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

LAURA R. KELLER, JEFFERY A. SCHLOSS, CAROLYN D. SILFLOW, and JOEL L. ROSENBAUM
Department of Biology, Yale University, New Haven, Connecticut 06511. Dr. Silflow's present address is Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108.

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}P]UTP 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}P]UTP 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.

Removal of the anterior pair of flagella from the unicellular green alga *Chlamydomonas* induces the synchronous regeneration of new flagella and synthesis of over 150 proteins (1–4). Many of these proteins, for example α- and β-tubulin and dynein, are localized in the flagellum (2, 5–7). In vitro translation and also hybridization analyses of total *Chlamydomonas* cell RNA using cloned DNA probes have demonstrated that increases in RNA coding for many of these proteins accompany the increased flagellar protein synthesis (8–10). These increases are rapid, detectable within 5–10 min of flagellar removal, and reach levels 10–40-fold above protein and RNA levels in nondeflagellated control cells (9, 10). It has not yet been determined whether the increase in flagellar protein messenger RNAs (mRNAs)[1] is due to increased nuclear transcription, RNA processing, or mRNA stabilization.

Almost nothing is known about the signal that initiates these responses following flagellar removal. The process of flagellar detachment itself does not appear to be responsible for the induction of new synthesis because cells that have been induced to resorb their flagella also turn on flagellar protein synthesis once the resorbing agent has been removed (2). Moreover, flagellar regrowth or outgrowth is not required to induce the synthesis because flagellar protein synthesis is 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.

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 × 10^6 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 $MgCl_2$, 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

[1] *Abbreviations used in this paper:* cDNA, complementary DNA; mRNA, messenger RNA; rRNA, ribosomal RNA; SSC, standard saline-citrate buffer; TCA, trichloroacetic acid.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 MARCH 1984 1138–1143

then repelleted by centrifugation. Nuclei were resuspended at a concentration of 1×10^9 nuclei (assuming 100% recovery of nuclei) per milliliter of solution II (2.5% Ficoll, 0.5 M sorbitol, 0.008% spermidine, 1 mM dithiothreitol, 5 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.5, 50% glycerol), and frozen 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 differences in DNA concentration were accounted for in each assay. Nuclei 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 epifluorescent 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 5×10^8 nuclei/ml at 25°C in 1.25% Ficoll, 0.25% sorbitol, 0.004% spermidine, 0.6 mM dithiothreitol, 12 mM $MgCl_2$, 45 mM Tris-Cl, pH 7.9, 30% glycerol, 150 mM NH_4Cl, 0.16 mM each ATP, GTP, CTP, 0.005 mM [α-^{32}P]UTP (20 Ci/mmol, Amersham Corp., Arlington Heights, IL), and 200 U/ml of the RNAse inhibitor RNAsin (Promega Biotec, Madison, WI). This protocol is a modification of that described by Landfear et al. (16) using isolated *Dictyostelium discoideum* nuclei. After 60 min, the reaction was terminated by addition of 1 vol of 2 mg/ml Na heparin, 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 $MgCl_2$, 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 × solution III, once with chloroform only, and precipitated with ethanol three times in the presence of 2 M CH_3COONH_4. In some cases, a 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 nucleic acids, aliquots of the assays were precipitated with 15% trichloroacetic acid (TCA), 5 mM $Na_4P_2O_7$, and collected on nitrocellulose filters (Type HAWP, Millipore Corp., Bedford, MA.). Filters were dissolved in 2-methoxyethanol and counted in Aquasol (New England Nuclear, Boston, MA) using a Beckman model 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 CH_3COONH_4, pH 7.0. Denatured plasmid DNA in 200-μl volumes was applied to nitrocellulose filters prewetted with 1 M CH_3COONH_4 using a Minifold sampling apparatus (Schleicher and Schuell, Inc., Keene, NH), and washed with 400 μl of 1 M CH_3COONH_4. The filters were washed with 5 × 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% deionized formamide, 5 × SSC, 50 mM Na phosphate, pH 6.5, 5 × Denhardt's solution (19), 0.1% SDS, 100 μg/ml denatured *E. coli* DNA, 100 μg/ml yeast transfer RNA, and 20 μg/ml polyr(A) RNA (9). In some experiments, the same number of counts per minute, from 0.5–5 × 10^6 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 A_{260} was determined spectrophotometrically after the third ethanol precipitation, and the same number of A_{260} units of nucleic acids from transcription assays using nuclei from deflagellated or nondeflagellated cells 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 dots 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 in two sets of dots at 0.2 μg plasmid 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 × SSC, 0.1% SDS, and for 40 min at 50°C with two changes of 0.1 × 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 72, 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 reinhardi* 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. 1 *c*). 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.

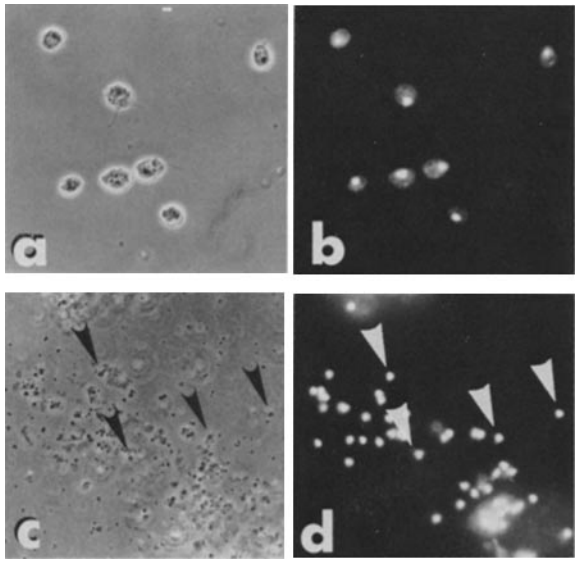

FIGURE 1 Phase-contrast and fluorescence microscopy of *Chlamydomonas* nuclei. Whole cells (*a* and *b*) and isolated nuclei (*c* and *d*) were stained with mithramycin and the same field was examined using phase-contrast (*a* and *c*) and fluorescence (*b* and *d*) microscopy. Nuclei are invisible in 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.

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-^{32}P]UTP. Concentrations of NH_4Cl or NaCl, which varied between 0 and 0.5 M, were tested in the assay: 0.15 M NH_4Cl resulted in maximum total incorporation of radioactive nucleotides. NaCl resulted in slightly lower incorporation over the concentration range tested. Monovalent cation concentrations >0.3 M resulted in lysis of the nuclei and dispersion of chromatin into the assay medium. Total incorporation was maximized by varying, one at a time, the concentrations of RNAsin, NH_4Cl, NaCl, KCl, and $MnCl_2$ to arrive at the assay conditions described in the Materials and Methods. Under optimal assay conditions, incorporation continues to increase at a nearly linear rate for at least 1 h (Fig. 2*A*). 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. 2*B*).

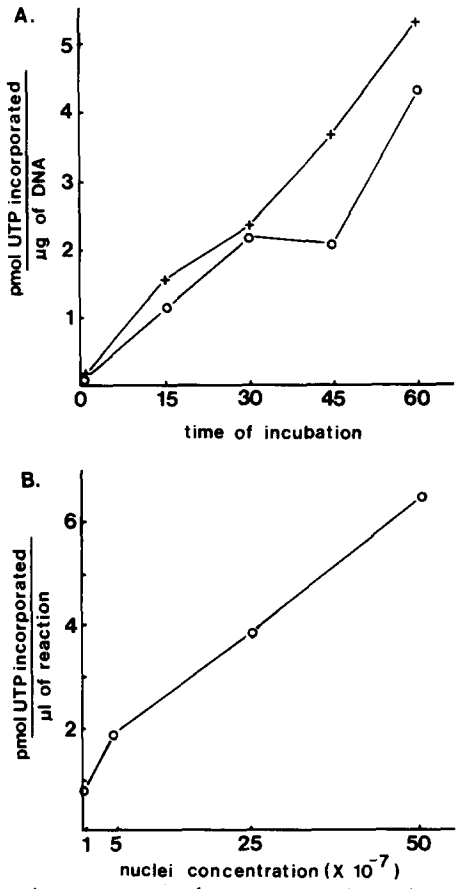

FIGURE 2 Characterization of in vitro transcription in isolated nuclei. (*A*) Time course showing increasing incorporation of [^{32}P]UTP into TCA-precipitable material with time. In the experiment shown, isolated nuclei were incubated in a 100-μl transcription reaction containing 0.16 mM each ATP, CTP, GTP, and 0.005 mM [α-^{32}P]-UTP (20 Ci/mmol). At the indicated minutes of incubation, triplicate aliquots of the assay were removed and precipitated with TCA, and the radioactivity in each aliquot was measured. DNA concentrations of the nuclei preparations used in this experiment were determined in a fluorometric assay using diaminobenzoic acid dihydrochloride (14). The difference in incorporation at 60 min by nuclei from deflagellated and nondeflagellated cells shown in this figure varies between assays and is not representative of all transcription assays. ×, assay using nuclei from deflagellated cells; O, assay using nuclei from nondeflagellated cells. (*B*) Incorporation into TCA-precipitable material when nuclei concentration per reaction is varied over a 50-fold range while the volume of the assay reaction remains constant.

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 A_{260} 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

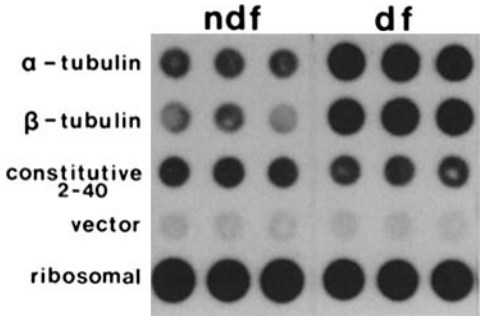

FIGURE 3 Analysis of RNAs transcribed in vitro by nuclei from deflagellated and nondeflagellated cells. Plasmids containing inserts of α-tubulin cDNA, β-tubulin cDNA, a cDNA to an mRNA that is synthesized constitutively during regeneration (*constitutive 2-40*), and 18S and 25S ribosomal genes (*ribosomal*) and a plasmid without an insert (*vector*) were spotted onto nitrocellulose filters. 2 μg of DNA per dot of the tubulins, the constitutive, and the vector plasmids and 0.2 μg per dot of the ribosomal plasmid were applied to the filters in the dots shown in this figure. Dots containing 10 μg per dot of ribosomal plasmid were also routinely included on the filters, but are not shown in this figure because of gross autoradiographic overexposure. Triplicate samples of dotted plasmid were hybridized with equal A_{260} units extracted from transcription assays using nuclei from deflagellated (*df*) or nondeflagellated (*ndf*) cells, and the resulting autoradiogram is shown. Hybridization to the filters shown here was also quantitated by scintillation counting (see Experiment 2 in Table I).

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-^{32}P]UTP and [α-^{32}P]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 A_{260} units of nucleic 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.

remains relatively constant. Background hybridization values detected when vector DNA without an insert was dotted and hybridized under the same conditions have been subtracted from values for specific RNAs. The data shown in Table I suggest a decrease in rRNA synthesis in deflagellated cell nuclei as compared with nuclei from nondeflagellated cells. However, DNA-excess hybridization conditions for the ribosomal sequences are difficult to achieve using this filter hybridization technique to compare transcription of several different genes simultaneously. To determine whether ribosomal RNA (rRNA) is transcribed at similar rates in nuclei from deflagellated and nondeflagellated cells, we therefore examined the kinetics of hybridization of RNA in transcription assays to dots of ribosomal DNA. Filters containing 10 μg of ribosomal DNA–containing plasmid were hybridized with RNA isolated from transcription assays using deflagellated or nondeflagellated cell nuclei for 12, 30, 48, and 72 h. The hybridization levels using these two RNA samples were virtually identical at each time point (data not shown). From this, we conclude that levels of hybridization of in vitro transcribed rRNA from deflagellated and nondeflagellated cell nuclei reflect their similar transcription rates (see next paragraph).

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 A_{260} 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.

TABLE II

Comparison of Methods to Determine the Stimulation of Tubulin Transcription in Deflagellated Cell Nuclei

	Average stimulation	Range of values for stimulation	Number of determinations
Method 1			
α-Tubulin	6.7	7.3–5.9	3
β-Tubulin	9.8	12.4–7.6	3
Constitutive	0.93	1.0–0.9	3
Ribosomal	0.5	0.6–0.3	3
Method 2			
α-Tubulin	3.7	8.4–2.1	10
β-Tubulin	4.4	8.2–1.9	10
Constitutive	1.4	2.4–0.5	5
Ribosomal	1.0	1.3–0.6	10

Values for the average stimulation of tubulin transcription for both methods were determined as described in Table I, by subtracting background cpm in vector DNA from cpm hybridized to filter dots containing tubulin, constitutive, or ribosomal gene sequenes, and comparing the cpm in DF samples to the cpm in NDF samples. For Method 1, the hybridization reactions contained the same A_{260} units of nucleic acid extracted from in vitro transcription assays containing nuclei from either deflagellated or nondeflagellated cells. For Method 2, the hybridization reactions contained equal cpm from transcription assays, and the cpm per filter dot were normalized to total incorporation and the DNA content of the preparations of nuclei used in the assays. These methods of determining stimulation of transcription are sufficiently different that the data have not been combined into a single average, although this table shows the data to be qualitatively similar. The stimulation of constitutive gene transcription using Method 2 is derived from fewer determinations because a different constitutive gene sequence that gave unreliably low levels of hybridization was used in some experiments. The stimulation using Method 1 combines the activities from two different preparations of nuclei, and Method 2 combines the activities of four different preparations of nuclei.

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

the effects of α-amanitin on in vitro transcription, we incubated isolated *Chlamydomonas* nuclei in transcription assays containing concentrations of α-amanitin from 0.001 to 10 μg/ml. Total incorporation into TCA-precipitable material, as well as specific incorporation into tubulin RNA, was measured. The results (Fig. 4) indicate that α-amanitin concentrations in the range of 0.1–1 μg/ml inhibit both total and tubulin specific incorporation. Incorporation into the constitutively synthesized RNA is also inhibited by these concentrations of α-amanitin (data not shown). Nuclei from nondeflagellated cells are less sensitive, by as much as 10-fold, to the drug than 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 resistent 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.

α-AMANITIN SENSITIVITY of in vitro TRANSCRIPTION

conc. (μg/ml)	% total incorporation		tubulin-specific incorporation	
	ndf	df	ndf	df
.001	100	100		
.01	98	71		
.1	72	77		
1	56	55		
10	40	39		

FIGURE 4 α-Amanitin sensitivity of transcription in isolated nuclei. The amount of total RNA transcribed in vitro in the presence of the indicated concentrations of α-amanitin was determined by TCA precipitation. Values in the table are expressed as percentages of the total amount of RNA synthesized in the presence of 0.001 μg/ml α-amanitin, and represent the average of five determinations. Tubulin-specific incorporation was determined by hybridizing the RNA synthesized in the presence of various concentrations of α-amanitin with tubulin cDNA plasmid that had been spotted onto nitrocellulose. For this experiment, each dot contains 0.2 μg of tubulin cDNA plasmid, and was hybridized in a reaction containing 4×10^6 cpm of labeled in vitro synthesized RNA (equivalent to 9.2 pmol UTP incorporated) in 0.2 ml volume.

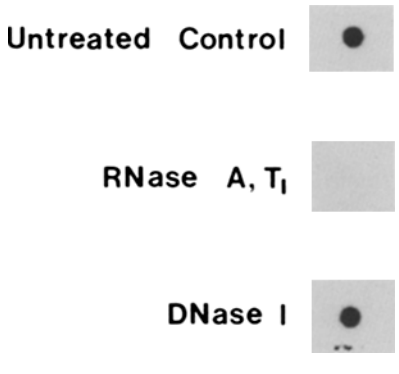

FIGURE 5 Specificity of the hybridization of in vitro synthesized RNA. Nucleic acids isolated from in vitro transcription assays by phenol extraction and a single ethanol precipitation were untreated or pretreated with DNAse I (0.1 μg/ml, Worthington Biochemical Corp.), or a mixture of RNAse A (0.15 μg/ml) and T_1 (50 U/ml, both from Sigma Chemical Co.). The samples were incubated at 37°C for 2 h and then hybridized with 0.2 μg of tubulin cDNA plasmid that had been spotted onto nitrocellulose in hybridization reactions containing 1×10^7 cpm (equivalent to 230 pmol UTP incorporated) in 1 ml of hybridization buffer.

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 T_1 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 occuring 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 protein gene activity.

We thank Drs. Scott Landfear and Paul Lefebvre for sharing their protocol for assaying transcription by nuclei isolated from *Dictyostelium discoideum* prior to publication. Dr. J. D. Rochaix kindly supplied his ribosomal DNA–containing plasmids for use in this work.

This work was supported by a reseach grant GM14642 from the National Institutes of Health to J. L. Rosenbaum. L. R. Keller was supported by a postdoctoral fellowship from the American Cancer Society, and J. A. Schloss by a U. S. Public Health Service postdoctoral fellowship.

Received for publication 9 December 1982, and in revised form 10 November 1983.

REFERENCES

1. Rosenbaum, J. L., J. E. Moulder, D. L. Ringo. 1969. Flagellar elongation and shortening in *Chlamydomonas*. I. The use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. *J. Cel Biol.* 41:601–619.
2. Lefebvre, P., S. A. Nordstrom, J. E. Moulder, and J. L. Rosenbaum. 1978. Flagellar elongation and shortening in *Chlamydomonas*. IV. Effects of flagellar detachment, regeneration, and resorption on the induction of flagellar protein synthesis. *J. Cell Biol.* 78:8–27.
3. Weeks, D., P. S. Collis, and M. A. Gealt. 1977. Control of induction of tubulin synthesis in *Chlamydomonas reinhardii*. *Nature (Lond.).* 268:667–668.
4. Weeks, D., and P. Collis. 1976. Induction of microtubule protein synthesis in *Chlamydomonas reinhardi* during flagellar regeneration. *Cell.* 9:15–27.
5. Piperno, G., and D. Luck. 1977. Microtubular proteins of *Chlamydomonas reinhardtii*. An immunochemical study based on the use of an antibody specific for the beta tubulin subunit. *J. Biol. Chem.* 252:383–391.
6. Piperno, G., B. Huang, and D. Luck. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralysed mutants of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA.* 74:1600–1604.
7. Huang, B., G. Piperno, and D. L. Luck. 1979. Paralysed flagella mutants of *Chlamydomonas reinhardtii* defective for axonemal doublet microtubule arms. *J. Biol. Chem.* 254:3091–3098.
8. Lefebvre, P. A., C. D. Silflow, E. D. Wieben, and J. L. Rosenbaum. 1980. Increased levels of mRNAs for tubulin and other flagellar proteins after amputation or shortening of *Chlamydomonas* flagella. *Cell.* 20:469–477.
9. Silflow, C. D., and J. L. Rosenbaum. 1981. Multiple alpha and beta tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation. *Cell.* 24:81–88.
10. Minami, S., P. S. Collis, E. E. Young, and D. P. Weeks. 1981. Tubulin induction in *C. reinhardii*: requirement for tubulin mRNA synthesis. *Cell.* 24:89–95.
11. Davies, D., and A. Plaskit. 1971. Genetic and structural analysis of cell wall formation in *Chlamydomonas reinhardi*. *Genet. Res.* 17:33–43.
12. Sager, R., and S. Granick. 1953. Nutritional studies with *Chlamydomonas reinhardii*. *Annu. NY Acad. Sci.* 56:831–838.
13. Witman, G., K. Carlson, J. Berliner, and J. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J. Cell Biol.* 54:507–539.
14. Kissane, J. M., and E. Robbins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* 233:;184–188.
15. Hill, B. T., and S. Whatley. 1975. A simple rapid microassay for DNA. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 56:20–23.
16. Landfear, S., P. A. Lefebvre, S. Chung, and H. Lodish. 1982. Transcriptional control of gene expression during development of *Dictyostelium discoideum*. *Mol. Cell. Biol.* 2:1417–1426.
17. Schloss, J. A., C. D. Silflow, and J. L. Rosenbaum. 1984. mRNA abundance changes during flagellar regeneration in *Chlamydomonas reinhardtii*. *Mol. Cell. Biol.* In press.
18. Marco, Y., and J.-D. Rochaix. 1980. Organization of the nuclear ribosomal DNA of *Chlamydomonas reinhardii*. *Mol. Gen. Genet.* 177:715–723.
19. Denhardt, D. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641–646.
20. Roeder, R. G., and W. J. Rutter. 1969. Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature (Lond.).* 224:234–237.
21. Lindell, T. J., F. Weinberg, P. W. Morris, R. G. Roeder, and W. J. Rutter. 1970. Specific inhibition of nuclear RNA polymerase II by alpha amanitin. *Science (Wash. DC).* 170:447–449.
22. Zylber, E., and S. Penman. 1971. Products of RNA polymerases in HeLa cell nuclei. *Proc. Natl. Acad. Sci. USA.* 68:2862–2865.
23. Guilfoyle, T. 1980. Transcription of the cauliflower mosaic virus genome in isolated nuclei from turnip leaves. *Virology.* 107:71–80.
24. Marzluff, W. F., E. C. Murphy, and R. C. C. Huang. 1973. Transcription of ribonucleic acid in isolated mouse myeloma nuclei. *Biochemistry.* 12:3440–3446.
25. Remillard, S., and G. Witman. 1982. Synthesis, transport, and utilization of specific flagellar proteins during flagellar regeneration in *Chlamydomonas*. *J. Cell Biol.* 93:615–631.
26. Smith, M. M., A. E. Reeve, and R. C. C. Huang. 1978. Analysis of RNA initiated in isolated mouse myeloma nuclei using purine nucleoside 5′ [gamma-S] triphosphates as affinity probes. *Cell.* 15:615–626.
27. Manley, J. L., P. A. Sharp, and M. L. Gefter. 1979. RNA synthesis in isolated nuclei: in vitro initiation of adenovirus 2 major late mRNA precursor. *Proc. Natl. Acad. Sci. USA.* 76:160–164.
28. Craine, B. L., and T. Kornberg. 1981. Activation of the major *Drosophila* heat shock genes in vitro. *Cell.* 25:671–681.
29. Bonner, J. J. 1981. Induction of *Drosophila* heat shock puffs in isolated polytene nuclei. *Dev. Biol.* 86:409–418.
30. Teyssot, B., L.-M. Houdebine, and J. Djiane. 1981. Prolactin induces release of a factor from membranes capable of stimulating beta-casein gene transcription in isolated mammary cell nuclei. *Proc. Natl. Acad. Sci. USA.* 78:6729–6733.