SYNTHESIS OF DNA, RNA, PROTEIN AND GLYCOPROTEIN IN MITOCHONDRIA OF CELLS TRANSFORMED WITH ROUS SARCOMA VIRUSES[1]

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Summary. Mitochondrial DNA and RNA synthesis in Rous sarcoma virus (RSV) transformed chick embryo fibroblast (CEF) cells pretreated with 10 mg per ml of camptothecin was elevated over that in normal CEF cells. Protein synthesis in the RSV-CEF cell mitochondria was also elevated when measured in the presence of 100 µg per ml of cycloheximide. Experiments with temperature sensitive mutants of RSV (TS-68) and RAV-CEF cells indicated the elevation in mitochondrial synthesis was directly related to cell transformation and not merely to cell viral infection. In related experiments, isolated pure mitochondrial preparations from the RSV-CEF cells synthesized greater amounts of glycoprotein autonomously than normal uninfected CEF controls.

Mitochondria from various cell types are thought to be capable under certain circumstances to synthesize various macromolecules autonomously without extramitochondrial input (1). Mitochondria contain DNA, RNA, protein, glycoprotein,lipid and glycolipid and for each macromolecule it is thought that a certain small percentage is synthesized by the mitochondria (1-11). Much interest has centered about identification of the products of the mitochondrial synthesizing system and the role such products play in the biogenesis of the mitochondrion and the correct integration of such mitochondrial macromolecules with extramitochondrial products to form the functional organelle.

Recently mitochondrial glycoproteins have been described in the intermembrane space (12) and intra-mitochondrial Ca^{2+}-binding glycoproteins have been partially purified (13,14). Glycoproteins on the mitochondrial surface have also been demonstrated both by techniques of lectin binding (15,16) and by particle microelectrophoresis (17).

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Because of recent interest in possible mitochondrial involvement in viral infection and oncogenesis (18-22) and suggestions of mitochondrial membrane alterations in neoplastic cells (23), we turned our attention to mitochondria of neoplastic cell models. Initially we reported that isolated SV-40 transformed 3T3 cell mitochondria exhibited a lower rate of protein and glycoprotein synthesis than did their normal 3T3 counterparts (24). Furthermore (25) mitochondria from virally transformed cells in continuous culture (MSV-3T3, RSV-3T3, PY-3T3 cells) were found to synthesize increased amounts of DNA, RNA and protein than their non-transformed control (3T3 cells). The present paper describes similar experiments in primary infected cultures of chick embryo fibroblasts (CEF) cell and Rous sarcoma virus transformants of these CEF cells. Use of various virus mutants in the present report allows the changes found to be attributed to cell transformation as opposed to mere virus replication or infection. Using cycloheximide to inhibit non-mitochondrial protein synthesis and camptothecin to inhibit non-mitochondrial nucleic acid synthesis, the present results were obtained.

Materials and Methods

Cells and Viruses: Three viruses were used. The Schmidt-Ruppin strain of subgroup A (SR-RSV) and a temperature-sensitive mutant of this virus, TS-68, which produces virus but does not transform cells at 41°C, were kindly supplied by Dr. H. Hannafusa (26). Rous associated virus of subgroup A (RAV-1) originally isolated from the Bryan strain (Br-RSV) was used as a non-transforming control virus.

Primary cultures of chick embryo cells derived from a leukosis-free flock which were tested for uniform susceptibility to RSV-subgroup A viruses were infected with the various viruses and incubated at 37°C or 41°C as noted in forced air-CO_2 incubators. The cultures were split on the second day after infection and were used for the biochemical studies on the fourth day when over 90% of the cells appeared visibly transformed by SR-RSV at 37°C or 41°C. An aliquot of the RAV-1 cells were tested at this time by superinfection with Br-RSV and were found to be uniformly resistant to transformation, indicating that they were infected. Uninfected cells for controls were handled in an identical manner.

Cells were prepared and maintained in F-12 medium and virus assays were carried out as previously described (27-29). Cultures were refed with fresh medium every 48 hours to ensure adequate nutrition.

All cells were harvested at the point where the control CEF and infected cells had formed an almost complete monolayer on the surface of the petri dish in which they were cultured, so that effects of cell contact would be the same in all cultures.

Materials: ^{3}H-leucine (sp. act. 50 Ci/mmole), ^{3}H-uridine (40 Ci/mmole), ^{3}H-thymidine (60 Ci/mmole), UDP-[^{14}C]glucose (240 Ci/mmole), and GDP-[^{14}C]-mannose (241 Ci/mmole), were purchased from New England Nuclear Corp. Biochemicals were purchased from Sigma Chemical Corp. (St. Louis, Mo., U.S.A.). Camptothecin was supplied by the National Cancer Institute.

In vivo Experiments: In vivo (i.e. measuring mitochondrial synthesis in intact cells) experiments were carried out utilizing radioactive precursor labelling of macromolecules in the presence of drug as given in the Table legends.

In vitro Experiments: Mitochondria were isolated as described previously (24); they were considered essentially pure on the basis of lack of contamination by membrane marker enzymes and high enrichment of mitochondrial enzymes (24). Mitochondria from the various cell lines were isolated by homogenization of the tissue and subsequent differential centrifugation in a medium consisting of 0.3 M sucrose, 2 mM EDTA, 30 mM nicotinamide, 0.7% bovine serum albumin, pH 7.4 as described previously (24,25). These preparations of mitochondria were found to have little plasma membrane and microsomal enzyme activity and were greatly enriched in succinic dehydrogenase and cytochrome c oxidase activity.

Mitochondrial acid insoluble macromolecule glycoproteins were labeled by incubation in a protein synthesis supporting medium (30) supplied with the appropriate radioactive precursors--UDP-[^{14}C]glucose (0.03 µCi/ml) or GDP-[^{14}C]-mannose (0.03 µCi/ml. Glycoprotein bound radioactivity was determined as previously described (5,24).

Results

Synthesis of Protein, DNA and RNA in Mitochondria in Intact Normal CEF Cells and SR-RSV-CEF and RAV-CEF Cells: The data of Table 1 demonstrate that protein synthesis was elevated slightly in the SR-RSV-CEF cells while RNA and DNA synthesis were markedly elevated in the transformed cells (SR-RSV-CEF). The data further demonstrate that infection with RAV virus which replicates but does not transform the CEF cells did not cause any elevations in mitochondrial macromolecule synthesis. These data suggest that the elevations found with the SR-RSV cells were truly the result of transformation and not merely viral infection and replication. With the SR-RSV-CEF cells the elevations in RNA and DNA synthesis were almost 2-fold.

The results presented in Table 2 indicate that elevation of the temperature of growth of the CEF cells from 37° to 41° resulted in slightly higher levels of mitochondrial macromolecular synthesis (e.g. 110% for DNA synthesis). Similarly when the SR-RSV-CEF cells were grown at 41° slightly higher eleva-

Table 1. Synthesis of Protein, DNA and RNA by Mitochondria in Intact Normal CEF Cells and RSV-CEF and RAV-CEF Cells

Cells were cultured and harvested as described previously (27-29). Cells were incubated in complete Dulbecco's medium for 30 min at 37° with the indicated concentration of antibiotic, then 10 μCi of [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine were added to the cell suspension as indicated and further incubation of the suspension was continued for 1 hour. Data are expressed as percentage of the CEF cell synthesis that the experimental cell mitochondrial synthesis represents, with the CEF cells arbitrarily set at 100%. Data are means ± S.D. for the transformed cells. For experiments with cycloheximide and [^{3}H]leucine the synthesis in all cells represented an average of 4.0% of that without any drug; for experiments with camptothecin and [^{3}H]thymidine the synthesis in all cells represented an average of 4.7% of that without any drug; and for experiments with camptothecin and [^{3}H]uridine the synthesis in all cells represented an average of 4.5%. Macromolecular bound radioactivity per mg cell protein was determined as given previously. Experiments were performed with 3 to 6 independent cell populations. Radioactivity which represents mitochondrially synthesized macromolecular bound moieties was determined by precipitation of whole cells with

Drug:precursor	Cell Line		
	CEF	SR-RSV-CEF	RAV-CEF
100 μg/ml cycloheximide:[^{3}H]-leucine	100 (2423±410)*	136 ± 10	102 ± 6
10 mg/ml camptothecin:[^{3}H]uridine	100 (1989±162)	186 ± 14	101 ± 4
10 mg/ml camptothecin:[^{3}H]-thymidine	100 (4686±515)	192 ± 16	98 ± 4

1% phosphotungstic acid, washing twice with 10% trichloracetic acid and once with ether:ethanol (2:1, v:v). The resultant precipitate was counted.

*In parenthesis are given actual number of cpm ± 1 S.D. which represent 100%.

tions in mitochondrial macromolecular synthesis occurred than was found with SR-RSV-CEF cells grown at 37° (Table 2). The temperature-sensitive mutant transformed cells (TS-68) grown at the permissive temperature of 37°C had greatly elevated levels of synthesis of mitochondrial macromolecules over the CEF cell levels which were comparable to the SR-RSV-CEF (37°) cell levels. However, when the temperature-sensitive virus TS-68 infected cells were grown at 41°C, a nonpermissive temperature, levels of mitochondrial macromolecule synthesis activity comparable to the CEF (41°C) levels were obtained.

Table 2. Synthesis of Protein DNA and RNA of Mitochondria in Intact Chick Embryo Fibroblasts Infected with Various Rous Sarcoma Virus Mutants. Experiments were performed as given in legend to Table 1 except cell lines noted were used.

Drug:Precursor	Cell Line					
	CEF(37°)	CEF(41°)	SR-RSV(37°) CEF	SR-RSV(41°) CEF	TS-68(37°) CEF	TS-68(41°) CEF
100 μg/ml cycloheximide: [^{3}H]leucine	100	101±2	136 ± 10	144 ± 14	148 ± 9	104 ± 16
10 mg/ml camptothecin: [^{3}H]uridine	100	106±4	186 ± 14	199 ± 16	198 ± 14	105 ± 8
10 mg/ml camptothecin: [^{3}H]thymidine	100	110±6	192 ± 16	207 ± 10	207 ± 8	112 ± 16

Data means ± 1 S.D.

Thus at the nonpermissive temperature, at which transformation does not occur, elevated levels of the synthesis of mitochondrial macromolecules further suggesting that the elevations seen in the SR-RSV-CEF cells are due to transformation of the cells and not merely viral infection and replication. Again with the TS-68 (37°) CEF cells very high elevations in mitochondrial nucleic acid synthesis occured; also protein synthesis was 148% control with the TS-68 (37°) CEF cells (Table 2).

Glycoprotein Synthesis by Isolated Mitochondria from Chick Embryo Fibroblasts and Virus Transformed Chick Embryo Fibroblasts: The data of Table 3

Table 3. Incorporation of Labelled Monosaccharide into Glycoprotein by Isolated Mitochondria from Chick Embryo Fibroblasts and Various Virus-Transformed Chick Embryo Fibroblasts. Data are cpm/mg mitochondrial protein; means ± S.D.

Cell line from which mitochondria were isolated	Precursor*	
	UDP-Glucose-^{14}C	GDP-Mannose-^{14}C
CEF	4320 ± 612	2610 ± 214
SR-RSV-CEF	8147 ± 627	5973 ± 406
RAV-CEF	4010 ± 421	2707 ± 297
CEF(37°)	4441 ± 707	2789 ± 244
CEF(41°)	5222 ± 412	3221 ± 286
SR-RSV-CEF(37°)	8284 ± 891	5642 ± 418
SR-RSV-CEF(41°)	10714 ± 973	6821 ± 910
TS-68-CEF(37°)	8249 ± 1076	5876 ± 219
TS-68-CEF(41°)	4689 ± 506	2888 ± 306

Experiments were performed as given in Materials and Methods.

*Radioactivity was present in macromolecules as given by precipitation in phosphotungstic acid, trichloroacetic acid and ether:ethanol (1:2, v:v). Hydrolysis of the reaction product and chromatography (4,5) resulted in 94% of the UDP-glucose-^{14}C being recovered as glucose-^{14}C and 99% of the GDP-mannose-^{14}C as mannose-^{14}C. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (38) of the reaction mixtures indicated the radioactivity stained for protein (39) and glycoprotein (40). Interestingly, for the increases in the glycoproteins synthesized, analysis by gel electrophoresis indicated that not only in the transformed cells were higher amounts of glycoproteins normally present in CEF synthesized but also amounts of different glycoproteins not normally present in CEF were synthesized.

clearly show that the transformed SR-RSV-CEF cell isolated mitochondria synthesized about twice as much glycoprotein utilizing either UDP-glucose or GDP-mannose as precursor as their non-transformed CEF controls or RAV-CEF controls. Furthermore the TS-68 (37°) CEF cells synthesized twice as much glycoprotein as the TS-68 (41°) CEF cells. Thus upon transformation (and not infection or virus replication) the CEF cell mitochondria tested *in vitro* have greatly accelerated rates of glycoprotein synthesis.

Discussion

The results presented herein demonstrate that upon transformation with the Rous sarcoma virus system mitochondria of chick embryo fibroblasts synthesize more protein, DNA and RNA in intact cells. Furthermore isolated mitochondria of the Rous sarcoma virus-transformed CEF cells can synthesize about twice as much glycoprotein as their non-transformed counterparts.

The present work is of special interest because of the work of Smith and Vinograd (20) and Clayton and Vinograd (31) who found increased frequency of dimeric forms of mitochondrial DNA in neoplastic cells and that of Nass (32) who found increased dimers and oligomeric forms of mitochondrial DNA in RSV-TS-68 cells grown only at the permissive temperature when cell transformation is manifested morphologically and functionally (32).

Mitochondrial DNA, RNA, and protein synthesis has been implicated in virus formation and oncogenic transformation by Richert and Hare (21). They found a decrease in focus formation and replication after treatment of chick embryo fibroblast cells with the mitochondrial inhibitors ethidium bromide, rifampicin, and chloramphenicol. Levine (22) found that during transformation of tissue culture cells by oncogenic virus (SV-40) mitochondrial DNA synthesis is accelerated, as is nuclear DNA synthesis. Vesco and Basilico (33) demonstrated the same phenomenon upon polyoma virus infection and showed with a temperature-sensitive mutant that the effect is a viral "early function." And Kára et al. (18) reported that Rous sarcoma virus particles can replicate within mitochondria, showing the feasibility of oncogenic viral information entering and staying within the mitochondrion. Whether or not this viral genome could be incorporated into the mitochondrial genome as it is in the nucleus (34) has not yet been investigated. However, Kára and Mach (35) have recently isolated subviral oncogenic particles (virosomes) from the mitochondria of Rous sarcoma cells as evidenced by electron microscopy. However, recent studies by Badir (37) with murine leukemia virus infected mouse cells and chick embryo fibroblasts infected with RSV have shown that ethidium bromide did not prevent replication of these viruses nor did it prevent cell transformation by RSV even though the mitochondria exhibited degenerative

changes by electronmicroscopy. Furthermore no virions or virion components were seen in mitochondria of RSV infected cells. Of particular interest is the recent demonstration that RNA of Venezuelan equine encephalomyelitis virus penetrated into isolated rat liver mitochondria, that replication of the virus occured within the mitochondria, and that upon infection, the mitochondria switched to production of virus-specified products as opposed to mitochondrion-specified products (19). Finally Küntzel et al. (36) have reported virus-like particles in mitochondria of Neurospora crassa. Some of the above data and that presented herein indicate that the mitochondrion may play a role in oncogenic transformation.

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