NON-RANDOM INCORPORATION OF 5-BROMODEOXYURIDINE IN RAT CELL DNA

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Received September 26,1974

Summary: Secondary cultures of rat embryo cells were exposed for 24 hrs. to 10^{-7}M [^{3}H] thymidine (TdR) or 10^{-7}M [^{3}H]5-bromodeoxyuridine (BrdU) in order to localize and compare the distribution of the isotopes in DNA. DNA was extracted, sheared, and centrifuged to equilibrium through neutral and alkaline CsCl density gradients. The DNA band from each gradient type was separated into a "heavy" and "light" fraction, and DNA-DNA reassociation hybridizations were performed on each sample. Renaturation profiles revealed that each fractionated DNA sample was representative of the complete rat cell genome, except for the "light" [^{3}H]BrdU-DNA prepared by centrifugation through alkaline CsCl gradients. This fraction was predominantly depleted of labeled late repetitive and intermediate sequences. Uncentrifuged rat DNA was sequentially fractionated during reassociation into rapidly, intermediate, and slowly reassociating sequences by hydroxyapatite chromatography. Relative specific activities of each component revealed a non-uniform distribution of [^{3}H]BrdU moieties as compared to [^{3}H]TdR. These results suggest a nonrandom incorporation of 10^{-7}M BrdU into rat cell DNA sequences.

The effects of the thymidine analog 5-bromodeoxyuridine (BrdU) on animal cells are manyfold (1-5). Suppression or activation of specific cellular functions are known to occur when bromouracil substitutes for thymine in DNA (1-6). Moreover, functional alterations between nuclear proteins and BrdU-treated DNA have recently been described suggesting that knowledge about the sites of BrdU integration in DNA may be relevant to the mechanism of BrdU effects (7-9).

We recently observed the induction and release of a latent type C virus from normal rat embryo cell cultures exposed to BrdU (6, 10). Reassociation hybridization profiles of rat DNA suggested that incorporation of bromouracil was nonrandom at low concentrations (10^{-7}M) of BrdU in contrast to equimolar concentrations of [^{3}H]TdR (11). For the present work, we fractionated rat cell DNA into "heavy" and "light" components following centrifugation through neutral and alkaline CsCl equilibrium density gradients. DNA-DNA reassociation studies of these samples, as well as hydroxyapatite-fractionated components, confirmed the selective and non-uniform distribution of [^{3}H]BrdU in rat DNA.

Materials and Methods

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

tiated and maintained as described (6, 10). Roller bottle cell cultures in log phase were exposed to 50 ml of medium 199 (Grand Island Biologicals Co.) made 10^{-7}M in [^{3}H]TdR (18.3 Ci/mM) or 10^{-7}M in [^{3}H]BrdU (12.7 Ci/mM) for 24 hrs. Cells were harvested with trypsin, washed, concentrated by low-speed centrifugation and DNA was extracted and purified as described (6,11). Purified DNA was then sheared at 4° in a Ribi cell fractionator at 50,000 p.s.i. (11, 12). This procedure consistently prepared DNA fragments 300-400 nucleotides long with a 5.4 S sedimentation coefficient as determined by the method of Studier (13). Unlabeled, bulk DNA was extracted from rat embryo cells, purified, sheared, and stored in the same manner.

Aliquots of labeled DNA preparations were thawed and centrifuged to equilibrium through neutral and alkaline CsCl density gradients. Optical grade CsCl was dissolved in either 0.08M sodium phosphate buffer (PB) (pH 6.8) or 0.3N NaOH (pH 13.0) to an initial overall density of 1.70 g/cm^3. Linear gradients were formed during centrifugation in a Spinco

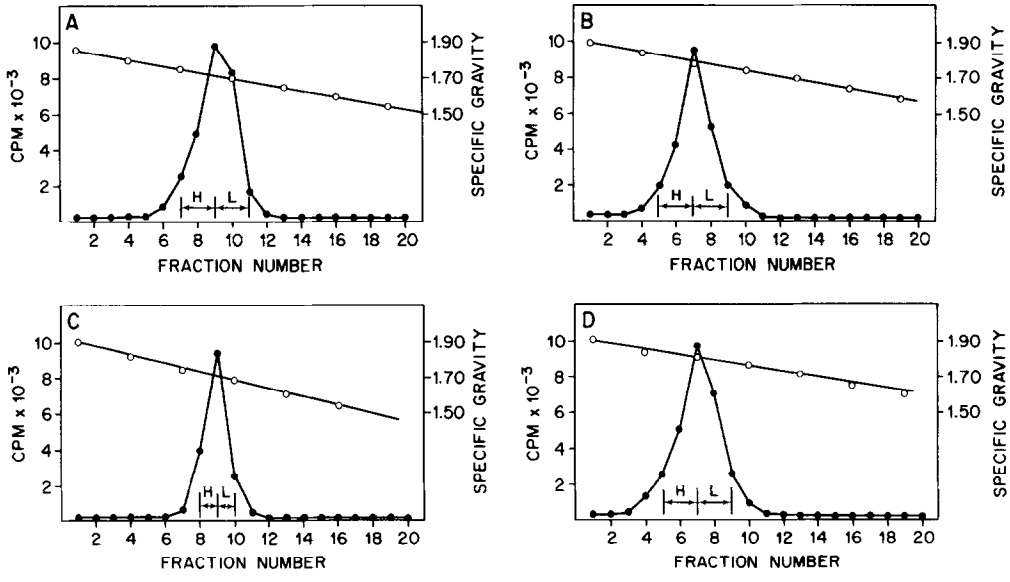


Fig. 1. Sedimentation and fractionation of rat embryo DNA through neutral and alkaline CsCl equilibrium density gradients. All samples were prepared in a Spinco SW-50.1 rotor at 165,000 x g for 48 hrs at 22°C. "Heavy" (H) and "light" (L) DNA aliquots were fractionated and pooled as shown. A; [^{3}H]TdR-labeled DNA in neutral CsCl. B; [^{3}H]TdR-labeled DNA in alkaline CsCl. C; [^{3}H]BrdU-labeled DNA in neutral CsCl. D; [^{3}H]BrdU-labeled DNA in alkaline CsCl. o—o; CsCl.

SW-50.1 rotor at 165,000 x g for 48 hrs at 22°. Aliquots were removed from the bottom of the tubes and assayed for radioactivity, A_{260}, and refractive index.

DNA-DNA reassociation experiments were performed and analyzed over a column of hydroxyapatite as described (11, 12, 14). Reaction mixtures were adsorbed to a column equilibrated with .08M PB at 60°C. Single-stranded DNA was eluted first with 3 column volumes of .18M PB. Double-stranded DNA was then eluted with a comparable volume of .4M PB.

Results

DNA from rat embryo cell cultures sedimented uniformly through neutral and alkaline CsCl gradients (Fig. 1.). The [^{3}H]TdR-labeled DNA banded at densities of 1.70 and 1.78 g/cm^3, whereas the [^{3}H]BrdU-labeled DNA sedimented as single density peaks of 1.72 and 1.80 g/cm^3 through neutral and alkaline CsCl, respectively. A_{260} profiles corresponded closely with the distribution of radioactivity in all instances. Fractions were combined from each gradient band to contain DNA of densities either greater or less than that of the peak region (Fig. 1). Thus, each band was fractionated into a "heavy" and "light" DNA fraction according to the distribution of radioactivity in CsCl equilibrium gradients. All "heavy" and "light" DNA samples were denatured and

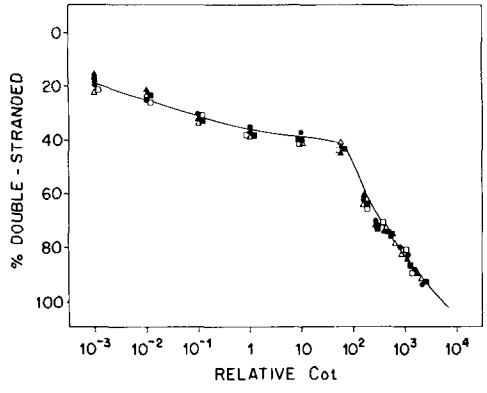
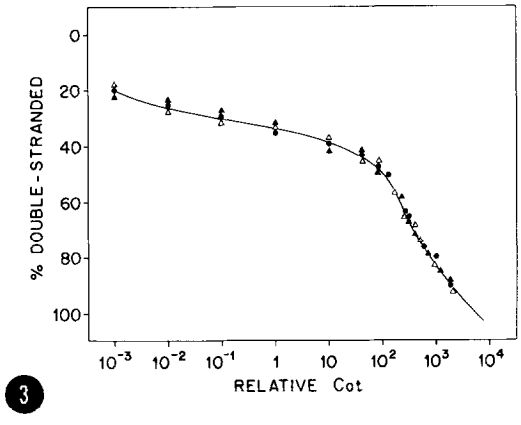


Fig. 2. DNA-DNA reassociation profiles of gradient-prepared [^{3}H]TdR-labeled DNA. Double stranded radioactivity determined over hydroxyapatite. All reaction mixtures maintained at 68°C in 0.40M PB, 1 mM EDTA, pH 6.8 following thermal denaturation at 100°C for 15 min. Relative Cot (mol-sec/l) values determined as before (11, 14). ●; 600 µg/ml, complete, [^{3}H]TdR-labeled rat DNA control. ▲; 820 µg/ml "heavy" [^{3}H]TdR-DNA from neutral CsCl. △; 817 µg/ml "light" [^{3}H]TdR-DNA from neutral CsCl. ■; 815 µg/ml "heavy" [^{3}H]TdR-DNA from alkaline CsCl. □; 817 µg/ml "light [^{3}H]TdR-DNA from alkaline CsCl.

individually reassociated in the presence of excess unlabeled rat DNA to determine whether segregation of particular sequences had occurred during centrifugation.

The reassociation pattern of unfractionated, complete [^{3}H]TdR-labeled DNA was typical for rat cells (11, 15) (Fig. 2). The rapidly reassociating component (approximately 24% of the genome) recombined by a Cot of 1. The intermediate (16% of genome), and slowly reassociating (60% of genome) DNA sequences hybridized extensively at a Cot value greater than 2,000. The "heavy" and "light" [^{3}H]TdR-labeled DNA each reassociated similar to the complete genome, regardless of whether fractionation occurred through neutral or alkaline gradients. These reassociation profiles suggested that each fractionated [^{3}H]TdR-DNA sample was representative of the entire rate genome. The "heavy" and "light" BrdU-treated DNA prepared through neutral CsCl gradients reannealed comparably to the [^{3}H]TdR-labeled DNA (Fig. 3). However, these reassociation profiles differed from the homologous [^{3}H]BrdU-DNA fractionated through alkaline CsCl (Fig. 4).

Although the "heavy" BrdU-DNA renaturation profile resembled the control plots, the "light" DNA fraction reassociated in a manner which suggested relatively fewer labeled late repetitive and intermediate sequences (15). Since bromouracil moieties sediment further than thymine



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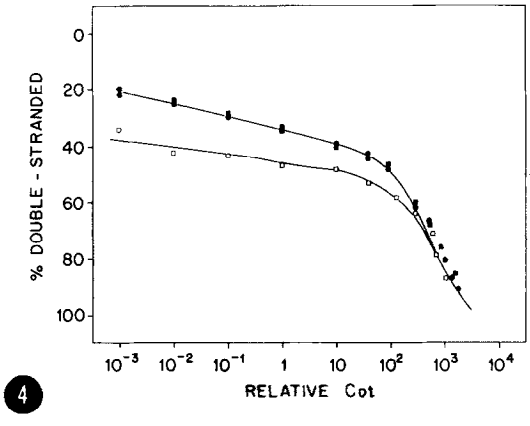

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Fig. 3. DNA-DNA reassociation profiles of [^{3}H]BrdU-labeled DNA prepared through neutral CsCl gradients. ●; 800 µg/ml complete [^{3}H]BrdU-DNA control. ▲; 750 µg/ml "heavy" [^{3}H]BrdU-DNA. △; 747 µg/ml "light" [^{3}H]BrdU-DNA.

Fig. 4. DNA-DNA reassociation profiles of [^{3}H]BrdU-DNA fractionated through alkaline CsCl gradients. ●; 800 µg/ml complete [^{3}H]BrdU-DNA control. ■; 616 µg/ml "heavy" [^{3}H]BrdU-DNA. □; 615 µg/ml "light [^{3}H]BrdU-DNA.

TABLE 1

DNA Source	[^{3}H]TdR-DNA			[^{3}H]BrdU-DNA		
	Relative Specific Activity	% of Genome	% of CPM	Relative Specific Activity	% of Genome	% of CPM
Complete rat genome	1.00	100	100	1.00	100	100
Repetitive sequences	.83	24	20	1.36	24	33
Intermediate sequences	.91	16	14	2.54	16	40
Unique sequences	.94	60	56	.35	60	21

Distribution of [^{3}H]TdR and [^{3}H]BrdU in rat embryo DNA after 24 hr label. Entire reaction mixtures were sequentially reassociated to Cots 1 (repetitive), 100 (intermediate), and 2,000 (unique) by fractionation over hydroxyapatite columns. Specific activities were recorded for each component and compared to original.

through linear CsCl density gradients (16), denaturation of BrdU-DNA in alkaline conditions apparently permitted a redistribution of single-stranded repetitive and intermediate from slowly reassociating sequences. Melting curve analyses performed on all hybrids revealed high thermal stability, implying accurate base pairing (11, 12, 14).

Complete [^{3}H]TdR and [^{3}H]BrdU-labeled rat DNA was quantitatively and sequentially fractionated over hydroxyapatite during reassociation. The entire reaction mixture was passed over hydroxyapatite at Cot 1. Double-stranded DNA (rapidly reassociating) was separated from single-stranded (intermediate and slowly renaturing). The latter material was incubated to Cot 100 and passed again over hydroxyapatite. This separated intermediate (double-stranded) from unique sequences among DNA. The single-stranded DNA was then reassociated to Cot 2000. Specific activities were measured for each fraction and compared to the initial value (Table 1). Unlike [^{3}H]TdR moieties which were apparently distributed uniformly throughout all DNA sequences, labeled bromouracil was apparently concentrated into rapidly and intermediate renaturing sequences, with light incorporation in the unique regions. These observations are consistent with the reassociation patterns of DNA populations prepared from CsCl gradients.

Discussion

The present DNA-DNA reassociation experiments further characterize the substitution of bromouracil following fractionation of DNA through neutral and alkaline CsCl equilibrium density gradients. All [^{3}H]TdR-labeled "heavy" and "light" DNA fractions reassociated with profiles representative of the complete rat embryo cell genome. Differences in renaturation plots only occurred between the "heavy" and "light" [^{3}H]BrdU-labeled DNA prepared by centrifugation through alkaline CsCl gradients. These differences were confirmed by the relative specific activities of hydroxyapatite-fractionated sequences derived from uncentrifuged DNA.

The overall labeling efficiency of [^{3}H]TdR and [^{3}H]BrdU into rat DNA was consistently similar as judged from initial specific activities. Had the [^{3}H]BrdU been uniformly incorporated through the rat DNA, segregation of multiple-copy from single-copy sequences during alkaline CsCl centrifugation would not be expected. Levitt and Dorfman (1, 2), and others (17) have proposed that the highly specific effects of BrdU stem most likely from non-random and differential substitutions by bromouracil into those DNA sequences responsible for the control of specialized cell functions. Our observations are in agreement with such an hypothesis.

Acknowledgement

This work was supported by Damon Runyon-Walter Winchell Research Grant #DRG-2F, USPHS Research Grant CA-14898, and USPHS Research Grant CA-14599.

References

1. Levitt, D., and Dorfman, S. (1972) Proc. Nat. Acad. Sci. USA 69: 1253-1257.
2. Levitt, D., and Dorfman, A. (1973) Proc. Nat. Acad. Sci. USA 70: 2201-2205.
3. Abbott, J., Mayne, R. and Holtzer, H. (1971) Devel. Biol. 28: 430-442.
4. Turkington, R.W., Mamjumder, G.C., and Riddle, M. (1971) J. Biol. Chem. 28: 1814-1819.
5. Teich, N., Lowy, D.R., Hartley, J.W., and Rowe, W.P. (1973) Virology. 51: 163-173.
6. Schwartz, S.A., and Kirsten, W.H. (1974) Virchows Archiv Ab. B. Zellpath. 15: 177-186.
7. Lin, S., and Riggs, A.D. (1974) Fed. Proc. 33, Abs. 1865.
8. Walther, B.T., Pictet, R.L., David, J.D., and Rutter, W.J. (1974) J. Biol. Chem. 249: 1953-1964.
9. Gordon, J.S., David, J.W., Bell, G., McCarthy, B.J., and Rutter, W.J. (1973) J. Cell Biol. 59: 1162.
10. Schwartz, S.A., Panem, S., Stefanski, E., and Kirsten, W.H. (1974) Cancer Res. 34: 2255-2259.
11. Schwartz, S.A., and Kirsten, W.H. (1974) Proc. Nat. Acad. Sci. In press.
12. Casey, J., Gordon, P., and Rabinowitz, M. (1974) Biochem. 13: 1059-1067.
13. Studier, F.W. (1965) J. Mol. Biol. 11: 373-381.
14. Kohne, D.E., and Britten, R.J. (1971) Proc. Nuc. Acid Res. 2: 500-512.
15. Holmes, D.S., and Bonner, J. (1974) Biochem. 13: 841-848.
16. Rownd, R. (1967) Biochem. Biophys. Acta 134: 464-467.
17. Preisler, H.D., Housman, D., Scher, W.S., and Friend, C. (1973) Proc. Nat. Acad. Sci. USA 70: 2956-2959.