Levels of Ribonucleotide Reductase Activity during the Division Cycle of the L Cell*

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SUMMARY

The levels of ribonucleotide reductase activity (measured as the conversion of 3H-cytosine monophosphate to 3H-dCMP) in cell-free extracts of partially synchronized cultures of L cells were measured as the cells moved through the division cycle. A strict parallelism was found between the measured enzyme levels and the fractions of the population that were forming DNA. This relationship between enzyme level and ability to replicate DNA indicated that the reductase activity fell rapidly when cells emerged from the DNA synthetic period, suggesting a lability of the enzyme in vivo. The lability in vivo was confirmed by measuring enzyme levels in cultures where further protein synthesis had been blocked with cycloheximide. Under these conditions, an apparent half-life of less than 2 hours was observed for ribonucleotide reductase in the L cell.

The mechanisms that control the initiation and termination of DNA replication in mammalian cells are not known. One element in the control process may be the level of enzymes associated with the synthesis and polymerization of the deoxyribonucleoside triphosphates.

In regenerating liver, the observed activities of thymidine and thymidylate kinases, thymidylate synthetase, deoxycytidylate deaminase, and DNA polymerase begin to increase at about the time that DNA synthesis begins (1-3). Similar increases related to the initiation of DNA replication were observed with thymidine kinase and DNA polymerase in kidney cells cultured directly from the rabbit (4). In these systems, DNA formation is also accompanied by an increase in the size of the deoxyribonucleoside pool (5) and in the rate of labeling of deoxycytidine nucleotides from added 3H-cytidine (6, 7).

Less information is available for permanently cultivatable cells. Both L (8) and HeLa (9, 10) cells show significant increases in the activity of thymidine kinase while DNA is being replicated. The level of DNA polymerase in the L cell, on the other hand, has been found to remain relatively constant during the division cycle (11, 12). Littlefield, McGovern, and Margeison (11) and Gold and Helleiner (12), however, have suggested that the distribution of the polymerase activity among the intracellular compartments may be a more meaningful criterion of function than the total level of activity.

Ribonucleotide reductase, an enzyme more closely associated with the pathway leading to DNA synthesis than thymidine kinase, has now been measured in partially synchronized cultures of L cells. The levels of enzyme activity were found to parallel the number of cells forming DNA as the cells move through the division cycle. The rapid fall in ribonucleotide reductase activity observed in L cells that had completed the replication of DNA suggested that the enzyme was relatively unstable in vivo. The results of experiments in which protein synthesis was blocked with cycloheximide did, in fact, indicate that the L cell reductase has an apparent half-life of less than 2 hours.

EXPERIMENTAL PROCEDURE

Materials—The compounds used include 3H-CMP and 3H-dCTP from Schwarz BioResearch, 3H-thymidine from New England Nuclear, calf thymus DNA from Worthington Biochemical Corporation, crystalline pyruvate kinase and unlabeled nucleotides from Sigma Chemical Company, and amethopterin (Methotrexate) from Lederle Laboratories.

Cell Cultures and Partial Synchronization—Monolayers of L cells were grown at 37° in basal medium (13) supplemented with 10% calf serum in 60-mm Petri dishes (3 ml/dish) or in 32-ounce culture bottles (50 ml/bottle).

DNA synthesis was partially synchronised by treatment with amethopterin (14) as adapted to L cells (15). Cultures were grown in bottles until they contained about 10⁷ cells. The medium was then replaced with fresh medium containing glycine (0.9 mM) and adenine (0.2 mM), and amethopterin (3 x 10⁻⁴ mM)
was added to block DNA synthesis. After 16 hours, DNA synthesis was reinitiated by replacing the culture medium with one that contained adenine and glycine as before, but thymidine (5 x 10⁻³ mM) was added in place of amethopterin.

The degree of cell synchrony was measured by the incorporation of ³H-thymidine (20 μCi per ml of medium). After 1 hour, the cells were harvested, and nuclei were isolated with 0.1 mM citric acid. The nuclei were counted and washed extensively in trichloroacetic acid, ethanol, and ether. Radioactivity was estimated by scintillation counting in a H2O2 phosphor solution.

Cell synchronization was also measured by radioautography after labeling with ³H-thymidine. The medium with amethopterin was replaced with one that was supplemented with adenine, glycine, and ³H-thymidine (0.4 μCi per ml of medium). After 1 hour, the attached cells were washed with medium containing unlabeled thymidine (5 x 10⁻³ mM), and the washed cells were detached from the surface of the culture bottle. The cells were then plated in 60-mm Petri dishes, and 45 min later the attached cells were fixed with 95% ethanol. After washing with 5% trichloroacetic acid, excess acid was removed with 70% ethanol. The cells were then covered with a thin layer of Kodak NTB-3 emulsion and exposed for 2 days. At this time, the cells were stained with hematoxylin, and 200 or more were scored as labeled or unlabeled.

Assay of Ribonucleotide Reductase—The L cells, detached from the glass surface by scraping, were washed by centrifugation in 0.14 M NaCl. Packed cells were stored at -70°C. Enzyme extracts were prepared from the frozen cells immediately before assay. The reduction of ³H-CMP to ³H-dCMP by cell-free extracts was measured by chromatography on Dowex 50 as previously described (16). With the use of the standard assay (16), all the detectable reductase activity of the homogenate was found in the soluble fraction obtained by centrifugation for 1 hour at 105,000 x g. A unit of enzyme was defined as the amount that catalyzed dCMP synthesis at a rate of 1 mpmole per hour.

Assay of DNA Polymerase—DNA polymerase was measured in the same extracts used for the reductase assay. Enzyme assays were carried out as previously described (4) with the following modifications. The reaction volume was reduced from 1.0 to 0.2 ml, and the time of incubation was reduced from 60 to 30 min. Each test mixture contained 0.25 to 0.7 mg of enzyme protein. A unit of enzyme was defined as the amount that catalyzed dCTP incorporation into DNA at a rate of 0.1 mpmole per hour.

RESULTS

Degree of Synchrony in Large Cultures—In order to obtain extract for the ribonucleotide reductase assay (0.3 to 0.9 mg of protein per 0.25 ml of test mixture), it was necessary to grow the L cells on a moderately large scale. With the large cultures (32-ounce bottles with 10⁹ cells per bottle) that were required, it was not possible to achieve the degree of synchronous replication of DNA that could be obtained consistently with smaller cultures (60-mm Petri dishes with 5 x 10⁶ cells per dish) (Fig. 2). Nevertheless, as the figure shows, the replacement of amethopterin with thymidine in the larger cultures

![Fig. 1. Effect of protein concentration on the conversion of ¹H-CMP to ¹H-dCMP by L cell extracts. The soluble extract (7.3 mg of protein per ml) was prepared from asynchronous L cells, and varying concentrations of extract were tested for ribonucleotide reductase activity as described under "Experimental Procedure".](http://www.jbc.org/)

![Fig. 2. DNA synthesis as a function of time in partially synchronized L cell cultures. Cultures in 60-mm Petri dishes (approximately 5 x 10⁶ cells per dish) and in 32-ounce bottles (approximately 10⁹ cells per bottle) were partially synchronized with amethopterin as described under "Experimental Procedure." The amethopterin was replaced by thymidine at zero time. The cultures were pulsed for 1 hour with ³H-thymidine (20 μCi per ml of medium) beginning at 30 min before the indicated times. Incorporation of ³H-thymidine into DNA and the percentage of labeled cells at each time point is shown in parentheses for the large scale culture. *, Petri dish cultures; ●, 32-ounce bottle cultures.](http://www.jbc.org/)
did result in a prompt initiation of DNA synthesis in 60 to 70% of the cells. By 9 to 12 hours after release of the block of DNA synthesis, only about 20% of the cells were incorporating 3H thymidine. After 20 hours, the number had risen to approximately 40%. The number of cells in the bottles increased by 70% between 33 and 203 hours.

Enzyme Levels and DNA Synthesis—The removal of amethopterin from the culture medium was followed by parallel changes in DNA synthesis and in the specific activity of ribonucleotide reductase. Thus, the data summarized in Table I indicate that measurable ribonucleotide reductase was obtained only from the cells that were in the process of replicating DNA. In contrast to the results with the reductase, the specific activity of DNA polymerase remained constant as the partially synchronized cultures passed through the division cycle.

That the reduced reductase activity at 12 hours was not the result of appearance of a soluble inhibitor was shown by assaying mixtures of extracts from 12- and 20-hour cultures. Observed activities of such mixtures were found to average 96 ± 16% of the calculated additive values.

Apparent Half-life of Ribonucleotide Reductase—A rapid fall in the specific activity of ribonucleotide reductase occurred between 33 and 93 hours after release of the amethopterin block (Table I). These results suggested that the enzyme had a short lifetime in the L cell and that enzyme synthesis ceased when DNA replication was completed. The apparent half-life of the reductase was determined by measuring the levels of activity in preparations from partially synchronized cultures (60 to 70% of the cells in the S phase) and in random cultures as a function of time after protein synthesis was stopped with cycloheximide (Fig. 3). The concentration of cycloheximide used, 1 μg per ml, inhibited cellular protein synthesis by 85 to 90% as judged by the incorporation of 3H-lysine at various times from 0 to 6 hours after the addition of the antibiotic to the cultures.

As shown in Fig. 3, in the presence of cycloheximide, the ribonucleotide reductase activity decayed exponentially with a half-life of approximately 2 hours. The level of DNA polymerase activity, on the other hand, remained constant during the 6-hour exposure of the cells to cycloheximide.

To test the possibility that cycloheximide acted by directly inhibiting the enzyme system, cell-free extracts were assayed for ribonucleotide reductase in the presence of cycloheximide. Rates of dCMP formation of 2.0, 3.2, and 2.9 mmoles/hour were observed in the presence of 0, 1, and 100 μg of cycloheximide per ml, respectively. Thus, the antibiotic had no direct effect upon the enzyme.

### Table I

<table>
<thead>
<tr>
<th>Time after thymidine addition</th>
<th>DNA synthesis</th>
<th>Ribonucleotide reductase</th>
<th>DNA polymerase</th>
<th>Ratio of percentage of labeled cells to ribonucleotide reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>krs</td>
<td>% labeled cells</td>
<td>units/mg protein</td>
<td>% labeled cells</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>33</td>
<td>68 ± 5 (3)</td>
<td>3.0 ± 0.7</td>
<td>4.3 ± 0.6</td>
<td>23</td>
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<tr>
<td>93</td>
<td>16 (1)</td>
<td>0.7</td>
<td>2.3</td>
<td>23</td>
</tr>
<tr>
<td>13</td>
<td>22 ± 5 (3)</td>
<td>0.9 ± 0.3</td>
<td>3.5 ± 0.1</td>
<td>24</td>
</tr>
<tr>
<td>203</td>
<td>41 ± 2 (3)</td>
<td>1.9 ± 0.1</td>
<td>2.8 ± 0.5</td>
<td>22</td>
</tr>
</tbody>
</table>

*FIG. 3. Effect of cycloheximide on the level of ribonucleotide reductase in L cells. Cycloheximide (1 μg per ml) was added to partially synchronized (33 hours after replacement of amethopterin by thymidine) and asynchronous cultures in 32-ounce bottles. Cells were harvested at the designated times after the addition of the antibiotic, and extracts were prepared and assayed for ribonucleotide reductase and DNA polymerase as described under "Experimental Procedure." ○, ribotide reductase (asynchronous culture); △, DNA polymerase (asynchronous culture); ●, ribonucleotide reductase (synchronous culture).*

### Table II

<table>
<thead>
<tr>
<th>Culture</th>
<th>Time after cycloheximide addition</th>
<th>Cell population</th>
<th>3H-Thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>krs</td>
<td></td>
<td>% labeled cells</td>
<td>cpm/10^6 cells</td>
</tr>
<tr>
<td>Random</td>
<td>0 (-)</td>
<td>1.20</td>
<td>46 6.85 14.0</td>
</tr>
<tr>
<td></td>
<td>1.5 (+)</td>
<td>1.25</td>
<td>37 1.93 5.2</td>
</tr>
<tr>
<td></td>
<td>4.5 (+)</td>
<td>1.42</td>
<td>40 0.92 2.3</td>
</tr>
<tr>
<td>Synchronous</td>
<td>0 (-)</td>
<td>0.93</td>
<td>80 11.3 14.1</td>
</tr>
<tr>
<td></td>
<td>6 (-)</td>
<td>1.03</td>
<td>16 1.76 11.0</td>
</tr>
<tr>
<td></td>
<td>6 (+)</td>
<td>1.02</td>
<td>42 1.48 3.5</td>
</tr>
</tbody>
</table>
The rates of disappearance of ribonucleotide reductase activity in a synchronized culture where the bulk of cells were in the replicative state at the time of cycloheximide addition and in asynchronous cultures were essentially identical (Fig. 3). In both types of culture, as shown in Table II, cycloheximide also markedly reduced the rate of DNA synthesis in replicative cells. Thus, in the cycloheximide-treated synchronized culture in asynchronous cultures were essentially identical (Fig. 3). the replicative state at the time of cycloheximide addition and activity in a synchronized culture where the bulk of cells were in 3728 Ribonucleotide control population had doubled.

In both types of culture, as shown in Table II, cycloheximide inhibited DNA synthesis and prevented progression of the cells through the replicative period (Table II) represents additional evidence that loss of reductase activity is associated with termination of DNA synthesis and not with subsequent cell division. In view of the limited cell synchrony and the fact that cycloheximide inhibited DNA synthesis as well as protein synthesis, it is not possible at present to decide whether (a) the enzyme is synthesized continuously during replication, maintaining a steady state balance between synthesis and degradation, or (b) the enzyme is synthesized rapidly at the beginning of the replicative period and is stable as long as normal replication continues.

The rapid degradation and the tight correlation of the reductase activity with DNA synthesis are in contrast to the properties of other enzymes associated with DNA synthesis that have been studied in mammalian cells. The solubility considered to be DNA polymerase is a stable enzyme with an activity level that remains constant throughout the division cycle. Increased levels of thymidine kinase activity do occur in cells that are synthesizing DNA, but there is only a limited correlation between the activity of the enzyme and the replicative period. Thus, with Chinese hamster fibroblasts synchronized in metaphase, Stubblefield and Murphy (17) found that the kinase levels increased continuously from a time shortly after the initiation of DNA synthesis into the subsequent mitotic period. Kinase levels appeared to decrease only at or shortly after cell division. Apparently similar results were obtained by Brent, Butler, and Crathorn (10) with metaphase synchronized HeLa cells. The apparent bursts of thymidine kinase synthesis found by Littlefield (8) to be associated with DNA synthesis in L cells synchronized with 5-fluorodeoxyuridine were not followed by enzyme loss when the cells left the replicative period. Finally, Eker (18), with Chang liver cells, observed that puromycin blocked any additional amethopterin-induced rise in the level of the kinase but did not cause a loss of the activity already present in the cells.

Instability in vivo may be a property of an important class of mammalian protein, those that the cell requires at high levels of activity for brief periods only. In addition to the ribonucleotide reductase that may be essential in the period of DNA replication, members of the class may include some liver enzymes for amino acid catabolism such as tyrosine a-ketoglutarate transaminase with an apparent half-life of 3 to 4 hours (19) and tryptophan pyrrolase with an apparent half-life of 2 to 4 hours (20), and the RNA polymerases of regenerating liver (apparent half-life, 24 to 3 hours (21) and of the estrogen-treated uterus (apparent half-life about 1 hour (22)).

Although ribonucleotide reductase may play an important role in endowing the mammalian cell with competency to form DNA, one cannot conclude on the basis of current knowledge that the enzyme is the critical regulatory agent. In view of the data presented in this report, it is not unreasonable to postulate that the reductase level constitutes one important element of a complex control system. It is unlikely, however, that the total cellular level of any single enzyme can be responsible for the quantitatively exact duplication that characterizes DNA replication.

**REFERENCES**

Levels of Ribonucleotide Reductase Activity during the Division Cycle of the L Cell

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