Detecting base pair substitutions in DNA fragments by temperature-gradient gel electrophoresis

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ABSTRACT

A vertical gel electrophoresis apparatus is described which can distinguish DNA fragments differing by single base pair substitutions. The system employs a homogenous polyacrylamide gel containing urea-formamide and a temperature gradient which runs either perpendicular or parallel to the direction of electrophoresis. The temperature-gradient system simplifies several features of the denaturant-gradient system (1) and is relatively inexpensive to construct. Eight homologous 373 bp DNAs differing by one, two, or nine base pair substitutions were examined. DNA electrophoretic mobility changed abruptly with the temperature induced unwinding of DNA domains. GC to AT substitutions at different locations within the first melting domain, as well as an AT to TA transversion were separated with temperature gradients parallel to the electrophoretic direction. The relative stabilities of the DNAs observed in the gels were compared to predictions of DNA melting theory. General agreement was observed however complete correspondence was not obtained.

INTRODUCTION

Denaturing gradient gel electrophoresis (DGGE) can separate two DNA fragments which differ in sequence by one base pair (1–3). The method employs a gradient of denaturing solvent in a polyacrylamide gel. Its ability to separate homologous sequences is attributed to two physical properties of DNA: the effect of base pair sequence on the helix-coil or 'melting' transition, and the electrophoretic mobility of duplex DNA with single stranded regions. Theoretical and experimental studies of the melting transition in solution show that DNA tends to unwind in a series of cooperatively melting domains (4,5). A DNA moving in a polyacrylamide gel with a gradient of denaturing solvent migrates until it reaches a denaturant concentration which induces a domain to unwind. At this point the mobility of the DNA abruptly decreases probably because of entanglement of its branched structure in the gel matrix. DNAs which differ in the stability of their first melting domain unwind at different positions in the gel.

In this work we introduce a method analogous to DGGE, vertical temperature-gradient gel electrophoresis (TGGE). This approach simplifies several features of the denaturing gradient system. A temperature gradient replaces the need for a gradient of denaturing solvent in the gel. The large buffer reservoir employed in DGGE is not required. It is relatively inexpensive to construct. A horizontal temperature-gradient system has been introduced by Rosenbaum and Reisner (6).

We have employed the temperature-gradient system to examine eight 373 bp DNAs differing by base substitutions. It is shown that single base pair substitutions, including an AT to TA transversion, can be detected. The resolving power of the system appears to be similar to that of DGGE. Decreasing the size of the temperature gradient increases the separation distance between DNAs. Previous results with the denaturing gradient gels suggested a close correspondence between the stability of DNA in gels and DNA melting theory (1,2). We have examined this correspondence with the temperature-gradient system. The relative thermal stabilities of the 373 bp DNAs in the gels were compared to the stabilities predicted by DNA melting theory. Two sets of stacking parameters available from solution studies were employed. The general features of DNA melting in the gel are predicted, however the detailed order of stability observed in the gel is not in agreement with predictions. The evaluation of stacking interactions using the thermal gradient gels is discussed.

DGGE has been used to detect base changes in human globin genes (7,8), and to map *in vitro* generated mutations (9). Recent modifications of the technique in which GC rich segments are synthesized onto fragment ends improve its potential as a tool for detecting DNA polymorphisms (10). The temperature-gradient system provides a simplified apparatus for these applications.

MATERIALS AND METHODS

DNA

DNA fragments were obtained from eight related plasmids. The plasmids were previously constructed by ligating 143 bp

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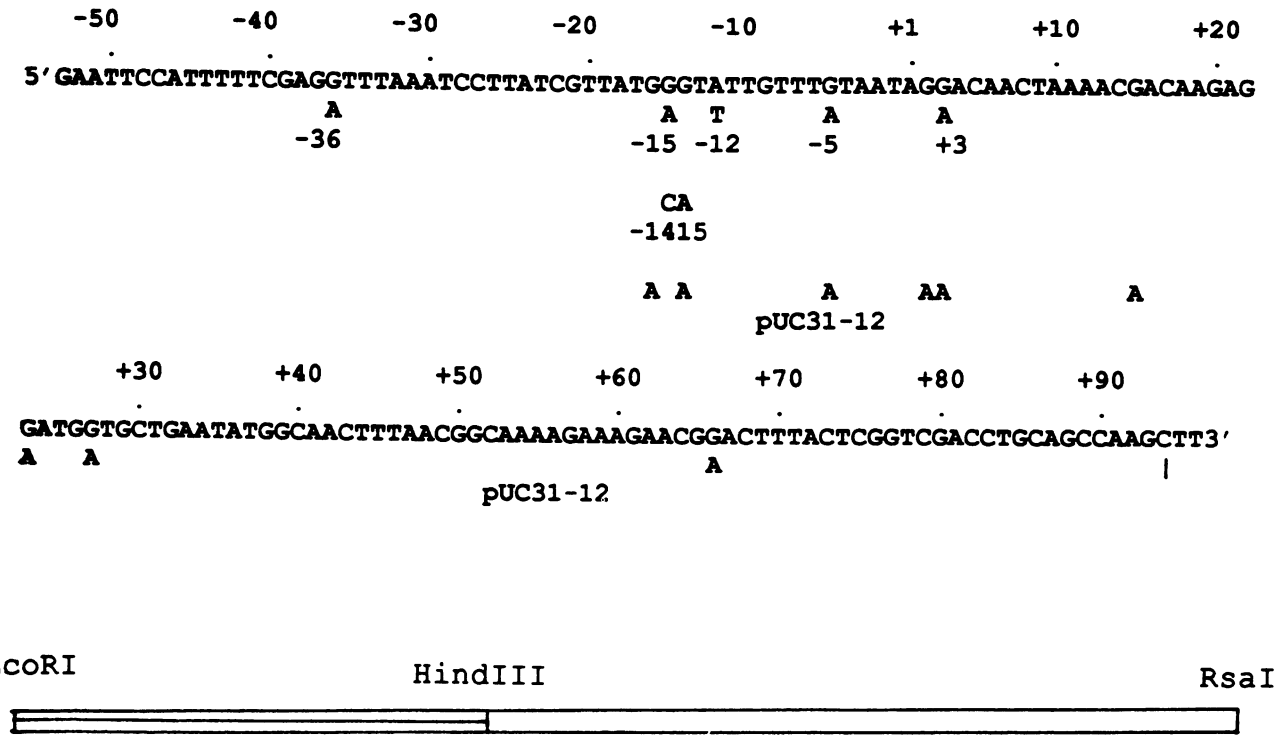

Figure 1. (**Top**) The *ctc* promoter region is shown along with the locations and designations of the mutations. The plasmid with the wild type *ctc* sequence was designated pUC8-31. Plasmids with single base pair substitutions are in the first row below the wild type sequence; pUC8-36,pUC8-15,pUC8-12,pUC8-5 and pUC8+3. The plasmid pUC8-1415 has a two base pair change. Plasmid pUC31-12 has nine GC to AT substitutions and one base pair deletion (|). (**Bottom**) Sketch of the 373 bp RsaI/EcoRI DNA fragment of the pUC plasmids showing the position of the *ctc* promoter region by the bisected segment.

EcoRI/HindIII DNA fragments containing the B. subtilis *ctc* promoter between the EcoRI and HindIII restriction sites of plasmid pUC8 (11−13). The plasmids are identical except for base pair changes in the 143 bp *ctc* promoter region. Figure 1 shows the wild type sequence of this region, the locations of the mutations, and plasmid designations. Five of the plasmids, pUC8+3, pUC8-5, pUC8-12, pUC8-15 and pUC8-36 have single base pair differences from the wild type sequence, pUC8-31. Plasmid DNAs were isolated from their host E. coli strain using previously described methods (11,14).

Each plasmid was digested with RsaI and EcoRI restriction endonucleases to produce three fragments; 1722, 676 and 373 bp in length. The 373 bp RsaI/EcoRI DNA contains the *ctc* region on one end as shown schematically in figure 1. The RsaI/EcoRI digest was selected since melting theory predicted that a large part of the *ctc* region is within the first melting domain of the 373 bp DNAs (see below).

Gel Electrophoresis

Temperature-gradient gel electrophoresis was carried out with a vertical acrylamide slab-gel apparatus. Figure 2 shows a picture and schematic of the plexiglass unit. It is a modification of a conventional vertical gel apparatus used in our lab. The changes enabled the glass plates containing the acrylamide gel to be sandwiched between two aluminum heating blocks. The glass plates are 3.5 mm thick (plate glass) and 25 cm×18.7 cm or 22.5 cm×18.7 cm. Both aluminum heating blocks are 15 cm×19 cm×1.25 cm. The cost of materials is about one hundred dollars for one gel apparatus.

Channels in the blocks allow circulating fluid to establish a temperature gradient from the top to bottom or from one side to the other. The channels running across the top and bottom are used to establish a temperature gradient in the same direction as electrophoretic migration. For a gradient perpendicular to DNA migration the fluid flows along the sides. Adhesive pipe tape is used to insulate the surfaces of the blocks not facing the glass. The rear block is placed against the main vertical support of the gel unit in the space formed by the overhanging upper buffer chamber. Both heating blocks rest on U-shaped plexiglass pieces which keep them above the buffer in the lower electrolyte chamber. Two thermostated fluid circulators (Haake Inc.) are employed to control the high and low temperatures.

The temperature gradient produced by the heating blocks was checked for linearity and uniformity at two temperature settings of the water circulators, 32°C/28°C and 44°C/18°C. A thermistor probe (± 0.5°C) inserted into a gel determined the gel temperatures at different depths and horizontal positions. For both of the above temperature settings the gradient in the gel was linear and uniform within the region covered by the blocks. 6.5% polyacrylamide gels (Sigma Chem. Co.) were employed with an acrylamide to bis-acrylamide ratio of 37.5/1. Gels were prepared by mixing two 6.5% acrylamide solutions with small volumes of ammonium persulfate and TEMED. The solvent of one acrylamide solution was 0.5 TBE (0.045 M sodium borate + 0.045 M Tris + 1mM EDTA pH 8.1). The solvent of the second solution was 0.5 TBE, 7.0 M urea and 40% formamide (v/v). The latter solvent is referred to as the '100% denaturing solvent'. Gels contained 58% 'denaturing solvent'. The electrophoresis buffer was 0.5 TBE. Both 0.8 mm or 1.5 mm thick spacers were used between the glass plates.

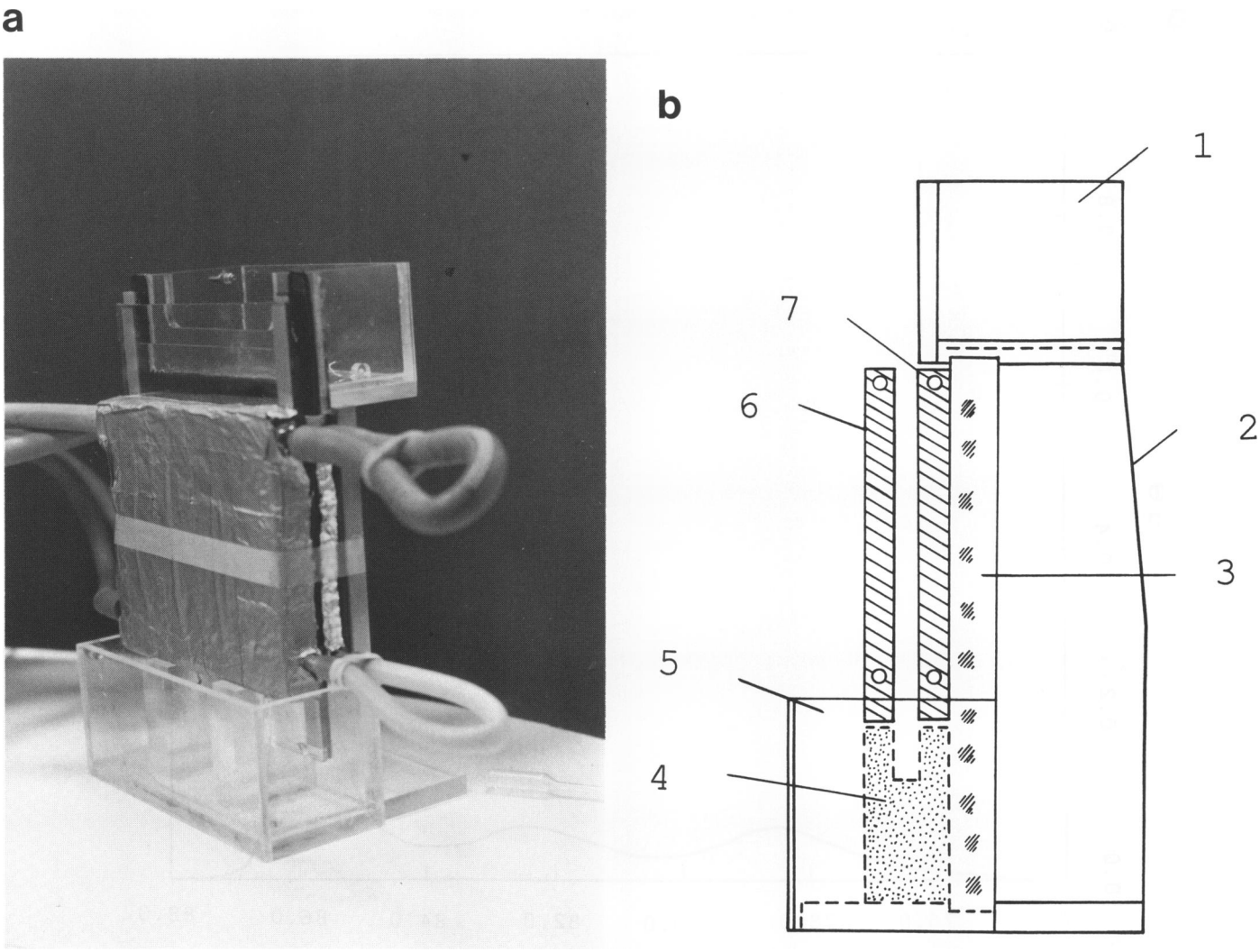

Figure 2. a) Picture of the plexiglass gel apparatus and temperature gradient heating blocks. b) Schematic of the gel apparatus and blocks. Items numbered are 1) upper buffer chamber, 2) support piece perpendicular to 3) main vertical support piece, 4)heating block standoffs in 5)lower buffer chamber.6)and 7) are aluminum heating blocks.

Gels were prepared from stock solutions in a manner similar to conventional polyacrylamide gels (15). After polymerization, gels were preelectrophoresed for 45 to 60 minutes prior to use. Digested plasmid DNA samples, approximately 1 μg, were mixed with ficol loading buffer, placed in the 10 mm wide gel lanes, and electrophoresed. The volume loaded per lane was 15−20 μl. Electrophoresis was generally carried out overnight at 80 volts. Gels were stained with an ethidium bromide solution for 10 minutes, destained for 5 minutes and photographed. For temperature-gradient gels in which the gradient was perpendicular to the direction of electrophoresis approximately 16 μg of digested plasmid was loaded in one long well across the top of the gel.

Calculation of Thermal Stability Properties

The model of the DNA helix-coil transition was used to calculate the thermal denaturation behavior of the DNA fragments (4,5,14). In addition to predicting the melting curve for a given DNA sequence (eg. figure 3a), the calculation can also produce melting profiles for the base pairs in a DNA sequence (figure 3b). A melting profile displays the probability that the n^{th} base pair of the sequence is melted, $\theta(n)$, at a given temperature. From a three dimensional display of melting profiles at a series of temperatures the lengths and locations of cooperatively melting domains can be visualized. The calculation of the melting profiles assumes that strand dissociation is negligible.

The nearest neighbor stacking parameters were from McCampbell et al. (14), and Gotoh and Tagashira (16). All other parameters, such as the loop entropy factor, strand dissociation parameters etc. were from McCampbell et al. (14). When only the first melting domain is of concern the dissociation parameters and loop entropy terms do not significantly influence theory-experiment comparisons. A solvent condition of 0.1 M Na^+ was assumed. Since Gotoh and Tagashira obtained their parameters in 0.02 M Na^+, an extrapolation was made to compare the calculations under a similar condition. Their parameters were extrapolated to 0.1 M Na^+ by scaling T_{AT} and T_{GC}, the average T_m's of AT and GC base pairs (17). Table I lists the stacking parameters employed. The sequence dependent melting behavior predicted with the Gotoh and Tagashira parameters previously produced a correspondence with results from the denaturing gradient gels (1,2).

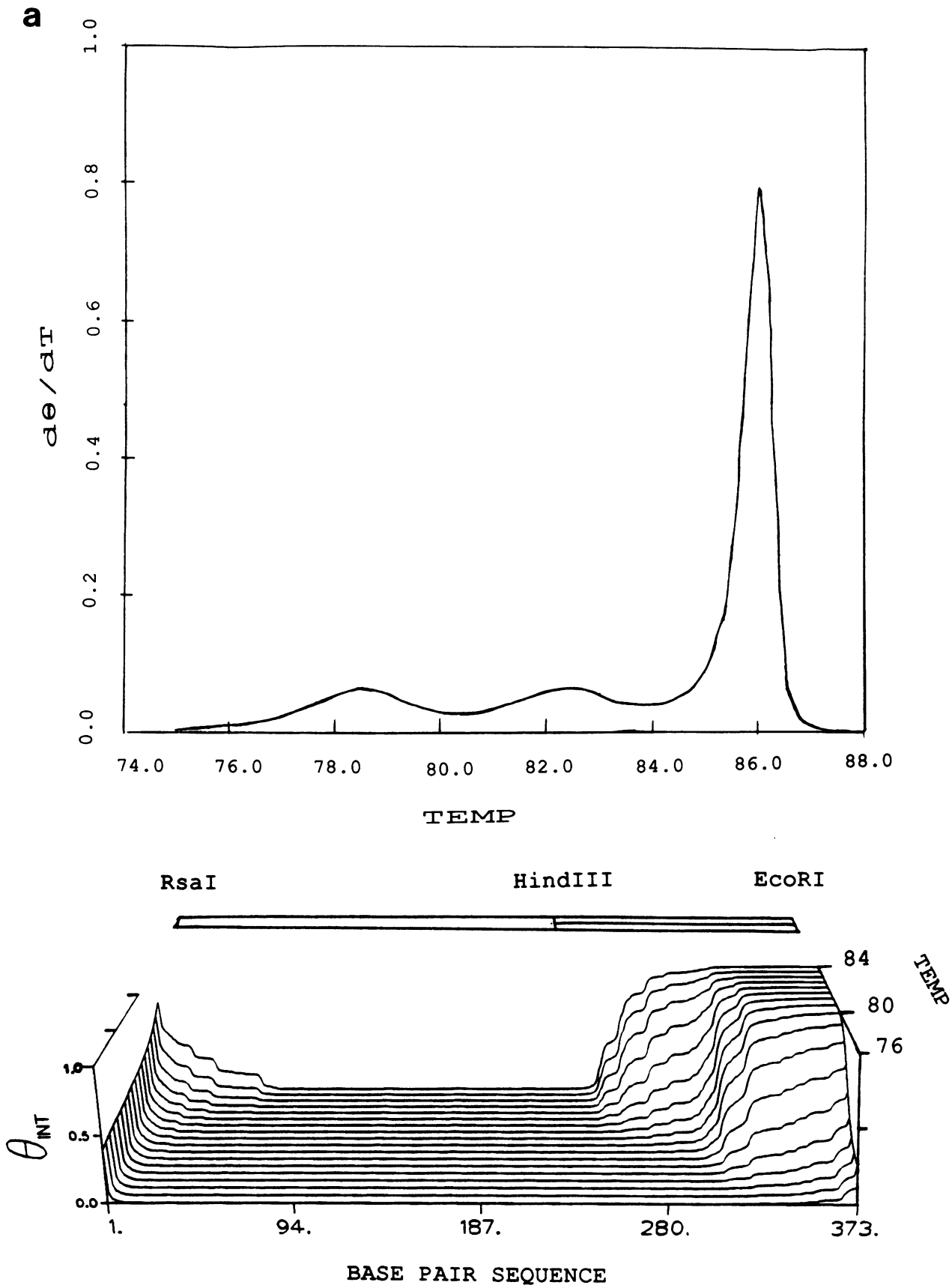

Figure 3. a) Predicted derivative melting curve of wild type sequence 373 bp DNA in 0.1 M Na^+. b) Predicted melting profile for the 373 bp DNA. θ_{INT} is the probability that the base pair is melted. The 373 bp DNA and its *ctc* region is indicated schematically above the profile. The orientation is opposite that shown in figure 1. The first melting domain includes about 70 bp of the *ctc* region.

Table I. Thermodynamic Stacking Interaction Energies Employed.

Base Pair Stack (5′ to 3′)	Gotoh & Tagashira ΔG(cal/mole)	McCampbell *et al.* ΔG(cal/mole)
AA	−50.4	−80.
AT	−113.4	60.
AG	385.6	0.
AC	−604.8	0.
TA	398.2	120.
TG	478.8	60.
TC	−320.0	0.
GG	224.3	−100.
GC	−1038.0	0.
CG	564.5	−150.

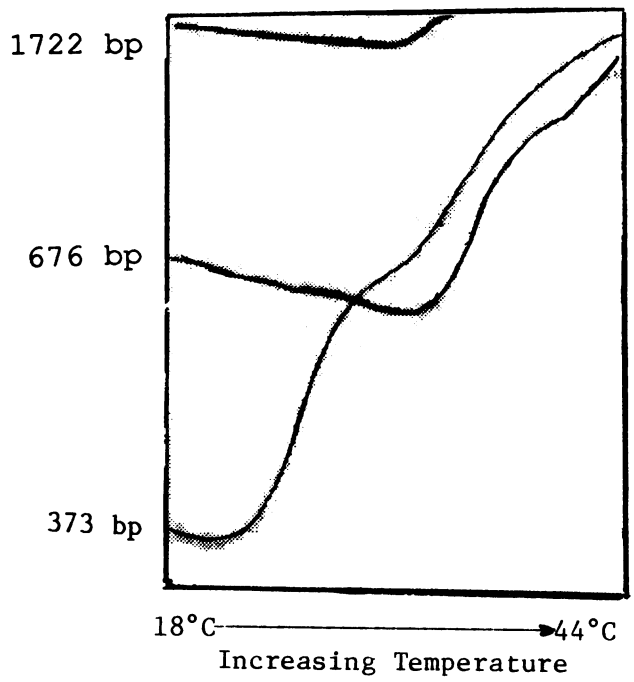

Figure 4. Temperature gradient gel of the RsaI/EcoRI digest of plasmid pUC8-31. Temperature gradient is perpendicular to the direction of electrophoresis. Picture is the negative image of ethidium bromide stained gel. 60% denaturant was used in a 6.5% polyacrylamide gel. The temperature gradient was 18°C to 44°C.

RESULTS

Denaturing gradient gel electrophoresis can detect single base pair differences in DNA fragments providing the differences are located in an early melting domain. To test the vertical temperature-gradient system, we sought a restriction digest of the pUC plasmids which would place the *ctc* region in an early melting domain of a DNA fragment. Melting curves were calculated for several plasmid restriction fragments containing the wild type *ctc* sequence in order to guide this selection.

Figure 3 illustrates the derivative melting curve and melting profile for the RsaI/EcoRI 373 bp restriction fragment. The derivative melting curve indicates that at least three cooperatively melting domains occur for this DNA. Peaks in the derivative melting curve have midpoint temperatures, T_m's, of 78.6°C, 82.5 ° C, and 86.1°C. The melting profiles,figure 3b, show that a large part of the *ctc* region melts in the first melting domain.

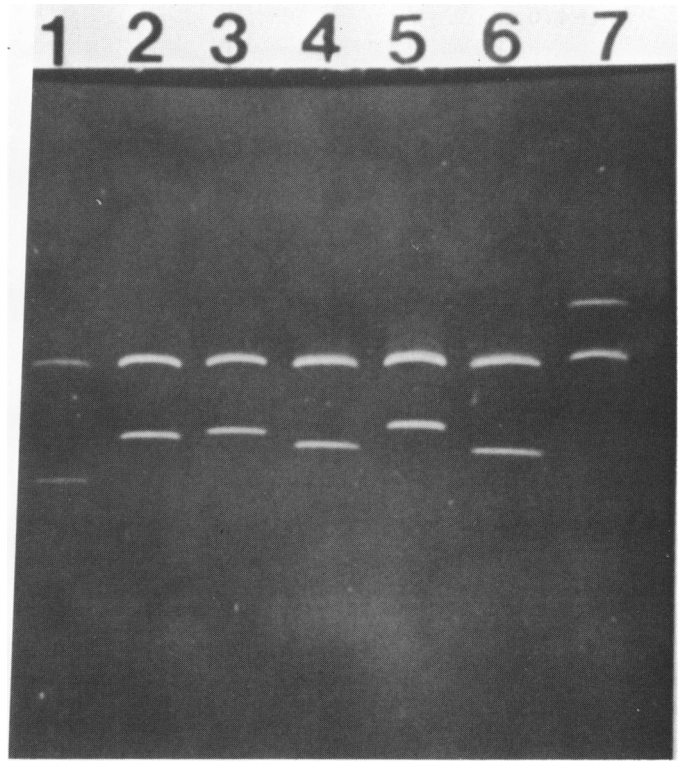

Figure 5. Temperature gradient gel with 10°C gradient parallel to direction of electrophoresis. DNA plasmids were digested with EcoRI and RsaI and loaded onto 6.5% polyacrylamide gels. The 676 and 373 bp DNAs are observed. Lanes and plasmids; 1)pUC8-31, 2) pUC8-36, 3)pUC8-15, 4)pUC8+3, 5)pUC8-5, 6)pUC8-1415, 7)pUC31-12.

All of the mutations except for pUC31-12 fall entirely within the first melting domain. The calculations employed the base pair stacking parameters from McCampbell et al. (14). The Gotoh and Tagashira (16) parameters gave similar results but with different T_m's for the domains. These findings suggested that mutations in the *ctc* region should be distinguishable from the wild type sequence by electrophoresis of 373 bp DNAs in a temperature-gradient gel.

Electrophoresis With A Perpendicular Temperature Gradient

The temperature-gradient system was first examined with a gradient perpendicular to the direction of electrophoresis. RsaI/EcoRI digests of pUC8-31 DNA were layered across one long lane at the top of the gel and electrophoresed. Figure 4 shows results obtained with the low temperature side of the gel at 18°C and the high temperature side at 44°C. The three DNA fragments from the digest undergo mobility transitions as a function of gel temperature. Two transition steps are observed for the 373 bp DNA and the 676 bp DNA. The first melting domain of the 676 bp DNA occurs between the first and second domains of the 373 bp DNA. The general behavior of the DNAs is similar to results obtained from solvent-gradient gels (2), and temperature-gradient gels run horizontally (10).

A gradual increase in mobility is observed for the 676 bp DNA prior to the onset of the large mobility decrease. This phenomena may be due to the effect of temperature on the average pore size of the polyacrylamide gel. A gradual mobility increase was also observed in the data presented with a horizontal temperature-gradient system (6). It is not apparent in the solvent-gradient gels which are maintained at a constant temperature (2).

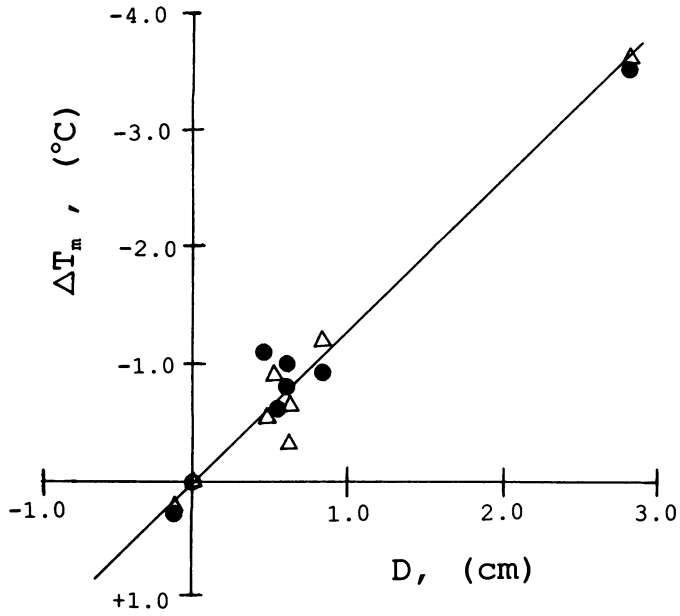

Figure 6. Plot of predicted T_m differences, ΔT_m, of first melting domains in 373 bp DNAs vs relative gel distances, D. The 373 bp DNA with the wild type sequence is used as the standard. Filled circles use McCampbell et al. parameters for ΔT_m, triangles use Gotoh and Tagashira parameters.

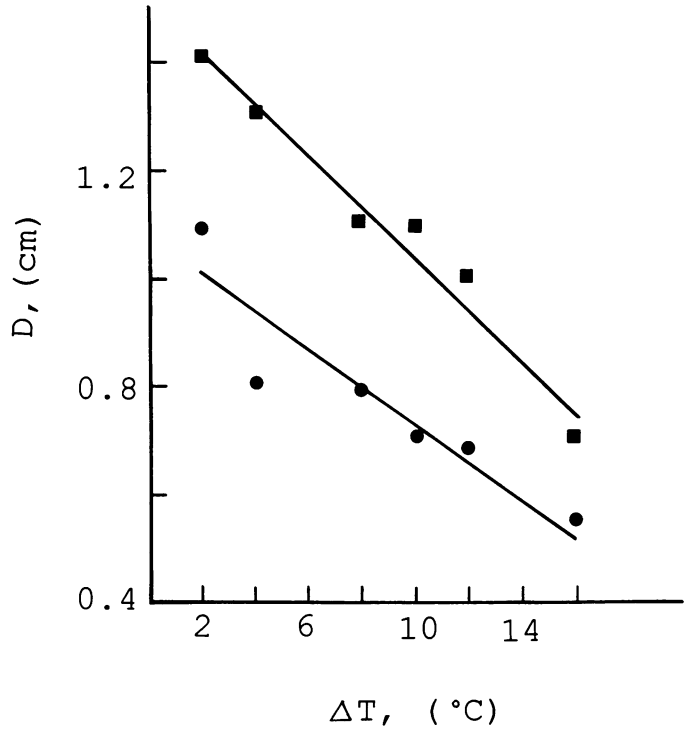

Figure 7. Plot of distance, D, between 373 bp DNAs from pUC8+3 (●) and pUC-5 (■) and wild type sequence 373 bp DNA vs. gel temperature gradient, ΔT. Midpoint temperature was 31.5°C. Gradients were parallel to electrophoretic direction. Other conditions are described in methods.

Temperature Gradients Parallel To Electrophoresis

The wild type and mutant 373 bp DNAs were analysed with temperature gradients parallel to the direction of electrophoresis. Figure 5 shows the 676 bp and 373 bp DNA bands resulting from a RsaI/EcoRI digest of seven of the plasmids. Single base pair changes can be detected, and one can distinguish GC to AT changes in different nearest neighbor environments. The wild type 373 bp DNA in lane 1 moved furthest in the gel. Fragments with single GC to AT base pair mutations are in lanes 2−5. Their *ctc* regions melted at temperatures lower than the wild type sequence, and have thus slowed in mobility higher up in the gel. The 373 bp fragment with ten base pair changes, lane 7, has denatured much higher in the gel. It is 28 mm above the wild type sequence in this gel. In this lane the 676 bp fragment has moved further than the 373 bp DNA.

The relative distances between the 373 bp DNAs from pUC8-36, pUC8-15, and pUC8+3 were quite small and their order were difficult to determined with a 10°C gradient gel. Gels with temperature gradients of 4°C were employed to increase the separation between the DNAs. Although the separation between the above three DNAs remained small (see below), the pUC8+3 DNA consistently moved further in the shallower gradient. The mutant DNAs from pUC8-36 and pUC8-15 were still not separable. Their mobilities were regarded as identical.

The experiment depicted in figure 5 employed a temperature gradient from 26.5°C to 36.5°C. The distances separating the mutant 373 bp DNAs from the wild type sequence are plotted vs the predicted T_m differences in figure 6. The circles correspond to calculations using the McCampbell parameters and the triangles correspond to calculations using the Gotoh Tagashira parameters. The 676 bp DNA was used as an internal standard for the distance measurements. Data for pUC8-12 obtained with similar gels was also included. Both parameter sets suggest a linear correlation between D and ΔT_m. Discussion of this data is presented later.

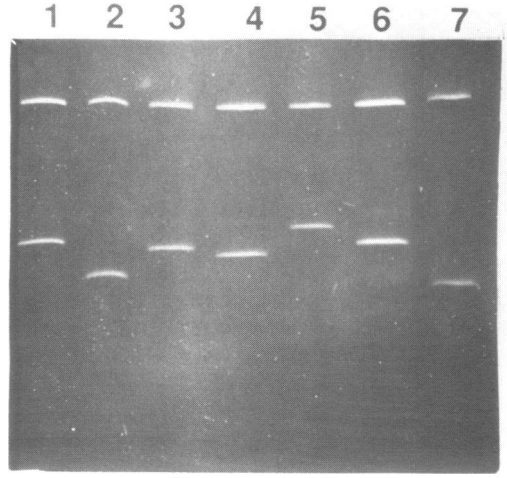

Figure 8. Temperature gradient gel with 4°C gradient parallel to direction of electrophoresis. DNA plasmids were digested with EcoRI and RsaI and loaded onto 6.5% polyacrylamide gels. The 676 and 373 bp DNAs are observed. Lanes and plasmids; 1) pUC8-15, 2) pUC8-31, 3)pUC8+3, 4)pUC8-1415, 5)pUC8-5, 6)pUC8-36, 7)pUC8-12.

The resolving power of the vertical gel system was examined as a function of the size of the temperature gradient. The 373 bp fragments from plasmids pUC8-31 (wild type), pUC8-5 and pUC8+3 were electrophoresed using temperature gradients from 2°C to 16°C. The midpoint temperature in the gel was 31.5°C. Figure 7 plots the distances between the wild type DNA and these two mutant DNAs as a function of the temperature gradient. As

expected the distances increase with shallower gradients. The displacements between the mutant fragments and the wild type DNA roughly double from a gradient of 16°C to 2°C. The displacements observed for two DNAs differing by a single base pair, 0.5−1.4 cm, are similar to results obtained using solvent-gradient gels with other DNAs (1,2).

Figure 8 shows a 4°C gradient gel of RsaI/EcoRI digests of seven of the pUC8 plasmids. The distances separating the mutant 373 bp DNAs from the wild type sequence is increased relative to figure 5. Of particular interest is the 373 bp DNA from pUC8-12 in lane 7. This DNA has an AT to TA transversion altering the sequence TAT to TTT (figure 1). It moved further into the gel than the wild type 373 bp DNA in lane 2, and is thus more thermally stable. This change in stability is consistent with previous analyses of AT polymers (18) and oligomers (19). The TpT base pair stack has a lower free energy and is thus more stable than the average of the ApT and TpA base pair stacks (Table I).

DISCUSSION

Applications

A vertical temperature-gradient gel system has been described which can detect single base pair differences in DNA duplexes. Applications similar to those pioneered by Lerman and co-workers (1−3) with denaturing solvent-gradient gels can be explored with this system. The temperature-gradient system may be more convenient since it avoids the requirements of a solvent gradient in the gel, and a large volume buffer reservoir. It also provides an easy way to alter the melting range in the gel.

The vertical system complements the horizontal-temperature gradient system described by Rosenbaum and Reisner (6). Advantages and disadvantages of these two systems appear to parallel conventional electrophoretic systems. The vertical system can distinguish between two DNAs which have the same base pair change at different locations, and can detect a single base pair transversion. A vertical thermal gradient system applied only across the direction of migration was described by Thatcher and Hodson (20). In addition to applications in molecular biology, the system we have described will also be useful for evaluating the thermodynamic parameters of base pair stacking.

Thermodynamic analysis

The data from figure 6 may be used to illustrate the evaluation of base pair stability parameters. Since the mutations we have examined in the 373 bp DNAs do not change the predicted sizes of the melting domains, the partially denatured DNAs should have the same mobilities. Distances between the DNAs should be related to their thermal stabilities. The scatter of the data points about the line in figure 6 indicates that neither stacking parameter set completely reproduces the order of stability observed in the temperature-gradient gels.

Adjustments to the parameters which improve the linear fit can be used to suggest a new set of satisfactory parameters. For example, the T_m differences predicted by McCampbell et al fit a straight line except for the DNAs from pUC8-1415 and pUC8-15. The predicted T_m's for the latter DNAs appear to be low relative to the wild type sequence. Decreasing the stacking energies of ApG and TpG can improve the fit. Adjustments to other nearest neighbor pairs can also improve the fit of the Gotoh and Tagashira predictions. In order to have confidence in the durability of a new parameter set, a large set of DNAs with single base pair changes needs to be examined. A study of this nature will refine the heirarchy of stacking interactions.

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