Salt dependence and thermodynamic interpretation of the thermal denaturation of small DNA restriction fragments

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ABSTRACT

The influence of cation concentration on the thermal denaturation of DNA restriction fragments from the *E. coli lac* regulatory region and from pVH51, ranging in size from 43- to 880- bp, is described. Upon increasing the ionic strength, the melting transitions broaden in a cooperative manner at salt concentrations characteristic for the specific fragment. For three fragments studied in detail, the salt concentration dependence at the midpoint varied between 0.03 and 0.19 M Na⁺. Along with the broadening, the melting transitions become more symmetrical. This result is discussed with respect to the irreversibility of melting transitions at low ionic strength. After a cooperative broadening, the shape of the melting curves remains constant up to salt concentrations of 0.5 M Na⁺. The $dT_M/d\log[Na^+]$ values for three fragments fall between 15.7 and 16.7. An easily applicable approximation of the van't Hoff equation is used to evaluate the enthalpies of 13 transitions arising from the denaturation of 43 to 600 bp. The results of this analysis are compared to calculations of the expected enthalpies based on calorimetric measurements. The T_M s of most transitions were directly related to the base composition, but several deviations from the predicted behavior were observed. The possible influences of fragment length and sequence on the thermal stability are discussed. The experimental and mathematical procedure to resolve a thermal denaturation transition with a width of $0.17 \pm 0.01^\circ$ and its distinction from another preceeding transition only approximately 0.15° away in an 880-bp *Hae* III fragment from pVH51 is described. This transition is about half as wide as the smallest one reported to date.

INTRODUCTION

The detection of fine structure in the thermal denaturation profiles of homogeneous viral DNAs has triggered new interest in determining the distribution of thermal stability along the DNA double helix (reviewed in 1,2). High resolution melting experiments have been carried out on large bacteriophage DNAs (>40 k bp) (3-6) as well as on smaller DNAs

(4-7 k bp) such as ϕ X174, fd, and the plasmid Col E1 (1,7-10). None of these studies has led to a straight-forward assignment of melting transitions in the derivative denaturation curves to segments of the DNA (1). One of the difficulties involved in this task is that melting transitions arising from different regions of the DNA frequently overlap. Computerized curve resolving programs can aid in interpreting this data. However, these methods generally assume that peaks in the derivative curves are symmetrical. Since studies (1,11) on single transitions have demonstrated that the peaks are asymmetrical and that the shape of the peaks is dependent on salt concentration, there remains a great deal of ambiguity when complicated thermal denaturation profiles are resolved into single transitions by these methods (1).

In an attempt to address the assignment problem experimentally, DNA restriction fragments of approximately 1-2 k bp in size from ϕ X174 and fd were melted individually, and their melting profiles were compared with those of the intact DNAs (12,13). The conclusion from these experiments was that the melting profiles of the large DNA molecules are not simply the sum of the melting profiles of their subfragments (13).

Theoretical attempts to derive stability maps have aided in the interpretation of denaturation profiles of viral DNAs in cases where the complete nucleotide sequence is known (reviewed in 1). While these studies have succeeded in predicting the overall shape of the melting profiles of ϕ X174 and fd DNAs, they differ significantly from the experimentally determined curves (14,15).

Improvements in DNA preparation procedures have made it possible to study the thermal denaturation of much smaller DNA restriction fragments, ranging in size down to as few as 80-bp (11,16). The denaturation profiles of small restriction fragments originating from the lactose control region of the *E. coli* genome were recently reported (11,17). Because of their small size, these fragments show rather simple denaturation profiles with a maximum of three subtransitions. All transitions are well separated from each other when the thermal denaturation is done at low ionic strength. Using these data

it was possible for the first time to develop a theoretical interpretation which led to a nearly complete agreement with the experimental results (18). The validity of this theoretical approach has been demonstrated by additional studies on a different set of small, sequenced restriction fragments. The experimental results in this case were exactly those predicted by the theory (17).

As mentioned above, an additional result obtained from the thermal denaturation study of small restriction fragments is that the transition width depends on the ionic strength. In all cases studied so far, the main transition in the melting profiles of small fragments widened between 0.01 and 0.105 M Na^+ (11,17). More detailed studies of this phenomenon as well as the dependence of T_M s of small fragments on the ionic strength may aid in the interpretation of the salt effects found in the denaturation of large viral DNAs (6,19).

We describe here the influence of cation concentration on the thermal denaturation of small restriction fragments derived from the lactose control region of E. coli and the plasmid pVH51. The alteration of transition shapes and T_M s with increasing ionic strength is studied, and experimental conditions are described that give high resolution for fragments as large as 880-bp containing subtransitions only half as wide as the smallest previously reported (20). Transitions involving approximately 43 base pairs up to approximately 600 base pairs are successfully interpreted using a straight-forward simplification of the van't Hoff equation (21).

MATERIALS AND METHODS

Preparation of DNA samples

The 95- and 301-bp fragments were prepared as previously described (22). The preparation of fragments (80-, 101-, 188-, 219-bp) derived from the 301-bp sequence is described elsewhere (16). The 203-bp fragment (22) was a gift of S.M. Stirdivant. The 43-, 69-, 84-, 360- and 880-bp fragments were prepared by digesting the plasmid pVH51 (23-26), linearized at the Eco RI site, with Hae III. The reaction products were separated by RPC-5 column chromatography (16,27). The purity of the frag-

ments was confirmed by acrylamide gel electrophoresis under load conditions which would have revealed the presence of any contaminating fragment over 2% of the total DNA. In all cases the samples used in this study did not contain detectable contaminating DNA (16). Further details on the preparation and characterization of these fragments are reported elsewhere (16). The DNA samples were concentrated to approximately $12 A_{260}/\text{ml}$ and dialyzed exhaustively into 10 mM Na cacodylate pH 7.0, 0.1 mM EDTA. The final salt concentration of each sample was then adjusted by mixing 40 μl of this stock solution into 1 ml of the desired NaCl (or CsCl) concentration containing 10 mM Na cacodylate pH 7.0 and 0.1 mM EDTA. The reported salt concentrations are corrected for this dilution. The final DNA concentration in these samples varied between 0.4 and 0.6 A_{260} . Prior to melting, the DNA samples were centrifuged to remove any dust particles and introduced into quartz cuvettes (one cm pathlength). The samples were saturated with helium by bubbling a fine stream of the gas through the solution for about 5 min at room temperature. The cuvettes were then tightly sealed with Teflon stoppers.

Measurement of absorption-temperature profiles

Absorbance-temperature transitions at 260 nm were measured with a Gilford 2000 spectrophotometer equipped with dual thermo spacers. Temperature readings were obtained from a platinum resistance probe mounted underneath the cuvette holder. Temperature calibration was done by comparing the platinum resistance thermometer to a quartz thermometer (Hewlett-Packard model 2804A) placed inside the insulated sample chamber. Repeat experiments showed the reproducibility of absolute T_M measurements to be $\pm 0.4^\circ\text{C}$. The measurement of internal temperature differences in a single melt, such as peak width and the distance between two subtransitions, was reproducible to within $\pm 5\%$; thus, transition width have an accuracy of a few hundredths of a degree. Temperature was controlled with a Neslab Exacal ex 100 thermal bath controlled by a Neslab ETP-3 temperature programmer. A second thermal bath maintained the temperature of the photomultiplier housing at 55°C . Heating rates were between 1°C per 12.5 min and 1°C per 50 min. The

1°C per 12.5 min heating rate was used most frequently and should be assumed unless stated otherwise. Control experiments were done at a heating rate of 1°C per 50 min for each fragment to ensure that no feature of the melting profile changed as a function of heating rate.

Handling of the data

The data were processed using a PDP 11/03 computer. The data were smoothed twice by fitting successive segments of 21 points to a cubic equation (11,20). Less rigorous smoothing revealed no difference in peak shape for the smaller fragments, however the shapes for both the 360- and 880-bp fragments were sensitive to the smoothing procedure. Thus, all data obtained for the 360-bp fragment was smoothed over segments of 11 points to a cubic equation. The data treatment for the 880-bp fragment is discussed in Results. Any deviation from the above smoothing conditions are described in the legends to the respective figures. The peak width was obtained from the derivative plots at half the maximum height of the transition. The width at 2σ was obtained in the same way at 0.61 times the maximum height of the transition (20). Areas under subtransitions were calculated from the θ vs. T plots without correcting for the different contributions of AT and GC base pairs to the hyperchromicity at 260 nm.

RESULTS

Collection and handling of data

Ansevin *et al.* have given a detailed description of procedures adequate for collecting and handling data in high resolution thermal denaturation experiments (20). The narrowest transition then observed was 0.3°C at 2σ of the first derivative of the melting curve. This peak could be resolved by collecting twenty data points per °C. The authors discussed the possibility of even narrower subtransitions in the melting profiles of natural DNAs according to different theoretical predictions (28,29). They also mentioned the necessity of adjusting the parameters of data collection and handling to these new situations. We describe below a procedure to measure a melting transition as narrow as 0.17°C at 2σ and to distin-

guish it from other subtransitions not more than 0.15°C away.

Fig. 1 displays different resolutions of the melting profile of the 880-bp Hae III fragment from pVH51. This fragment is the largest Hae III fragment in the plasmid and was designated fragment A in the restriction map (24). Fig. 1A shows the denaturation profile of this fragment at a heating rate of 1°C per 12.5 min, 1°C per 25 min, and 1°C per 50 min. Since the interval time of data reading was not changed, the number

Figure 1. Thermal denaturation curves of the 880-bp Hae III fragment from pVH51 at different resolutions. Panel A shows the influence of different heating rates on the appearance of the melting transition: dashed line, 1°C per 12.5 min; solid line, 1°C per 25 min; dotted line, 1°C per 50 min. Panel B shows the influence of the smoothing procedure on the resolution of the experiment: dashed line, same data as the dashed line in Panel A smoothed by fitting 21 point segments to a cubic equation; solid line, data from the dashed line smoothed by fitting segments of 11 points to a cubic equation; dotted line, the same data smoothed by fitting 7 point segments to a cubic equation. The curve shape seen in this last case is the same as that obtained by using this smoothing procedure on every fourth data point from the slow heating rate experiment (the dotted line in Panel A). All experiments shown in this figure were done at 0.105 M Na^{+} . The T_m of this fragment is 89.3°C .

of data points per degree increases from 25 to 50 to 100 points per degree for the respective heating rates. All three curves in this panel were smoothed by twice fitting successive 21 point segments to a cubic equation. In addition to the increased height of the main transition, two or possibly three subtransitions with T_M s very close to that of the main transition become more apparent with decreasing heating rate. Accordingly, the width of the transition (measured at half-height) drops from 0.43 to 0.19°C. This increase in resolution may be brought about by two different factors. First, the heating rate of 1°C per 12.5 min may be too fast to assure thermal and kinetic equilibrium at every point in the denaturation. Second, the increased resolution may be caused solely by the increased number of data points collected at the slower heating rate. In order to distinguish between these possibilities, the data of the fastest heating rate in Fig. 1A are shown in Fig. 1B when smoothed in the same way only over a smaller segment of points. The dashed line is the same as in Fig. 1A, obtained after smoothing the data twice by fitting successive segments of 21 points to a cubic equation. The solid line shows the same data smoothed twice by fitting successive 11 point segments, and the dotted line is derived from twice fitting successive 7 point segments. It can be seen that the width of the transition narrows using the less rigorous smoothing conditions. Also, the least rigorously smoothed data allows the fine structure of the melting profile to become more apparent, although the resolution of the slow heating rate experiment in Fig. 1A is not reached. In an additional experiment, however, when only every fourth data point from the dotted line in Fig. 1A (100 points/°C) is used with the same smoothing conditions that give the dotted line in Fig. 1B (25 points/°C), the same shape of the transition as the dotted line in Fig. 1B is obtained (result not shown). This direct comparison indicates that thermal equilibrium is reached even at the fastest heating rate and that only the increased number of data points obtained with the slower heating rate is required to give the enhanced resolution seen in Fig. 1A.

The time needed to switch a single beam instrument from

sample to blank limits the number of readings that can be taken in a given time. Therefore, the only way to get more readings is to decrease the heating rate, which increases the number of instrument fluctuations in any given part of the denaturation. Thus, in a long experiment such as the above where the sample temperature is raised at a rate of 1°C per 50 min, the short term fluctuations in optical resolution of the spectrophotometer become limiting. This fact, which has also been pointed out by Ansevin *et al.* (20), probably limits the resolution in our present apparatus at the level of the dotted line in Fig. 1A. For the same reason, smoothing these data over less than 21 point segments does not further improve the resolution due to the short term noise already inherent in the data.

Stability of DNA to high temperature

It was previously noted that the heating rate for DNA melting experiments was limited by the sensitivity of the DNA sample to damage by heat-induced depurination or other reactions (30-33). The dependence of the transition shape of the 880-bp melt on the number of data points and the smoothing procedure (Fig. 1) indicates that no extensive damage occurs to this DNA sample at heating rates as slow as 1°C per 50 min.

Influence of salt on the T_M of single transitions

The effect of salt concentration on the T_M and the shape of the thermal denaturation curves was determined in detail for three fragments. The three fragments were the 95- and 101-bp fragments from the *E. coli* lactose control region and the 360-bp *Eco* RI/*Hae* III fragment from pVH51 (designated B_1 in the restriction map, see ref. 24,25). Each of these fragments has melting profiles with sharp transitions (see ref. 11 and Fig. 4 herein for the 95- and the 101-bp fragment respectively) which are well separated from other preceeding transitions in the case of the 360-bp fragment (34). The T_M s follow the known linear relationship with the logarithm of the cation concentration. The $dT_M/d\log[Na^+]$ values derived from the data shown in Fig. 2 are 15.7°C for the 95- and 360-bp fragments, and 16.7°C for the 101-bp fragment. This difference might reflect the different behavior of the 95- and 101-bp sequences

Figure 2. Dependence of T_M on the salt concentration for the 95-, 101-, and 360-bp fragments. Closed boxes show the main transition of the 360-bp Eco RI/Hae III fragment, B_1 , from pVH51 (40% GC in the sequence melting under the main transition) (24,25); closed triangles, 95-bp fragment from the lactose control region of *E. coli* (47.4% GC) (44); open triangles, the same fragment with CsCl replacing NaCl; closed circles, the 101-bp fragment from the i-gene sequence of the lactose control region of *E. coli* (64% GC) (44).

toward changes in the salt concentration noted earlier (11). In order to test the possible influence of cation type, four determinations were carried out with the 95-bp fragment in the presence of CsCl instead of NaCl. The open triangles in Fig. 2 show that the T_M values obtained from these measurements all fall on the NaCl line. Thus, the thermal stability of this DNA fragment does not depend on the kind of cation that forms the ionic sphere surrounding it.

Influence of salt on the shape of single transitions

Fig. 3 displays the dependence of the width of the main transitions at half-height for the thermal denaturation profiles of the 95-, 101-, and 360-bp fragments. All three fragments show a sigmoidal increase of the width with the midpoint of this transition at a salt concentration which is characteristic for each fragment. The ionic strength at the midpoints and the corresponding T_M values at this ionic strength for the three

Figure 3. Dependence of the width at half-height (ΔT) on the salt concentration. The fragments and the symbols used to designate them are the same as in Fig. 2.

fragments are given in Table 1. Whereas the transition temperature is within 2.5°C for the 101- and 360-bp fragments, it is approximately 7°C lower for the 95-bp fragment. In order to result in the same temperature, the midpoint salt concentration for the 95-bp fragment would have to be 120 mM. Since this is far beyond the error in our measurements, the conclusion is that the transition of peak width with increasing salt concen-

TABLE 1

Midpoint salt concentrations of the peak broadening and the respective T_M s of the fragments.

Fragment size (bp)	Salt concentration (mM) at the midpoint of the transition (from Fig. 3)	Corresponding T_M ($^{\circ}\text{C}$) for the midpoint salt concentration (derived from Fig. 2)
95	41	74.5
101	30	80.0
360	186	82.5

tration does not occur at the same T_M for each of the three fragments.

The relative increase in peak width is approximately the same for all three fragments (72% for the 360-bp, 68% for the 101-bp, and 95% for the 95-bp fragment). The absolute increase varies from 0.23 to 0.8°C (interpretation of peak widths is discussed below). The open triangles in Fig. 3 are the result of CsCl replacing NaCl in the melting experiment. As shown above for the effect of salt on T_M , there is no influence of the cation type on either the shape of the melting curve or the salt concentration at the midpoint of change in peak width.

Fig. 4 shows the melting transition of the 101-bp frag-

Figure 4. Melting profiles of the 101-bp fragment at 10 and 50 mM Na^+ . The lower ionic strength (dashed line) is the last point before the transition in Fig. 3, and the higher ionic strength (solid line) is the first point after it. The T_M s in 10 mM and 50 mM Na^+ are 71.7 and 82.0°C, respectively. The vertical line through the $d\theta/dT_{\text{max}}$ values is drawn to demonstrate the reduced asymmetry of the melts at the higher salt concentration.

ment at 10 and 50 mM salt concentration. The lower ionic strength is just below the transition in peak width in Fig. 3 whereas the higher salt concentration is just above that transition. Therefore, the two melting curves in Fig. 4 demonstrate the decrease in height, increase in width, and loss of asymmetry of the denaturation profiles as the salt concentration is raised for the transitions in Fig. 3.

Effect of base composition on T_M s for single cooperative transitions

Results obtained from measurements on sheared chromosomal DNAs, consisting of inhomogeneous lengths and sequences, led to the Marmur-Doty equation relating the T_M to the GC content (35). Fig. 5 shows a similar plot for the fragments which allow the unambiguous assignment of a certain sequence to a cooperative

Figure 5. T_M as a function of base composition. Open circles represent a series of DNA restriction fragments prepared from an *Eco* RI/*Hae* III digest of pVH51: a is the main transition of the 360-bp fragment; b is the 43-bp fragment; c, the 69-bp fragment and d, the 84-bp fragment. (For a restriction map and the nucleotide sequence of pVH51 see references 24-26,45). Closed circles refer to fragments from the lactose control region of *E. coli*: a is the 80-bp fragment; b, the 95-bp fragment; c and d, the 203- and 188-bp fragments, respectively; e and f are the 219- and 301-bp fragments and g represents the 101-bp fragment. The T_M values were measured at 0.105 M Na^+ . (For a restriction map and the DNA sequence of the lactose control region see reference 44).

transition. The filled circles represent data from sequences which originate from the lac control region of E. coli and the open circles show data from fragments prepared from pVH51. When, in agreement with published results (1) and the data in Fig. 2, an average value of 16.2°C is used for $dT_M/d\log[Na^+]$, the equation relating the T_M with the salt concentration and GC content (X_{GC}) is:

$$T_M = (16.2)(\log[Na^+]) + (49 \pm 2)(X_{GC}) + (73 \pm 3)$$

In this equation, the slope and intercept differ from the best fit of other data (1).

Thermodynamic analysis of the melting curves

The enthalpy of the melting reaction of a single transition can be derived from the derivative melting profile using an approximation of the van't Hoff equation (21). The equation is:

$$\Delta H = 4RT_M^2/\Delta T$$

where T_M is the melting temperature in °K, ΔT is the width of the melting transition at half-height in °C and R is the gas constant. This equation is strictly valid only for monomolecular, one-step reactions where the opening of the first base pair in the cooperative unit is the rate-limiting step. The last transition in a DNA melting profile leads to strand separation and therefore is a biomolecular process. The use of this approximation despite this fact is justified by the finding that no concentration dependence of melting has been observed in the concentration range of UV measurements (36). In addition, the above equation is a rough approximation in that it assumes the melting transitions are symmetric and that their shape may be approximated (21) by a triangle such that $\partial\theta/\partial T|_{T_M} = 1/\Delta T$. Since derivative denaturation profiles of DNA fragments are not perfectly symmetric and triangular (see Figs. 1 and 4 and refs. 1,11), it is clear that only very approximate values can be expected from this analysis.

Table 2 lists the results obtained from our melting experiments published, in part, earlier (11,34) and compares them to the results obtained from calorimetric measurements (37,38). It is apparent that the agreement between the re-

Table 2. Comparison of the enthalpies derived from the optical melting studies and calculations based on calorimetric measurements.

No. of bp in transition ^a	Fragment melted (bp)	% GC in the transition	ΔT [°C] at half-height	ΔH [kcal/Mole] from melting curve ^b	ΔH [kcal/Mole] from calorimetry ^c	Percent Deviation
43	43	48.8	2.69	352	347	+1
64	219	47.5	1.85	502	516	-3
65	301	43.0	2.04	489	518	-6
69	69	52.2	2.08	477	561	-15
80	80	43.8	1.70	575	639	-10
81	301	47.0	1.68	603	652	-7
84	84	59.5	1.61	629	695	-9
95	95	47.4	1.60	618	765	-19
101	101	63.8	1.25	817	842	-3
155	219	60.0	0.70	1066	1310	-19
155	301	60.0	0.75	1024	1310	-22
270	360	40.0	0.33	1993	2138	-7
600 ^d	880	60.0 ^d	0.19	5497	4970	+11

^a These values were determined from area analyses of the differentiated melting curves as described (11).

^b The combined error of the values used to calculate the enthalpies is $\pm 6\%$.

^c These data were calculated from calorimetric measurements in refs. 37 and 38 as compiled in ref. 1. No correction was made for any dependence of ΔH on T_m .

^d Because the sequence of the 880-bp fragment is not known and due to the two preceding subtransitions close to the main transition (Fig. 1), the interpretation of this melt may be subject to a larger error.

sults from these different methods is fairly good, extending over a size range of the cooperative unit from 43 base pairs in the 43-bp fragment to 600 base pairs in the 880-bp fragment from pVH51. All the values given in Table 2 are derived from melting experiments done at 0.105 M Na⁺.

DISCUSSION

This paper describes the thermal denaturation of small DNA restriction fragments from the E. coli lactose control region and from the plasmid pVH51. The results obtained in these studies extend beyond the distribution of thermal stability in particular sequences and the relation of this stability to biological functions to allow a comparison of some physical properties of the melting transitions. The conclusions obtained in this study should be applicable to the thermal denaturation of all small and, in some cases, also large DNA fragments.

The 880-bp fragment from the Hae III digest of pVH51 shows the narrowest melting transition reported to date. With a width of $0.17 \pm 0.01^\circ$ at 2σ it is almost twice as narrow as the narrowest subtransition in bacteriophage λ DNA which was determined to be 0.30° at 2σ (6,20). The very small width together with a difference in T_M of only approximately 0.15° from the preceding subtransition required extreme care in data gathering and handling. Due to the presence of a subtransition so close to the main transition, the data density necessary to obtain resolution exceeds even the published values for a transition of this size (20). Because this thermal denaturation transition is even narrower than the transition found for homopolynucleotides (20), it should follow that the sequence of the 880-bp fragment must consist of an extremely even distribution of AT and GC base pairs. Unfortunately, the sequence of this segment of the pVH51 DNA has not been determined. Moreover, the length of the cooperatively melting region of this DNA must be longer than in homopolynucleotides because a small width indicates a high enthalpy which in turn arises from the thermal denaturation of a large number of base pairs (see below).

The two early melting transitions shown in Fig. 1A at the slowest heating rate may be the result of short term tempera-

ture fluctuations at the very slow heating rate of 1° per 50 min. However, since a shoulder appears when the data of the 1° per 12.5 min heating rate (Fig. 1B) is not excessively smoothed, this explanation is very unlikely. The interpretation that the increased data density at a 1° per 50 min heating rate results in the distinction between these subtransitions is further confirmed by the observation that the same melting curve as in the 1° per 12.5 min experiment is obtained from the 1° per 50 min experiment when only every fourth data point is used. This result also suggests that thermal and kinetic equilibrium is achieved even at the fastest heating rate. Thus, it is not necessary to perform high resolution melting experiments with stepwise increases of temperature as suggested previously (39). This observation also indicates that damage of the DNA that might occur from exposure to high temperature and alter the melting behavior does not seem to be a problem in this case.

The $dT_M/d\log[Na^+]$ values deduced from Fig. 2 are in agreement with published values (1) and, at least for the 95-bp fragment, do not depend on the type of the counterion (40).

It was noted earlier that the width of the melting transitions for small fragments increases with increasing salt concentration (11). Our present studies demonstrate the cooperative nature of the increase in width of a thermal transition with increasing salt concentration. Together with the sharpening of the transition at low salt concentration, the asymmetry of the melting transition increases (Fig. 4 and reference 11). This transition does not occur at the same melting temperature for the three fragments studied here. This finding makes a possible temperature dependence of ΔH as a reason for this behavior rather unlikely. It is difficult to derive the possible influence of fragment length or specific sequences from only three cases. However, one may note that the salt concentration at the midpoint of the transition in width increases with increasing AT content of the fragment, whereas there does not seem to be a uniform dependence on chain length.

A possible reason for this cooperative drop in transition width may be that an increase in cooperativity with decreasing

salt concentration (1) eliminates contributions from stepwise melting of the fragments at salt concentrations below the transition midpoint. On the other hand, the thermodynamic analysis at 0.105 M Na^+ is done for both the 95- and 101-bp fragments above their transition concentration and yet shows deviations similar to that obtained for the 270-bp cooperative unit in the 360-bp fragment which is evaluated below its transition concentration. This observation suggests that a change in the mechanism of melting is not responsible for the cooperative broadening of transitions with increasing salt concentration. As opposed to the above cases, where only a single melting transition is observed (see Fig. 4), the interpretation of the 360-bp data may be complicated by the presence of multiple melting transitions (34). It may be possible in this case that depending on the salt concentration, a rearrangement of the cooperatively melting regions contributes to the widening of the main transition.

The alteration in shape accompanying the broadening of the transition (Fig. 4) suggests another possible explanation. Since the melting curve at low salt shows more asymmetry than the one after broadening, it may be that a salt concentration above the transition in Fig. 3 is needed to assure kinetic equilibrium of the melting process. At salt concentrations below the transition, the fast reassociation of the single strands might be hindered by a high activation energy caused by the greater charge repulsion of the phosphates. In addition to the observed asymmetry of the melt (Fig. 4), the low ionic strength experiments give enthalpy values that are larger than those expected on the basis of the total number of base pairs in the fragment (see below). This may also be indicative of non-equilibrium conditions in these experiments.

The linear relationship between T_M and G+C content is valid for most but not all fragments. A dependence of T_M on the chain lengths of $(\text{dA-dT})_n$ and $(\text{dI-dC})_n$ levels off at approximately 50 base pairs, and is not very pronounced above a length of 40 base pairs (41). Thus, the large deviation of the 43-bp fragment from the line in Fig. 5 cannot be accounted for by its short chain length alone. On the other hand, inspection

of Fig. 5 shows that all fragments below the line are of short chain length and the ones above are of longer chain length. Thus, it is also possible that the polynucleotide data (41) predict the chain length dependence of the T_M rather inaccurately for DNA fragments. This may be due to the formation of hairpin and rosette structures for the alternating polynucleotides. In addition, since the Marmur-Doty equation which provides the best fit of published data (1) produces a line above the one in Fig. 5, it is possible that there is an influence of these short chain lengths on the T_M .

Sequence specific differences in thermal stability are known to arise from nearest neighbor and next nearest neighbor interactions (42). For example, the T_M of $(dG)_n \cdot (dC)_n$ is several degrees lower than the T_M of $(dG-dC)_n$ (42,43). The sequence of the 43-bp fragment, which shows the greatest deviation from the linear relationship in Fig. 5 has a strong strand bias for the distribution of purines (26) with 79% of all purines on the same strand. This strand bias may contribute to the observed deviation. On the other hand, a T_M slightly higher than the linear relationship in Fig. 5 is found for the overlapping 203- and 188-bp fragments from the lac control region which contain the same sequence giving rise to the main transition. Examination of the sequence of these fragments (44) shows that they have a rather even distribution of purines with many of them in an alternating purine-pyrimidine arrangement. Factors other than this, however, may contribute to the thermal stability of a fragment because the 80- and 95-bp fragments also have an even distribution of purines without showing increased stability (see Fig. 5) (44). Thus, it does not seem to be possible to distinguish clearly between length and sequence effects on the thermal stability.

Fig. 5 also shows the agreement of T_M values for the 188- and 203-bp as well as for the 219- and 301-bp fragments which contain the same sequences melting under their main transitions. This may be taken as an indication of the reproducibility of the data.

The thermodynamic interpretation (Table 2) of a large number of melting transitions using a rough approximation of

the van't Hoff relation shows good agreement with calculated ΔH values based on calorimetric measurements. This indicates the reasonableness of the assumptions made in the derivation of the equation used to calculate the enthalpies from the melts. This agreement over a large size range suggests that cooperatively melting regions in these fragments vary from 64 bp in the 219-bp fragment to approximately 600 bp in the 880-bp fragment. The enthalpy values are derived from melting transitions at 0.105 M Na^+ . Because most of the transitions become sharper at lower salt concentrations, it follows that a van't Hoff analysis at these salt concentrations results in enthalpies that are approximately 50% higher than the calorimetric data. It is likely that this is due to the non-equilibrium effects at low salt concentrations discussed above.

The ΔH values in Table 2 obtained from the melting experiments are generally lower than the calorimetric data. This is an expected result because a small broadening of the melting transitions may occur due to contributions from stepwise melting. In addition, the shorter fragments show extensive fraying from the ends of the double helix. As a result, a few percent of the base pairs are already open at the melting temperature. This effect also leads to a smaller ΔH value. Aside from the 880-bp fragment, all data agree with this observation. The 880-bp fragment is not sequenced yet which possibly leads to a larger error in the calculation of the calorimetric ΔH . As well, it is possible that the broadening of this melting transition takes place at higher salt concentrations than 0.105 M. This would also lead to a ΔH value which is too large (see above). The main transition of the 360-bp fragment is not yet broadened at 0.105 M Na^+ (Fig. 3). Its ΔH derived from the optical melting curve, however, is in good agreement with its ΔH derived from the calorimetric data and follows the trend observed for the other fragments. The presence of multiple transitions for this fragment may interfere with the thermodynamic analysis by altering the cooperative length as a function of the ionic strength as discussed above.

A straightforward simplification of the van't Hoff

equation allows reaction enthalpies to be calculated simply and directly from the derivative melting profiles. In the case of a fragment where the sequence or base composition is known, the enthalpy derived from the shape of the melting transition can be compared with a calculated enthalpy derived from calorimetric measurements. A large disagreement in these values (>50%) where the melting curve enthalpy is the smaller of the two suggests that multiple transitions are present under a single peak in the derivative melting profile. For example, if the 880-bp fragment is treated as having only a single transition, the observed peak width at half height (ΔT) in the highest resolution data is 0.41°C (see Fig. 1). This results in an enthalpy value of 2547 kcal/Mole which, when compared to an expected value of 7289 kcal/Mole calculated from calorimetric data, gives a -65% deviation. Since this type of comparison has been validated in the case of small restriction fragments, it may be especially valuable in the interpretation of complicated melting curves.

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FOOTNOTE

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