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RESEARCH ARTICLE

A General Method for Saturation Mutagenesis of Cloned DNA Fragments

Richard M. Myers, Leonard S. Lerman, Tom Maniatis

The development of procedures for introducing single-base substitutions into specific cloned DNA sequences has provided important tools for studying the molecular genetics of eukaryotes (*1, 2*) and prokaryotes (*3*). Two general strategies have been used for site-directed mutagenesis. First, single-base substitutions have been introduced into cloned DNA at specific nucleotide positions by oligonucleotide-directed mutagenesis procedures (*2, 4, 5*). Second, a variety of approaches have been developed for introducing random base substitutions into specific DNA sequences. One approach involves the use of various types of nucleotide misincorporation procedures (*6*). Alternatively, random mutations have been generated by treatment of single-stranded DNA with chemical mutagens followed by enzymatic synthesis of the complementary DNA strand (*7, 8*). For example, sodium bisulfite treatment of DNA molecules containing a single-stranded gap generates C to T transitions (*7*). However, unlike sodium bisulfite, chemicals that generate other transitions and transversions do not react preferentially with single-stranded DNA and therefore cannot be used to introduce mutations specifically at gaps. To overcome this problem the entire DNA molecule can be treated with these chemicals under conditions that minimize the frequency of multiple base substitutions in the sequence of interest. However, under these conditions, only a fraction of the target DNA fragments will contain a mutation. Therefore this approach is limited to situations where DNA molecules carrying a mutation can be identified by a genetic screen or selection. In particular, it is difficult to use these procedures to study DNA sequences involved in developmental or tissue-specific gene regulation, or to study structure-function relationships in proteins for which a genetic selection is not available. We have circumvented this difficulty by making use of a denaturing gradient gel electrophoresis procedure to identify and purify mutant DNA molecules in the absence of a phenotypic selection.

DNA fragments differing by single-base substitutions can be separated from each other by electrophoresis in polyacrylamide gels containing an ascending gradient of the DNA denaturants urea and formamide (*9–14*). DNA fragments of identical size, but differing by a single-base change, will initially move through the polyacrylamide gel at a constant rate. As they migrate into a critical concentration of denaturant, specific regions or "domains" within the fragment melt to produce partially denatured DNA. Melting of a domain is accompanied by an abrupt decrease in mobility, which is a consequence of the entanglement of

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branched DNA molecules in the acrylamide matrix. The position in the denaturing gradient gel at which the decrease in mobility is observed corresponds to the melting temperature (T_m) of the domain. Since a single-base substitution within the melting domain results in a T_m difference, partial denaturation of the mutant and wild-type DNA fragments will occur at different positions in the gel. DNA molecules can therefore be separated on the basis of very small differences in the T_m's of their melting domains.

A typical DNA fragment of around 400 base pairs (bp) will have more than one melting domain. Although a decrease in the mobility of the fragment will occur when the domain with the lowest T_m melts, the molecules continue to move slowly into higher concentrations of denaturant, where the other melting domains undergo strand separation. Since single-base mutations in any of these domains will alter their melting properties, these mutations will lead to differences in the pattern of electrophoresis. However, when the highest temperature domain melts, the fragment undergoes complete strand dissociation, and the resolving power of the gel is lost (*13, 14*). Thus, mutations in the melting domain with the highest T_m cannot be separated from the wild-type DNA fragment. This limitation of the denaturing gradient gel system has been overcome by attaching a GC-rich DNA sequence, designated a "GC-clamp," next to the DNA fragment of interest (*13, 14*). This GC-clamp makes it possible to detect single-base substitutions in all melting domains of an attached DNA fragment by preventing complete strand dissociation as it proceeds through the denaturing gradient gel.

We now describe a new chemical mutagenesis procedure for introducing all types of single-base substitutions into cloned DNA fragments. In addition, we demonstrate the feasibility of purifying large numbers of single-base mutations by preparative denaturing gradient gel electrophoresis. This approach to saturation mutagenesis is rapid and is applicable to any DNA fragment from 30 to 600 bp in length.

Mutagenesis strategy. The steps in the mutagenesis procedure are outlined in Fig. 1. (i) Single-stranded DNA containing the DNA fragment to be mutagenized (target DNA) is obtained with either an M13 bacteriophage DNA vector (*15*), or a GC-clamp vector (Fig. 2) that contains both a plasmid and a bacteriophage M13 origin of DNA replication (*17*). In the latter case the resulting plasmid is used to transform bacteria to ampicillin resistance, and single-stranded DNA containing the target sequence is produced by infecting the transformed cells with M13 phage. (ii) The single-stranded DNA is treated with a chemical under conditions in which approximately 10 to 20 percent of the target DNA sequences contain one hit. The use of more severe mutagenesis conditions produces multiple mutations in many of the target DNA fragments. (iii) After removal of the chemicals, an oligonucleotide primer is annealed to the DNA and then extended with avian myeloblastosis virus (AMV) reverse transcriptase. At a high frequency the primer extension products will contain an incorrect base at positions corresponding to the damaged base on the single-stranded template DNA. (iv) The duplex target-DNA fragments are excised by restriction enzymes (A plus B) and inserted into a plasmid that is suitable for genetic screening or selection, or into a GC-clamp vector. If the latter approach is taken, the ligated DNA is used to transform bacterial cells to ampicillin resistance, and plasmid DNA is isolated from pooled colonies. (v) The pooled plasmid DNA is digested with

Abstract. *A new procedure for generating and isolating random single-base substitutions in cloned DNA fragments is presented. The mutations are generated by treatment of single-stranded DNA with various chemicals, followed by the synthesis of the complementary strand with reverse transcriptase. Misincorporation frequently occurs when the enzyme encounters a damaged base in the mutagenized template DNA. The resulting duplex DNA fragments containing random single-base substitutions are cloned, amplified as a population, and isolated from wild-type DNA by preparative denaturing gradient gel electrophoresis. The physical separation of mutant DNA fragments makes it possible to isolate and characterize large numbers of site-directed single-base substitutions in the absence of a phenotypic selection. This procedure should be generally applicable to the fine-structure genetic analysis of regulatory and protein-coding sequences.*

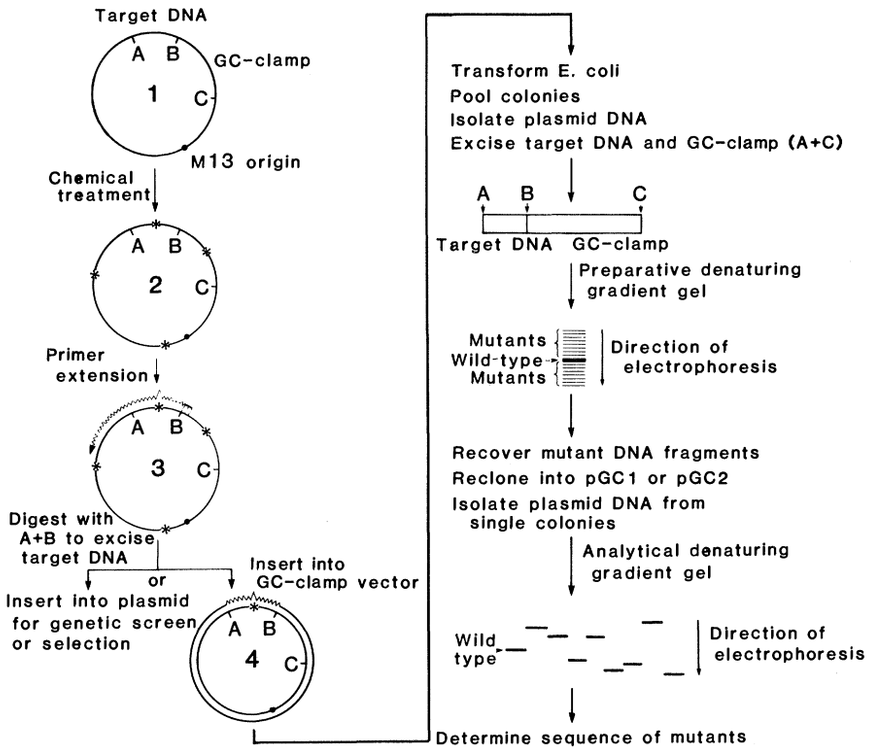


Fig. 1. Schematic diagram of the mutagenesis scheme. The circles labeled 1 through 3 represent single-stranded plasmid DNA containing the target sequence, the GC-clamp, the M13 origin of DNA replication and pBR322 DNA. The letters A, B, and C indicate restriction enzyme cleavage sites that are used to remove the target DNA (digestion with A + B) or target DNA + GC-clamp (A + C). The asterisks in circles 2 and 3 represent bases damaged by chemical treatment. The wavy arrow on circle 3 represents newly synthesized DNA primed by the GC-primer. Circle 4 represents the duplex pGC1 or pGC2 containing the damaged target DNA [see text and (*18, 22, 23*)].

restriction enzymes (C plus A) to excise a DNA fragment containing the target DNA and GC-clamp. The digestion products are then subjected to electrophoresis on a preparative denaturing gradient gel to separate the mutant and wild-type target DNA fragments. (vi) The mutant DNA fragments are recovered from the gel and ligated into a plasmid vector. The ligation mixture is used to transform *Escherichia coli* cells to ampicillin resistance, individual colonies are isolated, and the mutants are identified on an analytical denaturing gradient gel by again excising a DNA fragment containing both the target DNA and the GC-clamp. The DNA sequence of mutants is then determined. The details of each step in the mutagenesis procedure are described below.

Insertion of target DNA into M13 origin plasmids. Single-stranded DNA is required for the chemical mutagenesis step and serves as the template for the synthesis of a complementary DNA strand after chemical treatment. Moreover, separated single strands are useful for determining the sequence of the resulting mutants. Bacterial plasmids containing an origin of DNA replication from phage fd (*16*) or M13 (*17*) provide a convenient means of propagating the target DNA fragment as a plasmid, which can be used to generate large amounts of strand-specific single-stranded DNA. The plasmids pGC1 and pGC2 contain the bacteriophage M13 origin of replication (*17*), the ampicillin resistance gene, the GC-clamp, and a 65-bp polylinker (Fig. 2). The two plasmids differ only in the orientation of the polylinker. The target DNA fragment is inserted into the polylinker of pGC1 and pGC2 in opposite orientations with respect to the M13 origin so that each DNA strand can be separately mutagenized. In general, it is preferable to use naturally occurring restriction sites flanking the target DNA for the manipulations, so that the fragment can be reinserted into its original context after mutagenesis. However, if this is not essential, synthetic linkers can be used.

Table 1. The major targets for the three chemicals are shown and the time of incubation required to generate one damaged base every 1500 bp.

Compound	Primary targets	Time (minutes)
Nitrous acid	C, A, G	60
Formic acid	G, A	10
Hydrazine	C, T	10

Chemical mutagenesis. To maximize the chances of obtaining single-base substitutions in all positions of the target DNA, several different chemicals that damage DNA without breaking the phosphodiester backbone were tested (Table 1) (*18*). Nitrous acid is a widely used chemical mutagen that deaminates deoxycytosine, deoxyadenosine, and deoxyguanosine, changing these bases to deoxyuridine, deoxyhypoxanthine, and deoxyxanthine, respectively (*19*). Formic acid depurinates DNA by breaking the *N*-glycosyl bonds of purine bases, while hydrazine breaks pyrimidine rings (*20*).

To establish conditions for mutagenesis, the time of treatment with chemicals was varied, whereas the concentration of each chemical and the temperature were kept constant. If we assume that each mutagenic event is independent, the ratio of single to multiple base mutations in the target DNA follows a simple Poisson distribution. On the basis of this assumption, we adjusted the mutagenesis conditions so that approximately 10 to 20 percent of the target DNA fragments contain at least one mutation. This level of mutagenesis is sufficient to allow visualization and isolation of mutant DNA fragments on preparative denaturing gradient gels, yet only a small fraction (7 to 14 percent) of the mutant fragments will contain more than one base change. The level of mutagenesis was monitored by estimating the ratio of mutant and wild-type target DNA fragments by electrophoresis of pooled plasmid DNA on denaturing gradient gels. The time required to achieve approximately 10 to 20 percent mutagenesis in a target fragment of 135 bp varies from 10 to 60 minutes depending on the chemical (Table 1). Since the rate of base modification is linear with respect to time, treatment times should be doubled for fragments half this size.

Synthesis of duplex DNA from mutagenized single-stranded template. Site-directed chemical mutagenesis procedures usually involve treatment of single-stranded DNA with a chemical followed by synthesis of a complementary strand with DNA polymerase (*7, 21*). This step leads to misincorporation opposite the damaged base, and it generates a molecule that can be used to transform bacteria. If the target DNA is cloned into a single-stranded vector and the entire molecule is treated with the chemical, the frequency of damaged bases must be very low to avoid inactivating the phage vector (*21*). We have circumvented this problem by recloning the newly synthesized target DNA fragment into an untreated vector (*22*). With this approach it is possible to achieve a much higher frequency of single-base substitutions, and they are confined to the target DNA. AMV reverse transcriptase was chosen for second strand synthesis because previous studies indicated that depurination inhibits DNA synthesis by *E. coli* DNA polymerase I but not by reverse transcriptase (*21*). Indeed, we find that the reverse transcriptase copies

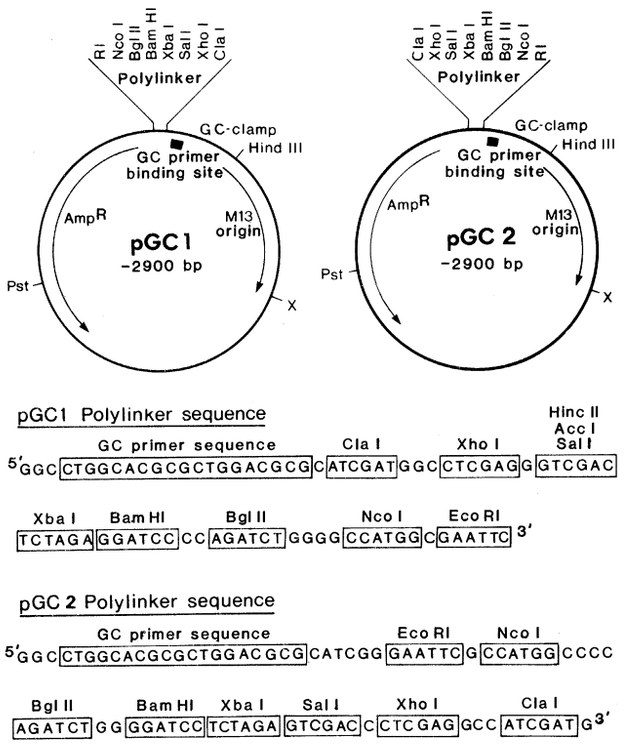


Fig. 2. Maps of GC-clamp plasmids. The plasmids pGC1 and pGC2 are composed of 1900 bp of pBR322 DNA which contains the ampicillin resistance gene and the colE1 origin (from nucleotides 2440 to 4362 on the pBR322 map), the bacteriophage M13 origin of replication (clockwise containing nucleotides 5330 to 6001 of M13), the GC-clamp (*13, 14*), and a 65-bp polylinker. The two plasmids are identical except for the orientation of the polylinker. A sequence homologous to the GC-primer used for the synthesis of second strand DNA and for dideoxy sequence analysis is located at the junction between the GC-clamp and the polylinker.

chemically treated and untreated DNA with equal efficiencies.

Denaturing gradient gel electrophoresis. Under optimal conditions, approximately 10 to 20 percent of the duplex target DNA fragments generated above contain single-base substitutions. Even with this relatively high efficiency of mutagenesis, it would be prohibitive to identify mutant DNA fragments by DNA sequence analysis. In some cases this problem can be solved by inserting the population of target DNA fragments into a plasmid that can be used in a genetic screen or selection. The alternative approach used here is to physically separate the fragments containing base substitutions from the wild-type fragments.

The first step in the denaturing gradient gel system is to establish optimal conditions for separating single-base substitutions in the target DNA fragment of interest (*23*). This optimization is accomplished by determining the concentration of denaturant at which melting domains within the target DNA undergo a helix-to-coil transition. This concentration can be easily derived by running the target DNA and the attached GC-clamp on a polyacrylamide gel containing a concentration gradient of denaturants perpendicular to the direction of electrophoresis (perpendicular gradient gel) (Fig. 3). When the mouse β-globin promoter fragment is attached to the GC-clamp in one orientation, a single melting transition is observed at a position in the gel corresponding to a denaturant concentration of 58 percent (Fig. 3A). The best resolution between wild-type and mutant DNA fragments is achieved when the domain of interest melts approximately in the middle of a gradient gel containing a total range of denaturant concentrations of 25 to 30 percent (*13*). In practice, gels containing a gradient of denaturants parallel to the direction of electrophoresis are used for preparative purposes and for the analysis of a large number of different mutants. Thus, from the data of Fig. 3A, a parallel gradient of approximately 45 to 70 percent denaturants would be used for the β-globin promoter fragment in this orientation.

Different gradient conditions are used when the β-globin promoter fragment is attached to the GC-clamp in the opposite orientation. As shown in the perpendicular gel in Fig. 3B, the promoter in this orientation displays two discrete melting transitions at denaturant concentrations of 38 and 68 percent. When a fragment attached to the GC-clamp melts in two domains, the best resolution is achieved with two types of parallel denaturing gradient gels. For mutants in the first melting domain, a gel with a 25 to 50 percent gradient of denaturants is used, whereas for the second domain mutants, a 55 to 80 percent gradient of denaturants is used (*13, 14*).

In practice, the target DNA of interest should be examined on perpendicular gels in both orientations with respect to the GC-clamp. The orientation with the fewest melting transitions should then be used in the preparative gradient gel electrophoresis step of the procedure. The time of electrophoresis is established by estimating the time required for the duplex DNA to reach the middle of the gel where melting begins (typically 5 to 6 hours for a 135-bp fragment attached to the GC-clamp), and then continuing electrophoresis for an additional 1 to 2 hours.

The results of a typical preparative gel of pools of mutagenized target DNA fragments are shown in Fig. 4A. Although most of the DNA migrates to the same position in the gel as the wild-type

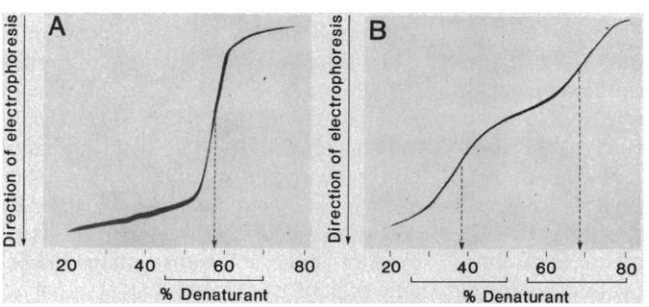
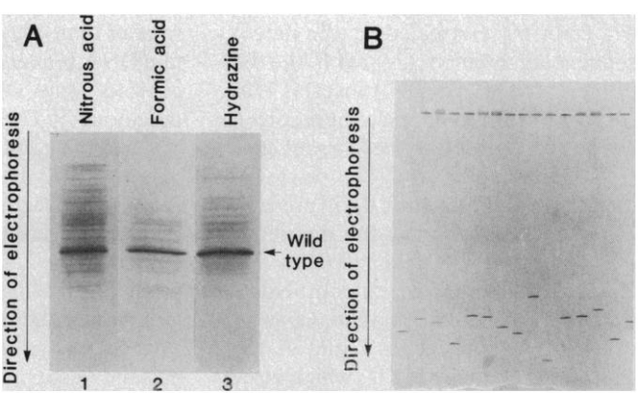


Fig. 3. Optimization of the denaturing gradient for maximal separation of mutant and wild-type target DNA fragments. (A) Perpendicular denaturing gradient gel of a fragment containing the GC-clamp and the mouse β-globin promoter fragment in one orientation. A gel containing a linear 20 to 80 percent gradient of denaturants perpendicular to the direction of electrophoresis was prepared as described (*13, 14*). The DNA sample was digested with restriction enzymes and layered across the top of the gel. Electrophoresis was carried out at 150 V for 5 hours, and the DNA was visualized by ethidium bromide staining. The midpoint of the melting transition corresponds to the T_m of the single melting domain of the attached target DNA fragment. The percentage of denaturant corresponding to the T_m can be determined by measuring the position of the midpoint of the transition on the horizontal axis of the gel. In the example shown, the T_m corresponds to a denaturant concentration of approximately 58 percent. The bracket indicates the range of denaturant concentrations to be used in the preparative denaturing gradient gel step in the mutagenesis procedure. (B) Perpendicular denaturing gradient gel of a fragment containing the GC-clamp and the mouse β-globin promoter fragment in the orientation opposite to that in (A). The gel and electrophoresis conditions were the same as those described in (A). In this orientation the promoter melts in two domains, as indicated by the presence of two distinct melting transitions. The T_m of the first domain corresponds to approximately 38 percent denaturant, whereas the T_m of the second domain corresponds to 68 percent denaturant. As in (A), the brackets indicate the optimal range of denaturant concentrations for each domain used in the preparative denaturing gradient gel.

Fig. 4. Preparative and analytical denaturing gradient gel electrophoresis of mutant and wild-type DNA fragments. (A) A preparative denaturing gradient gel of pooled mutagenized fragments containing the GC-clamp and the target fragment. (Lane 1) Nitrous acid pool; (lane 2) formic acid pool; (lane 3) hydrazine pool. The nitrous acid pool shown in this gel was composed of approximately 30 percent mutant and 70 percent wild-type DNA fragments. As was expected, a higher proportion (~20 percent) of the mutants isolated from this pool contained multiple (mostly two) base changes. The two other pools in this gel (representing approximately 10 and 15 percent mutagenesis efficiencies) resulted in fewer multiple mutants. (B) Analytical denaturing gradient gel of individual mutant DNA fragments purified from the preparative denaturing gradient gel and cloned into pGC1. The DNA in each lane was obtained from a plasmid miniprep of individual colonies and digested with restriction enzymes that release the target DNA fragment attached to the GC-clamp. The left lane contains the wild-type DNA fragment and the remaining lanes contain examples of different mutant DNA fragments.



	C→T	C→A	C→G	T→C	T→A	T→G	G→T	G→A	G→C	A→T	A→C	A→G
Nitrous acid	30	—	1	1	—	—	—	6	1	1	—	15
Formic acid	1	4	—	9	—	—	13	3	6	6	3	1
Hydrazine	11	1	—	18	1	4	—	—	1	1	—	11

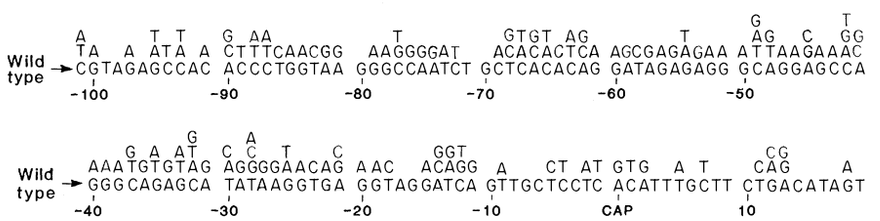


Fig. 5. Distribution of single-base substitutions in the mouse β-globin promoter region. (A) Compilation of the single-base substitutions obtained with the three chemicals discussed in the text. (B) Location of single-base substitutions in the mouse β-globin promoter region. The wild-type sequence is written as a continuous line, and the mutants appearing at each position are above the wild-type sequence.

DNA fragment, faint bands can be seen above and below the position of wild-type DNA. The mutant fragments are carefully excised and eluted from the gel and cloned as a population in the pGC1 or pGC2 vector. Individual colonies from the population are picked, and the plasmid DNA is examined by analytical denaturing gradient gel electrophoresis. An example of such an analysis is shown in Fig. 4B. Each transformant contains a different single-base substitution, and the separation from the wild-type DNA fragment ranges from 1 to 12 mm. Depending on the extent of mutagenesis and the care that is taken in recovering the mutant fragments from the preparative gel, anywhere from 35 to 100 percent of the colonies examined contain a mutant DNA fragment. Because of this variability we routinely check individual transformants to avoid unnecessary sequencing of wild-type DNA fragments.

Characterization of mutant fragments. Rapid sequence analysis of mutant DNA fragments is facilitated by the presence of the M13 origin in the pGC plasmids. After production of single-stranded DNA, the sequence of the target DNA is determined by the dideoxy sequencing method (*24*) with the GC-primer. If the target DNA insert is 150 to 200 bp in length or less, the entire sequence can be determined on one gel.

A tabulation of the 132 single-base mutations obtained thus far is presented in Fig. 5. Significantly, the distribution of the mutations obtained with each chemical appears to be random. Nitrous acid deaminates C, A, and G, which usually results in C to T and A to G transitions (deaminated G, which is xanthine, forms base pairs with C, so that no base substitution is expected). Indeed, the majority of the mutations observed with nitrous acid were the expected transitions. However, for reasons not understood, a few other transitions and transversions also occurred. Formic acid depurination resulted in a wide spectrum of transitions and transversions, including unexpected changes at pyrimidine residues. Finally, breakage of pyrimidine rings by hydrazine treatment resulted primarily in C to T and T to C transitions, but a number of other transitions and transversions were also observed. The mechanisms by which these unexpected base changes were generated are not understood. Nevertheless, by mutagenizing both strands of the target DNA with all three chemicals separately it should be possible to obtain nearly all base changes at every position in the target DNA.

Conclusion. We have described a rapid and efficient method for generating and isolating large numbers of random single-base substitutions. This procedure should be generally applicable to any DNA fragment in the size range of 30 to 600 bp in length. In practice, fragments smaller than 200 bp are chosen to facilitate DNA sequence analysis of the mutagenized target DNA. The generation of mutations by chemical treatment is simple, and the conditions are reproducible. In previously described chemical mutagenesis procedures, mild mutagenesis conditions were used to ensure viability of the vector (*21*). We circumvented this problem by using more severe chemical treatment conditions and then transferring the target DNA to an untreated plasmid vector. The target DNA recloning strategy was also used in oligonucleotide-directed and chemical-induced mutagenesis procedures (*25*). In conjunction with the chemical mutagenesis procedure this strategy allows the maximum possible frequency of single-base mutations, and it ensures that the mutations are exclusively localized in the target DNA.

The mutant DNA fragments produced by this procedure may be used directly in a genetic selection or screen, or they can be purified by gradient gel electrophoresis. The advantage of the latter approach is that base changes that do not affect function can be studied. We have estimated that 95 percent of all possible single-base substitutions can be resolved by denaturing gradient gel electrophoresis if the target DNA is attached to a GC-clamp (*13, 14*). The GC-clamp vectors (Fig. 2) were designed for easy movement of the target DNA between plasmids, and for the placement of the fragment in both orientations with respect to the clamp and M13 origin of replication.

Saturation mutagenesis in the absence of a phenotypic selection should be widely applicable to problems in molecular genetics and protein engineering. For example, this approach should be especially useful for analyzing elements involved in developmental or tissue-specific gene regulation. In the analysis of structure-function relationships of proteins, it is sometimes possible to predict the effect of specific amino acid changes on enzymatic activity (*26*). In this case, oligonucleotide-directed mutagenesis is preferable to random mutagenesis. However, in many circumstances it would be useful to generate large collections of random amino acid substitutions in specific regions of a protein.

References and Notes

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18. *Chemical mutagenesis procedures*: In three separate reactions for each target DNA, 40 μg of single-stranded DNA (a 1:1 mixture of plasmid DNA containing the target DNA and the M13 helper DNA) is treated with nitrous acid, formic acid, or hydrazine. Each reaction is perfomed in a volume of 100 μl at room temperature. Under the following conditions approximately one damaged base is introduced every 1500 nucleotides. Thus, if the target DNA is 150 nt in length, approximately 10 percent will contain one damaged base. Since the number of damaged bases increases as a linear function of time, a 10 percent mutagenesis efficiency can be achieved for target DNA fragments of any length by adjusting the reaction time. *Nitrous acid*: 1 hour in 250 m*M* sodium acetate, *p*H 4.3 and 1.0*M* sodium nitrite. A stock of 2*M* sodium nitrite is made in water and stored at +4°C for up to 1 week. *Formic acid*: 10 minutes in 12*M* formic acid. *Hydrazine*: 10 minutes in 60 percent hydrazine. The reactions are stopped by addition of 100 μl of 2.5*M* sodium acetate, *p*H 7, 20 μg of transfer RNA, 200 μl of water, and the DNA precipitated by the addition 1 ml of ethanol. The DNA is then precipitated with ethanol two more times to remove all traces of the mutagen. Samples are resuspended in 80 μl of TE buffer for the synthesis of double-stranded DNA as described below. Other chemicals have also been used successfully in this procedure. Treatment of the single-stranded DNA with 0.13 m*M* potassium permanganate resulted in transition mutations at both T and C residues. Depurination by the dimethyl sulfate (DMS) A and G reactions used in the chemical degradation DNA sequencing procedure (*20*) gave mutagenic specificities similar to that of formic acid. Permanganate and DMS treatment for 10 minutes at room temperature resulted in approximately 10 percent mutagenesis of the 135-bp β-globin fragment.
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22. *Synthesis of double-stranded DNA*: In a 100 μl reaction, 100 pmol of synthetic DNA GC primer (5′ CTGGCACGCGCTGGACGCG 3′) was annealed to each chemically treated single-stranded DNA sample in 7 m*M* tris-HCl, *p*H 7.5, 7 m*M* $MgCl_2$, 50 m*M* sodium chloride, and 2 m*M* dithiothreitol by treating the mixture at 85°C for 5 minutes and then at 40°C for 15 minutes; 10 μl of a solution containing (each at 1.25 m*M*) dGTP, dCTP, dATP, and dTTP was added. The reaction mixture was incubated at 37°C for 1 hour after the addition of 20 units of AMV reverse transcriptase (purchased from Life Sciences, Inc.). Carrier transfer RNA (20 μg) is added and the reactions are extracted with phenol and precipitated with ethanol. The double-stranded target DNA fragments are then excised from the plasmid by restriction enzyme digestion. The reaction mix is extracted with phenol, precipitated with ethanol and resuspended in 10 μl of water, and incubated at 37°C for 15 minutes with RNase A (100 μg/ml) to remove the carrier transfer RNA. The duplex target DNA fragments are then purified by agarose or polyacrylamide gel electrophoresis, and then inserted into pGC1 or pGC2 adjacent to the GC-clamp and used to transform *E. coli* strain MC1061 (*27*). Agar plates containing from 500 to several thousand colonies are scraped, and plasmid DNA is isolated from the pooled colonies. A DNA fragment carrying the target DNA and the GC-clamp is excised from the pooled plasmid DNA with the appropriate restriction enzymes, and 5 μg of the digest (approximately 500 ng of the excised fragment) is subjected to electrophoresis on a preparative denaturing gradient gel.
23. *Denaturing gradient gel electrophoresis*: The denaturing gradient gel system has been described (*11*), as well as the procedure for preparing and running the gels (*13*). The gel apparatus and necessary attachments can be obtained from Green Mountain Lab Supply, 86 Central Street, Waltham, Mass. 02154. After electrophoresis, the gel is stained with ethidium bromide, and mutant DNA molecules moving slower or faster than the wild-type fragment are eluted from the gel. These eluted fragments are then ligated into a plasmid backbone, and the mixture is used to transform an *E. coli* strain containing an F′ (LE392 F′ was used in these studies) (*17*). Small double-stranded DNA preparations made from individual colonies are digested with restriction enzymes to remove the target fragment attached to the GC-clamp and these molecules are examined to an analytical gradient gel. This step is included because occasionally up to 65 percent of the individual colonies may be wild type as a result of streaking of the DNA on the preparative denaturing gradient gel. Single-stranded DNA is made from individuals whose mobility is different from that of the wild type on the gradient gel, and the DNA sequence is determined by the dideoxy method, with the GC-primer.
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28. We thank S. Fischer for advice and discussions in the early stages of this work, B. Seed, S. Goodbourn, J. Posakony, D. DiMaio, and R. Brent for valuable discussions, Z. Larin-Goodbourn for technical assistance, and G. Brown for providing the GC-clamp primer. Supported by grants from the National Institutes of Health (L.S.L. and T.M.), a Damon Runyon–Walter Winchell Postdoctoral Fellowship (R.M.M.), and a Special Fellowship of the Leukemia Society of America (R.M.M.).

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