5-Hydroxymethyluracil in the DNA of a Dinoflagellate

(density gradient centrifugation/*Gyrodinium cohnii*/physical effects of base composition/satellite DNA)

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ABSTRACT During the characterization of DNA from the dinoflagellate *Gyrodinium cohnii*, a large discrepancy was detected between the estimation of guanine + cytosine content from the buoyant density of the DNA in CsCl (56.1% G+C) and from the midpoint (T_m) of its hyperchromicity induced by a thermal gradient (35.6% G+C). Composition analyses of ^{32}P-labeled nucleotides revealed an actual G+C content of 41.3%, and the presence of an unusual nucleotide amounting to about 37% of the expected thymidylate in unfractionated DNA—a feature that can explain the aberrant behavior of the DNA. The chromatographic properties of the unusual base and UV spectral analyses of the base and its corresponding nucleotide are consistent with its identification as hydroxymethyluracil. This base is not uniformly interspersed with thymine in the DNA. About 10% of *Gyrodinium* DNA is contributed by a fraction with low hydroxymethyluracil content, which behaves anomalously in Ag^+–Cs_2SO_4 density gradients but not in CsCl.

It has been suggested that dinoflagellate nuclei have a form intermediate between that of eukaryotes and the nucleoid of prokaryotes (1). This suggestion is based upon observations on the "coiled" or "banded" arrangement of DNA filaments in each of the hundred or so chromosomes per nucleus (1–3), the lack of cytochemically detectable histone-type protein associated with the DNA (1, 4), and the mechanism of nuclear division in these organisms (5), all of which resemble more closely properties characteristic of bacteria than of higher organisms. However, other aspects of nuclear structure and metabolism, such as the presence of a nucleolus and the processing of ribosomal RNA (6), and the appearance of the nuclear envelope in most species, are typically eukaryotic. Moreover, these organisms have a well-developed cytoplasm comparable with that found in other protophyta and protozoa (e.g., refs. 5 and 7).

Knowledge of the nature of dinoflagellate DNA, as it compares with that of prokaryotes and higher eukaryotes, is fundamental to a solution of the enigma dinoflagellate chromosomes present. In this article, some information is given indicating that the DNA of *Gyrodinium cohnii* has a complexity comparable with that in many eukaryotes. Emphasis is placed, however, on the fact that this complexity is superimposed upon another feature which, to the best of my knowledge, has been shown before only for certain bacteriophages—the contribution to total DNA of a large amount of an unusual base (8–10). In this case, an average of nearly 40% of the thymine is replaced by 5-hydroxymethyl-

uracil, and these bases are not uniformly interspersed throughout the DNA.

MATERIALS AND METHODS

Gyrodinium cohnii (a starter culture of which was kindly provided by Dr. D. Kubai) was grown axenically at 25°, with shaking, in flasks of AXM medium (11); cells were harvested at various stages in the growth curve by centrifugation. To obtain ^{32}P-labeled DNA, cells were grown for 4–6 doublings in the presence of ^{32}PO$_4$ (Amersham) at 2.5 μCi/ml.

Cells were lysed by freezing the cell pellet at −70°, then grinding them with a mortar and pestle in the presence of 0.05 M Tris·HCl (pH 7.6)–0.1 M EDTA. DNA was extracted from the viscous suspension with Sarkosyl and phenol–cresol (12). After further purification in CsCl or Cs$_2$SO$_4$, the DNA was pelleted, dissolved in 0.15 M NaCl–15 mM Na citrate or 0.01 M Na$_2$SO$_4$, then ethanol precipitated or dialyzed. The yield was up to 6 mg of DNA per 1-liter culture of stationary-phase cells.

Analytical Ultracentrifugation. Solutions of DNA in 0.02 M Tris·HCl (pH 8.0), were made to a density of about 1.715 gm cm^{-3} with CsCl. Runs (kindly performed by Dr. W. Hennig) were made in an M. S. E. Centriscan 75 ultracentrifuge (44,800 rpm, 25°, about 20 hr), and the buoyant density of *Gyrodinium* DNA was determined (13) relative to a marker of denatured *Pseudomonas aeruginosa* DNA (ρ = 1.737 gm cm^{-3}; ref. 14). The apparent G+C content was then determined according to Mandel *et al.* (13).

Preparative Ultracentrifugation was performed in a 60 Ti rotor at 40,000 rpm and 25° for about 60 hr, in gradients having a volume of about 11 ml. CsCl gradients had an initial density of 1.715 gm cm^{-3}. For Ag$^+$–Cs$_2$SO$_4$ gradients (12, 15, 16), DNA solutions were extensively dialyzed against 0.01 M Na$_2$SO$_4$; gradients were prepared to an initial density of 1.495 gm cm^{-3}, and an initial Ag$^+$ to DNA-phosphate ratio of 0.3.

Determinations of T_m were made in 15 mM NaCl–1.5 mM Na citrate on DNA that had been extensively dialyzed against the same solvent. Initial absorbances of about 0.25 were used. In determining the hyperchromicity at each of several elevated temperatures, the temperature of the cuvette holder was raised about 1° over a period of about 5 min with circulating water, and was held there a further 2 or 3 min to stabilize the temperature in the cuvettes before a reading was made and the melt was continued. A Zeiss PMQ II spectrophotometer, adapted to the purpose, was used to

Abbreviations: 5Me–dCMP and HOMedUMP, 5'-deoxyribonucleotides of 5-methylcytosine and hydroxymethyluracil; HOMeU, hydroxymethyluracil.
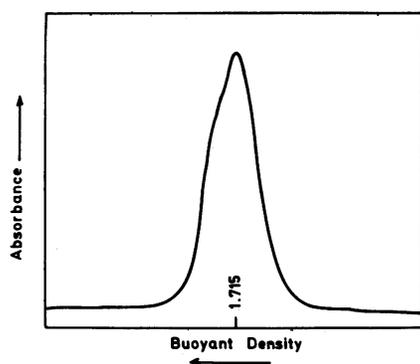
FIG. 1. Photoelectric scan of an equilibrium centrifugation of *G. cohnii* DNA in CsCl, the direction of the scan being from *right* to *left*. The peak buoyant density, as indicated, was determined in other gradients that included denatured *P. aeruginosa* DNA (ρ = 1.737) as a density marker. There is distinct asymmetry on both sides of the main band, indicative of at least three molecular populations.

obtain the melting curves. The temperature was monitored with a calibrated thermistor, the probe of which was in a separate cuvette filled with the same solvent. The absorbance at each temperature was corrected for thermal expansion of the solvent (17), and the apparent G+C content was determined from the midpoint of the hyperchromic shift by use of the equation provided by Mandel and Marmur (17). Derivative curves of the melting profiles were prepared according to Bernardi *et al.* (18).
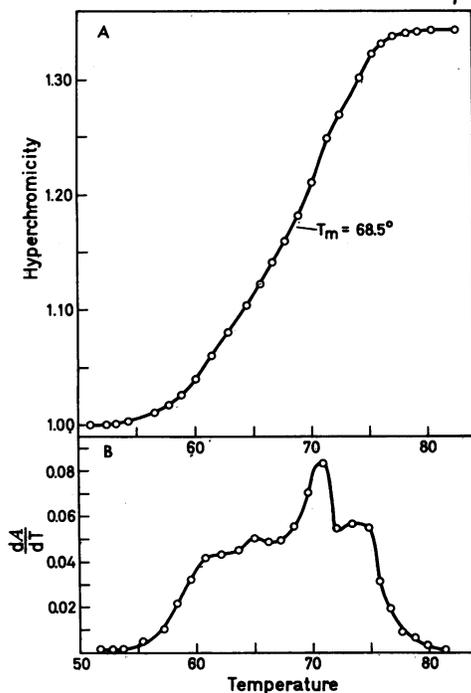

FIG. 2. (*A*) Melting curve of *Gyrodinium* DNA in 15 mM NaCl–1.5 mM Na citrate. The broad profile shows indications of heterogeneity in the melt. (*B*) Derivative plot of the melting curve: $y = \dfrac{A_{T1} - A_{T2}}{A_{max} - A_{min}} \Big/ (T1 - T2)$; $x = (T1 + T2)/2$. Such a treatment of the melting profile resolves it into at least four molecular populations.

Composition Analyses. Precipitated DNA was washed with ethanol and ether, dried under reduced pressure, and treated in one of the following ways: (*a*) *nucleotide analysis:* The DNA was dissolved at a concentration of 1 mg/ml in 0.01 M Tris·HCl (pH 7.6)–5 mM $MgCl_2$, and treated sequentially with DNase I and venom phosphodiesterase (both from Worthington), exactly as described by Ray and Hanawalt (19), except that each digestion was for at least 5 hr. Aliquots (usually 10–20 μl) of the digest were then spotted directly on 20 × 20 cm thin-layer plates of either unmodified cellulose (CEL 300; Macherey-Nagel & Co., Düren, Germany) or poly(ethyleneimine)-impregnated plates (CEL 300 PEI, from the same firm). Two-dimensional chromatography on unmodified cellulose plates, with the solvent front moving 15 cm from the origin in each direction, was performed with *iso*butyric acid–water–ammonia 66:20:1 and with saturated ammonium sulfate–1 M sodium acetate–*iso*propanol 80:18:2 (ref. 20). Ultraviolet-absorbing spots containing ³²P-labeled nucleotides were then excised and counted directly in toluene–PPO–POPOP. For UV spectrum analyses, spots of *Gyrodinium* nucleotides were excised and the UV-absorbing material was eluted with 0.1 N HCl onto filter paper (21). After overnight elution from this paper with the same solvent, spectra were determined in a Beckman DB spectrophotometer against appropriate blanks. The PEI plates were developed according to Randerath and Randerath's (21) procedure *2* for nucleotide mixtures. (*b*) *Base analysis:* 1 mg of DNA was hydrolyzed with 0.5 ml of 88% formic acid at 175° for 40 min (8). After rotary evaporation to dryness, the residue was dissolved in 0.1 ml of 1 N HCl. For R_F value determinations, 10-μl amounts were spotted on CEL 300 plates, and 5 μg each of uracil, thymine, and hydroxymethyluracil (Calbiochem) were spotted separately or together alongside. The plates were developed either in *iso*propanol–concentrated HCl–water 85:20.5:19.5 (ref. 22), or in *n*-butyl alcohol–acetone–water–ammonia 40:50:15:3 (ref. 9). For spectral analyses, 30 μl was spotted on washed Whatman
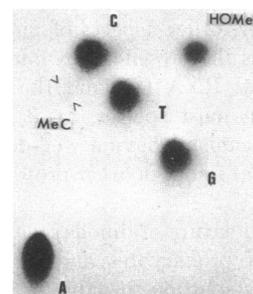

FIG. 3. Autoradiogram of [³²P]nucleotides from *Gyrodinium* DNA, chromatographed on a thin-layer cellulose plate. Chromatography in the first dimension (*right* to *left*) was with *iso*butyric acid–ammonium *iso*butyrate; the solvent for the second dimension (*bottom* to *top*) was ammonium sulfate–sodium acetate–*iso*propanol. The spot labeled HOMeU is that occupied by hydroxymethyldeoxyuridylic acid, identified by its spectral properties and the chromatographic behavior of the contained base. The 5-methyldeoxycytidylic acid spot (MeC) is bracketed by *arrowheads*. Radioactivity in this region amounts to 0.75% or less of the total. About 1500 cpm was applied to the chromatography plate, and autoradiographic exposure of the OSRAY (Agfa) x-ray film, tightly fixed to the chromatogram, was for 1 week.

no. 1 paper and run in the *iso*propanol–HCl solvent. The spots were excised and eluted with 0.1 N HCl; the eluate was measured in the spectrophotometer against blanks cut from the vicinity of the spots.

RESULTS

When DNA from *Gyrodinium cohnii* is centrifuged in a CsCl buoyant density gradient, the banding pattern shown in Fig. 1 is obtained. The absorbance profile is clearly non-Gaussian; rather, there is a prominent shoulder on the heavy side of the main band and a skewing on the light side. The peak buoyant density of *Gyrodinium* DNA in CsCl, 1.715 gm cm^{-3} relative to a marker of denatured DNA from *P. aeruginosa*, suggests an average G+C content of 56.1%.

Melting curves of *Gyrodinium* DNA yield broad hyperchromicity profiles, implying considerable heterogeneity in populations of DNA molecule (Fig. 2A). Indications for distinct phases in the melt are reinforced in the derivative plot of this curve (Fig. 2B), where four components can be discerned. Some of these likely correspond to DNA fractions causing asymmetry in the CsCl profile, but relationships have not been determined. The T_m of the DNA in 15 mM NaCl–1.5 mM Na citrate is 68.5°. According to the equation given by Mandel and Marmur (17), this represents a G+C content of 35.6%.

The discrepancy between the G+C content determined from CsCl (56.1%) and from the T_m (35.6%) could be accounted for in two ways. The most likely reason is that a large amount of an unusual base is present in the DNA (9, 10), a situation that heretofore had only been observed in certain bacteriophages. The second possibility is that the *sequence* of bases in a polynucleotide can have an influence on its density and melting properties (23). Some satellite DNAs in higher organisms show anomalous behavior with respect to density and melting characteristics, and in these cases the phenomenon appears to be a function of base sequence (16).

Although PEI-cellulose chromatography of [^{32}P]nucleotides showed essential equivalence of dAMP and dTMP, and of dGMP and dCMP, and a G+C content of about 41%, a possible explanation for the base-composition discrepancies

TABLE 1. *Gyrodinium* [^{32}P]*deoxyribonucleotide composition*

Nucleotide	Percent	Determinations*
dAMP	29.6 ± 0.3†	10
dTMP + HOMedUMP	29.2 ± 0.6	10
dGMP	20.9 ± 0.3	10
dCMP	20.4 ± 0.4‡	10
HOMedUMP	11.1 ± 0.3	4

*Three different DNA preparations were used, two of which were involved in HOMedUMP determination. Six of the determinations were done on PEI-cellulose plates before it was realized that HOMedUMP can be well separated by the conditions outlined in Fig. 4. With PEI plates, dTMP and HOMedUMP run very close together, giving an elongated "dTMP" spot.

†Values represent the means ± the standard errors.

‡This value can be considered as including 5-methyldeoxycytidylate, as it does in six of the 10 determinations. With the *iso*butyric acid–ammonium *iso*butyrate solvent, counts detectable in the 5Me-dCMP region amounted to 0.75% or less of the total.

TABLE 2. *Thin-layer chromatography of pyrimidines*

	R_F	
	HCOOH Hydrolysate	Reference
Thymine		
solvent A	0.85	0.85
solvent B	0.61	0.62
Uracil		
A	—	0.73
B	—	0.47
Hydroxymethyluracil		
A	0.67	0.67
B	0.35	0.35
Cytosine		
A	0.48	—
B	0.43	—

Reference compounds were from Calbiochem. Thin-layer plates were M-N CEL 300; the solvents were (A) *iso*propanol–concentrated HCl–water 85:20.5:19.5 and (B) *n*-butyl alcohol–acetone–water–ammonia 40:50:15:3, run for 10.5–11 cm beyond the origin.

was found with the chromatographic system used by Dawid *et al.* (20). With this system a fifth spot appears, migrating behind dTMP in the first dimension and ahead of it in the second (Fig. 3), and radioactivity in this spot plus the dTMP spot equals that in the dAMP spot. On PEI-cellulose, the fifth nucleotide chromatographs very similarly to dTMP. The nucleotide composition of *Gyrodinium cohnii* DNA is presented in Table 1 (5-methyldeoxycytidylic acid, a normal minor component of eukaryote DNA, is present in *Gyrodinium* DNA to the extent of about 0.75%, judging from ^{32}P counts recoverable from the appropriate region of chromatograms).

The fifth major component, which has been labeled HOMeU (hydroxymethyluracil) or HOMedUMP (hydroxymethyldeoxyuridylic acid), was determined to be such by its chromatographic and ultraviolet spectral properties:

(*a*) The behavior of this component in the two chromatographic systems used suggested dUMP (10) and HOMedUMP (9) to be likely candidates. Base chromatography was then performed with authentic uracil, hydroxymethyluracil, and thymine as R_F markers. The results are tabulated in Table 2, and show that the third major pyrimidine in a formic acid hydrolysate of *Gyrodinium* DNA comigrates with authentic HOMeU, while uracil and thymine migrate at considerably greater rates.

(*b*) According to spectral analyses summarized in Table 3, HOMeU and HOMedUMP from chemical and enzymatic hydrolysates of *Gyrodinium* DNA possess spectral properties consistent with these identifications.

(*c*) When the peak fractions from a CsCl gradient of *Gyrodinium* DNA (Fig. 4; 39.3% G+C, 40.1% substitution of T by HOMeU*, ρ = 1.715) are compared with unsub-

*In this instance, the HOMeU to T ratio is 0.67:1 (Fig. 4). The percent substitution of T by HOMeU, $\left(\dfrac{\text{HOMeU}}{\text{HOMeU} + \text{T}}\right) \times$

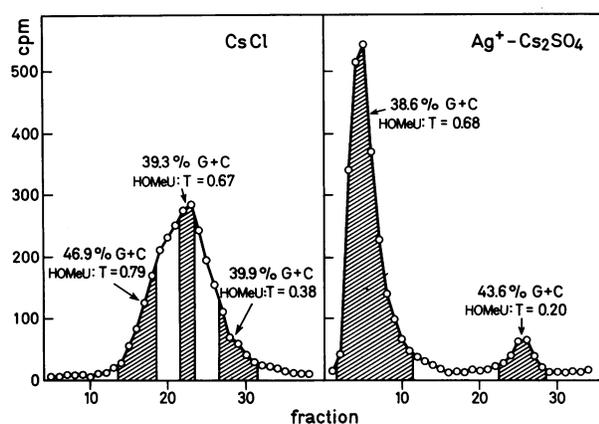100, is $\left(\dfrac{0.67}{0.67 + 1}\right) \times 100 = 40.1$.

Fig. 4. CsCl (*left*) and Ag$^+$-Cs$_2$SO$_4$ (*right*) density gradient centrifugation of ^{32}P-labeled *Gyrodinium* DNA. 0.3-ml Fractions were collected, from each of which 5 μl was counted to determine the profile. *Shaded* fractions were pooled, 200 μg of unlabeled *Gyrodinium* DNA was added to each as carrier, and the DNA was pelleted by centrifugation at 40,000 rpm for 15 hr. After the pellet was dissolved in 0.15 M NaCl-15 mM Na citrate, the DNA was precipitated with ethanol, then digested for nucleotide composition determination. The pelleting of DNA from the Ag$^+$-Cs$_2$SO$_4$ gradients was through 2 M NaCl in order to dissociate Ag$^+$-DNA complexes.

stituted DNA and phage SP8 DNA (HOMeU entirely replaces T, 43% G+C, ρ = 1.742), a linear relationship is found between the amount of substitution and the increase in buoyant density over that expected from the G+C content. The effect of HOMeU substitution on buoyant density follows the equation

$$\frac{\rho_{observed}}{\rho_{expected}} = 0.0236 \left(\frac{HOMeU}{HOMeU + T} \right) + 1,$$

where $\rho_{expected}$ is calculated from the equation ρ = 0.098 (G+C) + 1.660 (ref. 13). A linear relationship, but with steeper slope, also exists between halodeoxypyrimidine substitution and increase in DNA buoyant density (24).

The uniformity of HOMeU distribution in *Gyrodinium* DNA has been investigated by a determination of the HOMeU to T ratio in different buoyant density fractions. Base composition data from CsCl and Ag$^+$-Cs$_2$SO$_4$ gradients (Fig. 4) show that various populations exist with respect to HOMeU to T ratio, and that different ratios are not related to the overall base composition of the fractions. Populations very rich in HOMeU have not been detected, even with shearing of the DNA to low molecular weight before centrifugation. The DNA fraction having the *lowest* HOMeU to T ratio is a very-low-density satellite, amounting to about 10% of the total DNA, that appears in Ag$^+$-Cs$_2$SO$_4$ gradients (Fig. 4). In general, Ag$^+$ binds to G·C base-pairs to enhance buoyant density differences between G+C-rich and A+T-rich DNA (15). While the main band has a somewhat greater density than expected from its G+C content and the initial Ag$^+$ to nucleotide ratio, the low density of the satellite is not in keeping with its high G+C content and HOMeU to T ratio. However, when the satellite is recentrifuged in CsCl, it bands at a density of 1.708 gm cm^{-3}, consistent with its base composition (according to the equation above, DNA having 43.6% G+C and 16.7% replacement of T by HOMeU should

TABLE 3. *UV-Absorption properties of pyrimidines and nucleotides*

Pyrimidine	Absorbance in acid			
	A_{max}	A_{min}	250nm/260nm	280nm/260nm
Thymine*	264 nm	232 nm	0.54	0.68
Uracil†	257	226	0.18	0.87
Hydroxymethyluracil*	261	233	0.37	0.76
Hydroxymethyluracil†	261	232	0.36	0.75
Nucleotide				
dTMP*	267	240	0.75	0.65
dCMP*	280	241	2.08	0.42
HOMedUMP*	264	235	0.56	0.72
HOMedUMP‡	264	234	0.57	0.70

*Obtained from formic acid or enzymatic hydrolysates of *Gyrodinium* DNA.
†Standard obtained from Calbiochem.
‡Data taken from ref. 9.

band at 1.709 gm cm^{-3}). The reason the satellite binds an unexpectedly low amount of silver ion is not known.

DISCUSSION

In the DNA of higher organisms, dAMP, dTMP, dGMP, and dCMP account for about 95% or more of the total nucleotides. Plants have 3–6% of their DNA as 5Me-dCMP, replacing dCMP, and animals have less than half that amount; bacterial DNA can have about 0.5% 5-methylcytosine and 6-methylaminopurine as additions to the normal four bases (from data compiled by Shapiro, ref. 25).

Some bacteriophages are more bizarre with respect to their nucleotide compositions, and various large-scale substitutions have been reported. In the T-even series of coliphage, cytosine is replaced by 5-hydroxymethylcytosine (8), about 75% of which residues are glucosylated (26). Among phage that infect *Bacillus subtilis*, those in the PBS series have a complete substitution of thymine by uracil (10), and phage of the SP series replace thymine with hydroxymethyluracil (9).

About 37% of the expected thymine content of unfractionated *Gyrodinium* DNA is hydroxymethyluracil, but HOMeU to T ratios in fractions of different buoyant densities indicate that the distribution of this base is not uniform. In particular, a fraction that is also distinguished by an anomalously low affinity for silver ions contains only 17% of the bases complementary to adenine as HOMeU, while in the fraction of greatest buoyant density in CsCl, the value is 44%.

The complex nature of *Gyrodinium* DNA contributes to the enigmas in the structure of the dinoflagellate chromosome and its status in evolution. The proposition that these chromosomes (simply) reflect a step in the transition from prokaryote nucleoid to eukaryote nucleus is not supported by the HOMeU content of *Gyrodinium* DNA, a property hitherto restricted to some bacterial virus DNAs.† Data on the content

†The enzyme involved in the conversion of thymine to hydroxymethyluracil, thymine-7-hydroxylase, has been found in rat liver and *Neurospora*, where it appears to be involved in the production of RNA pyrimidines from thymine (27); hydroxymethyluracil has not been reported as being a component of the DNA.

and distribution of HOMeU suggest, in fact, that dinoflagellates possess rather special chromosomes, well out of the main line of evolution, but whether or not HOMeU is involved in the structure of the chromosome is not known.

The buoyant-density satellite appearing in Ag^+-Cs_2SO_4 gradients is of particular interest, not only for its anomalous physicochemical behavior, but also because it recalls *higher* organism satellite DNAs, which often exhibit similar behavior (16), that are localized in particular chromosome regions (constitutive heterochromatin; reviewed in ref. 28). The presence of such DNA in an organism without (typical) chromatin would present a novel situation for the evaluation of functional relationships between satellite DNA and the rest of the nucleus.

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