Transcription of α- and β-Tubulin Genes In Vitro in Isolated *Chlamydomonas reinhardi* Nuclei

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**ABSTRACT** Removal of the flagella of *Chlamydomonas* results in increases in both flagellar protein synthesis and tubulin messenger RNA accumulation. These observations led us to examine whether flagellar protein gene sequences are transcribed differentially in nuclei isolated before and after deflagellation. A nuclear isolation protocol was developed using the cell wall-less strain of *Chlamydomonas*, CW 15, after cell lysis with 0.5% Nonidet P-40. Transcriptional activity of isolated nuclei was determined by incorporating $^{32}$PUTP into TCA-precipitable and phenol-extractable RNA, and by hybridizing newly transcribed RNA to complementary DNA clones containing α- and β-tubulin sequences. Nuclei from deflagellated cells are more active in transcribing sequences that hybridize with α- and β-tubulin complementary DNA probes than are nuclei from nondeflagellated cells. In addition, while total $^{32}$PUTP incorporation is inhibited 45% by α-amanitin concentrations of 1.0 μg/ml, tubulin RNA synthesis in this system is completely inhibited by this concentration of α-amanitin. This demonstration of differential transcription in nuclei before and after cell deflagellation provides the means to study in vitro the mechanisms that signal and regulate flagellar protein gene activity.

**MATERIALS AND METHODS**

Nuclear Isolation: *Chlamydomonas reinhardi* cells, strain CW 15 (mating type −) (11), were grown in medium I of Sager and Granick (12) with aeration at 21°C on a cycle of 14 h light/10 h dark. For nuclear isolation, cells were concentrated to 1–10 x 10⁶ cells/ml and deflagellated by pH shock (13). At 20 min after deflagellation, cells were collected by centrifugation (650 g, 5 min, 4°C) and resuspended in solution I (25 mM HEPES-NaOH, pH 7.5, 20 mM KCl, 20 mM MgCl₂, 0.6 M sucrose, 10% glycerol, 5 mM dithiothreitol) at 0°C. Nonidet P-40 was added to 0.5% (vol/vol) to lyse the cells. Lysis was monitored by phase-contrast microscopy for up to 10 min, and nuclei were pelleted from the lysis solution by centrifugation (650 g, 5 min, 4°C). The nuclear pellet was resuspended in 10 ml of solution I without detergent and induced even when flagellar regeneration is blocked by drugs such as colchicine (2, 3). The nature of the signal that permits activation of only flagellar protein synthesis and how this signal is transmitted through the cytoplasm to the nucleus are not yet known.

To approach these problems, we have developed methods for isolating nuclei from *Chlamydomonas*, and have begun to study the synthesis of specific RNA transcripts in isolated nuclei. The data presented here show that nuclei from deflagellated *Chlamydomonas* cells are more active in transcribing α- and β-tubulin RNAs than are nuclei isolated from nondeflagellated control cells. This in vitro nuclear transcription system may now permit studies of the molecular mechanisms involved in signalling the regeneration response and in regulating the increase in mRNAs coding for flagellar proteins.

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Abbreviations used in this paper: cDNA, complementary DNA; mRNA, messenger RNA; rRNA, ribosomal RNA; SSC, standard saline-citrate buffer; TCA, trichloroacetic acid.
then repelletted by centrifugation. Nuclei were resuspended at a concentration of 1 x 10^8 nuclei per milliliter of solution II (2.5% Ficoll, 0.5% sorbitol, 0.008% spermidine, 1 mM dithiothreitol, 5 mM MgCl₂, 10 mM Tris-Cl, pH 7.5, 50% glycerol), and frozen and stored at -80°C until use. The DNA concentration of a sample of each preparation of nuclei was determined fluorometrically using diaminobenzoic acid dihydrochloride (14), and different DNA concentrations were selected for each in experiments. Nuclei were isolated and stored by this protocol support active transcription at the same level as freshly prepared, unfrozen nuclei for up to 8 mo. Samples of nuclei are visualized during isolation by fluorescence microscopy (Zeiss epifluorescence illumination, 450-490 nm excitation, 510-nm beam splitter, 520-nm barrier filter) after staining with the dye, mithramycin (gift of Pfizer, Inc., Groton, CT) (15).

**Transcription Assay:** Nuclei were assayed for transcriptional activity by incubation at a concentration of 5 x 10^8 nuclei/ml at 25°C in 1.25% Ficoll, 0.25% sorbitol, 0.004% spermidine, 0.6 mM dithiothreitol, 12 mM MgCl₂, 45 mM Tris-Cl, pH 7.9, 30% glycerol, 150 mM NaCl, 0.16 mM each ATP, GTP, CTP, 0.005 mM [α-32P]UTP (20 Ci/mmol, Amersham Corp., Arlington Heights, IL), and 200 U/ml of the RNAse inhibitor RNAasin (Promega Biotec, Madison, WI). This protocol is a modification of that described by Landfarer et al. (16) using isolated Dictyostelium discoideum nuclei. After 60 min, the reaction was terminated by addition of 1 vol of 2% sodium deoxyribonuclease, 1 mg/ml pronase (Calbiochem Behring Corp., La Jolla, CA) in solution III (0.4 M NaCl, 0.02 M Na acetate, pH 5.1, 2 mM MgCl₂, 2% SDS) and incubation for 30 min at 25°C. RNA was extracted three times with equal volumes of chloroform and redistilled phenol saturated with 0.5 x solution III, once with chloroform only, and ethanol precipitated with ethanol three times in the presence of 2 M NaCl.

In some cases, further digestion with DNase I (1 µg/ml, Worthington Biochemical Corp., Freehold, NJ) and another phenol extraction were performed (see Fig. 5). To determine incorporation of labeled UTP into nuclei acids, aliquots of the assays were precipitated with 15% trichloroacetic acid (TCA), 5 mM Na₃PO₄, and collected on nitrocellulose filters (Type HAWP, Millipore Corp., Bedford, MA). Filters were dissolved in 2-methoxyethanol and counted in an LS8000 scintillation counter (Beckman Instruments, Inc. Palo Alto, CA).

**Plasmid DNA Dots and Hybridization Conditions:** For dot hybridizations, plasmid DNA was denatured in 0.1 M NaOH for 20 min at 95°C, quick-chilled on ice, neutralized, and diluted to the desired concentration with 1 M CH₃COONa, pH 7.0. Denatured plasmid DNA in 200-µl volumes was applied to nitrocellulose filters prefretted with 1 M CH₃COONa using a Minifold sampling apparatus (Schleicher and Schuell, Inc., Keene, NH), and washed with 400 µl of 1 M CH₃COONa. The filters were washed with 5 x standard saline-citrate buffer (SSC, 0.6 M NaCl, 0.6 M Na citrate), dried, and then baked at 80°C for 2 h in vacuo. Plasmids containing α- and β-tubulin complementary DNAs (cDNAs) and the constitutive sequence cDNA will be described in detail elsewhere (17). The α- and β-tubulin cDNAs are nearly full length, 1,800 and 2,270 nucleotides long, respectively. The constitutive sequence cDNA (see Results) is 700 nucleotides long. pBR322 is the vector for these sequences. The ribosomal DNA gene-containing plasmid in pBR313 was obtained from Dr. J.-D. Rochaix (University of Geneva) (18). For hybridization of newly transcribed RNA to plasmid DNA dots, filters were prehybridized for 4-20 h and hybridized for 72 h at 40°C in buffer containing 50% denatured formamide, 5 x SSC, 50 mM Na phosphate, pH 6.5, 5 x Denhardt's solution (19), 0.1% SDS, 100 µg/ml denatured E. coli DNA, 100 µg/ml yeast transfer RNA, and 20 µg/ml poly(A) RNA (9). In some experiments, the same number of counts per minute, from 0.5-5 x 10⁶ cpm/ml, were added to each hybridization reaction and data were corrected for differences in total incorporation and the DNA content of the nuclei preparations used in the reactions. In other experiments, the A₂₆₀ was determined spectrophotometrically after the third wash with 1 M CH₃COONa, pH 7.0. Denatured plasmid DNA in 200-µl volumes was added to each hybridization reaction. To determine conditions for hybridization in DNA-excess, a series of plasmid DNA dots containing 0.2, 1, 2, 4, 7, and 10 µg DNA per dot was hybridized to in vitro transcribed RNA. Hybridization increased linearly from 0.2 to 2 µg DNA per dot, and further increases in hybridization were not detected above 2 µg DNA per dot for the tubulin and constitutive plasmid DNAs. Hybridization to the ribosomal DNA increased linearly with increasing concentrations of plasmid DNA from 1 to 10 µg DNA per dot, indicating that DNA excess conditions are not achieved even at 10 µg DNA per dot (which is near the DNA-binding capacity of the nitrocellulose). For quantitation, each dot of the tubulin and constitutive plasmids contained 2 µg plasmid DNA per dot. Ribosomal DNA was dotted at 5 µg DNA per dot (shown in Fig. 3 and Table I) and also 10 µg plasmid DNA per dot. After hybridization, filters were washed for 10 min at room temperature with two changes of 2 x SSC, 0.1% SDS, and for 40 min at 50°C with two changes of 0.1 x SSC, 0.1% SDS. Filters were wrapped in Saran wrap and exposed to Kodak XR or XAR film. Filters were cut, solubilized, and counted in a scintillation counter as described above.

To determine the efficiency of hybridization of the in vitro transcribed nuclear RNA sequences during the hybridization period, we added a second, fresh filter of plasmid dots to solutions of RNA that had been incubated as described above for 12, 30, 48, and 72 h, and hybridized for an additional 12, 54, 36, and 12 h, respectively. We found that hybridization to the second filter was <15% of hybridization to the initial filter of the initial 72-h incubation period for all the specific RNA sequences examined, demonstrating that hybridization had progressed essentially to completion.

**RESULTS**

**Isolation of Nuclei**

A protocol for isolating nuclei from Chlamydomonas reinhardtii was developed as a first step in constructing an in vitro system to study Chlamydomonas flagellar protein gene expression. Because wild-type Chlamydomonas are surrounded by a complex glycoprotein matrix that renders them difficult to lyse, the isolation protocol was developed using a mutant of Chlamydomonas, CW 15, that lacks this cell wall. Nuclei were identified among the products of cell lysis by using a fluorescent DNA-binding dye, mithramycin (15). Fig. 1 compares the appearance of isolated nuclei (c and d) with nuclei in whole cells (a and b) using phase-contrast and fluorescence microscopy. Isolated nuclei are uniform in size and their appearance is similar to nuclei in intact cells (Fig. 1, b and d), indicating that they remain intact during the isolation procedure. Nuclear preparations are contaminated by phase-dense particles (Fig. 1c). Approximately 20% of the nuclei are recovered from cells, based on the DNA content of the organism and the amount of DNA recovered in the final preparations.

**FIGURE 1** Phase-contrast and fluorescence microscopy of Chlamydomonas nuclei. Whole cells (a and b) and isolated nuclei (c and d) were stained with mithramycin and the same field was examined using phase-contrast (a and c) and fluorescence (b and d) microscopy. Nuclei are invisible in whole cells (a) but can be distinguished as bright dots after staining (b). Arrowheads pointing to nuclei emphasize the difficulty of distinguishing nuclei with phase contrast, and the ease of visualization after staining. Phase-dense particles are seen in the preparation of isolated nuclei (c). The appearances of isolated nuclei and nuclei in whole cells are similar, indicating that they remain intact during the isolation protocol. × 500.

**RAPID COMMUNICATIONS**

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RNA Synthesis in Isolated Nuclei

The transcriptional activity of nuclei isolated from deflagellated and nondeflagellated control cells was assayed in a medium containing unlabeled nucleotide triphosphates and [α-32P]UTP. Concentrations of NH₄Cl or NaCl, which varied between 0 and 0.5 M, were tested in the assay; 0.15 M NH₄Cl resulted in maximum total incorporation of radioactive nucleotides. NaCl resulted in optimal assay conditions, incorporation continues to increase at a nearly linear rate for at least 1 h (Fig. 2A). This incorporation rate is probably still less than the potential maximum incorporation rate since suboptimal concentrations of labeled nucleotide triphosphates were used to increase the specific activity of the newly transcribed RNA. A linear relationship exists between nuclei concentration and RNA synthetic activity in the assay (Fig. 2B).

Tubulin RNA Transcription In Vitro

To determine whether specific flagellar transcripts, such as α- and β-tubulin, are synthesized in greater amounts by nuclei from deflagellated cells than from nondeflagellated control cells, the RNA extracted from in vitro assays has been analyzed by hybridization to specific recombinant DNA plasmids (Fig. 3 and Table I). The plasmids used for hybridization include sequences for α- and β-tubulin, since tubulin mRNA levels in the cells increase dramatically after deflagellation (8, 9). Control hybridizations were performed with a plasmid containing cDNA corresponding to a “constitutive” mRNA (17), and a plasmid containing the genes for 18S and 25S ribosomal RNA. The abundance of these RNAs in cells does not change after deflagellation (17). Vector DNA without a recombinant insert also served as a control. The plasmids were spotted onto nitrocellulose filters and hybridized with the same A₂₆₀ units of nucleic acid extracted from in vitro transcription assays containing nuclei from either deflagellated or nondeflagellated cells. Each hybridization was allowed to continue essentially to completion, so that the differences in hybridization represent differences in tubulin and constitutive gene transcript concentrations. Scintillation counting of dots cut from these hybridized filters indicates that transcription of α- and β-tubulin RNA is stimulated in deflagellated cell nuclei 6–12-fold above levels in control, nondeflagellated cell nuclei (Table I). Fig. 3 and Table I also show that in vitro synthesis of RNA corresponding to the constitutive plasmid by both deflagellated and nondeflagellated nuclei.
The activity of different nuclear preparations. However, both sets of normalization and could also represent differences presented in Fig. 3 and Table I. The differences in stimulation the data using Method 1, the method of normalization presented in Table II Method 1 combines the activities from two different preparations of nuclei, and Method 2 combines the activities of four different preparations of nuclei.

Table I

| Calculated Stimulation of Tubulin Transcription in Deflagellated Cell Nuclei |
|-------------------------------|-------------------|-------------------|
|                               | cpm per filter dot | cpm minus background |
|                               | DF                | NDF               | DF                | NDF               | DF/NDF |
| **Experiment 1**              |                   |                   |                   |                   |        |
| α-Tubulin                     | 252.1             | 170.9             | 127.5             | 21.3              | 6.00    |
| β-Tubulin                     | 256.2             | 166.9             | 131.6             | 17.3              | 7.60    |
| Constitutive                  | 221.4             | 252.5             | 96.8              | 102.9             | 0.94    |
| Ribosomal                     | 2,831.6           | 4,343.2           | 2,707.0           | 4,193.6           | 0.64    |
| Vector                        | 124.6             | 149.6             | 0.0               | 0.0               | —       |
| **Experiment 2**              |                   |                   |                   |                   |        |
| α-Tubulin                     | 248.0             | 97.2              | 181.2             | 24.7              | 7.30    |
| β-Tubulin                     | 284.7             | 90.1              | 217.9             | 17.6              | 12.40   |
| Constitutive                  | 118.0             | 123.8             | 51.2              | 51.3              | 1.00    |
| Ribosomal                     | 474.2             | 699.7             | 407.4             | 627.2             | 0.76    |
| Vector                        | 66.8              | 72.5              | 0.0               | 0.0               | —       |

200 μl transcription assays contained 0.5 mM each ATP and CTP, and 0.05 mM (α-32P)UTP and (α-32P)GTP (4 Ci/mmol each). 1 cpm = 2.3 × 10^4 pmol NTP incorporated. Nitrocellulose filters containing dots of 2 μg of α-tubulin cDNA, β-tubulin cDNA, a cDNA to an RNA which is synthesized constitutively during regeneration, and plasmid DNA without an insert (vector) and 0.2 μg of ribosomal DNA were hybridized to equal A260 units of nuclear acids from in vitro transcription assays containing nuclei from either deflagellated (DF) or nondeflagellated (NDF) cells. The filters were sliced, solubilized, and counted in a liquid scintillation counter (see Materials and Methods). cpm per filter dot shows the average cpm in triplicate samples corrected for isotope decay. Background cpm in vector DNA samples are subtracted from these values. Stimulation is determined by directly comparing the cpm in DF samples with the cpm in NDF samples (DF/NDF). Hybridization to the tubulin and constitutive dots has proceeded essentially to completion under these conditions. Quantitation of hybridization to the 0.2 μg dots of ribosomal plasmid shown here gave the same relative stimulation as quantitation of dots containing 10 μg of ribosomal plasmid (not shown).

Different preparations of nuclei were used in Experiment 1 and Experiment 2.

We also determined the stimulation of tubulin transcription by including in the hybridization reactions equal counts per minute from transcription assays, and normalizing to total incorporation and DNA content of the preparations of nuclei used in the assays, rather than by the more straightforward analysis of including the same A260 units in the hybridization reactions (Table II, Method 2). Although the values for stimulation of tubulin transcription determined using this Method 2 of normalizing hybridized counts per minute with respect to total incorporation and DNA content are lower than values determined using Method 1, they are qualitatively similar to the data using Method 1, the method of normalization presented in Fig. 3 and Table I. The differences in stimulation shown in Table II could be the result of the different methods of normalization, and could also represent differences in activity of different nuclear preparations. However, both sets of data indicate that transcription of α- and β-tubulin genes increases 4–10-fold after deflagellation.

α-Amanitin Sensitivity of In Vitro Transcription

α-Amanitin has been shown to selectively inhibit activity of RNA polymerase II in higher plants and animals in the concentration range of 0.01–1 μg/ml (20–22). To determine
Results similar to these have been described for other systems. Synthetic activity in the assay that is resistant to α-amanitin of RNA polymerase II, while the remaining 40% of total RNA at 10 μg/ml α-amanitin probably represents inhibition of incorporation. The 60% inhibition of incorporation into total RNA are nuclei from deflagellated cells (Fig. 4, tubulin-specific incorporation). The 60% inhibition of incorporation into total RNA at 10 μg/ml α-amanitin probably represents inhibition of RNA polymerase II, while the remaining 40% of total synthetic activity in the assay that is resistant to α-amanitin probably represents transcription by polymerases I and III. Results similar to these have been described for other systems (23, 24), although the inhibitory activity of α-amanitin has not yet been tested directly on isolated Chlamydomonas cellular polymerases. Thus, synthesis of tubulin mRNA is completely inhibited at concentrations of 1 μg/ml of α-amanitin, while total transcription is inhibited 60% by concentrations of 10 μg/ml.

Characterization of In Vitro Transcripts

To demonstrate that hybridization to these plasmid dots indeed results from labeled RNA, nucleic acids that had been extracted from assays were pretreated with either DNase I or a mixture of RNases A and T1, before hybridization with dots of tubulin cDNA. As shown in Fig. 5, hybridization was completely abolished in the RNase-treated samples, whereas hybridization after DNase I or no treatment remains high.

**DISCUSSION**

The data presented here demonstrate that nuclei isolated from deflagellated Chlamydomonas cells are more active in producing α- and β-tubulin transcripts than are nuclei from nondeflagellated cells. This in vitro demonstration of differential gene activity in isolated nuclei relates to events occurring in vivo, where mRNAs coding for α- and β-tubulin accumulate to high levels in cells that have been deflagellated, but remain at low levels in nondeflagellated cells (9, 10). The 4-10-fold stimulation of in vitro transcription of tubulin genes reported here is similar to the 10-fold stimulation reported when flagellar proteins are labeled in vivo (25), when RNA is translated in vitro (8), or the 10-40-fold stimulation reported from quantitation of RNA gel hybridizations (9, 10). The increase in stimulation in isolated nuclei is specific for α- and β-tubulin (and possibly for other flagellar protein mRNAs). This is in contrast to in vitro transcription of the constitutive RNA, which remains at relatively constant levels in nuclei before and after deflagellation. In addition, rRNA is apparently transcribed at the same rate (although this remains to be rigorously demonstrated), and remains at constant abundance before and after deflagellation (17).

The increased activity of deflagellated cell nuclei is consistent with a model in which deflagellation of Chlamydomonas signals increased transcription of tubulin and flagellar protein genes. This result does not eliminate the possibility that an additional component in the mechanism for transcript accumulation is the stabilization or processing of tubulin gene transcripts in the nucleus. To determine the relative contributions of these two mechanisms to the observed transcript accumulation, one would have to demonstrate that nuclei from deflagellated cells initiate more new transcripts than nuclei from nondeflagellated cells, or determine the half-lives and transcription rates of tubulin mRNAs in cells before and after deflagellation in vivo. Results of in vivo pulse-labeling experiments in progress (E. Baker and J. Rosenbaum, personal communication) are quantitatively consistent with the results of in vitro transcription in isolated nuclei reported here. Our results do rule out a mechanism whereby the deflagellation-induced increase in tubulin mRNAs results solely from temporary inhibition of cytoplasmic mRNA degradation, since cytoplasmic influences have largely been eliminated in this in vitro nuclear assay.

If Chlamydomonas nuclei are to be useful in studying the signaling and regulation of flagellar protein gene expression in vitro, they must initiate synthesis of new RNA transcripts...
correctly, and produce transcripts of the correct size. It is not possible to determine from the data shown whether these isolated nuclei merely elongate preinitiated RNA transcripts or whether they also initiate new transcripts. However, we have preliminary evidence that RNA synthesized in vitro in isolated nuclei will hybridize to the 5' ends of digested α- and β-tubulin cloned probes which are nearly full-length. This evidence, in combination with the fact that these isolated nuclei continue to synthesize RNA at high rates for extended periods of time, suggests that reinitiation of new transcripts as well as completion of the transcripts initiated before nuclear isolation may be taking place. Experiments in progress should allow us to determine whether reinitiation does occur in our isolated nuclei. Initiation of RNA transcripts has been demonstrated in isolated mouse myeloma nuclei (26) and in nuclei from HeLa cells infected with adenovirus 2 (27). In addition, successful attempts have been made to induce gene activation in vitro in isolated nuclei using other systems, such as the heat shock response in Drosophila salivary gland nuclei (28, 29), and casein production in mouse mammary cells (30).

The capacity of isolated Chlamydomonas nuclei to differentially synthesize specific RNAs establishes an in vitro system in which the contributions of transcription and transcript maturation can be more easily assessed. This in vitro system may, in addition, provide the first step in identifying and characterizing the signals involved in initiating flagellar regeneration and the mechanisms which regulate flagellar gene activity.

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