Distribution of 5-Bromodeoxyuridine in the DNA of Rat Embryo Cells

(DNA·DNA reassociation)

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ABSTRACT Rat embryo cells were treated with ^3H -labeled 5-bromodeoxyuridine (BrdU) in order to determine the nature of substitution and distribution of the analog in DNA. At optimal oncornavirus-inducing doses of BrdU (10^{-4} M), density gradient centrifugation and DNA·DNA reassociation experiments revealed extensive ($>90\%$) and uniform base substitution in repetitive, intermediate, and unique DNA sequences. Suboptimal doses (10^{-7} M) of [^3H]BrdU resulted in less than 5% thymine replacement and nonrandom labeling of DNA. Treatment of cells with 10^{-7} M [^3H]dT resulted in relatively uniform labeling throughout all DNA sequences. At low concentrations, BrdU was incorporated predominantly within the repetitive and intermediate DNA of rat embryo cells. Moreover, the single-copy DNA sequences were lightly substituted and reassociated extensively only in the presence of excess unlabeled DNA. Such specificity of base substitution may be related to the unusually selective nature of BrdU effects on animal cells.

The thymidine analog 5-bromodeoxyuridine (BrdU) selectively suppresses differentiated functions as well as activates specific synthetic processes in animal cells (1-10). It is generally assumed that the analog is incorporated into DNA, and that bromouracil substitutes exclusively for thymine (1, 4, 5, 8, 11, 12). Hill *et al.* (13) and Gordon *et al.* (14) described functional alterations in chromatin transcription, and binding of regulator proteins in BrdU-substituted DNA. In the present work, we have attempted to analyze the distribution of radiolabeled BrdU within the DNA of rat embryo cells.

MATERIALS AND METHODS

Tissue Culture. Secondary cultures of 16-day-old Wistar/Furth (W/Fu) rat embryo cells were grown in medium 199 in Hank's salts (Grand Island Biologicals) supplemented with 10% (v/v) fetal-calf serum, 1% (w/v) L-glutamine, and 100 U/ml of penicillin (10, 15). Cells were dispersed into 670-cm² glass roller vessels (Bellco) and maintained in a 95% air, 5% CO₂ atmosphere at 37°. Replicate roller bottle cultures were grown to subconfluency. Half of the cultures in logarithmic phase were treated with 50 ml of medium 199 containing 10^{-4} M BrdU (Sigma) and 10 $\mu\text{Ci/ml}$ of [^3H]BrdU (12.7 Ci/mmol, New England Nuclear Corp.) (8, 10). The remaining cultures were exposed only to 50 ml of medium 199 containing 10 $\mu\text{Ci/ml}$ of [^3H]dT (18.3 Ci/mmol, New England Nuclear Corp.). Culture fluids were removed 24 hr later; cells were harvested with 0.25% (w/v) trypsin and concentrated by low-speed centrifugation.

Abbreviation: C₀t, product of concentration of nucleotide sequence of DNA and time of incubation.

Preparation of DNA. Concentrated cell pellets were suspended in 0.15 M NaCl-0.015 M trisodium citrate (pH 7.0) and made 1% in sodium dodecyl sulfate, 1% in 2-mercaptoethanol, and incubated at 60° for 10 min (16). An equal volume of cold chloroform-isoamyl alcohol (24:1, w/v) was added, and the suspension was extracted three times at room temperature. The aqueous phases were again extracted with equal volumes of phenol (equilibrated with NaCl-trisodium citrate) until no material remained at the interface. Three volumes of ice-cold ethanol were layered onto the aqueous phase, and DNA was spooled out of solution. The fibers were immediately dissolved in 80 mM sodium phosphate buffer (pH 6.8). The DNA solution was extensively dialyzed against 80 mM phosphate buffer, and adsorbed to a column of hydroxyapatite (HTP-DNA Grade, Bio Rad) equilibrated at 60° with 80 mM phosphate buffer (17, 18). Contaminating RNA was eluted with several column volumes of 0.18 M phosphate buffer, and the double-stranded DNA was subsequently eluted with 0.40 M phosphate buffer (17, 18). The column-purified DNA was diluted 3-fold, made 1 mM in EDTA, and mechanically sheared in a Ribi cell fractionator (Sorvall) at 50,000 lbs./inch² (17, 18). The procedure consistently prepared DNA fragments of uniform size (300-400 nucleotides in length) with a sedimentation coefficient of 5.4 S, according to the alkaline band method of Studier (19). Sheared, characterized DNA preparations were again purified over hydroxyapatite, assayed for A₂₆₀, frozen, and stored at -4° until use. Unlabeled, bulk DNA from W/Fu cell cultures was extracted, purified, sheared, and stored in the same manner.

Isopycnic CsCl Centrifugations. Aliquots of radioactive sheared DNA were thawed and layered onto preformed linear, 5-ml CsCl gradients (1.50-1.90 g/cm³ in 80 mM phosphate buffer, pH 6.8). The samples were centrifuged at 220,000 $\times g$ to equilibrium for 30 hr at 25° (10). Gradients were collected from below in 20 fractions. Each fraction was assayed for A₂₆₀ and refractive index, and counted in a Packard scintillation spectrometer (10).

Renaturation Kinetics. DNA preparations were thawed and made 0.40 M in phosphate buffer (pH 6.8) and 3 mM in EDTA (20). The initial A₂₆₀ of the reaction mixture was determined, and the samples were divided equally and heat-sealed into 100- μl capillary tubes. The capillaries were completely immersed in boiling water at 100° for 10-15 min (18, 20). After thermal denaturation, the samples were immediately transferred to and incubated in a 68° water bath (17, 20, 21). Capillary tubes were periodically withdrawn,

FIG. 1. Isopycnic CsCl density gradients of rat embryo cell DNA. (Upper panel) DNA from cell cultures labeled for 24 hr with 10 μ Ci/ml of [3 H]dT, sheared, and centrifuged to equilibrium at $220,000 \times g$ for 30 hr at 25°. Fractions were monitored for radioactivity, refractive index, and A_{260} . (Lower panel) DNA from cell cultures treated for 24 hr with 10^{-4} M [3 H]BrdU. Gradients were prepared and collected as before. ●, A_{260} ; ▲, radioactivity; ○, CsCl.

and each 100- μ l sample was diluted in 20 ml of cold 80 mM phosphate buffer and analyzed over hydroxyapatite.

Hydroxyapatite Thermal Chromatography. Hydroxyapatite (HTP-DNA Grade, Bio Rad) columns were equilibrated with 80 mM phosphate buffer and maintained at 60° (17, 18, 20, 21). DNA renaturation samples were layered onto the column. After thermal equilibration and adsorption, single-stranded DNA was eluted first with 3 bed volumes (3×5 ml) of 0.18 M phosphate buffer (17, 18, 20, 21). Double-stranded DNA was then collected in a similar manner with 0.40 M phosphate buffer (17, 18, 20, 21). Efficiency of adsorption and stepwise recovery of DNA was consistently >90%. Aliquots of single- and double-stranded DNA were analyzed for A_{260} as well as for radioactivity. C_{ot} values were calculated on the basis of the original A_{260} of the DNA mixture divided by 2 and multiplied by the time of reassociation in hours (21, 22). Corrections for the Na^+ concentration in the annealing mixture were made according to Britten and Kohne (17) and Wetmur and Davidson (23).

Melting Temperature Determination on Hydroxyapatite. Undenatured and reassociated (high C_{ot}) double-stranded DNA was adsorbed separately to hydroxyapatite columns equilibrated with 80 mM phosphate buffer at 60° as before. Single-stranded material was eluted with 3 bed-volume washes of 0.18 M phosphate buffer at each point as the column temperature was raised in small increments from 60° to 95° (21). Final washes were made with 0.40 M phosphate buffer to assure that no radioactivity remained adsorbed to the

FIG. 2. Reassociation of sheared rat embryo cell DNA measured over hydroxyapatite. [3 H]dT-labeled DNA (●, 850 μ g/ml in 0.40 M phosphate buffer, 3 mM EDTA, pH 6.8) was denatured by heating at 100° for 10 min and incubated at 68°. Samples were periodically assayed over hydroxyapatite for double-stranded radioactivity. DNA labeled with 10^{-4} M [3 H]BrdU (○, 725 μ g/ml in 0.40 M phosphate buffer, 3 mM EDTA, pH 6.8) was treated similarly. Results are plotted as a function of C_{ot} (mol-sec/liter) corrected for Na^+ concentration.

column after elution. The A_{260} and radioactivity of each fraction were determined.

RESULTS

Properties of Heavily Substituted DNA. It has been reported that bioactive, nontoxic doses of BrdU for animal cells range from 10^{-6} M to 10^{-3} M (1–10). A concentration of 10^{-4} M BrdU was initially chosen for the present work because this was the optimal dose for the induction of type-C viruses from rat embryo cell cultures (10). The degree of substitution and reassociation kinetics of analog-treated DNA were compared to DNA exposed to equimolar [3 H]dT.

The [3 H]dT-labeled DNA from rat embryo cell cultures migrated as a single peak with a buoyant density of about 1.70 g/cm³ after sedimentation through neutral CsCl gradients (Fig. 1). The DNA from cells treated with 10^{-4} M BrdU sedimented as two broad density regions between 1.84 and 1.73 g/cm³ (Fig. 1). The distribution of the [3 H]BrdU corresponded closely to the A_{260} profile of the heavily substituted (>90%) DNA (24). We concluded that at a concentration of 10^{-4} M, BrdU was extensively and uniformly distributed throughout the cellular DNA. The broad density peaks were probably due to asynchronous cell division, since many cells experienced at least two mitotic cycles during the 24-hr exposure to BrdU (10).

The reassociation profile of the [3 H]dT-labeled DNA was characteristic of rat DNA (25) (Fig. 2). The repetitive fraction (about 25%) reannealed by a C_{ot} value of 1, whereas the intermediate and early unique DNA reassociated by a C_{ot} value greater than 100. The remainder of the slowly reassociating (single copy) nucleotide sequences were extensively hybridized at a C_{ot} value of 2000. Comparable kinetics of self-renaturation were noted for the heavily substituted [3 H]BrdU-DNA (Fig. 2). The thermal stability of the native and BrdU-treated DNA samples revealed accurate base pairing with sharp melting profiles (Fig. 3). These results suggested that the BrdU moieties were uniformly distributed throughout the cellular genome, and that the BrdU-DNA reassociated in a manner similar to native DNA despite a high degree of analog substitution.

FIG. 3. Thermal stability of high C_0t renatured DNA hybrids as measured on hydroxyapatite. Radioactive samples were adsorbed to columns equilibrated at 60° with 80 mM phosphate buffer. Washes of 0.18 M phosphate buffer were assayed for radioactivity as the column temperature was raised in small increments. ●, native, undenatured [^3H]dT-labeled DNA from rat embryo cells; ○, high C_0t renatured DNA labeled with 10^{-7} M [^3H]BrdU; □, high C_0t renatured DNA treated with 10^{-4} M [^3H]BrdU.

Properties of Lightly Substituted DNA. The high concentration (10^{-4} M) used may mask critical areas of dT replacement if BrdU effects are indeed caused by nonrandom substitution into particular DNA sequences. In order to detect possible selective distribution, the BrdU was diluted to 10^{-7} M, and added for 24 hr to rat embryo cell cultures in logarithmic phase. As shown in Fig. 4, the lightly substituted DNA migrated as a single peak of absorbance and radioactivity after centrifugation through equilibrium CsCl gradients. The slight increase in density of the DNA from 1.70

FIG. 4. Isopycnic CsCl density gradient of DNA treated with 10^{-7} M [^3H]BrdU. Sheared DNA was layered onto preformed, 5-ml CsCl gradients and centrifuged at $220,000 \times g$ for 30 hr at 25°. ●, A_{260} ; ▲, radioactivity; ○, CsCl .

FIG. 5. Reassociation kinetics of rat embryo DNA treated with 10^{-7} M [^3H]BrdU. A sample (1.3 ml; 900 $\mu\text{g}/\text{ml}$) in 0.40 M phosphate buffer, 3 mM EDTA, pH 6.8 was denatured for 10 min at 100° and incubated at 68°. Aliquots were sampled and assayed over hydroxyapatite for double-stranded radioactivity (●) and A_{260} (○).

to 1.71 g/cm^3 represented about 2–5% substitution of dT by radiolabeled BrdU (24).

The reassociation kinetics of the lightly substituted DNA closely resembled the previous renaturation plots when absorbance was monitored at 260 nm (Fig. 5). However, the reaction failed to advance far beyond the 55–60% level (C_0t 550) when samples were assayed for double-stranded radioactivity. A portion of the rat DNA remained single-stranded at high C_0t values long after 92% renaturation was determined by spectrophotometry. This finding suggested that BrdU-substituted unique DNA sequences were sparse, or were physically damaged so that renaturation was impaired.

We eliminated the latter interpretation by the following experiments. Unlabeled homologous DNA was added in excess to the [^3H]BrdU-labeled DNA (Fig. 6). In this instance, reassociation of the radioactive DNA was driven nearly to completion. Moreover, the labeled unique sequences recombined with their complements with a relatively high degree of thermal stability (Fig. 3).

Direct, confirmatory evidence of the nonuniform nature of BrdU incorporation was obtained by determining the specific

FIG. 6. Reassociation kinetics of DNA treated with 10^{-7} M [^3H]BrdU and excess unlabeled, sheared rat embryo DNA as measured over hydroxyapatite for double-stranded radioactivity. ●, 850 $\mu\text{g}/\text{ml}$ of DNA treated with 10^{-7} M [^3H]BrdU; ○, 50 $\mu\text{g}/\text{ml}$ of DNA treated with 10^{-7} M [^3H]BrdU + 2.0 mg/ml of unlabeled rat embryo DNA.

TABLE 1. Distribution of [^3H]dT and [^3H]BrdU in W/Fu rat embryo DNA after it was labeled for 24 hr

W/Fu rat embryo DNA	10 $^{-7}$ M [^3H]dT DNA			10 $^{-7}$ M [^3H]BrdU DNA		
	Relative specific activity	% of Genome	% of cpm	Relative specific activity	% of Genome	% of cpm
Complete genome	1.00	100	100	1.00	100	100
Repetitive sequences	0.83	24	20	1.36	24	33
Intermediate sequences	0.91	16	14	2.54	16	40
Unique sequences	0.94	60	56	0.35	60	21

activities of repetitive ($< C_{ot} 1$), intermediate (C_{ot} values 1–100), and unique ($> C_{ot} 100$) DNA from [^3H]BrdU- and [^3H]dT-treated rat embryo cells (Table 1). Entire hybridization reaction mixtures were assayed quantitatively over hydroxyapatite columns at the desired C_{ot} values. The rat cell DNA was thereby totally fractionated according to reassociation rates. Table 1 shows [^3H]dT was distributed in a relatively uniform manner throughout each of the three DNA fractions, whereas equimolar concentrations of [^3H]BrdU were localized mainly in the repetitive and intermediate regions. The unique sequences contained only about $1/4$ of the label, although comprising nearly $2/3$ of the rat cell DNA. Thermal stability determinations of each sample revealed sharp melting profiles in all instances (data not shown). Hence, 10^{-7} M [^3H]BrdU was selectively distributed throughout the rat cell genome as compared to thymidine.

DISCUSSION

At BrdU concentrations optimal for bioactivity (10^{-4} M), greater than 90% of the thymine moieties were replaced by BrdU. The present DNA reassociation kinetics revealed a uniform incorporation of the analog into the DNA of rat embryo cells at this dose. At a dose of 10^{-7} M, fewer than 5% of the thymine moieties were substituted by [^3H]BrdU, and the analog was selectively incorporated into particular DNA fractions, as judged from DNA-DNA renaturation kinetics. Equivalent dilutions of [^3H]dT were incorporated more uniformly into the DNA of rat embryo cells under comparable experimental conditions. We have demonstrated that certain BrdU-substituted sequences in the single copy DNA differ from unsubstituted in that they reassociate extensively only in the presence of excess unlabeled DNA. Thus, [^3H]BrdU does not serve as a tracer of all unique sequences as does [^3H]dT. These findings are consistent with a hypothesis by Levitt and Dorfman (1, 2) who suggested that the specific effects of BrdU on animal cells stem from nonrandom substitutions for thymidine and that BrdU is preferentially incorporated into those DNA sequences involved in the control of specialized cell functions.

Exposure of animal cells to low levels of BrdU has been reported to have altered chromosome transcription (13), to affect binding of chromatin proteins (14), and to interfere with histone dissociation (26). Since these results were obtained with BrdU concentrations greater than 10^{-7} M, our observations may reflect a baseline, selective substitution of BrdU into critical regulatory sites. Investigations are in progress to determine minimal BrdU doses at which biological effects can still be detected. For example, exposure of synchronized cell cultures to low doses of BrdU during S phase may indeed amplify such effects.

Current evidence implies a critical role for unique DNA sequences in eukaryotic cells in that close relationships exist between the transcription of nonrepeated DNA sequences and the expression of differentiated cell functions (27–30). Furthermore, the presence of viral genes in the nuclear DNA of transformed animal cells has been deduced from molecular hybridization experiments (20, 31–33). Although the mechanism of viral gene insertion is presently unknown, viral gene copies are found predominantly in association with the single copy regions of the DNA (20, 31–33). Although most BrdU effects on animal cells are predominantly inhibitory in nature, the induction of latent oncornaviruses by BrdU has been regarded as a suppression of transcription of a mRNA that codes for a repressor of viral synthesis (34). Furthermore, Britten and Davidson (35) have proposed the presence of regulatory elements adjacent to structural genes. Affinity of BrdU for either of these hypothetical sites might affect regulation of, or interaction with, nuclear proteins responsible for repression or derepression of cell-specific function.

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