FORMATION OF dUMP-CONTAINING DNA BY Brij-58 LYSED CHO CELLS

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Received 9 July 1979

1. Introduction

Uracil is not detectable in the DNA of most organisms, although prokaryotic [1] and eukaryotic [2] DNA polymerases are able to incorporate dUTP in place of dTTP. As shown [1], prokaryotic cells efficiently dephosphorylate dUTP to dUMP by the enzyme dUTPase. In addition, dUMP-containing DNA is repaired. An initial event in this repair process appears to be uracil excision catalyzed by uracil-DNA glycosylase, followed by cleavage of DNA at the apyrimidinic site, resulting in the formation of relatively short DNA fragments (reviewed [3]). Based on these observations, it has been postulated that a portion of Okazaki-size DNA intermediates might normally be formed by excision repair of dUMP-containing DNA [4,5].

In subcellular systems prepared from HeLa cells [6] or lymphocytes [7], dUTP was shown to be dephosphorylated by an enzyme (or enzymes) located predominantly in cytoplasmic fractions. Nevertheless, dUTP was incorporated into DNA, and dUMP-containing DNA was converted, at least in part, to fragments of relatively small molecular weight [6,7]. In nuclei from lymphocytes [7], less dUMP-containing DNA was fragmented if the reaction mixture contained uracil known to inhibit uracil-DNA glycosylase [3]. On the other hand, uracil had little effect on degradation of dUMP-containing DNA in HeLa cell nuclei [6].

Recently, the synthesis of DNA molecules in 4 S and their conversion to DNA of > 25 S by CHO cells partially lysed by Brij-58 and incubated with a cell extract and dTTP have been described [8]. To evaluate the fate of dUTP and of dUMP-containing DNA in this system, the products formed during incubation of Brij-58 lysed cells with dUTP were analyzed. It will be shown that:

(1) In the presence of cell extract, dUTP is rapidly dephosphorylated to dUMP;
(2) Brij-58 lysed cells synthesize and subsequently degrade dUMP-containing DNA;
(3) Degradation is inhibited by uracil and, less efficiently, by other pyrimidine derivatives.

2. Materials and methods

2.1. *Materials*

dUTP was purchased from Sigma; all other deoxy- and ribonucleoside triphosphates, Brij-58 and uracil from Serva; PEI-cellulose plates and thymine from Merck; pyrimidine and dihydrouracil from Fluka; fluorouracil from Hoffmann-La Roche (Switzerland); d[5-^{3}H]UTP, d[5-^{3}H]TTP and [2-^{14}C]thymidine from The Radiochemical Centre (Amersham).

2.2. *Preparation of Brij-58 lysed cells and of cell extract*

Both methods have been detailed in [8]. To prepare Brij-58 lysed cells, CHO cells grown as monolayers as in [8] were removed from Petri dishes with trypsin, exposed for 10 min at 0°C to the lysis solution containing 0.01% Brij-58 and 80 mM KCl, and centrifuged. Lysed cells were washed, suspended in the incubation mixture, and incorporation of d[^{3}H]TTP (2×10^{-5} M, 1 Ci/mmol) or d[^{3}H]UTP (2×10^{-5} M, 1 Ci/mmol) at 30°C was assayed. To prepare cell extract, CHO cells were homogenized as in [8], the homogenate centrifuged at 100 000 × *g*, and the supernatant dialyzed overnight. This preparation will be referred to as 'cell extract'.

2.3. *Other methods*

To determine the radioactivity in DNA of Brij-58 lysed cells, acid-insoluble material was precipitated and collected on GF/C filters as in [8]. To measure the size of labeled DNA, Brij-58 lysed cells that had been incubated in the reaction mixture were suspended in 0.5 ml 0.4 M NaOH, layered on top of alkaline sucrose gradients and centrifuged in a Beckman SW-27 rotor as described [8]. Separation of labeled deoxyribonucleoside mono-, di- and triphosphates, was performed by thin-layer chromatography on PEI-cellulose plates according to [9]. After separation, the chromatograms were cut into strips, and the cellulose layers were scraped off into scintillation vials for determination of radioactivity.

3. Results and discussion

3.1. *Incorporation of d[^{3}H]UTP into DNA of Brij-58 lysed cells incubated without cell extract*

In a first experiment, Brij-58 lysed cells were incubated for different time intervals with either d[5-^{3}H]UTP or d[5-^{3}H]TTP. The results are shown in fig.1a. After 5 min incubation with d[^{3}H]UTP, the DNA contained ~50% of the radioactivity of DNA labeled with d[^{3}H]TTP. After 120 min incubation, however, the radioactivity of d[^{3}H]UTP-labeled DNA had decreased and represented ~5% of that obtained by labeling with d[^{3}H]TTP. This indicates that Brij-58 lysed cells both synthesized and degraded dUMP-containing DNA.

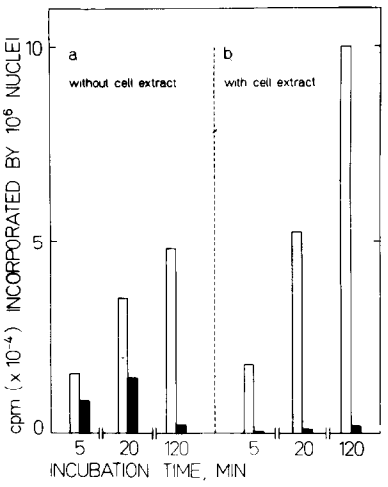

Fig.1(a,b). Incorporation of d[^{3}H]UTP and d[^{3}H]TTP into DNA of Brij-58 lysed cells. Partially lysed cells were incubated without cell extract (fig.1a) or with cell extract (fig.1b) at a concentration equivalent to 10^8 cells/ml. (□) Incubation with d[^{3}H]TTP. (■) Incubation with d[^{3}H]UTP.

3.2. *Incorporation of d[^{3}H]UTP into DNA of Brij-58 lysed cells incubated with cell extract*

As shown in fig.1b, cell extract added to the reaction mixture at a concentration equivalent to 10^8 cells/ml stimulated d[^{3}H]TTP-incorporation. In contrast, incorporation of d[^{3}H]UTP was almost undetectable. To examine the effect of cell extract on dUTP, d[^{3}H]UTP was incubated for 5 min at 30°C with cell extract at a concentration equivalent to 10^7 cells/ml. After incubation, the reaction mixture was subjected to thin-layer chromatography. About 95% of the radioactivity on the chromatogram migrated with dUMP. As control, d[^{3}H]UTP that had not been incubated with cell extract was chromatographed in parallel, and > 90% of the radioactivity migrated with dUTP. Thus, in the presence of 10-fold diluted cell extract, dUTP was rapidly dephosphorylated to dUMP. This may explain why d[^{3}H]UTP was incorporated very inefficiently by Brij-58 lysed cells incubated with cell extract. In addition, these results indicate that formation of replication intermediates by Brij-58 lysed cells incubated with cell extract and dTTP [8] would not be affected to any significant extent by traces of dUTP that were possibly present in the partially lysed cells and/or the cell extract.

3.3. *Size of d[^{3}H]UTP-labeled DNA as determined in alkaline sucrose gradients*

Brij-58 lysed cells were incubated for 5 or 20 min with d[^{3}H]UTP, and the size distribution of labeled DNA was analyzed in alkaline sucrose gradients. As shown in fig.2, most of the DNA formed during the first 5 min incubation with d[^{3}H]UTP sedimented with 4–9 S. After incubation for 20 min, DNA sedimented with a lower average rate, i.e., 4–6 S. In contrast, most of the DNA formed during incubation with d[^{3}H]UTP and 5 mM uracil sedimented with a higher average rate (~9 S), and more labeled DNA sedimented to the bottom of the tubes. After incubation with d[^{3}H]TTP (with or without uracil) for 20 min, most of the DNA sedimented with > 25 S

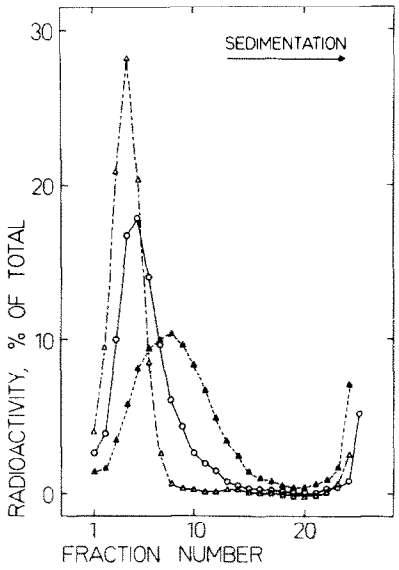

Fig.2. Radioactivity profiles in alkaline sucrose gradients of ^{3}H-labeled DNA formed by incubation of Brij-58 lysed cells with d[^{3}H]UTP for 5 min (○) or 20 min (△,▲), without uracil (○,△) or with 5 mM uracil (▲). DNA prelabeled by incubation of intact cells with d[^{14}C]Thd prior to lysis with Brij-58 sedimented to the bottom of the tubes in all gradients (not shown). Centrifugation was carried out at 20°C for 21 h.

(not shown). These results suggest that uracil partially inhibited degradation of dUMP-containing DNA, possibly by inhibition of uracil-DNA glycosylase [3].

3.4. *Effects of pyrimidine derivatives on degradation of dUMP-containing DNA*

To study the effects of uracil analogs, Brij-58 lysed cells were incubated with d[^{3}H]UTP and different pyrimidine derivatives for 5 or 120 min. As shown in table 1, addition of uracil, dihydrouracil, fluorouracil and thymine to the reaction mixtures caused an increase of label retained in DNA after 120 min incubation. This increase was not only attributable to differences in initial incorporation rates, as indicated by the similar amounts of ^{3}H-labeled DNA synthesized after 5 min incubation. Thus, besides uracil, other pyrimidine derivatives inhibited degradation of dUMP-containing DNA and may therefore inhibit uracil-DNA glycosylase in CHO cells, even though with a lower efficiency.

In conclusion, the results presented in fig.2 and table 1 indicate that uracil-DNA glycosylase is involved in the degradation of dUMP-containing DNA. Brij-58 lysed CHO cells may thus contain an enzyme (or several enzymes) involved in base-excision repair. As shown [8], they also contain enzymes involved in nucleotide-excision repair. This system may, therefore, be useful for the study of base excision as well as nucleotide excision and for the analysis of possible interactions between these types of DNA metabolism and DNA replication.

Table 1
Effect of uracil analogs on degradation of dUMP-containing DNA

Exp. no.	Pyrimidine derivative added (5 mM)	^{3}H cpm incorporated/ 10^{6} nuclei after	
		5 min	120 min
1	Uracil	5253	4379
	Thymine	7004	1051
	Fluorouracil	5428	875
	None	4727	350
2	Uracil	5154	6560
	Dihydrouracil	4920	1405
	Pyrimidine	3280	702
	None	3280	702

Acknowledgements

We thank Professor R. Schindler and Professor R. Braun for many helpful discussions and suggestions during the preparation of the manuscript. This work was supported by the Swiss National Science Foundation.

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