced per DNA circle. One semiquantitative method for assessing the average number of nicks per molecule is to denature the open circular DNA recovered from a reaction and then determine the ratio of single-stranded circles to single-stranded linears, either by gel electrophoresis or by counting these two forms by electron microscopy.[11] Finally, it should be noted that a small fraction of input DNA is linearized in this reaction. Presumably, these molecules have undergone a double-strand cleavage by DNase I in a "single hit" event[12]; therefore, their formation is not indicative of a high level of multiple nicks per molecule.

[10] L. Greenfield, L. Simpson, and D. Kaplan, *Biochim. Biophys. Acta* **407,** 365 (1975).
[11] R. W. Davis, M. N. Simon, and N. Davidson, this series, Vol. 21, p. 413.
[12] E. Melgar and D. A. Goldthwait, *J. Biol. Chem.* **243,** 4409 (1969).

Segment-Specific Nicking Procedure

Single nicks can be induced in defined segments of a circular DNA molecule with a two step, segment-specific nicking procedure.[3] In the first step, the *recA* protein of *E. coli* is used to catalyze the annealing of a unique, single-stranded DNA fragment to its complementary sequence on a covalently closed circular DNA.[13,14] The annealed fragment displaces one strand of the circular DNA, forming a single-stranded displacement loop, or D loop. Since one negative superhelical turn is removed for every 10 nucleotides displaced, the D loop is quite stable under the conditions of reaction. In the second step, a small amount of the single-strand specific S1 nuclease is used to nick the displaced strand, resulting in rapid breakdown of the D loop by spontaneous displacement of the fragment and termination of S1 nuclease action. The final product is an open circular DNA molecule with a nick located within the segment defined by the single-stranded fragment.

The reaction in which specific D loops are generated to provide a target for S1 nuclease nicking presents two nontrivial experimental tasks. First of all, a moderate quantity (0.5 to 2 μg) of a unique restriction fragment must be obtained in a single-stranded form. One of several possible solutions to this problem is described below. Second, *recA* protein catalyzed D-loop formation is an intrinsically complex reaction, one in which the stoichiometric ratios of the reactants (circular DNA, single-stranded fragment, and *recA* protein) must be carefully controlled. For a detailed discussion of the kinetics and probable mechanism of this reaction, the references cited in footnotes 13 and 14 can—and should—be consulted.

Exonuclease III Digestion of Isolated Restriction Fragments. A purified duplex DNA fragment can be converted to a heterogeneous mixture of single-stranded subfragments by limited digestion with exonuclease III. In this reaction, 1–2 μg of a restriction fragment is dissolved in 25 μl of 50 m*M* Tris-HCl, pH 8.0–0.5 m*M* $MgCl_2$–1 m*M* 2-mercaptoethanol–100 μg of gelatin per milliliter. After a 5-min preincubation of this solution at 45°, approximately 5 units of exonuclease III (New England BioLabs) is added and incubation is continued at 45° for 1 min per 70 base pairs of fragment length. The reaction is then terminated by adding 25 μl of 0.5 *M* NaCl–10 m*M* EDTA, pH 8.0, heating at 65° for 15 min, followed by phenol extraction and ethanol precipitation with 4 μg of carrier tRNA. The pellet is

[13] T. Shibata, C. DasGupta, R. P. Cunningham, and C. M. Radding, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1638 (1979).

[14] K. McEntee, G. M. Weinstock, and I. R. Lehman, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 2615 (1979).

dissolved in a small volume of 2 m*M* Tris-HCl, pH 8.0–0.2 m*M* EDTA, and the DNA concentration is calculated on the assumption of 50% hydrolysis of the restriction fragment. Prior to use in a D-loop reaction, this DNA mixture is heated by immersion in a boiling water bath for 30 sec.

Although exonuclease III is a double-strand specific exonuclease that degrades each strand in the 5′ to 3′ direction, it will slowly hydrolyze single-stranded DNA.[15] Therefore, it may be advisable to confirm that hydrolysis is not proceeding significantly beyond 50% by monitoring the release of acid-soluble counts from a radiolabeled fragment.

Alternatively, one specific strand can be completely degraded with exonuclease III by first incorporating an α-thiophosphate nucleotide (which is resistant to hydrolysis by this enzyme) onto one end of the fragment in a reaction with DNA polymerase.[16] With some restriction fragments, the two DNA strands can be separated electrophoretically and recovered from the gel matrix.[17]

recA Protein Catalyzed D-Loop Reaction. The stoichiometry of substrates must be controlled if this reaction is to be used reproducibly. One way to simplify this end is to hold constant the concentrations of circular DNA and single-stranded fragment and to make adjustments primarily in the *recA* protein concentration. For example, in a 30-μl volume of 20 m*M* Tris-HCl, pH 8.0–10 m*M* $MgCl_2$–1 m*M* dithiothreitol–1.3 m*M* ATP, efficient D-loop formation occurs between 150 ng of pBR322 and 30–50 ng of single-stranded fragment when 20–100 pmol of *recA* protein are added. Incubations are carried out in polypropylene tubes at 37° for 30 min. The fraction of circular DNA molecules that acquire a D-loop structure (typically between 40 and 90%) can be assayed either by nitrocellulose filter binding[13] or by electrophoresis of the reaction products (after addition of excess EDTA) on a standard agarose gel[3] *without* ethidium bromide (which destabilizes D-loop structures). This second assay is based on the fact that the circular DNA loses one negative superhelical turn for every 10 nucleotides of fragment annealed. Consequently, the electrophoretic mobility of D-looped molecules will depend on the size of the annealed fragment relative to the size of the circular DNA. Above a fragment size that eliminates all superhelical turns, the mobility of the circular DNA will remain constant at a value near that of open circular DNA. However, with fragments below a certain size, the mobility will not be detectably different from unreacted circular DNA; and therefore, a different assay

[15] B. Weiss, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 14. Academic Press, New York, 1981.

[16] S. D. Putney, S. J. Benkovic, and P. R. Schimmel, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7350 (1981).

[17] A. M. Maxam and W. Gilbert, this series, Vol. 65, p. 499.

may be necessary to detect D-loop formation (e.g., appearance of S1 nuclease-sensitive sites). The minimal fragment size that can form a stable D loop by *recA* protein catalysis with a small circular DNA such as pBR322 has not been determined.

The amount of *recA* protein required to catalyze D-loop formation is primarily a function of the amount of single-stranded DNA in the reaction mixture. Below a threshold *recA* protein level corresponding to approximately 1 enzyme molecule per 5 nucleotides, no reaction occurs.[13] Furthermore, at excessive levels of *recA* protein, the yield of D-looped molecules declines. Therefore, a titration series of reactions must usually be carried out to determine the enzyme's activity per nanogram of single-stranded DNA. Once this value has been established, it applies to all circular DNA molecules and homologous fragment substrates, provided that their ratios are held constant.

S1 Nuclease Reaction. In this reaction, the minimal amount of S1 nuclease necessary to convert 95–99% of D-looped molecules to a nicked circular form is used (see Fig. 2). The reaction is carried out by diluting 1 volume of the D-loop reaction mixture directly (i.e., without adding EDTA) into 9 volumes of solution (preheated at 45° for 10 min) containing 55 m*M* sodium cacodylate, pH 6.4–1.1 m*M* $ZnSO_4$–110 m*M* NaCl–0.44%

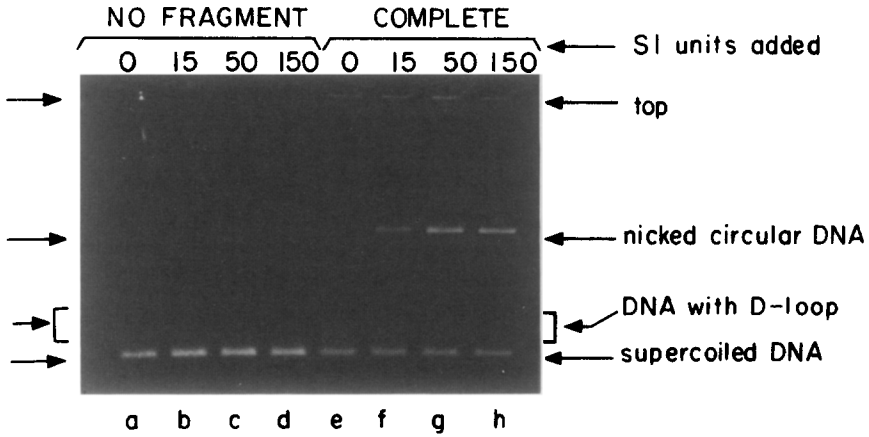

FIG. 2. Assay of the *recA* protein-catalyzed conversion of covalently closed circular pBR322 DNA to D-looped structures and their nicking by S1 nuclease. The single-stranded fragment, a 128 base-pair *Pvu*I-*Pst*I fragment digested with exonuclease III, was omitted from reactions a–d.

SDS–20–500 units of S1 nuclease (Miles Laboratories) per 100 ng of circular DNA. After incubation at 45° for 2 hr, S1 nuclease is inactivated by making the solution 150 m*M* Tris-HCl, pH 8.9–20 m*M* EDTA and incubating at 45° for an additional 30 min. The DNA is recovered by ethanol precipitation in the presence of carrier tRNA, followed by phenol extraction and a second ethanol precipitation. The extent of nicking of covalently closed circular DNA without D-loop structures should be assessed by running a control in which the single-stranded fragment has been omitted from the D-loop reaction (see Fig. 2). If the level of this side reaction is significant, the concentration of NaCl can be increased to 220 m*M*. If necessary, nicked circular molecules can be purified free of unreacted circular DNA by either agarose gel electrophoresis or acridine yellow polyacrylamide bead chromatography.[3]

Conversion of Nicks into Short Single-Stranded Gaps

After open circular DNA with a single, site-specific nick has been prepared, the next step is to convert the nick into a short gap by exonucleolytic removal of a small number of nucleotides. Of the exonucleases that initiate hydrolysis of one strand starting from a nick, two—*Micrococcus luteus* DNA polymerase I and T4 DNA polymerase—have properties that allow the extent of hydrolysis to be controlled. The *M. luteus* enzyme possesses both the 3′ → 5′- and 5′ → 3′-exonuclease functions typical of this class of bacterial polymerase.[18] However, both of these activities are relatively weak, particularly the 3′ → 5′-exonuclease. In the time-controlled reaction described below, an average of 5–6 nucleotides can be removed in predominantly the 5′ to 3′ direction from nicks,[19] although single-stranded gaps of 20 nucleotides or more in length may occasionally be generated. Alternatively, the exonuclease activity of T4 DNA polymerase can be used to remove nucleotides in the 3′ to 5′ direction from a nick.[20] This gapping reaction has the advantage that the extent of hydrolysis is controllable by addition of one or more deoxyribonucleoside triphosphates to the reaction mixture.[21]

In a 25-μl volume of 70 m*M* Tris-HCl, pH 8.0–7 m*M* $MgCl_2$–1 m*M* 2-mercaptoethanol, 0.5–5 μg of nicked circular DNA is incubated at room temperature for 60 min with 0.5 unit of *M. luteus* DNA polymerase I (Miles Laboratories) per microgram of DNA. (Note: Contrary to previous protocols,[2,3] the addition of one deoxyribonucleoside triphosphate to this

[18] L. K. Miller and R. D. Wells, *J. Biol. Chem.* **247,** 2667 (1972).
[19] D. Shortle, Ph.D. Dissertation, The Johns Hopkins University, Baltimore, Maryland, 1979.
[20] D. R. Rawlins and N. Muzyczka, *J. Virol.* **36,** 611 (1980).
[21] P. T. Englund, S. S. Price, and P. M. Weigel, this series, Vol. 29, p. 273.

reaction is not recommended.) The reaction is stopped by adding an equal volume of 0.5 *M* NaCl–25 m*M* EDTA, pH 8.0, followed by phenol extraction and ethanol precipitation. A simple assay to determine the yield of gapped molecules is to allow an aliquot of 100–200 ng of gapped DNA to react with T4 DNA ligase and ATP overnight at 0°. When electrophoresed on a standard agarose gel containing 0.5 μg of ethidium bromide per milliliter, molecules closed by ligation (i.e., ungapped) move as a band with a higher mobility than open circular molecules (i.e., gapped). Therefore, by running as controls on the same gel equal amounts of gapped, unligated DNA and nicked, ligated DNA, the percentage of total molecules which have acquired a single-stranded gap can be estimated.

Mutagenesis with Sodium Bisulfite

The reaction in which sodium bisulfite catalyzes the deamination of cytosine residues occurs in three steps. The bisulfite ion first adds to the double bond of cytosine via a covalent linkage to C-6. The resulting intermediate 5,6-dihydrocytosine 6-sulfonate then, in the rate-limiting step, undergoes hydrolytic loss of the amino group at C-4 to generate 5,6-dihydrouracil 6-sulfonate. Although this compound is stable at the pH of 6.0 used in the first two steps, it undergoes elimination of the bisulfite moiety at slightly alkaline pH to form uracil. At high concentrations (1–3 *M*) of sodium bisulfite, the initial kinetics of cytosine deamination are linear with time and approximately first order with respect to bisulfite concentration.[1] Consequently, the percentage of cytosine residues deaminated can most readily be controlled by adjustments in one or both of these two variables, with pH and temperature held constant.

Procedure. A solution of 4 *M* sodium bisulfite, pH 6.0, is prepared immediately prior to use by dissolving 156 mg of sodium bisulfite ($NaHSO_3$) plus 64 mg of sodium sulfite (Na_2SO_3) in 0.43 ml of distilled water. To obtain levels of cytosine deamination greater than 5–10%, three volumes of this solution are added to one volume of DNA solution (10–50 μg/ml in 15 m*M* NaCl–1.5 m*M* sodium citrate, pH 7.0) in a 6 × 50 mm or 7 × 70 mm glass tube, followed by 0.04 volume of a freshly prepared solution of 50 m*M* hydroquinone. After thorough mixing, 100 μl of paraffin oil are layered above the mixture, and the reaction is carried out at 37° in the dark. Under these conditions of 3 *M* sodium bisulfite, pH 6.0 at 37°, the rate of reaction is approximately 8–10% of susceptible cytosine residues deaminated per hour for the first 2–4 hr. To terminate the reaction and remove the sodium bisulfite, the mixture is transferred to a dialysis bag and dialyzed against the following sequence of five buffers: (*a*) 1000 volumes or more of 5 m*M* potassium phosphate, pH 6.8–0.5 m*M* hydroqui-

none at 0° (on ice) for 2 hr; (*b*) a repeat of (*a*); (*c*) 1000 volumes or more of 5 m*M* potassium phosphate, pH 6.8 without hydroquinone at 0° for 4 hr. To promote elimination of bisulfite ion from the 5,6-dihydrouracil 6-sulfonate present at this stage, dialysis is continued against (*d*) 1000 volumes or more of 0.2 *M* Tris-HCl, pH 9.2–50 m*M* NaCl–2 m*M* EDTA at 37° for 16–24 hr; (*e*) 1000 volumes of 10 m*M* Tris-HCl, pH 8.0–1 m*M* EDTA at 4° for 4 hr. At this point the DNA is recovered and concentrated by ethanol precipitation.

Bisulfite ion is readily oxidized by dissolved O_2 gas to generate free radicals.[1] Particularly at low concentrations of bisulfite and in the presence of divalent cations, this side reaction can cause extensive damage to DNA. During the incubation with 3 *M* bisulfite and the early stages of dialysis, a low concentration of hydroquinone serves as a free-radical scavenger. Nevertheless, degassing of the water used to make the dialysis solutions by 10 min of vigorous boiling is recommended as a precaution.

Recovery of Mutants

DNA molecules that have undergone cytosine deamination within a single-stranded region contain the nonphysiological base uracil. Before such molecules can give rise to pure mutant clones, the uracil base must function as template for incorporation of a complementary A residue during synthesis of the opposite strand, either *in vitro* or *in vivo* after DNA transformation. However, most if not all cells possess one or more repair enzymes that excise uracil from DNA, the enzyme DNA uracil *N*-glycosylase being the major repair activity in *E. coli*.[22] Not surprisingly, the transformation efficiency of plasmid DNA molecules with large single-stranded gaps drops appreciably after bisulfite treatment when an *Escherichia coli* strain with this enzyme (*ung*$^+$) is used as host.[7] In addition, single CG base-pair deletions have been identified at sites mutagenized with bisulfite among pBR322 mutants recovered on transformation of an *ung*$^+$ *E. coli* strain.[4] *In vitro* repair of mutagenized gaps before transformation by using DNA polymerase and T4 DNA ligase increased both the transformation efficiency and the efficiency of mutagenesis[7] and may reduce the incidence of deletions in the *E. coli* system.[4] Therefore, it is advisable to repair enzymically single-stranded gaps before transformation; and if using *E. coli*, an *ung*$^-$ strain should be considered as a recipient for transformation.

Finally, the authors would like to point out that the degree of single-strand specificity of bisulfite mutagenesis—under the conditions described in this chapter—is not yet known. At least one instance of a

[22] T. Lindahl, *Prog. Nucleic Acids Mol. Biol.* **22,** 135 (1979).

bisulfite-induced mutation occurring outside the single-stranded target site has been reported.[23] Therefore, the appearance of a mutant phenotype in an isolate recovered after directed mutagenesis with sodium bisulfite is only circumstantial evidence that the phenotypic change results from a mutation identified at the target site. Before this conclusion can be safely drawn, genetic mapping must be employed to confirm that the mutation responsible for the mutant phenotype maps to the expected region.

[23] R. Rothstein and R. Wu, *Gene* **15,** 167 (1981).

[32] Oligonucleotide-Directed Mutagenesis of DNA Fragments Cloned into M13 Vectors

By MARK J. ZOLLER and MICHAEL SMITH

The isolation and sequencing of genes has been a major focus of biological research for almost a decade. The emphasis has now turned toward identification of functional regions encoded within DNA sequences. A variety of *in vivo*[1] and *in vitro*[2-4] mutagenesis methods have been developed to identify regions of functional importance. These techniques provide useful information pertaining to the location and boundaries of a particular function. Once this has been established, a method is required to define the specific sequences involved by precisely directing mutations within a target site. Oligonucleotide-directed *in vitro* mutagenesis provides a means to alter a defined site within a region of cloned DNA.[5] This powerful technique has many far-reaching applications, from the definition of functional DNA sequences to the construction of new proteins.

[1] J. W. Drake and R. H. Baltz, *Annu. Rev. Biochem.* **45,** 11 (1976).
[2] W. Muller, H. Weber, F. Meyer, and C. Weissmann, *J. Mol. Biol.* **124,** 343 (1978).
[3] D. Shortle, D. DiMaio, and D. Nathans, *Annu. Rev. Genet.* **15,** 265 (1981).
[4] C. Weissmann, S. Nagata, T. Taniguichi, T. Weber, and F. Meyer, *in* "Genetic Engineering," (J. K. Setlow and A. Hollaender, eds.), Vol. 1, p. 133. 1979.
[5] M. Smith and S. Gillam, *in* "Genetic Engineering," (J. K. Setlow and A. Hollaender, eds.), Vol. 3, p. 1. 1981.

ISBN 0-12-182000-9