The correlation between the extent of RNA degradation and the level of MS I accumulated, though interesting, does not provide information as to the mechanism of NaCl-induced MS I accumulation. It is entirely possible that these phenomena are independent of each other. It is possible that NaCl at a high concentration causes the inhibition of uptake of amino acids and uracil which are required by the strains used in the present experiment. The resulting uracil starvation might then trigger RNA degradation (Lazzarini *et al.*, 1969). However, it is known that neither amino acid starvation nor uracil starvation causes the accumulation of MS I in relaxed strains (Cashel and Gallent, 1969; our unpublished data). The very transient nature of the NaCl-induced MS I accumulation and the concomitant degradation or cessation of RNA accumulation may be related to the rapid plasmolysis and deplasmolysis which occur in *E. coli* cells when placed in hypertonic NaCl solutions.

References

Bollum, F. J. (1968), *Methods Enzymol. 12 B*, 169.
Cashel, M. (1969), *J. Biol. Chem. 244*, 3133.
Cashel, M., and Gallant, J. (1969), *Nature (London) 221*, 838.
Cashel, M., and Kalbacher, B. (1970), *J. Biol. Chem. 245*, 2309.
Cashel, M., Lazzarini, R. A., and Kalbacher, B. (1969), *J. Chromatogr. Sci. 40*, 103.
Edlin, G., and Donini, P. (1971), *J. Biol. Chem. 246*, 4371.
Erlich, H., Laffler, T., and Gallant, J. (1971), *J. Biol. Chem. 246*, 6121.
Ezekiel, D. H. (1965), *Biochim. Biophys. Acta 95*, 54.
Harshman, R. B., and Yamazaki, H. (1971), *Biochemistry 10*, 3980.
Lazzarini, R. A., Cashel, M., and Gallant, J. (1971), *J. Biol. Chem. 246*, 4381.
Lazzarini, R. A., and Dahlberg, A. E. (1971), *J. Biol. Chem. 246*, 420.
Lazzarini, R. A., Nakata, K., and Winslow, R. M. (1969), *J. Biol. Chem. 244*, 3092.
Paranchych, W. (1966), *Virology 28*, 90.
Watson, R., and Yamazaki, H. (1972), *Biochemistry 11*, 611.

Nearest-Neighbor Frequencies of Mitochondrial Deoxyribonucleic Acid in Mouse Liver*

O. Antonoglou and J. G. Georgatsos

ABSTRACT: Nearest-neighbor frequencies of mouse liver mitochondrial DNA have been determined and compared to the dinucleotide frequencies of nuclear DNA from the same tissue. The results show that the distribution of dinucleotides in mitochondrial DNA is decidedly less random than in any other double-stranded DNA species studied to date.

There are three methods available at present for gaining an insight into the primary structure of a DNA molecule: the determination of isostichs as worked out in Chargaff's laboratory over the past 20 years (for review, see Chargaff, 1968), the analysis for nearest-neighbor frequencies introduced by Josse *et al.* (1961), and recently the application of the fingerprint method by Murray (1970) to DNA hydrolysis products following enzymic digestion. Even though the date of sequencing a DNA molecule, that Chargaff (1968) places in the 21st century, has hardly been brought any closer by these methods, they are still valuable tools for comparing either DNA preparations from different sources, or the same DNA molecules prior to and following the effect of various agents or processes on the genome. Furthermore, the smaller the size of the DNA molecule the less utopian a study of this nature is. mtDNA[1] seems to be one of the smallest functional DNA molecules known in living organisms. Its size as well as the possibility of being studied under identical conditions as its nuclear counterpart render mtDNA a comparatively attractive molecule for sequence studies. Furthermore, such studies might even contribute to answering the question of the phylogenetic origin of mitochondria as Cummins *et al.* (1967) have suggested.

In the present paper data are presented for the nearest-neighbor frequencies of all possible dinucleotides in mt- and nDNA of mouse liver.

Materials and Methods

Preparation of Nuclei and Mitochondria. Nuclei and mitochondria were isolated as previously described (Georgatsos *et al.*, 1970) from the livers of ether anesthetized mice of the pure inbred strain C3HAvy/HeSy. All livers were examined histologically by Dr. T. Sirmakesian of this Institute. The authors are aware that the trustworthiness of the results depends mainly on the total absence of nDNA in the preparations of mtDNA. Consequently extreme care was taken in the preparation of the mitochondrial fractions which were further processed only when the total absence of nuclei was

* From the Laboratory of Biochemistry, Theagenion Cancer Institute, Thessaloniki, Greece. *Received September 2, 1971.* This investigation has been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

[1] Abbreviations used are: mtDNA and nDNA, mitochondrial and nuclear DNA, respectively.

established by staining aliquots of the mitochondrial preparations by the standard Papanicolaou technique and scanning the slides microscopically.

Preparation of DNA. DNA was extracted from nuclei and mitochondria by a detergent phenol method essentially according to the method of Chatterjee *et al.* (1966), except that the medium where the subcellular particles were suspended was rendered 5×10^{-3} M with respect to EDTA. The DNA preparations thus obtained were dissolved in 1 ml of a solution of 0.015 M NaCl containing sodium citrate at a concentration of 0.0015 M and incubated first with 50 μg of crystalline pancreatic RNase for 30 min at 37° and then with 50 μg of Pronase for 1 hr. The mixtures were then treated with sodium lauryl sulfate and phenol and the DNAs were precipitated with alcohol according to the mentioned-above method. nDNA prepared in this manner was used directly as template in the RNA polymerase reaction. The mtDNA, however, before being used as template, was nicked by passage through a 25-gauge needle ten times, followed by heating at 100° for 10 min, and rapid cooling to 0°.

Enzymes and Substrates. DNA-dependent RNA polymerase was prepared by the method of Weiss (1968) from dried cells of *Micrococcus lysodeicticus*, purchased from Sigma, St. Louis, Mo. Crystalline pancreatic RNase from Mann Research Laboratories was heated for 5 min at 80° at a concentration of 1 mg/ml, prior to use. Pronase ex *Streptococcus griseus* was obtained from Koch-Light Laboratories, England, and heated to 80° at pH 5.0 as described by Stern (1968), before use. Ribonucleoside triphosphates were purchased from P-L Biochemicals. Crystalline bovine serum albumin was obtained from B.D.H., England. Carrier-free orthophosphate-^{32}P was purchased from C.E.A., France. α-^{32}P-labeled nucleoside triphosphates (approximate specific radioactivity 1 mCi/μmole) were prepared according to the method of Symons (1970a,b).

Transcription of DNA. The assay system of Weiss (1968) was employed for the transcription of DNA samples on ^{32}P-labeled RNA molecules. Five units of the enzyme was incubated with 100 μg of nDNA or 20 μg of mitochondrial DNA under optimal conditions. Under these conditions, approximately 50 μg of RNA was made from the nDNA template, while 20–30 μg of RNA was produced using mtDNA. Each DNA sample was employed four times as a template so that all four α-^{32}P-labeled nucleoside triphosphates might be employed in turn as the labeling precursors. The reactions were allowed to proceed for 1.5 hr in a final volume of 0.5 ml. The trichloroacetic acid precipitable material, instead of being filtered through a membrane filter according to the method employed (Weiss, 1968), was washed four times with 5% trichloroacetic acid, dissolved in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5), reprecipitated with 10% trichloroacetic acid, and washed twice with 5% trichloroacetic acid. This final step was found necessary to remove all traces of radioactive triphosphate nonspecifically adsorbed onto the precipitates.

Alkaline Hydrolysis of ^{32}P-Labeled RNA Products and Separation of Mononucleotides. The trichloroacetic acid washed pellets containing the labeled RNA products were dissolved in 0.3 ml of 0.3 M KOH and incubated at 37° for 16 hr. The solutions were neutralized to a congo red end point at pH 3.0 by means of 1 M $HClO_4$ and centrifuged at 8000g for 5 min. The supernatants were applied on Whatman No. 3-MM strips of paper along with markers of 0.4 μmole each of the four ribomononucleotides. The papers were subjected to electrophoresis in 0.05 M sodium citrate solution (pH 3.5), according to Markham and Smith (1952). The mononucleotide spots were eluted with 1.5 ml of H_2O and the radioactivity of the eluates was measured in a Tri-Carb liquid scintillation spectrometer. Electrophoresis of products was at first performed in duplicate. When, however, it was established that values were practically the same in both duplicate runs, this practice was abandoned.

TABLE I: Nearest-Neighbor Frequencies in RNAs Synthesized on Mouse Liver nDNA Templates, Expressed in Decimal Proportions of 1.

	Up	Ap	Cp	Gp	Sum
pA	0.055	0.101	0.075	0.066	0.297
pU	0.090	0.070	0.070	0.049	0.279
pG	0.073	0.075	0.011	0.055	0.214
pC	0.060	0.053	0.054	0.043	0.210
Sum	0.278	0.299	0.210	0.213	1.000
Sums	0.577		0.423		

A + U/G + C = 1.36

Chemical Estimation of Bases in DNA. The percent composition of bases in nDNA was determined by perchloric acid hydrolysis of the samples followed by separation of the bases by paper chromatography in a solvent composed of isopropyl alcohol–concentrated HCl–H_2O (170:41:39, v/v) as suggested by Wyatt (1951). Three separate attempts, with ten animals each, to determine the bases in mtDNA gave nonreproducible results, mainly because the amounts of bases isolated were extremely small and consequently significant errors were introduced in the absorbancy readings. Values for the mole per cent of guanine plus cytosine (% G + C) can be inferred from density determinations in CsCl. Thus, in a review written by Borst and Kroon (1969) a density of 1.701 g/cm^3 is listed for mouse liver mtDNA that corresponds to 42% G + C. However, the accuracy of this value is highly questionable since in the same review the per cent G + C of mouse L cells mtDNA is estimated to be 41 from direct analysis, 39 from density, and 38 from T_m.

Results

The frequencies of 16 dinucleotides of RNA synthesized on template of nuclear and mtDNA of mouse liver are presented in Table I and II, respectively. Table III shows the

TABLE II: Nearest-Neighbor Frequencies in RNAs Synthesized on Mouse Liver mtDNA Templates, Expressed in Decimal Proportions of 1.

	Up	Ap	Cp	Gp	Sum
pA	0.056	0.156	0.052	0.051	0.315
pU	0.169	0.065	0.050	0.039	0.323
pG	0.052	0.053	0.028	0.048	0.181
pC	0.047	0.040	0.052	0.042	0.181
Sum	0.324	0.314	0.182	0.180	1.000
Sums	0.638		0.362		

A + U/G + C = 1.77

TABLE III: Base Composition of nDNA Determined by Chemical Analysis and of RNA Product Synthesized on n- and mtDNA Templates and Estimated by the Nearest-Neighbor Technique.

Base	nDNA	RNA Synthesized on nDNA Template	RNA Synthesized on mtDNA Template
A	28.8	29.8	31.5
T(U)	29.2	27.8	32.4
G	20.7	21.0	18.1
C	21.3	21.4	18.0
A + T(U)/G + C	1.38	1.36	1.77

proportion of bases in n- and mtDNA as determined by chemical analysis and also as estimated from the data in Tables I and II by applying the calculations suggested by Josse and Swartz (1963). The base-incorporation factors were derived from the data of nearest-neighbor analysis. In Figures 1 and 2 are plotted the observed nearest-neighbor frequencies of the dinucleotides *vs.* the calculated frequencies for n- and mtDNA, respectively, assuming that the synthesized RNAs were true copies of the DNA templates. Even though only one experiment in each case is presented the results were confirmed by one additional analysis for nDNA and two additional experiments for mtDNA. In no case did values for individual dinucleotides deviate by more than 10% while in most cases the deviations were between 2 and 4%.

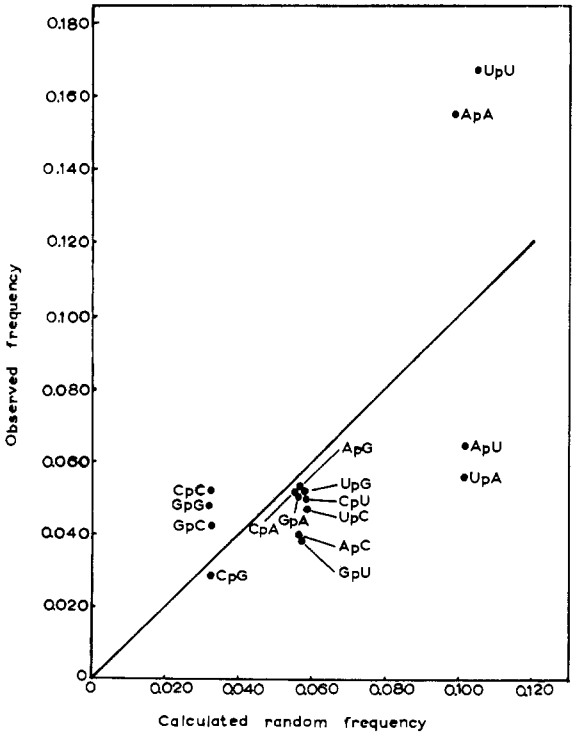

FIGURE 1: The observed nearest neighbor frequencies of RNA synthesized on mouse liver mitochondrial DNA template plotted against the expected random frequencies calculated from the base compositions of the samples (Table II).

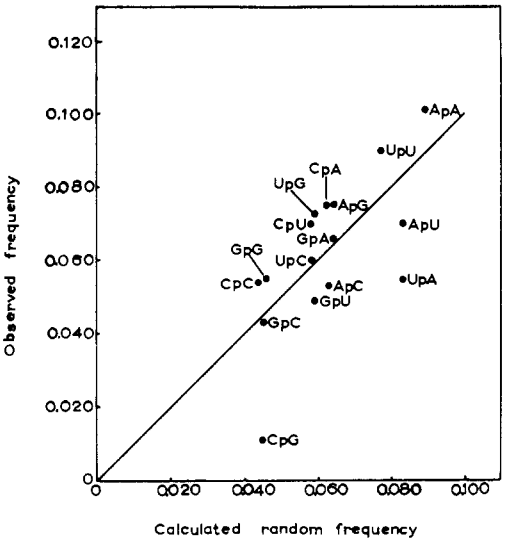

FIGURE 2: The observed nearest neighbor frequencies of RNA synthesized on mouse liver nuclear DNA template, plotted against the expected random frequencies calculated from the base compositions of the samples (determined by nearest neighbor analysis (Table II)).

Discussion

A number of investigators (Weiss and Nakamoto, 1961; Hurwitz *et al.*, 1962) have shown that when both strands of a DNA template are transcribed the nearest-neighbor frequencies of the newly synthesized RNA are equivalent to the nearest-neighbor frequencies of the DNA template. The excellent complementarity of A to U and G to C found in the RNA transcribed from the mtDNA in the present work (Table II) leads one to believe that both DNA strands are indeed transcribed and consequently the frequencies of dinucleotides in both DNA template as well as RNA product are the same.

Cummins *et al.* (1967) in an attempt to detect a possible phylogenetic link between bacterial and mtDNA determined the nearest-neighbor frequencies for dinucleotides ending in G of the RNA synthesized on templates of n- and mtDNA of *Physarum polycephalum*. These workers found that only the GpG frequency of mtDNA deviates significantly from randomness. The only finding in their results that might be of importance in the question of the phylogenetic origin of mitochondria is that the CpG frequency in mitochondrial DNA is very close to random while the same sequence deviates significantly from randomness in nDNA. From the data of Josse *et al.* (1961), in two strains of *Escherichia coli* it can be calculated that the CpG frequency in bacterial DNA is very close to random much like the CpG frequency in mtDNA. Our results confirm these findings of Cummins *et al.* (1967). However, by extending the study to all 16 dinucleotides we are at a more advantageous position for making comparisons. Thus in nDNA all dinucleotide sequences with the exception of CpG and UpA fall within 20% of the calculated random frequency. One should keep in mind at this point that the experimental error in nearest-neighbor analysis is approximately 10%. In mtDNA, on the contrary more than half of the dinucleotide sequences and specifically CpC, GpG, GpC, ApC, GpU, ApU, UpA, ApA, and UpU deviate by more than 20% from randomness. It is interesting to note in this respect that in *E. coli* DNA and *Micrococcus phlei* DNA (Josse *et al.*, 1961) only one dinucleotide deviates by more than 20% from randomness. We do not believe that any clear-cut phylogenetic correlations are justified when one takes into

account the present data as well as the data of Kornberg's group on animal, plant, and bacterial DNAs. The only justifiable conclusion that may be drawn, at least in the case of mouse liver mtDNA, is that the frequencies of dinucleotides are decidedly less random in mtDNA than in any other double-stranded DNA species studied thus far.

References

Borst, M., and Kroon, A. M. (1969), *Int. Rev. Cytol. 26*, 107.

Chargaff, E. (1968), *Progr. Nucl. Acid Res. Mol. Biol. 8*, 297.

Chatterjee, S. K., Dass, H. K., and Roy, S. C. (1966), *Biochim. Biophys. Acta 114*, 349.

Cummins, J. E., Rush, H. P., and Evans, T. E. (1967), *J. Mol. Biol. 23*, 281.

Georgatsos, J. G., Antonoglou, O., and Gabrielides, C. (1970), *Arch. Biochem. Biophys. 136*, 219.

Hurwitz, J., Furth, J., Anders, M., and Evans, A. (1962), *J. Biol. Chem. 237*, 3752.

Josse, J., Kaiser, A. D., and Kornberg, J. (1961), *J. Biol. Chem. 236*, 864.

Josse, J., and Swartz, M. (1963), *Methods Enzymol. 6*, 739.

Markham, R., and Smith, J. D. (1952), *in* The Nucleic Acids, Vol. 1, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic Press, p 267.

Murray, K. (1970), *Biochem. J. 118*, 831.

Stern, H. (1968), *Methods Enzymol. 12B*, 100.

Symons, R. H. (1970a), *Biochim. Biophys. Acta 209*, 296.

Symons, R. H. (1970b), *Biochem. Biophys. Res. Commun. 38*, 807.

Weiss, S. B. (1968), *Methods Enzymol. 12B*, 559.

Weiss, S. B., and Nakamoto, T. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1400.

Wyatt, G. P. (1951), *Biochem. J. 48*, 584.

Comparison of Some Reactions Catalyzed by Deoxyribonucleic Acid Polymerase from Avian Myeloblastosis Virus, *Escherichia coli*, and *Micrococcus luteus**

Robert D. Wells,† Rolf M. Flügel, Jacquelynn E. Larson, Paul F. Schendel, and Raymond W. Sweet

ABSTRACT: A comparative study was performed on the template specificities of the highly purified DNA polymerases from *Escherichia coli* and *Micrococcus luteus* and of a partially purified DNA polymerase from virions of avian myeloblastosis virus (AMV). The three DNA polymerases show approximately the same capacity to utilize twenty different high molecular weight templates. Thus, when tested with polymer templates (primers), the two bacterial DNA polymerases are at least as effective "reverse transcriptases" as the tumor virus associated DNA polymerase. However, $(rA)_n \cdot$oligo(dT) is a markedly better template than $(dA)_n \cdot$oligo(dT) for the AMV DNA polymerase, as reported previously. For the *M. luteus* DNA polymerase, the two templates are approximately equally effective. The AMV DNA polymerase provides faithful DNA synthesis when either DNAs or RNAs serve as templates (primers). DNA synthesis is dependent on the presence of a suitable primer strand and the newly synthesized DNA strand is covalently attached to the primer strand through a phosphodiester linkage. Thus, when a polyribonucleotide serves as a primer, the new DNA strand is joined to an RNA molecule. The AMV DNA polymerase apparently cannot initiate the synthesis of a new DNA strand. This behavior is identical with that observed for the two bacterial DNA polymerases.

A variety of nucleic acid metabolizing enzymes is associated with virions of RNA tumor viruses. These include DNA polymerase(s) (Baltimore, 1970; Temin and Mizutani, 1970; Spiegelman *et al.*, 1970), DNA ligase (Mizutani *et al.*, 1971), nuclease(s) (Mizutani *et al.*, 1970, 1971), nucleoside diphosphokinase (Miller and Wells, 1971), and other nucleotide kinases and phosphotransferases (Roy and Bishop, 1971; Mizutani and Temin, 1971). Other activities are also present (Mizutani and Temin, 1971).

Studies have indicated that some DNAs, RNAs, and DNA·RNA hybrids will serve as templates (primers) for the virion-associated DNA polymerases (Spiegelman *et al.*, 1970; Riman and Beaudreau, 1970; Fujinaga *et al.*, 1970; Mizutani *et al.*, 1970; Hatanaka *et al.*, 1970; Scolnick *et al.*, 1970, 1971; Duesberg *et al.*, 1971; Baltimore and Smoler, 1971). This and related work has led to the notion that these enzymes catalyze the flow of genetic information from RNA to DNA ("reverse transcription"). However, it is not certain if the "reverse transcriptases" have truly unique activities or have properties similar to well-characterized DNA polymerases.

Hence, a comparison was made of the catalytic capacities of a functionally pure viral polymerase (free of detectable contaminating activities) with two well characterized polymerases. Due to the comparative nature of this work, nucleic acid structural problems were obviated and empirical comparisons can be drawn.

* From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706. *Received August 23, 1971.* This work was supported by the National Science Foundation (GB-8786), the National Institutes of Health (Contract No. 71-2275), the Damon Runyon Fund (DRG-1049-A), and the Jane Coffin Childs Fund (No. 272). R. W. S. is a National Institutes of Health predoctoral fellow (GM 39327).

† To whom to address correspondence.