Cell Cycle Specific Fluctuations in Adenosine 3':5'-Cyclic Monophosphate and Polyamines of Chinese Hamster Cells

(ornithine decarboxylase/S-adenosylmethionine decarboxylase)

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ABSTRACT Chinese hamster V3 cells were synchronized by mitotic selection, which resulted in approximately 95% synchrony. The adenosine 3':5'-cyclic monophosphate level was elevated within 3 hr (G1 phase) and reached a level 2-fold higher than in early G1 within 6 hr (early S phase). An increase in ornithine decarboxylase activity (L-ornithine carboxyl-lyase, EC 4.1.1.17), the initial enzyme in the polyamine biosynthetic pathway, was detected within 4 hr and was maximal at 8 hr. Since about 20% of the cells were labeled with [3H]thymidine at 4 hr, ornithine decarboxylase exhibits cell-cycle specific activity starting in late G1 and continuing through middle S phase. The activity of S-adenosylmethionine decarboxylase (S-adenosyl-L-methionine carboxylase, EC 4.1.1.50) increased within 5 hr, i.e., early S phase. It is suggested on the basis of these data and other studies discussed herein that the increase in ornithine decarboxylase activity, which parallels closely the elevation in cyclic AMP, is an example of adenosine 3':5'-cyclic monophosphate-mediated protein synthesis.

Polyamine biosynthesis is greatly increased in mammalian tissues during embryogenesis (1, 2), after stimulation with hormones (3-10), following partial hepatectomy (11, 12), and during drug-induced hyperplasia (13). Parallel increases in the spermidine concentration and RNA content can be demonstrated in certain mammalian tissues (14).

There is no complete study of polyamine biosynthesis throughout the various phases of the mammalian cell cycle. In synchronously growing Don C cells, the activity of ornithine decarboxylase (L-ornithine carboxyl-lyase, EC 4.1.1.17), the first enzyme in the biosynthetic pathway of the polyamines, displays three peaks of activity during the cell cycle: one during mitosis, one during late G1-early S phase, and the third at late S phase (15). Only the activity at late G1-early S phase is abolished by inhibitors of RNA and of protein synthesis. Since the actual accumulation of polyamines was not studied, it is difficult to tell whether these changes in enzyme activity actually reflect changes in polyamine synthesis. In addition, polyamine concentrations have been assessed in AKR lymphoma cells from the mouse thymus that represent various phases of the cell cycle (16). Putrescine increased in late G1-early S, and spermidine and spermine concentrations in late S. These findings are consistent with an increase in ornithine decarboxylase (putrescine synthesis) only in late G1-early S. Furthermore, studies of polyamine accumulations in WI-38 human diploid fibroblasts, stimulated to divide, also suggest initiation of polyamine biosynthesis in late G1-early S phase (17, 18).

Recent studies have shown that dibutyryl-adenosine 3':5'-cyclic monophosphate can induce ornithine decarboxylase activity in the adrenal medulla and in numerous rat tissues (19-23). Further, it has been shown that cold exposure which leads to cholinergic stimulation of the adrenal medulla of the rat results in a marked increase in adenosine 3':5'-cyclic monophosphate (cAMP) levels, followed by a rapid increase in ornithine decarboxylase activity (24, 25). Methylxanthine derivatives, which increase cAMP levels by inhibiting phosphodiesterase activity, also result in marked increases in ornithine decarboxylase activity in rat tissues; i.e., liver, kidney, adrenal medulla, and cortex (26). Inhibitors of RNA and protein synthesis abolish the increase in ornithine decarboxylase, suggesting de novo synthesis is involved (25). Because both cAMP and polyamines are associated with the regulation of normal cellular growth, and because of the close temporal relationships between increases in cAMP and ornithine decarboxylase, the initial enzyme in the polyamine biosynthetic pathway, we have studied in detail ornithine decarboxylase activity, putrescine-stimulated S-adenosylmethionine decarboxylase (S-adenosyl-L-methionine decarboxylase, EC 4.1.1.50) activity, and the fluctuations in the endogenous concentrations of putrescine, spermidine, and spermine, as well as cAMP levels, at various times after mitotic selection of Chinese hamster V3 cells.

MATERIALS AND METHODS

[1-14C]Ornithine (11.9 mCi/mmol) and S-adenosyl-L-[carbony-14C]methionine (7.7 mCi/mmoll were obtained from New England Nuclear. Putrescine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride were obtained from Sigma. Dithiothreitol and pyridoxal phosphate were obtained from Cal Biochem.

Cell Culture and Synchronization. Chinese hamster V3 cells were cultured and maintained as previously described (27). For cell synchrony studies, cells were grown as monolayers in roller bottles. Prior to confluency, colcemid (0.06 µg/ml) was added for 1 and 1/2 hr and the medium then carefully decanted. Mitotic cells were collected by gently swirling Gey's balanced salt solution over the monolayer and pelleting the detached mitotic population by centrifugation for 2 min at 800 X g in a table top centrifuge. The cells were suspended in fresh medium and distributed in 10 ml aliquots to a series

Abbreviations: cAMP, adenosine 3':5'-cyclic monophosphate.
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of 100 mm tissue culture plates for enzyme and polyamine analysis. A parallel series of 5 ml aliquots in 60 mm tissue culture plates was established from the same population for cell cycle analysis. All of the above steps were carried out at 37°C.

To estimate the duration of G1, S, G2, and the generation time for each experiment, and to determine the degree of synchrony during cell cycle traverse, we labeled cells in the 60 mm plate series with [3H]thymidine (2 μCi/ml) for 10 min intervals after synchronization. They were washed, fixed with methanol:acetic acid (3:1) and prepared for autoradiography. The percent of labeled cells and the preparation of cells in mitosis were determined at each time interval from the same plate. A representative plot is shown in Fig. 1. The initial level of synchrony was always determined from the original mitotic cell suspension and yielded between 95 and 98% cells in metaphase. Disynchrony makes it difficult to evaluate changes occurring after 11 hr (Fig. 1).

Preparation of Cell Extracts. For enzymatic assays, the cells were sonicated with an E/MC Corp. ultrasonic cell disruptor equipped with a 4.5 inch probe in 100–200 μl of 0.05 M sodium-potassium phosphate buffer, pH 7.2, containing 1.0 mM dithiothreitol. Activity was optimal in sonicated homogenates, less in whole cells, and was not stable to freezing. There were no differences in the activities of these enzymes in crude homogenates as compared to supernatant solutions (100,000 × g). Therefore, sonicated homogenates routinely served as the source of the enzymes.

Assay of Ornithine Decarboxylase Activity. Enzyme activity was determined by measuring the release of 14CO2 from L-[1-14C]ornithine, as previously described with minor modifications (4, 11). Incubations were carried out in 13 ml centrifuge tubes equipped with rubber stoppers supporting a polypropylene center well (Kontes Glass Co.) that contained 0.2 ml of ethanolamine:2-methoxyethanol (2:1 v/v). Reaction mixtures consisted of 50–100 μl of cell extract, 20 μM pyridoxal phosphate, 0.1 mM L-[1-14C]ornithine, and 80–130 μl of 0.05 M sodium-potassium phosphate buffer, pH 7.2, containing 1 mM dithiothreitol, to make a total volume of 0.2 ml. After 60 min incubation at 37°C, 0.25 ml of 1 M citric acid was injected into the reaction mixture through the rubber cap to stop the reaction and release CO2 from the reaction mixture. The mixture was agitated for an additional 15 min at 37°C to ensure complete absorption of the 14CO2. The center well was removed and placed in a vial containing 2 ml of ethanol and 10 ml of an omnifluor-toluene scintillation medium. Radioactivity was assayed with a Beckman LS-150 liquid scintillation counter. All values were corrected against a boiled enzyme assay. Enzyme activity was linear for at least 1 hr and was proportional to the amount of cell extract added to the assay.

Assay for Putrescine-Stimulated S-Adenosyl-L-methionine Decarboxylase Activity. Enzyme activity was determined by measuring the release of 14CO2 from S-adenosyl-L-[carboxy-14C]methionine as previously described with minor modifications (28). Reaction mixtures consisted of 50–100 μl of cell extract, 20 μM pyridoxal phosphate, 2.5 mM putrescine dihydrochloride, 0.15 mM S-adenosyl-L-[carboxy-14C]methionine, and 70–120 μl of 0.05 M sodium-potassium phosphate buffer, pH 7.2, containing 0.1 mM dithiothreitol to make a final volume of 0.2 ml. Incubations were carried out and radioactivity measured as detailed above for the ornithine decarboxylase assay. Enzyme activity was linear for at least 1 hr and was proportional to the amount of cell extract added to the assay.

Determination of Polyamine Concentrations. Samples of 4 to 6 × 106 cells were prepared by sonication with an E/MC Corp. ultrasonic cell disruptor equipped with a 4.5 inch probe in 200 μl of 0.1 M HCl at 0–2°C. Dry sulfosalicylic acid was added to a final concentration of 4%. The homogenates were centrifuged at 100 × g for 15 min. The entire sample was then placed on a Beckman model 121 automatic amino-acid analyzer. This method has been described in detail previously (29).

Determination of cAMP Levels. For cAMP determinations, cells were collected by trypsinization, washed with balanced salt solution, and precipitated with 1 ml of cold 5% trichloroacetic acid containing about 5000 cpm/ml of [3H]cAMP to monitor recovery rate. The precipitates were dispersed, centrifuged, and the supernatants frozen on dry ice and ethanol. cAMP was purified from the other acid-soluble nucleotides as described by Mao and Guidotti (30); and its concentration determined by means of a protein kinase assay (31).

RESULTS AND DISCUSSION
The activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase increase in succession during the cell cycle (Fig. 2). Ornithine decarboxylase activity, which remains constant during the first 3 hr of the cycle, initially increases by the fourth hour when 20% of the cells incorporate [3H]thymidine; this indicates that the cells of the population are in G1–early S phase. The activity triples by 5 hr, and reaches a maximum by 8 hr that is nearly 8-fold greater than that at zero time. Thereafter, the activity declines and approaches the 1 hr value at 11 hr when the cell population is entering the second round of mitosis (Fig. 1), although disynchrony is high by this time. In contrast to the ornithine decarboxylase activity, the S-adenosylmethionine decarboxylase activity does not increase until the fifth hour when 40% of the cells become labeled with [3H]thymidine and its activity
Suspended, decarboxylase, and Ornithine
inhibitor, substrate of at least
of cell Putrescine.

Evidence from other systems would indicate that these increases are probably due to de novo synthesis of the enzymes (32, 33). However, in certain cases, changes in the half-lives of the enzymes have been found, e.g., in hepatoma cell cultures and in stimulated lymphocytes after the administration of inhibitor substances (34, 35).

The accumulation of polyamines during the cell cycle is described in Table 1. The concentration of putrescine doubles reaches a level by 8 hr that is 2-fold greater than that detected in early G1.

TABLE 1. Concentrations of putrescine, spermidine, and spermine during the cell cycle of synchronized Chinese hamster cells

<table>
<thead>
<tr>
<th>Phase</th>
<th>Time of cell cycle (hr)</th>
<th>Putrescine (nmol/4 × 10⁶ cells)</th>
<th>Spermidine (nmol/4 × 10⁶ cells)</th>
<th>Spermine (nmol/4 × 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>M</td>
<td>1.40 ± 0.10</td>
<td>13.70 ± 1.5</td>
<td>9.40 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>G1</td>
<td>0.35 ± 0.02</td>
<td>6.50 ± 0.8</td>
<td>5.14 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td>G1</td>
<td>0.72 ± 0.03*</td>
<td>6.50 ± 0.8</td>
<td>5.71 ± 0.7</td>
</tr>
<tr>
<td>6</td>
<td>S</td>
<td>1.00 ± 0.15*</td>
<td>6.04 ± 0.9</td>
<td>5.73 ± 0.7</td>
</tr>
<tr>
<td>8</td>
<td>S</td>
<td>2.30 ± 0.20*</td>
<td>8.50 ± 0.6*</td>
<td>7.23 ± 0.6*</td>
</tr>
<tr>
<td>10</td>
<td>S-G2</td>
<td>2.10 ± 0.23*</td>
<td>9.85 ± 0.5*</td>
<td>7.64 ± 0.5*</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>1.30 ± 0.17*</td>
<td>11.80 ± 1.3*</td>
<td>8.95 ± 0.9*</td>
</tr>
</tbody>
</table>

Each value represents the mean and SEM of four separate determinations. The amine concentrations were determined with an amino-acid analyzer as previously described (25).

* These values differ from the comparable value at 2 hr (P < 0.001).

between 2 and 4 hr, and within 8 hr, it is nearly 7-fold greater than that present at 2 hr. It appears from the putrescine level at zero hr versus the value at 2 hr, that either a significant amount of the putrescine is metabolized by the cells prior to division or that putrescine is excreted into the medium. This does not appear to be true for spermidine and spermine. The zero time concentrations have been adjusted to reflect the concentration for the same number of cells as present at the other time points. All the cells have divided within 45 min of mitotic collection after colcemid treatment. Spermidine concentrations show no significant increase until 8 hr which corresponds to middle to late S phase (Fig. 1). The pattern for spermine accumulation is very similar to that for spermidine, with significant elevations apparent within 8 hr.

These data would suggest that ornithine decarboxylase activity increases in G1–early S followed by enhancement of S-adenosylmethionine decarboxylase activity in early S phase. Increases in the biosynthetic activity of these two enzymes result in marked accumulation of putrescine by early to middle S phase, and accumulations of spermidine and spermine by middle to late S phase. It was also found that we could not use whole cell preparations to determine enzymatic activity, probably because of permeability problems. Maximal activity was obtained after sonication of the cells. Since whole cell were assayed in the study of ornithine decarboxylase activity of synchronously growing Don C cells (15), it is possible that some of the discrepancies between the two studies may be due to this factor. However, in this study it is apparent from both the ornithine decarboxylase activity measured during the cell cycle and the orderly accumulation of putrescine, that there is one marked peak of enzyme activity which is initiated in late G1–early S and continues through S phase. This datum is in good agreement with a study of polynamine content of AKR leukemic cells in various stages of the cell cycle (16). Thymus glands infiltrated with leukemic cells can be teased apart and cells separated according to density at unit gravity on a sucrose gradient. It was possible to show that the amounts of putrescine, spermidine, and spermine present per 10⁶ cells were markedly increased in cells at late G1–early S phase. Again, this would indicate that the precursor, putrescine, must be synthesized starting in late G1 or early S. The method of sucrose density gradient fractionation was applied to both normal thymic cells and AKR leukemic cells to separate them according to the phases of the cell cycle. Since the complications inherent in the utilization of synchronizing techniques can be eliminated by the use of sucrose density fractionation for separation of cells according to their position in the cell cycle, the comparison of that study with the study reported in this case, would indicate the validity of the findings reported herein.

The rates of total RNA synthesis during the cell cycle have been looked at in several cell types, but the results are conflicting and are confusing since most studies did not take into account changes in labeled precursor uptake or pool size during cell cycle (36–41). Similar conflicting evidence exists for rates of ribosomal RNA synthesis during the cell cycle (38, 42). The rate of uridine incorporation into total RNA has been reported by some investigators to increase linearly through the cell cycle (36–38), whereas others have described fluctuations in the rate of uridine incorporation, particularly an acceleration during early S phase (39–41). In a report describing the rates of RNA synthesis during the cell cycle of the Chinese hamster V79 cells used in the present study,


30. Mao, C. C. & Guidotti, A. (1974) “Simultaneous isolation of adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) in small tissue samples," Anal. Biochem. 59, 63–70.


