INCREASED INCORPORATION OF 5-BROMODEOXYURIDINE INTO THE DNA OF PROLIFERATING TISSUES IN PARTIALLY HEPATECTOMISED MICE

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1. Introduction

5-Bromodeoxyuridine (BrdU) has been widely used as a tool to investigate DNA replication in eukaryotic cells [1]. BrdU inhibits the expression of cellular differentiation in specialized cells in tissue culture, or in embryonic rudiments in organ cultures in vitro [2–6]. BrdU is incorporated into DNA as a structural analogue of thymidine in both bacterial [7, 8], and mammalian cells [9, 10] in vitro, but any attempt to study its effects on differentiated functions in vivo has been severely limited by the rapidity with which it is dehalogenated and catabolized [11]. Gross and Rabinowitz [12] in studying the synthesis of mitochondrial and nuclear DNA in rat liver found it necessary to inject 5-fluorouracil before and during treatment with BrdU, in order to obtain significant incorporation of the 5-halogenated uracil moiety into DNA. A recent observation by E. Farber and H. Sarma (personal communication) that BrdU was incorporated into regenerating rat liver, and the fact that BrdU is mainly catabolized in the liver [11] prompted us to hypothesize that the incorporation of BrdU into animal tissues could be markedly increased by removing two-thirds of the liver.

This paper reports that the incorporation of BrdU into the DNA of rapidly proliferating tissues is indeed markedly increased if BrdU injections are given in the first few hours after partial hepatectomy. The rapidly proliferating tissues in these experiments were the intestinal epithelial cells, the spleen and the isoproterenol-stimulated parotid glands of mice. This experimental method for increasing the incorporation of BrdU into DNA in vivo may be useful in investigating the in vivo effects of BrdU on cellular proliferation and differentiation, and elucidating the actual mechanisms involved.

2. Materials and methods

Fels A male mice bred in this laboratory and weighing approx. 30 g were used. Partial hepatectomy consisted of the removal under anesthesia of the median and left lateral lobes of the liver. Immediately after partial hepatectomy the mice received at hourly intervals 5 intraperitoneal (i.p.) injections of BrdU (0.4 μmoles/g body weight) in isotonic saline. Between the third and fourth injection of BrdU each mouse was given a single i.p. injection of 25 μCi of [^{3}H]methyl thymidine (specific activity 20 Ci/mmole, purchased from New England Nuclear Corporation). The mice, in groups of four animals, were killed, by cervical dislocation 30 min after the last injection of BrdU, and the intestine and spleen were removed. The intestinal mucosa was scraped free from the muscularis prior to the initial homogenization step for DNA extraction.

Another group of mice was injected with dl-isoproterenol (0.3 μmoles/g body weight, obtained from Winthrop Laboratories, New York) dissolved in water. Isoproterenol was injected 24 hr prior to partial hepatectomy so that the animals received the BrdU injections at the time of maximum DNA synthesis in the isoproterenol-stimulated parotid glands. The schedule of BrdU and [^{3}H]thymidine injections were exactly the same as for the animals described above. After sacrifice, the parotids were

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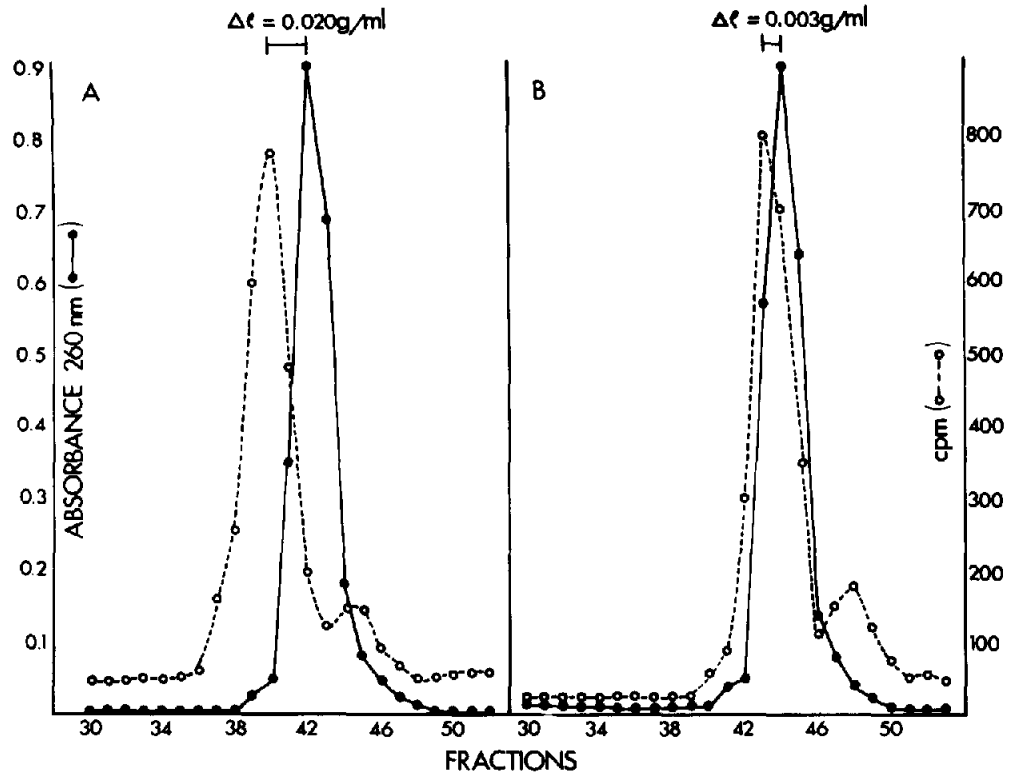

Fig. 1. Density profile in Cs_2SO_4 gradients of DNA isolated from the intestinal mucosa of mice that were given 5 injections of BrdU (0.4 μmoles/g body weight) and 25 μCi of [^{3}H] thymidine 90 min prior to sacrifice: A) Mice injected with BrdU and [^{3}H]Tdr after partial hepatectomy; B) Non-hepatectomized mice receiving the same treatment.

dissected out and freed of lymph nodes and fat. As controls, groups of mice, non-hepatectomized, received a similar number of injections of BrdU and [^{3}H]thymidine. Each experiment was repeated at least twice on two groups of animals.

In all cases DNA was extracted from each of the three tissues by the Marmur procedure [13].

The technique of Sato et al., [14] was followed for cesium sulfate density gradient centrifugation. Solid Cs_2SO_4 (obtained from Harshaw Cemical Co., Solon, Ohio, U.S.A.) was added to 20-ml samples containing 100–250 μg of DNA. The density was adjusted to 1.52 g/ml with a Bausch and Lomb refractometer. All samples were centrifuged in a Beckman 50 Ti rotor for 60 hr at 20°C and 45 000 rpm. After centrifugation the bottom of the tube was pierced and 10-drop fractions were collected and analysed for refractive index and absorbance at 260 nm. The radioactivity of each fraction, after precipitation with trichloroacetic acid and collection on a nitrocellulose membrane filter was determined in a Packard liquid scintillation counter, using a cellosolve–toluene scintillant [15].

3. Results and discussion

The density profile in Cs_2SO_4 gradients of DNA extracted from intestinal epithelial cells of mice, treated with repeated injections of BrdU is shown in fig. 1. Fig. 1A shows the density profile of DNA from animals receiving BrdU immediately after partial hepatectomy. Fig. 1B shows the density profile of DNA isolated from the intestine of non-hepatectomized animals. In partially hepatectomized animals there was a separation of the optical density and radioactivity bands. The optical density band corresponds to the

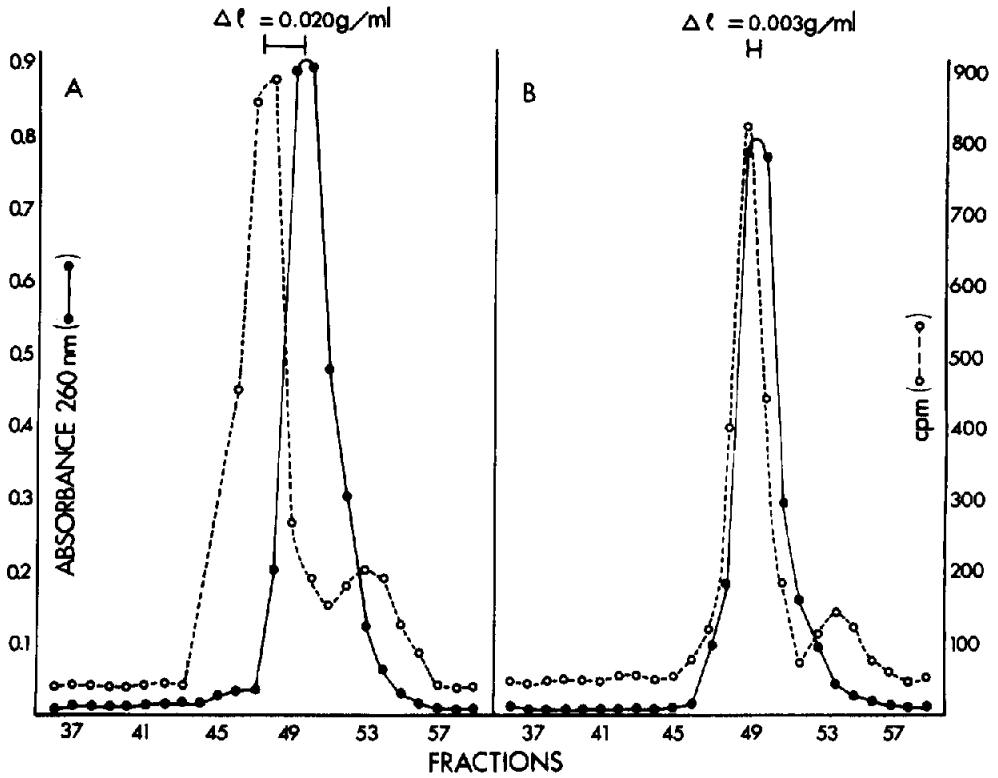

Fig. 2. Density profile in Cs_2SO_4 gradients of DNA isolated from the parotid glands of mice stimulated with isoproterenol (0.3 μmoles/g body wt). Twenty hours after isoproterenol the mice received 5 injections of BrdU (0.4 μmoles/g body wt) and 25 μCi of [^{3}H]thymidine 90 min prior to sacrifice: A) partial hepatectomy 20 hr after isoproterenol; B) no hepatectomy. Fractions 1–35 and 60–65 contained negligible radioactivity or absorbance and are not plotted in this figure. Each fraction contained 10 drops of the gradient.

bulk of the previously existing DNA, while the radioactivity band corresponds to the newly synthesized DNA containing BrdU. The separation of the peaks of these bands is equivalent to a difference in buoyant density of 0.020 g/ml, indicating that approx. 40% of the thymine residues have been replaced with bromouracil in one strand [16]. In animals not undergoing partial hepatectomy the separation between optical density and radioactivity bands was negligible, corresponding to a buoyant density difference of only 0.003 g/ml. This result indicates negligible replacement of thymine by BrdU in DNA under these conditions.

Similar results were obtained with DNA extracted from parotid glands of mice injected with isoproterenol. After a single injection of isoproterenol the maximum stimulation of DNA synthesis in parotid glands occurs between the 20th and the 28th hr [17]. By performing a partial hepatectomy in animals that had received isoproterenol 20 hr before, we therefore maximized the incorporation of BrdU into the parotid glands. Fig. 2 shows that in the stimulated parotid glands, just as in the intestinal mucosa, partial hepatectomy causes a considerable increase in the incorporation of BrdU into DNA.

In addition to the major radioactive peak, a second labeled peak with a density lower than hybrid DNA, was observed in all these samples. This bimodal profile of BrdU-substituted DNA has been previously reported after phytohemagglutinin [18] or antigenic stimulation [19] of lymphocytes, but was shown to disappear when DNA was broken up by sonication. This result was explained as an artifact of the gradient fractionation technique. BrdU-substituted DNA fragments, which are selectively large, were delayed from entering the hole through which the gradient is

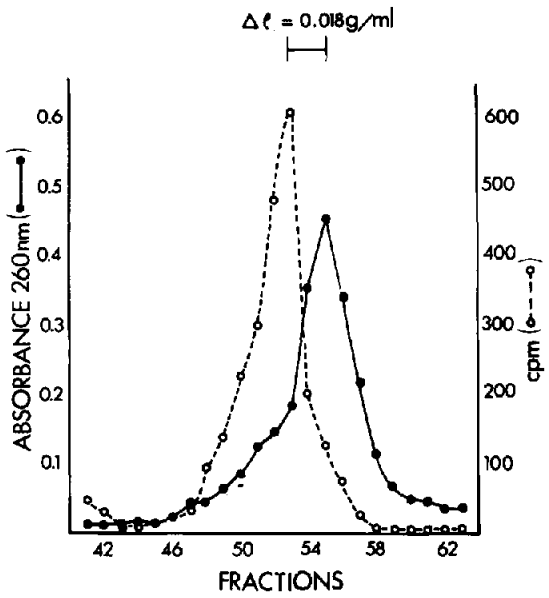

Fig. 3. Density profile in Cs_2SO_4 gradients of a sonicated extract of DNA isolated from the spleens of partially hepatectomized mice treated with 5 injections of BrdU (0.4 μmoles/g body weight) and 25 μCi of [^{3}H]thymidine 90 min prior to sacrifice. Fractions 1–40 and 64–70 which contained negligible radioactivity and absorbance were excluded from this graph.

collected [20]. Fig. 3 shows the density profile in Cs_2SO_4 gradients of DNA extracted from the spleens of partially-hepatectomized mice treated with BrdU and sheared by sonication (Branson sonicator, for 2 periods of 5 sec at 20 MHz at 50 W). Under these conditions, and confirming previous observations [20], the hybrid DNA is in the form of a single radioactive peak which exhibits a difference in buoyant density of 0.018 g/ml from the optical density band. No shift in buoyant density was observed in DNA isolated from the spleens of mice injected with BrdU, but without partial hepatectomy; the peaks of optical density and racioactivity coincided (data not shown).

In conclusion, these data demonstrate that after partial hepatectomy the incorporation of BrdU into DNA may be markedly increased in vivo. Incorporation has been demonstrated in rapidly proliferating tissues such as the small intestine, spleen and isoproterenol-stimulated salivary glands of mice. The reason for this increased incorporation is probably due to the fact that the dehalogenation of bromodeoxyuridine occurs in the liver of rats or mice. Removal of two-thirds of the liver by partial hepatectomy probably reduces the capability of the liver to dehalogenate the BrdU, thus allowing a considerable increase in its incorporation into the DNA of rapidly proliferating tissues. This technique may now be utilized to study in vivo the mechanisms by which BrdU preferentially inhibits the synthesis of differentiated products which are characteristic of certain cells.

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References

[1] Simon, E.H. (1961) J. Mol. Biol. 3, 101–109.
[2] Abbott, J. and Holtzer, H. (1968) Proc. Natl. Acad. Aci. U.S. 59, 1144–1151.
[3] Stockdale, F., Ozaki, K., Nameroff, M. and Holtzer, H. (1964) Science 146, 533–535.
[4] Wilt, F.H. (1965) J. Mol. Biol. 12, 331–341.
[5] Kreider, J.W., Del Villano, B., Shoff, W.H. and Davidson, E.A. (1972) Cancer Res. 32, 2148–2155.
[6] Silagi, S. (1971) In Vitro 7, 105–114.
[7] Zamenhof, S. and Griboff, G. (1954) Nature 174, 306–307.
[8] Dunn, D.B. and Smith, J.D. (1957) Biochem. J. 67, 494–506.
[9] Cheong, L., Rich, M.A. and Edinoff, M.L. (1960) J. Biol. Chem. 235, 1441–1447.
[10] Prusoff, W.H. (1959) Federation Proc. 18, 305.
[11] Barrett, H.W. and West, R.A. (1956) J. Am. Chem. Soc. 78, 1612–1615.
[12] Gross, N.J. and Rabinowitz, M. (1969) J. Biol. Chem. 244, 1563–1566.
[13] Marmur, J. (1961) J. Mol. Biol. 3, 208–218.
[14] Sato, S., Ariake, S., Saito, M. and Sugimura, T. (1972) Biochem. Biophys. Res. Commun. 49, 827–834.
[15] Gilman, A.G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305–312.
[16] Kohnlein, W., Thorsett, G. and Hutchinson, F. (1966) Biochim. Biophys. Acta 119, 195–197.

[17] Baserga, R. and Heffler, S. (1967) Exptl. Cell Res. 46, 571–580.
[18] Gottlieb, A., Taylor, L. and Sinsheimer, F. III (1970) Biochemistry 9, 4322–4328.
[19] Souleil, C. and Panijel, J. (1973) Exptl. Cell Res. 78, 462–466.
[20] Souleil, C. and Panijel, J. (1972) Eur. J. Biochem. 30, 234–241.