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Separation of coding sequences from structural DNA in the dinoflagellate *Crypthecodinium cohnii*

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Abstract

A protocol was developed that permitted mild restriction endonuclease digestion of intact nuclei isolated from the dinoflagellate *Crypthecodinium cohnii* while minimizing the activity of endogenous nucleases. These digestions released low molecular weight fragments of DNA but left considerable quantities of high molecular weight DNA uncut. Epifluorescent microscope examination of DAPI-stained nuclei before and after the digestions showed that the chromosomes and the nuclei remained intact throughout the incubations. Digestions of extracted, purified genomic DNA produced predominantly low molecular weight fragments. Southern blots of the two types of digestions (isolated nuclei, purified genomic DNA) were screened with three different cloned DNA probes: (1) heterologous dinoflagellate ribosomal DNA; (2) a single *C. cohnii* cDNA; and (3) a total *C. cohnii* cDNA library. The first two single-gene probes hybridized mainly to distinct fragments of low molecular weight material cleaved from the chromosomes in the isolated nuclei with relatively little labeling of the larger fragments of uncut DNA despite its high abundance. Comparable patterns were generally observed in blots of the purified genomic DNA. As expected, when the entire cDNA library was used as a probe of the two types of digestion, the hybridization signal was a broad smear including both low and high molecular weight DNA, with a few discrete bands visible as well. These data support the view that the main body of the dinoflagellate chromosome is structural or "silent" DNA, whereas coding sequences are preferentially accessible to mild restriction nuclease digestion, presumably due to their location at the periphery of the chromosomes on protruding filaments. The functional roles of these two classes of chromatin may thus be determined in part by their spatial organization. This differential accessibility to restriction enzymes makes it possible to design a cloning strategy that is optimized for dinoflagellate coding sequences.

Introduction

Of the many nuclear features that distinguish dinoflagellates from other eukaryotes, perhaps the most prominent is that dinoflagellates contain numerous permanently condensed chromosomes (Spector, 1984; Rizzo, 1987). This unusual compact configuration, plus the enormous DNA content (3–200 pg cell^{-1} versus 0.05–3.0 pg cell^{-1} in other unicellular eukaryotes) (Rizzo and Nooden, 1973), has led to the speculation that much of the DNA in dinoflagellate nuclei is not transcriptionally active (Babillot, 1970; Sigee, 1984). Evidence to date, much of it ultrastructural, has led to the hypothesis that the main body of the dinoflagellate chromosome consists of structural or "silent" DNA, with transcriptionally active sequences located on the periphery of this structure.

The first observations in this respect were those of Bouligand et al. (1968), Soyer (1969), and Soyer and Haapala (1973), who used a transmission electron microscope (TEM) to document fibers or filaments extending from the chromosomes into the nucleoplasm. In a more direct examination of the transcriptional activity of different DNAs, Babillot (1970) used [^{3}H]uridine and autoradiography to demonstrate that RNA synthesis occurred primarily in the nucleolus and on diffuse chromatin external

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to the condensed chromosomes in *Amphidinium carterae*, presumably on the protruding fibers. [^{3}H]adenine and high resolution electron microscope autoradiography were then used by Sigee (1984) to show that RNA transcription in *Prorocentrum micans* cells occurred on the DNA filaments and not on the structural DNA in the main body of the chromosomes. The cells used in that study were senescent, so it is not known how generally applicable the results are to other stages of growth.

Dinoflagellate chromosomes appear to be quite resistant to attack by nucleases, as suggested by Bodansky et al. (1979) and Herzog and Soyer (1981), who reported that only 10 to 13% of the DNA in isolated *Cryptothecodinium cohnii* and *P. micans* nuclei was digested by micrococcal nuclease. Taken together with the data of Sigee (1984) and others, these results suggest that the condensed DNA in the main chromosome body may be inaccessible to RNA polymerases and nucleases due to the compact configuration of that DNA. It is also possible that this condensed DNA does not contain coding sequences.

The general objective of this study was to conduct preliminary experiments to localize and separate dinoflagellate coding sequences from structural DNA. We demonstrated that coding sequences in intact nuclei are preferentially digested by restriction enzymes, and that bulk chromosomal DNA, which was inaccessible to these nucleases, contained few, if any, coding sequences. This differential accessibility was exploited to separate enriched fractions of restricted, clonable, coding regions. These efforts also provided an independent test of the claim that much of the dinoflagellate nuclear DNA is silent or structural in nature.

Results

To localize and separate coding sequences from structural DNA, specific procedures were developed for (1) isolation of dinoflagellate nuclei that were permeable to restriction enzymes while retaining the condensed state of the chromosomes; (2) digestion of the isolated nuclei with restriction enzymes and hybridization of the resulting digests with specific genes or complementary DNAs (cDNAs) to ascertain whether the easily cleaved, lower molecular weight fragments were enriched in expressed sequences; and (3) cloning of such fragments for future studies of gene structure and expression.

Isolation of nuclei

A number of different procedures were tested in the effort to obtain intact *C. cohnii* nuclei that would be permeable to restriction enzymes while retaining their native condensed chromosome configuration without visible changes from endogenous nucleases or salt effects. Initial attempts to use the isolation buffers of Rizzo and Nooden (1973) were unsatisfactory due to the activity of endogenous nucleases, which degraded the DNA and gave an unacceptable background of low molecular weight fragments in control (i.e., no enzyme added) incubations. For this reason, calcium was omitted from all buffer and sucrose cushion solutions and replaced by 10 mM $MgCl_2$. Small amounts of EDTA and EGTA were added to chelate contaminant calcium. After these changes, nonspecific digestion was further lowered to acceptable levels by reducing the incubation interval to 20 minutes at 37°C and by increasing the enzyme concentration to 160 U for each 2×10^6 nuclei (16 U/μg DNA). Furthermore, only enzymes capable of functioning in low salt buffer were used, because high salt concentrations (>25 mM NaCl) unwind the condensed DNA. Pretreatment of nuclei preparations with detergents was investigated as a means of partially disrupting the nuclear membranes, but this process proved to be unnecessary because the nuclei isolated with our standard procedure (which includes a Triton X-100 detergent wash) were permeable to restriction enzymes.

Epifluorescent observations of 4,6-diamidino-2-phenylindole (DAPI)–stained nuclei immediately after isolation showed that the typical condensed chromosome configuration was retained (data not shown). The DAPI staining highlighted individual chromosomes as small points of fluorescence clustered together within each nucleus. After 20 minutes of incubation at 37°C without restriction enzyme, the nuclei had not changed in size, nor had individual chromosomes lost their distinction as separate points of light. After 20 minutes of incubation with the restriction enzyme *Pvu*II at 37°C, the nuclei and chromosomes retained their intact appearance with perhaps a slight blurring of the chromosome intensity, presumably as a result of some loss of DNA fragments. *Pvu*II was a very effective enzyme for cleaving DNA from isolated *C. cohnii* nuclei (Figure 1). When *C. cohnii* chromosomes are physically sheared or exposed to high salt levels, the nuclear membranes rupture and DNA unwinds in a highly distended, fibrous configuration that was not observed under the conditions used for our digestions of isolated nuclei.

Digestion of purified genomic DNA and isolated nuclei

When purified *C. cohnii* genomic DNA was digested to completion with the enzymes *Pst*I and *Pvu*II,

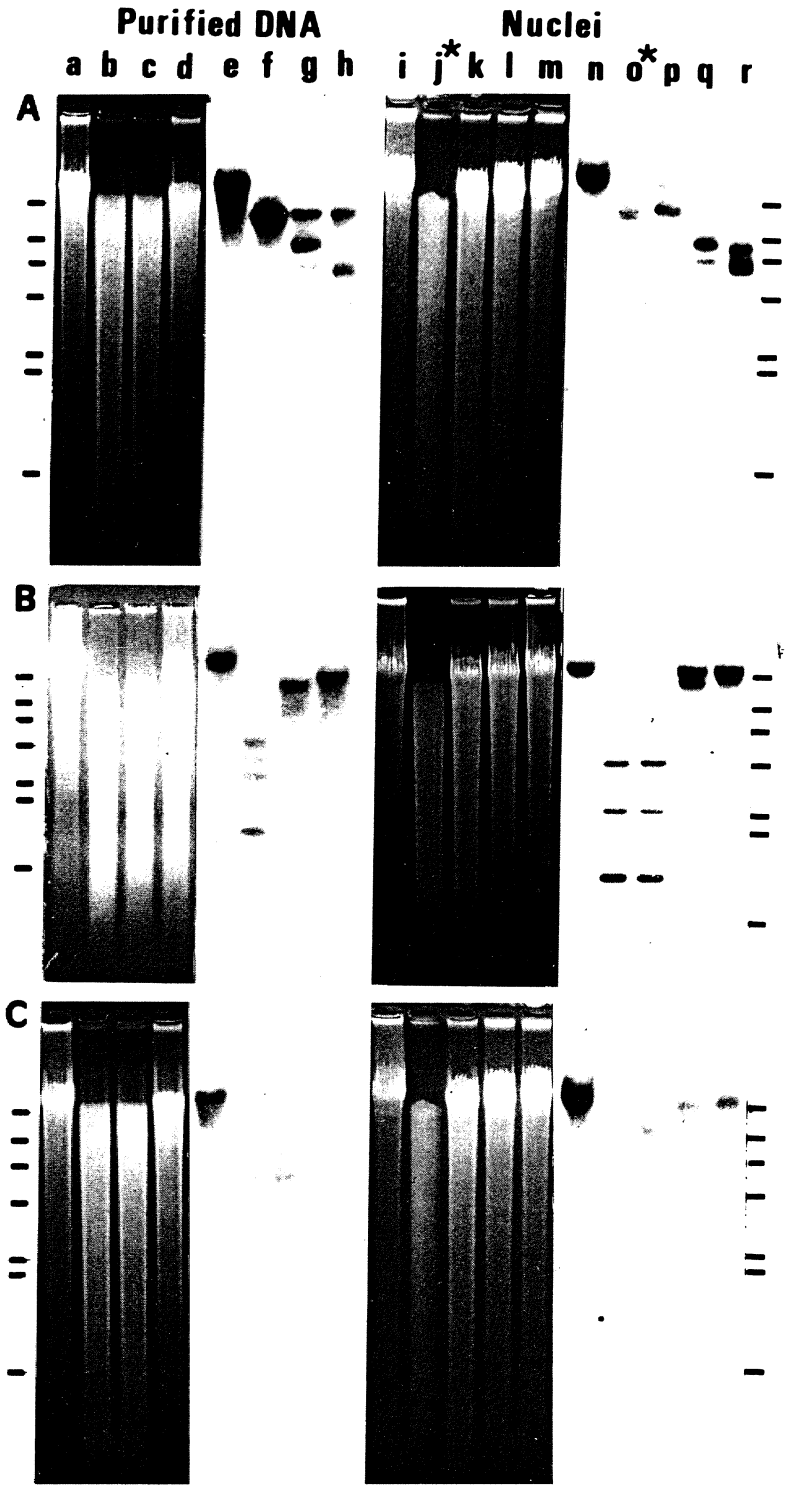

Figure 1. Agarose gel electrophoresis and Southern blot hybridization of various digestions of purified DNA (*left panel*) and isolated nuclei (*right panel*) from *Crypthecodinium cohnii*. (A) Probed with cloned ribosomal DNA from *Prorocentrum micans* (insert from plasmid *pM10H3.6*). (B) Probed with cDNA corresponding to a basic, nonhistone protein (HCcl) from *C. cohnii* (insert isolated from plasmid pHCc 1). (C) Probed with cDNAlib, the inserts of a *C. cohnii* cDNA library in phagemid pTZR19. The lane designations are as follows: a–d, ethidium bromide–stained gels of 10 μg purified genomic DNA with no added enzyme (a), or 100 U of *Pst*I (b), *Pvu*II (c), or *Kpn*I (d) digested overnight to completion. Lanes e–h show autoradiographs of filters obtained from the gels to the immediate left (a = e, b = f, c = g, d = h). Lanes i, k, l, and m are from digestions of isolated nuclei (approximately 8–10 μg DNA per lane). Lane j* is a purified genomic DNA control digested with *Pst*I, used as an internal standard. Lanes i, k, l, and m are digestions of nuclei using no enzyme, *Pst*I, *Pvu*II, and *Kpn*I, respectively. Lanes n–r are autoradiographs of hybridized Southern blots corresponding to the gels to the immediate left (i = n, j* = o*, k = p, l = q, m = r). *Hin*dIII fragments of bacteriophage lambda DNA are depicted to the left and right of the figure, from top to bottom: 23,130; 9,416; 6,557; 4,361; 2,322; 2,027; and 564 base pairs.

much of the initial high molecular weight material was transformed into smaller fragments that produced a continuous smear on ethidium bromide–stained gels; repeated sequences were visible as faint bands (see Figure 1, lanes b and c). Digestions with *Kpn*I mainly yielded high molecular weight fragments (see Figure 1, lane d). In contrast, mild digestions of isolated nuclei produced small DNA fragments, but the bulk of the material remained as high molecular weight fragments (see Figure 1, lanes

k–m). As a control, purified genomic DNA digested to completion with *Pst*I was added to these gels (see Figure 1, lane j*). *Kpn*I was again the least effective enzyme with respect to the production of small fragments. Endonuclease activity was reduced to acceptable levels, as evidenced by the low background of small fragments in the gel lanes for control incubations of nuclei with no added enzyme (see Figure 1, lane i).

Filter hybridizations using probes for coding sequences

When probed with cloned ribosomal DNA (rDNA) from *P. micans*, purified genomic DNA digested with the enzymes *Pst*I, *Pvu*II, and *Kpn*I revealed several distinct bands (see Figure 1A, lanes f–h, o*) that had migrated below the highest molecular weight components. In control digestions without added enzyme (see Figure 1A, lane e), the target sequences were all in the high molecular weight portion of the gel. When isolated nuclei were digested and hybridized with the rDNA probe (see Figure 1A, lanes n, p–r), the signals were essentially visible only in the lower molecular weight fragments and not in the large amount of slowly migrating high molecular weight DNA (see Figure 1A). Surprisingly, *Pvu*II and *Kpn*I were more effective in cutting rDNA in isolated nuclei than in purified genomic DNA, as evidenced by an increase in labeled low molecular weight fragments (e.g., compare lanes g and q or h and r of Figure 1A). This finding might be due to differences in the level of rDNA methylation between the genomic DNA preparation and the isolated nuclei used in this experiment.

When the filters were probed with HCcl cDNA encoding a nonhistone basic protein from *C. cohnii*, the banding patterns from mild digestions of isolated nuclei were essentially identical to those of the complete digestion of purified genomic DNA (Figure 1B). There were more *Pst*I restriction sites on this gene compared with the other enzymes, yielding 4 distinct bands in the digestions of both purified genomic DNA (see Figure 1B, lanes f and o*) and the isolated nuclei (lane p). In fact, with this enzyme, the labeling pattern from an overnight digestion of purified genomic DNA was identical to that from a short digestion of isolated nuclei. Fewer bands were observed with *Pvu*II and *Kpn*I digestions, yet the results still indicate that the HCcl-encoding DNA in isolated nuclei is accessible to digestions by these enzymes because the hybridization signals were slightly lower on the filters (see Figure 1B, lanes q and r) than for the uncut nuclear DNA (lane n).

When an entire cDNA library (cDNAlib) from *C. cohnii* was used as a probe, hybridization signals for both the purified genomic DNA and the isolated nuclei appeared as long smears with a few discrete bands, presumably corresponding to high copy number genes (Figure 1C). Some hybridization was also observed in the higher molecular weight material near the top of each lane of the nuclei digestions (see Figure 1C, lanes p–r), although the signal was considerably weaker than in the uncut nuclei control (lane n). *Kpn*I, which is very sensitive to DNA contaminants, was the least effective in cleaving the high molecular weight material (lane r).

Discussion

Two long-standing questions in dinoflagellate physiology are why these cells contain such enormous quantities of DNA and how they manage to replicate and transcribe that DNA given its highly condensed *in vivo* configuration. A growing body of ultrastructural evidence suggests that much of the dinoflagellate DNA is inactive or structural in nature, with transcriptionally active regions located on DNA filaments extending from the main body of the chromosome into the nucleoplasm (Babillot, 1970; Sigee, 1984). Furthermore, a current model of dinoflagellate DNA chromosome structure suggests that the DNA is configured as a helical array of nucleofilaments in a hierarchy of six levels of compaction, with little or no involvement of basic proteins (Herzog and Soyer, 1983; Herzog et al., 1984). This model provides an explanation for the replication and transcription of permanently condensed chromosomes by invoking local unwinding of select portions of the DNA. Our objective was to use molecular methods to examine the general location and accessibility of dinoflagellate coding sequences. In addition, we attempted to physically separate coding sequences from structural DNA, working from the assumption that accessibility to RNA polymerases (and, by analogy, restriction enzymes) would be determined in part by the architecture of the permanently condensed chromosomes. We reasoned that a mild restriction enzyme digestion of isolated nuclei would cleave protruding and surface DNA fibers of the chromosome into small fragments, leaving the bulk of DNA essentially untouched. Therefore, probing the resulting Southern blots with various cDNA or rDNA sequences should reveal whether the coding sequences are located in the lower molecular weight (accessible) or high molecular weight (inaccessible) fragments at the

bottom and top of the gels, respectively. Moreover, in the ideal case where all coding sequences are readily accessible and presumably exposed, the hybridization pattern from digestions of isolated nuclei should be quantitatively equivalent to that observed from complete digestions of purified genomic DNA, even though considerable amounts of unlabeled DNA remain as large fragments.

Consistent with this concept, we observed that the two single probes (pHCcl and pM10H3.6) hybridized almost exclusively to the smaller DNA fragments produced by the restriction enzymes (see Figure 1A, B), despite the fact that a considerable quantity of DNA from the digestions of isolated nuclei remained as high molecular weight material near the top of the gels. This finding was especially clear when genomic DNA and isolated nuclei were digested with *Pst*I and probed with pHCcl. The banding patterns were the same and of equal intensity (see Figure 1B, lanes o* and p), even though the ethidium bromide stain clearly showed that much of the DNA in the isolated nuclei remained uncut, whereas the genomic DNA had been digested to completion (see Figure 1B, lanes j* and k). Clearly, no copies of this gene were present in the large quantity of high molecular weight DNA left undigested in the nuclei preparations. These results are consistent with our hypothesis that coding sequences are far more accessible for restriction enzyme digestion than noncoding DNA.

The results from cDNAlib (see Figure 1C) deserve special comment. The diffuse hybridization signal over a broad range of molecular weights is to be expected because this complex probe represented a variety of unequally expressed genes. Hybridization should thus occur on restriction fragments of many different sizes, and banding intensities should vary with the level of the different messenger RNAs present in the cells when the cDNA library was constructed. This finding is in fact what was observed for both the isolated nuclei and the genomic DNA. With isolated nuclei, however, additional high molecular weight signals were observed (see Figure 1C, lanes q and r). Several possible explanations can be offered to explain this result, which at first appears to contradict our hypothesis. First, nonexpressed multiple copy genes or pseudogenes, possibly methylated, would not be accessible to digestion but would be labeled by the cDNAlib probe. Alternatively, due to the nonsynchronous nature of the cultures used in the nuclei isolations, some cells would have been in stages of division, DNA replication, or sexuality that could have protected certain genes from restriction enzyme cleavage. Unfortunately, no methods are known at present that permit synchronization of *C. cohnii* cultures. A final alternative is that this labeling of high molecular weight fragments from nuclei digestions may reflect the differential accessibility of the 99 *C. cohnii* chromosomes (Kubai and Ris, 1969) under the experimental conditions used. For example, chromosomes located near the center of the nucleus may be more slowly attacked by restriction enzymes than those on the periphery, yielding coding sequences in high molecular weight fractions. Results from the complex cDNA library are thus more difficult to interpret, yet are still consistent with our general hypothesis.

Bodansky et al. (1979) and Herzog and Soyer (1981) found that only 10 to 13% of the DNA in isolated dinoflagellate nuclei was accessible to micrococcal nuclease. A comparable situation was observed in this study; the ethidium bromide–stained gels showed a 4- 5-fold excess of uncut DNA of the isolated nuclei at the top of each lane relative to the smaller fragments below, whereas the purified genomic DNA was completely digested into small fragments using the same enzymes. These results argue that much of the DNA in dinoflagellate chromosomes is inaccessible to a variety of nucleases.

Because little if any cutting of the bulk DNA in the main chromosome bodies occurred during short digestions of isolated nuclei, and because our single cDNA and rDNA probes generally hybridized to lower molecular weight DNA fragments, we conclude that the coding sequences for those expressed genes were most likely external to the main chromosome bodies.

The methods described herein not only allowed us to confirm in a novel way the models that relate genome function to dinoflagellate chromosome architecture, but also allowed considerable enrichment of coding sequences, easily clonable in standard vectors. There is an obvious advantage to using this approach to obtain DNA directly from nuclei digestions, rather than relying on RNA extraction, polyadenylate selection, cDNA generation, and cloning. Furthermore, the screening of a coding sequence–enriched library produced using our methods would be much easier than the time-consuming screening of complete genomic libraries, especially when the targets are single or low copy number genes. This simplicity is particularly pertinant to the dinoflagellates, which have a genome size ranging from 10^9 to 10^{11} base pairs.

The general view of the dinoflagellate genome that emerges from this study is that most of the

nuclear DNA is noncoding. From an evolutionary standpoint, it remains a mystery why dinoflagellates would maintain so many permanently condensed chromosomes, because the nutritional and metabolic costs of synthesizing and replicating such large quantities of inactive DNA must be significant. We may not understand why dinoflagellates have so much DNA, but it appears that coding sequences are located external to the main chromosome bodies, allowing efficient gene regulation and expression. It is noteworthy that dinoflagellate chromatin is devoid of histones and nucleosomes (Rizzo, 1987). In this context, the presence of low levels of nonhistone basic nuclear proteins in dinoflagellates (the cDNA for which was recently cloned; Sala-Rovira et al., 1991) may function in a fundamentally different fashion from histones in other eukaryotes. Future combinations of immunocytochemistry to localize histone-like proteins (Geraud et al., 1991) and in situ hybridization to localize specific coding sequences will do much to explain the enigmatic structure of dinoflagellate chromatin.

Experimental Procedures

Isolation of nuclei

The heterotrophic dinoflagellate *C. cohnii* (strain WHd) was grown at 27°C in the dark in MLH medium (Tuttle and Loeblich, 1975). Typically, three 1-liter cultures were grown to a cell density of 3×10^5 cells mL^{-1} over 72 hours. Cells were harvested and carried through all subsequent steps up to the restriction enzyme incubations either on ice or in refrigerated centrifuges at 0 to 4°C.

The initial cultures were centrifuged in 500-mL bottles (10 min, 2,500 × g), and the pellet was rinsed with sterile, 0.2 μm filtered seawater. Centrifuging and washing were repeated several times until the supernatant was clear. The final pellet was resuspended in 30 mL of sterile filtered seawater, to which 3 mL of 10% Triton X-100 were added. After 10 minutes on ice with stirring, this mixture was layered onto two tubes, each containing 20 mL of 1.6 mol sucrose. (All sucrose solutions described herein contained 10 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethyl sulfonylfluoride [PMSF], and 10 mM Tris [pH 7.5]). These tubes were centrifuged at 3,200 × g for 10 minutes to concentrate intact cells. The pellets were combined in 50 mL of isolation buffer consisting of 10 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM EDTA, 1 mol hexylene glycol, 1 mM DTT, 1 mM PMSF, and 10 mM Tris (pH 7.5). This cell suspension was passed through a French Press at 1,500 pounds/in.^{2} and then filtered gently through 10 μm Nitex mesh. Triton X-100 was added to a final concentration of 0.8%, and the mixture was stirred for 10 minutes. This mixture was then divided into 4 aliquots, each of which was layered onto a step gradient (2.5 mL of 2.4 mol sucrose under 15 mL of 1.6 mol sucrose) and centrifuged at 4,200 × g for 20 minutes. The upper layers were removed by aspiration to a level just below the 1.6–2.4 mol interface. Material adhering to the tube wall at that interface was not removed because it contained numerous nuclei. The pellets and the residual material in the lower layer were combined and diluted with isolation buffer to 30 mL, divided in half, and then layered and centrifuged through the 1.6 and 2.4 mol sucrose cushion as previously done. The pellets were combined, resuspended in a total of 10 mL of low salt buffer (hereafter "L" buffer) containing 10 mM $MgCl_2$, 10 mM DTT, and 10 mM Tris HCl (pH 7.5), and centrifuged at 3,000 × g for 5 minutes. After removing the supernatant, the pellet was again resuspended and washed in L buffer. During this last wash, a 10-μL subsample of the resuspended nuclei was diluted with 10 μL DAPI (0.5 μg mL^{-1}) in 80 μL isolation buffer and the nuclei were counted in a Fuchs Rosenthal hemacytometer using an epifluorescence microscope. On the basis of the nuclei count, the pellet was then resuspended in sufficient L buffer so that 150 μl would contain 2×10^6 nuclei (approximately 8 μg DNA; Allen et al., 1975).

Isolation of genomic DNA

Total genomic DNA was obtained from late log-phase cells by isolating nuclei as described, except that the isolation buffer and the sucrose cushions contained 1 mM spermidine and 0.5 mM spermine instead of 10 mM $MgCl_2$. After being passed through the French Press, nuclei were counted as described and the suspension passed through a 1.6 mol sucrose cushion. The pellets were combined in 18 mL of 1 mol Tris (pH 7.5) and 20 mM EDTA and incubated at 60°C for 10 minutes. NaCl was added to 0.5 mol ribonuclease I (RNase) (20 μg mL^{-1}) and the mixture was incubated at 37°C for 30 minutes. SDS and proteinase K were added to final concentrations of 0.5% and 200 μg/ml of the mixture, respectively, and incubated at 37°C overnight. The mixture was gently extracted with phenol and chloroform, then chloroform alone, and the clear supernatant was combined with 0.6 vol isopropanol. The precipitate was washed with 70% ethanol and resuspended in

trace elements (TE) for several hours at 37°C until it dissolved. The DNA was purified in a CsCl gradient (Maniatis et al., 1982), dialyzed in TE, and quantified by absorbance at 260 nm. Approximately 800 μg of DNA was obtained from 10^9 cells.

Digestions and DNA extractions

Isolated nuclei and genomic DNA were digested with Boehringer Mannheim restriction enzymes *Pst*I, *Pvu*II, and *Kpn*I. Typical nuclei reaction concentrations were 2×10^6 nuclei (approximately 8–10 μg DNA) in 150 μL L buffer containing 160 U enzyme (4 μL at 40 U μL^{-1}). Concentrated enzymes were used to ensure that the glycerol in the stock enzymes was diluted to noninhibitory levels. After 20 minutes at 37°C, the nuclei digestions were terminated with 20 mM EDTA at 65°C for 10 minutes. The mixture was adjusted to final concentrations of 0.5% SDS and 200 μg ml^{-1} proteinase K and incubated at 37°C for 2 hours. The digests were gently extracted with phenol and chloroform, sodium acetate was added to 0.3 mol, and DNA was precipitated in 2 vol 95% ethanol. The tubes were held at −80°C for 30 minutes, and then centrifuged at 12,000 revolutions/min for 15 minutes. The pellets were washed with 70% ethanol, resuspended in 50 μL TE, and stored overnight at 4°C. When dissolution was complete, RNase was added to a final concentration of 20 μg ml^{-1} and the tubes were incubated at 37°C for 30 minutes.

Control digestions of purified DNA were also performed. Typical reaction volumes were 800 μL, containing 80 μg DNA (0.32 μg μL^{-1}) and 800 U restriction enzyme in L buffer. This mixture was incubated overnight at 37°C; additional enzymes (200 U) were added in the morning and the mixture underwent seven hours of additional incubation. The reaction was terminated with 20 mM EDTA for 10 minutes at 65°C, after which the DNA was precipitated, washed, and resuspended in TE as described to a final concentration of 0.2 μg μL^{-1}.

Electrophoresis and Southern blotting

Eight to 10 μg aliquots of uncut or digested DNA were heated to 65°C for 10 minutes and run horizontally on 0.8% agarose gels. After 4 hours at 60 to 80 V, the gels were stained with ethidium bromide and photographed. DNA on the gels was then transferred to nitrocellulose using the Southern blotting procedures described in Maniatis et al. (1982). Staining of the gels after the blotting confirmed that nearly all of the DNA was transferred to the filters.

Isolation of inserts and labeling of probes

The Southern blots were screened with three DNA probes. One, hereafter designated cDNAlib, is the entire cDNA library produced from *C. cohnii*, cloned into the phagemid vector pTZ19R (Sala-Rovira et al., 1991). Another, designated pHCcl, contains the cDNA corresponding to a basic, non-histone protein (HCcl) from *C. cohnii*, isolated from the cDNA library (Sala-Rovira et al., 1991). The third, pM10H3.6, contains a ribosomal RNA cistron from the dinoflagellate *P. micans* in vector pBR322 (Herzog and Maroteaux, 1986). It is a 3.6 Kb sequence coding for the 17S, 5.8S, and 24S rRNAs. Bacterial clones containing the inserts of interest were grown in LB medium and the plasmids were extracted and purified by CsCl gradient centrifugation. The inserts from the pTZ19R phagemids (cDNAlib and HCcl) were then cut out by restriction enzyme digestions of 10 μg plasmid DNA using *Bam*H1 and *Sph*I. The ribosomal rDNA insert was obtained by *Hin*dIII digestion of the pM10H3.6 plasmid. Fragments were separated on a gel and the inserts of interest electroeluted onto NA45 filter paper. The filters were washed in 1 mM EDTA, 20 μm Tris, and 0.15 mol NaCl, then placed in 250 μL of 1 mM EDTA, 20 mM Tris, and 0.5 mol NaCl at 55°C for 2 hours, thus allowing DNA to elute. Ethidium bromide was removed with isoamyl alcohol, and the DNA was purified with phenol and chloroform extractions. Approximately 2.0 to 4.5 μg of inserts were obtained from 10 μg plasmid DNA depending on the relative sizes of the insert and plasmid vector. The inserts were labeled with ^{32}P using the Boehringer Mannheim Random Primed DNA Labeling Kit (50 μCi deoxycytidine triphosphate [dCTP] for 0.1–0.2 μg DNA). Unincorporated dCTP was removed using the spun-column procedure of Maniatis et al. (1982).

Hybridizations and autoradiography

Prehybridization and hybridization conditions were generally those of Maniatis et al. (1982). The prehybridization solution (200 $\mu l/cm^2$ of filter) consisted of 6 × SSC, 50% formamide, 0.5% SDS, 5 × Denhardt's solution, and 200 μg ml^{-1} denatured salmon sperm DNA. The hybridization solution was as described with the addition of the ^{32}P-labeled denatured probe DNA and 10% dextran sulfate. Typically, 100 μL hybridization solution was added for each cm^2 of filter; the probe was adjusted to approximately 10^6 counts/min ml^{-1}. Filters were prehybridized overnight in a shaking water bath at 42°C and then hybridized for 16 to 20 hours at 42°C. The filters were washed with 1 × SSC and 0.1% SDS

two times, each for 15 minutes at room temperature, followed by 0.1 × SSC and 0.1% SDS at 68°C, two times, each for 15 minutes, and then several 15-minute washes in 0.1 × SSC and 0.5% SDS at 68°C. Filters were air dried and then exposed to radiographic film for autoradiography.

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