Nucleosome positioning in the somatic macronuclear genome of the ciliated protozoan *Tetrahymena thermophila* was analyzed by indirect end labeling. Nucleosomes were positioned nonrandomly in three different regions of the *Tetrahymena* genome. Nucleosome repeat length varied between adjacent nucleosomes. Nucleosome positioning in a histone H1 knockout strain was indistinguishable from that in a strain with wild type histone H1.

Native chromatin in eukaryotic cells is organized in nucleosomes consisting of 146 bp of DNA wrapped twice around an octamer of two each of the histone core proteins H2A, H2B, H3, and H4. The linker DNA between adjacent nucleosomes is associated with one molecule of a linker histone, usually histone H1. The nucleosome repeat length, the 146 bp of DNA associated with a histone octamer plus the linker DNA between adjacent nucleosomes, can vary with species, cell type, physiological state, and developmental stage, due to variability in the length of the linker DNA (1).

Nucleosomes are nonrandomly placed with respect to DNA sequence in diverse biological systems (2–4). Interactions between the nucleosome core octamer and signals in the nucleotide sequence are important determinants of nucleosome positioning (5, 6). In *vitro*, nucleosomes are tightly bound to certain sequences, called chromatin organizing regions (7, 8). It has been proposed that adjacent nucleosomes may then be aligned along the chromatin fiber in a regular array with reference to the chromatin organizing regions.

The role of linker histones in nucleosome positioning and alignment, if any, is not well understood. Early chromatin reconstitution experiments suggested that linker histones were responsible for spacing of adjacent nucleosomes and for spreading of nucleosome alignment along the chromosome (7, 9). However, *in vivo* experiments have failed to show direct correlation of changes in linker length with changes in linker histone type either in development or in artificial systems where the chromatin was challenged with novel histones (10–12).

A straightforward approach to determine whether linker histones are required for nucleosome positioning is to compare nucleosome positioning in a histone H1 knockout strain to that in cells with wild type histone H1. In most eukaryotes it is not possible to construct such strains because the protein is encoded by a family of repeated genes, which are often interspersed with the genes for the core histones.

The ciliated protozoan, *Tetrahymena*, contains two different nuclei, the germ line micronucleus and the transcriptionally active macronucleus. Micro- and macronuclear linker histones are encoded, respectively, by two unique genes, *MLH* (13) and *HHO* (14), both of which are transcribed in the macronucleus. Linker histone genes are nonessential in *Tetrahymena*. Strains in which the micronuclear and macronuclear linker histone genes have been knocked out, both individually and simultaneously, are viable and have normal fission rates (15). In the respective single knockout strains, the chromatin of the corresponding nucleus is visibly less condensed than in the parental strain with wild type linker histones.

In *Tetrahymena*, the only documented example of specific nucleosome positioning is on the rDNA minichromosome (16, 17). However, DNA reassociation experiments suggested that *Tetrahymena* nucleosomes may be positioned in relation to DNA sequences over the bulk of the *Tetrahymena* genome (18). The experiments described here extend the analysis of nucleosome positioning in *Tetrahymena* by analysis of the chromatin structure over specific sequences other than the rDNA minichromosome. Indirect end labeling experiments demonstrated that nucleosomes are specifically positioned in three different regions of the *Tetrahymena* macronuclear genome. Nucleosome repeat length appeared to vary with the particular nucleosome. No changes in nucleosome position or repeat length were detected in the absence of histone H1.

**Experimental Procedures**

**Cell Strains—** *Tetrahymena thermophila* strain CU428.1, *Mpr/Mpr* (6-methylpurine-sensitive, VII) and CU441, *ChxA/ChxA* (cytosine- methidime-sensitive, VI) of inbreeding line B were obtained from Peter Bruns (Cornell University, Ithaca, NY). *JH1*, a histone H1 knockout strain derived by disruption of the macronuclear *HHO* gene (15), was a generous gift from X. Shen and M. A. Gorovsky (University of Rochester, Rochester, NY). Strains were maintained in 1% and grown in 2% proteose peptone media prepared as described by Gorovsky *et al.* (19) and supplemented with penicillin/streptomycin. For *JH1*, 200 μg/ml paromomycin was added to the media for both strain maintenance and cell culture in order to ensure maintenance of the knockout gene.

**DNA Isolation—** Macronuclear DNA was isolated from CU428.1 and *ΔH1* by a modification of the method of Gorovsky *et al.* (19), as described by Capowski *et al.* (20).

**Micrococcal Nuclease Digestion of Nuclei—** *Tetrahymena* cells were grown to a density of 0.5–1.0 × 10⁶ cells/ml at 29 °C with shaking at 90 rpm. Nuclei were isolated from a 1.0–1.5-liter culture of cells by a modification of the methods described previously (21, 22). *Tetrahymena* were pelleted and resuspended in 90 ml of TMS (10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 3 mM CaCl₂, 250 mM sucrose) with 0.1 mM PMSF (phenylmethanesulfonyl fluoride) and 1 mM DTT (dithiothreitol) at 4 °C. The cells were lysed at 4 °C by the slow addition of Nonidet P-40 to a final concentration of 0.16% (v/v) with rapid stirring in a 250-ml glass beaker for 20 min at 4 °C. Sucrose was added to a concentration of 0.816 g/ml, and rapid stirring was continued for 50–60 min at 4 °C. The lysate was centrifuged in an HB-4 rotor at 9000 rpm (7500 × g) for 30 min at 4 °C. Pelleted nuclei were washed twice with cold Buffer A (15)

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† The abbreviations used are: bp, base pair(s); PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol.

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experiments in the map describe the fragments detected in the indirect end labeling experiments. Lines above and below the map describe the fragments detected in the indirect end labeling experiments in panels B and C, respectively, with the linker end of each successive fragment indicated by a vertical line along with the distance in kilobase pairs from each end to the restriction. B, indirect end labeling blot. MN, micrococcal nuclease digestion of nuclei (N) or purified DNA (D). Min., minutes of incubation in micrococcal nuclease buffer. Numbers to the left of the blot indicate the median size of fragments generated by micrococcal nuclease in lanes 1 and 3; numbers to the right indicate the sizes of fragments generated by digestion of those fragments with XbaI in lanes 2 and 4. C, indirect end labeling blot from the HindIII site. Notation is the same as in B.

mm Tris-HCl (pH 7.4), 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine (tri-HCl), 0.15 mM spermine (tetra-HCl), 2 mM CaCl2) at 6000 rpm for 2 min in a variable speed microcentrifuge and resuspended in a final volume of 1 ml of Buffer A containing 0.1 mM PMSF and 1 mM EDTA. DNA was quantitated by reading absorbance at 260 nm on a spectrophotometer and adjusted to a final concentration of 0.2 mg/ml with Buffer A containing 0.1 mM PMSF and 1 mM EDTA. Micrococcal nuclease (Worthington Biochemicals) was added to nuclei at a concentration of 30 units of micrococcal nuclease/mg of DNA. Digestions were performed at 30 °C for 3 and 6 min. The reaction was stopped by removing 2–5-ml aliquots of nuclei to 15-ml glass Corex tubes containing 15 ml EGTA and gentle mixing for 10 s. An equal volume of DNA preparation solution (20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% SDS) containing proteinase K (Promega) at 0.5 mg/ml was added, and samples were incubated at 65 °C for 5–16 h. Samples were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1) prior to precipitation in the presence of 0.33 volumes of 7.5 M NH4OAc and 2.5 volumes of EtOH at −20 °C. Control samples for endogenous nuclease activity consisted of intact nuclei incubated for 6 min at 30 °C in the absence of micrococcal nuclease and purified as described above. Control samples for micrococcal nuclease sequence specificity consisted of purified genomic DNA treated with micrococcal nuclease at a concentration of 15 units/mg of DNA for 3 or 6 min at 30 °C.

Southern Hybridization—Southern transfers were performed as described by Reed and Mann (23) with slight modifications. Following electrophoresis, TAE buffer was poured off, and gels were incubated in transfer buffer (5 M NaOH, 5 mM CaCl2) for 35 min with shaking. DNA was transferred onto GeneScreenPlus (Du Pont) as described for the “capillary blot procedure” in the GeneScreenPlus protocol manual except that the nylon membrane was placed in deionized H2O for 40 min, and then in transfer buffer for 2–5 min prior to transfer. Transfer was for 5–16 h. Following this, filters were rinsed for 40 min in neutralization buffer (2 M Tris-HCl (pH 7.0), 4 mM NaCl) with shaking. Filters were prehybridized at 65 °C for 5–16 h in 6× SSC (0.9 M NaCl and 0.09 M sodium citrate (pH 7.0)), 0.5% SDS, 1× Denhardt’s, and 0.1 mg/ml denatured salmon sperm DNA. Probes were random primer-labeled (24) and purified over a Sephadex G-50 (Sigma) spin column. Probe DNA was denatured by boiling for 10 min, placed on ice for 5–15 min, and then added to prehybridized filters at a concentration of 5 × 10⁶ cpml/ml of the prehybridization solution. Hybridizations proceeded for an additional 16–24 h at 65 °C with gentle shaking in a water bath or with rotation in a Hybaid oven. For indirect end labeling blots, both prehybridization and hybridization steps were performed at 55 °C. Following hybridization, filters were washed twice for 10 min with 2× SSC, then three times for 25 min with 2× SSC plus 1% SDS. All washes were performed at the hybridization temperature. The sizes of the hybridization fragments were estimated to the nearest 10 bp relative to fragments of pBR322 DNA digested with HindII on one side of the gel and Hi-Lo marker (Minnesota Molecular) on the other side.

RESULTS

Nucleosome Positioning in the Macronucleus of Tetrahymena—Indirect end labeling (25) was used to investigate nucleosome positioning at specific chromosomal sites in Tetrahymena. Three regions of the genome were selected for analysis as part of a study on the relationship between nucleosome positioning and DNA methylation. There is no known transcriptional activity at any of these sites; they are representative of generalized chromatin structure without modifications that might be associated with transcriptional regulation.

Nuclei were isolated from strain CU428.1 cells and digested

Fig. 1. Nucleosome positioning in Tlr1.R-B. A, restriction map of the macronuclear genome. H, HindIII; S, Sau3A; X, XbaI; bars, probes for the indirect end labeling experiments. Lines above and below the map describe the fragments detected in the indirect end labeling experiments in panels B and C, respectively, with the linker end of each successive fragment indicated by a vertical line along with the distance in kilobase pairs from each end to the restriction. B, indirect end labeling blot. MN, micrococcal nuclease digestion of nuclei (N) or purified DNA (D). Min., minutes of incubation in micrococcal nuclease buffer. Numbers to the left of the blot indicate the median size of fragments generated by micrococcal nuclease in lanes 1 and 3; numbers to the right indicate the sizes of fragments generated by digestion of those fragments with XbaI in lanes 2 and 4. C, indirect end labeling blot from the HindIII site. Notation is the same as in B.

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for three or six minutes with micrococcal nuclease. Micrococcal nuclease digestion occurred preferentially within linker DNA, generating the ladder of DNA fragments with the incremental size of approximately 200 bp characteristic of *Tetrahymena* chromatin (26).

The first region analyzed was originally cloned on a 4.0-kilobase pair *Bgl*II fragment, Tlr1.rB-B (27). Fig. 1A presents a partial restriction map of the macronuclear genome in this region. Fig. 1B shows an indirect end labeling experiment designed to assess nucleosome positioning to the left of the *Xba*I site. DNA from micrococcal nuclease-treated nuclei exhibited a ladder series of fragments due to preferential cleavage in linker DNA (Fig. 1B, lanes 1 and 3). Digestion of an aliquot of these samples with *Xba*I resulted in a new and specific pattern of fragments, consistent with specific nucleosome positioning over the macronuclear DNA in the Tlr1.rB-B region (lanes 2 and 4). The positions of linker DNA as determined in this experiment are indicated on the line above the restriction map in Fig. 1A.

Control experiments were performed to ensure that the banding pattern was attributable to digestion of the chromatin in linker DNA. To control for sequence preference of the enzyme, purified DNA was digested with micrococcal nuclease (lanes 5 and 6). In order to assess the level of endogenous nuclease activity, intact nuclei were incubated in the absence of micrococcal nuclease. The DNA was run on the gel without further treatment (lane 8) or after digestion with *Xba*I to determine the size of the restriction fragment (lane 7).

In order to confirm the positioning of nucleosomes assigned by the experiment in Fig. 1B, and to extend the localization of nucleosomes further to the right, an indirect end labeling experiment was performed (Fig. 1C) and probed with a DNA fragment near the *HindIII* site as indicated below the restriction map. As in the previous experiment, DNA from micrococcal nuclease-treated nuclei exhibited the ladder series of fragments due to preferential cleavage in linker DNA (Fig. 1C, lanes 1 and 3). Digestion of an aliquot of these samples with *HindIII* resulted in a small shift in the sizes of the hybridizing fragments (lanes 2 and 4). This new pattern maintained a discrete ladder and confirmed the positioning results, as indicated below the restriction map in Fig. 1A.

Evidence for specific nucleosome positioning was also found for two chromosomal regions originally cloned as sites of uniform DNA methylation, cyd1 and cyd2 (20). Fig. 2A presents a restriction map of the cyd1 locus. The indirect end labeling experiment shown in Fig. 2B revealed nucleosome positioning in the cyd1 region. Micrococcal nuclease treatment of the nuclei produced a series of fragments characteristic of internucleosomal cleavage of the DNA in chromatin (Fig. 2B, lanes 1 and 3). A ladder pattern of the hybridizing fragments was maintained after digestion with *HindIII* (lanes 2 and 4) indicating nucleosome positioning at the cyd1 locus. The ladder of hybridizing fragments detected with the cyd1 probe shifted only slightly upon digestion with *HindIII*, suggesting the *HindIII* site is close to or within linker DNA. Negative control samples of purified DNA treated with micrococcal nuclease showed that
the pattern of fragments observed did not result from micrococcal nuclease specificity for purified DNA (lanes 5 and 6). Similarly, a HindIII digest of DNA isolated from nuclei incubated in the absence of micrococcal nuclease demonstrated little to no endogenous nuclease activity within isolated nuclei (lane 7). Nucleosome positioning in this region was consistent in four independently isolated nuclei preparations (data not shown).

Fig. 3 shows the genomic restriction map and indirect end labeling experiment for the cyd2 region. The restriction enzyme used in this experiment, PstI, generates a 0.81-kilobase pair fragment in genomic DNA, allowing for the mapping of three linker regions and only two nucleosomes. Nonetheless, there is clear evidence for nucleosome positioning in this region.

Nucleosome Positioning in the Absence of Histone H1—Macronuclear histone H1 in Tetrahymena is encoded by the single copy gene, HHO (14). A knockout strain of the HHO gene, ΔH1, is viable, despite reduced chromatin condensation which is visible at the level of the light microscope (15). In order to determine whether the reduction in chromatin condensation in this strain is associated with a change in nucleosome positioning, indirect end labeling experiments were performed on the chromatin of the HHO knockout strain.

Indirect end labeling experiments of the Tlr1 and cyd1 regions are shown in Fig. 4, A and B, respectively. The sizes of the fragments were very similar to the sizes found in chromatin from the reference strain CU428.1 (Table I).

Analysis of the cyd2 region in chromatin from the ΔH1 cell line is shown in Fig. 4C. Lanes 1 and 3 contained DNA from nuclei incubated with micrococcal nuclease for 3 and 6 min, respectively. Aliquots of these samples digested with PstI (lanes 2 and 4) showed nucleosome positioning that is not significantly different from that in strain CU428. Lanes 5 and 6 contained DNA digested with micrococcal nuclease after purification from isolated nuclei. These samples displayed a discernible and reproducible pattern of bands. Thus there are preferred sites for micrococcal nuclease digestion in this region. However, those sites were not as susceptible to cleavage in chromatin, where the predominant cleavage is internucleosomal (lanes 2 and 4). The protection of inherently sensitive micrococcal nuclease sites in chromatin is one of the hallmarks of nucleosome positioning. The samples in lanes 7 and 8 showed little or no endogenous nuclease activity occurring under the conditions of incubation for micrococcal nuclease treatment. The DNA in lane 9 was digested with DpnI and PstI (DpnI digests a Sau3A site if the adenine residues are methylated). The coaggregation of this fragment with the smallest fragments in lanes 2 and 4 confirms the location of the Sau3A site in linker DNA (Fig. 3A).

The fragments detected by indirect end labeling in the ΔH1 knockout strain were strikingly similar in size to those from the wild type strain for all three regions examined (Table I). These experiments demonstrated that histone H1 is not required for nucleosome positioning in vivo.

### Table I

| Tlr | cyd.1 | cyd.2 |
|-----|-------|-------|
| CU428 | CU428 | CU428 |
| HindIII | HindIII | HindIII |
| XbaI | XbaI | XbaI |
| 0.22 | 0.34 | 0.23 |
| 0.18 | 0.21 | 0.21 |
| 0.18 | 0.20 | 0.21 |
| 0.19 | 0.17 | 0.16 |
| 0.17 | 0.20 | 0.20 |
| 0.24 | 0.26 | 0.20 |
| 0.21 | 0.20 |
| 0.35 | 0.35 |

**DISCUSSION**

Cot analysis previously suggested that nucleosomes are specifically positioned relative to DNA sequence in *Tetrahymena* (18). The data described here support that hypothesis and show specific nucleosome positioning for three separate regions of the macronuclear genome.

There appears to be considerable variability in the internucleosomal repeat length of adjacent nucleosomes in *Tetrahymena*. First, the bands of hybridization with the probes were sharper and had less lane background than the bands observed in the gels stained with ethidium bromide, suggesting that the variability in a particular region is less than the variability of nucleosome multimers over the genome as a whole (data not shown).

Second, the degree of sharpening of the hybridizing bands after digestion with the restriction enzyme suggests there is more variation between the fragments within a band than can be accounted for by imprecision of cutting of the micrococcal nuclease within the linker DNA. (Compare Fig. 1B, lanes 3 and 4, and Fig. 2B, lanes 3 and 4.) This would be expected if the four different fragments within the tetramer, for example, had different linker lengths due to variability in the length of the linkers of adjacent nucleosomes.

Measurements of DNA associated with individual nucleosomes support this interpretation. Although the nucleosome sizes determined in different experiments were quite repeatable, we measured internucleosomal distances ranging from 160 to 240 bp (Table I). In two cases, the internucleosomal distance appeared to be unusually large. In chromatin from both CU428.1 and ΔH1, there was about 350 bp of DNA between the fifth and sixth linker regions from the XbaI site in Tlr1.rB-B (Figs. 1B and 4A). Similarly, in blots of the cyd1 region, the distance between the last linker mapped and the HindIII site was 340 bp in both the presence and absence of histone H1 (Figs. 2B and 4B). These nucleosomes may have unusually long linkers or the regions may contain two closely packed nucleosomes such that the linker between them is relatively resistant to micrococcal nuclease. In any case, the data shown here do not support a model of equal spacing of nucleosomes between fixed boundaries. Instead, they indicate considerable variability in linker DNA length between adjacent nucleosomes. This may suggest that, in the absence of regulatory complexes that displace or reposition nucleosomes, the position of each individual nucleosome is determined by the most energetically favored position with respect to the associated DNA sequences (28).

The similarity of nucleosome positioning in strains with and without histone H1 suggests that macronuclear linker histones have a limited role, if any, in nucleosome spacing in vivo. One point to consider before reaching that conclusion is whether the macronuclear linker histones, encoded by the MLH gene, might substitute for histone H1, encoded by HHO. This is unlikely because for strains in which either the micro- or macronuclear linker histone genes have been knocked out, the chromatin decondenses only in the nucleus for which the corresponding linker histone gene has been deleted. Thus the two genes do not complement one another with respect to the chromatin condensation phenotype and, at least by this criterion, are not functionally redundant. The inability of the micro- and macronuclear linker histones to substitute for one another may be related to the fact that synthesis of the proteins is linked to DNA replication in the respective nuclei, which occurs at two different points in the cell cycle (29).

Micro- and macronuclear chromatin of *Tetrahymena* have
very different average internucleosomal repeat lengths of 175 and 202 bp, respectively (26). Prior to this study, that difference might have been ascribed to the very different structures of the micronuclear and macronuclear linker histones (13, 14, 30). Although histone H1 apparently does not determine nucleosome positioning in the macronucleus, we cannot eliminate the possibility that the unusual structure of micronuclear linker histones may contribute to the more compact nucleosome spacing in the micronucleus. However, differences also exist between the core histones of the two nuclei. Most notable of these in relation to generalized chromatin structure is the fact that a substantial proportion of the micronuclear histone H3 undergoes proteolytic processing, which removes six amino acids from the amino terminus (31).

Analysis of the function of histone H1 in vivo has been limited by the fact that, in most organisms, histones are encoded by a family of repeated and interspersed genes. Thus it is impossible to construct gene knockout strains. An obvious candidate for this kind of analysis is the yeast Saccharomyces cerevisiae. However, the question of whether or not yeast chromatin has histone H1 has been a matter of some debate. No linker histone proteins have been detected in yeast, but the gene HHO1 has homology to histone H1 genes of higher eukaryotes (32). Although recombinant HHO1 gene product had properties expected for a linker histone in chromatin reconstitution experiments, the absence of the HHO1 yeast protein in a knockout strain had no effect on the average nucleosome repeat length of bulk chromatin (33). The data presented here show that in Tetrahymena, where the presence of histone H1 is well established, there is similarly no detectable change in nucleosome repeat length in the absence of histone H1. Thus, it appears that in both Tetrahymena and yeast linker histones play no part in nucleosome positioning.

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