LOCALIZATION OF 5-BROMODEOXYURIDINE DISTRIBUTION IN RAT DNA AS DETERMINED BY SINGLE-STRAND SPECIFIC NUCLEASES

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Summary: Secondary cultures of rat embryo cells were synchronized by a double thymidine block. Following release from synchrony, the first entire S phase (7.5 hrs) was pulsed with either 10^{-7}M [^{3}H]thymidine (TdR) or 10^{-7}M [^{3}H]5-bromodeoxyuridine (BrdU) in order to determine the localization and distribution of the thymidine analog in DNA. The DNA was extracted, purified by hydroxyapatite column chromatography, and dialyzed against a sodium acetate buffer at pH 4.5. Each DNA sample was then reacted with the single-strand specific nuclease of Aspergillus oryzae (S1 nuclease) and assayed over hydroxyapatite. Nearly 25% of the DNA was physically lost from all the samples following nuclease treatment. However, 21% of the [^{3}H]TdR moieties were digested, as compared to 62% of the [^{3}H]BrdU residues. Reaction of native DNA samples with the Neurospora crassa endonuclease at pH 8.0 resulted in little degradation. Dialysis of [^{3}H]guanosine-labeled rat embryo DNA against the acetate buffer alone caused a 24% level of depurination. These results suggest that mild depurination creates artifactual single-stranded regions in rat DNA which are apparently enriched in [^{3}H]BrdU as compared to [^{3}H]TdR.

The thymidine analog 5-bromodeoxyuridine (BrdU) induces the release of latent endogenous type C RNA virus from normal cells of several animal species (1-5). Although BrdU must be incorporated into the nuclear DNA of host cells for virogenic induction (6), no detailed mechanism(s) of action has yet been described.

We recently observed the induction and release of a latent type C RNA virus from normal rat embryo cell cultures exposed to BrdU (7). DNA-DNA recombination studies showed that [^{3}H]BrdU was substituted in middle-repetitive DNA sequences to a greater extent than was equimolar [^{3}H]thymidine (TdR) (8,9). Because the effects of BrdU on other biological systems are equally highly specific (10), it was presumed that knowledge of the localization and distribution of BrdU in DNA sequences of eukaryotic cells may be relevant to the mechanism of action.

This work further characterizes the non-random pattern of [^{3}H]BrdU distribution in rat embryo DNA as determined by treatment with single-strand specific nucleases.

Materials and Methods

Secondary cultures of 16-day old Wistar Furth rat embryos were initiated

and maintained as described (7,8). In order to uniformly expose all cells equally to radioactive DNA precursors, cultures were synchronized by a double thymidine block (11-13). Following release from synchrony, cells were pulsed for a single S phase with either 10^{-7}M [^{3}H]TdR (18.3 Ci/mM), or 10^{-7}M [^{3}H]BrdU (12.7 Ci/mM) (8,13). At the cessation of S phase, the cells were immediately lysed in SSC buffer (0.15M NaCl, 0.015M trisodium citrate, pH 7.0) containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. DNA was extracted, purified, and concentrated as before (8,9,13). Aliquots of labeled DNA preparations were centrifuged to equilibrium through neutral CsCl density gradients as described (8,9) in order to ascertain the purity and specific gravity of each sample.

Enzymatic treatment of the native DNA samples was performed with both the single-strand specific nuclease S1 from Aspergillus oryzae (14,15) (Miles Laboratories, Inc.), as well as the Neurospora crassa endonuclease (16,17) (Miles Laboratories, Inc.). For the S1 nuclease assay, DNA samples were dialyzed for 24 hrs against several changes of sodium acetate buffer (0.3M NaCl, 0.03M sodium acetate, 1mM $ZnCl_2$, pH 4.5) at 4°C (14,15). The final enzyme reaction mixtures consisted of 1 unit of S1 nuclease/µg of DNA in the acetate buffer, and were incubated for 1 hr at 37°C (14,15). For the Neurospora crassa endonuclease reaction, DNA samples were dialyzed at 4°C for 24 hrs against several changes of Tris-HCl buffer (0.1M Tris-HCl, 0.01M $MgCl_2$, pH 8.0) (17,18). Final enzymatic conditions were 1 unit of endonuclease/50 µg of DNA incubated at 37°C for 30 min (17,18). Following both types of enzymatic digestion, reaction mixtures were dialyzed against 0.08M sodium phosphate buffer (pH 6.8) at 4°C overnight in preparation for hydroxyapatite column chromatography.

The enzymatic reaction mixtures were passed through hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) columns at 60°C (8,9). Single-stranded and double-stranded DNA was eluted, and specific activities of each respective fraction were determined (8,9).

Results

When released from thymidine blockade, the rat embryo cells resumed the cell cycle at the G_1/S interface (11,13). The DNA synthesis phase began immediately, peaked at 5 hrs, and was essentially complete by 7.5 hrs (Figure 1). A mitotic wave followed, and persisted for nearly 1 hr. The cell cycle for rat embryo cell cultures was similar to that reported for an established line of normal rat kidney cells: $(S+G_2)+M+G_1=8.5+(.5-1)+2.5=12$ hrs (12). Because we had previously determined that a dose of 10^{-7}M BrdU was sufficient for non-random incorporation in DNA as well as for maximal endogenous virus-specific expression (13), cell cultures were pulsed for

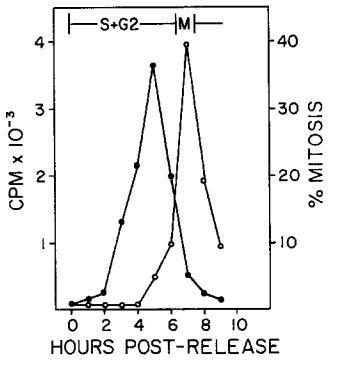

Figure 1. Synchronization of rat embryo cells. Replicate cultures were synchronized by a double thymidine block. Individual samples were pulsed for 30 min with 10 μCi/ml of [^{3}H]TdR and assayed for acid-insoluble radioactivity each hour following release (●). Coverslips of cells were fixed and stained hourly for mitotic figures (○).

the entire 7.5 hr S phase with either 10^{-7}M [^{3}H]TdR or equimolar [^{3}H]BrdU. This assured that the purified, radioactive DNA was nearly representative of a single, complete rat genome.

Following centrifugation to equilibrium through neutral CsCl density gradients, the [^{3}H]TdR- and [^{3}H]BrdU-labeled DNA samples banded as single peaks (Figure 2). The specific gravity of 1.72 of the [^{3}H]BrdU-labeled fraction represented less than 7% substitution of bromouracil for thymine when compared to the specific gravity (1.70) of the untreated DNA (18). The sharp profiles suggested that the DNA preparations were of high purity with nearly comparable buoyant densities.

Native DNA samples were adsorbed to hydroxyapatite columns, and only the double-stranded fractions were prepared for enzymatic digestion with both types of nuclease. For optimal S1 nuclease conditions, the DNA samples were made pH 4.5 in acetate buffer (14,15) by dialysis for 24 hrs at 4°C. To determine whether acid-induced degradation of DNA (depurination) resulted from this treatment alone, a sample of rat embryo cell DNA was prepared in the manner described following a single S phase pulse label with 10^{-7}M [^{3}H]guanosine. This DNA was made acidic in the sodium acetate buffer under identical conditions, and the extent of depurination (decrease in specific activity) was measured. Essentially all of the DNA was recovered following dialysis only, although approximately 76% of the [^{3}H]guanine remained (Table 1). Therefore, nearly 25% depurination occurred under the conditions of dialysis against the sodium acetate buffer.

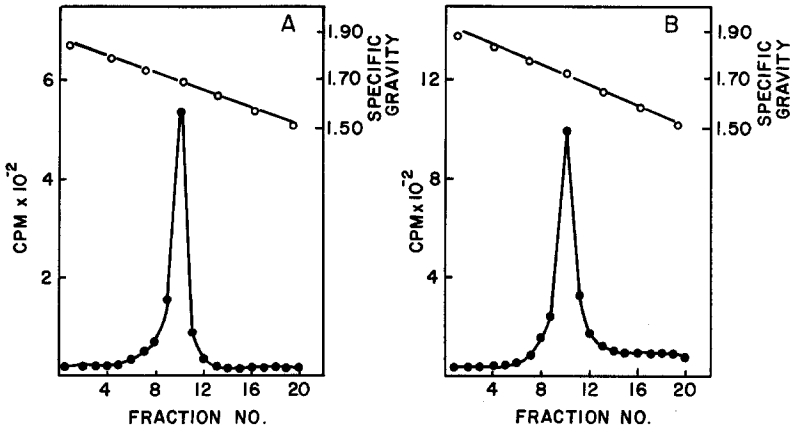

Figure 2. Isopycnic, neutral CsCl density gradients of rat embryo cell DNA. Gradients were formed during centrifugation to equilibrium in a Spinco SW-50.1 rotor at 220,000 x g for 48 hrs at 22°C. A, 10^{-7}M [^{3}H]TdR-DNA labeled during a single S phase. B, 10^{-7}M [^{3}H]BrdU-DNA labeled during a single S phase. ●, radioactivity. O, CsCl.

TABLE 1

	Before Dialysis	After Dialysis
Buffer	SSC, pH 7.0	Sodium acetate, pH 4.5
DNA Amount	59.2 µg	60.0 µg
Radioactivity	56,320 cpm	42,900 cpm
Specific Activity	945 cpm/µg	715 cpm/µg

Depurination of [^{3}H]guanosine-labeled rat embryo cell DNA following acid treatment. The entire DNA sample was dialyzed for 24 hrs against several changes of acetate buffer at 4°C. Specific activities were recorded before and after dialysis as a measure of depurination. SSC is 0.15M NaCl, 0.015M trisodium citrate, pH 7.0.

The types of DNA produced from digestion with the Aspergillus oryzae S1 nuclease and hydroxyapatite fractionation are summarized in Table 2. About 75% of the DNA was physically recovered following nuclease digestion, regardless of the type of labeled preparation used. Although the loss of

[^{3}H]thymine correlated with the corresponding loss of DNA, nearly 62% of the [^{3}H]bromouracil residues were missing from the [^{3}H]BrdU-treated sample. This represented a net decrease in radioactivity of nearly 40% when compared to [^{3}H]TdR-treated DNA. The corresponding specific activities of the double-stranded DNA fractions reflected this finding as well. The [^{3}H]TdR-DNA values were comparable before and after enzyme treatment, whereas the specific activity of the [^{3}H]BrdU-DNA had decreased nearly 50% following nuclease digestion.

In order to evaluate the influence of the depurination conditions on the above results, another single-strand specific nuclease with an optimal pH of 8.0 was used. The Neurospora crassa endonuclease assay required essentially no physical pretreatment which could influence DNA structural integrity. Following dialysis against the pH 8.0 Tris-HCl buffer, the native DNA samples were reacted with the enzyme as reported (16,17), and assayed over hydroxyapatite. Unlike the appreciable degradation observed with the S1 nuclease, the endonuclease from Neurospora crassa did not significantly digest DNA (Table 3).

The [^{3}H]TdR- and [^{3}H]BrdU-labeled DNA samples were recovered nearly quantitatively (Table 3). Furthermore, no single-stranded products were produced from the reactions with either DNA type, and the double-stranded specific activities were relatively unchanged as well. Therefore, when the native DNA samples were presented in a structurally intact form, few if any single-stranded regions were available to the endonuclease. To confirm the validity of these findings, both nuclease types were reacted with heat denatured rat embryo DNA under their respective optimal enzymatic conditions. Degradation was complete in both instances.

Discussion

The present work further characterizes the nature of incorporation and distribution of [^{3}H]BrdU in rat embryo cell DNA. The submaximal depurination which resulted from extensive dialysis in mild acid conditions produced artifactual single-stranded regions in native DNA. These sites were not available to a single-strand specific endonuclease if pretreatment under acid conditions was omitted. However, if the depurinated DNA was subsequently reacted with S1 nuclease, [^{3}H]bromouracil residues were removed to a relatively greater extent than was [^{3}H]thymine. Essentially no digestion occurred if the DNA was not previously depurinated.

We reported that 10^{-7}M [^{3}H]BrdU was incorporated in repetitive and middle-repetitive nucleotide sequences in rat DNA to a greater degree than was equimolar [^{3}H]TdR (8,9,13). Straus and Birnboim (19) recently described

TABLE 2

DNA	Enzyme	Single-Stranded DNA (μg)	Single-Stranded cpm	Double-Stranded DNA (μg)	Double-Stranded cpm	Double-Stranded DNA Lost	Double-Stranded cpm Lost	Double-Stranded Specific Activity (cpm/μg)
[^{3}H]TdR	-	28.75	4,960	89.00	124,184	———	———	1395
"	+	20.00	1,780	65.25	98,297	26%	21%	1506
[^{3}H]BrdU	-	25.28	63,029	92.00	185,312	———	———	2014
"	+	28.70	54,173	67.00	69,690	27%	62%	1040

Digestion of rat embryo cell DNA with S1 nuclease following dialysis against soldium acetate buffer, pH 4.5. Post-enzymatic reaction mixtures were made 0.08M in sodium phosphate buffer, pH 6.8, and assayed by hydroxyapatite chromatography at 60°C.

TABLE 3

DNA	Enzyme	Single-Stranded DNA (μg)	Single-Stranded cpm	Double-Stranded DNA (μg)	Double-Stranded cpm	Double-Stranded DNA Lost	Double-Stranded cpm Lost	Double-Stranded Specific Activity (cpm/μg)
[^{3}H]TdR	-	0	0	150.00	202,410	———	———	1349
"	+	0	0	140.00	193,077	6%	4%	1378
[^{3}H]BrdU	-	0	0	196.60	438,369	———	———	2229
"	+	0	0	188.30	420,620	4%	4%	2233

Digestion of rat embryo cell DNA with Neurospora crassa endonuclease following dialysis against Tris-HCl buffer, pH 8.0. Post-enzymatic reaction mixtures were made 0.08M in sodium phosphate buffer, pH 6.8, and analyzed by hydroxyapatite chromatography at 60°C.

the presence of long pyrimidine tracts in L-cell DNA which were associated with repeated nucleotide sequences. Similar tracts may exist in rat DNA, and mild depurination could conceivably generate clusters of single-stranded regions enriched in [^{3}H]BrdU. Their removal by S1 nuclease would be compatible with the results of hydroxyapatite analysis of Table 2. We are

presently studying the respective size and sequence complexity of the residual DNA species to determine whether nucleotide sequences of a particular repetition frequency were preferentially digested.

Case and Baker (20) have recently reported the position of regularly spaced single-stranded regions relative to BrdU-sensitive sites in sea urchin morula DNA. The authors found that BrdU was preferentially localized in these regularly spaced, single-stranded regions. It would be of interest to determine whether findings such as these are indicative of the native structure of genes, or whether their S1 nuclease reaction physical conditions revealed the non-random BrdU localization in a manner similar to our findings. Regardless of the mechanism involved, it appears that the single-strand specific nucleases are of great value in the elucidation of gene structure and organization.

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