Thermal denaturation of DNA from bromodeoxyuridine substituted cells.

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ABSTRACT

The thermal denaturation of DNA from cell lines extensively substituted with bromodeoxyuridine has been examined spectrophotometrically over a wide range in ionic strength and by thermal elution from hydroxyapatite columns. BrdU substitution stabilizes DNA at all ionic strengths between 7.5 mM and 1350 mM potassium ion concentration, although a plot of log ionic strength vs Tm deviates from linearity above 150 mM. This nonlinearity is most pronounced with BrdU-substituted DNAs, resulting in a lowered ΔTm between unsubstituted and substituted DNA with increasing ionic strength. DMSO is shown to decrease the Tm of both unsubstituted and BrdU-substituted DNA equally, at a rate of 0.5°C per 1% DMSO.

INTRODUCTION

5-Bromodeoxyuridine (BrdU) can be incorporated in place of thymidine into the DNA of mammalian cells grown in culture, generally resulting in several disruptive effects on cell growth and metabolism. For example, BrdU has been shown to act as a mutagen (1), to increase the binding affinity of chromosomal proteins to substituted DNA (2), and to generally inhibit the expression of differentiated cell functions (3). As an analog of thymidine, it is thought that many of the disruptive effects of BrdU are directly related to its incorporation into DNA. Cells which are unaffected by growth in the presence of BrdU are generally found to lack cytoplasmic thymidine kinase (4), and are therefore unable to supply the analog as a precursor for DNA synthesis.

We have reported on the isolation of two cell lines which survive with high levels of BrdU substitution for thymidine in nuclear DNA (5,6). One line, called B4, is BrdU-dependent and contains DNA which is ~60% substituted...
with BrdU. A second cell line, called HAB, is 99.8% substituted with BrdU.
Both cell lines have been maintained in continuous cultivation for over 300
generations without loss in viability. In light of this unusual property
it is of interest to characterize these cells in comparison to the disruptive
effects of BrdU established in other systems.

This report deals with the thermal stability of DNA extensively substi-
tuted with BrdU. It has previously been observed that such substitution
increases the thermal stability of DNA (7,8,9). However, no comprehensive
studies of melting behavior have been carried out with extensively substituted
DNA, nor over a wide range of ionic strengths. This latter point is particu-
larly relevant in light of the findings of Inman and Baldwin (10) that
while the synthetic copolymer poly dABU·dABU is approximately 9°C more stable
than poly dAT·dAT in 10 mM salt, this difference in Tm decreases with increas-
ing salt concentrations and disappears at concentrations greater than 500 mM.
It is thus conceivable that the observed increase in thermal stability of a
naturally occurring DNA substituted with BrdU might be substantially reduced
under physiological ionic conditions, causing no particularly disruptive
effects on chromatin structure or regulatory processes.

We have therefore examined the melting behavior of B4 and HAB cell DNA
in comparison to DNA from the unsubstituted parent cell line, both spectro-
photometrically between 5 mM and 900 mM potassium phosphate as well as by
thermal elution from hydroxyapatite columns in 80 mM potassium phosphate.
Further, the effect of dimethylsulfoxide (DMSO), a hydrophobic solvent known
to decrease the thermal stability of DNA (11), has been examined using both
unsubstituted and extensively substituted DNA. The thermal melting behavior
of the synthetic alternating copolymers poly dAT·dAT and dABU·dABU has also
been examined spectrophotometrically for comparison to the naturally occurring substituted DNAs.
MATERIALS AND METHODS

The cell lines and culture conditions used in this study have previously been described (5,6). Briefly, 3460 cells are derived from a Syrian hamster melanoma; the nuclear DNA is unsubstituted with BrdU and has a G + C content of 45%. B4 cells are derived from 3460 and the nuclear DNA has 62% of the thymidine residues replaced by BrdU. HAB cells are derived from B4 and the nuclear DNA is 99.8% substituted with BrdU. Methods of DNA isolation and the determination of base composition and levels of BrdU substitution have been described (5,6). Radioactively labeled DNA was obtained by growing the cells in the presence of carrier-free $^{32}$P (New England Nuclear) at 2 μc/ml for 72 hr prior to isolation of DNA. Unlabeled DNA was used throughout for the spectrophotometric melts and all samples had a double-strand molecular weight of $>25 \times 10^6$ daltons. $^{32}$P-labeled DNA was used for thermal elution on hydroxyapatite and was sheared prior to use to assure homogeneity in molecular weight between the DNA samples. DNA was sheared by six passages through a 27.5 gauge needle under maximum thumb pressure in 0.5 M NaCl. Double- and single-strand molecular weights for all DNA samples were determined to be approximately $7.5 \times 10^6$ daltons and $3.7 \times 10^6$ daltons, respectively. Molecular weights were determined in a Beckman Model E Ultracentrifuge according to Studier (12) [kindly performed by Dan Luk].

The synthetic alternating copolymers, poly dAT·dAT and poly dABU·dABU, were purchased from Grand Island Biologicals. DNA grade hydroxyapatite was purchased from Biorad. Prior to use it was boiled for 10 min in 10 mM phosphate buffer, pH 6.8, and fines removed by repeated decantation. The hydroxyapatite was stored in 1 mM phosphate buffer with a small amount of chloroform. Phosphate buffer (PB) was prepared as an equimolar mixture of mono- and dibasic potassium phosphate.

Thermal Denaturation. The absorbance of the DNA samples in potassium phosphate buffer was determined using a Gilford 2400 recording spectrophoto-
The temperature of the cuvette chamber was controlled by the circulation of ethylene glycol from a thermostated Haake bath through the cuvette housing and the temperature was raised at a constant rate of 0.5°C per min through a motorized drive attached to the Haake thermostat control. The spectrophotometer provides a direct read-out of the temperature within the cuvette chamber which was further calibrated by insertion of a thermistor into a cuvette containing only buffer, thus allowing for a correction of the Tm. 3 cm quartz cuvettes with a 1 cm light path were used throughout. Sample volume was 0.5 ml and the solution was overlaid with silicone oil to prevent evaporation. The cuvettes were degassed under vacuum for a minimum of 2 hr prior to beginning the melt. Solvent evaporation during all stages of the melt was determined to be less than 0.5%.

A jacketed column was used for all hydroxyapatite thermal elutions. The temperature was controlled by the circulation of water from a Haake thermostatically controlled water bath and measured by a calibrated thermometer immersed in the column buffer. The temperature could be read to 0.1°C and was raised in approximately 3°C increments. Column operation is essentially as described by Miyazawa and Thomas (13). The column bed consisted of 0.5 ml of hydroxyapatite (packed volume). To load the column, the sheared, labeled DNA was added at 60°C in 5 ml of 80 mM PB. After three washes with 80 mM PB at 60°C the temperature elution commenced. Greater than 95% of the DNA, as assayed by radioactivity, bound to the column under these conditions. For thermal elution, 5 ml aliquots of 80 mM PB were added and the temperature increased by about 3°C. Temperature equilibration took up to 5 min and after 10 min the solution was drained off and replaced by another 5 ml of buffer which was held for a further 10 min at the same temperature and then drained. This procedure removed approximately 90% of the radioactivity that could be eluted at that temperature. This was repeated up to the final temperature point of approximately 94°C, at which stage less than 3% of the DNA remained
on the column. Each 5 ml sample was precipitated by the addition of 1 ml of 50% TCA and 100 μl salmon sperm DNA (1 mg/ml) as carrier. Samples were collected on Whatman GF/A filters, washed with 5% TCA followed by ethanol, dried, and counted in LSC (Yorktown).

RESULTS

Optical Melting. Characteristic melting profiles determined spectrophotometrically for 3460, B4 and HAB cell DNA are shown in Fig. 1. Similar profiles are given for the synthetic copolymers, poly dAT-dAT and poly dABU-dABU. The salt concentration used for these melts ranges from 5 mM to 900 mM phosphate buffer (i.e., 7.5 mM to 1350 mM in potassium ion concentration). It is evident that at all salt concentrations examined the order of thermal stability is HAB>B4>3460 DNA. Similarly, poly dABU-dABU is consistently more stable than poly dAT-dAT.

The corrected Tm's of the cellular DNAs and synthetic copolymers at the potassium concentrations tested are given in Table I. These values are plotted against the log of the potassium concentration in Fig. 2. This relationship is a linear one for the natural DNAs up to 150 mM potassium after which an increasing deviation from linearity may be observed. This is particularly pronounced with HAB DNA. The effect of this deviation is that the ΔTm between the DNA samples decreases at higher ionic strengths. Thus, at 7.5 mM potassium the ΔTm between 3460 and HAB DNAs is 6.9°C, whereas this difference decreases to 3.8°C at 1350 mM potassium. At 200 mM PB, which is close to physiological ionic conditions, the ΔTm is 4.9°C. A similar, though lower magnitude, deviation is seen with B4 DNA which always falls intermediate to the unsubstituted and fully substituted DNAs.

A similar deviation is observed with the synthetic copolymers. The ΔTm for poly dAT-dAT and poly dABU-dABU decreases from 8.9°C at 15 mM potassium to 5.8°C at 1350 mM potassium. It is interesting to note that it is the BrdU substituted DNAs, both synthetic and naturally occurring, that deviate most...
Fig. 1. Thermal denaturation of BrdU-substituted DNA.
Melting profiles were determined spectrophotometrically in phosphate buffer: 900 mM (A,N), 500 mM (B,L), 200 mM (C,J), 100 mM (D,K), 50 mM (E,L), 10 mM (F,N), and 5 mM (G,N). The naturally occurring DNA from 3460 (•), B4 (○), and HAB cells (△) are in panels A-G, whereas poly dAT·dAT (■) and poly dABU·dABU (□) are in panels H-N.
TABLE I

Tm (°C)*

<table>
<thead>
<tr>
<th>Potassium Ion Concentration</th>
<th>HAB DNA</th>
<th>B4 DNA</th>
<th>3460 DNA</th>
<th>Poly dAT-dAT</th>
<th>Poly dABU-dABU</th>
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<tbody>
<tr>
<td>1350 mM</td>
<td>97.9</td>
<td>96.3</td>
<td>94.1</td>
<td>77.5</td>
<td>83.3</td>
</tr>
<tr>
<td>750 mM</td>
<td>96.8</td>
<td>94.7</td>
<td>92.2</td>
<td>73.6</td>
<td>80.2</td>
</tr>
<tr>
<td>300 mM</td>
<td>92.7</td>
<td>90.1</td>
<td>87.8</td>
<td>67.8</td>
<td>75.6</td>
</tr>
<tr>
<td>150 mM</td>
<td>89.6</td>
<td>86.7</td>
<td>83.9</td>
<td>63.4</td>
<td>71.3</td>
</tr>
<tr>
<td>75 mM</td>
<td>86.0</td>
<td>81.9</td>
<td>79.2</td>
<td>57.8</td>
<td>66.8</td>
</tr>
<tr>
<td>15 mM</td>
<td>75.2</td>
<td>71.8</td>
<td>68.2</td>
<td>47.2</td>
<td>56.1</td>
</tr>
<tr>
<td>7.5 mM</td>
<td>70.3</td>
<td>67.1</td>
<td>63.4</td>
<td>42.1</td>
<td>53.1</td>
</tr>
</tbody>
</table>

*Ts's are taken from Fig. 1 and corrected as described in Methods.

TABLE II

Hyperchromicity Range (°C)*

<table>
<thead>
<tr>
<th>Potassium Ion Concentration</th>
<th>HAB DNA</th>
<th>B4 DNA</th>
<th>3460 DNA</th>
<th>Poly dAT-dAT</th>
<th>Poly dABU-dABU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1350 mM</td>
<td>4.7</td>
<td>5.2</td>
<td>5.8</td>
<td>7.8</td>
<td>6.5</td>
</tr>
<tr>
<td>750 mM</td>
<td>4.9</td>
<td>5.8</td>
<td>6.3</td>
<td>7.8</td>
<td>6.0</td>
</tr>
<tr>
<td>300 mM</td>
<td>5.1</td>
<td>6.9</td>
<td>7.5</td>
<td>5.9</td>
<td>4.9</td>
</tr>
<tr>
<td>150 mM</td>
<td>5.9</td>
<td>7.4</td>
<td>7.6</td>
<td>5.5</td>
<td>4.5</td>
</tr>
<tr>
<td>75 mM</td>
<td>6.5</td>
<td>7.6</td>
<td>8.3</td>
<td>4.8</td>
<td>3.6</td>
</tr>
<tr>
<td>15 mM</td>
<td>6.7</td>
<td>8.0</td>
<td>8.2</td>
<td>3.9</td>
<td>2.6</td>
</tr>
<tr>
<td>7.5 mM</td>
<td>7.0</td>
<td>8.2</td>
<td>9.4</td>
<td>3.4</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*Range over which 80% of the hyperchromic shift occurs (10-90%) from linearity, causing a convergence of the curves for unsubstituted and substituted DNAs at higher ionic strengths.

The hyperchromic shift for the natural DNAs was approximately 31% with no significant difference between the 3 samples. The range over which 80% of the hyperchromic shift takes place for all DNA samples is given in Table
Fig. 2. Dependence of Tm on potassium ion concentration for 3460 (■), B4 (○), HAB (▲) cell DNA, and for poly dAT·dAT (●) and poly dABU·dABU (▲).

Fig. 3. Reduction in Tm by DMSO for 3460 (■), B4 (○) and HAB cell (▲) DNA, determined spectrophotometrically in 100 mM PB.
II. HAB DNA shows a sharper melting profile than 3460 DNA at all ionic strengths examined and B4 DNA is intermediate in all cases. As the ionic strength increases from 7.5 mM to 1350 mM potassium, the hyperchromicity range decreases by 32.8%, 36.1%, and 38.3% for HAB, B4, and 3460 DNAs, respectively. This is in sharp contrast to the synthetic copolymers which show a widening of the melting curve with increased potassium concentrations. Thus, on increasing the potassium concentration from 7.5 mM to 1350 mM, there is a 129% increase in the width of the melting curve for poly dAT-dAT and a 170% increase for poly dABU-dABU.

DMSO. Since BrdU substitution in DNA acts antagonistically upon DMSO induced differentiation in erythrocytes (14) and DMSO acts to decrease the Tm of DNA (11), it was of interest to compare the effect of DMSO on the melting behavior of unsubstituted and BrdU-substituted DNA. DMSO was added from 1-20% in 100 mM PB and the Tm's determined spectrophotometrically for 3460, B4 and HAB DNAs. These results are shown in Fig. 3 where it can be seen that DMSO decreases the thermal stability of all 3 DNA samples in a linear fashion at the rate of 0.5°C per percent DMSO.

Thermal Chromatography. Typical thermal elution profiles for 3460, B4, and HAB cell DNA on a hydroxyapatite column in 80 mM PB are shown in Fig. 4. BrdU substitution exerted a modest stabilizing effect on the thermal chromatography of the DNA samples, resulting in average Tm's of 80.45°C for 3460 DNA, 81.55°C for B4 DNA, and 82.30°C for HAB DNA.

These values are all lower than the Tm's anticipated at 80 mM salt from inspection of Fig. 2, which shows the melting behavior of the DNA samples in solution. This effect is more pronounced the higher the level of BrdU substitution. The Tm of 3460 DNA is approximately 2.0°C lower on hydroxyapatite column thermal elution than in solution, whereas the Tm's of B4 and HAB DNAs are lowered by 3.4°C and 6.3°C, respectively.
DISCUSSION

It has frequently been observed that BrdU substitution in DNA results in increased thermal stability. However, previous work has generally been performed on DNA with low or undetermined levels of BrdU substitution or only at low ionic strength. The B4 and HAB cell lines used in this study are unique in that extensive substitution by BrdU in DNA has not affected long-term cell viability. We have therefore characterized the melting behavior of the DNA from these cells over a range of ionic strengths so as to determine whether the increased thermal stability of DNA extensively substituted with BrdU is found to occur at ionic strengths close to physiological conditions. This point appeared particularly relevant in the light of the finding of Inman and Baldwin (10) that the ΔTm between poly dAT·dAT and poly dABU·dABU was substantially reduced on raising the ionic strength to 200 mM and above.

Our results show that BrdU substitution at 62% and 100% substantially increases the thermal stability of DNA in solution. It is notable, however, that this increased thermal stability declines relative to control DNA at
Ionic strengths greater than 150 mM in potassium concomitant with a deviation from linearity of the plot log ionic strength against Tm (Fig. 2). This deviation has been observed with other DNAs examined (15,16) and is generally attributed to denaturation of DNA by anions which, at high concentrations, may act as hydrophobic bond-breaking agents (16). It appears that increasing BrdU substitution accents this effect thus leading to convergence of the plots and a reduction in the observed ΔTms at ionic strengths above 150 mM. Nevertheless, the ΔTm between HAB and 3460 cell DNA at 300 mM potassium remains a substantial 4.9°C.

A similar convergence may be observed with the synthetic copolymers poly dAT·dAT and poly dABU·dABU, in partial agreement with the findings of Inman and Baldwin (10). We, however, observe a substantially less profound effect so that whereas Inman and Baldwin observe no difference in Tms at ionic strengths above 500 mM, we observe a ΔTm of 5.8°C at 1350 mM. This decline in ΔTm is due to a divergence from linearity of the plot of log ionic strength vs. Tm for poly dABU·dABU at higher ionic strengths. The plot for poly dAT·dAT remained linear over the range of ionic strengths examined. The reason for this discrepancy between our results and those of Inman and Baldwin is not clear at present, but may be related to the molecular weight of the copolymers used. It is noteworthy, however, that the Tm's we have determined for the synthetic copolymers at low ionic strength are in agreement with those of Inman and Baldwin.

It is interesting to note that the increase in thermal stability of the B4 and HAB DNA is not strictly a function of the extent of BrdU substitution. That is, when comparing B4 DNA to the extremes of 0 and 100% substitution (3460 and HAB DNA), we would predict only 42% substitution in B4 DNA if the relationship was strictly linear, rather than the value of 62% determined by base composition. It will require a comparison of a number of DNA samples ranging from 0 to 100% substitution in order to elucidate the exact relation-
Using the relationships of Schildkraut and Lifson (17) to calculate G + C content from Tm, we calculate that 3460 DNA has a G + C content of 40%. This value agrees well with typical mammalian DNAs (18) but is in sharp contrast to the value of 45% G + C we have routinely determined by direct base analysis (6,19). This discrepancy is the subject of continued investigation.

In agreement with previous results (10,15), we observe that the sharpness of melt of the natural DNAs increased with increasing ionic strength, while that for the artificial copolymers decreased (Table II). The reason for this latter point is not understood, although two theories have been put forward to account for the narrowing of the melting range of natural DNA. Gruenwedel and Hsu (15) have suggested that this is due to a destabilization of the G-C base pairs relative to the A-T base pairs by concentrated salt media. On the other hand, Dove and Davidson (16) postulate that this is due to a change in cooperativity of the helix to coil transition with changing salt concentrations. It should be noted that the replacement of A-T pairs by A-BU pairs in the DNA examined here appears to have little effect on the degree to which the melting curve is sharpened with increasing ionic strength, although the substituted DNAs consistently show a sharper melting range as would be expected due to the increased thermal stability of A-BU relative to A-T base pairs.

We have also examined the effect of DMSO on the melting behavior of substituted and unsubstituted DNA in solution. DMSO has been shown to enhance the expression of at least one differentiated function and BrdU antagonizes this effect (14). It has been suggested that these compounds might be mutually antagonistic perturbants of the transcriptional regulatory process (8). DMSO has the effect of lowering the Tm of DNA samples in solution and we have determined that this is a linear relationship in 100 mM PB with a gradient of 0.5°C per percent DMSO, regardless of the level of BrdU substitution of
the DNA examined. This is in contrast to the findings of Lapeyre and Bekhor (8) who observed a greater effect of DMSO on BrdU substituted DNA. Thus, in their studies at 0.25 mM EDTA, 1% DMSO lowered the Tm of unsubstituted DNA by 0.5°C but that of 6.7% BrdU substituted DNA by 1.3°C. The reason for these different findings is not known, although it may be related to the different ionic conditions used.

An alternative method of studying the melting behavior of DNA involves the use of hydroxyapatite chromatography. This method relies on the property of hydroxyapatite to retain double-helical DNA more tightly than single chains so that sequential elution from the column of less thermally stable DNA will occur on elevation of the column temperature. Examination by thermal chromatography of the 3 DNAs under study revealed modest differences in the Tms under the conditions employed. These results appear to indicate that BrdU substitution does not stabilize DNA on hydroxyapatite columns to the same extent as in solution. It should be pointed out, however, that while the differences in Tm are significant, some variation was found on using different DNA preparations and particularly different hydroxyapatite preparations, making this a less suitable method to examine altered thermal stability.

REFERENCES