SPECIFIC CHANGES IN THE SYNTHESIS OF MITOCHONDRIAL DNA IN CHICK EMBRYO FIBROBLASTS TRANSFORMED BY ROUS SARCOMA VIRUSES

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ABSTRACT

In chick-embryo fibroblasts infected with the Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A (wild type), or with a thermosensitive mutant of this virus, T5, the rates of mitochondrial DNA synthesis differ in cells that exhibit normal and malignant phenotypes. In wild type virus-infected cells grown at 36 or 41°C, morphological transformation is expressed, the rate of 2-deoxy-D-[^{3}H]glucose uptake is stimulated, and mitochondrial DNA synthesis in vivo is stimulated three- to fivefold over that in uninfected cells. In T5-infected cells these changes occur only at the permissive temperature (36°C); a shift to the nonpermissive temperature (41°C) causes the reversal of these effects, and the specific activity of purified mitochondrial DNA is characteristic of that from uninfected cells. In contrast, the specific activities of nuclear DNA purified from cells maximally transformed under the permissive conditions do not differ between wild type-infected and uninfected cells and do not change upon temperature shifts of the cells infected with the T5 virus. In parallel experiments with isolated mitochondria, the rate of mtDNA synthesis in vitro is again greater in mitochondria isolated from transformed cells. In addition, mitochondrial DNA synthesis in vitro in mitochondria from nontransformed and virus-transformed cells exhibits differential sensitivity to inhibition by mercaptoethanol. Furthermore, the mtDNA polymerase activity in mitochondrial extracts prepared from cells with transformed phenotypes is about sevenfold higher than in extracts from cells with nontransformed phenotypes.

Relatively few studies have been performed on the association of viruses with mitochondria and the consequences of viral infection on the functions of the mitochondrial genetic apparatus. Kára et al. (6, 7) have presented biochemical data suggesting that in certain cases mitochondria may be involved in Rous sarcoma virus replication. There have been reports that specifically mitochondrial DNA (mtDNA)[1] synthesis is stimulated by infection of monkey cells with SV40 virus (8), 3T3 cells with polyoma virus (26), and

[1] Abbreviations used in this paper are: mtDNA, mitochondrial DNA; CEF, chick embryo fibroblasts; RSV, Rous sarcoma virus; C36, C41, uninfected control CEF, cultured at 36 or 41°C respectively; WT36, WT41, CEF infected with the Schmidt-Ruppin strain of Rous sarcoma virus (wild type), cultured at 36 or 41°C, respectively; T536, T541, CEF infected with the temperature-sensitive mutant virus (T5), cultured at 36 or 41°C, respectively; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate; MCE, 2-mercaptoethanol.

HeLa cells (21), and mouse cells (22) with herpes simplex virus. Bosmann et al. (2) reported that the synthesis of mitochondrial RNA, DNA, and glycoprotein in chick embryo fibroblasts (CEF) transformed by Rous sarcoma viruses was elevated over that in normal CEF cells. However, in those experiments, radioactivity which was presumed to represent mitochondrially synthesized DNA was determined only by acid precipitation of whole cells labeled in the presence of large amounts of camptothecin. Analysis of purified labeled DNA from isolated mitochondria was not presented. Meaningful conclusions are difficult to draw with such an approach. Reports on the nonspecific acid precipitation of labeled nucleic acid precursors with cellular proteins in a manner not at all related to the synthesis of nucleic acids have appeared in the literature (3, 14).

From this laboratory it has recently been demonstrated (19) that there is a two- to threefold increase in the frequency of multiple-length mtDNA molecules in CEF transformed by oncogenic viruses as compared to nontransformed CEF. In view of this evidence, we have initiated a detailed study of the synthesis of mtDNA to determine whether and how this synthesis differs in CEF cells that express normal and in cells that express malignant phenotypes.

This communication presents clear and unambiguous evidence, obtained in well-controlled experiments, that there are distinct and specific changes in the synthesis of mtDNA in CEF made malignant by Rous sarcoma viruses.[1]

MATERIALS AND METHODS

Cell Culture and Virus Infection

Two virus strains were used in this study: the Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A (wild type) and the temperature-sensitive mutant of this virus (T5). The wild type virus transforms CEF both at 36°C (WT36) and 41°C (WT41). The transformed cells are characterized by alterations in cellular morphology and rates of sugar transport (13). The T5 virus, on the other hand, can infect and replicate at both temperatures (12, 13) but transforms CEF only at the permissive temperature, 36°C (T536). At the nonpermissive temperature, 41°C, the cells (T541) have the morphological appearance and behavior of normal uninfected cells (C36, C41).

Primary cultures of chick embryo fibroblasts were prepared from 10 to 11-day-old embryos (Spafas Inc., Norwich, Conn.) essentially as described (16, 23) and plated in Eagle's minimum essential medium with glutamine (Grand Island Biological Co., Grand Island, N.Y.), supplemented with penicillin (50 U/ml), streptomycin (100 μg/ml), 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), anti-pleuropneumonia like organism agent (Grand Island Biological Co.) and 5% fetal calf serum (Microbiological Associates, Bethesda, Md.). Cultures were fed within 24 h and trypsinized 48 h after plating. The secondary cultures were seeded at a cell density of 3×10^6 cells per Falcon flask (75 cm^2) (Falcon Plastics, Div. BioQuest, Oxnard, Calif.) in the above media except that the 5% fetal calf serum was replaced by 2% calf serum. Within 4 h the medium was removed and the cells were infected with 1.0 ml of the appropriate virus suspension. After 60 min at 36°C to allow virus adsorption, fresh medium containing 1% dimethylsulfoxide (DMSO) (24) was added to each flask. DMSO was routinely added since some of the infected cultures were also used for virus collection. Identical experimental results were obtained, however, when DMSO was omitted from the culture medium. Uninfected, control cultures were treated in the same manner except that the viral adsorption step was omitted. For these experiments, the cells from each embryo were cultured separately and in no case were the cells from different embryos pooled. The maintenance of each culture was carried out as indicated in the figure or table legend for each particular experiment.

Media used in these studies were periodically tested and found to be free of *Mycoplasma*.

Measurement of 2-Deoxyglucose Uptake

The procedure of Martin et al. (13) was slightly modified as described by Soslau and Nass (25). The isotope concentration used in our experiments was 0.5 μCi/ml of 2-deoxy-D-[^{3}H]glucose (New England Nuclear, Boston, Mass.; sp act 8.0 Ci/mmol). This isotope was added in 5 ml of glucose-free Hanks solution per Falcon flask (75 cm^2). All cells were plated at the same density of 5×10^6 cells per flask.

Purification of Mitochondrial and Nuclear DNA Labeled In Vivo

Radioisotopic labeling in vivo was performed by the addition of 5 μCi/ml of [*methyl*-^{3}H]thymidine (New England Nuclear; sp act 43.1 Ci/mmol) to cells growing in monolayer culture in the logarithmic phase of growth. After 3 h of incubation in the presence of this isotope, the cells were harvested by trypsinization and the mitochondria were isolated according to procedures previously described from this laboratory (19).

mtDNA was prepared as described (19) and centrifuged to equilibrium in cesium chloride-ethidium bromide-buoyant density gradients (17, 19, 20). In the experiments presented here, purified mtDNA labeled

in vivo refers to the covalently closed circular component of mtDNA which is isolated from the lower band of these gradients (component I, Fig. 1). This fraction is the major component of mtDNA. Analysis by electron microscopy indicates that this fraction contains only circular mtDNA molecules; no linear, nuclear DNA molecules were evident. The covalently closed circular mtDNA fraction is freed of ethidium bromide before the determination of DNA concentration by extensive dialysis at 4°C against saline-citrate-EDTA (0.15 M NaCl, 0.015 M Na-citrate, 0.001 M EDTA, pH 7.4) with Dowex 50 (Dow Chemical Co., Midland, Mich.). The quantitatively minor components of mtDNA in the intermediate and upper bands of the cesium chloride-ethidium bromide gradients will be described elsewhere in a study of replicative intermediates. Although the upper band (component II) is greatly enriched with nuclear DNA, this component also contains some nicked circular mtDNA molecules in various stages of replication.

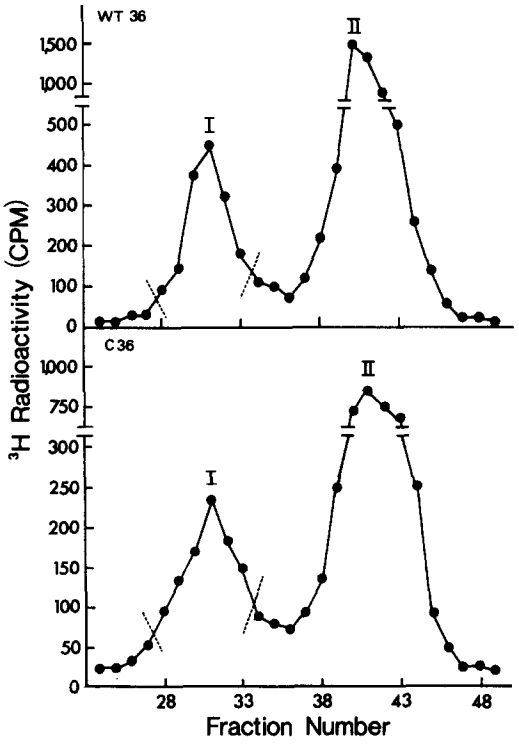

FIGURE 1 Purification of mitochondrial DNA by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. The specific activities of mtDNA which are reported in Tables I and II are determined on mtDNA preparations which have been purified by this method. Fractions are collected from the bottom of the tube and aliquots of each fraction are acid-precipitated and counted to localize the regions of the gradient which contain the labeled DNA. Purified mtDNA (component I) can be obtained by pooling the fractions between the dotted slashed lines. This cut is narrow enough to exclude the DNA in component II which consists of a mixture of nuclear DNA and nicked circular and linear mtDNA species. Component II was not used in the experiments presented here. Nuclear DNA was isolated separately from purified nuclei as described in Materials and Methods. (It should be noted that although it is valid to make quantitative comparisons between peak sizes on the same gradient, it may not be possible to directly quantitatively compare peak sizes between different gradients, e.g. C36 vs. WT36 profiles, until the appropriate fractions have been pooled, due to certain experimental variations in collecting different gradients, e.g., fraction size.)

Nuclear DNA was obtained according to the procedure described by Nass and Buck (15). The isolation was performed by chloroform/octanol extraction of SDS lysates of purified nuclear fractions, subsequent treatment with ribonuclease, and centrifugation of the DNA to equilibrium in neutral gradients of cesium chloride. The experimental results which were obtained by this procedure (Table II) were identical to those obtained when nuclear DNA was purified according to the same method as described above for mtDNA (data not shown).

Labeling of Mitochondrial DNA In Vitro

The cells were harvested by trypsinization and the mitochondria were isolated in the same manner as for the in vivo experiments (19) with one exception: homogenization of the cells was done in a solution of 0.7% bovine serum albumin, 0.025 M Tris-HCl, pH 7.4 (4°C), 0.002 M EDTA, and 0.03 M nicotinamide. Centrifugation was carried out in this solution containing 0.3 M sucrose.

After the final pelleting, the mitochondria were gently resuspended in the incubation mixture to which the unlabeled nucleoside triphosphates were subsequently added. The reaction was then immediately started by the addition of the radiolabeled nucleotide to the reaction vessel. The final incubation mixture contained per milliliter: $MgCl_2$ (10 μmol); KCl (2 μmol); KH_2PO_4/K_2HPO_4, pH 7.6 (10 μmol); ATP (2 μmol); Tris-HCl, pH 7.6 (36°C) (25 μmol); succinate (10 μmol); malate (1 μmol); nicotinamide (2 μmol); sucrose (80 μmol); dATP, dGTP, dCTP (15 nmol each); and [^{3}H]dTTP (New England Nuclear, sp act 43.44 Ci/mmol) and unlabeled dTTP (final concentration in the reaction mixture, 2 nmol [19 Ci/mmol]).

The incubations (1 ml) were performed in triplicate at 36°C. The amount of mitochondria assayed per milliliter was 200–400 μg. At the time points designated in the appropriate figures, 50-μl aliquots of the mitochondrial suspension were removed from the reaction vessel and placed in small sterile tubes which contained 50 μl

of lysis buffer (0.15 M NaCl, 0.10 M EDTA, 0.01 M Tris-HCl, pH 8.0 [36°C], 2% SDS). To this lysate, 250 μg of nuclease-free pronase (Calbiochem, San Diego, Calif.) which had been predigested for 2 h at 36°C was added in 10 μl of the above buffer without SDS. The lysates were then incubated for 60 min at 36°C and the labeled product (110 μl) was subsequently acid-precipitated on filter paper disks (Whatman 3 MM, Whatman Inc., Clifton, N. J.) and counted in a Packard liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.) under conditions where all the radioactivity is eluted from the disks and therefore the efficiency of counting is equalized in all samples (3). (The activity of the pronase solution was checked before use: 250 μg was sufficient to digest 1,000 μg of bovine serum albumin into acid soluble products at 36°C for 60 min under the conditions stated above.)

Preparation and Assay of Mitochondrial DNA Polymerase Activity in Mitochondrial Extracts Prepared from Nontransformed and Transformed Cells

Mitochondria were isolated in the same manner as for the in vitro experiments. The final mitochondrial pellet was resuspended in 0.01 M Tris-HCl, pH 7.8 (4°C), 5×10^{-3} M 2-mercaptoethanol (MCE) (T/M buffer) and frozen to −70°C three times and thawed by a 2-min incubation at 36°C. Upon the final thawing, freshly prepared 5% streptomycin sulfate (18) was added (0.1 ml/0.28 mg protein). The suspension was mixed, allowed to stand 10 min in ice, and centrifuged at 4°C at 20,000 *g* for 15 min. The resulting supernate was dialyzed against T/M buffer for 7 h with three changes of buffer. This preparation was termed freeze-thawed enzyme fraction 1 and was stored in 25–40-μl aliquots at −70°C (0.5 mg protein/ml).

Immediately before assay the appropriate enzyme aliquots were thawed and added to the reaction tubes. The final incubation mixture contained per milliliter: $MgCl_2$ (3.0 μmol); KCl (30 μmol); dATP, dGTP, dCTP (0.1 μmol each); Tris-HCl, pH 7.3 (36°C) (25 μmol); MCE (10 μmol); activated calf thymus DNA ([1 mg], prepared according to the procedure of Aposhian and Kornberg [1]); [^{3}H]dTTP (New England Nuclear, sp act 45 Ci/mmol) and unlabeled dTTP (final concentration in the reaction mixture, 0.1 μmol [12 Ci/mmol]).

The reaction was started by the addition of enzyme to the reaction vessel. The amount of freeze-thawed enzyme fraction 1 assayed per milliliter was 40 μg. The incubations were performed in duplicate at 36°C. At the designated time points, aliquots were removed from the appropriate reaction vessels, acid-precipitated on filter paper disks, and counted as described for the in vitro experiments.

RESULTS

Uptake of 2-Deoxyglucose

The development of transformation as reflected by increased rates of sugar transport in the transformed cells (13) was monitored at different times after infection. Fig. 2 indicates that the rate of 2-deoxyglucose uptake was significantly stimulated in all transformed cells (WT36, WT41, T536). This rate of increase started to stabilize at approximately 5 days after the infection. At this time also, more than 95% of these cells appeared to be uniformly morphologically transformed (24, and our unpublished observations). No increase in the rate of sugar uptake was seen in the nontransformed cells (C36, C41, T541). In addition, these cells (C36, C41, T541) exhibited normal phenotypes.

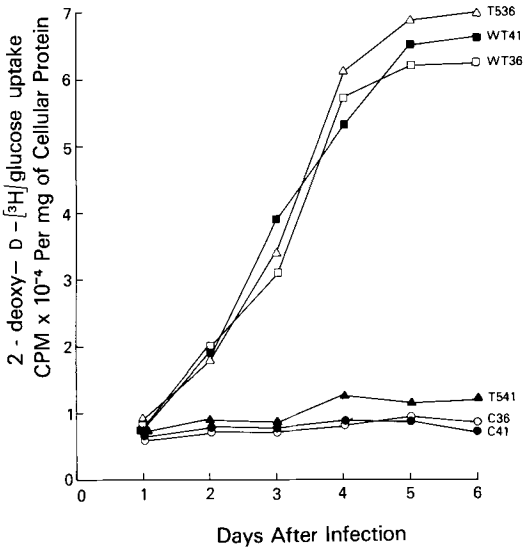

FIGURE 2 The uptake of 2-deoxyglucose in control and wild type-RSV or T5-RSV-infected chick embryo fibroblasts. CEF cultures were prepared and infected with either the wild type or T5 virus. Control uninfected CEF cultures were also prepared. All cells were immediately placed at the designated temperature. On the day each assay was to be performed, the flasks to be assayed were prepared by trypsinizing the appropriate cells and plating them at a cell density of 5×10^6 cells per flask (75 cm^2). 6 h after plating the uptake of 2-deoxy-D-[^{3}H]glucose was measured. Three flasks were used to determine each count per minute per milligram value. The variation was ±13%. The methods used are described in the text.

Synthesis of mtDNA In Vivo as a Function of Time after Infection

Several experiments were carried out to investigate the synthesis of mtDNA as related to the development of viral transformation. A comparison of the rate of uptake of 2-deoxyglucose and the specific activities of mtDNA purified from uninfected (C36) and wild type-infected cells (WT36) up to 6 days after viral infection (Table I) indicated that the specific activity of mtDNA increases in parallel with the increase in the rate of sugar transport in the transformed cells (WT36). In the uninfected, nontransformed cells (C36) no increase in either the rate of sugar transport or the specific activity of mtDNA was evident.

Cell Growth Rates

The cell culture conditions used in all the quantitative experiments presented in this paper (Tables II-IV, and Figs. 3-6) were slightly modified from Martin et al. (13) and described in the legend for Fig. 3. This figure demonstrates that both nontransformed (C36, C41, T541) and transformed (WT36, WT41, T536) cells, when grown under these specified conditions, were growing at equivalent rates and were in the logarithmic phase of growth 6 days after infection. This was the time-point at which the cells were harvested for labeling of mtDNA and nuclear DNA in vivo (Table II), for labeling of mtDNA in vitro (Figs. 4, 5 and Table IV), or for preparing and assaying mitochondrial extracts for mtDNA polymerase activity (Fig. 6).

Differential Synthesis of mtDNA and Nuclear DNA In Vivo

A comparison of the specific activities of extensively purified nuclear DNA and mtDNA was made in cells infected with the thermosensitive mutant virus and subsequently cultured at either the permissive (T536) or the nonpermissive temperature (T541). Purified DNAs from uninfected (C36, C41) and wild-type infected cells (WT36, WT41) were also examined.

The experimental data presented in Table II provide a clear demonstration that the increase in the specific activity of mtDNA is directly related

TABLE I

Synthesis of Mitochondrial DNA In Vivo in Control and Wild Type-RSV-Infected Chick Embryo Fibroblasts at Different Times after Infection

Cell type	Days after infection	Specific Activity* *cpm/μg mtDNA*		2-deoxy-D-[^{3}H]glucose uptake‡ *cpm/mg of cellular protein*	
			WT36/C36		WT36/C36
C36	0.25	200	1.09	6,400	1.02
WT36		217		6,500	
C36	2	195	1.25	6,900	3.00
WT36		244		20,700	
C36	3	175	1.87	7,000	5.00
WT36		327		35,000	
C36	4	160	2.83	7,500	7.00
WT36		452		52,500	
C36	5	150	3.73	6,700	8.40
WT36		560		56,280	
C36	6	160	4.08	7,000	8.50
WT36		652		59,500	

CEF cultures were prepared and infected with the wild-type virus and placed at 36°C immediately after infection (WT36). Control uninfected CEF cultures were also prepared and placed at 36°C (C36). On days 2 to 6 the appropriate cells were trypsinized and plated at a cell density of 5×10^6 cells per flask (75 cm^2). 3 h after plating, one-half of the control and wild-type infected cells were labeled with [^{3}H]thymidine in vivo (5 μCi/ml for 3 h). 6 h after plating these cells were harvested to determine the specific activities of their purified mtDNAs. In parallel, 6 h after plating, the remaining half were assayed for their uptake of 2-deoxy-D-[^{3}H]glucose. However, for the 0.25 day (6 h) after infection the originally plated secondary cultures (3×10^6 cells per flask) were used. The methods used are described in the text.

* The specific activity values represent the average of three determinations which agreed within 5%.

‡ Triplicate flasks were used for the 2-deoxyglucose measurement and the variation was ±15%.

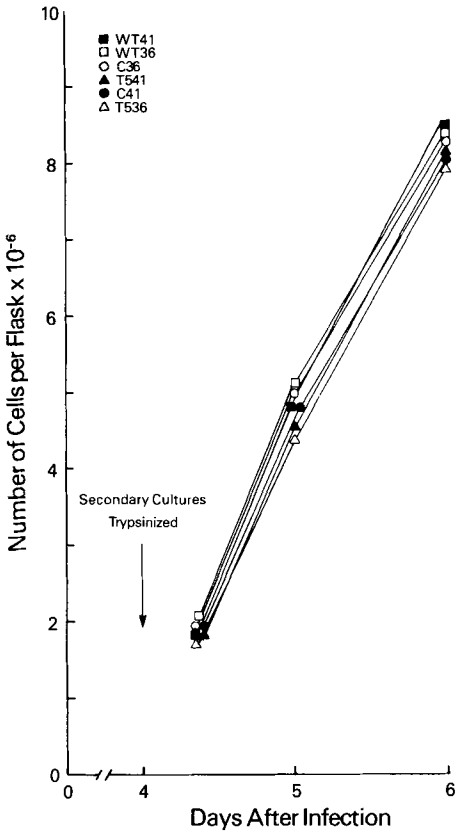

FIGURE 3 Rate of cell growth of control and wild type-RSV or T5-RSV-infected chick embryo fibroblasts. CEF cultures were prepared and infected with either the wild type or T5 virus. Control uninfected cultures were also prepared. The cells were subsequently cultured under growth conditions slightly modified from Martin et al. (13) in order that both the normal and transformed cells would be growing at the same rate at the time of radioisotopic labeling and cell harvesting. After infection, all cells were grown at 36°C for 4 days with daily medium change (media containing 2% calf serum and 1% DMSO). 4 days after infection these secondary cultures were trypsinized; for each experiment half of the cells were then shifted to 41°C and half remained at 36°C. These cultures were subsequently fed twice daily. At each time point designated in the figure, three flasks (75 cm^2) of each cell type were harvested by trypsinization after which the flasks were scraped with a rubber policeman. The cells were pelleted by centrifugation at 1,000 *g* for 20 min, resuspended in a small volume of phosphate-buffered saline, and counted manually with a hemocytometer under a phase microscope. Duplicate counts were taken for each flask. Therefore each point on the graph represents the average of six determinations. The variation was ±15%. Portions of the cells were also counted electronically in a Coulter Cell Counter (Coulter Electronics Inc.). These results generated a curve similar to the one presented here in Fig. 3.

TABLE II

Synthesis of Nuclear and Mitochondrial DNA In Vivo in Control and Wild Type-RSV or T5-RSV-Infected Chick Embryo Fibroblasts

Cell type	Specific activity,* *cpm/μg DNA*	
	Nuclear DNA	Mitochondrial DNA
Experiment A		
C36	12,845	150
C41	13,548	175
WT36	14,341	495
WT41	12,449	510
T536	12,298	601
T541	11,672	200
Experiment B		
C36	14,341	175
C41	12,730	190
WT36	12,233	652
WT41	13,868	675
Experiment C		
C36	12,449	190
C41	10,591	210
T536	10,981	584
T541	12,524	195

CEF cultures were prepared and maintained as described in the legend for Fig. 3. 6 days after infection the cells were labeled with [^{3}H]thymidine in vivo (5 μCi/ml for 3 h) and harvested. The appropriate organelles were isolated and the specific activities of their purified DNAs were determined. The methods used are described in the text.

* Each specific activity value represents the average of three determinations which agreed within 5%.

to the transformation of the cells and is not solely the consequence of viral infection. The specific activity of mtDNA isolated from fibroblasts transformed by the wild type virus (WT36, WT41) was three- to fivefold higher than that of mtDNA isolated from uninfected nontransformed cells (C36, C41) cultured at either temperature. In cells infected with the temperature-sensitive mutant virus (T536, T541), however, the increase in the specific activity of mtDNA was temperature-dependent and observed only when the cells were cultured at the permissive temperature (T536). At the nonpermissive temperature (T541), the specific activity of mtDNA was characteristic of that in the uninfected, nontransformed cells (C36, C41).

In contrast, the specific activity of purified nuclear DNA isolated from cells transformed by the wild type virus (WT36, WT41) was nearly the same as that isolated from nontransformed cells (C36, C41) grown under identical conditions in parallel cultures. In addition, the specific activity of nuclear DNA did not change when cells infected with the thermosensitive mutant virus were switched from the permissive (T536) to the nonpermissive temperature (T541). Since both nuclei and mitochondria were isolated concurrently from the same batch of cells, these observations suggested that the increase in the specific activities of mtDNA in the transformed cells was a mitochondria-specific occurrence. Although in these in vivo experiments the cells were labeled for 3 h with 5 μCi/ml of [^{3}H]thymidine as indicated in Materials and Methods, we obtained similar results when cells were labeled for 18 h in the presence of 2 μCi/ml of isotope (data not shown). Also recent experiments have been performed in which mtDNA was labeled in vivo with ^{32}P rather than ^{3}H. The difference in the specific activities of mtDNA between transformed and nontransformed cells was again observed.

Protein and DNA Content in Mitochondria

Table III indicates that the three- to fivefold increase in the specific activity of mtDNA from transformed cells (WT36, WT41, T536) is not the consequence of a comparable increase in the mass of mitochondria in the transformed cells (as measured by protein content) or the concentration of covalently closed circular mtDNA in these cells (as measured spectrophotometrically). Analysis of electron micrographs (not shown) of thin sections of normal and transformed cell preparations also suggests that the number of mitochondria in the transformed cells was nearly the same as in the nontransformed cells. The observed stimulation in mtDNA synthesis appears, therefore, to be due to an increase in the rate of synthesis of this DNA and not to a substantial increase in either the amount of mitochondria or mtDNA in the transformed cells.

Synthesis of mtDNA In Vitro

To dissociate the mtDNA synthetic system to some extent from extra-mitochondrial precursor pool effects which must be dealt with under in vivo labeling conditions, parallel experiments were performed where mitochondria were isolated from transformed and nontransformed cells and immediately assayed for their ability to synthesize mtDNA in vitro.

TABLE III

Protein and DNA Content in Mitochondria from Control and Wild-Type RSV or T5-RSV-Infected Chick Embryo Fibroblasts

Cell type	Mitochondrial protein*	Mitochondrial DNA‡
	mg/10^6 cells	*total μg of DNA* (isolated from the lower band of the CsCl-EB gradient) *per 10^6 cells*
Experiment 1		
C36	2.549	0.745
C41	2.511	0.756
WT36	2.500	0.750
WT41	2.509	0.755
Experiment 2		
C36	2.500	0.750
C41	2.530	0.747
T536	2.500	0.756
T541	2.507	0.746

CEF cultures were prepared and maintained as described in the legend for Fig. 3. 6 days after infection the cells were harvested and mitochondria were isolated as described in the text. Cells were counted in a Coulter Cell Counter (Coulter Electronics Inc.).

* Protein content represents the average of duplicate determinations.

‡ mtDNA was purified and the concentration was determined spectrophotometrically as described in the text.

Fig. 4 demonstrates that the rate of mtDNA synthesis in vitro was fivefold greater in mitochondria isolated from cells transformed with the wild type virus (WT36, WT41) than in nontransformed, uninfected cells (C36, C41) regardless of whether the cells from which the mitochondria were isolated were cultured at 36 or 41°C. On the other hand, in mitochondria isolated from cells infected with the thermosensitive mutant virus, this increased rate of synthesis was temperature-dependent and was observed only in the mitochondria isolated from cells cultured at the permissive temperature (T536). When these cells were switched to the nonpermissive temperature (T541), mitochondria isolated from them synthesized DNA at the same rate as mitochondria isolated from uninfected cells (C36, C41). The labeled product is completely digested with pancreatic deoxyribonuclease and its synthesis is inhibited by low concentrations of ethidium

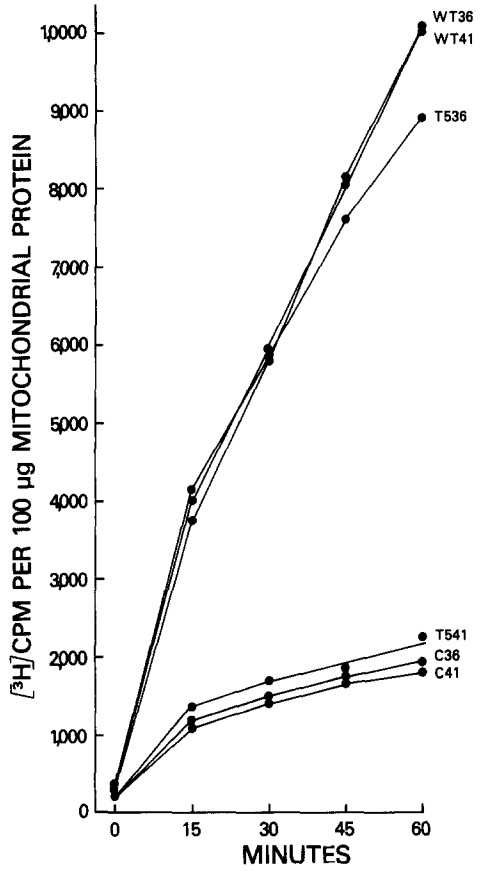

FIGURE 4 Synthesis of mitochondrial DNA in vitro in mitochondria isolated from control and wild type-RSV or T5-RSV-infected chick embryo fibroblasts. CEF cultures were prepared, infected, and subsequently maintained as described in the legend for Fig. 3. The cells were harvested 6 days after infection and the mitochondria were isolated. mtDNA was labeled in vitro with [^{3}H]thymidine-triphosphate. At each time point indicated in the figure, aliquots of the mitochondrial suspension were removed from the reaction vessel, the labeled mitochondria were lysed with SDS, and incubated for 60 min with nuclease-free, predigested Pronase. The labeled product was subsequently acid-precipitated on filter paper disks and counted as described in the text. All time points represent the average of triplicate determinations which agree within 5%.

bromide (not shown in figure). Preliminary analysis of the labeled products on cesium chloride-ethidium bromide-buoyant density gradients indicated that a major portion cobands with covalently closed circular marker mtDNA. (Detailed analysis and comparison of the labeling patterns of mtDNA synthesized in vitro by mitochondria isolated from transformed and nontransformed CEF will be presented elsewhere in a separate communication.)

An experiment was performed to investigate the possible effect of cell "age", or length of time in culture, on the increased rate of mtDNA synthesis in vitro observed in mitochondria isolated from transformed cells. It was tested whether the general health and viability of uninfected CEF in culture might decline more rapidly than that of the transformed cells, thereby giving a greater appearance of a quantitative difference between normal and malignant cells than actually exists. However, this was found not to be the case. When the synthesis of mtDNA in vitro by mitochondria isolated from uninfected cells (C36) maintained in culture only 4 days was compared in the same experiment to the synthesis by mitochondria isolated from cells infected with the wild type virus (WT36) and maintained in culture for 3 wk after infection, with medium changes twice daily, the increased rate of mtDNA synthesis in vitro in mitochondria isolated from the transformed cells was still approximately fivefold higher than the rate of synthesis in mitochondria from normal cells (data not shown).

A most interesting finding (Fig. 5) is that mtDNA synthesis in vitro in mitochondria isolated from nontransformed and transformed CEF exhibited differential sensitivity to inhibition by mercaptoethanol. In mitochondria isolated from normal cells (C36, C41) or T5-infected cells with nontransformed phenotypes (T541) mtDNA synthesis in vitro was significantly inhibited by mercaptoethanol. In contrast, the in vitro mtDNA synthesis by mitochondria isolated from morphologically transformed cells (WT36, WT41, T536) was only slightly inhibited by mercaptoethanol. This observation provides the first indication that there may perhaps be qualitative differences between the mtDNA synthetic system of nontransformed and transformed cells.

To demonstrate that the increased labeling of mtDNA in vitro was not the result of a differential nuclear contamination of the mitochondrial preparations between normal and malignant cells, an experiment was carried out in which WT36 mitochondria were isolated from an homogenate to which C36 nuclei were added and C36 mitochondria were isolated in parallel from an homogenate containing WT36 nuclei. Table IV indicates that the rate of mtDNA synthesis in vitro by mitochondria isolated from transformed

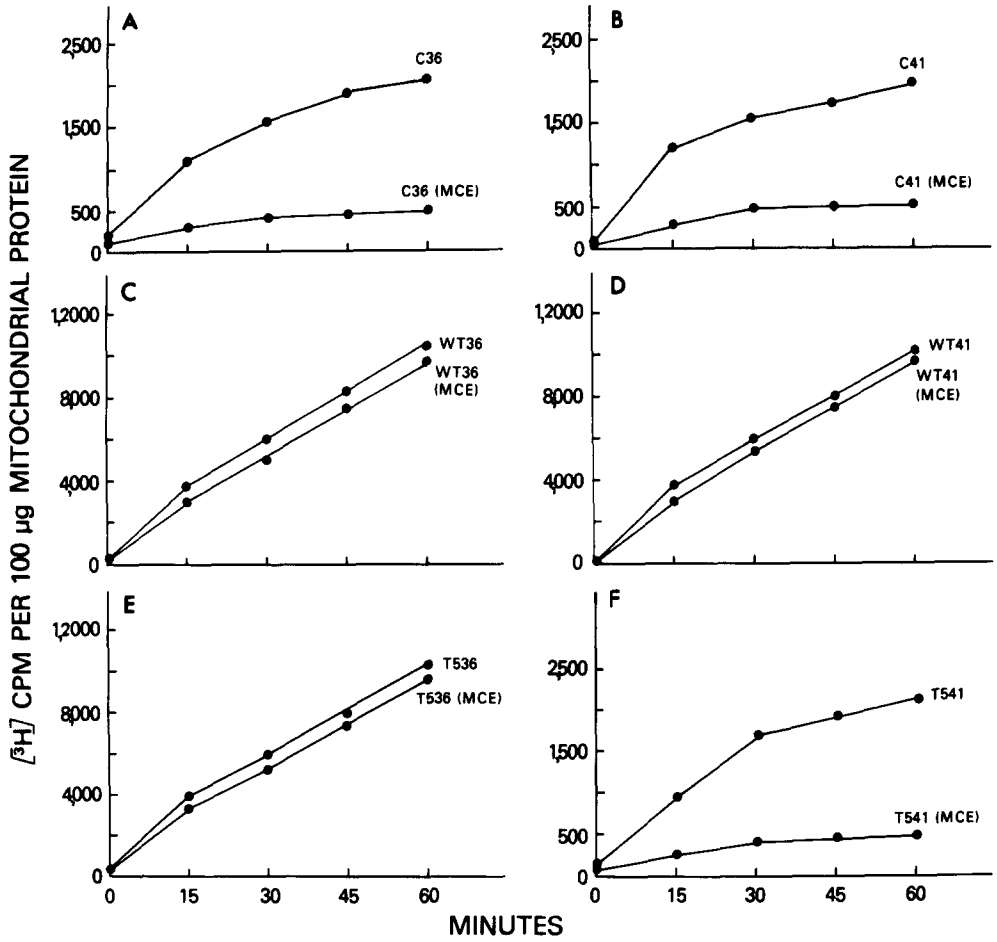

FIGURE 5 Synthesis of mitochondrial DNA in vitro in mitochondria isolated from control and wild type-RSV or T5-RSV-infected chick embryo fibroblasts in the presence and absence of mercaptoethanol. This experiment was performed exactly as described in the legend for Fig. 4. When mercaptoethanol was present it was added to the incubation mixture at a final concentration of 5 µmol/ml. (A) C36; (B) C41; (C) WT36; (D) WT41; (E) T536; (F) T451. All time points represent the average of triplicate determinations which agree within 5%.

cells (WT36) was threefold higher than that of mitochondria isolated from normal cells (C36), regardless of whether either mitochondrial preparation was isolated in the presence of C36 or WT36 nuclei. (The numbers in this table reflect the amount of mtDNA synthesis in vitro which has occurred in 30 min.)

Differential DNA Polymerase Activity in Mitochondrial Extracts Prepared from Nontransformed and Transformed Cells

To demonstrate that the increase in the synthesis of mtDNA in vitro in malignant cells is not merely the reflection of differences in intramitochondrial pool sizes or differential penetration of the labeled DNA precursor through the mitochondrial membranes, extracts were prepared from mitochondria isolated from normal and transformed cells and assayed for mtDNA polymerase activity. Fig. 6 indicates that the DNA polymerase activity was sevenfold greater in mitochondrial extracts prepared from cells transformed with the wild type virus (WT36, WT41) than in nontransformed, uninfected cells (C36, C41). In mitochondrial extracts prepared from cells infected with the temperature-sensitive virus (T536, T541), the increase in DNA polymerase activity occurred only at the permissive tempera-

TABLE IV

The Synthesis of Mitochondrial DNA In Vitro in Mitochondria of Control and Wild Type-RSV-Infected CEF after Pre-Exposure to Heterologous Nuclei

Cell type from which mitochondria were isolated	Additions to post-nuclear supernate obtained from first low-speed spin of cell homogenate	cpm [^{3}H]dTTP incorporated per 100 μg mitochondrial protein	
			WT/C
C36	None	6,000	3.07
WT36	None	18,400	
C36	C36 nuclei	7,500	3.06
WT36	C36 nuclei	23,000	
C36	WT36 nuclei	5,000	3.26
WT36	WT36 nuclei	16,300	

CEF cultures were prepared and infected with the wild-type virus. Infected and control uninfected cultures were subsequently maintained as described in the legend for Fig. 3. 6 days after infection the cells were harvested and lysed as described in Materials and Methods. Each cell preparation was then divided into three equal parts and the nuclear pellet was obtained from each by centrifugation at 700 *g* for 10 min at 4°C. The washed nuclear pellet obtained from the normal cells (C36) was subsequently mixed with the postnuclear supernate of the transformed cells (WT36). In parallel, the washed nuclear pellet obtained from the transformed cells was mixed with the postnuclear supernate of the control cells. After this mixing, both preparations underwent two low-speed centrifugations (700 *g*) for 10 min at 4°C to adequately remove the nuclei. This was followed by preparation of the mitochondria as described in Materials and Methods. The mitochondria were labeled in vitro and processed as described in the legend for Fig. 4. It was determined in a separate experiment that 2.3% of the respective mitochondrial preparations were carried over with the nuclei. This correction was accounted for in the calculations presented in this table.

ture (T536). At the nonpermissive temperature (T541), the DNA polymerase activity was characteristic of that in mitochondrial extracts prepared from uninfected cells. In the absence of exogenously added template, no incorporation occurred by any of the extract preparations (not shown in Fig. 6).

DISCUSSION

The procedures and methods of evaluation in published work on quantitative differences in metabolic events between normal and malignant cells are often not rigid or comparable. This makes it difficult to draw significant conclusions about the observed effects. However, in view of the degree of precision with which the data presented in this communication were obtained, these experiments establish a strong basis upon which to conclude that transformation of CEF by Rous sarcoma viruses to a malignant cell type results in specific, quantitative, and perhaps qualitative changes in the synthesis of mtDNA. The following technical considerations are of importance to support this conclusion.

Cell Culture Procedures

The experimental system is well controlled. The transformed cells can be directly compared to uninfected cells from which they are derived. In addition, infection of cells with the temperature-sensitive mutant virus makes it possible to

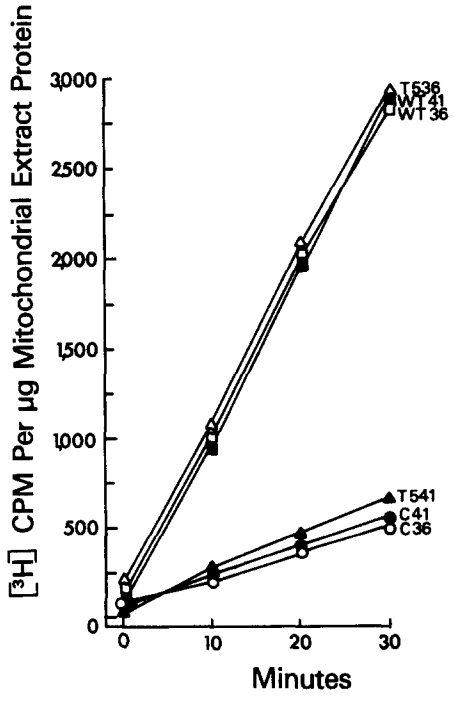

FIGURE 6 The incorporation of [^{3}H]dTTP by mitochondrial extracts prepared from control and wild type-RSV or T5-RSV-infected chick embryo fibroblasts. CEF cultures were prepared and maintained as described in the legend for Fig. 3. Preparation of mitochondrial extracts and assay of mtDNA polymerase activity is described in Materials and Methods. All time points represent the average of duplicate determinations which agree within 5%.

distinguish between events related to viral infection and events related to the infection-transformation process, simply by switching the transformed cells to the nonpermissive temperature.

All virus preparations used were derived within one to three infection cycles from freshly cloned virus. It has been reported that this precaution minimizes the number of temperature-resistant revertants and nontransforming variants in the virus stocks (13).

For each set of experiments, each chick embryo was cultured separately and the resultant cell populations were maintained separately throughout the viral infection and subsequent culturing procedures. This provided the opportunity to screen out those cell populations which might be somewhat resistant to viral infection or transformation and therefore avoided the problems associated with performing experiments on mixed populations of infected cells, all of which might not be transformed under the permissive conditions.

Cell Growth Rates

For each quantitative experiment, the cells were cultured under conditions where both the normal and malignant cells were growing at the same rate during the radioisotopic labeling and cell harvesting periods (Fig. 3). Also, the fraction of cells synthesizing nuclear DNA as determined by autoradiography was found to be about the same in all cell types at both temperatures (13, 19). This suggests that the quantitative differences observed in the synthesis of mtDNA were not merely a consequence of differences in growth rates between the transformed and nontransformed cells. In further support of this conclusion, the specific activities of nuclear DNA purified from the same batches of cells did not differ quantitatively (Table II).

Comparison of Specific Activities

The specific activities reported here have been calculated on extensively purified DNA preparations isolated from the appropriate organelles. In some cases, the absorbance data were checked and verified by a microfluorometric technique described by Nass (18). The relatively high specific activity levels of nuclear DNA (Table II) relative to those of mitochondrial DNA apparently reflect the separate metabolic characteristics of the two types of DNA.

Precautions with the In Vitro Assay

(*a*) For each in vitro experiment, all glassware used was sterile and all solutions used for the isolation and subsequent incubation were filter-sterilized with the exception of the isotope preparation which was added to the reaction vessel with a sterile syringe directly from the stock bottle. In this way, bacterial contamination was kept to a minimum. (The same precautions were taken in the preparation and assay of the mitochondrial extracts.) (*b*) To determine as accurately as possible the concentration of mitochondrial protein in each in vitro experiment or in each assay of the mitochondrial extracts, some precautions were taken. Aliquots of the appropriate mitochondrial suspension or enzyme preparation were acid precipitated in small, sterile, conical, centrifuge tubes and resuspended in sterile triple-distilled H_2O before the Lowry determination was begun. The bovine serum albumin standards were also treated in the same way. This was done in view of reports that various buffer components interfere with the Lowry analysis (e.g. references 4, 5) and render the results inaccurate. In addition, at least six points (in duplicate) within close range of the protein concentration of the sample were plotted for each standard curve. (*c*) We have observed that isolated mitochondria are capable of binding labeled DNA precursors to Pronase-sensitive molecules in a nonspecific manner during in vitro experiments designed to label mtDNA. The degree of nonspecific binding varies considerably depending on the cell type from which the mitochondria are isolated. The labeled product which results from the nonspecific binding process is acid precipitable. Acid precipitation of whole labeled mitochondria directly onto filter disks after in vitro labeling, a procedure widely used to determine the amount of mtDNA synthesized in in vitro experiments, is, therefore, an unsatisfactory method for accurate determination of quantitative amounts of mtDNA synthesized in vitro, especially if the synthesis of mtDNA from different cell types is to be compared. Treatment of the labeled mitochondrial preparations with SDS and nuclease-free pronase before the acid precipitation step, as done in the in vitro experiments presented here, eliminates this problem and allows accurate and meaningful quantitative differences to be detected. This is substantiated by the fact that all the radioactivity

presented in Figs. 4 and 5 represents DNase-sensitive molecules.

Nuclear Contamination

(*a*) In in vivo experiments, the specific activities of mtDNA (Tables I and II) were determined on preparations of covalently closed circular mtDNA molecules (component I) which had been purified by equilibrium centrifugation on cesium chloride-ethidium bromide gradients (Fig. 1). Examination of the component I fraction by electron microscopy revealed exclusively circular mtDNA molecules. Fig. 1 also demonstrates that the ratio of the radioactivity peaks of component II to component I was similar in both gradient profiles (C36 and WT36). This would not be the case if there existed a differential nuclear contamination of the mitochondrial preparations. Further purification of component I by rebanding on second cesium chloride-ethidium bromide gradients and subsequent comparison of the specific activities of the covalently closed circular mtDNA fractions obtained from the second gradient indicates that the difference in specific activities of mtDNA between transformed and nontransformed cells is preserved. In several recent experiments, mtDNA was purified on cesium chloride-propidium diiodide gradients where a greater separation of the component I and II bands was obtained than in cesium chloride-ethidium bromide gradients. Again, the difference in the specific activities of mtDNA between transformed and nontransformed cells is preserved. Finally, an experiment in which mtDNA was labeled in vivo was performed in parallel with the experiment presented in Table IV. During homogenization of the nontransformed (C36) and transformed (WT36) cells, the nuclei from a second, equal batch of WT36 cells were added to C36 homogenates and C36 nuclei to WT36 cell homogenates. It was found that the specific activity values of mtDNA derived from mitochondria of these mixed homogenates were not significantly altered over those obtained for mtDNA from corresponding homologous homogenates (data not shown).

(*b*) In in vitro experiments, the mitochondrial preparations which were to be labeled in vitro were routinely monitored by phase microscopy for the presence of contaminating nuclei. No gross contamination of the mitochondrial preparations with nuclear fragments was visible. Second, Table IV demonstrates that the increase in mtDNA synthesis in vitro observed in mitochondria isolated from transformed cells was not the result of differential nuclear contamination of the mitochondrial preparations. Furthermore, under the culture conditions used in these experiments (see legend for Fig. 3), we have not observed any evidence of nuclear fragmentation, which has been reported to occur under certain conditions in Rous sarcoma-infected CEF (9, 10), either upon observation of the cells by phase microscopy or upon analysis of numerous thin sections of both normal and transformed cells in the electron microscope.

Other Considerations

The increase in the rates of mtDNA synthesis in transformed cells has been confirmed both in in vivo experiments where the labeling of the extensively purified covalently closed circular fraction of mtDNA was compared, as well as in in vitro experiments where the labeling of the total DNA content of the mitochondria was compared. Since there appears to be no significant difference in the relative yields of mtDNA molecules from transformed and nontransformed cells (Table III), it is possible that the more highly labeled mtDNA molecules from transformed cells also reflect a greater rate of turnover for these molecules as compared to those from nontransformed cells and/or a difference in their replication mechanism.

The in vitro findings have been further substantiated and extended in the experiments with the mitochondrial extract preparations (Fig. 6). The mtDNA polymerase activity in mitochondrial extracts prepared from cells with transformed phenotypes (WT36, WT41, T536) was about sevenfold higher than that from cells with nontransformed phenotypes (C36, C41, T541). This rules out the possibility that merely changes in intra- and extra-mitochondrial nucleotide pools or mitochondrial membrane permeability could account for the stimulation. It is possible that the stimulation is due to a quantitative accumulation of DNA polymerase molecules in mitochondria of transformed cells, or to qualitative changes of the conformation of the polymerase molecules, possibly related to an association of the enzyme(s) with the transformed mitochondrial membrane, or to the induction of isozymes or changes in other enzymes active in DNA metabolism. These possibilities are under investigation.

The differential sensitivity to inhibition of mtDNA synthesis in vitro by mercaptoethanol between mitochondria isolated from normal and malignant cells (Fig. 5) might also prove to be a useful probe in analyzing possible differences in their mtDNA-synthesizing systems. Nass (18) has recently described a DNA methylase activity in mitochondrial preparations. This activity is inhibited by mercaptoethanol and dithiothreitol. In contrast, the enzyme from the nuclear fraction appeared to be indifferent to, or even slightly stimulated by, the thiol compounds (18). In addition, D'Agostino et al. (3) have reported that mtDNA synthesis in vitro in normal rat liver mitochondria was inhibited by mercaptoethanol and dithiothreitol. The addition to the in vitro system of cytoplasmic extracts prepared from solid rat or mouse tumors or from 22 h regenerating rat liver rendered these normal rat liver mitochondria partially resistant to the inhibition by MCE.

The function and possible role of mitochondria in malignant cells and in the transformation process is not known. Further studies are needed to show whether mtDNA synthesis is an essential factor in the transformation response of the host cell to the oncogenic virus infection or whether the observed mitochondrial effects are of a more peripheral nature.

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