Subroutines in the programme of *Chlamydomonas* gene expression induced by flagellar regeneration

By JEFFERY A. SCHLOSS

Department of Biology, Yale University, New Haven, CT 06511 U.S.A.

**TABLE OF CONTENTS**

**Summary**

Flagellar regeneration

A model system for studying gene expression

Flagellar regeneration requires *de novo* protein synthesis

Accumulation of specific mRNAs accounts for changes in protein synthesis patterns during regeneration

RNA accumulation is due largely to increased transcription

Subroutines in the programme of gene expression during flagellar regeneration

**References**

**SUMMARY**

The unicellular green alga *Chlamydomonas reinhardtii* possesses two anterior flagella that are rapidly replaced if they are lost. A cytoplasmic pool of flagellar precursors supports regeneration of partial length flagella, but complete regeneration requires *de novo* synthesis of flagellar proteins. This increase in protein synthesis is transient and is programmed by changes in the physical abundance of a set of RNAs. These changes were measured using cloned cDNAs. The curves that were generated with probes for a number of different RNAs were variations-on-the-theme of a rapid accumulation following deflagellation of the cells, followed by similarly rapid degradation. Differences in the characteristics of the accumulation curves suggest that several 'subroutines' for RNA metabolism appear to run concurrently during flagellar regeneration. A significant portion of the mRNA abundance regulation occurs at the transcriptional level. A group of cDNAs that encode mRNAs whose abundance remains at constant levels during regeneration provides an important internal control for the abundance and transcription measurements. Flagella contain over 200 different proteins, so the possibility exists that the cells coordinately and rapidly alter the transcription of the same number of genes. *Chlamydomonas* flagellar regeneration thus provides an opportunity to study cellular mechanisms for coordinating the expression of a large set of genes, and correlating these changes with an easily monitored morphogenetic process.

**FLAGELLAR REGENERATION**

_A model system for studying gene expression_

The use of organisms with flagella and cilia as eukaryotic model systems for the study of gene expression is based on the observation that regeneration of these
organelles following their experimental detachment induces biosynthetic alterations in the cells. The pertinent observations made on deciliated *Tetrahymena* (Guttman & Gorovsky, 1979; Marcaud & Hayes, 1979; Bird & Zimmerman, 1980), multiply deciliated *Strongylocentrotus* embryos (Stephens, 1977; Merlino, Chamberlain & Kleinsmith, 1978), differentiating *Naegleria* (Kowit & Fulton, 1974; Lai, Walsh, Wardell & Fulton, 1979), and deflagellated *Chlamydomonas* (see below) are that replacement of cilia or flagella is accompanied by changes in the synthesis of specific proteins and in the synthesis and/or accumulation of specific mRNAs.

This article will focus on *Chlamydomonas* because of the unique opportunities this cell provides for tracing the flagellar regeneration process from the level of organelle outgrowth (Lewin, 1953; Hagen-Seyfferth, 1959; Randall *et al.* 1967; Rosenbaum, Moulder & Ringo, 1969) to the synthesis and assembly of cytoplasmic precursor proteins (Rosenbaum *et al.* 1969; Weeks & Collis, 1976; Weeks, Collis & Gealt, 1977; Lefebvre, Nordstrom, Moulder & Rosenbaum, 1978; Remillard & Witman, 1982; Brunke, Collis & Weeks, 1982; L’Hernault & Rosenbaum, 1983), to the loading of flagellar mRNAs onto polysomes (Weeks & Collis, 1976), to the accumulation of flagellar mRNAs (Lefebvre, Silflow, Wieben & Rosenbaum, 1980; Silflow & Rosenbaum, 1981; Minami, Collis, Young & Weeks, 1981; Silflow *et al.* 1982; Brunke, Young, Buchbinder & Weeks, 1982; Schloss, Silflow & Rosenbaum, 1984), to transcriptional regulation (Keller, Schloss, Silflow & Rosenbaum, 1984), and finally to the structure of the flagellar protein genes (Silflow & Rosenbaum, 1981; Brunke *et al.* 1982). The choice to consider the process in this way — from the end product to the gene instead of the more conventional gene-to-gene-product view — can be justified on historical grounds: this is how the data were collected due to development of increasingly sensitive technologies. Moreover, this approach forms a useful paradigm for considering cellular control of the events involved, because it is presumably the direction of movement of cellular signals that modulate the response. It is these signals and their modes of action that we hope eventually to understand.

A corollary of the ability to explore the biosynthetic steps leading to organelle morphogenesis is that, because the organelle in question is biochemically heterogeneous, this should be an excellent system in which to ask whether particular elements of gene structure are important in controlling the expression of a large number of protein-coding templates. A typical eukaryotic flagellum contains nine outer doublet microtubules, each composed of a complete A-tubule and a partial B-subfibre, surrounding two complete central microtubules. In addition to microtubules, several other structures such as dynein arms, radial spokes, nexin links, and the central sheath are also present, regularly arrayed along the length of the flagellum. It is not surprising, then, that when flagella are isolated and analysed on polyacrylamide gels, they are found to contain, in addition to the major microtubule proteins alpha- and beta-tubulin, a large
number of other polypeptides. In *Chlamydomonas*, the ability to dissect flagella using functional, biochemical, morphological, and genetic approaches has led to the mapping of a number of these polypeptides to specific structures. For example, pfl4 cells are unable to swim and contain a mutation in a single locus (Ebersold, Levine, Levine & Olmsted, 1962). Electron microscopy demonstrates that the flagella of these cells are fully formed except for the lack of radial spoke structures. This correlates with the absence of several proteins, discerned by comparing polyacrylamide gels of flagella isolated from wild-type and pfl4 cells (Witman, Fay & Plummer, 1976; Piperno, Huang & Luck, 1977). Similar analyses, along with flagellar fractionation studies, have led to the assignment to specific flagellar structures of 60 of the over 200 different flagellar polypeptides that may be visualized on polyacrylamide gels (Witman, Carlson, Berliner & Rosenbaum, 1972; Witman, Plummer & Sander, 1978; Huang, Piperno & Luck, 1979; Piperno, Huang, Ramanis & Luck, 1981; Adams, Huang, Piperno & Luck, 1981; Luck, 1984). It is these proteins that are synthesized during flagellar regeneration and that provide the interesting possibility of studying cellular mechanisms for coordinately regulating the expression of a large number of genes.

*Flagellar regeneration requires de novo protein synthesis*

When *Chlamydomonas* cells are deflagellated by mechanical shear (Rosenbaum *et al.* 1969) or pH shock (Witman *et al.* 1972) rapid flagellar outgrowth begins immediately and then continues at a decelerating rate until flagella reach full length in 1 to 3 h. Both vegetative cells and gametes are capable of regenerating flagella. If cells are deflagellated in the presence of inhibitors of protein synthesis, partial regeneration occurs. While cells normally regenerate 12–15 μm long flagella, vegetative cells in cycloheximide regenerate only approximately 6 μm long flagella, while cycloheximide-treated gametes regrow flagella only about 3 μm long (Rosenbaum *et al.* 1969; Lefebvre *et al.* 1978). These experiments demonstrate that the cells contain a cytoplasmic pool of assembly competent flagellar precursors sufficient for assembling approximately half- or one-fifth-length flagella, depending on the culture conditions. Another estimate of the size of this pool was made by the completely independent criterion of immunoochemical determination of beta-tubulin content. These experiments show that deflagellated cell bodies contain just over half as much beta-tubulin protein as is present in a pair of flagella (Piperno & Luck, 1977), in close agreement with the vegetative cell assembly-competent-pool estimate derived from the inhibitor experiment.

If the cytoplasmic pool of flagellar precursors can supply components for regeneration of only partial length flagella, then deflagellation of these cells must induce a programme of flagellar protein synthesis to provide for the complete regeneration that normally occurs. Induction of protein synthesis has been demonstrated by pulse-labelling cells with [35S]sulphate before deflagellation (to
establish a synthetic rate 'baseline') and at successive intervals during regeneration, and analysing the labelled whole-cell proteins by polyacrylamide gel electrophoresis (Weeks et al. 1977; Lefebvre et al. 1978; Silflow et al. 1982; Remillard & Witman, 1982). These studies demonstrate that the synthetic rates of alpha- and beta-tubulin and several other polypeptides, some of which may be identified as flagellar components by comigration with bona fide flagellar proteins, are elevated in deflagellated cells, relative to their basal rates in non-deflagellated cells. Changes in the synthetic rates of these proteins occur over a background of constant synthetic rates, before and during regeneration, of the majority of the polypeptides visualized on the electropherograms.

Accumulation of specific mRNAs accounts for changes in protein synthesis patterns during regeneration

Polyribosomes isolated from regenerating cells direct the run-off translation of more tubulin than do polysomes from control, nonregenerating cells (Weeks & Collis, 1976). Is tubulin mRNA stored in control cells for subsequent loading onto polysomes? Experiments in which RNA was isolated from control and regenerating Chlamydomonas and then translated in vitro suggest that this is not the case; rather the amount of mRNAs encoding tubulin and numerous other flagellar proteins increases in response to deflagellation (Lefebvre et al. 1980).

To measure changes in mRNA abundance directly, recombinant DNAs complementary to the mRNAs have been cloned and selected. Using Chlamydomonas alpha- and beta-tubulin cDNAs (Silflow & Rosenbaum, 1980) or chicken alpha-tubulin cDNA and Chlamydomonas beta-tubulin cDNA (Minami et al. 1981; Brunke et al. 1982) to probe RNA transfers, a dramatic increase in tubulin mRNA has been demonstrated in regenerating cells. Accumulation of tubulin mRNAs, over their control cell levels, is detectable within 2 min after deflagellation (Fig. 1). The mRNA levels continue to increase, peaking about an hour after deflagellation, and then undergo a similarly rapid decrease. After flagella have reached full length, in about 2 h, tubulin mRNAs have returned to basal levels.

Chlamydomonas cells contain two alpha- and two beta-tubulin mRNAs, which correspond to the four tubulin genes of the organism, and which are coordinately regulated during flagellar regeneration (Fig. 1 and Silflow & Rosenbaum, 1981; Brunke et al. 1982). The alpha-tubulin mRNAs exhibit considerable homology, as do the beta-tubulin mRNAs, but there is no evidence for homology between alpha- and beta-tubulin mRNAs sequences. This cross hybridization accounts for the visualization of both bands in the RNA transfer experiments shown in Fig. 1, although each filter was probed with a cDNA corresponding to a single alpha-tubulin or beta-tubulin mRNA.

Although the tubulin mRNAs appear coordinately regulated on RNA transfers, there are differences in the accumulation kinetics of alpha- and beta-tubulin messages. In the experiment shown in Fig. 2, RNA was purified from samples of a synchronous gametic culture before and at closely spaced times after
Fig. 1. Relative concentrations of alpha- and beta-tubulin mRNAs during flagellar regeneration. RNA isolated from vegetative cells before (ndf) and at 2, 15, 30, 46, 60, 80, 100, and 130 min after deflagellation was resolved on denaturing agarose gels (2 μg total RNA/lane) and transferred to nitrocellulose filters. The filters were probed with $^{32}$P-labelled pcf10-2 (top panel) and pcf9-12 (bottom panel), which are full-length alpha- and beta-tubulin cDNA clones, respectively. Autoradiography of the filters reveals that tubulin mRNAs are present in control, non-deflagellated cells, accumulate for about an hour, and then return to control cell levels after regeneration is complete. The sizes (in numbers of nucleotides) of marker fragments are shown on the left. (Methods for this and subsequent figures are described in Schloss et al. 1984.)

deflagellation. The RNA was applied in 'dots' to nitrocellulose filters, probed with $^{32}$P-labelled cloned tubulin cDNAs, and hybridization was assayed by scintillation counting (Schloss et al. 1984). Alpha- and beta-tubulin mRNAs reach maximal levels, 11- to 12-fold higher than their control cell levels, at
Fig. 2. Different accumulation kinetics for alpha- and beta-tubulin mRNAs. Alpha-tubulin (□) and beta-tubulin (△) mRNAs were measured in RNA samples isolated before and after deflagellation of gametes. The values are plotted as a ratio of the amount of each mRNA present at each time point during regeneration relative to the amount of that mRNA present in control cells (before deflagellation). Thus a value of 1 indicates that an RNA species is present at the same level as in control cells. Maximal accumulation of alpha-tubulin mRNA occurs earlier than maximal accumulation of beta-tubulin mRNA. The abundance of a constitutive mRNA (○, pcf8-13, see also Fig. 3) remains constant through the experiment. In this experiment, flagella were 14.4 μm long in control cells and regenerated to 13.6 μm long by 190 min.

different times after deflagellation. The accumulation curves are complex, with two peaks (at 25 and 60 min for alpha and at 45 and 80 min for beta) separated by a decrease in abundance (at 45 and 60 min for alpha and beta, respectively). The differences between the accumulation kinetics are corroborated by the observations that (1) the abundance fluctuations occur at different times for alpha- and beta-tubulins, (2) similar curves were obtained for the same RNA experiment using a number of different tubulin cDNA probes (not shown), and (3) an RNA (pcf8-13) whose abundance remains constant during regeneration (Schloss et al. 1984) shows no fluctuation in this experiment (Fig. 2). Furthermore, differences in accumulation curves for alpha- and beta-tubulin mRNAs have been observed in two independent experiments (Schloss et al. 1984 and
unpublished observations). The significance of these observations for understanding control of tubulin gene expression remains to be determined.

Protein-labelling experiments and translation of purified mRNA in vitro suggest that many mRNAs other than tubulin undergo significant abundance changes during flagellar regeneration. To measure these changes, probes for some of these mRNAs were prepared (Schloss et al. 1984). Poly(A)^+ RNA from regenerating cells was reverse transcribed and inserted into a plasmid vector. The resulting recombinant DNA bank was screened to identify cDNAs whose homologous RNAs increase in abundance following deflagellation. Cloned cDNAs whose corresponding RNAs remain at constant abundance before and during regeneration were also selected. The latter serve as important controls for a number of experiments.

The regulation patterns for the newly selected clones were studied (Schloss et al. 1984). As in the experiments described above, RNA was isolated before and after deflagellation of a synchronous gamete culture and the RNA was applied to nitrocellulose filters. The cDNA probes were ^32P-labelled by nick translation, incubated with the RNA dots, and the extent of hybridization was determined by scintillation counting. In addition to tubulins, eight other cDNAs have been characterized that detect different mRNAs that accumulate following deflagellation (Fig. 3A). All of the RNAs are detected in control cells. The abundance of some of these mRNAs (corresponding to cDNA clones pcf6-2, 6-187, 9-26, and 6-100) changes after deflagellation only by a factor of two or three, while others (pcf3-21) undergo a 15-fold increase. Some of the RNAs attain maximal abundance at 30 min in this experiment, others do so at 50 min, while yet others probably peak sometime between 30 and 50 min. Most of the RNAs approach control cell levels by the time flagella regenerate to full length (Fig. 3E).

The differences in timing of maximal accumulation for various RNAs are probably not simply a function of size. For example, pcf6-8 peaks in abundance earlier than does pcf6-100, yet these RNAs are 2680 and 1150 nucleotides long, respectively (Schloss et al. 1984). The possibility remains, however, that the original transcript for pcf6-100 is longer than that for pcf6-8.

Three additional cDNA clones reveal an entirely different pattern of mRNA regulation. These RNAs increase two-to-four-fold in abundance within 10 min after deflagellation, and by 30 min have fallen to or below their levels in control cells (Fig. 3B). Thus the changes in abundance for these RNAs are completed while another set of RNAs (Fig. 3A) are still accumulating. At least two possibilities exist concerning the identities of these mRNAs. They may encode stress-induced proteins. A subset of the heat-shock proteins of Chlamydomonas is induced by deflagellation (G. May & J. Rosenbaum, manuscript in preparation), but the kinetics of this response have not been well characterized. They may alternatively be radial spoke protein mRNAs. Remillard & Witman (1982) demonstrated that radial spoke proteins are synthesized most rapidly during the first 15 min after gamete deflagellation. Attempts to identify the protein products
of these three mRNAs by hybrid selection and translation are in progress.

The synthetic rates of most of the proteins in the cells are not altered during regeneration. Several 'constitutive' cDNA probes were selected. The abundance measurements for each of these mRNAs vary from control cell levels by less than 25% during flagellar regeneration (Fig. 3C).

The cDNA probes described to this point represent poly(A)+RNAs and each hybridizes to a single band in RNA transfer experiments (Schloss et al. 1984). Several cDNA clones have been identified that hybridize to a very large number of different-sized poly(A)+RNAs. One example of such a clone is shown in Fig.

Fig. 3. Accumulation curves for regulated and constitutive RNAs. RNA samples isolated from control and regenerating gametes were dotted onto nitrocellulose filters and probed with labelled cDNAs. Hybridization was assayed by scintillation counting. The data are expressed as described in the legend to Fig. 2. (The beginning point for each curve is arbitrarily placed on the ordinate to provide a graphic display in which curves do not overlap.) The name of the cloned cDNA used to measure each RNA is given to the right of each curve. Panels A, B and D contain data for RNAs that show a number of differently shaped accumulation curves (see text). Results for constitutive RNAs are shown in panel C. Each cDNA clone represents a different RNA species except for clones 6-87 and 6-100, which contain portions of the same RNA sequence. Flagellar length measurements for this experiment are shown in panel E. Control cell flagellar length averaged 13.5 μm. Parts of this figure are redrawn from Schloss et al. (1984).
Programmed gene expression during flagellar regeneration

4. Some of these RNAs are regulated during flagellar regeneration. The accumulation kinetics for these RNAs differ from those of RNAs described above. Also present in control cells, these RNAs drop slightly below control cell levels soon after deflagellation and then increase dramatically between 10 and 50 min (Fig. 3D). They are lost from the cells, slowly at first and then more rapidly. Little more is known about these RNAs at this time. Experiments are in progress to determine their cytoplasmic localization and the organization of the genes that encode them.

RNA accumulation is due largely to increased transcription

A set of RNAs can accumulate because transcription of the genes encoding them increases or because the turnover rates of the RNAs themselves decrease. To determine whether deflagellation induces changes in transcription, nuclei were isolated from control and deflagellated *Chlamydomonas* cells, and transcription from these nuclei was carried out *in vitro* in the presence of labelled RNA precursors. This experimental approach is based on the observation that

![Fig. 4. A cloned cDNA that is homologous to a broad class of RNAs. RNA samples from control and regenerating gametes (same experiment as Fig. 3) were resolved on denaturing agarose gels (2 μg total RNA/lane), transferred to nitrocellulose filters, and probed with pcf5-14 (left panel) and an alpha-tubulin cDNA clone (right panel). Pcf5-14 contains a 1400 base pair cDNA insert and hybridizes to RNAs covering a large range of molecular sizes. The size range of homologous RNAs does not change during regeneration, although the hybridization intensity varies considerably (see Fig. 3D). Probing the same RNAs with a tubulin cDNA results in a discrete hybridization band in each gel lane. The sizes of marker fragments appear on the left.](image-url)
isolated nuclei maintain template specificity and are permeable to nucleotides, so that transcripts may be labelled (Smith & Huang, 1976).

Products of nuclear transcription \textit{in vitro} from control and regenerating cell nuclei were assayed by hybridization to cloned cDNAs. Regenerating cell nuclei are more active in transcribing alpha- and beta-tubulin RNAs than are control cell nuclei. Yet the intensity of hybridization to constitutive cDNAs (e.g. pcf2-40) is the same for \textit{in vitro} transcripts from both sets of nuclei (Keller \textit{et al.} 1984). These studies have recently been expanded to include several different cDNA clones that are homologous to RNAs that accumulate during regeneration (L. Keller & J. Schloss, unpublished observations). These experiments suggest that much of the RNA accumulation that occurs during regeneration is the result of increased transcription of a specific set of genes.

Nuclear transcription may provide an experimental system in which to test for molecules that signal the nucleus to alter its pattern of gene expression. Such a signal presumably arises in the cytoplasm and is related to flagellar length or some other as yet undetermined physiological parameter. By adding cytoplasmic fractions to isolated nuclei, it may be possible to reconstitute changes in transcription \textit{in vitro}. These experiments will probably require nuclei that are capable of initiating new transcripts. Recent experiments provide encouraging evidence that reinitiation occurs in the isolated nuclei (Keller \textit{et al.} 1984; and unpublished observations).

Experiments carried out in parallel with the isolated nuclei studies involve labelling of RNA synthesized by the cells \textit{in vivo}. Cells are pulsed with \textsuperscript{32}Porthophosphate before and after deflagellation, and then RNA is isolated and hybridized with cloned cDNAs of interest. In agreement with the results described above, these experiments demonstrate an increase in the transcription rate of alpha- and beta-tubulin RNAs, but no change in the rate of transcription of the constitutive RNA homologous to pcf2-40 (E. Baker, J. Schloss & J. Rosenbaum, manuscript in preparation). The change in tubulin RNA transcription is sufficient to account for the majority of the tubulin RNA accumulation, but is also consistent with the possibility that there may be changes in RNA stability. Direct measurements of RNA stability are in progress.

\textit{Subroutines in the programme of gene expression during flagellar regeneration}

The results described above suggest that the changes in gene expression that occur in response to deflagellation of \textit{Chlamydomonas} are more complex than just a coordinate increase in transcription of a collection of genes. Different RNAs undergo different patterns of accumulation, suggesting that subsets of the large group of genes whose transcription changes following deflagellation of the cells may respond to a variety of regulatory molecules. The observation that different RNAs peak in abundance at different times is particularly interesting in light of the suggestion that proteins that assemble into a particular flagellar structure are synthesized simultaneously, but proteins that assemble into different
Programmed gene expression during flagellar regeneration

Structures may be synthesized during various phases of the regeneration response (Remillard & Witman, 1982). Thus it will be very interesting to determine whether DNA sequence elements outside of the protein-coding regions of these genes are shared by genes that are ‘coordinately’ regulated during the flagellar response, and whether genes in different subsets actually have different regulatory sequences. The availability of probes for constitutively expressed sequences will permit us to eliminate from consideration as putative specific flagellar regulatory sequences, those regions of the DNA that may be shared by *Chlamydomonas* genes because they are involved in the basic mechanics of transcription.

If in addition to regulating transcription, the cells also regulate RNA stability, then additional regulatory molecules and receptors must be invoked. Preliminary data from the *in vivo* RNA-labelling experiments described above, and differences between the kinetics of abundance decreases for the various RNAs for which probes are now available, may be interpreted as indicating that specific groups of RNAs are subject to different degradation pathways, and that factors involved in these pathways may themselves be subject to regulated expression. Such speculation may be useful in devising testable hypotheses consistent with the experimental data.

The studies on flagellar regeneration that are described here provide the basis for continuing investigation in what has turned out to be a very tractable developmental model system. By combining the sensitivity of recombinant DNA technology with progress that has been made using genetic, biochemical, morphological, and functional approaches to studying *Chlamydomonas* flagella, we expect in the next few years to test theories that have broad relevance to understanding the basic programmes of animal and plant development.

The author thanks Joel Rosenbaum for the opportunity to participate in his laboratory’s ongoing studies of flagellar regeneration, and Ellen Baker and Laura Keller for permission to include some of their results prior to publication. The work was supported by U.S. Public Health Service awards to J. Rosenbaum. The author was a Muscular Dystrophy Association Fellow and a U.S. Public Health Service Fellow during parts of the work.

**REFERENCES**


Programmed gene expression during flagellar regeneration


