THE EFFECT OF ACTINOMYCIN ON THE SYNTHESIS OF MITOCHONDRIAL RNA IN HAMSTER CELLS

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There is considerable current interest in the nature of, and the site of synthesis of, mitochondrial RNA. We have recently found that "bulk" (metabolically stable) mitochondrial RNA from an established hamster cell line, BHK-21 (Stoker and MacPherson, 1964), consists of three centrifugal fractions: "27s" and "17s" components, which sediment slightly slower than analogous cytoplasmic (28s and 18s) species, and a 4s component (Dubin and Brown, 1967). The present report describes studies, using actinomycin D, which indicate that mitochondrial 17s RNA is synthesized on a special, presumably mitochondrial, template.

METHODS

The procedures for culturing, harvesting and fractionating cells, and for preparing and fractionating RNA, have been described (Dubin and Brown, 1967). As before, the final mitochondrial purification step, equilibrium density-gradient centrifugation, yielded a discrete band of RNA corresponding to the band of mitochondrial turbidity.

RESULTS

In these experiments low levels of actinomycin D (0.1 µg/ml) and long periods (22 hrs.) of exposure to the drug were used. This was done to (a) maximize any possible differential effects of the drug, and (b) allow the accumulation of measurable amounts of mitochondrial RNA, at best a very minor

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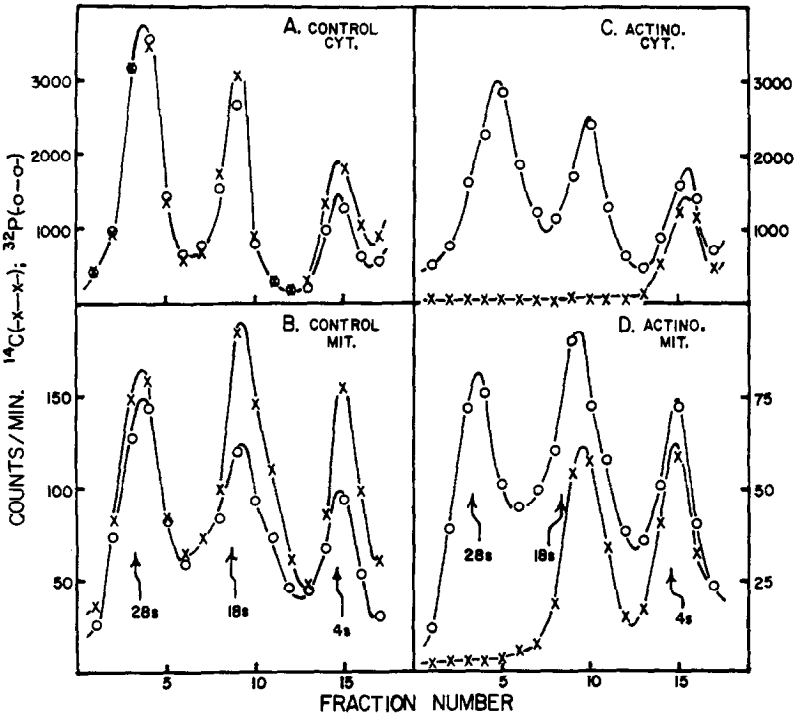
component of mitochondria. These conditions resulted in an approximately 90% inhibition of total cellular RNA synthesis in actinomycin-treated cultures. In a preliminary experiment, the yield of total mitochondrial RNA was con-



Legend to Fig. 1. Sucrose density-gradient centrifugation patterns of cytoplasmic and mitochondrial RNA from normal, and actinomycin-treated, hamster cells.

Cells were labeled with $^{32}P_i$ by growth for 26 hrs. (equivalent to about a 3-fold increase in cell number) in a modification (Dubin & Brown, 1967) of Eagle's medium, containing $^{32}P_i$, 4 μc/1.2 μmoles/ml. The cells were then sedimented and transferred to medium containing 11 μmoles of unlabeled P_i per ml, for a nineteen hour "chase" period. Portions (equivalent to approximately 100 ml of the starting culture) were then diluted into 400 ml of medium containing either ^{14}C-uridine 0.075 μc/0.06 μmoles/ml ("control"); or ^{14}C-uridine, 0.12 μc/0.06 μmoles/ml plus actinomycin D, 0.1 μg/ml ("actino"). (The difference in ^{14}C-uridine specific activity has been corrected for in the Figure.) After another 22 hrs. both cultures were harvested, RNA was prepared from the mitochondrial, and the cytoplasmic, fractions of the two cultures, and samples of each RNA preparation (as indicated on each Panel) were subjected to sucrose density-gradient centrifugation, all as described earlier (Dubin & Brown, 1967). Fractions were collected and assayed for 260 mμ absorbancy (not shown), and for ^{32}P and ^{14}C in RNA. The 260 mμ-absorbing peaks of the cytoplasmic patterns corresponded to the ^{32}P peaks; in addition, the magnitude of the "actino" 4s 260 mμ absorbancy peak indicated that the ^{14}C of this fraction represented largely net RNA synthesis rather than -CCA turnover. The mitochondrial RNA was processed together with carrier cytoplasmic RNA; in these patterns the positions of the 260 mμ-absorbing peaks are indicated by arrows. ^{14}C, (x——x); ^{32}P, (o——o).

siderably less affected; this apparent differential effect was therefore examined in a more elaborate study.

As detailed in the legend to Fig. 1, a culture was "pre-labeled" with $^{32}P_i$, subdivided into control and actinomycin-treated portions, and each culture was then incubated for a further 22 hrs. in the presence of ^{14}C-uridine. Cytoplasmic and mitochondrial RNA was prepared from each culture, and the RNA was fractionated by density-gradient centrifugation. The ^{32}P-labeled, "old" RNA was used as an internal control for artifacts such as gross differences in recoveries and in *in vitro* degradation among the various RNA preparations. The ^{14}C-labeled, "new" RNA served as a direct indicator of the effect of actinomycin on bulk RNA synthesis.

Fig. 1 shows the radioactivity patterns for the four RNA preparations. From these patterns, a more quantitative measure of the effect of actinomycin was obtained by determining the average $^{14}C/^{32}P$ ratio across each RNA peak (Table I).

Table I. Effect of Actinomycin on the Synthesis of Cytoplasmic and Mitochondrial RNA in Hamster Cells.

	CONTROL		ACTINOMYCIN			
RNA	CYT	MIT	CYT		MIT	
species	$^{14}C/^{32}P$	$^{14}C/^{32}P$	$^{14}C/^{32}P$	% of control	$^{14}C/^{32}P$	% of control
4s	1.4	1.5	0.81	58	0.81	54
18(17)s	1.2	1.5	0.04	3	0.62	42
28(27)s	1.0	1.1	0.02	2	0.04	4

The $^{14}C/^{32}P$ ratios have been calculated from the values across each of the peaks of Fig. 1. The specific radioactivities were such that a value of 1 represents approximately 1.5 parts of new RNA to 1 part of old. The cytoplasmic 28s and 18s, and the mitochondrial 27s, values represent maximum estimates since the ^{14}C in these regions appeared largely as a residual plateau.

The control cytoplasmic RNA yielded the usual 28s, 18s and 4s peaks (Fig. 1A), and, as expected in view of the long labeling periods, the ^{14}C and ^{32}P curves were quite similar.

The control mitochondrial RNA (Fig. 1B) resembled, to a first approximation, the corresponding cytoplasmic RNA preparation; the slight differences in sedimentation properties, and in the distribution of RNA among the three major centrifugal fractions, were as previously described (Dubin and Brown, 1967).

The "actinomycin" cytoplasmic RNA (Fig. 1C) yielded a ^{32}P pattern that also resembled the control, except for a slight suggestion of degradation (note the minor augmentation of the 18s and 4s peaks). However, this effect was negligible compared to the effect of actinomycin on new RNA synthesis. As shown by the ^{14}C values, synthesis of cytoplasmic 28s and 18s RNA was virtually nil, and synthesis of 4s RNA was about 50% of normal (Fig. 1C and Table I). (A sparing of 4s RNA synthesis in the presence of low levels of actinomycin has been described previously for rat liver (Revel and Hiatt, 1964); the mechanism of this effect is unknown.)

The "actinomycin" mitochondrial RNA resembled the corresponding cytoplasmic preparation insofar as the 27s and 4s peaks were concerned: there was no significant new 27s RNA synthesis, and new 4s RNA synthesis was approximately 50% of normal (Fig. 1D and Table I). However, in contrast to cytoplasmic 18s RNA, there was a marked sparing of mitochondrial 17s RNA: the $^{14}C/^{32}P$ ratios indicated that this component was also synthesized at a rate approaching 50% of normal (Table I).

DISCUSSION

A precedent for the preferential sparing of mitochondrial RNA synthesis in actinomycin-treated cells can be found in studies by Neubert and Helge (1965) on the RNA polymerase activity of isolated liver mitochondria. These authors observed an *in vitro* sparing effect that appeared to depend on the integrity of the mitochondrial membrane, and hence could be attributed

to exclusion of actinomycin by intact mitochondria. It thus seems reasonable to regard the actinomycin resistance of mitochondrial 17s RNA synthesis as evidence that this species arises within mitochondria, and to regard the apparent "normal" sensitivity of mitochondrial 27s and 4s RNA synthesis as evidence that these species arise outside, at the "normal" (nuclear) DNA templates.

This scheme is in general accord with other recent observations: (1) Mitochondrial 27s RNA resembles cytoplasmic 28s RNA in degree of methylation (Dubin and Brown, 1967); presumably the two would be subject to the same methylases during their common sojourn in the nucleus (see Greenberg and Penman, 1966). The slight differences between the two species might then be attributed to further minor modifications affecting 27s RNA only after it enters mitochondria. (2) Mitochondrial 17s RNA is considerably less methylated than cytoplasmic 18s RNA (Dubin and Brown, 1967); presumably the usual (nuclear) 18s methylases would never encounter an exclusively intramitochondrial species. (3) Bulk yeast RNA has been found to contain a single rapidly sedimenting component that hybridizes with mitochondrial DNA, and, like hamster mitochondrial 17s RNA, this yeast component sediments somewhat slower than the smaller of the two cytoplasmic ribosomal RNA species (Fukuhara, 1967). (4) Mitochondrial RNA from Tetrahymena appears to contain ribosomal components, but not 4s components, that hybridize efficiently with mitochondrial DNA (Suyama, 1967).

We must emphasize that the above scheme is no more than a working hypothesis. It seems clear, nevertheless, that actinomycin will prove to be useful tool for unraveling the intricacies of mitochondrial RNA synthesis.

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