Basal body-associated DNA: *In situ* studies in *Chlamydomonas reinhardtii*

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ABSTRACT We have explored the localization of the *uni* chromosome (LG XIX) of *Chlamydomonas reinhardtii* using the technique of *in situ* hybridization. Using standardized methods of cell fixation together with large chromosome-specific probes we have studied the position of *uni* DNA sequences in metaphase and interphase cells. We find that in dividing cells *uni* probes identify a condensed metaphase chromosome that shows no specialized orientation. In interphase cells *uni* hybridization signals occur on the anterior edge of the nucleus at a position where basal bodies are normally associated with the nuclear envelope. These data reveal an underlying spatial organization of *uni* chromosomal DNA within the interphase nucleus that may be significant in terms of the fact that this chromosome encodes numerous functions affecting basal body and flagellar assembly.

The *uni* linkage group in *Chlamydomonas reinhardtii* is rich in mutants that affect development of basal bodies and centrioles (1). It is clear from molecular analysis of one of these mutants at the *FLA10* locus that much will be learned about basal body function and flagellar assembly from characterization of the genes and gene products corresponding to *uni*-linked mutants (2). The density of markers relating specifically to basal body function led us to explore the *in situ* localization of *uni* chromosomal DNA. We found that DNA probes derived from the *uni* chromosome hybridized at basal body sites in paraformaldehyde-fixed cells (3). Although these signals were specific, the sensitivity of this technique for nuclear hybridization was too limited to provide insight into a possible nuclear localization of *uni* sequences.

In the present study we have reexamined the localization of *uni* DNA using conditions that allow complete access to nuclear DNA. We have pursued this analysis with mitotic cell populations from synchronized cultures and with interphase cells. We have employed *uni*-specific and non-*uni* probes in the form of yeast artificial chromosomes (YACs) whose large target size allows unambiguous identification and orientation of *in situ* hybridization signals. These experiments demonstrate that in dividing cells *uni* DNA forms a metaphase chromosome with no apparent orientation relative to the other *Chlamydomonas* chromosomes. This is in contrast to the picture in interphase cells. In the course of these experiments we have found that by using cross-linking fixation our published observations of *uni*-specific hybridizations at basal body sites are reproducible. With our new fixation conditions, which fail to preserve basal body structures, *uni*-specific signals are found at the anterior edge of the nucleus at the apparent position of basal body/nuclear association. The data suggest a model in which the *uni* chromosome is specifically oriented at the anterior end of the nucleus and segments of this chromosome may be in direct physical association with basal bodies in interphase cells.

MATERIALS AND METHODS

Cell Culture and Fixations. The cell-wallless strain of *C. reinhardtii* CW15.227.3C⁻ was synchronized by the method as described (2). Mitotic cells were harvested by centrifugation, resuspended in autolysin (4) for 30 min, recentrifuged, and then fixed in 3:1 methanol/acetic acid and dropped onto slides as described (5). Vegetative cells from liquid cultures of the CW strain were fixed in the same way. For samples with better preservation of structure, cells were allowed to attach to poly(D-lysine)-coated slides, then submerged in aqueous solution, and fixed in a gradient of increasing methanol/acetic acid to a final concentration of 3:1 (6).

DNA and Antibody Probes. YACs containing *Chlamydomonas* DNA from the *uni* chromosome or from chromosome VI were isolated, genetically mapped, and amplified (A. Infante, S. Lo, and J.L.H., unpublished data; M. Vashishtha, G. Segil, and J.L.H., unpublished data). Three independent *uni*-linked YACs were used: YAC B corresponds to one of our original subclones of the *uni* chromosome and maps in between *UNI* and *FLA9* (3); YAC GGG actually spans the *FLA10* locus; and YAC QQ is very closely linked to *FLA10* (M. Vashishtha, G. Segil, and J.L.H., unpublished data). The TOC1 clone was kindly provided by Michel Goldschmidt-Clermont (University of Geneva). DNA probes were labeled with biotin-11-dUTP or digoxigenin-11-dUTP by the method of nick translation (7). The mouse monoclonal antibody against acetylated α -tubulin (611B) was kindly provided by Gianni Piperno (Mt. Sinai School of Medicine).

***In Situ* Hybridizations.** *In situ* hybridizations were performed essentially as described by Ried *et al.* (8) except that samples were denatured in NaOH as described (9). Biotin-labeled sequences were detected with streptavidin conjugated to fluorescein. The digoxigenin-labeled sequences were detected with rhodamine-labeled anti-digoxigenin IgG. The 611B signal was visualized with a secondary anti-mouse IgG antibody conjugated either to fluorescein or Texas red.

Images were captured using a Zeiss epifluorescence microscope connected to a CH220 liquid-cooled charge-coupled device camera (Photometrics, Tucson, AZ). Image storage, pseudocoloring, and superimposition were accomplished using a Macintosh IIx computer and the GENE JOIN software as described (8).

RESULTS

To develop a technique for *in situ* hybridization to nuclear DNA in *Chlamydomonas* we adapted methods optimized for nuclear hybridization in other systems, such as methanol/acetic acid fixation. These techniques are commonly used for mapping genes on metaphase chromosomes in the mouse and human (8, 10). We used *Chlamydomonas* metaphase spreads prepared from synchronous cultures as *in situ* hybridization targets. As a probe we chose a dispersed moderately repetitive element named TOC1 (11). This element is ≈ 6 kb in length and occurs 22–24 times in the *C. reinhardtii* strain we used in

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Abbreviations: YAC, yeast artificial chromosome; DAPI, 4',6-diamidino-2-phenylindole.

these experiments. What followed was an extensive series of experiments aimed at developing fixation and hybridization conditions that gave reproducible results. Each attempt was critically evaluated by counting the number of TOC1 signals per nucleus. A high frequency of nuclei with the full complement of TOC1 signals indicated that the fixation and preparation conditions were successful.

An example of *in situ* hybridization to a *Chlamydomonas* metaphase spread using the TOC1 probe is shown in Fig. 1A. This image was obtained by fluorescence microscopy for which chromosomes were visualized by staining with the DNA fluorochrome DAPI. The TOC1 signals (seen in red) are dispersed and each chromosome has its own characteristic complement of TOC1 elements. Most of the signals appear as doublets, which is the expected result for the replicated and paired chromosomes in a metaphase cell. In this spread virtually all members of the TOC1 family are hybridized, proving that the DNA is accessible. Preparations such as this

were used to identify individual *Chlamydomonas* metaphase chromosomes by *in situ* hybridization.

Chromosome-specific probes were derived from DNA cloned in YACs. These clones were isolated from a *Chlamydomonas* genomic YAC library that was generated for the purpose of molecular genetic characterization of the *uni* chromosome (A. Infante, S. Lo, and J.L.H., unpublished data; M. Vashishtha, G. Segil, and J.L.H., unpublished data). The use of YACs as probes for *in situ* hybridization has two benefits: (i) the YACs are 100–200 kb in size, which generates a formidable hybridization signal, and (ii) such large clones contain dispersed repetitive DNA that generates a background of weak hybridization throughout the nuclear DNA, making it easy to discern the relative position of the full YAC signal.

The results of metaphase *in situ* hybridization with YAC probes are shown in Fig. 1C and D. In both experiments, two independent YAC probes were simultaneously hybridized to the sample. In Fig. 1C the red signal was generated by

FIG. 1. *In situ* hybridization to *Chlamydomonas* metaphase and interphase chromosomal DNA. Probes were labeled as described in the text, resulting in either red (rhodamine) or yellow (fluorescein) signals. DNA was visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. (A) Hybridization of the TOC1 dispersed repeat to a metaphase spread. (B) Hybridization of the TOC1 dispersed repeat to an interphase nucleus. Note the array of chloroplast nucleoids that are brightly stained with DAPI and the intense red background over the pyrenoid at the posterior end of the cell. (C) Hybridization of a *uni*-specific YAC (QQ, red signal) and a chromosome VI-specific YAC (114A, yellow signal) to a metaphase spread. (D) Hybridization of two *uni*-linked YACs (QQ, red signal; B, yellow signal) to a metaphase spread. In the YAC hybridizations shown in A, C, and D and in Fig. 2, the intensity of the signals has been significantly attenuated in order to clearly show the individual signals in their relative position on the DNA. (E–G) Dispersed background of hybridization from repetitive sequences in the YACs, which were used to ensure correct registration of the hybridization and DAPI images. (E) Unattenuated YAC B signal. (F) Unattenuated YAC GGG signal (also *uni*-linked). (G) Partial attenuation and superimposition of YAC GGG on DAPI image of nucleus. (Bar = 3 μ m.)

hybridization of a *uni*-specific YAC, whereas the yellow fluorescence signal was generated by a YAC belonging to chromosome VI. This YAC spans the *pf14* locus, which encodes a radial spoke protein of the flagellar axoneme (12, 13). It is used as a control to identify a second chromosome and to localize a non-*uni*-linked gene that encodes a flagellar function. The

image in Fig. 1D shows the simultaneous hybridization of two separate but *uni*-linked YACs. These signals colocalize on the same chromosome, confirming the presence of the *uni* chromosome on the metaphase plate.

Having defined conditions that give nuclear *in situ* hybridization in mitotic cells we employed precisely the same meth-

FIG. 2. *In situ* hybridization and immunofluorescence with *Chlamydomonas* interphase preparations. (A) Hybridization of two *uni*-linked YACs (GGG, red; B, yellow). (B) Hybridization of a *uni*-specific YAC (QQ, red) and a chromosome VI-specific YAC (114A, yellow). The background pattern of hybridization for QQ includes a number of dispersed secondary signals similar to those seen with GGG (Fig. 1F). These are sometimes visible in the composites shown here; note the tiny red dots in the nuclei in B and D. (C) Immunofluorescence with an anti-acetylated α -tubulin antibody (611B) showing flagellar structures. (D) Immunofluorescence showing flagellar remnants (yellow) and *in situ* hybridization with a *uni*-specific YAC probe (QQ, red). (E) Hybridization of a *uni*-specific YAC (QQ, red). For this preparation, cells were attached and then fixed in a gradient of methanol/acetic acid, better preserving the size and structure of the cells. (F) Second example of a gradient-fixed preparation hybridized with a *uni*-specific YAC probe (QQ, red). (G) Third example of a gradient-fixed preparation hybridized with a *uni*-specific YAC (QQ, red) and a chromosome VI-specific YAC (114A, yellow). (H) Immunofluorescence with the 611B antibody (red). This preparation was fixed with paraformaldehyde and shows an elongated nucleus (see text). (Bar = 3 μ m.)

ods for interphase cells. Accordingly, vegetative cells were fixed with methanol/acetic acid and dropped onto slides. The resulting interphase spreads were hybridized with the TOC1 probe and a representative image is shown in Fig. 1B. TOC1 hybridization signals are dispersed throughout the interphase nucleus, demonstrating excellent accessibility of the DNA. This image also reveals that the cell is quite spread out. This method of fixation yields a range of expanded cell images in which the nuclei are often more than four times their normal size. Despite the effects of spreading, the overall polarity and orientation of the cells are frequently retained. In an intact cell, the chloroplast forms a cup-shaped structure surrounding the nucleus. The position of the chloroplast can be made out in these preparations by the brightly staining nucleoids of chloroplast DNA. In addition, we often witnessed considerable background due to fluorescent reagents adhering to the pyrenoid. This structure is part of the chloroplast and is centrally located near the posterior end of the cell. This background is clearly seen in Fig. 1B and is advantageous in that it visualizes the position of the pyrenoid, which together with the nucleus defines the central axis of the cell.

These kinds of positional clues are essential to distinguish the posterior versus anterior poles of the nucleus, but to identify the subnuclear localization of hybridization signals we relied on the background hybridization generated by YAC probes. An example of how this background is used to enable correct registration is shown at the bottom of Fig. 1. These images, focusing on the nucleus of a single interphase cell, show the pattern of hybridization of a *uni*-linked YAC probe labeled with fluorescein (Fig. 1E) and the pattern of hybridization for a second independent *uni*-linked YAC probe labeled with rhodamine (Fig. 1F). Fig. 1G shows the superimposition of the signals in Fig. 1F on the DAPI image of this nucleus. Here the intensity of rhodamine signals has been attenuated and superimposed so that the dispersed repetitive hybridization pattern conforms to the shape of the nucleus indicated by DAPI staining. This reveals that the full YAC signal is on the lower edge of the nucleus. The dispersed background of hybridization caused by the YAC probe in Fig. 1E is weaker but just as useful for correct registration of the composite, which is shown in completed form in Fig. 2A.

Fig. 2 shows a series of hybridization and immunofluorescence experiments with interphase cells. The two topmost images show the results of double YAC hybridizations like those performed with metaphase spreads. Fig. 2A shows the results of an *in situ* hybridization with two *uni*-linked YACs. The signals are proximal and both are located at the anterior pole of the nucleus. In Fig. 2B a *uni*-specific YAC detected in red is paired with the chromosome VI-specific YAC detected in yellow. The *uni* sequence hybridizes at the anterior end of the nucleus opposite the chloroplast nucleoids and the pyrenoid. In contrast, the chromosome VI signal is localized toward the middle of the nucleus. The anterior polar localization of *uni*-specific sequences was conspicuous and reproducible: in cells that had retained the normal overall orientation of nucleus, chloroplast and pyrenoid *uni*-specific hybridizations occurred on the anterior edge of the nucleus. This position is exactly where basal bodies are normally positioned, so we sought to confirm our interpretation of the anatomy of the cells on these slides.

Methanol/acetic acid fixation is not generally effective for preserving proteins but immunofluorescence experiments gave evidence for persistence of flagellar components in these preparations. Using an antibody against acetylated α -tubulin (14) that prominently labels the flagellar axoneme, we found that remnants of flagella were clearly associated with some cells and that their relative position could be used to identify the anterior or basal body pole of the cell.

Two representative images are shown in the second row of Fig. 2. In Fig. 2C the acetylated α -tubulin epitope defines a pair

of flagellar structures. These are spread out like the rest of the cell but, nevertheless, serve to distinguish the anterior end of the cell. That *uni*-specific signals actually occur on this edge of the nucleus is demonstrated in Fig. 2D. Here, the antibody was applied to a sample that was previously hybridized with a *uni*-specific YAC probe. The flagellar structures are quite disrupted but their position clearly defines the anterior pole of the nucleus and the hybridization signal clearly occurs at this pole at a position expected for basal bodies.

The *in situ* hybridization experiments with interphase spreads yielded a large collection of images that strongly suggested that the position of the *uni* chromosome is confined to the front edge of the nucleus. Immunofluorescence data revealing the orientation of the flagella in these preparations confirmed our interpretation of cell polarity and further suggested that *uni*-specific hybridization signals are found near the attachment site of the basal body flagellar complex. To reproduce these observations in more intact cells that had not suffered the effects of spreading, but that were still fully accessible for hybridization, we modified our fixation protocol. Cells were first allowed to attach to poly(D-lysine)-coated slides while still alive and then slowly fixed in a gradient of increasing methanol/acetic acid concentrations. When successful, this method yielded preparations with small cells whose overall organization was intact. When hybridized with *uni*-specific probes, the results were consistent with those obtained with spread-out cells. Two sample images are shown in Fig. 2E and F. The *uni*-derived YAC hybridization signals seen in red are localized to the anterior edge of the nucleus. To demonstrate that this localization is a specific characteristic of the *uni* chromosome we repeated the double hybridizations including the *uni* and the chromosome VI YACs as probes. The result is shown Fig. 2G. The chromosome VI sequence is in the central area of the nucleus, whereas the *uni* sequence occupies its typical polar position. Because these cells closely resemble native *Chlamydomonas* with respect to their dimension as well as their orientation, we used these preparations to assess the frequency of *uni* hybridization events at the anterior edge of the nucleus. Cells with a defined longitudinal axis like those shown in Fig. 2E–G were examined and in a typical set of experiments we found *uni*-specific YAC signals occupied the basal body end of the nucleus in >80% of the samples ($n = 62$).

DISCUSSION

Using the technique of *in situ* hybridization with YAC probes we have directly examined the localization of the *uni* chromosome. The data show that in interphase cells segments of the chromosome corresponding to the YAC sequences are specifically localized at the anterior edge of the nucleus proximal to the basal body/flagellar complex. In mitotic cells, these probes identify *uni* sequences on a condensed metaphase chromosome. Examination of the hybridization pattern of these probes in metaphase reveals that they are located toward the physical center of the *uni* chromosome. On the genetic map of the *uni* linkage group, which is in fact linear (ref. 15; G. Segil and J.L.H., unpublished data), these YACs either encompass or are closely linked to flagellar assembly mutants. These data strongly suggest the existence of a spatial orientation for the *uni* chromosome within the interphase nucleus, placing genes involved in flagellar development close to the site of assembly. Further evidence in support of a polarized organization of the nucleus comes from a series of cell images like the one shown in Fig. 2H.

In the course of our experiments with various fixation protocols we found that under certain conditions as live cells attach to slides they undergo a deformation that reveals a feature of nuclear structure. In such cases the flagella continue beating momentarily after the posterior end of the cell sticks to the charged surface of the slide. The nucleus becomes

stretched out as a result. What is remarkable is that even after dramatic elongation of the nucleus it remains firmly attached to the cell body at one end and to the basal body/flagellar apparatus at the other end. The connection between the nucleus and the basal body/flagellar complex is known to be mediated at least in part by a contractile system of fibers containing the protein centrin (caltractin) (16–18). It is also likely that microtubules are involved, but whatever the complete nature of this connection it is quite firm and clearly involves nuclear DNA as well. As seen in Fig. 2H the elongated DAPI-stained nucleus is anchored at the cell body and at the basal bodies whose position is indicated by α -tubulin immunofluorescence. Our *in situ* hybridization studies suggest that there is an additional level of organization involving the nuclear DNA that is most closely attached to the anterior end of the nucleus. We propose that this aspect of the nucleus is not occupied by a random assortment of chromosomes but that the *uni* chromosome itself is specifically associated with this attachment point.

Our previously published data provided evidence that segments of the *uni* chromosome could be found in direct association with basal bodies themselves (3). In these experiments, lambda or plasmid-size probes from the *uni* chromosome hybridized at basal body sites in paraformaldehyde-fixed cells. We found that the intensity of the hybridization signal was proportional to the size of the *uni* probe that was used, which is the expected result for real hybridization events. As controls we used nuclear ribosomal and chloroplast probes, which labeled their respective *in situ* targets while at the same time showing no labeling at basal body sites. The case for direct basal body hybridization of *uni* DNA has been strengthened by more recent experiments in which we reproduced the result using an independent unique sequence probe from the *uni* chromosome and employing alkaline rather than heat denaturation of the sample. Again, all of our data suggest that the basal body hybridization signals are *uni*-specific, even though they were obtained with preparations that give limited access to nuclear DNA. Further experiments at higher resolution are necessary to reconcile the observations of extranuclear basal body signals seen following aldehyde fixation with our data showing anterior polar nuclear signals following methanol/acetic acid fixation. A final unexplained feature of these experiments concerns the high frequency of double signals, one per basal body, obtained with *uni*-specific probes in our earlier study (see figure 9 in ref. 3). The present study of hybridization to metaphase chromosomes and other data (ref. 19; A. Infante, S. Lo, and J.L.H., unpublished data) show that, in contrast to our earlier estimation, the *uni* chromosome is present at normal copy number.

The pattern of *uni*-specific hybridization at the anterior end of the nucleus, even allowing for the possibility of direct contact of the DNA with basal bodies through a specialization of the nuclear envelope, fits more into the broad problem of nuclear organization (reviewed in ref. 20) than into a strict conception of an organelle genome. The idea that the arrangement of chromosomes in the nucleus is not random, as scored by the configuration of telomeres and centromeres, is longstanding. It has also been suggested that chromosomes occupy discrete territories in interphase nuclei and that this organization is dynamic with respect to cell cycle and cell fate. The regulation of the location of an entire nuclear chromosome may be exemplified by the inactive X chromosome in female somatic cells (21, 22). But, with the exception of the nucleolus, little is known regarding the position of specific active genes. The localization of the *uni* chromosome may be an example of just this level of organization reflecting a specific orientation of DNA relative to a microtubule organizing center.

In this regard, the principle context for our observations is the occurrence on the *uni* linkage group of numerous mutants affecting basal body and flagellar assembly (1). The position of this cluster of genes at or near basal body sites may be required for the function of the corresponding gene products in the generation of the basal body/flagellar complex. Our current understanding of the genetics of this process and the structure of this chromosome appears to rule out a completely exclusive relation. For example, there are other loci on other linkage groups that influence the assembly of basal bodies and flagella (4). At the same time, several loci have been identified on the *uni* linkage group that have no apparent role in this process (23). The strongest evidence that the *uni* chromosome encodes functions directly associated with basal bodies comes from the characterization of the flagellar assembly mutant *FLA10* (2). This gene encodes a kinesin homologous protein (KHP1) that is likely to play a role in the active transport required for flagellar morphogenesis. More recently we have found that not only is KHP1 present in the flagellar axoneme but also within the interphase cell it is exclusively associated with basal bodies (M. Vashishtha, Z. Walther, and J.L.H., unpublished data). It will be interesting to determine whether the gene products of other *uni*-linked loci turn out to be basal body proteins.

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