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Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis

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ABSTRACT

Duplex DNA fragments differing by single base substitutions can be separated by electrophoresis in denaturing gradient polyacrylamide gels, but only substitutions in a restricted part of the molecule lead to a separation (1). In an effort to circumvent this problem, we demonstrated that the melting properties and electrophoretic behavior of a 135 base pair DNA fragment containing a β-globin promoter are changed by attaching a GC-rich sequence, called a 'GC-clamp' (2). We predicted that these changes should make it possible to resolve most, if not all, single base substitutions within fragments attached to the clamp. To test this possibility we examined the effect of several different single base substitutions on the electrophoretic behavior of the β-globin promoter fragment in denaturing gradient gels. We find that the GC-clamp allows the separation of fragments containing substitutions throughout the promoter fragment. Many of these substitutions do not lead to a separation when the fragment is not attached to the clamp. Theoretical calculations and analysis of a large number of different mutations indicate that approximately 95% of all possible single base substitutions should be separable when attached to a GC-clamp.

INTRODUCTION

A denaturing gradient gel electrophoresis procedure (3) has been used to identify restriction fragments of bacteriophage λ DNA bearing single base mutations (1). More recently this procedure has been used to detect single base substitutions in cloned human β-globin genes obtained from individuals with β-thalassemia (4,5), and the same mutations have been detected directly in total genomic DNA from β-thalassemia patients (5). The potential applications of the denaturing gradient gel system include the identification, localization, and diagnosis of naturally-occurring mutations, the mapping of induced mutations that can be phenotypically selected, the detection of new sequence polymorphisms for human genetic linkage analysis and for population genetic studies, and the purification of *in vitro*-generated point mutations.

At present, the utility of the denaturing gradient gel procedure is limited by the fact that not all single base substitutions lead to changes in the electrophoretic behavior of DNA fragments (1). This problem is a

consequence of the melting properties of DNA and the relationship between DNA melting and the electrophoretic behavior of a fragment in the denaturing gradient (6). As DNA fragments move through polyacrylamide gels containing an ascending gradient of denaturant, small regions called melting domains undergo a cooperative strand dissociation to produce partially denatured molecules which display a marked decrease in electrophoretic mobility. As these DNA molecules continue to move slowly into higher concentrations of denaturant, additional melting domains undergo strand dissociation. Because single base changes in any of these domains will alter their melting temperature (Tm), these changes will lead to differences in the pattern of electrophoresis in the denaturing gradient gel. However, when the final, or most stable, domain melts the fragment undergoes complete strand dissociation and the resolving power of the gel is lost. Therefore, base substitutions in the highest temperature melting domain of a DNA molecule cannot be detected by denaturing gradient gel electrophoresis. In an effort to circumvent this problem we attached a GC-rich DNA sequence (80% G+C) to a 135 base pair β-globin promoter fragment, and then analyzed the electrophoretic behavior of this hybrid molecule in denaturing gradient gels (2). We observed significant differences in the melting behavior of the promoter fragment when attached to the GC-clamp. In particular, the entire promoter can be melted while the GC-clamp remains in the duplex configuration, thus avoiding complete strand separation of the hybrid molecule. In principle it should now be possible to detect base substitutions in all regions of fragments attached to the GC-clamp.

In this paper we construct a number of single base substitutions in different regions of the β-globin promoter fragment, and then determine whether these substitutions can be detected when the fragment is attached to the GC-clamp. We find that every mutant tested can be detected by electrophoresis in denaturing gradient gels, including those located in the highest temperature melting domain of the promoter. These substitutions cannot be detected in the absence of the GC-clamp. Similar results from the analysis of a large number of other base substitutions and the results of theoretical melting calculations indicate that approximately 95% of all possible base substitutions in most fragments could be detected if the fragment is attached to the GC-clamp.

MATERIALS AND METHODS

Materials

The plasmid DNA molecules containing the GC-clamp and the wild type

mouse βmaj-globin promoter are described in the preceding paper (2). Mutant promoter molecules were prepared by a procedure involving random chemical mutagenesis that will be described later (in preparation).

Denaturing gradient gel electrophoresis

Electrophoresis on perpendicular and parallel denaturing gradient gels was performed as described in the preceding paper (2). Perpendicular gel electrophoresis of isolated promoter fragments was on 6.5% acrylamide gels with a 20%-80% denaturant range for 3 hr (100% denaturant = 7 M urea + 40% formamide; acrylamide/bisacrylamide = 37.5/1). Perpendicular gradient gel electrophoresis of GC-clamped promoter fragments was on 6.5% acrylamide gels with a denaturant range of 20%-80% for 5 hr. Parallel 'domain 1 mutant' gradient gels for GC-clamped promoters were 6.5% acrylamide with a 30%-60% denaturant range and were electrophoresed for 5 hr. Parallel 'domain 2 mutant' gradient gels for the GC-clamped promoters were 6.5% acrylamide with a 45%-80% denaturant range and were electrophoresed for 11 hr.

Computer-derived predictions of melting behavior

The theoretical separations of wild type and mutant DNA fragments in denaturing gradient gels were calculated as noted in the accompanying paper (2). These calculations made use of the sequence-specific statistical-mechanical theory of Poland (7), the computational improvement of Fixman and Friere (8), the nearest neighbor stability values of Gotoh and Tagashira (9), and a theoretical relation between the mobility of partly melted DNA molecules and the length of the melted region (6). The calculation assumes that the mobility declines exponentially as the number of melted flexible units increases. The number of melted bases constituting a flexible unit, 85, was inferred from analysis of perpendicular gradient gel patterns, using the defined linear relationship between denaturant concentration and temperature. There is a negligible difference between the initial rates of travel of the wild type and mutant DNA fragments as they begin moving through the gradient when both are completely double stranded. The rate difference becomes appreciable when one DNA fragment begins to melt earlier than the other. However, the resolution of the two partially melted DNA fragments decreases as they travel further into the gel.

A completely theoretical calculation requires some information that is not available. For example, the amount of denaturant lost from the top of the gel by diffusion, the initial fragment velocities, and an accurate temperature-denaturant calibration are unknown. We have therefore allowed some flexibility in the parameters characterizing the temperature-time cali-

bration. We used consistent values for the entire data set and experimentally derived relative running times. The expected shift values were based on a full calculation for each sequence and the theoretical gradient postion as a function of time, resulting in an interval verses time table for each single base substitution. Calculations were carried out mostly on a Charles River Data System model 68/05 computer.

RESULTS AND DISCUSSION

Analysis of wild type and mutant promoter fragments in the absence of a GC-clamp

DNA melting calculations using the sequence of a 135 bp fragment of DNA containing the mouse βmaj-globin promoter region predicted the existence of two melting domains. The region melting at the lower temperature (domain 1; ~71°C) extends from about -30 to +26 relative to the mRNA cap site. Domain 2, which melts at about 76°C, extends from -104 to -30. Single base substitutions located in either melting domain were constructed; most experiments presented here involved the use of a substitution at +8 (C to T; 'domain 1 mutant') and one at -60 (G to A; 'domain 2 mutant').

To determine whether either or both of these substitutions can be detected by denaturing gradient gel electrophoresis, a mixture of the wild type promoter fragment and the domain 1 mutant fragment was analyzed by electrophoresis in an acrylamide gel containing a concentration gradient of DNA denaturants perpendicular to the direction of electrophoresis (a 'perpendicular gradient gel'). As shown in Figure 1A separation of the two fragments occurs at a sharp transition corresponding to the melting of domain 1. Similar results were obtained with several other domain 1 mutants (data not shown). In contrast, when a similar analysis was performed with the domain 2 mutant, the wild type and mutant fragments are not resolved (Figure 1B). Other domain 2 mutants also failed to separate from the wild type fragment (data not shown).

Wild type and mutant promoter fragments were also analyzed on a gel containing a gradient of DNA denaturants parallel to the direction of electrophoresis (a 'parallel gradient gel'). Consistent with the perpendicular gradient gel analysis, the wild type (Figure 1C, lane 1) and the domain 1 mutant (Figure 1C, lane 2) fragments separate, but no resolution between the wild type and domain 2 mutant (Figure 1C, lane 3) fragments is observed. Similar results were obtained for several other fragments bearing different substitutions in either melting domain; in no case was a domain 2 mutant

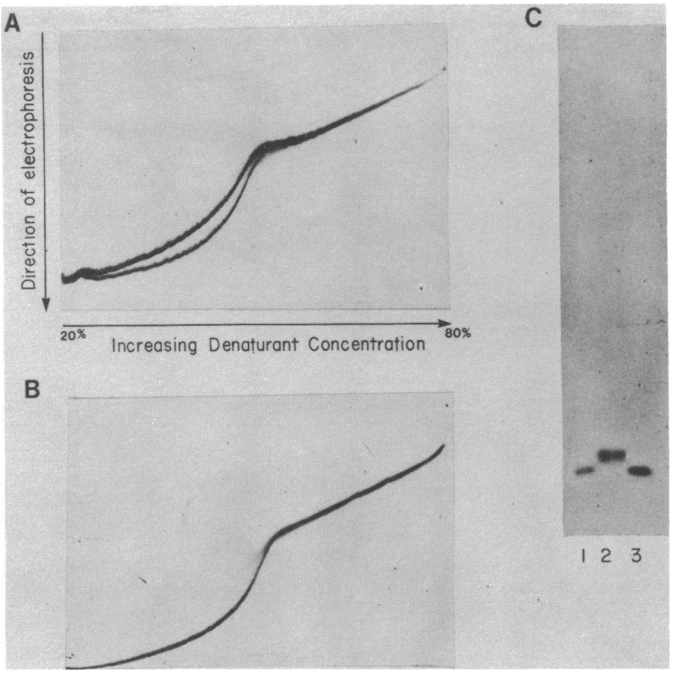

FIGURE 1: Denaturing gradient gel patterns of unclamped wild type and mutant β-globin promoter fragments.
(A) Perpendicular gel pattern of a mixture of the unclamped wild type and domain 1 mutant DNA fragments. The DNA mix was layered onto a 6.5% polyacrylamide gel containing a linear gradient of 20% to 80% denaturants and electrophoresed for 130 minutes at 150V. The DNA was visualized by staining with ethidium bromide; a negative image of the stained gel is shown.
(B) Perpendicular gel pattern of a mixture of the unclamped wild type and domain 2 mutant DNA fragments. The gel conditions were the same as those in panel (A).
(C) Parallel gel pattern of the unclamped wild type (lane 1), domain 1 mutant (lane 2), and domain 2 mutant (lane 3) DNA fragments. The DNA samples were layered onto a 14% polyacrylamide gel containing a linear gradient of 20% to 80% denaturants and electrophoresed for 5 hours at 150V.

fragment separated from the wild type fragment (data not shown). Thus, as predicted, domain 1 but not domain 2 mutant fragments can be separated from the wild type DNA fragment using both perpendicular and parallel gradient gels.

Analysis of wild type and mutant promoter fragments attached to the GC-clamp

Attachment of the GC-clamp to the β-globin promoter fragment significantly affects both the predicted and observed melting behavior of the fragment (2). When the clamp is attached at position -104 of the promoter frag-

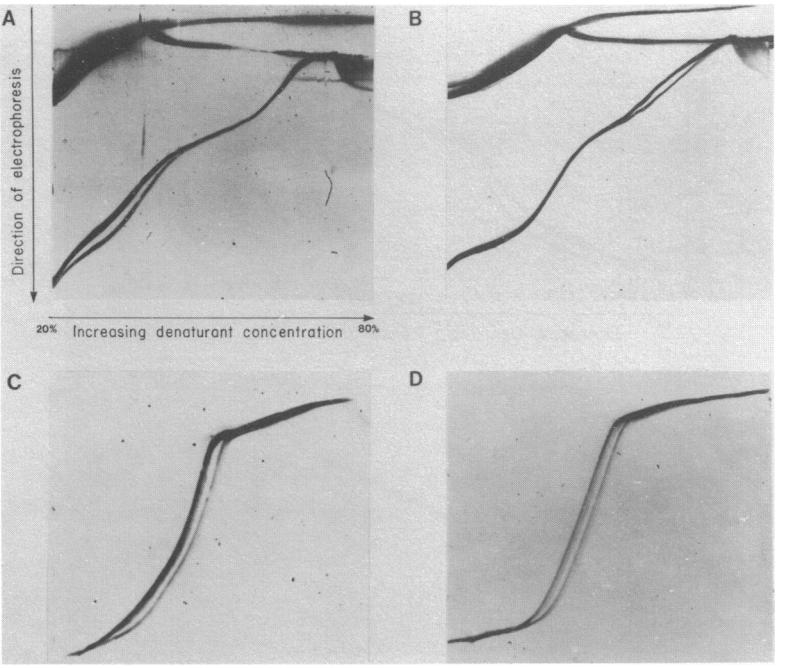

FIGURE 2: Perpendicular denaturing gradient gel patterns of wild type and mutant β-globin promoter fragments attached to the GC-clamp.
(A) Wild type and domain 1 mutant promoter fragments with the GC-clamp in the -104 orientation. The stained DNA at the top of the gel is from the plasmid vector.
(B) Wild type and domain 2 mutant promoter fragments with the GC-clamp in the -104 orientation.
(C) Wild type and domain 1 mutant promoter fragments with the GC-clamp in the +26 orientation.
(D) Wild type and domain 2 mutant promoter fragments with the GC-clamp in the +26 orientation.
The gel conditions for A-D were the same as those in Figure 1A, except that electrophoresis was for 5 hrs.

ment (the '-104 orientation') two melting domains are observed within the promoter sequence. The location and Tm of these domains is the same as that observed for the promoter fragment in the absence of the clamp. When the clamp is attached at position +26 of the promoter fragment (the '+26 orientation'), the promoter melts as single domain (2). To study the effect of domain 1 and 2 mutants on the melting behavior of the clamped promoter fragment the GC-clamp was attached in both the -104 and +26 orientations.

The perpendicular gel pattern of a mixture of wild type and domain 1 mutant DNA fragments clamped in the -104 orientation is shown in Figure 2A.

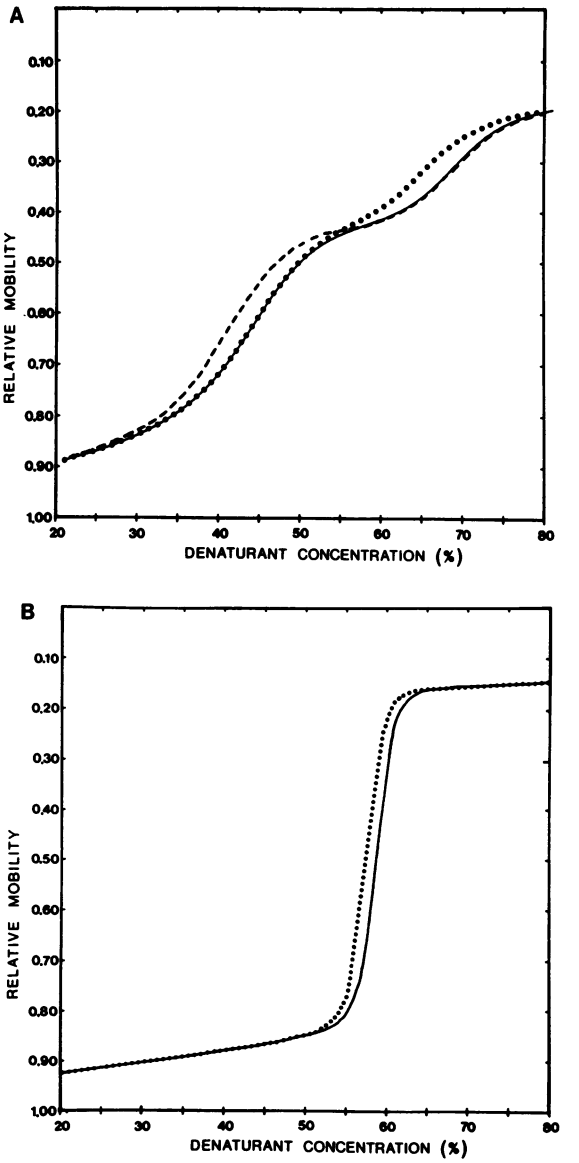

FIGURE 3: Calculated perpendicular denaturing gradient gel patterns of the wild type and mutant β-globin promoter fragments attached to the GC-clamp. (A) Calculated gel patterns for the promoter fragments attached to the GC-clamp in the -104 orientation. solid line: wild type fragment, dashed line: domain 1 mutant fragment, dotted line: domain 2 mutant fragment
(B) Calculated gel patterns for the promoter fragments attached to the GC-clamp in the +26 orientation. solid line: wild type fragment, dotted line: domain 1 and 2 mutant fragments

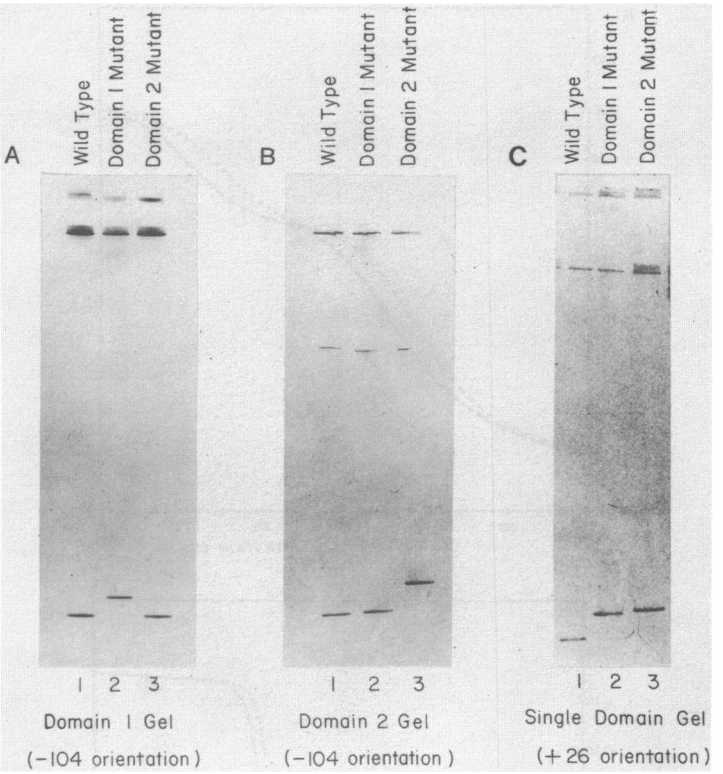

FIGURE 4: Parallel denaturing gradient gel patterns of wild type and mutant β-globin promoter fragments attached to the GC-clamp.
(A) Promoter fragments with the GC-clamp in the -104 orientation on a domain 1 gel. The DNA samples were loaded on a 6.5% polyacrylamide gel containing a parallel linear gradient of 30% to 60% denaturants and electrophoresed for 5 hours at 150V. lane 1: wild type, lane 2: domain 1 mutant, lane 3: domain 2 mutant
(B) Promoter fragments with the GC-clamp in the -104 orientation on a domain 2 gel. The DNA samples were loaded on a 6.5% polyacrylamide gel containing a parallel linear gradient of 45% to 80% denaturants and electrophoresed for 11 hours at 150V. lane 1: wild type, lane 2: domain 1 mutant, lane 3: domain 2 mutant
(C) Gel patterns of promoter fragments with the GC-clamp in the +26 orientation on a single domain gel. The DNA samples were loaded on a 6.5% polyacrylamide gel containing a parallel linear gradient of 37% to 67% denaturants and electrophoresed for 6 hours at 150V. lane 1: wild type, lane 2: domain 1 mutant, lane 3: domain 2 mutant

Separation between the two fragments is observed in the region of the gel where domain 1 melts. As expected, separation is not observed in the region of the gel where domain 2 melts. In contrast, when the domain 2 mutant is examined in the same manner, separation is observed only in the region of

the gel where domain 2 melts (Figure 2B). When the same mutant DNA fragments are examined in the +26 orientation, where only a single melting domain is observed in the promoter fragment, both the domain 1 and 2 mutants are clearly separated from the wild type DNA fragment (Figures 2C and 2D). Similar results were obtained with other domain 1 and 2 mutants (data not shown). These observations indicate that domain 2 mutants, which cannot be detected in the absence of the GC-clamp, are readily separable when the promoter fragment is attached to the clamp.

To determine whether the melting behavior of the clamped promoter fragments predicted by calculation are consistent with the observed gel patterns, we used the computer algorithm described in the accompanying paper to simulate the gel patterns of a mixture of wild type and domain 1 and 2 mutant fragments attached to the GC-clamp in the -104 orientation. As shown in Figure 3, the computed gel patterns for mobility as a function of denaturant are remarkably similar to those determined experimentally. Thus, it should be possible to use the algorithm to accurately predict the effect of the GC-clamp on the melting behavior of any DNA fragment and its mutant derivatives.

Parallel gradient gels are preferable to perpendicular gradient gels for the analysis of a large number of different mutants and for preparative purposes. For optimal resolution of mutants located in a particular melting domain, the denaturing gradient used in the parallel gel is adjusted so that the domain of interest melts approximately in the middle of the gel. In addition, the resolution is affected by the steepness of the gradient. In practice, the midpoint of the denaturing gradient for the parallel gel is chosen by first examining the melting transition for the domain of interest on a perpendicular gradient gel. For example, in the case of the β-globin promoter fragment clamped in the -104 orientation, the midpoint of the melting transition of domain 1 occurs at a denaturant concentration of 45%. In order to examine domain 1 mutants we prepared a parallel gel with a gradient of denaturants from 30% to 60%. These gradient conditions were chosen by taking the midpoint of the gradient as 45% denaturant and the total range of denaturant concentration as 30%. As shown in Figure 4A, the domain 1 mutant clearly resolves from the wild type fragment in the 'domain 1 gel', but the gel position of the domain 2 mutant is indistinguishable from the wild type fragment.

In order to examine domain 2 mutants clamped in the -104 orientation we used a parallel gradient of 45% to 80% denaturant, since the midpoint of

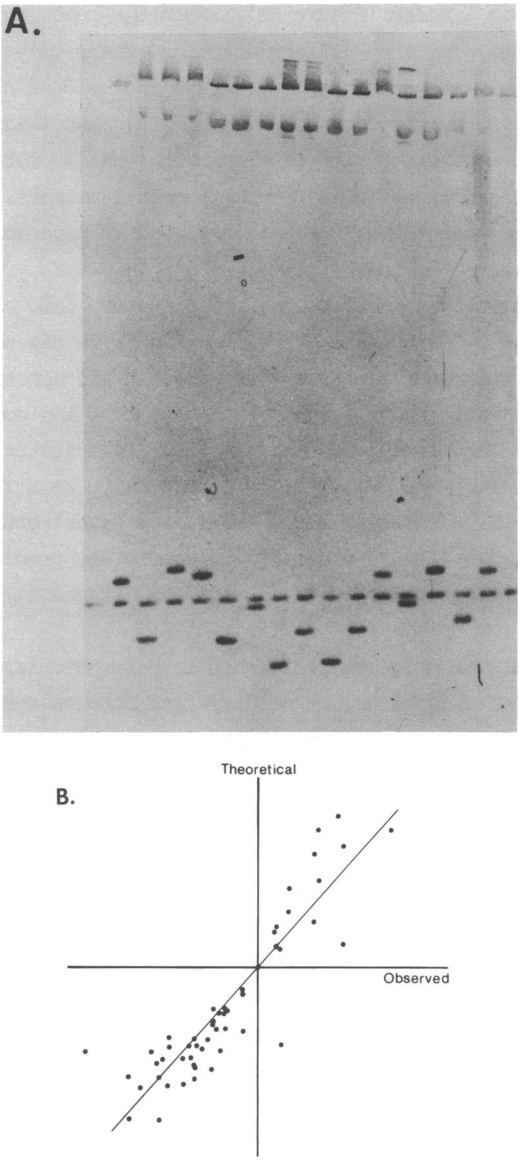

FIGURE 5: Observed and calculated parallel denaturing gradient gel patterns of mutant promoter fragments.
(A) Wild type and mutant promoter fragments with the GC-clamp in the -104 orientation on a domain 1 gel. A mixture of wild type and mutant DNA fragments was loaded in each lane. The first and last lanes contain only wild type DNA. Gel conditions were the same as those in Figure 4A.
(B) Correlation between the observed and calculated differences in gradient gel position of wild type and mutant DNA fragments.

the melting transition of domain 2 on the perpendicular gradient gel occurs at 63% denaturant. Under these conditions domain 1 melts as the DNA fragment enters the gel. These partially denatured molecules then travel through the gel until they reach the position where domain 2 melts. As shown in Figure 4B the domain 2 mutant separates from the wild type fragment, but since domain 1 melts at the top of the gel for both of wild type and mutant fragments, no separation of the domain 1 mutant is observed.

When the GC-clamp is attached to the promoter fragment in the +26 orientation, where the promoter melts as a single domain, both domain 1 and 2 mutants separate from the wild type DNA fragment on a single parallel gradient gel (Figure 4C). Based on the fact that the melting transition of the single domain occurs at a denaturant concentration of 53% in the perpendicular gradient gel (Figure 2C and D), we used a parallel gradient of 37% to 67% denaturant.

Comparison of the observed and calculated parallel denaturing gradient gel patterns of promoter mutants

We have demonstrated agreement between the calculated and observed denaturing gradient gel patterns of the wild type β-globin promoter fragment and of two mutants (Figures 1, 2 and 4). To determine whether such a correspondence can be extended to other mutants we have analyzed 38 different single base substitutions in the β-globin promoter fragment in parallel denaturing gradient gels. These mutants were obtained using a random chemical mutagensis procedure which will be described elsewhere (in preparation). An example of an analysis of 15 different mutants is shown in Figure 5A. To accurately determine the relative gel positions of the wild type and mutant fragments, a mixture of the two fragments was loaded in each lane. The interval between the gradient level of each of the mutant sequences and the level of the wild type molecule after an appropriate running time is plotted against the theoretical differences between the wild type and mutant sequences in Figure 5B. While the results are consistent with a simple proportionality between calculated and measured interval of equivalent temperature, the agreement is not as good as that reported in a previous study (2), and a few points deviate significantly from linearity. Although the reasons for this apparent discrepancy are unknown, it is possible that they are due to differences in the linearity of the denaturing gradients and the reliability of temperature and time controls in the two studies.

Thus far we have shown that the GC-clamp makes it possible to detect single base substitutions in each of the melting domains of the β-globin

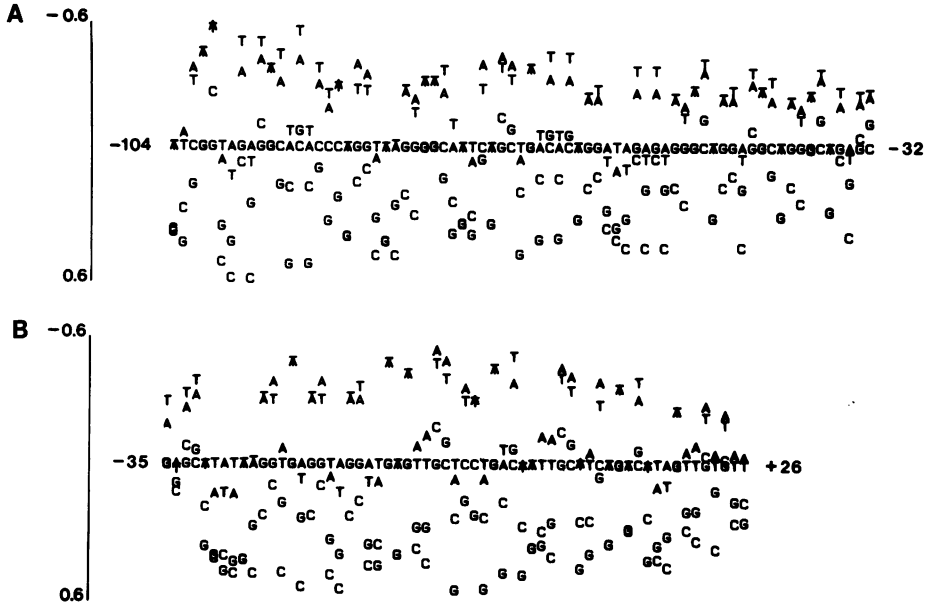

FIGURE 6: Calculated gel positions for all possible single base substitutions with the promoter fragment attached to the GC-clamp in the -104 orientation.
(A) Gel positions predicted for nucleotides -104 to -32 on a domain 2 denaturing gradient gel. The starting temperature in the calculations was set at 70°C, corresponding approximately to the denaturant concentration at the top of the gel. A displacement of 0.6 corresponds to 12 mm on the gel.
(B) Gel positions predicted for nucleotides -35 to +26 on a domain 1 denaturing gradient gel. The starting temperature in the calculations was set at 60°C, corresponding to the starting temperature in the gel.

promoter fragment. Moreover, we have shown that the predicted and observed gel patterns for several different mutants are similar. On this basis we attempted to calculate the fraction of all possible base substitutions in the promoter fragment that would lead to a separation from the wild type fragment on denaturing gradient gels. The alterations in DNA melting behavior and simulated gel patterns resulting from all possible single base substitutions at each position of the promoter fragment were calculated and displayed in the diagrams of Figures 6 and 7. The nucleotide sequence of the wild type promoter fragment is shown as a continuous line in each panel. The position of each of the substituted bases relative to the wild type nucleotide directly indicates the relative gel positions predicted by calculation. Base substitutions resulting in a higher or lower Tm are shown below or above the wild type sequence, respectively. The DNA melting calculations

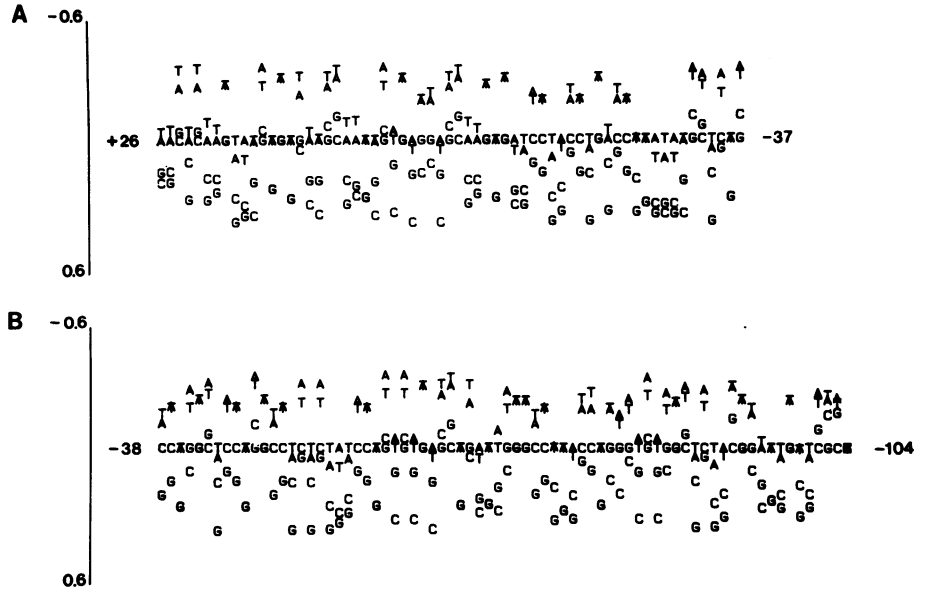

FIGURE 7: Calculated gel positions for all possible single base substitutions with the promoter fragment attached to the GC-clamp in the +26 orientation.
(A) Gel positions predicted for nucleotides +26 to -37 on a single domain denaturing gradient gel.
(B) Gel positions predicted for nucleotides -38 to -104 on a single domain denaturing gradient gel.

used to simulate the gel separations displayed in Figure 6 were obtained using the GC-clamp in the -104 orientation. Simulation of the gel patterns for changes in nucleotides -104 to -32 assumed that the running time and denaturant distribution of a domain 2 parallel gradient gel was used (Figure 6A). Similarly, the time and denaturant distribution for a domain 1 gel was assumed in the simulated gel patterns for changes in nucleotides -35 to +26 (Figure 6B). Examination of Figure 6 reveals that approximately 95% of the 405 possible single base substitutions in the promoter result in a discernable separation from the wild type DNA fragment. The few substitutions that do not lead to a separation are conservative transversions that do not change the base composition. Nevertheless, most of the conservative tranversions (105/135) should lead to separation on the gels. The range of predicted separations between mutant and wild type fragments is 1 to 12 millimeters.

The results of calculations used to simulate the gel separations shown in Figure 7 were obtained with the GC-clamp attached to the promoter in the +26 orientation. Because the promoter melts as a single domain in this orientation, the gel displacements were calculated for a single running time and gradient distribution. The effects of substitutions are seen to be more nearly uniform across the promoter, but the range of expected displacements is slightly smaller than that for the promoter clamped in the -104 orientation.

CONCLUSIONS

Attachment of a GC-clamp to the β-globin promoter fragment has increased the fraction of base substitutions that can be detected by denaturing gradient gel electrophoresis from an estimated 40% to 95%. In theory, the GC-clamp should make it possible to detect most single base substitutions in any DNA fragment in the size range of 25-500 bp. We estimate that the base composition of the attached fragment cannot exceed 75% G+C with the GC-clamp used in these studies. However, by using a GC-clamp that is 100% G+C it should be possible to resolve mutations in virtually any naturally occuring DNA fragment. The size limitation of approximately 500 bp for the attached fragment is a consequence of the decreased resolution of DNA molecules with large melted regions.

The increased sensitivity of the denaturing gradient gel system afforded by the GC-clamp can be exploited for a variety of applications. For example, the GC-clamp has been effectively utilized to purify DNA fragments generated by *in vitro* mutagenesis procedures (R.M.M. and T.M., in preparation). To date, the GC-clamp has been used to detect and isolate single base substitutions in the 135 bp mouse β-major globin promoter fragment, in a human β-interferon promoter fragment of 109 bp (R.M.M., S. Markowitz, and S. Goodbourn, unpublished), in a 300 bp DNA fragment from the adenovirus E1a gene (J. Lillie and M. Green, personal communication) and in several DNA fragments coding for the *E. coli* tryptophan synthase A gene (D. Milton, R.M.M., and J.K. Hardman).

The GC-clamp procedure can also be used to localize base substitutions in cloned genes carrying naturally-occurring sequence polymorphisms or mutations. Similarly, experimentally-induced mutations that have been phenotypically selected can be localized by this procedure. This could be accomplished by fragmenting the cloned genes with restriction enzymes, inserting

these fragments into a plasmid containing the GC-clamp, and then examining the behavior of the clamped fragments on denaturing gradient gels.

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