Increased Thermal Stability of Chromatin Containing 5-Bromodeoxyuridine-Substituted DNA

(pancreas/cytodifferentiation/gene expression/chromosome)

JOHN DAVID*, JOEL S. GORDON, AND WILLIAM J. RUTTER

Department of Biochemistry and Biophysics, University of California, San Francisco, School of Medicine, San Francisco, Calif. 94143

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ABSTRACT The replacement of thymidine by 5-bromodeoxyuridine (BrdU) inhibits cytodifferentiation in a number of systems. At concentrations that have a minimal effect on cell viability and proliferation, BrdU inhibits the synthesis and accumulation of cell-specific proteins in pancreatic rudiments, myoblasts, chondrocytes, and erythroblasts. BrdU probably exerts its effects only after it has been incorporated into DNA, although a non-DNA-linked effect has been reported (for review see refs. 1 and 2).

A plausible explanation for the effects exerted on cytodifferentiation is that the incorporation of BrdU into DNA affects the binding of certain regulatory proteins. This premise is supported by the demonstration by Lin and Riggs (3) that the lac repressor exhibits approximately 100-fold greater affinity for BrdU-containing DNA than for unsubstituted DNA. In addition, the rate of dissociation of the operator-repressor complex in the presence of a specific inducer (4) is markedly reduced if the operator DNA contains BrdU.

In this study we show that chromatin containing BrdU-substituted DNA isolated from HTC cells and developing rat pancreatic rudiments has altered properties. These changes are not manifest in the properties of BrdU-DNA. Thus, the altered properties of BrdU-chromatin must reflect changes in the affinities of the BrdU-DNA for other chromatin components, presumably proteins.

Abbreviations: HTC, hepatoma tissue culture; HAP, hydroxylapatite; $T_m$, temperature at which 50% maximal hypochromicity is reached.

* Present address: Division of Biological Science, University of Missouri, Columbia, Mo. 65201.

MATERIALS AND METHODS

All radioactive compounds were purchased from New England Nuclear, Inc., the hydroxylapatite (HAP) from Clarkson Chem. Inc., the BrdU from Calbiochem Inc., acrylamide and bis acrylamide from Eastman Kodak Co., and the sucrose (RNase free) from Schwarz/Mann.

Culture Conditions. Pancreas rudiments were dissected from 13-day-old Sprague–Dawley rat embryos and cultured in 7 times concentrated Eagle's Basal Medium as described by Rall et al. (5). Unless stated otherwise, the 13-day rudiments were grown for 48 hr in vitro and exposed to 10 $\mu$Ci/ml of [methyl-3H]thymidine or to [14C]BrdU (20 $\mu$g/ml, 6 $\mu$Ci/ml) during the last 24 hr of culture. Growth in a medium containing 20 $\mu$g/ml of BrdU resulted in about a 70% replacement of the thymidine in each newly synthesized strand of DNA.

The hepatoma tissue culture cells (HTC) (gift of Dr. Gordon Tomkins) were maintained in continuous logarithmic-phase growth with a generation time of 22 hr as detailed by Samuels and Tomkins (6), and were collected and washed as described by Baxter and Tomkins (7). Replacement of 80% of the thymidine in each newly synthesized DNA strand was achieved by growing the cells in a medium containing 15 $\mu$g/ml of BrdU.

Nuclei Preparation. (All operations were carried out at 0°.) Pancreas: The nuclei from pancreatic rudiments grown in different radioactive compounds were isolated separately. Embryonic pancreas rudiments (16–22) were placed in 0.3 ml of a solution of 0.25 M sucrose, 10 mM Tris·HCl, pH 7.6; 3 mM CaCl$_2$, 5 mM MgCl$_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), and 25 mM KCl; the rudiments were disrupted in a motorized Teflon-glass homogenizer, with a 2-ml capacity. The resulting homogenate was centrifuged for 1 min at 40 $\times$ g in a Sorvall centrifuge (HB4 rotor, 500 rpm). The supernatant was then centrifuged for 20 min at 1500 $\times$ g (HB4 rotor, 3000 rpm). The pellet, rich in nuclei, was suspended in 0.4 ml of the homogenizing buffer at pH 8.5. To prevent losses during subsequent handling, carrier nuclei, prepared from 6 g of adult pancreases by a modification of the procedure of Blobel and Potter (8) were added to the rudiments. A gradient composed of a 2 M sucrose upper phase and a 2.2 M sucrose lower phase was employed for the isolation of the carrier pancreas nuclei. All solutions contained 1 mM benzamidé·HCl (Eastman Kodak), a trypsin inhibitor. The sedimented carrier nuclei were suspended in a solution of 0.25 M sucrose, 10 mM Tris-
HCl, pH 8.5, 10 mM KCl, 5 mM MgCl₂, and 1 mM benzamidine·HCl. The carrier nuclei and the ³H- and ¹⁴C-labeled embryonic pancreas nuclei suspensions were combined and centrifuged at 1500 × g for 5 min (Sorvall HB4, 3000 rpm). The sedimented nuclei were suspended in 0.25 M sucrose, 10 mM KCl, 5 mM MgCl₂, 10 mM Tris·HCl, pH 7.1, and centrifuged as above. The nuclear pellet was then washed in 50 mM Tris·HCl, pH 7.0.

HTC Cells: The HTC cells grown in either [³H]thymidine or [¹⁴C]BrdU were separately suspended in 25 volumes of a hypotonic solution containing 10 mM Tris·HCl, pH 8.0, 1.0 mM MgCl₂, 10 mM KCl, and 0.5% of the non-ionic detergent NP 40 (Shell International Chem. Co.); they were then sedimented at 800 × g for 6 min in an International centrifuge. The cells were suspended in 25 volumes of the same solution, allowed to stand 15 min, and then gently homogenized with a Teflon-glass Potter-Elvehjem homogenizer. The nuclei were sedimented at 800 × g for 6 min and then sequentially washed with the following solutions: 25 volumes of 0.25 M sucrose, 3 mM CaCl₂, and 0.5% NP40 (1 time); 25 volumes in the same solution minus NP40 (2 times); and 10 volumes of 50 mM Tris·HCl, pH 8.0 (1 time).

Chromatin Preparation. The HTC and pancreatic nuclei preparations were separately suspended in 4 ml of 10 mM Tris·HCl, pH 8.0 and allowed to stand overnight at 4°C. The resulting gels were sonicated at 40 V for six 15-scc pulses in a Bio-Ouk 11 (Brouwill Scientific). Prior to combining the HTC chromatin samples containing BrdU-substituted and unsubstituted DNAs, aliquots were removed for analysis by hyperchromicity. The chromatin was then sheared in a French pressure cell at 12,000 lbs/inch² (8.3 × 10⁸ N/m²) (producing fragments of 8 to 12 × 10⁶ daltons). The insoluble material was removed by centrifugation at 10,500 × g for 10 min (Sorvall, HB4 rotor, 8000 rpm), prior to loading on the HAP columns.

DNA Preparation. For hyperchromic analysis: HTC DNA was separated from the chromatin proteins by centrifuging an aliquot of the HTC chromatin through a 3.0 M CsCl solution (9).

For thermal chromatography: Portions of the sonicated HTC or pancreas chromatin were extracted once with phenol, saturated with 50 mM Tris·HCl, pH 8.5, and twice with chloroform–octanol (10:1, v:v) (10).

Analysis of DNA and Chromatin. The hyperchromicity at 260 nm of a 2-ml sample of DNA or chromatin (0.3 absorbance units/ml in 0.01 M Tris·HCl or 0.012 M potassium phosphate, pH 8.0) was measured as a function of temperature in a Gilford recording spectrophotometer. The hyperchromicity profiles of the DNA and chromatin were independent of the buffers that were used.

The analysis of the DNA and chromatin by thermal chromatography on HAP was performed essentially as described by MeConaughy and McCarthy (11). The sheared DNA and chromatin solutions (20–35 A260 units), adjusted to 0.12 M potassium phosphate, pH 6.8, were added with stirring to a 1.0-ml HAP column prepared in a 6.0-ml disposable syringe and equilibrated with the same buffer. The columns were equilibrated for 5 min with 5.0 ml of 0.12 M potassium phosphate, pH 6.8, prior to elution at increasing 2.5°C temperature increments. After reaching 100°C, the column was eluted twice at 100°C with 5.0 ml of 8.0 M urea in 0.24 M potassium phosphate, pH 6.8, and 0.01 M ethylenediaminetetraacetate.
Fig. 3. The effect of BrdU incorporation on the thermal chromatography of DNA. BrdU-substituted DNA (C) was obtained from HTC cells incubated in [14C]BrdU (2.5 nCi/ml) during a second generation of growth in a medium containing 15 μg/ml of BrdU (see Materials and Methods). (A) Unsubstituted HTC cell DNA (C) was similarly labeled by incubating HTC cells in [methyl-3H]thymidine (10 nCi/ml), during the second generation of growth in BrdU-free medium. (B) Unsubstituted pancreas DNA, [3H]thymidine labeled (C), and [14C]BrdU-substituted pancreas DNA (C) were obtained as described in Materials and Methods. Twenty and 35 A260 units of pancreas and HTC DNA, respectively, were applied to the columns. The columns were eluted with 5 ml of 0.12 M KPO4 (pH 6.8) at every 2.5° increment in temperature from 60° to 100°. They were then eluted (UREA) with 8 M urea, 0.24 M KPO4 (pH 6.8), and 0.01 M ethylenediaminetetraacetate at 100°. The uneluted radioactivity (RESIDUAL) was determined after dissolving the HAP in 10% trichloroacetic acid. The derivative elution profiles, Δ% DNA/ΔT, for unsubstituted (A) and BrdU-substituted DNA (C) were calculated by dividing the percent DNA eluted at each elution step by the temperature increment for that step. 100 ± 10% of the DNA applied was recovered.

Subsequently, the HAP column was dissolved in 10% trichloroacetic acid and any residual DNA was collected on Whatman GF/C filters.

The percent BrdU substitution was calculated from the DNA density according to Flory and Vinograd (12). The DNA density was determined by equilibrium density centrifugation in a Spinco model E analytical ultracentrifuge with Micrococcus luteus DNA (ρ = 1.731 g/cm³) as a density marker.

Chromatin proteins were analyzed by gel electrophoresis (7.5% acrylamide) under denaturing conditions (1% sodium dodecyl sulfate) as described by Maizel (13).

RESULTS

Thermal Hyperchromic Analysis of Control and BrdU-Substituted DNA and Chromatin. Incorporation of BrdU into HTC cell DNA increases its thermal denaturation temperature. Similar results have been reported for DNA from other cells (14). Unsubstituted HTC DNA exhibited a melting temperature, Tm, of 67.5° (Fig. 1). BrdU-DNA isolated from HTC cells grown for 2 generations in 15 μg/ml of BrdU (80% substitution for thymidine in each newly synthesized DNA = 60% substitution overall, i.e., 50% of the molecules have BrdU in both strands, 50% have BrdU in a single strand) had a Tm of 71.5°. The denaturation curves (Fig. 1) of unsubstituted and BrdU-substituted HTC DNA are parallel, indicating that the stabilization by BrdU is uniformly observed throughout the DNA population. This is also evident from the first derivative denaturation curves (Fig. 1).

Chromatin isolated from HTC cells denatures at higher temperatures (Fig. 2) and over a broader range than does DNA isolated from the same source (Fig. 1). HTC chromatin isolated from cells grown in the presence of BrdU for 2 generations (as above) has a higher Tm (80.5°) than does chromatin isolated from cells grown in a BrdU-free medium (Tm 78.8°) (Fig. 2). However, the denaturation curves of BrdU-chromatin and unsubstituted chromatin are not parallel. The asymmetric stabilization of chromatin affected by BrdU is emphasized when the data is plotted as the first derivative (Fig. 2). A larger proportion of the BrdU-DNA-containing chromatin denatures at higher temperatures.

Thermal Chromatography of Control and BrdU-Substituted DNA and Chromatin. DNA and chromatin were isolated from HTC cells (80% substitution in newly synthesized strands = 60% overall) and 13-day-old rat pancreatic rudiments incubated in the presence of BrdU (70% substitution of newly synthesized strands = 35% substitution overall). The DNA and chromatin preparations were analyzed by thermal chromatography on hydroxyapatite columns as described in Materials and Methods. Differential labeling was used to facilitate accurate comparisons.

BrdU substitution exerted a modest effect on the thermal chromatography of the DNA preparations. The temperature at which 50% of the pancreatic or HTC BrdU-DNA was eluted was about 2–3° higher than that at which 50% of the unsubstituted pancreatic or HTC DNA, respectively, was eluted (Fig. 3). These differences in the thermal elution temp-

Fig. 4. The effect of BrdU incorporation on the thermal chromatography of chromatin. The chromatin that was the source of the DNA chromatographed in Fig. 3 was subjected to thermal chromatography under the same conditions. Forty A260 units of HTC chromatin (A) and pancreas chromatin (B) were applied to the HAP columns. The recovery of the [3H]-labeled unsubstituted DNA (C) was between 90 and 100% and that of the [14C]BrdU-substituted DNA (C) was between 75 and 90%. The derivative profiles of the unsubstituted (A) and BrdU-substituted samples (C) were determined as described in Fig. 3.
Properties of Chromatin with Bromodeoxyuridine-DNA

![Graph](https://via.placeholder.com/150)

**Fig. 5.** Effect of BrdU replacement on the delayed elution of BrdU-DNA from chromatin on HAP. Chromatin was prepared from pancreas rudiments that had been incubated as in Fig. 4 in media containing [3H]thymidine (○) and [3H]BrdU at concentrations of 0.3 µg/ml (△), 1.0 µg/ml (□), and 20 µg/ml (▲). The percent BrdU substitution of a newly synthesized DNA strand at BrdU concentrations of 0.3, 1.0, and 5.0 µg/ml are given in Table 1. There was a 70% replacement of thymidine by BrdU in each newly synthesized DNA strand of pancreas rudiments grown in 20 µg/ml of BrdU. The overall degree of substitution was approximately half the values of each newly synthesized strand.

The elution chromatography of chromatin containing unsubstituted and BrdU-substituted DNAs, as measured by hyperchromicity.

The thermal elution chromatography of chromatin containing unsubstituted and BrdU-substituted DNA is presented in Fig. 4. While most of the unsubstituted DNA was eluted before 100°, 100° and 8 M urea were required for elution of most of the BrdU-DNA. The recovery of the remaining BrdU-DNA required dissolution of the hydroxyapatite. The elution profiles of DNA from chromatin preparations varied somewhat; thus, we do not know whether the small differences observed in the thermal chromatographic profiles of HTC and pancreas DNA are significant.

Due to the loss of significant amounts of DNA during purification, it was necessary to determine if the differences in the behavior of BrdU-DNA and chromatin containing BrdU-DNA were due to selective losses of BrdU-rich sequences during extraction and isolation of the DNA. We, therefore, compared the pyrimidine ratios of HTC DNAs before and after purification. The ratio of [3H]thymidine to [3H]deoxyadenosine of a chromatin sample containing unsubstituted DNA was 3.6 ± 0.1 (mean ± average error). The ratio for unsubstituted DNA purified from that sample was 3.8 ± 0.1. The [3H]BrdU to [3H]deoxyguanosine ratio of a chromatin sample containing BrdU-substituted DNA was 1.17 ± 0.07 and the extracted DNA had a ratio of 1.18 ± 0.02. Thus, there was no obvious selective loss of DNA during purification.

**Dependence of Thermal Chromatographic Properties on Extent of BrdU Substitution.** Pancreatic rudiments were grown for 24 hr in the presence of various concentrations of BrdU. The elution profiles of the DNA of the chromatin are dependent on the BrdU concentration in the cultures and, therefore, are dependent on the degree of substitution of the DNA (Fig. 5). The concentration range of BrdU that affects the thermal elution chromatography of chromatin is similar to that which inhibits the accumulation of pancreas-specific proteins during organ culture (Table 1).

**Lack of Effect of BrdU on Chromatin Proteins.** Chromatin fractions containing both unsubstituted and BrdU-substituted DNA were subjected to sodium dodecyl sulfate gel chromatography and subsequently visualized by Coomassie Blue staining. No significant differences in the distribution of proteins were observed. Minor differences would not be detected in these experiments. BrdU also has no effect on the rate of leucine/tryptophan incorporation into the proteins of nuclei from HTC cells.

**Discussion**

BrdU substitution into DNA causes only a slight change in the thermal elution chromatography of purified DNA, but elicits a dramatic change in the thermal elution of DNA from chromatin. These differences must reflect an altered interaction between BrdU-DNA and other chromosomal components, probably proteins. We were unable to detect any major BrdU-induced changes in the population of nuclear proteins. BrdU substitution more likely increases the affinity of the DNA for certain normal chromosomal proteins, just as it increases the affinity of the lac operator for the lac repressor (3, 4). This implies that the methyl group of thymidine influences the binding of certain chromosomal proteins.

In contrast to the large changes in the thermal elution of BrdU-DNA from chromatin on hydroxylapatite, there were relatively small changes in thermal denaturation of the DNA of chromatin as measured by hyperchromicity. This is understandable, since different properties are being measured by these techniques. Hyperchromicity measures the stability of base pairs in the DNA duplex within the chromatin matrix. In the thermal chromatography of chromatin, the crucial step in the release of single-stranded DNA is either the dissociation of the DNA strands, or the resolution of the DNA from the associated proteins. If the destabilization of DNA-DNA bonds is limiting in this process, a single strand would be released only after the most stable bonds were dissociated. This contrasts to hyperchromicity which measures the cumulative destabilization of base pairs as it occurs. Therefore, the apparent greater stability by thermal chromatography of BrdU-DNA.

**Table 1.** The effect of BrdU on the accumulation of alpha-amylose by pancreatic rudiments in vitro

<table>
<thead>
<tr>
<th>Conc. of BrdU in culture media (µg/ml)</th>
<th>% Replacement of DNA thymidine by BrdU</th>
<th>Relative alpha-amylose activity (µg of maltose/µg of protein per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.00*</td>
</tr>
<tr>
<td>0.3</td>
<td>8</td>
<td>0.23</td>
</tr>
<tr>
<td>1.0</td>
<td>18</td>
<td>0.12</td>
</tr>
<tr>
<td>5.0</td>
<td>45</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The pancreatic rudiments were cultured and assayed for alpha-amylose as described by Walther et al. (20).

* Twenty-five micrometers of maltose released/µg of protein per min.

**DISCUSSION**

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DNA in chromatin would reflect the existence of a number of well distributed, short, and relatively BrdU-rich sequences that are selectively stabilized by an interaction with chromatin proteins. If, on the other hand, the dissociation of DNA–protein bonds is limiting, then the greater stability of BrdU-DNA-containing chromatin on thermal chromatography would reflect the increased stability of BrdU-DNA–protein bonds. Results of recent experiments support the latter alternative (J. S. Gordon, G. Bell, B. J. McCarthy, and W. J. Rutter, unpublished observations).

The altered properties of BrdU-chromatin may explain the specific effects of BrdU on cytodifferentiation. For example, an increased affinity for BrdU-DNA of the repressors of the genes that code for cell-specific products would result in decreased transcription of those genes. The observation that incorporation of BrdU into a single DNA strand can completely inhibit cytodifferentiation (15–18) could be explained if the putative regulatory proteins bind to both strands of the DNA. Adler et al. (19) have presented evidence suggesting that the lac repressor binds to both of the DNA strands of the lac operator. Furthermore, BrdUrd incorporation into either strand of the DNA molecule increases the affinity for the lac repressor (S. Lin and A. D. Riggs, personal communication).

The increased affinity of chromatin proteins for BrdU-DNA may be of considerable value in recognizing and isolating specific regulatory proteins. Elucidation of the nature of the BrdU-DNA interactions with these chromosomal proteins may contribute to an understanding of the regulation of transcription during cytodifferentiation.

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