Characterization of Chromatin Extensively Substituted with 5-Bromodeoxyuridine

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ABSTRACT: To investigate the mechanism of inhibition of differentiation by 5-bromodeoxyuridine (brUdRib), we have performed a compositional and structural analysis of chromatin from HeLa cells grown for one generation in 5-fluorodeoxyuridine and brUdRib. Both classes of chromatin proteins, histones and nonhistones, are present in identical amounts in control and brUdRib-chromatin and are similarly distributed on polyacrylamide gel electrophoresis for the two chromatins. brUdRib leads to a decreased rate of maturation of intermediate (20–30 S) DNA but no accumulation of immature or brUdRib-enriched fragments occurs in long-term experiments. While the melting temperature of brUdRib-chromatin is 2–3° higher than the control, this difference appears to be related to an inherently greater stability of brUdRib-substituted DNA and not to any altered stabilization of nucleic acid structure by different proteins or by different interactions of the same proteins. Proteins are dissociated from brUdRib-chromatin and control chromatin at identical concentrations of sodium phosphate in 5 M urea, further indicating similar protein-nucleic acid interactions in the two species. The conformation of brUdRib-containing DNA in chromatin is strikingly different from control chromatin DNA as judged by circular dichroism spectra.

The thymidine analog 5-deoxybromouridine (brUdRib)[1] appears to have a selective effect in blocking differentiation in a variety of cell types. At appropriate concentrations, brUdRib is incorporated into DNA, partially replacing thymidine, without significant effect on viability or cellular growth rate. However, the presence of brUdRib does block differentiative processes, for example, production of melanin by melanoma or retinal epithelial pigment cells (Coleman *et al.*, 1970; Silagi and Bruce, 1970), myosin synthesis, and myotubule formation by myoblasts (Bischoff and Holtzer, 1970), and synthesis of chondroitin sulfate by chondrocytes (Mayne *et al.*, 1971). Additionally, in certain cells, brUdRib induces alkaline phosphatase (Koyama and Ono, 1971, 1972) or virus synthesis (Hampar *et al.*, 1971). The mechanism of this selective effect of brUdRib on differentiation *in vitro* is not known, although there appears to be a consensus that the effect is directly related to the substitution of brUdRib for thymidine in DNA, rather than involving a non-DNA-linked mechanism. Rutter and collaborators (1973), in a recent review of this phenomenon, examined a number of possible alternative explanations, concluding that altered interactions between chromatin proteins and brUdRib-substituted DNA offered the most likely explanation for the effects of the analog on differentiation. In an attempt to further understand the mechanism through which brUdRib selectively blocks differentiation, we have carried out compositional and structural analyses of HeLa cell chromatin which has been extensively substituted with brUdRib.

Experimental Section

HeLa cells, clone S3, were maintained in spinner culture at 2–4 × 10^5 cells/ml in Eagle's minimal essential medium supplemented with 6% horse serum, 62 μg/ml of penicillin, and 135 μg/ml of streptomycin.

brUdRib was obtained from Sigma Chemical Corp. fUdRib was a product of Hoffmann-La Roche. Urea, sucrose, and CsCl were the Ultrapure Grade of the Schwarz Mann Corp. Isotopically labeled compounds were obtained from New England Nuclear Corp. and were the highest specific radioactivity products available.

Chromatin was prepared from HeLa cells by washing the cells twice in RSB (0.01 M Tris-Cl–0.01 M NaCl–1.5 mM $MgCl_2$ (pH 7.5)), lysis with 1% Triton X-100 in RSB, and sedimentation of nuclei. The nuclei were washed once in RSB, twice in 10 mM Tris-Cl (pH 8.0), and once each in 5 and 1 mM Tris-Cl (pH 8.0). The final pellet was suspended in 1 mM Tris-Cl (pH 8.0) and sheared briefly (Virtis 23 homogenizer, 60 V, 30 sec) to solubilize the chromatin. DNA was prepared by repeated chloroform–isoamyl alcohol (24:1) extractions of chromatin dissolved in 0.5% SDS, 0.5 M NaCl, followed by precipitation with ethanol. In some cases Pronase treatment (100 μg/ml, 37°, 4 hr) followed by further cycles of extraction and precipitation was employed, without any effect on the observed properties of the DNA.

RNA was determined by measurement of the A_{260} of material which became soluble in cold 10% perchloric acid following alkaline hydrolysis (0.1 N KOH, 100°, 10 min). Histones were extracted with 0.25 N HCl for 30 min at 0° and determined by the Lowry procedure as described by Layne (1957). Nonhistone protein was determined by the same method after dissolving the pellet from histone extraction in 1% SDS.

For electrophoretic analysis, histones were extracted from chromatin (Simpson and Reeck, 1973), dissolved in 1% SDS, 1% mercaptoethanol, 0.01 M sodium phosphate (pH 6.8), dialyzed against a tenfold dilution of the buffer containing 10% glycerol, and electrophoresed on 15-cm long 15% polyacrylamide gels containing 0.1% SDS as described (Levy *et al.*, 1972). Nonhistone chromatin proteins were prepared from the pellet remaining after histone extraction

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[1] Abbreviations used are: RSB, reticulocyte sedimentation buffer (composition defined); brUdRib, 5-bromodeoxyuridine; fUdRib, 5-fluorodeoxyuridine; SDS, sodium dodecyl sulfate.

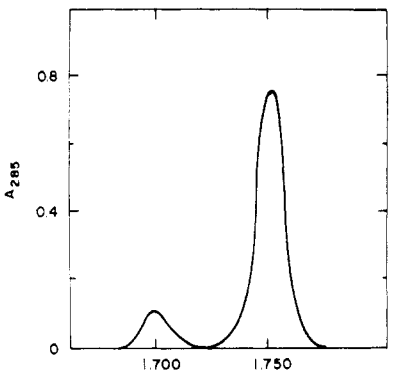

FIGURE 1: Isopycnic banding of brUdRib-labeled chromatin DNA. DNA from chromatin of HeLa cells grown for 22 hr with brUdRib and fUdRib was centrifuged to equilibrium in 58% (w/w) CsCl and 0.02 M Tris-Cl (pH 7.5). The figure is a direct tracing of the ultracentrifuge scanner pattern.

using the deoxyribonuclease I method of Wilson and Spelsberg (1973). The precipitated nonhistone proteins were dissolved in sample buffer and electrophoresed on 12-cm long discontinuous SDS-10% polyacrylamide gels as described by King and Laemmli (1971). All gels were stained with 0.25% Amido Black in 7% acetic acid-40% ethanol for 30 min and destained by soaking in the acetic acid-ethanol mixture containing a small amount of Dowex 1 resin. Gels were scanned in an ACTA III spectrophotometer (Beckman Instruments) equipped with a gel transport mechanism.

Thermal denaturation measurements and circular dichroism spectra were obtained for chromatin and DNA extensively dialyzed into 0.25 mM EDTA (pH 7.0) without or with various additions of 4 M NaCl, as previously described (Reeck *et al.*, 1972; Simpson and Sober, 1970). Analytical isopycnic banding in CsCl was performed at 44,000 rpm and 25° in the Beckman Model E Ultracentrifuge using the automatic photoelectric scanner system. DNA samples were sedimented to equilibrium in 58% (w/w) CsCl containing 0.02 M Tris-Cl (pH 7.5).

For alkaline sucrose gradient sedimentation, 2×10^6 cells (*ca.* 40 μg of DNA) were washed once in RSB, lysed by suspension in 0.25 ml of deionized water, and an equal volume of 1% sarkosyl, 0.4 N NaOH, and 20 mM EDTA was added. Samples were incubated at 37° for 25 min and then layered on linear 5-20% sucrose gradients containing 0.2 N NaOH, 1.0 M NaCl, and 10 mM EDTA, over a 75% (w/w) CsCl cushion. Samples were sedimented for 9 hr at 24,000 rpm in the Spinco SW 27.1 rotor at 15°. Gradients were collected by pumping from the bottom of the tube. Carrier DNA and albumin (100 μg each) were added and fractions were precipitated with 10% trichloroacetic acid. Precipitates were collected on Whatman GF/C filters, washed with 10% Cl_3CCOOH and then 95% ethanol, and dried at 100°. Samples were counted after overnight incubation at 37° with a mixture of 0.2 ml of NCS (Amersham-Searle)-water (9:1) and 10 ml of scintillation cocktail consisting of 4.2% rpi scintillator (Research Products International) in toluene. ^{14}C spill into the ^{3}H channel was corrected by the external standard technique.

Salt dissociation of chromatin proteins was analyzed by separation of dissociated proteins from the residual nucleoprotein complex by centrifugation. Chromatin samples were homogenized as described above at a DNA concentration of 0.25 mg/ml. Equal volumes of 10 M urea containing various concentrations of sodium phosphate (pH 5.5) were then added to aliquots of the chromatin and mixed vigorously. Sedimentation was carried out for 16 hr at 208,000*g* and 4°. After removal of the supernatant, the pelleted nucleoprotein was dissolved in 1% SDS. Aliquots (0.2 ml) of supernatants and resuspended pellets were counted with 0.8 ml of water and 10 ml of Aquasol (New England Nuclear Corp.) scintillation cocktail using a Beckman LS 250 liquid scintillation spectrometer.

TABLE I: Composition of Control and brUdRib-Substituted Chromatin.[a]

	Control Chromatin		brUdRib-Chromatin	
Preparation	I	II	I	II
Protein	1.45	1.51	1.48	1.45
Histone	1.02	1.05	1.04	1.03
Nonhistone	0.37	0.40	0.40	0.39
RNA		0.07		0.10

[a] All data are expressed as g/g of DNA.

Results

HeLa cells were grown for one generation (22 hr) in 1×10^{-5} M fUdRib (to block endogenous thymidine synthesis) and 1×10^{-5} M brUdRib. Isopycnic sedimentation of the DNA derived from such cells revealed the presence of two bands, a minor band at ρ 1.700 corresponding to unsubstituted DNA and a major band at ρ 1.750, representing DNA in which the thymidine of the strand synthesized during fUdRib-brUdRib treatment has been replaced by brUdRib (Figure 1). After correction for radial dilution, the band containing brUdRib was calculated to comprise 90% of the total chromatin DNA. Chromatin containing 85-90% of its DNA as this ρ 1.750 band was employed for all studies (other than pulse labeling) detailed in the remainder of the paper and will be called brUdRib-chromatin for purposes of comparison with control, or dT-chromatin.

The composition of dT- and brUdRib-chromatins are nearly identical. Averages of duplicate determinations of the content of total protein, histone, and nonhistone chromosomal protein for two separate preparations are detailed in Table I along with a single determination of RNA for the two chromatins. For both chromatins, total protein is present at concentrations of 1.4-1.5 g/g of DNA, histone content is 1.0-1.05 g/g of DNA, and the nonhistone protein content is 0.35-0.4 g/g of DNA. The histone content is that characteristic of a variety of eukaryotic chromatins (Bonner *et al.*, 1968) while the content of nonhistone chromosomal proteins is less than that determined by others for HeLa cell chromatin (Sadgopal and Bonner, 1970; Bhorjee and Pedersen, 1973). On the other hand, the amount of RNA associated with dT- and brUdRib-chromatin is similar and agrees with previous measurements of RNA content in the chromatin of metabolically active cells (Bonner *et al.*, 1968).

We have examined the distribution of both histones and nonhistone chromosomal proteins for both dT- and brUdRib-chromatins. Over 90% of the acid-extractable protein of both chromatins is present as the major histones of eukaryotic cells. As previously noted (Sadgopal and Bonner, 1970; Bhorjee and Pederson, 1973; Reeck *et al.*, 1974), two bands migrate with mobilities near those of f1 histone in

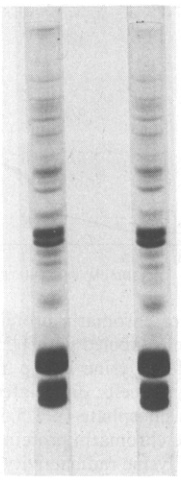

FIGURE 2: Acid-extractable proteins of brUdRib- (left) and dT- (right) chromatins. Proteins were electrophoresed on 15% acrylamide gels in the presence of SDS. Migration was from top to bottom. The major bands from the top are histones f1 (doublet), f3 and f2b, f2a2, and f2a1.

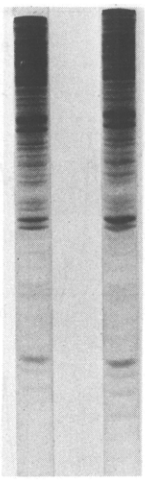

FIGURE 3: Nonhistone chromosomal proteins of brUdRib- (left) and dT- (right) chromatins. Proteins were solubilized from acid-extracted chromatin by deoxyribonuclease I treatment and electrophoresed on discontinuous SDS polyacrylamide gels. Migration was from top to bottom.

other cells (Figure 2). As judged by quantitative densitometry, the two chromatins contain similar amounts of any given histone. Further, all the minor acid-extractable proteins detected by SDS-polyacrylamide gel electrophoresis are present, and, in similar amount, in brUdRib-chromatin as well as dT-chromatin (Figure 2).

A similar situation prevails for the nonhistone chromatin proteins of the two chromatins. Over 50 bands are detectable in the nonhistone proteins of HeLa chromatin using the discontinuous SDS-polyacrylamide gel system (Figure 3). In several distinct preparations, all bands detected were present in both dT- and brUdRib-chromatins and again the staining intensities of the bands from the two chromatin types are similar if not identical. Thus, the protein contents of the two chromatins appear to be identical within the limits of resolution imposed by (1) the use of size criteria alone and (2) the limit of detection of proteins present in small amounts.

In a developing system, brUdRib has been shown to lead to defective DNA maturation with the accumulation of 8-15S (Kotzin and Baker, 1972) DNA segments. We have investigated whether a similar situation occurs in HeLa cells grown in the presence of the thymidine analog. Due to the photosensitivity of brUdRib and the possibility of radiation damage creating small nucleic acid fragments, all manipulations for these experiments were carried out in near darkness.

There appear to be quantitative differences in the rate of DNA processing in the absence and presence of brUdRib. HeLa cells were labeled continuously with [^{3}H]dA plus [^{14}C]brUdRib or [^{14}C]dT and the DNA was analyzed by alkaline sucrose gradient sedimentation after 20, 40, or 60 min of incubation (Figure 4). In controls label is broadly distributed, and primarily in the 20-30S region at 20 min. As incorporation continues the low molecular weight region (<30 S) reaches a constant level of radioactivity and is eventually obscured at 60 min by the accumulation of high molecular weight chromosomal DNA. Cells incubated with brUdRib, likewise, have a broad distribution of fragment sizes at 20 min. As incorporation proceeds, however, DNA with a modal size of 20-30 S accumulates and is not readily converted to high molecular weight DNA. When [^{3}H]dA was added together with [^{14}C]dT or [^{14}C]brUdRib, incorporated tritium was coincident with ^{14}C in either case. This indicates that there is no accumulation of brUdRib-rich intermediates in treated cells and that the overall rate of processing of intermediates into high molecular weight DNA in brUdRib-treated cells is impaired.

To investigate this further, the fate of pulse-labeled brUdRib-DNA fragments was examined during a prolonged chase also in the presence of brUdRib. Cells were labeled

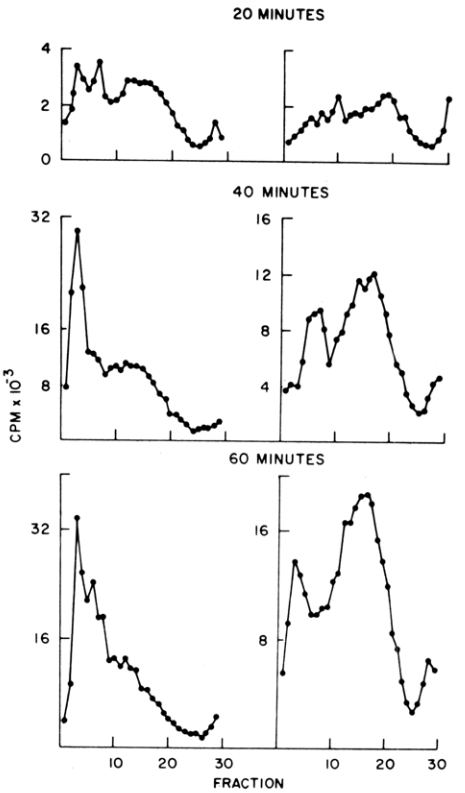

FIGURE 4: Processing of DNA in controls, left, and brUdRib-treated, right, HeLa cells. HeLa cells at 2×10^5 cells/ml were incubated for the indicated periods in 1×10^{-5} M fUdRib, 5 μCi/ml of [^{3}H]dA and left, 1×10^{-5} M dT with 1 μCi/ml of [^{14}C]dT or, right 1×10^{-5} M brUdRib with 1 μCi/ml of [^{14}C]brUdRib. Alkaline sucrose gradient sedimentation was performed as in the Experimental Section. Only the ^{14}C counts are shown. The bottom of the tube is fraction 1.

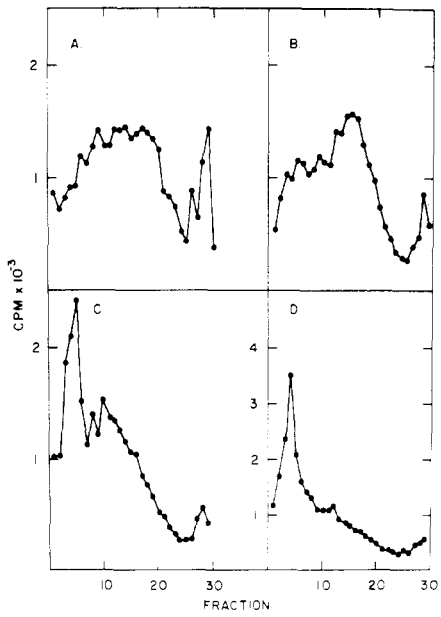

FIGURE 5: Pulse chase experiment with brUdRib-labeled chromatin DNA. HeLa cells at 2×10^6 cells/ml were incubated with 1×10^{-5} M fUdRib for 10 min then 10 μCi of [^{14}C] brUdRib was added to label cells for 30 min. After washing, the cells were then chased for varying lengths of time in 1×10^{-5} M both fUdRib and brUdRib. Length of chase period is A, 0 min; B, 30 min; C, 90 min; and D, 180 min. The bottom of the tube is fraction 1.

for 30 min with [^{14}C]brUdRib and, after washing, were incubated in brUdRib and fUdRib (Figure 5). At the end of the pulse period, label is present primarily in 20–30S fragments. With time, the label gradually chases into high molecular weight DNA and the conversion is essentially complete after 3 hr. Thus, the qualitative pathway of DNA synthesis in control and brUdRib-treated cells appears to be the same, albeit with a significantly decreased rate of conversion of 20–30S fragments into high molecular weight DNA in brUdRib treated cells. While processing is slowed by brUdRib, at the end of a full generation of labeling in the presence of the analog, the bulk of the DNA sediments to or near the sucrose–CsCl interface, as in controls.

Differences between dT- and brUdRib-chromatins were also detected in a comparison of the physicochemical properties of the two types of chromatin. The thermal denaturation pattern of HeLa dT-chromatin is similar to that observed for rabbit liver and other chromatins (Reeck *et al.*, 1972, 1974) with a broad melt extending overall from about

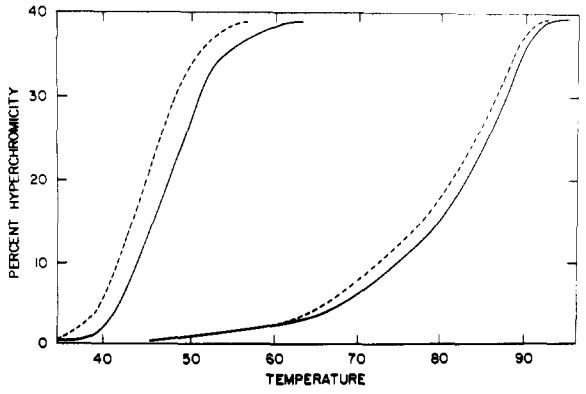

FIGURE 6: Thermal denaturation of dT- (- - - -) and brUdRib- (—) DNA and chromatins. The left-hand pair of melts are of DNA and the right-hand pair are of chromatin.

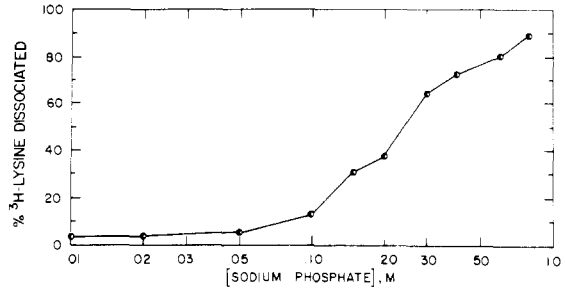

FIGURE 7: Dissociation of chromatin proteins by salt-urea mixtures. Chromatin from HeLa cells labeled with [^{3}H]lysine in the presence of brUdRib (○) and with [^{14}C]lysine in the absence of the analog (●) was isolated after mixing the cells, dissociated in 5 M urea and varying concentrations of sodium phosphate (pH 5.5), as indicated on the abscissa. The per cent of the chromatin proteins dissociated was calculated from measurements of lysine radioactivity.

55 to 90° and a small portion (<10%) of the DNA sequences melting below 70° (Figure 6). The midpoint for melting of dT-chromatin is at 80°. Substitution of brUdRib for nearly half the dT of HeLa chromatin results in a slight, but significant and reproducible, stabilization of the nucleoprotein to thermal denaturation. Little difference is observed in the early phase of the thermal transition but the major portion of the melt is displaced to slightly higher temperatures with the midpoint of the melt at 82° (Figure 6).

A similar situation obtains for the melting of dT- and brUdRib-DNAs. In the solvent employed, 0.25 mM EDTA (pH 7.0), HeLa cell DNA melts at 46°, a value similar to that for rabbit liver and calf thymus DNAs (Figure 6). The thermal denaturation of brUdRib-containing DNA is, however, displaced to higher temperatures with a midpoint at 49° (Figure 6). At higher salt concentrations, the melting points of both DNA samples are increased, for example, dT-DNA melts at 93° and brUdRib-DNA at 96° in 0.5 M NaCl–0.25 mM EDTA (pH 7.0) (data not shown). The magnitude of the shift in thermal stability is similar for dT- *vs.* brUdRib-DNA, on the one hand, and dT- *vs.* brUdRib-chromatin, on the other, suggesting that the higher thermal stability of brUdRib-chromatin derives from intrinsic differences in the structure and stability of the DNA double helix and not from differential stabilization by chromatin proteins.

The stability of protein-DNA interactions in dT- and brUdRib-chromatins was also assayed by salt–urea dissociation of the chromatin. The solvents employed, various concentrations of sodium phosphate (pH 5.5) in 5 M urea, are ones which the studies of Bartley and Chalkley (1972) suggest both prevents the generation of artefacts by the chromatin-associated protease (Furlan and Jericijo, 1967a,b; Bartley and Chalkley, 1970; Kurecki *et al.*, 1971; Garrels *et al.*, 1972) and prevents the aggregation and sedimentation of dissociated chromatin proteins. For the most accurate assessment of the dissociation of chromatin proteins, a pair label experiment was performed. After the initial washing, HeLa cells labeled with [^{3}H]lysine in the presence of fUdRib and brUdRib were mixed with control cells labeled with [^{14}C]lysine and chromatin isolation and further manipulations carried out on the single sample. Dissociation at a variety of concentrations of sodium phosphate in the presence of 5 M urea led to identical amounts of the two isotopes being solubilized for the chromatin sample, from a low value of about 3% of the label dissociated at 10

TABLE II: Dissociation of [^{3}H]Lysine Labeled Proteins from Control and brUdRib-Substituted Chromatin.[a]

[Sodium Phosphate], M	% [^{3}H]Lysyl Protein Dissociated	
	dT	brUdRib
0.1	19	17
0.2	37	35
0.3	65	65
0.4	72	72

[a] Separate preparations of control and dBrU-treated cells were labeled for 20 hr with [^{3}H]lysine, 0.5 μCi/ml in Eagle's medium, chromatin isolated, dissociated in 5 M urea, plus the indicated concentrations of sodium phosphate (pH 5.5) separated by centrifugation and analyzed as described in the Experimental Section.

mM sodium phosphate to nearly 90% of the input label dissociated at 0.8 M sodium phosphate (Figure 7).

To eliminate the possibility that such results derived from an equilibrium distribution of the two sets of labeled proteins on the mixed population of dT- and brUdRib-DNAs present in the dissociating mixture in the pair label experiment, several points of the experiment were repeated using separate samples of dT- and brUdRib-chromatins, labeled in both cases with [^{3}H]lysine and analyzed separately. The results (Table II) again indicate that the same proportion of the proteins of brUdRib-chromatin are dissociated at a given sodium phosphate concentration as are dissociated for the control, dT, sample.

The results obtained with lysine as label reflect binding of both histones and nonhistones, although favoring the former, highly basic proteins. The stability of nonhistone protein-DNA binding was investigated using chromatin labeled with [^{3}H]tryptophan. The dissociation of nonhistones is displaced to higher sodium phosphate concentrations (Table III) than that for lysine-labeled proteins. There is little difference in the relative stability of binding to control and brUdRib-chromatin over a range from <10 to >80% of labeled protein dissociated.

TABLE III: Dissociation of [^{3}H]Tryptophan Labeled Proteins from Control and brUdRib-Substituted Chromatin.[a]

[Sodium Phosphate], M	% [^{3}H]Tryptophanyl Protein Dissociated	
	dT	brUdRib
0.1	8.3	9.5
0.2	15.9	15.4
0.3	25.4	24.3
0.6	47.0	46.0
0.8	83.0	80.0

[a] Separate preparations of control and brUdRib-treated cells were labeled for 20 hr with [^{3}H]tryptophan, 0.66 μCi/ml in Eagle's medium, chromatin isolated, dissociated in 5 M urea, plus the indicated concentrations of sodium phosphate (pH 5.5) separated by centrifugation and analyzed by determination of the proportion of the ^{3}H which was insoluble after hydrolysis in 10% perchloric acid at 100° for 10 min.

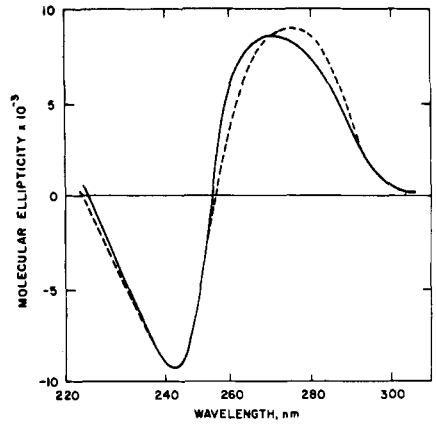

FIGURE 8: Circular dichroism spectra of dT-(- - - -) and brUdRib-(—) DNA.

Confirmation of differences in the structure of DNA in both the isolated state and in chromatin when partially substituted with brUdRib was obtained by study of the circular dichroism (CD) spectra of normal and brUdRib-substituted chromatin and its DNA (Figures 8 and 9). The CD spectrum of HeLa cell DNA and chromatin are similar to those previously reported for a variety of eukaryotic DNAs and chromatins (Permogorov *et al.*, 1970; Shih and Fasman, 1970; Simpson and Sober, 1970). DNA has a positive ellipticity maximum at 275 nm and a negative maximum at 245 nm, both with ellipticities of about 9000 deg cm^2/dmol (Figure 8). In chromatin, the positive maximum is split, slightly red shifted, and decreased to about half the magnitude of the corresponding band in protein-free DNA (Figure 9).

In contrast to the similarities of the CD spectra of HeLa cell chromatin and DNA with those of other tissue sources, the CD spectra of both brUdRib-chromatin and its constituent DNA differ from this common pattern. The positive maximum for brUdRib-DNA, while of the same magnitude as that of dT-DNA, is slightly blue-shifted to have a maximum at 270 nm (Figure 8). There is little if any alteration in the 245-nm negative ellipticity maximum (Figure 8). Even more marked changes are seen in the comparison of the CD spectra of dT- and brUdRib-chromatins. Below 250 nm the spectra of the two chromatins are identical within experimental error. Above this wavelength, however,

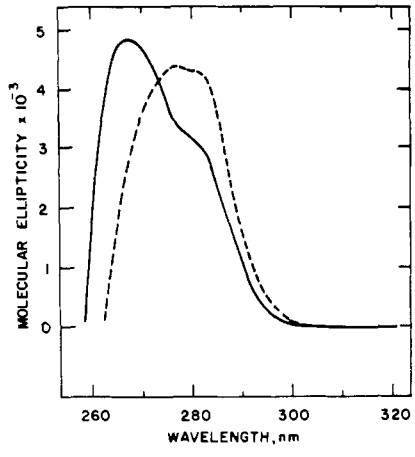

FIGURE 9: Circular dichroism spectra of dT-(- - - -) and brUdRib-(—) chromatin.

rather striking alterations are seen on substitutions of brUdRib for dT. The positive maximum for brUdRib-chromatin is seen to be definitely composed of two bands. The long wavelength band, at 285 nm, is diminished compared to control chromatin. In contrast, the shorter positive maximum, at 260 nm, is blue-shifted and markedly enhanced in brUdRib-chromatin when compared to dT-chromatin (Figure 9). The CD spectrum from 260 to 320 nm of brUdRib-DNA in 2 M NaCl is quite similar to that of brUdRib-chromatin in this spectral range (R. T. Simpson, unpublished observations). These data suggest a possibly different conformation for the DNA of the two systems and a further, more dramatic, difference in the conformation of DNA when in dT- and brUdRib-chromatin.

Discussion

The "specific" inhibition of differentiation by brUdRib has been noted for a large number of *in vivo* and *in vitro* differentiating systems. The effect is reversible on removal of brUdRib, is inhibited by the presence of exogenous dT, and generally requires the presence of brUdRib during a period of DNA synthesis, suggesting that brUdRib substitution for dT underlies the inhibition (for a review, see Rutter *et al.*, 1973). Inhibition of differentiation is observed after a single cycle of incorporation, demonstrating that unifilar substitution is sufficient (Bischoff and Holtzer, 1970). The rate of transcription of bacteriophage λ genes is markedly reduced when brUdRib substitutes for dT (Jones and Dove, 1972). However, there is no evidence for the production of altered proteins in cells treated with brUdRib (Stellwagen and Tomkins, 1971a,b). Additionally, brUdRib has been thought to lead to defective maturation of DNA in developing sea urchins where 8-15S DNA accumulates (Kotzin and Baker, 1972). Still another possible mechanism for the effect on brUdRib on cytodifferentiation derives from observations of Lin and Riggs (1972a,b) that purified lac repressor binds much more tightly to poly[d(A · brUdRib)] than to poly[d(A · T)]. Unpublished observations of David *et al.* (1970) (cited by Rutter *et al.*, 1973) that brUdRib substitution led to a shift in the melting of chromatin to higher temperatures, coupled with these observations of Lin and Riggs, led Rutter *et al.* (1973) to suggest that the most likely mechanism for the effects of brUdRib on differentiation was increased affinity of regulatory proteins for the substituted DNA.

In beginning our studies on the mechanism of brUdRib inhibition of eukaryotic cell differentation, we have examined the composition and structure of HeLa cell chromatin which has been extensively substituted *in vivo* with brUdRib. By blockage of endogenous thymidine synthesis with fUdRib, we consistently achieve substitution with brUdRib of nearly all the dT residues incorporated in a single cell cycle. The chromatin isolated from these brUdRib-treated cells has a composition of proteins and RNA which is similar, if not identical, to that of chromatin isolated from control cells. Further, the distribution of histones and nonhistone chromatin proteins detectable by SDS gel electrophoresis is the same for the two types of chromatin. Thus, if altered DNA-protein interactions underlie the effect of brUdRib on differentiation, these altered interactions do not lead to the presence of different amounts of the same, or of different proteins in chromatin. Of course, this conclusion must be limited by the sensitivity of the staining methods employed, which we estimate permit detection of a protein present in 50,000-100,000 copies per haploid genome.[2] Others have suggested limits of detection approximately tenfold lower than this estimate (Garrard *et al.*, 1974). Proteins present in less than this amount might, of course, differ strikingly for brUdRib- as compared to dT-chromatin.

Other methods of measuring the stability of protein-DNA interactions in chromatin also fail to provide support for the contention that brUdRib-DNA binds the major chromatin proteins more firmly than control DNA. The combination of a hydrogen bond breaking solvent with high salt concentrations effectively dissociates proteins from chromatin (Bartley and Chalkley, 1972). Increasing the concentration of sodium phosphate in the presence of 5 M urea leads to a gradual removal of nearly 90% of the proteins of chromatin and small or no differences are detected in the amount of either [^{3}H]tryptophan or [^{3}H]lysine-labeled protein removed from brUdRib- or dT-chromatin at the various salt concentrations employed (Figure 7, Tables II and III). Similarly, the melting profile of chromatin reflects, in addition to the inherent stability of the DNA double helix, additional stabilization due to electrostatic interactions with chromatin proteins. While the thermal denaturation of brUdRib-chromatin is displaced to somewhat higher temperatures than that of dT-chromatin, the displacement is similar to that observed for the isolated brUdRib-DNA when compared to dT-DNA (Figure 6).

A similar stabilization to thermal denaturation was observed in the case of brUdRib-containing synthetic polynucleotides by Inman and Baldwin (1962a,b). Thus, at low ionic strength, the mixed strand copolymer of poly[d(A-T)] · poly[d(A-brU)] melted 3° higher than the copolymer poly[d(A-T)] · poly[d(A-T)]. The data suggest that a higher stability of binding between brU and A stabilizes the DNA double helix in brUdRib-DNA over the stabilization observed for T· A pairing and that these two DNAs, differing inherently in their thermal stability, are identically further stabilized by their complement of proteins in chromatin. Taken together, the data suggest that the affinity of brUdRib-DNA and dT-DNA for chromatin proteins is identical at the current level of compositional and structural analyses.

As might be expected, the analog also leads to alterations in DNA synthesis, although the alterations we see in HeLa cells are not as marked as that previously reported for developing embryonic systems (Kotzin and Baker, 1972). DNA synthesis appears to proceed by the usual pathway established for eukaryotic systems with initial synthesis of small fragments, followed by their joining into intermediate 20-30S fragments and final maturation into high molecular weight, mature DNA. While there appears to be a delay in DNA processing in the presence of brUdRib in HeLa cells, all the fragments do chase into mature DNA eventually. The effect of brUdRib on maturation of DNA appears to be a generalized slowing of the transition from the 20-30S intermediates into mature DNA, since brUdRib-rich intermediates do not accumulate. In the time frame of studies of brUdRib inhibition of differentiation, this delay in processing would not be expected to have any effect, since such dif-

[2] The basis for this detection limit is as follows. A cell with a haploid DNA content of 3.5×10^{-12} g of DNA and a nonhistone chromatin protein to DNA ratio of 0.4 contains 1.4×10^{-12} g of nonhistones. Assuming a detection limit of 0.25% of the protein applied to a gel, for a protein of molecular weight 42,000, the minimal number of molecules per cell to be detected is $(1.4 \times 10^{-12} \times 6 \times 10^{-23} \times 0.0025)/4.2 \times 10^4 = 50{,}000$ copies per haploid genome.

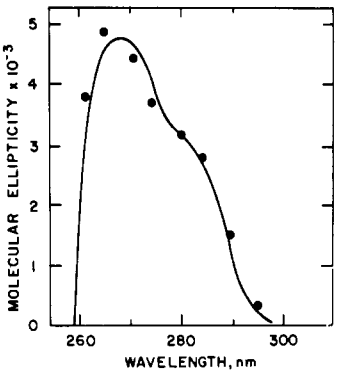

FIGURE 10: Fitting of the CD spectrum of brUdRib-chromatin. The solid line is the spectrum of brUdRib-chromatin. The symbols are calculated ellipticities based on a linear combination of 60% C, 6% B, and 34% A conformation of the DNA in chromatin. Ellipticities for the calculations were taken from Johnson *et al.* (1972) for the B and C conformations and from data of Tunis-Schneider and Maestre (1970) for the A conformation.

ferentiation experiments generally extend well beyond even a single cell cycle, and, at the end of a full generation, the DNAs of control and brUdRib-treated cells are quite similar in size distribution.

A rather striking difference between brUdRib- and dT-chromatins is apparent in the conformation of the nucleic acid in the nucleoprotein complex. While the circular dichroism spectra of dT- and brUdRib-DNA differ only slightly, the spectra of the two corresponding chromatins differ markedly in the range from 250 to 300 nm. These differences contrast with the essentially identical nature of the circular dichroism spectra of chromatins isolated from a wide variety of tissues (Johnson *et al.*, 1972; Permogorov *et al.*, 1970; Shih and Fasman, 1970; Simpson and Sober, 1970; Wagner and Spelsberg, 1971; Wilhelm *et al.*, 1970; and others). The origin of the differences between the CD spectra of control chromatin and that substituted with brUdRib remain a matter for conjecture; nevertheless, it is of interest to speculate what these changes may represent. Johnson and coworkers (1972) have treated the CD spectrum of chromatin as a simple linear combination of B and C DNA conformations, achieving an impressive fit to the experimental data using roughly equal amounts of the two structures. Hence, they have suggested that chromatin DNA may, in fact, exist as a mixture of these two DNA conformations although, alternatively, all the DNA could exist in an hypothetical intermediate conformation. The marked shift to lower wavelengths for the enhanced positive CD maximum in brUdRib-chromatin is not compatible with it existing as a mixture of the B and C forms of DNA. However, the spectrum of A form DNA is nonconservative with a maximum at about 270 nm (Tunis-Schneider and Maestre, 1970) and larger ellipticity than that of B form DNA. A rather reasonable fit to the experimental data for brUdRib-chromatin is obtained by a linear summation of 60% C, 6% B, and 34% A forms of DNA (Figure 10), suggesting that at least part of the effect of brUdRib substitution for dT may be to convert the conformation of a portion of chromatin DNA from the B to the A form.

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Mononucleotides in Aqueous Solution: Proton Magnetic Resonance Studies of Amino Groups†

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ABSTRACT: Proton magnetic resonance spectroscopy was applied to mononucleotides to obtain information on hydrogen-bonding properties as well as the rates of proton exchange between 5′-ribonucleotide amino groups and solvent water. Symmetrical acid–base catalysis of exchange rates was observed for GMP, AMP, and CMP. The rates of proton exchange are relatively slow and comparable to those of amides, in contrast to the diffusion-controlled exchange of imino (ring nitrogenous) protons. Chemical shift measurements provide evidence of extensive conjugation of CMP and AMP amino groups into the aromatic rings, since sigmoidal titration curves with midpoints at base pK were observed. In favorable cases, titration at the pK for phosphate ionization could also be monitored. Amino groups of mononucleotides interact with externally added phosphate, as judged from line width and chemical shift changes. This is attributed to the dual role of phosphate, as a proton exchange catalyst and a hydrogen-bond acceptor. From concentration-dependence studies, it appears that the phosphate group of one CMP molecule interacts with the amino group of another. In contrast, little or no association by hydrogen bonding was observed for cytidine. Restricted rotation of amino group was evident not only in cytidine but also in adenosine at acid pH. This implicates N-1 as the site of protonation in AMP. Comparisons with nicotinamide derivatives revealed significant similarities between nucleotide amino and amide protons with respect to restricted rotation and proton exchange characteristics. However, due to extensive charge delocalization into nucleotide amino groups—not evident in NMN^+—the pK for amino protonation might be lower than that for amides. These studies may give a better understanding of the factors that underlie the hydrogen-bonding specificity of nucleotide interaction.

In our previous communication, it was shown that the amino groups of mononucleotides in aqueous solution can participate in hydrogen bonding (Raszka and Kaplan, 1972). Proton magnetic resonance (pmr) spectroscopy revealed that the amino protons give rise to broad spectral lines; this broadening can be related to the lability of amino protons in aqueous solutions. This report examines the pH dependence of amino proton line widths and chemical shifts. Comparisons with amide protons of nicotinamide mononucleotide are undertaken in support of the view that nucleotide amino groups possess a significant amount of amide character.

Pmr chemical shift measurements can yield information on intermolecular and intramolecular association in solution. In the case of nucleotides, chemical shifts of amino protons show sensitivity to the protonation of ionizable groups, particularly base protonation. This is important for pmr studies of nucleotide hydrogen bonding, which rely on relatively small chemical shift changes.

Analyses of pmr line widths of the amino proton resonances can give detailed data on the rates of proton exchange between the amino groups and the solvent. Nitrogenous protons are labile and exchange with water at finite rates. In the region of intermediate exchange rates, amino proton resonances are broad; the contribution of exchange broadening to the line width is additive with that of spin–spin relaxation T_2

$$\pi \Delta \nu_{1/2} = \frac{1}{T_2} + \frac{1}{\tau}$$

where $1/\tau$ is the rate of exchange (Johnson, 1965). A more detailed treatment of line-width dependence, its relationship to acid–base catalytic rate constants, and a discussion of possible mechanisms of nucleotide amino group proton exchange, has already been presented (McConnell and Seawell, 1972).

Figure 1 displays the 220-MHz pmr spectra of the three mononucleotides at pH 7.5 and 0°. In water, the amino proton resonances are broad and sensitive to temperature; at higher temperatures they broaden rapidly and become unobservable above 50–60°. A positive temperature coefficient is characteristic of proton exchange and reflects the activation energy of the exchange process.

The chemical shifts of amino protons also change drastically with temperature, due principally to breaking of hydrogen bonds with water. In favorable cases, temperature-dependence studies can be used to infer intramolecular hy-

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