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*HYBRIDIZATION OF MITOCHONDRIAL RNA WITH MITOCHONDRIAL AND NUCLEAR DNA IN AGAR**

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Numerous observations have presented evidence for the occurrence of stranded DNA in the mitochondria.[1–5] Luck and Reich[6] have demonstrated the presence of a DNA-coupled RNA polymerase in the mitochondria of *Neurospora*. A growing body of evidence indicates that RNA is also present within the mitochondrial envelope.[7, 8] The simultaneous occurrence of DNA, a polymerase, and RNA within the mitochondrion strongly suggests that the mitochondrial DNA might be involved in RNA-coded protein synthesis independent of direct nuclear intervention. Such a hypothesis can be tested experimentally using the DNA-agar imbedding techniques as refined by Bolton, McCarthy, and Hoyer.[9, 10] If the nuclear and mitochondrial DNA's are different and unique, then it follows that the RNA's they code will also be different and unique, and the RNA from the mitochondrion would not recognize common base sequences in the nuclear DNA.

Evidence will be presented that RNA does indeed exist within the mitochondrion. Cross-matching evidence will be presented which indicates that some portion of the mitochondrial RNA has base sequences in common with nuclear DNA, and this observation opens the possibility that a portion of the mitochondrial RNA arises as a result of the action of nuclear DNA. The evidence presented, however, does not rule out the possibility that some portion of the mitochondrial RNA is coded only by mitochondrial DNA and that the RNA thus produced is unique to the mitochondrion.

Materials and Methods.—Mouse nuclear DNA was isolated from liver and brain by the method

of Burns and Thomas[11] as modified by McCarthy and Hoyer.[12] The mitochondrial DNA used in these experiments was supplied by Dr. Herman duBuy, to whom the authors express their appreciation. Both the nuclear and mitochondrial DNA, free of detectable RNA, were imbedded in 4% agar as described by McCarthy and Hoyer.[12]

Mitochondria were isolated from mouse liver and brain essentially by the method of Schneider[16] with the modification mentioned below.

In order to make comparisons between nuclear and mitochondrial RNA and to establish the existence of the latter, it was necessary to modify the standard isolation techniques to ensure little or no cross-contamination between nuclear and mitochondrial RNA. It was found that RNase treatment (50 γ/ml for 10 min at room temp.) of mitochondria which had been extracted in 10% sucrose (pH 7.4) had no effect upon the RNA contained in the mitochondria. Experiments performed with added radioactive RNA showed that the RNase treatment reduced the contamination of extramitochondrial RNA to less than 2% of the total RNA isolated. Accordingly, all mitochondria dealt with in this paper have received a pretreatment with RNase.

Nuclei were isolated from mouse liver and brain and washed repeatedly by low-speed centrifugation, with 0.25 *M* sucrose + bentonite. Nuclear and mitochondrial RNA's labeled with radioactive phosphorus were obtained by injecting mouse embryos *in utero* at about the 15th day of pregnancy with 400 μc per embryo of $P^{32}O_4$ neutralized with $NaHCO_3$. The embryos were removed 20 hr after injection, the nuclei separated by several low-speed centrifugations, and the mitochondria isolated, treated with RNase, and resedimented as described above. All classes of RNA were extracted by pH 5.2 acetate buffer treatment followed by deproteinization.[13] Contaminating DNA was removed by heating to 80°C in 0.5 *M* KCl, quenching in ice, and repeated passage through nitrocellulose filters to constant optical density. Diphenylamine reagent[14] and DNase treatment indicated little or no DNA contamination. Protein analyses by the Lowry[15] method showed essentially no protein present. All RNA's were passed through a Sephadex G-100 column to remove low-molecular-weight products, and only the front peak was collected and used. In all cases the 230/260 ratio was less than 0.5, and the 260/280 ratio was 2.0 or larger. All RNA samples were concentrated by pervaporation or reprecipitation and stored frozen until used. Cross-matching and competition experiments were carried out by the "tea bag" technique as described by McCarthy and Hoyer.[12] All counts were made in a Packard Tricarb scintillation counter.

Results.—Experiments involving mouse nuclear DNA imbedded in agar: P^{32}-labeled RNA from nuclei and from mitochondria was incubated with nuclear DNA imbedded in agar at 60°C. for 40 hr. Since RNA is known to bind nonspecifically to agar, blank agar controls were run for each RNA tested. In addition, in one series of experiments RNA was cross-matched against bacterial DNA in agar (*Brucella suis*). No difference was observed between blank agar and that containing nonhomologous DNA. In all data presented in Table 1, the nonspecifically bound RNA counts have been subtracted from the total counts bound.

TABLE 1

CROSS-MATCHING TO NUCLEAR DNA IN AGAR

Agar	Type of RNA	Total counts recovered	Counts bound	% Binding
nDNA[a]	nRNA[b]	198,199[c]	1,781	0.90
Blank[d]	nRNA	229,711	186	(−) 0.08
				0.82
nDNA	mRNA[e]	243,159	1,847	0.76
Blank	mRNA	365,982	941	(−) 0.26
				0.50

Incubation time, 40 hr at 60°, washed 10 times with 2 × SSC at 60°C, and eluted with 0.01 × SSC at 70°.

[a] 0.5 gm agar contained 120 γ nuclear DNA.

[b] 0.5 ml of P^{32}-labeled nRNA contained 11.4 γ RNA (2,570 cpm/γ).

[c] 10-min counts.

[d] 0.5 gm agar lacking DNA.

[e] 0.5 ml of P^{32}-labeled mRNA contained 24.8 γ RNA (1,510 cpm/γ).

The data in Table 1 indicate that both mitochondrial and nuclear RNA combine with nuclear DNA to the same extent. The low level of cross-matching obtained with steady-state labeled RNA has been discussed by McCarthy and Hoyer and is based on the observation that 99 per cent of the labeled RNA is ribosomal and soluble RNA and is coded by less than 1 per cent of the genome. It was found that doubling the amount of DNA in agar had no effect on the percentage of cross-matching, indicating that the availability of DNA sites was not the limiting factor. Increasing the amount of RNA increased the amount of RNA binding proportionally, suggesting that there is a consistent percentage of cross-matchable RNA in the total RNA extracted from mitochondrial as well as nuclear material.

Under the conditions of the hybridization experiment approximately 0.5 per cent of the total nuclear and mitochondrial RNA found combining sites on nuclear DNA. If the recovered material contains messenger RNA, a second application should show a greatly increased percentage of cross-matching because of the relative "enrichment" of messenger RNA.

Enrichment of nuclear and mitochondrial message corresponding to nuclear DNA was carried out as follows: 4 gm of agar containing 840 γ of DNA were incubated with 520 γ P^{32}-labeled mitochondrial RNA solution or with 580 γ of nuclear RNA. After removal of the nonbinding RNA by washing, the RNA which had combined with nuclear DNA was eluted at elevated temperature and low salt concentration. Both nuclear and mitochondrial eluted RNA's were reduced to a small volume and reincubated with nuclear DNA in agar (see Table 2).

From this evidence it can be concluded that both the nuclear and mitochondrial RNA's contain messenger RNA's having base sequences homologous to those found in nuclear DNA. From the preceding evidence it is not possible to determine whether the nuclear and mitochondrial RNA's are combining with the same or different regions of the nuclear DNA. To resolve this question, competition experiments were performed. In these experiments nonradioactive nuclear and mitochondrial RNA's at several concentrations were incubated in the presence of "enriched" radioactive nuclear and mitochondrial RNA in all combinations. A decrease in the binding of mitochondrial message in the presence of nuclear RNA would indicate that the mitochondrial and nuclear RNA were competing for the same sites on the nuclear DNA. The results of a typical competition experiment are shown in Figure 1.

The correspondence of the four curves indicates that the mitochondrial message

TABLE 2

CROSS-MATCHING ENRICHED NUCLEAR AND MITOCHONDRIAL RNA TO NUCLEAR DNA IN AGAR

Agar	RNA type	Total counts recovered	Counts bound	% Binding
nDNA[a]	enRNA[b]	7,229[c]	1,352	18.7
Blank	enRNA	6,064	497	(−) 8.2
				10.5
nDNA	emRNA[d]	4,376	716	16.4
Blank	emRNA	3,620	278	(−) 7.4
				9.0

[a] 0.5 gm agar contained 105 γ nuclear DNA.
[b] 0.5 ml enRNA contained 0.25 γ RNA (3,690 cpm/γ).
[c] 20-min counts.
[d] 0.5 ml P^{32}-labeled emRNA contained 0.20 γ RNA (2,715 cpm/γ).

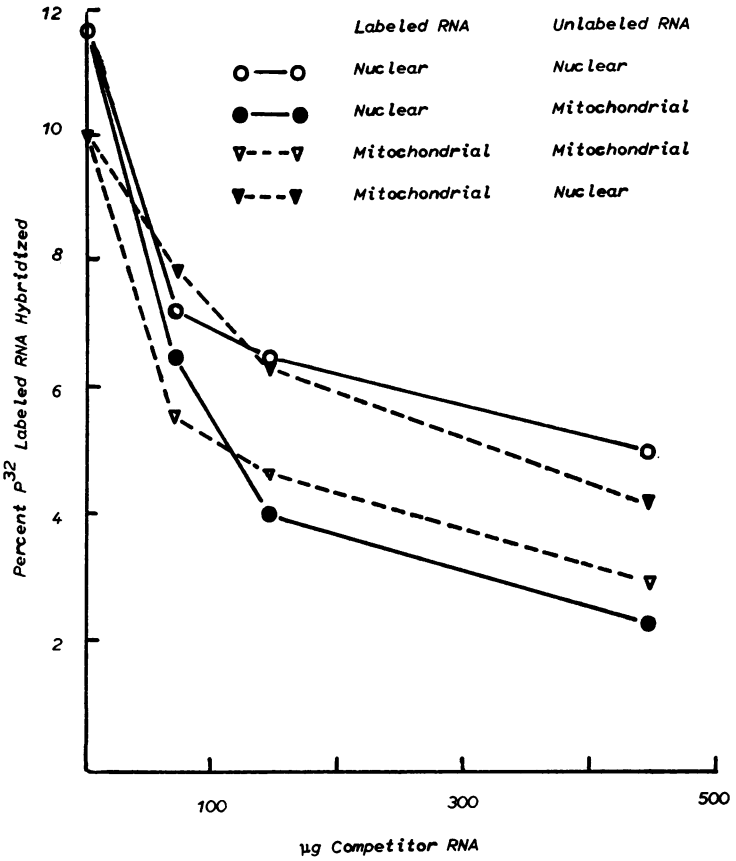

FIG. 1.—Competition by unlabeled mitochondrial and nuclear RNA's with P^{32}-labeled enriched mitochondrial and nuclear RNA's for sites on nuclear DNA in agar. Mitochondrial messenger RNA, 0.5 ml, containing 0.26 γ RNA (3,620 cpm/γP^{32}) or 0.5 ml of nuclear messenger RNA containing 0.38 γ RNA (4,920 cpm/γP^{32}) was added to agar containing 210 γ denatured DNA in the presence of unlabeled nuclear or mitochondrial RNA. Nonspecific counts bound by blank agar containing corresponding amounts of competitor RNA were subtracted from each reading.

enriched on nuclear DNA is combining with the same sites as the nuclear message. It can be concluded, therefore, that at least a part of the mitochondrial RNA has base sequences in common with nuclear RNA.

Experiments involving mitochondrial DNA in agar: When pure mitochondrial DNA became available in our laboratory through the courtesy of Dr. H. duBuy, it was imbedded in agar and hybridization experiments with sheared nuclear DNA and mitochondrial RNA were carried out.

The results of the hybridization of mitochondrial DNA in agar with nuclear DNA are presented in Table 3 along with the appropriate controls. It is apparent from these results that mitochondrial DNA has very few base sequences in common with nuclear DNA.

The results obtained from the hybridization of mitochondrial DNA in agar with mitochondrial RNA are also presented in Table 3. Although an apparent hybridization of 6.5 per cent is shown in the table, and this suggests that a portion

TABLE 3

CROSS-MATCHING TO MITOCHONDRIAL DNA IN AGAR

Agar	Cross-matched with:	Total counts recovered	Counts bound	% Binding
mDNA[a]	nDNA[b]	24,824[c]	74	0.29
nDNA[d]	nDNA[e]	2,088,521	156,000	7.5
B. suis DNA[f]	nDNA[e]	2,246,000	12,024	0.5
Blank	nDNA[e]	1,672,000	7,339	0.44
mDNA	mRNA[g]	1,366	90	6.5
nDNA	mRNA[h]	243,159	1,847	0.76
Blank	mRNA	365,982	941	0.26

[a] 2.75 γ mitochondrial DNA in 0.5 gm agar.
[b] 0.3 γ tritiated sheared nuclear DNA (7,800 cpm/γ).
[c] 20-min counts.
[d] 105 γ nuclear DNA in 0.5 gm agar.
[e] 30 γ tritiated sheared nuclear DNA (7,800 cpm/γ).
[f] 58 γ *B. suis* DNA in 0.5 gm agar.
[g] 0.12 γ P^{32}-labeled mitochondrial RNA (1,410 cpm/γ).
[h] 31 γ P^{32}-labeled mitochondrial RNA (2,014 cpm/γ).

of mitochondrial RNA has common base sequences with mitochondrial DNA, this figure should be regarded with some caution because of the high level of nonspecific binding encountered when very small quantities of RNA are hybridized. Accordingly, although the results shown suggest that hybridization has occurred, absolute evidence must await the availability of larger quantities of mitochondrial DNA.

Discussion.—From the data presented above, several conclusions may be drawn.

There exists in the mitochondrion a species of RNA which can be isolated essentially free of contamination by other cytoplasmic RNA's.

Since nonradioactive mitochondrial RNA competes for sites on nuclear DNA as efficiently as nuclear RNA, a considerable portion of the mitochondrial RNA must be coded for by nuclear DNA. The possibility of a second component of the mitochondrial RNA which combines to a very much greater extent with mitochondrial DNA than with nuclear DNA has not been eliminated.

The lack of cross-matching between nuclear and mitochondrial DNA indicates that these two materials have few base sequences in common. Since this is the case, it is logical to conclude that the mitochondrial DNA is in part a unique DNA and not related to its nuclear counterpart.

Summary.—Mitochondrial RNA has been isolated from mouse brain and liver, essentially free of contamination by other cytoplasmic RNA's.

Cross-matching and competition experiments have shown that two components may exist in the RNA from mitochondria, one of which is coded by nuclear DNA and the other by mitochondrial DNA.

No hybridization was found between mitochondrial and nuclear DNA.

It is suggested on these bases that the mitochondrial DNA represents a unique material, in part at least unrelated to nuclear DNA.

The following abbreviations have been used in the text: nDNA, nuclear deoxyribonucleic acid; mDNA, mitochondrial deoxyribonucleic acid; nRNA, nuclear ribonucleic acid; mRNA, mitochondrial ribonucleic acid; emRNA, enriched mitochondrial RNA; enRNA, enriched nuclear RNA; 1 × SSC, sodium chloride 0.15 *M*, sodium citrate 0.015 *M*.

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THE INFLUENCE OF COLLAGEN ON THE DEVELOPMENT OF MUSCLE CLONES

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Previous investigations have demonstrated that single embryonic muscle cells isolated in culture are capable of forming a macroscopic muscle colony within which the differentiation of multinucleated, cross-striated muscle fibers occurs.[1, 2] The development of such muscle clones from single cells, plated in relatively large volumes, is dependent upon the use of culture medium which has been previously exposed, for several days, to the presence of a dense population of cells.[2] Medium prepared in this manner ("conditioned medium") might be altered in many respects by the metabolic activities of the cells during the conditioning period. An almost limitless number of possible alterations to the medium might have been responsible for its ability to support the development of muscle clones. Therefore, it did not seem practical to engage immediately in a detailed biochemical analysis. We chose rather to reduce the complexity by defining more critically the biological parameters of the requirement for conditioned medium.

One approach which has been particularly fruitful was designed initially to examine the possibility that conditioned medium was required only during a particular phase of muscle clone development. In addition to answering this question, the results immediately suggested what the identity of the active component might be. These results[3] may be summarized as follows: (1) Single cells, plated initially in conditioned medium, which was then replaced by unconditioned medium after 3 days of cultivation, ultimately produced differentiated muscle colonies indistinguishable from companion cultures grown in conditioned medium throughout