The molecular complexity of Mu and Pi symbiont DNA of Paramecium aurelia

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ABSTRACT - The molecular size of mu and pi symbionts of Paramecium aurelia has been calculated from renaturation kinetic data. Observed values were 0.78×10^9 daltons for mu particle DNA and 0.81×10^9 daltons for pi particle DNA. Estimates of analytical complexity were 4.45×10^9 and 5.05×10^9 daltons respectively. Based on these data, mu and pi symbionts appear to possess multiple genomes and contain a minimum of 5 or 6 copies of each DNA sequence.

INTRODUCTION - Infectious, self-reproducing intracellular particles of unknown origin occur in a variety of biological forms and appear to be particularly wide spread among the protozoa, notably ciliates.[1,2] Among the best known, perhaps, are the symbiont particles found in certain strains of Paramecium aurelia.[3] Primarily on the basis of morphological considerations, the particles were thought to be bacterial in nature.[3] A recent study[4] on the size and structure of the genome of one of these symbiont types, i.e. lambda, suggests that, unlike most free-living bacteria which possess one or a few copies of each DNA genome, lambda, possibly as a consequence of prolonged intracellular residence, contains at least 10 copies of the genome. These results prompted us to examine the size and structure of the genomes of two other intracellular symbionts, mu and pi, which are found in stocks 138 and 139, respectively, of Paramecium aurelia.

METHODS - Culture techniques - Symbiont mu-bearing stock 138 and pi-bearing stock 139 of Paramecium aurelia were used in this investigation. The organisms were cultured in axenic medium at 27° in the dark. Animals from 25.6 l. of culture prepared in 1.6 l. batches of medium contained in 4 l. capacity Fernbach flasks or Roux bottles were harvested from early in the stationary phase of growth (6 or 7 days). Yields of mu- and pi-bearing animals averaged 24 ml and 34 ml of packed cells, respectively, per run. Methods for the preparation of the medium, maintenance of the stocks, mass cultivation procedures and harvesting of the organisms have been described elsewhere.[5]

Isolation and purification of mu and pi symbionts - Mu and pi symbionts were isolated from particle-bearing stocks 138 and 139 of P. aurelia, respectively, and purified by a procedure previously described.[6] Yields of mu particles ranged from 10 to 36 x 10^{10} per run, whereas yields of pi particles ranged from 37 to 76 x 10^{10} per run. Except for the presence of trichocysts in some preparations, the particles were essentially free from contamination with cell debris or other subcellular elements. When not immediately used for extraction of DNA, the particle preparations were stored at -70° for periods of not more than 4 weeks.

Extraction and purification of DNA - DNA was extracted from freshly isolated or frozen symbiont particle preparations by the method of Marmur.[7] Whole cell- and macronuclear-DNA[8] were obtained by a modification of the methods of Kirby[9] and Jones.[10] Details of this latter procedure are fully given in a previous paper.[4] Yields of highly purified DNA ranged from 0.5 to 1.0 mg from 20 x 10^{10} particles. The Marmur[7] procedure was used to extract DNA from stationary phase Escherichia coli strain K-12 cells cultured in 'C' medium of Roberts.[11] Final purification of the DNA preparations was accomplished by preparative CsCl gradient centrifugation Bacteriophage T_4 DNA was kindly supplied by Dr. Rudolf Werner of the University of Miami School of Medicine.

CsCl gradient centrifugation - Preparative and analytical CsCl gradient centrifugation was carried out as previously described. For preparative purposes, DNA samples containing 5 to 10 absorbancy units (260 nm) were centrifuged in 4.0 ml of CsCl solution (initial density = 1.700 g/cm^3) at 38,000 RPM for 72 hrs at ambient temperature in the Spinco SW 30 rotor. Fractions of two drops each were collected and the density determined from the refractive index of undiluted samples taken at regularly spaced intervals. Analytical CsCl gradient centrifugation was carried out in the Model E ultracentrifuge. Each sample contained E. coli K-12 DNA as a reference. The density of E. coli K-12 DNA was taken to be 1.710 g/cm^3.

Thermal transition and renaturation - DNA samples were denatured in 1 M NaCl, pH 7.0 by heating 10 min at 100°C, quenched in ice and renatured at T_m -25°C. The reaction was followed at 260 nm in the Beckman DK-2 recording spectrophotometer. The DNA was sheared to various sizes at 4°C in 1 M NaCl using the microprobe of the Branson model S-125 sonifier for periods of 30 to 60 seconds.

Alkaline sedimentation coefficients - Alkaline sedimentation coefficients were estimated by the method of Studier[12] using alkaline sucrose gradients calibrated by the general procedures outlined by Martin and Ames.[13]

Analytical methods - DNA was determined by the diphenylamine reaction[14] or by the p-nitrophenylhydrazine method of Webb and Levy.[15] Ribose was assayed by the orcinol procedure,[16] and protein by the method of Lowry, et al.[17] using Lab-trol as a standard. Total nucleic acid was estimated from U.V. absorbance at 260 nm using a coefficient of 24 for a 1.0 mg/ml solution, pH 7.0, 1.0 cm light path. Spectrophotometric measurements were made in the Beckman D. U. spectrophotometer.

Protein monolayer techniques and contour length measurements of DNA - The basic procedure of Kleinschmidt & Zahn[18] was used. 0.1 ml of a solution containing 1 to 2 µg/ml of DNA and 100 µg/ml of cytochrome c in 1 M ammonium acetate was delivered to the surface of a hypophase containing 0.1 M ammonium acetate by means of a wetted glass ramp. Samples of the protein film containing the DNA were picked up on carbon-coated grids, washed in ethanol, dried and rotary shadowed with platinum-palladium at an angle of 7°. To measure the length of the DNA strands, enlargements of the electron microscope negatives were printed on 11-1/2" x 14" semi-gloss photographic paper. The DNA strands were traced with the aid of a map reader and the molecular weight estimated from the contour length. A contour length of 1.96 µm is equivalent to a double-stranded molecular weight of 10^6 daltons.

RESULTS - Analytical CsCl gradient profiles - Following final purification by preparative CsCl gradient centrifugation, symbiont mu and pi DNA samples were subjected to analytical CsCl gradient centrifugation. Single peaks for both mu and pi DNA were obtained, corresponding to densities of 1.6952 g/cm^3 and 1.6942 g/cm^3, respectively (Fig. 1).

For comparative purposes, analytical CsCl gradient profiles for protozoan whole cell (symbiont-free) and macronuclear DNA were also included. Peak density (1.688 g/cm^3) of these DNA preparations differed significantly from those of symbiont mu and pi DNA.

Guanine plus cytosine content of symbiont DNA - Symbiont mu and pi DNA when heated in either SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) or 1 M NaCl, pH 7.0 melted sharply over a narrow temperature range and exhibited hyperchromic increases of 40% and 39% respectively (Fig. 2).

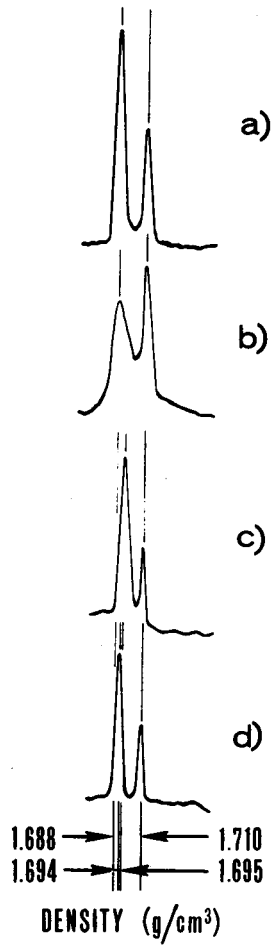

Fig. 1. Microdensitometer tracings of ultraviolet absorption scan of DNA preparations equilibrated in CsCl density gradients after 20 hours at 44,770 rev/min at 25°. (a) Whole cell P. aurelia (symbiont-free) DNA. (b) DNA from purified macronuclei. (c) DNA from purified mu symbionts. (d) DNA from purified pi symbionts. E. coli K-12 DNA (density = 1.710 g/cm^3) was used as reference.

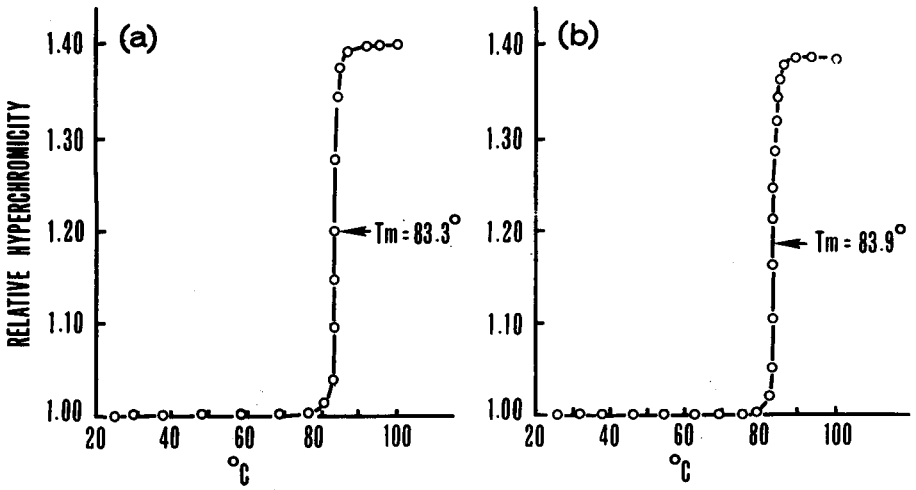

Fig. 2. Thermal transition profiles of a) Mu and b) Pi particle DNA in SSC.

The guanine + cytosine content calculated from both T_m and buoyant density data were in good agreement and are shown in Table 1.

Table 1

Guanine Plus Cytosine Content of Symbiont Particles and Whole Cell Paramecium aurelia (symbiont-free) DNA

DNA	T_m (SSC)	Hyper-chromicity (%)	Buoyant Density (g/cm^3)	G plus C Content (mole%) From T_m	Buoyant Density
Mu	83.3	40	1.695	34.2	35.7
Pi	83.9	39	1.694	35.6	34.7
P. aurelia (Whole Cell)	81.3*	42	1.688	29.3	28.6

*Data from reference #8

Kinetic complexity of symbiont mu and pi DNA - In order to determine the kinetic complexity of DNA it is necessary to determine both the sedimentation velocity coefficient ($S^{pH\ 13}_{20,w}$) of each DNA sample and its renaturation rate constant (k_2). Determination of k_2 values of a given sample may be obtained with an accuracy of about 95% ± 5%. Estimates of molecular weight, on the other hand, are often more difficult to obtain at this level of accuracy. For this reason, we compared single-stranded molecular weight values obtained from sedimentation velocity constant coefficients with those obtained from contour length measurements of both sheared and unsheared preparations of mu, pi and E. coli DNA (Table 2).

Table 2

A comparison of the single-stranded molecular weights determined for Mu, Pi and E. coli DNA

DNA	Sample	Contour length (μm)	$(S^{pH\ 13}_{20,w})$	Single-stranded Molecular Weight From EM	Single-stranded Molecular Weight From S value
Mu	Native	4.10*	23.7	4,010,000	4,280,000
	Sheared	0.213	6.8	209,000	191,000
Pi	Native	4.00	23.2	3,920,000	4,140,000
	Sheared	0.205	7.6	201,000	236,000
E. coli	Native	5.92	25.9	5,800,000	5,370,000
	Sheared	0.332	8.3	362,000	358,000

*Figures represent the average contour length of 50 native and 200 sheared DNA molecules as measured in the electron microscope. Alkaline S values were determined as described in METHODS. Single-stranded molecular weight was calculated from the following formula: $S^{pH\ 13}_{20,w} = 0.0528\ M^{0.400}$ (Ref. #12).

Values obtained by both methods were comparable. Renaturation rate plots (Fig. 3) revealed that symbiont mu and pi DNA followed second order kinetics up to approximately 50 or 60% of the course of the reaction, suggesting a single kinetic class. We have repeatedly observed deviations from second order kinetics for both mu and pi DNA as the reaction progressed beyond 70% of the total.

The relationship between the sedimentation coefficient $(S^{pH\ 13}_{20,w})$ and the renaturation rate constant (k_2) was linear as shown in Fig. 4.

The linear plot for both mu and pi DNA data was experimentally indistinguishable. The value of 0.8, obtained for the slope, was in good agreement with the value of 0.78 obtained previously by Wetmur and Davidson[19] for bacteriophage T_4 and T_7 DNA. Table 3 contains data for the renaturation rate constants and kinetic complexity of symbiont DNA. The data are normalized to

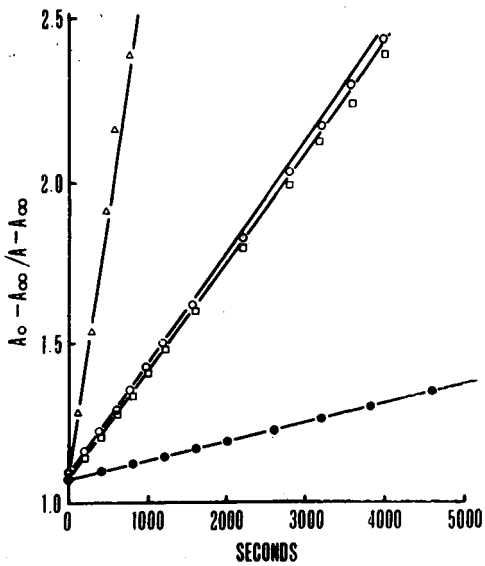

Fig. 3. Second order renaturation rate plots. The DNA samples at a concentration of 30 µg/ml were heated in 1 M NaCl, pH 7.0 at 100° for 10 min (E. coli was heated at 110° for 10 min) and renatured at (T_m -25°). The curves were adjusted to an ($S^{pH13}_{20,w}$) value of 10. T_4 Δ; mu o; pi □; E. coli K-12 ●.

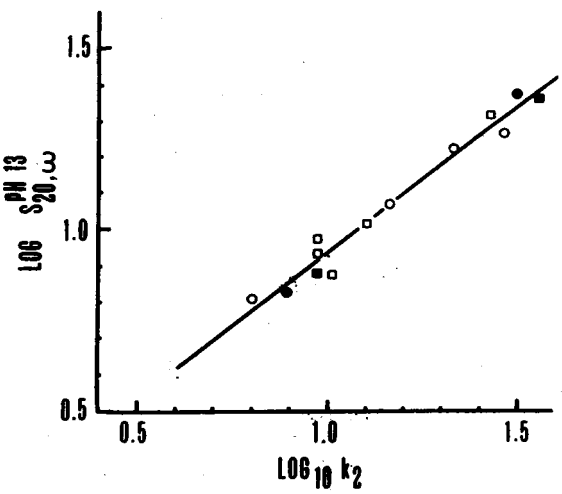

Fig. 4. The relationship between ($S^{pH\ 13}_{20,w}$) and k_2 of mu and pi DNA. The slope of the straight line plot = 0.8. o, pi DNA; ●, pi DNA (molecular weight determined in the electron microscope); □ , mu DNA; ■ , mu DNA (molecular weight determined in the electron microscope).

Table 3

Kinetic Complexity of Symbiont DNA

DNA	# Preparations	# Determinations	k_2 (l. $mol^{-1}sec^{-1}$)	Kinetic Complexity (Daltons)
E. coli	4	10	2.3	2.4×10^9
Mu	5	7	7.1	0.78×10^9
Pi	3	5	6.8	0.81×10^9
T_4	3	10	30.5	0.18×10^9

Kinetic complexity was determined in 1 M Na^+ at DNA concentrations ranging from 10 to 40 µg/ml.

$(S^{pH\ 13}_{20,w})^{1.25}$ values of 10. Actual $(S^{pH\ 13}_{20,w})^{1.25}$ values ranged from about 7 to 15. Kinetic complexity was calculated from the expression:

$$N_D = \frac{(S^{pH\ 13}_{20,w})^{1.25} \times 5.5}{k_2} \times 10^8 \quad \text{(Ref \#19)}$$

E. coli and T_4 DNA were included for comparative purposes. The average value obtained for the kinetic complexity of mu DNA was $0.78 \pm .16 \times 10^9$ daltons and for pi DNA $0.81 \pm .15 \times 10^9$ daltons. Data for the analytical complexity of symbiont DNA was estimated from direct measurements of the DNA content of mu and pi particle preparations isolated from protozoans taken from the early stationary phase of growth. The data are shown in Table 4. Values

Table 4

Analytical Complexity of Symbiont Mu and Pi DNA

Symbiont	DNA content per particle (pg $\times 10^{-3}$) Diphenylamine	p-Nitrophenylhydrazine	Average	Analytical Complexity (Daltons)
Mu	7.6	7.2	7.4	4.45×10^9
Pi	8.0	8.8	8.4	5.05×10^9

Each value represents the average of duplicate determinations of 4 preparations containing 6×10^{10} particles each.

obtained using two methods yielded comparable results. The average value obtained for the analytical complexity of mu DNA was 4.45×10^9 daltons and for pi DNA 5.05×10^9 daltons.

DISCUSSION - The narrow band widths observed in the analytical CsCl gradients and the sharpness of the thermal transition profiles suggest that the DNA's isolated from both mu and pi symbionts are native double-stranded molecules and are relatively homogenous with respect to G + C content. Values for the double-stranded molecular weight of the DNA isolated from the symbionts ranged from 8 to 10×10^6 daltons based on estimates of molecular weight calculated from both sedimentation coefficients and from contour length measurements in the electron microscope. Based on the linearity of initial slope of second order renaturation rate plots, mu and pi particle DNA appears to reassociate as a single kinetic class. However, deviations from linearity to higher orders in the later stages of the reaction, an effect observed by others for some bacterial DNA's,[20] may indicate a degree of compositional heterogeneity or may be due to catenane or network formation.[21]

The observed kinetic complexity determined for mu DNA was 0.78×10^9 and for pi DNA, 0.81×10^9 daltons. We consider these values to be experimentally indistinguishable. The renaturation rate constant (k_2) and hence the kinetic complexity is known to be affected by the G + C content of the DNA sample. For example, in our hands, we have consistently observed vaues for the kinetic complexity of T_4 DNA (average = $0.18 \pm 0.015 \times 10^9$ daltons) to be about 30% higher than the value 0.13×10^9 daltons reported for the analytical complexity. Because the G + C content of T_4 DNA (34 mole %) and those of the symbionts (35 mole %) are nearly identical, we have corrected our values for the kinetic complexity of mu and pi DNA to 0.56×10^9 and 0.59×10^9 daltons respectively. These values compare with the value of 0.39×10^9 daltons (corrected for G + C content) calculated for lambda symbionts of Paramecium aurelia, stock 299.[4] Based on these values, we estimate that the genome of mu and pi symbionts can code for a minimum of about 750 proteins. Lambda symbionts have been estimated to code for a minimum of about 500 proteins. Chemical analysis of mu and pi particle DNA by the diphenylamine method[14] and by the p-nitrophenylhydrazine procedure[15] indicate an analytical complexity of 4.45×10^9 and 5.05×10^9 daltons. respectively. Calculations based on these values and from values obtained for the observed kinetic complexity suggest that mu and pi particles possess multiple genomes and contain about 5 or 6 copies of each DNA sequence. Similar calculations based

on kinetic complexity values for molecular size corrected for G + C content suggest the presence of as many as 8 to 10 copies of the genome. These values may be compared to 10 to 20 copies of the genome estimated for lambda symbionts.[4] The presence of multicopy genomes in the symbionts appears to distinguish these entities from free-living bacteria, which normally contain only one or a few copies of each DNA sequence, and provides further support for the view that the symbionts may be representatives of a unique class of "organisms" in nature. Multicopy genomes have been reported for chloroplasts of Chlamydomonas reinhardi[23] and Euglena,[24,25] mitochondria of Tetrahymena[26] and for the kinetosomes of trypanosomes.[27]

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