Virus-like Particles Associated with the Mitochondria of Ethidium Bromide Treated Transformed Cells

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SUMMARY

A cell line resistant to ethidium bromide has been developed from baby hamster kidney cells transformed by Rous sarcoma virus. The parental cells are normally non-producers but the resistant cell line appears to produce virus-like particles that are associated with the mitochondria as visualized by electron microscopy and determined biochemically.

Virus-like particles have been reported to be associated with mitochondria of untreated plant (Harrison & Roberts, 1968; Kitajima & Costa, 1968; Weintraub & Ragetli, 1971), reptilian (Lunger & Clark, 1973, 1976), avian (Kara et al. 1971; Mach & Kara, 1970 and mammalian cells (Gazzolo et al. 1969). The role mitochondria may play in the synthesis and/or assembly of the virus, however, has not been clearly elucidated.

The present study describes virus-like particles associated with the mitochondria of baby hamster kidney cells transformed by Rous sarcoma virus. These cells are normally non-producers. They appear to become virus producers when treated with ethidium bromide. Ethidium bromide is a trypanocidal drug known to alter many aspects of the mitochondrion. (Knight, 1969; Zylber & Penman, 1969; Goldring, et al. 1970; Nass, 1970, 1972; Soslau & Nass, 1971).

There have been reports during the past few years describing both inhibition or lack of inhibition of retrovirus production by ethidium bromide. These differences may be due in part to the incomplete understanding of proviral DNA integration into the cellular genome and the difference in experimental design. Richert & Hare (1972) concluded from their studies with ethidium bromide that mitochondria were involved in virus replication. Bader (1973) demonstrated that ethidium bromide did not inhibit virus production and concluded that mitochondria were not involved in virus production. Roa & Bose (1974) demonstrated that the timing of exposure of cells to ethidium bromide with respect to virus infection was important. They suggested that ethidium bromide depleted the cell of some material involved in the establishment of virus infection. More recent studies by Guntaka et al. (1975) indicate that ethidium bromide is not depleting the cell of any particular factor involved in virus infection but alters the structure of proviral DNA by intercalation, thereby inhibiting its integration and replication. These studies demonstrate that the production of viruses is not inhibited by ethidium bromide in an established transformed cell line. The present report, in fact, indicates that ethidium bromide may induce virus production in some cells.

Baby hamster kidney cells transformed with the Bryan strain of Rous sarcoma virus (C13/B4) and the same cells resistant to ethidium bromide (B4EB82) were cultured under previously defined conditions (Soslau & Nass, 1975). Cells were prepared for electron microscopy by fixation in 3% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4) and 0.15 M-sucrose for 1 h at 4 °C. They were rinsed with the cacodylate buffer and fixed again in 1% osmic acid in the same buffer for 1 h. Cells were rinsed twice with the cacodylate buffer, removed from the flask with a rubber policeman, dehydrated in steps with 30, 50, 75, 95 and
100% ethanol and embedded in Araldite 506 from Ernest Fullam Co., N.Y. Sections were
stained with uranyl acetate and lead citrate and scanned on an Hitachi HU-12A electron
microscope.

Mitochondria were isolated and gradient purified by a previously reported procedure
(Buck et al. 1975). Mitochondria isolated in this fashion were found to be free of nuclei and
relatively free of membranous debris as viewed by electron microscopy. The gradient
purified mitochondria were suspended in 4 ml of buffer (TNE) containing 0.01 M-NaCl,
0.001 M-EDTA, 0.01 M-tris, pH 7.5, with 0.02 M-dithiothreitol (DTT) and 0.001 M-phenyl-
methyl sulphonylfluoride (PMSF). They were then sonicated twice at 4 °C for 15 s and
layered on a 30% glycerol pad. This was centrifuged at 50000 rev/min in an SW 50.1 rotor
(Beckman) for 1 h. The pellet was resuspended in the TNE buffer, layered on a 30 to 65%
linear sucrose gradient and centrifuged in the same rotor at 35000 rev/min for 18 h. The
tube was punctured at the bottom and 0.5 ml fractions collected and assayed for RNA-
dependent DNA polymerase activity.

The reaction mixture used to assay for the RNA-dependent DNA polymerase contained:
0.005 ml enzyme sample; 0.01 ml of a template (25 absorbance units/1.25 ml 0.001 M-EDTA,
0.01 M-tris, pH 7.5) plus Triton X-100 solution (0.5 ml Triton X-100, 2.5 g KCl plus 0.83 g
bovine serum albumin/100 ml), the template + Triton solution being prepared in a ratio of
1:1.5; and 0.085 ml reaction mixture (0.16 ml 1 M-tris, pH 7.8, + 0.3 ml 0.2 M-dithiothreitol
+ 2.72 ml H2O + 0.2 ml 0.01 M-MnCl2 or MgCl2). The 3H-thymidine triphosphate, 6 μmol
(49 Ci/mmol), was lyophilized to remove the ethanol and the reaction mixture was pre-
pared in the tube with the lyophilized isotope. The enzyme preparation was pre-incubated
with the template + Triton X-100 solution for 10 min at 4 °C and then the reaction mixture
added and incubated at 37 °C for 30 min. The reaction was stopped with 0.025 ml of 0.01 M-
tris, 0.02 M-EDTA, pH 8.0. The solution was absorbed on to 2.4 cm Whatman DE81 filter
discs (Blatti et al. 1970) and each disc was washed six times with 10 ml of 5% sodium
phosphate, twice with 10 ml of 70% ethanol, once with 5 ml of 100% ethanol and once
with 2 ml ether. The washings were performed with tippets to ensure consistency and each
filter was washed in a scintillation counting vial. After drying the discs 10 ml of toluene
liquifluor was added and the samples counted in an Intertechnique scintillation counter.

Evidence for the existence of virus-like particles associated with the mitochondria was
first obtained by electron microscopy. When the virus-transformed C18/B4 cells were grown
in the presence of 2 μg ethidium bromide/ml for 4 days it was observed that their mito-
chondrial matrix was less dense than the untreated cells and that virus-like particles were
associated with the mitochondria (Fig. 1a). Virus-like particles were never observed in the
mitochondria of the untreated cells. It is not known if the density of the mitochondrial
matrix in the untreated cell is obscuring any virus-like particles or if they are actually absent.
The number of sections scanned of the C13/B4 cells were far greater than those scanned of
the ethidium bromide treated C13/B4 cells. Mammalian cells cannot usually survive in the
presence of 2 μg ethidium bromide/ml for more than seven days due, at least in part, to the
loss of a functional respiratory system (Soslau & Nass, 1971). We have been able to establish
a cell line that is resistant to ethidium bromide (unpublished data). The resistant cell line has
been cultured and cloned in the presence of 2 μg ethidium bromide/ml for more than 2 years
and virus-like particles have been observed associated with the mitochondria in these
ethidium bromide resistant cells. The number of virus-like particles was increased, as
viewed by electron microscopy, when the cells were treated with iododeoxyuridine plus
dexamethasone, a treatment shown to enhance virus production (Fine et al. 1974). The
frequency of particles associated with mitochondria was also enhanced by exposing the cells
to 8 μg ethidium bromide/ml for 4 days. Fig. 1(b to f) demonstrates that these virus-like particles appear to be associated with mitochondria.

Enzyme studies assaying for an RNA-dependent DNA polymerase-type activity were performed on purified mitochondrial preparations to support the electron microscopic findings. The synthetic templates poly rA·dT₁₂₋₁₈ and poly dA·dT₁₂₋₁₈ were employed and mitochondria were purified from rat liver, rat kidney, RSV transformed baby hamster kidney cells (C₁₀/B₄) and the ethidium bromide resistant cells (B₄EB₉₂) grown in the presence of 8 μg ethidium bromide/ml. Fig. 2(a) shows the results of a reverse transcriptase assay with samples from ethidium bromide resistant cells, employing two different synthetic templates in the presence of a Mn²⁺ cation. The peak activity occurs in the 1.18 to 1.19 g/ml region. A higher activity is obtained with the synthetic RNA template than the synthetic DNA template. The activity was higher with the synthetic DNA template when Mg²⁺ was used as the cation, indicating the possible presence of a mitochondrial γ-like DNA polymerase (Gillespie et al. 1975; Bolden et al. 1977) in the same region of the gradient (not shown). The peak tubes (fractions 7 to 9) were pooled and diluted in the TNE buffer and re-centrifuged through a 30% glycerol pad. The pellet was resuspended in 0.35 ml of the TNE buffer and 10 μl of Triton X-100 was added followed by incubation at 4°C for 15 min. This material was then layered on a second sucrose gradient (30 to 65% sucrose in TNE) and centrifuged under the same conditions as the initial sucrose gradient. The exogenous assay of samples from the gradient is shown in Fig. 2(b). The reverse transcriptase-like activity was shifted to the 1.25 g/ml region and was apparently free of the γ-like DNA polymerase as there was greater propensity to use the synthetic RNA template than the DNA template in the presence of either the Mn²⁺ or Mg²⁺ cation. Similar results were obtained with the
Fig. 2. RNA-dependent DNA polymerase assay employing mitochondria obtained from: (a) B4EB16 cells grown in the presence of 8 μg ethidium bromide/ml for 5 days and centrifuged through a 30 to 65 % sucrose gradient as described in the text; (b) fractions pooled from the first gradient (tubes 7 to 9) subjected to Triton X-100 and re-centrifuged through a second 30 to 65 % sucrose gradient; (c) C13/B4 cells and centrifuged through a 30 to 65 % sucrose gradient as described in the text, and (d) B4EB16 cells exposed to 8 μg ethidium bromide/ml employing Poly rCm-oligo dG as a template. In (a) to (c) ○ — ○ represents the assay performed with a synthetic RNA template, • — •, the assay performed with a synthetic DNA template, all in the presence of Mn2+; □ — □ and ■ — ■ represent the same templates as the corresponding open or closed circles except that the assay was performed in the presence of Mg2+; △ — △, represent buoyant densities.
mitochondria prepared from C13/B4 cells except that slight differences were observed in the initial sucrose gradient. Fig. 2 (c) shows that there was some material already present in the 1.25 g/ml region of the gradient prior to Triton X-100 treatment. Controls were run with mitochondria isolated from rat liver and kidney and with mitochondria obtained from a rat kidney homogenate to which $10 \times 10^{10}$ Rauscher leukaemia virus particles per gram of tissue were added. [Kara et al. (1977) have demonstrated that both RSV and RLV appear to be associated with mitochondria.] All of the experiments were performed with approx. 10 mg of mitochondrial protein. All the controls were negative for the presence of a reverse transcriptase-like enzyme associated with the mitochondria.

Gerard (1975) reported the use of a new template, poly 2'-O-methylcytidylate-oligodeoxyguanylate (poly rCm-oligo dG) which is supposedly specific for virus reverse transcriptase. An assay was therefore performed with a mitochondrial preparation obtained from the B4EB2 cells grown in the presence of 8 /g ethidium bromide/ml employing the poly rCm-oligo dG template. The results depicted in Fig. 2(d) show a peak in the 1.17 g/ml region of the sucrose gradient.

Gradient purified mitochondria from the C13/B4 cells, the B4EB2 cells treated with 8 /g ethidium bromide/ml for four days and the corresponding ethidium bromide resistant mutants derived from control baby hamster kidney cells (C13EB2) were kindly tested for the avian p27 core protein by Dr D. P. Bolognesi (Department of Surgery, Duke University Medical Center). The results of the radioimmunoassay, indicated the presence of the avian p27 antigen associated only with the gradient purified mitochondria derived from the C13/B4 and B4EB2 cells. It is possible that the avian antigen is non-specifically adsorbed on to the mitochondrion and not actually associated with the observed virus-like particles.

The results presented indicate that a virus-like particle is induced by ethidium bromide. It is not clear at present whether these virus-like particles are derived from integrated RSV information or represent an endogenous species. The data for an RNA-dependent DNA polymerase (reverse transcriptase) associated with the mitochondrion suggests the presence of a virus enzyme. The enzyme activity observed is not related to a terminal transferase, it displays the characteristics of the virus reverse transcriptase with regard to template preference (poly rA-dT12-18 and poly rCm-oligo dG) and was found at the buoyant density of the presumptive virus in a sucrose gradient both before and after treatment with Triton X-100. The enzyme activity observed in the 1.17 to 1.19 g/ml region of the sucrose gradient, presumably in intact viruses, could be shifted to the 1.25 g/ml region upon treatment of the B4EB2 preparation with Triton X-100, indicating the removal of the virus envelope and permitting the virus core to migrate to a higher buoyant density. The sucrose gradient in Fig. 2(c) indicates that there may be a relatively large number of virus cores present in the untreated C13/B4 mitochondria as opposed to the case with the B4EB2 mitochondria.

A similar situation has been reported with RSV in transformed avian cells (Kara et al. 1971). The cores may be loosely associated with the mitochondrial membrane which has a buoyant density of about 1.17 g/ml (Kara et al. 1971) and upon sonication of the mitochondria both free virus cores and cores associated with membrane fragments are released, thereby resulting in reverse transcriptase activity spread throughout the 1.25 to 1.18 g/ml region of the gradient. The observed reverse transcriptase may also be that of a cellular enzyme or a mixture of both virus and cellular enzymes.

While it is possible that mitochondrial DNA is involved in virus production, no evidence for its involvement has been presented to date. RNA, on the other hand, has been shown to be transported across mitochondrial membranes (Attardi & Attardi, 1967, 1968; Georgatsos, 1972; Kyriakidis & Georgatsos, 1973; Kara et al. 1977). The transport of virus RNA
into the mitochondrion may account for virus-like particles associated with the mitochondria (Kara et al. 1977). However, it is too early to conclude that the mitochondrion may be a site of virus production. The electron micrographs of Fig. 1 are static pictures and may represent virus particles entering or leaving the mitochondrion. It should be noted that the virus-like particles in Fig. 1 are morphologically similar to the intramitochondrial virus presumed by Lunger and Clark (1976) to be synthesized in the mitochondrion, while similar pictures presented by Schumacher et al. (1974) were interpreted as representing viruses budding into the mitochondrion. Studies to clarify these points are presently under way.

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Short communications


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