112. *Tetrahymena* Histone Sequences and their Implications for Phylogeny of Protozoa

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Histones are basic proteins associated with the nuclear DNA of eukaryotes. Usually, histones consist of five molecular species, H1, H2A, H2B, H3, and H4. Their amino acid sequences were first determined for calf thymus H4^{1),2)} and H2B,³⁾ and then for calf thymus H2A^{4),5)} and H3,⁶⁾ followed by the sequencing of these histones from other animals and plants. Thus, it has been found that only two of the total 102 amino acid residues are different between the calf and pea H4 sequences,⁷⁾ and four of the 135 residues between the calf and pea H3 sequences,⁸⁾ which are the largest differences among the known sequences of these two histones. However, we found that the H2A and H2B sequences are much more different between calf and pea.⁹⁾

The ciliate protozoan, *Tetrahymena* histones were first isolated by us,¹⁰⁾ followed by the fractionation and characterization of the five molecular species.¹¹⁾ We have recently revised the isolation and fractionation procedures, and have now sequenced the H4 totally and the H2B partially. Strikingly, 20 residues differ between the *Tetrahymena* and calf H4 sequences, in spite of the fact that the histones from higher eukaryotes are the most highly conserved protein family known.¹²⁾ Differences in the H2B sequence between *Tetrahymena* and calf are also larger than those between calf and pea. These findings strongly suggest that the divergence of protozoa and higher eukaryotes is substantially earlier than that of animals and plants.

Materials and methods. An amiconucleate strain GL of *Tetrahymena pyriformis* was used. Cultures reaching the stationary state were continuously layered, at a flow rate of 1 liter/10 min, on 1.0 M sucrose-3 mM CaCl₂-1 mM MgCl₂-10 mM Tris-HCl, pH 7.5 containing 0.2% (v/v) Nonidet P-40 (1 liter), placed in a Hitachi RPRC 18 rotor, during centrifugation at 10,000 rpm (*ca.* 10,000×g). This procedure omits the cell harvest step, removing the cell debris onto the surface of the sucrose solution and yielding nuclei as precipitate. The nuclei were further purified and washed with 0.15 M NaCl, as described previously.¹³⁾

From the washed nuclei histones were extracted with 0.25 M

HCl, and the extract was fractionated with 0.5 M HClO_4 as described.¹⁴⁾ The precipitate fraction containing H2A, H2B, H3, and H4 was chromatographed on Bio-Gel P-60 with 20 mM HCl -0.02% (w/v) NaN_3 containing 0.25 M NaCl . The H4 fraction was highly pure. The H2B fraction was further purified by elution from the same column without NaCl .

The sequencing of the H4 was done by manual Edman degradation of the intact protein and of the tryptic peptides separated by repeating column chromatography. The amino-acid phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography and/or thin-layer chromatography. The H2B was sequenced by Edman degradation of the peptides preferentially cleaved at arginine residues with Clostripain (Worthington) and separated by repeating column chromatography. The larger peptides obtained by limited chymotrypsin digestion were also used.

Results. Fig. 1 shows the *Tetrahymena* H4 sequence deduced from the structure of the tryptic peptides, arranged by assuming homology with calf thymus H4.^{1),2)} The amino terminal of *Tetrahymena* H4 is not blocked; thus the first 17 residues were confirmed by manual Edman degradation of the intact protein. The first 53 residues are also consistent with similar results by automated degradation reported by Glover and Gorovsky.¹⁵⁾ The sequence of residue 54 through to the carboxy terminal is presented here for the first time, with the finding of microheterogeneity that about 14% of the total population has an additional arginine inserted between residues 88 and 89.

The *Tetrahymena* H2B sequence is shown in Fig. 2, which was deduced from the structure of the Clostripain peptides, arranged by assuming homology with calf thymus H2B.³⁾ The amino terminal of *Tetrahymena* H2B is blocked; thus the first 28 residues are not completely sequenced, but the sequence is likely to differ largely from that of the first 34 residues of calf H2B. The subsequent region through to the carboxy terminal is relatively homologous between *Tetrahymena* and calf H2B, with the same number of 91 residues. Besides the major component described above, some minor H2B variants appear to exist.

Further experiments for confirming the arrangement of tryptic or Clostripain peptides are now in progress. Nevertheless, the proposed sequences are sufficient to discuss the implications for the phylogeny of protozoa.

Discussion. Fig. 3 shows the number of differences in the H4 and H2B sequences between mammals, pea, and *Tetrahymena*. These sequences are compared for the major component of each histone. The mammal sequences are the same between calf,^{1),2)} pig,¹⁶⁾ and

TH		5	10	15	20
	H-Ala-Gly-	-Gly-Lys-Gly-Gly-Lys-Gly-Lys-Met-Gly-Lys-Val-Gly-Ala-Lys-Arg-His-Ser-Arg-Lys-			
CT	Ac-Ser-Gly-Arg-Gly-Lys-Gly-Lys-Gly-Lys-Gly-Lys-Gly-Gly-Lys-Arg-His-				-Arg-Lys-
		25	30	35	40
	Ser-Asn-Lys-Ala-Ser-Ile-Glu-Gly-Ile-Thr-Lys-Pro-Ala-Ile-Arg-Arg-Leu-Ala-Arg-Arg-				
	Val-Leu-Arg-Arg-Asn-Ile-Gln-Gly-Ile-Thr-Lys-Pro-Ala-Ile-Arg-Arg-Leu-Ala-Arg-Arg-				
		45	50	55	60
	Gly-Gly-Val-Lys-Arg-Ile-Ser-Ser-Phe-Ile-Tyr-Asp-Asp-Ser-Arg-Gln-Val-Leu-Lys-Ser-				
	Gly-Gly-Val-Lys-Arg-Ile-Ser-Gly-Leu-Ile-Tyr-Glu-Glu-Thr-Arg-Gly-Val-Leu-Lys-Val-				
		65	70	75	80
	Phe-Leu-Glu-Asn-Val-Val-Val-Arg-Asp-Ala-Val-Thr-Tyr-Thr-Glu-His-Ala-Arg-Arg-Lys-Thr-				
	Phe-Leu-Glu-Asn-Val-Ile-Arg-Asp-Ala-Val-Thr-Tyr-Thr-Glu-His-Ala-Lys-Arg-Lys-Thr-				
		85	90	95	100
	Val-Thr-Ala-Met-Asp-Val-Val-Tyr-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly-OH				
	Val-Thr-Ala-Met-Asp-Val-Val-Tyr-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly-OH				

Fig. 1. Amino acid sequence of *Tetrahymena* (TH) histone H4, compared to that of calf thymus (CT) H4.^{1),2)} The two sequences have been aligned to obtain maximum homology. A minor variant (ca. 14%) of *Tetrahymena* H4 has an additional arginine inserted between residues 88 and 89, and thus contains a total of 103 residues.

rat^{17),18)} for H4, and between human,¹⁹⁾ calf,³⁾ and mouse²⁰⁾ for H2B. The five total sequences now available for H4 are all composed of 102 amino acid residues, while the calf, pea, and *Tetrahymena* H2B sequences contain 125, 132 or 133, and 119 residues, respectively. These H2B sequences can be divided into a variable (not homologous) amino-terminal region (34, 39 or 40, and 28 residues, respectively)

Fig. 2. Amino acid sequence of *Tetrahymena* (TH) histone H2B, compared to that of calf thymus (CT) H2B.³⁾ The two sequences have been aligned to obtain maximum homology. The amino-terminal sequence of *Tetrahymena* H2B is tentative.

and a conserved middle to carboxy-terminal region (91, 93, and 91 residues, respectively).

Fig. 3. Percent difference in the H4 and H2B sequences between mammals (see text), pea, and *Tetrahymena*. The data for the H4 sequences are indicated outside the triangle, and show differences in the total sequence. The data for the H2B sequences, indicated inside, represent differences in the conserved middle to carboxy-terminal region (see text).

For the H4 sequences, the differences of only two residues (2% in the total sequence) between calf and pea are already known.⁷⁾ Now we have found differences of 20 residues (20%) between *Tetrahymena* and calf and differences of 19 residues (19%) between *Tetrahymena* and pea. For the H2B sequences, we have already shown differences of 19 residues (21%) in the conserved region between calf and pea, in addition to differences of maximal 34 to 40 residues in the variable region.⁹⁾ Now, the corresponding differences of 28+(28/34) residues (31% in the conserved region) between *Tetrahymena* and calf, and differences of 32+(28/40) residues (35%) between *Tetrahymena* and pea have been found. Thus in both the cases of the H4 and H2B sequences, the homology between the mammal and the plant is clearly higher than that between the protozoan and the mammal or plant.

These data strongly imply that the divergence of the lines leading to protozoa and to higher eukaryotes is ancient and substantially antedates the divergence of the lines leading to higher animals and plants. A similar conclusion is reached from cytochrome *c* sequences, including those of *Tetrahymena pyriformis* and three flagellates, suggesting that the divergence of protista is more ancient than the animal/plant divergence, and that *Tetrahymena* appears to have diverged even earlier than *Euglena* and *Crithidia*, 1.4 billion years ago.¹²⁾ For more detailed analysis of very distant evolutionary events, more abundant histone sequences, particularly of lower eukaryotes, need to be determined.

Summary. The total and partial amino acid sequences of *Tetrahymena* histones H4 and H2B, respectively, have been determined. Comparisons of these sequences with those of mammals and pea sug-

gest that the divergence of protozoa and higher eukaryotes is more ancient than the divergence of animals and plants.

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