THE KINETIC COMPLEXITY OF *EUGLENA GRACILIS* CHLOROPLAST DNA

Nelson S. SLAVIK and Charles L. HERSHBERGER
University of Illinois, Urbana, Department of Microbiology, 302 Burrill Hall, Urbana, Illinois 61801, USA

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1. Introduction

Intact chloroplast DNA (ct DNA) has been isolated from several higher plants as circular molecules with a mol. wt of 85–95 X 10^6 [1]. The molecular complexities of these ct DNA's agree with the analytical measurements of mol. wts. Discrepancy between the molecular weight derived from the analytical measurements and kinetics of reassociation for ct DNA from *Euglena gracilis* Z has generated confusion over the correct molecular weight of this ct DNA. Stutz calculated the kinetic complexity of ct DNA to be 180 X 10^6 daltons [2]. Manning and Richards calculated the analytical mol. wt to be 92 X 10^6 from electron microscopic measurement of a relaxed circular ct DNA molecule [3]. They explained the difference between the analytical and kinetically derived mol. wts through a graphical extrapolation of G+C mol percent dependency on DNA reassociation kinetics. However, the correction for G+C mol percent is invalid because ct DNA contains segments with heterogenous base compositions. The correction for mol percent G+C applies to a DNA molecule with a uniform base composition that is renatured at a temperature outside the range for its maximum rate of reassociation [4]. (James Wetmur, personal communication.) Therefore, the kinetic complexity [2] is a factor of two higher than the analytical mol. wt [3].

Due to internal base compositional heterogeneity along the length of the ct DNA molecule, carefully controlled renaturation conditions must be employed. The kinetics of renaturation of ct DNA were analyzed by a modification of the procedure developed by Wetmur and Davidson [5]. This analysis yields a kinetic complexity of 93 X 10^6 daltons, therefore agreeing with the size of the circular ct DNA molecules seen by Manning and Richards [3].

2. Materials and methods

Chloroplast DNA from heterotrophically grown *Euglena gracilis* Z was isolated as described [6]. Analytical ultracentrifugation of ct DNA was performed with a Spinco Model E Ultracentifuge equipped with a high intensity light source, monochrometer and four cell optics. Analytical band sedimentation velocities were determined at 30 000 rev/min, 20°C as described by Vinograd et al. [7]. Mol. wts were calculated from sedimentation coefficients as described by Studier [8]. Tracings of photographs were recorded with a Joyce-Loebl MK III B recording microdensitometer. Buoyant densities were calculated as described with the buoyant density of *Escherichia coli* taken as 1.710 g/cm^3 [9]. Denaturation and renaturation of ct DNA was performed in a Beckman Acta III Spectrophotometer with an automatic temperature programmer.

Bacteriophage λ and T_4 DNA's were a gift from J. Mielenz, University of Illinois.

3. Results and discussion

Chloroplast DNA from *Euglena gracilis* Z was isolated by a differential detergent extraction procedure and bands as a single homogeneous peak in an analytical CsCl gradient [6] (fig.1A). The probability graph of the ct DNA profile gives a single straight line indicating that the sample contains

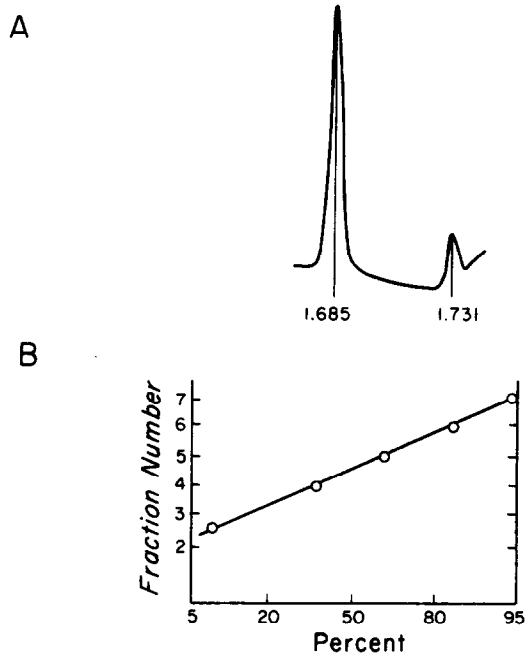

Fig.1. Analytical equilibrium density gradient ultracentrifugation of purified ct DNA and resulting probability graph. A) Chloroplast DNA was isolated as described [6] and centrifuged to equilibrium in CsCl at 44 000 rev/min at 25°C. *Micrococcus lysodeikticus* DNA was employed as a reference with a buoyant density of 1.731 g/cm^3. B) Probability graph of ct DNA from A) was prepared as described [10]. The single straight line indicates that the sample contains a single homogeneous species of DNA.

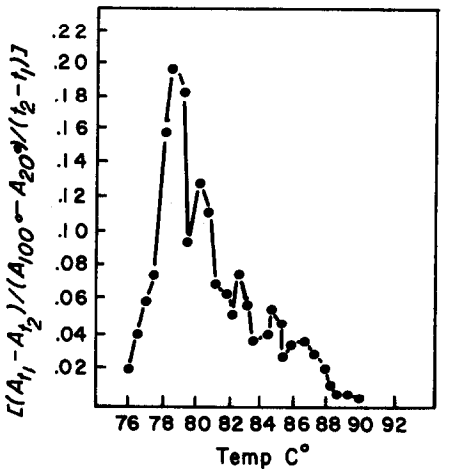

Fig.2. Thermal transition of ct DNA. The thermal transition of ct DNA was measured in buffer containing 0.15 M NaCl and 0.015 M sodium citrate at pH 7.0. Measurements were performed in a Beckman Acta III Spectrophotometer with automatic temperature programmer. The derivative of the thermal transition is shown: R versus temperature where R equals $(A_{t1}-A_{t2})/(A_{100°}-A_{20°}/t_2-t_1)$. Denaturation temperatures were equated into G+C mol percent according to Mandel [14].

a single species of DNA [10] (fig.1B). If the DNA contained two components with a buoyant density difference of 0.001 g/cm^3 or greater the probability graph would not be a straight line [10].

The thermal transition of ct DNA measured at 0.5°C intervals reveals extensive internal base composition heterogeneity within the ct DNA molecule (fig.2). There are five distinct regions varying in G+C content from 22% to 40%. Forty percent of the molecule has a G+C content of 22%. Four other differentially melting regions corresponding to 25%, 30%, 36% and 40% G+C comprise 27%, 16%, 10% and 7% of the ct DNA molecule, respectively. This differential denaturation profile is similar to that published by Stutz for DNA from isolated chloroplasts and suggests that all internal heterologous G+C regions are present in the ct DNA studied [11].

Chloroplast DNA was analyzed by band sedimentation to determine the likelyhood for loss of fragments during isolation. Shearing ct DNA from its native analytical mol. wt of 92 X 10^6 to piece sizes of 20 X 10^6 yields a fragment with a base composition of 32 mol percent G+C [11]. Further shearing to a mol. wt of 5 X 10^6 converts the 32% G+C fragment into two distinct components of 29 and 41 mole percent G+C [12]. Since the mol. wt of the ct DNA preparation is very crucial to the question of fragment loss, the mol. wt of the ct DNA was calculated from neutral analytical band sedimentation according to Studier [8]. Band sedimentation demonstrated a uniformly sedimenting band with a mol. wt of 46 X 10^6. Therefore, the ct DNA is almost ten times larger than the mol. wt needed to generate the 29 mol percent and 41 mol percent G+C components and two times larger than the mol. wt generating the 32 mol percent G+C fragment.

Band sedimentation of ct DNA through CsCl at pH 12.5 gives a single-stranded mol. wt of 2.1 X 10^6. The mol. wt of alkali denatured ct DNA was confirmed by sedimentation of denatured DNA at pH 8.0. Thermal

denaturation of ct DNA and subsequent neutral band sedimentation also gives a single-stranded mol. wt of 2.1×10^6. Therefore, thermal or alkali denaturation of high mol. wt ct DNA produces small single-stranded fragments. This suggests that there are 23 staggered, single-stranded nicks interspersed throughout the ct DNA molecule.

Conditions for ct DNA reassociation kinetic analysis must be carefully controlled due to the extreme base compositional heterogeneity along the ct DNA molecule [13]. Maximizing the reassociation kinetics of such a molecule can be achieved by two methods. Employing DNA fragments smaller than the size of the segments exhibiting the different base compositions will give a true complexity of the DNA molecule. Also, complexity must be calculated from data at the reassociation temperature where the reassociation kinetics are maximal. The rate of ct DNA renaturation was found to be maximal at temperatures from $T_m-25°C$ to $T_m-22°C$; therefore, ct DNA complexity was calculated from reassociation at 57°C or 25°C below the mean temperature of the thermal denaturation profile. The single-stranded mol. wt of ct DNA was 2.1×10^6 as determined by alkaline band sedimentation of samples before renaturation and after completion of renaturation.

The kinetics of renaturation of ct DNA were analyzed by a modification of the procedure developed by Wetmur and Davidson [5]. The temperature-jump method was abandoned because thirty to forty percent of the ct DNA renatured in the time it took for the temperature to go from T_m + 18°C to $T_m-25°C$ under our conditions. Christiansen et al. noted that the rate of reassociation is maximal only for the first thirty percent of renaturation [13]. Any rate derived after that point leads to a lower K_2 and thus a higher apparent kinetic complexity. It was imparative therefore, that the initial stage of the reassociation reaction be followed. Utilization of an alkali denaturation step followed by rapid re-neutralization of the warmed DNA solution enabled us to record the reaction from time zero. As seen in fig.3 the rate of reaction is linear when plotting $(A_0-A_\infty)/(A-A_\infty)$ versus time for the three species of DNA examined. Bacteriophage λ and T_4 DNA were employed as parallel controls bracketing the kinetic complexity of *Euglena gracilis* ct DNA. All reactions were measured at 0.15 M Na^+ concentrations and the rates corrected to 1.0 M Na^+ concen-

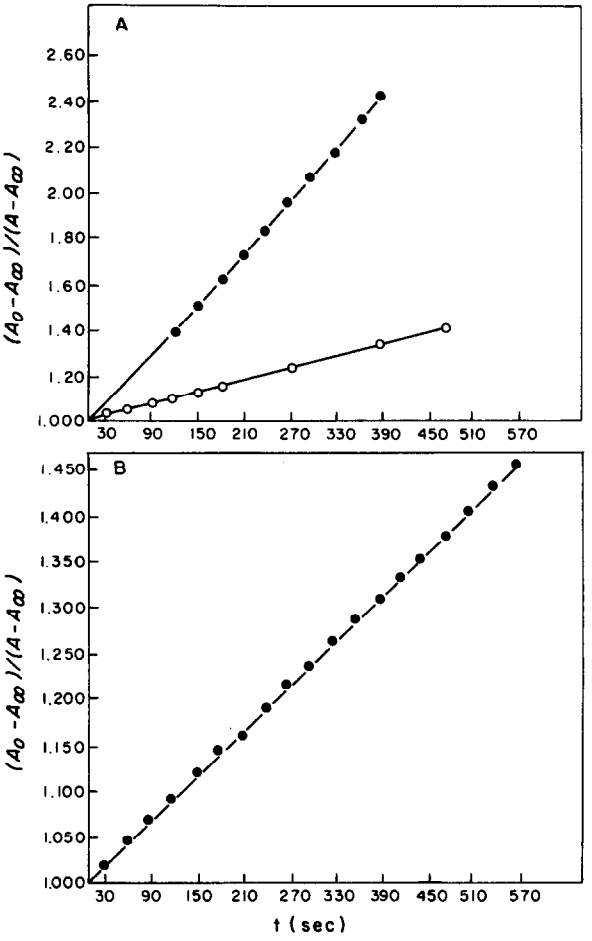

Fig.3. Reassociation kinetics of ct DNA. The reassociation kinetics were measured at 57°C in 0.15 M NaCl–0.015 M sodium citrate at pH 7.0 using a Beckman Acta III Spectrophotometer with an automatic temperature programmer. All DNA solutions were adjusted to 30 μg DNA/ml and warmed to renaturation temperature. Denaturation was accomplished by addition of 5 μl of concentrated NaOH. After 10–15 minutes at renaturation temperature, the solutions were reneutralized with 10 μl of concentrated KH_2PO_4. A) Second-order rate plot of λ DNA (-●-●-) and T_4 DNA (-○-○-). DNA was sheared 20 times through a 26G needle to a mol. wt of 5.0×10^6 for the denatured DNA as calculated by alkaline band sedimentation before renaturation and after completion of the reassociation reaction [8]. DNA from T_4 and λ were renatured at $T_m-19°C$ and $T_m-22°C$ respectively in 0.15 M Na^+, which falls within the temperature range for the maximum rate of reassociation for each DNA species [16]. B) Second-order rate plot of ct DNA. The mol. wt of denatured ct DNA was 2.1×10^6 daltons. Renaturation temperature for ct DNA was taken at 57°C, twenty-five degrees below the mean of the thermal denaturation profile and resulted in the maximum rate of reassociation.

Table 1
Comparison of molecular weights as derived from kinetic complexities and analytical measurements

DNA Species	$S^{pH\ 13}_{20,w}$ [8]	K_2 1M Na^+	$N_D - \frac{(5.5 \times 10^8)(S^{pH\ 13}_{20,w})\ 1.25}{K_2\ 1M\ Na^+}$ [5]	Analytical measurement
λ	24.6	1046	29×10^6	31×10^6 [15]
T_4	25.7	247	129×10^6	130×10^6 [16]
Euglena gracilis chloroplast	17.8	215	93.7×10^6	92×10^6 [3]

trations as described [5]. The kinetic complexity of each DNA was calculated according to Wetmur and Davidson and the results in table 1 [5].

As seen in table 1, the kinetic complexity of ct DNA does agree with the analytical molecular weight as calculated by Manning and Richards [3]. Renaturation conditions are extremely critical to ensure proper kinetic analysis. The conditions we have chosen make it possible to follow the initial rate of the kinetic reaction. The complexity obtained by Stutz was obtained under conditions in which a high percentage of DNA reassociation had occurred before the temperature for the maximum rate of renaturation had been reached, leading to a lower K_2 and a higher apparent complexity. Therefore, ct DNA from *Euglena gracilis* Z is within the range of molecular complexities seen for ct DNA's from species of higher plants

Acknowledgements

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