Sequence Homology between Mitochondrial DNAs of Different Eukaryotes†

Smilja Jakovcic, James Casey,‡ and Murray Rabinowitz*

ABSTRACT: The sequence divergence of mitochondrial DNAs (mtDNA) from rat, mouse, guinea pig, monkey, and chicken has been examined by DNA–DNA hybridization. mtDNAs, isolated as closed circular molecules by propidium iodide–CsCl centrifugation, were labeled in vitro by use of *Escherichia coli* DNA polymerase I, and renatured (T_m −35°) in the presence of a 2500-fold excess of heterologous mtDNA. Single-stranded and duplex DNA were separated by hydroxylapatite chromatography. The thermal stability of heteroduplexes was compared to the homoduplex by thermal elution chromatography on hydroxylapatite columns. Heteroduplex formation between the tritiated mtDNAs and a 2500-fold excess of rat mtDNA were 70, 59, 37, and 22%, respectively, for mouse, guinea pig, monkey, and chicken. Similar results were obtained in reciprocal hybridizations where one of the other mtDNAs was present in excess. Considerable mismatching of sequences in all the heterohybrids was indicated by a 18–24° depression in the t_{e50} of the heteroduplexes compared with the homoduplex. There was no apparent change in heteroduplex formation when the concentration ratio of driving DNA in excess to [^{3}H]mtDNA was varied between 1250 and 7500. Furthermore, a second renaturation with excess driving DNA after completion of the first reaction resulted in no detectable augmenting of heteroduplex formation. Similar sequences appear to be conserved preferentially in different organisms, since the presence of two or four different heterologous mtDNAs in excess resulted in only moderate and nonadditive increases in heteroduplex formation. Evolutionary divergence of mtDNA sequences appears to have occurred at rates similar to that for unique sequence nuclear DNA.

In the preceding paper (Jakovcic et al., 1975), we examined the sequence divergence of the mitochondrial leucyl-tRNA cistron in several eukaryotes by means of mitochondrial tRNA–mtDNA hybridization techniques. Hybridization of rat mitochondrial leucyl-tRNA with mtDNAs of rat, mouse, guinea pig, monkey, chicken, and yeast, and analysis of the melting profiles of the heterohybrids, indicated a wide range of sequence divergence of the mitochondrial leucyl-tRNA cistron. Whereas rat mitochondrial leucyl-tRNA sequences were well conserved in the mouse (T_m depression of the heterohybrid $\simeq$ 2–3°), greater divergence was noted in the guinea pig (T_m depression $\simeq$ 7–9°) and in the monkey (T_m depression $\simeq$ 15°). Chicken and yeast mtDNA did not hybridize with rat leucyl-tRNA indicating a sequence divergence greater than 21%. The degree of sequence divergence of the mitochondrial leucyl-tRNA cistron therefore appears to be considerably greater than that observed for cytoplasmic rRNA (Sinclair and Brown, 1971; Bendich and McCarthy, 1970), 5S RNA (Brown and Sugimoto, 1973), or hemoglobin mRNA (Gummerson and Williamson, 1974).

Transcripts of mammalian mtDNA include mitochondrial rRNA (Rifkin et al., 1967; Aloni and Attardi, 1971; Reijnders et al., 1972), at least 12 tRNAs (Wu et al., 1972), and poly(A) containing RNA (Perlman et al., 1973; Ojala and Attardi, 1974). The latter species presumably is mRNA possibly coding for some peptides of the cytochrome oxidase, cytochrome c_1, cytochrome *b*, and oligomyocin-sensitive ATPase complexes located in the inner mitochondrial membrane. The information necessary to transcribe mitochondrial rRNA and tRNA would utilize about 25% of the DNA sequences present in one 5-μ mtDNA strand (Aloni and Attardi, 1971). It has been suggested that a large proportion of the remaining sequences may represent spacer DNA. If this were so, rapidly evolving sequence divergence would be expected as is the case for spacer sequences in the rDNA of closely related species (Brown et al., 1972).

In this paper, we attempt to compare the sequence divergence of the mtDNA of several organisms with that observed for the leucyl-tRNA cistron. We used DNA–DNA hybridization in solution, with separation of single-stranded and duplex molecules by hydroxylapatite chromatography. The procedure is similar to that described by Britten and Kohne (1966, 1968) for examining sequence homology between repeated and unique sequences in chromosomal DNA. We conclude that the sequence divergence of total mtDNA may be greater than that for the leucyl-tRNA cistron. Approximately 70% hybridization was obtained between rat and mouse mtDNA, however, suggesting that a large fraction of the sequences is conserved, and that these sequences thus probably do not represent rapidly evolving spacer regions.

Methods

Preparation of Closed Circular mtDNA. Mitochondria were prepared from livers of decapitated adult female rats, guinea pigs, mice, and chickens, and from the liver of an anesthetized rhesus monkey as previously described (Jakovcic et al., 1971). Closed circular DNAs were then isolated

† From the Departments of Medicine and Biochemistry, University of Chicago, and The Franklin McLean Memorial Research Hospital,§ Chicago, Illinois 60637. *Received December 18, 1974.* This study was supported in part by Grants HL04442, HL09172, and HL05673 from the National Institutes of Health, U.S. Public Health Service, and the Louis Block Fund of the University of Chicago.

‡ Present address: Department of Chemistry, California Institute of Technology, Pasadena, California.

§ Operated by the University of Chicago for the U.S. Atomic Energy Commission.

according to the procedure of Hudson et al. (1969), as described in the preceding paper (Jakovcic et al., 1975).

Shearing and Concentration of Unlabeled mtDNAs. Mitochondrial DNA samples in 0.1 *M* sodium phosphate (pH 6.8)–1 m*M* EDTA were sheared at 50,000 psi in a Sorval Ribi cell fractionator at 7–10°. The average molecular size of the DNA fragments was 5.4 S, as determined by alkaline band sedimentation analysis according to Studier (1965), corresponding to a fragment size of about 400 nucleotides. After shearing, the DNA was applied to a 1 cm × 10 cm column containing 2 g of hydroxylapatite equilibrated in 0.05 *M* sodium phosphate (pH 6.8). The column was washed with 0.14 *M* sodium phosphate buffer (pH 6.8). The sheared DNA was then eluted with 0.4 *M* sodium phosphate (pH 6.8) and dialyzed extensively against 0.1 *M* sodium phosphate (pH 6.8).

Labeling of mtDNAs. In vitro labeling of mtDNAs was based on the nick translation with DNA polymerase I (Kelly et al., 1970) and was performed according to the procedure of Monoyama and Pagano (1973). The circular mtDNAs were first dialyzed in buffer containing 0.07 *M* KPO_4 (pH 7.4), 0.007 *M* $MgCl_2$, and 0.001 *M* mercaptoethanol, and were nicked randomly by the action of pancreatic DNase (0.02 g/g of DNA) at 37° for 15 min. Nick translation was then accomplished by in vitro incorporation of either ^3H- or ^{32}P-labeled thymidine triphosphate in the presence of equimolar ratios of nonradioactive nucleotides by the action of *E. coli* DNA polymerase I (gift from Dr. N. Cozzarelli) for 3 hr at 18°. The labeled DNA was separated from the radioactive nucleotides by Sephadex G-50 chromatography. The fractions containing the trichloroacetic acid precipitable counts were pooled, and the DNA solution was extracted with phenol. The specific activity of the DNA obtained varied from 3 to 5 × 10^6 cpm/μg of DNA. Each DNA corresponded to a molecular weight of 2 to 3 × 10^5 as determined by alkaline sucrose gradient analysis (Studier, 1965) and was therefore not subjected to shearing. The labeled DNA was dialyzed in 0.1 *M* sodium phosphate (pH 6.8) and applied to a hydroxylapatite column (1 cm × 10 cm) containing 2 g of hydroxylapatite equilibrated with 0.1 *M* sodium phosphate (pH 6.8). The free nucleotides in the sample were eluted with 0.14 *M* sodium phosphate, and the DNA was eluted with 0.4 *M* sodium phosphate (pH 6.8). The DNA was then dialyzed against 0.1 *M* sodium phosphate (pH 6.8), denatured at 110° for 5 min, and applied to a jacketed hydroxylapatite column (1 cm × 10 cm) at 50° containing 2 g of hydroxylapatite equilibrated with 0.14 *M* sodium phosphate (pH 6.8). The DNA eluted with 0.14 *M* sodium phosphate (pH 6.8) was dialyzed against 0.1 *M* sodium phosphate (pH 6.8) and used in renaturation studies. Duplex DNA formed at the "zero" time of the renaturation reaction, representing up to 15% of renaturated DNA, was removed by this procedure.

DNA Renaturation Kinetics. The procedure is based on that described by Britten and Kohne (1968), Hoyer and van de Velde (1971), and Kieff et al. (1972). A 2500-fold excess of unlabeled mtDNA was mixed with a small amount of homologous [^{32}P]mtDNA and heterologous [^3H]mtDNA. 50-μl aliquots in 0.1 *M* phosphate buffer (pH 6.8) were sealed in capillary tubes. The samples were heat-denatured at 110° for 5 min, cooled to 50° (T_m −35°), and placed in a constant-temperature bath at 50°. Sealed samples were removed after various time intervals and were quickly frozen at −20°. Single-stranded DNA was then separated from the duplex DNA by hydroxylapatite chromatography. Multiple samples were processed, with Pasteur pipets containing 0.4 ml of hydroxylapatite used as columns mounted on scintillation vials in the oven at 50°. Single-stranded DNA was eluted with 4 ml of 0.14 *M* sodium phosphate buffer (pH 6.8) at 50°. The double-stranded DNA was then eluted with 2 ml of 0.4 *M* sodium phosphate buffer (pH 6.8). Samples were mixed with 8 ml of instagel (Packard Instrument Co., Downers Grove, Illinois), and radioactivity was determined in a Packard scintillation counter. C_0t curves were plotted according to the method of Britten and Kohne (1968).

Hydroxylapatite Thermal Elution Chromatography. After hybridization of larger amounts (final volume 1 ml) of [^3H]mtDNA with [^{32}P]rat mtDNA, the samples containing renatured ^{32}P-labeled homohybrid and ^3H-labeled heterohybrid were applied to a jacketed column (at 40°) containing 4 g of hydroxylapatite equilibrated in 0.14 *M* sodium phosphate (pH 6.8). Buffer containing 0.14 *M* sodium phosphate (pH 6.8) and 8 *M* urea, which decreases the t_{e50} by about 10°, was then pumped through the column at 2.5 ml/min, while the column temperature was raised 30°/hr with a Haake F425 circulator coupled to a Haake PG11 linear temperature programmer. Column fractions of 5 ml were mixed with 8 ml of instagel and assayed for radioactivity. All solutions used for chromatography were extensively deaerated prior to use.

Optical Denaturation Analysis of Native mtDNAs. Native mtDNAs in 0.1 *M* sodium phosphate buffer were placed in a Gilford spectrophotometer Model 2400 at a temperature of 50°. The A_{260nm} was measured every 2 min as the temperature was raised at a rate of 12°/hr with a Haake PG11 linear temperature programmer coupled to a Haake temperature circulator as described previously (Casey et al., 1974).

Results

Optical Melting Curves of mtDNA. The buoyant densities and base composition of the mtDNAs studied are very similar (GC between 40 and 43%), except for yeast and chicken with G-C contents of 18 and 49%, respectively (Borst and Kroon, 1969). Thermal denaturation analysis of yeast mtDNA monitored optically, or by thermal elution hydroxylapatite chromatography of sheared DNA, showed a remarkably heterogeneous distribution of base composition (Bernardi et al., 1970; Casey et al., 1974). AT rich clusters are present in DNA segments smaller than 400–500 nucleotides (Bernardi et al., 1972; Casey et al., 1974). It was of interest to determine whether a heterogeneous distribution of bases also occurs in mtDNAs of higher organisms.

Thermal denaturation analyses of rat, mouse, guinea pig, and monkey mtDNAs were monitored by measurement of hyperchromicity at 260 nm. Cumulative and differential melting curves are shown in Figure 1A–D. The melting curves of T4 DNA are also shown for comparison. The mtDNAs of each of the higher eukaryotes studied melt over a wider range of temperatures than does T4 DNA, which has a sharp melting curve DNA. In this respect, their denaturation properties resemble those of yeast mtDNA, although the heterogeneous distribution of bases is not as marked, and there is no evidence for a large proportion of high AT segments as are present in yeast mtDNA.

Thermal elution hydroxylapatite chromatography of native rat [^3H]mtDNA (Figure 2) shows that DNA fragments sheared to 400–500 nucleotide pairs also display a

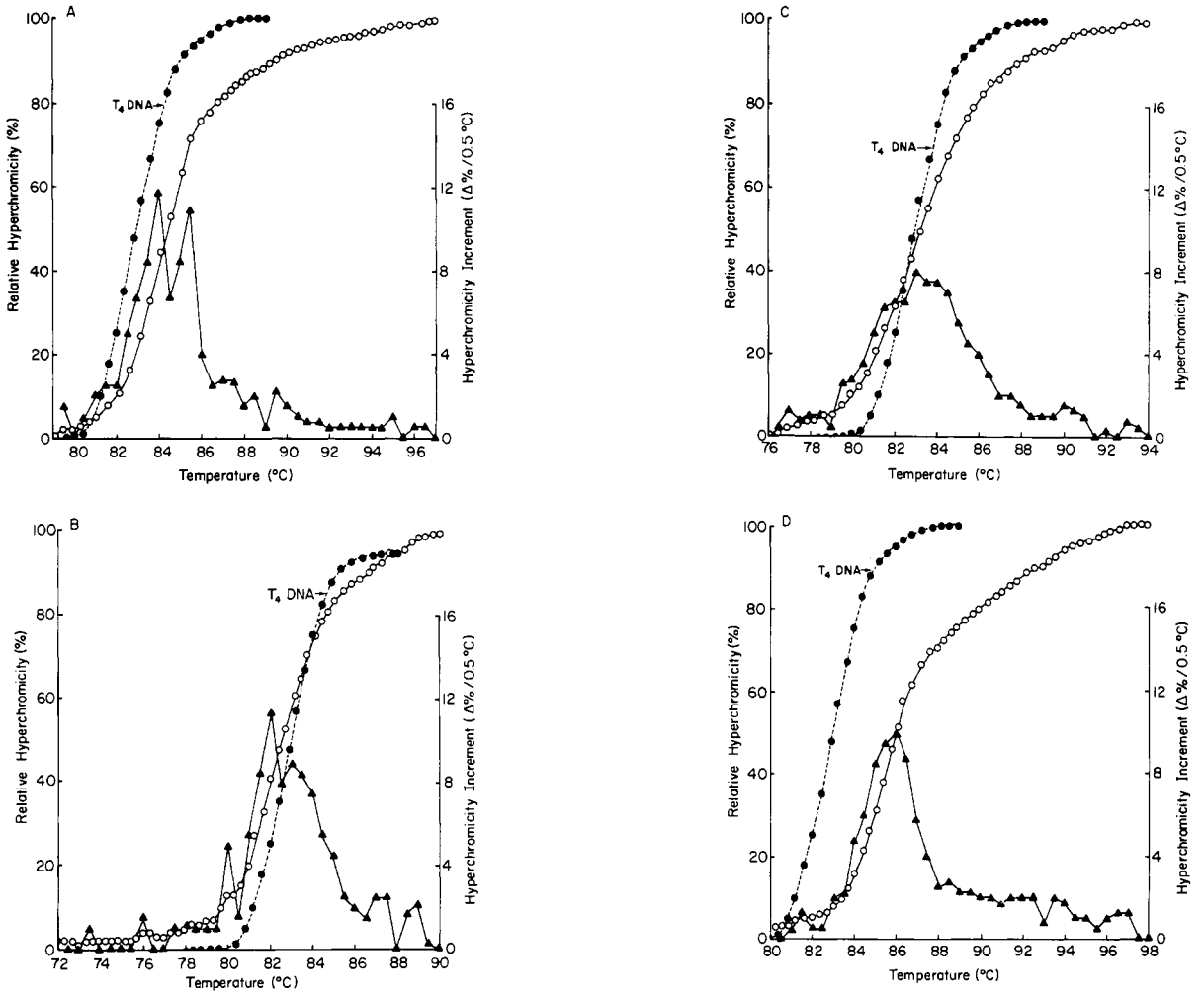

FIGURE 1: Optical melting profiles of mammalian mtDNAs; cumulative (○) and first derivative (▲) plots. Cumulative (●) plots for T4 DNA are also shown for comparison. (A) Rat mtDNA; (B) mouse mtDNA; (C) guinea pig mtDNA; (D) rhesus monkey mtDNA. DNA samples (25–40 μg/ml) were melted in 0.1 *M* phosphate buffer as described under Methods. Hyperchromicities were normalized to 100% and plotted as a function of temperature.

broad and heterogeneous melting curve which contrasts with the sharper curves for *E. coli* DNA and poly[d(A-T)].

Hybridization of [^{3}H]mtDNA with Excess Rat [^{32}P]mtDNA. mtDNA was labeled with [^{32}P]- or [^{3}H]TTP in vitro using *E. coli* DNA polymerase I after random nicking with pancreatic DNase as described under Methods. Incubation was at 18° to minimize branch formation (Dumas et al., 1971). The labeled mtDNA contained 5–15% "zero time renaturing" DNA, i.e., denatured DNA that behaved as a duplex structure on hydroxylapatite chromatography. This DNA probably represents self-annealing structures that were formed during the DNA polymerase reaction, as well as structures present in the original mtDNA. Prior to hybridization, "zero time renaturing" DNA was removed from all labeled mtDNA samples by hydroxylapatite chromatography as described under Methods. Denatured DNA eluting at 0.14 *M* phosphate buffer, and thus behaving as single-stranded DNA, was used in the hybridization experiments.

The hybridization of low concentrations of mouse [^{3}H]mtDNA in the presence of a 2500-fold excess of rat [^{32}P]mtDNA is shown in Figure 3. Hybridizations were carried out in 0.1 *M* PO_4 buffer at approximately 35° below the T_m of the homoduplex. The data are presented as C_0t plots according to the procedure introduced by Britten

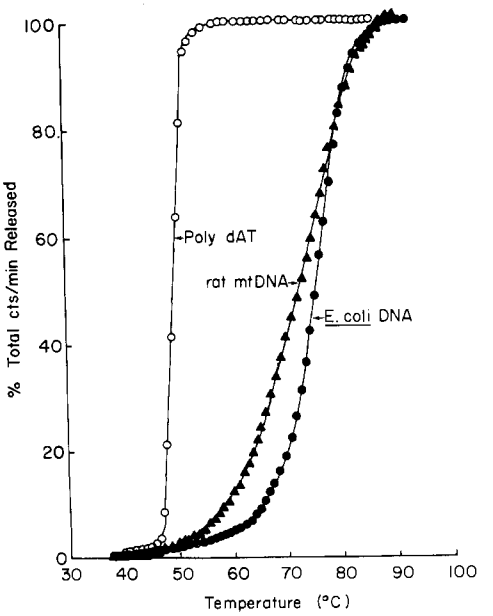

FIGURE 2: Cumulative melting profiles of rat [^{3}H]mtDNA obtained by thermal elution chromatography on hydroxylapatite columns. Rat [^{3}H]mtDNA (3×10^4 cpm; specific activity, 3.5×10^6 cpm/μg) was eluted from a hydroxylapatite column as a function of temperature, as described under Methods.

Table I: Heteroduplex Formation with Different Concentrations of Driving Rat mtDNA.[a]

	Concn of Rat mtDNA (μg/ml)				
	5.5 (C_0t = 1.0)[b]	11.0 (C_0t = 2.0)	16.5 (C_0t = 3.0)	22.0 (C_0t = 4.0)	33.0 (C_0t = 6.0)
	Heteroduplex Formation (%)				
Mouse [^{3}H]mtDNA (0.005 μg/ml)	58.5	59.7	61.8	60.4	60.1

[a] Data represent averages of two to four separate determinations. Conditions of hybridization are as described under Methods. [b] C_0t, (mol sec)/l.

Table II: Effect of a Second Renaturation with Excess Rat mtDNA on the Extent of Guinea Pig–Rat mtDNA Heteroduplex Formation.[a]

1st Renaturation (17 hr)			2nd Renaturation (8 hr)	
DNA in Low Concn (0.005 μg/ml)	DNA in High Concn (11.0 μg/ml)	% Duplex	DNA added (11.0 μg/ml)	% Duplex
[^{3}H] Guinea pig	[^{32}P] Rat	59	Rat	61
[^{3}H] Guinea pig	[^{32}P] Rat	59	Guinea pig	95
[^{3}H] Guinea pig	Guinea pig	94	Guinea pig	96
[^{32}P] Rat	Rat	90	Rat	96
[^{3}H] Guinea pig	None	6	None	9

[a] Renaturation reactions were carried out in duplicate in capillary pipets as described under Methods. At the end of the first renaturation period, appropriate samples were analyzed for duplex formation, and additional DNA was added to the others as indicated. The latter samples were then incubated in capillary pipets for an additional 8 hr. Duplicate determinations differed by no more than 2%.

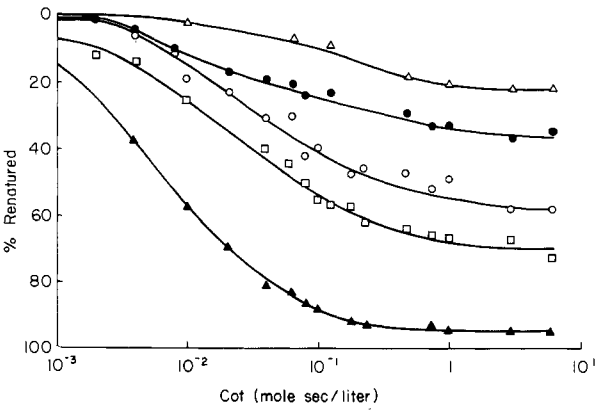

FIGURE 3: Renaturation of [^{3}H]mtDNA from different organisms in the presence of a 2500-fold excess of rat [^{32}P]mtDNA. Approximately 0.005 μg/ml (2 × 10^4 cpm) of rat (▲), mouse (□), guinea pig (○), monkey (●), or chicken (△) [^{3}H]mtDNA was renatured with 12.5 μg/ml of rat [^{32}P]mtDNA (2 × 10^4 cpm) in 0.1 *M* phosphate buffer (pH 6.8) at 50° (T_m −35°). At different times, 50-μl aliquots in sealed capillary tubes were removed, and the percent duplex DNA was analyzed on hydroxylapatite columns as described under Methods. In some cases DNA concentrations were decreased or increased, but the concentration rates of [^{3}H]DNA–driving DNA were kept constant. The data are presented as C_0t plots (Britten and Kohne, 1968).

and Kohne (1966, 1968). The concentration of mouse [^{3}H]mtDNA was kept sufficiently low so that less than 6% duplex DNA was obtained in control incubations containing no driving rat mtDNA. Thus, duplex mouse [^{3}H]mtDNA formed during the reaction predominantly represents heteroduplexes with homologous sequences in the rat mtDNA.

Rat mtDNA, followed by the measurement of duplex [^{32}P]DNA, renatured as unique species to greater than 95% of completion at C_0t values expected for a complexity of 1500 base pairs. About 70% of the mouse [^{3}H]mtDNA also became double-stranded at the end of the renaturation reaction. Thus, approximately 70% of DNA sequences in mouse mtDNA retain sufficient homology to hybridize with rat mtDNA.

Similar data obtained with guinea pig, monkey, and chicken [^{3}H]mtDNA annealed with a 2500-fold excess of rat mtDNA are also presented in Figure 3. When the renaturation of rat mtDNA is complete, approximately 59% guinea pig, 37% monkey, and 22% chicken [^{3}H]mtDNA have formed heteroduplexes with rat mtDNA sequences. Control renaturations (in the absence of rat mtDNA) for guinea pig, monkey, and chicken mtDNA were 3, 2, and 6%, respectively.

As will be illustrated in the next section, the heteroduplexes contain considerable mismatched sequences. Considerable base mismatching may result in a substantial decrease in the rate of formation of heteroduplexes (Sutton and McCallum, 1971; McCarthy and Farquhar, 1972; Bonner et al., 1973), and could lead to a depletion of rat mtDNA sequences homologous to [^{3}H]mtDNA of other species before heteroduplex formation is complete (McCarthy and Farquhar, 1972). This possibility was tested in the following manner.

First, we evaluated the effect of varying the concentration of driving DNA on heteroduplex formation. As seen in Table I, a sixfold difference in the ratio of the concentration of driving DNA to [^{3}H]mtDNA (ratios 1250–7500) results in no significant increases in the percent of heteroduplex formation. Thus the concentration of driving DNA used in most experiments (i.e., 1:2500) appears to be sufficient.

With these hybridization conditions, guinea pig–rat heteroduplex formation was not detectably enhanced (within the limits of the technique) by a second addition of driving rat mtDNA after the first homoduplex reaction had proceeded to completion (Table II). The unreacted single-stranded guinea pig [^{3}H]mtDNA was still capable of forming stable homohybrids since the addition of driving guinea pig mtDNA after the initial reaction between guinea pig and rat [^{3}H]mtDNA resulted in 93% duplex formation.

It therefore appears that few, if any, homologous sequences remain single stranded after renaturation with a 2500-fold excess of driving DNA. Thus the quantitative data obtained under these conditions of renaturation should provide a reasonable estimate of the degree of sequence homology.

Hybridization of [^{3}H]mtDNAs with Excess Mouse, Guinea Pig, Monkey, and Chicken mtDNAs. Hybridizations were also carried out with low concentrations of [^{3}H]mtDNAs and a 2500-fold excess of mouse, guinea pig, monkey, and chicken mtDNAs; the results are presented in

Table III: Heteroduplex Formation between Different Mitochondrial DNAs.[a]

DNA in Low Concn (0.005 μg/ml) (C_0t = 9.0 × 10^{-4} (mol sec)/l.)	% Duplex Formation			
	Driving DNAs (C_0t = 2.2 (mol sec)/l.)			
	Rat	Mouse	Guinea Pig	Monkey
Rat	96	74	52	35
Mouse	70	93	44	52
Guinea pig	59		97	49
Monkey	37		30	94
Chicken	22		16	36

[a] The concentration of driving DNAs was 12 μg/ml. Results are derived from complete C_0t curves, or from duplicate determinations at the C_0t incubated indicated.

Table III. The data obtained with excess rat mtDNA are also summarized for comparison. In almost all cases, good agreement is obtained in the reciprocal hybridizations; i.e., there was similar heteroduplex formation in reactions where one or the other of a pair of mtDNAs was present in excess.

We also tried to evaluate whether the same sequences were being conserved in different mtDNAs. In several experiments we compared heteroduplex formation in the presence of 1, 2, or 4 different mtDNAs in excess (Table IV). The extent of heteroduplex formations was not additive when two or more driving mtDNAs were used. Instead, the level of hybridization was similar (with mouse [^{3}H]mtDNA) or only slightly higher (with monkey and chicken [^{3}H]mtDNA) than obtained with a single driving DNA. Therefore it appears that, to some extent, similar sequences are preferentially conserved in the various mtDNAs.

Thermal Elution Chromatography of mtDNA Heterohybrids. To evaluate the degree of base sequence mismatching present in the mtDNA heterohybrids, we compared the melting curves of the heterohybrid with the curves for the rat mtDNA isohybrid. We used thermal elution chromatography on hydroxylapatite columns in the presence of 6 *M* urea, which lowers the t_{e50} by 10–12°. Melting curves for the rat isohybrids were highly reproducible, giving a t_{e50} of 72.2° ± 0.3 S.D. (Figure 4). Melting curves of the heterohybrids of rat mtDNA with mouse, guinea pig, monkey, and chicken mtDNA (Figure 4) showed a depression of the t_{e50} of 18–24°. The heterohybrid melting curves did not parallel the curves of the rat mtDNA homohybrids. Rather, the depression of the melting temperature was greater (as much as 30°) at lower melting temperatures, and considerably less (as little as 5°) at high melting temperatures. This effect is clearly seen in the differential melting curves of the heterohybrids (Figure 5). This pattern was particularly evident in the case of the rat–chicken mtDNA heterohybrid, and probably indicates different degrees of sequence divergence for different segments of the DNA. The extent of depression of melting temperatures appeared to be similar for the different heterohybrids.

We conclude that those sequences which retain sufficient homology with rat mtDNA to form heteroduplexes under our hybridization conditions still show a substantial degree of sequence mismatching.

Discussion

In this study we have estimated sequence homology among the mtDNAs of several mammalian and one avian species. Tritiated mtDNAs, labeled in vitro with DNA polymerase I, were annealed in the presence of a large excess of mtDNA of another species. Homologous sequences formed heteroduplexes which were separated from single-stranded DNA by hydroxylapatite chromatography. The thermal stability of the heterohybrids was analyzed by thermal elution chromatography on hydroxyalpatite columns, to provide a measure of the extent of sequence mismatching in the heterohybrids.

Table IV: Heteroduplex Formation in the Presence of One or Several Different Driving DNAs.

[^{3}H]DNA in Low Concn (0.005 μg/ml)	DNA in High Concn		
	Rat	Guinea Pig	Rat + Guinea Pig
	11 μg/ml (C_0t = 2.0 (mol sec)/l.)	11 μg/ml (C_0t = 2.0 (mol sec)/l.)	11 μg/ml for each DNA
	Heteroduplex (%)	Heteroduplex (%)	Heteroduplex (%)
Mouse	70	44	72
Monkey	37	29	43 (47[a])
Chicken	22	16	30

[a] Heteroduplex of 47% formed when rat, mouse, guinea pig, and chicken DNAs were present together in concentrations of 11 μg/ml for each DNA.

Table V: Hybridization of Rat Mitochondrial Leucyl-tRNA and Rat mtDNA with mtDNAs of Various Species.

mtDNAs	Rat Mitochondrial Leucyl-tRNA		Rat mtDNA (2500-fold excess)		
	T_m(°C) on 20% Formamide	ΔT_m(°C)	% Duplex Formation	t_{e50}(°C) in 8 *M* urea	Δt_{50}(°C)
Rat	54		95	72.5	
Mouse	51	3	70	54.0	18.5
Guinea pig	48	6	52	49.0	23.5
Monkey	39	15	33	54.6	17.9
Chicken		>25	19	54.8	17.7

As summarized in Table V, from 70 to 20% of the sequences in mouse, guinea pig, monkey, and chicken mtDNAs formed heteroduplexes with rat mtDNA. As can be seen from the C_0t curves (Figure 3), heteroduplex formation reached a maximum when rat homoduplex formation was complete, at which time single-stranded rat mtDNA sequences were removed from the reaction. In all cases heteroduplexes showed a 18–24° depression of the thermal elution profiles, indicating substantial sequence mispairing in the heteroduplexes. Because the rate of reassociation of mispaired sequences is considerably slower than for perfectly matched strands (Sutton and McCallum, 1973; McCarthy and Farquhar, 1972; Bonner et al., 1973), the magnitude of sequence homology may be underestimated if heteroduplex formation is incomplete when rat mtDNA homoduplex formation is complete. Depression of the rate of heteroduplex formation apparently had little effect on our results, since renaturation for a second time with large amounts of driving DNA led to no detectable additional hybridization.

The sequence divergence of mtDNA as a whole appears to be greater than the divergence observed for the mitochondrial leucyl-tRNA cistron. The t_{e50}'s of the mtDNA

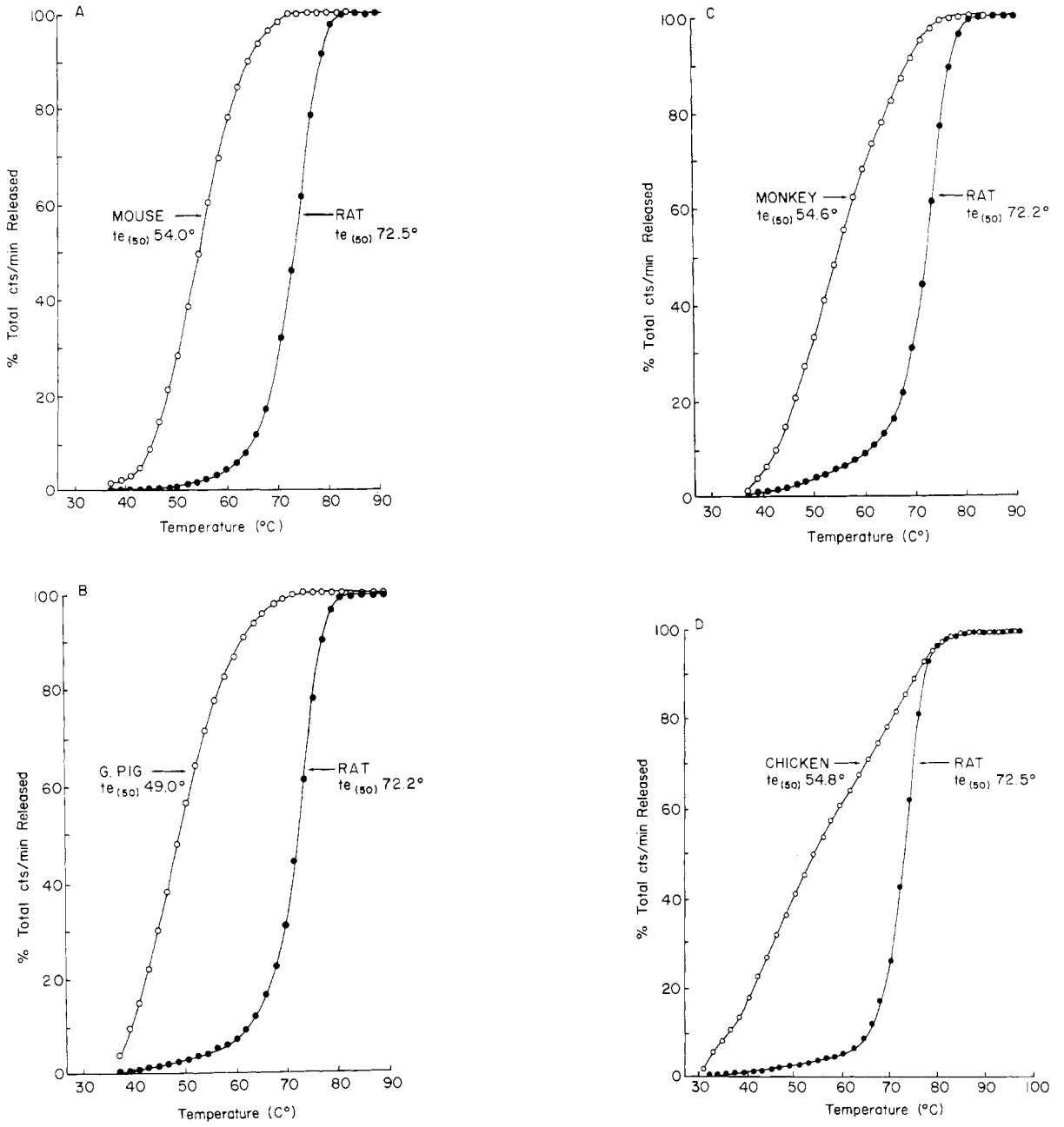

FIGURE 4: Thermal elution chromatography of [^{3}H]mtDNAs corenatured with about 2500-fold excess of rat [^{32}P]mtDNA. [^{3}H]mtDNAs and rat [^{32}P]mtDNA in phosphate buffer (pH 6.8) (volume 1.0 ml), were renatured to a C_0t of 2.0. The DNA concentrations and conditions were as described in the legend to Figure 3. The mixtures were applied to a hydroxylapatite column equilibrated with 0.14 *M* phosphate buffer (pH 6.8) and 8 *M* urea, and eluted as a function of temperature as described under Methods. (A) Mouse [^{3}H]mtDNA–rat [^{32}P]mtDNA; (B) guinea pig [^{3}H]mtDNA–rat [^{32}P]mtDNA; (C) monkey [^{3}H]mtDNA–rat [^{32}P]mtDNA; (D) chicken [^{3}H]mtDNA–rat [^{32}P]mtDNA.

heterohybrids were depressed about 18–24° compared to the rat–rat isohybrid. The depression varied from 5 to 30° in different parts of the melting curve. This compares with the smaller T_m depression for tRNA–mtDNA hybrids of 2–3°, 7–9°, and 15° respectively for the mouse, guinea pig, and monkey–rat heterohybrids (Jakovcic et al., 1975). Results for chicken mtDNA are more difficult to evaluate since its GC content is higher than that of rat mtDNA, and only 20% of its sequences form heteroduplexes with rat mtDNA. rRNA sequences comprise about 20% of the mitochondrial genome (Aloni and Attardi, 1971). If ribosomal sequences are relatively conserved, as has been found to be the case in *Xenopus* (Dawid, 1972), then they may account for a large fraction of chicken–rat heteroduplexes, but for a relatively smaller fraction of mouse–rat heteroduplexes. The larger proportion of sequences contributed by rDNA in the chicken–rat heteroduplexes may contribute to our apparently anomalous observation that t_{e50} depression of this heterohybrid was somewhat less than that of most of the others, although the latter retain a larger proportion of sequences homologous to the rat mtDNA.

Our results and conclusions are in several ways similar to Dawid's (1972) analysis of the divergence of the mitochondrial DNA sequences in two related amphibian species, *Xenopus leavis* and *Xenopus mulleri*. About 70% of the *Xenopus* mtDNA sequences formed heteroduplexes, comparable to our results for the rat–mouse heterohybrid. The *Xenopus* mtDNA heteroduplexes could be divided into two classes of sequences: one representing about 50% of the DNAs contained an average of 27% mismatched bases, and

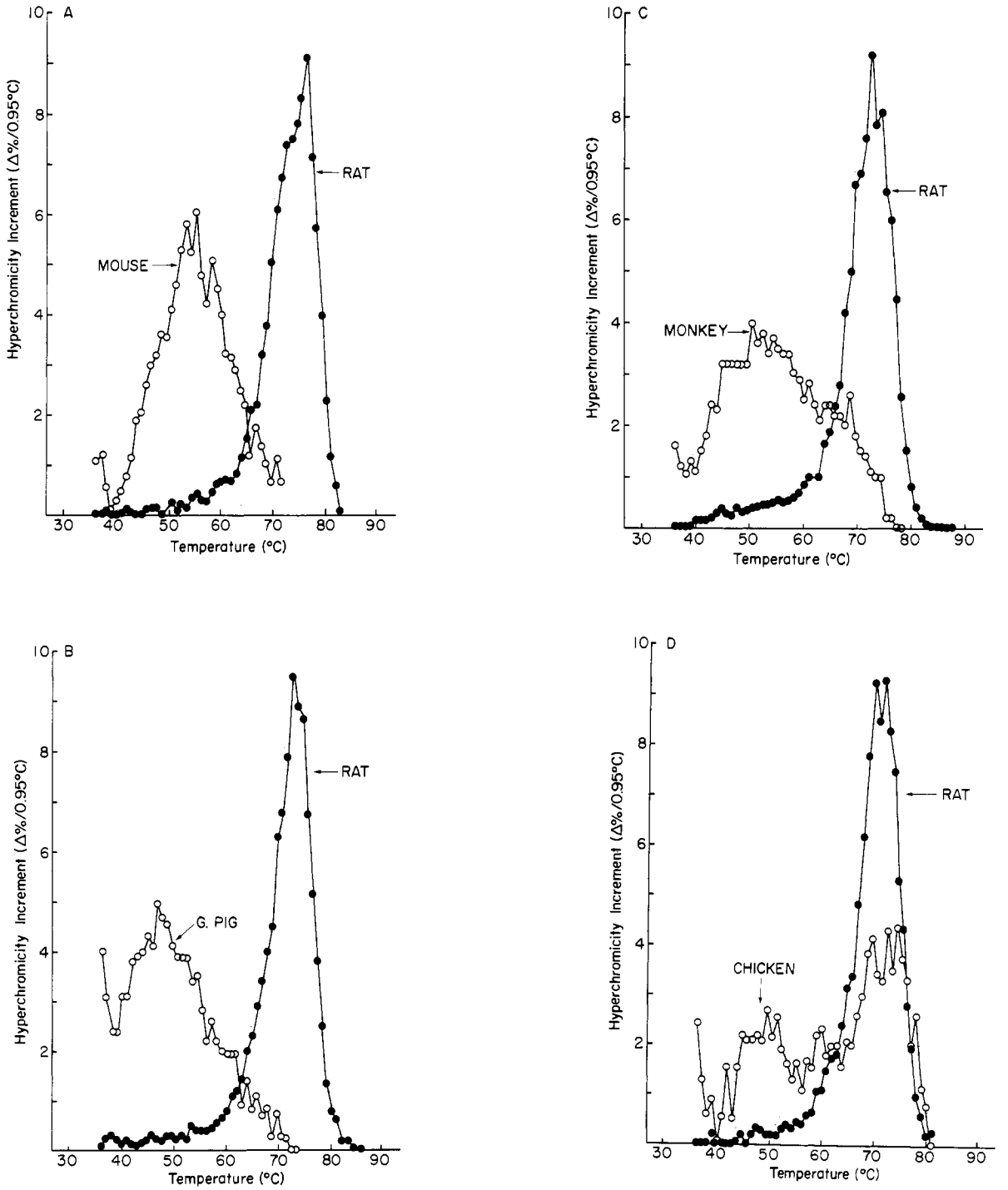

FIGURE 5: First derivative plots obtained from the data of the thermal elution chromatography of [^{3}H]mtDNAs corenatured with 2500-fold excess of rat [^{32}P]mtDNA under the conditions described in Figure 4. (A) Mouse [^{3}H]mtDNA-rat [^{32}P]mtDNA; (B) guinea pig [^{3}H]mtDNA-rat [^{32}P]mtDNA; (C) monkey [^{3}H]mtDNA-rat [^{32}P]mtDNA; (D) chicken [^{3}H]mtDNA-rat [^{32}P]mtDNA.

the second representing about 20% of the mtDNA sequences contained an average of only 6% mismatched bases. Cistrons for rRNA and tRNA accounted for most of the sequences in the DNA class that had diverged the least during evolution. We also found that the rat-mouse leucyl-tRNA cistron diverges little compared to changes in the bulk of the DNA. The magnitude of the sequence divergence that we observed in the mouse-rat heterohybrids was also similar to that in the class of *Xenopus* heteroduplex that contained the more divergent sequences. Dawid's (1972) suggestion that the class of rapidly evolving sequences might represent spacers, however, now seems unlikely in view of the recent demonstration of multiple poly(A) containing mitochondrial RNAs (Perlman et al., 1973; Ojala and Attardi, 1974) which would account for almost all the available sites on the mitochondrial genome.

From the data now available, the degree of sequence divergence noted here for mtDNA appears to be similar to that noted for unique sequence nuclear DNA in various rodents (Rice and Esposito, 1972). By a method essentially identical with that used in this study, Rice et al. noted 70% hybridization between rat and mouse nuclear DNA unique sequences, with 10-30° depression in the t_{e50}. About 30% homology was noted between hamster and rat DNA, with

about a 23° t_{e50} depression. Presumably the presence of multiple copies of the mitochondrial genome balances the much shorter generation time for mtDNA which, at least in somatic tissues, turns over rapidly with a $t_{1/2}$ of about 5–6 days (Rabinowitz and Swift, 1970). Quantitative comparison is further complicated by the fact that a much larger fraction of mtDNA than of nuclear DNA is involved in the transcription of rRNA and tRNA, and these sequences may be conserved to a relatively high degree.

Acknowledgments

We greatly appreciate the able technical assistance of Miss Rufina Nepomuceno, the generous gift of *E. coli* polymerase by Dr. Nicholas Cozzarelli, and the critical review of the manuscript by Dr. Godfrey Getz.

References

Aloni, Y., and Attardi, G. (1971), *J. Mol. Biol. 55,* 271.
Bendich, A. J., and McCarthy, B. J. (1970), *Proc. Natl. Acad. Sci. U.S.A. 65,* 349.
Bernardi, G., Fauves, M., Piperno, G., and Slonimski, P. P. (1970), *J. Mol. Biol. 48,* 23.
Bernardi, G., Piperno, G., and Fonte, G. (1972), *J. Mol. Biol. 65,* 173.
Bonner, T. I., Brenner, D. J., Neufeld, B. R., and Britten, R. J. (1973), *J. Mol. Biol. 81,* 123.
Borst, P., and Kroon, A. M. (1969), *Int. Rev. Cytol. 26,* 107.
Britten, R. J., and Kohne, D. E. (1966), *Carnegie Inst. Washington, Yearb. 65,* 78.
Britten, R. K., and Kohne, D. E. (1968), *Science 161,* 529.
Brown, D. D., and Sugimoto, K. (1973), *J. Mol. Biol. 78,* 397.
Brown, D. D., Wensink, P. C., and Jordan, E. (1972), *J. Mol. Biol. 63,* 57.
Casey, J., Gordon, P., and Rabinowitz, M. (1974), *Biochemistry 13,* 1059.
Dawid, I. B. (1972), *Dev. Biol. 29,* 139.
Dumas, L. B., Darby, G., and Sinsheimer, R. L. (1971), *Biochim. Biophys. Acta 228,* 407.
Gummerson, K. S., and Williamson, R. (1974), *Nature (London) 274,* 265.
Hoyer, B. H., and van de Velde, N. J. (1971), *Carnegie Inst. Washington, Yearb. 69,* 507.
Hudson, B., Upholt, W. B., Devinny, J., and Vinograd, J. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 813.
Jakovcic, S., Casey, J., and Rabinowitz, M. (1975), *Biochemistry,* preceding paper in this issue.
Jakovcic, S., Haddock, J., Getz, G. S., Rabinowitz, M., and Swift, H. (1971), *Biochem. J. 121,* 341.
Kelly, B. R., Cozzarelli, N. R., Deutscher, M. P., Lehman, I. R., and Kornberg, A. J. (1970), *J. Biol. Chem. 245,* 39.
Kieff, E., Hoyer, W., Bachenheimer, S., and Roizman, B. (1972), *J. Virol. 9,* 738.
Laird, C. D., McConaughy, B. L., and McCarthy, B. J. (1969), *Nature (London) 224,* 149.
McCarthy, B. J., and Farquhar, M. N. (1972), *Brookhaven Symp. Biol. 23,* 1.
Monoyama, M., and Pagano, J. S. (1973), *Nature (London) 242,* 44.
Ojala, D., and Attardi, G. (1974), *J. Mol. Biol. 82,* 151.
Perlman, S., Abelson, H., and Penman, S. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 350.
Rabinowitz, M., and Swift, H. (1970), *Physiol. Rev. 50,* 376.
Reijnders, L., Kleisen, C. M., Grivell, L. A., and Borst, P. (1972), *Biochim. Biophys. Acta 272,* 396.
Rice, N., and Esposito, P. (1972), *Carnegie Inst. Washington, Yearb. 72,* 197.
Rifkin, M. R., Wood, D. D., and Luck, D. J. L. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 1025.
Sinclair, J. H., and Brown, D. D. (1971), *Biochemistry 10,* 2761.
Studier, W. (1965), *J. Mol. Biol. 11,* 373.
Sutton, W. D., and McCallum, M. (1971), *Nature (London), New Biol. 232,* 83.
Wu, M., Davidson, N., Attardi, G , and Aloni, Y. (1972), *J. Mol. Biol. 71,* 81.