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Brief Communication

Comparative Incorporation of Tritiated Thymidine and Cytidine into the Mitochondrial and Nuclear DNA and RNA of Two Transplantable Hepatomas (3924A and H-35tc$_2$) and Host Livers[1]

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Summary

It has been found that the specific activity of thymidine-5-methyl-^{3}H incorporation into the mitochondrial DNA of two transplantable hepatomas (3924A and H-35tc$_2$) and host livers is less than the specific activity of the nuclear DNA. However, the ratio of mitochondrial DNA to nuclear DNA specific activity in both hepatomas and host livers is 10–100 times greater following cytidine-5-^{3}H administration than the ratio following thymidine-5-methyl-^{3}H administration. This marked difference in the ratios of the specific activities of thymidine and cytidine in mitochondrial and nuclear DNA of hepatomas and host livers appears to be the result of both an increase in cytidine incorporation into mitochondrial DNA compared to thymidine, and a decrease in cytidine incorporation into nuclear DNA compared to thymidine.

These differences in the relative incorporation of thymidine-5-methyl-^{3}H and cytidine-5-^{3}H into mitochondria and nuclei synthesizing DNA emphasize the need for caution in utilizing one labeled precursor as an index for DNA synthesis. These results also emphasize the need for the examination of differences in pool size and availability of these two labeled precursors and the need for examination of the permeability of the membranes of the two organelles.

Introduction

During an investigation of cytidine-5-^{3}H incorporation into RNA and DNA of mitochondria of Hepatoma 3924A, it was observed that the DNA specific activity found in the mitochondrial fraction was 25–50 times higher than that in its respective nuclear fraction. When the data were compared with those of thymidine-labeling experiments, it was noted that the rates of incorporation of these two labeled precursors for DNA synthesis in mitochondria and nuclei of the same tumor were markedly different. The observed disparity poses some interesting questions as to the different precursor pools and the biosynthetic pathways of nucleic acid synthesis in the mitochondria and nuclei of the hepatomas as well as of their host livers. The purpose of this communication is to report the relative rates of uptake of the labeled thymidine and cytidine into DNA, and the incorporation of cytidine into RNA of the mitochondrial and nuclear fractions of Hepatomas 3924A and H-35tc$_2$ and their host livers.

Materials and Methods

The growth patterns of transplantable rat hepatoma lines have been described by Morris (8, 9). Nowell *et al.* (13) described the chromosome number and karyotype of each of 41 transplantable hepatomas. Two of these hepatoma lines, 3924A and H-35tc$_2$, were used in this experiment.

Hepatoma 3924A is a solid, hypotetraploid tumor with a chromosome number of 72–73. This is a rapidly growing undifferentiated tumor with a generation time of approximately 0.6 month. Changes have been found in enzymatic activity involved in carbohydrate and lipid metabolism.

H-35tc$_2$ is a poorly differentiated, rapidly growing tumor with a generation time of approximately 0.7 month. It has a chromosome number of 52, and it appears to have less enzymatic and biochemical deviation from normal liver than 3924A.

The tumors were transplanted bilaterally in a subcutaneous position of the back. The animals were kept under uniform controlled conditions of temperature, humidity, and lighting.

Thymidine-5-methyl-^{3}H (3 c/mmole) and cytidine-5-^{3}H (6 c/mmole) were purchased from Schwarz BioResearch, Inc., Orangeburg, New York. One milliliter of labeled thymidine or cytidine, at a concentration of 0.017 micromole per ml in normal saline solution, was given to each rat by intravenous or intraperitoneal injection between 9 A.M. and 12 noon. The animals were sacrificed 1 hour after injection. Tissues were carefully dissected and cleaned. Schneider and Kuff's modified method (15) was used in the preparation of mitochondrial and nuclear fractions. The livers were excised rapidly, rinsed in chilled normal saline solution, blotted, and frozen quicky in a solid CO_2-acetone bath at −78°C. The livers from 6 rats were

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pooled and homogenized in an ice-bath (5 strokes for each small portion of the weighed liver) in a volume of 0.25 M sucrose equivalent to about 7 times the weight of the tissue. The homogenate was filtered through 4 layers of gauze and centrifuged for 15 minutes at 600 × *g* (International No. 958 horizontal rotor) to sediment the nuclei and large cellular fragments. The sediment was washed twice by resedimentation from 0.25 M sucrose before the isolation of nuclear DNA. The supernatant fluid from the first 600 × *g* was collected carefully and centrifuged once more to eliminate any possible nuclear contamination. The resulting supernatant fluid was centrifuged for 30 minutes at 5090 × *g* and 0°C (Servall type SS-34 rotor) to sediment the mitochondria. The mitochondrial fraction was washed twice by resedimentation from 0.25 M sucrose to remove the loosely packed, fluffy, pinkish microsomal layer, and, finally, by resedimentation from 0.15 M NaCl. The saline-washed mitochondria were resuspended in a volume of the original tissue used. In some instances, the washed mitochondrial fraction was purified further by sucrose density gradient according to Neubert (personal communication). The 4-times-washed mitochondrial fraction was resuspended uniformly in 0.32 M sucrose solution; 2.4 M sucrose was added to a final concentration of 1.75 M sucrose; 0.32 M sucrose was layered gently on top of the mitochondrial suspension. The preparation was then centrifuged at 24,000 rpm for 2 hours, using an SW rotor in the Spinco Ultracentrifuge. The mitochondria were collected at the interphase. The preparation was checked microscopically for nuclear fragments or intact nuclei during the period of standardization.

One ml of a 25 percent aqueous solution of sodium dodecyl sulphate was then added to each 14 ml of suspension. The mixture was heated for 10 minutes at 60°C, cooled to room temperature, and shaken for 30 minutes with an equal volume of 75 percent phenol. The aqueous phase was shaken again for 15 minutes with an equal volume of 75 percent phenol. The resulting aqueous phase was then extracted twice, each time with 2 volumes of ether. After the removal of the dissolved ether, the nucleic acids were precipitated by adding 2 volumes of absolute ethanol and storing the mixture in a freezer. Since mitochondrial DNA could not be collected by glass rod, the nucleates were sedimented by centrifugation. The DNA was dissolved in distilled water, and 10 N perchloric acid (PCA) was added to a final concentration of 0.6 N PCA. Acid-soluble materials were removed by centrifugation. The DNA was hydrolyzed twice, each time with a small amount of 0.6 N PCA for 10 minutes in a boiling water bath. The DNA content was determined by the diphenylamine reaction (3), and the radioactivity in the DNA was measured in a Packard Tricarb scintillation counter. Quenching was estimated by using an internal standard. The radioactivity measured was corrected for quenching and the counting efficiency (20 percent). The DNA content of the original homogenates was also determined to estimate the percentage of DNA recovered in the isolated nuclear and mitochondrial fractions. Duplicate samples from 2 mitochondrial fractions were subjected to deoxyribonuclease digestion, according to the method described by Luck and Reich (7) and Nass *et al.* (11). The DNA content of these fractions was compared with that of their controls.

The following criteria were used to evaluate the purity of the mitochondrial preparation: (*a*) The ratio of distribution of the total radioactivity between mitochondrial and nuclear DNA was very large, ranging from 1:35 in the normal liver and 1:1200 in the regenerating liver. (*b*) No appreciable changes in the amount of DNA and radioactivity were detected in the mitochondrial fraction after the deoxyribonuclease digestion. (*c*) An intact nucleus was rarely observed in the final mitochondrial suspension. Approximately 30 percent of the DNA in the homogenate was recovered in the isolated mitochondrial and nuclear DNA. Liver mitochondria prepared in this way account for about 1 percent or less of the tissue DNA, which is in agreement with the findings of others (10, 11, 15).

Results

Specific Activity of DNA. The incorporation of thymidine-^{3}H into mitochondrial and nuclear DNA is given in Table 1. The

Table 1

Isotope	Tissue	Specific activity (dpm/mg DNA) [A] Mitochondrial fraction	[B] Nuclear fraction	[A]/[B]	Specific activity (dpm/mg RNA) [A] Mitochondrial fraction	[B] Cell homogenate fraction	[A]/[B]	Specific activity ratios: Mitochondrial DNA / Mitochondrial RNA	Specific activity ratios: Nuclear DNA / cell homogenate RNA
Thymidine-5-methyl-^{3}H	Tumor 3924A	149,000	204,800	0.73					
	Tumor 3924A	133,000	177,200	0.75					
	Tumor H-35tc$_2$	88,000	736,200	0.12					
	Host liver 3924A	11,000	39,100	0.28					
	Host liver H-35tc$_2$	10,000	34,200	0.29					
Cytidine-5-^{3}H	Tumor 3924A	405,000	37,300	10.9	39,500	97,500	0.41	10.1	0.37
	Tumor H-35tc$_2$	1,490,000	91,800	16.2	55,500	258,800	0.22	205.2	0.35
	Host liver 3924A	930,000	21,300	43.5	8,000	36,300	0.22	116.2	0.58
	Host liver H-35tc$_2$	4,775,000	111,800	42.0	66,000	236,000	0.28	72.3	0.47

The incorporation of thymidine-5-methyl-^{3}H and cytidine-5-^{3}H into the mitochondrial and nuclear DNA and RNA of Morris hepatomas and host livers.

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specific activities of both mtiochondrial and nuclear DNA in the initial and repeat experiments on 3924A were in close agreement; the mitochondrial to nuclear DNA specific activity ratios were 0.73 and 0.75 respectively. There was less incorporation of labeled thymidine into the mitochondrial DNA of H-35tc_2, the mitochondrial to nuclear DNA specific activity ratio being 0.12. The specific activities for the mitochondrial and nuclear DNA in the host livers following thymidine-^{3}H administration were much less than for the tumors, being 0.28 for 3924A and 0.29 for H-35tc_2.

The specific activity of mitochondrial DNA was greater and the specific activity of nuclear DNA was less in the hepatomas following cytidine-^{3}H administration than it was following thymidine-^{3}H administration (Table 1). The ratio of mitochondrial DNA to nuclear DNA specific activity was 10.9 for 3924A and 16.2 for H-35tc_2. Furthermore, the ratio of mitochondrial and nuclear DNA specific activity was more pronounced in the host livers than in the tumors following cytidine-^{3}H administration, being 43.5 and 42 for 3924A and H-35tc_2 respectively.

Specific Activity of RNA. The specific activity of RNA extracted from tissue homogenate of the hepatomas and host livers was 2–70 times greater than that in the mitochondrial fraction when cytidine was used as the label. There was little difference among the ratios of the specific activities in the mitochondrial and tissue homogenate RNA fractions of both the hepatomas and host livers following cytidine-^{3}H administration; the ratios ranged from 0.22 to 0.41.

DNA/RNA Ratios (Specific Activity). The specific activities of DNA/RNA ratios of the tissue homogenate of these two lines of hepatomas and their host livers were very similar (0.35-0.58). On the contrary, a striking difference in the ratio of DNA/RNA was observed in the mitochondrial fraction. The radioactivity present in the tumor mitochondrial DNA was 10–200 times greater than that in the tumor mitochondrial RNA and 72–116 times greater in host liver mitochondrial DNA than in its corresponding mitochondrial RNA.

Discussion

The mitochondrial to nuclear DNA ratio of thymidine-^{3}H incorporation into the rapidly growing tumor 3924A was found to be 0.74 in this study; a ratio of 0.40 was reported by Bass *et al.* (1). The ratios for host livers were 0.28 for 3924A and 0.29 for H-35tc_2—considerably smaller than the normal liver ratio found by Bass *et al.*

The mitochondrial to nuclear DNA ratios of cytidine-^{3}H incorporation into the tumors were 15–130 times greater than the ratios of thymidine-^{3}H incorporation. This is the result of the marked increase in specific activity in the mitochondrial DNA and a decrease in specific activity in the nuclear DNA following cytidine-^{3}H administration compared to the specific activities following thymidine-^{3}H administration. The lower values for cytidine incorporation into nuclear DNA of 3924A are in agreement with Lea *et al.* (6), who reported lower values for deoxyuridine incorporation into nuclear DNA of 3924A compared with the values for thymidine incorporation.

The marked differences in the rates of incorporation of labeled thymidine and cytidine into the mitochondrial and nuclear DNA of both hepatomas and host livers might be explained in one of the following ways. First, the results would suggest that the mitochondrial pools for both cytidine and thymidine might be different from the nuclear pools. Schneider (14) showed that in normal liver the main deoxyribosyl constituent is deoxycytidine, whereas in regenerating liver and Novikoff hepatomas there is an enlarged pool of deoxyribonucleotides but not of deoxycytidine. Therefore, the difference in the relative uptake rates of cytidine may be the result of differences in the pool sizes of the intermediate compounds present in these two organelles. The differences also may be caused by the differential permeability of the membranes of these two organelles to thymidine and cytidine or their derivatives. Another possible contributing factor to these marked differences could be related to the times of availability of the labeled thymidine and cytidine for mitochondrial and nuclear DNA synthesis; thymidine is available for only 30 minutes to an hour in liver homogenate, while cytidine remains available for a number of hours (2, 4). However, the metabolism of nucleosides and nucleotides in the mitochondria is not known. Finally, differences in concentrations of enzymes involved in the conversion of labeled cytidine and thymidine for eventual utilization in mitochondrial and nuclear DNA may also contribute to the differences in the specific activities of the two precursors.

The ratio of the specific activity of mitochondrial RNA to the specific activity of the RNA in the cell homogenate fraction was 0.41 for 3924A and 0.22 for H-35tc_2. The ratios were similar for the host livers. The specific activity ratios between nuclear DNA and cell homogenate RNA in the tumors and host livers were similar, ranging from 0.35 to 0.58. It is interesting to note that the values for nuclear DNA/cell homogenate RNA ratios of hepatomas 3924A and H-35tc_2, in this *in vivo* cytidine incorporation, were 0.37 and 0.35 respectively (Column 10, Table 1); in Neubert's *in vitro* incorporation of uridine triphosphate-^{3}H into the nucleus of Hepatoma 3924A, the value was 0.24 (12). On the other hand, the incorporation ratios of mitochondrial DNA/mitochondrial RNA for hepatomas and host livers were significantly different in this experiment (Column 9, Table 1).

More information about the ratios of the specific activities for the different species of RNA's, in the mitochondria as well as in the nuclei and cytoplasm, should prove helpful in the interpretation of the DNA/RNA ratios in the different cellular organelles.

Addendum. Preliminary experiments have been carried out on the relative rates of incorporation of orotic acid-^{14}C into Hepatoma 3924A and its host liver, utilizing the same experimental design as for the tritium-labeled thymidine and cytidine experiments. The specific activity of mitochondrial DNA of the tumor was 9,000 dPM/mg DNA, and the specific activity for the nuclear DNA was 445 dPM/mg DNA. The specific activity in the host liver was approximately 50 times the specific activity of both the mitochondrial and nuclear DNA specific activity of the tumor. The specific activity of the mitochondrial DNA of the host liver was 470,000 dPM/mg DNA, and the specific activity of the nuclear DNA was 23,150 dPM/mg DNA. The ratios of mitochondrial to nuclear DNA specific activity for both tumor and host liver were approximately 20.

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