
Modification of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis

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Received 28 February 1985; Accepted 5 April 1985

ABSTRACT

The melting behavior of a DNA fragment carrying the mouse β major-globin promoter was investigated as a means of establishing procedures for separating DNA fragments differing by any single base substitution using the denaturing gradient gel electrophoresis procedure of Fischer and Lerman (1,2). We find that attachment of a 300 base pair GC-rich DNA sequence, termed a GC-clamp, to a 135 bp DNA fragment carrying the mouse β -globin promoter significantly alters the pattern of DNA melting within the promoter. When the promoter is attached to the clamp, the promoter sequences melt without undergoing strand dissociation. The calculated distribution of melting domains within the promoter differs markedly according to the relative orientation of the clamp and promoter sequences. We find that the behavior of DNA fragments containing the promoter and clamp sequences on denaturing gradient polyacrylamide gels is in close agreement with the theoretical melting calculations. These studies provide the basis for critical evaluation of the parameters for DNA melting calculations, and they establish conditions for determining whether all single base substitutions within the promoter can be separated on denaturing gradient gels.

INTRODUCTION

DNA molecules can be separated on the basis of their sequence by electrophoresis in polyacrylamide gels containing a gradient of formamide and urea (3). Duplex DNA fragments move through these gels with a constant mobility determined by molecular weight until they migrate into a denaturant concentration at which the DNA begins to melt. At this point the mobility of the DNA abruptly decreases. Thus, the final position of a DNA fragment in the gel is determined by its melting behavior. Fragments of identical size but differing sequences can therefore be separated on denaturing gradient gels. Single base substitutions alter the melting of a portion of the molecule strongly enough to effect substantial separations (1). Both transversions and double substitutions that leave the net base composition unchanged lead to significant changes in the final gradient position. The observed effects of single base substitutions are in close agreement with

theoretical expectations based on statistical mechanical melting and mobility calculations (2). However, the part of the molecule in which base substitutions can be detected and the sensitivity of detection are sequence-dependent. Consistent with theoretical expectations, only a fraction of known single base substitutions in a 536 bp bacteriophage λ DNA fragment were detected by denaturing gradient gel electrophoresis (1).

To extend the utility of the gradient gel electrophoresis system we have explored ways of altering the melting behavior of DNA fragments in such a way that all possible single base substitutions can be detected. Since formation of a partially melted molecule consisting of both helical and melted regions is the principal basis of large separations, we considered the possibility that molecules with the appropriate distribution of helix stability could be constructed by the attachment of the test sequence to a GC-rich sequence which we will refer to as a GC-clamp. If the GC content of the clamp is sufficiently higher than that of the test sequence, part of the DNA fragment will remain helical for a substantial interval in the gradient through which the test sequence melts. Thus, it should be possible to separate DNA fragments containing single base substitutions in every melting domain of the fragment. The same GC-dense clamp sequence should be appropriate for almost all test sequences if it is both sufficiently stable toward melting and is stably propagated in bacterial plasmids.

We have applied this principle to manipulation of the mouse β -major globin gene promoter as a step towards establishing procedures for obtaining an extensive set of promoter mutants. In this paper we present the theoretical consequences of attaching a clamp sequence to the promoter, and then demonstrate that the melting and mobility behavior of the clamped promoter fragment is in close correspondence with the theory. In the accompanying paper (4) we show that base substitutions in the promoter that cannot be separated in the normal sequence context are readily separated when the promoter is attached to the clamp.

MATERIALS AND METHODS

Plasmids

The GC-clamp was isolated from a region upstream from the human $\alpha 1$ -globin gene occurring between bases -113 to -409 from the mRNA start site (5,6,7). A HindIII and a BamHI linker was ligated to the 5' (base 1 in Figure 4) and 3' (base 305 in Figure 4) ends, respectively, of the GC-clamp. The GC-clamp can therefore be moved from one plasmid to another by digestion

with HindIII and BamHI. Similarly, a BamHI linker was attached to base -104 of the mouse β major-globin promoter region (8) and a BglII linker was attached to base +26 (bases 309 and 444 of Figure 4). Thus, the promoter fragment can be inserted into a plasmid adjacent to the GC-clamp in either orientation by BglII and BamHI cohesive ends. Plasmids containing the GC-clamp and the β -major globin promoter in either orientation are described in Figure 7.

Denaturing gradient gel electrophoresis

The system for running constant-temperature denaturing gradient gels has been described (9). The gels are run in a plexiglas apparatus that holds two glass plates such that the top of the plates forms an upper buffer chamber. The gel plates rest in a trough that is filled with 1% agarose in TAE buffer (40 mM tris, pH 7.4, 20 mM sodium acetate and 1 mM EDTA) to seal the bottom of the plates prior to pouring the gel. Gels are poured by mixing two solutions of different denaturant concentration in a linear gradient maker and dripping by gravity into the top or side of the plates for parallel or perpendicular gels, respectively. For pouring perpendicular denaturing gradient gels, the top of the gel is plugged with a Teflon spacer, which forms a single large well after polymerization.

Gels are composed of a fixed acrylamide concentration (acrylamide:bisacrylamide = 37.5:1) in TAE buffer with a linearly increasing concentration gradient of formamide and urea. Stock solutions of acrylamide, buffer, and 0% or 80% denaturant (100% denaturant = 40% formamide + 7 M urea) can be stored at +4°C for several months. These stock solutions are mixed to give two solutions for pouring a gradient with the desired denaturant concentration range. To each chilled solution, 1/200th volume 20% ammonium persulfate and 1/2000th volume TEMED is added immediately prior to pouring.

The apparatus containing the polymerized gel is placed in an aquarium of TAE buffer heated to 60°C, which serves as the lower electrophoresis chamber. Buffer is circulated by a peristaltic pump from the aquarium into the upper chamber, and is allowed to overflow from the upper chamber into the aquarium. Electrophoresis is performed by placing a cathode spanning the width of the gel in the upper chamber and a platinum anode 2-3' in length submerged at any position in the aquarium. Gels are electrophoresed at 150 V.

The 135 bp promoter fragment was examined on a perpendicular denaturing gradient gel containing 6.5% acrylamide and a 20% to 80% gradient of denaturants from left to right. In order to observe the promoter fragment

by ethidium staining, 25 μ g BamHI and BglII digested total plasmid DNA was loaded on the gel (total plasmid size was 2400 bp, thus, the gel contained \sim 1.2 μ g promoter fragment). Electrophoresis was for 130 min at 150 V.

The promoter attached to the GC-clamp was examined on a perpendicular gradient gel containing 6.5% acrylamide and a 20% to 80% gradient of denaturants. 15 μ g each restriction-digested plasmid DNA was loaded on the gel and electrophoresed for 5 hr at 150 V.

Calculation of fractional GC content

The variation in base composition along the sequence of each DNA molecule is represented in panel A of Figures 1,2,5, and 6 as the local fraction of G or C. The discontinuous function, 0 (for AT) or 1 (for GC) at every point, was converted into an interpretable pattern by cubic smoothing, replacing the center value in each set of 25 bp by the value of the least squares third order polynomial fitted to those 25 points, advancing one base pair at a time.

Calculation of melting maps and coherence maps

The theoretical expectation for the helix-random chain transition of each molecule was calculated as a function of temperature by the Poland-Fixman-Friere algorithms (10, 11). The coefficients for a ten-term series of exponentials representing a loop entropy exponent of 1.8 were provided by R.D. Blake. The entropy of melting was assumed to be uniform, at 24.5 Cal/degree, for all base pairs; the value of sigma was 3.5×10^{-6} . Stability values for the set of ten nearest neighbor base pairs were those given by Gotoh and Tagashira (12) and Gotoh (13) for melting in 0.022 M sodium (0.1 SSC), not identical with our solvent. A small increment in stability was applied at both ends of each sequence, corresponding to the weaker electrostatic field in the absence of a continued series of charged phosphates. The correction consisted of an exponentially declining increment with a decay length of 8 bp. The increment at the first and last nearest neighbor values was 8.8°C, which can be compared with the difference of 100 degrees between the hydrogen-bonded dinucleotides TA*AT and GC*CG (12,13). The slight change in melting depends mainly on the cumulative increment at the ends; it is not sensitive to the local distribution of the increment. The melting maps shown in panel B of Figures 1, 2, 5, and 6 represent the temperature, T_m , estimated by interpolation, at which each base pair in the molecule is expected to be at the midpoint of its equilibrium between helix and random chain. The Poland-Fixman-Friere calculation, carried out at 0.2°C intervals, provides $p(m)$, the probability at each temperature that a bp at posi-

tion m along the sequence is helical. The interpolation between $p(m)$ values bracketing 0.5 was calculated as a vant Hoff function, such that the interpolation parameter indicates the apparent ΔH of the transition. An apparent ΔS of the transition for each neighboring site is given by $\Delta H/T_m$. Since the entropy of melting for each bp introduced into the calculation is uniform and constant, the ratio of the apparent entropy inferred from the interpolation to the nominal entropy per bp, yields a dimensionless number which can be interpreted as the local length along the sequence over which there is close cooperation in melting. This value, termed coherence length, is shown in panel C of each melting diagram. It indicates inversely the breadth of the transition at each base pair, in that the lower the value given for the coherence at each site, the larger the temperature interval required to carry that particular base pair from a fully helical to a fully melted molecule.

Calculation of mobility in denaturing gradient gels

The ratio of the electrophoretic mobility of each partially melted molecule relative to that of the intact double helix was calculated by the relation $\mu/\mu_0 = \exp(-l_m/L_r)$, where l_m is the total number of non-helical base pairs inferred from the melting algorithms. L_r , the length of the flexible unit in the melted strand, was taken at 85 bases (2).

RESULTS AND DISCUSSION

A close correspondence has been established between the behavior of specific DNA molecules on denaturing gradient polyacrylamide gels and calculations based on the statistical mechanical theory for sequence-specific melting developed by Poland (10), the computational simplification by Fixman and Friere (11), and a hypothesis on the mobility of partially melted molecules in polyacrylamide gels (2). A diagram showing the calculated temperature at which each base pair is at the midpoint of its melting transition, which we term a melting map, provides a convenient representation of the distribution of helix stability. The strong cooperativity of helix melting, accurately represented by the theory, has the effect of imposing a locally uniform melting temperature over substantial lengths of helix despite considerable variation in base composition. Each region of uniform melting temperature is termed a domain and may contribute to a peak in the differential hyperchromicity profile measured by ultraviolet absorbance. Since the melting map represents only the unimolecular component of the melting equilibrium and ignores dissociation of the two strands, which is a

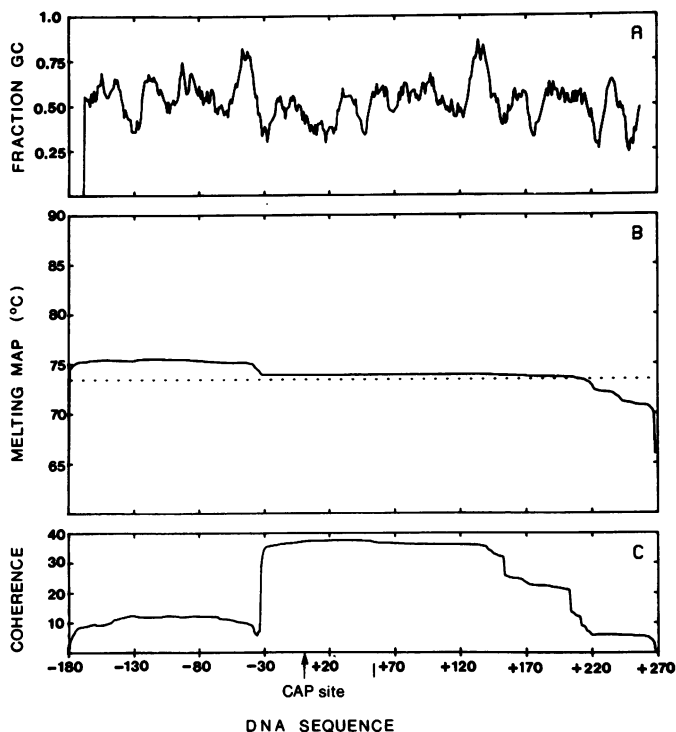


FIGURE 1: DNA melting calculations for a 450 bp DNA fragment located near the 5' end of the mouse β maj-globin gene.

The calculated base composition (A), melting temperature (T_m) (B) and a cooperativity function (C) are plotted as a function of sequence position numbered from bases -180 to +270 relative to the mRNA cap site of the mouse β -major globin gene. The local base composition shown in (A) was calculated as described in Materials and Methods. The solid line in (B) shows the calculated melting map of the sequence, the temperature at which each base pair is in 50:50 equilibrium between the helical and melted configurations. At temperatures below this line, a base pair will be helical, and at temperatures above the line, it will be melted. The dashed line shows the calculated temperature at which the dissociation constant for complete strand separation has the value 10^{-6} M. The coherence plotted in (C) is a measure of cooperativity and is explained in Materials and Methods.

bimolecular process, it applies rigorously only at indefinitely high DNA concentrations. At experimentally relevant concentrations, dissociation to give completely melted, separated strands will become significant at a temperature below the indicated melting of the highest domain. The temperature scale of the melting map bears a direct linear relation to denaturant concentration in the gel, and it is convenient to speak of denaturing potential

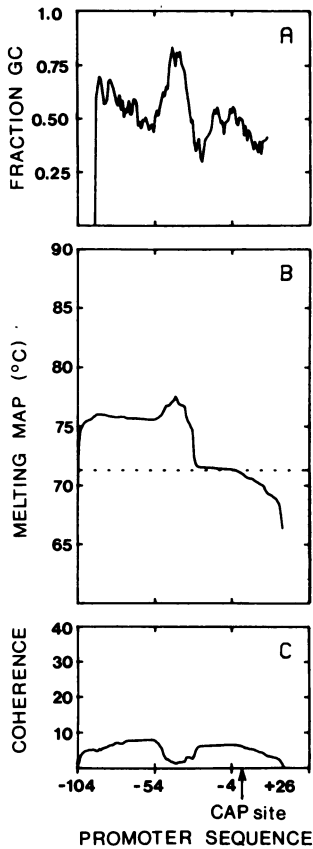
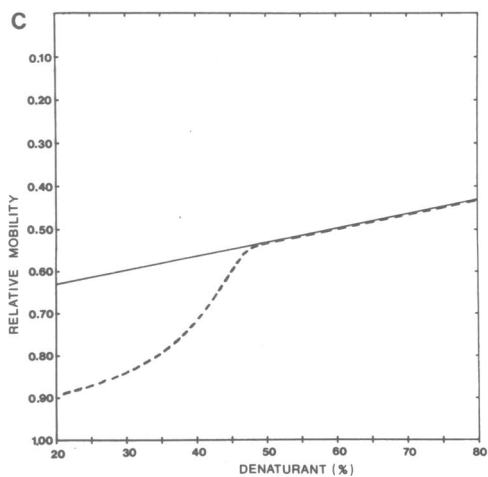
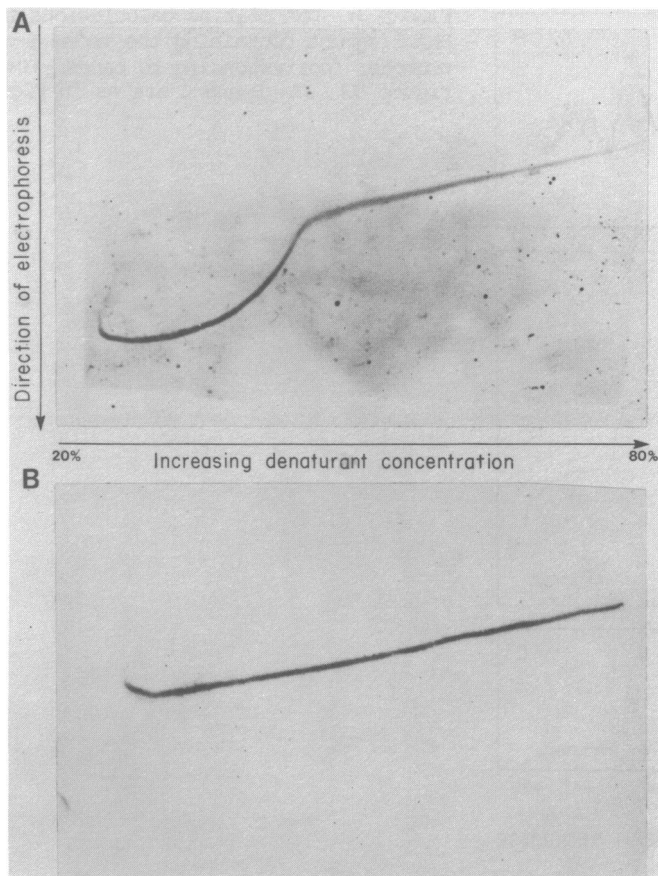


FIGURE 2: DNA melting calculations for a 135 bp DNA fragment containing the mouse β -major globin promoter (corresponding to bases -104 to +26 in Figure 1). A, B and C are as in Figure 1.

in the gel in terms of its equivalent temperature.

The separation of DNA fragments differing by a single base substitution is possible only under conditions that generate partially denatured molecules. Therefore, single base substitutions will not be discerned in DNA fragments containing a single melting domain. An example of this principle is provided by the mouse β -major globin promoter sequence (8). The three panels in Figure 1, calculated for a portion of the upstream sequence of the mouse β -major-globin gene, illustrate (top to bottom) the variation in base composition, the melting map, and the coherence. The dashed line in the melting map indicates the equivalent temperature at which the calculated strand dissociation constant is 10^{-6} M, comparable to the maximum fragment concentration in the gel. Two features of this melting map are significant. First, the melting map is nearly flat; there are two domains that are virtually indistinguishable. Second, melting and strand dissociation should oc-



cur simultaneously. For this fragment there is no temperature at which a partially melted molecule is favored by the equilibrium, and sensitivity to base substitutions cannot be expected.

The predicted melting behavior of DNA fragments changes significantly when they are separated from their normal flanking sequences by restriction endonuclease digestion. For example, the 135 bp sequence located between nucleotides -104 and +26 in Figure 1 melts within a small temperature interval in its normal sequence context. However the calculated melting map of the 135 bp fragment alone shows two small melting domains differing by about 5°C, and the temperature for $k_d = 10^{-6}$ M suggests that the second domain should remain in the double stranded configuration during melting of the first domain (Figure 2). We infer that substitutions in the right half of the DNA molecule might be separable, but not substitutions in the left.

To test the validity of the theoretical melting calculations we examined the 135 base pair β -globin promoter fragment on a polyacrylamide gel containing a gradient of denaturants perpendicular to the direction of electrophoresis. In these gels the DNA sample is loaded in a single well spanning the width of the gel. As shown in Figure 3A, a single melting transition is observed at a position in the perpendicular gel corresponding to a temperature of 71°C. This temperature can be calculated by measuring the position in the gel at which the midpoint of the steep increase in slope oc-

FIGURE 3: Experimental and calculated perpendicular denaturing gradient gel patterns of the 135 bp mouse β major-globin promoter fragment.

(A) A negative image of an ethidium-stained perpendicular denaturing gradient gel containing 6.5% acrylamide and a linearly increasing gradient of denaturants from left to right (20% denaturant on the left and 80% denaturant on the right). Electrophoresis was for 130 min at 150 V and 60°C. (B) An autoradiogram of the same gel shown in (A). A trace amount of an isolated single strand of the promoter fragment, labelled at the 5' end with 32 P, was loaded on the gel along with the unlabelled double stranded DNA fragment. When the ethidium-stained image is aligned with the autoradiogram, the curves coincide on the right half of the gel, indicating that the DNA is completely single stranded at the denaturant concentrations following the melting transition (see text). The labelled fragment used in this gel was the same sense as the promoter sequence shown in Figure 4; identical results were obtained with the opposite strand of the promoter (data not shown). No ethidium-staining material was observed when the isolated labelled single stranded DNA fragment was electrophoresed alone. (C) A simulation of the mobility versus temperature relations for the isolated promoter fragment. The dashed line represents the calculated ratio of the mobility of the partially melted molecule relative to the intact double helix, assuming that the mobility declines exponentially with a decay constant of 1/85 bp $^{-1}$. This curve ends at the temperature calculated to give a strand dissociation constant of 10^{-6} M. The solid line represents the mobility pattern expected for completely denatured DNA.

curs. This melting temperature is in excellent agreement with the T_m predicted for the first melting domain of the promoter fragment (Figure 2B). Since the concentration of denaturant increases from left to right in the gel, DNA molecules at the left side are double stranded, while the molecules at the right side should be completely melted. At the midpoint of the mobility transition in the gel the DNA molecules are melted throughout the first domain but remain in the duplex configuration in the second domain.

To show that the DNA to the right of the melting transition in the perpendicular gradient gel is in fact completely melted, we mixed a trace amount of one of the two DNA strands labelled with ^{32}P with the unlabelled duplex DNA of Figure 3A. As shown in the autoradiogram of Figure 3B, the position of labelled single stranded DNA at the right half of the gel coincides with the position of the ethidium-stained, initially duplex DNA, indicating identical behavior of both the single stranded and initially helical DNA molecules. As expected, the position of the initially duplex DNA at the left side of the gel does not coincide with that of the labelled single stranded DNA.

Additional correspondence between melting theory and the behavior of the promoter fragment on denaturing gradient gels was obtained by comparing a computer-generated gel pattern with the data of Figure 3A and 3B. The simulated gel pattern shown in Figure 3C was obtained by calculating the melting map of the fragment and then applying an exponential relation between the mobility of the partially and completely denatured molecules and the lengths of their single stranded regions (see (2) for a description of this simulation). The curve representing the calculated mobility of partially melted duplex DNA intersects the dashed line representing the estimated mobility of the dissociated single strands at an equivalent temperature of between 71°C and 72°C (Figure 3C). The most significant aspect of the similarity between the experimental (Figures 3A and 3B) and simulated (Figure 3C) gel mobility patterns is the fact that the equivalent temperature at which the DNA ceases to move through the gel as a branched molecule and begins to migrate as denatured single stranded DNA is the same in the two cases.

Based on the theoretical and experimental analysis described above, we conclude that single base substitutions occurring at approximately 40% of the positions of the 135 bp β -globin promoter fragment should be separated from the wild type DNA molecules on denaturing gradient gels. The substitutions located in the higher temperature melting domain should not separate from

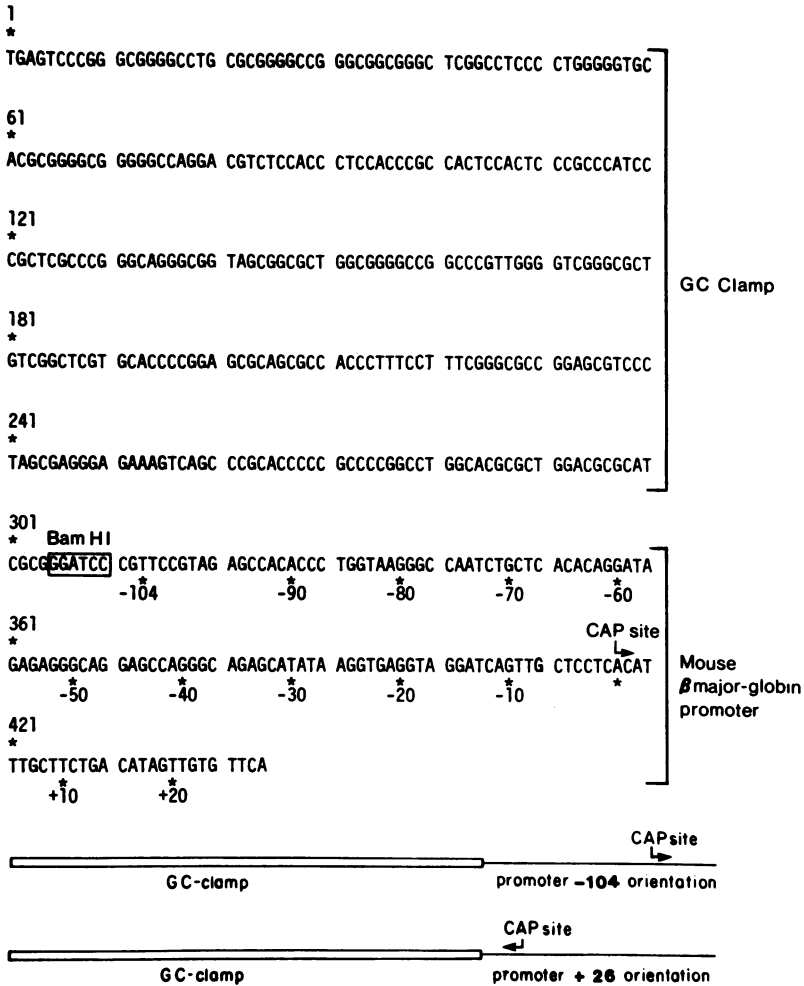


FIGURE 4: The nucleotide sequence of the GC-clamp attached to the mouse β major-globin promoter region in the -104 orientation. The numbers above the sequence refer to position along the entire length of the fragment. The GC-clamp extends from base 1 to the BamHI site at base 305. The promoter region extends from the BamHI site to base 444. The numbers below the sequence in the promoter region refer to the position in the promoter relative to the mRNA cap site of the β -globin gene which is indicated by the arrow. The schematic below the sequence shows the relationship between the GC-clamp and the promoter fragment in the -104 and +26 orientations.

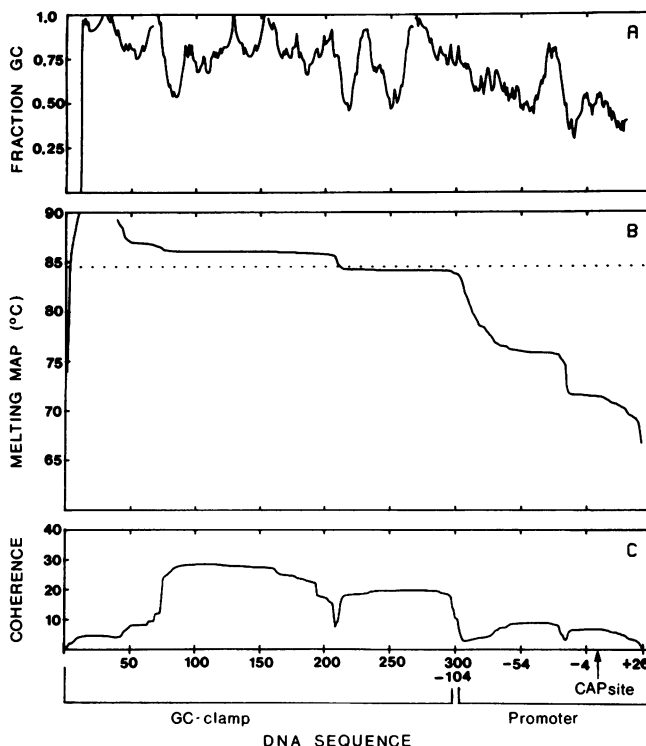


FIGURE 5: DNA melting calculations for a DNA fragment containing the GC-clamp and the mouse β -major promoter region in the -104 orientation. A, B, and C are as in Figure 1.

the corresponding wild type DNA fragments on the gel, since the strands would dissociate when the second domain is melted. In theory, attachment of a GC-rich DNA sequence to the promoter fragment could alter the melting map, and it should prevent strand dissociation upon complete melting of the promoter. To test these possibilities we calculated the melting behavior of the promoter fragment attached to a 300 bp GC-rich DNA fragment (GC-clamp). The nucleotide sequence of the promoter fragment attached at base -104 to the GC-clamp (the '-104 orientation') is shown in Figure 4. The local compositional map, the calculated melting map, and the coherence map for this clamped promoter fragment is shown in Figure 5. The corresponding calculations for the promoter attached to the same GC-clamp in the opposite orientation (the '+26 orientation') are shown in Figure 6.

To discuss these melting maps it is convenient to divide the promoter

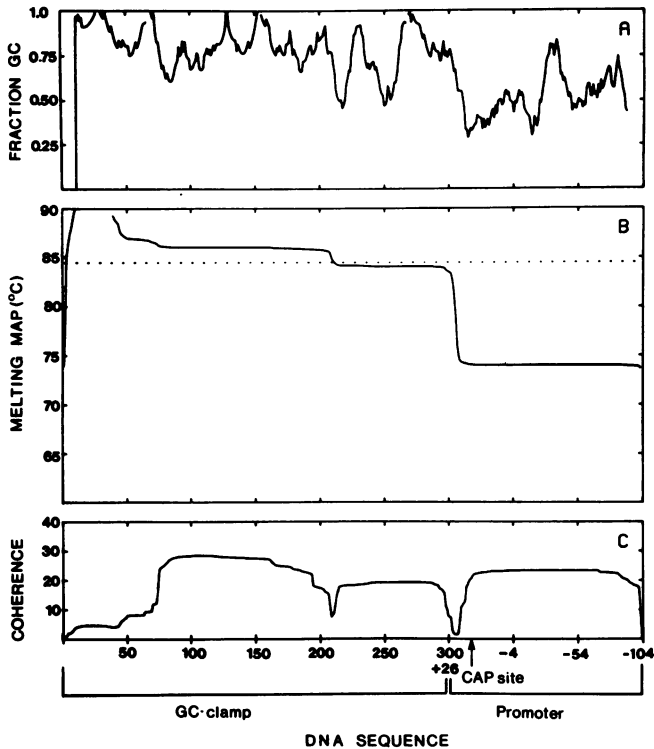


FIGURE 6: DNA melting calculations for a DNA fragment containing the GC-clamp and the mouse β -major promoter fragment in the +26 orientation. A, B, and C are as in Figure 1.

portion of these clamped molecules into two regions identifiable approximately with the two small domains in Figure 2. These regions define two distinct small domains with plateaus at 71°C and 76°C when the promoter is attached to the GC-clamp in the -104 orientation (Figure 5). However, in the +26 orientation both regions merge into a single long domain with an intermediate T_m of 73°C . Coherence in the promoter region is about double in the +26 orientation, indicating longer range cooperation and a sharper transition with a smaller temperature interval. Melting of the relatively AT-dense portion at the 3' end of the promoter, which establishes the lowest domain in the -104 orientation, is suppressed when that sequence is in the interior of the molecule when attached to the GC-clamp in the +26 orientation. The general melting behavior of the 300 bp clamp is independent of the orientation of the attached promoter fragment. Two broad domains melt-

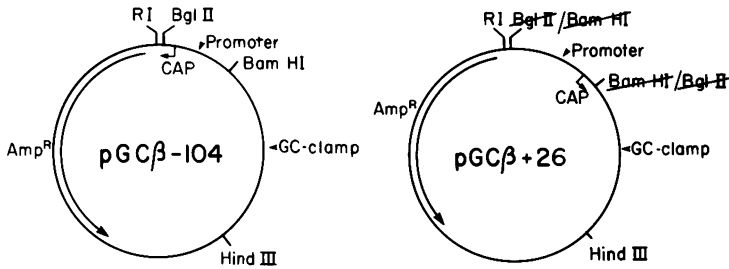


FIGURE 7: A diagram of the plasmids containing the GC-clamp sequence. The plasmid vector used in these constructs is composed of DNA sequences from the plasmid pBR322, from a HindIII site attached at nucleotide 2440 to the EcoRI site at 4363. Both plasmids are selected by growth on ampicillin.

ing at 87.0°C and 84.5°C are predicted (Figures 5 and 6). Although there are three regions of relatively high AT density near bases 70, 210, and 255, there is no indication that interior melting will occur at these sites.

The dotted lines in Figures 2B, 5B and 6B represent the temperatures at which strand dissociation is probable. In the case of the unclamped promoter fragment, the predicted temperature at which strand dissociation occurs is lower than the T_m calculated for domain 2, assuming an infinite DNA concentration (Figure 2B). In contrast, the predicted strand dissociation temperature for each clamped promoter fragment is well above the calculated T_m for the promoter sequences (Figures 5B and 6B). Single base substitutions in all regions of the promoter sequence should therefore result in an altered T_m which is lower than the strand dissociation temperature. Thus, in the presence of the GC-clamp, single base substitutions throughout the promoter sequence should result in separation of the mutant and wild type promoter fragments on denaturing gradient gels.

The theoretical effects of the GC-clamp on the melting behavior of the β -globin promoter fragment were tested by perpendicular gradient gel electrophoresis. DNA fragments containing the GC-clamp and promoter were obtained by constructing the plasmids shown in Figure 7. In the plasmid pGCβ-104, the promoter sequence is joined at -104 to the GC-clamp, while the promoter is attached to the clamp in the opposite orientation in the plasmid pGCβ+26. DNA fragments containing the GC-clamp and the promoter can be excised from both plasmids with appropriate restriction enzymes. The clamped promoter fragments were excised from both plasmids and run as a mixture on a single perpendicular denaturing gradient gel (Figure 8A). As anticipated from the melting map of Figure 5B, the fragment clamped in the -104 orienta-

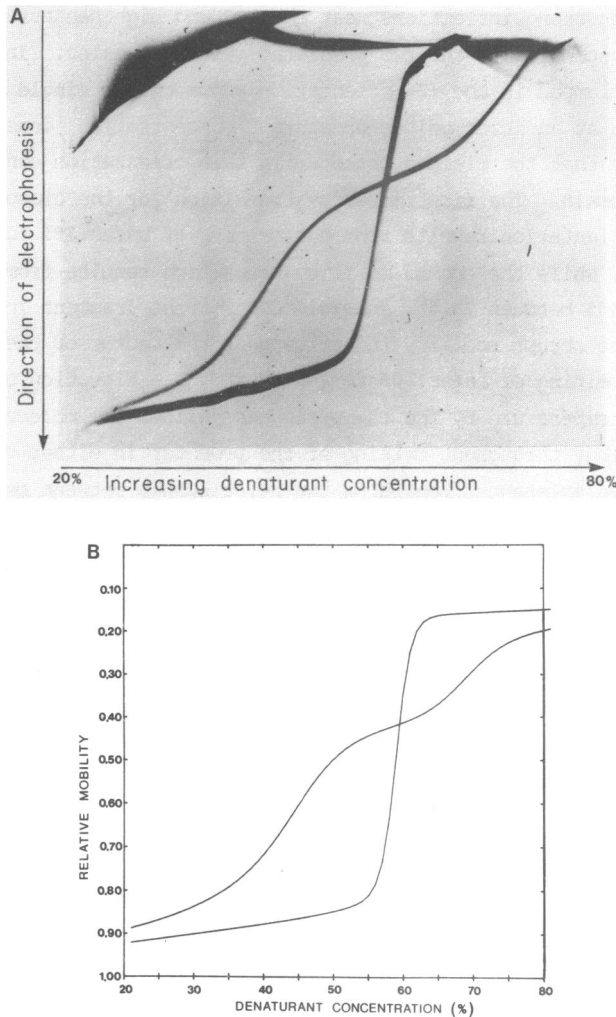


FIGURE 8: Experimental and calculated perpendicular denaturing gradient gel patterns for DNA fragments containing the GC-clamp and the mouse β -major promoter fragment in the -104 and +26 orientations.

(A) An ethidium bromide-stained perpendicular denaturing gradient gel in which equal amounts of the GC-clamp -104 promoter and the GC-clamp +26 promoter fragments were electrophoresed. The gel contained 6.5% acrylamide and a range of 20% to 80% denaturants from left to right. Electrophoresis was for 5 hr at 150 V and 60°C. The staining material that contains two inflections corresponds to the -104 orientation, whereas the staining material containing a single inflection corresponds to the +26 orientation.

(B) A simulation of the perpendicular denaturing gradient gel pattern for the GC-clamp attached to the promoter in the -104 and the +26 orientations. The calculated temperature for a dissociation constant of 10^{-6} M is higher than the right-hand edge of the plot and is not shown.

tion shows two strong inflections near the low and high denaturant ends of the gel, corresponding to the two predicted melting domains. In contrast, the fragment clamped in the +26 orientation shows only a single sharp mobility transition at an intermediate denaturant concentration, consistent with the prediction that the clamped promoter in this orientation contains a single melting domain. The first mobility transition for the clamped fragment in the -104 orientation results from the melting of bases 385 to 444 between 68°C and 72°C, while the second mobility transition results from melting of bases 305 to 385 between 75.5°C and 76.5°C. For the fragment in the +26 orientation the abrupt mobility reduction near the center of the gradient results from melting of bases 305 to 444 at 73.5°C. Elevation of the strand dissociation temperature by the clamp sequence allows the molecule to retain branched topology throughout melting of the promoter in either orientation.

The above inferences regarding the relationship between melting behavior and gel mobility are strengthened by comparing the observed perpendicular gradient gel patterns with those simulated from the melting calculations. Remarkably, the simulated patterns of electrophoresis for both of the clamped promoter fragments shown in Figure 8B are indistinguishable from the observed patterns shown in Figure 8A. The close simulation of the mobility functions for the promoter clamped in both orientations strongly supports both the melting theory and the formalism relating melting to mobility on gradient gels.

The perpendicular gradient gel patterns of the clamped promoter fragments provide the basis for critically evaluating the parameters used to calculate the relationship between DNA melting and mobility in gradient gels. The significant features of the gel patterns are: i. the temperature and gradient level at which both clamped molecules have the same mobility, ii. the amount of retardation of the clamped promoter fragments in the -104 orientation after melting of the first but before melting of the second domain, and iii. the denaturant concentration or equivalent temperature interval between the inflections caused by melting of the two domains of the clamped promoter fragment in the -104 orientation. In the calculation of the melting-mobility relationship, a slightly sloping baseline has been introduced to allow for the increasing viscosity of the denaturant solution across the gradient. One parameter that is varied in the calculation is the length of a flexible unit in the melted DNA chain. We find that a flexible unit of 85 melted bases fits the observed gel pattern better than larger or smaller values. The value is expected to resemble the Kuhn statistical

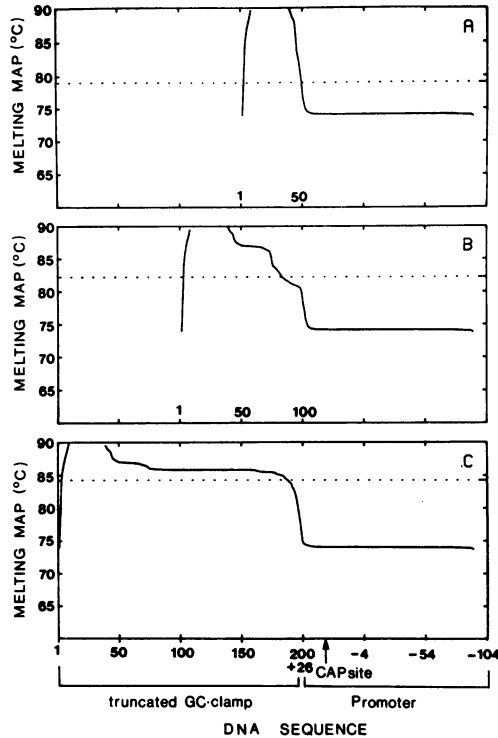


FIGURE 9: DNA melting calculations for hypothetical molecules containing the promoter fragment in the +26 orientation attached to GC-clamps 50, 100 and 200 bp in length.

(A) 50 bp, (B) 100 bp, and (C) 200 bp from the 5' end of the GC-clamp sequence in Figure 2 attached to the promoter. In all panels, the dashed line represents the calculated temperature at which the dissociation constant for strand separation is 10^{-6} M.

length, but that quantity is sensitive to the composition of the solvent for melted DNA strands, and no independent estimates under similar conditions are available for comparison. Another parameter that is varied in the calculation is the linear relationship between denaturant concentration in the gel and its temperature equivalent. Examination of the gel patterns of Figure 8A indicates that 0.312°C per % denaturant, agrees within 10% of the value inferred from the gel displacement of base substitutions in a bacteriophage λ fragment (1), and is within 30% of the value estimated from calorimetric and hyperchromicity studies (14, 15). However, published values represent pure rather than mixed solvents and dissimilar ion concentrations. The agreement between the predicted and experimental mobilities,

particularly for the promoter clamped in the -104 orientation, is conspicuously poorer if the correction for diminished electrostatic destabilization at the end of the molecule is omitted.

The GC-clamp used in the experiments described above is 300 bp in length. For certain applications of the gradient gel procedure a shorter clamp would be useful to reduce the time required for electrophoresis. It was therefore of interest to determine whether a shorter clamp sequence could be used to achieve similar changes in the melting behavior of the promoter fragment. The possible adequacy of shorter clamps for the promoter was calculated in the +26 orientation as shown in Figure 9. The three melting maps in panels A, B, and C correspond to the deletion of increasingly large segments from the 3' end of the clamp sequence, leaving respectively, 50, 100, and 200 bp of the 5' end of the clamp sequence. As before, the dashed line in each panel indicates the temperature at which the calculated dissociation constant for the complete separation of the two strands equals 10^{-6} M. It can be seen that the temperature interval between melting of the retarding domain and expected strand dissociation decreases as the length of the clamp is shortened. This temperature interval is 6, 8 and 11°C, respectively with clamps of 50, 100 and 200 bp. In principle each of these clamps should be adequate to achieve separation of single base substitutions throughout the promoter fragment. However the validity of this prediction remains to be experimentally established.

CONCLUSIONS

In this paper we have shown that the melting behavior of DNA molecules predicted by calculation strongly correlates with the pattern of electrophoresis on denaturing gradient polyacrylamide gels. This correlation between theory and experimental observations includes the location and T_m of melting domains, the effect of sequence context on the distribution of melting domains, and the behavior of partially denatured molecules during electrophoresis through denaturing gradient gels. The predictable alteration in the melting properties of duplex DNA by the GC-clamp provides strong evidence for the existence of melting domains. The practical implication of this work is that it should be possible to use the GC-clamp to achieve separation of single base substitutions in all regions of duplex DNA fragments. This possibility is confirmed in the case of the mouse β -major globin promoter in the accompanying paper.

ACKNOWLEDGEMENTS

We thank Karen Silverstein and Ronnie Serota for making the computer plots, and Zoia Larin-Goodbourn and Elizabeth Greene for technical assistance. R.M.M. was initially supported by a Damon Runyon-Walter Winchell Cancer Fund postdoctoral fellowship, and is presently a Special Fellow of the Leukemia Society of America. This work was supported by grants to L.S.L. and T.M. from the NIH.

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