

Rapid Changes in Tubulin RNA Synthesis and Stability Induced by Deflagellation in *Chlamydomonas*

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ABSTRACT Detachment of the flagella of *Chlamydomonas* induces a rapid accumulation of mRNAs for tubulin and other flagellar proteins. Measurement of the rate of alpha and beta tubulin RNA synthesis during flagellar regeneration shows that deflagellation elicits a rapid, 4–7-fold burst in tubulin RNA synthesis. The synthesis rate peaks within 10–15 min, then declines back to the predeflagellation rate. Redeflagellation of cells at times before the first flagellar regeneration is completed (and when cells have already accumulated elevated levels of tubulin RNA) induces another burst in tubulin RNA synthesis which is identical to the first in magnitude and duration. This finding indicates that the induction signal may act to simply reprogram the tubulin genes for a transient burst of maximal synthesis. Evidence is presented that the stability of the tubulin RNAs changes during regeneration. Stability changes include both an apparent stabilization during regeneration and accelerated decay following regeneration.

The eucaryotic biflagellate, *Chlamydomonas reinhardtii*, undergoes flagellar regeneration after mechanical detachment of its flagella. The response to deflagellation involves a large increase in the synthesis of flagellar proteins (14, 33) effected by an increased accumulation (6–40-fold) of their messenger RNAs (15, 20, 29, 31). Flagella are rapidly assembled, within 60–90 min, and subsequently flagellar-specific protein synthesis and mRNA abundance return to basal levels. Thus the response to deflagellation involves both an induction and a deinduction, the entire process spanning <3 h.

Recent evidence from this laboratory indicates that the increase in alpha and beta tubulin mRNA levels is due, at least in part, to a transcriptional activation of the tubulin genes. Nuclei isolated from deflagellated cells support an average fivefold more transcription of tubulin RNA sequences than do nuclei from nondeflagellated cells (12). We were interested in obtaining more detailed information about the extent and duration of the transcriptional induction and its termination. In particular, we wanted to know the maximum level of induction and when it occurs, the timing of the deinduction, and whether it can be correlated with completion of flagellar assembly, and whether the activation of tubulin RNA synthesis is sufficient to account for the large increase in abundance observed.

In this report, we describe the results of in vivo labeling experiments designed to elucidate the tubulin RNA synthetic and decay changes occurring throughout the regeneration process.

MATERIALS AND METHODS

Cell Growth and Preparation: *Chlamydomonas reinhardtii* cells, strain 21gr, were grown in a low phosphate variant of medium I (27) in which potassium phosphate salts have been reduced 20-fold (0.065 mM) and the media buffered with 10 mM HEPES. Cells were grown to $\sim 1 \times 10^6$ cells/ml except where noted. For deflagellation and labeling, cells were concentrated to $1\text{--}2 \times 10^7$ cells/ml in spent media or in fresh media containing no phosphate. Cells were deflagellated by mechanical shear (25) and stirred under fluorescent illumination during regeneration. Flagellar lengths were monitored by phase-contrast microscopy.

In Vivo Labeling: In all experiments cells were labeled with [^{32}P] orthophosphate (Amersham Corp., Arlington Heights, IL; PBS.11A in dilute HCl) at 80 $\mu\text{Ci/ml}$. For pulse labeling studies, aliquots of 2×10^8 cells were removed from cultures and transferred to centrifuge tubes containing 800 μCi of label. The cells were aerated under illumination during the 5-min labeling period. The pulse was terminated by addition of 2 vol of ice cold medium I, and cells were pelleted, lysed in 0.3 M NaCl-5 mM EGTA- 50 mM Tris-hydrochloride (pH 8.0)-2% SDS at a concentration of 1×10^8 cells/ml, and frozen in liquid nitrogen within 5 min from the end of the pulse period. For continuous label experiments, 2×10^8 cells were removed from the labeling culture and pelleted through 1.5–2 vol of ice cold medium I and processed similarly. Cell samples for nucleotide triphosphate pool analysis were pelleted through 20 vol of ice cold medium I, lysed in 62.5 mM Tris-hydrochloride (pH 6.8)-2% SDS-10% glycerol, 5% 2-mercaptoethanol, and frozen in liquid nitrogen.

[^{32}P]orthophosphate labeling in *Chlamydomonas* presents several problems. We have determined that the rate of uptake of the label (total internalized [^{32}P]PO $_4$) is variable and dependent upon small differences in the density to which the cell culture has grown (or the length of time cells have spent in the low phosphate media), and furthermore, is different in deflagellated and nondeflagellated cells. The rate of uptake of label is 1.2–6-fold slower in deflagellated cells and constantly changing during regeneration. The time for equilibration of label in the ribonucleotide triphosphate pools (analyzed by thin layer

chromatography on PEI-cellulose) is similarly affected (data not shown). The rate of labeling of the nuclear vs. the cytoplasmic ribonucleotide triphosphate pools, which cannot be separately determined, may also vary under these experimental conditions. Therefore rather than attempt to use the measured specific activity of the ATP pool to adjust all of the incorporation data, we have chosen to normalize all tubulin RNA synthesis and turnover measurements with respect to incorporation into ribosomal RNA (see following section). The effect of cell culture age on incorporation of [³²P]phosphate into ribosomal RNA in *Chlamydomonas* has been noted previously (34).

RNA Isolation: Cell lysates were incubated with 80–120 µg/ml proteinase K for 20 min at room temperature, extracted twice with buffer-saturated phenol/chloroform/isoamyl alcohol (50:49:1), and once with chloroform/isoamyl alcohol. RNA was purified by centrifugation through a CsCl cushion (9). Poly(A+) RNA was isolated by oligo deoxythymidylate(oligo dT)-cellulose chromatography (26).

Hybridizations: Plasmid DNA dots on nitrocellulose filters were prepared as previously described (29) except that 5 µg of each plasmid DNA were dotted (unless noted). pBR322 plasmids containing alpha tubulin (pcf4-2) and beta tubulin (pcf8-31), complementary DNAs (cDNAs) and a constitutively expressed sequence (pcf2-40) cDNA have been described (29). The genomic ribosomal DNA plasmid (2.35), containing a 25S-18S ribosomal repeat unit as a 2.35-kd BamHI fragment was provided by Dr. J. D. Rochaix, Departments of Molecular Biology and Plant Biology, University of Geneva, Switzerland (19). 2 µg (rather than 5) of this plasmid were dotted on most filters. Filters were prehybridized for 12–24 h and hybridized with 200 µg (100–133 µg/ml) of *in vivo* labeled RNA (total cellular) for 90–96 h at 42°C. Hybridization and prehybridization buffer contained 50% deionized formamide, 5 × SSC, 50 mM Na phosphate, pH 6.5, 5 × Denhardt's solution, 0.1% NaDodSO₄, 100 µg/ml denatured *Escherichia coli* DNA, 100 µg/ml yeast transfer RNA, and 20 µg/ml polyadenylic acid. DNA excess conditions for the tubulin and constitutive RNA sequences were demonstrated by hybridization of 200 µg of ³²P-RNA to filters containing 2, 4, 6, or 8 µg plasmid DNA dots under usual hybridization conditions. No increase in hybridization to these DNAs was observed beyond the 2 µg level, while hybridization to 2.35 (rDNA) increased linearly (not shown). Lengthening the hybridization time beyond 96 h results in no further increase in hybridization. Under these hybridization conditions it can be calculated that rRNA is in at least a 60-fold excess over hybridizable rDNA sequences. Since the same number of micrograms of RNA are included in each hybridization mixture (and rRNA levels do not change during regeneration [29]), the hybridization to 2.35 is proportional to the specific radioactivity of the ribosomal RNA. Relative hybridization to 2.35 has been used in most of these studies as a means of normalizing hybridization data from a number of different experiments and time points (see text). After hybridization, filters were washed twice for 5 min each in 2 × SSC-0.1% NaDodSO₄ at room temperature and twice for 30 min each in 0.1 × SSC-0.1% NaDodSO₄ at 50°C and dried under a heat lamp. After autoradiography individual dots were excised and hybridization quantified by scintillation counting in Liquifluor (New England Nuclear, Boston, MA). Counts per minute hybridizing to vector DNA (pBR322) were subtracted from each value.

RNA dot filters were prepared as described previously except that 5 µg of total cellular RNA were applied per dot. Nick-translation of plasmid DNA with ³²P-dCTP or ³²P-dATP was performed as described (29). 0.2 µg of nick-translated DNA was used for each hybridization.

RESULTS

Relative Rates of Synthesis of Tubulin RNA during Regeneration

Recent *in vitro* transcription studies, using nuclei isolated from regenerating *Chlamydomonas* cells, demonstrate that an elevated transcription rate of alpha and beta tubulin RNA follows deflagellation (12). However, additional information on the pattern and duration of the transcriptional induction is required to evaluate the contribution of new synthesis to the accumulation of these messages after deflagellation. Therefore, we have measured, *in vivo*, the rates of synthesis of alpha and beta tubulin RNA throughout regeneration relative to the rate in nondeflagellated, control cells. Aliquots of control cells and regenerating cells, deflagellated by mechanical shear, were pulsed for 5 min with [³²P]orthophosphate at intervals during regeneration. Total cellular RNA, prepared from pulse-labeled cells, was hybridized to nitrocel-

lulose filters containing duplicate dots of the following *Chlamydomonas* DNAs: complementary DNA sequences to alpha tubulin RNA (4-2), beta tubulin RNA (8-31), and a constitutive RNA (2-40), i.e., one whose abundance does not change during regeneration (29), a genomic rDNA repeat unit (2.35), and pBR322 DNA containing no insert. Hybridization conditions were in DNA excess with respect to the mRNA sequences (4-2, 8-31, and 2-40). Thus, hybridization to completion to these cDNAs is proportional to the relative rates of synthesis of their RNAs at each time point. The hybridization was in RNA excess with respect to ribosomal DNA sequences, and under our conditions hybridization to rDNA is proportional to the specific radioactivity of rRNA at each time point (see Materials and Methods). The amount of hybridization to each DNA dot was quantified by scintillation counting. Fig. 1A inset shows an example of an autoradiogram from such a hybridization, and Fig. 1A is a plot of the relative rates of synthesis of beta tubulin RNA derived from four separate experiments.

The counts per minute hybridizing to beta-tubulin DNA have been normalized, in each case, to the counts per minute hybridizing to rDNA. This was done because analysis of nucleotide triphosphate pools (by PEI-cellulose chromatography, data not shown) indicated that the rate of labeling of these pools was quite different in deflagellated and nondeflagellated cells (see Materials and Methods). In standardizing all measurements with respect to rRNA specific activity, there is an assumption that rRNA synthesis remains approximately constant during regeneration, and also, that polymerase I and II transcription draw from the same nucleotide precursor pool. Several observations support the validity of these assumptions. (a) *In vitro* transcription experiments indicate that nuclei from deflagellated cells transcribe rRNA at rates comparable to, or somewhat less than, nuclei from control cells (12). (b) The decrease in apparent rRNA specific activity in time points early after deflagellation is proportional to the decreased radioactivity in the ATP pool. (c) In experiments in which sufficient label has been incorporated into the constitutive RNA (2-40) to allow quantitation, normalization of the data with respect to this species or to ribosomal RNA yields essentially identical curves. Interpretation of the results in the light of a possible decrease in rRNA synthesis after deflagellation will be addressed in the Discussion.

Fig. 1 demonstrates that the rate of synthesis of tubulin RNA increases an average of sixfold within 10 min after deflagellation (the earliest time point taken), and declines back to control levels within ~90–100 min. While all the data suggest that the decline to control levels occurs by a series of decreasing rates, it is possible that the apparent intermediate rates are a reflection of cell asynchrony. However, the cells in each experiment were extremely synchronous with respect to flagellar regrowth. The data show that deflagellation elicits an immediate burst of tubulin RNA synthesis, followed by a fairly rapid attenuation in synthesis rate. During this period the rate of synthesis of the constitutive RNA remains constant. It is clear that reduction in the rate of synthesis occurs well before flagella are fully elongated (Fig. 1B), eliminating the possibility that completion of regeneration signals a deinduction of transcription.

While the kinetics of the return to the predeflagellation synthesis rate vary in the four experiments presented, there is a correlation between the rate of flagellar regeneration and the kinetics of deinduction. The two groups of cells exhibiting

the most rapid deinduction were also fully regenerated in a shorter time (ca. 60 vs. 90 min).

We cannot be certain that relative synthesis rates determined from 5-min pulse-labeled RNA are equivalent to transcription rates. Since the pulse period is brief relative to the half-life of tubulin RNA (see following half-life determination), cytoplasmic decay is probably an insignificant contributor to the final values. It is possible though that differences exist in very rapid posttranscriptional events which could

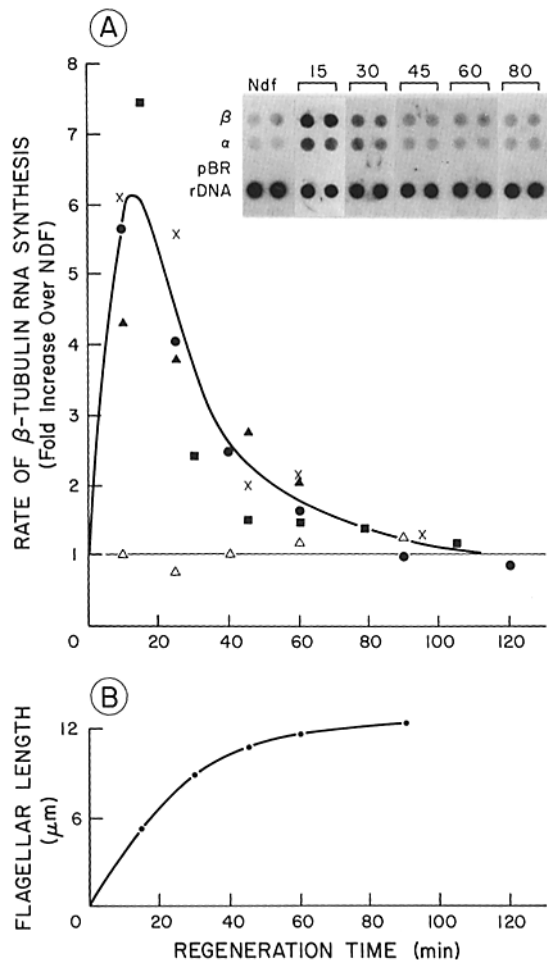


FIGURE 1 Relative rates of synthesis of tubulin mRNA during regeneration. ^{32}P -RNA, labeled in vivo with 5-min pulses of [^{32}P]-orthophosphate during regeneration, was hybridized to nitrocellulose filters containing duplicate dots of the following DNAs: 8-31 (beta tubulin), 4-2 (alpha tubulin), 2-40 (constitutive), 2.35 (ribosomal DNA), and pBR322 (vector). Hybridization was quantified by scintillation counting of individual dots, normalized with respect to hybridization to ribosomal DNA, and expressed as a fold increase over nondeflagellated values (NDF = 1). (A) The curve shows the fold increase in the relative rate of synthesis of beta tubulin mRNA during regeneration. The points on the curve represent data from four separate experiments (x, ●, ▲, ■). The curve for alpha tubulin RNA is similar to that shown for beta tubulin RNA. The open triangles (Δ) show hybridization to 2-40 (constitutive) DNA from one experiment. The relatively slow rate of labeling of 2-40 RNA compared with the tubulin RNAs, does not permit quantitative scintillation counting in all experiments. Inset shows a sample hybridization autoradiogram from one experiment (■). NDF, hybridization of 5-min pulse-labeled RNA from nondeflagellated cells. Remaining filters show hybridizations of RNA labeled for 10–15, 25–30, 40–45, 55–60, and 75–80 min after deflagellation. B shows a flagellar regeneration curve. Each point represents the average of the four experiments shown in A.

contribute to the increase in ^{32}P -hybridizable tubulin sequences in regenerating cells. The fact that the in vivo measured rate change following deflagellation is comparable to the change in transcription rate determined in vitro using isolated nuclei suggests that we are looking at mainly transcriptional differences.

Since the data shown in Fig. 1 are relative instantaneous rates of synthesis, integration under the curve provides a measure of the overall increase in synthesis during regeneration. The ratio of the area under the whole curve through any time, t (equivalent to synthesis in deflagellated cells) relative to the area under the curve defined by $y = 1$ (synthesis in nondeflagellated cells) yields the (average) fold increase in transcripts produced in regenerating vs. control cells during that time period. These values were determined for alpha and beta tubulin RNA synthesis from each of the four sets of data shown in Fig. 1 (and the corresponding alpha tubulin RNA synthesis data, not shown). These measurements, presented in Table I, show that there is an average 2.9-fold (range 2.5–3.3-fold) increase in tubulin RNA synthesized by regenerating cells over control cells during the 90 min following deflagellation.

Relative Rate of Tubulin RNA Synthesis after Multiple Deflagellations

Chlamydomonas can be redeflagellated during the course of regeneration (i.e., before full flagellar regrowth has occurred) and will begin assembly of another set of flagella (25). The flagellar elongations following each of multiple deflagellations proceed with similar kinetics. To determine whether the tubulin RNA synthetic response is identical after a second deflagellation, two in vivo pulse-label experiments were performed. In one, a portion of regenerating cells was redeflagellated early into regeneration when flagella were ~50% assembled; in the other, cells were redeflagellated when flagella were ~80% completed. In both experiments, a portion of cells was allowed to complete the first regeneration. Pulse-labeled ^{32}P -RNA isolated at various times from regenerating and control cells was hybridized to filter-bound cDNAs as described for Fig. 1. The flagellar regeneration curves from the two experiments are presented in Fig. 2, A and B, and the corresponding RNA synthesis data in Fig. 2, C and D. For clarity, only data for beta tubulin RNA synthesis are shown. The curves for alpha tubulin RNA synthesis are similar. In Experiment 1 (Fig. 2C), the rate of beta tubulin RNA synthesis was down to ~80% of its measured maximum at the time redeflagellation occurred, at which time it returned rapidly to its previous maximum rate. In Experiment 2 (Fig. 2D), the synthesis rate was down to 33% of its measured maximum at 50 min when the cells were redeflagellated, and again, the rate increased to

TABLE I
Increase in Synthesis of Tubulin RNA during Regeneration

Experiment no.	Alpha*	Beta*
1	3.2	2.6
2	3.3	3.2
3	3.0	2.6
4	2.5	2.6

* Area under DF (deflagellated) synthesis rate curve/area under NDF (nondeflagellated) curve ($y = 1$) from time 0 (deflagellation) to 90 min = average fold increase in tubulin RNA synthesized in deflagellated cells over nondeflagellated cells. The values for beta tubulin RNA were calculated from data presented in Fig. 1.

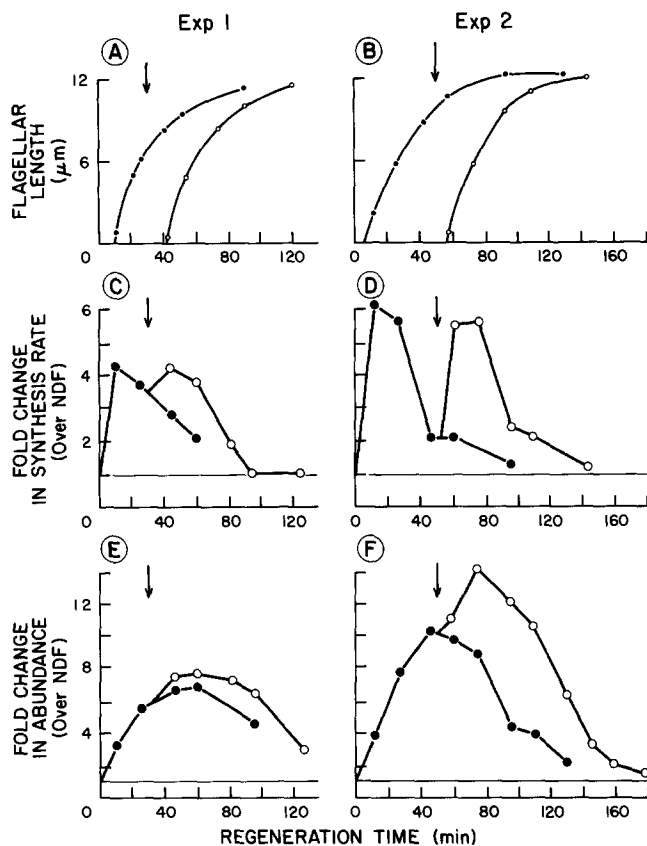


FIGURE 2 Relative rates of synthesis and accumulation of beta tubulin mRNA in double-deflagellated cells. *A* and *B* show flagellar regeneration curves from two different experiments in which regenerating cells were subjected to a second deflagellation. The arrows show the time at which the second deflagellation was performed on a portion of the regenerating cells. *C* and *D* show the relative rates of synthesis of beta tubulin RNA from the same experiments. Hybridization of 5-min pulse-labeled ^{32}P -RNA to tubulin cDNAs was performed and quantitated as described in Fig. 1. ●, single deflagellation; ○, double deflagellation. *E* and *F* show plots of the fold increase in accumulated beta tubulin RNA levels over nondeflagellated levels (NDF = 1) obtained by scintillation counting of the RNA dots shown in Fig. 3, averaging triplicate determinations (only duplicates are shown). Details are presented in the Fig. 3 legend. ●, single deflagellation; ○, double deflagellation.

the previous maximum within 10 min. It appears that deflagellation induces a maximal activation of tubulin RNA synthesis whatever the state of the flagella (during assembly or full length) or whatever the current tubulin RNA synthesis rate or abundance (see following section). It is possible that the extent of the transcriptional burst may not be subject to modulation; that is, the act of deflagellation may simply reprogram the tubulin genes to full transcriptional activity.

Accumulation of Tubulin RNAs during Regeneration

While the tubulin RNA synthesis rate peaks no later than 10–15 min after deflagellation, previously published work from this laboratory (15, 29, 31) and others (20) indicates that the accumulation of tubulin RNA peaks 30–40 min later. Furthermore, the peak accumulation levels reported (6–40-fold over nondeflagellated cell levels) are very high relative to the average 2.9-fold increase in synthesis just determined. The

broad range in accumulation levels reported could be due in part to differences in cell types used (e.g., gametes vs. vegetative cells) and variation in the time of day (cell cycle position) in which RNAs were prepared, as well as probable exaggerations of induced levels due to densitometric quantitation. However, even the lowest values obtained are high relative to the induced synthesis and suggest changes in stability of the tubulin RNAs in regenerating cells.

Due to the above-mentioned variation in accumulation levels reported, the importance of measuring synthesis and accumulation in the same group of regenerating cells is apparent. For the two experiments presented in Fig. 2, *C* and *D*, samples of unlabeled RNA were prepared simultaneously with each ^{32}P -RNA preparation. The unlabeled RNA was dotted onto nitrocellulose, and the relative abundance of alpha and beta tubulin RNA, as well as the constitutive RNA, was determined by hybridization with the corresponding nick-translated probe under DNA excess conditions. Autoradiograms of the dot hybridizations for alpha and beta tubulin RNA are shown in Fig. 3. For ease of comparison with corresponding synthesis rates (2, *C* and *D*), the changing beta tubulin RNA levels obtained by scintillation counting of excised dots are presented in Fig. 2, *E* and *F*. The data are presented as fold-increase in abundance over nondeflagellated beta tubulin RNA levels (nondeflagellated levels = 1), which remain constant throughout the 2–3 h of the experiments (data not shown). Note that the scales of Fig. 2, *C* and *D* are twice that of *E* and *F*.

Fig. 2, *E* and *F* show the maximum fold-increase in beta tubulin RNA abundance to be substantially greater than the 2.6 and 3.2-fold increases in synthesis determined for the same populations of cells (Experiments 1 and 2, Table I). The cells in Experiment 1 (Fig. 2*E*) attained a 6.8-fold increase in beta tubulin RNA sequences in single-deflagellated cells. The early redeflagellation resulted in only a somewhat elevated peak accumulation level (7.2-fold vs. 6.8-fold), but the higher level was maintained for a longer time. In Experiment 2 (Fig. 2*F*) beta tubulin RNA levels reached a 10.1-fold increase over predeflagellation levels, and a 14-fold increase for redeflagellated cells. In this experiment, redeflagellation occurred at just about the peak of tubulin RNA accumulation from the first deflagellation.

Comparison of rates of synthesis and accumulation can provide information on the stability of these RNAs. As mentioned previously, integration under the rate of synthesis curves gives the increase in total tubulin RNA synthesized over any period. The ratio of the area under each curve relative to the area under the control curve, $y = 1$, is equal to the fold increase in tubulin RNA synthesized over controls. These values were determined from time 0 (deflagellation) to the time point at which peak accumulation levels of alpha and beta tubulin RNA are reached (60 and 45 min for single-deflagellated cells in Experiments 1 and 2, respectively; 60 and 75 min for double-deflagellated cells). The maximum abundance increase cannot be expected to surpass the increase in synthesis if the stability of the tubulin RNA remains constant during regeneration.

Values for the increase in tubulin RNA synthesis up to the time of maximal accumulation are shown in Table II (column 1). The increase in synthesis of both alpha and beta tubulin RNA was less in cells from Experiment 1 than from Experiment 2, 3.1–3.6-fold vs. 4.4-fold, in single-deflagellated cells. Consistently, the Experiment 1 cells also accumulated less

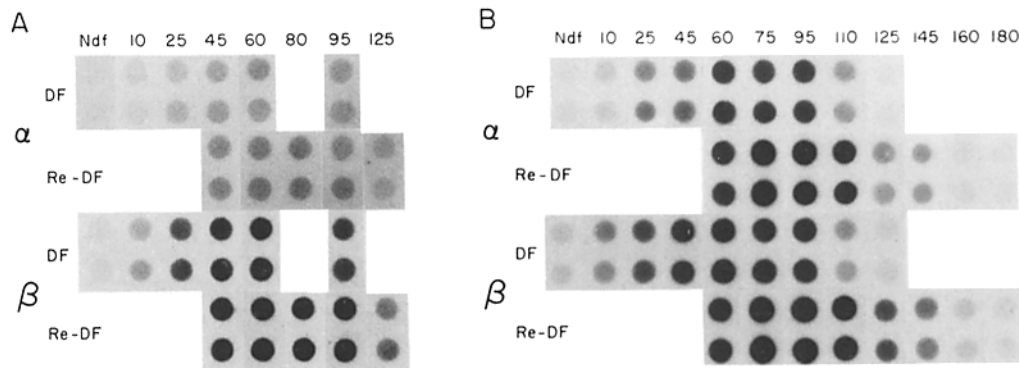


FIGURE 3 Tubulin RNA accumulation during regeneration in single- and double-deflagellated cells. Unlabeled total cytoplasmic RNA was prepared from cells at various times during regeneration after single and double deflagellations. Equal micrograms were immobilized on nitrocellulose filters in triplicate and hybridized to nick-translated plasmids 8-31 (beta tubulin), 4-2 (alpha

tubulin), and 2-40 (constitutive) under DNA excess conditions. *A* and *B* show autoradiograms of filters hybridized to alpha and beta tubulin cDNA probes. *A*, experiment 1; *B*, experiment 2. Hybridization to 2-40 (not shown) remained essentially constant. The relatively faint hybridization signal to alpha tubulin RNA in experiment 1 is due to use of a low specific activity probe in this hybridization. The RNAs used in these hybridizations were prepared from the same cells from which the Fig. 2 synthesis data were obtained. *DF*, single deflagellation; *ReDF*, redeflagellation. The second deflagellations occurred at 30 and 50 min in experiments 1 and 2, respectively. Plots of the fold increase in beta tubulin mRNA levels over nondeflagellated levels (NDF = 1) obtained by scintillation counting of the dots shown in *A* and *B*, are shown in Fig. 2, *E* and *F* for ease of comparison.

TABLE II
Stabilization of Tubulin RNA during Regeneration

	Synthesis* (Fold increase over NDF)	Accumulation† (Maximum)	Stability‡ increase
Single deflagellation			
Experiment 1—alpha	3.6	6.9 (60 min)	1.9
beta	3.1	6.8	2.2
Experiment 2—alpha	4.4	8.2 (45 min)	1.9
beta	4.4	10.1	2.3
Double deflagellation			
Experiment 1—alpha	4.3	6.9 (60 min)	1.6
beta	3.5	7.2	2.1
Experiment 2—alpha	4.7	12.0 (75 min)	2.5
beta	4.5	14.0	3.1

* Area under the DF synthesis rate curve/area under the NDF curve ($y = 1$) from time 0 (deflagellation) to time at which maximum accumulation occurs (indicated in parentheses in column 2). The values for beta tubulin RNA are calculated from the curves presented in Fig. 2, *A* and *B*.

† Maximum fold increase in tubulin RNA levels over NDF levels (NDF = 1) during regeneration, from Fig. 2, *C* and *D* and Fig. 3.

‡ Fold increase in accumulation/fold increase in synthesis.

tubulin RNA, 6.8–6.9-fold vs. 8.2–10.1-fold. The peak accumulation values after the first deflagellation are, in each case, about twice the predicted maxima (Table II, column 3). Thus, there must be at least a two-fold stabilization of the transcripts to account for the message levels attained. Whether the stability change is a cytoplasmic or nuclear (processing) phenomenon is not distinguished by this analysis.

Comparison of synthesis and accumulation values in double-deflagellated cells suggests similar stabilization of tubulin RNA during the second regeneration, although the values obtained (1.6–3.1-fold) show more variation than during the first accumulation. The higher values (2.5 and 3.1-fold increases in stability) calculated for Experiment 2 cells could be the result of the timing of the second deflagellation which occurred at or near the peak of tubulin RNA accumulation from the first induction. Although, in a number of other measurements of tubulin RNA accumulation following multiple deflagellations (unpublished data), we find the levels of

tubulin RNA induced by second (and third) deflagellations to be variable.

Half-life of Tubulin RNA

The rate of loss of tubulin RNA after 50–60 min of regeneration (Fig. 3 and Fig. 2, *E* and *F*) provides a measure (a maximum value) of the half-life of tubulin RNA during the period between the peak of accumulation and the return to basal levels. Relative abundance values taken from both sets of curves shown (Fig. 2, *E* and *F*), as well as those for alpha-tubulin RNA levels, were plotted logarithmically, and the curves are shown in Fig. 4. The data are best fit to two-component curves. The first component, extending to 30–35 min following the peak of accumulation and showing half-lives of 120–130 min for the tubulin RNAs, is due in whole or in part to the method of measurement. That is, unlike a true pulse-chase, synthesis of new molecules continues to contribute to the RNA abundance measured, and the rate of synthesis is still above the control rate during the first 30 min of this analysis. Although the two-component nature of the curve may indicate a change in stability of these RNAs at the inflection points, meaningful analysis is not possible. Unfortunately, we are unable to measure the half-life of tubulin RNA during the induction phase of the regeneration response, since an effective pulse-chase cannot be performed in these cells.

The second components of these curves, however, yield half-life values indicating a very rapid decay of the tubulin RNAs following regeneration, 27 min and 23 min for alpha and beta tubulin RNAs, respectively. Moreover, the half-lives calculated from this analysis are certainly overestimates due to the substantial contribution of synthesis to these values. Calculation of half-lives from other measurements of tubulin RNA loss following regeneration in *Chlamydomonas* (20, 29, and unpublished data) are consistent with the half-lives calculated here, with (maximum) values ranging from 14 to 26 min.

These half-life measurements were compared with the half-lives of alpha and beta tubulin RNAs in nonregenerating cells. To determine the normal half-lives of the tubulin RNAs in unperturbed vegetative cells, cells were exposed to [³²P]ortho-

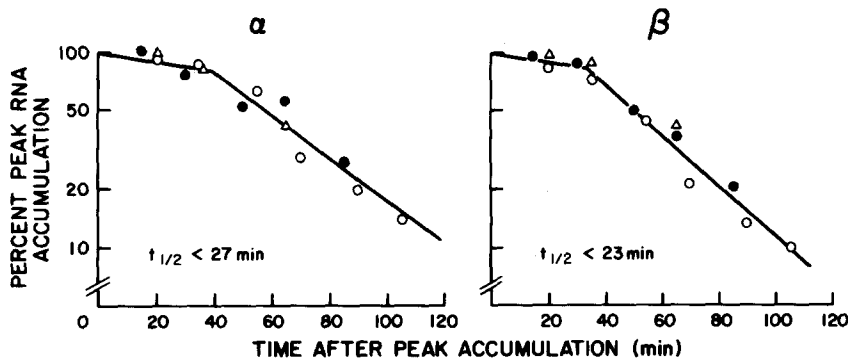


FIGURE 4 Half-lives of alpha and beta tubulin mRNAs following regeneration. The declining tubulin RNA levels after the peak in abundance in regenerating cells are plotted as the percent of peak levels. The data are taken from the abundance curves shown in Fig. 2, E and F (and corresponding alpha tubulin RNA data, Fig. 3). Δ , experiment 1 cells, double deflagellation, peak at 60 min (100%); \bullet , experiment 2 cells, single deflagellation, peak at 45 min; \circ , experiment 2 cells, double deflagellation, peak at 75 min. Best fits to the data were determined by linear regression analysis of the natural logarithms of the normalized values.

phosphate continuously for periods up to 2.5 h, removing aliquots of cells at intervals for RNA preparation. ^{32}P -RNA was hybridized to plasmid DNA dots and hybridization quantified as described previously. The data were plotted as the ratio of counts per minute incorporated into alpha and beta tubulin RNAs relative to hybridization to rDNA. It is assumed that rRNA is completely stable for the 2.5 h duration of this study (30); thus, any changes in the ratio of radioactivity incorporated into tubulin RNA, relative to ribosomal RNA, is a reflection of the instability of that species. A logarithmic plot of the decreasing ratio provides a direct measure of the half-life of the RNA.

The plots from two separate determinations of the half-lives of alpha and beta tubulin RNAs are shown in Fig. 5, A and B. The half-lives determined from these data are ca. 55 min (65 and 48 min) for alpha tubulin RNA, and ca. 45 min (47 and 39 min) for beta tubulin RNA.

From the same two experiments the half-life of the constitutive RNA (2-40) was determined to be at least 2.5 h (Fig. 5C). An accurate determination of the half-life of this longer lived mRNA cannot be made with only a 2.5-h labeling period. It is clear however, that the constitutive RNA is significantly more stable than either of the tubulin RNAs. Similarly, although we have not determined the actual average half-life of total poly (A+) RNA in *Chlamydomonas*, the ratio of incorporation into poly (A+) RNA relative to rRNA, indicates a half-life of at least 2.5 h (Fig. 5D).

Thus it appears that tubulin RNA decays with at least a two- to threefold shorter half-life following regeneration (<23–27 min) than during normal vegetative growth (45–55 min). One possible source of error in these measurements is the time required for the radioactivity in the nucleotide triphosphate pools to equilibrate. In the two experiments shown, cells were grown to different densities (2×10^6 cells/ml and 5×10^5 cells/ml). We had earlier determined that the rate of uptake of label and equilibration time of label into the nucleotide triphosphate pools is affected by the densities to which cells had grown (see Materials and Methods). These two cell culture conditions represented experimental extremes. The radioactivity in the ATP pool was essentially equilibrated within 15 min in the high density cells and required nearly 1 h to equilibrate in the low density cells (data obtained by thin layer chromatography, not shown). The difference in half-lives determined under these two conditions is ± 17 min for alpha tubulin RNA and ± 8 min for beta tubulin RNA. We conclude that neither the actual turnover time, nor our measurement of it by this method is severely altered by cell growth conditions. In neither case does the half-life determined in nonregenerating cells approach the low of ~ 20 min seen following regeneration.

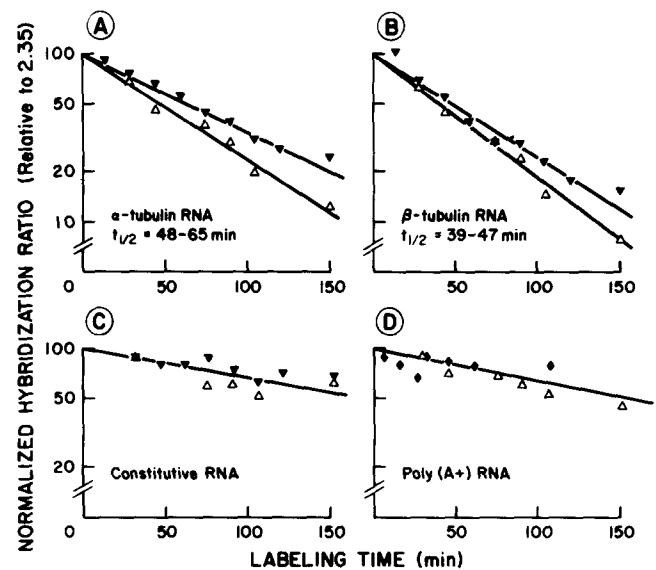


FIGURE 5 Half-lives of alpha and beta tubulin, and constitutive RNAs in nonregenerating cells. Vegetative cells were labeled continuously and RNA prepared from cells removed at intervals, as described in Materials and Methods. ^{32}P -RNA was hybridized to plasmid DNAs and hybridization quantified as described. Ratios of hybridization to 4-2 (alpha), 8-31 (beta), 2-40 (constitutive) DNAs, relative to 2.35 (rDNA), were determined. Ratios were plotted as a function of labeling time. Half-lives were determined as for Fig. 4. Separate experiments were normalized with respect to the y-intercept values which were set equal to 100. For the constitutive RNA and poly(A+) RNA, the two sets of data were combined. (A) Half-life of alpha tubulin RNA. The symbols represent data from two separate determinations. ∇ , Cells grown to 2×10^6 cells/ml; Δ , Cells grown to 5×10^5 cells/ml (see text). (B) Half-life of beta tubulin RNA; (C) half-life of constitutive (2-40) RNA; (D) stability of poly(A+) RNA. RNA samples from continuously labeled cells were separated into poly(A+) and poly(A-) fractions by oligo dT-cellulose chromatography. The specific activity of the poly(A+) RNA (counts per minute in poly(A+) per microgram of total RNA) was determined, and the normalized (y-intercept = 100) ratio of poly(A+) RNA specific activity relative to the specific activity of rRNA (counts per minute hybridizing to 2.35) is plotted. One set of data (\blacklozenge) is obtained from a different group of cells than those used for the preceding half-life determinations.

These half-life measurements indicate that alpha and beta tubulin RNAs decay at an accelerated rate during the late (deinduction) stage of regeneration (half-lives <27 and 23 min), relative to the rate in control, nonregenerating cells (half-lives ca. 55 and 45 min). While we cannot measure tubulin RNA half-life during the early (induction) phase of the regeneration response, we have inferred that the half-lives

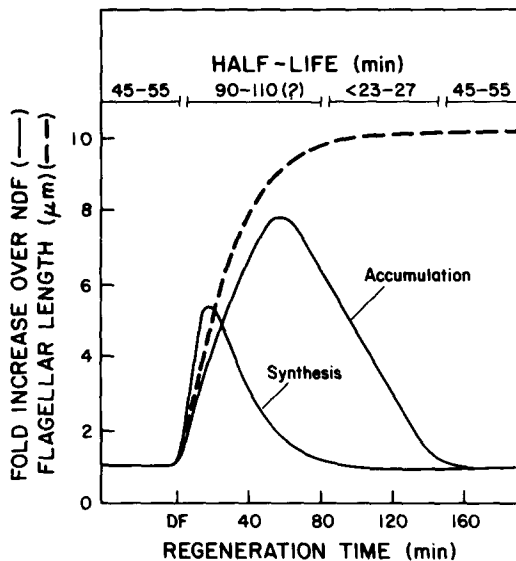


FIGURE 6 Changes in tubulin RNA synthesis rate, accumulation, and half-life during flagellar regeneration. A "typical" response to deflagellation is depicted in this diagram which summarizes the data on tubulin mRNA metabolism presented in this report.

must be lengthened at least 2-fold relative to the predeflagellation half-lives to account for the increase in tubulin RNA abundance observed (half-lives ca. 110 and 90 min). There is, then, at least a fourfold change in tubulin RNA stability during the course of regeneration.

Fig. 6 shows a diagram summarizing the changes in tubulin RNA synthesis, accumulation; and half-life during regeneration.

DISCUSSION

In this report we have demonstrated that deflagellation of *Chlamydomonas* cells signals a rapid, four to sevenfold increase in the rate of alpha and beta tubulin RNA synthesis. The maximum rate of synthesis is achieved very quickly, within 10–15 min, and the rate then declines back to the predeflagellation rate over the next 1–1.5 h. The subsequent accumulation of tubulin RNA peaks between 45 and 60 min following deflagellation. The maximum rate of tubulin RNA synthesis may actually occur even before 10 min. In all experiments, the first time point after deflagellation always showed the highest rate, and no pulse-labeling period ending earlier than 10 min was examined.

The brevity of the period during which tubulin RNA synthesis is maximal is as striking as the speed of activation. Whatever the signaling system is that causes the rapid induction of flagellar mRNA synthesis, its properties must include a mechanism for the rapid depression of synthesis that follows. It is clear that the deinduction is not signalled by completion of regeneration, since the decrease in synthesis rate begins when flagella are <50% assembled. A similar burst-attenuation pattern of transcriptional activation has been described for the stimulation of prolactin gene transcription by epidermal growth factor (21).

A second deflagellation before the completion of regeneration signals an activation of tubulin RNA synthesis that is virtually identical to the first activation; that is, it occurs with the same kinetics and to the same magnitude as the first (Fig. 2). These findings suggest that the function of the induction

signal may be simply to reprogram the flagellar genes for a transient burst of synthesis. The kinetics of the second activation in the two experiments shown were not affected by the different ongoing rates of synthesis, the levels of tubulin RNA already accumulated in the cells, or the state of assembly of the flagella at the time of redeflagellation. Apparent autoregulatory control of tubulin mRNA levels by unpolymerized tubulin pools has been reported to occur in a number of cell types (2, 5, 6). Since the flagellar protein precursor pools in *Chlamydomonas* cells vary considerably during regeneration (14), it is likely that there were quite different levels of unpolymerized tubulin in the cells at the first and second deflagellations and that these differences had no effect on the kinetics of activation. However, we are investigating the possibility of autoregulatory control of tubulin RNA synthesis and stability more carefully by methods that allow experimental manipulation of tubulin pool sizes.

We have shown that alpha and beta tubulin RNAs turn over rapidly, relative to most poly (A+) mRNAs in *Chlamydomonas*, with half-lives of ~55 and 45 min for alpha and beta, respectively. The half-life of total poly (A+) RNA is at least 2.5 h. Tubulin mRNA may have a short half-life relative to most cellular mRNAs in other systems too. A half-life of only 1–2 h for the tubulin mRNAs has been indicated in a number of mammalian cultured cells (2, 5).

Deflagellation induces the synthesis of not only tubulin, but a great many other proteins, many of which are identifiable flagellar structural proteins (15, 24). The return to basal rates of synthesis after regeneration is not precisely coordinate for all of these proteins, but is also relatively rapid (within 2–3 h) (24). It is possible that all flagellar mRNAs have relatively short half-lives in *Chlamydomonas*. Since we have cDNAs made to a number of these nontubulin, induced mRNAs (29), this possibility is easily investigated.

In addition to having short half-lives in nonregenerating cells, it is possible that many (or all) flagellar mRNAs are subject to accelerated degradation following regeneration. The finding that tubulin RNA decays with an apparent half-life of only ~20 min or less following regeneration, which is one-third to one-half the value determined under steady state conditions in nonregenerating cells (this study), indicates that tubulin RNA may be specifically degraded during the latter phase of regeneration. During this same period, the constitutive mRNAs, including 2–40, are maintained at constant levels (29). Interestingly, large increases in accumulated tubulin mRNA levels that accompany flagellate development in *Naegleria* are followed by exponential loss of tubulin RNA sequences with a half-life of only ~8 min (S. Remillard, personal communication). Similarly, a 40-fold accumulation of tubulin mRNA preceding mitosis in *Physarum* decays with a 19-min half-life following mitotic spindle formation (28). Tubulin RNA in *Chlamydomonas*, as well as in other cell types, may possess intrinsic features that allow its specific regulation by alterations in stability.

We also have demonstrated in this report that more tubulin RNA accumulates during regeneration than can be attributed to new synthesis, and infer that tubulin transcripts must be at least twofold more stable after deflagellation than in nonregenerating cells. The stabilization component of the accumulation may be even greater than twofold if we are overestimating the increase in tubulin RNA synthesis by normalization to rRNA synthesis. That is, if rRNA synthesis is actually somewhat depressed after deflagellation (12), then

the 2.9-fold average increase in tubulin RNA synthesis we have measured would be an overestimate. Dallman et al. (7) have made a related observation in *Chlamydomonas*. They reported that an approximate 2-fold increase in transcription of beta tubulin sequences preceding mitosis in permeabilized *Chlamydomonas* cells is insufficient to account for the 8–10-fold increase in abundance which occurs at this time. *Chlamydomonas* may use the same mechanisms to regulate tubulin RNA levels during the cell cycle and during flagellar regeneration.

It is possible that if the nuclear transit time of the tubulin RNAs were sufficiently long, significant amounts of new transcripts could accumulate in the nucleus following regeneration. Under these circumstances, part of the measured increase in accumulated tubulin RNA sequences would be a reflection of molecules not subject to cytoplasmic decay, and therefore lead to an erroneous inference of stabilization. However, the nuclear transit time for tubulin mRNA following deflagellation appears to be very short, as suggested by the rapid onset of elevated tubulin protein synthesis (a significant stimulation occurs during the first 10 min) (15) and the coincidence of the peak rate of tubulin protein synthesis and tubulin RNA accumulation (20).

Many studies have now documented a role of changing mRNA stabilities in the regulation of protein synthesis. For example, programmed destabilization of a large class of mRNAs in *Dictyostelium discoideum* is a fundamental regulatory feature of the differentiation cycle in this organism (4, 17, 18). Cell cycle regulation of histone mRNA levels in mammalian culture cells is mediated by both transcriptional rate changes and different half-lives during the S-phase histone RNA accumulation and in the absence of DNA synthesis (8, 11, 32). Histone mRNA levels in yeast cells also have been shown to be regulated by both transcriptional and posttranscriptional means (22, 23). There are many cases now in which hormones have been shown to dramatically alter specific mRNA half-lives (e.g., 3, 10, 13). Stabilization of nuclear transcripts has been demonstrated to effect the increase in dihydrofolate reductase mRNA accompanying stimulated growth in a mouse cell line (16).

Possible stabilization mechanisms for tubulin RNA following deflagellation in *Chlamydomonas* include (a) production of intrinsically more stable tubulin mRNPs during induction, e.g., by alterations in the transcripts themselves or by association with a different set of protective proteins; (b) nuclear stabilization of tubulin sequences usually destroyed before maturation; (c) a general cytoplasmic change in RNA degradation. The finding that the accumulated tubulin RNA decays with a very rapid half life following regeneration eliminates the first possibility, i.e., that the tubulin mRNPs induced by deflagellation are intrinsically more stable than the steady state population of tubulin mRNA. The second possibility, that a greater proportion of newly transcribed tubulin sequences are processed to maturity in regenerating cells than in nonregenerating cells, has some precedent (1, 16). The changing stability of tubulin mRNA during regeneration may be best explained by the third possibility, a general change in RNA degradation. Only those RNA species with normally rapid turnover times would show appreciable abundance changes with transient fluctuations in ribonuclease activity. Further specificity of mRNA stability changes could be facilitated by differential subcellular localization.

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