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The Mitochondrial Genome of *Euglena gracilis*

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Mitochondrial DNA from *Euglena gracilis* has been investigated in its chemical and physical properties. Its G + C content is equal to 25%; its buoyant density in a CsCl density gradient (1.690 g/cm^3) is higher, by 5 mg/cm^3, than expected for a bacterial DNA having the same base composition. The buoyant densities of denatured and renatured DNA are higher than that of native DNA by 10–12 mg/cm^3 and 6 mg/cm^3, respectively. The melting temperature, T_m, is 77 °C in standard saline citrate; the first derivative of the melting curve shows a striking multimodality. Degradation of the DNA by micrococcal nuclease indicates that about 40% of the DNA is formed by stretches lower than 10% in G + C. In all its properties the mitochondrial DNA from *Euglena gracilis* is strikingly similar to that of *Saccharomyces cerevisiae*.

The information currently available on the mitochondrial genome of *Euglena gracilis* is rather limited. Since Ray and Hanawalt [1] and Edelman *et al.* [2] independently reported that *Euglena* mitochondria contained a DNA having a buoyant density in CsCl gradients of 1.690–1.691 g/cm^3, only four papers have dealt with this subject. Manning *et al.* [3] have found that *Euglena* mitochondrial DNA shows a very wide distribution of lengths ranging from 1 to 19 μm with a mean length of 1.3 μm; Nass and Ben-Shaul [4], in contrast, found lengths of 0.6–0.9 μm; Crouse *et al.* [5] have calculated from the hybridization plateau of mitochondrial rRNA and its molecular weight, a genome complexity of $40 \cdot 10^6$, a value which agrees with the size of the longest molecules seen by Manning *et al.* [3]. Finally Stutz and Bernardi [6] showed that *Euglena* mitochondrial DNA could be separated from nuclear DNA by chromatography on hydroxyapatite.

In the present work, we have characterized in some of its chemical and physical properties the mitochondrial DNA from *Euglena*. In addition we have asked ourselves whether the mitochondrial genome of *Euglena* contains A+T-rich spacers. Such a question has been prompted by recent investigations on the organization of the mitochondrial genome of wild-type *Saccharomyces cerevisiae* cells [7] which have shown that this genome is made up, in equal parts, by "spacers" having a G + C content lower than 5% and by "genes" having an average G + C content of 32%. It was considered to be of interest to know whether the existence of spacers in mitochondrial DNA is unique to yeast, an organism which does not have an absolute requirement for functional mitochondria, or is of more general significance. In this case, spacers do not need to be of the A + T-rich type found in yeast. The methodology developed by Prunell and Bernardi [7] for the analysis of A + T-rich spacers led us, however, to consider first mitochondrial DNAs having very low G + C levels and which were, therefore, good candidates for containing A + T-rich spacers. Our first choice was the mitochondrial DNA of *Euglena gracilis*. This DNA was known to share with yeast mitochondrial DNA not only a very low buoyant density, but also the property of being eluted from hydroxyapatite columns at a higher phosphate molarity than nuclear DNA [6] a feature shown to be due, in the case of yeast DNA, to the nucleotide sequences of the spacers [8,9].

Definition. A_{260} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm, when measured in a 1-cm path-length cell.

Enzyme. Micrococcal nuclease (EC 3.1.4.7).

MATERIALS AND METHODS

Cultivation of Cells

Euglena gracilis, strain W_3BUL, an irreversibly bleached mutant, was grown heterotrophically with

Difco *Euglena* broth as a carbon source up to approximately 1.5×10^6 cells/ml. This strain does not contain any chloroplast-specific DNA [10].

Isolation of Mitochondria

Between 75 to 100 g of fresh wet packed cells were resuspended in 0.5 vol. of 0.05 M Tris-HCl, pH 7.9, 0.01 M EDTA, 0.25 M sucrose. Cells were opened by grinding with glass beads [11] and mitochondria separated by differential centrifugation and banding in Renographin [5].

DNA Isolation

DNA was isolated from purified mitochondria as reported [5]. The crude DNA samples were treated with ribonuclease B (bovine pancreas, type VII, Sigma) and chromatographed through a methylated serum albumin–kieselguhr column. Yields were 5–7 A_{260} units per batch. Nuclear DNA contaminating the preparation at levels of 5–10% was removed by hydroxyapatite chromatography [6].

Ultracentrifugation Experiments

Analytical density-gradient experiments in caesium chloride were done as previously described [9]. Sedimentation velocity experiments were done using the band sedimentation method, as described by Prunell and Bernardi [12].

Base Composition of DNA

This was determined by nucleoside analysis on Aminex A6 (BioRad, Richmond, Calif.) columns [13] after enzymic degradation.

Denaturation and Renaturation Experiments

DNA denaturation was carried out by heating the DNA solution (10 µg/ml in standard saline citrate; this solvent is 0.15 M NaCl, 0.015 M citrate, pH 7.2) at 100 °C for 10 min, followed by fast cooling (by immersion in an ice bath). Alternatively, DNA was denatured by adding 0.1 vol. of 1 M NaOH, followed by neutralization with 0.1 vol. of 1 M NaH_2PO_4. DNA renaturation was carried out keeping the DNA at 69 °C in standard saline citrate for 80 min.

Absorbance-Temperature Profiles

DNA solutions in standard saline citrate having an A_{260} close to 0.5 were used in this experiment which was done in duplicate. Absorbance-temperature profiles were determined using the automatic equipment of Prunell and Bernardi [7]. Heating rate was 45 °C/h; cooling rate was 60 °C/h.

Micrococcal Nuclease Digestions

DNA solutions were dialyzed against 0.1 M sodium borate, pH 8.8, and adjusted to an $A_{260} = 0.5$ and to 2.5 mM $CaCl_2$ by addition of 0.5 M $CaCl_2$. Digestions were performed either at 6 °C or at the melting temperatures (70 °C in this buffer), as described by Prunell and Bernardi [7].

Gel Filtration

DNA digests obtained at 6 °C or at the melting temperature were filtered on Sephadex G-25 (Pharmacia, Uppsala, Sweden) or Sephadex G-100 columns, respectively. 1-ml DNA solutions were loaded on 0.67×31-cm columns equilibrated with 0.05 M ammonium acetate, pH 5.5. Flow rate was kept at 6 ml/h using a Technicon (Chauncey, N. J.) peristaltic pump. All other conditions were those described by Prunell and Bernardi [7].

RESULTS

Physical and Chemical Properties of Mitochondrial DNA from Euglena gracilis

Chromatography on Hydroxyapatite. Chromatography on hydroxyapatite (Fig. 1) of the partially purified *Euglena* mitochondrial DNA described in the previous section showed that this DNA was eluted in a single peak centered at 0.29 M in sodium phosphate, pH 6.8. Density gradient centrifugation of the fractions revealed that the low-eluting half of the chromatographic peak was contaminated by decreasing amounts of nuclear DNA (Fig. 1). The low-eluting fractions were therefore rechromatographed on the same column (not shown). The high-eluting fractions (89–106, Fig. 1) of the chromatograms were pooled with the corresponding, nuclear-DNA-free fractions of the re-chromatography and used for the physical and chemical measurements reported below. As in the case of yeast mitochondrial DNA [14], a fractionation according to size was obtained across the chromatographic peak (Fig. 1).

Base Composition. The G + C content of mitochondrial DNA was found to be equal to 25% (Table 1). Glucosylation of the DNA can be ruled out since it hinders the enzymatic digestion by spleen exonuclease. No evidence for methylation of cytidine was found, the cytidine peak being symmetrical and showing no 5-methylcytidine shoulder. An analysis

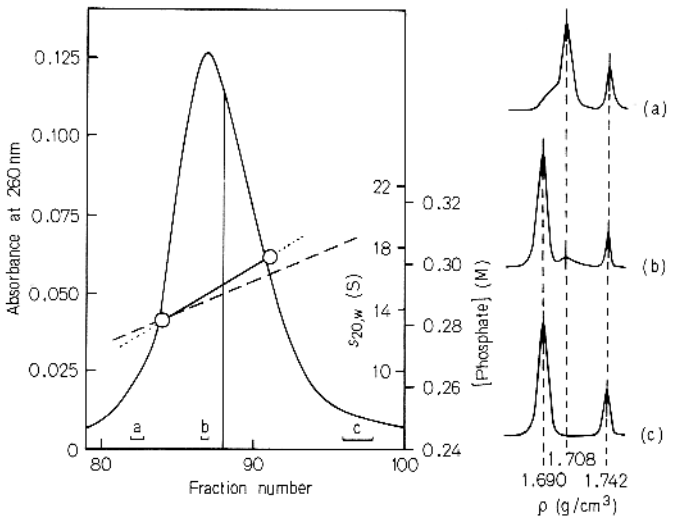

Fig. 1. *Chromatography of mitochondrial DNA from* Euglena gracilis. 16 ml of mitochondrial DNA solution in 0.1 M sodium phosphate buffer (8 A_{260} units) were loaded on a 1 × 12-cm hydroxyapatite column equilibrated with the same buffer. 10 ml of this buffer were used to wash the column and a 0.1 to 0.5 M gradient of sodium phosphate buffer (700 ml) was applied. 4-ml fractions were collected. Flow rate was 24 ml/h. Filled circles show the $s_{20,w}$ values of single fractions, the broken line indicates the phosphate molarity gradient. Horizontal bars indicate the pooled fractions used for analytical caesium chloride density-gradient experiments. (a), (b) and (c) show the scanner tracings of DNA from chromatographic fractions 82–83, 87, 96–98, respectively. DNA samples were centrifuged at 44000 rev./min for 21 h at 25 °C in a neutral CsCl density gradient. DNA from phage 2C (1.742 g/cm³) was used as density marker

Table 1. *Analysis of mitochondrial DNA and mitochondrial rRNA of* Euglena gracilis

Polynucleotide	Nucleoside	Amount
DNA		%
	dA	37.4
	dC	13.0
	dG	11.8
	dT	37.3
rRNA		
	rA	39.1
	rC	14.3
	rG	9.6
	rU	37.0

of mitochondrial ribosomal RNA of *Euglena* was also carried out (Table 1).

Buoyant Density. Unimodal, symmetrical bands, characterized by a buoyant density of 1.690 g/cm³, were obtained in CsCl density gradients. After heating and fast cooling, a buoyant density of 1.700 g/cm³ was found; a slightly higher value (1.702 g/cm³) was found after alkali treatment and neutralization. Renaturation under standard conditions led to a

Table 2. *Physical and chemical properties of* Euglena gracilis *mitochondrial DNA*

The sedimentation coefficient of the DNA preparation used was $s_{20,w}$ = 14 S.

Property	Material or method of measurement	Value
ϱ	native	1.69 g/cm³
	denatured:	
	heated, fast-cooled	1.700 g/cm³
	alkali, neutralized	1.702 g/cm³
	renatured	1.696 g/cm³
	alkaline CsCl	1.735 g/cm³
T_m		77 °C
Hyperchromicity		43%
G + C, from:	analysis	24.8%
	buoyant density	33%
	T_m	18.8%

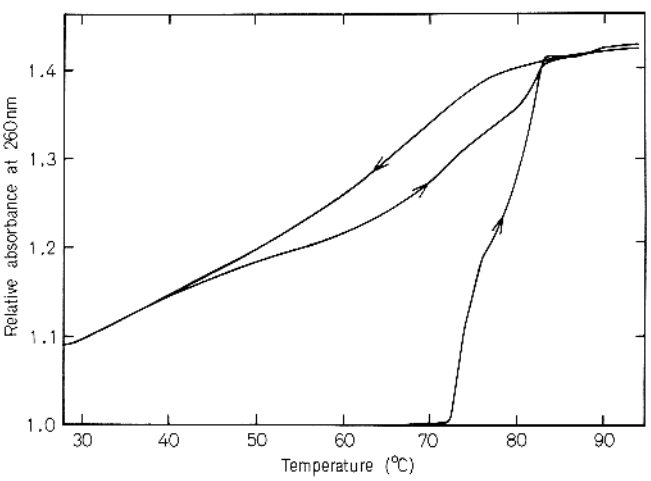

Fig. 2. *Ultraviolet-melting curve of mitochondrial DNA from* Euglena gracilis. The curve indicates the relative absorbance at 260 nm as measured during the first heating, the cooling down and the second heating. DNA solution in standard saline citrate having an A_{260} close to 0.5 was used in this experiment

decrease in buoyant density, (ϱ = 1.696 g/cm³) which, however, did not attain the native value. Finally, no strand separation was observed upon centrifugation in alkaline CsCl density gradient. All buoyant density results are shown in Table 2.

Melting. The melting profile of mitochondrial DNA is shown in Fig. 2, along with the cooling profile and the second melting curve. The hyperchromicity was 43%, and the melting temperature was 77 °C. The melting curve is characterized by a multimodality, which is clearly shown by the first derivative of the curve (Fig. 3). Two major components characterized by T_m values equal to 73 °C and 81.5 °C, respectively, could be seen, as well as two minor components melting at 75 °C and 79 °C, respectively. A minute

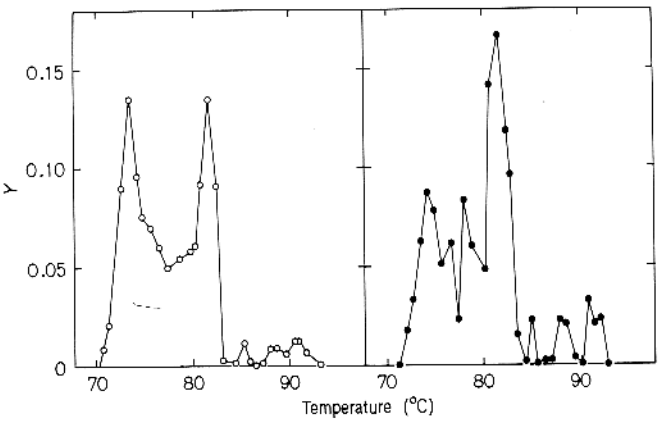

Fig. 3. *Differential melting curves of mitochondrial DNA from* Euglena gracilis. The ordinate indicates the increment in relative absorbance per degree:

$$Y = \frac{A_{t_2} - A_{t_1}}{(A_{100} - A_{25})(t_2 - t_1)}$$

where A_{t_1}, A_{t_2}, A_{100}, A_{25}, are absorbances measured at temperatures t_1, t_2, 100 and 25 °C, respectively. (○), 260 nm; (●), 280 nm

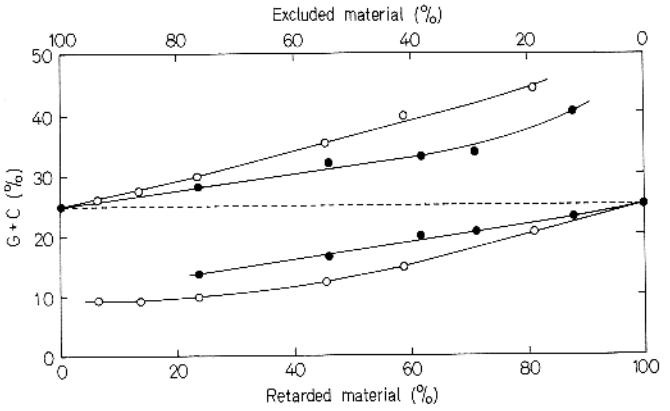

Fig. 4. *Plot of the G + C contents of the excluded and retarded fraction of* Euglena gracilis *mitochondrial DNA as degraded at 6 °C (○) and 70 °C (●), against the relative amount of excluded or retarded material*

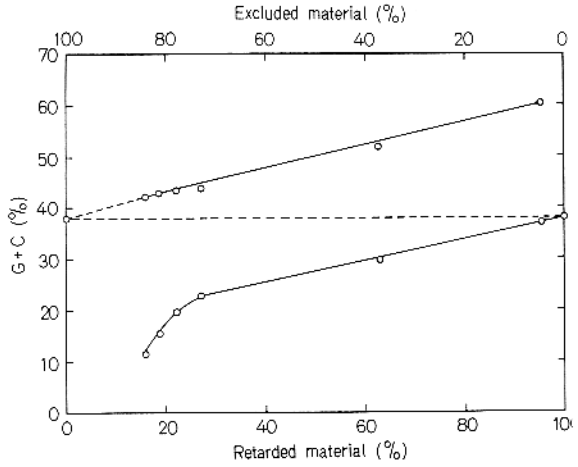

Fig. 5. *Plot of the G + C contents of the excluded and retarded fractions of* Haemophilus influenzae *DNA, as degraded at 6 °C, against the relative amount of excluded or retarded material*

amount of material melted between 87 °C and 93 °C. Fig. 3 also shows the melting transition, as obtained at 280 nm; as expected [15] under these conditions, the contribution of the G · C base pairs became more important, and, therefore, in addition, the high-melting components became more evident. The curve obtained upon cooling was largely shifted to the left and did not show any coincidence with the melting curve. The residual hyperchromicity was about 10%. The second melting curve showed an extremely broad transition.

Degradation by Micrococcal Nuclease

Fig. 4 shows the G + C contents of the excluded and retarded fractions obtained by running on Sephadex columns micrococcal nuclease digests of *Euglena* mitochondrial DNA, as obtained at low (6 °C) and at melting (70 °C) temperature, respectively. The general features of the curves resemble those of yeast mitochondrial DNA with, however, some interesting differences. At 6 °C, about 40% of the material could be obtained as small fragments having a G + C level lower or equal to 10%; the high-molecular-weight fragments increased in their G + C level in an essentially linear fashion as digestion proceeded; interestingly, they did not reach the high values shown by yeast mitochondrial DNA. At the melting temperature, the increase in G + C level of the small fragments was essentially linear from 10% to 25%, whereas the large fragments showed lower values compared to those exhibited at 6 °C.

The plots of Fig. 4 show a smaller curvature than in the case of yeast mitochondrial DNA, basically because the lowest G + C levels of the retarded material were higher and the highest G + C levels of the excluded material were lower than those found in the case of yeast. It seemed, therefore, interesting to compare the results obtained with *Euglena* mitochondrial DNA with those given by a bacterial DNA having a low G + C content. Fig. 5 shows the data obtained with *Haemophilus influenzae* DNA (G + C = 38%). The curve concerning the G + C content of the retarded material is strikingly different in its first part from that of *Euglena* mitochondrial DNA. The G + C content of the latter increases drastically from 10 to 25% in the range of 10–30% retarded material. The corresponding line extrapolates back to the origin. This initial upward trend in the plot indicates a lack of DNA stretches having a low G + C level in *H. influenzae* DNA.

DISCUSSION

The chromatographic properties of *Euglena* mitochondrial DNA on hydroxyapatite, already described [6], are characterized, by a very high elution molarity. This behavior is very similar to that of yeast mitochondrial DNA [8,9], in which case it was possible to show that the high elution molarity was due to the A + T-rich spacers [9,15]. Scarcity of material prevented us from checking whether the A + T-rich stretches (see below) of *Euglena* mitochondrial DNA are responsible for such a behavior, but it is likely that it is so in view of the very many similarities between the two DNAs. The dependence of the elution molarity of *Euglena* mitochondrial DNA upon molecular weight is a general phenomenon found with other DNAs as well [14]. Fractionation according to base composition in the chromatogram is a distinct possibility, shown to be realized in the case of degraded mitochondrial DNA [14]. In this case, it is not likely that a serious fractionation took place on the column because the buoyant densities of two highly separated fractions were the same (Fig. 1).

The G + C level of *Euglena* mitochondrial DNA was lower (25%) than that originally estimated (31%) on the basis of buoyant density (1,2]. The buoyant density of the DNA in CsCl density gradient, $\varrho = 1.690$ g/cm^3, is in good agreement with the value originally reported [1,2] and slightly higher than that (1.688 g/cm^3) previously found by Krawiec and Eisenstadt [16] and Stutz and Bernardi [6] for two different bleached mutants. Whether this difference in buoyant density reflects a real difference in the nucleotide sequences cannot be decided at the present time, but certainly this is an interesting possibility. The buoyant density of *Euglena* mitochondrial DNA is higher, by 5 mg/cm^3, than that expected on the basis of the relationship of Schildkraut *et al.* [17]. Such a discrepancy, which was already found in the case of yeast mitochondrial DNA [18], is not due to glucosylation nor to methylation for the reasons given in the previous section, and is in all likeliness, due to the buoyant density of the A + T-rich stretches, as shown for yeast mitochondrial DNA [14].

The melting curve was multimodal. The first major component corresponds, in all likeliness, to the A + T-rich stretches since its T_m of 73.5 °C indicates a G + C content of about 10% using Marmur and Doty's relationship [19] and its amount is about 40%. The second major component, melting at 81.8 °C, corresponds to about 30% of the genome and has an average G + C level of 27%. It is very tempting to assign the minor component melting at 79.2 °C to ribosomal cistrons, since it corresponds to the expected G + C level (24%) and amount (10%). This leaves unassigned the minor components melting at 76.2 °C and that melting in the 87–93 °C range; the amount of the latter, about 7%, rules out the possibility of a nuclear DNA contamination since this would have shown up in the buoyant density profile.

The renaturation properties of *Euglena* mitochondrial DNA again resemble those of yeast mitochondrial DNA in that the cooling curve did not follow the melting curve, the second melting showed a very broad transition and the buoyant density was very high, compared to the native one, both after fast cooling, or neutralization, and after reannealing. It should also be pointed out that the renaturation kinetics of *Euglena* mitochondrial DNA shows anomalies [20] very similar to those found in the case of yeast mitochondrial DNA [21].

Degradation by micrococcal nuclease indicated the presence in *Euglena* mitochondrial DNA of at least 40% of material only having a G + C content of 10% or less. In fact, the first figure may be underestimated and the second one overestimated because of "leakage" of G + C-rich material from other regions of the genome (see [7] for a discussion on this point); the intermediate, minor components seen in the differential melting curve are likely candidates for the "leaking" stretches, as in the case of the yeast mitochondrial DNA. The features seen in the micrococcal hydrolysates of *Euglena* mitochondrial DNA correspond to those found in the differential melting curves. In both cases, the major differences with yeast mitochondrial DNA are the higher G + C level of the A + T-rich spacers and the much greater compositional homogeneity of the bulk of the mitochondrial genes.

The main conclusion of the present investigations is that the mitochondrial genome of *Euglena gracilis* is organized in much the same way as that of *Saccharomyces cerevisiae*, namely as an interspersed system of A + T-rich spacers and G + C-rich genes. The relative amounts of spacers are very similar in both cases; the nucleotide sequences are quite different, however.

This conclusion has one important implication; namely, that the organization of the mitochondrial genome of yeast is not unique to this particular organism, but is also found in an evolutionarily rather distant organism like *Euglena*. The common organization of the mitochondrial genome in these two organisms is in agreement with the classical view [22] that fungi derive from protists, but argues against the idea [23] that the fungi and multicellular animal stems diverged from each other after the eukaryote stem gave rise first to the protozoan flagellate branch and later to the multicellular green plant branch.

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REFERENCES

1. Ray, D. S. & Hanawalt, P. C. (1965) *J. Mol. Biol. 11*, 760–768.
2. Edelman, M., Schiff, J. A. & Epstein, H. T. (1965) *J. Mol. Biol. 11*, 769–774.
3. Manning, J. E., Wolstenholme, D. R., Ryan, R. S., Hunter, J. A. & Richards, O. C. (1971) *Proc. Natl Acad. Sci. U.S.A. 68*, 1169–1173.
4. Nass, M. M. K. & Ben-Shaul, Y. (1972) *Biochim. Biophys. Acta, 272*, 130–136.
5. Crouse, E. J., Vandrey, J. P. & Stutz, E. (1974) *FEBS Lett. 42*, 262–266.
6. Stutz, E. & Bernardi, G. (1972) *Biochimie (Paris) 54*, 1013–1021.
7. Prunell, A. & Bernardi, G. (1974) *J. Mol. Biol. 86*, 825–841.
8. Bernardi, G., Carnevali, F., Nicolaieff, A., Piperno, G. & Tecce, G. (1968) *J. Mol. Biol. 32*, 493–505.
9. Bernardi, G., Piperno, G. & Fonty, G. (1972) *J. Mol. Biol. 65*, 173–189.
10. Schiff, J. A. & Epstein, H. T. (1965) in *Reproduction: Molecular, Subcellular and Cellular*, M. Lokke (ed.) pp. 131–189, Academic Press, New York.
11. Avadhani, N. G. & Buetow, D. E. (1972) *Biochem. J. 128*, 353–365.
12. Prunell, A. & Bernardi, G. (1973) *J. Biol. Chem. 248*, 3433–3440.
13. Thiery, J. P., Ehrlich, S. D., Devillers-Thiery, A. & Bernardi, G. (1973) *Eur. J. Biochem. 38*, 434–442.
14. Piperno, G., Fonty, G. & Bernardi, G. (1972) *J. Mol. Biol. 65*, 191–205.
15. Felsenfeld, G. & Hirschman, S. Z. (1965) *J. Mol. Biol. 13*, 407–427.
16. Krawiec, S. & Eisenstadt, J. M. (1970) *Biochim. Biophys. Acta, 217*, 120–131.
17. Schildkraut, C. L., Marmur, J. & Doty, P. (1962) *J. Mol. Biol. 4*, 430–443.
18. Bernardi, G., Faurès, M., Piperno, G. & Slonimski, P. P. (1970) *J. Mol. Biol. 48*, 23–42.
19. Marmur, J. & Doty, P. (1962) *J. Mol. Biol. 5*, 109–118.
20. Crouse, E. J. (1974) Ph. D. Thesis, Northwestern University, Evanston.
21. Christiansen, C., Christiansen, G. & Bak, D. L. (1974) *J. Mol. Biol. 84*, 65–82.
22. Whittaker, R. H. (1969) *Science (Wash. D. C.) 163*, 150–160.
23. McLaughlin, P. J. & Dayhoff, M. O. (1973) *J. Mol. Evol. 2*, 99–116.

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