REPLICATION OF ROUS SARCOMA VIRUS AND THE BIOSYNTHESIS OF THE ONCOGENIC SUBVIRAL RIBONUCLEOPROTEIN PARTICLES ("VIROSOMES") IN THE MITOCHONDRIA ISOLATED FROM ROUS SARCOMA TISSUE

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Received May 18, 1971

Studying the interaction of Rous sarcoma virus (strain Schmidt-Ruppin) with the target cell, we have recently found a surprisingly high titre of the infective Rous sarcoma virus in the mitochondria isolated by zonal and differential centrifugation from Rous sarcoma cells (Mach and Kára, 1971). The Rous sarcoma virus (RSV) was also detected in the isolated mitochondria by electron microscopy (Mach and Kára, 1971). The discrepancy between the relatively small number of viral particles observed by electron microscopy in the isolated mitochondria and the high titre of the infective RSV in the Rous sarcoma mitochondria (Mach and Kára,1971),indicated the presence of subviral infective particles inside the mitochondria and suggested that RSV most probably replicates within the mitochondria of Rous sarcoma cells. A similar hypothesis has been advanced by De-Thé et al.(1969) and Gazzolo et.al.(1969),based on the electron microscopic observation of viral nucleocapsides in the mitochondria of a hamster cell line transformed by RSV (Schmidt-Ruppin strain).

In this communication, we present the direct experimental evidence on the replication of RSV within the mitochondria of the Rous sarcoma cells. Further,we describe the isolation of the oncogenic subviral ribonucleoprotein particles ("virosomes"), synthesized within the Rous sarcoma mitochondria. The virosomes,

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sedimenting in the sucrose density gradient at a density of 1.28, contain the complete genome of Rous sarcoma virus and very probably represent an intermediary state in the development of RSV inside the mitochondria of Rous sarcoma cells.

Materials and Methods

Tumors were induced in 6-week-old Brown Leghorn chickens (free from avian leukosis viruses) by RSV (Schmidt-Ruppin strain) and excised 11 days after infection. Preparation of a cell-free extract from Rous sarcoma tissue was performed under sterile conditions as described previously (Kára,1968a,b). The mitochondria were isolated from the postnuclear supernatant by differential centrifugation for 10 minutes at 7,000 x g at 5°C in a MSE "High-speed 18" centrifuge under sterile conditions and purified by twice repeated resuspension in 50 ml of sterile minimal essential medium (Eagle,1959) containing 10% of inactivated calf cerum, centrifugation at 2,000 r.p.m. for 15 min. and sedimentation of the mitochondria from the supernatant at 7,000 x g for 10 min. at 5°C. The final mitochondrial pellet was suspended in sterile PBS solution (phosphate buffered saline,pH 7.4,without $Ca^{++}$and Mg^{++},Dulbecco, 1954) and the concentration of the mitochondrial proteins in the suspension was determined (Lowry et al.,1951).

Purified mitochondria isolated from 90 grams of Rous sarcoma tissue were labeled in vitro with uridine-5-^{3}H (sp.act.24.5 Ci/mmol, produced by ÚVVVR, Prague) under following conditions:

The incubation mixture contained 400 µCi uridine-5-^{3}H, 10 µmoles ATP (adenosine-5'-triphosphate,disodium,trihydrate, Calbiochem), 10 µmoles $MgCl_2$, 2 µmoles phosphoenolpyruvate (Boehringer), 0.8 ml sterile calf serum (inactivated at 56°C) and 75 mg of purified RS-mitochondria suspended in 5 ml of sterile PBS solution (pH 7.4), containing 1 ml of minimal essential medium(Eagle,1959). The incubation mixture (final volume 6 ml) in a sterile glass tube was incubated in a water thermostat at 39°C for 90 minutes.

After incubation the suspension was cooled in an ice bath, and centrifuged in a 10 ml sterile polypropylene centrifuge MSE tube at 7,000 x g for 10 min. The pellet was then resuspended in 10 ml of ice-cold,hypotonic sodium citrate buffer (0.01M, pH 7.0)and the mitochondria were disrupted by four times repeated freezing at -70°C in a mixture of ethanol and solid CO_2 and thawing in a water bath at 5°C. The mixture of disrupted, uridine-^{3}H labeled mitochondria

was then centrifuged under sterile conditioms at 9,000 x g for 20 min. at 5°C to sediment the mitochondrial membranes and the remaining undisrupted mitochondria. The supernatant (10 ml), containing ^{3}H-labeled mitochondrial RNA, ribosomal subunits and other ^{3}H-RNA-containing structures, was analyzed by zonal centrifugation (Mach and Kára, 1971).

Zonal runs were carried out in a MSE-65 Super-speed centrifuge fitted with MSE B-XIV zonal rotor which was sterilized before use by alcohol and UV-irradiation and loaded at 2,000 r.p.m. by the 550 ml of sucrose gradient (15 - 62 % sucrose) in 0.05M sodium citrate, pH 7.05, using the device described by Birnie and Harvey (1968). The sucrose solution and all buffers were sterilized before use by 0.1 per cent Baycovin. After the run at 36,000 r.p.m. at 5°C for 2 hours, the rotor was decelerated to 2,000 r.p.m., unloaded by 65 per cent sucrose, taking 15 ml fractions under sterile conditions. The fractions were stored at -70°C.

The distribution of radioactivity in the fractions after zonal centrifugation was analyzed by taking 6 ml aliquots from each fraction and adding an equal volume of 10 per cent trichloroacetic acid (TCA) and 0.1 ml of diluted (1:100) calf serum in stoppered tubes. After shaking, the mixture was allowed to stand overnight in a refrigerator (at +2°C) and then filtered on membrane filters (Schleicher-Schüll,BA 85, or Millipore,25 mm, HA 0.45 μ) using the Millipore Pyrex Microanalysis Filter Holder. The radioactive, acid-insoluble material was washed on the filter 3 times with 5 ml portions of ice-cold 5 per cent TCA and the radioactivity on the dried filters was measured in toluene solution of 2,5-diphenyloxazole (PPO) and 2-p-phenylene-bis(5-phenyloxazole)(POPOP),using Packard Tri-carb scintillation spectrometer.

The viral infectivity in the fractions collected after zonal centrifugation was determined as described in detail earlier (Mach and Kára,1971). Chick embryo fibroblast cultures in 60 mm dishes were infected with an aliquot (0.15 ml) of the fraction diluted in 1.5 ml of minimal essential medium with 10 per cent calf serum and incubated in a CO_2 thermostat at 39°C, changing the medium every day. The number of the transformed cells in one microscopic field was determined 3 days after infection using the inverted microscope and magnification 200x.

Isolation of the tumor tissue, preparation, purification and uridine-5-^{3}H labeling of the mitochondria was performed on the

same day, without storing or freezing the material, in order to reduce to minimum the mechanical damage to subcellular structures.

Results and discussion

When the uridine-5-^{3}H-labeled Rous sarcoma mitochondria were disrupted and analyzed by zonal centrifugation in a sucrose density gradient (15 - 62 per cent) as described in Materials and Methods, the incorporated radioactivity was mainly found in the fraction containing the mitochondrial membranes (fraction 12) at a density of 1.17 (fig. 1). Furthermore, radioactive material was present in fractions at a density of 1.16 (corresponding to RSV), but very low radioactivity was detected at a density of 1.19, characteristic for mitochondria (Mach and Kára,1971), indicating that practically all mitochondria were disrupted. Low radioactivity was

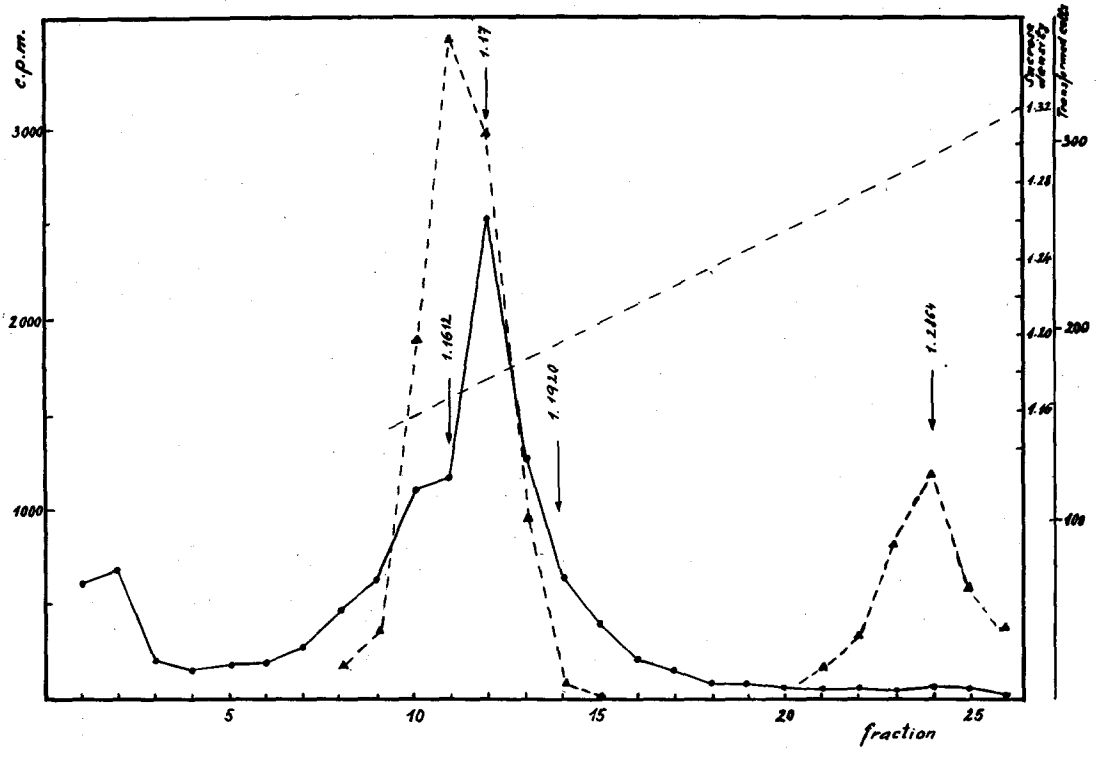

Fig.1. Sucrose density gradient zonal centrifugation of the disrupted, uridine-5-^{3}H labeled Rous sarcoma mitochondria (as described in the text). Full line- radioactivity (c.p.m.), dashed line - viral infectivity.

also found in the fraction 24 with a density of 1.2864 (fig. 1). Viral infectivity was present mainly in fractions 1.16 and 1.17, indicating the presence of the free RSV (density 1.16) and RSV bound to the mitochondrial membranes (density 1.17). As was detected by optical density (A_{260}), mitochondrial membranes were also present in fractions at a density of 1.16, thus interfering with RSV. Surprisingly, the cell transforming activity also was found in the fractions with a density of 1.28 - 1.29 (fig. 1).

In order to remove the mitochondrial membranes, which interfere with the zone of the free RSV (density 1.16), the uridine-5-^{3}H-labeled Rous sarcoma mitochondria, disrupted by freezing and thawinng in 0.01M sodium citrate, pH 7.0, were sedimented at 7,000 x g for 10 min. and the supernatant was analyzed by zonal centrifugation. As shown in fig. 2, a significant radioactivity was found in the fractions 32 - 41, with a maximum at a density of 1.28 (fraction 36 and 37). The chick embryo fibroblast transforming biological activity was also associated with this radioactive material, the highest biological activity coinciding with the maximal radioactivity (Fraction 37, density 1.28, fig. 2). Protein (50 µg/ml) was also detected in the fractions 36 and 37, indicating that the oncogenic viral ribonucleoprotein particles, synthesized in the isolated mitochondria, were present in these fractions.

Relatively low radioactivity, but significant viral infectivity, was found in fraction 20 (density 1.16,fig.2), indicating that the free Rous sarcoma virions (labeled with uridine-5-^{3}H in the viral RNA) were isolated from the Rous sarcoma mitochondria labeled in vitro with uridine-5-^{3}H for 90 minutes.

The oncogenic property of the ribonucleoprotein particles with a density of 1.28 (fig.2) was also proved by infecting one-week-old Brown Leghorn chickens with chick embryo cells infected in culture with fractions 36 and 37 (fig.2). The infected cultures (72 hours after infection) were treated with 0.05 per cent solution of trypsin in sterile PBS and the cell suspension (1 x 10^6cells in 0.5 ml) was injected intramuscularly to eight Brown Leghorn chickens. In 3 weeks 6 chickens (75% of the infected animals) developed tumors, which were characterized as typical, RSV-producing Rous sarcomas.

The presence of ^{3}H-labeled RSV in the extract from ^{3}H-labeled mitochondria was further proved by analyzing the pellet sedimenting at 10^5 x g after 1 hour centrifugation at 5°C of the membrane-free

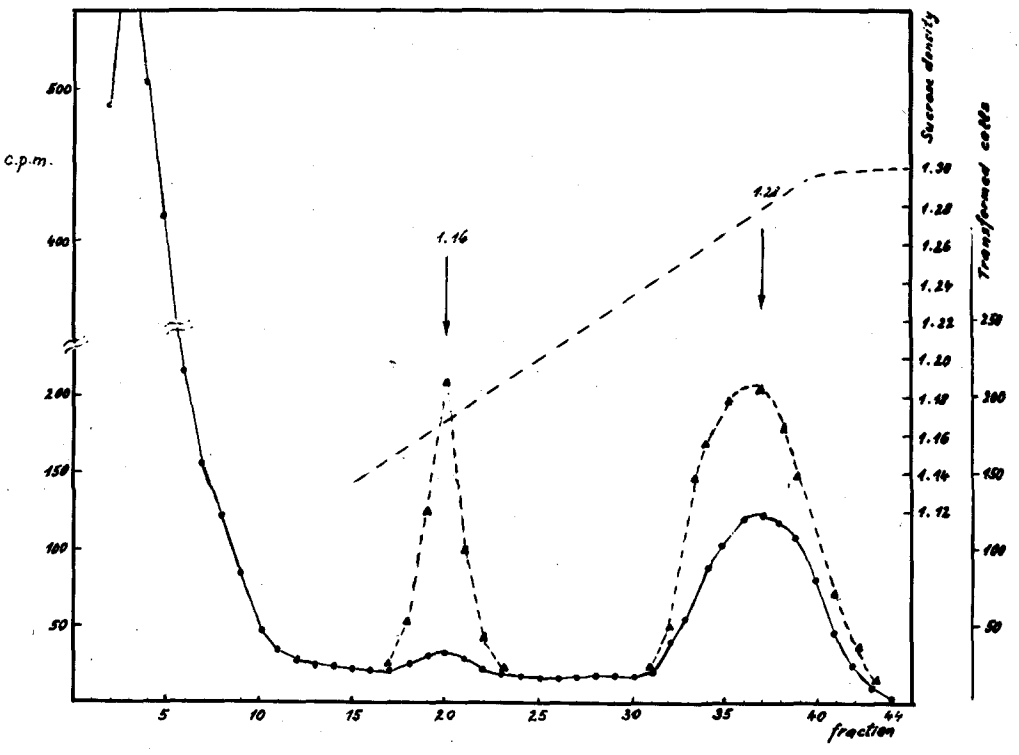

Fig.2. Sucrose density gradient zonal centrifugation of the membrane-free extract from uridine-5-^{3}H labeled RS-mitochondria. Uridine-5-^{3}H labeled RSV (density 1.16) and RS-virosomes (dens. 1.28) Full line- radioactivity(c.p.m.),dashed line- viral infectivity.

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extract from the ^{3}H-labeled Rous sarcoma mitochondria. The pellet was resuspended in 0.1 ml of sterile sodium citrate buffer (0.01 M, pH 7.0) and layered over the 15 - 60 per cent sucrose gradient in a 5 ml MSE polypropylene centrifuge tube and centrifuged in a 3 x 5 ml swing-out MSE rotor at 35,000 r.p.m. for 2 hours. As shown in fig. 3, the highest viral infectivity and radioactivity was present in fraction 12 (density 1.1690) which was clearly separated from ribosomal subunits (fractions 21 - 23, fig. 3).

The results presented here clearly show that Rous sarcoma virus is present and replicates inside the mitochondria isolated from Rous sarcoma cells and undoubtedly also in the mitochondria of the intact Rous tumor cells. These results confirm and extend our ear-

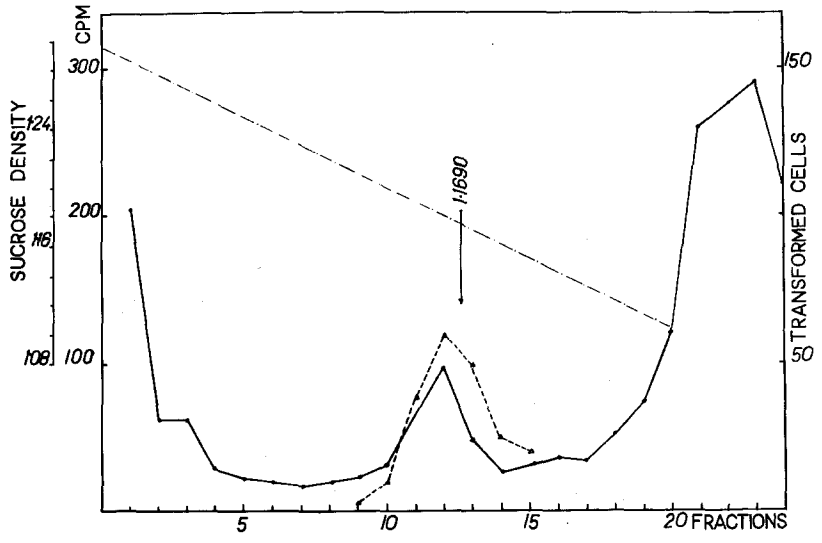

Fig.3. Sucrose density gradient sedimentation profile of the uridine-5-^{3}H labeled RSV, isolated from RS-mitochondria labeled in vitro with uridine-5-^{3}H for 90 min. (as described in the text).

lier findings (Mach and Kára,1971). An abnormal lability of the Rous sarcoma mitochondria studied by electron microscopy has been described earlier by Levine et al.(1961).

The oncogenic ribonucleoprotein particles, sedimenting in the sucrose density gradients at a density of 1.28, which we called "virosomes", very probably represent an intermediary state in the development of Rous sarcoma virus inside the Rous sarcoma mitochondria. The "virosomes", which are synthesized in the mitochondria isolated from Rous sarcoma cells, exhibit very similar physicochemical properties as the Rous sarcoma virus "cores" resulting, after removal of the lipoprotein envelope, from Rous sarcoma virions, which, however, were not infective (Bader et al.,1970). Our results also indicate that the rate of viral RNA biosynthesis in the isolated Rous sarcoma mitochondria and the time required for incorporation of viral RNA in complete virions is 90 minutes or less. Similar kinetics of viral RNA synthesis in cultured RSV-producing cells was found by Bader (1970).

The fact that the tumors induced by RS-virosomes in vivo are typical, RSV-producing Rous sarcomas indicates that the oncogenic Rous sarcoma virosomes carry the complete RSV genetic information. Therefore, beside viral RNA, the virus-specific DNA polymerases (Temin and Mizutani,1970, Mizutani et al.,1970, Spiegelman et al.,

1970, Garapin et al.,1970) and viral DNA (Levinson et al.,1970) may be also present in the RS-virosomes. Further studies on this problem are now in progress in this laboratory.

Results presented in this and previous communication (Mach and Kára,1971) clearly show that Rous sarcoma virus attacks the mitochondria in the target cell. The biochemical implications of these findings are particularly important. Increased aerobic glycolysis, a characteristic metabolic feature of Rous sarcoma cells (Morgan and Ganapathy,1963) and many other tumor cells, may be the consequence of the RSV-induced damage to the structure and function of the mitochondrial inner membrane, carrying the complex system of the respiratory enzymes (Green and Baum,1970, Smoly et al.,1970). In this connection, the data published by Fiala and Fiala (1967) are especially interesting, showing quantitative depletion of mitochondria and a depletion in the flavin and cytochrome c content of the mitochondrial soluble matrix in Morris Hepatoma 5123 A, in distinction to the normal rat liver.

Our findings open new interesting perspectives for studying the interactions of other RNA oncogenic viruses with the cellular mitochondria and suggest that the viral genome of RNA oncogenic viruses may persist in the mitochondria of the transformed, non-virus-producing mammalian cells. The importance of these studies for deeper understanding the mechanism of viral oncogenesis is obvious.

We wish to acknowledge the excellent technical assistance of Mr.M. Hrubeš throughout this work.

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