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RIBONUCLEOTIDES ARE CHANNELED INTO A MIXED DNA-RNA POLYMER BY PERMEABILIZED HAMSTER CELLS

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SUMMARY: Permeabilized CHEF/18 hamster cells incorporate label from [^{3}H] cytidine diphosphate (CDP) into material that we had designated as DNA. Spyrou and Reichard reported this label was incorporated only into RNA. To resolve this discrepancy the studies reported here were performed. We demonstrate incorporation into a DNA polymer with major interspersed RNA sequences. Incorporation into deoxycytidine isolated from this product was little diluted by a great excess of unlabeled dCTP, confirming channeling under these conditions of CDP into polymerized deoxyribonucleotides.

Lysolecithin permeabilized rodent cells rapidly incorporate deoxyribonucleoside triphosphates (dNTPs) into DNA, and also ribonucleoside triphosphates into RNA (1,2). [^{3}H] from CDP was reported also to be incorporated into DNA (3,4), as characterized by acid precipitability after two brief hydrolyses with NaOH (3). Spyrou and Reichard have reported in similar experiments that their product was completely hydrolyzed upon conventional longer alkaline hydrolysis, in contrast to the DNA made from dNTPs by these cells (5). In this experiment they found primarily CMP rather than dCMP hydrolysis products. We report here new experiments which identify our product as a DNA polymer with interspersed ribonucleotides. Furthermore, CDP was channeled into the dCMP of this product.

MATERIALS AND METHODS

Materials and methods for cell culture, permeabilization and alkaline hydrolysis were as described previously except that the incubation mixture (3) contained 0.075 mM $CaCl_2$.

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Chromatographic analysis: Material resistant to 30 min alkali treatment was acid precipitated and dissolved in 0.2 ml of 0.1 M Tris (base), then brought to pH 7.6 - 7.8 by adding 0.1 ml of 0.1 M Hepes and 0.1 M $MgCl_2$. DNase-I and phosphodiesterase were added at final concentrations of 100 µg/ml for each enzyme (6), and the sample was incubated overnight at 37°C. The material was brought to 7.5 ml, 5'-CMP and 5'-dCMP added as standards, and applied to a 0.7 x 15 cm column of Dowex 50 W-X8, 200-400 mesh (obtained from Dr. Reichard). Elution was with 0.2 M acetic acid (7). Fractions were collected and radioactivities determined.

Equilibrium density gradient analysis: Brief alkali resistant acid precipitable material was extracted in 2 ml of NET buffer (0.1 M NaCl/10 mM EDTA/10 mM Tris, HCl pH 8) with 1% sodium lauroyl sarcosine, sheared by ten passage through a 27 gauge needle and treated overnight with 300 µg/ml pronase at 37°C. Then the suspension was extracted 4 times with 5 ml of chloroform/isoamyl alcohol (24:1). One ml of CsTFA solution (8) (density 2 g/ml, purchased from Pharmacia Fine Chemicals) was added per every 0.38 ml of this sample and mixed thoroughly. 4.5 ml of this mixture in nitrocellulose tubes was centrifuged in a SW 50.1 rotor in a Beckman L5-50B ultracentrifuge at 44,000 rev/min at 20°C for 50 h. Fractions (0.15 ml) were collected, acid insoluble material in 50 µl aliquots were prepared (9) and the radioactivity determined. When material from intact cells that had incorporated [^{3}H] uridine was prepared as an RNA standard the alkaline hydrolysis step was completely omitted.

RESULTS AND DISCUSSION

Hydrolysis: We compared the adequacy of a brief hydrolysis with the more conventional long hydrolysis procedure (10). RNA of intact CHEF/18 cells was labeled with [^{3}H] uridine in the presence of hydroxyurea (HU) to block DNA synthesis. Acid precipitated (11) label was released by hydrolysis (Fig. 1), which was timed after the precipitate was well suspended for which at least 40 min was allowed. Brief hydrolysis had reached its end point within 30 min, and was as effective as conventional long hydrolysis for up to 18 h. Recovery was slightly higher if the precipitate was centrifuged rather than filtered (see 10,12). We conclude that one "brief" hydrolysis (30 min) adequately hydrolyzes authentic RNA, and this was used in the following experiments. But one 60 min hydrolysis or two 30 min hydrolysis as used previously (3) provide a safer margin.

DNA of intact cells was labeled with [^{3}H] thymidine. This label was relatively stable to alkali, although long hydrolysis caused up to 20% losses in some experiments. [^{3}H] dTTP incorporated by permeabilized cells using the dNTP system (3) also was not released by either brief or long hydrolysis (5).

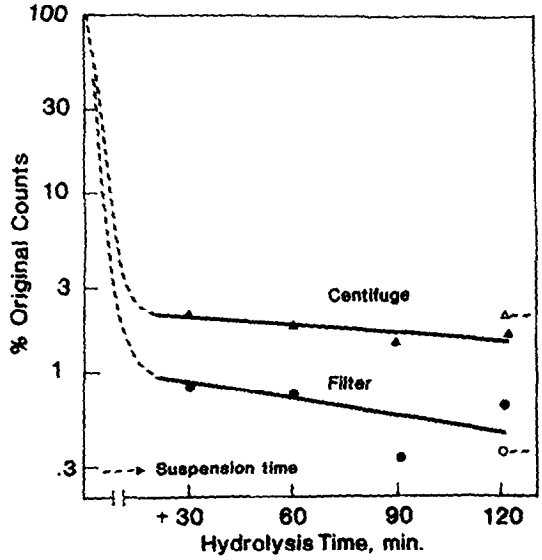

Fig. 1. Kinetics of RNA hydrolysis. CHEF/18 cells received 1 mM HU and 15 min later 5,6-[^{3}H] uridine for 1 h. Cells were precipitated with $HClO_4$ as described (3). The pellet was suspended in 0.3 ml 0.2 M NaOH until solubilized and then incubated at 37oC for the times indicated. After acid addition samples were separated on glass filters (GF/F) or centrifuged (3) (closed symbols). Other samples were treated by long hydrolysis (5) for 18 h. (open symbols) and then acidified and filtered or centrifuged.

Permeabilized cells: The critical experiment is incorporation of [^{3}H] CDP into permeabilized cells (Fig. 2). We used the same experimental design as Fig. 2 of Spyrou and Reichard (5). Our results were very different; 18

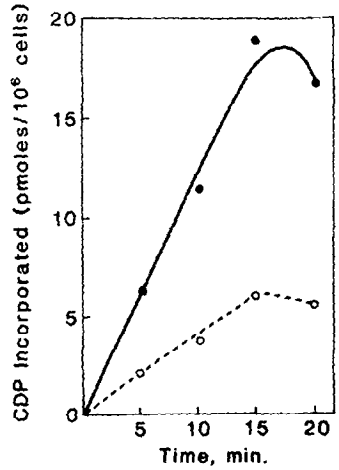

Fig. 2. Incorporation of CDP into alkali resistant material. Non-synchronous CHEF/18 cells were permeabilized and allowed to incorporate [^{3}H] CDP in the NDP reaction mixture (3) for the times indicated. Samples were acidified and centrifuged after 30 min brief alkaline hydrolysis (●) (see Fig. 1), or long alkaline hydrolysis (○) (5).

TABLE 1. Rates of precursor incorporation under various conditions

Labeled substrate	Ref.	System	Hydrolysis	Rate
CDP	*	NDP	none	10.
CDP	*	NDP + 1mM HU	none	11.
CDP	*	NDP	brief	1-2
CDP	*	NDP	long	0.5
CDP	*	NDP + 20mM HU	brief	0.1
thymidine	1,2	Intact cells	none	6
dTTP	1,2	dNTP	brief	3-4
dTTP	5	dNTP	long	3-4
dTTP	1	NDP	brief	1
dTTP	*	NDP	long	0.5

The rate is given as pmoles incorporated/min/10^6 cells; most data are for exponential non-synchronized cells. The dNTP system provides the 4 dNTPs and is low in dtt. The NDP system provides the 4 NDPs and 8 mM dtt, except that when the label is [^{3}H] dTTP in trace amounts and additional dtt is not provided. Asterisk (*) represents results from this article.

pmoles/10^6 cells/15 min were incorporated after brief hydrolysis. This is the amount we previously reported (3), and far below the 100 pmoles Spyrou and Reichard found (5). This material was much more stable to alkali than RNA. Much was stable to long alkaline hydrolysis, unlike Spyrou and Reichard's product (5).

When NDPs were provided as substrates, but a trace of [^{3}H]dTTP was used as label and dithiothreitol (ddt) was omitted, a similar amount of acid precipitable product was obtained, and its stability in alkali was like that obtained with [^{3}H] CDP (Table 1).

Characterization: Material remaining after short alkaline hydrolysis was hydrolyzed with enzymes to nucleotides (Fig. 3). Recovery was 85%. About two-thirds of the [^{3}H] cytosine was found in dCMP and one-third in CMP. The same ratio was obtained using Dowex AG1-X8 formate (5) followed by PEI chromatography to separate dCMP and CMP (13). In parallel experiments a very large excess of dCTP in the reaction mixture only slightly inhibited [^{3}H] CDP incorporation into the dCMP portion of the product (Fig. 3), as had been reported for the entire product (3).

The product obtained after brief hydrolysis was not a mixture of RNA and DNA. On a Cs tri-F-acetate gradient, which cleanly separates single stranded RNA and double stranded DNA (8), one sharp peak was found in the position of double stranded DNA (density 1.63), and far from the well-separated position

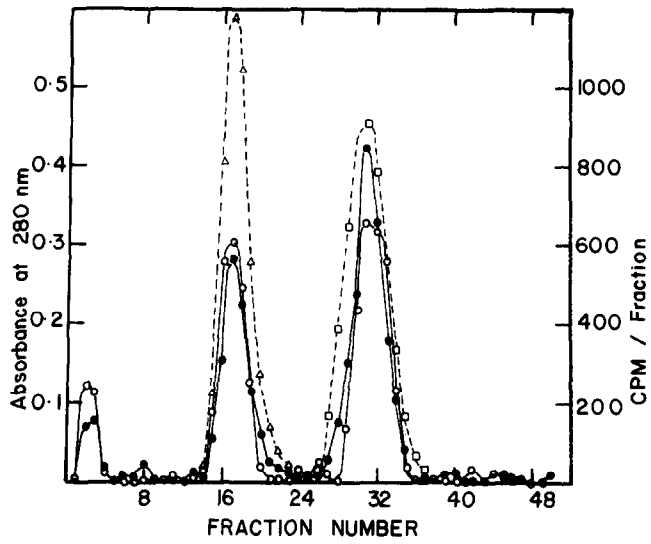

Fig. 3. Nucleotide chromatography. [^{3}H] CDP was incorporated for 10 min by permeabilized S phase cells, in the NDP reaction mixture without (●____●) or with (O____O) 200 μM dCTP. After brief alkali treatment the product was hydrolyzed with enzymes and run on a Dowex-50 W-X8 column (Materials and Methods). Locations of 5'-rCMP (Δ---Δ) and 5'-dCMP (---) standards are shown.

of RNA (density 1.84) (Fig. 4). Thus one double stranded product was made, which could be labeled with either [^{3}H] CDP or [^{3}H] TTP.

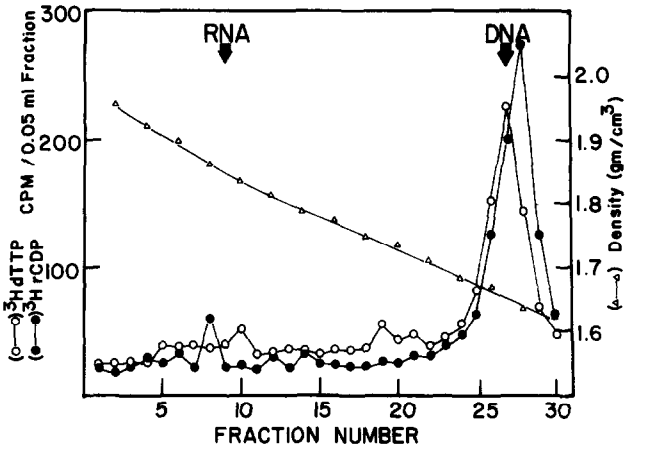

Fig. 4. Equilibrium density gradient analysis. S-phase cells were permeabilized and incubated for 10 min with [^{3}H] CDP in the NDP reaction mixture or [^{3}H] dTTP in the dNTP reaction mixture (3), and brief alkali resistant material was subjected to CsTFA centrifugation (Materials and Methods). TCA insoluble radioactivity was determined on filter papers (9). ●____●, [^{3}H] CDP and O____O, [^{3}H] dTTP incorporated material. As standards, S-phase cells were pulsed for 60 min with either [^{3}H] uridine or [^{3}H] thymidine, trypsinized, acid precipitated, extracted with NET buffer and centrifuged. Peak positions are shown at arrows.

Discussion: The NDP system we used incorporated CDP (with no hydrolysis) at about 10 pmoles/min/10^6 cells (Table 1), similar to the rate we obtained for in vivo incorporation into RNA (cells plus HU). Of these, 1.3 pmoles/min were resistant to brief alkaline hydrolysis, and 0.8 resistant to long alkaline hydrolysis. [^{3}H] dTTP incorporation under these conditions was similar. Hydroxyurea largely abolished [^{3}H] CDP incorporation into this briefly alkali stable material (1). Thus, permeabilized cells made a product of stability to alkali much greater than RNA, at about 1/6 the rate of RNA synthesis.

We conclude that Spyrou and Reichard's cells had incorporated [^{3}H]-CDP into RNA at 10 pmoles/min/10^6 cells, and their brief hydrolysis must have been ineffective in this particular experiment (5) (Fig. 2). Their Fig. 3 is consistent with RNA synthesis, since 3/4 of the hydrolysis product obtained from this same preparation was reported as 2' and 3' CMP. Dr. Spyrou, working with Dr. Reddy at the Karolinska Institute, repeated his earlier experiment, but obtained only 2.5 and 1 pmole/min/10^6 cells after brief hydrolysis, similar to the result reported here. Dr. Spyrou treated this material with a combination of pancreatic DNAse, snake venom phosphodiesterase and alkaline phosphatase; 70% of the radioactivity was recovered as cytidine and less than 1% as deoxycytidine (Dr. Reichard, pers. comm.). We conclude that their published experiment could not be repeated in either laboratory.

dCTP in great excess did not strongly inhibit incorporation of CDP into the dCMP peak (Fig. 3), which supports our original conclusion of channeling all the way from reductase to polymerase reactions (3). "Tight" channeling clearly is a property of this NDP system, depending in particular on 8 mM dtt which excludes incorporation of dNTPs (14). Certainly both precursors can be incorporated in vivo or in the presence of less dtt in permeabilized cells. Channeling could complement allosteric control of reductase. It could accelerate the de novo pathway when pools of dNTPs are low.

The data of Spyrou and Reichard (5) indicate that permeabilized cells do not make authentic DNA in the NDP system. Based on the data described here,

the product contains about two-thirds deoxynucleotides, which must be in sequences of at least 15 because they are precipitable (12). Interspersed and terminal (15) ribonucleotide sequences of various lengths are likely responsible for its composition and moderate alkali lability. This product presumably is an artifact; synthesis of authentic, ribonucleotide-free DNA by permeabilized cells from NDP precursors clearly requires experimental refinements.

ACKNOWLEDGMENT

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