High Resolution Experimental and Theoretical Thermal Denaturation Studies on Small Overlapping Restriction Fragments Containing the Escherichia coli Lactose Genetic Control Region*

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Wolfgang Hillen†‡§, Thomas C. Goodman§, Albert S. Benight¶, Roger M. Wartell¶, and Robert D. Wells§

From the †Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706 and the ¶School of Physics and Biology, Georgia Institute of Technology, Atlanta, Georgia 30332

The distribution of thermal stability in the Escherichia coli lac control region is evaluated from the melting behavior of 5 short (80–219 base pairs (bp)) sequenced DNA restriction fragments containing various parts of this sequence. The thermal denaturation of these fragments was measured at 3 salt concentrations. The previous notion that the melting curves for small fragments are sharp and asymmetric in 0.01 M Na\(^+\) and broadened and less asymmetric at 0.105 and 0.505 M Na\(^+\) is confirmed and the possible explanations are discussed. The existence of two thermodynamic boundaries in this region is also confirmed. The exact location of the boundary upstream of the cyclic AMP receptor protein (CAP) binding site is accurately determined from melting experiments at 250 and 282 nm. The second boundary located between the promoter and operator sequence is apparent at the two higher salt concentrations and begins to disappear at the lower salt concentration. The physical interpretation of the melting experiments is compared to the results of theoretical predictions derived from the known sequence of the fragments.

Improvements in instrumentation and data handling along with the techniques of gene cloning have increased the interest in thermal denaturation studies of DNA (for recent reviews, see Refs. 1 and 2). Instead of melting sheared chromosomal or viral DNAs from various sources which differ in GC content (3), recent work has concentrated on the thermal denaturation of DNA molecules that have been purified to homogeneity (1, 2). Also, theoretical melting curves calculated from viral DNA sequences have been compared with experimental transitions (4–11). Although general agreement exists, detailed features of differential melting curves are often not reproduced.

The use of small restriction fragments (80–800 bp) of known base pair sequence has several advantages over the viral DNAs (4–7 kilobase pairs long). (i) The melting curves are rather simple, allowing accurate determination of their shape parameters such as width, etc. (ii) Because only a few hundred or less base pairs are involved in the melting events, the accuracy in determining thermal stability boundaries is greatly increased. (iii) Possible sequence-specific features are more easily detectable. (iv) It is easier to evaluate theoretically the influence of sequence, strand dissociation, and base pair stability parameters on melting curves.

A previous study of the melting curves of restriction fragments containing the lactose operon control region showed that the promoter region was more thermolabile than the terminator region of the adjacent \(l\)-gene (12). This finding agrees with the notion of thermally unstable regions at gene boundaries. Melting curves calculated from the sequences of 4 lac operon restriction fragments predicted the observed transitions in 0.1 M Na\(^+\) concentration (13). Inclusion of duplex to single strand dissociation and variation of dissociation with fragment length were essential for providing good agreement between theory and experiment. These 2 studies form the basis for the current work.

We report the melting transitions of 5 restriction fragments originating from the Escherichia coli lactose operon. They range in size from 80–219 bp and contain portions of the sequences of fragments studied earlier. The thermal stability boundaries found previously (12) are not due to the short length of the fragments but rather an intrinsic characteristic of the sequences studied. Theoretical melting curves of the fragments calculated from their known sequence are in close agreement with the experimental transition curves for 0.105 and 0.505 M Na\(^+\) concentrations. Correct predictions of the \(T_m\) values, the widths of the melting transitions, and the boundaries between subtransitions substantiate the theoretical model and aid interpretation of the transitions.

MATERIALS AND METHODS

DNA—The 301- and 95-bp restriction fragments from the lactose control region were prepared as described (14–16). The 219-, 80-, 188-, and 101-bp fragments were prepared from the 301-bp fragment by preparative cleavage followed by RPC-5 column chromatography as described (17, 18). All 4 fragments were free of contaminating DNA after 1 column fractionation as judged by gel electrophoresis which would have allowed the detection of any contamination over 2%. Other materials and methods not specifically described were as reported previously (12–18).

Preparation of the DNA Fragments for Melting—The DNA samples were concentrated to between 0.45 and 0.65 A\(_{260}\)/ml and exhaustively dialyzed against 10 mM Na cacodylate (pH 7.0), 0.1 mM EDTA, and the indicated salt concentrations. All samples were dialyzed together so that each of the 3 Na\(^+\) concentrations tested was identical in the 4 different fragment samples. The absolute Na\(^+\) concentrations were determined in the last dialysate using a Radiometer CDM3...
conductive meter. The samples were centrifuged, introduced into quartz cuvettes (1 cm path length), and saturated with helium.

Melting Determinations—The absorption-temperature profiles were determined as described (18). Forty absorbance, blank, temperature readings were taken per degree. During the experiment, the photomultiplier housing was maintained at a temperature of 50°C. Processing of the data was as described (12, 10). Repeat experiments showed that the accuracy of the absolute \( T_m \) values is ±0.5°C. Internal temperature differences in the profiles are reproducible within ±0.04°C.

Theoretical Calculations—Theoretical melting curves were generated using the loop entropy model of the DNA helix → coil transition (13, 21). The melting temperatures were \( T_m = 64.3°C \), \( T_{dG} = 75.5°C \), \( T_G = 114.0°C \) for 0.1 M \( \text{Na}^+ \), \( T_m = 70.7°C \), \( T_{dG} = 100.0°C \) for 0.5 M \( \text{Na}^+ \), and \( T_{dG} = 44.0°C \), \( T_{dG} = 65.0°C \) for 0.01 M \( \text{Na}^+ \) (22, 23).

RESULTS

Location of the Fragments—The location of the fragments used in this study with respect to the 301-bp sequence (24) is shown in Fig. 1. Also, this figure displays the location of the thermodynamic boundary approximately 20 bp upstream of the CAP binding site (described in detail below under the melting behavior of the 219-bp fragment). The GC content of these fragments varies from 65% for the 101-bp fragment spanning the end of the i-gene sequence to 44% for the 80-bp fragment containing the repressor binding site. The 188-bp Hind II fragment contains the same sequence as the 203-bp fragment in our previous study (12) except for the absence of 9 bp from the z-gene end and 6 bp from the repressor gene end. It is of interest, therefore, to compare the thermal denaturation profile of the 188-bp DNA with that obtained from the melting of the 203-bp fragment.

The 219-bp Hpa II fragment extends from the repressor gene through the CAP binding site and the promoter. It contains the entire most stable portion of the sequence from the 301-bp fragment and an additional 61 base pairs of the lac regulatory sequence. The other fragment resulting from Hpa II digestion of the 301-bp fragment is 80 bp and contains a 14-bp inverted repeat sequence at the repressor binding site (24).

In addition to these new fragments, the thermal denaturation of the 95-bp fragment is re-examined for 2 reasons. (i) Previous shortages (12) in the amount of the fragment available have been overcome, allowing a larger number of repeat experiments; and (ii) the increased resolution of our apparatus enables us to measure melting profiles of short fragments at high ionic strength where the transition is broad and an increased signal-to-noise ratio is needed.

Melting Curves of the 80-, 95-, and 101-bp Fragments—The melting curves of the 80-, 95-, and 101-bp fragments are shown in Fig. 2, A–C, respectively. The melting transition of each fragment is shown at 0.01, 0.105, and 0.505 M \( \text{Na}^+ \). These transitions are offset on the vertical and horizontal scale to allow a direct comparison of their shape. Their \( T_m, dT_m/d\text{Na}^+ \), and width at half-height values are summarized in Table I in comparison with the values predicted by theory. The theoretical melting curves for these 3 fragments at 0.105 and 0.505 M \( \text{Na}^+ \) are shown in Fig. 2, A–C, as dashed lines in direct comparison with the experimental results.

The melting curves for these 3 fragments show a single transition under all salt conditions. Their variation in shape with increasing salt concentration is very similar. At 0.01 M \( \text{Na}^+ \), the transitions are sharp and clearly asymmetric with the high temperature side steeper than the low temperature side. The transitions are broadened at 0.105 and 0.505 M \( \text{Na}^+ \). In comparing the melting curves of the different fragments, it is clear that the width of the transitions at all 3 salt concentrations decreases with increasing chain length and GC content of the fragments. The asymmetry of the melting transition is most obvious in the sharpest transition belonging to the 101-bp fragment at 0.01 M \( \text{Na}^+ \) and becomes less apparent among the fragments with decreasing chain length and for a single fragment with increasing salt concentration.

Comparison of the dashed lines with the respective solid lines in Fig. 2, A–C, reveals the good agreement between theoretical predictions of the thermal denaturation of the fragments from their known sequence and the experiments at 0.105 and 0.505 M \( \text{Na}^+ \). This agreement is also evident from the comparison of the \( T_m \), peak height, and peak width values in Table I.

Melting of the 188- and 219-bp Fragments—In contrast to the fragments described above, the 188- and 219-bp fragments show more than 1 transition in their melting profiles. The solid lines in Fig. 3 display the differential melting curves of the 188-bp fragment at 0.01, 0.105, and 0.505 M \( \text{Na}^+ \). The lead-in shoulders of the melting curves are interpreted as an early melting transition. It is interesting to note that the low temperature melting region for the 188-bp fragment seems to move away from the main transition upon raising the ionic strength. This is opposite to the behavior observed for the middle transition in the 301-bp fragment and the early transition in the 219-bp fragment (see below). However, the same phenomenon is seen in the melt of the 203-bp fragment (12).

The shape of the main transition shows the same tendency as described above for the shorter fragments. It is sharp and clearly asymmetric at 0.01 M \( \text{Na}^+ \) and is broadened with decreasing ionic strength at 0.105 and 0.505 M \( \text{Na}^+ \). The dashed lines in Fig. 3 display the results of a calculation of the melting curve of the 188-bp fragment at 0.105 and 0.505 M \( \text{Na}^+ \). The comparison with the experiment demonstrates very good agreement and substantiates the biphase thermal denaturation of this fragment. The characteristic values for the experimental and theoretical results are compared in Table I. An analysis of the areas in this figure reveals that approximately 38 ± 10 bp melt in the early transition. This transition originates at the right end of the molecule as seen in Fig. 1. The number of base pairs ascribed to this early transition is nec-
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essarily rather inaccurate because the 2 transitions observed for this fragment are very close to each other. However, this estimate is in good agreement with the value obtained from the melting behavior of the previously studied 203-bp fragment where approximately 65 bp melt in the early transition. The difference in size of the early transition between these 2 molecules may be explained by the fact that in addition to cleaving 9 bp from the z-gene end, the 188-bp fragment lacks 3 GC pairs at the repressor end. The next 7 bp after the sticky end produced by Hinfl cleavage consist then of 1 GC bp and 6 AT bp in a row, ahead of a rather GC-rich block. These few base pairs might melt out in a broad early transition that is below the resolution of our data.

The melting profiles of the 219-bp fragment (solid lines) together with the results of the calculated thermal denaturation curves are shown in Fig. 4. These profiles clearly consist of two different transitions that are very well separated at 0.01 M Na⁺. At 0.105 and 0.505 M Na⁺, the two transitions move closer together. This observation is in contrast to the results obtained for the 188-bp fragment (see above and Fig. 3). A similar behavior for the same sequence has been observed in the thermal denaturation of the 301-bp fragment (12).

The shape of the 219-bp main transition shows the same features described above for the other fragments. It differs somewhat in the dependence of the broadening on the ionic strength. At 0.105 M Na⁺, the main transition is broadened as compared to the transition at 0.01 M Na⁺. Between 0.105 and 0.505 M Na⁺, the transition undergoes a further broadening that is not found in the other fragments. This observation resembles the dependence of transition width in the 301-bp

Fig. 2. Experimental and theoretical melting curve of the 80-, 95-, and 101-bp fragments. The 80- (A), 95- (B), and 101-bp (C) fragments denature in a single transition. The horizontal axis represents the temperature relative to the \( T_m \) values. \( T_m \) is the temperature of \( d\theta/dT \). The melting curves for 3 different ionic strengths are offset on the vertical axis. The lower curves are taken at 0.01 M Na⁺, the middle curves represent the 0.105 M Na⁺ melts, and the upper curves result from experiments at 0.505 M Na⁺. The dashed lines represent the results of the theory. The parameters of the melting curves in comparison to those obtained from the theory are listed in Table I.
Table I

| Fragment No. bp | Experiment (Tm°C) | Theory (Tm°C) | Experiment (ΔT°C) | Theory (ΔT°C) | Experiment (dn/dTm)° | Theory (dn/dTm)° |
|----------------|------------------|--------------|------------------|--------------|---------------------|------------------|
| 80             | 60.9             | 1.16         | 0.76             |              |                     |                  |
| 95             | 63.4             | 0.83         | 1.08             |              |                     |                  |
| 101            | 71.7             | 0.78         | 1.15             |              |                     |                  |
| 188            | 69.0             | 0.62         | 1.17             |              |                     |                  |
| 219            | 71.9             | 0.62         | 1.00             |              |                     |                  |
| 80             | 77.7             | 78.5         | 1.70             | 1.60         | 0.50                | 0.48             |
| 95             | 79.3             | 80.2         | 1.60             | 1.65         | 0.52                | 0.43             |
| 101            | 86.7             | 87.3         | 1.26             | 0.96         | 0.66                | 0.78             |
| 188            | 84.4             | 83.5         | 1.94             | 0.85         | 0.72                | 0.74             |
| 219            | 85.7             | 86.1         | 0.72             | 0.82         | 0.82                | 0.78             |
| 80             | 87.0             | 87.5         | 1.80             | 1.90         | 0.47                | 0.40             |
| 95             | 89.3             | 89.4         | 1.66             | 1.90         | 0.47                | 0.37             |
| 101            | 94.8             | 95.7         | 1.25             | 1.29         | 0.68                | 0.58             |
| 188            | 93.0             | 92.5         | 1.16             | 1.15         | 0.63                | 0.62             |
| 219            | 96.2             | 96.8         | 1.15             | 0.98         | 0.57                | 0.68             |

* Tm values are reproducible within ±0.5°C.
† ΔT values are reproducible within ±0.04°C.
‡ dn/dTm values are reproducible within ±0.03. Their value depends on the initial absorbance of the sample. In these experiments, the initial absorbance is usually read about 5°C below the first transition in the experiment. It is generally observed that in these small DNA fragments about 2 to 3% hyperchromicity occurs between 55°C and this temperature. This early hyperchromicity is attributed to fraying from the ends of the fragments. The overall transition shape should not be greatly affected by this phenomenon; however, the dn/dTm values could very well depend on the total hyperchromicity. This may account for some of the deviations between theory and experiment. Theoretical calculations produce values of 0.97 to 0.87 for θw at 5°C below the transition. Experimentally we assume θw = 1.0.

Fragment (12). It seems that a higher ionic strength is necessary to broaden the transitions to their final width for these 2 fragments.

Table I compares the experimental melting temperatures and the transition widths with the theoretical values. It should again be noted that very good agreement is reached between theory and experiment in this case also. In determining the number of base pairs melting in the early transition, only the melts at 0.01 and 0.105 M Na+ were used because the difference in the Tm values in the melt at 0.505 M Na+ is not large enough to allow an accurate distinction between the 2 transitions. The arrows in panel B of Fig. 1 indicate the location of the boundary between the early and late melting regions as determined from the data displayed in Fig. 4 after correcting for the larger contribution of AT base pairs to the hyperchromicity at 260 nm. The arrows in panel A of Fig. 1 result from melts done at 282 nm where the contribution of AT is negligible. The arrow in panel C of Fig. 1 indicates the result of the melting data on the 301-bp fragment. Taken together, these results very clearly confirm the existence of a thermal stability boundary next to the CAP binding site, which disconnects the stability of the regulatory sequence from the repressor gene sequence. The number of base pairs melting in the early transition of the 219-bp fragment is determined to be 65 ± 6 bp. Theoretically determined melting profiles of the 203-, 188-, and 219-bp fragments further support the above interpretation with regard to both the size and physical location of the early melting regions (see below).

Theoretical Analysis of the Melting Curves—Figs. 2, 3, and 4 show the comparison between theoretical and experimental transitions in 0.105 and 0.505 M Na+ for the 5 DNA fragments. The predicted shapes of the differential melting curves are generally within experimental error of the observed transitions. Table I summarizes a comparison of predicted and experimental parameters. Comparison of theory and experiment in 0.01 M Na+ did not give as good agreement as the higher Na+ concentrations and is not listed in Table I. Some of the results relevant to the low salt data are given below.
The overall agreement in 0.105 and 0.505 M Na⁺ substantiates an earlier calculation on the 95-, 144-, 203- and 301-bp lac fragments (13).

Fig. 5 shows the total fraction of hydrogen-bonded base pairs, $\theta_{\text{tot}}$, for the 95-bp fragment. $\theta_{\text{tot}}$ consists of the product of $\theta_{\text{un}}$, the fraction of bonded base pairs among duplex strands and $\theta_{\text{en}}$, the fraction of strands associated as a duplex. This analysis illustrates the dominance of the duplex to single strands dissociation (an “all-or-none” behavior) on the melting of the DNA fragments. The equilibrium constant for the dissociation of duplex to single strands is given by $1/\beta$, where $\beta$ is the ratio of partition functions for the external degrees of freedom of duplex and single strands. The simplest form of $\beta$ which provides a good fit to experiment is $\beta = kN^a$ where $a = a + b(1 - \theta_{\text{un}})$, $N$ is the number of base pairs, and $a$, $b$, and $b$ were $5 \times 10^3$, $-2.8$, and $-3.2$ for 0.105 M Na⁺ and $3 \times 10^3$, $-3.0$, and $-1.9$ in 0.505 M Na⁺. Analysis of the calculated melting curves of the 188- and 219-bp fragments corroborates the area analysis made earlier from the experimental curves.

![Fig. 5. Plots of $\theta_{\text{un}}, \theta_{\text{en}}$, and $\theta_{\text{tot}}$ versus temperature. $\theta_{\text{un}}$ (A), the total fraction of hydrogen-bonded base pairs, $\theta_{\text{en}}$ (B), the fraction of strands associated as a duplex with 1 or more intact base pairs, and $\theta_{\text{tot}}$ (C), the fraction of hydrogen-bonded base pairs among duplex strands are plotted versus temperature for the 95-bp fragment in 0.105 M Na⁺. The dominance of $\theta_{\text{en}}$ at $T_M$ (80.2°C) indicates the influence of duplex to single strand dissociation on melting.

Fig. 6 shows base pair opening profiles of the 188-bp fragment in 0.105 M Na⁺. The regions which have melted out by 82°C (the early melting subtransition) correspond to 14 bp from the i-gene side and 42 bp from the z-gene side. At higher temperature, these melting profiles become less meaningful because strand dissociation begins to dominate the melting process. A similar set of profiles of the 219-bp fragment shows that its low temperature subtransition in 0.105 M Na⁺ corresponds to 58 bp of the promoter region.

The theoretical analysis of the thermal denaturation of the 5 lac fragments predicts the observed decrease in main transition widths from high to medium salt concentration. Evaluation of the equilibrium constant for dissociation, $1/\beta$, at the peaks of the main transitions shows that this constant increases from 0.505 M Na⁺ to 105 M Na⁺. The sharpening of the transitions between 0.105 and 0.15 M Na⁺ can be also predicted by further increasing the strand dissociation constant approximately 500-fold for the lower salt concentration. Employing experimental estimates of $T_M$, $T_G$, and $T_S$ (see “Materials and Methods”), one can reproduce the sharpening of the transitions for all of the DNA fragments at 0.01 M Na⁺. However, $T_M$ values and peak widths are not within experimental error for several of the fragments.

**DISCUSSION**

The existence of a thermodynamic boundary located approximately 20 bp upstream of the CAP binding site was demonstrated previously with studies on the 301-bp fragment (12). It was theorized that CAP binding in this region could aid transcription by destabilizing the DNA (reviewed in Ref. 1). The 188- and 219-bp fragments derived from the 301-bp fragment contain the sequence forming this boundary. One of the goals of this study was to examine more closely the stability of the sequences on either side of that boundary. The presence of the boundary in the 219-bp fragment is demonstrated by the arrows in panels A and B in Fig. 1. Because the difference in GC content of the regions melting in two transitions is rather large (62% and 43%), the melting experiment at 282 nm was conducted. The good agreement of the 260- and 282-nm data and the confirmation of their area analysis by the results obtained from the theoretical evaluation substantiate the existence of this boundary.

Neither the 188- nor the 203-bp fragments contain this thermodynamic boundary despite the fact that its sequence is present in these fragments. The apparent reason for this observation is that both fragments lack approximately 100 bp of the GC-rich region on the leftward end of the 301-bp sequence (as shown in Fig. 1). The approximately 60 bp regions are 58% GC-rich, but do not contain enough base pairs to form a single cooperative unit. The gain in free energy derived from strand dissociation and coil formation for this sequence is, at the melting temperature of the early melting region, obviously larger than the contribution from keeping these 60 bp stacked and hydrogen bonded.

A second thermodynamic boundary revealed in the 301-bp fragment melting study (12) is located at the z-gene end of the promoter sequence. Because the 188- and 203-bp fragments
extend to the z-gene end of the 301-bp sequence, this boundary is expressed in their melting curves (Fig. 3 and Ref. 12). The area analysis for the 188-bp fragment is in agreement with the melting profile of this molecule obtained by theoretical analysis of the sequence. Due to the overlapping sequence of the 188- and 203-bp fragments, this result further confirms the interpretation of the 203-bp denaturation experiment reported earlier (12).

A major difference was found in the salt dependence of the melting curves of the 188- and 219-bp fragments. In going from the low to high ionic strength, the early melting transition of the 188-bp fragment moves away from the main transition. The opposite behavior was found for the 219-bp melt. At low salt, the 2 transitions were well separated and increasing ionic strength resulted in a decrease of the difference in $T_M$ between the 2 transitions. These observations agree with the results from the theoretical analyses of the sequences and can be explained by the different importance of 2 counteracting influences on these melting processes. If the difference in GC content between the sequences melting under the 2 transitions is large, the thermal denaturation process is dominated by the difference ($T_{GC} - T_{XT}$). This is the case for the 219-bp fragment with a sequence of 43% GC melting in the early transition, whereas the sequence giving rise to the main transition contains 62% GC. ($T_{GC} - T_{XT}$) decreases with increasing ionic strength, and hence, the difference in $T_M$ between the early and main transitions decreases also. On the other hand, if the difference in percent GC content of the sequences giving rise to the 2 transitions is small, as in the 188-bp fragment with 43% and 51%, the melting behavior is dominated by duplex to single strand dissociation. At low salt, the DNA double helix stiffens and the cooperative length increases. This tends to incorporate the early transition into the main transition at low ionic strength, which matches exactly the result found for the 188-bp melt (Fig. 3).

For the most part, the single transition melts of the 80-, 95-, and 101-bp fragments are predicted by the theory within the error limits of the experiment (Table I). The average melting process may be pictured as unwinding of base paired regions from the ends of the molecules (“fraying”) in the early stages of the transition, followed by a highly cooperative dissociation of partially duplex DNA to single strands. Previous studies indicated that the influence of dissociation on thermal transition curves increases with decreasing DNA length (25). This behavior is verified by the dependence of the theoretically calculated melting curves on the equilibrium constant for dissociation of duplex DNA to single strands, $1/\beta$. Predicted curves for the 80-, 95-, and 101-bp fragments are more sensitive to changes in $\beta$ than longer DNAs. In these 3 smallest fragments strand dissociation greatly dominates any partial melting of the molecules from the ends. The result of this is a single transition melting profile.

The general sharpening of the melting transitions between high and low ionic strength can be explained by the salt-dependent increase in the equilibrium dissociation constant, $1/\beta$. Theoretical analysis indicated that $1/\beta$ increases with decreasing ionic strength. Previous workers have shown that the strands to duplex association rate is highly dependent on salt concentration (26). It is not surprising that the equilibrium constant of this process should also show this behavior.

The lack of agreement between theory and experiment in 0.01 M Na+ may be due to several reasons. Electrostatic forces become more significant with decreasing ionic strength. These interactions have not been explicitly considered in the theory. The deviations of the theoretical predictions from the experiment for this low ionic strength may also be due to an experimental limitation that could become very important at 0.01 M Na+. Due to the increased charge repulsion of the phosphates at low ionic strength, the activation energy for the rennealing of the single strands may be too large to allow a sufficient rate of this reaction. As a result, the melting process would not occur at or near equilibrium and the theory would not be applicable. Effects such as this have been reported for DNA solutions in low ionic strength (2). The asymmetry of the melting transitions, which is larger at low salt as compared to high salt, could also be a result of this phenomenon.

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