Virogenic BrdU and BrdU-sensitive DNA sequences are disproportionately concentrated in the template-active chromatin of rat embryo cells

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Received 2 November 1978

ABSTRACT

In order to characterize the molecular mechanism responsible for the BrdU-mediated activation of endogenous retrovirus from normal rat embryo cells, the previously reported selective distribution of bromouracil in DNA was correlated with the corresponding organization of the nucleosome structure and template-active and -inactive chromatin. Following micrococcal nuclease digestion of chromatin labeled with either [^3H]thymidine or [^3H]BrdU, the amount and specific activities of the respective nucleosomal DNA were indistinguishable. Comparable findings were obtained following direct examination of the nuclease-sensitive, "spacer" DNA. However, when each chromatin type was fractionated into template-active and -inactive components, it was evident that [^3H]bromouracil was nonrandomly more concentrated in the template-active portion in comparison to the random distribution of [^3H]thymine moieties. Furthermore, it was apparent that the template-active chromatin fraction was substantially enriched in the nucleotide sequences of rat DNA known to be sensitive to the virogenic action of BrdU.

INTRODUCTION

The activation of endogenous retrovirus from a variety of normal mammalian cells in-vitro by 5-bromodeoxyuridine (BrdU) (1) has been regarded to be a useful model to study the regulation of expression of a naturally occurring eukaryotic gene. Reports from our laboratory (2-4), as well as from others (5-7), described the novel finding that BrdU was frequently incorporated in nuclear DNA in a manner distinctly different from that of the physiologic counterpart, thymidine. It is now apparent that under controlled conditions, bioactive levels of bromouracil are selectively concentrated to a relatively greater extent in repeated, and especially moderately repeated DNA sequences in several eukaryotic cell types (2-7). Furthermore, related studies have demonstrated that protein-nucleic acid interactions in chromatin were substantially altered whenever the DNA contained even low amounts of bromouracil

(8-10). These observations, therefore, have led several workers to postulate a simplified mechanism for BrdU-mediated effects, whereby regulatory proteins bind more tightly to certain repeated elements of the eukaryotic genome resulting in the commonly observed transcriptional modifications (9-12).

A great deal of information has rapidly accumulated with regard to the structure, function, and organization of eukaryotic chromatin (13-15). The nucleosome model of a repeating subunit approximately two hundred base pairs specifically associated with an octamer of histones most likely is a universally characteristic feature of animal cell chromatin. However, the functional correlation between this molecular arrangement and inherent transcriptional control is still unclear. On the other hand, novel experimental methods are becoming available to resolve some of these questions. For example, Gottesfeld and co-workers (16,17) have defined and characterized an experimental method to partially fractionate eukaryotic chromatin into template-active and -inactive constituents. Recently, this technique was successfully utilized to demonstrate that nucleotide sequences representing the globin gene in dimethylsulfoxide-treated Friend leukemia cells were localized in the template-active chromatin fraction (18,19). In light of the applicability of these new methods, the present work was undertaken to correlate the nonrandom distribution of virogenic BrdU in DNA with respect to the inherent organization of the corresponding nucleo-protein complex constituting rat embryo cell chromatin.

MATERIALS AND METHODS

Cell Culture Labeling: Secondary cell cultures of normal, 16-day old Wistar/Furth rat embryos were initiated and maintained as described (2-4). Only cells derived from mycoplasma-free cultures were utilized for subsequent labeling and extraction. Typically, several cell cultures in logphase of growth were treated overnight at 37°C with either 0.1 μ M [3 H]thymidine (18 Ci/mM, New England Nuclear Corp.) or an equimolar concentration of [3 H]BrdU (13 Ci/mM, New England Nuclear Corp.). The radiolabeled cells were then removed from the flasks by brief trypsinization, immediately rinsed several times in tissue culture medium made 10% in fetal calf serum, rinsed in phosphate-buffered saline, and finally sedimented by low-speed centrifugation prior to isolation of chromatin.

Chromatin Isolation: Cell pellets were resuspended in a cold solution of 10mM Tris-HCl, pH 7.5 made 6mM KCl, 5mM magnesium acetate, 1mM phenylmethylsulfonylfluoride (PMSF), and 0.1mM ethylene glycol bis (aminoethyl ether) tetracetate (EGTA) containing 0.5% NP-40 according to Wallace *et al.* (19). The subsequent pellets obtained following centrifugation at 10,000 x g for 10 minutes were prepared for chromatin isolation essentially according to the same authors. Frequently, chromatin suspensions were stored frozen at -70°C for several days in 25mM sodium acetate pH 6.6, and 0.1mM EGTA.

Nucleosome Fractionation: Chromatin suspensions were rinsed several times and brought up in a buffer containing 1mM Tris-HCl, pH 8.0, 0.1mM CaCl₂ at 5°C, and made 1 mg DNA/ml. Staphylococcal nuclease (Worthington Labs, Inc.) was added to each chromatin type to a concentration of 10 µg/ml, and reacted for periods of time ranging from 30 seconds to 90 minutes. Reaction mixtures were made 10% in ice-cold trichloroacetic acid (TCA) and prepared as before (10) for quantitation of acid-insoluble DNA. In order to quantitatively recover the DNA sequences otherwise digested by staphylococcal nuclease, a modification of the Bloch and Cedar method (20) was utilized. Briefly, chromatin resuspended in 1mM Tris-HCl, pH 7.9 was titrated with poly-D-lysine (Sigma Chemical Co.) and assayed as before following digestion with staphylococcal nuclease. [³H]thymidine- and [³H]BrdU-labeled chromatin-poly-D-lysine complexes were then digested with 1 µg/ml of pronase B (Calbiochem) for 4 hours. Reaction mixtures were brought up in a buffer containing 1mM Tris-HCl, pH 8.0, 0.1mM CaCl₂, 1mM PMSF, and digested for another 60 minutes with 10 µg/ml of staphylococcal nuclease. Acid-insoluble radioactivity was determined as above on a representative fraction. The majority of the material was retained for the extraction of DNA.

Chromatin Fractionation: In order to fractionate radiolabeled rat embryo chromatin into template-active and template-inactive constituents, the method of Gottesfeld *et al.* (16,17) was essentially followed. Briefly, each chromatin type was adjusted to be approximately 400 µg/ml of DNA in 25mM sodium acetate, pH 6.6, 0.1mM EDTA, and digested for 5 minutes at 24°C with 100 units/ml of DNase II (Worthington Biochem. Corp.). Template-active chromatin was subsequently recovered as above (16,17) by centrifugation and differential solubilization in MgCl₂.

DNA Extraction: Radiolabeled DNA was isolated and purified from

various chromatin subfractions as described (2-4) and specific activities were determined.

DNA-DNA Reassociation. Rat embryo cells were synchronized by a double thymidine block as before (3). Cultures were exposed to 10 μ Ci/ml of [3 H]thymidine (18 Ci/mM, New England Nuclear) during the 60 minute interval of the rat S phase in which the virogenic BrdU-responding nucleotide sequences have been shown to replicate (3). Following the labeling period, the cells were immediately lysed in a physiologic buffer containing 1% sodium dodecyl sulfate, and extracted as before for DNA (2-4). Purified DNA samples were mechanically sheared at 5°C to 450-500 nucleotide fragments in a Ribi Cell Fractionator (2-4). DNA-DNA reassociation reaction mixtures were heat-denatured and incubated in 0.40 M sodium phosphate buffer, pH 6.8, 1mM EDTA at 65°C, and assayed over hydroxyapatite (2-4). Cot values were corrected for Na⁺ concentration according to Britten *et al.* (21).

RESULTS

The [3 H]thymidine- and [3 H]BrdU-containing chromatin were digested with staphylococcal nuclease in order to determine whether the nonrandom distribution of [3 H]bromouracil (2-4) was correlated with protected or nuclease-sensitive regions of DNA. Figure 1 demonstrated that purified, [3 H]thymidine-labeled DNA was entirely sensitive to the action of micrococcal nuclease under identical conditions of digestion. On the other hand, radiolabeled chromatin of both isotopic types was digested to a limit in which almost half of the DNA was acid-soluble. Furthermore, the quantitation and kinetics of these digestions were nearly identical when purified nuclei were used in place of chromatin (data not shown).

Although the data from Figure 1 did not suggest significant differences in the extent of nuclease-resistant DNA in [3 H]thymidine- and [3 H]BrdU-labeled chromatin, it was desired to directly isolate and characterize the nuclease-sensitive DNA. The poly-D-lysine technique as utilized by Bloch and Cedar (20) was therefore selected to permit quantitative preparation of nuclease-sensitive chromatin DNA. Table I shows that titration of purified, radiolabeled DNA with poly-D-lysine and subsequent digestion with micrococcal nuclease resulted in a near-linear, direct proportionality of 1:1. As expected, comparable titration with radiolabeled chromatin required half as much poly-D-lysine to saturate the histone-free regions of DNA. Nuclease digestion of

Figure 1. Digestion of DNA and chromatin with staphylococcal nuclease. Radiolabeled DNA and chromatin samples were made 1 mg/ml as described in Methods and incubated at 37°C with 10 μ g/ml of nuclease for time periods shown. Δ , [3 H]thymidine-labeled rat embryo DNA; $\bullet$, [3 H]thymidine-labeled rat embryo chromatin; $\circ$, [3 H]BrdU-labeled rat embryo chromatin.

chromatin subsequent to maximal protection by polylysine revealed that the distribution of [3 H]thymidine and [3 H]BrdU was indistinguishable with respect to amount and specific activity of the "spacer" nucleotide sequences when compared to the histone-protected counterparts. Altogether, these findings suggested that the previously reported non-random distribution of bromouracil with regard to kinetic classes of eukaryotic DNA (2-7) was not reflected by disproportionate localization into either nucleosomes or "spacer" regions.

Inasmuch as the distribution of the respective radiolabeled nucleotides was comparable in histone-protected and histone-depleted DNA, the method of Gottesfeld *et al.* (16,17) was utilized to determine the isotopic distribution patterns in template-active and template-inactive chromatin. Following brief digestion with DNase II and solubilization in MgCl_2 , the chromatin subfractions were analyzed for mass of DNA as well as for amount of radioactivity (Table II). The first separations following nuclease treatment revealed substantial differences between the [3 H]thymidine- and [3 H]BrdU-labeled chromatin. Whereas nearly 81%

Table I.

Substrate	% poly-D-lysine	Pronase Digestion	% DNA Spared (+4%)	Relative Specific Activity
Purified DNA	0		0	
	20		22.2	
	40		44.5	
	60		58.8	
	80		88.7	
	100		98.4	
$[^3\text{H}]\text{TdR}$ Chromatin	0	-	55.2	0.95
	0	+	2.1	-
	50	-	98.5	0.98
	50	+	48.3	1.02
$[^3\text{H}]\text{BrdU}$ Chromatin	0	-	52.6	1.12
	0	+	<1	-
	50	-	98.4	0.97
	50	+	51.3	0.91

Protection of radiolabeled DNA and chromatin from staphylococcal nuclease by poly-D-lysine. Equal amounts of DNA (usually 100-200 μg) were titrated with poly-D-lysine prior to digestion with 10 $\mu\text{g}/\text{ml}$ of staphylococcal nuclease for 60 minutes as described in Methods. Chromatin was reproducibly spared from significant digestion by nuclease at the 50% level of polylysine. The % DNA spared values represent the average of four assays on different preparations with an overall standard deviation of $\pm 4\%$. The relative specific activities were calculated as such:

$$\frac{\text{final specific activity of the digested DNA subfraction}}{\text{initial specific activity of the respective unfractionated DNA sample}}$$

of the mass of the input DNAs pelleted as the template-inactive fraction (P1) for both series of rat chromatin, the distribution of the respective isotopes was disproportionate. Nearly 81% of the total $[^3\text{H}]\text{thymidine}$ residues co-fractionated with P1, as compared to 70% of the $[^3\text{H}]\text{BrdU}$ moieties. Although the absolute amounts of DNA in each fraction varied $\pm 4\%$ overall from preparation to preparation, the $[^3\text{H}]\text{BrdU}$ values were consistently 8-12% lower than the corresponding $[^3\text{H}]\text{thymidine}$ -labeled P1 counterparts.

When the S1 fractions were slowly made 2mM in MgCl_2 and recentrifuged, the distribution of the two isotopes differed again (Table II). Similar to the fractionation of other eukaryotic cell chromatin (16-19), the presumed template-active component S2 represented approximately 11-15% of the DNA mass regardless of isotopic precursor. As expected, a corresponding amount of $[^3\text{H}]\text{thymidine}$ was recovered from the S2 fraction.

Table II.

Chromatin fraction	% of total DNA mass(+4%)	% of total DNA C.P.M. (+2%)	Relative specific activity
[³ H]TdR template-inactive (P1)	83.2	81.3	.98
[³ H]TdR template-active (S2)	11.8	13.7	1.18
[³ H]BrdU template-inactive (P1)	80.1	70.7	.87
[³ H]BrdU template-active (S2)	12.4	23.5	1.92

Distribution of radiolabeled DNA precursors in template-inactive (P1) and template-active (S2) chromatin components as fractionated according to Gottesfeld et al (16,17). DNA was quantitatively extracted from chromatin subfractions and analyzed for A₂₆₀ as well as for radioactivity. The balance of the chromatin (P2) not included in the P1 or S2 fraction summations routinely constituted less than 5% of total chromatin DNA and was not analyzed further. The fractionations above represent the average of four analyses on different preparations of each chromatin type.

In contrast, more than 20% of the total radiolabeled bromouracil moieties were localized in S2. The relative specific activities of each component reflected the non-random nature of the comparative isotopic distributions.

We (3) and others (22) have shown that a finite identifiable portion of the synchronized S phase of rodent cell cultures was sensitive to the virogenic action of BrdU. In our rat embryo system, this interval of sensitivity represents <5% of the total DNA, and includes those genomic nucleotide sequences which replicate during a 60 minute period beginning 5 hours into the 7 1/2 hour rat cell S phase (3). In order to determine the relationship between this particular segment of genetic information and the template-active and -inactive chromatin fractions, the BrdU-sensitive DNA was pulse-labeled and used as a molecular probe for comparable sequences in the P1 and S2 components (Figure 2). The renaturation profiles obtained whenever radiolabeled, BrdU-responding sequences were hybridized to either total rat embryo DNA or P1 DNA were indistinguishable. However, at comparable mass ratios of labeled to unlabeled DNA, a different kinetic plot was generated from the hybridization of the S phase, pulse-labeled DNA to unlabeled S2 DNA. In this instance, the reaction was accelerated to an extent that sug-

Figure 2. DNA-DNA renaturation of the radiolabeled, BrdU-sensitive subfraction of S phase DNA with unlabeled DNA isolated from template-active and -inactive rat embryo chromatin. Samples were mechanically sheared to 450-500 nucleotides and renatured at a mass ratio of $10^3/1$, unlabeled/labeled DNA in 0.40 M sodium phosphate, pH 6.8 at 60°C, and assayed over hydroxyapatite as before (4). Corrections were made for Na^+ concentration according to Britten et al. (21). Pulse-labeled, BrdU-responding DNA sequences were hybridized with ●, complete, unlabeled rat embryo DNA; ■ unlabeled, template-inactive rat embryo chromatin DNA, and; ○, unlabeled, template-active rat embryo chromatin DNA.

gested substantial enrichment for the BrdU-responding sequences in the template-active S2 component. Although the radiolabeled 60 minute probe was composed of a complex and heterogeneous population of DNA kinetic classes, it was evident that this subfraction was disproportionately enriched in a great many sequences in common with the presumed transcriptionally-active chromatin.

DISCUSSION

During the past several years, a great deal of study has been devoted to the characterization of the kinetic classes of eukaryotic nucleotide sequences and their organization in purified DNA (21,23). More recently, comparable investigation into the structure and function of animal cell chromatin has been equally productive (13-15). Simplistic, well-characterized experimental systems have not been extensively

utilized, however, for the comprehensive and unified analysis of a distinct transcriptional process with regard to the relevant findings derived from the substantial nucleic acid and chromatin literature. Because of the data derived from previous intensive characterization of virus activation, the BrdU-mediated induction of latent retrovirus from normal rat embryo cells therefore, represented an excellent model in which to analyze the controlled expression of a naturally occurring gene with respect to the arrangement of finite DNA sequences and the associated nuclear proteins.

Our earlier work resulted in the identification of a restricted segment of rat S phase DNA sensitive to the virogenic effect of BrdU (3). Following treatment with staphylococcal nuclease, it was apparent that the non-random distribution of the analog was not reflected by disproportionate protection by chromosomal proteins. Similar observations were reported earlier by Lacy and Axel (24) in which the authors concluded that the nuclease-generated subunits from rat liver cells were random with respect to DNA sequence types. In a more related work, Panet and Cedar (25) recently reported that endogenous mouse proviral DNA sequences were randomly distributed between DNase I-sensitive and -resistant portions of the mouse genome. Interestingly, these workers suggested that the presence of integrated exogenous viral DNA affected the arrangement of proteins along the endogenous proviral genes. Groudine *et al.* (26) have described comparable findings for the DNase I sensitivity of endogenous retrovirus genomes in chick embryo fibroblasts.

In the present work, non-random distribution of BrdU was readily discernible only following chromatin fractionation into template-active and -inactive components. Although comparable portions of the rat genome partitioned into each respective P1 and S2 compartment, the increased specific activity of the [^3H]BrdU-treated S2 fraction relative to the [^3H]thymidine-labeled counterpart complemented the previously reported findings of disproportionate enrichment of bromouracil in moderately repeated DNA sequences (2-7). A more detailed kinetic characterization of the S2 chromatin constituents, irrespective of isotope, confirmed the previously reported findings of Gottesfeld *et al.* (16,17), wherein this DNA comprised a particular subset of nonrepeated sequences as well as a characteristic family of moderately repeated sequences (manuscript in preparation). Finally, the finding that transcriptionally-

active chromatin subunits were proportionately enriched in nascent RNA and non-histone proteins (27) is suggestive in light of our work (10) and that of others (12) regarding the strikingly altered interactions between BrdU-substituted DNA and the corresponding non-histone chromosomal proteins. Most recently we reported that [^3H]bromouracil moieties were relatively more protected than [^3H]thymine from DNase I following chromatin reconstitution in-vitro with purified non-histone proteins (28). Altogether, these observations are compatible with a molecular mechanism for virogenesis whereby BrdU exerts an inductive effect through disruption of physiologic nucleic acid-regulatory protein interactions especially restricted to moderately repeated regions of the eukaryotic genome.

Despite the requirement that BrdU must be incorporated in DNA for retrovirus activation to occur (3,22,29,30), the biological manifestations of the novel pattern of bromouracil distribution in DNA are unclear. Although conclusive evidence is currently lacking to implicate the non-random nature of analog localization with a distinct virogenic mechanism, it is highly suggestive that BrdU is disproportionately enriched in those particular nucleotide sequences and chromatin subfractions generally acknowledged to be involved in the regulation of gene expression. Work is in progress to gain further insight into the structural and functional correlation between finite, identifiable structural genes in eukaryotic DNA and the corresponding organization of chromosomal proteins.

ACKNOWLEDGEMENT

The expert technical assistance of Kathryn Miles is greatly appreciated. This work was supported in part by a grant from the Elsa U. Pardee Foundation, and by a Junior Faculty Clinical Fellowship from the American Cancer Society.

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