

Ribonucleotide Reductase Association with Mammalian Liver Mitochondria*

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Background: Mitochondrial DNA replication is supplied by precursor pools distinct from pools supplying nuclear DNA replication.

Results: Liver mitochondrial extracts contain significant ribonucleotide reductase activity, associated with nucleoids.

Conclusion: Intramitochondrial ribonucleotide reduction may be a significant source of mitochondrial dNTPs.

Significance: Knowing sources of mitochondrial dNTP pools may lead toward understanding mitochondrial genomic stability.

Deoxyribonucleoside triphosphate pools in mammalian mitochondria are highly asymmetric, and this asymmetry probably contributes to the elevated mutation rate for the mitochondrial genome as compared with the nuclear genome. To understand this asymmetry, we must identify pathways for synthesis and accumulation of dNTPs within mitochondria. We have identified ribonucleotide reductase activity specifically associated with mammalian tissue mitochondria. Examination of immunoprecipitated proteins by mass spectrometry revealed R1, the large ribonucleotide reductase subunit, in purified mitochondria. Significant enzymatic and immunological activity was seen in rat liver mitochondrial nucleoids, isolated as described by Wang and Bogenhagen (Wang, Y., and Bogenhagen, D. F. (2006) *J. Biol. Chem.* 281, 25791–25802). Moreover, incubation of respiring rat liver mitochondria with [¹⁴C]cytidine diphosphate leads to accumulation of radiolabeled deoxycytidine and thymidine nucleotides within the mitochondria. Comparable results were seen with [¹⁴C]guanosine diphosphate. Ribonucleotide reduction within the mitochondrion, as well as outside the organelle, needs to be considered as a possibly significant contributor to mitochondrial dNTP pools.

Control of DNA precursor pool sizes is closely linked to genomic stability (1, 2). Most analyses of dNTP pools in eukaryotic or bacterial cell extracts show a pronounced pool asymmetry, with dGTP strongly underrepresented (3). However, as shown by *in vitro* measurements of replication errors during DNA synthesis, this underrepresentation does not significantly affect replication fidelity (4). On the other hand, our analyses of dNTP pool sizes in mammalian tissue mitochondria have

yielded a different picture. In mitochondrial extracts, dGTP is greatly overrepresented, amounting to as much as 90% of total dNTP in rat heart mitochondria (5, 6). *In vitro* analyses of replication fidelity suggest that this dNTP pool asymmetry contributes to the mitochondrial mutation rate, which is 1–2 orders of magnitude higher than that for the nuclear genome (7, 8).

To assess the biological significance of mitochondrial dNTP pool asymmetry, it is important to understand the metabolic sources of dNTPs within the mitochondrion. Most attention has focused upon salvage synthesis within the mitochondrion (9) and cytosolic *de novo* synthesis, involving ribonucleotide reductase, followed by deoxyribonucleotide transport into the organelle (10, 11). The data of Rampazzo *et al.* (11) and Pontarin *et al.* (10) support the premise that in quiescent cells the salvage pathway predominates, whereas proliferating cells depend upon the *de novo* pathway beginning with ribonucleotide reductase (RNR)⁴ in the cytosol.

RNR is an oligomeric enzyme consisting of a homodimeric large subunit, R1, and a homodimeric small subunit, R2. The level of R2 is cell cycle-regulated, rising during the S phase to meet the increased demand for DNA precursors. More recently discovered is p53R2, an alternative small subunit that is not cell cycle-regulated and that is expressed in response to DNA damage. Pontarin *et al.* (12, 13) have presented evidence that RNR containing the p53R2 subunit plays an essential function in providing mitochondrial DNA precursors in quiescent cells.

The metabolic significance of both pathways is unquestioned. With respect to the salvage pathways, two of the four deoxyribonucleoside kinases in human cells—deoxyguanosine kinase and thymidine kinase 2—are localized within mitochondria (9), and genetic deficiency of either one has serious clinical consequences (14), resulting from depletion of mitochondrial DNA. With respect to the *de novo* pathway operating in the cytosol, Bourdon *et al.* (15) found that one member of the family of mitochondrial DNA depletion diseases results from a deficiency of p53R2, the DNA damage-inducible form of ribonucleotide reductase small subunit. Thus, with respect to the effects of genetic deficiency on human health, both the mito-

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⁴ The abbreviation used is: RNR, ribonucleotide reductase.

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chondrial salvage pathways and the RNR-related pathway that begins in the cytosol play significant metabolic functions.

In 1994 our laboratory reported evidence for RNR activity in mitochondria isolated from HeLa cells (16). However, because of the high RNR activity in the cytosol of these rapidly proliferating cells, we could not definitively rule out the possibility that the activity we were measuring resulted from cytosolic contamination of our mitochondrial preparations.

Our preliminary results (16) suggested the possibility of a third pathway to mitochondrial dNTPs, a pathway beginning with reduction of ribonucleotides within the mitochondrion. The issue is timely for several reasons. First, Pontarin *et al.* (10) briefly mentioned their inability to detect a mitochondrial RNR activity, and the apparent disagreement between our results and theirs needs to be resolved. Second, the possibility that RNR acts both in cytosol and mitochondria to produce mitochondrial dNTPs suggests a possibly wasteful duplication of function. Third, Anderson *et al.* (17) demonstrated a *de novo* pathway in mammalian mitochondria for dTMP biosynthesis from dUMP, involving intramitochondrial thymidylate synthase, dihydrofolate reductase, and serine hydroxymethyltransferase. Might other *de novo* pathways to deoxyribonucleotides exist within mitochondria?

For the present study, we first confirmed by an independent approach our previous finding of asymmetric dNTP pools in mitochondria. Next we examined ribonucleotide reductase activity in mitochondria and submitochondrial fractions from tissues in the adult rat, where we expected cytosolic RNR activity to be relatively low in nonproliferating tissues. Next, we used immunoprecipitation and mass spectrometry to search for RNR subunits in purified rat liver mitochondria. We also prepared nucleoids from rat liver mitochondria and analyzed these preparations for RNR. Finally, we asked whether isolated respiring rat liver mitochondria could take up radiolabeled ribonucleoside diphosphates and convert them *in situ* to deoxyribonucleotides.

EXPERIMENTAL PROCEDURES

Materials— $[^{14}\text{C}]$ Deoxycytidine diphosphate and $[^{14}\text{C}]$ deoxyguanosine diphosphate, both uniformly labeled, were obtained from Moravsek. Antibodies to RNR proteins R1, R2, and p53R2 were obtained from Santa Cruz Biotechnology, Inc. The antisera were raised against portions of the respective human proteins in goats, and the supplier certified that the antibodies cross-reacted with the corresponding mouse and rat proteins. Anti-goat IgG and protein A/G Plus-agarose beads were also obtained from Santa Cruz Biotechnology, Inc.

Methods—Mitochondria were isolated from livers of freshly sacrificed rats by rapid chilling of the organ, followed by homogenization in isolation buffer and differential centrifugation, as previously described (5, 6). The isolation buffer was 220 mM mannitol, 70 mM sucrose, 5 mM MOPS (pH 7.4), 2 mM EGTA, and 0.2 mg/ml BSA. The same procedure was used for isolating mitochondria from mouse liver. For some experiments, mitochondria were isolated from pig liver or pig heart. In these cases, the isolation procedures were essentially the same, but organs were obtained from freshly killed animals at a

local slaughterhouse. Mitochondria from HeLa cells were isolated as previously described (18).

Enzyme Assays—Enzyme assays were carried out on mitochondrial extracts or subfractions, as described under "Results." Ribonucleotide reductase activity was determined by conversion of $[^3\text{H}]\text{CDP}$ to $[^3\text{H}]\text{dCDP}$ as previously described (19). Mitochondrial and subcellular marker enzyme activities were determined by standard procedures: lactate dehydrogenase (20), citrate synthase (21), adenylate kinase (22), and cytochrome *c* oxidase (23).

Immunoprecipitation—All operations were carried out at 4 °C. Three aliquots of rat liver mitochondria, each representing ~0.75 g of tissue, were washed by two cycles of resuspension in isolation buffer, followed by centrifugation. Each pellet was resuspended in 0.75 ml of radioimmune precipitation assay buffer (Santa Cruz Biotechnology, Inc.) and disrupted by sonic oscillation. After centrifugation for 30 min at 14,000 rpm in a microcentrifuge, 0.5 μg of donkey anti-goat IgG was added to each supernatant along with 20 μl of protein A/G Plus-agarose bead suspension. After 30 min of standing, each mixture was centrifuged briefly (1 min at 5000 rpm), and to each supernatant was added 1.0 μg of primary antibody (R1, R2, or p53R2). Each mixture was allowed to stand for 2 h, following which 20 μl of protein A/G Plus bead suspension was added to each mixture, and the three mixtures were slowly rotated overnight. Each mixture was then centrifuged for 1 min at 5000 rpm, and the supernatants were discarded. Each pellet was washed by suspension followed by recentrifugation three times with radioimmune precipitation assay buffer and twice with 0.1 M Tris-HCl buffer (pH 8.0).

Digestion and LC/MS/MS Analysis of Immunoprecipitates—Proteomic analyses were performed in the Oregon State University Environmental Health Sciences Center mass spectrometry facility and core. In-solution digestion was performed using Promega protease enhancer and trypsin (modified, gold) following the manufacturer's protocol (Promega, Madison, WI). Tryptic peptides were trapped on a Michrom Peptide Cap-Trap column and a C18 column (Agilent Zorbax 300SB-C18, 250 \times 0.3 mm, 5 μm). A binary solvent system consisting of solvent A (2% aqueous acetonitrile with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) was used. Peptides were trapped and washed with 1% solvent B for 3 min. Peptide separation was achieved using a linear gradient from 3% B to 30% B at a flow rate of 4 $\mu\text{l}/\text{min}$ over 35 min. LC-MS/MS analysis was conducted on a Thermo LTQ-FT MS instrument coupled to a Waters nanoAcquity UPLC system. The LTQ-FT mass spectrometer was operated using data-dependent MS/MS acquisition with an MS precursor ion scan, performed in the ICR cell, from 350 to 2000 m/z with the resolving power set to 100,000 at m/z 400. MS/MS scans were performed by the linear ion trap on the five most abundant doubly or triply charged precursor ions detected in the MS scan.

Thermo RAW data files were processed with Proteome Discoverer v1.3.0. For database searching, Mascot (v2.3) and X!Tandem (embedded in Scaffold proteome analysis software) were used to search the *Rattus norvegicus* database, using the following parameters: the digestion enzyme was set to Trypsin/P, and two missed cleavage sites were allowed. The precur-

sor ion mass tolerance was set to 50 ppm, whereas fragment ion tolerance of 1.0 Da was used. Dynamic modifications that were considered: carbamidomethylation (+57.02 Da) for cysteine; oxidation (+15.99 Da) for methionine; phosphorylation (+97.98 Da) for serine, threonine, and tyrosine; deamidation (−0.98 Da) for asparagine and glutamine; and acetylation (42.02 Da) for lysine and the N terminus. Scaffold_3.3.1 (Proteome Software, Portland, OR) was used for search data compilation and data evaluation.

Sucrose Gradient Centrifugation—Mitochondria were suspended in isolation buffer plus 2 mM DTT, 0.2 mM PMSF, 2 mM pepstatin A, 5 mg/ml leupeptin, 2 mg/ml E-64, and 1% Triton X-100. The suspension was incubated for 10 min at 4 °C and then centrifuged at $18,000 \times g$ for 10 min at 4 °C. The supernatant was layered onto a 5–20% sucrose gradient in a Beckman SW41 rotor and centrifuged at 4 °C for 24 h at 26,000 rpm. The cytosol, analyzed in a separate gradient, was simply the postmitochondrial supernatant from the original fractionation of a liver homogenate.

Nucleoids—Nucleoids were isolated from rat liver and pig liver mitochondria essentially as described by Wang and Bogenhagen (24) for HeLa cell nucleoids. Briefly, mitochondria were isolated by centrifugation of a postnuclear supernatant through a Percoll step gradient and then purified by nuclease treatment and recentrifugation through sucrose. The purified mitochondria were next treated with digitonin to create mitoplasts. After washing and repelleting, the mitoplasts were lysed with Triton X-100 and subjected to brief centrifugation. The supernatant, containing lysed mitoplasts, was layered onto a 17 to 45% glycerol gradient over a pad of 30% Nycodenz and 30% glycerol and then centrifuged for 1.5 h at $186,000 \times g$ in a Beckman SW41 rotor.

Nucleotide Incorporation and Metabolism in Intact Mitochondria—For the experiments involving radiolabeled nucleotide uptake and reduction *in situ*, rat liver mitochondria, after isolation and washing, were aliquoted as a series of centrifugal pellets in microcentrifuge tubes, with each pellet representing ~1 g of wet weight of the original liver. Mitochondria were used on their day of isolation. Each pellet was suspended in 1.0 ml of an incubation mixture containing 70 mM sucrose, 220 mM mannitol, 5 mM MOPS (pH 7.4), 5 mM KH_2PO_4 , 5 mM MgCl_2 , 1 mM EGTA, 10 mM potassium glutamate, 2.5 mM sodium malate, 1.0 mM ADP, and 1.0 mg/ml bovine serum albumin. Each mixture was incubated at 37 °C for 10 min with occasional shaking. At that point 1.0 μCi of isotopic precursor ($[^{14}\text{C}]\text{CDP}$ or $[^{14}\text{C}]\text{GDP}$ as specified, uniformly labeled, without dilution) was added to each mixture. At each indicated time (5, 10, 20, and 30 min), one tube was removed and rapidly chilled to prevent further incorporation. After the final sample had been taken, all of the samples were centrifuged, and the mitochondrial pellets were washed by resuspension in incubation buffer followed by recentrifugation. Nucleotide extraction was carried out with 60% aqueous methanol followed by heating, as previously described (6).

Nucleotides were resolved, after the addition of nonradioactive marker nucleotides, by two-dimensional thin layer chromatography on PEI-cellulose sheets. The first dimension was 1 M lithium chloride saturated with sodium borate at pH 7, and

the second dimension was 0.35 M ammonium sulfate. Marker nucleotides were located under UV light, spots on the chromatogram were cut out, and radioactivity was measured by liquid scintillation counting.

RESULTS

dNTP Pool Asymmetry—As stated earlier, much of our interest in characterizing ribonucleotide reductase in mammalian mitochondria was to help illuminate factors influencing the pronounced dNTP pool asymmetry in these organelles (5, 6). These results have been questioned (25, 26), based partly upon possible nucleotide redistribution resulting from ATP loss during mitochondrial isolation and partly upon technical aspects of the DNA polymerase-based assay procedure used to measure dNTP pools. We found (6) that our ATP pools determined in isolated mitochondria conformed to values published by others, indicating that ATP loss during mitochondrial isolation was not a significant issue. Also, we confirmed the technical problem with the dNTP assay procedure but found that it does not apply under our assay conditions. Nevertheless, to resolve the issue, it seemed advisable to reconfirm our findings by an independent approach.

We normally assay dNTPs by the DNA polymerase-based method (27, 28). Although we have dealt with known sources of error (6, 26) in this assay, it seemed desirable to verify our measurements of dGTP levels in mitochondria by an independent approach. An obvious approach is HPLC, monitored by UV absorbance. In general, mitochondrial nucleotide pools are so low in relation to the amount of material available as to make their quantitation by HPLC problematical. However, our analyses of rat heart mitochondria by the enzymatic assay indicate that in this organ, mitochondrial dGTP levels are comparable to those of ribo-GTP (6), suggesting that dGTP can be quantitated by HPLC. As shown in Fig. 1 (*left panel*), two peaks in the analysis of a rat heart mitochondrial extract were identified as GTP and dGTP, respectively, by comparison of their elution times with standards. The extract was then passed through a boronate column, which retains ribonucleotides but not deoxyribonucleotides (29). Analysis of the flowthrough (*right profile*) showed disappearance of the peak identified as GTP, with the more abundant ATP not being quantitatively removed. More to the point, there was no change in the putative dGTP peak. The dGTP content of each extract, before and after boronate chromatography, was determined from the respective peak area, with reference to standards. Each fraction was also analyzed for dGTP by the enzymatic assay. Within experimental error the dGTP pool size, in pmol per mg of mitochondrial protein, was the same in all four cases, as shown in the data tabulated in Fig. 1. Hence, the unusually large dGTP pool in rat tissue mitochondria has been demonstrated by two independent approaches—by the DNA polymerase-based assay, as reported previously (6), and by HPLC, as shown here.

Ribonucleotide Reductase Activity in Tissue Mitochondria—As stated earlier, a concern about our earlier report (16) was the possibility that the RNR activity we were measuring in mitochondrial extracts represented contamination of our HeLa cell mitochondrial preparations with cytosol. For this reason we turned to tissue mitochondria from adult animals, where cyto-

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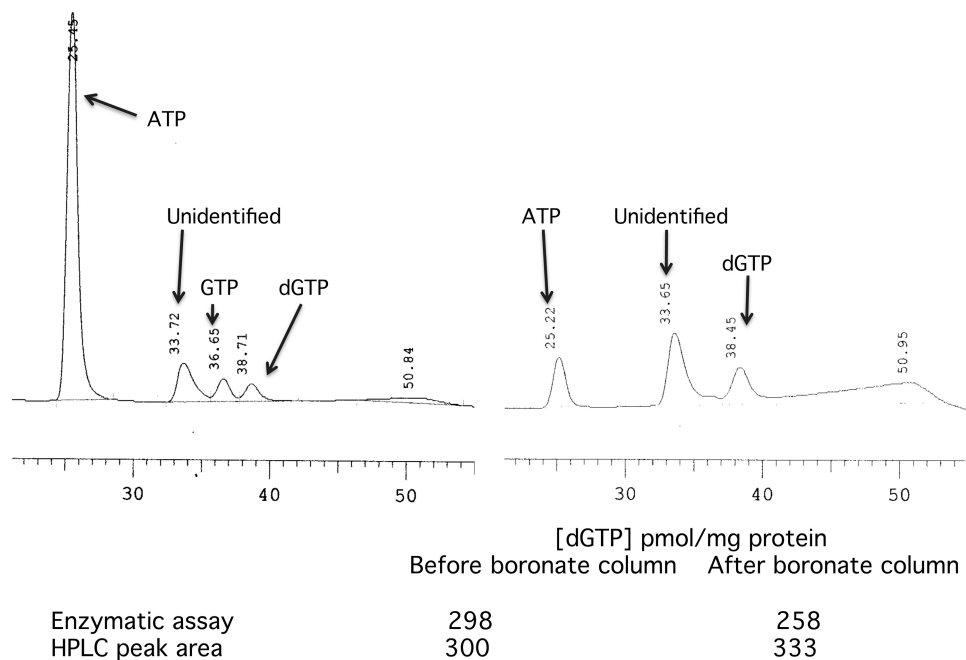


FIGURE 1. **Quantitation of dGTP in rat heart mitochondria by HPLC.** An extract of rat heart mitochondria was analyzed by HPLC before (*left panel*) and after (*right panel*) passage through a boronate column to remove ribonucleotides. Chromatography was carried out on a Whatman Partisil 10 SAX column as previously described for separation of ribonucleoside triphosphates (6). Nucleotides were identified by retention time and quantitated by peak area with reference to standards. dGTP content in the extracts before and after boronate chromatography was determined also by the DNA polymerase-based assay, and all of the determined values for dGTP are tabulated.

solic activity of RNR is expected to be low, and the contamination issue to be minimized. In the experiment of Fig. 2, we measured RNR activity in mitochondrial and cytosolic extracts from mouse, rat, and pig liver. In all cases the RNR specific activity in mitochondria was higher than that in cytosol, by factors of two to four. Also lactate dehydrogenase, representing a cytosolic marker, showed low activity in the mitochondrial fractions, indicating minimal cytosolic contamination. On the other hand, in HeLa cells the RNR specific activity in cytosol was higher than in mitochondria. This indicates that the RNR activity we earlier reported in HeLa cell mitochondria could have been a cytosolic contaminant. However, cytosolic contamination is ruled out in liver, where the mitochondrial specific activity is higher than that in cytosol.

The enzyme specific activity in mitochondrial extracts may appear low—approximately 10 pmol/min/mg of protein for rat liver extracts—but the values we observed are comparable to those reported for other crude tissue extracts. Earlier our laboratory reported ~3 pmol/min/mg of protein for BSC40 kidney cells and 20 pmol/min/mg for the same cells after infection by vaccinia virus, which induces a new RNR (19). Turner *et al.* (30) reported values between 15 and 50 pmol/min/mg of protein for mouse L cell extracts. Elford *et al.* (31) reported values ranging from below 1 to ~13 pmol/min/mg of protein for extracts from a series of rat hepatomas.

Ribonucleotide Reductase Activity in Submitochondrial Fractions—We next investigated the submitochondrial localization of RNR activity. In the experiment of Table 1, we divided rat liver mitochondria into a soluble fraction and a membrane fraction, essentially as described by Kang *et al.* (32). The fractionation was monitored by measurements of citrate synthase, a marker for soluble mitochondrial enzymes, and cytochrome *c*

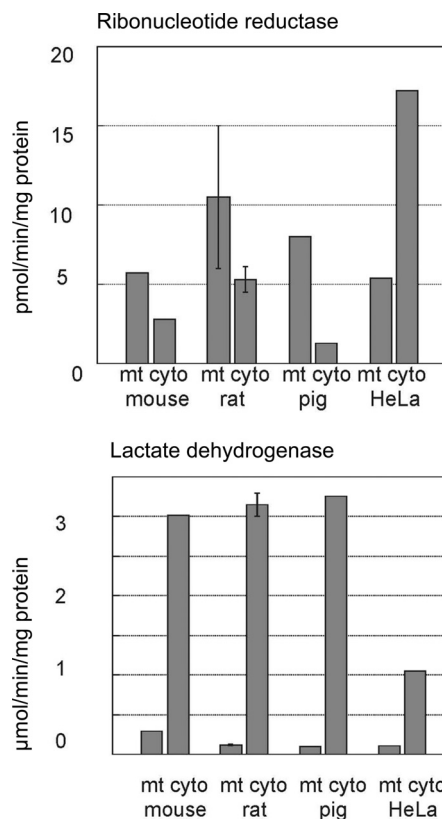


FIGURE 2. **Ribonucleotide reductase activity in mitochondria and cytosol of mammalian livers and of HeLa cells.** Lactate dehydrogenase was assayed as a marker for cytosolic contamination. The data for rat liver represent average values for duplicate assays on three different mitochondrial preparations \pm standard deviation. The values for the remaining assays represent averages of duplicate assays on single preparations. *cyto*, cytosol; *mt*, mitochondria.

TABLE 1

Enzyme activities in fractionated rat liver mitochondria divided into membrane *versus* soluble fraction

Mitochondria were divided into a soluble fraction and a membrane fraction, essentially as described by Kang *et al.* (32). Mitochondria were ruptured by sonic oscillation and then centrifuged at $320,000 \times g$ for 1 h at 4 °C. The supernatant was the soluble fraction, and the pellet, after resuspension, was the membrane fraction. Each value is a mean \pm standard deviation of two independent experiments with different rats, involving duplicate assays in each experiment. Both citrate synthase and cytochrome *c* oxidase activities are recorded as nmol/min/mg of protein. Ribonucleotide reductase activities are recorded as pmol/min/mg of protein.

Enzymes	Mitochondrial extract	Mitochondrial soluble fraction	Mitochondrial membrane fraction
Citrate synthase	126 \pm 21	292 \pm 88	31.2 \pm 12.7
Cytochrome <i>c</i> oxidase	157 \pm 13	20.3 \pm 1.1	421 \pm 69
Ribonucleotide reductase	13.6 \pm 0.5	49.8 \pm 16.3	4.6 \pm 1.9

oxidase, a membrane marker. RNR specific activity was enriched nearly 4-fold in the soluble fraction.

In another experiment we prepared mitoplasts from rat liver mitochondria, by solubilizing the outer membrane with digitonin (33). As shown in Table 2, 82% of total RNR activity was retained in the mitoplasts, comparable to what we saw with citrate synthase. Nearly all of the adenylate kinase activity, representing an intermembrane space marker, was absent from the mitoplasts. Together the combined data of Tables 1 and 2 indicate that in rat liver mitochondria, ribonucleotide reductase is largely localized in the matrix.

We hoped to purify the enzyme from pig liver mitochondria, because of the larger amount of starting material available, compared with rats. As in rodent tissues, pig heart and pig liver mitochondria both displayed enzyme activity and contained material immunoreactive with antibodies to human R1. However, much or most of the pig mitochondrial enzyme was membrane-bound (data not shown). The enzyme could be solubilized with some non-ionic detergents, but with low recovery of activity. By contrast, the enzyme activity in both rat liver and mouse liver was soluble. However, because of the difficulty of obtaining sufficient starting material from rat or mouse liver mitochondria, further purification was not attempted.

RNR Immunoreactive Material in Rat Tissue Mitochondria—Immunoblotting experiments were carried out with mitochondrial extracts and subfractions. Immunoreactive bands were seen with antibodies to both R1 and R2 (data not shown). However, the estimated molecular weights of immunoreactive material were lower than those reported for mammalian R1 and R2. Whether this results from degradation of enzyme subunits during mitochondrial isolation or from the possibility that the mitochondrial RNR is a distinct protein cannot be ascertained. Immunoblot experiments with purified mouse R1 and R2 proteins were inconclusive. Therefore we carried out immunoprecipitation with extracts of rat liver mitochondria and analyzed tryptic digests of the immunoprecipitates by mass spectrometry.

Table 3 presents results of this experiment. Washed mitochondria were extracted as described under "Experimental Procedures," and separate aliquots were treated with antibodies to mouse R1, R2, and p53R2. The table presents combined results from three immunoprecipitations. The R1 immunoprecipitate revealed four unique R1 peptides, covering 77 of the 792 residues in rat R1. The R2 immunoprecipitate also revealed four unique R1 peptides, which cover 64 of the 792 R1 residues. One of the four peptides was found in both immunoprecipitates. Neither R2- nor p53R2-derived peptides were detected with statistical assurance in any of the three immunoprecipitates.

TABLE 2

Enzyme activities in fractionated rat liver mitochondria divided into mitoplasts *versus* intact mitochondria

Mitoplasts were prepared as described by Ragan *et al.* (33). Briefly, a mitochondrial suspension at 25 mg/ml protein was mixed with digitonin at a ratio of 1 mg of digitonin to 10 mg of protein. After incubation for 15 min on ice, the sample was diluted with isotonic buffer and centrifuged at $15,000 \times g$ for 10 min, followed by gentle resuspension of the pellet. Each value is a mean of two independent experiments from different rats. Citrate synthase is a marker enzyme for mitochondrial matrix. Adenylate kinase is a marker enzyme for mitochondrial intermembrane space.

Enzymes	Total activity	
	Mitochondrial extract	Mitoplasts
	%	
Citrate synthase	100	79
Ribonucleotide reductase	100	82
Adenylate kinase	100	2

We assume that the R2 antibody precipitated a holoenzyme containing R1 but that any R2 peptides may have been present at levels too low to allow their unambiguous detection. The data identify R1 as a mitochondrial protein and suggest that R2 or an R2-related protein is present as well.

Because the p53R2 amino acid sequence is not in the rat protein database, we broadened the search to include sequences from the combined rat, mouse, and human amino acid sequences. This analysis did not reveal p53R2 peptides in any of the three immunoprecipitates nor did it reveal any R2 peptides that might have been missed through analysis of the rat database alone. Whether p53R2 might be present within mitochondria remains an open question; our analysis did not reveal it.

Sucrose Gradient Centrifugation of Mitochondrial RNR—Although it does not resolve the question of whether the activity in mitochondrial extracts represents a novel form of ribonucleotide reductase, we did analyze the RNR activity following sucrose gradient centrifugation of pig liver mitochondrial and cytosolic extracts (Fig. 3). The mitochondrial activity sedimented slightly more rapidly than human hemoglobin ($M_r = 65$ kDa), centrifuged separately as a marker, whereas the cytosolic activity sedimented approximately twice as rapidly as the marker. In this experiment, 96% of the mitochondrial activity and 73% of the cytosolic activity were recovered from the gradient. In another experiment (not shown), the activity in solubilized pig heart mitochondria behaved similarly to the behavior shown here for liver mitochondria.

Ribonucleotide Reductase in Mitochondrial Nucleoids—The mitochondrial nucleoid is a complex in the matrix containing tightly folded mitochondrial DNA and proteins, most of them connected with mitochondrial DNA replication and transcription. Mitochondrial genome replication evidently occurs at the nucleoid level, and much interest is focused upon this process

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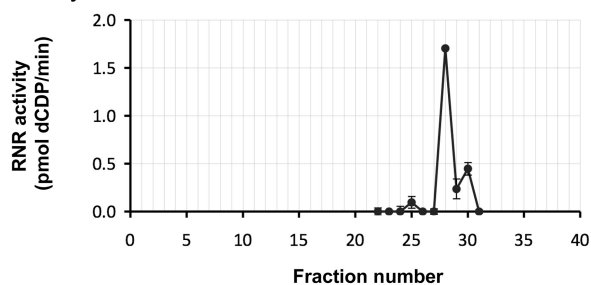
TABLE 3

Immunoprecipitation of ribonucleotide reductase subunits in rat liver mitochondrial extracts

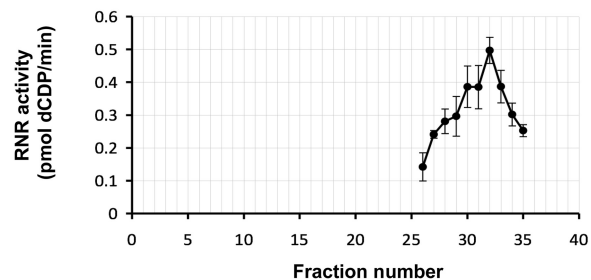
Antibodies to mouse R1, R2, and p53R2 proteins were used for immunoprecipitation analysis of washed rat liver mitochondrial extracts as described in the text. Peptides were identified with at least 95% probability, and protein identification based upon each individual peptide was at least 95%.

Antibody to	Rat R1 peptide in immunoprecipitate	Molecular mass of peptide	
		Theoretical	Observed
R1	SYLLKINGK	1177.8	1175.6
	NTGKEEQRAR	1231.7	1229.6
	SNQNLGTIKSNLCTEIVEYTSK	2774.3	2771.2
	NTAAMVCSLENR	1366.7	1364.6
R2	AQQLWYAIIESQTETGTPYMLYK	2933.4	2930.4
	NQIIACNGSIQSIPEIPEDLK	2424.1	2421.1
	ALKEEEEEK	1018.5	1016.5
	NTAAMVCSLENR	1366.7	1364.6

A. Pig liver cytosol



B. Pig liver mitochondria



C. Hemoglobin standard (A₅₄₀)

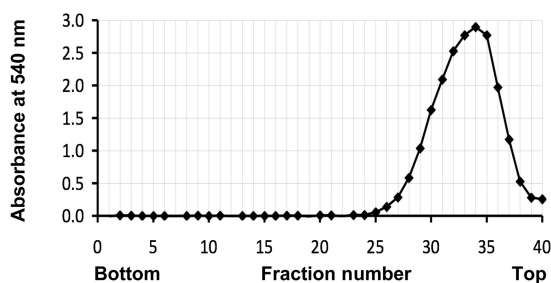


FIGURE 3. Sucrose gradient centrifugal analysis of ribonucleotide reductase activity in pig liver cytosol and mitochondrial extracts. The details are given under "Experimental Procedures." Human hemoglobin was centrifuged in a separate tube as a sedimentation rate marker. The direction of sedimentation is from *right* to *left*. Each data point in the analyses of RNR activity represents the average \pm standard deviation of triplicate assays. A, pig liver cytosol; B, pig liver mitochondria; C, hemoglobin standard.

as a basis for understanding inheritance of mitochondrial characteristics (34). Because of the role of ribonucleotide reductase in providing DNA precursors, it was of interest to know whether the enzyme in mitochondria is co-localized with the mitochondrial replisome.

To investigate this possibility, we isolated rat liver mitochondrial nucleoids by the procedure of Wang and Bogenhagen (24),

in which mitoplasts are isolated from purified mitochondria, followed by lysis of the mitoplasts and centrifugation of the lysate through a velocity gradient. As reported also by Wang and Bogenhagen, we observed two peaks of DNA and protein in the sedimentation profile (Fig. 4*b*), although in this experiment some of the heavy material sedimented to the bottom of the tube. Both the rapidly sedimenting and slowly sedimenting peaks contained material immunoreactive with human R2, the RNR small subunit protein, whereas R1-immunoreactive material was found only in the slowly sedimenting nucleoids (Fig. 4*a*). The fractions containing both R1- and R2-immunoreactive material also displayed enzyme activity, as shown in Fig. 4*c*. The physical association of RNR activity with nucleoids suggests that there may be a functional relationship as well. The strong association of R2 with the rapidly sedimenting nucleoids, in the apparent absence of R1, is of interest, but its significance is unknown. Note that in our hands the nucleoid markers RNA polymerase and HSP60 were found only in the slowly sedimenting peak (Fig. 4*a*).

We also investigated RNR association with nucleoids in pig liver mitochondria, which were prepared essentially identically to the procedure used in rat liver. The results were similar in that nucleoids sedimented in two forms, with R1 and R2 both in the slowly sedimenting form and R2 alone found in the rapidly sedimenting material (Fig. 5). A difference between rat and pig in these experiments was that in the pig liver nucleoids, the nucleoid markers that we analyzed were found in both rapidly and slowly sedimenting peaks. In this experiment, we also probed nucleoid fractions for p53R2, the DNA damage-inducible form of the RNR small subunit. We saw no evidence for the existence of this protein in nucleoids.

Ribonucleotide Reduction in Isolated Mitochondria—In preliminary experiments, we found that rat liver mitochondria readily took up radiolabeled cytidine diphosphate (35). It was of obvious interest to determine whether this RNR substrate could undergo reduction after being taken up into mitochondria.

For this study, we incubated respiring mitochondria with [¹⁴C]CDP and followed its fate by thin layer chromatographic separation of nucleotides on PEI-cellulose, followed by determination of radioactivity in the separated deoxyribonucleotides. In the earliest experiments, we carried out a preliminary separation of deoxyribonucleotides from ribonucleotides by boronate column chromatography followed by TLC analysis of the deoxyribonucleotides in the flowthrough. However, we

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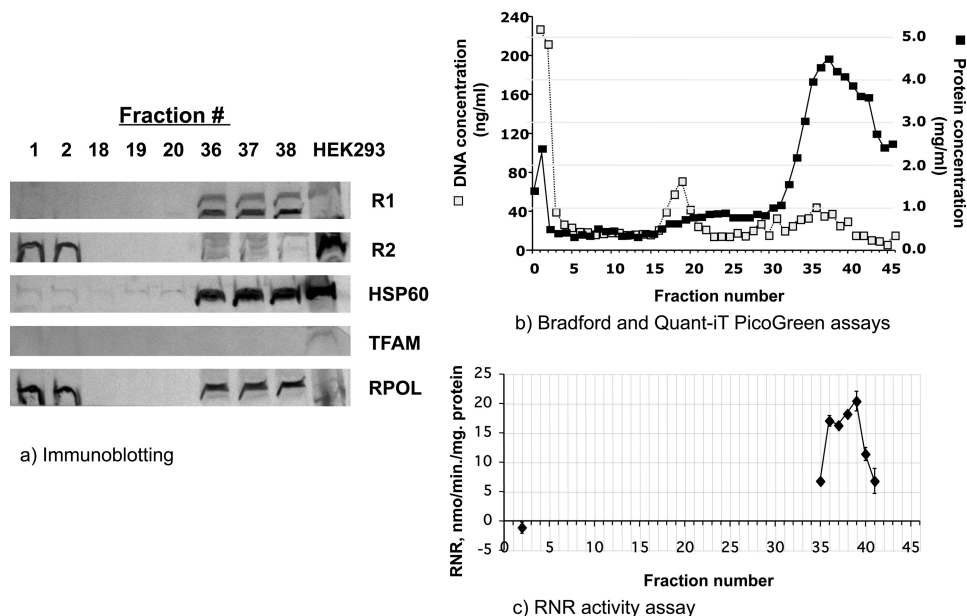


FIGURE 4. **Association of ribonucleotide reductase activity with mitochondrial nucleoids from rat liver.** Nucleoids were prepared and subjected to velocity gradient centrifugation as described by Wang and Bogenhagen (24). DNA and protein in the gradient fractions were analyzed by the Bradford and Pico-Green assays, as specified by the manufacturer. Selected gradient fractions were analyzed by immunoblotting for R1, R2, and selected nucleoid markers as shown. HEK293 depicts immunoblot analysis of an extract of cultured human kidney cells as a positive control. *a*, immunoblotting; *b*, DNA and protein concentrations; *c*, RNR activity.

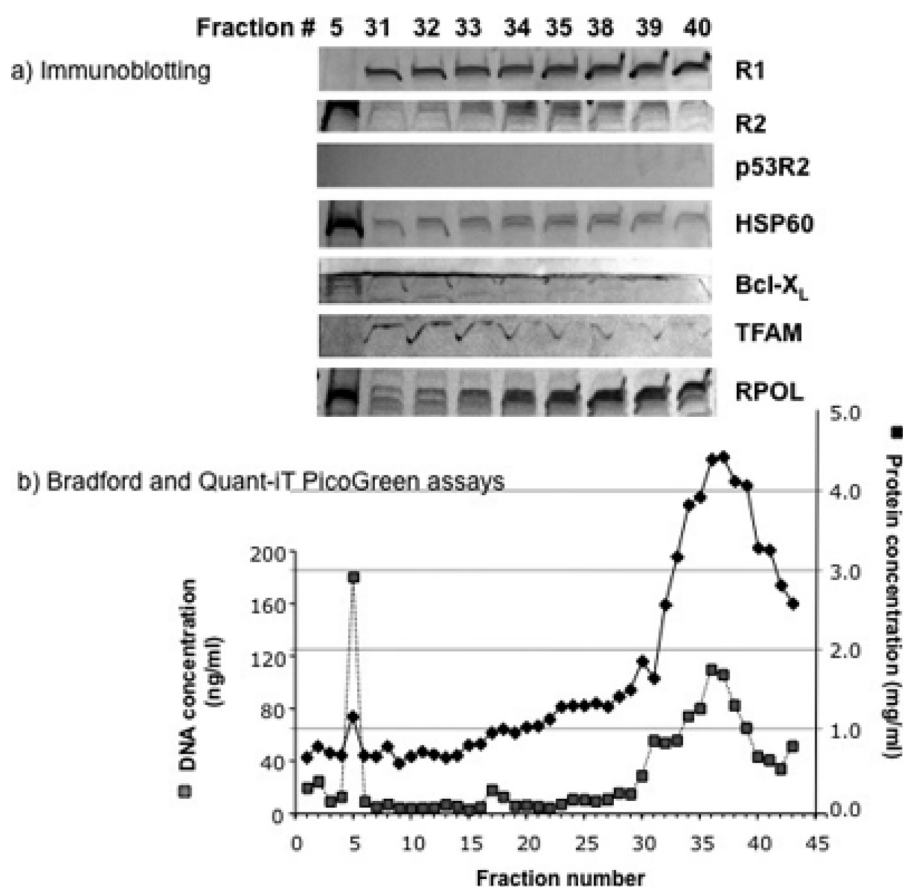


FIGURE 5. **Association of ribonucleotide reductase R1 and R2 proteins with nucleoids from pig liver mitochondria.** Nucleoids were isolated from pig liver and analyzed by velocity sedimentation as described by Wang and Bogenhagen (24). Selected fractions were analyzed by immunoblotting for R1, R2, p53R2, and selected nucleoid marker proteins. *a*, immunoblotting; *b*, DNA and protein concentrations.

Ribonucleotide Reductase in Mammalian Mitochondria

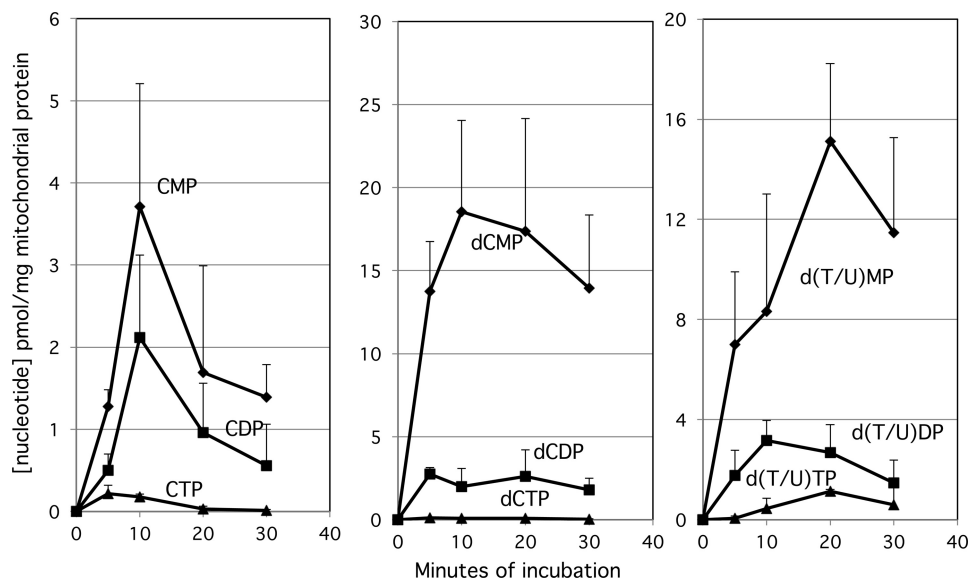


FIGURE 6. Conversion of [^{14}C]CDP to deoxyribonucleotides isolated rat liver mitochondria. For details, see "Experimental Procedures." Each data point is the average of five analyses of each sample \pm standard deviation from one experiment of three that were performed.

found that the nucleotides of interest could be separated by two-dimensional thin layer chromatography without prior fractionation. As shown in Fig. 6, substantial label from CDP appeared in dCMP, dCDP, dTMP, and dTDP, with smaller incorporation into dTTP and negligible radioactivity in dCTP. The PEI-cellulose chromatography, which resolves components by ion exchange, does not separate thymidine nucleotides from the corresponding deoxyuridine nucleotides. Therefore, the components migrating with marker dTMP, dTDP, and dTTP are labeled dT/UMP, dT/UDP, and dT/UTP, respectively.

From the total radioactivity found in deoxyribonucleotides in the first 5 min of incubation, when incorporation is maximal, we can estimate total ribonucleotide reductase activity as being ~ 5 pmol/min/mg of protein, approximately half the specific activity we observed in mitochondrial lysates (Tables 1 and 2). This may reflect a rate limitation at the mitochondrial uptake level under our conditions of incubation. Alternatively, degradation of deoxyribonucleotides by the mitochondrial nucleotidase (36) may yield nucleosides, which would be washed off of the TLC plates before chromatography.

With respect to the dGTP overrepresentation that we observe in mammalian mitochondria, this could result from relatively high activity of any biosynthetic reaction or limitation of any reaction that would consume mitochondrial dGTP. To determine whether intramitochondrial RNR might be such a rate-controlling step, we carried out identical uptake experiments with isolated respiring mitochondria, in which [^{14}C]GDP was incubated with mitochondria instead of CDP. As seen in Fig. 7, substantial reduction of the guanosine ribonucleotide occurred, with most of the deoxyguanosine nucleotides accumulating as dGMP. Total ribonucleotide reductase activity was considerably lower than what we observed in the CDP experiments: less than 2 pmol/min/mg of protein total conversion of GDP to deoxyguanosine nucleotides. Hence, this experiment confirms that active ribonucleotide reductase exists within mitochondria, but it does not identify ribonucleotide

reductase as an essential element in the processes leading to high dGTP accumulation in mitochondria.

DISCUSSION

Several lines of evidence described in this paper establish the presence of ribonucleotide reductase in mammalian mitochondria: 1) enzyme activity in mitochondrial extracts, at severalfold higher specific activity than seen in corresponding cytosolic extracts; 2) localization of enzyme activity to the mitochondrial matrix; 3) the presence of R1 protein in washed mitochondria, as determined by mass spectrometric analysis of immunoprecipitated mitochondrial proteins; 4) association of ribonucleotide reductase activity and immunoreactive material with liver mitochondrial nucleoids; and 5) the uptake of ribonucleoside diphosphates by respiring mitochondria and their conversion to deoxyribonucleotides.

With respect to the isotope uptake experiments, we would have expected to see the respective deoxyribonucleoside diphosphate (dCDP or dGDP) as the first labeled intermediate to appear. However, in both the CDP and GDP uptake experiments, the principal deoxyribonucleotides to accumulate were the monophosphates. Deoxyribonucleotide turnover in mitochondria is significant (36, 37), so it seems likely that ribonucleoside diphosphate initially taken into mitochondria was reduced to dNDP and rapidly dephosphorylated to dNMP. Might some other pathway have converted the radiolabeled ribonucleoside diphosphate to dNMP? Any such pathway would require conversion of the ribonucleoside diphosphate to the corresponding monophosphate and then to the nucleobase, followed by a nucleoside phosphorylase reaction using deoxyribose 1-phosphate as a deoxyribosyl donor. The resultant deoxyribonucleoside would then be phosphorylated to the dNMP. Aside from the question of whether mitochondria contain a significant deoxyribose 1-phosphate pool, these alternative pathways would require at least four reactions, with significant dNMP accumulation seen within the first 5 min of incubation. Because we have shown mitochondria to contain

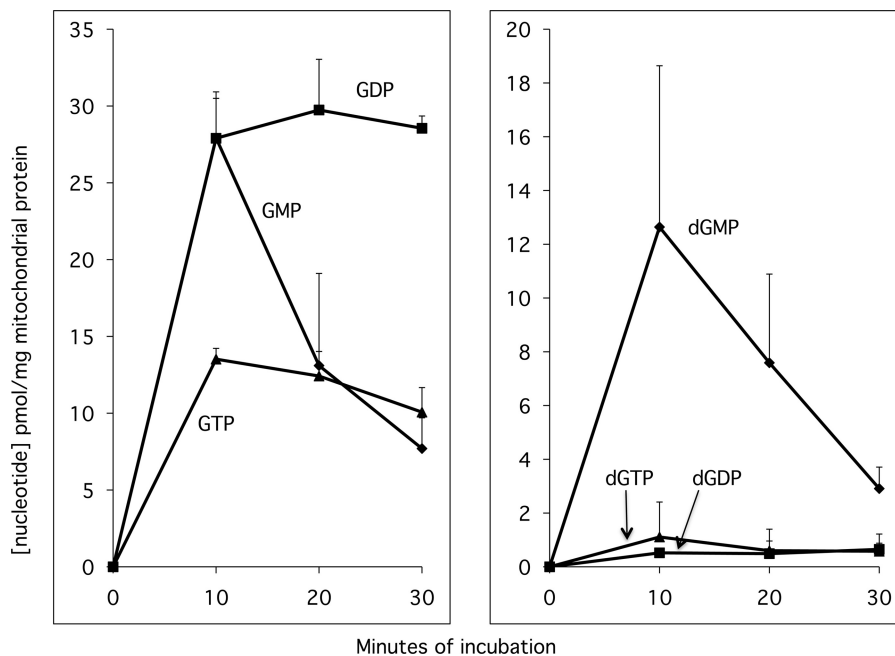


FIGURE 7. Conversion of [^{14}C]GDP to deoxyribonucleotides in isolated rat liver mitochondria. Each data point is the average of four analyses of each sample \pm standard deviation from an experiment that was done twice.

RNR activity, a pathway involving reduction of CDP or GDP followed by dephosphorylation of the resultant deoxyribonucleoside diphosphate seems more likely.

How might ribonucleotide reductase subunits have found their way into mitochondria? We have examined the sequences of rat R1, R2, and p53R2, and none contains an N-terminal targeting sequence. However, we note that pathways for mitochondrial protein incorporation have been described other than the classical presequence pathway (38, 39). Moreover, many of the 1098 proteins established in the mouse mitochondrial proteome in the survey of Pagliarini *et al.* (40) lack a classical targeting sequence. Finally, we note that mouse and Chinese hamster mitochondria have been shown to contain thymidylate synthase and a dihydrofolate reductase isoform, even though both proteins lack classical targeting sequences (17). Although we do not know the process whereby ribonucleotide reductase subunits are transported into mitochondria, it is reasonable to assume that it happens by a process other than the classical presequence pathway.

What is the molecular form of the mitochondrial ribonucleotide reductase? The presence of R1 is clearly established by the mass spectrometric analysis of immunoprecipitated protein, and, as noted earlier, the presence of four R1 peptides in the R2 immunoprecipitate suggests that an R1-R2 oligomer was precipitated by the R2 antibody. We did see one R2 peptide in the R2 immunoprecipitate, but with lower probability of correct peptide identification. p53R2 peptides were not seen at all. Our failure to detect either R2 or p53R2 with high assurance may mean that these proteins were present in our preparations in amounts too small to be detected. Unfortunately, the small amount of material available in rat liver mitochondria precluded a direct analysis with purified protein, and in an alternate source, namely pig liver mitochondria, the enzyme was in a membrane-bound form from which it could not be removed

without inactivation. Our failure to detect p53R2, either by immunoprecipitation or in the nucleoid experiments, does not mean that the protein is absent, just that we could not detect it.

Our detection of ribonucleotide reductase in mitochondria means that at least three pathways contribute nucleotides to mitochondrial dNTP pools: first, the well established salvage pathways starting with mitochondrial deoxyribonucleoside kinases; second, the equally well established cytosolic ribonucleotide reductase pathway using proteins R1 and p53R2; and third, as shown in this study, ribonucleotide reductase within the mitochondrion using R1 and possibly R2. Because previous studies were carried out with cycling or quiescent cells in culture and our work was done with a differentiated tissue, we are not in a position to estimate the relative contributions of each pathway under different conditions. However, given the importance of maintaining suitable dNTP pools within the mitochondrion, perhaps we should not be surprised to learn that there are multiple ways to supply these pools. In addition, the recent demonstration of a thymidylate synthesis cycle within mammalian mitochondria (17)—thymidylate synthase, dihydrofolate reductase, and serine hydroxymethyltransferase—makes it perhaps less surprising that ribonucleotide reductase is also present within mitochondria.

Any discussion of dNTP synthesis for mitochondrial DNA replication needs to take into account the findings of Morris *et al.* (41) that in mitochondria from perfused rat hearts, dCTP and dTTP are derived entirely from salvage pathways—a finding that appears to rule out a function for RNR in rat heart. Morris *et al.* worked with a different organ and a different experimental system from that described here and in previous investigations of mitochondrial nucleotide metabolism. Moreover, their analysis involved only the pyrimidine dNTPs. Whether dNTP synthetic pathways vary among different organs merits systematic investigation.

Ribonucleotide Reductase in Mammalian Mitochondria

One of our reasons for reopening an investigation of ribonucleoside diphosphate reductase in mitochondria was our hope to better understand the enzymatic basis for the unexpected dNTP pool asymmetry that we have described in mammalian mitochondria (6). That hope was not realized. The *in situ* reduction of GDP, observed in the experiments of Fig. 7, was approximately half the rate of CDP reduction under identical conditions. To the extent that freshly isolated, respiring rat liver mitochondria reflect conditions in intact cells, we might have expected the *in situ* activity of mitochondrial RNR to be higher with GDP than with CDP as the substrate. However, we have not pursued the possibility that the GDP reductase activity would be higher in the presence of proper allosteric modifiers. We did find that the activity for CDP reduction in our standard assay is sensitive to inhibition by dATP to the same extent as a cytosolic extract (data not shown). Although the mechanism by which mammalian tissue mitochondria maintain a striking dNTP pool asymmetry remains open, the data presented here indicate that ribonucleotide reductase activity within the mitochondrion needs to be considered as a possibly significant source of intramitochondrial dNTPs.

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