Incorporation of Labeled Precursors into Nuclear and Mitochondrial DNA of Cells Grown in Culture (36210)

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Mitochondrial and nuclear DNA differs not only in physical characteristics such as size and shape, but also in a number of parameters concerning their metabolism. Thus the two species of DNA are synthesized by different polymerases (1, 2) and their synthesis is inhibited to differing degrees by DNA intercalating agents (3) and hydroxyurea (4). Furthermore, the activity of the DNA polymerase in isolated mitochondria has been shown, to be independent of the mitotic cycle of *Physarum polycephalum* (2, 5).

In vivo studies have shown that the rates of incorporation of isotopically labeled precursors (6, 7) or analogues (8) into mitochondrial and nuclear DNA are different. Cell lines in culture have also been used in this respect (4, 9 10); and the tissue culture system is perhaps the simplest for studying the kinetics of labeling of mitochondrial DNA, although even here the usual reservations must be applied to interpreting incorporation of labeled precursor or analogue as a measure of DNA synthesis, because of transport of precursor and dilution in intracellular pools.

We have followed the incorporation of isotopically labeled precursors or analogues into nuclear and mitochondrial DNA in chick embryo fibroblasts (CEF) and BHK-21 cells. Under the conditions of our experiments, the kinetics of incorporation of label are different for mitochondrial and nuclear DNA; and, furthermore, our results suggest that the mitochondrial DNA which is synthesized, is translocated and becomes attached to water-insoluble structures.

Materials and Methods. Radioactive materials. Radioactive precursors were purchased from Amersham, Bucks, England.

Cell cultures. Primary cultures of chick embryo fibroblasts (CEF) were grown in 10 cm plastic petri dishes (NUNC) in Eagle's minimum essential medium (Dulbecco modification). The cultures were used after 4 days, at which time the cells were not dividing and were in G1. BHK/21 cells were grown in the same medium, and confluent nondividing cultures in G1 were used. Cultures were shown to be negative in PPLO checks including autoradiographic tests.

Isolation of DNA. To isolate mitochondrial and nuclear DNA, cells were washed with phosphate buffered saline, then scraped into 0.25 M sucrose containing 1.8×10^{-3} M $CaCl_2$ using a rubber policeman. The cells were homogenized; and the nuclei and mitochondria were separated as previously reported (8), and the specific radioactivity of each was determined. Specific radioactivity is defined as counts per minute per absorbancy unit at 260 nm (cpm/A_{260}). Briefly, the nuclei and mitochondria were separated by differential centrifugation in sucrose solutions, the nucleic acids were extracted and precipitated with alcohol. The precipitate was suspended in water and digested with pancreatic RNAse. The digests were centrifuged and the nuclear sediments were discarded while the mitochondrial sediments were saved for determinations of residual radioactivity. Nuclear DNA was isolated from the clarified supernatant solution by precipitation with alcohol; whereas mitochondrial DNA was separated from the ribooligonucleotides by filtration through Sephadex G-75.

It is apparent that this method of isolation of uncontaminated mitochondrial DNA depends entirely upon the total elimination of nuclei from the mitochondrial preparation. This was assured by periodically staining the

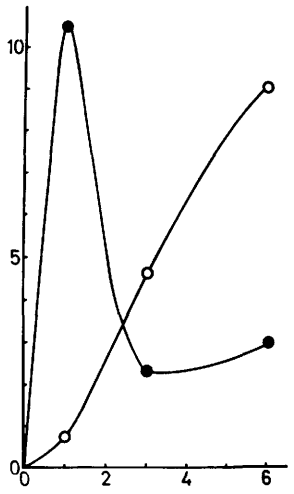

FIG. 1. CEF offered 5-IDU-^{125}I: Figs. 1 and 2 (abscissa) Time (hr); (ordinate) specific radioactivity of DNA (cpm/A_{260} × 10^3; (●) mitochondrial DNA; (○) nuclear DNA. The medium was removed from 4 day cultures and new medium containing 1 μCi/ml of precursor was added (4 ml/plate). At the times indicated, 10 plates were taken and the nuclear and mitochondrial DNA was isolated and the specific radioactivities were measured.

mitochondrial preparations by the routine Papanicolaou stain and searching for nuclei. In no case were there any nuclei detected. The present results furthermore constitute the best proof for the purity of the mitochondrial DNA preparations, since both the kinetics of incorporation of precursors, as well as the specific radioactivity of the DNA isolated from mitochondria are very different from those of nuclear DNA.

Results. Figures 1 and 2 show that precursors are incorporated quite differently into the DNA extracted from mitochondria and from nuclei of either CEF or BHK-21 cells. The specific activity of the nuclear DNA continues to increase with time after addition of label, while the specific activity of the mitochondrial DNA reaches a maximum and then decreases. Similar curves were obtained when thymidine-^{3}H, inorganic phosphate-^{32}P or 5-IDU-^{125}I were given to the cells in over a dozen different experiments employing both CEF, as well as BHK-21 cells. Maximal incorporation of label in mitochondria is reached at 1 hr, and less often at 3 hr, following change of medium. This result is in accord with other observations indicating that the metabolism of the two types of DNA is independent. Parenthetically it should be mentioned that the reason the results in the figures are given in terms of specific radioactivity rather than in absolute terms is that, in these experiments, mitochondrial DNA was not recovered quantitatively, since a considerable portion of the mitochondria were discarded at each centrifugation step to avoid nuclear contamination. Under these circumstances, absolute values are meaningless, while the most meaningful way of expressing incorporation of label is as specific radioactivity of the DNA.

The decrease in specific activity of mitochondrial DNA 3 hr after a medium change implies a decrease in the rate of incorporation of label into DNA, together with either breakdown or removal of newly formed DNA. The rate of incorporation of labeled precursor over 1 hr periods at various times after changing the medium was followed; and Fig. 3 shows that indeed there is a decrease in the rate of incorporation with time, which is not due to nonavailability of the precursor (since this is newly added) but rather to the inability of the mitochondria to further incorporate the precursor.

The stability of the newly labeled mitochondrial DNA was investigated by a method suggested by Attardi *et al.* (11) where DNA synthesis is inhibited by actinomycin D added at a point in time after incorporation of precursor into DNA had commenced. Figure 4 shows that both DNA species are stable, since their specific radioactivity remains constant

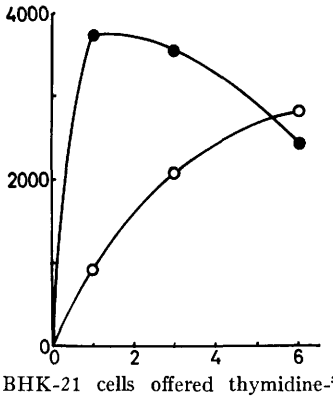

FIG. 2. BHK-21 cells offered thymidine-^{3}H; specific radioactivity of nuclear DNA is ×10^2.

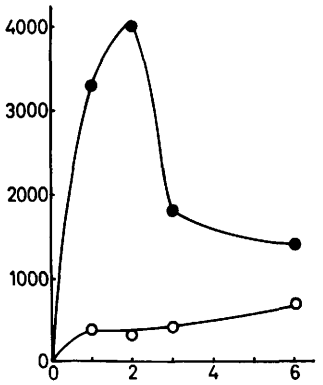

FIG. 3. The medium was changed in a series of 4 day cultures of CEF; and, at the times indicated, 10 μCi of thymidine-^{3}H were added to each of 10 plates. After 1 hr, the nuclear and mitochondrial DNA were isolated and the specific radioactivity was measured. (abscissa) Time (hr); (ordinate) specific radioactivity of DNA (cpm/A_{260}); (●) mitochondrial DNA; (○) nuclear DNA $\times 10^2$.

2 hr after addition of actinomycin D. If degradation of newly labeled DNA occurred, then the specific radioactivity of this DNA would be much lower than the respective DNA species immediately prior to the addition of the inhibitor.

One possibility, that of translocation of newly synthesized mitochondrial DNA, should be considered. In our methodology, only the water-soluble phenol-extracted DNA is studied. In fact when the residual radioactivity of the sediment obtained after RNase digestion of the phenol-extracted mitochondrial nucleic acids, was measured, it was noted, that a proportion of the total counts remained water insoluble. This proportion increases with time after addition of label and fresh medium. A large fraction of the insoluble radioactivity could be extracted by means of sodium deoxycholate (DOC) while the remainder could be solubilized by crystalline pancreatic DNase. The DOC-extracted radioactivity was associated with large molecular weight DNA as shown by chromatography on Sephadex G-75.

Discussion. The kinetics of incorporation of labeled precursor into mitochondrial DNA in cells grown in culture suggest that there is a burst of DNA synthesis which then decreases. Rabinowitz *et al.* (12) have reported an initial increase in mitochondrial DNA synthesis in the first few minutes in yeast undergoing aerobic adaptation, followed by a rapid decline in the rate of incorporation of precursor. It is difficult to compare animal cells growing in tissue culture to the yeast system, but the oxygen supply in the cells, will vary with time after addition of medium and with the volume of the medium (13). It is possible that the change of medium on nongrowing cells could induce some change in the metabolism of mitochondrial DNA.

Concerning the decrease in the specific activity of the mitochondrial DNA, our results resemble those of Wintersberger (14), who observed a decline in the incorporation of nucleotide triphosphates into isolated yeast mitochondria. This investigator considers the possibility that newly synthesized DNA is preferentially degraded by a nuclease specific for DNA lacking an ordered structure.

Our results presented in Fig. 4 exclude this possibility for the present case. Newly labeled mitochondrial DNA seems to be as stable as its nuclear counterpart. Translocation of newly-labeled DNA could however offer a possible explanation for the observed fall in

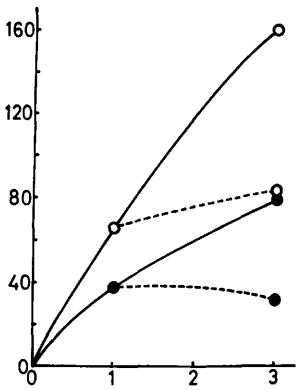

FIG. 4. Medium was removed from a series of 30 plates containing 4 day cultures of CEF and new medium with thymidine-^{3}H (3 μCi/ml) was added. After 1 hr, 10 of the plates were taken and the cells worked out for nuclear and mitochondrial DNA. Also at 1 hr, actinomycin D (5 μg/ml) was added to 10 additional plates; and, after a further 2 hr, these were harvested, as were the plates untreated with actinomycin D, and nuclear and mitochondrial DNA was isolated. The specific radioactivity of all DNA samples was determined. (abscissa) Time (hr); (ordinate) specific radioactivity of DNA (cmp/A_{260}). (●) mitochondrial DNA; (○) nuclear DNA.

specific radioactivity. In fact it appears now to be established that mitochondrial DNA is associated with membranes *in situ;* and it has been suggested that this may have functional implications with regard to replication and transcription (15).

Theoretically there could be a continuous *de novo* synthesis of DNA with a concomitant cessation of incorporation of an exogenous precursor leading to the observed decrease in specific radioactivity. However, this is highly unlikely since the kinetics of incorporation of orthophosphate-^{32}P do not differ from the kinetics of incorporation of the nucleoside precursor as mentioned in the Results.

Summary. Incorporation of labeled precursor into nuclear and mitochondrial DNA of cells grown in culture has been studied. The patterns of labeling of the nuclear and mitochondrial DNA are different, in that after change of medium, and addition of precursor, the specific activity of the nuclear DNA steadily increases, while the specific activity of the mitochondrial DNA increases and then decreases. Pulse-labeling experiments show that the rate of incorporation of precursor decreases with time. However, the decline in specific activity with time after addition of medium and precursor, cannot be attributed to degradation of newly formed DNA, which was shown to be stable in the presence of actinomycin D.

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