Labeling of Crithidia fasciculata DNA with [$^3$H]Thymidine

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SYNOPSIS. Attempts at continuous labeling of Crithidia fasciculata DNA with [$^3$H]thymidine led to a pulse-chase situation due to a cell-mediated conversion of thymidine to thymine in the medium. The uptake of thymine was slow compared to that of thymidine. Neither the addition of deoxyadenosine nor the sequential addition of several aliquots of [$^3$H]thymidine had an effect on the pattern of labeling.

Index Key Words: Crithidia fasciculata; thymidine; DNA; thymidine phosphorylase.

THE presence of the enzyme, thymidine phosphorylase, has been shown to be the cause of the relatively low extent of labeling of DNA by [$^3$H]thymidine in several different cell types, e.g. E. coli (2, 5, 8-11), certain murine and human tumor cells (13, 14) and normal mammalian liver (3, 4). This enzyme catalyzes a phosphorolytic cleavage of pyrimidine deoxyribonucleosides and is involved in the salvage pathway of thymine metabolism in these cells (7). The uptake of thymine is relatively slow compared to that of thymidine and thus the incorporation of label into DNA is effectively inhibited by the cleavage of [$^3$H]thymidine to [$^3$H]thymine.

In the course of a study of DNA replication in the parasitic protozoan, Crithidia fasciculata, we discovered an analogous situation. This report describes several preliminary experiments which indicate that continuous labeling of C. fasciculata DNA is hindered by the apparent presence of a thymidine phosphorylase-like enzyme activity.

MATERIALS AND METHODS

Culture of the cells.—The cells represent a clone of a Crithidia fasciculata culture originally obtained from Dr. Stuart Krassner. This clonal cell line has been growing in our laboratory for 3 years in Brain-Heart Infusion Medium (Difco Laboratories) with 10 /ig/ml hemin added after autoclaving. The cells were grown at 27 ± 0.5 C either in 3.5 liter quantities in a fermentator (Fermentation Industries, Allentown, Pa.) or in 150 ml to one liter quantities in Pyrex bottles rotating at 6 rpm. Antifoam B (Dow Corning) was added for defoaming as required. The experimental cultures were grown in either modified Trager's defined Medium C for Leishmania tarentolae (12) or in Kidder & Dutta's defined medium for Crithidia (G).

Cells were grown for at least 3 rapid subcultures in the appropriate defined medium prior to use in labeling experiments. The modification of Trager's Medium C consisted of the replacement of the purine-pyrimidine mixture by 20 /pg/ml adenine.

Labeling of the cells.—[Methyl-$^3$H]thymidine (18 C/mmc) and [methyl-$^3$H]thymine (14 C/mmc) were purchased from Schwarz/Manu Co. Experimental cultures were grown in bottles with rotation at 27 C. Incorporation of label was measured by spotting 100 ml of the cell culture onto Whatman 3 MM discs, drying and processing the discs through 5% (w/v) trichloroacetic acid, 70% (v/v) ethanol, 95% ethanol and ether. The discs were counted in toluene-Omnifluor (New England Nuclear) in a Nuclear Chicago Scintillation Counter.

Cell counts were performed by mixing samples of culture 1:1 with 3% (v/v) formalin and counting 200-500 cells in a hemacytometer.

Paper chromatography of [$^3$H]thymidine and [$^3$H]thymine.—Samples, which consisted of neutralized aliquots of the culture medium after removal of cells by centrifugation and precipitation with 0.5 N HClO₄, were spotted on Whatman #1 paper, and unlabeled thymine and thymidine were added as OD markers. The strips were equilibrated for 16 hr in the presence of the lower phase of the mixture ethyl acetate-water-formic acid (90:35:5); then descending chromatography was performed using the upper phase of this mixture. The spots were eluted overnight in 0.1 N HCl and then Aquasol (New England Nuclear) was added and the samples counted in a Nuclear Chicago Scintillation Counter.

Isolation of nuclear and kinetoplast DNA.—Kinetoplast DNA (K-DNA) was isolated from early stationary phase cells (2 × 10⁸ cells/ml). The cell pellet was washed once in 0.15 M NaCl, 0.02 M glucose, 0.02 M phosphate buffer, pH 7.9. The final pellet was resuspended in 0.15 M EDTA—0.15 M NaCl (pH 8.0) to a 5% (w/v) final concentration. Sodium Dodecyl Sulfate (Sarkosyl) 0.015 M sodium citrate, pH 7.4) was added to 1% (w/v) final concentration. Promase B grade or pronase CB B grade, Calbiochem, was added for 30 min at 37 C to a concentration of either 2 mg/ml or 0.5 mg/ml respectively. The solution was incubated at 56 C for 3-5 hr. The lysate was then passed through a #18 needle at 25 lb/lin² and then centrifuged at 20,000 rpm for 20 min in the SW 39 rotor at 4 C. The supernatant fluid was decanted and saline-sodium citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) was added to reisupend the pellet, and the solution was centrifuged as described previously. This final pellet was resuspended in saline-sodium citrate and subjected to RNase A + T₁ (20 /g/ml and 20 U/ml respectively) treatment for 30 min at 37 C. Pronase was added to 100 /g/ml and the solution incubated again at 37 C for 30 min. Deproteinization was performed with chloroform-isooamyl alcohol (24:1 v/v), and the DNA solution was dialyzed extensively against saline-sodium citrate.

This DNA was then subjected to ethidium bromide CsCl equilibrium centrifugation (40 hr, 40,000 rpm, 20 C, 6.5 ml, #50 rotor, p₁ 25C = 1.3876). The lower band consisting of covalently closed circular K-DNA networks and the upper band consisting of nuclear DNA (N-DNA) were recovered, and the dye was removed by extraction with iso-amyl alcohol and dialysis. The specific activities of the N-DNA and K-DNA were measured by reading the A₂₆₀ₕₐₜ and by spotting samples onto 3 MM discs for counting as described above.

RESULTS

Attempts at Continuous Labeling of DNA

Cells were grown in Medium C containing 10 /g/ml of [methyl-$^3$H]thymidine and different concentrations of unlabeled thymidine. Growth curves were followed and the specific activities of acid-precipitable [$^3$H] label were measured at various
Fig. 1. Incorporation of $[^{3}H]$thymidine into acid-insoluble material by C. fasciculata cells in a long term labeling situation in Medium C. $[^{3}H]$Thymidine (10 $\mu$g/ml, 18 C/\text{mM}) was added at time 0 and the specific activities (cpm/10$^6$ cells) of the incorporated material followed during growth. The concentration of unlabeled thymidine present in each culture was as follows: (a) 0; (b) 2 $\mu$g/ml; (c) 5 $\mu$g/ml; (d) 10 $\mu$g/ml; (e) 50 $\mu$g/ml. (••••••••••), acid insoluble cpm/10$^6$ cells ($\times 10^3$); 0 0 0, number of cells per ml; ( ), theoretical simple dilution curve assuming that no further synthesis occurs after the indicated point.

Fig. 2. Incorporation of $[^{3}H]$thymidine into acid-insoluble material by C. fasciculata cells in a long term labeling experiment in Kidder & Dutta's medium. $[^{3}H]$Thymidine (10 $\mu$g/ml, 18 C/\text{mM}) was added at time 0 and the specific activities (cpm/10$^6$ cells) of the incorporated material followed during growth. See Fig. 1 for description of symbols.

TABLE 1. Specific activities of purified N- and K-DNA after growth of cells for 3 days in $[^{3}H]$thymidine.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Specific Activity† (cpm/µg DNA)</th>
<th>K/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclear</td>
<td>30,394</td>
<td>1.09</td>
</tr>
<tr>
<td>kinetoplast</td>
<td>42,897</td>
<td></td>
</tr>
</tbody>
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† Concentration measured by A$_{260}$ assuming 1 OD = 41.5 µg/ml.
‡ Ratio corrected for 21.5% thymine in nuclear DNA vs 28% thymine in kinetoplast DNA (unpublished data).
Fig. 3. Conversion of [3H]thymidine (20 µc/ml, 18 C/mM) to [3H]thymine by C. fasciculata cells growing in Medium C. No unlabeled thymidine was added. Similar results were obtained using 5 µg/ml unlabeled thymidine with 20 µc/ml [3H]thymidine. Aliquots of the culture were removed and centrifuged at 2000 g for 10 min. The supernatant solution was made 0.5 N in perchloric acid and again centrifuged for 15 min at 5000 g. The resulting supernatant solution was neutralized with KOH and the precipitate of potassium perchlorate removed by centrifugation. Descending paper chromatography was performed on the final solution as described in Methods. The percent conversion of thymidine to thymine was calculated using the total cpm recovered from the thymine and thymidine spots at each time point.

Fig. 4. Effect of the sequential addition of several aliquots of [3H]thymidine on the labeling pattern of C. fasciculata in Medium C. [3H]Thymidine (5 µc/ml, 18 C/mM) was added at time 0, 5 hr and 12 hr during growth. The cell counts and specific activities of incorporated material were measured at each time. See Fig. 1 for description of symbols.

thymine in the medium was followed by descending paper chromatography. As shown in Fig. 3, the conversion proceeded linearly with time at least for 10 hr, by which time 78% of the exogenous thymidine originally present had been converted to thymine. This phenomenon would lead to the observed decreased incorporation of label provided thymine were not taken up to the same extent as thymidine. This was demonstrated to be the case by growing cells in Medium C in the presence of 3.3 µc/ml of [3H]thymine with 5 µg/ml of unlabeled thymine. After 3 days growth, the cells had attained a specific activity of $2.8 \times 10^{6}$ cpm/10^6 cells, which is equivalent to the basal levels attained by cells grown in [3H]thymidine after 60 hr growth.

Attempts to Increase Uptake of Thymidine

Deoxyadenosine (250 µg/ml), which is a known inhibitor of thymidine phosphorylase activity in E. coli (1), had no effect on the kinetics of labeling of DNA with [3H]thymidine in C. fasciculata.

Sequential addition of several aliquots of [3H]thymidine during the growth period also had no effect on the pattern of labeling. In this experiment cells were given 5 µc/ml [3H]thymidine at 0, 5 and 15 hr in the growth curve. As shown in Fig. 4, the specific activity reached a maximum at 5 hr and then decreased at 12 hr.

DISCUSSION

We have demonstrated that attempts at continuous labeling of C. fasciculata DNA by [3H]thymidine in either of 2 defined media led to an apparent pulse-chase situation explainable on the basis of a cell-mediated conversion of thymidine to thymine in the medium. Attempts to increase the amount of [3H]thymidine taken up, either by the addition of deoxyadenosine or by supplying additional aliquots of [3H]thymidine at 5 hr intervals, failed. A recognition of this labeling pattern is of obvious importance in any study of the replication of C. fasciculata DNA. Furthermore, this phenomenon, if it is of general occurrence among the parasitic hemoflagellates, may be significant in terms of limiting the chemotherapeutic value of thymidine analogues.

In our experiments, we did not distinguish between incorporation into N- or K-DNA, but the equality of the specific activities of purified N- and K-DNA from cells labeled with [3H]thymidine for 3 days implied that the labeling of the K-DNA also was following a pulse-chase pattern.

Similar results have been obtained independently by Gutteridge & Al Chalabi (5).

REFERENCES

LABELING OF CRITHIDIA