Coupled Ribonucleoside Diphosphate Reduction, Channeling, and Incorporation into DNA of Mammalian Cells*

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The replicative synthesis of DNA by mammalian cells exhibits numerous features that are not readily understandable if the reactions of precursor synthesis and polymerization are catalyzed by soluble enzymes. DNA synthesis is abruptly initiated in each cell cycle (at the beginning of S phase) after several hours of RNA and protein synthesis (during G1 phase), and yet neither DNA polymerase nor its substrates are limiting prior to initiation (1, 2). Chain elongation, in vivo, by incorporation of dNTPs' occurs at a rate several times faster than for most macromolecular biosynthetic processes, including the chemically similar process of RNA synthesis (see Refs. 3 and 4). The average concentrations of dNTPs existing in a cell during the S phase are quite low (5) relative to the average Km of DNA polymerase for in situ semiconservative DNA synthesis (6) which is 4- to 5-fold higher. Furthermore, radioactivity from rNTPs was incorporated into DNA of permeabilized cells more effectively than from dNTPs (7), under the conditions suitable for the incorporation of each nucleotide (8). Under conditions which included dithiothreitol as a reducing agent to permit reduction of rNTPs (7), dNTPs were incorporated even less rapidly.

These observations can be accounted for on the basis of a multi-enzyme complex which functions to channel rNTPs through a pathway starting with ribonucleotide reductase (2'-deoxyribonucleoside diphosphate:oxidized thioredoxin 2'-oxido-reductase, EC 1.17.4.1) and terminating with DNA polymerase. Evidence for such complexes has accumulated over 10 years, in prokaryotic and eukaryotic cells, both virus infected and uninfected (see Ref. 9; also Refs. 10-14). We have demonstrated that in mammalian cells more than a half-dozen enzymes of the DNA synthetic pathway can be co-sedimented on sucrose gradients. We proposed a multi-enzyme complex that we have named replitase (7). It was found in mammalian cells that were synthesizing DNA semiconservatively and located in the nuclear fraction. Cells that had not initiated DNA synthesis did not possess replitase, and the corresponding enzymes were found unassociated and in the cytoplasm. Such a complex might function to rapidly and specifically incorporate rNTPs into DNA (following their reduction) and exclude dNTPs.

We now report that permeabilized cells rapidly incorporate label from rNTPs into DNA. The rate is limited by the initial step, catalyzed by ribonucleotide reductase. The reduced nucleotide (rCDP) does not become mixed with an added pool of dCTP. We find that ribonucleotide reductase exists in the nucleus during S phase and that it can be sedimented with the other enzymes of replitase. These results indicate precursors are channeled through the replitase complex into DNA.

**Experimental Procedures**

Materials—All experiments were carried out with Chinese hamster embryo fibroblast cell line, CHEF/18 (15) (obtained from Dr. Ruth Sager, Sidney Farber Cancer Institute, Boston, MA).

dNTPs were purchased from P-L Biochemicals, and rNTPs and the other reagents were used were from Sigma. [3H]dCTP and [3H]dTTP and [3H]dCDP were from New England Nuclear, and [3H]rCDP was from Amersham Corp. [3H]dTTP and [3H]rCDP were from New England Nuclear, and [3H]rCDP was from Amersham Corp. [3H]dTTP and [3H]rCDP used in these experiments were about 80% pure at t = 0 as shown in Fig. 2. Less than 0.5% of free bases (either thymine or cytosine) were detected in these radiolabeled precursors.

Cell Culture and Synchronization—CHEF/18 cells were grown in Dulbecco's modified Eagle's medium (purchased from Flow Labora-

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The abbreviations used are: dNTP, deoxyribonucleoside 5'-triphosphate; rNTP, ribonucleoside 5'-diphosphate; rNTP, ribonucleoside 5'-triphosphate; HU, hydroxyurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
rNDP Channeling into DNA in Permeabilized Mammalian Cells

RESULTS

Incorporation of rNDPs into DNA of Permeabilized Cells—Incorporation of $[^3]H$rCDP into DNA was rapid (Fig. 1) as previously reported (7). The $V_{\text{max}}$ was 2.5 units, calculated from a double reciprocal plot that gave a straight line (Fig. 2). This value is comparable to the in vivo rate of about 5 units (17). The $K_m$ for this process was 100 $\mu$M, determined in terms of added rCDP. However, rCDP was rapidly converted to mono- and triphosphates in these reaction mixtures (Fig. 3). Thus, the average rCDP concentration was lower during this 10-min assay, and correspondingly the $K_m$ was nearer to 50 $\mu$M.

The incorporation of rNDPs into DNA could be limited by their rates of reduction. We, therefore, measured kinetics of ribonucleotide reductase in the same preparation. Using rCDP as a substrate $V_{\text{max}}$ was 3.0 units and $K_m$ was 100 $\mu$M (Fig. 2) (uncorrected). This value is similar to the $K_m$ of 94 $\mu$M reported for crude sonicates of DON hamster fibroblast cells (23) and 130 $\mu$M for permeabilized CHO cells (24) ribonucleotide reduc-

DNA polymerase activity was measured as described elsewhere (7). Protein concentration was estimated by the method of Lowry et al. (22).

![Fig. 1. Effect of ribo- and deoxyribonucleotide concentration upon rates of their incorporation into DNA in permeabilized cells. Synchronized CHEF/18 cells in S phase were permeabilized and incubated in the presence of rNDPs or dNTPs. The appropriate reaction mixtures are as described under “Experimental Procedures.” Incubations were carried out for 10 min at 37° C. Acid-precipitable and alkaline-resistant material was prepared and assayed for DNA synthesis.](image-url)

![Fig. 2. Double reciprocal plot of the rate of rCDP incorporation and reduction with rCDP concentration in permeabilized CHEF/18 cells. $\bullet$—$\bullet$, overall process of rCDP incorporation into DNA; $\bigcirc$—$\bigcirc$, rCDP reduction to deoxyribonucleotide. Activities are expressed as picomoles of dCDP formed or incorporated/10^9 cells/min.](image-url)
Ribonucleotide reductase obtained from Molt-4F cultured human cells (25). rADP have been reported for highly purified ribonucleotide tase. Lower values, ranging from 7 μM for rCDP to 80 μM for rADP have been reported for highly purified ribonucleotide reductase obtained from Molt-4F cultured human cells (25).

Comparison of these Kₚ values with reported intracellular ribonucleotide pool concentrations in the range of 0.2 to 1 mM (1, 26) leads to the conclusion that pools in cells could give a maximal rate for such an incorporation of rNDPs into DNA. If ribonucleotide reductase is rate limiting for incorporation of rNDPs into DNA, then any inhibition of this activity should decrease incorporation into DNA, and the two activities should be inhibited in proportion. We, therefore, tested inhi-

### TABLE I

**Effect of dCTP, dCDP, and dTTP on rCDP reduction and its incorporation into DNA by permeabilized S phase cells**

Numbers are units representing the rate in picomoles/min/10⁶ cells. When the rate of rCDP reduction was measured the incubation mixture used was the same as described for DNA synthesis in the presence of rNDPs. Therefore, the rate expressed is the sum of δC that appeared in the nucleotides and DNA.

| Condition       | Rate of reduction | Rate of incorporation |
|-----------------|-------------------|-----------------------|
| Control         | 2.42              | 2.16                  |
| + effector (200 μM) | 0.18              | 0.14                  |
| dATP            | 0.98              | 0.90                  |
| dTTP            | 2.21              | 1.98                  |
| dCTP            | 1.21              | 1.01                  |

Ribonucleotide reductase might reduce rNDPs and then release the dNDP products into a general metabolic pool upon which DNA polymerase draws. We would then expect decreased incorporation of [³⁷Cl]rCDP into DNA of permeabilized cells if nonradioactive dCTP is added, because of dilution of the labeled dCTP intermediate. But if rCDP is incorporated into DNA through a channeling process that excludes dNTPs there should be major effects due to dilution of label upon including dNTPs in the reaction mixture. This experiment must be done with rCDP and dCTP, since only dCTP does not strongly inhibit ribonucleotide reductase (Table I), in contrast to the other dNTPs (27). Addition of up to 200 μM dCTP to a reaction containing 200 μM [³⁷Cl]rCDP only slightly (8%) decreased incorporation into DNA (Fig. 4). Thus, rCDP must be converted to dCDP and incorporated into DNA without mixing with the general dCTP pool.

It is possible that dCDPs formed due to a “free” ribonucleotide reductase activity are released into a general metabolic pool in the incubation mixture, and these noncompartmentalized dNDPs are then channeled into DNA due to a tight coupling between deoxynucleoside diphosphate kinase (NDP kinase) and DNA polymerase. In order to clarify this and to understand if rNDP channelling into DNA was in fact due to the direct interaction between ribonucleotide reductase and the rest of the replication complex (containing DNA polymerase and NDP kinase) we have tested whether dNDPs added exogenously can equilibrate with radioactive dNDP’s produced by ribonucleotide reductase from the corresponding ribo-

### Intracellular Localization of Ribonucleotide Reductase—

The simplest explanation for the above channeling results is that ribonucleotide reductase is physically closely associated with later enzymes in the pathway leading to DNA synthesis. We have reported earlier that during the S phase a half-dozen of these enzymes are found in the nucleus and are in a rapidly sedimenting fraction which we named replitase (7). In con-

For rNDPs to be channeled into DNA through a sequence initiated by reduction, ribonucleotide reductase must be a component of replitase. Ribonucleotide reductase should then appear in the nucleus around the beginning of S phase, as do other replitase enzymes (7). Fig. 5 shows this is the case. In a synchronized population, S phase commenced at 6 h. Up to this time, ribonucleotide reductase activity was distributed between cytoplasm and nucleus. But after 6 h all extra en-

### FIG. 3.

Fate of nucleotides during DNA synthesis in situ. Incubation conditions are as described in Fig. 1. Quantitation of ribo-

### FIG. 4.

Effect of dCTP, dCDP, and dTTP on [³⁷Cl]rCDP incor-

| dNTP Concentration (μM) | dCTP | dCDP | dTTP |
|-------------------------|------|------|------|
| 0                      | <0.01| <0.01| <0.01|
| 200                    | 0.10 | 0.05 | 0.08 |
| 400                    | 0.15 | 0.10 | 0.12 |
| 800                    | 0.20 | 0.15 | 0.18 |

*Note: The above table represents the repeated experiment for the effect of dCTP.*
Cells cultured cell synchrony was measured by C3H]thymidine ([3H]Tdr) during releasing from isoleucine block and assayed for rCDP reductase and cytoplasmic lysates were prepared at various intervals after incorporation into acid-precipitable material as described elsewhere (7). From parallel bilized cells rapidly incorporate dNTPs into DNA, as previously reported (6, 17). From Fig. 1, V\text{max} was about 1.2 units, and K\text{m} was 50 \mu M in agreement with earlier reports (6). Stability of dTTP was relatively high compared to rCDP (Fig. 3). Deoxyribopyrimidine triphosphatase activity was reported to be absent from uninfected baby hamster kidney cells (28). The substrates added thus remained at relatively constant concentrations and provided a suitable basis for calculation of K\text{m}. The pools of dNTPs reported for Chinese hamster ovary cells in S phase (5 to 20 \mu M) (5) are well below this K\text{m} value and are inadequate to give a rate of incorporation comparable to the rate of DNA synthesis (5 units) maintained by intact cells.

The maximal rate of incorporation of rNDPs into DNA was more rapid than the rate of dNTP incorporation (Fig. 1), as previously noted (7). This result is hard to explain if a free pool of dNTPs must be created from rNDPs. When dNTPs were incorporated using the conditions devised for the rNTP assay the rate was decreased by about 2-fold (as previously observed) (7). Systematic changes from rNDP to dNTP assay conditions which differ in concentrations of six components, in duplicate experiments, revealed that the most important factor for inhibition of dNTP incorporation was dithiothreitol, with smaller effects from KC\text{1} or MgCl\text{2} (Table I1). Dithiothreitol was omitted or HU was present or when both changes were made. These results support the rate-limiting role of ribonucleotide reductase in the process. Pyrimidine nucleoside triphosphates did not compete with the diphosphates, indicating that they are not readily converted to the pools of dNTPs.

The effects of different conditions on incorporation of dNTPs, using [\text{3H}]TTP as a label, are shown in the first section of Table III. Incorporation in the absence of dithiothreitol was about half as rapid as for rNTP incorporation (plus dithiothreitol). In the presence of dithiothreitol, [\text{3H}]TTP incorporation was only about 40 to 50% of that observed in the absence of dithiothreitol. This inhibition of dithiothreitol cannot be because of competitive production of dNTPs from pyrimidine ribonucleoside triphosphates, since their presence or absence had practically no effect.

In the second section of Table III one sees that the rNTPs, using [\text{3H}]rATP as a label, are very slowly incorporated into DNA, again reflecting slow conversions to the diphosphates. Ribonucleosides, therefore, could not provide a precursor pool competitive with added dNTPs, even when ribonucleotide reductase was active. These overall rates are so small that conclusions from their variations are not possible.

The last column showed that [\text{14C}]rCDP incorporation was rapid in the presence of dithiothreitol and much slower when dithiothreitol was omitted or HU was present or when both changes were made. These results support the rate-limiting role of ribonucleotide reductase in the process. Pyrimidine nucleoside triphosphates did not compete with the diphosphates, indicating that they are not readily converted to rNTPs. Finally, HU dramatically increased the rate of dTTP incorporation in the presence of dithiothreitol, even when pyrimidine ribonucleotides were absent. This HU effect thus could not be to block de novo production of dNTPs. All of these results are consistent with the hypothesis that accessibility of DNA polymerase to dNTP substrates into these permeabilized cells is allosterically determined by the state of ribonucleotide reductase. They are not consistent with interaction of the two enzymes through modifications of free pools of dNTPs.

**Table II**

| Reaction mixture | Rate in units |
|------------------|--------------|
| dNTP reaction mixture | 1.49 |
| Complete | 0.88 |
| Minus dithiothreitol | 1.26 |
| Plus 80 mM KC\text{1} | 0.43 |
| 35 mM Hepes (instead of 50 mM) | 0.83 |
| Minus FeCl\text{3} | 0.63 |
| Plus 50 mM sucrose | 0.84 |
| 4 mM MgCl\text{2} (instead of 8 mM) | 0.64 |

**Discussion**

Our main conclusion from this work is that Chinese hamster cells synthesize DNA by a sequestered pathway that starts with rNDPs (see Fig. 6 for a schematic representation). The rate-limiting step is catalyzed by ribonucleoside diphosphate reductase. The metabolic intermediates of this pathway do not mix with free dNTPs, but rather are "channeled" directly into DNA. Free dNTPs, present in S phase cells at low concentrations, are not effectively incorporated. They may function as allosteric regulators of ribonucleotide reductase or as substrates for repair of DNA.

Kinetic data obtained with permeabilized cells provide evidence for these conclusions. These cells incorporated rNDPs into DNA approximately as rapidly as intact cells incorporated added nucleosides (see also Ref. 17). The K\text{m} for the...
**Incorporation of precursors into DNA; effects of dithiothreitol, hydroxyurea, and possible competing substrates**

Numbers are units (pmol/ml x 10 min) representing the rate of nucleotide incorporation into DNA of permeabilized S phase cells. Complete reaction mixture in this experiment is the rNDP reaction mixture, except for nucleotides added. 1) With [3H]dTPP as substrate the complete reaction mixture contained: 200 μM dATP, dGTP, dCTP, and dTTP; 4 mM rATP; and 33 μM rGTP, rCTP, and rUTP. 2) With [3H]rATP as substrate the complete reaction mixture contained: 200 μM rTPP; 33 μM rGTP, rCTP, and rUTP; and 200 μM dATP, dGTP, dCTP, and dTTP; and rATP; and 33 μM rGTP, rCTP, and rUTP. 3) With [3H]rCDP as substrate the complete reaction mixture contained: 200 μM rCDP, rGDP, rADP, and rTDP; 4 mM rATP; and 33 μM rGTP, rCTP, and rUTP. When hydroxyurea or dithiothreitol were added to the reaction mixture the final concentration of these components was 1 mM for HU and 8 mM for dithiothreitol.

### Table III

| Substrates | [3H]dTPP (200 μM) | [3H]rATP (200 μM) | [3H]rCDP (200 μM) |
|------------|------------------|------------------|------------------|
|            | Minus rCTP and rUTP | Plus rCTP and rUTP | Minus dGTP and dATP and dUTP | Plus dGTP and dATP and dUTP | Minus rCTP and rUTP | Plus rCTP and rUTP |
| Complete   | 0.50             | 0.50             | 0.25             | 0.10             | 2.30             | 2.20             |
| Minus dithiothreitol | 1.00             | 1.00             | 0.15             | 0.20             | 0.80             | 0.80             |
| Plus HU    | 0.75             | 0.90             | 0.10             | 0.10             | 0.80             | 0.80             |
| Minus dithiothreitol, plus HU | 1.00             | 1.40             | 0.15             | 0.20             | 0.55             | 0.50             |

*a Competing substrates.

### Fig. 6. A speculative model of the DNA-synthesizing enzyme complex in Chinese hamster embryo fibroblast cells. DTT, dithiothreitol.

Overall process is low enough that the pools of rNDPs found in S phase cells saturate the process to give maximal activity. This K_m was the same as the K_m for ribonucleotide reductase. The overall reaction required dithiothreitol and was inhibited by HU, a specific inhibitor of ribonucleotide reductase. It was also inhibited allosterically by dATP or dTTP, to a degree similar to inhibition of ribonucleotide reductase by these compounds. The incorporation of rCDP into DNA was little inhibited by added dCTP, not an allosteric inhibitor of ribonucleotide reductase. Incorporation of rCDP from added dNTPs in permeabilized cells in the presence of dithiothreitol. First, the dNTPs are slowly incorporated. Secondly, dCTP did not decrease incorporation of label from [3H]rCDP. An alternative explanation to the above is that the nuclear membrane provides a permeability barrier to dCTP. This seems very unlikely, since this barrier is permeable to many molecules including proteins (29). Various nucleotides also readily enter the nucleus of permeabilized cells. These cells allow rapid entry and incorporation into nuclear DNA of dNTPs in the absence of dithiothreitol and slower but quite appreciable incorporation in the presence of dithiothreitol. dATP, dTTP, and dGTP are effective nuclear ribonucleotide reductase allosteric inhibitors in the presence of dithiothreitol. Also, rNDPs and rATP are rapidly utilized in the synthesis of DNA, in the presence of dithiothreitol. Finally, according to this model HU would have to increase nuclear membrane permeability in order to increase dNTP incorporation (including dCTP). All of these results argue strongly against involvement of the nuclear membrane as a barrier to dNTPs.

The channeling of rNDPs into DNA, rate limitation by ribonucleotide reductase, and allosteric effects between this enzyme and DNA polymerase are best explained by a physical connection between these and other enzymes of the DNA synthesis pathway as in prokaryotic cells (30). We have previously proposed that these enzymes are associated into a complex which we have named replisome (7). Work in progress on properties of this complex in extracts support this idea.² The present studies on channeling are strengthened by demonstration that ribonucleotide reductase behaves like other enzymes of the pathway, appearing in the nucleus at the beginning of S phase and being rapidly sedimented along with nascent DNA.² The sedimentsed ribonucleotide reductase is not nonspecifically bound to some high molecular weight material as shown by cycle specificity of the sedimentation pattern.² Also a mutant that overproduces ribonucleotide reductase 10-fold has, in S phase, only a stoichiometric quantity of enzyme recovered in the nucleus; the great surplus remains in the cytoplasm (31). These results are consistent with close kinetic coupling of ribonucleotide reductase to the site of new DNA synthesis.

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