HYBRIDIZATION BETWEEN THE NUCLEAR AND KINETOPLAST DNA'S OF LEISHMANIA ENRIETTII AND BETWEEN NUCLEAR AND MITOCHONDRIAL DNA'S OF MOUSE LIVER

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The existence of extranuclear hereditary factors implies the presence of an extranuclear nucleic acid, to a certain degree different from nuclear DNA. The presence of DNA in mitochondria, plastids, and kinetoplasts with melting profiles, reannealing properties, and buoyant densities different from those of the corresponding nuclear DNA's has by now been demonstrated by various groups (e.g., see refs. 1–7). There are only a few reports regarding hybridization reactions between DNA from extranuclear sites with the corresponding nuclear DNA. Shipp *et al.*,[8] using tobacco-chloroplast DNA and nuclear DNA of matured tobacco leaves, found 10–15 per cent binding of P-labeled nuclear DNA fragments and 0.6 per cent binding of P-labeled chloroplast DNA fragments to nuclear DNA embedded in agar, indicating no detectable homology. Dawid[9] reported that frog-egg DNA fragments, apparently of mitochondrial origin,[10] (at the concentrations used) do not compete with C^{14}-labeled tadpole DNA in their reaction with homologous single-stranded frog DNA embedded in agar. He further showed in hybridization experiments that egg complementary RNA hybridized to a small degree with erythrocyte or liver nuclear DNA.

The present paper will present data on hybridization reactions between nuclear and kinetoplast DNA's of *Leishmania enriettii*, and nuclear and mitochondrial DNA's from mouse liver.

Materials and Methods.—Preparation of Leishmania DNA's: Kinetoplast and nuclear DNA of *Leishmania* were prepared by methods described previously.[7] Each batch of kinetoplast DNA was characterized by the formation of only one rapidly appearing band following density gradient centrifugation in CsCl (d = 1.699, 44,770 rpm, 18 hr).[11] C^{14}-labeled *Leishmania* nuclear DNA was obtained by the addition of C^{14}-thymidine (100 μc in 0.8 mg) to two Erlenmeyer flasks each containing 40 ml Senekjie medium, and inoculated with *ca.* 1.2×10^7 organisms each. The organisms were harvested after 3 days, filtered through cheesecloth, and centrifuged, and the pellet was washed once with water. The DNA was then prepared as previously described,[7] and fragmented by mechanical shear of $7.0–10.5 \times 10^6$ gm/cm^2 to a molecular weight of $3–5 \times 10^5$ (ref. 12).

Preparation of mouse-liver DNA's: Mouse mitochondrial DNA was prepared as described before,[11] with some modifications. The DNase treatment was eliminated. The final pellets were further purified by washing with saline, and centrifuged at 18,000 g for 20 min, and the bottom layer was discarded. After addition of the solution containing Tris, sodium lauryl sulfate, and pronase, as described before, the pellets were digested. The preparation was twice extracted with phenol, each time followed by centrifugation in a Spinco ultracentrifuge in a SW25 rotor at 16,000 rpm for 20 min.[3] The bottom layer and the interphase were discarded. Further purification proceeded as described before, except that the preparation was sheared at $7.0–10.5 \times 10^6$ gm/cm^2 before it was placed on the Sephadex column. Each batch of mitochondrial DNA was characterized by its degree of reannealing following thermal denaturation, as compared, under the same conditions, to mouse nuclear DNA. Mouse nuclear C^{14}-DNA fragments were prepared from C_{57} mouse embryo cultures. (The authors thank Dr. M. Martin of this laboratory for a gift of the C^{14}-labeled mouse nuclear DNA.)

Sedimentation-velocity centrifugation: The *Leishmania* DNA's were suspended in a solution

containing 1 *M* NaCl and 0.01 *M* Tris, pH 7.0, and adjusted to an OD_{260} of 0.5. The preparations were examined by sedimentation velocity centrifugation in a Spinco ultracentrifuge model E at 52,640 rpm at 20°C.

DNA-agar technique: In the first series of experiments, the DNA-agar hybridization technique of Bolton and McCarthy[13] was employed. Denatured nuclear DNA (heated to 100°C for 15 min in a solution containing a 1:100 dilution of SSC (hereafter SSC refers to a solution containing 0.15 *M* NaCl and 0.015 *M* sodium citrate)) was mixed with an equal volume of 8% Oxoid Ionagar #2, pressed through a stainless-steel screen, and washed in double-strength SSC. Assays of the amounts of trapped DNA were made spectrophotometrically by dissolving 0.5 gm DNA-agar in hot 5 *M* sodium perchlorate. Since only nuclear DNA could be adequately labeled with C^{14}-thymidine, competition experiments were performed with either nuclear or kinetoplast DNA fragments with C^{14}-labeled nuclear DNA fragments as the indicator. All fragments used for the competition reaction were diluted to the proper concentration, denatured in a 1:100 dilution of SSC at 100°C for 15 min, quenched in ice, and added separately to vials containing DNA-agar. The vials containing the reaction mixtures were incubated overnight at 70°C. The agars were then washed with double-strength SSC (70°C) and the bound fragments eluted at 78°C with water. After addition of 50 μg RNA-carrier the eluates were precipitated with trichloroacetic acid (TCA) (5% final), collected onto membrane filters, and counted in a Packard Tri Carb scintillation counter.

DNA-filter technique: A second series of experiments was performed with the DNA-filter technique of Gillespie and Spiegelman,[14] as modified by Denhardt,[15] to detect complementary DNA. Solutions of *Leishmania* nuclear and kinetoplast DNA's in double-strength SSC were adjusted to contain 65 and 100 μg/ml, respectively. The solutions were then heated to 100°C for 15 min and quickly quenched in ice for 10 min. Aliquots (1.0 ml) were then added to filters presoaked in double-strength SSC. The filters were again washed with 10 ml of the same solution. The filters were dried overnight in a vacuum desiccator at room temperature followed by 2 hr at 80°C in a vacuum oven. The filters, containing either nuclear or kinetoplast DNA, were incubated in separate vials, containing 1.0 ml preincubation medium, for 6 hr at 70°C. This medium contains[15]: 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine albumin (fraction V), in our case made up in double-strength SSC. The preincubation was followed by an additional 12 hr incubation at 70°C with denatured nuclear C^{14}-DNA fragments in the same medium. The filters were then washed in double-strength SSC at 70°C, dried, and counted. The eluted fragments were precipitated as above, collected onto membranes, dried, and counted.

Incubation temperature: Marmur and Doty[16] have reported that with a given DNA, the optimal renaturation was found to occur at about 25°C below its denaturation temperature (*Tm*). We have reported[11] that *Leishmania* nuclear DNA had a *Tm* of 92.8°C. Therefore, for the DNA-agar experiments, 70°C was selected as the highest practicable incubation temperature. This choice had a further advantage since the ability to discriminate between heterologous animal DNA's increases as the incubation temperature is elevated.[17] For reasons of comparison the same incubation temperature was used for the DNA-membrane experiments, although here higher temperatures could have been chosen.

Results.—The *Leishmania* DNA's used were examined by sedimentation-velocity centrifugation in order to determine whether they could be embedded in agar. The $s_{20,w}$ values were 30.3 for nuclear DNA, 5.5 for kinetoplast DNA, and 7.4 for the fragments, corresponding to molecular weights of 17.4×10^6, 1.6×10^5, 4.0×10^5, respectively (calculated by the formula of Doty *et al.*[18]). They are indicative of the sizes of the DNA's used. Only the nuclear DNA was of sufficient size to be effectively embedded in agar.

In the first series of experiments the DNA-agar technique was used to determine the degree of competition by unlabeled *Leishmania* nuclear or kinetoplast DNA fragments in the reaction between C^{14}-labeled nuclear DNA fragments and nuclear DNA embedded in agar. The results are given in Table 1. The data show that kinetoplast DNA fragments are about 60 per cent as effective as are unlabeled

TABLE 1

COMPETITION BY UNLABELED *Leishmania* NUCLEAR OR KINETOPLAST DNA FRAGMENTS IN THE REACTION BETWEEN NUCLEAR DNA-AGAR AND LABELED DNA FRAGMENTS

		DNA-Agar* with Competitor				DNA-Agar without Competitor (Controls)		
Expt. no.†	Competitor	μg added	Total count recovered‡	Total count bound	Count bound (%)	Total count recovered	Total count bound	Count bound (%)
1	Nuclear	150	17,938	694	3.9			
		250	17,791	407	2.3			
		350	17,581	504	2.9	15,624	2,189	14.0
	Kinetoplast	150	17,071	1,087	6.4			
		250	15,621	1,072	6.9			
		350	17,730	1,262	7.1			
2§	Nuclear	150	15,861	510	3.2			
		250	17,127	327	1.9			
		400	17,324	352	2.0	16,408	1,843	11.2
	Kinetoplast	150	12,132	929	7.7	16,558	1,900	11.5
		250	13,818	662	4.8			
		400	16,484	724	4.4			
3§	Nuclear	150						
		250	18,263	473	2.5			
		400				17,557	2,008	11.4
	Kinetoplast	150	14,210	1,296	9.1	17,451	2,007	11.5
		250	16,636	860	5.2			
		400	17,855	908	5.1			

* DNA (42 μg) per 0.5 gm agar.
† In all expts. 0.41 μg nuclear C^{14} DNA fragments (*ca.* 920 cpm) was added.
‡ Counts of front peak corrected by counts in tenth wash.
§ Fragments, not premixed, were added directly to the incubation vials.

nuclear DNA fragments in competing out nuclear C^{14}-DNA fragments. The data of the first experiment are graphically presented in Figure 1. It can be seen that the competition curve generated by the kinetoplast DNA did not converge with the nuclear competition curve as the concentrations of the competitors were increased.

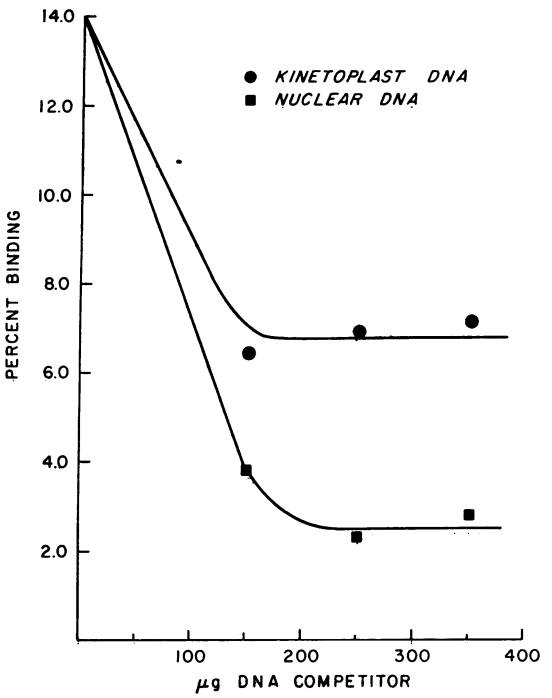

FIG. 1—Competition reactions between C^{14}-labeled nuclear DNA fragments and unlabeled *Leishmania* nuclear or kinetoplast DNA fragments for sites on nuclear DNA embedded in agar. Samples (0.5 ml) of a solution of 0.3 *M* NaCl + 0.03 *M* sodium citrate, containing 0.41 μg C^{14}-labeled *Leishmania* nuclear fragments (*ca.* 920 cpm) and various amounts of nuclear or kinetoplast DNA was incubated with 0.5 gm samples of *Leishmania* nuclear DNA-agar. The binding of labeled fragments to DNA-agar without competitor was stabilized by the addition of 100 μg of mouse nuclear DNA fragments. The per cent of labeled fragments bound vs. the various amounts of unlabeled competitor has been plotted.

The results of experiments with the *Leishmania* DNA's, using the membrane filter technique, are given in Table 2. They again show that the kinetoplast DNA contained nucleotide sequences similar enough to nuclear DNA to allow labeled nuclear DNA fragments to reassociate about 60–70 per cent as effectively with trapped kinetoplast DNA as with the corresponding nuclear DNA.

In order to determine whether an extranuclear DNA of another species would bind to the corresponding nuclear DNA, a similar series of experiments was performed with mouse-liver nuclear and mitochondrial DNA's, each immobilized on membranes, under the same conditions as described above for the protozoal DNA's, except that the incubation temperature of the reaction mixtures was 68°C.

It can be seen from Table 3 that, analogous to the results obtained with the protozoal DNA system, a higher proportion of labeled nuclear DNA fragments was bound to nuclear DNA than to mitochondrial DNA.

In order to determine whether the fragmentation of the mitochondrial DNA before immobilization on the membranes could account for the decreased binding with the nuclear C^{14} DNA fragments, the labeled nuclear fragments were also incubated with *fragmented* trapped nuclear DNA (Table 3, expt. 2). The fragmentation decreased the reassociation only to a limited degree.

To resolve the question whether the partial reassociation of extra-

TABLE 2

BINDING OF LABELED *Leishmania* NUCLEAR DNA FRAGMENTS TO NUCLEAR AND KINETOPLAST DNA, IMMOBILIZED ON MEMBRANES

Expt. no.	DNA Filters*					Blank Filters			
	Type of DNA embedded	Total counts recovered	Total counts on filter	Annealing (%)	Av. %	Similarity kin./nucl. DNA† (%)	Counts on blank filters	Total counts recovered in blanks	Binding to blanks (%)	Av. % binding to blanks
1	Kinetoplast	Not done	Not done	Not done			653	20,373	3.2	
	Nuclear	21,948	3,238	14.8			521	19,954	2.6	2.7
		20,649	4,026	19.5	17.1		453	19,614	2.3	
		20,652	3,552	17.2						
2	Kinetoplast	22,076	2,409	10.9	10.6		813	24,146	3.4	
		24,013	2,464	10.3			532	21,874	2.4	2.9
						60				
	Nuclear	21,929	3,298	15.0	15.7					
		23,305	3,818	16.4						
3	Kinetoplast	22,209	2,398	10.8	11.2		483	22,850	2.1	
		22,065	2,533	11.5			634	22,131	2.9	2.5
						74				
	Nuclear	23,557	4,005	17.0	14.2					
		22,195	2,522	11.4						

* *Ca.* 50 μg of either nuclear or kinetoplast DNA per filter, incubated with 0.5 μg nuclear C^{14} DNA fragments (2,300 cpm per μg). All samples were counted for 20 min.

† Percentage of nuclear DNA similar to kinetoplast DNA: $\frac{\text{Av. \% binding to kin. DNA} - \text{Av. \% binding to blanks}}{\text{Av. \% binding to nucl. DNA} - \text{Av. \% binding to blanks}} \times 100$.

TABLE 3

BINDING OF LABELED MOUSE NUCLEAR DNA FRAGMENTS TO NUCLEAR AND MITOCHONDRIAL DNA, IMMOBILIZED ON MEMBRANES

		DNA Filters*					Blank Filters			
Expt. no.	Type of DNA filter	Total counts recovered	Total counts on filter	Binding (%)	Av. % binding	Similarity mitochon./ nucl. DNA† (%)	Total counts recovered	Total counts on filter	Binding (%)	Av. % binding
1	Fragmented mitochondria	66,299	9,878	14.9	15.7		67,687	2,266	3.3	3.3
	"	58,659	9,764	16.6		42.3	70,849	2,250	3.2	
	Nonfragmented nuclei	63,591	20,443	32.1	32.7					
	"	61,732	20,531	33.2						
2	Fragmented mitochondria	58,843	8,969	15.2	15.5					
	"	60,298	9,543	15.8						
	Fragmented nuclei	60,828	16,771	27.6	25.8	45.9	58,484	1,490	2.5	2.6
	"	61,496	14,831	24.1			58,232	1,556	2.7	
	Nonfragmented nuclei	61,743	18,706	30.3	30.7					
	"	59,451	18,526	31.2						

Ca. 45 μg of either nuclear or mitochondrial DNA per filter, incubated with 0.17 μg nuclear C^{14} DNA fragments (20,500 cpm per μg). All samples were counted for 20 min. For method of determining percentage similarity, see Table 2.

nuclear with nuclear DNA was species-specific, the degree of reassociation was determined between immobilized mouse mitochondrial DNA and labeled *Leishmania* nuclear DNA, and between trapped *Leishmania* kinetoplast DNA and labeled mouse nuclear DNA, as compared with the appropriate controls. Table 4 shows that the extranuclear DNA's of *Leishmania* and mouse reassociated only with their corresponding nuclear DNA's, thus indicating species specificity.

Discussion.—In interpreting the above results regarding the 40–70 per cent similarity of nuclear and extranuclear DNA, the following should be considered. The relatively large size of nuclear DNA molecules implies a great heterogeneity of nucleotide sequences. The considerable degree of binding between the extranuclear and nuclear DNA's suggests, therefore, that the nuclear heterogeneity is reflected in the extranuclear DNA. However, two lines of evidence indicate homogeneity in the extranuclear DNA: (*a*) ultracentrifugal data and the degree of reannealing indicate a high degree of homogeneity and a low molecular weight; (*b*) functional studies[19] supply evidence indicating low protein-synthetic capacities (structural protein) of extranuclear DNA's as compared to nuclear DNA. In order to reconcile to a certain extent the implied complexity of the extranuclear DNA on one side, and the implied homogeneity on the other, it should be realized that a

TABLE 4

BINDING OF EITHER LABELED MOUSE OR *Leishmania* NUCLEAR DNA TO VARIOUS DNA'S IMMOBILIZED ON MEMBRANES

Vial no.	DNA Filters: Type of DNA immobilized*	DNA Filters: Labeled DNA used	DNA Filters: Total counts recovered	DNA Filters: Total counts on filter	DNA Filters: Annealing (%)	DNA Filters: Av. %	Blank Filters: Labeled DNA used	Blank Filters: Total counts recovered	Blank Filters: Total counts on filter	Counts bound (%)
1	Mouse mitochondria		27,244	770	2.8	2.8				
		C^{14} *Leishmania*† nuclear					C^{14} *Leishmania* nuclear	21,124	206	1.0
2	Mouse mitochondria		21,669	599	2.8					
3	Mouse mitochondria		60,693	8,390	13.8	13.3				
		C^{14} mouse‡ nuclear					C^{14} mouse nuclear	61,790	1,421	2.3
4	Mouse mitochondria		61,311	7,853	12.8					
5	*Leishmania* nuclear	C^{14} *Leishmania* nuclear	22,072	4,263	19.3	—				
6	Mouse nuclear	C^{14} *Leishmania* nuclear	21,664	1,214	5.6	—				
7	Kinetoplast	C^{14} mouse nuclear	60,570	2,289	3.8	—				

* Each filter contained about 45 μg DNA.
† C^{14} *Leishmania* nuclear DNA fragments (0.41 μg) added (*ca.* 920 cpm).
‡ C^{14} Mouse nuclear DNA (0.17 μg) added (*ca.* 3000 cpm).

40–70 per cent similarity of the nuclear and extranuclear DNA does not necessarily mean a perfect rematching of the original sequences. We prefer the term "reassociation," proposed by Waring and Britten[20] "which signifies the stable pairing of DNA strands by virtue of complementary nucleotide sequences, however imperfect the match." Further, we do not know what fraction of a fragment must form duplex structures before the whole becomes stably bound, or the degree of formation of "loose ends" or "loops."[21]

These considerations indicate that a certain degree of polynucleotide similarity, but not necessarily total complementarity of nuclear and extranuclear DNA is required in order to effect the reassociation observed. The degree of reassociation between the relatively homogeneous extranuclear DNA and the heterogeneous nuclear DNA, as reported here, might then be due to a similarity of polynucleotide strands of a given area of the extranuclear DNA, and a similar but not completely complementary area of short nucleotide sequences present in many copies in the nuclear DNA.

Another factor contributing to the degree of similarity of the two DNA's might be a certain amount of contamination of the cytoplasmic DNA's with nuclear DNA. However, the characterization of each batch of extranuclear DNA used (see *Materials and Methods*) minimizes this possibility. Also, if contaminating nuclear DNA were present in large proportion, the competition curve generated by kinetoplast DNA should have gradually converged with the nuclear competition curve as the concentrations were increased; also, the two curves should have been more divergent at low concentration. Neither of these situations fits the experimentally observed results.

An alternative explanation regarding the binding experiments of labeled mouse nuclear fragments to mitochondrial DNA has not been completely ruled out. The results with the mitochondrial system could also have been obtained if the mitochondrial DNA represented a reduplication of some nuclear DNA sequences similar enough to lead to duplex formation. The mitochondrial DNA would then be able to recognize only this fraction of the nuclear fragments, thus producing the observed decrease in binding. Although the degree of reannealing after denaturation of the mitochondrial DNA and its absence in the nuclear DNA does not favor this explanation, further experiments are in progress to study these alternatives.

The results of Table 4 indicate that the extranuclear DNA's of widely separated species are species specific. This supports the view that they have an origin common with other subcellular elements, contrary to the hypothesis of the exogenous origin of mitochondria.[19]

The evidence presented here for the existence of extranuclear DNA's in *Leishmania* and mouse cells, which are differentiable from their respective nuclear DNA's, is consistent with previously presented data, demonstrating the existence of extranuclear hereditary factors.[22–24]

Further, the significant proportion of similarity found by reassociation and competition reactions in the two systems is a suggestive indication of some degree of correspondence of the cytoplasmic DNA's with the nuclear components.

Summary.—Two methods were used to study the degree of hybridization between nuclear and kinetoplast DNA's of *Leishmania enriettii* and nuclear and mitochondrial DNA's of mouse liver. The first method involved the use of the DNA-

agar technique under conditions favoring high specificity of binding, i.e., elevated temperature. In this case, labeled nuclear fragments competed for sites on nuclear DNA-agar with either unlabeled nuclear or extranuclear DNA fragments. The second method involved the use of membrane filters with the improvements proposed by Denhardt. Here the degree of binding of labeled nuclear fragments to either membrane-immobilized nuclear or extranuclear DNA was studied. The results obtained by either method indicate that the extranuclear DNA's contain nucleotide sequences, similar enough to nuclear DNA fragments to allow labeled nuclear DNA fragments to reassociate 40–70 per cent as effectively with extranuclear DNA as with the corresponding nuclear DNA. The implications of these results are discussed.

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